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Full Length Research Paper

Isolation, identification and characterization of multiple enzyme producing actinobacteria from sediment samples of Kodiyakarai coast, the Bay of Bengal

P. Manivasagan*, S. Gnanam, K. Sivakumar, T. Thangaradjou, S. Vijayalakshmi and T. Balasubramanian

CAS in Marine Biology, Annamalai University, India.

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A total of twenty nine strains were isolated from the Kodiyakarai coastal sediments (Bay of Bengal) and ten of them exhibited multiple enzyme activity. Of these, the best (GK-22) was selected based on zone formation (amylase, cellulase and protease) and its growth conditions were standardized for better production of multiple enzymes. Present study on production of multiple enzymes (amylase, cellulase and protease) by GK-22 showed higher enzyme levels at the end of the stationary phase after incubation for 72 h at pH 7.0. Maximum activity of amylase, cellulase and protease (84, 88 and 89 IU/ml, respectively) was obtained at pH 7.0, temperature 45°C, sodium chloride concentration 2%, carbon compound sucrose, nitrogen compound beef extract, amino acid L-asparagine for amylase and cellulase and L-tyrosine for protease. The multiple enzymes were purified by precipitation with ammonium sulphate and ion exchange chromatography and the SDS-PAGE showed a single band for the purified enzyme, with an apparent molecular weight of 80 (amylase), 66 (cellulase) and 97 KDa (protease). The strain, GK-22 which showed higher multiple enzyme activity was tentatively identified as *Streptomyces alboniger*. These findings suggest that the strain can effectively be used in large scale production of multiple enzymes for commercial purposes, after testing and ascertaining the strain's capability in large scale fermentations.

Key words: Amylase, cellulase, protease, actinobacteria, Kodiyakarai, *Streptomycetes alboniger.*

INTRODUCTION

Programmes to select new microorganisms for enzyme production are increasing around the world. Actinomycetes are one of the most investigated groups since they constitute a potential source of biotechnologically interesting substances (Lealem and Gashe, 1994).

Starch is an abundant carbon source in nature, and -amylase (1, 4- -D-glucano hydrolase: EC 3.2.1.1), which hydrolyzes -1, 4-glucosidic linkages in starch- related molecules, is one of several enzymes involved in starch degradation (Paquet et al., 1991). Starch-degrading amylolytic enzymes are of great significance in biotechnological applications ranging from food fermentation, textile to paper industries (Lin et al., 1997; Pandey et al., 2000).

Complex cellulolytic enzymes, which convert cellulase to glucose, have been shown to be extracellular product of several microorganisms. Cellulase hydrolyzes -1, 4 glucosidic bonds in cellulase and its derivatives while it acts on the non-substituted cellulase leading to the formation of cellobiose, which can be further hydrolyzed by -glycosidase. The enzyme cellulase is also useful economically as it has the ability to convert the plant biomass into fuels and basic chemicals. Cellulase decomposing bacteria and fungi are widely distributed in the marine environment and they play an important role in mineralizing organic matter and also influencing the productivity of the sea (Kadota, 1956). Actinobacteria

*Corresponding author. E-mail: manimaribtech@gmail.com.

have also been found to be potential source for the degradation of cellulase. When compared to terrestrial streptomycetes, many of the marine isolates have been found to be active cellulase decomposers (Chandramohan et al., 1972).

Proteases catalyse the hydrolysis of proteins to peptides and amino acids. These enzymes are important from an industrial perspective and cater for the requirement of nearly 60% of the world enzyme market (Kalisz, 1988). Of these, proteases produced by alkalophiles are of interest from a biotechnological angle and are investigated not only in scientific areas like protein chemistry and protein engineering, but also find application in detergent, food, pharmaceutical and tannery industries (Kumar and Takagi, 1999).

Hence, the present study has been focused on the isolation, identification and characterization of multiple enzyme producing actinobacteria from sediment samples of Kodiyakarai coast, Bay of Bengal in order to determine its potential of actinomycetes for industrial application.

MATERIALS AND METHODS

Isolation of actinobacteria

Strains were isolated from the marine sediment samples collected at a depth of 10 m at a distance of 18 km off Kodiyakarai coast (Lat. 10°06'N; Lon. 79°51'E) of the Bay of Bengal. For the isolation, starch casein agar was used with the addition of 20 mg/l of nystatin and cycloheximide (100 mg/l), respectively (Kathiresan et al., 2005) to minimize bacterial and fungal contaminations. The strains were sub-cultured onto starch casein agar slant (medium with 50% sea water), incubated at 28°C for 28 days, to achieve good sporulation and preserved in 20% glycerol at -80°C.

Screening for multiple enzymes

Amylase activity was assessed by growing the isolates on Nutrient Agar Medium with 1% soluble starch at 36 ± 1°C for five days. After incubation, the plates were flooded with 1% iodine in 2% KI. A clear zone developed around the colony was considered positive for amylase activity (Collinas and Lynne, 1980). For protease activity (Cruickshank et al., 1975), the strains were screened qualitatively in Gelatin agar medium. After inoculation, the plates were incubated at 36 ± 1°C for five days. The plates were flooded with 20% aqueous solution of salphosalycylic acid. Formation of clear zone was indicative for the positive protease activity. For cellulase activity, the strains were grown on the cellulase activity medium. After incubation as above, the plates were flooded with a mixture of 0.1 ml hydrochloric acid and 5 ml 1% iodine in 2% KI. The clear zones around the colony against reddish-brown background indicated positive activity for cellulase (Yeoh et al., 1985).

