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*Full Length Research Paper*

# Production of exopolygalacturonase from wheat flour by *Aspergillus awamori* in submerged and surface culture fermentation

**Heidar Abbasi* and Seyyed Ruhollah Mortazavipur**

University of Applied Science and Technology of Khormuj, Bushehr, Iran.

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**Pectinases are a group of hydrolytic enzymes that degrade pectic substances. They are produced by a variety of microorganisms including filamentous fungi. Studies were carried out on the production of exoplygalacturonase (Exo-PGase) using wheat flour by *Aspergillus awamori* in submerged fermentation (SmF) and surface culture fermentation (SCF). Higher titres of was observed when medium was supplements with carbon (4 g/L Pectin for both SmF and SCF) and nitrogen (ammonium sulphate 6 g/L for both SmF and SCF) sources. SCF proved to be a better process than SmF for Exo-PGase production, giving the highest enzyme production rate in 96 h. Exo-PGase produced by *A. awamori* presented optimum activity at pH 5 for SmF and 5.5 for SCF. Use of continuous system for Exo-PGase production obtained a better result than the batch system.**

**Key words:** Exopolygalacturonase, *Aspergillus awamori*, wheat flour, surface culture fermentation, submerged fermentation, continuous production.

## INTRODUCTION

Pectic substances are major constituents of many plant tissues. They reduce the yield of juice in fruit processing plant and contribute in the product turbidity. Pectinases, which are produced by a variety of microorganisms, degrade pectic substances and play an important role in fruit and vegetable industries. Endopolygalacturonases (Endo-PGases) act on polygalacturonic acid randomly and releases oligogalactorunic acid. Lyases perform non-hydrolytic break down of pectates or pectinates. Pectin esterase catalyzes the de-esterification of methyl ester linkages of galacturonan backbone of pectic substances to release acidic pectins and methanol (Jayani et al., 2005). A combined action of pectinesterase and polygalacuronases is required for degrading pectic substances. Pectinlyase is the only enzyme capable of depolymerizing the pectin molecules without the prior action of the 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 and raw materials for chemical production. The fermentation industries that use glucose as a carbon source rely on hydrolysis of purified starch from cereals. However, the direct use of whole-wheat flour as a fermentation feed stock would remove the need to purify the starch to produce glucose, and would have the benefit of providing a more complete supply of nutrients. 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

*Corresponding author. E-mail: heidar.abbasi@yahoo.com. Tel: 0098 917 7755813. Fax: 0098 341 2118298.

**Abbreviations: SmF,** Submerged fermentation; **SCF,** surface culture fermentation; **Exo-PGase,** exoplygalacturonase.

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* is a popular species for pectinases production. *A. niger* synthesizes three types of pectolytic enzymes; polygalacturonase, pectinesterase, and pectinlyases (Taragano et al., 1997).

Pectinolytic enzyme preparations based on *A. niger* have been successfully used in practice (Pereira et al., 2002). In surface culture fermentation, filamentous fungi grow on the surface of a shallow liquid medium. The organisms uptake 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 the submerged fermentation in which vigorous aeration and mixing occur. Submerged fermentation has also been used for pectinases. Surface culture fermentation is usually done in batch mode. The present study reports on the batch and continuous production of exopolygalacturonase by *Aspergillus awamori* in submerged and surface culture fermentation employing wheat flour as substrate.

## MATERIALS AND METHODS

### Microorganism and inoculum

Fifteen fungal strains isolated from agricultural wastes in our laboratory, was used in the experiments. The microorganism was maintained on potato dextrose agar (PDA) at 4°C. A loop full 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, 2 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; $FeSO_4$, 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. Each liter of the mineral medium contained 975 ml of the basal mineral medium and 25 ml of the trace element solution. Citrus pectin was the carbon source. All the chemicals were of analytical grade.

### Fermentation

#### *Medium for surface culture fermentation*

A glass vessel was filled with 50 mL of the mineral medium plus wheat flour (desired concentration). It was sterilized in an autoclave at 120°C for 15 min. The liquid medium was then inoculated with the spores.

