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Integrated Utilization of Dairy Whey in Probiotic β-Galactosidase Production and Enzymatic Synthesis of Galacto-Oligosaccharides

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Abstract: This work established an integrated utilization of dairy whey in β-galactosidase production from *Lactobacillus bulgaricus* and prebiotics synthesis by the probiotic enzyme. A cost-effective whey-based medium was newly developed for culturing *Lactobacillus bulgaricus* to produce β-galactosidase. The medium was optimized through response surface methodology (RSM) involving a series of statistical designs, such as the Plackett–Burman design, steepest ascent experiment, and central composite design. Under the optimized medium, the β-galactosidase activity of *L. bulgaricus* reached 2034 U/L, which was twice that produced from the traditional MRS medium. The cells of *L. bulgaricus* harvested from the whey-based medium were subsequently treated with lysozyme. The resulting crude enzyme was used as an efficient catalyst, which catalyzed the synthesis of the prebiotic galacto-oligosaccharides (GOS) in a high yield of 44.7% by using whey (200 g/L) as the substrate. The sugar mixture was further purified by activated charcoal adsorption, thereby yielding a high-purity level of 77.6% GOS.

Keywords: whey; response surface methodology; β-galactosidase; galacto-oligosaccharides; synthesize

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1. Introduction

Galacto-oligosaccharides (GOS) are a well-known class of probiotics with functions resembling human milk oligosaccharide. GOS have various benefits for the human health, including the selective stimulation of the beneficial intestinal bacteria growth, maintenance of the normal flora balance in the intestine, increase in Ca^{2+} absorption, and decrease in serum cholesterol levels and cancer risks [1,2]. GOS can also act as receptor decoys and exhibit direct anti-infective functions by binding pathogens and helping flush them out of the gastrointestinal tract due to the structural similarity to the pathogen receptors [3]. GOS have higher inhibition of harmful bacteria. GOS also have more short-chain fatty acid production, and less gas generation than other prebiotics [4]. Correspondingly, GOS are regarded as one of the most popular prebiotics. The total prebiotics market is expected to reach approximately USD 10.55 billion in profits in the global market by 2025 [5]. Therefore, the synthesis of the popular GOS has been extensively investigated worldwide.

GOS are mixtures of linear and branched oligosaccharides with DPs of 2–8, which can be synthesized by microbial β-galactosidases by using lactose as a substrate [1]. During the β-galactosidase-catalyzed reaction, a covalent galactosyl-enzyme intermediate was firstly formed and combined with the release of glucose from lactose. When the acceptor is water, the hydrolysis reaction takes place and galactose is released from the enzyme. However, if sugars act as acceptors, then the transglycosylation reaction occurs with

sequential galactosyl transfer from the galactosyl-enzyme intermediate to sugars such as lactose substrate and hydrolysis byproducts (glucose and galactose); thus, the GOS mixture comprising transgalactosylated disaccharides and oligosaccharides is generated [6].

The β-galactosidases produced from a variety of micro-organisms have been applied in GOS synthesis [6,7]. Among them, the enzymes from probiotics, such as *Bifidobacteria* and *Lactobacilli,* have aroused particular interest in recent years because of their potential ability to produce GOS mixtures that are selective for metabolism by intestinal probiotics [1,8]. However, *Bifidobacteria* and *Lactobacilli* cell growth usually depends on the expensive MRS medium that contains 10 complex constituents, which increases the cost of industrial production of probiotic enzymes.

Whey is the liquid portion of milk left after casein precipitation and removal. Whey is a major by-product of the cheese industry, with an annual production of over 160 million tons worldwide and an estimated growth rate of 1–2%, yearly [9]. Whey is a valuable waste product retaining approximately 55% of the nutrients in milk, including lactose (4.5–5% *w/v*), soluble proteins (0.6–0.8% *w/v*), lipids (0.4–0.5% *w/v*), vitamins (B complex, etc.), citric acid (0.1%), lactic acid (0.05% *w/v*), and mineral salts (8–10% of the dry extract) [10–12]. Because of its high organic content, whey is considered as an important pollutant with high chemical and biological oxygen demand. If disposed of without prior treatment, whey will cause serious environmental risks [13]. Due to its high nutrition value and remarkable pollution capability, the reuse of whey has attracted worldwide attention. Concentrated whey powder has high lactose content (~80%); thus, it can be used as a cheap substitute for lactose to cultivate cells or make lactose-derived products [14,15].

In this work, an integrated whey utilization in probiotics growth, enzyme production, and GOS synthesis was established (Figure 1). A novel, low-cost whey-based medium was developed to replace the MRS medium for cultivating *Lactobacillus bulgaricus* L3 to produce β-galactosidase. The crude β-galactosidase from *L. bulgaricus* L3, which was simply prepared by lysozyme treatment without cell debris removal, acted as a considerably efficient catalyst for the first time to synthesize GOS by using inexpensive whey again as the substrate.

