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Production and partial purification of pectinase from *Streptomyces chartreusis*

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ABSTRACT

Pectinases usually familiar as pectic enzymes, are a main class of enzymes used in industries like wine, paper and food for the processing of fruits, vegetables, tea and coffee, can be extracted from filamentous bacteria *Streptomyces* sp. This study was conducted during January to July 2019 at Mehsana Urban Institute of Sciences, Gujarat, India to extract pectinase enzyme from S*treptomyces* sp. isolated from the soils of Mehsana District, Gujarat, India. Ammonium sulphate precipitation and ethanol precipitation methods were used for the purification of extracellular pectinase and spectrophotometric analysis was carried out for the measurement of enzyme activity. The specific activity was observed in enzyme extract, 192.16 U/mg. The selected isolate was sequenced and identified as a *Streptomyces chartreusis* through 16srRNA sequencing (Accession number-MN133919). The optimum pH and temperature for pectin hydrolysis was observed as 7 and 37°C, respectively. The thin layer chromatography analysis was investigated to identify degradation products of pectin hydrolysis.

Key Words : Enzyme activity, Pectinase, *Streptomyces chartreusis*, TLC

INTRODUCTION

Pectinase enzymes are used mainly to degrade pectic compounds, which are commonly used in the manufacture and clarification of fruit juices and drinks (Sudeep *et al.,* 2020). Pectin is a major component of middle lamella and primary cell wall of higher plants. Pectins are acidic heteropolysaccharide of high molecular weight, mainly made up of (1-4) associated residues of d-galacturonic acid (Kavuthodi and Sebastian, 2018). Three major classes of pectic polysaccharides are known, all of them containing d-galacturonic acid to a greater or a lesser extent with homogalacturonan (HG), rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII) (Bader *et al.,* 2018). Pectinases are a group of enzymes that according to their mechanism of action, degrade pectic substances and are classified. For instance, methylesterases remove highly or partially esterified galacturonan from methoxy groups. Polygalacturonases catalyse glycoside bond hydrolysis in a random manner (endopolygalacturonase) or from the non-reducing end of galacturonic or digalacturunic acid residue releasing homogalacturonase (exopolyglacturonases) (Cornuault *et al.,* 2018). Pectinolytic enzymes or pectinases are also categorised by their mode of action and substrate: polygalacturonases subclassified as endo-polygalacturonases (E.C. 3.2.1.15) and exo-polygalacturonases (E.C. 3.2.1.67), pectin methyl esterase (E.C. 4.2.2.9 and EC. 4.2.2.2) or pectin lyases (E.C. 4.2.2.10) and pectin methyl esterase (E.C. 42210) (E.C. 3.1.1.11). It is recommended that a mix of different pectinase forms, along with other enzymes such as cellulases and hemicellulases can degrade various polymers by multiple enzymes resulting in maximum degradation of pectin in different raw materials, such as in the production of citrus juice (Oumer and Abate, 2018). Studies have detailed that pectinase of microbial inception represents 25% of worldwide food and mechanical compounds deal

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and their market is expanding ceaselessly (Oumer, 2017).

Moreover, enzymes include a grounded worldwide market extended to arrive at USD 6.3 billion of every 2021 (Oumer and Abate, 2018). Microorganisms including bacteria and fungi are promising sources of enzymes. They deliver various extracellular proteins that have an uncommon effect in the deterioration of organic matter. These encompass pectinolytic enzymes which can be excreted to interrupt down the middle lamella in plants in order that it may insert fungal hyphae and extract nutrients from the plant (Gummadi and Panda, 2003). Pectinolytic enzymes are produced by using many different organisms like microorganism, insects, nematodes and protozoans (Khairnar *et al.,* 2009). Pectinases have essential roles in meals industries. These enzymes are useful for fruit juice extraction and wine clarification; tea, cocoa, and coffee fermentation; vegetable oil extraction; jam and jellies processing; and pickling (Kubra *et al.,* 2018). Moreover, they are utilized in paper and pulp industries, bleaching of paper, bio-scouring of cotton, retting and degumming of plant fibers, oil extraction, waste water remedy, chicken feed additives, protoplast fusion, and bioenergy production (Gummadi and Panda, 2003; Kubra *et al.,* 2018). Enzyme breakdown of the biomolecules relies upon the kind of microorganisms, fermentation conditions along with pH, incubation time or cultivation time, carbon and nitrogen source, types and substrate concentration, temperature, agitation, and use of enzyme (Kubra *et al.,* 2018). The utility of new enzymes with advantageous biochemical, physicochemical and low-cost production has constantly been seemed as critical studies. Based on this literature, a study on screening of pectinase producing *Streptomyces* sp. was carried out from the soil samples. Selected isolates were used for enzyme production and their applications.

