

Establishing the optimal conditions for fructose production from chicory inulin

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Abstract

In the present study, the Box-Behnken design was used to determine the optimal conditions for fructose production using the inulinase (enzyme) on chicory inulin (substrate). The inulinase concentration, pH, temperature and time of hydrolysis are the most important factors used to analyze the fructose production. Concentration of enzyme of 8-80 INU·g⁻¹, temperatures between 50 and 65°C, pH of 4.0-6.5 and hydrolysis time between 1 and 96 hours were studied to establish the optimal conditions for fructose production from chicory inulin. Enzyme concentration and hydrolysis time were the most important variables which have positive effects on enzymatic hydrolysis. For achieving high yields of fructose, for inulin hydrolysis, enzyme concentrations over 31.0 INU·g⁻¹ and hydrolysis time over 50 hours were necessary. The maximum yield of enzymatic hydrolysis was 0.959 g of fructose/g of inulin for the following conditions of inulin hydrolysis: enzyme concentration of 44 INU·g⁻¹, reaction temperature of 65°C, pH of 5.25 and 96 hours of hydrolysis.

Keywords: inulinase, fructose, response surface methodology.

Introduction

Inulin can be found as a reserve carbohydrate in plants such are Jerusalem artichoke, dahlia and chicory and in smaller amounts in garlic and onion [1, 2]. Inulins are a group of naturally occurring polysaccharides that can be hydrolyzed by two types of inulinases: exo-inulinases (β -D-fructanfructohydrolase, EC 3.2.1.80) and endo-inulinases (2,1- β -D-fructanfructanohydrolase, EC 3.2.1.7). Exo-inulinases split the terminal units of inulin in sucrose and inulin with a lower degree of polymerization and liberates fructose. Endo-inulinases hydrolyze inulin by breaking the bonds between fructose units that are located away from the ends of the polymer network, to produce oligosaccharides. Inulinases can be used in a wide range of industrial applications: for ultra-high fructose syrup obtaining from inulin, bioethanol production, inulo-oligosaccharide production, single-cell oil and single-cell protein production, some chemicals production like citric acid, butanediol, alcohols, lactic acid etc. [1,3,4].

During the years, many bacterial, filamentous fungi and yeast strains were used for inulinase production. Among the various microbial strains, *Kluyveromyces marxianus* and *Aspergillus niger* were reported as the most common and preferred sources for inulinase production [2,3].

Inulinases are enzymes that have different catalytic properties: molecular weight, optimum pH and temperature of action, stability, according to the sources. The inulinase produced by *Aspergillus niger* has optimum catalytic properties at pH of 4.4-5.0 and temperature of 40°C [5,6].

The aim of the present study was to establish the optimal conditions for hydrolysis of pure chicory inulin by using a commercial inulinase for production of a maximum quantity of fructose, based on statistical optimization analysis by applying Box-Behnken design and response surface methodology.

Materials and methods

Materials

Inulinase Novozym 960 from Novozymes A/S, Denmark was used in the study, being a liquid with a density of $1.17 \text{ g}\cdot\text{ml}^{-1}$ and having an activity of $400 \text{ INU}\cdot\text{g}^{-1}$ at $\text{pH} = 6.0$. One INU (inulinase units) is equivalent to the amount of enzyme that produces $1 \mu\text{mole}$ reducing carbohydrate per minute under the conditions of 50°C and $\text{pH} = 4.7$. The enzyme is produced by an *Aspergillus niger* strain.

The inulin from chicory was purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Germany).

