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Optimization of UV irradiation mutation conditions for cellulase production by mutant fungal strains of *Aspergillus niger* through solid state fermentation

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Abstract: Ultraviolet (UV) irradiation was used to induce mutagenesis in *Aspergillus niger* to provide a suitable mutant strain for overproduction of cellulase. Response surface methodology was successfully utilized to evaluate the effect of the mutation conditions, namely: UV exposure time (60–300 s) and distance of the strain from the UV source (0–20 cm) on created clear zone area around the strains. A maximum clear zone area ($600.525 \pm 15.537 \text{ mm}^2$) was obtained under the optimal mutation conditions of UV exposure time and distance of the 9 cm and 220 s. Rice and wheat straw were used as cellulosic substrates to produce cellulase by using the mutant strain through solid state fermentation. The results indicated that the total cellulase activity of the produced cellulase during 10 days of fermentation was 4.159 IU/ml, which was approximately twofold higher than that of the cellulase for the wild strain.

Keywords: *Aspergillus niger*; cellulase; response surface methodology; solid state fermentation; UV irradiation mutation.

1 Introduction

Plant biomass containing cellulose, called lignocellulosic waste, is a sustainable and feasible source for animal feed, and fuel and chemical production. One of the major potential benefits of biotechnology is the bioconversion of these

cellulosic wastes to valuable products using cellulase [1]. Cellulase as an important industrial hydrolytic enzyme is a complex enzyme system including: β -glucosidase (EC 3.2.1.21), endo-(1, 4)- β -D-glucanase (EC 3.2.1.4) and exo-(1, 4)- β -D-glucanase (EC 3.2.1.91) [2].

As compared to yeast and bacteria, filamentous fungi are the main microbial sources to produce more efficient enzymes industrially [2, 3]. Cellulases are mainly produced by the species of fungi such as *Chaetomium*, *Fusarium*, *Myrothecium*, *Trichoderma*, *Penicillium* and *Aspergillus* [4], and the most important species in cellulase production are *Aspergillus* and *Trichoderma* spp [5]. Studies showed that *Trichoderma reesei* is the most efficient producer of cellulase. However, it does not excrete a sufficient amount of β -glucosidase for enzymatic hydrolysis and subsequent converting of the exocellulase product into glucose [6, 7].

Recently, different strains of *Aspergillus* such as *Aspergillus niger*, *Aspergillus ustus*, *Aspergillus flavus*, *Aspergillus nidulans*, *Aspergillus oryzae*, *Aspergillus fumigatus*, *Aspergillus glaucus*, *Aspergillus clavatus* and *Aspergillus versicolor* have been widely used in biotechnology to produce various extracellular enzymes, and among them *A. niger* is a major producer of cellulolytic enzymes which can produce β -glucosidase higher than *T. reesei* [1, 8].

Different fermentation methods such as solid state fermentation (SSF) and submerged fermentation are industrially used to produce cellulase [9]. Among these two techniques, SSF is suitable for cultivation of filamentous fungi because of fungal growth in hyphae mode and their excellent tolerability in the growth media with low and high water activity and osmotic pressure, respectively. As a result, this leads to more efficient and competitive bioconversion of solid substrates for fungi compared to natural microflora [10, 11].

Due to overproduction of microbial enzyme, suitable strains can be genetically improved by induced mutagenesis through chemical and physical agents [4, 12, 13]. Studies showed that ultraviolet (UV) irradiation as a physical agent is more efficient and a preferable method to improve microbial strains such as *A. niger* compared

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to chemical agents [12]. There are several reports indicating the overproduction of cellulase by UV mutant strains of *A. niger* [12, 14, 15]. These studies revealed that the UV exposure time, distance of the microorganism to the UV source, composition and water activity of growth media and substrate are the main parameters which influence the yield of cellulase production.

