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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.

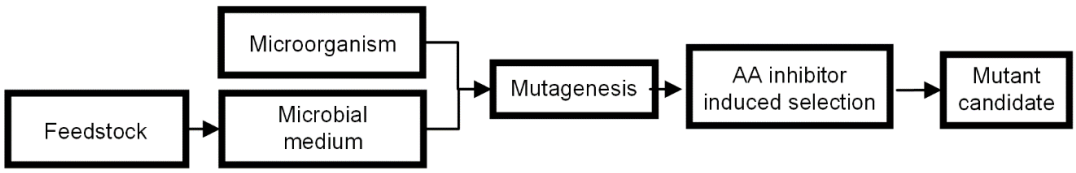

Figure 1. Scheme for the process of creating mutants.

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).

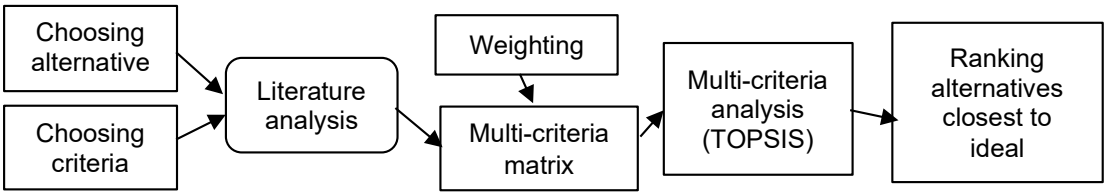

Figure 2. Methodologies algorithm.

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.

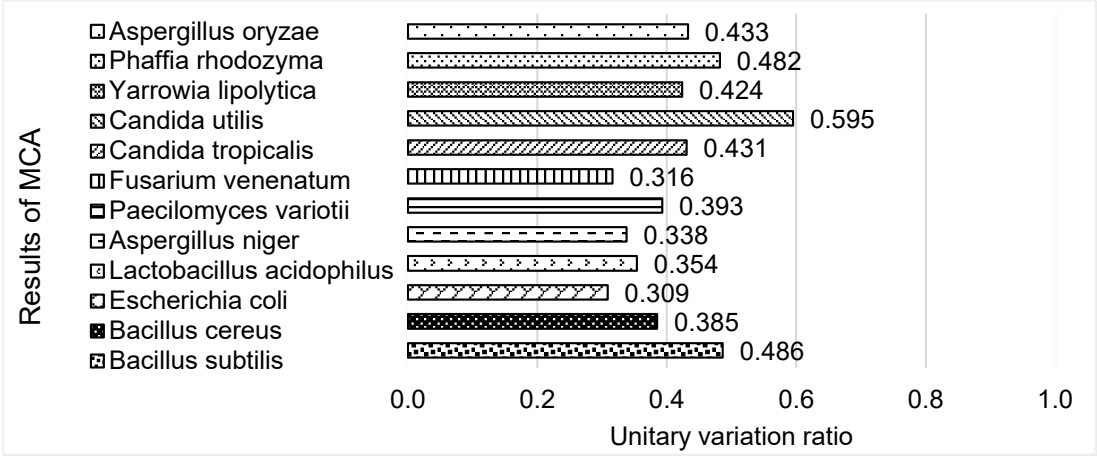

Figure 3. MCDA results of microorganisms.

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.

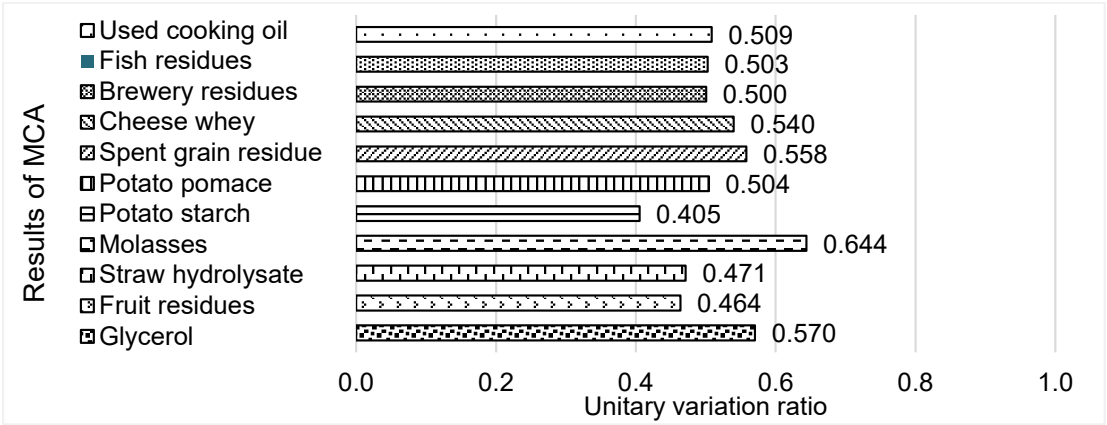

Figure 4. MCDA results of optimal feedstock.

