RIGA TECHNICAL UNIVERSITY

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A SCREENING METHOD FOR IMPROVED SINGLE-CELL PROTEIN PRODUCTION

Summary of the Doctoral Thesis

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RIGA TECHNICAL UNIVERSITY
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DOCTORAL THESIS PROPOSED TO RIGA TECHNICAL UNIVERSITY FOR PROMOTION TO THE SCIENTIFIC DEGREE OF DOCTOR OF SCIENCE

To be granted the scientific degree of Doctor of Science (Ph. D.), the present Doctoral Thesis has been submitted for defence at the open meeting of the RTU Promotion Council on 27 February 2025, at 14.00 at the Faculty of Natural Sciences and Technology of Riga Technical University, 12/1 Āzenes Street, Room 607.

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DECLARATION OF ACADEMIC INTEGRITY

I hereby declare that the Doctoral Thesis submitted for review to Riga Technical University for promotion to the scientific degree of Doctor of Science (Ph. D.) is my own. I confirm that this Doctoral Thesis has not been submitted to any other university for promotion to a scientific degree.

Svetlana Raita ……. (signature)
Date: ……

The Doctoral Thesis has been written in English. It consists of an Introduction, three chapters, Conclusions, 13 figures, 22 tables, and nine appendices; the total number of pages is 219, including appendices. The Bibliography contains 354 titles.

TABLE OF CONTENTS

ABBREVIATIONS

AAS – amino acid score
AEC – S-(2-aminoethyl)-L-cysteine
Ala – alanine
Arg – arginine
Asp ac – aspartic acid
ATX – astaxanthin
BCA – Bicinchoninic acid assay
Cys – cysteine
EAAI – essential amino acid index
Ex. – emission
EMS – ethyl methanesulfonate
Em. – excitation
FAO – Food and Agriculture Organisation
g – gravity, a unit of relative centrifugal force
GA – glufosinate-ammonium
Glu ac – glutamic acid
Gly – glycine
GPM – glycerine-peptone medium
GSM – glycerine-salt medium
His – histidine
Ile – isoleucine
Leu – leucine
Lys – lysine
M – mole
MCDA – multi-criteria decision analysis
Met – methionine
MSO – L-methionine sulfoximine
n/a – not analysed
OD – optical density
Phe – phenylalanine
Pro – proline
rpm – revolutions per minute
SCP – single-cell protein
Ser – serine
Thr – threonine
TOPSIS – a technique for order preference by similarity to an ideal solution
Tyr – tyrosine
Val – valine
YM – yeast medium
YNB – yeast nitrogen base

Abbreviations used for mutant strains (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]. Increasing the use of SCP, for example, in livestock feed could reduce the need for intensive farming while aligning with environmental strategies to reduce greenhouse gas emissions [8], [9].

SCP production technologies have undergone extensive research for decades [10] and have been widely used as a food supplement for humans and as a feed for animals [11]. Currently, SCP is being produced under different commercial names such as Brovile®, AlgaVia®, Quorn®, Vitam-R®, Pruteen®, Marmite®, and FermentIQ™, etc.[11], [12]. Although some products are already on the market, they remain a niche product that is not widely available or consumed [13]. 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 [14]. 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 that influence the competitiveness of technology. Improving and creating strains with superior characteristics can increase the competitiveness of SCP. Genetic engineering is the only way to improve protein biosynthesis in microorganisms [15], [16]. 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 [14], [17], [18].

This study proposes a new way to create mutant microorganisms with improved amino acid profiles. Random mutagenesis is suggested, 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 [19]–[21]. Interestingly, the widely used random mutagenesis to improve the desired properties of strains is not used to create protein-synthesised mutants. 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 the total protein concentration in microbial biomass is a significant factor, the concentration of essential amino acids is the main factor determining the value of the protein obtained. For example, essential amino acids, such as lysine, methionine, threonine, and tryptophan, are important in fish feed, as they are available in lower amounts in conventional protein sources such as soy [22], [23]. Thus, in creating SCP mutants, it is important to increase the total protein concentration in the 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 (active 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 biosynthesis 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 biosynthesis 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 biosynthesis 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 [24], 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 of 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 database of herbicides: generalised types of herbicides inhibiting amino acid synthesis in microorganisms, targeted amino acids, possible side effects (second publication – P2) and also summarised studies on the effectiveness of microorganism inhibition, the 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 a subsequent experimental study. The final step (S4) was experimental work on the creation of the *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 screening methodology (R1) and the mutants created (R2).

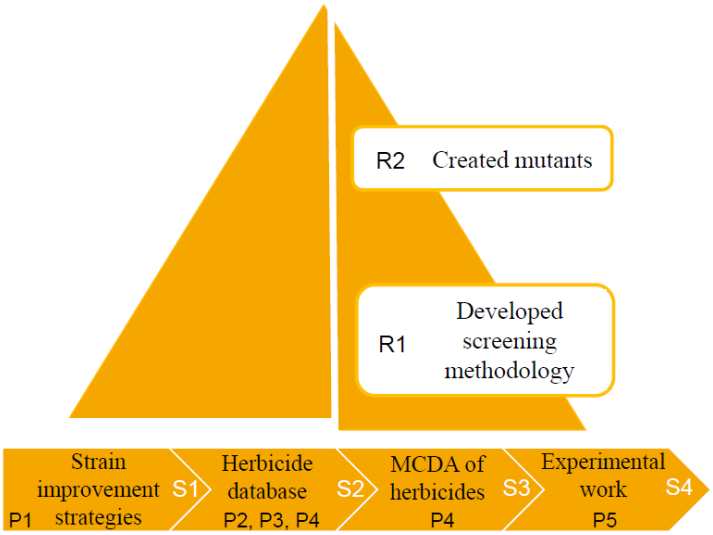

Fig. 1. The research framework of the Doctoral Thesis. The letters S, P, and R stand for steps, publications, and results, respectively.

