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# Statistical optimization of amylase production using grape fruit peels in submerged fermentation

**Nazia Iram[1], Hafiz Abdullah Shakir[1], Muhammad Irfan[2*], Muhammad Khan[1], Shaukat Ali[3], Arfa Anwer[1], Saba Saeed[1] and Javed Iqbal Qazi[1]**

[1]University of the Punjab, Lahore, Punjab, Pakistan. [2]University of Sargodha, University Road, 40100, Sargodha, Punjab, Pakistan. [3]Government College University, 54000, Lahore, Punjab, Pakistan. *Author for correspondence. E-mail: irfan.ashraf@uos.edu.pk; Irfan.biotechnologist@gmail.com

**ABSTRACT.** Amylase production by *Bacillus licheniformis* isolated from fish gut was statistically optimized using grape fruit peels in submerged fermentation. Nutritional parameters were optimized through one factor at a time approach. The selected parameters were grape fruit peels as carbon source, ammonium nitrate as nitrogen source, magnesium sulphate as mineral salt. These parameters along with initial medium pH was optimized through central composite design of response surface methodology. Maximum amylase production was observed at 5% grape fruit peels, 0.9% ammonium nitrate, 0.6% magnesium sulphate when initial pH of medium was adjusted 7. The amylase was optimally active at pH 9 at 80°C. The highest enzyme activity at 80ºC depicted the potential use of the strain especially in textiles and paper industrial processes which are performed at high temperatures.

**Keywords:** *Bacillus licheniformis*; grape fruit peel; amylase; optimization; response surface methodology; central composite design.

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## Introduction

Amylases help in breakdown of starch into simple carbohydrates maltose and glucose (Sarmiento, Peralta, & Blamey, 2015). The breakdown of internal α 1-4-glycosidic bond is done by α-amylase (1, 4-α-D-glucan glucanohydrolase; glycogenase). It is a metalloenzyme that contain calcium as metal co-factor. Amylase have several applications in variety of industries such as food, textiles, fermentation, paper, detergent and pharmaceutics. Due to advent in the field of biotechnology, amylase usage is very much widely dispersed in medical and analytical biochemistry (Kandra, 2003). The amylase producing bacteria help in industrial degradation of starch to produce dextrose syrup, glucose, maltodextrin etc. (Gopinath, Hilda, Annadurai, & Anbu, 2005). The demand of efficient and low-cost enzyme has been increased in various industrial processes (Cherry & Fidantsef, 2003; Nadeem, Qazi, Syed, & Gulsher, 2013). Production of amylase by bacterial strains, in general, is highly preferable in many industries. However, main limitation is the production cost of the enzyme. Therefore, its production and cost can be optimized by adopting various methodologies in the biotechnology field (Gopinath et al., 2005).

Bacterial amylase production can be increased by optimizing various nutrients. Traditional carbon sources like glucose, maltose, dextrin etc. are very costly so these can be substituted by agricultural wastes (Ghosh & Chandra, 1984; Gupta, Gigras, Mohapatra, Goswami, & Chauhan, 2003). Use of peels of different fruits as a carbon source in a medium for the growth of bacteria is getting popularity in both solid state and submerged fermentation. The bacteria are now being used to convert agro-wastes into profitable products (Awan, Jabeen, Manzoor, & Qazi, 2018). Use of fruit peels from grapefruit, orange, mango, apple etc was reported by several researchers (Haki & Rakshit, 2003; Sivaramakrishnan, Gangadharan, Nampoothiri, Soccol, & Pandey, 2006). For maximum production of amylase, pH is a main factor to be considered (Saxena & Singh, 2011). The optimization of fermentation parameters (physical and chemical) is highly required due to its large-scale usage in economy and practical application of this process. The optimization of culture media was traditionally performed by considering one parameter at a time. This method is time consuming and tedious to perform and interaction of various variables could not be measured simultaneously (Dey, Mitra, Banerjee, & Maiti, 2001). Response Surface Methodology

(RSM) is a recent procedure to optimize enzyme production by statistical way. It establishes a co-relation between controlled factors and experimental data. This technique requires experimental work to find interactive effect of different variables and can develop a link between variables of design and responses (Francis et al., 2003). Different bacterial strains can produce varying amount of amylase. Therefore, statistical approach like RSM is most suitable to optimize the rate of enzyme production (Pandey et al., 2000). The aim of present study was to screen amylase producing bacterial strains isolated from fish gut. The high yielding amylase producing bacteria was optimized on agro-waste (grape fruit peels) for minimizing the production cost employing central composite design (CCD) of RSM along with optimization of physical parameters for better enzyme activity.

## Material and methods

### Medium and strain

Peels of banana, mango, orange, grape fruit, pea, mosumbi, apple and banana peduncles were collected from local fruit shops and juice corners of Talagang city, District Chakwal Pakistan. Peels were washed, dried and ground to make fine powder, sieved (size of particles = 25 mm) and preserved in air tight jars. Three bacterial strains (*Roultella ornithinolytica, Bacillus amyloliquefaciens, B. licheniformis*) already isolated from fish gut were revived on nutrient agar plates and then screened for amylase production after culturing on selective medium (2% starch incorporated nutrient agar) at 37°C for 24h. Plates were observed for clear zone formation around colonies by flooding Gram's Iodine solution.

### Enzyme production

For the production of crude amylase, nutrient broth containing 2% starch were inoculated with 1% inoculum of each strain, incubated for 24h at 37°C under static condition. After incubation, culture medium was centrifuged at 10000 rpm for 10 minutes. The supernatant (crude enzyme) was used in enzyme assay. Strain yielded highest enzyme was further selected for optimization. All experiments were carried out in triplicates.

