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EXTRACELLULAR PECTINASE ACTIVITY FROM *Bacillus Cereus GC Subgroup A*: ISOLATION, PRODUCTION, OPTIMIZATION AND PARTIAL CHARACTERISATION

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ABSTRACT

A pectin degrading bacterium was isolated from a soil and identified as *Bacillus cereus GC subgroup A* based on its morphological, physiochemical and FAME-GC analysis. The Solid state fermentation at 37 °C for 72h, strain produced pectinase in different substrates; among the substrates orange peel powder and wheat bran powder were good for strain but mixture of orange peel powder and wheat bran powder enhanced enzyme production upto 804 IU/ml. The maximum pectinase production was studied at 37^0 C and pH 7 after 96h of incubation by *Bacillus cereus*. Regarding carbon sources, galactose (0.02%) was the best source for strain; while malt extract (0.1%) was found as the best nitrogen source for *Bacillus cereus.* The enzyme was partially purified by ammonium sulphate precipitation and dialysis was carried out to remove the excess salt. A Lineweaver-Burk analysis showed a *Km* value of 8.3 mg/ml and *Vmax* of 1428.5 IU/ml. The partially purified enzyme exhibited maximal activity at a temperature of 40°C and pH 6.0. The enzyme showed stability towards salts at different concentrations 1mM, 10mM, 50mM and 100mM. In addition to that $CaCl_2$ has increased activity of enzyme at higher concentration too. The amino acid composition of pectinase was also determined by BIOEDIT software. The molecular weight of pectinase was 38304.27 Da (348 amino acids) and was rich in glycine and valine.

Keywords: Pectinase, *Bacillus Cereus*, Fame-GC analysis, Amino acid composition

INTRODUCTION

Pectin or pectic substances are heterogeneous group of high molecular weight, complex, acidic structural polysaccharides with a backbone of galacturonic acid residues linked by α-(1-4) linkages **(Alphons *et al.* 2009; Biswapriya *et al.* 2011)**. They constitute major components of the middle lamella, a thin layer of adhesive extracellular material found between the primary cell walls of adjacent young plant cells **(Kapoor *et.al.* 2001; Hoondal *et.al.* 2000)**. Pectin is known to contain neutral sugars like xylose, Galactose and arabinose which are present in side chains. Pectinases are produced by many organisms such as bacteria **(Horikoshi 1972; Karbassi and Vaughan 1980),** fungi **(Aguillar and Huitron 1990)** and yeasts **(Gainvors and Belarbi. 1995)**. Pectic enzymes have two classes namely, pectin esterases and pectin depolymerases. Pectin esterase has the ability to de-esterifies pectin by the removal of methoxy residues. Pectin depolymerases readily breakdown the main chain of carbohydrate and it was further classified as polygalacturonase (PG) and pectin lyases (PL) Thus, pectinases are group of hydrolytic enzymes, which hydrolyze the pectin molecules and are readily soluble in water. Microbial pectinases account for 10-25% of the global food and industrial enzyme sales **(Singh *et al.* 1999a; Jayani *et al.* 2005; Murad and Azzaz 2011)** and their market is increasing day by day. These are used extensively for fruit juice clarification, juice extraction, manufacture of pectin free starch, refinement of vegetable fibres, degumming of natural fibres, wastewater treatment, curing of coffee, cocoa and tobacco and as an analytical tool in the assessment of plant products **(Alkorta *et al.* 1998)**. Optimization of media is very important to maximize the yield and productivity, and minimize the product cost **(Singh *et al.* 1999b)**. The aim of this study was to isolate the most prominent pectinolytic bacteria from soil samples with unique properties and optimize their fermentation conditions for maximum pectinase production. Owing to the expensive medium ingredients, solid state fermentation (SSF) was tried using wheat bran, orange peel as a carbon source separately and in combinations in order to find out cheap and suitable natural source for production of this industrially important pectinase enzyme.

