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Application of *Bacillus pumilus* β-xylosidase reaction and simulated moving bed purification to efficient production of high-purity xylobiose from xylose

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ABSTRACT

Xylobiose (X2) is recognized to possess great prebiotic function and to be highly favorable for application in food and prebiotic industries. In this study, we demonstrated that the cloned β-xylosidase of *Bacillus pumilus* IPO could be utilized to produce X2 via the reaction of xylose(X1) → xylobiose(X2). The use of such enzyme in the X1 → X2 reaction was found to give much higher X2 reaction yield and reaction efficiency, compared to those reported in the literature. Furthermore, we developed an efficient simulated moving bed (SMB) chromatographic process that could recover X2 from the reaction output with nearly 100% purity and 92% recovery on a continuous-separation mode. The developed SMB process could also recover the unreacted X1 almost completely, which leaves room for a further increase in the overall X2 reaction yield by reusing the recovered X1 from the SMB as the reactant of the upstream processing (i.e., *B. pumilus* IPO β-xylosidase X1 → X2 reaction). The results of this study will enable a highly economical and environmentally-friendly production of high-purity X2 from X1.

Introduction

Xylobiose (X2), which is an useful disaccharide that consists of two molecules of xylose (X1), has been recently highlighted as a high-value food supplement with outstanding prebiotic function [1–4]. In particular, X2 is recognized to possess the highest prebiotic activity in the proliferation of bifidobacteria, which plays an important role in the maintenance of the healthy intestinal microflora [1,5–8]. Furthermore, X2 is known to have the most favorable condition in terms of industrial applicability [5–8]. For these reasons, there has been significant interest in developing an economical method for the large-scale production of high-purity X2 (>99.9%) in food and prebiotic industries.

In regard to the production of X2, it is worth paying attention to the recent studies on the feasibility of converting X1 to X2 via the β-xylosidase reaction [9,10]. In these studies, the β-xylosidases from *Sporotrichum thermophile* and *Talaromyces thermophilus* were employed as the enzymes for the X1 → X2 reaction, and a buffer solution was used as the reaction solvent [9,10]. The highest X2 reaction yield was 5.65%, which was attained in the latest study [10]. The X2 reaction yield, however, was achieved by the addition of a third component (sorbitol) into the reaction solvent, which should be removed after the reaction and would thus be unfavorable to downstream processing. In addition, the reaction times (i.e., the times required for attaining the maximum X2 reaction yield) in the previous studies were all longer than 3 days, and the X1 loading concentrations were kept lower than 60% (w/v). More importantly, the recovery of X2 from the reaction output was not attempted in the previous studies.

To establish a highly economical X2 production process for industrial application, the efficiency of the aforementioned X1 → X2 reaction needs to be improved further in the following

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directions. First, the X2 reaction yield should be increased, and the reaction time should be shortened. Secondly, the applicable X1 loading concentration should be as high as possible for the purpose of ensuring high throughput. Thirdly, the addition of a third component into the reaction system needs to be avoided. Fourthly, using water rather than a buffer solution as the reaction solvent needs to be considered because it can reduce the separation load and separation costs for downstream processing. In addition to the above-mentioned requirements on the upstream processing (i.e., X1 → X2 reaction), it is essential to fulfill the task of separating the reaction product (X2) and the unreactant (X1) with high purities and high recoveries for both X2 and X1.

The goal of this study is to accomplish the aforementioned work, i.e. to propose an enhanced X1 → X2 reaction process that can lead to higher X2 reaction yield and shorter reaction time without using a third component and a buffer solution, and further to develop a highly efficient X2–X1 separation process that is based on a favorable operation mode for the large-scale X2 production (i.e., continuous-separation mode). As a first step for this work, we explored another enzyme source of β-xylosidase that could make a substantial improvement in the efficiency of the X1 → X2 reaction process. It was found in this study that the β-xylosidase from *Bacillus pumilus* IPO could surpass the previously adopted β-xylosidase enzymes [9,10] to a large extent in every respect. To date, the use of *B. pumilus* IPO β-xylosidase for the X1 → X2 reaction has not been reported in the literature. Furthermore, a continuous-mode chromatographic separation process for recovering high-purity X2 from the reaction output was developed in this study on the basis of the process structure and the operation principle of a simulated moving bed (SMB) technology [11–14]. It was confirmed from the experimental results that the developed separation process in this study was successful in recovering X2 from the reaction output on a continuous-separation mode, through which the X2 product of nearly 100% purity was obtained with 92% recovery. The unreacted X1 molecules were also recovered from the developed process without any loss, which is meaningful because they could be reused in the upstream X1 → X2 reaction.

