

Purification of bioxylitol by liquid–liquid extraction from enzymatic reaction mixture

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ABSTRACT

Xylitol purification is the most difficult step of the whole manufacturing process, and its purification being of commercial importance. This study aimed to purify bioxylitol by liquid–liquid extraction (LLE) from enzymatic reaction mixture. Bioxylitol extraction was conducted under various conditions of extraction time (45–105 min), sample to solvent ratio (SSR) (1:1–1:9 v/v), and number of extraction stages (NES) (1–9 n), and the response surface method was followed to optimize the variables. Xylitol separation was markedly affected by SSR and NES. Optimum time, SSR, and NES determined were 60 min, 1:4.5 (v/v), and 5 (n), respectively. Xylitol extraction yield attained was 78.14% (w/w) at these conditions. This research reveals the feasibility of clarifying bioxylitol produced via enzymatic reduction of xylose by LLE using ethyl acetate.

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Introduction

The polyol sweetener industries have registered an increasing demand for consuming sugar-free and low-calorie products. Among these sweeteners, xylitol is an important sugar substitute that has gained much attention globally because of its unique functional properties. Xylitol, also known as wood sugar, is a natural five-carbon sugar alcohol found in small amounts in fruits and vegetables. It is as sweet as sucrose, tastes good, has no aftertaste, and also has 40% less calories than sugar with a caloric value of 2.4 cal/g. Xylitol is widely used in food, pharmaceutical, odontological, and cosmetic industries as an ideal sugar for people with diabetics and obesity, to prevent ear and respiratory tract infections, osteoporosis, and dental caries, and to reduce gingivitis and control halitosis.^[1,2] Additionally, xylitol is identified as one of the 12 high-value specialty chemicals that can be produced from lignocellulosic biomass (LCB).^[3]

Nowadays, xylitol is commercially manufactured by an expensive chemical hydrogenation of pure D-xylose coming from acid hydrolysis of hardwood.^[1,4] Nevertheless, xylitol can also be manufactured biotechnologically from LCBs using microbes or isolated enzymes as catalysts of the xylose to xylitol bioconversion. The microbial production of xylitol has been studied extensively as an alternative to the chemical process.^[5–7] The advantage of the microbial process over chemical procedures is its lower cost due to

the non-necessity of extensive xylose purification.^[1] Xylitol bioproduction from xylose using xylose reductase (XR) has also been studied as an alternative to both chemical and microbial processes.^[8,9] One of the most significant advantages of enzymatic xylitol production is that it can afford an easy recovery of xylitol from reaction mixture.^[1] However, xylitol recovery from fermented broth and/or reaction mixture is still a great bottleneck, and there is no method available that allows an efficient purification and recovery of xylitol, which is necessary for xylitol production to become economically viable. Xylitol produced by enzymatic conversion instead of chemical reduction is called bioxylitol.

Purification and recovery of product exists as a very complicated step in various bioprocesses, which mainly depend on the nature of the product as well as on the complex composition of the fermented broth.^[10] To recover a pure product, it is often implied that important steps characterized by costs even higher than the production process are used. However, few reports are available on xylitol purification and recovery.^[2,11–13] Till now, on industrial scale, the obtained xylitol is purified and separated by chromatographic method. This technique of xylitol recovery is extensive and costly, and thus the final product is more expensive than other polyols.^[1] It is, therefore, worthwhile to explore an efficient and economically competitive strategy for xylitol

purification and recovery from xylitol-rich solution to diminish the manufacturing costs. A number of strategies have been employed to clarify and separate xylitol from fermented broth: activated charcoal, liquid–liquid extraction (LLE), precipitation, ion-exchange resins, membrane separation, and chromatographic methods, or a combination of these techniques.^[1,2,10,14]

LLE, also called solvent extraction, is a purification technique, which consists of the transfer of certain components (solutes) from one phase to another when immiscible or partly soluble liquid phases are brought into contact with each other. The solutes are separated based on their different solubilities in two different liquids.^[15,16] The advantages of LLE are that it is a simple, clean, faster, cost-effective, and energy-saving method for bioseparation. In addition, solvent recovery is easy from the process owing to their low boiling points, which is important to make the purification process economically feasible. However, LLE presents a tendency to form emulsion, thus slowing the purification process.^[10] The LLE strategy is widely employed in numerous industries (e.g. petroleum, hydrometallurgical, pharmaceutical, and nuclear industries) to recover dissolved substances or to remove undesirable impurities.^[2,15,16] It was pointed out that the purification of product by LLE depended on the characteristics of the desired product and solvent, composition of the reaction liquor, and a number of operating variables like sample to solvent ratio (SSR), extraction time, and stages.^[10,14] In this work, ethyl acetate (EA) was employed as solvent because it is one of the most efficient extractive agents for xylitol purification.^[14]

