Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS

J. Serb. Chem. Soc. 84 (10) 1093–1104 (2019)
JSCS–5249

UDC 664.644.5+579.832:66.011:519.22
Original scientific paper

Optimization of the thermostable alkaline and Ca-dependent α-amylase production from *Bacillus paralicheniformis* by statistical modeling

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(Received 25 September 2018, revised 4 March, accepted 6 March 2019)

Abstract: A novel amylolytic enzyme producing thermoalkaliphilic bacterium, the source of industrially used enzymes was isolated. Isolated strain was identified by morphological, physio-biochemical tests and the 16S rRNA gene sequence analysis. The optimal conditions of enzyme activity were determined. For higher α-amylase production, the variables such as yeast extract, starch, $CaCl_2$, $(NH_4)_2SO_4$, NaCl and $MgSO_4$ in the α-amylase production medium, the temperature and pH were screened by Plackett–Burman design and optimised using response surface methodology (RSM). The optimal conditions were found to be 0.15 g/L for starch, 0.15 mg/L for $CaCl_2$ and 60 °C for temperature. By using RSM model, amylase production increase was achieved sevenfold. It is showed that this method can be utilised to optimize α-amylase production in athermophilic bacteria such as *Bacillus paralicheniformis*.

Keywords: α-amylase; *Bacillus paralicheniformis*; optimization; response surface methodology.

INTRODUCTION

α-Amylases (1,4-α-D-glucan glucanohydrolase; E.C.3.2.1.1) belong to family 13 (GH-13) of the glycoside hydrolase group of enzymes that cleaves the internal α-1,4-glycosidic linkages in polysaccharides with the retention of α-anomeric configuration in the products.[1]

Amylases are among the most studied industrial enzymes accounting for nearly 30 % of enzyme production in the world. They have many applications including brewing, baking, fermentation, production of starch hydrolysis pro-

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https://doi.org/10.2298/JSC190227021B

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ducts such as glucose and fructose, biodegradation of *n*-alkanes, textile, paper, detergent, synthesis of nanoparticles[2-6] as well as use in medical and pharmaceutical industry.

In the industrial production of microbial-derived enzymes, scientists have focused on studying the isolation and characterization of new promising strains using different carbon and nitrogen sources. Microbial-derived enzymes have been preferred to enzymes isolated from complex eukaryotes[7] due to their biochemical diversity and ease with which enzyme concentrations may be increased by environmental and genetic manipulations.

Microorganisms are the most important sources for enzyme production. Almost all members of the *Bacillus* genus can synthesize α-amylase and are considered to be the most important sources of this enzyme because of: high biomass development rate, thermostability, less time and less space-consuming, cheap condition for production and easier process modification and optimization. Optimization of nutritional and physicochemical parameters is crucial to ensure that the industrial process is economically and viable cost-effective as the optimal conditions for maximum enzyme production vary widely depending on strain.[1,8,9]

Traditionally, one variable at a time approach (OVAT) has been often used by researchers to optimize the specific effects of the best parameters and variables for scanning physical and chemical parameters. The conventional method for optimizing enzyme production “one variable at the time” approach involves varying a single independent value, while maintaining the others at a constant level. However, many experimental tests have to be done for this and these processes will take more time. It is well known that when evaluating the interaction between variables it is quite difficult and time-consuming as well as unsuccessful in finding the optimum response results. For this reason, a more informative and practically accessible two-level factorial model can be used to easily analyze the interaction between factors. A factor-based statistical method called Plackett––Burman design (PBD) is used for evaluating the critical interactions of independent process variables, while to evaluate the interactions of the independent process variables response surface methodology (RSM) is mainly used.[10]

Bacillus paralicheniformis is known as Gram-stain-positive, rod-shaped, facultative anaerobic, mobile, endospore forming bacteria. Based on phylogenetic and phenotypic analyzes by Dunlap *et al.*,[11] it was concluded that this strain represents a new species from the *Bacillus* genus. The aim of this study is to enhance production of α-amylase from a newly isolated thermophilic *B. paralicheniformis* from Sorgun hot spring, Turkey.

