공학박사 학위논문

Bioprocess optimization of xylobiose synthesis using β-xylosidase from *Bacillus pumilus*

바실러스 퍼밀러스 유래의 베타자일로시데이즈를 이용한 자일로바이오스 합성공정 최적화

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바이오시스템·소재학부 바이오시스템공학 전공

경 명 옥

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Kyung Myungok

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지도교수 김 용 노

이 논문을 공학박사 학위논문으로 제출함

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바이오시스템·소재학부 바이오시스템공학 전공

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경명옥의 박사 학위논문을 인준함

2019년 1월

위 원 장 김 기 석 (인)

부 위 원 장 김 용 노 (인)

위 원 윤 세 왕 (인)

위 원 유 상 호 (인)

위 원 김 영 완 (인)

ABSTRACT

A desirable sugar complement is a functional sweetener that plays a role in inhibiting sucrase in the small intestine when taken along with sucrose. Xylobiose, a disaccharide with two xylose molecules, is a major component of xylooligosaccharides and is known as the best prebiotic supplement, which can selectively stimulate the growth of *Bifidobacterium* species. Recently, xylobiose has attracted attention as a bifunctional sweetener that can be used as a sugar complement having sucrase inhibition potency as well as a prebiotic ingredient. However, to produce high-purity xylobiose, the purification process involves the hydrolysis product of xylan which is extracted from the biomass (such as straw and corn cob); however, the production process is complicated and the cost is too high for a food ingredient. In this study, we have established an environment friendly and simple production process that can produce xylobiose from low-cost xylose by exploiting the condensation reaction of beta-xylosidase and have identified the characteristics of such a synthetic xylobiose. For enzymatic xylobiose synthesis, selection of beta-xylosidase having high xylobiose formation efficiency, optimization of synthesis conditions, and characterization of synthesized xylobiose were performed.

Among twelve beta-xylosidases, beta-xylosidase from *Bacillus pumilus* IPO, which has the highest efficiency of xylobiose synthesis, was selected and an enzyme expression system was constructed in *Saccharomyces cerevisiae* for its use in the food industry. To increase the productivity of beta-xylosidase, C/N ratio, N-source type, and glucose feeding speed of the medium were considered in a fed-

batch fermentation strategy. Through fed-batch fermentation, beta-xylosidase production was increased to 717.3 U/L (productivity 18.3 U/g_{DCW}), which was 7.1 times higher than the production by batch culture methods. Over 70% plasmid stability of the beta-xylosidase-expressing strain was maintained during fermentation. Optimization conditions for xylobiose synthesis were derived from the central composite design of response surface methodology. Xylobiose was produced at a maximum of 214.0 g/L using 6.7 M of xylose with 6.5 U/mL of beta-xylosidase at 50℃ for 24 hr, which was the highest quantity of xylobiose synthesized among production procedures employing beta-xylosidase and 9.4 times higher than the quantity reported in previous studies that employed fungal beta-xylosidase. Additionally, synthetic reaction could occur without additives such as buffer solutions or high sorbitol concentration, and no additional steps were required to remove the additive. The synthesized xylobiose was a mixture of beta-1,4- (51.5%) > beta-1,3- (35.9%) > beta-1,2-linkage (12.6%). The synthetic xylo-disaccharides were characterized by excellent acid resistance and thermal stability, which remained at least 90% even after 90 min of exposure at 100℃ under strongly acidic condition (pH 2). They also had a potency to inhibit intestinal rat sucrase similar to beta-1,4-xylobiose ($p < 0.05$).

Therefore, xylobiose, synthesized using beta-xylosidase from *B. pumilus* IPO, could be used as a sugar complement for inhibiting the activity of sucrase as well as for providing a basis for industrial production by simplifying the process and increasing productivity. Furthermore, because synthetic xylobiose has excellent properties of acid resistance and thermal stability, it can be used as a multi-functional sweetener.

Keyword: Sugar complement, Xylobiose, Beta-xylosidase, Condensation reaction, Response surface methodology, Central composite design, Simulated moving bed chromatography, Xylo-disaccharides

Student Number: 2015-30382

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ABBREVIATIONS

ADI	Acceptable Daily Intake
BBD	Box-Behnken Design
CCD	Central Composite Design
DCW	Dry Cell Weight
DP	Degree of Polymerization
GI	Glycemic Index
OD	Optical Density
pNP	p-Nitrophenol
pNPX	p-Nitrophenyl-beta-D-Xylopyranoside
RSM	Response Surface Methodology
SMB	Simulated Moving Bed
XOS	Xylooligosaccharides
XynB	Beta-xylosidase from *Bacillus pumilus* IPO
XynB-E	XynB expressed in *Escherichia coli*
XynB-S	XynB expressed in *Saccharomyces cerevisiae*

1. INTRODUCTION

1.1. Research background

The number of processed foods is steadily increasing with advances in food processing technology and consumer demand. Owing to an increased intake of processed foods, consumers unintentionally ingest a large amount of added sugars (Van and Seidell. 2007, Yang et al. 2014).[①] Many researchers have reported that excessive intake of added sugars is associated with increased prevalence of obesity and metabolic diseases (Tordoff and Alleva. 1990, Bray et al. 2004, McEwen BJ. 2018). These days, many countries have started focusing on the health problems caused by excessive sugars consumption. In 2015, the World Health Organization (WHO) released a new guideline that strongly recommends reducing free sugars intake to < 10% of the total energy intake (World Health Organization. 2015). The Ministry of Food and Drug Safety (MFDS, Republic of Korea) announced sugar reduction policy in 2016. The goal is to reduce the intake of added sugars through processed foods to within 10% of the total daily calorie intake by 2020 in order to improve public health (Ministry of Food and Drug Safety. 2016).

According to Lux Research, sugar accounted for 78% of $73.4 billion worth global sweetener market in 2014. High-fructose corn syrup (HFCS) and high-intensity sweeteners (HIS) contributed to 12% and 8% of this market share, respectively. While low-intensity sweeteners (LIS) and natural sweeteners accounted for a small percentage of sweeteners with a market share of 1% each (Figure 1-1, Lux Research. 2014).

① Sugar refers to sucrose and sugars refer to a wide range of saccharides.

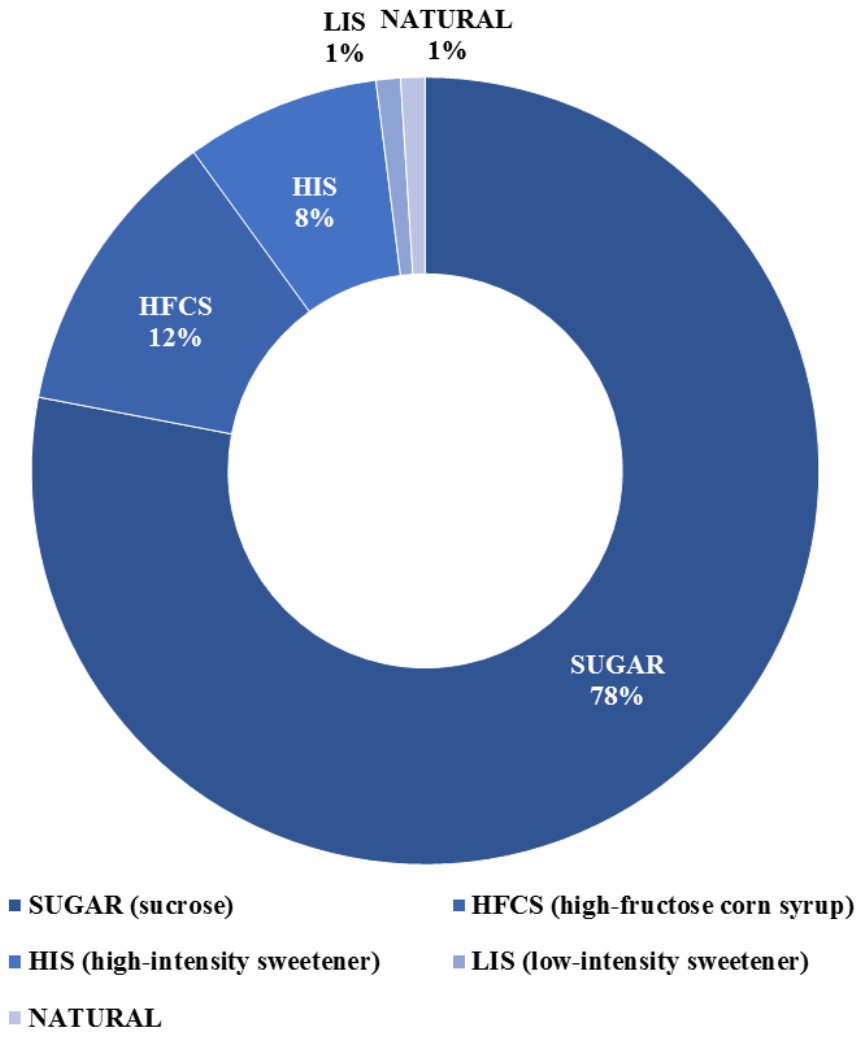


Figure 1-1. Global sweetener market (Lux research. 2014).

Sweeteners are classified as nutritive and non-nutritive. The predominant nutritive sweeteners are sugar (Sucrose, alpha-D-glucopyranosyl-1,2-beta-D-fructofuranoside) and high-fructose corn syrup (HFCS), which can be used as energy sources. Sugar is a natural sweetener made from sugar beet or sugar cane; it is the most widely used sweetener worldwide, and its sweetness quality is superior to that of any other sweeteners. In the last 5 years, the annual average of sugar consumption globally is maintained at > 160 million tons (United States Department of Agriculture. 2018). The annual domestic consumptions of sugar and HFCS are approximately 1 million tons and 0.3 million tons, respectively (Ministry of Food and Drug Safety. 2018).

Non-nutritive sweeteners have been developed to address the health risks associated with an increased intake of nutritive sweetener. They are alternatives to nutritive sweeteners, including zero- or low-calorie sweeteners such as sucralose, aspartame, saccharin, steviol glycosides, and sugar alcohols. The global market size of zero-calorie sweeteners was approximately $2 billion in 2016. Sucralose ($687 million), aspartame ($436 million), saccharin ($366 million), and steviol glycosides ($155 million) accounted for 80% of the total zero-calorie sweetener market (Figure 1-2, Technavio research. 2017).

Sucralose, one of the artificial high-intensity sweeteners, is 600 times sweeter than sugar. Sweetness quality and profile of sucralose are most similar to those of sugar. Aspartame and saccharin are representative artificial sweeteners and they are inexpensive. They are 200 times and 300 times sweeter than sugar, respectively. However, it is difficult to apply aspartame to various food products because aspartame has low thermal stability. Patients with phenylketonuria also

have a risk of neurological symptoms and should avoid consumption of aspartame-containing foods. Steviol glycosides, which are increasingly used in high-intensity sweeteners, are natural sweeteners extracted from stevia leaves. It is known to have sweetness approximately 200 times higher than that of sugar, but it has a bitter taste, and the appearance of sweetness is too late. Hence, steviol glycosides are mostly used in combination with other sweeteners (Shankar et al. 2013, Chattopadhyay et al. 2014, Edwards et al. 2016). However, the safety of high-intensity sweetener consumption remains controversial. High-intensity sweeteners may affect glucose homeostasis, evoking hunger signals, weight gain, and metabolic disturbance. Because high-intensity sweeteners are sweet and stimulate the digestive system, but they do not go through metabolic pathways as opposed to nutritive sweeteners. Recent studies have reported that high-intensity sweeteners affect waist circumference, glucose intolerance, insulin and glucagon-like peptide-1 secretion, microbiome, and hormones (Burke and Small. 2015, Suez et al. 2015, Soffritti et al. 2016, Sharma et al. 2016, Hess et al. 2018, Lertrit et al. 2018, Rother et al. 2018).

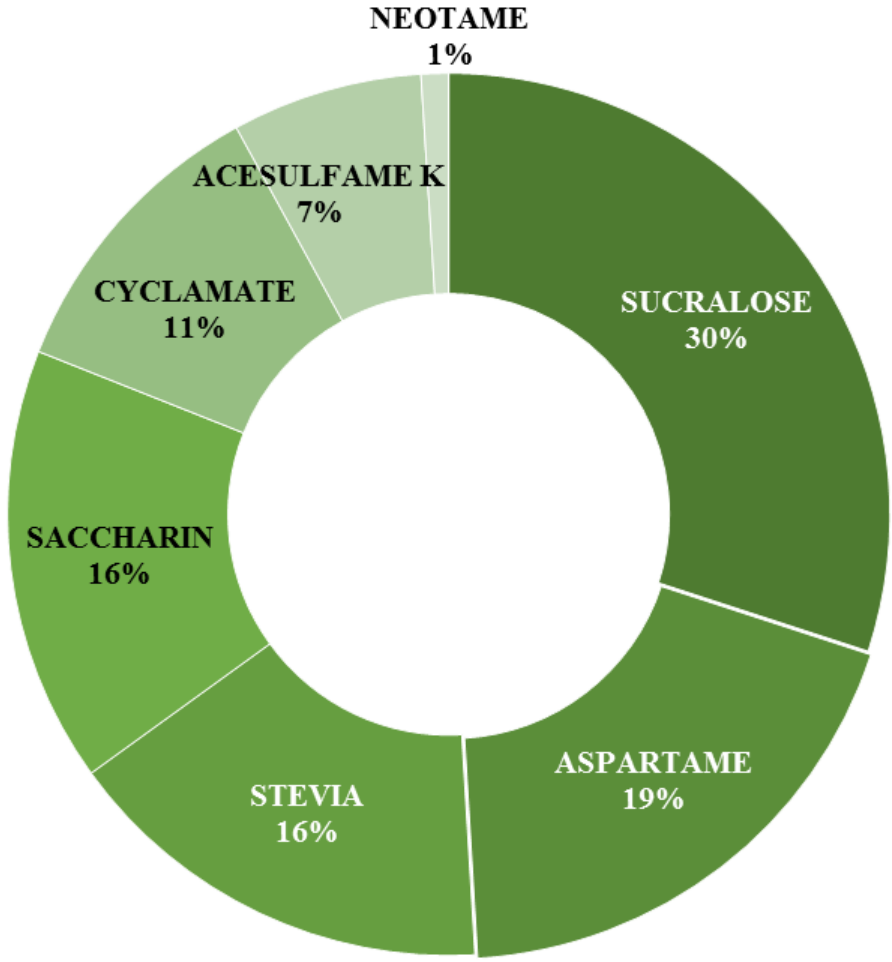


Figure 1-2. Global market share of zero-calorie sweeteners (Technavio research. 2017).

Low-intensity sweeteners contain lower calories than nutritive sweeteners (4 kcal/g). The major sugar alcohols are xylitol and erythritol. Xylitol is mainly used in chewing gum because it can inhibit the growth of oral bacteria and has sweetness similar to that of sugar. Erythritol contains lower calorie (0.2 kcal/g) than the other sugar alcohols (2.4 kcal/g), and its sweetness is 70% of that of sugar. Recently, several low intensity sweeteners, which are termed rare sugars, have been launched in the market. Although palatinose, tagatose and allulose are low-calorie sweeteners, they are expensive and relatively less sweet, and should be mixed with other high-intensity sweeteners. Hence, they have limitations in food industry applications. In addition, most of these low-intensity sweeteners can cause diarrhea when consumed excessive amounts (Mooradian et al. 2017). So far, dozens of alternative sweeteners have been developed but most sweeteners have low acceptable daily intake (ADI) because of their toxicity, disease prevalence, and side-effects. Humans have consumed alternative sweeteners for several decades. Long-term consumption of these substitutes is not sufficient to demonstrate safety. The most important characteristics of alternative sweeteners are sugar-like sweetness, low calorie, physicochemical stability, and safety. However, alternative sweeteners still cause side effects when consumed excessively, and their sweetness quality is also poor. There are no alternative sweeteners satisfies these request in the market yet.

Meanwhile, another attempt has been made to develop a functional sugar using a sugar complement to overcome the limitations of alternative sweeteners. Sugar complements are kinds of sweeteners that play a role in suppression of the elevation of blood glucose levels upon intake of pure sugar. They are required to

possess the physicochemical properties and sweetness like those of sugar. All sugar complements so far are pentose, such as arabinose, ribose, xylose, and xylooligosaccharides, as well as xylobiose. The types and characteristics of sugar complements are shown in Table 1-1.

Table 1-1. Clinical trial results of sugar complements

Sugar complement	Functionality	Additive ratio to sugar	Reference
L-Arabinose	Sucrase inhibition	4%	Shibanuma et al. (2011)
D-Ribose	Sucrase inhibition	10~14%	Kim et al. (2017)
D-Xylose	Sucrase inhibition	5~10%	Bae et al. (2011) Moon et al. (2012)
XOS powder [1)]	Sucrase inhibition Prebiotics	14~20%	Kyung et al. (2014) Nam et al. (2015) Jeon et al. (2015)
Xylobiose	Sucrase inhibition Prebiotics	10~14%	Lee et al. (2016) Lee et al. (2017)

[1)]: XOS powder contained 50% of anti-caking agent.

1.2. Research objectives

Sugar is the most widely used natural sweetener globally. It is used as a food material to introduce sweetness into many processed foods including cookies, breads, candies, chocolates, ice creams and beverages. Currently, excessive sugars consumption through processed foods has been considered to contribute to an increased risk of obesity and metabolic syndrome. Sugar complements, such as arabinose and xylose, are used to prevent health problems. It has sucrase-inhibiting effects and helps to maintain low blood glucose levels. Recently, xylooligosaccharides (XOS) and xylobiose have been recognized as a bifunctional food ingredient such as sucrase inhibitor and prebiotics. In addition, xylobiose-added sugar has the advantage of less hygroscopicity than XOS-added sugar. Therefore, Xylobiose is considered to be the best ingredient for sugar complements.

Xylan has to be used as raw material to produce xylobiose. It can be produced by chemical hydrolysis and ethanol precipitation from woody biomass. These xylan extraction processes have limitations to high production costs and large amounts of industrial waste. Although xylobiose is a good bifunctional sugar complement, it has not been produced as a food material to date. Some researchers have published studies involving xylobiose synthesis from xylose using enzymatic method, but the reaction takes more than a few days and xylobiose productivity is still low. Furthermore, optimal reaction conditions and purification processes for synthetic xylobiose have not yet been studied.

The main purpose of this research is to develop a process to synthesize xylobiose from D-xylose using an enzymatic method for environmentally friendly manufacturing of sugar complement.

The objectives of this study are as follows.

- Screening of high-efficiency beta-xylosidase producing wild-type strains for xylobiose synthesis and construction of cell lines that are generally recognized as safe (GRAS)
- Process development for the efficient production of beta-xylosidase by fed-batch culture
- Purification of beta-xylosidase and analysis of its characteristics
- Optimization of highly efficient xylobiose production through response surface methodology
- Applications of simulated moving bed chromatography for the separation process of synthetic xylobiose
- Identification of synthetic xylo-disaccharides and analysis of their characteristics

2. LITERATURE REVIEW

2.1. Sugar complements

2.1.1. Arabinose

L-arabinose is a saccharide comprising hemicellulose and aldopentose with an aldehyde group. Seri et al. (1996) reported on the inhibition potency of L-arabinose on porcine intestinal sucrase. The treatment of 10 mmol/L L-arabinose in 200 mmol/L sucrose inhibited sucrase activity by 65%. However, D-arabinose and L-arabinobiose were not effective in sucrase inhibition. In early 2000, sugar containing arabinose was first introduced in Japan. It has been reported that intake of sugar containing 4% arabinose contributes to the suppression of rapid increases in blood glucose levels as opposed to the intake of pure sugar in humans (Shibanuma et al. 2011). However, it was not implemented at a large scale production because arabinose yield, which was obtained from sugar beet pulp, was low and expensive to mass-production.

2.1.2. Ribose

D-ribose is an aldopentose that plays an important role as a constituent of DNA and RNA in vivo and is fermented from glucose by *Bacillus* species (De Wulf and Vandamme. 1997). D-ribose reportedly inhibits rat sucrase activity by as much as 70% in vitro (Asano et al. 1996). It has also been reported that unlike pure sugar, 10~14% D-ribose with sugar suppresses blood glucose levels in humans (Kim et al. 2017).

2.1.3. Xylose

D-xylose is an aldopentose monosaccharide that is a major component of xylan and is well-known source of xylitol. Tht treatment of 10 mmol/L D-xylose in 200 mmol/L sucrose inhibited sucrase activity by 58%. D-xylose has sucrase inhibitory effect similar to that of L-arabinose in vitro. However, compared with L-arabinose, D-xylose was found to be absorbed more rapidly in the small intestine after oral ingestion and was excreted 6.5 times more in the urine (Seri et al. 1996).

Sugar containing xylose was first developed in Korea (Park et al. 2007). XyloSugar™ (TS Corporation, Republic of Korea) and Xylose Sugar™ (CJ, Republic of Korea) were launched in the 2010. In clinical trials, the postprandial blood glucose and insulin levels were significantly lower in those who ingested 10% and 15% xylose-added sugar solutions than control group at 15, 30, and 45 min. However, there was no significant difference in the inhibitory effect of xylose concentration between those who ingested 10% and 15% xylose-added sugar solutions. The postprandial blood glucose area under the curve (0~120 min) was 21.4% smaller in the group taking 10% xylose-added sugar solution than the group taking the pure sugar solution (Bae et al. 2011). Moon et al. (2012) studied the effect of xylose-added sugar on glycemic index (GI) and suggested the appropriate xylose addition ratio.② GIs of 5 and 10% xylose-added sugar were 49.3 and 50.4, respectively, and classified as low GI foods (low GI ≤ 55). The inhibitory effects of postprandial blood glucose increase in those who ingested 5% and 10% xylose-

② Glycemic index (GI) was designed to reflect the absorption rate of carbohydrates. It is represented by comparing the degree of postprandial glucose response to the reference food with that to the test food (Jenkins et al. 1981).

added sugar were the same; thus, the recommended xylose addition ratio was 5%. GI of sugar with 5% xylose was approximately 30% lower than that of pure sugar. These products are being sold continuously in domestic and overseas markets.

2.1.4. Xylooligosaccharides

Xylooligosaccharides (XOS) is composed of 2~7 xylose units with beta-1,4-linkages, and is the most prevalent oligosaccharides with the least effective daily intake (0.7~7.5 g/day) among prebiotics (Ayyappan and Prapulla. 2011). XOS have been isolated by hydrolyzing biomass polysaccharides such as corn cob, cereal straw, and green coconut husk. It has been reported that XOS powder (XOS-MD), which is prepared using maltodextrin as an anti-caking agent, together with sugar, can inhibit a rapid increase in blood glucose levels. GIs of 14% and 20% XOS-MD-added sugar were 55.7 (56 ≤ medium GI food ≤ 69) and 48.0 (low GI food), respectively (Kyung et al. 2014). Nam et al. (2015) studied the GI of sugar added with XOS powder (XOS-RMD) using resistant maltodextrin as an anti-caking agent. GIs of sugar added 14% and 20% XOS-RMD were 54.7 and 52.5, respectively, thus classifying as low GI foods. XOS-MD and XOS-RMD comprised XOS and maltodextrin or resistant maltodextrin in 1:1 ratio. Considering that the content of pure XOS added to sugar is 7% or 10%, it can be considered to be similar to the suppression effect of blood glucose levels by sucrase inhibition of xylose. Young women who consumed 10 g/day of sugar with 0.7 g of XOS reportedly had increased bowel movements after one week, and their weekly bowel movements increased by more than twice (2.3/week → 4.8/week) after 4

weeks (Jeon et al. 2015). XylooligoSugar™ (TS Corporation, Republic of Korea) is a sweetener in which 14~20% XOS powder is mixed with sugar. It is the bifunctional sugar that can inhibit sucrase as well as improve constipation. However, XOS is expensive because its production process involves ethanol precipitation step for extracting xylan from woody materials. This manufacturing process is inefficient for industrial production owing to the need for complex chemical reactions and chromatographic purification of the mixture (McEwen et al. 1952, Akpinar et al. 2009, Samanta et al. 2014). In addition, XOS is composed of a mixture of oligosaccharides and it is a highly hygroscopic ingredient. Hence, manufacturing of XOS powder is disadvantageous owing to the involvement of anti-caking agents or moisture-proof packaging (Kyung et al. 2014, Nam et al. 2015, Zhang et al. 2018).

2.1.5. Xylobiose

Xylobiose is one of the components of xylooligosaccharides (XOS) and a disaccharide comprising two xylose molecules. It has gained popularity as a high-value functional sweetener with outstanding prebiotic functions (Jeong et al. 1998, Yuan et al. 2004, Lee et al. 2016). Xylobiose is known to have the highest prebiotic activity in the proliferation of intestinal *Bifidobacterium* species (Okazaki et al. 1990, Jeong et al. 1998). *Bifidobacterium adolescentis* uses xylobiose and xylotriose as energy sources. In addition, xylobiose is largely utilized by *B. infantis* and *B. longum*. However, most intestinal microbes do not use xylobiose. *Lactobacillus* species, *Bacteroides* species, as well as *Escherichia coli* and

Clostridium species, which are harmful bacteria, were found to be unable to use xylobiose as an energy source (Okazaki et al. 1990, Suwa et al. 1999).

Lee et al. (2017) reported that ingestion of xylobiose-containing sugar improves constipation in young women. The frequency of bowel movements in a group that ingested 10 g/day of 7% xylobiose-containing sugar increased steadily after sample ingestion and increased to 4 times/week at 6 weeks, which is twice the frequency at the before intake period. In addition, defecation amount, tenesmus, and abdominal pain were significantly improved, suggesting that intake of 0.7 g of xylobiose per day can improve constipation. In previous studies, it has been reported that the consumption of xylobiose-containing XOS increases not only the proliferation of beneficial bacteria in the intestines but also the production of organic acids, thereby inhibiting the proliferation of harmful bacteria. In addition, it has been shown that it maintains proper moisture content in the stool to help normal bowel movements (Tateyama et al. 2005, Childs et al. 2014). Therefore, xylobiose intake is considered to have excellent constipation-alleviating effect.

Another study has reported that xylobiose-containing sucrose has a suppressive effect on GI, which in turn leads to a decrease in postprandial blood glucose levels in healthy adults. Changes in blood glucose levels and GI after consuming 7%, 10%, and 14% xylobiose-containing sugar were significantly lower than those of associated with pure sugar intake. GI of 10% and 14% xylobiose-added sugar was classified as low GI food and lower than that of pure sugar (medium GI). Therefore, it was confirmed that xylobiose can be used as a sugar complement because it has an excellent effect of lowering sugar absorption into the body (Lee et al. 2016). Lim et al. (2016) reported that xylobiose supplementation

regulated miR-122a/33a expression in diabetic mice, diabetes-related metabolic control, and anti-inflammatory effects as well as decreased lipogenesis. The db/db mice were made to consume a diet comprising xylobiose which replacing only 5% of the sugar in the diet. The group to which xylobiose was given had lower levels of oral glucose tolerance, fasting blood glucose, insulin, and lipid metabolism (triglycerides, total cholesterol, and low-density lipoprotein cholesterol). In the liver, fatty acid synthetase, cholesterol-regulating enzyme, and oxidative stress marker levels were regulated along with a regulated expression of proinflammatory cytokines and tumor necrosis factor.

Recently, it has been reported that xylobiose can be a supplement to rats that are fed high-fat diet in order to inhibit fat accumulation and obesity-related metabolic diseases. The rats that were fed a high fat-diet with 10~15% xylobiose to substitute a portion of the sugar had a significantly decreased body weight, fat weights, fasting blood glucose, and blood lipid levels. Lipogenic, adipogenic, and beta-oxidation-related genes in mesenteric fat, proinflammatory cytokine levels, and lipolysis were downregulated in xylobiose-administered group. Therefore, xylobiose can facilitate the prevention of obesity because it has the ability to inhibit fat accumulation and obesity-related metabolism (Lim et al. 2018).

Xylobiose has been recognized as a bifunctional food ingredient such as sucrase inhibitor and prebiotics. In addition, xylobiose-added sugar has the advantage of less hygroscopicity than XOS-added sugar. Therefore, Xylobiose is considered to be the best ingredient for sugar complements.

2.2. Xylobiose production

Xylobiose is the best ingredient of the bifunctional sugar complement that has been developed. However, a method for the industrially production of xylobiose has not yet been developed. So far, xylobiose should be obtained by refining the hydrolyzate of xylan extracted from biomass, but it has not been used as a food material because of its complicated manufacturing process and high production cost (Jeong et al. 1998, Jiang et al. 2004, Tan et al. 2008, Lakshmi et al. 2012). These methods also had the inherent problem with the request for the raw material. As environmental regulations are strengthened, it is difficult to implement methods that generate large amounts of environmental waste. Therefore, it is necessary to develop an eco-friendly production method of xylobiose as a bifunctional sugar complement.

2.2.1. Chemical hydrolysis

Xylobiose can be obtained via a purification process using hydrolyzed XOS in combination with chemical and enzymatic methods. Some researchers have developed enzymatic digestion methods for increasing xylobiose content using extracted using xylan isolated from hemicellulose (Jeong et al. 1998) as well as strategies for separating xylobiose from XOS (Choi et al. 2016). However, depending on the type of lignocellulosic materials, hemicellulose content ranges between 2~35%. To produce xylobiose-containing XOS, only raw materials capable of obtaining high quality xylan can be used, which remains to be a challenging limitation (Alonso et al. 2003). To prepare xylan, deacetylation is

performed by subjecting ground lignocellulosic material to high-temperature steam treatment as well as to a treatment with acidic and alkaline solutions (Nabarlatz et al. 2005, Akpinar et al. 2007). Subsequently, xylan is extracted using the ethanol precipitation method. Further, xylan is hydrolyzed using enzymatic treatment, after which the oligosaccharides are separated using size-exclusion chromatography (Teleman et al. 2000).

2.2.2. Chemical synthesis

The initially followed chemical process for xylobiose synthesis is limited owing to an inability to easily produce xylobiose or its derivative in a large scale. However, the synthesis method developed by Aspinall and Ross (1961) was able to provide a high yield of D-xylobiose and its derivatives. This method utilizes the stereoselective nucleophilic opening of the anhydrous ring of the alkyl beta-D-ribopyranoside followed by stepwise condensation of bromosugar, acetylation, and deacetylation. An important step is the condensation of 2,3,4-tri-O-acetyl-alpha-D-xylopyranosyl bromide with benzyl 2,3-anhydro-3-D-ribobranoside (KOVÁČ P. 1979). The chemical synthesis method enabled the synthesis of various xylobiose derivatives efficiently, and it was used to study the mechanism of enzymatic reaction. However, chemically synthesized xylobiose should only be used for research purposes owing to the involvement of various toxic chemicals.

2.2.3. Enzymatic synthesis

Some researchers have investigated the synthesis of xylobiose using high-concentration xylose and fungal beta-xylosidase (Iizuka et al. 1993, Katapodis et al. 2006, Guergali et al. 2008). Iizuka et al. (1993) reported that xylobiose was synthesized using beta-xylosidase from *Aspergillus niger* and *Malbranchea pulchella*. Beta-xylosidase from *A. niger* was added to a 60% xylose solution and reacted at 50℃ for 14 days, resulting in approximately 12% of xylo-disaccharides production. Beta-xylosidase from *A. niger* produces beta-1,1-; beta-1,2-; beta-1,3-; and beta-1,4-xylobiose, whereas beta-xylosidase from *M. pulchella* did not produce beta-1,3-xylobiose. Beta-xylosidase from *Sporotricum thermophile* reportedly produced approximately 1% of xylobiose when mixed in 60% xylose at 40℃ for 28 days (Katapodis et al. 2006). Guerfali et al. (2008) observed sorbitol addition-based differences in xylobiose yield using beta-xylosidase from *Talaromyces thermophilus*. It was reported that 5.65% xylobiose was synthesized when 3M sorbitol was added to 40% xylose and reacted at 40℃ for 3 days. There is a 10-fold increase in yield as compared to the yield obtained in the absence of sorbitol. However, the removal of sorbitol after the reaction remains challenging. Previous studies have shown that not only is the yield of the synthesized xylobiose very low but also that the reaction time is too long. To use xylobiose as a functional material in food industry, it is necessary to screen an enzyme for high synthesis efficiency.

2.3. Beta-xylosidase

Beta-xylosidase (EC 3.2.1.37) is a beta-1,4-xylosyl linkage hydrolytic enzyme called xylan 1,4-beta-xylosidase. This enzyme has the property of hydrolyzing D-xylose from the non-reducing end. The main role of beta-xylosidase is to degrade soluble xylooligosaccharides (XOS). Howard (1957) reported that the cell extracts of bacteria isolated from sheep rumen could hydrolyze wheat flour pentosan to yield arabinose, xylose, and XOS (xylobiose, xylotriose, xylotetraose and xylopentaose), and found that beta-xylosidase was present in the mixture extracted from bacteria. Most microorganisms are known to possess beta-xylosidase, which helps them utilize the biomass material as a nutrient source (Bajpai P. 1997). Beta-xylosidase in microorganisms is bounded to the cell wall or secreted extracellularly because microorganisms can only utilize biomass broken down into the monosaccharide or disaccharide form (Biely P. 1985, Lenartovicz et al. 2003).

2.3.1. Classification of beta-xylosidases

The Carbohydrate active enzymes database has classified beta-xylosidase into glycoside hydrolases (GH) 3, 30, 39, 43, 52, 54, and 120 based on the amino acid sequence similarity (CAZy, http://www.cazy.org/). Fungal beta-xylosidases have been reported to belong mainly to GH 3, 43, and 54 (Ito et al. 2003, Wakiyama et al. 2008). Bacterial beta-xylosidase was found to belong to GH 3, 39, 43, 52, and 120. GH families with fugal and bacterial beta-xylosidase followed a retaining catalytic mechanism, except for the GH 43 (inverting catalytic

mechanism). The fungal hemicellulose hydrolase of this group is generally industrially useful because it does not perform transglycosylation (Song and Jacques. 1999, Yanase et al. 2002).

2.3.2. Properties of beta-xylosidase

Beta-xylosidase, which hydrolyzes xylan, has been found to be produced by many molds and bacteria. Among them, fungal beta-xylosidase has been studied extensively, and these enzymes reportedly showed to vary in terms of their purification, mode of action, properties and activity (Wong et al. 1988, Uffen 1997, Katapodis et al. 2006). In general, fungal beta-xylosidase showed optimal activity in the range of 30~60℃ (Pedersen et al. 2007), and the beta-xylosidase extracted from *Aspergillus phoenicis* reportedly exhibited optimal activity at 75℃ (Rizzatti et al. 2001). Although beta-xylosidase enzymes extracted from most fungi exhibited an optimal activity under weak acidic conditions (pH 4.0~6.0), beta-xylosidase from *Penicillium sclerotiorum* showed an optimal activity at pH 2.5 and that extracted from *Talaromyces thermophilus* showed an optimal activity at neutral pH 7.0 (Katapodis et al. 2006, Guerfali et al. 2008). Many fungal beta-xylosidases have broade substrate specificity. They reportedly exhibit alpha-L-arabinofuranosidase activity and also carry out hydrolysis of beta-xylosidase (Andrade et al 2004, Eneyskaya et al 2007, Yan et al. 2008).

The hydrolysis mechanisms of beta-xylosidase are classified into two types: inverting and retaining glycoside hydrolysis. Inverting hydrolysis is a process in

which two carboxylic side chains of the enzyme act as acids and bases, respectively, and the water molecule is directly involved in substrate degradation. On the other hand, retaining hydrolysis proceeds in two steps. First, glycosylation occurs in which the anomeric center of the substrate and the enzyme form an intermediate, then the deprotonated water molecule hydrolyzes while attacking (deglycosylation) the anomeric center (Sinnott 1990, Zechel and Withers 2000).

2.3.3. Industrial applications of beta-xylosidase

Beta-xylosidase is widely used in the conversion process during bioethanol production, pulp and paper industry, food ingredients production, and synthetic materials for research. Usually, fungal beta-xylosidase is more advantageous than that obtained from other microorganisms because of its high stability and low commercial production cost (Eneyskaya et al. 2003). Beta-xylosidases produced from *Trichoderma reesei* and *Humicola insolens* are the most widely used enzymes commercially (Sunna and Antranikian 1997). Over > 40 million tons of lignocellulosic biomass waste, which is one of the types of waste produced during agricultural and industrial activities, is being generated worldwide every year. Massive biomass wastes substantially increase environmental pollution and economic losses (Cano and Palet 2007). Biomass contains a large amount of renewable hemicellulose, and can be used as food, feed and energy sources, especially when xylan is being converted to XOS or xylose (Minic et al. 2004).

The most common method adopted for converting lignocellulosic biomass waste into renewable resources is hydrolysis using acids or enzymes. Buchholz et

al. (1981) developed a process for efficiently hydrolyzing lignocellulosic material via acid hydrolysis. However, this process reportedly produces toxic substances such as microbial growth inhibitors, furfural, and hydroxymethylfurfural as well as cause lignin degradation and generates a large amount of industrial waste water, in turn leading to environmental pollution. In addition, the acid hydrolysis process requires high-temperature, high-pressure, corrosion-resistant special devices and is associated with high operating costs and low economic efficiency (Ladisch 1979). On the other hand, enzymatic lignocellulosic material hydrolysis can selectively produce monosaccharides in mild conditions. In addition, this process has attracted much attention regardless of a low yield, because toxic materials are not generated and renewable resource production is possible in an environmentally friendly manner (Reilly 1981, Biely 1985).

Bioethanol production is commonly focused upon by industries that develop renewable resources using biomass waste. The acid treatment process as well as xylanase and beta-xylosidase reactions are applied in combination to obtain sugars such as xylose, which can be fermented by microorganisms using biomass waste. Bioethanol conversion is generally carried out via fermentation processes using such as *Pichia stipitis* and *Candida shehatae* (Screenath and Jeffries 2000, Katahira et al. 2004). In addition, xylanolytic enzymes such as xylanase and beta-xylosidase have been used as important raw materials in the pulp and paper industries (Biely 1985, Viikari et al 1994, Medeiros et al 2003, Polizeli et al 2005). The use of xylanolytic enzymes in the pulp pre-treatment process reportedly reduces the use of chlorine compounds by 30%, which in turn leads to a 20% reduction in the generation of toxic organochlorine-containing industrial waste water (Sunna and

Antranikian 1997, Raghukmar et al. 2004).

