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Isolation and Screening of Pectinase Producing Bacteria from Soil Sample

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***Abstract*: The vast majority of the industrial use of enzymes is covered from microorganisms. Microorganisms are favoured in industry because of their several advantages for example rapid growth, short life expectancy and simplicity in doing genetic alterations. Microbial enzymes are thus amply provided, very much standardized and promoted by many companies. Among various enzymes, *Pectinases* hold an exceptional place because of its different uses in various sectors like food, textile and biofuel industries.A total of 25% of total enzyme market is being shared by Pectinase alone.The current study was carried out to evaluate the pectinase activity of the pectinolytic bacteria. 40 Bacterial strains were isolated from different soil samples and screened for Pectinase production. Primary and Secondary screening showed 3 potential isolates I38 , I39 and I40 showing pectin degradation on Vincent's media. Further, extracellular pectinase was partially purified by ammonium sulphate precipitation and dialysis. Sequential ammonium sulphate saturations from 20-80% i.e. (20, 40, 60 and 80%) showed 60% ammonium sulphate was optimum for precipitation of intracellular enzyme whereas 80% was optimum for extracellular enzyme.**

***Keywords:* Soil bacteria, Screening tests, Ammonium sulphate, Pectinase**

I. INTRODUCTION

Enzymes are catalysts that speed up reactions in the body, such as the breakdown of complex carbohydrates and proteins into simpler substances, without modifying themselves in the process. Compared to chemical catalysts, enzymes are highly specific with great adjustable activity and high catalytic efficiency that promotes the use of enzymes in various industries like pharmaceutical, chemical and food industry. Among all industrially important enzymes, pectinase (EC number 3.2.1.15) is an enzyme which has a special significance and captures 25% of total enzyme market as it has many uses in different sectors such as food, wine making, textile and biofuel industries [1]. Production of pectinase is done by using microorganisms as they have high growth rate, short life span as well as genetic manipulations can be done easily. Thus, industries use microbial enzyme as they can be supplied in higher amounts, are well standardized and manufactured by several competing companies [2). Pectin is a heteropolysaccharide in primary cell wall of plants and is abundant in non-woody parts of terrestrial plants. It is a major component of middle lamella as it helps in binding cells together and also found in primary cell walls. Pectin is sedimented by exocytosis into cell all via vesicles produced in Golgi. Pectinase are present in higher plants and microbes. In plants, they help in cell wall extension and softening of some plant tissues during maturation and storage. Pectin is comprised of protopectins, pectins, pectinic acids and pectic acids. Pectin has a chain which is partially methyl esterified 1, 4-D-glacturonan and the demethylated pectin is called pectic acid or polygalacturonic acid. polygalacturonic acid splits into monogalacturonic acid, when a glycosidic linkage opens [3]. Depending upon their source of production pectinases can differ in nature. They can be alkaline or acidic. Pectinase commonly referred as pectic enzyme, is a heterogenous group of enzymes such as pectolyase, pectozymes and polygalacturonase that catabolizes complex polysaccharides of plant tissues into biomolecules like galacturonic acids. [4]. Prokaryotes produce alkaline pectinases while eukaryotic microorganisms produce acidic pectinases. Yeast is one such eukaryotic microorganism which has a distinct role in synthesis of pectinases. Their optimal pH and temperature are in the range of 3.5-11 and 40-75°C respectively. Pectinases enzymes are obtained from microorganisms such as bacteria, yeast, fungi and actinomycetes[5]Among pectinase enzyme producing microorganisms, the filamentous fungi are most common. *Aspergillus niger* is one such fungal species that is used for industrial production of Pectinase. On the basis of secretion pectinases are classified as extracellular pectinase and intracellular pectinase. Fungi produces both intracellular and extracellular enzymes however extracellular enzymes are preferred over intracellular as they can be easily extracted and purified and requires less capital [6].In comparison to animals, plants, viruses and fungal extracellular pectinases, bacterial extracellular pectinase is the most significant. *Bacillus subtilis* and *Cocci* produces extracellular pectinase which are of particular interest.These pectinases account for 10 percent of all enzyme activity worldwide [7,13]. Pectinolytic enzymes can be produced by both submerged and solid state fermentation. The substrates used for solid state fermentation are grains such as rice, corn, root, tubers and legumes. Pectinases form an important part of the process in industries producing fruit juices, textile, coffee and tea with various other biotechnological applications. About 10% of total enzyme production in the world market is occupied by microbial pectinases. Pectinases are mainly used for extraction and clarification of fruit juices and wines. Pectinases help increase yield of juices by pulp enzymatic liquefaction. Pulpy products are formed under the effect of pectinases by maceration of organized tissue

