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Process optimization for amylase production of *Bacillus subtilis* M4 mutant strain

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ABSTRACT

There are many factors that influence the character of bacterial metabolism and enzyme production.For the maximum production of the desired products, the media components and fermentation conditions should be optimized. In our investigation, we improved the amylase production of *Bacillus subtilis* M4 mutant strain by the combination of two optimization techniques. The cultural conditions (time period, temperature, pH, inoculum volume) and medium ingredients (various carbon, organic and inorganic nitrogen sources, chlorides, sulfates, phosphates, carbonates) were optimized by one factor at a time methodology (OFAT) and response surface methodology (RSM) to increase the amylase production. The optimum conditions for amylase production were found be the following: 35°C, pH range 7 and incubation time 72h, inoculum volume 8% (v/v). Optimum medium composition for amylase production was the following: starch 12.9 g, peptone 9.75 g, calcium carbonate 0.439 g, magnesium sulfate 0.464 g and potassium chloride 0.464 g per liter. When applied to our optimized medium in the fermentation process, the enzyme activity increased from 0.741 to 1.58 U/ml, which means a 2.1-fold increase compared to the original medium.

KEYWORD: Amylase activity, *Bacillus spp*., Statistical optimization, OFAT, RSM

INTRODUCTION

Amylases are glycoside hydrolases enzymes, which break starch into glucose, maltose, maltotriose, and dextrin by hydrolyzing process of glycosidic bonds. These enzymes have been widely used many years.First enzyme produced industrially was an amylase from a fungal source in 1894 and used as a pharmaceuticalaid for the treatment of digestive disorders [1]. Amylases are the major class of industrial enzymes, which constitute approximately 25% of the enzyme market [2]. The biological organisms such as plants, animals, and microorganisms are major sources of enzymes. Microorganism produces amylase more beneficially than other sources. The production rate of microorganisms is high and can be engineered to obtain enzymes of good characteristics [3]. The *Bacillus* genus trends to dominate the enzyme industry, because of almost all microorganisms of this genus synthesis α-amylase. Amylases obtained from *Bacillus licheniformis*, *Bacillus stearothermophilus*, and *Bacillus amyloliquefaciens* are used in many industrial processes such as in food, fermentation, textiles and paper industries [4]. The composition and concentration of fermentation media significantly influence bacterial growth and extracellular amylase production. Optimization of cultural conditions is essential for the maximum production of bacterial strains. Almost all forms of microorganisms grow differently with specificity to different substrates in the fermentation medium.

MATERIALS AND METHODS

Microorganism

B.subtilis M4 mutant strainobtained from physical and chemical mutagenesis in the Biosynthesis laboratory of School of Animal Science and Biotechnology, Mongolian University Of Life Sciences. The strain was kept on slantagar at 4ºC.

Culture condition

20 ml of basal media were sterilized and inoculated with 24 h bacterial slant and incubated in a shaking

incubator (110 rpm)at 37°C. When the absorbance of the culture broth reached at A600 nm to 0.15 (cell density about 1×10^6 CFU/ml) was used as a bacterial inoculum. The medium composition was the following: (g/l) starch 10, peptone 6, $MgSO_4$ 0.5, KCl 0.5 [6,7].

Enzyme production

The basal media was inoculated with 10% ($1x10^6$ CFU/ml) of bacterial inoculum and incubated at 37°C for 24 h in shaking incubator with shaking of 110 rpm. When reached the fermentation period, the culture medium was centrifuged at 5000 rpm for 10 min and supernatant was used as a crude enzyme [8].

Amylase Assay

Amylase activity was determined by spectrophotometric method according to Fisher and Stein [9]. 1.0 ml of the crude enzyme was taken in a test tube and 1.0 ml of substrate (starch) was added. The test tube was incubated at 45°C in the water bath for 30 min. Then 2.0 ml dinitro-salicylic acid reagent was added in the tube and kept in boiling water bath for 5 min. After cooling at room temperature, the absorbance was read at 540 nm by spectrophotometer. One unit of amylase activity was measured as the amount of amylase required to liberate reducing sugar equivalent to one mmol of D-glucose per minute at 45°C [10].

Bacterial growth

The growth of bacteria was determined by the optical density of culture broth at 600 nm ina spectrophotometer. Cells were isolated by centrifugation (5000 rpm for 5 min at 4ºC) of culture samples and washed two times with saline water (0.8% NaCl) [11].

Process Optimization for Amylase Production

One factor at a time methodology (OFAT)

Optimization of physicochemical parameters

The physicochemical parameters of the fermentation process, such as temperature, pH, inoculum volumeand incubation time were optimized. Enzyme activity was measured after incubation of bacterial strain at various temperatures ranging from 20˚C to 45˚C and pH from 3.0 to 10.0 and optimal temperature and pH were defined. The effect of inoculum volume on α-amylase activity was determined by inoculating the basal medium with different inoculum volumes ranging from 2% to 12% (v/v). To determine optimum incubation period, amylase activity and bacterial growth were observed during 120 h of incubation at the optimal temperature, pH and inoculum volume. Samples were taken every 24 h intervals [11].

