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Isolation and Characterization of Alkaline Pectinase Productive *Bacillus tropicus* from Fruit and Vegetable Waste Dump Soil

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HIGHLIGHTS

- Alkaline pectinase is very significant commercial enzyme used in bioscouring process.
- In traditional scouring, textile industry utilizes harmful alkaline chemicals to expel non-cellulosic material from the fabrics.
- Alkaline pectinase productive *Bacillus tropicus* was isolated from fruit and vegetable waste dump soil.
- *Bacillus tropicus* characterized by 16S rDNA sequences analysis.

Abstract: Alkaline pectinase is the utmost significant industrial enzyme of the bioscouring process. By considering bio scouring of cotton, 30 microbial isolates from fruit and vegetable waste rich dump soil of Solang Valley and Vasishta (Manali, Himachal Pradesh, India) were isolated and screened for the alkaline pectinase production in the current research work. Only four isolates P3, P16, P21, and P27 were capable to produce extracellular alkaline pectinase at pH 9. Further by applying submerged fermentation, the alkaline pectinase production was quantitatively screened. The most efficient isolate was P3 identified as *Bacillus tropicus*, based on morphological, biochemical, and molecular characterization. Molecular characteristics confirmed by 16S rDNA sequence analysis. The nucleotide sequence of the isolate was novel with a 97% similarity index and submitted to the GenBank with accession number MK332379. The *Bacillus* strain selected was active at broad pH range from 8-10.5 and a temperature range from 25-50 ^{o}C.

Optimum pH and temperature observed were 9 and 37 ºC respectively and can be suitably used for the bio scouring process for the pretreatment of the fabrics.

Keywords: Alkaline Pectinase; *Bacillus tropicus*; Bioscouring; vegetable waste; submerged fermentation.

INTRODUCTION

Pectinase is a group of one of the commercially important enzymes degrading pectin present in the plant cell wall. Pectinases degrade polysaccharide pectin to monogalacturonic acids and based on activity seven different classes of pectinases are pectinesterase (EC3.1.1.11), polygalcturonase (EC3.2.1.15), galacturan 1, 4-α-galactouronidase (EC3.2.1.67), exopoly-α-galactouronosidase (EC3.2.1.82), endopectate lyase (EC4.2.2.2), exopectate lyase (EC 4.2.2.9) and endopectin lyase (EC4.2.2.10) [1].

Pectic substances are complex polysaccharides with α (1-4) linkage in galacturonic acid backbone present as a cementing material in the middle lamella and the primary cell wall of all the plants [2,3]. Depending upon the different sources of the plant materials concentration of the pectic substances varies [4]. Commercial pectins are almost exclusively derived from the citrus peel or apple pomace, both by-products from juice manufacturing. Apple pomace contains 10-15% of pectin on a dry matter basis and citrus peel contains 20-30% [5,6].

Industrial enzyme's global market is growing day by day and in the world market, the pectinases production accounts for about 10% of the total enzyme production. By 2021 the market is estimated to reach around 35.5 million dollars [7]. In altering enzyme properties pH has a definite role. Acidic pectinases find a wide range of applications in the fruit processing industry for fruit juice clarification [8] and liquefaction of fruit juices [9] while alkaline pectinases find applications in various industrial processes such as fabric, pulp, and paper industry [10].

Alkaline pectinase is an emerging enzyme of commerce with primary employment in the textile and paper industries. Microorganisms are the main source of enzymes due to usage of low-cost substrates [11]. The textile industry is the utmost polluting industry due to maximum chemical usage. Traditional scouring involves alkaline chemicals to expel non-cellulosic material for soft and hydrophilic fiber, suitable for industrial applications. Due to the usage of the alkaline chemicals textile industries releases wastewater with high values of total dissolved solids, chemical oxygen demand, and biological oxygen demand [12-13]. Thus, need of the hour for sustainable development is the replacement of chemical processes to eco-friendly biochemical processes. Bioscouring employs enzymes to remove impurities without disturbing fiber's structure, strength, and environment [14-15].

Due to cheap production, easier gene manipulations, and faster product recovery, microbially derived pectinases find more use due to their advantage over a plant and animal-derived pectinases. The main microbial sources of pectinolytic enzymes are yeast [16], bacteria [17-18] and large varieties of fungi and particularly *Aspergillus* species [18-19]. Bacteria with well-characterized biology are well exploited as a source of industrially important enzymes. Bacterial pectinases are majorly extracellular enzymes with wide applications in bleaching of papers, wastewater treatment, and coffee fermentation [20-23]. One of the largest genera *Bacillus* from *Firmicutes* family covers a great diversity of strains. *Bacillus* is endospore-forming, gram-positive, rod shape motile bacteria; some are aerobes or facultative anaerobes. Spores are formed under the adverse condition of nutrition or temperature or both. Low cost production with new microbial isolates is fascinating the attention of many researchers nowadays [24]. According to Rehman and coauthors [25] alkaline pectinase produced by *Bacillus licheniformis* can be used for vegetables and food processing industries effluent treatment and used for bleaching of paper, textile industry for degumming of fiber and protoplast isolation [26]. Alkaline pectinase improves viscosity, breaking length, and porosity of the pulp [27-29]. It also reduces scouring chemicals in the scouring of plant fibers. According to Beg and coauthors [30] alkaline pectinase production is still underdeveloped as very fewer reports are available. According to Pilar and coauthors [31], common agro-industrial substrates such as wheat bran, sugarcane bagasse, rice bran, wheat straw, rice straw, corn cobs, sawdust, coconut coir pith, banana waste, tea waste, sugar beet pulp, apple pomace, orange peel, etc. are employed for pectinase production.