into suspension of intact cells [8,12].In wine industry also pectinases are used for decreasing astringency by solubilizing anthocyanins without leaching out polyphenols. Pectinases also increase pigmentation by extracting more anthocyanins [9]. Pectinases have application in fruit and vegetable industry and pectin is extensively used in food industry. Oils derived from peel have many useful applications in both food and pharmaceutical industry. Acidic pectinases are extensively used in fruit juice and wine making industries. The juices produced using pectinases commercially include: (a) Sparkling clear juices, (apple, pear and grape juices, (b) Juices with clouds (citrus juices, prune juices, tomato juice and nectars), and (c) Unicellular products where the intent is to preserve the integrity of the plant cells by selectively hydrolyzing the polysaccharides of the middle lamella. Pectinase produced by different organisms by submerged state fermentation are investigated severely and it has been found that they are cost-prohibitive because of high cost of process engineering [10**].**

MATERIALS AND METHODS

A)Chemicals and reagents : All the chemicals and reagents used were from procured from SRL Pvt. Limited, Hi- Media Limited, Merck India Limited and Sigma Chemicals Co. (USA).

B) Collection of samples:

The soil samples were collected from agriculture and vegetable waste dump areas from different locations of Mohali. The soil was collected with the help of sterile spatula, kept in clean polythene bags and were processed in the laboratory.

C) Isolation of pectinase producing bacteria:

1gm of soil sample was taken and serially 10 folds diluted with dilution 10^{-1} to 10^{-5}. After serially diluted, 300µl sample was taken from each dilution and plated on nutrient agar plates with control. The plates were incubated at 37°C for 24-48 hours. After 48 hours, different types of colonies were observed on plates. Colonies observed were counted under colony counter. Morphologically different colonies were transferred on nutrient agar plates. For further confirmation of pectinase producing bacteria, different isolates were spot inoculated on Vincent media at 37°C for 24-48 hours in an incubator.

D) Morphological characteristics:

After selecting bacteria with pectinolytic properties, they were streaked on NA plates and incubated at 35°C to produce isolated colonies. From the pure individual growth, colony morphology, biochemical studies, and molecular analysis were performed. On the basis of colony morphology different bacterial colonies were isolated from nutrient agar plate and streaked on nutrient agar plates containing pectin.

i) Staining of Isolated bacteria:

Gram and Endospore staining of morphologically different isolated colonies was done and observed under microscope.

ii) Screening:

All the pectinase positive isolates were screened by inoculating them into the screening media. Positive isolates of pectinase producing colonies were then incubated upto 2 weeks. The isolates with highest ratio of clear zone diameter to colony diameter were further selected for screening. Isolates with highest clear zone diameter to colony diameter ratio in the Pectin-Vincent's plates were subjected to submerged fermentation using Vincent's medium (The medium composition was (g/l): Peptone 0.5, Beef extracts 0.3, NaCl 0.5, Agar 15, Pectin 4.0, these contents dissolved in distilled water (pH 7.0))Samples were collected after every 24h and supernatant was used for measuring the enzyme activity.

iii) Pectinase Assay

Pectinase enzyme assay was based on the determination of reducing sugars produced as a result of enzymatic hydrolysis of pectin by dinitrosalicylic acid reagent (DNS) method [11].

iv) Partial Purification of Pectinase

Partial purification of pectinase was done by two methods a) using 90% ethanol and b) using 65% ammonium sulphate followed by Dialysis.

v) Quantitative Estimation of Enzyme

The protein content in the purified sample was determined by Folin-Lowry's method [13].

III. RESULTS

Isolation of bacteria

During the isolation process, the observed microbial population (cfu/ml) of soil samples obtained are shown in Table 1.

TABLE 1- MICROBIAL POPULATION IN DIFFERENT SOIL SAMPLES.