Production medium

All the experiments were carried out in 500 ml Erlenmeyer flasks containing amylase production broth, Carboxy methyl cellulase broth and gelatin broth. Sterile medium was inoculated with 2.0% of inoculum containing 3.1 - 4.7 x 10^4 CFU ml^{-1} incubated at 28°C and cultivated under agitation at 180 rpm for 5 days. Growth and enzyme activity were determined from the samples collected at 12 h intervals for which samples (5 ml) were taken from each of three replicate tubes. The cells were harvested by centrifugation at 10,000 rpm for 15 min, at 4°C. The mycelial mass was removed by vacuum filtration and dried in an oven at 80°C to measure the dry biomass weight and expressed as mg dry mass per 50 ml^{-1} of culture medium.

Optimization of culture conditions

The strain GK-22 was subjected to different culture conditions to derive the optimum conditions for amylase, protease and cellulase production. Growth and production were estimated at various temperatures (35, 40, 45, 50, 55 and 60°C), pH (4.0, 5.0, 6.0, 7.0, 8.0, and 9.0), concentrations of sodium chloride (0.05, 0.5, 1.0, 2.0, 3.0 and 4.0%), carbon sources (arabinose, xylose, inositol, fructose, rhamnose, sucrose and raffinose), nitrogen sources (peptone, yeast extract, beef extract, casein and potassium nitrate) and amino acids (L-glutamine, L- asparagine, L-tyrosine, L-lysine and L- histidine). All the experiments were carried out in shake flasks fermentation. All the experiments were carried out in triplicate and the average values have been reported. Standardization of culture condition was carried out one parameter at one time and the standardized values were used for subsequent experiments.

Temperature

Experiment was carried at various temperatures viz. 35, 40, 45, 50, 55 and 60°C to study their effect on enzyme production of GK-32. Experiments were carried out in triplicate and the average values were reported.

pH

Different pH concentrations viz. 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 of the broth were prepared using buffer solution. All the experiments were carried out in triplicate at optimum pH and average values were reported.

Sodium chloride

Various concentrations of sodium chloride (0.05, 0.5, 1.0, 2.0, 3.0 and 4.0%) were studied by changing the ratio of volume of sodium chloride solution to the broth and the flasks were incubated in triplicate and the average values were reported.

Carbon compounds

Culture broth was distributed into various flasks and 1% of each carbon source viz. arabinose, xylose, inositol, fructose, rhamnose, sucrose and raffinose, and was then added before inoculating of the strain and was incubated at the optimum pH, temperature and NaCl. The experiments were conducted in triplicate and the average values were reported.

Nitrogen compounds

Culture broth was used for studying the effect of various nitrogen compounds viz. peptone, yeast extract, beef extract, casein and potassium nitrate. The broth was distributed into various flasks and 1% of each nitrogen source was then added before strain inoculation. Cultures in triplicate were incubated at already standardized parameters.

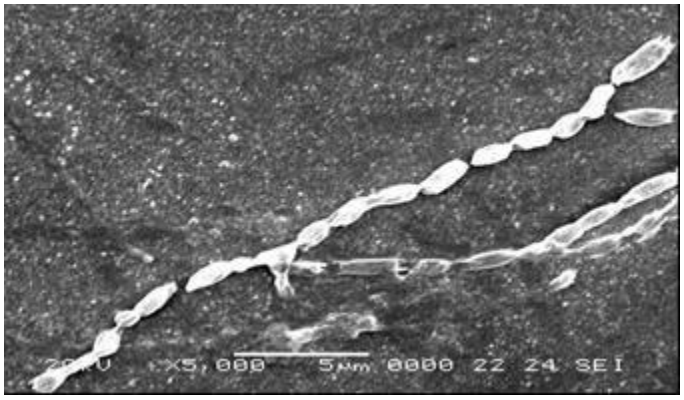

Figure 1. Spore chain morphology of strain GK-22.

Amino acids

Culture broth was used for studying the influence of amino acids such as L-glutamine, L- asparagine, L- tyrosine, L- lysine and L-histidine on enzyme production. The broth was distributed into various flasks and 0.8 ml of each amino acid was then added. Triplicate flasks were incubated, keeping all other conditions at their optimum level.

Purification of multiple enzymes

An extracted sample was precipitated with 80% ammonium sulphate saturation. The precipitate was dialysed against 20 mM potassium phosphate buffer for 12 h at 4°C. Further purification was carried out in ion exchange chromatography (DEAE-Cellulose). The dialysed protein was applied to a DEAE-Cellulose A-50 column (20 mm diameter × 60 mm long), pre-equilibrated with 20 mM potassium phosphate buffer (pH 7.0). After washing the column with 3 volume of equilibration buffer, bound proteins were eluted stepwise using phosphate buffers of increasing molarity and decreasing pH values at room temperature (approx. 25°C). The flow rate was adjusted to 24 ml h^{-1} and fractions (1 ml each) were collected. The fractions showing high enzyme activity were pooled and concentrated in lyophilizer.

Determination of protein

Protein concentration of the multiple enzymes in supernatant was determined by the method (Lowry et al., 1951), using bovine serum albumin as the standard.

Enzyme assay

Amylase activity was estimated by reducing sugar method (Miller, 1959) using 3, 5-dinitrosalicylic acid (DNS). The assay mixture contained 250 l of 50 mM Tris/HCl buffer (pH-7.5), 250 l of 1% soluble starch (substrate), and 500 l of appropriately diluted enzyme solution and the mixture was incubated at 50°C for 15 min. The reaction was stopped by adding 3 ml of DNS reagent and maintained in boiling water for 3 min and 1 ml of Rochelle salt solution was added finally. OD of the reaction mixture was measured at 540 nm. OD values were plotted in a standard graph prepared with different concentrations of D-glucose. One unit of enzymatic activity was defined as the amount of enzyme required to produce 1 M of glucose/min under the assay condition.

