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Enhanced alpha-amylase production using *Streptomyces gancidicus* ASD by process optimization

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The present study was focused on purification and optimization of amylase from marine actinomycetes. Among 101 actinomycetes isolates from Andaman & Nicobar islands, *Streptomyces sp gancidicus_ASD* was isolated and further studied. The enzyme activity was studied at various physical parameters like temperature, pH, carbon source, Nitrogen source, metal ions, NaCl concentration etc. by maintaining all the factors with 100 ml of crude extract. Also, media optimization with response surface methodology was used to ameliorate the bioprocess economics. A central composite design was conducted to optimize the four selected factors. Statistical analyses of the data of model fitting were done by using Design expert 10.0 (stat-Ease). Results show a maximum predicted amylase yield of 11460.34 IU/ml when using 1.05% sucrose, 0.608% beef extract, 7.1 pH and 40.35 °C temperature. The predicted value is approximately 1.24-fold much higher than the original production (9248 IU/mL) determined by the conventional one-factor-at-a-time optimization method which can be applied in bioprocess for increased amylase yield.

[Keywords: Actinomycetes; Andaman & Nicobar Islands; Purification; optimization; Enzyme activity; RSM.]

Introduction

Amylases are among the most important industrial enzymes with great significance in biotechnological studies. α-amylase (endo1,4-α-D-glucanglucohydrolase, EC 3.2.1.1), an extracellular enzyme, acts on starch and degrades it into disaccharide and tri-saccharide. This amylase, derived from microorganisms, has a major advantage as it is economical to produce in bulk amount and easy to manipulate to obtain enzymes of desired characteristics[1]. Marine environment is one of the

itself as an unrevealed platform for research. The microbial amylases could be potentially useful in various pharmaceutical and fine-chemical industries, with the event of new frontiers in biotechnology, clinical, medical and analytical chemistry, starch analytical chemistry and starch scarification[2]. Studies have shown that the aquatic actinomycetes can be an important source to new biological products such as industrial enzymes; amylase is produced too much by the aquatic Actinomycetes[3]. Amylases are also used in baking, brewing, textile, detergent, paper and distilling industry[4]. These uses have placed greater stress on increasing indigenous amylase production and search for more efficient processes.

There is increasing demand for enzymes with novel properties in various industries. About half of the marine heterotrophy listed in the Bergey's manual are characterized as being dietetic or starch hydrolyser's[5]. Each application of amylase requires unique properties with respect to specificity, stability, and temperature and pH dependence[6]. Screening of marine microorganisms with higher amylase activity discovery of novel biotechnological and industrial applications[7,8]. In the present study, we report the findings of novel alkaline amylase produced by newly isolated marine actinomycetes *Streptomyces sp gancidicus ASD_KT852565.*

Optimization of process variables following screening and isolation of potential amylase producer is a prerequisite to enhance production yields to suit the organism for industrial application. By optimization of process variables, one can find out the significant parameters that enhance the yields[9]. The best fermentation technique for optimization involves

submerged fermentation (SmF)[10]. The response surface method (RSM) is a statistical method that involves individual and interaction effects to account for curvature, to improve optimal process settings, to troubleshoot process problems and weak point[11] and to build models, evaluating the effects of factors for desirable response[12]. Hence, statistically-based experimental designs are preferred to evaluate the influence of medium components in fermentations for the production of industrially important enzymes, proteins[13]. Therefore, a combination of 'one-at-a-time' approach and RSM is well suited for the study of main and interaction effects of distinct factors in amylase production.

