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Review

Metabolic Engineering of Microorganisms to Produce L-Aspartate and Its Derivatives

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Abstract: Metabolic engineering is a promising strategy to realize green synthesis of valued chemicals derived from petroleum. According to the literature, cell factories for producing L-aspartate and its derivatives (β-alanine, ectoine, 3-hydroxypropionate, D-pantothenic acid and L-homoserine) have been developed. In this review, we firstly introduced the functions, applications and markets of L-aspartate and its derivatives. Then, the current research progress on microbial production of them was elaborated in detail. Finally, we have discussed the limiting factors and given some suggestions for realizing applications of engineered bacteria in the industry, including metabolic engineering of the bacteria to increase the titer, yield and productivity of the target products, fermentation condition optimization and downstream purification. With the development of novel technologies and increased investments in synthetic biology, it is promising to realize sustainable production of L-aspartate and its derivatives at the industrial scale in the future.

Keywords: metabolic engineering; L-aspartate; β-alanine; ectoine; 3-hydroxypropionate; D-pantothenic acid; L-homoserine

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1. Introduction

Metabolic engineering is a field of biotechnology that focuses on the manipulation and modification of metabolic pathways in cells to enhance the production of desirable products. This involves the use of genetic engineering techniques to alter the DNA of microorganisms, plants and animals in order to optimize their biochemical processes and improve the production of specific compounds. The main goal of metabolic engineering is to design or modify metabolic pathways to produce desired products in large quantities with high efficiency. This can involve introducing genes from other organisms or even synthesizing new genes to produce needed enzymes that enable the desired metabolic reactions [1]. Examples of products that can be produced using metabolic engineering include biofuels [2], bioplastics [3], pharmaceuticals [4], flavors [5], fragrances [6] and food additives [7]. Metabolic engineering is also used in the production of enzymes [8] and in the development of medical treatments for certain diseases [9]. The key advantages of metabolic engineering include the ability to produce large quantities of products in an environmentally sustainable manner with reduced dependence on non-renewable resources [10]. It also enables the creation of new and novel compounds with unique properties that can have a wide range of applications in different industries.

With the help of metabolic engineering, the microbial cell factories for the production of L-aspartate and its derivatives are realized. In this paper, we summarize the research progress on microbial production of L-aspartate and its derivatives: β-alanine, ectoine, 3-hydroxypropionate (3-HP), D-pantothenic acid and homoserine.

2. Metabolic Engineering of Microbials to Produce L-Aspartate and Its Derivatives

2.1. Developing Cell Factories to Produce L-Aspartate

L-aspartate is an amino acid that is naturally found in many fruits and vegetables, as well as in animal proteins. It has various functions, including its role as a precursor for the synthesis of other amino acids and for the production of energy in the body [11]. L-aspartate has various applications in the food and pharmaceutical industries. In the food industry, it is commonly used as a flavor enhancer and a sweetener in many diet and low-calorie products [8,12]. In the pharmaceutical industry, L-aspartate is used to treat symptoms of liver disease and to help improve brain function [13]. The market for L-aspartate is expected to grow in the coming years, as the demand for low-calorie and diet products continues to rise. The global market for L-aspartate was 93.15 million dollars in 2021 and is projected to grow at a CAGR of 6.20% from 2022 to 2029. The increasing demand for sports and energy drinks is also expected to drive the growth of the L-aspartate market. The Asia-Pacific region is anticipated to be the fastest-growing market for L-aspartate, due to the growing demand for low-calorie products and the increasing health awareness among consumers (https://www.databridgemarketresearch.com/reports/global-aspartic-acid-market, accessed on 3 May 2023).

There are several ways to produce L-aspartate at an industrial scale, including extraction from natural sources, chemical synthesis, enzymatic processes and fermentation. The choice of the production method depends on several factors, such as cost, efficiency and sustainability. Currently, the most widely used method is enzymatic conversion of the precursors, fumarate and ammonia, catalyzed by aspartase. For example, *E. coli* JCL1258/pBAW2/pASP400 produced over 77 g/L of L-aspartate from fumarate with a conversion yield of 83% [14]. Since fumarate is derived from petrochemicals, the enzymatic process is not an environmentally friendly synthetic technology [15]. For L-aspartate biosynthesis by fermentation, several types of bacteria have been engineered, including *Escherichia coli* (*E. coli*), *Corynebacterium glutamicum* (*C. glutamicum*) and *Brevibacterium flavum (B. flavum)* (Table 1). For L-aspartate cell factory construction, researchers focus on pathway modification [15,16] and overcoming the rate-limiting steps of L-aspartate biosynthesis (Figure 1) [13,17].

To date, the best cell factory for L-aspartate biosynthesis with glucose as a carbon source is the *E. coli* developed by Piao et al., producing 33.1 g/L of L-aspartate (Table 1). However, the yield was only 0.39 g/g, which was about 27% of the theoretical value when oxaloacetate/fumarate, the direct substrate for L-aspartate biosynthesis, was supplied by the reductive branch of the TCA cycle from glucose [15]. The researchers have focused on pathway modification to increase phosphoenolpyruvate (PEP), oxaloacetate (OAA), L-glutamate and CO_2 supply and optimizing fermentation conditions to improve L-aspartate biosynthesis [15]. For engineering *C. glutamicum* ATCC13032 to produce L-aspartate, several genes were inactivated since they consume pyruvate (*ldhA* and *avtA*) or fumarate (*sdhCAB*), and *aspB*, encoding L-aspartate aminotransferase, was overexpressed [18]. It could produce 5.72 g/L of L-aspartate with a yield of 0.75 g/g. As for *B. flavum* 70, it was developed after several rounds of mutations and could produce 22.6 g/L of L-aspartate (Table 1). When maleate is used as the substrate, it is firstly converted to fumarate by maleate cis-trans isomerase (MaiA) and then to L-aspartate by the engineered *E. coli*. In this process, a titer of L-aspartate of 419.8 g/L with a conversion ratio of 0.72 was achieved [13]. Above all, with glucose as the substrate, the yield of L-aspartate is much lower than the theoretical value (1.48 g/g). Low yield will waste the substrate and increase the production cost. For realizing cost-effective industrial production of L-aspartate by fermentation using low-cost substrates (glucose), there is still much work to do, such as improving cell growth by pathway modification and fermentation medium optimization.

Table 1. Summary of microbial production of L-aspartate.

Organism	Metabolic Engineering Strategies	Substrate	Titer (g/L)	Yield [a] (g/g)	Fermentation Strategy	Reference
Engineered cell factories						
E. coli XAR31	Introducing and overexpressing *CgaspC*, *Cgppc*, *Mspck*, *glk*, *bt-ca*, *acs*, *Cgasp*, *BsrocG* and CR, deleting genes involved in byproduct biosynthesis, developing a cofactor self-sufficient system, optimizing the fermentation conditions	glucose	33.1	0.39	fed-batch	[15]
C. glutamicum SLV. pEKEx3-aspB	Deleting genes involved in byproduct biosynthesis (*sdhCAB*, *ldhA* and *avtA*)	glucose	~5.72 [a]	0.75	flask	[18]
B. flavum 70	Developing several mutations: a citrate synthase-defective glutamate auxotroph, S-(2-aminoethyl)-L-cysteine-resistant mutant, a methionine-insensitive revertant and hosphoenolpyruvate carboxylase, a supplement of biotin	glucose	22.6	0.22	flask	[16]
Enzyme catalysis						
E. coli pMA-RBS4-G27A/G171A	Co-overexpressing maleate cis-trans isomerase (MaiA) mutant and aspartase (AspA) on the plasmid and optimizing their activity ratio by ribosome binding site (RBS) regulation	maleate	419.8	0.72	5-L fermenter	[13]
E. coli JCL1258/pBAW2/pASP400	Overexpressing *aspC* and *tyrB* on plasmid pASP400 and overexpressing *parB* and *aspA* on plasmid pBAW2	fumarate	77.60 [a]	0.83		[14]

[a] represents that the data were derived by calculating according to the literature.

