Production optimization using Plackett-Burman and Box-Behnken designs with partial characterization of amylase from marine actinomycetes

Sarah I Bukhari
King Saud University
Mohamed H Al-Agamy (malagamy@ksu.edu.sa)
King Saud University, Al-Azhar University https://orcid.org/0000-0001-9868-0355
Mahmoud S Kelany
National Institute of Oceanography and Fisheries
Mohammad R Al Hazani
King Saud University College of Pharmacy
Moaz M Hamed
National Institute of Oceanography and Fisheries

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Abstract

Amylase is an industrial enzyme that is used in the food and biofuel industries. We screened four actinomycetes strains for amylase biosynthesis. The *Streptomyces rochei* strain had a larger hydrolytic zone (24 mm) on starch agar plates, than the other isolates. Plackett-Burman's experimental design was implemented to optimize the conditions for amylase production by the selected strains. Growth under optimized culture conditions led to 1.7, 9.8, 7.7, and 3.12 -fold increases for the isolates *S. griseorubens*, *S. rochei*, *S. parvus*, and *Streptomyces* sp., respectively, in the specific activity measurement in comparison with growth under primary conditions. When applying the Box-Behnken design on *S. rochei* using the most significant parameters starch, K_2HPO_4, pH, and temperature, there was a 12.22-fold increase in the specific activity measurement: 7.37 U/mg. The optimal fermentation medium formula was kept at 30.6°C for seven days. The amylase from *S. rochei* was partially purified, and its molecular weight was determined using Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The molecular weight was found to be 45, 43, and 53 kDa. Amylase was particularly active at pH 6 and 65°C. The purified enzyme was most active at 65°C and a pH of 6, thermal stability of 70°C for 40 min and salt concentration of 1 M with a Km and Vmax of 6.58 mg/ml and 21.93 mg/ml/min, respectively. The amylase improved by adding Cu + 2, Zn + 2, and Fe + 2 (152.21%, 207.24%, and 111.89%). Increased production of amylase enzyme by *Streptomyces rochei* KR108310 attracts the production of industrially significant products.

Introduction

Marine habitats are regarded as a possible supplier of bioactive metabolites with enormous, unparalleled industrial value. Some 20,000 natural products, including nine licensed medicines and 12 under clinical trials, have been recorded from marine sources (Suthindhiran et al 2014). Extensive marine secondary metabolite varieties have been used for the production of medicinal products. Micro-organisms function as a complement to drug development, as they are an excellent source for synthesizing various useful enzymes. Recently, marine microbial enzymes have been found to be quick and cost effective to produce and environmentally sound (Asoodeh et al. 2013). However, in extreme circumstances, such as in alkaline and high temperature conditions, most enzymes are more frequently unstable in industrial processes. Therefore, researchers are interested in exploring marine microbes that are tolerant of extreme environmental conditions as a precious natural resource of new products, including different enzymes (Amador et al. 2003). Actinomycetes are Gram-positive and produce various commercial enzymes (Boovaragamoorthy et al 2019). Among Actinomyces, *Streptomyces* species are the most essential industrially-useful organism because of its ability to produce multiple enzymes (Al-Dhabi et al. 2019). Actinomycetes contain various enzymes, including protease, amylase, lipase, pectinase, cellulase, xylanase, glutaminase, and asparaginase. (Al-Dhabi et al. 2020). *Streptomyces* species produce various enzymes that are nutritionally essential for these Gram-positive bacteria, including *S. griseus*, S. *clavuligerus*, *S. thermoviolaceus*, *S. rimouses*, and *S. thermovulgaris*. Additionally, *Streptomyces* species release some extra cell enzymes (Al-Dhabi et al. 2016; Arasu et al 2019; Gupta et al. 1995), including amylase enzymes, which are considered a potential product for many fields, including manufacturing, clinical, medicinal, analytical chemistry, ethanol production, textile, food, brewing, and distillation industries. The manufacturing industries include the production of starch and syrup (Tonkova 2006) with divided associations to glucose, maltotriose, and maltose formation using chemical methods (Vidyalakshmi et al 2009). Amylases are commonly used as an agent to treat fruit, such as bananas, mangoes, citrus fruits, and papayas, and wash pots used in the fermentation of foodstuffs and the paper and textile industries. These enzymes come from different sources, such as actinomycetes and bacteria. The microbial source is favored among these amylases over other sources due to their wide availability and plasticity (Li et al 2011; Sadh et al 2018 and Vidyalakshmi et al., 2009). Also, α- Amylases can be used in the saccharification of starch, textiles, food, brewing, and distillation industries (Gupta et al. 2003 and Janaki, 2017).

The production of amylase enzymes rate from isolates has been investigated using horticulture methods, assessing pH and element requirements, incubation time, and the temperature of incubation. However, there are limited studies on the production of amylase from marine *Streptomyces* spp. Therefore, this study aimed to screen for the production of amylase from different marine actinomycetes isolates belonging to the genus *Streptomyces*, enhance amylase production and characterize this enzyme for various industrial processes.

Materials And Methods

Chemicals and nutrient media

All chemicals and cultivation media were obtained from the Al-Gomhoria Chemicals Company, Egypt. All laboratory tests were conducted using sterilized seawater. Earlier, several researchers isolated actinomycetes for the production of amylases from different sources.

Micro-organisms and cultural conditions

The marine actinomycetes isolates that were used in the study were *Streptomyces griseorubens* strain (MMH 9), *Streptomyces rochei* strain (HMM 13), *Streptomyces Parvus* strain (8), and *Streptomyces* sp. strain (M12). Dr. Moaz M. Hamed (Marine Microbiology Lab., Marine Environmental Division, National Institute of Oceanography and Fisheries, Red sea branch, Egypt) isolated all the strains from marine sediment samples from the Suez gulf, and all the isolates were deposited in Genbank with accession numbers KR133201, KR108310, KP675949, and MK388207, respectively. The marine actinobacterial isolates were maintained on slopes containing starch nitrate agar media, with the following composition per liter: 20 g of starch, 1 g of K_2HPO_4, 2 g of KNO_3, 0.5 g of $MgSO_4$, and 18 g of agar. The components were dissolved in 0.5 l of distilled water and 0.5 l of seawater (El-Sersy and Abou-Elela 2006). After autoclave and cementing, 50 and 20 µg/ml of tetracycline and nystatin were included as antibacterial and anti-hunger substances. After seven days, the strain was hatched at 30–32 °C. This isolate was protected for subsequent examination within the spore suspension of 20% (v / v) glycerol at −20 °C.

Primary screening of extracellular amylase enzyme

Amylase enzyme production was investigated using a starch casein agar plate with an active actinomycetes culture (Rathore et al 2019). The ingredients of the medium per 1 l were incubated at 32°C for seven days: 10 g of starch, 2 g of KNO_3, 1 g of Casein, 0.5 g of $MgSO_4$, 0.001 g of $FeSO_4$, 1 g of K_2HPO_4, 0.1 g of $CaCO_3$, 18 g of Agar, and 50/50 DW (Viswanathan and Rebecca 2019). The zone of plate hydrolysis after incubation indicated the positive activity of isolates for amylase production (Salgaonkar et al. 2019).

Enzyme assay

Amylase activity and protein production was measured using Di nitrosalysilic acid and folin reagent (Jain et al 2020 and Lowry et al 1951). The specific activity of amylase was calculated by dividing the activity into protein (Monteiro 2020).

Enhancement of the production of amylase enzyme

Statistical design for optimization of amylase enzyme

The Plackett-Burman experimental design (Plackett and Burman 1946) showed how necessary the medium components are to producing amylase enzymes through selected isolates using starch casein agar media. The Plackett-Burman design matrix had seven independent variables (Table 1) in eight combinations (Table 2). The baseline control was in

row No. 9. High (+1) and low (−1) values were evaluated for each component. Each experiment was replicated twice, and the mean of these values was used as the response.

Table 1: Factors of Plackett-Burman design with their high and lower levels.

Variables	Symbol	Ingredients per liter		
		Factors level	High level (+)	Lower level (-)
Starch	S	10	15	5
KNO_3	KN	2	3	1
Casein	Ca	1	1.5	0.5
$MgSO_4$	Mg	0.5	0.75	0.25
K_2HPO_4	K_2	1	1.5	0.5
pH	pH	7	8	6
Temperature °C	T	32	34	30

Both trials were conducted in triplicate. The main effect of each variable was estimated using the following equation:

$$E_{xi} = (\Sigma M_{i+} - \Sigma M_{i-}) / N$$

When Exi is the variable main effect, the amylase production radiuses for the tests were Mi+ and Mi-, where the independent variables were present in high and low concentrations, respectively, and N was used to measure the statistical t-values of the equal unpaired samples to determine the variable meaning by dividing by two using Microsoft Excel 2019.

Table 2. The test results for seven cultural variables of the applied design of the Plackett-Burman experimental design.

