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Pectinase Activity of some Micromycetes Isolated from Agricultural Soils

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Abstract

Pectinases have multiple nature and various forms which are necessary for the hydrolysis of pectin in several conventional industrial and natural processes. In this investigation fourteen fungal strains which isolated from agricultural soils ,were screened for pectinase activity. Pectinase activity were measured *in vitro* by assaying released reducing sugar from pectin as sole carbon source. *Aspergillus foetidus* and *Aspergillus aculeatus* showed highest pectinase activity. *Rhizoctonia solani* (AG-4) and *Trichoderma reesei* S578 showed the highest level of protein production. Reducing sugars concentration were different between 13th and 15th then reduced to 29th days after inoculation. Secreted proteins increased to 11th and 13th then decreased to 29th days. Optimum pH and temprature for pectinase activity were 5 and 50°C respectively. Glucose was detected as a degradation product by thin layer chromatography (TLC). Electrophoresis of the extracellular proteins of *A. foetidus* and *A. aculeatus* revealed two major proteins having a molecular mass of 79 and 63 kDa respectively.

Keywords: Enzyme activity; Pectinase; Fungal screening; In vitro

Introduction

Pectin is a structural polysaccharide found in primary cell wall and middle lamella of fruits and vegetables. The predominant structure of pectin is homopolymeric, made of a partially methylated poly-a-(1,4)-galacturonic acid [12]. The enzymes that hydrolyze pectic substances are broadly known as pectinases [15]. Pectinases are of multiple nature and various forms due to the complex nature of their substrates, which are necessary for the hydrolysis of pectin. Over the years, pectinases have been used in several conventional industrial processes such as textile, plant fiber processing, tea, coffee, oil extraction, treatment of industrial wastewater containing pectinacious material. They have also been reported to work in viruses purification , paper processing and de-pectinisation and clarification of fruit juices. They have'nt been commercialized yet [3, 4]. Environmental and legislative pressures have made pectinases a plausible solution for their industrial applications [21]. Furthermore, in combination with other enzymes like cellulases, arabinases and xylanases they have shown to increase the pressing efficiency enormously [31]. Many

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reports are available which describe the production and characterization of pectinolytic enzymes from a variety of microorganisms such as bacteria, fungi and yeasts . The microbial pectinases account for 25% of the global food enzymes sales. Almost all the commercial preparations of pectinases are produced from fungal sources [16] because their nature is generally regarded as safe (GRAS) [16]. Also as much as 90% of the enzyme can be excreted into the culture medium and they have high enzymatic excretion capacity [6]. Pectinase production by filamentous fungi differs along with the kind of strain, cultivation conditions (pH, temperature, aeration, agitation and incubation time) and the growth medium composition (particularly carbon and nitrogen sources) [5]. Therefore these must be specified separately for each and different strains. So, in order to meet this high demand, it is highly important to produce pectinolytic enzymes in a cost effective and productive way [31]. In this work the pectinase activity and enzyme production of some fungal strains isolated from soils of Iran by using citrus pectin as a carbon source were investigated. Some physico-chemical characteristics of the enzymes produced have been discussed.

Materials and Methods

Microrganisms

The strains (Table 1) used in this study were isolated from agricultural soils of different region of Iran. The strains were maintained on potato dextrose agar (PDA) slants as stock cultures at 4°C and subcultured monthly.

Fungi Identification

All isolated fungi were exposed to morphological and microscopic examination. All species of *Aspergillus* were identified to Klich [19].

Culture Medium and Sampling

The medium contained [9] (gL^{-1}): $(NH4)_2SO_4$: 2.64; $MgSO_4$: 0.14; KH_2PO_4: 0.34 and distilled water 1000 mL. The carbon source was 10g of citrus pectin. The pH was adjusted to 5.6. One hundred and fifty mL of medium in 500 mL erlenmeyer flasks were inoculated with spores (10^6 spores/mL) of 48 h-old culture of the fungi. For species of *Rhizoctonia* inoculation were performed using three pieses (6mm) of medium slant. The cultures were incubated at 25°C for 29 days without shaking. Sampling was started two days after inoculation and repeated every two days for protein and sugar assays. Erlenmeyer flask consisting culture medium without inoculation was used as witness.

