Continuous production of polygalacturonases (PGases) by *Aspergillus awamori* using wheat flour in surface culture fermentation

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Abstract
The continuous production of polygalacturonases (PGases) that Exo-polygalacturonase (exo-PGase) and Endo-polygalacturonase (endo-PGase) are two members of this group by a strain of *Aspergillus awamori* in surface culture fermentation was investigated. Surface culture fermentation is usually done in batch mode. Wheat flour acted as a good substrate for the cultivation of the fungus and production of PGases in surface culture fermentation. Fermentation started in batch mode until mycelia completely occupied the medium following growth of the microorganism, after which it was turned to the continuous mode by the introduction of fresh feed. The process continued for 34 days, and the thickness of the microbial layer on the surface of the liquid medium became almost constant after approximately one week. The production of PGases, however, continued throughout the experiment, and maximum activities of 1.2 U/ml and 0.014 U/ml were obtained for exo-polygalacturonase (exo-PGase) and endo-polygalacturonase (endo-PGase), respectively. An increase in production was observed when a similar system was used with a line for medium recycling. Lowering the residence time to 12 h decreased the exo-PGase and endo-PGase activities. Reducing the residence time from 24 h to 12 h almost halved the concentrations of the enzymes at the outlet.
***Keywords*:** Polygalacturonase; *Aspergillus awamori*; Wheat flour; Surface culture fermentation; Continuous production

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INTRODUCTION

Pectic substances are major constituents of many plant tissues. They reduce the yield of juice in fruit processing plants and contribute to product turbidity. Pectinases, which are produced by a variety of microorganisms, degrade pectic substances and play an important role in fruit and vegetables industries (Rombouts *et al*., 1989; Gomes *et al*., 1999).

Depending on their specificity and the type of reaction they catalyze, pectinases are classified into protopectinases, polygalacturonases, lyases, and pectin esterases. Protopectinases convert insoluble propectin to soluble pectin. Polygalacturonases (PGases) catalyze the hydrolytic cleavage of the polygalacturonic acid chain through the introduction of water across the oxygen bridge. They are the most extensively studied among the family of pectinolytic enzymes (Maria *et al*., 2006). Exo-PGases act on terminal monomers of polygalacturonic acid and release monogalacturonic acid. Endo-PGases act on polygalacturonic acid randomly and release oligogalactorunic acid. Lyases perform non-hydrolytic breakdown of pectates or pectinates. Pectin esterase catalyzes the de-esterification of methyl ester linkages of the galacturonan backbone of pectic substances to release acidic pectins and methanol (Jayani *et al*., 2005). A combined action of pectinesterase and polygalacturonases is required for degrading pectic substances. Pectinlyase is the only enzyme capable of depolymerizing the pectin molecules without the prior action of other enzymes (Taragano *et al*., 1997).

Generally, cereal grains are composed mainly of

starch plus other carbohydrates, protein, oil and fat. More importantly, they contain all the essential macro- and micronutrients, such as minerals and vitamins, to sustain microbial growth. This nutritional characteristic makes them potentially ideal as alternative, renewable, raw materials for chemical production Hence, overall production costs can be reduced. Moreover, the use of cereal grains as a starting material for the production of fine chemicals through fermentation would offer potentially cleaner and more environmentally friendly processes. Although some basal levels of pectinases are produced constitutively in many microorganisms, using pectic substances as substrates enhances pectinase production considerably. Various agro-industrial wastes, such as wheat bran (Taragano *et al*., 1997), sugarcane bagasse (Solis-Pereyra *et al*., 1993, 1996), coffee pulp (Boccas *et al*., 1994), lemon peel (Larios *et al*., 1989) and apple pomace (Hours *et al*., 1988) have been used for the microbial production of pectinases. Pectinases are secreted by different microbial species including bacteria, yeasts and filamentous fungi. Among filamentous fungi, Aspergillus niger and Aspergillus awamori are popular species for pectinase production. Aspergillus awamori synthesizes three types of pectolytic enzymes: polygalacturonase, pectinesterase, and pectinlyase (Botella *et al*., 2007). Pectinolytic enzyme preparations based on Aspergillus niger and Aspergillus awamori have been successfully used in practice (Pereira *et al*., 2002; and Botella *et al*., 2007).

