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Preparation of maltotriose from fermentation broth by hydrolysis of pullulan using pullulanase

Sheng-Jun Wu*, Jing Chen

School of Marine Science and Technology, HuaiHai Institute of Technology, 59 Cangwu Road, Xinpu, 222005, China

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ABSTRACT

In this study, we prepared maltotriose from the fermentation broth of *Auerobasidium Pullulans* CJ001 isolated from the sea mud by hydrolysis of pullulan with pullulanase. The fermentation broth was centrifuged to remove the microorganisms and then hydrolysed by pullulanase. The optimal hydrolysis conditions were obtained as follows: time, 9.40 h; pH, 4.92; temperature, 47.88 °C; pullulanase, 10 ASPU/g. Under these optimum hydrolysis conditions, the maximum dextrose equivalent value reached 31.86. The hydrolysates were filtrated through a filter membrane to separate any particle with molecular weight higher than 1000 Da, concentrated to ~20%, and precipitated with 8 volumes of absolute ethanol. The precipitate was dried at 80 °C for 2 h to yield the maltotriose product. The maltotriose content in the product and the yield of maltotriose were 92.13% and 90.23%, respectively. The results indicate that this was a promising way of maltotriose production.

1. Introduction

Pullulan, a biopolymer synthesized by the yeast-like fungus *Aureobasidium pullulans*, is a linear mixed linkage α-D-glucan consisting of majority of maltotriose and trace of maltotetraose repeating units interconnected by α-(1 ⟶ 6) linkages. Pullulan and its derivatives have numerous potential for food, pharmaceutical and other industrial applications (Singh, Saini, & Kennedy, 2008).

Pullulanase (EC 3.2.1.41) is a debranching enzyme which hydrolyses the α-1,6-glucosidic linkages in pullulan and other amylaceous polysaccharides (Matzke, Herrmann, Schneider, & Bakker, 2000). Complete hydrolysis of pullulan using pullulanase yields maltotriose as major product along with traces of maltotetraose.

Maltotriose possess many excellent properties, e.g. mild sweetness, ability to store well in moisture, and the prevention of retrogradation of starch in foodstuffs. These properties are useful in food and pharmaceutical industries (Singh et al., 2008). Maltotriose can be produced from starch by hydrolysis with maltotriose-producing amylases (Takasaki et al., 1991). However, this is not a suitable way for maltotriose production because the purity of maltotriose should be taken into consideration. For example, the typical sugar composition of hydrolysis products from liquefied starch with maltotriose-producing amylases was only about 55–60% of maltotriose (Takasaki et al., 1991). Thus, an alternative way for producing high maltotriose-containing product is worth to be investigated.

Therefore, it is of our interest to investigate the way for producing high maltotriose-containing product by hydrolyzing of the pullulan in the fermentation broth of *A. Pullulans* CJ001 isolated from sea mud from the east of China with pullulanase. The optimal hydrolysis and precipitation conditions were investigated and the product was partially characterized.

2. Materials and methods

2.1. Microorganism

A. Pullulans CJ001, isolated from the sea mud in the east of China, was maintained at 4 °C on potato dextrose agar (PDA) and subcultured every 2 weeks. Pullulanase (L-1000) was purchased from Genencor International, Inc., Wuxi, China.

2.2. Medium preparation

The medium contained 50 g sucrose, 2.0 g yeast extract, 0.77 g K_2HPO_4, 1.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, and 1.98 g NaCl in 1 L distilled water (Chen, Wu, & Pan, 2012). The pH was adjusted to 5.5, and the medium was autoclaved at 121 °C for 15 min.

* Corresponding author. Tel.: +86 0518 85895427; fax: +86 0518 85895428.
E-mail address: wushengjun008@sina.com (S.-J. Wu).

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2.3. Fermentation

Seed cultures were prepared by inoculating cells grown on a PDA agar slant into a 250-mL flask that contained 50 mL of the inoculum medium and subsequently incubated at 22 °C for 48 h with shaking at 200 rpm. 2.5 mL of the seed culture were transferred into the 250-mL flask containing 50 mL of the fermentation media. The culture was shaken at 22 °C and 200 rpm for 6 d (Wu, Chen, & Pan, 2012). Pullulan concentration in the resulting fermentation broth was 31.25 g/L (Wu et al., 2012).