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. *Agronomy Research*, 2021, 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. *Environmental and Climate Technologies*, 2022, vol. 26, no. 1, pp. 848–861. doi: 10.2478/rtuect-2022-0064
3. 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. *Agronomy Research*, 2024, vol. 22, no. S1, pp. 370–400. doi: 10.15159/AR.24.039
4. Raita, S., Berzina, I., Kusnere, Z., Kalnins, M., Kuzmika, I., Spalvins, K. Herbicide-based selection of mutants for improved SCP synthesis: Application and procedures. *Agronomy Research*, 2024, vol. 22, no. 2, pp. 913–938. doi: 10.15159/AR.24.060
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

1. 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
2. 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
3. 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
4. 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
5. 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 feed generally contains a combination of several protein sources to maintain the required amino acid balance for the target animal, and if necessary, addition pure amino acids can be supplemented [25]. Aquaculture feed formulations use fishmeal, legumes, oilseed crops, animal by-products, crustacean meal, and algal meal [26], [27]. Each of these sources separately has limiting essential amino acids. Plant-based proteins typically have less methionine, tryptophan, lysine, and cysteine, below 30 % of the mean fish requirement. Proteins of animal origin, except for fish meal, are generally the limiting amino acids as threonine, methionine, cysteine, isoleucine, and tyrosine [26], [27]. Algal protein contains the lowest concentrations of histidine and methionine [28]. In turn, fish meal is considered a more balanced source of essential amino acids for the fish diet. Fish meal is produced from small pelagic forage fish such as mackerel, herring, sardine, anchovy and only a small portion of fish by-products and wastes. Fish meal is considered the most nutritious and digestible source of protein for aquaculture feed [24], [29]–[31].

Fish meal was initially used as an effective and inexpensive protein-rich feed ingredient for farmed aquatic species. However, the decrease in fishing productivity and the increase in the demand for fish meal caused significant price changes [31]. The price of fish meal has increased in the past 20 years from 599 €/t in 2013 to 1673 €/t in 2023 and is expected to continue to rise [24], [32]. More than 80 % (86 % in 2020) of fish meal is used to produce feed for high-value aquaculture species, mainly to raise shrimp, salmon, and marine fish such as seabass [24]. Fish meal 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 basis, depending on the life stage [26], [33]. 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 the dependence on fishmeal and replace it with alternative protein sources [24], [34], [35].

Single-cell protein (SCP) is a promising alternative to fish meal and plant-based protein [36], [37]. SCP is dried biomass produced by various microorganisms such as algae, bacteria, fungi, yeast, and protists, which can metabolise different carbon and nitrogen sources [38], [39]. 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 [25], [40].

Recent experimental feeding trials have shown that yeast SCP can replace between 15 % and 60 % of fish meal in fish feed, corresponding to up to 345 g of yeast per kg of feed [37], [41]–[43]. For example, the results of the study [41] indicate that *S. cerevisiae* can replace up to 40 % of the fish meal in rainbow trout diets without compromising growth performance,

nutrient absorption, or health. The use of *W. anomalus* in the diet of rainbow trout at a rate of 20 % improved phosphorus absorption while reducing phosphorus consumption [41]. Interestingly, the appropriate amount of *S. cerevisiae* biomass added to the 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. In addition, there is an improvement in the conversion of feed to live weight [37]. SCP can also be successfully used to feed fish in the early stages of life. During this period, the fish require more protein in the feed due to the intense gain in body weight [44], [45]. In [44] , bacterial SCP replaced 50 % of the fish meal in the feed for the rainbow trout fry and improved the conversion ratio values compared to the standard feed [44].

Selected yeast species

The 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 salmonid feed formulations (e.g. Aquasta®, RedStar®) [41], [46]. This yeast has been studied for the past 50 years to develop competitive technology for natural astaxanthin production [47]. Interestingly, *P. rhodozyma* biomass is exclusively a source of astaxanthin but is not considered a protein or fatty acid source. Regulatory documents confirming the use of *P. rhodozyma* biomass in the formulation of aquaculture feed indicate permissible concentrations of astaxanthin and safety criteria for the additive [48]. According to the European Regulation 2015/1415, the astaxanthin content should not exceed 100 mg/kg of finished feed, while the biomass of concentration in the *P. rhodozyma* should be higher than 0.5 % [48]. Based on these conditions, the biomass of yeast *P. rhodozyma* can be used presumably in an amount of approximately 20 g per 1 kg of finished feed as a colouring component. No studies indicate that the biomass of this yeast 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 a rich supplement in astaxanthin. However, 100 g/kg of *P. rhodozyma* biomass in the diet was reported to provide similar growth rates for rainbow trout compared to an equivalent dose of a brewer's yeast-supplemented diet [49]. The protein content and amino acid composition of *P. rhodozyma* have been poorly studied. Several studies from the 80–90s reported that *P. rhodozyma* biomass contains approximately 25–47 % of protein, a balanced amino acid composition, and a high number of fatty acids [50]–[52].