Materials and Methods

Marine soil samples were collected from different coastal areas of Andaman & Nicobar Islands (*latitude* 60° and 14° N and *longitude* 91° and 94° E), St. Mary's island (*latitude* 13° 20' 60" N; *longitude* 74° 40' 60" E)-Udupi, Thekkumbad (latitude11.9765° N, longitude 75.2909° E) Kannur-Kerala and Panambur coast (*latitude*: 12º 54' 2' N. *longitude*: 74º 49' 25' E)-Mangalore, were placed in sterile plastic container. One mL from each diluted sample was cultured in 250 mL Erlenmeyer flask containing production media such as 50 mL of sterile molten starch casein agar medium (g/L Starch-10; Casein-0.3; KNO_3-2; NaCl-2; $K2HPO_4$; $MgSO_4.7H20$.05; $CaCO_3$-.02;$FeSO_4.7H_2O$-0.01; Agar-20), supplemented with antibiotics cyclohexamide (20 μg/mL) and nalidixic acid (100 μg/mL) to prevent fungal and bacterial growth respectively, poured into sterile petri-plates and incubated at 28 ^oC for 14 days. After 14 days, colonies of unicellular bacteria were counted on agar plates.

The agar plates were investigated in terms of colony appearance, colony colour, and conidia type (spore configuration) after incubation. The screening of the actinomycetes strains for amylase production was done by inoculating them on starch plate[14]. The organisms were spot inoculated on to the starch agar media and incubated at 28 °C for 72 hrs. Thereafter gram's iodine stain was spread on the plate and left for 5 min. The organism which secretes amylase produced zone of clearance or decolourization against the blue colour background. The selected isolates were further evaluated using biochemical tests and Gram staining and molecular characterization by 16s RNA analysis[15]. Extracellular secreted enzyme was recovered in 100 ml of 100 mMTris buffer (pH 10). By adding 100 ml of the buffer to the flask containing fermented media, the flask was shaken (150 rpm) for one hour. The suspension was filtered through whatmann filter paper No. 1 at 4 ^oC. The filtrate was transferred in centrifuge tubes and spun at 5000 rpm for five minutes at 4 ^oC. The supernatant was collected in a chilled beaker and treated as crude enzyme.

The crude enzyme was purified by ammonium sulphate precipitation upto 80% saturation and the precipitate was re-suspended in 10 ml of 100 mMTris buffer, pH 10, which was dialysed in the same buffer for 24 hour, with changes in buffer made every 2 hour. The dialysed sample was treated as purified extracellular alkaline amylase and assayed for the calculation of enzyme activity. SDS-PAGE was carried out in 12.5% resolving gel and 4.5% stacking gel for determination of molecular mass as per the method of Laemmli 1970. Protein bands were detected by de-staining the gel in a methanol-acetic acid-water solution (4:1:5) after the staining process with 1% Coomassie brilliant Blue R-250 and Zymogram was carried out to confirm amylase.

One of the major influencing factors in optimization is the temperature maintained for the organisms' growth, enzyme activity and its protein content. In the present study 50 ml of the crude enzyme was incubated at varying temperatures ranging from 15, 20, 28, 37, 40, 45 to 50 °C. pH (biocatalyst) is the most important factor which markedly influences the enzyme activity. In this study, pH varied from 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 to 9.0 under maintained temperature. On the other hand, culture broth was distributed into various flasks and 1% of each carbon source, viz., sucrose, fructose, dextrose, lactose, glucose, galactose, mannose, trehalose, melibose, and xylose was then added before inoculating the strain. Also, culture broth was used for studying the effect of various nitrogen compounds viz. peptone, yeast extract, ammonium sulphate, beef extract, sodium acetate, meat extract, urea, nutrient broth, potassium nitrate, and sodium citrate. The broth was distributed into various flasks and 1% of each nitrogen source was then added before strain inoculation. Cultures were incubated at already standardized parameters.

Even though amino acids are considered under trace minerals, they still enhance the yield of amylase and this was tested by varying the amino acids like L-glutamine, L-asparagine, L-tyrosine, L-lysine,

L-histidine, L-cysteine, L-arginine, L-therionine and L-tryptophan in the culture by maintaining other optimized parameters. The volume of medium decides the proper aeration to the culture which is reflected in the growth of the enzyme. The effect of medium volume was studied at different volumes in 250 ml flask, like 20 ml, 25 ml, 30 ml, 35 ml, 40 ml, 45 ml, 50 ml, 55 ml, 60 ml, and 70 ml under above optimized conditions. The incubation time of the culture decides the maturity of the cell for active enzyme production. Production of amylase is optimized by varying the incubation holding time from 12 hr, 24 hr, 36 hr, 48 hr, 60 hr, 72 hr, 84 hr, 96 hr, 108 hr and 120 hr. The utilization of NaCl determines the salinity level of the organism which also determines the nature of the organism (terrestrial or marine). Thus, the various concentrations of NaCl like 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 were used to find the optimizing level.