EXPERIMENTAL

Isolation and identification of amylase producing microorganism

The thermophilic amylase producing bacterial strain was isolated from water samples from Sorgun Hot Spring (39° 48' 14.0718" N, 35° 12' 31.0752" E), Yozgat (in the Central

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Anatolia Region of Turkey) using starch agar plates and incubated at 50 °C for 48 h. Amylase producing bacterial strain was obtained by the clearance test using Gram's iodine staining method. The selected amylase producing strain was phenotypically (shape, size, colour, Gram and spore staining, etc.) characterized and identified by the use of conventional biochemical methods (catalase, indole, oxidase, citritase and urease activity) and also molecular characterization was conducted by 16S rDNA sequencing. The selected strain was inoculated in Basal medium (BM, consisted (g/L) of yeast extract, 2.0; starch, 1.0; $CaCl_2$, 0.05; $MgSO_4·7H_2O$, 0.05; KH_2PO_4, 1.0; NaCl, 2.0; $(NH_4)_2SO_4$, 2.0) and incubated at 50 °C for 24 h in a shaker. The culture was centrifuged at 10.000 rpm at 4 °C for 10 min. and cell-free supernatant was used for enzyme activity. The α-amylase activity was determined using DNS method according to Bernfeld[12] and the protein content was determined by the Lowry method.[13]

One-variable-at-a-time approach

BM medium components were changed for enhancing the α-amylase activity by OVAT approach. To enhance the production of α-amylase, different nitrogen sources (0.05––0.02 %: peptone, yeast extract, ammonium chloride and ammonium sulphate), different carbon sources (0.05–0.02 % such as glucose, galactose, fructose, maltose and starch) and different metal ions in the form of salts (0.05–0.02 % such as $CaCl_2$, NaCl and $MgCl_2$) with the BM media were prepared and incubated at 50 °C for 24 h in a shaker. Similarly, influence of pH (6.0–9.0) and temperature (50–60 °C) on α-amylase production was tested. All the experiments were performed in triplicate.

Plackett–Burman design

PBD was used for identifying the significant variables and optimal level of each variable for higher α-amylase production. A total of six variables of medium (starch (X_1), yeast extract (X_2), $(NH_4)_2SO_4$ (X_3), $CaCl_2$ (X_4), $MgSO_4$ (X_5), NaCl (X_6)) and also two variables of culture, such as pH (X_7) and temperature (X_8) were studied to identify the most important variables for higher amylase production (Table I).

TABLE I. Experimental range and levels of the independent process variables to study on α-amylase activity

Variable	Symbol	Coded value	
		–1	+1
Concentration of starch, g L^{-1}	X_1	0.05	0.2
Concentration of yeast extract, g L^{-1}	X_2	0.1	0.5
Concentration of $(NH_4)_2SO_4$, mg L^{-1}	X_3	0.1	0.5
Concentration of $CaCl_2$, mg L^{-1}	X_4	0.05	0.2
Concentration of $MgSO_4$, mg L^{-1}	X_5	0.05	0.2
Concentration of NaCl, mg L^{-1}	X_6	0.05	0.2
pH	X_7	6.0	9.0
Temperature, °C	X_8	50	60

In this study, 12 experiments were performed to determine the most affected variables for amylase production (Table II). We used the following linear regression equation for the experimental data:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i \quad (1)$$

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In the equation each symbol used represents as follows. Y – a response for amylase enzyme activity (U mg^{-1}), β_0 – model intercept, β_i – linear coefficient.

TABLE II. Experimental design with independent variables applied in Plackett–Burman design for amylase production

Run	Variable								Amylase activity, U mg^{-1}		
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	Observed	Predicted	Residual value
1	–1	–1	–1	–1	–1	–1	–1	–1	127.68	137.53	–9.85
2	–1	1	1	1	–1	1	1	–1	180.42	196.37	–15.95
3	–1	–1	1	1	1	–1	1	1	230.28	218.69	11.59
4	1	1	–1	1	1	–1	1	–1	350.66	356.14	–5.48
5	1	1	1	–1	1	1	–1	1	264.50	274.35	–9.85
6	1	–1	–1	–1	1	1	1	–1	250.87	245.39	5.48
7	–1	–1	–1	1	1	1	–1	1	204.70	216.29	–11.59
8	–1	1	1	–1	1	–1	–1	–1	164.90	155.05	9.85
9	–1	1	–1	–1	–1	1	1	1	184.76	168.81	15.95
10	1	1	–1	1	–1	–1	–1	1	373.06	367.58	5.48
11	1	–1	1	–1	–1	–1	1	1	247.65	259.23	–11.58
12	1	–1	1	1	–1	1	–1	–1	300.35	284.39	15.96

By using p-value, significant variables can be measured for probability. The statistical significance is considered if p-value is less than 0.05 (Table III). Statistical analysis was carried out by the Minitab 15 Statistical Software (Minitab, Inc., State College, PA).

