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Purification and Characterization of the Pectin Lyase Produced by *Pleurotus ostreatus* Grown on Lemon Pulp Waste

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Abstract: Utilization of lemon pulp waste as an agroindustrial waste for production of pectin lyase (PL) [E.C.4.2.2.10] by *Pleurotus ostreatus* mushroom NRRL. 0366 was investigated using semi solid state culture. The enzyme was purified using DEAE-Cellulose column chromatography. The specific activity went up to 81.30 U/mg protein with a purification fold 15.6 and 30.24% recovery. The enzyme had molecular weight of (23 KDa) by SDS polyacrylamide gel electrophoresis and was mostly stable around (pH 7.5). The optimum pH and temperature for the enzyme activity were 7.5 and 60°C respectively. The Km value of PL was optained to be about 3.6 mM. The effect of some metal ions and inhibitors on PL activity was investigated, No stimulation in PL activity was observed in all the examined metal ions.

Key words: Pectin lyase, *Pleurotus ostreatus*, lemon pulpe waste, pectinases.

INTRODUCTION

Pectic transeliminases or pectin lyases are on among the pectinases that degrade the pectin polymers directly by b-elimination mechanism that results in the formation of 4,5-unsaturated oligogalacturonides while other pectinases act sequentially to degrade pectin molecule completely. Most of pectin lyases studied so far have been reported from microorganisms but there are scanty reports of their presence in plants and animals also (Whitaker 1990).

Pectin lyase (PL) E.C. 4.2.2.10 is mostly produced by fungal genera Aspergillus, Penicillium and Fusarium (Alana 1990). Yeasts like *saccharomyces cerevisiae* and *candida sp.* have been reported to produce pectinolytic enzymes such as Pectin lyase, polygalacturonases, and pectinesterases (Gainvors *et al*., 1994).

Colletotrichum lindemuthianum isolated from *Phaseolus vulgaris* was found to produce two forms of PL when grown in culture with sodium polypectate or in a medium having cell walls of *Phaseolus vulgaris* (Wijesundera *et al*., 1984). *Penicillium grieseoroseum* is able to produce PL in a medium containing yeast extract and sucrose (Baracat *et al*., 1994), tea extract (Baracat *et al*., 1997) and sugarcane juice (Piccoli-valle 2001). A pectin lyase was reported to be produced by *Pythium splendens* grown in infected cucumber fruits (Chen *et al*., 1998). An endophytic bacterial strain isolated from fresh cherries (Sakiyama *et al*., 2001) has been reported to produce pectin lyase.

Pectin lyases are the only known pectinases capable of degrading highly esterified pectins (like those found in fruits) into small molecules via b-elimination mechanism without producing methanol, in contract with the combination of PG and PE, which are normally found in commercial products (Whitaker 1990). This is important because methanol is toxic and may present health hazards. Methanol may be lost in vapour during juice concentration but a different condition occurs in viscous materials (purees, baby foods, etc.) or non-concentrated juices where methanol is only partially released during pasteurization. There are many reports of fruit juices clarification by pectin lyases (Yadav *et al*., 2008) PL has potential applications in cotton scouring (Hoondal *et al*., 2002), degumming of plant fibers (Bruhlmann *et al*., 1994) improve the quality of fiber (Beg *et al*., 2000), decreasing the cationic demond of Pectic solutions in paper processing (Reid and Ricard, 2000), purification of plant viruses (Salazar and Jayasinghe, 1999), treatment of effluents from food processing industries (Tanabe *et al*., 1987) and enhancing the fermentation step for tea and coffee processing (Godfrey, 1985).Rashad *et al*., (2009) studied the preparation and optimization of *Pleurotus ostreatus* medium using lemon peel as a food processing waste.

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The following studies were undertaken on isolation, purification and characterization of pectin lyase enzyme by using lemon pulpe waste in media for growing *pleurotus ostreatus* mushroom.

