RIGA TECHNICAL UNIVERSITY

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A SCREENING METHOD FOR IMPROVED SINGLE-CELL PROTEIN PRODUCTION

Doctoral Thesis

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RIGA TECHNICAL UNIVERSITY
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A SCREENING METHOD FOR IMPROVED SINGLE-CELL PROTEIN PRODUCTION

Doctoral Thesis

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ANNOTATION

The aquaculture sector has become increasingly essential in providing high-quality animal protein globally. Alternative protein sources are essential to address the growing demand for sustainable aquaculture feed. This is because traditional feed ingredients, such as fishmeal and plant-based proteins, are both limited in availability and environmentally burdensome. Single-cell proteins, derived from microbial biomass such as algae, bacteria, fungi, yeast, and protozoa, offer a promising alternative. Microorganisms can utilise diverse carbon and nitrogen sources, potentially reducing the need for intensive farming practices that threaten ecosystems and biodiversity worldwide. Despite substantial research and technological advancements, single-cell protein production requires further optimisation to meet large-scale demands effectively. Enhancing the properties of protein-synthesising microorganisms could improve the competitiveness of technology and contribute to the sustainable expansion of the feed market.

This Doctoral Thesis investigates a novel approach for developing microorganism mutants with improved essential amino acid profiles. Specifically, it utilises amino acid biosynthesis inhibitors (pure herbicidal compounds) to screen and select mutant strains with enhanced properties, produced through random mutagenesis. The Thesis is organised into three main chapters: Literature Review, Methodology, and Results and Discussion.

The Literature Review consists of two parts. The first part explores strategies to improve microbial protein synthesis, focusing on optimising fermentation processes and applying random mutagenesis. The second part presents a database of amino acid biosynthesis inhibitors, detailing their specificity, targeted amino acids, effects on microbial growth, treatment dosages, side effects, and usage conditions.

The Methodology chapter presents a multi-criteria decision analysis of amino acid biosynthesis inhibitors, accompanied by a comprehensive description of the materials, procedures, and equipment utilised in the experiments.

In the Results and Discussion chapter findings from the multi-criteria analysis and experimental studies are presented, highlighting the successful development of mutant strains with enhanced synthesis of essential amino acids. The amino acid profiles of the generated mutants were analysed and compared with those of the wild-type strain and single-cell protein-producing yeasts. The conclusions summarise the implications of these findings.

This Thesis, grounded in five scientific publications and presented at five international conferences, was conducted at the Biosystems Laboratory of the Institute of Energy Systems and Environment.

ANOTĀCIJA

Akvakultūras nozare kļūst arvien nozīmīgāka, nodrošinot augstas kvalitātes dzīvnieku izcelsmes proteīnus visā pasaulē. Lai apmierinātu pieaugošo ilgtspējīgas akvakultūras barības pieprasījumu, būtiski ir alternatīvi proteīnu avoti, jo tradicionālie barības komponenti, piemēram, zivju milti un augu izcelsmes proteīni, ir ierobežoti un rada lielu negatīvu ietekmi uz vidi. Vienšūnas proteīni, kas iegūti no mikrobiālās biomasas, piemēram, no aļģēm, baktērijām, sēnēm piedāvā daudzsološu alternatīvu. Mikroorganismi spēj izmantot dažādus oglekļa un slāpekļa avotus, kas potenciāli ļauj samazināt nepieciešamību pēc intensīvām lauksaimniecības praksēm, kas apdraud ekosistēmas un bioloģisko daudzveidību visā pasaulē. Lai gan vienšūnu proteīnu ražošanas tehnoloģijas ir plaši pētītas un pastāvīgi attīstās, plaša ražošana joprojām ir ierobežota. Proteīnu sintezējošo mikroorganismu īpašību uzlabošana varētu palielināt tehnoloģijas konkurētspēju un paplašināt barības tirgus piedāvājumu, tādā veidā veicinot akvakultūras nozares ilgtspējīgu izaugsmi.

Promocijas darbā jauna pieeja mutantu celmu radīšanai ar uzlabotu neaizstājamo aminoskābju profilu. Šī pieeja izmanto aminoskābju biosintēzes inhibitorus (tīrus herbicīdu savienojumus), lai atlasītu un izvēlētos mutantu celmus ar uzlabotām īpašībām, kas izveidoti ar nejaušas mutaģenēzes palīdzību. Promocijas darbs sastāv no trim galvenajām nodaļām: literatūras apskata, metodoloģijas, rezultātiem un diskusijas.

Literatūras apskata pirmajā daļā aplūkotas iespējas uzlabot mikroorganismu proteīnu biosintēzi, izmantojot divas stratēģijas: mikrobiālās fermentācijas procesa optimizāciju un mutaģenēzi. Literatūras apskata otrā daļa ir aminoskābju inhibitoru datubāze, kurā detalizēti aprakstīta to specifika, mērķa aminoskābes, ietekme uz mikroorganismu augšanu, devas, blakusparādības un lietošanas apstākļi.

Metodoloģijas nodaļu veido aminoskābju biosintēzes inhibitoru daudzkritēriju analīze un eksperimentālās daļas detalizēts darbā izmantoto materiālu, procedūru un iekārtu apraksts.

Rezultāti un diskusijas nodaļā ir analizēti un aprakstīti daudzkritēriju analīzes un eksperimentālā pētījuma rezultāti. Radīto mutantu aminoskābju profili analizēti un salīdzināti ar savvaļas tipa celmu un citiem vienšūnu proteīnu ražojošiem raugiem. Secinājumi par darba rezultātiem ir apkopoti promocijas darba beigās.

Promocijas darba pamatā ir piecas zinātniskās publikācijas un tās ir prezentētas piecās starptautiskās zinātniskās konferencēs. Praktiskais darbs ir izstrādāts Vides aizsardzības un siltuma sistēmu institūta Biosistēmu laboratorijā.

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TABLE OF CONTENTS

ABBREVIATIONS

AA – amino acid
AAS – amino acid score
AEC – S-(2-aminoethyl)-L-cysteine
AHAS – acetohydroxyacid synthase
Ala – alanine
ALS – acetolactate synthase
Arg – arginine
Asp ac – aspartic acid
ATX – astaxanthin
AVG – L-α-(2-aminoethoxyvinyl)glycine
B – bacteria
BCA – Bicinchoninic acid assay
BCAA – branched-chain amino acids
CFR – Code of Federal Regulations
CHG – 2-(1-cyclohexen-3(R)-yl)-S-glycine
CS – chlorsulfuron
Cys – cysteine
DCW – dry cell weight
DHQS – 3-dehydroquinate synthase
DNA – deoxyribonucleic acid
EAA – essential amino acid
EAAI – essential amino acid index
Em. – emission
EMS – ethyl methanesulfonate
EPSPS – 5-enolpyruvalshikimate-3-phosphate synthase
Ex. – excitation
F – fungi
FAO – Food and Agriculture Organisation
g – gravity, a unit of relative centrifugal force
GA – glufosinate-ammonium
Glu ac – glutamic acid
Gly – glycine
GP – glyphosate
GPM – glycerine-peptone medium
GS – glutamine synthetase
GSM – glycerine-salt medium
His – histidine
IGPD – imidazole glycerol-phosphate dehydratase
Ile – isoleucine
Leu – leucine
Lys – lysine
M – mole
MCDA – multi-criteria decision analysis
Met – methionine
MNNG – methylnitronitrosoguanidine
MOPS – 3-(N-morpholino)propanesulfonic acid
MSM – metsulfuron-methyl
MSO – L-methionine sulfoximine
n/a – not analysed
n/d – not defined
NaOH-SDS – sodium hydroxide-sodium dodecyl sulfate solution

NEAA – non-essential amino acid
OD – optical density
P5CR – δ1-pyrroline-5-carboxylate reductase
PAG – DL-propargylglycine
Phe – phenylalanine
Pro – proline
PT – phaseolotoxin
RNA – ribonucleic acid
ROS – reactive oxygen species
rpm – revolutions per minute
S/g – slow growth
SCP – single-cell protein
Ser – serine
Sign. – significant inhibition
SMM – sulfometuron-methyl
T – temperature
TBM – tribenuron-methyl
TCC – total carotenoid content
Thr – threonine
TOPSIS – a technique for order preference by similarity to an ideal solution
Tyr – tyrosine
UV – ultraviolet
v/v – volume per volume
Val – valine
YM – yeast medium
YNB – yeast nitrogen base

Abbreviations used for mutants (GA6/5-1; GA7/4-3; AEC2/1-2; AEC3/9-1, etc.).
The first letters indicate the amino acid biosynthesis inhibitor used in screening the mutants, the next digit corresponds to the inhibitor concentration, the number after an oblique stroke indicates the mutant's serial number, and the digit after the short hyphen indicates the number of subcultures.

INTRODUCTION

The relevance of the Doctoral Thesis

Aquaculture has become one of the most significant sectors in global food production; however, this growth presents serious challenges, particularly concerning traditional feeding practices. One of the most critical issues is the extensive use of fishmeal as the primary source of protein and lipids in aquaculture feed, with this fishmeal being predominantly derived from wild fish catches. Each year, more than 30 million tons of fish are redirected for non-food purposes, 80 % of which are used in aquaculture feed production, placing considerable pressure on wild fish stocks [1], [2]. The incorporation of soybean meal in aquaculture feed has partially replaced fishmeal; however, this shift has made the aquaculture industry increasingly reliant on agricultural resources. As a result, there is a pressing need for sustainable alternative protein sources that do not compete for resources with the food industry [3], [4].

Single-cell protein (SCP) is a promising alternative protein source for aquaculture feed. SCP is a dried biomass of microorganisms, primarily microalgae, bacteria, moulds, and yeasts with high protein content. The technology has many advantages over traditional dietary proteins since production is more environmentally friendly, consumes less water, requires smaller land areas, is not influenced by climatic conditions, and can be produced from agro-industrial by-products [5]–[7]. SCP is an alternative protein source that could help to improve sustainability and reduce the scarcity of proteins [8], [9]. Increasing the use of SCP in aquaculture and livestock feeds could reduce the need for intensive farming while aligning with environmental strategies for reducing greenhouse gas emissions [10], [11].

SCP production technologies have undergone extensive research for decades [12] and constantly expand into the market [13]. It has been used as a food supplement for humans and as a feed for animals [14]. Currently, SCP is being produced under different commercial names like Brovile®, AlgaVia®, Quorn®, Vitam-R®, Pruteen®, Marmite®, and FermentIQ™, etc.[14], [15]. Although some products are already in the market, they remain a niche product that is not widely available or consumed [16]. Existing limitations, such as insufficient processing and production capacity, poor infrastructure, high costs and expenses, etc., hinder the scaling up of the technology and achieving commercial viability [17]. In this regard, research aimed at developing SCP technologies is important. Microorganisms are the heart of SCP technology. Therefore, SCP productivity and quality are key factors influencing the competitiveness of the technology. Improving and creating strains with superior characteristics can increase the competitiveness of SCP. Genetic engineering is the sole way to improve protein biosynthesis in microorganisms [18], [19]. However, genetically modified microorganisms are prohibited from being used in food and feed without confirmation of safety in European countries, and approval of the safety of such microorganisms is difficult due to complex regulations and legislative barriers [17], [20], [21].

The Thesis proposes a new way to create mutant microorganisms with improved amino acid profiles. It is suggested to use random mutagenesis followed by a screening of mutants in a medium supplemented with amino acid biosynthesis inhibitors. This concept is well known in the selection of mutant microorganisms with improved synthesis of fatty acids and carotenoids,

where inhibition of the biosynthetic pathway of the target metabolite allowed the collection of overproducers [22]–[24]. Interestingly, the widely used random mutagenesis to improve the desired properties of strains is not used to create protein-synthesised mutants. However, there is evidence of the effect of random mutagenesis on increasing the protein content in microalgae biomass [25]. Several herbicides are amino acid biosynthesis inhibitors, which can be a tool for selecting improved SCP-producing mutants. It is assumed that microbial cells that have undergone mutagenesis and are capable of growing in media in the presence of an herbicide concentration that inhibits 100 % of the cells of the wild-type strain have a high probability of being protein-synthesising mutants.

Although total protein concentration in microbial biomass is a significant factor, the concentration of essential amino acids is the main factor determining the obtained protein's value. For example, essential amino acids, such as lysine, methionine, threonine, and tryptophan, are important in fish feeds, as they are available in lower amounts in conventional protein sources such as soy [26], [27]. Thus, in creating SCP mutants, it is important to increase the total protein concentration in microorganisms' biomass and the proportion of essential amino acids.

The aim and tasks of the Doctoral Thesis

The Doctoral Thesis aims to develop a method for screening mutant microorganisms with improved biosynthesis of essential amino acids. The screening method uses amino acid biosynthesis inhibitors to identify mutant overproducers.

To achieve the stated aim, the following main tasks are set:

1. study a single-cell protein improving strategies;
2. create a database of amino acid biosynthesis inhibitors and their effects on microorganisms;
3. conduct a multi-criteria decision analysis of amino acid biosynthesis inhibitors;
4. conduct experimental study and develop a screening methodology for mutants selecting with improved biosynthesis of essential amino acids.

The proposed hypothesis

Amino acid biosynthesis inhibitors (pure herbicide compounds) can be a tool in the selective screening of mutants with improved biosynthesis of essential amino acids created by random mutagenesis.

Scientific significance

The Thesis proposes a novel approach to creating microorganism strains with enhanced biosynthesis of essential amino acids. The methodology is based on random mutagenesis combined with selective screening of mutants using amino acid inhibitors. This methodology is flexible and applicable to improve the synthesis of various groups of amino acids in various microorganisms. The published articles in this Doctoral Thesis carry significant scientific impact, as they are the first to compile and analyse information on the effects of amino acid inhibitors on microorganisms as a potential tool for screening protein-enhanced mutants. Additionally, the theoretical framework is supported by an experimental study that

demonstrates the effective use of amino acid inhibitors to produce mutants with improved synthesis of essential amino acids.

A significant aspect of the Doctoral Thesis is the creation of *Phaffia rhodozyma* mutant strains. This yeast, typically regarded only as an astaxanthin feed supplement, was found to have unrecognised potential as an SCP (single-cell protein) producer. Protein analysis revealed that the mutants exhibited significantly increased synthesis of lysine, methionine, phenylalanine, isoleucine, and tryptophan, resulting in higher protein quality than traditional SCP-producing yeast species. This finding opens up new opportunities for developing technologies for simultaneously producing two microbial products, as in the case of *Phaffia rhodozyma* astaxanthin and protein.

Practical significance

According to the Food and Agricultural Organisation (FAO) statement [28], developing single-cell protein technology is important to reduce the burden on conventional feed protein production sectors. In this regard, improved commercial strains rich in amino acids, especially lysine and methionine, may provide a sustainable alternative source of protein in aquaculture diets. The developed and laboratory-validated methodology may have a direct practical application in biotechnology, where high-yielding strains can significantly increase the production efficiency of microbial products and reduce costs. Researchers and developers from the field of SCP production can use the data and methodology in the published articles in the Doctoral Thesis to create improved commercial strains or new strains that were not previously thought to be single-cell protein producers.

Research framework

The research framework consists of four main steps (Fig. 1). The first step (S1) reviewed strategies for improving single-cell protein production by optimising microbial fermentation conditions (first publication – P1). Then, the potential of random mutagenesis to create protein-improved mutants was reviewed (third publication – P3). The second step (S2) created a herbicide database: generalised types of herbicides inhibiting the amino acid synthesis in microorganisms, targeted amino acids, possible side effects (second publication – P2), and also summarised studies on the degree of microbial inhibition, doses used, and specific application conditions (P3). The third step (S3) performed a multi-criteria decision analysis of herbicides (MCDA) to select suitable amino acid biosynthesis inhibitors (fourth publication – P4) for subsequent experimental study. The final step (S4) was experimental work on creating *P. rhodozyma* mutant with improved synthesis of essential amino acids, including yeast mutagenesis, selective screening of mutants, and protein analysis of the selected mutants (fifth publication – P5). The main expected results were the developed screening methodology (R1) and the created mutants (R2).

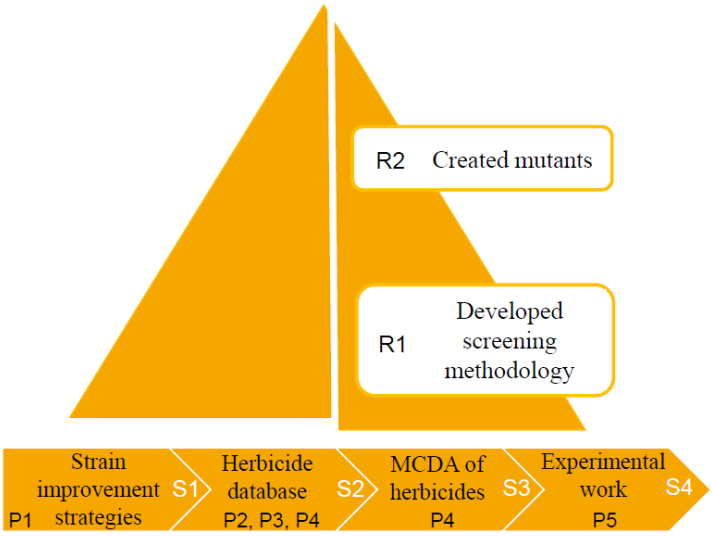

Fig. 1. The research framework of the Doctoral Thesis. The letters S, P, and R stand for steps, publications, and results, respectively.

Scientific approbation of the Doctoral Thesis

The Doctoral Thesis is based on the following scientific publications

1. Spalvins, K., Raita, S., Valters, K., Blumberga, D. Improving single cell protein yields and amino acid profile via mutagenesis: Review of applicable amino acid inhibitors for mutant selection. 2021. *Agronomy Research* vol. 19, no. 3, pp. 1285–1307. doi: 10.15159/ar.21.083
2. Raita, S., Kusnere, Z., Spalvins, K., Blumberga, D. Optimization of yeast cultivation factors for improved SCP production. 2022. *Environmental and Climate Technologies* vol. 26, no. 1, pp. 848–861. doi: 10.2478/rtuect-2022-0064
3. Raita, S., Berzina, I., Kusnere, Z., Kalnins, M., Kuzmika, I., Spalvins, K. Herbicide-based selection of mutants for improved single cell protein synthesis: application and procedures. 2024. *Agronomy Research* vol. 22, no. 2, pp. 913–938. doi: 10.15159/AR.24.060
4. Berzina, I., Raita, S., Kalnins, M., Spalvins, K., Kuzmika, I. In search of the best technological solutions for creating edible protein-rich mutants: a multi-criteria analysis approach. 2024. *Agronomy Research* vol. 22, no. S1, pp. 370–400. doi: 10.15159/AR.24.039.
5. Raita, S., Kuzmika, I., Geiba, Z., Mika, T., Spalvins, K. Enhanced amino acid biosynthesis in *Phaffia rhodozyma* via herbicide-induced selection. *Journal of Industrial Microbiology and Biotechnology*, (under review).

Other scientific publications

6. Raita, S., Spalvins, K., Blumberga, D. Prospect on agro-industrial residues usage for biobutanol production. *Agronomy Research*, 2021, vol. 19, no. 1, pp. 877–895. doi: 10.15159/ar.21.084
7. Raita, S., Spalvins, K., Raits, E., Silicka, I., Blumberga, D. Effect of polysorbates on the growth of *Rhodotorula glutinis* in oil rich medium. *Environmental and Climate Technologies*, 2021, vol. 25, no. 1, pp. 1075–1085. doi: 10.2478/rtuect-2021-0081
8. Raita, S., Feldmane, L., Kusnere, Z., Spalvins, K., Kuzmika, I., Berzina, I., Mika, T. Microbial carotenoids production: strains, conditions, and yield affecting factors. *Environmental and Climate Technologies*, 2023, vol. 27, no. 1, pp. 1027–1048. doi: 0.2478/rtuect-2023-0075
9. Spalvins, K, Kusnere, Z, Raita, S. Sustaining a Mars colony through integration of single-cell oil in biological life support systems. *Environmental and Climate Technologies*, 2023, vol. 27, no. 1, pp. 339–367. doi: 10.2478/rtuect-2023-0026
10. Berzina, I., Kalnins, M., Geiba, Z., Raita, S., Palcevska, J., Mika, T., Spalvins, K. Creating single-cell protein-producing *Bacillus subtilis* mutants using chemical mutagen and amino acid inhibitors. *Sientifica,* 2024, Nov. 8968295. 20 p. doi: 10.1155/sci5/8968295

Participation in scientific conferences

1. Spalvins, K., Raita, S., Valters, K., Blumberga, D. Improving single cell protein yields and amino acid profile via mutagenesis: a review of applicable amino acid inhibitors for mutant selection. XII International Conference of Biosystems Engineering, May 5–7, 2021, Estonia, Tartu.
2. Spalvins, K., Raita, S., Blumberga, D. Optimization of yeast cultivation factors for improved SCP production. XV International Scientific Conference of Environmental and Climate Technologies, 11–13 May, 2022, Latvia, Riga.
3. Raita, S., Berzina, I., Kusnere, Z., Kalnins, M., Kuzmika, I., Spalvins K. Herbicide-based selection of mutants for improved single cell protein synthesis: application and procedures. XV International Conference "Biosystems Engineering", May 8–10, 2024, Estonia, Tartu.
4. Berzina, I., Raita, S., Kalnins, M., Kuzmika, I., Spalvins, K. In search of the best technological solutions for creating edible protein-rich mutants: a MCDA approach. XV International Conference of Biosystems Engineering, May 8–10, 2024, Estonia, Tartu.
5. Saronova, J., Raita, S., Spalvins, K. Creation of single cell protein-producing mutants of *Phaffia rhodozyma*. XVII International Scientific Conference of Environmental and Climate Technologies, 15–17 May, 2024, Latvia, Riga.

Supervised and co-supervised bachelor and master's thesis

1. Elizabete Lošmele. Generation of UV mutants in oleaginous microorganisms. Bachelor thesis. RTU, 2022. (In Latvian).

2. Sintija Ozola. Generation of UV mutants in single-cell protein-producing microorganisms. Bachelor thesis. RTU, 2022. (In Latvian).
3. Iveta Kuzmika. Optimization of by-product media and culture conditions for *Phaffia rhodozyma.* Bachelor thesis. LU, 2023. (In Latvian).
4. Baiba Paegle. The creation of astaxanthin-producing mutants of *Phaffia rhodozyma.* Bachelor thesis. RTU, 2023. (In Latvian).
5. Linda Feldmane. Mutagenesis and property analysis of astaxanthin–producing microorganism *Phaffia rhodozyma*. Master's thesis. RTU, 2023. (In English).
6. Jeļizaveta Šaronova. Creation of single cell protein–producing mutants of *Phaffia rhodozyma.* Master's thesis. RTU, 2024. (In English).
7. Liega Krasovska. Assessment of technological developments in yeast-produced proteins and carotenoids. Master's thesis. RTU, 2024. (In English).

1. LITERATURE REVIEW

1.1. Potential of single-cell protein in aquaculture feed

Aquaculture is critical in providing a sustainable food source as wild fisheries are declining due to overfishing and adverse climate and environmental changes. Nowadays, aquaculture supplies more than half of the world's fish for human consumption [29]. To ensure high aquaculture productivity, it must be provided with balanced feed rich in high-quality protein [30], [31]. The quality of a protein source is assessed by amino acid (AA) composition, although its suitability primarily depends on the essential amino acid (EAA) requirements of the target species [32]–[35]. According to FAO, the diet of Atlantic salmon and rainbow trout should contain at least 42–50 % crude protein in dry feed, depending on the life stage [36], [37]. An important requirement for a fish diet is the content and balance of ten EAAs in the protein sources. These are histidine (His), threonine (Thr), arginine (Arg), valine (Val), methionine (Met), tryptophan (Trp), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), and lysine (Lys) [36], [37].

Aquaculture feed generally contains a combination of several protein sources to maintain the required AA balance for the target animal, and adding pure AAs can also be used if necessary [38]. Aquaculture feed formulations use fishmeal, legumes, oilseed crops, animal by-products, crustacean meal, and algal meal [37], [39]. Each of these sources separately has limiting EAAs. Plant-based proteins typically have less Met, Trp, and Lys, below 30 % of the mean fish requirement. Proteins of animal origin, except fishmeal, are usually the limiting are Thr, Met, and Ile [37], [39]. Algal protein contains the lowest concentration of His and Met [40]. In turn, fishmeal is considered a more balanced source of EAAs for the fish diet. Fishmeal is produced from small pelagic forage fish such as mackerel, herring, sardine, anchovy, and only a small portion from fish by-products and wastes. Fishmeal is considered the most nutritious and digestible source of protein for aquaculture feed [28], [32], [41], [42].

Fishmeal was initially used as an effective and inexpensive protein-rich feed ingredient for farmed aquatic species. However, decreased fishing productivity and increased demand for fishmeal caused significant price changes [42]. The price of fishmeal has increased over the past 20 years from 599 €/t in 2013 to 1673 €/t in 2023 and is expected to continue to rise [28], [43]. More than 80 % (86 % in 2020) of fishmeal is used to produce feed for high-value aquaculture species, mainly for farming shrimp, salmon, and marine fish like seabass [28]. Fishmeal is the main component in the formulation of fish feed. For example, rainbow trout feed contains 30–68 %, and Atlantic salmon feeds 28.8–60.5% fishmeal on a dry matter basis, depending on life stage [36], [37]. Expanding the aquaculture sector requires developing additional economically and environmentally viable solutions to meet the growing demand for feed and less dependence on conventional agricultural and marine ingredients. Therefore, for sustainable aquaculture farming, it is necessary to reduce dependence on fishmeal and replace it with alternative protein sources [28], [44], [45].

Single-cell protein (SCP) is a promising alternative to fishmeal and plant-based protein [46], [47]. SCP is dried biomass produced by various microorganisms such as algae, bacteria, fungi,

yeast, and protist, which can metabolise different carbon and nitrogen sources [48], [49]. Moreover, agro-industrial waste is successfully used to produce SCP, which ensures optimal management of food and agricultural organic waste [50], [51]. Nowadays, SCP, in particular biomass of *Chlorella vulgaris, Nannochloropsis gaditana, Schizochyrium* sp., *Saccharomyces cerevisiae, Candida utilis, Kluyveromyces marxianus, Wickerhamomyces anomalus, Spirulina maxima, Methylococcus capsulatus, Methylophilus methylotrophus* and *Methylobacterium extorguens* is successfully used by some companies in the formulation of fish feed [38], [52]. The SCP production technologies have been extensively researched for decades, and the SCP market in 2022 reached a total revenue of $ 13.1 billion [53].

Recent experimental feeding trials have shown that yeast SCP can replace between 15 % and 60 % of fishmeal in fish feed, corresponding to up to 345 g of yeast per kg of feed [46], [60]–[62]. For example, study [55] indicate that *S. cerevisiae* can replace up to 40 % of fishmeal in rainbow trout diets without compromising growth performance, nutrient absorption, or health. The use of *W. anomalus* in the rainbow trout diet at a rate of 20 % improved phosphorus absorption while reducing phosphorus consumption [55]. Interestingly, the appropriate amount of *S. cerevisiae* biomass added to feed depends on the fish species. Thus, brewer's yeast can replace 50 % of fishmeal protein for Sea bass and pacu, 20 % for Sea bream, and 15 % for Nile tilapia without negatively affecting growth. Moreover, there is an improvement in the conversion of feed to live weight [46]. SCP can also be successfully used to feed fish at early life stages. During this period, the fish requires more protein in the feed due to intensive body weight gain [58], [59]. In [58], bacterial SCP replaced 50 % of fishmeal in the feed for rainbow trout fry and improved the feed conversion ratio values compared to standard feed [58].

The main disadvantages associated with the inclusion of SCP in the fish diet in higher quantities are its middling digestibility, high nucleic acid content, and deterioration in the quality of feed pellets [38], [56]. The digestibility of SCP associated with the thick cell walls of microorganisms is increased due to biomass processing. In this case, complete purification from the cell wall is not required since it is rich in bioactive and immuno-stimulating compounds [60], [61]. For example, 200 g/kg of *C. utilis* added to feed containing soybean meal prevents the occurrence of soybean meal induced enteropathy in Atlantic salmon [62].

As many studies show, SCP digestibility depends on the species of yeast, the processing of yeast biomass, and the metabolic characteristics of the target fish species [55], [56], [60]. The content of nucleic acids, namely DNA and RNA, in microorganisms is higher than in a fish or plant-based meal. On average, the content of nucleic acids in fungi is 7–12 %, in bacteria is 15–16 %, in algae is 4–8 %, while in comparison, it is 1.32 % in fishmeal [32], [47], [63], [64]. Interestingly, fish have high activity of the enzyme urate oxidase (uricase), which allows them to catabolise high concentrations of nucleic acids [61], [63]. Atlantic salmon can digest bacterial biomass of 360 g/kg of the weight of the feed, which accounted for 39.96 g of nucleic acids, without negative effects on health [63]. These results are consistent with another study where 36 % bacterial biomass was a good substitute for fish meal in feed formulation for Atlantic salmon. This diet reduced the digestibility of nutrients but increased the feed efficiency ratio and reduced nitrogen and energy losses in fish [65]. It can be assumed that the amount of yeast biomass added to fish feed to replace fishmeal successfully depends on the final concentration

of nucleic acids in the feed. Several studies indicate that the acceptable threshold concentration of nucleic acids is close to 5–10 % in finished fish feed [38], [66]. In turn, in mammals, birds, and reptiles, the activity of this enzyme was lost during evolution, and the existing pathways of nucleic acid catabolism are less efficient. As a result, an excess of nucleic acids in the diet will cause metabolic disturbances and toxicological effects [61], [63]. However, microbial biomass processing methods have been developed to reduce the nucleic acid content in SCP to a safe level for addition to feed and food [67].

In general, the protein and AA profile concentration in microorganisms is mainly genetically predetermined [68]. However, the quantity and quality of single-cell protein in microorganisms can be improved using two strategies: using the biotechnological potential of the selected strain through optimisation of the microbial fermentation process [69]–[71] and improving the protein-synthesising properties of the microorganism through mutagenesis [72].

1.2. Improvement of yeast single-cell protein production through fermentation optimisation

Yeast-dried biomass has relatively high protein, amino acid, energy, and micronutrient content suitable for aquaculture diets [38], [70], [73]–[77]. Yeast has various advantages, including larger size, easier harvesting, and lower nucleic acid content, which reduces the cost of post-treatment compared to bacteria [78]. Yeasts can convert inexpensive, non-edible by-products from the food processing and agricultural industry into high–value protein with limited dependence on arable land, water, or changing climatic conditions [30], [79]. The substrate for the production of SCP should be accessible, nontoxic, abundant, renewable, low-cost, and able to support rapid growth and proliferation of the microorganisms, resulting in high-quality biomass [68], [80]. Common substrates for SCP are represented by fruit wastes [81], molasses [74], dairy industry by-products [82], [83], industry wastewater [84], [85], glycerol [75], natural gas, ethanol, methanol [47], etc.

SCP production yield and productivity strongly depend on culture medium composition, environmental conditions, and selected microorganisms [86], [87]. According to the literature, the main factors that affect yeast biomass growth and SCP content are pH, temperature, time of cultivation, and requirements for carbon, nitrogen, and trace elements [71], [76], [88]–[90]. Microorganisms can respond to environmental changes and the availability of nutrients and trace elements in the fermentation medium. This ability is essential for microorganisms' metabolism, growth, and reproduction. Yeast metabolism is a set of complex enzymatic reactions to substrate components penetrating the cell wall membrane. Therefore, it is crucial to consider that the lack of necessary elements in the fermentation medium or unfavourable conditions turns on the survival mechanisms in cells, which provoke the inhibition of anabolic processes, such as protein synthesis [91], [92]. The protein content in yeast cells can range from 10 % to over 79 % of dry matter (see Table 1.2). Yeast biomass comprises lipids (0.5–8 %), carbohydrates (18–43 %), minerals, and vitamins. Yeast biomass is rich in B vitamins, calcium, phosphorus, potassium, magnesium, copper, iron, zinc, manganese, and selenium [30], [47], [85], [93]. SCP is rich in EAAs, such as lysine, which are limited in most plant and animal

sources. Protein-rich yeast biomass can be used as an additive supplemented to the leading aquaculture and livestock diets instead of other sources, such as fish meal, soybean meal, and other plant-based protein sources [94]–[97].

Selection of yeast species

According to the studies, yeasts of the genus *Candida* are most often used for protein production, accumulating approximately 39–79% of the protein in dry cell biomass. Yeasts, such as *Candida utilis, Candida tropicalis, Candida pararugosa, Candida guilliermondii*, and *Saccharomyces cerevisiae* are capable of utilising various carbon-containing substrates, making them an excellent source of protein [74], [84], [94], [98], [99]. *Yarrowia lipolytica* is another yeast species commonly used for single-cell oil and protein production [75]. Studies report high protein content in this yeast, about 46–71 % [71], [75]. Other yeast species such as *Rhodotorula glutinis*, *Pichia stipitis*, *Blastobotrys adeninivorans,* and *Wickerhamomyces anomalus* show satisfactory results, 30–46 % of protein content [94], [100], [101].

Yeast biomass has been successfully used in fish feed formulations at low and higher inclusion amounts as fishmeal replacement [101], [57]. The nutritional value of yeast primarily depends on the protein content and its AA composition, as well as on the content of lipids, vitamins and minerals [102], [75]. AAs are significant biomolecules that serve as protein building blocks and are intermediates in various metabolic pathways in organisms [103]. The FAO report [104] shows that protein and AA requirements differ for animals. Fish diets have higher protein requirements than mammalian diets, and protein requirements decrease with age [104]. EAAs required in aquatic animal diets are lysine, methionine, and threonine, and these three AAs are limited in plant-based feed, such as soybean, corn, and rice [95]–[97]. SCP is generally well-digested by fish and crustaceans and comprises a protein content and AA profile similar to fishmeal [30], [105]. For example, *C. utilis* protein digestibility for Atlantic salmon (*Salmo salar*) was 88 % in a diet with 40 % yeast biomass [46], and for tilapia fry (*Oreochromis mossambicus*) reached 83.2 % in a diet with 35 % yeast biomass [106].

Table 1.1 summarises the composition of AAs in SCP of yeast biomass compared with other sources. The biomass of yeasts like *C. utilis*, *C. tropicalis*, *Y. lipolytica* and *S. cerevisiae* is rich in lysine, threonine, valine, and leucine [70], [73]–[75]. Although the concentration of EAAs in yeast protein varies between species and strains of the same species and depends on the substrate used in the studies, added trace elements and cultivation conditions [77], [92].

In general, yeasts have lower methionine content than fishmeal; however, some studies show that good results can be achieved under optimal fermentation process conditions. In studies by Gao et al. [74] and Umesh et al. [76], methionine content in yeast species *C. tropicalis* and *S. cerevisiae* was reported to be 2.27 and 3.2 g per 100 g of protein. Among the most commonly used crops, only rice protein comes close to the value of the EAA in fishmeal. Among plant proteins, rice also has a high content of 5.4 % arginine and 3.7 % phenylalanine, and corn has a higher content of 8.8% leucine [96]. Among plant proteins, rice also has a high content of 5.4 % arginine and 3.7 % phenylalanine, and corn has a higher content of 8.8 % leucine. In turn, soybean meal has a higher content of lysine and isoleucine but in a smaller amount compared to fish meal and yeast protein (Table 1.1).

Table 1.1

Essential Amino Acid Composition of Different Protein Sources

Protein source	Essential amino acids content, g/100 g of crude protein									Ref.
	Lys	Thr	Val	Met	Ile	Leu	Phe	His	Arg	
C. utilis	7.8	4.7	4.0	1.0	4.1	7.9	3.4	1.5	4.4	[70]
	5.14	4.1	5.5	1.58	4.8	7.12	4.1	n/a	3.2	[73]
C. tropicalis	6.91	4.35	4.58	2.27	4.00	6.24	3.71	n/a	n/a	[74]
Y. lipolytica	6.2	4.2	4.7	1.4	4.0	7.1	3.9	2.5	4.8	[75]
S. cerevisiae	6.5	4.6	4.9	1.4	3.7	6.4	3.3	2.4	4.7	[75]
	2.5	3.3	3.1	3.2	2.6	2.9	3.0	2.8	1.4	[76]
K. marxianus	n/a	6.94	7.5	0.77	5.48	7.74	3.58	1.9	n/a	[77]
W. anomalus	1.41	n/a	0.96	0.24	0.87	1.36	0.84	0.35	0.87	[101]
	3.06	1.89	1.95	0.33	1.84	2.90	1.63	1.12	2.57	[100]
*P. rhodozyma**	5.82	2.57	3.23	1.00	2.66	4.11	2.38	1.41	3.67	[107]
	4.70	3.90	3.70	1.10	2.90	5.10	2.80	1.70	6.30	[108]
Soybean meal	3.41	1.95	2.61	0.72	2.59	4.13	2.71	1.34	3.86	[109]
Corn protein isolate	1.0	1.8	2.1	1.1	1.7	8.8	3.4	1.1	1.7	[96]
Rice protein isolate	1.9	2.3	2.8	2.0	2.0	5.8	3.7	1.5	5.4	[96]
Fish meal	6.79	3.97	3.93	2.50	3.35	6.25	3.26	1.97	5.23	[46]

Note. n/a is not analysed. * non-conventional SCP yeast.

In addition to SCP production, *Y. lipolytica* is widely used in the production of lipids, erythritol, and citric acid [110]; *S. cerevisiae* for bioethanol and ergosterol production [111] [112]; *Kluyveromyces marxianus* for hydrolytic enzymes such as inulinase, lactase, pectinase, lipase production [113]; *Rhodotorula* sp. for carotenoids such as β–carotene, γ–carotene, torulene and torularhodin [114]; *Candida* sp. for biosurfactants and lipases production [115]–[117]; *P. kudriavzevii*, *B. adeninivorans* and *W. anomalus* for biogas production [101]. Consequently, SCP production can be integrated with the related industry to produce secondary cellular metabolites simultaneously. Additional production of other cellular components synthesised during fermentation is an excellent opportunity to increase the competitiveness of SCP technology [101].

Red yeast ***Phaffia rhodozyma*** was chosen for the study described in the experimental part of the Thesis. *P. rhodozyma*, or its teleomorph *Xanthophyllomyces dendrorhous*, is a well-known carotenoid-synthesising yeast used primarily as a natural astaxanthin supplement in salmonids feed formulations (e.g. Aquasta®, RedStar®) [55], [118]. This yeast has been studied for the last 50 years to develop competitive technology for natural astaxanthin production [119]. Interestingly, *P. rhodozyma* biomass is exclusively a source of astaxanthin but is not considered a protein and oil content. Regulatory documents confirming the use of *P. rhodozyma* biomass in the formulation of aquaculture feeds indicate permissible concentrations of astaxanthin and

safety criteria for the additive. According to European regulation 2015/1415, the astaxanthin content should not exceed 100 mg/kg of finished feed, while the concentration in the *P. rhodozyma* biomass should be higher than 0.5 % [120]. Based on these conditions, the biomass of the yeast *P. rhodozyma* can be used presumably in an amount of about 20 g per 1 kg of finished feed as a colouring component. No studies indicate that this yeast's biomass harms the health of salmonids or other animals. Experimental fish-feeding trials for *P. rhodozyma or X. dendrorhous* biomass are limited above the recommended levels for an astaxanthin-rich supplement. However, it was reported that 100 g/kg of *P. rhodozyma* biomass in the diet provided similar growth rates for rainbow trout compared to an equivalent dose of a brewer's yeast-supplemented diet [108].

The protein content and AA composition of *P. rhodozyma* have been poorly studied. There are several studies from the 80–90s reported that *P. rhodozyma* biomass contains about 25–47 % proteins, a balanced AA composition, and a high number of fatty acids [107], [121], [122]. According to a few studies, *P. rhodozyma* protein has a lower EAA content than conventional yeast SCP (Table 1.1). However, these studies did not aim to improve protein synthesis in *P. rhodozyma* biomass. Therefore, this yeast's potential as a producer of SCP should be further investigated. With satisfactory protein content in the biomass and moderate astaxanthin content (0.02–0.05 %), *P. rhodozyma* can become an excellent source of feed protein for salmon fish. Thus, red yeast biomass can be added to 200–350 g per kilogram of feed, which will partially replace traditional protein sources and provide the necessary level of astaxanthin in the diet of salmonids. This approach to obtaining SCP from *P. rhodozyma* allows for increasing the competitiveness of the technology and developing a new product for the feed market. Thus, it was decided to use *P. rhodozyma* in the experimental study. This study will examine and compare the amino acid profile of the wild strain and acquired mutants with conventional yeast species.

Carbon source

Many agro-industrial wastes and by-products have been used for the production of SCP and other metabolites, including glycerol [75], cheese whey [82], waste milk [83], different fruits peels [81], industrial waste cooking oil [123], salad oil manufacturing wastewater [70], potato processing wastewater [85], [124], olive mill wastewater [84], organic fraction of municipal solid waste [101]. Chosen substrates differ in many aspects, such as composition, structure, texture, complexity, etc. Table 1.2 summarises the biomass and protein content results using different substrates, cultivation conditions, and yeast species.

It is well established that most yeasts use sugars as their main carbon and energy sources; however, some can utilise non-conventional carbon sources such as starch, alcohols, polyols, hydrocarbons, and fatty acids [130]. For example, *Y. lipolytica* can metabolise a limited range of hexose sugars such as glucose, fructose, and mannose. Still, it can utilise acetate, alcohols, and hydrophobic substrates, including oils, alkanes, and fatty acids [110]. The ability of oleaginous yeast to utilise hydrophobic substrates is due to the presence of specific enzymes [131]. Noteworthy, protein biosynthesis in oleaginous yeasts such as *Y. lipolytica, R. glutinis, C. tropicalis* [132] cells is competitive to the lipid accumulation [79].

Table 1.2

Different Substrates Used as a Carbon Source and SCP Content in Different Yeast Species

Yeast species	Carbon substrate	Cultivation conditions			DCW, g/L	SCP, %	Ref.
		Time, h	T, °C	pH			
Saccharomyces cerevisiae	Glycerol	48	28	5.5	n/d	47.9	[75]
	Mango residue	30	30	4.0	15.28	79.1	[69]
Candida utilis	Opuntia ficus–indica HL	50	35	5.0	12.2	14.0	[98]
	Potato wastewater	48	28	5.0	5.65	48.9	[133]
Candida tropicalis	Soy molasses	30	30	5.5	10.83	56.4	[74]
	Sugarcane bagasse HL	96	30	5.0	16.97	60.1	[99]
	Sugar beet pulp	10	30	4.5	16.21	47.8	[94]
Candida pararugosa	Olive mill wastewater	96	30	n/d	21.68	39.4	[84]
Candida guilliermondii	Sugar beet pulp	10	30	4.5	15.5	49.2	[94]
Yarrowia lipolytica	Glycerol	48	28	5.5	n/d	46.7	[75]
	Waste cooking oil	120	28	n/d	57.37	12.6	[123]
	Olive fruits wastes	n/d	30	5.0	14.40	71.0	[71]
Kluyveromyces marxianus	Opuntia ficus–indica HL	50	40	5.0	11.1	10.0	[98]
Rhodotorula glutinis	Potato wastewater and 5 % glycerol	72	28	5.0	19.24	40.5	[87]
Pichia stipitis	Sugar beet pulp	10	30	4.5	19.54	45.6	[94]
Pichia kudriavzevii	Biogas substrate	12–15	30	7.0	7.36	32.7	[101]
Schwanniomyces etchellsii	Olive mill wastewater	96	30	n/d	15.11	35.9	[84]
Blastobotrys adeninivorans	Biogas substrate	12–15	37	7.0	14.83	30.5	[101]
	Spruce sugar HL	28	30	5.0	27.62	42.45	[100]
Wickerhamomyces anomalus	Biogas substrate	12–15	30	7.0	7.03	22.6	[101]
	Spruce sugar HL	24	30	5.0	29.78	41.22	[100]

Note. DCW – dry cell weight (grams per litre of medium); SCP – single-cell protein content (% of DCW); n/d – not defined; HL – hydrolysate.

A single carbon source is often used for microbial cultivation, although using multiple substrates can positively affect biomass yield and improve protein concentration. Generally, microbial metabolism varies significantly when a fermentation medium is presented with mixed carbon substrates compared to a single carbon source, as different nutrients interact in complex ways within the metabolic network [90]. Choosing suitable substrates in the optimal ratio for each species of microorganism. Kurcz et al. [133] found that the addition of 5 % glycerol to the potato wastewater medium increased yeast *C. utilis* biomass and protein yield compared to the glycerol-free medium. Still, when glycerol concentration in the medium is above 10 %, the opposite effect is viewed: biomass yield and protein content decreased. The authors suggest that a higher glycerol concentration inhibits the growth of *C. utilis*. On the other hand, the authors suggest that part of the glycerol assimilated by the yeast was probably used in the biosynthesis of other cell components, which led to a decrease in the proportion of protein components of the yeast biomass as a consequence [133].

Nitrogen source

During protein synthesis, nitrogen is one of the significant factors due to the structure properties of proteins [134]. Different sources of nitrogen, like ammonia, ammonium salt, nitrate, urea, and organic nitrogen in different substrates, such as potato and starch processing waste and cheese whey, are consumed by microorganisms [64], [68], [76]. Yeasts can utilise a range of different inorganic and organic nitrogen sources for incorporation into the cell's structural and functional nitrogenous components, such as AAs, peptides, proteins, polyamines, nucleic acids, and vitamins [91]. While yeast cells can use a variety of nitrogen-containing compounds as the sole nitrogen source, they show a hierarchical preference for those sources. Therefore, the growth rate and the type of synthesised metabolite depend on the quality and amount of available nitrogen [135]. Most yeast strains prefer glutamine or ammonia but use other nitrogen sources with a lowered growth rate [135]. This is because yeasts can use ammonium ions as the sole nitrogen source since they possess genes encoding enzymes for the biosynthesis of all AAs. The ammonium ions supplied as a nutrient or derived from the catabolism of other nitrogenous compounds can be directly assimilated and then serve as donors of the AA synthesis [91].

In the study by Arous et al. [84], the preferred nitrogen source for cultivation of *S. etchellsii* and *C. pararugosa* was ammonium salts (ammonium chloride and ammonium sulfate), in comparison with the addition of potassium nitrate, soy protein, and yeast extract gave 3–8 times lower biomass yield [84]. In another study by Umesh et al. [76], the highest biomass yield and protein content was obtained from *S. cerevisiae* when the medium contained beef extract and yeast extract compared with ammonium nitrate, ammonium sulfate, urea, and sodium nitrate [76]. Between inorganic nitrogen sources preferable for protein production by *C. utilis* were ammonium sulfate, urea, and ammonium chloride compared with potassium nitrate and sodium nitrate [89]. Interestingly, the selection of nitrogen sources can significantly improve the utilisation of xylose by yeast. Wu et al. [136] report that urea efficiently improved xylose consumption by *C. intermedia* in corncob and silver grass straw hydrolysate compared to

ammonium nitrate, ammonium chloride, and diammonium hydrogen phosphate had a negative effect on xylose consumption [136].

Carbon-to-nitrogen ratio

The initial carbon-to-nitrogen (C : N) ratio is a very important factor for substrate reduction, biomass production, and protein content [70], [84], [137]. C : N ratio of 10 : 1 is reported as the most appropriate result as the same ratio is presented in the microorganisms. A higher ratio will cause the disappearance of nitrogen before all carbon is consumed and the required biomass will not be obtained [64]. When nitrogen is limited, yeast cells slow their growth, while in the extreme case of nitrogen depletion, cells stop growing even with all other nutrients available in excess and enter a nitrogen-specific passive state [135]. At a ratio of 1 : 1, most nitrogen cannot enter cells and will be wasted [64]. For oleaginous yeasts, mechanisms are similar, i.e., with distinction at low nitrogen level, yeasts switch the metabolic pathway of protein synthesis to lipid synthesis [138]. Zheng et al. [70] recommended a C : N ratio from 5 : 1 to 8 : 1 for SCP production of *C. utilis* OZ993, at which protein content results in 48–49 %. However, between these ratios, the highest biomass was achieved at a 5 : 1 C:N ratio. On the other hand, lower C:N ratios adversely affected the cellular protein content of *C. utilis*. There was a substantial reduction in the protein level of cells, from 49 % to 18 %, when the C : N ratios gradually declined from 5 : 1 to 1 : 1 [70].

A similar conclusion was achieved in other studies. Arous et al. [84] report that the optimal C : N ratio was 8 : 1–10 : 1 for *S. etchellsii* and *C. pararugosa* with higher biomass production on oil mill wastewater-based medium with ammonium chloride supplementation [84]. In the study by Spalvins et al. [123], the highest SCP content in *Y. lipolytica* biomass was observed at 5 : 1–10 : 1 C : N ratio cultivated on waste cooking oil contained medium [123].

Supplementation of trace elements

An important role in SCP production is the trace elements addition to the fermentation medium. Yeasts require a range of metals for optimal growth, metabolism, and fermentation performance. The requirement for metal ions varies widely among strains. Therefore, the composition of the medium must be adjusted to avoid the inhibitory effects of trace elements on the growth of selected microorganisms [73]. The most requested are magnesium, calcium, potassium, zinc, and iron [74], [88], [130], [134], [139]. Yeast cells are starved for phosphate and sulfur arrest in a quiescent state in which fermentation of glucose is suppressed: external glucose is not depleted [135]. Daskalaki et al. [140] report that *Y. lipolytica* completely assimilates existing nitrogen in the medium within 48 hours; however, when the amount of carbon in the medium is depleted, the addition of nitrogen and magnesium causes an increase in the protein content of the biomass. This mechanism is explained in Dourou et al. [141], where the life cycle of oleaginous microorganisms has been described. After the depletion of the carbon source in the medium or due to a low uptake rate, the oleaginous microorganisms utilise their storage lipids as an energy source for maintenance purposes or as an intracellular carbon source for the production of new lipid-free cell components, provided that essential nutrients are available in the fermentation medium[141].

Gao et al. [74] showed that the addition of $CaCl_2$ in the medium is important for the production of SCP by *C. tropicalis*. The biomass production and total protein content increased when 0.05 g/L $CaCl_2$ was supplemented to soy molasses medium, where the addition of NaCl, $MgSO_4 \cdot 7H_2O$, K_2HPO_4, and $ZnSO_4 \cdot 7H_2O$ in the same amount had no notable effect [74]. In turn, the study by Nicolas et al. [73] provides a more detailed overview of the effect of different salt concentrations on the growth of the yeast *C. utilis*. Mineral salts at KH_2PO_4, $MgSO_4$, $FeSO_4$, and KCl at 0.2, 0.07, 0.002, and 0.8 g/L concentrations significantly increased biomass production [73].

Kieliszek et al. [92] optimised a medium with 0.02 g/L selenium supplementation enriched *S. cerevisiae* and *C. utilis* biomass functional diversity in terms of protein and AA content. Yeasts of both strains enriched with selenium contained a large amount of glutamic acid, aspartic acid, lysine, valine, histidine, and leucine. An analysis of the AA composition of *C. utilis* yeast biomass enriched with selenium showed that the concentration of the Lys was higher (8.3 %) than that of the biomass obtained without adding this element (5.6 %). Moreover, after cultivation in the medium supplemented with selenium, the total AA content for both *C. utilis* and *S. cerevisiae* strains was higher and increased by approximately 12 % and 5 %, respectively. However, the total protein content in the biomass of *S. cerevisiae* and *C. utilis* slightly decreased as compared to that in the control sample without additional selenium supplementation from 48.4 % and 42.1 % to 42.6 % and 37.0 %, respectively [92].

Temperature and pH

Another important factor in successful yeast cultivation is the choice of temperature and pH of medium. According to the literature, the most common temperature used for protein biosynthesis by yeast species is 25–30 °C [71], [75], [84], [123], [142]; however, for some yeasts, such *C. util*is and *K. marxianus* a temperature of 35 °C and 40 °C can be used [98]. Low temperature can inhibit nutrients from crossing the cell membrane, while a high temperature may inactivate enzymes of the metabolic pathway [84]. The optimal pH value for yeast growth is 4.0–7.0 [69], [81], [87], [94]; within this range, the most preferred pH of the medium for protein biosynthesis is 5.0–5.5, as described below.

Siddique et al. [81] report that the optimal conditions for the cultivation of *C. tropicalis* in 2 % watermelon peels hydrolysate medium were 37 °C and pH 7. In the other study by Gao et al. [74], the maximum biomass and protein content of *C. tropicalis* were attained when the initial pH was 5.5 at 30 °C. The highest biomass yield and protein content in Umesh et al. [76] work was obtained from *S. cerevisiae* cultivated at 25 °C and pH 5 compared with the lower results at 15, 37, 45 °C and pH 4, 6, 7, and 8. In the study by Jalasutram et al. [89], the *C. utilis* was cultivated in the pH 3 to 9 range at 30 °C; the maximum protein concentration was obtained at 6 pH and lowest at pH 3, 4, 8, and 9. The authors note that at highly alkaline and acidic cultivation conditions, repression of the enzyme activities is involved in protein production [89].

For *Y. lipolytica* the optimal conditions for protein production were 30 °C and pH from 5.0 to 6.0. In these conditions, the yeast produced 40–50 % of the protein in its dry cell weight cultivated on a medium based on wastes from biodiesel production (vegetable oils, degumming,

and glycerol fractions) [79]. These environmental conditions appropriate for *Y. lipolytica* cultivation in olive fruits waste-based medium is consistent with another study by Rages et al., [71], which observed the highest protein concentration of about 71 %, reached at 30 °C and pH 5. With an increase in pH from 5.5 to 7.5, a decrease in the amount of protein in the biomass was observed. In contrast, media with an initial pH of 6.5 had a higher biomass yield, with 55 % protein content [71]. The study [87] noted that the pH value in the range of 4.0–7.0 did not significantly affect the amount of protein in the *R. glutinis* biomass, which varied from 38.5 % to 41.3 % after 72 h of cultivation. However, maximum biomass production and protein content was obtained at pH 5.0 [87].

Time of cultivation

Zakhartsev et al. [143] described how the temperature of microorganism cultivation affects the kinetics of key molecular processes in the cell, thereby affecting the biomass-specific growth rate. On the other hand, a specific growth rate affects the macromolecular composition of growing microbial cells. For example, in carbon-limited conditions and at a low biomass specific growth rate (μ_{max} <0.1 h^{-1}) *S. cerevisiae* biomass contains up to 50 % of carbohydrate and up to 40 % protein, whereas at high growth rate (μ_{max} > 0.3 h^{-1}), the carbohydrates content linearly decreases to 15% and proteins content increases up to 60 % of the dry yeast biomass [143]. This may explain the high values of the protein content in a short cultivation time as described in the studies [94], [100], were 46–49 % SCP in the biomass of *C. tropicalis*, *P. stipites*, *C. guilliermondii*, and 47–51 % in *C. jadinii*, *W. anomalus*, *B. adeninivorans* were obtained after 10 and 12 h of cultivation, respectively.

One of the highest protein content of 69 % is mentioned in a study by Rages et al. [71], in cultured *Y. lipolytica* for 96 h. However, the biomass was not high, only 13.10 g/L. In the following days of cultivation, the amount of protein decreased, and at the time of the 8th day of incubation, it dropped to 45.63 %. In turn, the maximum biomass was reached on the 6th day of cultivation [71]. In studies by Dharumadurai et al. [80] and Rajendran et al. [142] *S. cerevisiae* reached higher biomass content after 168 h of fermentation, but the maximum protein content was recorded at 72 h of fermentation at a 5 % concentration of pineapple waste hydrolysate and papaw and banana fruit juices, respectively. Other results were obtained in the Umesh et al. [76] work when *S. cerevisiae* was cultivated on papaya waste hydrolysate. Higher biomass and protein content was obtained on 120 h of cultivation. In another study by Kurcz et al. [133], protein content in *C. utilis* dry biomass was higher after 48 h of fermentation in a potato wastewater-based medium. After 72 h it decreased from 43.5 % to 41.7 %, respectively. A similar tendency was observed when glycerol was added to the potato wastewater medium in the amounts of 5 % and 10 %. However, when glycerol was added to the medium in a volume of 15–25 %, the protein content was higher by 72 h, indicating that the protein accumulation time depends on the medium's constituent components [133].

The cultivation time of yeasts is an important parameter for harvesting protein-rich biomass since, in the later stages of fermentation when the maximum biomass is reached, the protein content already decreases. Therefore, it is necessary to perform experimental studies on the

influence of the quantity and quality of the substrate on the biomass growth rate and the rate of substrate consumption in order to assess the optimal time for biomass harvesting properly.

Inoculum size

The size of the inoculum (population of microorganisms or cells that is introduced in the fermentation medium) is another important factor in starting the fermentation process and influencing single-cell protein production [69], [84], [89], although studies describing the effect of different inoculum sizes on protein production are rare. The optimum inoculum size varies for different microorganisms [68] and depends on the total concentration of dissolved oxygen and nutrients in the fermentation medium [84], [89]. For example, in a study by Jalasutram et al. [89], for *C. utilis* inoculum ranging from 2 % to 10 % v/v protein production was increased with the increase of inoculum size with an optimum at 6 %, after which the level of SCP production was decreased. The authors note that cultivated yeast with an initial inoculum concentration of 6 %, dissolved oxygen, and consumed oxygen were at an equilibrium level [89]. Similar results were obtained in Arous et al. [84] when the inoculum size 2 %, 5 %, 7 %, and 10 % effect on the biomass production of *C. pararugosa* and *S. etchellsii* strains were tested. Under optimised cultivation conditions, adding 2 to 5 % inoculum of *C. pararugosa* led to maximum biomass production after 4 days of incubation. 7 % inoculum size addition caused a drop in yeast biomass to approximately 28 % compared to biomass obtained with 5 % inoculum size. Similarly, a slight decrease in the biomass production of *S. etchellsii* was observed when the inoculum size exceeded 7 %; however, there was no significant difference in biomass production of this strain when different inoculum sizes were tested. The authors concluded that large inoculum sizes (more than 7 %) lead to low yeast growth rates and cell survival due to the rapid consumption of nutrients in the medium [84]. In another study by Somda et al. [69], the optimisation of SCP production of *S. cerevisiae* was based on two parameters: a substrate concentration of 5 % to 10 % (g/L) and an inoculum size of 2 % to 12v% (v/v). The highest protein content (79 %) of yeast biomass was achieved using 8 % (v/v) inoculum and medium with 8 % (v/v) mango residue [69].

Thus, to optimise the protein production process, it is necessary to comprehensively consider the influence of different cultivation parameters, considering not only the fermentation medium's composition but also the preferences and characteristics of each yeast species.

