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Isolation, Molecular Identification and Characterization of the Culture Conditions for Extracellular Uricase Production by a New Strain of *Pseudomonas* sp.

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An uricase producing bacteria was isolated from soil through a screening program with a medium containing uric acid as sole carbon source. On the basis of the biochemical and phenotypic data, together with molecular analysis based on 16S rDNA gene sequences, this strain identified as a novel species of the genus Pseudomonas. The optimum conditions for uricase production by this Pseudomonas isolate were determined by studying the effect of some environmental and cultural factors. Among seven variables tested in our study to optimize uricase production by Pseudomonas sp., carbon and nitrogen sources, pH and $CuSO_4$ were selected based on their high significant effect on uricase production. The optimal combination of the major constituents of media for uricase production evaluated from the optimization algorithm of Box-Behnken was as follows: glucose, 0.1%; pH, 7; yeast extract, 0.5%; and $CuSO_4$, 0.001g.

Key words: Uricase; *Pseudomonas*; Optimization; Isolation.

Uricase or urate oxidase (urate: oxygen oxidoreductase, EC 1.7.3.3), a peroxisomal enzyme catalyzes the oxidative cleavage of the purine ring of urate and convert it to allantoin, carbon dioxide, and hydrogen peroxide. Uricase is not functional in some organisms including higher primates despite its role in converting highly insoluble uric acid into allantoin [1]. The increased uric acid concentration in blood can cause gout and renal failure and also it can increase the risk of tumor lysis hyperuricemia following cancer chemotherapy [2]. There is enough evidence to show that uricase can be used as protein drug to treat hyperuricemia [3,4]. Uricase also plays a crucial role as a diagnostic reagent for uric acid measurement *in vivo* [5].

Various organisms including most vertebrates animals, higher plants, fungi, and bacteria are able to produce uricase are able to produce uricase. Urate oxidase has been isolated from several sources, including *Rattus norvegicus* [6], *Glycine max* [7], *Aspergillus flavus* [8], *Pseudomonas aeroginosa* [9], *Pseudomonas acidovorance* [10], *Bacillus thermocatenulatus* [11], *Arthrobacter globiformis* [12], *Bacillus subtilis* [13], *Bacillus fastidiosus* [14], *Nocardia farcinica* [15], *Microbacterium* sp. [16].

Exploring new resources for urate oxidase is steel required due to its rising significance in therapy and in diagnostic tests and for finding

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more economic sources. In the present work, the local bacterial strains were screened for their uricase-producing ability to isolate the best microorganism which can produce higher amount of enzyme in shorter time. Then, the cultural and environmental conditions to increase the uricase production were optimized using experimental design approach.

MATERIALS AND METHODS

Sample collection, isolation and screening of bacterial isolates for uricase production

All reagents were used in this study is commercially available and of molecular biology or analytical grade. All the strains used in this study were isolated from soil samples collected from different regions of Shiraz (Fars province, south of Iran). To isolate the urate oxidase-producing microorganisms, 1 g of each sample was suspended in 10 ml of sterile distilled water and then the samples were vortexed for 1-2 min. Subsequently, one milliliter of each prepared dilution was transferred to nutrient broth as the basal medium and then incubated at 37°C for 24 h. Colonies were then picked up and subcultured in the screening medium containing uric acid as a sole carbon source (0.5% uric acid, 0.1% glucose, 0.4% NH_4NO_3, 0.5% KH_2PO_4, 1.5% microbiological agar). The pH was adjusted to 6.5±0.2. All the colonies were screened for enzyme production. Bacterial strains that were able to produce higher amount of uricase enzyme were isolated based on the size of clear zone appeared on the uric acid medium. Selected microorganisms were inoculated in broth medium. The supernatant obtained was used as the crude enzyme source and was assessed for extracellular activity of uricase. The broth media were incubated for 24 h at 37°C to obtain purified culture of uricase-producing organisms. The enzyme production was measured each 4 h up to 72 hours.

Urate oxidase assay

To determine uricase activity, the absorbance of quinoneimine dye formed by coupling with 4-aminoantipyrine, phenol, and peroxidase was measured at 505 nm by spectrophotometry. One unit of activity was defined as the formation of 1 µmol of hydrogen peroxide (0.5 µmol of quinoneimine dye) per minute at 37°C and pH 7.0.