Therefore, the potential of this yeast as a producer of SCP should be further investigated. With a satisfactory protein content in 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/kg 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 the development of 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.

2. METHODOLOGY

2.1. Multi-criteria decision analysis

This study aimed to compare and find the best amino acid biosynthesis inhibitors for selective screening of protein-improved mutants. To achieve this, the multi-criteria decision analysis (MCDA) methods TOPSIS (a technique for order preference by similarity to the ideal solution) and AHP (analytic hierarchy process) were used. The TOPSIS tool provides an optimal solution by calculating the relative closeness coefficient to the ideal solution, that is, identifying the best alternative depending on the criteria. The closeness coefficient is always between 0 and 1, where 1 is the preferred solution [53]. 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 [54]. The criteria weights obtained in the AHP method were used in the TOPSIS method for a more accurate evaluation of amino acid biosynthesis inhibitors.

The present study evaluates the following amino acid 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.

Amino acid biosynthesis inhibitors were evaluated based on four criteria: price, inhibition efficacy, inhibited amount of essential amino acids, and safety (Table 2.1).

Table 2.1

Criteria and AHP Weights Used for TOPSIS

No.	Criteria	Unit of measure	Weight
C1	Price* of amino acid biosynthesis inhibitors	€/100 mg	0.17
C2	Inhibition efficacy	-	0.28
C3	Inhibited essential amino acids	%	0.47
C4	Number of total health and environmental hazards	-	0.08
		Σ	1

*Local chemical distributors provided inhibitor prices.

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 extractwas used to prepare the inoculum. During experiments, *P. rhodozyma* was grown on glycerine-salt medium (GSM) that contained 40 g/L glycerine, 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. Glycerine-peptone medium (GPM)

contained 70 g/L glycerine, 32 g/L soy peptone, 10 g/L yeast extract, and 1.7 g/L YNB, pH 5.0±0.1.

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 a diameter of 0.22 µm pore size, and stored at 8 °C until experiments.

Microorganism strain and inoculum preparation

The yeast culture *P. rhodozyma* DSM 5626 was purchased from the German Collection of Microorganisms and Cell Cultures at the Leibniz Institute. Before experiments, fresh yeast culture was stored at 4 °C on a YM agar plate (Fig. 2.1. A). To prepare the inoculum, a 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.

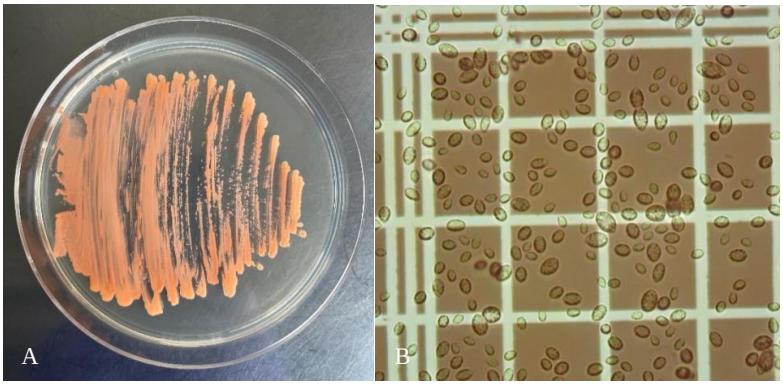

Fig. 2.1. *Phaffia rhodozyma* DSM 5626 (A) on a YM agar plate, (B) microscoping the sample at 400 × magnification.

Mutagenesis

Ethyl methanesulfonate (EMS, Sigma Aldrich) was used for *P. rhodozyma* DSM5626 cell mutagenesis at a concentration of 4 %. The target concentration of EMS that causes the death of 50–95 % of *P. rhodozyma* DSM 5626 cells was previously determined experimentally by Feldmane [55]. The EMS mutagenesis method was performed based on the literature with modifications [21], [55], [56]. Briefly, 48-hour inoculant cells were harvested, washed twice with 50 mM potassium phosphate buffer (pH 7.0), and then transferred to a 4 % EMS and 50 mM potassium phosphate buffer solution (EMS solution). Cells treated with EMS were incubated for 2 h at 22 °C on a rotary shaker at 250 rpm. After EMS was neutralised with 5 % sodium thiosulfate, treated cells were harvested and washed twice with potassium phosphate buffer and finally suspended with YM broth for night cultivation. The next day, cells were washed twice with potassium phosphate buffer to remove the YM medium. The cells were suspended with GSM medium, counted, and inoculated into the microplate.

Cell counting and growth determination

The following methods and equipment were used to determine the number of yeast cells and the growth dynamics:

- Cells were counted under a light microscope (micros Austria, MCX100LCD) with a 400 × magnification on a hemocytometer (Assistant, Neubauer improved, 0.1 mm depth camera) (Fig. 2.1. B).
- Culture optical density was measured at 600 nm in a microplate reader (TECAN Spark®) and processed with SparkControlMagellan^{TM} software.
- Cell dry weight was measured by the thermogravimetric method.
- The specific growth rate (μ) was calculated using the weight of dry biomass obtained during the exponential phase of yeast growth.

