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ORIGINAL PAPER

Enhanced inulinase production in solid state fermentation by a mutant of the marine yeast *Pichia guilliermondii* using surface response methodology and inulin hydrolysis

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Abstract In order to isolate inulinase overproducers of the marine yeast *Pichia guilliermondii*, strain 1, cells were mutated by using UV light and $LiCl_2$. One mutant (M-30) with enhanced inulinase production was obtained. Response surface methodology (RSM) was used to optimize the medium compositions and cultivation conditions for inulinase production by the mutant in solid-state fermentation. The initial moisture, inoculum, the amount ratio of wheat bran to rice bran, temperature, pH for the maximum inulinase production by the mutant M-30 were found to be 60.5%, 2.5%, 0.42, 30°C and 6.50, respectively. Under the optimized conditions, 455.9 U/grams of dry substrate (gds) of inulinase activity was reached in the solid state fermentation culture of the mutant M-30 whereas the predicted maximum inulinase activity of 459.2 U/gds was derived from RSM regression. Under the same conditions, its parent strain only produced 291.0 U/gds of inulinase activity. This is the highest inulinase activity produced by the yeast strains reported so far.

Keywords *P. guilliermondii* · Inulinase · Mutagenesis · Fermentation · Fructose · Response surface methodology

Introduction

Inulin is a natural storage carbohydrate mainly found in plants, such as Jerusalem artichoke, dahlia tubers or chicory root [18]. It is not a simple molecule, but a mixture of oligo- and/or polysaccharides composed of fructose unit chains (linked by β-(2→1)-D-fructosyl-fructose bonds) of various length, terminated generally by single glucose unit (linked by an β-D-glucopyranosoyl bond).

Inulinase (β-2,1-D-fructan fructanohydrolase (EC 3.2.1.7) targets the β-2,1 linkage of inulin and hydrolyzes it into fructose [9, 10]. Fructose is a GRAS (generally regarded as safe) sweetener, sweeter than sucrose (up to 1.5 times), with lower cost, and has functional properties that enhance flavor, color, and product stability, and is thus widely used in many foods and beverages instead of sucrose. Furthermore, fructose metabolism bypasses the known metabolic pathway of glucose and therefore does not require insulin [6]. Fructose also can be fermented into fuel ethanol [1, 14].

Fructose can be obtained by acid hydrolysis of inulin, but fructose is easily degraded at low pH and the process gives rise to coloring of the inulin hydrolysate and by-product formation in the form of difructose anhydrides [1, 4]. The complete hydrolysis of inulin using inulinase can yield 95% pure fructose. Therefore, inulin and inulinase can be used for production of either ultra-high fructose syrups, with D-fructose content over 95% by exo-enzymatic hydrolysis, or for production of ethanol by fermentation [3–5]. At present, it is very important to obtain the inulinase with high activity and the high inulinase producing microorganisms for efficient hydrolysis of inulin.

Solid state fermentation (SSF) offers numerous advantages for the production of bulk chemicals and enzymes due to low cost substrates, simplified downstream and environmental-friendly process, reduced energy requirement, reduced wastewater produced, high yield of fermentation products, high volumetric productivity, increased product recovery and simplicity of bioreactor design compared to submerged fermentation (SmF) [13]. In recent years, many researchers have studied inulinase production by SSF. Under the optimized conditions,

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inulinase activity of 391.9 U/g of dry fermented bagasse from *Kluyveromyces marxianus* NRRL Y-7571 was produced by SSF [11]. Selvakumar and Pandey [17] reported that the extracellular-inulinase concentration reached a peak (122.88 U/g of dry fermented substrate) with *K. marxianus* by SSF under the optimized conditions. After the optimization of solid state medium for production of inulinase by *Kluyveromyces* sp. S120 using surface response methodology, the average inulinase activity (409.8 U/g of initial dry substrate) was obtained [2].

In another study [5], we found that marine yeast strain 1, isolated from the surface of a marine alga and identified as a strain of *Pichia guilliermondii* could produce over 60 U/ml of inulinase under the optimal conditions. In this study, one mutant (M-30) with enhanced inulinase production was obtained from the marine yeast *P. guilliermondii* strain 1 using UV radiation and $LiCl_2$ treatment. Under the optimized conditions, 455.9 U/grams of dry substrate (gds) of inulinase activity was reached in the solid state fermentation culture of the mutant M-30. This is the highest inulinase activity produced by the yeast strains reported so far.

