GIDA
THE JOURNAL OF FOOD
E-ISSN 1309-6273, ISSN 1300-3070

Research / Araştırma
GIDA (2019) 44 (5): 802-809
doi: 10.15237/gida.GD19054

EFFECT OF SUBSTRATE CONCENTRATION AND SCALE UP ON THE POLYGALACTURONASE PRODUCTION

Nihan Göğüş[a], CananTarı[b]**
[a]Efes Vocational School, DokuzEylül University, Selcuk, Izmir, Turkey
[b]Department of Food Engineering, Izmir Institute of Technology, Gulbahce Campus, Urla, Izmir, Turkey

Received / Geliş: 11.03.2019; *Accepted* / Kabul: 07.07.2019; *Published online* / Online baskı: 23.09.2019

Göğüş, N., Tarı, C., (2019). Effect of substrate concentration and scale up on the polygalacturonase production. GIDA (2019) 44 (5): 802-809 doi: 10.15237/gida.GD19054

Göğüş, N., Tarı, C., (2019). Substrat konsantrasyonu ve ölçek büyütmenin poligalakturonaz üretimine etkisi. GIDA (2019) 44 (5): 802-809 doi: 10.15237/gida.GD19054

ABSTRACT
Pectinases have been used for many industrial applications since long time ago. The largest industrial application of these enzymes is in fruit juice and wine production for the extraction, filtration and clarification and for the maceration of fruits and vegetables. They work by enzymatic breaking down of the cell wall.In this study it was aimed to use the previously optimized shake flask media formulation in batch mode 1 L scale serial bioreactor system and 5 L scale in order to investigate the effects of substrate concentration and scale on PG activity and biomass production. In conclusion it was observed that average PG activity (101.29 U/ml) obtained in 5L scale bioreactor experiments was higher than the maximum PG activity (88.55 U/ml) at 40 g/L orange peel (OP) concentration obtained in the 1 L scale substrate concentration experiment. Furthermore,PG activity increased with an increase in substrate concentration except for 60 g/L orange peel concentration.
Keywords: Scale-up, polygalacturonase, Aspergillus sojae, bioreactor, orange peel

SUBSTRAT KONSANTRASYONU VE ÖLÇEK BÜYÜTMENİN POLİGALAKTURONAZ ÜRETİMİNE ETKİSİ

ÖZ
Pektinazlar, uzun zaman önceden beri bir çok endüstriyel uygulama için kullanılmıştır. Bu enzimlerin en büyük endüstriyel uygulaması meyve suyu ve şarap üretiminde ekstraksiyon, berraklaştırma ve filtrasyon ayrıca meyve ve sebzelerin maserayonudur. Hücre duvarının enzimatik parçalanması yoluyla etki ederler. Bu çalışmada, substrat konsantrasyonunun ve ölçek büyütmenin PG aktivitesi ve biyokütle üretimi üzerindeki etkilerini araştırmak için daha önce erlenlerde optimize edilmiş medya formülasyonunun kesikli tip 1 L ölçekli seri biyoreaktör sistemi ve 5 L ölçekte kullanılması amaçlanmıştır. Sonuç olarak, 5 L ölçekli biyoreaktör deneylerinde elde edilen ortalama PG aktivitesinin (101,29 U/ml), 1 L ölçekli substrat konsantrasyonu deneyinden elde edilen 40 g/L portakal kabuğu konsantrasyonundaki maksimum PG aktivitesinden (88,55 U/ml) daha yüksek olduğu belirlenmiştir. Ayrıca 60 g/L portakal kabuğu konsantrasyonu hariç substrat konsantrasyonu arttıkçaPG aktivitesi de artmıştır.
Anahtar kelimeler*:* Ölçek büyütme, poligalakturonaz, *Aspergillus sojae,* biyoreaktör, portakal kabuğu

[*] *Corresponding author / Yazışmalardan sorumlu yazar*;
✉nihan.gogus@deu.edu.tr, ✆(+90) 232 892 8776-77-82 (+90) 232 892 8784

