



Bacterial Polygalacturonase (PG) Production from Agro Industrial Waste by Solid State Fermentation

KEYWORDS

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ABSTRACT Agro-industrial wastes are mainly composed of complex polysaccharides that might serve as nutrients for microbial growth and production of enzymes. The aim of the current investigation was to study polygalacturonase (PG) production by *Bacillus licheniformis* cultured on media supplemented with the agro industrial waste (Potato peels). Total 40 bacterial isolates were isolated from clayed Potato peels collected from different restaurants in Nagpur district, MS, India. All bacterial isolates were then screened for their ability to produce polygalacturonase using potato peels by Solid state fermentation (SSF). Results showed that all of the isolates were found to have appreciable Pectinolytic ability of which 15 isolates showed good polygalacturonase producing potential using agro industrial waste at 370 C by pectin clearing zone. Five bacterial isolates were found to exhibit a higher polygalacturonase production by attacking potato peel. The bacterial isolates were identified on the basis of cell shape, cell arrangement, and relation to oxygen and physiological and biochemical tests as *Bacillus licheniformis*. The optimum incubation period observed was 96hrs at 6.6 pH. Out of all the nitrogen sources tested, Peptone, Urea and KNO₃ gave promising results. These results suggesting that, the polygalacturonase was produced from cheap raw material under solid state fermentation under all optimal conditions

Introduction:

The increasing energy demand has been focused worldwide attention on the utilization of renewable resources, particularly agricultural and industrial wastes. The agricultural and agro-industrial wastes, such as wheat bran, rice husk, corn straw, corn cob, fruit peels, paper industry wastes and orange and sugarcane bagasses have high organic matter content and their disposal arise both economic and environmental problems. On the other hand, their major components as cellulose, starch, lignin, xylan and pectin can be used by several microorganisms as carbon and energy sources producing enzymes and other products with high commercial value (Antranikian, 1992, and Bailey, 1990).

Pectic substances are characterized by long chains of galacturonic acid residues. On these residues are carboxyl groups, which are sometimes modified by the addition of methyl groups, forming methoxyl groups. Pectic enzymes act by breaking glycosidic bonds of the long carbon chains (polygalacturonase, pectin lyase and pectate lyase) and by splitting off methoxyl groups (pectin esterase) (Castilho, 1999a, Castilho, 1999b, Collee 1996, Gummadi 2007, Babu and Satyanarayana, 1995). Pectic substances are widely distributed in fruits and vegetables [10-30 %] in turnips, peels of orange and in pulps of tomato, pineapple and lemon), hence they form important natural substrates for pectinases (Gummadi 2007).

Enzymes which degrade pectic substances are pectinases or pectolytic enzymes and can be classified into three types. Pectin methyl esterase (PME) hydrolyzes the methyl ester of galacturonide chain liberating methanol. Polygalacturonases (PGases) and pectate lyases (PLases) split the molecular chains of the respective polymers (Goodman, Kiraly and Wood, 1986, Agrios, 1988 and Kashyap et.al., 2001). Few reviews have highlighted the biological and technological importance of pectinases (Ahlawat et.al., 2007, El-Sheekh et.al., 2008, Favela- Torres et.al., 2006, Kashyap et.al., 2001, Reid and Ricard, 2000).

In the current investigation, we report the nutritional and environmental conditions requirement for the production of polygalacturonase by *Bacillus licheniformis* under solid state fermentation conditions using Agro-waste (Potato peels).

Materials and Methods:

1. Isolation of Bacteria:

Bacterial isolates were obtained from clayed potato peels collected from various restaurants in Nagpur District. For this purpose, first potato peels were washed with distilled water, dried and ground. 1g of this ground peels was added in 10ml sterile distilled water. Serially diluted it in test tubes containing 10ml sterile distilled water. Then, 1ml of sample from last tube was transferred to sterile Petri plate containing autoclaved Czapek's Dox Pectin medium (HiMedia, Mumbai). The plates were incubated at 37°C for 24hrs. The colonies were then maintained on Czapek's Dox Pectin medium.

