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Original Article

Optimization of Catalase Production by *Kocuria* sp. ASB107 Using Response Surface Methodology and Molasses as the Carbon Source

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Abstract

Catalase is an antioxidant enzyme that has various applications in different industries, such as food, dairy, textile, etc. Alkaline catalase is suitable in this regard. *Kocuria* sp. ASB107 is a radio-resistant bacterium that has been isolated from Ab-e-siahspring in Ramsar (Mazandaran Province, Iran). This bacterium is able to produce relative high amounts of alkaline catalase and hence this catalase has the potential for industrial applications. Production of enzyme in a short time and with low expenses is of a great importance. Lag phase of *Kocuria* sp. ASB107 growth takes a long time in TSB medium (about 13 hours). In order to achieve the highest rate of bacterial growth, 5% inoculum size of cells was used in the middle logarithmic phase and as a result, bacterial lag phase decreased to 2 hours. On the other hand, for economic production of catalase, sugar beet molasses was used as a cheap carbon source. Several factors effecting bacterial growth and enzyme activity were selected for the optimization of catalase production by *Kocuria* sp. ASB107 using Response Surface Methodology (RSM) based on the Central Composite Design (CCD). Four variables (carbon source, nitrogen source, agitation, and inoculum size) were selected for the optimization studies. Results showed that *Kocuria* sp. ASB107 exhibits optimum rate of catalase activity (3399.08 U/ml) and bacterial biomass (6.904 g/L) with carbon source 3% v/v, nitrogen source 0.5% w/v, inoculum size 7.5% v/v, and agitation 200 rpm.

Keywords: Catalase, *Kocuria* sp. ASB107, Optimization, RSM, Sugar Beet Molasses

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Introduction

Catalase (CAT) (EC 1.11.1.6) is one of the components of the detoxification pathways in cells, that inhibits the formation of free radicals by catalyzing the breakdown of hydrogen peroxide (H_2O_2) into H_2O and O_2 [1, 2]. This enzyme has been purified from various sources, both eukaryotic and prokaryotic cells [3]. Catalases are widespread among aerobic bacteria, such as *Escherichia coli* and *Bacillus subtilis*. Some microaerophilic and anaerobic bacteria, for instance *Campylobacter jejuni* and *Desulfovibrio gigas* possess catalase, too [4, 5]. CAT has various applications in different industries, including food, dairy, textile, cosmetics, and paper. In these industries, a large amount of hydrogen peroxide is used. To prevent the interference of H_2O_2 with subsequent procedures, this is essential that the remained hydrogen peroxide be reduced with catalase [6, 7]. H_2O_2 is more active in alkaline conditions, so alkaline CATs is so important in the mentioned industries [8]. *Kocuria* sp. ASB107,which has been isolated from Ab-e-Siah mineral radioactive spring (Ramsar, Mazandaran Province, Iran), showed relatively high resistance to gamma radiation and UV light. The anti-oxidative barrier in this bacterium consists of enzymes such as catalase [9]. *Kocuria* sp. ASB107 is able to produce relatively high amounts of alkaline catalase, thus the bacterium is a suitable candidate for industrial applications.

Kocuria sp. ASB107 has a long lag phase in TSB as its regular medium. One of the biggest aims in industrial enzyme production is shortening the production time to diminish the costs. So, the bacterial lag phase must be reduced. Features such as inoculum size and growth phase reduce the lag phase duration [10, 11]. Media which are used in the cultivation of microorganisms contain all ingredients for the synthesis of cell components, including metabolic products. In industrial fermentations, indeterminate substrates are frequently used for economic reasons. Carbohydrate source is a main component of media making up 25 to 70% of the total cost of the fermentation process. In many cases by-products of other industries, such as molasses can be used as the carbohydrate source. Molasses is a by-product of sugar production and all types of molasses contain comparatively large amounts of total sugars or carbohydrates [12]. Response Surface Methodology (RSM) is a statistical approach for pursuing the optimum conditions for a multivariable system [13, 14]. Different parameters such as physicochemical conditions and the components of the cultivation media have effects on the enzyme production. Optimization of multiple parameters is time consuming, therefore, RSM can be used to assess the significance of several factors, particularly in the cases that interactions exist among factors and are difficult to determine their effects with one factor at a time [15].

