Research Article

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Novel pectinase from *Piriformospora indica*, optimization of growth parameters and enzyme production in submerged culture condition

Piriformospora indica’dan Yeni Pektinaz, Büyüme Parametrelerinin ve Enzim Üretiminin Batırılmış Kültür Koşulunda Optimizasyonu

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Abstract

**Background:** Pectinases are one of the upcoming enzymes in food processing industries and they hydrolyze pectin substances.

**Objectives:** This study was done to examine the production of pectinase by *Piriformospora indica* in submerged fermentation (SmF) along with growth parameters.

**Materials and methods:** The fungus *P. indica* was cultured on Kafer medium supplemented with pectin for 0–12 days and fungus growth, number of spores, total protein content, and pectinase activity were investigated.

**Results:** Firstly, pectinase secretion by *P. indica* was confirmed by cup-plate assay. The maximum dry cell weight (10.21 g/L), growth yield (0.65 g/g), specific growth rate (0.56 day⁻¹) and pectinase activity (10.47 U/mL) on pectin containing medium (P⁺) were achieved after 6 day of culture. In the case of pectin free medium (P⁻) all the parameters were less than P⁺ medium. The pectinase production by *P. indica* on P⁺ was 2.7 times higher than P⁻. The optimum pH and temperature for maximum polygalacturonase activity were 5 and 50°C, respectively.

**Conclusions:** For the first time, the work confirmed pectinase secretion by *P. indica* fungus and it could be a good source for pectinase production. Moreover, optimum pH 5, make it a potential candidate for future application in fruit juice industries.

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**Keywords:** *Piriformospora indica*; Galacturonic acid; Kafer; Pectin; Polygalacturonase.

**Introduction**

Pectinolytic enzymes or pectinases is a generic name for the family of enzymes is involved in pectin degradation
and they are widely distributed in higher plants and microorganisms such as fungi, bacteria and yeast. They play major roles in plant growth, maturation, storage and ecological balance maintenance. For many years pectinases have been used in several industrial processes including; degumming and maceration of plant fiber, tea and coffee fermentation, oil extraction, removing citrus fruit peels, treatment of sullage with citric and pectic material, viruses purification, protoplast fusion technology and plant pathology [1–3]. About 25% of the global food enzymes market is related to microbial pectinases [1].

Yeasts and filamentous fungi secrete an excess of important enzymes and other secondary metabolites in the growth medium. Due to hydrolytic activity, they are employed within food processing industries as well as in enhancement of food quality [4]. Nowadays, almost all of the microbial pectinolytic enzymes are obtained from Aspergillus niger [5]. During ripening of fruits, pectinases break down pectin and cause to cell wall weakness and softening tissue [6]. Most of the research in pectinase production has focused on the development of solid-state fermentation (SSF) and submerged fermentation (SmF) culture conditions [7].

Pectic substances as pectinases substrates are found in the middle lamellae and primary cell walls of higher plants with high molecular weight and negative charge [1, 2]. Pectin is composed of D-galacturonic acid residues linked together by α-1,4-glycosidic linkages to form homo galacturonan chains [8]. This backbone structure is branched and containing rhamnose, arabinanes and arabinogalactane as side chains [9].

High structural diversity of pectin caused to production of various types of pectinases by the same organism with different mechanisms of catalysis [10]. The pectinases are broadly classified into three groups based on mechanism of their action: pectin esterases, hydrolases and lyases [11].

It is reported that production of pectinase by Aspergillus and Fusarium genera in SmF is induced by pectin or some of its derivatives. Synthesis of polygalacturonase (EC 3.2.1.15) and pectinesterase (EC 3.1.1.11) by A. niger is induced by galacturonic or polygalacturonic acid at the transcription level [12].

Piriformospora indica as a root endophytic fungus, was isolated from the rhizosphere of the woody shrubs Prosopis juliflora and Zizyphus nummularia that growing in Indian Thar desert in 1997 [13]. This root endophyte fungus has wide spectrum of various substrate and able to create colony with large number of mono- and dicotyledonous plants including Arabidopsis thaliana, barley, wheat and tobacco [14].

Literature review is concerned; this study is the first investigation of the growth, sporulation and pectinase activity of P. indica on pectin containing medium (P+) in submerge fermentation condition have been studied.

