POLYGALACTURONASE (PG) PRODUCTION BY FUNGAL STRAINS USING AGRO-INDUSTRIAL BIOPRODUCT IN SOLID STATE FERMENTATION

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Abstract: Polygalacturonase production by isolated fungal strains was carried out in solid state fermentation. *Aspergillus niger (A. niger)* and *Penicillium sp* EGC5 produced polygalacturonase (PG) on mixture of apple bagasse and wheat bran. The mixture of apple bagasse and wheat bran acted as a good nutrient source and substrate for the cultivation of the microorganisms and polygalacturonase produced in solid state fermentation. In this respect, it was possible to obtain polygalacturonase activity at an acceptable yield, in comparison with a typical defined medium described in the literature for polygalacturonase production. Higher titres of polygalacturonase were observed when medium was supplemented with carbon (pectin) and nitrogen ($(NH_4)_2SO_4$ and peptone) sources. The maximum production of polygalacturonase was reached after 8 days cultivation. The temperature was 30°C and the relative humidity of the fermentation medium was 70%.

Keywords: *Polygalacturonase, Aspergillus niger, Apple bagasse, wheat bran, Solid state fermentation*

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1. Introduction

In the industrial production of fruit juices it is necessary to eliminate the pectin released during fruit processing in order to reduce the time of filtration and enhance the production at the end of the process [1]. Today, the application of pectinolytic enzymes plays an important role in food technology for the maceration of fruits and vegetables, as well as for the extraction, concentration and clarification of their juices [2].

Polygalacturonase comprises a heterogeneous group of enzymes that catalyze the breakdown of pectin-containing substrates. The major industrial applications of pectinases include extraction and clarification of fruit juices and grape musts, citrus fruit juice and wine technology, maceration of vegetables and fruits and extraction of olive oil. The fungus Polyporus squamosus is interesting from the viewpoint of simultaneous production of pectinases. The pectinase complex from P. squamosus does not contain pectin esterase and pectin lyase, the former makes it very suitable for application in cloudy citrus juice production and in wine making [3]. Furthermore, phytopathologic studies have reported that fungal endo-polygalacturonase (endoPGase) which is a major kind of pectinase has been shown to activate plant defense responses, including phytoalexin accumulation, lignification, synthesis of proteinase inhibitors, and necrosis [4]. Further research has confirmed that the mechanism is that the endoPGase can degrade the plant cell wall releasing pectic oligomers which can stimulate a wide array of plant defence responses [5]. With the increasing application of pectinase, decreasing its production cost has become one of the most important targets. For this purpose, the selection of carbon source and nitrogen source with low value is a practical consideration. Previous research has reported that many waste products from the agricultural industry containing pectin, such as sugar beet pulp (SBP), citrus pulp pellets, apple pomace, henequen pulp, lemon pulp and other related materials have been used as carbon source for induction of pectinase by many microorganisms [6].

Solid-state fermentation is traditionally defined as those processes in which microbial growth and products formation occur on the surfaces of solid substrates in the near absence of free water. Due to this low amount of water available in solid-state bioprocessing, the class of microorganisms that are most commonly used is fungi [7, 8]. Several agro-industrial waste and by-products such as orange bagasse [9], sugar cane bagasse [10], and other food processing waste [7] are effective substrates for depolymerizing enzyme production by solid-state fermentation. Recently, a large number of microorganisms, isolated from different materials, have been screened for their ability to degrade polysaccharide present in vegetable biomass producing pectinases on solid-state culture [11]. In

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light of the above points, the aim of this work was to study of polygalacturonase enzyme production by isolated strains of fungi by solid-state fermentation using agro industrial by-products. This investigation also is carried out studying the effect of various substrates, initial moisture content, carbon and nitrogen sources in solid state fermentation.

