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Production of alkaline pectinase by bacteria (*Cocci* sps.) isolated from decomposing fruit materials

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Abstract

Bacterial production of commercial enzymes, including pectinase, has always been the industrial choice. Two decomposing fruit materials (apple and oranges) were enriched to isolate pectinase producing bacteria and were screened for their pectinolytic activity. The best producer (O1*, i.e.* Orange 1) was characterized as *Cocci* sp. and studied for pectinase activity. The culture conditions were optimized for maximum enzyme production by isolate O1 and was found to be 35^{0}C at an alkaline pH of 8.0 with 120 rpm agitation (supporting aerobic conditions) and 72 hours of incubation time and required surfactant for achieving maximum enzyme activity of 13.96 U/ml in crude enzyme extracts. The study provides a strong bacterial candidate for potential industrial production of pectinase.

Keywords: Alkaline pectinase**,** Enrichment, Isolation, Screening, Extracellular

INTRODUCTION

Pectinase is a general term used for a mixture of several enzymes, which break down pectin. Pectin is a high molecular weight polysaccharide, primarily made up of α (1→ 4) linked D-galacturonic acids and are found in the middle lamella and primary cell wall of higher plants [1].

Pectinolytic enzymes are commonly used in processes involving the degradation of plant material and have a share of over 25% in the global scale of the food enzymes [2, 3]. These enzymes have numerous applications in various types of industries like food industries, paper and pulp industry, textile industry, etc [4, 5].

Several microbial strains, including bacterial and fungal strains have been shown to produce different types of pectinolytic enzymes [6, 7, 8, 9, 10, 11]. However, selection of a particular strain remains a tedious task and the choice gets tougher when commercially competent enzyme yields are to be achieved. 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]. The present investigation was carried out to enrich and isolate pectinase producing bacterial strains from decomposing and rottenning apples and oranges. These isolated bacterial strains were screened for pectinase production and the best producer of enzyme was primarily characterized for their morphology and the bioprocess parameters were optimized at shake flask level for the maximum production of pectinase by the isolated bacterium.

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MATERIALS AND METHODS

Sample Collection

For the isolation of pectinase producing bacteria, rottenning fruits (apple and orange) were collected in sterile plastics bags from the local fruit market of Gwalior city, Madhya Pradesh, India, and were stored at 4^{0}C till further investigation. All the chemicals and reagents used for the study were of analytical/microbiological grade and were obtained from commercial vendors.

Enrichment

20 gm of each sample [Apple (A1 and A2) and Orange (O1 and O2)] was dissolved in 100 ml of double distilled water and filtered with the help of sieve. 250 ml of sterile nutrient broth was separately inoculated with each of the filtrates and was incubated overnight at 30^{0}C and 120 rpm in rotary shaker incubator (CIS 24BL Remi, Mumbai). 5 ml each of the overnight cultures were then inoculated in 45 ml of sterile enrichment broth (0.5 g/l Pectin, 5% Yeast extract, 7.15 g/l NH_4Cl, 4.5 g/l $Na_2HPO_4.12H_20$, 6.3 g/l KH_2PO_4, 0.75 g/l KCl and 0.25 g/l $MgSO_4$; pH 7.2) and were subsequently incubated at 30^{0}C and 120 rpm in rotary shaker incubator for 36 hours.

The enriched cultures thus obtained, were subject to subsequent 5 rounds of subculturing using the same enrichment media and incubation conditions as for the 1st round but the concentration of yeast extract (carbon source) was serially decreased to 0.1% (w/v) and that of pectin was increased serially by 5g/l at every round of subculturing. The microbial growth was measured optically by measuring optical density (O.D.) at wavelength of 590nm using spectrophotometer (Systronic 106). All the experiments were conducted in triplicates.

Isolation

0.1ml of broths of 5th round enrichment cultures were spread on the basal agar medium plates with pectin gel surface and were

incubated at 30^{0}C for 36 hours to obtain the bacterial colonies. These colonies were picked and inoculated in fresh sterile pectin broth (5 g/l Pectin, 0.1% Yeast extract, 7.15 g/l NH_4Cl, 4.5 g/l $Na_2HPO_4.12H_20$, 6.3 g/l KH_2PO_4, 0.75 g/l KCl and 0.25 g/l $MgSO_4$; pH 7.2) and were subsequently incubated at 30^{0}C and 120 rpm in rotary shaker incubator for 36 hours, to obtain the pure cultures (A1, A2, O1 and O2).

