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Improved Production and Purification of Pectinase from *Streptomyces* sp. GHBA10 isolated from Valapattanam mangrove habitat, Kerala, India

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Abstract

Pectinases are enzymes that catalyze the hydrolysis of pectin (polygalacturonic acid) to galacturonic acid residues. Actinomycetes are efficient degraders of plant debris as they produce extracellular enzymes like cellulase, xylanase and pectinase. The present investigation focuses on improvement of pectinase production through media optimization, followed by its purification. Submerged fermentation was carried out using a mangrove isolate of Streptomyces sp. GHBA10, identified by 16S rDNA sequencing. The highest yield of the enzyme was obtained from 0.3% (w/v) pectin and 0.1% (w/v) tryptone at an initial medium pH of 8.5, when incubated at 30°C and 150 rpm for 6 days with an inoculum size of 5% (v/v). The effect of surfactants such as sodium dodecyl sulphate, Triton X-100, Tween-20, Tween-80 and cetrimide on the enzyme production revealed that cetrimide significantly enhanced the enzyme secretion. The crude pectinase was purified by salt precipitation, dialysis and gel filtration chromatography. The specific activity of the purified pectinase was estimated to be 2610 U/mg of enzyme protein. Purification revealed 3.5-fold increase in specific activity of the enzyme. Sodium dodecyl sulphate polyacrylamide gel electrophoresis revealed an apparent molecular weight of 32 kDa. This pectinase may be industrially used in extraction and clarification processes.

Keywords: *Streptomyces*, mangrove, pectinase, media optimization, specific activity.

Introduction

Pectin is a structural polysaccharide which forms an important component of middle lamella and primary cell wall of higher plants. It is primarily made up of α (1−4) linked D-galacturonic acid residues[1]. Depending on their mode of action, the enzymes hydrolyzing pectin are broadly known as pectinases, which include endo-polygalacturonase (E.C. 3.2.1.15), exo-polygalacturonase (E.C. 3.2.1.67), pectin lyase (E.C. 4.2.2.10) and pectin esterase (E.C. 3.1.1.11)[2,3]. Pectinases are extensively used in extraction and clarification of fruit juices, extraction of vegetable oil, processing of alcoholic beverages, fermentation of coffee beans and tea leaves, retting and degumming of fibers, etc[4, 5].

Pectinases are naturally produced by many organisms, including bacteria, fungi, yeasts, insects, nematodes, protozoa and plants. A quarter of the global food enzymes sale is met with microbial pectinases[6]. Although fungal pectinases are being industrially used, pectinase production from actinomycetes has also been reported earlier[7,8].

Actinomycetes belong to a distinct class of gram-positive bacteria which are of great biotechnological significance due to their ability to synthesize chemically diverse, commercially important bioactive compounds like antibiotics, enzymes, pigments, etc[9]. They constitute numerous unique species which are capable of growing in extreme, hostile and polluted environments[10]. Among the actinobacterial population, *Streptomyces* species have cosmopolitan distribution due to dispersion of their spores[11]. About 80% of the metabolites produced by actinomycetes originate from *Streptomyces* species[12].

Pectinases from streptomycetes have good thermal stability[3] and activity over a broad range of pH[2, 7] and may be desirable in various industrial applications that require usage of elevated temperatures, extremes of pH and ionic concentrations. Therefore, it is necessary to explore unique habitats and study the indigenous flora of streptomycetes with better enzymatic potential. Sediment from mangrove forests provides suitable niche for various actinomycetes due to abundance of moisture, salinity and organic debris of plants and animals. The present investigation focuses on isolation and screening of pectinolytic actinomycetes from mangrove ecosystem, enhanced production of pectinase through media optimization followed by its purification.

Material and Methods

Chemicals and reagents: Microbiological media used during this study were obtained from Himedia Laboratories Pvt. Limited (Mumbai, India). The analytical grade chemicals and reagents were purchased from Loba Chemie (Mumbai, India), Qualigens Fine Chemicals (Mumbai, India) and s. d. Fine-Chem Ltd. (Mumbai, India).

