Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 5 (8): 782-789, 2010
ISSN 1816-4935 / DOI: 10.3923/jm.2006.152.159

Pectin Lyase and Pectate Lyase from *Debaryomyces nepalensis* Isolated from Apple*

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Abstract: Yeast isolates GSP 101, 102 and 103 capable of producing both pectin lyase and pectate lyase were isolated from rotten apple. Among this, isolate GSP 101 exhibited higher levels of pectin lyase and pectate lyase. Based on 26S rDNA D1/D2 sequence analysis, the isolate was identified as *Debaryomyces nepalensis* (99.8% sequence identity). The isolate produced maximum 3.2 U mL^{-1} of pectin lyase and 2.3 U mL^{-1} of pectate lyase when grown in basal medium supplemented with pectin (5 g L^{-1}). The presence of glucose in medium did not show any negative effect on production of pectic lyases suggesting that there is no catabolite repression. Among the tested naturally available pectic substances, lemon peel was best inducer and carbon source for the production of PL and PGL. This is the first report on production of pectin lyase and pectate lyase by *Debaryomyces nepalensis*.

Key words: *Debaryomyces nepalensis,* pectin, polygalacturonic acid, pectin lyase, pectate lyase, lemon peels

Introduction

Pectic substances are naturally occurring heterogeneous macromolecular polyuronides widely distributed in plant tissues. The principle constituent of all pectic substance is rhamnogalacturonan. The primary chain in the polymer consists of α-D-galacturonate units linked through α-1, 4-glycosidic linkages. The side chains of pectin molecule consist of rhamnose, arabinose, galactose and xylose. In most plants, about 70% of the galacturonate units are esterified with methanol. Based on the degree of esterification, pectic substances are classified into three main types: protopectin, highly esterified parent pectic substance, which is insoluble in water; pectin where the degree of polymerization varies between 70-90% and polygalacturonic acid (unesterified) (Whitaker, 1991). The degradation of pectic substances involves the combined action of different pectinases, viz., esterases and depolymerases (hydrolases and lyases).

Pectic transeliminases or pectic lyases are one among the pectinases, which degrade pectic substances by β-elimination mechanism yielding 4:5 unsaturated oligogalacturonates. Pectin Lyase (PL) acting on pectin and polygalacturonate lyase or pectate lyase (PGL) acting on polygalacturonic acid are two important transeliminases acting on pectic substances. Fungal strains are mainly found to produce PL and bacterial strains were used for the production of PGL (Gummadi and Kumar, 2005). PL and PGL can be differentiated by their substrate requirement and the absolute requirement of calcium for PGL activity (Henrissat *et al*., 1995). Pectic lyases are extensively used in extraction and

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Originally Published in Research Journal of Microbiology, 2006

clarification of fruit juices, degumming of ramie and jute fibers, scouring of crude cotton fibers, pretreatment of wastewater from food processing industries (Bruhlmann, 1994; Naidu and Panda, 1998; Tanabe *et al*., 1998; Kashyap *et al*., 2001; Hoondal *et al*., 2002; Gummadi and Panda, 2003; Gummadi and Kumar, 2005). Since applications of pectic lyases are in an increasing trend, new strains capable of producing pectic lyases (both PL and PGL) are needed. The strain *Debaryomyces nepalensis* isolated from rotten apple was found to produce both PL and PGL. In this study we report for the first time the production of pectic lyases from *Debaryomyces nepalensis*. The isolate was able to use lemon peel as carbon source and inducer for production of PL and PGL.

Materials and Methods

Chemicals

Apple pectin (Sigma) was used as the substrate for PL and polygalacturonic acid (Sigma) was substrate for PGL. The other chemicals were of analytical grade procured in India.

Media

The screening medium had the following composition (g L^{-1}): $CaCl_2$, 0.05; KH_2PO_4, 0.2; $MgS0_4.7H_20$, 0.1; $(NH_4)_2SO_4$, 1.0; pectin, 0.5; agar, 15. The pH of the medium was maintained at 6.4. The Basal Medium (BM) had the following composition (g L^{-1}): $MgS0_4.7H_20$, 0.1; NH_4Cl, 2; Na_2HPO_4, 6; K_2HPO_4, 3; NaCl, 5. The initial pH was adjusted to 7.0. The minimal medium (MM) had the same composition of BM with glucose (8 g L^{-1}). The isolates were maintained in YEPD agar medium. YEPD medium has the following composition (g L^{-1}): yeast extract, 10; peptone, 20; dextrose, 20. For preparation of solid medium, YEPD medium was supplemented with agar (15 g L^{-1}).

