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RSM Mediated Optimization of Amylase Production from Marine *Bacillus* sp. VITRKHB

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ABSTRACT

Amylases (EC 3.2.1.1) hydrolyses the α-1,4 glycosidic linkages of starch. Microbes are the most preferred sources of enzymes due to their broad biochemical diversity. In the present study saltpan soils were collected from Ongole, Andhra Pradesh. Twenty bacterial isolates were isolated and screened for amylase production, Four isolates showed amylolytic activity. One isolate (N3) displaying the highest activity was selected for further study. The amylolytic bacterium was identified as *Bacillus* sp. VITRKHB. The maximum enzyme activity was observed in case of starch as carbon source and yeast extract as nitrogen source. Response surface methodology was employed to evaluate combined interactive effects of different variables, i.e., starch (0.21%), yeast extract (0.65%), pH (6.13), NaCl (4.63%) and temperature (45°C) on amylase production after 24h of incubation. Determination of co-efficient (R^2=0.8056) close to 1.0 indicated significant precision and reliability of the response data. The enzyme was purified to 1.45 fold by DEAE ion exchange chromatography and its specific activity was found to be 1.086 IU/mg.

Keywords: Enzyme, Marine Eubacteria, *Bacillus* sp VITRKHB, 16S rRNA, Response surface methodology, DEAE ion exchange chromatography

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INTRODUCTION

Enzymes have gained attention by researchers throughout the world because they play not only *in vivo* but possess potential industrial applications too. Amylases (EC 3.2.1.1) belong to a class of hydrolase associated with saliva and pancreatic juice, trigger hydrolysis of α-1,4 glycosidic linkages of starch polymer into dextrins and various gluco-oligosaccharide [1].They are of great value in a wide range of biotechnological applications in many industries such as sugar, textile, paper, brewing, baking, distilling and pharmaceutical industries [2]. They account for 25% of sales in enzyme market [3, 4].

There are various sources of enzymes ranging from unicellular to multicellular organisms, however, now-a-days due to their broad biochemical diversity, ease of mass culture and genetic manipulation, microorganisms are the most preferred sources [5]. Microbial enzymes are relatively more stable than the corresponding enzymes derived from plants and animals. Present enzyme proportion is not sufficient to meet the industrial demand [6]. Hence, there is a need to target extremophiles for unique and novel enzymes with advantageous properties and enhance their quality and quantity. Considerable screening techniques and process methodologies are devoted to the production of enzymes exhibiting tolerance to extreme conditions viz, temperature, salts and pH that are used in the industrial processes [7].

Marine environment harbors microbial groups that display adaptation to its extreme and variable pH, temperature, salinity, density, pressure and light. Consequently, these groups must be a better source of enzymes that exhibit optimal activities at a wide range of these parameters [8]. These enzymes have been observed to possess unusual characteristics due to their habitat related properties such as salt tolerance (above 1.7M) [9], hyperthermostability (80-108ºC), barophilicity (60 MpA), cold adaptivity (Esterase can retain its 50% activity at freezing point of water) and pH. They also have novel chemical and stereochemical properties [10]. Isolation of a number of extremophiles have been undertaken from marine environments that can thrive on a wide range of pH, salt concentration and pressure and they metabolize enzymes that can act upon carbohydrates, proteins and lipids [11,12,13]**.** Earlier few groups have reported the effective production of marine bacterial enzymes. The present study provides first report towards the optimized production of amylase isolated from the saltpan soil samples that were collected from the coastal areas of the Bay of Bengal at Ongole, Andhra Pradesh, India, employing Response surface methodology.

MATERIALS AND METHODS

Chemicals

Chemicals and media used in this study were purchased from Hi Media chemicals, Mumbai, India, Merck Specialities Private Limited and Sisco Research Private Limited, Mumbai, India.

Sample collection

The saltpan soil samples were collected from Ongole (15º30'N, 80º03'E), situated in coastal regions of Andhra Pradesh in sterile disposable bags from a depth of about 3-4 cm with the help of a sterile spatula. The bags were transferred to the Molecular and Microbiology Laboratory, VIT University, Vellore in sterile conditions and were subsequently stored at 4°C.