Effect of different carbon, nitrogen sources and mineral saltson amylase production

To determine effect of carbon and nitrogen sources on amylase production, different carbon sources (starch, glucose, lactose, dextrose, maltose and sucrose) at 1.0 % (w/v) concentration and different nitrogen sources (casein, peptone, yeast extract and ammonium chloride, ammonium nitrate, potassium nitrate, sodium nitrate and ammonium sulfate) at 0.5% (w/v) concentration were added to the fermentation medium [12,15]. To study the effect of mineral salts on amylase production, chlorides (manganese chloride, barium chloride, sodium chloride, potassium chloride, and magnesium chloride, calcium chloride, ferric chloride and ammonium chloride), sulfates (calcium sulfate, zinc sulfate, ferrous sulfate, magnesium sulfate, manganese sulfate, potassium sulfate and ammonium sulfate), phosphates (ammonium dihydrogen phosphate, dipotassium phosphate, monopotassium phosphate), carbonates (calcium carbonate, sodium carbonate) were employed at 0.05% (w/v) concentration [12].

Central composite rotatable design (CCRD)

In this experiment, we used the Central composite rotatable design method to find more accurate factor values to reach a good response. Five variables were selected from OFAT investigation results to evaluate their effects on amylase activity. We provided RSM analysis based on CCRD. Minitab version 18.1 program developed a five-level-five-variable CCRD with six replicates at the center point (13, 14). In total 32 experiments were carried out in triplicate. The coded levels of the independent variables are prescribed in Table 1. Experimental data were analyzed by the response surface regression (RSREG) methodology and the following second-order polynomial equation was calculated (1):

$$Y = \beta_{k0} + \sum_{i=1}^{5} \beta_{ki}\, x_i + \sum_{i=1}^{5} \beta_{kii}\, x_i^2 + \sum_{i=1}^{4} \sum_{i=i+1}^{5} \beta_{kij}\, x_i x_j$$

whereY is the response variable (amylase activity), β_{k0}, β_{ki}, β_{kii}, and β_{kij} are constant coefficients and xi the uncoded independentvariables[16]. MINITAB calculated the optimal values using equation (1).

Table 1.

Levels of the independent variables in CCRD

№	Variables	Symbol code	Coded levels				
			-2	-1	0	+1	+2
1	Starch (g/l)	X_1	5.0	10.0	15.0	20.0	25.0
2	Peptone (g/l)	X_2	5.0	7.5	10.0	12.5	15.0
3	$CaCO_3$ (g/l)	X_3	0.1	0.3	0.5	0.7	0.9
4	$MgSO_4$ (g/l)	X_4	0.1	0.3	0.5	0.7	0.9
5	KCl (g/l)	X_5	0.1	0.3	0.5	0.7	0.9

Validation of amylase productionin optimized media

We compared cell growth and amylase production in optimized media with growth and amylase production in basal media to verify our statistical calculation results. Bacterial cell growth, amylase activity and pH in the fermentation media were determined every 24 h to monitor the changes during 120 h fermentation of *B. subtilis* M4 strain.

RESULTS AND DISCUSSION

To enhance amylase production, we applied two different optimization technique and optimized culture condition and medium composition.

Incubation period: The Incubation period has an essential role in amylase activity. In our study, the incubation period for optimum production of amylase was 72h for *B. subtilis* M4. The enzyme activity reached a maximum of 0.799 U/ml (Fig 1), then decreased to 0.453 U/ml when the incubation time increased from 72 to 120 hours. Moreira et al. reported that different species of *Bacillus* have shown a similar cultivation period of 72 h [17] for maximum amylase production [18]. *A. oryzae* produced the maximum α-Amylase at 72 h of incubation period [19]. *B. amyloliquefaciens* reached the optimum α-Amylase activity after fermentation for 72 hours [15]. In our experiment, the optical density of the *B. subtilis* M4 strain reached a maximum also after 72h of fermentation (Fig. 1). It was noticed a positive correlation between growth and amylase activity for this strain that indicates the amylase production was growth associated with the *B. subtilis* M4 strain. Growth kinetics of the isolated *Bacillus* strain by Mishra and Behera, started lag phase right after inoculation. The stationary phase started from late 48 h which continued till 72 h and after that growth declined at 92 h [20].