Materials and methods

Cloning and expression of the β-xylosidase gene from B. pumilus *IPO*

The *B. pumilus* IPO (*BP*I) β-xylosidase, which was adopted as the enzyme for the X1 → X2 reaction in this study, was obtained through cloning and expressing its gene in *Escherichia coli*. This was carried out by referring to the procedures reported previously [15–19]. First, the information on the *BP*I XynB DNA (2207 bp) was acquired from the NCBI (national center for biotechnology information). Using this information, the *BP*I β-xylosidase DNA was synthesized at BIONEER Corporation (Daejeon, Korea). The synthesized β-xylosidase DNA was ligated into pET21a, and then transformed into *E. coli* BL21(DE3). The recombinant *E. coli* was cultured in Luria-Bertani (LB) medium that was supplemented with 100 μg/mL of ampicillin (Sigma-Aldrich Co., USA). When the OD_{600} of the culture medium became 0.8, IPTG at 0.5 mM (BIONEER Corporation, Korea) was added and the culture was continued at 20 °C and 100 rpm for 16 h.

After the culture, the cells were harvested by centrifugation and suspended in disruption buffer (50 mM Tris–HCl, 300 mM NaCl, 10 mM imidazole, pH 8.0). The cell suspension was sonicated by using a VC-505P ultrasonic processor (Sonic & Materials Inc., USA), followed by being centrifuged at 8000 rpm (6576 g-force) for 20 min. The supernatant obtained by the centrifugation was filtered through a 0.45 μm Minisart® RC filter (Sartorius Co., Germany), and then loaded on a Ni-NTA agarose column (Qiagen Co., Germany) for purification purpose. After then, the washing buffer (50 mM Tris–HCl, 300 mM NaCl, 20 mM imidazol, pH 8.0) and the elution buffer (50 mM Tris–HCl, 300 mM NaCl, 250 mM imidazol, pH 8.0) were introduced sequentially into the Ni-NTA agarose column in order to remove impurities and recover β-xylosidase. The active β-xylosidase fractions that were collected from the Ni-NTA agarose column were dialyzed with 50 mM sodium phosphate buffer, pH 7, and subsequently concentrated by ultrafiltration on an Amicon®Ultra system (Merck Millipore Co., Germany).

Implementation of the X1 → X2 reaction using the cloned β-xylosidase of B. pumilus *IPO (BPI)*

Xylose, which was supplied from Samin Chemical Co. (Siheung, Korea), was dissolved in distilled water by boiling in order to prepare 500 mL of aqueous xylose solution that contained xylose at 90% (w/v). The prepared xylose solution was placed in a drying oven at 50 °C. When the temperature of the xylose solution was equilibrated at 50 °C, the reaction began by adding 50 U of the *BP*I β-xylosidase (0.1 U/mL) to the solution. The reaction mixture consisting of the xylose solution and the β-xylosidase was incubated at 50 °C for 24 h. This reaction was stopped by heating the reaction mixture at 99 °C for 15 min. Then, the reaction output was diluted with distilled water and centrifuged at 7000 rpm for 20 min to remove the denatured β-xylosidase. The supernatant solution from the centrifugation was placed in a drying oven at 45 °C for 2 h, and then filtered through a 0.22 μm Stericup-GP (Merck Millipore Co., Germany). The resulting solution was then loaded into the downstream SMB separation process for recovery of high-purity xylobiose (X2).

Resin pretreatment and pulse-injection experiment for estimating the intrinsic parameters of the solutes in the reaction output

A commercial ion-exchange resin Dowex-50WX4, which was reported to be effective in oligosaccharide separation in the literature [20,21], was adopted in this study as the solid phase of the considered SMB process for recovery of X2 from the *BP*I β-xylosidase reaction output. The Dowex-50WX4 resin was supplied in hydrogen form from the Sigma-Aldrich Co. (St. Louis, MO), and it was converted to the sodium form prior to use. This resin was packed into an omnifit chromatographic column, which was purchased from the Bio-Chem Fluidics Co. (Boonton, NJ). The diameter and length of this column are 3.5 cm and 21.7 cm respectively. The bed voidage and particle porosity of the packed column were 0.3 and 0.629 respectively, which were obtained from a series of tracer-molecule pulse tests. The average diameter of the resin particle is 55.5 μm.

Using the column packed with the aforementioned Dowex-50WX4 resin, the pulse-injection experiment for estimating the intrinsic parameters was carried out in the following manner. First, the packed column was installed in a ÄKTA™ fast protein liquid chromatography (FPLC) system (Amersham Biosciences Co., USA), which consisted of two pumps (Amersham Biosciences P-920) and a fraction collector (Amersham FPLC Frac-900). One of the two pumps delivered deionized distilled water (DDW) and the other pump the mixture solution coming from the upstream process of the *BP*I β-xylosidase X1 → X2 reaction. Prior to the experiment, DDW was pumped into the column for a sufficiently long period of time. The experiment was started by switching the pump flow from DDW to the mixture solution and simultaneously collecting the column effluent in the fraction collector at intervals of 0.6 mL. The amount of the mixture solution loaded into the column was set at 20 mL. Immediately after the completion of such loading, the pump flow was switched back to DDW. The flow rates of both

pumps were kept constant at 2 mL/min throughout the experiment. In order to maintain temperature at 65 °C during the experiment, the DDW at a fixed temperature of 65 °C was continuously circulated through the jacket enclosing the packed column, which was carried out by a HST-250WL circulator (Hanbaek Co., Korea). In addition, both reservoirs containing DDW and the mixture solution respectively were immersed in a BW-20G water bath (Jeio Tech Co., Korea), which was also maintained at 65 °C. The concentrations of the column-effluent fractions that were obtained by the FPLC fraction collector were analyzed by a Waters HPLC system, which consisted of a Shodex Sugar KS-801 analysis column (300 × 8 mm) and Waters 2414 Refractive Index detector. During the HPLC analysis, the column temperature, flow rate, and injection volume were maintained at 80 °C, 0.6 mL/min, and 5 μL respectively while using DDW as mobile phase.