Isolation of Marine Eubacteria

Marine Eubacteria were isolated by serial dilution and spread plate method on Nutrient Agar medium dissolved in 50% distilled water and 50% marine water. The plates were incubated at 37°C for 24 h.

Screening of potent Amylase producing isolates

The isolates were screened for amylase production by growing them on Starch Agar medium at 37°C for 24 h. After incubation, starch agar was flooded with 1% iodine. The hydrolysis of starch resulting in the formation of clear zone around the colony indicates amylase production.

Identification of amylolytic bacteria

Cultural characterization

The isolates were characterized morphologically by observing their colour, shape, size, nature of the colony and pigmentation.

Microscopic observation

Gram staining, endospore staining and capsule staining were performed for the isolates and slides were observed under a high power magnifying lens in the light microscope to check the morphology of individual cells.

Biochemical characterization

Biochemical characterization was done by indole test, methyl red test, Voges Proskauer test and Simmons' citrate test.

Molecular characterization

The most efficient isolate was characterized based on 16S rRNA sequencing and the 16S rRNA sequence of the eubacteria was analyzed using online tool BLAST at NCBI gene bank. A neighborhood joining Phylogenetic tree was constructed using the Tree view software.

Production of Enzymes

Fermentation medium for amylase was prepared based on the following composition, (g/L) starch- 10, peptone-10, yeast extract -20, KH_2PO_4- 0.05, $MnCl_2.4H_2O$- 0.015, $MgSO_4.7H_2O$- 0.25, $CaCl_2.2H_2O$- 0.05 and $FeSO_4$.- 0.01 [13].

Optimization of carbon and nitrogen source

Effect of carbon source and nitrogen source was studied by using different carbon sources (sucrose, fructose, xylose, starch and lactose), nitrogen sources (beef extract, yeast extract, nutrient Broth, urea and casein) in the production medium [13].

Optimization using Response Surface Methodology (RSM)

Response surface methodology was employed in order to demonstrate the mutual interactive effect of the response variables on the activity of marine eubacterial amylase. The five independent variables to be optimized were starch (A), yeast extract (B), pH (C), NaCl (D) and temperature (E). A set of 31 experiments with five variables were required. The range of medium components selected for screening by RSM is shown in Table 1. Each variable was set at two levels (-1 and +1). Trace element solution was constantly maintained [14] .The incubation period was about 24 hours. The design was developed using a Design Expert, version 7.0.7.1.

Table 1.Range of media components for RSM

Variables	Levels	
	Low (-1)	**High (+1)**
A. Starch (%)	0.2	0.8
B. Yeast Extract (%)	0.5	4.0
C. pH	5.5	8
D. NaCl (%)	4.5	7.5
E. Temperature (^{O}C)	30	50

Protein Estimation

Protein estimation was done using Lowry's method [15]. Bovine serum albumin (BSA) was used as standard.

Enzyme Assay

The amylase activity was measured according to the method of Rick and Stegbauer [16]. One ml of enzyme was taken in the test tube and 1 ml of 1% starch were added, the mixture was incubated at 37°C for 15 minutes in a water bath. Then 2 ml of dinitrosalicyclic acid (DNS) reagent was added to stop the reaction and kept in boiling water bath for 5 minutes.

Subsequently, 1 ml of sodium potassium tartarate was added. Absorbance was taken at 570 nm and recorded.

Partial purification of enzyme

Amylase was partially purified by a standard procedure by ammonium sulphate precipitation. This was done in three sequential steps. One hundred ml of cell free extract was brought to 80% saturation with the help of ammonium sulphate slowly with gentle agitation at 4°C. The precipitate was removed by centrifugation (10,000 rpm at 4°C for 20 min). It was then dissolved in 0.1M Tris HCl buffer (pH 6.13) and protein content was estimated.

The precipitate was desalted by dialysis. The enzyme solution was poured in a bag of a selectively permeable membrane (Dialysis membrane-150, Himedia laboratories Ltd; Mumbai, India) and the membrane was tied from both the sides. Then the membrane was immersed in a large volume of 0.1M phosphate buffer that was stirred and maintained at about 4°C for 24 hours. Enzyme was stored till further use.