Determination of amino acid biosynthesis inhibitor concentration

Microorganism inhibition tests were performed at different concentrations of amino acid biosynthesis inhibitors, and mutant screening was performed in 48-well clear bottom microplates (SARSTEDT) in a microplate reader (Fig. 2.2. A) with an integrated cooling module (Laird Thermal Systems). The GSM medium was supplemented with 1.0×10^6 cells/mL of inoculum and an inhibitor at the appropriate concentration in triplicate. The concentrations of herbicides tested for growth inhibition ranged from 0.1–350 mM for glufosinate-ammonium, 0.1–50 mM for S-(2-aminoethyl)-L-cysteine, and 0.01–50 mM for methionine sulfoximine. The microplate was incubated for six days at 22 °C and 216 rpm in a humidity cassette (Fig. 2.2. B) to minimise drying of the medium. Additionally, to OD_{600} measurements, one sample from each triplet was microscoped, and its cell concentration was determined. The minimum concentrations of amino acid biosynthesis inhibitors causing complete inhibition of yeast growth were selected for further screening of mutants.

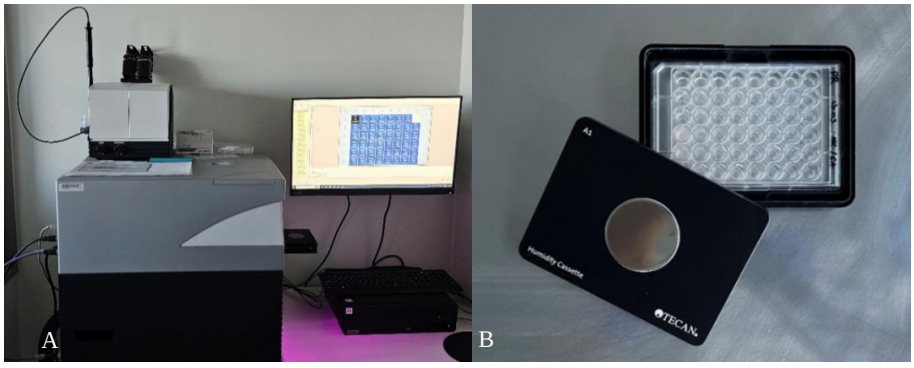

Fig. 2.2. A – Microplate reader (TECAN Spark®); B – 48-well microplate in humidity cassette.

Mutants screening

Mutagenised *P. rhodozyma* cells at a concentration of 2.0×10^6 cells/mL were inoculated into a 48-well microplate containing medium with appropriate inhibitor concentrations. Control

samples were a wild-type strain in a medium without an inhibitor, a wild-type strain in a medium with an inhibitor, and mutagenised cells in a medium without an inhibitor. The experiments were carried out at different times, the duration of 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 medium 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. 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 conditions

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 to 20 mL GSM medium 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 of GSM medium 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 medium 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.

2.3. Analytical methods for biomass evaluation

Determination of protein content

The bicycloninic acid (BCA) assay was used to determine protein concentration in the dry yeast biomass. The cell lysis method was based on a study [57] with significant modifications. The BCA test was performed with a BCA protein assay kit (Millipore, No. 71285-3). OD measurements were performed on a UV-Vis spectrophotometer (ThermoFisher, BioMateTM 160) using the “Protein BCA” programme.

Determination of amino acid profile

Identification and quantification of amino acids were done using high-performance liquid chromatography (HPLC). HPLC analysis was performed on the HPLC Shimadzu Nexera series using the YMC-Triart C18 column (150 mm × 3.0 mm, 3 µm) with a guard column of the same type in gradient mode. The chromatography conditions were done as follows [58]: 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 programme was 11 % B and 89 % A for 0–3 min, 22 % B and 78 % A for 12 min, 28 % B and 72 % A for 14 min, 30 % B and 70 %

A for 23 min, 65 % B and 35 % A for 27 min, 75 % B and 25 % A for 34 min, and 100 % B for 35 min. Fluorescence UV-Vis detection was performed on two channels: Ex. 350 nm and Em. 450 nm (0–29 min); for prolyne Ex. 266 nm and Em. 350 nm (29–35 min) [58].

Tryptophan was not determined in the study because it requires different chromatographic conditions (compared to other amino acids) and protein hydrolysis in an alkaline environment, since during acid hydrolysis (in a 6M HCI environment), it is destroyed [59], [60].

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 [28], [61]. An essential amino acid with an AAS below 100 % is considered limiting [62]. 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 [28], [61]. Protein quality is evaluated according to the following EAAI distribution: 70 % is low, 70 % to 89 % is medium, and above 90 % is high [61], [63]. The reference protein contains all the essential amino acids necessary to meet the requirements of a target animal, according to FAO [62].

Determination of astaxanthin content

The carotenoid extraction method was based on Luna-Flores et al. [21] with minor modifications. The contents in the biomass were determined by measuring the absorbance of the sample at wavelengths of 474 and 452 nm using a UV-Vis spectrophotometer (ThermoFisher, BioMateTM 160) “multiwavelength” program. A solution of hexane and ethyl acetate in a 1 : 1 ratio was used for a blank measurement. The amount of astaxanthin was calculated as described in [64].

