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Isolation, purification and characterization of alkaline pectinase from *Bacillus subtilis* isolated from soil

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ABSTRACT

Pectinase is an enzyme that breaks down pectin, a polysaccharide found in plant cell walls. Commonly referred to as pectic enzymes. Pectinase producing microorganisms were isolated from pectin rich sites using selective isolation techniques and were screened for their pectinolytic activity. The culture conditions like pH, temperature, carbon sources and nitrogen sources were optimized. The culture conditions were optimized for maximum enzyme production by isolate microorganisms present in soil to produce large quantity of pectinase the fruit extract saturate in a beaker containing soil for 7 days. The screening and isolation of bacteria were done by various biochemical tests. The purification of pectinase enzymes were done by dialysis and Ion exchange Chromatography.

Keywords: Pectinase, Bacillus, protein content, chromatographic technique.

INTRODUCTION

Pectinase is a complex enzyme preparation composed by consecutively active enzymes pectinesterase, polygalacturonase, pectinlyase ensuring a high level of decomposition of the pectin Substances .Pectin or other 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 [1]. 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. Pectinase is also obtain from Kinnow is one of the most important citrus crops of northern India especially of Punjab, which produces about 0.180 MMT of kinnow, accounting over 50% of the national produce[2]. Pectin is a polymeric material having carbohydrate group esterifies with methanol. It is an important component of plant cell wall. It is present in highest concentration in the middle lamella, where it acts as a cementing substance between adjacent cells. Plant pathogens attack target cells by producing number of cell degrading enzyme which facilitates the entry and expansion of pathogen in the host tissue [3]. The history of pectinases began with an understanding the structure of pectins substances and the mechanism by which pectolytic enzymes degrade pectic substances. Later the microbial production of pectinases became prominent for many decades. Many microorganisms viz.., bacteria, yeast, fungi could produce pectinases [4]. Evidence showed that pectinases are inducible and they can produce from different carbon sources. In the course of time, numerous reports have appeared on the optimization of fermentation and microbiological parameters and different fermentation strategies for the production of pectinases [5]. Apple and citrus fruits are the main source of commercial pectin at present. Pectin, a major constituent of cereals, vegetables, fruits; fibers are complex, high molecular weight heterogeneous and acidic structural polysaccharide [6]. Pectic substances are classified into four main types based on the type of modifications of the backbone chain which are; protopectin, pectic acid, pectinic acid and pectin. Pectic substances mainly consist of galacturonans and rhamnogalacturonans in which the C6 carbon of galactate is oxidized to a carboxyl group, the arabinans and the arabinogalactans [7]. Protopectin is the water insoluble parent pectin substance found in the middle lamella of plant tissues. It yields soluble pectic substances such as pectin or pectinic acid upon restricted hydrolysis. Pectic acid is a group designation applied to pectic substances mostly composed of

galacturonans containing negligible amounts of methoxyl groups. The salts of pectic acid are called pectates. Pectinic acids are the galacturonans containing various amounts of methoxyl groups. The salts of pectinic acids are either normal or acid pectinates. Under suitable conditions, pectinic acids are capable of forming gels with sugars and acids or if suitably low in methoxyl content, with certain metallic ions. Pectins are the soluble polymeric materials containing pectinic acids as the major component. They can form insoluble protopectins with other structural polysaccharides and proteins located in the cell wall. D-galacturonic acid is one of the major components of pectin [8]. The American Chemical Society classified pectic substances into four main types as follows [9].

(I) Protopectin: is the water insoluble pectic substance present in intact tissue. Protopectin on restricted hydrolysis yields pectin or pectic acids.
(II) Pectic acid: is the soluble polymer of galacturonans that contains negligible amount of methoxyl groups. Normal or acid salts of pectic acid are called pectates.
(III) Pectinic acids: is the polygalacturonan chain that contains >0 and <75% methylated galacturonate units. Normal or acid salts of pectinic acid are referred to as pectinates.
(IV) Pectin (Polymethyl galacturonate): is the polymeric material in which, at least, 75% of the carboxyl groups of the galacturonate units are esteriûed with methanol. It confers rigidity on cell wall when it is bound to cellulose in the cell wall.

