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Highly efficient enzymatic preparation of isomalto-oligosaccharides from starch using an enzyme cocktail

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ABSTRACT

Background: Current commercial production of isomalto-oligosaccharides (IMOs) commonly involves a lengthy multistage process with low yields.

Results: To improve the process efficiency for production of IMOs, we developed a simple and efficient method by using enzyme cocktails composed of the recombinant *Bacillus naganoensis* pullulanase produced by *Bacillus licheniformis*, α -amylase from *Bacillus amyloliquefaciens*, barley bran β -amylase, and α -transglucosidase from *Aspergillus niger* to perform simultaneous saccharification and transglycosylation to process the liquefied starch. After 13 h of reacting time, 49.09% IMOs (calculated from the total amount of isomaltose, isomaltotriose, and panose) were produced.

Conclusions: Our method of using an enzyme cocktail for the efficient production of IMOs offers an attractive alternative to the process presently in use.

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1. Introduction

Isomalto-oligosaccharides (IMOs) are glucose oligomers with α -D-(1,6)-linkages, with or without α -(1 \rightarrow 4) linkages [1], that include isomaltose, panose, isomaltotriose, isomaltotetraose, isomaltopentaose, nigerose, kojibiose, and higher branched oligosaccharides [2,3]. In addition, this definition has been extended to include glucooligosaccharides linked by α -(1 \rightarrow 6) linkage and/or a lower proportion of α -(1 \rightarrow 3) (nigerooligosaccharides) or α -(1 \rightarrow 2) (kojioligosaccharides) glucosidic linkages found in commercial IMO products [4,5,6,7] and IMO-like products. For example, branched IMOs known as glucooligosaccharides are produced using dextranase [8]; oligodextran by controlled-hydrolysis of dextran [9]; nonreducing IMO alditols by dextranase-catalyzed glucosylation of alditols such as glucitol, mannitol, maltitol, and Isomalt^R [10], isomaltulose or palatinose (α -D-Glc p-(1 \rightarrow 6)- α -D-Fru f) [11], and cyclic IMOs [12]. Thus, IMO structure is characterized by the IMOs degree of

polymerization (DP) (from 2 to ~10), type of linkages (α -1-2, 3, 4, or 6), and the proportion and position of each type of linkage. The most abundant and well-recognized functional components in IMOs are isomaltose/ α -D-Glc p-(1 \rightarrow 6)- α -D-Glc p, isomaltotriose/ α -D-Glc p-(1 \rightarrow 6)- α -D-Glc p-(1 \rightarrow 6)-D-Glc p, and panose/ α -D-Glc p-(1 \rightarrow 6)- α -D-Glc p-(1 \rightarrow 4)-D-Glc p, collectively abbreviated as iMTP [3].

IMOs are of considerable interest in the food, pharmaceutical, and cosmetic industries because of their unique properties such as low viscosity, resistance to crystallization, reduced sweetness, and bifidogenic effects. The most important property of IMOs and the key to their success is their prebiotic property that is responsible for their beneficial effects, including better intestinal health, mineral absorption, cholesterol regulation, and immunity and prevention of and resistance to various diseases such as dental caries. IMOs are also used as substitute sugars for patients with diabetes [13,14]. IMOs are normal components of the human diet and occur naturally in many fermented foods, including rice miso, soy sauce, and sake [15]. Isomaltose has also been identified as a natural constituent of honey [16].

Most commercial IMOs are enzymatically produced from starch through transglycosylation [2,3]. The typical commercially scaled starch-as-substrate process includes the following enzymatic processes: (1) starch is liquefied to form maltodextrin with bacterial

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or thermotolerant α -amylase (EC 3.2.1.1); (2) fungal α -amylase or β -amylase (EC 3.2.1.2) is used for saccharification to generate syrup with approximately 40–50% maltose and maltotriose [pullulanase (EC 3.2.1.41) may also be used in this step to obtain higher maltose solution with residual maltodextrins with low DP]; and (3) transglycosylase such as *Aspergillus niger* α -transglucosidase (EC 2.4.1.24) is used for IMO synthesis.