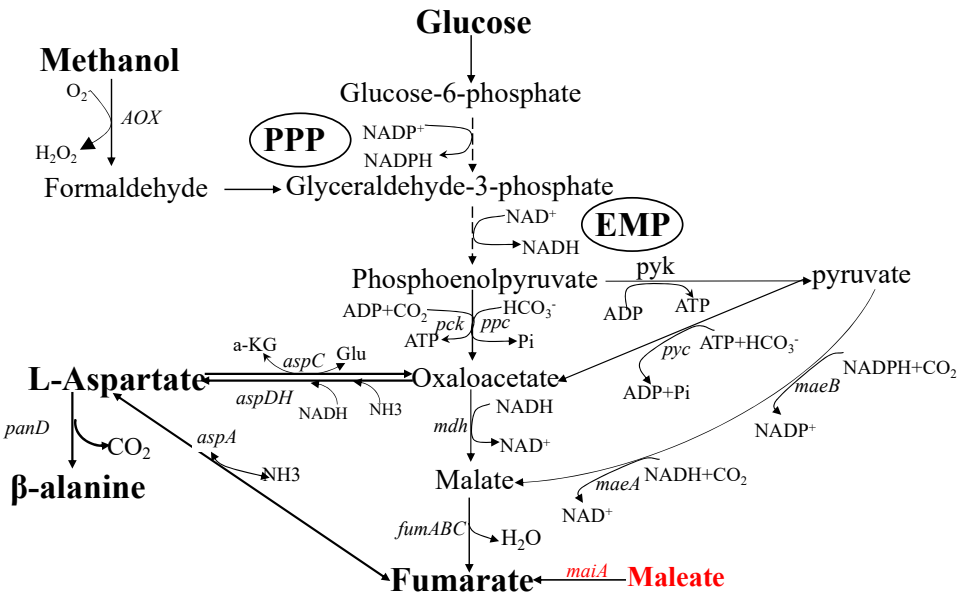

Figure 1. Biosynthetic pathways of L-aspartate and β-alanine. Here shows the metabolic pathways for L-aspartate and β-alanine biosynthesis with glucose as the substrate. Phosphoenolpyruvate is produced from glucose through EMP, then it can be converted to oxaloacetate or fumarate, which are the direct substrates for L-aspartate. Pyruvate derived from phosphoenolpyruvate can be metabolized to OAA or malate. Malate is catalyzed by fumarase to produce fumarate. β-alanine is derived from L-aspartate with aspartate decarboxylase [19]. *ppc*, phosphoenolpyruvate carboxylase; *pck*, phosphoenolpyruvate carboxykinase; *pyk*, pyruvate kinase; *pyc*, pyruvate carboxylase; *mdh*, malate dehydrogenase; *maeA*, malate dehydrogenase; *maeB*, malate dehydrogenase; *fumABC*, fumarase; *aspA*, aspartate ammonia-lyase; *aspDH*, NADH-dependent aspartate dehydrogenase; *aspC*, aspartate transaminase; AOX, alcohol oxidase; *maiA*, maleate cis-trans isomerase.

2.2. Developing Cell Factories to Produce β-Alanine

β-alanine is a non-essential amino acid that is naturally synthesized by the liver. It is used to synthesize the dipeptide carnosine, a powerful antioxidant [20]. β-alanine has various applications in the food and fitness industries. As a food additive, β-alanine is used as a flavor enhancer and acidity regulator. It is particularly useful in meat products, as it offers a pleasant taste and acts as a natural preservative [21]. In fitness industries, it is commonly used as a sports nutrition supplement to help increase endurance, delay fatigue and improve exercise performance [22]. The global market for β-alanine is expected to grow in the coming years, driven by the increasing demand for sports nutrition supplements and functional foods. The global market revenue of β-alanine was 75 million USD in 2019 and will reach 99 million USD in 2031, with a CAGR of 4.65% during 2023–2031 (https://www.marketwatch.com/press-release/beta-alanine-market-global-industry-share-trends-size-growth-opportunity-and-forecast-2023-2031-2023-04-14, accessed on 3 May 2023).

Currently, β-alanine is mainly produced via chemical synthesis, which involves toxic precursors and operates under harsh conditions [23]. For enzymatic processes, β-alanine can be derived from L-aspartate and fumarate. When L-aspartate, the precursor of β-alanine, was fed to *E. coli* expressing L-aspartate-α-decarboxylase, over 271 g/L of β-alanine was produced at a conversion rate of over 92% (Table 2). Although the highest conversion efficiency could be 97.2% when L-aspartate was used as the substrate, the cost for β-alanine biosynthesis is too high since the market price of L-aspartate is around $5000/ton, while the price for β-alanine is $6000/ton (Table 2). When fumarate is used as the substrate, it needs two enzymes, L-aspartate ammonia-lyase and L-aspartate-α-decarboxylase, to finish the β-alanine biosynthesis, and the highest titer can reach 200.3 g/L with a conversion efficiency of over 90% (Table 2). There have been a few studies on the production of β-alanine with bacteria through metabolic engineering. The bacteria used for constructing a β-alanine-producing cell factory include *E. coli*, *C. glutamicum*, *B. megaterium* and *Pichia pastoris* (Table 2). Glucose and methanol are used as carbon sources for β-alanine biosynthesis. Glucose is metabolized through the pentose phosphate pathway or EMP to produce PEP, which is converted to OAA, the substrate for L-aspartate biosynthesis. L-aspartate is converted to β-alanine by L-aspartate decarboxylase (Figure 1). With glucose as the substrate, the best strain for β-alanine biosynthesis is from *C. glutamicum*, and the highest reported titer of β-alanine was 166.6 g/L with a productivity of 1.74 g/(L.h) [24] (Table 2). However, the yield was only 0.28 g/g glucose (the maximum theoretical yield is 0.99 g/g glucose) due to the use of the pentose phosphate pathway and aerobic fermentation instead of anaerobic conditions for producing β-alanine. The metabolic engineering strategies they adapted were introducing L-aspartate 1-decarboxylases (encoded by *panD*) from *B. subtilis*, improving the supply of OAA and L-aspartate and speeding up the secretion of β-alanine. With glucose as the substrate, the highest yield was 0.75 g/g with engineered *E. coli* [15] (Table 2). When methanol was added to a culture of methylotrophic *Pichia pastoris* 2ADC-Spe, it was first converted to glyceraldehyde-3-phosphate with formaldehyde as an intermediate and then to β-alanine (Figure 1). However, the titer of β-alanine was only 5.6 g/L (Table 2). Except substrate optimization, some researchers tried to develop new methods to improve β-alanine production as well. For example, Dr. Alper's group has developed biosensor-assisted directed evolution and found ribonuclease E (encoded by *rne*) had a negative influence on β-alanine biosynthesis. The final strain, *E. coli* eBA32, could produce 34.8 g/L of β-alanine with fed-batch fermentation in 37 h [22]. Above all, this shows that a highly efficient β-alanine-producing cell factory can be realized in the future.

Table 2. Summary of microbial production of β-alanine.

Organism	Metabolic Engineering Strategies	Substrate	Titer (g/L)	Yield (g/g)	Productivity (g/(L.h))	Fermentation Strategy	Reference
Engineered cell factories							
E. coli XBR41	Introducing *BspanD*, deleting genes involved in byproduct biosynthesis, developing a cofactor self-sufficient system, optimizing the fermentation conditions	glucose	37.7	0.75 [a]		Fed-batch	[15]
E. coli NL-A13	High-throughput method to screen L-aspartate-α-decarboxylase variant ADCK43Y, evaluation and elevation cells' tolerance to β-alanine, improving fumarate supply and strengthening the pathway of fumarate and OAA to L-aspartate, optimizing culture medium	glucose glycerol	11.9			Fed-batch	[25]
E. coli W3110	Introducing *panD* from *Bacillus subtilis*, rerouting fluxes of the central carbon metabolism, relieving the inactivation of L-aspartate-α-decarboxylase, optimizing the fed-batch bioprocess	glucose	85.18	0.24	1.05	Fed-batch	[26]
E. coli ALA17/pTrc99a-$panD_{BS}$-$aspB_{CG}$	Introducing *panD* from *B.subtilis* and *aspB* from *C. glutamicum*, inactivating the β-alanine uptake system, the aspartate kinase I and III, *iclR*, *ptsG*, *aroG*, *galR*, overexpressing *ppc*, *aspC*, *aceB*, *aceA*, *glk*, and *gltBD* operon	glucose	43.94	0.20		Fed-batch	[23]
E. coli W FZβA-10	Introducing an L-aspartate a-decarboxylase gene from *Bacillus tequilensis*, a L-aspartate dehydrogenase gene from *Pseudomonas aeruginosa* and a pyruvate decarboxylase from *Corynebacterium glutamicum*, overexpressing *aspA*, deleting three native L-aspartate kinase genes and genes for byproduct biosynthesis (*ldhA*, *pflB*, *pta* and *adhE*)	glucose	43.12		0.89	Fed-batch	[27]
E. coli eBA32	Biosensor-enabled high-throughput screening, cofactor balancing and pathway modification	glucose	34.8			Fed-batch	
C. glutamicum XQ-5	Deleting byproduct biosynthesis pathway (lactate dehydrogenase and alanine/valine aminotransferases), replacing L-aspartate kinase (AK) with wild-type AK, introducing and overexpressing a mutated L-aspartate-α-decarboxylase ($BsADC^{E56S/I88M}$) from *B. subtilis*	glucose	56.5	39.5% [b]	0.79	Fed-batch	[28]
C. glutamicum BAL10 (pBA2_tr18)	Introducing *panD* from *B. subtilis*, overexpressing PTS-independent glucose uptake system, *ppc*, pyc, *aspB*, rocG from *B. subtilis*, *aspA* from *E. coli* and β-alanine exporter (NCgl0580), replacing the native *pck* with that from *Mannheimia succiniciproducens* (encoded by Mspck), deleting *odx* and *mdh*	glucose	166.6	0.28	1.74	Fed-batch	[24]
B. megaterium BMDBPG	Introducing a codon-optimized *panD* from *B. subtilis*, overexpressing *aspB*, *ppc* and NADH-dependent glutamate dehydrogenase (*gdh*)	glucose	17.60	0.23	0.78		[29]
methylotrophic Pichia pastoris 2ADC-Spe	Overexpressing *panD* from *B. subtilis* and *aspDH* from *S. proteamaculans*	methanol	5.6			Fed-batch	[30]