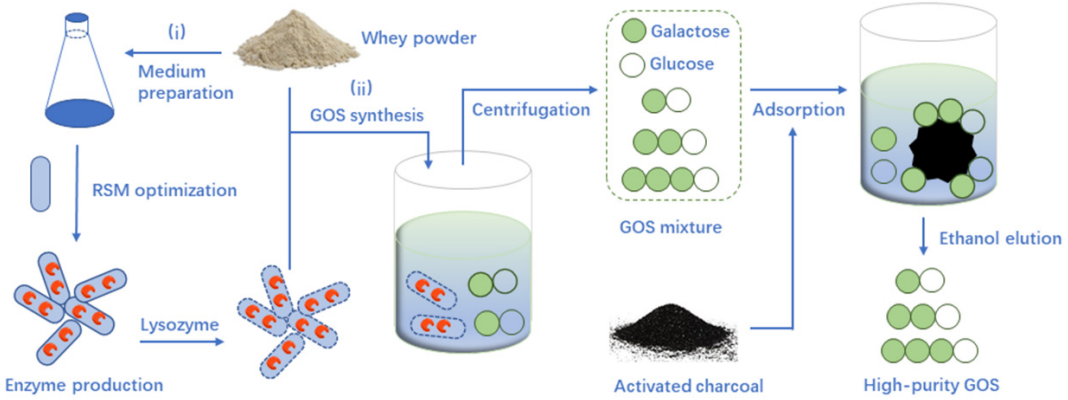

Figure 1. Outline of the integrated utilization of dairy whey in the β-galactosidase production and GOS synthesis followed by purification.

2. Results and Discussion

2.1. Whey-Based Medium Optimization by RSM for β-Galactosidase Production

The nutritional factors suitable for producing β-galactosidase by *L. bulgaricus* L3 were preliminarily screened by the individual addition of the MRS components to the whey broth in order to evaluate their effects on cell growth and enzyme production. Using whey alone or combining it with inorganic salts or yeast powder was insufficient to sustain cell growth. Nonetheless, the combination of whey, inorganic salts, and yeast powder resulted in higher biomass of bacteria and enzyme activity, compared with the MRS medium. Among inorganic salts, $CH_3COONa{\cdot}3H_2O$, triammonium citrate, and

K_2HPO_4 played important roles. Extra supplement with glucose, peptone, and beef extract exhibited no significant influence on cell growth, whereas the addition of corn steep liquor to the medium significantly enhanced enzyme production. Based on all of the results, the preliminary composition of the whey-based medium was determined as follows: 20 g/L whey powder; 2.5 g/L $CH_3COONa{\cdot}3H_2O$; 2 g/L triammonium citrate; 2 g/L K_2HPO_4; 4 g/L yeast powder; and 20 mL/L of corn steep liquor, all of which were subjected to optimization by RSM.

2.1.1. PB Design

PB design is an efficient technique for component optimization, which was first used to pick factors that significantly influenced enzyme production. Six variables, including whey powder, $CH_3COONa{\bullet}3H_2O$, triammonium citrate, K_2HPO_4, yeast powder, and corn steep liquor, were evaluated. Table 1 represents the PB experimental design for 12 trials and the corresponding enzyme activity. The effects of the variables on the response and the order of their importance are shown in Table S1 and Figure 2, respectively.

Table 1. Experimental design and results of the Plackett–Burman design.

Variables		Whey Powder (g/L)	CH_3COONa (g/L)	Triammonium Citrate (g/L)	K_2HPO_4 (g/L)	Yeast Powder (g/L)	Corn Steep Liquor (mL/L)	Enzyme Activity (U/L)
Symbol		A	B	C	D	E	F	
Coded levels	−1	16	2	1.6	1.6	3.2	16	
	+1	24	3	2.4	2.4	4.8	24	
Run	1	−1	+1	+1	−1	+1	−1	1002
	2	−1	+1	−1	−1	+1	+1	1229
	3	+1	−1	−1	−1	+1	+1	1456
	4	+1	+1	+1	+1	+1	−1	1420
	5	−1	−1	+1	+1	+1	+1	1205
	6	−1	−1	+1	+1	−1	+1	1313
	7	+1	+1	−1	+1	−1	+1	1861
	8	+1	+1	+1	−1	−1	+1	1682
	9	+1	−1	−1	+1	+1	−1	1324
	10	−1	−1	−1	−1	−1	−1	1038
	11	−1	+1	−1	+1	−1	−1	1157
	12	+1	−1	+1	−1	−1	−1	1253

