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Enzymatic Synthesis of Galacto-oligosaccharides in an Organic–Aqueous Biphasic System by a Novel β-Galactosidase from a Metagenomic Library

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ABSTRACT: Prebiotic galacto-oligosaccharides (GOS) were effectively synthesized from lactose in organic–aqueous biphasic media by a novel metagenome-derived β-galactosidase BgaP412. A maximum GOS yield of 46.6% (w/w) was achieved with 75.4% lactose conversion rate in the cyclohexane/buffer system [95:5 (v/v) cyclohexane/buffer] under the optimum reaction conditions (initial lactose concentration = 30% (w/v), T = 50 °C, pH 7.0, and t = 8 h). The corresponding productivity of GOS was approximately 17.5 g L^{-1} h^{-1}. The GOS mixture consisted of tri-, tetra-, and pentasaccharides. Trisaccharides were the chief component of reaction products. These experimental results showed that a low water content, a high initial lactose concentration, and an elevated reaction temperature could significantly promote the transgalactosylation activity of β-galactosidase BgaP412; at the same time, the enhanced GOS yield in an organic–aqueous biphasic system is because of the fact that thermodynamic equilibrium can be shifted to the synthetic direction by reversing the normal hydrolysis.

KEYWORDS: *Galacto-oligosaccharides, β-galactosidase, biphasic system, transgalactosylation, metagenome*

INTRODUCTION

Galacto-oligosaccharides (GOS), non-digestible carbohydrates derived from lactose, are composed of galactose monomers and a terminal glucose unit, varying in chain length and type of linkage. As a kind of prebiotics,[1−3] GOS are present naturally in mammalian milk. Injection of them can stimulate the growth of health-promoting bacteria[1,4] and offer some health benefits, such as improving mineral absorption,[5,6] inhibiting the adhesion of pathogenic bacteria to the colonic epithelium cells,[7,8] stimulating of the immune system,[9] and reducing the risk of colon cancer.[2] Therefore, GOS are widely incorporated in infant milk formulas, dairy products, beverages, clinical nutrition, bakery products, and pet food.[3]

GOS can be produced from lactose by the transgalactosylation activity of β-galactosidases (EC 3.2.1.23).[10,11] In the reaction mixture, β-galactosidase can catalyze hydrolysis and the transgalactosylation reaction concurrently. It first cleaves the β-1,4-D-glycosidic bonds between glucose and galactose and then transfers the galactosyl moieties to different acceptor molecules containing hydroxyl groups. When the acceptor is water, galactose is produced and the process is known as hydrolysis. On the other hand, GOS are formed when the galactosyl moieties are transferred to other carbohydrates, and the pathway is referred to as transgalactosylation.[10,11] Finally, transgalactosylation and hydrolysis will spontaneously approach a dynamic equilibrium. To date, the main drawback in aqueous media is that the catalysis equilibrium would be shifted to favor hydrolysis over transgalactosylation, which leads to a low GOS yield.[12] To overcome this disadvantage, enzymatic synthesis of GOS in an organic–aqueous system was developed, and it has been proven to be an effective strategy because the thermodynamic equilibrium can be shifted to the synthetic direction by reversing the normal hydrolysis as a result of the decrease of water activity in the system.[12−16]

In recent years, increasing attention has been paid to enzymatic synthesis of GOS from lactose, and the most widely studied β-galactosidases for GOS synthesis are from a microbial source. Enzymes from various species, including *Aspergillus*, *Bacillus*, *Kluyveromyces*, *Streptococcus*, *Bullera*, *Cryptococcus*, *Sporobolomyces*, *Sulfolobus*, and *Thermus*,[17−19] have been used for the synthesis of GOS from lactose, and it has been shown that the source of the enzyme has a direct impact on the optimal pH and temperature and the type of glycoside bond between D-galactose units and components in GOS.[18,19] However, thus far, all β-galactosidases applied for GOS synthesis were obtained by the traditional cultural approaches, which only focus on less than 1% of microorganisms in the environment, because more than 99% of microorganisms are uncultivable.[20] In addition, most β-galactosidases were less active and stable in the presence of organic solvents. Therefore, it is important to screen novel β-galactosidases with high production capacity for GOS and excellent tolerance to organic solvents. As a culture-independent method, metagenomics could be applied to discover novel genes for enzymes from both cultured and uncultured microorganisms within varieties of environments,[20,21] and it has been demonstrated as an effective approach in mining novel biocatalysts.[22] To date, a great variety of biotechnologically important genes for enzymes, such as esterase/lipase, protease, amylase, amidases, xylanase, and

