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Production of Extracellular Pectinase by Bacillus Cereus Isolated From Market Solid Waste

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Abstract

Pectin is a major component of primary cell wall of all land plants and encompasses a range of galacturonic acid rich polysaccharides. Pectinase or pectinolytic enzyme, hydrolyzes pectic substance, they have a share of 25% in the global sales of food enzymes. Production of this enzyme is affected by the nature of solid substrate, level of moisture content, presence or absence of carbon, nitrogen, minerals and vitamin supplements. Maximum enzyme production (44U/ml) is achieved when temperature is around 37°C, pH.8.5, pectin as a carbon source, yeast extract as a nitrogen source and incubation time is 36 hours. In addition to this, wheat bran acts as a better agro substrate and Magnesium chloride as supplement for better production of pectinase. The sequence analysis of 16S rDNA of the isolated organism was much similar when compared with gyrase B gene (gyrB) of Bacillus cereus. Pectinolytic enzyme is of significant importance in the application of apple juice industry in the current biotechnological era.

Introduction

Enzymes are biocatalysts which are synthesized by living cells. It is also defined as the catalyst that increases a velocity or rate of a chemical reaction. Many bacteria, fungi and higher plants are known to produce pectinolytic enzyme called pectinase, that breakdown pectin, a polysaccharide substrate that is found in the cell wall of plants. One of the most important and widely used commercial pectinase is polygalacturonase (PGA). Pectin was first isolated and described by Henri Braconnot (1825). Pectin is the jelly like matrix structural polysaccharide found in primary cell wall and middle lamella of fruits and vegetables [1]. The pectinases are required for extraction and clarification of fruit juices and wines, extraction of oils, flavors and pigments from plant materials, preparation of cellulose fibers for linen, jute and hemp manufacture, coffee and tea fermentations and novel applications in the production of oligogalacturonides as functional food components [2]. *Bacillus cereus* is an endemic, soil dwelling, Gram-positive, rod-shaped, beta hemolytic bacterium. *Bacillus sp* was discovered by Cohn and Koch in the 19th century. The *Bacillus cereus* group is composed of *B. anthracis, B. cereus, B. mycoides, B. pseudomycoides, B. thuringiensis* and *B. weihenstephanensis* [3,4]. The capacity of selected *Bacillus sp* to produce and secrete large quantities (20–25 g/L) of extracellular enzymes has placed them among the most important industrial enzyme producers [5]. Pectic substances are widely distributed in fruits and vegetables (10-30% in turnips peels of orange and in pulps of tomato, pineapple and lemon); hence they form important natural substrates for pectinases. Pectinases are mainly used for increasing filtration efficiency and clarification of fruit juices.

Material and Methods

Materials

Nutrient Agar Medium (NA), Luria Bertani Medium (LB), Pectate Agar Medium (PA), Growth medium (composition: Peptone - 3g, Yeast extract - 0.5g, KH_2PO_4 - 0.15g, $CaCl_2$ - 0.001g, Pectin - 0.5g, Na_2CO_3 - 0.5g at pH - 8.0) were the media used. The materials used were Agar, Cetyltrimethyl Ammonium Bromide (CTAB), Thiobarbituric Acid (TBA), Tris HCl, Polygalacturonic acid (PGA), NaOH, Bradford reagent, Glucose, Lactose, Sucrose, Maltose, Tryptone, Potassium nitrate and Ammonium sulphate, SDS, Triton-X 100, EDTA, zinc sulphate, Magnesium chloride, Sugarcane baggase, Orange baggase, Rice bran, Wheat bran, Green gram peel, 100 mM glycine buffer pH 10.0, 1.5 mM $CaCl_2$, 0.05% Ruthenium red (Sigma), 1% agarose, Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) reagents and Apple. The sample was collected from the market solid waste of Koyambedu market, Chennai, India in a sterile container.

Isolation, screening and identification of pectinase producing organism

The collected sample was serially diluted upto 10^7 dilutions. It was plated on NA medium and incubated at 37°C for 24 hours. The isolated pure strains were screened for extra cellular pectinase production using PA Medium containing PGA as a substrate [6]. The substrate utilized zone around the colony was observed using 3.3% CTAB solution overlaid on the medium and incubated for 10 min. The positive strain that produced maximum pectinase enzyme was selected and identified by biochemical and morphological test.