INTRODUCTION

In industry the most extensively used pectinases are the polygalacturonases (PG). (Jayani et al, 2005; Gummadiet al, 2007). As the pectinases are induced enzymes it is necessary to supplement the culture medium with pectin or with raw materials rich in pectin, like orange peel (Malvessi and Silveira, 2004). PGs can be industrially produced by submerged (SmF) and solid-state fermentations (SSF). It is known that about 90% of all industrial enzymes are produced by SmF using genetically modified microorganisms due to several process advantages over SSF. The drawbacks of SSF can be summarized as the difficulties in scale-up and control of process parameters such as pH, temperature, oxygen transfer and moisture. Additionally, low mixing efficiency and higher product impurities increases the product recovery costs of SSF (Nakkeeran et al., 2012). However Biz et al, 2016 show the potential use of solid-state fermentation to produce pectinases in pilot scale. Submerged fermentation (SmF) is generally used for the production of industrially important enzymes, employing mostly genetically modified strains (Pandey et al, 2000). In the investigation of Songulashvili et al, (2015) laccase was produced in a 120-L volume by SmF of *Cerrena unicolor* C-139 growing on wheat bran.It is known that different from the shake flask studies, bioreactors are a better controlled environment where the effect of common factors such as pH, agitation and dissolved oxygen tension can be observed and controlled. They also provide information required in scale up processes (Oncu et al, 2007).

Once a fungus has been decided to be used in the fermentation process, research starts under laboratory-scale conditions using 1-10 L fermenters for the examination of media formulation and feeding strategies (batch, fed-batch, continuous, etc.) and the selection of fermentation system (stirred tank, airlift, packed bed, solid state, hollow fibre, etc.). Additionally, reactor configuration, pH control, dissolved oxygen, foam and temperature should be considered. After the optimization of product yield, process scale-up is usually performed; primarily to pilot scale of 10-100 L and finally to industrial scale of 1000- 100 000 L or more. The conditions in the large-scale fermenters are not the same with the smaller scale or laboratory systems. Therefore during scale up, product yields decreases(Waites et al, 2001). Yield is affected by the following factors during scale up process; Choice of medium, cheaper nutrient sources are mostly used for large scale applications for a cost effective production, Inoculum type, quality, quantity and the inoculum propagation procedures, Degradation of heat-labile compounds in the industrial-scale sterilization protocols, which affects the final quality of the medium, Profiles of nutrient, temperature, pH and oxygen gradients are needed in larger scale fermenters although they are not experienced in smaller, well mixed fermenters, Foam generation, shear forces and carbon dioxide removal rate can be altered during scale up (Waites et al, 2001).

In our preliminary studies we have optimized the fermentation conditions and developed a low cost industrial media formulation for the production of polygalacturonase enzyme by using *Aspergillus sojae* mutant strain in shake flasks (Gogus et al, 2014). This study was performed in order to evaluate the performance of optimized media formulation in larger scales (1 L serial bioreactor system and 5 L bioreactor) in terms of maximum PG production by using submerged fermentation technique. Moreover effect of orange peel concentration in 1 L serial bioreactor system on PG activity and biomass was investigated.

MATERIALS AND METHODS

Materials

All chemicals were supplied by Sigma-Aldrich (www.sigmaaldrich.com), AppliChem (www. applichem.com), Merck (www.merck.com), or Riedel-de Haën (www. riedeldehaen.com). Orange peel was supplied by Jacobs University gGmbH, Bremen from local markets in Germany.

Microorganism

Aspergillus sojae ATCC 20235 was purchased from LGC Promochem Inc. (Teddington,Great Britain) an international distributor of ATCC (American Type of Culture Collection) in Europe. This wild type culture was randomly mutated

using ultraviolet light exposure by Jacobs University gGmbH, Bremen according to a modified procedure of De Nicolás-Santiago et al, (2006).The propagation of the cultures was done on YME agar plate medium containing malt extract (10 g/L), yeast extract (4 g/L), glucose (4 g/L) and agar (20 g/L) at 30°C until well sporulated, according to the procedure given by Goguset al, (2006).