The present study deals with the isolation of the distinct bacterial strains from various sources and further screened for the maximum alkaline pectinase generation and strain identified by conventional and molecular methods. Fruits and vegetable waste rich soil of Solang Valley and Vasishta (Manali, Himachal Pradesh, India) is recycled to produce the alkaline pectinase. They are today one of the upcoming enzymes

of the commercial sector. It has been estimated that microbial pectinases account for 25% of the global market. Very few reports are available on the production of alkaline pectinases from *Bacillus tropicus*.

MATERIAL AND METHODS

Isolation of bacteria

The bacteria were isolated from soil collected from nearby fruit and vegetable waste dump in Solang Valley and Vashisht (Manali, Himachal Pradesh, India, Lattitude: 32.2396ºN and Longitude: 76.9787º E). One gram of soil sample was mixed in 100 mL of sterile distilled water, afterward serially diluted up to 10 dilutions. A volume of 100 µL of each diluted sample was inoculated in pectin agar medium (Sigma Chemical Co.,USA) and incubated at 37 °C for 48 h with pH range from 8 to 10. The isolated colonies were selected from dilutions 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} to obtain pure bacterial cultures. The obtained pure colonies were subcultured in pectin agar medium (Sigma Chemical Co., USA) for further studies and again tested for their potential to use pectin as a growth substrate and cultures maintained in nutrient agar slants.

Screening of alkaline pectinase producing bacteria

The colonies on the serial dilution plates of 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} were transferred on to another pectin agar plates and incubated at 37 ºC for 24 hrs again. Enzyme activity was detected by a clear zone around the colony with the potassium-iodide solution (Merck Ltd., India) [32]. Pectin agar medium described by Soares and coauthors [33] was selected for pure bacterial isolation and composed of citrus pectin (1.0%), ammonium sulfate (0.14%), di-potassium hydrogen phosphate (0.6%), magnesium sulfate (0.01%), potassium dihydrogen phosphate (0.20%), agar-agar (2.0%) and pH set was 9.0 (Sigma Chemical Co., USA).

Employing submerged fermentation bacterial strain showing the zone of hydrolysis on pectin agar medium were screened using above mentioned broth medium (Sigma Chemical Co., USA). 20% (v/v) pure cultures inoculums was incubated at 37 ºC for 24 hrs. After 24 hrs inoculation transfer into 100 ml fermentation medium and again incubated for 24 hrs at 37 ºC. After 24 hrs incubation was centrifuged at 8,000rpm for 10min for biomass separation. The cell biomass obtained from 1.0 mL culture was washed with 0.01 M Tris-HCl buffer (pH 9.0). By sonication cells were lysed and cell debris was removed by centrifugation at 1800 rpm for 15 minutes. The supernatant was checked for pectinase activity.

Molecular Characterization of alkaline pectinase producing bacterial strain

For the molecular characterization of the selected strain, 16S rDNA sequence analysis was executed. Chen and Kuo [34] method with small changes was used for obtaining genomic DNA. 1.0% agarose gel electrophoresis was performed for confirmation of the extracted DNA. Then after visualizing under UV light, PCR amplification was performed along with 16S rDNA sequence analysis [35]. 50 microliters of the reaction mixture were prepared to contain dNTPs mix, 2.5 microliters genomic DNA template, 1.0 microliter of 10 pM primers, and 2.5U of DNA polymerase. Gene Amp PCR System 2700 Applied Biosystems was used for the amplification of the genomic DNA. 1.0% agarose gel electrophoresis was used for the PCR amplified production analysis. After visualizing under UV light, the purification of the PCR amplified product was performed according to the instructional protocol by manufacturer Promega. Amplified DNA fragment sequence analysis performed using 3130 Genetic Analyzer by Applied Biosystem DNA sequencer. MEGA 7 software was used for the phylogenetic and molecular evolutionary genetic analysis [36].

Production and separation of alkaline pectinase protein

The growth was developed on characterized fluid media as depicted by Bhardwaj [37] containing 1 % unadulterated Pectin (Sigma Chemical Co., USA), 0.1 % $(NH_4)2SO_4$, 0.6 % K_2HPO_4, 0.2 % KH_2PO_4, $MgSO_4 \cdot 7H_2O$, pH 6.0 and incubated at 37°C. Cell debris was expelled by utilizing filter paper. The filtrate was blended in with super cold acetone and permitted to stand 15 min at low temperature [38]. The whole substance was centrifuged at 6000 rpm for 10 min. The supernatant was disposed of. The precipitate was disintegrated in the least volume of ammonium hydroxide derived buffer (0.1 M, pH 9.2) and further exposed to Sephadex G-75 (Pharmacia Fine Chemicals, Sweden) with specific modifications. The purity and molecular weight were determined by SDS PAGE.

Enzyme assay

The 3,5-dinitrosalicylic acid (DNS) method given by Miller [39] with slight modifications was employed for the determination of partially purified alkaline pectinase activity by the measurement of the amount of galacturonic acid production. The reaction mixture was incubated at 37 °C for 10 minutes. In this experiment, the standard graph was prepared by using mono-D-galacturonic acid, and 1.0% citrus pectin (Sigma Chemical Co., USA) was used as a substrate.

Quantitative Assay of Protein Estimation

The concentration of total protein in the enzyme sample was estimated by Lowry's method using bovine serum albumin (Sisco Research Laboratories Pvt. Ltd., India) as a standard [40].

