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Toward Sustainable Amino Acid Production

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Abstract Because the global amino acid production industry has been growing steadily and is expected to grow even more in the future, efficient production by fermentation is of great importance from economic and sustainability viewpoints. Many systems biology technologies, such as genome breeding, omics analysis, metabolic flux analysis, and metabolic simulation, have been employed for the improvement of amino acid-producing strains of bacteria. Synthetic biological approaches have recently been applied to strain development. It is also important to use sustainable carbon sources, such as glycerol or pyrolytic sugars from cellulosic biomass, instead of conventional carbon sources, such as glucose or sucrose, which can be used as food. Furthermore, reduction of sub-raw substrates has been shown to lead to reduction of environmental burdens and cost. Recently, a new fermentation system for glutamate production under acidic pH was developed to decrease the amount of one sub-raw material, ammonium, for maintenance of culture pH. At the same time, the utilization of fermentation coproducts, such as cells, ammonium sulfate, and fermentation broth, is a useful approach to decrease waste. In this chapter, further perspectives for future amino acid fermentation from one-carbon compounds are described.

Keywords Amino acid, Coproduct, Fermentation, One-carbon compound, Raw material, Sub-raw material, Synthetic biology, Systems biology

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Contents

1 Systems Biology and Synthetic Biological Approaches

Since the genome sequence of the model and industrial organism *Escherichia coli* has been determined by Blattner et al. [1], the genome sequences of *Corynebacterium glutamicum* ATCC 13032 [2, 3]; *C. efficiens*, a near relative of *C. glutamicum* with different optimal growth temperatures [4]; *C. glutamicum* strain R [5]; *C. glutamicum* ATCC 14067 [formerly *Brevibacterium flavum*] [6]; and *C. glutamicum* AJ 1511 [formerly *Brevibacterium lactofermentum*] [7] have been determined. On the basis of genomic information, systems biology approaches have been applied to maximize the efficiency of amino acid production [8]. Genome breeding consisting of characterization and reconstitution of a mutation set essential for high-level production of amino acids was proposed by identifying mutations in the L-lysine-producer of *C. glutamicum* [9]. Omics analyses, such as transcriptomic, proteomic, and metabolomic analyses, have been extensively applied for improvement of amino acid producers [10, 11].

Metabolic flux analysis (MFA) using ^{13}C labeling is a powerful method for quantifying intracellular reaction rates within a metabolic network. In this approach, intracellular flux is calculated using a stoichiometric model for the major intracellular reactions and applying mass balances around intracellular metabolites using a set of measured extracellular fluxes, typically uptake rates of substrates and secretion rates of metabolites, as input for the calculations [12]. Since the first report on analysis of L-lysine production [13], MFA has been used for elucidating the in vivo metabolic state of cells during fermentation [14]. Recently, the development of MFA and its application to improvement of *C. glutamicum* amino acid producers has been reviewed in detail [15]. Integration of MFA data from different mutants and/or different cultural environments with different levels of omics analysis information using a systems biology approach is expected to reveal the roles of global regulators, which are quite important for metabolic regulation during amino acid fermentation [16]. Genome-scale metabolic models for *E. coli* [17] and *C. glutamicum* [18] have been developed and widely

used for estimation of, among other parameters, growth capability and amino acid production, using flux balance analysis.

Kinetic modeling is also valuable to industrial biotechnology and is able to assist in the rational design of cell factory properties or production processes in which they are utilized because kinetic models are capable of representing the complex biochemistry of cells more completely than most other types of models. However, several challenges must be overcome before kinetic modeling can reach the degree of maturity required for routine application in industry [19]. One of the targets of kinetic modeling has been the phosphotransferase system of *E. coli*, which is quite important for material production because it defines substrate uptake rates [20, 21]. Large-scale kinetic modeling and dynamic simulation, including the phosphotransferase system, glycolysis, and the pentose phosphate pathway but excluding the regulatory network of *E. coli*, has been reported [22]. Dynamic simulation of glutamate fermentation with large-scale kinetic modeling, including the central metabolic pathway regulatory network, has been reported [23], and extensive sensitivity analysis and validation have been performed [24].