Preparation of Inoculum

The inoculum for either shake flasks or bioreactor was obtained on molasses agar slants optimized by Goguset al, (2006) after the pre-activation step performed on YME agar using the stock cultures. The spore suspension was counted using Thoma bright line hemocytometer (Marienfield, Germany) and the suspensions were stored at 4°C until the inoculation. Inoculation rate was $2.8x10^3$ spore/mL.

Enzyme Activity, Total Protein and Total Carbohydrate Assays

Polygalacturonase (PG) activity analysis were performed according to the modified procedure given by Panda et al, (1999) using 2.4 g/L of polygalacturonic acid as substrate at pH 4.8 and 40°C. The amount of substrate and enzymes used were 0.4 and 0.086 mL, respectively. Galacturonic acid was used as standard. One unit of enzyme activity was defined as the amount of enzyme that catalyses the release of 1 micromole of galacturonic acid per unit volume of culture filtrate per unit time at standard assay conditions. The phenol-sulfuric acid method was used to determine the total carbohydrate content in fermentation broth where glucose was used as standard (DuBois et al, 1956).Thetotalprotein contents of the samples were determined according to the method described by Bradford, 1976 with BSA as a standard.

Biomass Determination

The biomass expressed as dry cell weight (mg/mL) was determined by means of gravimetric method. The fermentation broth was filtered through the dried and pre-weight Sartorius whatman filter discs grade: 389, followed by drying to constant weight at 95°C, overnight.

Effect of Substrate Concentration on PG Activity and Growth

Orange peel concentrations were decided according to our media optimization study in which optimum OP concentration was 33.98 g/L for maximum PG activity (Gogus et al, 2014). With this perspective six different orange peel concentrations were specified as followings; 60 g/L, 40 g/L, 20 g/L, 15 g/L, 10 g/L, 5 g/L. In order to have a cost effective media formulation additionally only 2.75 g/L ammonium sulphate was used as a nitrogen source. The other fermentation conditions were the optimized conditions in our previous study, as uncontrolled pH, 600 rpm agitation speed, 30^oC temperature, $2.8x10^6$ spore/L inoculation rate and 1 vvm aeration rate. Sartorius BIOSTAT Qplus-6 MO serial bioreactor was used for this experiment.

Effect of Scale up to 5L on Polygalacturonase Activity

The aim was to investigate the effect of scale up to 5L scale with a working volume of 4 L on the PG activity using the OP concentration which gave the maximum PG activity in 1 L scale serial bioreactor experiment which is explained in the previous section. Orange peel concentration at 40 g/Land ammonium sulphate 2.75 g/L was used. Experiments were performed with fully automated Biostat B plus, Sartorius, Gottingen, Germany bioreactor. The other fermentation conditions were as 600 rpm agitation speed, uncontrolled pH, 30^oC temperature, $2.8x10^6$ spore/L inoculation rate and 1 vvm aeration rate.

RESULTS AND DISCUSSION

Effect of Substrate Concentration on PG Activity and Growth

Performance of the bio-reaction system in submerged fermentation 1 L scale (750 mL working volume) on PG production and biomass was evaluated under batch mode. The aim of this study was to investigate the effect of different substrate concentrations on PG activity and biomass formation. Biomass was increased in first 24 h. for all substrate concentrations except for 5

g/L. It may be explained by the limited substrate concentration which is used for PG production instead of biomass production (Figure 1b). Additionally the use of particulated substrate such as OP may have caused some difficulties in biomass determination by dry weight method. However it is clear from the figure 1b that higher concentrations of substrate resulted higher biomass concentrations. Highest PG activity (88.55U/mL) was obtained at 95.h. with 40 g/L orange peel concentration (Figure 1a). It was clear from Figure 1a that PG activity increased with an increase in substrate concentration except for 60 g/L orange peel concentration. This case could be explained by catabolite repression. Maldonado and Saad, (1998) indicated that high sugar concentration stimulated pectinase production in solid state fermentation, whereas in submerged fermentation this production was inhibited, probably by catabolite repression. As the orange peel concentration increased, DO level decrease became faster after 24.h. as it can be observed from Figure 1c. At the 60, 40, 20 and 15 g/L orange peel concentrations the DO level dropped below 40% saturation level at the end of 48.h. (Figure 1c). At the beginning of fermentation pH started around 4.50 and decreased to 2.93 at the end of 95.h. (Figure 1d).