Preparation of inoculum for bacteria and production of extracellular enzymes

The inoculum for further enzyme modulation and other studies was prepared using LB medium. The pure culture was inoculated and incubated at 37°C in a rotary shaker over night. The fresh over night culture was used as an inoculum for enhanced enzyme production. Five ml of mother inoculum was cultured in 100ml Nutrient Broth, from that 5% of inoculum (0.5 @ 600 OD) was transferred aseptically into the Growth medium. The inoculated medium was incubated at 37°C for 48 hours by shake flask fermentation method at 200rpm [6].

Determination of extracellular enzyme activity

Aliquot of 10 ml of the culture suspension was centrifuged at 5000

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rpm for 15 minutes and cell free extract was subjected to enzyme assay. This extract was stored at 4°C for further analysis. The plate assay was performed using Pectate Agar plates amended with PGA. The Agar plates were prepared by mixing 0.5% PGA with 1.5% agar. A 10 mm diameter well was cut aseptically with the help of cork borer. The wells were filled with 50µl and 100µl of culture filtrate and incubated at 37°C overnight. The substrate utilized zone was observed around the wells for production of pectinase. CTAB solution (3.3%) was overlaid on the medium and kept for 10 minutes [7].

Pectinase activity was assayed by the pectin degradation method using TBA [8]. One ml of crude enzyme added to 1.5ml of substrate (1.2% pectin in 0.2M Tris –HCl pH 8.5) was incubated at 35°C for 1 hour. The reaction mixture was terminated by adding 1.5ml of (0.01M) TBA and 0.7ml of 1N HCL and the contents were boiled in water bath for 20 min. Absorbance was measured at 540nm in UV- VIS spectrometer (Beckmann).

The total protein content was measured using Bradford method (1976). One ml of culture filtrate was mixed with 5 ml of Bradford reagent. Absorbance was measured at 595 nm. The protein concentration was determined using BSA as standard.

Effect of ph, temperature and incubation time

Five percent bacterial inoculum added aseptically to 500 ml of sterile growth medium was incubated at 37°C at 150 rpm. Twenty ml of culture was added periodically for 48 hours at every 6 hours intervals. The pectinase activity was determined in the culture filtrate [7].

The effect of pH on pectinase activity was determined using 5% inoculum in 100 ml of growth medium at different pH (7, 7.5, 8, 8.5, 9, and 9.5) incubated at 37°C for 36 hours [9]. The effect of temperature on enzyme activity was determined using the inoculated medium (pH 8) incubated at different temperatures (28°C, 32°C, 37°C, 42°C, 47°C, and 52°C) for 36 hours[7].

Optimization of different Carbon, Nitrogen Sources and Supplements for pectinase production

The maximum pectinase activity was estimated using different carbon sources such as Glucose, Lactose, Sucrose, Pectin, PGA and Maltose in inoculated medium, (5% inoculum in 100 ml of Growth medium, pH-8.5) incubated at 37°C for 36 hours. Different nitrogen sources such as Yeast extract, Tryptone, Peptone, Potassium nitrate and Ammonium sulphate were used, similarly different supplements like SDS, Triton-X 100, EDTA, zinc sulphate and Magnesium chloride were used in the medium [10]. The Protein content and Enzyme activity was determined from the culture filtrate collected.

Effect of different crude substrates on pectinase production

The Growth medium (pH-8.5) was prepared and pectin was replaced by different natural products such as Sugarcane baggase, Orange baggase, Rice bran, Wheat bran, and Green gram peel were used as substrates. Five percent bacteria was inoculated in the medium with different substrates and incubated at 37°C for 36 hours [11]. The amount of total protein and Enzyme produced from different substrates were estimated using the culture filtrate.

Partial purification of the enzyme

The culture filtrate was precipitated using 80% w/v of Ammonium sulphate [12]. The precipitated protein was separated by centrifugation around 10,000 rpm for 10 min. The supernatant was discarded and the pellet was dissolved in 2 ml of 0.5 mM Tris-HCL (pH 8). The precipitated bacterial and fungal protein was transferred to the dialysis membrane, which was pre treated by immersing into warm 0.5mM Tris-HCL buffer (pH-8) for 10 minutes (Sigma). The pack was suspended freely into a large beaker, which contains around 500 ml of 0.5 mM Tris-HCL buffer (pH-8). The buffer was stirred slowly using magnetic stirrer and the entire setup was placed in the cold room for 48 hours. The buffer was changed periodically for every 12 hours for better dialysis. After dialysis the clip from one end of the membrane was removed and the sample was transferred in to the clean plastic vials.