Improved efficiencies of IMO preparations have been reported by optimizing the coupled effects of enzymes used in the manufacturing process. Maltogenic amylases (EC 3.2.1.133) from various bacteria have been shown to exhibit both α -(1,6)-transglycosylation and α -(1,4)-hydrolysis activities [17,18,19]. The coupled transglycosylation and hydrolysis activities of maltogenic amylases [20] have been used to produce IMOs from liquefied starch in a more efficient way than the conventional three-step process, resulting in a significant increase in the maximum yields of IMOs. Further improvement of IMO yields from liquefied starch was observed with the combined use of maltogenic amylase from *Bacillus stearothermophilus* ET1 and α -glucanotransferase (EC 2.4.1.25) from *Thermotoga maritima* with α -(1,4)-glycosidic bond transferring ability [21]. Production of long-chain IMOs from maltotriose has been reported using the thermostable amyloamylase and transglucosidase in combination, although, separately, amyloamylase resulted in products containing linear malto-oligosaccharides (MOSs) and transglucosidase resulted in short-chain IMOs [22]. In addition, *Corynebacterium glutamicum* amyloamylase was used to produce palatinose glucosides through the acceptor specificity of its intermolecular transglycosylation activity [23]. NMR analysis showed that the major products palatinose glucoside 1 and 2 were a tri- and tetra-saccharide, respectively, with the structure $[O-\alpha\text{-D-glucopyranosyl}-(1 \rightarrow 4)]_n-O-\alpha\text{-D-glucopyranosyl}-(1 \rightarrow 6)\text{-D-fructofuranose}$, where $n = 1$ or 2 [23]. No commercial application, however, has been reported using these improved processes or enzymes.

Although several new enzymes with desirable activities as described earlier have potential applications in IMO preparation, many aspects of the current IMO manufacturing need to be further improved or innovated because of its low substrate conversion rates, lengthy transglycosylation step, and undesirable enzyme usage/formula. In this study, using an enzyme cocktail composed of *Bacillus amyloliquefaciens* α -amylase (BAA), barley bran β -amylase (BBA), recombinant *Bacillus naganoensis* pullulanase in *Bacillus licheniformis* (PulA), and *A. niger* α -transglucosidase (An-TGase), the optimization of each step of IMO production including starch liquefaction, saccharification, and transglycosylation was investigated to develop novel processes for efficient IMO production.

2. Materials and methods

2.1. Enzymes and activity assay

The enzymes used in this study were BAA [24], thermostable α -amylase from *licheniformis* (BLA) [25], BBA, PulA [26], and An-TGase [27]. BLA (40,000 U/mL), BAA (2000 U/mL), and PulA (2000 U/mL) were obtained from Fujian Fuda Biotech Development Co., Ltd., China; BBA (700°DP/g) and An-TGase (360,000 U/g) were obtained from Jiangsu Ruiyang Biotech Co., Ltd., China.

The respective enzyme preparation activities were measured according to the methods described previously [25]. For BAA, one enzyme unit was defined as the amount of enzyme required to hydrolyze 1 g of starch in 1 h at pH 6.0 and 60°C. For BLA, one enzyme unit was defined as the amount of enzyme required to hydrolyze 1 mg of starch in 1 min at pH 6.0 and 70°C. PulA activity was determined according to the manufacturer instructions under the following conditions: the reaction mixture contained 0.1 mL of 0.5% (w/v) Red-Pullulan as substrate (MegaZyme, Co. Wicklow, A98 YV29, Ireland), 0.05 mol/L Tris-HCl buffer (pH 5.0), and 0.2 mL of enzyme

solution incubated at temperature of 40°C for 30 min. Then the reaction was stopped by adding 0.5 mL of ethanol; after holding for 10 min, the reaction mixture was centrifuged at 12000g for 2 min, and the absorbance of the supernatant was measured at 510 nm OD with water as the control. For PulA, one enzyme unit was defined as the amount of enzyme required to release reducing sugar equal to 1 μ mol glucose from 1 mL of 0.5% (w/v) Red-Pullulan in 1 min under the above reaction conditions. BBA activity was determined according to the protocol described in the fifth edition of Food Chemicals Codex [28]. One enzyme unit, expressed as degrees of diastatic power (°DP), was defined as the amount of enzyme in 0.1 mL of a 5% solution of the enzyme preparation that will produce sufficient reducing sugars to reduce 5 mL of Fehling's solution when the sample is incubated with 100 mL of the substrate for 1 h at 20°C. An-TGase activity was determined according to a previously described method [27]. One enzyme unit was defined as the amount of enzyme required to form 1 μ g of glucose in 1 h at pH 5.0 and 50°C.