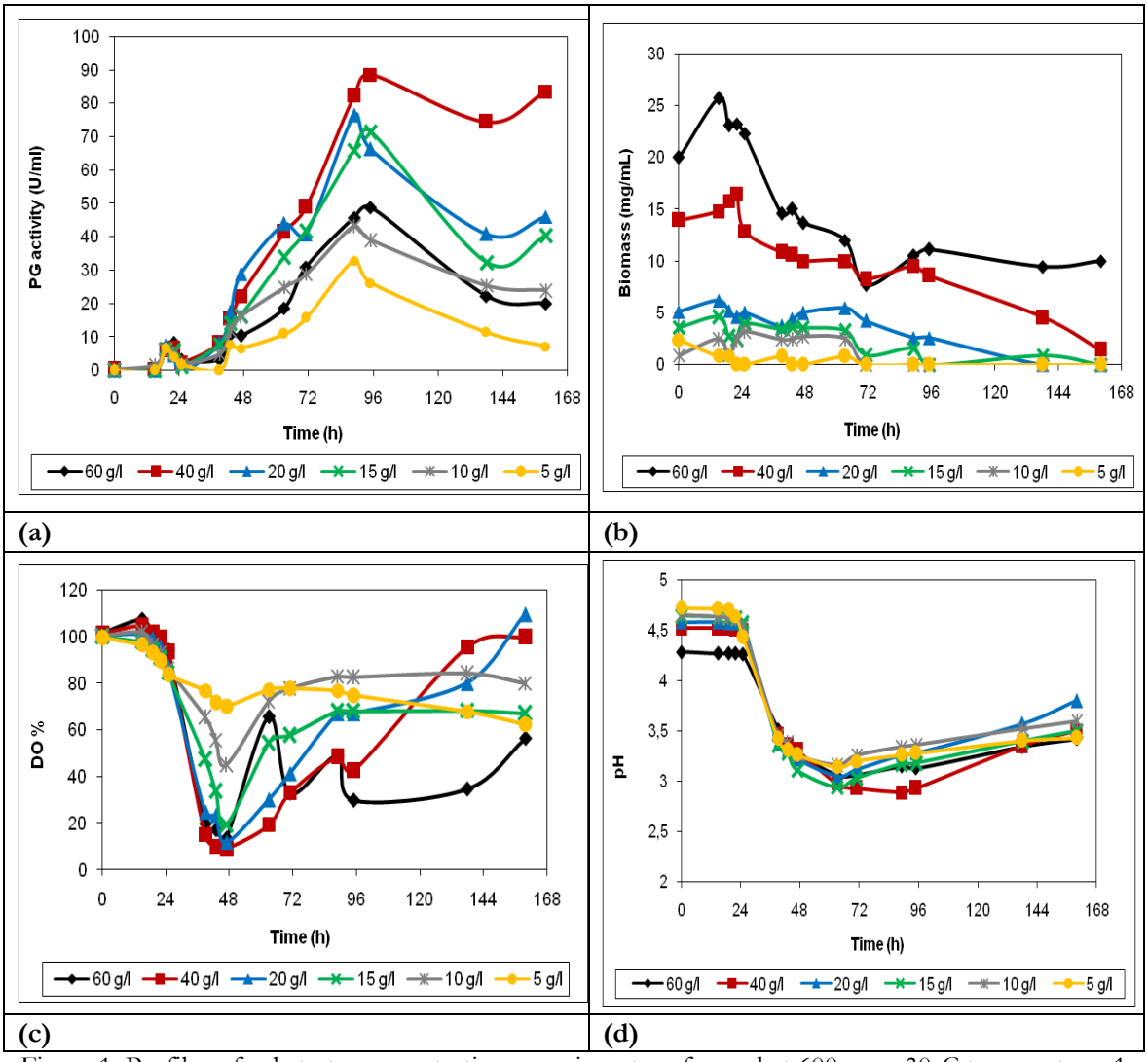

Figure 1. Profiles of substrate concentration experiment performed at 600 rpm, 30°C temperature, 1 vvm and uncontrolled pH conditions (a) PG activity, (b) biomass, (c) DO (%) and (d) pH.