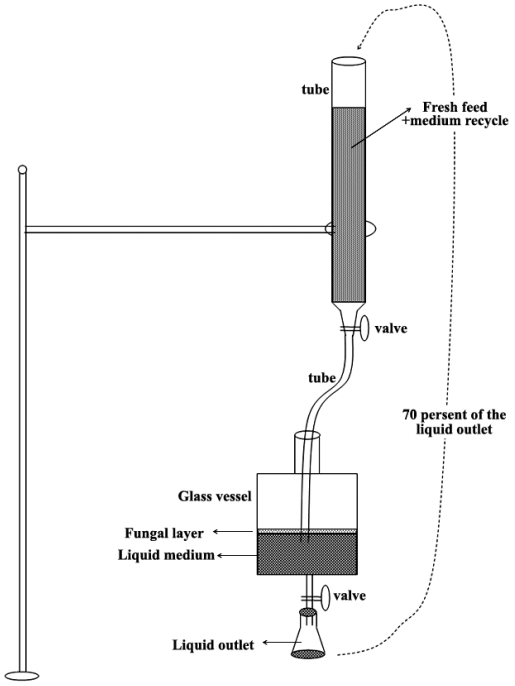

Figure 1. A schematic diagram of the continuous bioreactor.

Production of pectinase enzyme already was carried out in solid state fermentation and submerged fermentation and method of Surface culture fermentation is not carried out on pectinase production. In surface culture fermentation, filamentous fungi grow on the surface of a shallow liquid medium. These microorganisms take up necessary nutrients from the medium and release the products into it. This method of fermentation does not need aeration and mixing and the biomass is easily separated from the liquid medium. The bio-reaction rate, however, is slower than submerged fermentation in which vigorous aeration and mixing occurs. Because of this disadvantage, submerged fermentation is usually preferred when the fermentation medium is a liquid one. Nevertheless, the modification of surface culture fermentation to improve the productivity of the method is worthwhile to be considered. Surface culture fermentation is usually done in batch mode. In this research, the possibility of continuous surface culture fermentation for polygalacturonases production is investigated.

MATERIALS AND METHODS

Microorganism and inoculum: *Aspergillus awamori*, previously isolated from agricultural wastes in biotechnology laboratory in the Shahid Bahonar University of Kerman, was used in this study. The microorganism was maintained on potato dextrose agar (PDA) at 4°C. A loopfull of the spores was used as inoculum.

Fermentation medium: A mineral medium with the following composition was used as the basal medium for fermentation: citrus pectin 5 g/l, KH_2PO_4 3.4 g/l, K_2HPO_4 4.3 g/l, $(NH_4)_2SO_4$ 4 g/l, $MgCl_2.7H_2O$ 0.2 g/l, $CaCl_2.2H_2O$ 0.04 g/l, and FeSO4 0.03 g/l. The medium was supplemented with the following trace element solution: $MnCl_2$ 0.04 g/l, $NaMoO_4$ 0.08 g/l, $CuSO_4$ 0.006 g/l, H_3BO_3 0.013 g/l, $ZnSO_4$ 0.06 g/l.

One liter of the mineral medium contained 975 ml of the basal mineral medium and 25 ml of the trace element solution. Citrus pectin (5 g/l) and wheat flour (20 g/l) were used as the carbon source and substrate respectively. It was sterilized in an autoclave at 120°C for 15 min. All other chemicals were of analytical grade (Sigma, Germany).

Fermentation procedure: Batch production of PGase involving the method of surface culture was performed in 100 ml Erlenmeyer flasks with a working volume of 50 ml mineral medium plus wheat flour 20 (g/l). During the continuous production, a glass vessel was filled with 50 ml of the mineral medium plus wheat flour (desired concentration). The liquid medium was then inoculated with *Aspergillus awamori* spores. The vessel was provided with a valve for liquid outlet and a tube for introducing the fresh medium. The tube was inserted below the liquid medium surface so that the fresh medium could enter the bioreactor without disturbing the fungal layer on the surface. The bioreactor was placed in incubator at 35°C. A similar system was used to investigate the effect of medium recycling on fermentation (Fig. 1).