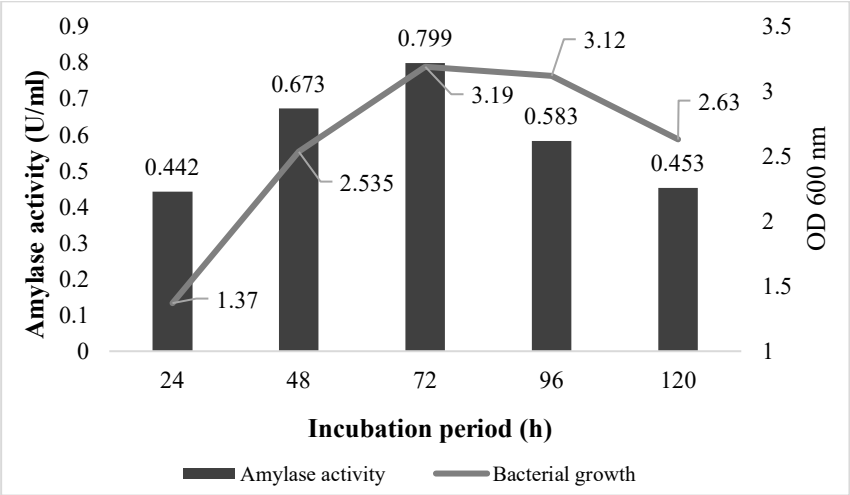


Figure. 1. Amylase activity at different time period

Temperature: Enzyme activity monitored at various temperatures displayed that *B. subtilis* M4 reached maximum amylase production at 35°C (Fig 2). The optimal temperature of 35°C was showed for amylase production by *B. subtilis* CBTK 106, isolated from banana wastes [21]. According to Pokhrel et al., 35°C was found as the optimum temperature at which enzyme activity was found to be higher, of the bacterial isolate identified as *Bacillus spp*. [22].

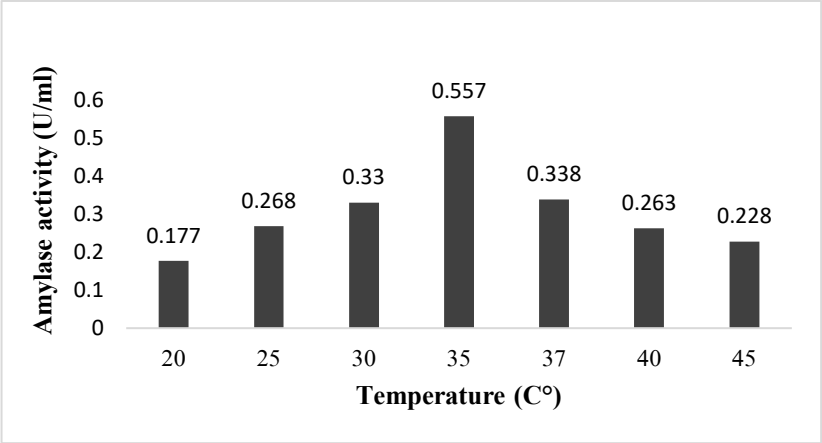


Figure 2. Amylase activity at different temperature

pH: *B. subtilis* M4 strain was cultured in media of different pH ranging from 5.0 to 10.0. The highest enzyme activity was registered in the medium of pH 7.0(Fig 3).According to Divakaran et al., amylases from *Bacillus* showed maximum activity at pH 7 [23]. Our results arein good agreement with this study.Behal et al., [24]investigated thermostable amylase producing *Bacillus spp* that revealed an optimum enzyme activity at pH 8.0 whereas in other species the optimum activity was at pH 7.0 [25].

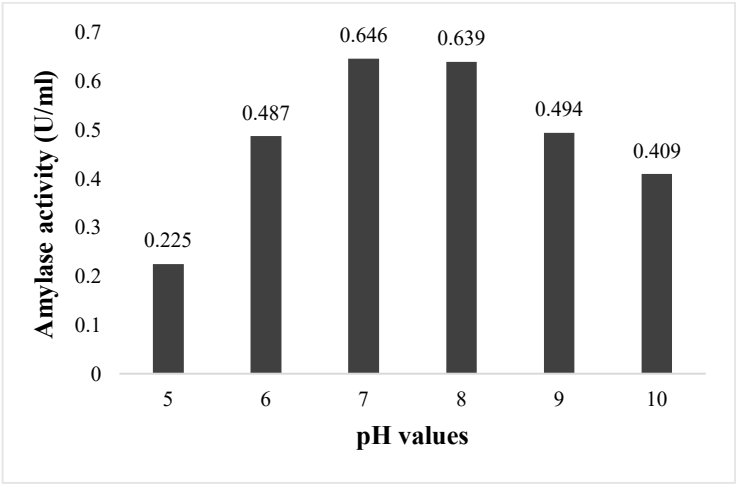


Figure 3. Amylase activity at different pH

Inoculum volume: In our experiment, the enzyme activity was increased as the inoculum volume increases and reached the optimum at 8% (Fig 4). When the inoculum volume was further increased, the enzyme activity slowly decreased. It may be caused by the initial speedy growth of bacteria and the lack of nutrients in the medium. According to Tsurikova et al., the optimal inoculum volume for the production of amylase was found to be 8% [26]. Our experiment repeated the results regarding the negative effects of high inoculum volume on amylase production of bacteria [27].