Effect of Scale Up to 5 L on PG Activity

For a fermentation process development scale-up is an important issue to be considered. In order to expand a process from lab-scale to commercial scale, it must be characterized and validated by some controls to evaluate its reliability and reproducibility. Generally it is known that large-scale fermentation processes give lower yield than laboratory scale due to the factors affecting the process yield.

As shown in Figure 2a, PG activity showed rapid increase after 24.h. and reached its maximum value at the end of 48.h. (101.38 U/mL).Furthermore biomass concentration reached to 13.42 mg/mL value at the end of 72.h. (Figure 2b). However biomass concentration dropped to 11.8 mg/mL level at 90.h and increased again to 13.7 mg/mL at the end of fermentation (96.h) which is the maximum biomass amount (Figure 2b). Same profile was also valid for PG activity profile where a decrease was observed after 48.h to 90.h and then activity increased to the end of fermentation (96.h). This will be due to the growth related PG activity production of the batch fermentation system. The maximum activity value was higher than the activity values obtained in the previous 1 L scale bioreactor experiments.

Specific PG activity similar to PG activity reached its maximum value at 48.h. with a value of 1103.09 U/mg protein as seen in Figure 2c. When the change in protein concentration over time was examined, it can be said that protein concentration profile was mostly constant between the 24.h and 64.h of the fermentation where rapid increase was observed in the PG activity between these hours. In addition, the stable protein concentration between 24-64.h, might be explained by the rapid increase in the enzyme production at this range which led dissolved oxygen level (DO) and pH fall quickly (Figure 2a,c). This decrease also stabilizes the biomass concentration between these hours as seen in Figure 2b.

