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Screening of bacterial strains for pectate lyase production and detection of optimal growth conditions for enhanced enzyme activity

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Abstract: In the present study, the pectate lyase production by fifty two bacterial strains isolated from ramie grown soils were studied and the strain RDSM01 showed maximum pectate lyase activity. According to sequence homology of Genbank, the strain RDSM01 was identified as *Bacillus subtilis* (Genbank Accession No. KX035109). Maximum pectate lyase activity of the strain was observed when 1.5% (v/v) inoculum was added to the growth medium and was incubated for 48 hours at 34-37^{0}C and at pH 7.0. The relative activity of the strain was 19% higher when apple pectin was used as carbon source compared to citrus pectin. Maximum enzyme production (149.1 – 153.4 IU/ml) was recorded when ammonium chloride or ammonium sulphate at 0.4% concentration was used as nitrogen source. Thus, *B. subtilis* strain RDSM01 possessing high pectate lyase activity may be effectively utilized for removal of gum from ramie fibre, which is primarily made of pectin and hemicellulose.

Keywords: *Bacillus subtilis*, Enzyme activity, Growth factors, Pectate lyase

INTRODUCTION

Pectin is a plant polysaccharide primarily made of α-(1-4)-linked D-galacturonic acid and is a component of the middle lamella and primary cell wall thereby contributing to the structure of plant tissues. Pectinolytic enzymes or pectinases degrade pectin through hydrolysis and trans-elimination and also by de-esterification reaction in which the ester bonds between the carboxyl and methyl groups in pectin are hydrolyzed (Uenojo and Pastore, 2007). Among the pectinases, pectate lyase (PEL) had been reported to be used for retting and degumming of bast fibres of flax, ramie, jute and kenaf (Hoondal *et al.*, 2000; Bhattacharya and Paul, 1976; Baracat *et al.*, 1989) as it can effectively degrade the pectin located in the middle lamella and in the primary cell wall of higher plants through trans-eliminative cleavage (Basu *et al.*, 2009).

The crude ramie fibre after mechanical extraction contains approximately 19-30% gum (w/w) which is primarily made of pectin and hemicelluloses and the gummy substances need to be reduced below 6% to obtain spinnable fibre (Mitra *et al.*, 2013). The gum in ramie fibre is removed conventionally by treating the fibre with strong (1%) hot alkali solution which decreases strength of the fibre as well as smoothness of the fibre surface and also pollutes the environment (Ray *et al.*, 2014). The major problems of enzymatic degumming using commercial grade enzymes is the low degumming efficiency, stability of the enzyme as well as high cost of production. In biochemical degumming process, the gum in ramie fibre can be effectively degraded by pectinolytic microorganisms or their enzymes and the fibre can be further treated with mild alkali solution to further reduce the residual gum content in it. Alkaline pectinases produced from *Bacillus sp.* had been efficiently utilized for degumming of bast fibre of ramie (*Boehmeria nivea* L.) (Basu *et al.*, 2009; Kapoor *et al.*, 2001) and buel (*Grewia optiva*) (Kashyap *et al.*,2001). Microbial retting of jute (*Corchorus* sp.) using consortium of *Bacillus pumillus* strains had effectively reduced the retting duration and improved fibre quality over conventional retting (Das *et al.,* 2015). Pectinase enzyme produced by *Rhizomucor pusillus* has hastened the retting of flax (*Linum usitatissimum* L.) (Henriksson *et al.*, 1999) while pectinase enzyme from *Fusarium sp.* could effectively remove the pectin and wax impurities from cotton surface and increase the tensile strength of cotton fabric compared to conventionally scoured fabric (Rajendran *et al.*, 2011). Thus the primary objective of the work was to isolate efficient strains of pectinolytic microorganism having high pectate lyase production potential from ramie grown soil and also to optimize the culture conditions for maximum PEL activity by the identified strains.