In the food industry, beta-xylosidase is used in the production of xylose and XOS. XOS is a sweetener in which 2~7 xylose units are composed of beta-1,4-linkages. Xylan can be extracted by employing physicochemical processes and then converted into oligosaccharides using xylanases and beta-xylosidases. Unlike the xylose production process, the process of separating intact xylan from biomass is complicated and results in the generation of a large amount of industrial waste; hence, the production amount is limited. Contrastingly, when a physicochemical method and acid hydrolysis are employed as the predominant processes, a majority of the xylose concentration reacts with beta-xylosidase, thereby increasing xylose production efficiency. Because xylose is relatively easier to produce than XOS, xylose is widely produced as a food material and commonly used as xylitol production (Parajo´ et al. 1998, Saha 2003). Beta-xylosidase is used in the clarification process to improve the final yield by increasing the ease of extraction in the juice-manufacturing process (Polizeli et al. 2005). In the brewing industry, beta-xylosidases and xylanases are used to lower viscosity and turbidity by carrying out hydrolysis of wheat flour. Moreover, the quality of wines can be improved by beta-xylosidase addition owing to the release of aroma and pigments from grape fiber (Manzanares et al. 1999). Furthermore, addition of beta-xylosidase to the feed mix administered to animals helps improve their nutrition levels by facilitating an increased degradation rate of grains or plants (Twomey et al. 2003, Polizeli et al. 2005).

2.4. Production of foreign proteins by *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is one of the generally recognized as safe (GRAS) microorganisms that has long been used in food and pharmaceutical industries for the production of heterologous proteins. *S. cerevisiae* is the first eukaryotic genome to be fully sequenced (Goffeau et al. 1996). It is easy to genetically manipulate yeast for expressing recombinant proteins that can be cultivated at a high-density cells depending on the expression system and culturing conditions used, without producing any harmful substances such as pyrogens, pathogens or viral compounds (Böer et al. 2007).

2.4.1. *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a commonly use microorganism in bread and wine making, which has been biotechnologically exploited to express a variety of heterologous recombinant proteins for decades. Selection of an expression system and its various aspects, such as plasmid copy number, promoter, selection marker, and signal sequence, is important to express foreign protein using *S. cerevisiae* as a host cell. Most yeast plasmids are shuttle vectors that can replicate in both *E. coli* and yeast, and their plasmid copy numbers are high. Yeast vectors contain a 2-micron vector based on an autonomously replicating sequence (ARS) or an integration vector that can insert a foreign gene into the chromosomal DNA of the yeast (Böer et al. 2007).

Genes related to amino acid synthesis, such as HIS4 (histidine), LEU2 (leucine), LYS2 (lysine), TRP1 (tryptophan), or URA3 (uracil) are used as markers for screening recombinant *S. cerevisiae*. These auxotrophic selectable marker-inserted strains have the disadvantage of using nutrient-deficient synthetic media; however, fed-batch cultures can regulate plasmid stability using C-source deficiency while using nutrient media (Rine J. et al. 1991, Zhang et al., 1996).

Promoters play an important role in regulating the expression of foreign proteins, and there are constitutive and inducible promoters. Strong constitutive promoters include alcohol dehydrogenase (ADH), glyceraldehyde-3-phosphate dehydrogenase (GPD, TDH), and phosphoglycerate kinase (PGK). Inducible promoters include galactose inducible (GAL) and acid phosphatase-regulated (PHO) systems (Mumberg et al. 1995, Partow et al. 2010). In addition, two promoters are used in combination to increase the expression level of foreign genes. Glucose oxidase from *Aspergillus niger* was inserted into the recombinant *S. cerevisiae* using the ADH2-GPD hybrid promoter, resulting in the expression of 9 g/L (Park et al. 2000).

Recombinant yeast is widely used in the production of foreign proteins because of its ease of gene operation, development of expression system, and ability to cultivate high-density cells. Hepatitis B is the first vaccine to be produced in yeast to combat the associated viral infections (McAleer et al. 1984). In addition, insulin, glucagon, hepatitis B surface antigens, antithrombin III, and human hemoglobin have been produced using *S. cerevisiae* (Demain and Vaishnav. 2009). A maximum of 320 mg/L of antithrombin III has been produced (Mallu et al. 2016), and the maximum production of human hemoglobin is 7% or more of the total cell

protein (Martinez et al. 2015).

2.4.2. Fed-batch fermentation

Yeast is more competitive in culture than other microorganisms because it tends to be more resistant to external stresses, such as low oxygen demand, during cultivation and owing to its ability to produce and re-consume ethanol depending on culture conditions. To improve the productivity of recombinant protein in yeast, process parameters including medium composition, C-source/N-source ratio, plasmid stability, temperature, pH, agitation, aeration, and medium feeding strategy should be considered (Celik and Calık. 2012). Fed-batch fermentation is widely used industrially to increase the productivity of recombinant proteins; this technique can prevent the accumulation of by-products such as ethanol and acetic acid and efficiently increase productivity via high-density cell cultivation (Zabriskie DW. 1987). However, the recombinant yeast cultured in a non-selective medium may exhibit plasmid instability. When culturing is prolonged, it proves to be disadvantageous because the total productivity decreases owing to an increase in the number of plasmid-free cells (Caunt et al. 1988). There are three reasons contributing to plasmid instability. First, there are defects in the system for distributing plasmid DNA during cell division. Second, the DNA of the inserted plasmid is structurally unstable; hence, its structure changes through deletion and rearrangement. Finally, the selection of plasmid-carrying cells is dependent on the ability of these cells to return to a cell state free of plasmids as well as on their ability to be genetically stable in non-selective media (Seo and Bailey. 1985). In

general, fed-batch cultures are maintained by controlling the growth and production stages of recombinant yeast as a strategy to overcome plasmid instability (Lee and Hassan. 1988, Hortacsu and Ryu. 1991). Fed-batch fermentation usually regulates the glucose feeding point through continuous glucose monitoring or by using off-gas monitoring system (Kangan et al. 1991). A fed-batch strategy to mitigate plasmid instability is to maintain a low glucose levels in the culture medium or to induce glucose starvation for a certain period (Huang et al. 1991, Saint et al. 1994).

2.5. Response surface methodology

Design of experiments (DOE) is the planning of experiments to collect information from variables. Planning is important for obtaining the maximum information from a minimum number of experiments in order to efficiently solve a problem. Response surface methodology (RSM) is a statistical analysis method that predicts the outcome at any variable level and optimizes the level of the variable to achieve the desired results. In particular, RSM can visually confirm results that vary depending on the degree of different variables by forming a two- or three-dimensional surface. The process of RSM occurs in three steps. First, the main factors that are considered to influence the response value need to be selected. Next, the experiment is performed using the Box-Behnken Design (BBD) or the Central Composite Design (CCD), and the conditions of the variables that optimize the reaction value in the region of interest are confirmed. Finally, it compares the predicted value with the actual result after conducting the experiment at optimized conditions using the model derived from RSM.

The maximum or minimum point on the surface obtained using RSM can be found and set as an optimal condition (Myers and Montgomery. 1995). These experimental design methods are based on a quadratic polynomial using the least-squares method. The BBD method is efficient because it can be involves a small number of experiments using only three levels; and these experiments can also be easily arranged and interpreted as opposed to those associated with other methods. CCD requires more experiments than BBD, but provides a more optimized value owing to the involvement of a higher number of experiments and is also more reliable (Ferreira et al. 2007, Martins et al. 2011).

2.6. Simulated moving bed

Simulated moving bed (SMB) separation process has gained popularity as a purification technique for fine chemical materials such as those used in pharmaceutical and biochemical industries. Nowadays, it is widely used in various industries. SMB is based on the principle of chromatography, usually in the form of a series of multiple columns containing adsorbents. SMB basically consists of two inlet ports for injecting feed and a desorbent, an extract port for discharging a substance having a relatively strong affinity for the adsorbent, and a raffinate port for releasing a substance having a relatively low affinity (Pais et al. 1998). Figure 2-1 shows a schematic illustration of the four-zone SMB unit (Choi et al. 2016).

SMB is characterized by the fact that the adsorbent is not actually moved and the flow of the valve connected to the column is periodically shifted in the same direction as the flow direction of the mobile phase; this ensures continuous low of the feed and separation of the target product. The number of columns used for SMB chromatography is known to be up to 32; this technique requires four to five pumps, and the connection of each column is equipped with a valve that can continuously change the flow of the mobile phase. In addition, pumps and valves require very precise control; hence, automatic control using a computer is required. Separate samples can be recovered through the valves attached to the corresponding column connections (Lee et al. 2005, Hur and Wankat. 2006, Suh et al. 2007). The most important advantage of SMB is that a continuous process can be performed and high-purity products can be obtained.

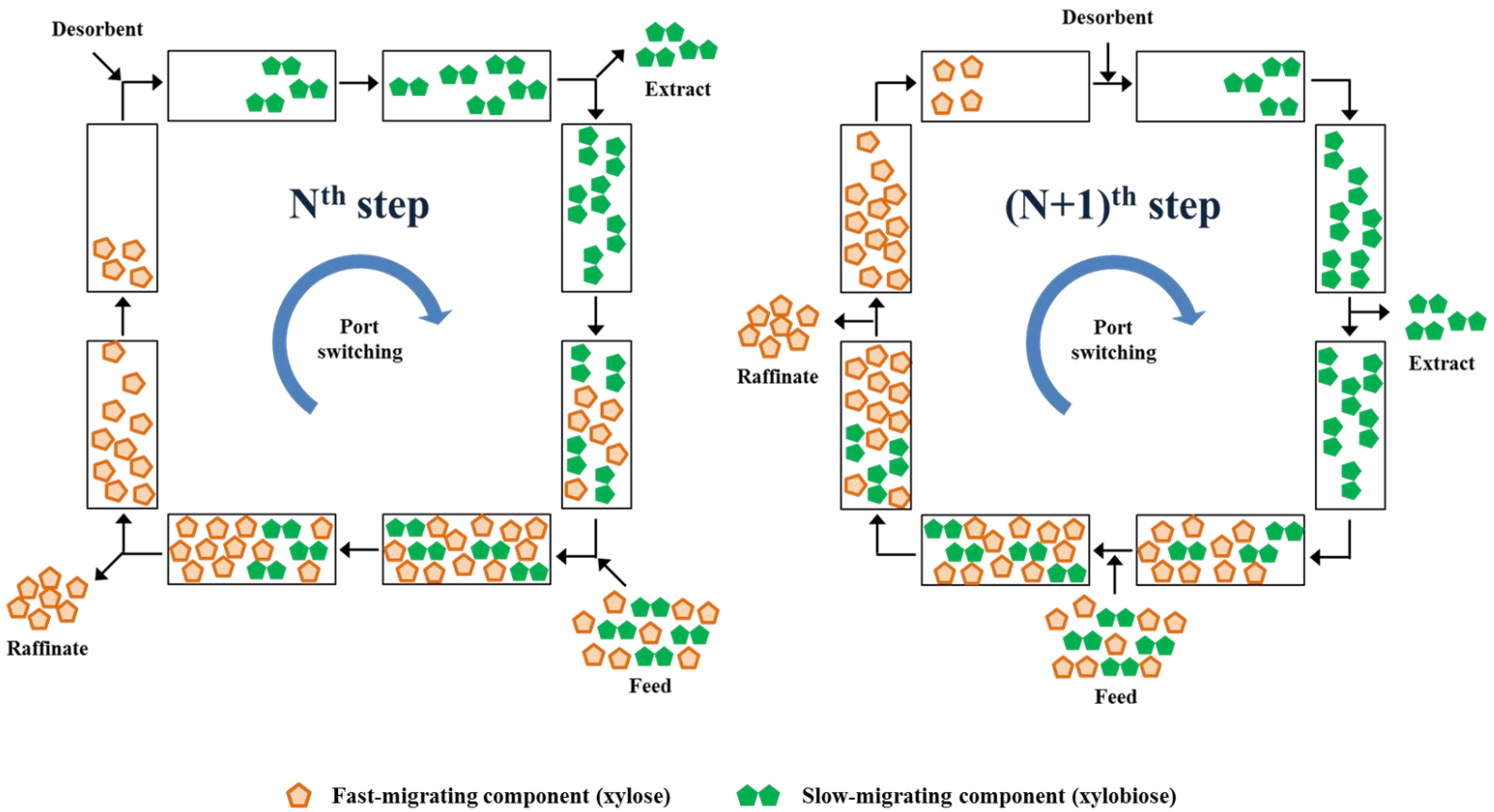


Figure 2-1. Schematic illustration of four-zone SMB unit (Choi et al. 2016).

3. MATERIALS AND METHODS

3.1. Construction of beta-xylosidase expression system

3.1.1. Cloning of beta-xylosidase from various microorganisms in *Escherichia coli*

To obtain beta-xylosidase with high xylobiose synthetic efficiency, candidates were selected through literature reviews. Twelve beta-xylosidases from various microorganisms were sorted by Glycoside Hydrolase family (Table 3-1). Nine microorganisms were purchased and genomic DNA was extracted from each microorganism grown using AccuPrep® Genomic DNA Extraction Kit (BIONEER, Republic of Korea). The 1,608 bp nucleotide sequence of beta-xylosidase from *Bacillus pumilus* IPO (Xu et al. 1991) was synthesized at BIONEER (Republic of Korea). Each primer pairs-DNA template was amplified with AccuPower® Taq PCR PreMix (BIONEER, Republic of Korea). After then, amplified products were separated by agarose gel electrophoresis and confirmed the DNA band by Digital Gel Documentation System (GDS-200D; Korea Lab Tech, Republic of Korea). Gel slice of target DNA was purified using AccuPrep® Gel Purification Kit (BIONEER, Republic of Korea). Each DNA fragment of twelve beta-xylosidase was ligated into the linear pET21a vector at 4℃ for 12 hr.

Table 3-1. Beta-xylosidase in various microorganisms and PCR primer sequences

DNA of beta-xylosidase (Origin)	Strain No.	Gene size (bp)	Restriction site	Primer sequence (5′ → 3′)	Tm (℃)
FgXyl3A (*Fervidobacterium gondwanense*)	DSM13020	2,328	BamH I Hind III	F: CGGGATCC ATG GAG ATA TAT AAG GAT TCT TC R: CCCAAGCTT GAA AGT GTA AAC TTT TGT AAA GAA	60
XynD (*Caldicellulosiruptor saccharolyticus*)	DSM 8903	1,311	Hind III Xho I	F: CCCAAGCTT ATG GGG GAA GGT ATA GTG AC R: CCGCTCGAG CCC CAT TTG GAT CCA AGT AC	60
XynB (*Thermoanaerobacterium saccharolyticum*)	ATCC49915	1,503	BamH I Hind III	F: CGGGATCC ATG ATT AAA GTA AGA GTG CCA G R: CCGCTCGAG ATA TCC ATT TAT CTT GCT ATC AT	60
Xsa (*Bacteroides ovatus*)	KCTC5827	975	BamH I Xho I	F: CGGGATCC ATG AAA ACA GAA AAA AGA TAT TTA G R: CCGCTCGAG TTC ATC TTT TCC CTC GAT AGT A	60
XynB (*Bacillus pumilus IPO*)	A synthetic	1,608	BamH I Xho I	F: CGCGGATCCATGAAGATTACTAATCCAGTGC R: CCGCTCGAGTTCAGTTGTTTCTTCGTATCG	53
XynB (*Bifidobacterium adolescentis*)	KCCM11206	1,629	BamH I Xho I	F: CGGGATCC ATG AAG ATT TCC AAC CCG R: CCGCTCGAG CTG GTT ATC GGA AAG CTC	53
XynD (*Bifidobacterium adolescentis*)	KCCM11206	1,035	EcoR I Xho I	F: CGGAATCC ATG AGC ATC GAC ACC CAG G R: CCGCTCGAG CAG GGC GAA GGC GGT TTC	60
XynB1 (*Lactobacillus brevis*)	ATCC14869	1,623	BamH I Xho I	F: CGGGATCC ATG AAG ATT CAA AAT CCA G R: CCGCTCGAG ATC TGG CAA CTC CTG ATA	51
XylC (*Paenibacillus woosongensis*)	KCTC 3953	1,425	BamH I Xho I	F: CGGGATCC ATG ACA AAA CAA GGT TTG AAT C R: CCGCTCGAG TTC CAG CGT GAA CGA GGC	59
Xsa (*Selenomonas ruminatium*)	ATCC12561	1,617	Hind III Xho I	F: CCCAAGCTT ATG AAC ATT CAA AAT CCC GT R: CCGCTCGAG GTC CAG TTC CTT GTA AGT GA	54
XylD (*Thermoanaerobacterium saccharolyticum*)	DSM8691	2,040	Hind III Xho I	F: CGGGATCC ATG ATA AGT AAA TCT TTT TAT GCG R: CCGCTCGAG TTT CAT CCA CAA TAC ACT TGT C	60
XylC (*Thermoanaerobacterium saccharolyticum*)	DSM8691	1,917	Hind III Xho I	F: CGGGATCC ATG GAA TAC CAT GTG GCT AAA A R: CCGCTCGAG CCA AAC TTT TAT GTA ATT ATT TCC	60

3.1.2. Transformation into *Escherichia coli*

The transformation refers to the protocol of NEB® 5-alpha competent *E. coli* (NEB, USA). Fifty μL of DH5-alpha competent cell thawed on ice and 2 μL of the ligated mixture was gently mixed and placed on ice for 30 min. Competent cell and ligation mixture were heat shocked at 42℃ for 30 sec, 950 μL Luria-Bertani (LB) medium was added and incubated at 37℃ for 60 min with shaking (250 rpm). Then, 50 μL of transformation mixture was spreaded on LB plate containing 100 μg/mL of ampicillin (Sigma-Aldrich Co., USA) and incubated overnight at 37℃. Transformed colony was cultured in 10 mL LB medium containing 100 μg/mL of ampicillin at 37℃ for 24 hr with shaking (200 rpm) and cells harvested by centrifugation (7,000 ×g, 4℃, 10 min). Each recombinant vector was prepared using AccuPrep® Plasmid Mini Extraction Kit (BIONEER, Republic of Korea). The prepared vector was transformed into NEB® BL21 (DE3) competent *E. coli* (NEB, USA) for beta-xylosidase expression.

3.1.3. Selection of beta-xylosidase for xylobiose synthesis

The recombinant *E. coli* was cultured in LB medium containing 100 μg/mL of ampicillin at 37℃ with shaking (200 rpm). When the OD600 of the culture medium became 0.8, IPTG (BIONEER, Republic of Korea) was added to a final concentration of 0.5 mM and culture was continued at 20℃ for 16 hr with shaking (100 rpm). The cells were harvested by centrifugation (7,000 ×g, 4℃, 10 min) and cell pellets were resuspended with 50 mM sodium phosphate buffer (pH 7.0), then

the cells were disrupted by sonication (VC-505P, SONICS & MATERIALS INC., USA). The crude extract was centrifuged (10,000 ×g, 4℃, 10 min) and supernatant was filtered through a 0.22 μm Minisart® RC filter (Sartorius, Germany). It was used for evaluation of xylobiose synthesis. Selection of beta-xylosidase was carried out by condensation reaction in 4M (60%, w/v) xylose with 50 mM sodium phosphate buffer (pH 7.0). Beta-xylosidase (0.1 U/mL) was added to a substrate solution, and then reaction mixture was incubated at 50℃ for 24 hr. The reaction was stopped by heat inactivation at 99℃ for 15 min. The amount of xylobiose was analyzed by HPLC with RID (Agilent 1100 series, USA). The mobile phase was used deionized distilled water (DDW) and flow rate was 0.5 mL/min at 80℃. The analytical column was used Waters Sugar-Pak I (6.5 mm × 300 mm, 10 μm).

3.1.4. Vector construction by different promoters into *Saccharomyces cerevisiae*

The nucleotide sequences of four promoters (ADH1, CYC1, TDH3, and TEF1) were obtained at NCBI site (https://www.ncbi.nlm.nih.gov/). These promoter genes were synthesized at BIONEER (Republic of Korea). Vector construction was performed using the p426GPD vector as a backbone. Each vector was ligated by replacing the GPD promoter. Promoter primer sequences are shown in Table 3-2. Six recombinant vectors were transformed into NEB® 5-alpha competent *E. coli* (NEB, USA), respectively. Each transformed colony was cultured in 10 mL LB medium containing 100 μg/mL of ampicillin at 37℃ for 24 hr with shaking (200 rpm) and cells harvested by centrifugation (7,000 ×g, 4℃,

10 min). Each recombinant vector was prepared using AccuPrep® Plasmid Mini Extraction Kit (BIONEER, Republic of Korea).

Table 3-2. Promoter primer sequences for vector construction in *S. cerevisiae*

Promoter	Sequence (5′ → 3′)
ADH1	F: CGA GCT CGC ATG CAA CTT CTT TTC R: CGGGATCCACTAGTTCTAGAAA
CYC1	F: CGAGCTCATTTGGCGAGCG R: CGGGATCCACTAGTTCTAGATTA
GAL1	p426GAL1 vector
GPD	p426GPD vector
TDH3	F: CGAGCTCAGTTTATCATTATC R: CGGGATCCACTAGTTCTAGAA
TEF1	F: CGAGCTCATAGCTTCAAAATG R: CGGGATCCACTAGTTCTAGAAA

3.1.5. Transformation into *Saccharomyces cerevisiae*

Six recombinant vectors containing the codon optimized beta-xylosidase gene were transformed into *Saccharomyces cerevisiae* (D452-2) using the Lithium-Acetate transformation method (Soni et al. 1993). *S. cerevisiae* D452-2 was cultured in 10 mL of YPD medium (glucose 20 g/L, yeast extract 10 g/L, peptone 20 g/L) at 30℃ for 24 hr with shaking (200 rpm). Seed culture broth (10 mL) was inoculated into 90 mL of YPD medium and cultured at 30℃ with 200 rpm. When the OD600 of the cells reached 0.7 to 1.2, they were harvested by centrifugation (7,000 × g, 4℃, 10 min). The pellet was resuspended with 5 mL of Lithium-Acetate mixture (500 mM Lithium acetate in 100 mM Tris-EDTA, pH 7.0) and 1 mL of cell suspension was transferred into 2 mL micro-tube. Then, the cell suspension was centrifuged at 7,000 × g (4℃, 30 sec) and the supernatant was discarded (Repeat 2 times). The carrier DNA (Deoxyribonucleic acid sodium salt from salmon testes, Sigma-Aldrich Co., USA) thawed on ice was boiled for 5 min and immediately cooled on ice. Sixt60 μL of competent cell resuspended with lithium acetate mixture, 5 μL of Carrier DNA, 5 μL of Plasmid, and 300 μL of 40% PEG 3500 (Sigma-Aldrich Co., USA) were transferred to a micro-tube and vortexed sufficiently. Competent cell and ligation mixture were placed at 30℃ for 30 min and heat shocked at 42℃ for 30 sec. Transformation mixture was resuspended with 1 mL of DDW and centrifuged at 7,000 × g (4℃, 30 sec), and the supernatant was discarded (Repeat 3 times). Then, 50 μL of transformation suspension with DDW was spreaded on Yeast Nitrogen Base Ura drop-out (YNB Ura-) agar plate and incubated at 30℃ for 24 hr.

3.2. Beta-xylosidase production

3.2.1. Culture condition

Flask culture condition is shown in Table 3-3. After the 1st & 2nd seed culture, the main culture was performed with YPD medium in a 1 L baffled flask. Sampling was carried out at 0, 12, 24, 36, 48, 60, 72, 96, and 120 hr. Dry cell weight (DCW), residual glucose, ethanol production, beta-xylosidase activity were measured. Batch and fed-batch culture were performed using 5-L jar fermenter (KoBioTech Co., Republic of Korea) and culture conditions is shown in Table 3-4. Seed culture proceeded in the same way as flask culture. The pH was maintained at 5.5 using 4 N HCl and 4 N NaOH during the fermentation. Sampling was carried out at intervals of 24 hr, and DCW, residual glucose, ethanol production, beta-xylosidase activity were analyzed.

Table 3-3. Flask culture

Condition	1st seed culture	2nd seed culture	Main culture
Media	YNB Ura-	YNB Ura-	YPD
Working volume	10 mL	50 mL	400 mL
Temperature (℃)	28	28	28
Shaking (rpm)	200	200	200
Time (hr)	48	24	120

Table 3-4. Five liter jar fermenter culture

Condition	Main culture
Media	YPD
Initial working volume (L)	3.0
Temperature (℃)	28
pH	5.5
Agitation (rpm)	350
Aeration (vvm)	1.0
DO (%)	> 20
Time (hr)	up to 140

3.2.2. Plasmid stability

One hundred μL of diluted culture samples (10^7~10^9) were spread on YPD plates and incubated at 30℃ for 24 hr. All grown colonies were transferred to a selective YNB Ura- plate by tooth-picking method (Wang Z and Da Silva NA, 1993). The plasmid stability was expressed as the ratio of surviving colonies on selective YNB Ura- plate.

3.3. Purification and analysis of beta-xylosidase

3.3.1. Purification of beta-xylosidase

The cells were harvested by centrifugation (7,000 ×g, 4℃, 10 min) and cell pellets were resuspended with lysis buffer (50 mM Tris-HCl 300 mM NaCl, 10 mM imidazole, pH 7.0), then the cells were disrupted by sonication (VC-505P, SONICS & MATERIALS INC., USA). The crude extract was centrifuged (10,000 ×g, 4℃, 10 min) and supernatant was filtered through a 0.22 μm Minisart® RC filter (Sartorius, Germany). The crude cell extract was loaded onto a Ni-NTA column (GE Healthcare, USA). The column bound to beta-xylosidase was washed with four column volumes using washing buffer (50 mM Tris-HCl, 300 mM NaCl, 50 mM imidazole, pH 7.0). Then, beta-xylosidase was eluted with three column volumes using elution buffer (50 mM Tris-HCl, 300 mM NaCl, 250 mM imidazole, pH 7.0). The elution fraction was dialyzed in a beaker filled with 50 mM sodium phosphate buffer (pH 7.0) in a Slide-A-Lyzer™ G2 Dialysis Cassettes (20K MWCO, Thermo-Fisher Scientific, USA). The dialyzed enzyme fraction was transferred to Amicon® Ultra 15 mL Centrifugal Filters (30 kDa MWCO, Millipore, Germany) and then concentrated using centrifugation (5,000 × g, 4 ° C, 20 min).

3.3.2. Determination of beta-xylosidase activity

The activity of beta-xylosidase was measured in a similar method using p-nitrophenyl-beta-D-xylopyranoside (pNPX, Sigma-Aldrich Co., USA) as substrate (John et al. 1979). The reaction mixture, composed of 1 mM pNPX (0.2 mL), 50 mM sodium phosphate buffer (pH 7.0, 0.2 mL), and diluted enzyme (0.2 mL), was incubated at 37℃ for 10 min with shaking (300 rpm). The reaction was stopped by 0.4 mL of 400 mM sodium carbonate. The amount of p-nitrophenol (pNP) liberated was measured at 405 nm using a SpectraMax Plus 384 Microplate Reader (Molecular Devices LLC., USA). One unit of enzyme activity was defined as the amount liberating 1 μmol pNP per min.

3.3.3. Bradford assay

The amount of protein was measured using a method of coloring the protein using coomassie brilliant blue G-250 (Bradford MM. 1976). Ten μL of sample and 500 μL of Bio-Rad Protein Assay reagent (BIO-RAD, USA) are mixed and vortexed. Then, 200 μL of the reaction mixture was transferred to a microplate and absorbance was measured at 595 nm using a SpectraMax Plus 384 Microplate Reader (Molecular Devices LLC., USA). Standard samples were prepared with 0 to 700 μg (100 μg intervals) of bovine serum albumin (Sigma-Aldrich Co., USA) and measured in the same manner as described above, and then a standard curve was prepared. The amount of protein in the sample was calculated.

3.3.4. SDS-PAGE analysis

Gel electrophoresis was performed in the manner described by Laemmli (Laemmli UK. 1970). Sample (10 μg) was mixed with NuPAGE® LDS Sample Buffer (4×, Thermo-Fisher Scientific, USA) and NuPAGE® Reducing Agent (10×, Thermo-Fisher Scientific, USA) and heated in a heat block at 80℃ for 10 min. NuPAGE® 4~12% Bis-Tris Protein Gels (1.0 mm, 10-well, Thermo-Fisher Scientific, USA) was immobilized on a gel caster and the diluted NuPAGE® MES SDS Running Buffer (20×, Thermo-Fisher Scientific, USA) was filled into the gel caster. Then, prepared samples and SeeBlue® Plus 2 Pre-Stained Standard (Thermo-Fisher Scientific, USA) were loaded into each well and the electrophoresis was run at 170 V for 55 min. After gel running, the gel was stained with a staining reagent containing coomassie brilliant blue R-250 for 30 min and rinsed several times with destaining reagent until the band appeared.

3.3.5. Size exclusion-high performance liquid chromatography (SEC-HPLC) analysis

SEC-HPLC analysis can confirm the presence of impurities. Analytical samples were prepared to be at least 100 μg/mL of protein and blank sample was prepared with buffer present in the beta-xylosidase. Each sample was filtered through a 0.22 μm Ministart® RC (Sartorius, Germany). The mobile phase was diluted with Phosphate-Buffered Saline (10×, pH 7.4, Thermo-Fisher Scientific, USA) and filtered through a 0.22 μm filter and degassed. The analytical instrument used Agilent 1100 series (Agilent, USA) and the UV detection wavelength was 220

nm. Analytical column was Superdex® 200 Increase 10/300 GL (8.6 μm, GE Healthcare, USA) and flow rate was 0.5 mL/min.

3.3.6. Liquid chromatography-mass spectrometry (LC-MS) analysis

LC-MS analysis was performed to determine the intact mass of beta-xylosidase. Analysis was performed by PROTEINWORKS (Republic of Korea). The native form sample was prepared at a concentration of 1 mg/mL of enzyme. Reducing form sample was prepared by adding 1 μL of DTT (GE Healthcare, USA) to 50 μg of enzyme and reacting at room temperature for 1 hr. The analytical instrument used UltiMate 3000 system (Dionex, USA) and the column was ZORBAX 300SB-C8 (2.1 × 50 mm, 3.5 μm, Agilent, USA). Mobile phase A was DDW with 0.2% formic acid and mobile phase B was acetonitrile containing 0.2% formic acid. Gradient conditions are as follows. Mobile phase B 5% for 3 min; mobile phase B 5 to 100% (linear gradient) for 22 min; mobile phase B 100% for 10 min; mobile phase B 5% for 10 min. The flow rate was 0.1 mL/min and the column oven temperature was 60℃. The MS instrument was a Micro Q-TOF III mass spectrometer (Bruker Daltonics, Germany) and the mode was ESI+. Source capillary voltage was 4,500, temperature was 190℃, and Ion Energy was 5.0 eV.

3.4. Characterization of beta-xylosidase

3.4.1. Effect of temperature on beta-xylosidase activity

The relative activity of beta-xylosidase was determined by various temperature ranges. The activity was measured at 20, 30, 35, 40, 45, 50, and 60℃. One mM pNPX (0.2 mL), 50 mM sodium phosphate buffer (pH 7.0, 0.2 mL) and the same amount of enzyme (0.2 mL) were mixed and reacted for 10 min at seven temperature conditions with shaking (300 rpm). The reaction was terminated by adding 0.4 mL of 400 mM sodium carbonate. The amount of p-nitrophenol (pNP) liberated was measured at 405 nm using a SpectraMax Plus 384 Microplate Reader (Molecular Devices LLC., USA).

3.4.2. Effect of pH on beta-xylosidase activity

The relative activity of beta-xylosidase was determined by various pH ranges. Fifty mM sodium citrate buffer (pH 5.0, pH 5.5), 50 mM sodium phosphate buffer (pH 6.0, pH 6.5, pH 7.0, pH 7.5), and 50 mM Tris-HCl buffer (pH 8.0, pH 8.5) were used. One mM pNPX (0.2 mL), various pH buffers and the same amount of enzyme (0.2 mL) were mixed and reacted for 10 min at 37℃ with shaking (300 rpm). The reaction was terminated by adding 0.4 mL of 400 mM sodium carbonate. The amount of p-nitrophenol (pNP) liberated was measured at 405 nm using a SpectraMax Plus 384 Microplate Reader (Molecular Devices LLC., USA).

3.5. Synthesis of xylobiose

3.5.1. Design of response surface methodology (RSM)

RSM was used to obtain for optimal conditions of xylobiose synthesis with a minimum number of experiments. Minitab 17 (Minitab Inc., USA) was used to derive experimental designs and regression models. Experimental design was based on the central composite design (CCD) method (Cochran and Cox. 1957, Box and Draper. 1987). The three independent variables are as follows: the substrate concentration (X_1), the amount of enzyme (X_2), and temperature (X_3). Each independent variable was a five code levels: -α, -1, 0, +1, and +α. A total of 40 combinations of test runs were generated in random order according to three independent variables and repeated experiment settings. Experimental results were analyzed by analysis of variance (ANOVA) for individual linear, quadratic terms, interaction terms, and regression coefficients. A *p*-value of less than 0.05 was considered statistically significant.

3.5.2. Xylobiose synthetic reaction

For the xylobiose synthesis, 7M (105%, w/v) xylose stock solution (prepared by boiling for 30 minutes or more) and 500 U/mL of beta-xylosidase were prepared. The reaction mixture was prepared according to the test run combination generated through RSM and reacted for 24 hr. The reaction was stopped at 99℃ for 15 min. The amount of xylobiose was analyzed by HPLC with RID (Agilent 1100 series, USA). The mobile phase was used DDW and flow rate

was 0.5 mL/min at 80℃. The analytical column was used Waters Sugar-Pak I (6.5 mm × 300 mm, 10 μm).

3.5.3. Large-scale production of xylobiose

For xylobiose synthesis, 1,900 g of D-xylose was placed in a 2,000 mL beaker and distilled water was added to a volume of 1.970 mL. D-xylose reaction solution was boiled until completely dissolved, then 6.5 U/mL beta-xylosidase and 50℃ DDW were added to reach a final volume of 2,000 mL when the temperature reaches equilibrium in the oven at 50℃. The reaction mixture was carefully mixed and reacted for 24 hr without stirring at 50℃. Then, the mixture was autoclaved to stop the reaction. The xylobiose synthesis product was diluted by adding DDW to a total solid content of 250 g/L and centrifuged (7,000 ×g, 4℃, 10 min) to remove denatured enzymes and impurities. And the supernatant was incubated in a 45℃ oven for 2 hr and filtered using a 0.22 μm Stericup-GP (Merck Millipore Co., Germany).

3.6. Purification of xylobiose

3.6.1. Purification of synthetic xylobiose using a simulated moving bed (SMB) method

Dowex-50WX4 (Sigma-Aldrich Co., USA) resin was used for oligosaccharides separation via the SMB method. The resin has been reported to convert the hydrogen form to sodium form to separate xylobiose from xylooligosaccharides (Wianiewski et al. 2014, Choi et al. 2016). The resin (average particle size 55.5 μm) converted to sodium form was loaded on an omnifit chromatographic column (35 mm × 217 mm, Bio-Chem Fluidics Co., USA). Purification of xylobiose was used a three-zone SMB unit (Chin and Wang. 2004, Choi et al. 2016) as shown in Figure 3-1. The main device consisted of Select-Trapping rotary valve (VICI Valco Instruments Co. Inc., USA), Waters 515 HPLC pump, and five columns. The operating parameters are shown in Table 3-5. SMB devices were controlled by Labview 8.0 software (National Instruments, USA). The desorbent was used DDW and maintained at 65℃ using CB407 heating mantle (Misung Scientific Co. Republic of Korea). The column with water jacket was maintained at 65℃° using a HST-250WL circulator.

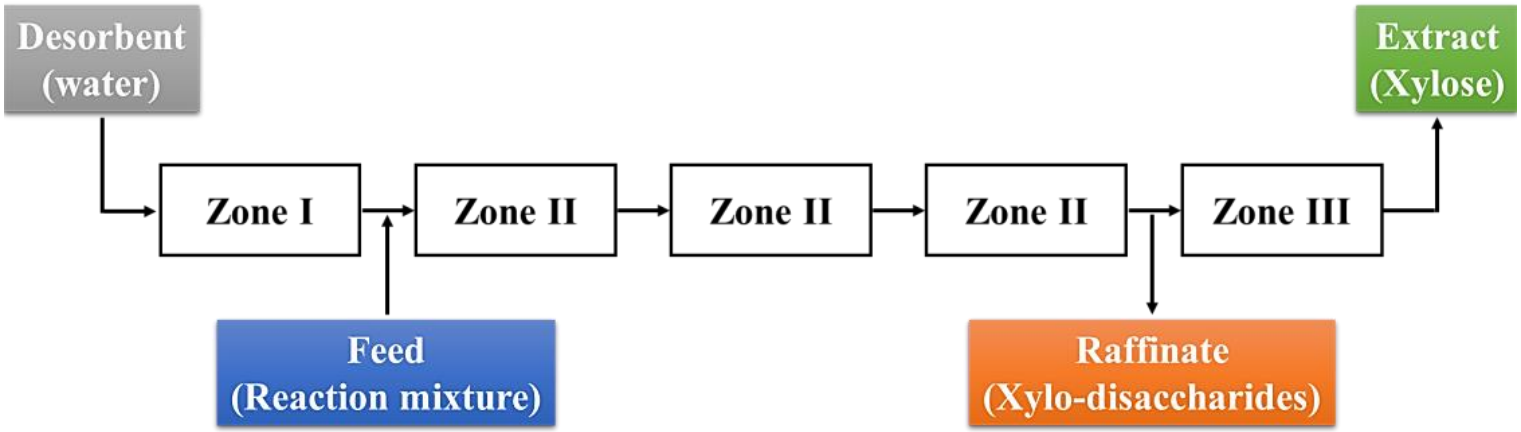


Figure 3-1. Schematic illustration of SMB unit for xylobiose separation.

