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Research Paper

Enhancing inulinase yield by irradiation mutation associated with optimization of culture conditions

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Abstract

A new inulinase-producing strain was isolated from rhizosphere soils of Jerusalem artichoke collected from Shihezi (Xinjiang, China) using Jerusalem artichoke power (JAP) as sole carbon source. It was identified as an *Aspergillus niger* strain by analysis of 16S rRNA. To improve inulinase production, this fungus was subjected to mutagenesis induced by ^{60}Co γ-irradiation. A genetically stable mutant (designated E12) was obtained and it showed 2.7-fold higher inulinase activity (128 U/mL) than the parental strain in the supernatant of a submerged culture. Sequential methodology was used to optimize the inulinase production of stain E12. A screening trial was first performed using Plackett-Burman design and variables with statistically significant effects on inulinase bio-production were identified. These significant factors were further optimized by central composite design experiments and response surface methodology. Finally, it was found that the maximum inulinase production (185 U/mL) could be achieved under the optimized conditions namely pH 7.0, yeast extract concentration of 5.0 g/L, JAP concentration of 66.5 g/L, peptone concentration of 29.1 g/L, solution volume of 49.4 mL in 250-mL shake flasks, agitation speed of 180 rpm, and fermentation time of 60 h. The yield of inulinase under optimized culture conditions was approximately 1.4-fold of that obtained by using basal culture medium. These findings are of significance for the potential industrial application of the mutant E12.

Key words: inulin, ethanol fermentation, inulinase, central composite design, response surface methodology.

Introduction

Jerusalem artichoke (*Helianthus tuberosus* L.) is a native plant of North America and its tubers contain about 75-85% (dry weight) of total sugars (Wang *et al.*, 2013). As an excellent source of sugar and renewable raw material, Jerusalem artichoke grows well in poor soil and even in sand, and has a high tolerance to frost and various plant diseases. Inulin is the primary storage carbohydrate of Jerusalem artichoke. As a natural carbohydrate, inulin occurs in plants mainly as an energy reserve and as a cryoprotectant with beneficial nutritional and technological properties (Saengthongpinit and Sajjaanantakul, 2005). Inulin has a polymerization degree (DP) 2-60 or higher, consisting of linear β-2, 1-linked polyfructose chains displaying a terminal glucose unit. Recently, the production of ethanol by fermentation of Jerusalem artichoke tubers has become a hot research issue (Yuan *et al.*, 2008; Zhang *et al.*, 2010; Hu *et al.*, 2012; Li *et al.*, 2013).

Recently, inulinases have received much attention as they can be widely applied to hydrolyze inulin for the production of fuel ethanol, fructose, and fructo-oligosaccharides (Gao *et al.*, 2009). Inulinases are a group of hydrolases which target on the β-2,1 linkage of inulin and hydrolyze it into fructose and glucose. Inulinases can be divided into endo-inulinase and exo-inulinase. The endo-inulinase hydrolyzes the internal linkages in inulin to produce inulotriose, inulotetraose, and inulopentaose as the main products. The exo-inulinase hydrolyzes inulin into fructose and glucose, then, the formed fructose and glucose can be further fermented into ethanol by specific microorganisms (Li *et al.*, 2013).

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Inulinases can be secreted by a variety of microbes including fungi, yeast, and bacteria. Among them, *Aspergillus* and *Kluyveromyces* strains are generally preferred choices for commercial applications (Zhang *et al.*, 2004). Recently, many studies have been conducted using inulinase from *Aspergillus* for enzymatic hydrolysis of inulin (Gill *et al.*, 2006; Sirisansaneeyakul *et al.*, 2006). Some efforts have been made to enhance enzyme activity of *Aspergillus* such as transgene expression (Zhang *et al.*, 2004) and coculture with other species (Ge *et al.*, 2009).

Irradiation mutagenesis by means of X-rays, ultraviolet rays, and γ-rays can cause morphological and biochemical alterations in microbial cells. Feng *et al.* (2014) found that nuclear irradiation could remarkably enhance the lipid yields of *Nitzschia* sp. Cheng *et al.* (2014) reported that the biomass and lipid yields of *Nitzschia* sp. were increased by 53.8% and 28.1%, respectively, through ^{60}Co-γ-ray-induced mutation at 900 Gy. However, research on the utilization of nuclear irradiation to enhance the inulinase activity of microbes is very scarce.

Thereupon, the objectives of the present study were (1) to isolate an inulinase-producing strain and re-mutate it by using ^{60}Co-γ radiation; (2) to optimize the conditions for inulinase production by the mutant.

Materials and Methods

Chemicals

Jerusalem artichoke dry powder was purchased from Xi'an Sinuote Biotechnology Co., Ltd., China. The Jerusalem artichoke powder (JAP) was used as carbon source for microbial screening, fermentation and inoculation. The JAP contains (w/w): 2.8% fructose, 3.2% sucrose, 80.7% inulin, 1.4% nitrogen, 4.9% crude fiber, 4.3% ash, and 2.7% water. Yeast extract and peptone, with nitrogen contents of 9.8% and 12.2% (w/w) respectively, were purchased from Aoboxing Biotechnology Co., Ltd. (Beijing, China). Inulin (≥ 98% purity) was obtained from Sigma-Adrich (St Louis, MO, USA). All other chemicals were of analytical grade and commercially available.