Characterization of the partially purified alkaline pectinase

Partially purified alkaline pectinase was used for the characterization of the alkaline pectinase. To determine the optimum temperature of alkaline pectinase, purified enzyme in the presence of 1% pectin in sodium borate buffer (pH 9.2) was incubated at a temperature range from 25-50 °C. And pH effect was studied by carrying reaction in the presence of 1% pectin (Sigma Chemical Co., USA) in a range of buffer systems such as sodium phosphate (7.2-8.2) buffer and sodium borate (9.2-10.2) buffer (Merck Ltd., India) at 37 °C for 10 minutes. Similarly, the effect of the substrate concentration was studied by considering 0.5-3% substrate concentration in the reaction mixture. Finally, incubation time effect determination was done by employing sodium borate buffer pH 9.2, at 37 °C in the presence on 1% pectin for the period range from 24-120 hr with the intervals of 24 hr.

Growth media effect on the alkaline pectinase production

The growth of the isolated bacterial strain was studied using different production media such as malt extract, Nutrient Broth, yeast extract media, Mueller Hinton Broth, Luria-Bertani Broth (Hi Media Laboratories Pvt Ltd., India) culture in fermenter (Scigenics Pvt. Ltd, India) at 37 °C for 24 hrs.

Alkaline pectinase production kinetics:

The kinetics of alkaline pectinase production was based on the Luedeking-Piret model, initially the growth of the bacterial strain using different production media [41]. This is an unstructured model, which combines to both the growth associated and non-growth associated contribution for pectinase production. Hence, the pectinase production depends upon the bacterial growth rate and biomass concentration can be written as follows:

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \tag{1}$$

Where α is growth-associated formation constant of pectinase (U/g), β is non-growth-associated formation constant of pectinase (U/g), P is the pectinase concentration (g/L), P is the pectinase concentration (U/g) and t is time (h).

RESULTS AND DISCUSSION

Isolation and screening of alkaline pectinase producing bacterial strain

Thirty different bacteria were isolated from the soil for primarily screened based on pectinolytic activity by using the spread plate method. Out of these 30 strains, 1- 3 were from cauliflower, 4 -7 were from apple waste, 8-12 was from citrus fruit and 12 -30 were from soil samples. Three isolates (P3, P16, and P27) were found in pectinolytic activity (Figure 1). The positive strain was selected based on the largest hydrolysis zone around its colony in the potassium-iodide assay and evaluate to the other strains. Among them, P-3 isolate showed the largest hydrolysis zone and thus selected for the production, and studies were continued with this strain.

Table 1. Bacterial isolates from domestic and natural sources

Bacterial Isolates	Domestic and natural sources
P1	Cauliflower waste
P2	Cauliflower waste
P3	Cauliflower waste
P4	Apple waste
P5	Apple waste
P6	Apple waste
P7	Apple waste
P8	Citrus fruit waste
P9	Citrus fruit waste
P10	Citrus fruit waste
P11	Citrus fruit waste
P12	Citrus fruit waste
P13	Soil sample from Vashisht Kund
P14	Soil sample from Vashisht Kund
P15	Soil sample from Vashisht Kund
P16	Soil sample from Vashisht Kund
P17	Soil sample from Vashisht Kund
P18	Soil sample from Vashisht Kund
P19	Soil sample from Solang Valley
P20	Soil sample from Solang Valley
P21	Soil sample from Solang Valley
P22	Soil sample from Solang Valley
P23	Soil sample from Solang Valley
P24	Soil sample from Solang Valley
P25	Soil sample from Solang Valley
P26	Soil sample from Solang Valley
P27	Soil sample from Solang Valley
P28	Soil sample from Solang Valley
P29	Soil sample from Solang Valley
P30	Soil sample from Solang Valley

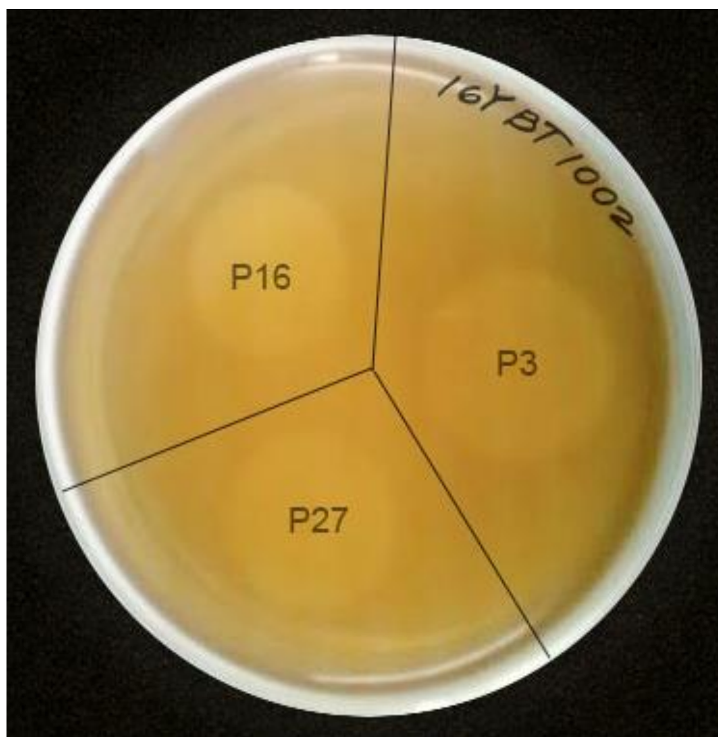

Figure 1. Pectin agar plate showing alkaline pectinase producing bacterial isolate stored after the qualitative screening

For the identification of the selected P-3 isolate the two conventional microbiological and current molecular technologies were used. The selected isolate based on the observed morphological and biochemical characterization (Table 2) compared with Bergey's [42] Manual of determinative bacteriology standard characterization, the isolate P-3 temporarily identified as *Bacillus* sp. [43].