2.2. Determination of pH and temperature optima

To determine the optimum temperature, the activities of the enzymes were measured at various temperatures ranging from 30°C to 80°C. To determine the optimum pH, the enzymatic activity was measured at the above temperatures under different pH conditions: pH 4.0–5.5 using acetate buffer and pH 6.0–7.5 using phosphate buffer.

2.3. Production of IMOs

For enzyme selection and parameter optimization, IMOs were prepared in a 500-mL reactor as follows: (1) Starch liquefaction: this step was performed using 25% (w/v, dry starch weight) corn starch slurry and 200 U/g (dried starch) of BLA at pH 5.8 and ~100°C for different time periods, and the samples were collected and the dextrose equivalent (DE) values were determined as described in Section 2.4. (2) Saccharification: When the reaction mixture was cooled to 55°C, a combination of enzymes was added to prepare MOS-rich syrup. The reference values of each enzyme (calculated based on dry substrate starch) are BBA 0.1°DP/g and PulA 0.1 U/g. (3) Transglycosylation: An-TGase was added to the reaction mixture (500 U/g dried starch), and the reaction was performed at 55°C for up to 30 h. For single parameter optimization, the above parameters for IMO preparation were fixed except for changes in one parameter. For the scaling-up preparation of IMOs in a 10-L reactor, 25% (w/v) liquefied corn starch with a DE value of approximately 25 was used as substrate and the last two steps, i.e., saccharification and transglycosylation, were combined and conducted at 55°C. The samples were collected, and the enzymes were inhibited by adjusting the pH to 9.0 with 1 mol/L sodium hydroxide and incubating at 100°C for 10 min.

2.4. Analytical methods

Total dry matter was determined as described previously [28]. The DE value of liquefied starch was determined by the titrimetric method with glucose as the reference, as described previously [29]. The content and components of IMOs and the sugar profiles during IMO preparation were analyzed according to a previously described method with some modification [30]. Briefly, the analysis was performed on an HPLC system (Agilent 1200 Series HPLC System) with an evaporative light-scattering detector (Alltech ELSD detector 2000s, Grace Co. Ltd.) using a TSKgel Amide-80 column (4.6 mm IDx250 mm, 5 μ m, Tosoh, Japan). Acetonitrile:water (67:33, v/v) was used as solvent at a flow rate of 1 mL min⁻¹ at 30°C. Glucose (G1) and maltose (G2) used as HPLC standards were purchased from Shanghai BioTech Co. Ltd., China. Isomaltose (IG₂), maltotriose (G3), isomaltotriose (IG₃), panose (P), maltotetraose (G4), maltopentaose

(G5), and maltohexaose (G6) used as HPLC standards were purchased from Sigma (St. Louis, MO). The MOS and IMO references were obtained from Jiangsu Ruiyang Biotech Co. Ltd., China.

iMTP value, a measure of the IMO content, was calculated as the percentage of the combined amounts of IG₂, IG₃, and P in the dried matter. The specific value of iMTP was calculated as iMTP (% w/w) divided by the reaction time.

3. Result and discussion

3.1. Effects of the degree of starch liquefaction on IMO yield

It is well established that the liquefaction degree of starch affects the quality and quantity of its final sugar products. For example, a certain degree of starch liquefaction (~DE 12) is required for higher glucose content formation during high-glucose syrup manufacturing, and a much lower degree of starch liquefaction (~DE 6 ~ 8) is preferred for higher maltose content during maltose syrup preparation [31]. However, in IMO preparation, not only maltose but also maltotriose and other MOSs formed during the starch liquefaction and saccharification are the main precursors of IMOs and favorite substrates for transglycosylation with An-TGase [32,33,34,35,36]. We therefore determined the effect of starch liquefaction degree on IMO formation. Starch slurry of 25% (w/v) was liquefied at different times using BLA at 100°C, and liquefied starch with DE values of 12, 20, or 30 was obtained. PuLA, BBA, and An-TGase were then added, and the reaction mixture was incubated at 55°C for up to 24 h. The sugar profiles were analyzed and are summarized in Fig. 1, which shows that increasing starch liquefaction degree shortened the transglycosylation time required for maximum yields of iMTP, indicating that well-liquefied starch was more beneficial to IMO formation with increased yields per reaction time.