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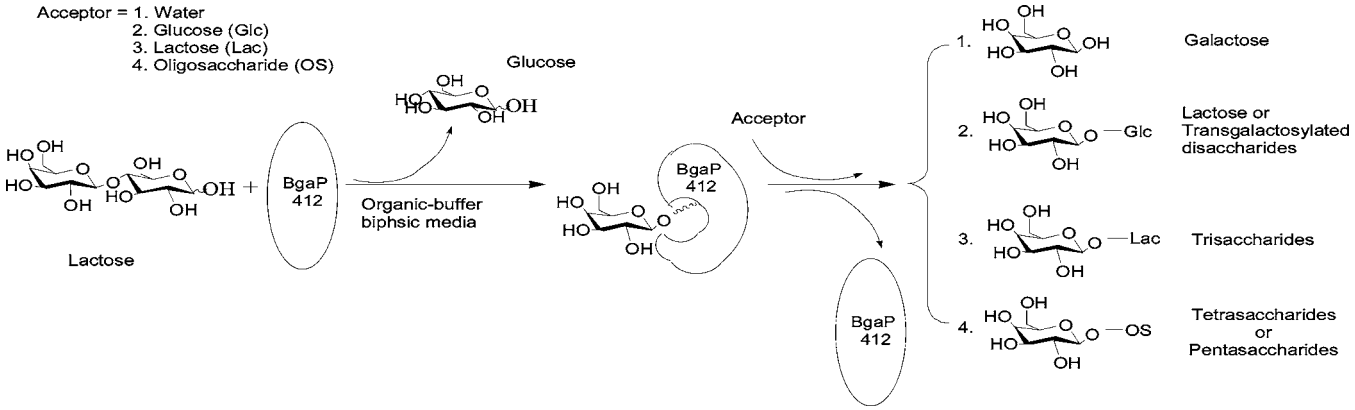

Figure 1. Enzymatic synthesis of GOS in organic−buffer media catalyzed by recombinant BgaP412.

cellulase, has been obtained through metagenomic approaches.[22−24]

Recently, a novel β-galactosidase gene (*bgaP412*, GenBank accession number JQ086357) was isolated by activity-based screening of a metagenome library, subsequently subcloned in *Escherichia coli* and recombinantly expressed in *Pichia pastoris*. The gene encoded a protein with a predicted molecular weight of 75 kDa. On the basis of sequence analysis, we know that BgaP412 belongs to the glycoside hydrolase 42 (GH-42) superfamily. BgaP412 exhibited high tolerance to water-immiscible organic solvents and displayed high transgalactosylation activity, which indicated that it may be useful in the production of GOS. This study was designed to investigate the enzymatic synthesis of GOS under the catalysis of BgaP412 in organic−aqueous media (Figure 1). To the best of our knowledge, this is the first report on GOS synthesis via a metagenome-derived β-galactosidase. The influence of organic solvents, water content, and initial lactose concentration in the biphasic system on the GOS yield was investigated. Operating parameters, including reaction temperature (T), pH, and reaction time (t), were optimized.

MATERIALS AND METHODS

Strains, Plasmids, and Materials. *E. coli* DH5α (Novagen, Madison, WI) were used as host for gene cloning. The *P. pastoris* GS115 *his*4 strain (Invitrogen, Carlsbad, CA) was used as a host for protein expression. The pPICZαB (Invitrogen, Carlsbad, CA) was employed to clone and express the target gene. *Eco*RI, *Kpn*I, *Pme*I, T4 DNA ligase, and DNA polymerase were purchased from Takara (Dalian, China). *o*-Nitrophenyl-β-D-galactopyranoside (*o*-NPG), lactose, glucose, and galactose were purchased from Sigma-Aldrich (St. Louis, MO). Cyclohexane [analytical reagent (AR) grade] was obtained from Guangzhou Chemical Reagent Factory (Guangzhou, China). All other chemicals and reagents were of analytical grade and were purchased from commercial sources, unless indicated otherwise.