Polygalacturonase activity assay: Liquid samples were taken from the fermentation medium at short interval after the end of the experiments. Exo-polygalacturonase (exo-PGase) and Endo-polygalacturonase (endo-PGase) are two members of polygalacturonases group. Endo-PGase activity was determined by viscometry. One milliliter of a suitably diluted sample was mixed with 18 ml of 20 g/l pectin solution in 0.1 M acetate buffer at pH 4.5, and reduction in viscosity of made solution was followed using the Ostwald viscometer (McGraw-Hill, Canada) (Ghyldyal *et al*., 1981). One endo-PGase unit (U) was defined as the amount of the enzyme that reduced the viscosity of the solution by 50% per min, under the conditions mentioned above. For exo-PGase activity, 0.3 ml of a suitably diluted sample was added to a solution containing 1 ml of 0.9% (9 g/l) pectin solution and 1 ml of 0.1 M acetate buffer (pH 4.5). Samples were incubated at 45°C for 30 min and reducing sugars were determined by the dinitrosalicylic acid (DNS) method (Miller, 1959). One exo-PGase unit (U) was defined as the amount of enzyme that liberated one µmole of galacturonic acid per min under the conditions mentioned above. All measurements were made in duplicate, and the average values were reported.

Biomass measurement: At the end of fermentation, the biomass was separated from the liquid medium using muslin cloth as filter. The biomass weight was then measured after being dried to a constant weight.

RESULTS

Batch production of polygalacturonases by surface culture fermentation: Some experiments were conducted in batch mode to determine biomass and PGase production as a function of time. After 24 h, the mycelia appeared on the surface, and at the end of the 4th day there was a rather thick layer of mycelia (about 2 mm in depth) on the surface of medium, which could not be broken into fragments easily. Figures 2A and B show the activity of PGases as a function of time in batch mode. The PGase activities reached a maximum value after approximately 5 days and constancy in the activity of the enzymes was observed after 9 days.

Effect of pectin and glucose addition on polygalacturonases production: The effect of pectin and glucose on PGases using wheat flour (20 g/l) as substrate was examined. When upto the 4 g/l of pectin was used,

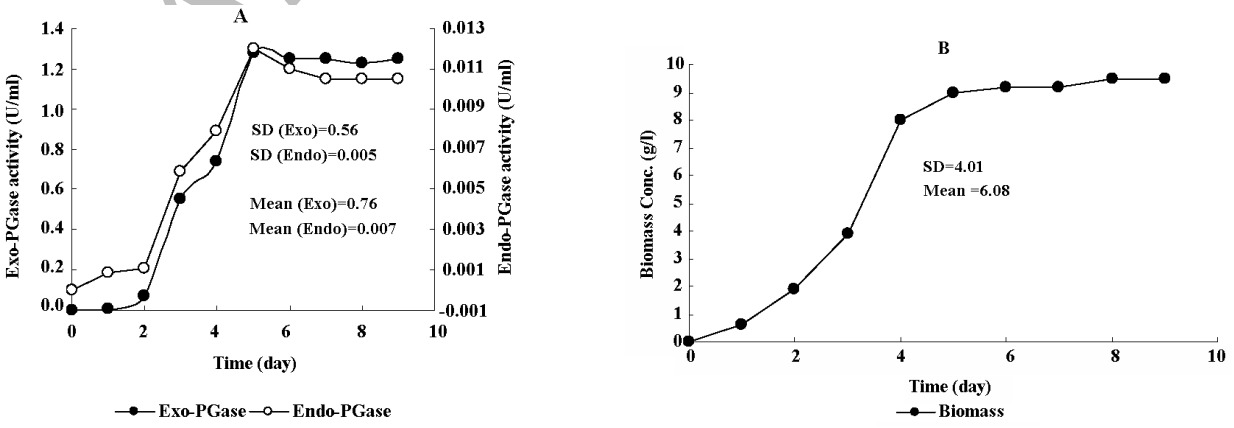

Figure 2. A: Pectinase production and B: Biomass concentration as a function of time in the batch system. Wheat flour concentration (60 g/l). Conc:Concentration.

maximum production of endo- and exo-PGase was observed (Fig. 3). The influence of glucose as an extra carbon source on the production of PGases under surface culture conditions is shown in Figure 4. For this purpose, the culture medium was supplemented with 0-8 g/l of glucose. However, when glucose was used as an extra carbon source, both the production of pectinase and the growth of the fungi (2.5 g\l) were found to be low (Fig. 4).