Isolation of inulinase-producing fungus strain

The rhizosphere soils of Jerusalem artichoke collecting from Shihezi (Xinjiang, China) were chosen as natural isolation sources of microbes. Enrichment of microorganisms was carried out from 5 g of individual soil samples with 50 mL of selecting medium (JAP 40.0 g/L, Na_2HPO_4 10.0 g/L, NH_4Cl 20.0 g/L, pH 7.0) in a 250-mL flask in a 28 °C incubator (180 rpm) for 4 days. Then an aliquot of culture medium was streaked onto selecting agar medium (selecting medium with 2% agar) and incubated for 3-5 days at 28 °C. After incubation, all the plates were observed and morphologically different colonies (fungus-like) were selected and purified by streaking over the same medium. For purification, each isolate was transferred at least three times. For further screening and identification, the isolates were maintained on Czapek yeast agar (CYA) medium (K_2HPO_4 1.0 g/L, yeast extract 5.0 g/L, sucrose 30.0 g/L, agar 15.0 g/L, Czapek concentrate 10.0 mL/L, distilled water 1000 mL) (Silva *et al.*, 2011). The composition of Czapek concentrate is (g/L): $NaNO_3$ 300.0, KCl 50.0, $FeSO_47H_2O$ 1.0, $ZnSO_47H_2O$ 1.0, $CuSO_45H_2O$ 0.5.

The isolates showing moderate to excellent activity were selected for secondary screening, which was performed by the spore cultivation method. For this reason, the isolates were transferred to potato dextrose agar (PDA) plates (peeled and sliced potato 200.0 g/L, glucose 20.0 g/L, agar 20.0 g/L). Following growth for 3-7 days at 28 °C, the mycelium was collected and mixed with sterile water (containing 0.05% (w/v) Tween 80) to prepare spore suspension (about 10^5 conidia per mL). After this, 1 mL of each conidial suspension was inoculated in a 250-mL shake flask containing 100 mL of fermentation medium (JAP 40.0 g/L, yeast extract 5.0 g/L, peptone 10.0 g/L, pH 7.0). The flasks were incubated in a rotary shaker (180 rpm) at 28 °C for 3-5 days, and then the microorganisms were re-isolated by serial dilution of the culture and plated on the selecting agar medium by adopting standard spread plate method. In this study, all media were autoclaved at 121 °C for 15 min.

Species identification

The preferred isolates were transferred to CYA plates. Each plate was inoculated with three 5-μL drops of freshly prepared conidial suspension, incubated at 28 °C for 7 days and observed for species identification by macroscopic and microscopic morphological characteristics as described by Maren (2002). The isolates were also sent to Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China) for molecular identification. Molecular identification was done by partial sequencing. Genomic DNA was isolated and about 500 bp rDNA fragments were amplified using primers CF1L (5'-GCCGACTCTTTGACYGAR GAR-3') and CF4 (5'-TTTYTGCATCATRAGYTGG AC-3') (Peterson, 2008). Sequencing PCR was done with the BigDye® Terminator v3.1 Cycle Sequencing Kit (ABI, Foster City, CA, USA). Sequence data was aligned with Lasergene sequence analysis software (DNASTAR, Inc., Madison, WI, USA) and homologies to sequences of reference strains were determined by blast search.

Determination of inulinase activity

The isolates were inoculated into 250-mL shake flasks, each containing 50 mL of fermentation medium, and then cultured at 28 °C and 180 rpm for 60 h on a rotary shaker. Afterwards, the fermented liquid broth was centrifuged at 12000 rpm and 4 °C for 10 min, and the supernatant was used as the crude enzyme preparation.

The inulinase activity was assayed by measuring the amount of reducing sugar released from inulin using a mod-

ified 3,5-di nitro salicylic acid (DNS) method (Sheng *et al.*, 2007). In brief, 1.0 mL of suitably diluted crude enzyme solution was mixed with 4.0 mL of acetate buffer (0.1 M, pH 5.0) containing 1.5% (w/v) inulin. After incubation at 55 °C for 10 min in a water bath, each reaction mixture was mixed with 5.0 mL of DNS reagent and then kept in boiling water for 5 min. The same mixtures but with inactivated enzyme crude extracts (heated at 100 °C for 10 min) were used as blank controls. After quick cooling on ice, the reaction mixture was assayed for reducing sugars at 540 nm using a UV-2100 UV/VIS spectrophotometer (UNICO, Shanghai, China). The calibrating curve was drawn for DNS using fructose. One unit of inulinase activity (U) was defined as the amount of enzyme responsible for the production of 1 mmol of reducing sugar per minute under the above conditions. All enzymatic reactions were performed in triplicates.

