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Amino Acid Production and Role of Genetic Engineering Approaches to Strain Improvement for Enhanced Production: Review

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Abstract
Amino acids, both proteogenic and non-proteogenic amino acids, are produced at industrial scale for diverse applications. The industrial amino acids production methods include extraction, enzymatic, chemical synthesis and microbial fermentation methods. However, microbial fermentation method has been found to be economically feasible and convenient production scheme for production of L-forms of amino acid in natural and safe way. The fermentation method has also been augmented by improvement of producing microbial strains to meet rapidly growing need for amino acids. The advanced high throughput technique in genetic engineering enables development of efficient strains for amino acid overproduction. With this review the historical development of amino acid production, different amino acid production methods, and strain improvement techniques are well reviewed and the way forward are recommended.
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1. Introduction

Amino acids, proteinogenic amino acids, are building blocks of protein during translation of genomic information into protein. Every amino acid contains nitrogen in the form of amino group (-NH2), carbon as carboxyl group(-COOH) and R group or side chain covalently liked to a carbon atom so called an α-carbon. Optical activity is common to all amino acids except glycine and have asymmetric carbon resulting in two forms - "D" and "L" forms. Moreover, proteinogenic amino acids possess L-forms only and are also referred to as metabolically active amino acids (Ayon, 2020).

In addition to their role as protein building blocks in living organisms, amino acids are used industrially in numerous ways. The primary use of amino acid is flavor enhancer in the form of monosodium glutamate produced from large seaweed and now being produced from bacteria via fermentation. In addition, amino acids used therapeutically for nutritional and pharmaceutical purposes. To mention a few certain diseases are treated and controlled with single amino acids such as L-dihydroxyphenylalanine (L-dopa) for Parkinson disease, glutamine and histidine to hill peptic ulcers, and arginine citrulline and ornithine to treat liver diseases (Reddy, 2022).

Amino acids, thus, are produced in surplus quantity and being sold worldwide. The amino acid industry shares large part of world market attributing to their vital role in various industries. Since 1908 amino acids have been produced in large quantity and the demand increases alarmingly from time to time. The worldwide amino acid market worth $8 billion that surged to $20.4 billion in 2020. US take the lead in amino acid market accounting to 20% of the global share close to $1.6 billion in 2011. The report further indicate that globally amino acid production reached 4.5 million tons in 2011, 6.68 million ton in 2014 and estimated to be 10 million ton in 2022 with a growth rate of 5.6%. The growing demand for amino acids includes markets for animal feed, health foods, pharmaceutical precursors, dietary supplements, artificial sweeteners and cosmetics. Among them, the animal feed supplement segment (involving L-lysine, DL-methionine, L-threonine and L-tryptophan) constitutes the largest share (56%) of the total amino acid market and it is expected to keep fueling market growth in the coming years. The annual demand for feed-grade amino acids globally is about 2.4 million tons with an estimated value of $6 × 10^9. Furthermore, there is strong commercial interest in developing new amino acid applications (Sanchez *et al*., 2017).

Moreover, production methods have shown remarkable revolution as the demand for amino acid increased since 1908. Several research findings and technology advancement has been reported in amino acid production since the discovery and large production of glutamic acid. This review, therefore, will provide information on historical development of amino acids production, research on amino acid producing microbes and the basic principle and advancement in strain improvement are reviewed from available research reports.

2. History of amino acid production

Amino acid production was started, when Professor Ikeda Kikunae purified the component of kelp responsible for a unique test of Japanese special dish, Kombu. While attending training in Germany at the center of organic chemistry, Professor Ikeda shared the aspiration with a German colleague mass production of a cheap source of nutrition. Justus von Liebig, the founder of the field, was renowned for his beef extract, which fed German armies— and incidentally made the inventor fabulously wealthy. Ikeda later recalled that he had received inspiration from an article by Japanese doctor Miyake Hide claiming that flavor facilitated digestion (Sand, 2005).

Since his childhood, Ikeda experienced a unique taste distinct from the four basic taste in kombu and other foods such as meat. He coined the term umami to name this putative taste. In 1907 he began a project to identify the responsible chemical substance that imparts umami test. The seaweed kelp was the focus of the research as to large quantities of kelp (*Laminaria japonica*), termed konbu, are harvested and consumed as food. Therefore, it was possible for Ikeda to find enough kelp for extraction of the substance and chemically identify. Ikeda used dried kelp to get rid of protein impurities by denaturing kelp protein through drying and are not extracted by water. The method Ikeda followed was water extraction and most of the mannitol and NaCl in the liquid was removed by crystallization making umami taste generating materials remain in the extract liquid. He suspected that the umami material to be salt of organic acid. Hence, he focused in separating the organic acid in consecutive steps. After repeated treatment he found organic salt precipitate with acid and salt, Ikeda reached to substance of molecular formula $C_5H_9NO_4$ and recognized to be glutamic acid. Even though glutamic acid was formerly described, it was not recognized by the scientists as a responsible for unique taste. This is because the taste arises when glutamate forms a sodium salt as monosodium glutamate (Kurihara, 2009).