MATERIALS AND METHODS

Microorganisms: Fifty two pectinolytic bacterial strains were isolated from the ramie grown soils of four locations belonging to two states of India situated

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in different agro-climatic conditions (Nagaon and Sorbhog of Assam; Coochbehar and Barrackpore of West Bengal). The climate of the locations is subtropical humid in nature, with hot and wet summers and cold winter. The first three locations, namely Nagaon, Sorbhog and Coochbehar are characterized with relatively hot summers (32-35^{0}C) and cold winter (7.5 - 22^{0}C), relative humidity being 85 - 97% and average annual rainfall varying from 1800 to 3300 mm, respectively. The soils are high in organic carbon content (7.3 - 7.8 g kg^{-1}) and acidic in nature (pH 5.3 – 5.8) in all the three locations. At Barrackpore, the average annual rainfall is around 1500 to 1600 mm, average maximum and minimum temperature being 31^{0}C and 21^{0}C, respectively with humidity varying from 51 to 93%. The soil is neutral (pH 6.8-7.1) and the organic carbon content is around 6.0 g kg^{-1}. The soils were treated in the laboratory with papaya (*Carica papaya* L.), a good source of pectin, to activate the pectinolytic strains present in those soils.

Bacteria were isolated on the yeast pectin media (0.5% NaCl, 0.4 % NH_4Cl, 1.0% yeast extract, 0.75% citrus pectin and 0.005% cycloheximide at pH 8.0) by pour plate method. Pectinolytic bacterial colonies (clear halo around the colonies) were selected, purified by repeated selection of single colonies following streaking on agar plate. Colonies showing maximum halo zone were selected for further study.

Production medium: The liquid medium of yeast pectin (pH- 8.0) was used for bacterial isolation and enzyme production. The yeast pectin media comprised of 0.5% NaCl, 0.4% NH_4Cl, 1.0% yeast extract, 0.75% citrus pectin and 0.005% cycloheximide at pH 8.0.

Screening of isolates for pectinolytic activity: The pectinolytic microbes were isolated from the pectin pre-enriched soil by standard pour plating method. Six distinct colonies among fifty two isolates producing maximum clear halo zone were selected and maintained for further study.

Identification of the selected isolates: The microbial characterization of the selected strain (RDSM01) was carried out by 16s rDNA method. The 16S rDNA gene was amplified using universal primer 515F

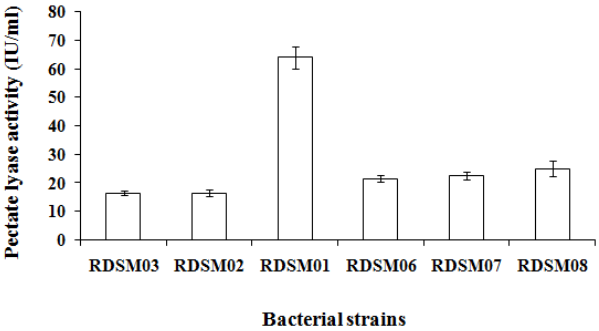

Fig. 1. *Pectate lyase production from isolated strains in yeast pectin media.*

Table 1. Effect of various carbon sources on pectate lyase activity.

Carbon sources	Pectate lyase activity* (IU/ml)
Sucrose	37.0±2.21
Citrus pectin	141.0±9.74
Apple pectin	168.5±6.09
Glycerol	35.5±2.28
Lactose	68.5±7.31
Xylose	72.5±4.19
Dextrose	83.0±5.23

*: mean values of 10 observations

(GTGCCAGCMGCCGCGGTAA) (Turner *et al.,* 1999)/1381R (CGGTGTGTACAAGRCCYGRGA) (Hodkinson and Lutzoni, 2009). According to the standard methodology, PCR amplification was carried out, the resolved amplified PCR products were gel excised and purified using MinElute Gel extraction kit (Qiagen Inc. USA). The purified PCR products were cloned into pGEM-T Easy vector system (Promega Corp., USA) as per the manufacturers protocol and transformed into competent cells of *E. coli* strain DH5α using the standard heat-shock method. The white colonies on Xgal-IPTG selection plate were considered as putative positive clones and confirmed for the presence of PCR product through EcoRI Restriction digestion. Three randomly selected positive clones were sequenced from the Xcelris labs limited, India. Sequenced data of cloned fragments were analyzed using BLASTN 2.3.1 (NCBI) and compared to the available NR database.

