Received: 1 March 2021 | Accepted: 3 May 2021

DOI: 10.1002/bab.2181

REVIEW ARTICLE

WILEY

Production of *myo*-inositol: Recent advance and prospective

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Invited review for the journal of *Biotechnology and Applied Biochemistry*

Abstract

Myo-inositol and its derivatives have been extensively used in the pharmaceutics, cosmetics, and food and feed industries. In recent years, compared with traditional chemical acid hydrolysis, biological methods have been taken as viable and cost-effective ways to *myo*-inositol production from cheap raw materials. In this review, we provide a thorough overview of the development, progress, current status, and future direction of *myo*-inositol production (e.g., chemical acid hydrolysis, microbial fermentation, and in vitro enzymatic biocatalysis). The chemical acid hydrolysis of phytate suffers from serious phosphorous pollution and intricate product separation, resulting in *myo*-inositol production at a high cost. For microbial fermentation, creative strategies have been provided for the efficient *myo*-inositol biosynthesis by synergetic utilization of glucose and glycerol in *Escherichia coli*. in vitro cascade enzymatic biocatalysis is a multienzymatic transformation of various substrates to *myo*-inositol. Here, the different in vitro pathways design, the source of selected enzymes, and the catalytic condition optimization have been summarized and analyzed. Also, we discuss some important existing challenges and suggest several viewpoints. The development of in vitro enzymatic biosystems featuring low cost, high volumetric productivity, flexible compatibility, and great robustness could be one of the promising strategies for future *myo*-inositol industrial biomanufacturing.

KEYWORDS
in vitro biocatalysis, industrial biomanufacturing, *myo*-inositol

1 | INOSITOL: PROPERTIES, FUNCTIONS, AND APPLICATIONS

Inositol (1,2,3,4,5,6-cyclohexanehexol) was originally isolated from muscle extracts by Johanes Joseph Scherer (1814–1869) in 1850.[1,2] Inositol is one of the most ancient components of living beings with multiple functions in eukaryotes.[3] In addition to its biological role, it can also be a crucial part of structural lipids and secondary messengers. The molecular formula and molecular weight of inositol are $C_6H_{12}O_6$ and 180.16 g/mol, respectively. The appearance of inositol is a white crystalline powder, which is relatively stable in the air. The sweet taste is about 50% of sucrose when compared with 10% solutions. It is highly soluble in water, slightly soluble in ice acetic acid, ethanol, glycol, and glycerin, cannot be soluble in chloroform, ether, and other organic solvents. Inositol has theoretically available stereoisomers in nine different ways, including *myo*-inositol, *scyllo*-inositol, *muco*-inositol, *epi*-inositol, *neo*-inositol, *allo*-inositol, D-*chiro*-inositol, L-*chiro*-inositol, and *cis*-inositol (Figure 1).