Trail	S	KN	Ca	Mg	K_2	pH	T
1	−1	−1	−1	1	1	1	−1
2	1	−1	−1	−1	−1	1	1
3	−1	1	−1	−1	1	−1	1
4	1	1	−1	1	−1	−1	−1
5	−1	−1	1	1	−1	−1	1
6	1	−1	1	−1	1	−1	−1
7	−1	1	1	−1	−1	1	−1
8	1	1	1	1	1	1	1
9	0	0	0	0	0	0	0

Experimental verification

Verification tests were conducted with double standards using the predicted optimized medium to validate the Plackett-Burman design statistical analysis results. The production of amylase enzyme was measured by dividing the activity into protein to determine the specific activity.

Optimization of the culture conditions using the Box-Behnken design

After assessing the relative importance of separate variables, the four most important variables were chosen to determine the optimum levels for amylase enzyme development. The Box-Behnken design (BBD) was used because it is a surface response methodology (Agrawal et al 2020 ; Box and Behnken 1960). This optimization method includes three key steps: coefficient estimation of a mathematical model, response prediction, and model adequacy verification. The four significant variables elucidated using the Plackett-Burman experimental design for the selected best amylase enzyme produced by the isolate of the *Streptomyces rochei* strain (HMM 13) were starch (X_1), K_2HPO_4 (X_2), pH (X_3), and temperature (X_4). The moo, center and tall levels of each variable were assigned as −1, 0, and +1, individually. A framework was built for the 27 trials, alongside the normal values for the four variables. The experiments were performed in triplicate and the mean values were determined for specific activities. The connection between the free factors and response functions was correlated using a second-order polynomial to actuate the optimal point. The condition for the four components was calculated using the formula below:

$$Y=B_0+B_1X_1+B_2X_2+B_3X_3+B_4X_4+B_{12}X_1X_2+B_{13}X_1X_3+B_{14}X_1X_4+B_{23}X_2X_3+B_{24}X_2X_4+B_{34}X_3X_4+B_{11}X_{11}+B_{22}X_{22}+B_{33}X_{33}+B_{44}+X_{44}$$

Where Y is the anticipated response, β_0 is the show constant, X_1, X_2, X_3, and X_4 are the free factors, β_1, β_2, β_3, and β_4 are the direct coefficients, β_{12}, β_{13}, β_{23}, and β_{24} are the cross-product coefficients, and β_{11}, β_{22}, β_{33}, and β_{44} are the quadratic coefficients. Microsoft Excel 2019 was used to examination of the experimental data collected using regression analysis.

Table 3. The Box-Behnken experimental design for four factors.

2	x3	x4	x1*x2	x1*x3	x1*x4	x2*x3	x2*x4	x3*x4	x1*x1	x2*x2	x3*x3	x4*x4
.	0	1	0	0	0	0	1	0	0	1	0	1
1	0	0	−1	0	0	0	0	0	1	1	0	0
)	1	−1	0	0	0	0	0	−1	0	0	1	1
)	0	1	0	0	1	0	0	0	1	0	0	1
.	−1	0	0	0	0	−1	0	0	0	1	1	0
)	−1	1	0	0	0	0	0	−1	0	0	1	1
)	1	1	0	0	0	0	0	1	0	0	1	1
)	1	0	0	1	0	0	0	0	1	0	1	0
)	1	0	0	−1	0	0	0	0	1	0	1	0
)	−1	−1	0	0	0	0	0	1	0	0	1	1
)	0	0	0	0	0	0	0	0	0	0	0	0
.	0	−1	0	0	0	0	−1	0	0	1	0	1
)	0	1	0	0	−1	0	0	0	1	0	0	1
.	1	0	0	0	0	1	0	0	0	1	1	0
.	0	0	1	0	0	0	0	0	1	1	0	0
1	0	−1	0	0	0	0	1	0	0	1	0	1
)	0	−1	0	0	−1	0	0	0	1	0	0	1
)	−1	0	0	1	0	0	0	0	1	0	1	0
1	0	0	1	0	0	0	0	0	1	1	0	0
)	0	−1	0	0	1	0	0	0	1	0	0	1
1	−1	0	0	0	0	1	0	0	0	1	1	0
1	1	0	0	0	0	−1	0	0	0	1	1	0
)	−1	0	0	−1	0	0	0	0	1	0	1	0
)	0	0	0	0	0	0	0	0	0	0	0	0
1	0	1	0	0	0	0	−1	0	0	1	0	1
.	0	0	−1	0	0	0	0	0	1	1	0	0
)	0	0	0	0	0	0	0	0	0	0	0	0

Statistical analysis

Numerous straight relaps were made utilizing Microsoft Excel predictions to determine the significance of the amylase protein (presented in specific activity) in terms of t-value, P-value, and confidence level. The level of significance (P-value) was resolved using the student's t-test. Every single impact t-test assesses the probability that finding the observed effect was pure chance. If this is highly unlikely, then the affect is thought to be caused by the variable when it is below the accepted level, such as 5%. The confidence level reflects a percentage of the P-value. The activities were assessed using the Microsoft Excel solver add-in program. Each response was simultaneously visualized in three-dimensional graphics created using STATISTICA 10.0 software for the four largest independent factors.

Model verification

Experimentally, optimal conditions were verified from the optimization experiments. The predictions were examined and compared to the basic conditions, near-optimal conditions, and conditions different to the optimum levels of the independent variables.

Purification of amylase enzyme

Amylase was purified from *Streptomyces rochei* HMM 13 using various steps, including precipitation of ammonium sulfate, dialysis, and Sephadex G-50 (Mohammed et al 2017). All purification steps were completed at 4°C. Overnight, the crude was accelerated by ammonium sulfate with precipitated proteins separated at different saturation percentages (25%, 50%, 75%, and 90%). The precipitated sample was dialyzed overnight against water and barium chloride (Beltagy et al 2018). The ultimate purification process was performed using Sephadex G-50, employing a test with 90% immersion precipitation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to calculate the atomic weight of the purified enzyme (Bano et al 2011).

Characterization of amylase enzyme

The pH (3–10) of purified *Streptomyces rochei* HMM 13 amylases were tested under standard assay conditions. The temperature effect was established on decontaminated amylase by hatching the improved amylase at a temperature extending from 30°C to 80°C utilizing phosphate buffer for 4h and measuring activity as portrayed already (Nithya et al 2017). Amylase thermal stability was determined when an enzyme was incubated at 55°C, 65°C, and 70°C for 4h. Enzyme activity was determined at regular time intervals of 20 min. Kinetic amylase activity parameters were calculated at soluble starch concentrations of 0,1 to 1,2 g/L at a pH of 6 and 65°C for 30 min (Hwang et al 2013). Double reciprocal plots obtained the Michaelis-Mentent constant (km) and maximum efficiency (Vmax) (Singh et al 2014). Optimum salinity for amylase activity was examined by activity at various NaCl concentrations (0.2–2.1 M) in phosphate buffer with pre-incubation of an enzyme at 65 °C and a pH of 6 for 30 min under standard conditions. Metal particles (Co+2, Cu+2, Mn+2, Zn+2, Mg+2, Fe+2, and EDTA) were contributed to distinguish their impact on amylase activity in 0.05 M phosphate buffer at a pH of 6. A measure of 5 mL of metal ion solution and reagents were combined with refined *S. rochei* HMM 13 amylases. This mixture was kept beneath 65°C for 30 min, and the enzyme activity was measured as depicted by (Amutha and Priya 2011).

Results

The marine actinomycetes strains were initially qualitatively assessed for extracellular enzyme development. Initial screening showed that amylases could be produced from isolated marine actinomycetes on culture plates. Among the isolates of actinomycetes, the *Streptomyces rochei* strain HMM 13 has been very active on starch agar (24 mm) when compared to other isolates (Table 4 & Fig. 1).

Table 4. Screening of amylase activity with specific activity by using marine actinomycetes strains

	Measuring per 100 ml of sample			
Actinomycetes strains	Activity (U/ml)	Protein (mg/ml)	SA (U/mg)	Agar plate (mm)
Streptomyces griseorubens KR133201	8.16	14.6	0.514	18
Streptomyces rochei KR108310	7.043	13.7	0.56	24
Streptomyces Parvus KP675949	6.89	15.88	0.434	22
Streptomyces sp. MK388207	5.86	13.22	0.443	20

The *Streptomyces rochei* HMM 13 strain selected was Gram-positive, mycelic coenocytic, and branched. It was catalase and protease positive, reduced nitrate, hydrolyzed starch, Voges Proskauer, indole, and H_2S production negative. Following flooding with Gram's Iodine on starch agar plates, the *Streptomyces rochei* HMM 13 strain showed a 24 mm zone. Based on 16S rRNA analysis, this organism was identified as *Streptomyces rochei*, the sequence was accessed by KR108310

Optimization of amylase production from actinomycetes isolates

The Plackett-Burman design has been used to assess the critical impact of utilizing starch casein agar medium components for the generation of amylase enzyme using *Streptomyces griseorubens strain* (MMH 9), *Streptomyces rochei* strain (HMM 13), *Streptomyces Parvus* strain (8), and *Streptomyces* sp. strain (M12). The most significant effect of the four isolates has been found for *Streptomyces rochei* HMM 13. The t-test showed that the most critical independent variable influencing amylase enzyme development was starch, k_2HPO_4, pH, and temperature. Consequently, specific activities were detected (Table 5).

