Food Chemistry 362 (2021) 130195

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

# Optimization and comparison of the production of galactooligosaccharides using free or immobilized *Aspergillus oryzae* β-galactosidase, followed by purification using silica gel

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ARTICLE INFO

*Keywords:*
Galactooligosaccharides
Optimization
Free enzyme
Immobilized enzyme
Purification
Economical evaluation

ABSTRACT

The aim of this study was to optimize and compare the production of galactooligosaccharides (GOSs) by free and cotton cloth-immobilized *Aspergillus oryzae* β-galactosidase, and perform economical evaluation of production of GOSs (100%) between them. Using the response surface method, the optimal reaction time (3.9 h), initial lactose concentration (57.13%), and enzyme to lactose ratio (44.81 U/g) were obtained for the free enzyme, which provided a GOSs yield of 32.62%. For the immobilized enzyme, the optimal yield of GOSs (32.48%) was obtained under reaction time (3.09 h), initial lactose concentration (52.74%), and temperature (50.0 °C). And it showed desirable reusability during five successive enzymatic reactions. The recovery rate of GOSs (100%) is 65% using silica gel filtration chromatography. The economical evaluation showed almost no difference in the manufacturing cost for the GOSs (100%) between these two systems, and that the recovery rate had a great impact on the cost.

## 1. Introduction

In the last century, commercial products containing galactooligosaccharides (GOSs) have been successfully launched in both Japan and Europe (Nauta, Bakker-Zierikzee, & Schoterman, 2009; Spherix consulting, Inc., 2010). They are usually applied to the food and beverage industry. As one of several approved prebiotics, GOSs have attracted a lot of attention due to their prebiotic activities such as their ability to stimulate the proliferation of beneficial bacteria and interact directly with intestinal epithelial cells (Rodriguez-Colinas, Poveda, Jimenez-Barbero, Ballesteros, & Plou, 2012; Hosono et al., 2003). In 2020, the global GOS market is expected to reach 175.7 kilo tons worth USD 1.01 billion, driven by the increasing attention on food nutrition (Panesar, Kaur, Singh, & Kennedy, 2018).

Enzymatic catalysis of lactose to GOSs by β-galactosidase is the most preferred mode of synthesis. Although this enzyme can originate from various microbial sources, *Aspergillus oryzae* has been shown to be the most promising enzyme source for commercial application in terms of food safety and expenditure (Albayrak & Yang, 2002; Zhou & Chen, 2001). At present, the enzymatic transglycosylation employed in the production of GOSs is an established technology, however, there are still several well-known challenges of it. At first, response parameters such as temperature, enzyme to lactose ratio (*E/S*), initial lactose concentration, pH, reaction time, and even the mode of reactor operation can all affect the GOS synthetic process (Vera, Guerrero, Conejeros, & Illanes, 2016). Neri et al. (2009), and Vera, Guerrero, and Illanes (2011) focused on the effects of several parameters on GOS yield, however, no any interactions between parameters were studied. In fact, the GOS synthesis is a result of the co-regulation of multiple parameters. Therefore, systematic research on these parameters, as well as combination of the response surface method (RSM) is necessary to understand the GOS production. Secondly, it has been found that the cost of the enzyme used for GOS synthesis is impossible to ignore especially when using a soluble enzyme in large quantities (Albayrak & Yang, 2002). In this regard, an immobilized

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https://doi.org/10.1016/j.foodchem.2021.130195
Received 21 June 2020; Received in revised form 25 April 2021; Accepted 23 May 2021
Available online 26 May 2021

enzyme has some advantages, including easy separation from the GOS mixtures and the ability to reuse it (Freitas, Marquez, Ribeiro, & Brandao, 2011). The cotton cloth has shown definite advantages in immobilization due to its high porosity, large specific surface, excellent mechanical strength, and ease of use (Albayrak & Yang, 2002). Although, the enzyme immobilized on cotton can be recycled, no study provides evidence that it is more practical and economical than free enzyme for the synthesis of GOSs, especially for purified GOSs (100%). It's urgent to optimize and compare the GOS production using these two forms of enzyme, followed by an economic evaluation. Thirdly, one of the major concerns in GOS production is that the purification of the its mixtures to remove the accompanying monosaccharides and the unreacted lactose, since downstream processing costs account for a substantial fraction of the total production cost (Vera et al., 2016). Both the Sephadex and Bio-gel have been used extensively to separate saccharides followed by structural characterization (Kumar, Prashanth, & Venkatesh, 2015). However, the packing material is quite expensive when gram-levels of GOSs are desired, which severely decreases its commercial value and will be an obstacle for its widespread application. The simulated moving bed chromatography (SMBC) has also been used in GOS purification, nevertheless, the high cost of the equipment and upkeep limits its application. Silica gel is one of the oldest and widely available chromatographic beads (Maruška & Pyell, 1997), and has been used successfully to separate polysaccharides (Krawczyk, Zalewski, Janeta, & Hodurek, 2018). To our knowledge, this is the first time to purify GOS mixtures by silica gel, which could greatly reduce the costs of packing material and equipment. In addition, preparation of efficient $\beta$-galactosidase with specific structure as well as utilization of the low-value co-product to produce GOSs are the new challenges of real-world applications. After all, the GOS yield and composition vary with the structure of the enzyme, and that will further affect its prebiotic function (Gosling, Stevens, Barber, Kentish and Gras, 2010). The whey permeate, co-product of cheese industry, can be the substrate of GOS production due to its high content of lactose, but the related researches are limited. Nevertheless, as to the *Aspergillus oryzae* $\beta$-galactosidase, Fischer and Kleinschmidt (2015) pointed out that results obtained with pure lactose solutions can be transferred to whey permeates.

Herein, we set out to optimize and compare the GOS production process using free and immobilized enzyme (*A. oryzae* $\beta$-galactosidase) systems. Meanwhile, the reusability of immobilized enzyme was also evaluated. Purification of GOSs (100%) was performed using a home-made silica gel column, and then was assessed using Fourier-transform infrared spectroscopy (FTIR). Finally, economic feasibility analysis for the production of purified GOSs (100%) by free and immobilized enzyme was conducted using SuperPro Designer.

## 2. Materials and methods

### 2.1. Enzyme and chemical reagents

*Aspergillus oryzae* $\beta$-galactosidase (EC 3.2.1.23, Genencor International, Rochester, NY, USA) with a declared activity of 100,000 fungal lactase units/g was used in this study. The amount of enzyme that produces 1 μmol o-nitrophenol per min at pH 4.5 and 37 °C is defined as one unit. Standards of D (+) glucose, D (+) galactose, D (+) lactose monohydrate, and o-nitrophenyl-$\beta$-galactopyranoside (ONPG) were purchased from Beijing Puxi Technology Co., Ltd, Beijing, China. Polyethyleneimine (PEI, No: 181978-5G) and glutaraldehyde (No: 01008335) were purchased from Sigma (St. Louis, MO, USA). Silica gel (200–300 mesh) was purchased from Macklin (Shanghai, China). Cotton cloth with 100% cotton fiber was obtained from a local market.

### 2.2. Construction of the immobilized enzymatic reaction system

The immobilized enzymatic reaction system was constructed using cotton cloth pretreated with PEI and was used to study various ratios of enzyme to PEI and enzyme to cotton cloth. The experimental procedure has been previously described by Albayrak and Yang (2002) and was used here with minor modification related to the determination of immobilized enzyme activity. In detail, a certain mass of cotton cloth was immersed in the PEI solution (pH at 8.0) for 5 min to completely wet the cloth, then the cloth was unfolded and the residual PEI solution was added to it. The enzyme solution (total 5 mL) was added to the cloth and transferred to a shaker at 150 rpm for 10 min. After the slurry became clear, the supernatant was discarded and a volume of glutaraldehyde equal to that of the PEI solution was added to cross-link the PEI-enzyme aggregate coated on the cloth. This step was conducted on a shaker at 150 rpm for 5 min. Finally, the cotton cloth was washed twice with distilled water and then three times with acetic acid buffer (pH 4.5). The activity of the immobilized enzyme on the cotton cloth was measured using a range of glucose concentrations versus enzyme activities, i.e. a standard curve of glucose concentrations (Y axis) and free enzyme activity (X axis) was generated. The glucose levels in the samples were measured using a kit (Rongsheng Biological Medicine Co. Ltd, Shanghai, China). Then the activity of the immobilized enzyme was calculated by referring to this standard curve using its corresponding glucose content. The immobilization yield can be obtained by following equation.