Effect of nitrogen sources on polygalacturonases production: The effect of sodium nitrate and ammonium sulphate on production of PGases, using wheat flour as substrate, is shown in Figures 5 and 6, respectively. An increase in the production of endo- and exo-PGases was observed upto 8 g/l of both nitrogenous compounds. However, the rise in the production of PGases was much lower with sodium nitrate, when compared with that in the presence of ammonium sulphate.

Continuous production of polygalacturonases under different wheat flour concentrations: One of the fratilities of batch bioreactors being is cleaning the bioreactor at the end of the experiment (about 4 days). Nevertheless, running the bioreactor under the continuous mode helps to avoid repeated cleaning and inoculations (about 30 days). Little information, however, exists regarding continuous fermentation in surface culture bioreactors. The main questions that should be answered regarding continuous surface culture fermentation are how thick a layer can a microbial strain grow on the surface, and does the layer of the microorganism still retain the ability to produce the enzymes after long periods of time?. To answer these questions, the second stage of experiments was conducted. The fermentation process was started in batch mode with an initial wheat flour concentration of 20 g/l. On the third day, when the surface was covered totally with the fungus, fresh medium with a wheat flour concentration of 20 g/l was made to pass through the bioreactor. The residence time was 1 day, and steady PGase activity was observed at the bioreactor outlet, implying the possibility of PGase production continuously. On the 9th day, the inlet wheat flour concentration increased to 40 g/l, and as expected the PGases activity increased accordingly. On the 14th day, the inlet wheat flour concentration increased to 60 g/l and a similar trend in PGase activity was observed at the outlet (higher wheat flour concentrations were not tested because the medium became too viscous to flow easily). The production continued constantly without any decrease due to the passage of time (the sum of data) (Fig. 7).

Continuous production of polygalacturonases under different wheat flour concentrations using medium recycling: The effect of residence time on the production of PGases in the continuous mode was investigated using the bioreactor without medium recycling. Three different residence times were applied: 24 h, 18 h, and 12 h (Fig. 9). After each change in residence time, a new steady state was observed during approximately 5 days. Reducing the residence time from 24 h to 12 h almost halved the concentrations of the enzymes at the outlet. After 31

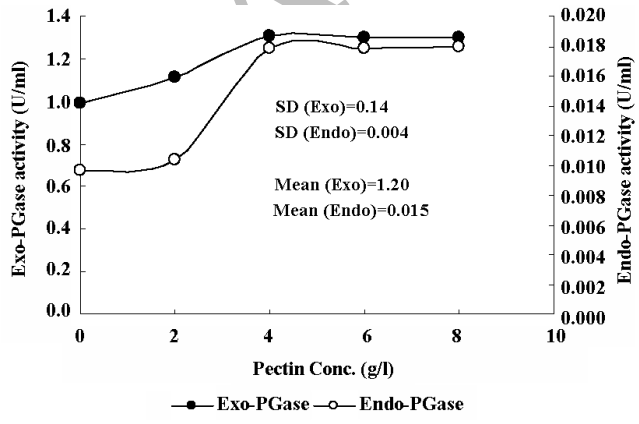

Figure 3. Effect of pectin on the production of PGases using wheat flour. Conc: Concentration.