Screening for Pectinase production

Pure cultures (A1, A2, O1 and O2) obtained after enrichment and isolation, were qualitatively screened for Pectinase production by inoculating them on agarified plates of screening media (1g/l $(NH_4)_2SO_4$; 6g/l Na_2HPO_4; 3g/l KH_2PO_4 and 5g/l Polygalacturonic acid). These inoculated plates were incubated at 30^{0}C for 24 hours to observe the zone of clearance as indicator of Pectinase production.

Estimation of Total extra cellular protein

The total extra cellular protein was estimated by Folin-Lowry's method (4, 9) for an indirect estimation of extracellular Pectinase production by the isolates using a standard curve of Bovine Serum Albumin (BSA) solution (20-100 µg/ml) and O.D. at wavelength of 660nm using double beam UV-Visible spectrophotometer (Systronic AU-2701). The analysis was done for the supernatant of culture broth of all the isolates (A1, A2, O1 and O2) after centrifugation at 8000rpm (Remi C-24BL), which were incubated for 48 hours at 30^{0}C and 120 rpm after inoculation in pectin broth.

Enzyme Activity Assay by Crude Pectinase by Isolates

The crude Pectinase enzyme produced by isolates was assayed using the standard protocol of Sigma [15]. The crude enzyme extracts of different isolates were prepared by the method used to estimate total extra cellular protein. Enzyme assay solution was prepared using 5 ml of 0.5% Pectin solution, 5ml of 500 mM iodine (200mM Potassium iodide), 1.0 ml of 1M Na_2CO_3 and 20 ml of 2M H_2SO_4. The enzyme assay solution thus prepared was mixed with 0.1 ml crude enzyme extracts (for samples) and with distilled water for Blank. These assay solution were incubated for 15 min at room temperature and then titrated against 100mM sodium thiosulphate solution to obtain a colourless solution with 1% starch solution indicator. The enzyme activity was calculated using following formula

$$\text{Units/ml Enzyme} = \frac{1 \times 100x \text{ (Vol. of Blank solution (ml) - Vol. of test solution (ml))} \times \text{Dilution factor}}{5 \times 0.1 \times 2}$$

Primary characterization of Isolate O1

The isolate O1, selected for further study after screened as best producer of pectinase, was studied primarily for its colony morphology, Gram's staining, negative staining and endospore staining.

Optimization of process parameters of Isolate O1 for Pectinase production

The Pectinase enzyme produced by isolate O1 was assayed for optimization of bioprocess parameters, like temperature, pH, incubation time, rotatory speed and requirement of surfactant.

A temperature range of 25^{0}C–50^{0}C and pH range of 6.0-9.0 was screened for optimization of temperature and pH parameter. Also, the incubation time of 24 hours, 48 hours, 72 hours, 96 hours and 120 hours, were used to study the optimal incubation time while the cultures were incubated at stationary and rotatory speeds of 110 – 150 rpm condition to determine whether agitation enhances enzyme production. Also, the isolate O1 was screened for requirement of added surfactant (Tween 80) for effect on pectinase production.

RESULTS

Enrichment, Isolation & Screening of Microorganisms

The enrichment culture of apple sample (A1 and A2) and oranges (O1 and O2) showed prolific microbial growth after 5th round of enrichment with optical density (λ_{590nm}) ranging from 0.250 to 0.333 (Figure 1). Of all the four enriched and isolated pure cultures (A1, A2, O1 and O2), sample O1 was selected for further study based on the prominence of zone of clearance on agarified pectin plates, which showed its maximum ability to produce enzyme pectinase as compared to others.

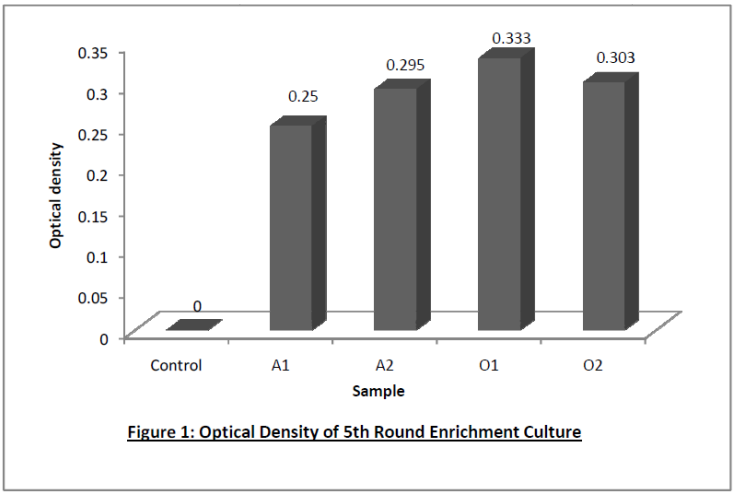

Figure 1: Optical Density of 5th Round Enrichment Culture

Estimation of total extra cellular protein

The selection of isolate O1, for further study, was also based on secondary screening of isolates for total extracellular protein assay by Folin-Lowry's method. The sample O1 showed the maximum extracellular protein concentration of 98 µg/ml after 48 hours of incubation as compared to other isolates (Figure 2).