SMB process experiment for continuous recovery of high-purity X2 from the reaction output

As a downstream process equipment for recovery of X2 from the upstream processing (i.e., the *BP*I β-xylosidase X1 → X2 reaction) output, a three-zone SMB unit with an open-loop structure was assembled in accordance with the guidelines of SMB hardware assemblage in the literature [22]. The main parts of the assembled unit are three rotary valves, three pumps, and four columns. The rotary valve used was the Select-Trapping (ST) valve from VICI Valco Instruments (Houston, TX), which was connected to each column for implementation of periodic port movement that occurs at the same time with the valve switching. All the actions associated with such a valve switching were controlled by a computer with Labview 8.0 software from National Instruments (Austin, TX).

Using the aforementioned three-zone SMB unit, two sets of SMB experiments were carried out at 65 °C. The first SMB experiment was aimed at the continuous removal of X1 from X2 under a favorable port-configuration mode for enriching the raffinate product containing X2. The second SMB experiment was aimed at the continuous removal of X3 from X2 under a favorable port-configuration mode for enriching the extract product containing X2. The mixture solution from the upstream process of the *BP*I β-xylosidase X1 → X2 reaction was loaded into the feed port of the first SMB unit (X2–X1 SMB unit). The raffinate product resulting from the first SMB experiment was stored in a glass container, and then used as the feed to the second SMB unit (X2–X3 SMB unit). The desorbent in both the first and the second SMB experiments was deionized distilled water (DDW). To maintain temperature at 65 °C during the SMB experiments, the DDW at 65 °C was continuously circulated through the jacket enclosing each of the four columns, which was implemented by the HST-250WL circulator. In addition, the reservoirs containing the feed and the desorbent were put in a CB407 heating mantle from Misung Scientific Co. (Yangju, Korea), which was also maintained at 65 °C. Each SMB experiment was continued for 240 steps (50 h), during which the effluent from the product port was collected and their concentrations were measured by the Waters HPLC system with the Shodex Sugar KS-801 analysis column.

Results and discussion

X1 → X2 reaction by the BPI β-xylosidase

Using the *BP*I β-xylosidase prepared in this study, we first sought to determine the optimal reaction time and temperature for the X1 → X2 reaction. In this task, the X1 loading concentration was set at 90% (w/v), which was much higher than in the previous studies [9,10] (Table 1), while the amount of β-xylosidase used was maintained below those in the previous studies [9,10]. This must be a more advantageous condition for the large-scale X2 production. In addition, water was employed as the reaction solvent, which would be more favorable to the downstream processing than the use of a buffer solution as in the previous studies (Table 1).

Under the aforementioned conditions, the X1 → X2 reaction was carried out at three different temperatures (40, 50, and 60 °C), and the resulting data are presented in Fig. 1. Note that the X2 reaction yield increases steadily with increasing time up to 24 h, beyond which the X2 reaction yield remains almost constant. It is also shown in Fig. 1 that the X2 reaction yield at 50 °C was better than those at 40 °C and 60 °C. Based on these data, the reaction time and temperature in this work were set at 24 h and 50 °C respectively, to achieve 17% reaction yield for X2. This result is compared with the results of the previous works [9,10] in Table 1, which clearly demonstrates that the X1 → X2 reaction process proposed in this study is far superior to those reported in the previous studies in all the upstream-performance factors such as X2 reaction yield, reaction time, and X1 loading concentration. It is thus expected that the X1 → X2 reaction process proposed in this study, which is based on the *BP*I β-xylosidase, will be of help to the establishment of the upstream process for the large-scale X2 production.

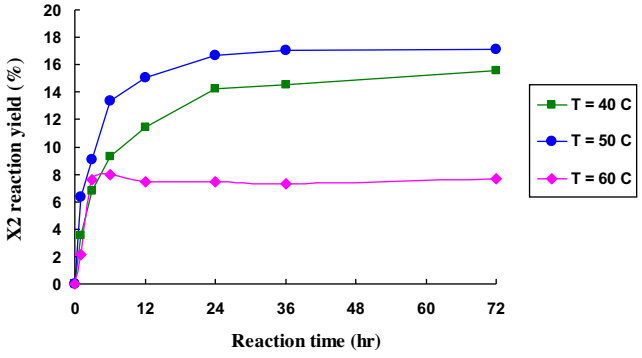

Fig. 1. Time course profiles of X2 reaction yield for the *BP*I β-xylosidase X1 → X2 reaction at three different temperatures.

