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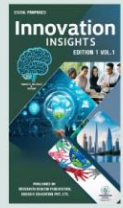

(RESEARCH ARTICLE)

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Screening of media constituents using Sequential Design of Experiments for production of Amylase from *Bacillus* Sp. Isolated from Potato Waste Dump Soil.

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Abstract

The present study was planned to isolate amylase producing microorganism from soil and to optimize conditions viz. for media constituents, temperature pH and incubation time for enzyme production. Different soil samples were collected from waste potato dump sites and were processed for isolation of amylase production. Morphological and biochemical characterization was carried out of purified cultures showing zone of clearance in starch agar plates. *Bacillus* sp. was isolated from 8 (80%) samples, out of which 6 (75%) belonged to *Bacillus subtilis* and 2 (25%) belonged to *Bacillus brevis*. Isolates were designated as APB 1-APB 8. Screening was done on the basis of the Design of Experiments where 7 factors were taken both chemical constituents and physical parameters were taken into account for all 8 different sample isolates and series of experiments were shown that in some isolates the optimized activity of amylase will shoot up to 71IU./ml. All 08 isolates were subjected to the design of experiment to address the selection of prime media constituents, in isolate AB7 we have seen a significant enzyme activity that can be used in future application for 10 fold production of enzymes. This study revealed that potato waste harbors' amylolytic *Bacillus* sp. and the amylase production can be anchored with the right constituents and physical parameters can be also adjusted for the optimized and enhanced growth used to treat this agricultural waste material.

Keyword: Optimization, Design Experiments, Amylases, *Bacillus,* Screening Model, Media constituents

1. Introduction

Microorganisms are the most important sources for enzyme production. Enzymes from fungal and bacterial sources have been increasingly applied in industrial sectors. Starch degrading amylolytic enzymes are most important in biotechnology industries with huge application in food, fermentation, textile and paper (Ahmad *et al.,* 2020). Amylase is an enzyme that catalyses or breakdown the starch into sugar. It can be derived from several sources like animals, plants and microbes. Amylase is present in human saliva. The pancreas also secretes amylase (alpha amylase) that hydrolyses the starch into fructose syrup that overcomes the acidic nature and maintains temperature (Tallapragada *et al*., 2018). All amylases are glycoside hydrolases and act on alpha 1, 4-glycosidic bonds (Sobia *et al*., 2023). Diastase, amylase was the first enzyme to be discovered and isolated by Anselme Payen in 1833. The EC no. of amylase is (3.2.1.1). Alpha-amylase is stable over a wide range of pH from 4 to 11. The optimum temperature for activity of Alpha-amylase is depending on the growth of microorganism Starch is major product of many economically important crops such as wheat, rice, maize and potato. Starch degrading amylolytic enzymes are most important in the biotechnology industries with large number of applications in the food fermentation, textile and paper production (Gojiya *et al.,* 2021). It has been possible for a long time to produce amylase with a variety of fungal and bacterial culture. As a rule bacterial amylases are more stable in regard to temperature than are amylases derived from fungal cultures. Bacterial amylases now are produced predominantly with genetically modified microorganisms (Various type of bacillus). In the production of amylases using fungi, cultures are most often used that are not regarded as preparation genetically modified. In one preparation genetically modified microorganism (trichoderma) are used. *Bacillus* species are

important sources of alpha amylase and have been used for the production of enzyme using solid substrate fermentation or submerged fermentation. The simple and cheap media for alpha amylase production was developed by (Ilori**,** *et al.,* 1997). Amylolytic enzyme can be divided into three groups: alpha-amylase, beta-amylase and Gama-amylase (Elyasi *et al.,* 2020). Out of these three, alpha-amylase (endo-amylase) is one of the most important industrial enzyme capable of hydrolyzing the 1,4-glycosidic linkages to glucose, maltose, and dextrin (Ullah *et al*., 2021). This imparts flavour and causes the bread to rise in fermentation, yeast ingest sugars and excrete alcohol. Amylase is also used in clothing and dish washer detergent to dissolve the starch strains from clothes or fabrics (Elmansy *et al.,* 2018).