Immobilization yield (%) = activity of immobilized enzyme/activity of total enzyme × 100%, where the total enzyme is the amount of the enzyme offered to the cotton for immobilization.

The cotton cloth loaded with enzyme was air dried, and a few cotton fibers were fixed using double faced adhesive tape followed by metal spraying. The microstructure of the surface of the fiber was examined using a scanning electron microscope (SEM) (Hitachi SU-8020, Tokyo, Japan).

### 2.3. Determination of the optimal parameters for GOS production using free and immobilized enzyme

A 50% (w/v) lactose solution (prepared by dissolving in sodium acetate buffer) with pH 4.5 and an enzyme to lactose ratio (*E/S)* of 30 U/g was used to evaluate the effect of reaction time on GOS synthesis in a thermostatic gas bath shaker (THZ-92A, Shanghai Boxun Industrial Co., Ltd., Shanghai, China) with the temperature set at 50 °C for up to 12 h. Using a 4 h reaction time, the effect of temperature on GOS production was evaluated by conducting the reaction from 30 to 70 °C in 10 °C increments. Similarly, the effect of different initial concentrations of lactose (10 to 60%, w/v), the effect of different *E/S* ratios at 10, 20, 30, 40, 50, and 60 U/g lactose, and the effect of pH ranging from 3.0 to 7.0 were also evaluated using several different shakers. All experiments were conducted in triplicate.

### 2.4. Response surface methodology (RSM) for GOS optimization

Based on the results obtained from the single factor experiments using the free and immobilized enzymatic action systems, reaction time (X), initial lactose concentration (Y), and enzyme to lactose ration (Z) in free enzyme system, as well as reaction time (X), initial lactose concentration (Y), and temperature (Z) in immobilized enzyme system were selected for further exploration using a three-level design in Design Expert (Version 7.0.0, Stat-Ease Inc., Minneapolis, MN, USA). A response surface method, with a Box-Behnken experimental design, was used to statistically optimize the above reaction conditions and evaluate the effects of these variables on GOS yield. Tables S1-S6 showed the arrangement of independent variables, design matrix, and results. The optimization trial was repeated twice.

### 2.5. Reusability of immobilized enzyme in GOS production

The reutilization experiment under the optimal conditions was performed to evaluate the performance of the immobilized enzyme for GOS production. The immobilized enzyme in the reactor was washed by

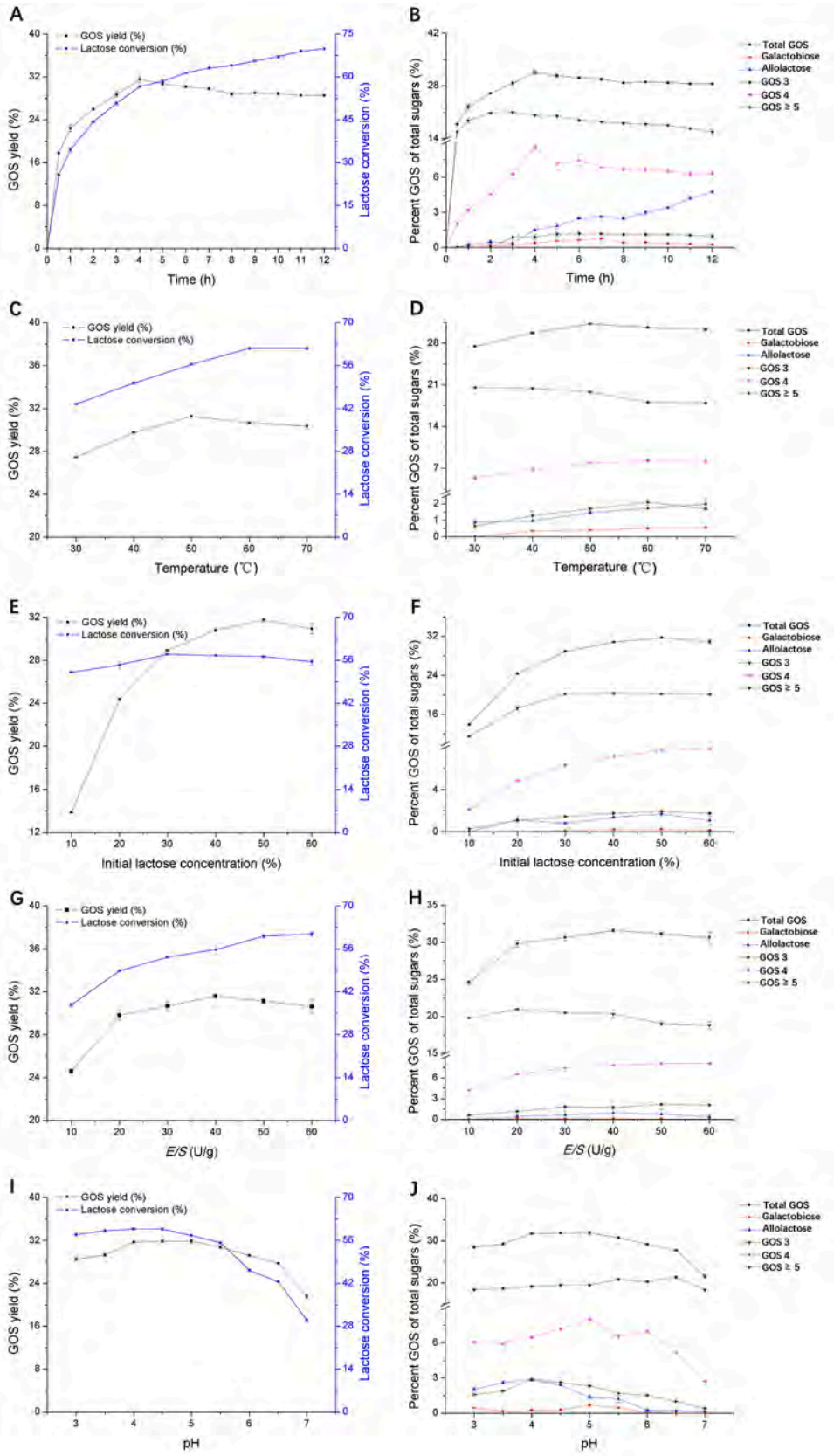


**Fig. 1.** Effect of different reaction parameters on GOS production in the free enzymatic reaction system. Panels A, C, E, G, and I showed the effects of reaction time, temperature, initial lactose concentration, enzyme to lactose ratio (*E/S*), and solution pH on total GOS yield and lactose conversion. Correspondingly, panels B, D, F, H, and J show the effects of the same parameters on the levels of the specific oligosaccharides indicated.

