

Full Length Research Paper

Isolation and biochemical characterization of bacterial isolates producing different levels of polygalacturonases from various sources

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Sixty-one bacterial strains were isolated from various sources including soil, water, rotted fruits and vegetables. Preliminary screening for pectinase producing bacterial strains was done by well plate method. Sixteen bacteria giving zones from 08 to 22 mm were selected and subjected to cellular, morphological and biochemical characterization. Further screening of selected strains was done quantitatively by 3,5-dinitrosalicylic acid (DNS) method for polygalacturonase production. Strain Z-AT23, Z-AT33 and Z-AT35 characterized as *Bacillus* sp. produced maximum amount of enzymes ranging from 1.04 to 2 U/ml. Selected *Bacillus* strains were also checked out at different temperatures and pH for the enzyme production. All *Bacilli* hydrolyze more pectin at pH 7.0 to 8.0 but strains Z-AT33 and Z-AT35 produced maximum enzyme at temperature of 42°C and Z-AT23 at 32°C.

Key words: *Bacillus*, 3,5-dinitrosalicylic acid (DNS) method, pectin, polygalacturonase.

INTRODUCTION

Enzymes which degrade pectic substances called pectinases or pectinolytic enzymes, comprises a heterogeneous group of enzymes, and can be classified into three main groups according to their mode of action on pectin containing substrates (Benen et al., 2002). Polygalacturonase (PGase) and pectate lyase (PLase) split the molecular chains of the respective polymers (Kashyap et al., 2001). Pectin methyl esterase (PME) hydrolyzes the methyl ester group of galacturonate unit and liberate methanol. Pectin substances are characterized as a long chain of galacturonic acid residues bounded with carboxyl group, which are sometimes modified by the addition of methyl groups forming methoxyl groups. Pectinolytic enzymes catalyzed pectic substances by breaking glycosidic bonds of the long carbon chains (Hydrolyses and lyases) and by splitting methoxyl groups (esterases). Pectinolytic enzymes having great industrial importance are required

for food processing industries, especially for extraction and clarification of fruit juices, extraction of oils, flavors and pigments from plant materials, textile (Phugare et al., 2011), pharmaceutical, leather, detergent and paper (Reid and Ricard, 2000).

Microbial enzymes are routinely used in many environment friendly and economic industrial sectors (Hoondal et al., 2002). Microbes are the best source of enzymes as they allow an economical technology with low resource consumption and low emission involving no social and political issues, as in the case of animal and plant sources (Dalvi et al., 2007). Such enzymes may be discovered by screening microorganisms sampled from diverse environments or developed by modification of known enzymes using modern methods of protein engineering or molecular evolution. As a result, several important food-processing enzymes such as amylases and lipases with properties tailored to particular food applications have become available (Olempska-Beer et al., 2006). New enzymes for commercial applications with desirable biochemical and physico-chemical characteristics and a low cost production have become a focus of research (Silva et al., 2002; Malvessi and

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Silveira, 2004; Phutela et al., 2005).

Pectinase is an enzyme group that catalyzes pectic substance by degradation reactions through depolymerization (hydrolases and lyases) and de-esterification (esterases) reactions. These polysaccharide degrading enzymes are suitable tools to study the structure of pectin. The main reason is the specificity of these enzymes in comparison to chemical methods, which are less-specific. Most of the plant cell wall degrading enzymes are encoded by a large multigenic family showing diverged expression pathways suggesting functional specialization (Coutinho et al., 2003). Pectinases are classified according to the mode of attack on their specific structural element of the pectin molecule (Benen et al., 2002). Alkaline pectinases are generally produced by bacteria, but are also made by some filamentous fungi and yeasts (Kapoor et al., 2001). They may be used in the pretreatment of waste water from vegetable food processing that contains pectin residues; the processing of textile fibers such as flax, jute and hemp, coffee and tea fermentation, vegetable-oil extraction and the treatment of paper pulp (Zhang et al., 2000).

This study was concerned with the searching of novel bacterial cultures from different habitats for the large scale commercial production of polygalacturonases. This effort can fulfill the national demand and strengthen the industry and economy because valuable foreign exchange is spent on import of industrial enzymes. The bacterial cultures, after screening by well plate method for maximum production of polygalacturonases were biochemically characterized.