Collection of mangrove sediment: Sediment samples were collected from the rhizosphere of *Avicennia officinalis* plants

inhabiting Valapattanam (11°56′0″ N, 75°18′0″ E) mangrove forest located at Kannur district in Kerala, India during October, 2010. Samples were transported to laboratory in sterile zip-lock polythene bags and stored for further processing.

Isolation of actinomycetes: Actinomycetes were isolated on starch casein nitrate (SCN) agar medium[13] supplemented with 50 µg/ml cycloheximide and 25 µg/ml streptomycin[14]. The plates were incubated at 30°C for 7-10 days. The actinomycete colonies were subcultured on SCN agar slants and stored at 4°C for further use.

Screening for pectinase production: Screening of actinomycetes for pectinolytic activity was carried out following the procedure of Pereira *et al.*[15]. Pure isolates of the actinomycetes were cultured on pectin agar and the plates were incubated at 28°C for 5-7 days[16]. Plates were then flooded with 1% (w/v) solution of polysaccharide precipitant cetrimide (cetyl trimethyl ammonium bromide) dissolved in 15% alcoholic solution and incubated at room temperature for 30-60 min. Pectinolytic activity was observed as a clear zone surrounding the colony against an opaque colour of the non-hydrolyzed medium. The diameters of the hydrolytic zones were measured to the nearest millimetre (mm) and a potent producer of pectinase was selected.

Molecular characterization of the pectinolytic actinomycete: The most potent pectinolytic actinomycete was cultured in SCN broth and incubated at 28°C for 7 days in an orbital shaker at 130 rpm. Genomic DNA was extracted using Fungal Genomic DNA Isolation Kit RKT 41/42 (Chromous Biotech Pvt. Ltd., Bangalore, India) according to the manufacturer instructions and visualized using 0.8% agarose gel (with ethidium bromide) electrophoresis.

The polymerase chain reaction (PCR) was performed with 25 µl of reaction mixture containing: 1.5 µl genomic DNA, 1 µl 10 pmol forward 16S rDNA primer (5'-AGAGTTTGATCCTGGCTCA-3'); 1 µl of 10 pmol reverse 16S rDNA primer (5'-ACGGCTACCTTGTTACGACT-3'); 1 µl of 30 mM deoxyribonucleoside 5'-triphosphate (N= A,T,G,C) (dNTP's); 2.5 µl of 10X PCR buffer and 1 µl Taq polymerase (1 U) (Chromous Biotech Pvt. Ltd., Bangalore, India). Sterile water was added to make up the volume to 25 µl. The thermal cycler (MJ Research PTC 200, USA) was programmed as follows: 2 min initial denaturation at 94°C, followed by 30 cycles that consisted of denaturation for 1 min at 94°C, annealing for 30 s at 57°C and extension at 74°C for 1 min and a final extension of 5 min at 74°C. The PCR-amplified product was detected by 1.2% agarose gel (with ethidium bromide) electrophoresis and the results were visualized under UV light using gel documentation system (Herolabs, Germany)[16]. The partial 16S rDNA sequencing of the PCR-amplified product was performed at Chromous Biotech Pvt. Ltd., Bangalore, India. The obtained nucleotide sequence was submitted to GenBank database. This nucleotide sequence data was manually aligned with the available nucleotide sequences retrieved from the NCBI database by using BLASTN[17].

Submerged fermentation of pectinase: Pectinase production from the selected actinomycete isolate was carried out in pectin broth containing (g/l): yeast extract, 1; pectin, 5; KH_2PO_4, 4; NaCl, 2; $MgSO_4.7H_2O$, 1; $MnSO_4$, 0.05; $FeSO_4.7H_2O$, 0.05; $CaCl_2.2H_2O$, 2; NH_4Cl, 2 and distilled water at pH 7.3. Fermentation was carried out in 500 ml Erlenmeyer flask containing 250 ml of production medium with 1% (v/v) inoculum (10^6 spores/ml) and incubated at 30°C under shaking condition (150 rpm) for 5 days[18]. The culture broth was centrifuged at 8,000 rpm for 30 min at 4°C. The supernatant was subjected to pectinase assay.