The trace elements which include metal ions also impact the production of amylase, which was studied by varying the metal ions like $CuSO_4$, $MgSO_4$, KNO_3 and $CaCO_3$. In the enzyme action, metallic cofactors are important because their presence or absence regulates enzyme activity. The presence of specific metallic ions along with essential nutrient source can inhibit or enhance amylase activity. Addition of substrate and its concentration plays a major role in the production of amylase. The effect of starch concentration was studied by varying its concentration such as 10 ml, 20 ml, 30 ml, 40 ml, 50 ml, 60 ml, 70 ml, 80 ml, and 90 ml per litre where all other parameters where under optimized state.

RSM is a collection of statistical and mathematical methods that are useful for modelling and analysing engineering problems. In this technique, the main objective is to optimize the response surface that is influenced by various process parameters[16,17,18]. RSM also quantifies the relationship between the controllable input parameters and the obtained response surfaces. The primary optimization stage study was done with various cheap carbon and nitrogen sources selected for one factor-at-a-time method (OFAT) study using *Streptomyces sp gancidicus_ASD* KT852565 for amylase production. To test the effect of carbon sources, each carbon source (sucrose, fructose, dextrose, lactose, glucose, galactose, mannose, trehalose, melibose, xylose purchased from Himedia, Mumbai) was added at 1% (w/v), by replacing starch and the influence of nitrogen sources by replacing casein with each nitrogen source (peptone, yeast extract, ammonium sulphate, beef extract, sodium acetate, meat extract, urea, nutrient broth, potassium nitrate, sodium citrate from Himedia, Mumbai) at 0.3% level. Central composite design (CCD):RSM was carried out using CCD design, optimized for further process to identify the interactions between the significant factors obtained from OFAT. The four variables chosen in this experiment were (C source) Sucrose, (N source) Beef extract, and pH and temperature with five coded levels ($-\alpha$,-1, 0, +1, $+\alpha$) were used for their combined influence on amylase production. Thirty experimental trials were carried out with 16 factorial points, 8 axial points with $\alpha=2$ and 6 replication of central points.

Results and Discussion

The selective isolation process resulted in isolation of 489 actinomycetes strains from 101 marine samples. Among 489 actinomycetes colonies isolated, 104 isolates were morphologically distinct; and among 104 isolates only 56 isolates exhibited the amylolytic activity. Hence, this strain was taken for further character analysis. The growth characteristics, presence of mycelium and soluble pigments were observed. Gram staining reveals that the active isolate was gram positive and chemical characterization showed it to be *Streptomyces*. Further, 16s RNA revealed it to be *Streptomyces sp gancidicus_ASD* with accession no. KT852565 by NCBI (Fig. 1).

The amylase activity and protein content was checked at various levels of purification such as crude, after ammonium sulphate saturation and finally after dialysis and the results are shown in Figure 2. Molecular weight of the purified enzyme was determined by SDS PAGE wherein wells were loaded with purified enzyme, and as can be seen from the figure below, a single band was observed slightly parallel to the band of the marker (BSA 44 KDa), thus the enzyme of interest is also with the molecular weight of 44 KDa (approx.). Hence, the enzyme was partially purified and the molecular weight of Streptomyces spp., PDS1 was found to be 44 kDA using SDS-PAGE[19]. Thus, the above molecular weight result is similar to that of the current finding with that of the *S. gancidicus_ASD*. Also, the enzyme under study was confirmed to be amylase by the zone formation due to the lysis of starch in Zymogram process shown in Figure 3.