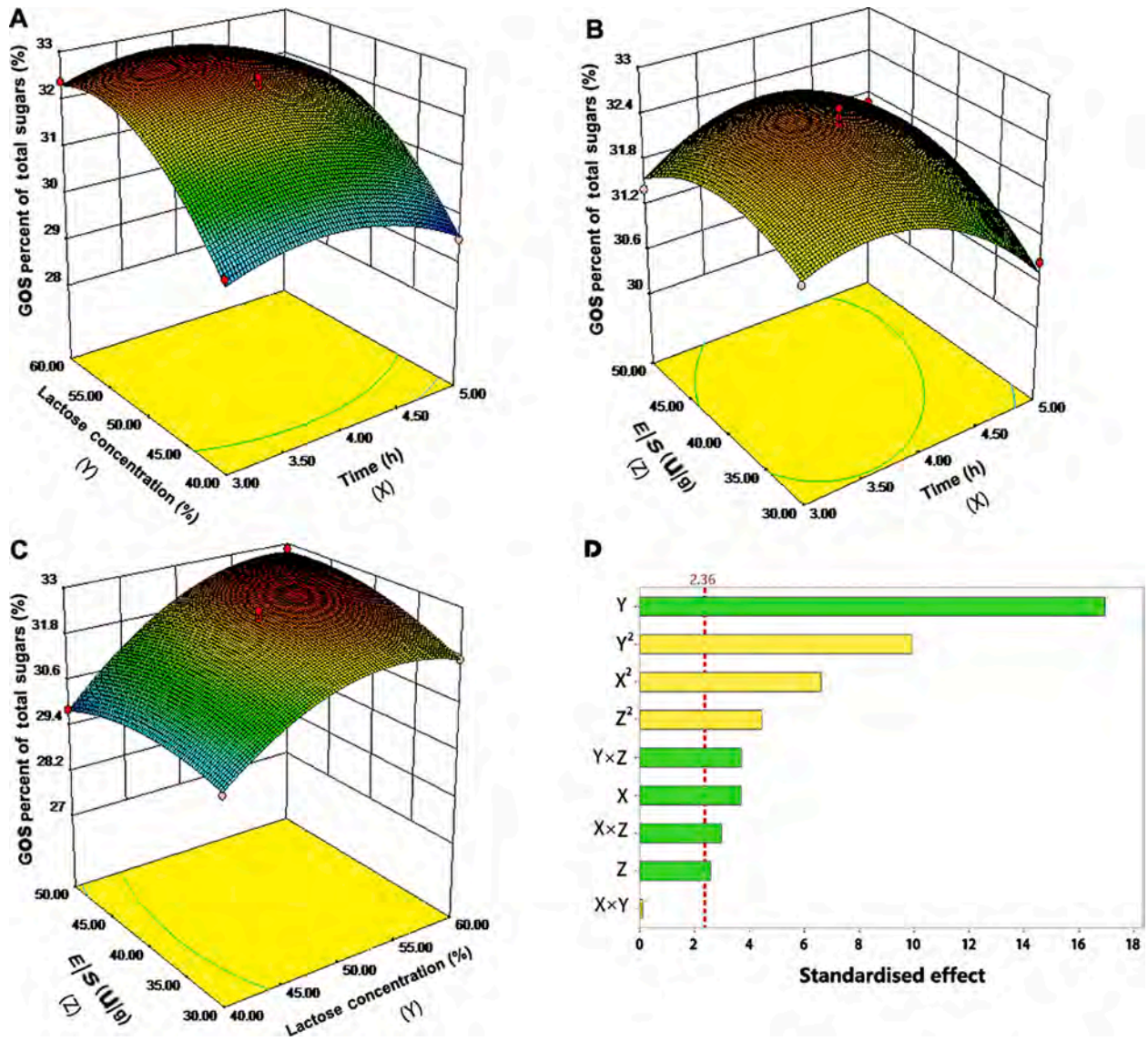


**Fig. 2.** A-C, response surface plot and the corresponding contour plot showing the effects of reaction time (X, h), initial lactose concentration (Y, %), and enzyme to lactose ratio (Z, *E/S*, U/g) on GOS (%) synthesis. D, Pareto charts showing the significance of X, Y, and Z on GOS yields. The significance line is shown (vertical line, $P < 0.05$). Positive (green columns) or negative (yellow columns) represent the effect of each factor on GOS yield. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

acetic acid buffer (pH at 4.5) to re-measure its activity after each reaction. The lactose content should be adjusted to prepare a 52.74% lactose solution according to the remaining enzyme activity to make sure the enzyme to lactose ratio (*E/S*) is constant for the next enzymatic reaction. This was repeated for a total of five consecutive reactions.

### 2.6. Chromatographic determination of carbohydrates

A quantitative analysis of GOS content was performed using a Waters e2695 (Milford, MA, USA). The HPLC system equipped with a Rezex™ RNM-Carbohydrate $Na^+$ (8%) column (300 mm × 7.8 mm, Agela Phenomenex, Tianjin, China) was used to detect the GOS components with the exception of galactobiose and allolactose, which could not be separated from lactose on this column. These disaccharides were further separated using a $NH_2$P-50 4E column (250 mm × 4.6 mm, Shodex, Tokyo, Japan). For the Rezex™ RNM-Carbohydrate $Na^+$ column, we set the temperature of the column and detector at 85 °C and 45 °C, respectively. Double distilled water was used as the mobile phase with a constant flow rate of 0.4 mL/min. For the $NH_2$P-50 4E column, the temperature of the column and detector were set at 30 °C and 40 °C, respectively. A mixture of acetonitrile:water (75:25) at 1.0 mL/min was used as the eluting solvent. A refractive index detector (Waters 2414) was connected to column. All the samples were filtered through a 0.22 μm membrane before analysis. The concentrations of saccharides including glucose, lactose, galactose and oligosaccharides were proportional to their peak areas. The GOS yield and degree of lactose conversion were calculated according to following equations.

$$\text{GOS yield (\%)} = (\text{final GOS concentration}/\text{initial lactose concentration}) \times 100$$

$$\text{Lactose conversion (\%)} = (\text{initial lactose concentration} - \text{final lactose concentration})/\text{initial lactose concentration} \times 100$$

### 2.7. GOS purification and thin-layer chromatography (TLC)

Two milliliters of the GOS mixtures were fractionated on a silica column (30 mm × 300 mm, loading of 30 g of silica gel), and eluted with

*n*-butanol:acetate:ethanol:water (3:1:1:1, v/v/v/v) at a flow rate of 0.6 mL/min. Two milliliter fractions were collected and monitored by thin-layer chromatography (TLC). TLC was carried out using a silica gel plate (GF254, Yantai Institute of Chemical Industry, Yantai, China), and *n*-butanol:acetate:ethanol:water (2:1:1:1, v/v/v/v) was used as the mobile phase with a sample loading volume of 1.5 μL. Ethanol:sulfuric acid (95:5, v/v) was sprayed onto the plate followed by baking at 110 ℃ for 10 min to visualize the bands. Finally, fractions containing the individual purified products were pooled and then freeze dried.

### 2.8. Fourier-transform infrared spectroscopy (FTIR)

The GOS powder obtained by lyophilization was homogenized with KBr at a ratio of 1:209 (mg/mg) and pressed into a 1-mm pellet. The FTIR spectrum of the sample was recorded over wavenumber ranging from 100 to 4000 $cm^{-1}$ on a FTIR-84000S (Shimadzu, Japan) with a 4 $cm^{-1}$ scanning resolution. The spectra obtained were the results of averaging 40 scans.

### 2.9. Economic feasibility analysis

The process for the production of purified GOSs (100%) in our lab was simulated using SuperPro Designer (Xiao et al., 2018) and following an economic feasibility analysis. The process flowsheet for production of GOSs (100%) in our lab is shown in Fig S7, and the detailed costs basis for materials were shown in Table S7.

## 3. Results and discussion

### 3.1. Optimization of GOS yield under free enzyme conditions

An HPLC system equipped with a $Na^+$ cation exchange column and an $NH_2$ column were initially used for the detection of total GOS yield and an analysis of its detailed composition. A typical GOS chromatogram is shown in Fig. S1. The main panel shows GOS polymers with DPs of 3, 4, and ≥5, as well as lactose, glucose, and galactose. The galactobiose and allolactose are generally masked by lactose when using common analytical methods (Urrutia et al., 2013). The left-hand panel shows that these two major disaccharides, i.e. galactobiose and allolactose could be detected using a packed $NH_2$ column. Accordingly, the combination of an $NH_2$ column and a $Na^+$ cation exchange column could completely separate the individual products.

Several reaction parameters, including reaction time, temperature, pH, initial lactose concentration, and the *E/S* ratio, were examined in the free enzyme system using *Aspergillus oryzae* *β*-galactosidase to investigate their effects on GOS yield and composition (Fig. 1). As expected, the degree of lactose conversion increased significantly with increasing time, increasing temperature, and with higher *E/S* ratios (Fig. 1A, C, G). The degree of lactose conversion was relatively independent of initial lactose concentration but decreased significantly as the pH was increased from 4.5 to 7. Wcith the exception of initial lactose concentration, GOS yield showed a similar pattern to the degree of lactose conversion. In contrast, there were large increases in GOS yield as the initial lactose concentration increased. It has previously been shown that increases in initial lactose concentration had a strong positive effect on GOS synthesis (Iwasaki, Nakajima, & Nakao, 1996). Of the total GOSs, GOS 3 was found to be the major component, accounting for at least 60% of the total, which agrees with the results of Neri et al., (2011). The levels of more highly polymerized GOSs were lower compared to GOS 3 levels due to the fact that transglycosylation and hydrolysis can occur simultaneously (Misson, Jin, & Zhang, 2017) and GOS 3 is the substrate for the higher polymerization of oligosaccharides.

Almost no changes in GOS yields were observed at different temperatures, as well as at pH ranging from 3 to 5.5 (Fig. 1D, J). This is consistent with the known enzymology of *Aspergillus oryzae* *β*-galactosidase as well as the work reported by Gao, Wu, and Wu (2019), which indicated that the optimal temperature for free enzyme activity ranges from 45 to 55 ℃, and pH is around 4.8. Thus, referring to our present results, we selected the reaction temperature (50 ℃) and pH (5.0) that showed the highest of GOS yield, for subsequent RSM design. The RSM study for the remaining three parameters, reaction time, initial lactose concentration, and *E/S* ratio are shown in Tables S1-S3. As a result, the fitted quadratic polynomial equation for our data was as follows:

$$\text{GOS yield (\%)} = -0.55750 + 3.52700 \times X + 0.88512 \times Y + 0.05758 \times Z - 0.00100 \times X \times Y + 0.02800 \times X \times Z + 0.00348 \times Y \times Z - 0.60525 \times X^2 - 0.00908 \times Y^2 - 0.00408 \times Z^2$$

where the X is reaction time (h), Y is the initial lactose concentration (%), and Z is the enzyme to lactose ratio (*E/S*, U/g) in the free enzyme system.