### Enzyme assay

For amylase assay, reducing sugar released at the end of reaction was measured by DNS method (Miller, 1959). Briefly, 0.5 mL of substrate solution (0.5 g starch in 100 mL of acetate buffer (pH 5) was incubated with 0.5 mL of crude enzyme at 40°C for 15 minutes. After incubation, 1 mL of freshly prepared DNS reagent was added and mixture were placed in boiling water bath for 15 minutes, then cooled at room temperature and the absorbance was recorded at 540 nm using spectrophotometer. Enzyme produced from 1 milligram of sugar per minute is called one unit of enzyme (Alkando & Ibrahim, 2011).

### Optimization of medium for maximum amylase production

### Effect of carbon source

Media comprising of 2% different carbon sources (banana peels, banana peduncles, mango peels, pea peels, grape fruit peels, orange peels and apple peels) were prepared, autoclaved, inoculated with 1% inoculum and incubated at 37°C for 24h in shaking incubator (100 rpm). The carbon source with highest amylase production was selected for further studies.

### Effect of organic and inorganic nitrogen sources on amylase production

The medium comprising of optimized 2% selected carbon source (grape fruit) supplemented with 1% organic and inorganic nitrogen sources (malt extract, peptone, beef extract, yeast extract, $NH_4Cl$, $KNO_3$, $(NH_4)_2SO_4$, $NH_4NO_3$) were prepared and proceeded as described above. The nitrogen source yielding highest enzyme was selected and used in medium for further experimentation.

### Effect of minerals

Media containing selected carbon source (2% grape fruit), nitrogen source (1% ammonium nitrate; $NH_4NO_3$) were prepared and supplemented with different salts including sodium chloride (NaCl), potassium chloride (KCl), potassium di hydrogen phosphate ($KH_2PO_4$), magnesium sulphate ($MgSO_4$),

calcium chloride ($CaCl_2$) and di potassium hydrogen phosphate ($K_2HPO_4$,). The salt with optimum enzyme production was used in subsequent study.

### Optimization of amylase production by response surface methodology

The optimization of different parameters was performed by using central composite design of response surface methodology. The selected four variables were grape fruit peel, ammonium nitrate, magnesium sulphate and pH. The coded values and their levels are mentioned in Table 1.

**Table 1.** Levels of Independent variables for CCD.

| Factors | Code | Level | | | | |
|---|---|---|---|---|---|---|
| | | -2 | -1 | 0 | +1 | +2 |
| Grape fruit peel (%) | X1 | 1 | 2 | 3 | 4 | 5 |
| Ammonium Nitrate (%) | X2 | 0.3 | 0.6 | 0.9 | 1.2 | 1.5 |
| Magnesium Sulphate (%) | X3 | 0.2 | 0.4 | 0.6 | 0.8 | 1.0 |
| pH | X4 | 3 | 5 | 7 | 9 | 11 |

A second order polynomial equation was applied to calculate the predicted enzyme value. The equation was as follows:

$$Y= ß_{\circ} + X_1 + X_2 + X_3 + X_4 + X_1^{\,2} + X_2^{\,2} + X_3^{\,2} + X_4^{\,2} + X_1 X_2 + X_1 X_3 + X_1 X_4 + X_2 X_3 + X_2 X_4 + X_3 X_4$$

Where Y is predicted response; $ß_{\circ}$ is model constant; $X_1$, $X_2$, $X_3$ and $X_4$ are coded values of four selected variables; $X_1^{\,2}$, $X_2^{\,2}$, $X_3^{\,2}$ and $X_4^{\,2}$are squared coefficients; $X_1 X_2$,$X_1 X_3$, $X_1 X_4$,$X_2 X_3$, $X_2 X_4$ and $X_3 X_4$ are interaction coefficients.

### Characterization of crude amylase

### Effect of pH on amylase activity

Effect of various pH ranged from 4 to 13 was tested to find optimum pH of amylase activity. The enzyme activity was measured as per standard assay procedures as described above.

### Effect of temperature on amylase activity

Optimum temperature of amylase was determined by incubating reaction mixture at 20, 30, 40, 50, 60, 70, 80 and 90°C for 15 minutes. The amylase activity was then measured as described above.

### Effect of substrate concentrations on amylase activity

The mixture containing 0.5 mL of different concentrations of substrate solutions (0.1-1% starch in optimized pH buffer) and 0.5 mL of crude enzyme was incubated at optimized temperature for 15 min. The amylase activity was then measured.

### Effect of incubation period on amylase activity

The reaction mixture containing 0.5 mL of substrate solution (0.5% starch in optimized pH buffer) and 0.5 mL of crude enzyme incubated at optimized temperature for 10-120 minutes. The amylase activity was then measured as described earlier.

### Statistical analysis

The data was analysed by applying multiple regression and analysis of variance (ANOVA). The significance of model was determined by comparing correlation coefficients.

## Results and discussion

Three strains showed clear zones for starch hydrolysis on selected medium (2% starch incorporated nutrient agar) when flooded with iodine solution. *B. licheniformis* showed the largest clear zone around the colony (Table 2; Figure 1). Maximum enzyme activity of 0.475±0.002 U $mL^{-1}$ was noted for *B. licheniformis*.

**Table 2.** Screening of bacterial isolates for amylase producing potential on the basis of diameter of clear zone and enzyme assay.

| Sr. No. | Bacterial isolates | Diameter (cm) | Enzyme activity (U $mL^{-1}$) |
|---|---|---|---|
| 1 | *Roultella ornithinolytica* | 1.2 | $0.44^{b}$±0.007 |
| 2 | *Bacillus amyloliquefaciens* | 1.8 | $0.42^{c}$±0.006 |
| 3 | *Bacillus licheniformis* | 2.5 | $0.47^{a}$±0.002 |

Means±standard deviation that do not share a letter in column are highly significantly different (P<0.001).