MATERIALS AND METHODS

Microorganism:

Cultures of *Pleurotus ostreatus* NRRL-0366 was obtained from Agricultural Research Service (Peoria, II). The stock culture was maintained on potato dextrose agar (PDA) slant for 1-2 weeks at 20-30°C and then stored at 4°C (Jodon and Royse 1979).

Agro-industrial Waste:

Citrus waste (Lemon Peel) was obtained from juice processing shops in the local market cairo.

Culture Media and Conditions:

The basal optimized medium described by Rashad *et al*., (2009) consisted of (gm%) 2.0 wet lemon waste 2.0, starch, 2.0 urea, extract of 20g of potato and 1.0g glucose 1cm^2 of inoculum mycelium containing 10mg cells was used to inoculate Erlenmeyer flasks (250 ml) containing 25ml of sterilized basal medium. The flasks were incubated in static incubator at 28-30°C for 4 days.

Enzyme Preparation:

The culture were harvested after 4 days (max. production day Rashad *et al*., 2009) and filtered using Whatmann filter paper No. 1, centrifuged at 13,000 rpm for 30 min at 4°C, dialyzed against distilled water and cell free filtrate (CFF) was used as crude enzyme source.

Enzyme Assay:

Enzyme activity of pectin lyase (PL) was assayed by the method reported by Martins *et al* 2002. The PL activity was determined by measuring the increase in absorbance at 235nm of substrate solution (0.8ml of 1% citrus pectin in 0.2M Tris-HCl buffer, pH 8.5) hydrolyzed by 0.2 ml enzyme solution, at 55°C for 5 min.

On unit of PLase was defined as the amount of enzyme releasing 1mmol of unsaturated uronide per min, based on the molar extinction coefficient (5500) of the unsaturated products (Manachini *et al*).

Protein Determination:

Protein was determined by the Lowry *et al*., (1951) method using bovin serum albumin as a standard or by measuring the absorbance at 280nm (Warburg and Christian 1942).

Purification of Extra Cellular P. ostreatus PL:

Several batches of the crude enzyme preparation were obtained and designated to purification procedure. Fractional precipitation with acetone (20-80%) and centrifugation was carried out for 15 min at 4°C and 13,000Xg.

Each precipitate was then dissolved in 0.05M Tris-HCL buffer, pH 8.0 and dialyzed against the same buffer, the dialyzed enzymes solution was loaded on DEAE cellulose column (25 x 1 cm i.d) previously equilibrated with 50mM Tris-HCl buffer pH 8.0. The proteins were eluted by applying linear gradient of NaCl (0.1-0.5M) in the same buffer was eluted. Fractions of 3ml were collected and the elution rate was 30ml/hr. The eluted fractions were monitored at 280nm for protein and assayed for enzyme activity.

Determination of Molecular Weight:

The molecular weight of the pure PL was determined either by sodium dodecyl sulphate-ploy acrylamide gel electrophoresis (SDS-PAGE) and gel filtration. SDS-PAGE was preformed by the method of (Laemmli 1970). The molecular weight markers for SDS-PAGE (phosphorylase b; 97KDa, Bovine albumin, 67KDa, ovalbumin 45KDa, carbonic anhydrase, 30 KDa, trypsin inhibitor, 20.1 KDa, and a-lactalbumin, 14.4 KDa), were obtained from pharmacia Co.

Gel filtration technique was done using sephadex G-200 column (25 x 1 cm i.d.) previously equilibrated and eluted with 50mM sodium acetate buffer, pH 5.0 (Andrews 1964). Blue dextran was used for determination the void volume (Vo). A calibration curve was obtained with standard proteins of known molecular weight, cytochrom (12.27KD), trypsin (24KD), pepsin (34.7KD) myoglobin (17KD) and bovine serum (66KD).