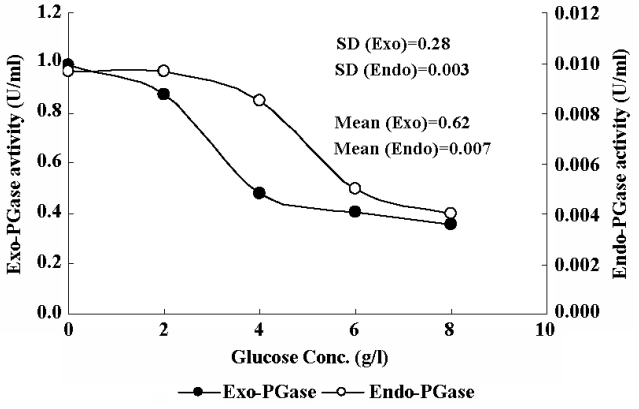

Figure 4. Effect of glucose on the production of PGases using wheat flour. Conc: Concentration.

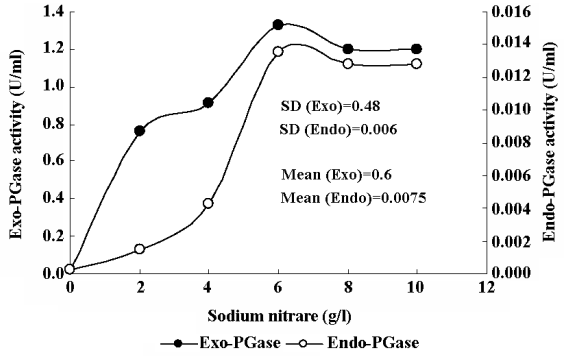

Figure 5. Effect of sodium nitrate, as the nitrogen source on PGase production .

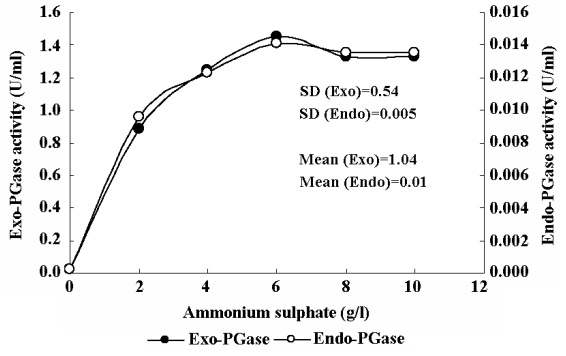

Figure 6. Effect of ammonium sulphate, as the nitrogen source on the production of PGase.

days of continuous fermentation, the biomass layer was separated from the liquid medium and dried to a constant weight of 7.8 g/l.

Continuous production of polygalacturonases under different residence times: The effect of residence time on the production of PGases in the continuous mode was investigetd using the bioreactor without medium recycling. Three different residence times were applied: 24 h, 18 h, and 12 h (Fig. 9). After each change in residence time, a new steady state was observed for five days. Reducing the residence time from 24 h to 12 h almost halved the concentrations of the enzymes at the outlet. After 31 days of continuous fermentation, the biomass layer was separated from the liquid medium and dried to a constant weight of 7.8 g/l.

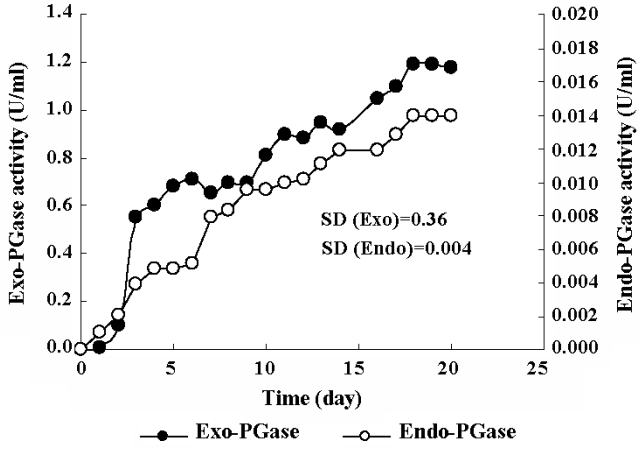

Figure 7. Continuous production of PGases in the surface culture bioreactor. Residence time: 1 day.