The three-dimensional surfaces for the three independent variables of GOS yield are shown in Fig. 2A-C. A pareto chart (Fig. 2D) was also used to show the significance of the different factors (González-Delgado, López-Muñoz, Morales, & Segura, 2016). Apart from the interaction between reaction time and initial lactose concentration which was not significant ($P > 0.05$), all the interaction coefficients for these three independent factors were significant ($P < 0.05$), which indicates that the main effects of reaction time and initial lactose concentration are independent. This provides further evidence that both parameters largely affect GOS yield. Also, it should be noted, that their quadratic effects ($X^2$, $Y^2$, and $Z^2$) had significant negative effects on GOS yield ($P < 0.05$), whereas their liner effects contributed to a positive GOS yield ($P < 0.05$). The data suggested that the initial lactose concentration had a strong positive impact on the GOS synthesis ($P < 0.05$), which is in agreement with the fact that higher concentrations of lactose can promote *trans*-glycosylation due to it being more accessible to the enzyme (Wang et al., 2020).

Taken together, all the parameters evaluated in the RSM design demonstrated significant effects on GOS yield, which suggests that more attention needs to be focused on these three parameters in actual production. Finally, the optimal independent variables to maximize the GOS yield were obtained at an initial lactose concentration of 57.13%, a reaction time of 3.9 h, and an *E/S* ratio of 44.81 U/g with a predicted yield of 32.89% (according to the equation). Following this, the experiment was repeated three times under the optimal conditions identified here and a mean yield of 32.62% was obtained, which validated the model. The GOS yield we achieved was 3.62% higher than the highest yield previously reported by Vera et al (2012), however, they did not detect the contents of galactobiose and allolactose. A higher concentration of GOS is crucial for reducing downstream processing costs associated with the removal of glucose, galactose, and lactose (Vera et al., 2016). In the industrial process, whey permeate is more suitable for GOS production because of it contains high content of lactose, meanwhile it is low-value byproduct. Further investigations on GOS production using whey permeate is needed.

### 3.2. GOS production using immobilized enzyme system

Immobilization of enzymes on support materials can not only stabilize enzymes against extreme conditions, but also reduce the cost of GOS production due to extension of the enzyme lifespan (Lu et al., 2012). At present, we constructed an immobilized *β*-galactosidase using cotton as a supporting material. As shown in Fig. S2C, the enzyme binds to the surface of the cotton fiber and no morphological changes were observed both pretreatment with double distilled water and PEI (Fig. S2A, B). According to the results in Fig. S2D, E, we selected 50 mg enzyme per gram of cotton for the next study. The enzyme coupling yield was lower than that reported by Albayark *et al.* (2002), one possible explanation for the lower coupling yield is the weak binding force between enzyme and glutaraldehyde, so that a lot of enzyme is lost during washing.

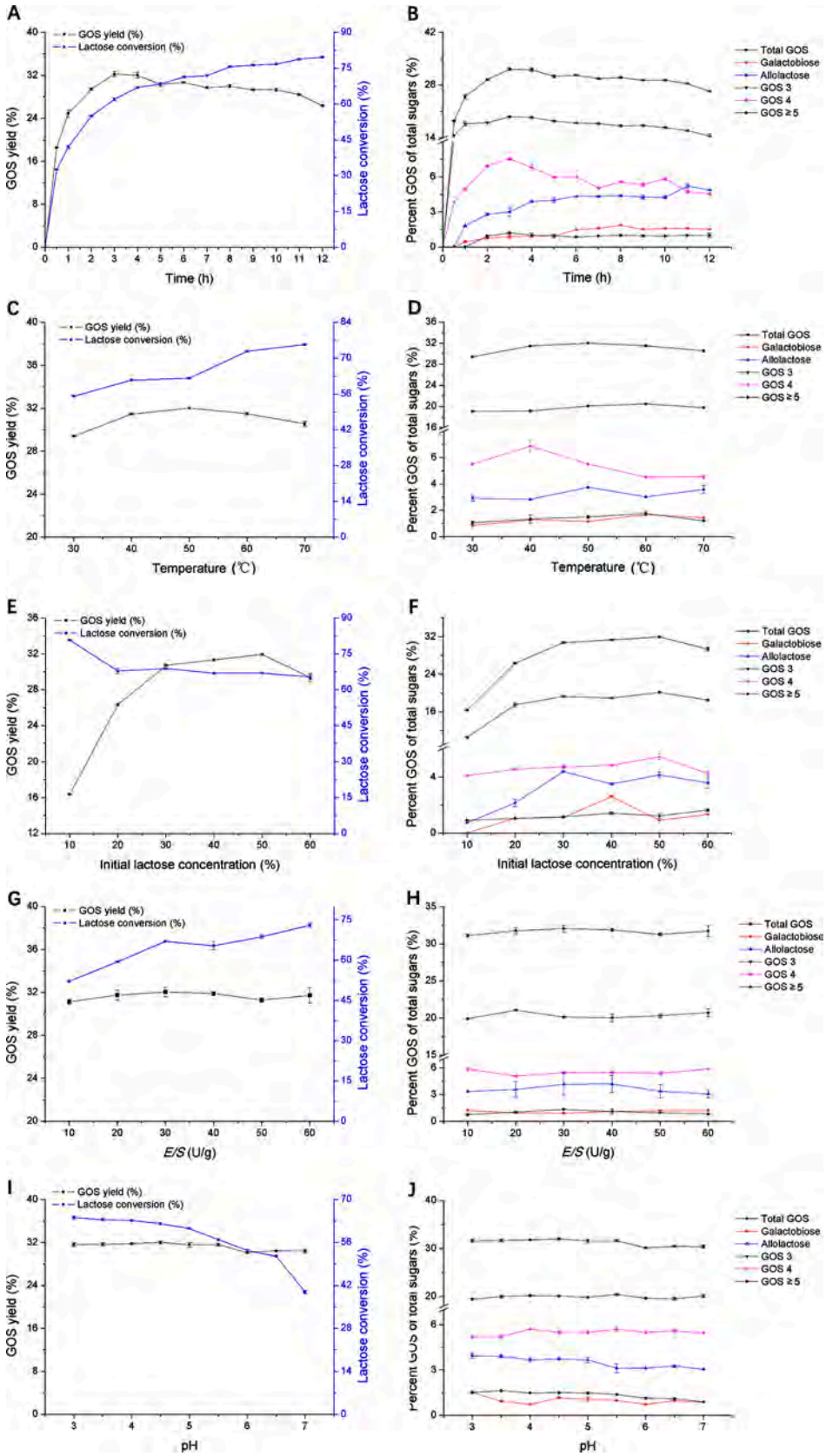


**Fig. 3.** Effect of different reaction parameters on GOS production in the immobilized enzymatic reaction system. Panels A, C, E, G, and I showed the effects of reaction time, temperature, initial lactose concentration, enzyme to lactose ratio (*E/S*), and solution pH on total GOS yield and lactose conversion. Correspondingly, panels B, D, F, H, and J show the effects of the same parameters on the levels of the specific oligosaccharides indicated.