Summary

The research results reviewed in this study show that yeast culture conditions such as temperature, pH, and time of cultivation greatly impact the protein content of yeast biomass. The most preferred pH and temperature for yeast SCP production are pH 5.0–5.5 and 28–30 °C. Time of cultivation is an important criterion for harvesting protein-rich biomass since in the later stages of fermentation, when the maximum biomass is reached, the protein content in it already decreases. This feature must be considered and investigated for each selected strain growing on a specific medium composition. Properly selected carbon to nitrogen ratio also has a strong influence on protein content, since the metabolic pathways of yeasts are directly related to the available amount of carbon and nitrogen. According to literature, the optimal C : N ratio

for protein production is 5 : 1–8 : 1. The addition of trace elements also has a positive effect on biomass growth and can affect the amino acid profile. At the beginning of the fermentation process, an important parameter is the size of the inoculum, which can affect the growth rate of the biomass. In conclusion, it should be noted that optimally selected cultivation conditions and skillfully designed culture medium composition allows the production of high protein content with a well–balanced amino acid profile.

1.3. Improvement of single-cell protein production through mutagenesis

Another approach to improve the production of SCP is to create a protein-improved mutant strain. Acquisition of better microorganism strains is very important for developing and improving technological solutions based on SCP production. Microorganisms can be improved using both classical mutagenesis and modern genetic engineering methods combined with advanced screening methods. Although the use of modern solutions is increasing classical mutagenesis and random screening methods are still considered to be simple and efficient for short-term strain development and are still widely used [22], [144]–[147].

Mutations are a natural process that occurs in all types of cells due to the influence of internal or external stimuli [148]. Mutations in microbial cells create new genetic variations that allow them to survive and adapt to rapidly changing environments [149]. Mutagenesis can be defined as the treatment of biological material with a mutagen, which results in an increase of mutation frequency above the level of spontaneous mutations [150]. This process accelerates the mutation frequency rate up to 100 times in the biological material compared to the natural mutation rate [151]. This knowledge has enabled the use of random mutagenesis to improve the productivity of commercially important microbial products (pigments, lipids, enzymes, surfactants, etc.) or increase tolerance to stressful conditions [24], [152]–[155]. Physical, chemical, or biological mutagenesis is widely used [152].

Ultraviolet (UV) irradiation is a widely used physical mutagen [156]–[159]. UV irradiation passes through cells, and both physical and chemical changes occur that can cause damage to cell structures such as membranes, enzymes, DNA, and others [160]. UV light causes mutation where cytosine is modified to thymine by base substitutions at dipyrimidine sites. UV irradiation also causes oxidative stress in cells by inducing the production of reactive oxygen species (ROS). ROS damages cellular DNA and can cause oxidative damage to DNA bases or even causes DNA breaks. It is known that some of these oxidative DNA and nucleotide damages can function as secondary mutagens. Therefore, it is possible to conclude that UV irradiation can induce specific primary DNA mutations and secondary mutations caused by oxidative stress [161]. UV mutagenesis is a relatively simple and effective method for obtaining random mutations in the genome of a microorganism. It is worth noting that each microorganism requires a mutagenesis optimisation step to determine the appropriate ratio of UV intensity to irradiation duration to achieve target cell mortality [162], [163].

Chemical mutagens can be divided into alkylating agents and base analogues [164]. Alkylating substances have a strong reaction with different matters, and working with them

should be done cautiously since they are toxic and have a similar effect to ionising radiation. Examples of such mutagen agents are ethyl methanesulfonate (EMS) and methylnitronitrosoguanidine (MNNG) [165]. Alkylation of genetic material generates triesters that degrade rapidly, producing alkyl groups that interfere with DNA replication. This process includes the hydrolysis of phosphate triesters, resulting in cleavage of the DNA backbone and the alkylation of nitrogenous bases, particularly guanine, which may lead to base-pairing errors during replication [150]. Other commonly used mutagens are base analogue 5-bromouracil and nitric acid. The 5-bromouracil can form a base pair with adenine, one of the DNA bases, but also can unexpectedly convert to an isomer that binds to another nucleotide base – guanine, causing variation in a single DNA base pair, which is called a transition mutation [166]. Nitric acid influences both replicating and non-replicating DNA and can induce the reversal of the mutant to the wild-type strain [167].

Mutagenesis involves treating cells with a mutagen sufficiently long to cause 50–95 % cell death [168]–[171]. It is necessary to find and choose an effective amount of mutagenic agent to mutate the target microorganism. The dose of mutagen may vary depending on the species, the time of mutagenesis, the environment temperature, and the composition of the medium. Therefore, preliminary tests are performed with different mutagen doses and exposure times to determine the optimal treatment of the target microorganism [150], [172]. The next step is to incubate the cells in the dark for 24 hours and then plate them on the preferred selective medium. Improved strains can be selected using screening appropriate to the desired phenotype [152], [173]. Selective agents that exert specific pressure on cells are widely used to select mutants with desired properties. For example, the metabolic inhibitors diphenylamine, β-ionone, and antimycin A are used to select *P. rhodozyma* mutants with improved astaxanthin biosynthesis. Additionally, astaxanthin-producing mutants are selected by visual assessment of the size and colour of colonies on agar [23], [24], [169], [174]–[176]. The fatty acid inhibitors cerulenin, isoniazid, and triclosan are used to select mutants with increased lipid biosynthesis [22], [154], [177]. Additionally, mutants with improved lipid synthesis can be screened using Sudan Black B stained cell microscopy, Nile red fluorescence microscopy, and spectrofluorimetry [154], [178].

Research on using random mutagenesis and selective agents to improve protein synthesis properties is limited. Significant improvements in protein biosynthesis have been reported in *Chlorella* sp. due to exposure to UV irradiation. Interestingly, the study aimed to improve biomass yield and lipid biosynthesis in microalgae, and the increase in protein content was determined by analysing the biochemical composition of the biomass [25]. In another study, UV irradiation was successfully used to create mutants of the bacteria *Bacillus megaterium* with improved lysine biosynthesis [179]. Therefore, it can be assumed that random mutagenesis can be used to create protein-rich mutants. Further screening of mutants must be used since protein synthesis is a non-obvious phenotype, and selecting such mutants is challenging. Analysing the total protein content in all candidates that survived mutagenesis is inefficient because it is time-consuming and labor-intensive [159]. Therefore, using selective pressure on protein synthesis by the AA biosynthesis inhibitors may be a good solution for the effective screening of protein-enhanced mutants.

1.4. Overview of amino acid biosynthesis inhibitors

Most AA biosynthesis inhibitors that are available are used in agriculture as herbicides, and this is also the intended application for most of the amino acid biosynthesis inhibitors that are currently in development. Therefore, the majority of research conducted on using these compounds is regarding their practical and cost-effectiveness in weed management [180]–[183]. Also, most of the research available on these herbicides is done on their inhibitory activity on plant biosynthetic pathways. In contrast, information on their activity on single-celled organisms such as bacteria, microscopic algae, yeasts, fungi, unicellular protists, archaea, etc., is limited. Although the information is lacking, most of the amino acid biosynthesis pathways are very similar, and many enzymes that are the main targets of inhibitors are the same in different kingdoms. Therefore, most of the inhibitors should promote an inhibitory response in microorganisms as well [182], [184]–[187]. One additional difference that might be encountered when applying these herbicides on microorganisms is that even if AA biosynthesis pathway is shared across kingdoms, in plants many AAs are synthesised in plastids, while bacteria and fungi does not have such structures, and the same pathways are localised in the cytosol [182], [184]. This fact most likely will affect the inhibitor concentration required to perform the mutant selection but also might affect some other properties of the inhibitor's effect or promote previously unnoticed side effects such as level of cytotoxicity, level of sensitivity to inhibitory effects, extent of DNA synthesis inhibition, extent of nutrient assimilation impairment, extent of pathway intermediates accumulation, extent of metabolic disruptions etc. [180], [182], [188]–[190]. For example, enzyme from histidine biosynthesis pathway imidazole glycerol-phosphate dehydratase can be inhibited by 2-hydroxy-3-(1,2,4-triazol-1-yl) propylphosphonate. This inhibitor is much more potent in yeasts than in plants, due to additional β-strand which enhances the binding of inhibiting compound to the yeast enzyme [191]. Therefore, inhibitor concentration should be decreased appropriately if used for selection of yeast mutants. Other inhibitors are isolated from microorganisms e.g., cornexistin is isolated from fungi *Paecilomyces variotii*. If this inhibitor is used for selection of related fungal strains, then it might be the case that these microorganisms show resistance to inhibitory effect [192]. Therefore, considering that the use of herbicides in the selection of microorganisms may lead to unpredictable side effects or render the inhibitor completely ineffective, each compound needs to be tested individually during mutant selection.

Ten of the twenty-one AAs found in living beings cannot be synthesised by the fish, these are: arginine, phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine and histidine [193]. Of these ten, four – lysine, methionine, threonine and tryptophan, are present at lower concentrations in plant-derived proteins than recommended in animal and fish feeds [26], [27], [194]. Therefore, if the produced SCP is intended for use in animal or fish feed, during the mutant selection it would be desirable to use AA biosynthesis inhibitors that inhibit the biosynthetic pathways of these four AAs. This way, selective pressure is applied directly to those mutants that can synthesise the relevant four AAs in larger quantities than respective wild types. If SCP is intended for other applications – human diet supplements, other animal and pet feeds, cosmetics, building block chemicals etc., the value of the produced SCP

can be increased also if the concentration of any other AA is increased via utilisation of appropriate inhibitor during the mutant selection. All AA biosynthesis inhibitors and their properties regarding mutant selection have been summarised in Table 1.3.

Table 1.3.

Properties of Amino Acid Inhibitors and their Target Enzymes [72]

Inhibitor	Target enzyme	Target present in	Inhibited AA	Pros (+), cons (–), side effects, etc.
Aromatic amino acid biosynthesis inhibitors				
Glyphosate	5-*enolpyruvyl*-shikimate 3-phosphate synthase (EPSPS)	Plants, archea, bacteria, fungi, algae	Phe, Trp, Tyr	(+) Inhibits microbes (+) Well studied effects (+) Widely available (–) Deregulates carbon metabolism (–) Shikimate accumulation
7-deoxy-sedoheptulase (7dSh)	3-dehydro-quinate synthase (DHQS)		Phe, Trp, Tyr	Cyanobacteria metabolite (+) Inhibits microbes (–) Limited availability
3-indoleacrylic acid	tryptophan synthase (TS)		Trp	Bacteria metabolite (+) Inhibits microbes (+) Widely available
Branched chain amino acid biosynthesis inhibitors				
Sulfonylureas Imidazolinones Triazolopyrimidines Pyrimidinyl (thio)benzoates Sulfonylaminocarbonyl triazolinones	Acetolactate synthase /acetohydroxy-acid synthase (ALS/AHAS)	Plants, archaea, bacteria, fungi	Ile, Leu, Val	(+) Inhibits microbes (+) Well studied effects (+) Widely available (–) Cause intermediates accumulation (–) DNA synthesis inhibition (–) Impaired assimilates transport
Histidine inhibitors				
2-hydroxy-3-(1,2,4-triazol-1-yl) propylphosphonate	Imidazole glycerol-phosphate dehydratase (IGPD)	Plants, bacteria, fungi, archaea	His	(+) Inhibits microbes (+) More potent in yeasts than plants (+) Widely availability (–) Further research required

Inhibitor	Target enzyme	Target present in	Inhibited AA	Pros (+), cons (–), side effects, etc.
3-(diethoxy-phophoryl)-3-(1H-1,2,3-triazol-4-one-1-yl)propan-2-ylcarboxylic esters				(–) Further research required
Monopyrrole aldehydes				(–) Further research required
S-1-(4-biphenyl)-4-(4-imidazolyl)-3-amino-2-butanone (BPIAB)	Histidinol dehydrogenase (HDH)			(–) Inhibitor activity unclear (–) Further research required
Glutamine inhibitors				
L-Phosphinothricin	Glutamine synthetase (GS)	Prokaryoteseukaryotes	Gln, Asp, Pro, Arg, Lys, Met, Thr, Ile	(+) Inhibits microbes (+) Well studied effects (+) Widely available (–) Ammonia accumulation
Methionine sulfoximine				
Tabtoxinine β-lactam				
Bialaphos				
Glutamate-derived amino acid biosynthesis inhibitors				
Cornexistin	Aspartate trans-aminase (AST)	Archaea, bacteria, eukaryotes	Asp, Met, Thr, Ile, Lys	Fungal metabolite (–) No activity in bacteria and fungi (–) Limited availability (–) Further research required
Phaseolotoxin	Ornithine carbamoyl-transferase (OCT)		Arg	Bacterial metabolite (+) Inhibits microbes (–) Limited availability (–) Further research required
Aminomethylene-bisphosphonates (AMBP)	δ1-pyrroline-5-carboxylate reductase (P5CR) and GS		Pro, Gln, Asp, Arg, Lys, Met, Thr, Ile	(+) More potent in bacteria than plants (+) Inhibits microbes (–) Ammonia accumulation (–) Limited availability
Aspartate-derived amino acid biosynthesis inhibitors				
2-(1-cyclohexen-3(R)-yl)-S-glycine (CHG)	Threonine deaminase (TD)	Plants, bacteria, fungi	Ile	(+) Inhibits microbes (–) Limited availability (–) Further research required
DL-Propargylglycine (PAG)	Cystathionine γ-synthase (Cγ-S)	Plants, bacteria, fungi	Met	(+) Inhibits microbes (+) Widely available

Inhibitor	Target enzyme	Target present in	Inhibited AA	Pros (+), cons (–), side effects, etc.
Rhizobitoxine	Cysteine-S-conjugate β-lyase (Cβ-L)	Plants, archaea, bacteria, fungi, animals	Met	Bacteria metabolite (–) Limited availability (–) Further research required
S-(2-aminoethyl)-L-cysteine (AEC)	Aspartate kinase (AK) and dihydrodi-picolinate synthase (DHDPS)	Plants, bacteria, archaea, fungi	Lys, Ile, Met, Thr	(+) Inhibits microbes (+) Widely available
L-α-(2-amino ethoxy-vinyl) glycine (AVG)	DHDPS		Lys	

Aromatic amino acid biosynthesis inhibitors

Aromatic amino acids are phenylalanine, tyrosine and tryptophan [182]. Aromatic AAs are synthesised in the shikimate pathway [195]. This pathway is found in plants, fungi, bacteria, archaea, microscopic algae and other eukaryotes and prokaryotes [196]. One of the pathway enzymes – 5-enolpyruvalshikimate-3-phosphate synthase (EPSPS), is a target for the widely used and commercially available herbicide glyphosate/N-(phosphonomethyl)glycine [197]. Glyphosate inhibits EPSPS, resulting in the synthesis cessation of all three aromatic AAs (Fig. 1.1, A) [184]. Although glyphosate is highly specific for EPSPS, it is suspected that it also causes shikimate accumulation, deregulating carbon metabolism [189]. Almost all species of fungi, bacteria, and algae are sensitive to glyphosate [198]–[200]. Some EPSPS isoforms are not sensitive to glyphosate [190]. While glyphosate-sensitive EPSPS class I is present in most bacteria [195], glyphosate-resistant EPSPS class II has been isolated from certain bacterial species, such as *Ochrobactrum anthropi* [201] and agrobacteria [190]. In general, the specific effect of glyphosate on EPSPS allows it to be used to select a large proportion of SCP-producing bacteria, fungi, and microscopic algae. However, potential adverse side effects and possible resistance may, in some cases, complicate mutant selection.

Brilisauer et al. 2019 reported on isolating a new inhibitor, 7-deoxy-sedoheptulose, from the cyanobacterium *Synechococcus elongates*. This inhibitor targets another shikimate pathway enzyme 3-dehydroquinate synthase (DHQS) (Fig. 1.1, A) [202]. Cyanobacteria treated with this inhibitor could be rescued by adding AAs to the medium, suggesting that the inhibitory effect was caused by AA starvation [182]. This observation suggests that using 7-deoxy-sedoheptulose may be more appropriate for mutant selection, as this inhibitor may not cause as pronounced side effects as glyphosate. The disadvantage of 7-deoxy-sedoheptulose is that it is not currently commercially available and requires chemoenzymatic synthesis and purification in the laboratory [202]. Although 7-deoxy-sedoheptulose has been isolated from cyanobacteria, studies to date have shown that this compound is able to inhibit the growth of plant, yeast, and even other cyanobacteria species [202], suggesting that resistance to this inhibitor is rare and is therefore likely to be effective in the selection of other microorganisms, although this has yet to be tested.

Another enzyme in the shikimate pathway, tryptophan synthase, is inhibited by 3-indoleacrylic acid, thus stopping tryptophan synthesis (Fig. 1.1, A) [182], [203], [204]. 3-indoleacrylic acid is commercially available because it is widely used to induce gene transcription. 3-indoleacrylic acid has been isolated from multiple species of bacteria [205]–[207], indicating that the use of this compound in the selection of some bacteria strains may be limited. In general, 3-indoleacrylic acid inhibition of the growth of bacteria, cyanobacteria and fungi have been reported [207], [208].

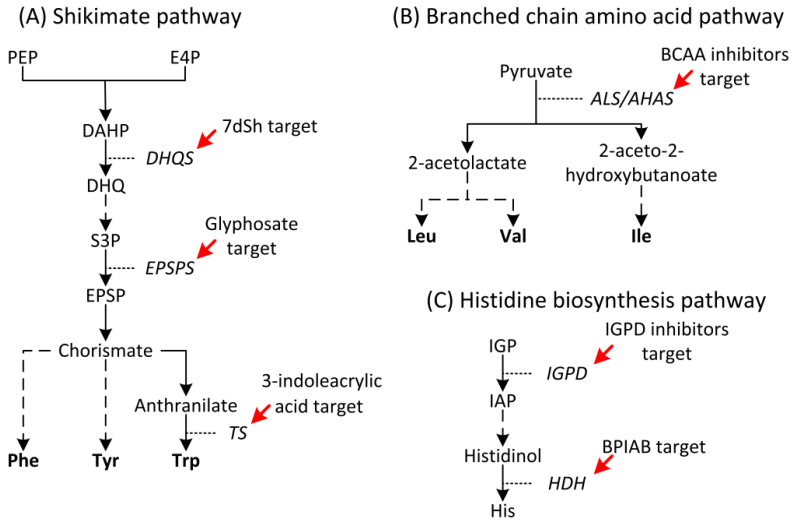

Fig. 1.1. (A) Simplified shikimate pathway, (B) simplified branched-chain AA biosynthesis pathway, (C) simplified histidine biosynthesis pathway.

In shikimate pathway (A), through condensation of phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P), 3-deoxy-arabino heptulosonate 7-phosphate (DAHP) is produced. DAHP is then converted to 3-dehydroquinate (DHQ) and this process is catalised by enzyme DHQ synthase (DHQS). DHQS can be inhibited by 7-deoxy-sedoheptulose (7dSh). Further down the shikimate pathway shikimate 3-phosphate (S3P) is converted to 5-enolpyruvyl-shikimate 3-phosphate (EPSP) and this reaction is catalised by EPSP synthase (EPSPS). EPSPS is target enzyme of popular herbicide – glyphosate. Inhibition of either DHQS or EPSPS causes ceasation of phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) biosynthesis. In tryptophan biosynthesis branch (A) intermediate anthranilate is converted to tryptophan by tryptophan synthase (TS). TS is targeted by inhibitor 3-indoleacrylic acid. In branched chain amino acid (BCAA) pathway (B) acetolactate synthase/acetohydroxyacid synthase (ALS/AHAS) catalyses condensation of pyruvate which produces 2-acetolactate. 2-acetolactate is early precursor of leucine (Leu) and valine (Val) biosynthesis. ALS/AHAS also catalyses reaction where pyruvate is used to produce 2-aceto-2-hydroxybutanoate which intermediate for isoleucine (ile) synthesis. There are various inhibitors available which specifically target ALS/AHAS. In histidine (His) biosynthesis pathway (C) initial step is dehydration of imidazole glycerol-phosphate (IGP) to produce imidazoleacetol phosphate (IAP) which is catalised by IGP dehydratase (IGPD). The last step of histidine biosynthesis is histidinol conversion to histidine by the enzyme histidinol dehydrogenase (HDH). HDH is targeted by inhibitor S-1-(4-biphenyl)-4-(4-imidazolyl)-3-amino-2-butanone (BPIAB). Figure 1.1 and pathway descriptions adapted from [182], [209].

Branched-chain amino acid biosynthesis inhibitors

Leucine, isoleucine, and valine are synthesized via branched-chain amino acids (BCAA) biosynthesis pathway [185]. This pathway is found in plants, bacteria, fungi, archaea, microscopic algae and other microorganisms [182], [185], [210], [211]. Although all three amino acids are synthesized in separate pathway branches, the synthesis of all three amino acid precursors is catalysed by the enzyme acetolactate synthase/acetohydroxyacid synthase (ALS/AHAS), which is also a target enzyme for various inhibitors (Fig. 1.1, B) [182]. Many of these inhibitors are commercially available and can be categorised into five groups: sulfonylureas, imidazolinones, triazolopyrimidines, pyrimidinyl(thio)benzoates and sulfonylaminocarbonyl-triazolinones [212]. In addition to amino acid depletion, all ALS/AHAS inhibitors are characterized by side effects such as branched-chain amino acid biosynthesis pathway intermediate accumulation, inhibition of DNA synthesis, and impaired assimilates transport [188]. These side effects can lead to errors in mutant selection because mutants with increased resilience to side effects might be selected rather than mutants with increased ability to synthesize more BCAAs. Using ALS/AHAS inhibitors, branched-chain amino acid biosynthesis has been successfully inhibited in yeasts [212]–[214], bacteria [215]–[217], microscopic algae [218], [219], moulds [214], [217] and in other microorganisms [220], which indicate that these inhibitors can be used effectively to select mutants with improved SCP production capacity, however, possible selection errors due to existing side effects must also be taken into consideration.

Histidine inhibitors

Histidine biosynthesis occurs in both plants and microorganisms [191], [221]. Although commercial histidine inhibitors are not available on the market, recent findings have reported on inhibitor called 2-hydroxy-3-(1,2,4-triazol-1-yl) propylphosphonate, targeting enzyme responsible for the sixth step in histidine biosynthesis named imidazole glycerol-phosphate dehydratase (IGPD) (Fig. 1.1, C) [182], [191]. Interestingly it has been demonstrated that this inhibitor is significantly more potent in yeasts than in plants [191]. Apart from IGPD inhibition there have been no reports on other side effects caused by 2-hydroxy-3-(1,2,4-triazol-1-yl) propylphosphonate, but it needs to be considered that as this novel inhibitor is further tested some cytotoxic effects might be found. Overall, 2-hydroxy-3-(1,2,4-triazol-1-yl) propylphosphonate is currently the only available inhibitor of histidine biosynthesis that could be used for improved SCP-rich mutant selection.

There have been reports on other inhibitors targeting IGPD, such as 1-(diethoxy-phosphoryl)-3-(4-one-1H-1,2,3-triazol-1-yl)-propan-2-yl carboxylic esters (Jin et al. 2015) and monopyrrole aldehydes [222], but further research is required to confirm their applicability in microorganism mutant selection.

Histidinol dehydrogenase is the last enzyme in histidine biosynthesis, which is targeted by inhibitor S-1-(4-biphenyl)-4-(4-imidazolyl)-3-amino-2-butanone (Fig. 1.1, C) [223]. The activity of this compound on histidinol dehydrogenase is also ambiguous and further studies are needed.

Glutamine inhibitors

Glutamine is the most abundant amino acid in living beings [224]. Therefore, it is reasonable to assume that selecting mutants for their glutamine synthesis capacity might result in discovery of strains with significantly increased total protein contents. Because glutamine is a major precursor in the glutamate-derived amino acid biosynthesis pathway, which results in the synthesis of aspartate, proline, and arginine, inhibition of glutamine synthesis results in the arrest of multiple amino acids biosynthesis (Fig. 1.2, D, E). Further cascading effect of glutamine inhibition will also prevent biosynthesis of aspartate-derived amino acids – lysine, methionine, threonine, and isoleucine (Fig. 1.2, F). Thus, by using only glutamine biosynthesis inhibitors, it is possible to inhibit the synthesis of eight amino acids, of which four are EAAs in animals. Thus, glutamine biosynthesis inhibitors in theory seem to be the most promising inhibitors to be used in the selection of mutants with increased capacity for the production of EAAs and increased protein content in general.

Glutamine biosynthesis occurs in both prokaryotes and eukaryotes [182], [225], [226]. To inhibit the glutamine biosynthetic pathway, all inhibitors target the enzyme glutamine synthetase (GS) (Fig. 1.2, D). GS differs between prokaryotes and eukaryotes, with prokaryotic GS having twelve active sites and eukaryotes ten [181], [227], [228], respectively, and differences in prokaryotic and eukaryotic GS susceptibility to different inhibitors have also been reported [229]–[233]. For example, the GS inhibitor phosphinothricin in soil at a concentration of 1 mM reduced the bacterial population by 40 % and the fungal population by 20 % [230]. Therefore, the effects of the same GS inhibitor may differ significantly from one species of microorganism to another. Several inhibitors are available for GS inhibition, which can be divided into four groups: methionine sulfoximine and its analogues, glufosinate isomer (phosphinothricin) and its analogues, bisphosphonates, and other GS inhibitors [181], [182]. As side effects for most of these inhibitors, ammonia assimilation disorders have been reported in both prokaryotes [229], [234] and eukaryotes [231], [233], [235], [236], which is rational because all these inhibitors target the same enzyme. Ammonia assimilation inhibition [235], [237] might cause errors in selection of mutants since it is likely that mutants with increased resilience to ammonia might be selected instead of those with increased glutamine synthesis capability. Therefore, vigorous testing of GS inhibitors is required for the SCP-producing microorganisms, followed by further analysis of the selected strains for their total protein content and amino acid composition. GS inhibitors have been described in detail by Berlicki [181].

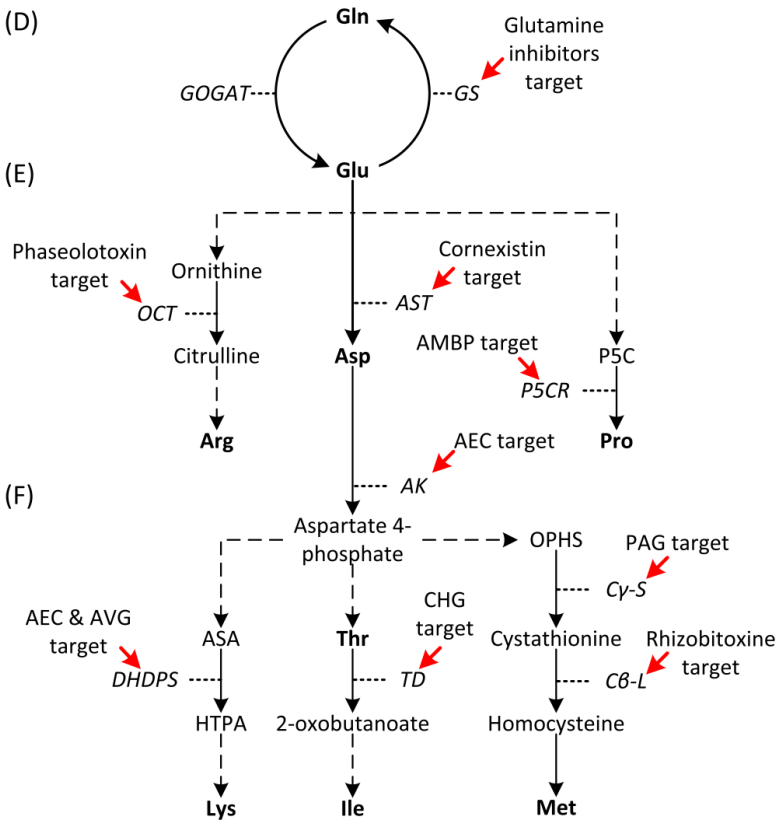

Fig. 1.2. (D) Simplified glutamine biosynthesis pathway, (E) simplified glutamate-derived amino acid biosynthesis pathway, (F) simplified aspartate-derived amino acid biosynthesis pathway.

In glutamine biosynthesis pathway (D), glutamine (Gln) is converted to glutamate (Glu) by glutamine oxoglutarate aminotransferase (GOGAT). Glutamate is converted back to glutamine by enzyme glutamine synthetase (GS), which is target of various GS specific inhibitors (Table 1). In arginine (Arg) synthesis branch from glutamate-derived amino acid biosynthesis pathway (E) one of the intermediate steps involves ornithine convertion to citrulline by ornithine carbamoyltransferase (OCT). OCT is targeted by inhibitor phaseolotoxin. Proline (Pro) is produced from δ1-pyrroline-5-carboxylate (P5C) by P5CR reductase. P5CR is target of various aminomethylene-bisphosphonates (AMBP). Some of AMBP are dual-target inhibitors which target P5CR and GS (D). Aspartate (Asp) is produced from glutamate by aspertate transaminase (AST) which is targeted by fungal metabolite cornexistin. Aspartate-derived amino acid biosynthesis pathway (F) starts with convertion of aspartate to aspartate 4-phosphate by aspartate kinase (AK). AK is targeted by S-(2-aminoethyl)-L-cysteine (AEC). If AK is inhibited it causes ceasation of methionine (met), threonine (thr), isoleucine (ile) and lysine (lys) synthesis. Further down the pathway one of the intermediates of lysine biosynthesis branch called aspartate semialdehyde (ASA) is converted to 4-hydroxy-tetrahydrodipicolinate (HTPA) by dihydrodipicolinate synthase (DHDPS). DHDPS can be inhibited by previously mentioned AEC or L-α-(2-amino ethoxy-vinyl) glycine (AVG). In isoleucine biosynthesis branch threonine is converted to 2-oxobutanoate by threonine deaminase (TD). TD is targeted by 2-(1-cyclohexen-3(R)-yl)-S-glycine (CHG). In methionine biosynthesis branch O-phosphohomoserine (OPHS) is converted to cystathionine by cystathionine γ-synthase (Cγ-S). Cystathionine is then converted to homocysteine by cysteine-S-conjugate β-lyase (Cβ-L). Cγ-S can be inhibited by DL-Propargylglycine (PAG) and Cβ-L can be inhibited by rhizobitoxine. Images and pathway descriptions adapted from [182], [238].

Glutamate-derived amino acid biosynthesis inhibitors

In glutamate-derived amino acid biosynthesis aspartate, proline and arginine are synthesized from glutamate via three separate pathways resulting in respective amino acids (Fig. 1.2, E) [182].

Similarly, as with glutamine inhibition, but to a smaller extent, inhibition of aspartate biosynthesis also affects the production of aspartate-derived amino acids [182]. Thus, by inhibiting aspartate biosynthesis, production of five amino acids – aspartate, lysine, methionine, threonine and isoleucine is prevented, which makes aspartate biosynthesis inhibitors promising candidates for use in SCP-rich mutant selection (Fig. 1.2, E, F). Aspartate biosynthesis is catalysed by an enzyme aspartate transaminase, which is targeted by fungal metabolite cornexistin (Fig. 1.2, E) [239], [240]. Low or no inhibitory activity of cornexistin has been reported in bacteria and fungi [192]. Therefore, cornexistin applicability for SCP-rich bacteria, yeast, fungi and microscopic algae mutant selection needs to be tested for each species of interest. Additionally, cornexistin is not commercially available and it need to be produced and purified in the laboratory [241]. If cornexistin or some other aspartate inhibitor will be proven to be viable for inhibition of aspartate biosynthesis in microorganisms, then this hypothetical inhibitor would be very useful in mutant selection, because, unlike glutamine inhibitors, aspartate inhibitors have not yet demonstrated adverse side effects such as ammonia accumulation.

In arginine biosynthesis one of the enzymes ornithine carbamoyltransferase is targeted by bacterial metabolite phaseolotoxin (Fig. 1.2, E) [242]. Its activity has been demonstrated in *E. coli* [243], but lack of analysis on other microorganisms suggests that similarly as in case with cornexistin, phaseolotoxin applicability for mutant selection needs to be checked on a case-by-case basis.

In proline biosynthesis enzyme δ1-pyrroline-5-carboxylate reductase (P5CR) can be inhibited by aminomethylene-bisphosphonates (Fig. 1.2, E) [244]. In study done by Forlani et al. [245], several of the evaluated bisphosphonates were more potent on bacterial P5CR than on plant P5CR. Fungi and bacteria inhibition has been confirmed in other studies as well [246], [247]. Most of these compounds are not readily available and require synthesis in laboratory [246], [247]. Two of the aminomethylene-bisphosphonates 3,5-dichlorophenylamino-methylenebisphosphonic acid and 3,5-dibromophenyl aminomethylenebis phosphonic acid simultaneously targeted P5CR and GS from glutamine biosynthesis pathway [248]. Both of these aminomethylene-bisphosphonates showed higher potency on GS inhibition than on P5CR inhibition [248] and as discussed previously (see section Glutamine inhibitors), for SCP-rich mutant selection GS inhibition might be preferable. As with other GS inhibitors toxic ammonia accumulation is expected.

Aspartate-derived amino acid biosynthesis inhibitors

Aspartate-derived amino acids are methionine, threonine, isoleucine and lysine (Fig. 1.2, F). In comparison to other herbicides, aspartate-derived amino acid biosynthesis inhibitors have been scarcely studied and no commercial herbicide is currently available on the market to

inhibit any of aspartate-derived amino acids [182]. However, there have been reports on compounds capable of inhibiting certain pathway enzymes [182], [249]–[251].

In isoleucine biosynthesis enzyme threonine deaminase can be inhibited by 2-(1-cyclohexen-3(R)-yl)-S-glycine (CHG) (Fig. 1.2, F) [182], [249], [251]. This herbicidal compound is not commercially available and needs to be synthesised in laboratory [249]. Threonine deaminase inhibition by CHG has also been observed in bacteria [249]. To confirm the use of CHG in SCP-rich mutant selection in the future, its efficacy in inhibiting other microorganisms needs to be tested.

In methionine biosynthesis the enzyme cystathionine γ-synthase can be inhibited by DL-Propargylglycine (Fig. 1.2, F) [250], [251]. It has been demonstrated that plants inhibited by DL-Propargylglycine can be rescued using methionine supplementation, thus indicating that the herbicidal activity comes from amino acid starvation [250], [251]. This observation is also a good indicator on the potential use of this compound in mutant selection. DL-Propargylglycine can be purchased as chemical reagent, thus no synthesis in the laboratory is required [252]. Studies have demonstrated propargylglycine inhibitory activity in microorganisms as well [253]–[256].

Another enzyme in methionine biosynthesis, cysteine-S-conjugate β-lyase, can be inhibited by the bacteria metabolite rhizobitoxine and its analogues (Fig. 1.2, F) [257], [258], but further assessment of its use on microorganisms is required.

In lysine biosynthesis enzyme dihydrodipicolinate synthase can be inhibited by S-(2-aminoethyl)-L-cysteine and L-α-(2-aminoethoxyvinyl)glycine (Fig. 1.2, F) [259]. Both compounds have also shown inhibitory activity in bacteria and fungi and can be used for the selection of mutants with improved lysine accumulation [260]–[264]. Both inhibitors are also commercially available.

In general, the use of aspartate-derived AA biosynthesis inhibitors in mutant selection may facilitate the discovery of new mutants with improved production capacity of EAAs. However, all compounds in this group inhibit only single amino acid, and if the goal is to improve the overall SCP production capacity as well as the ability to synthesise multiple EAAs, then a better approach would be to use either aspartate or glutamine inhibitors.

Summary

In total, six groups of amino acid biosynthesis inhibitors were reviewed in these subchapter, of which glutamine inhibitors are the most promising because it is possible to stop the synthesis of eight amino acids by using only one inhibitory compound. Many glutamine synthetase inhibitors have been introduced into the market, and their activity has been tested on plants, bacteria, fungi, yeasts, and microscopic algae. Therefore, finding the most suitable glutamine synthetase inhibitor to select the mutant microorganisms should be possible. As all glutamine synthetase inhibitors are also causing toxic ammonia accumulation, during selection, it should be taken into consideration that mutants with increased ammonia tolerance might be selected by mistake, and these false positives should be removed later during further mutant strain testing.

Glyphosate is the promising inhibitor for SCP-rich mutant selection, as this popular herbicide inhibits the synthesis of three aromatic amino acids, two of which are essential amino acids in animals. In addition, the effects of glyphosate have been extensively studied in various organisms, making it much easier to predict its effects on mutated microorganisms. As with glutamine inhibitors, glyphosate-induced side effects should also be considered when selecting mutant strains.

Another promising group of amino acid biosynthesis inhibitors is branched-chain amino acid biosynthesis inhibitors, which target acetolactate synthase/acetohydroxyacid synthase (ALS/AHAS). These inhibitors cease the synthesis of three essential amino acids. The effects of these inhibitors have been extensively studied on a wide variety of microorganisms, and ALS/AHAS inhibition can be achieved by using a wide range of inhibitors from five chemically distinct groups. The side effects of these inhibitors should also be considered during mutant selection.

Inhibition of aspartate biosynthesis also results in the arrest of several (five) amino acid syntheses. Unfortunately, only one compound has been found to inhibit aspartate biosynthesis, and it has low activity in both bacteria and fungi, so its use in SCP-rich mutant selection may be severely limited.

1.5. Effect of amino acid biosynthesis inhibitors on microbial growth

Further, it was necessary to analyse the available studies indicating the inhibitory effect of several herbicides (mentioned above) on bacteria and fungi. Investigate the conditions for using herbicides and applicable doses. The main effect of different AA biosynthesis inhibitors for plants, algae, bacteria, yeast, and moulds is similar: to inhibit the enzymatic activity responsible for the biosynthesis of AAs in cells [251], [265]–[269]. These inhibitors can lead to AA starvation, suppression of cell growth, and death at certain concentrations. However, other cellular responses are also observed, such as a complete lack of inhibition or stimulation of growth in some species [270]–[273]. The reasons for such reactions may lie in the individual characteristics of the species or strain of the microorganism and depend on environmental conditions and interactions in the microbiota [269], [274]. For example, the reason for the lack of inhibition may be associated with the activity of efflux pumps that release the herbicide from the cell or with a disruption of the transport system, such as a porin mutation [275]. Stimulation of growth may be caused by the ability of the microorganism to use the herbicide as a source of carbon or phosphorus, which has been described in several studies [273], [276], [277]. Studies show that some soil bacteria such as *Bacillus* sp., *Pseudomonas* sp., *Agrobacterium* sp., and fungi such as *Aspergillus* sp., *Trihoderma* sp. contribute to the biodegradation of herbicides. [276], [278].

The sources of carbon and nitrogen in the medium directly influence the response of microorganisms to the presence of AA biosynthesis inhibitors [274], [279], [280]. Studies report that the inhibitory effect is observed in minimal media containing an inorganic nitrogen source and is absent in protein or AA-rich media. If an inhibited AA is subsequently added to the minimal medium, the inhibition of the microorganism is reversed [274], [279]. Therefore,

minimal media should be used to select protein-producing mutants. Interesting results were achieved in a study of the ability of soil bacterial isolates to degrade the herbicide tribenuron-methyl (TBM). Isolate of *Serratia marcesens* in the presence of glucose, glycerol, or sucrose contributed to the complete degradation of 0.5 g/L tribenuron-methyl in 3 days. The addition of other carbon sources, such as sodium acetate, sodium succinate, sodium citrate, yeast extract, etc. supported the growth of the bacteria but did not affect the degradation of TBM. It was found that the bacteria cannot use the inhibitor as a carbon source, and its degradation is associated with microbial activity of a different nature. It turned out that the degradation of TBM molecules occurred due to acid hydrolysis caused by short-chain fatty acids, fermentation products of glucose, sucrose, and glycerol [280]. This is consistent with the finding of other studies that some herbicide degradation is caused by a pH decrease in a medium due to microbial metabolism [278], [281]. For example, the herbicide primisulfuron is stable in a neutral pH environment but lowering the pH below 6 during fermentation resulted in acidohydrolysis [281]. Herbicides metsulfuron-methyl and chlorsulfuron are sensitive to a decrease in pH. Significant degradation of these herbicides is reported after 15–48 hours in acidic aqueous solutions with a pH value of 2–5 [282]–[284]. In comparison, glyphosate and amitrole are stable to hydrolysis at pH 3–9 [285], [286]. Therefore, the medium must be buffered at neutral pH or, if necessary, used at pH 6–8 when using AA biosynthesis inhibitors. The importance of a media composition and pH stability is highlighted in the study reporting the degradation of chlorsulfuron and metsulfuron-methyl by *Aspergillus niger* in a rich-nutrient buffered media but not in a minimal buffered media [278].

The following Tables 1.3–1.7 summarise the results of the inhibition of bacteria and fungi by AA biosynthesis inhibitors. The tables contain information about thirteen AA biosynthesis inhibitors, a list of bacteria (B) and fungi (F), concentrations of inhibitors, percentage of inhibition, or other effects. Data are summarised for both commercial herbicides and pure active components.

Inhibitory effect of glutamine amino acid biosynthesis inhibitors

L-methionine sulfoximine (MSO) and glufosinate-ammonium (GA) are inhibitors of glutamine synthetase, an enzyme involved in nitrogen metabolism and the synthesis of glutamine, purines, and pyrimidines. Glutamine is a precursor to the biosynthesis pathway of the AAs: arginine (Arg), proline (Pro) and aspartate (Asp), lysine (Lys), methionine (Met), threonine (Thr), and isoleucine (Ile) [287]–[289]. The study [290] suggests that the use of sugars and a nitrogen source is a coordinated process in microbial cells and when ammonium assimilation and glutamine synthesis are impaired, a decrease in the rate of carbon catabolism is a natural outcome.

Despite similar activity, GA appears to be a more effective microbial inhibitor than MSO (Table 1.3). For 50 % growth inhibition of *Mycobacterium tuberculosis*, a concentration of 0.3 mg/L GA was required, while for MSO the concentration was 9.2 mg/L [289]. The fungus was less sensitive to these inhibitors; no inhibition of *A. niger* growth was detected when treated with 360.5 mg/L MSO, although 30 mg/L GA caused significant inhibition, *A. niger* colony diameters decreased by approximately threefold in the presence of GA [291]. Vallejo et al.

[269] assessed the effect of 10 mg/L GA on the growth and metabolism of wine yeast *Saccharomyces cerevisiae* during fermentation.

Table 1.3

Evaluation of Microbial Growth Inhibition by Glutamine Amino Acid Biosynthesis Inhibitors

Herb.	Microorganism	Inhibition		Ref.
		%	Conc, mg/L	
L-Methionine sulfoximine (CAS 15985-39-4)				
B	*Azospirillum brasilense*	100	5	[292]
	Mycobacterium tuberculosis	50	9.2	[289]
F	*Aspergillus niger*	0	360.5	[298]
	Gibberella fujikuroi	Sign.	360.5	[293]
Glufosinate-ammonium (CAS 77182-82-2)				
B	*Mycobacterium tuberculosis*	50	0.3	[289]
F	*Aspergillus niger*	Sign.	30	[291]
	Saccharomyces cerevisiae	55–63	10	[269]

Note. Sign. – significant inhibition. B – bacteria. F – fungi.

The presence of GA slowed down the rate of sugar metabolism, suppressed growth, and extended the lifespan of cells in the stationary phase. Biomass analysis showed an increase in AAs and polyamines in GA-treated cells compared to untreated cells. AAs such as Met, Ile, Leucine (Leu), phenylalanine (Phe), tryptophan (Trp), tyrosine (Tyr), etc. were significantly higher in the treated biomass. At the same time, the values of glutamine and asparagine decreased, and Lys, Arg, Pro, and Asp did not differ from untreated cells. This is one of the first studies to report the adaptation of a microorganism to the damaging effects of a herbicide by increasing the biosynthesis of AAs [269].

Inhibitory effect of aromatic amino acid biosynthesis inhibitor

Glyphosate or N-(Phosphonomethyl)glycine is an inhibitor of aromatic amino acids phenylalanine (Phe), tryptophan (Trp), and tyrosine (Tyr) [294]. Table 1.4 shows the required concentrations of glyphosate to completely inhibit the growth of pathogenic or beneficial intestinal bacteria and several fungi. Concentrations listed refer to the weight of glyphosate active ingredient in the commercial herbicide per liter of media. It is estimated that 75–5000 mg/L of glyphosate may be needed for complete inhibition of bacteria [295] and up to 1000 mg/L for fungi [296]. However, it should be noted that the inhibitory effect of glyphosate is stronger in the commercial formulation [297], [298]. Therefore, when using a pure substance, higher concentrations may be required.

Table 1.4

Evaluation of Microbial Growth Inhibition by Aromatic Amino Acid Biosynthesis Inhibitor

Herb.	Microorganism	Inhibition		Ref.
		%	Conc., mg/L	
Glyphosate (CAS 1071-83-6)				
B	*Bacillus* spp.	100	150–300	[295]
	Bacteriodes vulgatus	100	600	[295]
	Bifidobacterium adolescentis	100	75	[295]
	Campylobacter spp.	100	150	[295]
	Clostridium spp.	100	1200–5000	[295]
	Enterococcus faecalis	100	150	[295]
	Escherichia coli	100	1200	[295]
	Escherichia coli	100	80–160	[274]
	Lactobacillus spp.	100	600	[295]
	Riemerella anatipestifer	100	150	[295]
	Salmonella spp.	100	5000	[295]
	Staphylococcus spp.	100	300	[295]
F	*Geotrichum candidum*	100	1000	[296]
	Penicillium digitatum	100	240	[296]
	Penicillium italicum	100	240	[296]

Note. * Commercial herbicide was used. B – bacteria. F – fungi.

Inhibitory effect of aspartate-derived amino acid biosynthesis inhibitors

S-(2-aminoethyl)-L-cysteine (AEC), L-α-(2-aminoethoxyvinyl)glycine (AVG), and DL-propargylglycine (PAG) are aspartate-derived AA biosynthesis inhibitors (Table 1.5). AEC and AVG inhibit the biosynthesis of methionine (Met), lysine (Lys), threonine (Thr), and isoleucine (Ile) and PAG inhibits Met [72]. The sensitivity of bacteria to the AEC inhibitor varies. Treatment of *Bacillus subtilis* with 1000 mg/L of inhibitor resulted in complete inhibition, while 3000 mg/L caused 50 % inhibition of *Brevibacterium flavum*. Interestingly, the addition of 3000 mg/L L-threonine increased the inhibition of *B. flavum* from 50 % to over 90 % [263]. The effect of AEC on fungi has been poorly studied and there is no data on a 100 % inhibitory dose. Only one study reports that AEC inhibits yeast growth, however, the inhibition rate is too low; 83.3 % of the yeast isolates were resistant to the inhibitor [299]. Presumably, the sensitivity of bacteria to AEC is lower than that of fungi. No inhibition or inhibition of up to 50 % of bacteria was reported when treated with 590 mg/L AVG inhibitor [272]. In comparison, two species of fungi showed inhibition of up to 80 % at 0.3 mg/L commercial AVG inhibitor [253] and at 200 mg/L when using the pure substance [300]. It is worth noting that higher concentrations such as 1 g/L or more were not tested for this inhibitor. Therefore, it is expected that better inhibition may be achieved when using higher concentrations. For complete inhibition of *Fusarium oxysporum* fungi and the enzymatic activity of *Aspergillus flavipes* L-methioninase, about 1 g/L of PAG inhibitor is required [253], [301].

Table 1.5

Evaluation of Microbial Gowth Inhibition by Aspartate-Derived Amino Acid Biosynthesis Inhibitors

Herb.	Microorganism	Inhibition		Ref.
		%	Conc., mg/L	
S-(2-aminoethyl)-L-cysteine (CAS 2936-69-8)				
B	*Bacillus subtilis*	100	1000	[263]
	Brevibacterium flavum	50	3000	[263]
	Brevibacterium flavum	> 90	3000*	[263]
	Escherichia coli	100	1000	[263]
	Escherichia coli	100	1	[302]
F	yeast, the species were not specified	16.7	200	[299]
DL-Propargylglycine (CAS 64165-64-6)				
B	*Porphyromonas gingivalis*	100	6.8	[303]
F	*Aspergillus flavipes*	98**	1130	[301]
	Fusarium oxysporum	61–93	1000	[253]
L-α-(2-Aminoethoxyvinyl)glycine (CAS 49669-74-1)				
B	*Agrobacterium tumefaciens*	0	589.9	[272]
	Bacillus megaterium	34–38	589.9	[272]
	Erwinia amylovora	47–51	589.9	[272]
	Escherichia coli	0	589.9	[272]
	Pantoea agglomerans	0	589.9	[272]
	Pectobacterium carotovorum	0	589.9	[272]
	Pseudomonas spp.	0	589.9	[272]
	Xanthomonas hortorum	0	589.9	[272]
F	*Fusarium oxysporum*	24–81	0.29***	[253]
	Sclerotinia sclerotiorum	70-80	200	[300]

Note. * L-threonine supplementation; ** Enzyme L-methioninase inhibition; *** Commercial herbicide was used; B – bacteria; F – fungi.

Inhibitory effect of branched-chain amino acid biosynthesis inhibitors

Branched-chain AA biosynthesis inhibitors metsulfuron-methyl (MSM), sulfometuron-methyl (SMM), chlorsulfuron (CS), tribenuron-methyl (TBM), etc., belong to the group of sulfonylureas and imazapyr, imazapic, imazethapyr, and imazaquin, etc. belong to the group of imidazolinones. All of them inhibit the three AA isoleucine (Ile), leucine (Leu), and valine (Val). These inhibitors are considered the most effective herbicides required in micro-doses for complete inhibition of vegetation or microorganisms [276], [304], [305]. SMM appears more effective than other sulfonylureases in inhibiting microorganisms (Table 1.6). Inhibition of some bacteria and fungi by SMM has been achieved at relatively low concentrations. CS inhibited significantly against a range of bacteria, but the study was limited to 10 mg/L, and higher concentrations were not tested [306]. Another study used a concentration of 1.1 mg/L

CS, which did not inhibit various bacteria and only caused significant inhibition for *Azospirillum lipoferum* [270]. No studies have been reported on the inhibition of fungi by a CS inhibitor. The inhibitory effect of MSM on bacteria and fungi has been described in several publications (Table 1.6). The sensitivity of fungi to this inhibitor varies greatly. For example, to achieve 50 % inhibition of five yeast species, 5 to 200 mg/L of MSM was required, and for the resistant strain of *Candida mengyuniae* more than 5 g/L [304].

Table 1.6

Evaluation of Microbial Growth Inhibition by Branched-Chain Amino Acid Biosynthesis Inhibitors. Sulfonylureas

Herb.	Microorganism	Inhibition		Ref.
		%	Conc., mg/L	
Metsulfuron-methyl (CAS 74223-64-6)				
B	*Arthrobacter crystallopoietes*	100	50	[277]
	Bacillus subtilis	50	11.9	[216]
	Burkholderia spp.	50	1.19–47.7	[216]
	Mycobacterium avium	> 90	286.0	[307]
	Mycobacterium spp.	100	2.4–9.5	[216]
	Pseudomonas aeruginosa	90	95.3	[216]
F	*Candida mengyuniae* sp. nov.	50	> 5000	[304]
	Candida shehatae	50	10	[304]
	Pichia farinosa	50	200	[304]
	Saccharomyces cerevisiae	50	5	[304]
	Williopsis saturnus	50	200	[304]
Sulfometuron-methyl (CAS 74222-97-2)				
B	*Burkholderia pseudomallei*	50	74.7–182.2	[216]
	Mycobacterium avium	100	218.6	[307]
	Mycobacterium spp.	100	0.6–4.4	[271]
	Pseudomonas spp.	50	22.8–74.7	[216]
F	*Candida albicans*	80	12.5	[308]
	Saccharomyces cerevisiae	80	5	[308]
Chlorsulfuron (CAS 64902-72-3)				
B	*Agrobacterium tumefaciens*	Sign.	0.01–10	[306]
	Azospirillum lipoferum	S/g	1.1	[270]
	Azotobacter chroococcum	0	1.1	[270]
	Azotobacter spp.	Sign.	10	[306]
	Bacillus cereus	Sign.	1–10	[306]
	Bacillus spp.	0	1.1	[306]
	Bacillus subtilis	Sign.	0.001–10	[270]
	Bradyrhizobium sp.	Sign.	0.001–10	[306]

Herb.	Microorganism	Inhibition		Ref.
		%	Conc., mg/L	
	Brevundimonas vesicularis	0	1.1	[270]
	Cronobacter sakazakii	0	1.1	[270]
	Sinorhizobium meliloti	Sign.	0.001–10	[306]
	Enterobacter cloacae	0	1.1	[270]
	Escherichia coli	Sign.	0.1–10	[306]
	Micrococcus luteus	Sign.	0.1–10	[306]
	Mycobacterium avium	85	357.8	[307]
	Mycobacterium spp.	100	11.1–447.2	[271]
	Pantoea agglomerans	0	1.1	[270]
	Pectobacterium carotovorum	Sign.	0.1–10	[306]
	Pseudomonas aeruginosa	Sign.	0.01–10	[306]
	Pseudomonas luteola	S/g	1.1	[270]
	Rhizobium spp.	Sign.	0.001–10	[306]
	Serratia plymuthica	0	1.1	[270]
	Sphingomonas paucimobilis	0	1.1	[270]
	Stenotrophomonas maltophilia	0	1.1	[270]
	Streptomyces griseus	Sign.	0.1–1	[306]
Tribenuron-methyl (CAS 101200-48-0)				
B	*Mycobacterium tuberculosis* (3 strains)	0	49.4	[271]
F	*Alternaria triticina*	50	239.5*	[309]
	Pyrenophora tritici	50	238*	[309]

Note. * Commercial herbicide was used; B – bacteria; F – fungi; Sign. – significant inhibition; S/g – slow growth.

Complete inhibition of bacteria was observed when using 300–400 mg/L imazapyr, while lower concentrations of 26.13 mg/L and 32.66 mg/L imazapyr and imazethapyr had no inhibitory effect (Table 1.7). However, such a small concentration prolonged the generation time of *Bacillus cereus* [270], [273] and *B. circulans* [273]. Four yeast species that showed sensitivity to MSM were less sensitive to imazethapyr. For 50 % inhibition, 100, 25, 40, and 25 times more imazethapyr were required for *Candida shehatae, Pichia farinosa, Saccharomyces cerevisiae*, and *Williopsis saturnus*, respectively [304].

Table 1.7

Evaluation of Microbial Growth Inhibition by Branched-Chain Amino Acid Biosynthesis Inhibitors. Imidazolinones

Herb.	Microorganism	Inhibition		Ref.
		%	Conc., mg/L	
Imazapyr (CAS 81334-34-1)				
B	*Azospirillum lipoferum*	0	26.13	[270]
	Azotobacter chroococcum	0	26.13	[270]
	Bacillus cereus	100	300–400	[273]
	Bacillus spp.	S/g	26.13	[270]
	Brevundimonas vesicularis	0	26.13	[270]
	Cronobacter sakazakii	0	26.13	[270]
	Enterobacter cloacae	0	26.13	[270]
	Mycobacterium spp.	0	32.66	[271]
	Pantoea agglomerans	0	26.13	[270]
	Pseudomonas fluorescens	100	300–400	[273]
	Sphingomonas paucimobilis	0	26.13	[270]
	Stenotrophomonas maltophilia	0	26.13	[270]
Imazethapyr (CAS 81335-77-5)				
B	*Azospirillum lipoferum*	0	28.93	[270]
	Azotobacter chroococcum	0	28.93	[270]
	Bacillus spp.	0	28.93	[270]
	Bacillus subtilis	S/g	28.93	[270]
	Brevundimonas vesicularis	0	28.93	[270]
	Cronobacter sakazakii	0	28.93	[270]
	Enterobacter cloacae	0	28.93	[270]
	Mycobacterium spp.	0	36.17	[271]
	Pantoea agglomerans	0	28.93	[270]
	Serratia plymuthica	0	28.93	[270]
	Sphingomonas paucimobilis	0	28.93	[270]
	Stenotrophomonas maltophilia	0	28.93	[270]
	Candida mengyuniae sp. nov.	50	>5000	[304]
	Candida shehatae	50	1000	[304]
F	*Pichia farinosa*	50	>5000	[304]
	Saccharomyces cerevisiae	50	200	[304]
	Williopsis saturnus	50	5000	[304]
Imazaquin (CAS 81335-37-7)				
B	*Arthrobacter crystallopoietes*	100	300	[277]
	Bacteria (not specified)	100	10	[277]

Note. S/g – slow growth. B – bacteria. F – fungi.

Most of the data in the tables is devoted to inhibiting pathogenic or soil bacteria and fungi. It is reported that pathogenic microorganisms are less sensitive to the effects of AA biosynthesis inhibitors [295]. In summarising the data, concentrations of 0.01–1000 mg/L should be used to determine the complete inhibition of the target microorganism for each inhibitor. Subsequently, the tested concentrations can be reduced and narrowed around those mentioned in the literature to find the minimum concentration for complete inhibition of the target microorganism.

Side-effects and safety of amino acid biosynthesis inhibitors

When considering amino acid biosynthesis inhibitors as a selective substance for screening protein-synthesising mutants, it is important to take into account the possibility of false-positive selection, which characterises the risk associated with the side activity of inhibitors about other internal processes in microbial cells [254], [267], [275], [276], [310], [311]. According to the literature, all analysed inhibitors except AEC can disrupt or inhibit the biosynthesis of various metabolites. For example, GP deregulates carbon metabolism, inhibitors of the sulfonylurea and imidazolinone group inhibit DNA synthesis, and GA and MSO are cytotoxic and promote the accumulation of ammonia in the cell [72]. PT and PAG inhibit the synthesis of polyamines involved in cell proliferation and adaptation to stress factors. PAG also interferes assimilation of neutral AA like Lys [254], [310], [311]. Amitrole inhibits the biosynthesis of ergosterol and catalase [304], [312], [313]. This side activity may cause the microorganism to switch its metabolism to bypass the inhibitory effects of the substance or increase resistance to side effects, e.g. by increasing detoxification activity in the cell without any changes in the activity of biosynthesis of the target AA [267], [275], [276]. With this outcome, these AA biosynthesis inhibitors' use to select protein-producing strains will be less effective for selecting mutants after induced mutagenesis. Because non-target false-positive mutants can be selected with and instead of targeted protein-synthesising mutants, additional screening tools must be used.

The mechanisms of AA biosynthesis inhibitors' action on the metabolic pathways of microorganisms have not been well studied to provide a clear understanding of their use results. The safety of feed containing mutant biomass previously treated with herbicide is controversial. It is known that herbicides such as glyphosate, glufosinate-ammonium, sulfonylurea herbicides, and imidazolinones selectively inhibit the AA biosynthesis pathway in plant, fungal, and insect cells, but not in animals and humans [275], [314]. Studies report these herbicides' absence of mutagenicity and carcinogenicity, but they harm animal health at specific doses [275], [314], [315], [316]. DL-propargylglycine and L-methionine sulfoximine inhibit the AA biosynthetic pathway in animals. On the other hand, this effect of inhibitors can be used in therapy to minimise the consequences of several human diseases [317], [318]. AA biosynthesis inhibitor S-(2-aminoethyl)-Lcysteine hydrochloride is an analogue of lysine and is completely safe for animals [179], [319]. It can be concluded that the herbicide content in edible microbial biomass is not desirable. The safety of such mutants can be confirmed in the absence or acceptable levels of herbicides and their breakdown products in biomass using gas chromatographic analysis or another alternative technique. It is important to note that when using mutagens and AA biosynthesis inhibitors in research, the precautions specified by the manufacturer of the substances should be strictly followed and disposed of properly.

