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Optimization of inulinase production by a newly isolated *Aspergillus tubingensis* CR16 using low cost substrates

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ABSTRACT

Production of an extracellular, thermostable inulinase was carried out by a newly isolated strain of *Aspergillus tubingensis* CR16 using wheat bran and corn steep liquor (CSL) under solid state fermentation (SSF). Response surface methodology (RSM) involving Box Behnken design (BBD) was employed for the optimization of process parameters viz. time period of fermentation, % moisture content, inoculum size and pH of the medium. Maximum yield of inulinase was 257 ± 11.4 U/g, obtained by inoculating 5 g of wheat bran with 10^9 spores/ml, at initial 71.2% moisture content and pH 6.1 after 103 h of fermentation along with 1358.6 ± 0.8 U/g of invertase activity. Crude inulinase showed maximum activity at 60 °C and pH 5.0. The enzyme was found to be thermostable retaining about 90% of its activity for 4.5 h at 60 °C. Fructose was produced as an end product of inulin hydrolysis proving that the enzyme produced was exoinulinase.

1. Introduction

Inulin occurs as a reserved carbohydrate polymer mainly in the roots and tubers of jerusalem artichoke, chicory, dandelion, burdrock and dahlia (Chen, Chen, Chen, Xu, & Jin, 2011; Pandey et al., 1999a). It consists of linear chains of β-2,1-linked D-fructofuranose molecules terminated by a glucose residue (Vandamme & Derycke, 1983). Inulin has recently received a great interest as a relatively inexpensive and abundant substrate for the production of high fructose syrup. D-Fructose is occupying an increasingly important position in the modern world as a sweetener because of its higher sweetening value, its physiological metabolism in human body and its insignificant insulinogenic effects (Pandey et al., 1999a). Fructose production by enzymatic inulin hydrolysis is more advantageous than conventional process based on starch, which requires the action of α-amylase; amyloglucosidase and glucose isomerise and yields only 45% of fructose in the final product, due to thermodynamical equilibrium of the reaction (Sguarezi et al., 2009). Thus microbial inulinases are important class of industrial enzymes that have gained much attention in recent times. Inulinases can be produced by many of the microorganisms including strains of *Aspergillus* sp., *Penicillium* sp. and *Kluyveromyces* sp. Owing to the cost of pure inulin, alternate inulin containing raw, inexpensive substrates are preferred for microbial inulinase production. In recent years, inulinase production by solid state fermentation (SSF) has attracted much attention because of high productivity, simple operation, cost effectiveness and better product recovery (Singhania, Patel, Soccol, & Pandey, 2009). Moreover, the crude fermented products from SSF can be used directly as the enzyme source for biotransformation (Chen et al., 2011). Optimization of process by statistical experimental designs is very useful, as it helps in understanding the interactions among the process parameters at varying levels and in calculating an optimal level of each parameter for the maximal product yield (Reddy, Ramesh, Mrudula, Reddy, & Seenayya, 2003). RSM is a model consisting of mathematical and statistical techniques, widely used to study the effect of several variables and to seek the optimum conditions for a multivariable system (Xiong, Jinhua, & Dongsheng, 2007).

Although microbial inulinase production has been reported by many researchers, studies on inulinase production under SSF are relatively less. Assessment of fermentation conditions for inulinase production is of relevance since many fermentation parameters may significantly affect the productivity of the enzyme and thus production cost. In this context, the present work was focused to study the optimization of process parameters for inulinase production on low cost substrate under SSF using statistical design employing a newly isolated fungi *Aspergillus tubingensis* CR16. Properties of crude inulinase were also evaluated to predict its end applications.

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2. Materials and methods

2.1. Materials

Dry chicory roots were procured from Pioneer Chicory Factory, Anand, India. Wheat bran and sugarcane bagasse were bought from local vendors. Corn steep liquor (CSL) was provided by Anil Starch Ltd., Ahmedabad, India. Pure inulin (Chicory) and potato dextrose agar (PDA) were obtained from Hi-media (India). All other chemicals were of reagent grade.

2.2. Identification of microorganism

A new fungal strain CR16 was isolated from chicory rhizosphere soil. Preliminary identification of isolate was done by study of growth characters on PDA plate and microscopic observations. The isolate was sent for molecular identification to Agharkar Research Institute, Pune, India. Molecular identification was done by partial sequencing i.e. ITS. Genomic DNA was isolated and about 500 bp rDNA fragments were amplified using universal primers. Sequencing PCR was done with ABI-Big DYE@ Terminatorv3.1 Cycle Sequencing Kit (Part No. 4337455). Sequence data was aligned with publically available sequences and analyzed to reach identity. The strain was maintained on PDA slants at 4 °C.

2.3. Media

Fermentation medium-1 contained (g/l): peptone 15.0 and yeast extracts 15.0. Fermentation medium-2 contained (g/l): inulin, 10.0; K_2HPO_4, 1.0; $MgSO_4 7H_2$ 0, 0.5; $NaNO_3$, 1.5; KCl, 0.5; $FeSO_4$, 0.1; and $NH_4H_2PO_4$, 2.0.

2.4. Inoculum preparation

Spores of 72 h old culture of *A. tubingensis* CR16 were suspended in sterile distilled water containing 0.1% of Tween 80 and spore count was performed using Neuber's chamber.

2.5. Submerged fermentation using different carbon sources

Submerged fermentation was carried out in 50 ml of fermentation medium-1 containing different carbon sources in 1% concentration namely chicory root powder, wheat bran, sugarcane bagasse, inulin, sucrose, fructose and glucose. Medium was inoculated with 1% inoculum with 10^8 spores/ml.