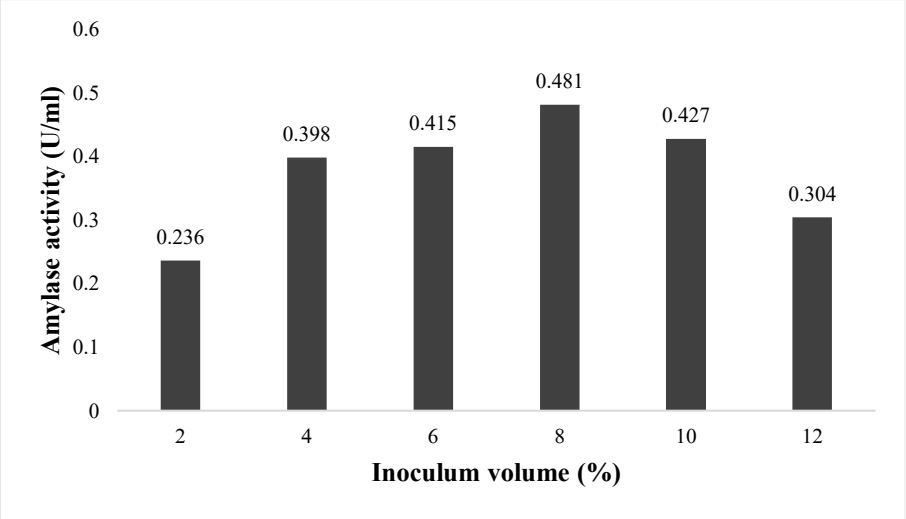


Figure 4. Amylase activity at different inoculum volume

Carbon Source: The effect of carbon sources on amylase production was determined by substituting the carbon sources of the basal media and culturing the bacteria in optimum conditions. Starch was revealed as the most suitable carbon source for amylase production by *B. subtilis* M4 strain (Fig. 5). Amylase is an inducible enzyme and is generally induced in the presence of starch or its hydrolytic product, maltose [28]. The utilization of soluble starch by *Bacillus spp*. was reported previously [29]. In the media containing lactose, the lowest amylase production was registered. Based on our experimental data, starch was supposed as the most suitable carbon source for *B. subtilis* M4 for amylase production.

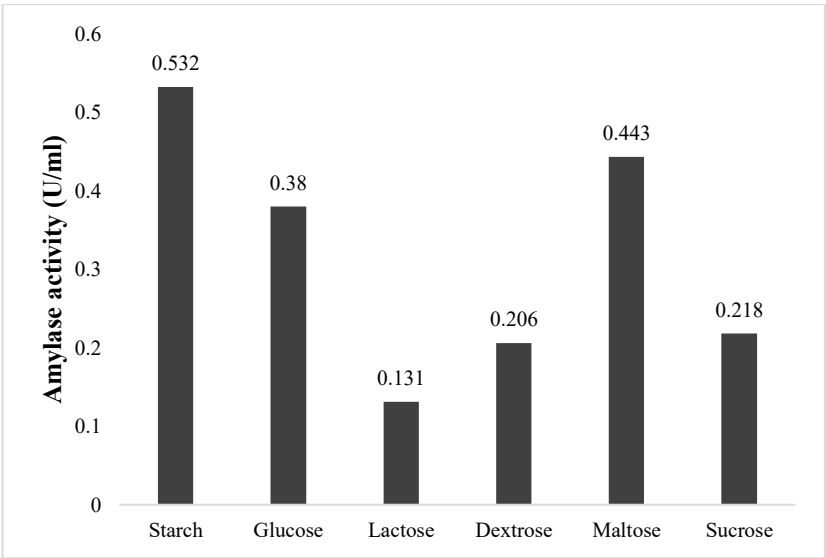


Figure 5. Amylase activity at different carbon sources

Nitrogen Sources: To determine the effect of nitrogen sources on amylase production we used different organic nitrogen sources with basal media. In the media containing peptone, the highest amylase production was recorded. Ammonium sulfate was revealed as the least suitable nitrogen source. According to our study, the B. subtilis M4 strain produced 6-fold more amylase in the media with peptone with comparing with ammonium sulfate (Fig. 6). Tryptone, peptone, and casein were registered as suitable sources for the production of amylase [30].