S.No	Soil sample	CFU
1	Agriculture soil	5.4×10^{-5}
2	Waste dump area soil	4.3×10^{-5}
3	Pot soil	7.3×10^{-4}

Morphological Characteristics

40 isolates were isolated (Fig. 1(a),(b)) from the different soil samples on the basis of cultural and morphological characteristics (Table 2). These colonies were designated as I1 to I40.

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TABLE 2: MORPHOLOGICAL CHARACTERISTICS OF BACTERIAL ISOLATES

Culture Code	Cell Shape	Colony Shape	Surface	Motility	Gram Reaction	Color	Odor	Endospore formation
I1	Cocci	Round	Smooth	Non-motile	+ve	Off-white	Odorless	No
I2	Cocci	Round	Smooth	Non-motile	-ve	Off-white	Odorless	No
I3	Cocci	Round	Smooth	Non-motile	+ve	Off-white	Odorless	No
I4	Cocci	Round	Smooth	Non-motile	-ve	Off-white	Odorless	No
I5	Cocci	Round	Smooth	Non-motile	-ve	Off-white	Odorless	No
I6	Rod shaped	Round	Smooth	Non-motile	-ve	Off-white	Odorless	No
I7	Cocci	Round	Smooth	Non-motile	-ve	Off-white	Odorless	No
I8	Cocci	Irregular	Smooth	Non-motile	-ve	Off-white	Odorless	No
I9	Cocci	Round	Smooth	Non-motile	-ve	Off-white	Odorless	No
I10	Cocci	Round	Smooth	Non-motile	-ve	Off-white	Odorless	No
I11	Cocci	Round	Smooth	Non-motile	+ve	Off-white	Odorless	No
I12	Cocci	Round	Smooth	Non-motile	+ve	Off-white	Odorless	No
I13	Cocci	Round	Smooth	Non-motile	+ve	Off-white	Odorless	No
I14	Cocci	Round	Smooth	Non-motile	-ve	Yellow	Odorless	No
I15	Cocci	Round	Smooth	Non-motile	+ve	Yellow	Odorless	No
I16	Cocci	Round	Smooth	Non-motile	-ve	Brown	Typical odor	No
I38	Rod shaped	Round	Rough	Motile	-ve	Off-white	Odorless	No
I39	Cocci	Round	Smooth	Motile	-ve	Off-white	Odorless	No
I40	Cocci	Round	Smooth	Motile	-ve	Off-white	Odorless	No

Fig. 1(a)

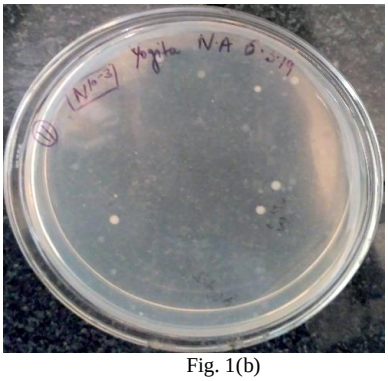

Fig. 1(b)

Fig. 1(a) &(b): Colonies of Isolates on nutrient agar medium

Screening

Isolates were spot inoculated on Vincent's media and iodine test was done to observe zone of clearance as shown in Fig. 2.

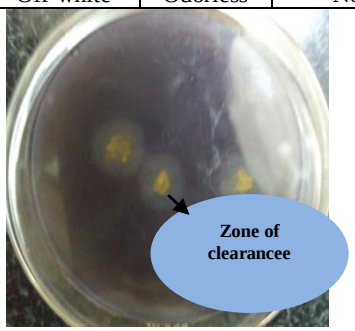

Fig. 2(a): Primary screening

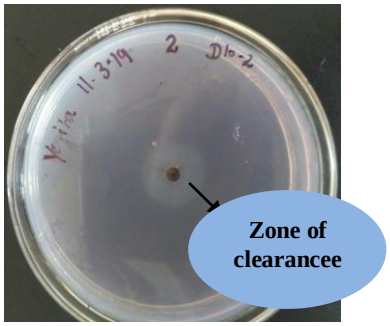

Fig. 2(b): Zone of clearance of 1.3cm

Fig. 2(c): No zone of clearance

Fig. 2(a,b,c): Different bacterial isolates showing zone of clearance.

Enzyme Activity

As shown in Table 3, I38 isolate had maximum enzyme activity i.e 32.53U/ml than I39 and I40.