Sugar and Protein Assay

Five hundred μL of broth medium were sampled for released sugar assays. Released sugars concentrations were determined using reducing sugares assay by Arsenate- Molybdate reagent [20]. Released fungal extracellular proteins concentration were determined by Bradford method [7], with bovine serum albumin (Sigma) as standard.

Partial Purification of Pectinase

Two hundred millilitre of cell-free supernatant was saturated with ammonium sulphate to 90% saturation. The saturated solution was left overnight at 4°C, centrifuged at 12000 g for 20 min at 4°C, dissolved in minimal amount of 100 mM Tris–HCl buffer (pH=8) and dialysed against the same buffer for 24 h at 4°C. The dialysed proteins thus obtained were treated as partially purified enzymes and consumed [21].

Optimal pH

Optimal pH for maximal activity of crude enzyme was determined by measuring enzyme activity using 50 mM Tris-HCl buffer of different pH 3, 4, 5, 6, 7, 8 and 9 [24].

Optimal Temperature

The enzyme was assayed at different incubation temperatures 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80°C with optimum pH [25].

TLC

TLC was performed with silica gel 60G (Merck) in a solvent system of butan-1-ol/acetic acid/water (2 : 1 : 1, v/v/v) and sugars were visualized by a heat treatment at 120 °C for 10 min after spraying 50% (v/v) H_2SO_4 in methanol [30].

Electrophoresis and Enzyme Assay

Crude enzyme sample was analysed by native poly acrylamid gel electrophoresis (native PAGE) by modifying the method that performed as described by Laemmli [22] and native-PAGE was carried out in the same way except sodium dodesyl sulphate (SDS) was excluded. Protein bands were observed by staining with

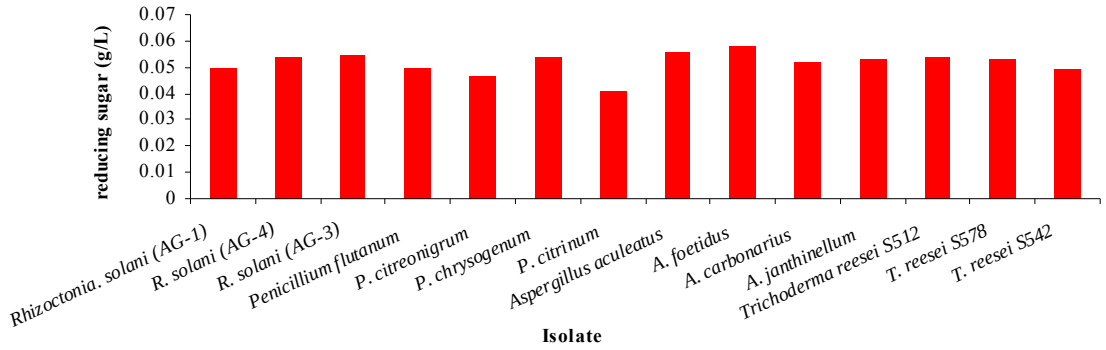

Figure 1. Variations of pectinolytic activities of different strains in pectin medium.