Screening and optimization of media constituents can be done by either varying one variable at a time or nonconventional using statistical methods. Conventional screening and optimization technique involve varying factor and their levels by maintaining the other factors at an unspecified constant level, and by doing so, the combined effect of the factors is generally neglected; moreover, the technique is time-consuming and requires a sufficiently large number of experimental runs. These limitations of a classical method can be eliminated by screening and optimizing all the affecting factors collectively by employing statistical experimental design and empirical model building using regression analysis. The most successfully used technique for optimization based on statistical methods is known as response surface methodology (RSM) (Chen 1994), which also helps in understanding the effects of individual variables and their interaction on final response (Montgomery 1991). To reduce the number of factors to be used in an optimization study, screening of factors is normally performed by employing another statistical design such as Plackett– Burman. Although RSM has been effectively used in optimization of various

biotechnological and industrial processes (Mohana et al. 2008; Reddy et al. 2008; Purama and Goyal 2008), the use of this powerful technique in screening followed by optimization of media constituents for enhancing enzyme production on an industrial scale Moreover we have found very less optimization studies form the isolates of bacillus that can be used in the waste management on an industrial scale has not yet been reported in the literature. In the present study, media constituents were, therefore, screened for their significant main effects using Plackett– Burman design to optimize concentration levels of the influential media constituents for enhancing of amylase production.

2. **Material and Methods**

Plackett–Burman design for screening of media constituents A Plackett–Burman design of total 17 experimental runs including three center-point replicates was used to screen the media constituents for their enzyme production based on previous reports (Production media was prepared which consists of Bacteriological peptone (6 gm/l), $MgSO_4$ (0.5 gm/l), KCl (0.5µgm/l), starch (1gm/l) (Vidyalakshmi *et al.,* 2009) , In this screening these media constituents along with temperature, pH and Incubation time were chosen as factors in this screening study. A minimum feasible concentration level was used for each factor, and correspondingly a maximum concentration level was chosen in such a way that the uncoded values represented the levels -1, 0 and 1, respectively, in which 0 is ascribed to the center-point value. Table 1 provides the range and levels of the factors tested and Table 2 provides the various experimental runs performed as per the design. In all the experimental runs, were recorded as the singular response of enzyme activity and the results analyzed using a statistical

software package, MINITAB® Release 21, PA, USA. The response enzyme activity was simply calculated.

Table 1. Design Table

Exp	Run	A	B	C	D	E	F	G
1		0	+	+	+	+	+	+
2		0	-	-	-	-	-	-
3		+	0	+	-	+	-	-
4		-	0	-	+	-	+	+
5		+	-	0	-	+	+	+
6		-	+	0	+	-	-	-
7		+	+	+	0	-	+	-
8		-	-	-	0	+	-	+
9		+	-	-	+	0	+	-
10		-	+	+	-	0	-	+
11		+	+	-	-	-	0	+
12		-	-	+	+	+	0	-
13		+	+	-	+	+	-	0
14		-	-	+	-	-	+	0
15		+	-	+	+	-	-	+
16		-	+	-	-	+	+	-
17		0	0	0	0	0	0	0

Design Summary Factors: 7 Replicates 1 Base runs: 17, Total runs: 17 Base blocks:1, Total blocks:1, Centre points1, Where A: Bacteriological Peptone ; B: MgSO4; C:KCl; D:Starch; E:Temperature (C); F:Incubation Time (hr); G: pH

Table 2. **Definitive screening design for** experimental runs performed.

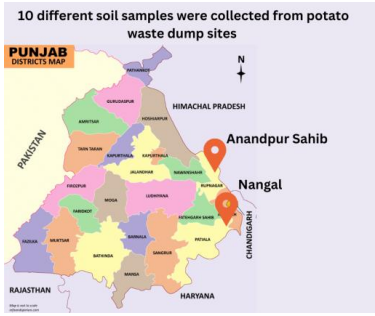
Run No*	**Bacteriological Peptone (g/L)**	**$MgSO_4$ (g/L)**	**KCl (μgm/L)**	**Starch (g/L)**	**Temperature (^{0}C)**	**Incubation Time (hr)**	**pH**
1	6	0.75	0.75	1.5	39	72	8
2	6	0.25	0.25	0.5	35	24	6

3	9	0.50	0.75	0.5	39	24	6
4	3	0.50	0.25	1.5	35	72	8
5	9	0.25	0.50	0.5	39	72	8
6	3	0.75	0.50	1.5	35	24	6
7	9	0.75	0.75	1.0	35	72	6
8	3	0.25	0.25	1.0	39	24	8
9	9	0.25	0.25	1.5	37	72	6
10	3	0.75	0.75	0.5	37	24	8
11	9	0.75	0.25	0.5	35	48	8
12	3	0.25	0.75	1.5	39	48	6
13	9	0.75	0.25	1.5	39	24	7
14	3	0.25	0.75	0.5	35	72	7
15	9	0.25	0.75	1.5	35	24	8
16	3	0.75	0.25	0.5	39	72	6
17	6	0.50	0.50	1.0	37	48	7