MATERIALS AND METHODS:

Isolation, Selection and Maintenance of culture

In view of getting efficient pectin degrading cultures, soils rich in pectic waste and fruit waste samples were scrutinized from fruit processing area, sewage of juice centres of different locations. The 1g of all sample were inoculated in 100 ml of pectin broth (0.1% w/v yeast extract, 0.2% w/v $NaNO_3$, 0.1% KH_2PO_4, 0.05% KCl , 0.05% $MgSO_4.7H_2O$, pH 6.0) supplemented with 0.2% w/v pectin and incubated on rotary shaker 120 rpm at 37 °C for 7 days. 0.1ml of previously enriched culture was inoculated in 100 ml of freshly prepared pectin broth supplemented with 0.5% w/v of pectin for next 7 days of enrichment. After several serial transfers in pectin broth, each time with increasing pectin concentration up to 2.0%, the soil sample was finally subjected for isolation of pectinase producers. After fifth week of enrichment, 0.1 ml of broth was spread on the surface of pectin containing agar medium plate and incubated at 37 °C for 24h. Different bacterial colonies were picked from each plate and streaked on the same medium. The pure cultures of bacteria used in the present investigation were transferred on to the slants of same composition and incubated at the same optimized conditions. Periodic transfers were made at regular intervals and every time.

Preliminary screening of pectinase producing bacterial isolates by well plate method

Purified bacterial isolates were preliminary screened for pectinase activity by well plate method **(Hannan *et al.* 2009)**. Supernatant from overnight incubated bacterial cultures (1.0 optical density at 600 nm) were used in all experiments to determine the pectinase activity. Modified medium containing 0.3% KH_2PO_4, 0.6% Na_2HPO_4, 0.2% NH_4Cl, 0.5% NaCl, 0.01% $MgSO_4.7H_2O$, 1.5% agar supplemented with 0.2 % pectin were prepared. After solidification of the medium, well of 5 mm in diameter were made in the agar with the help of cork-borer and filled with 50 µl of cell supernatant. After incubation for 24 h at 37°C, plates were flooded with iodine solution containing 0.25% iodine, 0.5% potassium iodide and 30 ml of 20% ethanol **(Cappuccino and Sherman 2002)**.

The pectinase activity was observed by a clear zone around the well. The result was observed by measuring the diameter of the clear zone.

Morphological and biochemical characterization of pectinase producing bacterial isolates

Biochemically, isolated strain of the bacterium was characterized by using carbohydrate utilization tests. Tests were also carried out to determine reaction with casein, starch, gelatine hydrolysis, catalase and determination of nitrate reduction **(Aneja 2007; Peter *et al.* 1986)**.

FAME-GC Analysis

The cultures were obtained from soil sample and pure cultured onto Trypticase Soy Broth Agar (TSBA) media. The fatty acids were extracted from loop full of culture with 65% chloroform and 35% methanol. 0.7% NaCl solution was added to the crude extract to remove methanol and water soluble components of extract. The aqueous layer was siphoned off and the organic phase was dried by anhydrous sodium sulphate. The pooled dried extract was concentrated under a stream of nitrogen. The recovered lipid was reconstituted in 0.5 M methanolic KOH and hydrolyzed. Hexane and methyl tert-butyl ether were used to quench the reaction. The recovered organic phase was pooled and analyzed by GC. As the bacteria are killed in the saponification step of the extraction, there is little infectivity concern with handling of the sample once this step is concluded. FAMEs are more volatile than their respective fatty acids and therefore more suitable to GC analysis. The Sherlock software automates all analytical operations and uses a sophisticated pattern recognition algorithm to match the unknown FAME profile to the stored library entries for identification **(Anju Rajan *et al.* 2011)**.

Optimization of pectinase production

The optimization study of the following parameters was done for better growth and production for enzyme.

Effect of pH on pectinase production

50 ml of production medium was prepared and pH of the medium was adjusted to 2, 3, 4, 5, 6, 7, 8 and 9.The sterilized production medium were inoculated and incubated for 3 days under the shaking condition at 37°C. The enzyme activity was studied. Sterile uninoculated production medium was used as blank for pectinase assay.

Effect of carbon and nitrogen source on pectinase production

Different carbon sources (0.2 g/l; Sorbitol, Lactose, Starch, Glucose, Sucrose, Fructose, maltose and Galactose) and nitrogen sources (1 g/l; Urea, Ammonium nitrate and ammonium sulphate, Malt extract, Beef extract, peptone and tryptone) were chosen .The effect of these different carbon and nitrogen sources on pectinase production under optimal pH 7.0 and temperature 37°C were examined.