Statistical analysis

The data presented 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. The astaxanthin concentrations, protein content, and amino acid profiles shown in the results and discussion chapter are based on samples collected on the 7^{th} day of cultivation. The results of the OD_{600} value were statistically analysed using a one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test. The results of the biomass, protein and astaxanthin values were statistically analysed using a one-way ANOVA followed by the Tukey 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 nonnormally distributed data and Pearson correlation analysis for normal data. Differences were considered statistically significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

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

Figures 3.1 and 3.2 represent the TOPSIS results of inhibitors selected for bacteria and fungi. The first three ranks are S-(2-aminoethyl)-L-cysteine, L-methionine sulfoximine, and glufosinate-ammonium for yeasts and fungi inhibition. This primacy may be because these inhibitors are leaders according to highly weighted criteria; they inhibit the largest amount of essential amino acids. S-(2-aminoethyl)-L-cysteine inhibits the biosynthesis of Met, Lys, Thr, Ile, and L-methionine sulfoximine and glufosinate-ammonium inhibit the biosynthesis of Arg, Lys, Met, Thr, Ile, and two non-essential amino acids. In addition, 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.

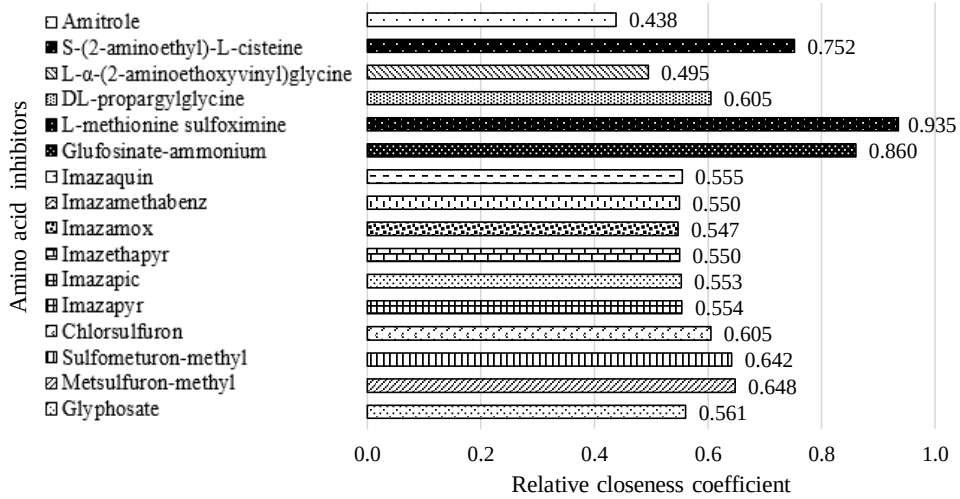

Fig. 3.1. TOPSIS results of amino acid biosynthesis inhibitors for bacteria.

The TOPSIS scores of sulfonylureas (metsulfuron-methyl, sulfometuron-methyl, chlorsulfuron) and glyphosate for fungi reached 0.533–0.581, which is significantly worse than the results of the above inhibitors. For bacteria, the results of sulfonylureas are slightly higher than those of imidazolinones, glyphosate, and DL-propargylglycine, corresponding to a unitary variation ratio. Therefore, using these inhibitors to inhibit bacteria to select mutants with increased synthesis of three EAAs, Ile, Leu, and Val, are more appropriate. Close to the worst solution was amitrole, which reached average values of 0.438 for bacteria and 0.462 for fungi.

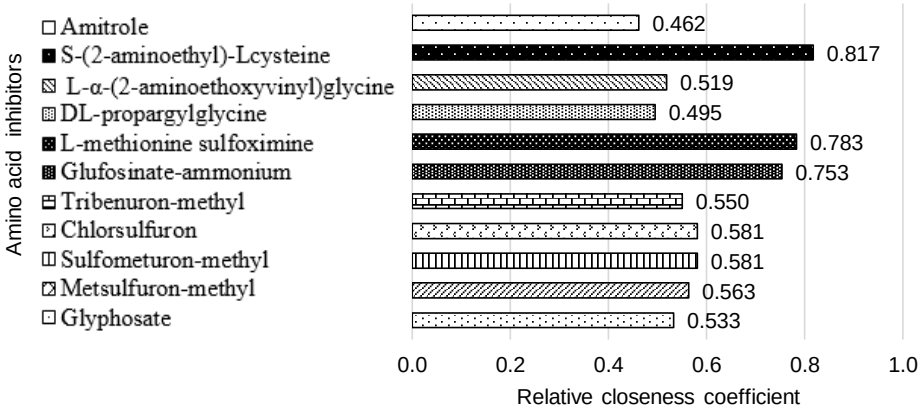

Fig. 3.2. TOPSIS results of amino acid biosynthesis inhibitors for fungi.

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 combinations of glyphosate + metsulfuron-methyl and glyphosate + DL-propargylglycine look more advantageous for selecting bacteria and fungi. Thus, combined inhibition would affect the biosynthesis of Phe, Trp, Tyr + Ile, Leu, Val, and Phe, Trp, Tyr + Met.

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

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

Seven concentrations from 0.1 to 50 mM of the amino acid biosynthesis inhibitor S-(2-aminoethyl)-L-cysteine (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 *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.

Table 3.1

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

Sample	AEC dose, 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	0.350	0.321
AEC3	2.5[c]	0.047	0.114	0.155	0.210	0.270	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	0.190	0.239	0.272

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

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 inhibitor concentrations 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 of each triplet (Fig. 3.3).

