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Microbial pectinases: Isolation, screening and production of pectinases by bacteria

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Abstract

Pectinases are the enzymes that break down polysaccharide pectin of plant tissues into simpler molecules like galacturonic acid. They are present in fruits of plants where they play a natural role in the ripening process; but microbial sources are preferred for large-scale production. Different microorganisms including bacteria, fungi, and also some actinomycetes are known to produce extracellular pectinases. Pectinases are frequently used in fruit and vegetable industry and various other industrial sectors wherever the degradation of pectin is required for a process. Owing to the importance of pectinase enzyme, a lot of researchers have studied and reported the isolation, identification and screening of bacteria from different sources for pectinolytic activity and their use for pectinase production.

Key words : Microbial pectinases, bacteria, isolation, screening.

Pectin is present in the middle lamella of plant cell and it is hetero polysaccharide. Henri Bracannot was the first who isolated and described the pectin[4]. Fruits and vegetables like peels of orange and pulps of tomato, pineapple and lemon contain more amount of pectic substances. Commercial source of pectin is citrus peel and apple pomace[19]. Pectic substance mainly consists of rhamnogalacturonan and galacturonan in which the C-6 carbon of galactate is oxidised to a carboxyl group arabinose and arabinogalactans[42].

Enzymes are highly used in pharmaceuticals, biofuels, food and beverages and in consumer products. It is a macromolecular biocatalyst[8]. The enzyme which hydrolyse pectin substances are called pectinases. Pectinase enzyme has several industrial applications such as in textile for plant fibre processing, in fruit juice clarification, in paper and pulp industry to depolymerize the polymers of galacturonic acid, in production of animal feeds, to remove the mucilage coat from the coffee beans, in tea fermentation to enhance

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the foam forming property of tea and waste water treatment[11,21]. Microorganisms are used for the production of certain enzymes because of low cost production for industries[38]. Pectinase comprises a heterogeneous group of enzymes that catalyze the breakdown of pectin containing substrates. The most important enzymes of the pectinase complex are polygalacturonase, pectin lyase, pectate lyase and pectin esterase. Pectinase enzyme constitutes the 25% of global food and industrial enzymes sales and market is increasing day by day[26].

Many microorganisms have the ability to produce pectinase enzymes. For pectinase production mainly *Bacillus* sp., and *Aspergillus* sp., are widely used[30,33]. Pectin degrading microorganisms produce pectinase and such pectinase producing microorganisms are isolated from different sources such as compost soil, agricultural waste, orange peel and banana peel, *etc*.[20]. Soil especially in and around compost as well as agricultural waste piles are good sources of biodegrading bacteria including pectin degrading bacteria. Pectinolytic bacteria were isolated and screened from agricultural waste dump soils in Vellore, Tamilnadu, India[16]. Pectinolytic bacteria were isolated from decomposing fruit materials[22], from fruit peel waste and agricultural waste[31] and from soil sample and rotten orange[14].

Isolation of pectinolytic bacteria and primary screening for pectinolytic activity is done by using nutrient agar or minimal media supplemented with pectin, pectinase screening agar medium (PSAM), Vincent's media or Citrus Pectin Agar (CPA)[1,31,34,40]. The pectinolytic activity is determined by the development of clear zone around bacterial growth after addition of iodine – potassium iodide solution. Plate assay, turbidity assay, cup diffusion assay and turbidometric assay are regularly used for screening of the pectinolytic activity of microorganisms including bacteria (primary screening)[4,5,12,14]. The bacteria are identified by morphological studies, biochemical tests and molecular methods (16S rDNA sequencing)[16,25,28]. After identifying the pectinolytic bacteria, the production of pectinase is studied by either submerged fermentation or solid-state fermentation and the growth and production parameters are optimized (secondary screening)[23,36]. Pectinase activity is determined by measuring the increase in reducing sugar formed by the enzymatic hydrolysis of pectin[35]. The enzyme is purified by ammonium sulfate precipitation and dialysis, and centrifugation methods and the molecular weight is determined by SDS-PAGE technique[7,36,37].

Many researchers have reported the isolation and screening of bacteria for pectinolytic activity, and production of pectinases by bacteria. In this review, the research works that have been reported in the last decade regarding this aspect are summarized.