2.6. Solid state fermentation (SSF)

SSF was performed using each of the three different substrates viz. chicory root powder, wheat bran and sugarcane bagasse. Substrates were taken in 5 g quantity in 250 ml Erlenmeyer flask and autoclaved at 121 °C for 15 min. The substrates were moistened with 10 ml of the separately sterilized fermentation medium-1, along with 10^8 spores/ml inoculum. Contents of the flasks were mixed thoroughly and were incubated at 30 °C under static conditions. To study the effect of different moistening agents, 5 g of wheat bran was moistened with 10 ml of different moistening agents which included tap water, 10% CSL, chicory root extract, fermentation medium-1 and fermentation medium-2, inoculated with 10^8 spores/ml inoculum and incubated at 30 °C under static conditions. Flasks were mixed intermittently and were removed at regular intervals for analyses.

2.7. Statistical optimization of inulinase production

RSM using BBD was applied for the optimization of inulinase production which involves full factorial search by observing simultaneous, systematic and efficient variation of important components on the fermentation process. Wheat bran was selected as a substrate for optimization studies and 10% CSL was used as a moistening agent. Particle size of wheat bran (5–7 mm) was kept constant in all the experimental runs. Four important process parameters namely fermentation period (X_1), % moisture content (X_2), inoculum size (X_3) and pH of the medium (X_4) were selected as independent variables and inulinase activity (Y) was the dependent variable response. Each of these independent variables was studied at three different levels as per BBD in four variables. The number of experiments (N) required for the development of BBD is defined as

$$N = 2k(k-1) + C_0$$

where k is the number of variables and C_0 is the number of central points (Ferreira et al., 2007). This was used to develop mathematical correlation between four variables on the production of inulinase with a total of 29 runs with five replicates at a central point to fit the polynomial model as per Eq. (1).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 + e \quad (1)$$

Specified range of four variables used for the optimization was selected as per shown in Table 2 along with the design of BBD in the coded and decoded levels of the four variables. Temperature was kept constant at 30 °C for all the experimental runs. Flasks were analysed for inulinase activity at specific time intervals as planned in BBD. Control reactors were also carried out to discount if enzyme activity already present prior fermentation.

Statistical analysis was done using the software MINITAB 16. Graphs were generated to highlight the roles played by various factors and to emphasize the roles played by physical constraints and biosynthetic aspects in the final yield of inulinase. Interpretation of optimum values of process parameters and curves showing their interactive effect was done using the same software.

2.8. Extraction of inulinase

After fermentation the enzyme was extracted in 0.2 M sodium acetate buffer (pH 5). Content of the flask was mixed thoroughly on a rotary shaker (150 rpm) at 30 °C for 30 min, filtered through muslin cloth and was centrifuged at 3000 rpm for 10 min at room temperature. Supernatant was considered as crude enzyme solution and was analyzed for inulinase, invertase and protein content.

2.9. Enzyme assays

Enzymes were assayed by measuring the reducing sugar concentration by DNS (Miller, 1939). Reaction mixture consisting of 0.1 ml of appropriately diluted enzyme and 0.9 ml of 1% inulin/sucrose in 0.2 M sodium acetate buffer (pH 5) was incubated at 60 °C for 20 min. The reaction was terminated by further incubation in boiling water bath for 10 min. One unit of inulinase activity was defined as the amount of enzyme necessary to release one micromole of fructose per minute under the above conditions. One unit of invertase activity was considered as the amount of enzyme which released one micromole of reducing sugar per minute under the above conditions.

Table 1
Effect of different carbon sources on inulinase production under submerged fermentation.

Carbon sources	Inulinase (U/ml)	Invertase (U/ml)	*I/S*	Protein (mg/ml)	Specific activity (U/mg)
Chicory root powder	21.9 ± 0.4	133.7 ± 1.0	0.16	8.9 ± 0.4	2.4
Inulin	18.9 ± 0.3	146.2 ± 1.3	0.12	7.3 ± 0.1	2.5
Sucrose	12.1 ± 0.1	67.2 ± 1.7	0.18	9.0 ± 0.1	1.3
Wheat bran	8.2 ± 0.6	45.5 ± 1.5	0.18	8.4 ± 0.1	0.9
Sugarcane bagasse	5.1 ± 0.4	18.8 ± 2.1	0.27	10.3 ± 0.1	0.4
Fructose	4.7 ± 0.5	15.6 ± 1.5	0.3	9.4 ± 0.0	0.5
Glucose	5.8 ± 0.0	28 ± 0.9	0.2	10.8 ± 0.4	0.5

2.10. Protein estimation

Protein estimation was done by Folin Lowry's method (Lowry, Rosebrough, Farr, & Randall, 1951).

2.11. Preliminary characterization of crude inulinase

To get the idea of optimum temperature and pH for inulinase action, assays were carried out at different temperature (50–80 °C) and pH (3–8) using 0.2 M citrate buffer for pH 3, 0.2 M sodium acetate buffer for pH 4 and 5 and 0.2 M sodium phosphate buffer for pH 6–8. To determine thermal stability of inulinase, the enzyme solution was kept at 60 °C in 0.2 M sodium acetate buffer (pH 5) in a temperature controlled water bath and the residual activity was measured at different time intervals up to 10 h.

2.12. Hydrolysis of inulin

For studying inulin hydrolysis, 0.1 ml of crude inulinase was added to 0.9 ml of 1% inulin in 0.2 M sodium acetate buffer (pH 5) and incubated at 60 °C. Samples were withdrawn every 30 min up to 120 min and were checked for the presence of reducing sugar by DNSA. Inulin hydrolysis was determined by an increase in reducing sugars in the reaction mixture.

2.13. Qualitative analysis of products of inulin hydrolysis

Products of inulin hydrolysis in the course of time were qualitatively analyzed by thin layer chromatography (TLC) using the solvent system isopropanol:ethyl acetate:water in ratio 5:2.5:2.5. Plate was sprayed with the spraying reagent and was incubated at 100 °C for color development.