Table 3-5. Operating parameters of xylobiose separation using SMB

Operating parameter	Condition
Port configuration	D → F → R → E
Column configuration	1 - 3 - 1
Desorbent flow rate (mL/min)	10.20
Feed flow rate (mL/min)	0.82
Extract flow rate (mL/min)	9.60
Raffinate flow rate (mL/min)	1.42
Port switching time (min)	12.12

3.6.2. Purification of synthetic xylobiose isomers

The mixture of xylobiose isomers purified through SMB was separated by paper chromatography. Sample was loaded onto GE Healthcare Whatman™ 3MM Chr (Thermo-Fisher Scientific, USA). A mixture of 1-propanol : ethyl acetate : water (7 : 2 : 1, v/v/v) was used as a developing solvent. Paper chromatography was developed three times for isomer separation. Xylobiose and isomers were detected using silver nitrate alkaline staining method (Trevelyan et al. 1950). The three isolated xylobiose isomers were eluted with DDW. Purity analysis of the isolated isomers was performed using HPLC with ZORBAX Carbohydrate column (4.6 mm × 250 mm, 5 μm), and over 98% of the purity fractions were collected.

3.6.3. Thin-layer chromatography (TLC) analysis

TLC Silica gel 60 (Merck Millipore Co., Germany) was activated at 110℃ for 30 min and then cooled to room temperature. The analytical sample was loaded onto the bottom of the TLC plate as a spot. A mixture of 1-propanol : ethyl acetate : water (7 : 2 : 1, v/v/v) was used as a developing solvent. The developed TLC plate was dried and the xylobiose isomer was detected using 10% H_2SO_4 in methanol (Cotta MA. 1993).

3.7. Identification and characterization of synthetic xylobiose

3.7.1. Liquid chromatography-mass spectrometry (LC-MS) analysis

LC-MS analysis was carried out by Korea Polymer Testing and Research Institute (Koptri, Republic of Korea). Agilent 1260 Infinity was performed using a ZORBAX Carbohydrate column (4.6 mm × 250 mm, 5 μm) at 35℃. The solvent was 70% acetonitrile and the flow rate was 1 mL/min. The xylobiose standard, synthesized xylobiose, and its isomers were dissolved in water and filtered through a 0.22 μm Ministart® RC (Sartorius, Germany). The sample injection was 5 μL. Detection was carried out on an API 3200 Mass spectrometry equipped with an electron ion spray source operating in the positive mode. Source temperature was 400℃. Analyst ™ software (Ver. 1.5.2) was used for the acquisition and processing of analytical data.

3.7.2. Nuclear magnetic resonance (NMR) analysis

NMR analysis was carried out by Research Institute of Standards and Analysis (Inha Univ., Republic of Korea). The ^{13}C NMR spectra were measured using a Bruker NMR 400 MHz (DIGITAL AVANCE III, Bruker, USA). The xylobiose standard, synthesized xylobiose, and its isomers were dissolved in D_2O at 24℃ and 5-mm probe tubes were used. Signal acquisition and processing were performed using Bruker's TopSpin™ software.

3.7.3. Effect of pH on thermal stability of xylobiose and its isomers

The test samples were prepared with 1% solution of xylobiose standard (Megazyme, USA), enzymatically synthesized xylobiose and two xylobiose isomers, respectively. pH 2, 6, and 10 solutions were prepared by mixing 0.2 N HCl and 0.2 N NaOH. Each test sample and three pH solutions were mixed at a ratio of 1 : 1 and stored at 100℃. Sampling was performed at 0, 10, 30, 60, 90, 120, 180, and 240 min. Each sample was diluted 5-fold in PBS buffer and analyzed by HPLC with Waters Sugar-Pak I column (6.5 mm × 300 mm, 10 μm). The residual ratio (%) was calculated as the peak area of each time relative to the initial peak area. The thermal stabilities of xylobiose and its isomers were measured in pH 2. The first order rate constants of thermal degradation kinetics for each material were calculated using the $ln\ (C_t/C_0) = -kt$ equation. C_t and C_0 represent the concentration at heating time (t) and initial state (0), and $-kt$ is reaction rate constant.

3.7.4. Kinetic parameters for hydrolysis of xylobiose isomers

The kinetic parameters of beta-xylosidase from *Bacillus pumilus* IPO were determined using p-nitrophenyl-beta-D-xylopyranoside (pNPX), beta-1,2-xylobiose, beta-1,3-xylobiose, and beta-1,4-xylobiose as substrates. Each concentration of pNPX was reacted with the enzyme at 37℃ and samples were taken for 5 min at 30 sec intervals. The absorbance was measured at 405 nm. Each concentration of xylobiose isomers was reacted with the enzyme at 37℃ and

samples were taken for 14 min at 2 min intervals. It was analyzed by HPLC. Kinetic parameters were calculated using the Michaelis-Menten equation.

3.7.5. Sucrase inhibition test

Extraction of intestinal acetone powder from rat was carried out using the method described in Kim et al. (2011). The control group was 2% sucrose solution. Test group 1 was supplemented with 5, 10, 15, and 20% of beta-1,4-xylobiose in 2% sucrose solution. Test group 2 was supplemented with 5, 10, 15, and 20% of synthetic xylo-disaccharides mixture (purified by SMB) in 2% sucrose solution. The crude sucrase extract from rat was added and the final concentration of the reaction mixture was 200 μg/mL based on the amount of protein. The sucrase inhibition reaction was carried out at 37℃ for 3 hr. Then, the reaction sample was heat-treated at 99℃ for 15 min to inactivate the enzyme. The amount of glucose produced in each sample was analyzed by HPLC. The relative sucrase inhibition (%) was calculated as 100 − (glucose production in the test group/glucose production in the control group × 100).

3.8. Statistical analysis

Experimental data were expressed as mean ± standard deviation. Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA) with post-hoc Tukey HSD (Honestly Significant Difference) using the Minitab 17 (Minitab Inc., USA). A p-value < 0.05 was considered statistically significant.

4. RESULTS AND DISCUSSIONS

4.1. Construction of beta-xylosidase expression system

4.1.1. Screening of beta-xylosidase in various strains for xylobiose synthesis

Twelve beta-xylosidase genes were inserted into each pET21a vector and transformants were obtained. These enzymes were expressed through IPTG induction, and cell extract was used for evaluating xylobiose synthesis. The result of xylobiose conversion ratio is shown in Table 4-1. All of the beta-xylosidase in GH 43 had a high xylobiose conversion ratio. Xsa from *Bacteroides ovatus* and XynD from *Bifidobacterium adolescentis* synthesized 4.0% and 4.6% xylobiose, respectively. In particular, conversion evaluation revealed that the beta-xylosidase from *Bacillus pumilus* IPO in *Escherichia coli* (XynB-E) synthesized up to 5% xylobiose. Contrastingly, the beta-xylosidase belonging to GH 3, GH 39, GH 52, and GH 120 showed a low xylobiose conversion ratio of 0.1~2.0%. GH 43 classified as an inverting enzyme was found to be more suitable for xylobiose synthesis than retaining enzymes. XynB-E was selected for enzymatic xylobiose synthesis, and its vector map is shown in Figure 4-1.

Table 4-1. Comparison of xylobiose conversion ratio by beta-xylosidase in various microorganisms

GH Family	Mechanism (Base/Donor) [1)]	DNA of beta-xylosidase (Origin)	Conversion (%)
GH 3	Retaining (Asp/Glu)	FgXyl3A (*Fervidobacterium gondwanense*)	0.1
GH 39	Retaining (Glu/Glu)	XynD (*Caldicellulosiruptor saccharolyticus*)	1.0
	Retaining (Glu/Glu)	XynB (*Thermoanaerobacterium saccharolyticum*)	0.1
GH 43	Inverting (Asp/Glu)	Xsa (*Bacteroides ovatus*)	4.0
	Inverting (Asp/Glu)	XynB (*Bacillus pumilus IPO*)	5.0
	Inverting (Asp/Glu)	XynB (*Bifidobacterium adolescentis*)	3.8
	Inverting (Asp/Glu)	XynD (*Bifidobacterium adolescentis*)	4.6
	Inverting (Asp/Glu)	XynB1 (*Lactobacillus brevis*)	1.5
	Inverting (Asp/Glu)	XylC (*Paenibacillus woosongensis*)	2.8
	Inverting (Asp/Glu)	Xsa (*Selenomonas ruminatium*)	3.0
GH 52	Retaining (Glu/Asp)	XylD (*Thermoanaerobacterium saccharolyticum*)	0.1
GH 120	Retaining (Asp/Glu)	XylC (*Thermoanaerobacterium saccharolyticum*)	2.1

[1)] Reference site: http://www.cazy.org/

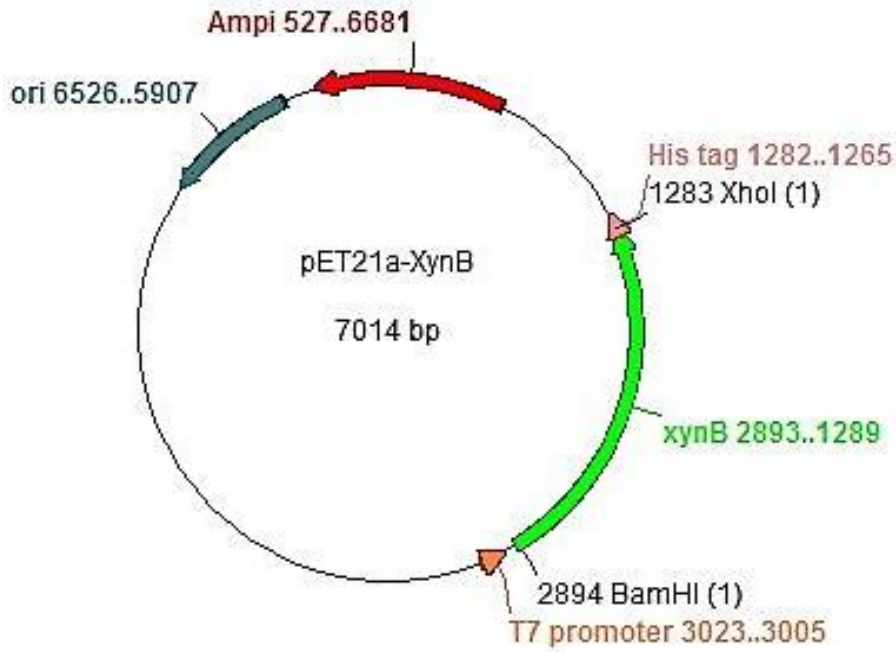


Figure 4-1. Vector map of beta-xylosidase from *B. pumilus* IPO in *E. coli* (XynB-E).

4.1.2. Beta-xylosidase expression levels in *Saccharomyces cerevisiae* under the influence of different promoters

Six different promoters (ADH1, CYC1, GAL1, GPD, TDH3, and TEF1) were evaluated in *S. cerevisiae* to obtain a high-expression system for beta-xylosidase production (Figure 4-2). The CYC1 and GPD promoter showed the highest expression levels of beta-xylosidase. The expression levels of CYC1 and GPD promoters were 59.1 ± 5.8 and 59.7 ± 5.4 $U/L_{culture\ broth}$. ADH1, TEF1, and TDH3 promoters expressed 51.6 ± 1.4, 51.2 ± 1.3, and 47.5 ± 9.7 $U/L_{culture\ broth}$, respectively, and the expression level of beta-xylosidase with regard to the GAL1 promoter was the lowest (21.4 ± 0.4 $U/L_{culture\ broth}$). There were no significant differences in the expression levels of beta-xylosidase among five promoters (ADH1, CYC1, GPD, TDH3, and TEF1). The GPD promoter was selected as the enzyme expression system because the expression level of beta-xylosidase was the highest among the six promoters. The vector map of beta-xylosidase from *Bacillus pumilus* IPO in *S. cerevisiae* (XynB-S) is shown in Figure 4-3. La Grange et al. (1997) constructed a pDLG11 vector with the *Bacillus pumilus* PLS beta-xylosidase gene (XynB) and the alcohol dehydrogenase 2 (ADH 2) promoter into *S. cerevisiae*. The transformant strain was cultured in 2-L fermenter, but no beta-xylosidase activity was observed. Other studies have reported no difference in the activity of beta-xylosidase (XylB) from *Bacillus* species. KK-1 expressed by the ADH1 and GAL promoters in recombinant *S. cerevisiae* (Kim et al. 2000, Heo et al. 2004). In this study, the expression level of beta-xylosidase produced by the ADH1 promoter was 2.4 times higher than that of the GAL promoter; however, the expression of beta-xylosidase from *Bacillus pumilus* IPO was less affected by the promoter, except the GAL1 promoter.

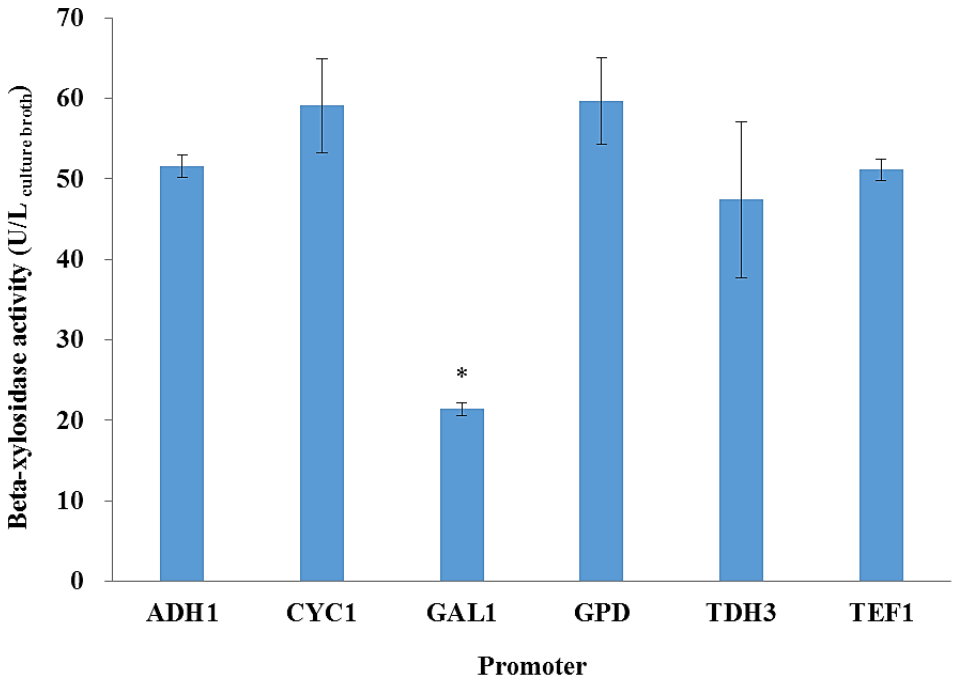


Figure 4-2. Comparison of beta-xylosidase expression levels by different promoters. Statistically significant difference (* $p < 0.05$).

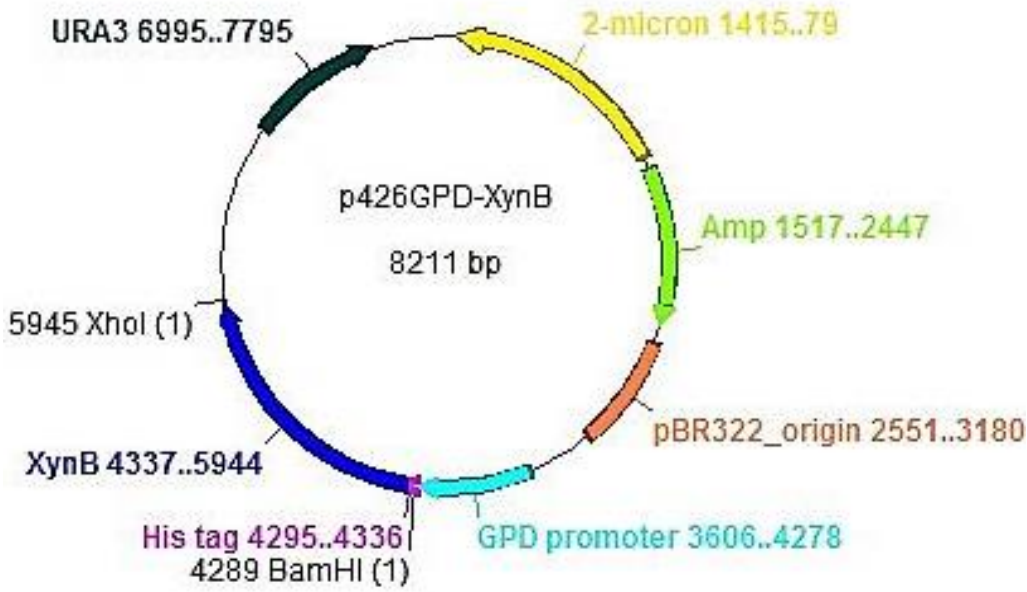


Figure 4-3. Vector map of beta-xylosidase from *B. pumilus* IPO in *S. cerevisiae* (XynB-S).

Twenty transformants were compared to select for high-expression XynB-S under a GPD promoter. Each colony was cultured in 10 mL YNB Ura- medium at 28℃ for 48 hr under shaking conditions (200 rpm) and then cultured in 50 mL YPD medium at 28℃ for 24 hr with continuous shaking. After the main culture, the expression levels of beta-xylosidase were evaluated in terms of enzyme activity and productivity (U/g_{DCW}). Most of the transformants, except #6, #7, #8, and #15, expressed beta-xylosidase at > 40 $U/L_{culture\ broth}$ (Figure 4-4). In particular, the amounts of beta-xylosidase expressed in #9 and #16 were the highest, i.e., 62.8 and 63.4 $U/L_{culture\ broth}$, respectively. The strains with the highest enzyme productivity were #13 (6.1 U/g_{DCW}) and #16 (6.2 U/g_{DCW}). Most strains showed a productivity value of 4.5 U/g_{DCW}. Four transformants (#6, #7, #8, and #15) with low beta-xylosidase expression levels and productivity were not found to be contaminated, but beta-xylosidase expression levels were $1/10^{th}$ of the expression levels of the other transformants and the cell growth was also less than half. These transformants are considered to have low expression levels of beta-xylosidase owing to unstable insertion of foreign vectors. Zhang et al. (1996) reported that it is important to maintain stable expression because transformed strains tend to lose recombinant plasmids.

In this study, #16 transformant having a high expression level of beta-xylosidase as well as a high productivity value was selected. To improve beta-xylosidase productivity using a high-concentration of cell mass, a certain level of plasmid stability should be maintained during culture.

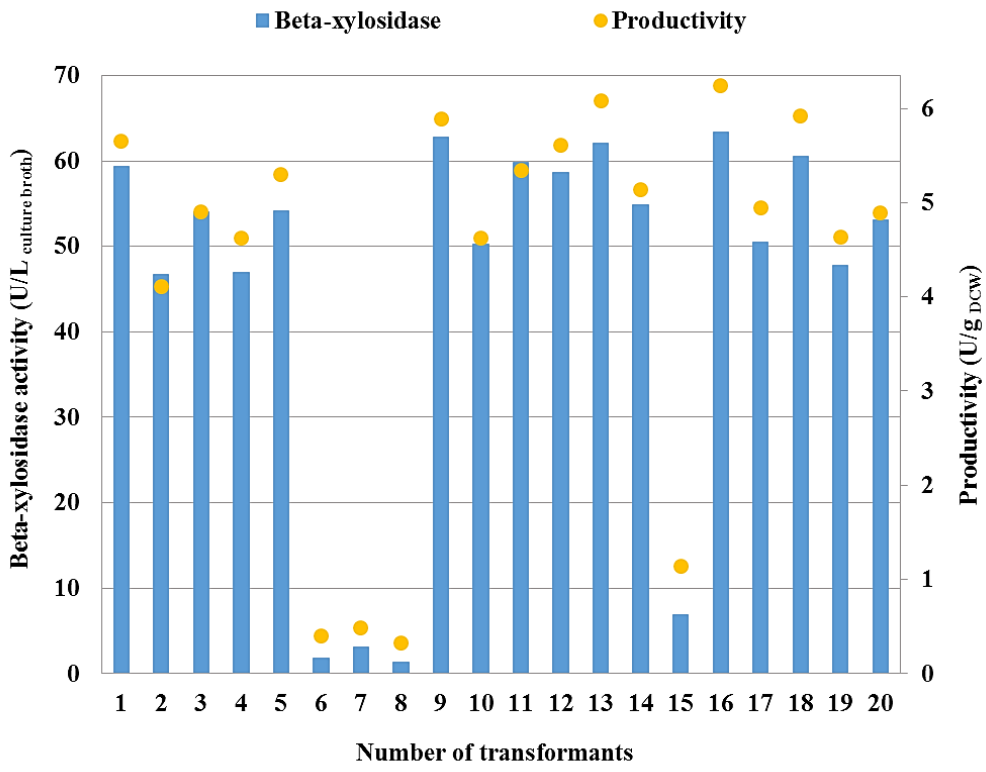


Figure 4-4. Beta-xylosidase expression levels of *S. cerevisiae* transformants.

In conclusion, beta-xylosidase having a high xylobiose synthesis efficiency was selected, and the expression system was constructed in a GRAS strain. Among the twelve beta-xylosidase genes, beta-xylosidase from *Bacillus pumilus* IPO, the most highly synthesized xylobiose, was screened. A yeast strain suitable for high-cell density cultivation and process improvement for recombinant protein productivity through fed-batch culture was selected as the host strain. The codon-optimized gene of beta-xylosidase was combined with several promoters, and the promoter-dependent differences in beta-xylosidase expression levels were compared. There were no significant differences among five promoters (ADH1, CYC1, GPD, TDH3, and TEF1) except the GAL1 promoter ($p < 0.05$). The plasmid with the highest expression level, namely p426GPD (59.7 ± 5.4 $U/L_{culture\ broth}$), was obtained. Beta-xylosidase expression in *S. cerevisiae* D452-2 was less affected by the promoter.

4.2. Improvement in beta-xylosidase productivity

4.2.1. Flask culture

As shown in Figure 4-5, flask culture was performed to determine the characteristics of beta-xylosidase expression in *S. cerevisiae* D452-2 using p426GPD. *S. cerevisiae* D452-2 consumed all of the glucose within 24 hr and produced 8.1 g/L ethanol over a 24 hr period. Cell growth rapidly increased until 24 hr (DCW 8.2 g/L), after which the stationary phase maintained from 24 hr to 120 hr (DCW 14.2 g/L) during ethanol consumption. Beta-xylosidase was continuously produced from the baseline to 120 hr (75.4 $U/L_{culture\ broth}$). The productivity of beta-xylosidase in flask culture was 3.9 U/g_{DCW} at 48 hr and 5.3 U/g_{DCW} at 120 hr.

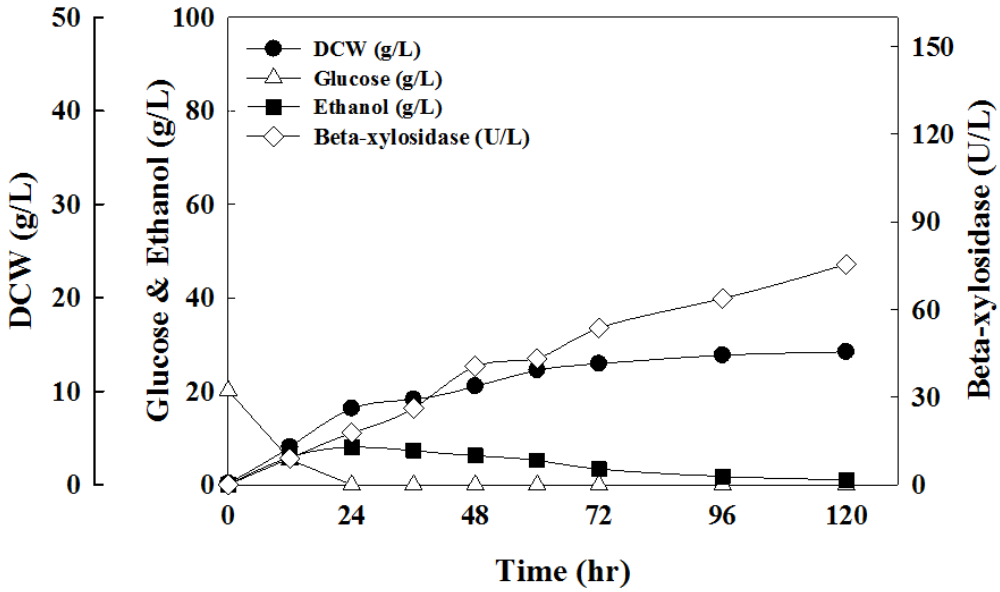


Figure 4-5. Profiles of flask culture of *S. cerevisiae* D452-2 with p426GPD on beta-xylosidase expression.

4.2.2. Comparison of beta-xylosidase productivity according to the C/N ratio in the medium

To establish a high-expression process for beta-xylosidase, a flask culture was performed according to the C-source/N-source ratio in the medium. The medium composition was as shown in Table 4-2. As shown in Figure 4-5, cells in flask culture consumed 20 g/L of glucose within 24 hr. Thus, the C-source of the C/N ratio test medium was set at 60 g/L. Flask culture was performed for 48 hr as shown in Table 3-3.

At the C/N ratios of 2 and 4, beta-xylosidase expression levels were the lowest and productivities were 3.6 U/g_{DCW} and 3.5 U/g_{DCW}, respectively (Figure 4-6). Such a medium composition is mainly utilized for cell growth owing to the lack of N-source. The medium compositions with a C/N ratio of < 1 not only showed a high-cell growth but also an increased beta-xylosidase expression level. At C/N ratios of 0.5 and 0.67, beta-xylosidase productivities were found to be similar, i.e., 13.1 U/g_{DCW} and 12.9 U/g_{DCW}, respectively, while beta-xylosidase expression levels were 287 U/$L_{culture\ broth}$ and 325 U/$L_{culture\ broth}$, respectively. At such a medium composition, an inhibition of cell growth and beta-xylosidase expression is considered to occur owing to an excessive N-source. Beta-xylosidase expression, DCW, and productivity were the highest at a C/N ratio of 0.67. The optimal C/N ratio was found to be 0.67 for flask culture, which was the same as the C/N ratio in YPD medium.

Table 4-2. Composition according to the C-source/N-source ratio in the medium

C/N ratio	C-source	N-source		
	Glucose (g/L)	Total (g/L)	Yeast extract (g/L)	Peptone (g/L)
4	60	15	5	10
2	60	30	10	20
1	60	60	20	40
0.67	60	90	30	60
0.50	60	120	40	80

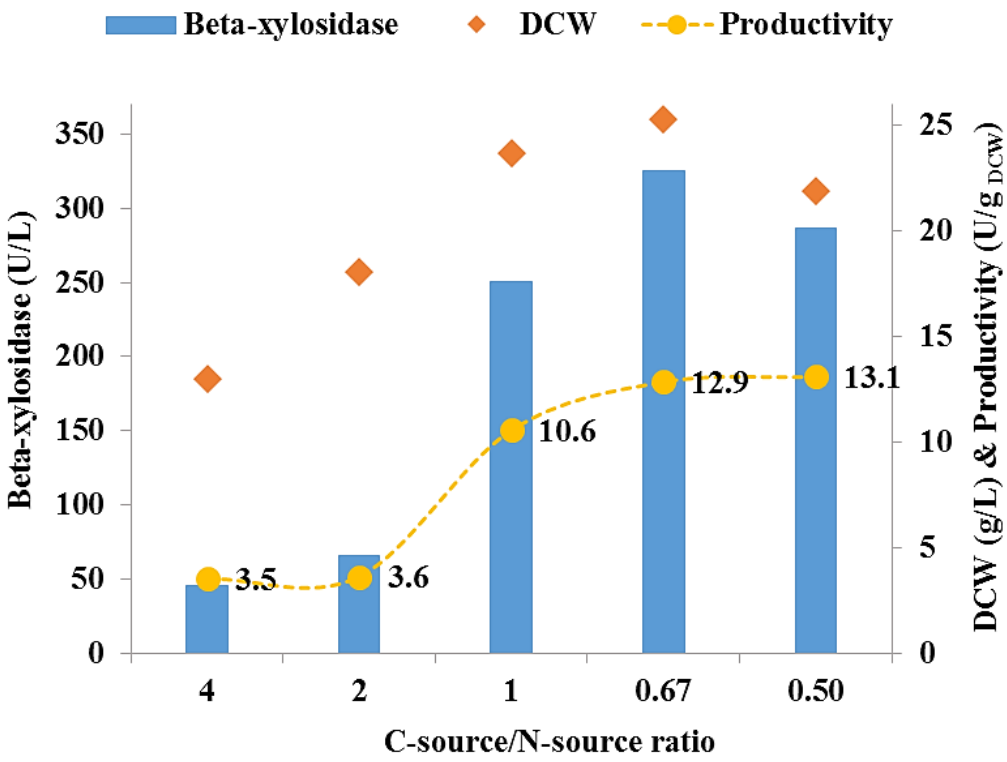


Figure 4-6. Effects of the C/N ratio in the medium on beta-xylosidase expression levels and productivities.

4.2.3. Batch and fed-batch cultures

Batch and fed-batch cultures were performed using a 5-L jar fermenter, and their results are shown in Figure 4-7. Beta-xylosidase productivity was the highest in the flask culture medium with a C/N ratio of 0.67; hence, YPD medium (glucose 20 g/L, yeast extract 10 g/L, peptone 20 g/L) was used in this experiment. In the batch culture, *S. cerevisiae* D452-2 consumed all of the glucose within 24 hr similar to the glucose consumption in the flask culture (Figure 4-5). On the other hand, ethanol production was 2.1 g/L at 24 hr, which was lower than that noted in the flask culture. It seems that the jar fermenter culture delivered oxygen more efficiently than the flask culture and provided an aerobic condition. Cell growth was 12.7 g/L at 24hr (1.5 times higher than in the flask culture) and 17.4 g/L at 48 hr. The expression levels of beta-xylosidase were 65.5 $U/L_{culture\ broth}$ at 24 hr and 101.6 $U/L_{culture\ broth}$ at 48 hr. Unlike the flask culture, the batch culture showed that the cells expressed beta-xylosidase until 48 hr after which the expression of this enzyme gradually decreased. Productivity of beta-xylosidase was 5.8 U/g_{DCW} at 48hr, which was 1.5 times higher than that noted in flask culture (3.9 U/g_{DCW}).

The fed-batch culture was performed in the same manner as the batch culture. Here, 20 g/L of glucose was fed every 24 hr after the start of cultivation. Cells tended to consume 20 g/L glucose within 24 hr. Ethanol accumulation was maintained below 3.6 g/L during fermentation. Cell growth was 12.6 g/L at 24 hr, which was the same as that in the batch culture. At 48 hr, cell growth was 22.6 g/L, which was 1.3 times higher than that in the batch culture; cell growth subsequently increased to 24.2 g/L at 72 hr. Beta-xylosidase was expressed at 58.9 $U/L_{culture\ broth}$ at 24 hr, which was lower than that in the batch culture. After 24 hr, the enzyme

expression level did not increase any more, and was maintained at > 60 $U/L_{culture\ broth}$. Productivity of beta-xylosidase was 2.8 U/g_{DCW} at 48hr, which was lower than in the batch culture. The fed-batch culture using YPD medium only showed an increase in cell growth.

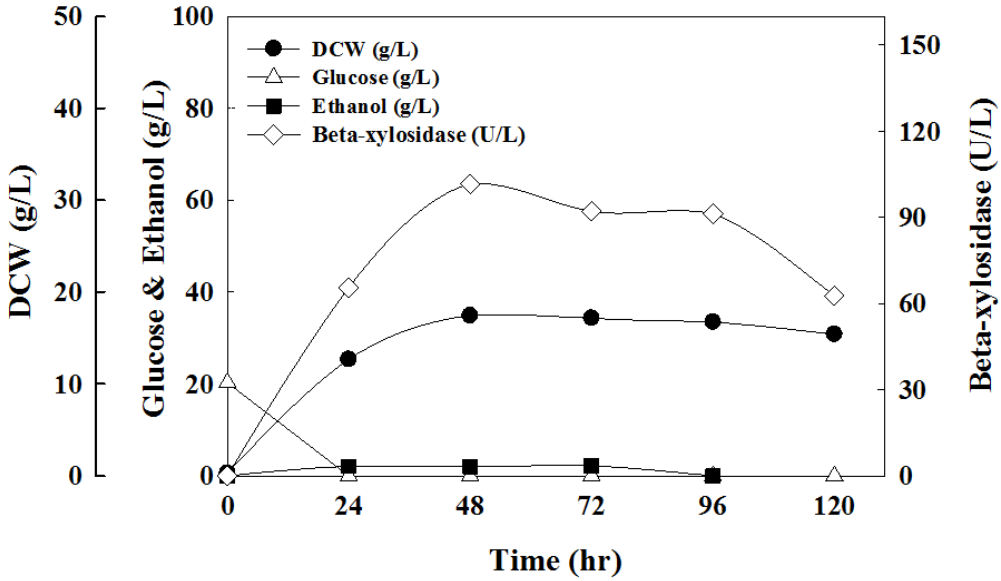
In another experiment, the optimal C/N ratio medium (0.67), used in the fed-batch culture to confirm that beta-xylosidase productivity can be increased. The nutrient level (glucose 60 g/L, yeast extract 30 g/L, peptone 60 g/L) is 3 times higher than that in the YPD medium and the C/N ratio is the same as that in the YPD medium. Consequently, DCW was 18.3 g/L at 24 hr, which was 1.5 times higher than that shown in Figure 4-7 (B). However, after 48 hr, DCW did not differ from the fed-batch culture shown in Figure 4-7 (B). Consequently, ethanol production was 16.1 g/L at 24 hr, which was 7.2 times higher than that shown in Figure 4-7 (B). The expression level of beta-xylosidase was 122.9 $U/L_{culture\ broth}$ at 48 hr, which was 2 times higher than that shown in Figure 4-7 (B). However, there was no difference in the beta-xylosidase expression level in the optimal C/N ration medium as compared with that in the batch culture [Figure 4-7 (A)] demonstrated (data not shown).

The optimal C/N ratio medium used in the flask culture was applied to jar fermenter cultivation; however, beta-xylosidase productivity did not improve and there was an increased accumulation of ethanol as a by-product. The production of ethanol seems to occur owing to a high-initial glucose concentration. Beta-xylosidase expression and activity of were considered to be inhibited owing to ethanol accumulation in the culture medium. In addition, the productivity of the enzyme also decreased to 4.8 U/g_{DCW} at 48 hr. Productivity was found to be

decreased when the initial medium concentration was high, despite using the optimal C/N ratio medium.

In this study, the growth and beta-xylosidase expression characteristics of *S. cerevisiae* D452-2 with p426GPD were confirmed through batch and fed-batch cultures. The optimal C/N ratio medium was applied as shown in Figure 4-6; although cell growth was higher in the optimal medium than that in flask culture, beta-xylosidase productivity decreased in jar fermenter cultivation. *S. cerevisiae* D452-2 with p426GPD seems to be unable to increase beta-xylosidase productivity owing to an insufficient N-source supply in fermenter culture performed using YPD medium. Therefore, based on the culture profile in Figure 4-7, N-source should be strengthened to increase initial cell growth, and glucose concentration should be decreased to reduce ethanol production.

(A)



(B)

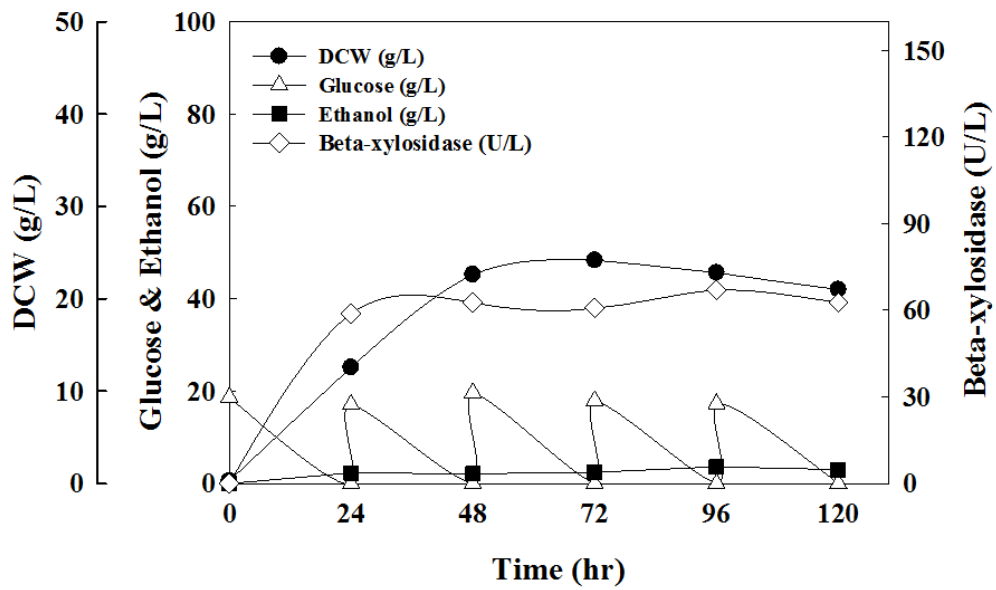


Figure 4-7. Profiles of batch (A) and fed-batch (B) cultures of *S. cerevisiae* D452-2 with p426GPD on beta-xylosidase expression.

The plasmid stability of the strains cultivated in a non-selective medium (YPD) was analyzed using the same samples of the flask (Figure 4-5), batch, and fed-batch cultures (Figure 4-7). The results are shown in Figure 4-8. The recombinant cells carrying the plasmid in the flask culture rapidly decreased to 36% at 24 hr and was maintained at approximately 40% over 120 hr. However, the plasmid stability of the batch and fed-batch cultures remained > 70% at 24 hr and was two times higher than that in the flask cultures. Subsequently, the plasmid stability of the batch and fed-batch cultures decreased slowly to 55% and 60%, respectively, at 120 hr. Wang and Da (1993) reported that recombinant yeast cultured in a selective medium maintained a high level of protein expression as well as consistent plasmid stability, but the proportion of plasmid-containing cells decreased from 54% to 34% when recombinant yeasts were cultured in a non-selective medium such as YPD, despite cell growth being higher than that in a non-selective culture medium. Cheng et al. (1997) reported that glucose starvation could be used in a fed-batch fermentation process as one of the strategies to maintain plasmid stability. It is difficult for recombinant yeast to maintain the inserted vector in a non-selective medium, and vector-free cells tend to increase the growth rate in a glucose-rich medium. In this study, the plasmid stability in 5-L jar fermenter culture was approximately 20% higher than that in a flask culture. Furthermore, the cell growth in batch and fed-batch cultures increased 1.5 times faster than the cell mass in the flask culture at the initial stage. It is considered that the plasmid stability in jar fermenter cultures is maintained at a higher rate than that in the flask culture because the cells quickly reached the glucose starvation state.