For a purification strategy, it is, hence, important to design and optimize the factors influencing product quality and recovery yield. Traditionally, parameter design has been conducted by observing the impact of one factor at a time (OFAT) on an experimental response while others are set at a constant value.^[17] OFAT is a simple, straightforward, and widely used experimental design, which does not require advanced statistical knowledge. However, the main drawback of this technique is that it is time-consuming and incapable of detecting the true optimal conditions due to the absence of the interaction effects among the factors.^[17,18] Response surface methodology (RSM) is also a simple and powerful statistical tool useful for modelling and optimizing process. Its major advantage is the reduced number of experimental trials needed to evaluate multiple parameters and their interactions.^[18]

No report is available in the literature regarding the optimization of LLE process for xylitol clarification. The present study was focused on the evaluation of LLE protocol for purifying bioxylitol obtained by enzymatic conversion of xylose present in the sawdust

hemicellulosic hydrolysate. The specific aims of this work are to explore the effects of factors, namely extraction time, sample to solvent ratio (SSR), and number of extraction stages (NES) on xylitol clarification from solution by LLE using OFAT, to optimize the process by RSM in order to maximize xylitol extraction yield.

Experimental

Raw material and chemicals

Xylose used as substrate was obtained from *Meranti* wood sawdust (MWS) through acid hydrolysis. MWS hydrolysis was performed at 124°C with 3.26% (w/w) H₂SO₄ for 80 min using a liquid to solid ratio of 8 g/g as reported.^[19,20] The solid and liquid phases were separated by filtration. The resulting filtrate, MWS hemicellulosic hydrolysate (MWSHH), was neutralized with CaO to pH 6.0 and its composition was determined by analytical methods. MWSHH was stored at 4°C and used in XR and bioxylitol production experiments. The synthetic xylitol was obtained from Merck Company (Darmstadt, Germany). All other medium components and chemicals used were of analytical grade and were purchased locally (Permula Chemicals Sdn Bhd, Terengganu, Malaysia).

Preparation of xylose reductase

Xylose reductase (XR) enzyme was prepared from an adapted strain of *Candida tropicalis* cultivated in hydrolysate growth medium. Yeast cells were harvested at the end of the log growth phase (20 h), re-suspended the washed pellet in potassium phosphate buffer (0.1 M; pH 7.0), and disrupted the cell suspension with a cell homogenizer according to the protocol outlined by Rafiqul and Sakinah.^[21] The cell homogenate was then centrifuged to obtain a supernatant solution. The supernatant was re-centrifuged and the refined supernatant was stored at –80°C, and used as crude XR enzyme. The XR having an activity of 11.2 U/mL was utilized for bioxylitol production experiment.

Preparation of samples

The samples were prepared using synthetic xylitol and bioxylitol obtained by enzymatic bioconversion of xylose present in the MWSHH.

Synthetic xylitol solution

The synthetic solution was prepared using commercial xylitol (99%, w/w) at concentration of 66 g/L in

ultrapure water followed by filtration (NY 0.45 μm , Membrane Solutions LLC, Texas, USA), and was utilized as model solution in LLE experiment.

Production of bioxytilol

Bioxytilol was manufactured from MWSHH using XR as described in detail in previous work.^[3] The reaction medium contained phosphate buffer (0.1 M; pH 7.0), XR enzyme, and NADPH in conical flask (50 mL). The reaction was started by adding MWSHH as substrate and the reaction mixture was mixed thoroughly. Preboiled XR was used as control. Production of bioxytilol was carried out in an incubator shaker at temperature 35°C, pH 7.0, using xylose concentration 18.8 g/L, NADPH concentration 2.83 g/L, enzyme concentration 3% (v/v), and agitation rate 100 rpm. After 12 h of incubation, the reaction was stopped by heating the reaction mixture in boiling water. The reaction mixture was centrifugation at 8000g for 10 min in order to separate the denatured protein and the supernatant was then filtered through 0.45 μm nylon syringe filter (Membrane solutions). The resulting filtrate was named as bioxytilol solution (reaction mixture) and analysed by HPLC for xylitol production. The concentration of bioxytilol in the solution was 13.37 g/L. The bioxytilol solution was stored at -20°C and used in subsequent LLE experiment.