The number of yeast cells after 116 h of cultivation in the 0.1 mM AEC sample was more than twice that of the original sample, while 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 was observed by half with the presence of autolysed cell particles observed in samples with a concentration of 2.5 mM and higher. Spearman's correlation analysis showed a statistically significant correlation between the last reading of the OD and the counted cell amount ($p < 0.05$).

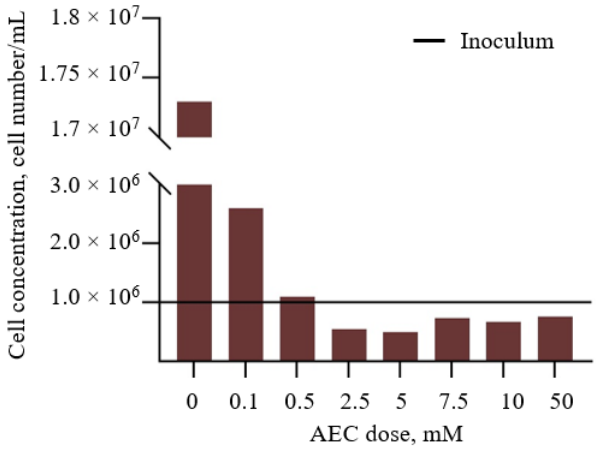

Fig. 3.3. Number of cells in samples containing different AEC concentrations after 116 h of cultivation. The horizontal line represents the initial concentration of cells in the samples.

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. Sano and Shiio (1970) reported a 100 % inhibition of *Bacillus subtilis* and *Escherichia coli* by adding 5 mM AEC to the medium, while 50 % inhibition of *Brevibacterium flavum* required 15 mM AEC [65].

Effect of glufosinate-ammonium on the *P. rhodozyma* growth

The glufosinate-ammonium (GA) inhibitor was tested twice. The first test consisted of 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 at the end of the test, the OD was higher than that of the control. Microscopy of

samples and counting cells revealed an interesting feature. The number of *P. rhodozyma* cells was higher in GA-containing samples 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 [66]–[70].

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 [71], [72]. In the present study, 100 mM GA had a complete inhibitory effect, and the number of cells decreased slightly compared to the amount inoculated.

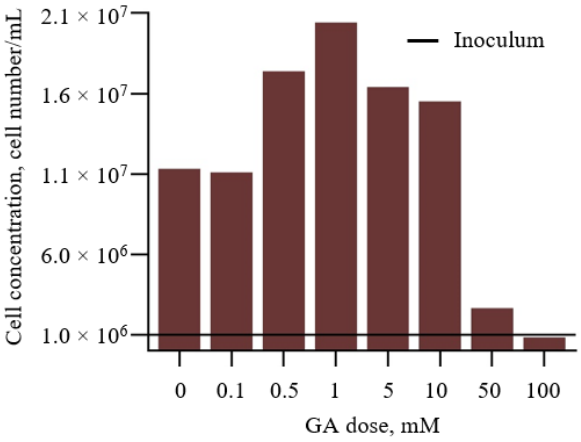

Fig. 3.4. Number of cells in samples containing different GA concentrations after 165 h of cultivation. The horizontal line represents the initial concentration of cells in the samples.

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 a

concentration of GA ranging from 50 mM to 350 mM inhibitory concentration, and a new test was performed with a concentration of GA ranging from 50 mM to 350 mM.

The second test did not reveal a statistically significant difference in OD among seven samples with concentrations from 50 mM to 350 mM 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.

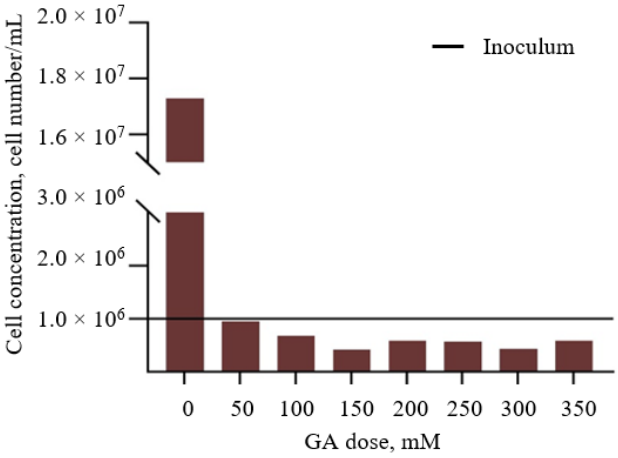

Fig. 3.5. Number of cells in samples containing different GA concentrations after 116 h of cultivation. The horizontal line represents the initial concentration of cells in the samples.

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 higher cause a 100 % inhibition of *P. rhodozyma* cells. This is confirmed by a decrease in the number of cells and the presence of autolyzed cell particles in the samples. The correlation between the final OD and the cells counted 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 approximately 100 % inhibitory doses.

Effect of L-methionine sulfoximine on the *P. rhodozyma* growth

Similarly to the GA inhibitor, MSO was tested twice. The first test included seven MSO concentrations from 0.1 mM 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 average value of the control sample was statistically significantly different from samples with the MSO inhibitor. At the same time, there was no statistical difference between inhibitor concentrations. The microscopy of the 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 doses to determine the minimal concentration for 100 % cell inhibition.

Both GA and MSO belong to the group of glutamine inhibitors [73]–[75]. GA is considered a more effective herbicide and is required in lower doses to inhibit microorganisms than MSO [72], [76]. For example, 0.05 mM MSO inhibits 50 % of *Mycobacterium tuberculosis* cells, while GA has the same effect at 0.0015 mM [72].