Screening and Identification of Strains Producing PL and PGL

Samples (soil, rotten orange, apple and guava and decaying wood) were used to screen for pectin degrading microorganisms. Each test sample (1 g) was mixed with 10 mL of sterile saline and incubated for 15 min, 100 μL of this was added to 900 μL of sterile saline and a serial dilution (10^{-1} to 10^{-6}) was prepared, of which 100 μL was added and distributed on an screening medium. The plates were incubated at 30°C for two days. Isolates capable of utilizing the pectin as sole source of carbon was picked and sub-cultured to single colony. Pure isolates were further screened based on their ability to produce PL and PGL by submerged fermentation in BM supplemented with 0.5 g L^{-1} pectin in test tubes. Pectinase producing cultures from National collection of industrial microorganisms (NCIM, Pune) and Institute of Microbial Type Culture Collection (IMTECH, Chandigarh) were also screened for PL and PGL production. Among all the tested strains, the best strain producing PL and PGL was further characterized by 26S rDNA D1/D2 sequence analysis, which was performed at National collection of yeast cultures (NCYC), Norwich, UK.

Production of PL and PGL by Submerged Fermentation

Prior to cultivation of isolated strains on production medium a loop of the strain from YEPD agar plates was transferred to 5 mL sterile YEPD medium and incubated on rotary shaker at 180 rpm and 30°C. After 12 h, 2% (v/v) of the seed culture was transferred into a 100 mL Erlenmeyer flask containing 25 mL of medium and incubated at 180 rpm and 30°C. Pectic lyases production was studied in BM and MM with and without pectin and polygalacturonic acid (5 g L^{-1}).

Effect of Different Naturally Available Pectic Substances on PL and PGL Production

The effect of different naturally available pectic sources (corn, peels of orange, lemon and banana, sugarcane bagasse and sugar beet) on the production of PL and PGL was studied. These natural sources were cut into small pieces, dried in oven at 70°C for two days and ground to fine powder (approximately 200 μm) and stored in airtight bags. The naturally available pectic substance used in this study act as an inducer and carbon source and was added to BM at 5 g L^{-1}.

Enzyme Assay

Supernatant was used as the source for enzyme assay. PL activity was assayed by measuring the formation of unsaturated oligogalacturonates at 235 nm (Albersheim, 1966). The reaction mixture contained 0.19% pectin in 100 mM citrate phosphate buffer (pH 5.2) and 100 μL of enzyme. The assay mixture was incubated for 3 min at 30°C and the increase in absorbance at 235 nm was measured using Perkin Elmer UV-Visible spectrophotometer. To test PGL activity pectin was replaced by 0.15% (w/v) polygalacturonic acid in 75 mM Tris-HCl buffer (pH-8.0) with 1 mM $CaCl_2$. One unit of enzyme activity was defined as an increase of 1.0 unit of absorbance at 235 nm of the reaction mixture per minute (Nakagawa *et al*., 2000).

Analytical Methods

Cell growth was monitored by measuring the absorbance at 600 nm (OD_{600} of 0.5 corresponds to 0.56 g dry weight L^{-1}). Protein was measured by the method of Lowry with bovine serum albumin as the standard. All the experiments were performed in triplicates and the activity values reported are mean values with ±5 to ±8% error.

