Open Science ID (OSID)

Advances in molecular modification and efficient expression of key enzymes for functional saccharide preparation

WU Jing (吴敬)[1,2,3*], TAO XiuMei (陶秀梅)[1,2,3], ZHANG Kang (张康)[1,2,3], SU LingQia (宿玲恰)[1,2,3] & CHEN Sheng (陈晟)[1,2,3]

[1] *State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China;*
[2] *School of Biotechnology and Key Laboratory of Industrial Biotechnology Ministry of Education, Jiangnan University, Wuxi 214122, China;*
[2] *International Joint Laboratory on Food Safety, Jiangnan University, Wuxi 214122, China*

Received December 10, 2017; accepted January 12, 2018

Abstract Functional saccharide is a general term that is often used to refer to the functional oligosaccharides, functional saccharide alcohols, and functional dietary fibers. These functional saccharides exhibit some health benefiting effects, such as having low calorie, preventing dental caries, and regulating intestinal disorders. Functional saccharides are widely used in food, health products, and the healthcare fields. The preparation of functional saccharides is accomplished mainly through reactions involving transglycosylation, isomerization, or hydrolysis catalyzed by glycosyltransferases, saccharide isomerases, and glycohydrolases, respectively. However, the poor catalytic properties of natural enzymes and low fermentation yields have restricted the large-scale industrial production of functional saccharides. Therefore, molecular modification and efficient expression of key enzymes for functional-saccharide preparation are very important for promoting the low-cost large-scale production of functional saccharides. In this report, the recent advances in functional optimization and expression preparation of enzymes related to functional saccharides are reviewed.

Keywords Functional saccharide; Enzyme; Molecular modification; Efficient expression
doi: 10.16262/j.cnki.1005-0841.2018.01.002

1 Introduction

With the rapid economic development and continuous improvements in living standards, foods with nutritional values, desirable flavors, and health benefiting functions have been gradually accepted by consumers and become a new trend in the food industry. Some saccharides, such as oligosaccharides, dietary fibers, and saccharide alcohols, are the special food components exhibiting special bioactive physiological effects. Functional oligosaccharides cannot be digested and absorbed by human gastrointestinal tract and, instead, directly enter into the large intestine, which will be digested by *bifidobacteria*. As a result, functional oligosaccharides are also considered to possess low calorie contents, which prevents the rapid increase of blood sugar and blood lipid levels [1]. Functional saccharide alcohols refer to polyhydroxyl alcohols obtained through hydrogenation of saccharides, resulting in polyol

* Corresponding author (Email: jingwu@jiangnan.edu.cn; Phone: 86-510-85327802)

sweeteners, predominantly composed of xylitol [2], sorbitol [3], and maltitol. Functional saccharide alcohols have multiple functions. For example, they are difficult to be fermented by acid-producing bacteria, and can prevent dental caries. Additionally, their metabolic process does not interfere with the function of pancreatic islets and glucose metabolism, and they can promote the proliferation of beneficial intestinal bacteria and improve intestinal function. Dietary fibers refer to the sum of the food components that can not be digested by human digestive enzymes. In recent years, dietary fibers have been considered the seventh nutrient and a bioactive ingredient besides the water, carbohydrates, proteins, fats, minerals, and vitamins, which is often referred to as the "life oasis".

In the preparation of functional saccharides, substrate hydrolysis, transglycosylation, and isomerization are the main procedures that are mainly catalyzed by biocatalysts/enzymes. Therefore, enzymes are the key factor in production of functional saccharides. In this context, the characterization, functional optimization, and efficient preparation of novel enzymes for functional saccharide preparation are of great significance for promoting the modernization of the production of functional saccharides so as to fit the rapid requirement of the public health industry.

Key enzymes involved in the preparation of functional saccharides include glycosyltransferases [4], saccharide isomerases [5], and glycohydrolases [6]. Glycosyltransferases for functional saccharide preparation include glucosyltransferase, galactosyltransferase, and fructosyltransferase, which transfer glycosyl groups from the donor to the acceptor. Saccharide isomerases include glucose isomerase, mannose isomerase, and sucrose isomerase, which enable the conversion of saccharide isomers. Glycohydrolases refer to enzymes, including various amylases and glucosidases, capable of degrading cellulose, starch, or other polysaccharides to monosaccharides, disaccharides, and even polysaccharides. Herein, we make a review of the recent advances in functional optimization and expression of enzymes preparation related to the functional saccharide production.

2 Molecular modification of enzymes

The key enzymes for functional saccharide production are usually the multifunctional enzymes capable of catalyzing saccharide hydrolysis, transglycosylation, and/or isomerization simultaneously. In addition, they do not exhibit the substrate specificity, instead, they are capable of producing complex and changeable products. Therefore, natural enzymes usually need to be modified to enhance their catalytic performance in order to improve their efficient synthesis of specific functional saccharides. Bioinformatics analysis of existing natural enzymes in combination with molecular biology techniques, such as directed evolution, site-directed mutagenesis, and truncated expression, are used to design and modify natural enzymes to achieve the desirable properties necessary for better and wider utilization.

2.1 Directed evolution

Directed evolution refers to the act of mimicking natural evolutionary mechanisms (e. g., random mutation, recombination, and natural selection) so as to construct a library containing numerous mutants. In combination with selection methods related to a pre-determined evolutionary direction, this method can be used to directionally screen and select valuable non-native protein molecules in order to obtain mutants exhibiting optimized performance. The most commonly used strategies for directed evolution of enzymes include error-prone polymerase chain reaction (PCR) [7] and DNA shuffling [8].