TABLE III. Statistically derived effects and coefficients by Plackett–Burman design for amylase production; R^2: 97.45 %, T: T-test value, P: probability value; *: indicates significant variables

Term	Effect	Coefficient	T	P
Constant	–	239.99	36.61	0.0
X_1	115.72	57.86	8.83	0.003*
X_2	26.13	13.06	1.99	0.140
X_3	–17.27	–8.63	–1.32	0.279
X_4	66.51	33.25	5.07	0.015*
X_5	8.66	4.33	0.66	0.556
X_6	–18.10	–9.05	–1.38	0.261
X_7	1.57	0.78	0.12	0.912
X_8	21.68	10.84	1.65	0.197

Response surface methodology

Face centred central composite design (FCCCD), which is employed to determine the effects of independent variables, was used to evaluate the optimal level of eight variables. Three major variables out of eight have been chosen to bring optimization study further (Table IV) and hereby twenty experimental runs were conducted (Table V).

A second order non-linear polynomial equation (2) shown below was used to fit the data:

$$Y = \beta_0 + \sum_{i=1}^{n}\beta_i X_i + \sum_{i=1}^{n}\beta_i X_i^2 + \sum_{i=1}^{n-1}\sum_{j=i+1}^{n}\beta_{ij} X_{ij} + \varepsilon \quad (2)$$

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where Y is response for the amylase activity (U/mg); independent variables are represented as X_i and X_j; β_0 is the model constant; β_i, β_{ii}, β_{ij} are denoted as the linear, square and the interaction effect respectively. X_i and X_j are independent variables and ε is the random error in the above equation. Design Expert 8.0 programme is used to perform response surface graphs.

TABLE IV. Independent variables of selected parameters for RSM–face centred central composite design

Variable	Level		
	–1	0	+1
Concentration of starch (X_1), g/L	0.05	0.1	0.15
Concentration of $CaCl_2$ (X_4), mg/L	0.05	0.1	0.15
Temperature (X_8), °C	50	55	60

TABLE V. Experimental design and result of reduced model CCD

Run no.	Coded variable			Amylase activity, U mg^{-1}		
	X_1	X_4	X_8	Observed	Predicted	Residual
1	1	1	1	367.52	371.76	–4.24
2	–1	1	1	142.22	153.02	–10.80
3	1	–1	1	253.53	241.45	12.08
4	1	1	–1	295.38	299.60	–4.22
5	1	–1	–1	193.96	185.98	7.98
6	–1	1	–1	104.91	119.81	–14.90
7	–1	–1	1	91.93	90.52	1.41
8	–1	–1	–1	75.44	74.01	1.43
9	–1	0	0	100.59	77.72	22.87
10	1	0	0	231.48	243.08	–11.60
11	0	–1	0	134.39	157.29	–22.90
12	0	1	0	279.51	245.34	34.17
13	0	0	–1	128.31	118.60	9.71
14	0	0	1	164.49	162.93	1.56
15	0	0	0	150.88	155.23	–4.35
16	0	0	0	151.66	155.23	–3.57
17	0	0	0	151.93	155.23	–3.30
18	0	0	0	151.42	155.23	–3.81
19	0	0	0	151.27	155.23	–3.96
20	0	0	0	151.66	155.23	–3.57

RESULTS AND DISCUSSION

The bacteria isolated from water samples were screened for amylase production on starch agar medium. From the water samples 4 bacterial strains were isolated, but only 1 strain showed amylase activity. The obtained isolate was designated as FMB2-1. The strain FMB2-1 was identified by morphological, physiological, biochemical and 16S rRNA gene sequencing. The isolate was observed under microscope and was found to be rod shape, motile, Gram-positive and spore forming bacteria. The isolate FMB2-1 was positive for catalase, indole, oxidase, citritase, urease, and starch hydrolysis, while it was negative for casein