Solid state fermentation

The effect of different substrates (orange peel, wheat bran and orange peel+ wheat bran) was studied. The substrates were obtained by drying the peels of orange in an oven maintained at 50°C till they were completely dried and whereas wheat bran was purchase dried form. The dried peels and the wheat bran were then grinded to powder. Solid state fermentation (SSF) was carried out in 250 ml Erlenmeyer flasks that contained 5 g of orange peel/wheat bran /wheat bran+ orange peel and 5 ml of distilled water (moistening agent). The flasks were sterilized at 121°C for 15 minutes. 1ml of inoculum was added, mixed well and incubated at 37°C for 72h.At the end of incubation period, the flasks were taken out and the content of each flask were extracted with 25 ml of sterile distilled water. Petri plates containing autoclaved modified MS medium supplemented with 0.2 % pectin were prepared. After solidification of the medium, well of 5 mm in diameter were made in the agar with the help of cork-borer and filled with 50 µl of cell supernatant. After incubation for 24 h at 37°C, plates were flooded with iodine solution. The pectinase activity was observed by a clear zone around the well. The activity of pectinase was measured according to standard method (Miller 1959).

Production of pectinase

Fermentations were carried out by inoculating the culture into a liquid medium composed of (0.2% $NaNO_3$, 0.1% KH_2PO_4, 0.05% KCl and 0.05% $MgSO_4$ at pH 7), 0.1% yeast extract and substrate as the main carbon source pectin at 0.5%. After incubation for 96 hours at 37°C under shaking conditions, the media was centrifuged. The supernatant was the source of enzymatic extract and was used for pectinase activity assay.

Pectinase Assay

Pectinase activity was measured according to Miller's method **(Miller 1959)**. Briefly, 0.5ml of cell free supernatant was incubated with 0.5ml of pectin in 0.1M acetate buffer with pH 6.0 and the reaction mixture was incubated at 40°C for 10 minutes in static condition. After adding 1ml of DNS reagent, the mixture was boiled for 5 minutes at 90°C. The reaction was stopped by adding 1ml of Rochelle's salt. Then the mixture was diluted by adding 2ml of de-ionized water. The absorbance was measured spectrophotometrically at 595 nm. A standard graph was generated using standard glucose solution. One unit of Pectinase activity was defined as the amount of enzyme which liberated 1µM glucose per minutes.

Determination of Protein Concentration

Protein of all enzymatic preparations was determined according to Lowry **[Lowry *et al.* 1951]** using bovine serum albumin as the standard. Readings were carried out in a spectrophotometer at 660 nm.

Purification of pectinase enzyme

Ammonium sulphate precipitation

Culture filtrate solution was treated with 70% of saturated ammonium sulphate solution. The crude enzyme was continuously stirred using magnetic stirrer and kept at 4°C for overnight, the enzyme was centrifuged at 10,000 rpm for 15 minutes, then supernatant was discarded and the pellets were dissolved in minimum volume of 0.5M phosphate buffer of pH 7.

Dialysis

About 8cm of the dialysis tube was cut and placed in 100 ml of 2% w/v sodium bicarbonate. 1 mM EDTA was added to chelate any metal ions. It was boiled for 10 minutes and was again washed in boiling distilled water for 10 minutes. The boiling process was repeated with distilled water again. The activated dialysis bag was filled with the enzymes and sealed from the both sides without any air bubbles. The bag was kept in 500ml of 50 mM phosphate buffer (pH 7.0) solution on a magnetic stirrer in ice cold condition for 8 hours. The buffer was changed frequently for every hour to avoid equilibration.

Effect of pH on catalytic activity of pectinase

The activity of pectinase was evaluated at different pH values. The partially purified enzyme was incubated using 0.1 M of buffers, in the range between pH 3 - 10, under assay conditions and the amount of glucose liberated was determined. Buffers used were sodium-acetate (pH 3.0 - 6.0), sodium-phosphate (pH-7), Tris-HCl (pH 8.0 - 10). The enzyme was incubated for 10 minutes at 40°C at different pH in the presence of substrate (10mg/ml) and the enzyme activity was determined by Miller's method.