Materials and methods

Yeast strain and media

The yeast strain *P. guilliermondii* strain 1 was isolated from the surface of a marine alga collected at 100 m depth of seawater at Bohai Sea, China [5]. This yeast strain was maintained in YPD medium (prepared with seawater) containing 2.0% glucose, 1.0% yeast extract, and 2.0% polypeptone at 4°C. The medium (prepared with seawater) for mutagenesis contained 2.0% glucose, 1.0% yeast extract, 0.5% $LiCl_2$, and 2.0% polypeptone. The liquid medium (prepared with seawater) for inulinase production contained 4.0% inulin, 0.5% yeast extract and initial pH 8. Yeast extract, polypeptone and inulin were all obtained from Sigma Chemical Co., St. Louis, MO, USA.

Formation of ascospores

Ascospore formation of *P. guilliremondii* strain 1 was carried out on 5.0% malt extract (Sigma Chemical Co., St. Louis, MO, USA) agar at 25°C for 5–15 days as described by Kurtzman and Fell [7].

Mutagenesis

The ascospores released from asci of *P. guilliremondii* strain 1 were suspended in sterile seawater and the ascospore concentration of the suspension was adjusted to 10^7 cells/ml. The ascospore suspension was diluted with sterile seawater to give 100 ascospores per ml and spread on plates containing 20 ml of the mutagenesis medium (plus 2.0% agar). A 15 W UV-lamp was used as the source of radiation. Ultraviolet mutagenesis was applied with exposure time of 5, 10, 15, 20, 25 and 30 s at a fixed distance of 25 cm. The mutated cells were fixed by further cultivating the treated cells in the medium for mutagenesis overnight in the dark. Approximately 0.1 ml of the UV and $LiCl_2$-treated cell suspensions was transferred to the YPD plates. Each colony that appeared within 72 h of incubation at 28°C was transferred to YPD slants, respectively. Potential mutant strains were cultured in 50 ml of the inulinase production medium (prepared with seawater) by shaking at 170 rpm and 28.0°C for 2 days. The culture was centrifuged at 4,000×*g* and 4°C for 5 min and the supernatant obtained was used as the crude inulinase preparation. Inulinase activity in the crude inulinase preparation was determined as described below. The mutants with enhanced inulinase production were selected for further studies. Finally, the mutant M-30 with 115.0 U/ml of inulinase activity in the liquid culture was obtained.

Determination of inulinase activity

The reaction mixture containing 0.1 ml of the crude inulinase preparation obtained above and 0.9 ml of phosphate buffer (0.1 M, pH 6.0) containing 2.0% inulin was incubated at 60°C for 10 min. The reaction was inactivated immediately by keeping the reaction mixture at 100°C for 10 min. The amount of reducing sugar released in the reaction mixture was assayed by the method of Nelson–Somogyi [19]. One inulinase unit (U) was defined as the amount of enzyme that produces 1 μmol of reducing sugar per min under the assay conditions used in this study.

DNA extraction, PCR and sequencing

The total genomic DNA of the wild type yeast strain and its mutant M-30 was isolated and purified using the methods as described by Sambrook et al. [16]. The specific primers for amplification of the inulinase gene (accession number: EU195799) in the yeasts were used, the forward primer: PP: 5′-ATGAGAGCTTTTCTTGCCTTAATT-3′ and the reverse primer PR: 5′-CTATGAAGTTGCCCTCAATTT TAA-3′. The reaction system (25 μl) was composed of 10 × buffer 2.5 μl, dNTP 0.8 μM, $MgCl_2$ 1.5 mM, PP or PR 0.5 μM, Taq DNA polymerase 1.25 U, template DNA 1.0 μl and H_2O 16.6 μl. The conditions for PCR amplification were as follows: initial denaturation at 94°C for 10 min, denaturation at 94°C for 1 min, annealing temperature at 53°C for 1.0 min, extension at 72°C for 2.0 min, final extension at 72°C for 10 min. PCR was run for 32 cycles and PCR cycler was GeneAmp PCR System

2400 made by Perkin–Elmer. PCR products were separated by agarose gel electrophoresis and recovered by using UNIQ-column DNA gel recovery kits (BIOASIA, Shanghai). The recovered PCR products were ligated into pGEM-T easy vector and transformed into the competent cells of *Escherichia coli* JM109. The transformants were selected on plates with ampicillin. The plasmids in the transformant cells were extracted by using the methods as described by Sambrook et al. [16]. In order to confirm that the PCR products had been ligated into the vector, the purified plasmids were used as templates for amplification of the inulinase gene in the yeast strains, respectively. The reaction system and the conditions for PCR amplification were the same as described above. The PCR products inserted on the vector were sequenced by Shanghai Sangon Company. Three clones from the mutant M-30 and its wild type were sequenced, respectively.