On optimization, the enzyme activity of amylase was found to be gradually increasing with increase in temperature and the maximum was maintained at 40 °C (Fig. 4). Thus, it reveals the organism to be mesophilic. Vasantha raj and Hemashenpagam[20] have also evaluated the influence the temperature on amylse production. In contrast to the present study Syed et al [21] found the optimum temperature to be 45 °C for amylase production by *Streptomyces gulbargensis*. Secondly, maintaining the temperature, the study on pH (Fig. 5) revealed that increase in the enzyme and protein content was obtained in the neutral pH of 7.0 and gradually the production decreased with increase in pH. If the pH was

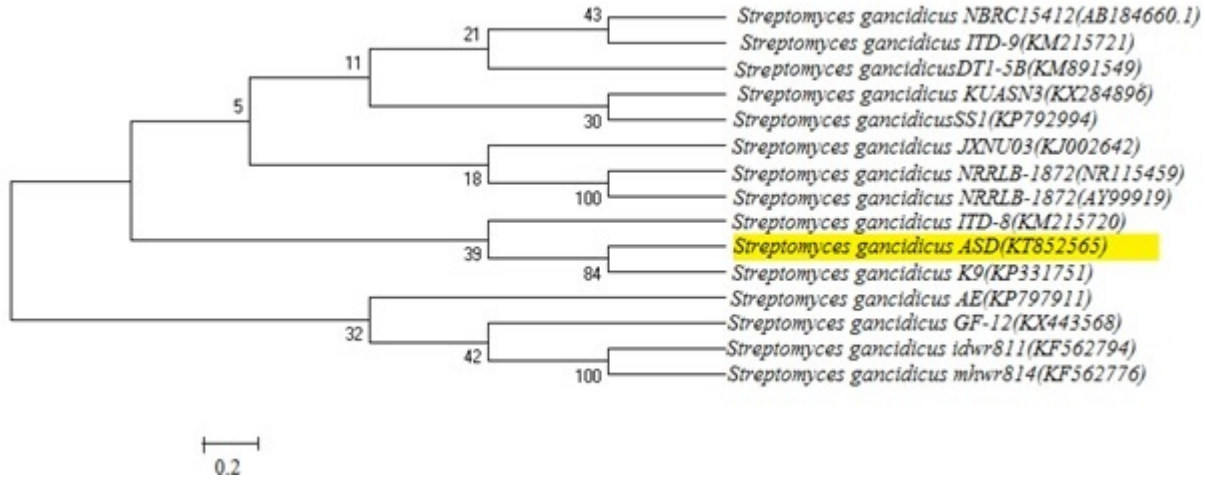

Fig. 1 — Phylogentic tree of *S.gancidicus ASD*

Purification step	volume (ml)	Total activity IU/ml/min	Total protein mg	specific activity (U/mg)	Purification fold (%)	Recovery (%)
Crude	80	9248	720	12.844	1	100
Ammonium sulphate	30	2868	60	47.7	3.713	29.122
Dialysis	10	516	2.5	206.4	4.327	17.991

Fig. 2 — Purification of amylase

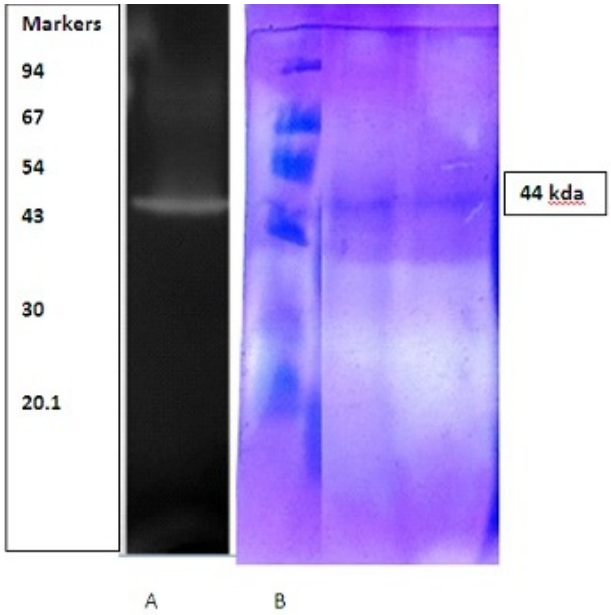

Fig.3 — A: Zymogram; B: SDS-PAGE of Amylase of interest.