Enzyme assay: The selected isolates were grown in yeast pectin broth for 48 hours at 35^{0}C temperature as stationary / shake (150 rpm) culture. The pectate lyase activity in the culture filtrate was determined by colorimetric method as described by Basu *et al.* (2009). One unit of activity was defined as the amount of enzyme that caused a change in absorbance of 0.01 under the conditions of the assay.

Optimization studies of PEL production

Optimization of temperature and pH: The optimum temperature for production of pectate lyase enzyme by the selected isolates was determined by incubating the production medium at different temperatures (25^{0}C, 28^{0}C, 31^{0}C, 34^{0}C, 37^{0}C, 40^{0}C, 43^{0}C, 46^{0}C and 50^{0}C) for 48 hours and enzyme activity was assayed. In another experiment, the pH of the medium was varied from 2 to 12. The medium was then inoculated with the selected strains at the optimum temperature (determined in the previous experiment) and was incubated at for 48 hours and the PEL activity was measured.

Optimization of inoculum size and incubation time: The effect of inoculum size on PEL production was studied by inoculating the production medium with different amount of inoculum (1.0%, 1.5%, 2.0%, 5.0%, 7.5%, 10%, 12.5% and 15%, v/v) at 34^{0}C for 48 hours under aseptic conditions and enzyme activity