In this study, we optimized the culture medium and conditions for catalase production by response surface optimization technique.

Materials and Methods

Microorganism and Chemicals

The microorganism used in this study, *Kocuria* sp. ASB107, is maintained in the microbial bank of National Laboratory of Microbiology at Alzahra University in Tehran-Iran. Yeast extract, lysozyme, $(NH4)_2SO_4$, and NaCl were obtained from Merck Company. Sugar beet molasses was provided by Iranian Sugar Factory.

The software Minitab version 17.1.0 (Minitab Inc, USA) was used for experimental design and data analysis.

Preparation of culture medium

Kocuria sp. ASB107 was grown on TSA (Tryptic Soy Agar) medium at 30ºC for 48 h and then aseptically transferred to a 250 ml flask containing 50 ml seed medium. The initial pH of the medium was adjusted to 9 before sterilization. The seed culture contained sugar beet molasses (as the main carbon source) 2% v/v, yeast extract (as the main nitrogen source) 1.5% w/v, NaCl 5 g/L, and $(NH_4)_2SO_4$ 0.5 g/L. The culture was incubated at 30°C with shaking at 150 rpm for 24 h. After that, the seed broth was aseptically added to a 250 ml flask containing 100 ml fermentation medium (sugar beet molasses (1%, 2%, 3% v/v), yeast extract (0.5%, 1.5%, 2.5% w/v), NaCl 5 g/L, and NH_4SO_4 0.5 g/L) with inoculum size of 5% (v/v) and were also incubated at 30°C with shaking at 150 rpm. In order to preclude Maillard reaction, molasses were sterilized separately at 121°C for 15 min.

Reduction of bacterial lag time

In order to investigate the effect of inoculum size and growth phase of seed culture on the reduction of bacterial lag phase, various amounts of culture broth (1.5%, 2.5%, 5% v/v) of *Kocuria* sp. ASB107 cells in middle logarithmic phase were introduced into the basal medium.

Optimization of experiments by Response Surface Methodology

The medium for catalase production was first optimized by a one-variable-at-a-time method. subsequently, the culture medium and conditions were optimized by Response Surface Methodology (RSM) using Central Composite Design (CCD). Four factors containing concentration of carbon source (1%, 2%, 3% v/v), nitrogen source (0.5%, 1.5%, 2.5% w/v), inoculum size (2.5%, 5%, 7.5% v/v), and agitation (100, 150, 200 rpm) were chosen as independent variables. Each factor was studied at three different levels as listed in Table 1. A total of 56 experiments designated with using the Minitab software (Table 2). Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA).

Desirability function

Desirability function is a general and recognized technique to concurrently determine the input variables that can lead to the optimum presentation levels for response. The desirability 1 is used for maximum and desirability 0 is used for minimum (or non-desirable) situations [16].

Statistical analysis

The experimental design and statistical analysis of the data were performed using Minitab software (ver. 17.1.0), at 95% level of significance.

Table 1. Ranges of the four independent variables used in RSM.

Code	Independent variables	Levels
A	Carbon source	1, 2, 3
B	Nitrogen source	0.5, 1.5, 2.5
C	Inoculum size	2.5, 5, 7.5
D	Agitation	100, 150, 200

Catalase assay

At late logarithmic phase, fermentation product was centrifuged at 4°C and 15000 g for 15 min. Bacterial biomass was divided into two parts, one part was dried at 65°C and weighed to determine the bacterial growth. The other part of the biomass was used to assay catalase activity, for which it was washed with phosphate buffer (50 mM). In order to extract the enzyme, biomass was treated with lysozyme and the cell debris were removed by centrifugation at 4°C and 20000 g for 30 min. The catalase activity in the supernatant was measured spectrophotometrically by checking the decrease in absorbance at 240 nm affected by the decomposing of hydrogen peroxide. The reaction mixture in a total volume of 1 ml was composed of 10 µl enzyme solution, 50 mM K_2HPO_4/KH_2PO_4 (pH 7.0), and 35 mM H_2O_2. One unit of catalase activity was defined as the amount of enzyme required to transform 1 µM of hydrogen peroxide to water and oxygen per min ($\varepsilon = 43.6/M\ cm$) [17, 18].