Table 5. Measurable investigations of the Plackett-Burman exploratory results

	Mean effect and t-value of all isolates							
	S. griseorubens		*S. rochei*		*S. parvus*		*Streptomyces* sp.	
Variable	ME	t-v*	ME	t-v*	ME	t-v*	ME	t-v*
Starch	1.17	2.55	1.43	8.44	1.17	2.55	0.88	1.73
KNO_3	0.42	0.93	0.26	1.57	0.42	0.93	0.05	0.1
Casein	0.08	0.18	-0.27	-1.61	0.081	0.18	0.15	0.3
$MgSo_4$	0.72	1.57	0.066	0.39	0.72	1.57	0.13	0.25
K_2HPO_4	-0.15	-0.33	-0.32	-1.77	-0.15	-0.33	-0.17	-0.34
pH	-0.8	-1.76	-0.46	-2.75	-0.8	-1.76	-0.61	-1.21
Temp	-0.37	-0.8	-0.43	-2.58	-0.37	-0.8	-1.15	-2.26

**t-value significant at the 1% level = 3.70*
t-value significant at the 5% level = 2.446
t-value significant at the 10% level = 1.94
t-value significant at the 20% level =1.372
Standard t-values are obtained following (Jones, 1994)

The primary effects of the examined factors on the specific activity results were estimated and are represented in (Fig 2). Based on these results, the positive (+) level of starch concentration in addition to the negative level (-) of K_2HPO_4, pH, and temperature supported production by *Streptomyces rochei* isolate. The positive (+) level of starch, casein, and K_2HPO_4, in addition to the negative level (-) of KNO_3, supported production by *Streptomyces griseorubens.* However, there was a positive (+) level of starch, KNO_3, and $MgSo_4$ in addition to the negative level of pH corroborative enzyme production by *Streptomyces parvus.* Finally, the positive (+) level of starch and negative level (-) of K_2HPO_4, pH, and temperature supported enzyme production by *Streptomyces sp.* Additionally, the t-value in Table 5 bolsters this perception. This approach has shown that the implemented design is correct. A verification experiment was used to evaluate the basic versus optimized medium.

Verification experiment

Verification was achieved by comparing the expected ideal levels of autonomous factors and basic conditions. Cultivation of *S. griseorubens* MMH9, *S. rochei* HMM13, *S. parvus* (8), and *Streptomyces* sp. M12 in the verified medium adjusted to a pH of six for seven days resulted in a 1.7, 9.8, 7.7, and 3.12 -fold increment in the specific activity measurement compared to the basal conditions (Table 6 and Fig. 3).

Table 6. Verification experiment: amylase enzyme production of strains grown in basal versus verified media using the Plackett-Burman design

	Actinomycetes strains	A (U/ml)	P (mg/ml)	SA (U/mg)	Agar plate (mm)	Purification fold
Medium	*S. griseorubens* MMH9	6.89	15.88	0.434	22	1
	S. rochei HMM13	8.16	14.62	0.56	24	1
	S. Parvus	7.04	13.69	0.514	18	1
	Streptomyces sp. M12	5.86	13.22	0.443	20	1
rified dium	*S. griseorubens* MMH9	8.12	13.69	0.59	27	0.37
	S. rochei HMM13	30.001	10.82	2.77	33	3.97
	S. Parvus	26.7	11.96	2.23	30	3.34
	Streptomyces sp. M12	9.92	10.85	0.91	28	1.06

Among the BBD design, The significant independent variables (Starch X_1, K_2HPO_4 X_2, pH X_3, and Temperature X_4) suggested by the Plackett-Burman design were used to investigate the optimum response region for amylase production by *Streptomyces rochei* HMM 13 at three levels (-, 0, +) in the BBD (Table 7). Table 8 presents the framework for the variables and the reaction of each trial. To determine the optimal solution, the experimental results were based on a second-order polynomial function (non-linear optimization algorithm):

$$Y= 4.921+ 0.46X_1 -0.17X_2 + 1.07X_3 + 0.46X_4 – 1.51X_1X_2 + 0.55X_1X_3 – 0.36X_1X_4 – 0.522X_2X_3 –0.33X_2X_4 + 0.94X_3X_4 - 1.15X_{11} – 1.12X_{22} – 0.32X_{33} – 2.57X_{44}$$

Table 7: The three levels of significant independent variables used in the Box-Behnken factorial experimental design for amylase production by *Streptomyces rochei* HMM 13.

Level	Starch (X1)	K_2HPO_4 (X2)	pH (X3)	Temperature °C (X4)
1	19 g	0.8 g	8	33
0	15 g	0.5 g	6	30
-1	11 g	0.2 g	4	27

Table 6: Box-Behnken factorial experimental design for amylase production by *Streptomyces rochei* HMM 13.

Trials	Starch (X1)	K_2HPO_4 (X2)	pH (X3)	Temp. °C (X4)	A (U/ml)	P (mg/ml)	SA (U/mg)
1	0	1	0	1	17.8227	10.8877	1.63696
2	1	-1	0	0	14.1576	63.8529	0.22172
3	0	0	1	-1	10.9595	13.2233	0.82880
4	1	0	0	1	13.0436	12.6484	1.03125
5	0	1	-1	0	12.0375	45.4552	0.26482
6	0	0	-1	1	16.6729	12.7562	1.30704
7	0	0	1	1	14.5888	71.8300	0.20310
8	1	0	1	0	14.5528	99.3906	0.14642
9	-1	0	1	0	16.3854	37.3703	0.43846
10	0	0	-1	-1	15.7027	12.9358	1.21388
11	0	0	0	0	18.7210	71.0754	0.26339
12	0	1	0	-1	15.5949	11.3548	1.37341
13	-1	0	0	1	18.6492	41.0354	0.45446
14	0	1	1	0	15.3793	68.8835	0.22326
15	1	1	0	0	19.3319	8.55205	2.26050
16	0	-1	0	-1	13.9779	13.7982	1.01302
17	1	0	0	-1	14.3372	18.1461	0.79009
18	-1	0	-1	0	13.0077	12.7562	1.01971
19	-1	-1	0	0	15.1277	24.8297	0.60926
20	-1	0	0	-1	13.4389	14.1935	0.94683
21	0	-1	-1	0	16.4932	29.3572	0.56181
22	0	-1	1	0	15.5949	71.2910	0.21875
23	1	0	-1	0	13.5108	44.7365	0.30200
24	0	0	0	0	12.4328	68.0930	0.18258
25	0	-1	0	1	11.5704	25.4405	0.4548
26	-1	1	0	0	14.1216	50.7014	0.2785
27	0	0	0	0	11.3189	62.1640	0.1820

At the model level, the correlation measures for estimating the regression equation are the multiple correlation coefficient *R* and the determination coefficient R^2. In this experiment, the value of R^2 was 0.824 for amylase enzyme production by *Streptomyces rochei* HMM 13, indicating a substantial degree of correlation between test values and the forecast values. The optimum levels of the four factors obtained from the polynomial model were calculated using the Microsoft Excel 2019 solver function and found to be Starch: 19 g, K_2HPO_4: 0.2g, pH: 8, and Temperature 30.6 °C. Also, Figure 3 - graph A-F displays the simultaneous effects with three-dimensional charts created using STATISTICA 10.0 software of each response's four most critical independent factors. As shown in the surface plots of the BBD, variations in Starch (X_1), K_2HPO_4 (X_2), pH (X_3), and Temperature (X_4) were effective within the examined concentration ranges and under the present experimental conditions. These figures suggest that increasing the starch concentration to (19 g/l) with a high level of temperature will promote the production of amylase enzyme. However, a higher level of enzyme production was accomplished with diminished K_2HPO_4 concentration.

Verification experiment

The optimum conditions obtained from the optimization experiment was experimentally verified and compared with measured data from the model. The positive relationship between anticipated and test values beneath ideal conditions demonstrates the precision and legitimacy of the model. Thus, we predicted that to produce the highest production of amylase enzyme by isolate *Streptomyces rochei* HMM 13, the medium formula should be kept at 30.6°C for seven days and formulated as follows (g/L): starch 19 g, KNO_3 3 g, casein 0.5 g, $MgSO_4$ 0.75 g, $FeSO_4$ 0.001 g, K_2HPO_4 0.2 g, $CaCO_3$ 0.1 g, agar 18 g, and 50/50 DW. An investigative study was conducted to compare the anticipated optimal levels of autonomous factors and fundamental conditions. Development of *Streptomyces rochei* HMM13 within the confirmed medium balanced to pH 8 for seven days resulted in a 12.22-fold increment within the specific activity estimation compared to the basal conditions (Table 7).

