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Production and characterization of a thermo-pH stable pectinase from *Bacillus licheniformis* UNP-1: A novel strain isolated from Unapdev hot spring

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An efficient thermostable pectinase producer was isolated from the hot water spring of Unapdev and identified as *Bacillus licheniformis* UNP-1 using culture-dependent techniques by its morphological, microscopic, biochemical, physiological and molecular characteristics. Thermostable pectinase production was optimized in a submerged fermentation system using modified fermentation medium (MFM). The optimized components of MFM performed by changing one parameter at a time were pectinase defined liquid medium containing (g/L) 10 pure Pectin, 2.0 KH_2PO_4, 6.0 K_2HPO_4, 2.0 $MgSO_4 \cdot 7H_2O$ Optimized culture conditions were used for thermostable pectinase production. *Bacillus licheniformis* UNP-1 produced 55.2 U/mL of pectinase. Optimum pH and temperature for the production were 9 and 60 ^oC, respectively with 48 hours of incubation. The pectinase enzyme showed maximum activity at pH 11 and at 80 ^oC. Pectinolytic activity was the highest in the presence of Fe^{3+} metal ion. Optimum catalytic activity was recorded at substrate concentration of polygalacturonic acid of 3.5% after 90 min of incubation. The molecular mass of the dialyzed thermostable pectinase was 35 kDa. Partially purified pectinase enzyme was used for fruit juice extraction and clarification.

[Keywords: Thermophiles; Thermostable Pectinase; Unapdev hot water spring]

Introduction

Microbial pectinases are used extensively for fruit juice clarification, juice extraction, degumming of fibers and waste water treatment[1]. In applications where these substrates are poorly soluble at ambient temperature, thermostable pectinases have proved more useful[2, 3]. Isolation of microorganisms to be used in industrial applications from sources such as hot water spring habitats is much preferred, as these strains produce enzymes that are stable under high temperature conditions[4]. In present study we selected Unapdev hot water spring which is located in Jalgaon district at Chopda taluka (21o 15' 0" N, 75o 18' 0"E), Maharashtra, India, in the foothills of Satpuda hills range. Unapdev is a holy place about 6 km from Advad village in Chopda taluka of Jalgaon district in Maharashtra state of India. The hot water flows throughout the year from the mouth of godly cow and is collected in a kund[5, 6]. There is a growing need to develop a cost-effective, environment-friendly method for synthesizing such enzymes[7]. The aim of the study was to optimize thermostable pectinase production by *Bacillus licheniformis* UNP-1, characterize the enzyme and investigate its biotechnological applications[2, 8].

Materials and Methods

Isolation and screening of thermostable pectinase producers

Water sample was collected from Unapdev hot spring, and pH measured. The 100µl water sample was inoculated into nutrient agar medium containing (g/L) peptone 5, beef extract 3, NaCl 5, and agar 15. The plates were incubated for 24 hours at 60 ^oC and morphologically different colonies on the medium were isolated[9,10], and further these bacterial colonies were screened for pectinase enzymatic activity. The bacterial cultures were spot inoculated on pectin agar plate containing (g/L) 10 pure Pectin, 2.0 KH_2PO_4, 6.0 K_2HPO_4, and 2.0 $MgSO_4 \cdot 7H_2O$. The plates were incubated at 60 ^oC for 24 hours. Cultures were incubated on pectin agar, and grams iodine solution was flooded onto the plates to screen for pectinase production. After incubation the highest zone of clearance showed isolate was selected as an efficient pectinase producer. Isolated colonies were cultivated on nutrient agar slants. Cells (1%, v/v) from each isolate were grown overnight and inoculated into modified fermentation medium (MFM) comprising (g/L) 10 pure Pectin, 2.0 KH_2PO_4, 6.0 K_2HPO_4, and

2.0 $MgSO_4 \cdot 7H_2O$ and incubated at 60 °C at 120 rpm for 48 hours. The cultures were then centrifuged at 10,000 rpm for 10 minute at 4 °C. Cell-free supernatants were subjected to the qualitative pectinase cup assay [11]. One unit activity was defined as the amount of enzyme forming 1 µmol of galacturonic acid per minute at 70 °C. Total protein content was determined with bovine serum albumin as the standard[12].