Liquid-liquid extraction (LLE) of xylitol

The LLE of xylitol was conducted in batch mode in separatory funnel (500 mL) at ambient temperature under atmospheric pressure. Fifty millilitres of either synthetic xylitol (66 g/L) or bioxytilol solution (13.37 g/L) was mixed with required amount of EA in different ratios (v/v) and the stopper was put into the neck of funnel. The funnel was shaken gently at every 15 min interval for a given period of time to equilibrate the solutes between the aqueous and organic phases. When shaking, the separatory funnel was vented frequently to release pressure. Later, the funnel was placed on a tripod stand. At the end of desired extraction time, the aqueous and organic fractions were separated carefully, stored at 4°C, and analysed by HPLC for xylitol and sugars. Xylitol extraction yield was calculated according to Eqs. (1) and (2), and expressed as weight percent (% w/w). All the tests were done in triplicate and the data were statistically analysed and expressed as the mean values \pm SD of three replicate experiments.

$$[X]_{\text{org}} = \left([X]_{\text{aq initial}} - [X]_{\text{aq final}} \right) \times \frac{V_{\text{aq}}}{V_{\text{org}}} \quad (1)$$

$$\text{Extraction yield (\%)} = \frac{[X]_{\text{org}}}{[X]_{\text{aq final}} + [X]_{\text{org}}} \times 100 \quad (2)$$

where $[X]_{\text{org}}$ is the total xylitol concentration (g/L) in organic phase, $[X]_{\text{aq initial}}$ is the initial xylitol concentration (g/L) in aqueous phase before extraction, $[X]_{\text{aq final}}$ is the final xylitol concentration (g/L) in aqueous phase after extraction, and V_{aq} and V_{org} are the volumes (L) of aqueous and organic phases, respectively.

Variable design procedure

The design of process variables is the initial step in the optimization studies on xylitol purification and is applied to determine the suitable range of each factor. It was executed with OFAT method by observing the effect of variables such as extraction time, SSR, and NES on the response, xylitol extraction yield (% w/w). The variation of factors and their ranges used for each study are presented in Table 1. At the beginning of the OFAT experiment, the levels of two variables out of three were held constant (SSR at 1:5 (v/v) and NES at 1 n) based on previous reports.^[14] The first factor was then changed until an optimum value was reached. This optimum value for the first variable was then used while the second factor was varied, and so on. To justify the OFAT outcomes, the experimental data were analysed by Minitab® program and statistically evaluated with analysis of variance (ANOVA), which comprised *F*-test, its associated probability ($P > F$), determination coefficient (R^2), and correlation coefficient (*R*).

Experimental design for model development

Central composite design (CCD) under RSM was employed to explore the interaction of variables, and to model and optimize them for maximum xylitol extraction yield as the experimental response. The effective ranges and levels of two major independent variables, SSR (*A*) and NES (*B*), were selected from the finding of OFAT study. Another factor extraction time was fixed at 60 min as its effect was found to be insignificant in xylitol purification. A 2² CCD with 5 levels coded (-2, -1, 0, +1, +2) leading to 11 experiments (3 runs at centre point) was

Table 1. Process variables and their ranges used in OFAT study for xylitol purification.

Variables	Change of variables		
	Time (min)	SSR (v/v)	NES (n)
Extraction time	45–105	1:5	1
Sample to solvent ratio (SSR)	60	1:1–1:9	1
Number of extraction stages (NES)	60	1:5	1–9

Table 2. CCD matrix with coded and actual variables along with the observed results.

Run	Coded variables		Actual variables		Xylitol extraction yield (Y, %)
	A	B	SSR (A, v/v)	NES (B, n)	
1	2	0	1:6	4	74.62 ± 7.10
2	-2	0	1:2	4	52.56 ± 4.93
3	0	-2	1:4	2	48.32 ± 4.59
4	0	0	1:4	4	80.94 ± 7.69
5	0	0	1:4	4	79.63 ± 7.56
6	1	1	1:5	5	83.23 ± 7.86
7	0	0	1:4	4	79.51 ± 7.55
8	0	2	1:4	6	72.44 ± 6.88
9	1	-1	1:5	3	66.73 ± 6.34
10	-1	-1	1:3	3	61.79 ± 5.87
11	-1	1	1:3	5	71.76 ± 6.82

SSR, sample to solvent ratio (v/v); NES, number of extraction stages (n).
Y = xylitol extraction yield (% w/w).

performed and their results were fitted to the second order model as expressed by Eq. (3). The design matrix of the coded and actual input variables and the corresponding response (extraction yield, Y) are listed in Table 2.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_{12} AB + \beta_{11} A^2 + \beta_{22} B^2 \quad (3)$$

Within Eq. (3), Y represents the response variable; A and B are the coded input variables (SSR and NES, respectively); β_0 is the interception coefficient; β_1 and β_2 are the linear coefficients; β_{11} and β_{22} are the quadratic coefficients; and β_{12} is the second order interaction coefficient. Design Expert (version 7.0, Stat Ease, Inc., Minneapolis, MN, USA) software was used for regression and graphical analyses of the data obtained. The model was evaluated by analysing the values of regression coefficients, ANOVA, P-, and F-values. The fit of the polynomial model was expressed by determination coefficient (R^2) and correlation coefficient (R). Six sets of experiments were conducted under proposed optimum conditions to validate the CCD model developed. A final experiment was also conducted to confirm the model.

