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Controlled Production of Fructose by an Exoinulinase from *Aspergillus Ficuum*

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Abstract An exoinulinase has been isolated, purified and characterised from a commercially available broth of *Aspergillus ficuum*. The enzyme was purified 4.2-fold in a 21% yield with a specific activity of 12,300 U mg^{-1}(protein) after dialysis, ammonium sulphate fractionation and Sephacryl S-200 size exclusion and ion exchange chromatography. The molecular weight of this enzyme was estimated to be 63 kDa by SDS-PAGE. It exhibited a pH and temperature optima of 5.4 and 50 °C respectively and under such conditions the enzyme remained stable with 96% and 63.8% residual activity after incubation for 12 h and 72 h respectively. The respective K_m and V_{max} values were 4.75 mM and 833.3 μmol min^{-1} ml^{-1}, respectively. Response surface methodological statistical analysis was evaluated for the maximal production of fructose from the hydrolysis of pure commercial chicory inulin. Incubation of the dialyzed crude exoinulinase (100 U/ml, 48 h, 50 °C, 150% inulin, pH 5.0) produced the highest amount of fructose (106.4 mg/ml) under static batch conditions. The purified exoinulinase was evaluated for fructose production and the highest amount (98 mg/ml) was produced after 12 h incubation at 50 °C, 150% inulin pH 5.0. The use of a crude exoinulinase preparation is economically desirable and the industrial production of fructose from inulin hydrolysis is biotechnologically feasible.

Keywords Fructose · Fructose synthesis · Exoinulinase · Response surface methodology · Production

Introduction

From a biotechnological point of view fructose is widely used in the food industry as a sweetener, as a special food for diabetics and as a diuretic and due to this importance it is therefore imperative to find optimal conditions for the production of fructose from renewable and readily available raw materials. Current methods that employ simple

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inexpensive raw materials, such as chemical hydrolysis of the inulin—a polymer of fructose with over 60 monomeric units—by treatment with organic or mineral acids or by acid-cation resins, zeolites or oxidised activated carbon, at high temperatures [1], often giving rise to a coloured hydrolysate and unwanted dimeric anhydride by-products that lower the yield and require a demanding downstream processing for its purification [1–3].

The economical disadvantage, the low pH, the high temperatures and environmentally unfriendly process with these chemical acids points towards the advantageous use of specific enzymes for the production of fructose as they operate at lower temperatures, produce less toxic and pollutant waste and produce fewer emissions and by-products [4]. Exoinulinase (EC 3.2.1.80), a member of glycoside hydrolase family (GHT) 32, is a fructofuranosyl hydrolase that splits fructose off the non-reducing end of inulin [5]. Most of the inulinolytic enzymes are thermophilic that allow fructose production at elevated temperatures such as 50 °C [4].

Inulin serves as a relatively inexpensive and abundant substrate for fructose production and consequently is of considerable industrial interest [5]. In order to enzymatically produce fructose, starting from a suitable, cheap, readily available, starting material it would be necessary to exercise careful control, strict protocol and experimental regime. In order to fully exploit this enzymatic reaction it was decided to investigate the experimental regime from a statistical standpoint and design a protocol using response surface methodology (RSM). “One-factor-at-a-time” experiments, where a single factor is varied while others are kept constant, are often expensive and time consuming and do not take into account the possible interaction of various independent factors that would skew the results. For these reasons, statistical methods have been developed to reduce the cost and duration of experiments that also allow for the observation of any interacting factors in the final process response. This technology uses quantitative data from appropriate experimental designs to determine, and simultaneously solve multivariate equations, in order to specify the optimum product for a specified set of factors through mathematical models [6–8]. RSM involves four important steps: (1) identification of critical factors for the product or process, (2) determination of the range of factor levels, (3) selection of specific test samples by the experimental design and (4) analysis of the data by RSM and data interpretation.

We now report on the isolation, purification and characterisation of an exoinulinase from a crude fermentation broth of *A. ficuum*. Furthermore, we examine this enzyme in conjunction with its role in the controlled enzymatic synthesis of fructose by means of response surface methodology. Analytical techniques such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC)-RI and MS-ESI were used to identify fructose and to quantitate and determine its rate of production.

Materials and Methods

Materials

Fructozyme L (Batch number KIN00046) a commercial enzyme preparation isolated from *A. ficuum* was obtained from Novozymes, South Africa dialysed against phosphate buffer (pH 6.5, 100 mM) and stored as crude exoinulinase enzyme extract at 4 °C until required. The Snake-Skin™ Pleated dialysis tubing with a MWCO of 10 kDa was purchased from Pierce Chemical Company, Rockford, USA. Sephacryl S-200, Bradford reagent, bis-acrylamide, polyacrylamide, inulin *(Cichorium intybus)* and dinitrosalicylic acid (DNS)

were purchased from Sigma (South Africa). Acetonitrile (HPLC grade) and lithium chloride were purchased from Merck, South Africa. All other chemicals and reagents were of analytical grade.