Table 2. *Cont.*

Organism	Metabolic Engineering Strategies	Substrate	Titer (g/L)	Yield (g/g)	Productivity (g/(L.h))	Fermentation Strategy	Reference
			Enzyme catalysis				
L-aspartate-α-decarboxylase (ADC) from C. glutamicum	Overexpressed in *Escherichia coli* BL21(DE3), optimal at 55 °C and pH 6 with excellent stability at 16–37 °C and pH 4–7	L-aspartate	12.85	97.2% [b]		Purified enzyme	[31]
E.coli BTW	Co-expressing two different types of L-aspartate-α-decarboxylase: one was from *B. subtilis* and the other was from *Tribolium castaneum*	L-aspartate	271.5	92.4% [b]		Whole cell	[32]
E.coli BTEW	Co-expressing three enzymes: two types of L-aspartate-α-decarboxylase (one was from *B. subtilis* and the other was from *Tribolium castaneum*) and one type of L-aspartase (AspA) from *E. coli*	fumarate	200.3	90.0% [b]		Whole cell	[32]
B. megaterium BMDA-6	Balancing the expression of L-aspartate-1-decarboxylases (ADC) from *B. subtilis* and aspartate ammonia-lyase (AspA) from *B. megaterium*, optimizing the cultivation conditions and biocatalysis process parameters	fumarate	11.68	0.78		Whole cell	[33]

[a] represents that the data were derived by calculating according to the literature. [b] the conversion efficiency of the substrate.

2.3. Developing Cell Factories to Produce Ectoine

Ectoine is a naturally occurring organic molecule. It functions as a protective agent, preventing damage to biological structures from harsh environmental conditions such as osmotic and thermal stress [34]. Ectoine also has water retention properties, allowing it to maintain hydration levels in cells, which is essential for the survival of organisms [35]. One potential application of ectoine is in the cosmetics industry, for its water-binding properties, which are key qualities for hydrating skin and hair. Besides this, ectoine has pharmaceutical applications for the treatment of skin disorders, eye diseases, and respiratory diseases as it has anti-inflammatory and antioxidant properties [36]. According to a report by businessresearchinsights, the market for ectoine was 20 million USD in 2021 and is expected to reach 31 million USD by 2028, growing at a CAGR of 6.6% from 2023 to 2028. This growth is driven by the increasing demand for natural-based cosmetics and personal-care products, as well as the growing awareness of the benefits of ectoine in healthcare (https://www.businessresearchinsights.com/market-reports/ectoine-market-100579, accessed on 3 May 2023).

Ectoine is currently produced by chemical synthesis, biocatalytic approach and fermentation. Ectoine can be chemically synthesized using chemical building blocks, such as glycine or sarcosine [37]. However, this method is not commonly used due to its low yield, high cost and low efficiency compared to biocatalytic and fermentation methods [37]. Ectoine can also be biosynthesized from its precursor, L-2,4-diaminobutyric acid (DABA), using an enzyme called ectoine synthase [38]. DABA is produced by certain bacteria and plants and can be chemically synthesized [39]. For fermentation, ectoine biosynthesis is realized in kinds of bacteria. Since ectoine is a compatible solute, it is produced by halophilic bacteria in response to high salt concentrations in their environments. Several halophilic bacteria are natural producers of ectoine in response to salt stress (Table 3). Among the natural producers, the best performer is *H. elongate* 1A01717, and this bacterium could produce 15.9 g/L of ectoine with glucose as the substrate. Some natural producers can convert glutamate to ectoine with L-aspartate as an intermediate (Figure 2). The key strategy was optimizing the fermentation conditions, such as the culture medium and NaCl concentration. With glutamate as the feedstock, the best performer is *H.salina* DSM 5928T, which could produce 14.86 g/L ectoine with a yield of 0.14 g/g at a productivity of about

0.32 g/(L.h) (Table 3). Polypeptone and yeast extract can also be the carbon sources for ectoine biosynthesis (Table 3). When glycerol was added to ectoine biosynthetic medium, it served as the source of acetyl-CoA (AcCoA) in the step converting L-2,4-diaminobutyrate to N-acetyl-2,4-diaminobutyrate (Figure 2). In the industry, halophiles are used to produce ectoine with fermentation on a large scale. However, high concentrations of salt could corrode the equipment [40]. It is urgent to realize ectoine biosynthesis under low-salt conditions. Luckily, with the development of metabolic engineering and new technologies, that is not a dream anymore.

Table 3. Summary of microbial production of ectoine.

Organism	Metabolic Engineering Strategies	Substrate	Titer (g/L)	Yield (g/g)	Productivity (g/(L.h))	Fermentation Strategy	Reference
Engineered cell factories							
E. coli ET11 (ectA:ectB:ectC = 1:2:1)	Introducing the *ectABC* gene cluster from *Halomonas venusta* ZH, regulating the copy number of *ectA*, *ectB* and *ectC*, eliminating byproduct metabolic pathways, optimizing the culture medium	glucose	53.2	0.33	1.11	fed-batch	[41]
E. coli Ect05	Introducing *ectABC* gene cluster from *Halomonas elongata* and a feedback-resistant L-aspartate kinase (lysC) from *Corynebacterium glutamicum*, deleting *thrA* and *iclR*, improving *ppc* expression by promoter replacement	glucose	25.1	0.11	0.84	fed-batch	[42]
E. coli S16-ectBAC	Introducing *ectABC* gene cluster from *Aestuariispira SWCN16*^{T} into *E. coli* BL21	sodium aspartate and glycerol	2.26			cell suspension bioconversion reactions in the optimum buffer	[43]
E. coli ET01	Introducing the *ectABC* operon from *Halomonas venusta* ZH, optimizing the fermentation process	glucose	47.8			fed-batch	[44]
E. coli BW25113	Introducing *ectABC* from *Halomonas elongata* and overexpressing these three genes with an arabinose-inducible promoter, optimizing the fermentation process	aspartate and glycerol	25.1	4.1 [b]	1.04	whole-cell catalysis	[45]
E. coli ECT2	Introducing the *ectABC* genes from *Halomonas elongata*, deleting *lysA*	glycerol and sodium aspartate	12.7	1.27	0.53	whole-cell catalysis	[46]
C. glutamicum ectABC^{opt}	Introducing the *ectABC* genes from *Pseudomonas stutzeri* and regulating their expression with different promoters and three linker elements	glucose, sucrose and fructose	65	0.19	2.3 [f]	fed-batch	[47]
C. glutamicum ECT-2	Introducing a codon-optimized synthetic *ectABCD* gene cluster from *Pseudomonas stutzeri*, inactivating the L-lysine exporter, optimizing the fed-batch process	glucose	4.5	0.24 [d]	0.28 [e]	fed-batch	[48]
C.glutamicum CB5L6	Introducing the *ectBAC* cluster from *Pseudomonas stutzeri*, deleting *pck*, *ldh* and *sugR*, improving the precursor supply (overexpression of Ecasd and CglysCS301Y), constructing repressor libraries (*BetI* from *E. coli* and *LmrA* from *B. subtilis*)	glucose	45.52	0.25		fed-batch	[49]

