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Journal of Advanced Scientific Research

Available online through http://www.sciensage.info

ISSN
0976-9595
Research Article

# SCREENING AND ISOLATION OF POLYGALACTURONASE (PGase) PRODUCING MICROORGANISMS BY USING AGRO-RESIDUES AS A SUBSTRATE

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**ABSTRACT**

Almost all the commercial preparations of polygalacturonase are produced from fungal sources. Only few reports are available for the cost effective production of polygalacturonase from bacteria. Therefore in the present study, a number of bacterial strains were isolated from soil sample and screened for polygalacturonase production by using agro-residues. KP10B producing maximum polygalacturonase activity (103.35±6.65 U/ml) was identified as *Bacillus subtilis*. Among all tested agro-residues, the maximum enzyme production (147.46±8.29 U/ml) was obtained by using wheat bran as a carbon source.

**Keywords:** Polygalacturonase, Pectin, *Bacillus subtilis,* Wheat bran.

## 1. INTRODUCTION

Pectin is a complex polysaccharide present in the middle lamella of plant cell walls and it has a backbone of - 1, 4-linked D-galaturonic acid residues, with the carboxyl group at C-6 esterified with a methyl group in some residues. It is completely digested by three major enzymes: pectin methylesterase (pectinesterase; EC:3.1.1.11), pectinase (polygalacturonase; EC: 3.1.1.15) and pectin lyase (EC: 4.2.2.10) to release galacturonic acids and its oligomers. Polygalacturonase (pectinase; EC.3.2.1.15) hydrolyzes the α-1, 4-glycosidic bond between galacturonic acid residues and releases oligomers of D-galacturonic acid [1]. PGase has various applications such as, in extraction and clarification of fruit juices, extraction of vegetable oil, processing of alcoholic beverages, fermentation of coffee beans and tea leaves, retting and degumming of fibers, pre treatment of waste water, paper and pulp industry etc.

Polygalacturonase has been reported in a large number of bacteria and fungi viz *Aspergillus niger* [1] *Aspergillus niveus* [2] *B. subtilis* [3] *B. licheniformis* [4] *B. vallismortis* [5] etc. but most commercial preparations of pectinase enzyme are obtained from fungal sources [6]. Hence, an attempt has been made to enrich and screen the bacterial strain for pectinase production and the best producer of enzyme was identified by 16s rRNA sequencing. An approach was also carried out to utilize the agro-residues as a substrate for the production of enzymes at a cheaper rate by using submerged fermentation.

## 2. MATERIAL AND METHODS

### 2.1. Chemicals and reagents

Pectin, polygalacturonic acid and D-galacturonic acid were purchased from Sigma-Aldrich Chemical Co., USA. Microbiological media, cetyl trimethyl ammonium bromide (CTAB) and 3, 5- Di-nitrosalicylic acid and other chemicals used in the study were of analytical grade procured from Hi-media Laboratories, Mumbai, India. Agro-residues and rotten fruits used in the study were collected locally (Aurangabad and Paithan, India).

### 2.2. Sample collection

Samples were collected from soil of fruit farms, different forest regions, fruit processing sites and garbage decomposing yard. Sampling has been carried out randomly using sterile plastic bags and the collected samples were stored at 4°C till further investigation.

### 2.3. Enrichment

One gm of each soil sample was inoculated in 100ml of nutrient broth (for bacterial), potato dextrose broth (for fungus) and starch casein broth (for actinomycetes) and incubated on rotary shaker (120 rpm) for 2 days (37°C), 7 days (30°C) and 5 days (30°C) respectively. For selective enrichment, 1ml of each previously enriched culture was inoculated in 100 ml of freshly prepared

pectin broth supplemented with 1% w/v of pectin and incubation conditions was same as for the 1st round. The enriched cultures thus obtained, were subject to subsequent second rounds of subculturing using the same enrichment media and incubation conditions as for the second round. The samples from last enrichment were subjected for isolation of pectinase producers.