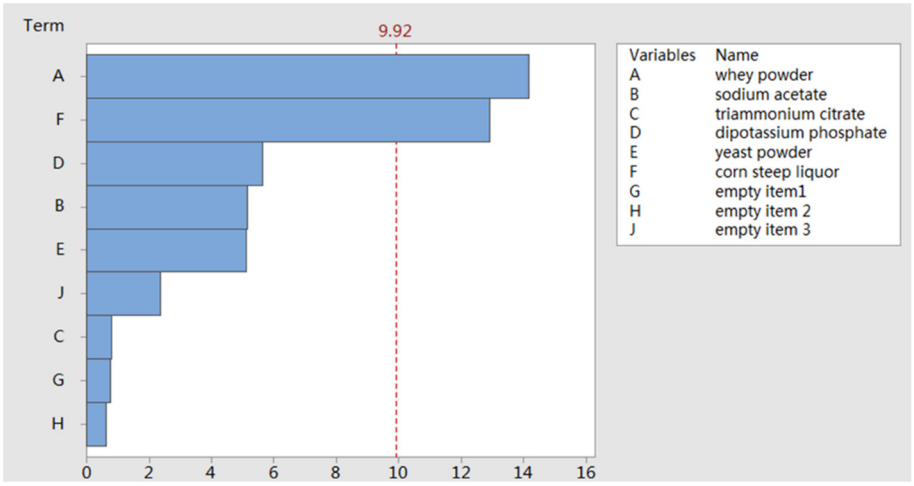

Figure 2. Pareto chart of standardized effects. Response is enzyme activity (U/L). α= 0.01.

Variables A and F exhibited the highest positive influences on the enzyme production because their *p* values were less than 0.001 and their T values were positive. In general,

large T values and low *p* values indicate the remarkable importance of a variable. The significance of variables A and F were also supported by the result of the Pareto chart of the standardized effects (Figure 2). By contrast, the *p* values of other variables (B–E) were larger than 0.005, suggesting their less significance for enzyme production (Table S1). According to the maximal response from PB results, the B–E concentrations were adjusted to +1, −1, +1, and −1 levels, as follows: 3 g/L CH_3COONa, 1.6 g/L triammonium citrate, 2.4 g/L K_2HPO_4, and 3.2 g/L yeast powder in the subsequent experiments.

To approach the neighborhood of the optimum response, we established the fitted first-order model equation for enzyme production from the PB design experiments:

$$Y = -334 + 42.75A + 127.0B - 39.6C + 129.2D - 69.6E + 28.74F \quad (1)$$

Statistical testing was performed for analysis of variance (ANOVA) according to the experimental data (Table S2). The test model had a high F value (45.06) and a low *p* value (<0.001), which was statistically significant at the 99.99% level. The accuracy of the fit of the polynomial model equation was expressed by the coefficient of determination (R^2), which showed that 98.18% of the variability in the response can be explained by the model. The adjusted R^2 (Adj R^2) value (0.96) showed the high significance of the model. All of these analyses revealed that the response equation was a suitable model for the PB design experiments.

2.1.2. Path of Steepest Ascent

The path of steepest ascent was employed to move rapidly towards the maximal optimum response by increasing A and F concentrations. The center point of the PB design was considered as the origin of the path. According to the coefficients of A and F in the model equation (i.e., Equation (1)) and the data obtained from pre-experiments, the whey powder concentration increased by 1.25-fold unit (5 g/L), while the corn steep liquor concentration improved by one design unit (4 mL/L). As shown in Table 2, maximum enzyme production was achieved in the third step, with 30 g/L whey powder and 28 g/L corn steep liquor. Meanwhile, continuously increasing A and F concentrations in the fourth and fifth steps decreased the enzyme activity. Thus, the levels of the third steps were used as the middle point for the second-order experiments.

Table 2. Experimental design and results of the steepest ascent.

Step	Whey Powder (g/L)	Corn Steep Liquor (mL/L)	Enzyme Activity (U/L)
1	20	20	1718
2	25	24	1849
3	30	28	1993
4	35	32	1885
5	40	36	1778

2.1.3. CCD and Response Surface Analysis

CCD was used to analyze the interaction among the significant factors of whey powder (X_1) and corn steep liquor (X_2) and to determine their optimal values. Table 3 shows the experimental condition together with the experimental responses. The following second-order polynomial equation was generated to explain the enzyme production by a multiple regression analysis of the experimental data:

$$Y = -4654.26785 + 255.09627X_1 + 193.80892X_2 + 0.30000X_1X_2 - 4.31857{X_1}^2 - 3.55479{X_2}^2 \quad (2)$$

Table 3. Experimental design and results of central composite design.

Run	Actual Level of Variables		Coded Levels		Enzyme Activity (U/L)
	Whey Powder (g/L)	Corn Steep Liquor (mL/L)	X_1	X_2	
1	25	24	−1	−1	1828
2	35	24	1	−1	1840
3	25	32	−1	1	1849
4	35	32	1	1	1885
5	22.93	28	$-\alpha$	0	1731
6	37.07	28	α	0	1821
7	30	22.34	0	$-\alpha$	1859
8	30	33.66	0	α	1897
9	30	28	0	0	1993
10	30	28	0	0	2007
11	30	28	0	0	2020
12	30	28	0	0	1981
13	30	28	0	0	2017

The established mathematical model was evaluated by ANOVA (Table 4). This model had large F (46.74) and small p values (<0.0001), indicating high significance and good correlation with the experimental results. The goodness of fit of the quadratic regression model equation can be checked by R^2. The closer the R^2 value is to 1, the better the model predicts the response. As listed in Table 4, the R^2 value was 0.9709, indicating that 97.09% of the variability in the response can be explained by the model. The Adj R^2 (0.9501) was also closely related to R^2. The p value (0.2138) of the lack of fit was insignificant, supporting the reliability of the model. The low coefficient of variation (1.09) indicated the remarkable reliability of the experimental data for the chosen model. All of these analyses confirmed that the response equation (Equation (4)) provided a suitable model for the CCD experiments. Therefore, the investigation of the response trends by using the model was reasonable.