While most of the studies on increasing cellulase production yield in the UV mutant of *A. niger* were focused strongly on optimization of the type and composition of the growth media, few researches have been conducted on the optimization of the UV irradiation conditions. The main aims of this study were (i) to optimize the UV exposure time and distance of the growth media inoculated with *A. niger* with respect to the UV source in order to obtain the improved fungal strain with highest cellulase production yield and (ii) to evaluate the total cellulase enzyme activity of the obtained mutant and wild strains of *A. niger* on the cellulosic substrates through SSF.

2 Materials and methods

2.1 Fungal strain and its morphological characteristics

A. niger (PTCC 5162) was obtained from the Persian Type Culture Collection (PTCC, Tehran, Iran). The strain was kept on slants of Sabouraud dextrose agar (SDA) (Sigma-Aldrich Inc., MO, USA). Morphological characteristics of cultured *A. niger* both in macroscopic and microscopic properties were investigated. Macroscopic features were checked during the fungi growth on the surface of SDA media and microscopic features were studied under an inverted microscope after staining the selected spores with lactophenol cotton blue at magnification of 40 \times .

2.2 Preparation of inoculum

A. niger was inoculated into the plates containing SDA and incubated at 28°C for 7 days to grow and produce spores. The spore suspension was provided by a collection of them from the surface of plates and mixed in 10 ml of normal saline (0.9% w/v). After that, spore suspension was diluted to concentrations of 1.0×10^7 spores per ml by counting in a Neubauer chamber using the method reported elsewhere. The prepared spore suspension was used as inoculum.

2.3 Lignocellulosic sources and pretreatment process

According to the literature, two different straw species namely, rice and wheat, were commonly used in the cellulase production [1]. Therefore, these lignocellulosic wastes were purchased from the local market and used as cellulosic substrates. These cellulosic

substrates were ground using a miller in order to reduce their size. Then, they were individually treated with NaOH (0.25 N) with a treatment ratio of 1 : 10 (w/v) for 1 h. After that, the pH was adjusted to 7 by washing thoroughly with tap water and dried in an oven at 80°C for 48 h [16].

2.4 UV mutagenesis and screening of mutated isolates

The prepared spore suspension of wild *A. niger* was discharged into the sterile plate and located under UV exposure (254 nm) at different distances from the UV source and for defined exposure time. The diameter of the plates was 90 mm. As screening media, Mandels mineral salts solution (Table 1) was supplemented with 17.5 g/l of agar (Merck Co., Darmstadt, Germany), 10 g/l of phosphoric acid (Merck Co.), swollen cellulose 0.1% (v/v), Triton X-100 (Merck Co.) and the pH was adjusted to 5 [10]. After UV mutagenesis, 20 μ l of treated spore suspensions was taken from the stock and poured into a small well which was created in the center of the plates containing Mandels screening medium. The plates were then incubated at 28°C for 7 days with daily monitoring of colonies and their surrounded clear zone diameter. Hypercellulolytic mutants were those which had the highest clear zone area around their colonies. The suitable mutant isolates were then selected for enzyme production through the SSF method to evaluate their total cellulase production activity compared to the wild strain.

2.5 Cellulase production through SSF

Fermentation medium was prepared by addition of 100 g/l cellulose (Sigma-Aldrich Inc., MO, USA) to Mandels mineral salts solution and its final pH was adjusted to 4.8 [10]. Some 5 g of each of the pretreated substrates were separately moistened with 10 ml of fermentation medium in Erlenmeyer flasks (250 ml) and sterilized at 121°C for 20 min. After that, the samples were cooled to room temperature, inoculated with 20% (v/w) of 1-day-old spore suspensions (wild and mutant strains) and incubated at 28°C for 2 weeks. In order to extract the enzyme, every 2 days, 50 ml of citrate buffer, which was prepared using citric acid (Merck Co., Darmstadt, Germany) and trisodium citrate (Merck Co., Darmstadt, Germany), was added into

Table 1: Mandels mineral salts solution components.