#### *Medium for submerged fermentation*

A glass vessel was filled with 100 ml of the mineral medium plus wheat flour (desired concentration). Medium components were same as surface culture fermentation components, and fermentation was carried out in this glass vessel by taking on rotatary shaker (300 rpm). The bioreactor was placed in an incubator at 35°C.

### Continuous bioreactors

For surface culture fermentation, a glass vessel was filled with 50 mL of the mineral medium plus wheat flour (30 g/L concentration). It was sterilized in an autoclave at 120°C for 15 min. The liquid medium was then inoculated with the spores of *A. awamori*. The vessel was provided with a valve for liquid outlet and a tube for introducing the fresh medium. The tube was fixed in a way that the fresh medium could enter the bioreactor without disturbing the fungal layer on the surface. A similar system was used for submerged fermentation, but this fermentation was carried out on a rotatary shaker (300 rpm).

### Polygalacturonase activity assay

Liquid samples were taken from the fermentation medium. For Exo-PGase activity, 0.3 ml of a suitably diluted sample was added to a solution containing 1 ml of 0.9% pectin solution and 1 mL of 0.1 molar 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 micromole 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 a filter. Then, it was dried to a constant weight, and finally its weight was measured.

## RESULTS AND DISCUSSION

### Selection of the strain with more Exo-PGase activity

Fifteen fungal strains, which were isolated from agro waste were employed in submerged and surface culture fermentation. Cultivation time was 7 days. Table 1 shows the results of Exo-PGase production by fungal strains in SmF and SCF. *A. awamori* produced the highest value of enzyme. *A. awamori* having the highest level of Exo-PGase production was selected.

### Evaluation of fermentation period

Production of Exo-PGase was evaluated up to 5 days

**Table 1.** Production of Exo-PGase by isolated fungal strains by SmF and SCF.

| Strain | SCF(U/mL) | SmF(U/mL) |
|---|---|---|
| *Asprgillus awamori* | 1.11 | 1.05 |
| *Aspergillus fuchensis* | 1.05 | 1.00 |
| *Trichoderma viride* | 1.01 | 0.93 |
| *Aspergillus niger* | 0.92 | 0.92 |
| Penicillium 4A.UMIDO.2 | 0.92 | 0.88 |
| Penicillium 2D.UMIDO | 0.86 | 0.87 |
| Aspergillus 2D.UMIDA.1 | 0.75 | 0.69 |
| Pencillium 4U.MIDO.2X | 0.70 | 0.58 |
| Trichoderma 2D.SECO.3 | 0.61 | 0.56 |
| Aspergillus Gel.14 | 0.59 | 0.49 |
| Aspergillus sp EG66F | 0.48 | 0.51 |
| Aspergillus 2C.SECO.4 | 0.39 | 0.43 |
| Aspergillus 4A UMIDA.1 | 0.39 | 0.39 |
| Trichoderma 4B.UMIDA.1 | 0.37 | 0.35 |
| Trichoderma 4B.SECO.7 | 0.33 | 0.28 |