Table 7: Progression of purification fold for amylase production using *Streptomyces rochei* HMM 13

Actinomycetes strains	A (U/ml)	P (mg/ml)	SA (U/mg)	Ap (mm)	P. fold
Basal Medium	0.227	0.407	0.56	24	1
Plackett-Burman	30.001	10.82	2.77	33	3.97
Box-Behnken	64.895	8.803	7.37	45	12.22
Ammonium Sulfate (90%)	67.91	7.22	9.403	-	15.79
Sephadex G-50	103.73	9.88	10.5	-	17.75

Amylase enzyme purification and characterization

Amylase was purified from *Streptomyces rochei* HMM 13 using ammonium sulfate, dialysis, and Sephadex G-50. The crude amylase showed 12.22-fold purification (Fig 5.), and the specific activity of amylase was 7.37 U/mg with activity of 64.895 U/ml.

At 90%, ammonium sulfate was ideal for the fractionation of amylase from *S. rochei* HMM 13, and the others showed no important amylase activity. The precipitated sample was dialyzed against buffer and water overnight, and the activity of the enzyme was inspected. After purification utilizing Sephadex G-50 chromatography, the specific activity of the enzyme was 10.5 U/mg with an activity of 103.73 U/ml. Dynamic amylase fractions were stacked onto the SDS-PAGE, and the atomic mass of decontaminated amylase was 45, 43, and 53 kDa at three peaks within the gel filtration chromatography (Fig. 6).

The digestion rate of starch with a purified enzyme was evaluated at different pH-values to assess the impact of varying pH on amylase activity. Enzyme characterization studies revealed stability in the wide pH range (3–10) of purified amylase with maximum strength and activity at a pH of 6 (Fig 7A).

Amylase was found to be profoundly dynamic at 65 °C (Fig. 7B). Decontaminated *S. rochei* amylase with optimum stability at 70°C was astoundingly steady inside the temperature extend 55–70°C *S. rochei* amylase was thermostable up to 70°C and held 52.8% initial activity after 100 min incubation at 70°C. The enzyme maintained its unique action for 40 min at 55°C, 65°C, and 70 °C (Fig 7C). It was uncovered that the amylase from *S. rochei* is thermostable. Moreover, the purified enzyme was characterized by the kinetical characteristics that illustrate a consistent Michaelis-Menten (Km) and Vmax of 6.58 mg/ml and 21.93 mg/ml/min, respectively (Fig. 7D). The purified enzyme seems to withstand saltiness up to 0.8 M NaCl, with the most elevated movement at 22.71 U/ml (Fig. 7E). The impact of different metal particles at concentrations of 0.05 M on purified *S. rochei* HMM 13 amylase activity is shown in Fig. 7F. The addition of Cu+2, Zn+2, and Fe+2 (152.21%, 207.24%, and 111.89%) upgraded enzyme activity. However, Mn+2 and Mg+2 had a limited impact on enzyme activity (91.47% and 90.96%).

Discussion

The diversity of marine actinomycetes is important in many areas of science and medicine (Magarvey et al 2004). They are a rich source of bioactive, chemically diverse substances (Bernan et al 1997). Gopinath has recently been using agar plates to determine the amylase activity of Penicillium sp. and Aspergillus versicolor (Gopinath et al 2017). Based on 16S rRNA analysis, this organism was identified as *Streptomyces rochei*; the sequence was accessed by KR108310 (Abd-Elnaby et al 2016). Amylase tests were conducted for *Streptomyces avermitilis* and *Streptomyces* BTSS 1001 (Acharyabhatta et al 2013 and Hwang et al 2013). Some studies have been conducted on the production of industrial enzymes by actinomycetes, such as by Selvin et al (2009), who isolated three types of actinomycetes from the southwest coast of India that had the potential to generate synthetic enzymes, such as cellulase, amylase, and lipase. The Bengal Gulf has been confirmed to be the possible source of aquatic actinomycetes that are capable of producing industrial enzymes, such as lipase, amylase, cellulase, caseinase, or gelatinase (Ramesh and Mathivanan 2009). The growth of actinomycetes and the production of enzymes are influenced by the composition of the medium. Therefore, there is a need to urgently change the productivity of enzymes by optimizing bacterial production and use on an industrial scale by physical factors and medium components (Al-Dhabi et al 2020). Studies of the enhancement of marine actinomycetes amylase development using statistical instruments have been recorded (Cotârleț and Bahrim 2012). They applied Plackett-Burman statistical experimental design to screen eleven medium components based on twelve experimental trials in developing the fermentation medium for amylase production by *Streptomyces* 4 ALGA, starch, glycerol, maltose, malt extract, glucose, urea, sodium caseinate, yeast extract, peptone, $CaCl_2$, and KCl concentrations. The factors that contributed most to maximum amylase activity were yeast extract and $CaCl_2$. The production of amylase enzymes increased to 23,96 U (Cotârleț and Bahrim 2012). Yahya (2016) reported that conditions in fungal strain *A. tubingensis* SY1 extracellular amylase production were factually optimized using the Plackett-Burman equation, with the most significant variables of amylase production being peptone, agitation, MgSO4.7H2O followed by inoculum size. The BBD is a diverse approach that involves the optimization of an answer based on various variable methods, including mathematical and statistical methods (Agrawal et al 2020). The BBD was utilized to optimize the culture conditions for amylase generation, and a value of 145,32 U/ml of amylase was obtained (Khusro et al 2017). Our results agree with Gupta, who said that after five days of incubation, the development of amylase was high at 37°C (108 ± 4.1 U/ml) with a maximum pH of 8.0 (118.1 ± 2.3). The optimal pH and amylase activity temperature vary from species to species (Gupta et al 2003). The overall production of amylase in submerged fermentation was 40°C (123.12.1 U/ml). Starch improved the production of amylase among carbon sources. There was less amylase activity in the other carbon sources chosen (Al-Dhabi et al 2020). The statistical approach on the surface response (RSM) using BBD resulted in a doubling of *Bacillus* sp. biomass yields and indicated the best conditions to establish a cost-efficient approach to enhance amylase production through variables optimized using BBD. Thus, RSM can be a useful tool for enzyme development by optimization the various independent factors involved in the fermentation method using a minimum number of experiments (Khusro et al 2017). The atomic mass of decontaminated amylase was 45, 43, and 53 kDa at three peaks within the gel filtration chromatography, Similarly, *Streptomyces fragilis* DA7-7 (Nithya et al 2017), *Streptomyces* sp. PDS1 (Ragunathan and Dhas 2013) and S. *gulbargensis* (Syed et al 2009) enrolled atomic masses of a-amylase 44, 51, and 55 kDa, respectively. Characterization of purified amylase have pH, temperature, salt concentration by 6, 65°C and 0.8 M NaCl, respectively. α-Amylases from actinomycetes have a pH optima in the alkaline to a neutral range (Al-Dhabi et al 2020), which is different from our findings. Amylase was found to be profoundly dynamic at 65°C (Fig. 7B). Comparable outcomes were found in another study (Al-Dhabi et al 2020a), in which most extreme amylase activity was notable at 60°C.It was uncovered that the amylase from *S. rochei* is thermostable. Numerous past studies have detailed that amylase from *Streptomyces* sp. MSC702 and *Streptomyces avermitilis* 5981 were steady at 60°C and 50°C, respectively (Hwang et al 2013 and Singh et al 2014). Among Km and Vmax constant, Singh et al (2014) detailed amylase from *Streptomyces* sp. MSC702. and found that the Km and Vmax values were 2.4 mg/ml and 21.85 mg/mL/min, respectively. The Km and Vmax of amylase produced by *Streptomyces fragilis* DA7-7 were 0.624 mU/mg and 0.836 mg/mL, respectively (Nithya et al 2017). The purified enzyme seems to withstand

saltiness up to 0.8 M NaCl, with the most elevated movement at 22.71 U/ml (Fig. 7E). Numerous past studies have described that a-amylase from *Streptomyces* sp. SNAJSM6 and *Nocardiopsis dassonvillei* KaS11 were steady at 3% and 9% concentrations of NaCl, respectively (Anand ety al 2019 and Rathore et al 2020). Finally, Cu + 2, Zn + 2, and Fe + 2 expanded amylase activity as reported by (Du et al 2018) and (Asgher et al 2007). The results of our study indicate the possible testing of the factors that have a positive impact on enzyme production using the Plackett-Burman and Box-Behnken designs. The amylase from *Streptomyces rochei* KR108310 strain HMM13 is steady at a wide range of temperatures and an acidic pH. Due to their importance, further study should concentrate on improving methods for using the enzyme in industrial processes. The findings obtained were consistent with other worldwide studies that suggest that the Streptomyces genus from marine sediments is suitable for the production of industrial enzymes.

Declarations

Consent for publication

Not applicable

Data Availability

All the data is presented in the manuscript and any additional data will be provided upon request. Please contact Mohamed Al-Agamy.

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Author details

[1]Department of Pharmaceutics, College of Pharmacy, King Saud University, 11451 Riyadh 2457, Saudi Arabia

2Department of Microbiology and Immunology, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt

[3]Marine Microbiology Department, National Institute of Oceanography and Fisheries, Egypt.

[4] Microbiology Unit, Central Lab, Research Center College of Pharmacy, King Saud University, 11451 Riyadh 2457, Saudi Arabia

Authors’ Contributions

Sarah I. Bukhari, Moaz M. Hamed, Mohamed H. Al-Agamy, Mahmoud S. Kelany, and Mohammad R Al Hazani performed the methodology. Moaz M. Hamed and Mahmoud S. Kelany drafted the manuscript and analyzed the data. Mohamed H. Al-Agamy contributed in data analysis, writing, review, and editing of the manuscript. All authors read and approved the final manuscript.