Components	Content	Specification
(NH ₄) ₂ SO ₄	1.4 g/l	Merck Co., Darmstadt, Germany
KH ₂ PO ₄	2.0 g/l	Merck Co., Darmstadt, Germany
CaCl ₂ ·2H ₂ O	0.4 g/l	Merck Co., Darmstadt, Germany
MgSO ₄ ·7H ₂ O	0.3 g/l	Merck Co., Darmstadt, Germany
FeSO ₄ ·7H ₂ O	5.0 mg/l	Merck Co., Darmstadt, Germany
ZnSO ₄ ·7H ₂ O	1.4 mg/l	Merck Co., Darmstadt, Germany
Tween 80	0.2 g/l	Merck Co., Darmstadt, Germany
Peptone	1.0 g/l	Quelab Co., Montreal, Canada
MnSO ₄ ·7H ₂ O	1.6 mg/l	Daejung Co., Shiheung, Korea
CoCl ₂ ·6H ₂ O	20.0 mg/l	Sigma-Aldrich Co., MO, USA
Urea	0.3 g/l	Sigma-Aldrich Co., MO, USA

each flask and shaken at 180 rpm and 28°C for 1 h. The samples were then centrifuged with an ultracentrifuge (Sorvall RC-B, DE, USA) at 9000 rpm and 4°C for 20 min. The collected supernatant was used to evaluate the activity of the extracted enzyme.

2.6 Total cellulase activity and glucose concentration assay

Total cellulase activity of the extracted enzyme was evaluated according to the filter paper activity (FPA) method which was described by Mandels et al. [17]. Aliquots of properly diluted clarified extracted enzyme were added in filter paper (Whatman No. 1) strips (1×6 cm; 50 mg) which were immersed in 1 ml of 0.05 M sodium citrate buffer (pH 5.0). The samples were then incubated at 50°C for 1 h and the concentration of the resulting glucose (reduced sugar) was measured according to the dinitrosalicylic acid method [18]. Total cellulase activity was expressed as filter-paper unit. One filter-paper unit activity is the amount of cellulose which can release 1 μmol glucose from 1 ml filter paper buffer solution/min.

2.7 Design of experiments and statistical analysis

The experiment was planned using a central composite design and response surface methodology used to evaluate the effects of two independent parameters, namely UV exposure time (60–300 s) (X_1) and distance from UV source (0–20 cm) (X_2), was applied to evaluate the obtained clear zone area (Y). Response surface methodology is a feasible method to estimate the interactions of independent variables on the responses making it highlighted as compared to the classical one-variable-at a time optimization [19]. A total of 13 experimental runs were generated with five different levels for each independent parameter using Minitab software (v.17 statistical package, Minitab Inc., PA, USA) (Table 2). All experiments were carried out throughout a day by using one block. In order to correlate the clear zone area (Y) to the studied variables, a second order polynomial equation (Eq. 1) was used:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (1)$$

where β_0 is a constant, β_1 and β_2 correspond to the linear effects, β_{11} , β_{22} represent the quadratic effects and β_{12} indicates the interaction effect. The suitability of the model was studied accounting for the coefficient of determination (R^2) and adjusted coefficient of determination (R^2 -adj). Analysis of variance was also carried out to provide the significance determinations of the resulted models in terms of p values; a small p value (<0.05) was considered as statistically significant. Based on the fitted polynomial equation, a three dimensional surface plot was designed to visualize the independent variable interactions. It should be considered that the response can be predicted thoroughly using the obtained model within the defined ranges for the independent variables. The contour plot was used to explain the optimum region and numerical response optimization was used to determine the optimum values of the UV exposure parameters for obtaining the desired response. For verification of the validity of the statistical experimental approaches, three additional approval tests were performed at obtained optimum mutation conditions [20].

Table 2: Central composite design (CCD) and response variables for final reduced model.