Cellulase was quantified by the method of Updegraff (1969). Cellulase activity against filter paper (FPase), carboxymethyl cellulase (CMCase) and -glucosidase were assayed using Ray et al., 1993 method. Reducing sugars released were determined by the dinitrosalicylic acid method (Miller, 1959). One unit of enzyme activity is defined as 1 mol glucose released/min/ml of culture supernatant.

500 l of 0.5% casein in Tris–HCl buffer with 100 l of enzyme solution was kept for 60 min at 370°C. Reaction was stopped by adding 500 l of 15% trichloroacetic acid solution with shaking. After 15 min, the mixture was centrifuged at 3,000 rpm for 5 min at 40°C. One ml of supernatant was added with one ml of 1 M NaoH and the absorbance was read at 440 nm. The enzyme activity was calculated from standard curve of L-tyrosine. One unit of enzyme activity is equivalent to 1 g of L-tyrosine min-1 ml-1 under the assay conditions.

Molecular weight determination in SDS-PAGE

The molecular weight of the multiple enzyme was determined by 15% Sodium dodecylsulfate polyacrylamide gel electrophoresis, (Laemmli, 1970) in LKB Bromma 2050 Midget electrophoresis units (Pharmacia Amersham Co). After electrophoresis, the gel was stained with Comassie Brilliant Blue R-250. Range moleculer markers (29–200 KDa) with five polypeptides were used as markers.

Taxonomic investigation

Genus level identification of the strain GK-22 was made using cell wall composition analysis and micromorphological studies (Lechevalier and Lechevalier, 1970). Characterization of the strain GK-22 was made, following the methods using standard yeast extract malt extract agar (ISP medium 2) (Okami, 1984). The species level identification of the strain was made using Nonomura keys and by using the Bergey's Manual of Determinative Bacteriology.

RESULTS AND DISCUSSION

Amylase, cellulase and protease are the industrially important enzymes having wider applications in pharmaceutical, leather, laundry, food, fermentation, textile, paper and waste processing industries. Industrial enzyme production would be effective only if the organism and the target enzyme are capable of tolerating different variables of the production processes. Primary screening for multiple enzyme producing actinomycetes was done in agar medium based on zone formation. A total of 29 strains were isolated from the Kodiyakarai coastal sediments and among them, ten strains exhibited multiple enzyme activity. Among them, the best (GK-22) was selected based on zone formation and the growth conditions for those strains were standardized for better production of multiple enzymes. The morphological (Figure 1), physiological and biochemical characteristics of the multiple enzyme producing strain GK-22 tested in the present study are given in Tables 1 and 2.

The strain, GK-22 exhibited multiple enzyme activity of (84 amylase, 88 cellulase and 89 protease IU/ml), at pH

Table 1. Comparison of morphological characteristics of strain GK-22 and *S. alboniger.*

Character studied	Strain GK-22	*Streptomyces alboniger*
Colour of aerial mycelium	White	White
Melanoid pigment	-	-
Reverse side pigment	-	-
Soluble pigment	+	+
Spore chain	Rectiflexibiles	Rectiflexibiles
Spore surface	Smooth	Smooth
Carbon source assimilation		
Arabinose	-	+
Xylose	±	±
Inositol	+	+
Mannitol	+	+
Fructose	±	±
Rhamnose	-	-
Sucrose	-	-
Raffinose	±	±

Table 2. Biochemical characteristics of the strain GK-22.

Biochemical property	GK-22
Utilization of sole amino acids	
L-glutamine	+
L- asparagine	+
L-tyrosine	+
L-lysine	+
L- histidine	+
Other characters	
Cellulase degradation	-
Hydrogen sulphide production	+
Melanin production	-
Gelatin liquefaction	-
Nitrate reduction	-
Starch hydrolysis	+
Milk coagulation	+
Growth in the presence of 0.1% phenol	+

7.0, sodium chloride 2%, and 45°C. Enzyme production was higher during the stationary phase of the growth of the organism (Figures 2 - 4), whereas maximum biomass occurred at 72 h.

Temperature and pH played an important role in cell growth as well as multiple enzyme production. Higher levels of enzyme production were recorded at 45°C (78 amylase, 68 cellulase and 75 protease IU/ml) (Figure 5), and relatively lower levels (15 amylase, 21 cellulase and 28 protease IU/ml) were recorded at 35°C. Regarding pH, maximum production (73 amylase, 89 cellulase and 72 protease IU/ml) was observed (Figure 6) at pH 7.0 and minimum level (10 amylase, 15 cellulase and 22 protease IU/ml) was observed at pH of 4.0. Present study has recorded 35°C as optimal, which is in agreement with the earlier findings of Stamford et al. (2001), Poornima et al. (2008), Vonothini et al. (2008), temperature optimum for amylase and protease was in the range of 25 and 50°C.

When different concentrations of sodium chloride were tested on the enzymatic activity of GK-22, good acidity was recorded at 2% sodium chloride concentration and there was no amylase production in the absence of