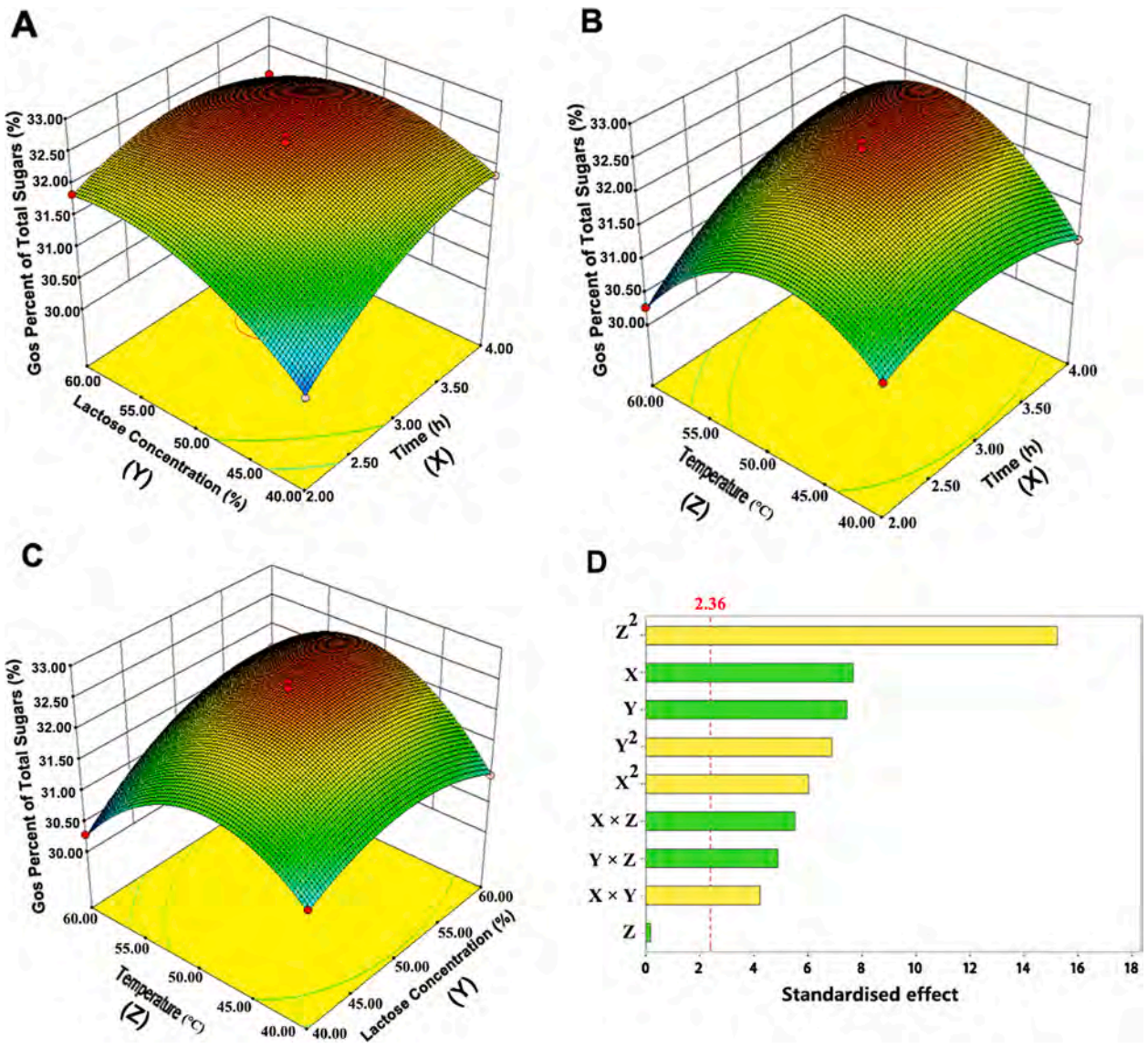


**Fig. 4.** A-C, response surface plot and the corresponding contour plot showing the effects of reaction time (X, h), initial lactose concentration (Y, %), and temperature (Z, ℃) on GOS (%) synthesis. D, Pareto charts showing the significance of X, Y, and Z on GOS yields. The significance line is shown (vertical line, $P < 0.05$). Positive (green columns) or negative (yellow columns) represent the effect of each factor on GOS yield. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Similarly, a RSM method was also applied to the optimization of GOS production in immobilized enzyme system. Firstly, we evaluate the effect of several reaction parameters mentioned in free enzyme system on its production. As shown in our results (Fig. 3A, C, E), the reaction time, temperature, and initial lactose concentration impacted significantly the GOS yield. The immobilized enzyme displayed better stability for the pH ranging from 3.0 to 7.0 (Fig. 3I), which was demonstrated by the comparable yields of GOS. It is beneficial to promote its application in industrial GOS production using the substrates of acid whey (pH ~ 4.5) and sweet whey (pH 6.0–6.5). What's more, there were slight of differences of amounts of total GOSs among different *E/S* (Fig. 3G). This result indicated that the transglycosylation efficiency of the $\beta$-galactosidase was improved by attaching it to the cotton cloth, especially for lower levels of *E/S*. In the immobilized enzyme system, the GOS 3 was also the main component of GOS mixtures.

Referring to the results of single factor experiments, the pH (4.5) and *E/S* (30U/g) were chosen firstly. Then, the reaction time, initial lactose concentration, and temperature were selected for subsequent RSM design (Tables S4-S6). Finally, a fitted quadratic polynomial equation for our data was as follows:

$$\text{GOS yield } (\%) = -1.03 + 2.32500 \times X + 0.41230 \times Y + 0.72940 \times Z - 0.02875 \times X \times Y + 0.03750 \times X \times Z + 0.00333 \times Y \times Z - 0.39900 \times X^2 - 0.00457 \times Y^2 - 0.01009 \times Z^2$$

where the X is reaction time (h), Y is the initial lactose concentration (%), and Z is the temperature (℃) in the immobilized enzyme system.

The results shown in three-dimensional surfaces and pareto chart (Fig. 4) indicated that the reaction time and initial lactose concentration affect positively GOS yields ($P < 0.05$), and their quadratic effects, as well as the interaction showed significant negative effects on GOS production ($P < 0.05$). It suggested that the main effects of these two independent factors on GOS yields are independent. Compared with above two factors, the effect of temperature on GOS yield is limited ($P > 0.05$). It may be the enhanced thermal stability of enzyme by immobilization on PEI composites (Albayrak & Yang, 2002). Then, the optimal factors to maximize the GOS yield were obtained at a reaction time of 3.09 h, an initial lactose concentration of 52.74%, and the temperature 50.0 ℃ with a predicted yield of 32.52%. Three times of independent

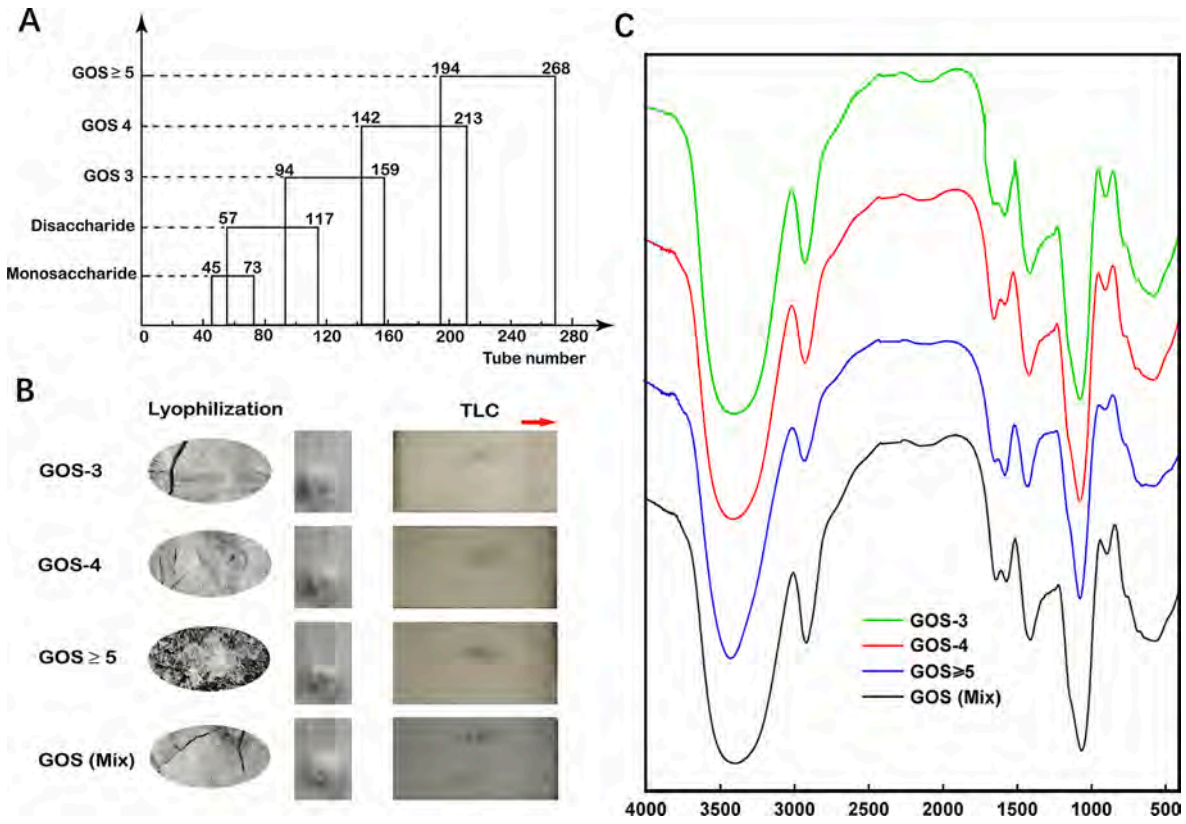


**Fig. 5.** Elution of GOS from the packed silica column followed by lyophilization and FTIR detection. A) The changes of sugar composition in eluent. Two milliliters of eluent was collected in tubes for further TLC analysis. Two tube numbers are at the top of each rectangle, which indicate the emergence (left) and disappearance (right) of corresponding sugar. The disaccharide contains lactose, allolactose, and galactobiose. The monosaccharide is a mixture of glucose and galactose. B) Specific fractions were pooled and lyophilized to obtain the pure GOS samples which were reanalyzed by TLC. The direction of eluent is indicated by the red arrow. C) FTIR spectra of the GOS mixture (black line), GOS 3 (green line), GOS 4 (red line), and GOS $\geq$ 5 (blue line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

experiments using above optimal conditions confirmed the validity of the model (with a mean yield of 32.48%).