Effects of added glucose on inulinase production

The mutant M-30 and its wild type were aerobically grown in the production medium containing different glucose concentrations (0, 1.0, 2.0, 3.0 and 4.0 g per 100 ml) for 48 h, respectively. Inulinase activity in the supernatant of the cultures was determined as described above. At the same time, OD value of the cultures was measured at 600 nm using spectrophotometer. The specific inulinase activity was defined as units of inulinase per OD_{600nm}. The inulinase activity of the cultures in the absence of added glucose was defined as 100%.

Screening of physical and chemical parameters using Central Composite Designs

The Central Composite Design for five variables which includes physical and chemical parameters at five levels (+2.38, +1, 0, −1, −2.38) (Tables 1, 2) [15] was used for screening. Among the physical and chemical parameters, moisture, the amount ratio of wheat bran and rice husk, temperature, inoculation size and pH were tested for their significance in inulinase production by the mutant M-30.

Solid state fermentation

Wheat bran and rice husk were used as the solid substrates. The substrates were supplemented with the liquid production medium containing 4.0 g inulin and 0.5 g yeast extract per 100 g dry substrate. Initial pH and moisture of the substrates were set at different levels according to Tables 1 and 2, respectively. All substrates were autoclaved at 15 psi for 20 min. Preliminary studies showed that no changes in moisture content of the substrates after autoclaving were detected. One loop of the cells of the yeast strain was transferred to 50.0 ml of YPD medium in 250 ml flask and aerobically cultivated for 24 h. The cell culture ($OD_{600nm} = 20.0$) was transferred to the solid state medium. Each flask was inoculated with different volume of the cell suspension ($OD_{600nm} = 20.0$) and incubated at different temperatures according to Tables 1 and 2.

A 5^5 factorial design was performed to assess the effect of moisture, the amount ratio of wheat bran to rice husk, temperature, inoculation size and pH on the inulinase production by the mutant M-30. A central point was carried out in triplicate plus two axial points for each independent factor for experimental error evaluation and second-order effects estimation, respectively. Table 1 shows the range of the studied factors and the correspondent coded levels.

Extraction of enzyme

A weighed quantity of the fermented matter was treated with acetate buffer (0.1 M, pH 5.0) and mixed thoroughly on a magnetic stirrer for 30 min at room temperature. The whole contents were centrifuged at 4,000×*g* and 4°C for 5 min and the supernatant obtained was taken as the crude inulinase preparation of the solid state fermentation. The inulinase activity in the crude inulinase preparation was determined as described above.

Statistical analysis of the data

The statistically planned experimentation is to identify the significant variables and their corresponding coefficients, so that the levels of variables can be managed to obtain a

Table 1 Range of the factors investigated in the experimental design for the inulinase production by the mutant M-30

Variables	Code	Levels				
		−2.38	−1	0	+1	+2.38
Moisture	*A*	56.93%	59%	60.5%	62%	64.07%
Inoculum	*B*	1%	1.31%	2%	2.5%	3.69%
The amount ratio of wheat bran to rice husk	*C*	0.21	0.33	0.42	0.5	0.62
Temperature	*D*	25.24	28	30	32	34.76
pH	*E*	5.31	6	6.5	7	7.69

Table 2 Experiments designs used in RSM by using five independent variables each at five levels showing observed values of inulinase production by the mutant M-30

Run	*A*	*B*	*C*	*D*	*E*	Inulinase activity (U/gds)
1	1	−1	1	1	−1	431.5 ± 1.1
2	1	1	1	−1	−1	459.1 ± 2.1
3	1	1	−1	−1	−1	420.4 ± 1.6
4	−1	−1	−1	−1	−1	459.2 ± 1.9
5	−1	−1	−1	1	1	407.1 ± 1.5
6	−1	1	1	−1	−1	431.5 ± 2.2
7	1	−1	1	−1	−1	395.4 ± 1.8
8	1	1	1	1	−1	430.2 ± 3.0
9	0	0	0	0	2.38	398.2 ± 1.4
10	0	0	0	2.38	0	422.6 ± 3.1
11	−2.38	0	0	0	0	390.4 ± 2.6
12	−1	1	1	−1	1	418.3 ± 2.3
13	0	0	−2.38	0	0	379.6 ± 3.3
14	0	0	0	0	0	404.2 ± 0.9
15	0	0	0	0	0	366.5 ± 0.7
16	1	−1	−1	−1	1	403.2 ± 2.7
17	−1	1	−1	1	−1	400.5 ± 3.4
18	−1	−1	1	−1	1	445.7 ± 3.3
19	0	0	0	0	0	399.6 ± 1.1
20	0	−2.38	0	0	0	425.2 ± 1.3
21	1	−1	−1	−1	−1	382.6 ± 1.5
22	−1	1	1	1	−1	409.5 ± 1.8
23	1	−1	1	−1	1	381.7 ± 1.1
24	1	−1	−1	1	1	408.7 ± 2.0
25	1	−1	1	1	1	371.5 ± 2.1
26	0	0	0	0	0	389.1 ± 3.0
27	−1	1	1	1	1	380.3 ± 2.7
28	0	0	2.38	0	0	371.9 ± 3.5
29	−1	1	−1	1	1	353.1 ± 2.1
30	−1	1	−1	−1	−1	371.4 ± 2.7
31	1	1	−1	−1	1	351.6 ± 2.8
32	−1	−1	−1	−1	1	349.3 ± 3.2
33	−1	−1	1	−1	−1	400.1 ± 3.3
34	1	1	1	−1	1	429.3 ± 2.3
35	−1	−1	1	1	−1	380.9 ± 2.3
36	1	1	−1	1	1	426.1 ± 2.5
37	−1	−1	1	1	1	391.4 ± 3.4
38	−1	1	−1	−1	1	407.5 ± 0.9
39	0	0	0	0	0	379.6 ± 1.4
40	−1	−1	−1	1	−1	381.9 ± 1.8
41	2.38	0	0	0	0	361.3 ± 1.3
42	0	0	0	0	−2.38	446.2 ± 3.0
43	0	0	0	0	0	446.2 ± 2.0
44	1	−1	−1	1	−1	446.2 ± 3.3
45	1	1	−1	1	−1	446.2 ± 2.6
46	0	0	0	0	0	446.2 ± 1.1
47	1	1	1	1	1	446.2 ± 1.5
48	0	2.38	0	0	0	446.2 ± 1.7
49	0	0	0	0	0	446.2 ± 3.0
50	0	0	0	−2.38	0	446.2 ± 2.0