Results and Discussion

Screening and Identification of Microorganism

Eleven isolates (2 fungal, 3 yeast and 6 bacterial isolates) were obtained from natural sources capable of degrading pectin when pectin was used as the sole source of carbon. In addition, five pure cultures from culture banks capable of producing pectinases were selected for screening of pectic lyases production. All the 16 isolates were tested for production of PL and PGL in submerged medium and their activities were shown in Table 1. All the fungal isolates produced only PL, whereas the bacterial isolates B4, B5, B8 produced only PGL. However, three yeast strains GSP 101, 102 and 103 produced both PL and PGL. Of this, isolate GSP 101 produced higher levels of PL and PGL. Hence, this isolate was used for further studies. The isolate GSP 101 was a Gram-positive with budding cells, showed creamy white smooth colonies on sabourand agar and negative for Germ tube test, suggesting that isolate GSP 101 was non-pathogenic yeast. The isolate was further characterized based on 26S rDNA analyses and the sequence was compared using EMBL database. The D1/D2 sequence of the strain shows 99.8% sequence identity for both *Candida* sp. BG02-6-6-2-1 and *Debaryomyces nepalensis*. However, the strain was found to sporulate on corn meal agar (CMA) after 17 days of incubation at 20°C, thus confirming its identity as *Debaryomyces nepalensis*. The 26 S rDNA D1/D2 sequence is given in the 5' to 3' direction (Fig. 1).

Many microorganisms have been reported to produce pectic lyases but most of them produce either PL or PGL. PL has been primarily produced by fungi (Alana *et al*., 1990; Manachini *et al*., 1998; Panda and Naidu, 2000; Piccoli-Valle *et al*., 2003) where as PGL by bacteria (Dave and Vaughn, 1971;

Table 1: Production of PL and PGL by different isolates and strains obtained from culture banks

Microorganism	PL activity (U mL^{-1})	PGL activity (U mL^{-1})
Isolate F1	0.04	-
Isolate F2	0.06	-
Aspergillus niger (MTCC 281)	0.03	-
Aspergillus niger (NCIM 505)	0.08	-
Aspergillus niger (NCIM 596)	0.06	-
Aspergillus niger (NCIM 1027)	0.05	-
Aspergillus niger (NCIM 514)	0.15	-
Isolate B4	-	0.01
Isolate B5	-	0.04
Isolate B6	-	-
Isolate B7	-	-
Isolate B8	-	0.09
Isolate B9	-	-
Isolate GSP 101	0.26	0.41
Isolate GSP 102	0.20	0.02
Isolate GSP 103	0.14	0.07

Table 2: Comparison of PL and PGL activities with and without calcium for crude and partially purified enzyme

	PL activity (U mL^{-1})		PGL activity (U mL^{-1})	
Fraction	With 1 mM Ca^{2+}	Without Ca^{2+}	With 1 mM Ca^{2}	Without Ca^{2+}
Crude	3.1	3.2	2.3	2.1
90% $(NH_4)_2SO_4$ precipitate	14.5	15.6	4.7	1.8
Elution of UNO Q6 column	3.2	3.6	1.6	0

McCarthy *et al*., 1985; Hayashi *et al*., 1997; Singh *et al*., 1999). However, very few reports were available where both the pectic lyases being produced by single microorganism (Nakagawa *et al*., 2000; Soriano *et al*., 2005). This is the first report on production of both pectic transeliminases by *Debaryomyces nepalensis*.

Effect of Different Media on PL and PGL Production

The effect of different media on the growth and production of PL and PGL by *Debaryomyces nepalensis* was studied. Maximum growth was observed when glucose was used as the carbon source (MM) followed by MM supplemented with pectin and polygalacturonic acid. The isolate was also able to grow in medium containing pectin and polygalacturonic acid as the sole carbon source (Fig. 2a). PL and PGL activities were detected in BM and MM suggesting that the enzymes are constitutive in nature. The production of pectic lyases was induced by the presence of pectic substances in the medium. The strain showed highest pectic lyases production when grown on basal medium supplemented with pectin than compared to polygalacturonic acid (Fig. 2b and c). The presence of glucose in the medium did not show any pronounced negative effect on the production of pectic lyases, suggesting that pectic lyases production in the isolate was not repressed by glucose. However, it has been reported that PL and PGL production by *Candida boidinii* and *Paenibacillus* sp. was repressed by the glucose in the medium (Nakagawa *et al*., 2000; Soriano *et al*., 2005). In all the media, maximum PL and PGL were produced within 24-36 h of fermentation and the production was growth associated (Fig. 2a-2c). Maximum activities of PL and PGL by *Debaryomyces nepalensis* obtained in this study were 3.2 and 2.3 U mL^{-1} respectively in 24-36 h of fermentation. These results were higher to those reported for pectic lyases production by different strains such as, 0.009 U mL^{-1} of PL by *Aspergillus niger* NCIM 548 (Panda and Naidu, 2000), 0.06 U mL^{-1} of PL *by Penicillium griseoroseum* CCT