**DOE as per the using a statistical software package, MINITAB® Release 21, PA, USA. Uncoded values are used in the table*

Collection and isolation of the strains from the Soil Sample

10 different soil samples were collected from potato waste dump sites from Nangal and Anandpur Sahib, Punjab, India. These soil samples were collected in the sterile polythene bags and brought these to laboratory of Sri Guru Teg Bahadur Khalsa College, Sri Anandpur sahib for isolation of *Bacillus* sp. Multiple visit to the sites has being done for the collection of the soil samples both the places we have found high production of potato and large amount of agricultural waste is alarming hence the selection was made on the basis of production.



Courtesy Map: https://agri.punjab.gov.in/

Soil samples were serially diluted by using distilled water from (10^{-1} to 10^{-9}). 1 gram of soil sample was added into 9ml of distilled water in 9 different test tubes. It was then cultured on starch agar medium (autoclaved) using the spread plate technique on petri plates. Incubation was done for overnight at 37^0C and colonies were observed on petri plates. The random individual colonies were now picked and cultured on nutrient agar medium. Starch hydrolysis test was performed for identifying the bacteria and iodine was used as an indicator. The plates were flooded with iodine, as in the presence of iodine, starch produces blue coloration of medium and clearance zone forming ability on starch nutrient agar plate where the starch was hydrolyzed, used for the primary selection of isolates (Luang *et al*., 2019). The isolates were further characterized on the basis of morphological and biochemical characteristics and their enzyme activity. Bacterial isolates were examined for cell shape, colony gram staining as per standard procedure as described in bergey's manual of determinative bacteriology (Bergey, 1957).The colonies were observed on characteristic such as size, margin, shape, pigmentation, gram's staining and elevation. The three most common cell morphologies are *cocci*, *bacilli* and *spirilla*. Under a high magnification microscope, these may be recognized according to their appearance the *cocci* are spherical in shape, bacilli are rod or cylindrical shaped and *spirilla*

are coil shaped. Results were recorded. Gram staining is a method of differentiating bacterial species into two large groups (gram-positive and gram-negative). Gram staining differentiates bacteria by the chemical and physical properties of their cell walls by detecting peptidoglycan, which is present in a thick layer in gram-positive bacteria. The smear was spread on the glass slide and was covered with few drops of methyl violet. After a minute, the slide was washed with water. Now, few drops of gram's iodine were added and kept for few minutes. The slide was again washed with water and was decolorized using ethyl alcohol. After decolorization, the slide was washed and few drops of neutral red were added. The slide was then washed, air dried, heat fixed and finally observed under microscope. A gram-positive result in a purple-blue color while a gram-negative results in a pink-red color. This test was performed to determine that the bacteria are motile or non-motile under a given temperature using a semi solid agar method. The organism was inoculated into the Centre of a glass tube with the help of inoculated loop. The test tubes were incubated at 37°C for 24-48 hours. After incubation checked the migration of bacteria away from the line if inoculation. Migration indicates positive results.

The bacterial isolates were characterized by series of biochemical tests as given in Bergey's manual of determinative bacteriology (1957). These tests performed are as follows: A bacterial isolate was inoculated into a test tube with a sterile loop. The tube was incubated at 35°C for 2-5 days. After this step, 2.5ml of medium was transferred to another tube. Around 5 drops of pH indicator methyl red were added to this tube. To mix methyl red, the tubes were rolled between the hands. Colour changes were observed. A fermentation broth was prepared which consisted of carbohydrate to be tested, nutrient broth and pH indicator i.e. phenol red. The test organism was