2. METHODOLOGY

2.1. Multi-criteria analysis of amino acid biosynthesis inhibitors for selective screening of protein-improved mutants

This study aimed to compare and find the best AA biosynthesis inhibitors for selective screening of protein-improved mutants. To achieve this, two multi-criteria decision analysis (MCDA) methods, TOPSIS (a technique for order preference by similarity to ideal solution) and AHP (Analytic Hierarchy Process), were used to identify the most suitable herbicides for yeast and bacteria inhibition.

Data collection for the evaluation of AA biosynthesis inhibitors was performed based on the available literature according to the following criteria:

- include AA biosynthesis inhibitors mentioned in Table 1.3 and additionally five–six inhibitors from sulfonylurea and imidazolinone groups;
- include an inhibitory effect on cells or directly on enzymes in vitro of bacteria, yeast, and fungi;
- include concentrations of AA biosynthesis inhibitors with 100 % inhibition;
- include concentrations of AA biosynthesis inhibitors with 50 %, 70 %, 90 % inhibition;
- include concentrations that provide significant inhibition of microbial growth;
- include results from studies using both commercial herbicides and their pure compounds.

Seventeen AA biosynthesis inhibitor data were successfully collected and analysed against four criteria for application to fungi and bacteria inhibition.

TOPSIS

The TOPSIS tool provides an optimal solution by calculating the relative closeness coefficient to the ideal solution, namely, identifying the best alternative depending on the criteria [320]. The advantages of the TOPSIS methodology are that it is the most significant approach to solving real-world problems, it is possible to recognise the proper alternative immediately, it can be used for situations with many alternatives and attributes, and it is suitable for use with quantitative or objective data [321]. Its disadvantage would be that it lacks provision to weigh elicitation, and TOPSIS determines the selected alternative based on its proximity to the ideal solution and the greatest distance from the "negative-ideal" solution; however, it does not consider the relative importance of the distances from these points [321].

The implementation of TOPSIS distinguishes six main steps – identifying the indicator matrix, calculating the normalised matrix, calculating the weighted normalised matrix, calculating ideal and anti-ideal values, calculating the relative closeness coefficient for each alternative and ranking the results [320]. The first step is identifying the indicator matrix. In this step, different alternatives are evaluated according to established criteria. The matrices for AA biosynthesis inhibitors are presented in Appendix 1–2.

TOPSIS method equations 2.1–2.5 are from [320]. A normalisation of these values should be carried out to calculate the criteria values for different alternatives correctly. Afterwards, the values are normalised using Eq. (2.1).

$$r_{ai} = \frac{x_{ai}}{\sqrt{\sum_{a=1}^{n} x_{ai}^2}} \tag{2.1}$$

where

a – alternative, a= 1,..,n;

i – criteria, i=1,...,m;

r_{ai} – normalised criteria value.

The next step is weighting, where a weighted normalised decision matrix is constructed by multiplying the normalised scores r_{ia} by their corresponding weights w_i (see Eq. (2.2)) given by AHP method.

$$v_{ai} = w_i * r_{ai}, \tag{2.2}$$

where

v_{ai} – weighted value;

w_i – criteria weight, ($w_{i1}+w_{i2}+\ldots+w_{im}=1$);

r_{ai} – normalised criteria value.

The fourth step is calculating ideal and anti-ideal values, where each weighted indicator is compared with the maximum and minimum values corresponding to the respective criterion. The sum of the squares of the criteria difference for each alternative from the maximum value is used to determine the total distance of the alternative to the ideal solution. The distance calculation for each action to the ideal solution is shown in Eq. (2.3), and the distance for each action to the anti-ideal solution in Eq. (2.4):

$$d_a^+ = \sqrt{\sum (v_i^+ - v_{ai})^2}, \tag{2.3}$$

where

d_a^+– distance for each action to the ideal solution.

$$d_a^- = \sqrt{\sum (v_i^- - v_{ai})^2}, \tag{2.4}$$

where

d_a^- – distance for each action to the anti-ideal solution.

The last two steps are calculating the relative closeness coefficient for each alternative by using Eq. (2.5) and ranking the final results.

$$C_a = \frac{d_a^-}{d_a^+ + d_a^-}, \tag{2.5}$$

where

C_a – relative closeness to the ideal solution coefficient.

The closeness coefficient is always between 0 and 1, where 1 is the preferred solution [320].

AHP

The AHP tool simplifies complex problems into a hierarchical structure by ranking alternatives through subjective judgment and scientific calculation. It assigns weights to criteria based on pairwise comparisons, assessing the importance of one criterion relative to another. Criteria are compared in pairs on a scale of importance or preference, where the rating is from 1 to 9, where 1 means equal importance, 3 means moderate, 5 means strong, 7 means very strong, and 9 means extreme. Intermediate 2, 4, 6, and 8 values are a compromise rating [322]. The values will be proportionally opposite when comparing criteria in reverse order [323]. Ten experts from our scientific team specialising in biotechnology performed a pairwise comparison of criteria for AA biosynthesis inhibitors. After evaluating the criteria, a normalisation of the matrix has been performed (Eq. (2.6)) [323]:

$$X_{ij} = \frac{C_{ij}}{\sum_{i=1}^{n} C_{ij}}, \tag{2.6}$$

where

C_{ij} – pairwise matrix elements (criteria), i=1, …, m; j=1, …, n.

$\sum_{i=1}^{n} C_{ij}$ – sum of value in a column.

Then, the sum of the columns of the normalised matrix was divided by the number of criteria used to obtain a priority vector according to Eq. 2.7 [323]:

$$W_{ij} = \frac{\sum_{j=1}^{n} X_{ij}}{n} \tag{2.7}$$

where

$\sum_{j=1}^{n} X_{ij}$ – normalised matrix column sum;

n – number of criteria.

The priority vector values can be used as weights for criteria in any multi-criteria analysis. The consistency index (CI) and consistency ratio (CR) are also calculated to validate the obtained criteria weights [322]. In this study, the CR was 0.0275, which is less than 0.10, confirming the consistency of the weights.

Alternatives and criteria

The present study evaluates the following seventeen AA biosynthesis inhibitor alternatives: glyphosate, metsulfuron-methyl, sulfometuron-methyl, chlorsulfuron, tribenuron-methyl, imazapyr, imazapic, imazethapyr, imazamox, imazamethabenz, imazaquin, glufosinate-ammonium, L-methionine sulfoximine, DL-propargylglycine, L-α-(2-aminoethoxyvinyl)glycine, S-(2-aminoethyl)-L-cysteine), and amitrole.

AA synthesis inhibitors were evaluated based on the four criteria: price of the inhibitor, inhibition efficacy, amount of inhibited EAA, and safety (Table 2.1). Analysis of the literature showed that the efficiencies of inhibition of bacteria and fungi differ significantly. Therefore, it was decided to conduct two separate TOPSIS analyses to evaluate inhibitor alternatives properly. Inhibition efficacies were categorised into microdose (0.001–0.009 g/L), low dose (0.01–0.09 g/L), moderate dose (0.1–0.9 g/L), and high dose (1–9 g/L) which corresponded to

the numbers 0, 1, 2, and 3, respectively. Several inhibitors belonging to the same chemical group or inhibiting the same enzyme in the AA biosynthetic pathway are included in the analysis. However, they do not have data on the inhibition dose for bacteria or fungi. These include inhibitory concentrations of imazapyr, imazamox, and imazamethabenz for bacterial assays and S-(2-aminoethyl)-L-cysteine for fungi. Therefore, to include the inhibitors of interest in the analysis, they were assigned dose values based on the group average (Annex 1 and 2).

Table 2.1

Criteria and AHP Weights Used for TOPSI

No.	Criteria	Unit of measure	Weight
C1	Price* of inhibitor	€/100 mg	0.17
C2	Inhibition efficacy	–	0.28
C3	Inhibited EAA	%	0.47
C4	Number of total health and environmental hazards	–	0.08
		Σ	1

*Local chemical distributors provided AA biosynthesis inhibitor prices.

The percentage of inhibited EAAs was calculated based on the importance of specific EAAs, maintaining a value of 100 % for the sum of nine EAAs. The significance of each EAA is based on its availability in conventional protein sources [3], [26], [27], [194]. Thus, Lys, Met, Thr, and Trp are rated as highly important EAAs (16.67 % for each), Val as moderately important (11.12 %), and His, Leu, Ile, Phe (5.55 % each). These EAA importance values are subjective and aim to compare the potential of inhibitors to select more beneficial protein-synthesising microorganism mutants for the feed industry. The last criterion, the safety of inhibitors, was assessed using a scoring system, where 0 is safe, and 1–7 is the total number of health and environmental hazards [324]. This criterion was included in the TOPSIS because it is necessary to consider the potential harm of inhibitors to health during use and utilisation.

2.2. Preparation for the experimental part of the study

Media and solutions preparation

Yeast medium (YM) broth containing 10 g/L glucose, 5 g/L peptone, 3 g/L malt extract, and 3 g/L yeast extract was used to prepare the inoculum. During experiments, *P. rhodozyma* was grown on glycerol-salt medium (GSM) that contained 40 g/L glycerol, 4.83 g/L NH4Cl, 1 g/L $K_2HPO_4 \cdot 3H_2O$, 0.88 g/L $MgSO_4 \cdot 7H_2O$, 0.2 g/L $CaCl_2 \cdot 2H_2O$, 20 g/L $C_8H_5KO_4$, and 1.7 g/L yeast nitrogen base (YNB) without AA, pH 6.0±0.1. GSM was additionally sterilised with a 0.22 µm pore size filter for microplate tests to minimise the risk of sample contamination. Glycerine-peptone-rich medium (GPM) contained 70 g/L glycerol, 32 g/L soy peptone, 10 g/L yeast extract, 1.7 g/L YNB, pH 5.0±0.1. YNB was dissolved in sterile distilled water and sterilised through a syringe filter with 0.22 µm pore size. Sterile YNB stock solution was added at a rate of 10 % to autoclaved media before adjusting the pH under sterile conditions.

Glufosinate-ammonium (CAS 77182-82-2) was purchased from Combi-Blocks (USA). S-(2-aminoethyl)-L-cysteine hydrochloride (CAS 2936-69-8) and L-Methionine sulfoximine (CAS 15985-39-4) were obtained from Sigma-Aldrich (USA). Herbicide stock solutions were dissolved in sterile water, sterilised through a syringe filter with 0.22 µm pore size, and stored at 8 °C until the experiments.

Microorganism strain and inoculum preparation

Yeast culture *P. rhodozyma* DSM 5626 was purchased from the German Collection of Microorganisms and Cell Cultures at the Leibniz Institute. It is a type strain isolated from Japanese beech (*Fagus crenata*) slime flux. The alternative strain numbers in other collections are ATCC 24202, CBS 5905, NBRC 10129, NRRL Y-10921, and IFO 10129. *P. rhodozyma* DSM 5626 strain is an anamorph strain [325] reproducing asexually, such as by budding or fission. This process results in genetically identical offspring. Because an anamorphic organism reproduces without genetic recombination, it tends to maintain stable traits across generations. This stability is beneficial when a specific strain with desired traits is used, ensuring these traits are maintained across successive cultures [326].

Before experiments, the fresh yeast culture was stored at 4 °C on a YM agar plate. To prepare inoculum, one whole 10 µL loop of culture was added to a 50 mL conical flask in a working volume of 20 mL of YM and cultivated for two days at 250 rpm, 22 °C. The following inoculant concentrations were used in the experiment: 1.0×10^6 cells/mL for inoculating a microplate in tests to determine the inhibitory effect of AA biosynthesis inhibitors, 2.0×10^6 cells/mL for an inoculant treated with a mutagen, followed by cultivation in a medium containing one of the inhibitors. Subculture of potential mutants occurred by transferring 20 % of the sample from the test microplate into a fresh microplate containing GSM with the same dosage of the corresponding inhibitor. Subculturing of potential mutants was repeated 2–3 times to strengthen the position of protein-synthesising mutant cells relative to the cells of the wild strain, which could survive the treatment of mutagenesis and inhibitor.

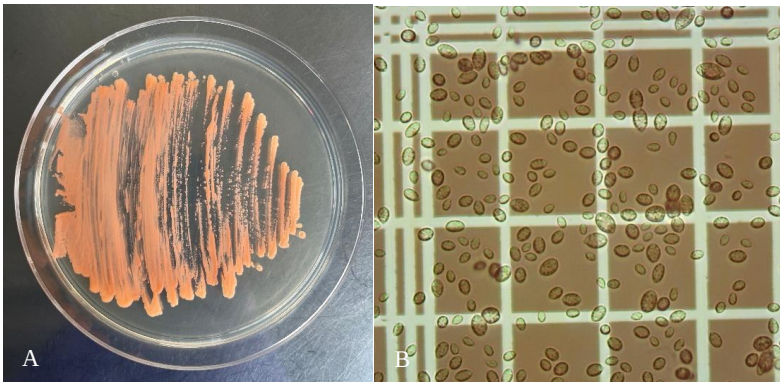

Fig. 2.1. *Phaffia rhodozyma* DSM 5626 (A) on YM agar plate, (B) microscoping the sample at 400× magnification.

Ethyl methanesulfonate mutagenesis

Ethyl methanesulfonate (EMS, Sigma Aldrich) was used for *P. rhodozyma* DSM5626 cell mutagenesis at 4 % concentration. The target concentration of EMS that causes the death of 50–95 % of *P. rhodozyma* DSM 5626 cells was previously determined experimentally by Feldmane [171]. EMS mutagenesis method was performed based on the literature with modifications [24], [151], [171]. The YM broth medium, agar plates, sterile 50 mM potassium phosphate buffer (pH 7.0), and sterile 5 % (w/v) sodium thiophosphate solution should be prepared beforehand. All instruments, materials, and surfaces contacted with EMS mutagen after work should be treated with 5 % sodium thiophosphate to inactivate the mutagen [150], [327]. The preparation of *P. rhodozyma* cells for mutagenesis and subsequent plating was performed as described below:

1. The inoculum was prepared in a 50 mL flask in the YM medium and incubated for 24–48 h at 22 °C.
2. Then, 5 mL of inoculum was transferred to a sterile 50 mL centrifuge tube and the cells were centrifuged for 10 min at 1800 × g, 22 °C. The supernatant was decanted.
3. The cells were washed twice in 5 mL of sterile 50 mM potassium phosphate buffer (pH 7.0). Centrifugation was performed for 10 min at 1800 × g, 22 °C.
4. After washing, the cells were resuspended in 5 mL potassium phosphate buffer supplemented with 209 μL sterile EMS solution. Cells were incubated in this solution for 2 h at 22 °C on an orbital shaker at 250 rpm.
5. 8 mL of sterile 5 % (w/v) sodium thiophosphate solution was added to the tube containing the treated cells to neutralise EMS. Then cells were gently shaked and centrifuged for 10 min at 1800 × g, 22 °C. The supernatant was decanted.
6. Treated cells were washed twice in potassium phosphate buffer as described in step 3.
7. Finally, all cells were resuspended in 15 mL of YM medium and incubated overnight at 22 °C on an orbital shaker at 250 rpm.
8. The next day, the cells were washed twice with potassium phosphate buffer to remove the YM media. Then, cells were suspended with GSM medium, counted, and inoculated into the microplate.

Cell counting in a hemocytometer

Cell counting in a hemocytometer directly counts cells under a microscope after diluting an appropriate sample. This technique ensures an equal amount of inoculum in each experiment and allows for comparing cell numbers before and after the experiment. The samples were viewed under a microscope (micros Austria, MCX100LCD) with a 400× magnification lens on a hemocytometer (Assistant, Neubauer improved, 0.1 mm depth camera). Four squares from the corners and one central square were counted using the “Hemocytometer sidekick” app to calculate the number of cells.

Microplate cultivation

Microorganism inhibition testing at different concentrations of AA biosynthesis inhibitors and mutant screening was performed in 48-well clear bottom microplates (SARSTEDT) in a microplate reader (TECAN Spark®) (Fig. 2.2. A) with an integrated cooling module (Laird Thermal Systems). The microplate was incubated for 6 days at 22 °C and 216 rpm in a humidity cassette (Fig. 2.2.B) to minimise drying out the medium. Optical density (OD) of samples was measured every hour during cultivation at 600 nm and processed with SparkControlMagellan^{TM} software. The OD_{600} measurement evaluates the cell density in the medium and monitors cell growth throughout the culture period. Multiple reads per well (MRW) were used due to the non-homogeneous distribution of cells in the well. The parameters for measurements were as follows: pattern circle (filled), size 6x6, flashes 10, and settle time 300.

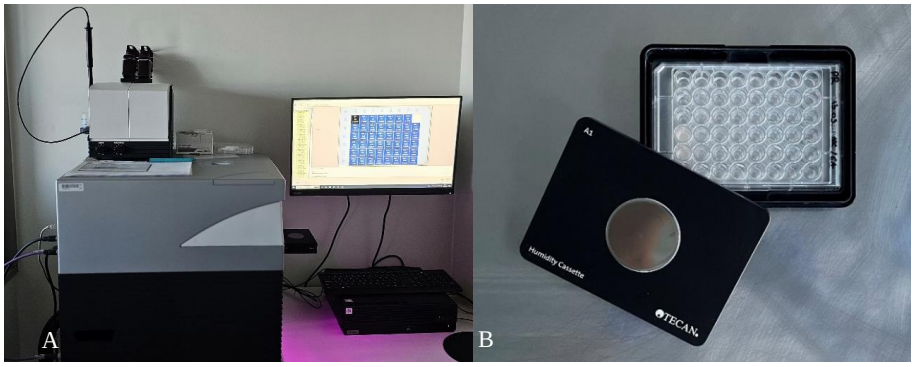

Figure 2.2. (A) Microplate reader (TECAN Spark®), (B) microplate in humidity cassette.

Determination of amino acid biosynthesis inhibitor concentration

Yeast inhibition testing at different concentrations of AA biosynthesis inhibitors was performed in microplates (Fig. 2.3) as described in previous section. The GSM medium was supplemented with 1.0×10^6 cells/mL inoculum and an inhibitor at the appropriate concentration in triplicates. The concentrations of herbicides tested for growth inhibition ranged from 0.1–350 mM for GA, 0.1–50 mM for AEC, and 0.01–50 mM for MSO. Additionally, to OD_{600} measurements, the cells from each sample triplet were microscoped and counted after cultivation. The minimum concentrations of AA biosynthesis inhibitors causing complete inhibition of yeast growth were selected for further screening of mutants.

Mutants screening

Mutagenised *P. rhodozyma* cells at a concentration of 2.0×10^6 cells/mL were inoculated into 48-well microplate containing medium with appropriate inhibitor concentrations. Control samples were a wild-type strain in a medium without an inhibitor (control 1), a wild-type strain in a medium with an inhibitor (control 2), and mutagenised cells in a medium without an inhibitor (control 3). The experiments were carried out at different times. The duration of yeast cultivation ranged from 91 h to 150 h, depending on the growth rate of the mutants in the microplate. Microscopy and cell counting were performed for each sample. Mutants with better

growth than in control wells with the wild-type strain in the inhibitor medium were selected for subculture into a new microplate. A 0.1 mL suspension of mutant cells was transferred to a new microplate containing 0.4 mL of GSM broth with an appropriate inhibitor. The mutants were cultured under the same conditions. After each subculture, the mutants with the highest growth were cultivated in 50 mL flasks with 15 mL YM broth without inhibitor. The cultures in the flasks with the highest and fastest growth were chosen for inoculum for mutant shake flasks cultivation tests.

Shake flasks cultivation

In the first shake flask test, potential mutants and wild-type yeast strains were inoculated at an initial concentration of 1.0×10^6 cells/mL into 20 mL of GSM broth and incubated for eleven days. In the second shake flask test, the inoculant concentration was doubled, and the working volume of the medium was increased to 25 ml GSM broth to obtain sufficient biomass for analysis. The third and subsequent tests were carried out using inoculants of 1.0×10^6 cells/mL in a 25 mL working volume of GPM broth and incubated for seven days. Inoculants were collected and washed twice in sterile water before each test. All shake flask tests were cultured on a rotary shaker (Elmi, DOS-20L) at 250 rpm in 250 mL baffled flasks in triplets at 22 °C. The biomass collected from the flask tests was subsequently used in the analytical measurements described in the next section.

2.3. Analytical methods for biomass evaluation

Determination of culture growth

Yeast growth was characterised by dry biomass determined in grams per litre of medium (g/L) for flask culture. The dry weight of the biomass was measured by the thermogravimetric method by drying the samples at 105 °C every day from the 3rd to the 11th or 7th day of the experiment. One millilitre of culture sample was taken at 24-hour intervals, centrifuged at 10 000 × *g*, 4 °C for 5 min, and the supernatant was removed. The wet biomass was then dried in a 1.5 mL centrifuge tube with the lid open at 105 °C overnight until a stable weight was reached before weighing.

The specific growth rate (μ) was calculated using the dry biomass weight obtained during the exponential phase of yeast growth, according to (2.8) equation [143],

$$\ln(X_t) = \ln(X_0) + \mu t \tag{2.8}$$

where

X_t – grams of yeast after a certain amount of time (g/L);

X_0 – grams of yeast at the beginning of exponential phase (g/L);

μ – specific growth rate (d^{-1});

t – time since the beginning of exponential phase (d).

Determination of protein content in biomass

Bicinchoninic acid assay (BCA) was used to determine protein concentration in dry yeast biomass. The cell lysis method was based on a study [328] with significant modifications. Dried

cells were crushed, and 0.01 g was weighed. One millilitre of MOPS (0.05M) was added to the sample and vortexed for a few minutes. Then, the sample was centrifugated at 16 000 × *g*, 5 °C for 10 min. After 900 µL of supernatant was decanted, 1 mL of NaOH-SDS (0.2M, 2.5 %) was added as well as glass beads (0.5 mm, amount of micro spoon). The sample was vortexed for one minute and placed in a water bath at 100 °C for five minutes. After the bath, the sample was placed in ice water. Then, the required amount of sample was taken and added to distilled water to obtain 750 µg/mL. BCA was performed with a BCA protein Assay kit (Millipore, No. 71285-3). OD measurements were performed in a UV-Vis spectrophotometer (ThermoFisher, BioMateTM 160) using the "Protein BCA" program. The concentration (%) of protein in the sample was calculated using the (2.9) equation,

$$\% = \frac{C \cdot 1000}{750} \cdot 100 \qquad (2.9)$$

where *C* is protein concentration (mg/L).

Biomass hydrolysis and determination of amino acid profile

Hydrolysis of samples: 20 mg to 50 mg of the yeast-dried cells were hydrolysed in 6M HCl/phenol solution at 110 ± 2 °C for 24 hours in so-called "sealed" tubes. Determination of methionine (as a methionine sulfone) and evaluation of cysteine and cystine AAs content (as a cysteic acid) was done using pre-oxidation of the samples for 16 h at 0 °C by performic acid before hydrolysis using the recommended protocol (EC 2009) with minor modification.

Identification and quantification of AAs were done using HPLC-grade reagents: AAs standard mix (product No. A9781, Supelco) and individual standards of methionine sulfone (product No. 64410, Sigma Aldrich) and cysteic acid (product No. 30170, Sigma Aldrich). An automated in-needle pre-column derivatisation of protein hydrolysates was done by orthophthalic aldehyde in the presence of mercaptopropionic acid (OPA/MPA) and 9-fluorenylmethyl chloroformate (FMOC) [329].

HPLC analysis was performed on the HPLC Shimadzu Nexera series using YMC-Triart C18 column (150 mm x 3.0 mm, 3 µm) with a guard column of the same type in gradient mode. The chromatography conditions were done as follows [330]: mobile phases: A eluent acetonitrile: methanol: H_2O = 45:40:15 (v/v/v) and B eluent 20 mM potassium phosphate buffer pH 6.9; flow rate 0.7 mL/min; column temperature – 35 °C, and the volume of the sample injection was 0.3 µL. The gradient elution program was 11 % B for 03min, 22 % for 12 min, 28 % for 14 min, 30 % for 23 min, 65 % for 27 min, 75 % for 34 min, and 100 % for 35 min. Fluorescence UV-Vis detection was done on two channels: Ex. 350 nm and Em. 450 nm (0–29 min); for prolyne Ex. 266 nm and Em. 350 nm (2935 min) [330].

Tryptophan was not determined in the study because it requires different chromatography conditions (compared to other AAs) and protein hydrolysis in an alkaline environment, since during acid hydrolysis (in a 6M HCI environment) it is destroyed [331], [332].

Evaluation of single-cell protein quality

Protein quality was assessed using two important chemical parameters: amino acid score (AAS) and essential amino acid index (EAAI). AAS evaluates the content of individual essential amino acids in SCP relative to a reference protein or dietary requirements of fish (see equation 2.10) [40], [333]. AAS was calculated for nine EAAs for fish diet. Tryptophan was not determined in this study. The reference protein contains all EAA necessary to satisfy the requirements of a target animal, according to the FAO. An EAA with an AAS below 100% is considered limiting [35].

$$\text{AAS}\ (\%) = \left(\frac{\text{mg of AA in 1 g of a test protein}}{\text{mg of AA in 1 g of a reference protein}}\right) \times 100 \quad (2.10)$$

where AA – amino acid.

The EAAI evaluates protein quality through the geometric mean value of the essential amino acid in SCP relative to the reference protein or dietary requirements of fish (see equation 2.11, 2.12) [40], [333].

$$\text{EAAI}\ (\%) = n^{\log(\text{EAA})} \quad (2.11)$$

where

EAA – essential amino acid;

n – the number of amino acids considered for the calculation (without Trp).

$$\log(\text{EAA}) = \frac{1}{n} \times \left[\log\left(100\frac{a1}{a1\text{R}}\right) + \ \ldots\ + \log\left(100\frac{an}{an\text{R}}\right)\right] \quad (2.12)$$

where

a – mg of amino acid in 1 g of a test protein;

aR – mg of amino acid in 1 g of a reference protein;

n – the number of amino acids considered for the calculation (overall 9 EAA).

The protein quality is evaluated according to the following EAAI distribution: 70 % is low, 70 % to 89 % is medium, and above 90 % is high [333], [334].

Biomass hydrolysis and determination of astaxanthin content in biomass

Starting from 7th day (10th day for samples grown on GSM medium) of cultivation 75 µL of each sample was collected in 2 mL tubes to determine astaxanthin concentration in the biomass. Tubes were centrifugated for 20 s while holding down the "Pulse" button, then supernatant was removed (ThermoFisher, Megafuge 16R). Samples were washed two times with 1 mL of distilled water, then stored at –20 °C till astaxanthin determination.

The carotenoid extraction method was based on Luna-Flores et al. [24] with minor modifications. Briefly, dimethyl sulfoxide (DMSO) was preheated in a water bath to 65 °C and 0.5 mL was added to each tube containing 75 µL of biomass sample. Samples were vortexed for 15 s and allowed to incubate in a water bath at 65 °C for 15 min. After this, the samples were shaken, and 0.1 mL of 0.01 M Na_3PO_4 (pH 7) and 1 mL of hexane: ethyl acetate solution in a 1 : 1 ratio were added. Samples were vortexed for 15 s and centrifuged at 14 000 rpm (21 694 × *g*) for 5 min to separate the organic and aqueous phases. The organic phase containing

dissolved carotenoids is on top of the aqueous phase. 600 µL of the organic phase was transferred to a glass cuvette with a lid for further measurement.

Astaxanthin contents in the biomass were determined by measuring the absorbance of the sample at wavelengths of 474 nm and 452 nm using a UV-Vis spectrophotometer (ThermoFisher, BioMateTM 160) "multi-wavelength" program. A solution of hexane and ethyl acetate in a 1:1 ratio was used for a blank measurement. After measuring all samples, the amount of astaxanthin and β-carotene in the organic phase of the sample was calculated using equation (2.13 and 2.14) [335]:

$$C_A = \frac{\varepsilon_{B,474} \times Abs_{452} - \varepsilon_{B,452} \times Abs_{474}}{[\varepsilon_{B,474} \times \varepsilon_{A,452} - \varepsilon_{B,452} \times \varepsilon_{A,474}]} \quad (2.13)$$

$$C_B = \frac{Abs_{452} - (\varepsilon_{A,452} \times C_A)}{\varepsilon_{B,452}} \quad (2.14)$$

where

C_A – astaxanthin concentration in the organic phase of the sample (M);

C_B is β-carotene concentration in the organic phase of the sample (M);

Abs_{452} – absorption at wavelength 452 nm;

Abs_{474} – absorption at wavelength 474 nm;

$\varepsilon_{B,452}$ – molar absorption coefficient of β-carotene at 452 nm (65 347 $mol^{-1}cm^{-1}$);

$\varepsilon_{B,474}$ – molar absorption coefficient of β-carotene at 474 nm (54 514 $mol^{-1}cm^{-1}$);

$\varepsilon_{A,452}$ – molar absorption coefficient of astaxanthin at 452 nm (74 179 $mol^{-1}cm^{-1}$);

$\varepsilon_{A,474}$ – molar absorption coefficient of astaxanthin at 474 nm (86 147 $mol^{-1}cm^{-1}$).

Statistical analysis

The presented data for biomass OD_{600} and dry weight, total protein, astaxanthin, and total carotenoid content are the means of three replicates. The amino acid composition of yeast protein is calculated as the mean of two replicates. OD_{600} value results were statistically analysed by a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. The results of the biomass, protein, astaxanthin, and amino acids were statistically analysed through a one-way ANOVA followed by Tukey's honestly significant difference (HSD) test using SPSS software (version 29.0). Correlation analyses were performed using Real Statistics Resource Pack for Excel (version Rel 8.9.1) using Spearman correlation analysis for non-normally distributed data and Pearson correlation analysis for normal data. Means with p-values less than 0.05 were considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Results of multi-criteria analysis of amino acid biosynthesis inhibitors

The effect of herbicidal treatment of microorganisms has not been well studied, and the available data do not provide clear answers. Studies evaluating the effect of AA biosynthesis inhibitors on the viability of rhizosphere microorganisms [276], [280] plant pathogens [267], and important food microorganisms [269], [297], [298] etc., were used for MCDA. It was found that the effect of AA biosynthesis inhibitors on the growth of fungi (yeast and mould) and bacteria in the rhizosphere is ambiguous and depends on the strain of the microorganism, the type of herbicide, and its formulation [298], [304]. Studies show that herbicides can be highly inhibitory to microorganisms at low dosages [271], [289], [302], weakly inhibitory at high dosages [299], growth stimulating [276] or having no effect [291], [307]. Inhibitory efficacy was assessed separately for bacteria and fungi (yeast and mould). Bacteria are generally more sensitive to AA biosynthesis inhibitors than fungi, although this does not apply to all inhibitors [336].

Therefore, categorisations were used for potently inhibitory or lethal concentrations to evaluate and compare the efficacy of bacterial and fungal inhibition. Bacteria were more sensitive to metsulfuron-methyl (MSM), sulfometuron-methyl (SMM), glufosinate-ammonium (GA), L-methionine sulfoximine (MSO) and DL-propargylglycine (PAG), where complete inhibition was achieved at micro-doses [254], [271], [289], [291], [303], [304]. According to the literature, chlorsulfuron (CS) completely inhibited bacterial growth at a low dose [270], [271]. Phaseolotoxin inhibited 97 % of the target bacterial enzyme (ornithine carbamoyl-transferase) involved in arginine biosynthesis at a low dose [270], [271], [337]. Glyphosate (GP), imidazolinones, L-α-(2-aminoethoxyvinyl)glycine (AVG), S-(2-aminoethyl)-L-cysteine (AEC) inhibited bacterial growth at medium doses, and amitrole at high doses [270]–[272], [274], [300], [302], [338], [339]. Fungi are more sensitive to sulfonylureas such as CS and SMM, which have a strong inhibitory effect at low concentrations. However, tribenuron-methyl (TBM) and MSM require a moderate dose [297], [304], [308]. Also, GA, MSO, AVG, AEC, and amitrole at moderate doses inhibit fungal growth by 80–100 % [291], [300], [308], [312], [338], [340], [341]. GP (the active ingredient of a commercial herbicide) and PAG seem to be less effective against fungi; they will be required in high doses [253], [296], [301], [342].

GA and MSO are capable of inhibiting up to 40 % of all AAs (Gln, Asp, Pro, Arg, Lys, Met, Thr, Ile), AVG and AEC up to 20 % (Met, Lys, Thr, Ile), GP (Phe, Trp, Tyr), sulfonylureas, imidazolinones slightly less up to 15 % (Ile, Leu, Val for both), and up to 5 % PAG (Met), PT (Arg) and amitrole (His). The evaluation of the percentage of inhibited EAAs showed the highest value for GA, MS, AVG, and AEC (56 %), the average value for GP and inhibitors from the group of sulfonylureas and imidazolinones (22 %) as well as PAG (17 %), amitrole received the lowest value (6 %) (see Annex 2, 3).

The safety of inhibitors or health and environmental hazards [324] received the lowest expert weight compared to other criteria – 0.08. This may be due to the experts' experience

working with such substances, the presence of the necessary laboratory equipment and personal protective equipment, and the practice of handling hazardous substances for disposal to a special company. Thus, using all the required precautions reduces the potential harm of inhibitors to a minimum and, as a result, has lower weight when assessing the criteria by experts.

Figures 3.1 and 3.2 represent TOPSIS results of inhibitors selected for bacteria and fungi. The first three ranks are AEC, MSO, and GA for yeasts and fungi. This priority may be because these inhibitors are leaders according to highly weighted criteria: they inhibit the largest amount of EAA. The prices of these inhibitors are 10.86–33.86 EUR/100 mg, which is much lower than 117.94 EUR/100 mg for imazamox or 2231.72 EUR/100 mg for L-α-(2-aminoethoxyvinyl)glycine.

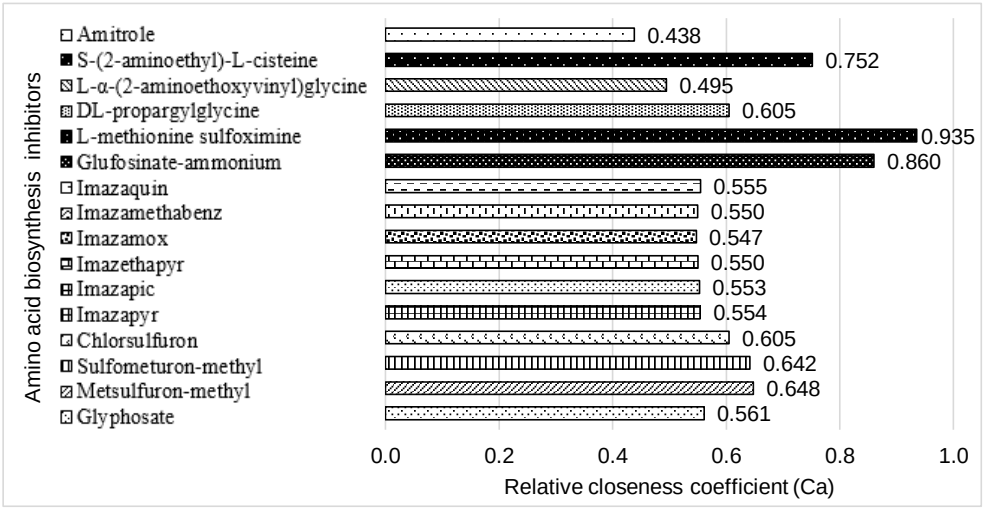

Fig. 3.1. TOPSIS results of amino acid biosynthesis inhibitors for bacteria.

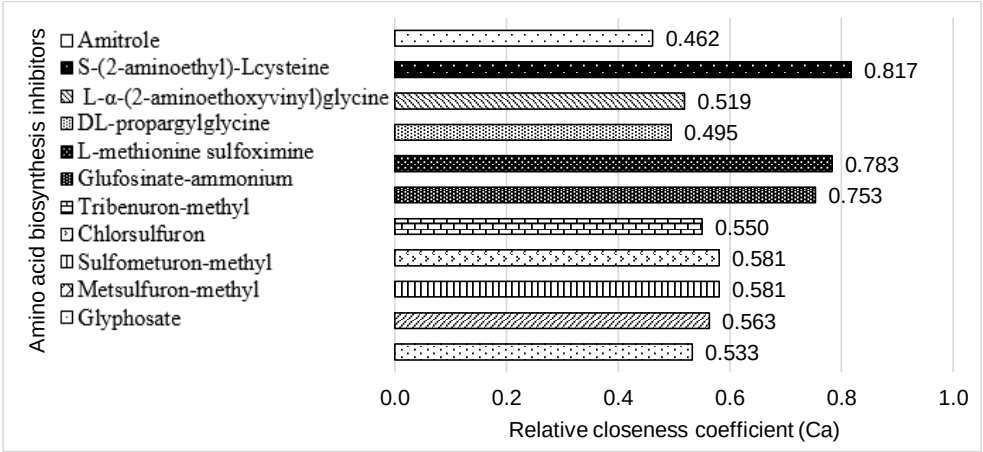

Fig. 3.2. TOPSIS results of amino acid biosynthesis inhibitors for fungi.

The results of sulfonylureas and GP for fungi reached 0.533–0.581, significantly worse than those of the tree inhibitors mentioned above. For bacteria, the results of sulfonylureas are slightly higher than those of imidazolinones, GP, and PAG, amounting to 0.561–0.605 unitary variation ratio. Therefore, using these inhibitors to inhibit bacteria to select mutants with increased synthesis of three EAAs, Ile, Leu, and Val is more appropriate. Close to the worst solution was amitrole, which reached average values of 0.438 for bacteria and 0.462 for fungi.

Although studies on the effects of herbicides and their active components on bacteria and fungi are limited, the MCDA results highlight potential inhibitors for further studies. It is necessary to understand the possibility of using AA biosynthesis inhibitors to select protein-producing strains after induced mutagenesis and the effectiveness of this method, considering the risk of false-positive selection. It is worth considering that the selected microorganism may be insensitive to a particular inhibitor. Therefore, creating a database combining industrially important microorganisms and the results of their successful inhibition or insensitivity to potential inhibitors or herbicides is advisable. To increase the amount of inhibited essential amino acids and the efficiency of inhibition, some inhibitors can be combined in a selective medium. From this perspective, the GP + MSM and GP + PAG combinations look more advantageous for selecting bacteria and fungi. Thus, combined inhibition would affect Phe, Trp, Tyr + Ile, Leu, Val, and Phe, Trp, Tyr + Met biosynthesis.

3.2. Experiments on growth inhibition of yeast *Phaffia rhodozyma*

Three AA biosynthesis inhibitors S-(2-aminoethyl)-L-cysteine (AEC), glufosinate-ammonium (GA), and L-methionine sulfoximine (MSO) were selected as the best herbicides for yeast *P. rhodozyma* inhibition based on MCDA results. In the present study, *P. rhodozyma* wild-type yeast was grown on GSM medium containing these three herbicides separately to determine concentrations with 100 % inhibition for each. GSM media with an inorganic nitrogen source was selected for yeast because an organic nitrogen source could counteract the inhibitory effects of herbicides [274], [279].

Effect of S-(2-aminoethyl)-L-cysteine on the *Phaffia rhodozyma* growth

Seven concentrations from 0.1 to 50 mM of the AA biosynthesis inhibitor AEC were tested on the wild-type strain *P. rhodozyma* DSM 5626 to determine the degree of inhibition. This inhibitor had a strong inhibitory effect on yeast (Table 3.1). The optical density results showed that an AEC concentration of 0.1 mM significantly inhibited the growth of *P. rhodozyma* and concentrations of 0.5 mM and higher reduced the OD by 2.5–3 times compared to the control without an inhibitor. Statistical analysis of the OD results showed that the mean value of the control sample was statistically significantly different from the samples with the AEC inhibitor, while there was only one sample between the concentrations of inhibitors with statistical differences. To confirm complete yeast inhibition, each sample was microscoped and cell proliferation after the last OD reading at 116 h was checked. Then the number of cells was counted for one sample from each triplet (Fig.3.3).

Table 3.1

OD Values of *P. rhodozyma* at Different Concentrations of the AEC Inhibitor

Sample	AEC conc., mM	Cultivation time, h					
		0	24	48	73	96	116
Control	0[a]	0.069	0.231	0.467	0.612	0.723	0.818
AEC1	0.1[b]	0.051	0.189	0.378	0.472	0.472	0.463
AEC2	0.5[c]	0.048	0.133	0.202	0.287	0.35	0.321
AEC3	2.5[c]	0.047	0.114	0.155	0.21	0.27	0.294
AEC4	5[c]	0.048	0.115	0.154	0.203	0.262	0.289
AEC5	7.5[c]	0.048	0.114	0.153	0.203	0.265	0.311
AEC6	10[c]	0.049	0.117	0.157	0.206	0.268	0.304
AEC7	50[c]	0.046	0.108	0.146	0.19	0.239	0.272

Note. Different letters indicate statistical significance among AEC concentrations according to Duncan's multiple range test at the 5 % level ($p < 0.05$).

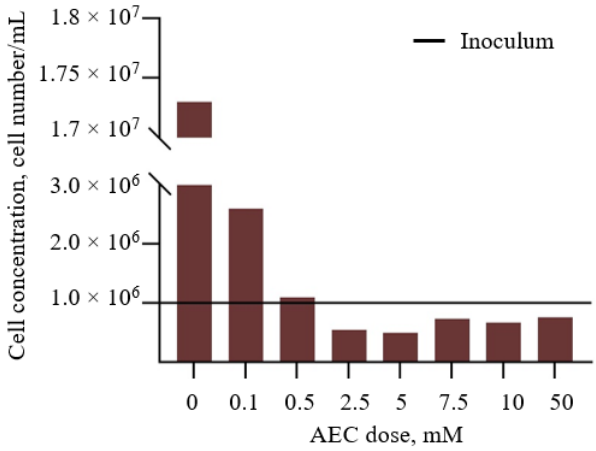

Fig. 3.3. Number of cells in samples containing different AEC concentrations after 116 h of cultivation. The horizontal line represents the initial concentration of cells in the samples.

The number of yeast cells after 116 h of cultivation in the 0.1 mM AEC sample was more than twice as high, whereas in the 0.5 mM AEC sample, it was equal to the initial inoculum, and cell budding was not observed. A reduction in the initial number of cells by half with the presence of autolysed cell particles observed in samples with a concentration of 2.5 mM and higher. The Spearman correlation analysis showed a statistically significant correlation between the last OD reading and the counted cell amount ($p < 0.05$). AEC concentrations of 0.5 mM and 2.5 mM were chosen for further experiments to select potential protein-synthesising mutants capable of reproduction in the presence of 100 % inhibitory doses. The effect of the AEC inhibitor on microorganisms has not been well-studied. Study [263] reported about 100 % inhibition of *Bacillus subtilis* and *Escherichia coli* by adding 5 mM AEC to the medium, whereas 50 % inhibition of *Brevibacterium flavum* required 15 mM AEC.

Effect of glufosinate-ammonium on the *Phaffia rhodozyma* growth

The GA inhibitor was tested twice. The first test comprised GA concentrations from 0.1 mM to 100 mM, of which significant inhibition occurred at the two highest concentrations (Table 3.2). Lower concentrations of 0.1–1 mM did not have statistically significant inhibition on yeast. In turn, 5–10 mM up to 96 h of cultivation caused growth inhibition, but by the end of the test, the OD was higher than that of the control. Microscopying of samples and counting cells revealed an interesting feature. The number of *P. rhodozyma* cells was higher in samples containing GA at concentrations from 0.1 mM to 10 mM relative to the control (Fig. 3.4). The growth-promoting effect of herbicides on some microorganisms has been described in numerous studies. Glyphosate, chlorsulfuron, imazaquin, and imazapyr have been reported to stimulate microbial growth at certain concentrations, but there is limited data on glufosinate-ammonium [273], [276], [306], [343], [344].

Table 3.2

OD Values of *P. rhodozyma* at Different Concentrations of the GA inhibitor

Sample	GA conc., mM	Cultivation time, h							
		0	24	48	73	96	120	144	165
Control	0[a]	0.006	0.075	0.349	0.623	0.738	0.819	0.876	0.916
GA1	0.1[a]	0.012	0.075	0.35	0.614	0.715	0.781	0.828	0.875
GA2	0.5[ab]	0.038	0.055	0.232	0.57	0.706	0.776	0.823	0.859
GA3	1[ab]	0.045	0.047	0.181	0.525	0.716	0.802	0.847	0.890
GA4	5[bc]	0.043	0.034	0.105	0.281	0.605	0.749	0.853	0.921
GA5	10[c]	0.039	0.031	0.081	0.201	0.481	0.699	0.855	0.956
GA6	50[d]	0.016	0.027	0.043	0.075	0.141	0.219	0.303	0.364
GA7	100[d]	0.012	0.026	0.036	0.046	0.059	0.081	0.104	0.121

Note. Different letters indicate statistical significance among GA concentrations according to Duncan's multiple range test at the 5% level ($p < 0.05$).

Interestingly, *P. rhodozyma* is more resistant to GA than *Saccharomyces cerevisiae* and *Aspergillus niger*, for which the presence of 0.05 mM and 0.15 mM GA, respectively, caused significant growth inhibition [269], [291]. The effect of GA on the metabolism of wine yeast *S. cerevisiae* during fermentation was studied by Vallejo et al. [269]. The presence of GA slowed down the rate of sugar metabolism, suppressed growth, and extended the lifespan of cells in the stationary phase [269]. The study [290] suggests that the use of sugars and a nitrogen source is a coordinated process in microbial cells and when ammonium assimilation and glutamine synthesis are impaired, a decrease in the rate of carbon catabolism is a natural outcome [54]. In the present study, 100 mM GA had a complete inhibitory effect, and the number of cells decreased slightly compared to the inoculated amount. Correlation analysis of OD and counted cells on the last day of the test showed a correlation coefficient of 0.52. This correlation was not statistically significant as the *p*-value (0.18). To ensure that 100 mM was the 100 % inhibitory concentration, a new test was performed with GA concentration ranging from 50 mM to 350 mM.

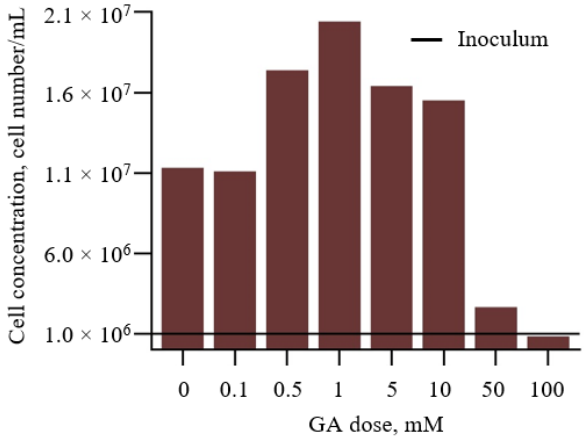

Fig. 3.4. Number of cells in samples containing different GA concentrations after 165 h of cultivation. The horizontal line represents the initial concentration of cells in the samples.

The second test did not reveal a statistically significant difference in OD among seven samples with 50 to 350 mM concentrations in the 50 mM increment step. Cell counting (Fig. 3.5) of one sample from each triplet showed that 50 mM GA had a stronger inhibitory effect on yeast than the previous test, and no cell proliferation was detected.

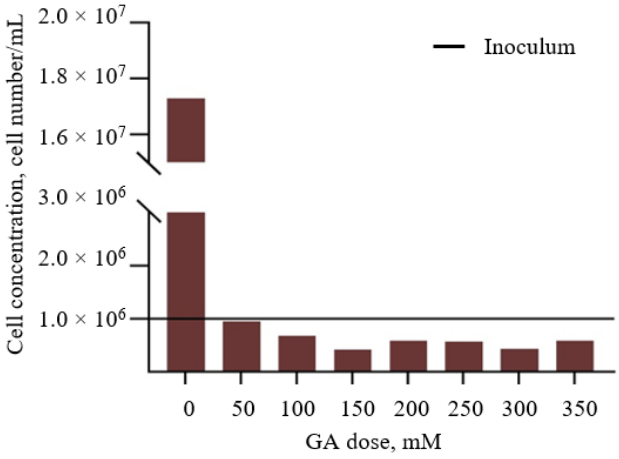

Fig. 3.5. Number of cells in samples containing different GA concentrations after 116 h of cultivation. The horizontal line represents the initial concentration of cells in the samples.

This result may be due to the test's time limit of 116 h compared to the first test of 165 h. However, this does not affect the conclusion that concentrations of 100 mM and above cause 100 % inhibition of *P. rhodozyma* cells. This is confirmed by a decrease in the number of cells and the presence of autolysed cell particles in the samples. The correlation between the final OD and counted cells was not statistically significant as the *p*-value (0.058) was slightly above the significance level (0.05). GA concentrations of 50 mM and 100 mM were chosen for further

experiments to select potential protein-synthesising mutants capable of reproduction in about 100 % inhibitory doses.

Effect of L-methionine sulfoximine on the *Phaffia rhodozyma* growth

Similar to the GA inhibitor, MSO was tested twice. The first test included seven MSO concentrations from 0.1 to 50 mM, of which all inhibitor concentrations showed a strong reduction in OD relative to the control. Statistical analysis of the OD results showed that the control sample's average value significantly differed from the samples with the MSO inhibitor. At the same time, there was no statistical difference between the inhibitor concentrations. Microscopy of samples and cell counting showed complete inhibition of yeast growth for all MSO concentrations tested after 138 h of culture (Fig. 3.6). The correlation coefficient between the last OD measurement and the counted cells was 0.32, which was not statistically significant ($p > 0.05$). It was decided to repeat the test with lower inhibitor dosages to determine the minimal concentration for 100 % cell inhibition.

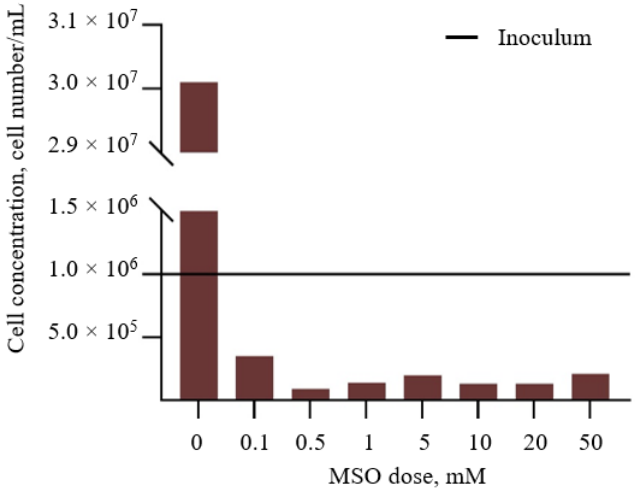

Fig. 3.6. Number of cells in samples containing different MSO concentrations after 138 h of cultivation. The horizontal line represents the initial concentration of cells in the samples.

Both GA and MSO belong to the group of glutamine inhibitors [287]–[289]. GA is considered a more effective herbicide and is required in lower doses to inhibit microorganisms than MSO [291], [293]. For example, 0.05 mM MSO inhibits 50 % of *Mycobacterium tuberculosis* cells, while GA has the same effect at 0.0015 mM [298]. This result is consistent with another study where 2 mM MSO had no inhibitory effect on *A. niger* while 0.15 mM GA had significant inhibition [293]. It is interesting that in the present study *P. rhodozyma* was more sensitive to the presence of MSO than to GA.

In the second MSO inhibition experiment, five concentrations from 0.01 mM to 0.1 mM were tested. OD increased in all samples during 97 h of cultivation, and the counted cells of the samples show (Fig. 3.7) the dependence of yeast growth on the concentration of the MSO inhibitor.

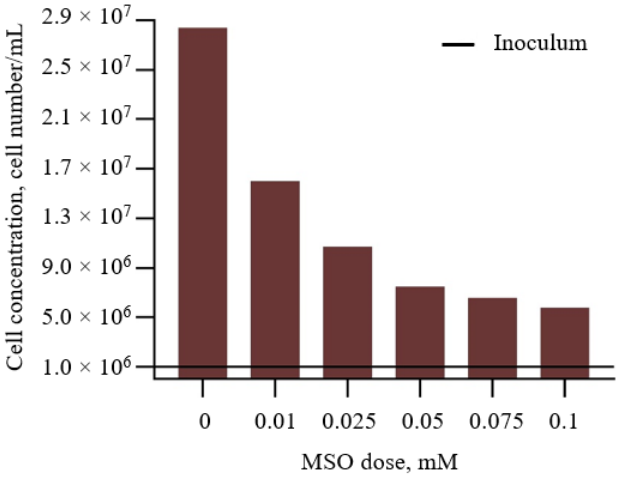

Figure 3.7. Number of cells in samples containing different MSO concentrations after 97 h of cultivation. The horizontal line represents the initial concentration of cells in the samples.

This time, a concentration of 0.1 mM did not cause complete cell inhibition as it did in the first test. Pearson correlation analysis determined the correlation coefficient (0.99) between the last OD measurement and the number of cells collected. The correlation was significant ($p < 0.05$). OD of all samples was similar to the control sample ($p > 0.05$). Therefore, an additional test must be performed in the 0.05–0.5 mM range to confirm the minimal concentration required for complete yeast inhibition (an additional test is not included in the present study).

It is worth noting that the OD values of the samples increased throughout the cultivation time, despite the absence of yeast cell proliferation. There are several assumptions about this appearance. Firstly, autolysis occurs in cells that are unable to maintain life activity at high concentrations of inhibitors, thereby forming many small cellular particles that enhance the scattering of light, increasing optical density [345]. Secondly, although the yeast was cultured in a microplate using a humidification cassette, evaporation of the medium in the samples was observed in the range of 1–5 % (v/v), depending on the laboratory's humidity level and the experiment's duration. Evaporation of water from the medium increases the density of suspended particles, thereby slightly increasing the OD of the samples.

3.3. Screening and analysis of selected mutants

Mutagenised *P. rhodozyma* cells at a concentration of 2.0×10^6 cells/mL were inoculated into 48-well microplates containing medium with 0.2 mM AEC, 5 mM AEC, 50 mM GA, and 100 mM GA concentrations. For further discussion, sample names AEC2, AEC3, GA6 and GA7 will refer to these concentrations (as listed in Tables 3.1 and 3.2). The experiments were performed at different times. The duration of cultivation was 91–150 h, depending on the growth rate of the mutants in the microplate. Microscopy and cell counting of samples GA6 and GA7 showed a strong reduction in the number of cells in the range from 5.0×10^4 cells/mL

to 3.7×10^5 cells/mL and from 1.0×10^4 cells/mL to 3.0×10^5 cells/mL, respectively. Cells subject to mutagenesis and in the presence of an AEC inhibitor were more viable. At the end of cultivation, the number of cells ranged from 3.0×10^4 cells/mL to 3.8×10^5 cells/mL and from 3.5×10^5 cells/mL to 1.0×10^6 cells/mL in samples AEC2 and AEC3, respectively. Then, all samples were re-inoculated under similar conditions at a concentration of 20 % (v/v) in GSM medium with the appropriate inhibitor in microplates. Samples GA6 and GA7 were subcultured three times, AEC2 twice, and AEC3 once. Cells were counted at the end of each cultivation. Samples GA6, GA7, and AEC2 showed improved growth after each subculture, and at the end of the 3rd and 2nd subcultures, the cell concentration in the microplate wells increased more than 10 or 100 times. AEC3 samples after subculture showed a decrease in the number of cells; thus, it was decided to stop at one subculture. The subculture of potential mutants was necessary to strengthen the potential mutants in the selective environment and reduce the risk of proliferation of non-mutated wild-type cells.

Further, samples with the best growth were selected from each subculture for the first cultivations in baffled flasks. One part of these samples showed satisfactory growth on the YM medium and was used as an inoculant for shake flask tests. The other part showed weak growth; therefore, it was not used in further experiments.

Microplate screening of yeast growth gives an approximate representation of the growth rate, but microscopy and cell counting are required to determine complete inhibition or poor growth. This applies both to the determination of the microorganism's minimum inhibitory concentration and the selection of potential mutants, described in the next section. This was confirmed by statistical analysis of the correlation between the OD value and the number of cells in the samples, indicating that the correlation is not statistically significant in most cases. Despite the microplate reader's limitations in detecting low concentrations of culture in a medium containing an AA biosynthesis inhibitor, this screening method is still more effective than screening on an agar medium. As shown in early experiments (data not shown), inhibition of colony-forming unit growth was observed using lower concentrations of AA biosynthesis inhibitors in agar medium than were used in liquid medium in this study. Screening of mutants on inhibitor agar plates was more difficult due to the large number of colony-forming units, small differences in size, and long incubation (up to seven days). Moreover, the further selection and analysis of biomass, such mutants did not significantly improve the synthesis of total protein or individual EAAs. Since microplate screening followed by viable cell counting allows for a more accurate determination of the 100 % cell inhibition concentration, the selection of potential mutants is more efficient.

Analysis of selected mutants cultivated on glycerine-salt medium

The first two experiments were performed in shaken flasks containing GSM medium. Experiments resulted in relatively low biomass and concentration of total proteins in both the wild-type strain and the selected mutants. Such results were expected for the wild-type strain because previous amino acid-free media selection experiments have shown the *P. rhodozyma* DSM 5626 limited ability to utilise ammonium chloride. However, other nitrogen sources, such as ammonium sulfate and potassium nitrate, were less suitable than ammonium chloride for

cultivating *P. rhodozyma* (data not provided). The biomass, total protein and astaxanthin concentrations of the wild-type strain were 10.83 g/L, 19.07 % and 0.049 %, similar to the three GA6 mutants selected from the first microplate subculture (Table 3.3). Although the specific growth rate of the GA6/1-1 and GA6/3-1 mutants was significantly higher 0.497 ± 0.057 and 0.506 ± 0.015 compared to 0.330 ± 0.078 for the wild strain (Annex 4). Mutagenesis and repeated subcultivation of mutant cells in the GSM medium containing GA inhibitors did not affect astaxanthin biosynthesis in biomass.

Table 3.3

Comparison of Wild-Type *P. rhodozyma* Strain with GA Mutants from the First Subculture

Strain*	Biomass, g/L	Protein, g/L	Protein, %	AXT, %
Wild strain	10.83 ± 0.97^{a}	2.07 ± 0.23^{a}	19.07 ± 0.53^{a}	0.049 ± 0.005^{a}
GA6/1-1	13.77 ± 0.59^{b}	2.45 ± 0.17^{a}	17.80 ± 0.60^{a}	0.051 ± 0.005^{a}
GA6/3-1	12.97 ± 0.15^{ab}	2.29 ± 0.15^{a}	17.69 ± 1.08^{a}	0.052 ± 0.003^{a}
GA6/5-1	11.90 ± 1.42^{ab}	2.28 ± 0.27^{a}	19.13 ± 0.60^{a}	0.053 ± 0.008^{a}

Note. * *P. rhodozyma* DSM 5626 wild-type strain and GA mutants were cultivated in shake flasks on GSM medium and analysed on day 11. Values in the same column with different superscript letters differ significantly ($p < 0.05$).