Recently, synthetic biological approaches have been applied to strain development [25]. A successful example wherein a new pathway was introduced to improve amino acid yield involves phosphoketolase (PKT). PKT catalyzes the following reactions:

$$\text{Fructose 6-phosphate} + P_i \rightarrow \text{Acetyl phosphate} + \text{Erythrose 4-phosphate} \quad (1)$$

$$\text{Xylulose 5-phosphate} + P_i \rightarrow \text{Acetyl phosphate} + \text{Glyceraldehyde 3-phosphate} \quad (2)$$

PKT can increase the maximum theoretical yield of L-glutamate from glucose up to 98.0% by weight (120% mol/mol L-glutamate produced/glucose consumed) by bypassing the CO_2-releasing pyruvate dehydrogenase reaction (Fig. 1). The *xfp* gene encoding PKT was cloned from *Bifidobacterium animalis* and overexpressed under a strong *cspB* promoter in the L-glutamate-producing strain of *C. glutamicum* (Δ*odhA* mutant). When cells of this producer strain with and without the *xfp* gene were cultivated in a controlled fermentation system, L-glutamate production from the *xfp*-expressing strain was much higher than that of the original strain coupled with suppressed CO_2 emission [26]. Recently, nonoxidative glycolysis has been suggested in which the PKT pathway enables complete carbon conservation in sugar catabolism to acetyl coenzyme A [27]. The enzyme mixture, PKT from *Bifidobacterium adolescentis*, transaldolase, transketolase, fructose 1,6-bisphosphate, ribulose 5-phosphate epimerase, ribose 5-phosphate isomerase, fructose 1,6-bisphosphate aldolase, and triose phosphate isomerase and ATP have been shown to convert one fructose 6-phosphate molecule to three acetyl phosphate molecules in vitro. Xylose was converted to acetate and other products under anaerobic conditions. The JCL118 strain (Δ*ldhA* Δ*adhE* Δ*frdBC* Δ*pflB*), which overexpresses PKT from *Bifidobacterium adolescentis* and fructose 1,6-bisphosphate from *E. coli*, produced acetate from xylose with a near theoretical