Purification of Exoinulinase

A fructozyme extract (50 ml, 2929 U mg^{-1} (protein)) was treated with solid ammonium sulphate (50% saturation) at 4 °C with gentle agitation for 4 h. The precipitate was collected (10,000×*g*, 20 min), redissolved in distilled water (10 ml) and dialysed overnight. Protein and enzyme assays were conducted on the sample before it was applied to a Sephacryl S-200 column (2.5×40 cm) equilibrated with citrate–phosphate buffer (pH 5.0, 100 mM). The unbound protein was washed from the column with the same buffer until the absorbance at $A_{280\ nm}$ of eluate had reached base line. The bound proteins were eluted by a stepwise 0–3.0 M NaCl in citrate–phosphate buffer (pH 5.0, 100 mM), at a flow rate of 2 ml min^{-1}. Fractions (5 ml) were collected, monitored for protein and exoinulinase activity and the active fractions collected and analysed by SDS [9] and native PAGE [10].

Assay of Exoinulinase

Exoinulinase activity was measured according to a modification of the procedure by Jing et al. [11] using pure non-hydrolysed commercial inulin as a model substrate. The assay mixture contained an aliquot (1 ml) of dialysed exoinulinase preparation and the inulin substrate (5%, *w*/*v*, 4 ml) dissolved in citrate–phosphate buffer (pH 5.0, 100 mM). The reaction was done in triplicate. The reaction mixture was incubated (50 °C, 1 h), after which the reaction was stopped by boiling for 10 min. The reaction products were centrifuged (13,000 rpm, 3 min), syringe filtered through a 0.45-μm Millipore nylon membrane and enzyme activity determined by measuring the mean amount of fructose liberated from a standard curve. One unit of exoinulinase unit was defined as the amount of enzyme catalysing the liberation of 1 μmol of fructose per minute under the experimental conditions.

Fructose concentration was estimated using the dinitrosalicylic (DNS) acid method [12]. An aliquot (500 μl) of the enzyme reaction mixture was treated with DNS reagent (3.0 ml), the tubes were boiled (5 min), cooled to room temperature and A_{540} was read. Any contribution of fructose from thermal hydrolysis of inulin was ruled out as none was found after incubating the inulin mixture (50 °C, 1 h) without any added exoinulinase enzyme. Furthermore, any residual fructose already present in the enzyme samples was estimated and an appropriate correction made in order to calculate the actual amount of sugar liberated by enzyme activity.

Gel Electrophoresis and Molecular Weight Determinations

A discontinuous native polyacrylamide gel, using 8% resolving gel and a 5% stacking gel, was prepared and samples (10 μl) applied and an electrophoresis run for 45 min. The gel was stained with Coomassie brilliant blue R-250 (30 min) followed by destaining overnight with methanol–acetic acid–water (1:1:8 *v*/*v*/*v*). To confirm the exoinulinase nature of the purified enzyme, the single band of the pure enzyme was cut out from the gel and tested for enzyme activity with 5% inulin under the assay conditions described before. The reaction products were analysed by HPLC and TLC as described below (Section "Analytical Procedures").

Samples (10 μl) and molecular weight markers (10 μl) [aprotinin, 6.5 kDa; lysozyme, 14.6 kDa; soybean trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; ovalbumin,

45 kDa; bovine serum albumin, 66.2 kDa; phosphorylase b, 97.4 kDa, and β-galactosidase, 116.3 kDa.] were applied to a 12% SDS-PAGE at 150 v for 45 min. The gels were stained using Coomassie brilliant blue R-250 followed by destaining overnight. The distance moved by the enzyme was measured and its corresponding molecular size calculated from the calibration curve of log molecular weight versus distance migrated.

pH, Temperature Optima and Stability of the Exoinulinase

The pH profile of the enzyme was evaluated by incubating the purified enzyme (300 μl, 123 U ml^{-1}) with inulin (1.2 ml, 5%, *w*/*v*) for 60 min at 50 °C in appropriate buffers: pH 3–5 (sodium acetate, 100 mM); pH 6–8 (citrate–phosphate, 100 mM) and pH 9–10 (sodium hydroxide-glycine, 100 mM). The optimum temperature for the endoinulinase was determined by incubating the enzymes (300 μl, 123 U ml^{-1}) with inulin (1.2 ml, 5%, *w*/*v*) for 60 min at different temperatures ranging from 30–80 °C at the optimum pH. In each case protein concentration and enzyme activity were determined as before. The stability of the enzyme was investigated, at respective optimum temperature and pH, by incubating the purified enzyme (300 μl, 123 U ml^{-1}) in citrate–phosphate buffer (100 mM, pH 5.0, 1.2 ml) over an incubation time of 72 h and recorded as the half life ($t_{0.5}$) or the time taken for the loss of 50% activity.