inoculated into the broth and was incubated result were observed. Inoculate Simmons citrate agar by using straight wire from an 18 to 24 hour old colony. Incubate at 35°C for up to seven days and result was observed. The presence of catalase enzyme in the test isolate is detected using hydrogen peroxide. If the bacteria possess catalase (i.e. are catalase-positive), when a small amount of bacterial isolate is added to hydrogen peroxide, bubbles of oxygen are observed. This test is a test to differentiate between bacteria based on their ability or inability to reduce nitrate (NO_3^-) to nitrite (NO_2^-) using anaerobic respiration. Inoculate nitrate broth with an isolate and incubate for 48 hours. Add 10-15 drops each of sulfanilic acid and *N*, *N*-dimethyl-1-naphthylamine. If the bacterium produces nitrate reductase, the broth will turn a deep red within 5 minutes at this step. If no color change is observed, then the result is inconclusive. Add a small amount of zinc to the broth. If the solution remains colorless, then both nitrate reductase and nitrite reductase are present. If the solution turns red, nitrate reductase is not present. This test is used to detect the enzyme which catalyzes the conversion of urea to ammonia and carbon dioxide. Ammonia raises the pH of the media if it is present. This change in pH is indicated by pH indicator called phenol red which is present in the media. A slope agar was heavily inoculated with a bacterial colony using a loop. Enzyme activity is defined as the amount of glucose produced per ml in the reaction mixture per unit time. The amylase producing ability from various isolates was done by checking the enzyme assay using the DNS method (Nigam and Ayyagari, 2007). 1ml of culture extract enzyme was pipetted into a test tube and 1 ml of starch was added to citrate phosphate buffer. Incubation was done at 40^0C for 30 minutes. A blank was set up which consisted of 2 ml of enzyme extract which had been boiled for 30 minutes, was added to starch solution and treated with the same reagent. The reaction was stopped by adding 2 ml of DNS

reagent (1.0 g of 3, 5, dinitrosalicyclic acid, 20 ml of NaOH and 30 grams of sodium potassium tartarate in 100 ml). Boiling was done for 5 minutes and after cooling 20 ml of distilled water was added to it. Colour intensity was determined at 540 nm. Enzyme activity was calculated by using the formula (Nigam and Ayyagari, 2007)

$$Enzyme\ Activity\ (IU) = \frac{Net\ Amount\ of\ Sugar\ Produced}{Molecular\ Weight\ of\ Sugar} \times \frac{Dilution\ Factor}{Incubation\ Time}$$

Production of amylase was estimated at different temperature, pH and incubation time. Temperature is an important role for the production of amylase. To study the effect of temperature on amylase production was carried out at different temperatures (25°C, 30°C, 35°C, 40°C, 45^{O}C, 50 ^{O}C). The enzyme assay was carried out after 24 hours of incubation (Kumar *et al.,* 2011).

The effect of pH for amylase production was determined by culturing the bacterium in the production media with different pH. The fermentation medium was prepared by varying the pH values (5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) for the production of amylase. The enzyme assay was carried out after 24 hours of incubation (Jakheng *et al*, 2020).

The effect of incubation period was determined by incubating production medium for different incubation periods (24, 36, 48, 60, 72, 84 and 96 hrs) at 37ºC (Akcan *et al.,* 2011). All chemicals are used in the study are from Sigma® Aldrich and 2023 Merck ©KGaA, Darmstadt, Germany.

3. Results and Discussion

The total numbers of the *Bacillus* sp.

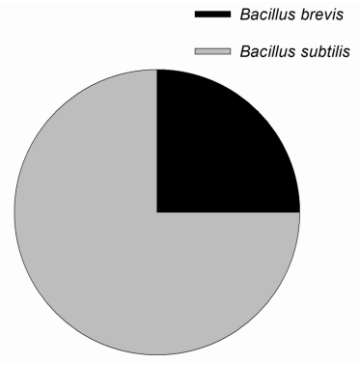


Fig 1. Total % of Isolates sp from APB1-APB 8 the percentage of the individual sp. More than 75 % of the isolates have *Bacillus subtillis* and 25% of *Bacillus brevis*

As per the design of experiments in the table 1 the enzyme activity was analyzed and we have seen a significant increase in the isolate 7 with marked enzyme activity as shown in the fig. 2. The amylase activity in the isolate 7 was noted as high as 71.2 IU/mL that was incongruent to the highest amylase activity reported *haloarchaea isolated* from Odiel saltern ponds in the southwest of Spain. The strain that exhibited the highest activity was selected and identified as *Haloarcula sp.* HS but in the bacillus sp. We have found the similar high enzyme activity. (Gómez-Villegas et al 2021). Also similar high enzyme activity was seen in the isolate 6 and 8 these isolates were then we can screen further for CCD optimization of the media and can also use in the industrial application for the batch production of amylase and use in agricultural bio waste management focused on the potato crops.