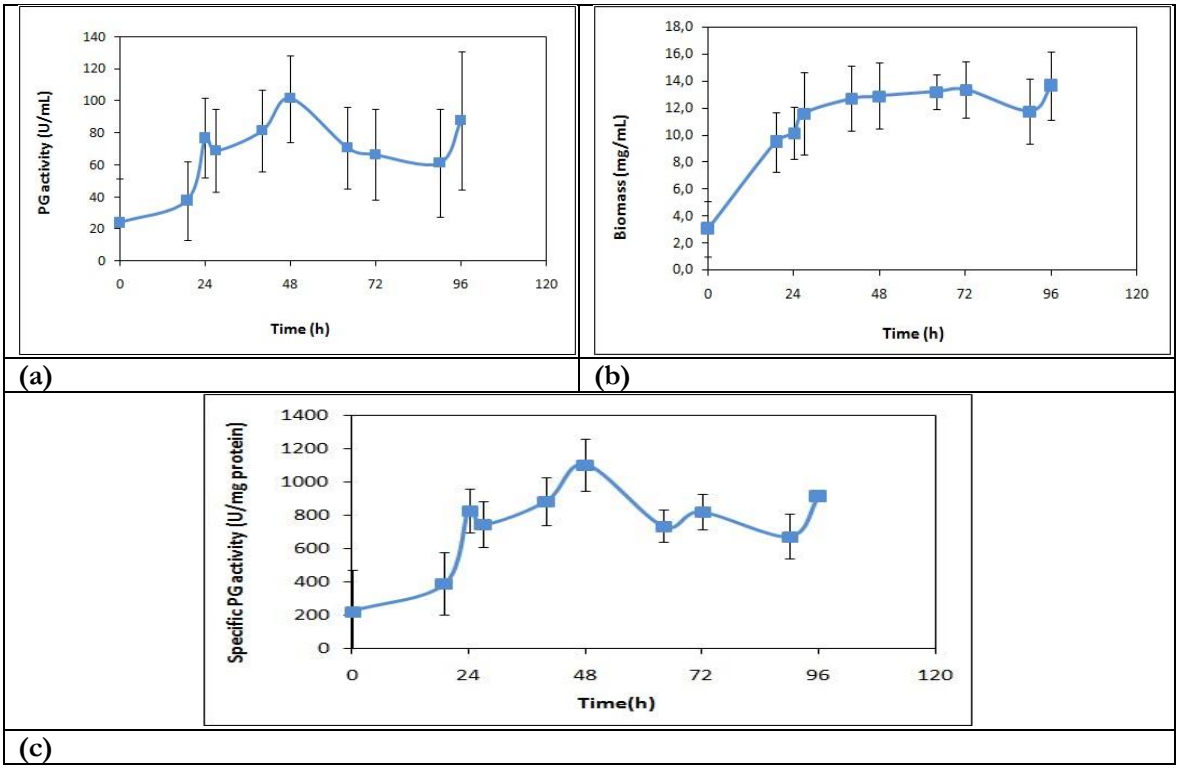

Figure 2. Profiles of first 5L scale bioreactor experiment performed at 600 rpm, 30°C temperature, 1 vvm and uncontrolled pH conditions (a) PG activity (U/mL), (b) Biomass (mg/mL), (c) Specific PG activity (U/mL).

In the study performed by Pollard et al, (2006) the scale up, from pilot scale to (0.07, 0.8, and 19 m^3) production scale (57 m^3) for the intracellular production of the pharmaceutically important secondary metabolite, pneumocandin from *Glarea lozoyensis* was described. They claimed that a scale up process requires the establishment of oxygen delivery and mixing efficiency in the bioreactor, coupled with process sensitivity and characterization studies that together define the process-limiting and critical scale-up parameters.

Junker et al, (2004) summarized the parameters to be considered for a successful initial pilot plant scale-up as followings, based on experiences during model cultivations: Minimization of culture pelleting; The use of media suitable for the large scale, (without solid particles, possessing a reasonable viscosity); Optimizing process parameters for pilot scale from laboratory scale; Establishment of reproducibility for similar fermentation vessels; Evaluation of data to determine the key parameters for improved process performance.

Junker et al, (2009) have developed pilot-scale fermentation for an antifungal compound produced by a filamentous fungus.The process was scaled up to the15.000 L working volume based on constant aeration rate (vvm) and peak impeller tip speed. In that study they concluded that although process scale up resulted with high productivity, high broth viscosity was a problem during fermentation which was also an important problem in our study.

Table 1 shows the effect of scaleon maximum PG activity, and biomass values with 40 g/L OP concentration. It is clear that scale up improved PG activity. As a result, maximum PG activity (101.29 U/mL) obtained at 48.h of 5L scale bioreactor experiment was higher than the maximum PG activity (88.55 U/mL) obtained at 96.h conducted in the previous section at 1 L scale. Higher scale caused PG activity to increase faster with a reasonably high activity value.

Meneghel et al,(2014)explained that unlimited oxygen availability in *A. niger* cultivation directed nutrient consumption to activities rather than biomass synthesis. According to the authors, fungi first adapt to the environment, and then startproteolysis and the oxidative stress produced in the cell leads to the release of hydrolyticenzymes to obtain substrate. The substrate consumption leads to either biomass or product formation. If consumption is fast, culturing time decreases at the without oxygen restriction condition. In the current study scale up improved PG activity production while limiting the biomass synthesis which might be explained with a better oxygen transfer in larger scales.

Table 1. Comparison of PG activity and biomass.

Scale	PG activity (U/mL)	Biomass (mg/mL)
*250 mL (shake flask)	110.93	-
1 L scale	88.55	25.71
5 L scale	101.29	13.42

*From shake flask experiments of our optimization study at 33.98 g/L (Gogus et al., 2014).

