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Purification and characterization of the pectin lyase and protease produced by *Penicillium velutinum* grown on *Eichhornia crassipes* under solid state fermentation

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Abstract: *Penicillium velutinum* van Beyma grown on water hyacinth *Eichhornia crassipes* (Mart.) Solms. wastes as a rich organic source under conditions of solid state fermentation was selected in a preliminary screening for producing interesting levels of pectin lyase (PL) and protease. Addition of some natural additives to the fermentation medium improved the potentiality of enzymes production. PL and protease secreted by *P. velutinum* under these conditions were purified to electrophoretic homogeneity using ammonium sulphate fractionation and 2-step-column chromatography. The purified PL expressed its maximal activity at 50 °C and pH value of 5.5, showed good stability in the pH range of 5.5 to 6.0 and its midpoint of thermal inactivation (Tm) was 65 °C after 45 min of exposure. Protease expressed its maximal activity at 65 °C and pH value of 7.0, showed good stability in the pH range of 6.5 to 7.5 and its Tm was 75 °C after 45 min of exposure. Ions of Ca, Na and K showed a stimulatory effect and ions of Zn and Mn showed an inhibitory effect. Moreover, Ag and Hg showed complete inhibitory effect on the two enzymes activity. Molecular mass of the purified PL and protease was found to be 42 and 63 kDa respectively.

Key words: *Eichhornia crassipes*; pectin lyase; *Penicillium velutinum*; protease; purification; solid state fermentation.

INTRODUCTION

Water hyacinth *Eichhornia crassipes* (Mart.) Solms. Pontederiaceae is a free-floating perennial aquatic plant native to tropical South America. In Egypt, it is considered as a serious nuisance invasive alien plant species that was introduced in the habitat of River Nile in the mid 19th century (Delchevalerie, 1871). The cell wall of *Eichhornia crassipes* consists of the cellulose, hemicellulose, pectin, and lignin (Abd-El-Naby, 1988). Moreover, it contains a high amount of protein (Louboudy *et al*., 2001; and Gunnarsson and Petersen, 2007). Several attempts were made to utilize this waterplant in the production of some fungal cellulases (Louboudy *et al*., 2001; Ali and Saad El-Dein, 2008; Deshpande *et al*., 2008) or bacterial and fungal pectinases (Louboudy *et al*., 2001; Bayoumi *et al*., 2008), etc.

Filamentous fungi such as *Penicillium* spp., *Aspergillus* spp., *Fusarium* spp., *Alternaria* spp., *Curvularia* spp. and *Trichoderma* spp. are the most important group of microorganisms used in solid state fermentation processes owing to their physiological and biochemical properties. (Han and Anderson, 1975; Reese, 1977; Gervais *et al*., 1988; Oriol *et al*., 1988a, 1988b; Fawzi, 2003). Solid state fermentation (SSF) started to replace the submerged or static fermentation methods since 1980s (Louboudy *et al*., 2001).

Among different pectinases, pectin lyase [poly (methoxygalacturonide) lyase, PL; E.C.4.2.2.10] seems to be the only pectic enzyme capable of breaking down pectin into smaller molecules and can work on methylated and/or non-methylated groups of galacturonic acid (Alana *et al*., 1990; Delgado *et al*., 1992).

Proteolytic enzymes are by far the most important group of enzymes produced commercially and are used in many areas of application, such as in detergents, brewing, photographic, leather and dairy industries (Yang *et al*., 2000).

In the present work, different fungal species were tested for their capability to grow on water hyacinth (*E. crassipes*) wastes as a probable organic substrate offering a cheap source of pectin and protein by SSF for the production of pectinase namely pectin lyase and protease. *Penicillium velutinum* exhibited higher values of enzymes activity than the other tested fungi. As far as the author is aware, PL and protease have not been previously purified or characterized from *P. velutinum* and this work is an attempt to bridge this gap of knowledge. The diversity of enzymes detected under these conditions was also studied.

