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AN EXTRACELLULAR ENZYME – PECTINASE: EFFICIENT PRODUCTION FROM THE BACTERIA *BACILLUS SP*.P15 ISOLATED FROM BANANA ORCHARD SOIL

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ABSTRACT

Pectinolytic enzyme can be applied in various industrial applications. Among the 27 bacterial strains isolated, 8 revealed pectinase activity from which we chose *Bacillus sp*. P15 based on significant zone of clearance. An attempt was made to optimize the partial purification methods by comparing lyophilisation (Concentrate) and ammonium sulfate precipitation followed by staining with Blue silver dye (Coomassie G-250). The lyophilized pectinase sample showed efficient enzyme activity and protein content. We propose to purify and commercialize this bacterial (*Bacillus sp*.P15) extracellular pectinase enzyme.

KEYWORDS: Extracellular pectinase enzyme, *Bacillus sp.* P15, Lyophilization, Ammonium sulfate precipitation, Blue Silver Staining.

INTRODUCTION

Microorganisms are the most effective producers of enzymes. These naturally - occuring enzymes can be used in a variety of agricultural and industrial processes. Enzymes are classified into 6 major groups of which hydrolytic enzymes like glucanase, protease, pectinase, amylase and cellulase are important for their industrial applications and play a key role in degradation of particulate organic matter in their natural environment.

Pectinase is a complex enzyme evidently composed of consecutively active enzymes pectinesterase, polygalacturonase, pectinlyase ensuring a high level of decomposition of the

pectin substances. There are evidences to show that pectinases are inducible and they can be produced 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.[11]

Pectic substances are complex colloidal acid polysaccharides, with a backbone of galacturonicacid residues linked by α (1→4) linkage. The side chains of the pectin molecule consist of L-rhamnose, arabinose, galactose and xylose. The carboxyl groups of galacturonic acid are partially esterified by methyl groups and partially or completely neutralized by Sodium, Potassium or Ammonium ions.[5] In our present study, we focused on highest producer of pectinase enzyme from bacterial flora and its streamline to enhance enzyme production.

MATERIALS AND METHODS

Soil sample collection

Soil was collected randomly from dense banana orchard and stored in polyethylene bag under refrigerated condition for isolating pectinase producing micro-organisms.

Isolation of microorganisms (Reetha *et al.*,[10])

Soil suspension was prepared by adding 1.0 g of garden soil in 10 ml sterile distilled water incubated in a shaker for 30 min. 1 ml of soil suspension was serially diluted up to 6 dilutions. 0.1 ml of each dilution was spread plated onto plates with pectin (pH 7), and incubated at 37ºC for 2 days for bacterial growth. Isolated bacterial cultures were maintained in nutrient agar medium.

Plate assay for pectinase enzyme

The isolates were screened for pectinase activity by growing on pectin agar medium (NaNO3 – 0.2%, MgSO4.7H2O – 0.05 %, K2HPO4 – 0.005%, FeSO4.7H2O – 0.001%, CaCl2 – 0.2%, Yeast extract – 0.03%, Pectin – 0.5%, Agar – 2%, pH – 7) containing pectin as carbon source. After incubation, (37°C for 24 hours) the plates were flooded with iodine solution (1.0 g Iodine; 5.0 g Potassium iodide; 330 ml Distilled water). After 10 – 15 min, pectinase activity was observed by the presence of measurable clear zone around the colony. The highest zone producing bacterial strain was chosen for further study.

Growth of *bacillus sp.*p15 isolate

The *Bacillus sp.* P15 culture was grown in the sterilized pectin broth medium incubated in an orbital shaker for 42 hrs, at 32°C for extracellular pectinase enzyme production. At the end of the incubation period, the broth was centrifuged at 4°C at 10,000 rpm for 10 min. The extracellular culture filtrate was used as source of enzyme.

