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

S. Raita, I. Berzina, Z, Kusnere*, M. Kalnins, I. Kuzmika and K. Spalvins

Riga Technical University, Institute of Energy Systems and Environment, Azenes street 12/1, LV 1048 Riga, Latvia
*Correspondence: zane.kusnere@rtu.lv

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

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

INTRODUCTION

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

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

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

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

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

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

EFFECT OF HERBICIDES ON THE GROWTH OF BACTERIA AND FUNGI

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

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

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

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

Glutamine AA inhibitors

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

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

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

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

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

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

Aromatic AA inhibitor

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

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

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

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

Aspartate-derived AA inhibitors

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

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

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

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

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

Branched-chain AA inhibitors

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

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

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

Table 4 (continued)

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

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

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

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

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

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

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

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

APPLICATION OF MUTAGENESIS AND AMINO ACID INHIBITORS

Mutagenesis

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

6. Wash the cells in potassium phosphate buffer twice.

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

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

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

Screening of mutants using AA inhibitors

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

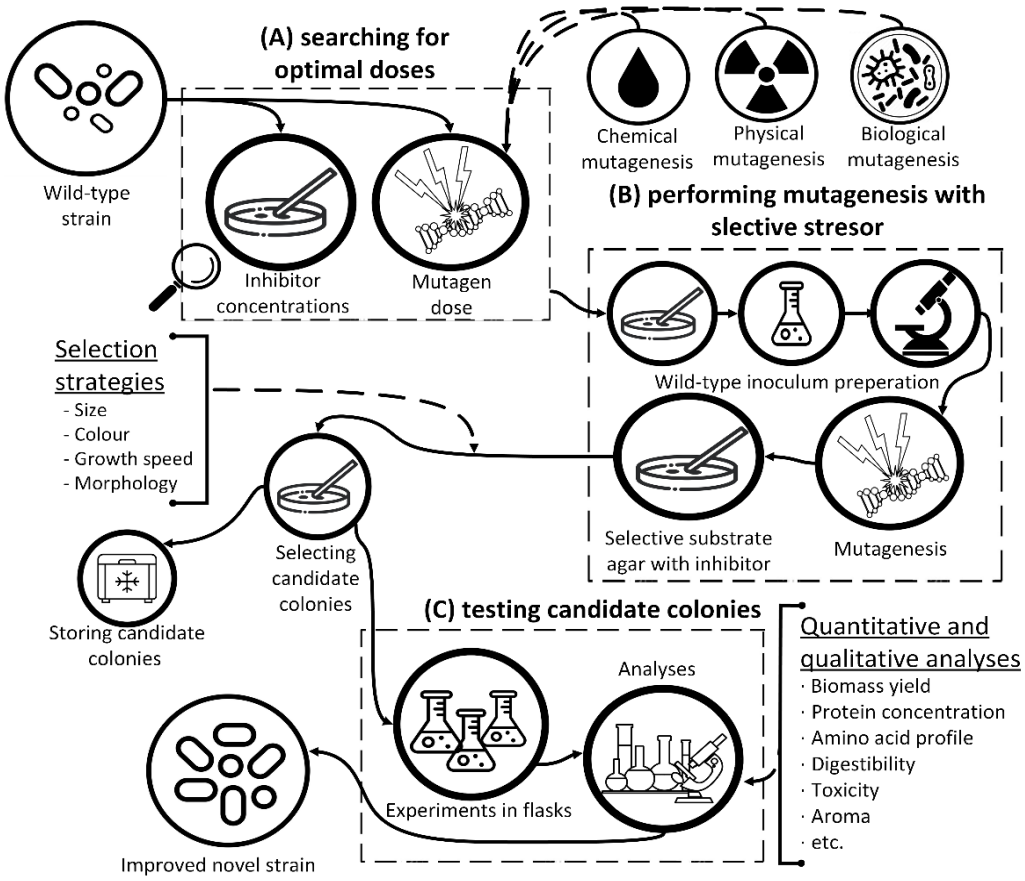

Figure 1. Process scheme for developing a novel mutant strain using mutagenesis and AA inhibitors.

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

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

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

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

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

Estimated effectiveness and safety of AA inhibitors

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

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

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

CONCLUSIONS

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

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

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

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