MATERIALS AND METHODS

Microorganisms and culture medium. Microorganisms used throughout this work were previously isolated from Egyptian

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soil, identified by Centraalbureau Voor Schimmelcultures, Netherlands, kept on yeast extract agar at 4 °C and routinely cultured. For enzyme preparation, a triplicate set of 250 ml capacity Erlenmeyer conical flasks was used (for each treatment) containing 8 ml of Waksman's media [KH_2PO_4 1 g/l; $CaC1_2$ 0.5 g/l, and $MgSO_4 \cdot 7H_2O$ 0.5 g/l (without organic sources i.e: peptone and dextrose)] and 20 g of fresh ground *Eichhornia crassipes* whole plants. The pH was adjusted at 5.0. Each group of three flasks was sterilized, inoculated with 2 ml of an evenly prepared spore suspension from each of the tested fungi ($\simeq 10^5$ ml^{-1} spores) and incubated for 7 days at 30 °C. The contents of each flask was thoroughly mixed with cooled distilled water (10 ml) and filtered off through a Buchner's apparatus. The filtrate was then subjected to an enzyme activity assay for the determination of pectin lyase and protease production of the tested fungi, in order to select the best fungus that was chosen for further studies.

Pectin lyase assay. Pectin lyase activity was evaluated by the method of Albersheim and Killias (1962) using citrus pectin as a substrate. The absorbance was read at 235 nm using a spectrophotometer (Spekol). One unit (U) of pectin lyase was defined as the amount of the enzyme that releases 1 µmole of 4,5-unsatmated digalacturonic acid per min.

Protease assay. Protease activity was determined according to Kunitz (1947) using casein as a substrate. The optical density of the supernatant was measured at 280 nm. One unit of protease was defined as the activity that produced an increase in optical density of 1.0 in 20 min at 280 nm.

Protein assay. Protein content was measured by UV absorption at 280 nm (Markwell *et al*., 1978) using bovine serum albumin as a standard.

Effect of enriching the water hyacinth *Eichhornia crassipes* medium. The enrichment with some natural additives i.e. malt, yeast and beef extracts, soybean meal, fish meal, molasses and corn-steep liquor (0.05%, w/v) was performed and the levels of enzyme production in the enriched media were statistically compared to that of the water hyacinth medium which served as control.

Purification of pectin lyase and protease. The enzymes were purified from the culture supernatant. All operations were done at 0 to 4 °C. The culture supernatant (200 ml) was freeze dried. The enzymatic protein precipitated by 60% $(NH_4)_2SO_4$ was collected, dissolved in 5 ml of 50 mM citrate phosphate buffer (pH 6.0) and dialyzed against the same buffer at 4 °C for 24 h. The enzymatic protein was fractionated through Sephadex G-100 column (18 x 2 cm) eluted with the same buffer at 20 ml/h. Five ml fractions were collected and assayed for protein (A_{280}), pectin lyase and protease activity (Plummer, 1978). The active fractions with the highest specific activity of each enzyme were pooled and dialyzed. The pooled fractions of each enzyme were separately further fractionated by passage through DEAE-Cellulose column (Diethylaminoethyl-Cellulose) eluted with a gradient of 0-0.8 M NaCl in citrate phosphate buffer (240 ml) at a flow rate of 10 ml/h. For each enzyme, five ml fractions were collected and assayed for protein and enzymes activity. The most active fractions were collected and dialyzed once again to remove Na^+ and Cl^- (Peterson and Sober, 1962; Palmer, 1991). The prepared enzymes were freeze dried and stored at 0 °C for further investigations.

Characterization of the purified pectin lyase and protease.