MATERIALS AND METHODS

Sample collection and isolation of bacterial strains

Samples including soil, water, rotten fruit and vegetables were collected and stored in air tight polythene bags at 20°C. Different dilutions of each sample were prepared in autoclaved distilled water and spreaded on LA (Lauria agar) plate containing 0.5% yeast extract, 0.5% NaCl, 1% trypton, 1.5% agar having pH 7 (Gerhardt et al., 1994). The plates were incubated for 24 h at 37°C. Bacterial colonies were picked from each plate and streaked on LA plates for further purification. The plates were incubated at the same conditions as above for 24 h.

Screening of bacterial isolates for pectinolytic activity

Bacterial cultures of about 100 ml were incubated at 37°C from starter culture of optical density 0.1 at 600 nm in 20 ml LB (Luria broth) containing 0.5% yeast extract, 0.5% NaCl, 1% Trypton having pH 7 (Gerhardt et al., 1994). After 24 h the broth was centrifuged and supernatant was obtained. The resultant supernatant was used for the screening of bacterial isolates for pectinase activity by well plate method. Petriplates containing autoclaved modified MS medium containing 0.3% KH₂PO₄, 0.6% Na₂HPO₄, 0.2% NH₄Cl, 0.5% NaCl, 0.01% MgSO₄ · 7H₂O, 1.5% agar (Gerhardt et al., 1994) supplemented with 0.2% pectin were prepared. After solidification of the medium, four wells of 5 mm in

diameter were cut in the agar with the help of cork borer. Each well was filled with 25 µL of cell supernatant. After incubating for 24 h at 37°C, plates were observed for pectinase activity by flooding them with iodine solution containing 0.25% iodine, 0.5% potassium iodide and 31 ml of 20% ethanol (Cappuccino and Sherman, 2007). The enzyme activity was observed by measuring the diameter of clear zone around the well in millimeter.

Biochemical characterization of bacterial strains

Bacterial isolates which hydrolyzed the pectin in well plate assay were morphologically and biochemically characterized. The pectinolytic bacteria isolates were biochemically characterized by different tests (Table 2) such as catalase, oxidase, urease, mannitol motility, SIM agar deep tube, starch hydrolysis, nitrate reduction and IMVIC (Cappuccino and Sherman, 2007).

Quantitative assay for polygalacturonase

Reducing sugar released by enzyme action was determined by DNS (Dinitrosalicylic acid) method (Miller, 1959; Bailey, 1988; Hannan et al., 2009). A standard curve of galacturonic acid was constructed and used for estimating the polygalacturonic acid activities in terms of mg/ml and then converted in units (U). One unit is defined as the amount of enzyme which catalysis the formation of 1 µmol of galacturonic acid per minute at 40°C (pH 5.0).

Parameters controlling the polygalacturonase production

Effect of different temperature

The most potent bacterial isolates Z-AT23, Z-AT33 and Z-AT39 were allowed to grow on the L-broth containing 0.5% yeast extract, 0.5% NaCl, 1% Trypton having pH 7 (Gerhardt et al., 1994) at 37°C on continuous shaking (150 rpm) for starter culture. After 24 h, 100 µL bacterial culture of optical density 0.1 at 600 nm was incubated in 100 ml conical flask containing 20 ml L-broth supplemented with 0.2% pectic substrate and incubated for 24 h at 4, 20, 32 and 42°C on continuous shaking (150 rpm). Level of polygalacturonase production at different temperatures was performed by DNS method (Miller, 1959; Bailey, 1988; Hannan et al., 2009).

Effect of different pH values

L-broth for the most potent isolates was prepared as mentioned before. The pH was adjusted ranging from 5 to 9. Inoculum size and incubation conditions were carried as previously mentioned. Quantification of polygalacturonase was done by DNS method to check the maximum level of enzyme of each isolate at different pH.