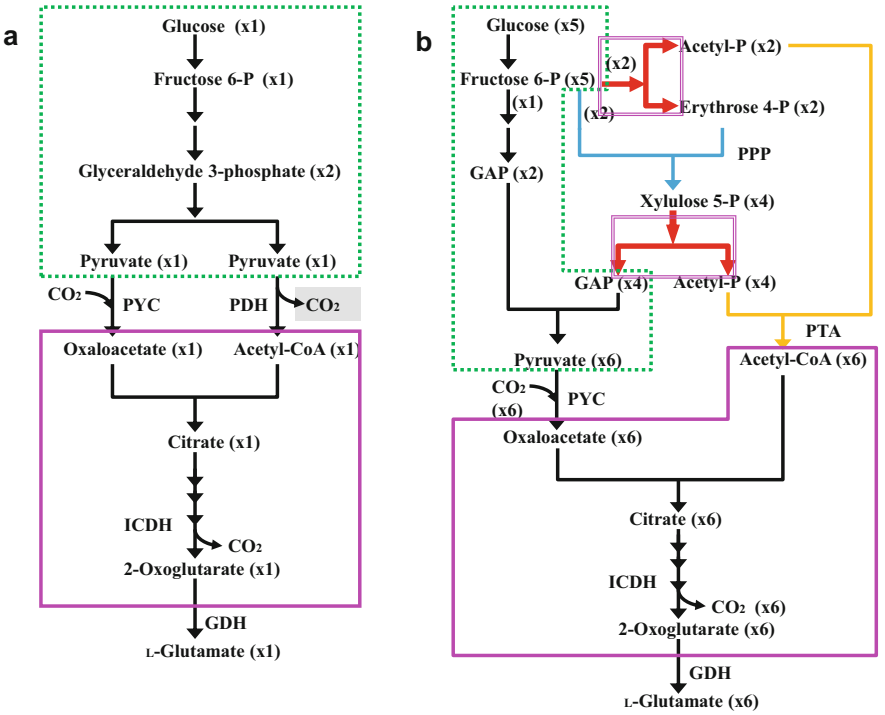

Fig. 1 The phosphoketolase (PKT) pathway. Biosynthesis of L-glutamate from glucose (modified from Chinen et al. [26]). Glycolysis and the oxidative branch of the tricarboxylic acid cycle are enclosed by *dotted line* and *solid line*, respectively. The number of compounds involved in each metabolic reaction under ideal conditions is shown in *parentheses*. (**a**) Metabolic pathway of wild-type *Corynebacterium glutamicum* showing maximum theoretical yield of L-glutamate from glucose. Emission of CO_2 induced by pyruvate dehydrogenase (PDH) is indicated by the *panel*. (**b**) Metabolic pathway engineered by introduction of phosphoketolase (indicated and enclosed by *double line*) to bypass CO_2 emission. *PPP* pentose phosphate pathway, *PTA* phosphotransacetylase. This pathway produces 6 mol of L-glutamate from 5 mol of glucose without the loss of carbon via CO_2 release

ratio of acetate/xylose in vivo [27]. Utilization of the PKT pathway enables complete carbon conservation in sugar catabolism to acetyl coenzyme A and is expected to be used in conjunction with CO_2 fixation and other one-carbon (C1) assimilation pathways to achieve a 100% carbon yield from various substances.

2 Alternative Raw Materials

Currently, most amino acids are produced mainly from sugars, such as glucose or sucrose, which are also used as foods. From a sustainability viewpoint, raw materials used for amino acid fermentation should avoid competition with food resources. The candidates for nonfood raw materials are glycerol, a valuable by-product in biodiesel production, and glucose and C5 sugars from cellulosic biomass by enzymatic hydrolysis.

Glycerol, also known as glycerin, is a by-product of a transesterification reaction used in biodiesel factories. Glycerol produced from biodiesel factories is crude and contains various impurities. Crude glycerol obtained from biodiesel factories consists of glycerol, water, organic and inorganic salts, soap, alcohol, and traces of glycerides. However, crude glycerol could be used as an organic carbon substrate for the production of value-added chemicals, such as 1,3-propanediol, organic acids, or polyols, by microorganisms [28].

C. glutamicum, which cannot utilize glycerol naturally, was engineered for glycerol utilization by heterologous expression of *E. coli* aerobic glycerol utilization genes encoding a glycerol facilitator (*glpF*), glycerol kinase (*glpK*), and glycerol-3-phosphate dehydrogenase (*glpD*). *C. glutamicum* strains expressing these genes show fast growth with glycerol as the sole carbon source and similar L-glutamate and L-lysine production properties to those of glucose [29]. Meiswinkel et al. [30] reported growth of recombinant strains expressing *glpF*, *glpK*, and *glpD* from *E. coli* fed crude glycerol from biodiesel factories. Besides growth, production of the amino acids L-glutamate, L-lysine, and L-arginine was shown to be dependent on the quality of crude glycerol from biodiesel factories [30]. Because *E. coli* has been known to utilize glycerol, many amino acids produced by *E. coli* can also be produced from glycerol.

Lignocellulosic materials containing cellulose, hemicellulose, and lignin are the most abundant renewable organic resource on earth. Conversion of both cellulose (glucose) and hemicellulose (hexose and pentose) in the production of ethanol has been studied intensively with the aim of developing a technically and economically viable bioprocess for fuel production. Thus, utilization of lignocellulosic materials as a renewable resource for energy and various chemicals is expected to increase.

Xylose and arabinose are the major pentose constituents in hemicellulose. However, wild-type *C. glutamicum* strains cannot utilize the pentose fractions of lignocellulosic hydrolysates. Previously, introduction of the xylose operon from *E. coli* was shown to enable growth of *C. glutamicum* on xylose [31], and implementation of the *E. coli araABD* gene cluster enabled growth and production of amino acids on this carbon source [32]. Meiswinkel et al. [33] found that introduction of the *Xanthomonas campestris* xylose isomerase gene and *C. glutamicum* xylulokinase gene doubled the growth rate and increased glutamate productivity of *C. glutamicum* from that of the strain solely expressing the *E. coli* xylose isomerase gene. Furthermore, Gopinath et al. [34] reported that recombinant pentose-utilizing