3.2. pH and temperature optima for simultaneous saccharification and transglycosylation in the preparation of IMOs

During the saccharification and transglycosylation steps of IMO production, BBA or PuLA can be used to generate more maltose and MOSs, which serve as substrates for An-TGase. However, each enzyme has its own pH and temperature optimum. Studies have shown that BBA shows maximum activity at pH 5.5 and 55°C [35], PuLA at pH 4.5 and 60°C [36], and An-TGase at pH 4.5 and 60°C [32]. We evaluated the properties of these commercially available enzymes in this study (Fig. 2) and found that the values either agreed with or deviated only

slightly from the published values. An-TGase and PuLA showed the pH optimum at pH 5.0 and BBA at pH 5.5. All three enzymes retained more than 50% of their maximum activity at pH 5.0–6.0 (Fig. 2a). As for their temperature optima, PuLA, BBA, and An-TGase displayed the maximum activity at 60°C, 50°C, and 55°C, respectively. All three enzymes retained more than 50% of the maximum activity at temperatures from 45°C to 65°C (Fig. 2b).

To further examine the possibility of requiring pH and temperature control during IMO preparation, yields of iMTP were evaluated at different initial pH values and temperatures. As shown in Fig. 2c and Fig. 2d, saccharification and transglycosylation were only slightly affected by pH between 4.5 and 6.0 and temperatures between 45°C and 60°C. An optimal pH of 5.8 and optimal temperature of 55°C were selected for subsequent experiments.

3.3. Dosage of An-TGase affects IMO formation efficiency

The effects of the amount of An-TGase on iMTP preparation were examined, and the results are summarized in Fig. 3. The use of An-TGase at dosages of 500, 1000, and 2000 U/g resulted in maximum IMO yields of 46.47% at 13 h, 45.80% at 8 h, and 45.46% at 4 h, respectively. These results show that high dosage of An-TGase can significantly reduce the transglycosylation time. However, from the economic point of view (An-TGase is one of the most expensive enzymes used in IMO production), 500–1000 U/g dosage is more suitable for industrial-scale preparation of IMOs.

3.4. Roles of PuLA, BBA, and BAA in IMO preparation

Pullulanase is an enzyme that specifically hydrolyzes α -1,6-linkages in starch molecules and is known as a debranching enzyme [32]. To determine its effect on IMO preparation, different amounts of PuLA were added to the reaction mixture. Although the control showed a maximum of 35% iMTP formation, addition of PuLA significantly increased iMTP synthesis, achieving a maximum value of 46% when 0.9 U/g of PuLA was added (Table 1). However, treatment of IMO products with the same amount of PuLA under similar conditions showed no significant effects on IMO quality, as analyzed by HPLC (data not shown). From this, it can be said that PuLA is beneficial for IMO formation as it debranches liquefied starch, thus facilitating the consequent saccharification, but hardly hydrolyzes α -1,6-linkages in oligosaccharides.

β -Amylase specifically hydrolyzes α -1,4-linkages in starch molecules to form maltose [35]. To clarify the effect of BBA on IMO preparation, different amounts of BBA were added to the reaction mixture. In the absence of BBA, only about 33% IMOs were formed. The addition of 0.5°DP/g of BBA increased both the percentage of iMTP and the specific value of iMTP (Table 1). These results indicate that BBA enhances IMO formation by enhancing the yields of maltose and maltotriose and thus promotes transglycosylation by An-TGase. Furthermore, the activity of BBA, which catalyzes the hydrolysis of maltodextrin to form MOSs, is necessary for IMO production as re-confirmed in this study (Table 1). With the addition of a certain amount of BBA (0.3–0.7°DP/g), iMTP values could be significantly increased, and the maximum yield was obtained when $\geq 0.5^\circ$ DP/g of BBA was added.