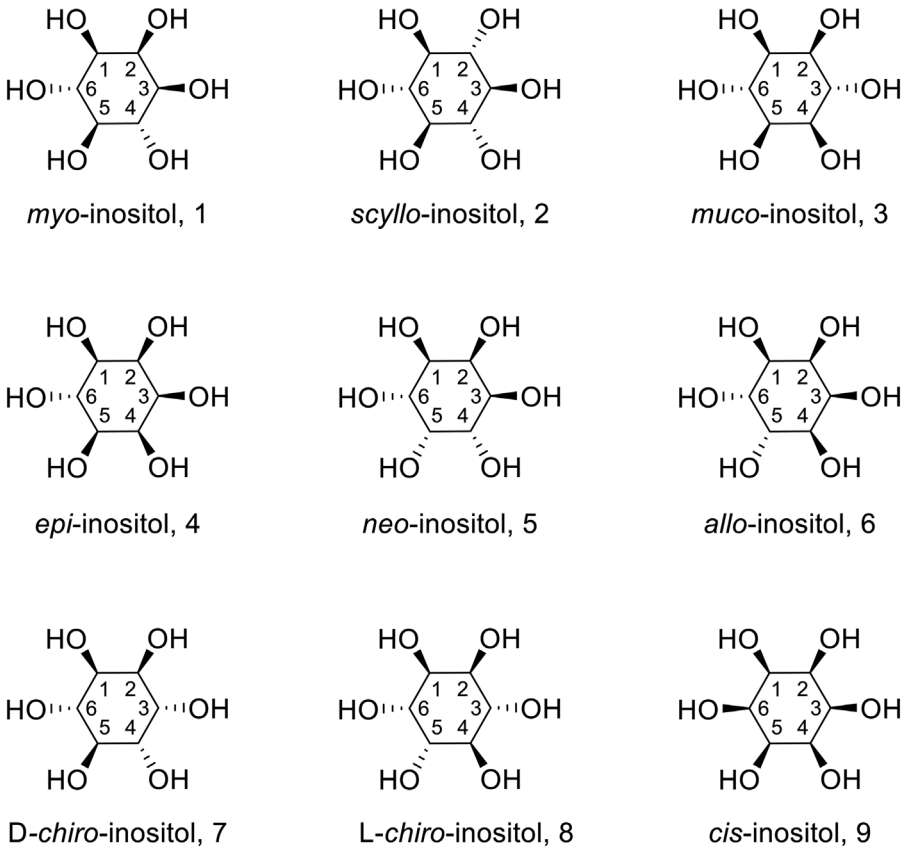

FIGURE 1 The structures of nine isomers of *myo*-inositol

Myo-inositol (*cis*-1,2,3,5-*trans*-4,6-cyclohexanehexol), the most abundant form among inositol isomers, is far and wild distributed in the tissues without coma, mammals, plants, fungi, and some bacteria.[4] In humans, *myo*-inositol and its derivatives play very important roles in many fields, such as adjustment of glucose metabolism, chromatin and cytoskeleton remodeling, gene transcription, proliferation, and apoptosis.[5,6] That is to say, the *myo*-inositol network possesses many biological functions. It enables the cells to have proper responses to many stress conditions.[7] The treatment with different inositol isomers has been demonstrated to generate prominent therapeutic effects in several illnesses, for instance, polycystic ovary syndrome,[2,8] cancer,[9] diabetes,[10,11] and neurological disorders.[12,13] Furthermore, the toxicity of *myo*-inositol has been investigated as the lethal dose 50 in mouse is 10,000 mg/kg body weight with oral administration.[14] The safety of *myo*-inositol has also been assessed at the given doses of *myo*-inositol ranging from 4 to 30 g/day for nearly 1 year.[15]

Based on its various functions, *myo*-inositol is extensively used in the fields of food and feed, pharmaceutics, cosmetics, and industry. In the pharmaceutical industry, it is a universal adulterant for plenty of pharmacons.[16] *Myo*-inositol is also applied to treat cancer,[9] liver cirrhosis,[17] fatty liver,[18] and diabetes.[6,19] In the cosmetics industry, *myo*-inositol can be used to promote cell growth and prohibit cell aging. In the food industry, it is primarily applied as an additive in high-end energy drinks and foodstuffs because of its functions as a nutraceutical and functional sweetener. *Myo*-inositol is also an important aquatic feed additive for aquatic animals lacking its synthesis ability.[20] Additionally, as an important intermediate in the industrial field, *myo*-inositol is used to produce glucuronic acid,[21] glucaric acid,[22] and *scyllo*-inositol.[23–25]

Overall, the demand for *myo*-inositol is increasing and the current worldwide market is about 15 thousand tons per year, affording a global market of around USD 60 million by 2020. For the production of *myo*-inositol, different methods including the traditional chemical acid hydrolysis, microbial fermentation, and in vitro enzymatic biosystems have been applied (Table 1).

2 | CHEMICAL PRODUCTION OF *MYO*-INOSITOL

At present, China and Japan are the main countries for *myo*-inositol production. The main raw material for *myo*-inositol production is phytate (inositol hexakisphosphate,

TABLE 1 Comparison of the different methods for *myo*-inositol production

Methods	Chemical acid hydrolysis	Microbial fermentation	in vitro enzymatic biosystems
Advantages	1. Abundant resources of raw materials 2. Matured production experience	1. Green and sustainable production 2. Advanced biological techniques 3. Varieties of bio-production platform	1. Decreased phosphorous pollution 2. High production titer and yield 3. Much less production cost
Disadvantages	1. Strict requirements on equipment 2. Serious phosphorus-rich wastewater 3. Complicated production steps 4. Low production yield 5. High production cost	1. Hard to modulate flux distribution 2. Hard to accumulate precursor G6P	1. High cost for enzyme production 2. Enzyme instability

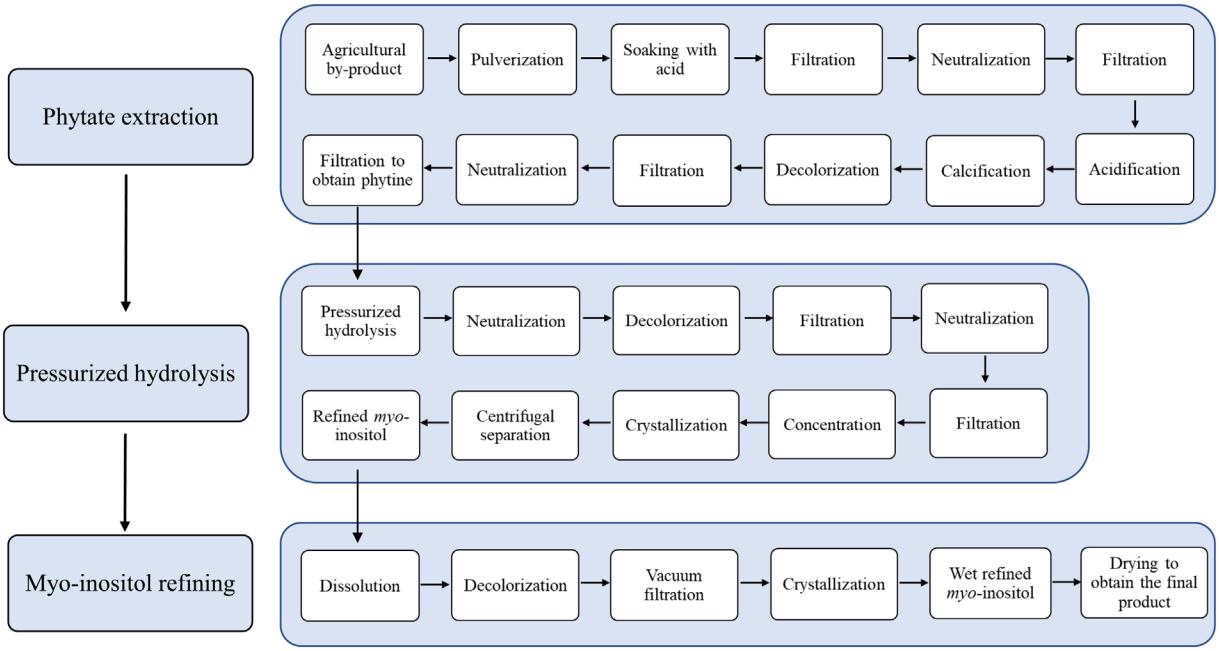

FIGURE 2 The flow chart of pressurized hydrolysis method for *myo*-inositol production