2. Material and Methods

2.1. Microorganisms

The strains of fungi, which had been isolated from agricultural wastes and soil sample in our laboratory, were used in the experiments. Samples (2 g) from agricultural soil and decaying fruit were pooled and homogenized in sterile mineral medium containing 1% of citrus pectin; 0.14% of $(NH_4)_2SO_4$; 0.20% of K_2HPO_4; 0.02% of $MgSO_4 \cdot 7H_2O$; 0.10% of nutrient solution (5 mg/L $FeSO_4 \cdot 7H_2O$; 1.6 mg/L $MnSO_4 \cdot H_2O$; 1.4 mg/L $ZnSO_4 \cdot 7H_2O$; 2.0 mg/L $CoCl_2$), pH 5.0. The mixture was incubated at 30°C for 24 h and a loop of the homogenized one was then streaked onto the same medium, containing agar 3% and incubated at 30°C for 24 to 72 h. All morphological contrasting colonies were purified by repeated streaking.

2.2. Media, cultivation of microorganism and polygalacturonase production

The solid substrates were prepared as follows:

1. Apple bagasse. The pellet of apple bagasse (pressed mixture of pulp and peel) was provided by Fazael Agro-industrial Ltda/Bushehr/SP/ Iran. Chemical analysis revealed that the dry material was composed (% on dry basis): 11.8% fiber, 6.3% nitrogen, 6.7% ash, 3.8% protein, 19% total sugar (9% reducing sugar) and 0.1% pectin. . The material was ground and particles sieved by a Bender UUS 230 strainer and dried at 80°C.

2. Wheat bran. Wheat bran was obtained commercially, dried and used untreated. Chemical analysis revealed that the dry material was composed (% on dry basis): 8.12% fiber, 6.5% nitrogen, 4.57% ash, 6.23% protein, 16.7% total sugar (5.22% reducing sugar) and 0.08% pectin. The percent of water is not effective on the production of polygalacturonases.

The substrates for fermentations consisted of apple bagasse and wheat bran mixture in proportion of 1:1.

Solid-state fermentation (SSF) was carried out using a 250 mL Erlenmeyer flask containing 5 g of sterilized substrate (120°C/40min) inoculated with 10 mL aliquots of conidia suspension (approx.107 spore/g dry substrate) which was obtained from a 7-day agar slant culture suspended in sterile Tween 80 solution. After inoculation, 10 mL of nutrient solution, composed of 0.1% NH_4NO_3; 0.1% $NH_4H_2PO_4$; 0.1% $MgSO_4 \cdot 7H_2O$, were added to each of the flasks. The final moisture content of the medium was approximately 70%. The cultivation was carried out at 30°C for 10 days. At 24 h intervals the solid fermented material, corresponding to one Erlenmeyer flask, was mixed with 30 mL of distilled water (6 g of fermented material/mL), stirred for 40 min, filtered under vacuum and centrifuged. The supernatant was used as crude enzyme solution.

An adequate supply of carbon and nitrogen as energy sources is critical for optimum growth affecting the growth of organism and its metabolism. Factors such as initial moisture and temperature of fermentation medium do very important for growth of microorganism and enzyme production. To study the effect of supplementation of carbon and nitrogen sources, were added.

2.3. Polygalacturonase activity assay

Polygalacturonase (PG) activity was determined by measuring the release of reducing groups from citrus pectin using the 3,5-dinitrosalicylic acid (DNS) reagent assay [12]. The reaction mixture containing 0.8 mL 1% citric pectin 67% methoxylated in 0.2 M acetate buffer, pH 5.0 and 0.2 mL of crude enzyme solution, was incubated at 40°C for 10 min. One unit of enzymatic activity (U/g) was defined as the amount of enzyme which released one mol of galacturonic acid per minute per gram of substrate (mixture of apple bagasse and wheat bran). All measurements were made in duplicate, and the average values were reported.

3. Results and Discussion

3.1. Selection of the strain with more polygalacturonase activity

Initially seventeen strains of fungi isolated from decaying fruit and soil sample were cultivated in mixture of apple bagasse and wheat bran in proportion of 1:1 for 10 days. Data of Table 1 showed that synthesis of polygalacturonase by all the fungi tested depended upon the proportion of mixture used. Among the fungi studied *Aspergillus niger (A. niger)* and *Penicillium sp* EGC5 presented high production of PG in solid state fermentation (Table 1) being selected for a second stage of assays which both the fungi were cultivated in mixture composed by apple bagasse and wheat bran in proportion of 1:1 for 12 days.