3. Results

3.1. Identification of microorganism

The isolate showed mycelial growth with black sporulation on PDA plate. Microscopic examination of fungus showed the presence of upright conidiophores that terminated in swollen vesicle, with conidiospores on it, which is typical for *Aspergillus* sp. Molecular identification reports revealed that isolate CR16 showed 99% sequence similarity with the strain of *A. tubingensis* (Gene Bank accession no. GQ461899.1). Thus newly isolated fungal strain CR16 was designated as a strain of *A. tubingensis* (Gene Bank accession no. JQ982501, NFCCF accession no. NFCCI 2061).

3.2. Submerged fermentation using different carbon sources

Various carbon sources including pure sugars and agro industrial substrates were checked for their effect on inulinase production. Considerable inulinase production was achieved on pure as well as raw substrates. However maximum inulinase production (21.9 U/ml) was obtained using chicory root powder as a carbon source, followed by pure inulin (18.9 U/ml) (Table 1). Sucrose and wheat bran supported inulinase production up to 12.1 U/ml and 8.2 U/ml respectively. Other carbon sources viz. glucose, fructose and sugarcane bagasse were found to be poor inducers for inulinase production with 5.8 U/ml, 5.1 U/ml and 4.7 U/ml of inulinase yield respectively. Significant amount of invertase was also produced on all the carbon sources. *I/S* value obtained using different carbon sources was in the range of 0.1–0.3.

3.3. Solid state fermentation

Solid state fermentation was carried out using three different low cost substrates including wheat bran, sugarcane bagasse and chicory root powder for different time interval. Among the three, wheat bran was found to be the most suitable substrate for SSF giving maximum inulinase production 165.5 ± 1.5 U/g, followed by sugarcane bagasse which gave 152.2 ± 4.7 U/g and chicory root powder which gave 67.4 ± 6.2 U/g inulinase yield.

Among the different moistening agents studied, CSL was found to be the most suitable; giving maximum inulinase yield of 198.4 ± 0.1 U/g (Fig. 1), followed by fermentation medium-1 giving 165.5 ± 1.5 U/g and chicory root extracts giving 115.5 ± 3.5 U/g inulinase yield. Use of tap water as a moistening agent also showed considerable, 73.3 ± 3.6 U/g, inulinase production.

3.4. Optimization of process parameters using BBD

As wheat bran was found to be the most suitable substrate and CSL, the most suitable moistening agent, for inulinase production by *A. tubingensis* CR16 under SSF, they were used for the optimization studies.

Four important process variables which included fermentation period, % moisture content, inoculum size and pH were investigated for their optimum combination using BBD. Design and results of the experiments are shown in Table 2. Results were analyzed by ANOVA (Table 3) and the second order regression equation provided the levels of inulinase activity as the function of fermentation period, % moisture content, inoculum size and pH. By applying multiple regression analysis on the experimental data the second order

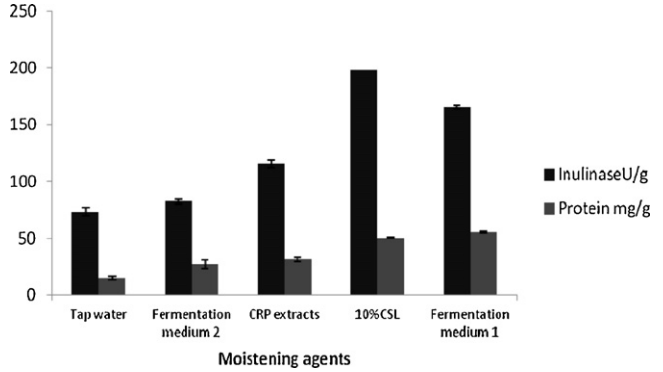

Fig. 1. Effect of moistening agents on inulinase production by *Aspergillus tubingensis* CR 16 under SSF using wheat bran as a substrate.

Table 2
BBD with coded and decoded levels for inulinase production by *Aspergillus tubingensis* CR-16 under SSF.

RSM no.	Fermentation period (h)	% moisture content	Inoculum size (LOG spores/ml)	pH	Predicted inulinase (U/g)	Experimental inulinase (U/g)
1	−1(48)	−1(60)	0(5)	0(5)	67.6	86.3
2	−1(48)	1(80)	0(5)	0(5)	20.9	36.9
3	1(144)	−1(60)	0(5)	0(5)	56	42.1
4	1(144)	1(80)	0(5)	0(5)	119.9	103.3
5	−1(48)	0(70)	−1(1)	0(5)	75.7	100.8
6	−1(48)	0(70)	1(9)	0(5)	165.2	163.6
7	1(144)	0(70)	−1(1)	0(5)	130.2	128.7
8	1(144)	0(70)	1(9)	0(5)	198.1	170
9	−1(48)	0(70)	0(5)	−1(2)	111.3	81.6
10	−1(48)	0(70)	0(5)	1(8)	61.8	33.5
11	1(144)	0(70)	0(5)	−1(2)	153	182.4
12	1(144)	0(70)	0(5)	1(8)	107.5	138.3
13	0(96)	−1(60)	−1(1)	0(5)	105.7	73.7
14	0(96)	−1(60)	1(9)	0(5)	151.5	118.5
15	0(96)	1(80)	−1(1)	0(5)	81.4	115.6
16	0(96)	1(80)	1(9)	0(5)	193	226.1
17	0(96)	−1(60)	0(5)	−1(2)	101.8	127.4
18	0(96)	−1(60)	0(5)	1(8)	87.7	122.5
19	0(96)	1(80)	0(5)	−1(2)	143.8	105.9
20	0(96)	1(80)	0(5)	1(8)	62.8	34.2
21	0(96)	0(70)	−1(1)	−1(2)	208.8	201.4
22	0(96)	0(70)	−1(1)	1(8)	62.9	44.8
23	0(96)	0(70)	1(9)	−1(2)	189.1	209.3
24	0(96)	0(70)	1(9)	1(8)	240	249.6
25	0(96)	0(70)	0(5)	0(5)	204.1	199.2
26	0(96)	0(70)	0(5)	0(5)	204.1	187.2
27	0(96)	0(70)	0(5)	0(5)	204.1	190.4
28	0(96)	0(70)	0(5)	0(5)	204.1	238.8
29	0(96)	0(70)	0(5)	0(5)	204.1	205

polynomial equation was obtained in terms of process variables as per Eq. (2).