Sds page analysis and zymography of pectinase

The protein profile and the presence of enzyme were confirmed by SDS PAGE analysis [13]. The SDS-PAGE was performed in 13 % (w/v) gels and samples were heated for 10 min at 45°C in the sample buffer before loading on wells. After electrophoresis, gels were soaked in 2.5% (w/v) Triton X-100 for 30 min, washed in 100 mM glycine buffer pH 10.0, 1.5 mM $CaCl_2$ for 30 min and overlaid with 1% agarose gel containing 0.1% polygalacturonic acid in the same buffer as above. After 30 min of incubation at 45°C, gels were stained with 0.05% (w/v) Ruthenium red (Sigma) for 10 min and washed with water until pectatelyase bands became visible [14].

Pcr amplification of 16s rDNA

The PCR amplification of 16s rDNA was carried out [15], using synthetic primers such as **Forward primer:** (5′ - ATT GGT GAC ACC GAT CAA ACA - 3′) and **Reverse primer:** (5′ - TCA TAC GTA TGG ATG TTA TTC - 3′).The amplified product was run in 1% agarose gel electrophoresis to confirm the amplification [16].

Determination of pectinase activity on fruits

The efficiency of extra cellular activity of pectinase enzyme was determined Ranveer et al. [17]. Finely chopped apple pieces of 20g weight were added in two beakers. In one beaker 5ml of partially purified pectinase enzyme and in the other beaker 5ml of distilled water was added. This setup (after stirring the contents well) was placed inside the boiling water bath at 40°C for 20 minutes. With the help of paper coffee filter the juicy watery part was filtered from the beakers. The activity of enzyme on apple was determined. Distilled water with apple pieces acts as control (Figure11).

Results and Discussion

Seventeen bacterial strains were isolated from the waste samples,

Plate 1: Screening of *Bacillus cereus* for Pectinases activity in pectate agar plate.

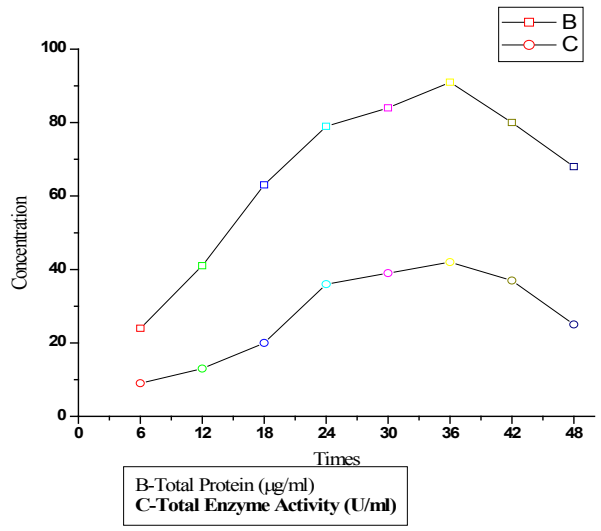

Figure 1: Enzyme Production and Total Protein Content of Bacterial Isolate at Different Incubation Time.

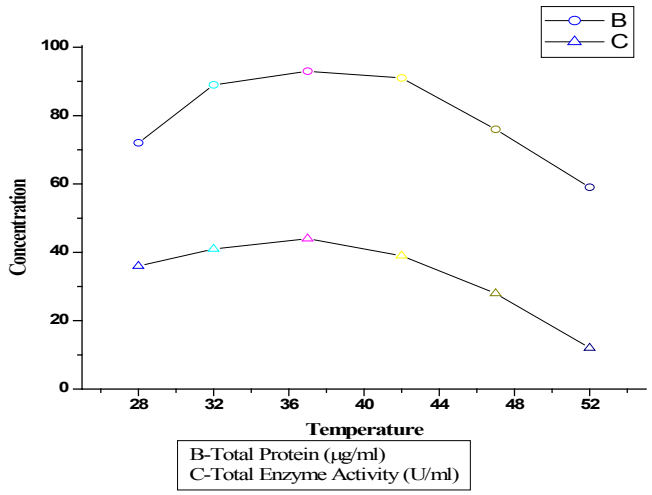

Figure 2: Effect of temperature on total protein and enzyme production by bacterial isolate. The protein concentration is obtained at different temperature (28°C - 52°C). The optimum temperature for total protein content and total enzyme activity is found to be 37°C.

but later during screening it was found that only 6 bacterial strains showed positive results on pectinase production. Among 6 bacterial strains the better zone forming bacterial strain was identified as Bacillus cereus, by using standard biochemical tests (Plate 1).