### 2.4. Primary Screening

For the isolation of bacteria, fungus and actinomycetes, the final enriched cultures were subjected to serial dilution and 0.1 ml aliquots of samples from appropriate dilutions were inoculated onto sterilized nutrient agar (NA), potato dextrose agar (PDA) and starch casein agar (SCA) medium by using spread plate method and the inoculated plates were incubated for 2 days (37°C), 7 days (30°C) and 5 days (30°C) respectively. Isolated colonies on NA, PDA and SCA were picked up and spot inoculated on yeast extract pectin medium (1% yeast extract , 0.25% pectin , 2.5% agar and pH was adjusted to 7.0±0.5) [7], PDA (containing 1% pectin) and pectin agar media respectively under same incubation condition. After incubation, plates were flooded with 1% cetyl trimethyl ammonium bromide (CTAB) to observe the zone of clearance, which indicated the presence of pectinase.

### 2.5. Secondary Screening

Bacterial, fungal and actinomycete isolates with clear zone were subjected to submerged fermentation using yeast extract pectin medium (YEP), Czapek's medium [8] with 1% pectin (pH was adjusted to 5.5) and pectin broth (g/l: yeast extract, 1; pectin, 5; $KH_2PO_4$, 4; NaCl, 2; $MgSO_4.7H_2O$, 1; $MnSO_4$, 0.05; $FeSO_4.7H_2O$, 0.05; $CaCl_2.2H_2O$, 2; $NH_4Cl$, 2 and distilled water at pH 7.3.) [9] respectively. Polygalacturonase production from the selected bacteria, fungus and actinomycete isolates were carried out in 50mL of YEP , modified Czapek's and pectin broth production medium in 250mL Erlenmeyer flask which were inoculated with 2% inoculums (130 $x10^4$CFU/ml), one disc (cork borer diameter was 5mm) of fungal hyphae from growing edge of actively growing culture and 1% (v/v) inoculum ($10^6$spores/ml) respectively. Flasks were incubated at 37°C (bacterial, for 48hrs), 30°C (fungal, for 7 days) and 30°C (actinomycetes, for 5 days) under shaking condition. After incubation, bacterial broth was centrifuged at 10000rpm for 10mins and actinomycete culture broth was centrifuged at 8,000 rpm for 30mins. The supernatant was subjected to enzyme assay. Fungal cultures were harvested by filtration through Whatman No.1 filter paper. The filtrate was stored at 4°C and used as the crude enzymes for quantitative polygalacturonase assay.

### 2.6. Enzyme assay

PGase activity was determined by measuring the release of reducing groups from polygalacturonic acid using the 3, 5, dinitrosalicyclic acid reagent (DNSA) assay [11]. The reaction mixture containing 200µl of enzyme and 1000 µl substrate (0.05% polygalacturonic acid in 0.1M glycine NaOH buffer, pH, 8.2) was incubated at 40°C for 20mins. After incubation, 2ml of DNSA was added and the tubes were placed in boiling water bath for 10min. The tubes were cooled and the reducing sugar was determined by measuring the absorbance at 540nm using UV/Vis spectrophotometer. One unit (U) of enzyme activity is defined as the amount of poly-galacturonase required to release 1µmol of galacturonic acid per minute under standard assay condition. Enzyme activity was calculated by using standard graph prepared by different concentrations of galacturonic acid solutions. The PGase activity (in (µM/mL/min or U/ml) was calculated by the following equation [12]:

Enzyme activity (µM/mL/min) = {(Concentration of galacturonic acid (µg/ml) × dilution factor X 1000)/ (Molecular weight of galacturonic acid × time of incubation in mins)}.