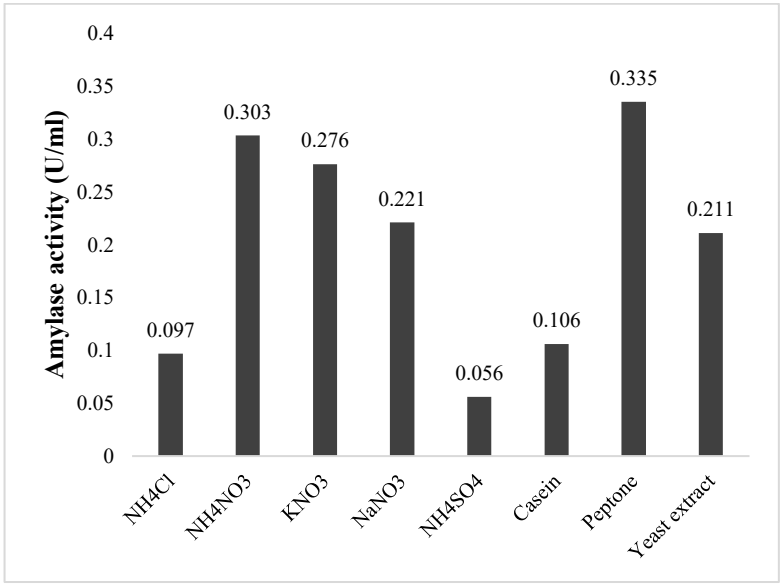


Figure 6. Amylase activity at different nitrogen sources

Chlorides: We have found that calcium chloride affects the highest amylase production and potassium chloride is the second suitable chloride for *B.subtilis* M4 (Fig 7). The addition of salts of some metal ions resulted in good growth of microorganisms and so better enzyme production (most α-amylases are recognized as metalloenzymes). Ca^{2+} ions are revealed being present in the majority of these enzymes. The addition of $CaCl_2$ to the culture media enhanced enzyme production [31].

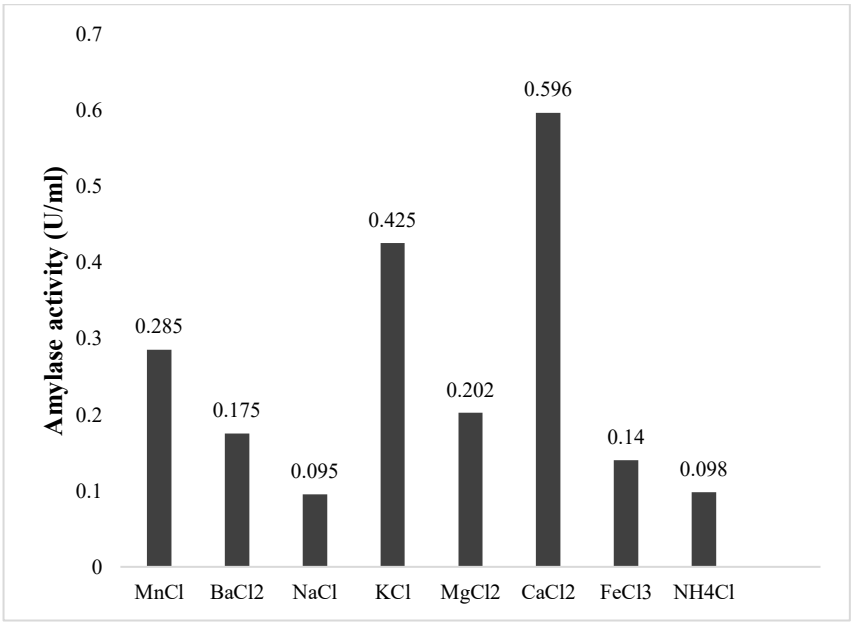


Figure 7. Amylase activity at different chlorides

Sulfates: In our study, calcium sulfate was registered as the best sulfate source for amylase production (Fig 8). The next one was found to be magnesium sulfate. Mg^{2+} had a great role and enzyme production was decreased to 50% when Mg^{2+} was eliminated from the medium. Na^{+} and Mg^{2+} jointly stimulated enzyme production by *Bacillus spp*. CRP strain [32].