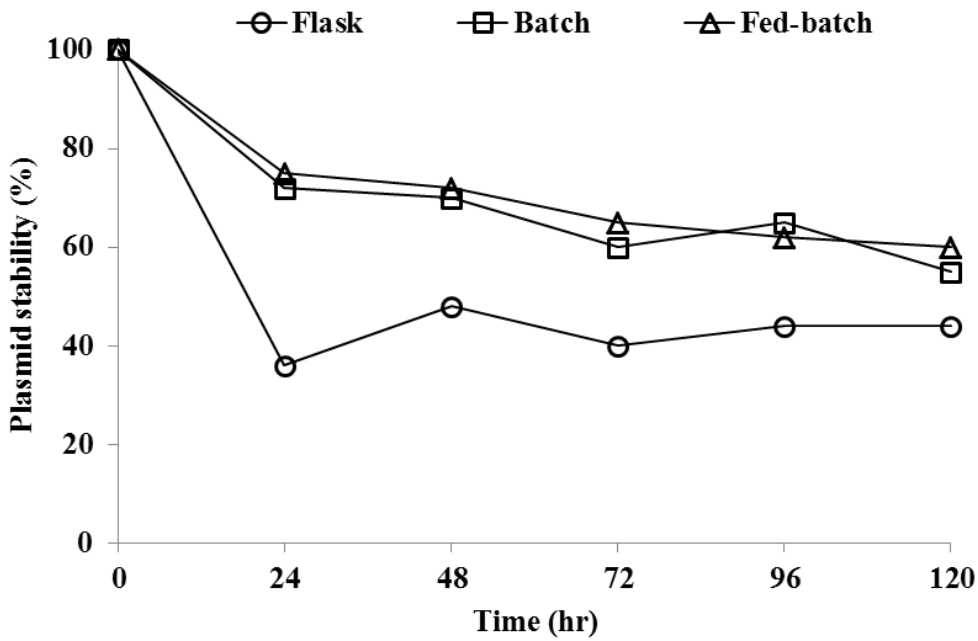


Figure 4-8. Comparison of plasmid stability by culture methods.

4.2.4. Effect of C/N ratio in the medium on beta-xylosidase production in fed-batch culture

Figure 4-9 shows the results of fed-batch culture using the YPD medium enhanced nitrogen source (glucose 20 g/L, yeast extract 20 g/L, peptone 40 g/L). The C/N ratio in the medium was 0.33 at baseline, which was lower than that in the YPD medium; furthermore, 20 g/L glucose was fed every 24 hr. Residual glucose and ethanol accumulation were similar to those associated with (B) in Figure 4-7. The cell growth in the fed-batch culture having an enhanced nitrogen source was 23.8 g/L at 48 hr, which was the same as that for (B) in Figure 4-7 (fed-batch culture using YPD medium). However, DCW was 38.1 g/L at 72 hr, which was 1.6 times higher than that seen for (B) in Figure 4-7. The expression level of beta-xylosidase was 158.2 $U/L_{culture\ broth}$ at 48 hr and maintained approximately at 160.0 $U/L_{culture\ broth}$ until 120 hr. The beta-xylosidase productivity was 6.6 U/g_{DCW} at 48 hr, which was 2.4 times higher than that in the fed-batch culture of (B), as shown in Figure 4-7. However, this value is half as low as the optimal productivity (13.1 U/g_{DCW}), as shown in Figure 4-6. This study confirmed that the appropriate initial C/N ratio for expressing beta-xylosidase in the fed-batch culture was 0.33. Therefore, to increase the beta-xylosidase expression level of *S. cerevisiae* D452-2 with p426GPD, it is necessary to investigate the optimization condition by controlling glucose feeding along with fixing the initial C/N ratio in the medium at 0.33.

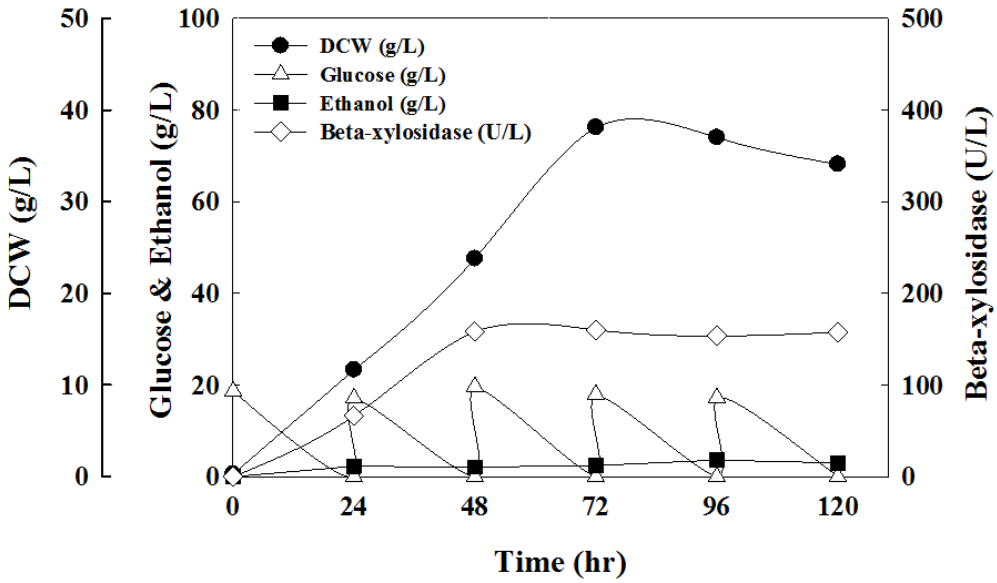


Figure 4-9. Effect of C/N ratio in the medium on beta-xylosidase production of *S. cerevisiae* D452-2 with p426GPD in fed-batch culture.

4.2.5. Effect of N-source composition on beta-xylosidase production in fed-batch fermentation

To confirm the difference in beta-xylosidase productivity based on N-source type, fed-batch cultures were divided into two types. (A) was a peptone-enhanced medium (glucose 20 g/L, yeast extract 20 g/L, peptone 40 g/L) and (B) was a yeast extract-enhanced medium (glucose 20 g/L, yeast extract 40 g/L, peptone 20 g/L). The initial C/N ratio in the medium was fixed as 0.33 (C-source 20 g/L, N-source 60 g/L), and 20 g/L glucose was fed every 12 hr after 24 hr from the start of cultivation.

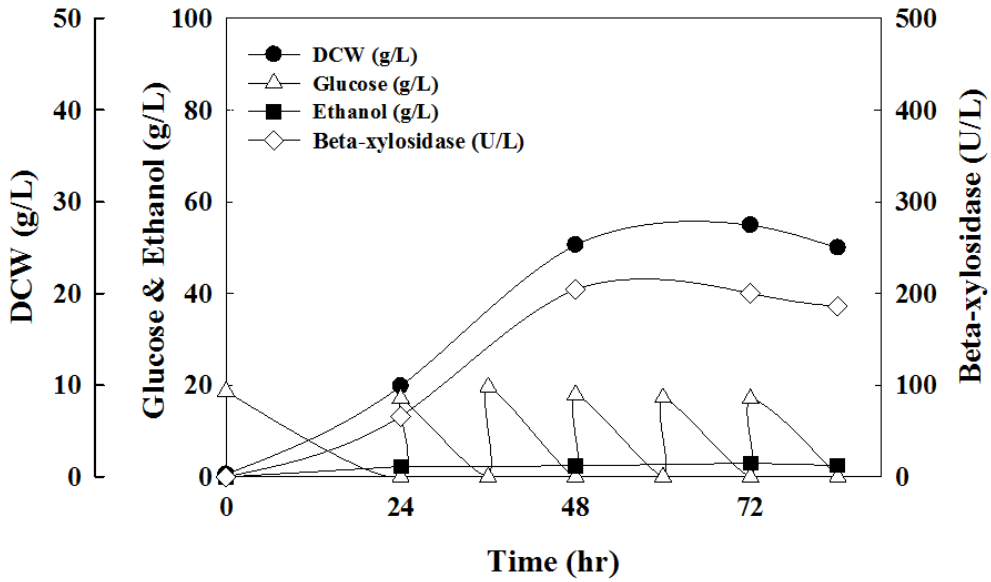
As shown in Figure 4-10, there was a difference in cell growth and beta-xylosidase expression level according to N-source composition. DCW of (A) was 9.9 g/L at 24 hr and 25.3 g/L at 48 hr. Cell growth in (A) was maintained at an exponential phase until 48 hr. DCW was the highest at 72 hr (27.5 g/L). However, cell growth of (B) was maintained at the exponential phase up to 60 hr. DCW of (B) was 12.5 g/L at 24 hr and 30.2 g/L at 48 hr. DCW was the highest at 84 hr (38.3 g/L), which was 1.5 times higher than the DCW of (A). Similar to the composition of the YPD medium, the peptone-enhanced medium (A) maintained ethanol accumulation at a level of 2.3~3.0 g/L, while the yeast extract-enhanced medium (B) had 1.3~2.3 g/L ethanol accumulation. The cell growth of *S. cerevisiae* D452-2 with p426GPD strain could be increased by using an N-source medium having a high yeast extract ratio.

The beta-xylosidase expression levels of (A) and (B) were 204.5 $U/L_{culture\ broth}$ and 372.0 $U/L_{culture\ broth}$ at 48 hr, which were 1.3 times and 2.4 times higher than

those when glucose was fed at intervals of 24 hr (Figure 4-9), respectively. It was confirmed that glucose feeding at 12 hr intervals resulted in a higher beta-xylosidase expression level as compared with that achieved after feeding at 24 hr intervals; in addition N-source medium with a high yeast extract ratio could greatly increase beta-xylosidase expression level. The productivity values of (A) were 8.1 U/g_{DCW} at 48 hr and 7.3 U/g_{DCW} at 72 hr, while those for (B) were 12.3 U/g_{DCW} at 48 hr and 10.1 U/g_{DCW} at 72 hr. The productivity value of (B) was similar to that associated with the optimal C/N-ratio medium (13.1 U/g_{DCW}), as shown in Figure 4-6. The plasmid stability in both (A) and (B) was maintained at > 70%.

In this study, it was found that glucose feeding at 12 hr intervals had a positive effect on cell growth and beta-xylosidase productivity. Further, it was confirmed that the beta-xylosidase production of *S. cerevisiae* D452-2 with p426GPD could be effectively increased depending on the N-source composition even if the C/N ratio in the medium remains the same.

(A)



(B)

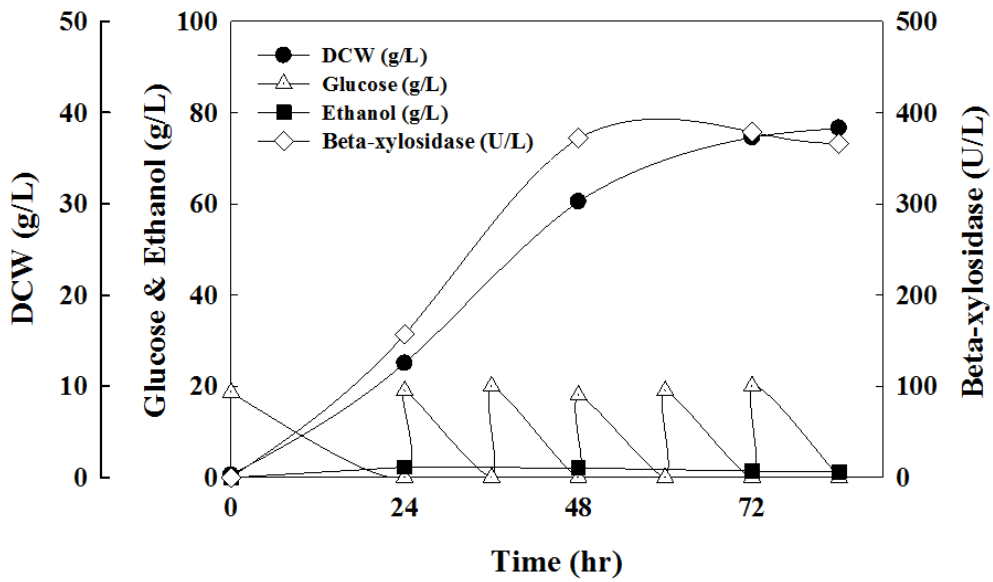


Figure 4-10. Comparison of beta-xylosidase productivity according to N-source composition in fed-batch fermentation. (A) Peptone enhanced medium: glucose 20 g/L, yeast extract 20 g/L, peptone 40 g/L; (B) Yeast extract enhanced medium: glucose 20 g/L, yeast extract 40 g/L, peptone 20 g/L.

4.2.6. Effect of glucose feeding speed on beta-xylosidase production in fed-batch fermentation

Recombinant proteins using yeast in a fed-batch culture are produced to maximize productivity by employing a high-cell density culture and by controlling the glucose feeding rate. An effective glucose feeding strategy is to add limited amount of glucose, which in turn minimizes ethanol formation rate, maintains a certain level of plasmid stability, and increases yeast growth and target protein productivity. Mendoza-Vega et al. (1994) reported that optimum productivity was achieved when the glucose feeding rate was adjusted to maintain ethanol levels at 2~6 g/L in the culture medium. In this study, an optimized glucose feeding rate, which was suitable for improving beta-xylosidase productivity, was confirmed.

The glucose feeding speed was 1, 2, 3, and 4 g/L in each fed-batch fermentation culture and glucose was fed at 24 hr after the start of cultivation. The total amount of glucose fed through the fed-batch was 96 g/L. As shown in Table 4-3, when the glucose feeding rates were 3 g/L/hr and 4 g/L/hr, the productivity values and beta-xylosidase were 15.2 U/g_{DCW} and 17.4 U/g_{DCW}, respectively. Considering the productivity of beta-xylosidase per the amount of glucose, the feeding speed was found to be the best at 3 g/L/hr.

Table 4-3. Comparison of beta-xylosidase productivity by glucose feeding speed

Feeding speed (g/L/hr)	Glucose feeding (g/L)	Feeding interval (hr)	Maximum productivity (U/g_{DCW})
1	24	24	4.6 at 120 hr
2	24	12	9.0 at 96 hr
3	12	4	15.2 at 72 hr
4	16	4	17.4 at 72 hr

4.2.7. Optimization of beta-xylosidase production in fed-batch fermentation

Optimum conditions for beta-xylosidase production in fed-batch cultivation were confirmed using yeast extract-enhanced medium (glucose 20 g/L, yeast extract 40 g/L, peptone 20 g/L) and a glucose feeding rate of 3 g/L/hr. Based on these optimization conditions, beta-xylosidase expression profiles were analyzed. It was also confirmed that by doubling the N-source concentration in the initial medium, enzyme productivity could be further improved (Figure 4-11).

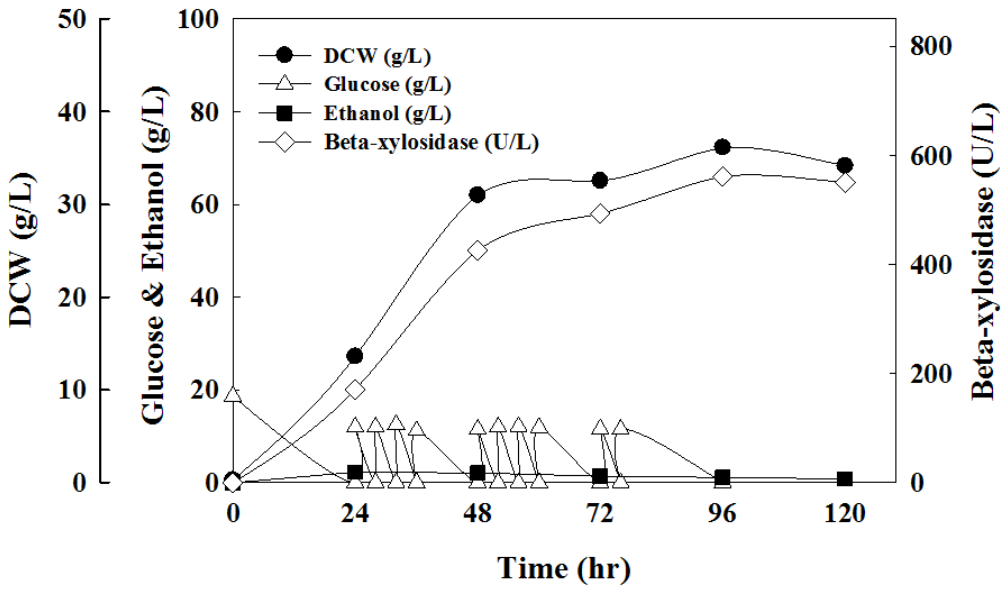
(A) was used for culturing in the above-mentioned yeast extract-enhanced medium. Twenty-four hours after the start of cultivation, 12 g/L of glucose was fed 10 times at 4 hr intervals. Cell growth was in the exponential phase until 48 hr, at which DCW was 31.0 g/L. Cells slowly increased until 96 hr and DCW increased to 36.1 g/L. Ethanol accumulated at a concentration of 1.1~2.5 g/L, suggesting that the feed rate of glucose was adequate. Beta-xylosidase expression levels in (A) were 425.9 $U/L_{culture\ broth}$ and 561.5 $U/L_{culture\ broth}$ at 48 hr and 96 hr, respectively. This level slightly decreased to 550.0 $U/L_{culture\ broth}$ at 120 hr. The productivity values for beta-xylosidase were 13.7 U/g_{DCW} at 48 hr and 15.6 U/g_{DCW} at 96 hr. This is similar to the maximum productivity at glucose feeding speed of 3 g/L/hr, which was confirmed to be an optimal feeding speed in Table 4-3.

(B) showed an improvement in the productivity of beta-xylosidase owing to the N-source enhancement in the initial medium under culture condition of (A). Cell growth in (B) was maintained at an exponential phase until 48 hr and DCW was 28.9 g/L. Cell growth reached its maximum at 96 hr, and DCW was 39.2 g/L.

During the fed-batch fermentation of (B), ethanol accumulation was maintained at 1.1~2.4 g/L in the culture broth. Beta-xylosidase levels in (B) were 544.2 $U/L_{culture\ broth}$ and 717.3 $U/L_{culture\ broth}$ at 48 hr and 96 hr, respectively. This was confirmed to be 1.3 times higher than those in (A). N-source concentration in the initial (B) medium was 2 times higher than that in (A), which did not affect the cell growth but affected the increase in beta-xylosidase expression. Beta-xylosidase productivity values of (B) were 18.8 U/g_{DCW} at 48 hr and 18.3 U/g_{DCW} at 96 hr, which was 1.4 times and 1.2 times higher than those of (A), respectively. It is the highest value among all other experimental results obtained so far. Expression of beta-xylosidase in *S. cerevisiae* D452-2 with p426GPD increased with increasing cell growth. The plasmid stability of both (A) and (B) was maintained at > 70%.

In this study, it was found that the optimal conditions for effectively improving beta-xylosidase expression in *S. cerevisiae* D452-2 with p426GPD were enhancement of the medium with yeast extract and maintenance of glucose feeding speed at 3 g/L/hr. As shown in Table 4-4, the amount of beta-xylosidase expression by fed-batch optimization was 9.5 times and 7.1 times higher than that of flask and batch culture, respectively. The beta-xylosidase productivity of the fed-batch culture was increased 4.7-fold and 3.2-fold higher than that of flask and batch culture, respectively.

(A)



(B)

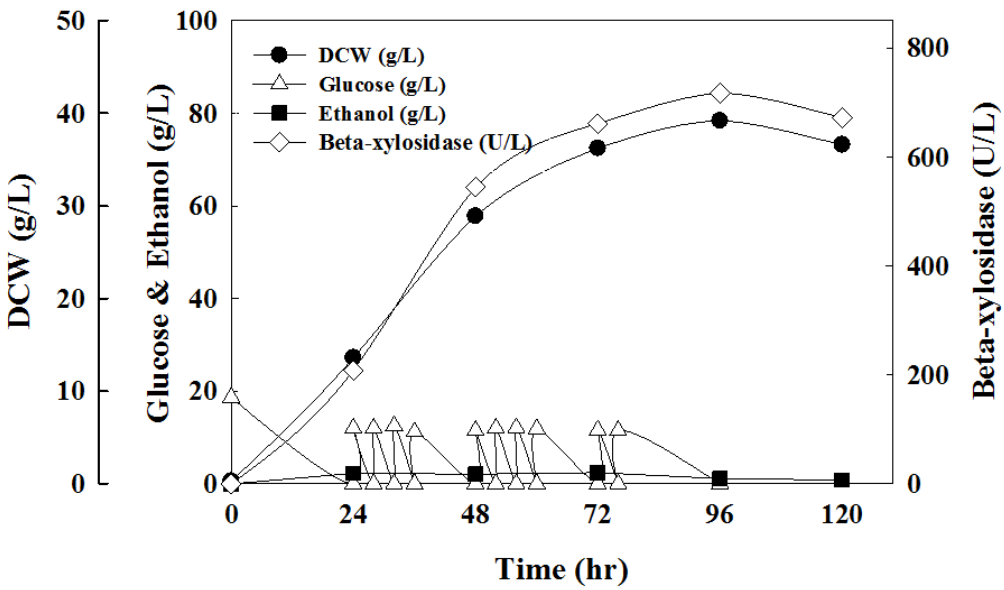


Figure 4-11. Profile of beta-xylosidase production under optimized medium conditions. (A) Optimized medium (glucose 20 g/L, yeast extract 40 g/L, peptone 20 g/L); (B) N-source enhanced optimized medium (glucose 20 g/L, yeast extract 80 g/L, peptone 40 g/L).

Table 4-4. Comparison of beta-xylosidase production

	S. cerevisiae		
	Flask	Batch	Fed-batch (optimization)
Beta-xylosidase (U/L)	75.4	101.6	717.3
Productivity (U/g_{DCW})	3.9	5.8	18.3
Culture time (hr)	120	48	96

In conclusion, to improve the productivity of beta-xylosidase, the fed-batch strategy was established by confirming the C-source/N-source ratio in the medium, N-source type in the medium, and glucose feeding speed. The expression and productivity of beta-xylosidase in flask cultivation was the highest at a C-source/N-source ratio of 0.67 in the medium. At this time, the expression level of beta-xylosidase was 325 $U/L_{culture\ broth}$, and the productivity was 12.9 U/g_{DCW}. The C/N ratio in the optimal medium was equal to that in the YPD medium. Beta-xylosidase production of *S. cerevisiae* D452-2 with p426GPD was found to be efficient in yeast extract-enhanced medium (20 g/L of glucose, 40 g/L of yeast extract, 20 g/L of peptone). To increase the productivity of beta-xylosidase through high-density cell cultivation in fed-batch, optimization experiments were conducted to control glucose metabolic rate. The best glucose feeding speed was 3 g/L/hr. Maximum productivity of beta-xylosidase was 18.8 U/g_{DCW}, and plasmid stability was over 70% during cultivation.

4.3. Purification of beta-xylosidase

4.3.1. Purification of beta-xylosidase expressed in *Escherichia coli* and *Saccharomyces cerevisiae*

Table 4-5 and Table 4-6 show the results of purification of the beta-xylosidase from *Bacillus pumilus* IPO (XynB) produced from the recombinant strains of *E. coli* and *S. cerevisiae*. XynB expressed in *E. coli* (XynB-E) was obtained through flask culture, and 1 L of the culture broth was purified. The total activity of crude cell extract was 920 $U/L_{culture\ broth}$, and after the Ni-NTA purification, 503.2 $U/L_{culture\ broth}$ of beta-xylosidase was obtained. Its specific activity was found to be 6.2 U/mg. XynB expressed in *S. cerevisiae* (XynB-S) was obtained through fed-batch fermentation and 1 L of the culture broth was purified. The total activity of crude cell extract was 680.0 $U/L_{culture\ broth}$, and after the Ni-NTA purification, 354.5 $U/L_{culture\ broth}$ of beta-xylosidase was obtained. Its specific activity was 6.3 U/mg, similar to that of *E. coli*.

Table 4-5. Purification table of beta-xylosidase from *B. pumilus* IPO expressed in *E. coli* (XynB-E)

	Total volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification fold
Cell extract	100.0	920.0	460.0	2.0	100	1
Ni-NTA	8.0	503.2	80.8	6.2	55	3

Table 4-6. Purification table of beta-xylosidase from *B. pumilus* IPO expressed in *S. cerevisiae* (XynB-S)

	Total volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification fold
Cell extract	200.0	680.0	880.0	0.8	100	1
Ni-NTA	5.0	354.5	56.0	6.3	52	8

4.3.2. Analysis of beta-xylosidase purity

The purified beta-xylosidase was analyzed using SDS-PAGE, as shown in Figure 4-12. Lane 1 is a sample obtained by purifying beta-xylosidase from *Bacillus pumilus* IPO expressed in recombinant *E. coli* (XynB-E) with Ni-NTA affinity resin, and Lane 2 is a sample obtained by purifying beta-xylosidase from *Bacillus pumilus* IPO expressed in recombinant *S. cerevisiae* (XynB-S) using the same method. Both samples were loaded with 10 μg protein and analyzed. The theoretical molecular weight of beta-xylosidase was 62 kDa, and two samples had bands of molecular weight ranging between 50 kDa and 75 kDa. This indicates that the molecular weight of beta-xylosidase is approximately 62 kDa. Highly purified beta-xylosidase was obtained, as confirmed by its appearance as a single band, indication the absence of any impurities.

SEC-HPLC was performed to confirm the purity of XynB-S as shown in Figure 4-13. The analytical sample was prepared at 200 μg/mL of protein concentration. Beta-xylosidase appeared as a single peak, as confirmed by SDS-PAGE. The retention time was 20 min and purity was 98%.

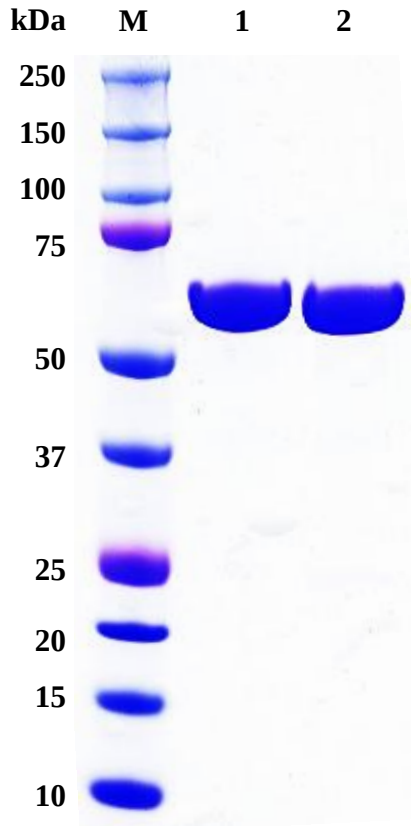


Figure 4-12. SDS-PAGE analysis of beta-xylosidase from *B. pumilus* IPO (XynB) expressed in different hosts. Lane M: marker; lane 1: purified XynB expressed in *E. coli* (XynB-E); lane 2: purified beta-xylosidase expressed in *S. cerevisiae* (XynB-S).

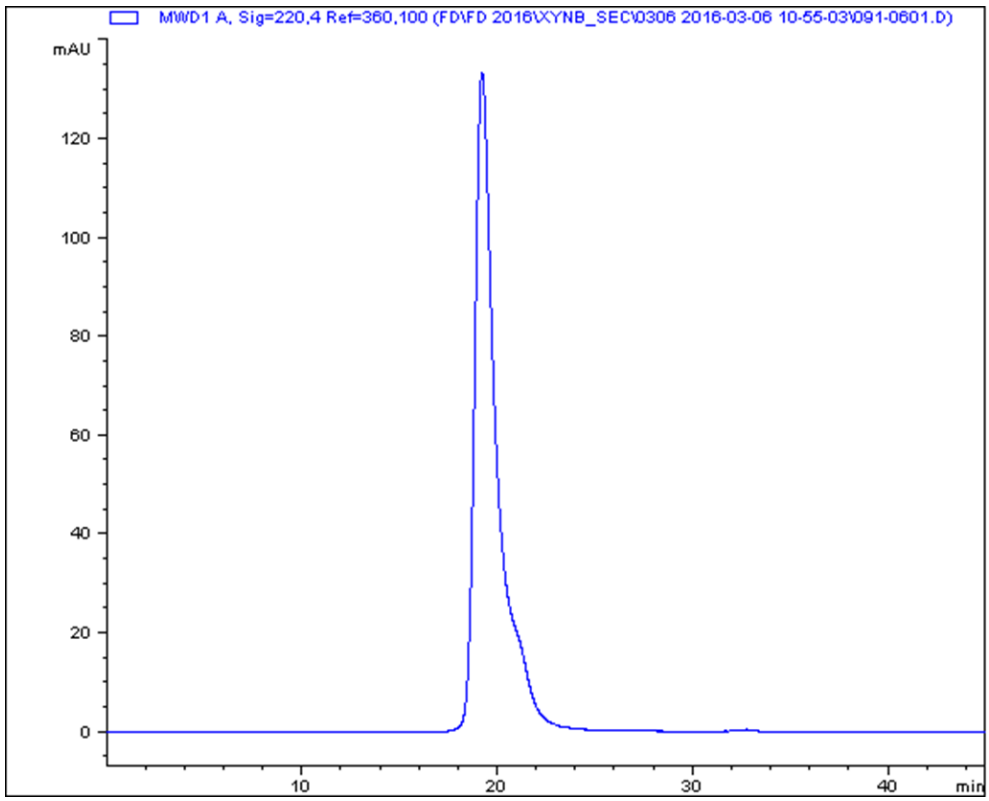
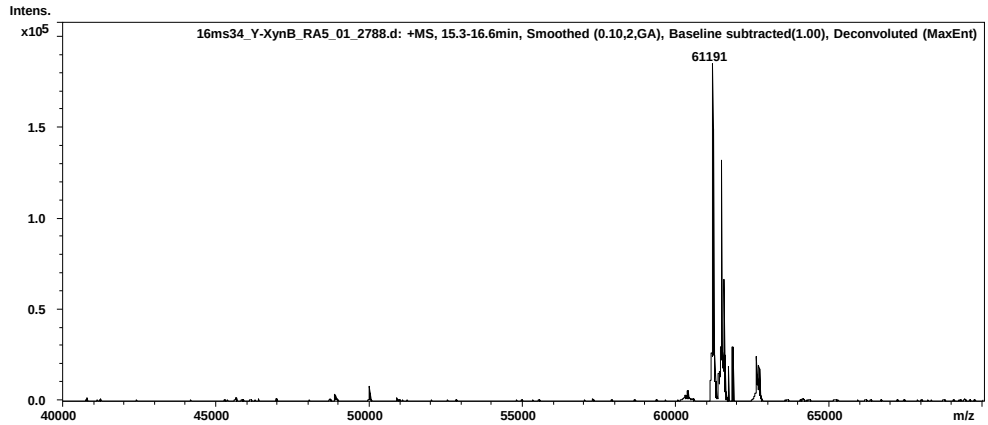


Figure 4-13. SEC-HPLC analysis of beta-xylosidase from *B. pumilus* IPO expressed in *S. cerevisiae* (XynB-S).

4.3.3. Determination of beta-xylosidase molecular weight

Figure 4-14 shows intact molecular weight and disulfide bond formation of beta-xylosidase from *Bacillus pumilus* IPO expressed in recombinant *S. cerevisiae* (XynB-S). (A) is the analysis result for the native form of enzyme, and its intact molecular weight was found to be 61,191 Da. (B) is the result of disulfide bond digestion by carrying out DTT reagent treatment and analyzing the monomer form of the enzyme. The molecular weight of the reduced form of the enzyme was 61,191 Da, which was the same as that of the native form (A). XynB-S was found to be in its monomeric form having no structural changes, and its intact molecular weight was found to be 61,191 Da. The molecular weight determined by LC-MS analysis was slightly smaller than the theoretical molecular weight, but this is considered an acceptable error range.

(A) Native form



(B) Reducing form

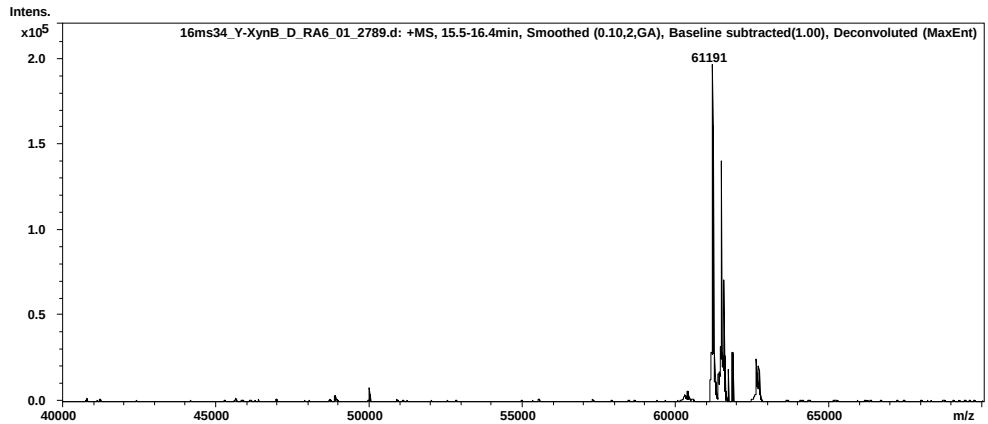


Figure 4-14. HPLC-ESI-MS analysis of beta-xylosidase from *B. pumilus* IPO expressed in *S. cerevisiae* (XynB-S). Native form (A) and reducing form (B).

4.4. Characterization of beta-xylosidase

4.4.1. Effect of temperature on beta-xylosidase activity

The optimal temperature of beta-xylosidase from *Bacillus pumilus* IPO expressed in recombinant *E. coli* and *S. cerevisiae* was confirmed. As shown in Figure 4-15, there was no difference in the activity of beta-xylosidase between the two hosts. The optimum temperature of beta-xylosidase was 30℃. The relative activity at 35℃ was approximately 90% as compared with the optimal activity, and while the relative activity at 40℃ represented approximately 85% of the optimal activity. However, the activity of beta-xylosidase at 45℃ dramatically decreased to less than half of the optimal activity. The activity of the enzyme was found to be unstable at 60℃, and its relative activity was 20%. Xu et al. (1991) reported that the optimum temperature for hydrolysis of beta-xylosidase from *Bacillus pumilus* IPO was 37℃ and the stability of the enzyme was maintained at 40℃. In this study, the optimal temperature for hydrolysis of beta-xylosidase from *Bacillus pumilus* IPO was 30℃, which was slightly different from that mentioned in a previous study (Xu et al. 1991).

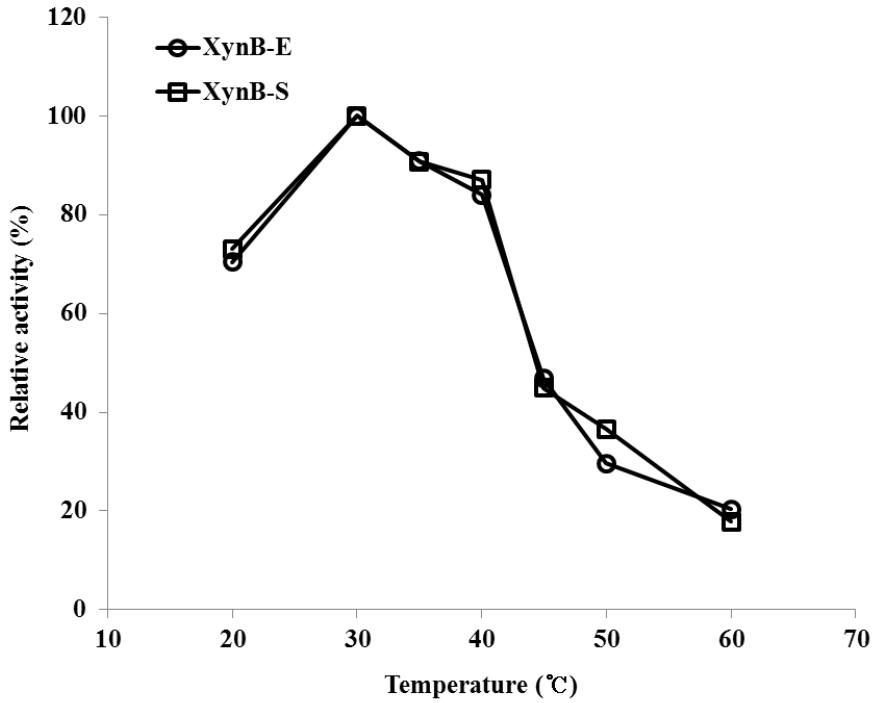


Figure 4-15. Optimum temperature of beta-xylosidase from *B. pumilus* IPO. The relative activity is calculated on the basis of beta-xylosidase activity observed at 30℃. XynB-E: beta-xylosidase expressed in *E. coli*; XynB-S: beta-xylosidase expressed in *S. cerevisiae*.

4.4.2. Effect of pH on beta-xylosidase activity

The optimal pH of beta-xylosidase from *Bacillus pumilus* IPO expressed in recombinant *E. coli* and *S. cerevisiae* was confirmed. As shown in Figure 4-16, the beta-xylosidase activity exhibited by the two hosts. At pH 5.0, the relative activity of beta-xylosidase was significantly reduced to 20% of the optimal activity. However, both enzymes were found to be very stable and maintain relative activity above 96% at pH 6.0~7.0. The optimum pH for both enzymes was 6.5. The relative activity of the enzyme at pH 8.5 was lowered to half compare with the optimal activity. Furthermore, the relative activity of beta-xylosidase expressed in *S. cerevisiae* tends to be lower than that of *E. coli* at pH 7.0~8.5. Xu et al. (1991) reported that the optimum pH for hydrolysis of beta-xylosidase from *Bacillus pumilus* IPO was 7.0, and this finding was consistent with that observed in the current study.