Inulinase assay

A quantity of 0.2 g of pure chicory inulin was mixed using V1 Plus mixer (Boeco, Germany) with 10 ml phosphate-citrate buffer ($\text{pH} = 4.00, 5.25$ or 6.50), according to Table 2, in graduated tubes, until inulin dissolution. The pH was checked with the pH -meter (Mettler Toledo, UK). $8.0\text{-}80.0 \text{ INU/g}$ inulinase was added to the inulin solution and the mixture was incubated at different temperatures according with experimental design: 50°C and 65°C respectively, using Stericell 111 oven (Münchener Medizin Mechanik GmbH, Germany) and at 57.5°C using BF400 incubator (Binder GmbH, Germany) for 1 to 96 hours. The enzymatic reaction was stopped by boiling the samples for 10 minutes on a water bath. After cooling, the amount of fructose was determined using 3,5-dinitrosalicylic acid (DNSA) method based on a standard calibration curve [7].

All the samples were made in triplicate. All the values presented in the tables are the mean values.

Statistical analysis

The levels of the significant parameters and the interaction effects between various parameters, which significantly influence the enzymatic hydrolysis, were analyzed and optimized using the Unscrambler X software (version 10.1, Camo Software, Norway). A four factor Box-Behnken design, with 3 levels for each factor, was used for design. The particularity of this type of design is that extreme levels are not included in the design, so the experiments combine only 3 levels of each design variable. The mid-levels of some variables are combined with extreme levels of others [8]. A total of 27 experiments were necessary to estimate the coefficients of the model.

The quadratic model used by software to describe the tested experimental conditions is a second degree polynomial equation (1).

$$Y = b_0 + b_1A + b_2B + b_3C + b_4D + b_5AB + b_6AC + b_7BC + b_8AD + b_9BD + b_{10}CD + b_{11}A^2 + b_{12}B^2 + b_{13}C^2 + b_{14}D^2 \quad (1)$$

where Y is the response (dependent variable), the amount of fructose released from inulin hydrolysis, respectively; A, B, C and D are independent variables, b_0 is the intercept and b_1 to b_{14} are the regression coefficients.

Results and discussion

The influence of four independent variables: concentration of enzyme, temperature, hydrolysis time and pH, upon enzymatic hydrolysis was investigated, in order to increase the yield of reducing sugar released from substrate.

The matrix of variation of independent variables is presented in Table 1.

Table 1. The independent variables and their levels for the Box-Behnken design

Independent variables	Code	Levels of variations		
		-1	0	+1
Concentration of enzyme, [INU·g ⁻¹]	A	8.00	44.00	80.00
Temperature, [°C]	B	50.00	57.50	65.00
Hydrolysis time, [h]	C	1.00	48.50	96.00
pH	D	4.00	5.25	6.50

The experimental design is shown in Table 2.

Table 2. The experimental design of the enzymatic hydrolysis of chicory pure inulin using *Aspergillus niger* commercial inulinase

Run	Independent variable variation				Fructose amount, [g·g ⁻¹ chicory inulin]
	A	B	C	D	
1	0	0	-1	-1	0.415
2	0	0	+1	-1	0.741
3	0	0	-1	+1	0.450
4	0	0	+1	+1	0.746
5	-1	-1	0	0	0.780
6	+1	-1	0	0	0.812
7	-1	+1	0	0	0.490
8	+1	+1	0	0	0.786
9	-1	0	0	-1	0.416
10	+1	0	0	-1	0.609
11	-1	0	0	+1	0.471
12	+1	0	0	+1	0.702
13	0	-1	-1	0	0.535
14	0	+1	-1	0	0.536
15	0	-1	+1	0	0.852
16	0	+1	+1	0	0.959
17	0	-1	0	-1	0.619
18	0	+1	0	-1	0.605
19	0	-1	0	+1	0.735
20	0	+1	0	+1	0.579
21	-1	0	-1	0	0.292
22	+1	0	-1	0	0.566
23	-1	0	+1	0	0.725
24	+1	0	+1	0	0.915
25	0	0	0	0	0.844
26	0	0	0	0	0.915
27	0	0	0	0	0.846

As it can be seen in Table 2, a fructose amount between 0.292 to 0.959 g·g⁻¹ inulin was produced by enzymatic hydrolysis of chicory inulin by the *Aspergillus niger* inulinase. The maximum quantity of fructose released from substrate was produced when the concentration of enzyme was 44 INU·g⁻¹, the temperature was 65°C, the hydrolysis time was 96 hours and the pH was 5.25. The minimum fructose amount of 0.292 g·g⁻¹ inulin was obtained at an enzyme concentration of 8 INU·g⁻¹, temperature of 57.5°C, the hydrolysis time of 1 hour and pH of 5.25. Smaller amounts of fructose were due to the reduced enzyme concentration and hydrolysis time.