Similarly Fontana et al, 2009 tested *Aspergillus oryzae* polygalacturonase with a soluble medium in STR and concluded that after 96 h, maximum enzymatic activity values achieved for exo- and endo-PG were 65.2 U/mL and 91.3 U/mL, in the STR, with similar activity values of 60.6 U/mL and 86.2 U/mL, respectively, being in the airlift bioreactor. Our study achieved similar activities again after 96. h. Pectinase production by *Aspergillus oryzae* performed in bioreactor resulted in 43 U/mL activity according to Meneghel et al, 2014.Study performed by Wolf-Marquez et al, 2017 revealed that the scaling-up of the process at a 5 L-bioreactor improved endo- and exopectinase production 10 and 40 times, respectively, when compared to the values recorded at the 1 L-stirred tank. Similarly current study achieved improved polygalacturonase production with scaling-up of the process from 1 L to 5 L scale. However maximum activity (110.93 U/mL) was produced in shake flask experiment as given in Table 1which may be explained with high hydro mechanical stress generated in stirred

tanks. In a study performed by Rocha-Valadez et al, (2006) it was reported that shake flasks generate lower levels of hydro mechanical stress than stirred tanks where that higher shear rates triggered an earlier synthesis of 6-pentyl-a-pyrone in the stirred tank cultures.

CONCLUSION

Consequently, scale up to 5 L improved PG production rather than biomass which is explained with the unlimited oxygen transfer in larger scales that leads enzyme production. However, higher pectinase activity obtained in batch fermentation than bioreactor experiments may be explained with high hydro mechanical stress generated in stirred tanks which limits PG production. Additionally in 1 L scale due to the oxygen limitation PG activity started to increase after 48.h where in 5 L scale PG activity started to increase after 24.h which was earlier than 1 L scale. Furthermore in 1 L scale maximum PG activity was achieved at 40g/L OP concentration, similar with the 5 L scale OP concentration.

In general conclusion, the present study underlines the need to develop a correct and consistent scaling-up process for a high production of polygalacturonase using cheap substrates in order to obtain enough data for the studies about the contribution of the enzyme in different industrial food applications.

ACKNOWLEDGEMENTS

The authors are grateful to Izmir Institute of Technology, Biotechnology and Bioengineering Research and Application Center for the Sartorius BIOSTAT Qplus-6 MO serial bioreactor system. The authors also acknowledge Prof. Dr. Marcello Fernandez LAHORE from Jacobs University gGmbH, Bremen, Germany for sharing their mutant fungal strains.

REFERENCES

Jayani, R.S., Saxena, S., Gupta R. (2005). Microbial pectinolytic enzymes: A review. *Process Biochem*,40(9):2931-2944, doi: 10.1016/j.procbio. 2005.03.026.

Gummadi, S.N., Manoj, N., Kumar, D.S. (2007). Structural and Biochemical Properties of Pectinases. *In Industrial Enzymes*, Polaina, J., MacCabe, A.P., Eds.; Springer: Heidelberg, pp. 99-115.

Malvessi, M.,Ve Da Silveira, M.M. (2004). Influence of medium composition and pH on the production of polygalacturonases by *Aspergillus oryzae*. *Braz Arch BiolTechnol*, 47(5):693-702.

Nakkeeran, E., Gowthaman, M.K., Umesh-Kumar, S., Subramanian, R. (2012). Techno-economic Analysis of Processes for *Aspergillus carbonarius* Polygalacturonase Production. *J Biosci Bioeng,* 113(5):634–640.

Biz,A., Finkler,A.T.J., Pitol, L.O., Medina,B.S., Nadia K., Mitchell,D.A.(2016). Production of pectinases by solid-state fermentation of a mixture of citrus waste and sugarcane bagasse in a pilot-scale packed-bed bioreactor. *Biochem Eng J*, 111:54–62.

Pandey, A., Soccol, C.R., Mitchell, D.(2000). New developments in solid state fermentation: I-bioprocesses and products. *Process Biochem*, 35(10):1153-1169, http://dx.doi.org/10.1016 /S0032-9592(00)00152-7.