2. Inoculum built up:

40g potato peels were taken in conical flask to which Basal medium (Sucrose - 10g, KNO₃ - 0.6g, KH₂PO₄ - 1g, MgSO₄ - 0.25g, CaCl₂ - 0.1g, Citrate phosphate buffer- 1000ml, pH - 7) was added just to soak the peels. The flask was autoclaved at 15lbs pressure at 121°C for 15minutes. The flask was then inoculated with the previously isolated Bacterial colonies, incubated at 37°C for 96hrs to get more inoculums.

3. Qualitative screening test:

• Step I:

Pectinolytic enzyme production medium (HiMedia, Mumbai) having composition of **Part A:** NaNO₃ - 2g, KH₂PO₄ - 1g, KCl - 0.5g, MgSO₄ - 0.5g, Yeast extract - 1g, Distilled water - 1000ml, pH - 7, **Part B:** Pectin - 5g, Distilled water - 1000ml was taken. Part A and part B medium was autoclaved separately at 15lbs pressure for 20minutes and then mixed together after autoclaving. This autoclaved media was then inoculated with bacterial colonies, incubated 37°C for 96hrs. The flask was then centrifuged at 5000rpm for 20minutes. The supernatant was then collected.

• Step II:

The autoclaved Polygalacturonase Production and activity assay medium (Pectin - 1g, Arabic gum - 5g, Agar agar - 15g, Citrate phosphate buffer - 1000ml, pH - 7) was poured in sterile Petri plate. Three wells were punctured in it by using sterile cork borer. 0.1ml of previously obtained supernatant was added in each well. Plate was incubated at 37°C for 24hrs. After 24hrs, the results were obtained by adding Lugol's Iodine solution.

4. Construction of Galacturonic acid standard curve:

The protocol followed was as follows-

Sr. No.	Reagents	Blank	T ₁	T ₂	T ₃	T ₄	T ₅	T ₆	T ₇
1	Working standard Galacturonic acid	0	0.5	1	1.5	2	3	4	5
2	Distilled water (ml)	5	4.5	4	3.5	3	2	1	0
3	DNS (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

Table 1: Protocol for Galacturonic acid standard curve

All the tubes were heated in boiling water bath for 5-10 min, then cooled at room temperature. After cooling, the Optical density was taken at 510nm. The graph of concentration of Galacturonic acid was then plotted against absorbance.

5. Estimation of polygalacturonase activity:

• For experimental:

0.125g of pectin was dissolved in 25ml of 0.2M citrate phosphate buffer of pH 6.4. To that 0.5ml of enzyme solution (Supernatant) was added. Flask was then incubated at 37°C for 30min. It was heated in boiling water bath for 5min. The reaction was stopped by using 3ml DNS reagent. Absorbance was recorded at 570nm.

• For Control:

0.125g of pectin was dissolved in 25ml of 0.2M citrate phosphate buffer of pH 6.4. Flask was then incubated at 37°C for 30min. Flask was heated in boiling water bath for 5min. The reaction was stopped by using 3ml DNS reagent. Absorbance was recorded at 570nm. Galacturonic acid standard curve was plotted and Galacturonic acid concentration was measured per unit to find out enzyme activity.

6. Optimization of Polygalacturonase productivity:

• At different pH:

The production medium for most potent isolate were prepared as previously mentioned. The pH was adjusted to different pH values (5, 5.6, 6, 6.4, 7, 7.6, 8, 8.8) by using citrate phosphate buffer (pH ranging from 3-8) and sodium carbonate bicarbonate buffer (pH 9.5). The medium were inoculated with bacterial isolates and incubated. After incubation, the flasks were centrifuged and supernatant was collected for estimating polygalacturonase enzyme activity.

• At different nitrogen sources:

The production medium was supplemented with different nitrogen sources at an equimolar amount of nitrogen that present in sodium nitrate (0.2%W/V) in basal medium. Control was devoid of any nitrogen source. The applied nitrogen sources were KNO₃, Ammonium molybdate, NH₄SO₄, Urea and peptone. The flasks were inoculated and incubated. After incubation, it was centrifuged and supernatant was collected for estimating polygalacturonase enzyme activity.

• At different Incubation periods: Effect of different incubation periods on the polygalacturonase productivity using potato peels under solid state fermentation condition by *Bacillus licheniformis* was tested at time interval of 6, 12, 24, 48, 72, 96, 120, 144 and 168 hrs respectively. After incubation the medium was centrifuged and supernatant was collected for further processing.