Preliminary and molecular identification

Morphological and biochemical identification of the isolate was carried out according to Bergey's manual of Determinative Bacteriology[13]. The UNP-1 was also subjected to 16S rRNA molecular analysis. DNA was extracted by Insta Gene Matrix (Bio-Rad, USA) treatmentfrom cell pellets and the 16S rRNA gene was amplified in a thermo cycler (Applied Biosystems, USA) with pair of primers as- 27F AGA GTT TGA TCM TGG CTC AG, 1492R TAC GGY TAC CTT GTTACGACTT,[14] 785FGGATTAGATA CCCTGGTA, 907RCCGTCAATTCMTTTRAGTTT[15] used for sequencing purpose only. Sequencing was performed by using big dye terminator cycle sequencing kit. Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). The amplified 16S rDNA PCR product was gel-purified with a QIA quick Gel Extraction kit (Qia-gen, USA) and sequenced in an ABI PrismTM377 automated DNA sequencer (Applied Biosystems, USA). The 16S rDNA sequences were initially analyzed using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi)[18.] Phylogenetic tree was drawn using MEGA 6.06 to determine the genetic affiliation of the isolate of query with other closely related bacteria[16, 17, 19].

Optimization of physico-chemical parameters for maximum thermostable pectinase production

Effect of carbon, nitrogen, phosphorus sources, pH and temperature on enzymes activities were assessed by varying one parameter at a time. A culture of UNP-1 was grown overnight into MFM and incubated with shaking at 120 rpm speed under 60 °C for 48 hours. Samples were withdrawn aseptically after every 24 hours, and the catalytic activity of thermostable pectinase was determined under standard assay conditions[11].

The parameters tested for maximum pectinase production were carbon sources in 0.5% (w/v) such as glucose, lactose, maltose, fructose, D-arabinose, sucrose, D-mannitol, D-galactose, D-ribose, and trehalose. Nitrogen sources in 0.5% (w/v), such as peptone, ammonium sulfate, gelatin, ammonium chloride, ammonium nitrate, ammonium molybdate, urea, 4-aminoantipyrin, potassium nitrate, and sodium nitrate. Phosphorus sources in 0.5% (w/v), such as sodium di-hydrogen phosphate, disodium hydrogen phosphate, potassium hydrogen phosphate, di-ammonium hydrogen phosphate, ammonium hydrogen orthophosphate and some agricultural wastes were purchased from local market verities and used as a substrate sources in 1% (w/v), such as Gram bran (*Lens culinaris*) of Reliance agro, Wheat bran (*Triticum aestivum*) of D-mart, coconut peel, Black gram peel (*Vigna mungo*) of Reliance agro, Sorghum bran (*Sorghum bicolor*) of Reliance agro, Green gram (*Vigna radiata*) of D-mart, banana peel powder and used tea powder[10]. For bulk production, 500 mL of optimized modified fermentation medium was prepared in a 1-L Erlenmeyer flask. After sterilization, an optimized quantity of prepared inocula was inoculated and incubated under optimized culture conditions[10, 11].

Purification and characterization of thermostable pectinase

After production, a cell-free supernatant of UNP-1 was extracted by centrifuging whole fermented broth at 10,000 rpm and 4 °C for 10 min. Enzyme precipitation was carried out with concentrations of 70% ammonium sulphate. The enzyme precipitate was collected by centrifuging the ammonium sulphate fractions at 10,000 rpm for 20 min at 4 °C. The catalytic activity of the enzyme solution was determined under standard assay conditions. The active fractions were dialyzed against 0.2 mol/L glycine–NaOH buffer (pH 10.0), and the enzyme activity was determined. The total protein content of the enzyme was determined by the method of Lowry. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the molecular mass by comparison with the standard broad-range protein markers (Merck Biosciences)[11].

The catalytic activity of the partially purified enzyme was screened at pH 4.0-13.0 in an appropriate buffer system. Optimum temperature of the pectinase activity of reaction mixture was determined by incubating as per standard assay for pectinase enzyme in temperature ranges of 30 °C-100 °C. Optimum

substrate concentration was determined by varying in the range of 0.5% - 4%. The effect of incubation time on enzymatic activity was determined by incubating reaction for various time intervals such as 30 min to 180 min. The effect of various metal ions were studied on pectinase enzyme activity by using 10 mM metal ions, such as Mg^{2+} ($MgCl_2$), Fe^{3+} ($FeCl_3$), Zn^{2+} ($ZnCl_2$), Li ($LiCl_2$), Cu^{2+} ($CuCl_2$), and Na^+ (NaCl).