Error-prone PCR generates a large number of random mutants containing a certain gene through mismatches, from which the desired individuals are selected. DNA shuffling uses DNAases to hydrolyze the parent gene or mutated gene to produce random fragments, which are then subjected to PCR without primers to generate a large number of mutants. Table 1 lists the enzymes with excellent performance obtained using error-prone PCR and *in vitro* homologous-recombination technology. Shim et al. [9] screened a M234T/F259I/V591A mutant of cyclodextrin (CD) glucosyltransferase (CGTase) by error-prone PCR, resulting in an enzyme exhibiting a 10-fold decrease in cyclization activity and a 15-fold increase in hydrolyzing activity. The addition of CGTase reduced the retrogradation rate of bread by as much as the level that was observed by the commercial anti-staling enzyme novamyl during a 7-day storage at 4 ℃. No CD was detected in bread treated with CGTase, whereas 21 mg of CD per 10 g of bread was

produced in bread treated with wild-type CGTase. Rentschler et al. [10] constructed a mutant library of β-galactosidase by error-prone PCR and obtained a mutant exhibiting a >2-fold increase in specific activity. Tang et al. [11] screened two highly thermostable mutants after three rounds of shuffling of maltogenic amylase from *Bacillus thermoalkalophilus* ET2, each having optimal reaction temperatures of 80 ℃, which was 10 ℃ higher than that of wild-type. To create xylanase variants possessing both thermal and alkaline stability in a single enzyme, Stephens et al. [12] used one mutant with high thermostability and the other with high alkaline stability from *Thermomyces lanuginosus* xylanase DSM 5826 (xynA) as templates for DNA shuffling, resulting in the screening of two xylanase mutants (S340 and S325) with both thermal and alkaline stability. Melzer et al. [13] obtained a CGTase mutant library of alkaliphilic *Bacillus* sp. G-825-6 by a combination of error-prone PCR and DNA-shuffling technology, resulting in a mutant with seven substituted amino acids. The CD_8-synthesizing activity of the mutant was increased by 1.2 fold, and the product ratio of CD_7 : CD_8 shifted to 1 : 7 as compared with 1 : 3 from the wild-type enzyme. Another CGTase variant with nine amino acid substitutions was active at a pH range of 4.0 to 10.0. Compared with the wild-type enzyme, which was inactive at pH<6.0, the mutant retained 70% of its CD8-synthesizing activity at pH 4.0.

Table 1 Enzymes obtained using error-prone PCR and DNA shuffling

Improved property	Enzyme	Site	Improvements	Secondary structure or subsite	Ref.
Selectivity	Cyclodextrin Glucosyltransferase	M234T/F259I/V591A	The hydrolyzing activity of the mutant was increased 15-fold, and the cyclization activity was reduced 10-fold as compared with wild-type	+1,+2	[9]
Specific activity	β-Galactosidase	S432T/A762V	The specific activity of the mutant was increased 2-fold	α-helix, Loop	[10]
Thermo-stability	Maltogenic amylase	N147D/F195L/N263S/D311G/A344V/F397S/N508D	The optimal temperature was 10 ℃ higher than that of wild-type, and the half-life was 20-fold greater than that of wild-type at 78 ℃.	Loop, α-helix, β-sheet	[11]
pH stability	Cyclodextrin Glucosyltransferase	E39K/T66S/L71P/I101L/S461G/E472G/V605A/N606K/R684H	Compared with the wild-type enzyme, which is inactive below pH 6.0, the mutant retained 70% of its CD8-synthesizing activity at pH 4.0	Loop, β-sheet	[13]
Thermostability and pH stability	Xylanase	A54T/K30E/W40R/T57A/K80R (S325),W40R/T57A/K80R(S340)	S340 retained 54% stability at 80 ℃ and 60% stability at pH 10; S325 displayed 85% stability at 80 ℃ and 60% stability at pH 10	Loop, β-sheet	[12]
Product specificity	Cyclodextrin Glucosyltransferase	N187D/A248V/V252E/H352L/D465G/E560V/E687G	Increased γ-cyclodextrin specificity; the product ratio of CD7 : CD8 changed from 1 : 3 to 1 : 7	+1, +2	[13]

2.2 Site-directed mutagenesis

Site-directed mutagenesis is an important approach to modify enzyme genes. Enzymes with excellent properties can be obtained after experimental validation through the introduction of beneficial mutations at specific sites, with mutations at either single or multiple sites. Site-directed mutagenesis is a rational design that can only be successfully implemented on the basis of a full understanding of enzyme structure and function, as well as their catalytic mechanisms and active site(s). Table 2 lists the enzymes with desirable properties obtained by site-directed mutagenesis. The improved properties of these enzymes include increased thermostability and substrate conversion, selectivity, and product specificity. To improve the enzyme thermostability, Duan et al. [14] introduced amino acids with high B-factors into sucrose

Table 2 Enzymes with excellent properties obtained by site-directed mutagenesis

Improved property	Enzyme	Site	Improvements	Secondary structure, subsite, or domain	Ref.
Thermostability	Sucrose isomerase	E175N, K576D, K174D, G176D, S575D and N577K	The half-lives of E175N, K576D, and E175N/K576D mutants were 2.30-, 1.78-, and 7.65-fold longer than that of the wild-type enzyme at 45 ℃	loop	[14]
	Pullulanase	D503F, D437H, D503Y, D437H/D503Y	The thermostability of the D437H/D503Y double mutant increased to an optimal temperature 60 ℃, and the half-life at 60 ℃ was 4.3-fold longer than that of the wild-type enzyme	loop	[15]
Substrate conversion	L-arabinose isomerase	K320R/N475K, V408A/N475K, K428N/N475K, C450S/N475K	D-tagatose conversion by the C450S/N475K mutant was 20% higher than that of wild-type	Loop, α-helix, β-sheet	[16]
	β-Galactosidase	F359Q, F441Y	Yields of GOS reached 50.9% for the wild-type enzyme, 58.3% for F359Q, and 61.7% for F441Y	−1, +1 site	[23]
Selectivity	maltooligosyltrehalose synthase (MTSase)	Y290F, Y367F, F405Y and Y409F	Compared with wild-type MTSase, MTSase F405Y showed decreased hydrolysis: transglycosylation rations, whereas MTSases Y290F, Y367F, and Y409F showed increased ratios.	+1 site	[18]
	α-Glucosidase	Asn694 was replaced by Ala, Leu, Phe and Trp	N694F and N694W mutations led to the accumulation of larger amounts of isomaltose and isomaltotriose relative to those achieved with the wild-type enzyme	+2 site	[19]
Product specificity	Sucrose isomerase	Q299E, ΔQ299 and Y296D	The Q299E mutant increased the conversion rate of isomaltulose from 90.28% to 94.16%, Q299E, ΔQ299, and Y296D showed significant effect on product specificity	Not available	[20]
	Branching enzyme	M349T, M349S, M349H and M349Y	M349T and M349S showed 24.5% and 21.1% increases in specific activity as compared with that of wild-type GBE, respectively. Additionally, M349T and M349S displayed 24.2% and 17.6% enhancements in the α-1, 6-glycosidic linkage ratio of potato-starch samples, respectively	Catalytic domain	[21]
	Cyclodextrin Glucosyltransferase	D145A, R146A/D147P, R146P/D147A	The R146A/D147P and R146P/D147A double mutants exhibited α-CD to total CD production ratios of 75.1% and 76.1%, which exceeded 63.2% from the wild-type enzyme	−7 site	[22]