Pectinase production by bacteria :

Seventy four bacterial strains were isolated from soil and rotten vegetables and studied for the production of polygalactouronase. Enzyme production was achieved by using citrus pectin as carbon source. The maximum activity was showed by PG-31 strain and was identified as *Bacillus sphaericus*. 16h old culture produced the maximum amount of polygalactouronase

in 72 h of incubation in rotary shaker (160 rpm) at 30°C and pH 6.8. The supplementation of media with 0.9% D-galacturonic acid increased the activity by 23%[17]. Ten bacterial strains were isolated from agricultural waste dump soils. Three strains that showed high polygalacturonase activity were identified based on the morphology and biochemical characterization as *Bacillus* sp. (SH1), *Bacillus* sp. (SC1) and *Bacillus* sp. (ST1). The polygalacturonase activity was found to be maximum at 30°C for 24 hour incubation by *Bacillus* sp. (SH1) (886 U/ml), *Bacillus* sp. (ST1) (908 U/ml) and *Bacillus* sp. (SC1) (988 U/ml). The maximum yield was obtained from *Bacillus* sp. (SC1). In solid state fermentation, *Bacillus* sp. (SC1) produced maximum pectinase activity with wheat bran as a substrate (1431 U/ml) and yield of 1199 U/ml by using rice bran as a substrate[16].

Bacillus sp. and *Pseudomonas* sp., isolated from fruit peel wastes, showed appreciable production of pectinolytic enzymes, such as pectin esterase and pectin lyase. Both the strains showed maximum pectinase activity at one percent pectin concentration. Comparatively *Bacillus* sp., expressed maximum pectate lyase and pectin esterase activity[13]. Pectinolytic bacteria were isolated from decomposing fruit materials (apple and oranges). A cocci bacteria was isolated that showed the maximum pectinolytic activity. Pectinase production was carried out at 35°C, at an alkaline pH 8.0 with 1200 rpm agitation for 72h. The maximum enzyme activity of crude enzyme extract was 13.96 U/ml[22]. A bacterial strain isolated from fruit market wastes was characterized by 16S rDNA sequencing and identified as *Bacillus* sp. MFW7. Using Cassava waste as substrate, after 72 h incubation at pH 6.5 and temperature 35°C, maximum pectinase production was observed by *Bacillus* sp. MFW7. Purified pectinase had a molecular weight of 37 KDa as identified by SDS PAGE[25].

Mineral medium was used for the production of pectinase from batch fermentation using *Bacillus subtilis* EFRL09, isolated from agricultural waste. In mineral medium, waste date syrup was used as carbon source and yeast extract as nitrogen source. pH (8.0), temperature (45°C), concentration of carbon (15 g/L) and nitrogen (7.5 g/L) sources were identified as optimum for the maximum production of pectinase (2,700 U/mL) by *Bacillus subtilis* EFRL09 strain in batch culture[31]. The pectinolytic bacteria were isolated from carrot waste and screened using minimal media. A strain of *Bacillus* sp., was identified based on morphological, biochemical tests, and16s RNA gene sequencing studies. This strain was used for the bulk production of pectinase (maximum yield of 49.58%) at pH 9.0, 50°C temperature using carrot waste[28].

Optimization of pectinase production by *Bacillus firmus* isolated from local cow was carried out by growing on basal media containing pectin. The optimum conditions for the pectinase production by *B. firmus* were pH 7.0 – 8.0, 40 - 50°C and fermentation time of 18 h. While the optimum conditions for pectinase activity were pH 7.0, 50°C and reaction times of 30 min. The Zn^{2+}, Mg^{2+}, and K^{+} ions were found to be enzyme inhibitors[6]. *Enterobacter aerogenes* NBO2 isolated from rotten oranges was studied for pectinase

production under submerged fermentation. The optimum fermentation conditions were pH 6.5, 37°C, inoculums size of 3% (v/v) and agitation speed of 250 rpm, with 1.5% of pectin and 0.26% of yeast extract as carbon and nitrogen sources. These optimum conditions resulted in the highest pectinase production of about 18.54 U/mL at 24 hours incubation time[10].

Bacillus circulans strain was isolated from dump yards of vegetable wastes and screened for pectinase production in submerged fermentation (SmF) condition. The maximum pectinase production was observed with galactose (256 U/ml), yeast extract (130 U/ml), ammonium sulphate (72 U/ml), pH 7.0 (236 U/ml), temperature 40°C (126 U/ml), Tween-80 (144 U/ml), incubation time 48 hours (166 U/ml) and inoculum size level 5% (136 U/ml)[32]. A bacterial strain *Bacillus subtilis* MTCC 441 was studied for pectinase production. The optimum conditions for the pectinase production by *B. subtilis* MTCC 441 were pH 6.5, temperature 37°C, incubation period of 96 h, and the best carbon and nitrogen source were lactose and peptone, respectively. The pectinase enzyme was purified initially by ammonium sulphate precipitation and dialysis method and finally by SDS-PAGE[29].