Effect of Temperature on catalytic activity of pectinase

The pectinase activity was tested at different temperatures viz., 10 to 80°C. Sodium-phosphate buffer (pH-7) to maintain optimum pH of enzyme was used in the system and the enzyme substrate reaction was carried out at different temperatures, after which the velocity of enzyme reaction was measured.

Effect of Different Salts on catalytic activity of pectinase

The effect of salts (i.e. NaCl, $CaCl_2$ and NH_4Cl) on the enzyme activity were tested at different concentrations (1mM, 10mM, 50mM and 100mM) by incubating with the purified enzyme. After the exposure, enzyme activity of each sample was measured by Miller's method.

Determination of K_m and V_{max} of pectinase

The Michaelis constant K_m and maximal velocity V_{max} of the purified enzyme were determined using pectin as substrate in the range of 5-30 mg with the help of Lineweaver-Burk plot **(Lineweaver and Burk 1934)** relating 1 / [V] to 1 / [S]**.**

Amino Acid Composition

Amino acid sequences of *Bacillus sp.* pectinase was retrieved from NCBI database (http://www.ncbi.nlm.nih.gov/) in FASTA format. Amino acid composition of retrieved sequences was done using BIOEDIT software.

RESULTS AND DISCUSSION

Pectinase positive colonies were screened by formation of clear zone around colonies. Figure 1 shows the bacterium producing pectinase enzyme, a clear zone around the well as the pectin near the well is utilized. Depending upon the zone of clearance, the isolate was selected for further experimental studies. Morphologically, it is gram positive and rod shaped bacterium, 3.0 – 5.2 µm in length and 0.4 – 0.7 µm in width growing at pH 7 and temperature 37°C. It produced oxidase, catalase, lipase, protease, gelatinase and amylase (Table 1). The Microbial Identification System (MIDI) for fatty acid methyl ester (FAME) analysis is a standard method for identification of microorganisms **(Schutter *et al.*2000)**. Whole cell fatty acids are converted to methyl esters and analyzed by gas chromatography. The fatty acid composition of the unknown is compared to a library of known organisms in order to find the closest match. The list of the fatty acids composition like straight Chain fatty acids 15.63%, branched chain fatty acid 83.34%, Mono Unsaturated Fatty Acid 0.34% and oleic acid (C18:1) w9c 0.69 % was given clearly according to the GC report **(Anju Rajan *et al.* 2011).** The chromatogram obtained in this experimental analysis (Figure 2) is more descriptive and elaborative. Our experimental data matches and establish the similar result mentioned in the report of MIDI Sherlock software databases and the similarity was matched with *Bacillus-cereus-GC subgroup A.* The mostly concerned genus was *Bacillus* and it reported 0.736 similarity indexes. Based on morphological, physiological, biochemical characteristics and FAME-GC analysis it was identified as *Bacillus Cereus subgroup A*. The production of enzyme was carried out at the optimized condition 37° C for 96 h at pH 7. The enzyme was purified using 70% ammonium sulphate precipitation and dialyzed against 50mM phosphate buffer of pH 7 for overnight at 4°C. The results of purification steps of pectinase of *Bacillus Cereus* are presented in (Table 2). After the two steps of purification, the purification fold was 2.5. The pH optimization study has reported, pectinase production in *Bacillus cereus* in a modest range (pH 6 to 8) (Figure-3). There was near about loss of pectinase activity at pH values less than 4.0 and more than 9.0. As per literature study, optimal pH range (8.9 to 9.4) reported for pectinase of *Bacillus polymyxa* **(Nagel and Vaughn 1961)**; pH 9.0 for *Bacillus stareothermophillus* **(Karbassi and Vaughn 1980)** and pH 10.0 reported for *Bacillus sp.* RK9 **(Fogarty and Kelly 1983)**. The isolate was capable of utilizing a wide variety of carbon sources. However, galactose was the best carbon source in the present study (Figure-4). Six different nitrogen sources such as Beef extract, Malt extract, Peptone, Ammonium sulphate and tryptone were tested for pectinase production in *Bacillus cereus.* Among them Malt extract supported a maximum enzyme activity (Figure-5). Partial characterization study shows that *Bacillus cereus* could grow well at 37°C (optimum growth temperature), at pH 6 (Figure-6). The temperature stability profile of pectinase activity revealed that the enzyme is maximally active at moderately high temperatures ranging from 40 to 60ºC with highest activity detected at 40ºC incubation temperature for 1 hour (Figure-7). This temperature stability of *Bacillus sp.* DT7 is higher than the values reported by **(Nagel and Hasegawa 1968)** for the crude enzyme of a *Bacillus sp.* (below 30°C) and by **(Nagel and Vaughn 1961)** for the crude enzyme of *B. polymyxa* (45ºC) but less than the values reported by **(Horikoshi 1972)** for *Bacillus* (65°C) and by **(Karbassi and Vaughn 1980)** for *B. stearothermophilus* (70ºC). A further increase in the reaction temperature caused significant drop in the pectinase activity. Similarly, temperatures lower than 40°C resulted in decrease in the pectinase activity. The purified enzyme was sensitive to some of the salts tested at a concentration of 1mM, 10 mM, 50mM and 100mM. The presence of $CaCl_2$, NaCl acted as a stimulator of pectinase activity resulting in an increase of in the enzyme activity, whereas NH_4Cl decreased pectinase activity (Table-3).The K_m *and* V_{max} of pectinase towards pectin were determined. The apparent K_m *and* V_{max} of the pectinase for pectin were 8.3 mg/ml and 1428.5 IU/ml, respectively (Figure-8). The primary sequence of the enzyme was retrieved from NCBI database http://www.ncbi.nlm.nih.gov/) having GenBank accession No: AGK82226.1. The sequence obtained is given below.