Data are given as mean ± SD, $n = 3$

gds grams of initial dry substrate

desired output. Hence, the coefficients, sum of squares in percentage (SS%) and coefficient of variation (CV) were analyzed using the experimental results of the inulinase activity produced by the mutant M-30. Design-Expert® (Stat-Ease, Inc., Minneapolis, MN, USA. Version 7.0.0, 2005), was used to design the experimental plan and conduct the analysis of the results.

Inulin hydrolysis

Inulin hydrolysis was carried out by incubating the reaction mixture containing 2.0% inulin in 0.1 M phosphate buffer (pH 6.0) and 20 µl of 360 U/ml of the crude inulinase preparation of solid state fermentation at 60°C for 12 h. The end products of inulin hydrolysis after 12 h of the incubation at 60°C were sampled and identified to ascertain the extent of hydrolysis by ascending thin layer chromatography (Silica gel 60, MERCK, Germany) with the solvent system of *n*-butanol–pyridine–water (6:4:3) and a detection reagent comprising 2.0% diphenylamine in acetone-2.0% aniline in acetone-85% phosphoric acid (5: 5: 1 by volume) [10]. Glucose was used as a standard since it has the same Rf as fructose.

Results and discussion

Mutagenesis and screening for inulinase hyperproduction

In the screening experiment described in "Materials and methods", the cultures of the parent *P. guilliermondii* strain 1 and its mutant derivatives were grown in the liquid production medium for two days. Inulinase activity of the cultures was determined as described in "Materials and methods". About 1,000 potential mutant derivatives were screened in this way. We found that only the mutants M-31 and M-30 were improved with respect to inulinase production while other mutants showed decreased inulinase production compared to its wild type (data not shown). Among those, maximum inulinase activity (115.0 U/ml)

was achieved by the mutant M-30 with approximate 2.3-fold increase in inulinase activity when compared to that of the wild type while the mutant M-31 produced 52.6 U/ml of inulinase activity. The mutant M-30 was obtained when the lethal death rate was over 90% and treatment time was 20 s (data not shown). Therefore, the mutant M-30 was used in the subsequent studies.

Although inulinases from different microorganisms have been confirmed to have high potential for applications in different biotechnological fields, they have not been applied commercially so far. The main reason for this is that the inulinase activity available is too low. Therefore, it is very important to find microbial strains that produce very high inulinase activity during fermentation. In this study, the inulinase activity (115.0 U/ml) in liquid production medium by the mutant M-30 was the highest reported so far. the Inulinase activity reported in the literature by any other yeasts is less than 85.0 U/ml [1, 5, 18, 21, 22]. Therefore, the inulinase produced by the mutant M-30 of the marine yeast strain has potentially for use in biotechnological fields.

In order to know if the enhanced inulinase production in the mutant M-30 is related to the mutated inulinase gene, the PCR products encoding the inulinase from the parental cultures of *P. guilliermondii* strain 1 and its mutant M-30 were sequenced. The results in Fig. 1 demonstrate that the sequence of the inulinase gene (accession number: EU195799) from the parental culture of *P. guilliermondii* strain 1 was completely the same as that (accession number: EU195800) of the inulinase gene from the mutant M-30, indicating that the inulinase gene in the mutant M-30 was not mutated.