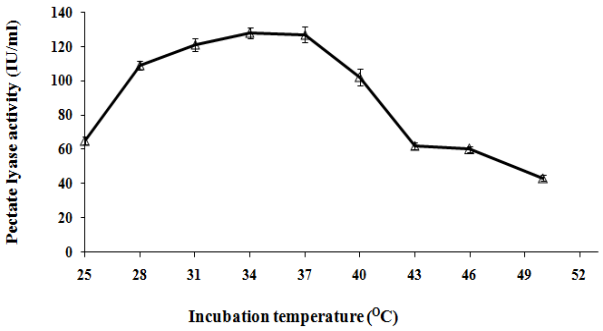

Fig. 2. *Effect of temperature on pectate lyase activity.*

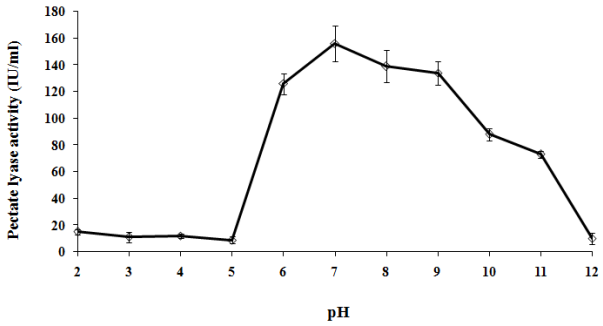

Fig. 3. *Effect of pH on pectate lyase activity.*

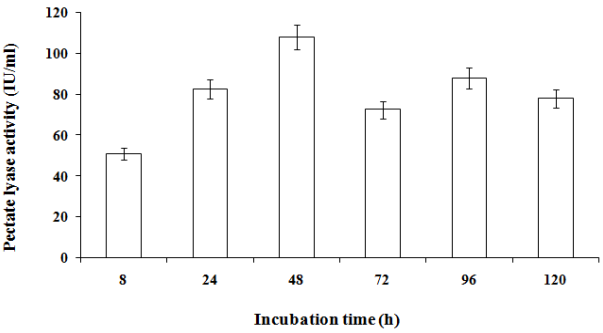

Fig. 4. *Effect of incubation time on pectate lyase activity.*

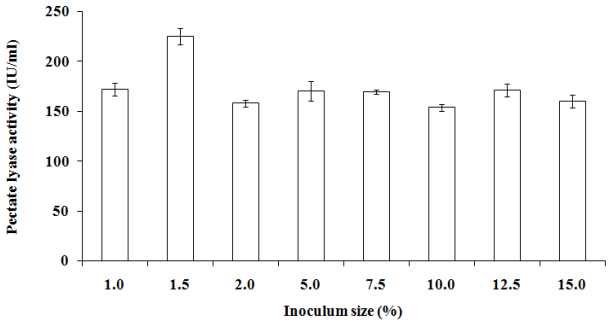

Fig. 5. *Effect of inoculum size on pectate lyase activity.*

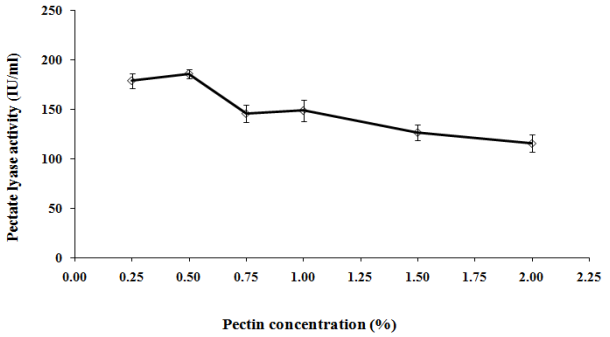

Fig.6.*Effect of pectin concentration on pectate lyase activity.*

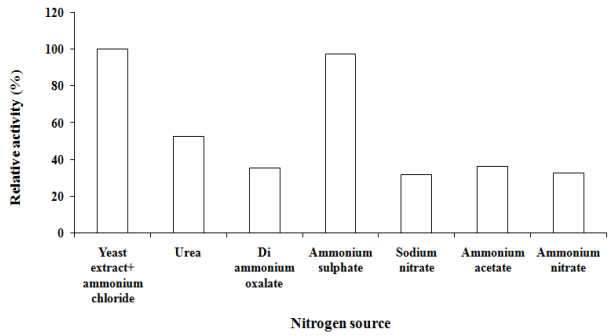

Fig. 7. *Effect of various nitrogen sources on relative pectate lyase activity.*

was assayed. Similarly, the medium was inoculated with the selected isolate at 34^{0}C and was incubated for different time periods (24, 48, 72, 96 and 120 hours) and PEL activity was measured.

Optimization of pectin concentration: The PEL activity was measured at different pectin concentrations (0.25%, 0.5%, 0.75%, 1.0%,1.25, 1.5%, 1.75 and 2.0% w/v) to determine the optimum concentration of carbon source in the production medium for getting maximal enzyme production.

Optimization of carbon and nitrogen sources: Various carbon sources, viz. citrus pectin, apple pectin, glycerol, sucrose, lactose, xylose and dextrose were used in the production medium at a concentration of 1.0% w/v to study the effect of carbon source on pectate lyase production. The medium was incubated at 34^{0}C for 48 hours and the culture supernatants were assayed for PEL activity.

The production medium was supplemented with different nitrogen sources including ammonium chloride, urea, di-ammonium oxalate, ammonium sulphate, sodium nitrate, ammonium acetate, ammonium nitrate and yeast extract at a concentration of 0.1% w/v. The medium was then incubated at 34^{0}C for 48 hours and the PEL activity was determined.

RESULTS AND DISCUSSION

Six out of fifty two bacterial isolates grown in pectin agar plate were primarily chosen based on the size of halo zone produced around the colonies (data not shown) and pectinolytic activity of these six isolates was studied. The isolate RDSM01 recorded maximum pectate lyase activity (60 IU/ml) among the six and was finally selected (Fig. 1).

According to a 16s rDNA sequence homology analysis from BLASTN 2.3.1 (NCBI) results, the strain (RDSM01) was identified as *Bacillus subtilis*. The cloned 16s rDNA sequence was deposited in the NCBI with GenBank Accession No. KX035109. Further, the

optimum condition for production of pectate lyase (PEL) enzyme by the strain RDSM01 was studied.