The next experiment used increased amounts of inoculum and medium, as described previously in the Methodology chapter. This improved the biomass yield, protein and astaxanthin content in the studied strains, but no significant difference was observed between the wild strain and the mutants. The five mutants selected from the second subculture did not have statistically significant differences in biomass and protein concentration from the wild-type strain (Table 3.4). Therefore, it was decided to select six mutants from both tests with higher biomass or protein content relative to the wild-type strain and analyse them for AA profile.

Table 3.4

Comparison of Wild-Type *P. rhodozyma* Strain with GA Mutants from the Second Subculture

Strain*	Biomass, g/L	Protein, g/L	Protein, %	ATX, %
Wild strain	14.20 ± 0.40^{a}	2.91 ± 0.17^{a}	20.49 ± 1.50^{a}	0.054 ± 0.004^{a}
GA6/1-2	13.83 ± 0.57^{a}	3.16 ± 0.19^{a}	22.89 ± 1.52^{a}	0.054 ± 0.002^{a}
GA6/2-2	13.90 ± 0.62^{a}	2.71 ± 0.13^{a}	19.47 ± 0.13^{a}	0.054 ± 0.003^{a}
GA6/3-2	13.97 ± 0.31^{a}	2.84 ± 0.15^{a}	20.33 ± 0.87^{a}	0.052 ± 0.010^{a}
GA6/4-2	14.23 ± 0.35^{a}	3.01 ± 0.22^{a}	21.11 ± 1.09^{a}	0.053 ± 0.002^{a}
GA7/4-2	14.23 ± 0.06^{a}	2.92 ± 0.05^{a}	20.49 ± 0.28^{a}	0.056 ± 0.003^{a}

Note. * *P. rhodozyma* DSM 5626 wild-type strain and GA mutants were cultivated in shake flasks on GSM medium and analysed on day 11. Values in the same column with different superscript letters differ significantly ($p < 0.05$).

The wild-type strain served as a control to evaluate changes in the AA profiles of the mutants for each test. Analysis of AA profiles showed a high Arg content in all tested strains, which is not typical for yeast. Wild-type *P. rhodozyma* contained 14.23–16.54 g of Arg per

100 g crude protein, and the GA6 mutants contained 14.08–19.24 g Arg per 100 g crude protein, which was 3–5 times higher than the reported in the literature for *P. rhodozyma* and SCP yeasts species [46], [112], [113]. Higher concentrations of Ile and Leu and lower concentrations of Lys were observed in all samples (Tables 3.5 and 3.6), which differed from results in other studies [107], [108]. The content of non-essential AAs such as glutamic acid (Glu/E) plus glutamine (Gln) and proline (Pro) in *P. rhodozyma* protein was higher in our study. Presumably, such an atypical AA profile in both the wild-type strain and potential mutants is a consequence of cultivation on the GSM medium. The influence of a medium on the AA profile was reported in Ezekiel study[346], where the Met content in *C. utilis* biomass varied depending on the type of substrate pretreatment. For example, the Met content was 0.87 g per 100 g crude protein in yeast biomass grown on Cassava peel acid hydrolysate, while the Met content on enzymatic hydrolysate was 0.36 g per 100 g crude protein [346]. Therefore, to confirm the influence of the medium on the AA profile, further tests were performed on the glycerine-peptone-rich medium. It is assumed that the AA profile of yeast grown under favourable conditions may be more balanced.

Table 3.5

Amino Acid Profile of Pure Protein in GA Mutants from the First Subculture

Strain*	Wild strain	GA6/1-1	GA6/3-1	GA6/5-1
Pure protein, %	16.82 ± 0.20	17.94 ± 0.31	16.96 ± 0.23	16.92 ± 0.22
EAAs, g/100 g of pure protein				
His	2.32 ± 0.04^{a}	2.21 ± 0.14^{a}	2.38 ± 0.02^{a}	2.40 ± 0.13^{a}
Thr	4.83 ± 0.13^{c}	4.37 ± 0.01^{a}	4.42 ± 0.01^{ab}	4.66 ± 0.05^{bc}
Arg	16.13 ± 0.29^{ab}	16.65 ± 0.54^{ab}	17.02 ± 0.12^{b}	15.69 ± 0.13^{a}
Val	5.24 ± 0.05^{a}	5.34 ± 0.07^{ab}	5.33 ± 0.05^{ab}	**5.51 ± 0.00^{b}**
Met	0.96	1.12	1.03	1.25
Try	n/a	n/a	n/a	n/a
Phe	3.83 ± 0.04^{ab}	3.73 ± 0.04^{a}	3.73 ± 0.02^{a}	3.89 ± 0.03^{b}
Ile	4.30 ± 0.27^{a}	4.23 ± 0.02^{a}	4.24 ± 0.07^{a}	4.26 ± 0.05^{a}
Leu	7.26 ± 0.05^{a}	7.00 ± 0.15^{a}	7.08 ± 0.14^{a}	7.31 ± 0.03^{a}
Lys	4.63 ± 0.01^{a}	4.80 ± 0.17^{a}	4.64 ± 0.00^{a}	4.70 ± 0.10^{a}
NEAAs, g/100 g of pure protein				
Asp ac	9.39 ± 0.47^{ab}	8.82 ± 0.15^{a}	9.93 ± 0.14^{b}	9.64 ± 0.12^{ab}
Glu ac	15.25 ± 0.44^{a}	15.49 ± 1.03^{a}	15.21 ± 0.10^{a}	14.68 ± 0.19^{a}
Ser	5.04 ± 0.31^{b}	4.22 ± 0.14^{a}	4.21 ± 0.06^{a}	4.84 ± 0.19^{ab}
Gly	4.72 ± 0.06^{a}	4.82 ± 0.08^{a}	4.91 ± 0.09^{a}	4.75 ± 0.10^{a}
Ala	6.50 ± 0.15^{b}	5.98 ± 0.05^{a}	6.03 ± 0.06^{a}	6.27 ± 0.02^{ab}
Tyr	3.30 ± 0.01^{a}	3.19 ± 0.17^{a}	3.17 ± 0.04^{a}	3.27 ± 0.04^{a}
Cys	0.23 ± 0.49	2.19 ± 0.04	0.92 ± 0.05	0.77 ± 0.00
Pro	6.07 ± 0.15^{a}	5.84 ± 0.38^{a}	5.73 ± 0.33^{a}	6.12 ± 0.42^{a}

Note. * *P. rhodozyma* DSM 5626 wild-type strain and GA mutants were cultivated in shake flasks on GSM medium and analysed on day 11. Values in the same row with different superscript letters differ significantly ($p < 0.05$).

Analysis of the AA profile of crude protein showed that the mutant strain GA6/1-1 has a significantly higher content of Arg, Val and Lys, and strain GA6/3-2 has a significantly higher content of Arg and Lys compared to the wild-type strain. However, when assessing the AA content in pure protein, only the GA6/5-1 mutant strain has a significantly higher Val content compared to the wild-type strain (Table 3.5). The AA profile of the three GA mutants from the second subcultured microplate did not differ significantly from the wild-type strain (Table 3.6).

Table 3.6

Amino Acid Profile of Pure Protein in GA Mutants from the Second Subculture

Strain*	Wild strain	GA6/1-2	GA6/2-2	GA6/3-2
Pure protein, %	18.16 ± 0.05	17.38 ± 0.38	18.03 ± 0.05	19.78 ± 0.20
EAAs, g/100 g of pure protein				
His	2.41 ± 0.04[a]	2.44 ± 0.17[a]	2.17 ± 0.06[a]	2.32 ± 0.02[a]
Thr	4.33 ± 0.17[a]	4.13 ± 0.10[a]	3.93 ± 0.16[a]	4.36 ± 0.23[a]
Arg	18.64 ± 0.03[a]	18.85 ± 0.08[a]	20.47 ± 0.94[a]	19.53 ± 0.10[a]
Val	5.19 ± 0.10[a]	5.36 ± 0.15[a]	5.17 ± 0.18[a]	4.88 ± 0.22[a]
Met	0.82	0.66	0.60	0.64
Try	n/a	n/a	n/a	n/a
Phe	3.64 ± 0.10[a]	3.66 ± 0.03[a]	3.51 ± 0.03[a]	3.49 ± 0.07[a]
Ile	4.30 ± 0.06[a]	4.42 ± 0.00[a]	4.25 ± 0.01[a]	4.10 ± 0.16[a]
Leu	7.02 ± 0.04[a]	7.10 ± 0.14[a]	6.79 ± 0.07[a]	6.82 ± 0.05[a]
Lys	4.91 ± 0.21[a]	4.84 ± 0.26[a]	5.08 ± 0.04[a]	5.36 ± 0.12[a]
NEAAs, g/100 g of pure protein				
Asp ac	8.90 ± 0.45[a]	9.14 ± 0.21[a]	8.98 ± 0.02[a]	8.53 ± 0.09[a]
Glu ac	14.89 ± 0.43[a]	15.45 ± 0.25[a]	15.25 ± 0.03[a]	15.17 ± 0.09[a]
Ser	4.17 ± 0.29[a]	3.64 ± 0.04[a]	3.75 ± 0.27[a]	4.50 ± 0.37[a]
Gly	4.80 ± 0.20[a]	4.91 ± 0.03[a]	4.70 ± 0.05[a]	4.39 ± 0.16[a]
Ala	5.66 ± 0.04[a]	5.65 ± 0.01[a]	5.44 ± 0.16[a]	5.69 ± 0.13[a]
Tyr	3.23 ± 0.05[ab]	3.28 ± 0.08[b]	3.13 ± 0.01[ab]	3.08 ± 0.03[a]
Cys	0.70 ± 0.02	0.41 ± 0.01	0.47 ± 0.00	1.19 ± 0.00
Pro	6.40 ± 0.22[a]	6.06 ± 0.17[a]	6.32 ± 0.19[a]	5.95 ± 0.12[a]

Note. * *P. rhodozyma* DSM 5626 wild-type strain and GA mutants were cultivated in shake flasks on GSM medium and analysed on day 11.Values in the same row with different superscript letters differ significantly ($p < 0.05$).

Table 3.7 provides the results of AAS and EAAI for mutants compared to wild-type strains. Anchovy fishmeal was used as a reference protein [102] in AAS and EAAI calculations (incl. for comparative studies) because it is recognised as the best protein source for fish diets, including salmon [31], [334], [347]. The AA profile of the reference protein, expressed as g amino acid per 100 g crude protein, was as follows: 2.4 g His, 4.3 g Thr, 5.6 g Arg, 5.4 g Val, 3.0 g Met, 1.2 g Trp, 4.1 g Phe, 4.7 g Ile, 7.6 g Leu, and 7.8 g Lys [102]. However, it is worth noting that there is no EU regulation clearly defining the quality of fishmeal protein used for fish feed, and there is also no classification of protein quality based on the amount of ten EAAs contained. In general, fishmeal can be of varying quality depending on the species of fish used, whether whole fish or by-products are used [28], [32], [41]. For example, fishmeal from sprat

caught in the Caspian Sea contained on average 0.73 g Arg, 1.97 g Met, 1.53 g Phe, 2.80 g Leu and 5.50 g Lys per 100 g crude protein [41], which is much lower than that of anchovy fishmeal used as a reference protein in our study [102]. In another study [31], fishmeal had lower levels of all nine EAAs, and only Trp was higher by 0.2 g per 100 g crude protein than anchovy fishmeal [102]. Therefore, it can be assumed that the protein quality of mutants may be more suitable for replacing fishmeal if the reference protein is taken to be a fishmeal of less than the highest quality.

Generally, the AASs of the examined strains were higher than reported in the literature for this yeast species (Table 3.7). Although the AASs of Met and Lys were lower in the protein of wild-type strain and four of the six GA mutants. The first limiting AA in all *P. rhodozyma* strains was Met, which is consistent with the literature [107], [108]. The protein of *P. rhodozyma* DSM 5626, ATCC 2420, and the commercial Red Star® yeast strain have an EAAI of less than 70 %, which is considered low quality. The two GA6 mutants have EAAI values between 70 % and 89 %, which are considered to be of medium-quality protein. A high-quality protein should have an EAAI value of 90 % or higher [333], [334]. The mutant strain GA6/1-1 had higher protein quality than the other strains analysed. The amino acid scores of Val, Met, and Lys in the protein of the mutant GA6/1-1 were 14 %, 10 %, and 9 % higher than those of the wild-type strain.

Table 3.7

Comparison of AAS and EAAI in *P. rhodozyma* Wild-Type Strain and GA Mutants Grown on GSM Medium

Strain*	Wild strain	GA6/1-1	GA6/3-1	GA6/5-1	ATCC 24202	Red Star®
Crude protein, %	19	18	18	19	47	22
AAS (%) of crude protein						
His	85	90	96	93	59	71
Thr	99	104	99	96	60	91
Arg	254	296	296	251	66	113
Val	86	100	96	91	60	69
Met	28	38	34	37	33	37
Phe	82	92	88	84	58	68
Ile	81	92	86	80	57	62
Leu	84	93	89	86	54	67
Lys	52	61	58	53	75	60
EAAI (%)	66	75	73	69	47	56

Note. * *P. rhodozyma* DSM 5626 wild-type strain and GA mutants were cultivated in shake flasks on GSM medium; *P. rhodozyma* ATCC 24202 was cultivated in peat hydrolysate media in 2 L bioreactor [107]; The study does not contain data on the strain and cultivation conditions of the commercial product *P. rhodozyma* Red Star® [108].

For EAAI calculations, Met might be combined with Cys and Phe with Tyr since fish metabolism allows these EAAs to be interchangeable [40], [348]. Feeding trials on fingerling carp with a diet containing Met and Cys in different proportions showed the ability of Cys to

replace up to 40 % of the required Met [349]. In the present study, the Cys content of the samples was not used to calculate EAAI because the obtained values were not precise. This is because of the reverse phase liquid chromatography conditions in which the cysteic acid peak overlaps with the aspartic acid peak. Therefore, the approximate amounts of Cys were calculated as the difference between the aspartic acid peaks in the oxidised and non-oxidised samples, considering the difference in the weight of the samples.

Analysis of selected mutants cultivated on glycerine-peptone medium

The third and fourth experiments were performed in shaken flasks containing GPM medium. GA and AEC mutants were selected from the third and first microplate subcultures, respectively. After the third subcultivation of the GA mutants on the microplate, it was possible to grow a sufficient amount of inoculant to include GA7 mutants in the flask experiment. Biomass and protein concentration increased significantly, while astaxanthin concentration decreased for all tested strains on glycerine-peptone-rich medium (Tables 3.8 and 3.11). The decrease in astaxanthin concentration in biomass grown on a rich nutrient medium under favourable conditions was expected. Improvement in carotenoid biosynthesis occurs under stressful conditions, which was caused by the previously used GSM medium [119]. GA mutants showed no statistically significant difference in biomass, protein, astaxanthin and total carotenoid values compared to the wild-type strain. On the other hand, a significant difference was observed between some mutants in biomass values and total protein concentration.

Table 3.8

Comparison of Wild-Type *P. rhodozyma* Strain with GA Mutants from the Third Subculture

Strain*	Biomass, g/L	Protein, g/L	Protein, %	AXT, %
Wild strain	39.43 ± 0.67^{ab}	12.22 ± 0.09^{a}	30.98 ± 0.41^{ab}	0.029 ± 0.002^{a}
GA6/1-3	40.20 ± 1.01^{ab}	12.22 ± 0.30^{a}	30.40 ± 1.54^{ab}	0.030 ± 0.003^{a}
GA6/2-3	38.10 ± 1.08^{a}	11.93 ± 1.34^{a}	31.33 ± 2.60^{ab}	0.030 ± 0.001^{a}
GA6/3-3	40.33 ± 0.68^{ab}	12.34 ± 0.43^{a}	30.62 ± 0.54^{ab}	0.034 ± 0.003^{a}
GA6/4-3	39.57 ± 1.07^{ab}	11.95 ± 1.13^{a}	30.22 ± 2.01^{a}	0.029 ± 0.002^{a}
GA7/4-3	40.90 ± 0.56^{b}	13.33 ± 0.62^{a}	32.58 ± 1.11^{ab}	0.028 ± 0.001^{a}
GA7/5-3	40.53 ± 0.40^{b}	14.15 ± 0.79^{a}	34.89 ± 1.90^{b}	0.028 ± 0.002^{a}

*Note: * P. rhodozyma* wild-type strain and GA mutants were cultivated in shake flasks on GPM medium and analysed on day 7. Values in the same column with different superscript letters differ significantly ($p < 0.05$).

Analysis of the AA profile of yeast strains cultivated on a GPM medium showed an improvement in the AA pattern. The concentration of Arg in the protein of the wild-type and mutants decreased by more than two-fold and was consistent with another study [36]. Concentrations of other EAAs in protein, on the contrary, increased. Three GA mutants showed significantly higher concentrations of Lys (GA6/3-3) and Met (GA6/4-3 and GA7/5-3) compared to wild-type strain (Table 3.9). The three strains, GA6/1-3, GA6/2-3 (data not shown), and GA7/4-3 have not significant differences in amino acid profile compared to the wild-type strain.

Table 3.9

Amino Acid Profile of *P. rhodozyma* GA Mutants from the Third Subculture

Strain*	Wild strain	GA6/3-3	GA6/4-3	GA7/4-3	GA7/5-3
Pure protein, %	30.27 ± 0.12	29.44 ± 1.04	30.28 ± 0.41	29.24 ± 0.34	29.36 ± 0.26
EAAs, g/100 g of pure protein					
His	2.46 ± 0.14^{a}	2.48 ± 0.09^{a}	2.52 ± 0.25^{a}	2.45 ± 0.03^{a}	2.41 ± 0.16^{a}
Thr	5.51 ± 0.26^{a}	4.93 ± 0.24^{a}	5.38 ± 0.09^{a}	5.13 ± 0.12^{a}	5.43 ± 0.12^{a}
Arg	6.77 ± 0.26^{abc}	7.41 ± 0.07^{c}	7.19 ± 0.05^{bc}	6.10 ± 0.02^{a}	6.38 ± 0.06^{ab}
Val	5.94 ± 0.02^{ab}	6.02 ± 0.04^{ab}	5.72 ± 0.09^{a}	6.08 ± 0.02^{b}	5.80 ± 0.02^{ab}
Met	1.24 ± 0.09^{a}	1.23 ±0.02^{a}	**1.70 ± 0.05^{b}**	1.41 ± 0.12^{ab}	**1.56 ± 0.09^{b}**
Trp	n/a	n/a	n/a	n/a	n/a
Phe	4.34 ± 0.10^{a}	4.24 ± 0.27^{a}	4.41 ± 0.03^{a}	4.49 ± 0.06^{a}	4.43 ± 0.06^{a}
Ile	5.14 ± 0.21^{a}	5.40 ± 0.21^{a}	4.96 ± 0.03^{a}	5.36 ± 0.08^{a}	4.99 ± 0.09^{a}
Leu	8.38 ± 0.10^{a}	8.19 ± 0.10^{a}	8.08 ± 0.19^{a}	8.38 ± 0.08^{a}	8.34 ± 0.07^{a}
Lys	4.90 ± 0.12^{a}	**5.60 ± 0.13^{b}**	5.20 ± 0.18^{ab}	4.90 ± 0.09^{a}	4.94 ± 0.02^{a}
NEAAs, g/100 g of pure protein					
Asp ac	10.70 ± 0.39^{a}	10.82 ± 0.30^{a}	11.07 ± 0.15^{a}	10.74 ± 0.13^{a}	10.51 ± 0.09^{a}
Glu ac	14.93 ± 0.01^{a}	14.83 ± 0.58^{a}	14.58 ± 0.26^{a}	14.97 ±0.17^{a}	14.91 ± 0.13^{a}
Ser	5.60 ± 0.38^{a}	4.81 ± 0.11^{a}	5.55 ± 0.07^{a}	4.72 ± 0.38^{a}	5.63 ± 0.08^{a}
Gly	5.79± 0.01^{ab}	6.01±0.03^{ab}	5.59 ± 0.16^{a}	6.19 ± 0.13^{b}	5.67 ± 0.08^{ab}
Ala	7.45 ± 0.22^{a}	7.24 ± 0.04^{a}	7.26 ± 0.04^{a}	7.28 ± 0.06^{a}	7.34 ± 0.08^{a}
Tyr	3.79 ± 0.13^{a}	3.72 ± 0.00^{a}	3.70 ± 0.05^{a}	3.79 ± 0.04^{a}	3.69 ± 0.03^{a}
Cys	0.31 ± 1.80	0.16 ± 0.66	0.81 ± 0.02	1.27 ± 0.08	1.39 ± 0.04
Pro	6.81 ± 0.22^{a}	6.93 ± 0.36^{a}	6.33 ± 0.24^{a}	6.56 ± 0.01^{a}	6.51 ± 0.41^{a}

Note. * *P. rhodozyma* DSM 5626 wild-type strain and GA mutants were cultivated in shake flasks on GPM medium. Values in the same row with different superscript letters differ significantly ($p < 0.05$).

It was unexpected to detect that the AA profile of *P. rhodozyma* DSM 5626 is not inferior to those of the three best SCP producers, *C. utilis, S. cerevisiae*, and *K. marxianus,* except for Lys and partially Met [46]. All strains tested had lower amounts of Lys to 5.41 g per 100 g crude protein compared to 6.95 g, 6.19 g, and 6.51 g Lys per 100 g crude protein in *C. utilis, S. cerevisiae,* and *K. marxianus*, respectively [46]. The Met content was also higher in *C. utilis* and *K. marxianus* than in *P. rhodozyma* and in three GA6 mutants. The mutant strains GA6/4-3 and GA7/5-3 showed statistically significant increases in Met by 37 % and 26 % compared to the wild-type strain. The Met content was higher in these two mutants than in the three SCP yeast species when calculated per 100 g crude protein. However, SCP commercial yeast strains have a higher protein concentration in the biomass (Table 3.10). Thus, *P. rhodozyma* is inferior in AA content to an equivalent amount of biomass. To achieve increased protein concentrations in biomass, a series of best *P. rhodozyma mutant* fermentations must be carried out in a bioreactor under optimal conditions for protein synthesis [350].

Table 3.10

Comparison of AAS and EAAI in *P. rhodozyma* GA Mutants from the Third Subculture

Strain	Wild strain	GA6/3-3	GA6/4-3	GA7/4-3	GA7/5-3	CU	SC	KM
Crude protein, %	31	31	30	33	35	56	46	51
AAS, %								
His	100	99	113	93	89	82	71	76
Thr	125	110	125	110	105	111	104	98
Arg	118	127	131	98	97	83	75	73
Val	107	107	108	102	91	84	75	73
Met	38	39	55	45	47	45	51	41
Phe	104	99	108	98	91	101	90	80
Ile	107	110	106	102	89	90	83	73
Leu	108	104	106	99	92	91	80	72
Lys	61	69	69	58	53	89	83	79
EAAI, %	74	76	80	71	66	69	64	59

Note. CU – *Candida utilis,* SC – *Saccharomyces cerevisiae,* and KM – *Kluyveromyces marxianus* are commercial SCP yeast strains (Borregaard ASA, Norway) [46].

According to the literature, GA has an inhibitory effect on the biosynthesis of seven AAs [287], [288], [289], of which five are EAAs for fish. Interestingly, in the study of Vallejo et al., the treatment of wine yeast with 0.05 mM GA significantly increased the synthesis of Met, Ile, Leu, Phe, Trp, and Tyr compared to untreated cells [269]. At the same time, Phe, Trp, and Tyr are not targets for this inhibitor, which may indicate the switching-on side effects in the microorganism cells in the presence of the herbicide [72].

Since the culture medium in the test flasks was different for the GA mutants, it was impossible to conclude the effect of repeated microplate subcultures of mutant cells in a selective medium on the amino acid profile of the selected mutants. In the future, it is necessary to conduct a comparative experiment with mutant cells selected from 3–4 microplate subcultures to assess the effect of the duration of herbicide treatment on the selection of mutants with the best amino acid profile.

In the fourth flask experiment, eight AEC3 mutants and one AEC2 mutant did not have significantly higher biomass, protein, and astaxanthin content than the wild-type strain (Table 3.11). Several mutants showed significantly lower biomass, protein and astaxanthin concentration values. Therefore, as in previous tests, the AA profile was analysed for mutants with higher biomass and protein concentration.

The AA profile was analysed for five mutants: AEC2/1-2, AEC3/3-1, AEC3/5-1, AEC3/8-1, and AEC3/9-1. All tested AEC mutants had lower Arg content compared to the wild-type strain. The protein of the AEC2/1-2 mutant contained a significantly higher concentration of Thr, Phe, Ala, and Tyr. AEC3/9-1 mutant had a significantly increased content of Met, Phe, Ile, and Lys compared to the wild-type strain (Table 3.12).

Table 3.11

Comparison of Wild-Type *P. rhodozyma* Strain with AEC Mutants from the First or Second Subculture

Strain*	Biomass, g/L	Protein, g/L	Protein, %	ATX, %
Wild strain	38.95 ± 0.35^{bcd}	11.98 ± 0.04^{ab}	30.75 ± 0.15^{bc}	0.032 ± 0.012^{bc}
AEC2/1-2	38.73 ± 1.24^{bcd}	11.02 ± 0.59^{a}	28.45 ± 1.28^{ab}	0.039 ± 0.003^{c}
AEC3/1-1	39.70 ± 1.56^{bcd}	10.53 ± 0.76^{ab}	26.52 ± 1.06^{a}	0.034 ± 0.003^{bc}
AEC3/3-1	40.70 ± 0.70^{d}	11.65 ± 0.43^{ab}	28.62 ± 0.77^{ab}	0.032 ± 0.003^{bc}
AEC3/4-1	35.75 ± 0.25^{a}	10.55 ± 0.43^{ab}	29.51 ± 1.20^{abc}	0.019 ± 0.001^{a}
AEC3/5-1	39.13 ± 0.95^{bcd}	11.57 ± 0.79^{ab}	29.57 ± 1.31^{abc}	0.038 ± 0.001^{bc}
AEC3/6-1	37.93 ± 0.32^{abc}	11.89 ± 0.64^{ab}	31.35 ± 1.89^{bc}	0.027 ± 0.002^{ab}
AEC3/7-1	39.83 ± 1.10^{bcd}	11.77 ± 0.53^{ab}	29.55 ± 1.35^{abc}	0.034 ± 0.006^{bc}
AEC3/8-1	40.43 ± 1.12^{cd}	12.31 ± 0.55^{b}	30.45 ± 1.81^{abc}	0.037 ± 0.003^{bc}
AEC3/9-1	37.30 ± 0.26^{ab}	12.20 ± 0.59^{ab}	32.71 ± 1.78^{c}	0.018 ± 0.002^{a}

Note. * *P. rhodozyma* DSM 5626 wild-type strain and AEC mutants were cultivated in shake flasks on GPM medium. Values in the same column with different superscript letters differ significantly ($p < 0.05$).

Table 3.12

Amino Acid Profile of *P. rhodozyma* AEC Mutants from the First and Second Subculture

Strain*	Wild strain	AEC2/1-2	AEC3/5-1	AEC3/9-1
Pure protein, %	30.92 ± 0.18	26.77 ± 0.40	29.12 ± 0.57	31.35 ± 0.51
EAAs, g/100 g of pure protein				
His	2.67 ± 0.04^{a}	2.27 ± 0.06^{a}	2.40 ± 0.07^{a}	2.39 ± 0.02^{a}
Thr	4.93 ± 0.13^{ab}	$\mathbf{5.43 \pm 0.02^{c}}$	5.23 ± 0.06^{bc}	4.79 ± 0.19^{ab}
Arg	8.30 ± 0.26^{c}	5.85 ± 0.10^{a}	7.16 ± 0.01^{b}	6.59 ± 0.29^{b}
Val	6.59 ± 0.00^{b}	6.66 ± 0.04^{b}	6.68 ± 0.01^{b}	6.50 ± 0.08^{b}
Met	1.23 ± 0.08^{ab}	1.34 ± 0.02^{b}	1.26 ± 0.06^{ab}	$\mathbf{1.66 \pm 0.05^{c}}$
Trp	n/a	n/a	n/a	n/a
Phe	4.17 ± 0.05^{a}	$\mathbf{4.43 \pm 0.05^{c}}$	$\mathbf{4.35 \pm 0.00^{bc}}$	$\mathbf{4.40 \pm 0.04^{c}}$
Ile	4.88 ± 0.05^{a}	4.89 ± 0.05^{a}	4.91 ± 0.04^{a}	$\mathbf{5.27 \pm 0.10^{b}}$
Leu	7.98 ± 0.08^{ab}	8.19 ± 0.07^{b}	8.01 ± 0.03^{ab}	8.18 ± 0.10^{b}
Lys	5.91 ± 0.00^{a}	5.49 ± 0.12^{a}	5.60 ± 0.28^{a}	$\mathbf{7.31 \pm 0.17^{b}}$
NEAAs, g/100 g of pure protein				
Asp ac	10.39 ± 0.05^{ab}	11.12 ± 0.17^{b}	11.17 ± 0.22^{b}	10.34 ± 0.17^{ab}
Glu ac	15.32 ± 0.01^{a}	15.45 ± 0.03^{a}	15.94 ± 0.61^{ab}	15.52 ± 0.29^{a}
Ser	4.87 ± 0.37^{ab}	5.80 ± 0.10^{b}	5.39 ± 0.04^{ab}	4.27 ± 0.50^{a}
Gly	5.48 ± 0.08^{a}	5.44 ± 0.09^{a}	5.42 ± 0.14^{a}	5.87 ± 0.10^{a}
Ala	6.68 ± 0.02^{a}	$\mathbf{7.26 \pm 0.05^{b}}$	6.83 ± 0.01^{a}	6.73 ± 0.08^{a}
Tyr	3.49 ± 0.03^{a}	$\mathbf{3.80 \pm 0.06^{b}}$	$\mathbf{3.72 \pm 0.07^{b}}$	3.67 ± 0.06^{ab}
Cys	1.26 ± 0.02	0.64 ± 0.00	0	0.29 ± 0.01
Pro	5.77 ± 0.05^{a}	5.91 ± 0.12^{a}	6.00 ± 0.51^{a}	6.28 ± 0.12^{a}

Note. * *P. rhodozyma* DSM 5626 wild-type strain and AEC mutants were cultivated in shake flasks on GPM medium. Values in the same row with different superscript letters differ significantly ($p < 0.05$).

Mutant AEC3/9-1 had improved Met content by 35 %, Lys by 24 %, Ile by 8 % and Phe by 6 % compared to the wild strain. Strain AEC3/5-1 contained significantly higher amounts of Phe and Tyr compared to the wild-type strain. Two strains AEC3/3-1 and AEC3/8-1 did not show significant differences in amino acid profile compared to the wild-type strain (data not shown).

The EAAI and AAS of His, Arg, Val, Phe, Ile, and Leu were higher in all *P. rhodozyma* strains compared with three commercial SCP strains (Table 3.13). The strain AEC3/9-1 with improved Lys and Met content was the best strain of all mutants tested in this work. The AAS of Lys at 95.59 % is higher than that previously reported in yeasts such as *Yarrowia lipolytica* [75], [351], *Cyberlindnera jadinii, Blastobotrys adeninivorans* [56], *Wickerhamomyces anomalus* [56], [100], *Saccharomyces cerevisiae* [46], [55], [75], *Candida utilis* [46], [360], *Kluyveromyces marxianus* [46], [359], and fishmeal of not the highest quality [31], [41]. Interestingly, AEC is an inhibitor of the biosynthesis of the four EAAs Met, Lys, Thr and Ile [353]; however, in the present study, an increase in the biosynthesis of non-target AA biosynthesis inhibitors was noted.

Table 3.13

Comparison of AAS and EAA in *P. rhodozyma* AEC Mutants from the First and Second Subculture

Strain	Wild strain	AEC2/1-2	AEC3/5-1	AEC3/9-1	CU	SC	KM
Crude protein, %	31	28	30	33	56	46	51
AAS, %							
His	112	92	102	96	82	71	76
Thr	115	120	120	112	111	104	98
Arg	149	98	128	118	83	75	73
Val	123	117	124	115	84	75	73
Met	41	43	41	54	45	51	41
Phe	102	102	106	112	101	90	80
Ile	104	98	105	121	90	83	73
Leu	106	102	106	109	91	80	72
Lys	76	68	74	96	89	83	79
EAAI, %	79	73	78	82	69	64	59

Note. CU – *Candida utilis*, SC – *Saccharomyces cerevisiae*, and KM – *Kluyveromyces marxianus* are commercial SCP yeast strains (Borregaard ASA, Norway) [46].

Doctoral Thesis demonstrates the potential of AA biosynthesis inhibitors in screening of protein-improved mutants. Using a selective medium reduced the number of mutants studied, allowing for compact tests in flasks. Although the analysis of the mutant biomass did not show an increase in the synthesis of total protein, an increase in the synthesis of essential AAs was achieved. Screening of mutagenised yeast cells on GA selective medium resulted in selecting two mutants with significantly improved Met synthesis and one mutant with significantly improved Lys synthesis. Screening of mutagenised yeast cells on an AEC selective medium resulted in selecting three mutants with significantly improved synthesis of two to four AAs. The best mutant in this study, AEC3/9-1, had significantly improved synthesis of four essential

AAs for fish diets: Met, Phe, Ile, and Lys. The increase in synthesising the first two limiting AAs in yeast – Met and Lys – was especially valuable. This proves that AA biosynthesis inhibitors are a good tool for selecting improved protein-quality mutants. Thus, the hypothesis proposed in the Doctoral Thesis was confirmed.

In the future, it is necessary to isolate mutant strains from the best mutant samples and to test the genetic stability of the created mutant strains to maintain the improved synthesis of certain amino acids through multi-generational cultivation. Since it has been observed that strains may lose the ability to produce products when repeatedly transferred in batch culture, and the production rate may decrease during long-term fermentation [354], it is important to take multiple preventive measures to maintain the improved ability of the mutant strain as long as possible and not to lose the newly acquired strain.

An important aspect of this study is disclosing the potential of *P. rhodozyma* as an SCP producer. *P. rhodozyma* DSM 5626 biomass has about 31 % protein and 0.029 % astaxanthin when cultivated in glycerol-peptone-rich medium with pH 5 in shaken flasks at 22 °C for seven days. Under these conditions, *P. rhodozyma* DSM 5626 has a moderate quality protein similar to fishmeal and EAA profile, except for the limiting content of Met and Lys. The *P. rhodozyma* DSM 5626 protein is superior in EAA content to other SCP producers such as *C. jadinii, W. anomalus, B. adeninivorans*, or fishmeal of not the highest quality. This was unexpected since studies on the potential of *P. rhodozyma* as an SCP producer have not been conducted and are limited. Further studies are needed to optimise culture conditions to increase protein concentration in the biomass and determine the competitiveness of both *P. rhodozyma* DSM 5626 and the best mutants as simultaneous protein and astaxanthin producers.

CONCLUSION

1. The multi-criteria decision analysis identified three amino acid inhibitors – glufosinate-ammonium, L-methionine sulfoximine, and S-(2-aminoethyl)-L-cysteine – as optimal for selective screening of bacteria and fungi. They inhibit many essential amino acids, including Met and Lys, which is especially valuable since they are limited in plant-derived protein and yeast. Additional inhibitors, such as DL-propargylglycine, sulfonylureas, and imidazolinones, demonstrated effective bacterial inhibition. Combining inhibitors like glyphosate with metsulfuron-methyl or DL-propargylglycine could apply selective pressure on a broader range of essential amino acid biosynthesis pathways, enhancing inhibitory effects and expanding the scope of microorganisms amenable to selective screening.
2. The glufosinate-ammonium inhibitor is a suitable tool for the selective screening of yeast mutants with increased Met and Lys synthesis. The two mutants with significantly improved Met biosynthesis were isolated using 50 mM and 100 mM glufosinate-ammonium concentrations. These mutants showed equal or greater Met content in the crude protein than conventional SCP yeasts such as *Y. lipolytica, C. utilis, S. cerevisiae*, and *K. marxianus*.
3. The S-(2-aminoethyl)-L-cysteine inhibitor promoted the selection of two mutants with significantly improved biosynthesis of Thr, Phe, Ala and Tyr using 0.5 mM S-(2-aminoethyl)-L-cysteine and Phe, Ile, Lys, and Met using 2.5 mM S-(2-aminoethyl)-L-cysteine. The mutant AEC3/9-1 with increased amino acid content, including Lys and Met, demonstrated superior content of nine essential amino acids, thus outperforming the conventional yeast SCP protein.
4. The use of glufosinate-ammonium and S-(2-aminoethyl)-L-cysteine resulted in the selection of mutants with improved synthesis of Met and Lys. Specifically, mutants GA6/4-3 and GA7/5-3 exhibited 37 % and 26 % higher Met levels, respectively, while GA6/3-3 had a 14 % increase in Lys compared to the wild strain. The AEC3/9-1 mutant demonstrated a 35 % increase in Met, 24 % in Lys, 6% in Phe, and 8% in Ile, underscoring the efficacy of this screening approach in enhancing essential amino acid content.
5. Amino acid biosynthesis inhibitors are a good tool for screening mutants with improved biosynthesis of essential amino acids and protein quality. Thus, the hypothesis proposed in the Doctoral Thesis was confirmed.
6. This experimental study is the first to assess the protein content and amino acid profile of the *P. rhodozyma* DSM 5626 strain. Findings reveal that this strain produces medium-quality protein with an essential amino acid profile similar to that of high-quality fishmeal, except for limited levels of Met and Lys. Notably, the protein quality of *P. rhodozyma* DSM 5626 is higher than that of some other yeasts studied as SCPs, such as *C. jadinii, W. anomalus*, and *B. adeninivorans*.

ANNEX

Annex 1

TOPSIS Matrix for Evaluating Herbicides for Bacteria Selection

Herbicides	Criteria for herbicide evaluation				Sources*
	C1	*C2*	C3	C4	
GP	2.61	2	22.22	2	[1], [2], [3], [4], [5], [6]
MSM	2.66	0	22.22	2	[7], [8], [9]
SMM	18.55	0	22.22	4	[8], [9], [10], [11]
CS	87.48	1	22.22	2	[8], [9], [11], [12], [13]
Imazapyr	45.36	2	22.22	3	[8], [9], [11], [12], [14]
Imazapic	87.48	2	22.22	2	[9], [11], [15]
Imazethapyr	90.07	2	22.22	3	[8], [9], [11], [12], [15]
Imazamox	117.94	2	22.22	3	[9], [11], [15]
Imazamethabenz	163.62	2	22.22	0	[9], [11], [15]
Imazaquin	43.03	2	22.22	3	[9], [11], [15], [16]
GA	33.86	0	55.56	7	[11], [15], [17], [18], [19], [20]
MSO	15.07	0	55.56	3	[11], [15], [18], [19], [20]
PPG	8.99	0	16.67	3	[11], [15], [21], [22]
AVG	2231.72	2	55.56	3	[11], [15], [23], [24], [25]
AEC	10.86	2	55.56	0	[11], [15], [26], [27], [28]
Amitrole	0.432	3	5.55	3	[11], [31], [32], [33], [34], [35], [36]
Preference value	MIN	MIN	MAX	MIN	

Note. * Sources used in creating the matrix are listed in Annex 3.

Criteria:

C1 – Price of inhibitor provided by local distribution company, EUR per 100 mg.

C2 – Bacteria inhibition efficacy (micro-dose (0), low dose (1), moderate dose (2), high dose (3).

C3 – Inhibited EAA, %.

C4 – Hazards (safety 0, 1–7 number of total health and environmental hazards).

Annex 2

TOPSIS Matrix for Evaluating Inhibitors for Yeast Selection

Herbicides	Criteria for herbicide evaluation				Sources*
	C1	*C2*	C3	C4	
GP	2.61	3	22.22	2	[2], [4], [5], [6], [11], [37]
MSM	2.66	2	22.22	2	[7], [9], [11], [38]
SMM	18.55	1	22.22	4	[9], [10], [11], [39]
CS	87.48	1	22.22	2	[9], [11], [13]
TBM	74.52	2	22.22	4	[9], [11], [15], [40], [41]
GA	33.86	2	55.56	7	[11], [15], [17], [20], [42]
MSO	15.07	2	55.56	3	[11], [15], [20], [43]
PPG	8.99	3	16.67	3	[11], [15], [22], [44], [45], [46]
AVG	2231.72	2	55.56	3	[11], [15], [23], [24], [47]
AEC	10.86	2.5	55.56	0	[11], [15], [27]
Amitrole	0.432	2	5.55	3	[11], [32], [33], [34], [35], [36], [39]
Preference value	MIN	MIN	MAX	MIN	

Note. * Sources used in creating the matrix are listed in Annex 3.

Criteria:

C1 – Price of inhibitor provided by local distribution company, EUR per 100 mg.

C2 – *Bacteria* inhibition efficacy (micro-dose (0), low dose (1), moderate dose (2), high dose (3).

C3 – Inhibited EAA, %.

C4 – Hazards (safety 0, 1–7 number of total health and environmental hazards).

Sources used in creating matrices for TOPSIS analysis

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P. rhodozyma DSM 5626 and GA Mutant Biomass Analysis

Strain*	DSM 5626	GA6/1-1	GA6/3-1	GA6/5-1
Biomass, g/L	10.83 ± 0.97^{a}	13.77 ± 0.59^{b}	12.97 ± 0.15^{ab}	11.90 ± 1.42^{ab}
Protein, g/L	2.07 ± 0.23^{a}	2.45 ± 0.17^{a}	2.29 ± 0.15^{a}	2.28 ± 0.27^{a}
Protein, %	19.07 ± 0.53^{a}	17.80 ± 0.60^{a}	17.69 ± 1.08^{a}	19.13 ± 0.60^{a}
Total carotenoid, mg/L	6.35 ± 0.84^{a}	8.07 ± 1.22^{a}	7.67 ± 0.45^{a}	6.99 ± 1.62^{a}
Astaxanthin, mg/L	5.28 ± 0.71^{a}	7.03 ± 0.91^{a}	6.72 ± 0.33^{a}	6.27 ± 1.50^{a}
Astaxanthin, %	0.049 ± 0.005^{a}	0.051 ± 0.005^{a}	0.052 ± 0.003^{a}	0.053 ± 0.008^{a}
μ, day^{-1}	0.330 ± 0.078^{a}	0.497 ± 0.057^{b}	0.506 ± 0.015^{b}	0.440 ± 0.073^{ab}

Note. * *P. rhodozyma* and GA mutants from the first subcultured microplates.

Strain**	DSM 5626	GA6/1-2	GA6/2-1	GA6/3-2	GA6/4-2	GA7/4-2
Biomass, g/L	14.20 ± 0.40^{a}	13.83 ± 0.57^{a}	13.90 ± 0.62^{a}	13.97 ± 0.31^{a}	14.23 ± 0.35^{a}	14.23 ± 0.06^{a}
Protein, g/L	2.91 ± 0.17^{a}	3.16 ± 0.19^{a}	2.71 ± 0.13^{a}	2.84 ± 0.15^{a}	3.01 ± 0.22^{a}	2.92 ± 0.05^{a}
Protein, %	20.49 ± 1.50^{a}	22.89 ± 1.52^{a}	19.47 ± 0.13^{a}	20.33 ± 0.87^{a}	21.11 ± 1.09^{a}	20.49 ± 0.28^{a}
Total carotenoid, mg/L	9.53 ± 0.99^{ab}	8.86 ± 0.38^{ab}	8.90 ± 0.28^{ab}	7.70 ± 1.58^{a}	9.00 ± 0.06^{ab}	10.46 ± 0.47^{b}
Astaxanthin, mg/L	7.60 ± 0.73^{a}	7.43 ± 0.27^{a}	7.56 ± 0.08^{a}	7.29 ± 1.28^{a}	7.61 ± 0.21^{a}	8.01 ± 0.43^{a}
Astaxanthin, %	0.054 ± 0.004^{a}	0.054 ± 0.002^{a}	0.054 ± 0.003^{a}	0.052 ± 0.010^{a}	0.053 ± 0.002^{a}	0.056 ± 0.003^{a}
μ, day^{-1}	0.264 ± 0.044^{bc}	0.220 ± 0.008^{abc}	0.304 ± 0.024^{cd}	0.364 ± 0.000^{d}	0.183 ± 0.016^{a}	0.215 ± 0.015^{ab}

Note. * *P. rhodozyma* and GA mutants from the second subcultured microplates.

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The publications included in the Doctoral Thesis

The Doctoral Thesis is based on the following scientific publications:

1. Raita S., Kusnere Z., Spalvins K., Blumberga D. Optimization of yeast cultivation factors for improved SCP production. 2022. *Environmental and Climate Technologies* vol. 26, no. 1, pp. 848–861. doi: 10.2478/rtuect-2022-0064
2. Spalvins K., Raita S., Valters K., Blumberga D. Improving single cell protein yields and amino acid profile via mutagenesis: Review of applicable amino acid inhibitors for mutant selection. 2021. *Agronomy Research* vol. 19, no. 3, pp. 1285–1307. doi: 10.15159/ar.21.083
3. Raita S., Berzina I., Kusnere Z., Kalnins M., Kuzmika I., Spalvins K. Herbicide-based selection of mutants for improved SCP synthesis: application and procedures. 2024. *Agronomy Research* vol. 19, no. 3, pp. 1285–1307. doi: 10.15159/AR.24.060.
4. Berzina I., Raita S., Kalnins M., Spalvins K., Kuzmika I. In search of the best technological solutions for creating edible protein-rich mutants: a multi-criteria analysis approach. 2024. *Agronomy Research* vol. 22, no. S1, pp. 370–400. doi: 10.15159/AR.24.039.
5. Raita S., Kuzmika I., Geiba, Z., Mika T. Spalvins K. Enhanced amino acid biosynthesis in *Phaffia rhodozyma* via herbicide-induced selection. *Journal of Industrial Microbiology and Biotechnology*, (under review).

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Optimization of Yeast Cultivation Factors for Improved SCP Production

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***Abstract* – Yeast Single-Cell Proteins (SCP) production using various agro-industrial by-products and wastes have significant potential as an alternative to the soy meal, and fish meal protein used for livestock and aquaculture feeds. The use of organic wastes as a substrate in the fermentation processes can be accepted as one of the solutions to reduce the total price of the culture and an environmentally friendlier method of removing these residues. This review article focuses on the yeast biomass yield and protein content increase strategies, which is impossible without understanding metabolic pathways and switching mechanisms. The present work discusses optimization strategies for protein-enriched yeast biomass production, such as fermentation medium composition, including a selection of carbon and nitrogen sources and their ratio, supplemented trace elements, and cultivation conditions such as pH, temperature, time of cultivation, and inoculum size. This review summarizes the theoretical knowledge and experimental results of other researchers that provide an overview of the achievements of the last decades in the production of SCP.**

***Keywords* – Amino acids; carbon to nitrogen ratio; cultivation conditions; single-cell protein; yeast**

Nomenclature		
h	Hours	–
g_{dw}	Gram of the dry biomass	–
μ_{max}	The maximum specific growth rate of biomass	h^{-1}
v/v	Volume per volume	%

1. INTRODUCTION

The aquaculture industry's importance in providing humankind with high–quality animal protein gradually increases over time. To meet the growing demand for aquafeed, alternative protein sources are essential for the sustainable development of the aquaculture industry. Since conventional aquaculture feed sources such as fishmeal and plant origin protein are limited and have a negative impact on the environment [1]–[4]. Single–cell protein (SCP) is widely used as a valuable feed additive, replacing costly conventional sources like soymeal and fishmeal [5]–[7]. Single–cell protein is microbial biomass produced by various microorganisms such as algae, bacteria, fungi, yeast, and protists, which can metabolize different carbon and nitrogen sources [8], [9]. The production of single-cell proteins involves the growth of microorganism in a fermenter and includes downstream processes such as

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separation and concentration of microbial biomass, drying, and mixed up with animal feed or directly used [10], [11].

Yeast dried biomass is commonly used as a supplement in animal feed due to its relatively high protein, amino acid, energy, and micronutrient content compared with feed grains and oilseed meals [12]. Yeast has various advantages including larger size, thus easier harvesting, and lower nucleic acid content, which reduces the cost of post-treatment [13]. Yeasts are able to convert inexpensive non-edible by-products from the food processing and agricultural industry into high-value protein with limited dependence on arable land, water, or changing climatic conditions [14], [15]. Substrate for production of SCP should be accessible, nontoxic, abundant, renewable, low-cost, and able to support rapid growth and proliferation of the organisms resulting in high-quality biomass [16], [17]. Common sources of biomass for SCP are represented by fruit wastes [18], molasses [19], dairy industry by-products [20], [21], industry wastewater [22], [23], glycerol [24], natural gas, ethanol, methanol [25], etc.

The selection of yeast strain, fermentation medium composition, trace element supplementation, cultivation conditions, and harvested biomass treatment can all alter the final SCP composition [14], [26]–[28]. The protein content in yeast cells can range from 10 % to over 79 % of dry matter (see Table 1). Yeast biomass is also composed of lipids (0.5–8 %), carbohydrates (18–43%), minerals, and vitamins. Yeast biomass is rich in B vitamins, calcium, phosphorus, potassium, magnesium, copper, iron, zinc, manganese, and selenium [14], [23], [25], [29]. SCP is rich in certain essential amino acids, such as lysine and methionine, which are limited in most plant and animal sources. Studies have shown that yeasts such as *Saccharomyces cerevisiae*, *Candida utilis*, *Candida tropicalis*, *Yarrowia lipolytica* and *Kluyveromyces marxianus* have suitable amino acid composition as protein sources in diets for aquaculture [19], [24], [30]–[33]. Protein–rich yeast biomass can be used as an additive supplemented to the leading aquaculture and livestock diets instead of other sources, such as fish meal, soybean meal, and other plant–based protein sources [5], [34]–[36].

Although yeast SCP has various advantages, it also has some limitations. The main challenge is the nucleic acid content in cells. The biomass of most microorganisms contains 4–20 % of nucleic acid [1], [37]. Most yeasts contain 5–8 % of nucleic acid in dry matter which is less than bacteria contain 8–15 % [37], [38]. The consumption of single–cell protein with high contents of nucleic acids could cause serious problem with health, therefore, yeast biomass needs a reduced number of nucleic acids to acceptable level about 1 % for using as feed or food. SCP with high nucleic acid content is recommended only for feeding animals with short life spans [10], [37]. Another challenge can be considered the poor digestibility of the SCP, since yeast has a complex and thick cell envelope [39]. Therefore, for improvement of yeast SCP digestibility and reduction or removal of nucleic acids content, the cell wall of yeast should be processed by mechanical disruption, chemical, physical, enzymatic treatment, or combinations of these methods [37], [39]. Despite the increase in total production costs due to the use of methods for disrupting the walls of yeast cells, the final processed yeast presents nutrient digestibility coefficients similar to those of high quality protein for the food and feed industry [40].

Optimization of yeast cultivation factors is an extremely important step to obtain the highest efficiency of protein production and an appropriate amino acid profile in yeast cell biomass [37]. The present work discusses the optimization strategies for protein–enriched yeast biomass production that may result in a balanced, renewable, high–protein ingredient that is a suitable alternative to classic feed proteins.

2. INFLUENCING FACTORS ON YEAST SCP PRODUCTION

The concentration of protein and amino acids profile in yeast cells mainly genetically predetermined [16]; however, to fully exploit the biotechnological potential of the selected yeast strain, it is necessary to optimize microbial fermentation process [41]. The yield and productivity of SCP production are strongly dependent on culture medium composition, environmental conditions, and selected microorganisms [6], [42]. According to the literature, the main factors that affect yeast biomass growth and SCP content are pH, temperature, time of cultivation and requirements for carbon, nitrogen, and trace elements. [32], [43]–[46].

Microorganisms can respond to environmental changes and the availability of nutrients and trace elements in the fermentation medium. This ability is essential for the metabolism, growth, and reproduction of microorganisms. Yeast metabolism is a set of complex enzymatic reactions to substrate components penetrating the cell wall membrane. Therefore, it is crucial to consider that the lack of necessary elements in the fermentation medium or unfavourable conditions turns on the survival mechanisms in cells, which provokes the inhibition of anabolic processes, such as protein synthesis [47], [48].

2.1. Selection of yeast species

Various yeasts species have been used for numerous industrial applications. SCP qualifies as an excellent source of nutritive proteins, but other cellular components synthesized during fermentation also add value to the resulting biomass [6]. Besides SCP production, *Yarrowia lipolytica* is widely used in the production of lipids, erythritol and citric acid [49], *Saccharomyces cerevisiae* for bioethanol and ergosterol production [50], [51], *Kluyveromyces marxianus* for hydrolytic enzymes such as inulinase, lactase, pectinase, lipase production [52], Rhodotorula sp. for carotenoids such as β-carotene, γ-carotene, torulene and torularhodin [53], Candida sp. for biosurfactants and lipases production [54]–[56], *Pichia kudriavzevii*, *Blastobotrys adeninivorans* and *Wickerhamomyces anomalus* for biogas production [57]. Consequently, the production of SCP from yeast biomass can be widely used in related industries for the production of other cellular metabolites. For example, in the study by Ohlsson *et al.* [57] biomass of three yeast species, *P. kudriavzevii*, *B. adeninivorans* and *W. anomalus,* were examined for yeast SCP and biogas co–production. The authors concluded that this technology has potential after further optimizing cultivation parameters [57].

According to the studies summarized in Table 1, yeasts of the genus Candida are most often used for protein production, which are able to accumulate approximately 39–79 % of protein in dry cell biomass. Yeasts, such as *Candida utilis, Candida tropicalis, Candida pararugosa,* and *Candida guilliermondii*, are capable of utilizing various carbon containing substrates, making them an excellent source of protein [19], [22], [34], [58]–[60]. *Yarrowia lipolytica*, another specie of yeast, commonly used for single-cell oil production, however, can also be used for single–cell protein production [24]. Studies report high protein content in the cells of the preceding species, about 46–71 % [24], [46]. Other yeast species such as *Rhodotorula glutinis*, *Pichia stipitis*, *Blastobotrys adeninivorans* and *Wickerhamomyces anomalus* show satisfactory results, 30–46 % of protein content [28], [34], [57]. However, when evaluating the protein content of yeast species, biomass yield and the factors influencing them should be considered, which is described in the sections below.

2.2. Carbon source

Production of single-cell protein by fermentation process has been mentioned by many researchers (see Table 1). It is well established that most yeasts use sugars as their main

carbon and energy sources; however, there are some yeasts that can utilize non–conventional carbon sources such as starch, alcohols, polyols, hydrocarbons, and fatty acids [61]. For example, *Y. lipolytica* can metabolize a limited range of hexose sugars such as glucose, fructose, and mannose, but it can utilize acetate, alcohols, and hydrophobic substrates, including oils, alkanes, and fatty acids [49]. The ability of oleaginous yeast to utilize hydrophobic substrates is due to the presence of specific enzymes [62]. Noteworthy, the biosynthesis of protein in oleaginous yeasts such as *Y. lipolytica, R. glutinis, C. tropicalis* [63] cells is competitive to the lipid accumulation [15] therefore, it is especially important to observe certain culture conditions such as the ratio of carbon to nitrogen, as described below.

A number of agro-industrial waste and by-products have been used for the production of SCP and other metabolites, including glycerol [24], cheese whey [20], waste milk [21], different fruits peels [18], industrial waste cooking oil [64], salad oil manufacturing wastewater [30], potato processing wastewater [23], [65], olive mill wastewater [22], organic fraction of municipal solid waste [57]. Table 1 summarizes studies with the biomass and protein content using different substrates, cultivation conditions, and yeast species.

For microbial cultivation, a single carbon source is often used, although the use of multiple substrates can positively affect biomass yield and improve protein concentration. Generally, microbial metabolism varies significantly when fermentation medium is presented with mixed carbon substrates compared to a single carbon source, as different nutrients interact in complex ways within the metabolic network [45]. It is important to choose suitable substrates in the optimal ratio for each species of microorganism. Kurcz *et al.* [60] found that the addition of 5 % glycerol to the potato wastewater medium increased yeast *C. utilis* biomass and protein yield compared to the glycerol–free medium, but when glycerol concentration in the medium is above 10 %, the opposite effect is viewed, biomass yield and protein content decreased. The authors suggest that a higher concentration of glycerol inhibits the growth of *C. utilis*. On the other hand, the authors suggest that part of the glycerol assimilated by the yeast was probably used in the biosynthesis of other cell components, which led to a decrease in the proportion of protein components of the yeast biomass as a consequence [60].

TABLE 1. DIFFERENT SUBSTRATES USED AS A CARBON SOURCE AND SCP CONTENT IN DIFFERENT YEAST SPECIES

Yeast specie	Carbon substrate	Cultivation conditions			DCW, g/L	SCP, %	Ref.
		Cultivation time, h	T, °C	pH			
Saccharomyces cerevisiae	Glycerol	48	28	5.5	n/d	47.9	[24]
	Mango residue	30	30	4.0	15.28	79.1	[41]
Candida utilis	*Opuntia ficus-indica* hydrolysate	50	35	5.0	12.2	14.0	[58]
	Potato wastewater	48	28	5.0	5.65	48.9	[60]
Candida tropicalis	Soy molasses	30	30	5.5	10.83	56.4	[19]
	Sugarcane bagasse hydrolysate	96	30	5.0	16.97	60.1	[59]
	Sugar beet pulp	10	30	4.5	16.21	47.8	[34]
Candida pararugosa	Olive mill wastewater	96	30	n/d	21.68	39.4	[22]

Candida guilliermondii	Sugar beet pulp	10	30	4.5	15.5	49.2	[34]
Yarrowia lipolytica	Glycerol	48	28	5.5	n/d	46.7	[24]
	Waste cooking oil	120	28	n/d	57.37	12.6	[64]
	Olive fruits wastes	n/d	30	5.0	14.40	71.0	[46]
Kluyveromyces marxianus	*Opuntia ficus-indica* hydrolysate	50	40	5.0	11.1	10.0	[58]
Rhodotorula glutinis	Potato wastewater and 5 % glycerol	72	28	5.0	19.24	40.5	[42]
Pichia stipitis	Sugar beet pulp	10	30	4.5	19.54	45.6	[34]
Pichia kudriavzevii	Biogas substrate	12–15	30	7.0	7.36	32.7	[57]
Schwanniomyces etchellsii	Olive mill wastewater	96	30	n/d	15.11	35.9	[22]
Blastobotrys adeninivorans	Biogas substrate	12–15	37	7.0	14.83	30.5	[57]
	Spruce sugar hydrolysate	28	30	5.0	27.62	42.45	[28]
Wickerhamomyces anomalus	Biogas substrate	12–15	30	7.0	7.03	22.6	[57]
	Spruce sugar hydrolysate	24	30	5.0	29.78	41.22	[28]

Note: DCW – dry cell weight (grams per liter of medium); SCP – single–cell protein content (% of DCW); n/d – not defined.