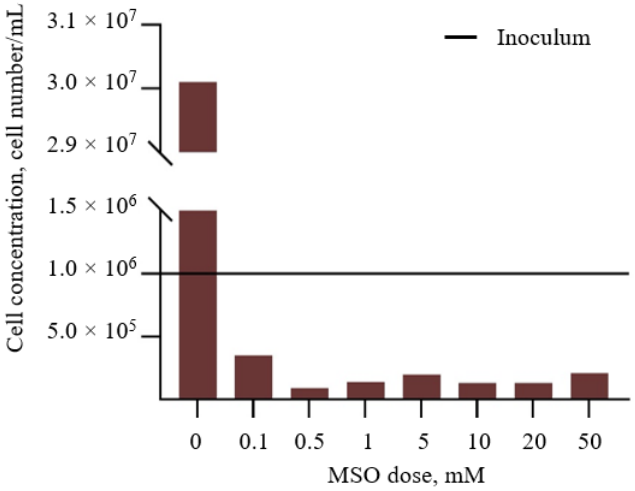

Fig. 3.6. Number of cells in samples containing different concentrations of MSO after 138 h of cultivation. The horizontal line represents the initial concentration of cells in the samples.

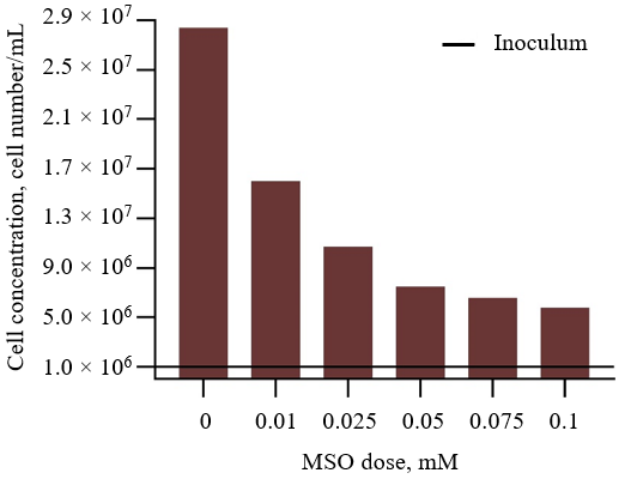

Fig. 3.7. Number of cells in samples containing different MSO concentrations after 97 h of cultivation. The horizontal line represents the initial concentration of cells in the samples.

This result is consistent with another study in which 2 mM MSO had no inhibitory effect on *A. niger* while 0.15 mM GA had significant inhibition [76]. It is interesting that in the present study, *P. rhodozyma* was more sensitive to the presence of MSO than to GA. Five concentrations from 0.01 mM to 0.1 mM were tested in the second MSO inhibition experiment

(Fig. 3.7). 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. 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).

3.3. Screening and analysis of selected mutants

Analysis of selected mutants cultivated on glycerine-salt medium

The first two experiments were performed in shaken flasks containing GSM medium. Experiments resulted in slow growth, 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 that *P. rhodozyma* DSM 5626 has a limited ability to use an inorganic nitrogen source – ammonium chloride. However, other nitrogen sources, such as ammonium sulphate and potassium nitrate, were less suitable than ammonium chloride for the cultivation of *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). Mutagenesis and repeated subcultivation of strains in GSM medium containing GA inhibitors did not affect astaxanthin biosynthesis.

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 row 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 performance of the wild-type strain. 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 a higher biomass or protein content relative to the wild-type strain and analyse them for the amino acid 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 amino acid profiles of the mutants for each test. Analysis of amino acid profiles showed a high Arg content in all strains tested, which is not typical for yeast. Wild-type *P. rhodozyma* contained 14.23–16.54 g of Arg per 100 g crude protein, and 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 yeast species [37], [49], [51]. Higher concentrations of Ile and Leu and lower concentrations of Lys were observed in all samples, which differed from the results in other studies [49], [51]. The content of non-essential amino acids such as glutamic acid (Glu/E) plus glutamine (Gln) and proline (Pro) in the *P. rhodozyma* protein was higher in our study. Presumably, such an atypical amino acid profile in both the wild-type strain and potential mutants is a consequence of cultivation in the GSM medium. The influence of a medium on the amino acid profile was reported in Ezekiel's study [77], where the Met content in *C. utilis* biomass varied depending on the type of substrate pretreatment. Therefore, further tests were performed on the glycerine-peptone-rich medium. It is assumed that the amino acid profile of yeast grown under favourable conditions may be more balanced.

Table 3.5

Amino Acid Profile of Pure Protein in GA Mutant Strains 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				

Table 3.5 continued

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 amino acid profile of pure protein showed that only the GA6/5-1 mutant strain has a significantly higher Val content than the wild-type strain (Table 3.5). The amino acid profile of the three GA mutant strains from the second subcultured microplate did not differ significantly from the wild-type strain.