isomerase to obtain a mutant with a half-life 7.65-fold longer than that of wild-type. They also screened a pullulanase D437H/D503YT mutant exhibiting a half-life 4.3-fold longer than that of the wild-type variant [15] through sequence alignment and structural simulation. With regard to the increased substrate conversion, Oh et al. [16] screened an L-arabinose isomerase C450S/N475K mutant exhibiting a tagatose-conversion rate increased by 20% as compared with the wild-type variant following a combination of random and site-directed mutagenesis. Wu et al. [17] obtained a β-galactosidase F441Y mutant capable of a galactooligosaccharide yield of 61.7% (wild-type: 50.9%) through multiple-sequence alignments. To increase the enzyme selectivity, mutations located near subsite +1 of maltooligosyltrehalose synthase were performed by Tsueiyun et al. [18] in order to alter the ratio of hydrolysis to transglycosylation. MTSase selectivity could be changed by altering hydrogen bonding and/or hydrophobic interactions between substrate and enzyme at positions near subsite +1 of the enzyme-substrate complex. Mutation of amino acids at subsite +2 of α-glucosidase performed by Min et al. [19] altered the hydrolysis and transglycosylation reactions, resulting in accumulation of large amounts of isomaltose and isomaltotriose. Besides, Liu et al. [20] obtained sucrose isomerase Q299E, ΔQ299, and Y296D mutants exhibiting significant improvements in product specificity. Liu et al. [21] also found that a branching-enzyme M349T mutant obtained through multiple-sequence alignment and structural analysis displayed a 24.2% enhancement in the α-1,6-glycosidic linkage ratio in potato-starch samples. Wang et al. [22] masked subsite −7 of the active site by removing hydrogen-bonding interactions between the enzyme and substrate to block the formation of larger CDs, resulting in increased specificity for the product α-CD.

2.3 Truncated expression

Previous studies demonstrated that certain regions of enzyme-encoding genes are not required for enzymatic activity [24; 25]; therefore, modification methods involving random or specific truncation of genes are often used to increase enzyme-expression levels or to improve enzyme properties. Truncation can be selected at single site or multiple sites, and the truncated enzyme can be directly obtained by truncation at specific sites, or enzymes with excellent properties can also be screened by random truncation and construction of a truncation library. Duan et al. [26] used a D437H/D503Y (DM) mutant of pullulanase from *Bacillus deramificans* as a starting strain to construct three N-terminal truncation variants from the original DM that lacked the CBM41 domain (DM-T1), the CBM41 and X25 domains (DM-T2), or the CBM41, X25, and X45 domains (DM-T3). After the expression, DM-T3 existed as an inclusion body, whereas 72.8% and 74.8% of the total pullulanase activities of DM-T1 and DM-T2, respectively, were secreted into the medium. The activities of the truncated enzymes were 2.8- and 2.9-fold higher than that of the DM enzyme, respectively. Bai et al. [27] reported that an N-terminal truncation (amino acids 1—733) of 4,6-α-glucanotransferase (4,6-α-GTase; GTFB) enzymes from *Lactobacillus reuteri* was strongly enhanced in its soluble expression of GTFB-ΔN in *Escherichia coli*, which was ~75-fold higher than that of full-length wild-type GTFB.

The structural modifications of key enzymes involved in functional saccharide production have been analyzed. Mutation sites that can increase the thermostability and pH stability of enzymes are usually located in loops and β-sheets, with enzyme stabilities increased by altering interactions between amino acids. In contrast, mutation sites that change enzyme selectivity are usually located in the substrate-binding regions of catalytic domains, whereas mutations near the catalytic center alter the specific activity of the enzyme. Most mutation sites that change product specificity are located in the catalytic domains or substrate-binding sites adjacent to the catalytic domains, which could significantly impact the specificity of the products. Truncation of amino acid residues that are not required for enzyme activity can be used to improve the soluble expression of the enzyme.

3 Efficient expression of enzymes

To meet consumers' need, reducing the cost of functional saccharide production is particularly important. The cost of key enzymes required for functional saccharide preparation accounts for a large

proportion of the total cost of this process. Therefore, increasing the enzyme yield to reduce costs is of great significance for the large-scale preparation of functional saccharides. Because the key enzymes involved in functional saccharide production are food enzymes, the expression systems for these enzymes are usually required to be generally recognized as safe (GRAS) microorganisms. In this context, prokaryotic expression systems mainly include *Bacillus* expression systems [28], and eukaryotic expression systems are mainly yeast [29] and mold expression systems [30].

3.1 Prokaryotic expression systems

The most well-studied and widely used prokaryotic expression system for saccharide enzymes is *Bacillus subtilis* (Table 3) [31].

Table 3 Expression of key enzymes involved in functional saccharide production in prokaryotic expression systems

Host	Enzyme	Source	Enzyme unit	Ref.
B. subtilis	alkaline amylase	*Alkaliphilic bacterium* N10	591.4 U/mL	[55]
B. subtilis	β-CGTase	*Bacillus circulans* 251	571.2 U/mL	[33]
B. subtilis	α-amylase	*Geobacillus stearothermophilus*	2300 U/mL	[56]
B. subtilis	α-amylase	*Bacillus licheniformis* CICC 10181	1352 U/mL	[56]
B. subtilis	D-psicose 3-epimerase	*Ruminococcus* sp. 5_1_39BFAA	95 U/mL	[57]
B. subtilis	4-α-glucanotransferase	*Thermus scotoductus*	6.0 U/mL	[39]
B. subtilis	β-Mannanase	*Bacillus licheniformis* DSM13	2207 U/mL	[40]
B. subtilis	Alkali-tolerant xylanase	*Bacillus pumilus* BYG	327.2 U/mL	[41]
B. licheniformis	trehalose synthase	*Thermomonospora curvata* JCM3096	24.7 U/mL	[44]
B. licheniformis	α-amylase	*B. licheniformis*	155 U/mL	[43]
B. amyloliquefaciens	α-amylase	*B. amyloliquefaciens*	4800 U/mL	[46]
B. amyloliquefaciens	glutaminase	*B. amyloliquefaciens* SWJS22	2690.02 U/mg	[48]
B. megaterium	amylase	*B. megaterium*	1666.6 U/mL	[51]
B. megaterium	levansucrase SacB	*B. megaterium*	0.42524 U/mL	[53]
B. megaterium	Levansucrase	*Lactobacillus reuteri* 121	413.7 U/mL	[54]