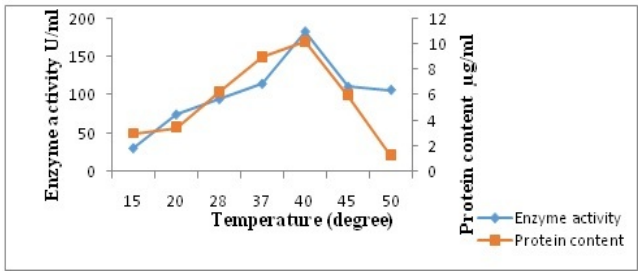

Fig. 4 — Effect of temperature on Enzyme activity and Protein content

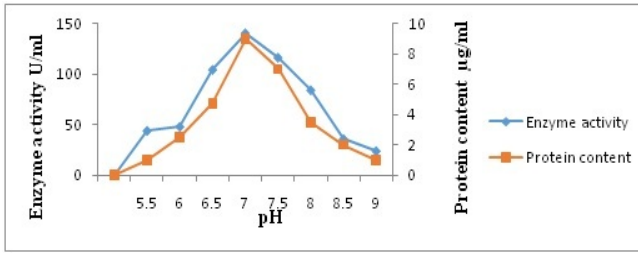

Fig. 5 — Effect of pH on Enzyme activity and Protein content

increased or decreased the activity decreased. Different organisms have different optima pH and increased or decreased pH will result in poor microbial growth[22]. With pH 7.0, optimum enzyme production resulted by *Streptomyces sp*. SLBA-08 in recent studies[23].

Investigation with carbon source, one among the essential nutrients when added at the concentration of 1% the amylase growth was maximum with sucrose followed by lactose and fructose (Fig. 6). The sucrose usage by *Streptomyces sp gancidicus_ASD* indicates simple sugar as its carbon source. One of the other major nutrients, nitrogen source was added in 1% concentration and the optimized was obtained with Beef extract followed by nutrient broth, and the third position was shared by both urea and peptone (Fig. 7). The inhibitory effect of inorganic nitrogen was well demonstrated by Akcan[24].

Maintaining the optimized parameters, the influence of amino acids was studied revealing that the consumption of L-asparagine was the highest followed by L-alanine and lowest consumption was found with L-tyrosine (Fig. 8). The space for the culture is determined by the volume of the medium in the flask. In the present study, when the volume of the medium was at 50 ml per 250 ml flask as in Figure 9 the enzyme production and activity was found to be maximum.

The age of the culture determines the maturity of the organism in producing the enzyme. In this case, Figure 10 showed that 96 hour old fermented culture showed good growth after which increase in incubation led to the destruction in the activity and also protein content. Results of Dhanya[25] are in coincidence with that of the soil actinomycetes. Enzyme production is related to the growth of the organism. Microorganism growth would have reached its optimum (due to insufficient nutrients) that indirectly stimulates the growth of secondary metabolites with decreased enzyme production of interest[26]. To study the salinity of the strain, the NaCl concentration was varied and the yield increased when the NaCl concentration was maintained to 1.5 % (Fig. 11). Gradual increase in NaCl concentration resulted in decrease in the yield.