Enzyme assay

1. DNS (Dinitro salicylic acid) Assay (Miller *et al.*,[6])

The reaction mixture contained 0.5ml ml of the enzyme and 1 ml of 0.3% pectin as substrate with Phosphate buffer (pH 7.2, 0.2 M). The mixture was incubated at 37°C for 30 minutes and then the reaction was stopped by adding 3 ml of DNS reagent followed by boiling the tubes exactly for 5 minutes. The absorbance of the solution was measured at 540 nm using UV-Vis spectrophotometer.[6] Pure pectinase enzyme was used as the standard.

2. Protein assay

Protein concentrations were determined by the method of Bradford, using Bovine Serum Albumin as the standard.[1] The absorbance of the protein concentration was measured at 595nm using -Vis spectrophotometer.

PARTIAL PURIFICATION

1. Ammonium sulfate fractional (AMS) precipitation

The culture filtrate was used as an enzyme source. Briefly, 100 ml of the pectinolytic crude extract of the *Bacillus* sp. P15 grown using pectin (0.5%) as the substrate to which solid ammonium sulfate (80%, w/v) was added with gentle stirring and the resultant solution was kept at 0 to 4°C at for 24 h after which the formed precipitate was collected by centrifugation at 10,000 rpm for 20 minutes at 4°C. Pellet was dissolved in phosphate buffer (50 mM, pH 7.2) and stored at 4°C. All the purification steps were carried out at 4°C to prevent the denaturation of enzymes.

2. Lyophilization (LP) method

Lyophilization process was carried out at -55°C. 100 ml of the culture filtrate was lyophilized. During lyophilization, the water molecules were removed to concentrate the sample. The lyophilized crude enzyme (4 ml) was stored at 4°C for further studies.

DIALYSIS

The crude enzyme from AMS and LP method were purified by Dialysis followed by method of Wingfield *et al*.[13] The dialysis bag was activated by boiling it in distilled water for 30 min, followed by pouring off the lyophilized crude enzyme into it. The bag was placed in beaker filled with glass distilled water and phosphate buffer (1:1) (10mM, pH 7.2) and incubated overnight at 4°C. After incubation, set up was constantly stirred for 4 h with a change of the phosphate buffer (10mM, pH 7.2) every 30 minutes to obtain the partially pure enzyme.

NATIVE - POLYACRYLAMIDE GEL ELECTROPHORESIS (NATIVE – PAGE)

The crude enzyme and partially purified enzyme were detected by NATIVE – Polyacrylamide Gel Electrophoresis method.[4] The gel was prepared in 8% concentration (Resolving gel: Water 2.30ml, 30% acrylamide stock 1.30ml, 1.5M Tris-Hcl pH 8.8 1.30ml, 12% Ammonium per sulfate 0.05µl, TEMED 0.003µl. Stacking gel: Water 1.40ml, 30% Acrylamide stock 0.33µl, 0.5M Tris-Hcl pH 6.8 0.25µl, 10% Ammonium per sulfate 0.02µl, TEMED 0.002µl). 30 µl of protein containing crude sample was dissolved in 10 µl (3:1) of sample buffer (Bromophenol blue 0.05%, Glycerol 1ml, Stacking gel buffer 1.25 ml, Distilled water 7.25 ml) and the gel was fixed and stained with Blue silver staining.

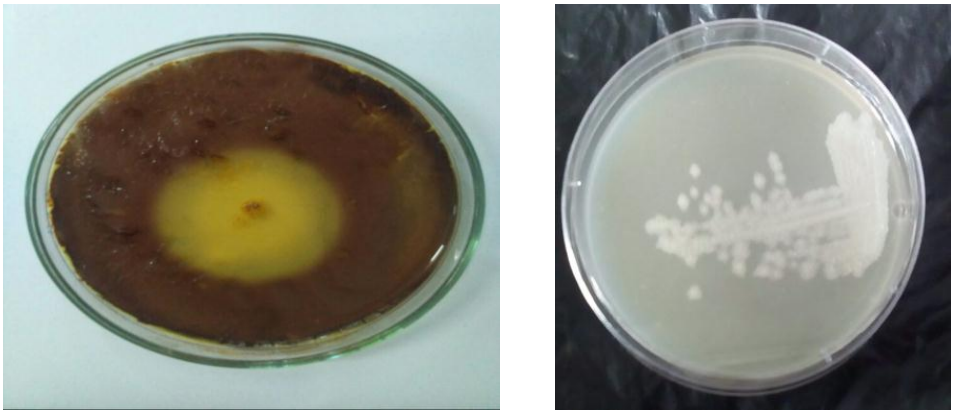
PREPARATION OF BLUE SILVER STAINING DYE AND STAINING GEL[9]