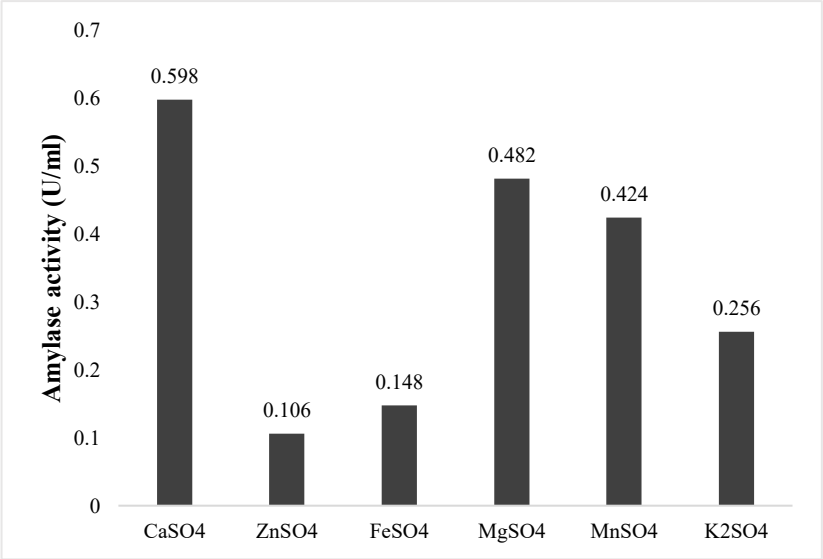


Figure 8. Amylase activity at different sulfates

Phosphates and carbonates: From different phosphates, monopotassium phosphate highly stimulated amylase production by *B.subtilis* M4 (Fig 9). Phosphate has a significant regulatory role in the synthesis of primary and secondary metabolites in microorganisms [33] and affects the growth of the organism and amylase production. Above 0.2 M phosphate levels significantly increased enzyme production and conidiation in *A. oryzae* [34]. In this experiment, calcium carbonate greatly increased the production of amylase (Fig. 9), so we defined it to be the best calcium source for *B. subtilis* M4 strain for amylase production.

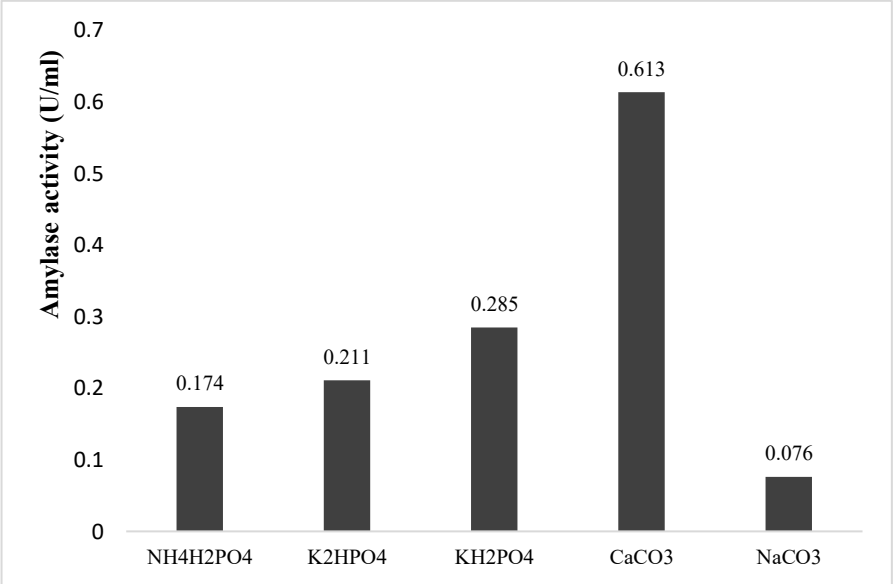


Figure 9. Amylase activity at different phosphates and carbonates

Central composite design (CCD)

We have determined the effect of the selected 5 variables on amylase activity by the response surface methodology. CCRD matrix with experimental and predicted data are described in Table.2. Amylase activity was identified as the response, starch, peptone, calcium carbonate, magnesium sulfate, and potassium chloride were taken as factors impacting amylase production of *B. subtilis* M4 strain.

Table 2.

CCRD and Experimental Data for RSM

Run №	X1	X2	X3	X4	X5	Amylase activity (U/ml)	
						Experimental*	Predicted
1	-1	-1	-1	-1	1	0.960	0.952
2	1	-1	-1	-1	-1	1.160	1.162
3	-1	1	-1	-1	-1	1.235	1.245
4	1	1	-1	-1	1	1.059	1.020
5	-1	-1	1	-1	-1	1.315	1.285
6	1	-1	1	-1	1	1.469	1.391
7	-1	1	1	-1	1	1.399	1.330
8	1	1	1	-1	-1	0.956	0.896
9	-1	-1	-1	1	-1	1.163	1.202
10	1	-1	-1	1	1	1.045	1.035
11	-1	1	-1	1	1	0.851	0.850
12	1	1	-1	1	-1	1.117	1.124
13	-1	-1	1	1	1	1.172	1.132
14	1	-1	1	1	-1	1.187	1.156
15	-1	1	1	1	-1	1.380	1.357
16	1	1	1	1	1	1.032	0.961
17	-2	0	0	0	0	1.335	1.346
18	2	0	0	0	0	1.104	1.194
19	0	-2	0	0	0	1.358	1.386
20	0	2	0	0	0	1.180	1.253
21	0	0	-2	0	0	1.013	0.963
22	0	0	2	0	0	1.041	1.192
23	0	0	0	-2	0	1.262	1.348
24	0	0	0	2	0	1.217	1.232
25	0	0	0	0	-2	1.207	1.200
26	0	0	0	0	2	0.902	1.010
27	0	0	0	0	0	1.546	1.537
28	0	0	0	0	0	1.549	1.537
29	0	0	0	0	0	1.557	1.537
30	0	0	0	0	0	1.598	1.537
31	0	0	0	0	0	1.547	1.537
32	0	0	0	0	0	1.527	1.537