Ion Exchange Chromatography

The dialyzed enzyme solution obtained from the previous step was loaded into a DEAE cellulose column. The column was pre-equilibrated with 10 mM Tris - HCl buffer (pH 6.13). The column was again washed with 10 mM Tris - HCl buffer (pH 6.13). Proteins were eluted with 10 mM Tris - HCl and 0 to 2.0 M NaCl gradient. Eluted fractions (3 ml) were collected and absorbance was measured at 280 nm. The amylase activity and protein concentration of fractions was measured and specific activity was calculated.

Brine shrimp hatchability test

The brine shrimp hatchability test was performed based on the method reported by Karthik et al. [17]. The brine shrimp (*Artemia salina*) eggs or cysts were hatched in sterile seawater (1 g cyst per liter) at 28°C, under conditions of continuous lighting and strong aeration. The different concentration of marine eubacterial amylase (250, 500, 750, 1000 µg/ml) was added along with the eggs. The number of free *nauplii* in each treatment was calculated. This test was carried out to check the toxicity of the enzyme.

RESULTS AND DISCUSSION

Isolation and Screening of Amylase producing Marine Eubacteria

In the present investigation, a total of 5 marine sediments was collected and processed. A total of 20 (N1 – N20) different bacterial strains were isolated from the marine sediments of the Bay of Bengal in the coastal regions of Andhra Pradesh. The number of isolates was lesser as compared to earlier reports on isolation of marine bacteria [18, 19, 20, 21]. Increase in the

amount of sediments and repeating the sampling might have resulted in those numbers of isolates. In this study, samples were collected, processed and analyzed only once. Among the 20 isolates, only 4 isolates showed amylase activity. Among these only one (N3) showed highest activity, which was selected for further study (Fig 1). The ability of marine eubacteria to produce amylase is a well known phenomenon and has been documented previously [21]

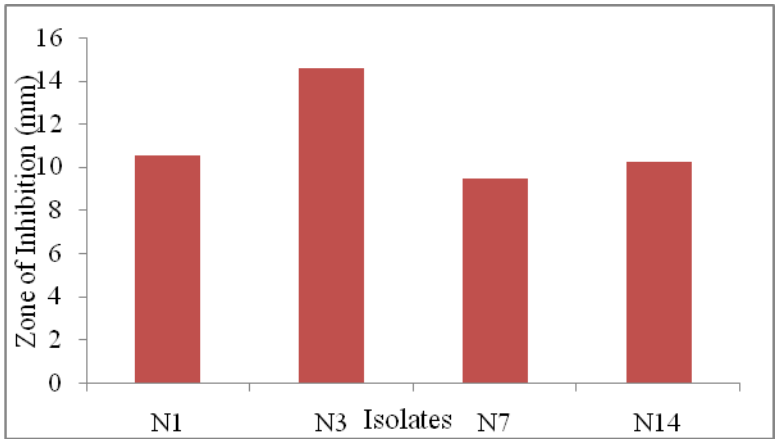

Fig 1: Zone of inhibition of different isolates for amylase

Identification of Amylase producing eubacteria

The 16S rRNA sequence analysis showed 99% identity to that of *Bacillus* sp. The morphological and biochemical properties of N3 were also consistent with those of *Bacillus.* sp. (Table 2). Hence, the isolate was identified as *Bacillus* sp. and designated as *Bacillus* sp. VITRKHB (Accession number: JF960957). A neighborhood joining Phylogenetic tree was constructed using the Tree view software (Fig 2).

Table 2: Morphological and Biochemical characteristics of N3 isolate

	Characterization of bacteria	Result
Culture characteristics	Colony Morphology on Nutrient Agar Medium	Medium, circular, off white Mucoid, colonies.
Microscopic characters	Gram staining Endospore staining	Gram positive, rods Terminal Endospore forming
Biochemical characters	Indole Methyl Red Voges Proskauer Citrate Utilization Catalase test Oxidase test Starch Hydrolysis	Negative Negative Positive Positive Positive Positive Positive