Cloning, Expression, and Preparation of Purified Recombinant β-Galactosidase. The complete *bgaP412* sequence from the positive clone was subcloned into the pPICZαB vector (*Eco*RI and *Kpn*I sites) in DH5α and expressed in *P. pastoris*. *E. coli* transformants were cultivated at 37 °C in low-salt Luria−Bertani (LB) medium containing zeocin (25 μg/mL). *P. pastoris* transformants were cultivated at 30 °C in yeast extract−peptone−dextrose (YPD) medium containing zeocin (25 μg/mL). After confirmation by sequencing, the correct recombinant *P. pastoris* cells were inoculated to 100 mL of phosphate-buffered minimal methanol medium supplemented with 1% yeast extract (BMMY) and grown to an OD_{600} of 6.0 at 30 °C with agitation, and then the cells were collected at 3000*g* for 5 min and resuspended in several 100 mL of BMMY medium to an OD_{600} of 1.0. Cultures were supplemented with 0.5% methanol every 24 h, and cultivation continued at 30 °C for 4 days with agitation. After that, the extracellular β-galactosidase in the cultures was purified using the His•Bind Purification Kit (Novagen, Madison, WI) according to the instructions of the manufacturer. The purified β-galactosidase was stored in glycine−sodium hydroxide buffer (50 mM, pH 9.0) at 4 °C and remained fully active throughout. Protein concentrations were determined according to the method of Bradford using bovine serum albumin as the standard.

Standard β-Galactosidase Assay. β-Galactosidase activity was assayed at 52 °C using *o*-NPG as the substrate. The reaction mixture was composed of 100 μL of the enzyme solution and 400 μL of *o*-NPG solution (2.5 g/L in 50 mM potassium phosphate buffer at pH 6.8). After incubation at 52 °C for 10 min, the reaction was terminated by adding 500 μL of 10% sodium carbonate. The absorbance of *o*-nitrophenol was measured at 405 nm using a spectrophotometer. A total of 1 unit of β-galactosidase activity is defined as the amount of enzyme needed to produce 1 μmol of *o*-nitrophenol per minute under the assay condition.

Enzymatic Synthesis of GOS. The synthesis of GOS was carried out with lactose monohydrate as the substrate and recombinant β-galactosidase as the catalyst. The reaction mixture (25 mL) containing purified enzyme (10 units), lactose monohydrate, buffer, and organic solvents was added into a 100 mL conical flask with a ground-in glass stopper, and the reaction was carried out in a thermostatic oscillator. When the reaction was terminated, the mixture was centrifuged at 12000*g* for 5 min at room temperature and the aqueous phase was collected and diluted with deionized water. The as-prepared syrup was used for further analysis.

The influence of organic solvents with different log P values [dimethylsulfoxide (−1.35), acetonitrile (−0.34), ethyl acetate (0.73), *n*-butanol (0.84), butyl acetate (1.78), heptanol (2.62), cyclohexane (3.44), heptane (4.66), and octane (5.18)] on the synthesis of GOS was investigated using the aqueous buffer solution (50 mM potassium phosphate buffer at pH 7.0), which contained 10 units of recombinant enzyme and 30% lactose (w/v). The ratios of aqueous/organic phases were all 5:95 (v/v). The reaction mixtures were placed at 50 °C, and samples (0.5 mL) were withdrawn at regular intervals terminated by heating at 100 °C for 10 min to inactivate the enzyme.

To optimize synthetic conditions in cyclohexane/buffer biphasic media, the reactions were studied at seven different water contents (5, 10, 15, 20, 25, 30, and 35%), six different initial lactose concentrations (5, 10, 20, 30, 40, and 50%), six different pH values (6, 7, 8, 9, 10, and 11), and six different reaction temperatures (30, 35, 40, 45, 50, and 55 °C) for 8 h. Aliquots (0.5 mL) were withdrawn at regular intervals and terminated by heating at 100 °C for 10 min.

The effects of variations in the reaction time on GOS synthesis were determined in the optimum conditions [cyclohexane/potassium phosphate buffer (50 mM) = 95:5 (v/v), enzyme activity = 10 units, initial lactose concentrations = 30% (w/v), pH 7.0, and T = 50 °C]. Aliquots (0.5 mL) were withdrawn at regular time intervals (t = 2, 4, 6, 8, 10, and 12 h) and by heating at 100 °C for 10 min.

Carbohydrate Analysis. *Thin-Layer Chromatography (TLC).* For qualitative analysis of carbohydrate composition, the syrups mentioned before were diluted to 5% (w/v) with deionized water and then the diluents were analyzed by TLC on aluminum sheets coated with silica gel 60 (Merck, Darmstadt, Germany) using butanol/ethanol/water (5:3:2, v/v/v) as the mobile phase. The different spots were visualized by spraying a solution containing 0.5% (w/v) 3,5-dihydroxytoluene and 20% (v/v) sulfuric acid and heating for 20 min at 120 °C.[25] Afterward, the different spots were quantitated by the software ImageJ 1.40.[25] Assays were performed in triplicate.