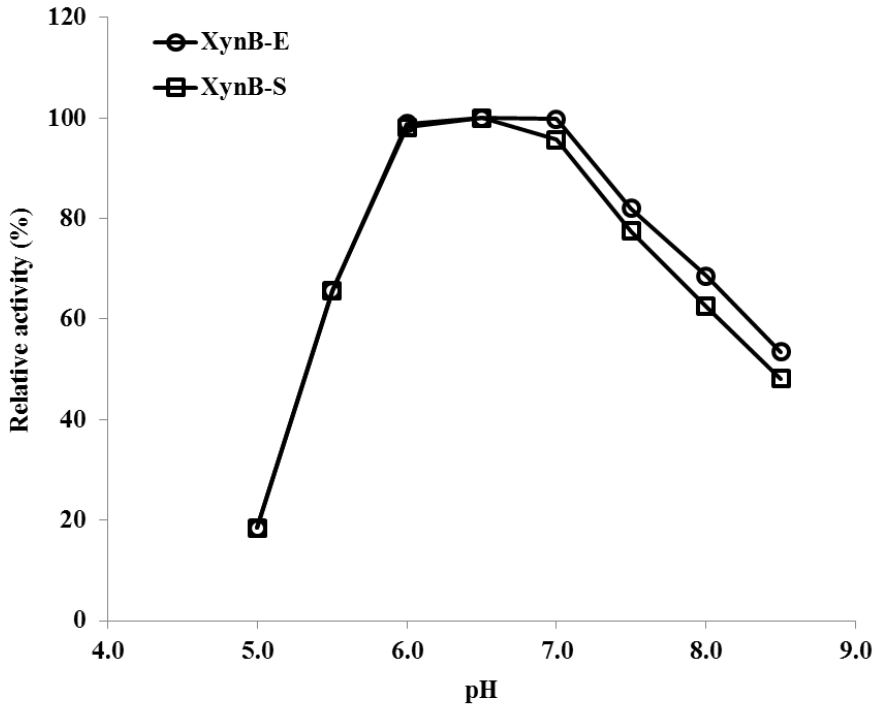


Figure 4-16. Optimum pH of beta-xylosidase from *B. pumilus* IPO. The relative activity is calculated on the basis of beta-xylosidase activity observed at pH 6.5. XynB-E: beta-xylosidase expressed in *E.coli*; XynB-S: beta-xylosidase expressed in *S. cerevisiae*.

In conclusion, the beta-xylosidase from *Bacillus pumilus* IPO (XynB) was expressed using recombinant strains of *E. coli* and *S. cerevisiae*. Beta-xylosidase expressed in *S. cerevisiae* was purified using Ni-NTA and the purity was over 98%. The intact molecular weight of beta-xylosidase was measured to be 61.2 kDa by LC-MS, which was similar to the theoretical molecular weight (62 kDa). The optimal conditions for the activity of beta-xylosidase were 30℃ and pH 6.5.

4.5. Enzymatic synthesis of xylobiose

4.5.1. Preliminary experiment on xylobiose synthesis

A preliminary experiment of xylobiose synthesis involving beta-xylosidase from *Bacillus pumilus IPO* expressed in *E. coli* by varying pH, temperature, xylose concentration, and enzyme amount to identify the main factors influencing xylobiose formation. As shown in Figure 4-17 (A), the optimum pH was 6 and the conversion rate of xylobiose was 9.6% when 1 U/mL beta-xylosidase was added to 4 M xylose. At pH 7 and pH 8, xylobiose conversion was 7.5%, which was slightly lower than at pH 6. The Figure 4-17 (B) shows temperature-dependent comparative analysis of xylobiose conversion ratio in the presence of 4 M xylose and 1 U/mL beta-xylosidase at pH 7. The conversion ratio of xylobiose at 40℃ was 8.7%, which was the highest. The conversion ratios at 30℃ and 50℃ were similar at 7.4% and 7.5%, respectively. The Figure 4-17 (C) shows that xylobiose was synthesized by varying xylose concentration in the presence of 1 U/mL beta-xylosidase. The xylobiose conversion was carried out at 50℃ for 24 hr at pH 7. The xylobiose conversion ratio tended to increase with increasing xylose concentration. Its conversion was up to 16.4% at 6.7 M (100%, w/v) xylose. However, xylobiose conversion ratio decreased to 15.6% at 7 M (105%, w/v) xylose. Katapodis et al. (2006) reported that xylobiose synthesis using 0.1 U/mL of fungal beta-xylosidase (*Sporotrichum thermophile*) can increase to 1.2% at a substrate concentration up to 4 M (60%, w/v) xylose. The bacterial beta-xylosidase (*Bacillus pumilus* IPO) selected in this study was found to carry out a condensation reaction at considerably higher xylose concentrations than those used in previous studies, and

along with a high conversion ratio of xylobiose. (D) shows xylobiose conversion based on the amount of enzyme added, while the substrate concentration was fixed at 6 M (90%, w/v). The conversion of xylobiose was evaluated by adding 0.1 U/mL, 1 U/mL, 5 U/mL, and 10 U/mL of beta-xylosidase. The xylobiose conversion ratio rapidly increased between 0.1 U/mL and 1 U/mL. On the other hand, the conversion ratios of xylobiose at 1 U/mL, 5 U/mL, and 10 U/mL of beta-xylosidase were 14.7%, 15.4%, and 14.9%, respectively. When beta-xylosidase level was above 1 U/mL, the conversion ratio of xylobiose did not increase significantly. Among the above-mentioned factors, xylobiose synthesis was considered to be most significantly affected by substrate concentration and enzyme amount.

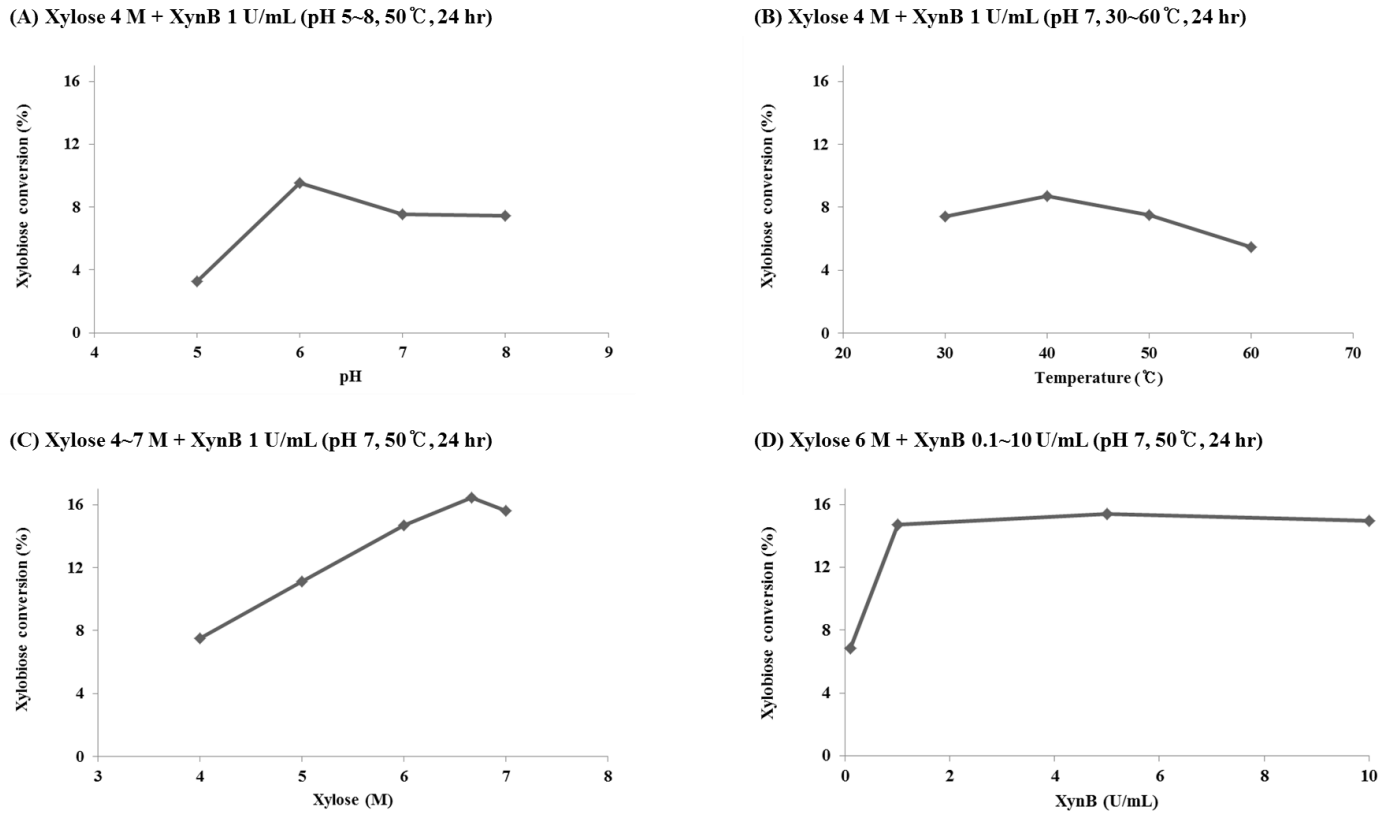


Figure 4-17. Comparison of xylobiose conversion according to pH (A), temperature (B), xylose concentration (C), and amount of beta-xylosidase (D).

4.5.2. Design of response surface methodology for enzymatic synthesis of xylobiose

CCD was used to efficiently optimize the conditions of xylobiose synthesis. As shown in Table 4-7, three independent variables were selected at the following ranges based on preliminary experiments conducted for xylobiose synthesis: xylose concentration, 4.0~6.0 M; beta-xylosidase amount, 0.5~5.0 U/mL; and reaction temperature, 45~55℃. Each independent variable comprised five levels ($-\alpha, -1, 0, +1, +\alpha$), and 20 test runs were designed with 14 experimental combinations and 6 center point replications. A total of 40 tests were performed by two sets of repeated experiments (Table 4-8). The reactions for xylobiose synthesis were all performed in a random order. Figure 4-18 shows CCD for xylobiose synthesis. CCD consists of eight cube points, six axial points, and one center point. Table 4-8 shows the results of xylobiose production and the experimental conditions for each factor established using CCD. Xylobiose production (Y, g/L) is represented by an empirical second-order polynomial equation (1).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

X_1, X_2, and X_3 : the level of independent variables

β_0 : the model constant term

β_1, β_2, and β_3 : the linear coefficients

β_{11}, β_{22}, and β_{33} : the quadratic coefficients

β_{12}, β_{13}, and β_{23} : the interactive coefficients

Table 4-7. Levels of independent variables for central composite design

Independent variables	Symbol	Coded level				
		$-\alpha$[1)]	−1	0	+1	$+\alpha$
Substrate concentration (M)	X_1	3.3	4.0	5.0	6.0	6.7
Enzyme amount (U/mL)	X_2	−1.0[2)]	0.5	2.75	5.0	6.5
Reaction temperature (℃)	X_3	41.6	45.0	50.0	55.0	58.4

[1)] α = 1.68179.

[2)] −1.0 was adjusted to zero in the reaction experiment.

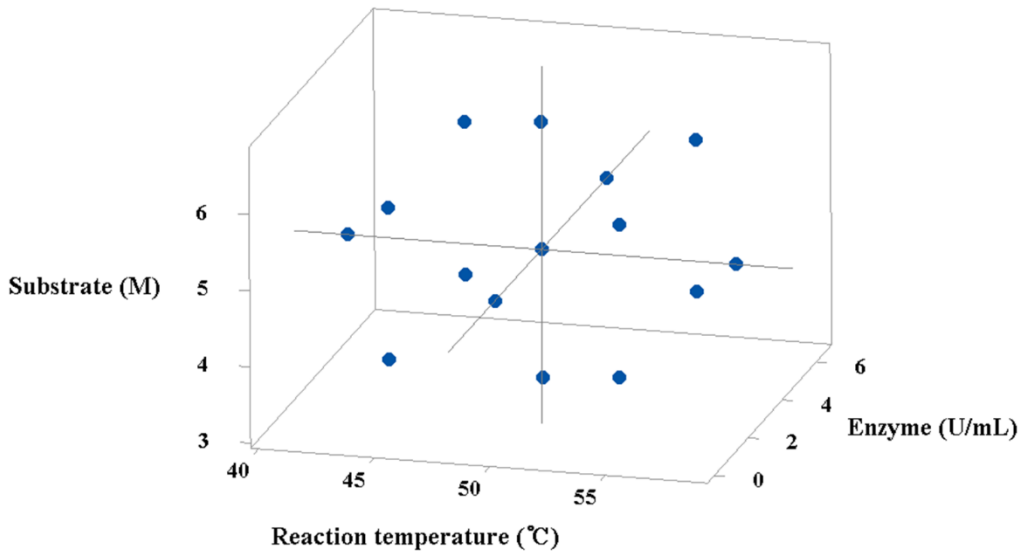


Figure 4-18. Central composite design (CCD) for optimization of xylobiose synthesis.

Table 4-8. Central composite design and observed experimental data for xylobiose production

Run [1]	block	X_1	X_2	X_3	Conversion (%)	Production (g/L)	Productivity [2]
1	1	5.0	2.8	50.0	12.3	92.2	0.03
2	1	3.3	2.8	50.0	6.9	34.4	0.01
3	1	5.0	2.8	50.0	12.4	92.9	0.03
4	1	5.0	6.5	50.0	12.7	95.5	0.01
5	1	5.0	2.8	50.0	12.4	92.8	0.03
6	1	5.0	2.8	50.0	12.4	93.3	0.03
7	1	5.0	2.8	50.0	12.5	93.4	0.03
8	1	4.0	0.5	45.0	8.0	48.0	0.10
9	1	6.7	2.8	50.0	12.4	124.0	0.05
10	1	4.0	5.0	55.0	9.1	54.7	0.01
11	1	5.0	2.8	58.4	12.1	91.0	0.03
12	1	5.0	2.8	50.0	12.4	93.0	0.03
13	1	6.0	5.0	55.0	17.6	158.8	0.03
14	1	4.0	0.5	55.0	7.8	46.8	0.09
15	1	6.0	0.5	55.0	11.6	104.2	0.21
16	1	6.0	0.5	45.0	8.8	78.8	0.16
17	1	5.0	0.0	50.0	0.0	0.0	0.00
18	1	6.0	5.0	45.0	17.5	157.5	0.03
19	1	4.0	5.0	45.0	6.6	39.7	0.01
20	1	5.0	2.8	41.6	12.0	90.2	0.03
21	2	6.0	0.5	45.0	9.0	80.8	0.16
22	2	5.0	2.8	50.0	12.4	92.8	0.03
23	2	5.0	2.8	50.0	12.3	92.5	0.03
24	2	3.3	2.8	50.0	7.0	35.1	0.01
25	2	5.0	2.8	41.6	12.3	92.1	0.03
26	2	4.0	0.5	55.0	7.9	47.4	0.09
27	2	5.0	2.8	58.4	12.2	91.8	0.03
28	2	5.0	2.8	50.0	12.4	93.4	0.03
29	2	5.0	2.8	50.0	12.6	94.4	0.03
30	2	5.0	2.8	50.0	12.7	95.5	0.03
31	2	6.0	5.0	45.0	17.2	155.2	0.03
32	2	4.0	5.0	45.0	9.1	54.7	0.01
33	2	6.7	2.8	50.0	11.5	114.9	0.04
34	2	5.0	2.8	50.0	12.6	94.6	0.03
35	2	5.0	0.0	50.0	0.0	0.0	0.00
36	2	6.0	0.5	55.0	11.8	106.4	0.21
37	2	4.0	5.0	55.0	9.0	54.1	0.01
38	2	4.0	0.5	45.0	8.4	50.4	0.10
39	2	5.0	6.5	50.0	13.1	98.3	0.02
40	2	6.0	5.0	55.0	17.7	159.4	0.03

[1] Experimental run was performed in a random order.

[2] Productivity (g/U) = Production (g/L) / X_2 (U/mL) × 1 L/1000 mL.

4.5.3. Development of the response surface methodology model and statistical analysis for xylobiose production

Model in Table 4-9 was derived from 40 experimental results that were planned using a combination of xylose concentration (X_1), beta-xylosidase amount (X_2), and reaction temperature (X_3). The response value (Y) was xylobiose production (g/L), and productivity was applied as a weight to increase the reliability of the model. The degree of freedom was 37 because two abnormal observations (Run 17 and Run 35) with standardization residuals outside the range of ± 3.0 were excluded. Moreover, R-squared of the model was 0.9643, suggesting high accuracy of the model for predicting xylobiose production; moreover, the reliability of the response could be expected to be 96.4%. The correlation between xylobiose production (Y, g/L) and the three independent variables is shown by a second-order polynomial equation (2).

$$\text{Xylobiose production (g/L)} = 126.00 + 24.70\,X_1 - 1.37\,X_2 - 7.79\,X_3 - 6.97\,X_1^2 - 0.28\,X_2^2 + 0.03\,X_3^2 + 5.88\,X_1 X_2 + 1.26\,X_1 X_3 - 0.39\,X_2 X_3 \quad (2)$$

The linear coefficients of the three independent variables in the regression model equation are X_1 = 24.70 (xylose concentration, M), X_2 = −1.37 (beta-xylosidase amount, U/mL), and X_3 = −7.79 (reaction temperature, ℃). The order of independent variables affecting xylobiose production was X_1 (xylose concentration) > X_3 (reaction temperature) > X_2 (beta-xylosidase amount).

Analysis of variance (ANOVA) is used to verify the hypotheses associated the parameters of the model. The mean squared value was determined based on a 95% confidence interval ($\alpha = 0.05$). A *p*-value is used as a means of confirming the importance of each source. A *p*-value of less than 0.05 indicates that the model is significant, while a value greater than 0.10 is considered insignificant (Xu et al. 2008, Francesc and Julia. 2014). The results of ANOVA for model equation are shown in Table 4-10. The *p*-value was less than 0.002 in model, linear, square and interaction coefficients. This model was highly accurate for predicting xylobiose production. In particular, it was shown that the *p*-value is less than 0.001 in X_1 (xylose concentration), X_2 (beta-xylosidase amount), X_1^2 (xylose concentration × xylose concentration), $X_1 \cdot X_2$ (xylose concentration × beta-xylosidase amount), $X_1 \cdot X_3$ (xylose concentration × reaction temperature) was very significant. Among them, the independent variable X_1 showed a very high influence in this model.

Table 4-9. Model summery statistics

Source	Degree of freedom	Standard deviation	R-squared	Adjusted R-squared	Predicted R-squared
Model	37	1.43	0.9643	0.9529	0.9239

Table 4-10. Analysis of variance (ANOVA) for the xylobiose production model

Source	Degree of freedom	Adjusted sum of squares	Adjusted mean of square	F-value	*p*-value
Model	9	1548.6	172.1	84.1	0.000
Linear	3	1327.6	442.5	216.4	0.000
X_1	1	795.8	795.8	389.2	0.000
X_2	1	155.7	155.7	76.1	0.000
X_3	1	1.9	1.9	0.9	0.341
Square	3	39.1	13.0	6.4	0.002
$X_1 \cdot X_1$	1	32.5	32.5	15.9	0.000
$X_2 \cdot X_2$	1	1.0	1.0	0.5	0.497
$X_3 \cdot X_3$	1	0.4	0.4	0.2	0.646
Interaction	3	160.7	53.6	26.2	0.000
$X_1 \cdot X_2$	1	99.7	99.7	48.8	0.000
$X_1 \cdot X_3$	1	46.6	46.6	22.8	0.000
$X_2 \cdot X_3$	1	14.7	14.7	7.2	0.012
Residual error	28	57.3	2.0	-	-
Leak of fit	4	52.7	13.2	69.8	0.000
Pure error	24	4.5	0.2	-	-
Linear	3	1327.6	442.5	216.4	0.000
Total	37	1605.8	-	-	-

The residual analysis is based on the assumption that the results obtained from the experiment satisfy the linear condition, i.e., the mean and the variance, the mutual independence, and the relationship between independent and dependent variables satisfy the linear condition (Park and Row. 2013). To investigate the suitability of the prediction model for xylobiose production, a residual analysis, which is the difference between the measured value of the dependent variable and the value predicted by regression equation, was performed for the model by following the prediction equation (2).

The normal probability plot of the standardization residuals in Figure 4-19 showed a linear regression and could assume a normal distribution. The scatter plot of the residuals versus log of fit was randomly distributed within the horizontal bands with normalized residuals and fitted values within ± 3.0, which suggested that the variance of the residuals is constant. The residual histogram indicates whether the data is biased toward a specific direction or whether the data has any abnormal value. The histogram in Figure 4-19 followed a normal distribution. The residuals versus order indicates that the residuals are correlated according to the order of experiments. It was confirmed that the scatter plot did not show any direction with regard to order, and that the residuals did not interact with each other.

Therefore, it is considered that the predicted model can be applied to the optimization of xylobiose synthesis because statistical analysis proved that its results were reliable.

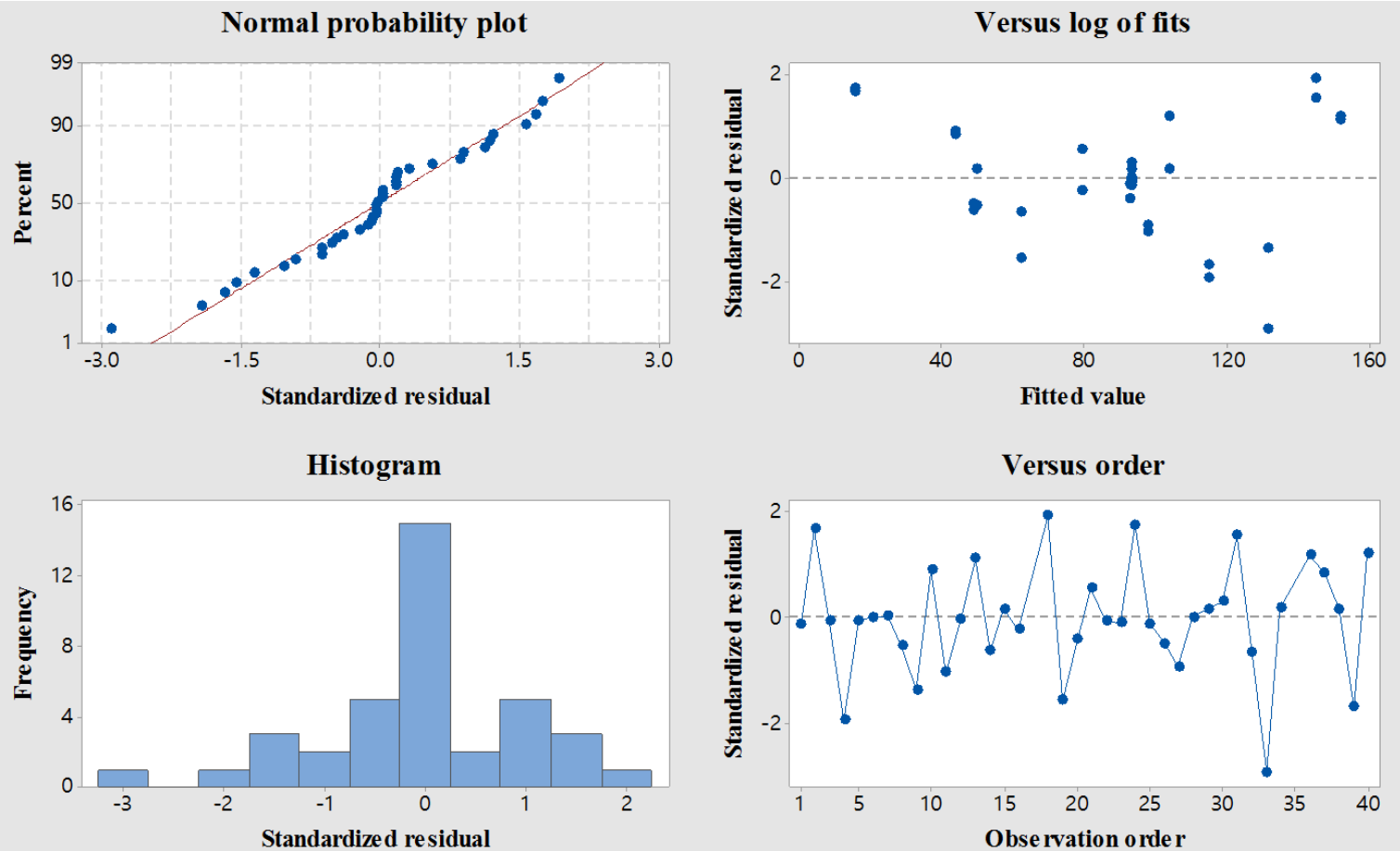


Figure 4-19. Residual plots for xylobiose production.

4.5.4. Effects of independent variables and their interactions

Xylobiose synthesis results based on 20 combinations were used to obtain three-dimensional response surface and two-dimensional contour plots as shown in Figure 4-20, Figure 4-21, and Figure 4-23. These plots show the regression equation used for obtaining xylobiose synthesis results; these plots could further be used to demonstrate the characteristics of reaction parameters.

Figure 4-20 shows the interaction effects of X_1 (xylose concentration, M) and X_2 (beta-xylosidase amount, U/mL) on xylobiose production (g/L). (A) is the three-dimensional response surface plot and (B) is the two-dimensional contour plot. Xylose concentration and beta-xylosidase amount were positively correlated with xylobiose production. In the xylose concentration range of 3.0~4.6 M, xylobiose production remained constant despite an increase in the amount of beta-xylosidase. However, when xylose concentration exceeded 5.0 M, xylobiose production proportionally increased with an increase in beta-xylosidase level. Xylose concentration had a greater effect on xylobiose production. On the other hand, xylobiose production tended to decrease with an increased in the amount of beta-xylosidase only when xylose concentration was < 3.5 M. The presence of high beta-xylosidase levels at low substrate concentrations may be attributed to dominant hydrolysis reaction rather than condensation reaction. The results of xylobiose production analysis based on the relationship between xylose concentration and beta-xylosidase amount revealed that xylobiose production of over 150 g/L required more than 6.0 M xylose and 4 U/mL beta-xylosidase.

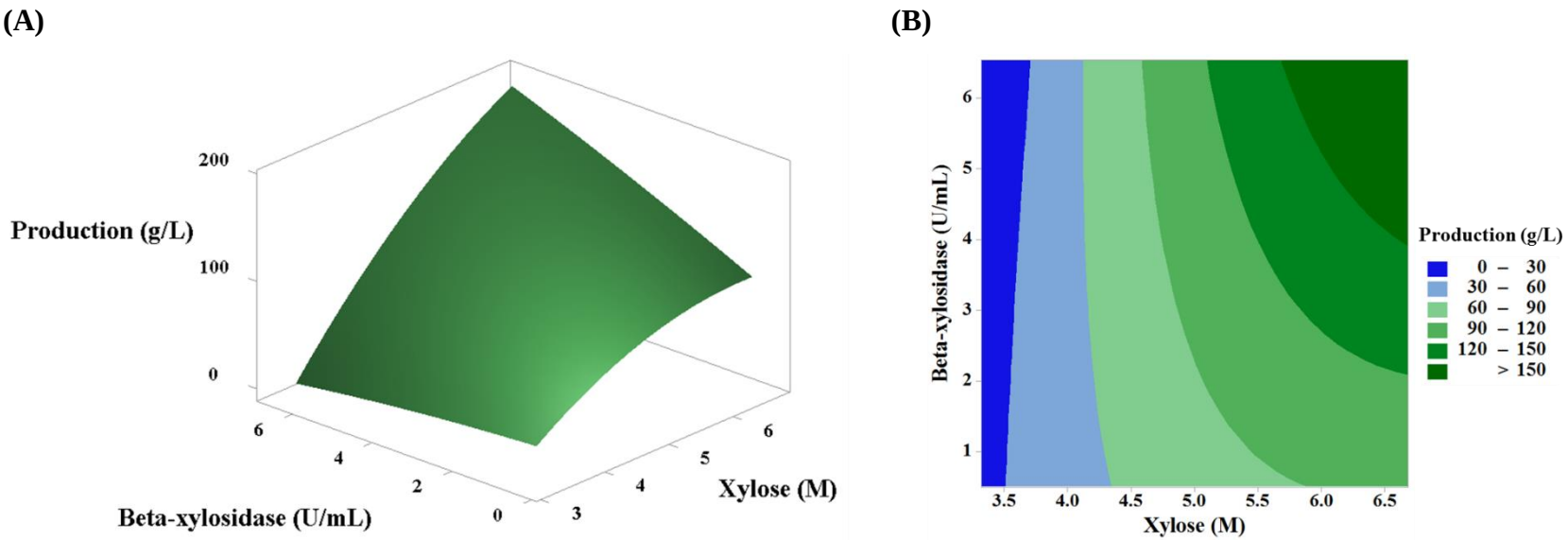


Figure 4-20. Response surface plot (A) and contour plot (B) for the relationships between xylobiose production and experimental variables (Xylose vs Beta-xylosidase).

Figure 4-21 shows the effects of interaction between X_1 (xylose concentration, M) and X_3 (reaction temperature, ℃) on xylobiose production (g/L). (A) is the three-dimensional response surface plot and (B) is the two-dimensional contour plot. Xylose concentration and reaction temperature were positively correlated with xylobiose production. In the xylose concentration range of 3.0~5.5 M, xylobiose production remained constant even when the reaction temperature was increased. Xylobiose production increased with increasing temperature at a xylose concentration of > 5.5 M. In particular, maximal xylobiose production (150 g/L) occurred at a very narrow range xylose concentration, i.e., of 6.5 M, and at a temperature range of 57~58℃. The investigation of the relationship among the xylose concentration, reaction temperature, and xylobiose production revealed that xylobiose production more likely to be influenced by xylose concentration. This could explain the reason why the temperature constant is the smallest among the linear coefficients of the model equation (2). In addition, xylobiose production tended to decrease with an increase in the reaction temperature when xylose concentration was < 3.7 M; this phenomenon could be attributed to the thermal stability of the enzyme because the optimum temperature for beta-xylosidase from *Bacillus pumilus* IPO was 30℃ (as shown in Figure 4-15). The relative activity of beta-xylosidase was approximately 85% at 40℃, however, the enzyme activity was dramatically decreased at a temperature of > 40℃, and its relative activity was found to be below < 20% at 60℃. As shown in Figure 4-21 (A), beta-xylosidase at a low xylose concentration led to a decreased xylobiose production with increasing temperature, but beta-xylosidase at high xylose concentration led to an increased xylobiose production with increasing temperature. This phenomenon indicates that

xylose concentration affects the stability of the enzyme depending on the temperature conditions. Beta-xylosidase present at a high xylose concentration was able to increase xylobiose production by improving its thermal stability even at elevated temperatures.

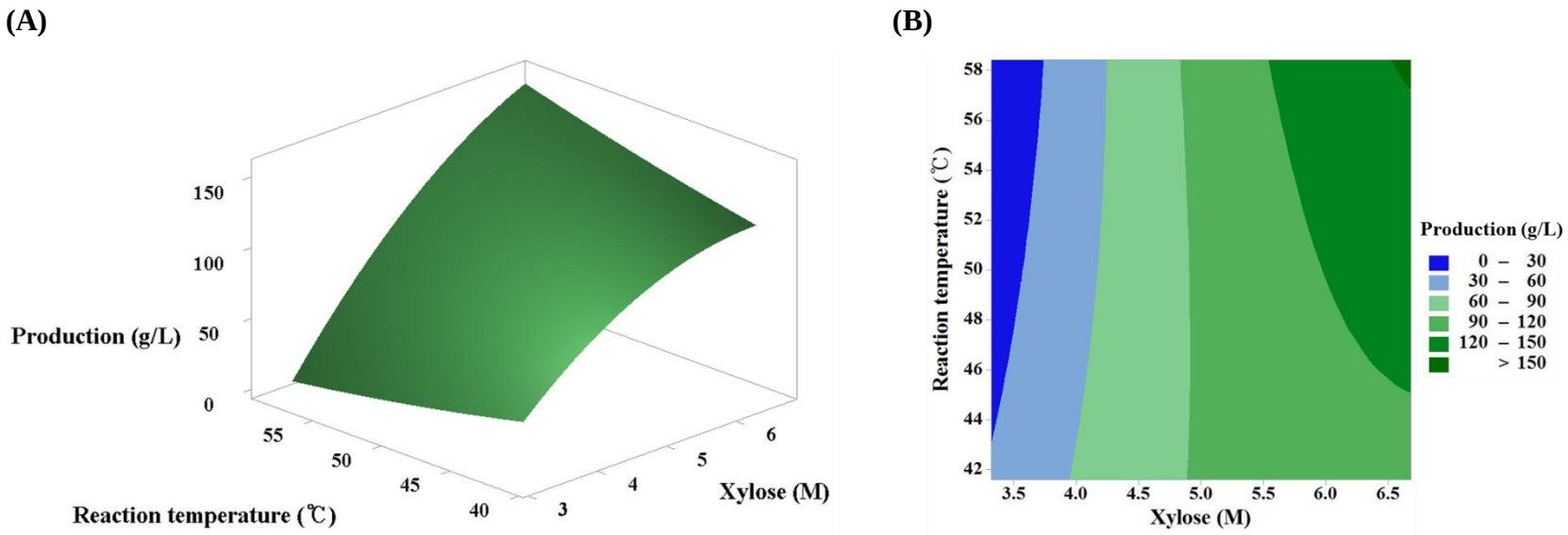


Figure 4-21. Response surface plot (A) and contour plot (B) for the relationships between xylobiose production and experimental variables (Xylose vs Reaction temperature).

Figure 4-22 shows the enzyme thermal stability with regard to temperature and xylose. As shown in (A), the relative activity of beta-xylosidase at 40℃ in the absence of xylose was maintained at 80% of the initial activity after 24 hr. However, the relative activity of beta-xylosidase in the presence of high-xylose concentration (5 M) was 60% of the initial activity after 1 hr, and decreased steadily thereafter, with approximately 20% activity remaining after 24 hr. (C) and (D) show that the relative activity of beta-xylosidase in the absence of xylose was rapidly decreased to 30% and 10%, respectively, within 1 hr, and the enzyme activity was undetectable after 6 hr in both conditions. However, when beta-xylosidase was present with 5 M xylose, 50% and 40% of enzyme activity remained at 1 hr at 60℃ and 70℃, respectively. Interestingly, when beta-xylosidase was incubated for 6 hr at 50℃, residual activity remained the same at 30% regardless of the absence or presence of 5 M xylose as shown in (B). The beta-xylosidase activity in the absence of xylose was relatively high until 6 hr, and the relative activity of the enzyme in the presence of 5 M xylose remained high after 6 hr. In addition, beta-xylosidase in the absence of xylose was inactivated at 18 hr, but enzyme activity in 5 M xylose remained at 20%.

This experiment confirmed the effect of high-concentration xylose on the thermal stability of the enzyme over time with the same amount of beta-xylosidase (1 U/mL). However, the hydrolytic activity of pNPX was measured in the presence of 50 mM xylose (100-fold dilution). Therefore, the hydrolytic activity in the presence of xylose could be reduced to a greater extent by the effect of high-concentration xylose than with the activity of beta-xylosidase in the absence of xylose. As shown in (A), the relative decrease of beta-xylosidase in the presence of

5 M xylose at 40℃ appears to be due to the negative effect of high-concentration xylose on activity measurement. Nevertheless, beta-xylosidase showed a greater tendency to maintain relative activity for a long time with the presence of 5 M xylose at 50-70℃, than with the absence of xylose. It seems that high concentrations of xylose may have an effect on improving the thermal stability of beta-xylosidase as a stabilizer. As shown in Figure 4-15, beta-xylosidase is a weakly thermostable enzyme, but the presence of high concentrations of xylose enhances the heat resistance of beta-xylosidase as shown in Figure 4-22 (B) to (D). This suggests that this phenomenon is similar to that reported in previous studies, showing that the high stability of saccharides in combination with proteins improves the thermal stability of proteins. Lee and Timasheff (1981) reported the interaction between protein and sucrose solution. The effect of sucrose concentration was confirmed by measuring the thermodynamic and kinetic density of alpha-chymotrypsin, chymotrypsinogen, and ribonuclease. The presence of sucrose along with the protein did not induce a structural change in the protein, and the thermal denaturation of alpha-chymotrypsin decreased with increasing sucrose concentration. Gianfreda and Scarfi (1991) reported that one of the techniques for enhancing enzyme stability is the addition of saccharides. Usually, sucrose, glycerol, sorbitol or ethanol can be added and stored at a temperature below 0℃ for facilitating stable storage without denaturation of the purified enzyme and protein. The presence of proteins together with stabilizers (sucrose, lactose, polyhydric alcohols, etc.) increases stabilization, thereby aiding the maintenance of a stable structure with the least surface area; additionally, the effects of undesirable free energy changes and interactions with water are minimized.

Consequently, it was confirmed that the appropriate synthesis temperature of xylobiose was higher than the optimal hydrolysis activity temperature. The transglycosylation reaction of inverting glycosidase, which is the reverse reaction of the hydrolysis reaction, requires higher activation energy (Ea) than hydrolysis does. Therefore, the enzyme with improved heat resistance is expected to be more advantageous at high temperature reaction than the low temperature.