Assay of pectinase: Polygalacturonase activity was determined by quantifying the amount of reducing groups expressed as galacturonic acid units, liberated during the incubation of 1 ml of 1% (w/v) citrus pectin, prepared in 0.2 M phosphate buffer (pH 7.2) with 500 µl of the enzyme at 37°C for 30 min, by DNSA method[19]. One unit of polygalacturonase activity was defined as the amount of enzyme required to release 1 µmol of galacturonic acid per minute under standard assay conditions[20] and expressed as units per litre (U/l). Specific activity was defined as the amount of enzyme required to release 1 µmol of galacturonic acid per minute per milligram of total enzyme protein and expressed as units per milligram (U/mg).

Effect of nutritional parameters: The effect of substrate concentration on pectinase synthesis was investigated by incorporating various concentrations of pectin (0.05, 0.1, 0.3, 0.5, 1, 3 and 5% w/v) into the production medium. The effect of different organic (beef extract, yeast extract, peptone, tryptone, casein) and inorganic (ammonium chloride, ammonium nitrate, ammonium sulphate, sodium nitrate, sodium nitrite) nitrogen sources were determined. The concentrations of the best nitrogen supplement were also varied (0.1, 0.2, 0.3, 0.5, 1, 2, 3 and 5% w/v).

Effect of physico-chemical parameters: The effect of pH was determined by adjusting the initial pH of the medium to 4.5, 5.5, 6.5, 7.5 and 8.5. The production of pectinase was noted at 25, 30 and 37°C. The effect of inoculum size was evaluated using different volumes of spore suspension such as 0.5, 1, 3, 5 and 10% (v/v). The role of surfactants such as sodium dodecyl sulphate (SDS), Triton X-100, Tween-20, Tween-80, cetrimide (CTAB) and sodium deoxycholate (SDC) at 0.05% concentration was investigated. The effect of incubation time affecting pectinase production was also determined after every 24 h of incubation for a period of 7 days.

Purification of pectinase: The crude enzyme supernatant was added to an equal volume of ice cold 80% (w/v) saturated solution of ammonium sulphate, mixed well and kept for precipitation overnight at 4°C. The resulting precipitate was collected by centrifugation at 5000 rpm for 30 min at 4°C. The

precipitate was dissolved in 5 ml of 0.2 M phosphate buffer (pH 7.5) and dialyzed against distilled water. The dialyzed sample was purified by gel filtration chromatography using the protocol of Keller *et al.*[21] with certain modifications. The dialyzed sample was loaded to Sephadex G-200 column (1.5 x 10 cm) equilibrated with 0.2 M phosphate buffer (pH 7.5) and eluted with the same buffer. About 10 fractions were collected at a flow rate of 0.1 ml/min. The fractions showing pectinase activity were pooled. The specific activity of the purified pectinase was determined and compared with that of the crude enzyme.

Determination of protein content: The protein contents of the crude and purified pectinases were determined by the method specified by Lowry *et al.*[22], using bovine serum albumin as the standard.

Determination of molecular weight: The molecular weight of the purified pectinase was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using broad range protein marker (NEW ENGLAND Biolabs, UK)[23].

Statistical analysis: Effect of each parameter was studied in triplicate and the data have been graphically presented as mean ± standard deviation of triplicates (n = 3).

Results and Discussion

Mangrove sediment contains large amounts of detritus (plant material converted to dead organic matter) from autolysis and/or microbial degradation of fallen leaves, flowers, fruits and twigs[24]. These organic materials supply energy and nutrients for the sustenance of several living forms found in the mangrove habitat[25]. Microorganisms such as bacteria, fungi and actinomycetes, which degrade the plant based polysaccharides, plentifully thrive in the mangrove sediment[26]. In the present study, the rhizospheric sediment samples showed pH values ranging between 6.4-8.3.