Determinations of Kinetic Parameters and Inhibition of Exoinulinase

Different concentrations (5–20 mM) each of inulin in citrate–phosphate buffer (pH 5.0, 100 mM) were chosen for the purified inulinase to give measurable reaction rates and the reactions were performed in triplicate. An average M_r for inulin was estimated to be 5,500 from the average degree of polymerisation of 34 due to the polydisperse nature of the compound [13]. The affinity constant, K_m and the rate of reaction, V_{max} were determined using linear regression plots of Cornish-Bowden [14].

To examine the effect of fructose as an end-product inhibitor for the enzyme the exoimulinase (1.0 ml, 100 U ml^{-1}) was treated with inulin (5–20 mM) suspended in citrate–phosphate buffer (pH 5.0, 100 mM) in the presence of added initial fructose (5–20% *w*/*v*) at 50 °C, 1 h. The reaction was terminated by boiling (10 min) and final fructose concentrations determined as described before.

To substantiate whether Michaelis–Menten kinetics was being followed the reaction mixture was dialysed against deioinised water (4 °C, 24 h) and the enzyme activity determined as before.

Enzymatic Synthesis of Fructose using Response Surface Methodology

Maximum fructose production was optimised by response surface methodology (RSM) with central composite design (CCD). Since the conditions were unknown at the start of the experiments it was important to rotate the regression coefficients in order to allow equality in all directions. Initially the critical parameters, that were simultaneously varied for fructose production in the first RSM, were pH (5.0 to 6.2), temperature (30 to 50 °C) and inulin concentration (50 to 150 mg ml^{-1}) (Table 1). These conditions were established and the variables coded according to Eq. 1.

$$x_i = \left(X_i - X_{cp}\right)/\Delta X_i \quad (1)$$

Table 1 Generation of coded levels using central composite design for preliminary fructose production using response surface methodology by a commercial exoinulinase preparation.

Independent variable	Symbol	Coded levels		
		−1	0	+1
pH	X_1	5.0	5.6	6.2
Temperature (°C)	X_2	30	40	50
Inulin (mg ml^{-1})	X_3	50	100	150

Incubation time and enzyme dosage was kept constant at 8 h and 100 U/ml respectively

Where x_i is the coded level of the variable, X_i is the real level of the variable, X_{cp} is the real level of the variable at the centre point and ΔX_i is the step change value at the real level. The experimental run treatments are also presented (Table 2).

An aliquot (1 ml) of the enzyme (100 U/ml) was added to pure inulin (4 ml) at the appropriate concentration, temperature and pH and incubated for 8 h. The tubes were boiled for 10 min to stop the enzyme activity and fructose concentration was determined by a fructose assay kit and standard curve as described above. In the second RSM experimental runs, the most important independent variables—incubation time (4 to 12 h) X_1, enzyme dosage (20 to 100 U ml^{-1}) X_2 and inulin concentration (50 to 150 mg ml^{-1}) X_3 were simultaneously varied (Table 2) while the pH and temperature were kept constant at 5.0 and 50 °C respectively. A third set of experiments simultaneously varied reaction time (24 to 48 h), temperature (30 to 50 °C), and inulin concentration (50 to 150 mg ml^{-1}) (Table 3) while initial pH and enzyme dosage were kept constant at 5.0 and 100 U ml^{-1} respectively.

An aliquot of inulin solution of appropriate concentration, (50 to 150 mg ml^{-1}, 1,200 μl) suspended in citrate–phosphate buffer (100 mM, pH 5.0) was allowed to equilibrate at the respective temperatures 30–50 °C before adding an appropriate concentration of exoinulinase enzyme (20 to 100 U.ml^{-1}, 300 μl). After the necessary incubation time (4 to 12; 24 to 48 h), the reactions were stopped by boiling the tubes for 10 min. Any effects of thermal and acid hydrolysis were ruled out after fructose was not detected after incubating the inulin substrate alone at 50 °C, at pH 5.0 for the duration of the experiment. The samples were centrifuged, syringe filtered (0.45 μm nylon membranes) and fructose concentration was determined by fructose assay kit and a standard curve.

Analytical Procedures

Concentrations and rate of production of fructose were quantified using high performance liquid chromatography (HPLC) coupled to a refractive index (RI) detector. The HPLC

Table 2 Generation of coded levels using central composite design for fructose production using response surface methodology by an exoinulinase preparation.