Effect of different process parameters for pectinase production

Time interval on enzyme production: The culture was checked for enzyme activity for 48 hours at every 6 hours interval. The results revealed that there is gradual increase in production from 24th hour and higher production occurred at 36th hour (Figure 1). This shows that bacterial isolate should have maintained its log phase from 24th hour to 36th hour. Besides, it is believed that the higher production of pectinase has occurred in extreme log phase because even though the log phase was maintained around 24th to 36th hour, the followed drop of production has indicated that the organism should have entered the stationary phase of growth. This variation of log phase timing is based on the nutrients present in the medium and the cultural condition of the organism. This result correlated with the work of [7] by using some Gram positive bacteria like *Bacillus sp.*

Temperature and pH

From our study, it was found that the mesophlic organism has the potential to produce higher amount of pectinase (44U/ml) at 37°C (Figure 2). This indicates that the optimum temperature for better

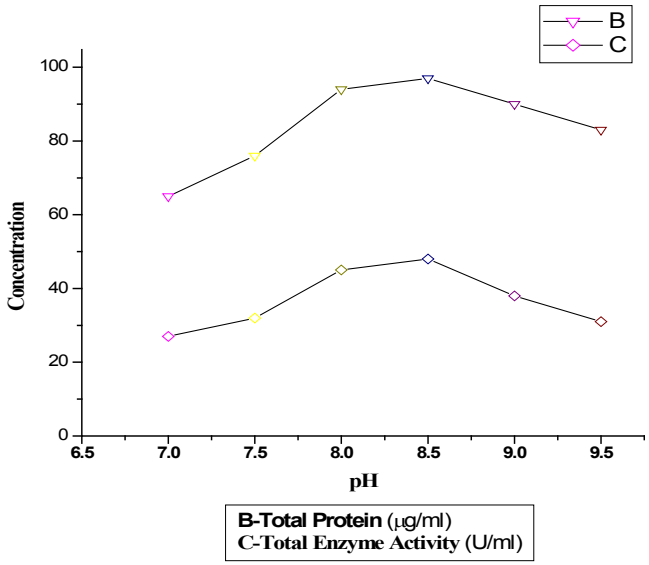

Figure 3: Effect of pH on total protein and enzyme production of bacterial isolate. The total protein content and total enzyme activity was measured at different pH (7.0 - 9.5). The optimum pH for higher amount of total protein content and total enzyme activity was found to be 8.5.

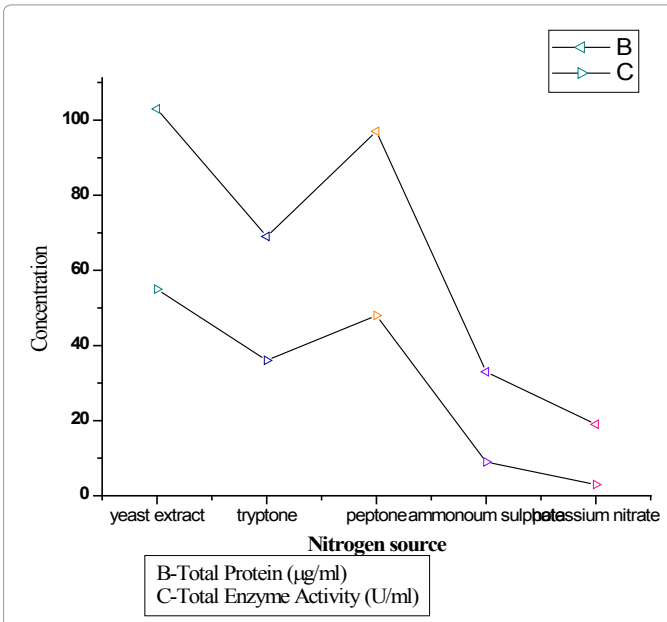

Figure 4: Enzyme production and total protein production on different nitrogen sources. Different nitrogen sources were used. The best nitrogen source for over production of enzyme is found to be yeast extract.