3.1.1 The *B. subtilis* expression system

B. subtilis has long been used in food production. The earliest fermented food, "Natto", is made from soybeans fermented with *B. subtilis* [32]. *B. subtilis* is widely used in food-enzyme production as a *Bacillus* model strain and has characteristics including non-pathogenicity, efficient protein expression, and a clear genetic background. Most saccharide enzymes can be expressed well in *B. subtilis*. For example, β-CGTase, which is prone to forming inclusion bodies in *E. coli*, can be well secreted extracellularly in *B. subtilis*, resulting in the highest enzyme activity reaching 571.2 U/mL [33]. Recent research on the *B. subtilis* expression system has mainly focused on modification of host bacteria and expression elements and optimization of expression genes.

Wild-type *B. subtilis* produces a series of proteases, with the widely used *B. subtilis* WB600, WB700, and WB800 strains derived from *B. subtilis* strain 168 through knockout of six, seven, and eight proteases, respectively. The expression levels of food enzymes in *B. subtilis* can also be enhanced by overexpression of chaperones or removal of specific chaperone-regulatory elements. Overexpression of the chaperone prsA improved the folding of amylase in *B. subtilis*, thereby enhancing its resistance to protease degradation [34].

Modification of expression elements has been focused on the screening and optimization of promoters and signal peptides. Promoters used for expression in *B. subtilis* are mainly divided into constitutive, inducible, stage-specific, and self-inducible promoters. The expression via a constitutive promoter does not require the addition of an inducer that can be activated continuously at different stages of cell growth. The P43 and HpaII promoters are commonly used constitutive promoters. The expression strength associated

with constitutive promoters is usually strong, allowing proteins to be highly expressed during cell growth and thereby affect cell growth. Inducible promoters need certain inducers to initiate the protein expression, which can be well regulated to optimize cell growth and enzyme production. Isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoters [35], sucrose-inducible promoters [36], and xylose-inducible promoters [37] are commonly used. The IPTG-inducible promoter does not represent a strict inducible promoter, as the induction strength is somewhat weak, and the inducer IPTG is toxic, resulting in its limited industrial application. The industrial application of sucrose-inducible promoters is also limited because of its weak expression strength. Compared with the aforementioned two inducible promoters, xylose-inducible promoters are widely used in the expression of food enzymes. The expression of D-psicose 3-epimerase using a xylose-inducible promoter in a 7.5-L fermenter reached 2.6 g/L; however, the problem is xylose is expensive. Stage-specific promoters are those that are active only when the cell is grown to a specific stage and do not require the addition of an inducer. Self-inducible promoters can initiate expression under conditions associated with thermal changes, ethanol stress, and salt, acid, or glucose deprivation without the addition of inducers [38]. P_{manP} and P_{srf} are common self-inducible promoters that can be used to express proteins during high-cell-density fermentation. Recently, double-promoter-expression systems have been studied based on their effectiveness at improving the expression of foreign proteins. Compared with the single promoter $P_{amyQ'}$, the dual promoter $P_{Hpa\,II}$-$P_{amyQ'}$ can express α-CGTase, β-CGTase, and pullulanase at 2.9-, 1.3-, and 1.5-fold greater levels, respectively [33]. Dual promoters consisting of an amylase promoter from *B. subtilis* NA64 or *Bacillus licheniformis* and the single promoter $P_{Hpa\,II}$ can express 4-α-CGTase at 11- and 12-fold higher levels relative to the single promoter $P_{Hpa\,II}$, respectively [39].

Since optimal signal peptides for different proteins are usually not the same, food-enzyme expression can be improved through high-throughput screening of signal-peptide libraries for the optimal signal peptide. The expression of β-mannanase in *B. subtilis* was optimized by screening the optimal signal peptide, SP_{lipA}, from six signal peptides. Combined with the overexpression of related chaperones and optimization of the promoters, the final yield reached 2207 U/mL [40]. To optimize the expression of alkali-tolerant xylanase in *B. subtilis*, the optimal signal peptide, SP_{phoB}, was determined by screening 114 Sec-type signal peptides and 24 Tat-type signal peptides, followed by replacement of the original promoter, P_{43}, with the P_{glvm} promoter, resulting in the enzyme yield increasing to 327.2 U/mL [41].

The expression levels of the same types of saccharide enzymes in *B. subtilis* from different sources can differ greatly. For example, the yield of amylase from *B. licheniformis* and *B. stearothermophilus* was 90 U/mL and 111 U/mL, respectively; however, the specific activity of amylase from *B. stearothermophilus* was 5.1-fold higher than that from *B. licheniformis*. To improve the enzyme expression in *B. subtilis*, strategies including codon optimization, error-prone PCR, homologous recombination, and gene truncation are often used.

3.1.2 Other *Bacillus* expression systems

In addition to the *B. subtilis* expression system, other *Bacillus* expression systems are used for food-enzyme expression, including *B. licheniformis*, *Bacillus amyloliquefaciens*, and *Bacillus megaterium*. Strategies for improving the expression of saccharide enzymes in these expression systems are similar to those used in *B. subtilis*, which include protease knockdown in host bacteria and/or overexpression of chaperones, optimization of promoters and signal peptides in expression plasmids, codon optimization, and mutation of the expression gene.

B. licheniformis is a saprophytic bacterium widely distributed in the environment [42]. Due to its moderate growth rate, correct folding of foreign proteins can be guaranteed. Currently, saccharide enzymes that were previously expressed in *B. licheniformis* include α-amylase [43], β-glucanase, trehalose synthase [44], and CD CGTase [45]. In contrast, *B. amyloliquefaciens* is commonly present in soil and animal gut and can be easily separated and cultivated [31]. Food enzymes expressed in *B. amyloliquefaciens* include amylase [46], α-glucosidase [47], pullulanase [47], and glutaminase [48]. *B. megaterium* has been studied for >100 years [49], and although it is not classified as a generally

recognized as safe microorganisms, it is completely non-pathogenic and was classified as safe by the German Federal Ministry of Food and Agriculture in 2013 [50]. *B. megaterium* does not produce extracellular alkaline protease, presents plasmids that are genetically stable, and can use a wide range of carbon sources, making them suitable for industrial fermentation. Currently, food enzymes expressed in *B. megaterium* include amylase [51], β-galactosidase [52], and levansucrase [53; 54].