Results:

1 Isolation of bacteria:

Forty bacterial isolates were isolated from fermented potato peel. These isolates were purified and subjected to a screening in order to examine their Pectinolytic activities on the basis of mean diameters of clearing zones (mm). All the 40 isolates have Pectinolytic activities while 15 isolates of them were considered to give good producers. The fifteen isolates were subjected to attack potato peel waste under solid state fermentation. Five bacterial isolates namely BC1, BC2, BC9, BC12 & BC15 out of 15 gave higher polygalacturonase pro-

ductivity by attacking potato peels. These five potent bacterial isolates were subjected to an identification program to the species level. The morphological characteristics and stain reaction led to the fact that the given bacterial isolates were suggestive to being belonging to the genus *Bacillus*, Gram positive aerobes to facultative anaerobes. Consulting Bergey's manual of Systematic bacteriology, the isolates were found to be *Bacillus licheniformis*.

2 Innoculum built up:

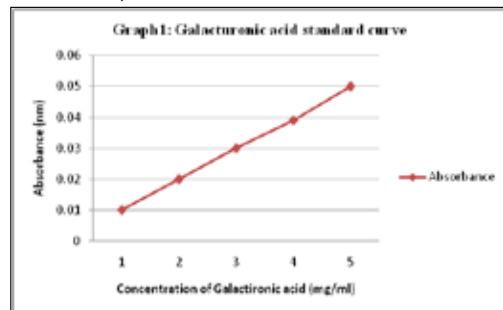
These isolates were inoculated in Basal medium containing potato peels, Heavy growth of organism was observed after 96hrs of incubation.

3 Quantitative screening test:

Later on for quantitative screening test, Pectinolytic enzyme production medium was then inoculated with bacterial colonies, incubated 37°C for 96hrs. The flask was then centrifuged at 5000rpm for 20minutes. The supernatant was then collected. The autoclaved Polygalacturonase Production and activity assay medium was poured in sterile Petri plate. Three wells were punctured in it by using sterile cork borer. 0.1ml of previously obtained supernatant was added in each well. Plate was incubated at 37°C for 24hrs. After 24hrs, by adding Lugol's Iodine solution, clearing zones of the medium was observed.

4 Construction of galacturonic acid standard curve:

When the graph of galacturonic acid concentration (mg/ml) was plotted against Optical density, straight line was observed (Graph 1).



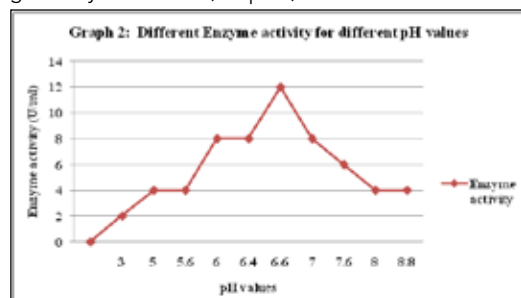
5. Determination of polygalacturonase Enzyme activity:

Absorbance at 570 nm was found to be 0.02 which when plotted on galacturonic acid standard curve, galacturonic acid concentration was found to be 2mg/ml. Then the enzyme activity was calculated by using following formula:

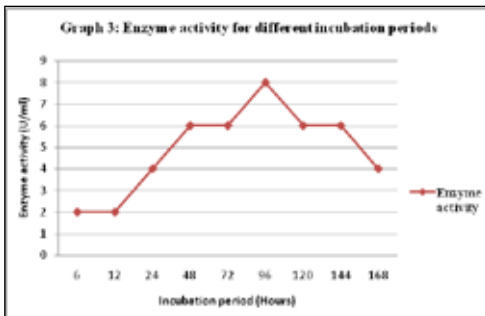
$$\text{Enzyme activity} = \frac{\text{Amount of substrate produced}}{\text{ml of enzyme added}} = \frac{2}{0.5} = 4\text{U/ml}$$

6. Optimization of Polygalacturonase productivity:

• At different pH: The Polygalacturonase productivity by *Bacillus licheniformis* reached its maximum level at pH 6.6. Below and above this optimal pH value, the enzyme activity gradually decreased (Graph 2).