Fig. 1: The 26 S rDNA D1/D2 sequence of the isolated strain *Debaryomyces nepalensis*

6421(McCarthy *et al*., 1985), 0.07 U mL^{-1} of PL by *Penicillium italicum* CECT 2294 (Alana *et al*., 1990), 0.4 U mL^{-1} of PGL by *Bacillus licheniformis* (Singh *et al*., 1999), 0.96 U mL^{-1} of PGL by *Pseudomonas marginalis* (Hayashi *et al*., 1997) and 1.4 U mL^{-1} of PGL by *Bacillus pumilis* (Dave and Vaughn, 1971). However, *Paenibacillus* sp. (47.2 U mL^{-1} of PL and 23.4 U mL^{-1} of PGL) and *Bacillus* sp. (53 U mL^{-1} of PL) showed higher activities than reported in the present study (Kashyap *et al*., 2000; Soriano *et al*., 2005). The specific activities of PL (4.3 U mg^{-1}) and PGL (3.3 U mg^{-1}) reported in this report were much higher than reported for *Candida boidinii* (0.673 U mg^{-1} of PL and PGL) (Nakagawa *et al*., 2000). Production of pectic lyases in shorter fermentation time is advantageous and alternative to fungal production.

PL and PGL Production Using Naturally Available Pectic Substances

The ability of the isolate to produce PL and PGL using naturally available pectic substances was studied to reduce the overall cost of fermentation (using nutrient medium with pectin). For this purpose, corn, sugar beet and wastes containing pectic substances such as orange, banana and lemon peels and sugar cane bagasse (5 g L^{-1}) was used as the sole carbon source and inducer for production of PL and PGL. The isolate *Debaryomyces nepalensis* showed maximum PL production in lemon peel medium, which is equal to that obtained in BM, supplemented with pectin (Fig. 3a). The production of PGL was same when BM was supplemented with lemon peel and orange peel but lower than compared to BM supplemented with pectin (Fig. 3b). From this result, it is clear that lemon peel was the best carbon source and inducer for production of both PL and PGL among the tested pectic substances. Hence, pectin in the medium can be replaced by lemon peel.

Characterization of Lyase Activities

In order to confirm whether the pectinase activity obtained in this study was due to pectic lyases, the enzymatic reaction was performed as discussed in materials and methods. The product of reaction was scanned from 200 nm to 500 nm using UV-Visible spectrophotometer. The results clearly showed that the product of enzymatic reaction has maximum absorbance around 235 nm for both PL and PGL (result not shown), which is due to the formation of unsaturated oligogalacturonates by transelimination mechanism (Albersheim, 1966). It is know that PGL has absolute requirement of calcium for its activity whereas PL is independent of calcium. To study the effect of Ca^{2+} on PL and PGL activity, the enzymatic reaction was performed with (1 mM) and without Ca^{2+} for PL and PGL activity. It was found that Ca^{2+} has no effect on PL activity in the crude culture broth. But the absence