References

1. Abd-Elnaby HM, Abo-Elala GM, Abdel-Raouf UM, Hamed MM (2016) Antibacterial and anticancer activity of extracellular synthesized silver nanoparticles from marine *Streptomyces rochei* MHM13. The Egyptian Journal of Aquatic Research 42(3): 301–312
2. Acharyabhatta A, Kandula SK, Terli R (2013) Taxonomy and polyphasic characterization of alkaline amylase producing marine actinomycete *Streptomyces rochei* BTSS 1001. International journal of microbiology 2013 (276921):1–8
3. Agrawal DC, Yadav A, Kesarwani R, Srivastava O, Kayastha AM (2020) Immobilization of fenugreek β-amylase onto functionalized graphene quantum dots (GQDs) using Box-Behnken design: Its biochemical, thermodynamic and kinetic studies. Int J Biol Macromol 144:170–182
4. Al-Dhabi NA, Esmail GA, Duraipandiyan V, Arasu MV (2019) Chemical profiling of Streptomyces sp. Al-Dhabi-2 recovered from an extreme environment in Saudi Arabia as a novel drug source for medical and industrial applications. Saudi Journal of Biological Sciences 26(4):758–766
5. Al-Dhabi NA, Esmail GA, Duraipandiyan V, Arasu MV, Salem-Bekhit MM (2016) Isolation, identification and screening of antimicrobial thermophilic *Streptomyces* sp. Al-Dhabi-1 isolated from Tharban hot spring, Saudi Arabia. Extremophiles 20(1):79–90
6. Al-Dhabi NA, Esmail GA, Ghilan, Ghilan A-KM, Arasu MV (2020) Isolation and screening of *Streptomyces* sp. Al-Dhabi-49 from the environment of Saudi Arabia with concomitant production of lipase and protease in submerged fermentation. Saudi Journal of Biological Sciences 27(1):474–479
7. Al-Dhabi NA, Esmail GA, Ghilan A-KM, Arasu MV, Duraipandiyan V, Ponmurugan K (2020) Isolation and purification of starch hydrolysing amylase from *Streptomyces* sp. Al-Dhabi-46 obtained from the Jazan region of Saudi Arabia with industrial applications. Journal of King Saud University-Science 32(1):1226–1232
8. Amador ML, Jimeno J, Paz-Ares L, Cortes-Funes H, Hidalgo M (2003) Progress in the development and acquisition of anticancer agents from marine sources. Ann Oncol 14(11):1607–1615
9. Amutha K, Priya KJ (2011) Effect of pH, temperature and metal ions on amylase activity from *Bacillus subtilis* KCX 006. International. Journal of Pharma Bio Sciences 2(2):407–413
10. Anand J, Ramamoorthy K, Muthukumar G, Nagaraj S (2019) Production and partial purification of α-amylase producing *Streptomyces* sp. SNAJSM6 isolated from seaweed Sargassum myriocystum J. Agardh. Indian Journal of Geo Marine Sciences 48(8):1245–1251
11. Arasu MV, Arokiyaraj S, Viayaraghavan P, Kumar T, Duraipandiyan V, Al-Dhabi NA (2019) One step green synthesis of larvicidal, and azo dye degrading antibacterial nanoparticles by response surface methodology. J Photochem Photobiol B 190:154–162
12. Asgher M, Asad MJ, Rahman S, Legge R (2007) A thermostable α-amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing. Journal of food engineering 79(3):950–955
13. Asoodeh A, Alemi A, Heydari A, Akbari J (2013) Purification and biochemical characterization of an acidophilic amylase from a newly isolated *Bacillus* sp. DR90. Extremophiles 17(2):339–348
14. Bano S, Qader SAU, Aman A, Syed MN, Azhar A (2011) Purification and characterization of novel α-amylase from *Bacillus subtilis* KIBGE HAS. Aaps Pharmscitech 12(1): 255–261
15. Beltagy EA, Rawway M, Abdul-Raouf UM, Elshenawy MA, Kelany MS (2018) Purification and characterization of theromohalophilic chitinase producing by halophilic *Aspergillus flavus* isolated from Suez Gulf. The Egyptian Journal of Aquatic Research 44(3):227–232

16. Bernan V, Greenstein M, Maiese W (1997) Marine microorganisms as a source of new natural products. Adv Appl Microbiol 43:57–90
17. Boovaragamoorthy GM, Anbazhagan M, Piruthiviraj P, Pugazhendhi A, Kumar SS, Al-Dhabi NA (2019) Clinically important microbial diversity and its antibiotic resistance pattern towards various drugs. J Infect Public Health 12(6):783–788
18. Box GE, Behnken DW (1960) Some new three level designs for the study of quantitative variables. Technometrics 2(4):455–475
19. Cotârleț M, Bahrim GE (2012) Application of plackett-burman experimental design to optimize the cold-active alpha amylase biosynthesis by psychrotrophic *Streptomyces* 4 Alga. The Annals of the University Dunarea de Jos of Galati. Fascicle VI-Food Technology 36(2):9–19
20. Du R, Song Q, Zhang Q, Zhao F, Kim RC, Zhou Z (2018) Purification and characterization of novel thermostable and Ca-independent α-amylase produced by *Bacillus amyloliquefaciens* BH072. International journal of biological macromolecules 115:1151–1156
21. El-Sersy NA, Abou-Elela GM (2006) Antagonistic effect of marine *Nocardia brasiliensis* against the fish pathogen *Vibrio damsela*: Application of Plackett- Burman experimental design to evaluate factors affecting the production of the antibacterial agent. International journal of oceans oceanography 1(1):141–150
22. Gopinath SC, Anbu P, Arshad MM, Lakshmipriya T, Voon CH, Hashim U (2017) Biotechnological processes in microbial amylase production. BioMed research international 2017 ID 1272193:1–9
23. Gupta J, Yadav B, Gupta H (1995) Nutritional value of jack bean for broiler. Indian Journal of Poultry Science 30:112–116
24. Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B (2003) Microbial α-amylases: a biotechnological perspective. Process biochemistry 38(11):1599–1616
25. Hwang SY, Nakashima K, Okai N, Okazaki F, Miyake M, Harazono K (2013) Thermal stability and starch degradation profile of α-amylase from *Streptomyces avermitilis*. Bioscience, biotechnology, and biochemistry 77(12): 2449–2453
26. Jain A, Jain R, Jain S (2020) Quantitative Analysis of Reducing Sugars by 3, 5-Dinitrosalicylic Acid (DNSA Method). In: Basic Techniques in Biochemistry, Microbiology and Molecular Biology. Springer Protocols Handbooks. Humana, New York, pp 181–183
27. Janaki T (2017) Enzymes From Actinomycetes - Review. International Journal of ChemTech Research 10(3):326–332
28. Khusro A, Barathikannan K, Aarti C, Agastian P (2017) Optimization of thermo-alkali stable amylase production and biomass yield from *Bacillus* sp. under submerged cultivation. Fermentation 3(1):7
29. Li X, Zhang J, Zhu S (2011) Improved thermostable α-amylase activity of *Bacillus amyloliquefaciens* by low-energy ion implantation. Genet Mol Res 10(3):2181–2189
30. Lowry O, Rosebrough N, Farr A, Randall R (1951) Protein measurement with the folin phenol reagent. The Journal of Biological Chemistry 193(1):265–275
31. Magarvey NA, Keller JM, Bernan V, Dworkin M, Sherman DH (2004) Isolation and characterization of novel marine-derived actinomycete taxa rich in bioactive metabolites. Appl Environ Microbiol 70(12):7520–7529
32. Mohammed TU, Murtadha JH, Razzaq IHA (2017) Measurement and purification of Alanine aminotransferase (ALT) enzyme activity in patients with celiac disease. Baghdad Science Journal 14(3):557–563
33. Monteiro MCP, Tavares DG, Nery EM, Queiroz MV, Pereira OL, Cardoso PG (2020) Enzyme production by *Induratia* spp. isolated from coffee plants in Brazil. Brazilian Archives of Biology Technology 63:1678–4324