Effect of pH and pH stability. The effect of pH on enzyme activities was assessed by adding respectively 1 ml of pectin lyase solution to 1 ml of 1% citrus pectin and 1 ml of protease solution to 1 ml of 1% casein, at different pH values (3.5-9.0) obtained by using 0.05 M citrate-phosphate and 0.05 M Tris-HCl buffer. After incubation at 50 °C for 30 min for pectin lyase and at 30 °C for 20 min for protease, the reaction was stopped and each enzyme activity was measured under the standard assay conditions. To determine pH stability, each enzyme was incubated in the presence of pH values within the above cited range for two time intervals of 20 and 60 min. The residual activity for each enzyme was assayed.

Effect of temperature and thermal stability. The effect of temperature on enzymes activity was assessed by incubating each enzyme with the corresponding substrate at various temperatures ranging from 30 to 80 °C. Each enzyme activity was measured to determine the optimum temperature for activity. However, for determination of thermal stability, the enzymes were incubated for variable durations (0 to 60 min) at fixed temperatures (50 to 70 °C for PL; 65 to 85 °C for protease).

Effect of different metal ions and some enzyme inhibitors. The effect of different metal ions and some enzyme inhibitors on the relative activity of the purified PL and protease was performed by adding the investigated metal ions as chloride at 1, 5 or 10 mM and was incubated with the two enzymes before adding substrate. Activity of the enzyme in complete absence of such compounds served as control (100% activity).

Molecular mass. The molecular mass of the enzymes was estimated by gel filtration on Sephadex G_{75} using standard proteins with sample volume of 5 ml (Palmer, 1991). Elution of standard proteins was monitored at 280 nm while elution of Blue Dextran, used to determine the void volume, was monitored at 540 nm.

Statistical validation of treatment effects. The mean, standard deviation, T-score and probability "P" values of 3 replicates of the investigated factors and the control were computed according to the mathematical principles described by Glantz (1992). Results were considered highly significant, significant or non-significant where $P < 0.01$, > 0.01 and < 0.05, > 0.05, respectively.

RESULTS AND DISCUSSION

A strain of *Penicillium velutinum* van Beyma CBS nr 428.65 was selected in a preliminary screening of 15 fungal species. This fungus released interesting levels of PL (4.35 U/g) and protease (10.74 U/g) activities when grown on water hyacinth *Eichhornia crassipes* wastes as a rich organic source under solid state conditions. The aforementioned finding can be correlated to a faster rate of degradation of pectin in this waste combined with the increase of accumulation of protein hydrolysates (Hamdy, 2005). Moreover, Ammar *et al*. (1994) stated that *Penicillium* spp. produces highly pecteolytic and proteolytic enzymes when grown on agricultural wastes. Furthermore, Louboudy *et al*. (2001) reported the utilization of *Eichhornia crassipes* for pectinases and cellulases enzymes production under solid state fermentation by different microorganisms.