Therefore, increasing the dosages of PuLA and BBA and the degree of starch liquefaction resulted in elevated levels of substrates for transglycosylation, especially higher contents of maltose and maltotriose. This leads to an improved IMO production efficiency through transglycosylation by An-TGase, which rapidly transfers glucosyl residues to MOSs, followed by gradual hydrolysis of both α -(1 \rightarrow 4) linkages and α -(1 \rightarrow 6) linkages at the nonreducing end to generate smaller molecules with mainly α -(1 \rightarrow 6) linkages [33,34]. Given that BAA catalyzes the hydrolysis of maltodextrin to form MOSs, it is expected that the use of BAA will generate more preferred

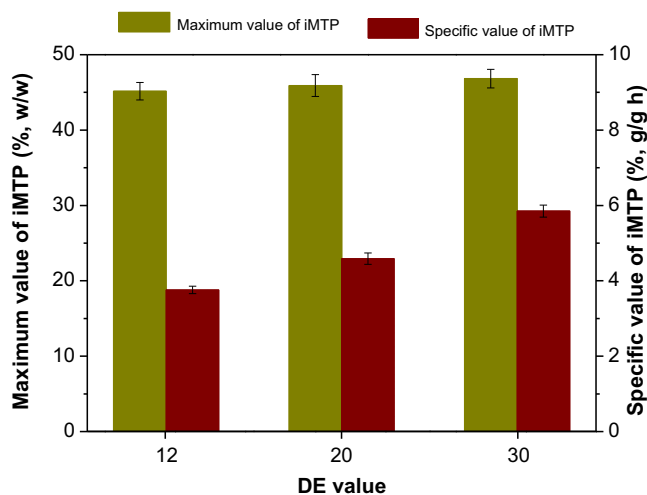


Fig. 1. Effect of starch liquefaction degree (DE) on the yield and specific production of isomaltose, isomaltotriose, and panose (iMTP).