Experiment no.	UV exposure time (s)	Distance from UV lamp (cm)	Clear zone area (mm ²)	
			Exp ^a	Pre ^b
1	60	10.000	376.380	345.418
2	180	10.000	586.395	576.630
3	180	10.000	624.263	576.630
4	95	2.900	302.225	346.714
5	180	10.000	593.460	576.630
6	180	10.000	502.400	576.630
7	95	17.000	302.225	295.277
8	265	2.900	477.280	459.999
9	180	20.000	302.225	292.125
10	180	10.000	257.480	576.630
11	300	10.000	334.606	499.972
12	180	0.000	376.800	364.938
13	265	17.000	376.800	404.513

^aExperimental values of studied responses.

^bPredicted values of studied responses.

3 Results and discussion

3.1 Morphological characteristics of fungal strain

Microscopic characteristics of cultured *A. niger* under the microscope, including spore formation, are shown in Figure 1. This figure clearly indicates the growing process of *A. niger* while surface colony color is initially white (Figure 1A), becoming black to deep brown with conidial production (Figure 1B). Stained *A. niger* with lactophenol cotton blue is also illustrated in Figure 1C. As clearly observed, the surface of vesicle was covered with phialides which were surrounded with many formed spores. These spores had the potential to produce cellulase enzyme.

3.2 Response surface model

According to the obtained values for the experimental runs (Table 2) and by applying multiple regression analysis, the second order polynomial model for studying two independent parameters was fitted. The estimated regression coefficients and corresponding significance of regressions for the model are given in Table 3.

In the final reduced model, non-significant effects were removed. However, the non-significant main term of the studied independent variables could not be removed from the model, if either their quadratic or interaction

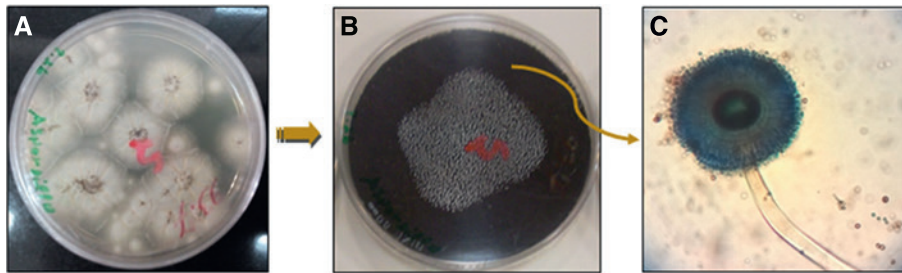


Figure 1: Initial white colonies (A), and 1-week-old black colonies of *Aspergillus niger* on Sabouraud dextrose agar (SDA) (B) and microscopic features of *A. niger* stained with lactophenol cotton blue at magnification of $40\times$ (C).

Table 3: Regression coefficients, R^2 , adjusted R^2 (R^2 -adj) and probability values for the final reduced model.

Regression coefficient ^a	Clear zone area (mm ²)
β_0 (constant)	- 97.3
β_1 (main effect)	4.49
β_2 (main effect)	45.98
β_{11} (quadratic effect)	- 0.01069
β_{22} (quadratic effect)	- 2.481
β_{12} (interaction effect)	-
R^2	0.921
R^2 -adj	0.869
Lack of fit (p value)	0.715

β_1 and β_2 are the coefficients of main effects of UV exposure time and distance from UV lamp, respectively. β_{11} and β_{22} are the coefficients of quadratic effects of UV exposure time and distance from UV lamp, respectively. β_{12} is the coefficient of interaction effect of UV exposure time and distance from UV lamp.

effects were significant ($p < 0.05$) (Table 4) [21]. F ratio and p values of all the main, quadratic and interaction terms of the obtained final models are also shown in Table 4. Generally, lower p values and higher F ratios indicate higher importance of the chosen term on the responses [22]. High values of the R^2 and adjusted R^2 (0.92) and R^2 -adj (0.87) for the obtained model showed the suitability and

high accuracy of the suggested model. Moreover, obtained non-significant lack of fits for the achieved model confirmed its sufficient fitness to the UV exposure parameters effects (Table 3). As shown in Table 4, even though the UV exposure time had a significant ($p < 0.05$) effect on the clear zone area, the main effect of distance from the UV source and the interaction effect of the independent variables had non-significant effects. Due to the significant ($p < 0.05$) effect of distance quadratic term, its main term was kept in the final reduced model.