Analytical procedures

Xylitol, xylose, glucose, acetic acid, and arabinose concentrations were determined at 80°C by HPLC using an Agilent 1200 chromatograph (Agilent, Santa Clara, CA, USA) equipped with a refractive index detector (RID) and a Rezex RHM Monosaccharide (300 mm × 7.8 mm; Phenomenex, Torrance, CA, USA) column. The optimized chromatographic conditions were ultrapure water as mobile phase, with a flow rate of 0.6 mL/min, temperature of 80°C, and an injection volume of 20 μ L. Furfural and hydroxymethylfurfural (HMF) were also determined by HPLC, but with an UV-DAD set at 276 nm and a Zorbax eclipse XDB-C₁₈ (5 μ m; Agilent)

column at 25°C. In this case, the mobile phase was acetonitrile/water (1:8) with 1% (v/v) glacial acetic acid at a flow rate of 0.8 mL/min. The eluents were previously vacuum filtered (NY 0.45 μ m, Membrane solutions) and degassed. Samples were diluted with ultrapure water when necessary prior to injection. All the samples and standard solutions were filtered with syringe filter (NY 0.45 μ m) into HPLC vials before running analysis. The aqueous and organic fractions obtained in the LLE were analysed for xylitol and other compounds using HPLC.

The total contents of lignin degradation products (LDPs) were estimated spectrophotometrically according to the modified Prussian blue method^[22] using tannic acid as standard. The cell dry weight was measured by dry weight method, and XR activity was determined spectrophotometrically at 340 nm as reported previously.^[21] One unit (U) of XR is referred to as the amount of enzyme required to catalyse the oxidation of 1 μ mol of NADPH per min at pH 7.0 and 25°C.

Results and discussion

Composition of MWS hydrolysate

To achieve a xylose-rich hydrolysate with minimum by-products, the hemicellulose part of MWS biomass was hydrolysed at 124°C with 3.26% H₂SO₄ for 80 min using a liquid/solid ratio (w/w) of 8. This optimized condition yielded a hemicellulosic hydrolysate containing 18.8 g/L xylose as the main constituent, together with a low content of glucose, acetic acid, arabinose, LDPs, furfural, and HMF. Table 3 shows the chemical composition of MWSHH used for culture and reaction media preparation.

Composition of bioxylitol solution

The enzymatic production of bioxylitol from MWSHH was conducted for a period of 12 h at 35°C, pH 7.0 using 18.8 g/L crude xylose, 2.83 g/L NADPH, 3% XR, and 100 rpm agitation.^[3] Xylitol production of 13.37 g/L with a yield of 71% was attained under these conditions. At the end of xylose to xylitol bioconversion,

Table 3. Composition of MWS hydrolysate used for culture and reaction media preparation.

Constituents	Concentration of constituents (g/L)
Xylose	18.8 ± 1.51
Glucose	4.64 ± 0.39
Acetic acid	4.14 ± 0.33
Arabinose	2.55 ± 0.21
LDPs	1.55 ± 0.13
Furfural	0.55 ± 0.04
HMF	0.08 ± 0.007

LDPs, lignin degradation products; HMF, hydroxymethylfurfural.

the content of residual xylose in the reaction mixture was 5.42 g/L. The concentrations of glucose, arabinose, LDPs, furfural, and HMF (Table 3) remained almost the same throughout the reaction period. Acetic acid concentration slightly decreased with increasing time probably due to its partial evaporation during the reaction. However, the presence of these undesirable compounds in the reaction mixture necessitates the development of inexpensive and efficient purification methods for recovering xylitol. Thus, the extraction of xylitol from these impurities in the reaction mixture is important to facilitate the xylitol recovery in a later step.

Xylitol purification by LLE

The removal of desired solute from one phase into the solvent phase is referred to as extraction. LLE is a low-cost and faster separation strategy and is used extensively in industrial processes for the extraction of biomolecules.^[16] One of the primary advantages of LLE process is the ability to operate in a continuous, multistage, and countercurrent flow mode. Xylitol extraction was carried out by LLE using EA as extracting solvent, and the process was sequentially optimized by OFAT and RSM approaches.