Table 1
Comparison of the β-xylosidase X1 → X2 reaction processes of the previous and present studies in terms of the key upstream-performance factors for the large-scale X2 production.

Source for the β-xylosidase used in the X1 → X2 reaction	Reaction solvent	Enzyme (U/mL)	X1 loading concentration (%, w/v)	Reaction time (day)	X2 reaction yield (%)
Sporotrichum thermophile in the previous study [9]	Buffer solution	0.1	60	28	1.2
Talaromyces thermophilus in the previous study [10]	Buffer solution	14.7	40	6	1.4
Talaromyces thermophilus in the previous study [10]	Buffer solution	14.7	40 with 3 M sorbitol	3	5.7
Bacillus pumilus IPO in the present study	Water	0.1	90	1	17

SMB separation for recovery of high-purity X2 from the reaction output

The solutes in the output from the aforementioned *BP*I β-xylosidase X1 → X2 reaction were mostly X2 (the product of interest) and X1 (the unreacted component). Additionally, a small amount of xylotriose (X3) (<0.5%) was formed as a side-product. To establish an efficient downstream process for the large-scale X2 production, we developed an SMB process that could fulfill the recovery of high-purity X2 from the reaction output on a continuous mode. As a first step for this work, the intrinsic parameters of the reaction-output solutes on the selected resin (Dowex-50WX4 in sodium form), which were the essential prerequisites for the SMB design and optimization, were determined on the basis of the pulse-injection experimental data, the solute movement theory [23], and the literature correlations [24–27]. The resulting intrinsic parameters of the reaction-output solutes (X2, X1, and X3) are listed in Table 2, which indicates that the migration velocities of the solutes through the Dowex-50WX4 column are virtually governed by size-exclusion