2.3. Nitrogen source

During protein synthesis, nitrogen is one of the significant factors due to the structure properties of proteins [37]. Different sources of nitrogen like ammonia, ammonium salt, nitrate, urea, and organic nitrogen in different substrates such as potato and starch processing wastes and cheese whey, are consumed by microorganisms [1], [16], [32]. Yeasts are capable of utilizing a range of different inorganic and organic sources of nitrogen for incorporation into the structural and functional nitrogenous components of the cell, such as amino acids, peptides, proteins, polyamines, nucleic acids, and vitamins [47]. While yeast cells can use a variety of nitrogen–containing compounds as the sole nitrogen source, they show a hierarchical preference for those sources. Therefore, the growth rate and the type of synthesized metabolite depend on the quality and amount of available metabolizable nitrogen [66]. Most yeast strains prefer glutamine or ammonia but will use other nitrogen sources, although with a lowered growth rate [66]. This is due to the fact that yeasts can use ammonium ions as the sole source of nitrogen since they possess genes encoding enzymes for the biosynthesis of all amino acids. The ammonium ions that are either supplied as a nutrient or are derived from the catabolism of other nitrogenous compounds can be directly assimilated and then serve as donors of the amino acid synthesis [47].

In the study by Arous *et al.* [22] preferred carbon source for cultivation of *S. etchellsii* and *C. pararugosa* was ammonium salts (ammonium chloride and ammonium sulphate), in comparison with the addition of potassium nitrate, soy protein, and yeast extract gave 3–8 times lower biomass yield [22]. In another study by Umesh *et al.* [32], the highest biomass

yield and protein content was obtained from *S. cerevisiae* when the medium contained beef extract and yeast extract as nitrogen sources over ammonium nitrate, ammonium sulfate, urea, and sodium nitrate [32]. Between inorganic nitrogen sources preferable for protein production by *C. utilis* were ammonium sulfate, urea, and ammonium chloride compared with potassium nitrate and sodium nitrate [44]. Interestingly, the selection of nitrogen sources can significantly improve the utilization of xylose by yeast. Wu *et al.* [67] report that urea efficiently improved xylose consumption by *C. intermedia* in corncob and silver grass straw hydrolysate compared to ammonium nitrate, ammonium chloride, and diammonium hydrogen phosphate had a negative effect on xylose consumption [67].

2.4. Carbon to nitrogen ratio

The initial carbon to nitrogen (C:N) ratio is a very important factor for substrate reduction, biomass production, and protein content [22], [30], [68]. C:N ratio of 10:1 is reported as the most appropriate result as the same ratio is presented in the microorganisms. A higher ratio will cause the disappearance of nitrogen before all carbon is consumed and the required biomass will not be obtained [1]. When nitrogen is limited, yeast cells slow their growth, while in the extreme case of nitrogen depletion, cells stop growing even with all other nutrients available in excess and enter a nitrogen–specific passive state [66]. At the ratio of 1:1, most nitrogen cannot enter cells and will be wasted [1]. For oleaginous yeasts, mechanisms are similar, i.e., with distinction at low nitrogen level, yeasts switch the metabolic pathway of protein synthesis to lipid synthesis [69]. Zheng *et al.* [30] recommended a C:N ratio from 5:1 to 8:1 for SCP production of *C. utilis* OZ993, at which protein content results in 48–49 %. However, between these ratios, the highest biomass was achieved at 5:1 C:N ratio. On the other hand, lower C:N ratios adversely affected the cellular protein content of *C. utilis*. There was a substantial reduction in the protein level of cells, from 49 % to 18 %, when the C:N ratios gradually declined from 5:1 to 1:1 [30].

A similar conclusion was achieved in other researchers works. Arous *et al.* [22] report that the optimal C:N ratio was 8:1–10:1 for *S. etchellsii* and *C. pararugosa* with higher biomass production on oil mill wastewater–based medium with ammonium chloride supplementation [22]. In the study by Spalvins *et al.* [64], the highest SCP content in *Y. lipolytica* biomass was observed at 5:1–10:1 C:N ratio cultivated on waste cooking oil contained medium [64].

2.5. Supplementation of trace elements

An important role in SCP production is the trace elements addition to the fermentation medium. Yeasts require a range of metals for optimal growth, metabolism, and fermentation performance. The requirement for metal ions varies widely with the different strains, so it is necessary to adjust the composition of the medium to avoid the inhibitory effects of trace elements on the growth of selected microorganisms [31]. The most requested is phosphate, magnesium, calcium, potassium, zinc, and iron [12], [34], [37], [55], [70]. Yeast cells are starved for phosphate and sulphur arrest in a quiescent state in which fermentation of glucose is suppressed: external glucose is not depleted [66]. Daskalaki *et al.* [71] report that *Y. lipolytica* completely assimilates existing nitrogen in the medium within 48 hours; however, when the amount of carbon in the medium is depleted, the addition of nitrogen and magnesium causes an increase in the protein content of the biomass [71]. This mechanism is explained in the work of Dourou *et al.* [72], where the life cycle of oleaginous microorganisms has been described. After the depletion of the carbon source in the medium or due to a low uptake rate, the oleaginous microorganisms utilize their own storage lipids as an energy source for

maintenance purposes or as an intracellular carbon source for the production of new lipid–free cell components, provided that essential nutrients are available in the fermentation medium [72].

Gao *et al.* [19] showed that the addition of $CaCl_2$ in the medium is important for the production of SCP by *C. tropicalis*. The biomass production and total protein content increased when 0.05 g/L $CaCl_2$ was supplemented to soy molasses medium, where the addition of NaCl, $MgSO_4 \cdot 7H_2O$, K_2HPO_4, and $ZnSO_4 \cdot 7H_2O$ in the same amount had no notable effect [19]. In turn, the study by Nicolas *et al.* [31] provides a more detailed overview of the effect of different salt concentrations on the growth of the yeast *C. utilis*. Mineral salts KH_2PO4, $MgSO_4$, $FeSO_4$, and KCl at 0.2, 0.07, 0.002, and 0.8 g/L concentrations significantly increased biomass production [31].

Kieliszek *et al.* [48] optimized medium with 0.02 g/L selenium supplementation enriched *S. cerevisiae* and *C. utilis* biomass functional diversity in terms of protein and amino acid content. Yeasts of both strains enriched with selenium contained a large amount of glutamic acid, aspartic acid, lysine, valine, histidine, and leucine. An analysis of the amino acid composition of *C. utilis* yeast biomass enriched with selenium showed that the concentration of the most important amino acids lysine was at a higher level (8.3 %) as compared to that of the biomass obtained without the addition of this element (5.6 %). Moreover, after cultivation in the medium supplemented with selenium, the total amino acid content for both *C. utilis* and *S. cerevisiae* strains was higher and increased by approximately 12 and 5 %, respectively. However, the total protein content in the biomass of *S. cerevisiae* and *C. utilis* slightly decreased as compared to that in the control sample without additional selenium supplementation from 48.4 % and 42.1 % to 42.6 % and 37.0 %, respectively [48].

2.6. Temperature and pH

Another important factor in successful yeast cultivation is the choice of temperature and pH of medium. According to the literature, the most common temperature used for protein biosynthesis by yeast species is 25–30 °C [22], [24], [46], [64], [73]; however, for some yeasts, such *C. util*is and *K. marxianus* a temperature of 35 and 40 °C can be used [58]. Low temperature can inhibit nutrients from crossing the cell membrane, while a high temperature may inactivate enzymes of the metabolic pathway [22]. The optimal pH value for yeast growth is 4.0–7.0 [18], [34], [41], [74]; within this range, the most preferred pH of the medium for protein biosynthesis is 5.0–5.5, as described below.

Siddique *et al.* [18] report that the optimal conditions for the cultivation of *C. tropicalis* cultivated in 2 % watermelon peels hydrolysate were 37 °C and pH 7. In the other study by Gao *et al.* [19], the maximum biomass and protein content of *C. tropicalis* were attained when the initial pH was 5.5 at 30 °C. The highest biomass yield and protein content in Umesh *et al.* [32] work was obtained from *S. cerevisiae* cultivated at 25 °C and pH 5 in comparison with the lower result at 15, 37, 45 °C and pH 4, 6, 7, and 8. In the study by Jalasutram *et al.* [44], the *C. utilis* was cultivated in the range of pH 3 to 9 at 30 °C; the maximum protein concentration was obtained at 6 pH and lowest at pH 3, 4, 8, and 9. The authors note that the activity of enzymes involved in protein production decreases under strongly alkaline and acidic conditions of cultivation [44].

For *Y. lipolytica* the optimal conditions for protein production were 30 °C and pH from 5.0 to 6.0, where the highest biomass was obtained at pH 5.0. In these conditions the yeast produced 40–50 % of the protein in its dry cell weight cultivated on medium based on wastes from biodiesel production (vegetable oils, degumming, and glycerol fractions) [15]. These environmental conditions appropriate for *Y. lipolytica* cultivation in olive fruits waste–based medium is consistent with another study by Rages *et al.*, [46], which observed the highest

protein concentration of about 71 %, which has been reached at 30 °C and pH 5. With an increase in pH from 5.5 to7.5, a decrease in the amount of protein in the biomass was observed. In contrast, media with an initial pH of 6.5 resulted in a higher biomass yield, with 55 % content of protein [46]. Kot *et al.* [74] noted that the pH value in the range of 4.0–7.0 did not significantly affect the amount of protein in the *R. glutinis* biomass, which varied from 38.5 % to 41.3 % after 72 h of cultivation. However, maximum biomass production and protein content was obtained at pH 5.0 [74].

2.7. Time of cultivation

Zakhartsev *et al.* [75] described how the temperature of microorganism cultivation affects the kinetics of key molecular processes in the cell, thereby affecting the biomass specific growth rate[†]. On the other hand, a specific growth rate affects the macromolecular composition of growing microbial cells. For example, in carbon limited continuous cultures of yeast *S. cerevisiae* at low biomass specific growth rate ($\mu_{max} < 0.1$ h^{-1}) the carbohydrates content is up to 50 % and proteins content is up to 40 % of the dry biomass, whereas at high growth rate ($\mu_{max} > 0.3$ h^{-1}), the carbohydrates content linearly decreases to 15 % and proteins content increases up to 60 % of the dry biomass [75]. This may explain the high values of the protein content in a short cultivation time as described in the studies of Patelski *et al.* [34] and Lapena *et al.* [28], were 46–49 % SCP in the biomass of *C. tropicalis*, *P. stipites*, *C. guilliermondii*, and 47–51 % in *C. jadinii*, *W. anomalus*, *B. adeninivorans* were obtained after 10 and 12 h of cultivation, respectively.

One of the highest protein content of 69% is mentioned in a study by Rages *et al.* [46], in cultured *Y. lipolytica* for 96 h. However, the amount of biomass was not high, only 13.10 g per liter. In the following days of cultivation, the amount of protein decreased, and at the time of the 8th day of incubation, it dropped to 45.63%. In turn, the maximum biomass was reached on the 6th day of cultivation [46]. In studies by Dharumadurai *et al.* [17] and Rajendran *et al.* [73] *S. cerevisiae* reached higher biomass content after 168 h of fermentation, but the maximum protein content was recorded at 72 h of fermentation at a 5 % concentration of pineapple waste hydrolysate and papaw and banana fruit juices, respectively. Other results were obtained in the Umesh *et al.* [32] work when *S. cerevisiae* was cultivated on papaya waste hydrolysate. Higher biomass and protein content was obtained on 120 h of cultivation. In another study by Kurcz *et al.* [60], protein content in *C. utilis* dry biomass was higher after 48 h of fermentation in a potato wastewater–based medium, and after 72 h it decreased from 43.5 to 41.7 %, respectively. A similar tendency was observed when glycerol was added to the potato wastewater medium in the amount of 5 % and 10 %. However, when glycerol was added to the medium in a volume of 15–25 %, the protein content was higher by 72 h, which indicates that the time of protein accumulation depends on the constituent components of the medium [60].

The cultivation time of yeasts is an important parameter for protein production, so it is necessary to perform experimental studies on the influence of the quantity and quality of the substrate on the biomass growth rate and the rate of substrate consumption in order to properly assess the optimal time for biomass harvesting.

2.8. Inoculum size

The size of the inoculum (population of microorganisms or cells that is introduced in the fermentation medium) is another important factor in starting the fermentation process and

[†] μ_{max} – maximum specific growth rate of biomass [g_{dw}/ (g_{dw} · h)] or [h^{-1}].

influencing single–cell protein production [22], [41], [44], although studies describing the effect of different inoculum sizes on protein production are rare. The optimum inoculum size varies for different microorganisms [16], and depends on the total concentration of dissolved oxygen and nutrients in the fermentation medium [22], [44]. For example, in a study by Jalasutram *et al.* [44], for *C. utilis* inoculum ranging from 2 to 10 % v/v protein production was increased with the increase of inoculum size with an optimum at 6 %, after which the level of SCP production was decreased. The authors note that cultivated yeast with an initial inoculum concentration of 6 %, dissolved oxygen, and consumed oxygen were at an equilibrium level [44]. Similar results were obtained in Arous *et al.* [22], when the effect of inoculum size 2 %, 5 %, 7 %, and 10 % on the biomass production of *C. pararugosa* and *S. etchellsii* strains were tested. Under optimized cultivation conditions, the addition of 2 to 5 % inoculum of *C. pararugosa* led to maximum biomass production after 4 days of incubation. 7 % inoculum size addition caused a drop in yeast biomass to approximately 28 % as compared to biomass obtained with 5 % inoculum size. Similarly, a slight decrease in the biomass production of *S. etchellsii* was observed when the inoculum size exceeded 7 %; however, there was no significant difference in biomass production of this strain when different inoculum sizes were tested. The authors concluded that with large inoculum sizes (higher than 7 %), the nutrients in the growth medium might be rapidly consumed which results in low rate of yeast growth and cell survival [22]. In another study by Somda *et al.* [41], the optimization of SCP production of *S. cerevisiae* was based on two parameters: a substrate concentration of 5 to 10 % (g/L) and an inoculum size of 2 to 12 % (v/v). The highest protein content of 79 % was achieved at 8 % inoculum and 8 % mango residues concentration [41].

Thus, to optimize the protein production process, it is necessary to comprehensively consider the influence of different cultivation parameters, taking into account not only the composition of the fermentation medium, but also the preferences and characteristics of each species of yeast.

3. SCP AMINO ACID COMPOSITION

Yeast biomass has been successfully used in fish feed formulations, both at low and at higher inclusion amount as fishmeal replacement [57], [76]. The nutritional value of yeast primarily depends on the content of protein and its amino acid composition, as well as on the content of lipid, vitamin and minerals [4], [24]. Amino acids are significant biomolecules that serve as protein building blocks and are intermediates in various metabolic pathways in organisms [77]. According to the FAO report [78], the protein and amino acids requirements for different animal species are different. Fish diets have higher protein requirements than mammalian diets, and protein requirements decrease with age [78]. Essential amino acids required in aquatic animal diets are lysine, methionine, and threonine, and these three amino acids are limited in plant–based feed such as soybean, corn, and rice [5], [35], [36]. Single-cell proteins are generally well digested by fish and crustaceans and comprise a protein content and amino acid profiles similar to fishmeal [14], [79].

Table 2 summarizes the composition of amino acids in SCP of yeast biomass compared with other sources. The biomass of yeasts like *C. utilis*, *C. tropicalis*, *Y. lipolytica* and *S. cerevisiae* is rich in lysine, threonine, valine, and leucine [19], [24], [30], [31]. Although the concentration of essential amino acids in yeast protein varies between species and strains of the same species and depends on the substrate used in the studies, added trace elements and cultivation conditions [33], [48].

TABLE 2. ESSENTIAL AMINO ACID COMPOSITION OF DIFFERENT PROTEIN SOURCES

Protein source	Essential amino acids content, g/ 100 g of protein									Ref.
	Lys	Thr	Val	Met	Ile	Leu	Phe	His	Arg	
C. utilis	7.8	4.7	4.0	1.0	4.1	7.9	3.4	1.5	4.4	[30]
	5.14	4.1	5.5	1.58	4.8	7.12	4.1	n/a	3.2	[31]
C. tropicalis	6.91	4.35	4.58	2.27	4.00	6.24	3.71	n/a	n/a	[19]
Y. lipolytica	6.2	4.2	4.7	1.4	4.0	7.1	3.9	2.5	4.8	[24]
S. cerevisiae	6.5	4.6	4.9	1.4	3.7	6.4	3.3	2.4	4.7	[24]
	2.5	3.3	3.1	3.2	2.6	2.9	3.0	2.8	1.4	[32]
K. marxianus	n/a	6.94	7.5	0.77	5.48	7.74	3.58	1.9	n/a	[33]
W. anomalus	1.41	n/a	0.96	0.24	0.87	1.36	0.84	0.35	0.87	[57]
	3.06	1.89	1.95	0.33	1.84	2.90	1.63	1.12	2.57	[28]
Soybean meal	3.41	1.95	2.61	0.72	2.59	4.13	2.71	1.34	3.86	[80]
Corn protein isolate	1.0	1.8	2.1	1.1	1.7	8.8	3.4	1.1	1.7	[36]
Rice protein isolate	1.9	2.3	2.8	2.0	2.0	5.8	3.7	1.5	5.4	[36]
Fish meal	6.79	3.97	3.93	2.50	3.35	6.25	3.26	1.97	5.23	[81]

Note: n/a – not analysed

In general, yeasts have lower methionine content than fishmeal; however, some studies show that good results can be achieved under optimal fermentation process conditions. In studies by Gao *et al.* [19] and Umesh *et al.* [32], methionine content in yeast species *C. tropicalis* and *S. cerevisiae* was reported to be 2.27 and 3.2 g per 100 g of protein. Among the most commonly used crops, only rice protein comes close to the value of the essential amino acid in fishmeal. Among plant proteins, rice also has a high content of 5.4 % arginine and 3.7 % phenylalanine, and corn has a higher content of 8.8 % leucine [36]. Among plant proteins, rice also has a high content of 5.4 % arginine and 3.7% phenylalanine, and corn has a higher content of 8.8 % leucine. In turn, soybean meal has a higher content of lysine and isoleucine but in a smaller amount compared to fish meal and yeast protein [19], [24], [30], [31], [33], [80], [81]. Therefore, protein-rich yeast biomass appears to be a sustainable alternative to fishmeal and plant origin proteins.

4. CONCLUSIONS

The research results reviewed in this study show that yeast culture conditions such as temperature, pH, and time of cultivation have a huge impact on the protein content of yeast biomass. The most preferred pH and temperature of the medium for SCP production by yeasts are pH 5.0–5.5 and 28–30 °C. Time of cultivation is an important criterion for harvesting protein–rich biomass since in the later stages of fermentation, when the maximum biomass is reached, the protein content in it already decreases. This feature must be considered and investigated for each selected strain growing on a specific medium composition. Properly selected carbon to nitrogen ratio also has a strong influence on protein content, since the metabolic pathways of yeasts are directly related to the available amount of carbon and nitrogen. According to literature, the optimal C:N ratio for protein production is 5:1–8:1. The addition of trace elements also has a positive effect on biomass growth and can affect the amino acid profile. At the beginning of the fermentation process, an important parameter is

the size of the inoculum, which can affect the growth rate of the biomass. In conclusion, it should be noted that optimally selected cultivation conditions and skilfully designed culture medium composition allows the production of high protein content with a well–balanced amino acid profile.

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Improving single cell protein yields and amino acid profile via mutagenesis: review of applicable amino acid inhibitors for mutant selection

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Abstract. Single cell protein (SCP) is a good alternative for substituting plant and animal derived dietary proteins, since SCP production is more environmentally friendly, consumes less water, requires smaller land areas and its effect on climate change is much less pronounced than it is in the case of agriculturally derived proteins. Another advantage of SCP is that it is possible to use a wide variety of biodegradable agro-industrial by-products for the cultivation of SCP producing microorganisms. However, to make single cell protein technology more widely available and improve its economic viability in such markets as animal and fish feed industries, it is necessary to improve the protein yields and amino acid profiles in microorganism strains capable of using agro-industrial by-products. One way to improve the strains used in the process is to create and select SCP-rich mutants. In this review authors propose a novel approach to create SCP-rich mutants with improved total protein content and essential amino acid profiles. In this approach amino acid inhibitors are used to create selective pressure on created mutants. It is expected that mutants with the most pronounced growth would either have higher total protein content, increased essential amino acid concentrations or both, when cultivated on selective plates containing one or multiple amino acid inhibitors. This paper reviews the most suitable groups of amino acid inhibitors that could be used for selection of new strains of SCP-producing microorganisms.

Key words: mutagenesis, microbial protein, essential amino acids, amino acid inhibitors, herbicides, low-cost substrate, agricultural residues.

INTRODUCTION

Single cell protein as sustainable feed ingredient

Proteins are a group of nutrients that both humans and animals use as a major source of amino acids. Of the twenty-one amino acids found in living creatures, nine are not synthesized in the human body and need to be consumed via diet to maintain proper functionality of the human body. Four of these nine essential amino acids - lysine, methionine, threonine, and tryptophan, are not available in sufficient quantities in plant-derived products (Wang et al., 2017), thus animal sourced foods need to be included in

diet to prevent various health problems related to protein deficiency in the long term (Wang et al., 2017).

Livestock products are the main source of complete protein worldwide (Martin, 2001). Today, livestock products (meat, milk, eggs) provide more than 33% of the total protein intake in human diets (Martin, 2001). Rapidly growing human populations and growing consumer demand are generating large demand for animal products (Chadd et al., 2002). This leads to the need to identify alternative sources of protein to ensure a sustainable supply of animal feeds.

Although livestock farming is a major source of protein, calorie wise it is a very inefficient industry. Only 3–7% of the calories consumed by farm animals are converted into live weight (Shepon et al., 2016). For this reason, 83% of the world's agricultural land is used for production of livestock feeds (Poore & Nemecek, 2018). For example, in order to produce 1 kg of meat (beef), it requires 25 kg of grain and 15,000 litres of water (Mekonnen & Hoekstra, 2010). If these areas were used to grow direct human foods, our planet could feed an additional 3.5 billion people (Cassidy et al., 2013). Increasing the use of single cell protein in livestock feeds would reduce the need for intensive farming, which has a very negative impact on local ecosystems and species diversity worldwide.

Globally, seafood is also a very important source of protein for humans. On average, fish and crustaceans account for 17% of the world's protein intake (FAO, 2014). Since 2014, most of the fish and crustaceans consumed by humans are produced in captivity (FAO, 2016). This increase in farmed fish and crustaceans is mainly driven by rapidly growing demand for fish products, the depletion of fish populations in the wild and rapidly developing aquaculture industry (Tacon & Metian, 2015). Although the aquaculture industry has surpassed the wild capture fisheries in terms of production volume (Tacon & Metian, 2015), wild capture fisheries is still the main source of feed for aquaculture industry. Wild capture has remained stagnant over the last 20 years and now is no longer able to adequately supply the aquaculture industry with fishmeal (protein source) used as feed for farmed fish (Tacon & Metian, 2015). Thus, aquaculture had to look for new sources of feeds and currently the main source of protein for aquaculture fish is soy (Tacon & Metian, 2015). Soy lacks essential amino acids recommended for use in animal feeds. As a result, the aquaculture industry, same as livestock industry, has also become dependent on agricultural inputs. In addition, plant derived feeds are unsuitable for intestinal tract of predatory fish (salmonids etc.), which is one of the causes of poor health of aquaculture fish, fish are more likely to die and large amounts of antibiotics are needed to treat the various diseases (FAO, 1980).

In general, both livestock and aquaculture industries need to find new sources of protein-rich feeds that contain all the amino acids needed for a complete diet for farmed animals. Single cell proteins produced by using agro-industrial by-products are considered a very promising alternative. The authors have reviewed applicability of most of these by-products in previous articles (Spalvins et al., 2017; Spalvins et al., 2018; Spalvins & Blumberga 2018; Spalvins & Blumberga 2019; Spalvins et al., 2019). This technology is based on the cultivation of protein-producing microorganisms (bacteria, yeasts, fungi, and microalgae) using biodegradable agricultural residues and production by-products as the main source of nutrients for microbial growth. Not only would this technology produce protein-rich feed containing all the essential amino acids, but the feed itself would be cheaper, since inexpensive by-products of other industries would be

used as production substrates (Spalvins et al., 2017; Spalvins et al., 2018; Spalvins et al., 2020). Additionally, by replacing agriculture derived feeds with SCP, the health of the fish is considerably increased because these proteins are more easily digested (FAO 1980) and in its composition SCP is much more similar to feed these species of fish can acquire in wild (plankton: microalgae, bacteria, fungi etc.) (Finco et al., 2017; Spalvins & Blumberga 2018).

Creation of improved SCP-producing strains via mutagenesis

The single-cell protein production technologies have been extensively researched for decades, and the single-cell protein market in 2017 reached a total revenue of $ 5.3 billion (P&S Intelligence 2018). Although SCP market has been steadily growing and more and more SCPs produced from by-products are being introduced to the market (Ritala et al., 2017), there is always room to improve novel or already well-known SCP-producing microorganism strains. Regarding increased SCP yields and production efficiency, various properties can be improved in microorganism strains, such as, biomass concentration/cell density, protein concentration in biomass, growth speed, utilization efficiency of the selected by-products, tolerance to harmful compounds present in selected by-products etc.

Existing strains can be replaced by novel species or different strains by isolating microorganisms from areas which have been polluted with contaminants (agro-industrial by-products) that could be used as the main feedstock for SCP production. By isolating strains from contaminated areas it is possible to find strains that have adapted to utilize specific by-products more efficiently. Examples of such approach has been widely reported (Mehta 1973; Wong & Chan, 1980; Kim & Lebeault, 1981; Ivarson & Morita, 1982; Baldensperger et al., 1985; Kornochalert et al., 2014; Yadav et al., 2016; De Gregorio et al., 2002). In this approach appropriate strains can be selected from nature by looking for various beneficial factors such as: growth speed, protein content, temperature optimum, maximum tolerated temperature, salinity, shear tolerance, growth on particular substrates, growth in selective environment etc. (Borowitzka & Moheimani, 2013).

Another approach is to introduce the desired properties in existing strains. Acquisition of better microorganism strains is very important for the development and improvement of technological solutions based on SCP production. Microorganisms can be improved using both classical mutagenesis and modern genetic engineering methods combined with advanced screening methods. Although use of modern solutions is increasing (Yan et al., 2018; Leavell et al., 2020; Tatenhove-Pel et al., 2020), classical mutagenesis and random screening methods are still considered to be simple and efficient for short-term strain development (Rowlands, 1984; Anderson, 1995; Winston, 2008; Atzmüller et al., 2019) and are still widely used (Sivaramakrishnan & Incharoensakdi, 2017; Yamada et al., 2017; Zhu et al., 2018; Atzmüller et al., 2019). Mutagenesis accelerates the mutations frequency rate up to 100 times when compared to natural mutation rate (Winston, 2008). Mutagens can be divided into physical, such as UV-light, gamma and X-rays, and chemical, such as ethyl methane sulphonate (EMS), nitrosomethyl guanidine (NTG), etc. (Rowlands, 1984; Anderson, 1995). After treatment with the mutagen, the surviving strains must be selected, and this can be done by screening as many mutants as practically possible or by using selective media in which only those mutants that have acquired the desired properties will develop. For example,

UV-mutagenesis has been widely used for creation of mutants with improved lipid production capabilities (Sivaramakrishnan & Incharoensakdi, 2017; Yamada et al., 2017; Zhu et al., 2018; Atzmüller et al., 2019). It is possible to select suitable mutants using fatty acid biosynthesis inhibitors such as cerulenin (Omura, 1976; Tapia et al., 2012; Katre et al., 2017; Atzmüller et al., 2019), and it has been shown that a mutant with a higher lipid production capacity will form larger colonies on a selective plate, thus making mutant selection quick, convenient and simple. If produced lipids are used for example as a feedstock for biodiesel production, changes in fatty acid composition due to mutation is not that critical for the biodiesel production process (Atzmüller et al., 2019). If lipids are used as feed, then changes in fatty acid composition need to be accounted for as that directly affect health and feed conversion ratios of the farm animal (Long et al., 2020). It is also the same in case with SCP when used for animal or fish feed. Although total protein concentration in microbial biomass is a significant factor, the concentration of essential amino acids is the main factor that determines the value of the obtained protein. For example, such essential amino acids as lysine, methionine, threonine and tryptophan are very important components in fish feeds, as they are available in lower amounts in conventional protein sources such as soy (Al-Marzooqi et al., 2010; Hardy et al., 2018). Thus, in creating SCP mutants, it is important not only to increase the total protein concentration in the biomass of microorganisms, but also to increase the proportion of essential amino acids. Just as in the case with using cerulenin to select mutant strains with enhanced fatty acid synthesis capabilities (Omura, 1976; Tapia et al., 2012; Katre et al., 2017; Atzmüller et al., 2019), it could be possible to use amino acid inhibitors to select for improved SCP-rich mutants. Mutants with increased protein and specific amino acid concentrations would form larger colonies on selective plates that contain one or multiple amino acid inhibitors. The largest colonies could be then picked for further screening, i.e., testing of growth rate, cell density, protein content, amino acid composition etc. Unlike fatty acid inhibitors, there are no universal inhibitors of amino acids that inhibit the synthesis of all amino acids, so it is necessary to select different amino acid inhibitors, each individually or in various combinations to select for mutants not only with increased protein concentration but also with improved essential amino acid concentrations.

To the best of the authors knowledge this is the first review to analyse the possibility of using amino acid inhibitors to select for SCP-rich mutants.

AMINO ACID INHIBITORS

Most amino acid inhibitors that are available are used in agriculture as herbicides and this is the intended application also for most of the amino acid inhibitors that are currently in development (HRAC 2002; Berlicki, 2008; Cobb & Reade, 2010; Hall et al., 2020). Therefore, majority of research conducted on using these compounds are regarding their practical and cost effectiveness in weed management (Llewellyn et al., 2016; Hall et al., 2020). Also most of the research available on these herbicides is done on their inhibitory activity on plant biosynthetic pathways, while information on their activity on single-celled organisms such as bacteria, microscopic algae, yeasts, fungi, unicellular protists, archaea etc. is limited. These aspects need to be considered when selecting an amino acid inhibitor for use in SCP-producing mutant selection, as the actual inhibitor response may differ significantly from what was expected. Although

information is lacking most of the amino acid biosynthesis pathways are very similar and many enzymes that are the main targets of inhibitors are the same in different kingdoms (Herrmann & Weaver, 1999; Joshi et al., 2006; Shearer, 2007; Binder, 2010; Hall et al., 2020), therefore most of the inhibitors should promote inhibitory response in microorganisms as well. One additional difference that might be encountered when applying these herbicides on microorganisms is that even if amino acid biosynthesis pathway is shared across kingdoms, in plants many amino acids are synthesized in plastids (Herrmann & Weaver, 1999; Hall et al., 2020), while, for example, bacteria and fungi does not have such structures and the same pathways are localized in cytosol. This fact most likely will affect the inhibitor concentration required to perform the mutant selection, but also might affect some other properties of the inhibitor's effect or promote previously unnoticed side effects such as level of cytotoxicity, level of sensitivity to inhibitory effects, extent of DNA synthesis inhibition, extent of nutrient assimilation impairment, extent of pathway intermediates accumulation, extent of metabolic disruptions etc. (Zhou et al., 2007; Cobb & Reade, 2010; Fucile et al., 2011; Orcaray et al., 2012; Hall et al., 2020). For example, enzyme from histidine biosynthesis pathway imidazole glycerol-phosphate dehydratase can be inhibited by 2-hydroxy-3-(1,2,4-triazol-1-yl) propylphosphonate (Rawson et al., 2018). This inhibitor is much more potent in yeasts than in plants, due to additional β-strand which enhances the binding of inhibiting compound to the yeast enzyme (Rawson et al., 2018), therefore inhibitor concentration should be decreased appropriately if used for selection of yeast mutants. Other inhibitors are isolated from microorganisms e.g., cornexistin is isolated from fungi *Paecilomyces variotii*. If this inhibitor is used for selection of related fungal strains, then it might be the case that these microorganisms show resistance to inhibitory effect (Takahashi et al., 1994). Therefore, considering that the use of herbicides in the selection of microorganisms may lead to unpredictable side effects or render the inhibitor completely ineffective, each compound needs to be tested individually during mutant selection.

Ten of the twenty-one amino acids found in living beings cannot be synthesized by the fish, these are: arginine, phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine and histidine (Andersen et al., 2016). Of these ten, four - lysine, methionine, threonine and tryptophan, are present at lower concentrations in plant derived proteins than recommended in animal and fish feeds (FAO, 1980; Al-Marzooqi et al., 2010; Finco et al., 2017; Hardy et al., 2018). Therefore, if the produced SCP is intended for use in animal or fish feed, during the mutant selection it would be desirable to use amino acid inhibitors that inhibit the biosynthetic pathways of these four amino acids. In this way, selective pressure is applied directly to those mutants that are able to synthesize the relevant four amino acids in larger quantities than respective wild types. If SCP is intended for other applications - human diet supplements, other animal and pet feeds, cosmetics, building block chemicals etc., value of the produced SCP can be increased also if the concentration of any other amino acid is increased via utilization of appropriate inhibitor during the mutant selection. All amino acid inhibitors and their properties regarding mutant selection have been summarized in Table 1.

Table 1. Properties of amino acid (AA) inhibitors and their target enzymes

Inhibitor	Target enzyme	Target present in	Inhibited AA	Pros (+), cons (–), side effects, etc.
Aromatic amino acid inhibitors				
Glyphosate[1]	5-*enolpyruvyl*-shikimate 3-phosphate synthase (EPSPS)[1]	Plants, archea, bacteria, fungi, algae[1]	Phe, Trp, Tyr	(+) Inhibits microbes[41-43] (+) Well studied effects[2,40] (+) Widely available (–) Deregulates carbon metabolism[2] (–) Shikimate accumulation[2]
7-deoxy-sedoheptulase[3] (7dSh)	3-dehydro-quinate synthase (DHQS)[4]		Phe, Trp, Tyr	Cyanobacteria metabolite[5] (+) Inhibits microbes[4] (–) Limited availability[5]
3-indoleacrylic acid[6]	tryptophan synthase (TS)[4]		Trp	Bacteria metabolite[7] (+) Inhibits microbes[7,44] (+) Widely available
Branched chain amino acid inhibitors[8]				
Sulfonylureas Imidazolinones Triazolopyrimidines Pyrimidinyl (thio)benzoates Sulfonylaminocarbonyl triazolinones	Acetolactate synthase /acetohydroxy-acid synthase (ALS/AHAS)[4]	Plants, archaea, bacteria, fungi[10]	Ile, Leu, Val	(+) Inhibits microbes[45-49] (+) Well studied effects[45-49] (+) Widely available (–) Cause intermediates accumulation[4,9] (–) DNA synthesis inhibition[4,9] (–) Disrupted assimilates transport[4,9]
Histidine inhibitors				
2-hydroxy-3-(1,2,4-triazol-1-yl) propylphosphonate[16]	Imidazole glycerol-phosphate dehydratase (IGPD)[16]	Plants, bacteria, fungi, archaea[17]	His	(+) Inhibits microbes[16] (+) More potent in yeasts than plants[16] (+) Widely availability (–) Further research required
3-(diethoxy-phophoryl)-3-(1H-1,2,3-triazol-4-one-1-yl)propan-2-ylcarboxylic esters[18]				(–) Further research required
Monopyrrole aldehydes[19]				(–) Further research required
S-1-(4-biphenyl)-4-(4-imidazolyl)-3-amino-2-butanone[20] (BPIAB)	Histidinol dehydrogenase (HDH)[20]			(–) Inhibitor activity unclear[20] (–) Further research required
Glutamine inhibitors[15]				
L-Phosphinothricin[11] Methionine sulfoximine[14] Tabtoxinine β-lactam[14] Bialaphos[4]	Glutamine synthetase (GS)[11]	Prokaryotes, eukaryotes [12,13]	Gln, Asp, Pro, Arg, Lys, Met, Thr, Ile	(+) Inhibits microbes[50] (+) Well studied effects[50-54] (+) Widely available (–) Ammonia accumulation[11]

Table 1 continued

Glutamate-derived amino acid inhibitors				
Cornexistin[21,22]	Aspartate trans-aminase (AST)[21,22]	Archaea, bacteria, eukaryotes [4,38,39]	Asp, Met, Thr, Ile, Lys	Fungal metabolite[21] (–) No activity in bacteria and fungi[23] (–) Limited availability (–) Further research equired
Phaseolotoxin[24]	Ornithine carbamoyl-transferase (OCT)[24]		Arg	Bacterial metabolite[24] (+) Inhibits microbes[55] (–) Limited availability (–) Further research required
Aminomethylene-bisphosphonates[25] (AMBP)	δ1-pyrroline-5-carboxylate reductase (P5CR) and GS[25]		Pro, Gln, Asp, Arg, Lys, Met, Thr, Ile	(+) More potent in bacteria than plants[26] (+) Inhibits microbes[26] (–) Ammonia accumulation[56] (–) Limited availability
Aspartate-derived amino acid inhibitors				
2-(1-cyclohexen-3(R)-yl)-S-glycine[27] (CHG)	Threonine deaminase (TD)[27]	Plants, bacteria, fungi[28]	Ile	(+) Inhibits microbes[57,58] (–) Limited availability[27] (–) Further research required
DL-Propargylglycine[29,30] (PAG)	Cystathionine γ-synthase (Cγ-S)[29,30]	Plants, bacteria, fungi[35]	Met	(+) Inhibits microbes[59-62] (+) Widely available
Rhizobitoxine[31,32]	Cysteine-S-conjugate β-lyase (Cβ-L)[31,32]	Plants, archaea, bacteria, fungi, animals[36]	Met	Bacteria metabolite[31] (–) Limited availability[34] (–) Further research required
S-(2-aminoethyl)-L-cysteine[33] (AEC)	Aspartate kinase (AK) and dihydrodi-picolinate synthase (DHDPS)[33]	Plants, bacteria, archaea, fungi[37]	Lys	(+) Inhibits microbes[63-67] (+) Widely available
L-α-(2-amino ethoxy-vinyl) glycine[33] (AVG)	DHDPS			

[1](Herrmann & Weaver, 1999); [2](Orcaray et al., 2012); [3](Schultz & Coruzzi, 1995); [4](Hall et al., 2020); [5](Brilisauer et al., 2019); [6](Widholm, 1981); [7](Wang et al., 2013); [8](HRAC 2002); [9](Zhou et al., 2007); [10](Binder, 2010); [11](Cobb & Reade, 2010); [12](Forde & Lea, 2007); [13](Patrick et al., 2018); [14](Maughan & Cobbett, 2003); [15](Berlicki, 2008); [16](Rawson et al., 2018); [17](Shearer, 2007); [18](Jin et al., 2015); [19](Schweitzer et al., 2002); [20](Dancer et al., 1996); [21](Amagasa et al., 1994); [22](Nakajima et al., 1991); [23](Takahashi et al., 1994); [24](Mitchell & Bieleski, 1977), [25](Forlani et al., 2013); [26](Forlani et al., 2012); [27](Szamosi et al., 1994); [28](Joshi et al., 2006); [29](Ravanel et al., 1998b); [30](Ravanel et al., 1998a); [31](Okazaki et al., 2007); [32](Giovanelli et al., 1971); [33](Soares da Costa et al., 2018); [34](Okazaki et al., 2007); [35](Goyer et al., 2007); [36](Cooper et al., 2011); [37](Pearce et al., 2017); [38](Singh et al., 2019); [39](Zúñiga et al., 2002); [40](Steinrücken & Amrhein, 1980); [41](Leino et al., 2020); [42](Funke et al., 2006); [43](Morjan et al., 2002); [44](Nonomura et al., 1996); [45](Jia et al., 2000); [46](Kreisberg et al., 2013); [47](Landstein et al., 1995); [48](Lee et al., 2013); [49](Burnet & Hodgson, 1991); [50](Ahmad & Malloch, 1995); [51](Myrold & Posavatz, 2007); [52](Ahmad et al., 1995); [53](Kim & Rhee, 1987); [54](Kulkarni et al., 2006); [55](Staskawicz, 1979); [56](Giberti et al., 2017); [57](Keller-Schierlein et al., 1969); [58](Szamosi et al., 1994); [59](Jin et al., 2004); [60](Piotrowska & Paszewski, 1986); [61](Johnston et al., 1979); [62](Lockwood & Coombs, 1991); [63](Zabriskie & Jackson 2000); [64](Ekwealor & Obeta, 2006); [65](Han et al., 1991); [66](Sano, 1970); [67](Rupp et al., 1989).

1. Aromatic amino acid inhibitors

Aromatic amino acids are phenylalanine, tyrosine and tryptophan (Hall et al., 2020). Aromatic amino acids are synthesized in the shikimate pathway (Tohge et al., 2013). This pathway is found in plants, fungi, bacteria, archaea, microscopic algae and other eukaryotes and prokaryotes (Hall et al., 2020). One of the pathway enzymes - 5-enolpyruvalshikimate-3-phosphate synthase (EPSPS), is a target for the widely used and commercially available herbicide glyphosate/N-(phosphonomethyl)glycine (Steinrücken & Amrhein, 1980). Glyphosate inhibits EPSPS, resulting in the synthesis cessation of all three aromatic amino acids (Fig. 1, A) (Herrmann & Weaver, 1999). Although glyphosate is highly specific for EPSPS, it is suspected that glyphosate also causes shikimate accumulation, which in turn deregulates carbon metabolism (Orcaray et al., 2012). Almost all species of fungi, bacteria and algae are sensitive to glyphosate (Morjan et al., 2002; Funke et al., 2006; Leino et al., 2020). Some EPSPS isoforms are not sensitive to glyphosate (Fucile et al., 2011). While glyphosate-sensitive EPSPS class I is present in most bacteria (Tohge et al., 2013), glyphosate-resistant EPSPS class II has been isolated from certain bacterial species, such as *Ochrobactrum anthropi* (Tian et al., 2010) and agrobacteria (Fucile et al., 2011). In general, the specific effect of glyphosate on EPSPS allows it to be used to select for a large proportion of SCP-producing bacteria, fungi and microscopic algae, however, potential adverse side effects and possible resistance may in some cases complicate mutant selection.

Brilisauer et al., 2019 reported on the isolation of a new inhibitor, 7-deoxy-sedoheptulose, from the cyanobacterium *Synechococcus elongates* (Brilisauer et al., 2019). This inhibitor targets another shikimate pathway enzyme 3-dehydroquinate synthase (DHQS) (Fig. 1, A) (Brilisauer et al., 2019). Cyanobacteria treated with this inhibitor could be rescued by adding amino acids to the medium, suggesting that the inhibitory effect was caused by amino acid starvation (Hall et al., 2020). This observation suggests that the use of 7-deoxy-sedoheptulose may be more appropriate for mutant selection, as this inhibitor may not cause as pronounced side effects as glyphosate. The disadvantage of 7-deoxy-sedoheptulose is that it is not currently commercially available and requires hemoenzymatic synthesis and purification in laboratory (Brilisauer et al., 2019). Although 7-deoxy-sedoheptulose has been isolated from cyanobacteria, studies to date have shown that this compound is able to inhibit the growth of plant, yeast, and even other cyanobacteria species (Brilisauer et al., 2019), suggesting that resistance to this inhibitor is rare and is therefore likely to be effective in the selection of other microorganisms, although this has yet to be tested.

Another enzyme in the shikimate pathway, tryptophan synthase, is inhibited by 3-indoleacrylic acid, thus stopping tryptophan synthesis (Fig. 1, A) (Widholm, 1981; Sachpatzidis et al., 1999; Hall et al., 2020). 3-indoleacrylic acid is commercially available because it is widely used to induce gene transcription. 3-indoleacrylic acid has been isolated from multiple species of bacteria (Wikoff et al., 2009; Wang et al., 2013; Zhang & Davies 2016), indicating that the use of this compound in the selection of some bacteria strains may be limited. In general, 3-indoleacrylic acid inhibition of the growth of bacteria, cyanobacteria and fungi have been reported (Nonomura et al., 1996; Wang et al., 2013).

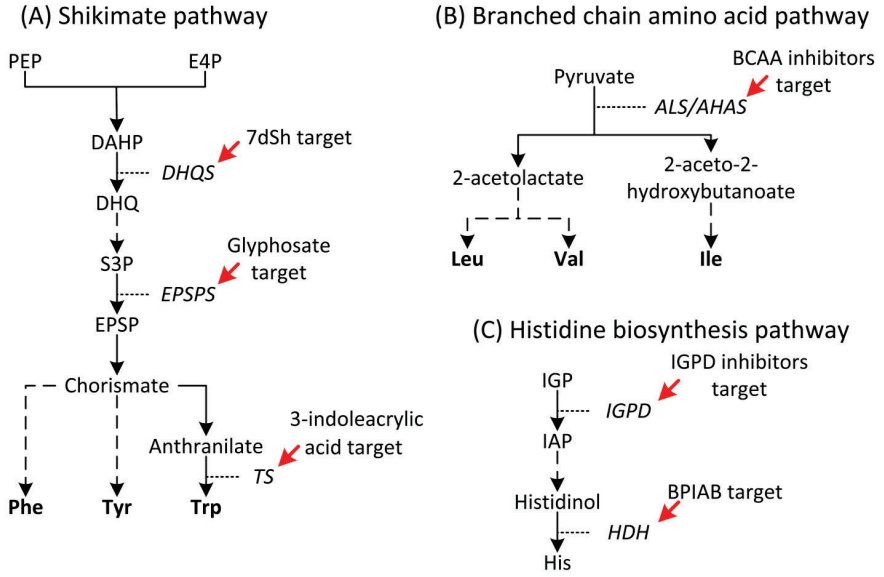

Figure 1. (A) Simplified shikimate pathway, (B) simplified branched chain amino acid biosynthesis pathway, (C) simplified histidine biosynthesis pathway. In shikimate pathway (A), through condensation of phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P), 3-deoxy-arabino heptulosonate 7-phosphate (DAHP) is produced. DAHP is then converted to 3-dehydroquinate (DHQ) and this process is catalized by enzyme DHQ synthase (DHQS). DHQS can be inhibited by 7-deoxy-sedoheptulose (7dSh). Further down the shikimate pathway shikimate 3-phosphate (S3P) is converted to 5-enolpyruvyl-shikimate 3-phosphate (EPSP) and this reaction is catalized by EPSP synthase (EPSPS). EPSPS is target enzyme of popular herbicide - glyphosate. Inhibition of either DHQS or EPSPS causes cessation of phenylalanine (phe), tyrosine (tyr) and tryptophan (trp) biosynthesis. In tryptophan biosynthesis branch (A) intermediate anthranilate is converted to tryptophan by tryptophan synthase (TS). TS is targeted by inhibitor 3-indoleacrylic acid. In branched chain amino acid (BCAA) pathway (B) acetolactate synthase/acetohydroxyacid synthase (ALS/AHAS) catalyzes condensation of pyruvate which produces 2-acetolactate. 2-acetolactate is early precursor of leucine (leu) and valine (val) biosynthesis. ALS/AHAS also catalyzes reaction where pyruvate is used to produce 2-aceto-2-hydroxybutanoate which is intermediate for isoleucine (ile) synthesis. There are various inhibitors available which specifically target ALS/AHAS (Table 1). In histidine (his) biosynthesis pathway (C) initial step is dehydration of imidazole glycerol-phosphate (IGP) to produce imidazoleacetol phosphate (IAP) which is catalized by IGP dehydratase (IGPD). There are various IGPD inhibitors available (Table 1). Last step of histidine biosynthesis is histidinol conversion to histidine by enzyme histidinol dehydrogenase (HDH). HDH is targeted by inhibitor S-1-(4-biphenyl)-4-(4-imidazolyl)-3-amino-2-butanone (BPIAB). Images and pathway descriptions adapted from Hall et al., 2020, Tzin & Galili, 2010.

2. Branched chain amino acid inhibitors

Leucine, isoleucine and valine are synthesized via branched-chain amino acids biosynthesis (BCAA) pathway (Binder, 2010). This pathway is found in plants, bacteria, fungi, archaea, microscopic algae and other microorganisms (Singh & Shaner, 1995; Binder, 2010; Duan et al., 2019; Hall et al., 2020). Although all three amino acids are synthesized in separate pathway branches, the synthesis of all three amino acid

precursors is catalyzed by the enzyme acetolactate synthase/acetohydroxyacid synthase (ALS/AHAS), which is also a target enzyme for various inhibitors (Fig. 1, B) (Hall et al., 2020). Many of these inhibitors are commercially available, which can be categorized into five groups: sulfonylureas, imidazolinones, triazolopyrimidines, pyrimidinyl(thio)benzoates and sulfonylaminocarbonyl-triazolinones (HRAC 2002). Full list of ALS/AHAS inhibitors are provided by (HRAC 2002). In addition to amino acid depletion, all ALS/AHAS inhibitors are characterized by side effects such as branched chain amino acid biosynthesis pathway intermediate accumulation, inhibition of DNA synthesis, and impaired assimilates transport (Zhou et al., 2007). These side effects can lead to errors in mutant selection, because mutants with increased resilience to side effects might be selected rather than mutants with increased ability to synthesize more BCAAs. Using ALS/AHAS inhibitors, branched chain amino acid biosynthesis has been successfully inhibited in yeasts (Lee et al., 2013; Jia et al., 2000; Duggleby et al., 2003), bacteria (Massey et al., 1976; Allievi & Gigliotti, 2001; Kreisberg et al., 2013), microscopic algae (Landstein et al., 1995, 1993), fungi (Allievi & Gigliotti, 2001; Lee et al., 2013) and in other microorganisms (Burnet & Hodgson, 1991), which indicate that these inhibitors can be used effectively to select mutants with improved SCP production capacity, however, possible selection errors due to existing side effects must also be taken into consideration.

3. Histidine inhibitors

Histidine biosynthesis occurs in both plants and microorganisms (Rawson et al., 2018; Stepansky & Leustek, 2006). Although commercial histidine inhibitors are not available on the market, recent findings have reported on inhibitor called 2-hydroxy-3-(1,2,4-triazol-1-yl) propylphosphonate, targeting enzyme responsible for the sixth step in histidine biosynthesis named imidazole glycerol-phosphate dehydratase (IGPD) (Fig. 1, C) (Rawson et al., 2018; Hall et al., 2020). Interestingly it has been demonstrated that this inhibitor is significantly more potent in yeasts than in plants (Rawson et al., 2018). Apart from IGPD inhibition there have been no reports on other side effects caused by 2-hydroxy-3-(1,2,4-triazol-1-yl) propylphosphonate, but it needs to be considered that as this novel inhibitor is further tested some cytotoxic effects might be found. Overall, 2-hydroxy-3- (1,2,4-triazol-1-yl) propylphosphonate is currently the only available inhibitor of histidine biosynthesis that could be used for improved SCP-rich mutant selection.

There have been reports on other inhibitors targeting IGPD, such as 1-(diethoxy-phosphoryl)-3-(4-one-1H-1,2,3-triazol-1-yl)-propan-2-yl carboxylic esters (Jin et al., 2015) and monopyrrole aldehydes (Schweitzer et al., 2002), but further research is required to confirm their applicability in microorganism mutant selection.

Histidinol dehydrogenase is the last enzyme in histidine biosynthesis, which is targeted by inhibitor S-1-(4-biphenyl)-4-(4-imidazolyl)-3-amino-2-butanone (Fig. 1, C) (Dancer et al., 1996). The activity of this compound on histidinol dehydrogenase is also ambiguous and further studies are needed.

4. Glutamine inhibitors

Glutamine is the most abundant amino acid in living beings (Cruzat et al., 2018). Therefore, it is reasonable to assume that selecting mutants for their glutamine synthesis capacity might result in discovery of strains with significantly increased total protein

contents. Because glutamine is a major precursor in the glutamate-derived amino acid biosynthesis pathway, which results in the synthesis of aspartate, proline, and arginine, inhibition of glutamine synthesis results in arrest of multiple amino acids biosynthesis (Fig. 2, D, E). Further cascading effect of glutamine inhibition will also prevent biosynthesis of aspartate-derived amino acids - lysine, methionine, threonine, and isoleucine (Fig. 2, F). Thus, by using only glutamine biosynthesis inhibitors, it is possible to inhibit the synthesis of eight amino acids, of which four are essential amino acids in animals. Thus, glutamine biosynthesis inhibitors in theory seem to be the most promising inhibitors to be used in the selection of mutants with increased capacity for the production of essential amino acids and increased protein content in general.

Glutamine biosynthesis occurs in both prokaryotes and eukaryotes (Forde & Lea, 2007; Patrick et al., 2018; Hall et al., 2020). To inhibit the glutamine biosynthetic pathway, all inhibitors target enzyme glutamine synthetase (GS) (Fig. 2, D). GS differs between prokaryotes and eukaryotes, with prokaryotic GS having twelve active sites and eukaryotes ten (Unno et al., 2006; Almassy et al., 1986; Berlicki, 2008), respectively, and differences in prokaryotic and eukaryotic GS susceptibility to different inhibitors have also been reported (Kim & Rhee 1987; Ahmad & Malloch, 1995; Ahmad et al., 1995; Kulkarni et al., 2006; Myrold & Posavatz, 2007). For example, the GS inhibitor phosphinothricin in soil at a concentration of 1 mM reduced the bacterial population by 40% and the fungal population by 20% (Ahmad & Malloch, 1995). Therefore, the effects of the same GS inhibitor may differ significantly from one species of microorganism to another. A number of inhibitors are available for GS inhibition, which can be divided into four groups: methionine sulfoximine and its analogues, glufosinate isomer (phosphinothricin) and its analogues, bisphosphonates, and other GS inhibitors (Berlicki, 2008; Hall et al., 2020). As side effects for most of these inhibitors, ammonia assimilation disorders have been reported in both prokaryotes (Myrold & Posavatz, 2007;) and eukaryotes (De Block et al., 1987; Boussiba & Gibson, 1991; Ahmad et al., 1995; Maughan & Cobbett, 2003; Kulkarni et al., 2006), which is rational because all these inhibitors target the same enzyme. Ammonia assimilation inhibition (Maughan & Cobbett, 2003; Cobb & Reade, 2010) might cause errors in selection of mutants since it is likely that mutants with increased resilience to ammonia might be selected instead of those with increased glutamine synthesis capability. Therefore, vigorous testing of GS inhibitors is required for the SCP-producing microorganisms, followed by further analysis of the selected strains for their total protein content and amino acid composition. GS inhibitors have been described in detail by Berlicki, 2008.

5. Glutamate-derived amino acid inhibitors

In glutamate-derived amino acid biosynthesis aspartate, proline and arginine are synthesized from glutamate via three separate pathways resulting in respective amino acids (Fig. 2, E) (Hall et al., 2020).

Similarly as with glutamine inhibition, but to smaller extent, inhibition of aspartate biosynthesis also affects production of aspartate-derived amino acids (Hall et al., 2020). Thus, by inhibiting aspartate biosynthesis, production of five amino acids – aspartate, lysine, methionine, threonine and isoleucine is prevented, which makes aspartate biosynthesis inhibitors promising candidates for use in SCP-rich mutant selection (Fig. 2, E, F). Aspartate biosynthesis is catalysed by an enzyme aspartate transaminase which is targeted by fungal metabolite cornexistin (Fig. 2, E) (Amagasa et al., 1994;

Nakajima et al., 1991). Low or no inhibitory activity of cornexistin has been reported in bacteria and fungi (Takahashi et al., 1994). Therefore, cornexistin applicability for SCP-rich bacteria, yeast, fungi and microscopic algae mutant selection needs to be tested for each species of interest. Additionally, cornexistin is not commercially available and it need to be produced and purified in laboratory (Steinborn et al., 2020). If, cornexistin or some other aspartate inhibitor will be proven to be viable for inhibition of aspartate biosynthesis in microorganisms, then this hypothetical inhibitor would be very useful in mutant selection, because, unlike glutamine inhibitors, aspartate inhibitors have not yet demonstrated adverse side effects such as ammonia accumulation.

In arginine biosynthesis one of the enzymes ornithine carbamoyltransferase is targeted by bacterial metabolite phaseolotoxin (Fig. 2, E) (Mitchell & Bieleski, 1977). Its activity has been demonstrated in *E. coli* (Staskawicz, 1979), but lack of analysis on other microorganisms suggests that similarly as in case with cornexistin, phaseolotoxin applicability for mutant selection need to be checked on case by case basis.

In proline biosynthesis enzyme δ1-pyrroline-5-carboxylate reductase (P5CR) can be inhibited by aminomethylene-bisphosphonates (Fig. 2, E) (Forlani et al., 2013). In study done by Forlani et al., 2012, several of the evaluated bisphosphonates were more potent on bacterial P5CR than on plant P5CR. Fungi and bacteria inhibition has been confirmed in other studies as well (Kunda et al., 2012; Shaik et al., 2020). Most of these compounds are not readily available and require synthesis in laboratory (Kunda et al., 2012; Shaik et al., 2020). Two of the aminomethylene-bisphosphonates - 3,5-dichlorophenylamino-methylenebisphosphonic acid and 3,5-dibromophenyl aminomethylenebis phosphonic acid simultaneously targeted P5CR and glutamine synthetase (GS) from glutamine biosynthesis pathway (Giberti et al., 2017). Both of these aminomethylene-bisphosphonates showed higher potency on GS inhibition than on P5CR inhibition (Giberti et al., 2017) and as discussed previously (see section 4. Glutamine inhibitors), for SCP-rich mutant selection GS inhibition might be preferable. As with other GS inhibitors toxic ammonia accumulation is expected.

6. Aspartate-derived amino acid inhibitors

Aspartate-derived amino acids are methionine, threonine, isoleucine and lysine (Fig. 2, F). In comparison to other herbicides, aspartate-derived amino acid inhibitors have been scarcely studied and no commercial herbicide is currently available on the market to inhibit any of aspartate-derived amino acids (Hall et al., 2020). However, there have been reports on compounds capable of inhibiting certain pathway enzymes (Keller-Schierlein et al., 1969; Ravanel et al., 1998b, 1998a; Szamosi, Shaner & Singh 1994; Hall et al., 2020).

In isoleucine biosynthesis enzyme threonine deaminase can be inhibited by 2-(1-cyclohexen-3(R)-yl)-S-glycine (CHG) (Fig. 2, F) (Ravanel et al., 1998a; Hall et al., 2020; Szamosi et al., 1994). This herbicidal compound is not commercially available and needs to be synthesized in laboratory (Szamosi et al., 1994). Threonine deaminase inhibition by CHG, has also been observed in bacteria (Keller-Schierlein et al., 1969; Szamosi et al., 1994). To confirm the use of CHG in SCP-rich mutant selection in the future, its efficacy in inhibiting other microorganisms needs to be tested.