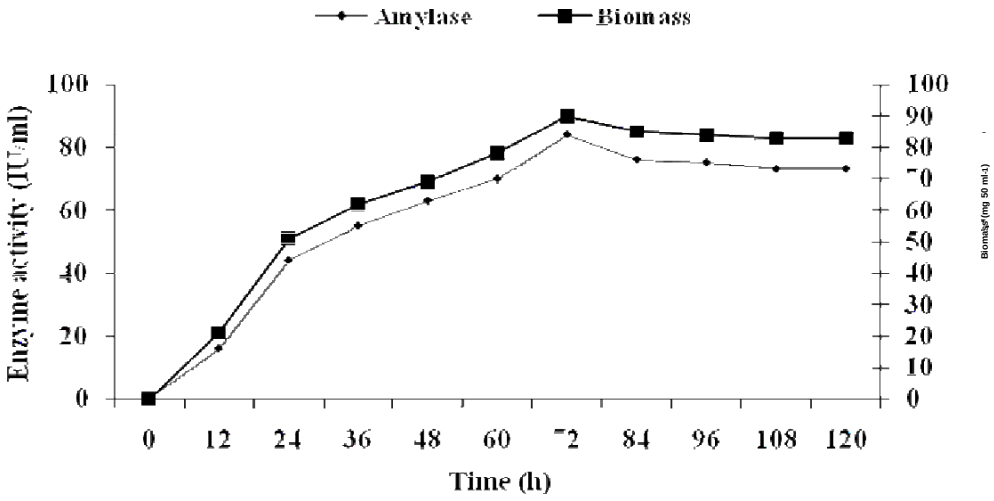

Figure 2. Biomass and amylase production.

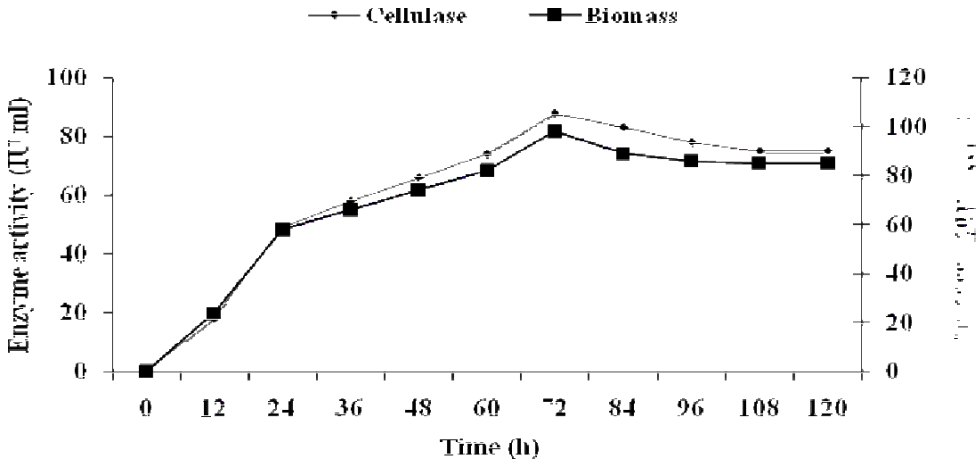

Figure 3. Biomass and cellulase production.

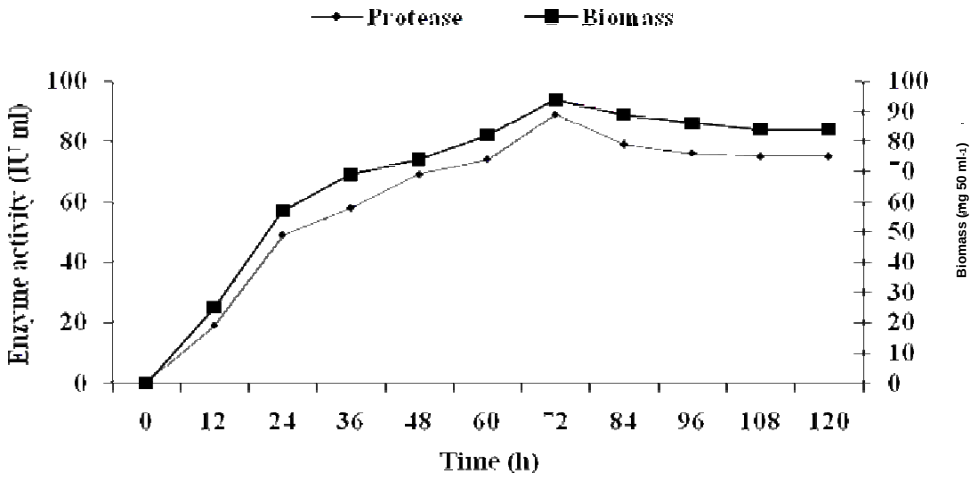

Figure 4. Biomass and protease production.

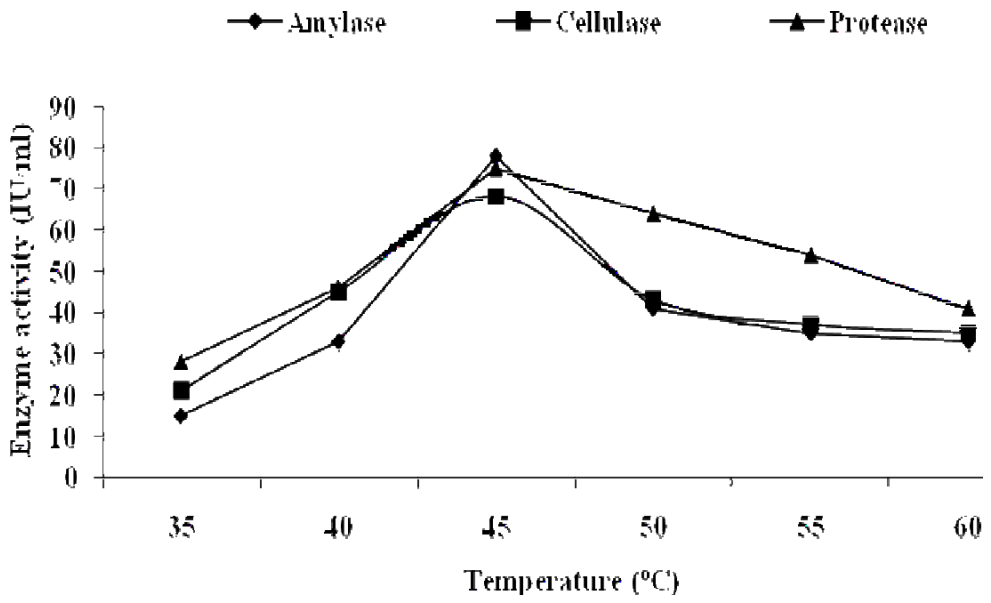

Figure 5. Effect of temperature on multiple enzyme activity.