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and gelatine hydrolysis. The total 16S rRNA sequence analysis of the FMB2-1 strain (GenBank accession number is KP992870) indicates that the strain is a member of genus *Bacillus* and showing a high similarity to *Bacillus paralicheniformis* (99.65 %). The neighbour-joining method was utilised to construct a phylogenetic tree for the novel isolate FMB2-1 showing the position within the species of the *Bacillus* genus (Fig. 1).

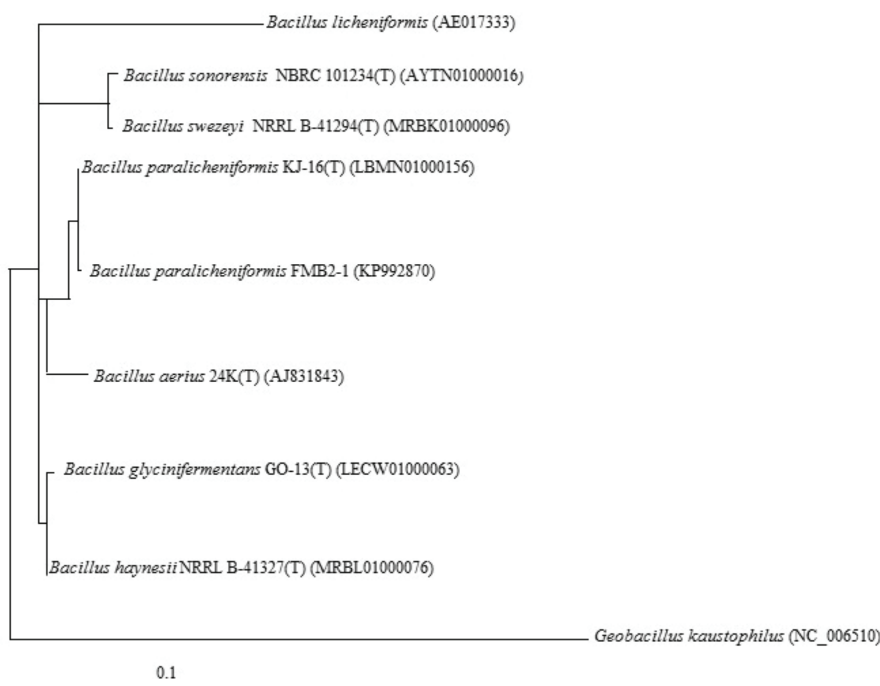

Fig. 1. Partial 16S rDNA sequence-based phylogenetic neighbour-joining tree showing the phylogenetic relationship of strain FMB2-1 relative to other strains of the genus *Bacillus*. The tree topology was obtained by calculation using the CLC Sequence Viewer 6 program. *Geobacillus kaustophilus* is used as the out-group. Bar indicates 0.1 nucleotide substitutions per position.

One variable at a time approach and Plackett–Burman design

In this study, different carbon sources (glucose, galactose, fructose, maltose and starch), nitrogen sources (peptone, yeast extract, ammonium chloride and ammonium sulphate), metal ions ($CaCl_2$, NaCl and $MgCl_2$), pH (6.0–9.0) and temperature (50–60 °C) were evaluated for optimal amylase production. In our study, among carbon and nitrogen sources, starch (356.1 U/mg), yeast extract (325.4 U/mg) and ammonium sulphate (322.8 U/mg) supported bacterial growth and were important factors for the synthesis of amylase by the isolate. In previous studies, the yeast extract was found to reduce the lag phase by promoting

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faster bacterial growth.[14,15] In addition, the utilization of soluble starch by *Bacillus* sp. were reported previously.[16-18]

In this study, amylase production was increased in the presence of $CaCl_2$ (407.4 U/mg), NaCl (321.1 U/mg) and $MgCl_2$ (210.5 U/mg) for *B. paralicheniformis* FMB2-1. Most of α-amylases are known as metalloenzymes that require calcium ions (Ca^{2+}) for their activities, structural integrity and thermal stability. The crystal structure of α-amylase shows that calcium ion is involved in ionic interaction between domain A and domain B between which α-amylase active site is located and thus calcium ion form an ionic bridge between these domains promoting α-amylase stability and catalytic activity.[19] In previous studies, other *Bacillus* species such as *B. cereus*,[20] *B. licheniformis*,[21] *B. licheniformis* AT70,[18] *B. licheniformis* ATCC 9945a[22] and *B. stearothermophilus*[23] have maximum amylase production in the presence of $CaCl_2$.