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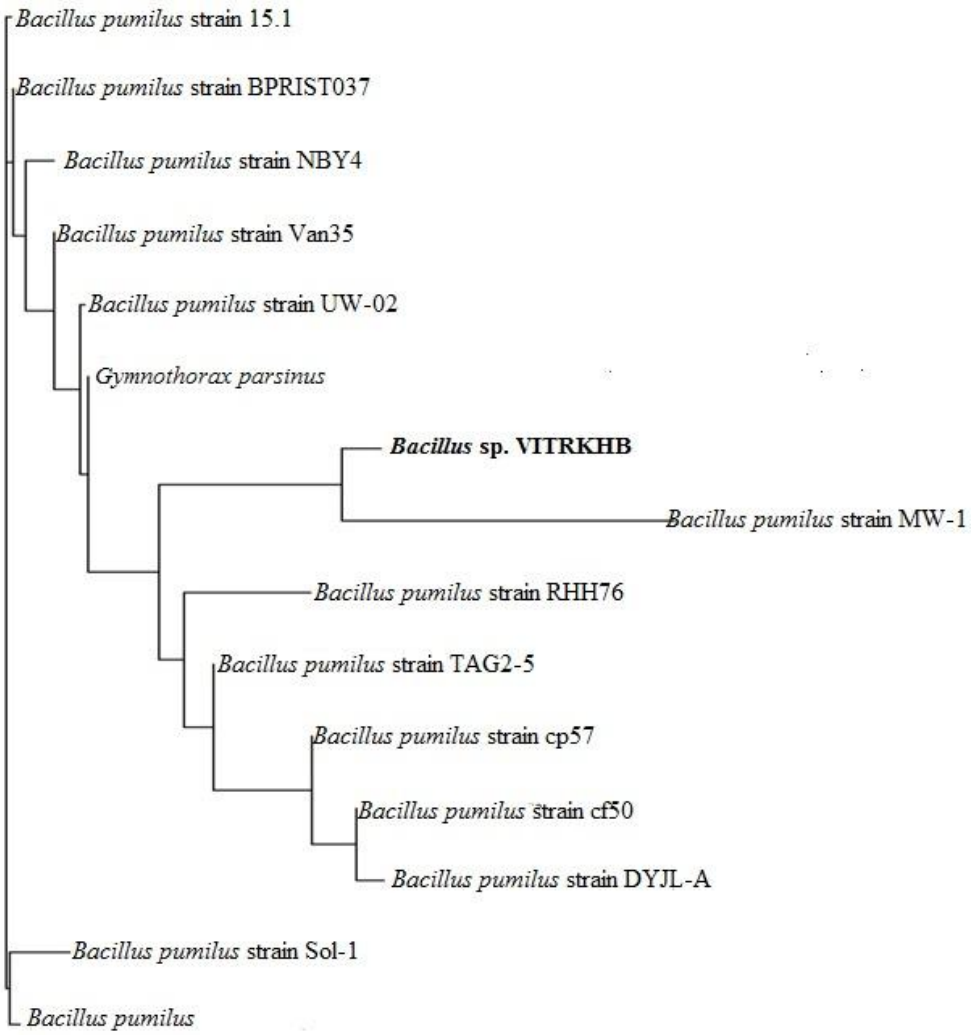

Fig 2: Neighborhood joining phylogenetic tree 16S rRNA sequence of *Bacillus* sp.VITRKHB and other relevant sequences from database

Effect of different Carbon sources

Bajpai [22] reported that different carbon sources can greatly influence the production of amylase. Effect of different carbon sources on amylase activity and enzyme production was studied by using 5 different carbon sources namely lactose, sucrose, xylose, fructose and starch in production media (Fig 3). Maximum enzyme production was seen in the presence of xylose, however, maximum enzyme activity was observed in the presence of starch. Starch is generally accepted as nutritional component for induction of amylolytic enzymes. One of the report [23] recommended sucrose as a best carbon source for amylase production by marine bacterium *Halobacterium salinarum* MMD047. There are also reports on maltose as a best carbon source for amylase production from *Bacillus* sp. [24, 25]. They mentioned that induction of α – amylase requires starch and maltose as substrate. Starch was used as a carbon source [26] for amylase production from *Bacillus licheniforms*. Besides this, there are reports [27] that suggested the use of sodium citrate and sucrose as a carbon source for the strains of *Bacillus subtilis* and

Bacillus amyloliquifaciens. In another study, *Bacillus mairini* showed the maximum enzyme activity in the presence of starch as carbon source; whereas, the minimum enzyme activity and protein content were observed in the presence of dextrose [13]. Likewise, *Thermomyces lanuginosus* ATCC34626 the maltodextrin was found to be the best carbon source [28]. These results indicate that the disaccharides are more suitable carbon source than polysaccharides.