Table 4. ANOVA for response surface quadratic model.

Source	Sum of Squares	df	Mean Square	F Value	p-Value Prob > F	
Model	99,800.07	5	19,960.01	46.74	<0.0001	significant
X_1	3840.09	1	3840.09	8.99	0.0200	-
X_2	1792.11	1	1792.11	4.20	0.0797	-
X_1X_2	144.00	1	144.00	0.34	0.5797	-
${X_1}^2$	81,044.50	1	81,044.50	189.76	<0.0001	-
${X_2}^2$	22,542.03	1	22,542.03	52.78	0.0002	-
Residual	2989.62	7	427.09	-	-	-
Lack of Fit	1906.42	3	635.47	2.35	0.2140	not significant
Pure Error	1083.20	4	270.80	-	-	-

C.V. % = 1.09; R^2 = 0. 9709; Adj R^2 = 0. 9501; Pred R^2 = 0.8517.

Table 4 also shows the significance of each model term and interaction among variables. The primary coefficients X_1 and X_2 had high F values and small p values (<0.1), indicating that the whey powder and steep liquor had significant effects on β-galactosidase production. The p values of the quadratic coefficients ${X_1}^2$ and ${X_2}^2$ were also less than 0.001, further suggesting the remarkable influence of the two factors on the response. Their trace changes might significantly affect enzyme activity. Based on the regression model, whey powder was more important for enzyme production due to its larger linear and quadratic coefficients, compared with those of the corn steep liquor. The effect of the interaction between the two variables on the response was insignificant because the quadratic coefficient of ${X_1}^2{X_2}^2$ had a large p value of >0.5.

The optimal level of each variable and the effect of their interaction on enzyme production were further explored by constructing the response surface plot and contour plot according to the regression model. As shown in Figure 3, the response surface graph appeared convex, indicating that the model covered the optimal response area. The shape of the contour plot was close to cycle, suggesting that the interaction between the two variables was not obvious, which was consistent with the significant test results described above.

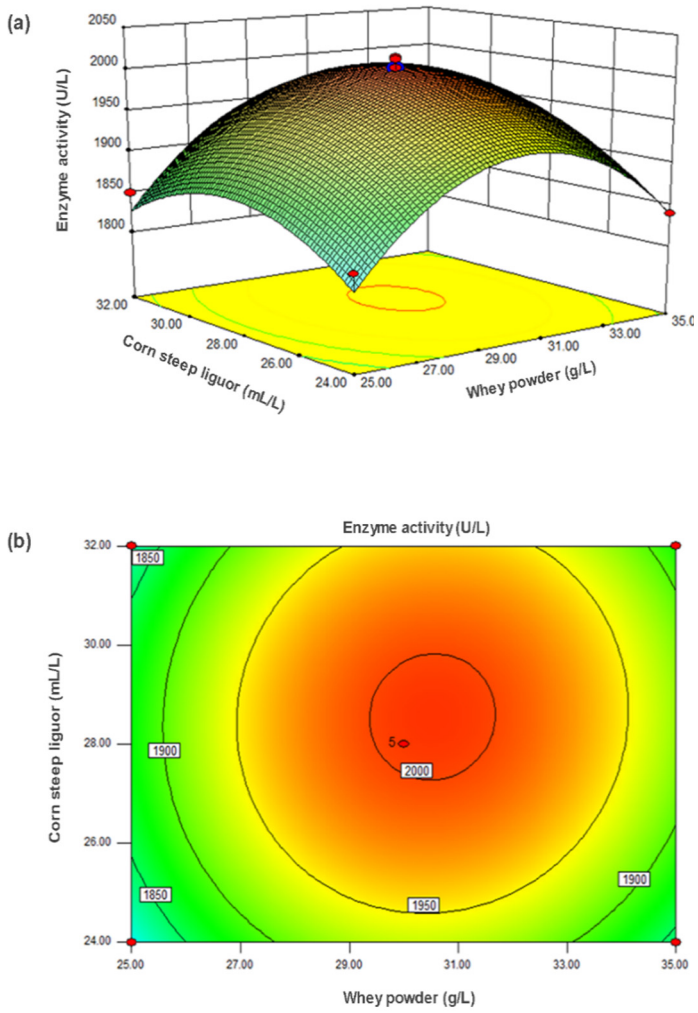

Figure 3. Effects of whey powder and corn steep liquor on enzyme production by *L. bulgaricus* L3; (**a**) response surface plot; (**b**) contour plot.