Morphological, biochemical and molecular identification of the isolated strain

The morphological, biochemical and physiological traits of the strain were identified according to Bergey's manual of systematic bacteriology [17]. Gram staining, Simmons citrate, Indol and Triple Sugar Iron test were conducted. For further identification, 16S ribosomal DNA was analyzed. The 16S rDNA of the strain was amplified using universal 16S ribosomal DNA primers (F: 5'-CAGCCGCGGTAATAC-3' and R: 5'-ACGGGCGGTGTGTAC-3').

Screening of main factors influences uricase production by new isolate

For optimizing purpose, various medium components and culture parameters that affect uricase production have been evaluated. The effect of incubation time, temperature, pH, different carbon and nitrogen sources, uric acid concentration, and different ions were studied. One factor at a time method was used in this step.

Optimization of uricase production by new isolate

Response surface methodology (RSM) using the Box-Behnken design of experiments was used to evaluate correlation between four independent variables on production of uricase. The variables were studied in triplicate fashion at 3 levels and were designated as "1, 0 and +1 (coded values), respectively. Table 1 represents design matrix of 27 trials experiments in four independent variables containing carbon source, pH, nitrogen source, and ions. For these four factors, the equation is:

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

where Y is the predicted response, β_0 is model constant; X_1, X_2,$X_3$and $X_4$are independent variables; β_1, β_2, β_3 and β_4 are linear coefficients; β_{12}, β_{13}, β_{14} , β_{23}, β_{24} and $\beta_{34}$are cross product coefficientsand β_{11}, β_{22}, β_{33} and β_{44} are the quadratic coefficients. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2. Minitab 15 software

was used for the analysis of the experimental data obtained. Experiments were performed in triplicate fashion and mean values are given as responses.

RESULTS

Isolation and screening of microorganisms

To isolate uricase-producing bacteria, 90 soil samples were collected from different regions of Shiraz (Fars province, south of Iran). Most samples have bacterial strains were able to grow on the screening media and make clear zones in different size. The observed clear zones indicate that microorganisms were capable to degrade the uric acid of the screening media and use it as carbon source. Consumption of uric acid demonstrates that grown microorganisms could produce uricase enzyme. Fig. 1 indicates one of the evaluated strains with uricase production ability in solid medium. To assay the amount of uricase produced by each organism, the colonies which made bigger clear zones was transferred to the broth media. After 24 h incubation at 37°C, the uricase activity of each medium was measured using UV-Visible spectrophotometry. As it is revealed in Fig. 2, samples which were capable of producing uricase in broth medium, degraded uric acid leading to color change. The sample was shown the higher ability to produce uricase was chosen and considered for further studies.

Identification of isolated strain

Biochemical tests as the first step of identification revealed that the isolated strain was rod-shape gram negative bacterium. Other biochemical properties of the strain are listed in Table 3. Molecular identification of isolated strain was also performed using universal 16S ribosomal DNA primers. The obtained sequence was compared to the NCBI sequence database using BLAST algorithms, showed more than 98% identity with the homologous fragments of the strains belongs to the genus *Pseudomonas*.

Determination of the factors affecting uricase production

Evaluation of the effect of change in each variable on uricase production was carried out under situation where other mentioned factors were remained constant. The time course of uricase production was presented in 72 h. The maximum enzyme production was observed at 24 h incubation (Fig. 3). The influence of different carbon sources was studied at 24 h of incubation. It can be seen that among equal concentration of different carbon sources, glucose is the most effective carbon source of broth medium for uricase production (Fig. 4). Since nitrogen source has a significant effect on uricase production, we conducted an experiment to determine the most suitable nitrogen source for uricase production by *Pseudomonas* sp. some organic and inorganic nitrogen source were evaluated in the broth medium. The results indicates that the efficiency of different nitrogen sources are in order of yeast extract > KNO_3> NH_4NO_3> Trypton> Pepton (Fig. 5). To determine the optimum temperature for bacterial growth to produce higher amount of uricase, broth media were incubated at different

Table 1. Box-Behnken experimental design representing the response of uricase enzyme activity affected by glucose and yeast extract, pH and $CuSO_4$. R = 89.97 %.