$$Y = 204.12 + 21.842X_1 + 4.292X_2 + 39.342X_3 + (-23.758)X_4 + (-64.31)X_1^2 + (-73.685)X_2^2 + 2.515X_3^2 + (-31.385)X_4^2 + 27.65X_1X_2 + (-5.375)X_1X_3 + 1.0X_1X_4 + 16.425X_2X_3 + (-16.7)X_2X_4 + 49.225X_3X_4 \quad (2)$$

where Y is inulinase yield (U/g), X_1 is fermentation period, X_2 is %moisture content, X_3 is inoculum size and X_4 is pH of the medium.

ANOVA of the second order regression model demonstrated that the model was highly significant with very low P value of 0.001. Lack of fit was >0.05 (Table 3). As predicted by the model, among the parameters, maximum interaction occurred between inoculum size and pH with the P value 0.01 (Table 4). The predicted R^2 value obtained in this study was 85.9%. The fitted response for the above regression model was plotted in Fig. 2a and b for the pair wise combination of the most interactive variables while keeping others at their predicted optimum levels. The optimum conditions found for maximum inulinase production were 103 h of fermentation period, 71.2% of moisture content, inoculum size of 10^9 spores/ml and pH 6.1. By substituting the levels of the factors into the regression equation, maximum predictable response for inulinase production was calculated to be 253.3 U/g (Fig. 2b).

Table 3
Analysis of variance for inulinase.

Source	DF	Seq. SS	Adj. SS	Adj. MS	F	P
Regression	14	103,926	103,926	7423.3	6.08	0.001
Linear	4	31,292	31,292	7823.1	6.41	0.004
Square	4	57,569	57,569	14392.2	11.79	0.000
Interaction	6	15,065	15065	2510.8	2.06	0.125
Residual error	14	17084	17084	1220.3		
Lack of fit	10	15382	15382	1538.2	3.61	0.114
Pure error	4	1702	1702	425.6		
Total	28	121010				

3.5. Validation of the experimental model

In order to determine accuracy of the model and to verify the optimization results, experiments were repeated in triplicates under optimized culture conditions i.e. 71.2% of moisture content, 10^9 spores/ml of inoculum size and pH of the medium 6.1 for 103 h of fermentation. Under these conditions, 257.2 ± 11.4 U/g of inulinase was obtained. This value of enzyme yield corresponds very well to the values predicted by the model (253.3 U/g). After statistical optimization, inulinase yield was increased to 1.3 fold.

We had also studied sucrose hydrolytic activity of inulinase produced by *A. tubingensis* CR16 under optimized conditions. Enzyme units obtained using 1% sucrose solution as a substrate under the reaction conditions were 1358.6 ± 0.8 U/g giving I/S value 0.18 which proves that enzyme produced by *A. tubingensis* CR16 was a true inulinase.

3.6. Preliminary characterization of crude inulinase

Optimum temperature for inulinase activity was 60 °C and optimum pH was pH 5.0. Thermostability studies at 60 °C showed that crude inulinase was thermostable retaining more than 90% of its activity for about 4.5 h and about 50% of its activity even after 9 h (Fig. 3).

Hydrolysis of inulin was studied using a combination of crude inulinase and pure inulin (chicory). There was a rapid increase in the reducing sugars in initial 30 min of the reaction which continued to increase up to 120 min but at a slower rate (data not shown here). To determine exo- or endoacting nature of crude inulinase, TLC analysis of the reaction products of inulin treated with inulinase was done (Fig. 4). Fructose was the only sugar released during hydrolysis, supported the view that inulinase was an end group

Table 4
Regression coefficients for inulinase (U/g).

Term	Coefficient	SE coefficient	*T*	*P*
Constant	204.120	15.62	13.066	0.000
Fermentation period	21.842	10.08	2.166	0.048
% moisture content	4.292	10.08	0.426	0.677
Inoculum size	39.342	10.08	3.901	0.002
pH	−23.758	10.08	−2.356	0.034
Fermentation period × fermentation period	−64.310	13.72	−4.689	0.000
% moisture content × % moisture content	−73.685	13.72	−5.372	0.000
Inoculum size × inoculum size	2.515	13.72	0.183	0.857
pH × pH	−31.385	13.72	−2.288	0.038
Fermentation period × % moisture content	27.650	17.47	1.583	0.136
Fermentation period × inoculum size	−5.375	17.47	−0.308	0.763
Fermentation period × pH	1.000	17.47	0.057	0.955
% moisture content × inoculum size	16.425	17.47	0.940	0.363
% moisture content × pH	−16.700	17.47	−0.956	0.355
Inoculum size × pH	49.225	17.47	2.818	0.014

cleaving enzyme. Thus inulinase produced by *A. tubingensis* CR16 was an exoinulinase.

4. Discussion

The genus *Aspergillus* contains more than 260 species and has great impact in various fields of research and biotechnological applications. *A. tubingensis* belongs to the *Aspergillus niger* complex (black aspergilli) and is commonly found on plant products and in processed foods, such as coffee, grapes and cereals. This species is morphologically indistinguishable from *A. niger* and can be reliably identified only by molecular methods (Susca, Stea, Mule, & Perrone, 2007). *Aspergillus* species are known to be one of the predominant inulinase producers; however inulinase production by *A. tubingensis* has not been reported so far.

