Establishing the Optimum Conditions for Inulin Hydrolysis by Using Commercial Inulinase

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In the present study, the central composite design was used to determine the effect of inulinase concentration, pH, temperature and time of hydrolysis of chicory inulin by using the inulinase produced by Novozymes. Concentration of enzyme of 0.10-1.00% (v/w), temperatures between 50 and 65°C, pH of 4.0-6.5 and hydrolysis time between 1 and 96 h were studied to establish the optimal conditions of the chicory inulin. Enzyme concentration and hydrolysis time were the most important variables which have positive effects on enzymatic hydrolysis. When enzyme concentrations over 0.35% and hydrolysis time over 68 h are used for inulin hydrolysis, high yields of enzymatic hydrolysis can be achieved. The maximum yield of enzymatic hydrolysis was 0.979 g of reducing sugars/g of substrate using the following conditions for inulin hydrolysis: enzyme concentration 0.55%, reaction temperature 65°C, pH 5.25 after 96 h of hydrolysis.

Keywords: inulinase, central composite design (CCD), fructose, chicory inulin, response surface methodology.

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 hydrolysed 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 hydrolyse inulin by breaking the bonds between fructose units that are located away from the ends of the polymer network, to produce oligo-saccharides. 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 *p*H and temperature of action, stability, according to the sources. The inulinase produced by *Aspergillus niger* has optimum catalytic properties at *p*H 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, based on statistical optimization analysis by applying central composite design and response surface methodology.

Experimental part

Materials and methods

Inulinase Novozym 960 from Novozymes A/S, Denmark was used in the study, being a liquid with a density of 1.17 g/mL and having an activity of 400 INU/g at *p*H = 6. One INU (inulinase units) is equivalent to the amount of enzyme that produces 1 μmole reducing carbohydrate per minute under the conditions of 50°C and *p*H = 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 (*p*H = 4.00, 5.25 or 6.50), according to table 2, in graduated tubes, until inulin dissolution. The *p*H was checked with the pH-meter (Mettler Toledo, UK). 0.1-1 % inulinase (v/w, 8.0-80.0 INU/g) was added to the inulin solution and the mixture was incubated at different temperatures according to experimental design: 50 and 65°C respectively, using Stericell 111 oven (Münchener MedizinMechanik GmbH, Germany) and at 57.5°C using BF400 incubator (Binder GmbH, Germany) for 1 to 96 h. The enzymatic reaction was stopped by boiling the samples for 10 min on a water bath. After cooling, the amount of reducing sugars, expressed as fructose, were determined using 3,5-dinitrosalicylic acid (DNSA) method based on a standard calibration curve [7]. All the samples were made in triplicate.

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 central composite design of experiments (CCD) and response surface methodology, using the Unscrambler X software (Version 10.1, Camo Software, Norway). A 4-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).

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Independent variables	Code	Levels of variations		
		-1	0	+1
Concentration of enzyme, [%]	A	0.100	0.550	1.000
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

Table 1
INDEPENDENT VARIABLES AND THEIR LEVELS FOR THE BOX-BEHNKEN DESIGN

Run	Independent variable variation				Yield of hydrolysis, [g fructose/g substrate]
	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	0.979
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

Table 2
EXPERIMENTAL DESIGN OF THE ENZYMATIC HYDROLYSIS OF CHICORY PURE INULIN USING *ASPERGILLUS NIGER* COMMERCIAL INULINASE

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

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 discussions

The influence of four independent variables: concentration of enzyme (%), temperature (°C), hydrolysis time (h) and *p*H, 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.

The experimental design is shown in table 2.

As it can be seen in table 2, a fructose yield between 0.292 to 0.979 g fructose/g inulin was produced by enzymatic hydrolysis of pure inulin by the commercial inulinase. The maximum quantity of fructose released from substrate is produced when the concentration of enzyme is 0.550%, the temperature is 65°C, the hydrolysis time is 96 h and the *p*H is 5.25 and the minimum fructose amount, 0.292 g/g inulin respectively, at an enzyme concentration of 0.10%, temperature of 57.5°C, the hydrolysis time of 1 hour and *p*H of 5.25. The small amounts of fructose are due to the reduced enzyme concentration and hydrolysis time.

Sirisansaneeyakul et al. [9] using inulinases of A. niger TISTR 3570, and the inulin hydrolysis parameters: inulin concentration of 100 $g \cdot L^{-1}$, temperature of 40°C, *p*H = 4.5

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	-	-	-	-

Table 3
ANALYSIS OF VARIANCE (ANOVA) FOR YIELD OF ENZYMATIC HYDROLYSIS EVALUATION

and after 25 h of hydrolysis, obtained a fructose quantity of 723 $g \cdot kg^{-1}$. In this case, the low fructose yield produced can be due to the reduced hydrolysis time. Cruz et al. [10] using an inulinase from A. niger-245, hydrolysis *p*H between 4...4.5, at 60°C and after 60 h of chicory inulin hydrolysis, produced a fructose yield of 90% (90 g fructose in 100 g inulin). Comparing with our experimental data, it can be concluded that the enzymatic inulin hydrolysis parameters are influenced by the type of the A. niger strain used 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 (97.90%) are close to the superior yields found in literature.