It has been reported that fructose or glucose available in the medium represses inulinase biosynthesis [20]. Therefore, effects of different concentrations of added glucose in the liquid production medium on the inulinase production by the mutant M-30 and its parent strain were examined. The results in Fig. 2 indicate that glucose repression on the inulinase production by the mutant M-30 was relieved in some degree compared to that in its parent strain when the added glucose concentrations in the media were higher than 2.0%. This may imply that the regulation system for

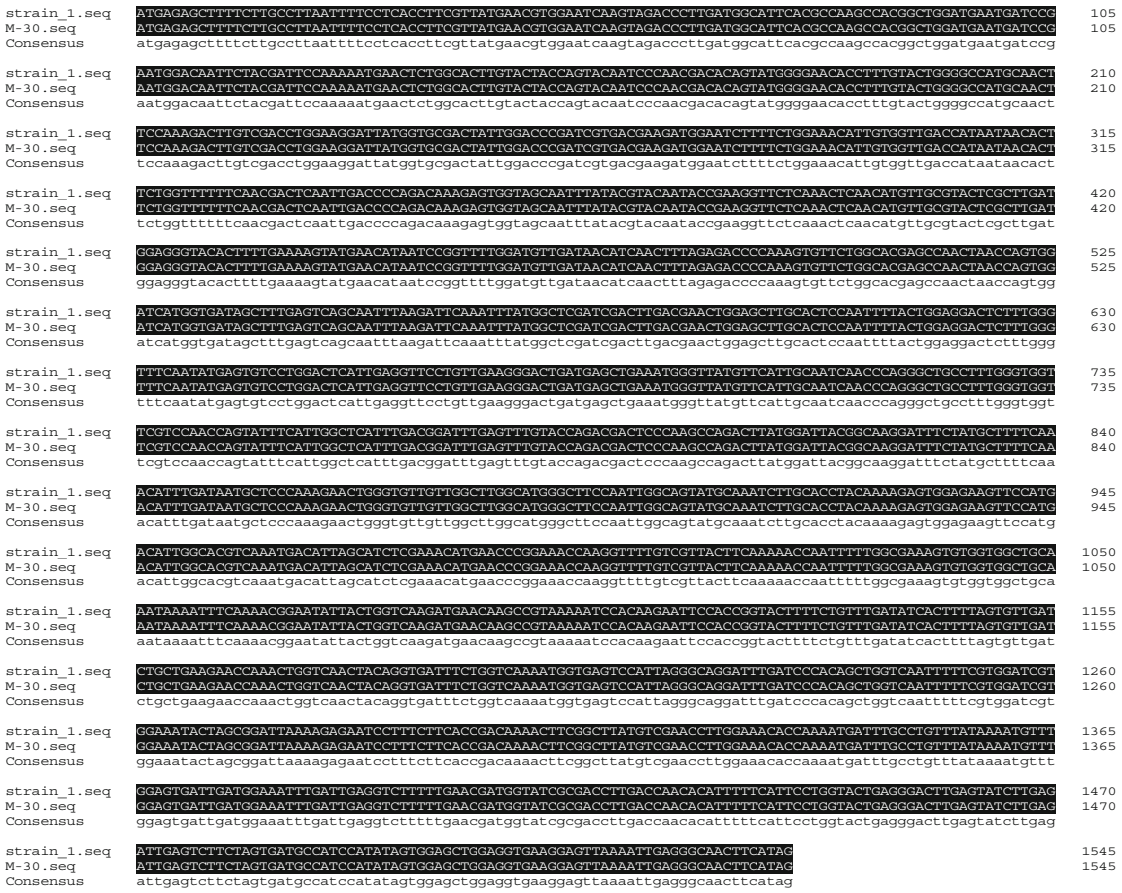

Fig. 1 Multiple alignment of the inulinase gene (EU195799) from the wild type *P. guilliermondii* strain 1 (strain_1) with the inulinase gene (EU195800) from the mutant M-30 (M-30). Multiple sequence alignment of DNAs was carried out using the DNAMAN 6.0