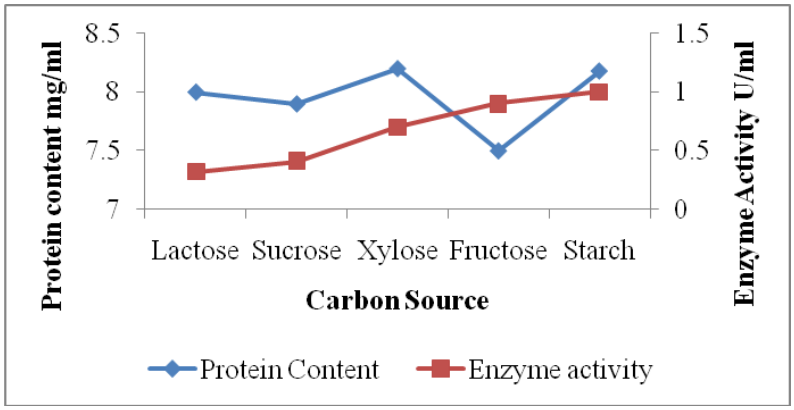

Fig 3: Effects of different carbon sources on enzyme activity and production

Effect of different Nitrogen sources

Highest enzyme activity and production were found when yeast extract was used as nitrogen source (Fig 4). This result was in accordance with the results obtained from some of the previous reports [28, 29, 30]. One study revealed that for *Halobacterium salinarum*, the MMD047 beef extract was the best Nitrogen source and Yeast extract showed the inhibitory effect on enzyme production [23]. Likewise, in another study on *Bacillus megaterium* revealed that peptone was the best nitrogen source for amylase production [22]. Teodoro et al. [31] reported the use of yeast extract as a nitrogen source in combination with peptone. This study shows yeast extract as the best nitrogen source for amylase production. According to previous reports, organic nitrogen is the best source of nitrogen as compared to inorganic nitrogen for higher α-amylases yield from several *Bacillus* sp. The same inclination was observed in our study.

Optimization using Response Surface Methodology (RSM)

To examine combined effect of the independent variables starch (A), yeast extract (B), pH (C), NaCl (D) and temperature (E) on the activity of amylase from *Bacillus* VITRKHB, an experiment of 31 runs were designed and performed with an incubation period of 24 hours. ANOVA analysis was carried out using a response surface quadratic model that gave the following equation.

$$R= -0.47-0.057*A+0.38*B+0.093*C+0.19*D-0.16*E-0.25*A*B+0.048*A*C-0.32*A*D-0.045*A*E+0.15*B*C-0.22*B*D-0.44*B*E+0.14*C*D-6.057E-003*C*E-0.059*D*E+1.09*A^2+0.42*B^2-1.57*C^2+0.86*D^2+0.72*E^2$$

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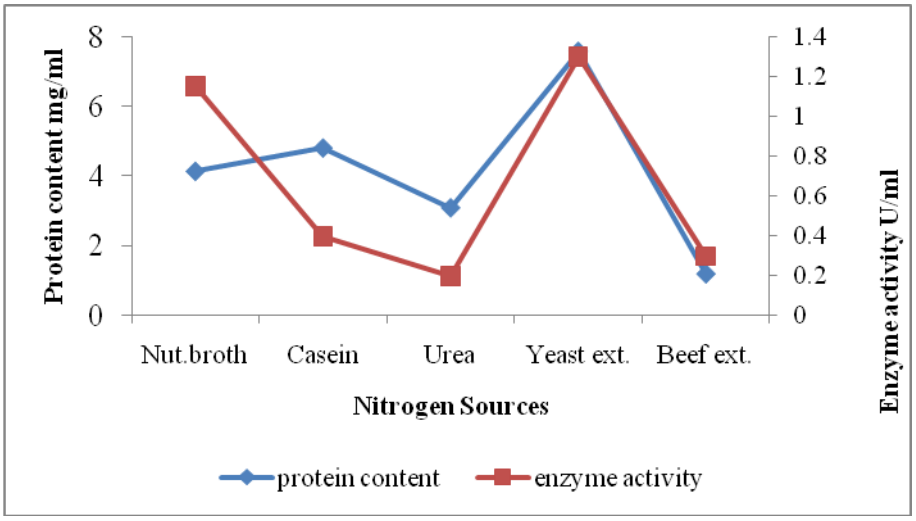