Run Order	X_1	X_2	X_3	X_4	Measured Activity	Predicted Activity
1	1	1	0	0	0.18	0.182083
2	-1	-1	0	0	0.17	0.167083
3	1	0	-1	0	0.18	0.18875
4	-1	0	-1	0	0.19	0.19875
5	0	-1	1	0	0.17	0.164167
6	1	-1	0	0	0.18	0.182083
7	0	0	-1	1	0.21	0.205417
8	-1	0	0	-1	0.17	0.169167
9	0	0	0	0	0.17	0.173333
10	-1	1	0	0	0.19	0.187083
11	0	1	0	1	0.19	0.19375
12	0	1	-1	0	0.19	0.1875
13	0	-1	0	-1	0.18	0.185417
14	1	0	0	-1	0.23	0.224167
15	0	-1	0	1	0.17	0.17875
16	0	1	0	-1	0.19	0.190417
17	1	0	0	1	0.18	0.1725
18	0	0	1	-1	0.19	0.19375
19	-1	0	0	1	0.22	0.2175
20	0	0	0	0	0.18	0.173333
21	1	0	1	0	0.19	0.190417
22	0	-1	-1	0	0.19	0.1825
23	-1	0	1	0	0.17	0.170417
24	0	1	1	0	0.18	0.179167
25	0	0	1	1	0.18	0.182083
26	0	0	0	0	0.17	0.173333
27	0	0	-1	-1	0.20	0.197083

temperatures (15, 25, 37, 45°C) for 24h. Fig. 6 shows the optimum temperature for the growth of *Pseudomonas* sp. to produce uricase enzyme is 37°C. The optimum pH-value of broth medium suitable for uricase production was determined. The initial pH of different media was adjusted at 4, 5, 6, 7, 8, 9, 10, and 11. The results indicated that the maximum of uricase production by *Pseudomonas* sp. can be observed at pH 7(Fig. 7). Also, the results of evaluation of different concentration of uric acid (as an inducer) on uricase production showed that the optimum uric acid concentration is 0.4% (Fig. 8). To examine the suitability of different ions added to the broth media, different ions were studied. The results presented in Fig. 9 indicated that *Pseudomonas* sp. produce more uricase enzyme at the presence of $CuSO_4$.

Optimization of uricase production using response surface methodology

The results of analysis of data from the Box-Behnken experiments are given in Table 4. The

Fig. 1. A strain with uricase production ability in solid medium

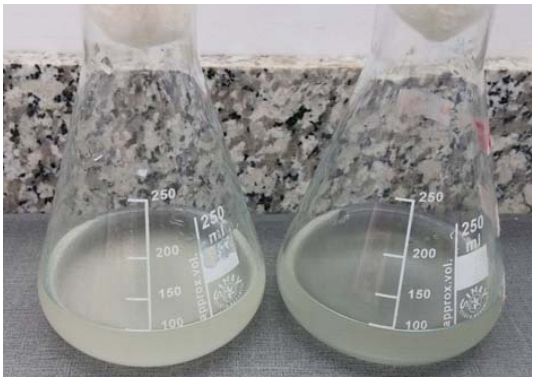

Fig. 2. A strain with uricase production ability in the broth medium: before the growth (left) and after the growth (right)

polynomial model describing the correlation between 4 factors and uricase activity could be presented as follows:

$$Y = 42.166 + 1.216X_1 + 2.433X_2 - 3.244X_3 - 0.405X_4$$

$$-1.404X_1X_2 + 2.107X_1X_3 + 0.702X_2X_3 - 7.022X_1X_4$$

$$+0.702X_2X_4 - 1.404X_3X_4 + 2.433X_1^2 - 0.405X_2^2 + 2.027X_3^2 + 4X_4^2$$

where x_1, x_2, x_3 and X_4 are carbon source, pH, nitrogen source, and $CuSO_4$, respectively. The regression analysis of the data showed coefficient of determination (R^2) value of 0.8997, and adjusted R^2 value was 0.7827, which were in excellent agreement ensuring a satisfactory adjustment of the proposed model. Consequently, only~10% of the total variance could not be justified by the model. This demonstrated that obtained Box-Behnken equation was a well-suited model to describe the response of the experiment pertaining to uricase production. To determine the optimal condition of uricase production and the relationship between the response and the significant variables, statistical analyses of variance (ANOVA) was performed through a joint

Table 2. The levels of variables chosen for the Box-Behnken Design

Variables	Variable Code	-1	0	1
Glucose (%)	X_1	0.1	0.3	0.5
pH	X_2	6.0	7.5	9
Yeast extract (%)	X_3	0.5	1	1.5
$CuSO_4$ (g)	X_4	0.0	0.001	0.002