34. Nithya K, Muthukumar C, Kadaikunnan S, Alharbi NS, Khaled JM, Dhanasekaran D (2017) Purification, characterization, and statistical optimization of a thermostable α-amylase from desert actinobacterium *Streptomyces fragilis* DA7-7. 3 Biotech 7(5): 350
35. Plackett RL, Burman JP (1946) The design of optimum multifactorial experiments. Biometrika 33(4):305–325
36. Ragunathan R, Dhas RP (2013) Exploitation of novel Actinomycetes isolated from Wetern ghats of Tamilnadu and production of Xylanase by *Streptomyces* spp. pds1. Science Journal of Microbiology 174:1–6
37. Ramesh S, Mathivanan N (2009) Screening of marine actinomycetes isolated from the Bay of Bengal, India for antimicrobial activity and industrial enzymes. World Journal of Microbiology Biotechnology 25(12):2103–2111
38. Rathore DS, Malaviya K, Dobariya A, Singh SP (2020) Optimization of the Production of an Amylase from a Marine Actinomycetes *Nocardiopsis Dassonvillei* Strain Kas11. Proceedings of the National Conference on Innovations in Biological Sciences (NCIBS) 2020, Available at SSRN: https://ssrn.com/abstract=3567722
39. Rathore DS, Sheikh M, Gohel S, Singh SP (2019) Isolation strategies, abundance and characteristics of the marine actinomycetes of Kachhighadi, Gujarat, India. Journal of the Marine Biological Association of India 61(1):71–78
40. Sadh PK, Duhan S, Duhan JS (2018) Agro-industrial wastes and their utilization using solid state fermentation: a review. Bioresources Bioprocessing 5(1):1–15
41. Salgaonkar BB, Sawant DT, Harinarayanan S, Bragança JM (2019) Alpha-amylase Production by Extremely Halophilic Archaeon *Halococcus* Strain GUVSC8. Starch Starke 71(1800018):1–10
42. Singh R, Kumar V, Kapoor V (2014) Partial purification and characterization of a heat stable α-amylase from a thermophilic actinobacteria, *Streptomyces* sp. MSC702. Enzyme research 2014 ID 106363:1–8
43. Suthindhiran K, Jayasri MA, Dipali D, Prasar A (2014) Screening and characterization of protease producing actinomycetes from marine saltern. J Basic Microbiol 54(10):1098–1109
44. Syed DG, Agasar D, Pandey A (2009) Production and partial purification of α-amylase from a novel isolate *Streptomyces gulbargensis*. Journal of industrial microbiology biotechnology 36(2):189–194
45. Tonkova A (2006) Microbial starch converting enzymes of the α-amylase family. In: Ray RC, Word OP (eds) Microbial biotechnology in horticulture, vol 1, Science Publishers. Enfield pp 421–472
46. Vidyalakshmi R, Paranthaman R, Indhumathi J (2009) Amylase production on submerged fermentation by *Bacillus* spp. World Journal of Chemistry 4(1):89–91
47. Viswanathan K, Rebecca LJ (2019) Screening of Amylase and Cellulase Enzymes from Marine Actinomycetes. Research Journal of Pharmacy Technology 12(8):3787–3790
48. Yahya S, Jahangir S, Shahid S, Muhammad S, Shakeel AK (2016) Production optimization by using plackett-burman design and partial characterization of amylase from *Aspergillus Tubingensis* sy 1. Pak J Bot 48(6):2557–2561

Figures

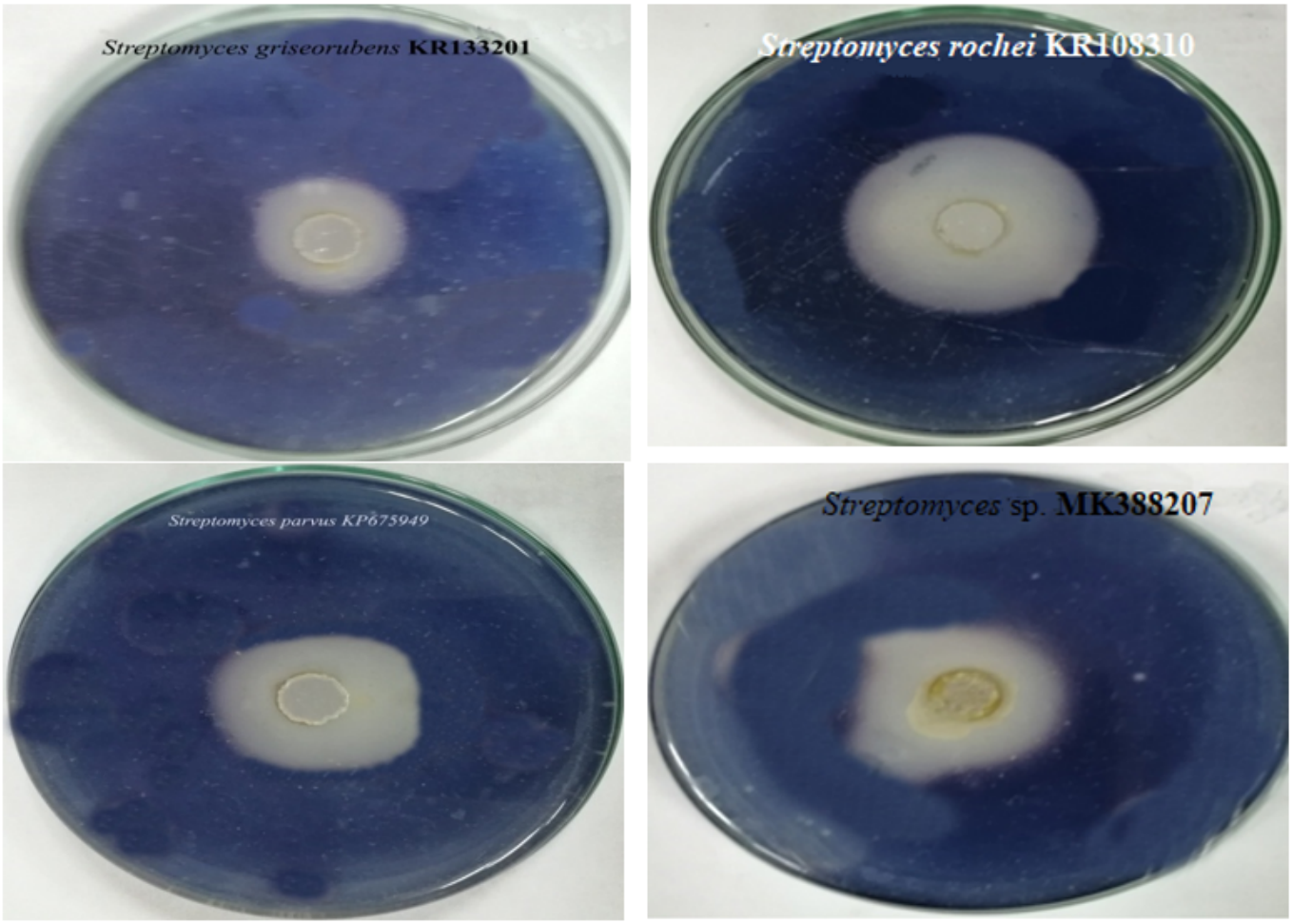

Figure 1

Amylase production by different actinomycetes isolates using the agar diffusion technique

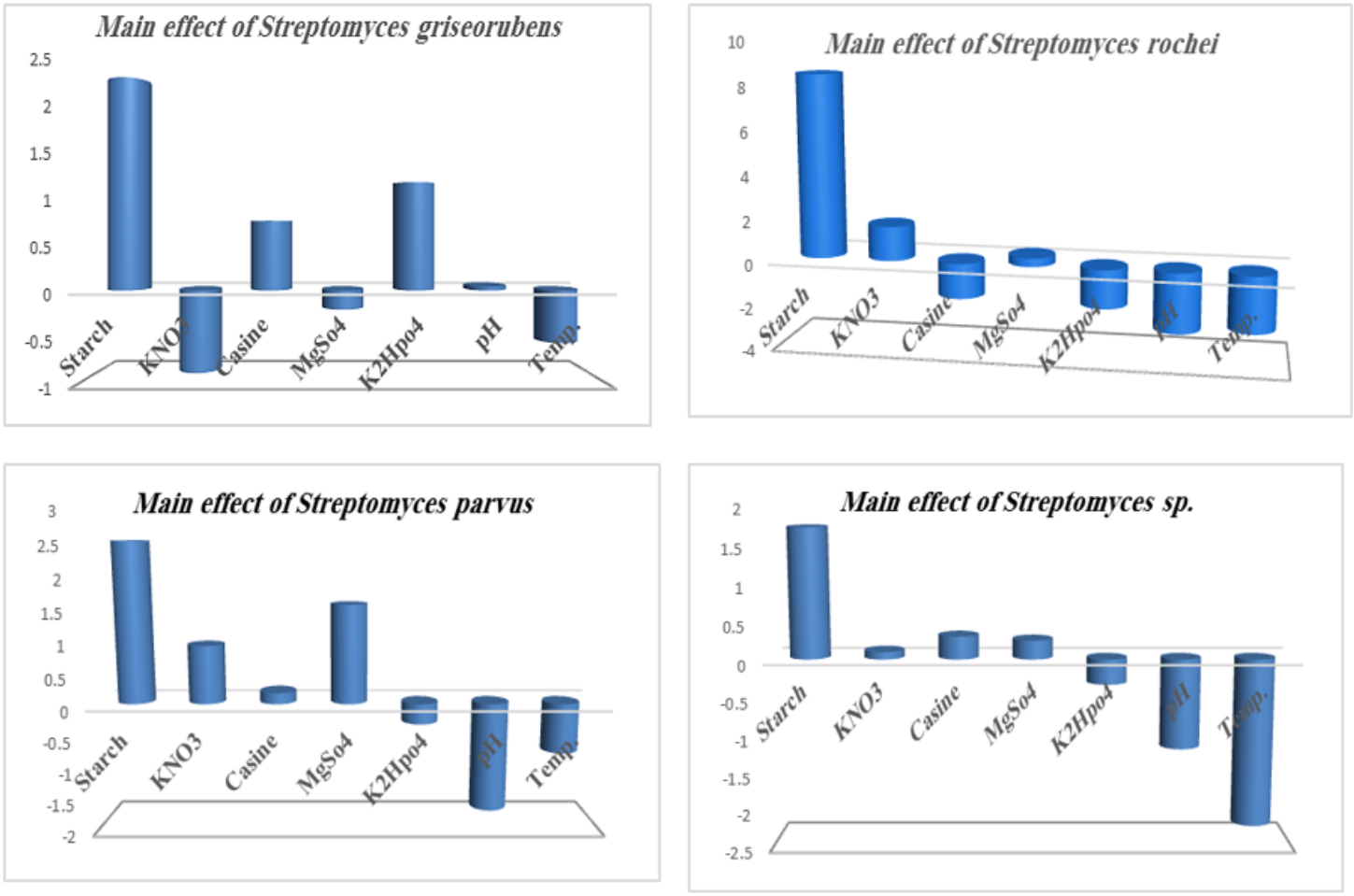

Figure 2

Illustration of fermentation conditions influencing the generation of amylase enzyme by strains