Two bacterial strains were isolated from rotten orange and were identified as *Staphylococcus aureus* and *Bacillus* sp. Using minimal media, the pectinolytic activity of organism was tested by Kirby Bauer well diffusion method. Both bacterial strains showed nearly similar pectinolytic activity but comparatively *Bacillus* sp. exhibited the highest pectinolytic activity at the highest tested concentration of 30 µl with clearance zone of 25 mm[14]. Six pectinase producing bacterial strains were isolated from soil samples of fruit market using pectinase screening agar medium (PSAM). One strain showing maximum zone (clear zone of 6 mm) was selected and this strain was identified by morphological and biochemical characterization as *Staphylococcus* sp.[34]. Pectinase production by *Bacillus aureus* under submerged fermentation using production medium supplemented with citrus pectin and wheat bran was studied. Production parameters were optimized and maximum enzyme production was observed at pH 8.0 (41.60 IU/ml), at 37 °C (36.3 IU/ml), at incubation for 24 h (36 IU/ml) and in fructose supplemented medium (69 IU/ml). The suitability of this pectinase for juice clarification was investigated. A pectinase dose of 500 µl enzyme with 1 g of fruit pulp exhibited optimum release of reducing sugar and decrease in viscosity (54 %) at pH 7.0, after 24 h of treatment at 37ºC[23].

A total of 70 bacterial strains were isolated from 12 soil samples and screened for pectinase production by spot inoculation on Vincent's agar medium containing pectin as sole carbon source. 36 isolates showed pectinolytic activity with clear zones ranging from 7 - 35 mm. By Standard Conventional methods the pectinase producing isolates were found to be *Bacillus* spp., *Pseudomonas* spp., and *Staphylococcus aureus*. Further four pectinase producing isolates were identified up to species level as *Bacillus firmus* (P1), *Bacillus coagulans* (P13), *Bacillus endophyticus* (P57) and *Bacillus vietnamensis* (P58)[1]. A bacterial strain isolated from maize field soil was identified as *Bacillus axarquiensis* MRRP128 (KF621022) by 16S rRNA sequencing. Maximum enzyme production by

B. axarquiensis MRRP128 (KF621022) was observed at 32°C temperature and pH 7.0, at 72 hours of incubation, with 1% pectin under static conditions. The bacterium produced good amount of polygalacturonase activity (2122 U/lit) at neutral pH[27].

Paenibacillus lactis NRC1 isolated from mangrove habitat was assessed for pectinase production. Primary screening of pectinolytic activity was done and the activity was confirmed by the clear zones on the pectin medium plates. In submerged fermentation *P. lactis* NRC1 produced 3.20 U/ml after 2 days of incubation at pH 7, 40°C, 5 g/L pectin and yeast extract 1 g/L. The enzyme was purified by chromatography and the molecular weight of the purified pectinase was determined by SDS-PAGE as 45 kD[36]. *Bacillus megaterium, Bacillus bataviensis, Paenibacillus* sp., isolated from Lagos Lagoons showed an appreciable pectinase production under submerged fermentation (SMF) conditions. *Paenibacillus* sp., showed more pectinase prodcution compared to other two species. The optimum pH and temperature for the pectinase production by the *Bacillus megaterium* and *Bacillus bataviensis* was 8.0 and 60°C and by *Paenibacillus* sp., was 6.5 and 40°C. The pectinase activity was improved by metal ions such as Na^+ and K^+. Gel filtration was used to check the molecular weight of enzymes and found to be 29,512 Da, 32,359 Da and 25,119 Da for *Paenibacillus* sp., *B. megatreium* and *B. bataviensis* respectively[3].