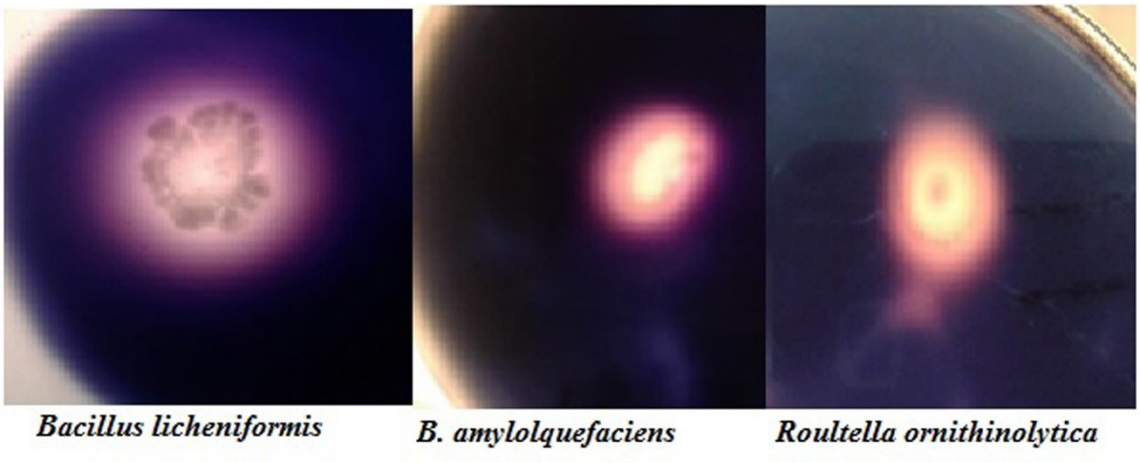


**Figure 1.** Clear zone around the colony of different strains.

## Selection of medium ingredients

Microbial amylase production has been greatly affected by composition and physical conditions of culture medium. Saito (1975) used *B. licheniformis* for amylase production and explained regulatory factors affecting the production of enzyme. The main component of culture medium is carbon because bacteria require a weighty quantity of carbon source in growing medium. In present study, the focus was to grow bacteria on a medium which contain a carbon source that could easily be available on low cost. Fruit peels were selected as carbon source for production of extracellular amylase. In present study, maximum enzyme production (1.327±0.004 U $mL^{-1}$) was recorded by *B. licheniformis* with grape fruit peels and minimum enzyme production (0.181±0.006 U $mL^{-1}$) was recorded for banana peels. Therefore, grape fruit was selected as carbon source for further experimentation (Figure 2). Abd-Elhalem, El-Sawy, Gamal and Abou-Taleb (2015) has reported same type of results of amylase production by *Bacillus* species using agro-wastes. Similarly, maximum amylase (7.26 IU $mL^{-1}$ $min^{-1}$) of *Bacillus* species noted by Unakal, Kallur and Kaliwal (2012) using 50 g banana peels as carbon source.

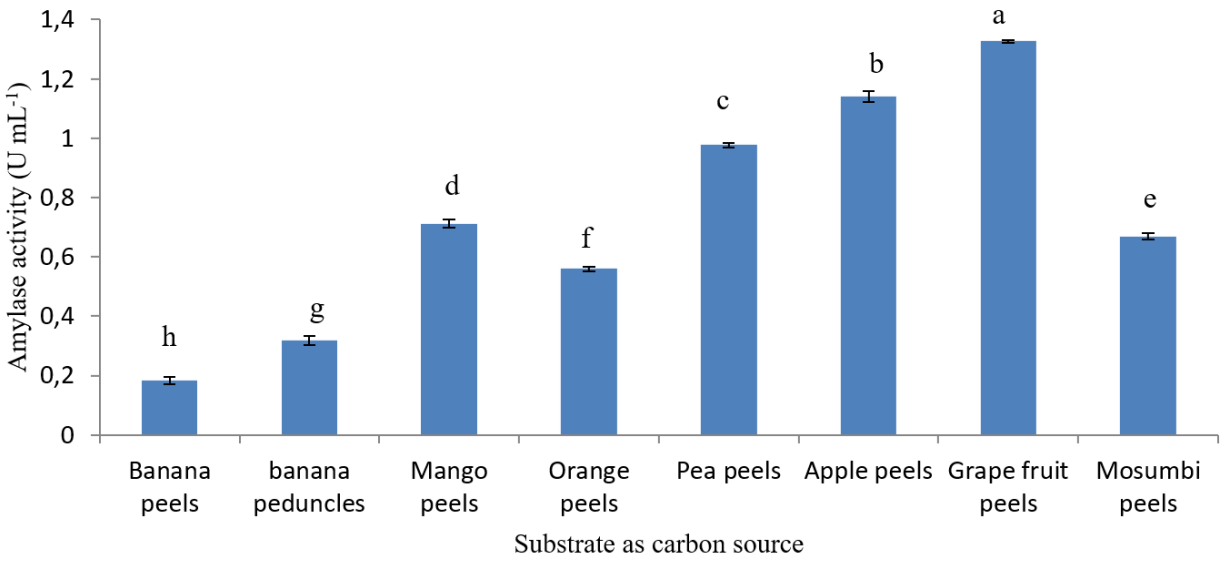


**Figure 2.** Effect of different agro-wastes (carbon source) on enzyme production. Different alphabet showed that means are highly significantly different ($p < 0.001$).