Materials and methods

Microorganism, media and culture conditions

Piriformospora indica was obtained from Department of Plant Pathology, School of Agriculture, Tarbiat Modares University, Tehran, Iran. Specific medium for P. indica growth is Hill and Kafer [15] or modified Aspergillus medium. It was cultured on modified Kafer [16] medium (glucose was replaced with the pectin) with the following composition: 5.0 g/L pectin, 10 g/L agar, 3.0 g/L peptone, 3.0 g/L yeast extract. 1.83 g/L KH2PO4, 0.65 g/L MgSO4·7H2O, 2.5 mL/L microelements stock solution, 10 mL/L of vitamin stock solution, 1.0 mL/L of 0.1 M CaCl2 solution, 1.0 mL/L of 0.1 M FeCl3 solution and 2.5 mL/L of 5.0 g/L FeSO4·7H2O. The microelements stock solution contained (g/L): ZnSO4·7H2O, 22.4; H3BO3, 11.0; MnCl2·4H2O, 5.0; CoCl2·6H2O; CuSO4·5H2O, 1.6; (NH4)6 Mo7O27·4H2O, 1.0; Na2EDTA, 50.0. The vitamin stock solution consisted of 0.5 g/L of biotin, 1.0 g/L of p-aminobenzoic acid, 5.0 g/L of nicotinamide, 1.0 g/L of pyridoxal phosphate solution and 2.5 g/L riboflavin solution. Pectin-containing medium is shown as P+.

For submerged cultivation of P. indica, 10 mm of agar discs were transferred to 250 mL flasks containing 50 mL of modified Kafer medium at 29°C and shacked at 200 rpm for 12 days. Medium without pectin (P−) was used as control.

Measurement of cell fresh and dry weight, growth yield, specific growth rate and spore yield

Every other day, three flasks were removed, filtered through Whatman no. 1 paper and parameters including; fresh and dry cell weight, medium pH, spore yield determined. The cell growth, growth yield (Yx/S), specific growth rate (μ) and spore yield were calculated according to Kumar et al. [17].

Measurement of spores

Spore measurement was done according to the Kumar et al. [17] method with some modifications. Briefly, the
pear-shaped chlamydospores of \textit{P. indica} were separated and released by adding Tween 80 to the culture medium and vortexing for 10 min. Detached spores were counted by a hemocytometer.

**Total protein determination**

The filtrate was centrifuged (at 10,000 rpm at 4°C for 15 min) and analyzed for protein content and pectinase activity. Determination of total protein content was performed according to the Bradford’s method, using bovine serum albumin (BSA) as standard [18]. Absorbance of the sample was measured at 595 nm using spectrophotometer (UV–Vis 1800 spectrophotometer Shimadzu).

**Pectinase activity**

PGase activity was assayed by chemical methods: cupplate assay, using ruthenium red for staining; determination of released reducing sugars, using 3,5-dinitrosalicylic acid (DNS). To confirm polygalacturonase production, 20 μL of \textit{P. indica} suspension was inoculated on solid Kafer medium containing 1% of polygalacturonic acid and incubated at 30°C for 2 days. Then, plates were stained with 10 mL of 0.1% ruthenium red aqueous solution for 5 min, rinsed with deionized water and the clear zone around the fungal colony was considered as positive pectinase activity [19].

To investigate pectinase activity, DNS method with some modification was used [20]. Multiple dilutions of galacturonic acid were used as standard. The enzymatic reaction mixture included 0.25 mL of cell-free supernatant and 0.75 mL of 1% pectin in 0.2 M phosphate buffer pH 6.5 as substrate. The mixture was incubated at 60°C for 5 min. One unit (U) was expressed in term of the enzyme quantity which would yield 1 μmol galacturonic acid per minute during the standard assay condition.

**Determination of optimum pH and temperature for pectinase activity**

To determine the optimum pH and temperature for pectinase activity, the mixture was incubated at different pH and temperatures for 5 min. To achieve optimal pH, the substrate was incubated with varying buffers including; citrate buffer (pH 3.0–5.0), phosphate buffer (pH 6.0–8.0), Tris–HCl buffer (pH 9.0) and glycine-NaOH buffer (pH 10.0). To find optimum temperature, the pectinase activity was determined at 25–60°C (25, 30, 35, 40, 45, 50, 55 and 60°C).