Mutagenesis

The cells of isolate exhibiting the highest inulinase activity were mutated by nuclear irradiation. For this, the mycelium was harvested and suspended in sterile water. Five mL of the suspension in aliquots was transferred to sterile tubes and irradiated at room temperature using a ^{60}Co γ-ray irradiator, with dosages of 800, 1000 and 1200 Gy respectively (92-99% kill rate). Five μL of each conidial suspension were inoculated to the solid fermentation culture medium for single cell separation after irradiation. After incubation at 28 °C for 48 h, formed individual colonies were then picked up into 96 micro-well-plates with 0.2 mL of sterile water. Afterwards, the cell suspension was inoculated to the solid selecting culture medium, incubated at 28 °C for 36 h. The mutants were grouped to three categories (forward mutation, negative mutation and non-mutation) on the basis of colony diameters. The inulinase activity of mutants was measured as described above.

Plackett-Burman experimental design

Factors including JAP concentration, yeast extract concentration, peptone concentration, duration, temperature, solution volume, inoculation size and pH were examined for the fermentation production of inulinase in 250-mL Erlenmeyer flasks. The level of each factor was first estimated through single factor experiments. All experiments were carried out in triplicate and the averages of inulinase activity were taken as the response. The fermentation time was 60 h.

An initial screening test was conducted on all eight components of the medium and culture conditions using Plackett-Burman experimental design. Each variable was defined at two levels, namely a low level (coded by -1) and a high level (coded by 1) as listed in Table 1. The three dummy variables (X_4, X_8 and X_{11}) were adopted to investigate the experimental errors. The results of Plackett-Burman experimental design were fitted by the first-order model as follows (Reddy *et al.*, 2008):

$$Y = \beta_0 + \sum \beta_i X_i \ (i = 1,2,\ldots k) \quad (1)$$

where Y is the estimated target function, β_0 the model intercept, β_i the regression coefficient, X_i the coded independent factor.

Path of steepest ascent method

Based on the results of Plackett-Burman experimental design, the optimum level scope of each selected factor was examined by path of steepest ascent method. A path of steepest ascent method was designed using the direction of Plackett-Burman experimental value as the uphill direction. The changing step size was confirmed according to the effect value of Plackett-Burman design. High value was selected when the effect value of variable was positive, and low value was selected when the effect value of variable was negative.

Table 1 - The Plackett-Burman design for screening the variables, and the statistical analysis of variables.

Factor	Variable	Low level (-1)	High level (1)	F value	p value
JAP (g/L)	X_1	40.0	60.0	23.24	0.0170*
pH	X_2	5.0	7.0	0.65	0.4790
Yeast extract (g/L)	X_3	5.0	7.5	4.78	0.1168
Fermentation time (h)	X_5	60	96	0.33	0.6080
Peptone (g/L)	X_6	10.0	15.0	50.43	0.0057**
Temperature (°C)	X_7	27	29	0.48	0.5385
Solution volume (mL)	X_9	40	60	25.97	0.0146*
Inoculation size (%)	X_{10}	4	6	0.14	0.7352
Dummy variable	X_4, X_8, X_{11}	-1	1	-	-
-	Model	-	-	13.25	0.0285*

*Significant at 95% confidence degree ($p < 0.05$). ** Extremely significant level at 99% confidence degree ($p < 0.01$).

Central composite design

A central composite design with three variables (Table 2) was used to evaluate the response pattern and to determine the optimum combination of JAP content, peptone content and solution volume for maximizing inulinase production (Plackett-Burman design had shown significant curvature and confirmed the significance of all four parameters). The variables with that were predicted to produce highest inulinase activity by the path of steepest ascent method were used as center-point for the central composite design. Experimental results were fitted to a predictive quadratic polynomial equation as the correlation between the response variable and the independent variables (Xie *et al.*, 2014):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (2)$$

where Y is the predicted response; β_0, intercept; β_1, β_2, β_3, linear coefficients; β_{11}, β_{22}, β_{33}, quadratic coefficients; and β_{12}, β_{13}, β_{23} are interactive coefficients. The experimental design was developed using Design Expert 8.0.7.1 (Statease, Inc., Minneapolis, MS, USA).

Results and discussion

Isolation and identification of the *Aspergillus*

After the isolation period, a total of 12 inulinase-producing isolates were recovered from 42 soil samples. These strains could solubilize inulin particles in the agar plates and form clear surrounding zones. This was further confirmed by growing the isolates in liquid medium containing inulin as sole carbon source. Among the 12 isolates tested, one gave maximum enzyme activity (34.4 U/mL) and it was designated as G-60. The partial 1400 bp 16S rRNA gene sequence of the G-60 isolate exhibited 99.3% homology with *Aspergillus niger* strain AF10 (Gen-Bank number AF369388). Thus the strain was identified as *A. niger* G-60.

Screening single-celled mutants with high inulinase productivity

Compared with the limited penetration effect of ultraviolet rays, γ-rays can generate free radicals, which may alter the composition of biological cells (Kovacs and Keresztes, 2002). Thereupon, *A. niger* G-60 was mutated with ^{60}Co-γ-rays in this study because of their strong penetration capability. Random selection and screening of mutants is a time-consuming laborious process. To reduce labour intensity, the rational selection approach was adopted basing on the selection of mutants having higher growth capability in selecting medium agar plates than the parental strain. After two days of incubation, the diameter of mutant colonies was measured and compared with the colony diameter of parental strain G-60. A single colony of mutant with a diameter ≥ 3.3 cm, ≤ 2.7 cm, or between 2.7 and 3.3 cm was considered a forward-, negative- or non-mutation strain, respectively.