2.4. Hydrolysis of the pullulan in the fermentation broth

The culture was centrifuged at 15,000 × *g* for 20 min to remove the microorganisms. Different amounts of pullulanase (4, 6, 8, 10, 12, and 14 ASPU/g, respectively) were added to a reactor containing 100 mL of the fermentation broth, and the reactor was incubated in a thermostatic water bath at different temperatures for designated time periods (Table 1). Aliquots were periodically taken from the reaction mixture and mixed with the same volume of a 0.4 M trichloroacetic acid solution to terminate the reaction. The DE values of the reaction mixture were used to evaluate the degree of hydrolysis.

2.5. Precipitating with ethanol

The reaction mixture was filtrated through a 1000 Da-interception-filter membrane, concentrated to ~20% and then precipitated with different (2, 4, 6, 8, 10 and 12) volumes of ethanol. The precipitates were dried at 80 °C in an oven to a constant weight. The optimum volume of ethanol was obtained according to the highest recovery and content of maltotriose (Wu, Chen, Tong, Xu, & Jin, 2009).

2.6. Analytical methods

The reducing sugars were estimated by the method of Somogyi (Nelson, 1944). The pH of the solution was recorded using a digital pH meter (Model: PHS-3C, CD Instruments, China). Ash, moisture, and protein content of the samples were determined as per standard methods (Hou, 2004). The composition of the sugar in the sample of maltotriose was analysed by Water 600 HPLC equipped with a double column system. The first column (Sugarpark 1, 6.5 mmid × 300 mm) used pure water as mobile phase at a flow rate of 0.5 mL/min and the column temperature was maintained at 85 °C. The second column (SpherisorbNH$_2$, 4.6 mmid × 250 mm) used acetonitrile/water (70/30, v/v) as mobile phase at a flow rate of 1 mL/min and the column temperature was 30 °C. The detector sensitivity was 4 and the injection volume was 10 μL (Wu et al., 2009).

Table 1
The central-composite design for optimizing hydrolysis conditions.

	X_1	X_2	X_3	Y
1	8.00	48.00	3.32	19.37
2	6.00	50.00	4.00	15.24
3	8.00	44.64	5.00	21.56
4	10.00	46.00	4.00	26.53
5	10.00	50.00	4.00	22.67
6	10.00	50.00	6.00	22.86
7	6.00	46.00	4.00	14.92
8	8.00	48.00	5.00	29.93
9	10.00	46.00	6.00	27.15
10	8.00	48.00	5.00	30.22
11	6.00	46.00	6.00	14.27
12	8.00	51.36	5.00	19.69
13	6.00	50.00	6.00	15.46
14	8.00	48.00	6.68	19.64
15	8.00	48.00	5.00	30.56
16	8.00	48.00	5.00	30.37
17	8.00	48.00	5.00	30.45
18	11.36	48.00	5.00	30.46
19	8.00	48.00	5.00	30.61
20	4.64	48.00	5.00	12.98

X_1 = Time (h), X_2 = Temperature (°C), X_3 = pH.

2.7. Experimental design

A central composite design (CCD) was used to optimize the hydrolysis conditions using pullulanase and for fitting a polynomial model as follows:

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

where Y is the DE value, β_0 is the intercept term, β_1, β_2 and β_3 are linear coefficients, β_{12}, β_{13} and β_{23} are interaction coefficients, β_{11}, β_{22} *and* β_{33} are squared coefficients and X_1, X_2 and X_3 are coded independent variables. Design Expert software (version 8.0.5b, State-Ease Inc., Minneapolis, USA) was used for the experimental design, data analysis, and model building.

2.8. Statistical analysis

All data are presented as mean ± S.D. Statistical analysis was performed using Statgraphics Centurion XV version 15.1.02. A multifactor ANOVA with posterior multiple range test was used for determining statistical significance.