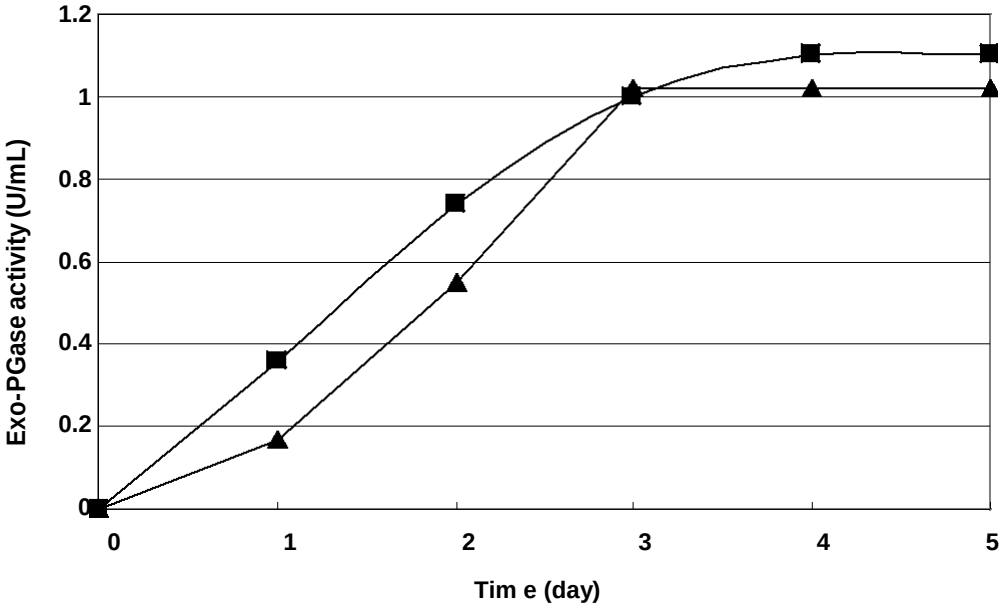


**Figure 1.** Evaluation of fermentation period for the production of Exo-PGase from wheat flour by *A. awamori* in SmF (triangle) and SCF (square).

(Figure 1). A gradual increase in the production of Exo-PGase over a period of 3 days was observed in submerged and was up to 4 days in surface culture fermentation (Figure 1). The production of Exo-PGase was high, in surface culture fermentation (1.09 U/ml) when compared to submerged fermentation (1.02 U/ml). It is known that the period of fermentation was upon the nature medium, fermenting organism, concentration of nutrients and the process physiological conditions. Generally, the period of fermentation in synthetic medium by pectinolytic fungi vary from 48 to 72 h. The optimized fermentation provided by several researchers (Taragano et al., 1997; Solis-Pereyra et al., 1993) indicate a wide range in submerged (40-120 h).

### Effect of nitrogen sources on PGases production

The effect of sodium nitrate and ammonium sulphate on the production of PGases from wheat flour in both submerged and surface culture fermentation is as shown in Figures 2 and 3, respectively. Both showed better

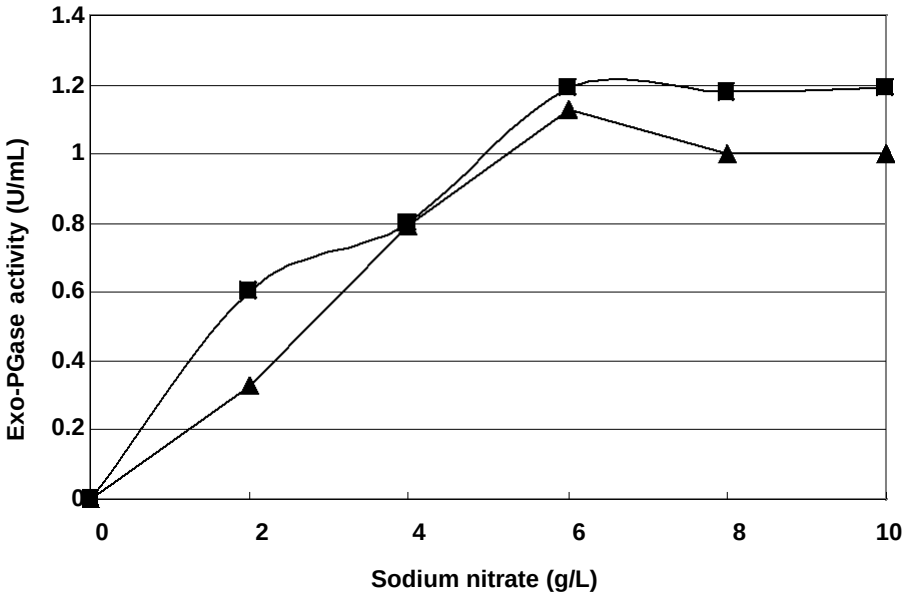


**Figure 2.** Effect of sodium nitrate as nitrogen source on the production of Exo-PGase by *A. awamori* in SmF (triangle) and SCF (square).