High-Performance Liquid Chromatography (HPLC). Quantitative analysis of individual carbohydrates was performed with HPLC with a refractive index detector via a system equipped with a degassing system (Agilent Technologies, Cheshire, U.K.) and a pump (Agilent Technologies, Cheshire, U.K.). The column used was a 300 × 7.8 mm inner diameter, 8 μm, Rezex RNM carbohydrate Na^+ (8%), with a 50 × 7.8 mm inner diameter guard column of the same material (Phenomenex Co., Los Angeles, CA), a refractive index detector 830-RI (Jasco Co., Tokyo, Japan), and a chromatography data system (SIS Co., Livermore, CA). HPLC-grade water was used as the mobile phase at a flow rate of 0.5 mL/min. The injection volume was 20 μL. Individual sugars in the mixtures were identified by comparing the retention times to those of standard sugars, including Gal(β1−6)-Gal(β1−6)-Gal(β1−6)-Gal(β1−4)-Glc, Gal(β1−6)-Gal(β1−6)-Gal-(β1−4)-Glc, 6′-galactosyl-lactose, lactose, glucose, and galactose. Quantitative measurement of each peak was performed using standard calibration curves of sugar standards.[26]

Determination of the GOS Yield and Conversion Rate. The GOS yield and lactose conversion rate were calculated using the following formulas:

$$\text{GOS yield (\%)} = \frac{\text{GOS formed}}{\text{total sugars}} \times 100$$

$$\text{lactose conversion rate (\%)} = (\text{initial lactose content} - \text{residual lactose content}) / \text{initial lactose content} \times 100$$

Total sugars is the sum of all sugars (glucose, galactose, residual lactose, and GOS), and it is also equal to the initial lactose content.

RESULTS AND DISCUSSION

Effect of Different Organic Phases on GOS Synthesis. As is well-known, the nature of the organic solvents may greatly affect the activity of biocatalysts. Log *P* (logarithm of partition coefficient of a substance in the standard 1-octanol/water two-phase system) has been the most commonly used to describe solvent polarity and gives the best correlation with the enzyme activity.[27] To evaluate the effect of different organic phases on transgalactosylation of recombinant BgaP412, several organic solvents were selected. The log *P* values of these solvents vary from −1.35 to 5.18. Potassium phosphate buffer (50 mM, pH 7.0) was employed as the control. Figure 2 shows that the lactose conversion rate in buffer was the highest (92.6%) and the GOS yield was approximately 34.6% (w/w). In contrast, higher GOS yields were obtained with lower lactose conversion rates in the media with cyclohexane, *n*-heptane, butyl acetate, ethyl acetate, and heptanol, respectively. A maximum GOS yield of 46.6% (w/w) was achieved at 75.4% lactose conversion rate in the cyclohexane/buffer system (95:5, v/v). The conclusive contrast between a high lactose conversion rate and a low GOS yield in buffer suggested that hydrolysis occupied a dominant position in the aqueous environment. In contrast, GOS yields were enhanced in water-immiscible organic solvent media because the reaction equilibrium was shifted to favor transgalactosylation over hydrolysis by lowering the water activity. However, the GOS yield and lactose conversion rate were both low in the presence of dimethyl sulfoxide and acetonitrile. The difference in the production of GOS can be attributed to the direct effect of each solvent on the operational performance of the enzyme. Generally, solvents can strip the essential water that maintains the active conformation of enzymes, and hydrophilic organic solvents (−2.5 < log *P* < 0) tend to strip more essential water than hydrophobic organic solvents (2 < log *P* < 4), which leads to enzyme denaturation and inactivation.[28−30] Therefore, hydrophobic solvents are usually superior to hydrophilic solvents as enzymatic reaction media.[12,14,31] In addition, solvents can directly influence the activity of enzymes by altering hydrogen bonds and hydrophobic interactions.[29,30] Consequently, there was no clear linear correlation between the water solubility of organic solvents and yields of the transglycosidation product. This was consistent with the previous reports.[12−14,32]

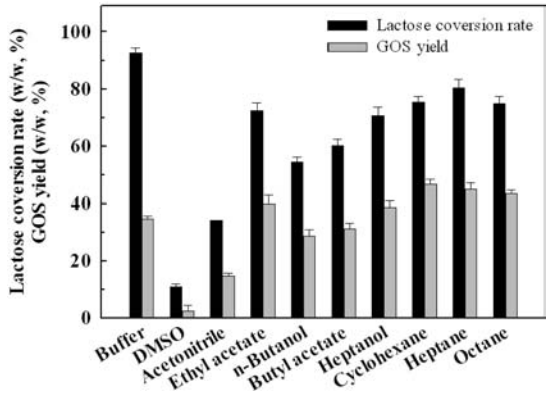

Figure 2. GOS yield and lactose conversion rate in different organic−aqueous two-phase media [β-galactosidase activity = 10 units, initial lactose concentrations = 30% (w/v), *T* = 50 °C, pH 7.0, and *t* = 8 h]. Bars indicate standard deviations. DMSO = dimethyl sulfoxide.