MATERIALS AND METHODS

The present investigation was conducted during January to July 2019 at Mehsana Urban Institute of Sciences, Gujarat, India to screen pectinase producing *Streptomyces* sp. from the soil samples; optimize for enzyme production, partial purification and detection of enzyme activity; and also study the application of pectinases.

Isolation of *Streptomyces* Consortia from Soil

Soil samples were collected from 3 sites of Mehsana District, Gujarat, India which includes Visnagar (23.7°N 72.55°E, 117 m), Bechraji (23.498°N 72.043°E, 58.52 m), Vijapur (23°33′39.39′′N 72°45′3.93′′E, 116 m). Transferred 1 gram of soil samples into 100ml of starch casein broth media containing calcium carbonate - 0.02 g, ferrous sulphate - 0.01 g, magnesium sulfate - 0.05 g, potassium nitrate - 2 g, potassium phosphate - 2 g, sodium chloride - 2g, soluble starch – 10 g, casein - 0.3 g and incubated on rotary shaker at 33°C for 7 days. The isolated colonies on starch casein agar plate were subculture and maintained at 4°C until further use.

Screening and Identification of Pectinolytic Isolates

Screening of pectinase producing streptomyces sp. was carried out by using MP-5 medium. The plates were incubated at 37°C for 3 days and Iodine solution was flooded in the plates to observe zone of hydrolysis of pectin (Bharadwaj and Udupa, 2019). The selected isolates were maintained at 4°C for enzyme production. Furthermore, the *Streptomyces* strains were identified by using Gram Staining method to observe their morphology, mycelia characteristics, the arrangement under the bright field microscope under 40X magnification.

Fermentative Production of Pectinase

The inoculum was prepared by inoculating selected strain AB-4 in the medium containing soluble starch - 10g, potassium nitrate - 2 g, potassium phosphate dibasic - 2 g, NaCl - 2g, casein - 0.3 g, magnesium sulfate - 0.005 g, calcium carbonate - 0.0002 g, ferrous sulfate - 0.01 g, agar - 16 g, PH- 7. All the flasks were incubated at 37°C at 150 rpm for 7 days in aerobic conditions. The Submerged fermentation was carried out for production of Pectinase enzyme by selected isolate AB-4 in fermentation medium containing Pectin - 1%, peptone - 0.5g,

Meat extract - 0.3g and incubated the flask 37°C at 150 rpm for 3 days under agitation. 2 mL of the fermented broth were pipette out into a sterile tube and centrifuged at 8000 rpm for 20 min. The supernatant obtained was used as the crude enzyme and used for analysis.

Partial Purification of Pectinase Enzyme

The fermentation broth was subjected for centrifugation at 5000 rpm for 15min. The cells and other debris were separated. Cell free supernatant was collected as an enzyme supernatant for purification as described below. The saturated ammonium sulfate solution was added in enzyme sample with rapid stirring and the beaker was incubated at 4°C for overnight. After 24 hr the content was transferred into centrifuge tube from beaker and precipitated at 3000rpm for 30min. Carefully the solution was transferred to dialysis tubing and dialyzed versus in 0.08% Sodium azide and stored at -80°C for further process.