After the identification of active taste substance in kombu from kelp as monosodium glutamate, Ikeda appealed a patent right as a new seasoning agent. Later on, a known entrepreneur Saburousuke Suzuki in the chemical and pharmaceutical industry agreed to commercialize the new seasoning agent with trade name of AJI-No-MOTO in 1909 (Sano, 2009).

Following the finding of active ingredient of kelp as flavor additive, amino acid production and need has been increased. And to meet the growing demand of monosodium glutamate and other amino acid, various production methods have been developed. In the first commercial production process for MSG in 1909, L-glutamic acid was separated as hydrochloride from wheat gluten hydrolyzed with hydrochloric acid. Production of MSG by isolation from protein hydrolyzate continued for some 50 years. In 1955, the microbial production of L-glutamic acid from simple materials such as glucose and ammonia were developed in Japan. Since then, this fermentation method has been applied to the production of a variety of amino acids. At the same time, methods of synthesis and optical resolution were also studied, and the synthetic production of glutamic acid were launched in 1960. Meanwhile, other amino acids such as DL-methionhe, glycine, and L-tryptophan are also manufactured in this way (Izumi *et al*., 1978).

Currently, amino acid become a multibillion-dollar business and a lot of countries and companies are involved in amino acid market. Some companies are major players in the amino acid production industry. Among them are Ajinomoto, Archer Daniels Midland, Cargill Inc., Daesang Corporation, Evonik Industries AG, Kyowa Hakko Kogyo, Nippon Soda, Prinova U.S. LLC, Royal DSM, Showa Denko KK and Zhejiang Chemicals. Small-scale participants include Iris Biotech GmbH, Nanjing Liang Chemical, Sunrise Nutrachem Group, Tokyo Chemical Industry, Novus International Inc., Anaspec Inc., CJ Cheil Jedang Corporation and Adisseo France SAS (Sanchez *et al*., 2017).

3. Methods of amino acid production

3.1. Extraction

The first industrial production process was an extraction method by which amino acids are extracted from different sources. The process is based on the hydrolysis of protein sources using acid. The protein sources hair, feature, nail and blood are treated with acid in order to denature the protein and amino acids are extracted. The method is not widely used as to it depends on the availability of resources. Nevertheless, some amino acids cysteine and cystine are still produced by extraction method (Ivanov *et al*., 2013).

The method was first proposed by Ikeda using wheat gluten as the source of L-glutamate because it has the highest content of L-glutamine among industrially available raw materials. L-Glutamine becomes L-glutamic acid after protein hydrolysis; the total L-glutamate content (L-glutamate + L-glutamine) of hydrolyzed wheat gluten is 30 g/100 g protein. Gluten was first separated from wheat flour by washing the starch from dough. The resulting crude gluten was transferred to pottery vessels (termed Domyoji-game), combined with hydrochloric acid, and heated for 20 h. A variety of vessels were tested, and old-fashioned Domyoji-game proved to be the most resistant to hydrochloric acid and heat. The protein hydrolysate was filtered to eliminate a black residue (termed humus) that resulted from the reaction of amino acids with carbohydrates and was then returned to the Domyoji-game to be concentrated for 24 h. This concentrate was transferred to another Domyoji-game and

stored for 1 month to allow the L-glutamic acid hydrochloride salt to crystallize (Sano, 2009).

Some researchers have tried to extract amino acids from fish products. Yoshida, *et al*. (1999) extracted cystine, alanine, glycine, leucine and Pyroglutamic acid from fish meat. Although the production of amino acid by acid or alkaline hydrolysis of potential source is possible, the process may result in partial hydrolysis or racemization of some amino acids, including the essential ones (Widyarani *et al*., 2016).

3.2. Chemical synthesis method

As it is described by Ault (2004) before World War II there was no chemical synthesis that could compete with the extraction methods. After the War, the discovery of the oxo reaction, and its application to acrylonitrile, available from either acetylene plus HCN or from propylene by oxidation in the presence of ammonia, made possible the synthesis of β-cyanopropionaldehyde, the key intermediate for the synthesis of L-glutamic acid.

β-Cyanopropionaldehyde was then converted to L-glutamic acid by the Strecker process in which the aldehyde is converted to the amino analog of a cyanohydrin, which is then hydrolyzed to L-glutamic acid (Iwanaga *et al.*, 1961).