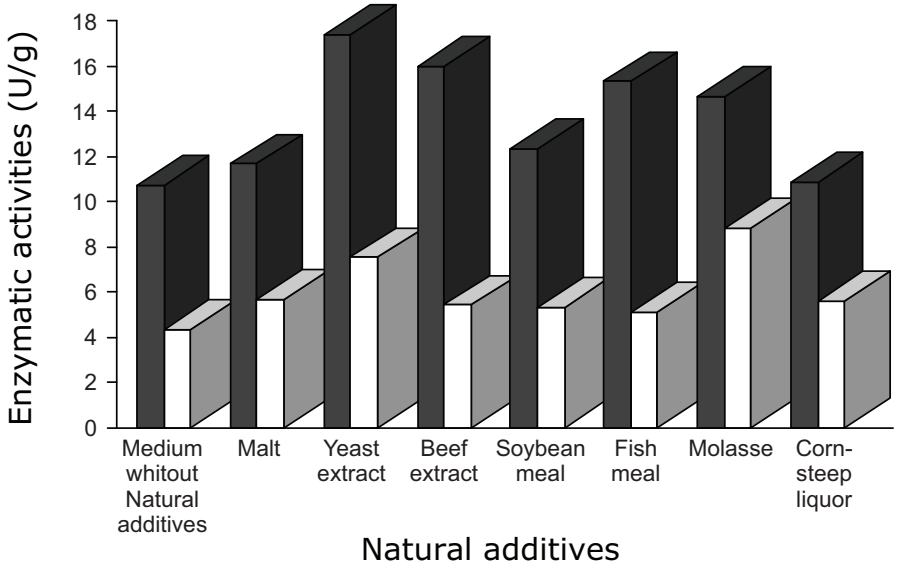

FIG. 1 - Effect of enriching the water hyacinth *Eichhornia crassipes* medium with some natural additives on the production of pectin lyase (-□-) and protease (-■-) from *Penicillium velutinum.*

Cheap agro-industrial by-products (corn-steep liquor, molasses and fish meal) and some additives (yeast, malt and beef extracts) were used to increase the pectin lyase and protease enzymes production by *P. velutinum* grown on water hyacinth medium (Crueger and Crueger, 2000). It was found that the maximum productivities of pecteolytic (8.38 U/g) and proteolytic enzymes (17.83 U/g) were recorded in the presence of molasses and yeast extract (0.05% w/v) respectively (Fig. 1). In this regard, El-Gindy *et al*. (2008) had previously stated that these additives promote the production of enzymes on solid state cultures.

A summary of purification steps of PL and protease produced by *P. velutinum* was recorded in Tables 1 and 2 respectively. The results showed that PL was purified 42.31 fold obtaining a final specific activity of 688.51 U/mg protein (Table 1). Protease was purified 59.32 fold to a specific activity of 1467.54 U/mg protein (Table 2). PL and protease have not been previously purified or characterized from *P. velutinum* grown on *E. crassipes*.

Testing the pH-dependence of the activity of PL and protease revealed that 5.5 was optimum for activity of PL while 7.0 was optimum for activity of protease (Fig. 2A) and there was a sharp decrease in activity at the acidic values and a minor decline at the basic one of the two enzymes. This optimum pH value (5.5) was identical to that found for PL from *Aspergillus niger* (Spagna *et al*., 1995) and *Curvularia inaequalis* (Afifi *et al*., 2002) while alkali value (7.5) was found optimal for PL from *Rhyzopus oryzae* (Hamdy, 2005). The optimum pH value of protease (7.0) was similar to that found for the same enzyme from *Fusarium prolif-*

TABLE 1 - Purification steps of pectin lyase from *Penicillium velutinum* grown on Water hyacinth medium with molasses

Treatment	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Recovery (%)	Purification fold
Cell-free filtrate (200 ml)	84.58	1376	16.27	100	1.0
Cell-free precipitate [60% $(NH_4)_2SO_4$)].	56.31	1158	20.56	84.16	1.26
Gel filtration (Sephadex G-100)	6.54	756	115.60	54.94	7.11
Ion-exchange chromatography (DEAE-Cellulose)	0.87	599	688.51	43.54	42.31

TABLE 2 - Purification steps of protease from *Penicillium velutinum* grown on Water hyacinth medium with yeast extract

Treatment	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Recovery (%)	Purification fold
Cell-free filtrate (200 ml)	97.00	2400	24.74	100	1.0
Cell-free precipitate [60% $(NH_4)_2SO_4$)].	58.31	2156	36.97	89.83	1.49
Gel filtration (Sephadex G-100)	6.56	1848	281.71	77.00	11.39
Ion-exchange chromatography (DEAE-Cellulose)	1.14	1673	1467.54	69.7	59.32