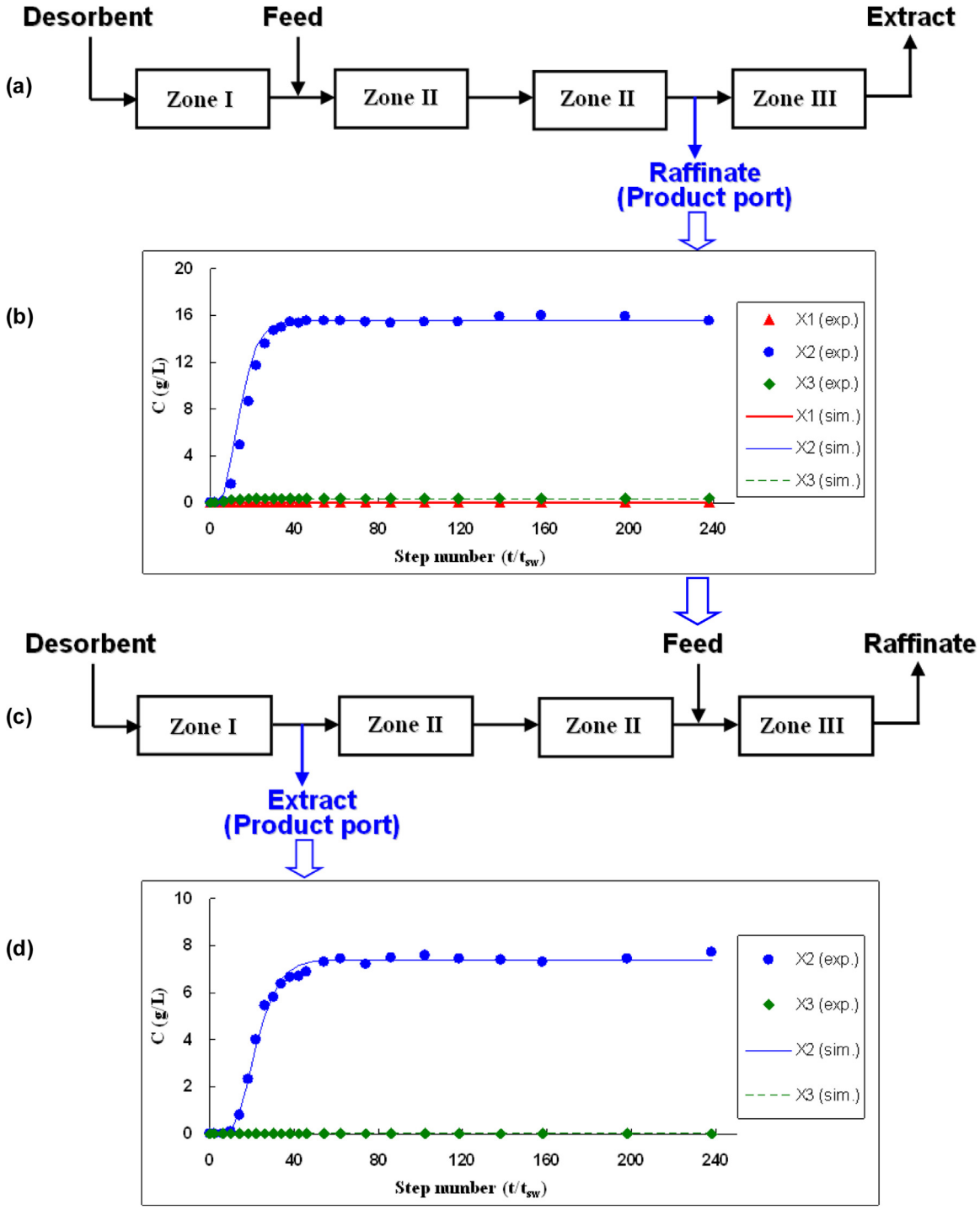

Fig. 2. Downstream SMB process for recovery of X2 from the reaction output and the experimental concentration profiles from the product port. (a) Schematic illustration of the X2–X1 separation SMB. (b) Product concentration profiles in the X2–X1 separation SMB. (c) Schematic illustration of the X2–X3 separation SMB. (d) Product concentration profiles in the X2–X3 separation SMB.

Table 2
Intrinsic parameters of the solutes in the output from the *BP*I β-xylosidase X1 → X2 reaction under the adopted chromatographic condition (solid phase: Dowex-50WX4 resin in sodium form, liquid phase: water, temperature: 65 °C).

Intrinsic parameters	X1	X2	X3
Size-exclusion factor	0.842	0.613	0.458
Molecular diffusivity[a] (cm^2/min)	1.23×10^{-3}	8.35×10^{-4}	6.61×10^{-4}
Intra-particle diffusivity[b] (cm^2/min)	5.26×10^{-6}	6.84×10^{-6}	1.02×10^{-5}
Axial dispersion coefficient (cm^2/min)	Chung and Wen correlation [24]		
Film mass-transfer coefficient (cm/min)	Wilson and Geankoplis correlation [25]		

[a] The value of the molecular diffusivity was calculated from the Wilke and Chang correlation [26].
[b] The initial value of the intra-particle diffusivity was calculated first from the Mackie and Meares correlation [27], and then fine-tuned by fitting the simulation with the experimental data.

mechanism. Comparison of the size-exclusion factors of the two major components shows that the desired reaction-product component (X2) migrates faster than the unreacted component (X1).

Using the above-determined intrinsic parameters of X2 and X1 (Table 2), we carried out the optimal SMB design for X2–X1 separation, which included the following two tasks: (i) finding the column, zone, and port configurations, which can give low equipment cost, simple robust operation, high X2 purity, and high X2 product concentration; and (ii) finding the optimal operating parameters (flow rates and port switching time), which can lead to high purities and high recoveries for both X2 and X1. For the first task, it was found that the best column configuration is 1 – 2 – 1 (i.e. one column in Zone I, two columns in Zone II, and one column in Zone III) based on a three-zone and open-loop SMB structure and the best port configuration is desorbent → feed → raffinate → extract (Fig. 2a). Under such configurations, the second task was to optimize the operating parameters such that the X2 recovery could be maximized, while the X2 purity on a X3-free basis was maintained higher than 99.95%. This task was accomplished by using the SMB optimization tool based on an up-to-date genetic algorithm [28,29] and a lumped mass-transfer model [11,29–31]. The resultant operating parameters from the SMB optimization, including the results for the column and port configurations, are listed in Table 3.

On the basis of the optimized operating parameters and the selected configurations in Table 3, the SMB experiment for continuous separation between X2 and X1 was carried out at 65 °C. In this experiment, the actual output from the upstream processing (i.e., *BP*I β-xylosidase X1 → X2 reaction) was loaded into the feed port while water was introduced into the desorbent port. The concentrations of X2, X1, and X3 in the solution loaded into the feed port (i.e. feed solution) were 31.5, 152.1, and 0.82 g/L respectively, which were identified by the HPLC analysis chromatogram for the feed solution (Fig. 3). The results for the SMB experiment are presented in Figs. 2b, 4a, and b . It is clear that only X2 and a small amount of X3 were continuously recovered from the product (raffinate) port (Fig. 2b), in which there was no trace of X1 (Fig. 4a). Simultaneously, the amount of X2 loss in the X1-recovery port (extract port) was very small (Fig. 4b). As a consequence, the X2 purity reached 98%, which could amount to 100% on a X3-free basis, and the X2 recovery was also as high as 97%. It is also worth mentioning that the X1 of 99% purity was continuously recovered from the extract port without any loss in the other outlet port (Fig. 4a and b), which leaves room for a further increase in the overall X2 reaction yield by reusing the recovered X1 from the SMB as the reactant of the upstream processing (i.e., *BP*I β-xylosidase X1 → X2 reaction).