Changes in pH may lead to a breakage in the ionic bonds that hold the tertiary structure of the enzyme which in turn causes the enzyme to lose its function. pH changes may also shift the amino acids charges in the active-site leading to enzyme–substrate complex disruption.[9] Optimum amylase production was obtained at pH 9.0 compared with some other amylases from *Bacillus* species including *B. mojavensis* SA,[24] *Bacillus* sp. BCC 01-50[25] and *B. subtilis* AS-SO1a,[26] the optimum pH of our enzyme is more favourable for industrial production. Amylases have activity at alkaline pH ranging from 9.0 to 11.0, due to their stability under detergent conditions and the oxidative stability of amylases makes it good ingredient for detergents. In addition, stability of alkaline conditions is an important criteria for their use in detergents where the washing environment is very oxidizing and removal of starch from surfaces in providing a whiteness benefit.[27-29] Bacterial amylases are produced at a much wider range of temperature. Burhan,[28] Oyeleke *et al.*,[30] Sharma and Satyanarayana[31] and Afrisham *et al.*[18] demonstrated that the maximum amylase enzyme was produced at the temperature of 60 °C by *Bacillus* sp. A3-15, *B. megaterium*, *B. acidicola* and *B. licheniformis* AT70, respectively. Maximum enzyme production was at 60 °C by *B. paralicheniformis* FMB2-1. Thermostable α-amylases are desired as they minimize contamination risk and reduce reaction time, thus saving considerable amount of energy.[32] Thermostable amylases are used in the industry such as bakery industry, biocatalysis, clarification of beer or fruit juices, detergents, textile, pretreatment of animal feed to improve the digestibility of fiber and hydrolysis and modification of starch to produce glucose and fructose syrup, crystalline dextrose, dextrose syrup, maltose and maltodextrins.[2,33-36]

In Table III, the effects of each variable are shown according to the PBD. It consists of 12 runs and their corresponding amylase activities, starch, $CaCl_2$ and temperature showed positive effects, while the other variables showed negative effects.

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In previous studies, other researchers have tried amylase optimization using the RSM method. Roy and Mukherjee,[37] Keharom *et al.*[38] and Stergiou *et al.*[39] found positive effects of starch, Zambare[40] and Mustafa *et al.*[41] found positive effects of temperature, and Gangadharan *et al.*[42] found positive effects of $CaCl_2$ on amylase production using RSM method. The variables of probability p-value <0.05 were considered significant, and p-value higher than 0.05 were not considered significant. Using the experimental data in terms of actual values of the tested variables in this work the following linear regression model (Eq. (3)) was developed:

$$Y = 239.992 + 57.862X_1 + 13.066X_2 - 8.63X_3 + 33.259X_4 + + 4.333X_5 - 9.053X_6 + 0.787X_7 + 10.840X_8 \quad (3)$$

The model validated by the correlation coefficient (R^2) which was found 97.45 %, indicates that there is only 0.2 % variation in the data that could not be explained by the model (Table III).

For studies on the amylase production optimization, temperature, starch and $CaCl_2$ are considered to be the most important variables. Since the other components and conditions resulted in a negative effect, they were kept at a lower level (–1) in the medium used for production.

Response surface methodology

The FCCCD was used to find the effects of starch (X_1), $CaCl_2$ (X_4) and temperature (X_8) on the amylase production. Table IV shows the variables and their levels. Twenty experiments were carried out for three independent variables at three levels (–1, 0 and +1), based on the FCCCD (Table V).

