**Optimization of the Thermostable Alkaline and Ca-dependent -Amylase Production from *Bacillus paralicheniformis* by Statistical Modeling**

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*Abstract:* A novel amylolytic enzyme producing thermoalkaliphilic bacteria was isolated and used in the present study since they are the source of industrially used enzymes. Isolated strain identified by morphological, physio-biochemical tests and the 16S rRNA gene sequence analysis. The optimal conditions of enzyme activity were determined. For higher -amylase production, the variables such as yeast extract, starch, CaCl2, (NH4)2SO4, NaCl and MgSO4 in the -amylase production medium and 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 CaCl2 and 60 ºC for temperature. By using RSM model, amylase production increase was achieved up to seven-fold. It is showed that this method can be utilised to optimize -amylase production in a thermophilic bacteria such as *Bacillus paralicheniformis*.

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

RUNNING TITLE: A-AMYLASE PRODUCTION FROM *BACILLUS PARALICHENIFORMIS*

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INTRODUCTION

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

Amylases are one of 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 products such as glucose and fructose, biodegradation of n-alkanes, textile, paper, detergent, medical and pharmaceutical industry, synthesis of nanoparticles.2-6

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. Traditionally, biochemical varieties and enzyme concentrations have been preferred by microbial-derived enzymes because of their ease of enrichment by environmental and genetic manipulation instead of enzymes isolated from complex eukaryotes.7

Microorganisms are the most important sources for enzyme production. Almost all members of the *Bacillus* genus can synthesize-amylase and are considered to be the most important sources of this enzyme because of high biomass development rate, thermostability, take 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 cost-effective viable 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. 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 unsuccess 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 is concluded that this strain represents a new species from the *Bacillus* genus. The aim of this study is to enhance production of -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 Anatolia Region of Turkey) using starch agar plates and incubated at 50 °C for 48 hours. 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; CaCl2, 0.05; MgSO4.7H2O, 0.05; KH2PO4, 1.0; NaCl, 2.0; (NH4)2SO4, 2.0;] and incubated at 50 °C for 24 hours in a shaker. The culture was centrifuged at 10.000 rpm at 4 oC for 10 min. and cell free supernatant was used for enzyme activity. The -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 -amylase activity by OVAT approach. To enhance the production of -amylase, different nitrogen sources such as (0.05-0.02% (w/v): peptone, yeast extract, ammonium chloride and ammonium sulphate), different carbon sources (0.05-0.02% (w/v) such as glucose, galactose, fructose, maltose and starch) and different metal ions in the form of salts (0.05-0.02% (w/v) such as CaCl2, NaCl and MgCl2) with the BM media were prepared and incubated at 50 °C for 24 hours in a shaker. Similarly, different pH (6.0-9.0) and temperature (50-60 °C) on -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 -amylase production. A total of six variables of medium [starch (*X*1), yeast extract (*X*2), (NH4)2SO4 (*X*3), CaCl2 (*X*4), MgSO4 (*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 1). In order to screen the major influencing variables for the amylase production listed in Table 1.

TABLE 1: Experimental range and levels of the independent process variables to study on α-amylase activity

|  |  |  |  |
| --- | --- | --- | --- |
| **Variables** | **Symbols** | **Coded Values** | |
| **-1** | **+1** |
| Concentration of starch, g L-1 | *X1* | 0.05 | 0.2 |
| Concentration of yeast extract, g L-1 | *X2* | 0.1 | 0.5 |
| Concentration of (NH4)2SO4, mg L-1 | *X3* | 0.1 | 0.5 |
| Concentration of CaCl2, mg L-1 | *X4* | 0.05 | 0.2 |
| Concentration of MgSO4, mg L-1 | *X5* | 0.05 | 0.2 |
| Concentration of NaCl, mg L-1 | *X6* | 0.05 | 0.2 |
| pH | *X7* | 6.0 | 9.0 |
| Temperature,  oC | *X8* | 50 | 60 |

TABLE 2: Experimental design with independent variables applied in Plackett–Burman design for amylase production

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Run** | **Variables** | | | | | | | | **Amylase activity, U mg-1** | | |
|  | *X1* | *X2* | *X3* | *X4* | *X5* | *X6* | *X7* | *X8* | **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 | 11.58 |
| **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 | -11.58 |
| **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 | 15.95 |

In this study, 12 experiments were performed to determine the most affected variables for amylase production (Table 2). We used the following linear regression equation for the experimental data.