Moreover, it is reported by Ivanov *et al*. (2013) that Glycine and L-alanine are produced by chemical synthesis by series of chemical reaction, Strecker amino acid synthesis, an amino acid is synthesized from an aldehyde (or ketone). The process starts with condensing ammonium chloride in the presence of potassium cyanide, resulting in an α-aminonitrile followed by hydrolyzed to form amino acid. The original Strecker reaction combines acetaldehyde, ammonia, and hydrogen cyanide to give alanine after hydrolysis as shown in figure 1.

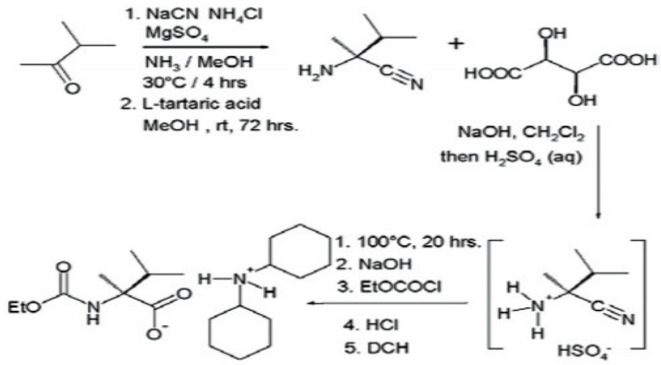

Figure 1 The Strecker synthesis is the industrial chemical synthesis of an L-valine derivative starting from 3-methyl-2-butanone (Ivanov et al., 2014).

According to Mueller and Huebner (2003) and Okafor (2007) the chemical synthesis method of amino acid is not feasible for industrial production since it results in racemic forms of amino acids and it is expensive to purify racemic mixture. An additional step involving the use of an immobilized enzyme, aminoacylase, produced by *Aspergillus niger*, is required to obtain the biologically active L-form. Ault (2004) mentioned that chemical synthesis is widely applied to produce alanine, glycine, methionine, phenylalanine, threonine, tryptophan and valine.

3.3. Enzymatic method

The core of fermentation process in producing amino acids is the metabolic pathways catalyzed by enzymes in the producing strains. Now, it is possible to synthesize amino acid using microbial enzymes from appropriate intermediates. The production of alanine, for example as summarized by Izumi *et al*. (1978), can be produced by suspension of *Escherichia coli*. At industrial scale L-alanine is produced from L- asparatic using L-aspartate 0-decarboxylase of *Pseudomonas dacunhae* cells as it is illustrated by figure2.

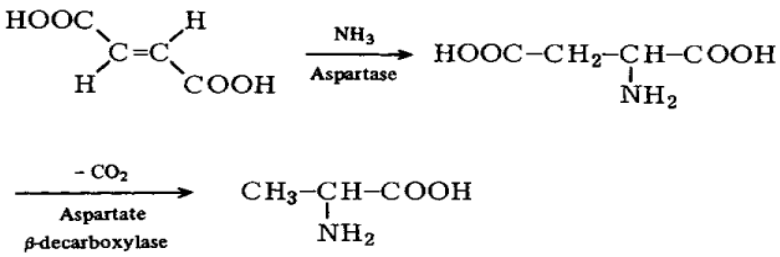

Figure 2 Enzymatic formation of alanine from fumaric acid via aspartic acid (Izumi et al., 1978)

The enzymatic method uses pure enzymes to convert precursor substance into amino acids. This bypasses the long fermentation process and reduce production costs. The production of amino acid by enzymatic conversion of substances started 40 years ago in Japan by the separation of D and L form by immobilized acylase. Amino acids including alanine, aspartic acid, cysteine, cystine, methionine, phenylalanine, serine, tryptophan and valine are also produced by enzymatic method. Enzymatic production of amino acids results in L-form of amino acids and thus avoid the expensive purification step in chemical method (Ivanov *et al*., 2013).

The enzymatic method has now been supplanted by a continuous microbiological process in which the reacting solution passes over a fixed bed of an immobilized microorganism (Ault, 2004). Rodríguez-Alonso *et al*. (2017) produced optically active L-amino acid through double-racemase hydantoinase process. The process is a system of four enzymes used to produce optically pure L-amino acids from a racemic mixture of hydantoins. The first enzyme, D,L-hydantoinase, preferably hydrolyzes D-hydantoins from D,L-hydantoins to N-carbamoyl-D-amino acids. The remaining L-hydantoins are racemized by the second enzyme, hydantoin racemase, and continue supplying substrate D-hydantoins to the first enzyme. N-carbamoyl-D-amino acid is racemized in turn to N-carbamoyl-L-amino acid by the third enzyme, carbamoyl racemase. Finally, the N-carbamoyl-L-amino acid is transformed to L-amino acid by the fourth enzyme, L-carbamoylase. Therefore, the product of one enzyme is the substrate of another. Perfect coordination of the four activities is necessary to avoid the accumulation of reaction intermediates and to achieve an adequate rate for commercial purposes.