Characterization of the Pure Enzyme:

Optimum pH:

The pH optimum was determined using the same assay method with 0.1M sod. acetate buffer (for pH range 3.0-5.0) phosphate buffer (for the pH range 6.0-7.0) and 0.1M tris-HCl buffer (for the pH 8.0-9.0). The pure enzyme was incubated in different pH values at 55°C for 10min and then the activity was measured under the standard conditions.

pH Stability:

The pH stability of the enzyme was studied by exposing the enzyme to buffers of different pHs for 30 min at 30°C. After this period, the activity of PL was measured under the standard assay conditions. Then relative activity was calculated.

Optimum Temperature:

Optimum temperature for the enzyme activity was determined by assaying the activity of the enzyme at different temperatures in the range 20-90°C.

Thermostability:

Thermal stability of the enzyme was tested by incubating the purified enzyme at various temperature (20-70°C) for 1h and the remaining enzyme was then assayed using the standard assay conditions.

Effect of Pectin Concentration of PL Activity and Michaelis Constant:

The Michaelis constants (K_m and V_{max} values) of PL were determined by measuring the activity of pure enzyme at different concentrations of citrus pectin (Engel 1977).

Effect of Some Metals and Reagents on PL Activity:

The enzyme activity of purified PL enzyme was determined in the presence of various metal ions, namely (Ba^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}, Ca^{2+}, Cu^{2+}, Mg^{2+}, Hg^{2+} and Fe^{3+}) under optimum reaction conditions. Residul activities in the presence of the salts were compared with the controls (without salts).

RESULTS AND DISCUSSION

The results of partial purification of the crude pectin lyase enzyme produced by *P. ostreatus* NRRL-0366 showed that the highest specific activity (35.72 U/mg protein) and best recovery (68.6%) was achieved by using the fraction of 50% acetone (Table 1). Further purification steps was carried out using ion exchange (DEAE-cellulose) column with elution of 0.05M Tris-HCl buffer pH 8.0 and linear gradient of NaCl. The most active enzyme fraction was achieved with the buffer giving a specific activity of 81.3 U/mg protein with a purification fold 15.6 and 30.24% recovery (Table 1, Fig. 1). Also, the data in Fig. (1) revealed the single peak having higher PL activity was attained which indicates its purity and homogeneity. This was also illustrated by SDS polyacrylamide gel electrophoresis. (Fig. 2). Few reports mentioned the purification steps of PL enzyme. Hamdy, 2005 reported that higher *Rhizopus oryzae* PL activity was attained with successive precipitation with ammonium sulphate (65%) with a purification fold of 2.04 and a specific activity of 209.6U/mg with a yield of 70.8% of the original activity. Then he used 2-step column chromatography for further purification which achieved final pure PL enzyme with SP-activity 2313 U/mg protein and 22.5 fold purification.

Table 1: Purification scheme of extracellular Pectin lyase PL from *P. ostreatus* grown on lemon peel medium.

Purification steps	Total units	Total protein (mg)	Specific activity (U/mg protein)	Recovery %	Fold purification
Crude filtrate	887.2	170.0	5.22	100	-
Acetone					
(0.50%)	609.0	17.05	35.72	68.64	6.85
(50-80%)	43.03	8.50	5.06	4.85	0.97
DEAE cellulose column	268.30	3.30	81.30	30.24	15.60

The purified pectin lyase showed a single protein band on SDS-PAGE (Fig. 2) and the molecular weight of the enzyme was (23 KDa) by SDS-PAGE and 24 KDa by gel filtration. This value was coincide with to the value recorded of *Pythium splendens* PL (23 KDa) (Alana *et al*., 1989) and close to the value of *Penicillium italicum* (22 KDa) (Alana *et al*., 1990). While it was lower than these reported by Nikaidou *et al*., 1995 from *Pseudomonas marginalis* (34 KDa) estimated by SDS, from *Rhizopus oryzae* (31 KDa) estimated by gel filtration (Hamdy, 2005).