Many useful enzymes are produced using industrial fermentation of *Aspergillus niger* [10]. Many studies have been reported that the enzyme preparations used in the food industry are of fungal origin because fungi are the potent producers of pectic enzymes. [11]. Bacterial strain producing commercial enzymes are always preferred over fungal strains because of ease of fermentation process (for production) and implementation of strain improvement techniques or any modern technique to increase the yield of production [12]. A great variety of strains of bacteria (al., and mold are capable of producing pectic enzymes [13].

MATERIALS AND METHODS

Materials

Fruit peels, beaker, Nutrient Agar Media, Petri plates, Test tubes, Measuring cylinders, Jars Micropipettes (100 µl), Distilled water: 100 ml NaCl: 0.85 gm, Crystal Violet, Iodine, and Ethyl alcohol (95%), Saffranin, green, and, Hydrogen Peroxide (3%), Mannitol fermenting broth, PAM (Pectin Agar Media), ruthenium red solution [14-16].

Isolation of bacteria from the soil by using serial dilution method

Soil samples were collected from an area of ITLS campus. The soil samples were taken from depth of 5-10 cm and kept in plastic bag until drying was performed immediately. The soil samples were air dried at room temperature at 27^0C for a week and grind it using a mortar pestle. Then soil sample were sieved with 0.5mm sieve to remove larger particles such as stone and plant debris in order to obtain a consistent soil particle soil for isolation using the soil dilution technique.

1.5 ml of distilled water was taken in a jar. Peptones, NaCl, yeast extract were added into it. Volume was maintained 100 ml by adding distilled water into it. Further pH was maintained at 7.2 by using acid or base. Agar was added to it and then stirred it for 2-3 min with the help of magnetic stirrer. Then it was autoclaved for 20 minutes at 121°C at 15 lb/inch pressure**.**

Identification of bacteria

The bacteria were identified by cultural and microscopic characteristics (Gram positive and gram negative) by Grams staining techniques. Further the presence of endospore in the given bacterial master plate was identified by staining techniques.

Isolation of test bacteria, *Bacillus subtilis*

The test bacteria was isolated from fruit peels in NAM (nutrient agar media) media and incubated at 37^0C for two days. Individual bacterial colonies were singled out and further purified by sub culturing on NAM media [17-20].

Biochemical test

Check the presence of Catalase enzyme in the bacterial master plates

Two-three drops of 3% hydrogen peroxide were taken on a clean glass slide. One loop full of the culture was just kept over the hydrogen peroxide. Slide was than observed for the appearance or absence of gas bubbles.

MRVP test
Added 5 drops of methyl red indicator in 2 test tubes with the help if dropper. The change in colour was observed. Added 12 drops of V-P 1 and 2-3 drops of V-P 2 in the remaining test tubes along with control test tube. The test tubes were shacked gently for 30 seconds with the cap off to expose the media to oxygen. Allowed the reaction to complete for 15-30 minutes. The change ibn colour was observed.

Mannitol fermentation test
Manitol, peptone, NaCl and phenol red were added in 100ml of distilled water. The pH was maintained at 7.4. Then broth was autoclaved for 20 minutes at 121°C and 15 lb/inch pressure. After cooling, the broth was poured into 5 test tubes. Four of them were inoculated with the four of the master plates. Label it according to the master plate from which inoculation was done. One of the test tubes was remained un-inoculated and used as control. Further the mouth of the test tubes was closed with the help of cotton plugs. Kept it for incubation for 24 hours at 37°C.

Citrate utilization test
Weighed the required quantity of media content**.** The content was dissolved in 100 ml distilled water by continuously stirring it in a stirrer. Maintain the pH of medium to 6.9**.** Now the medium was autoclaved at 121^0C for 15 minutes. Poured the medium in test tubes which have been previously sterilized by autoclaving at 15 psi for 15 minutes at 121^0 C. Further the test tubes were left in slanted position for making an agar slant tube. After sometime, the test tubes were inoculated with specific microbial colonies. Then incubated the tubes at 37^0C for 48 h.