Carbon compounds are the sources of carbon skeleton and energy for microorganisms. In the present studies, inulinase was produced on all the tested carbon sources but inulin rich raw substrate namely chicory root powder showed the highest inulinase production followed by pure inulin (chicory), sucrose and wheat

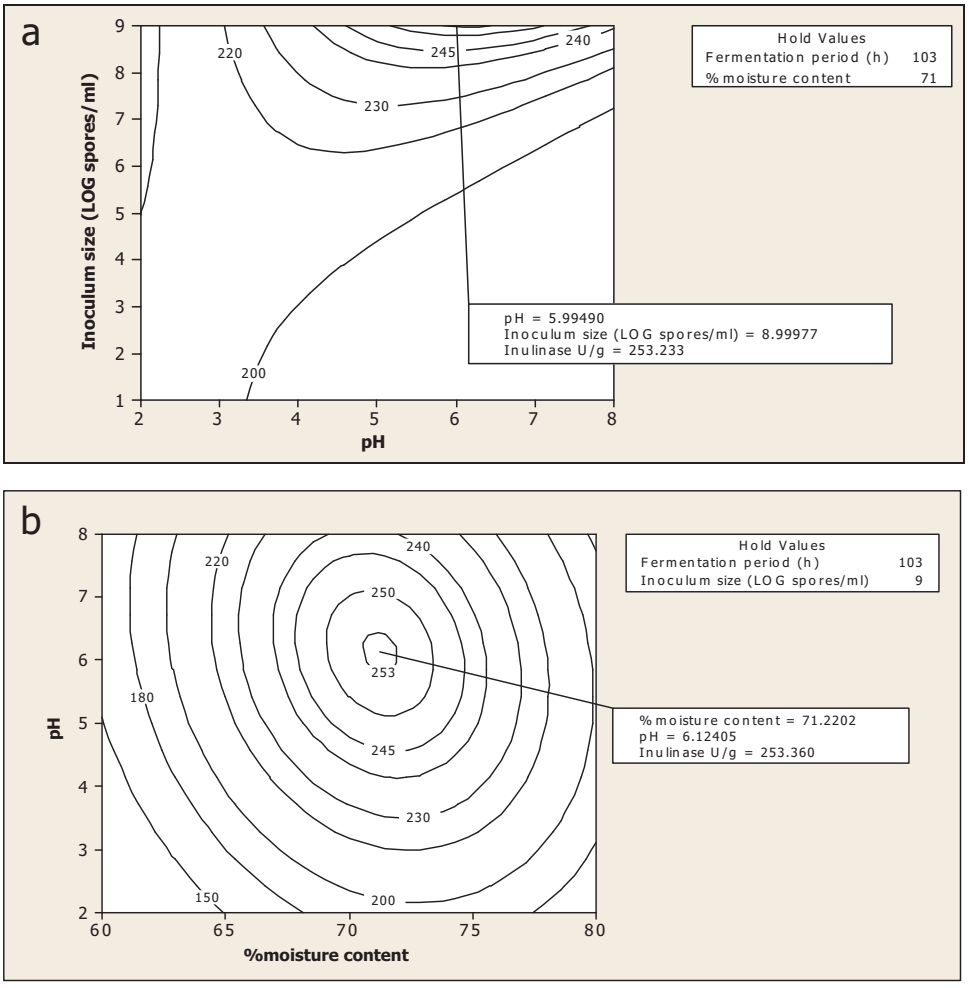

Fig. 2. (a) Counter plot of interaction of inoculum size (LOG spores/ml) and pH. (b) Counter plot of interaction of % moisture content and pH.

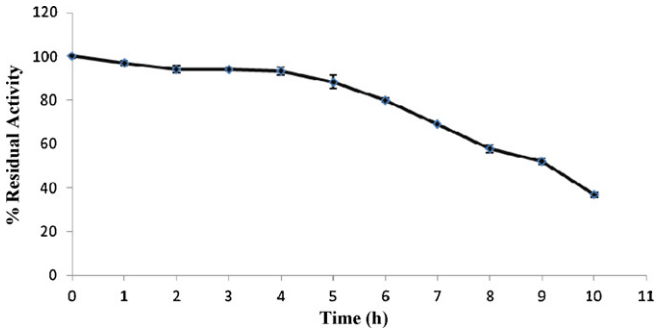

Fig. 3. Thermostability of inulinase at 60 °C.

bran in descending order (Table 1). Chicory roots are known to store about 15–20% inulin as a reserve carbohydrate and wheat bran contains 1–4% inulin (Van, Coussement, Leenheer, Hoebregs, & Smits, 1995). There are many reports on the use of raw inulin containing substrates for inulinase production. Kango (2008) has reported higher inulinase production by *A. niger* using dandelion tap root extracts as a carbon source compared to pure chicory inulin. Park and Yun (2001) utilized chicory roots for endoinulinase production and obtained 15 U/ml of inulinase by *Xanthomonas* sp. Inulinase produced by most of the microorganisms are inducible (Singh & Bhermi, 2008). However, in the present study, fructose which is believed to be a primary inducer for inulinase (Saber & El-Naggar, 2009), was not found to be effective as the yield was decreased by about four fold as compared to inulin. Moreover, the results of the present study revealed that this enzyme may have constitutive nature. Saber and El-Naggar (2009) has reported constitutive production of inulinase by *Aspergillus tamarii* AR-IN9 which was induced by the presence of inulin and inulin containing substrates. There are number of reports on constitutive production of inulinase by various isolates (Bernardo, Silva-Santisteban, Converti, & Maugeri Filho, 2009; Cruz, Belote, Belline, & Cruz, 1998).