### 2.7. Identification

The strain (KP10B) which produced maximum poly-galacturonase activity in secondary screening was identified on the basis of 16S rRNA gene sequence analysis at microbiology laboratory of NCCS, Pune and deposited in their culture collection. The 16S rRNA gene sequence was amplified using universal primers (27 f: 5'-AGAGTTTGATCCTGGCTCAG-3' and 149 2 r: 5'-TACGGCTACCTTGTTACGACTT-3') as per procedure established by Gulati A *et al.,* [10]. The amplified product was sequenced directly on a 3730xl Genetic Analyzer using the ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Bio Systems, USA). The sequence data retrieved was constructed and interpreted using Lasergene SeqMan Pro Pro DNA sequence assembly software (DNASTAR Inc.). A similarity search was conducted for newly created 16S rRNA gene sequences towards the prokaryotic species type strains with validly published names accessible in the EzBio Cloud database. Phylogenetic tree was constructed by

neighbor-joining method using the Molecular Evolutionary Genetics Analysis (MEGA 6, PSU, USA) software with 1000 bootstrap replications.

### 2.8. Effect of nutrient media

The effect of nutrient media on the production of PGase in submerged fermentation was studied by using Yeast extract, Luria-Bertani broth, Nutrient broth, Peptone, Tryptone soybean meal, and Yeast malt broth extract. Each nutrient medium was supplemented with 0.25% (w/v) citrus pectin and pH was adjusted to 7.0±0.5. A 50 ml of nutrient media in 250mL Erlenmeyer flasks were inoculated with 2% (v/v) of inoculums ($80\times10^4$ CFU/ml) and incubated at 37°C, 120 rpm for 48 hours. After incubation, cultural broth was centrifuged at 10000 rpm for 5mins at 4°C. The supernatant was used as crude enzyme extract and subjected to measuring the enzyme activity.

### 2.9. Selection of agro-residues

Agro-residues such as wheat bran, rice bran, bajra bran, jwar bran, nachani bran, sugarcane baggase, apple peel, sweet lime peel, lemon peel, pineapple peel, orange peel, corn cob, deoiled soya meal, deoiled sesame cake, ground nut deoiled cake and tuar fali cover were used as a substrate for the production of PGase in submerged fermentation. All agro-residues were dried and ground finally in electric grinder and then passed through sieve. All the agro-residues and control (citrus pectin) were added separately in YEP medium (Pectin is replaced by 1% agro-residue) and autoclaved at 121°C for 15 minutes. Each flask was incubated with 2% inoculums ($86\times10^4$ CFU /ml) of KP10B and incubated at 37°C for 48hrs. After incubation each flask was analyzed for PGase production. All experiments were conducted in triplicate and the mean values of all the sets of observations were taken for evaluation of results.

## 3. RESULTS AND DISCUSSION

### 3.1. Isolation, screening and identification

In the current study several bacterial strains were isolated from various sources. Total 65 cultures were selectively isolated by enrichment method on nutrient agar, PDA and SCA. Among 60 isolated microorganisms, about 45 isolates were showing pectinolytic activity. The 45 isolates were selected based on the zone of clearance seen around them on YEPA and pectin agar medium after flooding with1% CTAB. Fig.1 shows pectinolytic activity of some bacterial isolates. On the basis of zone of clearance on YEPA and pectin agar plate, 50 isolates were selected for quantitative screening. It was found that all 50 isolates were able to produce poly-galacturonase and their activity ranged from 10±8 U/ml to 103.35±6.65 U/ml. KP10B showed maximum polygalacturonase activity (103.35±6.65 U/ml), so it was selected for further study. The observation of wrinkled, irregular and opaque colonies (KP10B) on nutrient agar indicated the presence of *Bacillus sp*. Actinonmycetes and fungi were also been isolated but they showed low polygalacturonase activity. Fig. 2 shows PGase activity of some isolates.