(1)

In the equation each symbol used represents as follows;

*Y* - a response for amylase enzyme activity (U mg-1),

**0 - model intercept,

*i* - linear coefficient

By using *p*-value, significant variables can be measured for probability. The statistical significance is considered if *p*-value is less than 0.05. Statistical analysis was carried out by the Minitab 15 Statistical Software (Minitab, Inc., State College, PA) (Table 3).

TABLE 3: Statistically derived effects and coefficients by Plackett–Burman design for amylase production

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Term | Effect | Coefficient | *T* | *P* |
| Constant |  | 239.99 | 36.61 | 0.0 |
| *X1* | 115.72 | 57.86 | 8.83 | 0.003\* |
| *X2* | 26.13 | 13.06 | 1.99 | 0.140 |
| *X3* | -17.27 | -8.63 | -1.32 | 0.279 |
| *X4* | 66.51 | 33.25 | 5.07 | 0.015\* |
| *X5* | 8.66 | 4.33 | 0.66 | 0.556 |
| *X6* | -18.10 | -9.05 | -1.38 | 0.261 |
| *X7* | 1.57 | 0.78 | 0.12 | 0.912 |
| *X8* | 21.68 | 10.84 | 1.65 | 0.197 |

R2: 97.45%, *T*: T-Test value, *P*: probability value \* Indicates significant variables

*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 4) and hereby twenty experimental runs were conducted (Table 5).

TABLE 4: Independent variables of selected parameters for RSM-Face centred central composite design

|  |  |  |  |
| --- | --- | --- | --- |
| Variables | Levels | | |
| -1 Levels | 0 Levels | +1 Levels |
| Concentration of starch (*X1*), g/L | 0.05 | 0.1 | 0.15 |
| Concentration of CaCl2 (*X4*), mg/L | 0.05 | 0.1 | 0.15 |
| Temperature (*X8*), oC | 50 | 55 | 60 |

TABLE 5. Experimental design and result of reduced model CCD

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Run no | Coded variables | | | Amylase activity, U mg-1 | | |
| *X1* | *X4* | *X8* | Observed | Predicted | Residual |
| 1 | 1 | 1 | 1 | 367.52 | 371.76 | -4.24 |
| 2 | -1 | 1 | 1 | 142.22 | 153.02 | -10.79 |
| 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 | -14.89 |
| 7 | -1 | -1 | 1 | 91.93 | 90.52 | 1.40 |
| 8 | -1 | -1 | -1 | 75.44 | 74.01 | 1.42 |
| 9 | -1 | 0 | 0 | 100.59 | 77.72 | 22.86 |
| 10 | 1 | 0 | 0 | 231.48 | 243.08 | -11.59 |
| 11 | 0 | -1 | 0 | 134.39 | 157.29 | -22.89 |
| 12 | 0 | 1 | 0 | 279.51 | 245.34 | 34.16 |
| 13 | 0 | 0 | -1 | 128.31 | 118.6 | 9.71 |
| 14 | 0 | 0 | 1 | 164.49 | 162.93 | 1.55 |
| 15 | 0 | 0 | 0 | 150.88 | 155.23 | -4.34 |
| 16 | 0 | 0 | 0 | 151.66 | 155.23 | -3.56 |
| 17 | 0 | 0 | 0 | 151.93 | 155.23 | -3.29 |
| 18 | 0 | 0 | 0 | 151.42 | 155.23 | -3.81 |
| 19 | 0 | 0 | 0 | 151.27 | 155.23 | -3.95 |
| 20 | 0 | 0 | 0 | 151.66 | 155.23 | -3.56 |

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

 (2)

where *Y* is response for the amylase activity (U/mg); independent variables are represented as *Xi* and *Xj*; *β0* is the model constant; *βi, βii, βij* are denoted as the linear, square and the interaction effect respectively. *Xi* and *Xj* are independent variables and is the random error in the above equation. Design Expert 8.0 programme is used to perform response surface graphs.