IP6). Phytate is abstracted from corn-soaking water and rice bran-soaking water during the processing of agricultural products. The traditional method of producing *myo*-inositol is pressurized acid hydrolysis of phytate. The production process of *myo*-inositol is shown in Figure 2. Phytate is firstly purified through a chain of steps, comprising acid soaking, neutralization, and filtration. Then, phytate is hydrolyzed to generate *myo*-inositol with the help of inorganic acid under high temperature and high pressure. The crude *myo*-inositol is concentrated and crystallized to obtain the refined *myo*-inositol. However, these production steps are complicated and cumbersome, with low yield and high production input; the steps such as acid soaking and pressurization have strict requirements on equipment; the utilization rate of raw materials is limited by the pressure in the process; it has a serious impact on the ecological environment because six phosphate ions are generated accompanying with one *myo*-inositol molecule production. Therefore, this production method by the pressurized acid hydrolysis of phytate suffers from serious pressure for phosphorus-rich wastewater treatment and complicated steps for the product of *myo*-inositol separation.

In recent years, a new process of atmospheric hydrolysis has been developed. The raw material is still phytate. Under the atmospheric conditions, a catalyst composed of glycerin, urea, and calcium carbonate is added into a 40% aqueous solution of phytate at a mass ratio of 1.0:5.5. After heating at 140°C for 3 h, the hydrolysate is cooled down to about 90°C, filtered, and crystallized at room temperature, and the crystals are washed with absolute ethanol and dried at 90°C for 50 min to obtain the refined *myo*-inositol. Compared with the conventional method, the atmospheric hydrolysis method avoids some restrictions and losses caused by pressurization, but the use of

catalyst brings many new problems. Although it saves costs compared with the traditional pressurized acid hydrolysis method, it is difficult to recover and reuse the catalyst. Meanwhile, the separation of product *myo*-inositol from the catalyst is also a big problem, which is still leading to a high production cost.

3 | MICROBIAL FERMENTATIVE PRODUCTION OF *MYO*-INOSITOL

Microbial fermentative production of *myo*-inositol is taken as a viable and cost-effective method.[26] The initial research on the production of *myo*-inositol by microbial fermentation began in 1945 when the *myo*-inositol auxotrophic strain was isolated from fungal mutants by Beadle and Tatum.[27] Subsequently, the Makoto Shirai team from Japan did important research on the breeding of *myo*-inositol-producing microorganisms belonging to the genus *Candida*. Three mutant strains were selected after mutation breeding by resistance to a glucose metabolism antagonist in a medium. Among them, the *myo*-inositol production of *Candida boidini* DGR1-14 was 0.4–3 g/L by aerated fermentation for 34–96 h with very low yields.[28] As the naturally selected strains usually had low *myo*-inositol titers, many people began to focus on the theoretical research for *myo*-inositol microbial biosynthesis in *Saccharomyces cerevisiae* to improve the production of *myo*-inositol. In 1976, Culbertson et al.[29] reported the *myo*-inositol biosynthesis of *S. cerevisiae* was regulated and controlled, the key enzymes for the biosynthesis of *myo*-inositol were inhibited by *myo*-inositol. In 1981, Greenberg et al.[30] found that there were at least three genes of OPI1, OPI2, OPI4 in *S. cerevisiae* to inhibit the synthesis of inositol 1-phosphate synthase (IPS), which was the key enzyme for the biosynthesis of *myo*-inositol. In 1990, Henry and coworkers[31,32] used the genetic engineering technology to obtain the mutant strain *S. cerevisiae* YS2 (ATCC-74033), where the negative regulator OPI1 gene was deleted and multiple copies of INO1 gene was overexpressed, providing a powerful engineering method to produce *myo*-inositol.

The biosynthesis of *myo*-inositol from glucose 6-phosphate (G6P) is catalyzed by IPS and inositol monophosphatase (IMP). First, IPS catalyzes the generation of inositol 1-phosphate (I1P) from G6P. Then, IMP catalyzes the dephosphorylation of I1P to generate *myo*-inositol. However, *myo*-inositol production by microorganisms has received limited success by using this way. For example, an *Escherichia coli* strain that was constructed by metabolic engineering strategies can synthesize 21 g/L of *myo*-inositol and 4 g/L of I1P from glucose as the substrate with only 11% combined yield (mol/mol).[33] Moreover, an effective dynamic regulation

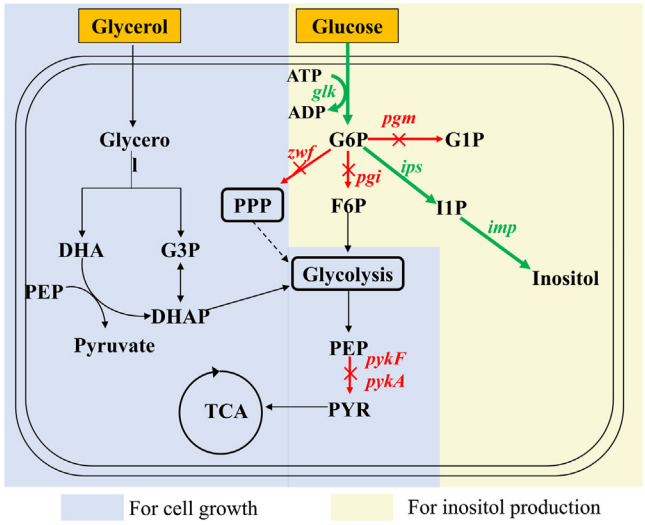

FIGURE 3 Schematic representation of *myo*-inositol production via the synergetic utilization of glucose and glycerol. Red arrows and crosses indicate gene knockouts; green arrows indicate gene overexpression; blue square indicates glycerol utilization for cell growth; yellow square indicates glucose utilization for *myo*-inositol production. DHA, dihydroxyacetone; DHAP, glycerone phosphate; G3P, glycerol 3-phosphate; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; G1P, glucose 1-phosphate; I1P, inositol 1-phosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; PPP, pentose phosphate pathway; TCA, tricarboxylic acid cycle. *glk*, glucokinase; *pgm*, phosphoglucomutase; *ips*, inositol 1-phosphate synthase; *imp*, inositol monophosphatase; *zwf*, glucose 6-phosphate dehydrogenase; pgi, glucose 6-phosphate isomerase; pykF/pykA, pyruvate kinase