The response surface plot showed the presence of a maximum point of the β-galactosidase production in the response region. Estimation by Design Expert 8.0 showed that the maximal enzyme activity would achieve 2005.8 U/L when whey powder and corn steep liquor concentrations were 30.5 g/L and 28.6 mL/L, respectively.

Hence, the theoretically optimal medium for β-galactosidase production by *L. bulgaricus* L3 consisted of 30.5 g/L whey powder, 3 g/L $CH_3COONa\bullet 3H_2O$, 1.6 g/L triammonium citrate, 2.4 g/L K_2HPO_4, 3.2 g/L yeast powder, and 28.6 mL/L of corn steep liquor.

2.1.4. Model Verification

To validate the adequacy of the model equation for the predicted maximal enzyme activity, we conducted confirmation experiments by cultivating *L. bulgaricus* L3 under the predicted optimal medium conditions. The enzyme activity reached 2034 U/L, which was consistent with the predicted model value. The good correlation between the theoretical and experimental results justified the validity of the response model and the existence of

an optimum point. When cultured in the optimal whey-based medium, *L. bulgaricus* L3 had a high cell amount of 6.2×10^8 cfu/mL and produced a two-fold amount of enzyme versus that cultivated in the MRS medium (1125 U/L).

The actual access to the high enzyme activity from *L. bulgaricus* L3 in the predicted medium proved the efficiency and accuracy of RSM in the medium optimization. RSM helped save time and effort, compared with the traditional method of optimizing multi-factorial system by one factor at a time, because it combined statistical and mathematical techniques for designing experiments, building models, and exploring the relationships among several independent variables on a system response [16,17].

2.2. GOS Synthesis from Whey Powder by β-Galactosidase

The cells of *L. bulgaricus* L3 cultivated in the optimal medium were harvested and treated with lysozyme. The resulting suspension was used as crude β-galactosidase for the GOS synthesis. The reaction was performed by incubating the enzyme with 200 g/L whey (containing ~160 g/L lactose), at 45 °C. Figure 4a shows the time course of the GOS synthesis by *L. bulgaricus* L3. The lysozyme-treated cells proved to be an efficient catalyst form of β-galactosidase because they produced high GOS yield (>30%) when the reaction was initiated at 1 h. The GOS produced by *L. bulgaricus* L3 accumulated increasingly with the extended reaction time, and it reached the maximum at 9 h when approximately 90% lactose substrate was utilized and converted into hydrolysis and transglycosylation products. The result of the HPLC analysis revealed that the product mixture at 9 h contained 10.1% lactose, 45.2% monosaccharides (including glucose and galactose), and 44.7% GOS comprising 26.3% transglycosylated disaccharides, 15.9% trisaccharides, and 2.5% tetrasaccharide (Figure 5a).

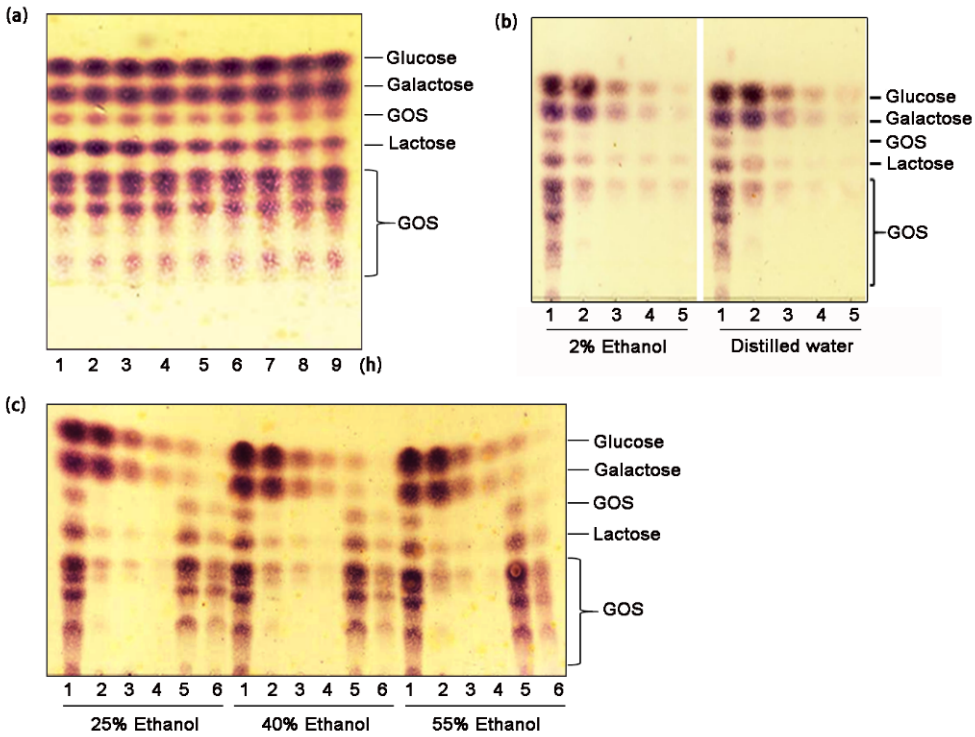

Figure 4. Processes of GOS synthesis and purification. (**a**) GOS synthesis by *L. bulgaricus* L3 β-galactosidase within 9 h. (**b**) Elution of monosaccharides from activated charcoal particles. 1, GOS products; 2, samples after charcoal adsorption; 3–5, sequential elution of monosaccharides by 2% ethanol (left) or distilled water (right). (**c**) GOS purification by activated charcoal adsorption. 1, GOS products; 2, samples after adsorption; 3 and 4, sequential elution of monosaccharides by distilled water; 5 and 6, sequential elution of oligosaccharides by high concentrations of ethanol.