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 and gelatine hydrolysis. The total 16S rRNA sequence analysis of the FMB2-1strain (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 FMB2-1showing the position within the species of the *Bacillus* genus (Figure 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 (CaCl2, NaCl and MgCl2), pH (6.0-9.0) and temperature (50-60 oC) 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 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 CaCl2 (407.4 U/mg), NaCl (321.1 U/mg) and MgCl2 (210.5 U/mg) for *B. paralicheniformis* FMB2-1. Most of -amylases are known as metalloenzymes that require calcium ions (Ca2+) 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* AT7018, *B. licheniformis* ATCC 9945a22 and *B. stearothermophilus*23 have maximum amylase production in the presence of CaCl2.

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* SA24, *Bacillus* sp. BCC 01-5025 and *B. subtilis* AS-SO1a26, the optimum pH of our enzyme is more favorable 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, have potential ingredients in 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. Burhan28, Oyeleke et al.30, Sharma and Satyanarayana31 and Afrisham et al.18 demonstrated that the maximum amylase enzyme was produced at the temperature of 60 oC by *Bacillus* sp. A3-15, *B. megaterium*, *B. acidicola* and *B. licheniformis* AT70, respectively. Maximum enzyme production was at 60 oC 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, biocatalytic, 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 3, the effects of each variable are shown according to the PBD. Consists of 12 runs and their corresponding amylase activities, starch, CaCl2 and temperature showed positive effects, while the other variables showed negative effects.

In previous studies, other researchers have tried amylase optimization using the RSM method. Roy and Mukherjee37, Keharom et al.38 and Stergiou et al.39 found positive effects of starch, Zambare40 and Mustafa et al.41 found positive effects of temperature, Gangadharan et al.42 found positive effects of CaCl2 on amylase production using RSM method. The variables of probability p-value <0.05 were considered significant, and *p*-value was higher than 0.05 were not considered significant. Using the experimental data in terms of actual values of the tested variables we developed the following linear regression model [Eq. (3)]. The model validated by the correlation coefficient (*R2*) which was found 97.45 %, indicates that there was only 0.2 % variation in the data that could not be explained by the model (Table 3).

Y = 239.992+ 57.862*X*1 + 13.066*X2* - 8.63*X3* + 33.259*X4* + 4.333*X5* - 9.053*X6* + 0.787*X7*

+10.840*X8*. (3)

For studies on the amylase production optimization, temperature, starch and CaCl2 are considered to bemost 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 (*X1*), CaCl2 (*X4*) and temperature (*X8*) on the amylase production. Table 4 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 5).

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:

Enzyme activity = 155.23 + 82.67*X1* + 44.02*X4* + 22.169*X8* + 16.95*X1X4* + 9.739*X1X8*

+ 4.17*X4X8* - 5.170*X*12 + 46.086*X*42 - 14.46*X*82.(4)

ANOVA and Fisher’s *F*-test were used for model fitness and its adequacy. The results can be seen in Table 6.

TABLE 6: Analysis of variables for amylase activity

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Amylase activity, U mg-1 | | df | F value | Prob > F |
| Source | Sum of square | Mean square |
| Model | 104600.00 | 11626.20 | 9 | 37.28 | < 0.0001 |
| *X1-*Starch | 68356.78 | 68356.78 | 1 | 219.18 | < 0.0001 |
| *X4-*CaCl2 | 19385.58 | 19385.58 | 1 | 62.16 | < 0.0001 |
| *X8-*Temperature | 4914.70 | 4914.70 | 1 | 15.76 | 0.0026 |
| *X1X4* | 2299.52 | 2299.52 | 1 | 7.37 | 0.0217 |
| *X1X8* | 758.87 | 758.87 | 1 | 2.43 | 0.1498 |
| *X4X8* | 139.39 | 139.39 | 1 | 0.4469 | 0.5189 |
| *X12* | 73.51 | 73.51 | 1 | 0.2357 | 0.6378 |
| *X42* | 5840.93 | 5840.93 | 1 | 18.73 | 0.0015 |
| *X82* | 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 |  |  |

df: degree of freedom, Prob > F: significance level

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 model terms are significant. In this case *X1*, *X4*, *X8*, *X1X4*, *X82* 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 R2 (0.7838) indicate a high correlation between predicted and experimental values.