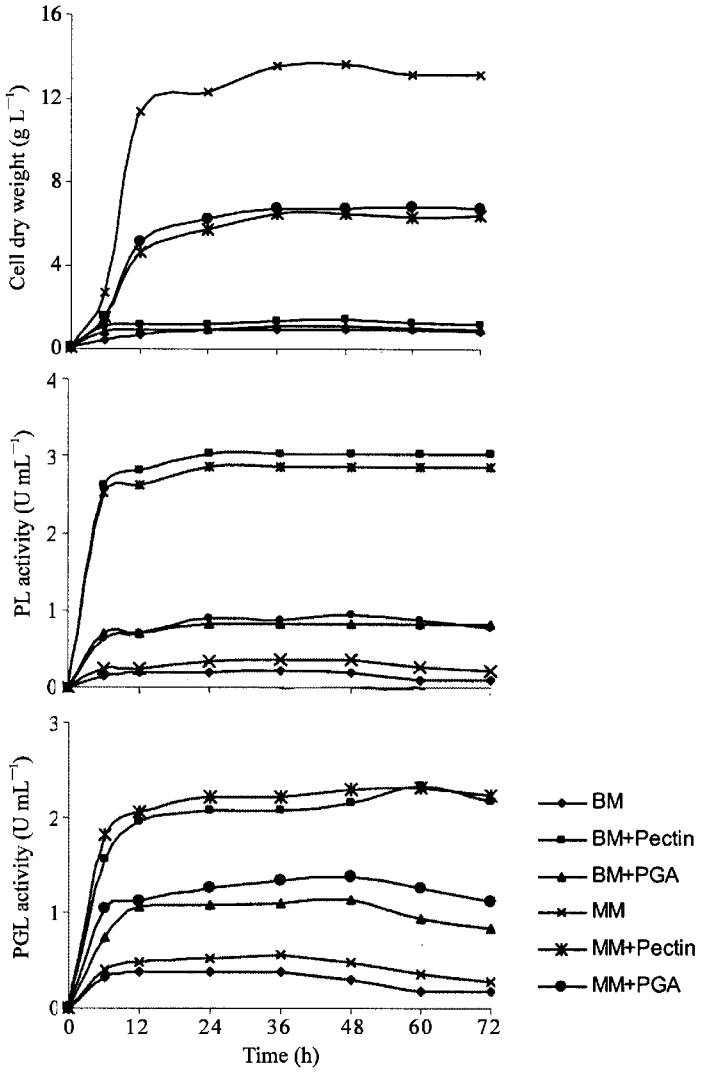

Fig. 2: Effect of different media on growth and production of PL and PGL by *Debaryomyces nepalensis* (a) Growth profile (b) PL activity profile © PGL activity profile. PGA: polygalacturonic acid

of Ca^{2+} in the assay mixture slightly reduced the PGL activity. However, PGL activity was not completely abolished in the absence of Ca^{2+}. Calcium is found abundantly in the cell walls of plants which is required for the *in vivo* activity of PGL in plants (Hepler and Wayne, 1985). The calcium ions present in the cell walls of lemon peel might have interfered with the PGL activity and hence enzyme assay were performed with both crude and partially purified enzyme (90% ammonium sulfate saturation and elute of anion exchange column). It was found that activity of PL was not affected in

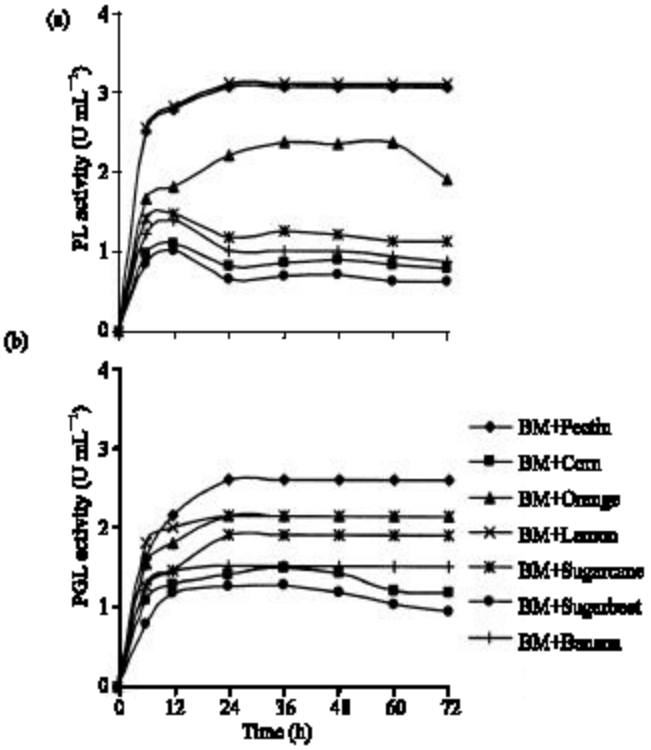

Fig. 3: Effect of naturally available pectic substances on production of PL and PGL by *Debaryomyces nepalensis* in BM (a) PL activity profile (b) PGL activity profile

the presence and absence of Ca^{2+} in crude and partially purified fractions. However, ammonium sulfate fraction shows reduction of PGL activity drastically by almost less than half without Ca^{2+} whereas in elute of Q6 PGL activity is completely abolished (Table 2). These results clearly indicate that presence of Ca^{2+} is absolutely necessary for PGL activity whereas calcium is not required for PL.

Acknowledgements

This study was supported by research grant from Department of Science and Technology, India. Authors thank National Collection of Yeast Cultures, Norwich, UK for their help in strain identification.

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