Sirisansaneeyakul et al., 2007, using inulinases produced by *Aspergillus niger* TISTR 3570 strain and the inulin hydrolysis parameters: inulin concentration of 100 g·l⁻¹, temperature of 40°C, pH = 4.5 and after 25 hours of hydrolysis, obtained a fructose quantity of 723 g·kg⁻¹ [9]. In this case, the low fructose yield produced can be due to the reduced hydrolysis time. Cruz et al., 1998, using an inulinase from *Aspergillus niger* 245, hydrolysis pH between 4...4.5, at 60°C and after 60 hours of chicory inulin hydrolysis, produced a fructose yield of 90% [10]. Compared with our experimental data, it can be concluded that the enzymatic inulin hydrolysis parameters are influenced by the type of the *Aspergillus niger* strain and also by the degree of enzyme purification.

The maximum fructose yield of the microbial exo-inulinases is between 95...98%, as many researchers found [2,6,11,12]. The maximum values of fructose yields obtained in this study (95.90%) are in the range of the limits found in scientific literature.

The statistical significance of model equation was checked using F-test analysis of variance (ANOVA) (Table 3).

Table 3. Analysis of variance (ANOVA) for enzymatic hydrolysis evaluation

Source	Sum of squares	Degrees of freedom	Mean squares	F-ratio	p-value
(Enzyme concentration) A	0.1232	1.000	0.1232	38.1898	0.0000
(Temperature) B	0.00107	1.000	0.00107	3.3101	0.0939
(Hydrolysis time) C	0.3902	1.000	0.3902	120.9468	0.0000
(pH) D	0.0064	1.000	0.0064	1.9960	0.1831
(Enzyme concentration · Temperature) AB	0.0174	1.000	0.0174	5.4002	0.0385
(Enzyme concentration · Hydrolysis time) AC	0.0018	1.000	0.0018	0.5467	0.4739
(Temperature · Hydrolysis time) BC	0.0040	1.000	0.0040	1.2301	0.2891
(Enzyme concentration · pH) AD	0.0004	1.000	0.0004	0.1119	0.7438
(Temperature · pH) BD	0.0050	1.000	0.0050	1.5623	0.2351
(Hydrolysis time · pH) CD	0.0002	1.000	0.0002	0.0697	0.7962
(Enzyme concentration · Enzyme concentration) A ²	0.0881	1.000	0.0881	27.2939	0.0002
(Temperature · Temperature) B ²	0.0067	1.000	0.0067	2.0831	0.1745
(Hydrolysis time · Hydrolysis time) C ²	0.0588	1.000	0.0588	18.2238	0.0011
(pH · pH) D ²	0.1885	1.000	0.1885	58.4219	0.0000
Model	0.7940	14.000	0.0567	17.5781	0.0000
Error	0.0387	12.000	0.0032	-	-
Corr. total	0.8328	26.000	-	-	-
Linear	0.5306	4.000	0.1326	41.1107	0.0000
Interaction 2	0.0288	6.000	0.0048	1.4868	0.2627

Quadratic	0.3421	4.000	0.0855	26.5057	0.0000
Lack of fit	0.0354	10.000	0.0035	2.1691	0.3566
R-squared	0.9535	-	-	-	-
Adjusted R-squared	0.8993	-	-	-	-
Adeq. Precision	13.64407	-	-	-	-

The model F-value of 17.57 implies that the model was significant. A p-value lower than 0.05 indicates that the model terms are also significant. In this case the correlative effects of the enzyme concentration, the hydrolysis time, the pH, the temperature were significant model terms. Also, p-values greater than 0.05 indicate that the model terms were not significant. The lack of fit F-value of 2.16 implies that the lack of fit is not significant relative to the pure error. There is a 35.66% chance that a lack of fit F-value this large could occur due to the noise. Adeq. Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. In this experiment the ratio value of 13.64 indicates an adequate signal.