Independent variable	Symbol	Coded levels		
		−1	0	+1
Time (h)	X_1	4	8	12
Exoinulinase (U ml^{-1})	X_2	20	60	100
Inulin (mg ml^{-1})	X_3	50	100	150

pH and temperature were kept constant at 5 and 50 °C respectively

Table 3 Response surface methodology and CCD for fructose production at pH 5 and using 100 U ml^{-1} exoinulinase preparation.

Independent variable	Symbol	Coded levels		
		−1	0	+1
Time (h)	X_1	24	36	48
Temperature (°C)	X_2	30	40	50
Inulin mg ml^{-1}	X_3	50	100	150

The coded levels were generated using Eq. 1

system consisted of a Beckman pump, a 20-μl Beckman injector loop, a Prevail Carbohydrate guard and analytical columns (Alltech, South Africa), a Knauer RI detector and 32 Karat peak integration software with a mobile phase of 73% (*v*/*v*) HPLC grade acetonitrile in Milli-Q distilled water. All samples and mobile phase were syringe filtered using 0.45-μm Millipore membrane discs before injection. All analyses were carried out in triplicate and values reported as the means with standard deviations. Qualitative determination of reaction products in the reaction mixture was performed by using thin layer chromatography. An aliquot (2–4 μl) of the reaction mixture was spotted on the TLC plate and then developed with a solvent system of butanol/acetic acid/water (5:4:1) (*v*/*v*/*v*) in a TLC developing tank. In order to get good separation of the reaction products, ascending development was repeated twice at room temperature. The plate was allowed to dry in a fume hood and then developed by spraying with anisaldehyde spray reagent using an Aldrich® flask-type sprayer. The plate was then dried in an oven at 110 °C for 15 min to visualise the reaction spots. Protein concentration was determined according to the method of Bradford [15].

Statistical and Data Analyses

In order to fully describe the response surface around the optimum region, a CCD was used with three coded levels as in Tables 1, 2, and 3. The quadratic model for predicting the optimal yield was calculated according to Eq. 2 provided that a measurable response is obtained.

$$y = b_0 + \sum b_r x_i + \sum b_{ir} x_i^2 + \sum b_{ij} x_i x_j \quad (2)$$

Where y is the response variable, b_0 is the constant, b_r is the coefficient for the linear effect, b_{ir} is the coefficient for the quadratic effect, b_{ij} is the coefficient for the interaction

Table 4 Purification table for exoinulinase.

Fraction	Volume (ml)	[Protein] (mg ml^{-1})	Total protein (mg)	Activity (U ml^{-1})	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	P. Fold
Crude	50	0.04	20.0	117	5 857	2 929	100	1.0
AS[a]	10	0.02	0.2	99	990	4 950	17	1.7
SS-200[b]	10	0.01	0.1	123	1 230	12 300	21	4.2

[a] Dialysed pellet after 50% ammonium sulphate precipitation

[b] Sephacryl S-200 with NaCl linear gradient elution (0–3 M)

effect and x is the coded level of the respective independent variable. The data generated was statistically analysed using response surface regression using Statistica 7 for first, second order and response surface regression models to predict the yield as the response factor. Mean and standard deviation calculations and comparison of data sets using analysis of variance (ANOVA) was performed with Microsoft Excel 2003 statistical tools. The ANOVA was performed to 5% level of significance.

Results

Purification of Exoinulinase

The purification of the exoinulinase is summarised (Table 4). Crude exoinulinase (117 U/ml) was precipitated with 50% saturation ammonium sulphate at 4 °C and the reconstituted pellet fractionated on a Sephacryl S-200 molecular exclusion column. Gradient elution with 0 to 3 M NaCl gave two major protein peaks with fractions 17 to 26 eluting at 0.5 M NaCl, containing exoinulinase activity (Fig. 1). The enzyme was purified 4.2-fold, in 21% yield and a specific activity of 12,300 U mg^{-1}.

Gel Electrophoresis and Molecular Weight Determinations

A homogeneous monomeric exoinulinase band was observed by both SDS and discontinuous native-PAGE with a molecular mass of 63 kDa (Figs. 2 and 3). The exoinulinase was resolved by native PAGE and the pure band was cut-out to determine exoinulinase activity in order to confirm its authenticity as an exoinulinase. HPLC and TLC analysis of the reaction products of the pure band confirmed that it was an exoinulinase because it produced fructose, inulobiose, sucrose and traces of 1-kestose in the reaction mixture.