**Results**

**Effect of pectin on growth parameters**

According to the results, fungi dry cell weights on P⁺ medium were significantly nearly two times more than P⁻ medium (Table 1). In addition to, the maximum dry cell weight for P⁺ and P⁻ was achieved after 6 and 8 day of culture, respectively. In P⁺ medium the maximum values of growth yield (Yₓ/S) measured were 1.7 fold higher than P⁻ ones.

The pear-shaped chlamydospores were appeared after 6 and 9 day of growth on P⁺ and P⁻ media, respectively. Maximum spore yield on P⁺ and P⁻ media was observed approximately 3 day after beginning of sporulation (Table 1).

Initial pH of medium was adjusted to 6.5 and pH of P⁺ medium revealed interesting results which have a consistent pattern with growth and pectinolytic activity of \textit{P. indica} (Figure 1B). On the other hand, the pH of P⁻ medium slightly increased by the time (Figure 1A). At this time total protein content of P⁺ nearly was two-fold higher than P⁻ medium.

**Table 1:** Effect of pectin on growth, spore yield and pectinase activity of \textit{P. indica}.

| Medium | Maximum DCW (g/L) | Maximum FW (g/L) | Yₓ/S (g/g) | χ (day⁻¹) | Maximum pectinase specific activity (U/mg) | Maximum spore yield (no. of spores/mL) | Time required to achieve maximum (day) |
|--------|------------------|------------------|------------|----------|------------------------------------------|----------------------------------------|---------------------------------------|
| P⁺     | 10.21±0.26       | 222±16.5         | 0.65±0.03  | 0.56±0.01| 52.35±0.41                               | (3.42±0.66)×10⁷                        | 6                                    |
| P⁻     | 5.70±0.15        | 105±23.6         | 0.38±0.02  | 0.45±0.02| 19.3±0.54                                | (2.29±0.33)×10⁷                        | 8                                    |

P⁺, Kafer medium without pectin; P⁻, Kafer medium supplemented with pectin.
Pectinase production

The PGase activity was significantly confirmed by cup-plate assay as shown in Figure 2. The plates were positive for pectinase activity in a cup-plate assay, as evidenced by clear hydrolysation halos. In this study, the maximum pectinolytic activity of \(P. \text{indica}\) on \(P^+\) medium (52.35 U/mg) was observed on 6 day after cultivation and about three fold higher than that of \(P^-\) (19.3 U/mg).

Effect of pH on pectinase activity

As seen in Figure 3 the enzyme was active over a wide range of pH values, displaying over 80% of its activity in the pH range 5.0–7.0. The activity was decreased by decreasing the pH from 5.0 to 3.0 and decreased by increasing the pH from 5 to 10. According to the results the optimum pH for both \(P^+\) and \(P^-\) medium was 5 and in pH 3 and 10 enzyme lost about 57% and 82% of its optimum activity, respectively.

Effect of temperature on pectinase activity

Enzyme activity vs temperature were tested and plotted in Figure 4. Results revealed that 50 °C was optimum temperature for the maximum production of pectinase from \(P. \text{indica}\) in both conditions. The enzyme was active over a broad temperature range of 25–60°C.

Discussion

Pectinases are the growing enzymes of biotechnological sector and are ecofriendly tools of nature that being used extensively in various industries. The present work makes up a first contribution to evaluate the potential of \(P. \text{indica}\) to synthesize polygalcturonase. Due to solubility problems, 5 g/L of the apple pectin was used. Pectin...
caused to increased fungal growth and spore yield during submerged culture compare with P−. Delayed sporulation on P− related to slower growth and late arrival to stationary phase in comparison to P+. It is established that the exhaustion of the nutrients induces the sporulation and increase of cell density [21]. Thus the slow consumption of nutrients and less growth may result in delay in sporulation and decrease in spore yield.

Kumar and his coworkers reported that the glucose depletion possibly stimulate sporulation of P. indica, as other nutrients were in excess and similar results have been reported by Monteiro et al. [21], in the case of Bacillus subtilis by increasing the glucose concentration, vegetative cell growth was increased and then its depletion enhanced sporulation. Moreover, Hood and Shew [22], described that depletion of nutrients in the culture environment of Thielaviopsis basicola fungus stimulated spore formation [17]. The higher protein content on P+ medium is possibly related to maximum pectinolytic activity could be explained as a result of P. indica ability to induce production of pectinolytic enzymes for degradation of pectic substances as a carbon source in nutrient and carbon scarcity condition.