In present study, amylase production increased many folds after supplementation of medium with nitrogen containing compound. When different nitrogen sources were added to the grape fruit medium, ammonium nitrate improved the rate of amylase production. Maximum enzyme production up to 1.601±0.015 U $mL^{-1}$ was recorded by *B. licheniformis* (Figure 3). Karataş Uyar, Tolan, and Baysal (2013) reported similar results when medium was supplemented by ammonium sulphate. Yeast extract was selected as nitrogen source by Khusro, Barathikannan, Aarti, and Agastian (2017). In present study, ammonium nitrate play very important role in medium to increase the amylase production. This finding was in line with other researchers that used different organic and inorganic nitrogen sources and peptone was selected with maximum amylase by *Bacillus* species (Simair et al., 2017).

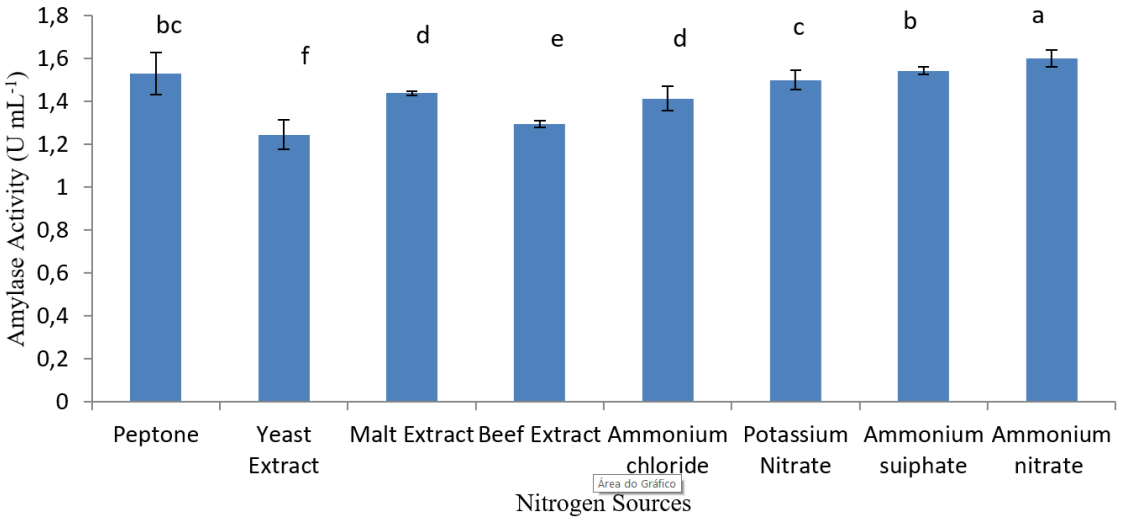


**Figure 3.** Effect of different nitrogen sources on enzyme production. Different alphabet showed that means±SD are highly significantly different ($p < 0.001$).

The use of mineral salts can also increase the amylase production. In present study, amylase production increased up to 1.643±0.029 U $mL^{-1}$ after addition of magnesium sulphate as mineral salt into the medium already containing carbon (graph fruit peels) and nitrogen (ammonium nitrates) sources. Similar findings regarding magnesium sulphate reported for maximum production of amylase by other researchers (Unakal et al., 2012). Sodium chloride and calcium chloride were the two other mineral salts used in present study. Both showed positive effect on amylase production by *B. licheniformis* (Figure 4). Asghar, Azhar, Rafiq, Sheikh, and Asad (2002) also described that maximum yield of amylase could be achieved by adding calcium chloride in the wasted bread medium for growth of *Arachniotus* species.

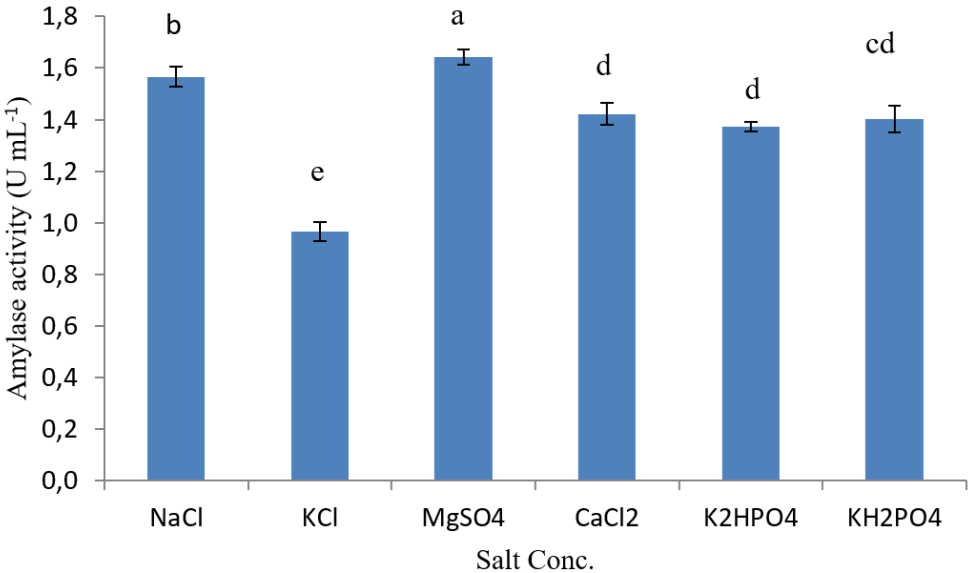


**Figure 4.** Effect of salt on enzyme production. Different alphabet showed that means±SD are highly significantly different ($p < 0.001$).