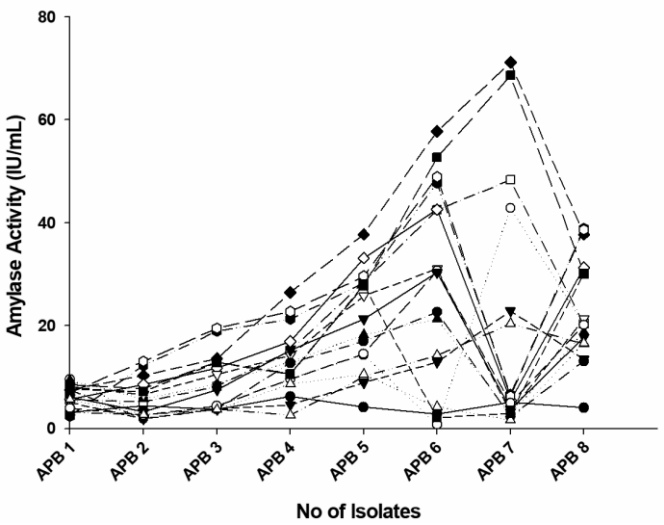


Fig 2. Design of experiments in the table 1 the enzyme activity was analyzed and we have seen a significant increase in the isolate 7 with marked enzyme activity as shown.

The starch hydrolysis test was performed to check the ability of bacteria to break down starch. This test differentiates between the bacteria that produces amylase from those which are non-amylases producing. Iodine was used as an indicator. The isolates were observed for the starch hydrolysis test. A clear zone was formed which showed the positive hydrolysis test for amylase (Fig 3). 8 isolates were tested positive for the amylase producing bacteria and were designated as APB1-APB 8.

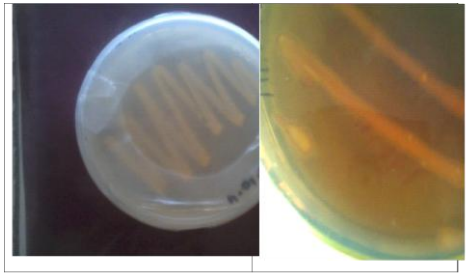


Fig 3. A. Bacterial growth on media containing starch B. Amylase Producing Isolates Showing Clear Zone

The use of starch nutrient agar and iodine for detecting amylase have been reported by Forgarty and Kelly, 1979 and also by Iverson and Millis, 1974 that starch hydrolysis can be detected on plates as a clear zone surrounding a colony. The procedure employed showed a positive result for the *Aspergillus* strain isolated. Isolates were morphologically characterized according to the shape, margin, size, elevation, pigmentation gram's staining and motility test. The results are shown in the Table 3.

3.1 Biochemical Test of Bacterial Isolates: Various biochemical tests were performed to characterize the screened isolates. All the isolates showed their biochemical properties as given in Table 2

Table 2. Biochemical Characteristics from Different Isolates

Isolates	Methyl red Test	Carbohydrate Fermentation Test	Citrate Utilization Test	Catalase test	Nitrate reduction Test	Urease Test	Probable identity
APB 1	-	Glucose + Fructose -	+	+	+	-	*Bacillus subtilis*
APB 2	-	Glucose + Fructose -	+	+	+	-	*Bacillus subtilis*
APB 3	-	Glucose +	+	+	+	-	*Bacillus*

		Fructose -					*subtilis*
APB 4	-	Glucose + Fructose -	+	+	+	-	*Bacillus subtilis*
APB 5	-	Glucose + Fructose -	-	+	+	-	*Bacillus brevis*
APB 6	-	Glucose + Fructose -	+	+	+	-	*Bacillus subtilis*
APB 7	-	Glucose + Fructose -	-	-	-	-	*Bacillus brevis*
PB 8	-	Glucose + Fructose -	+	+	+	-	*Bacillus subtilis*

Out of the 8 samples, six isolates were recognized as *Bacillus subtilis*, and two isolates were recognized as *Bacillus brevis* (Table 2).

The Table 2 and Figure 1 showed that out of 8 isolates, 6 isolates (75%) belonged to *Bacillus subtilis* and 2 of them (25%) belonged to *Bacillus brevis.*

Screening results all the 8 isolates are subjected for the statistical screening and then the response (Enzmye Activity) were calculated. In the APB1 all 17 runs were done as shown in the **Table S1 and S2** were done. Analysis of variance and

Regression Equation in Encoded. As per the mathematical regression the equation for the isolate. **Fig S1**: Pareto charts and Residual Plot for Screening design model: APB 1 versus Bacteriological peptone, MgSO4, KCL, Starch, Temperature, Incubation Time, pH were shown the effect between the response and the factors.