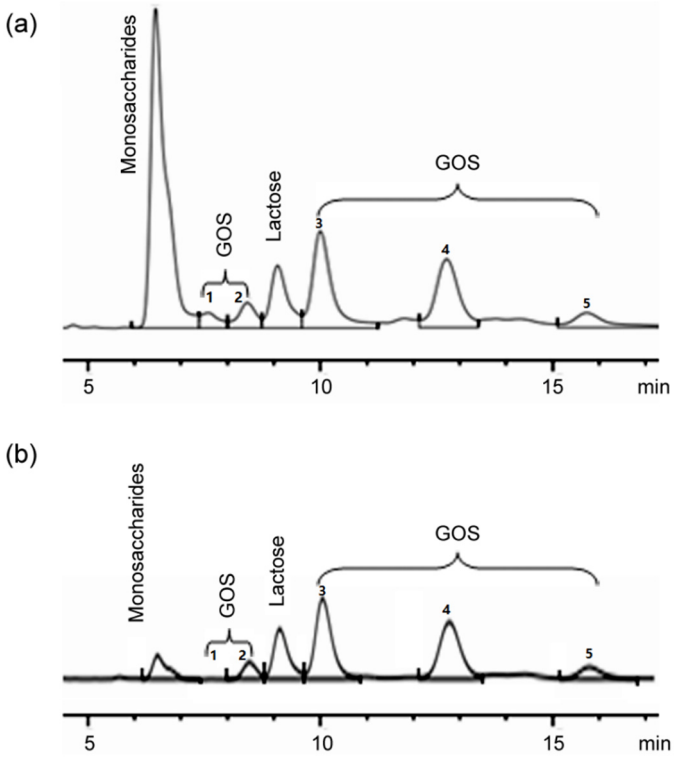

Figure 5. HPLC analysis of GOS synthesized by *L. bulgaricus* L3 (**a**) and treated after activated charcoal adsorption (**b**). The peaks at 6.4 min represent monosaccharides including galactose and glucose, while the peak at 9.1 min represents lactose. Peaks at 7.6, 8.4, 9.9, 12.7, and 15.7 min are all GOS products, including transglycosylated disaccharides (peaks 1, 2, and 3), trisaccharides (peak 4), and tetrasaccharide (peak 5).

The conversion of whey into GOS has been investigated in various cases. For example, the recombinant β-galactosidase from *Paenibacillus barengoltzii,* which was purified from *Escherichia coli,* exhibited high transglycosylation activity, and produced GOS with a maximum yield of 47.9% (*w/w*) when incubated with whey at a lactose concentration of 350 g/L, for 8 h [18]. In another example, the crude β-galactosidase from the yeast *Bullera singularis* KCTC 7534, which was obtained from sonication treatment, formed a 34% GOS yield after 27 h incubation with 200 g/L whey permeate [19].

In this work, the highly efficient whey conversion to GOS was achieved by the lysozyme-treated crude β-galactosidase from a beneficial lactic acid bacterium with probiotic functions, i.e., *L. bulgaricus*, which is a well-known excellent health and longevity source in our daily diet [20–22]. *L. bulgaricus* was originally discovered in Bulgaria and named after the country [21]. It serves as the main bacteria in yogurt production and is commonly used alongside *Streptococcus thermophiles*.

Probiotic lactic acid bacteria are of considerable interest for the enzyme-involved production and process because of their generally recognized as safe status [21]. Recently, β-galactosidases from various *S. thermophilus* strains isolated from different dairy products have been investigated for GOS synthesis by using whey supplemented with lactose as the substrate. The crude enzyme of *S. thermophiles* resulting from cell disruption by microfluidizer produced a 5.3% yield of GOS with 300 g/L lactose, for 5 h, at 40 °C. The use of crude β-galactosidase extract from *S. thermophiles* can significantly reduce production cost because it avoids laborious and expensive chromatographic steps for enzyme purification [23]. However, unfortunately, the GOS yield by the crude enzyme was modest. By contrast, the crude enzyme from *L. bulgaricus* L3, which was prepared by lysozyme treatment, acted as a greatly efficient catalyst. It resulted in a 44.7% yield of GOS, which was significantly higher than those produced by *S. thermophiles* and comparable with those obtained by pure β-galactosidases when using whey as the substrate [18,23].