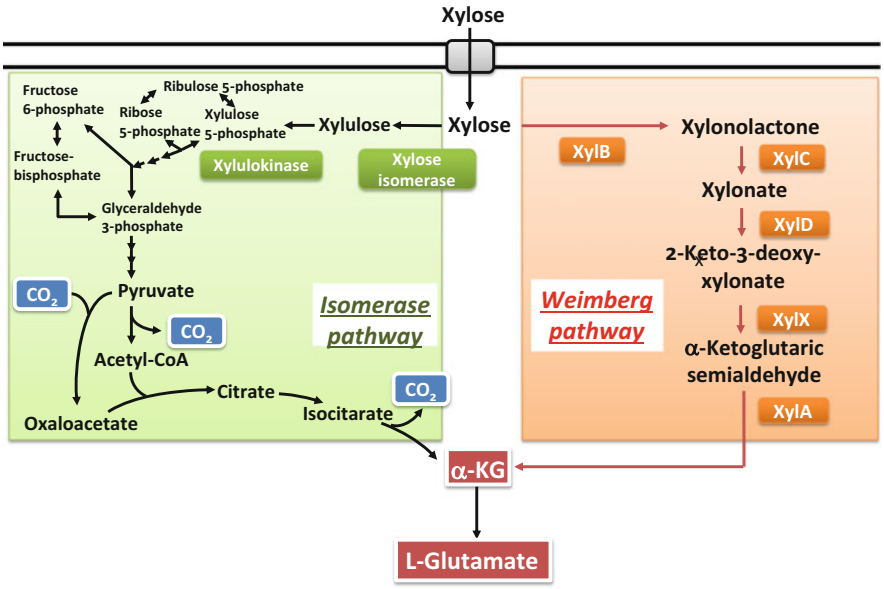

Fig. 2 Xylose utilization pathways. Biosynthesis of L-glutamate from xylose. The isomerase pathway is accompanied by carbon loss (*left*). The Weimberg pathway encoded by the *xylXABCD* operon produces 1 mol of L-glutamate from 1 mol of xylose without the loss of carbon via CO_2 release

strains derived from an L-lysine-producing *C. glutamicum* strain utilized arabinose and/or xylose, which were present in acid hydrolysates of rice straw or wheat bran.

In contrast to the commonly known isomerase pathway that leads to significant carbon loss in the form of CO_2, the Weimberg pathway (Fig. 2), first discovered in *Pseudomonas fragi* and later in *Haloferax volcanii* and *Caulobacter crescentus* [35–37], is an attractive alternative for xylose assimilation. In this five-step oxidative pathway, xylose is exclusively oxidized to the C5 compound α-ketoglutarate without carbon loss (Fig. 2). Previously, the Weimberg pathway encoded by the *xylXABCD* operon from *Caulobacter crescentus* was introduced into *C. glutamicum* and enabled a recombinant *C. glutamicum* strain to utilize xylose where it is the sole carbon source and in xylose/glucose mixtures [38]. The recombinant strain efficiently produced L-glutamate from xylose, and its L-glutamate productivity was higher than that of a strain carrying a gene encoding xylose isomerase by which *C. glutamicum* could assimilate xylose (Yamada et al. unpublished).

Direct utilization of cellulose or hemicellulose is one major goal. Adham et al. [39] expressed xylanase Xys1 and the cellulase Cel1 from the straw-decomposing *Streptomyces halstedii* JM8 in *C. glutamicum* ATCC 13869. Hyeon et al. [40] reported development of a *C. glutamicum* strain expressing functional minicellulosomes containing chimeric endoglucanase E consisting of the endoglucanase E catalytic backbone of *Clostridium thermocellum* fused with the endoglucanase B dockerin domain of *Clostridium cellulovorans*. The engineered

strain degraded carboxymethyl cellulose efficiently by substrate targeting via the carbohydrate-binding module [40]. Tsuchidate et al. [41] expressed endoglucanase from *Clostridium cellulovorans* 743B in *C. glutamicum* using the *E. coli torA* signal sequence. The secreted endoglucanase produced 123 mg of reducing sugar from 5 g of β-glucan after 72 h at 30°C. Moreover, L-glutamate fermentation from β-glucan with the addition of *Aspergillus aculeatus* β-glucosidase produced by recombinant *Aspergillus oryzae* resulted in 178 mg/L of L-glutamate from 15 g of β-glucan [41]. Recently, Kim et al. [42] reported that cellulase complexes containing two cellulolytic enzymes, endoglucanase E and β-glucosidase A from *Clostridium thermocellum*, anchored to the surface of *C. glutamicum* by a mechanosensitive channel synergistically lead to a 3.1- to 6.0-fold increase in the direct conversion of biomass (rice straw and *Miscanthus* and rape stem pretreated under high temperature with alkaline chemicals) to reducing sugars relative to conversion by secreted cellulase complexes.