3.2. Kinetic of polygalacturonase production in solid state fermentation

This experiment was conducted in batch mode to determine the polygalacturonase production as a func-

Table 1: Production of polygalacturonase by isolated fungal strains by SSF

Strain	PG (U/g)
A. niger	7.93
Penicillium sp EGC5	7.72
Aspergillus sp EG66F	6.88
Aspergillus sp EGC4	6.49
Penicillium 2D.UMIDO	6.49
Trichoderma 2D.SECO.3	6.31
Aspergillus Gel.14	6.18
Aspergillus awamori	6.01
Penicillium 4U.MIDO.2X	5.73
Aspergillus 2D.UMIDA.1	5.56
Aspergillus 4A UMIDA.1	5.52
Aspergillus 2C.SECO.4	5.46
Trichoderma 4B.UMIDA.1	4.66
Trichoderma 4B.SECO.7	4.37
Penicillium 2A.SECO	4.36
Thermoascus sp 179. 5	4.11
Aureobasidium sp RE	3.55

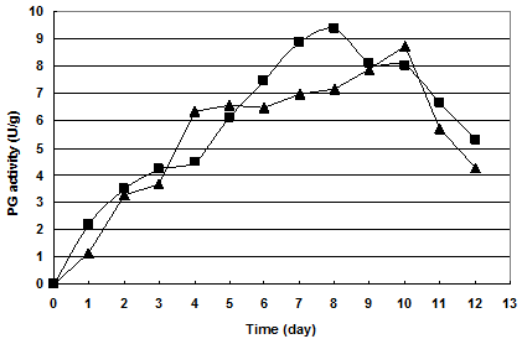

Figure 1: Evaluation of fermentation period for the production of PG from mixture of apple bagasse and wheat flour by *A. niger* (square) and *Penicillium sp* EGC5 (triangle).

tion of time. After about one day, the mycelia appeared on the surface, and at the end of the 4th day (about 2mm) there was a rather thick layer of mycelia on the surface. The layer was woven and could not be broken into fragments easily. Figure 1 shows the polygalacturonase activity as a function of time in batch mode.Production of polygalacturonase was evaluated up to 12 days. Polygalacturonase production by A. niger peaked between 7th and 8th days of cultivation, with the maximum activity of PG 9.36 U/g. when *Penicillium sp* EGC5 was used maximal polygalacturonase activity (8.73 U/g) was detected at the 10th day. A *A. niger* having the highest level of Polygalacturonase production was selected for the following of presented tests.

3.3. *Effect of temperature on plygalacturonase production solid state fermentation*

The effect of different temperatures on polygalacturonase production by *A. niger* was examined (Figure 2). A significant amount of polygalacturonase was produced by *A. niger* at temperatures between 25 and 35°C. The highest production rate was observed at 30°C. polygalacturonase production was detected in cultures incubated at temperatures between 15 and

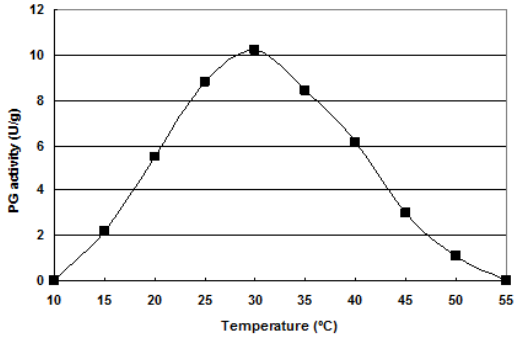

Figure 2: Effect of temperature on polygalacturonase production by *A. niger*

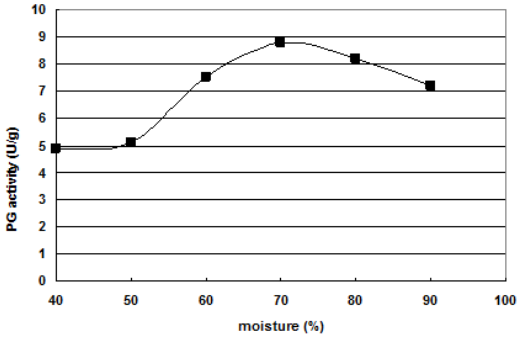

Figure 3: Effect of initial moisture on polygalacturonase production by *A. niger*

50°C. The highest value (10.23 U/g) was attained at 30°C, where no enzyme production was observed at 10 and 55°C. Cultivation time: 10 days.