As shown in Figure 1, the colony counts with forward-mutation (namely having enhanced enzyme-producing ability) were 5, 6 and 2 for 800, 1000 and 1200 Gy irradiation, respectively. Similarly, the colony counts with negative mutation (namely having decreased enzyme-producing ability) were 22, 8 and 33 for 800, 1000 and 1200 Gy irradiation, respectively. Thus it can be seen, low to moderate doses of γ-ray irradiation (800 and 1000 Gy) could produce more forward mutants relative to high dose of irradiation (1200 Gy). Both the lowest rate of forward mutation (2.1%) and the highest rate of negative mutation (34.4%) occurred under 1200 Gy irradiation. This indicates that high irradiation doses seriously damage cell metabolism regulation system, and cell growth deteriorates due to the loss of self-repair ability of cells during damage recovery (Agarwal *et al.*, 2008).

To further examine inulinase productivity of mutants, six forward mutants (designated A1, C10, D6, E12, F3 and F4 respectively) and three negative mutants (designated C2, D11 and G3 respectively) were selected for enzyme activity assays. Figure 2 demonstrates inulinase activity produced by both parental strain G-60 and 9 mutants obtained with different doses of irradiation. As shown, the three negative mutants (C2, G3 and D11) had significantly lower enzyme productivity than strain G-60. The other six forward mutants exhibited higher inulinase activity than the parental one. Among them, mutant E12 possessed the maximum inulinase productivity (128 U/mL), about 2.7 times higher than that of the parent strain *A. niger* G-60. Thereupon, the mutant E12 was finally selected for optimization of inulinase production conditions in the following experiments.

Table 2 - Range of variation of the independent factors used in the central composite design.

Variable	Code	Level of variation				
		-1.68	-1	0	1	1.68
JAP (g/L)	A	53.2	60.0	70.0	80.0	86.8
Peptone (g/L)	B	9.9	15.0	22.5	30.0	35.1
Solution volume (mL)	C	33.2	40.0	50.0	60.0	66.8

Plackett-Burman experimental design

The Plackett-Burman experimental design for 12 trials with two levels for each variable and the corresponding inulinase production are presented in Table 3. To approach the neighborhood of the optimum response, a fitted first-order model for inulinase production was obtained from the Plackett-Burman experimental design as follows:

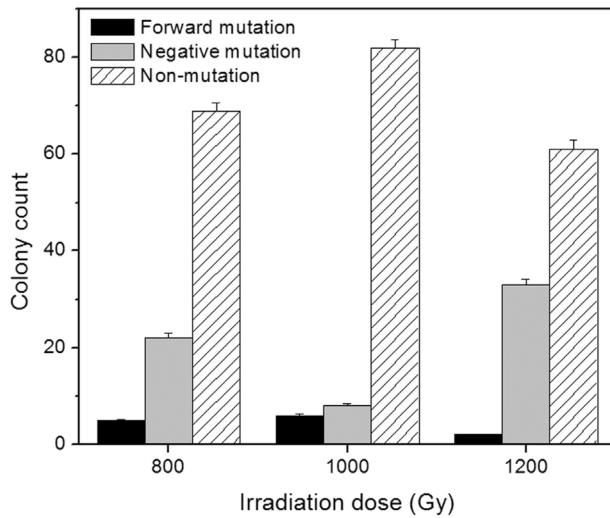

Figure 1 - Colony number of surviving cells grown in selecting medium agar plates after two days of incubation under different irradiation doses. Error bars represent the standard error associated with triplicate experiments. A single colony of mutant with a diameter ≥ 3.3 cm, ≤ 2.7 cm, or between 2.7 and 3.3 cm was considered a forward-, negative- or non-mutation strain, respectively.

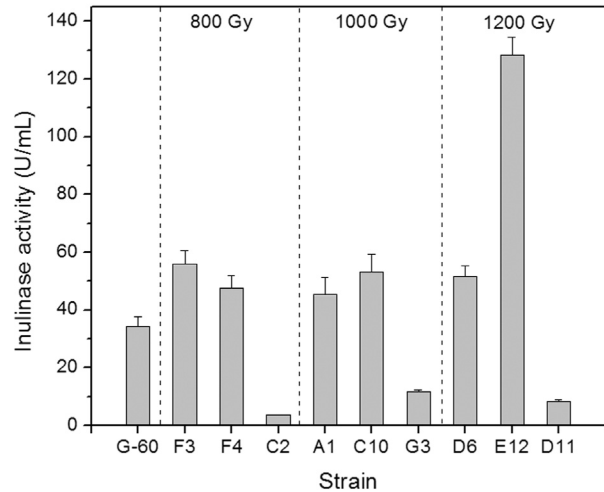

Figure 2 - Inulinase yield of parental strain G-60 and mutants after cultivation at 28 °C and 180 rpm for 60 h using the fermentation medium.