3.4. Microbial fermentation

The last way of amino acid production is biological process such as enzymatic and fermentation. The former is mediated by enzymatically catalyzed process by the action of an enzyme to catalyzed the formation of the desired amino acid. Numerous enzymes have been applied to synthesis the required amino acid like hydrolytic enzymes, ammonia lyases, NAD+-dependent L-amino acid dehydrogenases. The sources of these enzymes are microorganisms such as *Escherichia coli*, *Saccharomyces cerevisiae*, *Pseudomonas dacunhae, Crypotococcus lurendi* (D'Este *et al.*, 2018).

Amino acids are among primary metabolites and are supposed to be utilized by cells to make proteins and as sources of energy. In addition, unlike secondary metabolites amino acids are thought to be unable to be excreted to external environment. Hence, it was considered impossible to ferment amino acid using microorganisms. Later on, in 1957 Kinoshita and colleagues successfully isolated from soil, sewage soil, sewage and animal feces bacterial strain, *Corynebacterium glutamicum*. The discovery of the strain became a paradigm shift in amino acid production through fermentation. Besides, it was more valuable that wild strain could be used in large scale production of amino acid under optimized fermentation conditions. The fermentation process commenced by sterilizing fermentation tank which is filled with appropriate substrate containing suitable carbon, nitrogen sulfur, phosphorus and other trace elements. Then, the substrate is inoculated with producing strain. The tank is stirred under specified conditions (temperature, pH, aeration). The suitable recovery technique shall be applied to obtain the amino acid of interest in the required form (Leuchtenberger *et al*., 2005).

Currently, the most widely applied and economically viable method of amino acid production is fermentation. The microorganisms are provided with growth substrate rich in adequate carbon, nitrogen and growth factor. Fermentation can be carried out under aerobic or anaerobic condition by which the substrate is converted in to a diverse array of amino acid by the producing strain. The process of fermentation is advantageous over other methods because it produces L-form of amino acids and bypass additional purification steps, it can be conducted at conditions enabling the amino acids to sustain active forms and omit maintenance costs. However, fermentation method has inherent disadvantage: sterilization and energy source for oxygen transfer to maintain aerobic conditions, agitation to mix substrates significantly increased capital and production costs. In addition, establishment cost is further escalated by bigger reactor required for fermentation at large scale production (D'Este *et al*., 2018).

Generally, amino acid production investment depends on the method of production which determined by analyzing several criteria such as available technologies and capital investments, product market size and revenues, raw materials and operation costs as well as environmental impact of each specific process. However, mainly due to economic and environmental advantages, fermentation is the most used process at industrial scale (Ikeda, 2003). Nevertheless, the fermentation method is further upgraded by improving the effectiveness of producing strain through high-tech genetic engineering techniques and the capability of strains in growing on cheap substrates (wastes and agro-industrial byproducts) makes fermentation method remain the best production scheme in amino acid business.

4. Strain improvement and amino acid production

The fermentation method was first recognized by the isolation of soil bacterium *Corynebacterium glutamicum* by Kinoshita *et al*. in 1957. The bacterium was able to excrete remarkable amount of L-glutamine while growing on sugar and ammonia. Later on, *C. glutamicum* produced a large amount of L-lysine by fermentation and other

amino acids include L-valine, L-isoleucine, L-threonine, L-aspartic acid and L-alanine (Ivanov *et al*., 2013).
The wild type microbes are less efficient in producing commercially important metabolites. The metabolic feature of wild microbes is not suited for the environment at which metabolites are produced in surplus amount. This urges improvement of the gens that control metabolic and genetic machineries. Thus, in order to improve the wild strains, there must be means to alter the gen composition responsible for metabolic pathways for excessive production of metabolites of interest. There are situations where enzymatic structural change is required which enhances its catalytic activity. In contrast, there will be opportunities of changing particular region of genome that can result in deregulation of gene and metabolite overproduction (Sanghavi *et al*., 2020).
The usual method to design amino acid producing strain are well reviewed by Hirasawa and Shimizu (2016) and are: (i) amplification of biosynthesis pathway enzymes for the target amino acids, (ii) reduction of byproducts formation, (iii) release of feedback regulation of key enzymes by the target amino acid, (iv) increased supply of reducing equivalents such as NADPH, (v) reduction of metabolic fluxes to the TCA cycle, because most target amino acids are produced from intermediate metabolites in the glycolysis and the pentose phosphate pathways, and (vi) increased export of target amino acids out of the cells (Figure 3).