3.2 Eukaryotic expression systems

Eukaryotic expression systems for food-enzyme expression mainly consist of yeast and mold expression systems (Table 4).

Table 4 Expression of key enzymes involved in functional saccharide production in eukaryotic expression systems

Host	Enzyme	Source	Enzyme unit (U/mL)	Ref.
P. pastoris	xylanase	*Streptomyces sp. FA1*	1788	[61]
P. pastoris	Maltooligosyltrehalose synthase	*Sulfolobus acidocaldarius* ATCC 33909	747.7	[62]
P. pastoris	β-mannanase	*Rhizomucor miehei*	85200	[64]
P. pastoris	β-mannanase	*A. niger* GIM3.452	222.8	[75]
P. pastoris	α-galactosidase	*A. niger*	1299	[60]
P. pastoris	α-galactosidase	*Rhizomucor miehei*	1953.9	[76]
P. pastoris	endo-polygalacturonase	*Aspergillus aculeatus*	2408.7	[65]
S. cerevisiae	β-1,3-1,4-glucanase	*B. subtilis*	45.1	[63]
A. niger	xylanase	*A. niger* CICC2462	4495.9	[74]
A. niger	glucoamylase	*A. niger*	274	[70]
A. niger	glucoamylase	*Talaromyces stipitatus*	800	[71]

3.2.1 Yeast expression systems

Yeasts are eukaryotic, single-celled microorganisms that exhibit similar advantages to those of prokaryotic and eukaryotic hosts. They are safe, reliable, and grow rapidly with simple cultivation. The most prominent feature of yeasts is their ability to recognize eukaryotic genes and transcribe and translate them into active proteins. As protein-expression systems, yeast has been widely used in the field of genetic engineering, with several expression systems developed, including those associated with *Pichia pastoris*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, and *Candida utilis*. Among them, *S. cerevisiae* and *P. pastoris* are most commonly used [58; 59]. Most eukaryotic food enzymes can be expressed in yeast expression systems, which allow easy cultivation, enable mutagenesis and gene-function studies, and possess the capacity for protein post-translational processing.

Many types of saccharide enzymes are currently expressed in the yeast expression systems. One study reported optimization of α-galactosidase expression in *P. pastoris* through codon optimization, signal-peptide replacement, and comparative selection of host strains and saturation mutagenesis of protease sites, resulting in enzyme activity of 1299 U/mL from a 2-L fermentation and which was 12-fold higher than that in unaltered recombinant *P. pastoris* [60]. Additionally, optimization of induction temperature and time, as well as methanol concentration, enhanced the activity of xylanase expressed in recombinant *P. pastoris* in a 3.6-L fermenter to 1374 U/mL after 132 h of fermentation [61]. Similarly, the activity of maltooligosyltrehalose synthase expressed in recombinant *P. pastoris* in a 3.6-L fermenter reached 747.3 U/mL after a 96-h fermentation by optimizing the same expression parameters [62]. Other food enzymes expressed in yeast expression systems include β-1,3-1,4-glucanase [63], β-mannanase [64], and endo-polygalacturonase [65].

3.2.2 Mold expression systems

Aspergillus niger is a well-studied and commonly used expression system for the expression of saccharide enzymes. *A. niger* has a clear genetic background and strong ability to secrete extracellular proteins, including amylase, and have long been used in the industrial production of food and medicine,

given their classification as a safe strain [66]. The use of filamentous fungi, such as *A. niger*, to express enzymes has become a primary method for enzyme production in China since 2013. Among 52 types of food-enzyme preparations in China (GB2760-2011), 17 are produced using the genetically modified *Aspergillus* strains; however, endogenous enzymes, including glucoamylase and α-amylase, from *A. niger* affect the purity of expressed proteins, impeding its use for further exogenous enzyme separation and purification. The background expression of amylases can be reduced by knocking out the *amyR* gene in the genome [67]. Most proteins expressed in *A. niger* undergo glycosylation, which can affect their functions and properties, including half-life [68]. Strategies to improve the exogenous protein expression in *A. niger* include use of strong homologous promoters, increasing the copy number of expressed genes, co-expression of chaperones, knockout of proteases, selection of random mutations, gene-fusion expression, and optimization of fermentation conditions [69].

Many types of food enzymes have been expressed using the *A. niger* expression system. A previous study reported that glucoamylase and glucose were produced using potato waste fermented by *A. niger*. Using the following optimized medium, expression of the two products increased by 126% and 98% to 274 U/mL and 41.7 g/L, respectively [70]. Fourteen predicted amylase genes and nine predicted glucoamylase genes were identified and recombinantly expressed in *E. coli* and *A. niger*, resulting in screening of glucoamylase from *Talaromyces stipitatus* capable of raw starch digestion, with subsequent enzyme expression in *A. niger* reaching 800 U/mL in a 20-L fermenter [71]. Studies on the effect of carbon repressors on food-enzyme secretion during solid-state fermentation of *A. niger* revealed that certain concentrations of glucose help cells produce moonlighting proteins, which are essential in the classical secretory pathway. Compared with the medium containing only starch, amylase and glucoamylase activities increased 8-fold and between 20- and 29-fold in the medium containing starch and glucose (60—120 g/L) [72]. Other food enzymes expressed in *A. niger* include β-mannase [73], α-galactosidase, and xylanase [74].

References

[1] Daubioul CA, Taper HS, De Wispelaere, LD, et al. Dietary oligofructose lessens hepatic steatosis, but does not prevent hypertriglyceridemia in obese Zucker rats. *Journal of Nutrition*, 2000, 130(5): 1314—1319.

[2] Salim-ur-Rehman, Mushtaq, Z, Zahoor, T, et al. Xylitol: a review on bioproduction, application, health benefits, and related safety issues. *Critical Reviews in Food Science & Nutrition*, 2015. 55(11): 1514.