APB 1 =8.9 + 0.175 Bacteriological peptone - 2.91 MgSO4 - 0.71 KCL - 0.20 Starch + 0.146 Temperature - 0.0189 Incubation Time - 0.893 pH

Units the enzyme activities were calculates as per the model equation vs. the experimental value. In case of APB2 the results were slight different as shown the **Table S3 and S4** with the regression equation

In the APB2 all 16 runs were done as shown in the **Table S3 and S4** were done. Analysis of variance and Regression Equation in Encoded. As per the mathematical regression the equation for the isolate. **Fig S3**: Pareto charts and Residual Plot for Screening design model: APB 2 versus Bacteriological peptone, MgSO4, KCL, Starch, Temperature, Incubation Time, pH were shown the effect between the response and the factors.

APB 2*=-0.1 + 0.042 Bacteriological peptone - 0.07 MgSO4 + 1.64 KCL - 0.01 Starch + 0.052 Temperature + 0.0185 Incubation Time + 0.29 pH*

Units the enzyme activities were calculates as per the model equation vs. the experimental value. In case of APB2 the results were slight different as shown the **Table S3 and S4** with the regression equation

In the APB3 all 16 runs were done as shown in the **Table S5 and S6** were done. Analysis of variance and Regression Equation in Encoded. As per the mathematical regression the

equation for the isolate. **Fig S5**: Pareto charts and Residual Plot for Screening design model: APB 3 versus Bacteriological peptone, MgSO4, KCL, Starch, Temperature, Incubation Time, pH were shown the effect between the response and the factors.

APB 3*=6.3 + 0.176 Bacteriological peptone - 1.91 MgSO4 + 2.29 KCL - 0.47 Starch - 0.053 Temperature + 0.0025 Incubation Time + 0.53 pH*

Units the enzyme activities were calculates as per the model equation vs. the experimental value. In case of APB3 the results were slight different as shown the **Table S5 and S5** with the regression equation.

In the APB4 all 16 runs were done as shown in the **Table S7 and S8** were done. Analysis of variance and Regression Equation in Encoded. As per the mathematical regression the equation for the isolate. **Fig S7**: Pareto charts and Residual Plot for Screening design model: APB 4 versus Bacteriological peptone, MgSO4, KCL, Starch, Temperature, Incubation Time, pH were shown the effect between the response and the factors.

APB 4*= 37.9 + 0.334 Bacteriological peptone + 2.03 MgSO4 + 4.04 KCL + 1.27 Starch - 0.62 Temperature - 0.0337 Incubation Time - 0.94 pH*

Units the enzyme activities were calculates as per the model equation vs. the experimental value. In case of APB4 the results were slight different as shown the **Table S7 and S8** with the regression equation.

In the APB5 all 16 runs were done as shown in the **Table S9 and S10** were done. Analysis of variance and Regression Equation in Encoded. As per the mathematical regression the equation for the isolate. **Fig S9**: Pareto charts and Residual Plot for Screening design model: APB 5 versus Bacteriological

peptone, MgSO4, KCL, Starch, Temperature, Incubation Time, pH were shown the effect between the response and the factors.

APB 5*= 53.4 + 0.17 Bacteriological peptone - 2.0 MgSO4 + 3.0 KCL - 0.18 Starch - 0.85 Temperature - 0.078 Incubation Time + 0.19 pH*

Units the enzyme activities were calculates as per the model equation vs. the experimental value. In case of APB5 the results were slight different as shown the **Table S9 and S10** with the regression equation.

In the APB6 all 17 runs were done as shown in the **Table S11 and S12** were done. Analysis of variance and Regression Equation in Encoded. As per the mathematical regression the equation for the isolate. **Fig S11**: Pareto charts and Residual Plot for Screening design model: APB 6 versus Bacteriological peptone, MgSO4, KCL, Starch, Temperature, Incubation Time, pH were shown the effect between the response and the factors.

APB 6*= 49 + 0.75 Bacteriological peptone - 5.3 MgSO4 + 17.5 KCL + 0.4 Starch - 1.33 Temperature + 0.002 Incubation Time + 2.37 pH*

Units the enzyme activities were calculates as per the model equation vs. the experimental value. In case of APB6 the results were slight different as shown the **Table S11 and S12** with the regression equation.

In the APB7 all 17 runs were done as shown in the **Table S13 and S14** were done. Analysis of variance and Regression Equation in Encoded. As per the mathematical regression the equation for the isolate. **Fig S13**: Pareto charts and Residual Plot for Screening design model: APB 7 versus Bacteriological peptone, MgSO4, KCL, Starch, Temperature, Incubation Time, pH were shown the effect between the response and the factors.