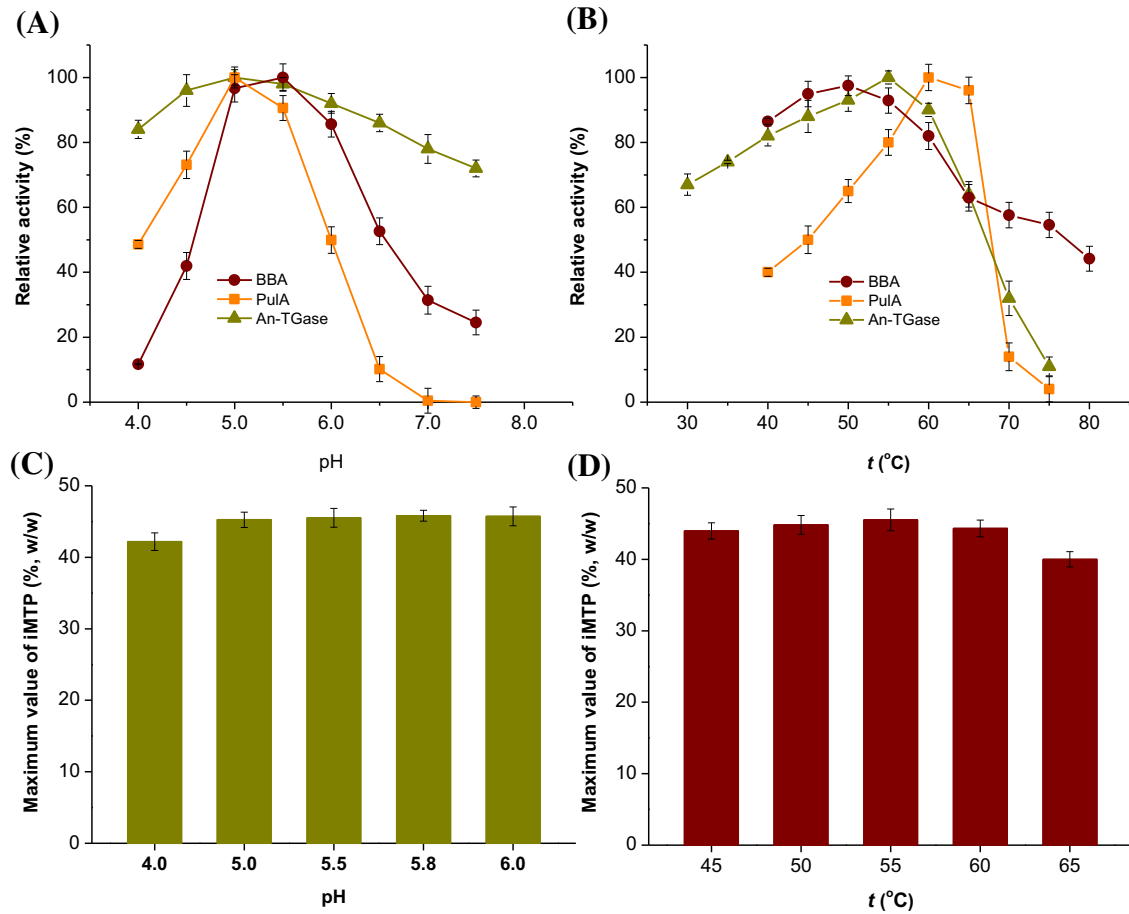


Fig. 2. Optimal pH and temperature used for IMO preparation. (a) optimum pH profile of enzymes, (b) optimum temperature profile of enzymes. (c) optimum pH for saccharification and transglycosylation. (d) optimum temperature for saccharification and transglycosylation.

substrates for BBA, which in turn produces higher levels of suitable substrates for An-TGase, thus accelerating the transglycosylation process during IMO preparation. Therefore, the effects of BAA (10–18 U/g) on IMO production were investigated with the use of 0.5°DP/g of BBA. As expected, increasing the dosage of BAA greatly enhanced the iMTP yield and especially the specific value of iMTP and shortened

the transglycosylation process (Table 1). The use of 16 U/g of BAA resulted in improved iMTP yield, with a 22% increase in the specific value of iMTP (from 3.01 to 3.68) (Table 1). These results also suggest a novel application of BAA in the preparation of long-chain or middle-chain isomalto/malto-polysaccharides, a novel soluble dietary fiber prepared through the enzymatic conversion of starch [37,38].

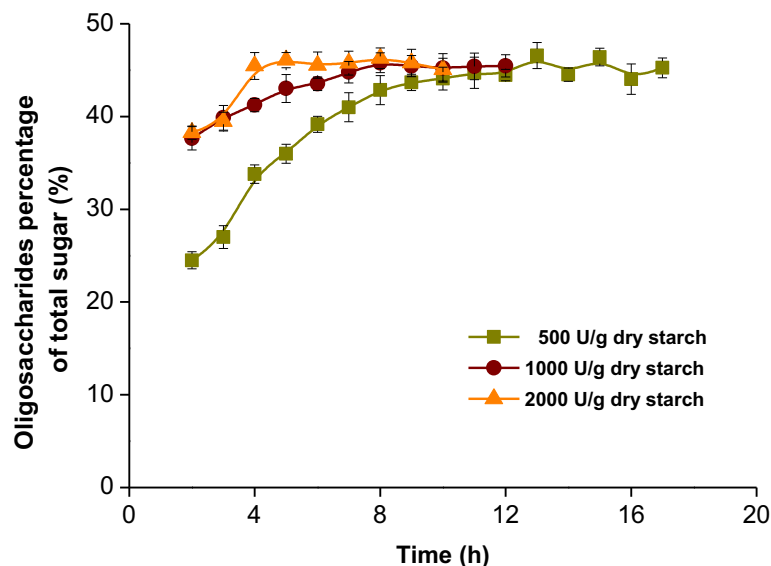


Fig. 3. Effects of α-glucosyltransferase dosage on IMO formation.

Table 1

Effects of pullulanase, BBA, and BAA on IMO formation during saccharification and transglycosylation stages.

Dosage of enzyme	Maximum value of iMTP (%, w/w)	Specific value of iMTP (%, g/g·h ⁻¹)
PulA (U/g)		
0	35.01 ± 1.33	2.33 ± 0.09
0.3	40.42 ± 1.23	2.70 ± 0.08
0.6	43.85 ± 1.42	2.92 ± 0.09
0.9	45.38 ± 1.33	3.03 ± 0.09
1.8	45.51 ± 1.23	3.03 ± 0.08
BBA (°DP/g)		
0	33.18 ± 1.33	2.21 ± 0.05
0.3	43.45 ± 1.23	2.90 ± 0.07
0.4	45.04 ± 1.42	2.92 ± 0.08
0.5	46.29 ± 1.33	3.01 ± 0.06
0.6	46.02 ± 1.23	3.07 ± 0.09
0.7	45.74 ± 1.13	3.05 ± 0.06
BAA (U/g)		
10	46.55 ± 1.25	3.58 ± 0.09
12	46.80 ± 1.36	3.60 ± 0.08
14	47.07 ± 1.29	3.62 ± 0.07
16	47.79 ± 1.20	3.68 ± 0.08
18	47.32 ± 1.09	3.64 ± 0.09