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 mutant strains were selected from the third and first microplate subcultures. After the third subcultivation of the GA mutants on the microplate, growing a sufficient amount of inoculant to include GA7 mutants in the flask experiment was possible. Biomass and protein concentration increased significantly, while astaxanthin concentration decreased for all tested strains on glycerine-peptone-rich medium (Tables 3.6 and 3.9). The decrease in astaxanthin concentration in biomass grown in a rich nutrient medium under favourable conditions was expected. Improvement in carotenoid biosynthesis occurs under stressful conditions caused by previously used GSM medium [47]. The GA mutants showed no statistically significant differences in biomass, protein, astaxanthin, and total carotenoid values compared to the wild-type strain.

Table 3.6

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 amino acid profile of yeast strains grown in a GPM medium showed an improvement in the amino acid pattern. The Arg concentration in the protein of the wild-type and mutant strains decreased by more than two-fold and was consistent with another study [36]. On the contrary, the concentrations of other essential amino acids in protein increased. Three GA mutants showed significantly higher concentrations of Lys (GA6/3-3) and Met (GA6/4-3 and GA7/5-3) compared to the wild-type strain (Table 3.7). The three strains, GA6/1-3, GA6/2-3 (data not shown), and GA7/4-3 have no significant differences in amino acid profile compared to the wild-type strain.

Table 3.7

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. * The strains 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 amino acid 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 [37]. All mutants tested had lower amounts of Lys at 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 [37]. The Met content was also higher in *C. utilis* and *K. marxianus* than in *P. rhodozyma* and 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, commercial yeast

strains of SCP have a higher protein concentration in the biomass (Table 3.8). Thus, *P. rhodozyma* is inferior in amino acid content to an equivalent amount of biomass.

Table 3.8

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) [37].

According to the literature, GA has an inhibitory effect on the biosynthesis of seven amino acids [73]–[75], of which five are essential amino acids for fish. Interestingly, in the study by 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 synthesis compared to untreated cells [71]. At the same time, Phe, Trp, and Tyr are not targets for this inhibitor, which may indicate switching-on side effects in microorganism cells in the presence of the herbicide [78].

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 3.9). Several mutant strains showed significantly lower concentration values of biomass, protein, and astaxanthin. Therefore, as in previous tests, the amino acid profile was analysed for mutants with higher biomass and protein concentration.

Table 3.9

Comparison of Wild-Type *P. rhodozyma* Strain with AEC Mutants from the First and 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}

Table 3.9 continued

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 ($p < 0.05$).

The amino acid profile was analysed for five mutant strains: 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, and the AEC3/9-1 mutant had a significantly higher content of Phe, Ile, and Lys compared to the wild-type strain (Table 3.10). 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 than the wild-type strain. Two strains, AEC3/3-1 and AEC3/8-1, did not show significant differences in amino acid profile compared to wild-type strain (data not shown).

Table 3.10

Amino Acid Profile of *P. rhodozyma* AEC Mutant 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}	**5.43 ± 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}	**1.66 ± 0.05^{c}**
Trp	n/a	n/a	n/a	n/a
Phe	4.17 ± 0.05^{a}	**4.43 ± 0.05^{c}**	**4.35 ± 0.00^{bc}**	**4.40 ± 0.04^{c}**
Ile	4.88 ± 0.05^{a}	4.89 ± 0.05^{a}	4.91 ± 0.04^{a}	**5.27 ± 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}	**7.31 ± 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}	**7.26 ± 0.05^{b}**	6.83 ± 0.01^{a}	6.73 ± 0.08^{a}
Tyr	3.49 ± 0.03^{a}	**3.80 ± 0.06^{b}**	**3.72 ± 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 ($p < 0.05$).

The EAAI and AAS of His, Arg, Val, Phe, Ile, and Leu were higher in all *P. rhodozyma* strains compared to three commercial SCP strains (Table 3.11). The strain AEC3/9-1 with an improved Lys and Met content was the best strain of all mutants tested in this study. The AAS of Lys at 95.59 % is higher than previously reported in yeasts such as *Yarrowia lipolytica* [79], [80], *Cyberlindnera jadinii, Blastobotrys adeninivorans* [42], *Wickerhamomyces anomalus* [42], [81], *Saccharomyces cerevisiae* [37], [41], [79], *Candida utilis* [37], [82], *Kluyveromyces marxianus* [37], [80], and even fish meal of not the highest quality [83], [29]. Interestingly, AEC is an inhibitor of the biosynthesis of the four essential amino acids, Met, Lys, Thr and Ile [84]; however, in the present study, an increase in the biosynthesis of non-target amino acid biosynthesis inhibitors was observed.

Table 3.11

Comparison of AAS and EAAI 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) [37].

This study demonstrates the potential of amino acid biosynthesis inhibitors in the screening of protein-improved mutants. Using a selective medium reduced the number of mutants studied, allowing compact tests in flasks. Although the analysis of the mutant biomass did not show an increase in total protein, an increase in the synthesis of essential amino acids was achieved. The selection of mutagenized yeast cells on GA selective medium resulted in the selection of 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, AEC3/9-1, had a significantly improved synthesis of four essential amino acids for fish diets: Met, Phe, Ile, and Lys. The increase in synthesis of the first two limiting amino acids in yeast – Met and Lys – was especially valuable. This shows that amino acid biosynthesis inhibitors are a good tool for selecting improved protein-quality mutants. Thus, the hypothesis proposed in the Doctoral Thesis was confirmed.

CONCLUSIONS

1. The multi-criteria decision analysis identified three amino acid biosynthesis 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 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 both amino acid biosynthesis inhibitors 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, 8 % in Ile, and 6 % in Phe, 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*.

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