Furthermore, fatty acids represent an alternative carbon source derived from biodiesel production or cooking oil wastes or produced by microalgae. Doi et al. [43] reported that fatty acids can be used as raw materials for L-lysine fermentation and that reduction of hydrogen peroxide stress derived from fatty acid β-oxidation improved fatty acid utilization in *E. coli*. Acetate and ethanol are also expected to be alternative carbon sources because *C. glutamicum* can grow on these substances [44, 45].

3 Reduction of Sub-raw Materials by a New Fermentation System

3.1 Concept of the New Fermentation System

Because L-glutamate is an acidic amino acid, the bacterial growth medium is acidified in accordance with L-glutamate accumulation during fermentation. Generally, L-glutamate fermentation in *C. glutamicum* is performed at a neutral pH. Therefore, addition of a large amount of alkali, usually ammonia, is necessary to maintain the pH of the medium. L-Glutamate in the culture medium is stored as ammonium salt. After fermentation, L-glutamate is crystallized by addition of acid, usually sulfuric acid or hydrochloric acid, utilizing the low solubility of this amino acid in acidic conditions (Fig. 3). The final product, monosodium glutamate, is produced by neutralizing L-glutamate with sodium hydroxide. In this traditional manufacturing process, large amounts of alkali and acid are used in the fermentation and crystal isolation steps, respectively, and a large amount of by-product salt, such as ammonium sulfate, is produced.

In recent years, production of L-glutamate by integrating fermentation and isolation steps has been called "L-glutamate crystallization fermentation." In this novel method, crystallization of L-glutamate occurs during the fermentation

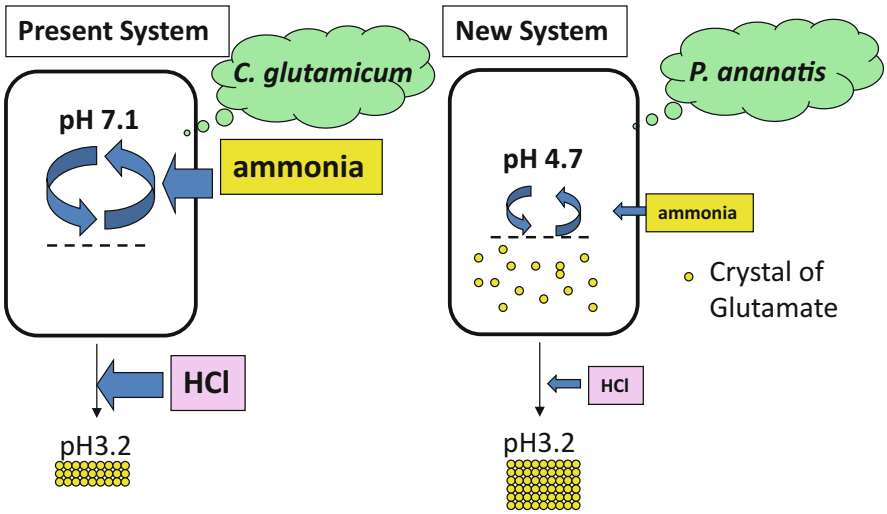

Fig. 3 New fermentation system for reduction of sub-raw materials. Fermentation under acidic conditions leads a decreased input of ammonia and hydrochloric acid. In addition, because the solubility of glutamate is low in acidic conditions, a large amount of glutamate accumulates as crystals in the fermentation tank

process. L-Glutamate has two carboxyl groups and one amino group, and its theoretical isoelectric point is pH 3.22. Due to its acidity, the solubility of L-glutamate in acidic conditions is low. If L-glutamate fermentation is performed under acidic conditions, a large portion of the L-glutamate would lose its electrical charge and precipitate as crystals. Thus, much of the counter ion ammonia, as well as the acid added and salt produced in the purification step, would be reduced. For example, when fermentation is performed at pH 4.5 and 7, the amount of ammonia and acid needed for fermentation and crystallization would be decreased by 40% and 80%, respectively.