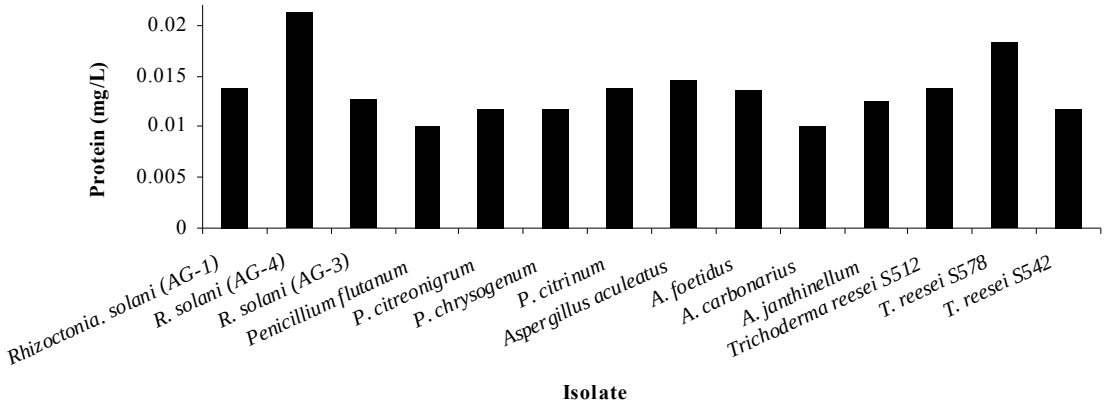

Figure 2. Variations of released proteins of different isolates in pectin medium.

coomasie R-250 brilliant blue (Merck). Also molecular mass of them were specified by Fermentas molecular weight pattern. Protein bands on the electrophoresis gel was cuted with a sharp razor and were subjected to pectin solution (1%) overnight. The treatments were incubated in 45°C for 1 h. The solution objected to measurement sugar assay by Kossem and Nanni pieri [20] method.

Statistical Analysis

Analysis of variances were conducted by SAS (v. 9.1) statistical software.

Results

The growth process started twelve hours after inoculation. Significant differences were existed for measurements of sugar assays and secreted proteins of different strains (Table 1, Fig. 1 and 2).

Sugar and Protein Assay

There were significant difference in content of released sugars between different genera and species. *A. foetidus* and *A. aculeatus* showed highest and *Penicillium citrinum* showed lowest pectinase activity respectively (Tables 1 and 2, Fig. 1). There were significant difference in proteins concentration between different isolates. *R. solani* (AG-4) and *T. reesei* S578 showed highest and *Penicillium flutanum* showed lowest protein concentration respectively (Tables 1 and 2, Fig. 2). In strains with high pectinase activity and high protein production, reducing sugars concentrations were different between 13th and 15th then reduced to 29th days (Fig. 3). For secreted proteins in culture medium the considerable increment was observed in initial days after inoculation and then decreased, as in *R. solani* (AG-4) and *A. aculeatus* increased to 11th whereas *A. foetidus* and *T. reesei* S578 increased to 13th respectively and decreased to after days (29th) (Fig. 4). On the basis of correlation analysis of sugar and protein,

there was a very signficant negative covariance between sugar and protein assay ($P<0.01$).

Optimum pH for Enzyme Aactivity

The enzyme activity was gradually increased with increasing pH till 5 for *A. foetidus* and *A. aculeatus*, then enzyme activity was declined. In lower or higher than optimum pH, enzyme activity was decreased (Fig. 5).

Optimum Temperature for Enzyme Activity

Temprature increase till 45°C and 50°C was caused increase enzyme activity of *A. foetidus* and *A. aculeatus* respectively. Enzyme activity increased by increasing temprature. In below or above than optimum temprature, enzyme activity was decreased. Also, optimum temperature of *A. foetidus* was higher than *A. aculeatus* (Fig. 6).

Identification of Degradation Products by TLC

To investigate the ability of fungal pectinase to degrade pectin, citrus pectin used as a carbon source. Pectinase readily degraded pectin and the degradation products were investigated by TLC too. Glucose was detected as a degradation product. Glucose spot of *A. foetidus* was more colourful than *A.aculeatus* (Fig. 7).

Electrophorestic Stydies and Enzyme Assay

Electrophoresis of the extracellular proteins of *A. foetidus* and *A. aculeatus* revealed two major proteins of 79 and 63 kDa (Fig. 8), having strong pectinolytic activities, confirmed by sugar assay. Other proteins, between 25 and 45 kDa and below 25 kDa were also observed.