TABLE 3: ENZYME ACTIVITY OF BACTERIAL ISOLATES

S.No.	Isolates	Enzyme activity U/ml
1.	I38	32.53
2.	I39	30.12
3.	I40	21.68

IV DISCUSSION

Pectinase is used vastly in number of industries and results in reduction of production cost as it enhances the extraction efficiency of fruits, textiles, coffee, paper and pulp. Pectinase originated from microorganisms can be isolated from pectin rich soil. 40 isolates were isolated from pectin rich soil samples collected from seven different regions of Mohali as shown in Fig. 1 and these isolates were cultured, preserved and named as I1 to I40 for further identification on basis of morphological characteristics and other parameters. The isolates were screened for their pectinase producing capability. In a similar study by [13] ninety-five isolates were screened from thirty samples based on characterization on the selective growth media and were classified as actinomycetes, bacteria and fungi. [14] isolated thermophilic actinomycetes and *Thermoactinomyces spp*. from different mushroom compost. All the 40 isolates were examined on the basis of morphological characteristics. They exhibit a variation in shape, colour, odor amongst different strains as shown in table 2, 5% isolates are rod shaped rest 95% are cocci in cell shape. Colony shape of isolates is observed as round and smooth in surface only 2.5% of isolates were having irregular shape and rough surface. Out of 40 isolates 3 were observed as motile. Colour of colonies varied from off- white, yellow to brown. All isolates were odorless except I16 which had a typical odor.Endospore formation was not observed in any of the isolates. According to a research [15] morphological fingerprinting of the colonies revealed that 19% were white in color, 56% were cream and 25% were yellow in color. As far as shape of the colonies was concerned, 82% of the colonies were circular in form, 12% wrinkled while 6% were punctiform. On the basis of the colony elevation and margin, 63% of the isolates were flat, while 37% were each raised whereas 74% were entire, 13% were undulated and 13% were lobate. Based on the cell shape, 81% of isolates were rod shaped, while 19% were bacilli whereas, on the basis of texture, 63% colonies were smooth, 30% were rough and 7% were filamentous. In present study, the isolated bacterial strains were qualitatively screened for pectinase production on Vincent's medium and only nine strains I6, I7, I8, I10, I11, I12, I38, I39, I40 were observed to produce extracellular pectinase and in primary screening, 3 isolates were found to be positive for pectinase production as shown in Fig. 2 & Fig. 3. While in secondary screening, identified isolates with higher pectinase activity. One isolate, I38 showed good zone of clearance as shown in Fig. 3.1 thus, selected for further study as it was considered to be high enzyme producers as zone of clearance of 1.3 cm was observed using iodine. [20] the ratio between clear zone diameter and colony diameter was calculated. The highest ratio observed was 4.7 ± 1.2 by isolate Btk27. Those isolates which scored higher than or equal to 2.0±1.5 (Mean ± SD) which accounts for 33.3% of the pectinase positive isolates. I38 has maximum enzyme activity i.e. 32.53U/ml whereas enzyme activity of I39 and I40 was recorded as 30.12U/ml and 21.68 U/ml respectively. Ammonium sulphate precipitation was done using different concentration of ammonium sulphate to identify optimum concentration of ammonium sulphate for precipitation of intracellular and extracellular protein. In this study, pellet and supernatant was collected and sequential ammonium sulphate saturations from 20-80% i.e. (20, 40, 60 and 80%). Similarly, cell free extracts of *B. parabrevis* C1 and pectinase was precipitated from 30-50% saturation of ammonium sulphate, with 27.5 mg protein and a specific activity of 31.86 IU/mg. 1.66 fold purification of enzyme was achieved with 73.3% pectinase yield at 50% ammonium sulphate fractionation. [16] [17] [18] it has been that observed 30-50% saturation as optimum for efficient precipitation of pectinase from bacterial strains. A 2.31 fold increase in pectinase after ammonium sulphate precipitation was achieved from *Bacillus subtilis* by [15] 50% saturation of ammonium sulphate has been adjudged as best for precipitation of water melon pectinase by [19] In this study, by using different ammonium sulphate concentrations result observed was that 60% ammonium sulphate is optimum for precipitation of intracellular enzyme whereas 80% was optimum for extracellular enzyme as shown in graph 3.

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