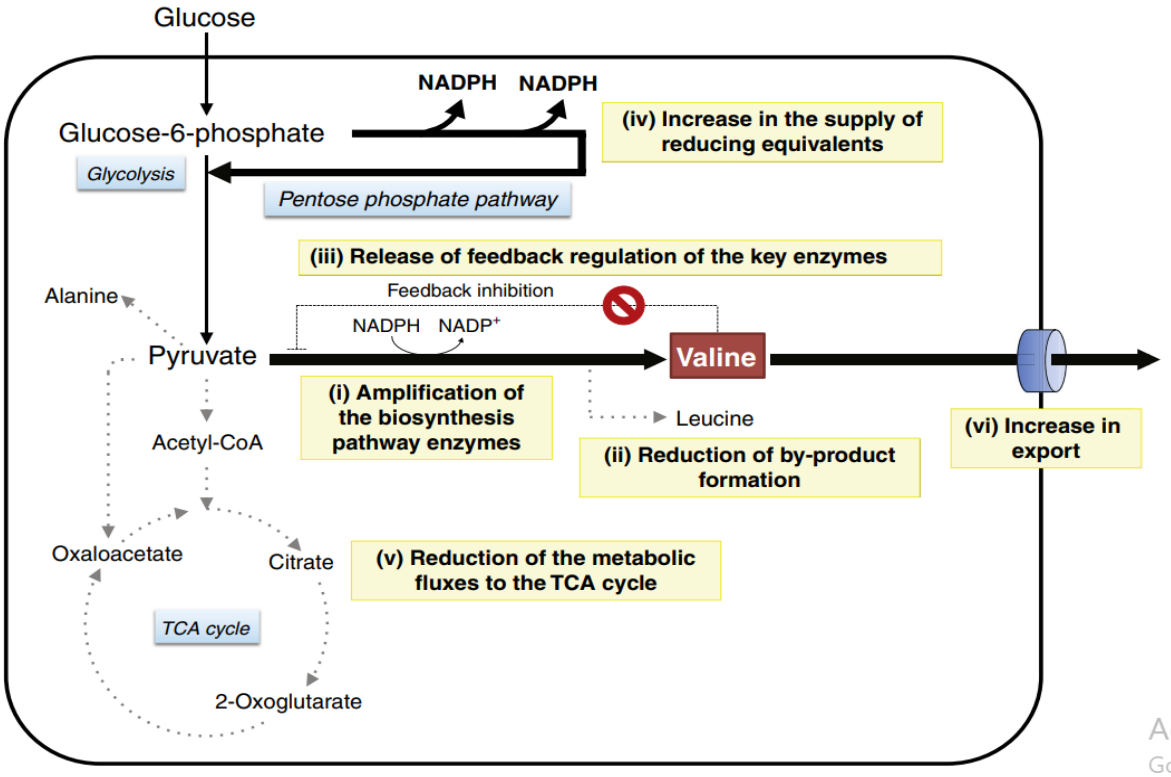

Figure 3 Common metabolic engineering strategies for amino acid production. A summary of strategies used for breeding host microbial cells for valine production is given as an example. In the case of arginine production, strategy (v) should not be adopted because this amino acid is produced from 2-oxoglutarate, an intermediate metabolite of the TCA cycle (Hirasawa and Shimizu, 2016).

According to Sanghavi *et al*. (2020) strain improvement for higher production of desired metabolites helps to design strain with properties of: I)Proficient assimilation of low-cost and complex raw materials, ii) Removal of byproducts and change in the product ratios, iii) Overproduction and excretion of native and foreign products, iv) Short duration of fermentation time and easy scale-up, v) Tolerance of various metabolites produced and vi) Provide morphological changes in cell which is suitable for product separation. As it is described in figure 4 there are five strain improvement methods: mutagenesis, genetic engineering, metabolic engineering, omics and physiological engineering.