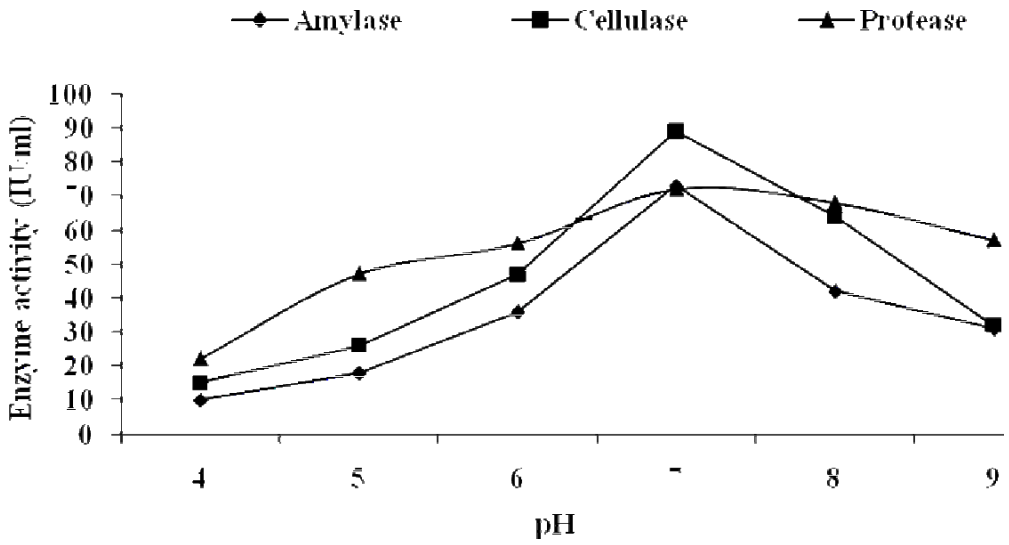

Figure 6. Effect of pH on multiple enzyme activity.

sodium chloride (Figure 7). Likewise, Kundu (2006) also observed the maximum amylase activity at 1 - 2% sodium chloride concentration by the actinomycete strain isolated from estuarine fishes.

Different carbon sources were used in the production medium for determining the highest yield of enzyme production. The highest activity was obtained when sucrose was used as the carbon source (Figure 8), while the activity was minimum with arabinose. Poornima et al. (2008) tested enzyme activity in various carbon sources and found the highest amylase activity in rhamnose.

Maintaining the physical factors and the carbon sources at optimized condition, nitrogen sources of varying nature were also tested. Of the different nitrogen sources, the test strain showed maximum activity in the presence of beef extract (Figure 9), but Kathiresan and Manivannan (2006) have observed maximum enzyme activity in the yeast extract and peptone as nitrogen sources in *Penicillium fellutanum*.

When different amino acids were tested, amylase and cellulase enzyme production was higher in L-asparagine and minimum in L-histidine (Figure 10). Protease enzyme

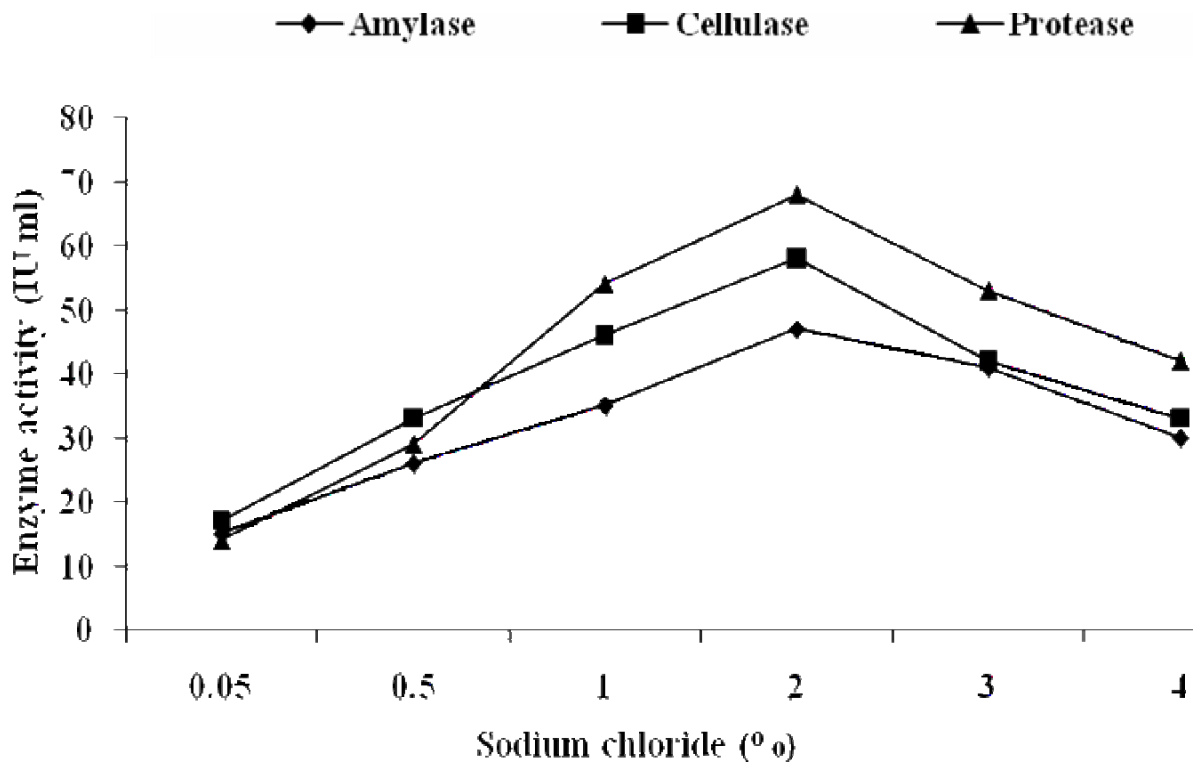

Figure 7. Effect of sodium chloride on multiple enzyme activity.