3. Results and discussion

3.1. Effect of time, temperature, and pH on pullulan hydrolysis

A combination of three variables, i.e. time, temperature, and pH, was optimised using CCD. The design and results of the experiments performed using CCD are presented in Table 1. The results were subjected to statistical analysis using ANOVA, and the regression model was obtained using Eq. (2):

$$Y = 2223.70197 + 28.94923 \times X_1 + 85.19580 \times X_2 + 36.77943 \times X_3 - 0.30187 \times X_1 X_2 + 0.077500 \times X_1 X_3 + 0.027500 X_2 X_3 - 0.77068 \times {X_1}^2 - 0.86746 \times {X_2}^2 - 3.86584 \times {X_3}^2 \quad (2)$$

where Y is the DE values, X_1 is the time (h), X_2 is the temperature (°C) and X_3 is pH. The statistical significance of Eq. (2) for the response surface quadratic model was determined using ANOVA. The data presented in Table 2 indicate that the model was highly significant, as demonstrated by the F-value and the probability value [$(P > F) < 0.0001$]. The accuracy of fit was verified on the basis of the high multiple correlation coefficient ($R^2 = 99.16\%$), which indicated that the response model can explain 99.16% of the total variations. The value of the adjusted multiple correlation coefficient ($R^2_{Adj} = 99.74\%$) was even high enough to indicate the significance of the model. In general, a regression model with an R^2 value greater than 0.9 can be considered to have a very high correlation (Haaland, 1989).

The interaction between time and temperature was significant [$(P > F) < 0.0001$], and this was consistent with the general nature of enzyme, i.e. the optimum temperature is related with time (Table 2). The interaction between time and pH and that between temperature and pH were not significant (Table 2). The optimal time, temperature, and pH for the maximum DE value (based on

Table 2
Analysis of variance for the experimental results of the central-composite design.

Factor*	Sum of square	Degree of freedom	*F*-value	*P* > *F*	Significance
X_1	345.77	1	810.46	< 0.0001	**
X_2	7.01	1	63.55	< 0.0001	**
X3	0.051	1	0.46	0.5122	
X_1^2	139.65	1	1241.48	< 0.0001	**
X_2^2	173.51	1	1572.89	< 0.0001	**
X_3^2	215.37	1	1952.37	< 0.0001	**
$X_1 \times X_2$	11.66	1	105.74	< 0.0001	**
$X_1 \times X_3$	0.19	1	1.74	0.2163	
$X_2 \times X_3$	0.024	1	0.22	0.6496	
Model	804.64	9	810.46	< 0.0001	**
Lack of fit	0.79	5	2.50	0.1686	
Pure error	0.32	5	0.063		

X_1 = Time (h), X_2 = Temperature (°C), X_3 = pH.
* Statistically significant at 95% of probability level.
** Statistically significant at 99% of probability level.

the model) were 9.40 h, 47.88 °C, and 4.92, respectively. In contrast, other reports have described optimal conditions for pullulan hydrolysis as 6 h, 45 °C, and pH 5 (Wu et al., 2009) and 6 h, 50 °C, and pH 5 (Singh, Saini, & Kennedy, 2010). The different optimal pullulan hydrolysis conditions reported in the literature may be due to the differences in the sources of pullulan and pullulanase. The maximum predictable response for DE was calculated by substituting the levels of the factors into the regression equation. The maximum DE obtained experimentally using the optimised conditions was 31.86. This was consistent with the predicted value of 32.42 obtained using the response surface methodology regression study.

3.2. Effect of pullulanase amount on pullulan hydrolysis

The effect of pullulanase amount on hydrolysis of pullulan is shown in Fig. 1. The DE values initially increased sharply as pullulanase amount in the reaction mixture increased, and then leveled off. The maximum DE was obtained when 10 ASPU/g of pullulanase was added to the reaction mixture. The results indicated that all pullulan was saturated by 10 ASPU/g of pullulanase, and this was consistent with the previous report (Wu et al., 2009). Thus, the optimum pullulanase amount under this condition was 10 ASPU/g.