Screening of pectinolytic actinomycetes: Ten isolates of actinomycetes were obtained from the mangrove sediments and were designated as A, 4, 6, 7, 10, 12A, A-11, E, I-7 and I-23. The observation of dry, compact/powdery, chalk-like colonies on SCNA indicated the presence of streptomycetes. The screening for pectinase production revealed that all the isolates were pectinolytic. Isolates A, 4, 6, 7 and 10 demonstrated good pectinolytic potential with the isolate number 10 as the highest producer (data not shown). On the other hand, isolates E, I-7, I-23 and 12A showed moderate enzymatic potential, whereas, isolate number A-11 was found to produce the lowest zone of pectin hydrolysis. Mangrove isolates of actinomycetes have good pectinolytic potential, probably due to the abundance of organic remains in the detritus. This observation is supported by findings from a previous investigation wherein 61.1% of the mangrove isolates of streptomycetes demonstrated good pectinolytic potential[14].

Molecular characterization of the pectinolytic actinomycete: The 616 bp partial 16S rDNA sequence of the actinomycete strain GHBA10 was obtained and submitted to GenBank database. This submitted nucleotide sequence was provided a GenBank accession number JX683187.1. Based on the results of BLASTN search using the sequence data the actinomycete isolate was found to be closely related to *Streptomyces* sp.

Effect of nutritional parameters: The success of a fermentation process depends greatly on various process parameters that influence the microbial growth and metabolite production[27]. Submerged fermentation is extensively used for the industrial production of microbial enzymes. Availability of good quantity of an utilizable substrate in the fermentation medium supports high production of the enzyme. It was elucidated from an earlier investigation that pectinase production from *Streptomyces* sp. was stimulated by pectin[7]. The optimization of substrate concentration demonstrated the highest pectinase activity of 1520 U/l from 0.3% (w/v) pectin. Pectin percentages either lower or higher than 0.3% demonstrated lesser production of the enzyme, as shown in figure 1.

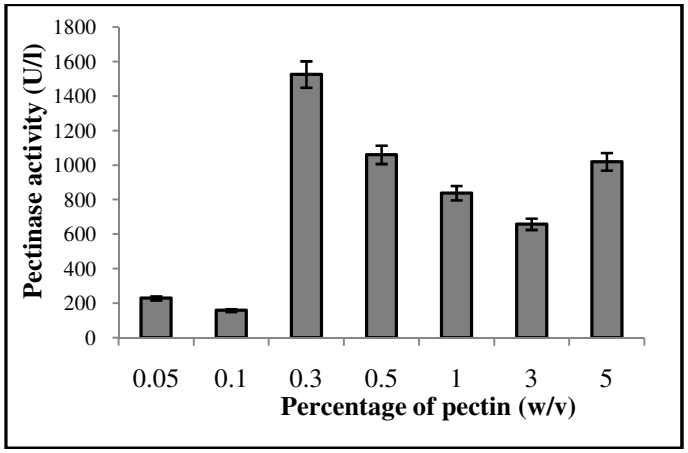

Figure-1
Effect of pectin concentration on pectinase production, Error bars represent the standard deviation calculated from three independent experiments

Nitrogen supplements, when incorporated into the production medium, facilitate better biomass production and subsequently higher metabolite secretion. Nitrogenous compounds are utilized by the microbial cells for the synthesis of nucleotides, amino acids, proteins, enzymes and other metabolites[28]. The effect of various organic and inorganic nitrogen supplements was studied. Organic nitrogen supplements such as beef extract, yeast extract, peptone, tryptone and casein showed good yield of pectinase. Among these, tryptone supported the maximum pectinase production (260 U/l). This may be attributed to the vitamins and accessory growth factors naturally present in these organic supplements[28]. Among the inorganic nitrogen sources, both ammonium chloride and sodium nitrate supported higher

