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

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

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

INTRODUCTION

Single cell protein as sustainable feed ingredient

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

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

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

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

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

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

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

Creation of improved SCP-producing strains via mutagenesis

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

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

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

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

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

AMINO ACID INHIBITORS

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

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

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

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

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

Table 1 continued

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

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

1. Aromatic amino acid inhibitors

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

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

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

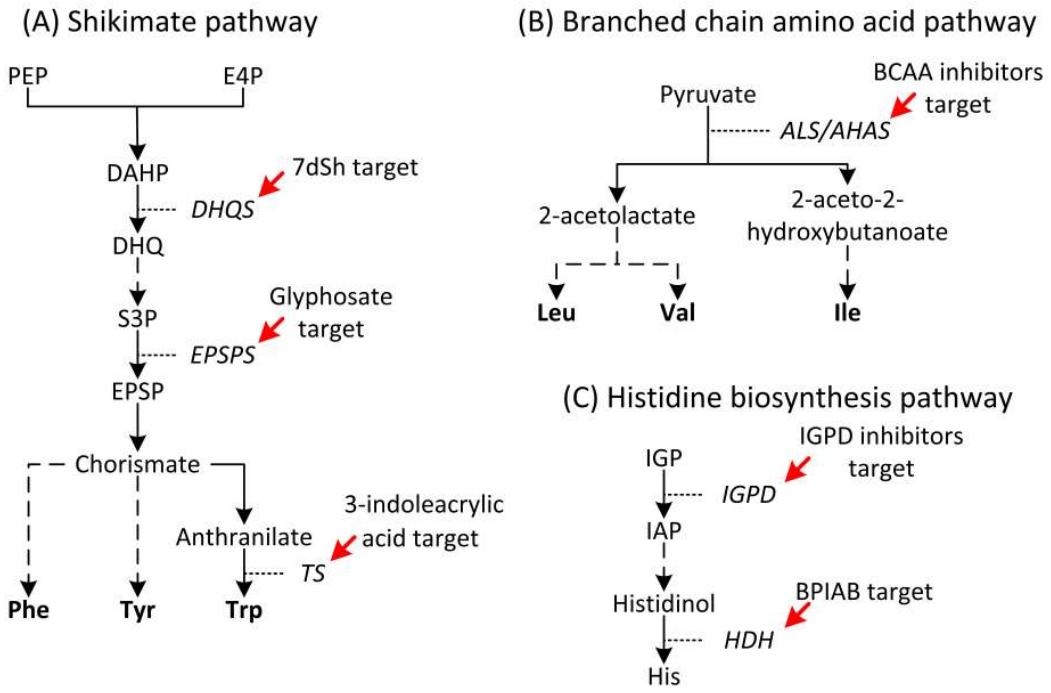

Figure 1. (A) Simplified shikimate pathway, (B) simplified branched chain amino acid biosynthesis pathway, (C) simplified histidine biosynthesis pathway. In shikimate pathway (A), through condensation of phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P), 3-deoxy-arabino heptulosonate 7-phosphate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

2. Branched chain amino acid inhibitors

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

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

3. Histidine inhibitors

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

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

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

4. Glutamine inhibitors

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

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

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

5. Glutamate-derived amino acid inhibitors

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

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

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

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

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

6. Aspartate-derived amino acid inhibitors

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

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

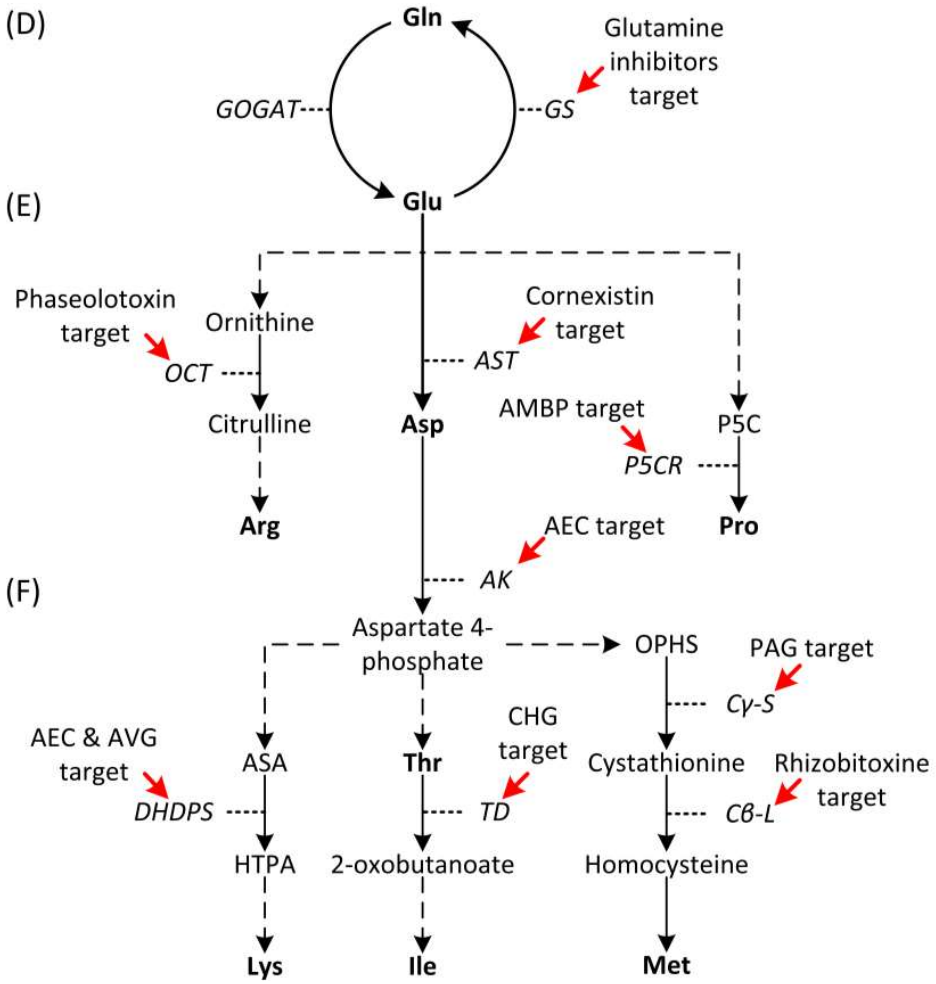

Figure 2. (D) Simplified glutamine biosynthesis pathway, (E) simplified glutamate-derived amino acid biosynthesis pathway, (F) simplified aspartate-derived amino acid biosynthesis pathway. In glutamine biosynthesis pathway (D), glutamine (gln) is converted to glutamate (glu) by glutamine oxoglutarate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

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

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

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

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

CONCLUSIONS

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

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

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

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

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

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

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

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

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