3.2.1 Analysis of response surface model for clear zone area

Clear zone area values (Y_2) varied from 275.800 to 624.263 mm² (Table 2). Table 3 indicates that the main and quadratic effects of both UV exposure parameters had positive and negative effects on the clear zone area, respectively. In fact, the results indicated that at lower levels of UV exposure time, with increase in the UV exposure time, the formed clear zone area increased, while at higher levels, this increase was not significant. It is more pronounced when the quadratic effect of time is incorporated due to its higher F ratio. The obtained results are also demonstrated by the three dimensional response surface plot for better visualization of the main and quadratic effects of the studied UV exposure parameters on the clear zone area (Figure 2). The results are in agreement with several studies reported elsewhere [12, 23, 24]. In these studies, results showed that at constant distances of UV source, the growth of different species of *Aspergillus* is much affected in low levels of UV exposure time, in that increasing the time of exposure led to a greater clear zone area, however, the clear zone area decreases with higher levels of exposure time. The observed decline in clear zone area can be related to the back mutation. It seems that at higher UV exposure time, most of the microorganisms are inactivated; therefore, the created clear zone area

Table 4: Significance probability (p value, F ratio) of regression coefficients in final reduced second order polynomial model.

Main effects	Main effects		Quadratic effects		Interacted effects
	x_1	x_2	x_1^2	x_2^2	x_1x_2
Clear zone area (mm ²)					
p value	0.037	0.157 ^a	0.014	0.001	-
F ratio	7.09	2.62	11.72	45.66	-

^aNot significant ($p > 0.05$).

1: UV exposure time; 2: Distance from UV lamp.

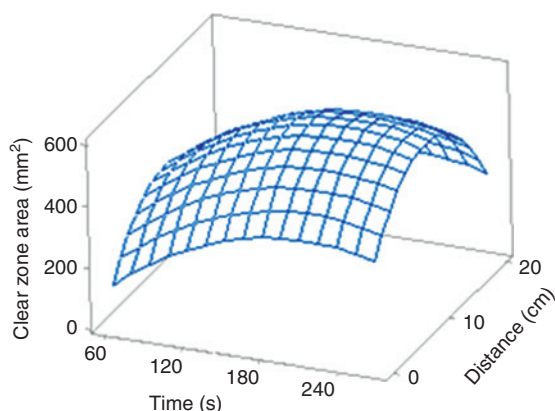


Figure 2: Response surface plot for clear zone area as a function of significant ($p < 0.05$) interaction effects between UV exposure time and distance from UV source.

as a consequence of decomposition of available cellulose by cellulase enzyme of *A. niger* spores decreases [23].

By contrast, as clearly observed in Figure 2, at constant UV exposure times, by increasing the distance from the UV source, the clear zone area increased and then decreased. It seems that at higher distances from the UV source, UV irradiation intensity decreases, which in turn, could not induce mutagenesis in the fungus spores.

3.3 Optimization of UV irradiation mutation conditions

In order to determine the optimum condition for producing cellulase enzyme (characterized by the largest clear zone area) through UV irradiation mutation of *A. niger*, a

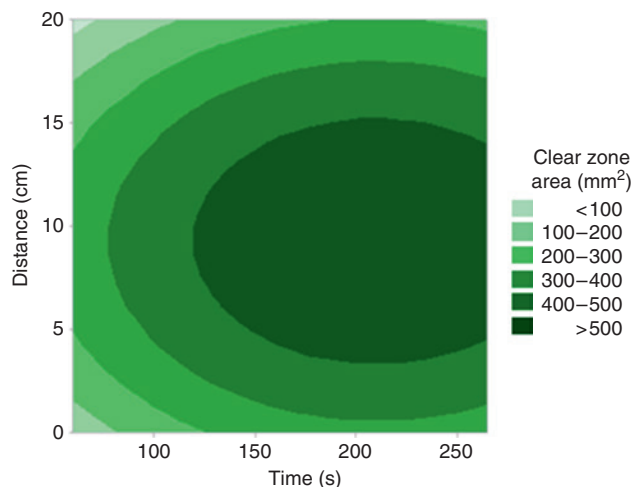


Figure 3: Contour plot for clear zone area as a function of significant ($p < 0.05$) interaction effects between UV exposure time and distance from UV source.