means was applied to modulate carbon flux distribution to enhance *myo*-inositol production from glucose. Nevertheless, the production yield was low, indicating that only a small quantity of G6P is utilized to produce *myo*-inositol.[34] The main reason is the competitive distribution of carbon flux. G6P is the initial substance of the pentose phosphate pathway, saccharide biosynthesis, and glycolysis, making it the key node of central metabolism. It is presumed that the high yield of *myo*-inositol from glucose would not be available on metabolic engineering of strain. However, massive efforts have been devoted recently to improve *myo*-inositol production via the biosynthetic pathway of *myo*-inositol from different substrates.[35,36]

The great challenge for effective *myo*-inositol biosynthesis is the appropriate distribution of carbon flux. Two research teams have constructed similar microbial pathways for *myo*-inositol production (Figure 3). Herein, Yuan and coworkers[35] had overcome this challenge for efficient *myo*-inositol biosynthesis by synergetic utilization of glucose and glycerol in *E. coli*. Specifically, cell metabolism can be artificially divided into two modules including cell growth and *myo*-inositol biosynthesis. Therefore, the PGI

encoding gene *pgi* and the G6PDH encoding gene *zwf* were first deleted to intercept glycolysis and pentose phosphate pathways in *E. coli* BW25113. Consequently, glucose was conserved to facilitate the production of *myo*-inositol while glycerol was catabolized for cell growth. Second, the pyruvate kinase (PYK) encoding gene *pyk* was knocked out to block the phosphoenolpyruvate-consuming reaction, to promote the glycerol catabolism. Until then, both cell growth and *myo*-inositol production modules were optimized to obtain effective *myo*-inositol production. It was observed that the optimal enzyme combination of IPS from *S. cerevisiae* (encoded by *INO1*) and endogenous IMP from *E. coli* (encoded by *SuhB*) was beneficial to enhancing *myo*-inositol production and better cell growth. The inducible $P_L$lacO1 promoter of pCS-INO1-suhB was displaced by a strong constitutive promoter BBa_J23100 to circumvent the usage of expensive inducer. In addition, the suppression of glycerol utilization from glucose was relieved by overexpression of glycerol kinase. The final *E. coli* strain TEJ-41 produced a high titer of *myo*-inositol with these efforts. In 3-L fed-batch cultivation, the production of *myo*-inositol was 76 from 71 g/L of glucose and 41 g/L of glycerol, indicating the scaling-up potential. This study provided a creative way to obtain the rational distribution of carbon flux.

Meanwhile, Tao and coworkers[36] had designed a *myo*-inositol biosynthesis pathway by synergetic utilization of glucose and glycerol in *E. coli*. Here, both improvements of G6P supply and optimization of high-density fermentation were the key means to improve *myo*-inositol production. A series of recombinant strains were constructed by knocking out several key genes, including the glucose 6-phosphate isomerase encoding gene *pgi*, the phosphoglucomutase (PGM) encoding gene *pgm*, the 6-phosphofructokinase encoding *pfkA*, and PYK encoding gene *pykF*. The expression levels of IPS and IMP were optimized to be the plasmid combination pR01+p03. The expression level of *zwf* gene was regulated by the screening of different promoters or RBSs to enhance *myo*-inositol production. About 48 mM *myo*-inositol was produced in recombinant strain R15 (SG104, Δ*pgi*, Δ*pgm*, and RBSL5-*zwf*) transformed with plasmids pR01+p03. In this study, the gene *pgi* was deleted to redirect the carbon flux from glucose to produce *myo*-inositol. However, it led to the restriction of cell growth. Glycerol was selected to relieve the inhibition of cell growth. The high-density fermentation was detected by synergetic utilization of glucose and glycerol and strain R04 (SG104 and Δ*pgi*) transformed with plasmids pR01+p03 reached the highest density. A titer of 106.3 g/L (590.5 mM) *myo*-inositol was produced with a yield of 0.82 mol/mol glucose, during the scaled-up bioconversion in situ. To our limited knowledge, the titer of *myo*-inositol was the highest than previously reported. This work provided a paradigm to acquire an appropriate assignment of carbon flux. It had a much higher *myo*-inositol production titer and high-density than that of Yuan and his coworkers by different genetic manipulations.[35] Therefore, it is a promising method for *myo*-inositol production.