Statistical analysis

Mean values were derived from experiments performed in triplicate and used for graphical and tabular representation of data. Error is indicated as standard deviation (n = 3) calculated with MS-Excel 2013 software[14].

Application of thermostable pectinase in fruit juice extraction and clarification

For the extraction of juice from apple, the apples were chopped into cubes of roughly 5 mm in size. Then different enzymatic treatments were given and added in the beakers and they were stirred with a separate plastic spoon and wrapped with plastic. Then these beakers were put in water bath for 2 hours. After removing the beakers from water bath they were gently squeezed. Finally these juices were filtered by using paper coffee filters and measured by using 100 mL graduated cylinders. For studing the fruit juice clarification, 10 mL portion of juice was taken to remove the cloudy pulp particles and centrifuged at 3000 rpm for 10 min. The clarity of fruit juice was determined by measuring at 450 nm using UV-VIS spectrophotometer and distilled water was used as a blank[13].

Results

Isolation, screening and identification of pectinase producing bacteria

The pH of the Unapdev hot spring water samples was 9. Total 107 colonies were found on the agar plates out of which 13 morphologically distinct colonies were isolated. Of these, 10 showed zone of clearance on pectin agar plates. The isolate with the largest zone of clearance and with significantly higher activity (55.2 U/mL) was designated UNP-1. A cell-free supernatant of UNP-1 showed a zone of clearance around the well in a pectin agar plate. The phenotypic, biochemical and physiological characteristics of the UNP-1 isolate are shown in Tables 1, 2, 3, and 4. The 16S rRNA gene sequence was submitted to the GenBank nucleotide repository under accession number KT 447683. *Bacillus licheniformis* UNP-1 was also deposited in the Microbial Culture Collection at National Centre for Cell Science and was allocated the accession number MCC 3026. The nucleotide sequence of the16S rRNA gene of the isolate showed greatest homology (100%) with the previously published sequence of *Bacillus licheniformis* strain DSM 13 (NR 118996). On the basis of the morphological, microscopic, biochemical and physiological attributes, antibiotic sensitivity and molecular analysis, UNP-1 was identified as *Bacillus licheniformis*. Evolutionary relationship of *Bacillus licheniformis* strain UNP-1 in relation to other closely related members of the phylum *Firmicute* was determined from the phylogenetic tree (Fig. 1).

Optimization of physico-chemical parameters for maximum thermostable pectinase production

Bacillus licheniformis UNP-1 exhibited maximum growth and pectinase production at pH 9

Table 1 — Morphological characterization of isolate

Sr.no.	Charactors	UNP-1
1	Size	0.3mm
2	Shape	Circular
3	Color	White
4	Opacity	Opaque
5	Elevation	Raised
6	Surface	Smooth
7	Consistancy	Non-sticky
8	Margine/Edge	Entire
9	Gram's nature	+ve rod
10	Motility	Highly motile

Table 2 — Biochemical characterization of isolate

Sr. No.	Biochemical test	UNP-1
1	Nitrate reduction	+
2	Indole production	-
3	Methyl red test	++
4	Voges-Proskauer test	-
5	Citrate utilization	-
6	H_2S Production	-
7	Esculin hydrolysis	+
Enzyme Profile		
8	Catalase test	+
9	Amylase	++
10	Protease	-
11	Gelatinase	-
12	Pectinase	++
13	Lipase	+
14	Cellulase	-
15	Urease test	++

Table 3 — Carbohydrate utilization of isolates
+ Positive; - Negative

Sr. no.	Sugar Profile	UNP-1
1	Dextrose	++
2	Maltose	+
3	Mannose	+
4	D-Mannitol	-
5	Glucose	+
6	D-Galactose	+
7	D-Xylose	-
8	Inositol	-
9	Lactose	+
10	Sucrose	+
11	Fructose	+
12	Ribose	+
13	Sorbitol	+
14	Arabinose	-
15	Trehalose	+
16	Raffinose	-
17	Salicin	+
18	Dulcitol	-
19	Adonitol	-
20	Sodium citrate	-
21	Starch	+
22	Glycerol	+
23	Chitin	-
24	Pectin	-
25	Cellulose	-
26	Aesculin	-
27	D-Chloralose	-
28	Citrate	-
29	Ethanol	-
30	Methanol	-