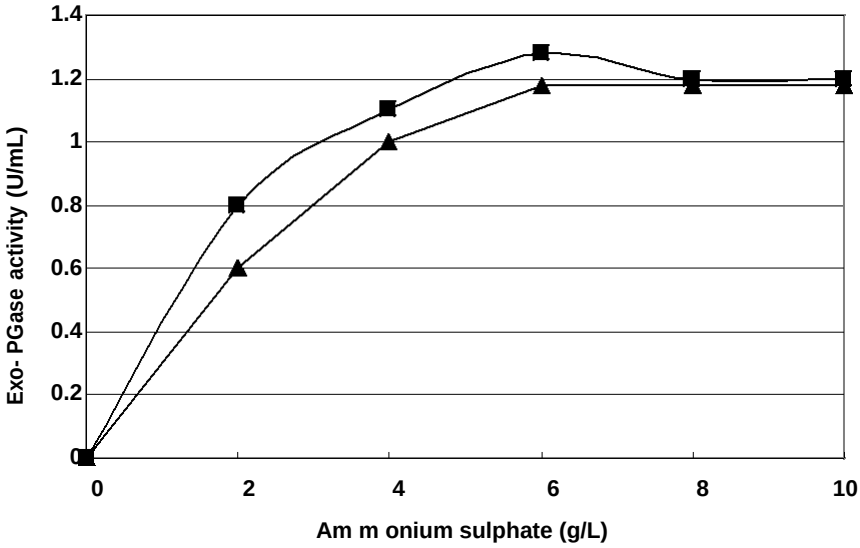


**Figure 3.** Effect of ammonium sulphate as nitrogen source on the production of Exo-PGase by *A. awamori* in SmF (triangle) and SCF (square).

effect on production of Exo-PGase in surface culture fermentation when compared to submerged fermentation. Increase in the production of Exo-PGases was observed which was up to 6 g/L concentration of both the nitrogenous compounds.

However, the increase was very less with sodium nitrate when compared to ammonium sulphate. Our investigation revealed that both sodium nitrate and ammonium sulphate did influence production of PGase positively in both submerged and surface culture fermentation.

**Effect of carbon sources on Exo-PGase production**

Effect of glucose and pectin as a source of carbon on the production of Exo-PGase from wheat flour was examined. The production of Exo-PGase was high by both carbon source in submerged fermentation as well as surface culture fermentation (Figures 4 and 5). However,

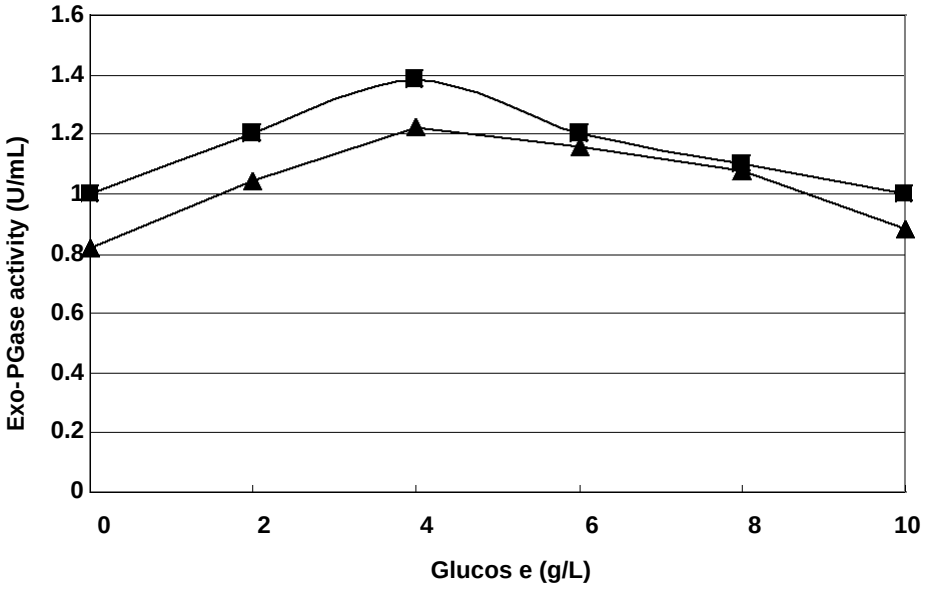


**Figure 4.** Effect of glucose as carbon source on the production of Exo-PGase by *A. awamori* in SmF (triangle) and SCF (square).