Because the highest GOS yield was attained in the immiscible cyclohexane/buffer system and the subsequent recycling of organic solvent and separation of reaction products are easy to operate in such a system, cyclohexane was selected as the organic phase. The further experiments was optimized in the cyclohexane/buffer system.

In our study, the reaction products were analyzed by both TLC and HPLC and the results of TLC analysis were consistent with that of HPLC analysis. Furthermore, TLC analysis figures were more intuitive, concrete, and authentic than line charts, which represented the results of HPLC analysis. Thus, the TLC analysis figures were used to present the effect of the reaction parameters on the GOS synthesis.

Effect of the Water Content on GOS Synthesis. Water is necessary to maintain the active conformation of the enzyme. The amount of water in the biphasic system plays an important role in the synthesis of GOS. The effect of the water content on GOS synthesis was investigated in a range of 5−35% in the cyclohexane/buffer media. As shown in Figure 3A, the maximum yield was obtained with 5% (v/v) of water in the system. As the water content increased, both the lactose conversion rate and GOS yield decreased. When the water content increased to 35%, the GOS yield has been reduced to 39% and the lactose conversion rate was also reduced to 63%, indicating that the stability and catalytic activity of recombinant BgaP412 decreased slightly in the presence of a higher water

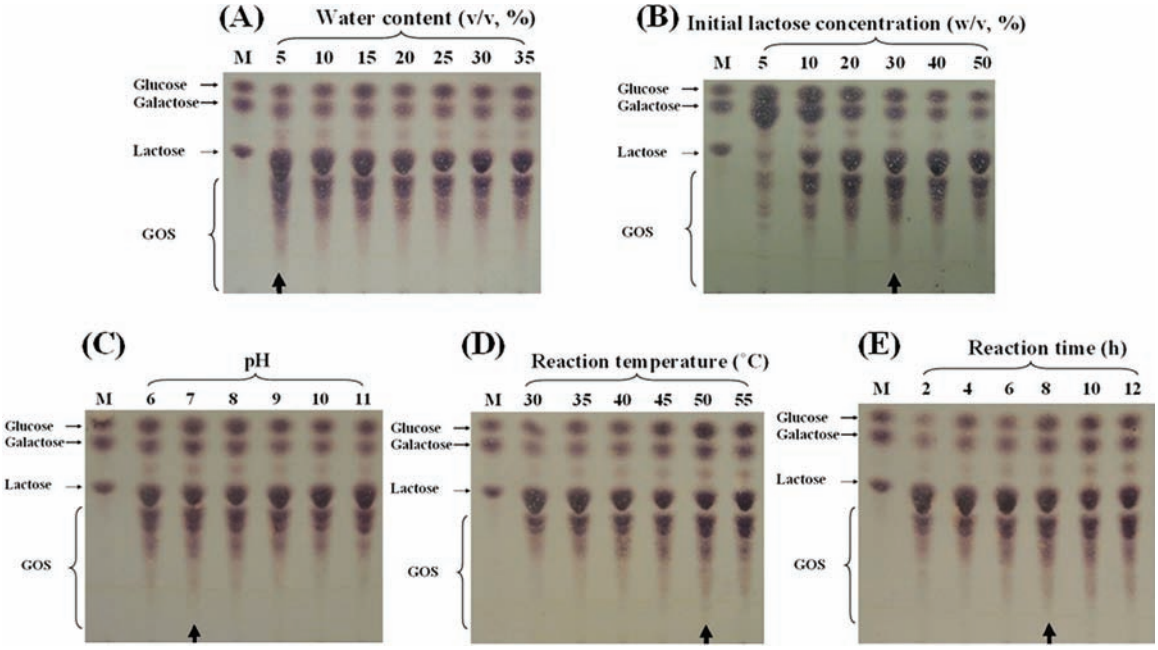

Figure 3. Qualitative analysis by TLC of GOS synthesis in cyclohexane/buffer media catalyzed by recombinant BgaP412 with lactose as the substrate. The conditions used in each group were (A) varied water contents (5−35%, v/v), β-galactosidase activity = 10 units, initial lactose concentrations = 30% (w/v), T = 50 °C, pH 7.0, and t = 8 h; (B) varied initial lactose concentrations (5−50%, w/v), water content = 5%, β-galactosidase activity = 10 units, T = 50 °C, pH 7.0, and t = 8 h; (C) varied pH (pH 6−11), water content = 5%, β-galactosidase activity = 10 units, initial lactose concentrations = 30% (w/v), T = 50 °C, and t = 8 h; (D) varied temperatures (30−55 °C), water content = 5%, β-galactosidase activity = 10 units, initial lactose concentrations = 30% (w/v), pH 7.0, and t = 8 h; and (E) varied time (2−12 h), water content = 5%, β-galactosidase activity = 10 units, initial lactose concentrations = 30% (w/v), T = 50 °C, and pH 7.0.

content. This tendency observed in our study differed from the report by Chen et al.,[12] who reported that the dependence of the GOS yield and water content was a bell-shaped curve and the maximum yield was attained at 10% water content. However, our observation was similar to the report by Hua et al.,[32] who found both the yield of the transgalactosylation product and the lactose conversion rate progressively decreased as the water content increased, but their decline rates were much more obvious. Another reason for the reduction of the GOS yield was that the thermodynamic equilibrium was shifted from transgalactosylation toward hydrolysis when the water content rose.

It is particularly worth noting that neither the hydrolysis nor the transgalactosylation reaction proceeded in the absence of water (neat cyclohexane; data not shown). There are two main reasons for this phenomenon. First, as described above, enzymes require some essential water bound to their surface to exhibit both conformational flexibility and enzymatic activity. Therefore, enzymes are more rigid and less active in anhydrous organic solvents than in organic−aqueous mixtures.[29,30] Second, lactose cannot be dissolved in the neat organic solvent, which prevents its contact to the active site of the enzyme.[32] Hence, the following experiments were carried out in the cyclohexane/buffer system (95:5, v/v).