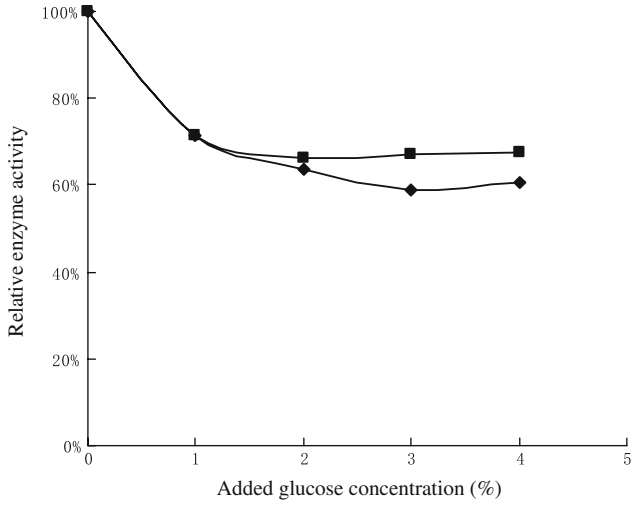

Fig. 2 Effects of added glucose on inulinase production by the mutant M-30 (*filled square*) and its parent strain (*filled diamond*). Specific inulinase activity (U/ml/OD_{600nm}) was obtained in the culture containing different glucose concentrations. The specific inulinase activity (U/ml/OD_{600nm}) in the culture without added glucose was regarded as 100%. Data are given as mean ± SD, $n = 3$

biosynthesis of the inulinase in the mutant M-30 was changed. However, the exact mechanisms of the enhanced inulinase production by the mutant M-30 will be further elucidated. A mutant of *Kluyveromyces* sp. Y-85 resistant to catabolite repression was also found to produce more inulinase (68.9 U/ml) compared to its parent strain [20].

Optimization for inulinase production in solid state fermentation using surface response methodology

As the SSF offers numerous advantages for the production of bulk chemicals and enzymes, the technique was used to produce inulinase by the mutant M-30. Response surface methodology (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 [15]. In RSM, the number of experimental runs required is very few, leading to saving of time, chemicals, glassware and manpower. Experimental design and data analysis using appropriate software make the analysis easier. Many results [13] have shown that temperature, initial moisture content of substrate, inoculum size, the amount and type of solids used and pH were identified as most influential among physical parameters for production of enzyme. Therefore, a Central Composite Designs were employed to analyze the interactive effect of these parameters and to arrive at an optimum for the inulinase production by the mutant M-30. The base points for the design were selected from a single-parameter study (data not shown). A summary of the variables and their variation levels are given in Table 1.

Table 3 Analysis of Variance(ANOVA) for regression

Source	Sum of squares	*df*	Mean squre	*F*-value	Prob > *F*
Model	42980.72	20	2149.04	19.45	<0.0001
Residual	3204.00	29	110.48		
Lack of fit	3204.00	22	145.64		
Pure error	0	7	0		
Core total	46184.72	49			

SSF was carried out according to the design (Table 1) for 120 h. The fermented samples were extracted and assayed for inulinase activity as described in "Materials and methods". The results were analyzed on a PC running under Windows OS, using the Design-Expert® 7.0.0 statistical software and the response surface generated using STATISTICA (StatSoft Inc., Tulsa, USA). The design and results (inulinase activity) of the experiments carried out with the Central Composite Designs are given in Table 2.

The analysis of variance (ANOVA) was employed (Table 3) for the determination of the significant parameters. ANOVA consists of classifying and cross classifying statistical results and testing whether the means of a specified classification differ significantly. This was carried by Fisher's statistical test for square due to regression to the mean square due to error and indicated the influence (significance) of each controlled factor on the tested model. The results obtained were submitted to ANOVA on SAS package and the regression model was given as Eq. 1:

$$\begin{aligned} Y = & -17443.75924 + 367.09634 \times A + 313.2327 \\ & \times B + 557.47425 \times C + 213.40123 \times D + 946.29092 \\ & \times E - 1.20417 \times A \times B - 2.03431 \times A \times C - 1.16146 \\ & \times A \times D - 3.72083 \times A \times E + 0.36765 \times B \times C \\ & - 0.34688 \times B \times D - 0.61250 \times B \times E \\ & + 5.71691 \times C \times D + 4.48529 \times C \times E \\ & - 1.99688 \times D \times E - 2.45582 \times \mathrm{A2} - 46.21476 \\ & \times \mathrm{B2} - 910.37031 \times \mathrm{C2} - 2.31611 \times \mathrm{D2} \\ & - 52.57872 \times \mathrm{E2} \end{aligned} \quad (1)$$

where Y was inulinase yield, A was moisture, B was inoculum size, C was the amount ratio of wheat bran to rice bran, D was temperature, E was pH. The ANOVA of the quadratic regression model demonstrates that Eq. (1) was a highly significant model, as was evident from the Fisher's F-test with a very low probability value (F-value = 19.45) (Table 3). Values of "Prob > F" less than 0.0001 (Table 3) indicate that model terms were significant. The Model F-value of 19.45 implies that the model was significant. There was only a 0.01% chance that a "Model F-Value" could occur due to noise. Values of "Prob > F" less than 0.05 indicate that model terms were significant. The goodness of fit of the model was checked by