$$Y = -78.60 + 9.23X_1 + 1.54X_2 - 33.46X_3 + 0.12X_5 + 54.39X_6 + 1.33X_7 - 0.98X_9 + 0.71X_{10} \quad (3)$$

The coefficient of each variable in Eq. (3) represents the influential degree of this variable on inulinase yield. The results of statistical analyses are shown in Table 1. A factor with a confidence level greater than 95% ($p \leq 0.05$) was considered to have a significant effect on inulinase yield and was selected for further study. The linear regression coefficient R^2 was 0.9350 and the p value was 0.0285 (< 0.05) for the model. These results indicate that the model was suitable for Plackett-Burman experimental design. The p value for peptone concentration (X_6) was less than 0.01 with a 99% confidence, indicating that peptone concentration had an extremely significant effect on inulinase yield. The p values for JAP concentration (X_1) and solution volume (X_9) were both less than 0.05 with a 95% confidence. These results clearly suggest that, in all the factors examined, peptone concentration, JAP concentration and solution volume were the most significant for inulinase production, and other factors had no significant effect on inulinase production. Peptone concentration was found to be the most influential factor, followed by solution volume and JAP concentration. Of the three significant variables identified, solution volume exerted a negative influence, while peptone concentration and JAP concentration exerted a positive influence on inulinase yield. Thus, these variables were selected as studied objects for the following experiments.

Table 3 - The design and results of Plackett-Burman design.

No.	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	Inulinase yield (U/mL)
1	-1	-1	1	-1	1	1	-1	1	1	1	-1	125
2	1	1	-1	-1	-1	1	-1	1	1	-1	1	91.0
3	1	-1	1	1	-1	1	1	1	-1	-1	-1	156
4	1	1	-1	1	1	1	-1	-1	-1	1	-1	110
5	-1	1	1	1	-1	-1	-1	1	-1	1	1	77.4
6	-1	1	1	-1	1	1	1	-1	-1	-1	1	133
7	-1	-1	-1	1	-1	1	1	-1	1	1	1	93.3
8	-1	1	-1	1	1	-1	1	1	1	-1	-1	122
9	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	107
10	1	-1	-1	1	1	-1	-1	-1	1	-1	-1	104
11	1	-1	-1	-1	1	-1	-1	1	-1	1	1	115
12	1	1	1	-1	-1	-1	1	-1	1	1	-1	126

The path of steepest ascent

The above results indicate that compared with other factors, peptone concentration, JAP concentration, and solution volume can significantly influence inulinase yield. Moreover, Eq. (3) shows that the coefficient of X_9 was negative while the coefficients of X_1 and X_6 were positive, which means that the decrease in solution volume and the increase in concentrations of JAP and peptone can exert positive effect on inulinase production. The path of the steepest ascent was used to search for the proper direction to change the levels of these three factors while keeping the other factors constant at the levels found in basal fermentation medium. The experimental design and corresponding results are listed in Table 4. The results indicate that the yield of inulinase was maximum when the concentrations of JAP and peptone, and solution volume were selected to be 70.0 g/L, 22.5 g/L and 50.0 mL, respectively. These results suggest that this level for each of the three factors was near the region of maximum inulinase production response.

Central composite design and response surface methodology

To further enhance inulinase production by the mutant E12, central composite design and the response surface methodology were employed to analyze the interactive effect of JAP concentration, peptone concentration and solution volume and to obtain an optimum for the inulinase production by the mutant E12. The values of the three variables in the run 3 obtained from the steepest ascent path (Table 4) were taken as the center points with the other variables fixed at low level (Table 1). The design and results of the experiments carried out with the central composite design are listed in Table 5.

The analysis of variance (ANOVA) was employed for the determination of the significant parameters. The results obtained were submitted to ANOVA on Design-Expert 8.0 and the regression model was given as the following equation:

$$Y = -3650.52 + 55.28A + 4246B + 54.08C - 0.45A^2 - 1.01B^2 - 0.55C^2 + 0.29AB - 0.12AC - 036BC \quad (4)$$

where Y is inulinase activity, A is JAP concentration, B is peptone concentration, C is solution volume (Table 2). The ANOVA of the quadratic regression model demonstrates that Eq. (4) is a highly significant model, as is evident from the Fisher's F-test with a very low probability value (F value = 7.70) (Table 6). The p value (0.0013) was less than 0.01 with a 99% confidence (Table 6), indicating model terms were extremely significant. Of the first terms (A, B, C), JAP concentration (A) had significant effect on inulinase yield ($p < 0.05$). Of the quadratic terms (A^2, B^2, C^2), concentrations of JAP and peptone and solution volume had extremely significant effect on inulinase yield ($p < 0.01$). However, the interaction terms (AB, AC, BC) had no significant effect on inulinase yield ($p > 0.05$).