The pectic substances are well known as inducers of pectinolytic enzymes production [23, 24]. Several studies confirmed the stimulatory effect of carbon source starvation on expression of fungal enzymes involving in degradation of various plant polysaccharides as well as pectic substances [25]. To quantify the potency of P. indica in bioconversion of pectin, the amount of P+ and P− media pectinase activity for 12 days have been reported in Figure 1. The time of maximum growth and pectinase production by P. indica coincided and the maximum pectinase activity was achieved in late log or early stationary phase on medium containing 0.5% (w/v) pectin as a carbon source. It was similarity in accordance with the day of reached to the highest amount of biomass production and the least pH value (Table 1).

Nowadays a wide variety of biotechnological activities focus on apple pectin due to its ease of consumption as substrate to induce pectinase production by various types of microorganisms, especially filamentous fungi [26]. D-galacturonic acid due to catabolic pathway, degradation by filamentous fungi, and induction of gene expression of fungal pectinolytic enzymes, could be considered as a carbon source which can be particularly consumed by microorganisms [27–29]. Studies about production of extracellular pectinases by Aspergillus sp. confirmed that existence and vicinity of pectic materials in the medium act as pectinase inducer [30, 31].

The pectinolytic enzymes of fungal origin are globally used in fruit processing. Such enzymes have been industrially produced by the filamentous fungi including A. niger, Coniothyrium diploidiela, Sclerotinia libertiana and species of Botrytis, Penicillum and Rhizopus. Pectinase production by filamentous fungi varies according to the strain, the composition of the growth medium and the cultivation conditions (pH, temperature, aeration, agitation and incubation time) [32]. The specific activity of the PGases obtained from different microbial sources including A. niger (pH=0.8–4.3, T=43°C), Aspergillus awamoni (pH=5, T=40°C), Kluyveromyces marxianus, Fusarium oxysporum (pH=11, T=69°C), Penicillum frequentans (pH=5.8, T=50°C), Bacillus sp. KSM-P410 (pH=7, T=50°C) and Saccharomyces pastorains (pH=4.2, T=50°C) is 982, 487, 97, 209, 185, 54 and 0.62 U/mg, respectively [1].

All over the results (Figure 2 and Table 1) confirmed the stimulatory role of apple pectin to enhance the pectinolytic activity, growth yield and spore yield of P. indica.
Our results on both media were coincided with several reports evaluate the optimum pH of fungal pectinases, including: 4.8 for polygalacturonase from A. niger, 5.0 for A. awamori, 4–5.5 for Aspergillus japonicas and 5 Aspergillus sojae [33]. Most of the microbial polygalacturonases have an optimal pH range from 3.5 to 5.5 and mostly produced by fungal sources, especially from A. niger [33].

The maximum activity of the most fungal endoPGs is at acidic pH usually around pH 4.0–6.0 and alkaline endoPGs mostly produced by bacteria. Endo-PGs with different properties are useful in different fields. Acidic endo-PGs are broadly used in fruit juice industries for clarification of fruit and vegetable juices by degrading pectin and decreasing viscosity, and in the feed industry to qualify animal feeds with other hydrolyses [34]. Due to optimum pH 5, P. indica could be used in fruit juice industries. The enzyme was active over a broad temperature range of 25–60°C. Temperature as a key regulator of the metabolic rate of the organism, determines the amount of the end products [35].

The results obtained in the present study have shown the potential for the conversion of apple pectin (a renewable source of carbon and energy) into valuable products and confirmed pectinase secretion by P. indica fungus. Moreover, optimum pH 5, make it a potential candidate for future application in fruit juice industries.

Chemical compounds studied in this article

Casein (PubChem CID: 73995022); Coomassie Brilliant Blue (PubChem CID: 633920); 3,5-Dinitrosalicylic acid (PubChem CID: 5251731); Glycine (PubChem CID: 750); Nicotinic Acid (PubChem CID: 938); Pyridoxine (PubChem CID: 1054); Thiamine (PubChem CID: 1130).

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