Table 4 Test of significance for regression coefficient

Model terms	Coefficient estimate	*df*	Standard error	95% CI low	95% CI high	*P* value
Intercept	446.25	1	3.69	438.70	453.79	–
A	10.59	1	1.60	7.32	13.85	<0.0001
B	−2.46	1	1.60	−5.73	0.80	0.1337
C	−10.71	1	1.60	−13.43	−6.90	<0.0001
D	−14.62	1	1.60	−17.88	−11.35	<0.0001
E	−10.96	1	1.60	−14.23	−7.69	<0.0001
AB	−0.90	1	1.86	−4.70	2.90	0.63
AC	−0.26	1	1.86	−4.06	3.54	0.89
AD	−3.48	1	1.86	−7.28	0.32	0.07
AE	−2.79	1	1.86	−6.59	1.01	0.14
BC	0.016	1	1.86	−3.78	3.82	0.99
BD	−0.35	1	1.86	−4.15	3.45	0.85
BE	−0.15	1	1.86	−3.95	3.65	0.93
CD	0.97	1	1.86	−2.83	4.77	0.60
CE	0.19	1	1.86	−3.61	3.99	0.92
DE	−2.00	1	1.86	−5.80	1.80	0.29
A^2	−5.53	1	1.41	−8.41	−2.64	0.0005
B^2	−11.55	1	1.41	−14.44	−8.67	<0.0001
C^2	−6.58	1	1.41	−9.46	−3.69	<0.0001
D^2	−9.26	1	1.41	−12.51	−6.38	<0.0001
E^2	−13.14	1	1.41	−16.03	−10.26	<0.0001

determination coefficient (R^2) (data not shown). In this case, the value of the determination coefficient ($R^2 = 0.93$) indicates that only 6.94% of the total variations was not explained by the model. The value of the adjusted determination coefficient (Adj$R^2 = 0.88$) was also very high to advocate for a high significance of the model. At the same time, a relatively lower value of the coefficient of variation (CV = 2.59%) indicates a better precision and reliability of the experiments carried out.

The "Pred R-Squared" of 0.70 is in reasonable agreement with the "Adj R-Squared" of 0.88. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable (Table 3). The ratio of 16.04 indicates an adequate signal (data not shown). Therefore, this model can be used to navigate the design space. Among model terms, moisture (*A*), the amount ratio of wheat bran to rice husk (*C*), and pH (*E*) were very significant with a probability of over 99.9%; temperature (*D*) was also significant with a probability of over 99.9% (Table 4) while inoculum size (*B*) had no obvious effects on the inulinase production by the mutant M-30. Table 4 also indicate that the interaction between *A* and *A*, *B* and *B*, *D* and *D* and *E* and *E*, had very significant influence on the inulinase yield by the mutant M-30. However, the interaction between *A* and *B*, *A* and *C*, *A* and *D*, *A* and *E*, *B* and *C*, *B* and *D*, *B* and *E*, *C* and *D*, *C* and *E*, *D* and *E*, *A* and *A* had no clear influence on the inulinase yield by the mutant M-30. Furthermore, the interaction between *C* and *C* and *B* and *B*, *D* and *D*, *E* and *E* had great influence on the inulinase yield by the mutant M-30 (Table 4).

The fitted response for the above regression model was plotted in Fig. 3. The three-dimensional graph was generated for the pair-wise combination of the five variables, while keeping the other one at their optimum levels for the inulinase production by the mutant M-30. The graph was given here to highlight the roles played by the various variables.

The predicted maximum inulinase activity (459.2 U/gds) derived from RSM regression was obtained when the initial moisture, inoculum, the amount ratio of wheat bran to rice bran, temperature, pH was 60.5%, 2.5%, 0.42,