Table 2. Characteristics of the potent alkaline pectinase producing isolate

Colony Morphology	**Opaque, dull, finally wrinkled and adherent colonies**
Cellular Characteristics	
Gram's staining	Positive
Motility	Motile
Morphology	Rods with rounded ends
Size	0.5-1.0 µm in length
Biochemical Reactions	
Amylase	+++++
Oxidase	+++++
Catalase	+++++
Indole production	--------
Voges-Proskauer	+++++
Citrate utilization	--------
Urea	--------
H_2S production	--------
Fermentation Reaction	
Glucose	Acid production
Galactose	Acid production
Maltose	Acid production

Isolate's genomic DNA was employed as a template for 16S rDNA amplification by PCR and agarose gel electrophoresis was performed for the examination of the PCR product (Figure 2). The PCR product was first purified to receive the correct sequence and sequenced. To GenBank database 16S rDNA nucleotide sequence was submitted with accession number MK332379 (Figure 3).

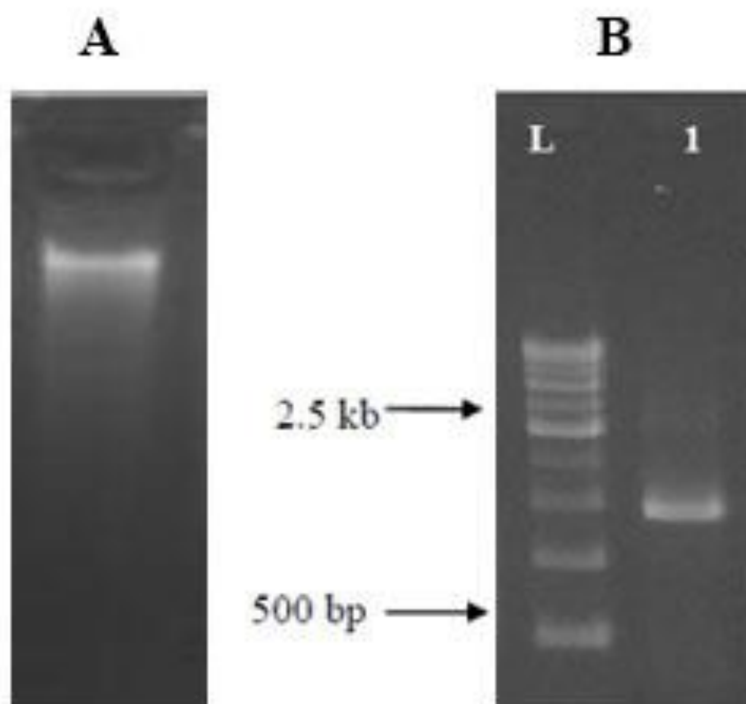

Figure 2. (A) DNA obtained from *Bacillus tropicus* strain MCCC 1A01406 (B) *Bacillus tropicus* 16S rDNA PCR product in agarose gel electrophoresis

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   1 tgctcttatg agttagcggc ggagggtgag taacacgtgg gtaacctgcc cataagactg
  61 ggataactcc gggaaaccgg ggctaatacc ggataacatt ttgaaccgca tggttcgaaa
 121 ttgaaaggcg gcttcggctg tcacttatgg atggacccgc gtcgcattag ctagttggtg
 181 aggtaacggc tcaccaaggc aacgatgcgt agccgacctg agagggtgat cggccacact
 241 gggactgaga cacggcccag actcctacgg gaggcagcag tagggaatct tccgcaatgg
 301 acgaaagtct gacggagcaa cgccgcgtga gtgatgaagg ctttcgggtc gtaaaactct
 361 gttgttaggg aagaacaagt gctagttgaa taagctggca ccttgacggt acctaaccag
 421 aaagccacgg ctaactacgt gccagcagcc gcggtaatac gtaggtggca agcgttgtcc
 481 ggaattattg ggcgtaaagc gcgcgcaggt ggtttcttaa gtctgatgtg aaagcccccg
 541 gctcaaccgt ggagggtcat tggaaactgg gggacttgag tgcagaagag gagagtggaa
 601 ttccatgtgt agcggtgaaa tgcgtagaga tgtggaggaa caccagtggc gaaggcgact
 661 ttctggtctg taactgacgc tgaggcgcga aagcgtgggg agcgaacagg attagatacc
 721 cttgggtagt ccacgccgta aacgatgagt gctaagtgtt agagggtttc cgccctttag
 781 tgctgcagtt aacgcattaa gcactccgcc tggggagtac ggtcgcaaga ctgaaactca
 841 aaggaattga cgggggcccg cacaagcggt ggagcatgtg gtttaattcg aagcaacgcg
 901 aagaacctta ccaggtcttg acatcctctg acaaccctag agatagggct tccccttcgg
 961 gggcagagtg acaggtggtg catggttgtc gtcagctcgt gtcgtgagat gttgggttaa
1021 gtcccgcaac gagcgcaacc cttgatctta gttgccagca ttcagttggg cactctaagg
1081 tgactgccgg tgacaaaccg gaggaaggtg gggatgacgt caaatcatca tgccccttat
1141 gacctgggct acacacgtgc tacaatgggc agaacaaagg gcagcgaagc cgcgaggcta
1201 agccaatccc acaaatctgt tctcagttcg gatcgcagtc tgcaactcga ctgcgtgaag
1261 ctggaatcgc tagtaatcgc
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Figure 3. 16S rDNA nucleotide sequence of *Bacillus tropicus* 1A01406

In the NCBI database, BLAST showed significant alignment of *Bacillus tropicus* with 97% similarities. And finally, the phylogenetic tree determined the isolate is *Bacillus tropicus* (Figure 4) [44].