Fig 4: Effects of different nitrogen sources on enzyme activity and production

The F value was 2.07. The model F value explained 88.18% significance and chance of noise was only 11.82%. Values of Probability > F (< 0.050) also indicated that the model terms were significant. In this case B, AD, BE, C^2 were significant model terms. In general, values greater than 0.1000 indicate the model terms are not significant. The Lack of Fit for F-value was 2.19 and this indicated that Lack of Fit is not significant relative to the pure error. There was a 20.44% chance that "Lack of Fit F-value" this large could occur due to noise. Non significant lack of fit was good. In the present work, all the linear, interactive effects of AB, AC, AD, AE, DE, BC, BD, BE, CD, CE and DE were significant for enzyme activity. The coefficient of determination (R^2) for enzyme activity was calculated as 0.8056. This indicated that the statistical model explained 80.56% of the variability in response and only 19.44% of variance was not explained by the model. Value of R^2near to 1.0 indicated that the model was strong and it can predict the response in a better manner [32] and this supports our results. The adjusted R^2 value corrects the R^2 value for the sample size and for the number of terms in the model. The value of the adjusted determination coefficient, R^2 was 0.4167. This implied a higher significance of the model applied for analyzing the data [33, 34]. In the present study, the adjusted R^2 value (0.4167) was lesser than the R^2 value (0.8056). The smaller value of adjusted R^2 as compared to that of the R^2 may be due to the presence of multiple terms but small sample size. The interactive effects of independent variables on enzyme were studied by plotting 3D surface curves. The 3D curves of the calculated enzyme activity for the interactions between the variables are shown in Figure 5. Optimal level of the significant variables for the maximum amylase production were: pH - 6.13, starch - 0.21%, yeast extract - 0.65%, temperature -45°C, and NaCl – 4.63%.

The statistical experimental designs and mathematical methods have wide application in the field of biotechnology. Response surface methodology (RSM) is one such method that is applied for modeling problems with the aim to optimize responses that were influenced by multiple variants. Performing statistically designed experiments, filling experimentally

determined response data into a quadratic model, predicting response and checking significance of the model are the major steps involved in this process [35]. RSM was advantageous for industrial purposes as it requires fewer numbers of experimental trials for prediction and quantification of combined interactions between the variables [36] and hence eases the process of optimization. The 3D plots for response surface enables visualization of parameter interaction and it was often applied to satisfactory optimization of microbial enzyme production [37]. In the vision of wider application of enzymes, its economical production becomes a primary concern. RSM mediated optimization of amylase production from *Bacillus* sp. has previously been reported by many researchers. This method was previously used to evaluate the effect of pH, temperature and inoculum size on production of amylase from *Bacillus* sp by applying a full factorial central composite design [38]. In another report on optimization of α-amylase production by *Bacillus* sp using RSM, combined interaction of starch, glycerin, peptone and yeast extract on the production of enzymes was evaluated [39]. Optimized level of variables for the maximum α-amylase yield from *Bacillus subtilis* 168 were starch 2.55 g/l, yeast extract 8.4 g/l, sodium chloride 8.1% and 48 h of incubation [40]. In the present study, we employed RSM to examine the combined interaction of different variables viz, starch, yeast extract, pH, NaCl and temperature. This technique was found to be efficient for the optimization of process parameters for amylase production. After optimization, production media for amylase from marine *Bacillus* sp. VITRKHB was found to be economical and an enhancement in enzyme activity was achieved [41].

Enzyme production was carried out by submerged fermentation and was partially purified by Ammonium sulphate precipitation, dialysis and DEAE Ion Exchange chromatography. Enzyme activity and total protein were measured, specific activity and purification fold was calculated (Table 3) after each step of purification.