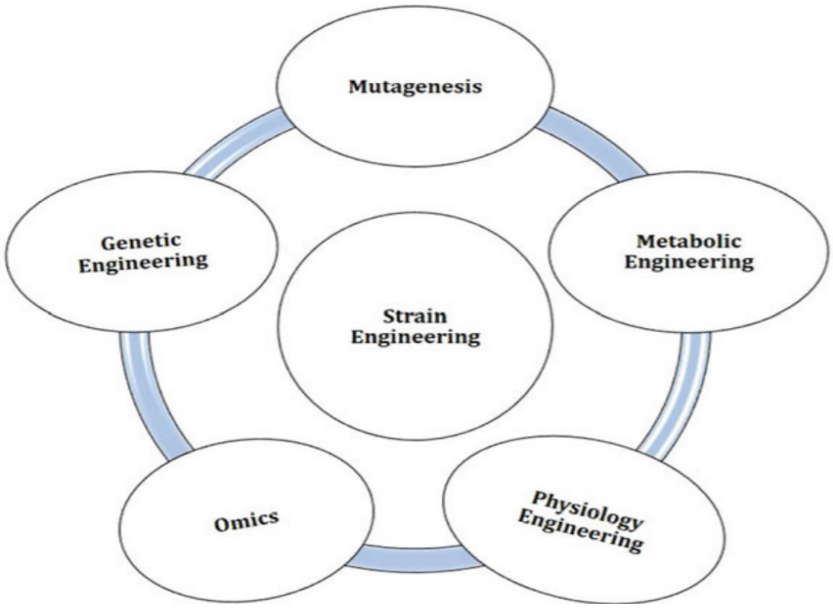

Figure 4 Approaches of strain engineering (Sanghavi et al, 2020)

4.1. Mutagenesis

So far, random mutation has been widely applied in strain improvement to obtain efficient amino acid producing microorganisms. According to Ivanov *et al*. (2013) the mutants are differentiated by increased membrane permeability, regulation defects, or biosynthesis enzymes with altered kinetic characteristics. Moreover, it is vital to understand the microbial amino acid metabolisms to boost amino acid productivity and exhaustive analysis permits the quantification of the flow of metabolites in a fermentation process as a function of time (metabolic flux analysis). In this way the addition of nutrients can be optimized, and yields can be increased as a result. Metabolic flux analysis also makes it possible to model the metabolism of a given production strain (metabolic modeling).

Using mutagenesis strain improvement requires understanding of underlying metabolic pathways and genomic content of the microbe under study and it is very important to have robust technique and plan. Choosing appropriate technique depends on amount of time it takes, cost, and need of skill labor. Physical and chemical mutagenesis are among the common approaches for strain improvement. The former method includes treating the cells with ionizing and non-ionizing radiation. The physical mutagenesis can either be indirect or direct. The direct effect involves the direct ionizing of DNA strands owing to ejection or excitation of electrons to a higher energy level resulting in the formation of reactive oxygen species. The reactive oxygen species bring about nitrogen base modification and/or single/double-stranded breaks in the DNA causing deletions and translocations (Morita *et al*. 2009). In some cases, point mutation can arise owing to single-strand breaks (SSBs), or due to nitrogen base switchover for example, the conversion of pyrimidine bases to 5-(hydroxymethyl) uracil, 5-formyluracil, 5-hydroxycytosine, and 5-hydroxyuracil (Min *et al*. 2003).

Chemical mutagenesis is mainly based on chemical agents, also known as chemical mutagens, such as base analogs, deaminating agents, alkylating agents, and intercalating agents. The treatment of cells with chemical mutagens results in either site specific mutation or change DNA structure/or its sequence. The chemical mutans are of two types: base analogs and DNA intercalating agents. A base analog can replace a DNA base during replication and can result in transition mutations. Whereas, intercalating agents are molecules that may get inserted in between DNA bases, resulting in frameshift mutation during replication. Other chemical mutagens may act by the generation of reactive oxygen species (ROS), deamination and alkylation (Sanghavi *et al*., 2020).

Over all, Shin and Lee (2014) explained that random mutation has inherent disadvantage in developing unwanted genetic change in microorganisms being mutated. Unfortunately, either of induced or spontaneous mutations are of little use as the exact mechanism of mutation needs to be thoroughly understood for its repetitive and specific usage. Thus, to manipulate specific gene that to makes cell to synthesis interest of amino acid and avoid risk of unwanted genetic change a new technique has been developed, metabolic engineering. This emerging approach enable to form cellular factories not only for amino acid but also bio-fuels, pharmaceuticals, bio-plastics, platform chemicals and even silk proteins.

4.2. Metabolic engineering

The production of amino acid through microbial fermentation needs controlling metabolic pathways for over

production of amino acids. For this reason, metabolic engineering of producer microbes focuses on manipulating central metabolic pathways and branch pathways in order to eliminate feedback inhibition and competing disrupting pathways. However, metabolic pathways responsible for biosynthesis of specific product (amino acid) are affected by other several factors: regulatory proteins, by-product formation, and other related biosynthesis pathways (Liu *et al*., 2019).

Metabolic engineering aimed at removing certain enzymes that hinders the formation of desired product or by products/co-products which inhibits catalytic activity of enzymes in the metabolic pathways. Liu *et al*. (2022) summarized the metabolic engineering of the native strain *A. oxydans HAP*-1 for industrial production of L-alanine. The strain produces racemic mixture of LD-alanine while only L-form is required in the market. The gene responsible for racemase was inactivated and the yield for production of L-alanine was remarkably boosted. Similarly, the review indicates the co-product formation extremely hamper L-alanine production which was addressed by deletion of genes related to the competitive metabolic pathways. Jojima *et al*. (2010) genetically engineered *Corynebacterium glutamicum* by inactivating genes associated with production of organic acids in *C. glutamicum* to direct carbon flux from organic acids to alanine.