Variable design by OFAT

OFAT is a simple statistical plan where the individual effects of parameters on the response can be seen on graphs. The OFAT study aimed to explore the impact of factors on xylitol extraction from solution and to design them for further optimization. The concentrations of synthetic xylitol and bioxylitol in the sample solutions were 66 and 13.37 g/L, respectively. The influences of three variables extraction time, SSR, and NES on the clarification of xylitol, evaluated by OFAT, are detailed in the following sections.

Influence of extraction time

To evaluate the effect of extraction time on xylitol separation, different levels of extraction time varying from 45 to 105 min were employed (Table 1). Figure 1a depicts the influence of extraction time on xylitol separation from its synthetic solution at fixed SSR (1:5 v/v) and NES (1 n). The yield of xylitol extraction increased with increase in extraction time up to 60 min with a maximum value of 47.16% (w/w). Further increase in time did not enhance xylitol separation at all. The linearity in xylitol extraction during extraction time above 60 min might be due to its lower concentration in the aqueous phase. The shorter extraction time is more beneficial in terms of product separation and recovery. Hence, extraction time was maintained at 60 min in the subsequent studies as it did not show significant effect on xylitol purification at high level.

Influence of SSR

SSR in the experiment was maintained at five different levels ranging from 1:1 to 1:9 (v/v) to examine its influence on the extraction of xylitol from solution, and is shown in Fig. 1b. It was noticed that xylitol extraction increased with increasing SSR to a certain extent and resulted in the highest value of 47.16% at 1:5 (v/v) when extraction time and NES were kept constant (Table 1). The obtained results are closely similar to those of Misra et al.^[10] who reported the extraction of xylitol from fermented broth by LLE. Xylitol extraction yield diminished drastically on further increase of SSR, and at 1:9 (v/v), it reached to 39.11% (Fig. 1b). The decrease in xylitol extraction into the organic phase might be attributed to the greater liquid-liquid film resistance formed accompanied by an increase in emulsion formation as the solvent (EA) volume is increased, which contributed greater barrier for xylitol to be extracted effectively.^[23,24] A considerable amount of xylitol was extracted within SSR 1:1–1:5 (v/v) that was further investigated by RSM for optimizing the significant factors. Thus, the

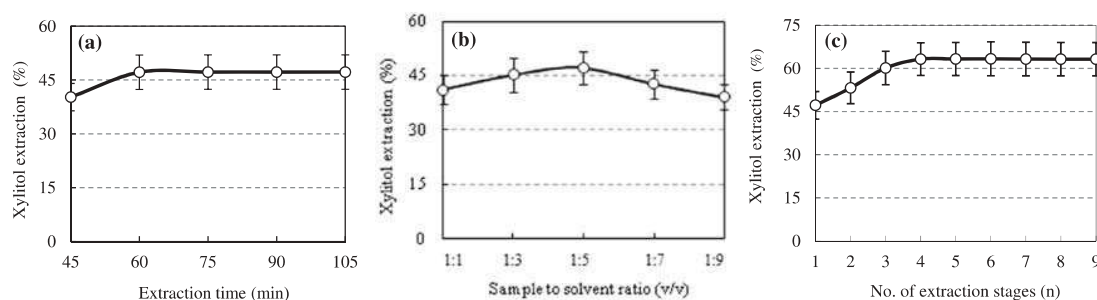


Figure 1. Influence of the three parameters on xylitol purification: (a) extraction time, (b) sample to solvent ratio, and (c) number of extraction stages. Xylitol was separated from synthetic xylitol solution by LLE using ethyl acetate as solvent. The initial xylitol concentration was 66 g/L. The error bars indicate the standard deviations of the mean values.

possible optimum SSR was found to be 1:5 (v/v) for further experiments to monitor the effect of NES on xylitol clarification.

Influence of extraction stages

NES was varied between 1 and 9 (n) to select the suitable NES for maximum extraction of xylitol. Figure 1c illustrates the effect of various NES on xylitol extraction from its synthetic solution at constant values of extraction time (60 min) and SSR (1:5 v/v). From the figure, it was evident that the removal of xylitol gradually increased with the increase of NES up to four stages of extraction with a maximum yield of 63.20% (w/w). The yield of xylitol extraction remained almost constant with further increase of NES (Fig. 1c). This finding indicated that some amount of xylitol was extracted to the fresh solvent added in each of the four stages. It was pointed out that the multiple stages yielded higher extraction efficiency, as it increases the partition coefficient by decreasing the concentration of solute in aqueous layer by batch addition of fresh ethyl acetate. In addition, the presence of lesser xylitol in the aqueous phase could slow or stop its transfer to the organic (EA) phase. Similar outcomes have been reported for the purification of xylitol from fermented broth.^[14] The observe optimum NES was 4 n and its effect was further statistically estimated in the range of 2–6 (n) using RSM.