Table 3. *Cont.*

Organism	Metabolic Engineering Strategies	Substrate	Titer (g/L)	Yield (g/g)	Productivity (g/(L.h))	Fermentation Strategy	Reference
H. hydrothermalis Y2/ΔectD/ΔdoeA	Identifying the pathways for ectoine synthesis and catabolism, deleting genes involved in ectoine catabolism (EctD and DoeA) and Na+/H+ antiporter (Mrp), optimizing the culture medium	monosodium glutamate	10.5	0.21		fed-batch	[50]
H. bluephagenesis TD-ADEL-58	Overexpressing three clusters related to ectoine biosynthesis, including *ectABC*, *lysC* and *asd*, deleting byproduct biosynthetic pathways	glucose	28	0.21	1.0	fed-batch	[51]
		Natural producers					
Chromohalobacter salexigens	Optimizing the medium composition, especially the C/N ratio, to regulate the metabolic pattern	glucose	4.21			fed-batch	[52]
Chromohalobacter salexigens	Producing ectoine with two continuously operated bioreactors, regulating the hyperosmotic conditions and thermal stress	glucose	8.2		2.1	fed-batch	[40]
Brevibacterium sp. JCM 6894	Inducing ectoine biosynthesis with 2 M NaCl, fermentation with non-sterilized medium	polypepton and dried yeast extract	2.4			flask	[53]
Brevibacterium epidermis DSM20659	Optimizing the fermentation conditions and the extraction technology	monosodium glutamate	8	0.05	0.08 [e]	fed-batch	[54]
H. boliviensis LC1^T	Optimizing NaCl concentrations and the medium for fed-batch cultivations	glucose, monosodium glutamate	4.3	0.07 [c]	0.12 [e]	fed-batch	[35]
H. boliviensis LC1^T	Optimizing the nutrient parameters in the fed-batch fermenter	glucose, monosodium glutamate	9.2		0.26 [e]	fed-batch	[55]
H. salina BCRC17875	Optimizing the agitation speed and medium composition	yeast extract	13.94			fed-batch	[56]
Sinobaca sp. H24	Isolating an ectoine producer from soil, optimizing culture medium, identifying the genes involved in ectoine biosynthesis	yeast extract, glycerol	0.01			flask	[57]
H. salina DSM 5928	Optimizing the culture medium and NaCl concentration	monosodium glutamate	6.9		0.33 [e]	batch	[58]
H. elongata DSM2581	Two nanostructures, multiwalled carbon nanotube (MWCNT) and iron oxide nanoparticle (Fe2 O3 NPs), to increase the availability of the substrate	glucose	14.25			batch	[59]
Marinococcus sp. *MAR2*	Optimizing the culture condition with response surface methodology (RSM) and a fed-batch strategy	yeast extract	5.6		0.16 [e]	fed-batch	[60]
H. salina DSM 5928T	Optimizing the two-step fermentation conditions: growing of cells and production of ectoine by resting cells	monosodium glutamate	14.86	0.14	0.32 [e]	batch	[61]
Marinococcus sp. *ECT1*	Developing semi-synthesized medium (YAMS medium), optimizing the yeast extract concentrations	yeast extract	2.5			batch	[62]
H. brevibacterium sp. JCM 6894	Optimizing the conditions for ectoine biosynthesis	polypepton, glucose, yeast extract	2.5				[63]

Table 3. *Cont.*

Organism	Metabolic Engineering Strategies	Substrate	Titer (g/L)	Yield (g/g)	Productivity (g/(L.h))	Fermentation Strategy	Reference
H. campaniensis G8-52 (CCTCCM2019777)	Developing a higher ectoine producer by multiple rounds of UV mutation, identifying the key mutations (orf00723 and orf02403 (lipA)) related to ectoine biosynthesis	sodium L-glutamate	1.51			flask	[64]
H. elongata DSM 2581^{T}	Testing NaCl influence on ectoine biosynthesis, revealing higher NaCl concentration activating genes involved in the pentose phosphate pathway, Entner–Doudoroff pathway, flagellar assembly pathway, ectoine metabolism, repressing genes involved in the tricarboxylic acid cycle and fatty acid metabolism		12.91				[65]
H. boliviensis DSM 15516(T)	Optimizing the conditions for two-step fermentation and producing ectoine with milking process	glucose	8.9		0.38 [e]	fed-batch	[66]
P. halophilum DSM 102817^{T}	Optimizing the culture medium, developing strategies for ectoine isolation	glucose	0.41 [a]			flask	[67]
H. elongate 1A01717	Optimizing the ectoine extraction and purification process	glucose	15.9			fed-batch	[68]

[a] represents that the data were derived by calculating according to the literature. [b] g/g DCW. c g/g DCW calculated according to the literature. [d] g/g substrate calculated according to the literature. [e] calculated according to the literature. [f] at the beginning of the feed phase.

For metabolic engineering of bacteria to produce ectoine, two strategies are used. One approach is to introduce the genes that encode the enzymes involved in the ectoine biosynthetic pathway into a bacterial host that has a relatively clear genome background and well-developed gene operation method. For example, the *ectABC* genes, encoding the three enzymes required for ectoine biosynthesis, are cloned from *Halomonas elongata* and introduced into a bacterial host such as *E. coli* [44]. This is combined with additional manipulation to increase precursor L-aspartate production. According to the literature, the best engineered strain for ectoine biosynthesis is *C. glutamicum* ectABC^{opt}, carrying the ectoine pathway from *Pseudomonas stutzeri* that was expressed from synthetic promoters. After fermentation condition optimization, *C. glutamicum* ectABC^{opt} produced about 65 g/L of ectoine with a productivity of 2.3 g/(L.h) at the beginning of the feed phase [47]. An engineered *E. coli* strain ET11 produced 53.2 g/L of ectoine with a yield of 0.33 g ectoine/g glucose during fed-batch fermentation [41] (Table 3). The metabolic engineering strategies they used were introducing the *ectABC* gene cluster from *Halomonas venusta* ZH, regulating the copy numbers of *ectA*, *ectB* and *ectC* and eliminating byproduct metabolic pathways. For improving the ectoine production further, they optimized the fermentation medium as well. In summary, metabolic engineering strategies have yielded promising results for realizing ectoine biosynthesis for industrial use.

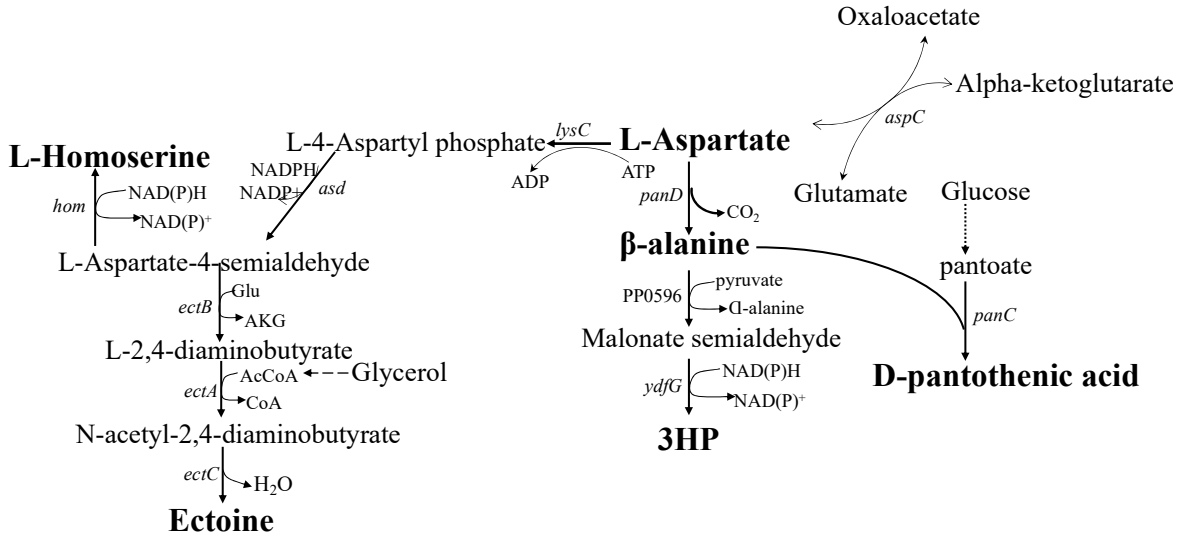

Figure 2. Pathways for producing L-aspartate derivatives with L-aspartate as the substrate. *lysC*, aspartokinase; *asd*, aspartate-semialdehyde dehydrogenase; *hom*, L-homoserine dehydrogenase; *ectB*, L-2,4-diaminobutyrate transaminase; *ectA*, 2,4-diaminobutyrate acetyltransferase; *ectC*, ectoine synthase; *panD*, aspartate decarboxylase; PP0596, β-alanine-pyruvate transaminase; *ydfG*, 3-hydroxyacid dehydrogenase; *panC*, pantothenate synthetase; *aspC*, aspartate transaminase.

2.4. Developing Cell Factories to Produce 3-Hydroxypropionate

3-hydroxypropionate (3-HP) is a naturally occurring organic acid and a precursor chemical to produce various value-added chemicals such as acrylates, acrylic acid, malic acid and 1,3-propanediol [69]. One potential application of 3-HP is as a building block chemical for biodegradable polymers, potentially replacing petroleum-based plastics in environmentally conscious products [70]. The global market for 3-HP and its related compounds is expected to grow significantly in the coming years. According to a report by marketwatch, the market for 3-HP was 117.14 million USD in 2022 and is expected to reach 153.81 million USD by 2028 with a CAGR of 4.64% (https://www.marketwatch.com/press-release/3-hydroxypropionic-acid-market-research-2023-2030-2023-06-15, accessed on 3 May 2023). This growth is driven by the increasing demand for sustainable and eco-friendly materials, as well as the increasing investment in renewable chemicals and biofuels.