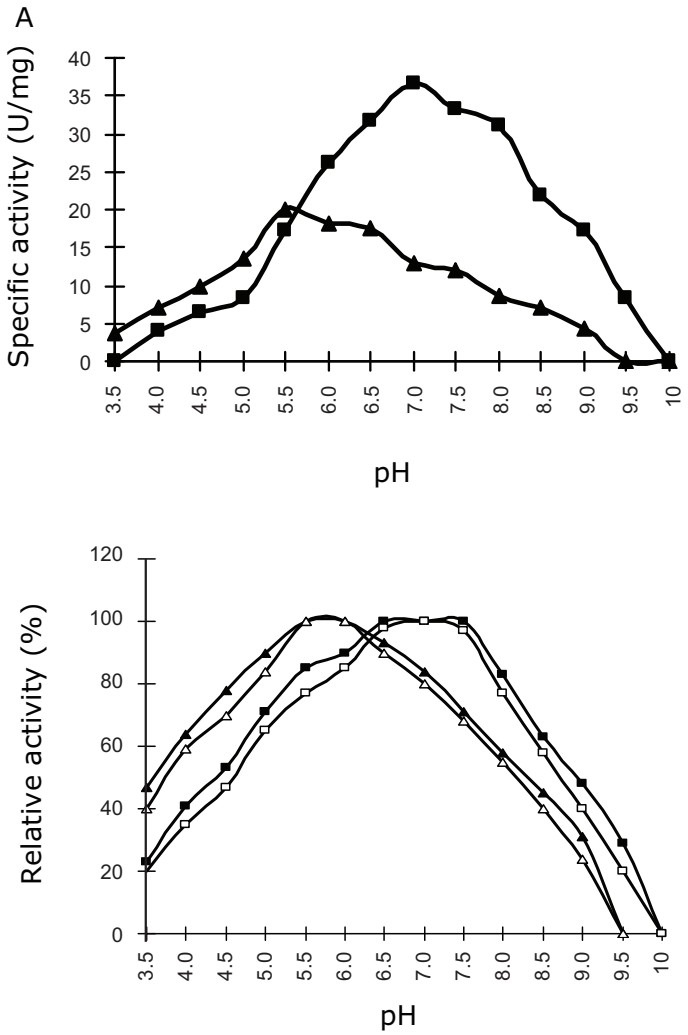

FIG. 2 - The effect of pH (A) and pH stability (B) on the activity of the purified pectin lyase (PL) and protease from *Penicillium velutinum*. A: PL (-▲-) and protease (-■-). B: PL after 20 min (-▲-), PL after 60 min (-Δ-), protease after 20 min (-■-), protease after 60 min (-□-).

eratum (Fawzi, 2003). As the pH value diverged from the optimum level, the efficient functioning of the enzyme was affected. This could be ascribed to the decreased saturation of the enzyme with substrate due to a decreased affinity and/or due to the effect of pH on the stability of the enzyme (Dixon and Webb, 1979).

Incubation of each enzyme preparation at different pH values for either 20 or 60 min demonstrated that PL was almost unaffected by incubation in the pH range 5.5-6.0 for either 20 or 60 min (Fig. 2B). These results were in accordance with the results of Afifi *et al*. (2002), while protease was almost unaffected by incubation in the pH range 6.5-7.5 for either 20 or 60 min, and completely stable after 60 min of incubation at pH 7.0 (Fig. 2B). These results were in accordance with that of Rhodes *et al*. (1983) and Datta (1992).

Concerning the effect of temperature on the enzymes activity, the results showed that both PL and protease achieved a high hydrolyzing effect at 50 and 65 °C respectively (Fig. 3A). PLs from other sources have been reported to be optimally active at this range i.e 40-50 °C (Wijesundera *et al*., 1984; Manachini *et al*., 1988; Afifi *et al*., 2002) and that of 65 °C was reported also for protease obtained from *Pleurotus ostreatus* (Palmieri *et al*., 2001).