A quadratic non-linear polynomial equation (4) which was developed using the experimental results and the independent variables in terms of actual values is as follows:

$$\text{Enzyme activity} = 155.23 + 82.67X_1 + 44.02X_4 + 22.169X_8 + 16.95X_1X_4 + 9.739X_1X_8 + 4.17X_4X_8 - 5.170X_1^2 + 46.086X_4^2 - 14.46X_8^2 \quad (4)$$

ANOVA and Fisher's F-test were used for model fitness and its adequacy. The results can be seen in Table VI.

The model was found to be significant with a model F-value of 37.28. It means that F-value being so large could occur due to noise with only a 0.01 % chance. Values of "Prob > F" less than 0.0500 indicates that the model terms are significant. In this case X_1, X_4, X_8, X_1X_4 and X_8^2 are significant model terms. The determination coefficient (0.9711) indicating that 97.1 % of experimental data were compatible with the model, and the values of adjusted (0.9450) and predicted R^2 (0.7838) indicate a high correlation between predicted and experimental values.

In this work, three-dimensional response surface graphs showing the interaction between the variables tested and their effects on the responses for presenting the results clearly (Fig. 2a–c) were constructed.

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TABLE VI. Analysis of variables for amylase activity; *df*: degree of freedom, Prob > *F*: significance level

Source	Amylase activity, U mg^{-1}		*df*	*F* value	Prob > *F*
	Sum of square	Mean square			
Model	104600.00	11626.20	9	37.28	< 0.0001
X_1 – Starch	68356.78	68356.78	1	219.18	< 0.0001
X_4 – CaCl2	19385.58	19385.58	1	62.16	< 0.0001
X_8 – Temperature	4914.70	4914.70	1	15.76	0.0026
X_1X_4	2299.52	2299.52	1	7.37	0.0217
X_1X_8	758.87	758.87	1	2.43	0.1498
X_4X_8	139.39	139.39	1	0.4469	0.5189
X_1^2	73.51	73.51	1	0.2357	0.6378
X_4^2	5840.93	5840.93	1	18.73	0.0015
X_8^2	575.29	575.29	1	1.84	0.2043
Residual	3118.78	311.88	10		
Lack of fit	3118.10	623.62	5		
Pure error	0.6778	0.1356	5		
Cor total	107800.00		19		