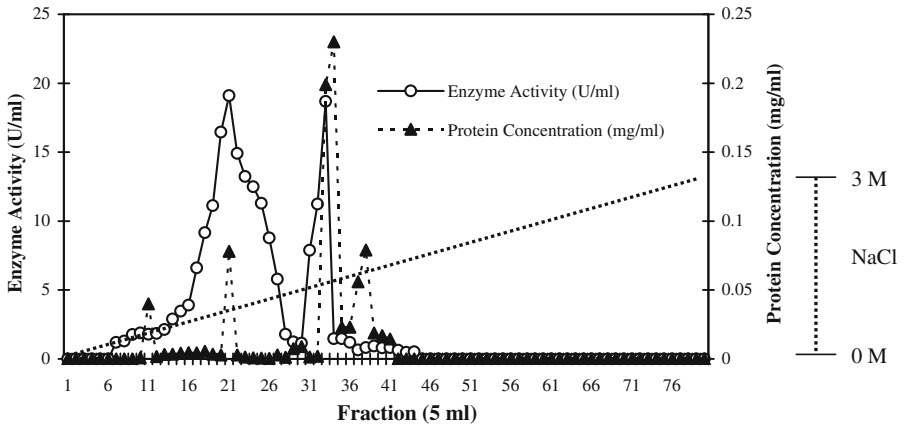

Fig. 1 Gradient elution (0 to 3 M NaCl) profile of exoinulinase using Sephacryl S-200 molecular exclusion chromatography. Fractions (5 ml) were collected and protein concentration was monitored using the Bradford method and exoinulinase activity was determined by incubating the enzyme fraction with 5% inulin at 50 °C for 1 h and reducing sugars were assayed by the DNS method

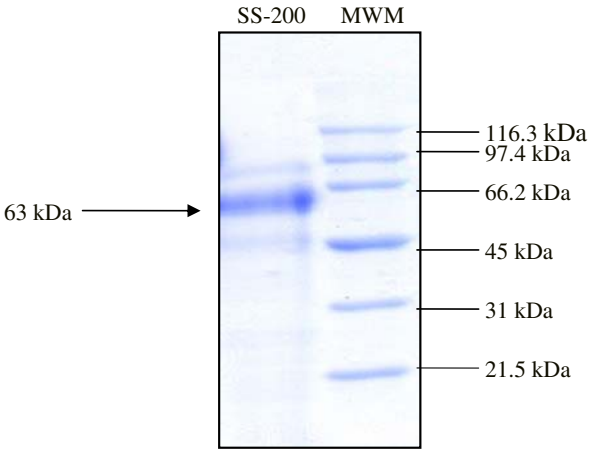

Fig. 2 SDS-PAGE of the purified exoinulinase using 8% separating gel and a 5% stacking gel. *Lane SS-200* was loaded with the pooled fractions 17 to 26 from Sephacryl S-200 molecular exclusion chromatography and *lane MWM* was loaded with molecular weight markers ranging from 6.5 to 116.3 kDa. The gel was stained with Coomassie Brilliant R-250 for 30 min and destained with glacial acetic acid destaining solution overnight with gentle shaking

pH, Temperature Optima and Stability of the Exoinulinase

The pH and temperature optima of the exoinulinase were 5 and 50 °C respectively. The exoinulinase was stable at 50 °C since after 12 h, 96% activity remained while after 72 h of incubation, the enzyme had 63.8% residual activity.

Kinetic Parameters and Inhibition of Exoinulinase

The kinetic parameters K_m and V_{max}, determined by the initial rate experiments were estimated, respectively, as 4.75 mM and 833.3 μmol min^{-1} ml^{-1} at 50 °C and pH 5.0 (Fig. 4).

The addition of initial fructose to the reaction mixture had a profound effect on the yield of fructose from inulin hydrolysis by the crude exoinulinase indicating that fructose is

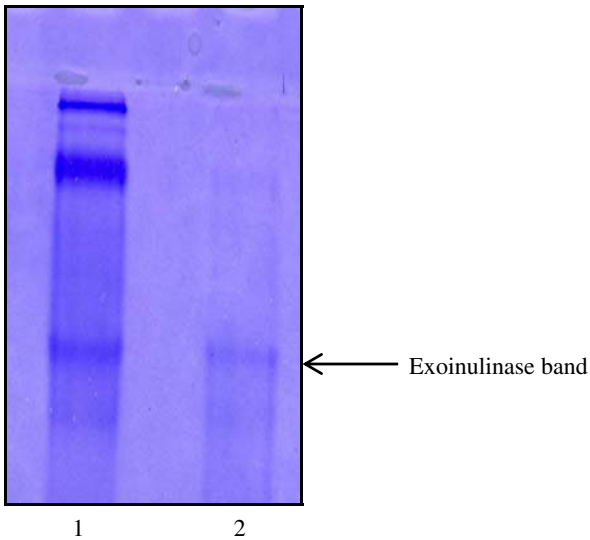

Fig. 3 Activity PAGE of the pure exoinulinase after Sephacryl S-200 size exclusion chromatography using 8% separating gel and a 5% stacking gel. *Lane 1* dialysed crude Fructozyme, *lane 2* pooled fractions 17 to 26 after Sephacryl S-200 size exclusion chromatography. The gel was stained with Coomassie Brilliant Blue R-250 for 30 min and destained with glacial acetic acid destaining solution overnight with gentle shaking