Identification of pectinase producing bacteria in the master plate culture
Pectin Agar Medium (PAM) was prepared by using, 0.5g agar in 1.5ml of composition of media. This solution was used as a gel to inoculate culture in petri plates having media of PAM. The bacterial suspension was prepared after inoculating each microbial colony (1 to5). From each strain, (1 to 5) taken 0.25 microlitre suspension and transfer thick drop on petriplates (middle). The plates were incubated for for 3 days at 37^0 C. Flooding was done by ruthenium red for one hour, a clear zone was found.

Extraction and purification of pectin from pectinase `producing bacteria
Prepared pectin production broth and inoculated with bacterial culture having better growth. Incubated for 5 days. Separated 10 ml of broth in a test tube. Test tube was inoculated with the help of master plates. Kept it for inoculation for 24 hours at 37°C. Kept it for incubation for 24 hours. Observed the growth of culture. Supernatant was taken as a crude enzyme. Take 4 sterilized centrifuge tubes and poured the culture into it. Centrifuge it for 15minutes at 10,000 rpm. Supernatant was taken & pellet was discarded. Supernatant contained enzyme in crude form so called crude enzyme. Measure the volume. 0.6 gm of ammonium sulphate per ml was added in the crude sample pinch by pinch followed with constant stirring on magnetic stirrer in an ice bath. Ice bath was prepared by placing the crude sample into a small beaker and further it into a large beaker with ice into it. Further the sample was kept for 24 hours. Further the sample was centrifuged to 10,000 rpm for 20 minutes. Proteins get settled down as pellet. Supernatant was discarded and further the pellet was taken for further procedure [21].

Purification of sample by osmosis to perform dialysis and ion exchange chromatography
Dialysis bag was dipped into 10 ml of distilled water & put it into water bath for 10 minutes. Water was discarded and 10 ml of 2% $NaHCO_3$ was added after placing in water bath for 10 minutes. Again add dialysis bag into 10 ml of distilled water. Now dialysis bag was tied with a thread from one side & protein sample was poured into it. Tied it from both the sides. 100 ml of T.E. buffer was taken into a jar and dialysis bag was dipped into it. Kept this arrangement for 24 hours at 4°C. Dialysis bag was taken out and again dipped into 100 ml of sodium citrate buffer and kept it to constant stirring for 30 minutes at magnetic stirrer. Then T.E. buffer was removed and fresh 100 ml of Sodium citrate buffer was added. Stirred it for 30 minutes at magnetic stirrer [22].

Dialysis
Activate the dialysis bag by boiling it in distilled water for 30 min, then in 2% sodium bicarbonate for 30 min and then in distilled water for 30 minutes. Poured the crude extract in the bag and tie its end. Place the dialysis bag in beaker filled with sodium citrate buffer (pH 7.1). Incubated it for overnight at 4^0C. After incubation stir the set up constantly for 3 h and change the sodium citrate buffer (pH, 7.1) buffer after every 30 minutes, pure crude extract is obtained [23].

Ion-Exchange Chromatography
DEAE, Ion-exchange chromatography was performed with the help of variable buffer. The burette was washed with distilled water followed by methanol, and finally with distilled water. DEAE bedding was introduced in the burette. Taken this in one test tube. Run 6 tubes of variable buffer of ionic strength 25mM, 50mM, 75mM, 100mM, 125mM and 150mM Sodium citrate. Taken elution buffer of respective ionic strength in respective test tube.

Estimation of protein content in the purified enzyme by Follin- Lowry's Method

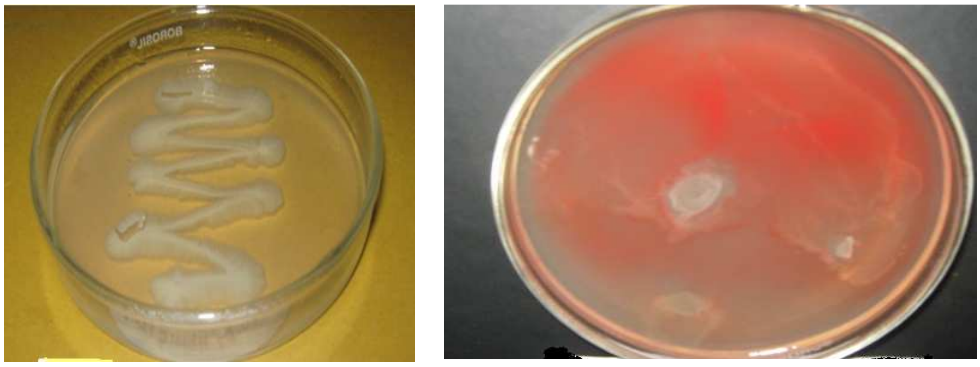
The protein content was determined by Follin-Lowry's method. Protein solution Stock standard: 50 mg of bovine serum albumin was dissolved in distilled water and the volume was made up to 50 ml in a standard. Working standard: 10 ml of stock is diluted to 40 ml distilled water [24].