[3] Silveira, MM, Jonas, R. The biotechnological production of sorbitol. *Applied Microbiology & Biotechnology*, 2002, 59(4—5): 400—408.

[4] Palcic, MM. Glycosyltransferases as biocatalysts. *Current Opinion in Chemical Biology*, 2011, 15(2): 226—233.

[5] SH, B, MB, R, VV, D. Molecular and industrial aspects of glucose isomerase. *Microbiological reviews*, 1996, 60(2): 280.

[6] Sathya, TA, Khan, M. Diversity of glycosyl hydrolase enzymes from metagenome and their application in food industry. *Journal of Food Science*, 2014, 79(11): 2149—2156.

[7] Chen, KQ, Arnold, FH. Enzyme engineering for nonaqueous solvents: random mutagenesis to enhance activity of subtilisin E in polar organic media. *Bio/technology*, 1991, 9(11): 1073.

[8] Stemmer, WP. DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution. *Proc Natl Acad Sci U S A*, 1994, 91(22): 10747—10751.

[9] Shim, JH, Kim, YW, Kim, TJ, et al. Improvement of cyclodextrin glucanotransferase as an antistaling enzyme by error-prone PCR. *Protein Engineering Design & Selection*, 2004, 17(3): 205—211.

[10] Rentschler, E, Schwarz, T, Stressler, T, et al. Development and validation of a screening system for a β-galactosidase with increased specific activity produced by directed evolution. *European Food Research and Technology*, 2016, 242(12): 2129—2138.

[11] Tang, SY, Le, QT, Shim, JH, Y et al. Enhancing thermostability of maltogenic amylase from Bacillus thermoalkalophilus ET2 by DNA shuffling. *Febs Journal*, 2006, 273(14): 3335—3345.

[12] Stephens, DE, Khan, FI, Singh, P, et al. Creation of thermostable and alkaline stable xylanase variants by DNA shuffling. *Journal of Biotechnology*, 2014, 187: 139—146.

[13] Melzer, S, Sonnendecker, C, Föllner, C, et al. Stepwise error - prone PCR and DNA shuffling changed the pH activity range and product specificity of the cyclodextrin glucanotransferase from an alkaliphilicBacillus sp. *Febs Open Bio*, 2015, 5(1): 528.

[14] Duan, X, Cheng, S, Ai, Y, et al. Enhancing the hermostability of Serratia plymuthica Sucrose Isomerase Using B-Factor-Directed Mutagenesis. *Plos One*, 2016, 11(2): e0149208.

[15] Duan, X, Chen, J, Wu, J. Improving the thermostability and catalytic efficiency of Bacillus deramificans pullulanase by site-directed mutagenesis. *Applied & Environmental Microbiology*, 2013, 79(13): 4072.

[16] Oh, HJ, Kim, HJ, Oh, DK. Increase in D-tagatose production rate by site-directed mutagenesis of L-arabinose isomerase from Geobacillus thermodenitrificans. *Biotechnology Letters*, 2006, 28(3): 145.

[17] Pohl, S, Bhavsar, G, Hulme, J, et al. Proteomic analysis of *Bacillus subtilis* strains engineered for improved production of heterologous proteins. *Proteomics*, 2013, 13(22): 3298—3308.

[18] Fang T, Tseng W, Chung Y, et al. Mutations on Aromatic Residues of the Active Site To Alter Selectivity of the Sulfolobus solfataricus Maltooligosyltrehalose Synthase. *Journal of Agricultural & Food Chemistry*, 2006, 54(10): 3585—3590.

[19] Min, M, Okuyama, M, Sato, M, et al. Effects of mutation of Asn694 in Aspergillus niger α -glucosidase on hydrolysis and transglucosylation. *Applied Microbiology & Biotechnology*, 2017, 101(16): 6399.

[20] Liu, H, Xing, X, Lu, F, et al. Functional Modification of the Substrate-Binding Site for Isomaltulose Production Based on Predicted Structure of Sucrose Isomerase from Pantoea dispersa UQ68 J. *International Conference on Applied Biotechnology*. Springer. 2016, 59—68.

[21] Liu, Y., Ban, X., Li, C., et al. Met349 mutations enhance the activity of 1, 4-α-glucan branching enzyme from Geobacillus thermoglucosidans STB02. *Journal of Agricultural & Food Chemistry*, 2017, 65(28).

[22] Wang, L., Duan, X., Wu, J. Enhancing the α-Cyclodextrin Specificity of Cyclodextrin Glycosyltransferase from Paenibacillus macerans by Mutagenesis Masking the Subsite -7. *Applied & Environmental Microbiology*, 2016, 82(8): AEM. 03535-15.

[23] Y, W, S, Y, S, C, D, W., J, C., J, W. Enhancing the production of galacto-oligosaccharides by mutagenesis of Sulfolobus solfataricus β-galactosidase. *Food Chemistry*, 2013, 138(2—3): 1588—1595.

[24] Shima, S, Igarashi, Y, Kodama, T. Nucleotide sequence analysis of the endoglucanase-encoding gene, celCCD, of *Clostridium cellulolyticum*. *Gene*, 1991, 104(1): 33—38.

[25] Lo, H. F., Lin, L. L., Chiang, W. Y., et al. Deletion analysis of the C-terminal region of the alpha-amylase of *Bacillus sp* strain TS-23. *Archives of Microbiology*, 2002, 178(2): 115—123.

[26] X, D., J, W. Enhancing the secretion efficiency and thermostability of a Bacillus deramificans pullulanase mutant (D437H/D503Y) by N-terminal domain truncation. *Applied and Environmental Microbiology*, 2015, 81(6): 1926.

[27] Bai Y, Kaaij R M V D, Leemhuis H, et al. Biochemical Characterization of the Lactobacillus reuteri Glycoside Hydrolase Family 70 GTFB Type of 4,6-α-Glucanotransferase Enzymes That Synthesize Soluble Dietary Starch Fibers. *Applied and Environmental Microbiology*, 2015, 81(20): 7223—7332.

[28] Yansura, DG, Henner, DJ. Use of the *Escherichia coli* lac repressor and operator to control gene expression in *Bacillus subtilis*. *Proceedings of the National Academy of Sciences of the United States of America*, 1984, 81(2): 439—43.

[29] Spiegelman, S, Sussman, RR, Pinska, E. On the cytoplasmic nature of "long-term adaptation" in yeast. *Proceedings of the National Academy of Sciences of the United States of America*, 1950, 36(11): 591—606.