Metal ions acts as one of the trace elements which also help in enhancing the growth of the organism

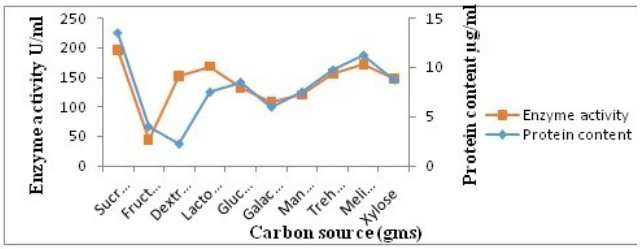

Fig. 6 — Effect of Carbon source on Enzyme activity and Protein content

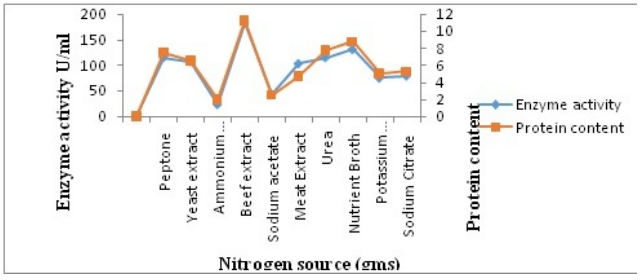

Fig. 7 — Effect of Nitrogen source on Enzyme activity and protein content

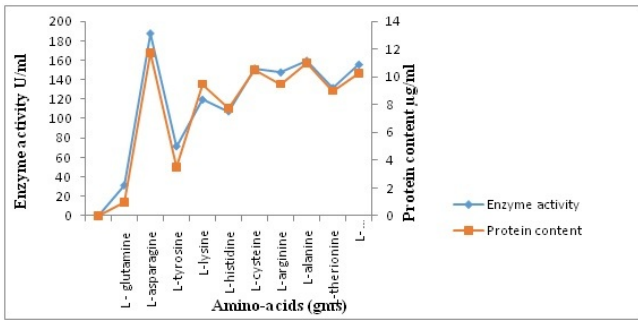

Fig. 8 — Effect of Amino-acids on Enzyme activity and Protein content

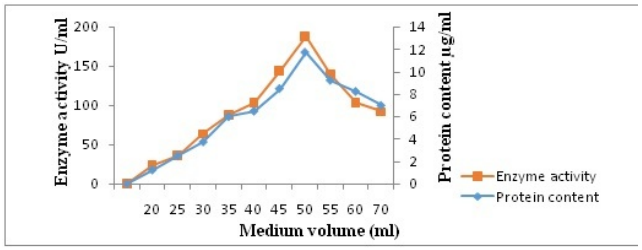

Fig. 9 — Effect of Medium volume on Enzyme activity and Protein content

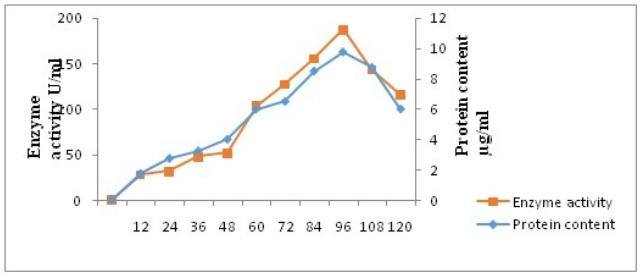

Fig. 10 — Effect of Incubation time on Enzyme activity and Protein content

among the five metal ions used for investigation. Figure 12 depict $CuSO_4$ to be the highest amylase enhancer while $CaCO_3$ was found to be the least enhancer of amylase by *Streptomyces sp gancidicus_ASD*. The amylase production by the organism is directly proportional to substrate utilization in the medium. In the present study starch was used as the substrate and the concentration of the substrate was found to be 50 ml/l as shown in Figure 13 above, which shows that there was a gradual decrease in the enzyme activity with increase in the starch concentration. Similar results were observed from fungal isolates by Ominyi Matthias[27].

Marine sediments may harbor a great diversity of culturable Actinobacteria[28]. In this study, a large culturable biodiversity of actinobacteria was obtained. *Streptomyces, Rhodococcus*, and *Micromonospora* are readily cultured actinobacterial genera in marine sediments[29]; however, this observation relies on the influence of the culture methods used. In this study, isolates of Streptomyces was focused. Amoung various actinomycetes isolated, they are distinct strains due to their differences in morphological features.