Discussion

The enzymes preparations used in the food industry are of fungal origin because fungi are potent producers of pectic enzymes and the optimal pH of fungal enzymes are very close to the pH of many fruit juices, which range from pH 3 to 5.5 [29]. *Aspergillus niger, Aspergillus awamori, Penicillium restrictum, Trichoderma viride, Mucor piriformis* and *Yarrowia lipolytica* are the fungi which are used in both submerged and solid state fermentation for production of different industrial products like citric acid, ethanol. Meanwhile some like *Aspergillus oryzae* and *Penicillium expansum* are normally regarded as safe by United States Food and Drug Administration (USFDA) are used in food industry. However, the filamentous fungi are the most common used fungi in industry [4]. Growth of strains started afew hours after inoculation. It confirmed that all of 14 fungal strains were capable of pectinase production and degradation of pectin polymer as carbon source. This result showed different fungi can produce extracellular enzymes to prepare their growth requirements and all tested strains had positive pectinase activity. Strains belonging to *A. foetidus* and *A. aculeatus* showed highest level of reducing sugar respectively. Like *A. niger* and *A. flavus* that showed highest pectinolytic activity [25]. In *R. solani* (AG-4) and *T. reesei* S578 were showed lowest reducing sugars. This result suggested that the concentration of enzyme in secreted proteins is low or pectinases of these strains have enzyme activity.

Table 1. Means comparison of sugar and protein assay in different tested isolates

Isolates	Means	
	Sugar (g/L)	Protein (mg/L)
Apergillus foetidus	0.057578 (A)	0.13577 (B-D)
Aspergillus aculeatus	0.055867 (AB)	0.01415 (B)
Rhizoctonia solani (AG-3)	0.054667 (BC)	0.012756 (B-D)
Penicillium chrysogenum	0.054067 (BC)	0.011733 (B-D)
Trichoderma reesei S512	0.053867 (B-D)	0.013733 (BC)
Aspergillus janthinellum	0.053333 (CD)	0.012422 (B-D)
Rhizoctonia solani (AG-4)	0.053267 (CD)	0.021311 (A)
Trichoderma reesei S578	0.052867 (CD)	0.017664 (AB)
Aspergillus carbonarius	0.051644 (DE)	0.010000 (D)
Rhizoctonia solani (AG-1)	0.049933 (EF)	0.013711 (BC)
Penicillium flutanum	0.049889 (EF)	0.010089 (CD)
Trichoderma reesei S542	0.049156 (H)	0.011711 (B-D)
Penicillium citreonigrum	0.046200 (G)	0.012467 (B-D)
Penicillium citrinum	0.041121(F)	0.013778 (B)

Means with the same letter are not significantly different (p=0.01)

Table 2. Analysis of variance of protein and sugar assay on pectin culture medium

Source of Variance	Df	Mean Squre	
		Sugar assay	*Protein assay*
Isolate	13	0.00007262**	0.00000539*
Total error	26	0.00001557	0.00000193
CV		7.6726	0.1937

$^{**}p$= 0.01, $^{*}p$= 0.05

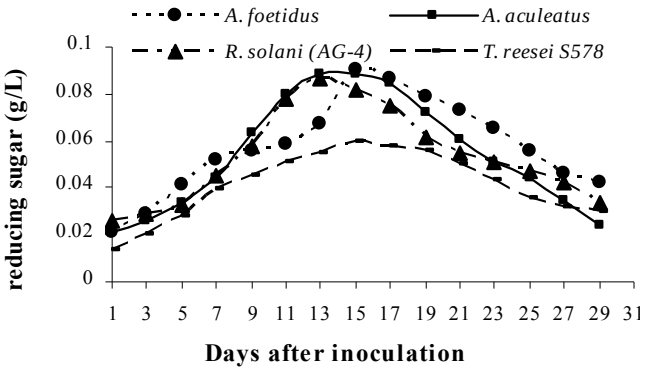

Figure 3. Variations of released sugars of four tested strains with highest sugar and protein production.