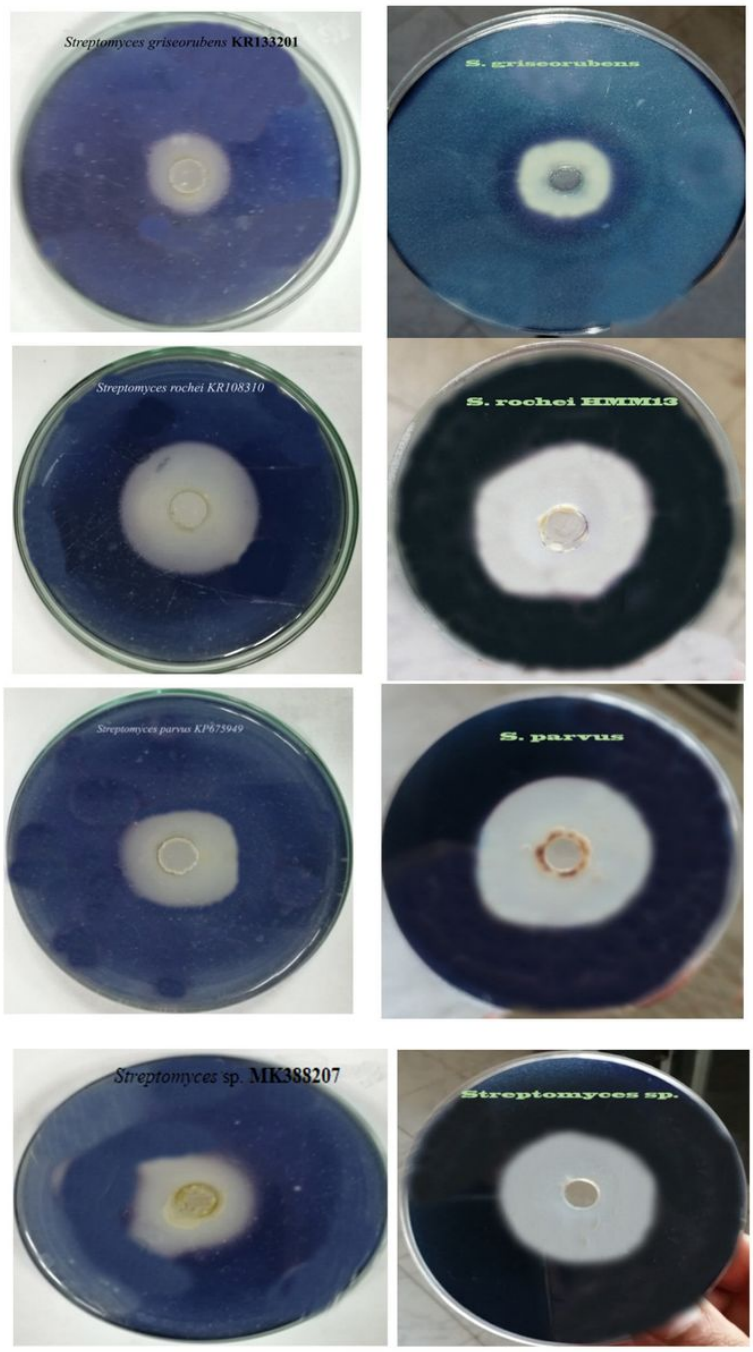

Figure 3

Comparison of chitinase agar well plate after Plackett-Burman with basal medium

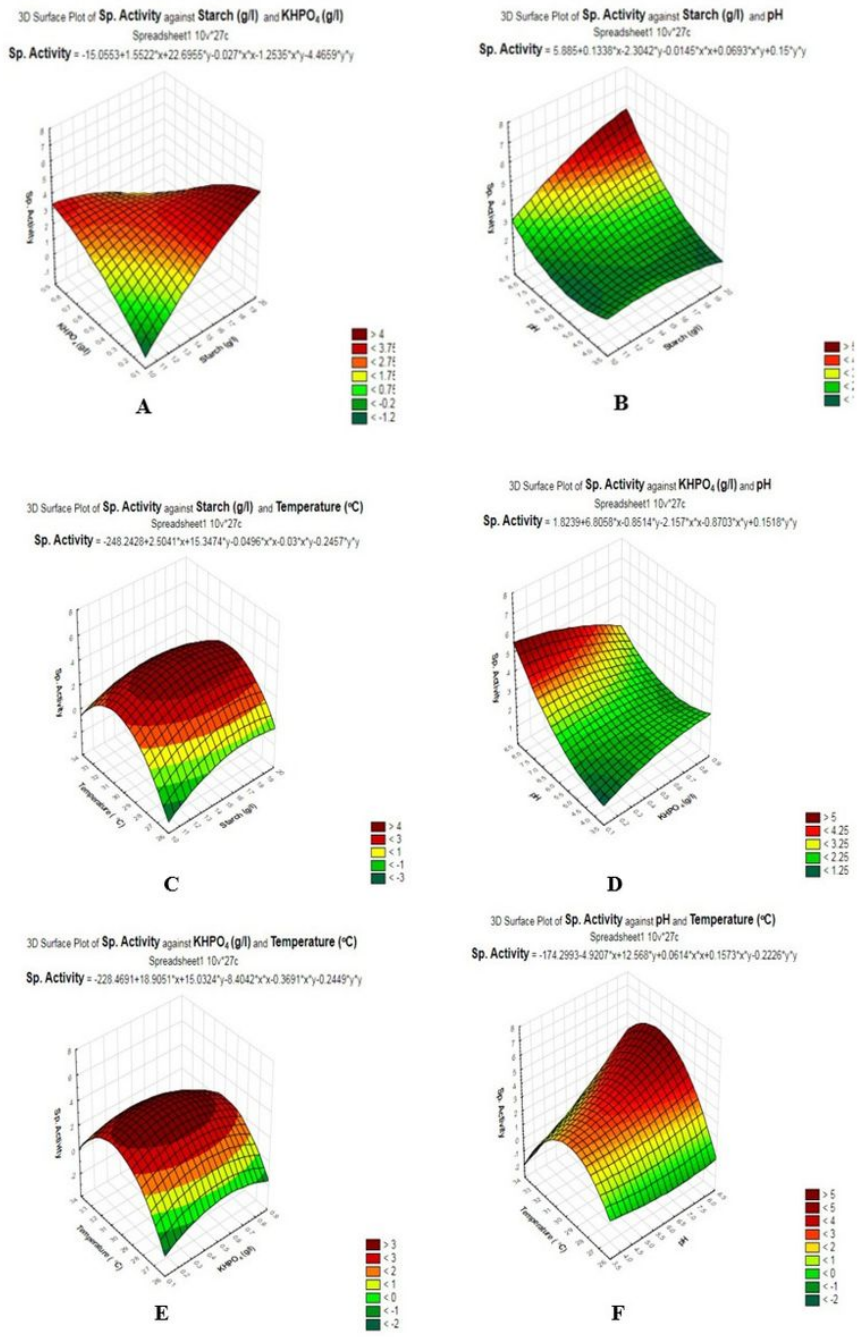

Figure 4

Box-Behnken design: (A), starch, K2HPO4 and specific activity; (B), starch, pH, and specific activity; (C), starch, temperature, and specific activity; (D), K2HPO4, pH, and specific activity; (E), K2HPO4, temperature, and specific activity; (F), pH, temperature, and specific activity.