The interactions of the three components and their optimum level in inulinase production were further analyzed through response surface methodology. The three-dimension response surface curves and respective contour plots are presented in Figures 3-5. Three-dimensional graphs were generated for the combination of the two variables, while keeping the other one at their optimum levels determined by the path of steepest ascent for inulinase production by the mutant E12. It can be seen that the response surface was convex, suggesting that the optimum conditions are well-defined and there existed a maximum for each variable. According to the results of the response surface analysis, the predicted maximum production of inuli-

Table 4 - The designs and results of path of steepest ascent.

No.	JAP (g/L)	Peptone (g/L)	Solution volume (mL)	Inulinase yield (U/mL)
1	50.0	12.5	60	97.0
2	60.0	17.5	55	138
3	70.0	22.5	50	166
4	80.0	27.5	45	120
5	90.0	32.5	40	104
6	100.0	37.5	35	81.7

Table 5 - The designs and results of central composite design.

No.	JAP (A)	Peptone (B)	Solution volume (C)	Inulinase yield (U/mL)
1	1	-1	1	151
2	0	0	0	166
3	0	0	0	166
4	1.68	0	0	118
5	0	-1.68	0	131
6	0	0	0	166
7	-1	-1	-1	81.1
8	1	-1	-1	137
9	-1	1	-1	117
10	0	0	0	166
11	0	0	-1.68	113
12	-1.68	0	0	152
13	1	1	1	109
14	0	0	0	166
15	-1	-1	1	144
16	0	1.68	0	137
17	0	0	0	166
18	1	1	-1	138
19	0	0	1.68	127
20	-1	1	1	132

nase was 193 U/mL when the concentrations of JAP and peptone, and solution volume were 66.5 g/L, 29.1 g/L and 49.4 mL, respectively, and the rest of the variables were kept at zero level (Table 2). In the present study, inorganic

Table 6 - Analysis of variance (ANOVA) for regression of central composite design.

Variance origin	*F* value	p value
A	6.97	0.0247*
B	0.094	0.8295
C	0.74	0.4099
A^2	16.55	0.0023**
B^2	26.17	0.0005**
C^2	24.34	0.0006**
AB	2.18	0.1708
AC	0.041	0.8434
BC	3.20	0.1040
Model	7.70	0.0013**
First term	0.58	0.6375
Quadratic term	18.71	0.0002
Interaction term	0.35	0.7864
Lack of fit	2.22	0.2007

*Significant at 95% confidence degree ($p < 0.05$). **Extremely significant level at 99% confidence degree ($p < 0.01$).