PEL activity of the strain RDSM01 was studied over a temperature range of 25-50^{0}C and higher enzyme activity (127-128 IU/ml) was noticed at temperature range of 34-37^{0}C which declined steadily with further increase in temperature (Fig. 2). The reduced PEL activity of the strain at higher temperature may be due to thermal denaturation of the enzyme possibly arising from breaking of non-covalent linkages (Georis *et al*., 2000; Bhatti *et al*., 2006). The optimum temperature for pectate lyase production by *Bacillus subtilis* strain had been reported earlier as 40^{0}C (Nasser *et al*., 1990). The PEL activity of *Bacilus subtilis* RDSM01 was significantly influenced by the pH of the growth media and maximum activity was recorded at pH 7.0 (Fig. 3). The reduction in enzyme activity at pH values, both lower or higher than the optimum pH, could be possibly due to the effect of pH on the stability of the enzyme (Al Balaa *et al*., 2014) or due to the alteration in the solubility of the substrates in the medium and their availability for the bacetrial growth (Bruhlman, 1995).

A rapid elevation of extracellular pectate lyase production was observed upto an incubation period of 48 hours and it became almost constant thereafter (Fig. 4). The enzyme production by a micoorganism depends primarily on its growth rate in the substrate and the time for maximal enzyme production in a given medium is a characteristic for that strain (Ramesh and Lonsane, 1990). Higher pectate lyase activity by the strain RDSM01 was recorded when the broth was inoculated with 1.5% v/v inoculum (Fig. 5) and further increase in the inoculum size did not considerably increase enzyme production. The inoculum size of 1.5% v/v could possibly provide adequate biomass during the log phase of the culture thereby leading to higher enzyme secretion by the strain while the decline in enzyme production at higher inoculum levels might be due to the competetion among the bacterial cells for nutrients. Similar relationship between alkaline pectinase secretion and inoculum size was observed with *B. pumilus* dcsr1 by Sharma and Satyanarayana (2012).

The PEL activity of *B. subtilis* RDSM01 was significantly higher at 0.25% (179 IU/ml) and 0.5% (186 IU/ml) pectin concentration and the extracellular pectinase enzyme production decreased significantly with further increase in pectin concentration (Fig. 6). The result indicated enzymatic repression possibly due to the presence of some pectin degradation products acting as enzyme inhibitors at higher substrate concentration (Teixeira *et al*., 2000). Similar inhibition of enzyme activity at higher pectin content was reported in pectinase production by *Aspergillus niger* MTCC 281 by using natural substrates i.e., wheat and corn flour (Palaniyappan *et al.,* 2009).

Maximum enzyme secretion by the strain (168.5 IU/ml) was observed when apple pectin was used as carbon source and the relative activity was 19% higher as compared to that of citrus pectin (Table 1). This suggests that the organism utilized apple pectin more efficiently compared to other carbon sources. The low PEL activity (relative activity of 25 – 59%) of *Bacillus subtilis* RDSM01 in sucrose, lactose, xylose, dextrose and glycerol indicated the possible susceptibility of the strain to catabolic repression while utilizing these carbon sources. Efficient utilization of citrus and apple pectin by *Bacillus sphaericus* as compared to other sugars as carbon sources was also observed earlier (Jayani *et al.*, 2010).

Among the different nitrogen sources used, relative activity of the strain was higher in 0.4% ammonium chloride (control) and 0.4% ammonium sulphate (97.2 - 100%) while it was quite low (31.5 – 52.7%) in other nitrogen sources (Fig. 7). The results can be explained on the basis of possible growth promotion of *B. subtilis* RDSM01 due to addition of ammonium chloride or ammonium sulphate in the medium thereby resulting in higher production of pectate lyase by the strain. The results corroborated the earlier findings of Kashyap *et al*. (2003).

Conclusion

The pectinolytic bacterial strain *Bacillus subtilis* RDSM01 isolated from soil of Barrackpore, West Bengal, India showed higher pectate lyase activity when incubated at 34-37^{0}C temperature for 48 hours and at pH 7.0. The strain prefered apple pectin as carbon source and the optimal pectin concentration in the growth medium was found to be 0.5%. Maximum enzyme production was observed when ammonium chloride or ammonium sulphate at a concentration level of 0.4% was used as nitrogen source in the medium. Since *Bacillus subtilis* RDSM01 had shown considerably higher pectate lyase activity and the optimum conditions for maximum enzyme production had been determined, this strain can be effectively used for removal of gum of ramie fibre which is primarily made of pectin and hemicellulose.

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