[30] Ophuls, W. Further observations on a pathogenic mould formerly described as a protozoon (coccidioides immitis, coccidioides pyogenes). *The Journal of Experimental Medicine*, 1905, 6(4—6): 443—85.

[31] de Boer, AS, Diderichsen, B. On the safety of *Bacillus subtilis* and *B. amyloliquefaciens*: a review. *Appl Microbiol Biotechnol*, 1991, 36(1): 1—4.

[32] Kiers, JL, Van Laeken, AEA, Rombouts, FM, et al. In vitro digestibility of *Bacillus* fermented soya bean. *International Journal of Food Microbiology*, 2000, 60(2—3): 163—169.

[33] Zhang, K, Su, L, Duan, X, et al. High-level extracellular protein production in *Bacillus subtilis* using an optimized dual-promoter expression system. *Microbial Cell Factories*, 2017, 16(1): 32.

[34] Vitikainen, M, Hyyrylainen, HL, Kivimaki, A, et al. Secretion of heterologous proteins in *Bacillus subtilis* can be improved by engineering cell components affecting post-translocational protein folding and degradation. *Journal of Applied Microbiology*, 2005, 99 (2): 363—375.

[35] Phan, TTP., Nguyen, HD, Schumann, W. Novel plasmid-based expression vectors for intra- and extracellular production of recombinant proteins in *Bacillus subtilis*. *Protein Expression and Purification*, 2006, 46(2): 189—195.

[36] Zukowski, MM, Miller, L. Hyperproduction of an intracellular heterologous protein in a sacUh mutant of *Bacillus subtilis*. *Gene*, 1986, 46(2—3): 247—255.

[37] Kim, L, Mogk, A, Schumann, W. A xylose-inducible *Bacillus subtilis* integration vector and its application. *Gene*, 1996, 181(1—2): 71—76.

[38] Guan, C, Cui, W, Cheng, J, et al. Construction and development of an auto-regulatory gene expression system in *Bacillus subtilis*. *Microbial Cell Factories*, 2015, 14.

[39] Kang, HK, Jang, JH, Shim, JH, et al. Efficient constitutive expression of thermostable 4-alpha-glucanotransferase in *Bacillus subtilis*

using dual promoters. *World Journal of Microbiology & Biotechnology*, 2010, 26(10): 1915—1918.

[40] Song, Y, Fu, G, Dong, H, et al. High-efficiency secretion of beta-mannanase in *Bacillus subtilis* through protein synthesis and secretion optimization. *Journal of Agricultural and Food Chemistry*, 2017, 65(12): 2540—2548.

[41] Zhang, W, Yang, M, Yang, Y, et al. Optimal secretion of alkali-tolerant xylanase in *Bacillus subtilis* by signal peptide screening. *Applied Microbiology and Biotechnology*, 2016, 100(20): 8745—8756.

[42] Singh, A, Van Hamme, JD, Ward, OP. Surfactants in microbiology and biotechnology: Part 2. Application aspects. *Biotechnology Advances*, 2007, 25(1): 99—121.

[43] He, P, Zhang, Z, Cai, D, et al. High-level production of alpha-amylase by manipulating the expression of alanine racamase in *Bacillus licheniformis*. *Biotechnology Letters*, 2017, 39(9): 1389—1394.

[44] Li, Y, Gu, Z, Zhang, L, et al. Inducible expression of trehalose synthase in *Bacillus licheniformis*. *Protein Expression and Purification*, 2017, 130: 115—122.

[45] Craynest, M, Jorgensen, S, Sarvas, M, et al. Enhanced secretion of heterologous cyclodextrin glycosyltransferase by a mutant of *Bacillus licheniformis* defective in the D-alanylation of teichoic acids. *Letters in Applied Microbiology*, 2003, 37(1): 75—80.

[46] Milner, JA, Martin, DJ, Smith, A. Oxygen transfer conditions in the production of alpha-amylase by *Bacillus amyloliquefaciens*. *Enzyme and Microbial Technology*, 1996, 18(7): 507—512.

[47] Castro, GR, Mendez, BS, Sineriz, F. Amylolytic enzymes produced by *Bacillus amyloliquefaciens* MIR-41 in batch and continuous culture. *Journal of Chemical Technology and Biotechnology*, 1993, 56(3): 289—294.

[48] Zhou, C, Cui, C, Zhao, M. Optimization on glutaminase production from *Bacillus amyloliquefaciens* SWJS22 by solid-state fermentation. *Food and Machinery*, 2015, 31(6): 33—37.

[49] Ehrlich, HL, Watson, DW. Lysogenesis of *Bacillus megatherium*. *Journal of Bacteriology*, 1949, 58(5): 627—632.

[50] Biedendieck, R. A *Bacillus megaterium* system for the production of recombinant proteins and protein complexes. In: *Advanced Technologies for Protein Complex Production and Characterization*, (Ed.) M.C. Vega, 2016, 896: 97—113.

[51] Saxena, R, Singh, R. Contemporaneous production of amylase and protease through CCD response surface methodology by newly isolated *Bacillus megaterium* strain B69. *Enzyme Research*, 2014, 2014: 601046—601046.

[52] Rygus, T, Hillen, W. Inducible high-level expression of heterologous genes in *Bacillus megaterium* using the regulatory elements of the xylose-utilization operon. *Applied Microbiology and Biotechnology*, 1991, 35(5): 594—599.

[53] Biedendieck, R, Gamer, M, Jaensch, L, et al. A sucrose-inducible promoter system for the intra- and extracellular protein production in *Bacillus megaterium*. *Journal of Biotechnology*, 2007, 132(4): 426—430.

[54] Malten, M, Biedendieck, R, Gamer, M, et al. A *Bacillus megaterium* plasmid system for the production, export, and one-step purification of affinity-tagged heterologous levansucrase from growth medium. *Applied and Environmental Microbiology*, 2006, 72(2): 1677—1679.

[55] Ma, Y, Shen, W, Chen, X, et al. Significantly enhancing recombinant alkaline amylase production in *Bacillus subtilis* by integration of a novel mutagenesis-screening strategy with systems-level fermentation optimization. *Journal of Biological Engineering*, 2016, 10.