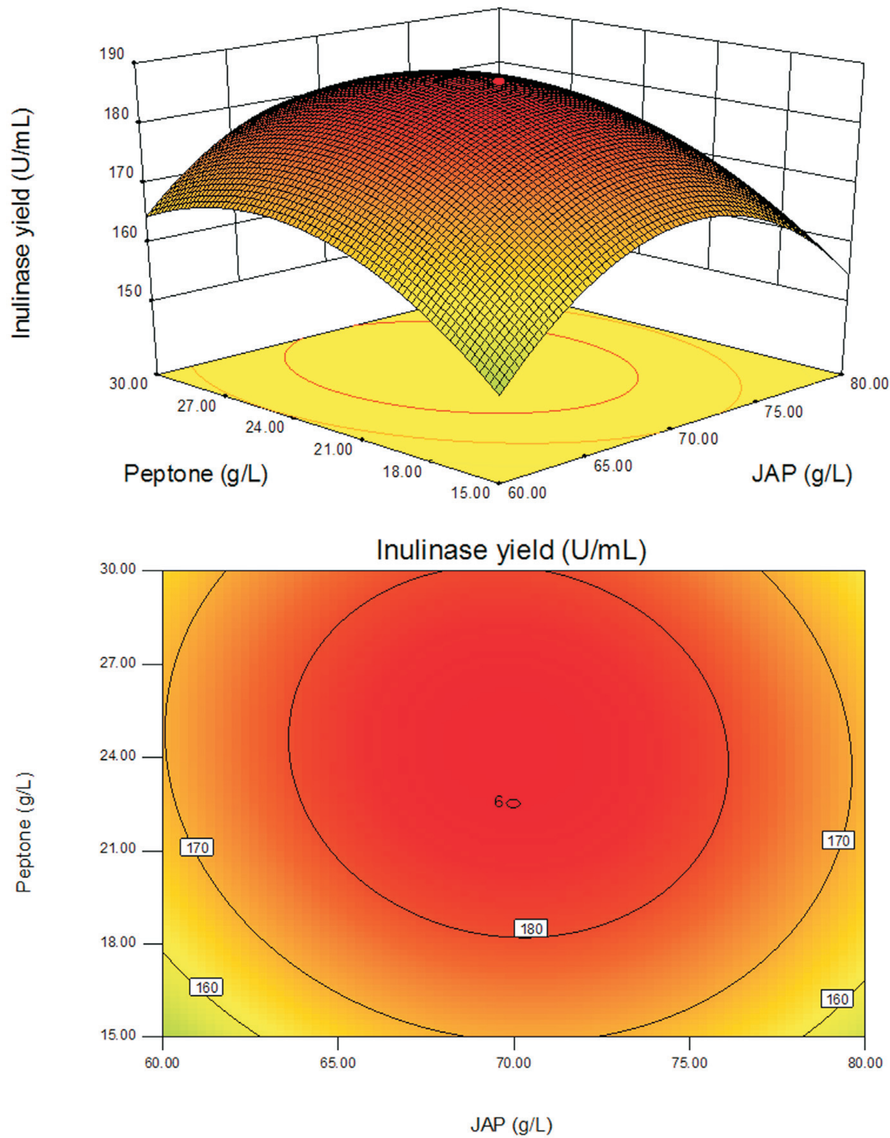

Figure 3 - The response surface plot and the corresponding contour plot showing the effects of JAP and peptone on inulinase production by mutant E12. Solution volume level, 50.0 mL.

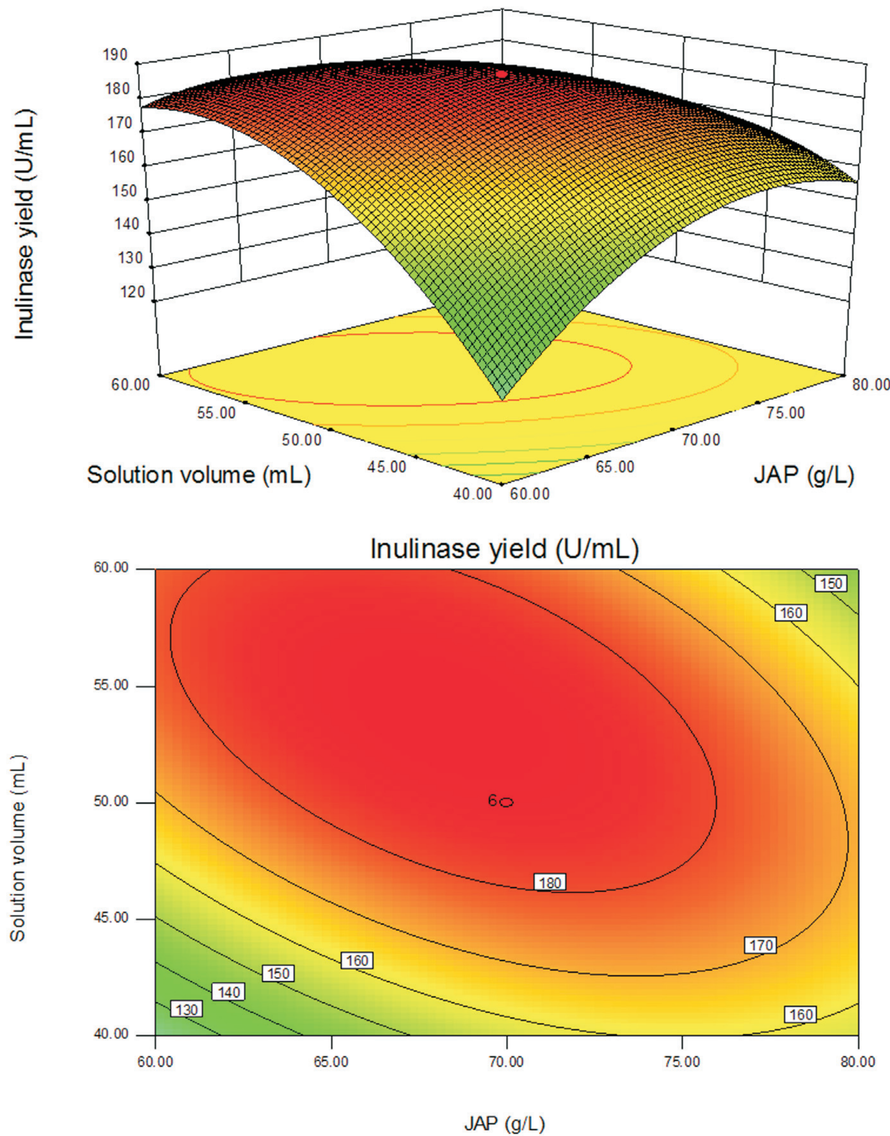

Figure 4 - The response surface plot and the corresponding contour plot showing the effects of JAP and solution volume on inulinase production by mutant E12. Peptone level, 22.5 g/L.

salts such as ammonium, magnesium and sulfate ions were not included in the fementation medium composition. This is because the carbon source used (JAP) is a crude material containing a variety of inorganic nutrients.

The mutant E12 was cultured with the optimized medium to examine the effectiveness of the model equations in successfully predicting the optimum response values. The set of conditions predicted by response surface methodology as optimum was used for experimental validation. The inulinase yield obtained from experiments using the mutant E12 culture was 185 U/mL, a value that was similar to that predicted by response surface methodology model (193 U/mL), indicating that the model can be indeed employed for optimizing culture conditions for inulinase production. Thus, the yield of inulinase using the optimized culture medium was approximately 1.4-fold of that obtained when basal culture medium was used.