## Optimization of medium concentration using central composite design

Central composite design (CCD) of RSM was used for optimization of concentration of medium ingredients and physical factor (pH). The selected four variables were grape fruit peels as carbon source, ammonium nitrate as nitrogen source, magnesium sulphate as salt and media prepared at different pH values. The results of 26 runs were represented in Table 3. The response was calculated by 2nd order polynomial regression equation (equation 2). Maximum enzyme (1.740 U $mL^{-1}$) recorded with 5 % grape fruit peels, 0.9 % ammonium nitrate, 0.6 % magnesium sulphate at pH 7. Mushtaq, Irfan, Tabassum and Qazi (2017) obtained initial medium pH of 5 for maximum amylase production from *B. subtilis* K-18 optimized through CCD.

**Table 3.** CCD design for optimization of different variables.

| Exp. No. | X1 | X2 | X3 | X4 | Enzyme value (U $mL^{-1}$) | | |
|---|---|---|---|---|---|---|---|
| | | | | | Observed | Predicted | Residual |
| 1 | 1 | 0.9 | 0.6 | 7 | 0.297 | 0.666 | -0.369 |
| 2 | 3 | 0.9 | 0.6 | 11 | 0.323 | 0.688 | -0.365 |
| 3 | 2 | 1.2 | 0.8 | 5 | 1.217 | 1.201 | 0.016 |
| 4 | 3 | 0.9 | 0.2 | 7 | 1.369 | 1.469 | -0.100 |
| 5 | 3 | 0.9 | 0.6 | 7 | 1.379 | 1.416 | -0.037 |
| 6 | 5 | 0.9 | 0.6 | 7 | 1.740 | 1.554 | 0.186 |
| 7 | 2 | 0.6 | 0.8 | 9 | 0.833 | 0.761 | 0.072 |
| 8 | 3 | 0.9 | 1.0 | 7 | 1.431 | 1.515 | -0.084 |
| 9 | 3 | 0.3 | 0.6 | 7 | 1.445 | 1.484 | -0.039 |
| 10 | 2 | 0.6 | 0.8 | 5 | 1.394 | 1.274 | 0.120 |
| 11 | 2 | 0.6 | 0.4 | 5 | 1.319 | 1.282 | 0.037 |
| 12 | 4 | 1.2 | 0.4 | 9 | 1.430 | 1.425 | 0.005 |
| 13 | 2 | 1.2 | 0.4 | 9 | 1.284 | 1.00 | 0.280 |
| 14 | 4 | 1.2 | 0.8 | 9 | 1.501 | 1.480 | 0.021 |
| 15 | 4 | 0.6 | 0.8 | 9 | 1.580 | 1.428 | 0.152 |
| 16 | 3 | 0.9 | 0.6 | 3 | 1.499 | 1.318 | 0.181 |
| 17 | 4 | 1.2 | 0.4 | 5 | 1.528 | 1.542 | -0.014 |
| 18 | 2 | 1.2 | 0.8 | 9 | 1.091 | 0.889 | 0.202 |
| 19 | 2 | 0.6 | 0.4 | 9 | 0.942 | 0.763 | 0.180 |
| 20 | 2 | 1.2 | 0.8 | 5 | 1.243 | 1.201 | 0.042 |
| 21 | 4 | 1.2 | 0.8 | 5 | 1.537 | 1.591 | -0.054 |
| 22 | 3 | 1.5 | 0.6 | 7 | 1.432 | 1.576 | -0.144 |
| 23 | 4 | 0.6 | 0.4 | 5 | 1.502 | 1.579 | -0.077 |
| 24 | 3 | 0.9 | 0.6 | 7 | 1.453 | 1.416 | 0.037 |
| 25 | 2 | 1.2 | 0.4 | 5 | 1.295 | 1.322 | -0.027 |
| 26 | 4 | 0.6 | 0.8 | 5 | 1.517 | 1.739 | -0.222 |

$$Y = 0.701^{*} + 0.435X_1 - 0.609X_2 - 0.752X_3 + 0.130X_4 - 0.076X_1^2 + 0.317X_2^2 + 0.473X_3^2 - 0.026X_4^2 - 0.063X_1X_2 + 0.212X_1X_3 + 0.025X_1X_4 - 0.468X_2X_3 + 0.084X_2X_4 + 0.004X_3X_4$$

The statistical analysis was performed by using ANOVA (analysis of variance) to find out influence of selected four parameters by CCD. The F and P values of model indicated that the proposed model was significant (Table 4). The $R^2$ value for multiple regression was 0.789 which indicated that there was 78.9% variability found in production of amylase. The closeness between predicted $R^2$ and adjusted value of $R^2$ indicated precision of results (Hassan & Karim, 2015). If the $R^2$ value is close to 1 there is more correlation between experimental and predicted values as in this case it was 0.789 which was close to 1 which proved the goodness of model. A significant correlation was observed between observed and predicted values of enzyme. The dissimilarities between two values were due to different coefficient factors (Prajapati, Trivedi, & Patel, 2015; Zhao, Zheng, Wang, & Zhou, 2011).

Figure 5 shows relationship between grape fruit peel as carbon source (X1), Ammonium sulphate (X2), $MgSO_4$ (X3) and pH(X4) on amylase production. These plots indicated that each parameter had significant effect on amylase production in submerge fermentation.