[56] Chen, J, Fu, G, Gai, Y, et al. Combinatorial sec pathway analysis for improved heterologous protein secretion in *Bacillus subtilis*: identification of bottlenecks by systematic gene overexpression. *Microbial Cell Factories*, 2015,14.

[57] Chen, J, Zhu, Y, Fu, G, et al. High-level intra- and extra-cellular production of D-psicose 3-epimerase via a modified xylose-inducible expression system in *Bacillus subtilis*. *Journal of Industrial Microbiology & Biotechnology*, 2016, 43(11): 1577—1591.

[58] Gerngross, TU. Advances in the production of human therapeutic proteins in *yeasts* and *filamentous fungi*. *Nature Biotechnology*, 2004, 22(11): 1409—1414.

[59] Gellissen, G, Hollenberg, CP. Application of *yeasts* in gene expression studies: a comparison of *Saccharomyces cerevisiae*, *Hansenula polymorpha* and *Kluyveromyces lactis*—a review. *Gene*, 1997, 190(1): 87—97.

[60] Zheng, X, Fang, B, Han, D, et al. Improving the secretory expression of an α-Galactosidase from *Aspergillus niger* in *Pichia pastoris*. *PLoS One*, 2016, 11(8): e0161529.

[61] Xu, Y, Wu, J, Zheng, K, et al. A xylanase from *Streptomyces sp.* FA1: heterologous expression, characterization, and its application in Chinese steamed bread. *Journal of Industrial Microbiology & Biotechnology*, 2016, 43(5): 663—670.

[62] Han, C, Su, L, Hong, R, et al. A comparative study of maltooligosyltrehalose synthase from *Sulfolobus acidocaldarius* expressed in *Pichia pastoris* and *Escherichia coli*. *Process Biochemistry*, 2017, 60: 35—41.

[63] Sathya, TA, Khan, M. Diversity of glycosyl hydrolase enzymes from metagenome and their application in food industry. *Journal of Food Science*, 2014, 79(11): R2149—R2156.

[64] Li, Y, Yi, P, Wang, N, et al. High level expression of beta-mannanase (RmMan5A) in *Pichia pastoris* for partially hydrolyzed guar gum production. *International Journal of Biological Macromolecules*, 2017, 105: 1171—1179.

[65] Abdulrachman, D, Thongkred, P, Kocharin, K, et al. Heterologous expression of *Aspergillus aculeatus* endo-polygalacturonase in *Pichia pastoris* by high cell density fermentation and its application in textile scouring. *Bmc Biotechnology*, 2017, 17.

[66] Raistrick, H, Clark, AB. On the mechanism of oxalic acid formation by *Aspergillus niger*: report to the medical research committee. *The*

Biochemical Journal, 1919, 13(4): 329—44.

[67] Zhang, H, Wang, S, Zhang, XX, et al. The amyR-deletion strain of *Aspergillus niger* CICC2462 is a suitable host strain to express secreted protein with a low background. *Microbial Cell Factories*, 2016. 15.

[68] Nevalainen, H, Peterson, R. Making recombinant proteins in *filamentous fungi*-are we expecting too much?. *Frontiers in Microbiology*, 2014, 5.

[69] Archer, DB, Jeenes, DJ, Mackenzie, DA. Strategies for improving heterologous protein-production from *Filamentous fungl*. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology*, 1994, 65(3): 245—250.

[70] Izmirlioglu, G, Demirci, A. Strain selection and medium optimization for glucoamylase production from industrial potato waste by *Aspergillus niger*. *Journal of the Science of Food and Agriculture*, 2016, 96(8): 2788—2795.

[71] Xiao, Z, Wu, M, Grosse, S, et al. Genome mining for new alpha-amylase and glucoamylase encoding sequences and high level expression of a glucoamylase from *Talaromyces stipitatus* for potential raw starch hydrolysis. *Applied Biochemistry and Biotechnology*, 2014, 172(1): 73—86.

[72] Carrillo-Sancen, G, Carrasco-Navarro, U, Tomasini-Campocosio, A, et al. Effect of glucose as a carbon repressor on the extracellular proteome of *Aspergillus niger* during the production of amylases by solid state cultivation. *Process Biochemistry*, 2016, 51(12): 2001—2010.

[73] Naganagouda, K, Salimath, PV, Mulimani, VH. Purification and characterization of endo-beta-1,4 mannanase from *Aspergillus niger* gr for application in food processing industry. *Journal of Microbiology and Biotechnology*, 2009, 19(10): 1184—1190.

[74] Li, J, Zhang, H, Zhang, Y, et al. Construction of food-grade xylanase engineering strain of *Aspergillus niger*. *Journal of Northeast Agricultural University*, 2013, 44(11): 7—13.

[75] Chen, X, Zhou, B, Xu, M, et al. Enhancing the expression of *Aspergillus niger* beta-mannanase in *Pichia pastoris* by coexpression of protein disulfide isomerase. *Turkish Journal of Biology*, 2015, 39(2): 312—319.

[76] Chen, Z, Yan, Q, Jiang, Z, et al. High-level expression of a novel alpha-galactosidase gene from *Rhizomucor miehei* in *Pichia pastoris* and characterization of the recombinant enyzme. *Protein Expression and Purification*, 2015, 110: 107—114.

Wu Jing

Dr. Wu obtained her bachelor's and master's degree of Fermentation Engineering from Wuxi Institute of Light Industry, her doctoral degree of Microbial and Biochemical Pharmacy from China Pharmaceutical University. She performed post-doctoral training of Biochemistry at the University of Michigan, and is now a full professor in the State Key Laboratory of Food Science and Technology, Jiangnan University. Dr. Wu's work focuses on the development of basic theories and key technologies for gene identification, functional reconstruction and high-yield production of enzymes utilized in food industry. So far, she has published more than 160 papers and about 110 of them were collected by SCI. She has chief-edited 3 scientific books, and obtained 46 authorized patents (3 international). Dr. Wu received the First Prize of Progress in Science and Technology awarded by the Ministry of Education (ranking No. 1). Moreover, she was elected to the Young Scientific and Technological Innovation Leader Program by the Ministry of Science and Technology and the Excellent Talents in New Century Program by the Ministry of Education, and received the National Outstanding Researcher in Science and Technology Award. In addition, she was supported by the National Science Fund for Distinguished Young Scholars and was elected as distinguished professor of "Yangtze River Scholar Program" by the Ministry of Education.