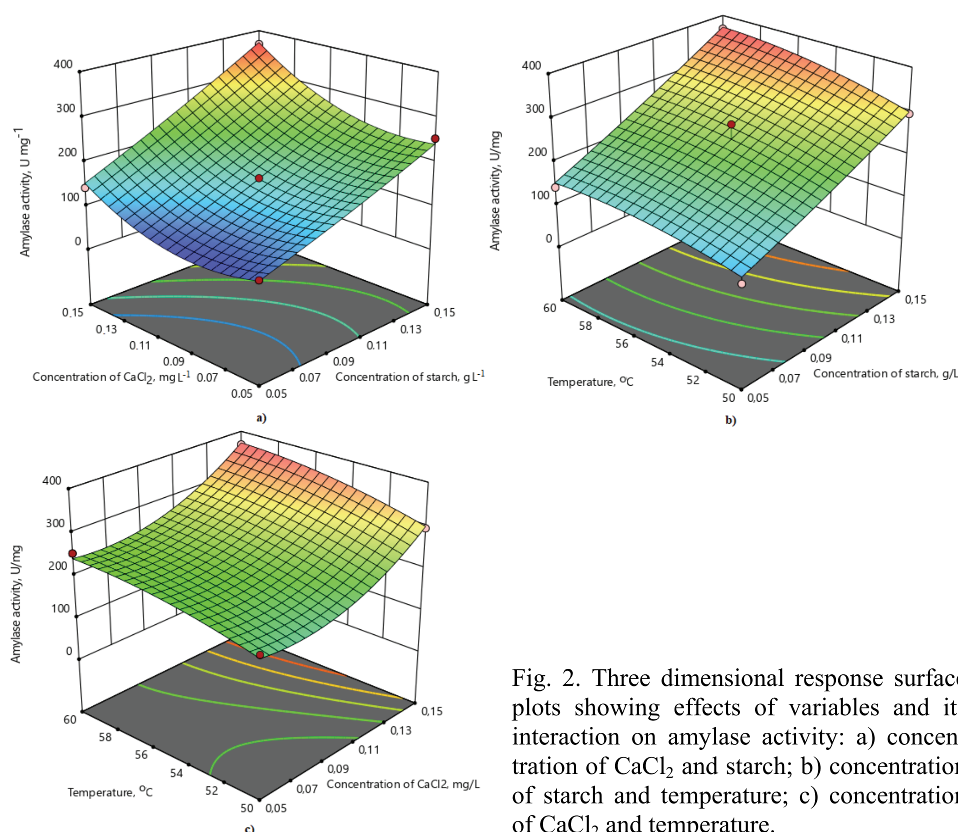

Fig. 2. Three dimensional response surface plots showing effects of variables and its interaction on amylase activity: a) concentration of $CaCl_2$ and starch; b) concentration of starch and temperature; c) concentration of $CaCl_2$ and temperature.

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The 3D graphs show the amylase activity plotted on *z*-axis against any two parameters, in this case other variables were kept at a constant level, particularly at its centre level. A strong interaction between the independent variables can be clearly seen in the graphs (Fig. 2a–c).

$CaCl_2$ is a crucial factor for the amylase production in this study. Fig. 2a represents the interaction between starch and $CaCl_2$ concentration. The optimal conditions were found to be 0.15 g/L for starch, 0.15 mg/L for $CaCl_2$ at 60 °C. The shape of the contour shows a positive interaction between the two variables. Most probably, the higher enzyme production at higher starch concentrations is due to stabilizing effect of Ca^{2+}.[42] The response surface curve for the interaction of starch concentration and temperature is represented in Fig. 2b. Enzyme production increases with starch concentrations, but temperature at lower or higher levels did not lead to higher enzyme production. Fig. 2c demonstrates the interaction of temperature and various $CaCl_2$ concentrations. The amylase activity increases with $CaCl_2$ (Fig. 2a–c) at higher starch concentrations and at moderate temperature.

CONCLUSION

In conclusion, a Gram-stain-positive, rod shape, motile, endospore-forming and amylase producer thermophilic strain was isolated from Sorgun Hot spring water. The FMB2-1 strain identified as *Bacillus paralicheniformis* was deposited in Dicle University, Molecular Biology Laboratory, while 16S rRNA gene sequence of this strain was deposited at GenBank. To select the variables for amylase production and to obtain a higher α-amylase production OVAT approach was used. PBD was utilised for identifying the significant six variables of medium (starch, yeast extract, $(NH_4)_2SO_4$, $CaCl_2$, $MgSO_4$, NaCl), as well as two variables of culture pH and temperature. By using RSM based face centred central composite design these variables were further optimized. The amylase production increased as much as 7-fold by optimizing the media in comparison to the unoptimized media. The present study indicated that temperature, starch and $CaCl_2$ concentrations among various process parameters significantly influence the amylase production and yield.

И З В О Д

ОПТИМИЗАЦИЈА ПРОИЗВОДЊЕ ТЕРМОСТАБИЛНЕ И КАЦИЈУМ-ЗАВИСНЕ α-АМИЛАЗЕ ИЗ *Bacillus paralicheniformis* ПРИМЕНОМ СТАТИСТИЧКОГ МОДЕЛОВАЊА

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Изолована је нова врста термоалкалофилних бактерија која производи амилолитичке ензиме корисне као извор ензима у индустрији. Изоловани сој је идентификован

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морфолошки, применом физичко-биохемијских тестова и анализом генске секвенце 16S rRNA. Утврђени су оптимални услови ензимске активности. Испитан је утицај различитих промењљивих на принос α-амилазе: концентрације екстракта квасца, скроба, $CaCl_2$, $(NH_4)_2SO_4$, NaCl и $MgSO_4$ у медијуму, температуре и pH, применом Plackett–Burman дизајна, а процес је оптимизован применом методе одзивних површина (RSM). Оптимални услови за производњу су били 0,15 g/L скроба, 0,15 mg/L $CaCl_2$ и 60 °C. Користећи RSM модел, повећан је принос амилазе седам пута, чиме је показано да се описани метод може користити за оптимизацију производње α-амилазе у термофилним бактеријама као што је *Bacillus paralicheniformis*.

(Примљено 27. фебруара, ревидирано 4. марта, прихваћено 6. марта 2019)

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