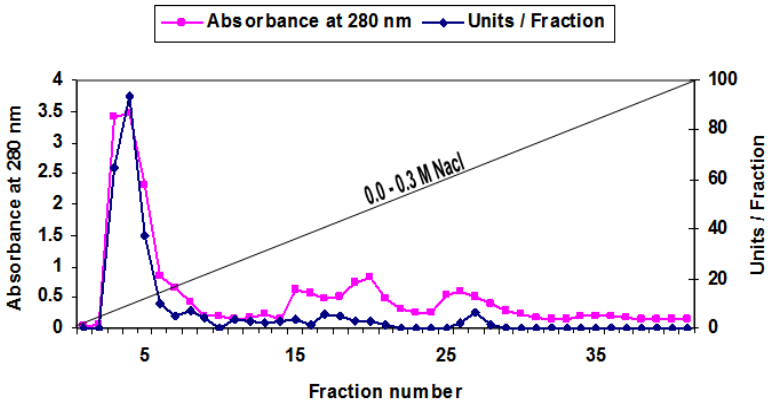

Fig. 1: A typical elution profile of pectin lyase from *P.ostreatus* on DEAE cellulose column (1cmx25) previously equilibrated with Tris-HCL buffer pH 8, at a flow rate of 30 ml /h and 3 ml fractions.

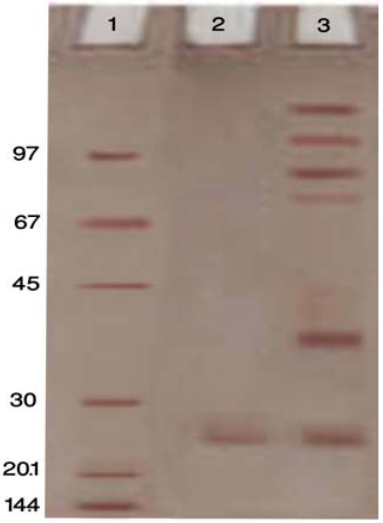

Fig. 2: SDS polyacrylamide gel electrophoresis of *P.ostreatus* PL. Lane(1) standard molecular weight marker., Lane(2) pure PL enzyme., Lane (3) crude PL extract enzyme

The variation in activity of purified pectin lyase with pH of the reaction medium is shown in (Fig. 3). The pH optimum of *P. ostreatus* pectin lyase was 7.5 which is lower than obtained for PLs from *Pythium splendens*, *Penicillium oxalicum*, *Cystofilobasidium capitatum* and *Aspergillus flavus* (Chen *et al*., 1998, Yadav and Shastri 2007, Nakagawa *et al*., 2005 and Yadav *et al*. 2008).

The results of the present studies on pH stability of purified pectin lyase are shown in (Fig. 4). PL enzyme kept about 90% of its activity at pH (6-7) and at pH 8.5. At higher pH value (9.0) and at acidic one, the enzyme lost up to 68% of its activity. These results were in agreement with that mentioned by Manachini *et al*. (1988).

The effect of incubation temperature on the activity of the pure enzyme was tested at pH 7.5 in the range of 20-90°C. As shown in (Fig. 5), the optimum temperature was found to be 60°C, which was in the same range 40-60°C as reported in the literature for *Penicillium italicum*, *Aspergillus japenocus*, *Penicillium adamatezii*, *Bacillus subtilis* and *Aspergillus flavus* (Alana *et al*., 1991, Dinnella *et al*., 1994 Soriano *et al*., 2000 and Yadav *et al*., 2008).

On the studying of the heat stability of *P. ostreatus* pectin lyase (PL), the results showed marked stability at 30°C for 60 min (Fig. 6). It retained 72% of its activity at 60°C for 30 min while complete inhibition of the activity was recorded after heating the enzyme at 70°C for 60 min. Similar results of temperature stabilities have been reported in literature for *pythium splendens* (Chen *et al*., 1998) an for pectin lyase *penicillium italicum* (Alana 1991).