**Table 4.** Analysis of variance (ANOVA) of different parameters for amylase production.

| Effect | df | SS | MS | F | P |
|---|---|---|---|---|---|
| Model | 14 | 2.386748 | 0.170482 | 2.946436 | 0.039305 |
| Intercept | 1 | 0.006040 | 0.006040 | 0.104384 | 0.752690 |
| X1 | 1 | 0.046902 | 0.046902 | 0.810608 | 0.387227 |
| $X1^2$ | 1 | 0.101839 | 0.101839 | 0.760077 | 0.211498 |
| X2 | 1 | 0.008372 | 0.008372 | 0.144700 | 0.710895 |
| $X2^2$ | 1 | 0.014202 | 0.014202 | 0.245448 | 0.630047 |
| X3 | 1 | 0.005672 | 0.005672 | 0.098035 | 0.760058 |
| $X3^2$ | 1 | 0.006240 | 0.006240 | 0.107847 | 0.748776 |
| X4 | 1 | 0.014705 | 0.014705 | 0.254143 | 0.624117 |
| $X4^2$ | 1 | 0.186023 | 0.186023 | 3.215032 | 0.100474 |
| X1* X2 | 1 | 0.005487 | 0.005487 | 0.094836 | 0.763870 |
| X1* X3 | 1 | 0.027105 | 0.027105 | 0.468448 | 0.507866 |
| X2* X3 | 1 | 0.011927 | 0.011927 | 0.206135 | 0.658641 |
| X1* X4 | 1 | 0.038267 | 0.038267 | 0.661364 | 0.433329 |
| X2*X4 | 1 | 0.038128 | 0.038128 | 0.658964 | 0.434141 |
| X3 * X4 | 1 | 0.000038 | 0.000038 | 0.000652 | 0.980084 |
| Error | 11 | 0.636465 | 0.057860 | | |

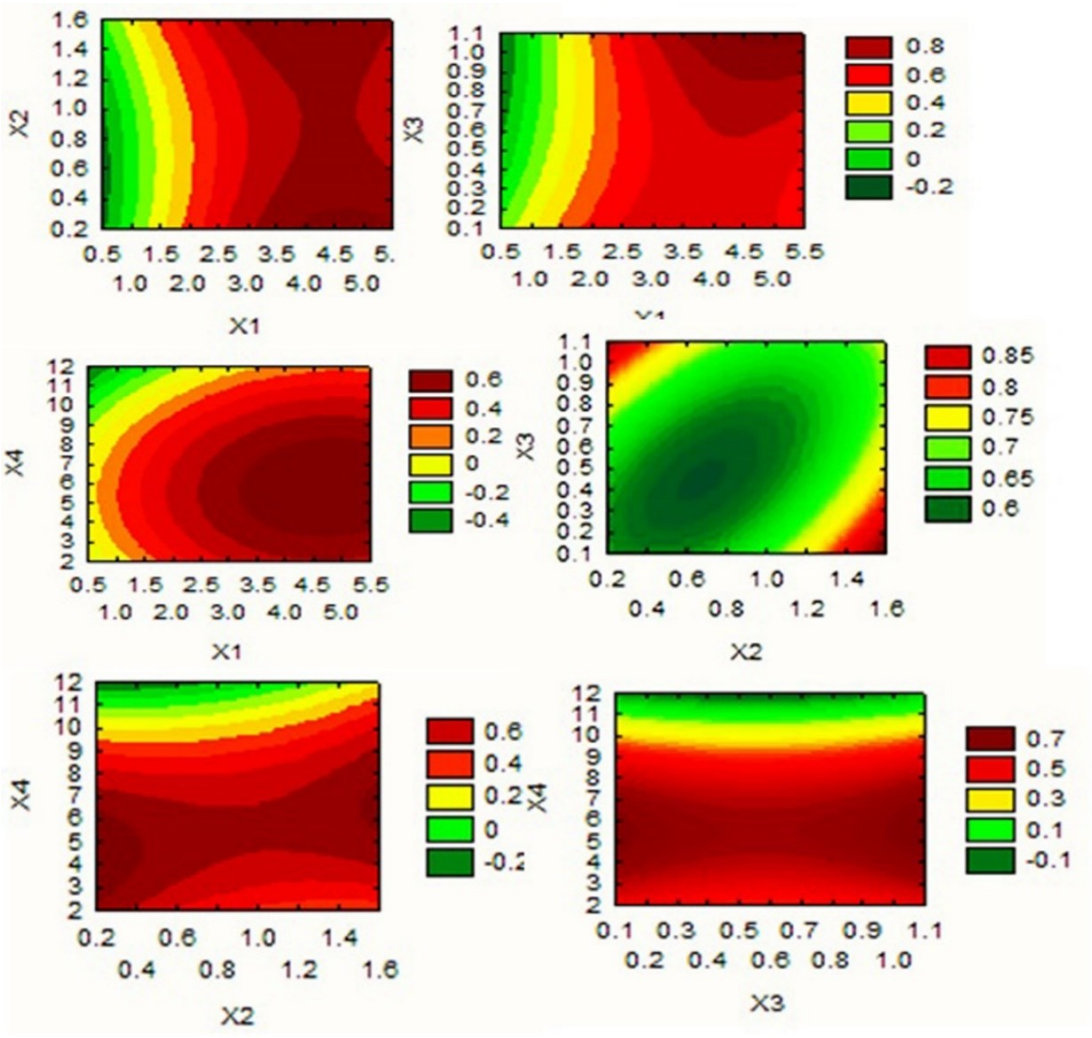


**Figure 5.** Contour plots for grape fruit (X1), ammonium sulphate (X2), $MgSO_4$ (X3) and pH (X4) on amylase production.

The desirability chart for amylase production (Figure 6) indicated the validation of model by repeated experiments. Results obtained were in line with predicted values as revealed by the model.