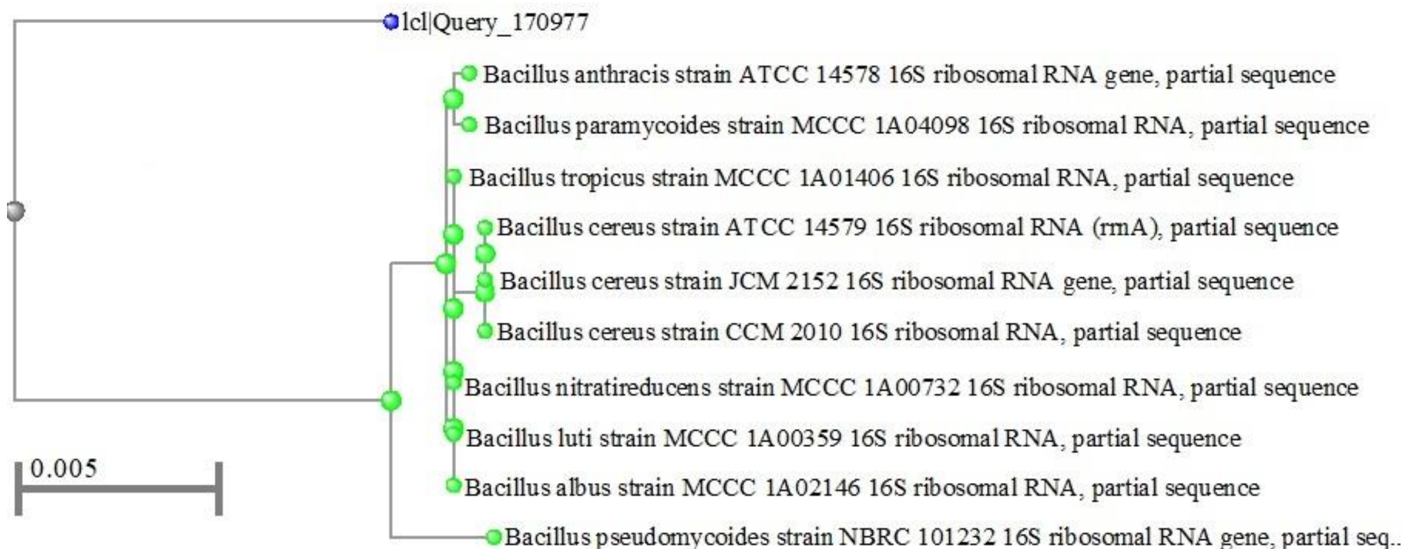

Figure 4. Phylogenetic tree exhibiting the lineage of *Bacillus tropicus* MCCC 1A01406 with another species

Pectinase Activity

The pectinase activity of *Bacillus* sp. was evaluated after purification steps. The enzyme unit was defined that the amount of enzyme required to synthesis one µmol of product per minute under standard assay conditions. The alkaline pectinase activity of isolate P-3, P-16, and P-27 were obtained 31.5, 5.25, and 10.02 U/mg respectively at 37 ºC for 24 hrs. The maximum pectinase activity was found by isolate P-3. The present result is supported by the reports of Kashyap and coauthors [45] where they have reported maximum activity 15.4 U/ml in YEP medium.

Effect of growth media:

Different media such as Malt extract, Nutrient Broth, yeast extract media, Mueller Hinton Broth, Luria-Bertani Broth were used to determine optimum media for alkaline pectinase production. Bacterial isolate was inoculated into 50 mL of these broths. The maximum growth was observed in Yeast Extract media supplemented with pectin proved to be the finest for the alkaline pectinase production under shaking condition about 43 U/mL of alkaline pectinase was produced while the alkaline pectinase production was minimum in Luria-Bertani Broth (Figure 5). The findings are also supported by the results of Oumer and Abate, they have also received highest pectinase production using yeast extract medium 10.1±1.44 U/mL [46].