Likewise, *E. coli* was metabolically engineered for fermentative production of L-trypthophan as reviewed by Liu *et al*. (2019). In particular, metabolic pathway engineering allows enhanced product titers by inactivating/blocking the competing pathways, increasing the intracellular level of essential precursors, and overexpressing rate-limiting enzymatic steps.

Although metabolic engineering technique replace random mutation to modify strain for better production, it suffers from limitation of being unable to consider the whole metabolic process, only focus few gens/ metabolic pathways. The systems metabolic engineering, another emerging approach, is a multidisciplinary area that integrates systems biology, synthetic biology and evolutionary biology. It is an efficient approach for strain improvement and process optimization, and has been successfully applied in the microbial production of various chemicals including amino acids. Systems metabolic engineering strategies including pathway focused approaches, systems biology-based approaches, evolutionary approaches and their applications in two major amino acid producing microorganisms: *Corynebacterium glutamicum* and *Escherichia coli* (Ma *et al*., 2017).

According to Lee *et al*. (2011) system metabolic engineering apply genome, transcriptome, proteome, metabolome, and fluxome, together with gene regulatory and signaling information for understanding of cellular and metabolic characteristics of microorganisms. This helps to identify and manipulate specific genes. System metabolic engineering has two strategies, as reviewed by Ma *et al*. (2017) based on the situation either the target gene is identified or remain unknown. The first strategy is the rational intuitive which covers typical metabolic engineering process of the synthetic pathway of a certain product, that from the uptake of carbon source, elimination of byproducts, enrichment of precursors, to the reconstruction of related metabolic pathways, supply of cofactor when the target gene is identified well. The second strategy focuses mainly on omics-based metabolic engineering techniques and various evolution approaches when no obvious target genes are known Figure 5.

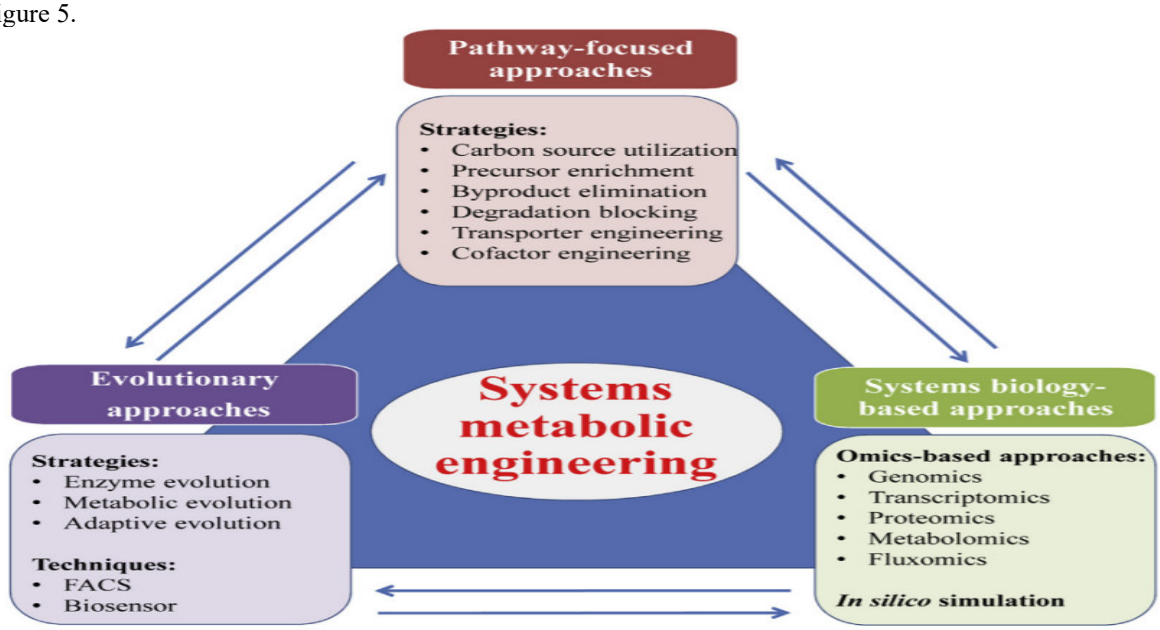

Figure 5 The constitution and strategies of systems metabolic engineering (Ma et al., 2017).