Currently, 3-HP is produced via chemical synthesis and cell factory. 3-HP can be produced through chemical synthesis using acrolein, formaldehyde and hydrogen cyanide [71]. This method is not economically feasible due to the high cost of raw materials and environmental concerns. For realizing 3-HP biosynthesis with bacteria, many researchers have focused on optimizing the 3-HP biosynthetic pathway and tried different feedstocks to increase the titer and yield of 3-HP during fermentation (Table 4). The feedstocks can be a single sugar (such as glucose, xylose, glycerol, malonate, acrylic acid, 1,3-propanediol, ethanol and sorbitol), a sugar combination (glycerol and acetate, glucose and cellobiose) or a complex mixture (such as fatty acids (FAs), mechanically refined corn stover hydrolysate) (Table 4). After β-alanine is produced from L-aspartate, it is converted to 3-HP via malonate semialdehyde, an important intermediate for 3-HP biosynthesis. Except 1,3-propanediol and acrylic acid, all of the substrates mentioned above can join 3-HP biosynthesis via malonate semialdehyde (Figure 3). Glycerol is the most promising substrate for 3-HP biosynthesis since it is a byproduct of biodiesel and just needs two steps to complete the biosynthetic process. *K. pneumoniae* is the natural producer of 3-HP. After overexpressing of PuuC, the best engineered performer of *K. pneumoniae* could produce 102.61 g/L of 3-HP with glycerol as the carbon source [72]. *E. coli* is the most popular strain used for engineering, and it is modified to produce 3-HP from kinds of sugars as well (Table 4). To date, the best one is introducing *dhaB1234*, *gdrAB* and *ydcW* from *K. pneumoniae* into *E. coli* to realize 3-HP biosynthesis with glycerol as substrate, and the titer has reached

76.2 g/L at a productivity of 1.89 g/(L.h) [73]. That is promising for industrial use. Yeast has also been engineered to produce 3-HP via the malonyl-CoA pathway, and the titer has reached 71.09 g/L, which is the highest value with glucose as the substrate [74]. The interesting thing is that 3-HP biosynthesis was finished in the mitochondria. Except overexpressing malonyl-CoA reductase (MCR), they also optimized the expression of POS5 and IDP1 to improve NADPH supply. In addition, they found an ACC1 mutant could improve 3-HP production as well. When 1,3-propanediol is used as the substrate, there are only two steps needed to realize 3-HP biosynthesis, and the best performer is engineered *Halomonas bluephagenesis* TD27, which could produce 154 g/L of 3-HP with a yield of 0.93 g/g 1,3-propanediol (Table 4). The metabolic engineering strategies were deleting the 3-HP degradation pathway and overexpressing alcohol dehydrogenases (AdhP) to improve 3-HP biosynthesis [73]. *Halomonas bluephagenesis* is promising for industrial use since it can be cultured under an open and unsterile condition with continuous process [75]. Except the organisms mentioned above, 3-HP biosynthesis has also been realized in *Schizosaccharomyces pombe*, *Lactobacillus reuteri*, *Debaryomyces hansenii*, *Rhodococcus erythropolis*, *Lentilactobacillus diolivorans* and *Gluconobacter oxydans* (Table 4). The chassis cells and its cultivation conditions have great influence on 3-HP biosynthesis. Above all, it is promising to realize green biosynthesis of 3-HP via metabolic engineering in the industry.

Table 4. Summary of microbial production of 3-HP.

Organism	Metabolic Engineering Strategies	Substrate	Titer (g/L)	Yield (g/g)	Productivity (g/(L.h))	Fermentation Strategy	Reference
Engineered cell factories							
E. coli FA08	Optimizing the FA utilization pathway and fermentation conditions, introducing 3-HP biosynthesis module and balancing the carbon flux to maximize 3-HP production	fatty acids (FAs)	52	1.56		fed-batch	[76]
E. coli ZJU-3HP01	Developing a dual-substrate fermentative strategy, balancing the activity between glycerol dehydratase and aldehyde dehydrogenase with glucose added	glycerol and glucose	17.20			fed-batch	[77]
E. coli WL (pTac15kBAB, p100Rkyd)	Introducing a glycerol-dependent 3-HP biosynthetic pathway (*dhaB1234*, *gdrAB* and *ydcW*) from *Klebsiella pneumoniae*, regulating the expression *of ydcW*, optimizing the fed-batch fermentation conditions	glycerol	76.2	0.457	1.89	fed-batch	[73]
E. coli W DUBGK	Identifying the 3-HP-tolerant *Escherichia coli* strain among nine strains according to their growth in the presence of 25 g/L of 3-HP, introducing the 3-HP biosynthetic pathway into *E. coli* W, overexpressing them on plasmids	glycerol	41.5	0.61 [a]	0.86	fed-batch	[78]
E. coli PSO119	Overexpressing pyruvate aminotransferase, 3-hydroxyacid dehydrogenase, L-aspartate-1-decarboxylase, L-alanine aminotransferase, phosphoenolpyruvate carboxylase and alanine racemase, adaptive evolution, deleting L-valine transaminase, developing a dual-substrate fermentative strategy	glucose and xylose	29.1	0.22		fed-batch	[79]

Table 4. *Cont.*

Organism	Metabolic Engineering Strategies	Substrate	Titer (g/L)	Yield (g/g)	Productivity (g/(L.h))	Fermentation Strategy	Reference
E. coli JHS01304	Overexpressing *galP* and *gpsA*, analyzing the metabolome, deleting exogenous GPD1	glucose and xylose	37.6	0.17	0.63	fed-batch	[80]
Aspergillus niger An3HP9/pyc2/ ald6aΔ/3HP-6	Introducing the β-alanine biosynthetic pathway, identifying and modifying the genetic targets according to proteomic and metabolomic analysis, optimizing the fermentation conditions	corn stover hydrolysate	36.0	0.48 [b]			[81]
E. coli C43 (DE3) ZXP05	Developing malonic acid transporter mutants via directed evolution and enzyme-inhibition-based high throughput screening approach	malonate	20.08	1.55 [a]			[82]
E. coli Q2186	Directed evolution of rate-limiting enzyme MCR-C and fine tuning of MCR-N expression level, optimizing the fermentation conditions	glucose	40.6	0.19		fed-batch	[83]
E. coli SH-BGK1	Modulating the expression level of glycerol dehydratase (DhaB), alpha-ketoglutaric semialdehyde dehydrogenase (KGSADH) and glycerol dehydratase reactivase (GDR)	glycerol	38.7			fed-batch	[84]
E. coli JHS00947 expressing *L. brevis dhaB* and *dhaR* and *E. coli aldH*	Overexpression of *dhaB* and *dhaR* from *Lactobacillus brevis* KCTC33069 and *aldH* from *E. coli*, two-step feeding strategy	glycerol	14.3		0.26	fed-batch	[85]
E. coli SH501_E209Q/ E269Q	Developing variants of an aldehyde dehydrogenase (GabD4) from *Cupriavidus necator*	glycerol	71.9		1.8	fed-batch	[86]
E. coli JHS01300/pELDRR + pCPaGGRm	Deleting *ptsG*, overexpressing *xylR*, *GPD1* and *GPP2* genes from *S.cerevisiae*, *dhaB1B2B3* and *dhaR1R2* from *Lactobacillus brevis* and *aldhH* from *Pseudomonas aeruginosa*	glucose and xylose	29.4	0.36	0.54	fed-batch	[87]
E. coli JHS_Δgypr-PT7	Overexpressing *puuC* with a strong promoter, deleting *puu* operon repressor gene, *puuR*	co-fermentation of glucose and xylose	53.7			fed-batch	[88]
E. coli BEP113	Overexpressing *AdhEMut*, *mcr* from *Chloroflexus aurantiacus* and *dtsR1*, *accBC* from *Corynebacterium glutamicum*, modulating *pntAB* expression	ethanol	13.17	0.57			[89]
S. cerevisiae ST687	Integrating multiple copies of malonyl-CoA reductase (MCR) from *Chloroflexus aurantiacus* and phosphorylation- and acetyl-CoA carboxylase ACC1 genes into the chromosome, overexpressing native pyruvate decarboxylase PDC1, aldehyde dehydrogenase ALD6 and acetyl-CoA synthase from *Salmonella enterica* SEacs (L641P), engineering glyceraldehyde-3-phosphate dehydrogenase to increase NADPH supply, ^{13}C metabolic flux analysis	glucose	9.8	0.13 [a]		fed-batch	[90]