2.3. GOS Purification by Activated Charcoal Adsorption

The GOS mixture produced by *L. bulgaricus* L3 was deproteinized and treated by adsorption with activated charcoal, which was performed by directly mixing sugar samples with charcoal particles without using a column. As shown in Figure 4b, the charcoal particles showed poor adsorption ability for monosaccharides but was good for oligosaccharides since most monosaccharides combined with trace oligosaccharides remained in the mixture after adsorption treatment. Even the binding force of the minor monosaccharides with charcoal particles was vulnerable as they can be easily eluted from the particles by distilled water, which was comparable with the use of 2% ethanol as the eluent. The effect of ethanol concentrations on the elution of the sugar samples from the activated charcoal was investigated. The result showed that the oligosaccharides were almost fully eluted by 55% ethanol (Figure 4c). The finally purified GOS mixture was composed of 2.9% monosaccharides, 19.5% lactose, and 77.6% GOS, as analyzed by HPLC (Figure 5b).

Thanks to the reduction in the saccharides that increase the level of postprandial glucose and calorie, high-purity GOS have expanded applications in food and pharmaceutical industries, including consumption by diabetic patients and therapeutic treatment of intestinal diseases [24].

To date, various methods have been studied for the removal of monosaccharides and lactose in the GOS mixture. These methods include ion-exchange chromatographic process, activated charcoal adsorptions, size exclusion chromatography, nanofiltration, and yeast selective fermentation [1]. Considering the feasibility and low cost of industrialized production, the GOS syrup synthesized by *L. bulgaricus* L3 was simply mixed with the charcoal particles for purification without using the complex column chromatography that was commonly reported. The method was easy to operate and resulted in high-purity oligosaccharides. The activated charcoal showed higher affinity for GOS, compared with mono- and disaccharides. It is reported that the main surface area of the activated charcoal is hydrophobic, and the hydrophobicity of the solute contributes to adsorption. As the hydrophobicity of sugars is increased with the number of CH groups, their adsorption intensity will depend on their molecular weight [24,25].

3. Materials and Methods

3.1. Materials

Whey powder was supplied by Davisco Foods (Le Sueur, MN, USA), with the components listed as following: moisture (%) 4.7 ± 0.2, total Protein (N × 6.38) (%) 6.0 ± 1.0, fat (%) 0.9 ± 0.3, ash (%) 9.8 ± 1.0, lactose (%) 80.0 ± 1.5, scorched particles 7.5 mg/25 g, pH 6.2 ± 0.2. *o*-Nitrophenyl-β-D-galactopyranoside (*o*NPGal) was purchased from Sangon (Shanghai, China). Lysozyme was obtained from Amresco (Englewood, NJ, USA). Silica gel 60 F254 plates were purchased from Merck (Darmstadt, Germany). Activated charcoal was obtained from Sigma-Aldrich. HPLC grade acetonitrile was supplied by Honeywell Burdick and Jackson (Muskegon, MI, USA). Other chemicals were of analytical grade.

3.2. Strains and Mediums

L. bulgaricus L3 was cultured for 12 h at 37 °C in the MRS broth or whey medium that was developed in this study. The typical MRS medium contained 20 g/L glucose, 10 g/L peptone, 10 g/L beef extract, 4 g/L yeast extract, 1 mL/L of Tween 80, 5 g/L $CH_3COONa{\cdot}3H_2O$, 2 g/L triammonium citrate, 2 g/L K_2HPO_4, 0.2 g/L $MgSO_4{\cdot}7H_2O$, and 0.05 g/L $MnSO_4{\cdot}4H_2O$ at native pH. The initial whey medium included 20 g/L whey powder, 2.5 g/L $CH_3COONa{\cdot}3H_2O$, 2 g/L triammonium citrate, 2 g/L K_2HPO_4, 4 g/L yeast powder, and 20 mL/L of corn steep liquor.

3.3. β-Galactosidase Assays

The β-galactosidase activity was measured by adding the wet cells of *L. bulgaricus* L3 to 450 μL of 2 mM *o*NPGal. Wet cells were prepared by centrifugation of 0.6 mL of liquid culture at 12,000 rpm for 1 min and suspended in 50 μL of phosphate buffer (pH 7.0). The

reaction was performed at 37 °C, for 10 min, and then stopped by adding 1 mL of 500 mM Na_2CO_3. The amount of *o*-nitrophenol released was measured at 420 nm. One unit of enzyme activity (U) was defined as the amount of enzyme required to liberate 1 µmol of *o*-nitrophenol per minute under the assay conditions [7].

3.4. Plackett–Burman (PB) Design

The effects of six independent factors, including whey powder, $CH_3COONa{\cdot}3H_2O$, triammonium citrate, K_2HPO_4, yeast powder, and corn steep liquor on β-galactosidase production were investigated by a PB design using the software Minitab 17. Each variable was represented at two levels, high and low, which were denoted by (+) and (−), respectively. The concentrations of the high levels of each factor were 1.5-fold of the low levels. The data were analyzed based on the first-order model, as follows:

$$Y = \beta_0 + \Sigma\ \beta_i X_i \quad (3)$$

where Y is the response, β_0 is the model intercept, β_i is the linear coefficient, and X_i is the level of the independent variable [26]. Table 1 lists the levels of each variable used in the experimental design. All assays were performed in triplicate.

