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Research article

Screening, isolation and identification of pectinase producing bacterial strains from rotting fruits and determination of their pectinolytic activity

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Abstract
Naturally occurring polysaccharide pectin, the methylated ester of polygalacturonic acid, is very importance in both scientific and commercial world due to its biodegradability. A large group of pectinase enzymes causing breakdown of pectin polysaccharides of plants and fruit are used in industrial sector to increase the yield and clarity of fruit juices. In this study, two bacterial strains were isolated using dilutions of 10^{-4} and 10^{-6} of rotten oranges. Isolated organisms were identified based on staining and biochemical tests. The pectinolytic activity was determined using pectin containing minimal essential medium. The methodology applied was Kirby Bauer agar well diffusion method at the temperature is 35 ± 2^0C. Based on Gram staining, spore staining and biochemical tests, two bacterial strains L (*Staphylococcus aureus*) and M (*Bacillus cereus*) were isolated and identified. Both strains showed different pectinolytic zones depending on the concentration of inoculum and the largest pectinolytic zone of 25 mm was observed by both strains. These two strains are efficient and have potential to be implicated commercially to increase the clarity and quality of fruit juices.
Keywords: Pectin, polygalacturonic acid, Kirby Bauer method, iodine reagent, pectinase

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Introduction

Pectin or other pectic substances are heterogeneous group of high molecular weight polysaccharides with galacturonic acid residues linked by (1-4) linkages [1]. Pectins constituting middle lamella found between the primary cell walls of adjacent young plant cells [2]. There are four main types of pectic substances protopectin, pectic acid, pectinic acid and pectin. Pectins are the soluble polymeric materials containing pectinic acids as the major component. They can form insoluble protopectin with other structural polysaccharides and proteins located in the cell wall. There are basically three types of pectic enzymes: de-esterifying enzymes (pectinesterases), depolymerizing enzymes (hydrolases and lyases), and protopectinases. They can be further classified as: endo-liquefying or -depolymerizing enzymes or exo-saccharifying enzymes [1]. Pectic enzymes contribute to the degradation of pectin by various mechanisms. Elimination of pectic substances is an essential step in many foods processing and wine industries. These enzymes are mainly synthesized by plants and microorganisms [3]. Fungi synthesize polygalacturonases, polymethyl galacturonases, pectin lyases and pectin esterases. The biotechnological potential of pectinolytic enzymes from microorganisms has drawn a great deal of attention for use as biocatalysts in a variety of industrial processes [4]. Compared to non-enzymatic chemical catalysts, biocatalysts (enzymes or whole-cell microorganisms) are known to present some interesting and advantageous features: high efficiency, mild environmentally-friendly operation conditions, versatility and high selectivity (chemo-, region- and stereo-selectivity) [5]. The low cost substrates are used to reduce production costs to produce industrial enzymes [6]. For the industrial production of pectinolytic enzymes, it is important to improve the fermentation conditions for the better production of extracellular enzymes on inexpensive carbon sources such as apple pomace, citric peels, and other agricultural wastes which contain appreciable quantities of pectin [7] and are degradable by lyases and hydrolases of pectinases [8]. Pectinases have extensive commercial importance for various industrial food applications like in fruit juice industries in order to improve fruit juice yield and clarity. The use of liquefying enzymes for mash treatment results in improvement of juice flow, as maceration of tea leaves [9], processing of cotton fabric [10], leading to a shorter press-time, without the necessity for pressing aids. At the same time pectin is broken down into such an extent that the viscosity of mash is reduced as viscosity relates to molecular weight [11-13].