APB 7 *= 157 + 2.09 Bacteriological peptone + 1.0 MgSO4 + 10.1 KCL - 8.8 Starch - 3.05 Temperature + 0.136 Incubation Time - 5.92 pH*

Units the enzyme activities were calculates as per the model equation vs. the experimental value. In case of APB7 the results were slight different as shown the **Table S13 and S14** with the regression equation.

In the APB8 all 17 runs were done as shown in the **Table S15 and S16** were done. Analysis of variance and Regression Equation in Encoded. As per the mathematical regression the equation for the isolate. **Fig S15**: Pareto charts and Residual Plot for Screening design model: APB 8 versus Bacteriological peptone, MgSO4, KCL, Starch, Temperature, Incubation Time, pH were shown the effect between the response and the factors.

APB 8= *64.2 + 0.13 Bacteriological peptone - 7.2 MgSO4 - 0.6 KCL - 4.56 Starch - 0.86 Temperature + 0.019 Incubation Time - 0.40 pH*

Units the enzyme activities were calculates as per the model equation vs. the experimental value. In case of APB8 the results were slight different as shown the **Table S15 and S16** with the regression equation.

Fig S16: Optimized valued as per the Model decoded the Isolates with Response we have seen that maximum Enzyme activity recoded and with final equation is for the culture will be

Bacteriological peptone: 6.61978, MgSO4: 0.75, KCL 0.25, Starch: 1.5, Temperature: 35 Incubation Time: 72 and pH: 6.

Further optimization of these isolates can be achieved through CCD or RSM and they can be produced in large quantities for biotechnology applications.

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Conclusions: A total of 10 potato waste dump site soil samples were collected from potato waste dump areas and were processed for isolation of amylase production. Morphological and biochemical characterization was carried out of purified cultures showing clear zone in petriplates. *Bacillus* sp. was isolated from 8 (80%) samples, out of which 6 (75%) belonged to *Bacillus subtilis* and 2 (25%) belonged to *Bacillus brevis*. Isolates were designated as APB 1-APB 8. The amylase producing ability was checked for isolates by using the DNS method and enzyme activity was calculated. The maximum enzyme activity was shown by APB 4 (6.21 IU/ml), so this isolate was considered to optimize conditions for amylase production.

Isolate APB 4, 7 and 8 based on the highest amylase producing ability can be selected for further to see the effect of substrate concentration, nitrogen source, carbon source, surfactants etc. for the maximum amylase production. The crude enzyme extracts can be used further for different industrial application after purification. Enzyme technology is presently going through a phase of maturation and evolution. Enzymes will clearly be more widely used in the future and this will be reflected in the number enzymes available on an industrial and research scale, the variety of reactions catalyzed and the range of environmental conditions under which they will operate. These Isolates can be used for further optimization via CCD or RSM and can be used for mass production which can be used for biotechnological processes.

References

1. Akcan, N. Uyar, F. and Guven, A. (2011) Alpha-Amylase Production by *Bacillus subtilis* RSKK96 in Submerged Cultivation. *Kafkas Univ Vet Fak Derg.* **17:** 17-22. DOI: https://doi.org/10.9775/kvfd.2010.3036
2. Ahmed, N.E., El Shamy, A.R. & Awad, H.M.(2020) Optimization and immobilization of amylase produced by *Aspergillus terreus* using pomegranate peel waste. *Bull Natl Res Cent* **44**, 109 DOI: https://doi.org/10.1186/s42269-020-00363-3
3. Breed S. R, Murray E.G.D, Smith N.R, (1957) (7thed.) Bergey's *Mannual of Determinative Bacteriology.*Seventh edition, American Society for Microbiology. Williams and Willkins Co. Publishers, Baltimore, USA.
4. Elmansy, E.A., Asker, M.S., El-Kady, E.M. *et al.*(2018) Production and optimization of α-amylase from thermo-halophilic bacteria isolated from different local marine environments. *Bull Natl Res Cent* **42**, 31.DOI: https://doi.org/10.1186/s42269-018-0033-2
5. Elyasi Far B, Ahmadi Y, Yari Khosroshahi A, Dilmaghani A.(2020) Microbial Alpha-Amylase Production: Progress, Challenges and Perspectives. Adv Pharm Bull. Jul;10(3):350-358. DOI: https://doi.org/0.34172/apb.2020.043
6. Fogarty, W and Kelly, C. (1979) Starch degrading enzyme of Microbial origin. *Journal of Progress in Industrial Microbiology.* **15**: 87-150. DOI: https://doi.org/10.34172/apb.2020.043
7. Iverson, W. and Millis, N. (1974) A Method for the detection of starch hydrolysis by bacteria. *J. Appl. Biotechnol.* **37**: 443-446 DOI: https://doi.org/10.1111/j.1365-2672.1974.tb00460.x