Results

The results showed that inoculum size has a strong effect on the duration of the lag phase of *Kocuria* sp. ASB107 so that increase in inoculum size, decreases the duration of lag phase (Fig. 1 & 2). The results of optimization with response surface methodology showed that the maximum catalase production (3399.08 U/ml) and bacterial biomass (6.904 g/L) could be achieved under the following conditions: carbon source 3% v/v, nitrogen source 0.5% w/v, inoculum size 7.5% v/v, and agitation 200 rpm (Table 2).

Central composite design revealed that four independent variables, including carbon source, nitrogen source, agitation, and inoculum size significantly influenced the catalase production (P-Value < 0.05) (Table 3). The mathematical model relating to the catalase production with the independent process variables, A, B, C, and D is given in the second-order polynomial equation.

$$X=5580+ 351\ A+ 2522\ B+ 771\ C- 117.24\ D- 6.7\ A^2- 485.5\ B^2- 71.07\ C^2+ 0.4118\ D2 - 184.7\ AB - 22.92\ AC + 1.952\ AD + 26.61\ BC - 4.680\ BD + 0.698\ CD$$

$$Y=-0.0697- 0.0127\ A+ 0.0573\ B+ 0.00268\ C+ 0.001432\ D+ 0.00218\ A^2+ 0.00243\ B^2+0.000109\ C^2+ 0.000001\ D^2+ 0.00277\ AB+ 0.001143\ AC+ 0.000036\ AD- 0.003542BC- 0.000236\ BD + 0.000018\ CD$$

Where X is the catalase activity yield, Y is bacterial growth, A is carbon source, B is nitrogen source, C is inoculum size, and D is agitation. Significant P-values suggests that the obtained experimental data fits well with the model. The fitness of the model was also checked by determination of coefficient (R^2) with $R^2$of 96.97% and 97.06% for catalase activity and bacterial growth, respectively. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA).

This analysis is carried out for a level of significance of 95%. According to Table 2 every four factors are important for catalase production (P-Value <0.05). The desirability values for the responses are shown in Figure 3. The number in bracket indicates the optimal level of the related parameter. The desirability value varies between 0 and 1, depending on the closeness of the outputs towards the target. Figure 4 shows the response surface plots of catalase production.

Table 2. Central composite design with responses for catalase production and bacteria biomass for *Kocuria* sp. ASB107.

Run	Carbon source (% v/v)	Nitrogen source (% w/v)	Inoculum size (% v/v)	Agitation (rpm)	Enzyme activity (U/ml)	Biomass (g/L)
1	1	0.5	2.5	100	791.28	2.02
2	3	0.5	2.5	100	1757.39	2.36
3	1	2.5	2.5	100	1690.94	3.34
4	3	2.5	2.5	100	1655.95	3.66
5	1	0.5	7.5	100	1397.39	2.6
6	3	0.5	7.5	100	1766.06	2.66
7	1	2.5	7.5	100	2740.83	3.3
8	3	2.5	7.5	100	2332.57	3.94
9	1	0.5	2.5	200	1621.56	5.46
10	3	0.5	2.5	200	2424.46	5.46
11	1	2.5	2.5	200	1661.96	5.88
12	3	2.5	2.5	200	2064.22	6.51
13	1	0.5	7.5	200	2483.95	6
14	3	0.5	7.5	200	3368.95	6.904
15	1	2.5	7.5	200	2619.27	5.9
16	3	2.5	7.5	200	2988.53	6.36
17	1	1.5	5	150	1866.97	4.06
18	3	1.5	5	150	2136.24	4.5
19	2	0.5	5	150	1476.86	3.84
20	2	2.5	5	150	1653.67	4.66
21	2	1.5	2.5	150	1357.65	4.12
22	2	1.5	7.5	150	2143.66	4.4
23	2	1.5	5	100	2674.31	3.26
24	2	1.5	5	200	3291.97	5.26
25	2	1.5	5	150	1885.05	4.32
26	2	1.5	5	150	1966.69	4.48