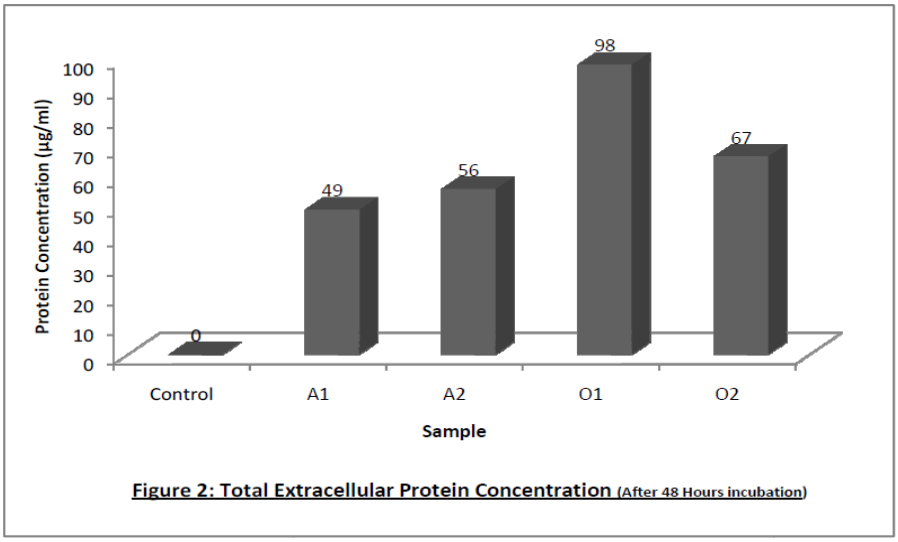

Figure 2: Total Extracellular Protein Concentration (After 48 Hours incubation)

Pectinase activity of crude enzyme extract of isolates

The filtrates of 48 hour cultures of the isolates (A1, A2, O1 and O2) were used as crude enzyme extracts and the isolate O1 again showed the maximum enzyme activity as compared to the other isolates (Figure 3). This was also supportive evidence to further study the isolate O1.

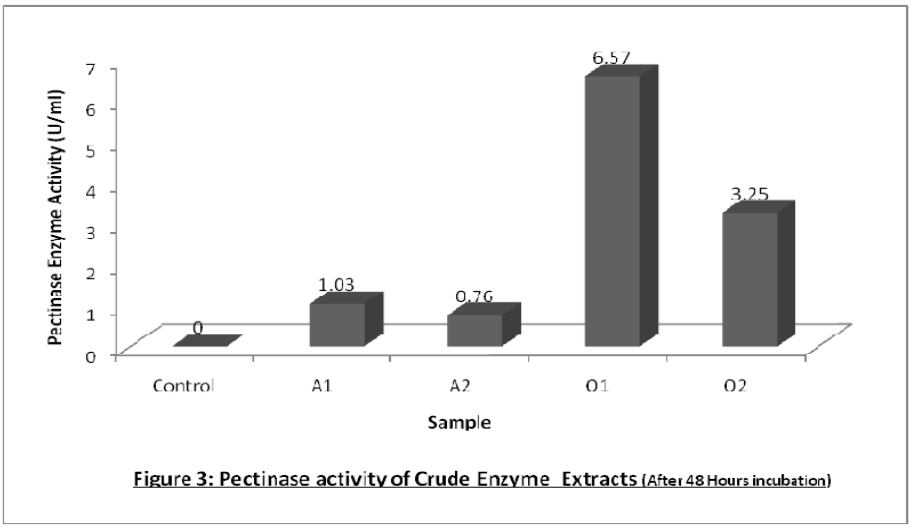

Figure 3: Pectinase activity of Crude Enzyme Extracts (After 48 Hours incubation)

Morphological characteristics of isolate O1

The colonies of isolate O1 was found to be light cream coloured, circular or rhizoid, covering the entire margins and were elevated. The different staining of isolate O1 revealed it as Gram negative *Cocci* and non-endospore formers.