We have constructed 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).

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).

CaCl2 is a crucial factor for the amylase production in this study. Fig. 2a represents the interaction between starch and CaCl2 concentration. The optimal conditions were found to be 0.15 g/L for starch, 0.15 mg/L for CaCl2 at 60 oC. 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 Ca2+ ions.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 CaCl2 concentrations. The amylase activity increases with CaCl2 (Fig. 2a–c) at higher starch concentrations and at moderate temperature.

|  |  |
| --- | --- |
|  |  |

Fig 2.Three dimensional response surface plots showing effects of variables and its interaction on amylase activity. **(a)** Concentration of CaCl2 and Starch; **(b)** Concentration of starch and temperature; **(c)** Concentration of CaCl2 and temperature

CONCLUSION

In conclusion, a Gram-stain-Positive, rod shape, motile, endospore-forming and amylase producer thermophilic strainwas 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, OVAT approach was used and to obtain a higher -amylase production PBD was utilised for identifying the significant six variables of medium [starch, yeast extract, (NH4)2SO4, CaCl2, MgSO4, NaCl], as well as also two variables of culture, such as 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 CaCl2 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, произвођач амилолитичких ензима, који се могу користити као извор ензима у индустрији. Изолован сој је идентификован морфолошки, применом физичко-биохемијских тестова и анализом генске секвенце 16S рRNК. Утврђени су оптимални услови ензимске активности. Испитан је утицај различитих променљивих на принос α-амилазе: концентрације екстракта квасца, скроба, CaCl2, (NH4)2SO4, NaCl и MgSO4 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 CaCl2 и 60 ºC. 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*.