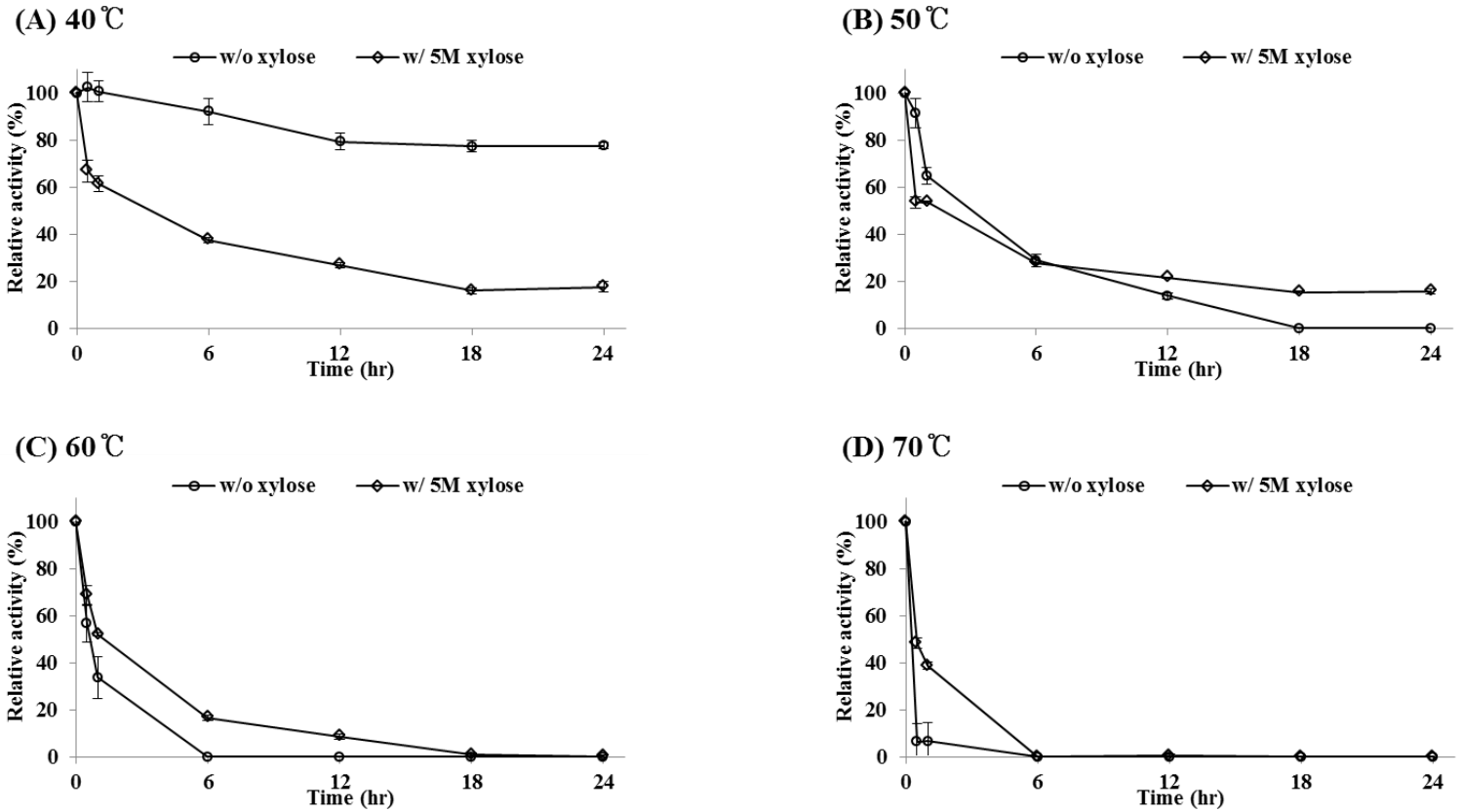


Figure 4-22. Comparison of beta-xylosidase thermal stability by temperature and xylose concentration.

Figure 4-23 shows the effect of interaction between X_2 (beta-xylosidase amount, U/mL) and X_3 (reaction temperature, ℃) on xylobiose production (g/L). (A) is the three-dimensional response surface plot and (B) is the two-dimensional contour plot. Beta-xylosidase amount and reaction temperatures positively correlated with xylobiose production, but the two independent variables had little effect on xylobiose production. Xylobiose production tended to increase sharply as the amount of beta-xylosidase increased under low-temperature conditions (40~50℃), but xylobiose production tended to increase steadily at a high-temperature range (50~60℃) even when the amount of beta-xylosidase increased. On the other hand, xylobiose production decreased with increasing temperature at a beta-xylosidase amount of 6.0~6.5 U/mL. This result is attributable to the stability of the enzyme with temperature at a constant xylose concentration. Xylobiose production increased with increasing beta-xylosidase level. In particular, xylobiose production was highest at a beta-xylosidase concentration of 5.6~6.5 U/mL and a temperature range of 42~46℃.

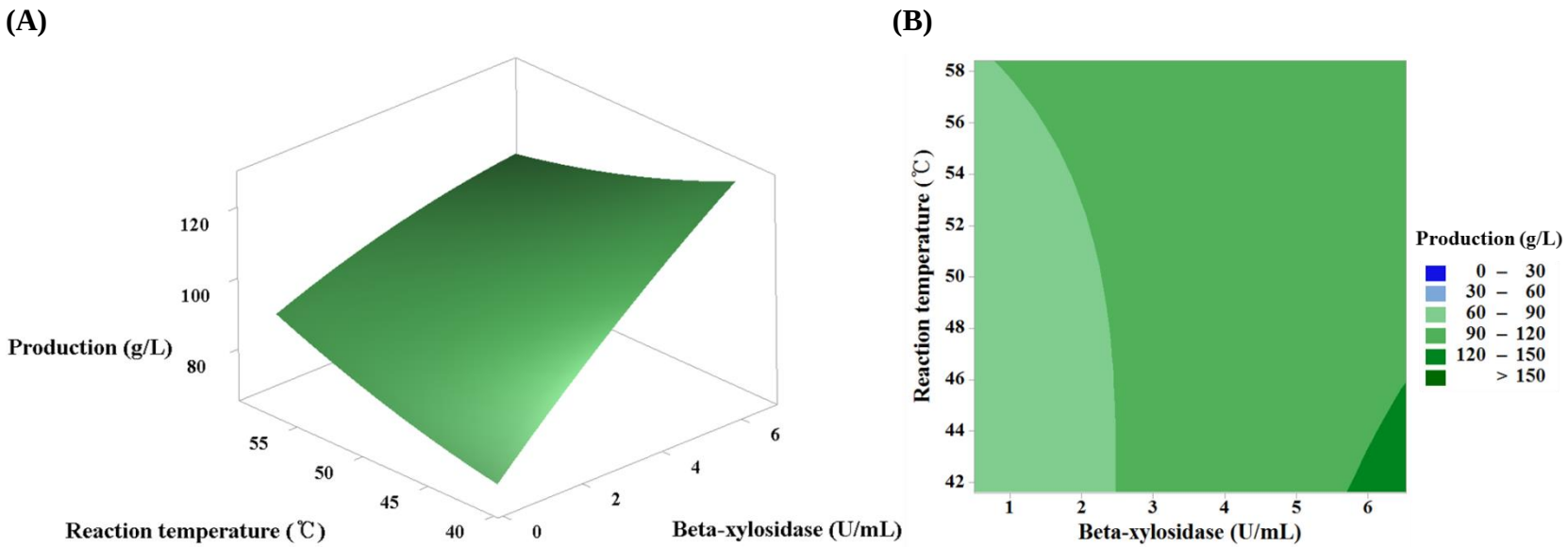


Figure 4-23. Response surface plot (A) and contour plot (B) for the relationships between xylobiose production and experimental variables (Beta-xylosidase vs Reaction temperature).

4.5.5. Optimization of xylobiose production

The reaction optimization diagram for xylobiose production was obtained using the Minitab 17 as shown in Figure 4-24. By adjusting the three independent variables of the optimization diagram, the maximum response value (Y, g/L) and desirability (D) are predicted. The red vertical line in the graph represents the current settings of each independent variable, and the blue horizontal dotted line represents the predicted response value. The gray area represents the reaction condition of the independent xylobiose production variable, which was < 150 g/L. Three xylobiose production conditions were predicted using the optimization diagram shown in Table 4-11. X_1 (xylose concentration, M) and X_2 (beta-xylosidase amount, U/mL) values were set at 6.7 M (100%, w/v) and 6.5 U/mL, respectively, and xylobiose production levels were predicted according to the three temperature conditions.

Xylobiose production was predicted to increase with increasing temperature. It was predicted to be 199.2 g/L at 58.4℃. The results of xylobiose production are shown in Table 4-11 and Figure 4-25. Xylobiose produced 159.8 g/L at 45℃, which was 13.8% lower than the predicted value. During the reaction at 45℃, xylose precipitated to > 50% of the reaction volume, which is considered to produce a lower level of xylobiose than expected. As a result of xylose solubility determination, it was confirmed that xylose concentration of 4 M (60%, w/v) and 6 M (75%, w/v) were dissolved at 30℃ and 60℃, respectively. The strategy to maximize xylobiose production is to maximize xylose concentration as the strongest independent variable. The xylose concentration was fixed at 6.7 M (1,000

g/L). When the reaction was carried out at 40℃, xylose in the initial reaction mixture existed at a supersaturated state, but it was slowly precipitated as the reaction proceeded for 24 hr. This suggests that xylobiose was produced at a significantly smaller amount than the predicted value. In addition, the standard deviation of xylobiose production at 45℃ was large owing to the difference in the amount of xylose precipitation between the repeated test groups.

Xylobiose was produced at concentrations of 207.5 g/L and 206.1 g/L at 50℃ and 58.4℃. These concentrations were 9.2% and 3.2% higher than the predicted levels, respectively. The amount of precipitated xylose at 50℃ and 58.4℃ was less than 5% of the total reaction volume over 24 hr. The reaction solution for xylobiose production was mixed with supersaturated xylose solution, beta-xylosidase, and buffer. The solid content excluding xylose was less than 1%. At a lower temperature, high-purity supersaturated xylose solution may increase the precipitation amount. The solubility of xylose was relatively increased and the content of xylose decreased rapidly as the amount of xylobiose increased. Xylobiose synthesis at 50℃ and 58.4℃ could prevent xylose precipitation because beta-xylosidase produced more amount of xylobiose than that produced at 45℃.

As shown in Figure 4-21, xylobiose production increased with increasing temperature when the amount of beta-xylosidase was fixed, but the amount of xylobiose production in the optimization experiment was similar to that produced at 50℃ and 58.4℃. Furthermore, xylobiose production tended to decrease at 58.4℃

than at 50℃. The xylobiose production reached an equilibrium between 50℃ and 58.4℃. The relative hydrolysis activity of beta-xylosidase was the highest at 30℃ and the enzyme stability was maintained at 85% at a temperature of 40℃. An optimum temperature for xylobiose production should be considered for the stability of beta-xylosidase and minimum xylose precipitation. Therefore, the production temperature of xylobiose should be set at 50℃.

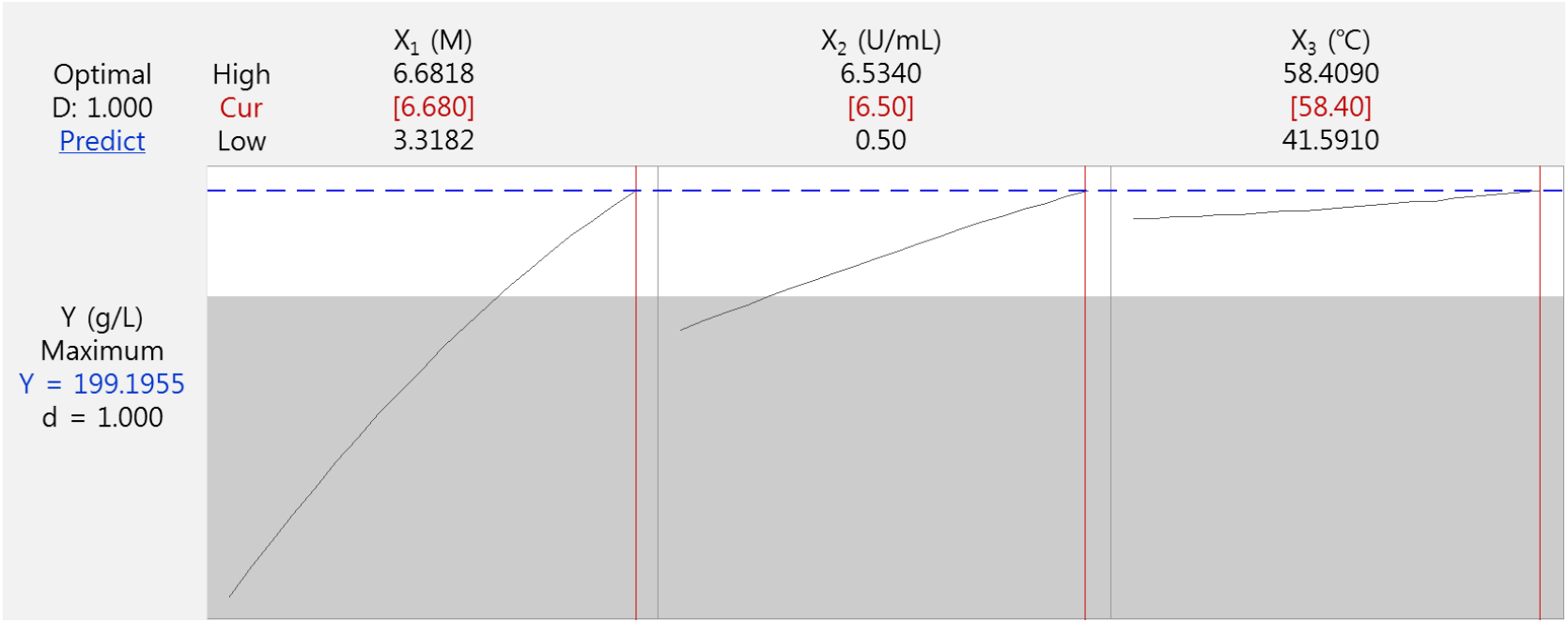


Figure 4-24. Response optimization plot for xylobiose production.

Table 4-11. Validation of response optimization trial for xylobiose production

Independent variable			D [4]	Production (g/L)		Error
X_1 [1]	X_2 [2]	X_3 [3]		Predicted value	Experimental value	
6.68	6.5	45.0	1.000	185.5	159.8 ± 13.5 [5]	-13.8%
6.68	6.5	50.0	1.000	189.4	207.5 ± 0.6	9.2%
6.68	6.5	58.4	1.000	199.2	206.1 ± 0.5	3.2%

[1] Xylose (M).

[2] XynB-E: Beta-xylosidase from *Bacillus pumilus* IPO expressed in *E. coli* (U/mL).

[3] Reaction temperature (℃).

[4] Desirability.

[5] Mean ± SD.

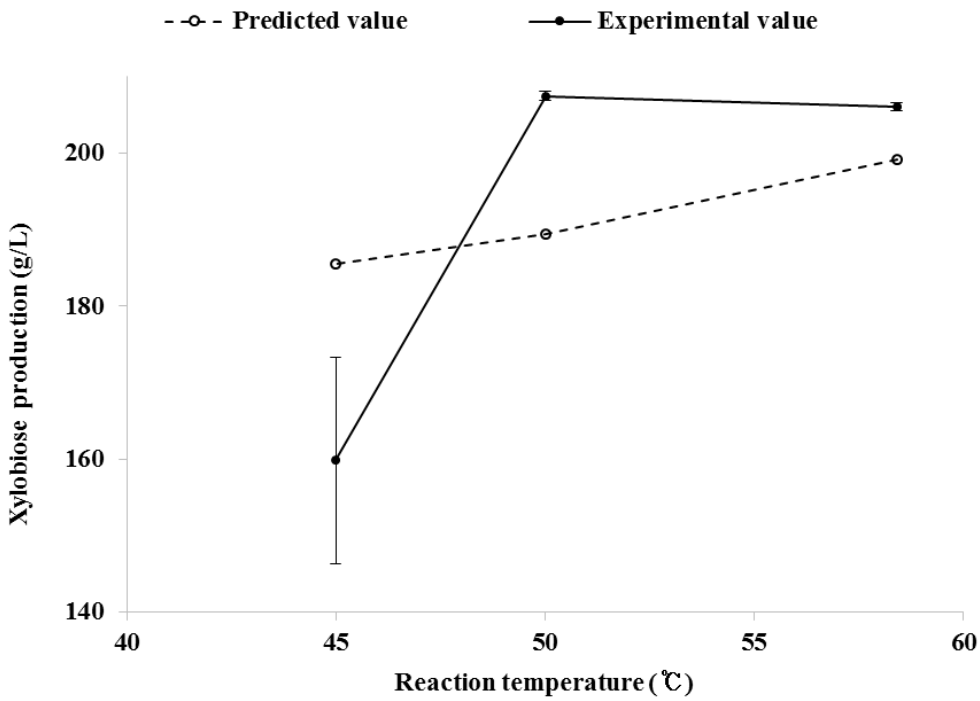


Figure 4-25. Comparison of predicted- and experimental-value of xylobiose production.

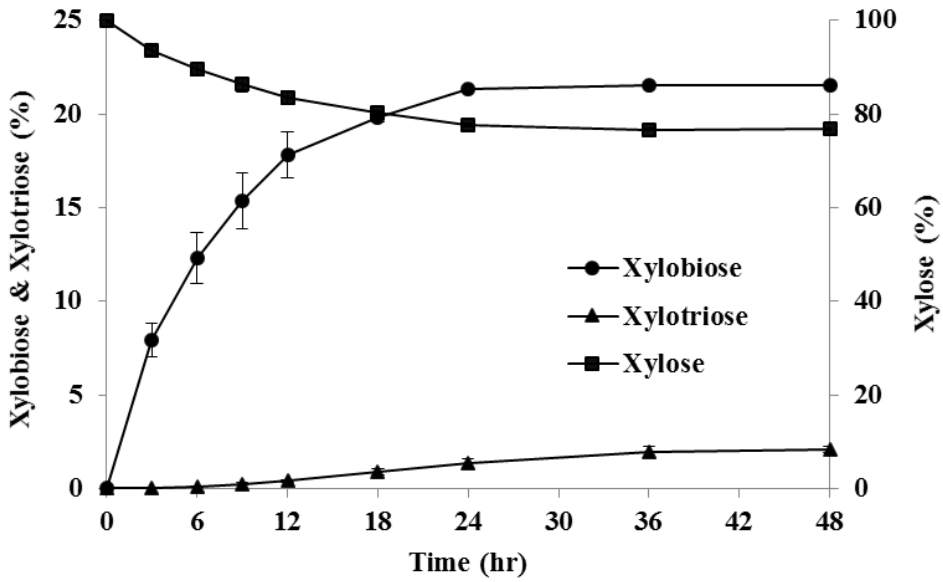
4.5.6. Xylobiose synthesis reaction under optimized conditions

Optimization conditions for xylobiose production were determined as shown in Table 4-12 using RSM analysis. Figure 4-26 (A) shows the result of reaction under the 6.7 M xylose and 6.5 U/mL beta-xylosidase at 50℃ for 24 hr as a final optimization condition. (B) depicts a further analysis for determining if xylobiose production can be increased after increasing beta-xylosidase to 10.0 U/mL under optimized conditions. Because the amount of xylobiose synthesis using beta-xylosidase from *Bacillus pumilus* IPO did not differ on using a buffer or water in preliminary experiments, the current optimization reaction involved the use of DDW as a solvent. To determine the optimum reaction time for xylobiose production, time-dependent xylobiose synthesis pattern was analyzed.

(A) showed that xylose was converted to 12.3% xylobiose and 0.1% xylotriose at 6 hr; subsequently, xylobiose increased by approximately 3% at 3 hr intervals. Xylobiose and xylotriose were converted to 21.3% and 1.3% for 24 hr, respectively. Between 24 hr and 48 hr, xylobiose reached equilibrium at 21.5%, but xylotriose slowly increased to 2.1%. The amount of xylobiose production noted in (A), which involved the use of water as a reaction solvent, was slightly higher than that reported in Table 4-11, which involved the use of buffer as a reaction solvent. (B) showed that xylose was converted to 13.3% xylobiose and 0.2 % xylotriose at 6 hr; which was slightly higher than that seen in (A). Subsequently, xylobiose increased by approximately 3% at 3 hr intervals up to 12 hr. Xylobiose and xylotriose were converted to 21.3% and 2.0% for 24 hr, respectively. Between 24 hr and 48 hr, xylobiose production reached a stage of equilibrium at 21.3%, but xylotriose production slowly increased to 2.7%.

The xylobiose production seen in (A) and (B) was 214.0 g/L and 213.7 g/L for 24 hr. Xylobiose production time was found to be suitable for 24 hr. (B) showed that xylobiose and xylotriose production rates were faster than (A), but did not increase production yield. The optimal conditions for xylobiose synthesis involved 6.7 M xylose, 6.5 U/mL beta-xylosidase, reaction temperature at 50℃, and a reaction time of 24 hr. The maximum production of xylobiose was 214.0 g/L, which was higher than the production level reported in a previous study which involved xylobiose synthesis up to 5.7% (22.8 g/L) using the fungal beta-xylosidase from *Talaromyces thermophiles* in 40% xylose solution containing 3 M sorbitol (Guerfali et al. 2008). The optimal production of xylobiose in this study was 9.4 times higher than that reported in previous studies. In addition, it proves to be economically advantageous as a desalting process after the reaction becomes unnecessary, because pure water can be used as the reaction solvent instead of a buffer. Therefore, such a process of functional xylobiose synthesis can be considered as the basis for industrial production.

(A)



(B)

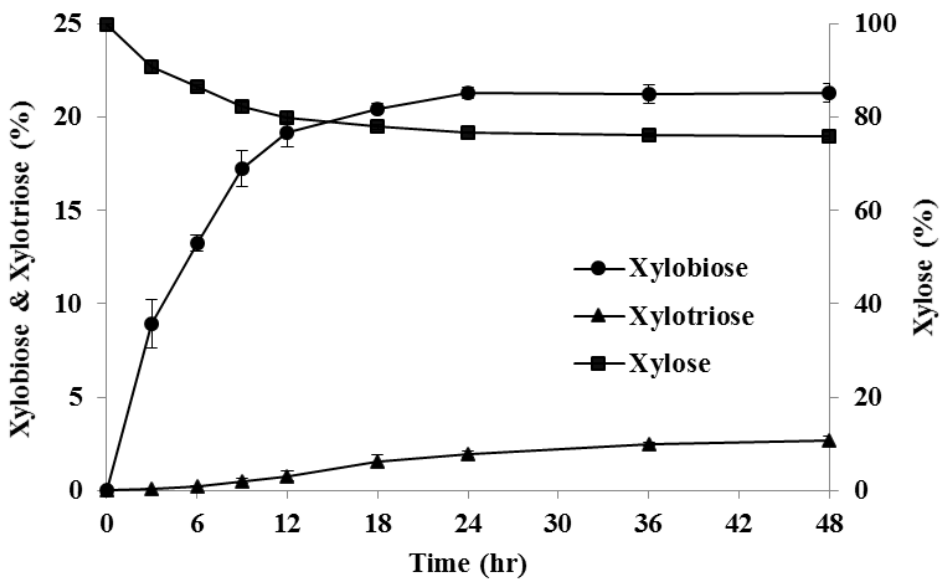


Figure 4-26. Time course of xylobiose production using beta-xylosidase from *B. pumilus* IPO expressed in *S. cerevisiae*. (A) XynB-S 6.5 U/mL; (B) XynB-S 10 U/mL.

Table 4-12. Summary of optimal reaction for xylobiose

Independent variables					Experimental results	
X_1 [1)]	X_2 [2)]	X_2' [3)]	X_3 [4)]	X_4 [5)]	Conversion (%)	Production (g/L)
6.7	6.5	XynB-S	50.0	24	21.0 ± 0.1	214.0 ± 0.6
6.7	10.0	XynB-S	50.0	24	21.3 ± 0.3	213.7 ± 2.9

[1)] Xylose (M).

[2)] Beta-xylosidase (U/mL).

[3)] Type of beta-xylosidase.

[4)] Reaction temperature (℃).

[5)] Reaction time (hr).

[6)] Mean ± SD.

In conclusion, to establish the optimization conditions for xylobiose synthesis, the experimental design was based on CCD of RSM. The independent variables were xylose concentration (X_1, M), beta-xylosidase amount (X_2, U/mL), and reaction temperature (X_3, ℃), and the reaction values were set to xylobiose production (Y, g/L). An empirical second-order polynomial equation of xylobiose production was obtained through CCD and R-squared of the model was 0.9643. The order of independent variables affecting xylobiose production was X_1 (xylose concentration) > X_3 (reaction temperature) > X_2 (beta-xylosidase amount). Although the optimum temperature for beta-xylosidase hydrolysis activity was 30℃, it was confirmed that high concentrations of xylose and high temperature were suitable to increase the amount of xylobiose synthesis using beta-xylosidase from *Bacillus pumilus* IPO. High concentration of xylose is considered to play an important role in maintaining beta-xylosidase stability during xylobiose synthesis.

4.6. Purification of synthetic xylobiose

4.6.1. Purification of synthetic xylobiose using simulated moving bed

The simulated moving bed (SMB) operating system was based on previous research. Ion exchange resin (Dowex-50WX4) in sodium form tends to have higher binding force as the degree of polymerization (DP) increases. It was reported to be most suitable for xylobiose separation from XOS (Choi et al. 2016). Therefore, the port configuration (Desorbent → Feed → Raffinate → Extract) was applied in the same way as that implemented in a previous research (Figure 3-1). The reaction mixture was prepared as a feed with a total solids content of 251.05 g/L, of which xylobiose was 45.44 g/L (18.1%), as shown in Table 4-13. The movement of xylose was the fastest. Then, xylobiose and xylotriose were passed through the column in this order, as shown in Figure 4-27. Xylose was passed through the extract port and xylobiose was collected through the raffinate port. The purity of xylose in the extract fraction was 99.6%, which can be reused as a material for xylobiose synthesis. The purity and recovery of xylobiose were 98.2% and 80%, respectively. This study confirmed that xylobiose can be purified from 18.1% to 98.2% in a one step process using 1-3-1 column configuration. This study has established a one-step purification process to produce high-purity xylobiose.

Table 4-13. Summary of the purification results using SMB

	Component	Feed	Raffinate	Extract
Concentration (g/L)	Xylose	204.35	-	20.57
	Xylobiose	45.44	19.34	0.08
	Xylotriose	1.26	0.35	-
Purity (%)		-	98.2	-
Recovery (%)		-	80.0	-

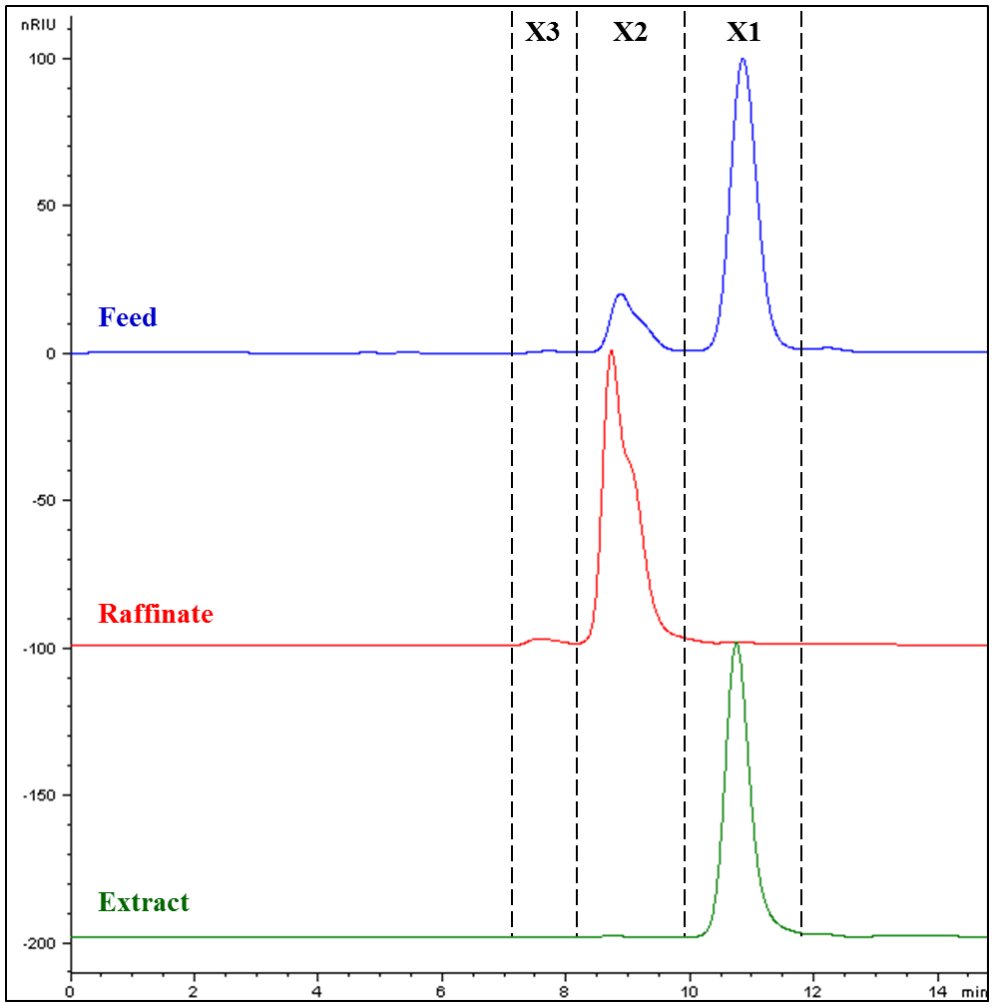


Figure 4-27. HPLC chromatogram with Waters Sugar-PakI column of purified synthetic xylobiose using SMB process. X1: xylose; X2: xylobiose; X3: xylotriose.

4.6.2. Purification of xylobiose isomers using paper chromatography

TLC analysis of xylobiose isomers was conducted using a solvent mixture containing 1-propanol : ethyl acetate : water (7 : 2 : 1, v/v/v). It was confirmed that xylobiose and two isomer spots were separated as shown in Figure 4-28. The movement of the two isomers was faster than that of beta-1,4-xylobiose. The synthesized xylobiose isomers were named X2-P1, X2-P2 and X2-P3 (beta-1,4-xylobiose). The intensity of each spot on the TLC plate was in the order of X2-P3 > X2-P1 > X2-P2. Compared with the peak area in Figure 4-30, X2-P1 and X2-P2 were expected to be Unknown 1 and Unknown 2, respectively. In order to obtain each isomer, xylobiose isomer mixture was purified by paper chromatography. In the three samples separated by paper chromatography, only the fractions having a purity of 98% or more were eluted by deionized distilled water and then lyophilized. HPLC analysis of the isomers showed that the retention times of X2-P1 and X2-P2 were 6.3 min and 6.9 min, respectively. X2-P3 was confirmed to be beta-1,4-xylobiose at RT 7.5 min (Figure 4-29).

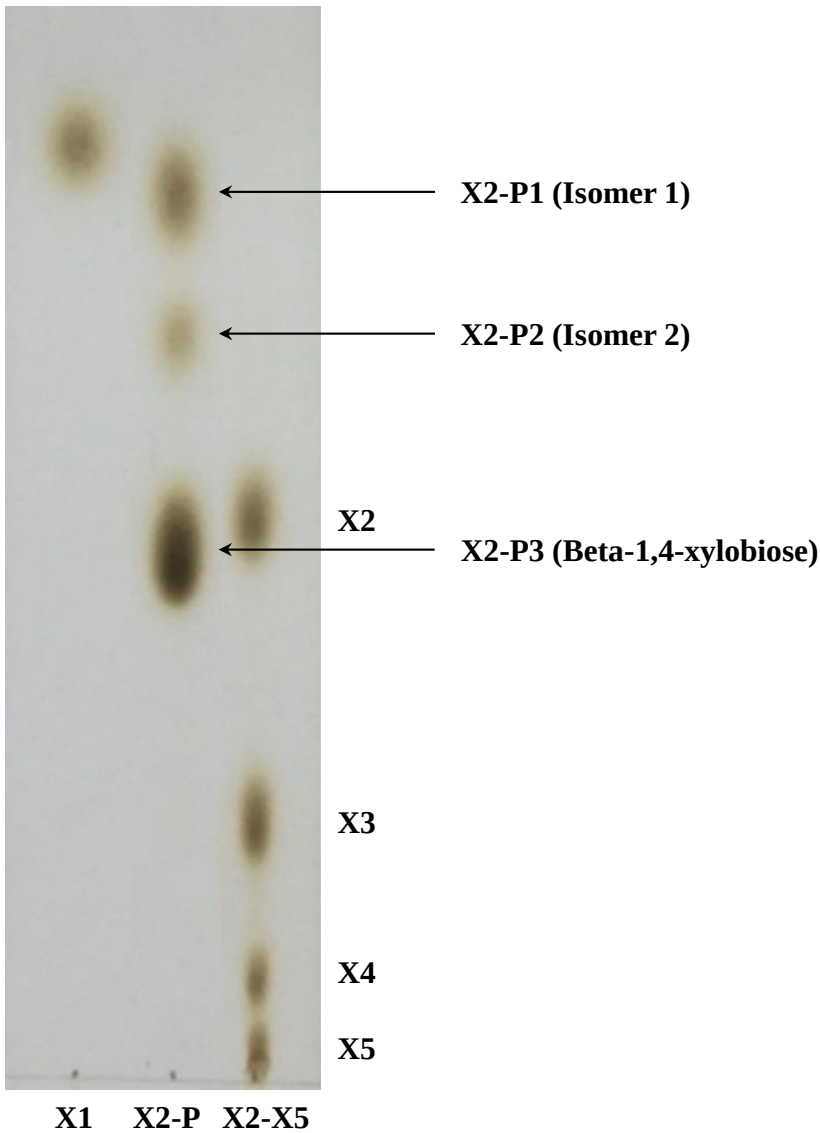


Figure 4-28. TLC analysis of purified synthetic xylo-disaccharides. Lane X1: beta-1,4-xylose standard; lane X2-P: purified synthetic xylo-disaccharides; lane X2-X5: beta-1,4-xylobiose (X2), beta-1,4-xylotriose (X3), beta-1,4-xylotetraose (X4), beta-1,4-xylopentaose (X5).

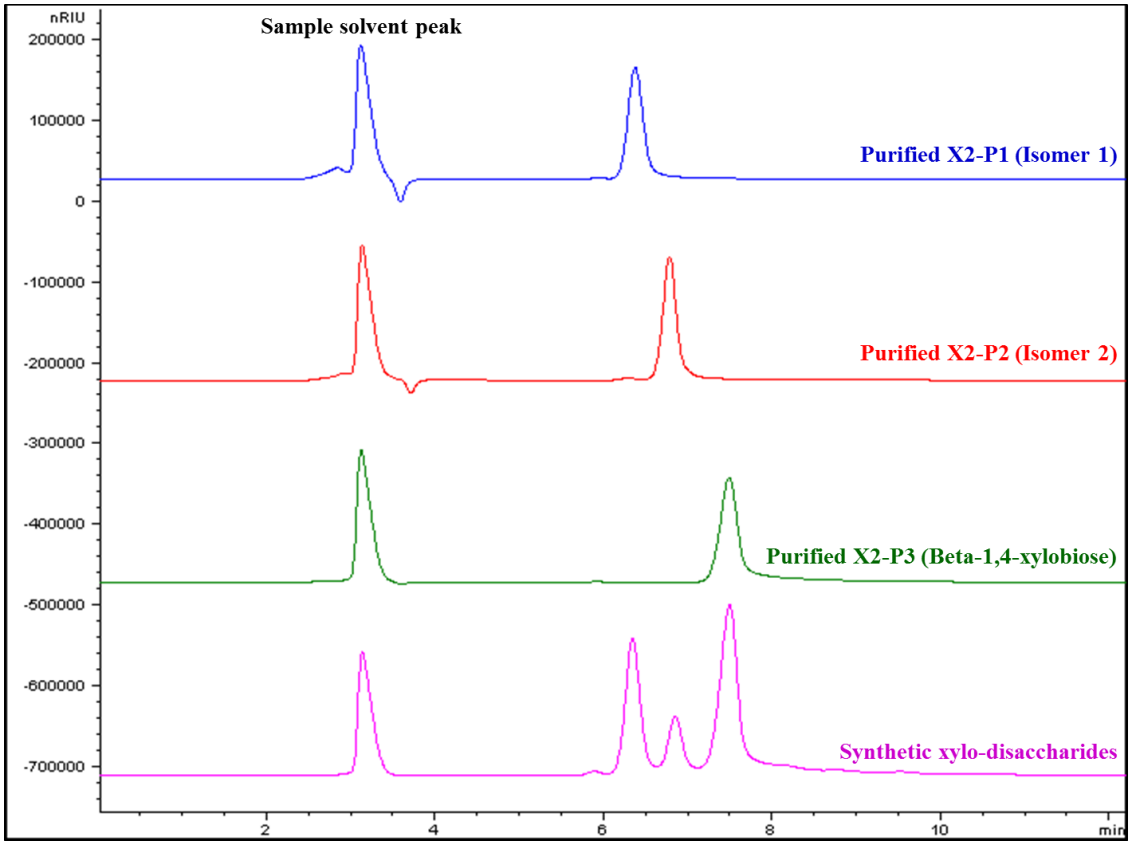


Figure 4-29. HPLC chromatogram of purified synthetic xylobiose and its isomers.

In conclusion, the xylo-disaccharides synthesized by bacterial beta-xylosidase from *Bacillus pumilus* IPO was separated by a one step process, providing 98.2% purity using simulated moving bed (SMB) chromatography. In paper chromatography, the xylobiose isomer mixture was separated in the same pattern as that done in TLC.

4.7. Identification and characterization of synthetic xylobiose

4.7.1. Molecular weight of xylobiose isomers by liquid chromatography-mass spectrometry (LC-MS) analysis

^{13}C-NMR analysis was conducted to identify xylobiose isomers synthesized by beta-xylosidase from *Bacillus pumilus* IPO. On comparing with the NMR analysis report published by Petráková and Kováč (1981), it was expected that three different linkages of xylobiose were mixed. Figure 4-27 shows that the xylobiose peak has a slight shoulder when analyzed using cation-exchange gel resin in calcium form. It is considered that the peak was not separated because the isomer was mixed. SMB operation was performed using the cation-exchange resin in sodium form; hence, no problems were found to be caused by the presence of xylobiose isomers. The ion exchange resins were found to be able to effectively separate the monosaccharide and disaccharide, but not the isomer. Iizuka et al. (1993) reported that the fungal beta-xylosidase from *Aspergillus niger* could synthesize four types of xylobiose (beta-1,4- >> beta-1,3- > beta-1,2- ≥ beta-1,1-linkage), whereas beta-xylosidase from *Malbranchea pulchella* could synthesize three types of xylobiose (beta-1,4- ≥ beta-1,1- >> beta-1,2-linkage). Xylobiose synthesis aided by fungal beta-xylosidase using xylose showed broad specificity for beta-xylosyl linkages.