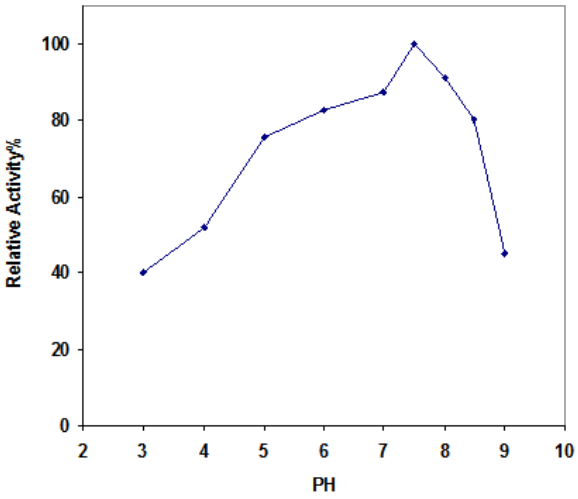

Fig. 3: pH optimum of *P.ostreatus* pectin lyase activity.

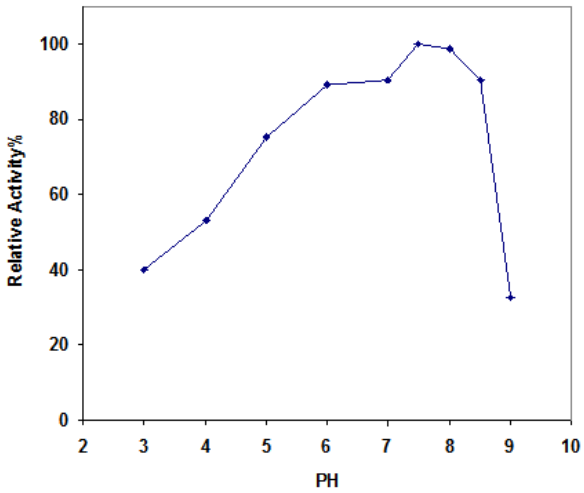

Fig. 4: pH stability of *P.ostreatus* pectin lyase

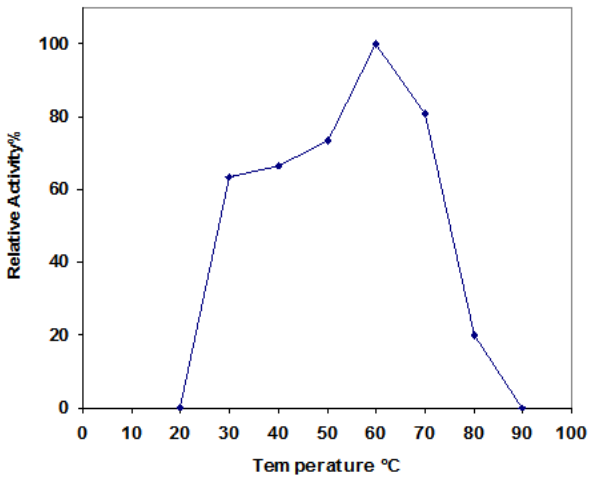

Fig. 5: Temperature optimum of *P.ostreatus* pectin lyase.

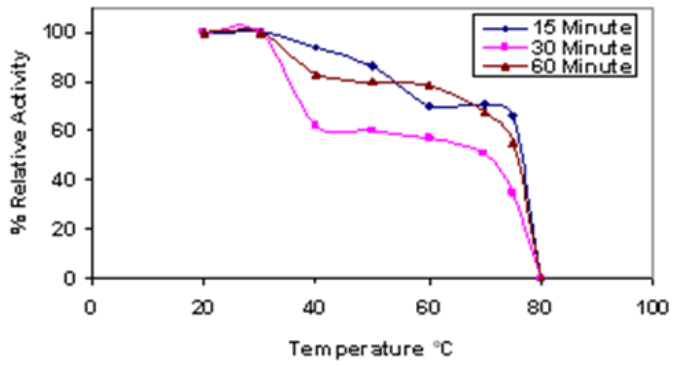

Fig. 6: Thermal stability of *P. ostreatus* pectin lyase.