pectinase synthesis, whereas, ammonium sulphate showed the lowest enzyme production, as shown in figure 2. Interestingly, 0.1% tryptone supported highest yield of pectinase (630 U/l), as depicted in figure 3. Further increase in the tryptone percentage resulted in moderate level of the enzyme synthesis. It is evident from these results that carbon to nitrogen (C/N) ratio of 3.0 used in the fermentation medium demonstrated significant level of pectinase production. On the other hand, a very high C/N ratio substantially decreased the enzyme production. Optimization of pectinase production by *Aspergillus niger* NCIM 548 reported maximal productivity (22.87 U/ml) at a C/N ratio of 5.94[29]. Our result is in close proximity with that of a previous study which reported the maximum lipase production (13 U/ml) from *Penicillium aurantiogriseum* with a C/N ratio of 2.5[30]. A similar study on pectinase production from a thermophilic *Bacillus* sp. reported maximum polygalacturonase synthesis (39 U/ml) in broth containing 0.5% (w/v) apple pectin and 0.3% (w/v) corn steep liquor with a C/N ratio of 1.6[31].

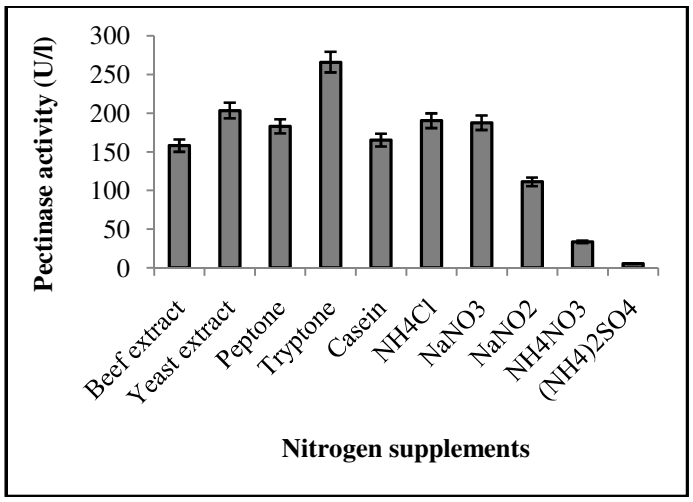

Figure-2
Effect of nitrogen supplements on pectinase production, Error bars represent the standard deviation calculated from three independent experiments

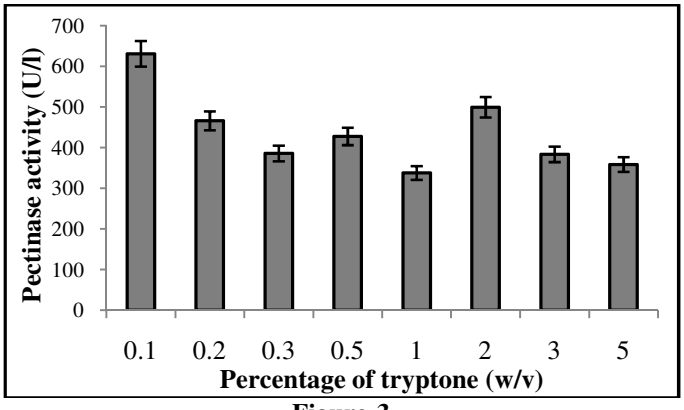

Figure-3
Effect of tryptone concentration on pectinase production, Error bars represent the standard deviation calculated from three independent experiments

Effect of physico-chemical parameters: pH of the fermentation medium plays a vital role in determining the level of metabolite synthesis. The stability of the microbial metabolite is also dependent on the hydrogen ion concentration of the medium[32]. In the present study, an initial medium pH of 8.5 supported maximum pectinase production (1340 U/l). Mangrove isolates of actinomycete generally prefer neutral to alkaline pH for their growth and enzyme production. In the present study the pectinase production gradually increased from initial pH of 5.5 to 8.5, as shown in figure 4.