contour plot was generated. Figure 3 shows the contour plot for distance and time of UV exposure for finding the optimum mutation conditions in terms of increasing cellulase enzyme production by the mutant form of *A. niger*. Numerical optimization was also used to find the exact optimum levels of mutation condition variables. The results showed that the optimum UV exposure distance and time were obtained at 9 cm and 220 s, respectively.

3.4 Validation of the response surface model

Verification experiments were performed using the optimum mutation conditions. Comparison of the predicted (586.765 mm^2) and obtained experimental ($600.525 \pm 15.537 \text{ mm}^2$) values of clear zone area at optimum mutation conditions indicated that there were non-significant differences between the predicted and experimental values, which was reconfirmed by the adequacy of the fitted model for the studied responses.

3.5 Cellulase production through SSF at optimum mutation conditions

3.5.1 Selection of a suitable substrate

In order to screen the suitable cellulosic substrate, cellulase production using the wild type of *A. niger* spores (1.0×10^7 spores/ml) was carried out by SSF using both rice and wheat straw as lignocellulosic wastes. The main macro cellulosic compounds of the substrates were converted into glucose using the produced cellulase. The



Figure 4: The absorbance of glucose released from both rice and wheat straw as substrates of cellulase enzyme. The experiments are performed in triplicate and the values are represented as mean \pm SD.

higher released glucose concentration of the substrate is an indicator of the high cellulase production. Figure 4 shows the absorbance of the released glucose as a manifestation of its concentration during the fermentation time (6 days). As can be seen in Figure 4, initial screening of the two substrates indicated that the concentration of the released glucose was significantly ($p < 0.05$) higher when rice straw was used as the substrate than that of results observed for wheat straw. Therefore, rice straw was selected as a suitable lignocellulosic substrate for determining the produced cellulase activity through SSF.

3.5.2 Total cellulase activity of produced enzyme by wild and mutant microorganism

The cellulase activity of mutant and wild types of *A. niger* spores, which is extracellularly produced in optimum UV irradiation conditions, was assessed and the results are shown in Figure 5. The produced cellulase mutant strain showed higher activity than that observed for the wild strain during the entire experiment (i.e. 10 days). The results revealed that the maximum FPA was obtained at day 7 of SSF with a value of 4.159 IU/ml for the mutant strain, which was approximately twofold higher than that of the wild type strain. There was an increasing trend in FPA up to day 7 of fermentation, while it decreased during the following days of fermentation for both mutant and wild strains. This reduction in FPA can be interpreted based on the idea that the susceptible part of the cellulosic molecules is quickly digested and as a result, the remaining portion in crystalline form cannot be used for enzyme production. In addition, this decrease may be related to accumulation of some compounds that have inhibitory roles for enzymes like cellobiase [25, 26].

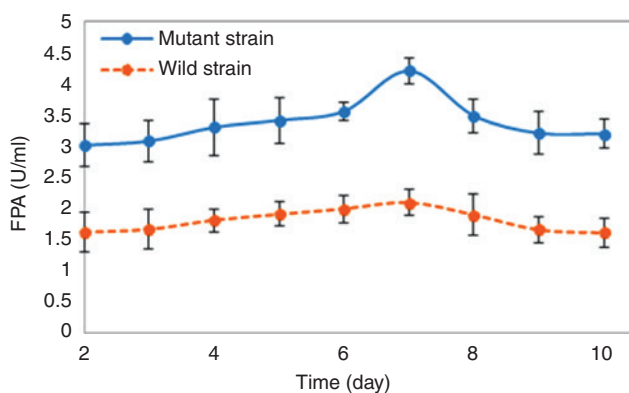


Figure 5: Comparison of cellulase activity by the wild and UV-induced mutants of *Aspergillus niger* using SSF expressed as FPA (U/ml). The experiments are performed in triplicate and the values are represented as mean \pm SD.