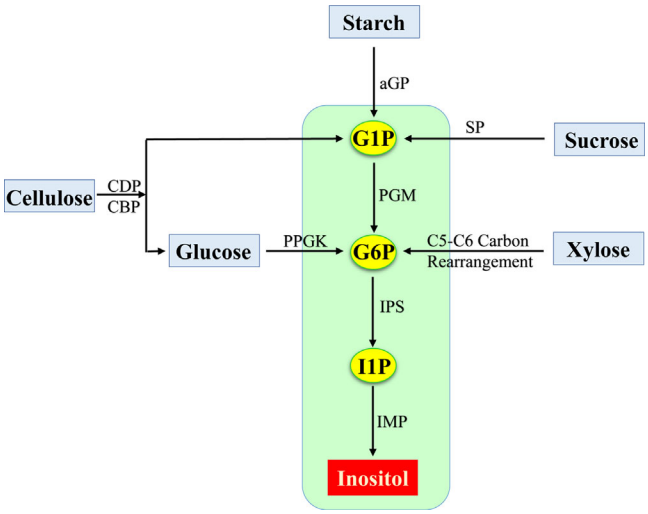

FIGURE 4 in vitro enzymatic biosystems for *myo*-inositol production from various substrates. G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; I1P, inositol 1-phosphate; αGP, alpha-glucan phosphorylase; PGM, phosphoglucomutase; IPS, inositol 1-phosphate synthase; IMP, inositol monophosphatase; CBP, cellobiose phosphorylase; CDP, cellodextrin phosphorylase; PPGK, polyphosphate-dependent glucokinase; SP, sucrose phosphorylase; Enzymes used in C5-C6 carbon rearrangement: TK, transketolase; RPE, ribulose 5-phosphate epimerase; RPI, ribose 5-phosphate isomerase; TIM, triosephosphate isomerase; ALD, fructose bisphosphate aldolase; FBP, fructose 1,6-bisphosphatase; PGI, glucose 6-phosphate isomerase

4 | in vitro ENZYMATIC PRODUCTION OF *MYO*-INOSITOL

The in vitro enzymatic biosystems are assembled by multiple purified/partially purified enzymes from different sources and/or (biomimetic) coenzymes in one pot for implementing biological cascade reactions.[37] In contrast to microbial fermentation, in vitro enzymatic biosystems present plenty of advantages, for instance, high product yield and titer, high product quality, easy process control and optimization, easy product separation, easy scale-up, and so on.[38] Numerous in vitro enzymatic biosystems have been verified to produce *myo*-inositol with high titers and yields from various sugars, including starch,[39,40] cellulose,[41] sucrose,[42] xylose,[43] and glucose.[44] These in vitro approaches to biotechnical production of *myo*-inositol are summarized in Figure 4 and Table 2.

TABLE 2 *Myo*-inositol production from various substrates by biocatalysis and biotransformation

Substrate (g/L)	Biocatalyst	Reaction time (h)	*Myo*-inositol (g/L)	Conversion rate (%)	Reference
Glucose (50)	Aerated fermentation of *Candida boidini* DGR1-14	48	3.2	6.4	[28]
Glucose (216)	Fed-batch fermentation of *E. coli* JWF1/pAD1.88A	54	21	9.7	[33]
Glucose (71) + glycerol (41)	3-L fed-batch cultivation of *E. coli* TEJ-41	96	76	107	[35]
Glucose (130) + glycerol	Whole cells of *E. coli* R04 harboring plasmids pR01+p03	23	106.3	82	[36]
Maltodextrin (10, DE = 4–7)	in vitro synthetic enzymatic bioreaction using 6-enzyme cocktail including hyperthermophilic aGP, PGM, IPS, IMP, IA, and 4GT.	48	9.89	98.9	[39]
Maltodextrin (125, DE = 10)	in vitro synthetic enzymatic bioreaction using 5-enzyme cocktail including hyperthermophilic aGP, PGM, IPS, IMP, and IA.	48	95	63.3	
Amylose (14.5)	in vitro synthetic enzymatic bioreaction using 4-enzyme cocktail including hyperthermophilic MalP, PGM, IPS, and IMP.	6	14.5	100	[40]
Soluble starch (14.5)	in vitro synthetic enzymatic bioreaction using 5-enzyme cocktail including hyperthermophilic MalP, PGM, IPS, IMP, and pullulanase.	6	10.4	72	
Raw potato (150)		6	26.3	17.5	
Cellodextrins (10)	in vitro synthetic enzymatic bioreaction using 6-enzyme cocktail including thermophilic CBP, CDP, PPGK, and hyperthermophilic PGM, IPS, IMP.	24	9.9	99	[41]
Avicel (containing 50 g/L cellodextrins)		36	36.3	72.6	
Sucrose (17.1)	in vitro synthetic enzymatic bioreaction using 4-enzyme cocktail including thermophilic SP, and hyperthermophilic PGM, IPS, IMP.	108	8.64	50.5	[42]
Xylose (3)	in vitro synthetic enzymatic bioreaction using 11-enzyme cocktail including hyperthermophilic XI, XK, TK, RPE, RPI, TIM, ALD, FBP, PGI, IPS, and IMP.	72	2.9	96.7	[43]
Glucose(9)	in vitro synthetic enzymatic bioreaction using 3-enzyme cocktail including mesophilic PPGK, IPS, and IMP.	24	8.2	91	[44]

Note: MalP, maltodextrin phosphorylase; PGM, phosphoglucomutase; IPS, inositol 1-phosphate synthase; IMP, inositol monophosphatase; αGP, alpha-glucan phosphorylase; IA, isoamylase; 4GT, 4-a-glucanotransferase; CBP, cellobiose phosphorylase; CDP, cellodextrin phosphorylase; PPGK, polyphosphate-dependent glucokinase; SP, sucrose phosphorylase; XI, xylose isomerase; XK, D-xylulose kinase; TK, transketolase; RPE, ribulose 5-phosphate epimerase; RPI, ribose 5-phosphate isomerase; TIM, triose phosphate isomerase; ALD, fructose bisphosphate aldolase; FBP, fructose 1,6-bisphosphatase; PGI, glucose 6-phosphate isomerase.