*The observed data of amylase activity are the average of triple experiments

The secondary order polynomial equation (2) is the following:

$$Y = 1.5373 - 0.0380\,X1 - 0.0333\,X2 + 0.0574\,X3 - 0.0290\,X4 - 0.0473\,X5 - 0.0668\,X1*X1 - 0.0545\,X2*X2 - 0.1150\,X3*X3 - 0.0619\,X4*X4 - 0.1081\,X5*X5 - 0.0596\,X1*X2 - 0.0496\,X1*X3 + 0.0050\,X1*X4 + 0.0559\,X1*X5 - 0.0193\,X2*X3 + 0.0042\,X2*X4 - 0.0104\,X2*X5 - 0.0080\,X3*X4 + 0.0621\,X3*X5 - 0.0605\,X4*X5 \quad (2)$$

The calculated response surface for the most important factors is demonstrated by CCRD in a 3-dimensional graph (Fig. 10). 3-dimensional graph of starch and peptone (A), calcium carbonate and magnesium sulfate (B), starch and potassium chloride (C) against amylase activity demonstrate the outcomes of the statistical and mathematical analyses.

According our study the optimum concentrations of selected factors were as following (Fig. 11): starch -0.42 (12.9 g/l), peptone -0.10 (9.75 g/l), calcium carbonate 0.30 (0.439 g/l), magnesium sulfate -0.18 (0.464 g/l), potassium chloride -0.18 (0.464 g/l), respectively. At this optimized condition, our model predicted 1.563 U/ml of amylase activity.

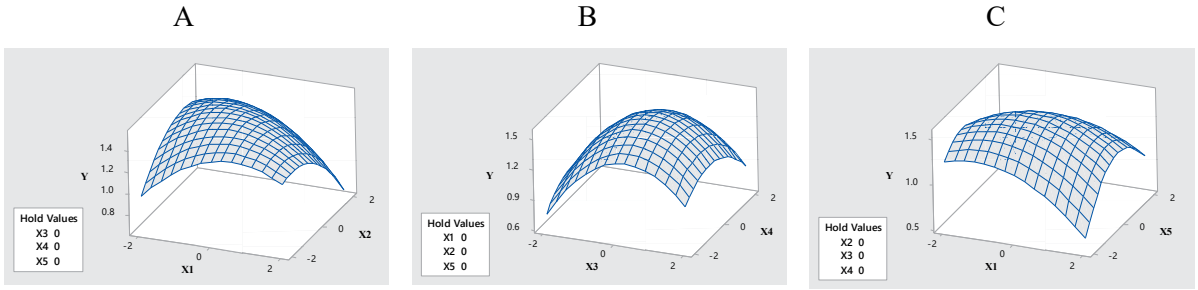


Figure. 10. Three-dimensional graph for amylase production: A unction of starch and peptone(A), calcium carbonate and magnesium sulfate (B), starch and potassium chloride(C)

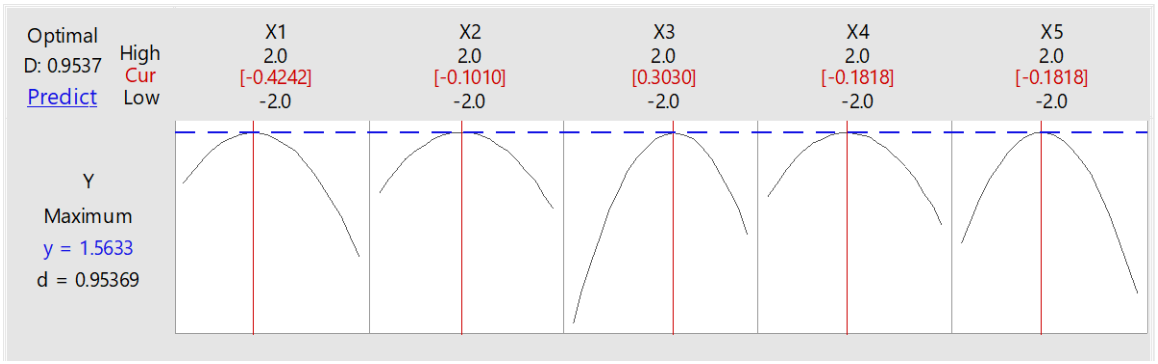
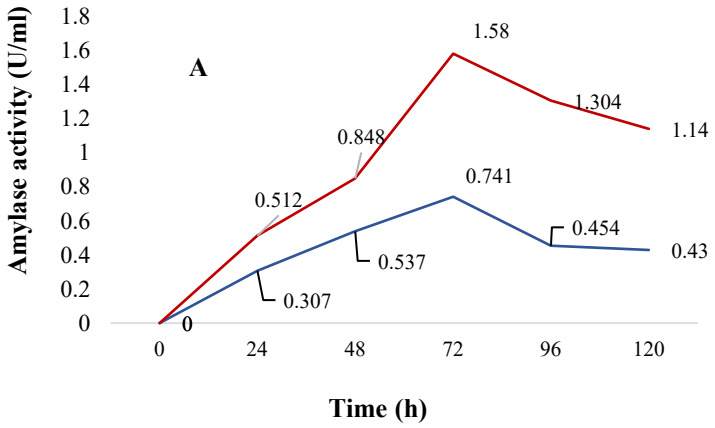