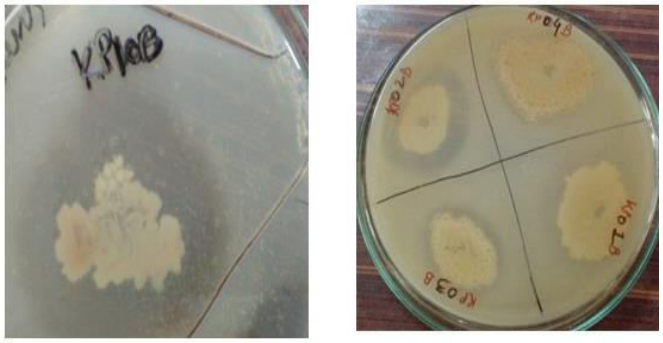

**Fig. 1: Pectinolytic activity of some bacterial isolates on YEPA medium**

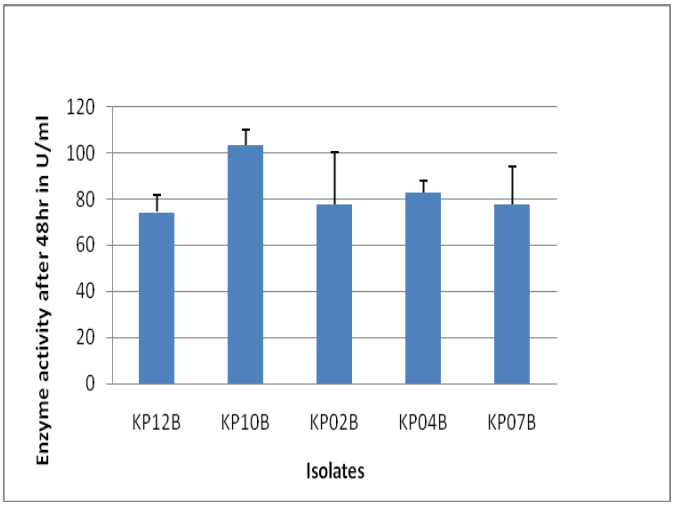


**Fig. 2: Polygalacturonase activity of top 5 isolates**

### 3.2. Identification

16SrRNA gene sequence of KP10B showed 99.85% homology with already reported gene of *Bacillus subtilis* strain HR-4 (16SrRNA gene). The sequence was deposited in Gene bank database with the accession number MW585606. The phylogenetic tree was constructed *in silico* for 16S rRNA gene sequences of the isolate (KP10B) with the closest sequences found within

the NCBI GenBank and *E. coli* as an out-group. The phylogenetic analysis revealed that this isolate (KP10B) had the closest genetic relationship with the *B. subtilis* strain HR-4 with high bootstrap support value. Phylogenetic tree of KP10B with different *Bacillus sp.* and *E.coli* has been presented in Fig.3.