Pectinase Enzyme Activity

Pectinase activity was measured based on the determination of reducing sugars produced as a result of enzymatic hydrolysis of pectin by dinitro salicylic acid (DNSA, IUPAC name 2-hydroxy-3,5-dinitrobenzoic acid) method (Miller, 1959). The supernatant (100 µL) from the culture broth was served as the source of enzyme. In additional, substrate was prepared by mixing 0.5% (W/V) citrus pectin in 0.1 M of pH 7.5 phosphate buffer. From the prepared substrate, 900 µL was added to 3test tubes (one for enzyme, one for enzyme blank and one for reagent blank). Then, 100 µL of crude enzyme was added to test tube and labeled as enzyme and 100 µL of distilled water was added to test tube labeled as reagent blank while test tube labeled as enzyme blank remained as it was. All 3 test tubes were incubated at 50°C for 10 min. in shaking water bath. After incubation 2000 µl of dinitrosalicylic acid reagent (DNSA) was added in all 3 test tubes to stop the reaction. Test tube no.2 labeled as enzyme blank 100 µL of crude enzyme was added after the DNSA reagent. Then all the 3 test tubes were incubated in boiling water bath (92°C) for 10 min. After incubation allow cool down all 3 test tubes and measure optical density (OD) by using spectrophotometer at 540 nm.

Effect of Temperature on Enzyme Activity

Selected isolate AB4 was streaked on SCA containing pectin and incubated at different temperature like 25°C, 30°C, 32°C, 34°C, 36°C, 38°C and 40°C for 72hrs after incubation diameter of zone of hydrolysis was measured (Singh and Mandal *et al.,* 2012).

Effect of pH on enzyme activity: Selected isolate AB4 was streaked on SCA containing pectin and incubated at different pH like 6, 6.5, 7.0, 7.5, 8.0, 8.5, 9.5 and 10 using 1N HCl and NaOH were adjusted. After 72 hr. after incubation diameter of zone of hydrolysis was measured (Singh and Mandal *et al.,* 2012).

Effect of NaCl on Enzyme Activity

Selected isolate AB4 was streaked on SCA containing pectin and incubated at different NaCl concentration 0%, 1%, 1.5%, 2%, 2.5%, 3% and 3.5% using NaCl. After 72 hr. of incubation diameter of zone of hydrolysis was measured (Singh and Mandal *et al.,* 2012).

Molecular Identification of Selected Isolate AB4

Genomic DNA was isolated from the isolate. The ~1.3 kb/1.5kb, 16s-rDNA fragment was amplified using high–fidelity PCR polymerase. The PCR product was sequenced Bi-directionally. The sequence data was aligned and analyzed to identify the bacterium and its closest neighbors.

Clarification of Apple Juice by Using Pectinase

Clarification of apple juice was performed in 250 ml Erlenmeyer flask containing apple juice extract in 100ml of basal mineral salt medium (g/L) NaNO3(2.0 g/L), NaCl (0.8 g/L), KCl (0.8 g/L), CaCl2. 2H2O (0.1 g/L), KH2PO4 (2.0 g/L), Na2HPO4.12 H2O (2.0 g/L), MgSO4 (0.2 g/L), FeSO4 (0.2 g/L). 20 ml of cell free supernatant from 48hrs culture broth was added in the flask. Control flask was prepared without inoculation of enzyme extract. The flasks were shaken at room temperature for 12hr. Juice clarity was measured according

to the methods of Ough and Crowell (1979).

Identification of Reducing Sugars by TLC

Identification of products was detected by TLC analysis. silica gels 60 F254 purchased from Merck were utilized for separation of products. The silica gel was activated at 110 - 120°C for 1 hr. Reductive sugars were separated in a solvent system including butanol/acetic acid/water (3:1:1). After separation, the surface of TLC sheets was coated by 25% (v/v) H_2SO_4 in methanol and was treated at 120°C for 30 - 45 minutes. Rf values of the spots belonging to sugars were detected by comparison those of the sugar standards (1%).