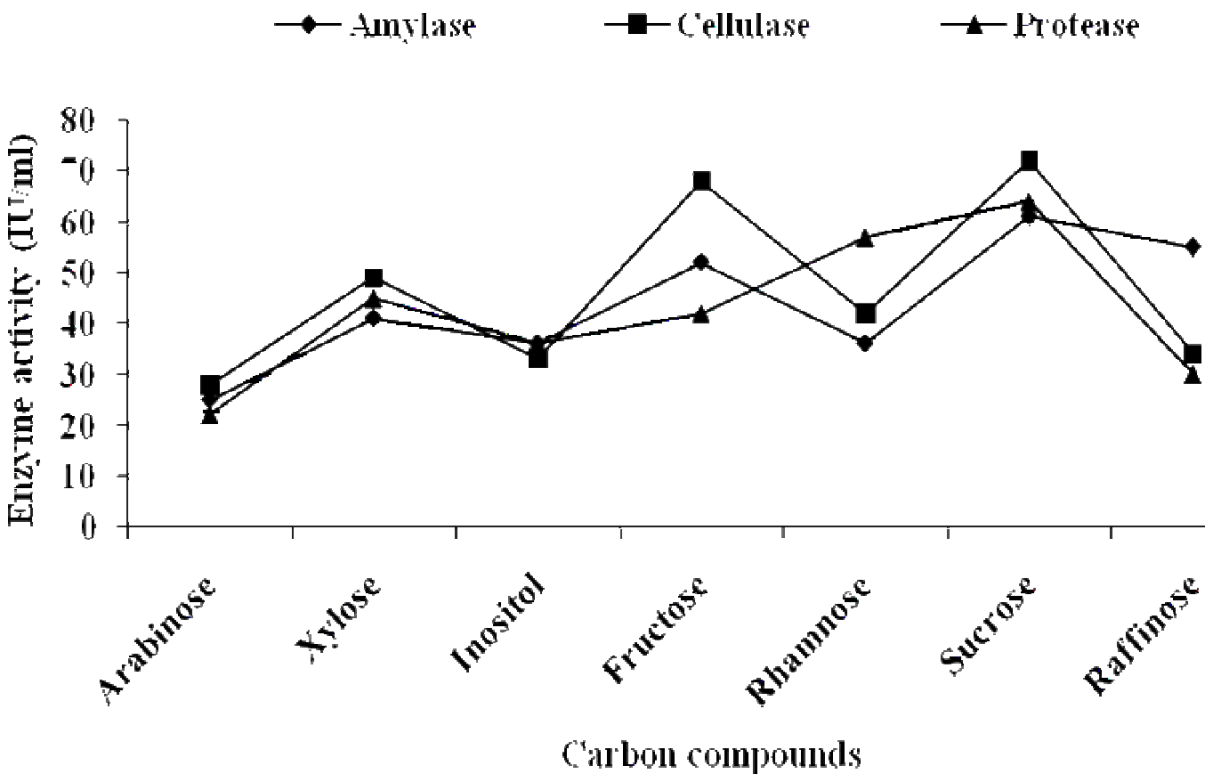

Figure 8. Effect of carbon sources on multiple enzyme activity.

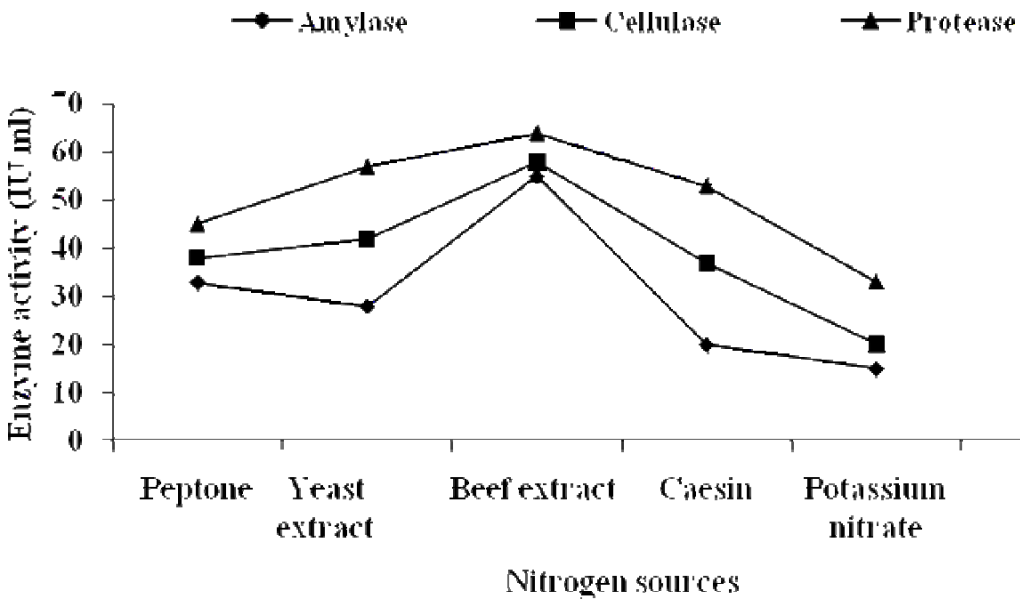

Figure 9. Effect of nitrogen sources on multiple enzyme activity.