Moreover, midpoint of thermal inactivation (Tm) was determined and recorded at 65 °C after 45 min of exposure. The PL enzyme retained its original activity after heating up to 50 °C for 1 h while no activity was recorded after heating the enzyme at 70 °C for 45 min (Fig. 3B). PL from *P. velutinum* has a similar stability to PL of *Pythium splendens* (Chen *et al*., 1998). The protease enzyme retained its original activity after heating up to 65 °C for 45 min while no activity was recorded after heating the enzyme at 80 °C for 45 min and 85 °C for 30 min (Fig. 3C). Midpoint of thermal inactivation (Tm) was recorded at 75 °C after 45 min of exposure. The thermal inactivation of enzymes is nearly always due to the denaturation of the enzyme proteins (Dixon and Webb, 1979).

Effect of some metal ions and enzyme inhibitors was investigated and the data are presented in Table 3. PL and protease activity were significantly increased in presence of Ca^{2+}, Mg^{2+}, Na^{+} or K^{+}. Potassium ions were found to stimulate PL secreted by *Curvularia inaequalis* (Afifi *et al*., 2002) and substrate binding (Palmer, 1991). Ca^{2+} appears to play a role in maintaining the structural integrity required for catalytic activity of PL (Liao *et al*., 1994, 1997; Henrissat *et al*., 1995). Activity of PL and protease was inhibited in presence of Zn^{+} or Mn^{+} and severely inhibited in presence of Ag^{2+} and Hg^{2+}. Similar findings were reported for other PLs from *Pythium splendens* (Chen *et al*., 1998) and *Aspergillus sp*. (Delgado *et al*., 1992) and protease from *Pleurotus ostreatus*