RESULTS

Isolation and screening of bacterial isolates for pectinolytic activity

Sixty-two bacterial isolates were isolated from the collected samples. These isolates were purified by frequently restreaking them on L-agar plate (Gerhardt et al., 1994) and subjected to preliminary screening by well plate method on modified MS medium (Gerhardt et al.,

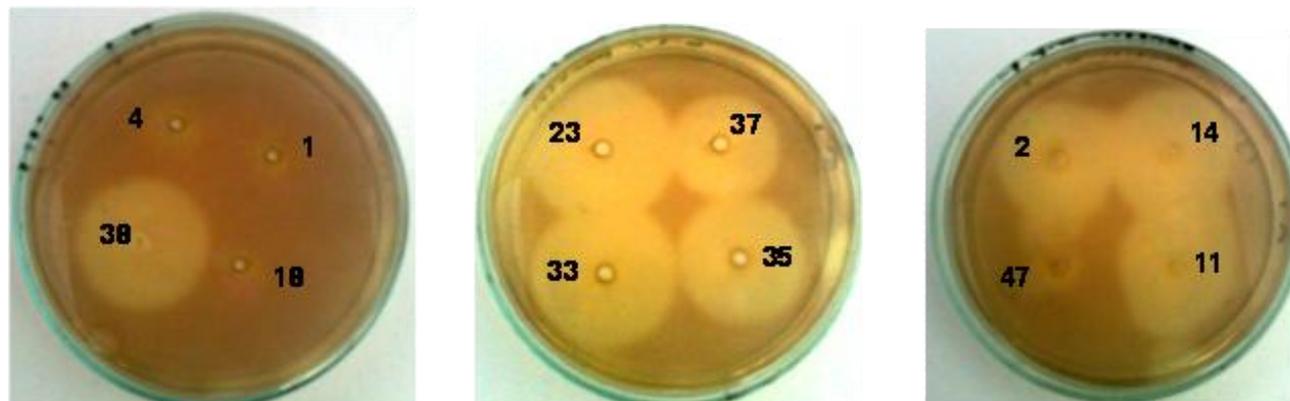


Figure 1. Zone formation by different bacterial cultures showing pectin depolymerization on MS medium supplemented with 0.2% pectin and flooded with iodine solution.

Table 1. Size of zones in mm by selected pectin depolymerizing bacterial isolates in well plate assay.

Isolate no.	Zone size (mm)	Isolate no.	Zone size (mm)
Z-AT2	10	Z-AT35	21
Z-AT6	08	Z-AT37	16
Z-AT11	14	Z-AT38	18
Z-AT14	13	Z-AT39	10
Z-AT23	22	Z-AT45	08
Z-AT27	08	Z-AT46	12
Z-AT33	22	Z-AT57	13
Z-AT34	08	Z-AT63	12

1994) supplemented with 0.2 % pectin (Figure 1). Sixteen isolates listed in Table 1 were selected for pectinolytic activity on the basis of zone size ranging from 08 mm to 22 mm after flooded the plate with iodine solution. One isolate from the juice factory liquid, two from grapes and two from potatoes were giving very good pectin depolymerizing zones of more than 20 mm. Three isolate from sewage water, rotten grapes and cucumber were giving zones of more than 10 mm in size. Eight out of sixteen isolates were giving less than 10mm size zones. Remaining isolates were unable to give any zone in pectin plates.

Characterization of pectinolytic bacterial isolates

Sixteen bacterial strains which were giving pectinolytic zones in well plate assay were subjected to cellular and biochemical tests (Table 2) and characterization as *Bacillus* sp.(Z-AT6, Z-AT11, Z-AT23, Z-AT33, Z-AT34, Z-AT35, Z-AT38, Z-AT39) *Corynebacterium* sp.(Z-AT27, Z-AT37, Z-AT 46) and *Staphylococcus* sp.(Z-AT14, Z-AT14, Z-AT45, Z-AT57, Z-AT63).

Quantitative analysis of polygalacturonase

Exopectinolytic activity in cell free filtrates was assayed by the quantification of reducing sugars using DNS method (Miller, 1959; Bailey, 1988; Hannan et al., 2009). Z-AT11, Z-AT23, Z-AT33, Z-AT35 and Z-AT38 characterized as *Bacillus* sp. showed maximum enzyme activity (1.4, 1.9, 2.0, 1.9, and 1.7 U/ml, respectively) at 37°C as they also showed maximum zones in plate assay. *Corynebacterium* and *Streptococcus* sp. are comparatively less producers as compare to *Bacillus* species (Figure 2).

Parameters controlling polygalacturonase productivity

Isolates of *Bacillus* sp. (Z-AT23, Z-AT33, Z-AT35) giving maximum zone of inhibition (Table 1) and enzyme production subjected to different pH and temperatures showed that response of different isolates is variable at different pH and temperature. Z-AT23 showed more enzyme production at pH 8 and 32°C, Z-AT33 at pH 7 and 42°C, whereas Z-AT 35 gave more

Table 2. Biochemical tests for the characterization of bacterial isolates capable of hydrolysis pectin in well plate assay.