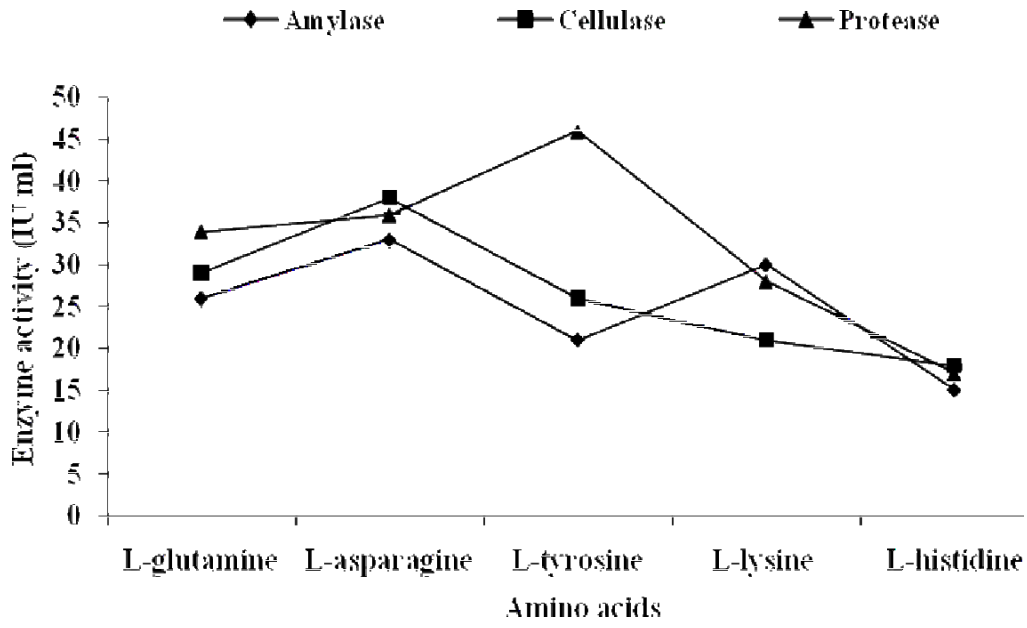

Figure 10. Effect of amino acid sources on multiple enzyme activity.

production was higher in L- tyrosine. Patel et al. (2005) and Kundu (2006) have also observed maximum enzyme activity in L-Phenylalanine in *Aspergillus oryzae,* Aspergillus *awamori and Streptomyces galilaeus.*

In the purification protocol, the enzymes were concentrated from the growth medium by ammonium sulphate precipitation method and were separated by using ion-exchange chromatography followed by hydrophobic interaction chromatography. The overall yield and activity are summarized in Tables 3 - 5. The remits indicates that the purification steps increased the purity of the enzymes at 23.79, 24.45 and 11.93 fold but reduced the enzyme recovery by ¼, that is, 26.43%, 26.88% and 27.44%. As the purification steps removes

Table 3. Summary of the purification steps of amylase from the culture supernatant of GK-22.

Purification step	Volume (ml)	Total activity (IU/ml)	Total protein (mg)	Specific activity (IU/mg)	Purification (fold)	Recovery (%)
Crude extract	100	4884.55	182.8	26.72	0.0	100
Ammonium sulphate precipitation (80% saturation) and dialysis	25	3191.61	43.2	73.88	2.76	65.34
DEAE-Cellulose chromatography	5	843.69	0.48	1757.70	23.79	26.43

Table 4. Summary of the purification steps of cellulase from the culture supernatant of GK-22.

Purification step	Volume (ml)	Total activity (IU/ml)	Total protein (mg)	Specific activity (IU/mg)	Purification (fold)	Recovery (%)
Crude extract	100	3737.42	161.2	23.18	0.0	100
Ammonium sulphate precipitation (80% saturation) and dialysis	25	1748.45	38.2	45.77	1.97	46.78
DEAE-Cellulose chromatography	5	469.95	0.42	1118.93	24.45	26.88

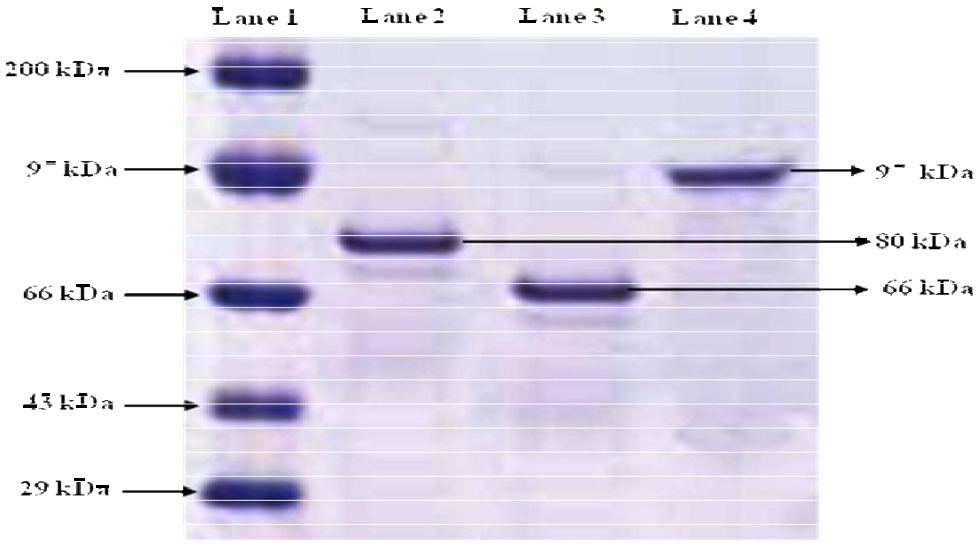

Figure 11. SDS- PAGE of the purified enzymes: Lane 1, molecular weight markers; lane 2, amylase enzyme; lane 3, cellulase enzyme; lane 4, protease enzyme.

the unwanted proteins from the crude extract, the protein content of the enzyme also reduced in all the steps.