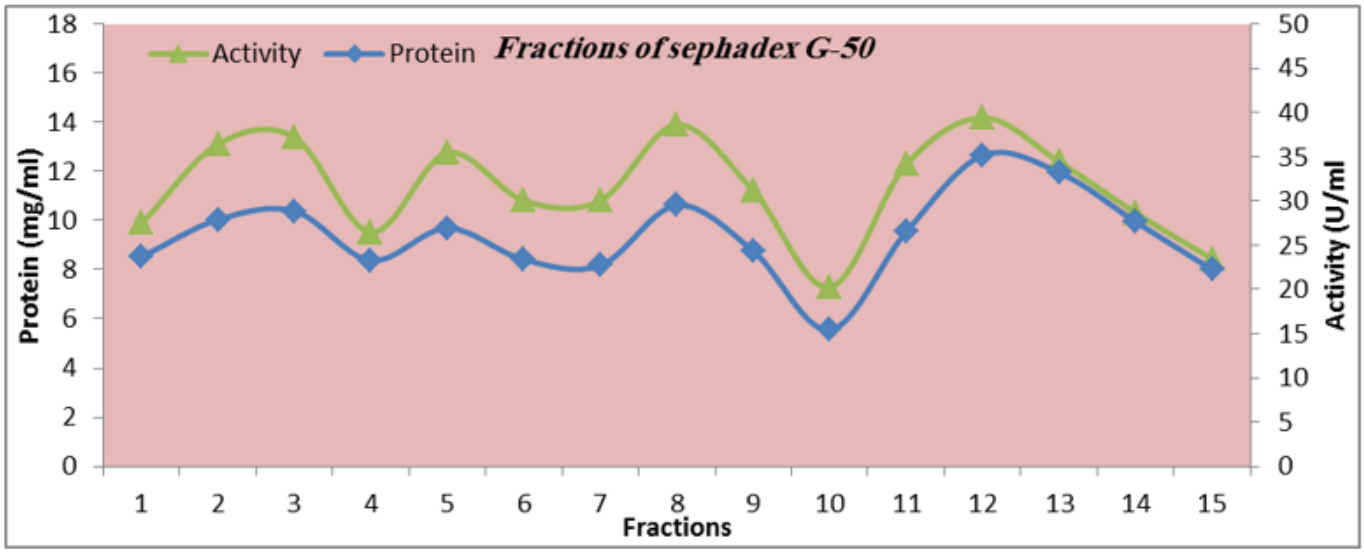

Figure 5

Fractions of amylase production by Sephadex G-50 using Streptomyces rochei HMM 13

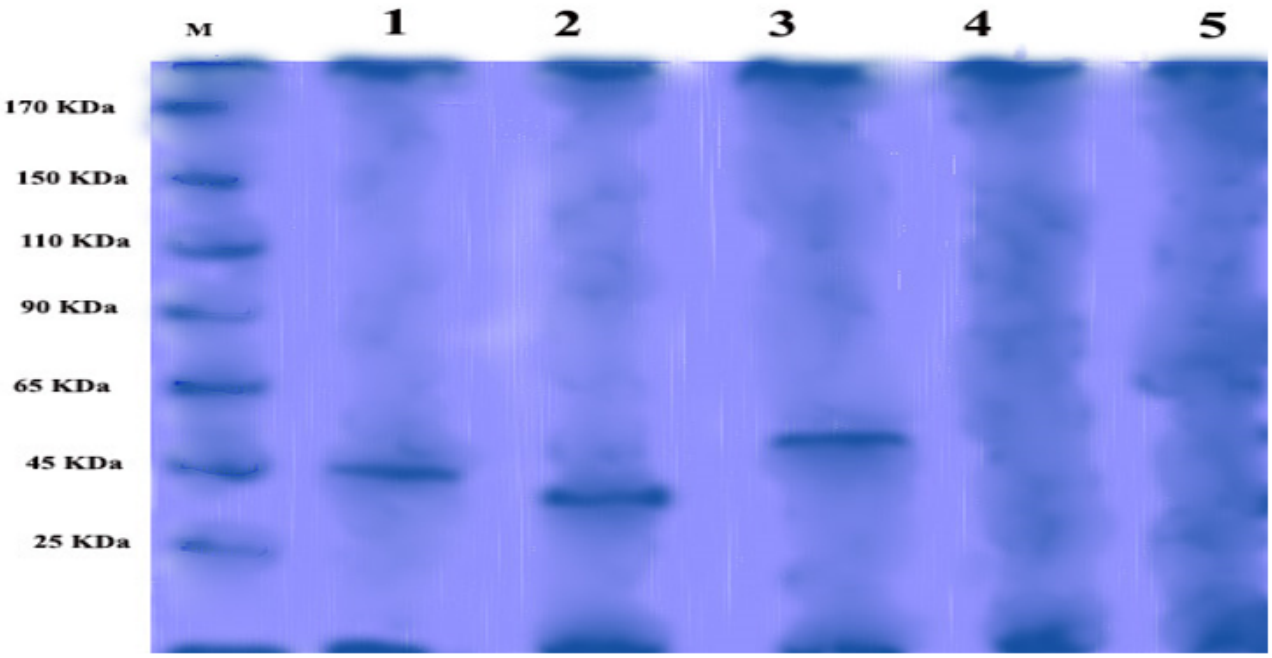

Figure 6

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis for purified amylase enzyme where Lane 1: marker standard protein with range (25–170 KDa) and Lane 2–6: loading samples.

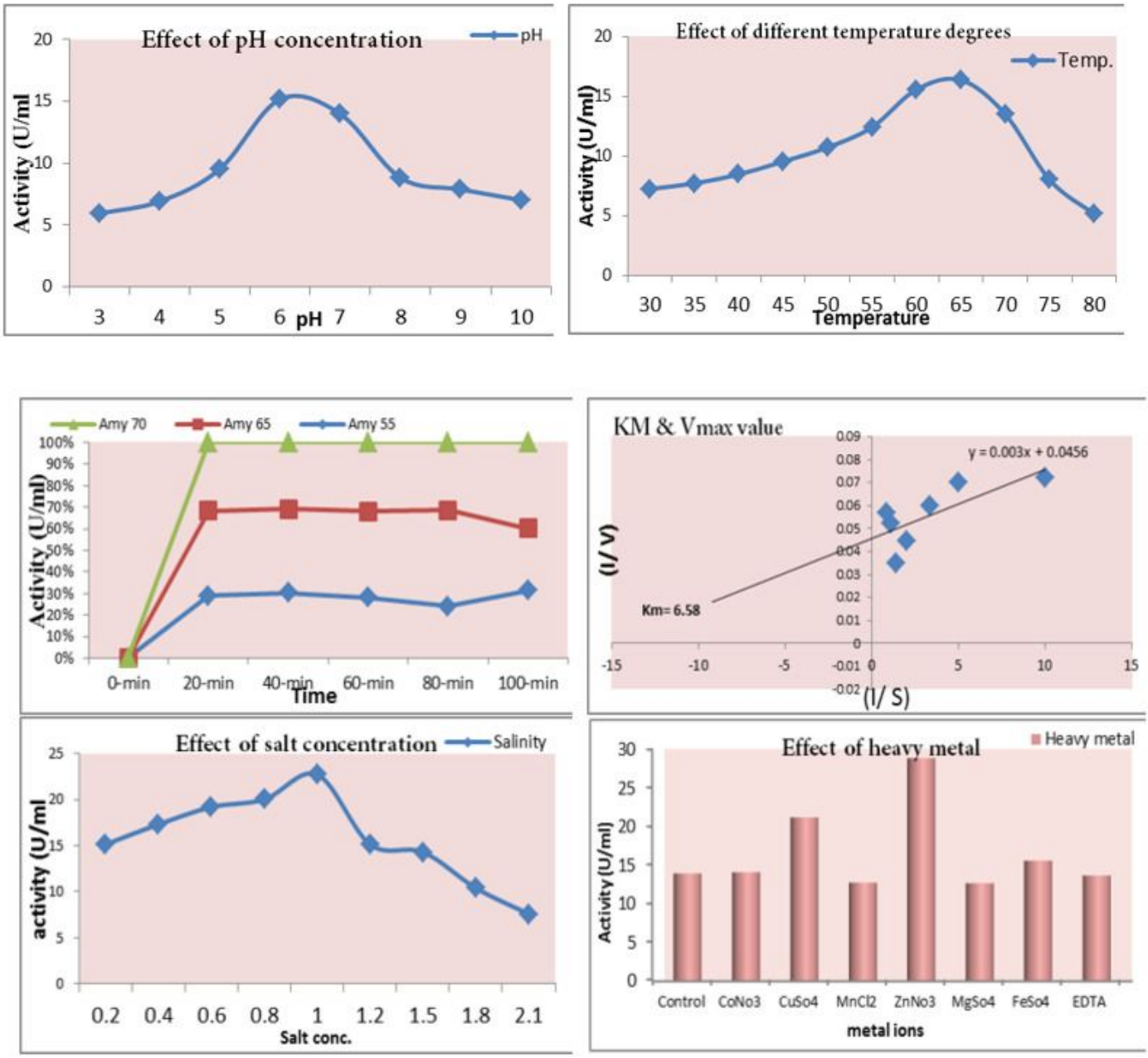

Figure 7

Characterization of purified amylase enzyme: (A), pH effect; (B), Temperature effect; (C), Thermal stability; (D), Km and Vmax value; (E), Effect of salt concentration; (F), Metal ion effect