Optimal medium composition is vital for increasing inulinase production using submerged cultures. Statistical experimental design provides a systematic and efficient means of realizing desired goals, and it helps in understanding the interactions among the process parameters at varying levels and in calculating an optimaum level of each variable for the maximum product yield (Trivedi *et al.*, 2012). Many previous studies have demonstrated that inulinase production by *Aspergillus* species could be remarkably enhanced by the optimization of cultivation conditions and medium compositions (Skowronek and Fiedurek, 2004; Kumar *et al.*, 2005; Trivedi *et al.*, 2012; Abd El Aty *et al.*, 2014).

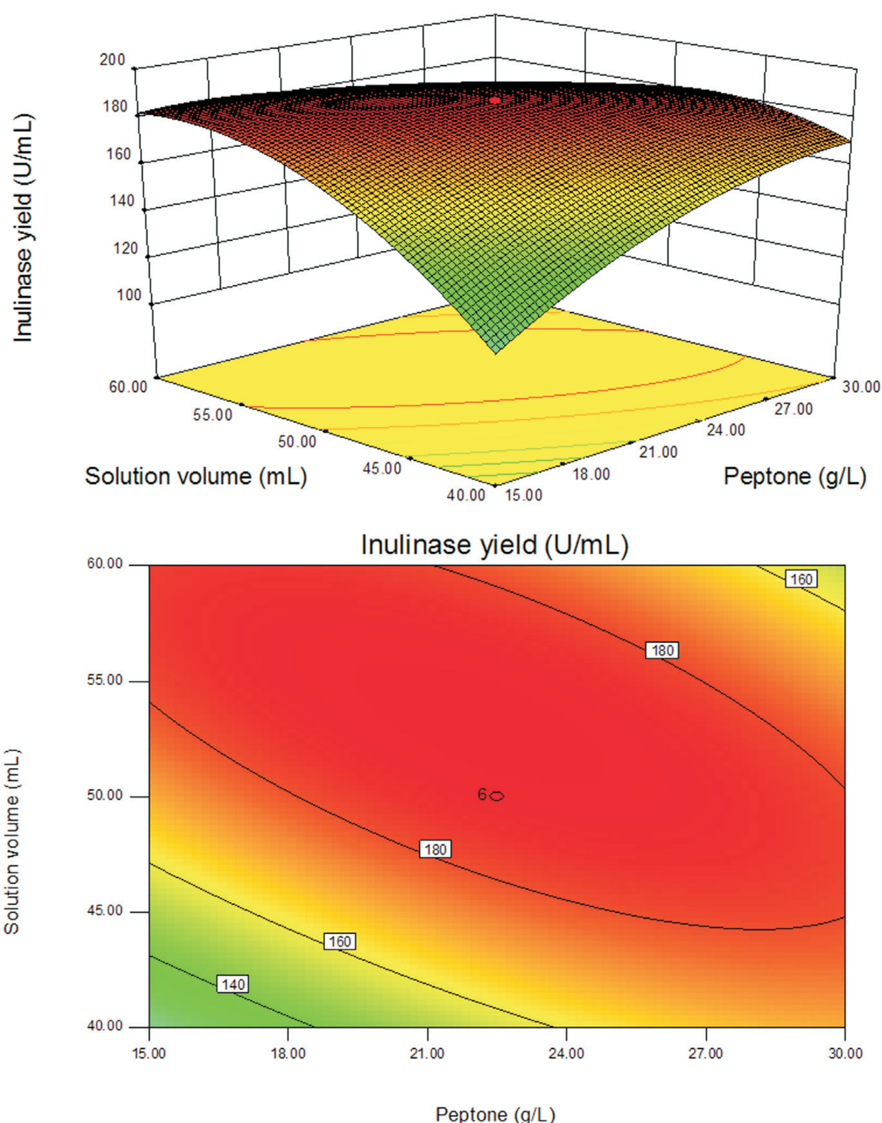

Figure 5 - The response surface plot and the corresponding contour plot showing the effects of peptone and solution volume on inulinase production by mutant E12. JAP level, 70.0 g/L.

In this study, the mutant E12 showed higher inulinase production ability than some other *Aspergillus* species. For example, the highest extracellular enzyme activity of *A. niger* 13/36 (Skowronek and Fiedurek, 2004) and *A. niger* AUP19 (Kumar *et al.*, 2005) was found to be 80 and 176 U/mL, respectively. Moreover, the mutant E12 had a lower nutrient demand than strains 13/36 and AUP19, as they needed sucrose or galactose as additional carbon source.

Conclusions

Results from this study have demonstrated that *A. niger* G-60 was an efficient inulinase producer. Also, it was effective to enhance inulinase yield of the strain by nuclear irradiation mutation. A mutant strain of *A. niger* G-60 (designated as E12), with inulinase yield improved more than 2.7-fold compared to the parental strain, was obtained by ^{60}Co-γ mutagenesis treatment in this study. The application of response surface methodology design resulted in an improvement of inulinase yield. The mutant E12 could produce inulinase at a high level and therefore had significant commercial potential. Further research is needed to explore the transformed functional genes and regulated metabolic network, which are related to inulinase porduction in *A. niger* G-60 cells mutated by nuclear irradiation.

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