Selection of a good support material that supports the development of microorganism is a key aspect in SSF and thus involves

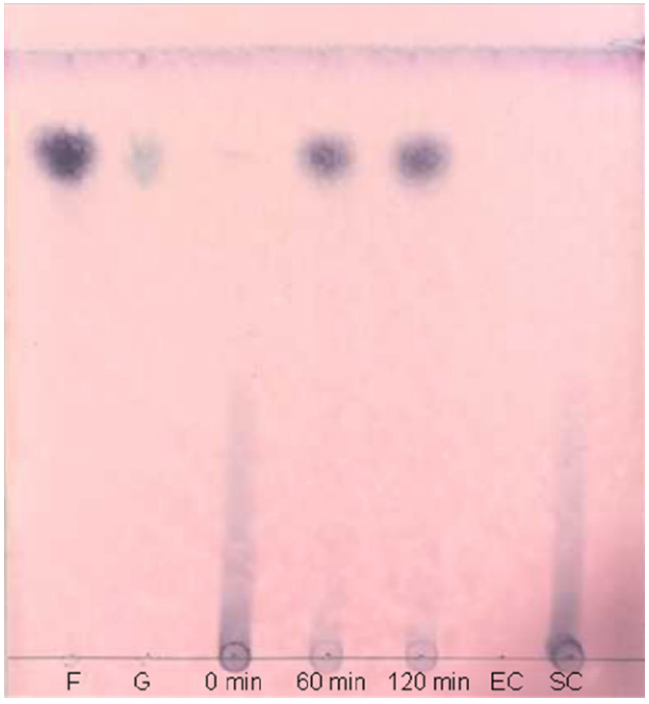

Fig. 4. Thin layer chromatogram of hydrolyzed products of inulin.

screening of number of agro industrial materials for microbial growth and product formation. This material can act as a source of nutrients and/or as support to microbial growth (Mazutti, Ceni, Luccio, & Treichel, 2007). From the present study it was observed that under SSF conditions wheat bran proved to be a better substrate when compared with sugarcane bagasse and chicory root powder. Wheat bran is considered as the universal substrate because it acts as a complete nutritious feed for microorganisms, having all the ingredients and remains loose even under moist conditions providing a large surface area. Moreover it contains various soluble sugars which help the initiation and growth of microorganisms. Wheat bran has been employed as a substrate for the production of many enzymes like xylanase, cellulases, etc. under SSF but there are few reports on the use of wheat bran as a substrate for inulinase production. Xiong et al. (2007) and Chen et al. (2011) have reported the use of wheat bran as a substrate for inulinase production by *Kluyveromyces* S120 by SSF. Sugarcane bagasse has also been reported as a substrate for inulinase production (Bender, Mazutti, de Oliveira, Luccio, & Treichel, 2006; Mazutti, Bender, Treichel, & Luccio, 2006; Treichel, Mazutti, Filho, & Rodrigues, 2009). No reports were found in the literature regarding the use of chicory root powder as a substrate for solid state fermentation. In the present study, chicory root powder was proved to be the best carbon source for inulinase production under submerged conditions, but the yield of inulinase was reduced remarkably when used for SSF.

Among various moistening agents, CSL was found to be the most suitable moistening agent (Fig. 1). CSL is well known nitrogen rich by-product of starch industry and it is also the source of minerals, vitamins, amino acids and other essential nutrients for the growth of microorganisms (Mazutti et al., 2007). In a similar study, Xiong et al. (2007) reported the use of CSL for inulinase production under SSF using wheat bran as a substrate by *Kluyveromyces* S120. Gill, Sharma, Harchand, and Singh (2003) also reported CSL to be the best nitrogen source for inulinase production by *Actinomycete* strain.

Conventional change-one-factor-at-a-time approach has been used for medium optimization, but it is laborious and time consuming. Also it can lead to misinterpretation of results, as interactions between different factors are overlooked. RSM is a statistically designed protocol in which several variables are simultaneously varied and the experimental responses of design of experiments are fitted to quadratic function (Chen et al., 2011). Hence BBD was applied for the optimization of inulinase production by *A. tubingensis* CR16 under SSF. ANOVA consists of classifying and cross classifying statistical results, and testing whether the means of a specified classification differ significantly (Sheng et al., 2008). ANOVA was carried out by Fisher's statistical test for square due to regression to the mean square due to error and indicates the significance of each controlled factor on the tested model. The model computed for the R^2 value showed 85.9% indicating that it is appropriate and can be useful to predict the effect of fermentation period, % moisture content, inoculum size and pH on the production of inulinase and reaching optimum conditions of factor for desirable response. Graphs were given here to highlight the roles played by various factors in the final yield of inulinase. Among the variables, inoculum size was the most significant linearly and had a positive effect (Table 4). The effect of inoculum on inulinase production can be observed in Fig. 2a which shows, increased inulinase yield with increasing inoculum size. Usually size of inoculum plays a significant role in the production of metabolites under SSF. It is important to provide an optimal size of inoculum in fermentation process, since a lower inoculum size may give insufficient biomass for the product formation (Pandey, 2003). Significant interaction occurred between inoculum size and pH (Table 4). Increase in pH above 6.1 decreased the inulinase yield. Present findings are in accordance with the fact that most of the fungi have their optimum

growth pH in an acidic range. Fermentation period also had significant influence on the product formation. Inulinase yield increased with an increase in fermentation period but extended fermentation decreased the product yield. These results are important for large scale inulinase production in industries because faster fermentations give higher productivity. Moreover shorter fermentation time is desirable to decrease the risk of contamination (Mazutti et al., 2007). Substrate moisture is a critical factor in SSF and its importance for enzyme production has been well established. It is reported that low substrate moistures in SSF resulted in suboptimal product formation due to reduced mass transfer process such as diffusion of solutes and gas to the cell during fermentation (Pandey & Selvakumar, 1999b). In the present study, moisture content was not significant linearly; instead it had a quadratic effect with a negative regression coefficient (Table 4). Thus increase in moisture content beyond 71.2%, decreased the yield of inulinase (Fig. 2b). Experimentally obtained value of inulinase production (257 ± 11.4 U/g) under the optimized conditions was in complete accordance to the predicted value (253.3 U/g) stating that the model was significant. By statistical optimization, inulinase yield was enhanced to 1.3 fold. Chen et al. (2011) also applied BBD for the media optimization for inulinase production by *Aspergillus ficuum* JNSP5-06 under SSF using wheat bran as a substrate and has achieved maximum of 205.63 U/g of inulinase yield under optimized conditions.