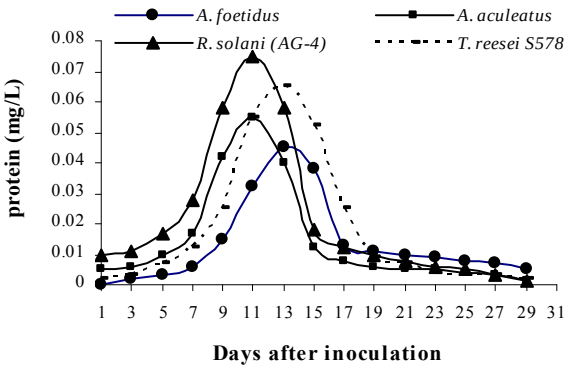

Figure 4. Variations of proteins of four tested species with highest sugar and protein production.

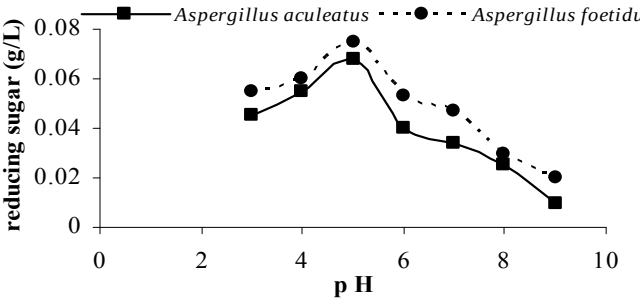

Figure 5. pH effect on enzyme activity of *A. foetidus* and *A. aculeatus*.

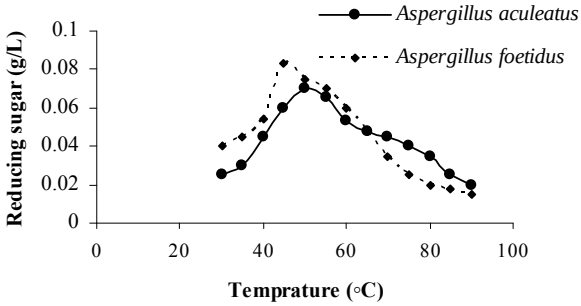

Figure 6. Temperature effect on enzyme activity of *A. foetidus* and *A. aculeatus*.

Concerning to the time course for reducing sugars for pectin culture medium in the first stage, a continuous increase in reducing sugars concentration was observed. In the second stage, a significant decrease was seen. In presence of released sugares after pectin degradation, pectinase production and pectinase activity decreased, whereas with exhaust of released sugars the pectinase activity was recovered. This result suggested that released sugares act as repressor after pectinase activity. Since pectin is a high level molecular weight polysaccharide, the question arises as to how induction takes place or how the cells can sense the substrate in the outer environment. It has been suggested that some microorganisms can produce low levels of basal constitutive activities that degrade the polymeric substance, and that low levels of the reaction serve as inducers or energy sources to promote cell growth and pectinase production as demonstrated in some bacteria and fungi [8, 23]. The production of pectinolytic enzymes is induced by low concentrations of galacturonic acid [16]. The highest concentration (5%) of the glucose gives lower production levels in stationary phase, indicating that at high concentrations either galacturonic acid or one of its metabolites exhibit self catabolite repression. In the presence of glucose, the enzyme production reduces to basal levels [16].

Optimum pH for Enzyme Activity

The pH influences on pectinase activity clarified that the enzymes were active for pH range 2-8 in both strains with optimum pH of 5 for maximum enzyme activity in *A. foetidus* and *A. aculeatus*. These results were in correlation with several earlier reports showing that the best pH of 4.8 for exo-polygalacturonase from *A. niger,* and optimum pH of 5 for *Rhizopus stolonifer* [14] *Mucor pusillus* [2] and optimum pH of 3.8 for *Sclerotium rolfsii* [8]. In lower or higher than optimum pH enzyme activity was decreased. This is a well establish fact that each enzyme has a characteristic optimum pH for its own activity [24]. Acidic pectic enzyme used in the fruit juice and wine making that come from fungal source [17].