Isolate no.	1	2	3	4	5	6	7	8	9	10	11
	CATA.	OXID.	CITR.	UREA.	INDO.	METH.	VOGU.	NITR.	STAR.	H ₂ S	MANN.
Z-AT2	+	-	-	-	-	+	+	-	-	-	-
Z-AT6	+	-	-	-	-	+	+	-	-	-	-
Z-AT11	+	-	-	-	+	+	+	+	+	-	+
Z-AT14	+	-	+	-	-	+	-	-	-	-	+
Z-AT23	+	-	-	-	+	-	+	+	+	-	+
Z-AT27	+	-	-	-	+	+	+	+	+	-	-
Z-AT33	+	-	-	-	+	-	+	+	+	-	+
Z-AT34	+	-	-	-	+	+	-	+	+	-	+
Z-AT35	+	-	-	-	+	-	+	+	+	-	+
Z-AT37	+	-	-	-	-	+	-	-	+	-	+
Z-AT38	+	-	-	-	-	+	-	-	-	-	+
Z-AT39	+	-	-	-	+	+	-	+	+	-	+
Z-AT45	+	-	+	-	-	+	-	-	+	-	+
Z-AT46	+	-	+	-	-	+	-	-	-	-	-
Z-AT57	+	-	+	+	+	+	-	+	-	-	-
Z-AT63	+	-	-	-	+	+	-	+	+	-	+

Cata., Catalase Test; Oxid., Oxidase test; Citr., Citrate utilization; Urea., Urease test; Indo., Indole; Meth., Methyl red test; Vogu., Vogus proskaur test; Nitr., Nitrate reduction; Star., Starch hydrolysis test; H₂S, Hydrogen sulphide test; Mann., Mannitol test.

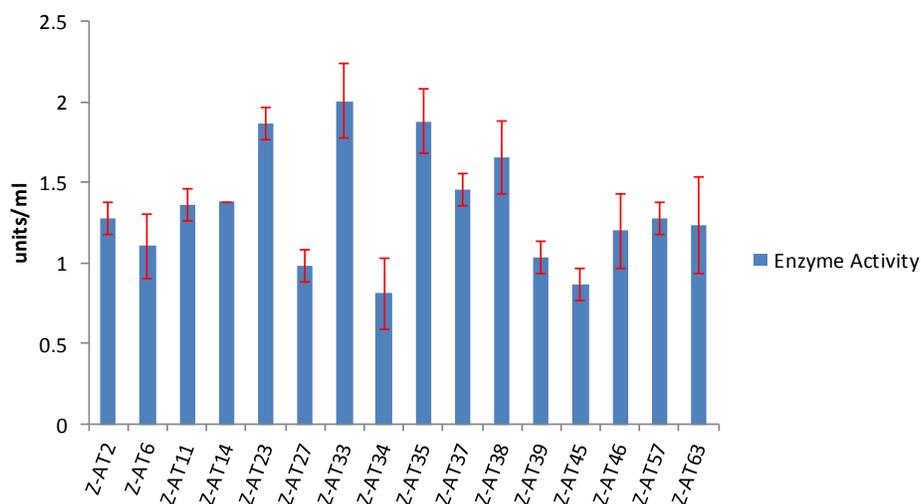


Figure 2. Quantification of polygalacturonase (U/ml) in the overnight cultures of selected pectinolytic bacteria, *Bacillus* sp. (Z-AT6, Z-AT11, Z-AT23, Z-AT33, Z-AT34, Z-AT35, Z-AT38, Z-AT39) *Corynebacterium* sp.(Z-AT Z-AT27, Z-AT37, Z-AT 46) and *Staphylococcus* sp. (Z-AT14, Z-AT45, Z-AT57, Z-AT63).

polygalacturonase production at pH 8 and 42°C (Figure 3).