The results indicated a specific activity of 0.878 U/mg after ammonium sulfate precipitation with a purification fold of 1.18. After dialysis the activity was found to be 0.884 U/mg with a purification fold of 1.19. The final purification step presented 1.45-fold enzyme purification with a specific activity of 1.086 (Figure 6). Effectiveness of each purification step was indicated in these results. A good yield of 56.03% was seen after Ion Exchange chromatography. The fifth peak (Fig 6) shows the highest protein concentration (0.73 mg/ml). A previous report [23] showed 37% recovery using DEAE cellulose and Sephadex G-200 columns with a purification fold of 2.74 for purification of amylase from marine bacterium *Halobacterium salinarum* MMD047.

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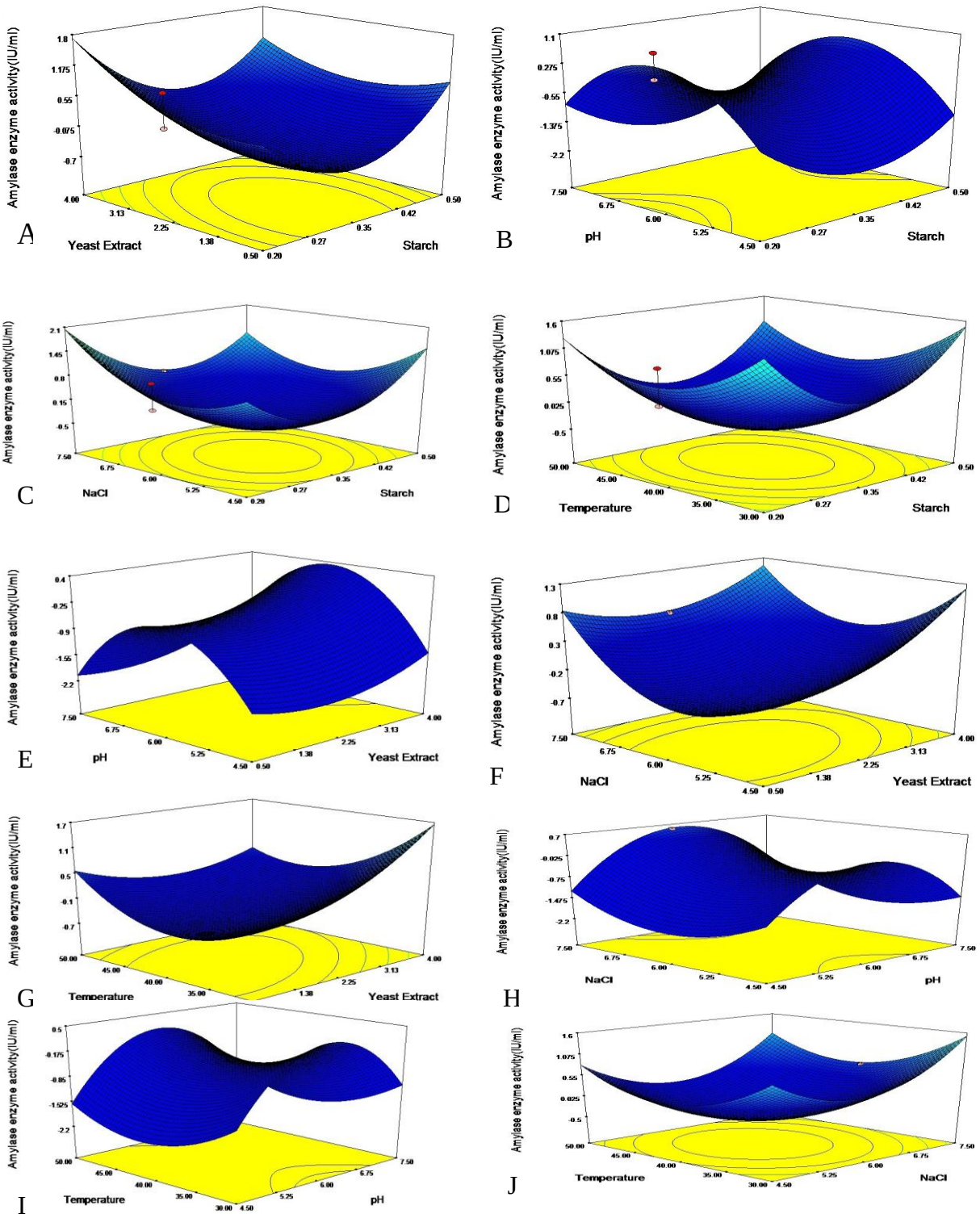