3.2 Isolation of the Host Strain

Because L-glutamate crystallization fermentation requires acidic fermentation conditions, it is essential that the host be able to grow at a low pH and resist high concentrations of glutamate. After screening various strains for these properties, *Pantoea ananatis* strain AJ13355 isolated from the soil of a tea plantation was selected. In the current study, the complete genomic sequence of *P. ananatis* AJ13355 was determined and found to consist of a single, circular chromosome consisting of 4,555,536 bp (DDBJ: AP012032) and a circular plasmid (pEA320) with 321,744 bp (DDBJ: AP012033). After automated annotation, 4,071 protein-coding sequences were identified in the *P. ananatis* AJ13355 genome [46].

3.3 Construction of Glutamate-Producing Bacteria

Although *P. ananatis* AJ13355 possesses all of the genes needed for biosynthesis of glutamate, L-glutamate accumulation was not induced by penicillin or surfactants, unlike in *C. glutamicum*. Therefore, a strain which overproduces glutamate by modifying the L-glutamate biosynthetic pathway in *P. ananatis* AJ13355 was bred. To do so, enhancement of the biosynthetic pathway and knock out of degradation pathway genes of the target substance were used. Then, the L-glutamate producer was constructed through the following steps: (1) decreased extracellular polysaccharide production, (2) weakened 2-oxoglutarate dehydrogenase complex activity, and (3) enhanced activity of key enzymes in glutamate biosynthesis, such as citrate synthase, phosphoenolpyruvate carboxylase, and glutamate dehydrogenase. However, glutamate production deteriorated, and accumulation of by-products, such as acetoin and 2,3-butanediol, was observed, especially when cultured at an acidic pH. L-Glutamate loses its electrical charge under acidic conditions and can easily pass through the hydrophobic cell membrane. When the cellular pH was maintained at near 7, L-glutamate flowed into the cell and reionized, making it unable to cross the membrane again. Consequently, L-glutamate was concentrated inside the cell, which led to feedback inhibition of L-glutamate biosynthesis.

There are two types of citrate synthase enzymes. Type I enzymes are found in *C. glutamicum* and other Gram-positive bacteria, and type II enzymes are found in Gram-negative bacteria. Type II enzyme activity is inhibited by NADH allosterically and α-ketoglutarate. In a recent study, type II citrate synthases were found to be inhibited by high concentrations of glutamate, while type I enzymes were not, and introduction of type I citrate synthase from *C. glutamicum* effectively increased glutamate production.

3.4 Glutamate Production with Crystallization

L-glutamate production was improved by enhancement of type I citrate synthase activity. However, an extremely high concentration of L-glutamate in the medium still had a negative effect on cell growth and sugar consumption. Therefore, a mutant *P. ananatis* AJ13601 strain with glutamate resistance was isolated. This mutant strain produced 82 g/L of glutamate at pH 4.5 with glucose as the carbon source. Because the solubility of glutamate at that pH is 41 g/L, a large amount accumulated as crystals. This is the first example of L-glutamate crystallization fermentation. L-glutamate crystallization fermentation not only decreases the cost of acid and alkali but also the burden on the environment. Thus, this new process is a promising method of sustainable amino acid production.

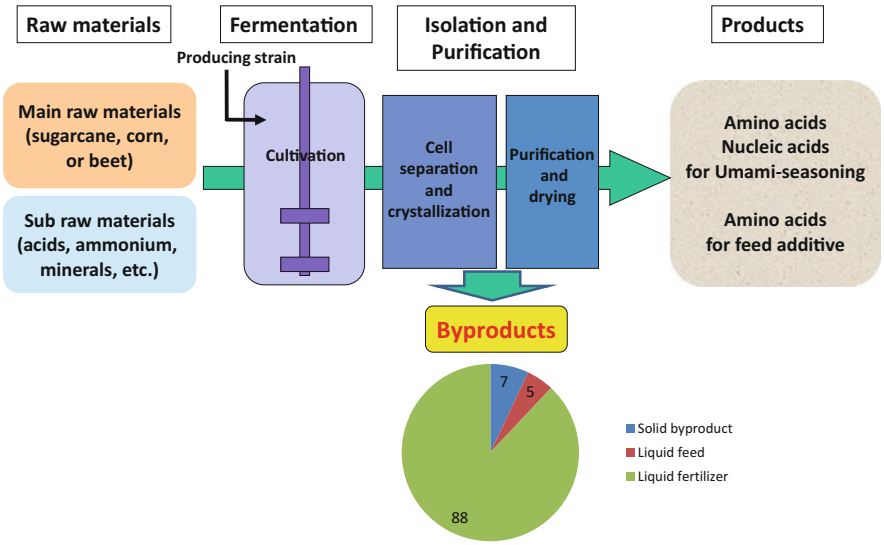

Fig. 4 Utilization of amino acid fermentation coproducts. Amino acid by-products are generated during the purification and isolation process. Coproducts are typically distributed as solid by-products (7%), liquid feeds (5%), and liquid fertilizers (88%)