4.1 | in vitro enzymatic production of *myo*-inositol from starch

Starch is one of the most abundant renewable resources, which is produced by plant photosynthesis and mostly harvested from cultivated seeds and tubers.[45,46] An in vitro enzymatic pathway was established to convert starch to *myo*-inositol by You and coworkers.[39] It has four steps for *myo*-inositol generation: (i) G1P generation from starch and phosphate catalyzed by alpha-glucan phosphorylase (αGP) from the hyperthermophilic bacterium *Thermotoga maritima;* (ii) G6P generation from G1P transformed by phosphoglucomutase from the hyperthermophilic archaeon *Thermococcus kodakarensis*; (iii) I1P generation from G6P catalyzed by IPS from the hyperthermophilic archaeon *Archaeoglobus fulgidus*, and (iv) both *myo*-inositol and phosphate generation from I1P catalyzed by IMP from the hyperthermophilic bacterium *T. maritima*. Here, the overall Gibbs energy was calculated to be −80.1 kJ/mol by the consolidation of four reaction steps from starch to *myo*-inositol, driving the overall reaction toward completeness. The proof-of-concept reaction was conducted on 10 g/L maltodextrins catalyzed by the above-mentioned four enzymes at 70°C without NAD^+ addition. Only 4.5 g/L *myo*-inositol was produced along with some maltose accumulation. Next, two auxiliary enzymes, namely, 4-a-glucanotransferase (4GT) from *Thermococcus litoralis* and isoamylase (IA) from *Sulfolobus tokodaii* were added to the reaction solution, obtaining a very high titer of 9.89 g/L with a yield of 98.9%. Furthermore, this in vitro reaction has been successfully conducted on 20,000-L bioreactor by the core four enzymes plus IA at 70°C. The final production of *myo*-inositol was over 95 from 125 g/L maltodextrins (DE = 10).

Similarly, Atomi and coworkers[40] have constructed an in vitro enzymatic biosystem to produce *myo*-inositol from starch. This pathway was constructed the same as mentioned above by You et al.,[39] where four hyperthermophilic enzymes were used including maltodextrin phosphorylase (MalP) from *T. kodakarensis*, PGM from *T. kodakarensis*, IPS from *A. fulgidus*, and IMP from *T. maritima*. It's worth noting that, NAD^+ was used as the cofactor of IPS in the third reaction step. First, the four enzymes were individually cultured in *E. coli*. Second, they were harvested and purified by heat treatment. Third, the reaction was conducted by using the four enzymes at 90°C with amylose, phosphate, and NAD^+, obtaining approximately 74 mM *myo*-inositol with a yield of 93%. Furthermore, by accompanying the addition of 1 mM NAD^+ every 2 h, 2.9 g *myo*-inositol was produced from 2.9 g amylose with a molar conversion of 96%. Finally, the pullulanase from *T. maritima* was integrated into the system and the production values of *myo*-inositol from soluble starch and raw potato were approximately 10.4 and 26.3 g/L, corresponding to yields of 73% and 61%, respectively. However, to obtain a higher level of efficiency, several key problems needed to be solved, such as the high cost and multiple steps in enzyme preparation, the single use and stability of the enzymes, and the thermal degradation of NAD^+.

The construction of in vitro biosystems for *myo*-inositol production from starch affords an alternative to the conventional chemical extraction method. By using MalP (or αGP) and PGM reactions, the phosphorylated glucose was introduced to avoid the consumption of ATP, which was more beneficial to its industrial scale-up. Moreover, it has many biomanufacturing advantages over both the acid hydrolysis of phytate and the microbial fermentation: (1) much less costly and more abundant substrate, (2) lower capital investment, (3) decreased phosphorous pollution, (4) easy product separation. However, the biggest shortcoming in the production of inositol from starch catalyzed by multienzyme in vitro is the instability and low activity of the enzymes. To further increase *myo*-inositol production efficiency from starch, several issues need to be considered: (1) gene mining and protein engineering for enzymes with better thermostability and activity, especially for IPS, which is the rate-limited enzyme. Protein engineering methods, including directed evolution, rational design, and combination, could be utilized to obtain better IPS. (2) Enzyme immobilization for higher system stability and quicker separation of these enzymes from the reaction solution, thereby stimulating the industrial application of biocatalysts. (3) Build stoichiometric mathematical models for quick optimization of the *myo*-inositol production system. Taken all in all, this way of in vitro *myo*-inositol production from starch has great potential and will open a new era of industrial biomanufacturing of *myo*-inositol.

4.2 | in vitro enzymatic production of *myo*-inositol from cellulose

Cellulose is a natural biopolymer comprised of anhydroglucose units. It is the most abundant renewable resource on the earth, which can be a promising source for manufacturing biochemicals.[47–49] Meng et al.[41] designed the in vitro stoichiometric enzymatic biosystems for *myo*-inositol production from cellulosic biomass. For the pathway design, three cascade phosphorolysis enzymes, cellodextrin phosphorylase (CDP) from *Clostridium thermocellum*, cellobiose phosphorylase (CBP) from *C. thermocellum*, and polyphosphate-dependent glucokinase (PPGK) from *Thermobifida fusca* YX, were first used for the generation of G1P and G6P from the substrate cellodextrins. To convert all the glucose units of cellulose to

produce *myo*-inositol, three enzymes including PGM from *T. kodakaraensis*, IPS from *A. fulgidus*, and IMP from *T. maritime*, were supplemented into the reaction to produce *myo*-inositol and phosphate from G1P and G6P. The released phosphate is recycling for G1P and G6P generation by CDP and CBP. The one-pot reaction was conducted on 10 g/L cellodextrins at 55°C with the six-enzyme cocktail added at different times. Only 6.91 g/L *myo*-inositol was produced with a yield of 69.1%. Next, the concentration of Mg^{2+} was optimized to enhance the *myo*-inositol production of 9.81 g/L at 60 h. Preferably, the reaction temperature was shifted from 55°C to 70°C at 8 h and the final *myo*-inositol production was about 9.90 g/L at 24 h with a high yield of 99%. The reaction time was shortened to 24 h from 60 h, as well. Moreover, the high concentration of pure cellodextrins and natural biomass hydrolysates were all used as the substrates in this in vitro enzymatic biosystems to produce *myo*-inositol, leading to yields of 72.6%, 27.2%, and 30.6%, respectively.