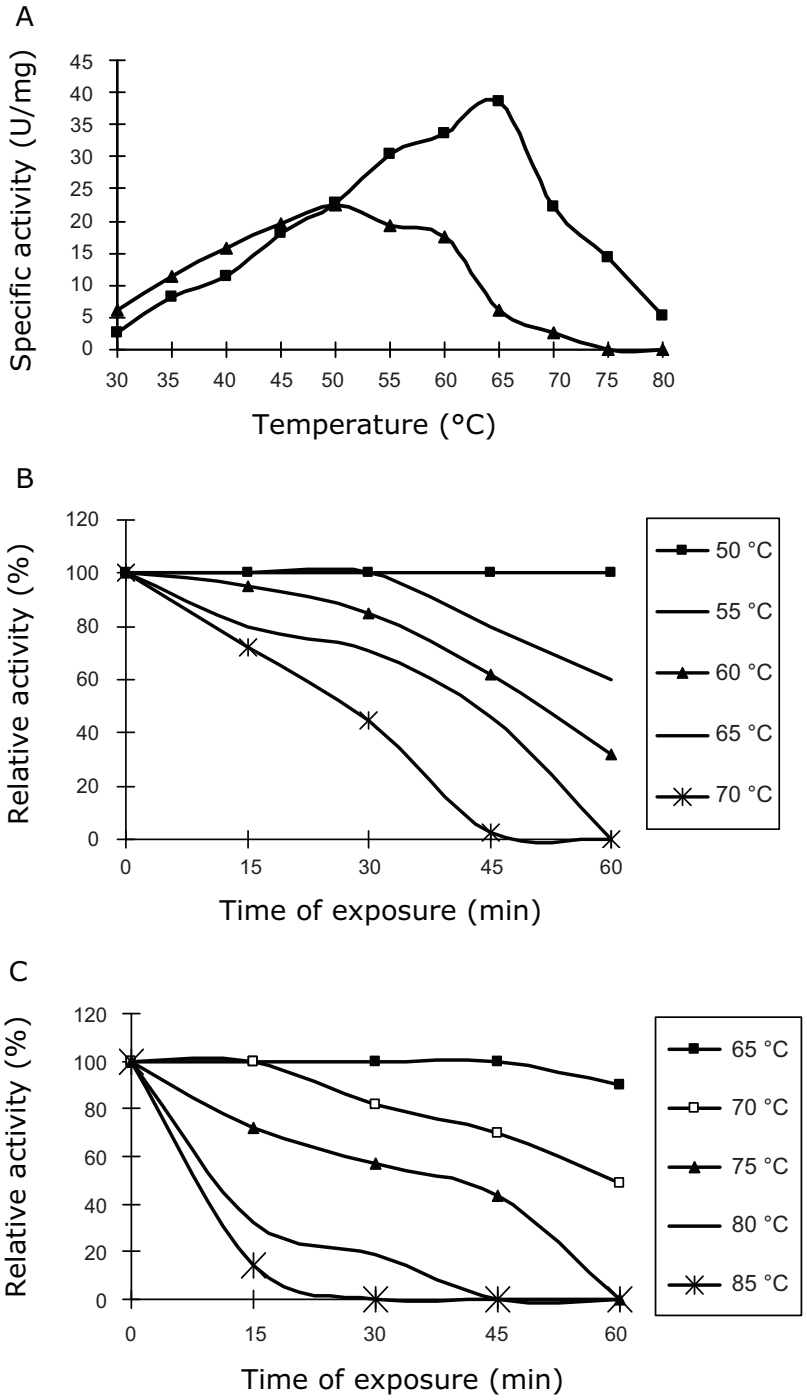

FIG. 3 - A: The effect of different temperature on the activity of the purified pectin lyase (-▲-) and protease (-■-) from *Penicillium velutinum.* B: Thermal stability of the purified pectin lyase. C: Thermal stability of the purified protease.

(Palmieri *et al*., 2001). Possible participation of SH group in enzyme structure was evidenced from the severe inhibitory effect caused by Ag^{2+} and Hg^{2+}, sodium arsenate in addition to the recorded inhibition in presence of iodoacetic acid (SH-group specific inhibitor) (Table 3). Inhibitory effect of iodoacetic acid upon PL from P. *fluorescence* and P. *viridiflava* was previously stated (Liao *et al*., 1997). Addition of EDTA resulted in a highly significant inhibition for the activity of the enzyme (Table 3) and this is in contrast with the findings of Chen *et al*. (1998) who reported no effect for EDTA upon PL from *Pythium splendens*.

Molecular mass of the purified PL and protease from *P. velutinum* was found to be 42 and 63 kDa respectively as estimated by gel filtration, while only 23 kDa was recorded for PL from *Pythium splendens* and *Colletotrichum lindemuthianum* (Wijesundera *et al*., 1984; Chen *et al*., 1998) and 38 kDa for PL from *A. niger* (Spagna *et al*., 1995). Moreover Hossain *et al*. (2006) found that the molecular mass of protease from *Aspergillus niger* was 46 kDa. As a matter of fact, comparison of molecular mass of different pectinases from the same and different sources should be undertaken with full care due to the great variations in the molecular weight of the pectins which may vary from 20,000 to

TABLE 3- Effect of different metal ions and some enzymes inhibitors on the relative activities of the purified PL and protease from *Penicillium velutinum*

Metal ions or inhibitors	Relative activity					
	PL			Protease		
	1 mM	5 mM	10 mM	1 mM	5 mM	10 mM
Ag^{2+}	0.0	0.0	0.0	0.0	0.0	0.0
Ba^{2+}	115	105	98	105	100	95
Ca^{2+}	111	125	133	107	110	136
Cu^{2+}	85	79	65	92	83	75
Fe^{3+}	108	95	85	118	99	87
Hg^{2+}	0.0	0.0	0.0	0.0	0.0	0.0
K^{+}	121	129	138	119	123	129
Mg^{2+}	116	108	105	106	100	98
Mn^{+}	75	61	52	85	72	63
Na^{+}	112	120	128	108	116	130
Zn^{+}	73	61	58	66	52	22
EDTA	79	60	52	80	69	55
Iodoacetate	34	25	16	52	41	33
Sodium arsenate	68	56	42	63	48	35
L-Cysteine	80	71	65	78	69	57

400,000 (Merck, 1989), distinct culture conditions and the several methods used in assessing the enzyme activities (Friedrich *et al*., 1989).

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