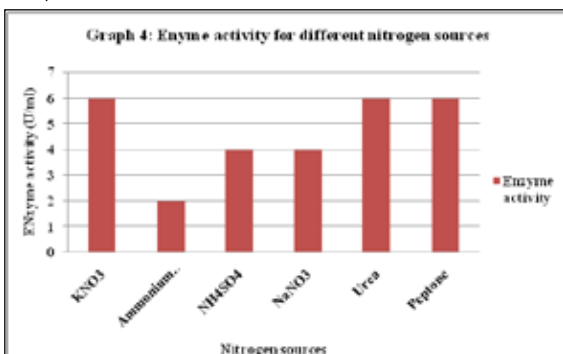


• **At different incubation period:** Effect of different incubation periods on the polygalacturonase productivity using potato peels under solid state fermentation conditions by *Bacillus licheniformis* was tested at time intervals of 6, 12, 24, 48, 72, 96, 120, 144 and 168 hrs. The level of polygalacturonase increased gradually with increasing the incubation period up to a maximum of 96hrs. Then gradually decreased after these periods (Graph 3)



• **Different nitrogen sources:**

Effect of different organic as well as inorganic nitrogen sources on polygalacturonase productivity by the most potent Bacterial isolates of *Bacillus licheniformis* were studied. Five different nitrogen sources were applied. The maximum value of polygalacturonase productivity reached up to 6U/ml in presence of Peptone, Urea and KNO_3 . While in presence of Ammonium molybdate it showed lowest productivity (Graph 4)



Discussion:

5 Enzyme production is a growing field of biotechnology and the world market for enzymes is over 1.5 billion and it is anticipated to double by the year 2015 (Lowe, 2002). The majority of the industrial enzymes are of microbial origin (Sharma, Chisti and Banerjee, 2001). In the present study, forty bacterial isolates were isolated from potato peels collected from different restaurants in Nagpur district, MS, India. These bacterial isolates were grown at 37 °C to be able to produce a polygalacturonase which is favorable to be used as additive for clarification of the juices. A screening of pectinolytic productivities of the 40 bacterial isolates showed that, twenty bacterial isolates gave a good pectinolytic productivity. The nature of solid substrate is the most important factor in solid state fermentation (SSF). This not only supplies the nutrients to the culture but also serves as an anchorage for the growth of microbial cells (Sneath, 1986). The selection of substrate for SSF process depends upon several factors mainly related with the cost of availability and this may involve the screening of several agro-industrial residues. An optimum solid substrate provides all necessary nutrients to the microorganism for optimum function. However, some of the nutrients may be available in sub-optimal concentrations or even not present in the substrates. In such cases, it would be necessary to supplement them externally (Sneath, 1986). Indeed 30- 40 % of the production cost for industrial enzymes are accounted for the cost of the culture medium.

In order to reduce medium costs we screen different low-cost substrates and in the course of this we identified potato peels for cost-effective production of the enzyme under study. SSF is receiving a renewed surge of interest, primarily because of increased productivity and prospect of using a wide range of agro-industrial residues as substrates (Park et al., 2002). From an industrial point of view, in order to achieve production of low cost enzymes these bacterial isolates under study were allowed to grow on natural substances such as *Solanum tuberosum* (ST) (Potato) peels. SSF are usually simpler and can use wastes of agro-industrial substrates for enzyme production. The minimal amount of water allows the production of metabolites in a more concentrated form making the downstream processing less time consuming and less expensive (Pandey et al., 2001 and El-Sheekh et al. 2008). Higher production of pectinase in SSF process may be due to the reason that solid substrate not only supplies the nutrient to the microbial cultures growing in it, but also serves as anchorage for the cells allowing them to utilize the substrate effectively (Pandey et al., 2000). This trial appeared that only five bacterial isolates BC1, BC2, BC9, BC12 & BC15 were considered to be the best for pectinase production by growing on potato peels under solid state fermentation (SSF) conditions. They all were identified as *Bacillus licheniformis*. These results agree with that obtained by Kapoor et al., (2001) who reported that, members of the genus *Bacillus* and related genera are known to produce extracellular pectinases, which have applications in the fiber industry. Bayoumi (1997) produced polygalacturonase from *Bacillus cereus*. Kobayashi et al. (2000) purified the first bacterial Exo-PG from *Bacillus* sp. strain KSM-P443. In the present study, *Bacillus licheniformis* was a Gram-positive rod, catalase positive, spore forming bacteria and grew in both aerobic and anaerobic conditions. These results are in accordance with that recorded by Kapoor et al., (2000) who found that, *Bacillus* sp. MG-CP-2 produce an alkaline and thermostable PG in degumming of ramie (*Boehmeria nivea*) and Sunn hemp (*Crotalaria juncea*).