The blue silver staining dye contains the following (final concentration in the ready- to- use solution): 2% H_3PO_3, 10% $(NH_4)2SO_4$, 20% methanol, and 0.1% Coomassie G-250. The gel was fixed with 30% methanol and 10% acetic acid for 30 min. After fixation, the gel was washed (4 times) with glass distilled water and stained. After 30mins to 24 hrs the protein bands was visualized. The above method was performed at room temperature condition.

RESULTS AND DISCUSSION

ISOLATION AND SCREENING OF PECTINOLYTIC BACTERIA USING PLATE ASSAY

We isolated 27 bacterial cultures from garden soil (Table. 1). These bacterial cultures were screened for pectinolytic activity using plate assay on pectin agar medium with pectin as the sole carbon substrate. Among these 8 bacterial cultures showed highest pectinolytic activity (Table. 2). Only one bacterium *Bacillus sp*. P15 was selected that showed significant zone of clearance (Figure. 1a). The selected isolate was maintained on pectin agar medium. (Figure.1b).



(a) **(b)**

Figure 1: (a): Plate assay of Pectinolytic activity of *Bacillus sp*.P15, (b): Morphology of *Bacillus sp.* P15.

Table. 1: 27 Soil bacterial isolates with pectinolytic activity using plate assay.

Bacterial Isolates	Pectinase activity
B1	+
B2	-
B3	+
B4	+
B5	-
B6	+
B7	-
B8	-
B9	-
B10	+
B11	-
B12	+
B13	-
B14	-
B15	-
16	+
17	+
B18	+
B19	+
20	++
B21	+
CP1	++
CP3	++
CP5	++
CP7	+
P15	++
F20	++

Table 2: Preliminary screening of pectinolytic bacteria.

Bacterial isolates	Pectinolytic activity (Zone of inhibition mm)
16	32
17	38
20	42
CP1	46
CP3	43
CP5	42
F20	42
P15	47

PECTINASE CONCENTRATION/ PARTIAL PURIFICATION

The total protein content in partially purified crude enzyme of *Bacillus* sp. P15 was evaluated by Bradford assay. In ammonium sulfate precipitation method, the total protein was 0.427 mg/ml. The lyophilized crude enzyme contains 0.585 mg/ml. The enzyme activity was calculated and tabulated in Table. 3.

Table 3: Pectinase activity from lyophilized, Ammonium sulfate, Dialysis crude enzyme sample.

Enzyme From *Bacillus sp.*P15	Lyophilized crude		Ammonium sulfate crude		Dialysis of lyophilized crude		Dialysis of Ammonium sulfate crude	
	Protein mg/ml	Enzyme activity U/ml	Protein mg/ml	Enzyme activity U/ml	Protein mg/ml	Enzyme activity U/ml	Protein mg/ml	Enzyme activity U/ml
Pectinase	0.585	448.20	0.427	315.36	0.807	632.84	0.206	435.24