DISCUSSION

In the cell walls of fruits and vegetables, pectin is the

predominant component (Anuradha et al., 2010) which supports the growth of pectinolytic isolates. Hence vegetables and fruits are good sources of pectinolytic isolates. Although many fungi and yeast (Birgisson et al., 2003) produced exopolygalacturase, both are slow growing and processing microorganisms. Thus, this study was aimed at searching for fast growing and processing

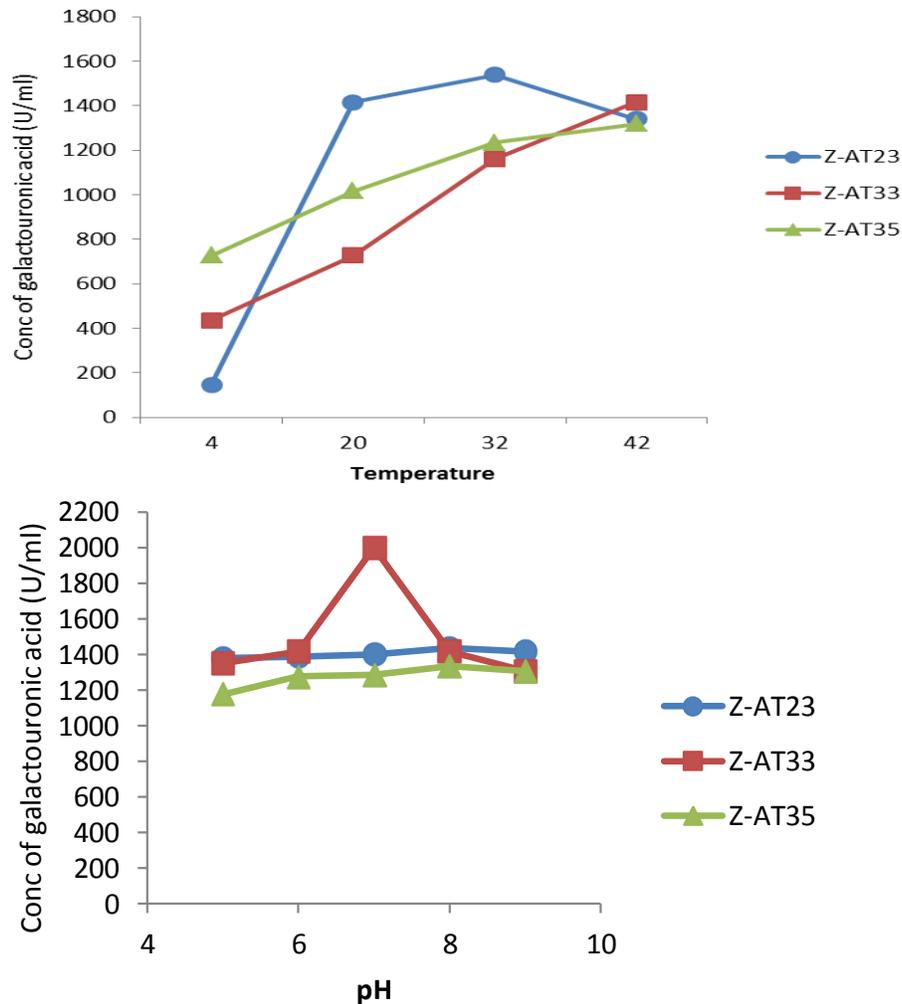


Figure 3. Effect of temperature and pH on the production of polygalacturonases.

bacteria which produce high level of polygalacturonase to fulfill the national demand. There is a dreadful need to uplift national demand by searching the beneficial microorganism on new and novel lines to meet with the national needs required for different industries.

In this study, we have isolated 62 bacterial isolates from different sources, preliminary screened out by well plate method on the Minimal Salt medium plates containing 0.2% pectin. Sixteen pectinolytic isolates that depolymerized pectin, producing a colorless hydrolytic zone in the well plate assay (Soares et al., 1999) were selected. Size of maximum zone was 22 mm visible after flooding the plate with potassium iodide solution (Janani et al., 2011; Rashmi et al., 2008). Poor producers showed no pectinolytic activity and no clear lysis zones. The biochemical and morphological characteristics showed that maximum isolates were *Bacillus* sp. while few were *Clostridium* sp. and *Staphylococcus* sp. Bacterial species isolated from the grapes peel have the ability to produce large quantity of polygalacturonase.