(76±0.866025) (Fig. 2) and 60 °C (420±8.164966) (Fig. 3). *Bacillus licheniformis* UNP-1 showed maximum pectinase production after 48 hours of incubation with shaking at 120 rpm. The result of optimization showed the constitution of the most suitable fermentation medium as: D-Ribose (0.5% w/v), peptone (0.5% w/v), Potassium di-hydrogen phosphate (0.5% w/v) and coconut peel (1% w/v). However, it was not used after complete optimization hence not included in this study manuscript. Enhanced growth and production were seen in the medium supplemented with coconut peel (1% w/v) (58.8 U/mL) as a substrate (Fig. 4), D-Ribose as a carbon source (51 U/mL) (Fig. 5), and maximum growth and pectinase production were found with peptone (30.6 U/mL) as a nitrogen source (Fig. 6). Potassium di-hydrogen phosphate (27 U/mL) had shown maximum production when used as a phosphorus source (Fig. 7)

Table 4 — Antibiotic susceptibility pattern
R-resistant; S-sensitive

Antibiotics	Results
Bacitracin	R
Chloramphenicol	R
Penicillin	R
Polymyxin	R
Gentamycin	S
Neomycin	R
Ampicillin	R
Streptomycin	R
Sulphatriad	R
Tetracyclin	R
Piperacillin	R
Linezoid	R
Ciprofloxacin	S
Teicoplanin	S
Vancomycin	S
Augmentin	R
Norfloxacin	R
Co-Trimaxole	R
Tobramycin	S
Cephalothin	R
Gentamycin	S
Cephoxitin	R

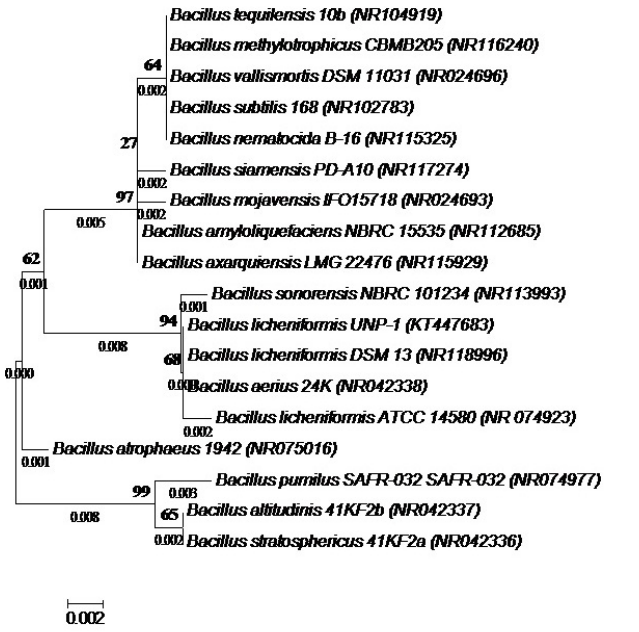

Fig. 1 — The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths (below the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 18 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 634 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.06.