Molecular weight of the multiple enzymes was determined by SDS-PAGE gel electrophoresis (Figure 11). Single protein band was observed when stained with Comassie blue, and it clearly indicated the purity of the protein. The molecular weight was 80, 66 and 97 KDa for amylase, cellulase and protease, respectively. Das et al. (2004) obtained purified protein with 42 kDa molecular weight in *Bacillus subtilis*. Lin et al. (1998) reported amylase with 42.8 kDa molecular mass from *Bacillus* sp. Thus, many investigators have reported different molecular masses of enzymes isolated from microorganisms.

Present investigation on the determination of optimal process parameters for the strain GK-22 for its better enzyme activity has yielded important results, indicating that the strain can be used for large scale industrial production of the enzymes. However, these parameters have to be tested in mass cultures in automated

incubators so as to confirm the optimum conditions for enzyme production for industrial application.

REFERENCES

Lealem F, Gashe BA (1994). Amylase production by a gram positive bacterium isolated from fermenting tef (*Eraglostis tef*). J. Appl. Bacteriol., 77: 348-352.

Paquet V, Croux C, Goma G, Soucaille P (1991). Purification and characterization of the extracellular alpha amylase from *Clostridium acetobutylicum* ATCC 824. Appl. Environ. Microbiol., 57:212-218.

Lin LL, Hsu WH, Chu WS (1997). A gene encoding for -amylase from thermophilic *Bacillus* sp., strain TS-23 and its expression in *Escherichia coli*. J. Appl. Microbiol., 82: 325-334.

Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D, Mohan R (2000). Advances in microbial amylases. Biotechnol. Appl. Biochem., 31: 135-152.

Kadota H (1956). A study on the marine aerobic cellulase- decomposing bacteria, Memories of the College of Agric, Kyoto Univ., 74: 1-178.

Chandramohan D, Ramu S, Natarajan R (1972). Cellulolytic activity of marine Streptomycetes. Curr. Sci., 41: 241-246.

Kalisz HM (1988). Microbial proteinases. Advances in Biochem Engineering Biotechnol., 36: 783-791.

Kumar CG, Takagi H (1999). Microbial alkaline proteases: From a bio-industrial viewpoint. Biotechnol. Adv., 17: 561-594.

Kathiresan K, Balagurunathan R, Masilamaiselvam M (2005). Fungicidal activity of marine actinomycetes against phyotopathogenic fungi. Indian J. Biotechnol., 4: 271-276.

Collinas CH, Lyne PM, (1980). Microbiological Methods, 4th ed., Butterworths, London. p. 89.

Cruickshank R, Duguid JP, Marmion BP, Swain RHA (1975). Medical Microbiology. Vol. 12th ed, Churchill Livingstone, London. p. 74.

Yeoh HH, Khew E, Lim G (1985). A simple method for screening celluloytic fungi. Mycol., 77: 161-162.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the folin-phenol reagent. J. Biol. Chem., 193: 265-275.

Updegraff DM (1969). Semi micro determination of cellulase biological materials. Analyt. Biochem., 32: 420-424.

Ray L, Pal A, Ghosh AK, Chattodhyay P (1993). Cellulase and -glucosidase from *Aspergillus niger* and saccharification of some cellulosic wastes. J. Microbiol. Biotechnol., 8: 85-94.

Miller GL (1959). Use of DNS reagent for the determination of reducing sugars. Anal. Chem., 31: 426-428.

Laemmlli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature (London) 227: 680-685.

Lechevalier MP, Lechevalier H (1970). Chemical composition as a criterion in the classification of aerobic actinomycetes. Int. J. Syst. Bacteriol. 20: 435-443.

Okami Y (1984). Marine microorganisms as a source of bioactive agents In. Current Perspective in Microbial ecology. Klug MJ, Reddy CA (eds) American society for microbiology, Washington DC. pp. 615-655.

Stamford TLM, Stamford NP, Coelho LCBB, Araujo JM (2001). Production and characterization of a thermostable -amylase from *Nocardiopsis* sp. Endophyte of yam bean. Biores. Technol., 76: 137-141.

Poornima R, Maloy KS, Sivakumar K, Pushpavalli V (2008). Optimization of -amylase production by Actinomycete strain AE-19 isolated from shrimp pond. Trends in Appl. Sci. Res., 3(1): 45-52.

Vonothini G, Murugan M, Sivakumar K, Sudha S (2008). Optimization of protease production by an actinomycete Strain, PS-18A isolated from an estuarine shrimp pond. Afr. J. Biotechnol., 7(18): 225-3230.

Kundu S (2006). Isolation and identification of actinomycetes from estuarine fishes and optimization of -amylase enzyme. M.Phil. Thesis, Annamalai University, India. p. 46.

Kathiresan K, Manivannan S (2006). -Amylase production by *Penicillium fellutanum* isolated from mangrove rhizosphere soil. Afr. J. Biotechnol., 5(10): 829-832.

Patel AK, Nampoothiri KM, Ramachandran S, Szakacs G, Pandey A (2005). Partial reification and characterization of -Amylase produced by *Aspergilus oryzae* using spent-brewing grains. Ind. J. Biotechnol., 4: 336-341.