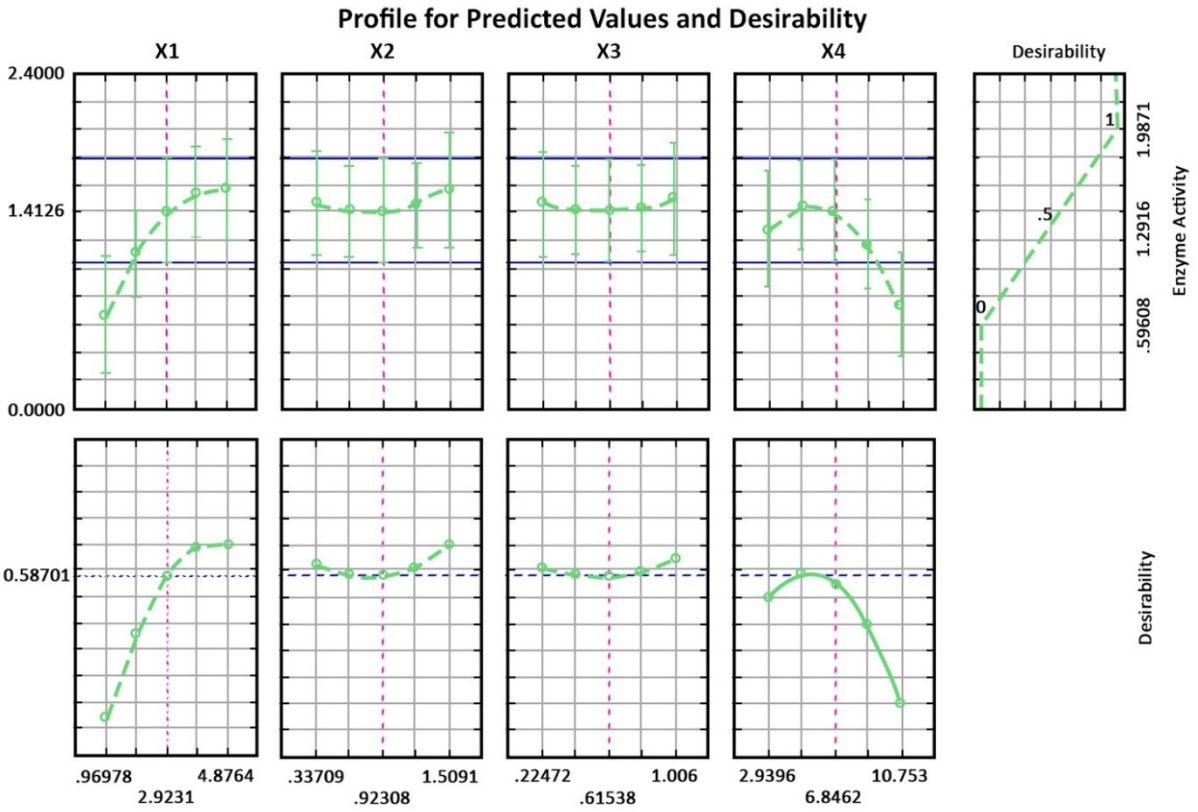


**Figure 6.** Desirability profile for amylase production.

## Characterization of crude amylase

Enzyme activity was checked for different pH values and maximum amylase activity (3.551±0.059 U $mL^{-1}$) by *B. licheniformis* was recorded at pH 9 while minimum activity (2.703±0.016) was recorded at pH 4 (Figure 7). Similar finding was recorded by Khusro et al. (2017) at pH 9 and another study on *B. licheniformis* by Vaseekaran. Balakumar and Arasaratnam (2010) reported optimum pH 7 for maximum amylase activity. The change in pH of medium can change the amylase activity because proteins or enzymes denature at altered pH. Simair et al. (2017) reported maximum amylase activity of *Bacillus* species at pH 8.

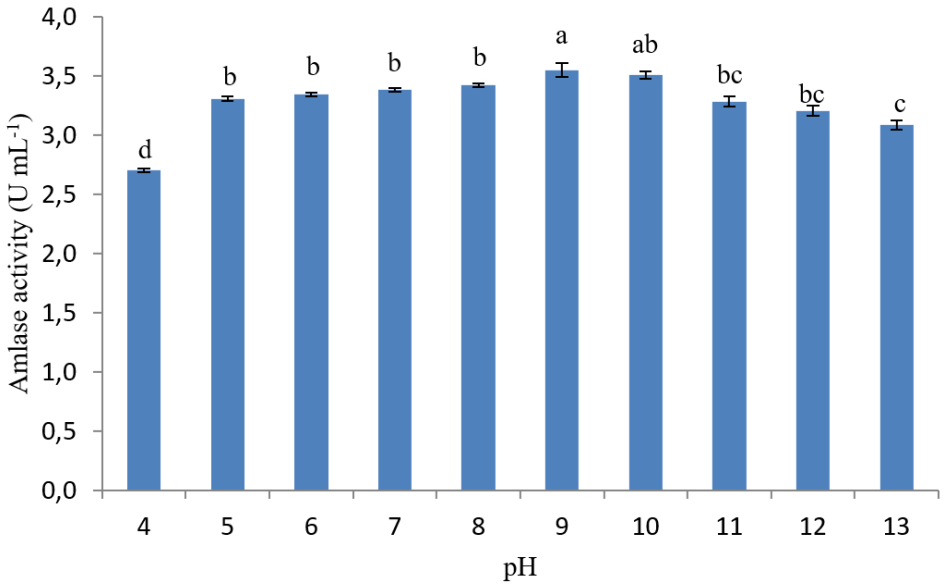


**Figure 7.** Effect of pH on amylase activity. Different alphabet showed that means are highly significantly different ($p < 0.05$).

Amylase activity is greatly influenced by change in temperature. In present studies it was found that amylase of *B. licheniformis* remained active at high temperature. Maximum amylase activity (3.566±0.025

U $mL^{-1}$) by *B. licheniformis* was recorded at 80°C while minimum activity (3.260±0.012 U $mL^{-1}$) was recorded at 20°C (Figure 8). Further increase in temperature of incubation decreased its activity. So, this enzyme might be proved a best source of starch hydrolysis in those industrial processes which are performed at high temperatures. Vaseekaran et al. (2017) proposed that same *Bacillus* species could produce amylase which remain active at 90°C. This variety in temperature range might be due to change in isolation source of bacteria. Amylase produced from *Bacillus subtilis* strains exhibit optimum temperature of 60°C (Irfan, Gulsher, Nadeem, & Syed, 2009; Irfan, Nadeem, Syed, Shakir, & Qazi, 2016).