Using OFAT method, the possible ideal conditions for xylitol extraction were determined as extraction time 60 min, SSR 1:5 (v/v), and NES 4 (n). The highest xylitol extraction of 63.2% was attained under these conditions. The OFAT study demonstrated that two input variables, SSR and NES, notably influenced the separation of xylitol by LLE from solution. Therefore, these factors were further optimized by RSM to obtain the true optimal conditions for enhanced xylitol recovery. Other factor extraction time was set at 60 min throughout the investigations due to its insignificant effect on xylitol purification and to shorten downstream processing period. The observed data were statistically analysed by linear regression using Minitab® software to verify the OFAT results. The ANOVA for the impacts of different variables examined by OFAT on xylitol purification is tabulated in Table 4. The output of ANOVA demonstrated that SSR and NES imparted significant effects on the purification of xylitol (as their $P > F$ values are less than 0.05). In addition, these variables revealed strong relationship with xylitol purification and showed the satisfactory R values of 0.952 and 0.941 (Table 4), respectively. The impact of reaction time was insignificant ($P > F$ above 0.05 indicates variables are

Table 4. ANOVA for the impacts of variables examined by OFAT on xylitol purification.

Variables examined	Description of regression	Values	Results
Extraction time (h)	Regression model	Linear	Insignificant
	F -value	2.35	
	$P > F$	0.081	
	R^2	0.605	
Sample to solvent ratio (SSR; v/v)	Regression model	Linear	Significant
	F -value	5.49	
	$P > F$	0.002	
	R^2	0.907	
Number of extraction stages (NES; n)	Regression model	Linear	Significant
	F -value	21.69	
	$P > F$	0.0001	
	R^2	0.886	
	R	0.941	

$P > F$ less than 0.05 indicates factors and models are significant. R^2 , determination coefficient; R , correlation coefficient.

insignificant). It is pointed out that the significant parameters were found consistent with the graphical presentations of OFAT studies. Therefore, the single-factor experiment proved to be important aiming at the search of the possible optimal conditions for a purification process for maximum extraction yield.

Process optimization using RSM

Optimization study was further continued with CCD of RSM aiming to: (i) maximize xylitol extraction; (ii) develop model and determine which variables have higher effect on extraction yields; (iii) show interactions among variables; and (iv) give an insight on the robustness of the approach close to the optimum conditions. Based on OFAT study, the two variables namely SSR (A) and NES (B) were opted for further evaluation of their main and interaction effects on xylitol purification using CCD. The results of response surface study on xylitol separation including model evaluation and validation are discussed hereafter.

The best-fitting model was determined by the regression analysis and the quadratic model was evaluated by ANOVA. The results of ANOVA for the quadratic model representing xylitol extraction yield are listed in Table 5. The fit summary analysis denoted that the developed model was significant at 95% confidence level (CL) to represent the response. The model adequacy was tested through F -test, $P > F$, R^2 , and R . The values of probability $P > F$ less than 0.05 indicate that the model terms are statistically significant. The F - and P - values of model were 179.40 and <0.0001, respectively, which implied that the model was highly significant with low probability. These results also implied that the obtained model was in good prediction of the observed data and the variables have significant impacts on the response extraction yield

Table 5. ANOVA for the quadratic model representing xylitol extraction yield (Y).

Source	Sum of squares	Degree of freedom	Mean square	F-value	$P > F$
Model	1352.81	5	270.56	179.40	<0.0001 ^a
A	305.32	1	305.32	202.44	<0.0001
B	465.13	1	465.13	308.41	<0.0001
AB	10.66	1	10.66	7.07	0.0450
A^2	325.38	1	325.38	215.74	<0.0001
B^2	465.00	1	465.00	308.32	<0.0001
Residual	7.54	5	1.51		
Lack of fit	6.28	3	2.09	3.33	0.2396 ^b
Pure error	1.26	2	0.63		
Corresponding total	1360.35	10			
R^2	0.9945				
R	0.9972				
Adjusted R^2	0.9889				

$P > F$ less than 0.05 indicates model terms are significant.

^aModel is significant.

^bLack of fit is not significant.