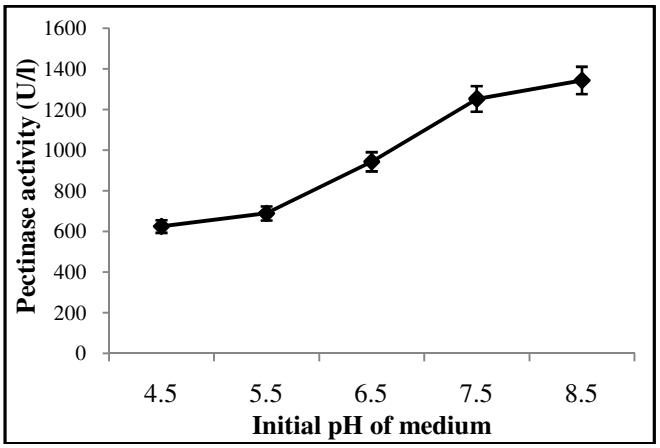

Figure-4
Effect of initial pH of medium on pectinase production, Error bars represent the standard deviation calculated from three independent experiments

The incubation temperature greatly affects the microbial growth rate, enzyme secretion, enzyme inhibition and protein denaturation[33]. 30°C supported maximum pectinase synthesis (980 U/l), as presented in figure 5. Owing to the mesophilic nature of the *Streptomyces* isolate, this temperature might have facilitated better membrane permeability and therefore maximum pectinase secretion into the medium. A decrease in pectinase production was observed when the incubation temperature was 37°C, which might be due to the impaired metabolic activity of the isolate.

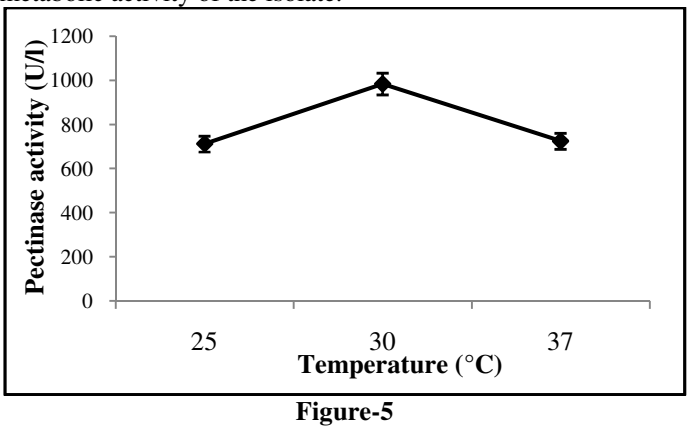

Figure-5
Effect of incubation temperature on pectinase production, Error bars represent the standard deviation calculated from three independent experiments

The initial load of microorganisms also influences the final level of the enzyme synthesized. 5% (v/v) inoculum resulted in highest pectinase production (450 U/l). With an increase in the inoculum percentage the enzyme production steadily increased, probably due to effective utilization of the substrate by the actively growing microbial cells, as depicted in figure 6. A decrease in the enzyme production was noticed when 10% (v/v) inoculum was used. This could be due to rapid depletion of nutrients and development of oxygen stress resulting from a high microbial load.

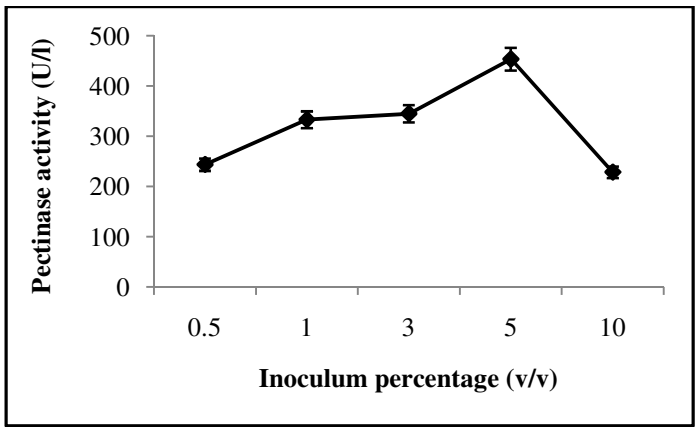

Figure-6
Effect of inoculum size on pectinase production, Error bars represent the standard deviation calculated from three independent experiments

In order to determine the effect of surfactants on the enzyme production, various compounds were used. Among these, 0.05% (w/v) cetrimide demonstrated the highest activity of pectinase (190 U/l). SDS and Triton X-100 supported moderate level of pectinase production, as illustrated in figure 7. As reported previously, surfactants such as Tween-80 and Tween-20 had stimulatory effects on pectinase activity[34].