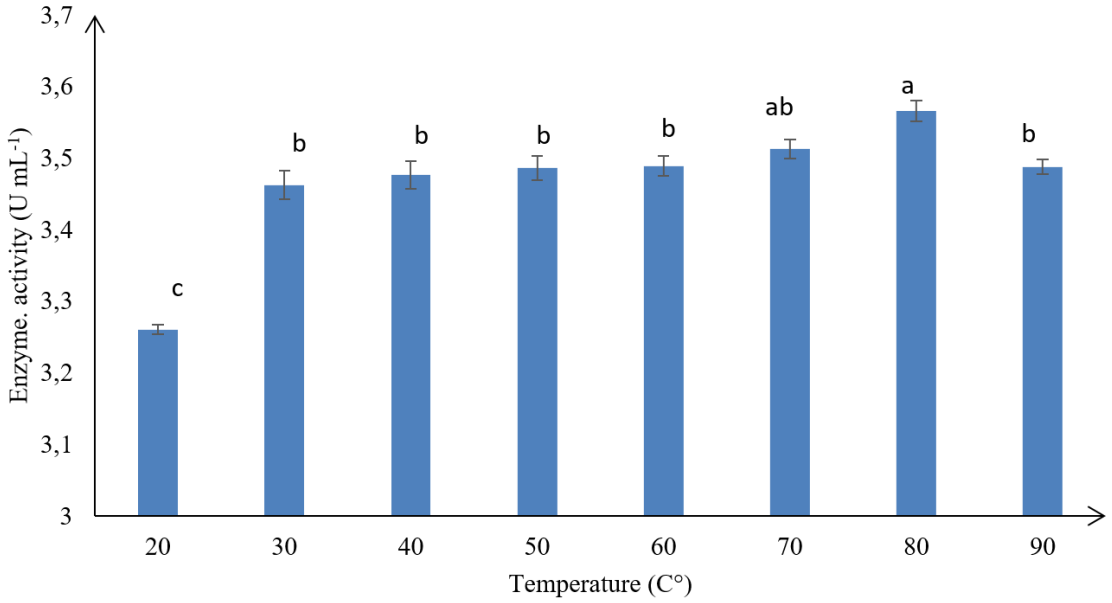


**Figure 8.** Effect of temperature on enzyme activity.

Amylase may remain active under different incubation times depending on the source of isolation. In recent study, crude amylase of *B. licheniformis* was incubated at 80 °C for different time periods ranging from 10 to 120 minutes. Maximum amylase activity up to 5.132±0.013 U $mL^{-1}$ by *B. licheniformis* was recorded when reaction mixture was incubated for 30 minutes while minimum activity up to 3.899±0.032 U $mL^{-1}$ was recorded for incubation period of 120 minutes (Figure 9). The enzyme activity increased with increase in time interval after that it became decreased for longer time of incubation. Vaseekaran et al. (2010) proposed that *B. licheniformis* isolated from soil receiving bakery wastes showed maximum enzyme activity when incubated for 5 minutes at 90°C. Maximum amylase activity was recorded for 4h of incubation at 60°C by Khusro et al. (2017).

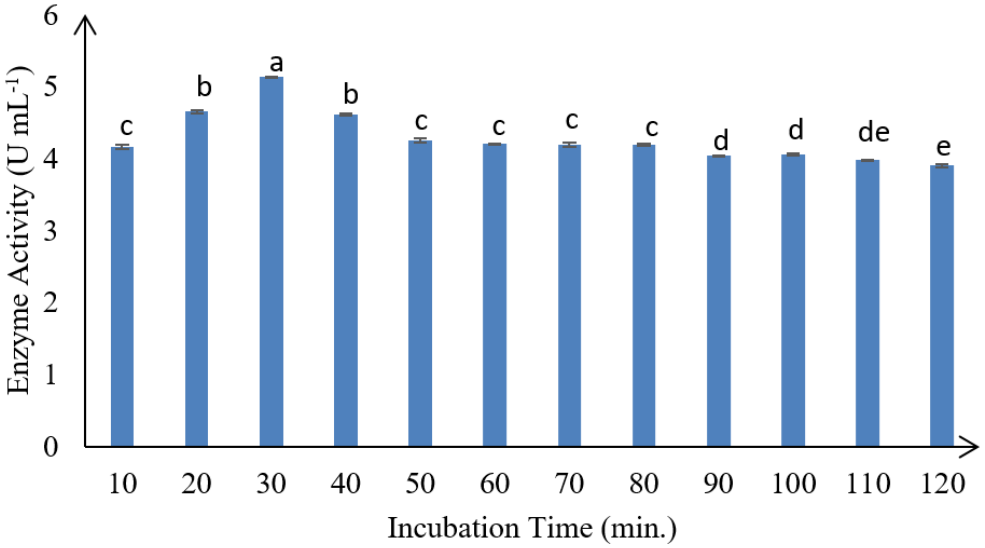


**Figure 9.** Effect of duration of incubation period on enzyme activity.

## Conclusion

*Bacillus licheniformis* already isolated from fish gut had capability to produce amylase in submerged fermentation. Central composite design of response surface methodology was useful for optimization of various process parameters for enhanced production. The amylase produced in this study was alkaline and thermostable which could be exploited in industrial application.

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