Effect of the Initial Lactose Concentration on GOS Synthesis. The substrate concentration in the reaction media can markedly affect the enzyme activity via influencing the water solubility of the solvent. The effect of the initial lactose concentration was studied in a weight percent range of 5−50% (w/v), and the results are displayed in Figure 3B. It was found that the yield of GOS increased with an increasing initial lactose concentration from 5 to 30% and decreased slightly afterward. A maximum GOS yield was attained when the initial lactose concentration was 30%. It is worth mentioning that GOS yields were kept at a high level (exceeded 40%) in high lactose concentrations (10−40%). The main reason for this phenomenon is that a high initial lactose concentration made donor and acceptor molecules of the galactosyl moieties more available in the reaction mixture and, thus, decreased the availability of water molecules as acceptors of the galactosyl moieties; hence, more transgalactosylation proceeded while less hydrolysis took place. Gosling et al.[10] suggested that an increase in transgalactosylation seemed more important than a decrease in hydrolysis for explaining the enhanced yield of GOS at raised initial lactose concentrations. Various studies have indicated that the initial lactose concentration in the reaction mixture (aqueous and organic−aqueous) is a main factor influencing GOS synthesis.[10,12,14,16,33] This work reconfirmed the importance of employing a high initial lactose concentration for achieving a high GOS yield. The optimal initial lactose concentration of recombinant BgaP412 was 30% (w/v) under the conditions used, whereas the highest lactose conversion rate was achieved when the initial lactose concentration was 5%, and progressively decreased with the increase of lactose supply. The reason for this change may be that the oversupplied lactose has caused the inhibition of recombinant BgaP412.

Effect of the pH on GOS Synthesis. As presented in Figure 3C, the maximum GOS yield was obtained at pH 7.0 and the pH value showed minimal effect on GOS production in a broad range from 6.0 to 9.0. Within this range, the GOS yield and lactose conversion rate held above 41.5 and 68.5%, respectively. The lactose conversion rate changed concurrently with the GOS yield throughout the entire pH range. The optimum pH for GOS production is 2 units lower than the pH (9.0) optimal for this enzyme to exhibit the highest hydrolytic activity, which was previously mentioned. The effect of pH on

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the GOS production observed in this study was in accordance with the publication by Hua et al.[32] and vastly different from the report by Srisimarat and Pongsawasdi.[16] The former found that a wide pH range from 6.0 to 9.0 was applicable to the synthesis reaction using immobilized lactase and immobilized glucose isomerase. The latter reported that GOS production, catalyzed by β-galactosidase from *Aspergillus oryzae*, dropped dramatically when pH $\geq$ 6.0 and no GOS was produced when pH $\geq$ 8.0. These discrepancies can be attributed to the different sources of β-galactosidases. In general, β-galactosidases from yeast or bacterial origin have more neutral pH optima between 6 and 7.5, while fungal β-galactosidase from *A. oryzae* showed optimum GOS formation at pH as low as 3.5.[19]