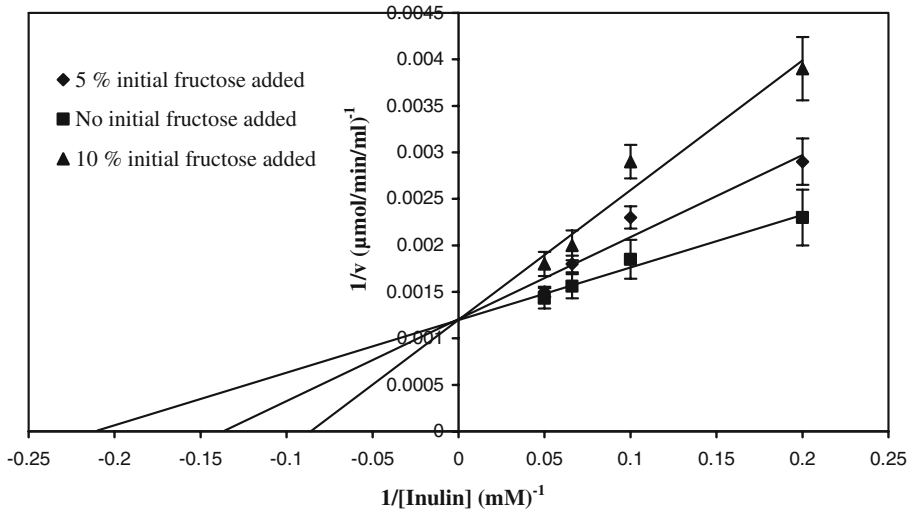

Fig. 4 Lineweaver–Burk double reciprocal plot of the exoinulinase in the presence and absence of initial fructose (5%). Each point on the graph represents the mean ± SD of three replicate determinations

acting as an end product inhibitor to this enzyme. In the presence of initial fructose (5%), the K_m was 7.3 mM and the V_{max} was 833.3 µmol/min/ml while in the presence of 10% initial fructose, the K_m increased to 11.58 mM and the V_{max} remained unchanged at 833.33 µmol/min/ml. The increase in K_m with the increase in initial fructose concentration and the unchanging V_{max} in the presence or absence of the inhibitor indicate that fructose acts as a competitive inhibitor of the exoinulinase. The exoinulinase showed reversible inhibition suggesting that it followed Michaelis–Menten kinetics.

Production of Fructose by the Exoinulnase

The main objective of response surface methodology is to develop the optimum conditions for the maximum rate of production of fructose. Since this is unknown at the start of the experiments, it is necessary to ensure that regression coefficients are rotated, thereby allowing for equality in all directions. In order to chose important parameters that are crucial for fructose production from inulin hydrolysis by an exoinulinase, three factors were chosen, pH (5.0 to 6.2), temperature (30 to 50 °C) and inulin concentration (50 to 150 mg ml^{-1}). The highest production of fructose was 76.2 mg after 8 h incubation at pH 5.0, temperature 50 °C and 150 mg ml^{-1} inulin (Table 5) while response surface regression predicted 70 mg of fructose (R^2 of 98.67, p=0.034) under similar experimental conditions. A response surface methodology experimental design was chosen in place of a classic factorial design experiment. The RSM allowed for the investigation of factor interaction as well as greatly reducing the number of experiments needed to determine factor significance on the rate of fructose production, thereby reducing costs and increasing experimental efficiency. The 2^3 standard Box–Wilson central composite design used in these experiments includes the use of eight classic factorial design points (samples 1–8), six axial points (samples 9–14) and one central point (sample 15) [16]. This affords uniform precision and variance of prediction equal for all points about a centre point.

Since the maximum fructose production was from incubation at pH 5 and a temperature of 50 °C these two parameters were kept constant in a second RSM strategy. In this case the

Table 5 Coded combinations of independent variables at different levels using RSM with CCD.