Bacillus subtilis strain BTK27 showed maximum pectinase production at pH 7.5 and 50°C. The enzyme activity was stimulated with Mg^{2+} and Ca^{2+} ions. *Bacillus subtilis* Btk 27 enzyme was stable in surfactants used in various industries. The enzyme completely removed the mucilage from coffee beans within 24 h treatment in coffee processing[26]. Production of polygalacturonase enzyme by pectinolytic bacterial strain *Bacillus licheniformis* GD2a using banana peel waste was studied. Optimum conditions for the production of polygalacturonase by *B. licheniformis* GD2a was 65°C and pH 7.0 wherein highest enzyme activity of 0.204 U/ml was observed[43].

Fifty microorganisms were isolated from spoiled fruits and vegetables screened for pectinase production using pectin agar plates. Seven strains were found to have pectinolytic activity and the most efficient bacterial strain, MPTD1, was identified as *Bacillus sonorensis*. The maximum enzyme activity obtained *B. sonorensis* was 2.43 µM/mL/min. Pectinase production was high at low to middle value of pH (2.5-8.0), pectin concentration of 19 - 23% and $MgSO_4$ concentration of 0.08 - 0.09%[24]. A total of forty bacterial isolates were isolated from vegetable dump waste soil. Primary screening was done by hydrolysis of pectin and the pectinase activity was assessed by determining the reducing sugar increase resulting from the enzymatic hydrolysis of pectin. The isolate K6 that showed higher pectinase activity in broth medium was identified as *Chryseobacterium indologenes* strain SD and selected for further studies. The *C. indologenes* strain SD produced maximum pectinase production at 37°C, pH 7.5 and incubation for 72 hours, with citrus pectin and yeast extract as carbon and nitrogen sources. The pectinase activity on citrus pectin as substrate was found to be best at pH 8.0 and 40°C[35].

Bacillus strains were isolated from the soil dumped with decomposed fruits from mango processing industry and screened for pectinase production by well diffusion assay using Citrus Pectin Agar (CPA) medium. The strain which showed best pectinolytic activity (15mm clear zone) was characterized with the assistance of IMTECH, Chandigarh, India and identified as *Bacillus megaterium* MTCC 10773[40]. The four bacteria *viz., Bacillus subtilis, Bacillus cereus, Bacillus megaterium* and *Bacillus thuringiensis* were isolated from four soil samples and studied for pectinase production using wheat bran, pigeon peas husk and grams husk as substrates. Out of these four bacteria, *Bacillus cereus* and *Bacillus thuringiensis* showed highest potential of pectinase production. *Bacillus cereus* produced highest pectinase enzyme 111.10 IU/ml/min from pigeon peas husk. *Bacillus megaterium* produced highest pectinase enzyme 113.87 IU/ml/min from wheat bran. Comparatively, *Bacillus megaterium* produced highest pectinase enzyme from wheat bran[41].

Seventeen bacterial strains were isolated from soil and screened for pectinase production using pectinase screening agar (PSAM) medium. Strain showing maximum pectin degradation was identified as *Streptomyces thermocarboxydus* by 16S rRNA analysis. *S. thermocarboxydus* showed optimum production at pH 4.0 and 50°C while the bacterial enzyme activity was optimum at neutral and alkaline pH and 60°C[9]. *Bacillus cereus* and *Staphylococcus aureus* were isolated from the citrus orchard soil and rotten fruits. Both bacteria exhibited maximum growth at 72 hrs of incubation and the optimum conditions for pectinase production were 37°C and pH 8.0. Comparatively *Bacillus cereus* showed the highest potential of pectinase production with clear zone of 12 mm[12].

A thermostable pectinase was isolated from a thermophilic bacterium and studied. The bacterium was isolated from the hot water spring and identified on the basis of its morphological, microscopic, biochemical, physiological and molecular characteristics as *Bacillus licheniformis* UNP-1. Thermostable pectinase production by this bacterium was optimized in a submerged fermentation system using modified fermentation medium (MFM). *B. licheniformis* UNP-1 produced 55.2 U/mL of pectinase. For the production of pectinase, optimum pH was 9.0 and temperature 60°C with 48 hours of incubation. The maximum activity of pectinase enzyme was observed at pH 11.0 and 80°C. The molecular weight of the purified pectinase was 35 kDa. The *B. licheniformis* UNP-1 pectinase resulted in significant fruit juice extraction and fruit juice clarification when apple without peel / with peel were treated[15]. A total of 65 bacterial colonies were isolated from the rhizosphere of a medicinal plant *Andrographis paniculata* Nees and screened for pectinase activity. In plate assay using pectin as a sole source of carbon, 62 (95.38%) out of the 65 isolates showed varying degree of pectinase activity. Among these 62 strains, strain JBST36 showed best pectinase activity followed by the JBST22 and JBST27 (clear zone ≥ 15 mm). Based on the morphological characterization, biochemical test and 16S rRNA analysis, JBST22 was identified as *Bacillus flexus* and the other two (JBST27 and JBST36) were identified as *Bacillus subtilis*[18].