RESULTS AND DISUSSION

Isolation of *Streptomyces* sp. and Screening of Pectinase Producing Isolates

Total 11 *Streptomyces* sp. were isolated on starch casein agar medium (Fig. 1). All the isolates are filamentous in structure confirmed with Gram staining (Fig. 2). The isolates were screened for Pectinase activity by using MP-5 medium. Out of 11 isolates isolate A1, A3, A8, A18, AB1 and AB4 were showing clear zones by flooding the plate with Iodine solution in MP-5 medium (Fig. 3).

Pectinase Enzyme Activity

The pectinase activity from selected isolate was performed in extra cellular enzyme supernatant from fermentation broth. Unit activity of enzyme, total protein content and specific activity were calculated for crude

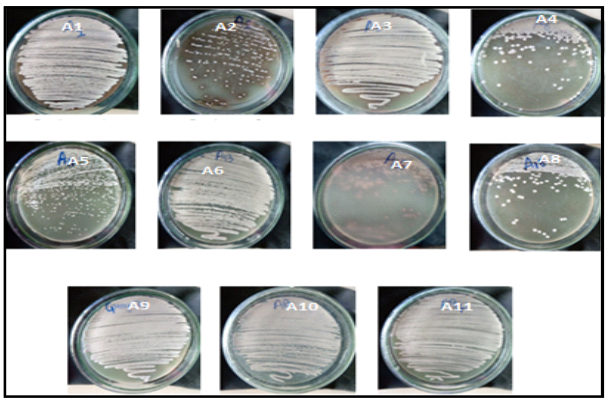

Fig. 1. Isolation of *Streptomyces* sp. on SCA agar.

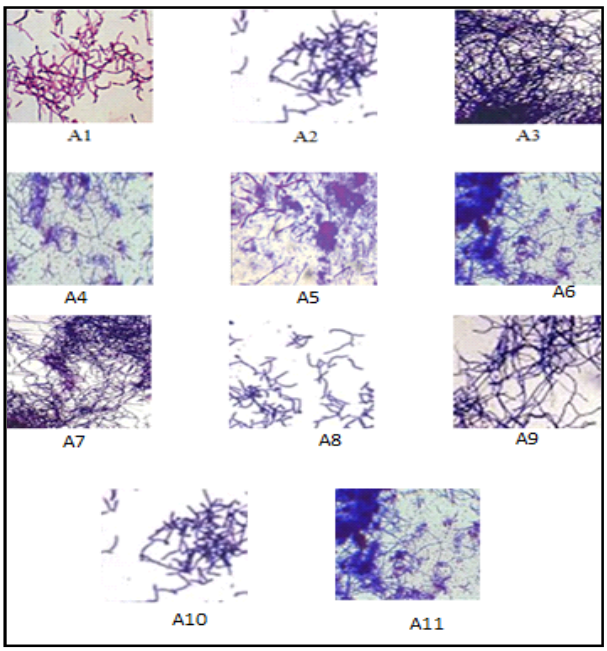

Fig. 2. Gram staining of *Streptomyces* sp.

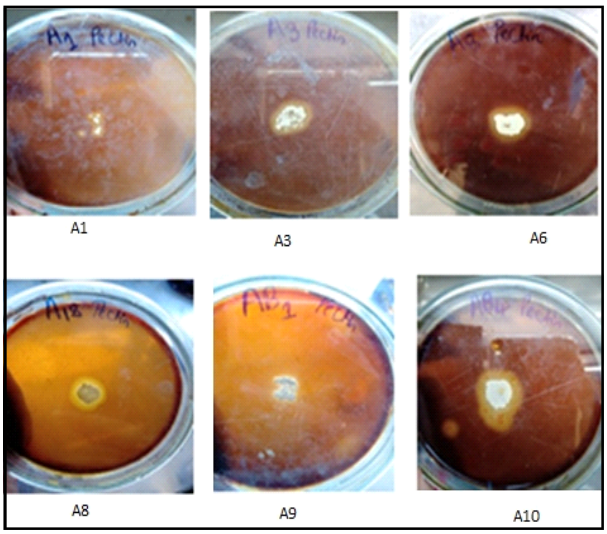

Fig. 3. Screening of pectinase producing *Streptomyces* sp.