27	2	1.5	5	150	1940.37	4.68
28	2	1.5	5	150	1978.76	4.58
29	1	0.5	2.5	100	779.82	2.02
30	3	0.5	2.5	100	1766.06	2.44
31	1	2.5	2.5	100	1685.78	3.34
32	3	2.5	2.5	100	1676.85	3.66
33	1	0.5	7.5	100	1400.48	2.6
34	3	0.5	7.5	100	1834.86	2.66
35	1	2.5	7.5	100	2740.83	3.16
36	3	2.5	7.5	100	2389.91	4
37	1	0.5	2.5	200	1600.92	5.46
38	3	0.5	2.5	200	2372.71	5.46
39	1	2.5	2.5	200	1621.56	5.88
40	3	2.5	2.5	200	2019.74	6.51
41	1	0.5	7.5	200	2545.87	6
42	3	0.5	7.5	200	3399.08	6.904
43	1	2.5	7.5	200	2493.12	5.74
44	3	2.5	7.5	200	2814.22	6.36
45	1	1.5	5	150	1862.39	4
46	3	1.5	5	150	2185.78	4.5
47	2	0.5	5	150	1518.35	3.84
48	2	2.5	5	150	1678.65	4.74
49	2	1.5	2.5	150	1384.17	4.02
50	2	1.5	7.5	150	1863.53	4.4
51	2	1.5	5	100	2645.83	3.26
52	2	1.5	5	200	3277.52	5.26
53	2	1.5	5	150	1956.78	4.9
54	2	1.5	5	150	1936.45	4.48
55	2	1.5	5	150	1984.75	4.48
56	2	1.5	5	150	1988.46	4.54

Table 3. Analysis of variance for catalase production.

Terms	Coefficient	F-Value	P-Value
Constant	0.21779	-	0.000
Carbon Source	0.01123	27.82	0.000
Nitrogen Source	0.01702	63.84	0.000
Inoculum size	0.00873	16.82	0.000
Agitation	0.07365	195.94	0.000
Carbon* Carbon	0.00218	0.15	0.700
Nitrogen * Nitrogen	0.00243	0.19	0.668
Inoculum size*Inoculum size	0.00068	0.01	0.904
Agitation * Agitation	0.00193	0.12	0.733
Carbon*Nitrogen	0.00277	1.50	0.227
Carbon*Inoculum size	0.286	1.60	0.213
Carbon*Agitation	0.00179	0.63	0.432
Nitrogen*Inoculum size	-0.00886	15.37	0.000
Nitrogen*Agitation	-0.01179	27.26	0.000
Inoculum size*Agitation	0.00229	1.03	0.316
Lack- of- fit	-	1.09	0.31

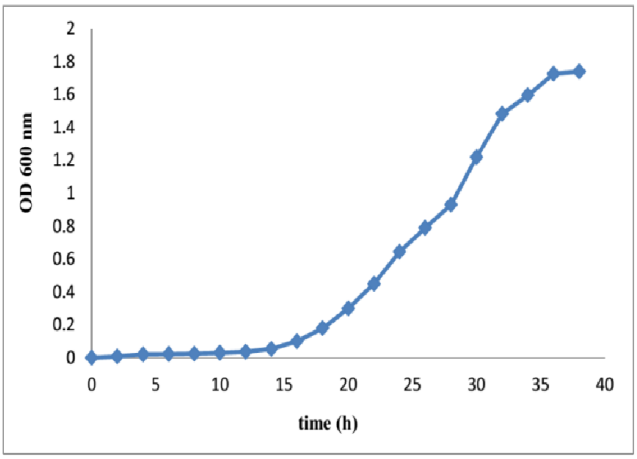

Figure 1. Growth curve of *Kocuria* sp. ASB107.