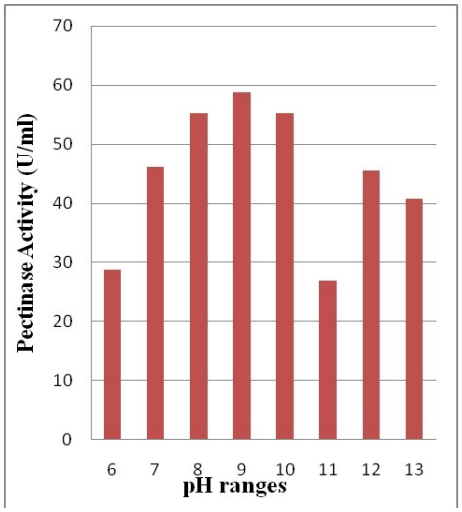

Fig. 2 — Effect of pH on pectinase production

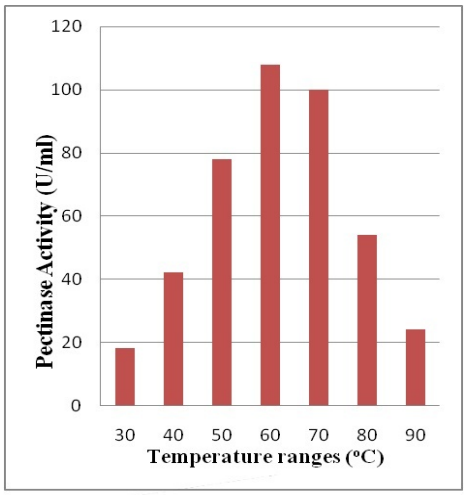

Fig. 3 — Effect of temperature on pectinase production

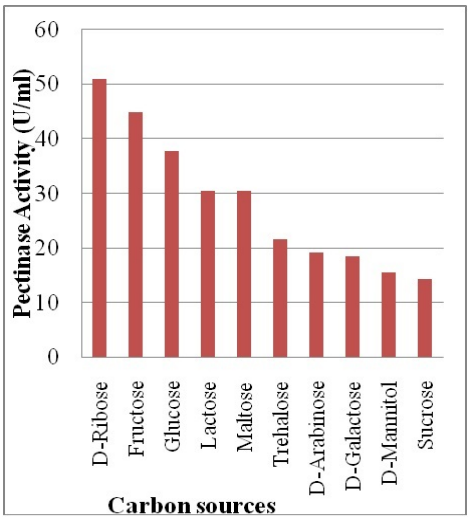

Fig. 4 — Effect of carbon sources on pectinase production

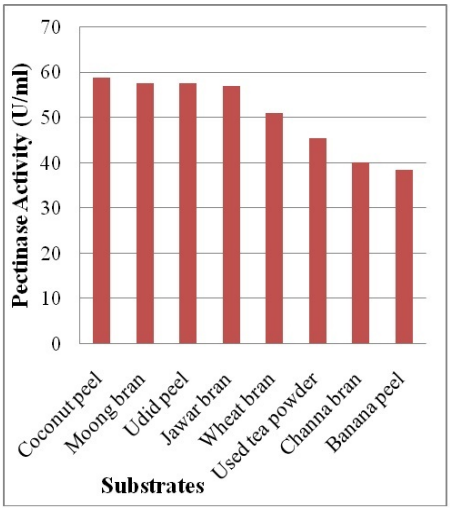

Fig. 5 — Effect of substrates on pectinase production

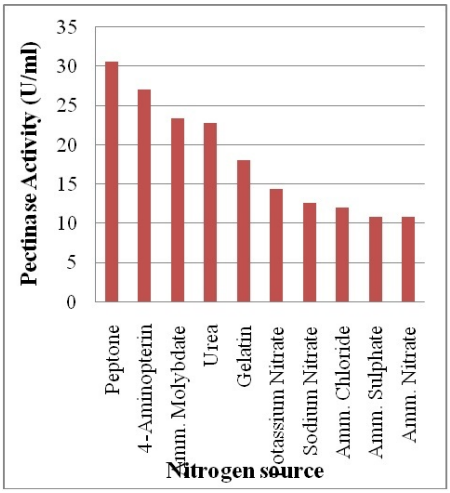

Fig. 6 — Effect of nitrogen on pectinase production

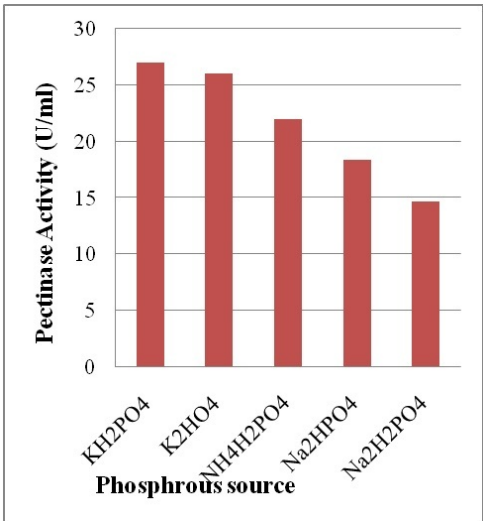

Fig. 7 — Effect of phosphorous on pectinase production

Production, partial purification and characterization of thermostable pectinase

The entire experiment of molecular weight determining was carried out as outsourcing experiment (Royal Life Sciences Pvt. Ltd., Hydrabad, India), where purification of pectinase was carried out prior to molecular weight determination. The molecular weight determined of partially purified pectinase enzyme was approximately 35 kDa. Catalytic activity of partially purified pectinase was determined at pH 4 to 13 and was the highest at pH 