Table 4. *Cont.*

Organism	Metabolic Engineering Strategies	Substrate	Titer (g/L)	Yield (g/g)	Productivity (g/(L.h))	Fermentation Strategy	Reference
S. cerevisiae N3IP_2	Producing 3-HP in the mitochondria by overexpressing malonyl-CoA reductase (MCR) in the mitochondria, overexpressing POS5 and IDP1 to improve NADPH supply, overexpressing of an ACC1 mutant to improve 3-HP production	glucose	71.09	0.23	0.71	fed-batch	[74]
S. cerevisiae SH18	Genome integration of MCR-C encoding C-terminal of MCR, improving supply of malonyl-CoA and NADPH by overexpressing MPCox, RtCIT1, YHM2, MmACL/AnACL, ACC1, MDH3, RtME, PYC1, IDP2, ZWF1, GND1, TKL1 and TAL1, modulating the expression of a fatty acid synthase gene FAS1 with a glucose concentration-sensitive promoter PHXT1	glucose	56.5	0.31	0.53	fed-batch	[69]
Pichia pastoris PpHP6	Introducing and engineering the *mcr* gene from *Chloroflexus aurantiacus*, improving NADPH and malonyl-CoA supply by overexpressing the ACC_{Yl} and $cPOS5_{Sc}$, optimizing the fermentation conditions	glycerol	24.75	0.13	0.54	fed-batch	[91]
K. pneumoniae with *YneI* overexpression	Overexpressing aldehyde dehydrogenase, YneI and YdcW	glycerol	2.4			shake-flask culture	[92]
K. pneumoniae with aldehyde dehydrogenases (ALDH) from *Bacillus subtilis*	Introducing aldehyde dehydrogenases (ALDH), DhaS from *B.subtilis*	glycerol	18				[93]
K. pneumoniae Q1643	Overexpressing glycerol dehydratase, its reactivation factor (*dhaB123*, *gdrA* and *gdrB* from *K. pneumoniae*), aldehyde dehydrogenase (*aldH* from *E. coli*), deleting *dhaT* and *yqhD*	glycerol	2.03			flask culture	[94]
K. pneumoniae ΔadhPΔpflB (pTAC-puuC)	Deleting *adhP* and *pflB*, overexpressing *puuC*	glycerol	66.91	1.40		fed-batch	[95]
K. pneumoniae -T7 (pET28a-puuC)	Developing the T7 expression system and overexpressing *puuC*	glycerol	67.59	0.5632		fed-batch	[96]
K. pneumoniae	Overexpressing *ald4* and *dhaB*, optimizing the fermentation conditions	glycerol and glucose	3.77			flask	[97]
K. pneumoniae with *L. reuteri pduP* overexpression	Overexpressing a *pduP* gene from *Lactobacillus reuteri*	glycerol	1.38			batch fermentation	[98]
K. pneumoniae ΔdhaTΔyqhD overexpressing both *PuuC* and *DhaB*	Deleting *dhaT* and *yqhD*, overexpressing *puuC* and *dhaB*	glycerol	>28	>0.4		fed-batch	[99]
K. pneumoniae (p3tac-PuuC)	Overexpressing *puuC*, optimizing fermentation conditions, mathematical model analysis	glycerol	102.61			fed-batch	[72]

Table 4. *Cont.*

Organism	Metabolic Engineering Strategies	Substrate	Titer (g/L)	Yield (g/g)	Productivity (g/(L.h))	Fermentation Strategy	Reference
K. pneumoniae Δldh1Δldh2Δpta (pTAC-puuC)	Overexpressing *puuC*, deleting the pathways for lactate and acetate biosynthesis according to metabolix flux analysis, optimizing fermentation conditions, describing a flux distribution model of glycerol metabolism	glycerol	83.8	0.54		fed-batch	[100]
Schizosaccharomyces pombe overexpressing Cut6p and CaMCR	Overexpressing the *S. pombe* acetyl-CoA carboxylase (Cut6p) and the malonyl-CoA reductase from *Chloroflexus aurantiacus* (CaMCR) with the *S. pombe* hsp9 promoter, optimizing the fermentation conditions	glycerol and acetate	7.6				[101]
Schizosaccharomyces pombe	Dissecting the *mcr* gene from *Chloroflexus aurantiacus* into two functionally distinct fragments and balancing the activity of them, overexpressing aldehyde dehydrogenase, acetyl-CoA synthase and pantothenate kinase, introducing beta-glucosidase	glucose and cellobiose	11.4	0.11 [b]		fed-batch	[102]
engineered *Halomonas bluephagenesis* TD27	Deleting the 3-HP degradation pathway, overexpressing alcohol dehydrogenases (AdhP)	1,3-propanediol	154	0.93	2.4	fed-batch	[75]
		Natural producers					
Lactobacillus reuteri	Optimizing fermentation conditions	glycerol	5.2		1.3	fed-batch	[103]
Lactobacillus reuteri DSM17938	Comparing the ability of three *Lactobacillus reuteri* strains to produce 3-HP, analyzing the influence of glycerol and metabolites on strains' physiological states and survival	glycerol			2		[104]
Debaryomyces hansenii WT39	Selecting strains with propionic acid as the substrate, making mutations with the low-energy ion N^+	glucose	62.42		1.30	flask	[105]
Rhodococcus erythropolis LG12	Isolating strains with acrylic acid as the substrate, optimizing the fermentation conditions	acrylic acid	17.5	1.11 [a]	0.22		[106]
Lentilactobacillus diolivorans	Optimizing the fermentation conditions	0.025 mol/mol glucose/glycerol	67.7			fed-batch	[107]
Gluconobacter oxydans ZJB09112	Optimizing the fermentation conditions	1,3-propanediol	76.3		1.5	fed-batch	[108]
K. pneumoniae and *Gluconobacter oxydans*	Developing a two-step process to produce 3-HP with glycerol	glycerol	60.5	0.50			[109]

[a] represents that the data were derived by calculating according to the literature. [b] the unit is C-mol 3-HP/C-mol sugars.

2.5. Current Process for Developing Cell Factories to Produce D-Pantothenic Acid

D-pantothenic acid is a water-soluble B-vitamin (Vitamin B5) that plays a crucial role in energy metabolism and the synthesis of various compounds, such as fatty acids, cholesterol and steroid hormones [110]. The global market of D-pantothenic acid was valued at about 460.3 million USD in 2020 and is expected to exhibit a CAGR of 6.19% over the forecast period (2021–2028). It is primarily driven by the application of D-pantothenic acid as an ingredient in dietary supplements and animal feed. It is also used in the production of cosmetics, pharmaceuticals and food additives. (https://www.globenewswire.com/en/news-release/

2021/12/14/2351835/0/en/At-6-2-CAGR-Global-Pantothenic-Acid-Market-to-Reach-US-750-7-Million-by-2028-Says-Coherent-Market-Insights-CMI.html, accessed on 3 May 2023).

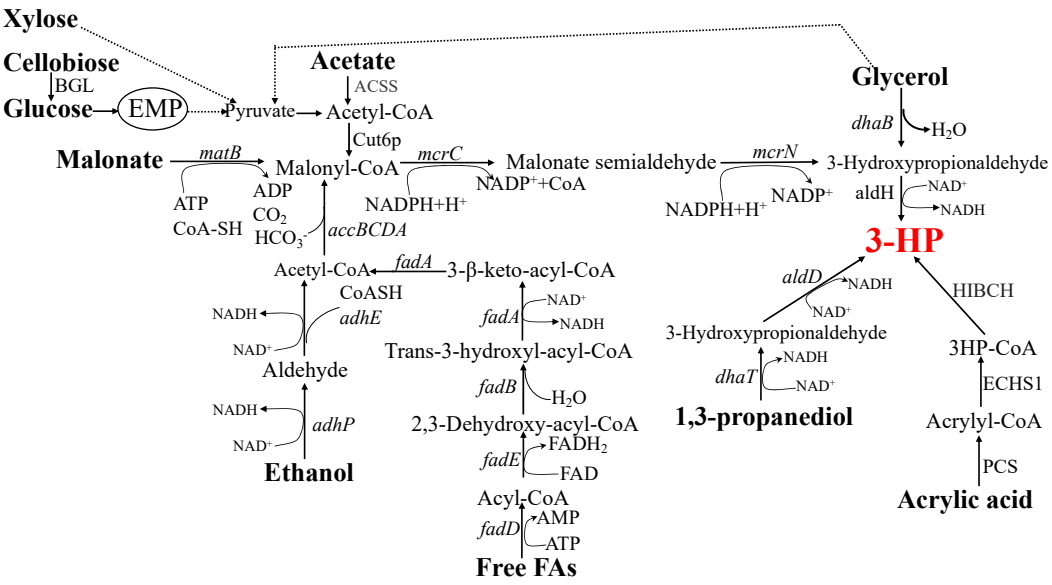

Figure 3. Biosynthetic pathways of 3-HP. The substrate used for 3-HP biosynthesis is marked in bold. *dhaB*, glycerol dehydratase; *aldH*, aldehyde dehydrogenase; *matB*, malonyl-CoA synthetase; *mcrC*, malonyl-CoA reductase C-domain; *mcrN*, malonyl-CoA reductase N-domain; *fadD*, fatty acyl-CoA synthetase; *fadE*, acyl-CoA dehydrogenase; *fadB*, α component of the fatty acid oxidation complex; *fadA*, β component of the fatty acid oxidation complex; *accBCDA*, acetyl-CoA carboxyltransferase complex; *adhP*, alcohol dehydrogenase; *adhE*, alcohol/aldehyde dehydrogenase; ACSS, acetyl-CoA synthetase; Cut6p, acetyl-CoA/biotin carboxylase; BGL, beta-glucosidase; *dhaT*, alcohol dehydrogenase; *aldD*, aldehyde dehydrogenase; PCS, acrylyl-CoA (propionyl-CoA) synthetase; ECHS1, enoyl-CoA hydratase, HIBCH, 3-hydroxyisobutyryl-CoA hydrolase.