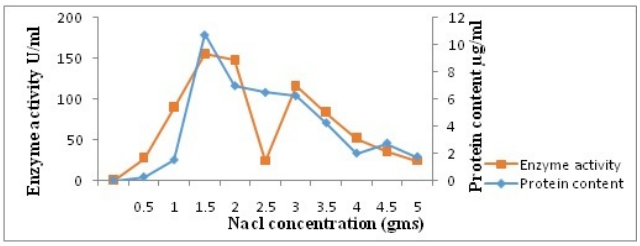

Fig. 11 — Effect of NaCl concentration on Enzyme activity and Protein content

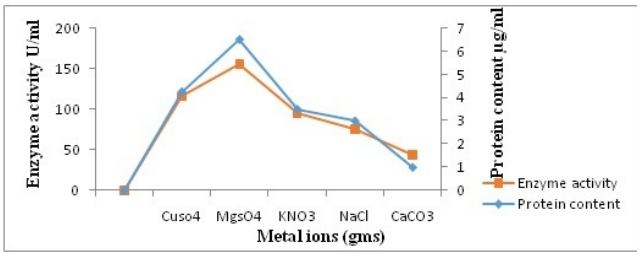

Fig. 12 — Effect of Metal ions on Enzyme activity and Protein Content

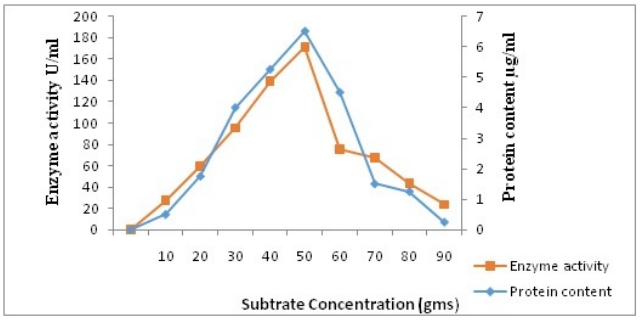

Fig. 13 — Effect of Substrate concentration on Enzyme activity and Protein Content

Primary optimization stage: One-factor-at-a-time method study

The maximum amylase activity was determined as 9248 IU/ml for sucrose and fructose as carbon and nitrogen source respectively with control medium (traditional actinomycetes medium-starch casein agar) observed to be 394 IU/mL. Similar result was reported for by marine actinomycetes Streptomyces showing 391.45 IU/ml as its amylase activity with starch-casein agar[30]. It was evident that sucrose was found to be the best carbon source for the enhanced amylase activity when compared to starch and other sugars tested (Fig. 14). When compared to the other nitrogen sources, beef extract enhanced the amylase activity given in (Fig. 15). Similar result was obtained by Shaktimay[31]. Ours is the first study on enhanced amylase production using sucrose and beef extract as simple source in modified actinomycetes media. Hence sucrose and beef extract were selected as the potential carbon and nitrogen source, respectively, for production of amylase from *Streptomyces sp gancidicus*_ASD.

Experimental design of RSM: Central composite design (CCD)

Based on the result of OFAT approach, optimum levels of significant factors and the effect of their