Songulashvili,G., Spindler,D., Jimene´z-Tobo´n,G.A., Jaspers,C., Kerns,G., Penninckx,M.J. (2015).Production of a high level of laccase by submerged fermentation at 120-L scale of *Cerrena unicolor* C-139 grown on wheat bran. *C R Biologies,* 338:121–125, http://dx.doi.org /10.1016/j.crvi.2014.12.001.

Oncu, S., Tari, C., Unluturk, S. (2007). Effect of various process parameters on morphology, rheology, and polygalacturonase production by *Aspergillus sojae* in a batch bioreactor. *Biotechnol Prog*, 23:836-845.

Waites, M.J., Morgan, N.L., Rockey J.S., Gary H. (2001). Industrial Microbiology: An Introduction, Blackwell Science Ltd, pp. 107-108.

Göğüş,N., Hakgüder Taze,B., Demir,H., Tarı,C., Ünlütürk,S., Lahore,M.F. (2014). Evaluation of orange peel, an industrial waste, for the production of *Aspergillus sojae* polygalacturonase considering both morphology and rheology effects. *Turk J Biol*, 38:537-548, doi:10.3906/biy-1308-47

Panda, T., Naidu, G.S.N., Sinha, J. (1999). Multiresponse analysis of microbiological parameters affecting the production of pectolytic enzymes by *Aspergillus niger*: A statistical view. *Process Biochem*, 35, 187-195.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F. (1956). Colorimetric Method for Determination of Sugars and Related Substances. *Anal Chem*, 28(3):350-356.

Bradford, M. (1976). A Rapid and Sensitive Method for the Quantitation of microgram Quantities of Protein Utilizing the Principle of Protein-dye Binding. *Anal Biochem,*72:248-254.

Maldonado, M.C., Strasser de Saad A.M.(1998). Production of pectinesterase and polygalacturonase by *Aspergillus niger* in submerged and solid state systems. *J Ind Microbiol Biotechnol*, 20(1):34–38,doi: 10.1038/sj.jim. 2900470.

Pollard, D.J., Kirschner, T.F., Hunt, G.R., Tong, I-T., Stieber, R., Salmon, P.M. (2006).Scale Up of a Viscous Fungal Fermentation: Application of Scale-Up Criteria with Regime Analysis and Operating Boundary Conditions. *Biotechnol Bioeng*, 96(2):307-317,doi:10.1002/bit.21112.

Rocha-Valadez, J.A., Estrada, M., Galindo, E., Serrano-Carreon, L. (2006). From shake flasks to stirred fermentors: Scale-up of an Extractive Fermentation Process for 6-pentyl-a-pyrone Production by *Trichoderma harzianum* using Volumetric Power Input. *Process Biochem,* 41(6):1347–1352,doi:10.1016/j.procbio. 2006.01.013.

Junker, B.H., Hesse, M., Burgess, B., Masurekar, P., Connors, N., Seeley, A. (2004). Early Phase Process Scale-Up Challenges for Fungal and Filamentous Bacterial Cultures. *Appl Biochem Biotechnol*, 119(3):241-277,doi: 10.1007/s12010-004-0005-x.

Junker, B., Walker,A., Hesse,M., Lester,M., Vesey,D., Christensen,J., Burgess,B., Connors,N. (2009). Pilot-scale process development and scale up for antifungal production. *Bioprocess Biosyst Eng*, 32(4):443–458,doi: 10.1007/s00449-008-0264-y.

Fontana, R.C., Polidoro, T.A., da Silveira, M.M.(2009). Comparison of stirred tank and airlift bioreactors in the production of polygalacturonases by *Aspergillus oryzae*. *Bioresour Technol*, 100(19):4493–4498, http://dx.doi.org/ 10.1016/j.biortech.2008.11.062.

Meneghel, L., Reis, Guilherme, P., Reginatto, C., Malvessi, E., da Silveira, M.M. (2014). Assessment of pectinase production by *Aspergillus oryzae* In growth-limiting liquid medium under limited and non-limited oxygen supply. *Process Biochem*, 49:1800–1807, http://dx.doi.org/10.1016/ j.procbio.2014.07.021.