Synthetic methods for producing D-pantothenic acid include chemical synthesis, enzymatic catalysis and microbial fermentation. Currently, D-pantothenic acid is mainly produced by chemical synthesis and enzymatic catalysis [111]. Chemical synthesis involves several steps and requires some toxic chemicals, such as hydrocyanic acid and sodium cyanide, which cause wastewater pollution [112]. For enzymatic catalysis, pantothenate synthetase can catalyze pantoate and β-alanine to produce D-pantothenic acid. For example, when pantothenate synthetase was overexpressed in *E. coli* or in *Bacillus megaterium* (*B. megaterium*), D-pantothenic acid was biosynthesized after pantoate and β-alanine were added into the culture medium (Table 5). The titer of D-pantothenic acid in *E. coli* was 97.1 g/L at a productivity of 3.0 g/(L.h) [113], while that in *B. megaterium* was about 45.56 g/L with fed-batch fermentation [114]. However, since pantoate is much more expensive from commercial sources, enzymatic process is not a good choice for D-pantothenic acid synthesis in the industry [115]. For microbial fermentation, D-pantothenic acid biosynthesis was realized with glucose and β-alanine as feedstocks since glucose could be converted to pantoate through the valine biosynthetic pathway combined with overexpression of *panB* from different kinds of organisms (Figure 2). With this strategy, *E. coli* DPAL 8 could produce 66.39 g/L of D-pantothenic acid with a yield of 0.27 g/g glucose after optimizing the fermentation conditions (Table 5). For genome modification, several genes involved in pantoate biosynthesis were overexpressed, such as *pck*, *maeB*, *ilvD*, *ilvBN* and *cycA*. Pathways for byproduct biosynthesis were deleted or downregulated in *E. coli* DPAL 8 [116]. L-isoleucine and citric acid are used for D-pantothenic acid biosynthesis also since they can increase ATP and NADPH supply via the TCA cycle [117]. L-isoleucine is also beneficial for improving the availability of CoA. When L-isoleucine and glucose were used to feed the engineered strain, the best performer, *E. coli* ECPA, could produce 39.1 g/L

of D-pantothenic acid with a yield of 0.175 g/g glucose at a productivity of 0.58 g/(L.h) (Table 5). Furthermore, some engineered strains can use glucose as the only substrate for D-pantothenic acid biosynthesis also, including *E. coli*, *C. glutamicum* and *Saccharomyces cerevisiae* (Table 5). Moreover, the highest titer of D-pantothenic acid reached 68.3 g/L with a yield of 0.36 g/g and a productivity of 0.794 g/(L.h) in *E. coli* DPA02/*pT-ppnk*. The metabolic engineering strategies were overexpressing *ppnk* and deleting genes involved in byproduct biosynthesis, such as *aceF* and *mdh* [118]. Overall, metabolic engineering is a powerful tool for realizing D-pantothenic acid commercialization.

Table 5. Summary of microbial production of D-pantothenic acid.

Organism	Metabolic Engineering Strategies	Substrate	Titer (g/L)	Yield (g/g)	Productivity (g/(L.h))	Fermentation Strategy	Reference
Engineered cell factories							
E. coli DPAL 8	Overexpressing *pck*, *maeB*, *ilvD*, *ilvBN* and *cycA*, decreasing the expression of *gdhA*, deleting *pta*, optimizing the fermentation conditions	glucose β-alanine	66.39	0.27 [b]		fed-batch	[116]
E. coli DPA02/pT-*ppnk*	Overexpressing *ppnk*, deleting *aceF* and *mdh*, optimizing the fermentation conditions	glucose	68.3	0.36	0.794	fed-batch	[118]
E. coli BL21(DE3) strain expressing pantothenate synthetase from *C.glutamicum*	Overexpressing pantothenate synthetases from *C. glutamicum*	pantoate and β-alanine	97.1		3.0	substrate added at the beginning	[113]
E. coli W3110 DPA-11/pTrc99A-*panB*-K25A/E189S-*panC*	Protein engineering of ketopantoate hydroxymethyltransferase from *C. glutamicum*, overexpressing *panB*, *CgKPHMT-K25A/E189S* and *panC*	glucose β-alanine L-isoleucine	41.17		0.65	fed-batch	[119]
E. coli W3110/pTrc99A-panB-panC	Optimizing the fermentation conditions, overexpressing *panB* and *panC*	L-isoleucine glucose	31.6	0.17 [a]	0.55 [a]	fed-batch	[120]
E. coli DPA-9/pTrc99a-panBC(C.G)	Overexpressing *panB*, *panC*, *panE* and *ilvC*, making mutations of *ilvG* and *coaA*, deleting *avtA* and *ilvA*, deregulating *ilvE*	β-alanine glucose,	28.45		0.40	fed-batch	[121]
Escherichia DPA21	Decreasing *ilvE* expression, overexpressing *ilvBN*, *glyA*, *pntAB*, *cyo*, *cyoA* and *serA*^{fbr}, optimizing the fermentation conditions according to comparative transcriptome and metabolomics analysis	citric acid, glucose, β-alanine	45.35	0.31	0.50 [b]	fed-batch	[122]
Bacillus megaterium BM-4	Overexpressing *panBC*, *panE*, *ilvBNC*, *ilvD*, *serA* and *glyA*	glucose, β-alanine	19.70	0.26	0.78 [b]	fed-batch	[114]
B. megaterium BM-1 (pantoate-β-alanine ligase (PBL))	Overexpressing *panC* from *B. subtilis*	pantoate and β-alanine	45.56			fed-batch	[115]
C. glutamicum Pan-4/pXtuf-*panBCD*$_{Bsu}$	Overexpressing *panBCD* from *B. subtilis*, *ilvBNC*, *aspB* and *aspA*, deleting *avtA*, *ilvE* and *ilvA*	glucose	18.62			5 L bioreactor	[123]
Saccharomyces cerevisiae DPA171	Enhancing the D-pantothenic acid biosynthetic pathway by adjusting the copy numbers of key genes, deleting bypass genes, balancing cofactor utilization, optimizing GAL-inducible system	glucose	4.1				[124]
Natural producers							
E. coli ECPA	Optimizing the fermentation conditions	L-isoleucine glucose	39.1	0.175	0.58 [a]	fed-batch	[117]

[a] represents that the data were derived by calculating according to the literature. [b] the yield is g D-pantothenic acid/g glucose.

2.6. Developing Cell Factories to Produce L-Homoserine

L-homoserine is an amino acid and functions as an intermediate in multiple metabolic pathways, including the synthesis of various essential amino acids, such as methionine and threonine, and the production of certain pharmaceuticals and specialty chemicals [125]. The market for L-homoserine is relatively small compared to other amino acids due to its inefficient production and expensive price [126].

In the industry, L-homoserine can be produced via chemical synthesis, enzymatic synthesis and microbial fermentation. Chemical synthesis is expensive and complicated, and enzymatic synthesis has limited scalability. Therefore, microbial fermentation is the most promising method for producing L-homoserine. Microbial sources of L-homoserine biosynthesis are bacteria such as *E. coli* and *C. glutamicum*, and glucose is usually used as the feedstock. For producing L-homoserine from glucose, the biosynthetic pathway is shown in Figure 2. Glucose is metabolized to L-aspartate (Figure 2) and then to L-homoserine catalyzed by aspartokinase (*lysC*), aspartate-semialdehyde dehydrogenase (*asd*) and L-homoserine dehydrogenase [127] (Figure 2). To date, the best producer of L-homoserine is *E. coli* W-18/pM2/pR1, and the titer could reach 110 g/L with a yield of 0.64 g/g at a productivity of 1.82 g/(L.h) (Table 6). The metabolic engineering strategies were improving precursor supply, such as OAA and L-aspartate, by overexpressing *glf*, *ppc*, *aspA*, *glk*, *asd*, *metL* and *rhtA* and decreasing byproduct biosynthesis, such as lactate and acetate, by deleting *lysA*, *thrB*, *metA*, *ldhA*, *adhE*, *pflB*, *ptsG*, *iclR* and *arcA*. They also deleted *lacI* and regulated key genes' expression with the lac promoter [126]. The fermentation strategy was fed-batch and two stage bioreaction: the growth stage and the production stage [126]. For L-homoserine biosynthesis, the highest productivity was 1.96 g/(L.h), and it was realized by engineering *E. coli* BW25113 after redox balance regulation and competitive and degradative pathway deletion [128]. Since *C. glutamicum* is successfully engineered to produce kinds of amino acids, some researchers have also engineered it to produce L-homoserine with different sugars. Among them, the best performer is *C. glutamicum* Cg18-1, which could produce 63.5 g/L of L-homoserine with a yield of 0.25 g/g glucose (Table 6). Their work focused on improving NADPH supply by regulating specific genes' expression, such as *pntAB* and *ppnK*, and pathway modification, such as enhancing the pentose phosphate pathway (PPP) and introducing the Entner–Doudoroff (ED) pathway [129]. Since the productivity was lower than the industry demand ($\geq$2 g/L/h) and the yield is less than 50% of the theoretical value, there is still a distance to achieve L-homoserine biosynthesis with a cell factory in the industry.