(Y , %). Moreover, the model having an insignificant lack of fit ($P > F = 0.2396$) and a satisfactory R^2 value of 0.9945 (Table 5). The value of R was 0.9972, indicating a good agreement among the experimental and predicted values. The ranking of model terms (based on the F -value) having significant effect on xylitol recovery is $B > B^2 > A^2 > A > AB$. These results were consistent with ANOVA for the effects of variables studied by OFAT (Table 4) where the variable NES (B) has the highest F -value. Fitting of the data to the model and their subsequent ANOVA showed that the LLE of xylitol was most suitably described with a quadratic model as Eq. (4), where Y is the xylitol extraction yield; A the SSR and B the NES.

$$Y = +79.97 + 5.04A + 6.23B + 1.63AB - 4.11A^2 - 4.91B^2 \quad (4)$$

Interaction of variables

The interaction and response surface plots are the graphical representations of the regression model equation used to understand the interaction between variables and to locate their optimal levels for maximum xylitol extraction using LLE. The interaction and surface plots illustrated in Fig. 2 were constructed based on the function of two input variables SSR (A) and NES (B). The significant interaction among the variables is indicated by an elliptical nature of the contour diagram. It was found that the yield of xylitol extraction gradually increased when the SSR and NES increased to maximum level. The enhancement in extraction yield from 70.51% (w/w) at 1:3 (v/v) to 83.86% at a SSR of 1:5 (v/v) brought by increasing SSR appeared to be larger at higher NES (5 n) (Fig. 2a,b). It was highlighted that a notable improvement in extraction yield due to the interaction between SSR and NES as the NES increases from 3 to 5 (n), depicting that this factor has the largest impact on the yield, which is consistent with ANOVA output (Table 5). These results indicated a positive correlation with both of these variables. These results also interpreted that high level of SSR and NES will give the maximum xylitol extraction yield of 83.23% as obtained in run 6 (Table 2). However, at relatively high SSR (1:6 in run 1) and NES (6 n in run 8), extraction yield considerably decreased to 74.62% and 72.44%, respectively.

Validation and confirmation of model

Validation is the final step of optimization studies and is important to confirm the prediction accuracy. According to the developed model, numerical

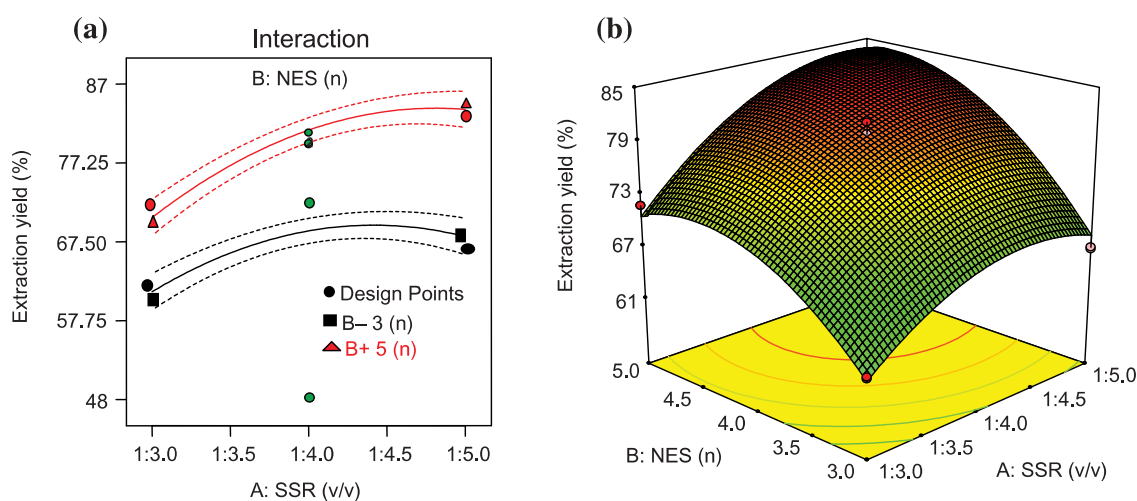


Figure 2. The combined effects of sample to solvent ratio (SSR) and number of extraction stages (NES) on xylitol purification by LLE: (a) interaction; and (b) response surface plots.

Table 6. Experimental layout, and results of verification and confirmation run.