Table 3. Biochemical properties of isolated strain

Biochemical Properties Of Isolated Strain	Result
Gram Staining	-
Glucose	-
Maltose	-
Lactose	-
Mannitol	-
Galactose	-
Fructose	-
Nitrate Reduction	+
Indol Production	-
Motility Test	+

test of four parameters (Table 2). ANOVA for uricase production shows that fitted second-order response surface model is highly significant with *F*-test =6.46 (p = 0.005) as shown in Table 4. The p-value is used for the evaluation of model significance. A very significant model has a p-value below 0.01 and a significant model below 0.05.

The optimal levels of the four factors achieved at glucose, 0.1%; pH, 7; yeast extract, 0.5%; and $CuSO_4$, 0.001g. Surface plots were presented when data from experimental design were supplied into the statistical design carried out using Minitab software. In these results, surface plots represent effect of two independent variables on enzyme production (Fig. 10).

As the regression analysis demonstrated, all variables have different effects on uricase production. According to the results, the effect of yeast extract and pH are more significant. Based on the presented correlation table, increasing of pH lead to increasing of uricase production, while the decreasing of nitrogen source from 1.5% to 0.5% lead to increasing the uricase production. Moreover, analysis of six surface plots (Fig. 10) indicated that the interaction of the two variables including carbon source and $CuSO_4$ were very high.

DISCUSSION

To date, many bacterial sources have been documented to be capable of producing uricase. *Pseudomonas acidovorans* [9], *Arthrobacter globiformis* [12], *Bacillus subtilis* [13], *Bacillus fastidious* [18], *Microbacterium* sp. [19] and *Bacillus thermocatenulatus* [11] are some of these sources. In this study, a total of 90 samples with bacterial strains that were able to grow in media containing uric acid as sole carbon source were screened. Among these samples, the 20 isolated strains with the higher ability to produce uricase based on the uricase assay data were selected for