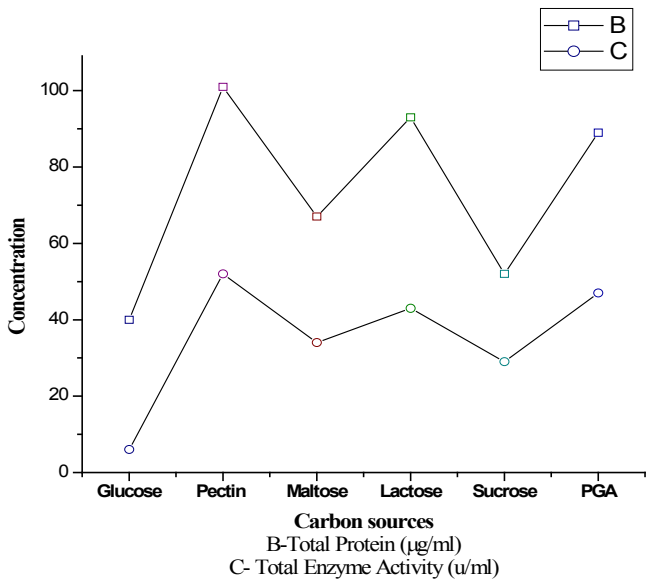

Figure 5: Enzyme production and total protein production on different carbon source. The total protein content and enzyme activity is found to be higher by using.

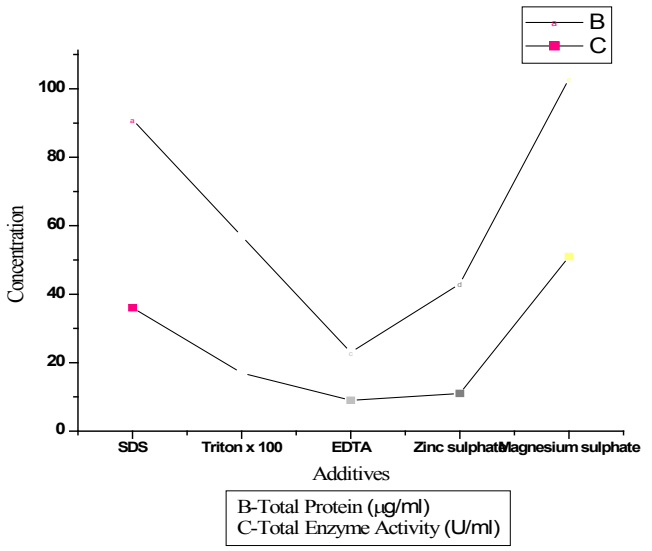

Figure 6: Enzyme and total protein production on different additives.

production of bacterial isolates is 37°C. Many *Bacillus spp.* needs 32-37°C for better production of pectinase [7].

Similarly the study results revealed that the pH around 8.5 is optimum for enhanced pectinase production (Figure 3). Most of the *Bacillus spp.* produce high amount of pectinase between pH 7-9 [6].

Carbon, nitrogen source and supplements

Pectin was found to be the right carbon source for bacterial strain for higher production of pectinase (Figure 4) than glucose, due to feed back inhibition. The yeast extract was found to be the best nitrogen source for bacterial isolate to produce pectinase enzyme compared to other nitrogen sources like Ammonium sulphate, tryptone, peptone and potassium nitrate (Figure 5). Magnesium chloride has enhanced the pectinase production in bacteria (Figure 6). This induction may be varying from organism to organism based on the mineral requirement and tolerance against the ionic detergents. *Bacillus sp*. has produced considerable amount of pectinase from the medium amended with 0.5 ppm of Ca^{2+} [7].

Crude substrates

The results revealed that wheat bran was found to enhance pectinase production (Figure 7). Most of the *Bacillus* spp produce higher quantity of enzyme in wheat bran substrate [12].

SDS PAGE and Zymogram

The protein profile analyzed by SDS-PAGE showed the presence of multiple protein bands, around molecular weight 30 - 50 KDa, which confirms the presence of pectinase enzyme. This result resembled with the previous work [13], (Figure 8). In zymogram the SDS- PAGE containing pectinase was detected by gel coat. A clear yellow zone appearance in SDS PAGE shows the presence of partially purified pectinase (Figure 9).