4 Utilization of Coproducts

Amino acid manufacturing processes generate waste, atmospheric emissions, and drainage water. To minimize the environmental impact and ensure production sustainability, utilization of by-products of amino acid fermentation is critically important. These by-products are nutrient-rich and used as coproducts in agricultural, animal, and aquatic products industries around the world. The examples sold by Ajinomoto Co., Inc., described below were also shown in the Ajinomoto Group Sustainability Report 2012 [47]. A schematic representation of by-product formation in the amino acid production process, as well as the ratio of solid (7%) and liquid (93%) content in coproducts, is shown in Fig. 4. By-products are generated during purification and isolation after fermentation. Approximately 90% of the by-products are transformed into coproducts which are mainly used in slow-release solid fertilizer, foliar fertilizer, dried bacterial cells, and silage modifying agents.

Solid by-products contain salts, waste activated carbon, excess sludge, and waste filter aids. These can be used in, for example, animal and fish feeds, raw material for solid fertilizers, soil conditioners, raw material for cement, and fuels. AJIFOL® foliar fertilizer enables plants to effectively absorb nutrients through their leaves and is a prime example of a value-added coproduct. AJIFOL® liquid fertilizers are produced from the fermentation of L-glutamate and contain macro- and micronutrients, nitrogen and potassium, as well as several additional amino acids. These fertilizers were first launched in 1988 in Brazil and have since been used in many countries, including Brazil, Peru, the United States, Thailand, Vietnam,

Indonesia, and Japan. The benefits of AJIFOL® include (1) increased plant growth and development, as well as improved crop production and quality, especially under stressful conditions, by effectively providing macro- and micronutrients required by plants, and (2) increased resistance to diseases due to the elicitor activity of amino acids.

In Japan, the liquid fertilizer AMIHEART®, derived from the fermentation of inosine using natto bacteria (*Bacillus amyloliquefaciens*), was launched in 2011. This fertilizer is rich in inosine and has been proven effective in fostering the rooting of plants, promoting rapid growth, and increasing crop yields. It is mainly used for melons, strawberries, tomatoes, bell peppers, eggplant, and tea cultivation and is now beginning to be used in rice nursery production.

AJITEIN®, high value-added protein feeds containing bacterial cells by using protein-rich bacterial cells separated from coproducts that are often used as liquid fertilizers and feeds (Fig. 3), is distributed throughout Indonesia as an alternative to fish and soybean meal. One of the advantages that AJITEIN® possesses over the other protein sources is that it contains β-glucan, which can stimulate and enhance the immune system of livestock. The liquid fertilizers AMI-AMI® and AMINAR® are representative coproducts of Ajinomoto group companies in Thailand, Vietnam, and Indonesia. These fertilizers are mainly utilized for cultivation of major crops, such as rice, corn, soybeans, and sugar cane, as well as vegetables and fruits. They derive from the liquid coproducts of monosodium glutamate production, which are rich in protein and free amino acids. Thus, they can be applied as an alternative source of protein in the manufacture of animal and fish feed.

5 Toward Further Sustainable Amino Acid Fermentation

For more than 50 years, the fermentation of amino acids has continuously improved. However, most amino acids are still mainly produced from sugars, such as glucose or sucrose, which are food raw materials. The candidates for nonfood raw materials are glycerol, a valuable by-product in biodiesel production by transesterification, as well as glucose and C5 sugars from cellulosic biomass (Sect. 2).