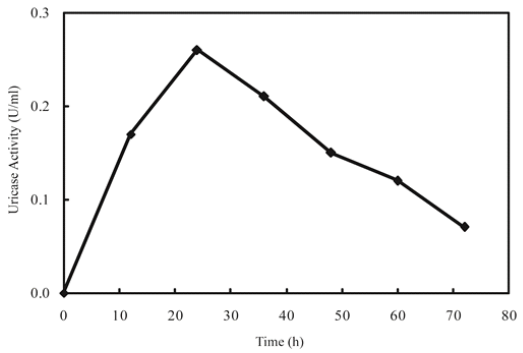

Fig. 3. Time course of uricase production

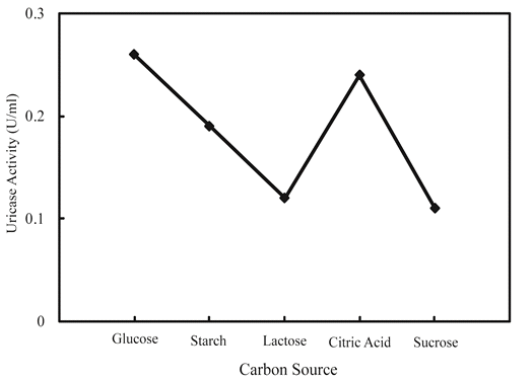

Fig. 4. The effect of different carbon source on the uricase production

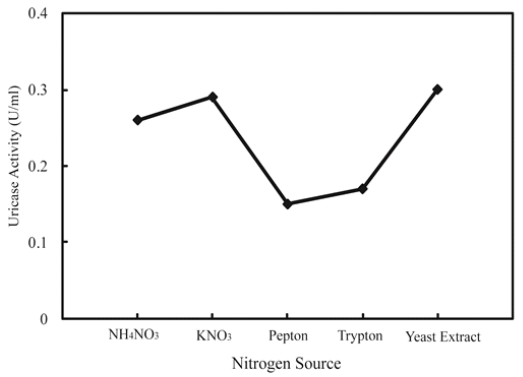

Fig. 5. The effect of different nitrogen source on the uricase production

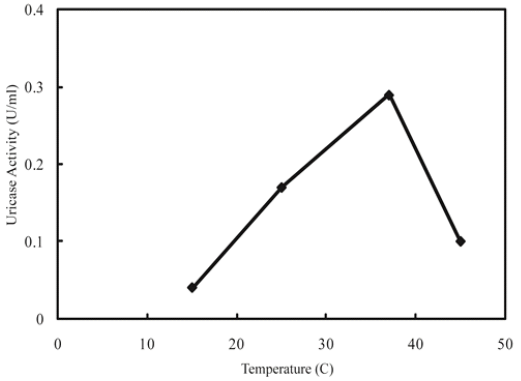

Fig. 6. Thermal stability of crude uricase

further studies. Biochemical, phenotypic and molecular analyses on the basis of 16S rDNA gene sequencing showed that *Pseudomonas* sp. is the best uricase producer. This *Pseudomonas* strain represented high uricase activity and a higher level of enzyme activity was obtained by optimization of the culture conditions. In contrast to the study was conducted on uricase production by other *Pseudomonas* species [20], the results showed that yeast extract and $CuSO_4$ are the most important variables of bacterial medium and optimum pH for uricase production in this medium is 7. Also we reached to the uricase production in 24 h cultivation, which was an important characteristic of this isolated strain. As it mentioned before, previous studies showed the maximum productivity of uricase after 30-36 hours cultivation [11, 21].

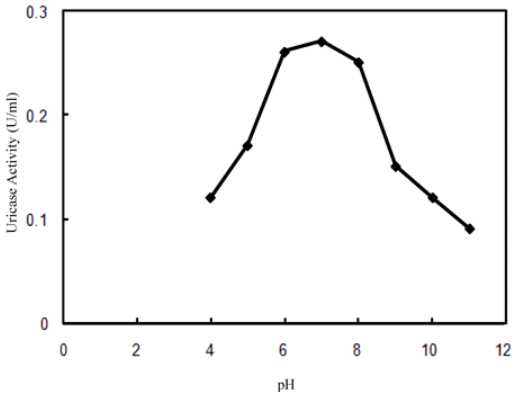

Fig. 7. The effect of different initial pH on uricase production

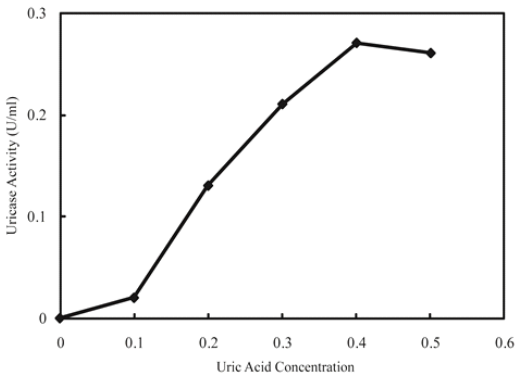

Fig. 8. The effect of uric acid concentration on uricase activity

The influence of different cultural and environmental factors was measured using two procedures including one-factor-at-a-time and Box-Behnken design. At the first step, seven factors including incubation time, temperature, pH, ions, carbon and nitrogen source, and uric acid (inducer) concentration have been evaluated. Our results showed that carbon and nitrogen sources, pH of the medium and concentration of $CuSO_4$ in the medium were important in terms of production of uricase. Among organic and inorganic nitrogen sources, it was revealed that yeast extract as an organic nitrogen source, has the most influence on uricase production. The finding of this study is in consistence with Zhou and colleagues [21], who observed that uricase production by

Table 4. Statistical analysis of Box-Behnken design

Term	Coefficient	SE Coefficient	T	P
Constant	0.173333	0.004111	42.166	0.000
X_1	0.0025	0.002055	1.216	0.247
X_2	0.005	0.002055	2.433	0.032
X_3	-0.00667	0.002055	-3.244	0.007
X_4	-0.00083	0.002055	-0.405	0.692
X_1*X_1	0.0075	0.003083	2.433	0.032
X_2*X_2	-0.00125	0.003083	-0.405	0.692
X_3*X_3	0.00625	0.003083	2.027	0.065
X_4*X_4	0.015	0.003083	4.865	0.000
X_1*X_2	-0.005	0.00356	-1.404	0.186
X_1*X_3	0.0075	0.00356	2.107	0.057
X_1*X_4	-0.025	0.00356	-7.022	0.000
X_2*X_3	0.0025	0.00356	0.702	0.496
X_2*X_4	0.0025	0.00356	0.702	0.496
X_3*X_4	-0.005	0.00356	-1.404	0.186