REFERENCES

1. S. Sivaramakrishnan, D. Gangadharan, K.N. Madhavan, A. Pandey, *Food Technol. Biotechnol.* **44** (2006) 269. <http://www.ftb.com.hr/images/pdfarticles/2006/April-June/44-269.pdf>
2. M.J.E.C. Van der Maarel, B. Van der Veen, J.C.M. Uitdehaag, H. Leemhuis, L. Dijkhuizen, *J. Biotechnol*. **94** (2002) 137. <https://doi.org/10.1016/S0168-1656(01)00407-2>
3. R. Gupta, P. Gigras, H. Mohapatra, V.K. Goswami, B. Chauhan, *Process Biochem*. **38** (2003) 1599. <https://doi.org/10.1016/S0032-9592(03)00053-0>
4. M. Karimi, D. Biria, *Chemosphere*. **152** (2016) 166. <https://doi.org/10.1016/j.chemosphere.2016.02.120>
5. P. Arunkumar, M. Thanalakshmi, P. Kumar, K. Premkumar, *Colloids Surf. B Biointerfaces*. **103** (2013) 517. <https://doi.org/10.1016/j.colsurfb.2012.10.051>
6. D. Mehta, T. Satyanarayana, *Front. Microbiol.* **7** (2016) 1129. [https://doi.org/10.3389/fmicb.2016.01129](https://dx.doi.org/10.3389%2Ffmicb.2016.01129" \t "pmc_ext)
7. T. Panneerselvam, S. Elavarasi, *Int. J. Curr. Microbiol. Appl. Sci.* **4** (2015), 543. <https://www.ijcmas.com/vol-4-2/T.%20Panneerselvam%20and%20S.%20Elavarasi.pdf>
8. M. Schallmey, A. Singh, O.P. Ward, *Can. J. Microbiol.* **50** (2004) 1. <https://doi.org/10.1139/w03-076>
9. A. Deljou, I. Arezi, T.  *Period. Biol.* **118** (2016), 405. https://doi.org/10.18054/pb.v118i4.3737
10. D. C.Sharma, Satyanarayana, *Bioresour. Technol.* **97** (2006) 727. <https://doi.org/10.1016/j.biortech.2005.04.012>
11. C. A.Dunlap, S.J.Kim, S.W. Kwon, A. Rooney, *Int. J. Syst. Evol. Microbiol.***65** (2015) 2104. <https://doi.org/10.1099/ijs.0.000226>
12. Bernfeld, 1955 Bernfeld, P. Amylases α and β. Methods in Enzymology. Colobick, S.P.; Kalpan, N.O. Ed., 1955, 1, 149–158. <http://dx.doi.org/10.1016/0076-6879(55)01021-5>
13. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall*, J. Biol. Chem.* **193** (1951) 265. <http://www.jbc.org/content/193/1/265.long>
14. R.P. Van Hille, L. Bromfield, S. Botha, G. Jones, A. W. Van Zyl, S. T. Harrison. *Adv. Mater. Res.* **71** (2009) 413. <https://doi.org/10.4028/www.scientific.net/AMR.71-73.413>
15. S. Suman, K. Ramesh, *Pharm. Sci. & Res.* **2** (2010) 149. <https://www.jpsr.pharmainfo.in/Documents/Volumes/Vol2Issue3/jpsr02031002.pdf>
16. K. Das, R. Doley, A.K. Mukherjee, *Biotechnol. Appl. Biochem.* **40** (2004) 291. <https://doi.org/10.1042/BA20040034>
17. K. R. Jetendra, A. K. Mukherjee, *Biochem. Eng. J.* **77** (2013) 220. <https://doi.org/10.1016/j.bej.2013.06.012>
18. S. Afrisham, A. Badoei-Dalfard, A. Namaki-Shoushtari, Z. Karami, *J. Mol. Catal. B Enzym.* 132 (2016) 98. <https://doi.org/10.1016/j.molcatb.2016.07.002>
19. R. Lifshitz, A. Levitzki, *Biochemistry*. **15** (1976),1987. <https://www.ncbi.nlm.nih.gov/pubmed/817737>
20. R. Vaikundamoorthy, R. Rajendran, A. Selvaraju, K. Moorthy, S. Perumal, *Bioorg. Chem.* **77** (2018) 494. [https://doi.org/10.1016/j.bioorg.2018.02.014](http://dx.doi.org/10.1016/j.bioorg.2018.02.014)
21. S. De Cordt, K. Vanhoof, J. Hu, G. Maesmans,  M. Hendrickx, P.Tobback, *Biotechnol. Bioeng.* **40** (1992), 396. [https://doi.org/10.1002/bit.260400309](https://doi.org/10.1002/bit.260400309" \t "_blank)
22. N. Bozic, J. Ruiz, J. Lopez-Santin, Z. Vujcic, *J. Serb. Chem. Soc.*  **76** (2011), 965. doi: 10.2298/JSC101010098B
23. Z. Li, L. Su, X. Duan, D. Wu, J. Wu, *BioMed Research International.*2017:5479762. https://doi.org/10.1155/2017/5479762.
24. A. Hammami, N. Fakhfakh, O. Abdelhedi, M. Nasri, A. Bayoudh, *Int. J. Biol. Macromol.***108** (2018) 56. <https://doi.org/10.1016/j.ijbiomac.2017.11.148>
25. A.A. Simair, A.S. Qureshi, I. Khushk, C.H. Ali, S. Lashari, M.A. Bhutto, G. S. Mangrio, C. Lu, *Biomed. Res. Int.* 2017, https://doi.org/10.1155/2017/9173040
26. J. K. Roy, S. K. Rai, A. K. Mukherjee, *Int. J. Biol. Macromol.* **50** (2012) 219. <https://doi.org/10.1016/j.ijbiomac.2011.10.026>
27. O. Kirk, T.V. Borchert, C.C. Fuglsang, *Curr. Opin. Biotechnol*. **13** (2002) 345-51. [https://doi.org/doi.org/10.1016/S0958-1669(02)00328-2](https://doi.org/10.1016/S0958-1669(02)00328-2" \o "Persistent link using digital object identifier" \t "_blank)
28. A. Burhan, *Bioresour. Technol.* **99** (2008) 3071. <https://doi.org/10.1016/j.biortech.2007.06.019>
29. S. Murakami , H. Nishimoto , Y. Toyama , E. Shimamoto , S. Takenaka , J. Kaulpiboon, M. Prousoontorn, T. Limpaseni, P. Pongsawasdi , K. Aoki, *Biosci. Biotechnol. Biochem*. **71** (2007) 2393-401. <https://doi.org/10.1271/bbb.60666>
30. B. Oyeleke, S. H. Auta, E. C. Egwim, *Niger State J. Microbiol. Antimicrob.* **2** (2010), 88. <https://academicjournals.org/article/article1380022186_Oyeleke%20et%20al2.pdf>
31. A.Sharma, T. Satyanarayana, *Extremophiles* **16** (2012) 515. <https://doi.org/10.1007/s10529-010-0322-9>
32. Z. Konsoula, M. Liakopoulou-Kyriakides, *Biores. Technol.* **98** (2007) 150-157.