8. Jakheng S. P. E. , Thomas J , Umar M, Ojeleye F.S , Albert A.K , Oko J.O , Ojo S.A, Sagir M.R, Amuta I.C , Gaiya J.D and Kutman N.A (2020) Screening of Locally Isolated Penicillium Species from the Soil for Amylase Production. Journal of Applied Life Sciences International 23(12): 76-83 DOI: https://doi.org/10.9734/jalsi/2020/v23i1230207
9. K Ashwin, Kumar G, L Karthik and K.V. R Bhaskara (2011) Optimization, production and partial purification of extracellular alpha amylase from Bacillus sp. marini. 3(1):33-42 DOI: https://doi.org/10.1111/j.1365-2672.1974.tb00460.x
10. Kumar, A., Kumar, G., Karthik, L. and Bhaskara Rao, K.V. (2011) Optimization, production and Partial Purification of Extracellular a- amylase from *Bacillus* sp. Marini. *Archives of Applied Science Research*. **3**(1): 33-42.
11. Luang-In V, Yotchaisarn M, Saengha W, Udomwong P, Deeseenthum S, Maneewan K. (2019) Isolation and Identification of Amylase-producing Bacteria from Soil in Nasinuan Community Forest, Maha Sarakham, Thailand. Biomed Pharmacol J 2019;12(3) DOI: https://dx.doi.org/10.13005/bpj/1735
12. Nigam, A. and Ayyagri, A. (2007*)* First edition, *Lab Mannual Biochemistry, Immunology and Biotechnology*. Tata McGraw Hill Publishing Company Limited, New Delhi. **pp**: 183.
13. Ilori, M.O., Amund, O.O. & Omidiji, O. (1997) Purification and properties of an α-amylase produced by a cassava-fermenting strain ofMicrococcus luteus . Folia Microbiol 42, 445–449. DOI: https://doi.org/10.1007/BF02826551
14. Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D, Mohan R. (2000) Advances in microbial amylases.

Biotechnol Appl Biochem. Apr;31(2):135-52. DOI: http://doi.org/10.1042/ba19990073.

15. R Gojiya, P. andhare, F Marchawala, I Bhattacharya and D Upadhyay (2021) Production of amylase from microbial sources: a review. 10(4): 341-350 DOI: http://dx.doi.org/10.31032/IJBPAS/2021/10.4.1038
16. Shankar T, Isaiarasu L (2011) Cellulase production by *Bacillus pumilus* EWBCM1 under varying cultural conditions. Middle-East J Sci Res 8:40–45
17. Sobia Sharif, Asad Hussain Shah, Anila Fariq, Sammyia Jannat, Sajida Rasheed, Azra Yasmin (2023) Optimization of amylase production using response surface methodology from newly isolated thermophilic bacteria, Heliyon, Volume 9, Issue 1, ISSN 2405-8440 DOI: https://doi.org/10.1016/j.heliyon.2023.e12901
18. Tallapragada Padmavathi, Rayavarapu Bhargavi, Purushothama Rao Priyanka, Naige Ranganath Niranjan, Pogakul Veerabhadrappa Pavitra (2018) Screening of potential probiotic lactic acid bacteria and production of amylase and its partial purification, Journal of Genetic Engineering and Biotechnology, Volume 16, Issue 2. DOI: https://doi.org/10.1016/j.jgeb.2018.03.005
19. Irfan Ullah, Mohammad Sayyar Khan, Shahin Shah Khan, Waqar Ahmad, Lingjie Zheng, Syed Usman Ali Shah, Mazhar Ullah, Aqib Iqbal (2021) Identification and characterization of thermophilic amylase producing bacterial isolates from the brick kiln soil, Saudi Journal of Biological Sciences, Volume 28, Issue 1, Pages 970-979, ISSN 1319-562X. DOI: https://doi.org/10.1016/j.sjbs.2020.11.017
20. Patricia Gómez-Villegas , Javier Vigara, Luis Romero , Cecilia Goto , Sara Raposo , Brígida Gonçalves and Rosa Léon (2021) Biochemical Characterization of the Amylase Activity from the New Haloarchaeal Strain Haloarcula sp.