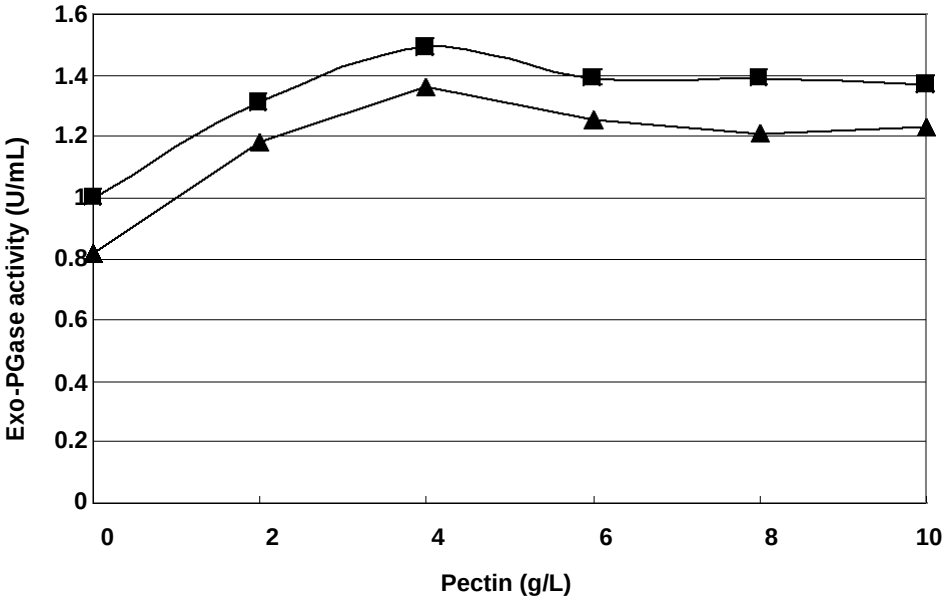


**Figure 5.** Effect of pectin as carbon source on the production of Exo-PGase by *A. awamori* in SmF (triangle) and SCF (square).

effect of these sources was more in surface culture fermentation when compared to submerged fermentation. Maximum production of Exo-PGase (1.36 U/mL for glucose and 1.49 U/mL for pectin) were recorded in surface culture fermentation when compared to Exo-PGase (1.26 U/mL for glucose and 1.34 U/mL for pectin) in submerged fermentation under equal condition. An adequate supply of carbon as energy source is critical for optimum growth affecting the growth of organism and its metabolism. At low concentration of carbon sources, an increasing substrate concentration usually favours product synthesis.

### Effect of pH on Exo-PGase production

The effect of pH on Exo-PGase production by *A. awamori* is shown in Figure 6. Different pHs were obtained by