Effect of the Temperature on GOS Synthesis. The influence of the reaction temperature on GOS synthesis was investigated by varying the temperature from 30 to 55 °C. As shown in Figure 3D, both the GOS yield and lactose conversion rate increased with the increase of the reaction temperature in the range of 30−50 °C and reached their maxima at T = 50 °C. This observation was consistent with the report by Hsu et al.,[33] whose results demonstrated that, as the reaction temperature increased from 25 to 45 °C, the GOS yield increased from 13 to 32.5% and the lactose conversion rate increased from 24.1 to 59.4%. The primary reason for this behavior is that high temperatures were able to speed the reaction, increase the solubility of the lactose, and reduce the viscosity of the reaction medium, so that the transgalactosylation activity was favored over the hydrolytic activity. However, a further increase of the reaction temperature resulted in a reduction in both the GOS yield and lactose conversion rate. While the reaction temperature was over 55 °C, both the GOS yield and lactose conversion rate significantly reduced (data not shown). This may be attributed to the unstability of the enzyme at a temperature higher than 55 °C. In particular, it was found that the reaction temperature had little effect in a wide range from 40 to 55 °C; the GOS yield maintained 43%, and the lactose conversion rate remained 72%. This suggested that recombinant BgaP412 showed excellent thermostability in the biphasic system.

Effect of the Reaction Time on GOS Synthesis. Figure 3E showed the effects of the reaction time on GOS synthesis. Figure 4 displayed a time course of GOS synthesis catalyzed by recombinant BgaP412, including the content variations of GOS, glucose, galactose, and lactose. In the initial stage of the reaction, the amount of lactose declined rapidly, while contents of GOS, glucose, and galactose increased in varying degrees. Furthermore, the content of glucose in the reaction media was found to be higher than that of galactose consistently throughout the whole process, indicating the participation of galactose in GOS synthesis. The highest GOS yield of 46.6% was obtained when the gap between the two monosaccharides reached a maximum after 8 h of reaction. This indicated that transgalactosylation was dominant in the early period. After that, the GOS yield began to decrease, which was similar to those reports previously published.[11,12,14,16,32,33] The decrease suggested that the formed GOS started to hydrolyze at a faster pace than they were synthesized.

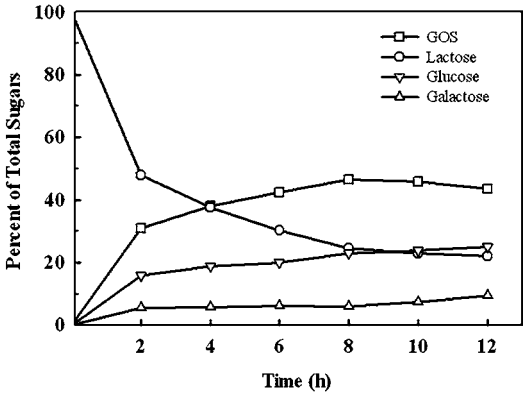

Figure 4. Time course of GOS synthesis in cyclohexane/buffer media catalyzed by recombinant BgaP412 [cyclohexane/buffer = 95:5 (v/v), β-galactosidase activity = 10 units, initial lactose concentrations = 30% (w/v), pH 7.0, and T = 50 °C].

GOS Synthesis in Optimum Conditions. GOS synthesis by recombinant BgaP412 was performed in the cyclohexane/buffer system [95:5 (v/v) cyclohexane/buffer] under the optimum conditions [initial lactose concentrations = 30% (w/v), β-galactosidase activity = 10 units, T = 50 °C, pH 7.0, and t = 8 h], and the reaction product was analyzed by TLC and HPLC. Figure 5 showed the TLC analysis of the product mixture. Figure 6 is a typical HPLC profile of the reaction product. The tri-, tetra-, and pentasaccharides were noted. Yields of them were 30.7, 10.1, and 5.8%, respectively. This result was in agreement with the research by Srisimarat and Pongsawasdi,[16] who reported that three types of GOS were produced by β-galactosidases from *A. oryzae* in organic solvents with cyclodextrin. However, our results were different from the reports by Hsu et al.[33] and Osman et al.[11] The former found that only two types of GOS were formed by β-galactosidases from *Bifidobacterium longum* BCRC 15708, while the latter reported that four types of GOS were produced by whole cells of *Bifidobacterium bifidum* NCIMB 41171, including transgalactosylated disaccharides. It should be noted that the HPLC analytical system used in this study was unable to differentiate lactose and transgalactosylated disaccharides. Currently, almost all of the reports demonstrated that trisaccharides were the major type of GOS produced.[10−16,33] That is because trisaccharides are synthesized in one step by transferring