Pectinases are produced during the natural ripening process of some fruit and act in combination with cellulase. A large number of microbial strains have been studied for the production of pectinase

[14]. The main sources for the pectinolytic complex enzymes are yeast, bacteria and a large number of filamentous fungi of which the most relevant ones are *Aspergillus* sp. The pectinase production in yeast has received less attention due to less yield obtained in comparison to bacteria [15]. A range of bacterial and fungal strains produce a variety of pectinolytic enzymes [16, 17]. The selection of high yield pectinase producing strain is difficult, however studies revealed that bacterial strains usually have been shown to produce more yield of pectinolytic enzymes than those of fungal ones [18]. The objectives of the present study were the screening, isolation and identification of pectinase producing bacterial strains from rotten citrus fruit samples (oranges) and determination of their pectinolytic activity.

Materials and methods

Sample collection

For the isolation of pectinase producing bacterial strains, partially decayed fruit (oranges) were taken in sterile polythene bags from the local market behind UVAS outfall road, Lahore. Till further proceedings, samples were stored at 4^{o}C.

Preparation of pectin substrate

Fresh orange peels were washed with distilled water several times to wash away extra adhering substances. Peels were cut into small pieces and dried overnight at 65^{o}C with shaking at intervals to prevent peels burn. The dried peels were grinded into fine powder that was weighed (23g) and taken in 100mL flask in which 20 ml of 0.5N HCl was added and boiled at 100^{o}C for nearly an hour. It was filtered and the procedure was revised two times for more clearance and quantity. Filtrates were combined and cooled following the addition of 2× of absolute ethanol to precipitate pectin which after being dried at ambient temperature and weighed (4.1g), was placed in sterile bag and stored at 4^{o}C till further investigation. The pectin yield was about 17.8% from the partially rotten orange peel.

Isolation of pectinolytic bacteria

Rotten oranges were used to isolate the pectinase producing bacterial strains. 1 ml of rotten pulp was added in 0.9% saline as a diluent and made 10 fold serial dilutions. A total of 0.1 ml of each dilution was spread on the plates of minimal essential agar medium (MEM) with 2% pectin as a sole source of carbon for bacterial growth [19, 20]. These plates were incubated for 24 to 48 hours at 35^{o}C along with control with 0.9% saline only. The bacterial colonies appeared on plates were further purified on same medium under the same conditions. The control has no growth. Two colonies named as L and M strains were preserved in 30% glycerol at -70^{0}C till further investigation.

Identification of bacterial strains

Identification of both strains was done on the basis of colonial morphology (Size, shape, edges and color of colonies), staining (Gram and endospore) and biochemical testing (catalase, oxidase, IMViC, nitrate reduction, urease and mannitol fermentation) following the previous methods [21, 22]. .

Refreshment of bacterial strains

Three sterile tubes of LB broth with 2% pectin were used for refreshing the preserved bacterial strains. Two tubes were inoculated with each of *Bacillus cereus* (L) and *Staphylococcus aureus* (M) strains leaving the third one as such as control and incubated at 35^{o}C for 24 hrs. The inoculated tubes were turbid and control was without turbidity.

Kirby Bauer method and measurement of zones

The pectinolytic activities of the bacterial strains were determined by Kirby Bauer (KB) disc diffusion method on the minimal essential medium containing 2% pectin as sole source of carbon [23]. Two medium plates were used. A total of 30 µl of each of strain was aseptically poured in 5 bored wells of MEM plates. Plate was then incubated at 35^{o}C for 24 hours. After incubation, plate was taken out and flooded with 50 mM iodine reagent [24]. Plates were then left undisturbed for 5-10 minutes. Then iodine solution was decanted and clear zones around the wells were seen while control plate well with saline showed no pectinolytic activity.