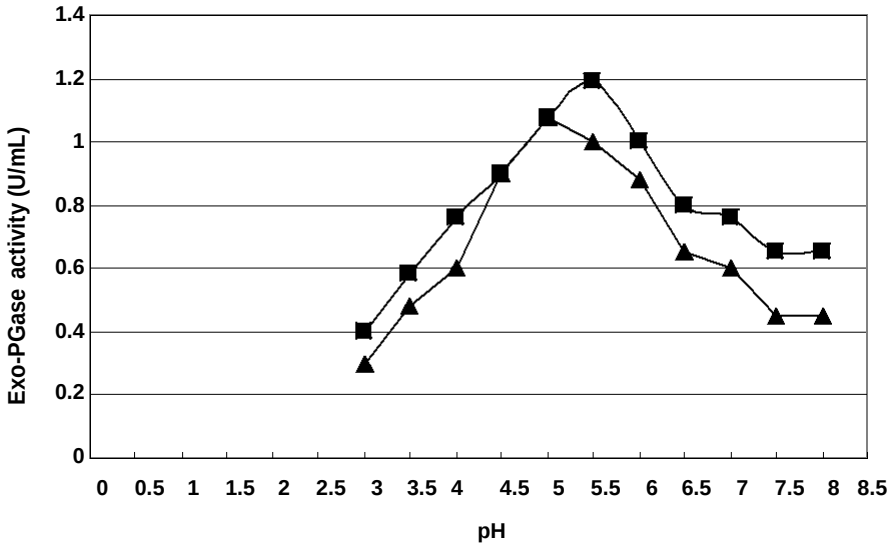


**Figure 6.** Effect of pH on the production of Exo-PGase by *Aspergillus awamori* in SmF (triangle) and SCF (square).

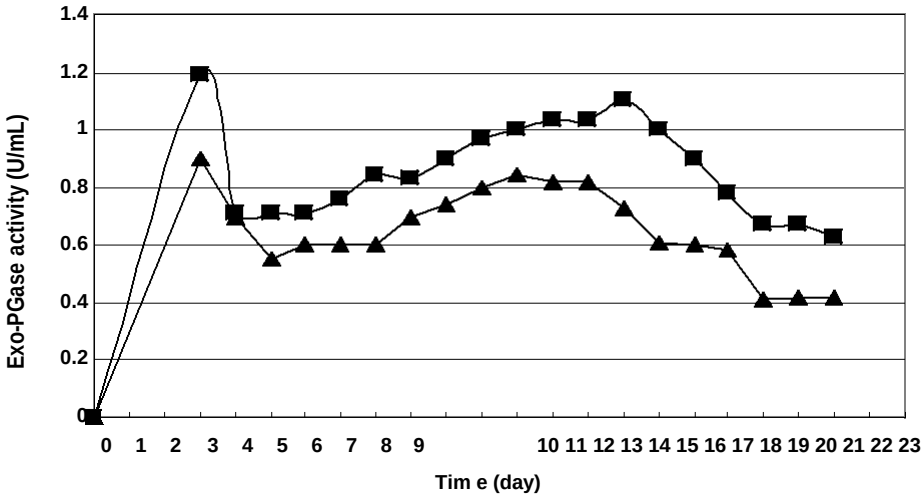


**Figure 7.** Continuous production of Exo-PGase by *Aspergillus awamori* in SmF (triangle) and SCF (square).

changing the values of $K_2HPO_4$ and $(NH_4)_2SO_4$ in mineral medium. A significant amount of Exo-PGase in both fermentation methods was produced by *A. awamori* pHs between 4.5 and 6. The highest value of Exo-PGase production was observed at pH 5 for submerged fermentation and pH 5.5 for surface culture fermentation.

## Continuous production of Exo-PGase

Running the bioreactor under continuous mode helps avoiding repeated cleaning and inoculation. Little information, however, exists on continuous fermentation in surface culture and submerged bioreactors. The main questions that should be answered, regarding the continuous surface culture and submerged fermentation is, can the microorganism retain its ability to produce the enzymes after long times? The fermentation for surface culture started in batch mode. On the third day, when the surface had been covered totally with the fungus, the fresh medium with the wheat flour concentration of 30 g/L started to pass through the bioreactors. The residence time was 1 day. The results are in Figure 7. The process continued for 21 days and the enzyme was produced by

the microorganism after this time.

## Conclusion

To our knowledge, this is the first report on continuous Exo-PGases production on surface culture fermentation and submerged fermentation. After careful analysis, the following results were obtained: wheat flour could be an attractive and promising substrate in submerged fermentation and surface culture fermentation for the production of Exo-PGase by *Aspergillus awamori*, and using a defined mineral medium, it is possible to produce PGases in a surface culture and submerged bioreactors continuously.


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