11 as 1.38 U/mL (11.84±0.070146) (Fig. 8). Catalytic activity of partially purified pectinase was determined at temperature 30 to 100 °C, when it showned the highest activity 1.44 U/mL at 100 °C (6.54±0.152944) (Fig. 9). Catalytic activity of partially purified crude pectinase in presence of 10 mM metal ions were recorded where Fe^{3+} ions showed 5.7 U/mL as the highest activity (Fig. 10). Catalytic activity of partially purified pectinase was 2.7 U/mL when 3.5% (20.16±0.47916) substrate concentrations were used (Fig. 11). Catalytic activity of partially purified pectinase was recorded 1.48U/mL after 90 min (6.72±0.152206) of incubation (Fig. 12).

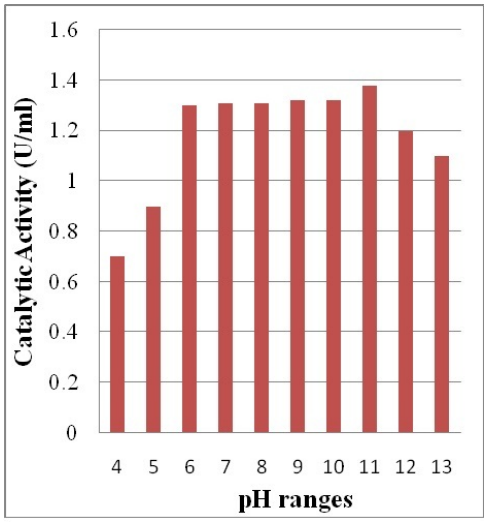

Fig. 8 — Effect of pH on catalytic activity of pectinase

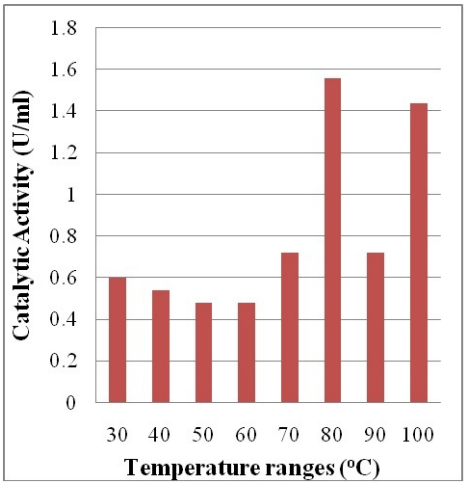

Fig. 9 — Effect of temperature on catalytic activity of pectinase

Thermostable pectinase for fruit juice extraction and fruit juice clarification

The fruit juice extraction was carried out by using the pectinase enzyme as well as the other enzyme along with pectinase. 23 mL of fruit juice was