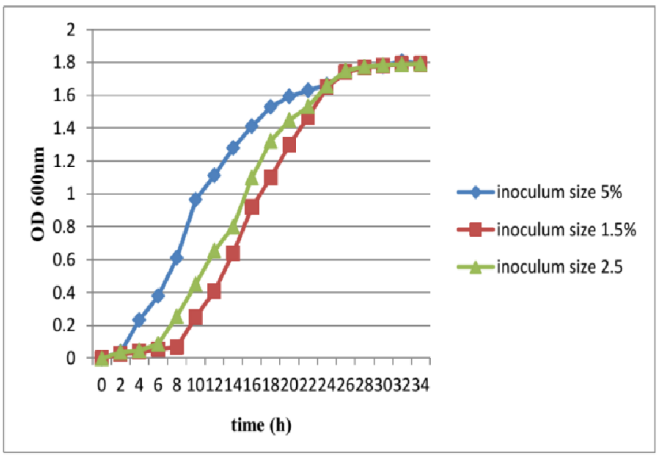

Figure 2. Effect of inoculum size on the lag phase reduction of *Kocuria* sp. ASB107.

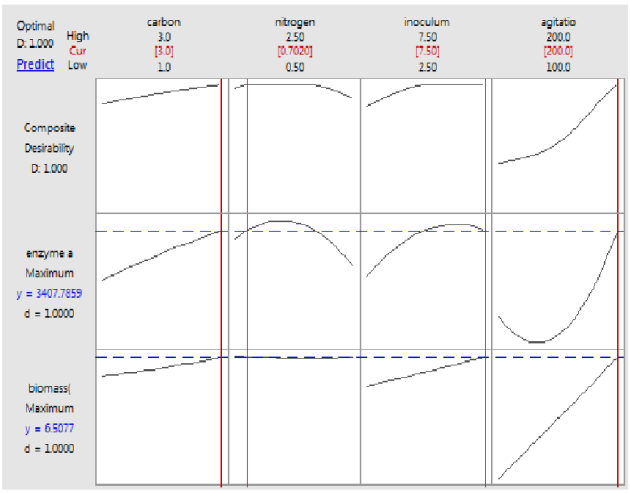

Figure 3. Maximized desirability for response: Biomass and enzyme activity are presented. The numbers in bracket indicate the optimal level of the related parameter. The desirability value varies between 0 and 1 depending on the closeness of the outputs towards the target.

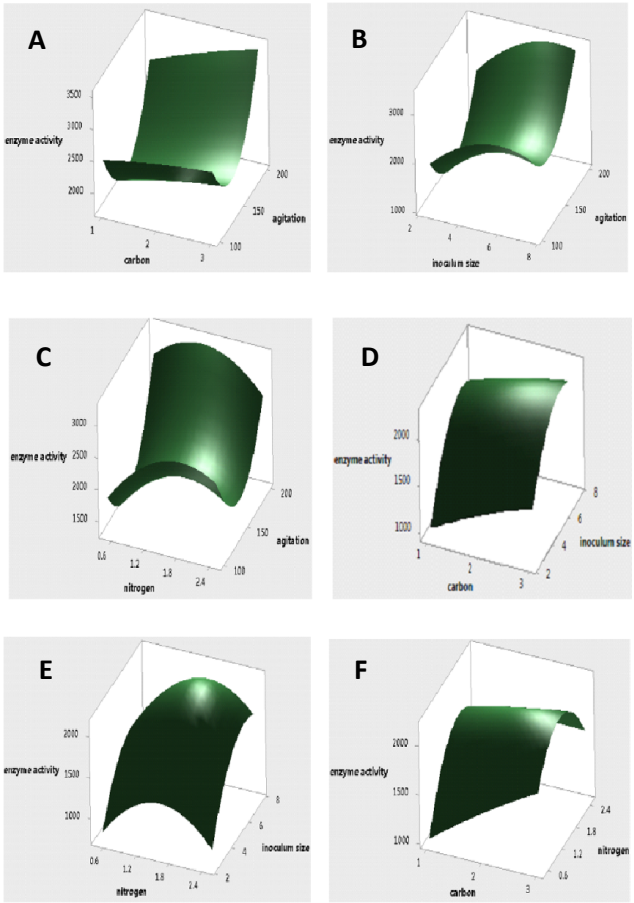

Figure 4. Surface plot for catalase production showing the interactive effects of carbon source and agitation (A), inoculum size and agitation (B), nitrogen source and agitation (C), carbon source and inoculum size (D), nitrogen source and inoculum size (E), and carbon source and nitrogen source (F).