enzyme sample and purified enzyme sample (Table 1). The specific pectinase activity was obtained from enzyme supernatant, ethanol precipitation and ammonium sulphate precipitation 51.04U/mg, 192.16 U/mg, and 42.09 U/mg, respectively. The maximum yield of pectinase was acquired by ethanol precipitation. As indicated in previous studies, the isolation, purification and characterization of pectinase from *Streptomyces thermocarboxydus* was investigated (Bharadwaj and Udupa, 2019). The enzyme activity of pectin enzyme was found as 0.03 U/mL in *Streptomyces*

Table 1. Pectinase activity

	Total activity (U)	Total protein (mg)	Specific activity (U/mg)
Crude	6432	126	51.04
$(NH_4)_2SO_4$ fraction (30% - 60%)	1347	32	42.09
Ethanol Precipitation	7110	37	192.16

coelicoflavus GIAL86 (Salehghamari *et al.*, 2019). In another study thermostable alkaline pectinase was produced from alkalophilic *Streptomyces* sp. RCK-SC. The Pectinase production was optimal at 45 °C in shaking conditions (200 rev/min) and shown activity 76,000 IU/L (Kuhad *et al.*, 2004). The unit activity of pectin belonging to *Streptomyces* sp. was isolated on Horikoshi medium supplemented with 1% w/v wheat bran was reported as 46 IU/mL (Beg *et al.*, 2000). In another study, the specific activity of the purified pectinase was estimated to be 2610 U/mg of enzyme protein (Arijit *et al.*, 2013). Compared to the literature, it is clear that extracellular pectinase obtained from *Streptomyces* sp. demonstrates high activity and has the potential to be used as an agent for clarification of fruit juice after careful examinations.

Effect of Temperature, pH and NaCl on Enzyme Activity

The maximum zone of hydrolysis of pectin was found at 37°C (Fig. 4). At pH 7 optimum enzyme activity was observed (Fig. 5). 1.05% NaCl concentration is optimum for maximum pectin hydrolysis by pectinase enzyme (Fig. 6).

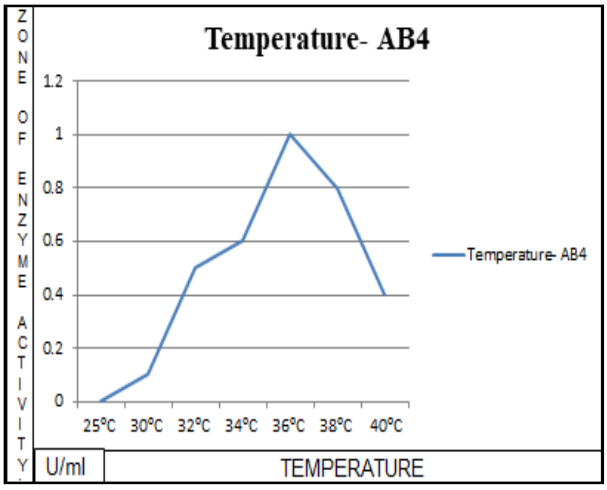

Fig. 4. Effect of temperature on enzyme activity.