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

The model F-value of 17.58 implies the model is significant. There is only 0.01% chance that a model F-value this large could occur due to noise. A p-value lower than 0.050 indicates that the model terms are also significant. In this case the enzyme concentration, the hydrolysis time, the enzyme concentration, temperature,

the enzyme concentration . enzyme concentration, the hydrolysis time . hydrolysis time and the pH . pH are significant model terms. p-values greater than 0.0500 indicate that the model terms are not significant. The lack of fit F-value of 2.17 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 13.644 indicates an adequate signal.

According to the data presented in table 3, the quadratic model fits well for variable optimization as the p-value is lower than 0.05. For fructose release after pure inulin hydrolysis by the commercial inulinase, the summary shows that the model is 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 has the greatest influence on the yield of enzymatic hydrolysis. Also, the concentration of enzyme is an important factor that increases the yield of substrate hydrolysis. The temperature effect on inulin hydrolysis is insignificant for the studied range, as the p-value is higher than 0.05. The tested inulinase seems to be higher active at temperature of 50°C, but the influence of the temperature on enzyme activity is not significant in the temperature range studied (50-65°C). The *p*H does not influence the yield of hydrolysis, for the range of the *p*H value tested in this study (4.0 – 6.5), as the p-value is also higher than 0.05.

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

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

Table 4
b-COEFFICIENT OF MODELS FOR PURE INULIN HYDROLISIS WITH COMMERCIAL INULINASE FROM FUNGAL SOURCE

The regression equation 1 becomes:

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

after the analysis of variance, which gives the level of reducing sugars amount as a function of the investigated factors affecting the hydrolysis process.

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 are 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.

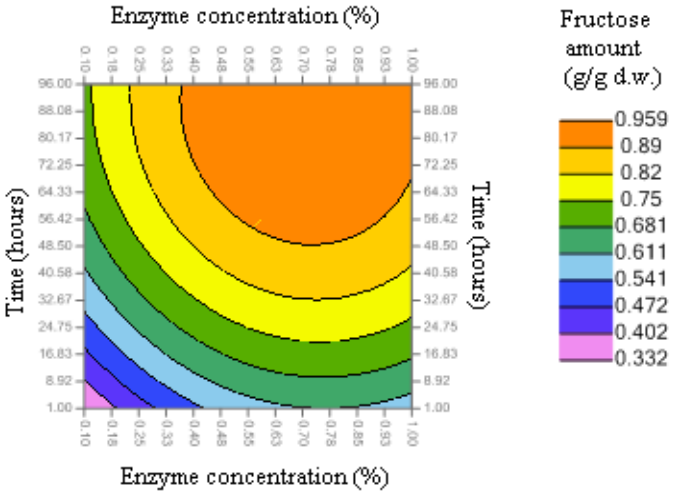

Fig. 1. Response surface for enzymatic hydrolysis of pure chicory inulin as a function of the concentration of enzyme and hydrolysis time.

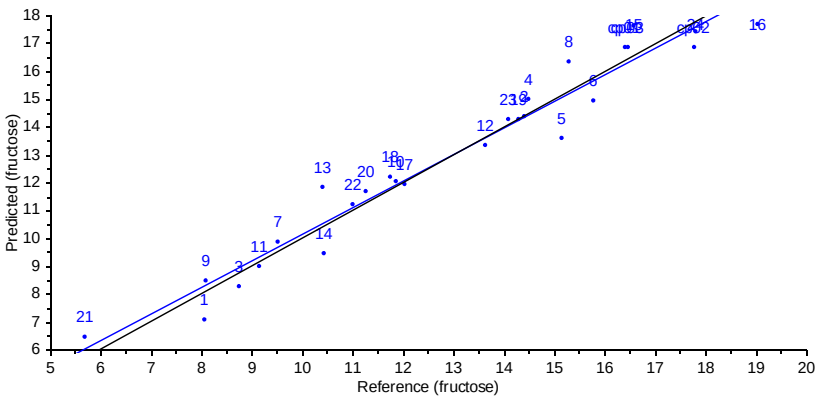

Fig. 2. Predicted values versus experimental values

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

This plot shows the correlations between the studied variables. The fructose amount released after hydrolysis is higher when the enzyme concentration and the time exceed 0.35% and respectively 68 h of hydrolysis. The enzymatic production of fructose is 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 is increasing when the enzyme concentration is higher than 0.35%, the temperature of hydrolysis is higher than 50°C, and the time of hydrolysis exceeds 68 h. In our case, the *p*H of the reaction medium is not an important factor. The maximum fructose yield of 97.90% 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. [13] 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. The model system estimated optimal *p*H and temperature values of 5.4 and 52°C, respectively for the immobilised inulinase and 4.9 and 56°C, respectively for the free inulinase.

On the other hand, Burkert et al. [14], by studying the effects of substrate concentration (sucrose and inulin), *p*H 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 U/mL at temperature between 50 and 51°C.

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

From the data presented in figure 2 it can be stated that the quadratic model is relevant, as the correlation between the predicted data and real data values is 0.976 and the Bias value is close to zero.

Conclusions

The enzymatic hydrolysis conditions for pure chicory inulin using commercial fungal inulinase were optimised by applied statistical optimisation and response surface methodology. Hydrolysis time and the concentration of enzyme have significant influence on the yield of hydrolysis, while temperature and *p*H had an insignificant effect on reducing sugars releasing from inulin hydrolysis. By using enzymes concentrations over 0.35% 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 0.55%, reaction temperature 65°C, *p*H 5.25 and time of 96 h. The mathematical optimisation 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 correlative effect of different factors.

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