MMRSSIVKLHAFSEVVIQLWLYGVSSVTADLSTPNLGLQGFATLDGGTTT
VGGLGGEIVFVRNELINALKSKNPNRPLTIYVNGTITPSNTSNSKISDKDVS
NVSILGVGTNGRLNGIGIKVIIRNIHFEGFYMEDDPRGKKYDFDYINVENS
HHIWIDHCTFVNGNDGAVDIKKYSNYITVAYNITFSHDKVSLGGSSDKEG
NSEAGHYDRNITFHHNYFKTLNSRVPARFFGKAHLCSNYFENMRTGVSG
NVFRAEMLVEHNVFENATNPLGFPIYGVAGAMGAKVHVEGFYYCKEPE
VRPVEEGKPALDPREYYDYTLDPVQDVPKIVVDGAGAGKLVFGLITIA

The molecular weight obtained from BIOEDIT data (348 amino acids) was 38304.27 Da and the amino acid composition of pectinase was mentioned in (Table 4**).** The data shows, pectinase enzyme was rich in glycine and valine (Figure-9). In concern with the use of various agricultural waste and agro-industrial by-products, in the present study suggested that mixture of orange peel and wheat bran found to be the best substrate for pectinase production by *Bacillus cereus.* The Enzyme activity of the pectinase enzyme was obtained with 0.5% of Orange peel powder and 0.5% of wheat bran powder 166 IU/ml and 333IU/ml respectively after 72 h of incubation at pH 7 and temperature 37°C whereas enzyme activity of pectinase enzyme was obtained 804 IU/ml with mixture of both the substrate after 72 h of incubation at pH 7 and temperature 37°C (Figure-10). The similar studies were also carried out by **(Silva *et.al.* 2002)**. In order to use enzyme from the isolates for commercial applications, it must have desirable biochemical, physiochemical characteristics and low cost of production. Orange bagasse, wheat bran is very cheap, abundantly available. Its dumping in nature causes pollution problem; hence its eco-friendly utilization is essential which tempted to use agro-waste for pectinase production by solid state fermentation.

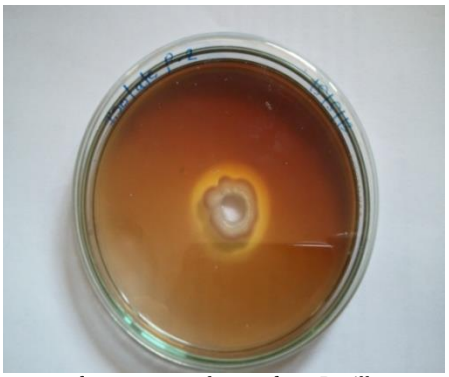

Figure 1 Screening of pectinase production from *Bacillus sp.* using pectin as substrate