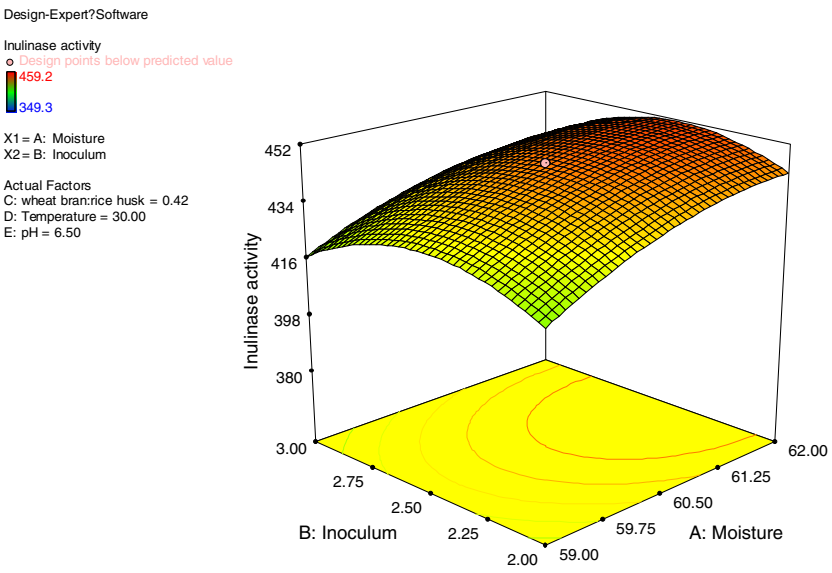

Fig. 3 Response surface for inulinase activity

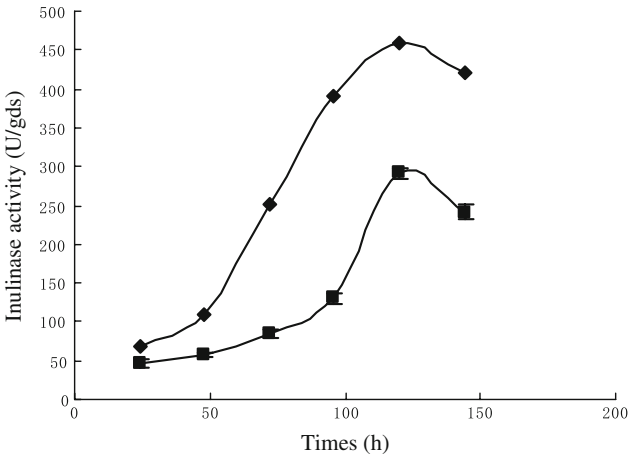

Fig. 4 The time course of inulinase production by the mutant M-30 and its parent strain under the optimal conditions of SSF. Data are given as mean ± SD, $n = 3$

30°C and 6.50, respectively (Fig. 3). The time course of the inulinase production by the mutant M-30 and its parent strain was examined during SSF under the optimal conditions obtained from RSM (Fig. 3). The results in Fig. 4 show that the highest inulinase activity (455.9 U/gds) was reached within 120 h of the solid state fermentation. The results also suggest that the actual inulinase activity (455.9 U/gds) in the optimized medium from three replications was close to the predicted value (459.2 U/gds) and the model was proven again to be adequate (Fig. 3). However, under the same conditions, its wild type only produced 291.0 U/gds of inulinase activity. To our knowledge, this is the highest inulinase activity produced by the yeast strains reported so far. The inulinase activity produced by any other yeasts during the solid-state fermentation is less than 410 U/gds [2, 11, 12].

Hydrolysis products

The hydrolysis products of inulin by the crude inulinase obtained during the solid state fermentation were analyzed by thin layer chromatography (TLC). The results in Fig. 5 show that a large amount of monosaccharides and a trace amount of disaccharides were detected after the hydrolysis. This means that the crude inulinase produced by the mutant M-30 had a high exoinulinase activity. This characteristic may find potential applications in ultra-high fructose syrup production and high ethanol production because a large amount of monosaccharides will be released from inulin during the hydrolysis. In order to determine the exact hydrolysis products of inulin, HLPC will be used in future studies. In our previous study [5], a large amount of monosaccharides and a trace amount of oligosaccharides were also observed after the hydrolysis of inulin by the crude inulinase of the wild type *P. guilliermondii* strain 1.

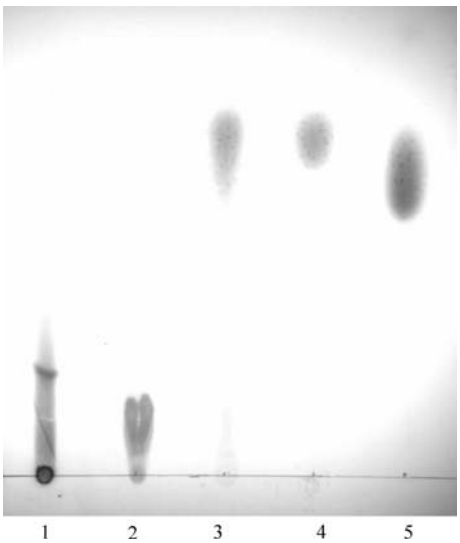

Fig. 5 Thin layer chromatography of hydrolysis products of inulin by the crude inulinase produced by the mutant M-30. *1* Inulin, *2* control (2.0% inulin + 20 µl of the inactivated crude inulinase), *3* hydrolysis products, *4* glucose, *5* maltose

This means that as in the wild type *P. guilliermondii* strain 1, the mutant M-30 also produced exoinulinase. The monosaccharides and oligosaccharides were detected after inulin hydrolysis for more than 2 h by the purified exoinulinase produced by *K. marxianus* var. *bulgaricus* [8].

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