Statistical analysis

Bivariate correlation statistical analysis using Pearson's test was applied on results to find relationship between concentration of bacterial inoculum in wells and pectinolytic clear zones using SPSS software version 20.0**.**

Results and discussion

The strain named as "L" was identified as *Bacillus cereus*. Large, irregular and flat colonies with undulate margins were appeared on Lauria Bertani (LB) agar. Gram-positive, rod-shaped (streptobacilli) on Gram staining, arranged in short chains, spore-forming on spore staining of glycerol preserved culture. The strain was catalase, Voges-Proskauer, glucose fermentation and

citrate tests positive, and reduces nitrate to nitrite. While other tests showed negative results. The strain M was identified as *Staphylococcus aureus*. Golden yellow rounded small colonies on mannitol salt agar medium, Gram-positive, cluster-forming coccus, non-spore forming, fermentation of glucose produces mainly lactic acid, ferments mannitol, catalase positive and coagulase positive (Table 1) [22]. Although, the biochemical and staining results identified both strains as *Bacillus cereus* (L) and *Staphylococcus aureus* (M) but 16S rRNA sequence analysis is required for confirmation. Therefore, both strains will be regard as *Bacillus* sp. and *Staphylococcus* sp. in results.

Table 1 Biochemical test results of isolated strains with pectinolytic activity.

Strains	Catalase	Oxidase	Indole	Methyl Red	VP	Citrate	Nitrate	Urease	Pectinase	Mannitol
L	+	-	-	-	+	+	+	-	+	-
M	+	-	-	-	+	+	+	-	+	+

VP = Voges-Proskauer

On an average, both bacterial strains showed nearly similar pectinolytic activity clear zones but *Bacillus* sp. showed the highest potential of pectinase production at the highest tested concentration of 30 µl with clearance zone of 25 mm (Table 2). The advantage of using microorganisms for the production of enzymes is that these are not influenced by climatic and seasonal factors typically the *Bacillus cereus* producing bulk amylase [25, 26]. The Pearson correlation test also showed significant correlation between the concentration of inoculum and pectinolytic zones (Table 3).

The success of this experiment is the isolation of pectinase producing bacterial strains from natural sources like rotten oranges. Citrus peels especially orange peels are rich source of pectin favoring the most microbes including bacteria and fungi. Orange waste, peels and pomace are also the source of pectin substances. Orange peels have been reported to contain 18.3% pectin and is utilized for commercial extraction of pectin. Hence, the isolation and identification of such microbes is very important for studying their activity [27]. Pectinase activity of different pectinase producing bacteria is determined by growing them on minimal essential medium containing 2% pectin as sole source of carbon. Small concentration of pectin was used, otherwise, at high concentration of pectin bacteria may come under stress and it causes inhibition to pectinase activity. For the measurement of pectinolytic zones, KB method is preferred because it helps in both qualitative as well as quantitative assay of pectinase activity of isolated strains. Greater the diameter, greater will be the ability of bacteria to produce pectinase. It is also easiest method to perform *in vitro*. Major achievement of this study are to screen out most efficient pectinases producing strains of bacteria from the natural environment to fulfill the requirement of local demand as both are potent producers.

Table 2 Pectinolytic zones pattern of various concentration of *Bacillus* sp. and *Staphylococcus* sp.

Name of Bacterium	Conc. (ul/well)	Pectinolytic zones (mm)
Bacillus sp.	10	15.1
	15	17.4
	20	19.9
	25	23.4
	30	25
Staphylococcus sp.	10	14.4
	15	16.2
	20	18.4
	25	21.7
	30	24.2

Table 3 Correlation between concentration of strains inoculum and pectinolytic zones around wells determined by KB method.

	Statistical tests	Conc.	Zones
Conc. of inoculum (ul/well)	Pearson correlation	-	0.982**
	Sig. (2-tailed)		0.000
	N	10	10
Pectinolytic zones (mm)	Pearson correlation	0.982**	-
	Sig. (2-tailed)	0.000	
	N	10	10

** = Correlation is significant at the $P = 0.01$ level (2-tailed).

Conclusions

Bacillus sp. and *Staphylococcus* sp. were isolated from the rotten fruit and were found as efficient pectinase producing bacterial strains that can be used for various industrial applications including extraction and clarification of fruit juices, processing of cotton fabric in textile industries, bleaching of paper, removal of pectic waste waters and maceration of tea leaves.

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