3.3. Effect of the ethanol volumes on the recovery and content of maltotriose

After the particle with molecular weight higher than 1000 Da was separated by filter membrane filtration, the hydrolysates were concentrated to about ~20% and then subjected to precipitation. Considering that too ethanol amount causes considerable maltotriose lose and that too high ethanol amount decreases

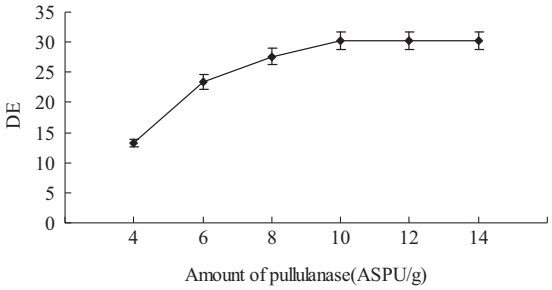

Fig. 1. Effect of pullulanase amount on hydrolysis of pullulan. Bars represent the standard deviation. Data are shown as mean ± S.D. (n = 3).

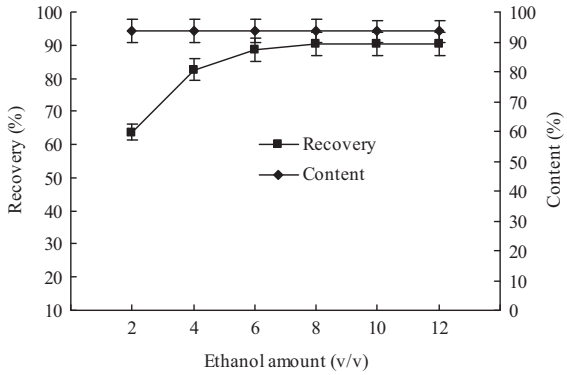

Fig. 2. Effect of absolute ethanol volumes on the recovery and purity of maltotriose. Bars represent the standard deviation. Data are shown as mean ± S.D. (n = 3).

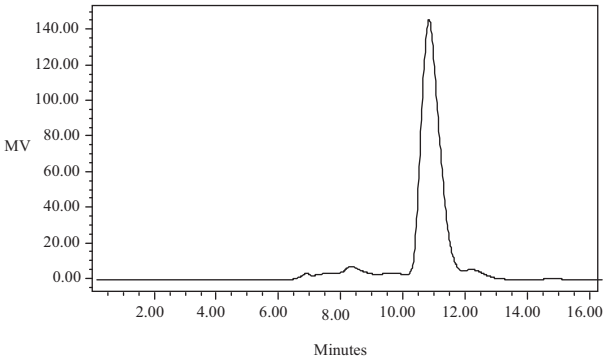

Fig. 3. HPLC spectra of the resulting maltotriose sample.

maltotriose content, it is of our interest to investigate the effects of ethanol volumes ranging from 2 to 12 on the recovery and content of maltotriose. Maximum maltotriose recovery was observed when 8 volumes of ethanol was used for the precipitation of maltotriose (Fig. 2), this was also consistent with the previous report (Wu et al., 2009). However, maltotriose content was affected by ethanol amount, indicating that glucose did not precipitate under such conditions.

3.4. Characterization of the product

The maltotriose product obtained was partially characterised. The contents of ash, moisture, maltotetraose, and maltotriose were 0.51%, 2.74%, 4.73%, and 92.13%, respectively. Therefore, the maltotriose content in the product was rather high (Fig. 3). The yield of maltotriose was 90.23%. The product did not contain any protein and was white powder and soluble in water.

4. Conclusions

High content of maltotriose product can be prepared by hydrolysis of pullulan in the fermentation broth using commercial pullulanase. Maximum DE was obtained under the optimum hydrolysis conditions, i.e. time, 9.40 h, 47.88 °C, pH 4.92, and 10 ASPU/g of pullulanase. The yield of maltotriose product and the content of maltotriose in the product were 92.13% and 90.23%, respectively. The results indicate that this may be a promising way of preparation of maltotriose in the future.

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