Yeasts are also known to produce pectinases especially polygalacturonases (Birgisson et al., 2003). With interest in only polygalacturonase production, the isolates were further subjected to secondary selection, assessed with the reducing sugar method (DNS). Quantification of the released reducing sugars of selected supernatants was done to analyze the enzymatic activity. Evaluation of the activity in the selected strains was done in culture supernatants. The maximum values for hydrolase activities were observed by Soriano et al. (2005) for *Bacillus* sp. on different substrates (at 40°C), polygalacturonic acid 0.05 U/ml (pH = 5.0) and pectin 0.10 U/ml (pH = 7.0) (Cabeza et al., 2011). After the primary selection, secondary selection was carried out on the criteria of the highest activity at 37°C, assessed with the reducing sugar method (DNS). Three bacterial isolates (Z-AT23, Z-AT33 and Z-AT35) biochemically characterized as *Bacillus* sp. were found to be the most potent isolates for polygalacturonase production ranging from 1.04 to 2 U/ml.

Determination of polygalacturonase (PG) activity through quantification of reducing sugars can detect both exo and endo PG activity. The only soluble product released in the hydrolysis of pectin and polygalacturonic acid was monogalacturonic acid. Exo-PGs can be classified into two types: fungal exo-PGs, which produce monogalacturonic acid as the main end product, and the bacterial exo-PGs, which produce digalacturonic acid as the main end product (Jayani et al., 2005). PG from *A. giganteus* is a typical fungal exo-PG. Janani et al. (2011) showed that maximum production of pectinase was obtained from *Bacillus* species. Temperature is known to control the metabolic rate of the organism involved in the process, which in turn determines the quantity of end product (Patil and Dayanand, 2005). Strain Z-AT23 (*Bacillus* sp.) gave better production of pectinase at pH 8 and 30°C. The limit of polygalacturonase activity was noted at a temperature of 30°C by Mathew et al. (2008). Increase in temperature thereafter caused a decrease in enzyme production, indicating a mesophilic nature of the microorganisms.

However, a moderate optimal temperature for a longer incubation period is favorable as to reduce the cost of production. Z-AT33 (*Bacillus* sp.) produced maximum pectinase production at pH 7 and 42°C while Z-AT35 (*Bacillus* sp.) produced at pH 8 and 42°C. In case of fungi, the maximum rate was obtained at a pH of 5.0. A change in pH beyond the optimum may have a severe effect on enzyme production (Phutela et al., 2005). The optimum pH of mesophilic pectinases has been established to range between 4.0 to 5.5 and temperature optimum between 36 to 45°C (Favela-Torres et al., 2006). Hence, polygalacturonases can be produced under both acidic and alkaline conditions. From our studies, it is concluded that isolates of *Bacillus* sp. produce different levels of polygalacturonase at variable pH and temperatures ranges from 6 to 8 and 32 to 42°C, respectively.