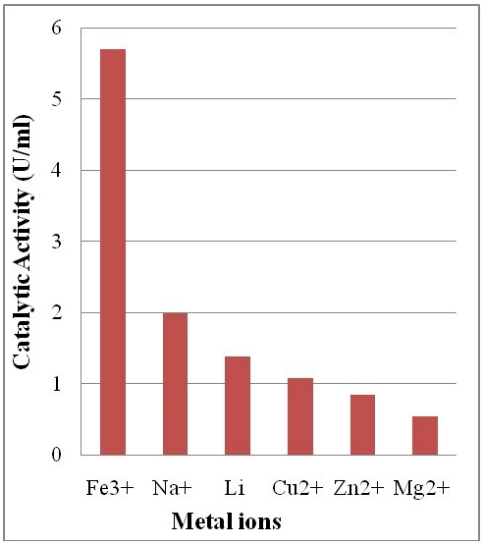

Fig. 10 — Effect of metal ions on catalytic activity of pectinase

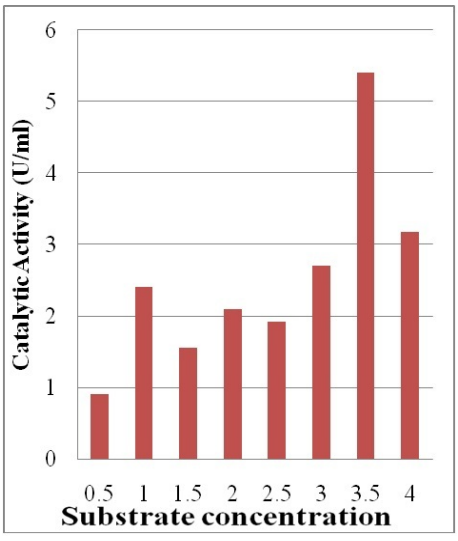

Fig. 11 — Effect of Substrate concentration on catalytic activity of pectinase

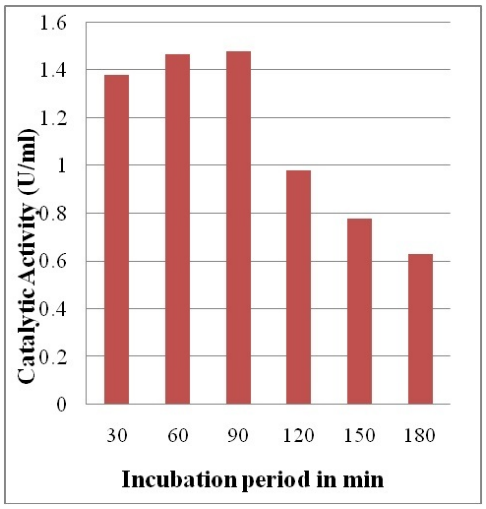

Fig. 12 — Effect of incubation period on catalytic activity of pectinase

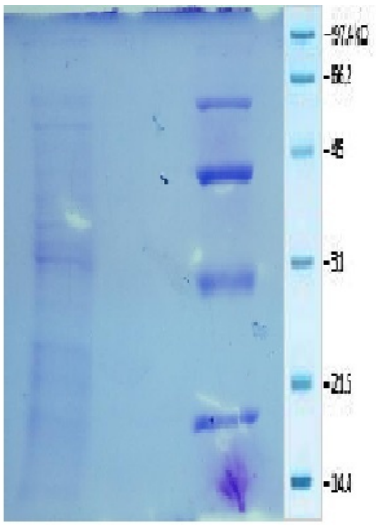

Fig. 13 — Molecular weight determination of pectinase 1-enzyme 2-molecular markers

extracted when apple without peel was treated with crude pectinase and 19 mL juice was extracted when treated with pure pectinase. 12 mL juice was extracted when crude pectinase treatment was given to apple with peel. 22 mL juice was extracted by the treatment of amylase on the fruit without peel and 16 mL was extracted from apple with peel. 18.5 mL juice was extracted when treatment of crude pectinase along with crude amylase given to the fruit without peel and 17 mL was extracted from the same treatment on the fruits with peel. And the apple juice which was extracted by the treatment of crude pectinase and amylase enzyme had more clarity as compared to that extracted using other enzymatic treatment (Fig. 13).

Discussion

Optimum pH and temperature of pectinase was at 9 and 60 °C respectively, however, in 2014, thermophilic pectinase from *Bacillus Licheniformis* reported maximum catalytic activity at similar range of pH23,25 while similar temperature range was reported in 2013 pectinase from *Bacillus halodurans* [26]. Optimization of catalytic activity by pectinase shown was the highest at pH 10 and at temperature 80 °C. In the present finding, the optimum catalytic activity shown by *Bacillus licheniformis* was the highest at pH 11 and 80 °C temperature. UNP-1 and t*Bacillus licheniformis* UNP-1 showed Fe^{3+} dependant pectinase activity, while in 2013 it was reported that most of the pectinase from *Bacillus halodurans* showed Ca^{2+} dependant pectinase activity[25]. Coconut peel was the best suitable substrate for highest catalytic activity of Pectinase while in 2014 it was reported that orange peel was the best suitable substrate for maximum catalytic activity of pectinase from *Bacillus Licheniformis*[25]. The highest catalytic activity of thermophilic pectinase was noticed in 3.5 % of substrate concentration after 90 min of incubation. The *Bacillus licheniformis* UNP-1 pectinase gives maximum production in fruit juice extraction and shows good result in fruit juice clarification. Similar study was carried out by Cao et al. in 1992 and Kashyap et al. in 2000[20-23].

Conclusion

An efficient thermostable pectinase producer was isolated from Unapdev hot water spring and identified as *Bacillus licheniformis* UNP-1. Maximum thermostable pectinase production was achieved by media optimization process of all constituents of medium and some agricultural wastes as a substrate. Hence, the UNP-1 isolate and its thermostable pectinase could find potential applications in biotechnological industries.

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