The result of the present study are in agreement with findings of similar previous studies [13–15, 27–29]. In a study by Gokhale et al. [27], mutants of *A. niger* NCIM 1207 produced a twofold increase in β -glucosidase production over the wild strain using a shake flask culture. In another study, for mutagenesis of *A. niger*, UV and γ -ray were applied for producing β -glucosidase. The maximum increase in β -glucosidase was obtained by *A. niger* KK2 mutant when it was grown on the basal medium for 7 days [13].

In a further study, mutants of *A. niger* KKS through UV and γ -ray treatment were used to investigate the production of cellulase and hemicellulase by SSF. The maximum FPA for *A. niger* KK2 mutant grown on rice straw was 19.5 IU/g in 4 days, while in the case of carboxymethyl cellulase (CMCase) and β -glucosidase after 5–6 days of fermentation, the values were 129 IU/g and 100 IU/g, respectively [14].

The other mutant strains were obtained from *A. niger* UAM-GS1 by UV radiation in order to enhance the cellulolytic and hemicellulolytic activities of the produced cellulase. Mutant strains, namely GS1-S059 and GS1-S067, increased the amount of cellulase production significantly as compared to their wild strains using SSF [15]. Vu et al. [29] successfully obtained a hyper-producing cellulase mutant form of *Aspergillus* named *SU14* which was mutated using UV and γ -ray, and N-methyl-N'-nitro-N-nitrosoguanidine (NTG). By optimizing SSF conditions, the resulting cellulase yield achieved was 8.5-fold more than that of the wild type strain using basal wheat bran medium.

All of the mentioned studies indicated the useful effects of physical mutation methods, especially UV radiation, for fungal strains. Furthermore, these studies demonstrated that environmental accumulation of lignocellulosic wastes could be solved by mutated fungal which, can convert them to valuable products like enzymes during SSF or submerged fermentation.

4 Conclusions

A simple, rapid and one step mutation process was developed to induce mutagenesis in *A. niger* using UV irradiation. The obtained mutant strain had high potential to overproduce the cellulase. The results indicated the usefulness of central composite design for studying the effects of the mutation conditions on the production of cellulase. Second order regression model, with high coefficient of determination values ($R^2 > 0.92$ and adjusted $R^2\text{-adj} > 0.87$), were significantly ($p < 0.05$) fitted for predicting the mutant cellulase activity as a function of UV

exposure time and distance. The statistically non-significant differences between experimental and predicted values of the studied response, verified the validity of the generated model by response surface methodology.

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Bionotes



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Hoda Jafarizadeh-Malmiri

Hoda Jafarizadeh-Malmiri received his BSc and MSc degrees in food engineering (Iran). He obtained his PhD in food science from Universiti Putra Malaysia in 2012. His PhD thesis was extension of shelf life of bananas using edible coating conjugated with silver nanoparticles. He joined SUT, Iran in 2012 and is currently working as Assistant Professor in the faculty of Chemical Engineering. His fields of interest include nanobiotechnology, food biotechnology, organic and inorganic nanoparticles green synthesis.

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**Mohammad Adibpour**

Mohammad Adibpour received his Bachelor's degree in lab sciences from Tabriz University of Medical Sciences in 1990. He obtained his Master's degree in the field of medical mycology from Tehran University of Medical Sciences (1993). In 1993, he was hired as a member of the Faculty of Medicine, Tabriz University of Medical Sciences. Currently, he is working as an instructor in the School of Medicine. His research fields include diagnosis of yeast like fungi, infectivity of the yeast-like mouth disease and nanoparticles synthesis using fungi.