This study gave a paradigm to get a high yield of *myo*-inositol from a low concentration of cellodextrin, but the yields from a high concentration of pure cellodextrins and natural biomass hydrolysates were less than that from a low concentration of cellodextrins. For further improvement of the product's titer and yield from cellulose, several issues needed to be solved in the future: (1) To obtain a powerful CDP with high efficiency for phosphorylation of long-chain cellodextrins. (2) To construct an enzyme complex including CDP and PGM for enhancement of G6P production. (3) To improve the thermostabilities of CDP, CBP, and PPGK. (4) To alleviate the Maillard reaction for mitigation of enzyme deactivation, whatever this in vitro enzymatic biosystem provides a cost-effective method to produce value-added products from cellulosic biomass.

4.3 | in vitro enzymatic production of *myo*-inositol from sucrose

Sucrose is the most abundant disaccharide composed of glucose linked to fructose, and one of the key components of the bioeconomy. The actual difficulties in the sugar industry, such as fluctuating profits and shrinking market, have motivated it to exploit much more value-added products.[42] Recently, *myo*-inositol was produced from sucrose via a coenzyme-free enzymatic biosystem containing four thermophilic enzymes (sucrose phosphorylase [SP], PGM, IPS, and IMP) by Zhong et al.[42] Except for the first step that G1P and fructose generation from sucrose and phosphate catalyzed by SP from *Thermoanaerobacterium thermosaccharolyticum*, the last three catalytic steps are the same as that of You's work.[39] This pathway has an overall Gibbs energy of −108.7 kJ/mol, showing its ability to obtain a theoretical yield of *myo*-inositol. The reactions were firstly conducted on 50 mM sucrose catalyzed by the four-enzyme cocktail at a given unit ratio of 1:1:2:1.5 at 50°C or 70°C. Whatever, the production increased gradually until 18.5 mM *myo*-inositol at 50°C for 72 h, while 12 mM *myo*-inositol at 70°C for 2 h, owing to the fast deactivation of SP at evaluated temperature. These two reactions indicated that the biosynthesis of *myo*-inositol from sucrose can be implemented by using these four enzymes, but the process is limited by the thermostability inconsistency between thermophilic SP and three hyperthermophilic enzymes. It is necessary to develop a thermal cycling biocatalysis system to avoid this thermostability inconsistency. Creatively, SP was immobilized on cellulose-containing magnetic nanoparticles. By using a switch of the magnetic field, this immobilized SP was added into the reaction at 50°C and removed at 70°C to maintain its activity. Finally, 48 mM *myo*-inositol was produced with a yield of 0.98 mol/mol sucrose.

4.4 | in vitro enzymatic production of *myo*-inositol from xylose

Xylose is the most abundant pentose obtained from hemicellulose in lignocellulosic biomass, which is often used as a food sweetener and the feedstock of xylitol. Recently, the utilization of xylose has been paid much attention to the development of high-value biochemicals. In this study, Cheng et al.[43] constructed an in vitro cofactor-free pathway for *myo*-inositol production from xylose. This pathway is comprised of three steps: (1) xylose phosphorylation. D-Xylulose 5-phosphate (Xu5P) generation from xylose and ATP catalyzed by xylose isomerase (XI) and D-xylulose kinase (XK); (2) carbon–carbon (C–C) rearrangement and circulation. G6P generation from Xu5P catalyzed by ribulose 5-phosphate epimerase (RPE), ribose 5-phosphate isomerase (RPI), transketolase (TK), transaldolase (TAL), triosephosphate isomerase (TIM), fructose bisphosphate aldolase (ALD), fructose 1,6-bisphosphatase (FBP), and glucose 6-phosphate isomerase (PGI); (3) dephosphorylation. *Myo*-inositol generation from G6P catalyzed by IPS and IMP. Here, all these 12 enzymes were selected from hyperthermophilic microorganisms. The one-pot reaction was firstly conducted on 20 mM xylose by using a 12-enzyme cocktail and only 4.25 mM *myo*-inositol was produced at 70°C, suggesting the reaction conditions were not suitable for *myo*-inositol production. Next, the elements of the reaction conditions were optimized one by one, obtaining 16.1 mM *myo*-inositol from 20 mM xylose with a yield of 96.6% at 70°C. However, 5 mM ATP was further added into this reaction to get the high product yield,

indicating that ATP was not stable and degraded at 70°C. In this study, because XK also had a low activity based on polyphosphate,[50] less costly feedstock-polyphosphate was used as the phosphoryl donor to conduct the reaction and only 3.43 mM *myo*-inositol was produced with a yield of 17.1%.