Sample run	pH [X_1]	Temperature [X_2]	Inulin (mg ml^{-1}) [X_3]	Fructose production (mg/ml)
1	5.0 (−1)	30 (−1)	50 (−1)	33.4
2	6.2 (+1)	30 (−1)	50 (−1)	27.2
3	5.0 (−1)	50 (+1)	50 (−1)	62.8
4	6.2 (+1)	50 (+1)	50 (−1)	54.4
5	5.0 (−1)	30 (−1)	150 (+1)	60.1
6	6.2 (+1)	30 (−1)	150 (+1)	57.4
7	5.0 (−1)	50 (+1)	150 (+1)	76.2
8	6.2 (+1)	50 (+1)	150 (+1)	55.5
9	5.0 (−1)	40 (0)	100 (0)	54.3
10	6.2 (+1)	40 (0)	100 (0)	52.6
11	5.6 (0)	30 (−1)	100 (0)	42.3
12	5.6 (0)	50 (+1)	100 (0)	57.3
13	5.6 (0)	40 (0)	50 (−1)	52.1
14	5.6 (0)	40 (0)	150 (+1)	44.7
15	5.6 (0)	40 (0)	100 (0)	51.8

The coded levels (values in parentheses) were generated as described in Eq. 1. Incubation was for 8 h at 100 U ml^{-1} exoinulinase

three variable parameters were time (4 to 12 h), enzyme concentration (20 to 100 U ml^{-1}) and inulin concentration (50 to 150 g l^{-1}) (Tables 2 and 6).

The highest yields of fructose were 84.5 mg after 12 h incubation with 100 U ml^{-1} enzyme and 150 mg ml^{-1} inulin and 83.7 mg with the same inulin concentration, for 8 h incubation and 60 U ml^{-1} enzyme. The corresponding predicted rate of production of fructose by response surface regression were 84.1 and 83.7 mg respectively (R^2 of 0.99, p= 0.25) indicating that there were no significant differences between the observed and the predicted fructose yield.

Table 6 Coded combinations of independent variables at different levels using RSM with CCD.

Sample run	Time (h) [X_1]	Exoinulinase [X_2]	Inulin (mg ml^{-1}) [X_3]	Fructose production (mg/ml)
1	4 (−1)	20 (−1)	50 (−1)	33.2
2	12 (+1)	20 (−1)	50 (−1)	36.7
3	4 (−1)	100 (+1)	50 (−1)	32.1
4	12 (+1)	100 (+1)	50 (−1)	32.2
5	4 (−1)	20 (−1)	150 (+1)	81.6
6	12 (+1)	20 (−1)	150 (+1)	82.1
7	4 (−1)	100 (+1)	150 (+1)	81.4
8	12 (+1)	100 (+1)	150 (+1)	84.5
9	4 (−1)	60 (0)	100 (0)	63.6
10	12 (+1)	60 (0)	100 (0)	64.7
11	8 (0)	20 (−1)	100 (0)	60.1
12	8 (0)	100 (+1)	100 (0)	60.1
13	8 (0)	60 (0)	50 (−1)	34.4
14	8 (0)	60 (0)	150 (+1)	83.7
15	8 (0)	60 (0)	100 (0)	66.3

The coded levels (values in parentheses) were generated as described in equation 1. Incubation was at pH 5 and a temperature of 50 °C

In the third RSM experimental runs, three parameters, time (24 to 48 h), temperature (30 to 50 °C) and inulin concentration (50 to 150 mg ml^{-1}) were varied simultaneously with the initial pH of 5.0 and enzyme concentration of 100 U ml^{-1} being fixed (Tables 3 and 7). Experimental run 8 gave the highest fructose concentration (106.4 mg) after 48 h incubation at 50 °C at 150 mg ml^{-1} inulin concentration. Response surface regression predicted a maximal of 105 mg of fructose (R^2 of 0.98, p=0.096) under same experimental conditions. Experimental run 14 also gave a high fructose yield (104.9 mg) after 36 h incubation, at 40 °C with 150 mg ml^{-1} inulin concentration. Experimental runs 5 and 7 also gave fairly high fructose yields, 103.3 and 102.7 mg respectively.

Discussion

The principal aim of this study was to use a crude and purified exoinulinase isolated from a commercial enzyme cocktail, fructozyme extracted from *A. ficuum* for fructose production. Temperature and pH profiles as well as stability of the purified exoinulinase were investigated on enzyme activity. The purified exoinulinase was further evaluated for production of fructose using response surface methodology.

Inulin hydrolysis using enzymes is usually carried out at 60 °C and mild pH conditions and the temperature is especially critical because it prevents microbial contamination of the final product, lower viscosity, improved transfer rates and also that it allows for the use of higher concentrations of inulin due to increased solubility [2, 17]. Under these conditions it is therefore crucial to employ highly thermostable inulinolytic enzymes from microbial sources.