According to the data presented in Table 3, the quadratic model fitted well for variable optimization as the p-value was lower than 0.05. For fructose release after pure inulin hydrolysis by the commercial inulinase, the summary showed that the model was globally significant, so it can be interpreted.

Studying the parameters from the ANOVA statistical analysis, it can be easily observed that hydrolysis time of pure inulin had the greatest influence on the yield of enzymatic hydrolysis. Also, the concentration of enzyme was an important factor that increased the yield of substrate hydrolysis. The temperature effect on inulin hydrolysis was insignificant for the studied range, as the p-value was higher than 0.05. The tested inulinase seemed to be higher active at temperature of 50°C, but the influence of the temperature on enzyme activity was not significant in the temperature range studied (50-65°C). The pH does not influence the yield of hydrolysis, for the range of the pH values tested in this study (4.0 – 6.5), as the p-value was also higher than 0.05.

In Table 4 the most important b-coefficients for the quadratic model describing the enzymatic hydrolysis process of the present study were presented.

Table 4. The b-coefficients of models for pure inulin hydrolysis with commercial inulinase from fungal source

b-coefficients	Values
b ₀	0.8683
b ₁	0.1013
b ₃	0.3902
b ₅	0.066
b ₁₁	-0.1285
b ₁₃	-0.105
b ₁₄	-0.188

After the analysis of variance, which gives the level of fructose amount as a function of the investigated factors affecting the hydrolysis process, the regression equation 1 becomes:

$$\text{Fructose release, g}\cdot\text{g}^{-1} \text{ substrate} = 0.8683 + 0.1013A + 0.1803C + 0.066AB - 0.1285A^2 - 0.1050C^2 - 0.1880D^2 \quad (2)$$

The terms that do not contribute significantly to the prediction of Y were omitted from the full model and a reduced second-order polynomial equation was obtained. This implies

that the effects of enzyme concentration and hydrolysis time were significant as it is obvious from their high coefficients.

The results of multiple linear regression analysis (reduced model) reveal that, by increasing the time of hydrolysis and the enzyme concentration, the amount of fructose (expressed by Y) will increase, as the b_1 and b_3 coefficients are positive.

Figure 1 shows the interactions and optimal levels of the studied variables that were determined by plotting the response surface curves.