Optimization of culture conditions of isolate O1 for Pectinase production

The pectinase production profile of isolate O1 was maximised by optimization of culture conditions. The enzyme activity was observed maximum and thus optimal culture condition at pH 8.0, with 72 hours incubation time and 120 rpm agitation at 35^{0}C temperature (Figure 4, 5, 6 and 7). The inclusion of surfactant (Tween 80) elevated the enzyme activity.

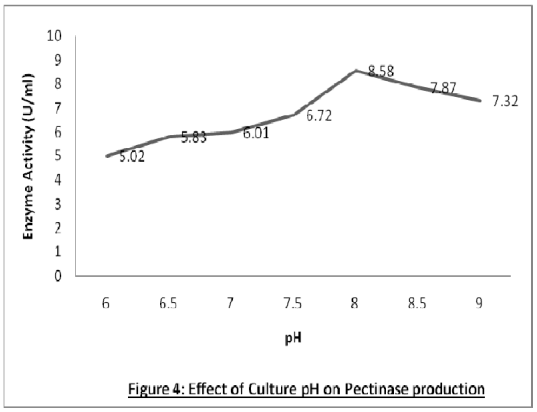

Figure 4: Effect of Culture pH on Pectinase production

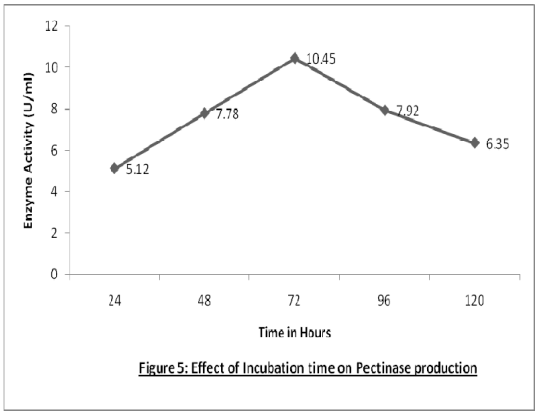

Figure 5: Effect of Incubation time on Pectinase production

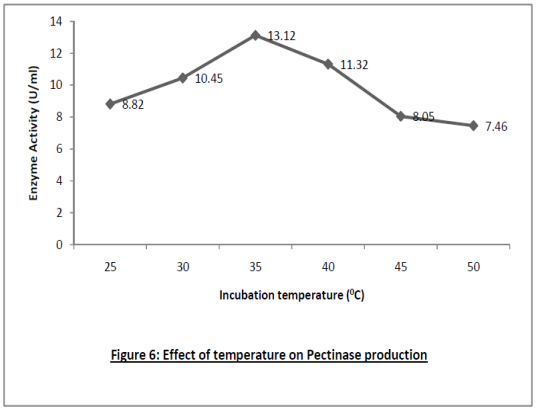

Figure 6: Effect of temperature on Pectinase production

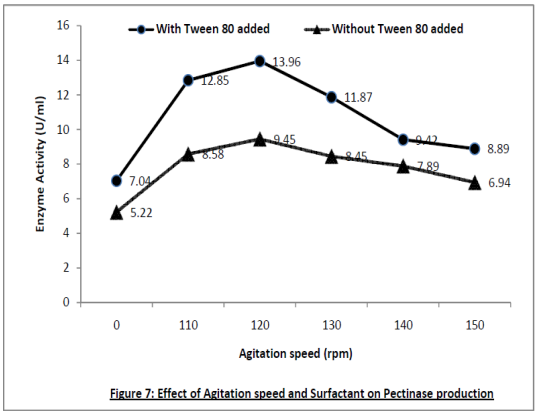

Figure 7: Effect of Agitation speed and Surfactant on Pectinase production

DISCUSSION

The present study made a successful primary attempt to enrich and isolate the potential bacterial strain from the natural reservoirs (rotten apple and oranges) producing industrially important pectinase enzyme. Among all, the isolate from orange (O1) showed the maximum pectinase enzyme activity and was primarily identified as Gram negative *Cocci*. The optimum culture condition for pectinase production by isolate O1 was found to be 35^{0}C at an alkaline pH of 8.0 with 120 rpm agitation (supporting aerobic conditions) and 72 hours of incubation time. The elevation of enzyme activity with addition of surfactant (Tween 80) reflects the extracellular nature of enzyme. Thus, the present study is in good accordance with previously reported studies [14, 16, 17, 18] and is probably the first report of *Cocci* sp. producing pectinase. The study also provides a good platform for further investigations into microbial identification, biochemical characterization of enzyme and optimization of culture conditions to scale up further for its commercial implications.

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