Fig 5: Effects of (A) Yeast Extract and starch, (B) pH and starch, (C)NaCl and starch, (D)Temperature and starch, (E) pH and yeast extract, (F) NaCl and yeast extract, (G)Temperature and yeast extract, (H) NaCl and pH, (I) Temperature and pH, (J) Temperature and NaCl

Purification of Enzyme

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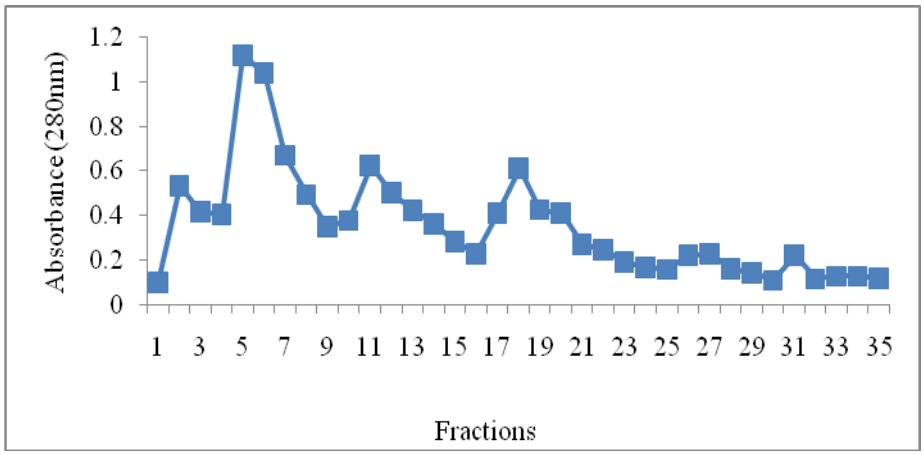

Fig 6: Purification of amylase using DEAE-Cellulose column

Table 3: Purification of amylase from *Bacillus* sp.VITRKHB

Purification steps	Total protein (mg/ml)	Enzyme Activity (U/ml)	Specific Activity U/mg	Fold Purification	Yield (%)
Crude Extract	4.30	3.20	0.744	1	100
Ammonium precipitation	3.30	2.90	0.878	1.18	90.6
Dialysis	2.25	1.99	0.884	1.19	62.18
Ion Exchange	0.73	0.793	1.086	1.45	56.03

Brine shrimp hatchability test

The percentage of toxicity was checked at 12 h and 24 h of exposure to the isolated marine eubacterial amylase. No significant change in hatchability of *Artemia* embryos up to 1000 µg was found. It is usually highly sensitive to toxins at the early developmental stages. Hence, marine *Bacillus* sp. VITRKHB amylase extract didn't show any toxicity. And hence it can be believed as safe for use as food additives in food industry, brewing and pharmaceutical industry and in distillation.

CONCLUSION

This study reports the economical production and partial purification of amylase from marine eubacteria *Bacillus* sp VITRKHB. This strain showed maximum enzyme activity in the presence of starch as carbon source and yeast extract as a nitrogen source. Response surface methodology was applied to evaluate combined interactive effect of different variables, i.e., Starch (0.21%), yeast extract (0.65%), pH (6.13), NaCl (4.63%) and temperature (45°C) on amylase production after 24h of incubation. Results generated by RSM allowed rapid screening of large experimental process for determining the optimum range of parameters. The amylase produced was purified to 1.45 fold and its specific activity was found to be 1.086 U/mg. Moreover, the isolated enzyme did not show a toxic effect. This shows that this enzyme can retain its activity at a high salt concentration and temperature and is non-toxic. Hence, it can be

efficiently used for various applications in food industry, brewing and pharmaceutical industry and in distillation. The optimized media and physicochemical parameters were suitable for a good yield of enzyme with less consumption of time, material and effort.

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