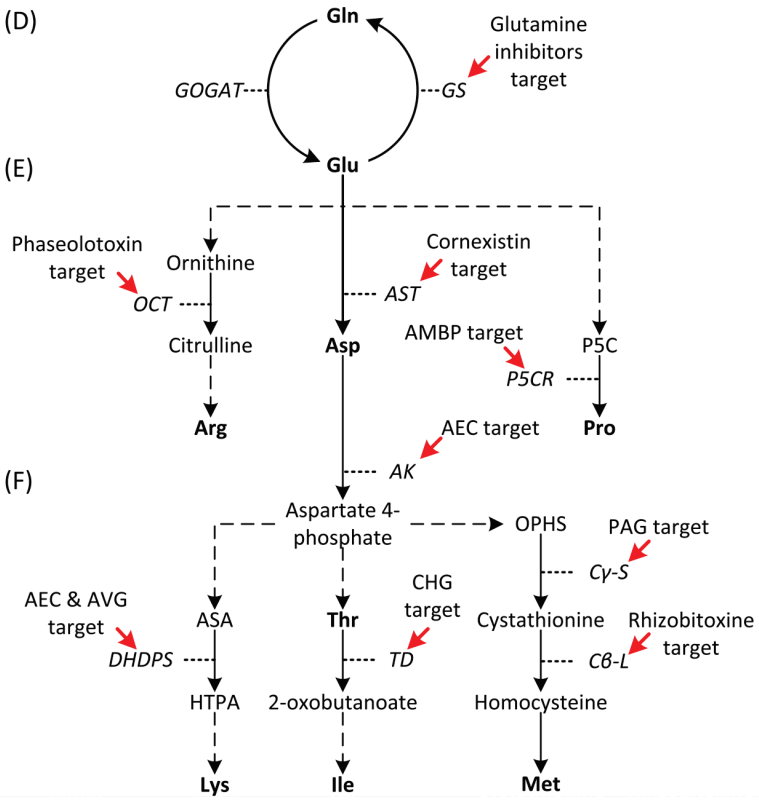

Figure 2. (D) Simplified glutamine biosynthesis pathway, (E) simplified glutamate-derived amino acid biosynthesis pathway, (F) simplified aspartate-derived amino acid biosynthesis pathway. In glutamine biosynthesis pathway (D), glutamine (gln) is converted to glutamate (glu) by glutamine oxoglutarate aminotransferase (GOGAT). Glutamate is converted back to glutamine by enzyme glutamine synthetase (GS), which is target of various GS specific inhibitors (Table 1). In arginine (arg) synthesis branch from glutamate-derived amino acid biosynthesis pathway (E) one of the intermediate steps involves ornithine convertion to citrulline by ornithine carbamoyltransferase (OCT). OCT is targeted by inhibitor phaseolotoxin. Proline (pro) is produced from δ1-pyrroline-5-carboxylate (P5C) by P5CR reductase. P5CR is target of various aminomethylene-bisphosphonates (AMBP). Some of AMBP are dual-target inhibitors which target P5CR and GS (D). Aspartate (asp) is produced from glutamate by aspertate transaminase (AST) which is targeted by fungal metabolite cornexistin. Aspartate-derived amino acid biosynthesis pathway (F) starts with convertion of aspartate to aspartate 4-phosphate by aspartate kinase (AK). AK is targeted by S-(2-aminoethyl)-L-cysteine (AEC). If AK is inhibited it causes cessation of methionine (met), threonine (thr), isoleucine (ile) and lysine (lys) synthesis. Further down the pathway one of the intermediates of lysine biosynthesis branch called aspartate semialdehyde (ASA) is converted to 4-hydroxy-tetrahydrodipicolinate (HTPA) by dihydrodipicolinate synthase (DHDPS). DHDPS can be inhibited by previously mentioned AEC or L-α-(2-amino ethoxy-vinyl) glycine (AVG). In isoleucine biosynthesis branch threonine is converted to 2-oxobutanoate by threonine deaminase (TD). TD is targeted by 2-(1-cyclohexen-3(R)-yl)-S-glycine (CHG). In methionine biosynthesis branch O-phosphohomoserine (OPHS) is converted to cystathionine by cystathionine γ-synthase (Cγ-S). Cystathionine is then converted to homocysteine by cysteine-S-conjugate β-lyase (Cβ-L). Cγ-S can be inhibited by DL-Propargylglycine (PAG) and Cβ-L can be inhibited by rhizobitoxine. Images and pathway descriptions adapted from Hall et al., 2020, Choi & Coloff, 2019.

In methionine biosynthesis enzyme cystathionine γ-synthase can be inhibited by DL-Propargylglycine (Fig. 2, F) (Ravanel et al., 1998a, 1998b). It has been demonstrated, that plants inhibited by DL-Propargylglycine can be rescued using methionine supplementation, thus indicating that the herbicidal activity comes from amino acid starvation (Ravanel et al., 1998b, 1998a). This observation is also a good indicator on the potential use of this compound in mutant selection. DL-Propargylglycine can be purchased as chemical reagent, thus no synthesis in laboratory is required (Yoshioka et al., 2014). Studies have demonstrated propargylglycine inhibitory activity in microorganisms as well (Johnston et al., 1979; Piotrowska & Paszewski, 1986; Lockwood & Coombs, 1991; Jin et al., 2004).

Another enzyme in methionine biosynthesis - cysteine-S-conjugate β-lyase, can be inhibited by bacteria metabolite rhizobitoxine and its analogues (Fig. 2, F) (Giovanelli et al., 1971; Okazaki et al., 2007), but further assessment on its use on microorganisms is required.

In lysine biosynthesis enzyme dihydrodipicolinate synthase can be inhibited by S-(2-aminoethyl)-L-cysteine and L-α-(2-aminoethoxyvinyl)glycine (Fig. 2, F) (Soares da Costa et al., 2018). Both compounds have also shown inhibitory activity in bacteria and fungi and can be used for selection of mutants with improved lysine accumulation (Sano, 1970; Rupp et al., 1989; Han et al., 1991; Zabriskie & Jackson 2000; Ekwealor & Obeta, 2006). Both inhibitors are also commercially available.

In general, the use of aspartate-derived amino acid inhibitors in mutant selection may facilitate the discovery of new mutants with improved production capacity of essential amino acids. However, all compounds in this group inhibit only single amino acid, and if the goal is to improve the overall SCP production capacity as well as the ability to synthesize multiple essential amino acids, then a better approach would be to use either aspartate or glutamine inhibitors.

CONCLUSIONS

Single cell protein is a good alternative to substitute plant-derived proteins in animal and fish feeds. SCP production technologies offer a number of environmental benefits over conventional protein sources, and these proteins can be produced from biodegradable agro-industrial by-products from other industries. SCP-producing microorganism strains are at the heart of all SCP technological solutions, so improving the properties and productivity of these strains is vital to increasing the competitiveness of SCP. In order to create microorganisms with better properties for the production of SCP, one of the possible approaches is the creation and selection of mutants. After treatment with mutagen, to select mutants with the highest protein and essential amino acid synthesis capacity, in this article we have reviewed various amino acid inhibitors that could theoretically be used in the selection of such mutants. Most amino acid inhibitors are designed for use as herbicides in weed control in agriculture, so information on the effects of these inhibitors on microorganisms is often limited. To the best of our knowledge, using amino acid inhibitors to select SCP-producing mutants with increased total protein content and improved essential amino acid profiles is a novel idea.

In total, 6 groups of amino acid inhibitors were reviewed in the article, of which glutamine inhibitors are the most promising, because it is possible to stop the synthesis of eight amino acids by using only one inhibitory compound. Many glutamine synthetase

inhibitors have been introduced into the market and their activity has been tested not only in plants, but in bacteria, fungi, yeasts and microscopic algae as well. Therefore, it should be possible to find the most suitable glutamine synthetase inhibitor for the selection of the mutant microorganisms. As all glutamine synthetase inhibitors are also causing toxic ammonia accumulation, during selection it should be taken into consideration that mutants with increased ammonia tolerance might be selected by mistake and these false positives should be removed later during further mutant strain testing.

Other promising inhibitors for SCP-rich mutant selection are glyphosate, as this popular herbicide inhibits the synthesis of three aromatic amino acids, two of which are essential amino acids in animals. In addition, the effects of glyphosate have been extensively studied in a variety of organisms, making it much easier to predict its effects on mutated microorganisms. As with glutamine inhibitors, glyphosate induced side effect should also be considered during the selection of mutant strains.

Another promising group of amino acid inhibitors are branched chain amino acid inhibitors, which target acetolactate synthase/acetohydroxyacid synthase (ALS/AHAS). These inhibitors cease synthesis of three essential amino acids. The effects of these inhibitors have been extensively studied on a wide variety of microorganisms, and ALS/AHAS inhibition can be achieved by using wide range of inhibitors from five chemically distinct groups. The side effects of these inhibitors should also be considered during mutant selection.

Inhibition of aspartate biosynthesis also results in arrest of several (five) amino acid syntheses. Unfortunately, currently only one compound has been found to inhibit aspartate biosynthesis, which has low activity in both bacteria and fungi, so its use in SCP-rich mutant selection may be severely limited.

In addition to the selection of individual inhibitors, it is possible to use combinations of multiple inhibitors in the selective medium to pick mutants with specifically improved amino acid profiles for use in specialized animal feeds or other higher value-added market segments. However, it should also be taken into consideration that the combination of inhibitors can be very time consuming until optimal concentrations are found for each applied inhibitor, and the combination of inhibitors may cause previously unobserved side effects or disproportionately amplify known effects, which would again complicate mutant selection.

In general, due to the lack of studies to date on the use of amino acid inhibitors in the selection of SCP-rich mutants, most of the hypotheses proposed here will need to be tested in a practical laboratory setting.

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Herbicide-based selection of mutants for improved single cell protein synthesis: application and procedures

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Abstract. Enhancement of industrially important microbial strains using random mutagenesis is widely used. Screening of potential mutants accelerates the selection of mutants with desired properties such as improved synthesis of lipids, carotenoids, enzymes, or increased tolerance to unfavourable conditions. However, random mutagenesis has not been used to improve protein biosynthesis in microorganisms, and a method for screening these mutants has not yet been developed. The present work reviews the new concept of using herbicides as tools for selecting mutant microorganisms with improved protein biosynthesis. Several pure herbicide substances are amino acid inhibitors whose specific action can be used as a selective pressure for screening protein-rich mutants. The article summarises information about thirteen amino acid inhibitors that inhibit microorganisms and provides data on applicable doses and specifics of use. The article contains mutagenesis protocols and mutant selection strategies, supplemented by theoretical considerations for practical application.

Key words: amino acid inhibitors, herbicide, mutagenesis, mutant selection, protein production, single cell protein.

INTRODUCTION

Agriculture and industrial by-products are commonly employed for energy production, and it is crucial to explore and exploit their potential to create higher-value products. Based on added value, the bioresource pyramid categorises products into energy, bulk chemicals, food, animal feed, special fibres, pharmaceuticals, and fine chemicals. Approximately 46% of the globally produced waste is organic, with food waste presenting a valuable resource (Chavan et al., 2022). Recovering and transforming food waste into high-value products can be more profitable and ecological than conventional processing, contributing to the transition to zero-waste management (Arnold, 2018; Narisetty et al., 2022).

One such higher-value product is microbial protein, called single-cell protein (SCP). SCP is an alternative protein source that offers a sustainable solution to reduce protein scarcity (Najafpour, 2007; FAO, 2020). Wider use of SCP, particularly in livestock and aquaculture feeds, could reduce the need for intensive farming and align with environmental strategies to reduce greenhouse gas emissions (European

Commission, 2012, 2019). Today, 1 in 3 people worldwide struggle with moderate to severe food insecurity, yet 33% of food produced is wasted (Arnold, 2018; United Nations, 2023). Using food waste as a feedstock to produce SCP would help provide a sufficient protein supply to meet growing demand. According to the United Nations Sustainable Development Goals 2023 report, since waste substrates can be used as raw materials, they will help solve current environmental problems (United Nations, 2023).

SCP technology has many advantages over conventional feed protein sources. It is acknowledged for its environmental friendliness, reduced water consumption, resilience to climatic conditions, smaller land requirements, and utilisation of agro-industrial by-products as feedstock (Singh & Mishra, 1995; García-Garibay et al., 2014; El-Sayed, 2020; Berzina et al., 2024). Although SCP production technologies have undergone extensive research and are steadily expanding in the market (Ritala et al., 2017; P&S Intelligence, 2018), there exists potential for further enhancing the properties of SCP-producing microorganisms. Improving and creating strains with superior characteristics can boost the competitiveness of the SCP and contribute to the advancement of a circular bioeconomy by maximising the efficient utilisation of available resources for high-value-added product production (Spalvins et al., 2021).

The present study proposes a new way to create mutant microorganisms with higher protein biosynthesis or an improved amino acid (AA) profile. We suggest using random mutagenesis followed by a screening of mutants in media supplemented with AA inhibitors. This concept is well known in the selection of mutant microorganisms with improved synthesis of fatty acids and carotenoids, where inhibition of the biosynthetic pathway of the target metabolite allowed the collection of overproducers (Ducrey Sanpietro & Kula, 1998; Atzmüller et al., 2019; Luna-Flores et al., 2022). Interestingly, the widely used random mutagenesis to improve the desired properties of strains is not used to create protein-synthesised mutants. Although there is evidence of the effect of random mutagenesis on increasing the protein content biomass of microalgae (Liu et al., 2015). Several herbicides are AA inhibitors, which can be a tool for selecting improved SCP-producing mutants. We assume that microbial cells that have undergone mutagenesis and are capable of growing in media in the presence of an herbicide concentration that inhibits 100% of the cells of the wild-type strain have a high probability of being protein overproducers.

The first part of the study summarises the results of inhibition of bacteria and fungi treated with commercial herbicides and pure active ingredients. Evaluation of microbial growth inhibition and the herbicide doses used are presented in 5 tables. The second part of the study presents protocols for the mutagenesis of microorganisms and subsequent screening of protein-synthesising mutants on selective media supplemented with AA inhibitors. The protocols and accompanying description of the screening procedure for potential mutants consider the details of working with treated yeast cells based on our experience creating SCP yeast mutants.

EFFECT OF HERBICIDES ON THE GROWTH OF BACTERIA AND FUNGI

Many studies report that AA inhibitors, which are the active ingredients of herbicides, affect the growth of microorganisms (Table 1–5). The main effect of different AA inhibitors for plants, algae, bacteria, yeast, and moulds is similar: to inhibit

the enzymatic activity responsible for the biosynthesis of AAs in cells (Kumada et al., 1993; Ravanel et al., 1998; Grant Pearce et al., 2017; Vallejo et al., 2017; Sardrood & Goltapeh, 2018; Lonhienne et al., 2020; Tall & Puigbò, 2020). These inhibitors can lead to AA starvation, suppression of cell growth, and death at specific concentrations. However, other cellular responses are also observed, such as a complete lack of inhibition or stimulation of growth in some species (Forlani et al., 1995; Grandoni et al., 1998; Xuedong et al., 2005¸ Halgren et al., 2011). The reasons for such reactions may lie in the individual characteristics of the species or strain of the microorganism and depend on environmental conditions and interactions in the microbiota (Vallejo et al., 2017; Nielsen et al., 2018). For example, the reason for the lack of inhibition may be associated with the activity of efflux pumps that release the herbicide from the cell or with a disruption of the transport system, such as a porin mutation (Thiour-Mauprivez et al., 2019). Stimulation of growth may be caused by the ability of the microorganism to use the herbicide as a source of carbon or phosphorus, which has been described in several studies (Xuedong et al., 2005; Wang et al., 2007; Łozowicka et al., 2021).

The sources of carbon and nitrogen in the medium directly influence the response of microorganisms to the presence of AA inhibitors (Schloss, 1990; Wang et al., 2012; Nielsen et al., 2018). Studies report that the inhibitory effect is observed in minimal media containing an inorganic nitrogen source and is absent in protein or AA-rich media. If an inhibited AA is subsequently added to the minimal medium, the inhibition of the microorganism is reversed (Schloss, 1990; Nielsen et al., 2018). Therefore, minimal media should be used to select protein-producing mutants. Interesting results were achieved in a study of the ability of soil bacterial isolates to degrade the herbicide tribenuron-methyl (TBM). Isolate of *Serratia marcesens* in the presence of glucose, glycerol, or sucrose contributed to the complete degradation of 0.5 g L^{-1} tribenuron-methyl in 3 days. Adding other carbon sources, such as sodium acetate, sodium succinate, sodium citrate, yeast extract, etc., supported the growth of the bacteria but did not affect the degradation of TBM. It was found that the bacteria could not use the inhibitor as a carbon source, and its degradation is associated with microbial activity of a different nature. It turned out that the degradation of TBM molecules occurred due to acid hydrolysis caused by short-chain fatty acids, fermentation products of glucose, sucrose, and glycerol (Wang et al., 2012). This is consistent with the finding of other studies that a pH decrease in a medium due to microbial metabolism causes some herbicide degradation (Braschi et al., 2000; Boschin et al., 2003). For example, the herbicide primisulfuron is stable in a neutral pH environment, but lowering the pH below six during fermentation resulted in acidohydrolysis (Braschi et al., 2000). Herbicides metsulfuron-methyl and chlorsulfuron are sensitive to a decrease in pH. Significant degradation of these herbicides is reported after 15–48 hours in acidic aqueous solutions with a pH value of 2–5 (PubChem, 2024a, 2024b, 2024c). In comparison, glyphosate and amitrole are stable to hydrolysis at pH 3–9 (PubChem, 2024d, 2024e). Therefore, the medium must be buffered at neutral pH or, if necessary, used at pH 6–8 when using AA inhibitors. The importance of a media composition and pH stability is highlighted in the study reporting the degradation of chlorsulfuron and metsulfuron-methyl by *Aspergillus niger* in a rich-nutrient buffered media but not in a minimal buffered media (Boschin et al., 2003).

In this article, we summarised the results of the inhibition of bacteria and fungi by AA inhibitors (Tables 1–5), selecting AA inhibitors with the best-ranked value according to multicriteria data analysis (MCDA) in our previous study (Berzina et al., 2024). The tables contain information about thirteen AA inhibitors (CAS number of the substance which AA inhibits), a list of bacteria (B) and fungi (F), concentrations of inhibitors, percentage of inhibition, or other effects. Data for both commercial herbicides and pure active components are summarised.

Glutamine AA inhibitors

L-methionine sulfoximine (MSO) and glufosinate-ammonium (GA) are inhibitors of glutamine synthetase, an enzyme involved in nitrogen metabolism and the synthesis of glutamine, purines, and pyrimidines. Glutamine is a precursor to the biosynthesis pathway of the AAs: arginine (Arg), proline (Pro) and aspartate (Asp), lysine (Lys), methionine (Met), threonine (Thr), and isoleucine (Ile) (Mowbray et al., 2014; Joo et al., 2018; Gong et al., 2020). The study (Hernandez & Mora, 1986) suggests that using sugars and a nitrogen source is a coordinated process in microbial cells. When ammonium assimilation and glutamine synthesis are impaired, a decrease in the rate of carbon catabolism is a natural outcome.

Table 1. Evaluation of microbial growth inhibition using herbicides and pure active components. Glutamine inhibitors

Herb. Microorganism		Inhibition		References
		%	Conc, mg L^{-1}	
L-Methionine sulfoximine (CAS 15985-39-4)				
B	*Azospirillum brasilense*	100	5	(Van Dommelen et al., 2003)
	Mycobacterium tuberculosis	50	9.2	(Mowbray et al., 2014)
F	*Aspergillus niger*	0	360.5	(Ahuja & Punekar, 2008)
	Gibberella fujikuroi	Sign.	360.5	(Muñoz & Agosin, 1993)
Glufosinate ammonium (CAS 77182-82-2)				
B	*Mycobacterium tuberculosis*	50	0.3	(Mowbray et al., 2014)
F	*Aspergillus niger*	Sign.	30	(Ahuja & Punekar, 2008)
	Saccharomyces cerevisiae	55–63	10	(Vallejo et al., 2017)

Sign. – significant inhibition; B – bacteria; F – fungi.

Despite similar activity, GA appears to be a more effective microbial inhibitor than MSO (Table 1). For 50% growth inhibition of *Mycobacterium tuberculosis*, a concentration of 0.3 mg L^{-1} GA was required, while for MSO, the concentration was 9.2 mg L^{-1} (Mowbray et al., 2014). The fungus was less sensitive to these inhibitors; no inhibition of *Aspergillus niger* growth was detected when treated with 360.5 mg L^{-1} MSO, although 30 mg L^{-1} GA caused significant inhibition, *A. niger* colony diameters decreased by approximately threefold in the presence of GA (Ahuja & Punekar, 2008). Vallejo et al. assessed the effect of 10 mg L^{-1} GA on the growth and metabolism of wine yeast *Saccharomyces cerevisiae* during fermentation. The presence of GA slowed down the rate of sugar metabolism, suppressed growth, and extended the lifespan of cells in the stationary phase. Biomass analysis showed an increase in AAs and polyamines in GA-treated cells compared to untreated cells. AAs such as Met, Ile, Leucine (Leu),

phenylalanine (Phe), tryptophan (Trp), tyrosine (Tyr), etc. were significantly higher in the treated biomass. At the same time, the values of glutamine and asparagine decreased, and Lys, Arg, Pro, and Asp did not differ from untreated cells. This is one of the first studies to report the adaptation of a microorganism to the damaging effects of a herbicide by increasing the biosynthesis of AAs (Vallejo et al., 2017).

Aromatic AA inhibitor

Glyphosate or N-(Phosphonomethyl)glycine is an inhibitor of aromatic amino acids phenylalanine (Phe), tryptophan (Trp), and tyrosine (Tyr) (Hertel et al., 2021). Table 2 shows the required concentrations of glyphosate to completely inhibit the growth of pathogenic or beneficial intestinal bacteria and several fungi. Concentrations refer to the weight of glyphosate active ingredient in the commercial herbicide per litre of media. It is estimated that 75–5,000 mg L^{-1} of glyphosate may be needed for complete inhibition of bacteria (Shehata et al., 2014) and up to 1,000 mg L^{-1} for fungi (Tahiri et al., 2022). However, it should be noted that the inhibitory effect of glyphosate is stronger in the commercial formulation (Braconi et al., 2006; Clair et al., 2012). Therefore, when using a pure substance, higher concentrations may be required.

Table 2. Evaluation of microbial growth inhibition using herbicides and pure active components. Aromatic amino acid inhibitor

Herb.	Microorganism	Inhibition		References
		%	Conc., mg L^{-1}	
Glyphosate (CAS 1071-83-6)				
B	*Bacillus* spp.	100	150–300	(Shehata et al., 2014)
	Bacteroides vulgatus	100	600	(Shehata et al., 2014)
	Bifidobacterium adolescentis	100	75	(Shehata et al., 2014)
	Campylobacter spp.	100	150	(Shehata et al., 2014)
	Clostridium spp.	100	1,200–5,000	(Shehata et al., 2014)
	Enterococcus faecalis	100	150	(Shehata et al., 2014)
	Escherichia coli	100	1,200	(Shehata et al., 2014)
	Escherichia coli	100	80–160	(Nielsen et al., 2018)
	Lactobacillus spp.	100	600	(Shehata et al., 2014)
	Riemerella anatipestifer	100	150	(Shehata et al., 2014)
	Salmonella spp.	100	5,000	(Shehata et al., 2014)
	Staphylococcus spp.	100	300	(Shehata et al., 2014)
F	*Geotrichum candidum*	100	1,000	(Tahiri et al., 2022)
	Penicillium digitatum	100	240	(Tahiri et al., 2022)
	Penicillium italicum	100	240	(Tahiri et al., 2022)

* Commercial herbicide was used; B – bacteria; F – fungi.

Aspartate-derived AA inhibitors

S-(2-aminoethyl)-L-cysteine (AEC), L-α-(2-aminoethoxyvinyl) glycine (AVG), and DL-propargylglycine (PAG) are aspartate-derived amino acid inhibitors (Table 3). AEC and AVG inhibit the biosynthesis of methionine (Met), lysine (Lys), threonine (Thr), and isoleucine (Ile), and PAG inhibits Met (Spalvins et al., 2021). The sensitivity of bacteria to the AEC inhibitor varies. Treatment of *Bacillus subtilis* with 1,000 mg L^{-1}

of inhibitor resulted in complete inhibition, while 3,000 mg L^{-1} caused 50% inhibition of *Brevibacterium flavum*. Interestingly, adding 3,000 mg L^{-1} L-threonine increased the inhibition of *B. flavum* from 50% to over 90% (Shiio, 1970). The effect of AEC on fungi has been poorly studied, and there is no data on a 100% inhibitory dose. Only one study reports that AEC inhibits yeast growth; however, the inhibition rate is too low; 83.3% of the yeast isolates were resistant to the inhibitor (Odunfa et al., 2001). Presumably, the sensitivity of bacteria to AEC is lower than fungi's. No inhibition or inhibition of up to 50% of bacteria was reported when treated with 590 mg L^{-1} AVG inhibitor (Halgren et al., 2011). In comparison, two species of fungi showed up to 80% inhibition at 0.3 mg L^{-1} commercial AVG inhibitor (Jin et al., 2004) and at 200 mg L^{-1} when using the pure substance (Al-Masri et al., 2006). It is worth noting that higher concentrations, such as 1 g L^{-1} or more, were not tested for this inhibitor. Therefore, it is expected that better inhibition may be achieved when using higher concentrations. For complete inhibition of *Fusarium oxysporum* fungi and the enzymatic activity of *Aspergillus flavipes* L-methioninase, about 1 g L^{-1} of PAG inhibitor is required (Jin et al., 2004; El-Sayed, 2011).

Table 3. Evaluation of microbial growth inhibition using herbicides and pure active components. Aspartate-derived amino acid inhibitors

Herb.	Microorganism	Inhibition		References
		%	Conc., mg L^{-1}	
S-(2-aminoethyl)-L-cysteine (CAS 2936-69-8)				
B	*Bacillus subtilis*	100	1,000	(Shiio, 1970)
	Brevibacterium flavum	50	3,000	(Shiio, 1970)
	Brevibacterium flavum	> 90	3,000*	(Shiio, 1970)
	Escherichia coli	100	1,000	(Shiio, 1970)
	Escherichia coli	100	1	(Ataide et al., 2007)
F	yeast, the species were not specified	16.7	200	(Odunfa et al., 2001)
DL-Propargylglycine (CAS 64165-64-6)				
B	*Porphyromonas gingivalis*	100	6.8	(Kandalam et al., 2018)
F	*Aspergillus flavipes*	98**	1,130	(El-Sayed, 2011)
	Fusarium oxysporum	61–93	1,000	(Jin et al., 2004)
L-α-(2-Aminoethoxyvinyl) glycine (CAS 49669-74-1)				
B	*Agrobacterium tumefaciens*	0	589.9	(Halgren et al., 2011)
	Bacillus megaterium	34–38	589.9	(Halgren et al., 2011)
	Erwinia amylovora	47–51	589.9	(Halgren et al., 2011)
	Escherichia coli	0	589.9	(Halgren et al., 2011)
	Pantoea agglomerans	0	589.9	(Halgren et al., 2011)
	Pectobacterium carotovorum	0	589.9	(Halgren et al., 2011)
	Pseudomonas spp.	0	589.9	(Halgren et al., 2011)
	Xanthomonas hortorum	0	589.9	(Halgren et al., 2011)
F	*Fusarium oxysporum*	24–81	0.29***	(Jin et al., 2004)
	Sclerotinia sclerotiorum	70–80	200	(Al-Masri et al., 2006)

* L-threonine supplementation; ** Enzyme L-methioninase inhibition; *** Commercial herbicide was used; B – bacteria; F – fungi.

Branched-chain AA inhibitors

Branched-chain AA inhibitors metsulfuron-methyl (MSM), sulfometuron-methyl (SMM), chlorsulfuron (CS), tribenuron-methyl (TBM), etc., belong to the group of sulfonylureas and imazapyr, imazapic, imazethapyr, and imazaquin, etc. belong to the group of imidazolinones. All of them inhibit the three AA isoleucine (Ile), leucine (Leu), and valine (Val). These inhibitors are considered the most effective herbicides required in micro-doses for complete inhibition of vegetation or microorganisms (Chen et al., 2009; Zabalza et al., 2013; Łozowicka et al., 2021; Berzina et al., 2024). SMM appears more effective than other sulfonylureases in inhibiting microorganisms (Table 4).

Table 4. Evaluation of microbial growth inhibition using herbicides and pure active components. Branched-chain amino acid inhibitors. Sulfonylureas

Herb.	Microorganism	Inhibition		References
		%	Conc., mg L^{-1}	
Metsulfuron-methyl (CAS 74223-64-6)				
B	*Arthrobacter crystallopoietes*	100	50	(Wang et al., 2007)
	Bacillus subtilis	50	11.9	(Kreisberg et al., 2013)
	Burkholderia spp.	50	1.19–47.7	(Kreisberg et al., 2013)
	Mycobacterium avium	> 90	286.0	(Zohar et al., 2003)
	Mycobacterium spp.	100	2.4–9.5	(Kreisberg et al., 2013)
	Pseudomonas aeruginosa	90	95.3	(Kreisberg et al., 2013)
F	*Candida mengyuniae* sp. nov.	50	> 5,000	(Chen et al., 2009)
	Candida shehatae	50	10	(Chen et al., 2009)
	Pichia farinosa	50	200	(Chen et al., 2009)
	Saccharomyces cerevisiae	50	5	(Chen et al., 2009)
	Williopsis saturnus	50	200	(Chen et al., 2009)
Sulfometuron-methyl (CAS 74222-97-2)				
B	*Burkholderia pseudomallei*	50	74.7–182.2	(Kreisberg et al., 2013)
	Mycobacterium avium	100	218.6	(Zohar et al., 2003)
	Mycobacterium spp.	100	0.6–4.4	(Grandoni et al., 1998)
	Pseudomonas spp.	50	22.8–74.7	(Kreisberg et al., 2013)
F	*Candida albicans*	80	12.5	(Kingsbury & McCusker, 2010)
	Saccharomyces cerevisiae	80	5	(Kingsbury & McCusker, 2010)
Chlorsulfuron (CAS 64902-72-3)				
B	*Agrobacterium tumefaciens*	Sign.	0.01–10	(Petrovickij-Angerer, 2009)
	Azospirillum lipoferum	S/g	1.1	(Forlani et al., 1995)
	Azotobacter chroococcum	0	1.1	(Forlani et al., 1995)
	Azotobacter spp.	Sign.	10	(Petrovickij-Angerer, 2009)
	Bacillus cereus	Sign.	1–10	(Petrovickij-Angerer, 2009)
	Bacillus spp.	0	1.1	(Petrovickij-Angerer, 2009)
	Bacillus subtilis	Sign.	0.001–10	(Forlani et al., 1995)
	Bradyrhizobium sp.	Sign.	0.001–10	(Petrovickij-Angerer, 2009)
	Brevundimonas vesicularis	0	1.1	(Forlani et al., 1995)
	Cronobacter sakazakii	0	1.1	(Forlani et al., 1995)

Table 4 (continued)

	Sinorhizobium meliloti	Sign.	0.001–10	(Petrovickij-Angerer, 2009)
	Enterobacter cloacae	0	1.1	(Forlani et al., 1995)
	Escherichia coli	Sign.	0.1–10	(Petrovickij-Angerer, 2009)
	Micrococcus luteus	Sign.	0.1–10	(Petrovickij-Angerer, 2009)
	Mycobacterium avium	85	357.8	(Zohar et al., 2003)
	Mycobacterium spp.	100	11.1–447.2	(Grandoni et al., 1998)
	Pantoea agglomerans	0	1.1	(Forlani et al., 1995)
	Pectobacterium carotovorum	Sign.	0.1–10	(Petrovickij-Angerer, 2009)
	Pseudomonas aeruginosa	Sign.	0.01–10	(Petrovickij-Angerer, 2009)
	Pseudomonas luteola	S/g	1.1	(Forlani et al., 1995)
	Rhizobium spp.	Sign.	0.001–10	(Petrovickij-Angerer, 2009)
	Serratia plymuthica	0	1.1	(Forlani et al., 1995)
	Sphingomonas paucimobilis	0	1.1	(Forlani et al., 1995)
	Stenotrophomonas maltophilia	0	1.1	(Forlani et al., 1995)
	Streptomyces griseus	Sign.	0.1–1	(Petrovickij-Angerer, 2009)
Tribenuron-methyl (CAS 101200-48-0)				
B	*Mycobacterium tuberculosis* (3 strains)	0	49.4	(Grandoni et al., 1998)
F	*Alternaria triticina*	50	239.5*	(Sameer, 2019)
	Pyrenophora tritici	50	238*	(Sameer, 2019)

* Commercial herbicide was used; B – bacteria; F – fungi; Sign. – Significant inhibition; S/g – Slow growth.

SMM's inhibition of some bacteria and fungi has been achieved at relatively low concentrations. CS had significant inhibition against a variety of bacteria, but the study was limited to a concentration of 10 mg L^{-1}, and higher concentrations were not tested (Petrovickij-Angerer, 2009). Another study used a concentration of 1.1 mg L^{-1} CS, which did not have an inhibitory effect on various bacteria and only caused significant inhibition for *Azospirillum lipoferum* (Forlani et al., 1995). No studies have been reported on the inhibition of fungi by a CS inhibitor. The inhibitory effect of MSM on bacteria and fungi has been described in several publications. The sensitivity of fungi to this inhibitor varies greatly. To achieve 50% inhibition of five yeast species, 5 to 200 mg L^{-1} of MSM was required, and for the resistant strain of *Candida mengyuniae,* more than 5 g L^{-1} (Chen et al., 2009).

Complete inhibition of bacteria was observed when using 300–400 mg L^{-1} imazapyr, while lower concentrations of 26.13 and 32.66 mg L^{-1} imazapyr and imazethapyr had no inhibitory effect (Table 5). However, such a small concentration prolonged the generation time of *Bacillus cereus* (Forlani et al., 1995; Xuedong et al., 2005) and *B. circulans* (Xuedong et al., 2005). Four yeast species that showed sensitivity to MSM were less sensitive to imazethapyr. For 50% inhibition, 100, 25, 40, and 25 times more imazethapyr were required for *Candida shehatae, Pichia farinosa, Saccharomyces cerevisiae*, and *Williopsis saturnus*, respectively (Chen et al., 2009).

Table 5. Evaluation of microbial growth inhibition using herbicides and pure active components. Branched-chain amino acid inhibitors. Imidazolinones

Herb.	Microorganism	Inhibition		References
		%	Conc., mg L^{-1}	
Imazapyr (CAS 81334-34-1)				
B	*Azospirillum lipoferum*	0	26.13	(Forlani et al., 1995)
	Azotobacter chroococcum	0	26.13	(Forlani et al., 1995)
	Bacillus cereus	100	300	(Forlani et al., 1995)
	Bacillus spp.	S/g	26.1	(Forlani et al., 1995)
	Bacillus spp.	0	26.13	(Xuedong et al., 2005)
	Brevundimonas vesicularis	0	26.13	(Forlani et al., 1995)
	Cronobacter sakazakii	0	26.13	(Forlani et al., 1995)
	Enterobacter cloacae	0	26.13	(Forlani et al., 1995)
	Mycobacterium spp.	0	32.66	(Grandoni et al., 1998)
	Pantoea agglomerans	0	26.13	(Forlani et al., 1995)
	Pseudomonas fluorescens	100	300–400	(Xuedong et al., 2005)
	Bacillus cereus	100	400	(Xuedong et al., 2005)
	Pseudomonas luteola	0	26.13	(Forlani et al., 1995)
	Serratia plymuthica	0	26.13	(Forlani et al., 1995)
	Sphingomonas paucimobilis	0	26.13	(Forlani et al., 1995)
	Stenotrophomonas maltophilia	0	26.13	(Forlani et al., 1995)
Imazethapyr (CAS 81335-77-5)				
B	*Azospirillum lipoferum*	0	28.93	(Forlani et al., 1995)
	Azotobacter chroococcum	0	28.93	(Forlani et al., 1995)
	Bacillus spp.	0	28.93	(Forlani et al., 1995)
	Bacillus subtilis	S/g	28.9	(Forlani et al., 1995)
	Brevundimonas vesicularis	0	28.93	(Forlani et al., 1995)
	Cronobacter sakazakii	0	28.93	(Forlani et al., 1995)
	Enterobacter cloacae	0	28.93	(Forlani et al., 1995)
	Mycobacterium spp.	0	36.17	(Grandoni et al., 1998)
	Pantoea agglomerans	0	28.9	(Forlani et al., 1995)
	Pseudomonas luteola	0	28.9	(Forlani et al., 1995)
	Serratia plymuthica	0	28.9	(Forlani et al., 1995)
	Sphingomonas paucimobilis	0	28.9	(Forlani et al., 1995)
	Stenotrophomonas maltophilia	0	28.9	(Forlani et al., 1995)
F	*Candida mengyuniae* sp. nov.	50	>5,000	(Chen et al., 2009)
	Candida shehatae	50	1,000	(Chen et al., 2009)
	Pichia farinosa	50	>5,000	(Chen et al., 2009)
	Saccharomyces cerevisiae	50	200	(Chen et al., 2009)
	Williopsis saturnus	50	5,000	(Chen et al., 2009)
Imazaquin (CAS 81335-37-7)				
B	*Arthrobacter crystallopoietes*	100	300	(Wang et al., 2007)
	Bacteria (not specified)	100	10	(Wang et al., 2007)

S/g – Slow growth; B – bacteria; F – fungi.

Most of the data in the tables is devoted to inhibiting pathogenic or soil bacteria and fungi. Pathogenic microorganisms have been reported to be less sensitive to the effects of AA inhibitors (Shehata et al., 2014). Therefore, it can be expected that herbicides may be more effective in inhibiting industrially important microorganisms. To summarise the data, concentrations of 0.01–1,000 mg L^{-1} should be used to determine each inhibitor's complete inhibition of the target microorganism. Subsequently, the tested concentrations can be reduced and narrowed around those mentioned in the literature to find the minimum concentration for full inhibition of the target microorganism.

APPLICATION OF MUTAGENESIS AND AMINO ACID INHIBITORS

Mutagenesis

Mutations are a natural process that occurs in all types of cells as a result of the influence of internal or external stimuli (Pacher & Puchta, 2017). Mutations in microbial cells create new genetic variations that allow them to survive and adapt to rapidly changing environments (Boyce, 2022). This knowledge has enabled the use of random mutagenesis to improve the productivity of commercially important microbial products (pigments, lipids, enzymes, surfactants, etc.) or increase tolerance to stressful conditions (Katre et al., 2017; Bouassida et al., 2018; Vasylkivska et al., 2020; Bleisch et al., 2022; Luna-Flores et al., 2022). Physical, chemical, or biological mutagenesis is widely used for these purposes (Bleisch et al., 2022).

Ultraviolet (UV) irradiation is a widely used physical mutagen (Yamada et al., 2017; Ardelean et al., 2018; Yu et al., 2020; Li et al., 2021). UV irradiation passes through cells, and both physical and chemical changes occur that can cause damage to cell structures such as membranes, enzymes, DNA, and others (Ho, 1975). UV light causes a mutation in which cytosine is modified to thymine by base substitutions at the dipyrimidine sites. UV irradiation also causes oxidative stress in cells by inducing the production of reactive oxygen species (ROS). ROS damages cellular DNA and can cause oxidative damage to DNA bases or even cause DNA breaks. It is known that some of these oxidative DNA and nucleotide damages can function as secondary mutagens. Therefore, it is possible to conclude that UV irradiation can induce specific primary DNA mutations and secondary mutations caused by oxidative stress (Ikehata & Ono, 2011). UV mutagenesis is a relatively simple and effective method for obtaining random mutations in the genome of a microorganism. It is worth noting that each microorganism requires a mutagenesis optimization step to determine the appropriate ratio of UV intensity to irradiation duration to achieve target cell mortality (Shibai et al., 2017; Suryadi et al., 2022).

Chemical mutagens can be divided into alkylating agents and base analogues (Leitão, 2012). Alkylating substances have a strong reaction with different matters and working with it should be done cautiously since they are toxic and have a similar effect to ionizing radiation. An example of such mutagen agents is ethyl methanesulfonate (EMS) and methylnitronitrosoguanidine (MNNG) (Manti & D'Arco, 2010). Alkylation of genetic material leads to the generation of triesters that degrade rapidly, producing alkyl groups that interfere with DNA replication. This process includes the hydrolysis of phosphate triesters, resulting in cleavage of the DNA backbone, and the alkylation of

nitrogenous bases, particularly guanine, which may lead to base-pairing errors during replication (Kodym & Afza, 2003). Other commonly used mutagens are the base analogue 5-bromouracil and nitric acid. The 5-bromouracil can form a base pair with adenine, one of the DNA bases, but also can unexpectedly convert to an isomer that binds to another nucleotide base - guanine, causing variation in a single DNA base pair which is called a transition mutation (Ross et al., 1987). Nitric acid influences both replicating and non-replicating DNA as well can induce the reversal of the mutant to the wild-type strain (Weiss, 2006).

Mutagenesis involves treating cells with a mutagen long enough to cause 50–95% cell death (Chumpolkulwong et al., 1997; Ang et al., 2019; Khan et al., 2020). It is necessary to find and choose an effective amount of mutagenic agent to mutate the target microorganism. The dose of mutagen may vary depending on the species, the time of mutagenesis, the environment temperature, and the composition of the medium. Therefore, preliminary tests are performed with different mutagens doses and exposure times to determine the optimal treatment of the target microorganism (Kodym & Afza, 2003; Demirkan & Özdemir, 2020). The next step is to incubate the cells in the dark for 24 hours and then plate them on the preferred selective medium. Improved strains can be selected using screening appropriate to the desired phenotype (Spencer & Spencer, 1996; Bleisch et al., 2022). Selective agents that exert specific pressure on cells are widely used to select mutants with desired properties. For example, the metabolic inhibitors diphenylamine, β-ionone, and antimycin A are used to select *P. rhodozyma* mutants with improved astaxanthin biosynthesis. Additionally, astaxanthin-producing mutants are selected by visual assessment of the size and colour of colonies on agar (An et al., 1989; Chumpolkulwong et al., 1997; Ducrey Sanpietro & Kula, 1998; Lin et al., 2012; Xie et al., 2014; Luna-Flores et al., 2022). The fatty acid inhibitors cerulenin, isoniazid, and triclosan are used to select mutants with increased lipid biosynthesis (Arora et al., 2020; Atzmüller et al., 2019; Katre et al., 2017). Furthermore, mutants with improved lipid synthesis can be screened using Sudan Black B stained cell microscopy, Nile red fluorescence microscopy, and spectrofluorimetry (Katre et al., 2017; Demirkan & Yıldırım, 2023).

Research on using random mutagenesis and selective agents to improve protein synthesis properties is limited. Significant improvements in protein biosynthesis have been reported in *Chlorella* sp. due to exposure to UV irradiation. Interestingly, the study aimed to improve biomass yield and lipid biosynthesis in microalgae, and the increase in protein content was determined by analysing the biochemical composition of biomass (Liu et al., 2015). In another study, UV irradiation successfully created *Bacillus megaterium* mutant with improved lysine biosynthesis (Li et al., 2015). Therefore, it can be assumed that random mutagenesis can be used to create protein-rich mutants. Further screening of mutants must be used since protein synthesis is a non-obvious phenotype, and selecting such mutants is challenging. Analysing the total protein content in all candidates that survived mutagenesis is not efficient because it is time-consuming and labour-intensive (Yu et al., 2020). Therefore, the use of selective pressure on protein synthesis by the AA inhibitor may be a good solution for effectively screening protein-rich mutants.

Mutagenesis using UV irradiation. A UV light source (254 nm) will be needed to perform mutagenesis with UV irradiation. The UV lamp should be turned on in the laminar flow hood 20 minutes before the start of treatment. Safety glasses should be worn during work to protect the eyes from UV light. The process steps listed below are designed for the yeast *Yarrowia lipolytica* (Winston, 2008; Atzmüller et al., 2019; Ozola, 2022):

1. Prepare an inoculum in a 50 mL flask in a universal yeast medium and incubate for 24 at 28 °C.
2. Collect 1 mL of the inoculum cell suspension and transfer it to a sterile 1.5–2 mL microcentrifuge tube.
3. Centrifuge cells at room temperature for 10–15 seconds using the 'pulse' function and decant the supernatant under aseptic conditions.
4. Resuspend the cells in 1 mL sterile distilled water, vortex, and centrifuge as described in step 3. Wash cells in sterile water twice.
5. Then, determine and dilute the cell concentration in the aqueous solution to obtain a concentration of 1.0×10^6 cells per mL.
6. Distribute the resulting suspension among sterile 1.5 mL tubes, each filled with 1 mL of the suspension (use enough tubes so that there is enough for the experiment and leave one tube that will not be treated. It will act as a control).
7. Place the vortex stand for microcentrifuge tubes 20 cm away from the UV lamp (the distance can also be optimised, with specific durations).
8. Treat the cells with UV light for different durations (e.g., 5, 10, 20, 30, 40, 50, and 60 seconds) while the tubes are vortexing at medium speed in the stand. For other microorganisms, a new combination of distance to the source and irradiation time should be selected to achieve the required percentage of cell mortality.
9. After treatment, dilute samples to 2,000 cells mL^{-1}. Plate 100 µL of each sample on agar in triplicate (respectively 200 cells per plate) and incubate at the microorganism's optimal temperature.
10. Count colony-forming units after 48–96 hours and determine the percentage of dead cells. Conditions that ensure the death of 50–95% of cells are recommended.

Notes: UV mutagenesis of cells can be performed on agar plates, considering the following features. Some components of the environment, such as vitamins, may be sensitive to UV irradiation. Treating cells with open plate lids is more practical, significantly reducing processing time.

Mutagenesis using MNNG. This MNNG mutagenesis protocol is based on methods described in the literature (Winston, 2008; Luna-Flores et al., 2022) and modified in our laboratory for mutagenesis of the red yeast *Phaffia rhodozyma* (Feldmane, 2023). These materials should be prepared in advance - universal yeast broth medium and agar plates, 0.1 M sterile citrate buffer (pH 5.5), and sterile 0.1 M phosphate buffer (pH 7.0). The half-life of EMS in water at pH 7.0 and 20 °C is 93 hours, and at 30 °C, the half-life is 26 hours. MNNG solution also is not stable for an extended period (Luna-Flores et al., 2022). Therefore, the mutagen solutions should be prepared before use and not stored (Kodym & Afza, 2003). It is also important to rinse and wipe all instruments, materials, and surfaces that were in contact with EMS mutagen after work with 5% sodium thiophosphate to inactivate it (Rowlands, 1984). The protocol includes step-by-step instructions for preparing microorganism cells for mutagenesis and subsequent plating:

1. Prepare an inoculum in a 50 mL flask in a universal yeast medium and incubate for 24–48 hours at 22 °C.

2. Transfer 1 mL of the inoculum cell suspension to a sterile 1.5–2 mL microcentrifuge tube. Centrifuge cells at room temperature for 10–15 seconds using the 'pulse' function. 20–22 °C will be the preferred centrifuging temperature for *P. rhodozyma* cells. Decant the supernatant and then resuspend the cells in 1 mL of sterile 0.1 M citrate buffer (pH 5.5). Wash cells in citrate buffer twice.

3. Prepare a 1.25 g L^{-1} (0.125%) MNNG solution using 0.005 g MNNG crystals. Dissolve them in 4 mL of sterile citrate buffer (pH 5.5). Vortex until the crystals are completely dissolved.

4. Dilute the MNNG solution to a concentration of 0.167 g L^{-1} (0.0167%). For example, transfer 200 µL of MNNG stock solution and 1,300 µL of citrate buffer into a 1.5–2 mL microcentrifuge tube.

5. Resuspend the washed cells without supernatant in 1 mL of MNNG solution (0.0167%) and incubate at 22 °C for 30 min. Gently shake the microcentrifuge tube a few times every 10 min to ensure that the cells are mixed evenly.

6. Centrifuge the cells and decant the MNNG solution, then wash them twice in 0.1 M phosphate buffer (pH 7.0).

7. To prepare the inoculum, resuspend all cells in approximately 15 mL of the universal yeast medium. Incubate cells overnight at 22 °C on an orbital shaker at 250 rpm.

8. The next day, dilute and count samples to 3,000 cells mL^{-1}. Plate 100 µL of each sample on agar in triplicate (300 cells per plate) and incubate at 22 °C.

9. Count colony-forming units after 48–96 hours and determine the percentage of dead cells. It is recommended that conditions ensure the death of 50–95% of cells.

Mutagenesis using EMS. This protocol is based on the method used by Luna-Flores et al. (Winston, 2008; Luna-Flores et al., 2022). These materials should be prepared in advance - medium plates, sterile 0.5 mM potassium phosphate buffer (pH 7.0), sterile 5% (w v^{-1}) sodium thiophosphate solution, and microbial medium. EMS concentration should be previously determined to cause 50–95% cell death in the test organism. All instruments, materials, and surfaces that were in contact with EMS mutagen after work should be treated with 5% sodium thiophosphate to inactivate the mutagen. In this description, the concentration was determined for the yeast strain *Phaffia rhodozyma* (Feldmane, 2023):

1. Prepare an inoculum in a 50 mL flask in a universal yeast medium and incubate for 24–48 hours at 22 °C.

2. Transfer 5 mL of inoculum to a sterile 50 mL centrifuge tube, then centrifuge the cells for 10 min at 1,800 × g, 22 °C. Decant the supernatant and add 5 mL of sterile 50 mM potassium phosphate buffer (pH 7.0).

3. Resuspend the cells then perform centrifugation as described in step 2. Repeat the washing twice.

4. Resuspend the cells in 5 mL potassium phosphate buffer and add 209 µL sterile EMS solution; the EMS concentration in the solution is 4%. Incubate the cells in this solution for 2 h at 22 °C on an orbital shaker at 250 rpm.

5. Then add 8 mL of sterile 5% (w v^{-1}) sodium thiophosphate solution to neutralise EMS, gently vortex and centrifuge the cells as described in step 3. with potassium phosphate buffer.

6. Wash the cells in potassium phosphate buffer twice.

7. To prepare the inoculum, resuspend all cells in approximately 15 mL of the universal yeast medium. Incubate cells overnight at 22 °C on an orbital shaker at 250 rpm.

8. The next day, dilute and count samples to 3,000 cells mL^{-1}. Plate 100 µL of each sample on agar in triplicate (300 cells per plate) and incubate at 22 °C.

9. Count colony-forming units after 48–96 hours and determine the percentage of dead cells. It is recommended that conditions ensure the death of 50–95% of cells.

Screening of mutants using AA inhibitors

Preparation of agar plates with AA inhibitors. The wild-type strain must be cultivated on selective agar with different inhibitor concentrations from 0.01 to 1,000 mg L^{-1} to determine the 100% cell inhibition dose. It should be noted that the selective agar does not have organic nitrogen in its composition, and the aqueous solubility of AA inhibitors is closely related to the pH of the solution. For example, the water solubility of sulfometuron-methyl at pH 5 is 0.00642 g L^{-1}, and at pH 8.6, it is 12.5 g L^{-1}, resulting in a 1947-fold increase in solubility with increasing pH (PubChem, 2024f). However, some AA inhibitors are practically insoluble in water; thus, they need to be dissolved in polar solvents. This study reports that the stock solution of the inhibitor imazaquin was prepared with methanol since its solubility in water is three times lower than in methanol (Wang et al., 2007; PubChem, 2024g). Polar organic solvents can also promote hydrolysis of herbicides such as chlorsulfuron, so it needs to be dissolved in dimethyl sulfoxide (DMSO) to prepare the stock solution (MedchemExpress, 2024; PubChem, 2024b). It is, therefore, important to study the available information on the properties of the inhibitor before preparing the solutions. The protocol for preparing an agar medium supplemented with 5 doses of inhibitor is as follows:

1. Prepare an AA inhibitor stock solution for the 420–540 mL of agar medium.

2. Sterilize the inhibitor stock solution using a membrane filter with a pore size of 0.22 µm.

3. Prepare 6 bottles of minimal agar medium for approximately 70–90 mL of medium for 6 plates each. Adjust pH to neutral and autoclave.

4. After sterilisation, cool the media to approximately 45–50 °C and add the required amount of inhibitor solution (add vitamins, salts, or antibiotics if necessary). Add AA inhibitor solution in different concentrations from 0.01 to 1,000 mg per litre of agar medium into 5 bottles.

5. Mix the agar thoroughly and carefully to avoid the formation of bubbles and pour into Petri plates.

6. Prepare an inoculum in a 50 mL flask in a universal yeast medium and incubate for 24–48 hours under optimal conditions.

7. Transfer 1 mL of the inoculum to a sterile 1.5–2 mL microcentrifuge tube. Centrifuge cells at room temperature for 10–15 seconds using the ‘pulse’ function.

8. Decant the supernatant and then resuspend the cells in 1 mL of 0.1 M phosphate buffer (pH 7.0). Wash the cells in phosphate buffer twice.

9. Prepare a cell suspension with 3,000 cells mL^{-1}. Plate 100 µL of cell suspension on agar in triplicate with 5 different concentrations of AA inhibitor and as a control on agar without inhibitor.

10. Incubate the plates for 2–7 days and count the surviving colonies to determine the approximate dose of complete inhibition. Repeat the test using narrow-range concentrations to find the minimum dose to completely inhibit the target microorganism.

Even though most inhibitor solutions can be stored at room temperature for 30 days, except for imazethapyr and imazapyr, these inhibitor solutions have a half-life of 2 and 6 days (PubChem, 2024c, 2024h), respectively, it is also better to store the solutions in the dark as they degrade rapidly in sunlight. All solutions can be stored in a freezer at -20 °C for 1 month or at -80 °C for 6 months ('MedChemExpress', 2024).

Selection of potential candidates on selective plates. When the needed dose for mutagen (~50–95% cell death) and inhibitor (100% inhibited growth) is found, these two factors should be combined. Plates with selective agar should be prepared so that there are at least three identical samples. In an experiment where mutagenesis is combined with an inhibition, many inhibitor concentrations should be tested. This will increase the chances of selecting potentially better mutants. For yeast, use a two-day-old inoculum and add 300 cells to each plate.

After incubation of the plates for two to three days, the potential mutant candidates are chosen and transferred to a new plate with the same inhibitor and concentration as the previous plate on which it was placed. The colonies can be selected by multiple strategies or characteristics: size, colour, growth speed, morphology, etc. When these candidates have grown on the new plates, those colonies that better fit the selection criteria should again be transferred to a regular agar plate, saved in storage, and further used in experiments. To determine whether mutant selection was successful chosen candidates should be used in a flask test obtaining biomass which can be used in detailed analyses. It is expected that the mutants will be able to synthesize more total protein or increase the synthesis of inhibitor-targeted AA.

As seen in Fig. 1. new mutants from the mutagenesis step should be placed in cold permanent storage. Although mutations will be inherited by the offspring of mutants, if possible, number of generations should be reduced as much as possible. The mutant microorganism has better chances to keep their gained quality if used under environmental conditions corresponding to employed selective stressors or, in this case, AA inhibitors (Lenski, 1991). Otherwise, those abilities can decline in time and may eventually be lost (Lenski, 1991; Peng & Liang, 2020). Other stressful environments can also promote oxidative stress to microorganisms that can damage DNA and trigger SOS response and adaptive mutation response (Peng & Liang, 2020). Genetic stability should be one of the factors that are tested after mutant creation by determining whether the new strain maintains obtained traits after multiple generations. It has been observed that strains can lose the ability to produce products upon repeated transfers in batch culture and the production rate can decrease during long-time fermentation (Peng & Liang, 2020). Therefore, it would be important to employ multiple preventive actions to maintain the enhanced abilities as long as possible for the mutant strain and not lose the newly acquired strain.

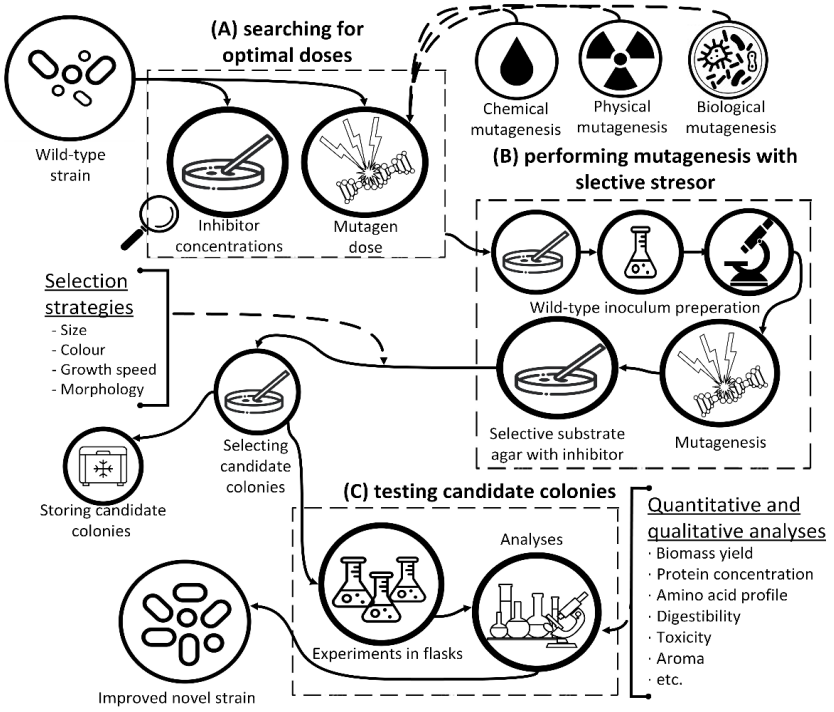

Figure 1. Process scheme for developing a novel mutant strain using mutagenesis and AA inhibitors.

In stage (A) (Fig 1, A), inhibition doses are searched where mutagenesis cell death is 50–90% and for AA inhibitor 100% growth inhibition. In stage (B) (Fig. 1, B), a wild-type strain inoculum is prepared to perform mutagenesis. Afterward, the cell aliquot with a concentration of 300 cells per 100 μL is inoculated on multiple selective substrate agar plates with AA inhibitors. Candidate colonies are selected after two to three days according to their size, colour, morphology, or other criteria and transferred on agar plates with the same properties as the previous one. When the new candidate colonies have grown, they are stored and used in experiments. In stage (C) (Fig. 1, C), the candidate colonies are used to perform experiments, which can take the form of, for example, flask tests where the candidates are compared to each other by determining which has higher quantitative properties, such as biomass yield or cell density. Later, gathered biomass can be used for further analysis where other quantitative and qualitative properties can be determined. According to the study results, the colonies with the best improved and desirable properties are considered new novel strains.

Determination of microbial growth inhibition using a microplate reader. An alternative approach to screening mutants on selective agar plates is to measure a cell suspension's optical density (OD) using a microplate reader. The use of 48-well or 96-well microplates increases screening throughput while reducing time and resource costs (Alcalde et al., 2005; Yu et al., 2020). However, OD measurements with a microplate

reader cannot distinguish between dead and viable cells. Therefore, cell counting at the end of cultivation is required (Hazan et al., 2012). The following protocol is applicable for screening potential mutants, where they are inoculated into a microplate after mutagenesis and placed in a medium supplemented with a selected inhibitor concentration.