4.5 | in vitro enzymatic production of *myo*-inositol from glucose

The biosynthesis of *myo*-inositol from glucose was validated nearly a century ago.[51,52] It was constituted of three steps: (1) G6P generation from glucose and ATP catalyzed by ATP-dependent glucokinase; (2) I1P generation from G6P catalyzed by IPS; (3) *myo*-inositol and phosphate generation from I1P catalyzed by IMP. Herein, Tao and coworkers[44] constructed an in vitro pathway to produce *myo*-inositol from glucose by using three enzymes, polyphosphate-dependent PPGK from *Arthrobacter sp*. OY3WO11, IPS from *Trypanosoma brucei* TREU927, and IMP from *E. coli*. Instead of ATP, polyphosphate was chosen as the feedstock for G6P generation because of its low cost. The conversion ratios were both over 90% when the reactions were performed at low concentrations of glucose/sodium hexametaphosphate, respectively. However, the conversion ratio decreased drastically with the increased concentration of glucose /sodium hexametaphosphate. Sodium hexametaphosphate could inhibit the activity of IPS, restricting the biotransformation of G6P to I1P. TbIPS activity was decreased by 50% in the presence of 15 mM sodium hexametaphosphate, and it was almost completely deactivated at 20 mM. A two-step cascade reaction was constructed to alleviate the inhibitory effect of sodium hexametaphosphate on IPS, and 45.2 mM *myo*-inositol was generated from 50 mM glucose with a conversion ratio of 90.4%. However, the *myo*-inositol production did not improve with increased glucose concentration, as G6P generated in the first step was not completely converted to inositol. The inhibitory effect of phosphate on IPS was detected to find that TbIPS activity was decreased by about 50% at 50 mM phosphate, and almost completely abolished by 125 mM phosphate. Furthermore, phosphate showed the same inhibitory effect on the other five purified IPSs, which could not obtain much higher *myo*-inositol titer and yield. In the future, much work needs to be devoted to eliminating the inhibitory effects of sodium hexametaphosphate and phosphate, including the enhancement of both activity and tolerance of IPS by enzyme engineering, and the reduction of the enzyme production costs by using hyperthermophilic enzymes and/or enzyme immobilization.

5 | CONCLUSION AND OUTLOOK

Nowadays, *myo*-inositol can be well produced by the methods of chemical acid hydrolysis of phytate, microbial fermentation, and in vitro enzymatic cascade reactions. Phytate, the substrate for producing *myo*-inositol, is abstracted from corn-soaking water and rice bran-soaking water during the processing of agricultural products. Phytate is hydrolyzed to *myo*-inositol by acid hydrolysis in chemical production, resulting in high production costs and serious pollution. However, because of the abundant resources of raw materials and the mature industrial production experience, inositol is still produced by this chemical method in China and Japan. Great achievements with high titers and yields in microbial production of *myo*-inositol are obtained by using biotechnological approaches. For *myo*-inositol production, the biosynthesis pathways from G6P in both *S. cerevisiae* and *E. coli* are catalyzed by IPS and IMP. As G6P is the key node of central metabolism, only a small amount of G6P can be used as the precursor for inositol production. The difference is that the *myo*-inositol biosynthesis of *S. cerevisiae* is rigorously regulated and controlled, owing to the key enzymes are inhibited by *myo*-inositol and the synthesis of I1P is inhibited by at least three genes of OPI1, OPI2, OPI4, thus the titer of inositol produced by *S. cerevisiae* is only 9 g/L by OPI1 gene deletion and fermentation optimization,[53] while the titer of inositol produced by *E. coli* could reach to a high level of 106.3 g/L by synergetic utilization of glucose and glycerol as carbon sources.[36] The synthetic utilization of glucose and glycerol may be a hopeful method for high-titer of *myo*-inositol production and high-density fermentation. Moreover, the method of scaffold-free enzyme assemblies for the construction of enzyme complex is an effective strategy to prevent intermediate diffusion, improve product yield and control the flux of metabolites in vivo and in vitro.[54] Some pairs of short peptide tags from the dock-and-lock peptide interacting family, such as RIAD and RIDD, SpyTag and SpyCatcher, could be used to create scaffold-free enzyme complexes.[55] By scaffold-free assembling the key enzyme IPS with the cascade enzymes in the *myo*-inositol biosynthetic pathway, it will lead to the formation of a pathway node and the improvement of myo-inositol production. On the other side, *myo*-inositol biosynthesis by using *E. coli* mutant strains should always be taken seriously. To avoid food safety problems, food-grade expression hosts such as *Bacillus subtilis*, *Pichia pastoris*, *S. cerevisiae*, or *Lactococcus lactis* need to be taken into consideration in the future. Last but not the least, high titers and/or theoretical yields of *myo*-inositol have been obtained through the in vitro enzymatic synthetic biosystems from various sugars. Most importantly, the way of in vitro *myo*-inositol production

from starch has been applied to market manufacturing. It is noteworthy that, the novel in vitro enzymatic reaction method has been conducted for *myo*-inositol industrial production in Sichuan Bohaoda Biotechnology Co., Ltd. However, the in vitro enzymatic platform does still need to be improved in several aspects as follows, enzyme immobilization for a prolonged lifetime and easy separation, fast conversion implementation at high substrate concentrations, multienzyme complexes construction to accelerate the reaction rate, and so on. Moreover, the activity of key enzyme IPS needs to be improved through site-directed mutagenesis or directed evolution for efficient *myo*-inositol biosynthesis.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (Grant No. 31700033, 21706274, and 32022044), High-Tech Innovation Fund of Chinese Academy of Sciences (GQRC-19-11), and Tianjin Synthetic Biotechnology Innovation Capacity Improvement Project (TSBICIP-KJGG-003).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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How to cite this article: Li Y, Han P, Wang J, Shi T, You C. Production of *myo*-inositol: recent advance and prospective. Biotechnol Appl Biochem. 2022;69:1101–1111.
https://doi.org/10.1002/bab.2181