Many microbial preparations of inulinase possess remarkable invertase activity (S) accompanying inulinase activity (I); hence, their catalytic activity is described in terms of I/S ratio. Thus, I/S (inulin/sucrose) is used to characterize the enzymes: inulinase ($I/S > 10^{-2}$) and invertase ($I/S < 10^{-4}$). The naming of inulinase or invertase as β-fructosidase is based on its relative hydrolytic capacity for inulin and sucrose (I/S) (Ettalibi & Baratti, 1987). In the present study, I/S value is 0.18. Hence the enzyme can be defined as true inulinase. The enzyme yield based on sucrose hydrolytic capacity was very high (1358.6 ± 0.8). To the best of our knowledge, we are reporting highest yield of inulinase under SSF. There are some reports showing higher yield in SSF (Mazutti et al., 2006; Treichel et al., 2009; Xiong et al., 2007) but their results are based on sucrose hydrolytic activity.

The knowledge of temperature and pH influence on the activity of enzyme preparations is very important for the application of enzyme. Inulinase produced by *A. tubingensis* CR16 had temperature optima of 60 °C. Gill, Manhas, and Singh (2006) also showed 60 °C as an optimum temperature for inulinase enzyme obtained from *A. fumigatus*. Optimum pH for inulinase enzyme action was 5.0 which was in agreement with the general range of many fungal inulinases reported so far (Pandey et al., 1999a). Overall, these properties i.e. low pH and high temperature optima offer advantages for industrial fructose syrup production, as it will prevent microbial contamination (Vandamme & Derycke, 1983). Higher thermostability of industrially important enzymes brings down the cost of production (Saber & El-Naggar, 2009). Industrial process for the production of fructose from inulin is carried out at 60 °C, which is necessary to obtain high hydrolysis rate. Most of the reported inulinases lose their activity after few hours at this temperature. Therefore there is a growing interest to produce thermostable inulinases (Gill et al., 2006). Inulinase produced by *A. tubingensis* CR16 was found to be highly stable at 60 °C retaining most of its activity even after 4.5 h (Fig. 3). Thermostability of inulinase was much better as compared to *A. tamarii* AR-IN9 inulinase which was inactivated up to 89% after 3 h at 45 °C (Saber & El-Naggar, 2009). Singh and Gill (2006) has reported that inulinase produced by *A. ficuum* and *Aspergillus versicolor* was inactivated up to 74% after 6 h and 28% at 60 °C respectively.

Inulinases belong to the group of fructanohydrolases and can be classified as endoinulinase (2,1-β-D-fructan fructanohydrolase) which hydrolyze internal β-2,1-fructofuranosidic linkages to yield inulotriose, -tetraose and pentaose as the main products. In contrast, exoinulinase (β-D-fructan fructohydrolase) splits terminal fructose units. Analysis of the products of inulin hydrolysis by TLC showed the presence of fructose (Fig. 4). Release of fructose as an end product of inulin hydrolysis states that inulinase was an exoacting enzyme. Fructose is an important ingredient in food and pharmaceutical industry (Gill et al., 2006). Fructose is considered as a safe alternative to sucrose because it has beneficial effects in diabetic patients, increases iron absorption in children, high solubility, low viscosity, higher sweetening capacity and thus can be used as a low calorie sweetener (Pandey et al., 1999a).

5. Conclusion

The present work demonstrates the production of thermostable inulinase by a newly isolated fungal strain *A. tubingensis* CR16 on low cost substrates namely wheat bran and CSL under SSF. The applicability of statistical approach proved useful for the optimization of culture conditions for inulinase production. The maximum yield of inulinase achieved was 257 ± 11.4 U/g. Enzyme yield based on sucrose hydrolytic activity was 1358.6 ± 0.8 U/g which was the highest yield reported so far. Characteristics of crude inulinase such as its action at high temperature and low pH, thermostability and fructose producing capacity make it an attractive source for industrial application. However, further studies on its purification may provide other unexplored information regarding this enzyme.

References

Bender, J. P., Mazutti, M. A., de Oliveira, D., Luccio, M. D., & Treichel, H. (2006). Inulinase production by *Kluyveromyces marxianus* NRRL Y-7571 using solid state fermentation. *Applied Biochemistry and Biotechnology*, *06*, 129–132.

Bernardo, O., Silva-Santisteban, Y., Converti, A., & Maugeri Filho, F. (2009). Effects of carbon and nitrogen sources and oxygenation on the production of inulinase by *Kluyveromyces marxianus*. *Applied Biochemistry and Biotechnology*, *152*, 249–261.

Chen, H. Q., Chen, X. M., Chen, T. X., Xu, X. M., & Jin, Z. Y. (2011). Optimization of solid-state medium for the production of inulinase by *Aspergillus ficuum* JNSP 5-06 using response surface methodology. *Carbohydrate Polymers*, *86*, 249–254.