Estimation of activity of crude and pure pectinase enzyme by DNS method

The activity of the pectinase enzyme was quantified by taking the absorbance at 540 nm in UV-VIS spectrophotometer. A standard curve was prepared between absorbance at 540 nm and concentration of D-galacturonic acid in mg/ml. The elutes obtained by ion exchange chromatography was also subjected to DNS assay. The amount of reducing sugar released in the reaction mixture for each of elutes was determined by using standard curve [25].

RESULTS

The cultures obtained on petriplates were pure form of bacteria which can be taken as master plates for further experimentation. The red color of ruthenium red solution decolorize while the area of bacterial growth is white in color. Clear zone obtained indicates the pectinase enzyme produced by the bacterium. The results are shown in **Figure 1**.



(a) **(b)**

Figure 1: (a) Master plate of *Bacillus subtilis*; (b) Pectinase screening color produce by *Bacillus subtilis*

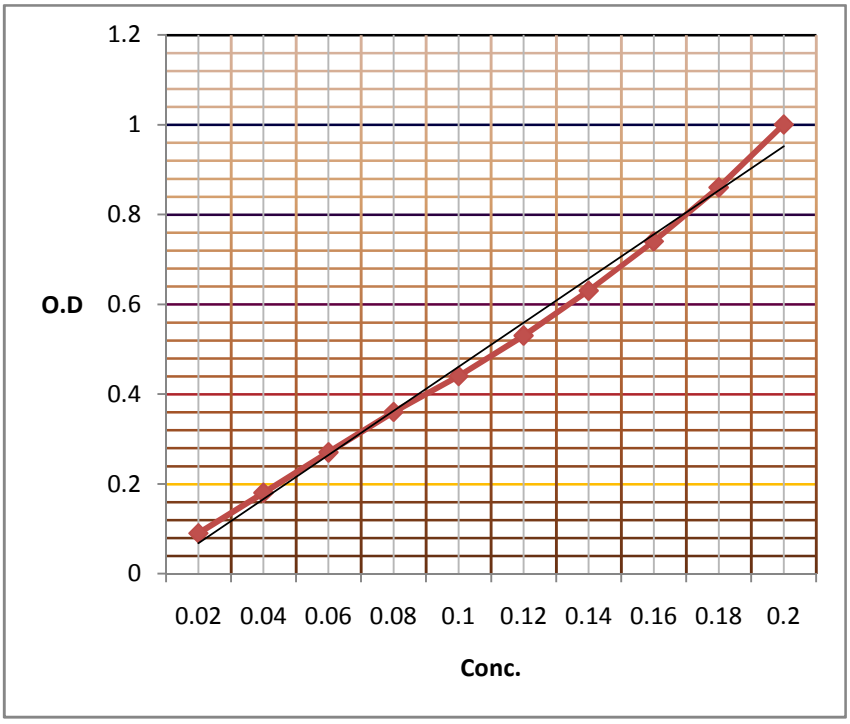


Figure 2: Standard plot for determination of enzyme activity

Pectinase enzyme activity and protein concentration in the samples

As per the results, protein concentration in the crude enzyme was found to be 0.0644 mg/ml while in purified enzyme, the protein concentration was found to be 0.0444 mg/ml. The activity of crude enzyme was found to be 1.1065 U/ml while the activity of purified enzyme was found to be 0.7628 U/ml. The results are shown in **Figure 2**. The results of effect of concentration of substrate and temperature are shown in **Figure 3** and **4**.