1. Dissolve needed inhibitors.
2. Prepare optimal medium for the microorganism (buffered medium at 6–7 pH with inorganic nitrogen source should be used).
3. Divide the medium and add inhibitors in different concentrations determined previously. Use the 24-well, 48-well, or 96-well microplate with a working volume of 1mL, 0.5 mL, or 0.2 mL, respectively.
4. Inoculate each well of the triplet with an inoculum concentration of 1.0×10^6 cells mL^{-1} for each inhibitor dose.
5. Carefully fill the microplate with samples (each medium/inhibitor concentration should be done in triplicate).
6. Set the microplate reader to measure OD every hour and incubate the microplate at the optimal temperature with agitation for 48 to 120 hours.
7. Microscope and count the viable cells from each sample at the end of the test.
8. Based on the OD and number of cells, select the minimum concentration of inhibitor that completely inhibits the growth of the microorganism.

It is important to note that a significant portion of the inoculum cells may be lost after mutagenesis and buffer washing. Therefore, it is necessary to prepare a larger amount of inoculant, for example, 50–100 mL, depending on the cell density in the culture. This is necessary to subsequently be able to inoculate each well of the microplate with 1.0×10^7 cells per mL. Since, theoretically, after chemical mutagenesis, 10% of viable cells will remain in the inoculum. The optimal cell concentrations may vary depending on the microorganism and the used microplate reader. Consistency in the cell concentration directly inoculated into the microplate is crucial for identifying a complete growth-inhibiting inhibitor dose and seeding cells post-mutagenesis. Mutagenesis should be carried out on cells collected from the entire volume of the inoculum. Resuspend the cells in 5 mL of potassium phosphate as described in the EMS mutagenesis protocol and then follow the specified volumes of solutions. In the case of MNNG, it is necessary to increase the volumes of all solutions used in the protocol by at least five times. Further, all actions are performed according to the microplate protocol, with the only correction for the inoculation of cells in mL being ten times higher than that used in the previous test to search for a complete growth-inhibiting dose. After completing the microplate test, it is necessary to take samples from the wells with confirmed microbial growth for further inoculation into flasks for protein analysis from the collected biomass.

Estimated effectiveness and safety of AA inhibitors

Determining which of the mutagens (UV radiation, EMC, or MNNG) is more effective is difficult. Improved strains have been obtained using each of them, according to numerous publications (Lin et al., 2012; Katre et al., 2017; Arora et al., 2020; Bleisch et al., 2022; Demirkan & Yıldırım, 2023). However, a comparison of these three mutagens according to such criteria as methods probability of success, approximate induced mutation frequency, methods toxicity to environment, price of the required

amount of mutagen per run, etc., using the MCDA was rated higher for EMS and lower for MNNG (Berzina et al., 2024). It is worth noting that combining these mutagens leads to a more effective result (Agrawal et al., 2013; Kumar et al., 2015; Zhang et al., 2016; Gopinath et al., 2020). The effectiveness of AA inhibitors as a selective agent for screening protein-rich mutants can be assessed after experimental tests. On the other hand, a preliminary MCDA analysis of AA inhibitors according to criteria such as inhibition efficacy, inhibited EAA, possibility of false positive selection, price of inhibitor, etc., showed potentially the best inhibitors for bacteria and fungi. According to the analysis, glufosinate-ammonium, L-methionine sulfoximine, L-α-(2-aminoethoxyvinyl) glycine, and S-(2-aminoethyl)-L-cysteine are potentially the best AA inhibitors for both bacteria and fungi. However, considering the high cost of L-α-(2-aminoethoxyvinyl) glycine, using this AA inhibitor for mutant screening seems irrelevant (Berzina et al., 2024). Promising results were obtained in a study of the effects of herbicides on wine yeast. Biochemical analysis of *Saccharomyces cerevisiae* biomass treated with the inhibitor glufosinate-ammonium showed a significant increase in some essential AA compared to untreated biomass (Vallejo et al., 2017).

Currently, the mechanisms of AA inhibitors' action on the metabolic pathways of microorganisms have not been well studied to provide a clear understanding of the results of their use. The safety of feed containing mutant biomass previously treated with herbicide is controversial. It is known that herbicides such as glyphosate, glufosinate ammonium, sulfonylurea herbicides (Table 4), and imidazolinones (Table 5) selectively inhibit the amino acid biosynthesis pathway in plant, fungal, and insect cells, but not in animals and humans (Gupta, 2018; Thiour-Mauprivez et al., 2019). Studies report these herbicides' absence of mutagenicity and carcinogenicity, but they harm animal health at specific doses (Gupta, 2018; Thiour-Mauprivez et al., 2019; Berry, 2020; Peillex & Pelletier, 2020). DL-propargylglycine and L-methionine sulfoximine inhibit the amino acid biosynthetic pathway in animals. On the other hand, this effect of inhibitors can be used in therapy and minimisation of the consequences of several human diseases (Brusilow & Peters, 2017; Zhou et al., 2018). AA inhibitor S-(2-aminoethyl)-Lcysteine hydrochloride is an analogue of lysine and is completely safe for animals (Friedman & Gumbmann, 1981; Li et al., 2015). It can be concluded that the herbicide content in edible microbial biomass is not desirable. The safety of such mutants can be confirmed in the absence or acceptable levels of herbicides and their breakdown products in biomass using gas chromatographic analysis or another alternative technique. It is important to note that when using mutagens and AA inhibitors in research, the precautions specified by the manufacturer of the substances should be strictly followed and disposed of properly.

CONCLUSIONS

Many microorganisms, such as bacteria, fungi, and microalgae, are excellent SCP producers. Microbial growth rate, protein productivity, and protein quality are significant determining factors for the successful production of SCP on a commercial scale. Improved microbial strains capable of synthesising more proteins, mainly by increasing the content of amino acids that are limiting in traditional plant-based protein sources, will increase the potential of SCP and contribute to the commercial development of technology.

In the present study, we examined thirteen herbicides with the potential to exert selective pressure on mutant microbial strains to select improved SCP producers. Literature data are summarised in five tables and contain concentrations of herbicides inhibiting bacteria, yeast, and moulds. Microalgae, protists, and other microorganisms were not considered. However, the presented protocols may apply to additional inhibitory dose studies. The article contains protocols for random mutagenesis and describes the preparation of a selective medium for subsequent screening of mutants with desired properties on plates or with a microplate. The article is valuable for its detailed description of both critical aspects of the methodology and recommendations based on our experience working with AA inhibitors in the laboratory.

In the future, a series of experiments must be conducted to confirm the effectiveness of AA inhibitors in selecting protein-synthesizing mutants and the stability of the mutation. If the result is positive, the growing conditions must be optimised, and the quality of the biomass and protein, including its safety for use in feed or food, must be analysed. Thus, using this technology to create SCP-improved strains on a broad scale could reduce pressure on conventional protein production sectors such as agriculture and fishery.

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In search of the best technological solutions for creating edible protein-rich mutants: a multi-criteria analysis approach

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Abstract. Single-cell protein (SCP) is a promising alternative for replacing plant and animal-derived dietary proteins. SCP contains essential nutrients and high levels of essential amino acids (AA). Given the versatility of microbial strains and waste substrates that can be used as feedstocks, many variations of production processes can be explored. Improving these microorganism strains by enhancing their properties and productivity is vital to increasing SCP competitiveness. One of the options to enhance microorganism strains would be by creating mutants with better AA profiles. By using mutagenesis and AA inhibitors it should be possible the create novel strains with improved AA-producing properties. The use of AA inhibitors to promote selective pressure on SCP-producing strains is a novel concept and is not a widely explored approach, therefore, the further development of this method should be explored. This paper used a multi-criteria decision analysis method to evaluate different technological factors vital for creating protein-rich mutants. These factors are microorganism strains, agro-industrial waste substrates used as process feedstocks, AA inhibitors, and mutagenesis methods. Microorganisms *Candida utilis* and *Bacillus subtilis* showed the highest potential for being used. Molasses was the 'closest to the ideal' substrate to be used as feedstock for SCP production. As the most promising mutagenesis method ethyl methane sulphonate was selected. Glufosinate ammonium and methionine sulfoximine for both bacteria and fungi were identified as the best inhibitors for SCP-rich mutant selection. Identified combinations of optimal solutions for microorganisms, substrates, inhibitors, and mutagenesis techniques should be further investigated and evaluated in laboratory settings. This could help to increase SCP's competitiveness as a sustainable protein source.

Key words: agro-industrial waste, amino acids, amino acid inhibitors, biomass, herbicides, low-cost substrate, microbial protein, microorganisms, multi-criteria analysis, MCDA, mutagenesis, proteins, residues, single-cell proteins, SCP, TOPSIS, waste biomass.

INTRODUCTION

Proteins have always played a significant role in maintaining human health. They contain amino acids (AA) which are crucial for various physiological processes in the body (Martin, 2001). Livestock products contribute over 33% of the total protein intake in human diets (Martin, 2001), and approximately 83% of the world's agricultural land

is used to produce feed for livestock (Mekonnen & Hoekstra, 2012; Poore & Nemecek, 2018). This area could be potentially used to grow food to feed an additional 3.5 billion people (Cassidy et al., 2013). Meanwhile, fish and crustaceans account for 17% of the world's protein intake (FAO, 2014). The use of fish and crustaceans has caused overfishing by depleting marine fish resources leading to 391 species threatened with extinction (Øverland et al., 2013; Dulvy et al., 2021). This has spurred the rapid expansion of aquaculture in the past two decades to meet the increasing demand for fish (Yarnold et al., 2019), necessitating the provision of essential nutrients for farmed fish. While aquaculture has surpassed wild-capture fisheries in production volume, it still heavily relies on wild capture for fishmeal (Tacon & Metian, 2015). This dependence poses challenges, showing the need for more sustainable solutions such as single-cell protein (SCP) (Spinelli, 1980; Yarnold et al., 2019). SCP is an alternative protein source that could help to improve sustainability and reduce the scarcity of proteins (Najafpour, 2007; FAO, 2020). Increasing the use of SCP, for example, in livestock feeds could reduce the need for intensive farming while aligning with environmental strategies for reducing greenhouse gas emissions (European Commission, 2012, 2019a).

SCP are known as bioproteins, microbial proteins, or microbial biomass. The technology has many advantages over traditional dietary proteins, since production is more environmentally friendly, consumes less water, requires smaller land areas, is not influenced by climatic conditions, and can be produced from agro-industrial by-products (Singh & Mishra, 1995; García-Garibay et al., 2014; El-Sayed, 2020). Each microorganism has its own capabilities to consume waste substrates as feedstocks and the ability to synthesize proteins and AA. For choosing the best microorganism for SCP production it should be capable of synthesizing large amounts of proteins, and essential AA (EAA), as well as the ability to grow in large density and consume various substrates as feed. The use of different waste substrates can be environmentally friendly, resource and cost-efficient (Pogaku et al., 2009). Waste substrates can be used as carbon sources and nitrogen sources for the microorganism. Carbohydrates typically contribute to about 0.5 g of dry biomass per gram of substrate and the carbon source can account for approximately 60% of the production costs significantly influencing the outcome and costs of SCP (García-Garibay et al., 2014). Nitrogen source can be one of the most important factors that can directly influence protein synthesis by microorganisms (Vethathirri, et al., 2021). The use of waste substrates in the production of value-added products is in line with multiple European Union goals (Tutto, 2017; European Commission, 2018, 2019b; Vidal-Antich et al., 2022).

The SCP production technologies have been extensively researched (P&S Intelligence, 2018), and are steadily growing as more products are being introduced into the market (Ritala et al., 2017). It has been widely used as a food supplement for humans and as a feed for animals (Kumar et al., 2024). Currently, SCP is being produced under different commercial names like Brovile®, AlgaVia®, Quorn®, Vitam- R®, Pruteen®, Marmite®, and FermentIQ™, etc. (Wikandari et al., 2021; Kumar et al., 2024). Although there already are some products in the market, they remain a niche product that is not widely available or consumed (Salazar-López et al., 2022). However, a report published by Market Research Intellect evaluated that the SCP market size was USD 6.64 billion in 2023 and that it is expected to reach USD 10.4 billion by 2031, growing at a 4.42% CAGR from 2024 to 2031 (Intellect, 2024).

To better introduce new SCP products to the market requires efforts by various actors, particularly by different businesses, investors, and engineers, who can help solve the different challenges that this industry is facing (Van Der Weele et al., 2019; Wada et al., 2022). Several challenges need to be overcome before more large-scale SCP processes are introduced in the market, necessitating more pilot-scale demonstrations to increase technology readiness level (Sekoai et al., 2024), as well as challenges in terms of consumer acceptance and market adoption (Van Der Weele et al., 2019; Salazar-López et al., 2022). More studies should be conducted to assess the technical and economic feasibility of SCP processes, especially using food waste as a carbon source (Sekoai et al., 2024). To increase the diversity of the technology, cheaper carbon sources and optimal process parameters are still being researched as well as applicable microorganisms (Salazar-López et al., 2022; Kumar et al., 2024). New scientific tools are being used to enhance strain performance by targeting SCP-producing biochemical pathways (Sekoai et al., 2024). Enhancing and creating a strain with superior properties can increase SCP competitiveness (Spalvins et al., 2021). Classical mutagenesis and random screening methods are simple and efficient methods for strain development (Rowlands, 1984; Anderson, 1995; Winston, 2008; Atzmüller et al., 2019) and are still widely used (Yamada et al., 2017; Zhu et al., 2018; Atzmüller et al., 2019; Soedarmodjo & Widjaja, 2021). After treating the microorganism with a mutagen, the surviving cells must be selected for desired traits, for example, by using a selective media (Spalvins et al., 2021). This strategy would help to create improved SCP-producing strains that have higher total protein and AA content. EAAs such as lysine, methionine, threonine, and tryptophan are very important, as they are available in lower amounts in conventional plant-derived protein sources (Spinelli, 1980; Al-Marzooqi et al., 2010; Finco et al., 2017; Hardy et al., 2018).

AA inhibitors were selected as potential selective agents for the selection of improved SCP-producing mutants. AA inhibitors are the active ingredients in commercial herbicides developed for weed control. The main principle of herbicides is the inhibition of the enzymatic activity responsible for the biosynthesis of AA in cells, as a result of which the treated weeds die (Kumada et al., 1993; Ravanel et al., 1998; Vallejo et al., 2017; Lonhienne et al., 2020; Tall & Puigbò, 2020). Herbicides have been used ubiquitously for over 50 years in agriculture and during this time the effects of herbicides on the agroecosystem have been studied. Studies have shown that herbicide treatment reduces the numerical population of certain microorganisms in the soil and on the surface of cultivated plants (Wang et al., 2012; Sardrood & Goltapeh, 2018; Łozowicka et al., 2021). Almost all herbicides are nonspecific and have an inhibitory effect on the enzymatic activity of fungi, molds, bacteria, and algae, suppressing their growth at certain concentrations (Kumada et al., 1993; Ravanel et al., 1998; Grant Pearce et al., 2017; Lonhienne et al., 2020; Tall & Puigbò, 2020; Couchet et al., 2021). It is expected that the use of AA inhibitors may identify protein-synthesizing mutants capable of increased protein synthesis, similar to the successful use of fatty acid inhibitors to select single-cell oil-synthesizing mutants (Atzmüller et al., 2019). The use of AA inhibitors to promote selective pressure on SCP-producing strains is a novel concept (Spalvins et al., 2021), and is not a widely explored approach. Consequently, the further development of this method is scientifically innovative. It is important to note that varieties of induced mutant microorganisms are widely used in the food industry (Molzahn, 1977), pharmacy (Butler, 2011), biofuel production (Raita et al., 2021),

enzyme production (Kumar et al., 2014), and many other industries. The creation and distribution of induced mutants is not restricted and the use of induced mutants in human and animal consumption is considered safe (Yamada et al., 2017), therefore mutagenesis and AA inhibitors can be used to create SCP-rich mutants.

After mutagenesis and selective screening using AA inhibitors, the microorganism needs to be reevaluated for its safety. A status such as GRAS (Generally Recognized as Safe) or being on the Qualified Presumption of Safety (QPS) list helps to speed this process (Galano et al., 2021). For example, in the EU microorganisms from the QPS list are considered safe, and mutated microorganisms have fewer requirements to prove their safety. After confirming that the genetic modifications have been evaluated and do not raise any safety issues, the generated mutant strain is deemed safe once more (Galano et al., 2021).

By summarizing the SCP technology and SCP-producing mutant creation, firstly, it is important to choose the microorganism and feedstock that are applicable to each other and can provide significant results in biomass and protein concentrations (Spalvins et al., 2018b, 2018a). By using mutagenesis microorganism cells are damaged and mutations in them can be induced. A mutagen dose should be found that causes 50–90% of cell death. By applying the treated cells in its selected feedstock medium with AA inhibitor, which creates a selective pressure allowing only those cells that are more capable of AA synthesis to grow. It is necessary to choose an inhibitor concentration that causes 100% growth inhibition for the wild-type strain. It should be noted that at this stage the microbial medium should be without organic nitrogen to improve the AA inhibition effectiveness (Raita et al., 2024). Candidates can be selected according to various criteria such as their size, colour, morphology, etc. When the new candidate colonies have grown, they are stored and used in experiments.

A scheme of the process is visualized in Fig. 1.

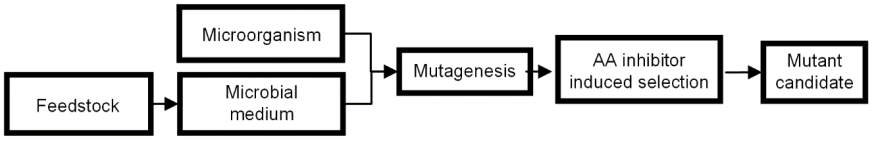

Figure 1. Scheme for the process of creating mutants.

A more detailed description of the process has been provided by the authors in (Raita et al., 2024) review paper.

This study aims to compare and find the best alternatives for creating edible protein-rich mutants in four technological aspects: microorganism strain, waste substrates used as process feedstock, AA inhibitor, and mutagenesis method. To achieve that multi-criteria decision analysis (MCDA) was used to identify the most suitable sets of appropriate microorganism strains, mutagenesis techniques, applicable AA inhibitors, and low-cost medium feedstock by comparing alternatives in each group and finding the 'closest to ideal'. Finding the potentially best solution could be beneficial for developing a methodology for creating new SCP-producing mutant strains. From MCDA selected sets should be evaluated in laboratory settings verifying the possibility of creating enhanced mutant strains that would be superior to the currently used strains. Hopefully,

with this MCDA the authors will find the best potential alternatives, with which in the future it will be possible to create an enhanced strain that can compete as a product for aquaculture feed, with a superior AA profile and protein quality than fishmeal (Cho & Kim, 2011).

MATERIALS AND METHODS

In this study, the methodology includes the MCDA method TOPSIS (Technique for Order Preference by Similarity to Ideal Solution) which is used to compare different technological alternatives. The TOPSIS tool provides an optimal solution by calculating the relative closeness coefficient to the ideal solution (Tzeng & Huang, 2011), namely, identifying the best alternative depending on set criteria. The implementation of TOPSIS distinguishes six main steps - identifying indicator matrix, calculating normalized matrix, calculating weighted normalized matrix, calculating ideal and anti-ideal values, and calculating relative closeness coefficient for each alternative and ranking the results. The closeness coefficient is always between 0 and 1, where 1 is the preferred action or solution (Tzeng & Huang, 2011). The methodologies algorithm is represented in Fig. 2. The methodology of performing TOPSIS can be found in more detail described by (Behzadian et al., 2012).

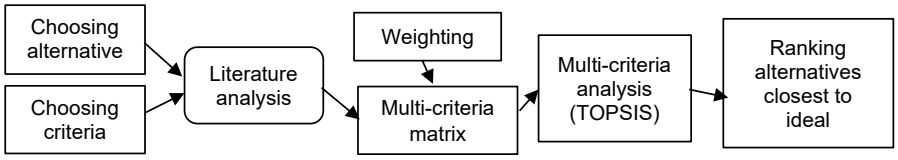

Figure 2. Methodologies algorithm.

The advantages of the TOPSIS methodology are that it is the most significant approach to solving real-world problems, it is possible to immediately recognize the proper alternative, it can be used for situations with many alternatives and attributes, and is suitable for use with quantitative or objective data (Alsalem et al., 2018). Its disadvantage would be that it lacks provision to weigh elicitation and TOPSIS determines the selected alternative based on its proximity to the ideal solution and the greatest distance from the 'negative-ideal' solution; however, it does not consider the relative importance of the distances from these points (Alsalem et al., 2018).

Criteria weights for microorganisms, waste substrates, and mutagenesis methods were based on expert evaluation. People who have studied or at the moment work in biology, environmental engineering, and food technology were targeted as potential experts. Together thirty-two experts participated in the evaluation. Of these experts, five were with doctoral degrees, sixteen with master's degrees, and eleven with bachelor's level degrees. Eighteen of the participants were from the biology or biotechnology fields, nine – were environmental engineers, two - were food and technology engineers, and one representative each from chemistry, molecular genetics, and pharmacology fields. For questionnaire the Google Forms was used (Annex A). Weights for AA inhibitors criteria were provided and determined by the 10 researchers of Riga Technical University, Institute of Energy Systems and Environment with expertise in microbiology

and biotechnologies who have been researching this novel idea of using herbicides as AA inhibitors for developing mutant strains. Each person gave the evaluation without consultation with others to provide a discrete individual evaluation. The weighted sum for all criteria in each analysis was one. Sensitivity analysis was not performed because criteria weights were based on expert evaluation.

The alternatives and criteria used will be described, discussed, and evaluated in further sections for each factor. Data and formulas for the multi-criteria matrix can be seen in Annex A.

For microbial strain evaluation, twelve alternatives and thirteen criteria were chosen based on the literature review. Data for criteria were acquired from publications with two principles to ensure a balance of data. The first principle was that the authors used unselected, unmodified microorganisms (wild-type) and the second principle was that the authors used batch fermentation. For strain evaluation 54 literature sources were used, including 42 publications, six sources from Food and Drug Administration database (fda.gov), one source from the Google patents database (patents.google.com), and six sources from chemical supplier websites. While evaluating the substrate factor, eleven alternatives and ten criteria were chosen based on the literature review. Data for criteria were acquired from publications with a principle that the fermentation process was performed using batch fermentation with unmodified microorganisms. For substrate evaluation 37 literature sources were used, including 35 publications and two internet sources such as The Food and Agriculture Organization database. For mutagenesis methods evaluation three alternatives and six criteria were chosen based on a literature review which included 11 sources from which seven were publications and internet resources from various chemical suppliers. Data collection for the evaluation of amino acid inhibitors was carried out based on the available literature according to the following criteria:

- include 33–37 amino acid inhibitors mentioned in a previous publication (Spalvins et al., 2021), incl. 5–6 inhibitors from each group such as sulfonylureas and imidazolinones;
- include an inhibitory effect on cells or directly on enzymes in vitro of bacteria, yeast, and fungi;
- include concentrations of AA inhibitors with 100% inhibition;
- include concentrations of AA inhibitors with 50%, 70% and 90% inhibition;
- include concentrations that provide significant inhibition of microbial growth;
- include results from studies using both commercial herbicides and their pure compounds.

Data were successfully collected for 17 amino acid inhibitors and then MCDA was carried out separately for fungi and bacteria. To summarize, 11 and 17 AA inhibitors were analysed according to 7 criteria for application to fungi and bacteria, respectively. The literature review of bacteria AA inhibitors consisted of 31 publications while the review of fungal AA inhibitors consisted of 27 publications.

RESULTS AND DISCUSSION

Evaluation of microorganisms

For microbial strain evaluation, twelve alternatives were chosen from which four were bacteria (*Bacillus subtilis, Bacillus cereus, Escherichia coli, Lactobacillus acidophilus*), four were fungi (A*spergillus niger, Aspergillus oryzae, Paecilomyces variotii, Fusarium venenatum*) and four were yeasts (*Candida tropicalis, Candida utilis, Yarrowia lipolytica, Phaffia rhodozyma*). The choice of strains to be included in this study was made based on previously conducted studies and reviews (Spalvins et al., 2018a, 2018b; Raita et al., 2022). These strains have shown that they can synthesize an impressive amount of biomass with protein content as high as 71% of the dry biomass (Spalvins et al., 2018b). They are also capable of utilising different low-cost substrates as feedstock which is a beneficial advantage for SCP production. The different species were evaluated by thirteen criteria (see Table 1). In the data search for criteria, batch experiment fermentation parameters were used, excluding continuous or fed-batch fermentation to increase comparability between microorganism species as continuous or fed-batch data for many of them were not available. In addition, only information about wild-type strains was used and data about mutants or genetically modified organisms were not included. Microorganism GRAS status was evaluated with values 0 or 1, where 0 was attributed to strain with no GRAS status and 1 was attributed to strain with GRAS status. The ability to produce valuable secondary metabolite criterion was evaluated with values 0 or 1, where strain with no ability to produce a valuable secondary product was attributed zero and strain with the said ability with value 1. Quantitative values for other criteria values were acquired from the literature.

Table 1. Indicators and weights used in MCDA of microorganisms

Criteria and unit of measurement	Unit of measure	Weight
Biomass concentration	g biomass L^{-1} medium	0.082
Protein content	% of total biomass	0.104
Yield efficiency	g biomass g^{-1} medium	0.093
Fermentation time	h	0.089
Protein production rate	g biomass L^{-1} medium h^{-1}	0.097
Optimal temperature	°C	0.069
Approximate mutagenetic resistance (EMS concentration with a survival rate of 10% to 1% and exposure time from 15 to 60 minutes)	M	0.010
EAAs content	% of total protein	0.090
Content of AAs that are lacking in the plant-derived protein	% of total protein	0.101
Microorganism GRAS status	-	0.066
Ability to produce valuable secondary metabolite	-	0.067
Revenue from metabolite production using 1 ton fermenter	Euro day^{-1}	0.073
Nucleic acid content	% of total biomass	0.058
	Σ	1

The criteria for alternatives were assessed by experts in the following order of importance: protein content > content of AAs that are lacking in the plant-derived protein > protein production rate > yield efficiency > EAAs content > fermentation time > biomass concentration > revenue from metabolite production > optimal temperature >

ability to produce valuable secondary metabolite > microorganism GRAS status > nucleic acid content > approximate mutagenetic resistance. Approximate mutagenetic resistance was evaluated only by the authors and was not added to the questionnaire due to it being relevant only during the initial development of mutagenesis protocols (see Annex A), this criterion does not affect the SCP production itself. The results are visualized in Fig. 3.

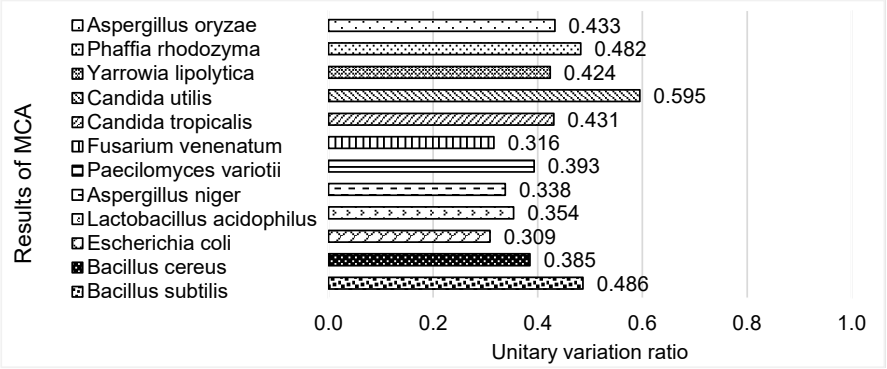

Figure 3. MCDA results of microorganisms.

Candida utilis, *Bacillus subtilis,* and *Phaffia rhodozyma* all showed great potential as SCP producers, and as can be seen in Fig. 3 *Candida utilis* were the closest to the ideal solution reaching 0.595. *C utilis* ranked second in protein content, first in protein production rate, and third in AAs that are lacking in the plant-derived protein criterion (Annex A), all criteria were deemed as crucial for SCP producers by experts. *C. utilis* have been researched for SCP production on various waste substrates such as wine lees, potato waste, pineapple cannery effluent, and salad oil factory wastewater (Carranza-Méndez et al., 2022). In batch fermentation, *C. utilis* can achieve a growth rate of 0.68 g L^{-1} h^{-1} and a protein production rate of 0.51 g L^{-1} h^{-1} (Rajoka et al., 2006), however in continuous fermentation, the growth rate can reach 1.62 g L^{-1} h^{-1} and the protein production rate 0.63 g L^{-1} h^{-1} (Lucca et al., 1995). This makes the microorganism promising for SCP production as growth rate and protein production rate are important to successfully commercialize SCP production. It should be noted that different *Candida* species are opportunistic human pathogens, which includes one of the assessed alternative strains *C. tropicalis* which does not have GRAS status due to possible health risks (Bajić et al., 2023). Even though opportunistic pathogen status does not forbid microorganism use for SCP production it will increase post-treatment costs and can cause wariness in costumers for derived product's safety. GRAS status not only helps with commercialization but also with documentation as novel foods need to go through accreditation of safety and it can cause problems when the chosen microorganism can pose health risks to humans. Another vital parameter of SCP is digestibility. For *C. utilis* protein digestibility varies between target organisms for weaned piglets it is reported as 80% with a diet of 40% crude protein from *C. utilis* (Cruz et al., 2019), for Atlantic salmon (*Salmo salar*) 88% with a diet of 40% biomass from *C. utilis* (Øverland et al.,

2013) and for tilapia fry (*Oreochromis mossambicus*) reached 83.2% with a diet of 35% biomass from *C. utilis* (Olvera-Novoa et al., 2002).

Second runner-up *B. subtilis* ranked 3rd in protein content, 5th in protein production rate, and 9th in the amount of AAs that are lacking in the plant diet criterion (Annex A). *B. subtilis* is an aerobic, gram-positive soil bacterium that has been frequently employed in biotechnology. It secretes a variety of enzymes that can degrade a wide range of substrates (Su et al., 2020). This includes groundnut, walnut, and melon shells, ram horn, and soybean hull (Omogbai & Obazenu, 2017; Bratosin et al., 2021). The growth rate of *B. subtilis* in batch fermentation can reach 0.15 g L^{-1} h^{-1} and a protein production rate of 0.11 g L^{-1} h^{-1} (Kurbanoglu & Algur, 2002). As previously mentioned, to successfully compare all chosen microorganisms only batch fermentation data were used and one of the reasons was that wild-type *B. subtilis* has not been used in continuous fermentation for SCP production thereby research in this section could be beneficial. A noteworthy aspect is the reported resistance and biodegradation capabilities of some herbicides such as nicosulfuron (Z. Zhang et al., 2020), tribenuron-methyl (Zeinali Dizaj et al., 2023), and glyphosate (Yu et al., 2015) which are AA inhibitors. Therefore, using AA inhibitors as selective agents for increased AA content could be complicated due to this reported resistance. *B. subtilis* for now is mostly added as an additive and acts as a probiotic (Félix et al., 2010) and while there is little to no data on *B. subtilis* use as feed, it can be expected that *B. subtilis* would show similar results as other bacteria. *Methylophilus methylotrophus* in a diet for Rainbow trout (*Oncorhynchus mykiss*) with 28% concentration has reached 84% digestibility, while *Methylococcus* with *Alcaligenes* and *Bacilus* have shown various results from 88 to 85% digestibility (Glencross et al., 2020).

P. rhodozyma resulted as third in the MCDA while ranked 9th in protein content, 9th in protein production rate, and first in AAs that are lacking in the plant diet criteria (Annex A). It can utilise various carbon-rich substrates such as molasses, peat hydrolyses, eucalyptus hydrolysates, sugarcane juice, corn wet-milling, and corn starch hydrolysate (Roy et al., 2008; Luna-Flores et al., 2022). Another criterion where *P. rhodozyma* scored the highest was approximate revenue from industrial-grade metabolite production, as astaxanthin is a high-value substance with high market demand (Patel et al., 2022). Even though the majority of studies of *P. rhodozyma* have been focused on astaxanthin production (Mussagy et al., 2022), there have been successful attempts at the simultaneous production of biomass and astaxanthin (Moriel et al., 2004). Most improvements in astaxanthin production were developed with mutagenesis (Xie et al., 2014; Mussagy et al., 2022), and simultaneous screening for protein and astaxanthin production could result in industrially suitable strains. In batch fermentation, *P. rhodozyma* can obtain a growth rate of 0.13 g L^{-1} h^{-1} and protein productivity of 0.06 g L^{-1} h^{-1} while in fed-batch fermentation growth rate of 0.38 g L^{-1} h^{-1} and protein productivity of 0.18 g L^{-1} h^{-1} was achieved (Zhang et al., 2023). Similar to *B. subtilis* also *P. rhodozyma* is mostly used as a feed supplement with less than one percentage concentration (Bjerkeng et al., 2007) with no available data on digestibility tests.

Another prospective SCP producer is *Yarowia lipolytica* which resulted in a very close MCDA ranking with *Aspergillus oryzae*. One of the drawbacks of *Y. lipolytica* use in SCP production is the insufficient protein content of the biomass and in the case of *A. oryzae* - inadequate amounts of biomass production. Even though both microorganisms have limitations for becoming effective SCP producers, during the strain creation beneficial mutations could emerge that can remedy these limitations.

Metabolite production would add another revenue stream alongside SCP production. However, extraction could potentially cause degradation of SCP quality such as the use of organic solvent extraction (Kim et al., 2021; Zhang et al., 2023). But while there are risks of lowering the quality of protein using harsh extraction methods there are methods with minimal effects on protein quality such as an aqueous two-phase system (Santos et al., 2022). Another perspective is metabolites that do not require extraction, such as astaxanthin, where metabolite production does not affect protein quality as biomass of microorganisms has a dual purpose – source of SCP and source of astaxanthin (Lim et al., 2018).

Evaluation of waste substrates for microbial medium

Food wastes and by-products from food industries have a great potential for being used as a feedstock for protein production (Muniz et al., 2020). The approach of using different substrates improves cost-effectiveness and resource effectiveness when implemented at scale (Pogaku et al., 2009). Eleven different alternative substrates that can be used either as a carbon source or nitrogen source were evaluated: glycerol (from biodiesel production), straw hydrolysate (agricultural residue), molasses, potato starch, and pulp, fruit, brewery and spent grain residue, and liquid cheese whey, fish residues and waste cooking oil (from food and beverage processing industries).

Chosen substrates differ from each other in many aspects such as composition, structure, texture, complexity, etc. Molasses, cheese whey, some fruit wastes, and straw hydrolysate can be classified as monosaccharides and disaccharides-rich sources, while fruit residues that are rich in fibre, potato residues, brewery residues, and spent grains are structural polysaccharides-rich sources (Spalvins et al., 2018a). Polysaccharides-rich sources can be more difficult to incorporate in mediums than mono- and disaccharides-rich sources. They often need to be pre-treated or the used microorganism must be able to hydrolyze it. It can be difficult for some microorganisms to use polysaccharides as feedstock if they cannot produce the necessary enzymes or the optimal conditions for growth and enzymatic activity differ and both actions cannot be done simultaneously (Berzina & Spalvins, 2023). Waste cooking oil as a lipid-rich source has the potential to be used as a carbon source for microorganisms that can produce extracellular emulsifiers (Garti et al., 2001; Patel et al., 2015; Spalvins et al., 2020).

Substrate alternatives were evaluated according to ten criteria with weight provided by experts (see Table 2). Values for criteria such as expected protein yield, expected biomass and protein concentration protein content, average biomass production rate, availability, and pre-treatment cost were obtained from published papers (Annex A). Such criteria as shelf life and the energy required for storage were evaluated based on whether the substrate could be stored at room temperature (1), cold storage (5), or freezing (10). These values were chosen based on current rent prices for storage facilities and energy expenses (rent per volume is approximately five times cheaper than storing in refrigerated (cold) storage and freezing storage is two times more expensive than cold storage) (Høyli & Aarsæther, 2023). Similarly, the seasonality of waste product generation was evaluated, considering whether the substrate could be produced year-round or only during certain months (1–12). Therefore, these three criteria assumptions were made to assign values.

Table 2. Indicators and weights used in MCDA of waste substrates

Criteria	Unit of measure	Weight
Expected biomass yields	g g^{-1}	0.105
Expected biomass concentration	g L^{-1}	0.103
Expected protein content in biomass	%	0.123
Average biomass production rate	g L^{-1} h^{-1}	0.110
Substrate availability	million t $year^{-1}$	0.099
Shelf life	-	0.074
Substrate seasonality	-	0.084
Storage cost	-	0.078
Substrate pre-treatment costs	EUR t^{-1}	0.104
Substrate price	EUR t^{-1}	0.121
	Σ	1

The criteria are listed in descending order according to expert evaluation: protein content > substrate price > average biomass production rate > expected biomass yield > pre-treatment cost > protein concentration > availability > seasonality > storage cost > shelf life. Substrates' availability in different regions can differ and for each region accessibility for industrial by-products should be evaluated.

MCDA analysis results are represented in Fig. 4.

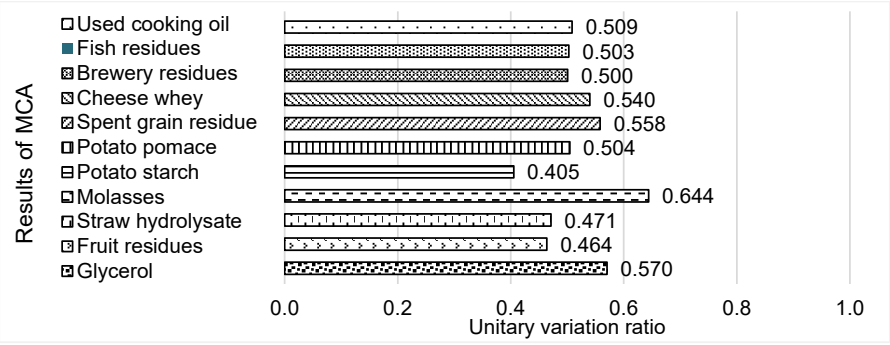

Figure 4. MCDA results of optimal feedstock.

The MCDA results for choosing the 'closest to ideal' waste substrate that could be used as feedstock in SCP production show that molasses is superior to other substrates. From a practical point of view, molasses is easy to incorporate into the culture medium, because of its liquid form, and solubility. It does not need to be pre-treated (Spalvins et al., 2018a). It contains about 50% of sucrose and glucose which microorganisms can easily use (Feliatra et al., 2022; Corrado et al., 2023; Koukoumaki et al., 2023), and therefore achieve high biomass conversation value. There have been studies where yeasts, fungi, and bacteria have been grown using molasses as a substrate to produce various products (Gao et al., 2012; Hashem et al., 2013; Favaro et al., 2019; Coimbra et al., 2021; Feliatra et al., 2022; Corrado et al., 2023). The fact that molasses can be used in the production of several products with added value can create competition between them, therefore, it should be evaluated from the bioeconomy point of view which product

is more valuable to produce. Storage and transportation could be an issue (Corrado et al., 2023), although the substrate does not need to be frozen, it should be stored in a refrigerator. The reason why molasses gained such a high result is that high biomass (0.635 g g^{-1}) and protein (54.3%) yields were achieved when this substrate was used (Hashem et al., 2013)

Glycerol is often used to cultivate microorganisms (Morais et al., 2019; Bajić et al., 2023; Koukoumaki et al., 2023). It is easy to store and use in microbial mediums. Crude glycerol that is left from bio-diesel production would be an attractive alternative to purified glycerol (Attarbachi et al., 2023). The MCDA result may be lower than molasses because there were not many published data showing high biomass and protein results while using batch fermentation. The highest biomass yield found was 21.8 g L^{-1} (Odriosolla dos Santos et al., 2012) even though with an optimized process it would be possible to get higher results. Pan et al. in a fed-batch fermentation using glycerol gained 173.3 g L^{-1} biomass (Pan et al., 2023), showing high prospects of using this waste substrate.

The composition of spent grains can be very different from one plant to the other, and the composition can also vary within a single production unit (Duarte et al., 2008). Spent grain is rich in various valuable components, including starch, cellulose, hemicellulose, protein, and lignin, which could be utilized to develop various high-value products (Duarte et al., 2008; Parchami et al., 2023), thereby pre-treatment should be considered (Plaza et al., 2017). By hydrolysing the waste substrate, it is possible to significantly increase the concentrations of simple sugars that are available to the microorganism (Duarte et al., 2008). This industrial waste is available throughout the year, at low cost, and in large amounts, unlike the seasonal agricultural crops (Plaza et al., 2017). For example, the brewing industry produces a great volume of residues, and brewers' spent grain is about 85% of them (Mussatto & Roberto, 2005). One of the reasons why this substrate gained such a high unitary variation ratio was the biomass yield (0.74 g g^{-1}), concentration (64.8 g L^{-1}), and protein content (32%) that Parchami et al. managed to gain by cultivating *Aspergillus oryzea* brewer's spent grain (Parchami et al., 2023), showing the high prospects of substrates application to the technology.

Even though straw hydrolysate in consistency has similarities with molasses (high sugar content, viscose, and easy to solubilize in medium), pre-treatment costs are very high (Baral & Shah, 2017). Pre-treatment itself can be a crucial part of utilizing waste substrates (Eloka-Eboka & Maroa, 2023). Substrates such as fruit, rape seed, and brewery residues can be pre-treated by acidic, alkali, or enzymatic hydrolysis (Baral & Shah, 2017; Plaza et al., 2017; Guardia et al., 2019; C. Zhang et al., 2020), and steam explosion can be applied to produce straw hydrolysate (Tan et al., 2021). Using fungi to hydrolysate substrates such as food and brewery residues can also present a cost-effective and environmentally friendly approach (Guo et al., 2014; El Gnaoui et al., 2022; Berzina & Spalvins, 2023).

Potato starch theoretically is a great substrate for SCP production, but due to some properties such as gelatinization, it may be difficult to use it practically in the preparation of microbial mediums (Fonseca et al., 2021). By thermally processing starch liquid medium gelation is induced and its viscosity increases as starch molecules swell (Blas & Gidenne, 2020), making it impractical to work with. Because of the gelatinization and high viscosity, it can be hard for the microorganism to digest the substance (Berzina, 2023). The organism should have a high amylase-producing capacity, or the starch liquid

should be hydrolysed to improve the process (Spalvins et al., 2018a, 2018b). Because starch can also be sold as a product to food industries it has a comparatively higher price than other substrates thus reducing its unitary variation ratio.

Other substrates gained quite similar results to each other. Each of them has their advantages and disadvantages. Fish residue, used oil, and cheese whey advantage would be the ease of implementation in the medium. Potential biomass and protein yields achieved when using some of the substrates such as fruit wastes (9.4 g L^{-1}) could be higher (Annex A) (Salem Awad et al., 2021), but it would be necessary to study these feedstocks further. With fruit and potato residues the year-round availability could be problematic on a large scale, but if the substrate is generated abundantly and can be stored inexpensively, it becomes a non-issue.

Overall, it is important to note that those substrates that function as carbon and nitrogen sources can be combined in mediums, for example, molasses with cheese whey. By optimizing and increasing waste substrate concentration in the growth medium, the production costs can be significantly reduced (García-Garibay et al., 2014). These combinations and different concentrations should be evaluated and researched further in a laboratory setting. The best microorganisms from each class from the previous section could be potentially tested with these waste substrates. It should be emphasized that each substrate and microorganism combination can show its advantages and disadvantages when cultivating together and upscaling the process. The rising issues could be, e.g., during cultivation extensive foaming could occur due to used substrates, the substrate itself could be impractical to use in large-scale production, e.g. while using different oils as feedstock emulsifiers might be needed, etc.

Evaluation of mutagenesis methods

Mutagenesis can be defined as the treatment of biological material with a mutagen, which results in an increase in mutation frequency above the level of spontaneous mutations (Kodym & Afza, 2003). This process accelerates the mutation frequency rate up to 100 times in the biological material when compared to the natural mutation rate (Winston, 2008). Mutagens can be divided into three groups - chemical (base analogues, base altering agents, intercalating agents), physical (heat, ionizing radiation, non-ionizing radiation), and biological (transposons, insertion sequences, TALENs, ZNFs, CRISPR/Cas9, etc.) (Rowlands, 1984; Anderson, 1995; Winston, 2008). For this study, one physical mutagenesis method (using UV light) and two chemical mutagenesis methods (ethyl methane sulphonate (EMS), and nitrosomethyl guanidine (MNNG)) were evaluated. These methods were chosen for their ease of use, low costs, maturity of the procedures, and safety. Defining criteria for different mutagenesis methods posed challenges as the mechanism of action was not the same between them. All defined criteria for evaluating mutagenesis methods with corresponding weights are listed in Table 3. The first criterion was defined as the method's probability of success which describes the chance of a successful mutation in the microorganism population which is expressed as a percentage using data from argE3 → Arg (+) mutation revision tests (Aaron et al., 1980; Śledzieska-Gójska et al., 1992; Fabisiewicz & Janion, 1998). Both criteria 'the possibility to combine method' and 'methods toxicity to the environment' were defined as qualitative. The possibility to combine methods ranged from 0 to 1 or respectively can (1) or cannot (0) be combined. And methods' toxicity to the environment ranged from 0 to 2, respectively, has non-environmental toxicity (0), is toxic to the

environment, but easily disposable (1), and toxic to the environment with special utilization requirements (2).

Table 3. Indicators and weights used in MCDA of mutagenesis methods

Criteria	Unit of measure	Weight
Methods probability of success	(%)	0.205
Possibility to combine methods	-	0.177
Methods toxicity to the environment	-	0.149
Price of the required amount of mutagen per run	EUR	0.162
Process time for the method	h	0.146
Approximate induced mutation frequency	mutations/gene/cell division	0.160
	Σ	1

The criteria for alternatives were assessed by experts in descending order of importance: methods probability of success > possibility to combine methods > price of the required amount of mutagen per run > approximate induced mutation frequency > methods toxicity to environment > process time for the method. Because all three methods can be combined and used successively this criterion was not considered mathematically important. The least important factor is the processing time for the method as many thousands of mutants are generated per run creating bottlenecks in mutant testing not in the mutant generation.

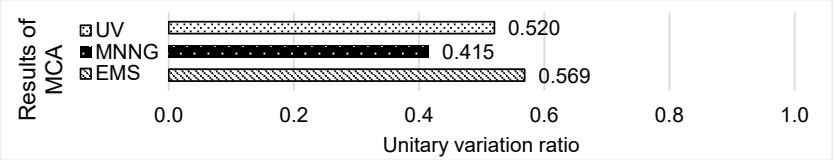

Figure 5. MCDA results of mutagenesis methods.

MCDA ranked EMS mutagenesis as the closest to an ideal solution, following UV mutagenesis, and as the last MNNG mutagenesis which is represented in Fig. 5. One of the key reasons for the high EMS mutagenesis rank was the method's success rate. Both MNNG and EMS are strong alkylating substances with an identical mode of action (Izumi & Mellon, 2016; Greim, 2024). Surprisingly, EMS probability of success was five times higher than MNNG methods (Annex A), possibly due to some chemical or structural differences. Experts chose price per run as the second most important criterion for mutagenesis methods, which was the highest for the EMS approach. Even though experts deemed price per run as an important criterion, the price can be affected by many unknown factors and could fluctuate greatly. For example, the selected microorganism may require more or less substance for mutagenesis and cause the price per run to change. Needed EMS concentration for different microorganism strains can range from 0.002 M to 0.48 M (Sarachek & Bish, 1976; Shafique et al., 2009; Leonard et al., 2013; Demırkan & Özdemır, 2020).

The UV mutagenesis method greatly differs from the rest as it causes DNA change through photochemical reaction introducing DNA lesions instead of alkylation as it was with EMS and MNNG methods (Ikehata & Ono, 2011). UV method's probability of

success was ten times smaller than EMS methods, which perhaps is caused by the cell's natural defense against UV radiation. UV mutagenesis methods' price per run was low as the running cost consists of electricity consumption by the UV bulb.

Evaluation of AA inhibitors

Most AA inhibitors that are available are used in agriculture as herbicides and this is the intended application also for most of the AA inhibitors that are currently in development (Berlicki, 2008; Cobb & Reade, 2010a; Cobb, & Reade, 2010b; Hall et al., 2020). Therefore, most of the research conducted on using these compounds is regarding their practical and cost-effectiveness in weed management (Llewellyn et al., 2016; Hall et al., 2020). Most of the research available on these herbicides is done on their inhibitory activity on plant biosynthetic pathways, while information on their activity on microorganisms is limited. These aspects need to be considered when selecting an AA inhibitor for use in SCP-producing mutant selection, as the actual inhibitor response may differ from what was expected.

The effect of herbicidal treatment of microorganisms has not been well studied, and the available data do not provide clear answers. Studies evaluating the effect of AA inhibitors on the viability of rhizosphere microorganisms (Wang et al., 2012; Łozowicka et al., 2021), plant pathogens (Sardrood & Goltapeh, 2018), and important food microorganisms (Braconi et al., 2006; Clair et al., 2012; Vallejo et al., 2017) etc. were used for further evaluation and MCDA of AA inhibitors. The present study evaluates the following AA inhibitors: aromatic AA inhibitor (glyphosate), branched-chain AA inhibitors (sulfonylureas: metsulfuron methyl, sulfometuron methyl, chlorsulfuron, tribenuron methyl; imidazolinones: imazapyr, imazapic, imazethapyr, imazamox, imazamethabenz, imazaquin), glutamine inhibitors (glufosinate ammonium, methionine sulfoximine), aspartate-derived AA inhibitors (propargylglycine, L-α-(2-aminoethoxyvinyl)glycine, S-(2-aminoethyl)-L-cysteine), glutamate-derived AA inhibitor (phaseolotoxin), and histidine inhibitor (amitrole) (Rose et al., 2016; Vallejo et al., 2017; Spalvins et al., 2021).

Amino acid inhibitors were evaluated based on criteria such as price, inhibition efficacy, selectivity, amount of inhibited AA and EAA, safety, and possibility for false positive selection (Table 4). The criteria for alternatives were assessed by experts in descending order of importance: inhibited EAA>possibility of false positive selection>inhibited AA>inhibition efficacy>price of inhibitor>selectivity>safety.

Table 4. Indicators and weights used in MCDA of AA inhibitors

Criteria	Unit of measure	Weight
Price of inhibitor	EUR 100 mg^{-1}	0.130
Inhibition efficacy	-	0.143
Selectivity	-	0.128
Inhibited AA	%	0.153
Inhibited EAA	%	0.214
Number of total health and environmental hazards	-	0.077
Possibility of false positive selection	-	0.155
	Σ	1

It was found that the effect of AA inhibitors on the growth of fungi (yeast and mold) and bacteria in the rhizosphere is ambiguous and depends on the strain of the microorganism, the type of herbicide, and its formulation (Chen et al., 2009; Clair et al., 2012). E.g., commercial herbicide formulas often have a stronger effect on inhibiting the growth of microorganisms than the active substance itself (Braconi et al., 2006; Clair et al., 2012). Studies show that herbicides can be highly inhibitory to microorganisms at low dosages (Grandoni et al., 1998; Ataide et al., 2007; Mowbray et al., 2014), weakly inhibitory at high dosages (Odunfa et al., 2001), growth stimulating (Łozowicka et al., 2021) or having no effect (Zohar et al., 2003; Ahuja & Punekar, 2008). Inhibitory efficacy was assessed for bacteria and fungi (yeast and mould) separately (Annex A). In general, bacteria are more sensitive to AA inhibitors than fungi, although this does not apply to all inhibitors (Tripathi et al., 2020). Moreover, within a domain and even a genus, the range of concentrations for inhibition varies greatly (Ahuja & Punekar, 2008; Chen et al., 2009). E.g., in a study by Chen et al., 2009, 50% inhibition of the yeasts *Pichia farinosa, S. cerevisiae, Williopsis saturnus, C. shehatae* was obtained when treated with metsulfuron methyl at concentrations of 0.005, 0.2, 0.01 and 0.2 g L^{-1} of medium, respectively, while inhibition of growth of *C. mengyuniae* sp. nov. was not achieved at concentration of 5 g L^{-1} (Chen et al., 2009). Moreover, it is known that some soil bacteria (*Bacillus* sp., *Pseudomonas* sp., *Agrobacterium* sp.) and fungi (*Aspergillus* sp., *Trihoderma* sp.) can use herbicides as a source of carbon, promoting biodegradation of herbicides (Boschin et al., 2003; Łozowicka et al., 2021).

Therefore, categorizations were used for concentrations that were potently inhibitory or lethal to evaluate and compare the efficacy of bacterial and fungal inhibition. Thus, the inhibition efficacy is divided into micro-dose (0.001–0.009 g L^{-1}), low dose (0.01–0.09 g L^{-1}), moderate dose (0.1–0.9 g L^{-1}), and high dose (1–9 g L^{-1}). Several inhibitors belonging to the same chemical group or inhibiting the same enzyme in the amino acid biosynthetic pathway are included in the MCDA, although they do not have data on the lethal dose for bacteria or fungi. These include inhibitory concentrations of imazapyr, imazamox, and imazamethabenz for bacterial assays and S-(2-aminoethyl)-L-cysteine for fungi. Therefore, to include the inhibitors of interest in the MCDA, they were assigned dose values based on the group average.

Bacteria were more sensitive to metsulfuron methyl (MSM), sulfometuron methyl (SMM), glufosinate ammonium (GA), methionine sulfoximine (MS) and propargylglycine (PAG), where complete inhibition was achieved at micro-doses (Piotrowska & Paszewski, 1986; Grandoni et al., 1998; Ahuja & Punekar, 2008; Chen et al., 2009; Mowbray et al., 2014; Kandalam et al., 2018). According to the literature, chlorsulfuron (CS) completely inhibited bacterial growth at a low dose (Forlani et al., 1995; Grandoni et al., 1998). Phaseolotoxin (PT) inhibited 97% of the target bacterial enzyme (ornithine carbamoyl-transferase) involved in arginine biosynthesis at a low dose (Templeton et al., 1984; Forlani et al., 1995; Grandoni et al., 1998). Glyphosate (GP), imidazolinones, L-α-(2-Aminoethoxyvinyl) glycine (AVG), S-(2-aminoethyl)-L-cysteine (AEC) inhibited bacterial growth at medium doses, and amitrole (AT) at high doses (Bamford et al., 1976; Forlani et al., 1995; Grandoni et al., 1998; Al-Masri et al., 2006; Ataide et al., 2007;Halgren et al., 2011; Nielsen et al., 2018; Bak et al., 2021). Fungi as well as bacteria are more sensitive to sulfonylureas such as CS and SMM, which have a strong inhibitory effect at low concentrations, although tribenuron methyl (TM) and MSM require a moderate dose (Braconi et al., 2006; Chen et al., 2009; Kingsbury &

McCusker, 2010). Also, GA, MS, AVG, AEC, and AT at moderate doses inhibit fungal growth by 80–100% (Hilton, 1960; Muñoz & Agosin, 1993; Al-Masri et al., 2006; Ahuja & Punekar, 2008; Kingsbury & McCusker, 2010; Chen et al., 2019; Bak et al., 2021). Glyphosate (the active ingredient of a commercial herbicide) and PAG seem to be less effective against fungi; they will be required in high doses (Jin et al., 2004; Tanney & Hutchison, 2010; El-Sayed, 2011; Tahiri et al., 2022). It is worth noting that this assessment of inhibitory effectiveness against bacterial and fungal enzymes of AA biosynthesis is relative due to limited research and includes only those inhibitors that had an inhibitory effect on bacteria and fungi. AA inhibitors with no inhibitory effect on the target microorganism or with anti-algae activity were not included in further analysis.

Initially, eighteen AA inhibitors were evaluated for selective activity against bacterial and fungal amino acid precursor enzymes. According to the literature, all target enzymes of these inhibitors are present in microorganisms of both domains (Kumada et al., 1993; Ravanel et al., 1998; Van Rooyen et al., 2006; Min et al., 2015; Grant Pearce et al., 2017; Lonhienne et al., 2020; Tall & Puigbò, 2020). Therefore, the weight of this criterion was not considered mathematically important and was not included in the herbicide analysis. The percentage of inhibited AA when using the analysed inhibitors was calculated relative to the total possible amount (twenty) (Spalvins et al., 2021; Annex A). Thus, GA and MS are potentially capable of inhibiting up to 40% of all AAs (Gln, Asp, Pro, Arg, Lys, Met, Thr, Ile), AVG and AEC up to 20% (Met, Lys, Thr, Ile), GP (Phe, Trp, Tyr), sulfonylureas, imidazolinones slightly less up to 15% (Ile, Leu, Val for both), and up to 5% PAG (Met), PT (Arg) and AT (His) (Spalvins et al., 2021; Annex A). The percentage of inhibited EAAs was calculated based on the importance of specific EAAs, maintaining a value of 100% for the sum of nine EAAs. The importance of each EAA is based on its availability in conventional protein sources (Spinelli, 1980; Al-Marzooqi et al., 2010; Finco et al., 2017; Hardy et al., 2018). Thus, Lys, Met, Thr, and Trp are rated as highly important EAAs (16.67% for each), Val as moderately important (11.12%), less important His, Leu, Ile, Phe (5.55% each). These EAA importance values are subjective and are aimed at comparing the potential of inhibitors to select more beneficial protein-synthesizing mutant strains for food, feed, cosmetics, etc. industries. Essentially, this assessment combines the quantitative and qualitative values of EAA inhibition. This distribution of percentages resulted in the highest value for GA, MS, AVG, and AEC (56%), the average value for GP and inhibitors from the group of sulfonylureas and imidazolinones (22%) as well as PAG (17%), the lowest value was received by AT (6%). PT was rated 0% because it inhibits one non-essential AA (see Annex A).

The safety of inhibitors was assessed using a scoring system, where 0 is safe and 1–7 is the total number of health and environmental hazards (PubChem, 2023). This criterion was included in the MCDA because it is necessary to consider the potential harm of inhibitors to health during use and utilization. This criterion received the lowest expert weight compared to other criteria - 0.077. This may be due to the experience of experts in working with such substances, the presence of the necessary laboratory equipment, and personal protective equipment, and the practice of handing over hazardous substances for disposal to a special company. Thus, the use of all necessary precautions reduces the potential harm of inhibitors to a minimum and, as a result, has lower weight when assessing the criteria by experts.

The last criterion is the possibility of false positive selection, which characterizes the risk associated with the side activity of inhibitors to other internal processes in microbial cells. According to the literature, all analysed inhibitors except AEC are capable of disrupting or inhibiting the biosynthesis of various metabolites. E.g., GP deregulates carbon metabolism, inhibitors of the sulfonylurea and imidazolinone group inhibit DNA synthesis, GA and MS are cytotoxic and promote the accumulation of ammonia in the cell (Spalvins et al., 2021). PT and PAG inhibit the synthesis of polyamines involved in cell proliferation and adaptation to stress factors. PAG also interferes assimilation of neutral AA like Lys (Piotrowska & Paszewski, 1986; Bachmann et al., 2004; Kalamaki et al., 2009). AT inhibits the biosynthesis of ergosterol and catalase (Hilton, 1960; Chen et al., 2009; Rocha et al., 2021). This side activity may cause the microorganism to switch its metabolism to bypass the inhibitory effects of the substance or increase resistance to side effects, e.g. by increasing detoxification activity in the cell without any changes in the activity of biosynthesis of the target AA (Sardrood & Goltapeh, 2018; Thiour-Mauprivez et al., 2019; Łozowicka et al., 2021). With this outcome, the use of these AA inhibitors for the selection of mutants after induced mutagenesis to select protein-producing strains will be less effective. Because non-target false-positive mutants can be selected together with and/or instead of targeted protein-synthesizing mutants, additional screening tools need to be used.