Table 1 Morphological and biochemical characterization of isolated strain

Characters	Strain
Colonies	Large, irregular and flat with undulate margin
Morphology	Rod, Beta haemolytic
Gram nature	+
Motility	+
Oxidase	+
Catalase	+
pH Range	5-9
Temperature	20-60
Urease	-
Nitrate reduction	+
H_2S Production	-
Indole Production	-
Lipase	+
Protease	+
Hydrolysis of:	
Casein	+
Gelatin	+
Starch	+
Utilization of:	
Glucose	+
Lactose	-

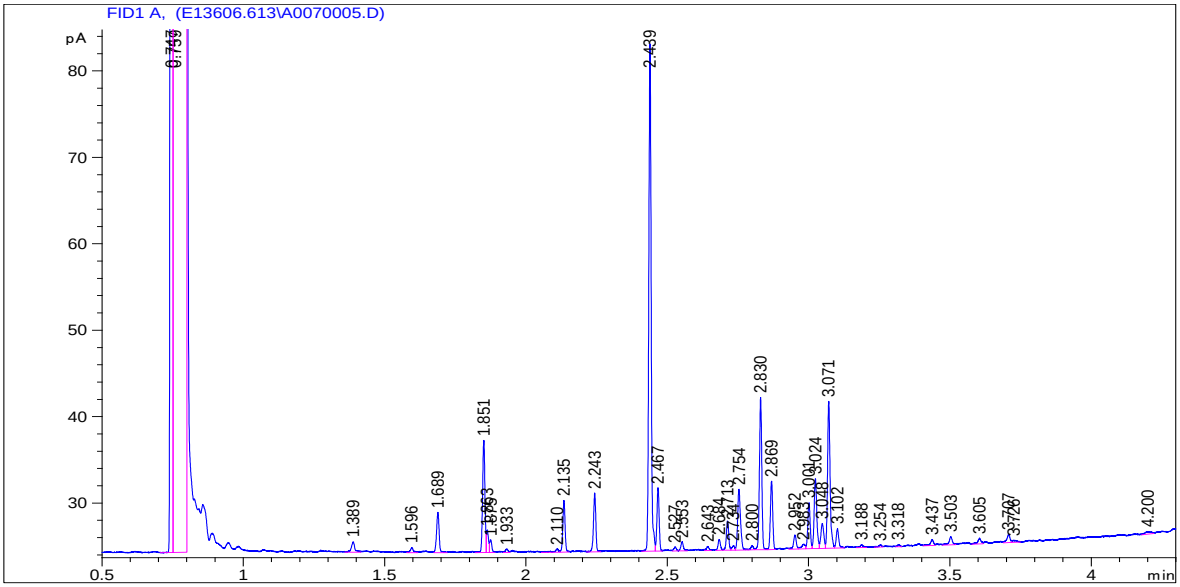

Figure 2 Chromatogram of bacterial sample one showing the fatty acid peaks through Agilent GC 6850