3.4. *Effect of initial moisture on polygalacturonase production*

In order to understand the effect of water availability, substrate swelling and the oxygen diffusion, different moisture level were tested (40-90%, on dry weight basis). Sterile distilled mineral medium was used as the moistening agent. Polygalacturonase activity was less low when the moisture content was higher or lower than 70% (Figure 3). The low enzyme activity at high substrate moisture levels could be attributed to the decreased porosity, alteration in particle structure, gummy texture, lower oxygen transfer or increased formation of aerial hypha. Likewise, lower moisture levels lead to reduced diffusion of the nutrients in the solid substrate, lower degree of swelling and higher water tension.

3.5. *Effect of carbon sources on polygalacturonase production*

Different carbon sources: glucose, pectin, galactose, sucrose and polygalacturonic acid (PGA) were supplemented separately to a final concentration of 2%

Table 2: Production of polygalacturonase using various carbon sources by *A. niger*

Carbon source	PG (U/g)
Glucose	9.59
Pectin	11.41
Galactose	6.79
Sucrose	7.92
Polygalacturonic acid	8.41

Table 3: Production of polygalacturonase using various nitrogen sources by *A. niger*

Nitrogen source	PG (U/g)
Yeast extract	7.29
Peptone	8.05
Trypton	6.93
$(NH_4)_2SO_4$	8.44
$NaNO_3$	5.66
NH_4Cl	6.78

(g/L) in solid media. Polygalacturonase activity produced by *A. niger* presented in Table 2. Among the different carbon sources studied pectin was produced high value of polygalacturonase in solid state fermentation (11.41 U/g). The effect of various synthetic carbon sources such as pectin, glucose, sucrose, fructose, galacturonic acid and many other such on the production of polygalacturonase in solid state condition were studied by Taragano et al.[13](pectin(8.49 U/g) and glucose (12.66 U/g)); Solis-Pereyra et al[14] glucose (4.65 U/g) and galaturonic acid(21.88 U/g)); Boccas et al[15] glucose(4.12 U/g), sucrose(13.49 U/g) and fructose(9.22 U/g)); and Hours et al[16] glucose(10.28 U/g) and pectin(14.33 U/g))) indicating a wide range of concentrations. Cultivation time: 10 days.

3.6. Effect of nitrogen sources on polygalacturonase production

Different organic and inorganic nitrogen sources: yeast extract, peptone, trypton, $((NH_4)_2SO_4$, $NaNO_3$ and NH_4Cl were supplemented separately to a final concentration of 2% (g/L) in solid media. Of the various nitrogen compounds tested for polygalacturonase production (Table 3), high polygalacturonase activity were obtained $(NH_4)_2SO_4$ and peptone. Our investigation revealed that both $(NH_4)_2SO_4$ and peptone did influence production of polygalacturonase positively in solid state fermentation. However the influence was less peptone as compared to $(NH_4)_2SO_4$. Cultivation time: 10days.

3.7. Effect of different materials on polygalacturonase production

The effect of different materials on polygalacturonase production by *A. niger* are shown in Table 4. Data of Table 4 showed that mixture of apple bagasse and wheat bran was a good substrate for production of polygalacturonase and the highest production was observed in this mixture.

Table 4: Production of polygalacturonase using different materials by *A. niger*. Mixture proportion 1:1

Carbon source	PG (U/g)
Apple bagasse and wheat bran	11.43
Orange bagasse and wheat bran	9.36
Wheat bran and sugar can bagasse	7.48
Apple bagasse and orange bagasse	8.66
Grape pomace and wheat bran	8.41

4. Conclusion

In conclusion, it is feasible to use agricultural byproducts for polygalacturonase production. Moreover, as mixture of apple bagasse and wheat bran is a cheap and readily available by product, the production of polygalacturonase using solid state fermentation may be a cost effective affair. After the initial growth of microorganism on the surface, the growth becomes very slow and the thickness of the mycelia layer becomes almost constant throughout the fermentation. Among filamentous fungi *A. niger* is a popular species for pectinases production. The highest production rate was observed at 30°C. Pectin and ammonium sulphate as carbon and nitrogen sources respectively was produced high value of polygalacturonase in solid state fermentation.

5. Conclusion

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