Analytical condition was established to confirm xylobiose isomers using a ZORBAX Carbohydrate column packed with porous silica microspheres. As shown in Figure 4-30, purified xylobiose through SMB showed two unknown compounds besides beta-1,4-xylobiose. LC-MS analysis was performed to confirm that the two

unknown peaks were xylobiose isomers. As shown in Table 4-14, the xylobiose standard, synthesized xylobiose, unknown 1, and unknown 2 had the same molecular weight of 305.114 Da. LC-MS analysis results for each sample are shown from Figure 4-31 to Figure 4-34. The theoretical molecular weight of xylobiose is 282.24 Da. In LC-MS analysis, the total molecular weight increased because the sample was ionized using Na^+ (22.99 Da). Bacterial beta-xylosidase from *Bacillus pumilus* IPO was able to synthesize three kinds of xylobiose. It is necessary to identify the structure of xylobiose using NMR analysis.

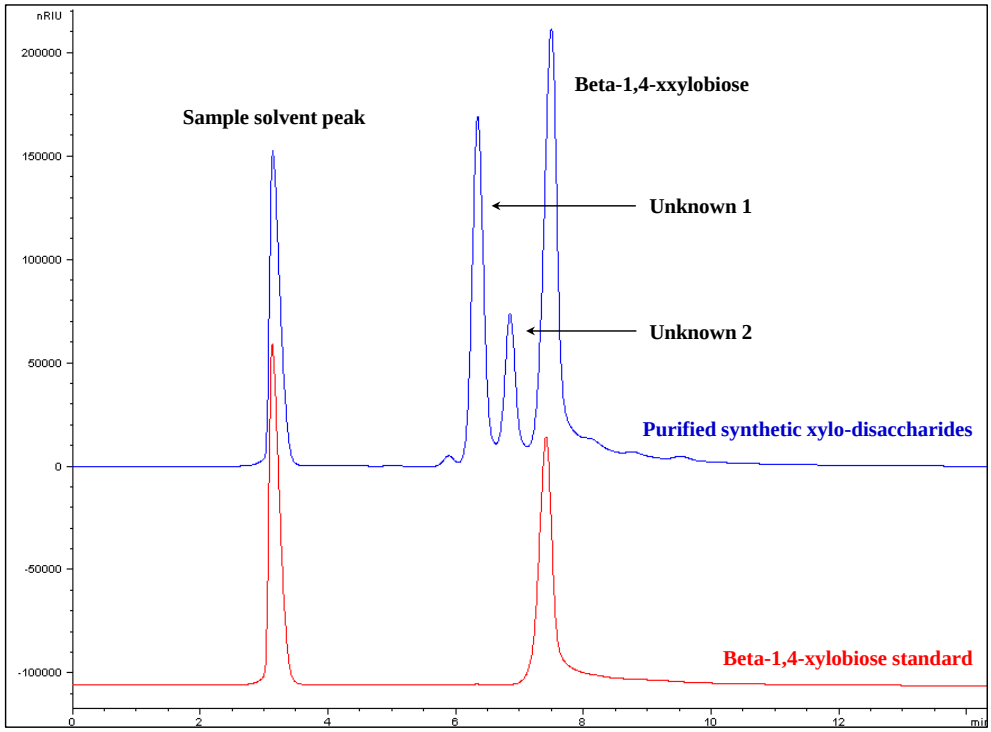


Figure 4-30. HPLC chromatogram of synthetic xylo-disaccharides purified using SMB.

Table 4-14. Characteristics of synthetic xylo-disaccharides

Sample		Peak RT (min)	Peak ratio (%)	Molar mass (Da)
Standard	Beta-1,4-xylobiose	7.5	-	305.114
Purified synthetic xylo-disaccharides	Unknown 1	6.3	35.9	305.114
	Unknown 2	6.9	12.6	305.114
	Beta-1,4-xylobiose	7.5	51.5	305.114

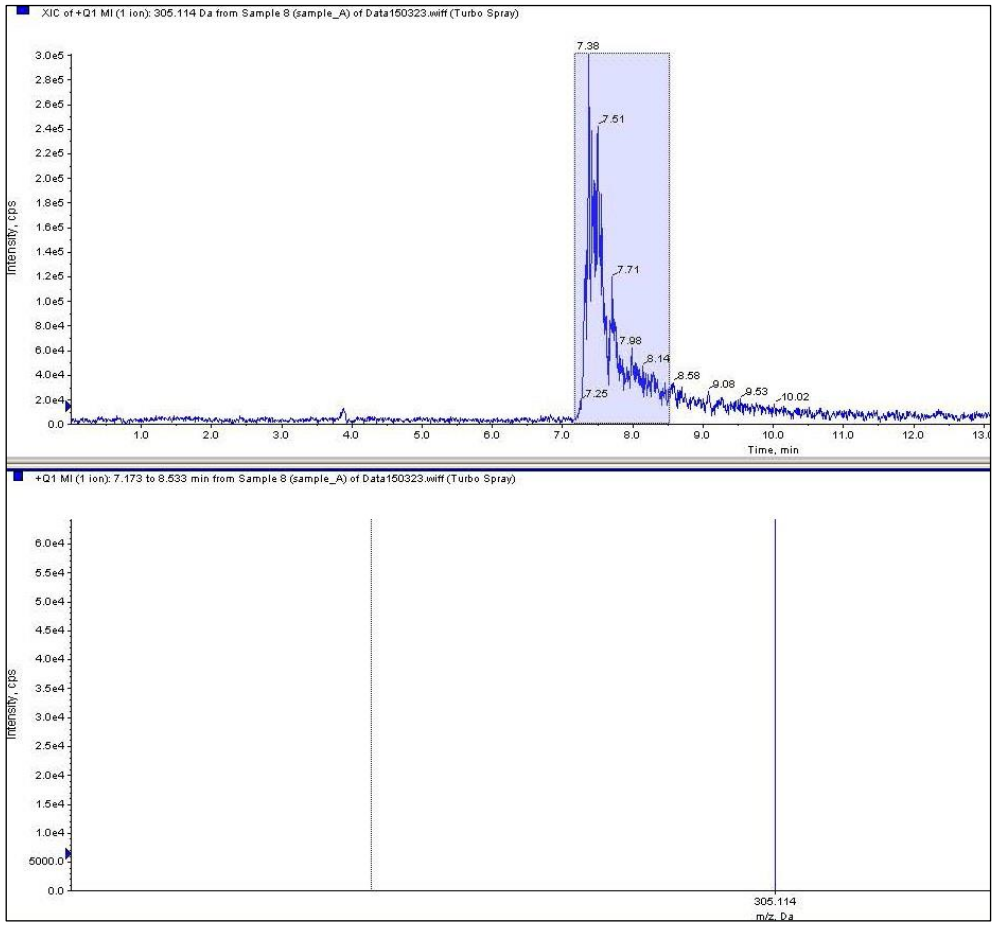


Figure 4-31. HPLC-ESI-MS chromatogram of xylobiose standard.

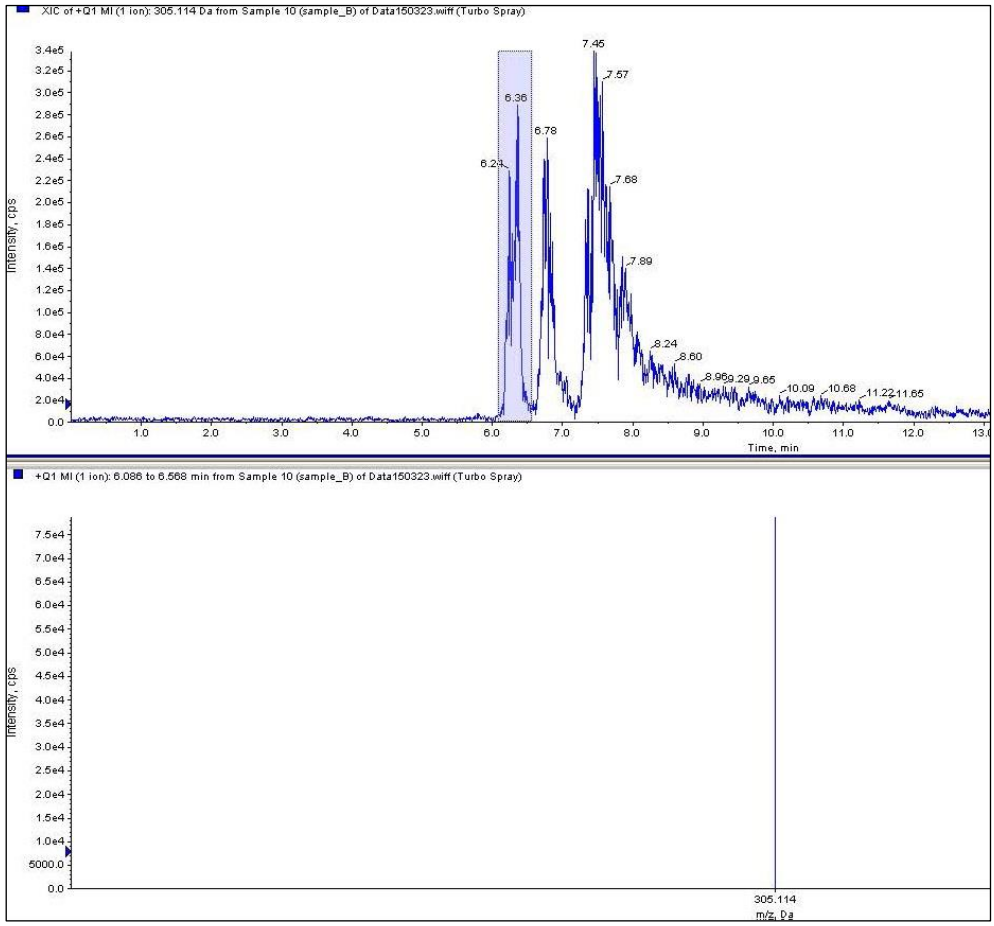


Figure 4-32. HPLC-ESI-MS chromatogram of purified synthetic xylo-disaccharide (X2-P1, unknown 1).

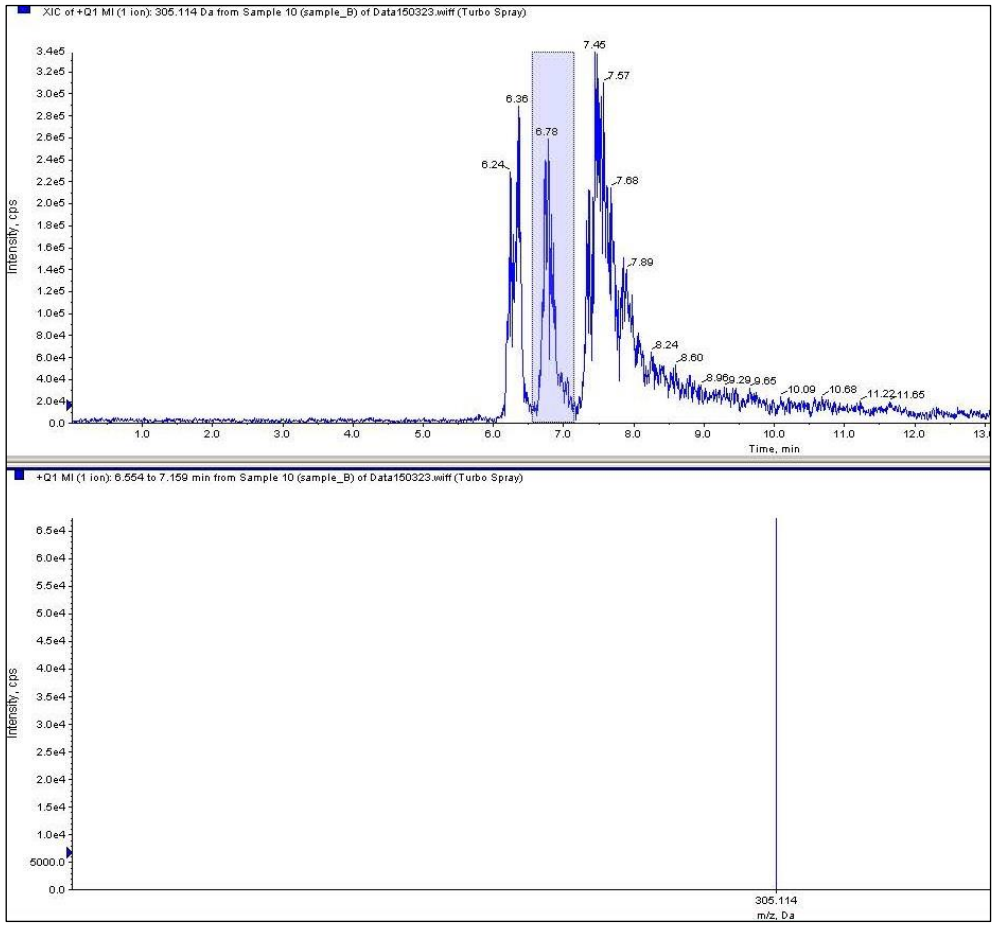


Figure 4-33. HPLC-ESI-MS chromatogram of purified synthetic xylo-disaccharide (X2-P2, unknown 2).

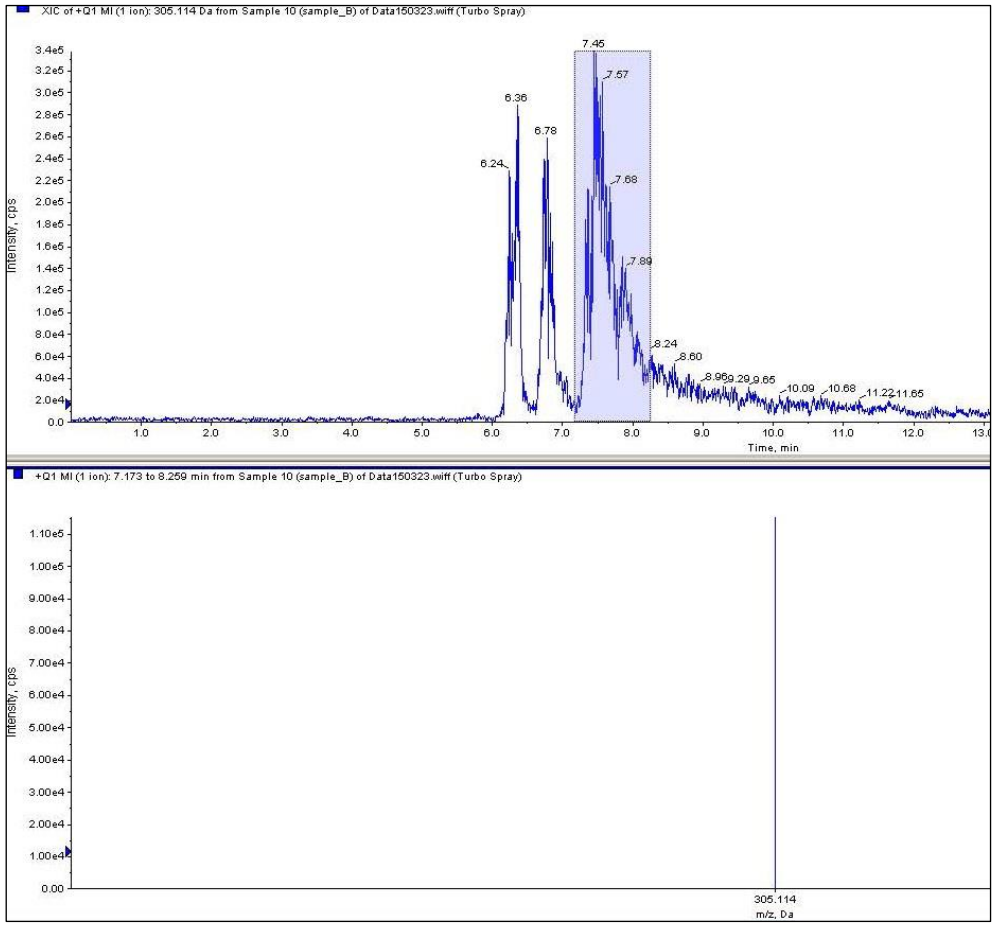


Figure 4-34. HPLC-ESI-MS chromatogram of purified synthetic xylo-disaccharide (X2-P3, Beta-1,4-xylobiose).

4.7.2. Determination of xylobiose isomers using nuclear magnetic resonance (NMR) analysis

The ^{13}C NMR spectra have facilitated the identification of linkages and stereochemistry of saccharides in carbohydrate research (Shashkov and Chizhov. 1976, Kovač et al. 1980). NMR analysis was conducted to confirm the structure of xylobiose isomers synthesized using beta-xylosidase. NMR spectra of xylobiose isomers were interpreted by referring to the results of a study published by Petráková and Kováč (1981). As shown in Table 4-15, the difference between the NMR spectra described in the previous studies and that obtained in this study was very small, at approximately 1 ppm. The peak position and intensity of each carbon showed the same tendency as that reported in a previous study. A slight difference can be interpreted as the difference between the analysis equipment and analysis conditions.

The chemical shifts of X2-P1 were observed at C'-1 (103.5 ppm) of the non-reducing unit and at Cα-3 (81.7 ppm) and Cβ-3 (84.2 ppm) of the reducing unit. As shown in Figure 4-35, the signal intensity of the Cβ anomer in aqueous solution was stronger than that of the Cα anomer. X2-P1 was identified as beta-1,3-xylobiose. The chemical shifts of X2-P2 were observed at C'α-1 (104.7 ppm) and C'β-1 (103.8 ppm) of the non-reducing unit as well as at Cα-2 (80.7 ppm) and Cβ-2 (81.8 ppm) of the reducing unit. As shown in Figure 4-36, the signal intensity of the Cα anomer in aqueous solution was stronger than that of Cβ anomer. This characteristic was reversed to that of beta-1,3-xylobiose, which was consistent with the characteristic revealed by the NMR spectra results published by Petráková and Kováč (1981). X2-P2 was identified as beta-1,2-xylobiose. The chemical shifts of

X2-P3 were observed at C'-1 (101.9 ppm) of the non-reducing unit as well as at Cα-4 (76.6 ppm) and Cβ-4 (76.5 ppm) of the reducing unit. As shown in Figure 4-37, the signal intensity of the Cβ anomer in aqueous solution was stronger than that of Cα anomer. It showed the same characteristics as that of beta-1,3-xylobiose in aqueous solution. X2-P3 was identified as beta-1,4-xylobiose. Bacterial beta-xylosidase from *Bacillus pumilus* IPO was found to be able to synthesize xylobiose predominantly in the order of beta-1,4- > beta-1,3- > beta-1,2-linkage with xylose condensation reaction. These properties were also observed in the condensation reaction with beta-glucosidase. It has been reported that beta-glucosidase from various origins can generate disaccharides through condensation reaction occurring at a high glucose concentration (Prade et al. 1998, Bhatia et al. 2002). The major product was gentiobiose (beta-1,6-linkage), whereas the minor products were sophorose (beta-1,2-linkage), laminaribiose (beta-1,3-linkage), and cellobiose (beta-1,4-linkage). Synthesis ratios of these disaccharides varied depending on the origin of beta-glucosidase (Ajisaka et al. 1987, Srisomsap et al. 1999). Bacterial beta-xylosidase from *Bacillus pumilus* IPO also showed broad specificity in the reverse reaction as fungal beta-xylosidase. The beta-xylosidase used in this study was found to produce beta-1,4-xylobiose as a major product.

Table 4-15. Chemical shifts in the ^{13}C-NMR spectra of synthetic xylo-disaccharides

Compound	Ring[1)] or anomer	Chemical shift					Identification
		C-1	C-2	C-3	C-4	C-5	
X2-P1 (Synthetic xylo-disaccharide 1)	Cα	92.2 (93.3) [2)]	71.0 (72.1)	81.7 (82.9)	67.8 (68.9)	61.1 (62.1)	Beta-1,3-xylobiose
	Cβ	96.5 (97.6)	73.7 (74.9)	84.2 (85.3)	67.8 (68.9)	64.9 (65.5)	
	C'	103.5 (104.7)	73.4 (74.6)	75.7 (76.8)	69.3 (70.4)	65.2 (66.3)	

Table 4-15. (Continued)

Sample	Ring[1)] or anomer	Chemical shift					Identification
		C-1	C-2	C-3	C-4	C-5	
X2-P2 (Synthetic xylo-disaccharide 2)	Cα	92.0 (93.1)	80.7 (81.9)	71.9 (73.0)	69.2 (70.4)	60.7 (61.7)	Beta-1,2-xylobiose
	Cβ	95.4 (96.5)	81.8 (82.9)	75.5 (74.5)	69.2 (70.4)	65.0 (66.2)	
	C'α	104.7 (105.9)	73.5 (74.3)	75.6 (76.7)	69.2 (70.4)	65.1 (66.2)	
	C'β	103.8 (104.9)	72.2 (74.0)	75.7 (76.8)	69.2 (70.4)	65.2 (66.4)	

Table 4-15. (Continued)

Sample	Ring[1)] or anomer	Chemical shift					Identification
		C-1	C-2	C-3	C-4	C-5	
X2-P3 (Synthetic xylo-disaccharide 3)	Cα	92.0 (93.2)	71.0 (72.1)	71.4 (72.6)	76.6 (77.7)	58.9 (60.0)	Beta-1,4-xylobiose
	Cβ	96.5 (97.7)	73.9 (75.1)	74.0 (75.1)	76.5 (77.6)	63.0 (64.1)	
	C'	101.9 (103.0)	72.8 (74.0)	75.6 (76.8)	69.2 (70.4)	65.2 (66.4)	

[1)]: C-reducing unit, C'-non reducing unit

[2)]: Values in parentheses taken from Reference (Petráková and Kováč. 1981)

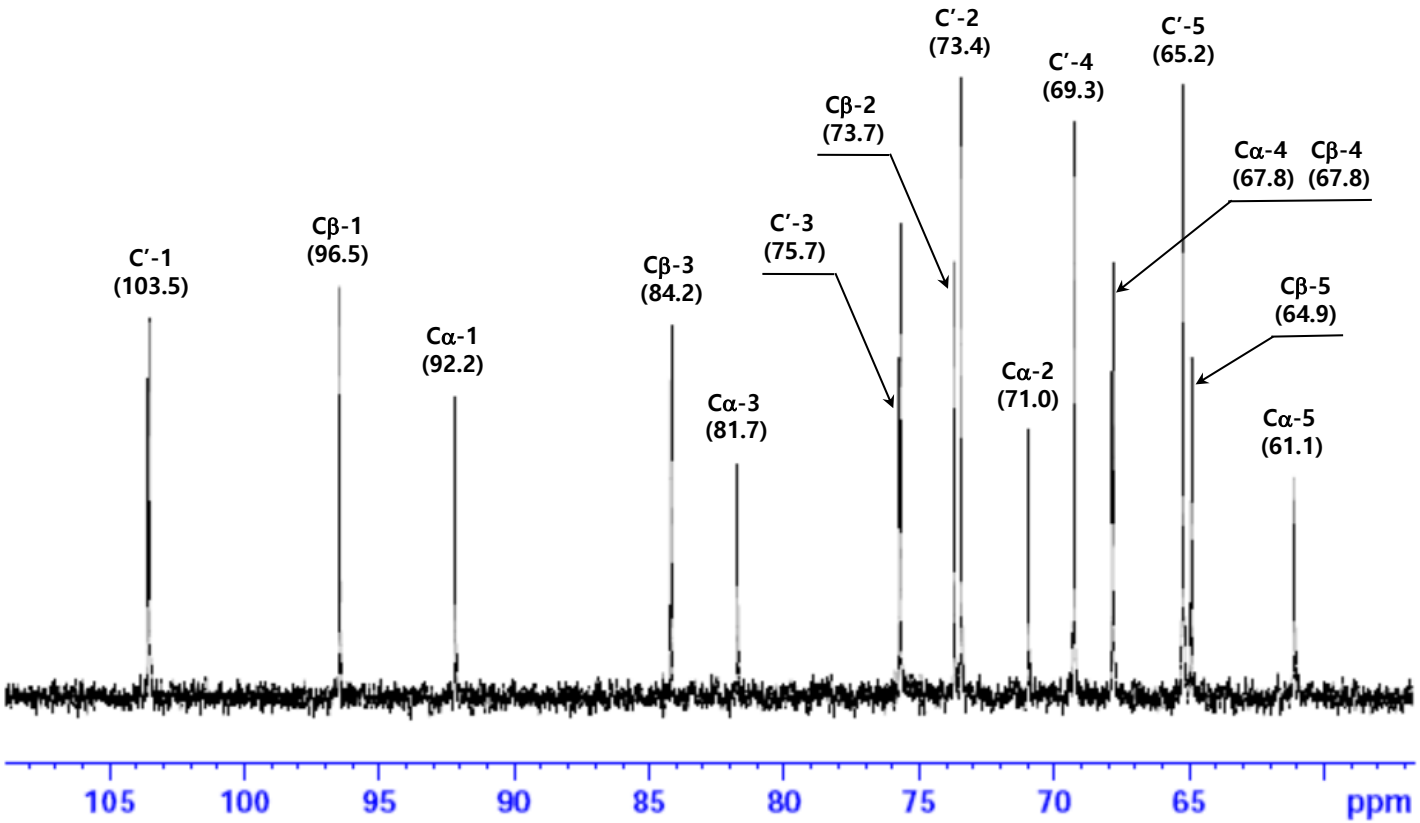


Figure 4-35. ^{13}C-NMR spectrum of synthetic xylo-disaccharide (X2-P1, Isomer 1).

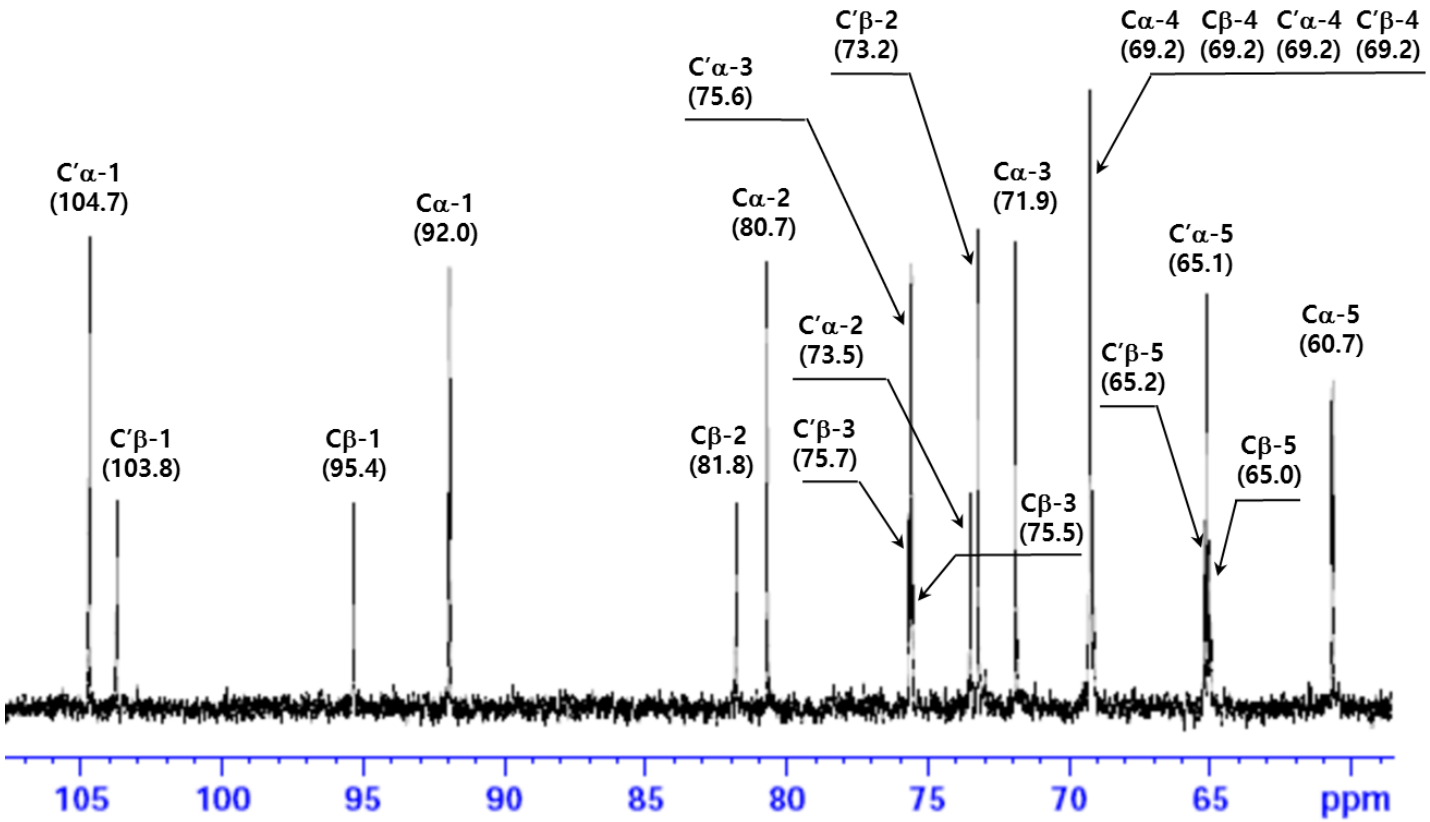


Figure 4-36. ^{13}C-NMR spectrum of synthetic xylo-disaccharide (X2-P2, Isomer 2).

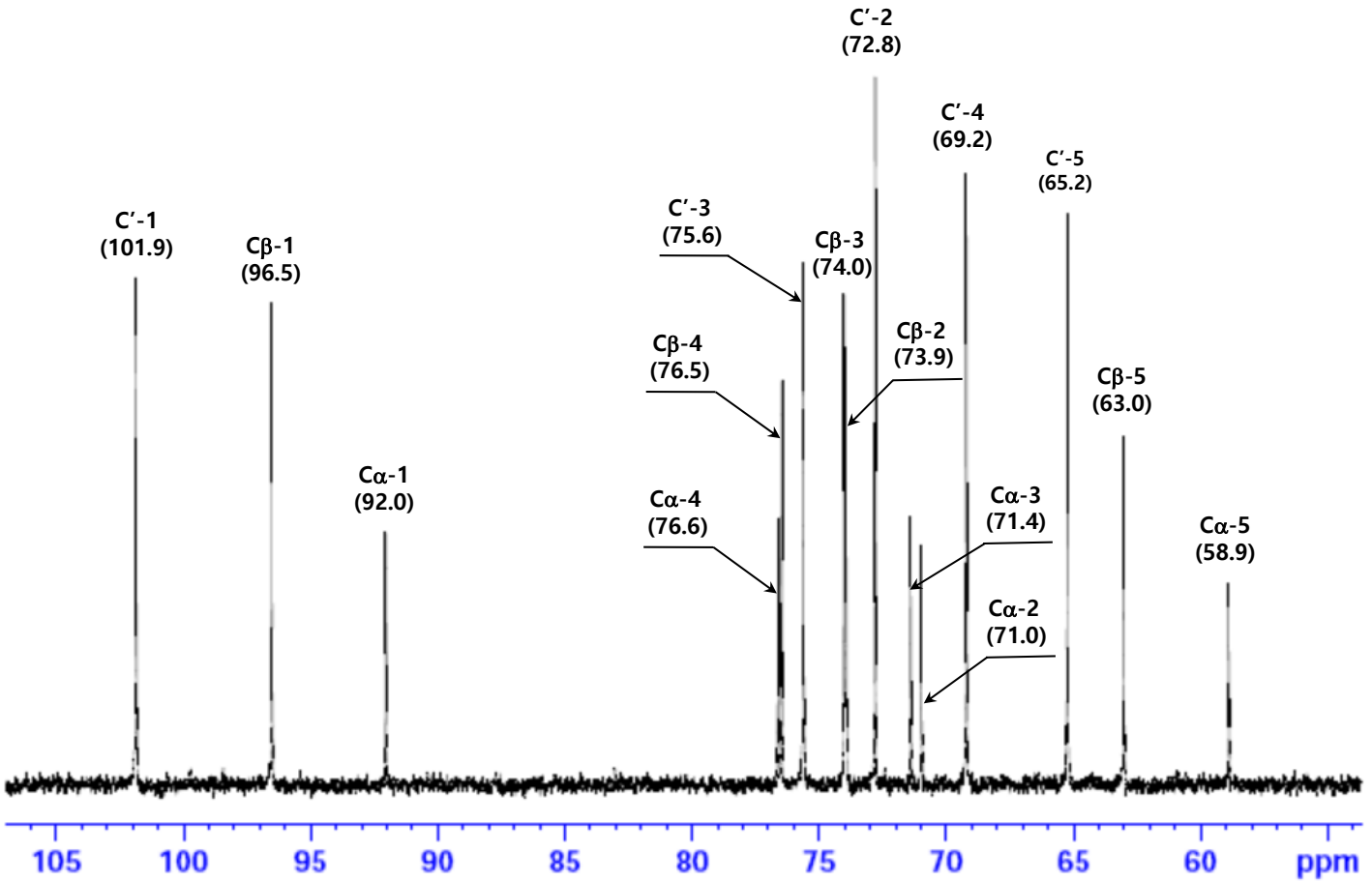


Figure 4-37. ^{13}C-NMR spectrum of synthetic xylo-disaccharide (X2-P3, Beta-1,4-xylobiose)

4.7.3. Kinetic parameters of synthetic xylo-disaccharides

Kinetic parameters were measured to understand the hydrolysis-related characteristics of xylobiose isomers which were subjected to the action of beta-xylosidase from *Bacillus pumilus* IPO. K_m and k_{cat} of p-nitrophenyl-beta-D-xylopyranoside (pNPX), beta-1,2-xylobiose, beta-1,3-xylobiose, and beta-1,4-xylobiose were measured as shown in Table 4-16. There was no statistically significant difference ($p < 0.05$) in the kinetic parameters of pNPX acted upon by beta-xylosidase expressed in different hosts (*E. coli* and *S. cerevisiae*). The K_m values of xylo-disaccharides were in the order of beta-1,4-xylobiose > beta-1,2-xylobiose > beta-1,3-xylobiose, and there was a statistically significant difference ($p < 0.05$). The substrate affinity of beta-xylosidase was lowest for beta-1,4-xylobiose. The k_{cat} values were also observed in the order of beta-1,4-xylobiose > beta-1,2-xylobiose > beta-1,3-xylobiose, and a statistically significant difference existed among them ($p < 0.05$). The k_{cat}/K_m values were in the order of beta-1,2-xylobios $\geq$ beta-1,4-xylobiose > beta-1,3-xylobiose, but statistically significant differences were found only with regard to beta-1,3-xylobiose ($p < 0.05$). Among xylo-disaccharides, beta-1,3-xylobiose had the highest substrate-affinity for beta-xylosidase, but its k_{cat} was very low, i.e., at $1/10^{th}$ of the k_{cat} values of other isomers. Thus, the k_{cat}/K_m of beta-1,3-xylobiose was the lowest. However, the K_m of beta-1,2-xylobiose and beta-1,4-xylobiose differed, but there was no statistically significant difference with regard to k_{cat}/K_m.

The synthetic ratio of xylo-disaccharides in the reverse reaction of beta-xylosidase was in the order of beta-1,4-xylobiose (51.5%) > beta-1,3-xylobiose (35.9%) > beta-1,2-xylobiose (12.6%). On taking the characteristics of xylo-

disaccharides synthesis and kinetics parameters of beta-xylosidase into account, beta-1,4-xylobiose, which had low-substrate affinity and high-catalytic efficiency, was found to be the most abundant in the condensation reaction. Moreover, beta-1,3-xylobiose, which had high-substrate affinity and low-catalytic efficiency, was abundantly produced in the second order. Beta-1,2-xylobiose had lower substrate affinity than beta-1,3-xylose and higher catalytic efficiency than beta-1,4-xylobiose but the least amount of synthesis productivity.

Table 4-16. Kinetic parameters of p-nitrophenyl-beta-D-xylopyranoside (pNPX) and synthetic xylo-disaccharides

Enzyme	Substrate	K_m (mM)	V_{max} (μM/min)	k_{cat} (min^{-1})	k_{cat}/K_m ($mM^{-1}min^{-1}$)
XynB-E [1)]	pNPX	1.9 ± 0.2[3) c 4)]	17.2 ± 0.9 [c]	1211 ± 65 [b]	649 ± 29 [a]
XynB-S [2)]	pNPX	1.9 ± 0.2 [c]	18.1 ± 1.0 [c]	1243 ± 72 [b]	647 ± 30 [a]
XynB-S	Beta-1,2-xylobiose (X2-P2)	2.7 ± 0.1 [b]	86.2 ± 1.2 [a]	1186 ± 17 [b]	448 ± 15 [b]
XynB-S	Beta-1,3-xylobiose (X2-P1)	2.0 ± 0.1 [c]	44.5 ± 0.2 [b]	153 ± 1 [c]	78 ± 1 [c]
XynB-S	Beta-1,4-xylobiose (X2-P3)	4.2 ± 0.2 [a]	77.0 ± 6.3 [a]	1588 ± 130 [a]	381 ± 15 [b]

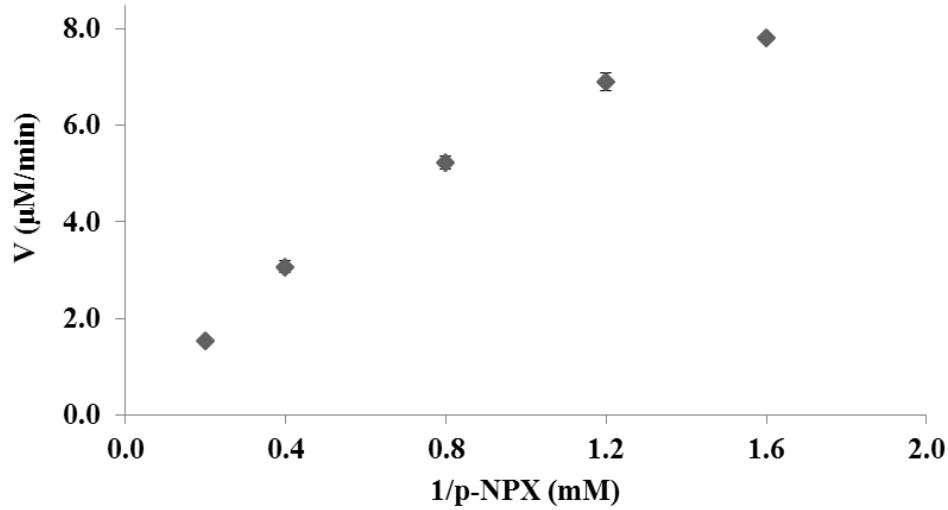
[1)] Beta-xylosidase from *B. pumilus* IPO expressed in *E. coli*.