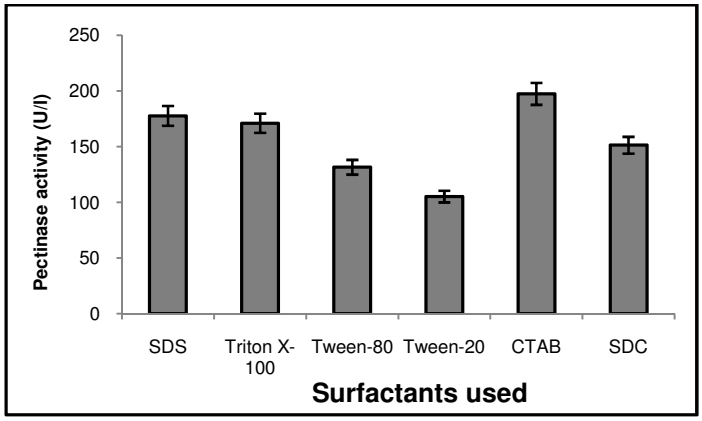

Figure-7
Effect of surfactants on pectinase production, Error bars represent the standard deviation calculated from three independent experiments

The level of enzyme production varies with the time duration of the fermentation process[28]. Maximum pectinase production from *Streptomyces* sp. was observed after 6 days of incubation (450 U/l). A gradual increase in the enzyme level was detected till the 6th day of the fermentation process, whereas, there was a steep decline in the pectinase activity after the 7th day of incubation, as shown in figure 8. Beyond this period the enzyme production drastically reduced, probably due to the depletion of essential nutrients in the medium and/or accumulation of toxic secondary metabolites.

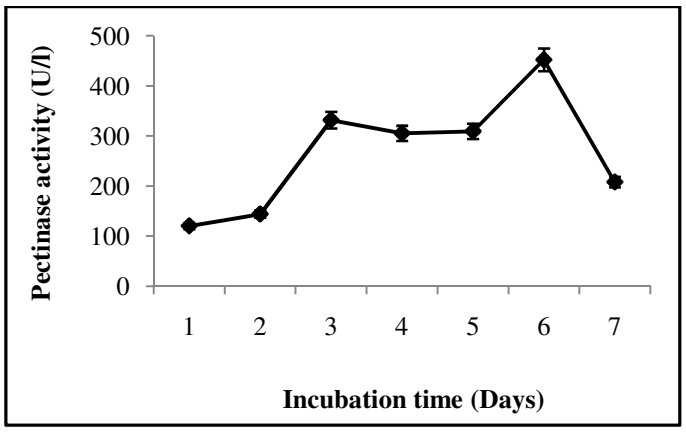

Figure-8
Effect of incubation time on pectinase production, Error bars represent the standard deviation calculated from three independent experiments

Purification of pectinase: The crude pectinase was purified by ammonium sulphate precipitation, dialysis and gel filtration chromatography. The activity of the crude and purified pectinase was evaluated as 250 U/l and 658 U/l, respectively. The specific activity of the crude and purified pectinase was recorded as 744 U/mg and 2610 U/mg, respectively. After column chromatography, 3.5-fold increase in the specific activity was noted.

The apparent molecular weight of the purified pectinase from *Streptomyces* sp. GHBA10 was found to be 32 kDa by SDS-PAGE. This result is in close proximity with an earlier study that reported a pectinase from *Penicillium chrysogenum* having an apparent molecular weight of 31 kDa[35]. In another study, the molecular weight of purified exo-polygalacturonase from *Streptomyces lydicus* was determined as 43 kDa[36].

Conclusion

Actinomycetes possess excellent enzymatic potential besides their proven antimicrobial properties. Mangrove sediment offers suitable habitat for isolating different *Streptomyces* sp. with many unique properties. The present study focuses on improvement of pectinase production from *Streptomyces* sp. GHBA10.

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