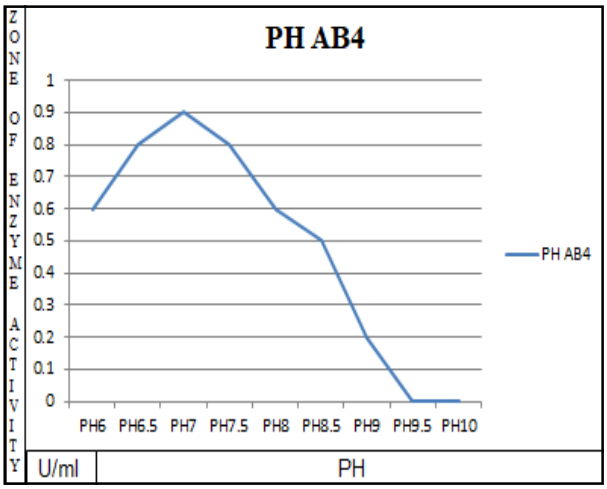

Fig. 5. Effect of pH on enzyme activity.

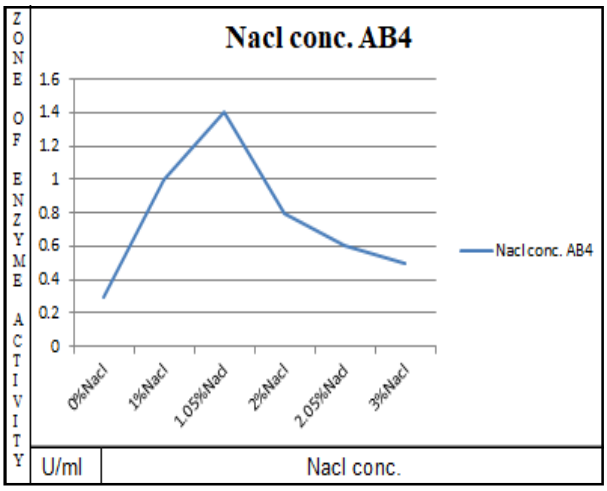

Fig. 6. Effect of NaCl on enzyme activity.

Molecular Characterization of Selected Isolate

Isolate AB4 had the biggest clear zone among all the isolates in the pectinase screening test. The chosen organism was sent for sequencing using rRNA technology. The isolate was found, by this rRNA technology, to be *Streptomyces chartreusis*. The accession number provided from NCBI Bank it is MN133919.

Clarification of Apple Juice

After 12 hr. of incubation the mineral

medium having apple juice with Purified enzyme becomes less viscous. It indicates presence of pectinase degrades pectin in Apple juice (Fig. 7).

Identification of Reducing Sugars by TLC

TLC analysis was performed for identification of reducing sugars. It was detected that glucose, fructose, and saccharose standards migrated with Rf values 0.29, 0.27, and 0.20 on TLC plates. In comparison with the spots of standards, only one spot, at Rf value 0.28, belonging to glucose was observed (Fig. 8). The lack of spots relating to the other sugars can be explained because that glucose concentration was higher than fructose and saccharose in the reaction mixture. In TLC analysis, the observation of reducing sugars indicates the degradation of pectin polymer by pectinase during fermentation.

Fig. 7. Clarification of Apple Juice.

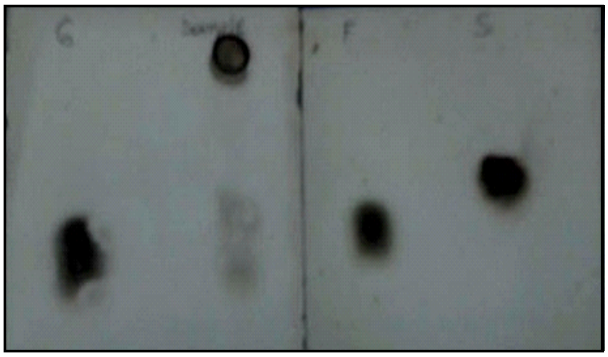

Fig. 8. Identification of reducing sugars on TLC.

CONCLUSION

Identification of the significant factors affecting pectinase production is essential for large scale production. *Streptomyces chartreusis* is able to produce pectinase enzyme. The specific pectinase activity was obtained from enzyme supernatant, ethanol precipitation and ammonium sulphate precipitation of 51.04U/mg, 192.16 U/mg and 42.09 U/mg, respectively. So, it should be used for cost effective production of pectinase.

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