Figure 11. The optimum concentrations of selected factors, founded by solving the secondary order polynomial equation (2)

Comparison between basal and optimized media

Fig.11 describes changes of amylase production (A), cell density (B), and pH (C) during the incubation period of *B. subtilis* M4 in the optimized and basal medium. Cells grew more and reached the highest level at 72 h of incubation, then the growth reduced slightly. The bacterial growth kept stable after 48 h of cultivation. The culture broth pH during the cultivation was between 6.5-7.5. In the optimized medium, the enzyme activity increased from 0.741 to 1.58 U/ml, which means a 2.1 fold. According to Deljou and Arezi, in the result of medium optimization, amylase production by *B. licheniformis*AZ2 increased by 2.4 fold [11]. Another study reports that for optimization of the fermentation medium components and environmental factors was used OFAT approach and Plackett-Burman design. As a result of this work amylase production by *Bacillus lichineformis* AH214, was enhanced 2.0 fold compared to the original medium [35].



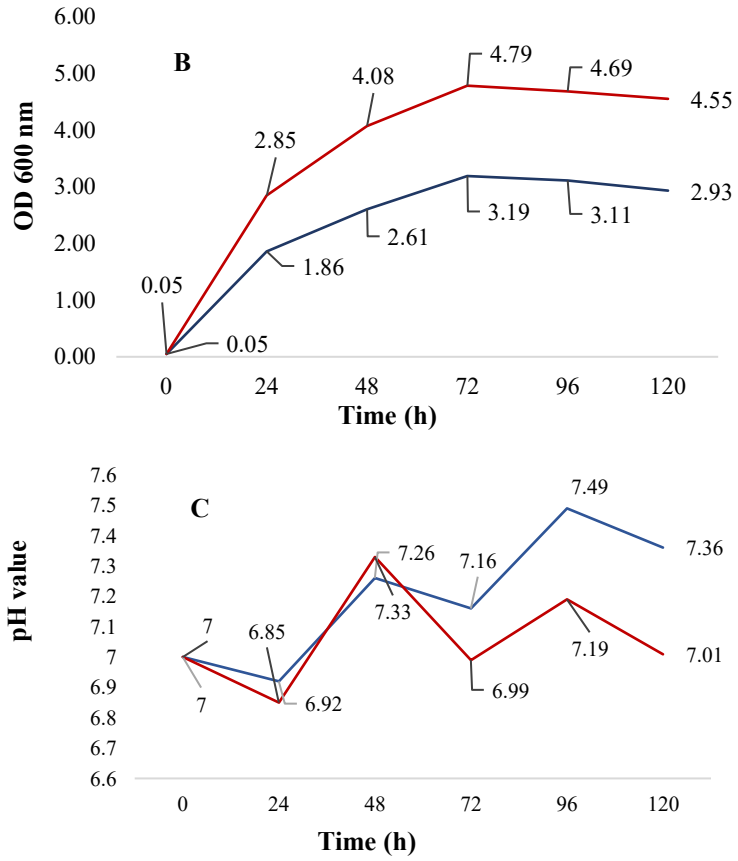


Figure.12.Change in amylase production (A), cell density(B), and pH (C) during fermentation of *B.subtilis* M4. Optimized medium (-----)and basalmedium (-----). Each data states the mean of triple independent assays (the standard errors were less than 5% of the means).

CONCLUSION

In this study, we aimed to increase the amylase production by the *B. subtilis* M4 mutant strain. For this purpose, the cultural conditions and medium ingredients were optimized by one factor at a time approach and response surface methodology. The optimal culture conditions for amylase production were the following: temperature 35ºC, pH value 7, incubation time 72 h, inoculum volume 8% (v/v). Optimal fermentation medium for amylase production contained starch 12.9, peptone 9.75, calcium carbonate 0.439, magnesium sulfate 0.464 and potassium chloride 0.464 g/l. In the optimized fermentation media, the enzyme activity reached 1.58 U/ml, which means a 2.1 fold compared to the basal medium. To be applied commercially, further study is needed for the improvement of this enzyme production.

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