Thereafter, we compared the differences in GOS synthesis with free and immobilized enzyme systems under respective optimal conditions, and evaluated the reusability of immobilized enzyme in five batches of enzymatic reactions. Both forms of the enzyme showed comparable levels of total GOS (Fig. S3A). Huerta et al, (2011) also reported no differences in GOS yield were found when employing free or immobilized enzymes. The levels of GOS with higher degrees of polymerization (GOS 3, GOS 4, and GOS $\geq$ 5) were lower in the mixture produced by the immobilized enzymatic reaction system (Fig. S3A), which may be caused by steric hindrance, resulting from overlap of enzymes fixed on the cotton thereby restricting GOS extension (Zhang, Yuwen, Li, & Li, 2012). In addition, the free enzyme synthesized less galactobiose and allolactose than the enzyme immobilized on the cotton cloth. This study reports for the first time the differences in GOS production between these two forms of $\beta$-galactosidase from *A. oryzae*. Earlier works (Haider & Husain, 2009; Ansair & Husain, 2010) only investigated variations in enzyme activity when addressing the reusability of the enzyme and ignored GOS synthesis. At present study, there were slight decreases in yields of total GOS, GOS 4 and GOS $\geq$ 5 with the increase of reaction batches (Fig. S3B). This may be related to the reduction of lactose conversion. A total of 63% of the initial activity remained on the cotton after five successive enzymatic reactions (Fig. S3C). Eskandarloo and Abbaspourrad, (2018) reported that the activity of $\beta$-galactosidase immobilized on glass beads remained at 90% after ten uses. However, the absolute quantity of the enzyme retained on bead was less than observed in our study, in other words, considerably more $\beta$-galactosidase was acquired when the cotton was used for a support. Importantly, the enzyme activity in our study was measured after a practical application i.e. the synthesis of GOSs, instead of merely using ONPG as the substrate to evaluate its activity. The similar shortcoming was also found in the studies of Haider and Husain, (2009) as well as Ansari and Husain (2010). The decrease of enzyme activity may be explained by the mass loss of the outer layer of enzymatic derivative (multilayer enzyme system was achieved in the present study), because of the scouring of lactose solution. Collectively, immobilization of the enzyme on cotton is easy to perform and yield functional enzyme for GOS production.

### 3.3. *Purification of GOS using silica gel filtration chromatography*

Fig. 5 shows the purification of GOS using a home-made column packed with silica gel. One of the drawbacks of using $\beta$-galactosidase for GOS production is the presence of monosaccharides and lactose in the product, which makes the GOS unsuitable for patients with diabetes and people with lactose intolerance (Mattar, Mazo, & Carrilho, 2012; Hernández, Ruiz-Matute, Olano, Moreno, & Sanz, 2009). Additionally, it masks the prebiotic function of GOSs because a low purity GOS can be non-selectively fermented by most bacteria. Therefore, it is necessary to remove lactose and monosaccharides from GOSs. The silica gel column-based separation (Fig. 5A) exhibited a clear ability to separate GOSs from lactose hydrolysate (Fig. S4 shows initial TLC results). Initially, the GOS mixtures were attached to the silica gel, then the eluent containing *n*-butanol:acetate:ethanol:water was applied to elute them at a flow rate of 0.6 mL/min. Each 2 mL of fractions were collected to determine the sugar composition by TLC method. However, it is difficult to completely separate them from each other, as a mixture of the two sugars was consistently obtained with several tubes. In detail, no saccharides were

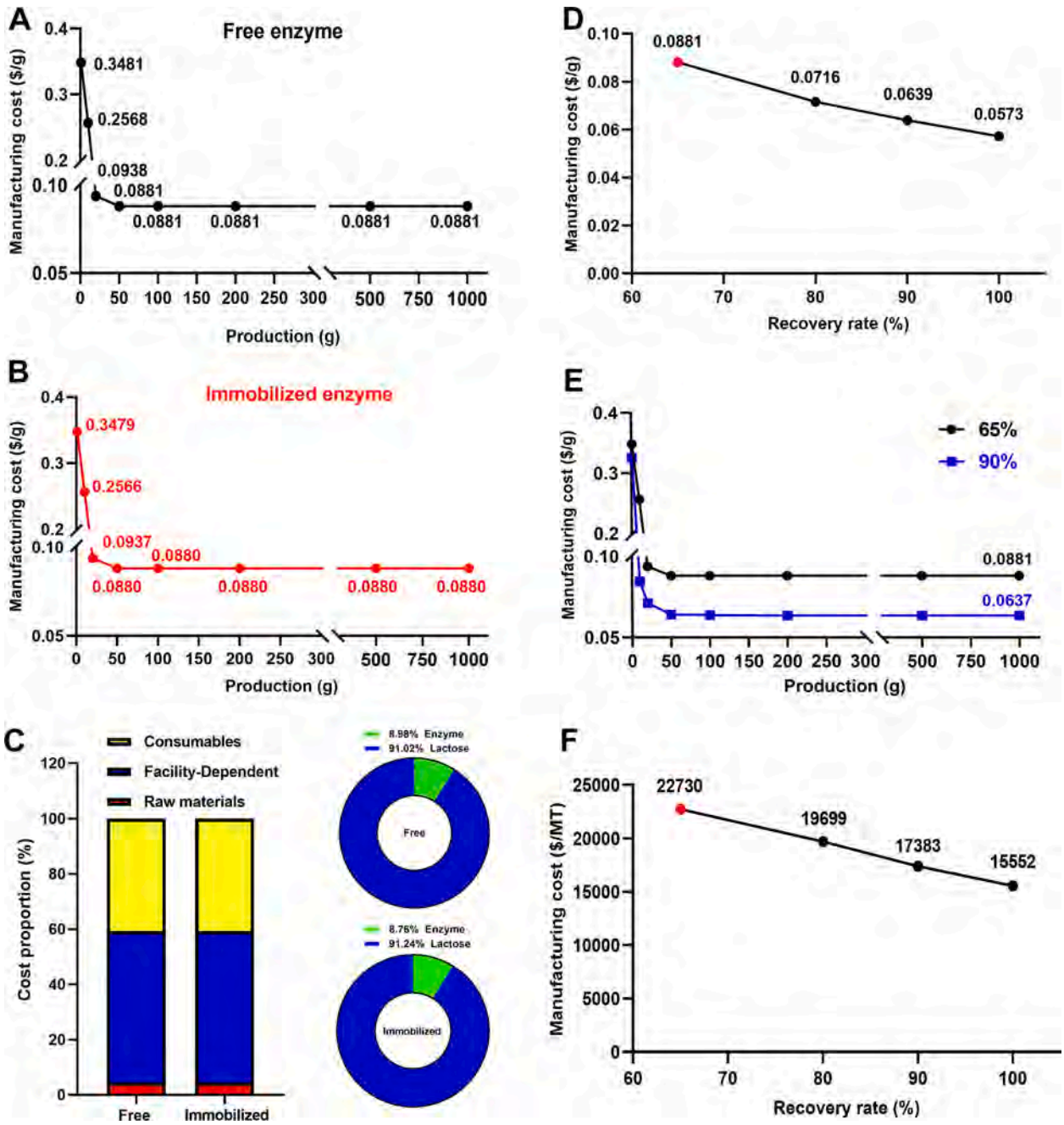


**Fig. 6.** Economic feasibility analysis for production of purified GOSs (100%). A) Effects of production scale on manufacturing cost in free enzyme system, the recovery rate is 65%. B) Effects of production scale on manufacturing cost in immobilized enzyme system, the recovery rate is 65%. C) Cost proportion for 1 kg of GOSs production, costs for raw materials were shown in pie chart. D) Effects of recovery rate of GOSs on manufacturing cost, the yield is 100 g. E) The comparison of manufacturing costs between 65% and 90% recovery rate. F) Effects of recovery rate of GOSs on manufacturing cost, the yield is 100 MT.