REFERENCES

- Anuradha K, Padma PN, Venkateshwar S, Reddy G (2010). Fungal isolates from natural pectic substrates for polygalacturonase and multienzyme production. *Indian J. Microbiol.* 50:339-344.
- Bailey MJ (1988). A note on the use of dinitrosalicylic acid for determining the products of enzymatic reactions. *Appl. Microbiol. Biotechnol.* 29:494-496.
- Benen JAE, Vinken JP, Alebeek GWM (2002). Pectins and their manipulation. Blackwell Publishing, Oxford. pp. 174-180.
- Birgisson H, Delgado O, Arroyo LG, Hatti-kaul R, Mattiasson B (2003). Cold adapted yeasts as producers of cold-active polygalacturonases. *Extremophiles* 7:185-193.
- Cabeza F, Baca L, Punteros EM, Loto F, Baigori MD, Morata VI (2011). Selection of psychrotolerant microorganisms producing cold active pectinases for biotechnological processes at low temperature. *Food Technol. Biotechnol.* 49(2):187-195.
- Cappuccino J, Sherma N (2007). *Microbiology: A Laboratory manual*. 7th. Ed. Pearson Education.
- Coutinho PM, Stam M, Blanc E, Henrissat B (2003). Why are there so many carbohydrate-active enzyme-related genes in plants? *Trends Plant Sci.* 8:563-565.
- Dalvi P, Anthappan P, Darade N, Kanoongo N, Adivarekar R (2007). Amylase and pectinase from single source for simultaneous desizing and scouring. *Ind. J. Fiber Text. Res.* 32:459-465.
- Favela-Torres E, Volke-Sepúlveda T, Viniestra-González G (2006). Production of Hydrolytic Depolymerising Pectinases. *Food Technol. Biotechnol.* 44(2):221-227.
- Gerhardt P, Murray RGE, Wood WA, Kreig NR (1994). *Methods for general and Molecular Bacteriology*. ASM, Washington, DC.
- Hannan A, Bajwa R, Latif Z (2009). Status of *Aspergillus niger* strains for pectinases production potential. *Pak. J. Phytopathol.* 21:77-82.
- Hoondal GS, Tiwari RP, Tewari R, Dahiya N, Beg QK (2002). Microbial alkaline pectinases and their industrial applications: A review. *Appl. Microbiol. Biotechnol.* 59:409-418.
- Janani L, Karthik G, Kumar KV, Rao B (2011). Screening of Pectinase Producing Microorganisms from Agricultural Waste Dump Soil. *Asian J. Biochem. Pharm. Res.* 2(1):2231-2560.
- Jayani RS, Saxena S, Gupta R (2005). Microbial pectinolytic enzymes: a review. *Process Biochem.* 40:2931-2944.
- Kapoor M, Beg QK, Bhushan B, Singh K, Dadhich KS, Hoondal GS (2001). Application of an alkaline and thermostable polygalacturonase from *Bacillus* sp. MG-cp-2 in degumming of ramie (*Boehmeria nivea*) and sunn hemp (*Crotalaria juncea*) bast fibers. *Proc. Biochem.* 36:803-807.
- Kashyap DR, Voha PK, Chopra S, Tewari R (2001). Application of pectinases in the commercial sector. A Review. *Bioresour. Technol.* 77:216-285.
- Malvessi E, Silveira MM (2004). Influence of medium composition and pH on the production of polygalacturonase by *Aspergillus oryzae*. *Braz. Arch. Biol. Technol.* 47:693-702.
- Mathew A, Eldo AN, Molly AG (2008). Optimization of culture conditions for the production of thermostable polygalacturonase by *Penicillium* SPC-F 20. *J. Ind. Microbiol. Biotechnol.* 35:1001-1005.
- Miller GH (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* 31:426-429.
- Olemska-Beer ZS, Merker RI, Ditto MD, DiNovi MJ (2006). Food processing enzymes from recombinant microorganisms-A review. *Regul. Toxicol. Pharmacol.* 45:144-158.
- Patil NP, Dayanand A (2005). Optimization of process for the production of fungal pectinases from deseeded sun Xower head in submerged and solid state conditions. *Bioresour. Technol.* 97:2340-2344.
- Phugare SS, Kalyani DC, Patil AV, Jadhav JP (2011). Textile dye degradation by bacterial consortium and subsequent toxicological analysis of dye and dye metabolites using cytotoxicity genotoxicity and oxidative stress studies. *J. Hazard. Mater.* 186:713-723.
- Phutela U, Dhuna V, Sandhu S, Chadha BS (2005). Pectinase & polygalacturonase production by a thermophilic *Aspergillus fumigates* isolated from decomposing orange peels. *Braz. J. Microbiol.* 36:63-69.
- Rashmi RS, Murthy KR, Sneha G, Shabana S, Syama A, Radhika VS (2008). Partial purification and biochemical characterization of extracellular pectinase from *Aspergillus niger* isolated from groundnut seeds. *J. Appl. Biosci.* 9(1):378-384.
- Reid I, Ricard M (2000). Pectinase in paper making: solving retention problems in mechanical pulps bleached with hydrogen peroxide. *Enzyme Microb. Technol.* 26:115-123.
- Silva D, Martins ES, Silva R, Gomes E (2002). Pectinase production by *Penicillium viridicatum* RFC3 by solid state fermentation using agricultural wastes and agro-industrial by-products. *Braz. J. Microbiol.* 33:318-324.
- Soares MMCN, Da Silva R, Gomes E (1999). Screening of bacterial strains for pectinolytic activity: characterization of the polygalacturonase produced by *Bacillus* sp. *Revista de Microbiologia.* 30(4):299-303.
- Soriano M, Díaz P, Pastor FIJ (2005). Pectinolytic systems of two aerobic sporegenous bacterial strains with high activity on pectin. *Curr. Microbiol.* 50:114-118.
- Zhang J, Henriksson G, Johansson G (2000). Polygalacturonase is the key component in the enzymatic retting of flax. *J. Biotechnol.* 81:85-89.