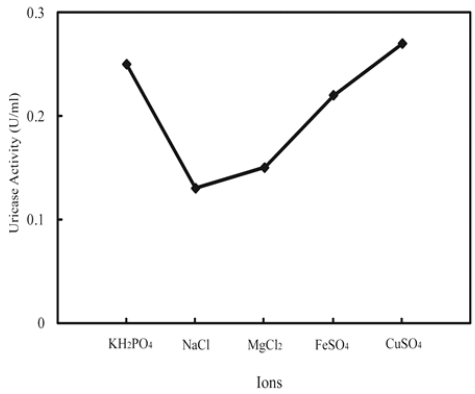

Fig. 9. The effect of different ions on uricase production

Microbacterium sp. was more enhanced by using the organic nitrogen than the inorganic nitrogen. According to Lotfy [11], *Bacillus thermocatenulatus* showed the highest level of uricase production in the presence of corn steep liquor as an organic nitrogen source. Peptone has shown the highest effect on uricase production by fungal sources like *Mucor hiemalis* [22]. In general, it seemed that as organic nitrogen sources contain most kinds of amino acids and growth factors for the growth of bacterium that could be metabolized directly by cells, uricase production was more enhanced by using organic nitrogen source than the inorganic nitrogen.

Our screening procedure revealed that the optimum pH of medium for uricase production was 7. In a similar work, *Bacillus thermocatenulatus* supported maximum production of enzyme at pH 7 [11]. However, Yazdi et al. [22] figured out that pH 6 yielded the highest uricase