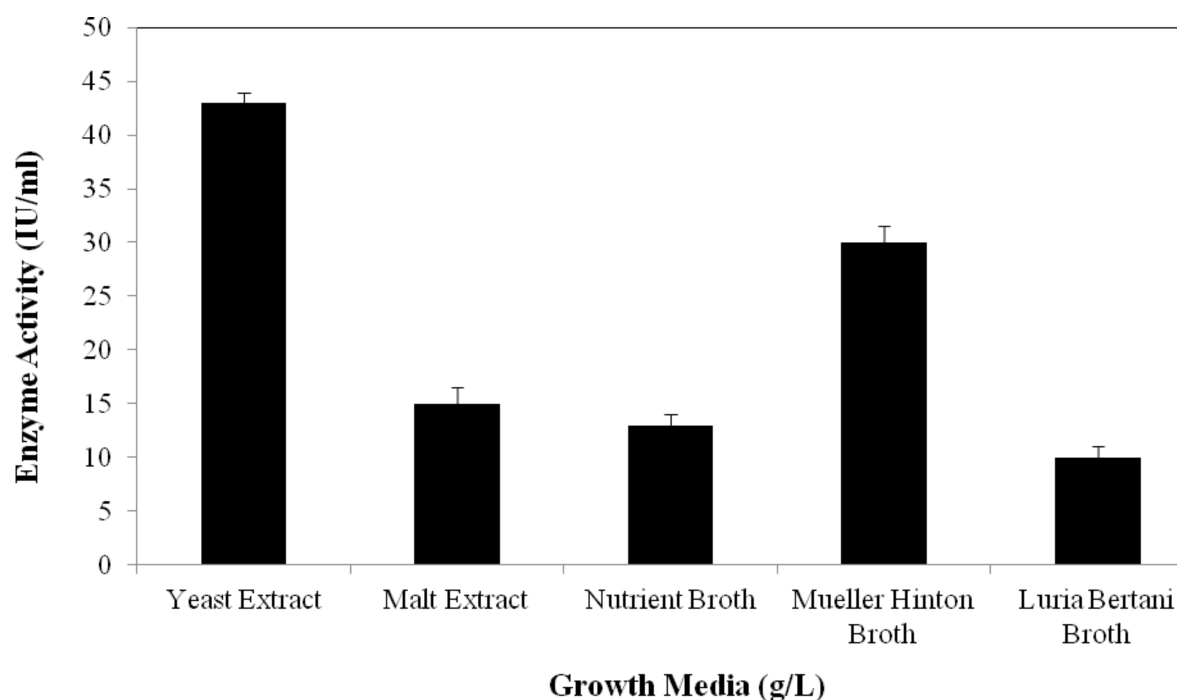

Figure 5. Effect of growth media on alkaline pectinase production. Bars correspond to standard deviation.

Production and purification of alkaline pectinase

The characterized liquid medium was employed for the alkaline pectinase production. From 48 h old culture the crude enzyme was collected, and acetone precipitated later. After that gel filtration was performed using Sephadex G-75 column chromatography. According to results enzymes were purified 1.4-fold with a specific activity of 9.21 U/mg by using cold acetone purification method and 9.5-fold with the specific activity of 65 U/mg by using column chromatography. Present results are supported by the findings of Mei and coauthors; they have reported increase in specific activity with the increased purification up to 142 U/mg [47].

Characterization of alkaline pectinase

Effect of pH and temperature

Factors such as pH and temperature have a great influence on enzyme activity. In the present study, alkaline pectinase was highly active at pH range from 7.5-10.5 with the optimum pH 9.0 and confirms the alkaline nature of pectinase (Figure 6). A few previous findings also suggest that the maximum pectinase production from most of the Bacillus spp. is between pH 7-9 [48]. Similarly, alkaline pectinase activity was found at the temperature range 25-50 °C with the optimum activity at 37 °C (Figure 7). All the same, Namasivayam and coauthors [49] observed optimum temperature at 37 °C for maximum pectin production from *B. cereus*.