Cruz, V. D., Belote, J. G., Belline, M. Z., & Cruz, R. (1998). Production and action pattern of inulinase from *Aspergillus niger*-245: Hydrolysis of inulin from several sources. *Revista de Microbiologia*, *24*, 301–306.

Ettalibi, M., & Baratti, J. C. (1987). Purification, properties and comparison of invertase, exoinulinases and endoinulinases of *Aspergillus ficuum*. *Applied Microbiology and Biotechnology*, *26*, 13–20.

Ferreira, S. L. C., Bruns, R. E., Ferreira, H. S., Matos, G. D., David, J. M., Brandao, G. C., et al. (2007). Box-Behnken design: An alternative for the optimization of analytical methods. *Analytica Chimica Acta*, *597*, 179–186.

Gill, P. K., Manhas, R. K., & Singh, P. (2006). Comparative analysis of thermostability of extracellular inulinase activity from *Aspergillus fumigates* with commercially available (Novozyme) inulinase. *Bioresource Technology*, *97*, 355–358.

Gill, P. K., Sharma, A. D., Harchand, R. K., & Singh, P. (2003). Effect of media supplements and culture conditions on inulinase production by an actinomycete strain. *Bioresource Technology*, *87*, 359–362.

Kango, N. (2008). Production of inulinase using tap roots of dandelion (*Taraxacum officinale*) by *Aspergillus niger*. *Journal of Food Engineering*, *85*, 473–478.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *The Journal of Biological Chemistry*, *31*, 426–428.

Mazutti, M., Bender, J. P., Treichel, H., & Luccio, M. D. (2006). Optimization of inulinase production by solid-state fermentation using sugarcane bagasse as substrate. *Enzyme and Microbial Technology*, *39*, 56–59.

Mazutti, M., Ceni, G., Luccio, M. D., & Treichel, H. (2007). Production of inulinase by solid state fermentation: Effect of process parameters on production and preliminary characterization of enzyme preparations. *Bioprocess and Biosystems Engineering*, *30*, 297–304.

Miller, G. L. (1939). Use of dinitrisalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, *31*, 426–428.

Pandey, A. (2003). Solid state fermentation. *Biochemical Engineering Journal*, *13*, 81–84.

Pandey, A., & Selvakumar, P. (1999b). Solid state fermentation for the synthesis of inulinase from *Staphylococcus* sp. and *Kluyveromyces marxianus*. *Process Biochemistry*, *34*, 851–855.

Pandey, A., Soccol, C. R., Selvakumar, P., Soccol, V. T., Krieger, N., & Fontana, J. D. (1999a). Recent developments in microbial inulinases—Its production, properties and industrial applications. *Applied Biochemistry and Biotechnology*, *81*, 35–52.

Park, J. P., & Yun, J. W. (2001). Utilization of chicory roots for microbial endoinulinase production. *Letters in Applied Microbiology*, *33*, 183–187.

Reddy, R. M. P., Ramesh, B., Mrudula, S., Reddy, G., & Seenayya, G. (2003). Production of thermostable β-amylase by *Clostridium thermosulfurogenes* SV2 in solid-state fermentation: Optimization of nutrient levels using response surface methodology. *Process Biochemistry*, *39*, 267–277.

Saber, W. I. A., & El-Naggar, N. E. (2009). Optimization of fermentation conditions for the biosynthesis of inulinase by the new source: *Aspergillus tamarii* and hydrolysis of some inulin containing agro-wastes. *Biotechnology*, *8*, 425–433.

Sguarezi, C., Longo, C., Ceni, G., Boni, G., Silva, M. F., Luccio, M. D., et al. (2009). Inulinase production by agro-industrial residues: Optimization of pretreatment of substrates and production medium. *Food and Bioprocess Technology*, *2*, 409–414.

Sheng, J., Chi, Z., Yan, K., Wang, X., Gong, F., & Li, J. (2008). Use of response surface methodology for optimizing process parameters for high inulinase production by the marine yeast *Cryptococcus aureus* G7a in solid-state fermentation and hydrolysis of inulin. *Bioprocess and Biosystems Engineering*, *32*, 333–339.

Singh, P., & Gill, P. K. (2006). Production of inulinases: Recent advances. *Food Technology and Biotechnology*, *44*, 151–162.

Singh, R. S., & Bhermi, H. K. (2008). Production of extracellular exoinulinase from *Kluyveromyces marxianus* YS-1 using root tubers of *Asparagus officinalis*. *Bioresource Technology*, *99*, 7418–7423.

Singhania, R. R., Patel, A. K., Soccol, C. R., & Pandey, A. (2009). Recent advances in solid-state fermentation. *Biochemical Engineering Journal*, *44*, 13–18.

Susca, A., Stea, G., Mule, G., & Perrone, G. (2007). Polymerase chain reaction (PCR) identification of *Aspergillus niger* and *Aspergillus tubingensis* based on the calmodulin gene. *Food Additives and Contaminants*, *24*, 1154–1160.

Treichel, H., Mazutti, M. A., Filho, F. M., & Rodrigues, M. I. (2009). Technical viability of the production, partial purification and characterisation of inulinase using pre-treated agro industrial residues. *Bioprocess and Biosystems Engineering*, *32*, 425–433.

Van, L. J., Coussement, P., Leenheer, L. D., Hoebregs, H., & Smits, G. (1995). On the presence of inulin and OF as natural ingredients in the western diet. *Critical Reviews in Food Science and Nutrition*, http://dx.doi.org/10.1080/10408399509527714

Vandamme, E. J., & Derycke, D. G. (1983). Microbial inulinases fermentation process, properties and application. *Advances in Applied Microbiology*, *29*, 139–176.

Xiong, C., Jinhua, W., & Dongsheng, L. (2007). Optimization of solid-state medium for the production of inulinase by *Kluyveromyces* S120 using response surface methodology. *Biochemical Engineering Journal*, *34*, 179–184.