3.5. Improved processes for IMO production

The above results show that several microbial amylases are capable of catalyzing maltodextrin hydrolysis to form MOSs during enhanced IMO formation, and this prompted us to develop an enzyme cocktail-based process for IMO preparation. The reaction mixture in a 10-L reactor contained 25% (w/v) liquefied starch with a DE value of 25 and an enzyme cocktail. In the proposed process (Table 2) for IMO production using an enzyme cocktail of BAA, BBA, PulA, and An-TGase, 49.09% iMTP was obtained in 13 h, a significant improvement over the currently used process for commercial production with 39–41% iMTP yields (personal communication). Interestingly, the process proposed here can be easily modified to accommodate a Do-It-Yourself (DIY) style by increasing the dosage of An-TGase (Table 2), which would enable people to prepare their own IMOs using an enzyme cocktail of BAA, BBA, PulA, and An-TGase (2000 U/g). This rapid preparation of IMOs was completed in 4 h, making it suitable for the production of IMOs at home.

In the traditional method for the production of IMOs, α-amylase is added to starch slurry (30%), followed by maltogenase such as β-amylase for saccharification and then transglucosidase for transglycosylation. The resulting IMOs are finally purified with yeast fermentation and chromatography [39]. The entire process requires approximately 120 h to yield a mixture with 58% IMOs [39]. However, in the currently used process for the commercial-scale production of IMOs, a thermostable α-amylase is added to starch slurry, followed by a jet liquefaction process at 107–109°C. After cooling to 55°C, fungal α-amylase is used for saccharification for 4 h and subsequently transglucosidase is added to perform transglycosylation for another 40–60 h. The resulting IMOs are finally physically or biotechnologically purified to prepare IMOs of different grades. A lengthy process and low yields are the main disadvantages of this process, which takes 45–65 h to produce 39–41% IMOs (unpublished data from a Chinese IMO manufacturer, personal communication). The new process developed in this study uses PulA, BAA, BBA, and An-TGase to perform

simultaneous saccharification and transglycosylation to process the liquefied starch. After 13 h of reaction time, 49.09% IMOs (calculated from the total amount of isomaltose, isomaltotriose, and panose) were produced. A rapid process for IMO preparation was also tested for a DIY purpose, with a yield of 45.5% IMOs in 4 h (Table 2).

An alternative method has been developed in which *Geobacillus stearothermophilus* maltogenic amylase and *T. maritima* α-glucanotransferase were simultaneously used to process the liquefied starch solution. After 14 h of reaction, a yield of 68% IMOs was obtained [20]. This relatively lower yield is possibly due to the formation of DP₄- or larger IMOs, which are not calculated as the contents of IMOs.

4. Conclusion

In the present study, a novel process for high-efficiency production of IMOs was developed based on the combined functions of commercially available *B. naganoensis* pullulanase, *B. amyloliquefaciens* α-amylase, barley bran β-amylase, and *A. niger* α-transglucosidase. Pullulanase is a crucial factor to accelerate saccharification and MOSs formation and significantly shortened the duration of transglycosylation. Saccharification and transglycosylation can be done simultaneously with acceptable IMO productivity, thus providing a new possibility to simplify and improve the IMO production from starch with an enzyme cocktail.

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Conflict of Interest Statement

The authors declare that they have no competing interests.

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Table 2

IMO production using different processes.

Enzyme cocktails	Maximum value of iMTP (%, w/w)	Specific value of iMTP (%, g/g·h ⁻¹)	Process parameters
BAA/BBA/PulA/An-TGase	49.09 ± 1.56	3.78 ± 0.12	16.0 U/g BAA, 0.9 U/g PulA, 0.5°DP/g BBA, 500 U/g An-TGase at pH 5.8 and 55°C for 13 h
BAA/BBA/PulA/An-TGase (DIY)	45.52 ± 0.91	11.38 ± 0.23	16.0 U/g BAA, 0.9 U/g PulA, 0.5°DP/g BBA, 2000 U/g An-TGase at pH 5.8 and 55°C for 4 h

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