Pathway focused approach of metabolic engineering enable microbes to overproduce the required metabolite by combined metabolic engineering tools including: increased expression of enzymes and effective

utilization of carbon sources. In addition, avoiding feedback inhibition, transcriptional attenuation, and blocking bypass pathway are simultaneously employed in strain improvement. Meiswinkel *et al*. (2012) successfully transferred and expressed xylose isomerase genes and xylulokinase genes in combination in *Corynebacterium glutamicum* for better production of glutamate, lysine and ornithine and for effective utilization of carbon source. For enhanced production of amino acid elimination of competent pathways, byproduct formation and controlling feedback inhibition in metabolic pathway is crucial. Qin *et al*. (2014) developed a strain from *Corynebacterium glutamicum* strain ATCC13032 to produce L-methionine. They employed metabolic engineering that involve: (i) deletion of the gene *thrB* encoding homoserine kinase to increase the precursor supply, (ii) deletion of the gene *mcbR* encoding the regulator *McbR* to release the transcriptional repression to various genes in the L-methionine biosynthetic pathway, (iii) overexpression of the gene *lysCm* encoding feedback-resistant aspartate kinase and the gene *homm* encoding feedback-resistant homoserine dehydrogenase to further increase the precursor supply, and (iv) overexpression of the gene cluster *brnF* and *brnE* encoding the export protein complex *BrnFE* to increase extracellular L-methionine concentration.

Evolutionary approach of strain improvement is directly linked to phenotypes viz. growth and stress tolerance. It is being utilized for strain selection with specific trait of interest with industrial applications (Stella *et al*., 2019). Evolutionary engineering is further explained by Lee *et al*. (2011) that the process of maximizing performance of cells either by adaptive or random evolution under particular environmental or genetic conditions, and when it is accompanied by metabolic engineering it is termed as metabolic evolution. Adaptive evolutionary strategies have been widely used as a means to develop cells with unique properties without the need for metabolic or regulatory details of the strain, which can be accompanied by metabolic engineering for better outcome (Jiang *et al*., 2013). Evolutionary metabolic engineering consists of enzyme evolution, metabolic evolution, and adaptive evolution. Cellular biosensor and fluorescence activated cell sorting are among the techniques employed to select mutants with the trait of interest (Ma *et al*., 2017).

Corynebacterium glutamicum was engineered using adaptive evolution by Jiang *et al*. (2013) for improved production of L-ornithineis. The bacterial cells were subjected to growth based adaptive evolution by adding inhibitory agents and the survival mutant cells were further selected by growth on other supplements. Then, the transcriptional levels of genes coding enzymes for L-ornithine pathway were analyzed and all of the gens were upregulated except one gene. To improve the expression of the remaining gene, the mutant cells were genetically modified. The application of evolutionary engineering coupled with metabolic engineering enables *Corynebacterium glutamicum* strain to overproduce L-ornithine. Thus, evolutionary engineering strategy can be employed to bridge adaptive evolution and metabolic engineering to increase the production of target products. However, evolutionary engineering requires much time and effort to obtain the improved strains, especially when the criteria for selecting cells that have the desired phenotypes are not clear. Nevertheless, as described above, there is no doubt that evolutionary engineering can have a high impact on metabolic engineering for the enhanced production of desired chemicals and materials with optimized cellular growth (Lee *et al*, 2011).

5. Conclusion

Amino acids of both proteogenic and non-proteogenic are being produced worldwide for various reason. The production method varies greatly according to the type of amino acid of interest and the economic feasibility. Three methods have widely been applied to meet the growing demand of amino acids. But microbial fermentation has been given due attention since only L-form of amino acids are produced which are more preferable than D-forms. Moreover, the synthesis of merely L-form of amino acids eliminates expensive cost dedicated to purification of racemic mixture of DL-forms and make fermentation method more economically feasible. Besides, microbial fermentation can be carried out using cheap and easily available by products of agriculture and industry. The challenge facing microbial fermentation is obtaining efficient microbial strain for overproduction of amino acids. Recently, techniques have been developed to enhance amino acid synthesis and secreting ability of wild microbial strains. Random mutagenesis was the first technique to trigger amino acid production though it suffers from disadvantage of irregulated gene expression. Latter on, metabolic engineering focusing on specific metabolic pathway was introduced. This enable to develop strains capable of synthesizing amino acid, but the result was not satisfactory as to it doesn't consider the whole biological system. Currently, a more advanced and wholistic method is in place, systems metabolic engineering, which comprises of pathway focused approach, evolutionary approach and systems biology-based approach. The integration of all these approaches is now employed to make strain improvement more reliable and effective.

All in all, microbial fermentation of amino acid is the primary source of metabolically active amino acids. The most intensively studied strains are *Corynebacterium glutamicum* and *Escherichia coli*, and are being genetically modified to produce more metabolites. However, consumers prefer amino acids produced from wild strains than genetically modified microorganisms and exploring nature for efficient microorganisms is underway. Therefore, it is strongly recommended to exhaustively exploit untapped microbial genetic resource for competent microbes to meet the ever-increasing demand for amino acids.

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