MCDA results of inhibitors selected for bacteria and fungi are represented in Fig. 6 and Fig. 7. MS is ranked first place, GA second, AEC third, and AVG fourth. This primacy may be due to the fact that these 4 inhibitors are leaders according to highly weighted criteria: they inhibit the largest amount of AA and EAA. Interestingly, the results of AEC and AVG for fungi reached almost equivalent values, although other criteria such as price and possibility of false positive selection are strikingly different in favour of AEC.

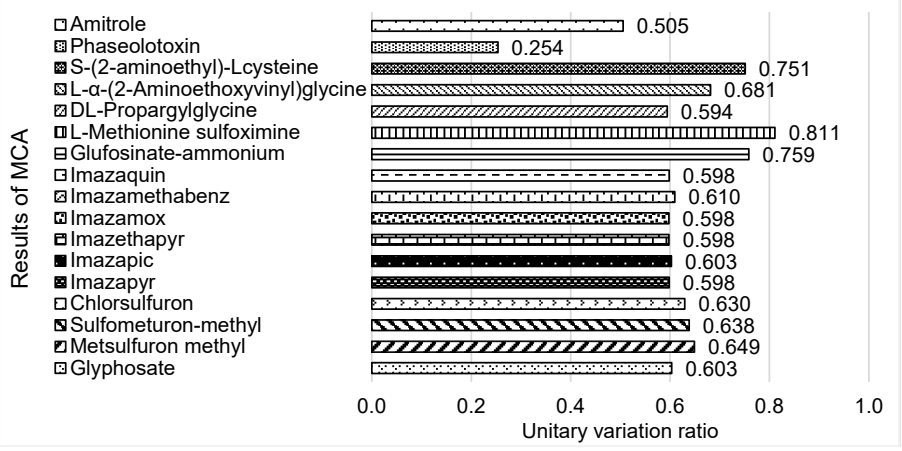

Figure 6. MCDA results of AA inhibitors for bacteria.

The results of sulfonylureas and GP for fungi reached 0.325, which is significantly worse than those of the four inhibitors mentioned above. For bacteria, the results of sulfonylureas are slightly higher than those of imidazolinones, GP, and PAG amounting to 0.594–0.649. Therefore, it is more appropriate to use these inhibitors to inhibit bacteria with the goal of selecting mutant strains with increased synthesis of three EAAs: Ile, Leu, and Val. AT reached average values of 0.505 for bacteria; for fungi, on the contrary, it turned out to be the worst solution, reaching 0.033. Close to the worst solution were PT for bacteria and PAG for fungi.

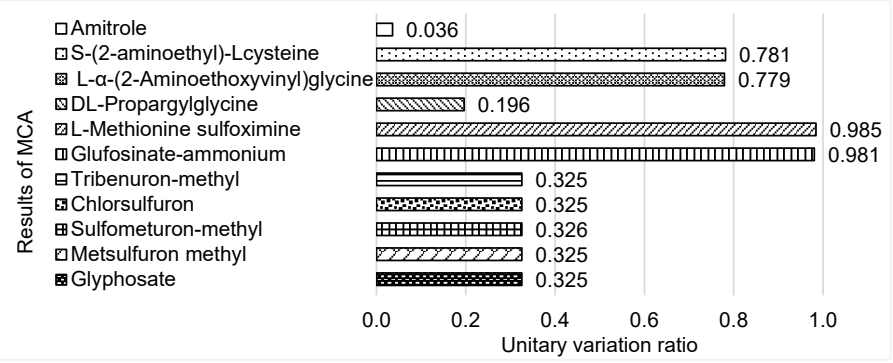

Figure 7. MCDA results of AA inhibitors for fungi.

Although studies on the effects of herbicides and their active components on bacteria and fungi are limited, the MCDA results highlight potential inhibitors for further study. It is necessary to understand the possibility of using AA inhibitors for the selection of protein-producing strains after induced mutagenesis and the effectiveness of this method, taking into account the risk of false-positive selection. It is worth considering that the selected microorganism may be insensitive to a particular inhibitor. Therefore, it is advisable to create a database combining industrially important microorganisms and the results of their successful inhibition or insensitivity to potential inhibitors or herbicides. To improve the results of MCDA, some inhibitors can be combined, which will theoretically increase the amount of inhibited AA and EAA and increase the efficiency of inhibition. From this perspective, the combinations of GP + MSM and GP + PAG look more advantageous for the selection of both bacteria and fungi. Thus, combined inhibition would affect Phe, Trp, Tyr + Ile, Leu, Val, and Phe, Trp, Tyr + Met biosynthesis.

CONCLUSIONS

SCP technology has a high potential to reduce protein scarcity. The technology can increase resource efficiency because agricultural and industrial wastes can be used as feedstock and overall technology is more environmentally friendly. To increase its competitiveness new microbial mutant strains with enhanced protein production abilities should be developed.

In this study, MCDA was performed to determine which of the four technological aspects are the closest to ideal solutions for creating protein-rich mutant strains for SCP production. From TOPSIS results two of the highest results were achieved by yeast species *C. utilis* and *P. rhodozyma*. They excelled in their ability to produce protein. Both had a high protein content in their total biomass and had high protein productivity. *P. rhodozyma* stood out with its AA profile as it had the highest AA content which is lacking in the plant-derived proteins. From bacteria species *B. subtilis* gained the highest result and from fungi *A. oryzae*. *B. subtilis* showed considerable protein content while *A. oryzae* excelled in protein productivity.

From waste substrates molasses showed to be theoretically the best feedstock for SCP production because it can be easily implemented in microbial mediums, it is applicable for cultivating bacteria, fungi, and yeast, and there have been reports of achieving high yields of biomass when using this substrate. Glycerol had the second-highest score. Other evaluated substrates had more or less similar unitary variation ratios, which indicates that they have similar prospects of being used in production.

For mutagenesis techniques three different alternatives were evaluated - UV, EMS, and MNNG. Mutagenesis with EMS was ranked as the closest to ideal by TOPSIS while UV mutagenesis was second and MNNG was last. EMS excelled in methods probability of success while UV and MNNG success rates were ten and five times lower, respectively. The cost and time were deemed to be non-essential criteria because the price can depend on the used microorganism strain and the time used for mutagenesis is insignificant when considering the time consumed in mutant evaluation.

The results of the MCDA analysis showed that the best solution for both bacteria and fungi are four AA inhibitors: glufosinate ammonium, methionine sulfoximine, L-α-(2-aminoethoxyvinyl) glycine, and S-(2-aminoethyl)-L-cysteine since they inhibit a high amount of AA and EAA. Propargylglycine and inhibitors of the sulfonylurea and imidazolinone groups showed acceptable results for bacteria, but the unitary coefficient for fungi was unsatisfactory. Therefore, further research is needed on the combinations of more advantageous inhibitors such as glyphosate with metsulfuron methyl or another sulfonylurea, and glyphosate with propargylglycine. Such combinations will allow selective pressure to be exerted on the biosynthesis of a larger variety of important EAAs.

Following these MCDA results, identified potential combinations of microorganisms, substrates, mutagenesis methods, and inhibitors should be tested in a laboratory setting. While testing microorganism and waste substrate compatibility, technical problems can potentially arise, such as extensive foaming, oil layering, etc. Mutagenic methods and AA inhibitors should also be evaluated, and concentrations and doses should be optimized. These parameters can differ for each organism. Each of these combinations would require thorough testing and evaluation. The results of these tests should become the focus of future research papers.

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ANNEX: doi.org/10.15159/eds.art.spl.24.01

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Enhanced amino acid biosynthesis in *Phaffia rhodozyma* via herbicide-induced selection

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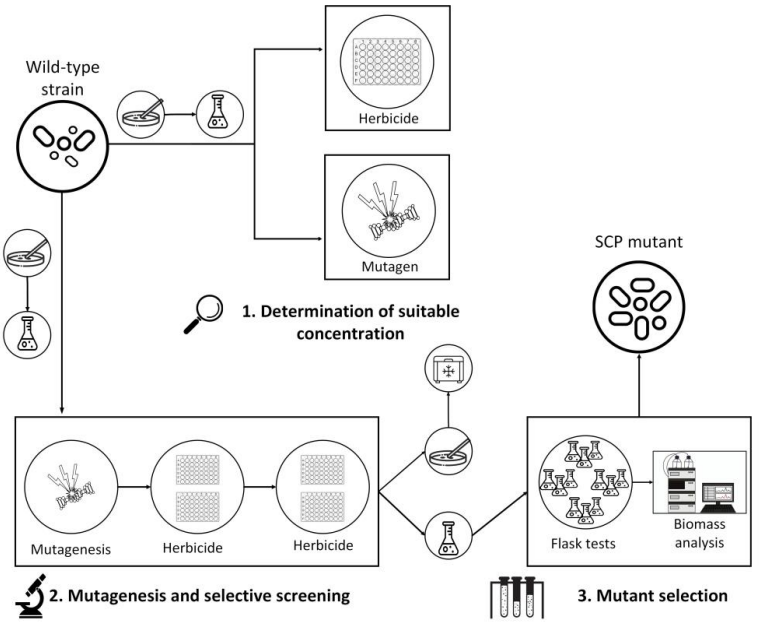
***Abstract* – According to the Food and Agricultural Organization 2024 statement, the development of single-cell protein technology is important to reduce the burden on conventional feed protein production sectors. In this regard, improved commercial strains rich in amino acids, especially Lys and Met, may provide a sustainable alternative source of protein in aquaculture diets. The developed and laboratory-validated methodology for creating protein-synthesising mutants will strengthen the competitiveness of SCP production technology. The present work provides unique results on improving the protein-producing properties of wild-type *Phaffia rhodozyma* DSM 5626 by mutagenesis and screening on herbicide-containing medium as a selective agent for amino acid biosynthesis inhibition. Inhibitory concentrations of pure herbicide actives were determined for S-(2-aminoethyl)-L-cysteine (AEC) and glufosinate-ammonium (GA) for complete inhibition and strong inhibition of the DSM 5626 strain. GA at a concentration of 50 mM and 100 mM and AEC at 0.5 mM and 2.5 mM were chosen for mutant selection after chemical mutagenesis. The use of herbicides resulted in the selection of mutants with significantly improved synthesis of Met and Lys. Specifically, mutants GA6/4 and GA7/5 exhibited 37% and 26% higher Met levels, respectively, while GA6/3 had a 14% increase in Lys compared to the wild–type strain. The AEC3/9 mutant demonstrated a 35% increase in Met, 24% in Lys, 8% in Ile, and 6% in Phe, underscoring the efficacy of this screening approach in enhancing essential amino acid content. The protein quality parameters EAAI and AAS of these mutants became higher in comparison with commercial strains of SCP yeast as *C. utilis, S. cerevisiae, K. marxianus,* etc.**

***Keywords* – mutagenesis; essential amino acids; S-(2-aminoethyl)-L cysteine; glufosinate-ammonium; single-cell protein; amino acid score; essential amino acid index.**



The pathway for enhancing amino acid biosynthesis in yeast via mutagenesis and selective screening using herbicides.

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INTRODUCTION

Aquaculture plays a critical role in providing a sustainable food source as wild fisheries are declining due to overfishing and adverse climate and environmental changes. Nowadays, aquaculture supplies more than half of the world's fish for human consumption (Mair et al., 2023). To ensure high aquaculture productivity, it must be provided with balanced feed rich in high-quality protein (Felix et al., 2023; Margareth & Anders, 2016). The quality of a protein source is assessed by amino acid composition, although its suitability primarily depends on the essential amino acid requirements of the target species (Cho & Kim, 2011; Kurpad, 2012; Wolfe et al., 2018). According to the Food and Agricultural Organisation (FAO), the diet of Atlantic salmon and rainbow trout should contain at least 42–50% crude protein in dry feed, depending on the life stage. An important requirement for a salmon and trout diet is the content and balance of essential amino acids in the protein sources. These are histidine (His), threonine (Thr), arginine (Arg), valine (Val), methionine (Met), tryptophan (Trp), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), and lysine (Lys) (FAO, 2013a, 2013b). Fishmeal is considered the most nutritious and most digestible source of protein for aquaculture feed (Cottrell et al., 2020; FAO, 2022; Janbakhsh et al., 2018; Jannathulla et al., 2021).

Fishmeal was originally used as an effective and inexpensive protein-rich feed ingredient for farmed aquatic species. However, decreased fishing productivity and increased demand for fishmeal caused significant price changes (Cottrell et al., 2020). The price of fishmeal has increased over the past 10 years from 599 EUR/t in 2013 to 1673 EUR/t in 2023 and is expected to continue to rise (FAO, 2022; www.indexmundi.com). More than 80% of fishmeal is used to produce feed for high-value aquaculture species, mainly for farming shrimp, salmonids and marine fish like seabass (FAO, 2022). The expansion of the aquaculture sector requires the development of additional economically and environmentally viable solutions to meet the growing demand for feed and less dependence on conventional agricultural and marine ingredients. Therefore, for sustainable aquaculture farming, it is necessary to reduce dependence on fishmeal and replace it with alternative protein sources (FAO, 2022; Gasco et al., 2018; Jannathulla et al., 2019).

Single-cell protein (SCP) is a promising alternative to fishmeal and plant-based protein (Bratosin et al., 2021), (Øverland et al., 2013). It has less negative impact on the environment and requires fewer resources compared to agricultural crops (Nyyssölä et al., 2022; Salazar-López et al., 2022; Sharif et al., 2021). Moreover, agro-industrial waste is successfully used to produce single-cell protein, which ensures optimal management of food and agricultural organic waste (Spalvins, Ivanovs, et al., 2018; Spalvins, Zihare, et al., 2018). Nowadays, SCP in particular biomass of *Chlorella vulgaris, Nannochloropsis gaditana, Schizochyrium* sp., *Saccharomyces cerevisiae, Candida utilis, Kluyveromyces marxianus, Wickerhamomyces anomalus, Spirulina maxima, Methylococcus capsulatus, Methylophilus methylotrophus and Methylobacterium extorguens* is successfully used by some companies in the formulation of fish feed, albeit in small volumes (Glencross et al., 2020; Nyyssölä et al., 2022). Recent experimental feeding trials have shown that SCP can replace between 15 and 60% of fishmeal, corresponding up to 345 g of yeast per kg of fish feed without compromising growth performance, nutrient absorption, or health (Agboola et al., 2022; Huyben et al., 2017; Øverland et al., 2013; Vidakovic et al., 2020).

The present study proposes a novel approach to create SCP-rich mutants with improved essential amino acid profiles. In this approach, amino acid biosynthesis inhibitors are used as a tool to select mutants with desirable properties created through random mutagenesis. This concept is well known in the selection of mutant microorganisms with improved biosynthesis of fatty acids and carotenoids, where inhibition of the biosynthetic pathway of the target metabolite allowed the collection of overproducers (Atzmüller et al., 2019; Ducrey Sanpietro & Kula, 1998; Luna-Flores et al., 2022). Interestingly, the widely used random mutagenesis to improve the desired properties of strains is not used to create protein-synthesised mutants. Although, there is evidence of the effect of random mutagenesis on increasing the protein content in microalgae biomass (Liu et al., 2015). Several herbicides are amino acid biosynthesis inhibitors, which can be a tool for selecting improved SCP-producing mutants. It is assumed that

microbial cells that have undergone mutagenesis and are capable of growing in medium in the presence of an herbicide concentration that inhibits about 100% of the cells of the wild-type strain have a high probability of being mutants with an improved amino acid profile (Raita et al., 2024; Spalvins et al., 2021).

The present study used the red yeast *Phaffia rhodozyma* to evaluate the potential of amino acid inhibitors in screening mutants with improved synthesis of essential amino acids. *P. rhodozyma* is a well-known carotenoid-synthesising yeast used primarily as a natural astaxanthin supplement in salmonids feed formulations (e.g. Aquasta®, RedStar®) (Bjerkeng et al., 2007; Vidakovic et al., 2020). This yeast has been studied for the last 50 years to develop competitive technology for natural astaxanthin production (Raita et al., 2023). Interestingly, *P. rhodozyma* biomass is exclusively a source of astaxanthin but does not consider the protein content. The protein content and amino acid composition of *P. rhodozyma* have been poorly studied. There are several studies from the 80–90s reported that *P. rhodozyma* biomass contains about 25–47% proteins, a balanced amino acid composition, and a high number of fatty acids (Acheampong, 1993; Johnson et al., 1980; Martin et al., 1993). The present study compares the total protein content, amino acid profile, and protein quality in biomass of created *P. rhodozyma* mutants with the wild-type *P. rhodozyma* strain and conventional SCP-producing yeast species.

MATERIALS AND METHODS

Media and solutions preparation

Yeast medium (YM) broth contained glucose 10 g/L, peptone 5 g/L, malt extract 3 g/L, yeast extract 3 g/L was used for inoculum preparation. For yeast inhibition tests and mutant screening, *P. rhodozyma* was cultivated on glycerine-salt medium (GSM) that contained 40 g/L glycerol, 4.83 g/L NH_4Cl, 1 g/L $K_2HPO4 \cdot 3H_2O$, 0.88 $MgSO_4 \cdot 7H_2O$, 0.2 g/L $CaCl_2 \cdot 2H_2O$, 20 g/L $C_8H_5KO_4$, and 1.7 g/L yeast nitrogen base without amino acid (YNB), pH 6.0±0.05. Glycerine-peptone-rich medium (GPR) contained 70 g/L glycerol, 32 g/L soy peptone, 10 g/L yeast extract, 1.7 g/L YNB, pH 6.0±0.1 was used for flask experiments. YNB was dissolved in sterile distilled water and filtersterilised through a syringe filter with 0.22 μm pore size. Sterile YNB stock solution was added at rate of 10% to autoclaved media before adjusting the pH under sterile conditions.

Herbicide stock solutions were prepared before experiments by dissolving in sterile water and filtersterilising through a syringe filter with 0.22 μm pore size. Glufosinate-ammonium (GA, CAS 77182-82-2) was purchased from Combi-Blocks (USA) and S-(2-aminoethyl)-L-cysteine hydrochloride (AEC, CAS 2936-69-8) from Sigma-Aldrich (USA). Ethyl methanesulfonate (EMS) (Sigma Aldrich, USA) was used for *P. rhodozyma* cell mutagenesis at 4% concentration. EMS solution preparation and mutagenesis protocol is described in (Raita et al., 2024).

Microorganism strain

Yeast culture *P. rhodozyma* DSM 5626 was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ) at the Leibniz Institute. It is a type strain isolated from Japanese beech (*Fagus crenata*) slime flux. The alternative strain numbers in other collections are ATCC 24202, CBS 5905, NBRC 10129, NRRL Y-10921, and IFO 10129. *P. rhodozyma* DSM 5626 strain is an anamorph strain (DSMZ homepage) reproducing asexually. This process results in genetically identical offspring. Because an anamorphic organism reproduces without genetic recombination, it tends to maintain stable traits across generations. This stability is beneficial when a specific strain with desired traits is used, ensuring these traits are maintained across successive cultures (Gerrish & Lenski, 1998). The yeast culture was maintained on a yeast medium agar (YMA) plate containing 10 g/L glucose, 5 g/L peptone, 3 g/L malt extract, 3 g/L yeast extract, and 15 g/L agar. For all experiments, the inoculums were grown on yeast medium broth (YMB) at 22 °C on a rotary shaker at 250 rpm for 48 h.

Microplate cultivation

The GSM medium with 1.0×10^6 cells/mL of inoculum and an inhibitor at the appropriate concentration was used. Microplate cultivation lasted 116 h for yeast inhibition tests and up to 150 h for mutant screening at a specific inhibitor concentration. At the end of microplate cultivation, the cells from all wells were counted.

Determination of herbicide inhibitory concentration

Two amino acid biosynthesis inhibitors S-(2-aminoethyl)-L-cysteine (AEC) and glufosinate ammonium (GA) were selected as the best herbicides for yeast inhibition based on multi-criteria decision analysis results in our previous study (Berzina et al., 2024). GA is an inhibitor of glutamine, a precursor to the biosynthesis of the seven amino acids arginine (Arg), proline (Pro), aspartate (Asp), lysine (Lys), methionine (Met), threonine (Thr) and isoleucine (Ile) (Gong et al., 2020; Joo et al., 2018). AEC is an inhibitor of the biosynthesis of the four amino acids Met, Lys, Thr and Ile (Pearce et al., 2017). In the present study, wild-type yeast was grown on GSM broth containing these herbicides separately to determine inhibition concentrations of about 100% for each. GSM with an inorganic nitrogen source was selected for *P. rhodozyma* because an organic nitrogen source could counteract the inhibitory effects of herbicides (Raita et al., 2024).

Seven concentrations of the S-(2-aminoethyl)-L-cysteine (AEC) ranged from 0.1 to 50 mM (0.1, 0.5, 2.5, 5, 7.5, 10, and 50 mM) and twelve concentrations of the GA ranged from 0.1 to 350 mM (0.1, 0.5, 2.5, 5, 7.5, 10, 50, 100, 150, 200, 250, 300, and 350 mM) were tested for wild-type strain inhibition. The yeast inhibition test was conducted at different time points for GA and AEC inhibitors under identical conditions. The tests were performed in 48-well microplates with a working volume of 0.5 mL. The GSM medium, was supplemented with inhibitors and 1.0×10^6 cells/mL inoculum and incubated for 116 h. At the end of the test, the cells were microscopically examined and counted. The concentrations of amino acid biosynthesis inhibitors causing complete inhibition of yeast growth were selected for further screening of mutants.

Mutants screening

Screening of mutagenised *P. rhodozyma* cells at a concentration of 2.0×10^6 cells/mL was conducted in GSM medium containing four concentrations of inhibitors: 2.5 mM AEC (AEC2), 5 mM AEC (AEC3), 50 mM GA (GA6), and 100 mM GA (GA7). Control samples were: (1) a wild-type strain in a medium without an inhibitor (control-1), (2) a wild-type strain in a medium with an inhibitor (control-2), and (3) mutagenised cells in a medium without an inhibitor (control-3). Microscopy and cell counting were performed for all samples. Mutants exhibiting superior growth compared to control-2 were selected for subculture. Control-1 and control-3 were included to ensure consistent culturing conditions across different microplates and time points.

Then subculturing was performed to strengthen the potential mutants in the selective medium and minimise the risk of non-mutated wild-type cell proliferation. A 0.1 mL suspension of mutant cells was inoculated into 0.4 mL of GSM medium containing the corresponding concentration of inhibitor. Samples GA6 and GA7 were subcultured three times, AEC2 twice, and AEC3 once.

Cells were counted at the end of each subcultivation. Samples GA6, GA7, and AEC2 showed improved growth after each subculture, and at the end of the 3rd and 2nd subcultures, the cell concentration in the microplate wells increased more than 10 or 100 times. AEC3 samples after subculture showed a decrease in cell number, leading to a stop at one subculture. The mutants with higher cell concentration from microplate wells were transferred into 50 mL Erlenmeyer flasks with 15 mL YMB without inhibitors and cultivated for up to four days. Cultures that reached cell concentrations of 1.0×10^6 cells/mL and higher were selected as inoculum for mutant shake flasks cultivation tests.

Shake flasks cultivation

The shake flask tests were conducted using inoculants of 1.0×10^6 cells/mL in a 25 mL working volume of GPM broth and incubated for seven days. Inoculants were collected and washed twice in sterile water before each test. All shake flask tests were cultured in 250 mL baffled flasks in triplets at 22 °C on a rotary shaker at 250 rpm. The biomass collected from the flask tests was subsequently used in the analytical measurements described in the next section.

Analytical methods

Determination of yeast growth

Yeast growth was assessed by dry cell weight, culture optical density, and cell concentration. Dry biomass was determined by thermogravimetric analysis. Yeast biomass samples were collected daily from the 3rd to the 7th day of the experiment and calculated as dry cell weight per liter of medium (g/L) for flask culture.

Optical density at 600 nm (OD_{600}) was measured every hour at six points per well during cultivation, using a microplate reader (TECAN Spark®, Switzerland) equipped with an integrated cooling module and compatible 48-well clear-bottom microplate (SARSTEDT, Germany). To prevent medium evoporation, the microplate was cultured in a humidity cassette (TECAN Spark®, Switzerland).

Cell concentration was determined for inoculants and samples at the end of microplate cultivation by counting from all wells using a hemocytometer (Assistant, Neubauer improved, 0.1 mm depth camera, Germany) and light microscope (Micros Austria, MCX100LCD, Austria) at 400× magnification.

Determination of total protein content in biomass

Total protein content in dry yeast biomass was determined by Bicinchoninic acid (BCA) assay kit (Merck, Germany) according to the manufacturer's protocol. Absorbance measurements were performed at a wavelength of 562 nm in a UV-Vis spectrophotometer (ThermoFisher, BioMate™ 160, USA). The dry cell lysis procedure was adapted from a previously described method (Guerlava et al., 1998).

Determination of amino acid profile

20 mg to 50 mg of dry yeast biomass were hydrolysed in 6M HCl and 0.1% phenol solution at 110 ± 2 °C for 24 h in sealed tubes. Determination of methionine (as a methionine sulfone) and cysteine and cystine amino acids content (as a cysteic acid) was done using pre-oxidation of the samples for 16 h at 0 °C by performic acid before hydrolysis using the protocol (EC 2009) with minor modification.

Identification and quantification of amino acids were done using HPLC-grade reagents: amino acid standard mix (Supelco, USA) and individual methionine sulfone and cysteic acid standards. An automated in-needle pre-column derivatisation of protein hydrolysates was done by orthophthalic aldehyde (OPA) in the presence of mercaptopropionic acid (MPA) (Merck, Germany) and 9-fluorenylmethyl chloroformate (FMOC) (Soma et al., 2022).

High-performance liquid chromatography (HPLC) analysis was conducted using a Shimadzu Nexera Series HPLC system (Shimadzu, Japan) equipped with a YMC-Triart C18 column (150 mm × 3.0 mm, 3 µm) and a guard column of the same type. The chromatography was performed in gradient mode described in the protocol (GL Sciences, n.d.). Tryptophane was not determined in this study. Chemicals were purchased from Sigma Aldrich, USA unless otherwise noted.

Evaluation of protein quality

The study focused on assessing the amino acid profile of mutants and evaluating the nutritional value of their protein for potential use in aquaculture feed. To achieve this, protein quality was analysed using two important chemical parameters: amino acid score (AAS) and essential amino acid index (EAAI).

AAS evaluates the content of individual essential amino acids relative to a reference protein or dietary requirements of fish (see equation 3) (Astorga-España et al., 2016; Machado et al., 2020):

$$\text{AAS (\%)} = \left(\frac{\text{mg of AA in 1 g of a test protein}}{\text{mg of AA in 1 g of a reference protein}}\right) \times 100 \quad (3)$$

where
AA – amino acid.

The EAAI evaluates protein quality through the geometric mean value of the essential amino acid relative to the reference protein or dietary requirements of fish (see equation 4, 5) (Astorga-España et al., 2016; Machado et al., 2020).

$$\text{EAAI (\%)} = n^{\log(\text{EAA})} \quad (4)$$

where
EAA – essential amino acid;
n – the number of amino acids considered for the calculation (overall 9 EAA).

$$\log(\text{EAA}) = \frac{1}{n} \times \left[\log\left(100\frac{a1}{a1\text{R}}\right) + \ldots + \log\left(100\frac{an}{an\text{R}}\right)\right] \quad (5)$$

where
a – mg of amino acid in 1 g of a test protein;
*a*R – mg of amino acid in 1 g of a reference protein;
n – the number of amino acids considered for the calculation (overall 9 EAA).

Anchovy fishmeal was used as a reference protein (Agboola et al., 2021) in AAS and EAAI calculations (including for comparative studies) because it is recognised as the best protein source for fish diets, including salmon (Bunda et al., 2015; Felix et al., 2023; Tibbetts et al., 2017). The AA profile of the reference protein, expressed as g amino acid per 100 g crude protein, is as follows: 2.4 g His, 4.3 g Thr, 5.6 g Arg, 5.4 g Val. 3.0 g Met, 1.2 g Trp, 4.1 g Phe, 4.7 g Ile, 7.6 g Leu, and 7.8 g Lys (Agboola et al., 2021).

Determination of astaxanthin content in biomass

The carotenoid extraction method was adapted from (Luna-Flores et al., 2022) and the determination of astaxanthin and total carotenoid content followed the protocol described in (Feldmane, 2023). The measurement procedure for astaxanthin and total carotenoid concentration was based on (Ni et al., 2005). For absorbance measurements, a UV-Vis spectrophotometer (ThermoFisher, BioMate™ 160) was used with the "multi-wavelength" detection program set to 474 nm and 452 nm.

Data processing

The data for biomass dry weight, total protein, astaxanthin, and total carotenoid content are presented as the means of three replicates. The amino acid composition of yeast protein is calculated as the mean of two replicates. The astaxanthin concentrations, protein content, and amino acid profiles shown in the results and discussion chapter are based on samples collected on the 7th day of cultivation.

Statistical analysis of biomass, protein, and astaxanthin values was performed using a one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test in SPSS software (version 29.0). OD_{600} value results were statistically analysed by ANOVA followed by Duncan's multiple range test. Differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Wild-type Phaffia rhodozyma growth inhibition

AEC caused a strong inhibitory effect on wild-type strain growth. At the lowest tested concentration (0.1 mM), significantly inhibited yeast growth. Increasing the concentration to 0.5 mM resulted in a slight

cell increase compared to initial cell concentration from inoculum. AEC concentrations of 2.5 mM and higher resulted in complete cell inhibition, accompanied by cell autolysis at the end of cultivation. These findings are consistent with study by Sano and Shiio (1970) reported about 100% inhibition of *Bacillus subtilis* and *Escherichia coli* at 5 mM AEC, whereas for 50% inhibition of *Brevibacterium flavum* required 15 mM AEC (Sano & Shiio, 1970).

Phaffia rhodozyma showed less sensitivity to GA compared to AEC inhibitor, moreover, at 0.1–10 mM, growth stimulation was observed. The growth-promoting effect of herbicides on some microorganisms has been described in numerous studies. Glyphosate, chlorsulfuron, imazaquin, and imazapyr have been reported to stimulate microbial growth at certain concentrations (Araújo et al., 2003; Łozowicka et al., 2021; Petrovickij-Angerer, 2009; Wang et al., 2007; Xuedong et al., 2005), but there is limited data on glufosinate-ammonium. The strong inhibition was observed at 50 mM GA concentration and complete cell growth inhibition was determined from 100 mM and higher. In present study *P. rhodozyma* demonstrated a higher tolerance to GA compered to *Saccharomyces cerevisiae* and *Aspergillus niger*, where significant growth inhibition was observed at concentrations of 0.05 mM and 0.15 mM GA, respectively (Ahuja & Punekar, 2008; Vallejo et al., 2017).

0.5 mM AEC, 2.5 mM AEC, 50 mM GA, and 100mM GA concentrations were selected for mutant screening as they represent strong inhibitory doses necessary for selecting mutants with enhanced target amino acid synthesis.

Selected mutant analysis

In the present study was conducted two flask experiments for six mutants selected from GA-contained media (hereafter referred to as GA mutants) and nine mutants selected from AEC-contained media (AEC mutants). *P. rhodozyma* wild-type strain on 7th day cultivation reached a 38.95–39.43 g/L biomass contained about 30.75–30.98% and 0.029–0.032% protein and astaxanthin content, respectively. GA mutants showed no statistically significant difference in biomass, protein, astaxanthin and total carotenoid values compared to the wild strain (Table 1). However, a significant difference was observed between some mutants in biomass values and total protein concentration.

Table 1. Comparison of biomass parameters in wild-type *P. rhodozyma* strain and GA mutants

Strain*	Biomass, g/L	Protein, g/L	Astaxanthin, mg/L	Total carotenoid, mg/L	Protein, %	Astaxanthin, %
Wild stain	39.43 ± 0.67^{ab}	12.22 ± 0.09^{a}	11.54 ± 1.16^{a}	16.41 ± 0.66^{a}	30.98 ± 0.41^{ab}	0.029 ± 0.002^{a}
GA6/1	40.20 ± 1.01^{ab}	12.22 ± 0.30^{a}	12.18 ± 1.03^{a}	15.04 ± 0.94^{a}	30.40 ± 1.54^{ab}	0.030 ± 0.003^{a}
GA6/2	38.10 ± 1.08^{a}	11.93 ± 1.34^{a}	11.31 ± 0.60^{a}	14.62 ± 0.76^{a}	31.33 ± 2.60^{ab}	0.030 ± 0.001^{a}
GA6/3	40.33 ± 0.68^{ab}	12.34 ± 0.43^{a}	13.51 ± 1.31^{a}	15.93 ± 0.52^{a}	30.62 ± 0.54^{ab}	0.034 ± 0.003^{a}
GA6/4	39.57 ± 1.07^{ab}	11.95 ± 1.13^{a}	11.49 ± 0.34^{a}	15.20 ± 0.86^{a}	30.22 ± 2.01^{a}	0.029 ± 0.002^{a}
GA7/4	40.90 ± 0.56^{b}	13.33 ± 0.62^{a}	11.60 ± 0.19^{a}	15.86 ± 0.38^{a}	32.58 ± 1.11^{ab}	0.028 ± 0.001^{a}
GA7/5	40.53 ± 0.40^{b}	14.15 ± 0.79^{a}	11.45 ± 0.88^{a}	16.23 ± 0.61^{a}	34.89 ± 1.90^{b}	0.028 ± 0.002^{a}

* – *P. rhodozyma* DSM 5626 wild-type strain and GA mutants. Values in the same column with different superscript letters differ significantly ($p < 0.05$).

The AEC mutants did not have significantly higher biomass parameters compared to the wild-type strain (Table 2). Moreover, the AEC3/4 mutant showed significantly lower biomass concentrations, total carotenoid and astaxanthin contents. The AEC3/9 mutant had a two-fold decrease in astaxanthin in the biomass compared to the wild-type strain.

Table 2. Comparison of biomass parameters in wild-type *P. rhodozyma* strain and AEC mutants

Strain*	Biomass, g/L	Protein, g/L	Astaxanthin, mg/L	Total carotenoid, mg/L	Protein, %	Astaxanthin, %
DSM 5626	38.95 ± 0.35^{bcd}	11.98 ± 0.04^{ab}	12.43 ± 3.42^{bc}	17.96 ± 1.44^{bcd}	30.75 ± 0.15^{bc}	0.032 ± 0.012^{bc}
AEC2/1	38.73 ± 1.24^{bcd}	11.02 ± 0.59^{a}	15.11 ± 0.92^{d}	19.33 ± 1.10^{d}	28.45 ± 1.28^{ab}	0.039 ± 0.003^{c}

AEC3/1	39.70 ± 1.56^{bcd}	10.53 ± 0.76^{ab}	13.54 ± 0.75^{bc}	17.75 ± 1.29^{bcd}	26.52 ± 1.06^{a}	0.034 ± 0.003^{bc}
AEC3/3	40.70 ± 0.70^{d}	11.65 ± 0.43^{ab}	13.22 ± 1.36^{bc}	16.23 ± 1.52^{abcd}	28.62 ± 0.77^{ab}	0.032 ± 0.003^{bc}
AEC3/4	35.75 ± 0.25^{a}	10.55 ± 0.43^{ab}	6.76 ± 0.45^{a}	12.60 ± 1.98^{a}	29.51 ± 1.20^{abc}	0.019 ± 0.001^{a}
AEC3/5	39.13 ± 0.95^{bcd}	11.57 ± 0.79^{ab}	14.76 ± 0.48^{d}	19.30 ± 1.42^{d}	29.57 ± 1.31^{abc}	0.038 ± 0.001^{bc}
AEC3/6	37.93 ± 0.32^{abc}	11.89 ± 0.64^{ab}	10.16 ± 0.65^{ab}	14.32 ± 1.71^{abc}	31.35 ± 1.89^{bc}	0.027 ± 0.002^{ab}
AEC3/7	39.83 ± 1.10^{bcd}	11.77 ± 0.53^{ab}	13.61 ± 2.13^{bc}	18.77 ± 0.98^{cd}	29.55 ± 1.35^{abc}	0.034 ± 0.006^{bc}
AEC3/8	40.43 ± 1.12^{cd}	12.31 ± 0.55^{b}	14.91 ± 1.44^{d}	19.37 ± 1.41^{d}	30.45 ± 1.81^{abc}	0.037 ± 0.003^{bc}
AEC3/9	37.30 ± 0.26^{ab}	12.20 ± 0.59^{ab}	6.83 ± 0.90^{a}	13.40 ± 2.53^{ab}	32.71 ± 1.78^{c}	0.018 ± 0.002^{a}

*– *P. rhodozyma* DSM 5626 wild-type strain and AEC mutants. Values in the same column with different superscript letters differ significantly ($p < 0.05$).

According to the literature, GA has an inhibitory effect on the biosynthesis of seven amino acids, of which five are essential for fish (Gong et al., 2020; Joo et al., 2018; Mowbray et al., 2014). Interestingly, in the study of Vallejo et al., the treatment of wine yeast with 0.05 mM GA significantly increased the synthesis of Met, Ile, Leu, Phe, Trp, Tyr compared to untreated cells (Vallejo et al., 2017). At the same time, Phe, Trp, Tyr are not targets for this inhibitor, which may indicate the switching-on side effects in the microorganism cells in the presence of the herbicide (Spalvins et al., 2021). In the present study, three mutants shown a significant improvement of GA-specific amino acid. GA6/3 had a higher amount of Lys by 14%, GA6/4 and GA7/5 have higher amount of Met by 37% and 25%, respectively (Table 3). The amino acid pattern of *P. rhodozyma* DSM 5626 was found to be more consistent with SCP compared to the pattern of other strains of this yeast species. Comparison of protein quality parameters between the strains is presented in Tables 5 and 6.

Table 3. Amino acid profile of *P. rhodozyma* GA mutants

Strain*	DSM 5626	GA6/1	GA6/2	GA6/3	GA6/4	GA7/4	GA7/5
Pure protein, %	30.27 ± 0.12	29.45 ± 0.89	30.77 ± 0.41	29.44 ± 1.04	30.28 ± 0.41	29.24 ± 0.34	29.36 ± 0.26
EAAs, g/100 g of pure protein							
His	2.46 ± 0.14^{a}	2.41 ± 0.04^{a}	2.31 ± 0.08^{a}	2.48 ± 0.09^{a}	2.52 ± 0.25^{a}	2.45 ± 0.03^{a}	2.41 ± 0.16^{a}
Thr	5.51 ± 0.26^{a}	4.90 ± 0.23^{a}	5.37 ± 0.17^{a}	4.93 ± 0.24^{a}	5.38 ± 0.09^{a}	5.13 ± 0.12^{a}	5.43 ± 0.12^{a}
Arg	6.77 ± 0.26^{abc}	7.20 ± 0.26^{bc}	6.72 ± 0.51^{abc}	7.41 ± 0.07^{c}	7.19 ± 0.05^{bc}	6.10 ± 0.02^{a}	6.38 ± 0.06^{ab}
Val	5.94 ± 0.02^{ab}	6.02 ± 0.00^{ab}	6.01 ± 0.18^{ab}	6.02 ± 0.04^{ab}	5.72 ± 0.09^{a}	6.08 ± 0.02^{b}	5.80 ± 0.02^{ab}
Met	1.24 ± 0.09^{a}	1.21 ± 0.13^{a}	1.19 ± 0.10^{a}	1.23 ± 0.05^{a}	1.70 ± 0.05^{b}	1.41 ± 0.12^{ab}	1.56 ± 0.09^{b}
Trp	ND	ND	ND	ND	ND	ND	ND
Phe	4.34 ± 0.10^{a}	4.26 ± 0.07^{a}	4.25 ± 0.02^{a}	4.24 ± 0.27^{a}	4.41 ± 0.03^{a}	4.49 ± 0.06^{a}	4.43 ± 0.06^{a}
Ile	5.14 ± 0.21^{a}	5.39 ± 0.13^{a}	5.48 ± 0.37^{a}	5.40 ± 0.21^{a}	4.96 ± 0.03^{a}	5.36 ± 0.08^{a}	4.99 ± 0.09^{a}
Leu	8.38 ± 0.10^{a}	8.27 ± 0.00^{a}	8.40 ± 0.07^{a}	8.19 ± 0.10^{a}	8.08 ± 0.19^{a}	8.38 ± 0.08^{a}	8.34 ± 0.07^{a}
Lys	4.90 ± 0.12^{a}	5.42 ± 0.07^{ab}	4.90 ± 0.27^{a}	5.60 ± 0.13^{b}	5.20 ± 0.18^{ab}	4.90 ± 0.09^{a}	4.94 ± 0.02^{a}
NEAAs, g/100 g of pure protein							
Asp ac	10.70 ± 0.39^{a}	11.18 ± 0.35^{a}	10.99 ± 0.15^{a}	10.82 ± 0.30^{a}	11.07 ± 0.15^{a}	10.74 ± 0.13^{a}	10.51 ± 0.09^{a}
Glu ac	14.93 ± 0.01^{a}	15.21 ± 0.11^{a}	14.89 ± 0.45^{a}	14.83 ± 0.58^{a}	14.58 ± 0.26^{a}	14.97 ± 0.17^{a}	14.91 ± 0.13^{a}
Ser	5.60 ± 0.38^{a}	4.65 ± 0.40^{a}	5.41 ± 0.27^{a}	4.81 ± 0.11^{a}	5.55 ± 0.07^{a}	4.72 ± 0.38^{a}	5.63 ± 0.08^{a}
Gly	5.79 ± 0.01^{ab}	6.07 ± 0.05^{ab}	5.85 ± 0.29^{ab}	6.01 ± 0.03^{ab}	5.59 ± 0.16^{a}	6.19 ± 0.13^{b}	5.67 ± 0.08^{ab}
Ala	7.45 ± 0.22^{a}	7.30 ± 0.01^{a}	7.37 ± 0.07^{a}	7.24 ± 0.04^{a}	7.26 ± 0.04^{a}	7.28 ± 0.06^{a}	7.34 ± 0.08^{a}
Tyr	3.79 ± 0.13^{a}	3.78 ± 0.12^{a}	3.84 ± 0.05^{a}	3.72 ± 0.00^{a}	3.70 ± 0.05^{a}	3.79 ± 0.04^{a}	3.69 ± 0.03^{a}
Cys	0.31 ± 1.80	0	0.27 ± 0.00	0.16 ± 0.66	0.81 ± 0.02	1.27 ± 0.08	1.39 ± 0.04
Pro	6.81 ± 0.22^{a}	6.73 ± 0.25^{a}	6.82 ± 0.44^{a}	6.93 ± 0.36^{a}	6.33 ± 0.24^{a}	6.56 ± 0.01^{a}	6.51 ± 0.41^{a}

*– *P. rhodozyma* DSM 5626 wild-type strain and GA mutants were cultivated in shake flasks on GPM medium. Values in the same row with different superscript letters differ significantly ($p < 0.05$). ND – not determined.

AEC is an inhibitor of the biosynthesis of the four EAAs: Met, Lys, Thr and Ile (Pearce et al., 2017). In the present study, an increase in these amino acids and non-target AAs such as Phe, Ala and Tyr was found. The protein of the AEC2/1 mutant contained a significantly higher concentration of Thr, Phe, Ala

and Tyr. The AEC3/9 mutant had improved Met content by 35%, Lys by 24%, Ile by 8% and Phe by 6% compared to the wild-type strain (Table 4).

Table 4. Amino Acid Profile of *P. rhodozyma* AEC mutant

Strain*	DSM 5626	AEC2/1	AEC3/3	AEC3/5	AEC3/8	AEC3/9
Pure protein, %	30.92 ± 0.18	26.77 ± 0.40	27.85 ± 0.23	29.12 ± 0.57	28.78 ± 0.18	31.35 ± 0.51
EAAs, g/100 g of pure protein						
His	2.67 ± 0.04^{a}	2.27 ± 0.06^{a}	2.40 ± 0.00^{a}	2.40 ± 0.07^{a}	2.51 ± 0.30^{a}	2.39 ± 0.02^{a}
Thr	4.93 ± 0.13^{ab}	5.43 ± 0.02^{c}	4.73 ± 0.09^{a}	5.23 ± 0.06^{bc}	4.99 ± 0.11^{abc}	4.79 ± 0.19^{ab}
Arg	8.30 ± 0.26^{c}	5.85 ± 0.10^{a}	8.00 ± 0.16^{c}	7.16 ± 0.01^{b}	7.93 ± 0.01^{c}	6.59 ± 0.29^{b}
Val	6.59 ± 0.00^{b}	6.66 ± 0.04^{b}	6.64 ± 0.11^{b}	6.68 ± 0.01^{b}	6.03 ± 0.03^{a}	6.50 ± 0.08^{b}
Met	1.23 ± 0.08^{ab}	1.34 ± 0.02^{b}	1.43 ± 0.11^{bc}	1.26 ± 0.06^{ab}	1.08 ± 0.07^{a}	1.66 ± 0.05^{c}
Trp	ND	ND	ND	ND	ND	ND
Phe	4.17 ± 0.05^{a}	4.43 ± 0.05^{c}	4.19 ± 0.04^{ab}	4.35 ± 0.00^{b}	4.20 ± 0.04^{ab}	4.40 ± 0.04^{c}
Ile	4.88 ± 0.05^{a}	4.89 ± 0.05^{a}	4.97 ± 0.08^{a}	4.91 ± 0.04^{a}	4.95 ± 0.00^{a}	5.27 ± 0.10^{b}
Leu	7.98 ± 0.08^{ab}	8.19 ± 0.07^{b}	7.81 ± 0.09^{a}	8.01 ± 0.03^{ab}	8.10 ± 0.11^{ab}	8.18 ± 0.10^{b}
Lys	5.91 ± 0.00^{a}	5.49 ± 0.12^{a}	5.89 ± 0.09^{a}	5.60 ± 0.28^{a}	5.43 ± 0.23^{a}	7.31 ± 0.17^{b}
NEAAs, g/100 g of pure protein						
Asp ac	10.39 ± 0.05^{ab}	11.12 ± 0.17^{b}	10.44 ± 0.09^{ab}	11.17 ± 0.22^{b}	10.03 ± 0.43^{a}	10.34 ± 0.17^{ab}
Glu ac	15.32 ± 0.01^{a}	15.45 ± 0.03^{a}	15.79 ± 0.09^{a}	15.94 ± 0.61^{ab}	17.02 ± 0.21^{b}	15.52 ± 0.29^{a}
Ser	4.87 ± 0.37^{ab}	5.80 ± 0.10^{b}	4.36 ± 0.32^{a}	5.39 ± 0.04^{ab}	4.85 ± 0.07^{ab}	4.27 ± 0.50^{a}
Gly	5.48 ± 0.08^{a}	5.44 ± 0.09^{a}	5.72 ± 0.14^{a}	5.42 ± 0.14^{a}	5.52 ± 0.11^{a}	5.87 ± 0.10^{a}
Ala	6.68 ± 0.02^{a}	7.26 ± 0.05^{b}	6.66 ± 0.05^{a}	6.83 ± 0.01^{a}	6.75 ± 0.04^{a}	6.73 ± 0.08^{a}
Tyr	3.49 ± 0.03^{a}	3.80 ± 0.06^{b}	3.47 ± 0.03^{a}	3.72 ± 0.07^{b}	3.85 ± 0.03^{b}	3.67 ± 0.06^{ab}
Cys	1.26 ± 0.02	0.64 ± 0.00	1.05 ± 0.01	0	0.81 ± 0.00	0.29 ± 0.01
Pro	5.77 ± 0.05^{a}	5.91 ± 0.12^{a}	6.47 ± 0.03^{a}	6.00 ± 0.51^{a}	5.96 ± 0.04^{a}	6.28 ± 0.12^{a}

*– *P. rhodozyma* DSM 5626 wild-type strain and GA mutants were cultivated in shake flasks on GPM medium. Values in the same row with different superscript letters differ significantly ($p < 0.05$). ND – not determined.

Aquaculture feed generally contains a combination of several protein sources to maintain the required amino acid balance for the target animal, and the addition of pure amino acids can also be used if necessary (Glencross et al., 2020). Basically, aquaculture's feed formulations use fishmeal, legumes, oilseed crops, animal by-products, crustacean meal, algal meal. Each of these sources separately has limiting essential amino acids. Plant-based proteins typically have less methionine, tryptophan, lysine, and cysteine below 30% mean fish requirement. Proteins of animal origin except fishmeal are usually the limiting are Thr, Met, Cys, Ile, and Tyr. The SCP quality of the amino acids depends on the microorganism type. The limiting amino acid for *Saccharomyces cerevisiae*, *Candida utilis* is Met, for *Spirulina maxima* is Met, Cys, and Lys, and for *Methylophilus methylotrophus* is Cys (FAO, 2013a; Tacon, 1987). Algal protein contains the lowest concentration of His and Met (Astorga-España et al., 2016). In turn, fishmeal is considered a more balanced source of essential amino acids for the fish diet. Fishmeal is produced from small pelagic forage fish such as mackerel, herring, sardine, anchovy, and only a small portion from fish by-products and wastes (Cottrell et al., 2020; FAO, 2022; Janbakhsh et al., 2018; Jannathulla et al., 2021).

The first limiting amino acid in all *P. rhodozyma* strains was Met, which is typical for yeasts and algae (FAO, 2013a; Tacon, 1987). The EAAI of *P. rhodozyma* DSM 5626 protein indicated moderate quality from 70 to 89%. This contrasts with many commercial SCP-yeast proteins, which generally exhibit low EAAI values (<70%), reflecting suboptimal protein quality for fish feed applications (Tables 5 and 6). Notably, the GA6/4 and AEC3/9 mutant strains demonstrated the highest EAAI values among the tested mutants, reaching levels approaching high-quality protein, which requires an EAAI of ≥ 90% (Bunda et al., 2015; Machado et al., 2020). An EAA with an AAS below 100% is considered limiting (Kurpad, 2012). However, it is worth noting that there is no EU regulation clearly defining the quality of fishmeal protein used for fish feed, and there is also no classification of protein quality based on the amount of ten EAAs contained. In general, fishmeal can be of varying quality depending on the species of fish used,

whether whole fish or by-products are used (Janbakhsh et al., 2018; Jannathulla et al., 2021; FAO, 2022). For example, fishmeal from sprat caught in the Caspian Sea contained on average 0.73 g Arg, 1.97 g Met, 1.53 g Phe, 2.80 g Leu and 5.50 g Lys per 100 g crude protein (Janbakhsh et al., 2018), which is much lower than that of anchovy fishmeal used as a reference protein in our study (Agboola et al., 2021).

Table 5. Comparison of AAS and EAAI in *P. rhodozyma* wild strain and GA mutant strains

Strain	DSM 5626	GA6/1	GA6/2	GA6/3	GA6/4	GA7/4	GA7/5	PR[1]	PR[2]	CU[3]	SC[3]	KM[3]
Crude protein, %	30.98	30.40	31.33	30.62	30.22	32.58	34.89	47.10	22	56.0	46.0	51.1
AAS (%)												
His	100.36	100.18	97.78	99.24	113.44	93.29	88.99	58.75	70.83	81.67	71.25	75.83
Thr	125.20	116.59	121.01	110.25	125.10	109.62	105.33	59.77	90.70	110.93	103.95	98.37
Arg	118.19	123.87	112.51	127.19	130.52	98.43	97.05	65.54	112.50	83.39	75.00	72.68
Val	107.46	110.20	112.61	107.17	108.43	101.68	90.67	59.81	68.52	83.52	74.81	73.33
Met	38.26	39.11	37.44	39.82	54.90	49.95	46.75	33.33	36.67	45.33	51.33	41.00
Phe	103.53	103.92	102.26	99.45	108.38	98.19	90.52	58.05	68.29	101.46	90.49	80.24
Ile	106.94	111.60	121.17	110.31	106.30	102.19	88.81	56.60	61.70	89.79	82.98	72.55
Leu	107.77	107.56	110.19	103.68	105.80	99.09	92.39	54.08	67.11	91.32	79.61	71.58
Lys	61.35	69.35	64.76	69.09	69.11	57.68	53.47	74.62	60.26	89.10	83.46	79.36
EAAI (%)	74.37	75.87	75.20	74.71	80.37	71.12	66.39	47.16	56.03	68.72	63.87	59.43

PR – *P. rhodozyma:* [1]–ATCC 24202 was cultivated in peat hydrolysate media in 2 L bioreactor (Acheampong, 1993) and [2] – commercial yeast product *P. rhodozyma* Red Star® (Sanderson & Jolly, 1994); [3] – CU – *Candida utilis*, SC – *Saccharomyces cerevisiae*, and KM – *Kluyveromyces marxianus* are commercial yeast strains (Borregaard ASA, Norway) (Øverland et al., 2013).

Table 6. Comparison of AAS and EAA in *P. rhodozyma* wild strain and AEC mutant strains

Strain	DSM 5626	AEC2/1	AEC3/3	AEC3/5	AEC3/8	AEC3/9	PR[1]	PR[2]	CU[3]	SC[3]	KM[3]
Crude protein, %	30.76	28.44	28.62	29.56	30.44	33.47	47.10	22	56.0	46.0	51.1
AAS (%) of crude protein											
His	111.96	91.77	97.90	101.94	107.61	96.32	58.75	70.83	81.67	71.25	75.83
Thr	115.33	120.42	108.97	120.41	108.52	112.22	59.77	90.70	110.93	103.95	98.37
Arg	149.04	98.09	141.79	127.51	134.55	118.10	65.54	112.50	83.39	75.00	72.68
Val	122.57	116.76	118.85	123.45	105.75	115.11	59.81	68.52	83.52	74.81	73.33
Met	43.46	43.88	47.03	39.20	33.93	50.36	33.33	36.67	45.33	51.33	41.00
Phe	102.31	101.99	99.40	105.94	97.92	111.50	58.05	68.29	101.46	90.49	80.24
Ile	104.44	98.18	102.32	104.88	100.04	120.98	56.60	61.70	89.79	82.98	72.55
Leu	105.57	101.95	99.71	105.54	102.16	109.37	54.08	67.11	91.32	79.61	71.58
Lys	76.10	67.97	74.71	74.21	68.02	95.59	74.62	60.26	89.10	83.46	79.36
EAAI (%)	79.91	73.21	77.26	77.50	73.18	81.37	47.16	56.03	68.72	63.87	59.43

PR – *P. rhodozyma:* [1] – ATCC 24202 was cultivated in peat hydrolysate media in 2 L bioreactor (Acheampong, 1993) and [2] – commercial product *P. rhodozyma* Red Star® (Sanderson & Jolly, 1994); [3] – CU – *Candida utilis*, SC – *Saccharomyces cerevisiae*, and KM – *Kluyveromyces marxianus* are commercial yeast strains (Borregaard ASA, Norway) (Øverland et al., 2013).

Similarly, other studies reported that fishmeal can have lower levels of all nine EAAs except for Trp, which may exceed reference values by 0.2 g per 100 g crude protein (Felix et al., 2023). These variations highlight the potential of *P. rhodozyma* mutants to replace lower-quality fishmeal, particularly when the reference protein is not of the highest standard. The suitability of SCP protein quality can vary depending on the reference protein chosen. For example, using the essential amino acid requirements of salmonids as an etalon standard (Agboola et al., 2021; Hua & Bureau, 2019), the EAAI of *P. rhodozyma* DSM 5626 protein could approach 100%. In this study, the AAS values for His, Arg, Val, Phe, Ile, and Leu were consistently higher in all *P. rhodozyma* strains compared to three commercial SCP yeast strains. Among the mutants, AEC3/9 exhibited the most improved Lys and Met content, making it the best-performing

strain. The Lys AAS of 95.59% was significantly higher than values reported for other yeasts such as *Yarrowia lipolytica* (Michalik et al., 2014; Yang et al., 2022), *Cyberlindnera jadinii*, *Blastobotrys adeninivorans* (Agboola et al., 2022), *Wickerhamomyces anomalus* (Agboola et al., 2022; Lapeña et al., 2020), *Saccharomyces cerevisiae* (Øverland et al., 2013), (Vidakovic et al., 2020), (Michalik et al., 2014), *Candida utilis* (Marius et al., 2018; Øverland et al., 2013), *Kluyveromyces marxianus* (Øverland et al., 2013; Yang et al., 2022), and fishmeal of not the highest quality (Felix et al., 2023), (Janbakhsh et al., 2018).

Conclusion

The present study demonstrates the potential of amino acid biosynthesis inhibitors in screening of protein-improved mutants. Using a selective medium reduced the number of mutants studied, allowing for compact tests in flasks. Although the analysis of the mutant biomass did not show an increase in the synthesis of total protein, an increase in the synthesis of essential amino acids was achieved. Screening of mutagenised yeast cells on GA selective medium resulted in selecting two mutants with significantly improved Met synthesis and one mutant with significantly improved Lys synthesis. Screening of mutagenised yeast cells on an AEC selective medium resulted in selecting three mutants with significantly improved synthesis of two to four amino acids. The best mutant in this study, AEC3/9, had significantly improved synthesis of four essential amino acids for fish diets: Met, Phe, Ile, and Lys. The increase in synthesising the first two limiting amino acids in yeast – Met and Lys – was especially valuable. This proves that amino acid biosynthesis inhibitors are a good tool for selecting improved protein-quality mutants.

An important aspect of this study is disclosing the potential of *P. rhodozyma* as an SCP producer. *P. rhodozyma* DSM 5626 biomass has about 31% protein and 0.029% astaxanthin when cultivated in glycerol-peptone-rich medium with pH 5 in shaken flasks at 22 °C for seven days. Under these conditions, *P. rhodozyma* DSM 5626 has a moderate quality protein similar to fishmeal and essential amino acid profile, except for the limiting content of Met and Lys. The *P. rhodozyma* DSM 5626 protein is superior in essential amino acid content to other SCP producers such as *C. jadinii*, *W. anomalus*, *B. adeninivorans*, or low quality fishmeal. This was unexpected since *P. rhodozyma* has not been previously considered as an SCP producer. Further research is required to optimise culture conditions to increase protein concentration in the yeast biomass and to assess the competitiveness of both *P. rhodozyma* DSM 5626 and the best mutants as simultaneous producers of protein and astaxanthin.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Svetlana Raita developed the study methodology, analysed data, and wrote the manuscript. Iveta Kuzmika prepared the statistical analysis of data, tables, and part of the methodology description. Zane Geiba, Iveta Kuzmika, and Taras Mika performed laboratory experiments and sample analysis. Kriss Spalvins is the study leader. All the authors approved the final manuscript.

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