Table 6. Summary of microbial production of L-homoserine.

Organism	Metabolic Engineering Strategies	Substrate	Titer (g/L)	Yield (g/g)	Productivity (g/(L.h))	Fermentation Strategy	Reference
E. coli W-18/pM2/pR1	Overexpressing *glf*, *ppc*, *aspA*, *glk*, *asd*, *metL* and *rhtA*, deleting *lysA*, *thrB*, *metA*, *lacI*, *ldhA*, *adhE*, *pflB*, *ptsG*, *iclR* and *arcA*, optimizing the fermentation conditions	glucose	110.8	0.64	1.82	fed-batch	[126]
E. coli HOM-14	Overexpressing *thrB*, $thrA^{fbr}$, *ppc*, *aspA8*, *pntAB* and *rhtA*	glucose	60.1	0.42	1.25	fed-batch	[130]
E. coli H28	Overexpressing $thrA^{fbr}$, *thrABC*, *ppc*, *aspC*, *aspA* $lysC_{cgl}^{fbr}$, *rhtA pntAB*, asd_{tmo} and adh_{pae}, deleting *lacI*	glucose	85.29	0.43	1.78	fed-batch	[131]
E. coli LJL12	Overexpressing *thrA*, deleting *lysA*, *metA*, *thrBC*, *iclR*, *gltA*, *pykA* and *pykF*	glucose	35.8	0.35	0.82	fed-batch	[132]
E. coli SHL17	Overexpressing *pntAB*, *rhtA*, *ppc*, *thrA* and *asd*, introducing a hok/sok toxin/antitoxin system	glucose	44.4	0.21	0.93	fed-batch	[133]

Table 6. *Cont.*

Organism	Metabolic Engineering Strategies	Substrate	Titer (g/L)	Yield (g/g)	Productivity (g/(L.h))	Fermentation Strategy	Reference
E. coli HS15	Overexpressing *pntAB*, *rhtB*, *glk*, *zglf*, *ppc*, *aspC*, *gdhA*, *thrA*, *asd* and *aspA*, deleting *lysA*, *metA*, *thrB*, *lacI*, *ldhA*, *poxB*, *pflB* and *iclR*	glucose	84.1	0.50	1.96	fed-batch	[128]
C. glutamicum Cg18-1	Overexpressing $thrA^{S345F}$, *aspC*, pyc^{P458S}, $lysC^{T311I}$, *asd*, hom^{V59A}, *brnFE*, icd^{M1V}, $dapA^{M1V}$ and *gapN*, deleting *mcbR*, *metD*, *thrB*, *NCgl2688* and *metY*	glucose	63.5	0.25		fed-batch	[129]
C. glutamicum *Cg09−1*	Overexpressing *lysC*, *asd*, *hom*, *pyc*, *brnFE*, *lysCT311I* and *asd* from *C. glutamicum* and *aspC* and *thrAS345F* from *E. coli*, deleting *thrB*, *mcbR* and *metD*, decreasing the expression of *dapA* and *icd*	glucose	8.8			batch	[134]
Corynebacterium sp. 9366-EMS/329	Developing a mutant *Corynebacterium* sp. requiring threonine	sucrose	14.5			batch	[135]

3. Perspective

Metabolic engineering is a promising method for realizing desired products biosynthesis with a cell factory. Nowadays, except the natural producers, four kinds of microbials, *E. coli*, *B. subtilis*, *C. glutamicum* and *S. cerevisiae*, are popular for producing kinds of compounds with metabolic engineering since their genome backgrounds are relatively clear and the gene editing methods are well developed. There are several factors that block the commercializing of the engineered strains, and the detailed information is described as the following:

(1) metabolic engineering of the bacteria to increase the titer, yield and productivity of the target products

After the biosynthetic pathway is clear, the next step is optimizing the pathway to improve the titer and yield of the target product, usually by balancing the supply and consumption of the cofactors (NADH, NADPH, $FADH_2$), deleting the competitive pathways, regulating the expression of genes involved in the biosynthesis and increasing the key enzymes' activity as well as specificity. Shi et al. have developed a cell factory to produce isobutanol under anaerobic conditions with a high yield of 0.92 mol/mol glucose [136]. The strategies they applied were deleting competitive pathways, such as biosynthetic pathways of ethanol, acetate and lactate, regulating key genes' expression (*alsS*, *ilvC*, *ilvD*, *kivD* and *adhA*) with strong artificial promoters and increasing the conversion speed between NADH and NADPH by activating transhydrogenase and NAD kinase together. With the development of bioinformatics, pathway optimization becomes more rational and more accurate.

Aspartate ammonia-lyase, an important enzyme for L-aspartate biosynthesis, is allosterically regulated by L-aspartate. In addition, the activity of phosphoenolpyruvate carboxylase, catalyzing phosphoenolpyruvate to oxaloacetate, is also inhibited by a high concentration of L-aspartate. This problem should be solved for developing a high-performance cell factory for L-aspartate biosynthesis. Protein engineering is relatively difficult since it is time-consuming and usually unsuccessful. Luckily, with the development of new technologies (e.g., Alphafold), it becomes easier and more predictable. Fei et al. have developed a dual-fluorescence reporter system to screen L-aspartate-α-decarboxylase variants with a high-throughput method and found one mutant with increased activity and stability [137]. This mutant was further applied for β-alanine biosynthesis in *E. coli* Nissle 1917 [25]. The growth of the engineered strain is another factor that the researchers need to consider since it will influence the productivity of the target products. The engineered

strain for L-aspartate biosynthesis needs relatively enriched fermentation medium (yeast extract added) since the bacteria could not grow well with mineral medium only [15].

After the target product is biosynthesized in the cell, the next step is to transfer it from intracellular to extracellular in order to release its inhibition to the enzymes involved in its biosynthesis. To realize this, the secretion mechanism of the target product needs to be investigated. For L-aspartate, we only know that its uptake is realized by the C4-dicarboxylate transporter [138]. However, little information is given for its secretion. Ghiffary et al. have found a β-alanine exporter in *C. glutamicum*, which had a great influence on the titer of β-alanine [24].

(2) fermentation condition optimization

Some bacteria are natural producers of the target product, while the fermentation strategies of those bacteria are not well developed. For example, many halophilic bacteria, such as *Halomonas* sp. [51] and *Sinobaca* sp. [57], have the ability to produce ectoine, but we have little data about its scale-up fermentation.

The engineered strain cannot enter into the industry until the fermentation cost is competitive with the up-to-date synthetic method. The fermentation cost includes the medium cost for strain growth, the substrates for target product biosynthesis and the fermentation conditions, such as sterile treatment, pH control, dissolved oxygen (DO) control and feeding strategy. Some researchers have focused on optimizing the fermentation process. For example, *E. coli* ET01 is an engineered producer for ectoine biosynthesis. For improving ectoine production, Dong et al. have optimized the fermentation condition, such as feeding strategies and DO levels. Finally, 47.8 g/L of ectoine has been produced with two-stage fermentation [44]. A *Clostridium pasteurianum* strain for 1,3-propanediol biosynthesis developed by Dr. Zeng's group could be fermented with medium of low cost and unsterile treatment. The fermentation cost was decreased by 50% [139]. As a renewable energy source, biomass is promising to be the substrate for valuable product biosynthesis. Nowadays, many researchers try to find an economic way to break the biomass into monosaccharides with less toxic side products produced. If it is successful, the fermentation cost will be dramatically decreased.

(3) downstream processing

The purification cost is another important factor needing to be taken into account. The methods for purification are determined by the characteristic of the target product, the culture medium and the side products. Ectoine was produced by *H. elongata* with fermentation. Chen et al. have designed a strategy with multiple steps to purify it, including microfiltration, desalination, cation exchange, decolorizing with activated carbon, refining with methanol, crystallization and centrifugation. However, the yield is only 43%, and the process is time consuming. The method is not ready for commercial use [68].

Above all, there are many scientific problems that should be solved before a biosynthesized product moves into commercialization. Luckily, with the development of novel technologies, such as synthetic biology and bioinformatics, the engineering process becomes predictable and faster. According to the literature, to improve 3-HP production in *Aspergillus niger*, several genes were selected and modified according to proteomic and metabolomic analysis [81]. After protein engineering of ketopantoate hydroxymethyltransferase from *C. glutamicum* and overexpressing *panB*, *CgKPHMT-K25A/E189S* and *panC*, *E. coli* W3110 DPA-11 could produce 41.17 g/L of D-pantothenic acid [119]. Moreover, the fermentation condition of D-pantothenic acid in *Escherichia* DPA21 was optimized according to comparative transcriptome and metabolomics analysis [122]. In the future, with the development of genome editing technology such as CRISPR/Cas9, some natural producers of 3-HP can be engineered. As a result, this gives us more choice to realize 3-HP biosynthesis in the industry.

The cost of fermentation and downstream purification has great influence on the product's selling price and are of great concern to the fermentation company. We believe these problems will be solved by the cooperation of researchers from different areas.

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