[2)] Beta-xylosidase from *B. pumilus* IPO expressed in *S. cerevisiae*.

[3)] Mean ± SD.

[4)] Different alphabets mean significant differences between samples ($p < 0.05$).

(A)



(B)

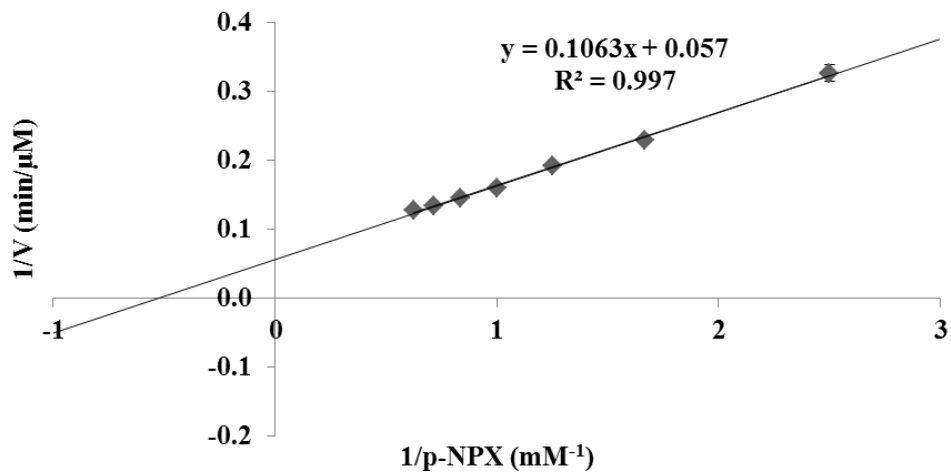
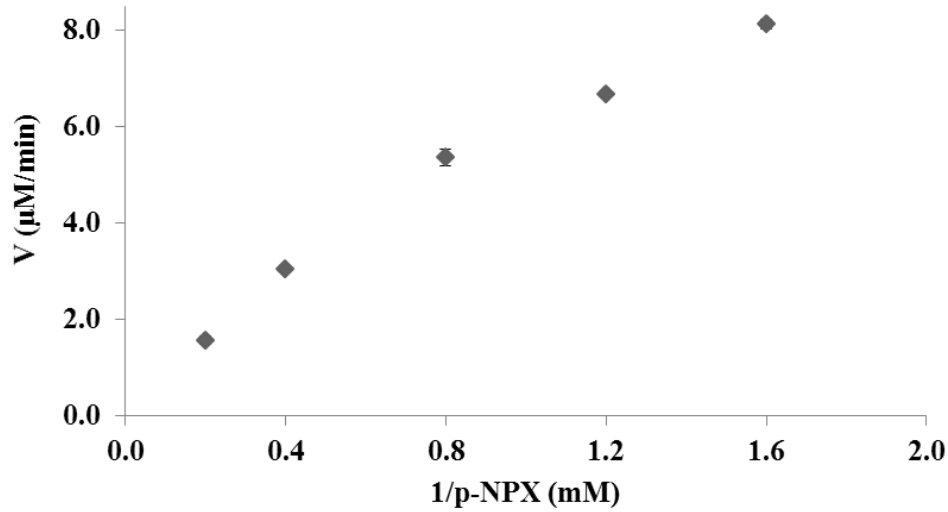


Figure 4-38. p-Nitrophenyl-beta-D-xylopyranoside (pNPX) hydrolysis reaction by beta-xylosidase from *B. pumilus* IPO expressed in *E. coli* (XynB-E). (A) Michaelis-Menten plot; (B) Lineweaver-Burk plot.

(A)



(B)

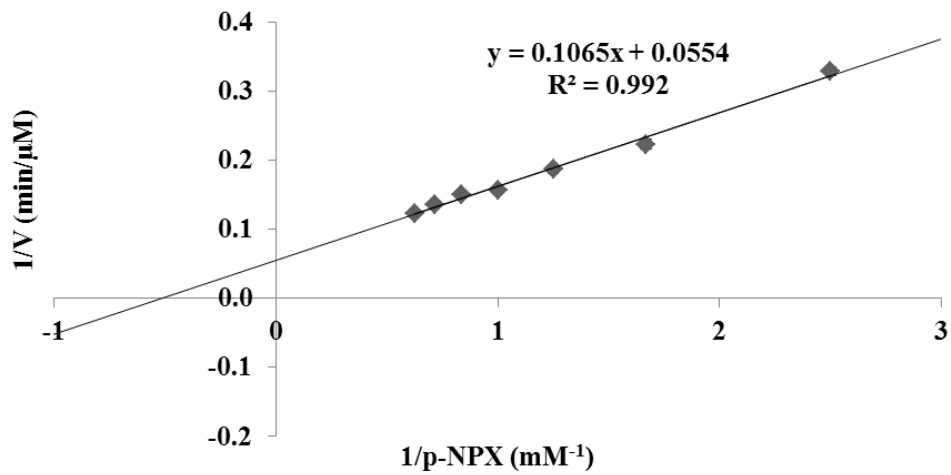
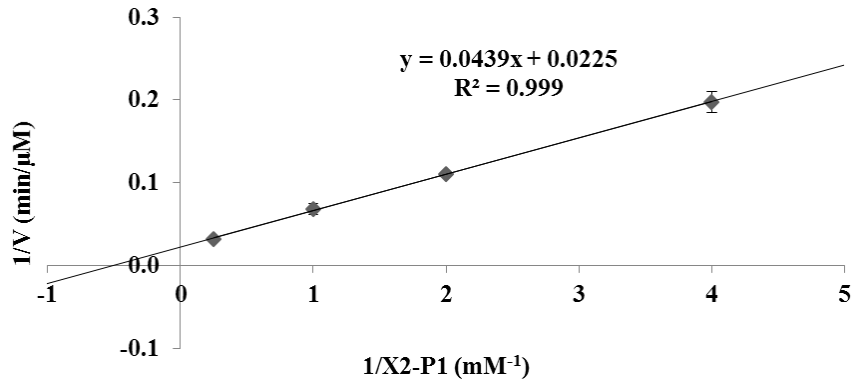
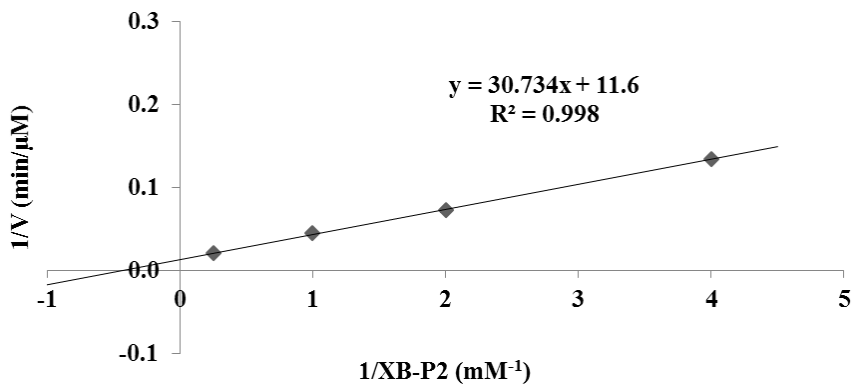


Figure 4-39. p-Nitrophenyl-beta-D-xylopyranoside (pNPX) hydrolysis reaction by beta-xylosidase from *B. pumilus* IPO expressed in *S. cerevisiae* (XynB-S). (A) Michaelis-Menten plot; (B) Lineweaver-Burk plot.

(A)



(B)



(C)

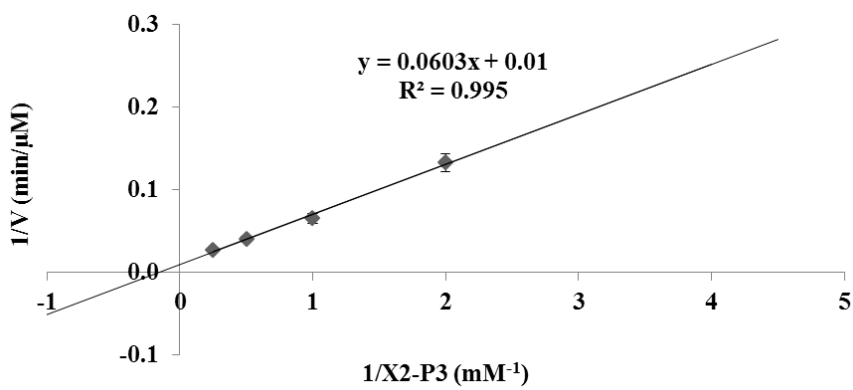


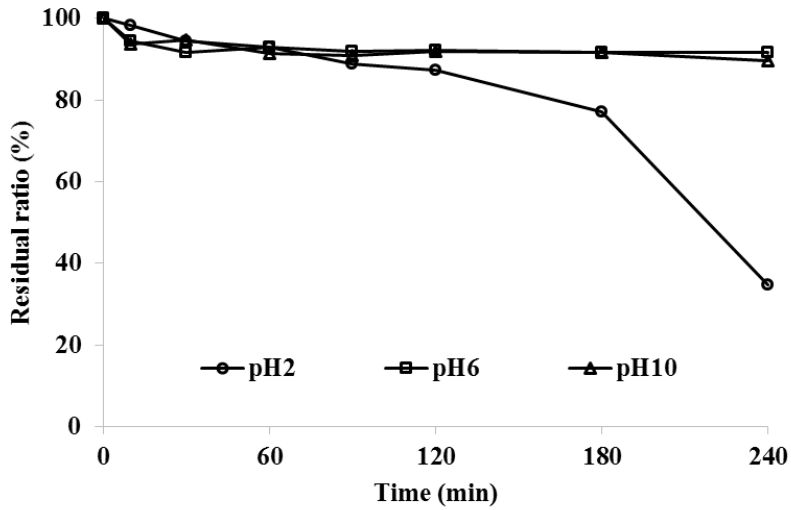
Figure 4-40. Lineweaver-Burk plot of purified synthetic xylo-disaccharides hydrolysis reaction by beta-xylosidase from *B. pumilus* IPO expressed in *S. cerevisiae*. (A) X2-P1 (Beta-1,3-xylobiose); (B) X2-P2 (Beta-1,2-xylobiose); (C) X2-P3 (Beta-1,4-xylobiose).

4.7.4. Effect of pH on the thermal stability of synthetic xylo-disaccharides

The thermal stability of xylobiose isomers synthesized by beta-xylosidase was measured at 100℃ under three pH conditions (pH 2, pH 6, pH 10). As shown in Figure 4-41 and Figure 4-42, three xylobiose isomers composed of beta-bonds showed excellent thermal stability at pH 6 and pH 10. These isomers did not decompose for 4 hr, regardless of the bonding structure. Beta-1,3-xylobiose [Figure 4-41 (B)] and beta-1,4-xylobiose [Figure 4-42 (A)] remained almost similar to their initial amounts for 240 min at 100℃ and showed very high thermal stability. Beta-1,2-xylobiose concentration [Figure 4-41 (A)] also remained over 90% for 240 min at 100℃ and was highly thermostable.

On the other hand, the thermal stability in acidic conditions varied according to the beta-linkage structure. The residual amounts of beta-1,2-xylobiose and beta-1,3-xylobiose were maintained at approximately 90% for 90 min at 100℃ and then rapidly decomposed. Both materials decreased to approximately 35% at 240 min. Beta-1,3-xylobiose was found to be more degraded than beta-1,2-xylobiose at pH 2. Beta-1,4-xylobiose was maintained at over 90% for 120 min at 100℃ and at 70% for 240 min. Among the three kinds of beta-linkages, beta-1,4-xylobiose showed the highest thermal stability even at pH 2.

(A)



(B)

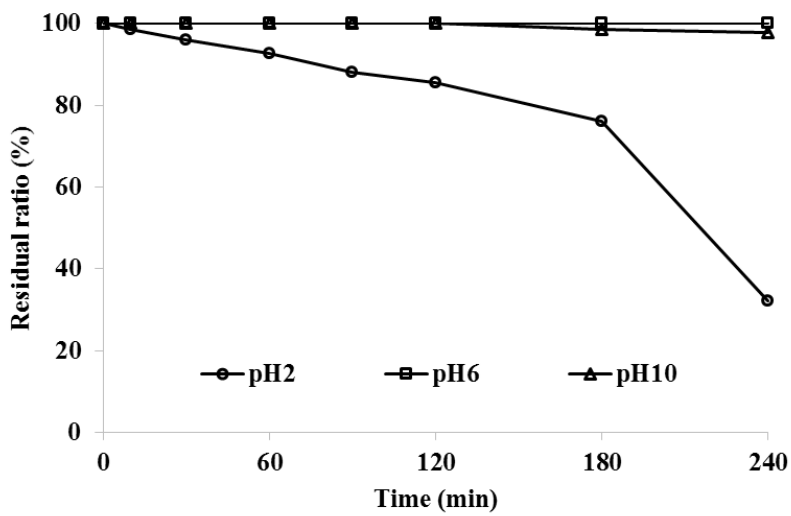
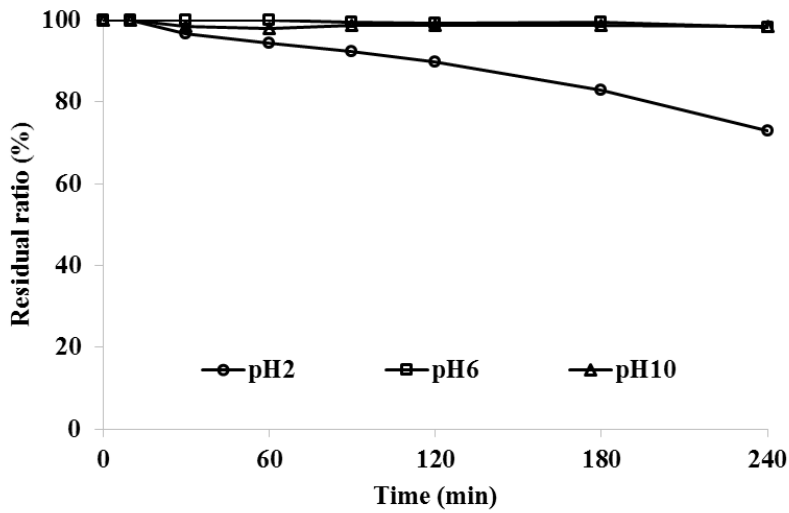


Figure 4-41. Effect of pH on the thermal stability of purified synthetic xylo-disaccharides. Beta-1,2-xylobiose (A); Beta-1,3-xylobiose (B).

(A)



(B)

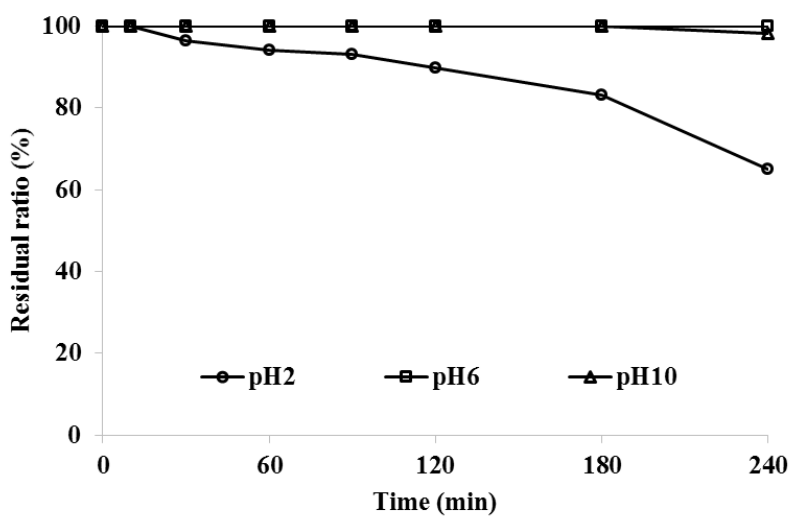


Figure 4-42. Effect of pH on the thermal stability of purified synthetic xylo-disaccharides. Beta-1,4-xylobiose (A); Beta-1,4-xylobiose standard (B).

The kinetics parameters for thermal decomposition of xylobiose isomers at pH 2 are shown in Figure 4-43 and Table 4-17. The degradation rate constants of beta-1,2-xylobiose and beta-1,3-xylobiose were 1.3 and 1.5 times higher than those of beta-1,4-xylobiose, respectively. The degradation rate constants of synthetic beta-1,4-xylobiose and the beta-1,4-xylobiose standard were the same. The time taken for degrading 50% of the substance is expressed as half-lives (T½). The half-life value of beta-1,3-xylobiose was the fastest (192 min) and that of beta-1,2-xylobiose was 205 min. Thermal stability at pH 2 was in the order of beta-1,4-xylobiose > beta-1,2-xylobiose > beta-1.3-xylobiose. Sweeteners with high thermal stability have an advantage because they can be widely applied to the processed food industry. Sucrose, the most commonly used sweetener, was decomposed to 63% for 30 min at 55℃ when 0.1 M sulfuric acid was added. Sucrose was reported to have low thermal stability under acidic conditions (Hartofylax et al. 1989). However, xylo-disaccharides exhibited high thermal stability at 100℃ under low pH condition. In fact, most processed food manufacturing processes require a heat treatment process for < 30 min. The three isomers were investigated for their thermal stability under severe conditions; xylo-disaccharides have excellent thermal stability. Therefore, when xylo-disaccharides were applied to the food industry, they can be maintained without degradation.

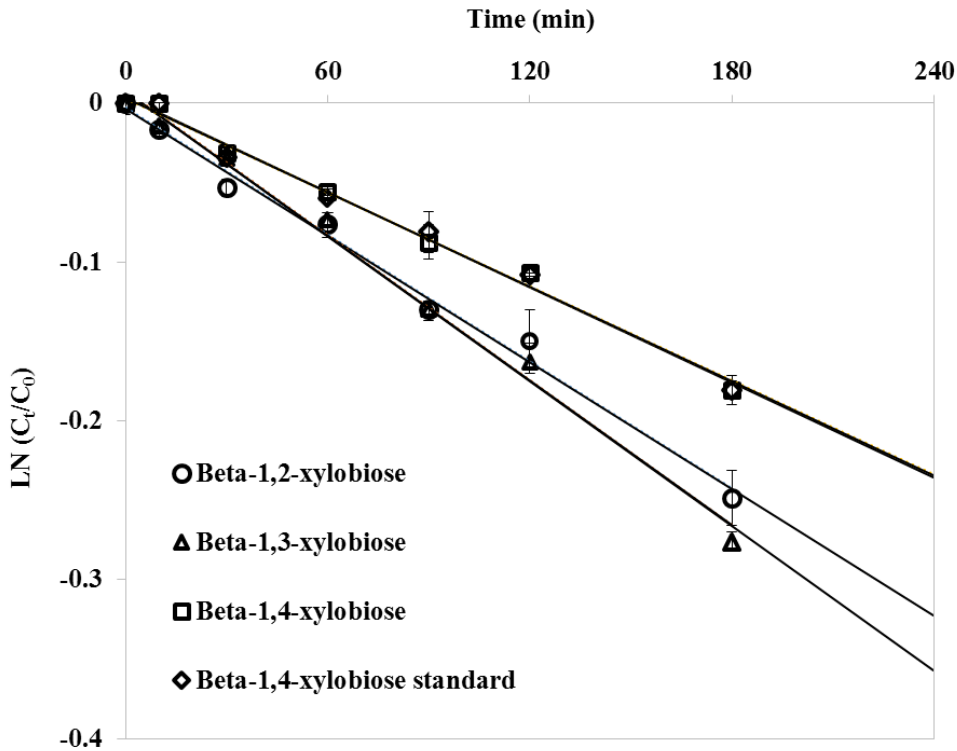


Figure 4-43. Thermal degradation kinetics of synthetic xylo-disaccharides at pH 2.

Table 4-17. Thermal degradation rate constant (*k*) of synthetic xylo-disaccharides

Xylo-disaccharides	Rate constant (k, min^{-1})	R^2	T½ (min)
Beta-1,2-xylobiose (X2-P2)	1.33×10^{-3}	0.992	205
Beta-1,3-xylobiose (X2-P1)	1.52×10^{-3}	0.992	192
Beta-1,4-xylobiose (X2-P3)	1.00×10^{-3}	0.992	695
Beta-1,4-xylobiose standard	0.99×10^{-3}	0.991	701

4.7.5. Inhibitory potency of synthetic xylo-disaccharides on intestinal rat sucrase

The inhibitory effect of xylo-disaccharides synthesized by beta-xylosidase on rat sucrase is shown in Figure 4-44. The addition of 5~20% inhibitor to the sucrose showed dose-dependent inhibitory potency of intestinal rat sucrase that takes part in hydrolysis activity. Xylobiose (beta-1,4-xylobiose standard) showed higher inhibitory activity against rat sucrase at all concentrations than synthetic xylo-disaccharides. This means that beta-1,3-xylobiose and beta-1,2-xylobiose have a slightly lower inhibitory effect on rat sucrase than beta-1,4-xylobiose. However, there was no statistically significant difference between xylobiose and xylo-disaccharides at all concentrations except for group subjected to 15% inhibitor concentration ($p < 0.05$). For the same inhibitor, the effect of rat sucrase inhibition was significantly different when added at a concentration of more than 10% ($p < 0.05$). Addition of 20% xylo-disaccharides based on the amount of sucrose (16.7% of the actual mixture, 20/120) could inhibit the activity of rat sucrase by 30% more than the inhibition associated with the use of the pure sugar. Therefore, synthetic xylo-disaccharides are highly functional and can be used as a sugar complement.

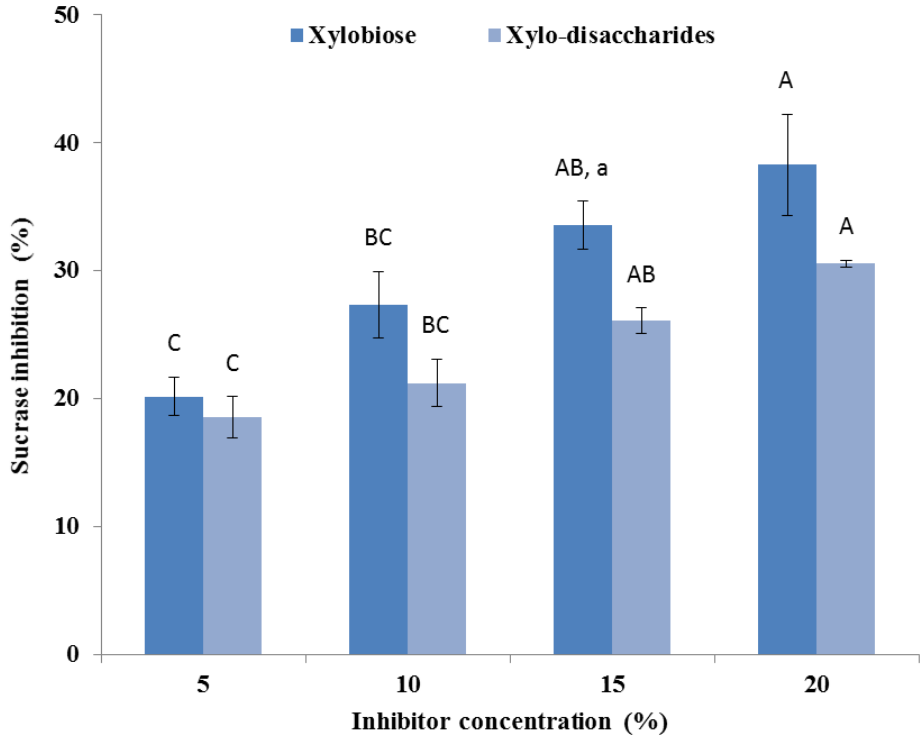


Figure 4-44. Intestinal rat sucrase inhibitory potency of synthetic xylo-disaccharides. Upper case letters indicate the difference between the concentrations of the same sample ($p < 0.05$). Lower case indicates differences between different samples ($p < 0.05$).

In conclusion, analysis of NMR data showed that three kinds of xylobiose isomer were identified. Beta-1,2-xylobiose was predominantly in the Cα anomeric form in aqueous solution, while beta-1,3-xylobiose and beta-1,4-xylobiose were predominantly present in the Cβ anomeric form. The major product of xylo-disaccharides synthesized by beta-xylosidase was beta-1,4-xylobiose (51.5%); in addition beta-1,3-xylobiose (35.9%) and beta-1,2-xylobiose (12.6%) were produced. Kinetic parameters were measured to understand the characteristics associated with the hydrolysis of xylo-disaccharides by beta-xylosidase from *Bacillus pumilus* IPO. Among the three xylo-disaccharides, beta-1.3-xylobiose (2.0 mM) was the best substrate affinity, and beta-1,2-xylobiose (448 $mM^{-1}min^{-1}$) was the highest in k_{cat}/K_m. However, among the three isomers of synthetic xylo-disaccharides, the most abundant one was beta-1,4-xylobiose, which had the lowest substrate affinity (4.2 mM) and 5 times higher k_{cat}/K_m value (381 $mM^{-1}min^{-1}$) than that of beta-1,3-xylobiose (78 $mM^{-1}min^{-1}$). Xylo-disaccharides were highly thermostable, and it was confirmed that at least 90% of the xylo-disaccharides continued to exist after storage at 100℃ for 90 min even under acidic condition (pH 2). In addition, xylobiose and xylo-disaccharides were found to have similar inhibitory potency on the activity of intestinal rat sucrase. Addition of 20% xylo-disaccharides based on the amount of sucrose inhibited the activity of intestinal rat sucrase by 30%.

5. OVERALL DISCUSSIONS AND RECOMENDATIONS

The eating habits of modern society have evolved in terms of the perception of food not only as an energy source but also as a means of culinary delight. With the advancements in the food industry, various processed foods have been developed, and sweeteners are used to impart sweetness to most processed foods. Sucrose made from sugar cane juice is most commonly used in the food industry because it has the best sweetness quality. However, excessive intake of sugars causes obesity. An increased prevalence of obesity and metabolic diseases has increased the need for alternative sweeteners. Thus, alternative sweeteners such as artificial high-intensity sweeteners and low-calorie sugar alcohols, which are not used as energy sources, have been developed. However, alternative sweeteners should be used with caution because they cause side effects when consumed in excess. Recently, a sugar complement that can function as an inhibitor of sucrase activity in the body when ingested with sugar is proposed as another alternative.

Pentose (such as xylose, arabinose, and ribose), which is a sugar complement, has sucrase-inhibition activity. Sugar complement is added to substitute 5~20% of the sugar; in this manner, sweetness of sugar can be maintained; however, the digestion and absorption of sugar in the body can be reduced (Park et al. 2007, Krog-Mikkelsen et al. 2011, Kim et al. 2017). In particular, xylobiose is a bifunctional sweetener and has been shown to be effective for inhibiting sucrase as well as for contributing toward the proliferation of beneficial intestinal bacteria (Lee et al. 2016, Lee et al. 2017). Xylobiose can produce xylan as a raw material; however, xylan is not yet used as a food material because of the complicated process of extraction and purification. This study developed an environmentally friendly and effective process for the synthesis of

xylobiose using xylose and beta-xylosidase, and identified the properties and functionality of the synthesized products.

The enzyme for xylobiose synthesis was selected as beta-xylosidase from *Bacillus pumilus* IPO which had the highest efficiency for xylobiose production yield. The beta-xylosidase gene was introduced into *Saccharomyces cerevisiae* (GRAS strain used in the food industry which can be cultivated at a high-cell density), and a fed-batch culture was selected as a strategy for increasing enzyme production. To increase the productivity of the enzyme, the C/N ratio, N-source type, and glucose feeding speed in the medium were considered. The recombinant yeast strains into which the foreign gene is introduced can easily lose the plasmid owing to prolonged incubation in the non-selective medium. In addition, yeast constantly produces and consumes ethanol during fermentation. When the amount of ethanol production increases, the productivity of the target protein may decrease. Thus, the supply of carbon sources should be appropriately controlled. In this study, the plasmid stability was maintained at over 70% by controlling the glucose feeding speed, and the ethanol production also did not exceeded 3 g/L. As a result, beta-xylosidase production through fed-batch culture optimization was 7.1 times higher than that produced by batch culture. In this study, only the medium composition and glucose feeding speed were considered as a strategy to increase the productivity of beta-xylosidase, but various other parameters also influence the fed-batch culture.

The synthesis of xylobiose using xylose is based on the reverse reaction of hydrolysis catalyzed by xylosidases. Xylose concentration, enzyme amount, and the reaction temperature were selected as factors to improve the xylobiose

synthesis. To maximize the yield of the xylobiose synthesis, a reaction prediction model was obtained using central composite design combined with the response surface methodology. As shown in Figure 4-20 and 3-21, xylose concentration contributed to the greatest influence on xylobiose synthesis. Investigations performed for optimization condition revealed that xylobiose up to a level of 214.0 g/L could be synthesized (Table 4-12). This value was 9.4 times higher than the synthesized amount reported in a previous study (Guerfali et al. 2008). To the best of our knowledge, xylobiose synthesis methodology adopted in this current study produced the best results in comparison with all previously conducted studies. Interestingly, the optimum temperature for hydrolysis activity of beta-xylosidase was 30℃, but the optimum temperature for xylobiose synthesis was 50℃. Beta-xylosidase from *Bacillus pumilus* IPO was found to improve thermal stability in the presence of high concentrations of xylose. The addition of a stabilizer such as saccharides to a protein has been reported to prevent protein surface changes and maintain protein stability with minimal interaction with water (Gianfreda and Scarfi. 1991). Therefore, increase in xylobiose production at above optimal hydrolysis temperature of beta-xylosidase can possibly be attributed to the high concentration of xylose, which functions as a stabilizer of the enzyme. Moreover, such high temperature might provide sufficient energy to overcome high activation energy barrier of the reverse hydrolysis reaction. In this study, we have developed a batch-type reaction process for xylobiose synthesis. In fact, to industrially produce xylobiose, it is necessary to establish conditions for maximizing xylobiose production taking into account the efficiency of xylobiose formation per unit enzyme and immobilization of enzyme or cell permeability. Therefore, to increase the efficiency of xylobiose synthesis, further studies on the stability of the enzyme

using the immobilization method as well as on the frequency and duration of enzyme immobilization are required.

Analysis of NMR data revealed that three kinds of xylobiose isomers were produced. The synthesized xylo-disaccharides in this study were found to be mixed with beta-1,4-xylobiose > beta-1,3-xylobiose > beta-1,2-xylobiose. Beta-1,2-xylobiose was predominantly in the Cα anomeric form in aqueous solution, whereas beta-1,3-xylobiose and beta-1,4-xylobiose were predominantly in the Cβ anomeric form. Given the kinetic analysis for the hydrolysis of xylobiose isomers, k_{cat}/K_m for beta-1,3 xylobiose was the lowest, and that for beta-1,2 and beta-1,4-xylobiose was comparable (Table 4-16). Thus, beta-1,3-xylobiose might be the relative resist against rehydrolysis by beta-xylosidase from *Bacillus pumilus* IPO. In the case of beta-1,2 and beta-1,4-products, the k_{cat}/K_m values were not significantly different; however, beta-1,4-product was produced more than beta-1,2 product. Even though the K_m of beta-1,4-product was the double of that for beta-1,2 product, the end point of xylobiose synthesis was the almost saturated condition (Xylobiose concentration > 10 K_m), leading to V_{max} for hydrolysis. Thus rehydrolysis for beta-1,2 and beta-1-4 products might be the same. The reason for the higher production of beta-1,4-xylobiose than that of beta-1,2-xylobiose could be faster synthesis of beta-1,4-xylobiose. This hypothesis could be supported by the thermal stability results. As seen in Table 4-17, thermal degradation rate constant for beta-1,2-xylobiose was faster than beta-1,4-xylobiose, suggesting that the energy level of beta-1,2-xylobiose is higher than that of beta-1,4-xylobiose. Given the similar k_{cat}/K_m for both isomers, we can expect Ea for both hydrolysis to be almost the same. As the products of the hydrolysis of both isomers are the same

(two xylose molecules), the energy level of the products of the hydrolysis of beta-1,2 and beta-1,4-xylobiose is the same. This situation gives higher Ea for the reverse reaction of beta-1,2-xylobiose than that of beta-1,4-xylobiose, resulting in a faster reaction rate of beta-1,4-xylobiose formation than that of beta-1,2-xylobiose formation.

Xylobiose is a bifunctional sweetener, which functions as a sugar complement and a prebiotic ingredient. Blood glucose levels and glycemic index in a group of healthy adults administered with sucrose supplemented with beta-1,4-xylobiose were lower than those consumed with the group that was administered pure sugar (Lee et al. 2016). In addition, administration of sucrose supplemented with beta-1,4-xylobiose among women having constipation improved the constipation status within 2 weeks (Lee et al. 2017). The synthesized xylo-disaccharides showed inhibitory effects on rat sucrase similar to those exerted to beta-1,4-xylobiose. Therefore, these synthetic xylo-disaccharides can be expected to be applied as a sugar complement. However, further experiments will be needed to determine whether beta-1,3-xylobiose or beta-1,2-xylobiose is a prebiotic material that can selectively increase the proliferation of *Bifidobacterium* species.

This study has developed a simple and environmentally friendly manufacturing process to effectively synthesize xylobiose from xylose using bacterial beta-xylosidase. The rat sucrase inhibitory effect of the synthesized xylo-disaccharides was confirmed to be the same as that exerted by beta-1,4-xylobiose. Furthermore, such a synthetic xylobiose could be used as a sugar complement and as a functional material in various food-related industries because of its high acid resistance and thermal stability. Therefore, although xylo-disaccharides contain

isomers, they can be used as a sugar complement in the form of xylobiose. Moreover, synthetic xylo-disaccharides are expected to be functional sweeteners that can be applied to a variety of processed foods.

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국 문 초 록

바실러스 퍼밀러스 유래의 베타자일로시데이즈를 이용한 자일로바이오스 합성공정 최적화

경 명 옥
바이오시스템공학 전공
서울대학교

바람직한 설탕보완재 (Sugar complement)는 설탕과 함께 섭취할 경우 설탕의 감미특성은 유지시키고, 체내 설탕분해효소의 활성을 억제하는 기능이 있는 감미료이다. 자일로바이오스 (Xylobiose)는 자일로오스 두 분자가 결합된 이당류로 자일로올리고당의 주요 성분이자 비피도박테리움 속에 의해서 선택적으로 이용되는 가장 좋은 프리바이오틱스로 알려졌다. 최근 자일로바이오스는 설탕분해효소를 저해하는 기능이 밝혀지면서 설탕보완재와 프리바이오틱스로 활용될 수 있는 양기능성 감미료로 주목 받게 되었다. 하지만 고순도 자일로바이오스를 생산하려면 목질계에서 분리한 자일란의 가수분해 산물로부터 분리공정을 거쳐야 하기 때문에 생산공정이 복잡하고 비용이 높은 한계가 있어 아직까지 식품소재로 활용되지 못하고 있다. 본

연구는 베타자일로시데이즈 (Beta-xylosidase)의 축합반응 (Condensation reaction)을 이용해서 가격이 저렴한 자일로오스에서 자일로바이오스를 생산할 수 있는 친환경적이고 단순한 생산공정을 확립하고, 합성된 자일로바이오스의 특성을 규명해서 식품소재로 활용할 수 있는 기반을 마련했다.

열두 종의 베타자일로시데이즈 중에서 자일로바이오스 합성효율이 가장 우수한 *Bacillus pumilus* IPO 유래의 베타자일로시데이즈를 선별했고, 식품산업에 사용할 수 있는 효모 (*Saccharomyces cerevisiae*)에 효소 발현시스템을 구축했다. 효소의 생산성을 높이기 위한 유가배양 (Fed-batch culture) 전략으로 배지의 탄소원/질소원 비율, 질소원 종류, 포도당 공급속도를 고려했다. 유가배양을 통해서 베타자일로시데이즈의 생산량은 회분배양 (Batch culture) 보다 7.1 배 높은 717.3 U/L (생산성 18.3 U/g_{DCW})까지 증가시켰다. 효소생산 균주의 플라스미드 안정성은 70% 이상으로 유지되었다. 자일로바이오스의 합성을 위한 최적화 조건은 반응표면설계 (Response surface methodology, RSM)의 중심합성계획법 (Central composite design, CCD)을 통해서 도출했다. 6.7 M 자일로오스, 6.5 U/mL 베타자일로시데이즈, 50℃ 반응 조건에서 최대 214.0 g/L의 자일로바이오스가 생산되었다. 이것은 지금까지 보고된 생산량 중에서 가장 높았고, 곰팡이 유래 베타자일로시데이즈를 이용한 선행연구보다 9.4 배 높았다. 게다가 반응물은 완충용액 및 솔비톨과 같은 첨가물을 사용하지 않았기 때문에 염분이나 첨가제를 제거하는 공정을 생략할 수

있는 장점이 있다. 합성된 자일로바이오스는 베타-1,4 결합 (51.5%) > 베타-1,3 결합 (35.9%) > 베타-1,2 결합 (12.6%) 로 구성된 자일로-이당류 (Xylo-disaccharides)로 밝혀졌다. 자일로-이당류는 pH 2의 산성 조건아래 100℃에서 90 분 동안 노출시켰을 때 최소 90% 이상 유지될 만큼 내산성과 열 안정성이 매우 우수한 특징이 있었다. 또한, 이들은 쥐 소장에서 추출된 설탕분해효소에 대한 활성 저해기능이 베타-1,4 자일로바이오스와 유사한 것으로 나타났다 ($p < 0.05$).

따라서 베타자일로시데이즈를 이용한 자일로바이오스 합성공정 개발은 반응조건의 최적화로 인한 생산성 증대 및 친환경적 공정의 단순화를 통해서 산업적으로 생산할 수 있는 기반을 마련했다. 또한, 자일로-이당류는 설탕분해효소의 활성을 저해하는 설탕보완재로 사용이 가능할 뿐만 아니라 내산성과 열 안정성이 우수하기 때문에 다양한 기능성 감미료로 활용이 가능할 것이다.

주제어: 설탕보완재 (Sugar complement), 자일로바이오스 (Xylobiose), 베타자일로시데이즈 (Beta-xylosidase), 축합반응 (Condensation reaction), 반응표면설계 (Response surface methodology), 중심합성계획법 (Central composite design), 모사이동상크로마토그래피 (Simulated moving bed chromatography), 자일로-이당류 (Xylo-disaccharides)

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