It exhibited a pH and temperature optima of 5.4 and 50 °C respectively and under such conditions the enzyme remained stable with no decrease in activity after 60 min. Characterisation of inulinases has been carried out and generally, an average optimum temperature of 50 °C has been reported though 60 °C is recommended for industrial application to avoid microbial contamination of reactors and final product and also to

Table 7 Coded combinations of independent variables at different levels using RSM with CCD.

Sample run	Time (h) [X_1]	Temperature [X_2]	Inulin (mg ml^{-1}) [X_3]	Fructose production (mg/ml)
1	24 (−1)	30 (−1)	50 (−1)	38.2
2	48 (+1)	30 (−1)	50 (−1)	40.1
3	24 (−1)	50 (+1)	50 (−1)	36.5
4	48 (+1)	50 (+1)	50 (−1)	38.7
5	24 (−1)	30 (−1)	150 (+1)	103.3
6	48 (+1)	30 (−1)	150 (+1)	81.8
7	24 (−1)	50 (+1)	150 (+1)	102.7
8	48 (+1)	50 (+1)	150 (+1)	106.4
9	24 (−1)	40 (0)	100 (0)	91.6
10	48 (+1)	40 (0)	100 (0)	82.6
11	36 (0)	30 (−1)	100 (0)	76.6
12	36 (0)	50 (+1)	100 (0)	64.4
13	36 (0)	40 (0)	50 (−1)	40.1
14	36 (0)	40 (0)	150 (+1)	104.9
15	36 (0)	40 (0)	100 (0)	79.8

The coded levels (values in parentheses) were generated as described in equation 1. Initial pH=5.0 and 100 U ml^{-1} exoinulinase

increase solubility of the inulin substrate (5% maximal solubility at 50 °C) with the main disadvantage of a reduction in enzyme stability at these elevated temperatures [18]. The optimal pH of most inulinases ranges from 4.5 to 5.0 with the advantage of reduced colour formation because of the mild conditions. Inulinases are generally stable in the pH range 4.0 to 6.0.

The fructose yield from the controlled hydrolysis of inulin in all the RSM trials was affected by various factors such as pH, enzyme dosage, incubation time, temperature and inulin concentration. The second and third RSM, the exoinulinase produced highest fructose concentrations, 84.51 mg and 106.4 mg, respectively.

The addition of initial fructose to the reaction mixture resulted in the decrease of fructose production from 100% relative fructose produced with no added initial fructose to 39.98% relative fructose produced at 20% initial fructose suggesting that fructose is acting as an end product inihibitor to the exoinulinase. The K_m was 11.58 mM in the presence of 10% added initial fructose, 7.3 mM in the presence of 5% added initial fructose and 4.75 mM with no added initial fructose. The high V_{max} and low K_m show the affinity of the exoinulinase for the inulin substrate in the absence of added initial fructose. The V_{max} of this exoinulinase (833.3 μmol/min/ml) remained unchanged both in the absence and presence of the inhibitor (fructose) suggesting that fructose is a competitive inhibitor of the exoinulinase with respect to inulin as the substrate. Dialysis of the reaction mixture completely removed fructose and showed that the type of inhibition was reversible which shows true Michaelis–Menten kinetics for the exoinulinase enzyme. Zhang et al. [19] showed that glucose was a competitive inhibitor to enzyme activity for the hydrolysis of inulin with a degree of polymerisation not less than 10. Furthermore, these workers established that fructose had no inhibitory effect on the exoinulinase from *Kluyveromyces marxianus* for the hydrolysis of oligosaccharides with a DP≤9. According to Diaz et al. [3] the inhibitory nature of fructose can be explained by the reaction equilibrium thermodynamics, or by the diffusional competition between large substrate molecules and small product ones.

The exoinulinase was purified to eletrophoretic homogeneity and it was a monomeric band on SDS-PAGE with a molecular weight of 63 kDa. The molecular weight of the purified exoinulinase falls in the range of molecular weight of microbial exoinulinases that have been purified to date [20, 21]. Native PAGE afforded the confirmation of the exoinulinase nature of this enzyme. The optimal conditions for fructose production were established to be temperature 50 °C, pH 5.0, enzyme dosage 100 U ml^{-1} and inulin 150 mg ml^{-1}. A long incubation time was ideal for high yield of fructose for complete hydrolysis of inulin since the enzyme showed good pH and thermostability. Consequently it can be speculated that these conditions could be incorporated into an industrial process for fructose production using this enzyme preparation. In addition, the pH optimum (5.0) observed for the exoinulinase is advantageous for industrial fructose syrup preparations as it prevents undesired colour formation and formation of unwanted by products such as fructose dianhydrides [2].

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