C1 compounds occur abundantly in nature. Methane and methanol are two of the most important C1 compounds from a biotechnological and bulk chemical viewpoint. The possibility of utilizing the C1 substrates methane and methanol as alternative nonfood feedstocks has attracted high scientific interest. This interest is largely based on economic considerations (i.e., low methanol costs and increased capacity for methanol production worldwide) and technological advances, which allow for production of important industrial chemicals from methanol as a feedstock using microbes. Generally, methanol is prepared by reaction of a mixture of carbon oxides (CO and CO_2) with hydrogen; the CO/CO_2 mixture, in turn, derives from methane generally obtained from natural gas (e.g., shale gas). Methanol is being increasingly produced from renewable sources. The biomass is gasified, and

the resulting gas, a mixture of CO, H_2, and CO_2, satisfies the quality requirements of methanol synthesis. In the long term, more renewable/bio-based methanol use in, for example, shipping and transportation fuels is envisioned. However, methane and methanol utilization has not yet been commercialized because the price of methanol still makes it a more expensive substrate than sugar for bulk amino acid production.

Methylotrophs comprise a large number of both aerobic and anaerobic microorganisms that can grow in methane and methanol. Obligate methylotrophs can exclusively utilize C1 compounds as a sole carbon and energy source, while facultative methylotrophs can utilize both C1 and multicarbon compounds. Genetic tools for many methylotrophs have been established, and engineering of methylotrophs leading to overproduction of different amino acids has been reported [48]. For example, the Gram-negative obligate methylotroph *Methylophilus methylotrophus* synthesized 1 g/L of L-lysine at 37°C through expression of a mutant gene encoding dihydrodipicolinate synthase that is deregulated by L-lysine inhibition [49]. By coexpressing a mutant gene encoding an L-lysine transporter, LysE from recombinant *C. glutamicum* accumulated 11.3 g/L of L-lysine from methanol [50]. A recombinant mutant of the Gram-negative obligate methylotroph *Methylobacillus glycogenes* overexpressing a dihydrodipicolinate synthase that is partly desensitized to inhibition by L-lysine was reported to produce approximately 8 g/L of L-lysine and 37 g/L of L-glutamate from methanol at 37°C [51].

Bacillus methanolicus is a Gram-positive, facultative methylotrophic and thermophilic bacterium considered to be one of the few candidates with the potential to convert methanol to value-added products and amino acids at high temperatures [48, 52]. The *Bacillus methanolicus* classical mutant with homoserine auxotrophy and resistance to amino acid analogs derived from strain NOA2 has been reported to secrete 37 g/L of L-lysine in fed-batch bioreactors [53]. *Bacillus methanolicus* MGA3 (ATCC 53907) has been shown to secrete 55 g/L of L-glutamate at 50°C with methanol as a carbon source in fed-batch bioreactors [54]. Considering its ability to produce high concentrations of L-glutamate and L-lysine at 50°C, *Bacillus methanolicus* represents a promising microorganism for industrial-scale production processes.

As an alternative approach to engineering methylotrophs for production of amino acids from methanol, it may be possible to exploit the ability to utilize methanol as a carbon source in naturally nonmethylotrophic, amino acid-producing bacteria by introducing suitable heterologous pathways, such as the ribulose monophosphate or serine pathway. Witthoff et al. [55] showed the capability of *C. glutamicum* to oxidize methanol to CO_2 and identified the key enzymes involved in this endogenous pathway as a first step toward making *C. glutamicum* a methylotroph.

Direct conversion of CO_2 to amino acids is one of ultimate methods from an environmental viewpoint. Matsunaga et al. [56] reported the production of L-glutamate from CO_2 by the marine cyanobacterium *Synechococcus* sp. NKBG040607A using a biosolar reactor; the maximum CO_2-to-glutamate conversion ratio was 28% at a cell density of 3×10^8 cells/mL. L-glutamate

productivity using the biosolar reactor has been reported to be 15 μmol/L/h. Furthermore, Ryu et al. [57] reported a new type of artificial photosynthetic system that integrally and efficiently couples biocatalytic redox reactions with photocatalytic water splitting. Efficient coupling is achieved using tetracobalt polyoxometalate and a rhodium-based organometallic compound as hole and electron scavengers, respectively, for photoexcited $[Ru(bpy)_3]^{2+}$ that successively photosynthesize L-glutamate as a model compound using a model redox enzyme (L-glutamate dehydrogenase) upon in situ photoregeneration of cofactors. By unlocking new, beneficial amino acid functions, usage of amino acids has prevailed and is expected to expand in the future. Thus, efficient production of amino acids will continue to be explored further to help conserve and improve the global environment and maintain sustainable production.

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