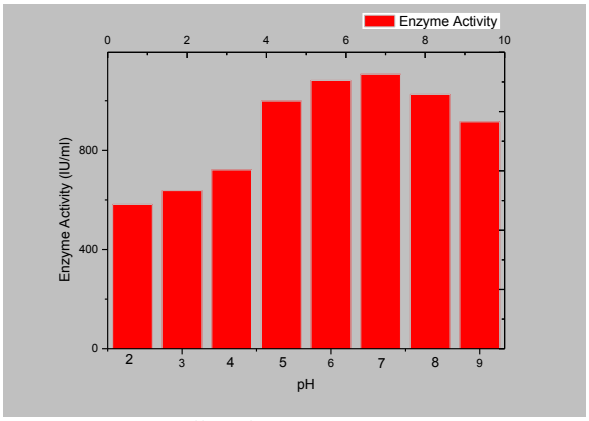

Figure 3 Effect of pH on pectinase production

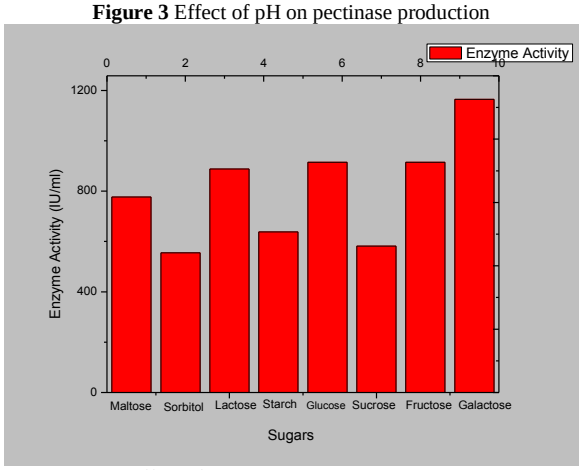

Figure 4 Effect of carbon source on pectinase production

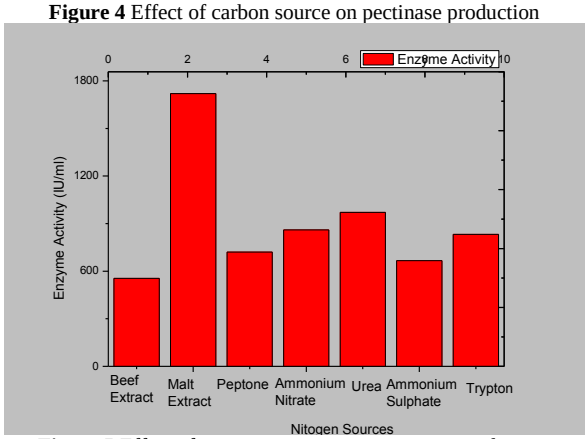

Figure 5 Effect of nitrogen sources on pectinase production

Table 2 Purification and recovery of pectinase from *Bacillus Cereus*

Steps	Pectinase activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude extract	888	6	116.4	1	100
70% Ammonium sulphate precipitation	582	5	148	1.2	83.33
Dialysis	582	2	291	2.5	40

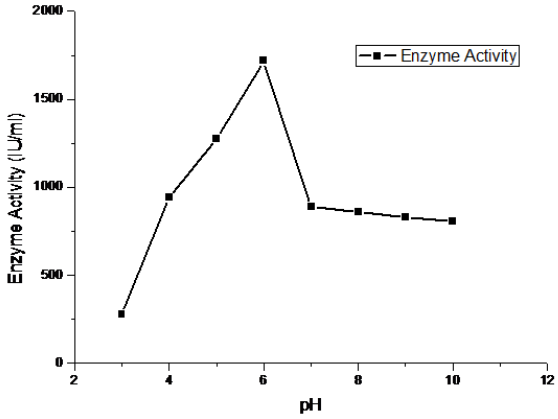

Figure 6 Effect of pH on purified pectinase

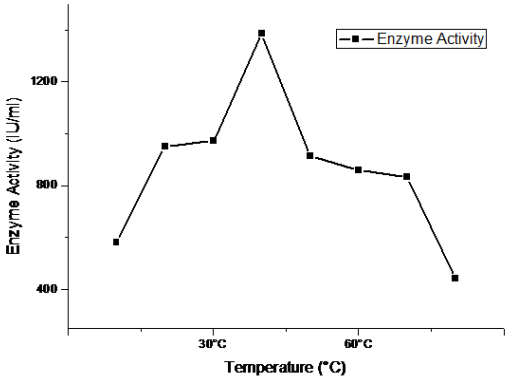

Figure 7 Effect of temperature on purified pectinase

Table 3 Effect of different salts on pectinase activity

Activator or Inhibitor	Relative Activity (%)			
	10^{-3}M	1×10^{-2}M	5×10^{-2}M	10^{-1}M
Control	100	100	100	100
NaCl	100±0.12	106±0.28	120±0.34	140±0.36
NH_4Cl	147±0.21	142±0.28	128±0.14	123±0.23
$CaCl_2$	110±0.11	147±0.06	305±0.32	324±0.16