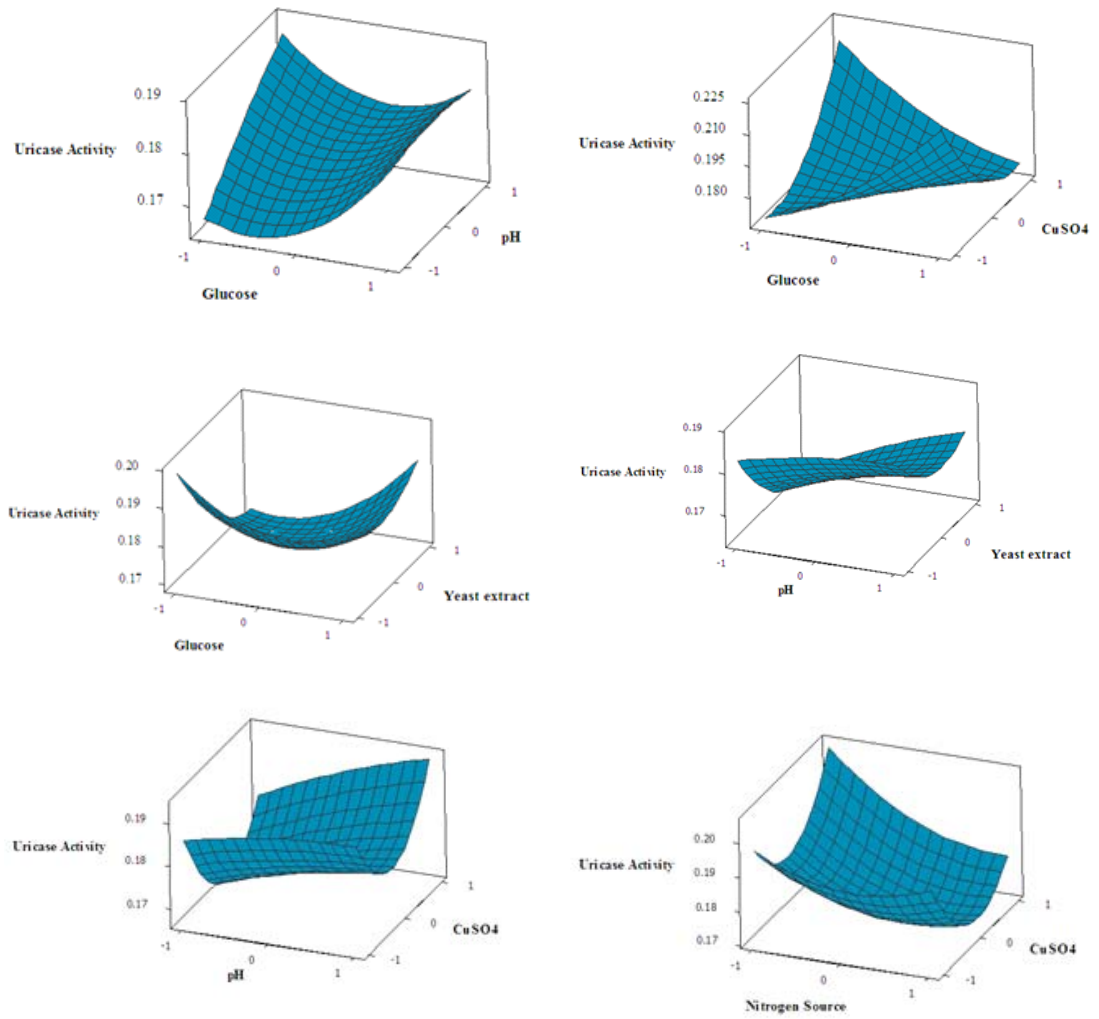

Fig. 10. Response surface plots showing the effects between either culture medium components of glucose, yeast extract and $CuSO_4$, and pH on uricase enzyme production (U/ml) by isolated strain. (a) the effect of glucose and pH; (b) the effect of glucose and $CuSO_4$; (c) the effect of glucose and yeast extract; (d) the effect of yeast extract and pH; (e) the effect of pH and $CuSO_4$; (f) the effect of yeast extract and $CuSO_4$. In each plot, the other values were hold at zero level

production for fungal producers. In this study, the maximum uricase production was achieved in the presence of $CuSO_4$. This finding is in agreement with other studies that reported the production of uricase by *Mucor hiemalis* was increased in the presence of $CuSO_4$ [20, 22]. In contrast, Saeed et al. [9] found that NaCl and $CaCl_2$ enhance uricase production by *Pseudomonas aeroginosa*.

The study showed that the highest amount of uricase production can be attained after 24 h incubation at 37°C in the broth medium adjusted to pH 7. As we mentioned before, in none of previous studies they reach such a short time. In fact, the time of incubation for obtaining high yield of uricase was varied, depends on the microorganism. Zhou and colleagues [16] reported that the highest level of uricase production was at 30°C, at pH 7.5 for 30-36 h. In another, the highest enzyme production was attained at pH 7.0 after 30-36 h incubation at 30°C [11]. In other studies on fungal resources), uricase it was expressed that the rate of uric acid consumption in the reaction mixture was increased with increasing the incubation time [22].

Also, the optimum conditions for uricase production by this *Pseudomonas* isolate were evaluated by examining the effect of most significant environmental and cultural factors. Among seven variables tested in our study to optimize uricase production by *Pseudomonas* sp., carbon and nitrogen sources, pH and $CuSO_4$ were selected based on their high significant effect on uricase production. The optimal combination of the major constituents of media for uricase production evaluated from the optimization algorithm of Box-Behnken was as follows: glucose, 0.1%; pH, 7; yeast extract, 0.5%; and $CuSO_4$, 0.001g.

As the Box-Behnken model suggested, mentioned factors had different effects on uricase production. The effect of yeast extract and pH are more significant, individually. Furthermore, prepared surface plots indicated that the effect resulted from interaction of the two variables including carbon source and $CuSO_4$ were more significant compared to the situation where their influences were evaluated individually (Table 4). Applying Box-Behnken design to optimize the selected factors for maximal production is an efficient method that tests the effect of factors interaction.

The R value of Box-Behnken model was 89.97 % for the activity of uricase, which shows a good correlation between calculated and predicted values. Satisfactory adjustment of the proposed model with about 90 % variability in response was explained by the proposed model and only 10 % of the total variance was not explained by the model. Besides, this model converts the bioprocess factor correlations into a mathematical model that predicts where the optimum is likely to be located. It is worthwhile to advise the microbial industries to apply such experimental designs to maintain high efficiency and profit bioprocesses.

In conclusion, production of a relatively high amount of extracellular uricase in a simple medium and in a relatively short time is the chief advantages of uricase production by *Pseudomonas* sp. The production yield of uricase can be enhanced by altering cultivation conditions. These characteristics make *Pseudomonas* sp. a good candidate for large-scale production of uricase, and further characterization of the biochemical and structural properties of the enzyme are warranted.

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