Discussion

Kocuria sp. ASB107, isolated from the Ab-e-Siah mineral radioactive spring (Ramsar, Mazandaran Province, Iran), is able to produce relatively high amounts of alkaline catalase, which is suitable for industrial purposes.

Kocuria sp. ASB107 has a long lag phase (13 h) in TSB as its regular medium. When molasses was used as the cabone source in the culture medium, the effect of inoculum size and growth phase of seed culture on the duration of the lag phase was investigated. Using 5% inoculum size (when cells are in mid logarithmic phase), bacterial lag phase decreased to 2 hours. In other bacteria, such as *Bacillus* [19] and *Salmonella* (with sucrose as the carbon source) [20] the duration of the lag phase is also dependent on the inoculum size. As molasses are mainly composed of sucrose and glucose, it is a suitable source for bacterial growth. On the other hand, molasses as a carbon source might have contributed better growth due to the presence of growth promoting components [21, 22]. So, the carbon source can be considered as a factor in reducing the duration of bacterial lag phase. The amount of inoculum affects the lag phase because many bacterial species use quorum sensing to organize gene expression and hence by communicating with each other using signaling molecules, stimulate bacterial growth [23].

Using products such as molasses from other industries is desirable for their low cost [24]. In a research, medium components were optimized for enhance acetoin production by *Bacillus subtilis* from molasses. Results showed that molasses are more productive than pure sucrose in acetoin fermentation [25]. In another study, the production of alkaline protease from *Bacillus pantotheneticus* using molasses as a substrate was investigated. The results showed, among the four studied carbon sources (Glucose, Glycerol, Starch, and molasses), molasses is the best source for the protease production [26].

Nowadays, optimizing the process using Response Surface Methodology will function as a potential example for the applications used in industrial microbial fermentations [25].

In this study, we optimized the culture medium and conditions for catalase production through Response Surface optimization techniques. The P-value showed that all selected factors (carbon source, nitrogen source, inoculum size, and agitation) and their interactions were significant ($p<0.05$). On the other hand, agitation factor, which has highest F-Value among other factors, is the most significant factor in the production of the catalase. Also, the P-value > 0.05 for lack of fitness represents a good model. P-values for lack of fitness in catalase production was 0.31, which is more than 0.05 ($p> 0.05$). These results properly validate the model. The catalase production (3399.08 U/ml) by *Kocuria* sp. ASB107 using RSM in the present study was higher than the catalase produced by *Serratia marcescens* SYBC-01 (621.8 ± 68.5 U/ml) [1] and *Bacillus* sp. F26 (29.9 U/ml) [8]. The results showed that molasses is a suitable and inexpensive substrate for catalase production, because it contains proteins, minerals, vitamins, etc. Catalase production (3399.08 U/ml) in *Kocuria* sp. ASB107 using molasses was higher than the

catalase produced (621.8 ± 68.5 U/ml) *by Serratia marcescens* SYBC-01 with starch as carbon source [1]. In the present study, the catalase production by *Kocuria* sp. ASB107 using RSM (3399.08 U/ml) was higher than the catalase produced via one-variable-at-a-time method (2136/25 U/ml) [27]. So, the application of response surface methodology resulted in an enhancement in catalase production and also reduces the cost of production.

Due to the importance of catalase in various industries and the need to produce this enzyme at the lowest cost and the shortest possible time, it is essential that adequate resources for the production of the enzyme be identified.

Conclusion

The results of the present study indicate that *Kocuria* sp. ASB107 is a promising candidate for the low-cost, large scale catalase enzyme production in the industry.

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