<https://doi.org/10.1016/j.biortech.2005.11.001>

1. G.D. Haki, S.K. Rakshit, *Biores. Technol*. **89** (2003) 17-34. [https://doi.org/10.1016/S0960-8524(03)00033-6](https://doi.org/10.1016/S0960-8524(03)00033-6" \o "Persistent link using digital object identifier" \t "_blank)
2. T. Satyanarayana, D. Mehta, Thermophilic microbes in environmental and industrial biotechnology: Biotechnology of thermophiles, Springer Science+Business Media, Dordrecht, 2013 <https://doi.org/10.1007/978-94-007-5899-5>
3. T.F.A. Abu, V.N. Enujiugha, D M. Sanni, O.S. Bamidele*, Int. J. Life Sc. Bt. & Pharm. Res*. **3** (2014) 1-18.
4. A. Sundarram, T.P.K. Murthy, *J. App.& Env. Microbiol*. **2** (2014) 166-175. <https://doi.org/10.12691/jaem-2-4-10>
5. J. K. Roy, A. K. Mukherjee, *Biochem. Eng. J.***77** (2013) 220. <https://doi.org/10.1016/j.bej.2013.06.012>
6. S. Keharom, R. Mahachai, S. Chanthai, *Int. Food Res. J.* **23** (2016), 10. <http://www.ifrj.upm.edu.my/23%20(01)%202016/(2).pdf>
7. P.Y. Stergiou, A. Foukis, L. Theodorou, M. Papagianni, E. Papamichael, *Braz. Arch. Biol. Technol.* **57** (2014), 421. <http://dx.doi.org/10.1590/S1516-8913201401485>
8. V. P. Zambare, *Emir. J. Food Agric.* **23** (2011), 37. http://ejfa.info
9. S.R.Mustafa, A. Husaini, C. N. Hipolito, H. Hussain, N. Suhaili, H. A. Roslan, *Braz. Arch. Biol. Technol.* **59** (2016) e16150632.http://dx.doi.org/10.1590/1678-4324-2016150632

1. [D. Gangadharan](https://www.sciencedirect.com/science/article/pii/S0960852407005779?via%3Dihub" \l "!), [S. Sivaramakrishnan](https://www.sciencedirect.com/science/article/pii/S0960852407005779?via%3Dihub#!), [K. M. Nampoothiri](https://www.sciencedirect.com/science/article/pii/S0960852407005779?via%3Dihub#!), [R. K. Sukumaran](https://www.sciencedirect.com/science/article/pii/S0960852407005779?via%3Dihub#!), [A. Pandey](https://www.sciencedirect.com/science/article/pii/S0960852407005779?via%3Dihub#!). *Bioresour. Technol.*  [**99** (2008](https://www.sciencedirect.com/science/journal/09608524/99/11)),  4597. DOI:[10.1016/j.biortech.2007.07.028](https://doi.org/10.1016/j.biortech.2007.07.028)