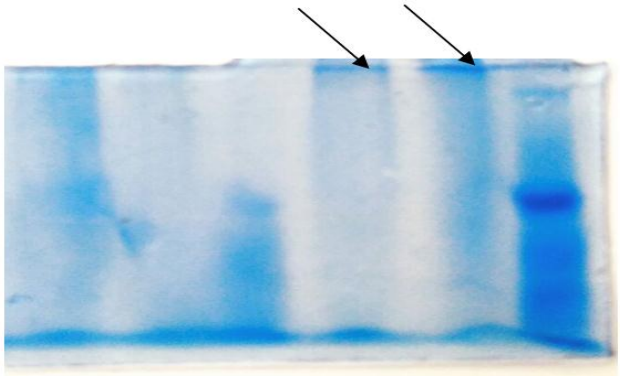
DIALYSIS

The crude enzymes obtained from ammonium sulfate and lyophilization were subjected to further purification by dialysis. The dialyzed enzymes were evaluated for total protein content and enzyme activity. The AMS dialyzed enzyme showed 0.206 mg/ml yield whereas LP dialyzed enzyme showed 0.807 mg/ml yield. The enzyme activity was also high in according to their yield (Table 3).

NATIVE-PAGE GEL

The electrophoretic patterns of *Bacillus sp.* P15 crude extract was performed to obtain protein bands. The culture filtrate of *Bacillus* sp., crude enzymes from AMS and LP method and the dialyzed AMS and LP enzymes were loaded in wells. Commercially purified Pectinase enzyme was loaded as standard. The crude enzymes and partially purified enzymes showed band formation in the gel. The clear and concentrated band was observed in LP dialyzed

enzyme. There was gradual increase in clear band visibility revealed that the enzyme was purified in each purification process (Figure 3).



(a) (b) (c) (d) (e) (f)

Figure 3: NATIVE- PAGE gel: (a) Lyophilized crude (LP), (b) culture filtrate, (c) Ammonium sulfate (AMS), (d) dialyzed enzyme (LP), (e) dialyzed (AMS) and (f) Pure pectinase enzyme (fungal) (Standard).

DISUSSION

Although pectinase production is an inherent property of most organisms, only those microbes that produce a substantial amount of extracellular pectinase are of industrial importance and have been exploited commercially. Among 27 isolates studied by us, only 8 bacterial strains possess pectinase activity. Varghese *et al.*,[12] reported 12 isolates that were potent pectinase producers among the 47 isolates.

Pectinase activities of various bacteria were determined by growing them on minimal essential medium containing 2% pectin as sole source of carbon. Small concentration of pectin was used, otherwise, bacteria may come under stress causing inhibition in production of the enzyme as reported by Jabeen A *et al.*[3] The chosen bacteria *Bacillus* sp. P15 was able to produce maximum pectinase at lowest pectin substrate concentration of about 0.5% containing medium at 32°C for 42 hrs.

Here in partial purification, lyophilization method was used to concentrate the culture filtrate. According to Gatlin and Nail[2], lyophilization provides a valuable tool by permitting dehydration of heat-sensitive enzymes and protein-drugs at low temperature. The final product is quickly and easily reconstituted, and the process is compatible with aseptic operations. In our study, purification process using lyophilization, maximum protein yield was observed when compared with ammonium sulfate precipitation method.

In NATIVE Gel Electrophoresis, it is evident that the crude enzyme from both AMS and LP method got purified in each purification process such as Precipitation and Dialysis. In electrophoresis, the gel was stained with blue silver staining dye. The blue silver exhibits a much faster dye uptake during the first hour of coloration even at equilibrium (24 hrs), the blue silver exhibits a much higher sensitivity than all other recipes[7], but somewhat lower sensitivity of blue silver as compared to silver stain protocols.[8]

CONCLUSION

Microbial Pectinase can be stated as the most important enzyme for the many industrial and biotechnological applications. In this study, an efficient pectinase producer from banana orchard soil, *Bacillus sp* P15 was identified for these applications. Also this study has enabled the ideal formulation of media composition and unique lyophilized technique for maximum pectinase yield by every *Bacillus* sp. Owing to the enormous potential of pectinase, it is important to undertake further research, as we investigate optimal conditions for purification of microbial pectinase and to commercialize the same.

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