To make a further improvement in the X2 purity, the design of an additional SMB process for separation between X2 and X3 was carried out. The column and port configurations of the X2–X3 separation SMB were determined on the basis of the same issues that were considered in the design of the preceding X2–X1 separation SMB. Since X2 is an extract product in the X2–X3 SMB, it is appropriate to adopt the port configuration of desorbent → extract → feed → raffinate (Fig. 2c) while keeping the same column

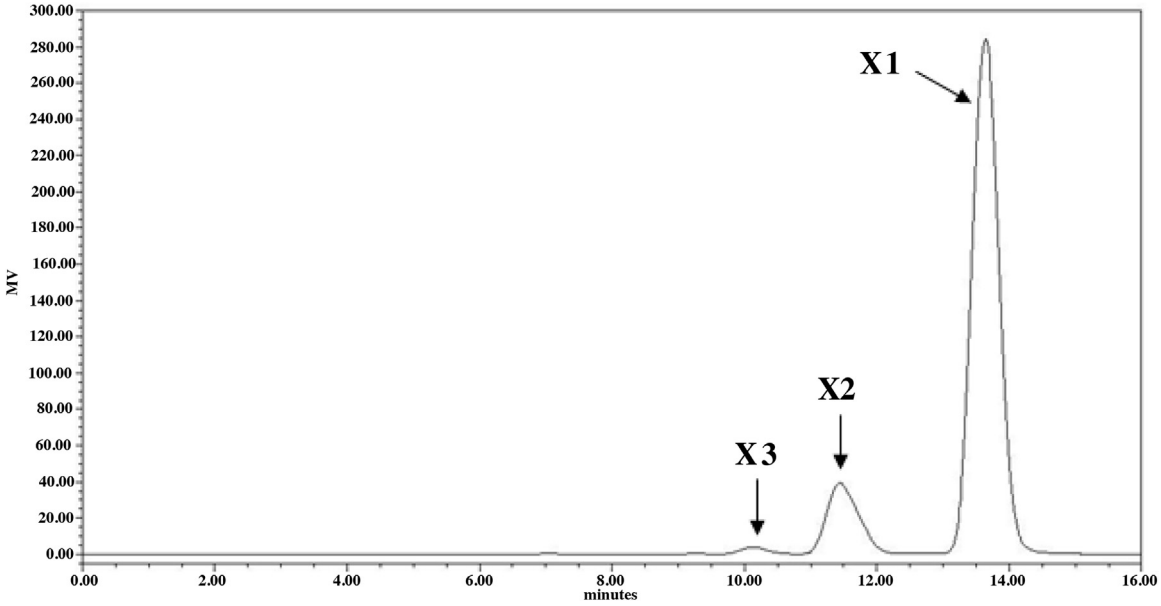

Fig. 3. HPLC analysis chromatogram for the feed solution of the X2–X1 separation SMB process (Fig. 2a) that was obtained from the *BP*I β-xylosidase X1 → X2 reaction.

Table 3
Results from the optimal designs of the X2–X1 separation SMB and the X2–X3 separation SMB.

Operating parameter	X2–X1 separation SMB	X2–X3 separation SMB
Port configuration[a]	D → F → R → E	D → E → F → R
Column configuration	1 – 2 – 1	1 – 2 – 1
Desorbent flow rate (mL/min)	10	10
Feed flow rate (mL/min)	1	0.7
Raffinate flow rate (mL/min)	1.96	9.32
Extract flow rate (mL/min)	9.04	1.38
Port switching time (min)	12.41	12.50

[a] D: desorbent, F: feed, R: raffinate, E: extract.

configuration as in the preceding X2-X1 SMB. This is because such configurations can allow the placement of the enrichment zone for X2 (extract product) as seen in Fig. 2c. Under such configurations of the X2–X3 SMB, its optimal operating parameters were determined in such a way that the X2 recovery could be maximized under the constraint of X2 purity ≥99.95%. The optimal operating parameters for the adopted column and port configurations are summarized in Table 3.

The SMB experiment for X2–X3 separation was carried out at 65 °C on the basis of the above-determined configurations and operating parameters (Table 3). In this experiment, the product solution from the preceding X2–X1 SMB (Fig. 4a) was loaded into the feed port while water was introduced into the desorbent port. The concentrations of X2 and X3 in the feed solution to the X2–X3 SMB were 15.6 and 0.35 g/L respectively (Fig. 4a). The X2–X3 SMB experiment was continued for 240 steps (50 h), and the results are presented in Figs. 2d, 5a, and b . The effluent from the product (extract) port contains only X2 without a trace of X3 (Figs. 2d and 5a). In addition, Fig. 5b shows that the amount of X2 loss in the other port (raffinate port) was very small. As a result, the X2 purity reached nearly 100%. The X2 recovery was also as high as 94% for the X2–X3 SMB, and 92% for the entire downstream process (i.e., X2–X1 SMB + X2–X3 SMB).