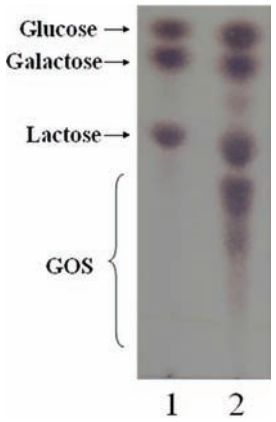

Figure 5. TLC analysis of the reaction product formed by recombinant BgaP412 in the cyclohexane/buffer system [95:5 (v/v) cyclohexane/buffer] under the optimum reaction conditions [initial lactose concentrations = 30% (w/v), β-galactosidase activity = 10 units, pH 7.0, T = 50 °C, and t = 8 h]. Lane 1, enzymatic synthesis reaction mixture; lane 2, lactose, galactose, and glucose standard mixtures.

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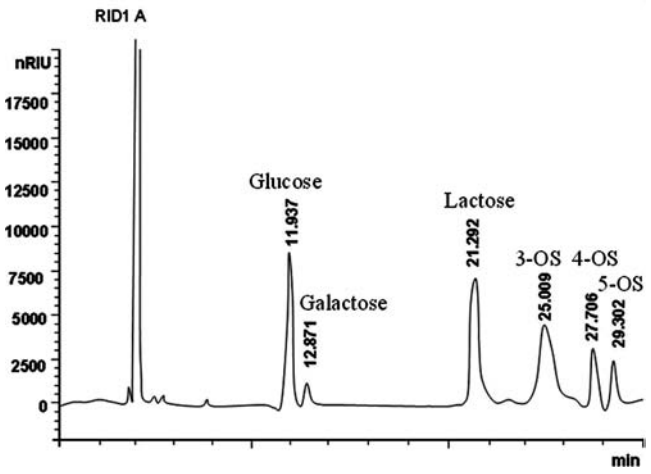

Figure 6. HPLC profiles of the reaction product formed by recombinant BgaP412 in the cyclohexane/buffer system [95:5 (v/v) cyclohexane/buffer] under the optimum reaction conditions [initial lactose concentrations = 30% (w/v), β-galactosidase activity = 10 units, pH 7.0, T = 50 °C, and t = 8 h]. The peaks were glucose, 11.9 min; galactose, 12.9 min; lactose, 21.3 min; trisaccharides (3-OS), 25.0 min, tetrasaccharides (4-OS), 27.7 min; and pentasaccharides (5-OS), 29.3 min.

galactosyl moieties to lactose, whose concentration is the highest in the reaction mixture at the beginning.

In conclusion, GOS were effectively synthesized via a novel metagenome-derived β-galactosidase in organic−aqueous biphasic media. In comparison to other β-galactosidases employed in GOS production, BgaP412 from the metagenomic library exhibited high tolerance to water-immiscible organic solvents and displayed high transgalactosylation activity in wide ranges of initial lactose concentrations (10−40%, w/v), pH values (pH 6−9), and reaction temperatures (40−55 °C) in the cyclohexane/buffer biphasic system. This approach has several advantages over aqueous media. First, it can shift the thermodynamic equilibrium toward the transgalactosylation reaction over hydrolysis by lowering water activity. Second, the hydrophilic interactions between water and organic solvent increase the lactose concentration in micro-aqueous core of organic−aqueous complexes. Third, the presence of organic solvent offers the advantage of limiting potential for microbial contamination within the process. All of these characteristics made BgaP412 a potential candidate for industrial production of GOS. This study highlights the utility of the organic−aqueous biphasic system in improving the synthesis of GOS and demonstrates that the metagenomic approach is a valuable tool in discovering potential novel biocatalysts.

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Notes

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ABBREVIATIONS USED

GOS, galacto-oligosaccharides; Lac, lactose; Glc, glucose; OS, oligosaccharides; *o*-NPG, *o*-nitrophenyl-β-D-galactopyranoside; YPD, yeast extract−peptone−dextrose; BMMY, phosphate-buffered minimal methanol supplemented with 1% yeast extract

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