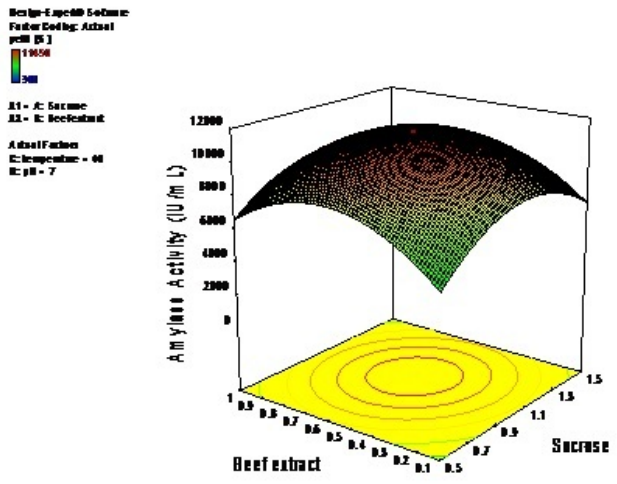

Fig.14 — 3D surface showing the effects between sucrose and Beef extract

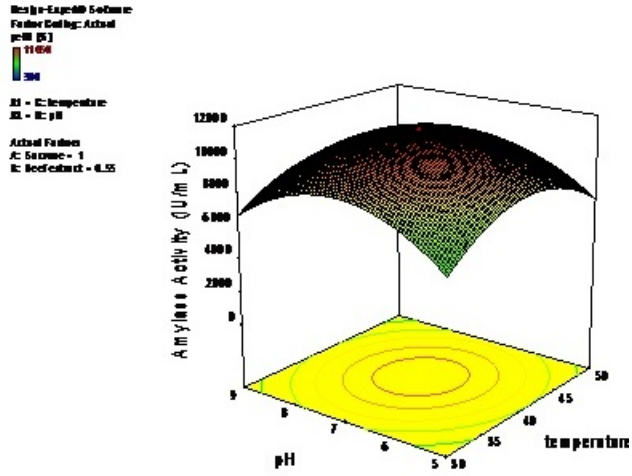

Fig.15 — 3D surface showing the effects between pH and Temperature

interactions on amylase production were determined by CCD experiments. The variables sucrose and beef extract were selected based on the result of the OFAT method; further pH and temperature were added for optimization by RSM. The experimental design was carried out to determine the parameter ranges together with coded and actual values of the four independent variables for amylase production. The maximum experimental value for amylase production was 11450 IU/mL based on RSM. The regression analysis data were fitted to a quadratic model and the second order regression equation was obtained. Full actual model on amylase production is shown in Eq. (3)

$$Y = +11450 + 441.67 * A + 316.67 * B + 150.00 * C + 58.33 * D - 237.50 * AB + 100.00 * AC + 125.00 * AD - 650.00 * BC + 75.00 * BD - 212.50 * CD - 2352.08 * A2 - 2614.58 * B2 - 2252.08 * C2 - 2614.58 * D2 \quad \ldots (3)$$

Where Y is enzyme (amylase) activity IU/mL, A is sucrose (g/L), B is beef extract (g/L), C is temperature (ºC), and D is pH.

Validation of the optimum condition

To verify the optimization results and to determine the accuracy of the experiment run by RSM (activity of 11450.34 IU/ml), a laboratory test was conducted in duplicate with the optimized media containing 1.054% sucrose, 0.573% beef extract, 6.9 pH and 40.4 ºC. On experimentation, the observed response of amylase yield from the *Streptomyces sp gancidicus_ ASD* KT852565 was 11460.00 IU/mL, which strongly proves the suitability of the developed model.

Conclusion

Streptomyces is one the most widely present actinomycetes, but the study of actinomycetes from marine soil on amylase is quite rare. The present study revealed that the *Streptomyces sp gancidicus_ASD* KT852565, a newly isolated species, is in hand with teressstial organism in chemical characterization and also can withstand salinity compared to terrestrial organisms. Being optimized by different parameters, it is ready to be used in the industrial application of amylase production and also in various starch hydrolysis, detergents and paper industries. The results further lead to new fermentation parameters and easy recovery of the amylase in short time. All the ingredients utilized by the *S.guancidicus_ASD* also showed to be easily available and simple nutrients which can be readily degraded. Even though the organism was collected from marine soil sample, the results prove it to be with anonym's character, like it is mesophilic by its temperature where as many marine organisms are thermophilic; it has neutral pH while others are alkaline; the last with just 1.5 % NaCl concentration with normal salinity compared to other highly saline marine organisms; and finally, validation of the enzyme showed the enzyme activity to be distinct from others. All these different characteristics is make *Streptomyces sp gancidicus_ASD* to be unique.

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