Run	Operating factors		Xylitol extraction yield (% w/w)			
	SSR (A, v/v)	NES (B, n)	Predicted	Actual	Residual	Error (%)
1	1:5	4	80.85	78.72	2.13	2.71
2	1:5	5	81.26	82.48	-1.22	1.47
3	1:4.5	5	81.25	82.51	-1.25	1.52
4	1:4	4	79.18	81.64	-2.46	3.01
5	1:4	5	81.21	79.18	2.03	2.56
6	1:3	5	76.12	72.44	3.68	5.08

optimization was carried out using “Design Expert program” and six suggested optimal conditions were achieved, which are recorded in Table 6. To verify the optimization results, LLE experiment was performed at the predicted optimal conditions. The residuals and percent errors between the experimental and predicted values are calculated.^[20] The best result for xylitol extraction obtained was 82.51% at SSR 1:4.5 (v/v) and NES 5 (n) (shown in boldface in Table 6). The percent errors, ranging from 1.52% to 5.08%, indicated that the constructed model was noticeably adequate as the errors were well within acceptable value (5%). These findings also suggested that the model is reasonably accurate at 95% of CL. Optimum SSR and NES were determined from the model to be 1:4.5 (v/v) and 5 (n), respectively. At these conditions, the model predicted a maximum xylitol extraction yield of 81.25%. To prove this yield, a confirmation experiment was conducted and the result is also shown in boldface in Table 6. The observed xylitol extraction of 82.51% was very close to the predicted value, indicating the model to be rationally reliable within 95% CL. Thus, the created model could reliably predict ideal solvent extraction conditions with respect to maximal recovery of xylitol from solution.

For xylitol purification, the optimum conditions established were extraction time of 60 min, SSR 1:4.5 (v/v), and NES 5 (n). These optimized conditions gave a xylitol extraction yield of 82.51% (w/w). It was mentioned that xylitol extraction yield of 82.51% with ideal conditions was achieved by the CCD, which was 1.31-fold higher than the yield obtained by the OFAT (63.2%). These outcomes demonstrated the importance and applicability of the sequential optimization strategies for improving xylitol separation from solution by LLE.

Xylitol purification from bioxylitol solution

Faveri et al.^[12] and Mussatto et al.^[14] reported that xylitol purification from fermented hemicellulosic hydrolysate is very difficult due to the presence of various impurities resulting from biomass hydrolysis. The optimal conditions achieved were applied to

maximize xylitol extraction from bioxylitol solution (enzymatic reaction mixture). This work revealed that xylitol can be clarified from enzymatic reaction mixture through LLE. Since extraction solvent dose is expensive, the LLE-based purification of bioxylitol (from enzymatic reaction mix) requires recycling of solvent (EA) to make the extraction approach economically viable. This can be easily accomplished by recovering solvent, via evaporation because of their low boiling points, for the recycling of them in the LLE process. Using optimum conditions (time 60 min, SSR 1:4.5, and NES 5 n) for extracting xylitol from bioxylitol solution resulted in extraction yield of 78.14%. It was seen that there was a diminution of 4.37% in yield (Y) for the experiments using bioxylitol solution, compared with the optimization result (82.51%) with commercial xylitol. The decrease in Y might be attributed to the low xylitol concentration in the enzymatic reaction mixture. These findings pointed out that the reaction mixture containing xylitol, xylose, glucose, acetic acid, arabinose, LDPs, furfural, and HMF (in the concentrations of 13.37, 5.42, 4.64, 4.14, 2.55, 1.55, 0.55, and 0.08 g/L, respectively) did not hinder the purification courses.

Xylitol separation and purification from fermented broth by LLE using ethyl acetate has been reported^[10,14] with the highest extraction yield of 33.26% (w/w, calculated), obtained at 25°C with 1:5 (v/v) SSR using a NES of 1 (n).^[10] In this study, bioxylitol extraction yield (78.14%) is higher than the value achieved from fermented broth probably due to the increased NES, and the absence of microbial media ingredients and cell debris from the reaction mixture. Thus, the LLE method using ethyl acetate proved to be efficient for xylitol purification from the untreated reaction mixture that reduces the cost of its recovery thereby making the process more feasible. This is the first report on the purification of xylitol from enzymatic reaction mixture using LLE strategy. Therefore, the obtained optimum conditions will be selected for subsequent generation of purified bioxylitol solution to facilitate its recovery through crystallization.

Conclusion

The current study reveals the feasibility of clarifying bioxylitol produced via enzymatic reduction of xylose present in the hemicellulosic hydrolysate by LLE using ethyl acetate. LLE is a simple, efficient, and rapid purification strategy that can be used for bioxylitol clarification. The influence of various experimental parameters (extraction time, SSR, and NES) in xylitol extraction was assayed using two optimization methods, namely OFAT and CCD to ensure its high

extraction recovery. A 2^2 CCD was adopted to design experiments and to optimize the process with respect to xylitol extraction yield. Xylitol separation was significantly influenced by SSR and NES. The ideal conditions for bioxylitol purification were obtained as extraction time 60 min, SSR 1:4.5 (v/v), and NES 5 (n). These conditions led to an extraction yield of 78.14%. This work will provide a sound basis for the industrial production and purification of xylitol, a high-value specialty chemical, to improve the yield and quality of xylitol crystals in a later step.

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