3.5. Steepest Ascent Experiment

Steepest ascent experiment helps to move towards the largest response rapidly. The center point of the PB design was taken as the origin for the path of steepest ascent. Meanwhile, the ascent direction and length of the ascent pace were determined based on the first-order model, as shown in Equation (3), which was obtained from the PB design. A series of experimental runs was carried out along the path, until no additional increase was observed in the response.

3.6. Central Composite Design (CCD)

A 2^2 factorial central composite design (α = 1.414) was conducted to optimize the concentration of whey powder (X_1) and corn steep liquor (X_2), which showed a significantly positive effect on enzyme production. All assays were performed in triplicate.

The second-order polynomial coefficient was calculated and analyzed by using Design Expert software 8.0. The role of each variable, their interactions, and the value of predicted response were explained by applying the following quadratic equation:

$$Y = \beta_0 + \Sigma\ \beta_i X_i + \Sigma\ \beta_{ij} X_i X_j + \Sigma \beta_{ii} {X_i}^2 \quad (4)$$

where Y is the predicted response, β_0 is the offset term, β_i is the linear effect, β_{ii} is the squared effect, β_{ij} is the interaction effect, and X_i is the dimensionless coded value of X [26].

3.7. GOS Synthesis by Lysozyme-Treated Cells from Whey Powder

L. bulgaricus L3 was cultured in 2 L of the optimal whey medium at 37 °C, for 12 h. Subsequently, cells were harvested from the culture by centrifugation at 12,000 rpm, for 5 min, and suspended in 50 mM potassium phosphate buffer (pH 7.0), in a ratio of 1:1 (U/mL). The cell mixture was subsequently incubated with 5 mg lysozyme (10,000 U/mg) at 37 °C, for 1 h. The resulting suspension was used as the crude β-galactosidase and supplemented with whey powder at 200 g/L at 45 °C. After 1–10 h incubation, the reaction mixture was stopped by boiling for 5 min to inactivate the enzyme and then centrifuged at 12,000 rpm, for 30 min. The cell debris was removed, and the sugar supernatant was analyzed by TLC and HPLC, as described below.

3.8. GOS Purification by Activated Charcoal Adsorption

The GOS mixture produced by *L. bulgaricus* L3 was precipitated with ethanol in a ratio of 1:2 (*v/v*) at 4 °C, for 2 h, followed by centrifugation at 12,000 rpm, for 10 min, to remove proteins. The ethanol in the sample was subsequently removed by rotary

evaporation. The remaining sugar sample devoid of proteins was diluted with distilled water to 3.5% and absorbed on to 80 g/L activated charcoal. After stirring at 45 °C, for 1 h, the mixture was filtered, and the remaining activated charcoal loaded with sugars was washed and eluted with 2% (*v/v*) ethanol or distilled water to remove monosaccharides. Next, ethanol elution at different concentrations (25–55%, *v/v*) was conducted to investigate the oligosaccharide release from the activated charcoal. The eluted sugars by using 55% ethanol were further concentrated by rotary evaporation, with the ethanol removed and tetrasaccharide recovered at the same time.

3.9. TLC and HPLC Analysis

TLC was performed by loading samples on the Silica gel 60 F254 plates and using a mixture of *n*-butanol: ethanol: water (5: 3: 2, *v/v/v*) as the developing solvent. Sugars separating on the TLC plate were detected by spraying with 0.5% (*w/v*) 3,5-dihydroxytoluene in 20% (*v/v*) sulfuric acid and heated at 120 °C, for 5 min. HPLC was performed using an Agilent ZORBAX carbohydrate column (4.6 × 250 mm) with the column oven temperature remaining at 30 °C. Sugar samples were filtered through a 0.22 μm polypropylene and the mobile phase acetonitrile/water (7:3, *v/v*) was degassed in an ultrasonic bath before use. The samples were eluted with a flow rate at 1 mL/min. Sugar was detected through Agilent 1200 refractive index detector. Data analysis was performed by Agilent Chemstation B.04.01 [7]. The yield of GOS was defined as the mass ratio of GOS to the total saccharides.

4. Conclusions

This work provided an integrated utilization of whey waste in *L. bulgaricus* cell growth, β-galactosidase production, and GOS synthesis. The newly-developed whey-based medium, which was optimized by RSM, contained less composition but yielded a higher amount of β-galactosidase, compared with the MRS medium. The following incubation of the crude enzyme of *L. bulgaricus* with whey as the substrate provided a facile, efficient, and low-cost approach for GOS synthesis. The results reused whey waste and laid a foundation for the industrial cultivation of probiotics and production of GOS by probiotic enzymes.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/catal11060658/s1, Table S1: Statistical analysis of variables based on PB design, Table S2: ANNOVA for PB design model.

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