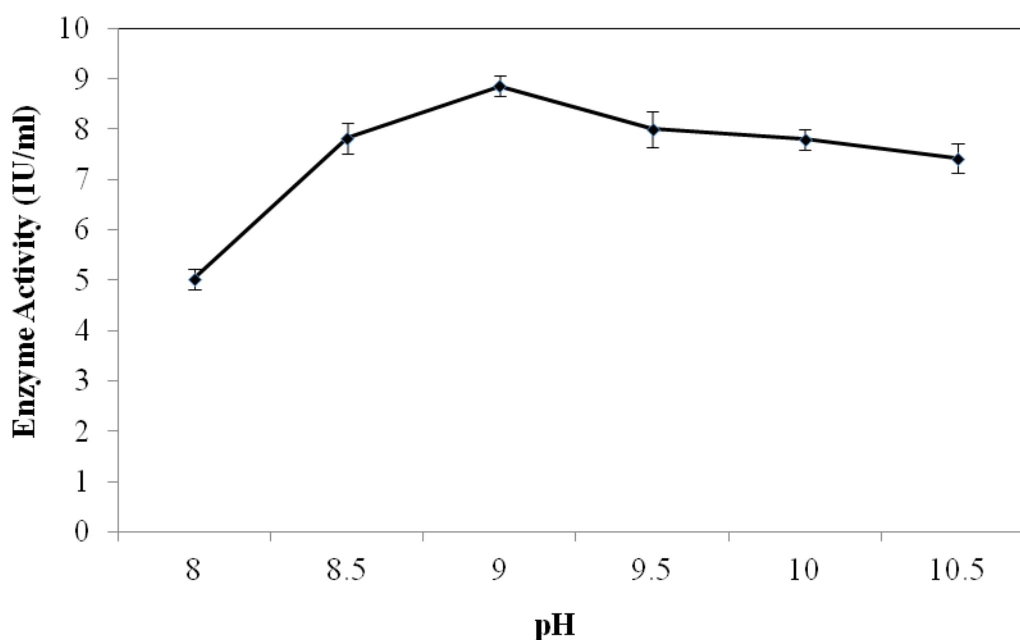

Figure 6. Effect of culture pH on enzyme activity

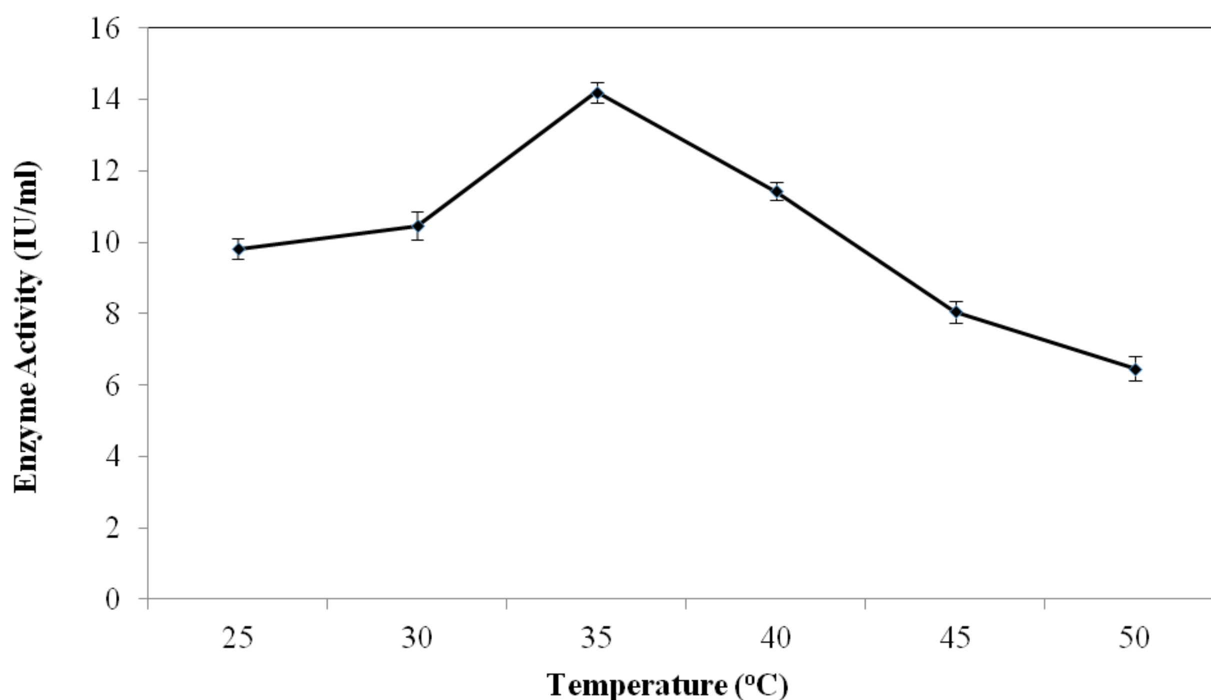

Figure 7. Effect of temperature on enzyme activity

Effect of Incubation time and substrate concentration

At the 2.5% pectin concentration, alkaline pectinase showed its maximum activity, with an increase in substrate concentration there was no significant change in alkaline pectinase activity (Figure 8). This finding is also supported by the results of the other study [50]. Same way, the optimum incubation time for alkaline pectinase was observed at 37 ºC for 72 hrs which is like the outcome of Loera and coauthors [51] (Figure 9).