Totally 66 strains isolated from rotten

fruits and vegetables (tomato, mango, peach and cabbage) on Luria agar were screened for pectinolytic activity. Out of 66, 7 strains produced potential pectinolytic activity. Based on morphological and biochemical test, the bacteria were identified as *Bacillus* spp.[11]. *Bacillus tequilensis* CAS-MEI-2-33 isolated from cigar wrappers was screened for alkaline pectinase production using tobacco stalk (TS). *B. tequilensis* CAS-MEI-2-33 exhibited optimal pectinase activity (1370 U/mL) at fermentation period of 40 g, pH 7.0, 40 g/L TS concentration of TS, and 3% inoculum amount[44]. 100 bacterial strains were isolated from Maranda city farmland and screened by MP medium. Three bacteria that exhibited better pectinase activity were identified based on biochemical and molecular studies as *Enterobacter* MF41, *Enterobacter* MF84, and *Enterobacter* MF90. Comparatively *Enterobacter* MF84 was best having optimum activity at 30°C and pH 9.0. By optimization procedures pectinase production by *Enterobacter* MF84 was increased up to 12 times (from 1.16 to 14.16 U/mg)[20].

Forty Bacterial strains were isolated from different soil samples (agriculture and vegetable waste dump areas) and screened for pectinase production. Out of 40, three potential isolates numbered as 138, 139 and 140 showed better pectinolytic activity on Vincent's media. Isolate 138 was rod shaped while other two bacteria were cocci. Isolate 138 had maximum enzyme activity (32.53 U/ml) than 139 (30.12 u/ml) and 140 (21.68 u/ml). Pectinase was partially purified by ammonium sulphate precipitation and dialysis. Sequential ammonium sulphate saturations from 20-80% showed 60% ammonium sulphate was optimum for precipitation of intracellular enzyme whereas 80% was optimum for extracellular enzyme[37]. Twenty nine bacteria were isolated from forest soil samples. Out of 29, 17 were found to be pectinolytic and among them only four bacteria (S-5, S-10, S-14, and S-17) showed high pectin hydrolysis zones. Based on colony morphology, microscopic characterization, biochemical characteristics, and 16S rDNA sequencing, these four bacteria were identified as *Streptomyces* sp. (S-5, S-14), *Cellulomonas* sp. (S-10), and *Bacillus* sp. (S-17). Pectinase extracted from these bacteria exhibited significant activity in oil extraction from sesame seeds and juice extraction from ground apples[39].

Bacillus pumilus bacterium was isolated from tomato vegetables. pectin lyase enzyme was purified from *B. pumilus* and characterized. Using SDSPAGE, PL enzyme was determined to have a single subunit, and molecular weight was defined as 32.88 kDa with gel chromatography technique. Immobilization of purified PL enzyme onto nanoflower chitosan/calcium pyrophosphate yielded hybrid NPs. The effects of pure PL and hNF-PL enzymes on the clarification and cleavage rate of fruit juices obtained from black grape, pomegranate, peach, red apple, and plum were investigated. Highest clarity rates were observed in the production of peach juice, which were 76.6% and 78.7% for pure PL and hNF- PL, respectively[7].

In recent years, the potential of using bacteria as the source of industrially relevant enzymes has increased the interest on exploring several bacteria for extracellular

enzyme production including pectinase. There are a lot of industrial processes to which pectinases can be applied to improve the quality and the yield of final products. Applications of pectinase include fruit juice clarification, juice extraction, manufacture of pectin free starch, refinement of vegetable fibers, degumming of natural fibers, *etc*. Research on pectinase has progressed very rapidly over the last decade and novel industrial applications of the enzyme especially in solid waste management have been identified. To conclude, it must be noted that though a lot of research work is going on in this field, still it is very necessary for screening a large number of bacteria from many unexploited habitats for highly active pectinase enzymes with unique properties like more resistant to higher temperatures, activity at broad pH ranges, and thereby more preferred for industrial processes. Other advantage of bacteria for pectinase production is that they can be subjected to genetic and environmental manipulations to increase the yield.

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