DISCUSSION

Our study confirmed that method of continuous production of polygalacturonases enzyme offer gigantic advantage over the batch production. In this way, utilizing wheat flour as a cheap and readily available cereal raw material, it was possible to obtain polygalacturonase activities at a acceptable yield (1.5 U/ml and 0.014 U/ml for exo- and endo- PGases respectively in a batch system and 24.3 U/ml and 0.328 U/ml for exo- and endo-PGases respectively in a continuous bioreactor). When glucose was used as an extra carbon source, both the production of polygalacturonase and the growth of the fungi were low. This indicated that the microorganism was unable to hydrolyze enough glucose to support mycelium formation. The effect of various synthetic carbon sources such as pectin, glucose, sucrose, fructose, galacturonic acid and many other such on the production of polygalacturonas were studied by Taragano *et al*., 1997 (pectin (1.49 U/ml) and glucose (1.26 U/ml); Solis-Pereyra *et al*., 1996 (glucose (0.65 U/ml) and galaturonic acid 1.88 U/ml); Boccas *et al*., 1994 glucose (4.12 U/g), sucrose (13.49 U/g) and fructose(9.22 U/g); and Hours *et al*., 1988 glucose(10.28 U/g) and pectin (14.33 U/g) indicating a wide range of concentrations.

Using a defined mineral medium and wheat flour as the substrate, it was possible to produce PGases in a surface culture bioreactor continuously. The ability of the microorganism to produce PGases did not decrease with the passage of time throughout the experiment. After the initial growth of the microorganism on the surface, growth rate decreased and the dried weight of the mycelia layer became almost constant throughout the fermentation. Using a recycle line was useful in increasing PGase concentrations in the liquid medium.

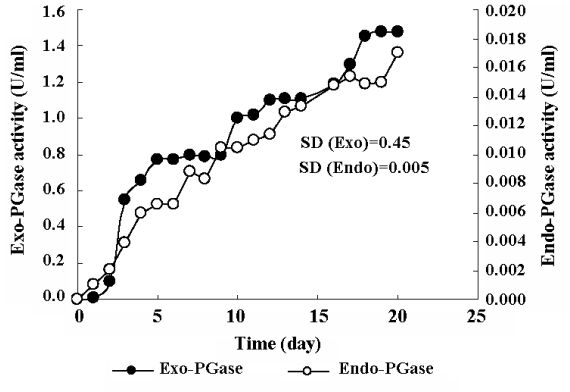

Figure 8. Continuous production of PGases in the surface culture bioreactor. 70% of the bioreactor outlet is mixed with fresh feed.

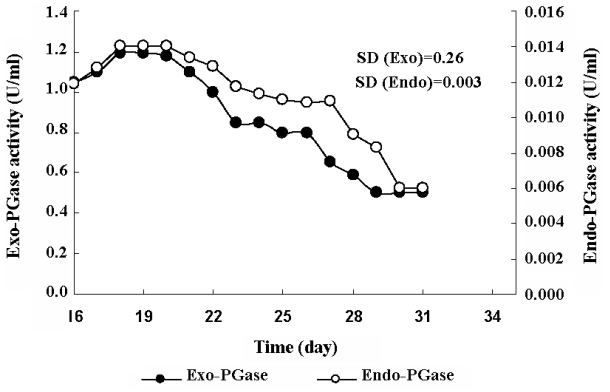

Figure 9. Continuous production of PGases in the surface culture bioreactor under different residence times (day 16-20; residence time = 24 h, day 21-26; residence time = 18 h, day 27-31; residence time = 6 h). Inlet wheat flour concentration: 60 g/l.

Investigation of the effect of nitrogen sources revealed that both sodium nitrate and ammonium sulphate influenced the production of PGases positively in surface culture fermentation. The observations of (Hours *et al*., 1988) suggested that lower levels of ammonium sulphate (1.6 g/l) added to medium did not influence enzyme yield. The inorganic nitrogen on the production of pectinase was also reported by (Boccas *et al*., 1994; Larios *et al*., 1989). Both sodium nitrate and ammonium sulphate influenced the production of PGase positively during surface culture fermentation, however, the effect of sodium nitrate on the production of PGases was less than that of ammonium sulphate.

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