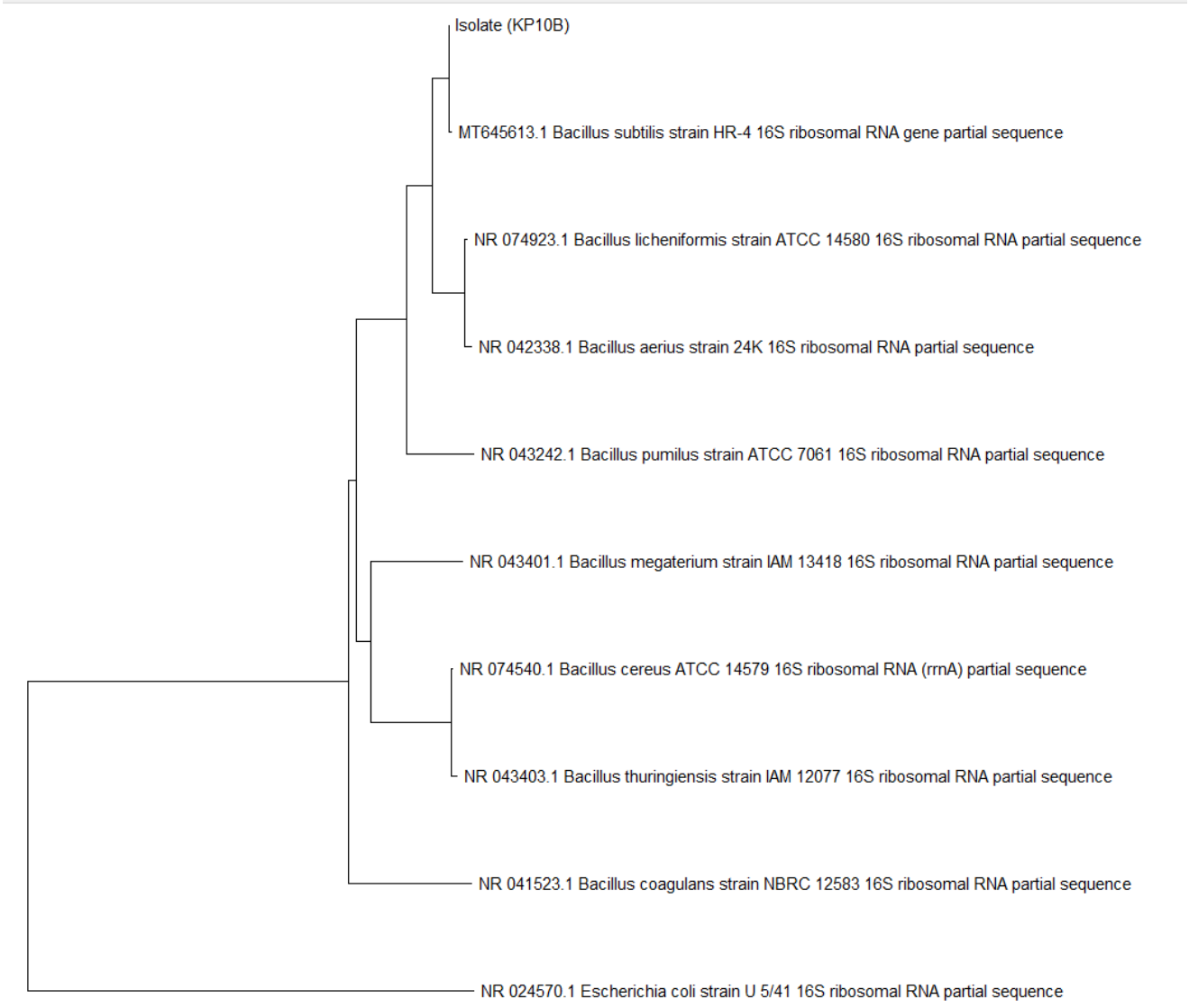


**Fig. 3: Phylogenetic tree based on 16S rRNA gene sequence showing the relationship of KP10B with other organisms belong to *Bacillus sp.* and *E. coli* as an out group**

### 3.3. Nutrient media

At the end of incubation, inoculated nutrient media were assayed for polygalacturonase production. The highest polygalacturonase production was achieved by using yeast extract (105.25±4.92 U/ml). The production of polygalacturonase in yeast extract was higher than any other media (Table 1), so it was used for further study. Yeast extract was the best nitrogen source for polygalacturonase production, possibly due to its high content in minerals, vitamins (especially those belonging to B complex), coenzymes and nitrogen components [13].

**Table 1: Polygalacturonase activity of different nutrient media**

| Nutrient media | Enzyme activity (U/ml) |
|---|---|
| Yeast extract | 105.25±4.92 |
| Luria- bertani | 65.33±10.96 |
| Peptone | 88.54±7.72 |
| Tryptone soyabean meal | 68.36±3.06 |
| Nutrient broth | 68.93±9.18 |
| Yeast malt broth | 86.48±10.77 |

### 3.4. Effect of Agro-residues

Due to the fact that agro-residues are cost effective, KP10B was tested for the production of poly-galacturonase by using different agro-residues as a substrate. It was found that medium containing wheat bran (1% concentration) resulted in high PGase production (147.46±8.29 U/ml) in comparison to any other agro-residues after 48 hrs of incubation. Fig. 4 shows the effect of agro-residues on the production of polygalacturonase.