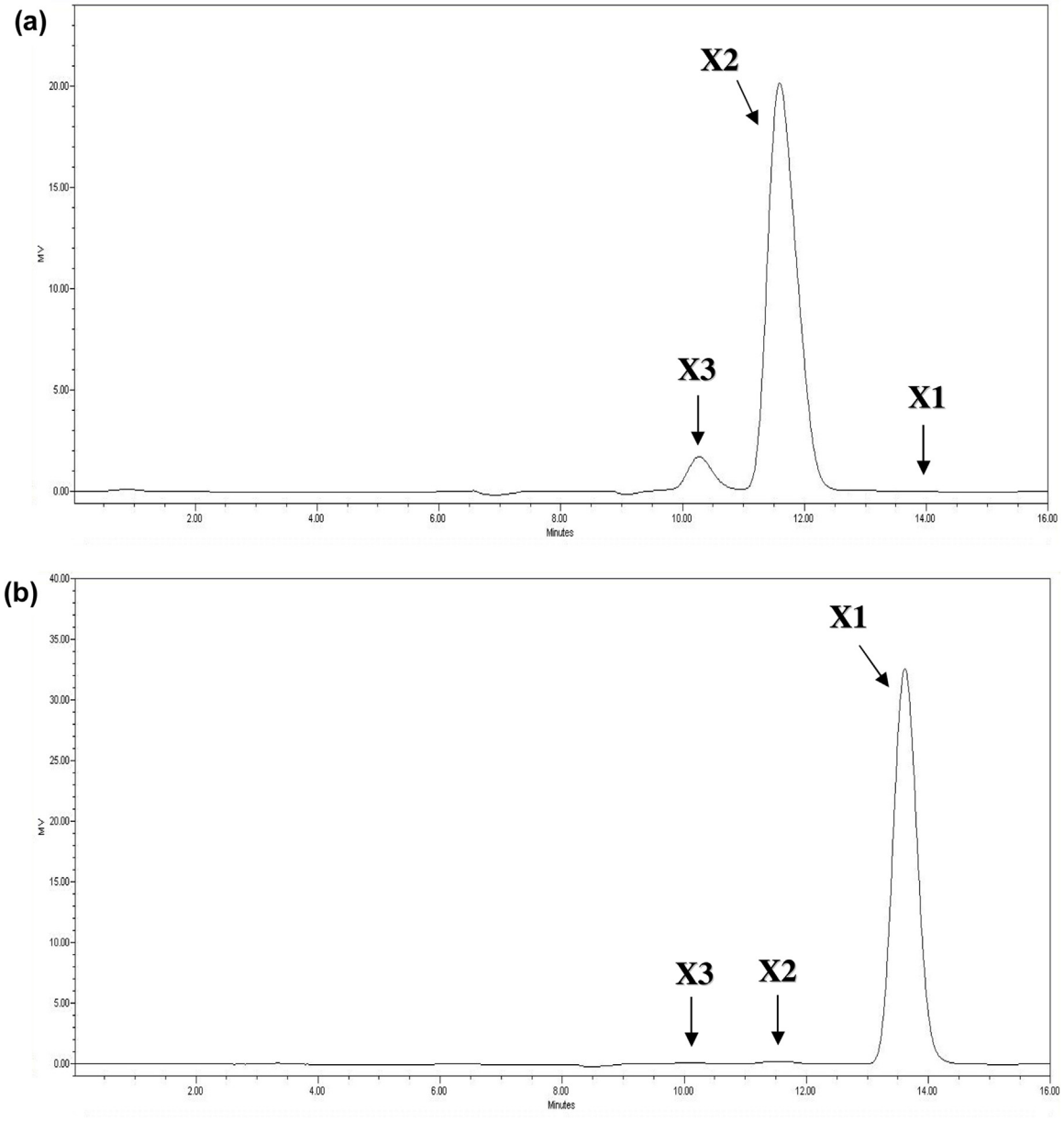

Fig. 4. HPLC analysis chromatograms for the samples collected from the two outlet ports at the final step in the X2–X1 separation SMB process (Fig. 2a). (a) Raffinate (product). (b) Extract.