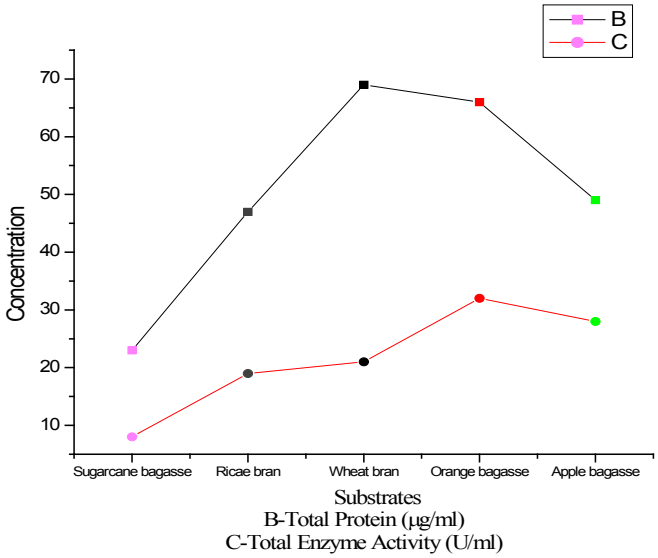

Figure 7: Enzyme and total protein production on different crude substrates.

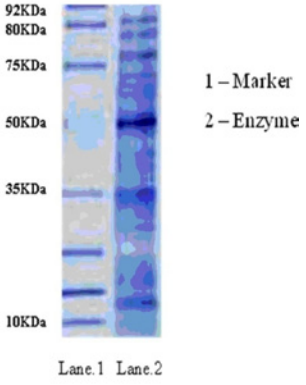

Figure 8: Enzyme profile in SDS- PAGE, lane1 (Marker), lane 2 (Enzyme).

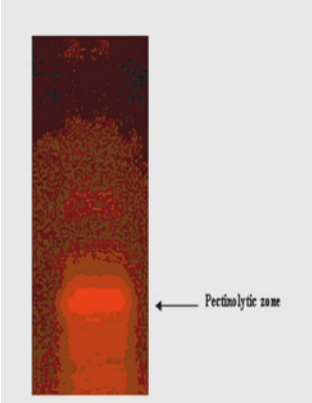

Figure 9: Zymography of pectinases.

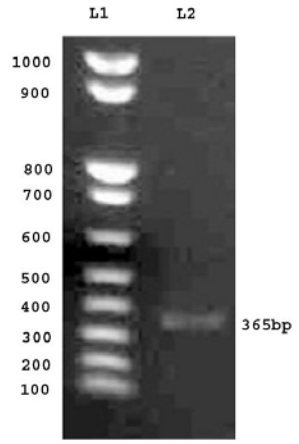

Figure 10: PCR Amplification DNA Lane 1(Marker), Lane 2(Enzyme), 365bp band was observed.

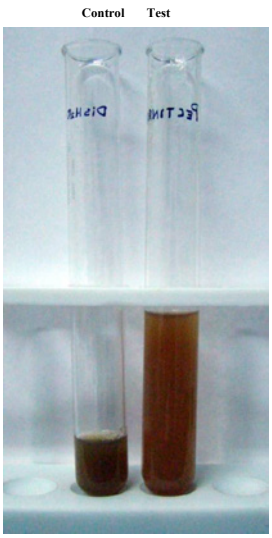

Figure 11: Apple Juice Extraction with Partialy Purified Pectinase.

Dna profile

The 16S rDNA primer was designed for the specific detection of *Bacillus cereus* and was tested with genomic DNA of *Bacillus cereus*. A PCR product of desired size of 365 bp was obtained in reactions containing genomic DNA of the targeted organisms. In DNA sequence analysis of *Bacillus cereus*, the amplified result was much similar when compared with pectinase producing *Bacillus cereus* isolated from the market solid waste. Similar work was done in 16S rDNA sequence analysis of *Bacillus cereus* and closely related species using gyrase B gene (gyrB) as a molecular diagnostic marker (Figure 10) [17].

Conclusion

The pectinase enzyme producing bacterial strain was isolated from market solid waste (Koyambedu market, Chennai, INDIA). The isolated bacterial strain is identified as *Bacillus cereus*. The production and optimization studies revealed that *Bacillus cereus* requires 37°C, pH 8.5, pectin (carbon source), yeast extract (nitrogen source), and 36 hours of incubation time for higher pectinase enzyme production. In addition to this, for enhanced production of pectinase, wheat bran and Magnesium chloride acts as a good agro waste substrate and supplement respectively. The partial purification of pectinase was employed by ammonium sulphate precipitation. The partially purified pectinase was characterized and the protein profile was analyzed in SDS-PAGE and Zymogram by showing the suitable bands. The genomic DNA extraction was performed for further work on 16S rDNA Sequencing. The present study clearly indicates that the microbes can be used in fruit juice manufacturing and in allied industry for cost effective production of pectinase. Another important advantage is that the enzyme's properties can be altered by altering the genes responsible through rDNA technology for enhanced production.

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