The Michaelis constant (Km) value of the pure *P. ostreatus* PL was 3.6 mM using pectin as a substrate. This results of Km (3.6 mM) was nearly at Km PL value of *Rhizopus oryzae* as reported by Hamdy (2005) while it was higher than that obtained for Km PL from *Aspergillus joponicus* and *Aspergillus oryzae* as reported by Ishii and Yokotsuka (1975), Lim *et al*., (1983) respectively and it was lower than that Km PL value of *Penicillium italicum* as Alana *et al*., (1991).

The results of the present study of substrate specificity for PL was made by using pectins with different degree of esterification (DE) (Table 2). The enzyme activities with different pectins sources were measured and calculated relative to the activity with citrus peel pectin (63.66%) which regarded as 100% activity. The extent of hydrolysis of different pectins was increased with increasing of its DE. This results were in agreement with that mentioned by Nakagawa *et al*., (2005).

Table 2: Relative activities of *P. ostreatus* PL toward different estrified pectins.

Substrates	% Relative activity
Citrus Pectin (DE 63-66%)	100.0
Citrus Pectin (DE 89%)	110.2
Pectin apple (methyl 7.8%)	66.3

The activity with pectin citrus (DE 63-66%) was taken as 100% activity. Each value represents the average of two experiments.

The effect of different metal cations at the concentration of 1mM on *P. ostreatus* PL assay system is shown in (Table 3). All the examined cations showed different and partial inhibitory effects on the PL activity. The effectiveness of metal cations as inhibition for PL was in the order of Zn^{2+} < Mg^{2+} < Co^{2+} < Ca^{2+} < Ni^{2+} < Ba^{2+} < Cu^{2+} < Fe^{3+} < Hg^{2+} with 14.2, 15.3, 19.2, 25.2, 30.0, 34.3, 36.4, 52.9 and 81.0% inhibition at control respectively. Similar results was noticeable in the PL activity of *Rhizopus oryzae* in which PL was inhibited in the presence of Zn^{2+}andCo^{2}. Severely inhibition was found in the presence of Hg^{2+} (Hamdy, 2005). While in contrast with the metal ions (Ba^{2+}, $Ca^{2+}$and Mg^{2+} affected stimulatory the PL activity. Afifi *et al*., (2000) stated that *curvularia inaequalis* PL activity was inhibited by Co^{2+} Mn^{2+} and Zn^{2+} and completely inhibited by Ag^{2+} and Hg^{2+} whereas it was activated in the presence of Ca^{2+}, Cu^{2+}, Mg^{2+}, $Na^{+}$and K^{+}. Similar findings were reported for other PLs form *Pythium splendens* (Chen *et al*., 1998) and Aspergillus Sp. (Delgado *et al*., 1992).

Table 3: Effect of metal cations on *P. ostreatus* pectin lyase

Metal ions (1mM Conc.)	Relative activity %
Control	100
Ba^{2+}	65.7
Co^{2+}	80.8
Zn^{2+}	85.6
Ni^{2+}	70.0
Ca^{2+}	74.8
Cu^{2+}	63.6
Mg^{2+}	84.7
Hg^{2+}	18.9
Fe^{3+}	47.1

Control: The enzyme activity without added metal ions was taken as 100% activity. Each enzyme was pre-incubated with each salt at 30°C for 30 min and residual activity was assayed.

Conclusion:

Pure *P. ostreatus* pectin lyases had a high level of activity, good affinity towards citrus pectin (DE 63-66%). It has alkaline pH(7.5). high optimum temperature (60°C). As a result, the poperties of *P. ostreatus* PL may be suitable for fruit processing industries.

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