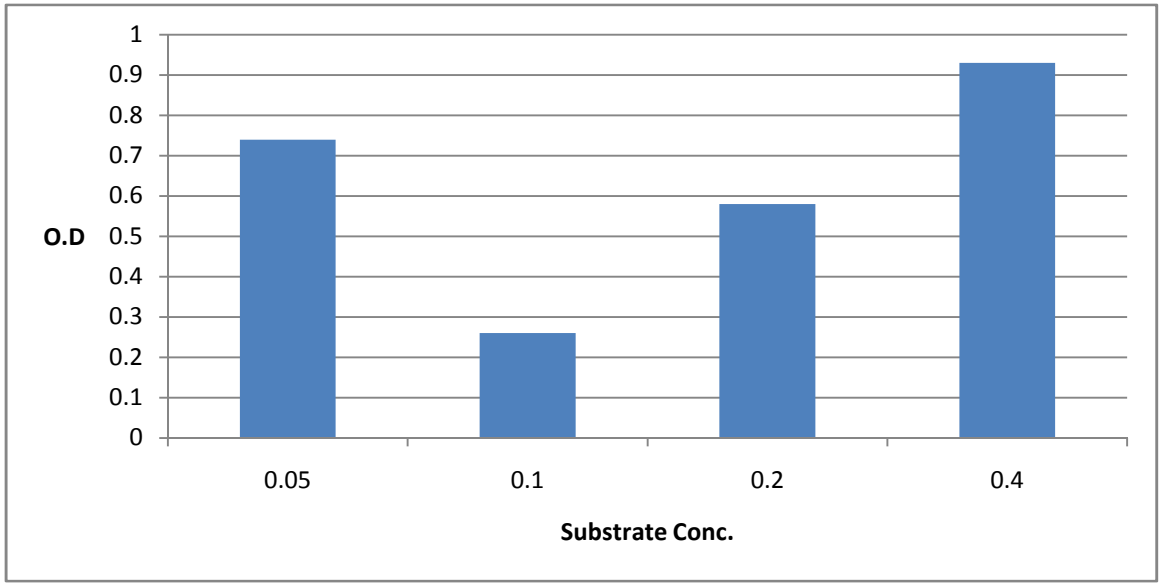


Figure 3: Effect of substrate concentration on enzyme activity

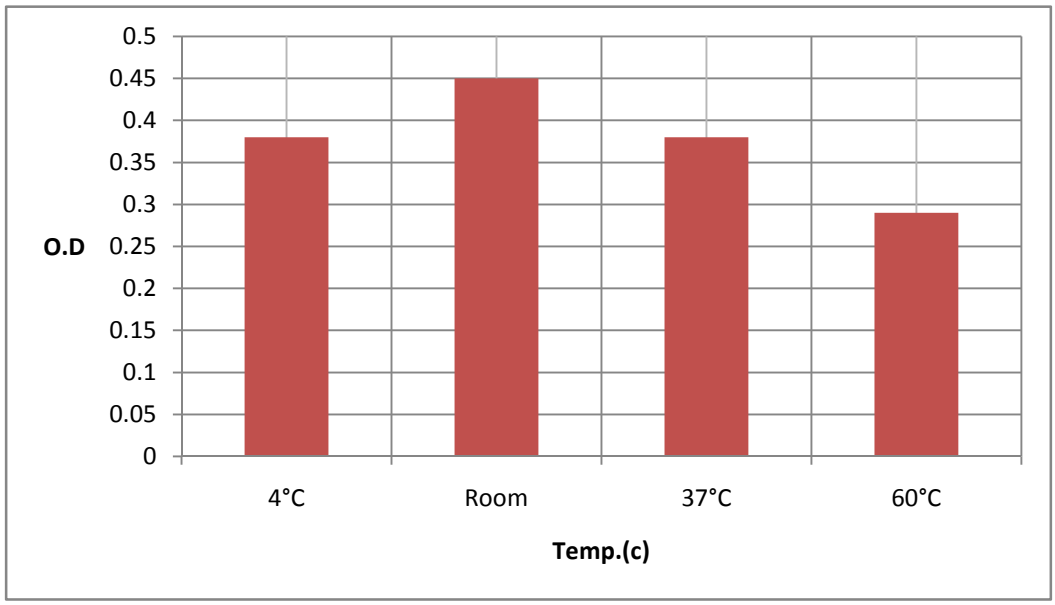


Figure 4: Effect of temperature on enzyme activity

CONCLUSION

The aim of the present work was to isolate and identify a high pectinase producer from soil Bacillus subtilis. Optimum temperature and pH were determined as 37 ^{0}C, and 8-9 pH and best carbon sources was glucose. This information has enabled the ideal formulation of media composition for maximum pectinase production by this organism. The protein concentration in the crude enzyme was found to be 0.0644 mg/ml while in purified enzyme, the protein concentration was found to be 0.0444 mg/ml. The activity of crude enzyme was found to be 1.1065 U/ml while the activity of pure enzyme was found to be 0.7628 U/ml at pH, 9.0. The results obtained in the present study will be useful for commercial production of pectinase.

REFERENCES

[1] Z.I. Kertesz, Interscience, New York, **1951**.
[2] G.H. Joseph, Pectin: Bibliography of pharmaceutical literature. (Ontario: Sunkist Growers), **1956**.
[3] E.A. McComb, R.M. McCready, *Analytical Chemistry*, **1957**, 29,819–821.
[4] D.A. Rees and A.W. Wright, *Journal of Chemical Society*, **1971**, Section B, 2, pp. 1366-1372.
[5] K. Horikoshi, *Agricultural and Biological Chemistry*, **1972**, 36, 285-293.
[6] F. Wilson and J. Dietschy, *Biochimica et Biophysica Acta*, **1974**, 34, pp. 1034.
[7] T.A. Miettinen, S. Tarpila, *Clinica Chimica Acta*, **1977**, 60, pp. 1429-1431.
[8] A.M. Mayer and E. Harel, *Phytochemistry*, **1979**, 18, 193-215.