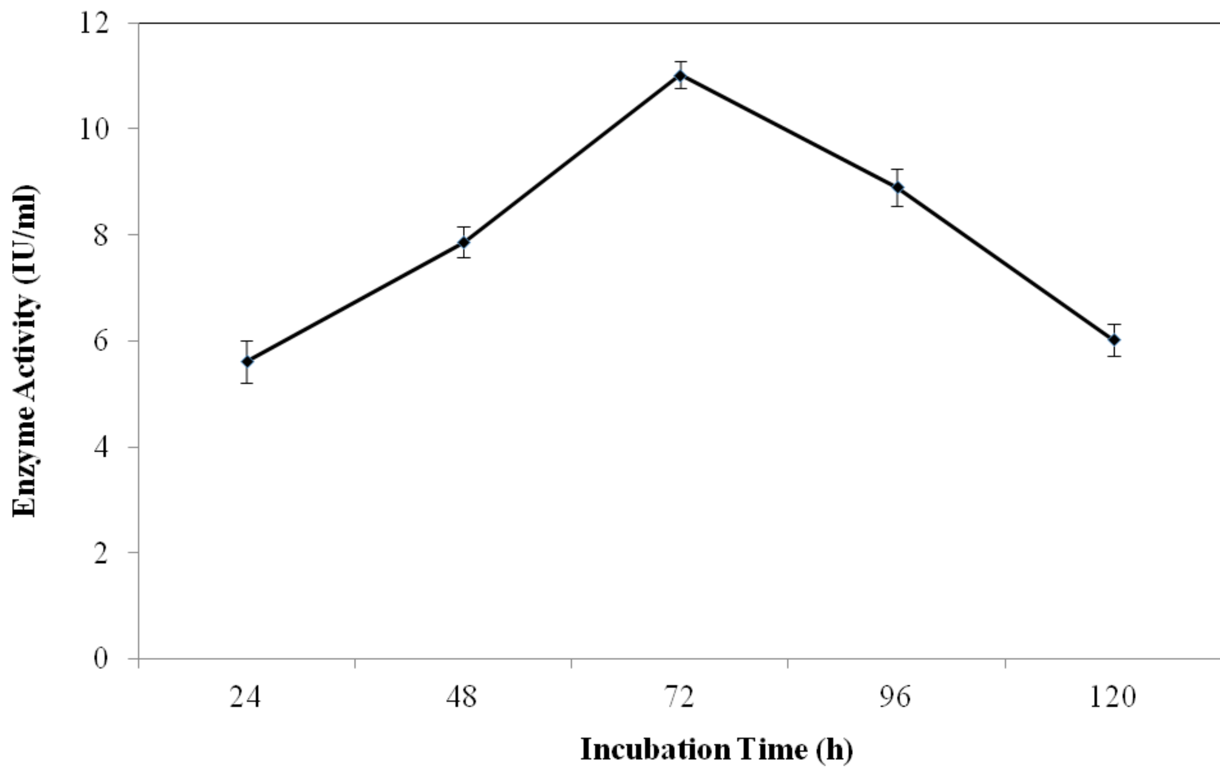

Figure 8. Effect of incubation time on enzyme activity

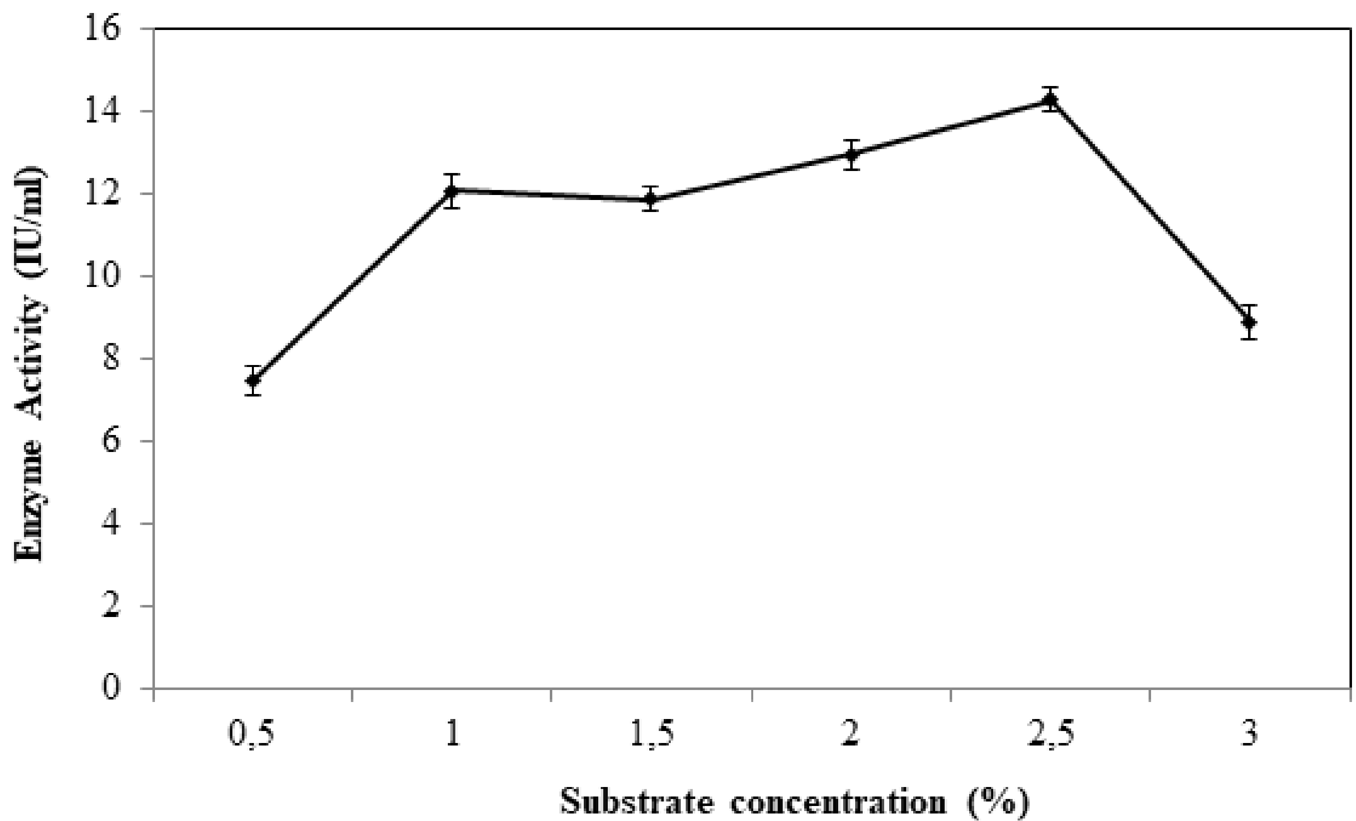

Figure 9. Effect of substrate concentration on enzyme activity

Enzyme production kinetics

Alkaline pectinase production kinetics was studies by experiment conditions. The Equation 1 is applied to simulate the production of alkaline pectinase by *Bacillus tropicus*. The highest concentration of pectinase was produced in 72 h (43 U/mL) by experimental results. The pectinase production obtained from the experiments was fitted to the equation under all experimental conditions. The experimental results match with the model predicted values reasonably well with R^2 value of 0.90.

The Michaelis-Menten Kinetics explained the rate of the reaction with respect to the substrate concentration with K_m and V_{max} values of 2.2 gm/L mM and 144 U/mg were determined under standard reaction conditions.

CONCLUSION

After screening various bacterial isolates to produce alkaline pectinase, only four isolates showed pectinolytic activity. By using the submerged fermentation technique these four isolates were further screened to produce alkaline pectinase. P-3 was observed to produce higher amount of alkaline pectinase. Based on morphological and biochemical characteristics the isolate was identified as *Bacillus tropicus.* The sequence of the isolated strain was 97% similar to 16S rDNA sequences of *Bacillus tropicus* from different sources of the GenBank database. Yeast extract media supplemented with pectin proved to be the best to produce alkaline pectinase by the isolated bacterial strain. Further to increase its industrial applicability biochemical characterization of alkaline pectinase from *Bacillus tropicus* MCCC1A01406 will be required. In the present study optimized pH, incubation time, substrate concentration and temperature to produce alkaline pectinase was obtained by using the potential isolate *Bacillus tropicus*.

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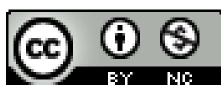
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