Wheat bran supported enhance enzyme production and the reason might be the presence of essential nutrients that include pectin, different proteins, vitamins and other sugar contents [14]. It has been previously reported that the wheat bran act as a good substrate for the production of polygalacturonase [14, 15]. Namasivayam E *et al.,* was working on *B. cereus* isolated from market solid waste, reported that pectinase production was enhanced by wheat bran [16].

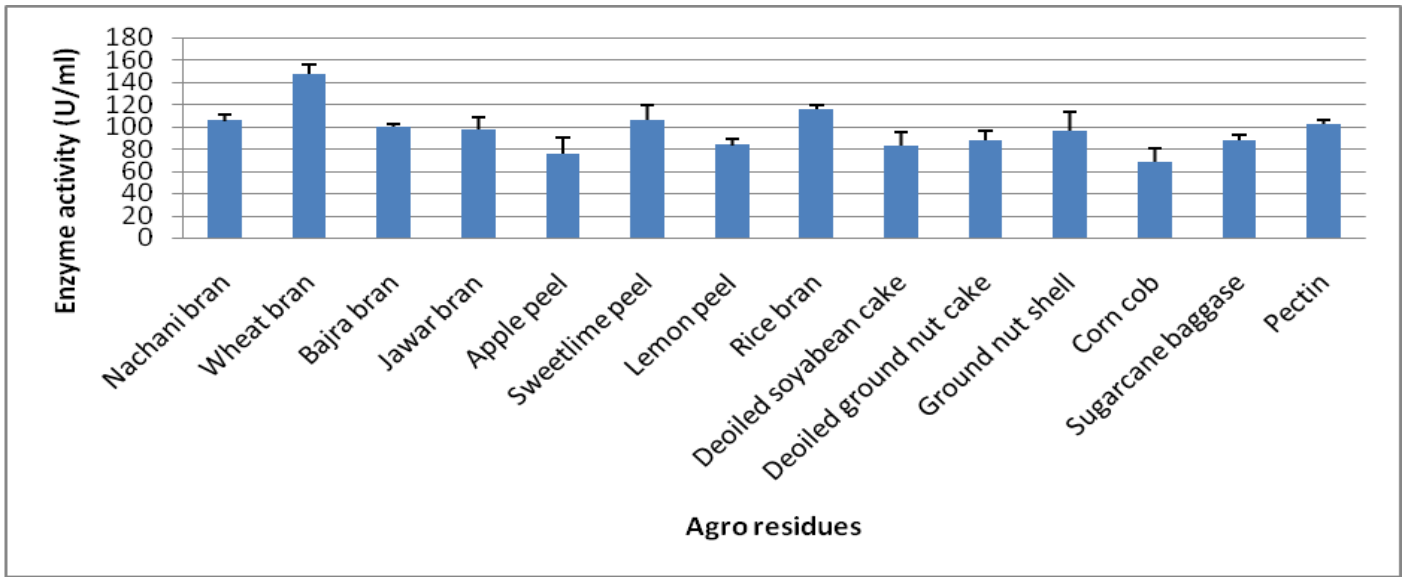


**Fig. 4: Effect of agro-residues on the production of polygalacturonase**

## 4. CONCLUSION

In the present study, KP10B, a bacterium isolated from soil, produced good amount of polygalacturonase after 48 hrs of incubation in production medium at 37°C and pH 7.0. Maximum enzyme production was obtained with wheat bran used as carbon source in comparison to commercial pectin and yeast extract used as nitrogen source. It is feasible to use wheat bran for the production of polygalacturonase in industrial scale, as it reduces costs and aggregates value to the organic material, bringing benefits to the environment as well as the industry and readily available.

### *Conflict of interest*

None declared

## 5. REFRENCES

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