Values are mean ± Standard deviation, n=3

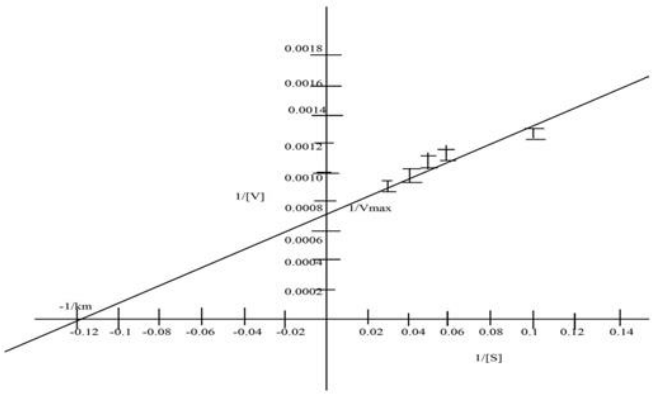

Figure 8 Determinutesation of *Km* and *Vmax* of pectinase from. *Bacillus Cereus GC subgroup A.*

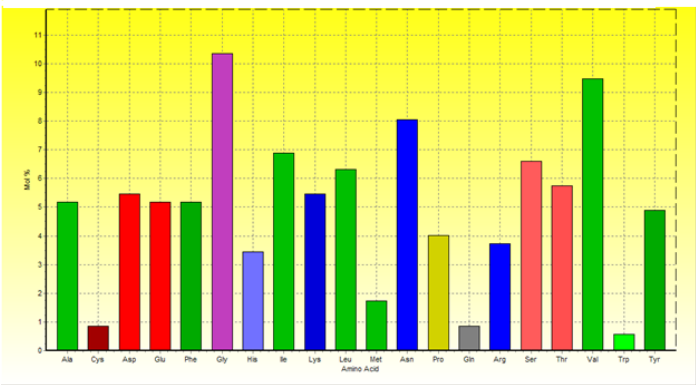

Figure 9 Amino Acid Composition Of pectinase from *Bacillus sp.*

Table 4 Amino acid contents (mole %) of *Bacillus sp.* Pectinase protein

Protein: pectinase
Length = 348 amino acids
Molecular Weight = 38304.27 Daltons

Amino Acid	Number	Mole (%)
Ala A	18	5.17
Cys C	3	0.86
Asp D	19	5.46
Glu E	18	5.17
Phe F	18	5.17
Gly G	36	10.34
His H	12	3.45
Ile I	24	6.90
Lys K	19	5.46
Leu L	22	6.32
Met M	6	1.72
Asn N	28	8.05
Pro P	14	4.02
Gln Q	3	0.86
Arg R	13	3.74
Ser S	23	6.61
Thr T	20	5.75
Val V	33	9.48
Trp W	2	0.57
Tyr Y	17	4.89

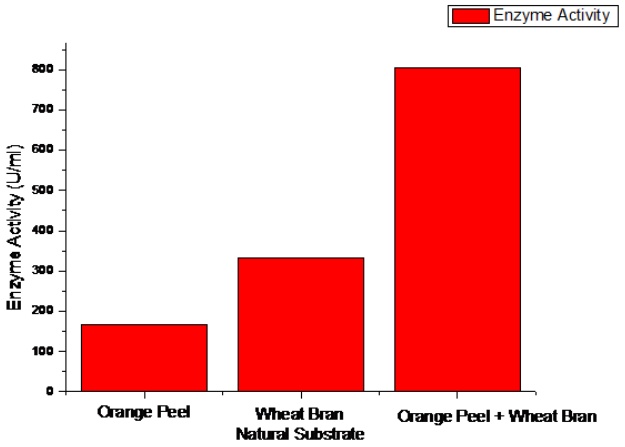

Figure 10 Effect of Natural substrate on pectinase production

CONCLUSION

In this study, the isolate *Bacillus cereus GC subgroup A* was identified by morphological, biochemical and FAME-GC Analysis. The culture conditions were optimized for pectinase production. The bacterium Bacillus *cereus subgroup-A* were produced significant amount of pectinase after 72 h of incubation in fermentation medium at 37°C and pH 7. Previous reports on the production of pectinase by *Bacillus sp.* under solid state fermentation is less. Since the Solid state Fermentation has of special economic interest of countries with large amount of agro-industrial residues such as wheat bran and orange peel can be used as carbon source for pectinase production in large scale. Large scale-up studies are needed for better output for commercial production. Some additional features like enhanced production with the addition of $CaCl_2$; shorter period of incubation for pectinase production, lesser amount of pectin in the growth medium indicate the potential of this organism to be used at commercial level for degumming of ramie, pre-treatment of waste water from fruit juice-processing industries.

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