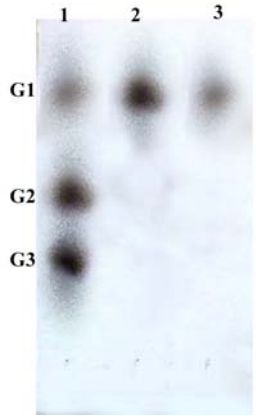

Figure 7. Substrate specificity and degradation products of *Aspergillus aculeatus* and *Aspergillus foetidus* by TLC, Lane 1, standard sugars: glucose (G1), fructose (G2) and cellobiose (G3) Lane 2, *A. foetidus*. Lane 3, *A. aculeatus*.

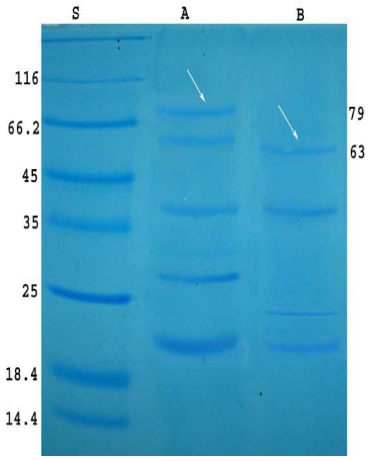

Figure 8. Electrophoretic patterns of the extracellular proteins produced by *Aspergillus foetidus* (lane A), *Aspergillus aculeatus* (lane B) and protein size marker (KDa) (lane S).

Optimum Temperature for Enzyme Activity

The strains pectinases were active over a wide temperature range of 30–90°C. The optimum temperature of 50°C is necessary for the highest enzyme activity in *A. foetidus* and *A. aculeatus* respectively. In fact, whenever more than optimum temperature applied enzyme activity whould be decreased. This result suggested that every enzyme is optimally active and stable upto a certain temperature and may gets denatured at higher temperatures. As pectinases from *Aspergillus* strains have been described to be susceptible to denaturation at temperature (heating) about 50ºC [3, 13]. For *Penicillium frequentans* and *R. solani* optimum temperature for enzyme activity were 50°C [10]. *S. rolfsii* in 55°C had maximum enzyme activity that was close to 50°C [8] while on the basis of some stydies optimum tempratute for enzyme activity in *A. niger, A. flavus, A. japonicus, and Chaetomium globosum* was lower than 50°C [1, 26].

Identification of Degradation Products by TLC

Glucose spots were observed in both *Aspergillus* strains. Pectinase readily degraded pectin and glucose was detected as a degradation product. Cellulose, hemicellulose and pectin can be hydrolyzed using pectinase, cellulase and β-glucosidase enzymes to produce glucose, fructose, galactose, arabinose, xylose, rhamnose and galacturonic acid [32]. Glucose spot of *A. foetidus* was more colourful than *A. aculeatus*. Maybe glucose concentration in *A. foetidus* was higher than from *A. aculeatus*. This result suggested that the enzyme activity of *A. foetidus* was higher than as compared *A. aculeatus*.

Electrophoretic Studies

The molecular weight of pectinases 79 kDa and 63 kDa in *A. foetidus* and *A. aculeatus* respectively, determined by native-PAGE were similar to those reported for some fungal extracellular pectinases, such as the 68 kDa enzyme from *Sclerotinia sclerotiorum* [27], the 60 kDa enzyme from *Cochliobolus carbonum* [28], the 78 kDa enzyme from *Aspergillus tubingensis* [18], and the 74 kDa enzyme from *Fusarium oxysporum* [11].

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