found until fraction 45, after which they were eluted in order of their degree of polymerization from low to high. For example, GOS 3, 4, and ≥5 appeared in fractions 94-159, 142-213, and 194-268, respectively. Based on the results of the TLC analysis (Fig. 5A), we pooled the fractions containing the GOS species of interest and obtained powders by lyophilization (Fig. 5B). The recovery of GOSs from the separation column was 65%, and the primary loss results from the partial GOS 3 as well as the whole disaccharides were coeluted with lactose. Thus future work is to optimize the eluent composition and the elution velocity to improve its recovery. As a result, pure GOS 3, GOS 4, and a mixture of GOSs with higher DP (GOS ≥ 5) were obtained. To further characterize the GOS samples, we also measured their purity by assessing the saccharide levels using a phenol sulfuric acid assay (Fig. S5B) with minor modifications. Specifically, we replaced glucose as substrate with galactose, due to the dominance of galactose over glucose in our GOS samples. The results showed that the saccharide contents were over 99.69% (data not shown). In addition, an HPLC analysis of GOS 3, GOS 4, and GOS ≥ 5 also suggested that the purification process was successful (Fig. S6). Finally, an FTIR analysis (Fig. 5C) was conducted to analyze the characteristic chemical bonds. Both the single components and the GOS mixture exhibited broad intense peaks near 3400 $cm^{-1}$ and 2922 $cm^{-1}$ from O—H and H—C—H stretching vibrations, respectively. These absorption peaks are characteristic of polysaccharides. Meanwhile, the deformation vibration of the C—H bond at 1415 $cm^{-1}$ (Shi et al., 2019) was clearly present in our FTIR spectra. In addition, the region ranging from 1200 to 950 $cm^{-1}$ is indicative of the C—O—C

bonds, C—O—H bonds, and the hydroxyl of the pyranose ring (Wang et al., 2017, Wu, Hu, Li, Huang, & Jiang, 2014). In the present study, absorption peaks were observed in the same region, suggesting an intact glycoside structure, i.e. galactose and glucose. Most of the previous studies regarding GOSs purification by Sephadex G-25 or Bio-gel *P*2 were focused on the structural characterization of specific DP of oligosaccharide, and they did not report the recovery rate of GOSs. Although the Sephadex G-25 or Bio-gel *P*2 can be reused, the cost (the unit price is 100 times higher than silica) of it as well as time and reagent required for cleaning and maintenance are substantial, which result in the restriction of its large-scale application in purification of GOSs. Overall, this study provided a simple and feasible way to purify GOS mixtures using silica-gel column chromatography. Further studies are required to define the chemical structure of the GOSs, which are important in their role as prebiotics.

### 3.4. Economic feasibility analysis for purified GOSs production

The process for manufacturing purified GOSs (100%) from lactose by free and immobilized enzyme includes preparation of galactosidase for hydrolysis of lactose to produce low purity of GOS mixtures, followed by silica gel adsorption to remove monosaccharides and lactose, combination of rotary evaporateion and lyophilization to acquire GOS powder. The SuperPro Designer was used for simulation of above process in our lab (Fig. S7A), and following an economic feasibility analysis (Fig. 6). In present bench scale testing, the manufacturing cost is sensitive to GOS yield for free (Fig. 6A) and immobilized enzyme (Fig. 6B) systems, when the yield is lower than 50 g. The manufacturing costs for 1 kg of GOSs were US $88.1 (free) and US $88.0 (immobilized), which were both much lower than market price US $155 in china. The results in section 3.3 indicated that enzyme immobilized on cotton cloth could reduce its loss, and enzyme used for GOS synthesis account for part of the cost (Albayrak & Yang, 2002). However, almost no differences on cost between these two kinds of enzyme systems were observed. One of the interpretations is that the GOSs prepared in present study is high purity (100%), cost of silica gel is much greater than the enzyme. This is consistent with the results shown in Fig. 6C that the cost for consumables (silica gel and eluent) takes up around 40%, which is much more than raw materials (enzyme and lactose). We can also see that cost for lactose is much higher than the enzyme (Fig. 6C, right inset) for production of 1 kg of GOSs in our lab. Meanwhile, we found that improving the recovery rate of GOSs would decrease significantly the manufacturing cost (Fig. 6D). And 27.7% of cost for preparation of 1 kg of GOSs would be saved if the recovery rate increased from 65% (present study) to 90% (Fig. 6E). Thus, it's necessary to improve the recovery rate of GOSs in purification. Although, referring to the study reported by Grand View Research, Inc. global GOS market is expected to reach 175.7 kilo tons in 2020, it is still unclear to the demand for high purity of GOSs (especially for 100% GOSs). To our knowledge, there is no research associated with the production of 100% GOSs. On the basis of our process flowsheet (Fig S7B), we mainly regulated equipment dimensions and pipeline flow to simulate the industrial production of GOSs (100%) with an annual yield of 100 MT. The results shown in Fig. 6F indicated that the cost for 1 MT of GOSs would be US $ 22,730 (i.e. US $22.73/kg), which is much lower than present bench scale testing (~US $88/kg). And the manufacturing cost is also sensitive to the recovery rate. In the future work, we need to scale up 100% GOS production to provide practical evidence for industrial production.

## 4. Conclusions

The GOS production was optimized using response surface method in the free and immobilized enzyme system, giving the maximum yields of 32.62% and 32.48%, respectively. As to the immobilized enzyme, there were only slight decreases in GOS yields among the five consecutive enzymatic reactions. And>63% of initial enzyme activity were remained, which suggested its desirable reusability. Meanwhile, the GOS (100%) mixtures and specific GOS 3 and 4 were obtained following silica gel-filtration chromatography. Finally, this work shows practical evidence for cost-effective production and improved purification of GOSs.

## CRediT authorship contribution statement

**Geng Wang:** Conceptualization, Methodology, Investigation, Writing - original draft. **Haidong Wang:** Formal analysis, Visualization. **Yucheng Chen:** Methodology, Formal analysis. **Xun Pei:** Resources, Writing - review & editing. **Wanjing Sun:** Visualization, Conceptualization. **Lujie Liu:** Writing - review & editing, Validation. **Fengqin Wang:** Investigation, Formal analysis. **Muhammad Umar Yaqoob:** Resources, Writing - review & editing. **Wenjing Tao:** Resources, Formal analysis. **Zhiping Xiao:** Formal analysis. **Yuyue Jin:** Visualization. **Shang-Tian Yang:** Visualization. **Dongqiang Lin:** Formal analysis. **Minqi Wang:** Project administration, Funding acquisition, Writing - review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

We acknowledge financial support from The National Key Research and Development Program of China (2018YFE0112700) and The Science and Technology Key Projects of Zhejiang Province, China (2019C02005).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.130195.

## References

Albayrak, N., & Yang, S. T. (2002). Production of galacto-oligosaccharides from lactose by *Aspergillus oryzae* β-galactosidase immobilized on cotton cloth. *Biotechnology and Bioengineering, 77*, 8–19.

Ansari, S. A., & Husain, Q. (2010). Lactose hydrolysis by β-galactosidase immobilized on concanavalin A-cellulose in batch and continuous mode. *Journal of Molecular Catalysis B: Enzymatic, 63*(1-2), 68–74.

Eskandarloo, H., & Abbaspourrad, A. (2018). Production of galacto-oligosaccharides from whey permeate using β-galactosidase immobilized on functionalized glass beads. *Food Chemistry, 251*, 115–124.

Freitas, F. F., Marquez, L. D. S., Ribeiro, G. P., Brandão, G. C., Cardoso, V. L., & Ribeiro, E. J. (2011). A comparison of the kinetic properties of free and immobilized *Aspergillus oryzae* β-galactosidase. *Biochemical Engineering Journal, 58-59*, 33–38.

Fischer, C., & Kleinschmidt, T. (2015). Synthesis of galactooligosaccharides using sweet and acid whey as a substrate. *International Dairy Journal, 48*, 15–22.

Gosling, A., Stevens, G. W., Barber, A. R., Kentish, S. E., & Gras, S. L. (2010). Recent advances refining galactooligosaccharide production from lactose. *Food Chemistry, 121*(2), 307–318.