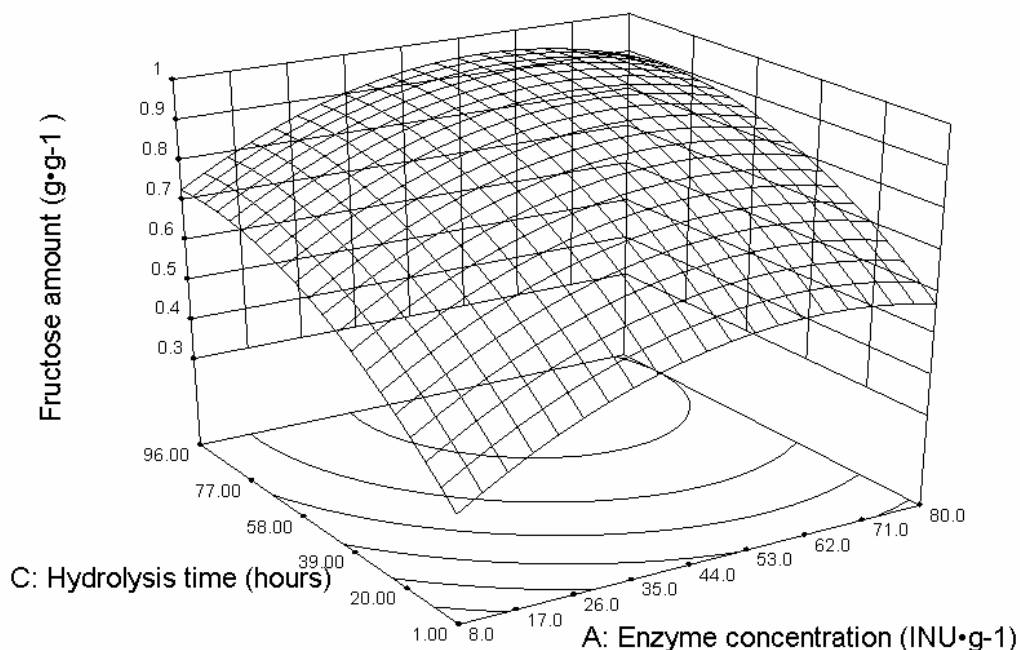


Figure 1. Response surface for enzymatic hydrolysis of pure chicory inulin as a function of the concentration of enzyme and hydrolysis time

This plot shows the correlations between the studied variables. The fructose amount released after hydrolysis were higher when the enzyme concentration and the time exceed 31.0 INU·g⁻¹ and respectively 68 hours of hydrolysis. The enzymatic production of fructose was highly influenced by enzyme concentration, which is one important factor for the fructose production by inulin hydrolysis.

The obtained central composite model and the statistical analysis of the results showed that the quantity of fructose produced was increased when the enzyme concentration is higher than 31.0 INU·g⁻¹, the temperature of hydrolysis is higher than 50°C, and the time of hydrolysis exceeds 68 hours. In our case, the pH of the reaction medium is not an important factor. The maximum fructose yield of 95.90% indicates that the commercial inulinase acts at the end of the polymer chain, the enzyme having an exo-inulinase activity. These results are in accordance with those obtained by other researchers which reported the significant parameters for inulin hydrolysis using the statistical analysis, using different inulinase producers. Rocha et al., 2006, established by factorial design and surface response methodology, the optimal conditions for inulin hydrolysis using a commercial free and immobilised inulinase produced by *A. niger* [13].

On the other hand, Burkert et al., 2006, by studying the effects of substrate concentration (sucrose and inulin), pH and temperature on inulinase activity produced by *Kluyveromyces marxianus* ATCC 16045 using four factorial design and surface response analysis, found that using inulin as substrate, the temperature was the only variable statistically significant and the maximum activity of the inulinase was at the concentration of $7.3 \text{ U}\cdot\text{mL}^{-1}$ at temperature between 50°C and 51°C [14].

Nasab et al., 2009, investigated the effects of acidic and alkaline pH on the inulin hydrolysis using the central composite rotatable design to model, and the response surface methodology [15]. The statistical analysis of the results confirmed that pH, temperature and time are significant variables at acidic pH, and the maximum amount of inulin hydrolysis is obtained at the pH lower than 2,0, temperature higher than 90°C after 1 hour. Fructose can be produced by chemical hydrolysis, not only enzymatic, by chemical means the advantage being the reduced hydrolysis period.

Conclusions

The enzymatic hydrolysis conditions for pure chicory inulin using commercial fungal inulinase were optimized by applying statistical optimization and response surface methodology. Hydrolysis time and the concentration of enzyme have significant influence on the yield of hydrolysis, while temperature and pH had an insignificant effect on reducing sugars releasing from inulin hydrolysis. By using enzyme concentrations over $31.0 \text{ INU}\cdot\text{g}^{-1}$ and hydrolysis times over 68 h, high inulin hydrolysis yields can be achieved. The maximum fructose yield was obtained using the inulin hydrolysis parameters: enzyme concentration $44 \text{ INU}\cdot\text{g}^{-1}$, reaction temperature 65°C , pH 5.25 and time of 96 hours. The mathematical optimization of enzymatic hydrolysis process assures a good opportunity to increase the efficiency of biotechnological conditions for polymer bioconversion in simple sugars by evaluation of the correlation effect of different factors.

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