[9] A. Karbassi, R.H. Vaughn, *Canadian Journal of Microbiology*, **1980**, 26, 377±384.
[10] G. Dunaif and B.O. Schneeman, *American Journal of Clinical Nutrition*, **1981**, 34, pp. 1034-1035.
[11] R.S. Jayani, S. Saxena and R. Gupta, *J. Biological Chemistry*, **1985**, 43, 23-31.
[12] F.M. Rombouts and W. Pilnik, *Symbiosis*, **1986**, 2, 79-89.
[13] H.S.S. Sharma, *Applied Microbiology and Biotechnology*, **1987**, 26, 358-362.
[14] H. Tanabe, K. Yoshihara, K. Tamura, Y. Kobayashi and P. Footrakul, *Journal of Fermentation Technology*, **1987**, 65, 243-246.
[15] G.E. Trease and W.C. Evans, Pharmacogonasy, **1989**, 14th Edition, Brown Publication.
[16] G. Aguillar and C. Huitron, *Biotechnology Letters*, **1990**, 12, 655-660.
[17] V.K. Joshi and V.P. Bhutani, *Sciences Des Aliments*, **1991**, 11(3): 491-496.
[18] M.D.L.T.M. Polizeli, J.A Jorge, H.F. Terenzi, *J. Gen Microbiology*, **1991**, 137, 1815-1823.
[19] D.G. Oakenful, In The chemistry and technology of pectin, ed. R.H. Walter. (New York:Academic Press), 1991.
[20] J.R. Whitaker, Microbial Enzymes and Biotechnology (Fogarty, W. M., and Kelly, C. T., eds.) Elsevier Applied Science, London-New York, pp. 133- 175, **1991**.
[21] J. Cao, L. Zheng and S. Chen, *Enzyme and Microbial Technology* , **1992**, 14, 1013-1016.
[22] C. Perez, C. Anesini, *Journal of Ethnopharmacology*, **1993**, 44, 41-46.
[23] A. Gainvors and A. Belarbi, *Yeast*, **1995**, 10, 1311-1319.
[24] P. Sriamornsak, MSc Thesis, Mahidol University, **1996**.
[25] I.L. Novosel'skaya, Chemistry of Natural Compounds, **2000**, 36, pp. 1-10.