González-Delgado, I., López-Muñoz, M.-J., Morales, G., & Segura, Y. (2016). Optimisation of the synthesis of high galacto-oligosaccharides (GOS) from lactose with β-galactosidase from *kluyveromyces lactis*. *International Dairy Journal, 61*, 211–219.

Gao, X., Wu, J., & Wu, D. (2019). Rational design of the beta-galactosidase from *Aspergillus oryzae* to improve galactooligosaccharide production. *Food Chemistry, 286*, 362–367.

Hosono, A., Ozawa, A., Kato, R., Ohnishi, Y., Nakanishi, Y., Kimura, T., & Nakamura, R. (2003). Dietary fructooligosaccharides induce immunoregulation of intestinal IgA secretion by murine Peyer's patch cells. *Biosci. Biotech. Bioch., 67*(4), 758–764.

Haider, T., & Husain, Q. (2009). Immobilization of β-galactosidase from *Aspergillus oryzae* via immunoaffinity support. *Biochemical Engineering Journal, 43*(3), 307–314.

Hernández, O., Ruiz-Matute, A. I., Olano, A., Moreno, F. J., & Sanz, M. L. (2009). Comparison of fractionation techniques to obtain prebiotic galactooligosaccharides. *International Dairy Journal, 19*(9), 531–536.

Huerta, L. M., Vera, C., Guerrero, C., Wilson, L., & Illanes, A. (2011). Synthesis of galacto-oligosaccharides at very high lactose concentrations with immobilized β-galactosidases from *Aspergillus oryzae*. *Process Biochemistry, 46*(1), 245–252.

Iwasaki, K.-I., Nakajima, M., & Nakao, S.-I. (1996). Galacto-oligosaccharide production from lactose by an enzymic batch reaction using β-galactosidase. *Process Biochemistry, 31*(1), 69–76.

Kumar, V. P., Prashanth, K. V. H., & Venkatesh, Y. P. (2015). Structural analyses and immunomodulatory properties of fructo-oligosaccharides from onion (Allium cepa). *Carbohydrate Polymers, 117*, 115–122.

Krawczyk, T., Zalewski, M., Janeta, A., & Hodurek, P. (2018). SEC Separation of Polysaccharides Using Macroporous Spherical Silica Gel as a Stationary Phase. *Chromatographia, 81*(10), 1365–1372.

Lu, L., Xu, S., Zhao, R., Zhang, D., Li, Z., Li, Y., & Xiao, M. (2012). Synthesis of galactooligosaccharides by CBD fusion β-galactosidase immobilized on cellulose. *Bioresource Technology, 116*, 327–333.

Maruška, A., & Pyell, U. (1997). Capillary electrochromatography: Normal-phase mode using silica gel and cellulose-based packing materials. *Journal of Chromatography A, 782*(2), 167–174.

Mattar, R., Mazo, D. F. D. C., & Carrilho, F. J. (2012). Lactose intolerance: Diagnosis, genetic, and clinical factors. *Clin. Exp. Gastroenterol., 5*, 113–121.

Misson, M., Jin, B.o., & Zhang, H.u. (2017). A recirculating spiral bioreactor for galactooligosaccharide production using polymer Nanofiber-β-galactosidase assembly. *Industrial and Engineering Chemistry Research, 56*(44), 12479–12487.

Nauta, A., Bakker-Zierikzee, A. M., & Schoterman, M. H. C. (2009). Galacto-Oligosaccharides, in: Cho, S.S., Finocchiaro, T. (Eds), Handbook of Prebiotics and Probiotics Ingredients: Health Benefits and Food Applications (pp. 75-94). Boca Raton.

Neri, D. F. M., Balcão, V. M., Costa, R. S., Rocha, I. C. A. P., Ferreira, E. M. F. C., Torres, D. P. M., … Teixeira, J. A. (2009). Galacto-oligosaccharides production during lactose hydrolysis by free *Aspergillus oryzae* β-galactosidase and immobilized on magnetic polysiloxane-polyvinyl alcohol. *Food Chemistry, 115*(1), 92–99.

Neri, D. F. M., Balcão, V. M., Cardoso, S. M., Silva, A. M. S., Domingues, M.d. R. M., Torres, D. P. M., … Teixeira, J. A. C. (2011). Characterization of galactooligosaccharides produced by β-galactosidase immobilized onto magnetized Dacron. *International Dairy Journal, 21*(3), 172–178.

Panesar, P. S., Kaur, R., Singh, R. S., & Kennedy, J, F. (2018). Biocatalytic strategies in the production of galacto-oligosaccharides and its global status. Int. J. Biol. Macromol. 111, 667-679.

Rodriguez-Colinas, B., Poveda, A., Jimenez-Barbero, J., Ballesteros, A. O., & Plou, F. J. (2012). Galacto-oligosaccharide synthesis from lactose solution or skim milk using the β-galactosidase from *Bacillus circulans*. *Journal of Agriculture and Food Chemistry, 60*(25), 6391–6398.

Spherix Consulting Inc., 2010. Generally Recognized as Safe (GRAS) determination for the use of galacto-oligosaccharides in foods and infants formulas. Yakult Pharmaceutical Industry Co., Ltd, Kunitachi-shi.

Shi, L., Fang, B., Yong, Y., Li, X., Gong, D., Li, J., … Ju, X. (2019). Chitosan oligosaccharide-mediated attenuation of LPS-induced inflammation in IPEC-J2 cells is related to the TLR4/NF-κB signaling pathway. *Carbohydrate Polymers, 219*, 269–279.

Urrutia, P., Rodriguez-Colinas, B., Fernandez-Arrojo, L., Ballesteros, A. O., Wilson, L., Illanes, A., & Plou, F. J. (2013). Detailed analysis of galactooligosaccharides synthesis with β-galactosidase from *Aspergillus oryzae*. *Journal of Agriculture and Food Chemistry, 61*(5), 1081–1087.

Vera, C., Guerrero, C., & Illanes, A. (2011). Determination of the transgalactosylation activity of *Aspergillus oryzae* β-galactosidase: Effect of pH, temperature, and galactose and glucose concentrations. *Carbohydrate Research, 346*(6), 745–752.

Vera, C., Guerrero, C., Conejeros, R., & Illanes, A. (2012). Synthesis of galacto-oligosaccharides by β-galactosidase from *Aspergillus oryzae* using partially dissolved and supersaturated solution of lactose. *Enyzme and Microbial Technology, 50*(3), 188–194.

Vera, C., Córdova, A., Aburto, C., Guerrero, C., Suárez, S., & Illanes, A. (2016). Synthesis and purification of galacto-oligosaccharides: State of the art. *World Journal of Microbiology & Biotechnology, 32*, 197–216.

Wu, G.-H., Hu, T., Li, Z.-Y., Huang, Z.-L., & Jiang, J.-G. (2014). In vitro antioxidant activities of the polysaccharides from pleurotus tuber-regium (Fr.) Sing. *Food Chemistry, 148*, 351–356.

Wang, M., Zhu, P., Zhao, S., Nie, C., Wang, N., Du, X., & Zhou, Y. (2017). Characterization, antioxidant activity and immunomodulatory activity of polysaccharides from the swollen culms of zizania latifolia. *International Journal of Biological Macromolecules, 95*, 809–817.

Wang, G., Zhu, J., Liu, L., Yaqoob, M. U., Pei, X., Tao, W., … Wang, M. (2020). Optimization for galactooligosaccharides synthesis: A potential alternative for gut health and immunity. *Life Sciences, 245*, 117353. https://doi.org/10.1016/j.lfs.2020.117353.

Xiao, Z., Cheng, C., Bao, T., Liu, L., Wang, B., Tao, W., … Wang, M. (2018). Production of butyric acid from acid hydrolysate of corn husk in fermentation by *Clostridium tyrobutyricum*: Kinetics and process economic analysis. *Biotechnology for Biofuels, 11*(1). https://doi.org/10.1186/s13068-018-1165-1.

Zhang, D.-H., Yuwen, L.-X., Li, C., & Li, Y.-Q. (2012). Effect of poly (vinyl acetate–acrylamide) microspheres properties and steric hindrance on the immobilization of Candida rugosa lipase. *Bioresource Technology, 124*, 233–236.

Zhou, Q. Z. K., & Chen, X. D. (2001). Effects of temperature and pH on the catalytic activity of the immobilized β-galactosidase from Kluyveromyces lactis. *Biochemical Engineering Journal, 9*, 33–40.