The environmental conditions in solid-state fermentation can stimulate the microbe to produce the extracellular enzymes with different properties other than those of enzymes produced by the same organism under the conditions performed in submerged fermentation (Pandey et al., 2000). In this field, many workers dealt with the main different factors that affect the enzymes production such as temperature, pH, aeration, addition of different carbon and nitrogen sources. Although such factors were previously studied by many authors, (Lee et al., 1999) Still, we need for more investigation seems to be continuously required to give a chance to isolate more organisms for enzyme production. The purpose of the present work is to determine the optimum conditions for the enzyme (s) productivities by *Bacillus licheniformis*. On the other hand, the economic feasibility of the microbial enzymes production application generally depends on the cost of its production processes. In order to obtain high and commercially viable yields of PG enzymes, it was essential to optimize the fermentation medium used for bacterial growth and enzymes production. Optimal parameters of the PG enzymes biosynthesis from microbial origin, varied greatly, with the variation of the producing strain, environmental, and nutritional conditions (Sharaf and Ammar, 2000).

In the present study, incubation period has an obvious effect on polygalacturonase by *Bacillus licheniformis*, it seems from the results that a lag phase was observed during the first 24h when spore germination took place with practically no enzyme synthesis. Maximal PG productivity on potato peels was observed at the end of 96 h, after which a decline in enzyme activity was observed. This might be due to denaturation and/or decomposition of PG as a result of interaction with other compounds in the fermented medium (Ramesh and Lonsane, 1987) or due to sugar consumption (Garzon and Hours, 1992). In accordance with the present results, Martins et al., (2002) found that, PG production peaked between the 2nd and 4th days of cultivation when wheat bran

or orange bagasse was used as substrate for *Thermoascus aurantiacus*. On the other hand, different incubation periods were recorded by any authors for *Aspergillus foetidus* e.g. 36h (Garzon and Hours, 1992) might be due to who use *Aspergillus foetidus* growing on citrus waste but we use *Bacillus licheniformis* growing on potato peels. The highest PG activity was obtained after 40 days by *Lentinus edodes* growing on straw berry pomace medium under SSF conditions (Zheng and Shetty, 1999). These results are in accordance with those recorded by Kaur et al., (2003) who reported that, PG production by the thermophilic mould *S. thermophile* Apinis was high after 4 days in SmF. As regarded the effect of pH on PG production the present results showed that the optimal pH for highest yield of the PG enzyme from *B. licheniformis* was 6.6. Below and above this optimal pH, the enzyme productivity decreased gradually. This might be due to the growth rate of the *B. licheniformis* was decreased above this pH and the acidity and alkalinity was harmful for bacteria. These results agree with that obtained by Zheng and shetty, (1999) who reported that, PG produced from *Lentinus edodes* has a relatively lower pH optimum (pH 5.0). Kobayashi et al., (2000) reported that, PG produced by *Bacillus* sp. Strain KSM-P443 growing on a pectate agar plate in 100 mM tris-HCl buffer

(pH 7.0). In accordance to the present results, Debing et al., (2005) found that the optimal formula for pectinase production from *A. niger* by solid fermentation under the conditions of natural aeration, natural substrate pH (about 6.5), and environmental humidity of 60 % is rice dextrose 8%, wheat bran 24%, ammonium sulfate [(NH₄)₂ SO₄] 6%, and water 61%, Tween 80 was found to have a negative effect on the production of pectinase in solid substrate. Concerning the effect of the addition of different nitrogen sources for the purpose of biosynthesis and production of PG, it was found that, peptone, urea and KNO₃ were the best PG inducers for *B. licheniformis*. This might be due to peptone is a natural nitrogen source. In this respect, Kashyap et al., (2003) found that, when various nitrogen sources were supplemented in wheat bran medium, yeast extract (YE), peptone and ammonium chloride were found to enhance pectinase production up to 24%.

In conclusion, agro industrial waste such as potato peels induces high levels of a polygalacturonase by *Bacillus licheniformis*. Finally, the use of these residues on industrial enzymatic production would aggregate value to waste and would reduce the environmental pollution.

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