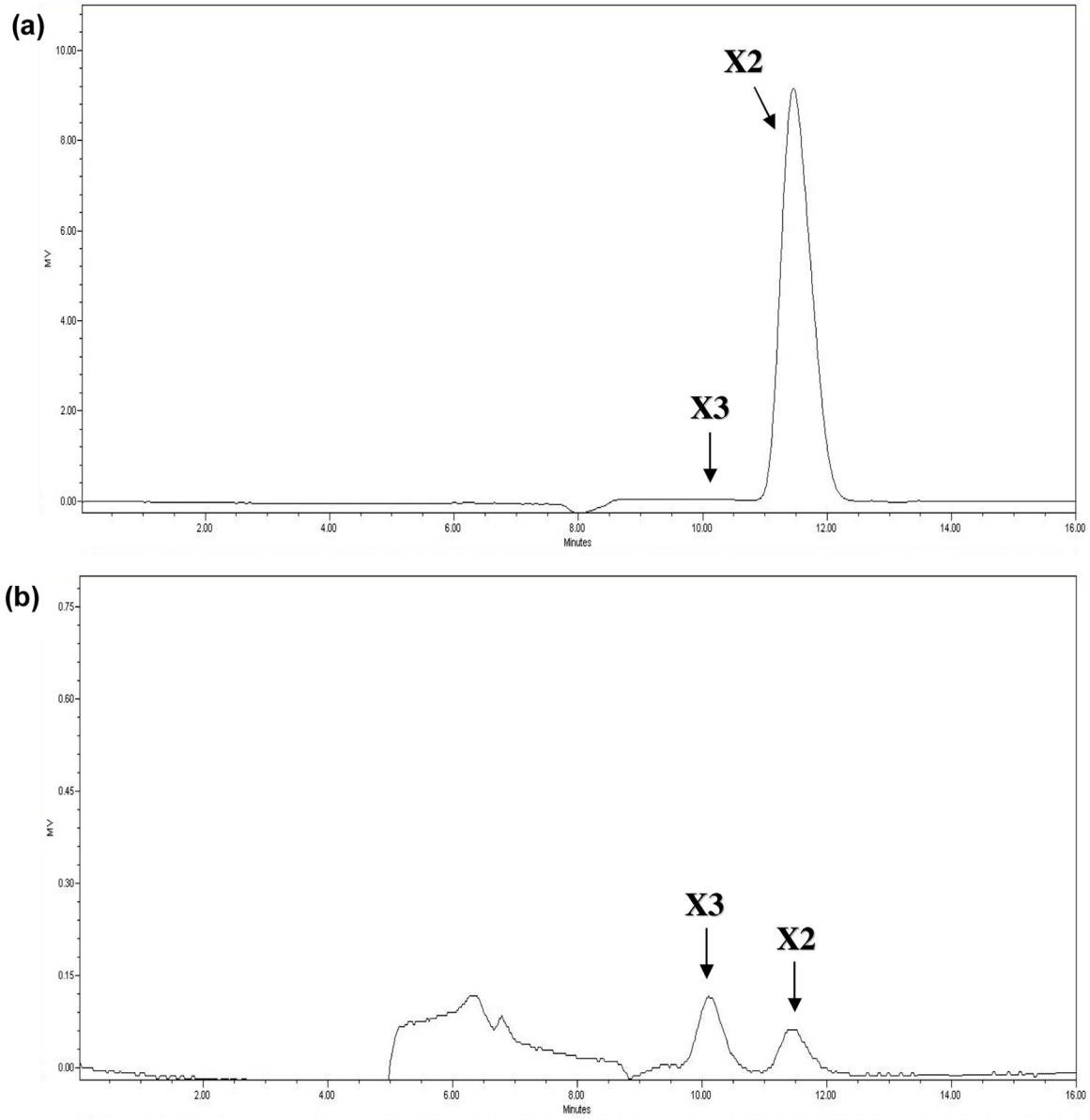

Fig. 5. HPLC analysis chromatograms for the samples collected from the two outlet ports at the final step in the X2–X3 separation SMB process (Fig. 2c). (a) Extract (product). (b) Raffinate.

Potential merits of the proposed X2 production method (BPI β-xylosidase X1 → X2 reaction + SMB separation of X2 from the reaction output)

Overall, the results of this study showed that the proposed X2 production method is quite promising. This method is based on the *BP*I β-xylosidase X1 → X2 reaction followed by the SMB recoveries of both X2 (reaction product) and X1 (unreactant) with high purities. The high enzyme productivity and high reaction yield for X2, high recovery for high-purity X2, and the collection of high-purity X1 without loss will be able to promote the large-scale production of high-purity (>99.9%) X2 on an industrially competitive mode. Economical production of X2 with excellent prebiotic function will make this product widely available to the general public and help the growth of the prebiotic industries.

Furthermore, the X2 production method developed in this study is environmentally-friendly, and it enables the industries to meet strict environmental regulations. The current xylooligosaccharides (XOS) production method suffers from the use of harsh, highly concentrated acids for extracting xylan from hemicelluloses [4], whereas the proposed method does not use any environmentally-unfriendly processes. Moreover, high-purity X2 is more effective in prebiotic function than XOS. If high-purity X2 can be produced at a large scale using a more environmentally-friendly process at a lower cost, X2 has potential to become a major substitute of XOS. The method developed in this study can become the major method of producing high-purity xylobiose (X2) in the future.

Conclusions

In this study, we developed the upstream and downstream processes that can have sufficiently high efficiencies for the large-scale production of high-purity xylobiose (X2) from xylose (X1). First, it was found that the cloned β-xylosidase of *B. pumilus* IPO could be applied to the X1 → X2 reaction, in which the X2 reaction yield of 17% was attained 24 h after the reaction at 50 °C. Such X2 reaction yield and enzyme productivity were much higher than

those reported in the literature. To recover X2 from the reaction output with high purity and high recovery, an efficient downstream process that could be operated on a continuous-separation mode in favor of the large-scale X2 production was developed on the basis of a simulated moving bed (SMB) technology. The experimental results showed that the developed SMB process in this study could recover X2 from the reaction output with nearly 100% purity and 92% recovery. It was also confirmed that the unreacted X1 molecules could be recovered with 99% purity from the reaction output without any loss. The results of this study will help pave the way for establishing a large-scale production of high-purity X2 in an environmentally-friendly way.

Acknowledgments

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