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.

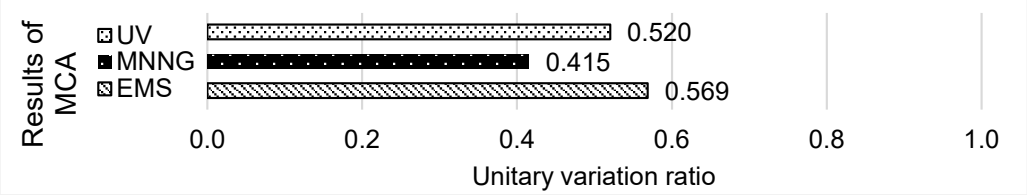

Figure 5. MCDA results of mutagenesis methods.

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.

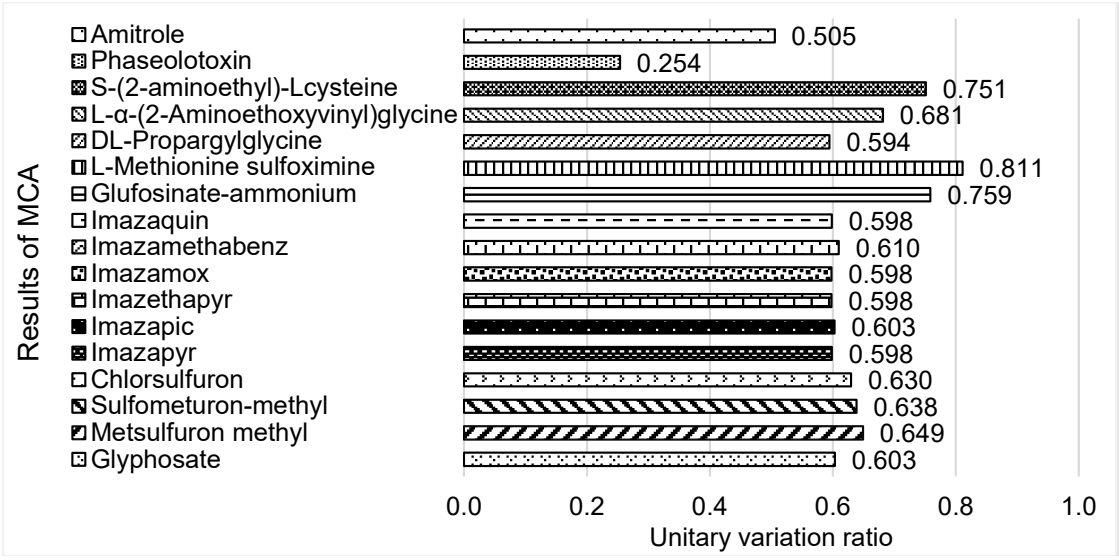

Figure 6. MCDA results of AA inhibitors for bacteria.

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.

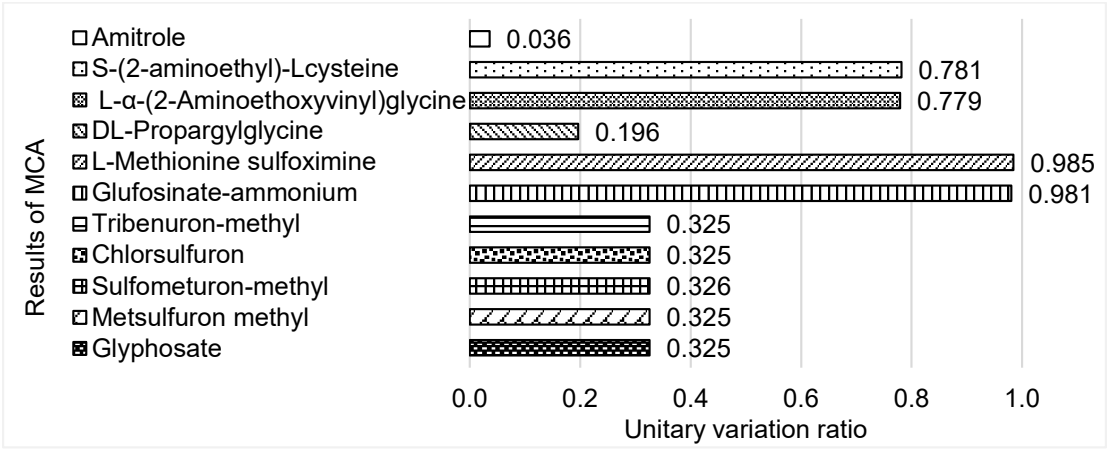

Figure 7. MCDA results of AA inhibitors for fungi.

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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