Vol. 16, No. 2 (2017) 399-404
Revista Mexicana de Ingeniería Química

ENDOPOLYGALACTURONASE FROM *Kluyveromyces marxianus* CDBB-L-278: A COMPARATIVE STUDY OF GENE EXPRESSION AND ENZYME ACTIVITY UNDER AEROBIC AND ANAEROBIC CONDITIONS

ENDOPOLIGALACTURONASA DE *Kluyveromyces marxianus* CDBB-L-278: UN ESTUDIO COMPARATATIVO DE LA EXPRESIÓN DEL GEN Y DE LA ACTIVIDAD ENZIMÁTICA BAJO CONDICIONES AEROBIAS Y ANAEROBIAS

A. Rivera-Noriega[1], A.E. Cruz-Guerrero[1], A.I. Morales-Estrada[1], L. Gómez-Ruiz[1], M. López-Perez[2], M. García-Garibay[1,3]*

[1]*Departamento de Biotecnología, Universidad Autónoma Metropolitana, Iztapalapa, Av. San Rafael Atlixco 186 Col. Vicentina, Ciudad de México, 09340 México.*

[2]*Departmento de Ciencias Ambientales, Universidad Autónoma Metropolitana, Lerma, Av. Hidalgo Poniente 46, Col. La Estación, Lerma Edo. de México, 52006.*

[3]*Departmento de Ciencias de la Alimentación, Universidad Autónoma Metropolitana, Lerma, Av. Hidalgo Poniente 46, Col. La Estación, Lerma Edo. de México, 52006 México.*

Received January 21, 2017; Accepted March 16, 2017

Abstract

The yeast strain *Kluyveromyces marxianus* CDBB-L-278 is an outstanding producer of endopolygalacturonase (EC. 3.2.1.15); it has been reported that its production is repressed under high dissolved oxygen tension. The expression of the endopolygalacturonase gene (*EPG*) from this yeast was compared under both aerobic and anaerobic conditions and was related to the enzyme activity. Specific enzyme production under anaerobic conditions (0.233 uPG mg^{-1}) was five times higher than that obtained under aerobic conditions (0.048 uPG mg^{-1}), demonstrating that anaerobic growth promotes the synthesis of endo-PG; relative quantification of *EPG* gene expression was 21.19 times higher in anaerobic culture, suggesting that production of endo-PG under anaerobic culture conditions is a consequence of the regulation of the *EPG* gene by dissolved oxygen saturation in the growth medium. Results point out that the higher production of endo-PG under anaerobic culture conditions is a consequence of the regulation of *EPG* gene.

Keywords: dissolved oxygen regulation, endopolygalacturonase, *EPG* gene, *Kluyveromyces marxianus*, pectinase.

Resumen

La cepa de levadura *Kluyveromyces marxianus* CDBB-L-278 es una extraordinaria productora de endopoligalacturonasa (EC. 3.2.1.15); se ha reportado que esta producción se reprime bajo una alta tensión de oxígeno. La expresión del gen de la endopoligalacturonasa (*EPG*) de esta levadura se comparó bajo condiciones aerobias y anaerobias y se relacionó con la actividad enzimática. La producción específica bajo condiciones anaerobias (0.233 uPG mg^{-1}) fue cinco veces mayor que la actividad específica obtenida bajo condiciones aerobias (0.048 uPG mg^{-1}), demostrando que el crecimiento anaerobio promueve la síntesis de la endo-PG; la cuantificación relativa de la expresión del gen *EPG* fue 21.19 veces mayor en el cultivo anaerobio, sugiriendo que la producción de la endopoligalacturonasa bajo condiciones anaerobias es consecuencia de la regulación del gen *EPG* por las condiciones de saturación de oxígeno disuelto en el medio de cultivo. Los resultados demuestran que el aumento de la producción de endo-PG bajo condiciones anaerobias es consecuencia de la regulación del gen *EPG*.

Palabras clave: dissolved oxygen regulation, endopolygalacturonase, *EPG* gene, *Kluyveromyces marxianus*, pectinase.

* *Corresponding author. E-mail*: jmgg@xanum.uam.mx
Tel. +(52)(55)5804-4720

1 Introduction

Kluyveromyces marxianus is a food grade yeast that has been used commercially for the production of single cell protein, alcohol, lactase and other goods, and its potential in food biotechnology is even wider (Fonseca *et al*., 2008; Pérez *et al*., 2013; Martínez-Corona *et al*., 2015). For instance, it has a great potential in the production of enzymes (Espinoza *et al*., 1992), such as endo-polygalacturonase (endo-PG, EC. 3.2.1.15), an efficient pectin-degrading enzyme that cleaves the internal α-1,4-D glycosidic linkages displaying macerative properties in vegetable tissues (Espinoza *et al*., 1992; Alimardani *et al*., 2011); this pectinase has been studied since long ago (Luh and Phaf, 1951; Luh and Phaf, 1954) and its use in the process of clarification of apple juice has been successfully demonstrated (Gómez-Ruiz *et al*., 1988). Particularly, the strain *K. marxianus* CDDB-L-278 has demonstrated to be an exceptional producer of endo-PG showing notably higher activity of pectinase than any other *K. marxianus* strain tested by our group (Espinoza *et al*., 1992). Production studies of endo-PG by *K. marxianus* have demonstrated that synthesis is repressed by dissolved oxygen (DO) in the growth medium (Wimborne and Rickard, 1978; García-Garibay *et al*., 1987). A later study by Cruz-Guerrero *et al*., (1999) found that 3.3 mgL^{-1} of DO was the threshold for the repression of enzyme production in the strain *K. marxianus* CDBB-L-278.

Beyond these physiological studies, there are no reports regarding the expression of *EPG* gene related to DO level in the medium; the current paper deals with the effect of DO in the expression of the *EPG* gene of *K. marxianus* CDBB-L-278 and the resulting activity of endo-PG. The study of the influence of DO on expression of *EPG* gene is important in order to allow an improved control on the production of endo-PG by the yeast, and will contribute to understand the interesting mechanism of gene control expression by oxygen, which, as far as we know, has not been studied.

2 Materials and methods

2.1 Strain

K. marxianus CDBB-L-278 was obtained from de Culture Collection of the Centre for Research and Advanced Studies (CINVESTAV) at the National Polytechnic Institute, Mexico City. This strain was maintained on potato-dextrose agar slants (Bioxon, Mexico).

2.2 Culture medium

A synthetic medium was used containing (g 100 ml^{-1}): glucose 2, $(NH_4)_2SO_4$ 0.2, KH_2PO_4 0.05, $MgSO_4$ 0.05 and yeast extract 0.2; glucose and salts were from J.T. Baker (Mexico), and yeast extract was from Bioxon (Mexico). Medium pH was adjusted to 5 and autoclaved for 15 min at 121°C.

2.3 Culture conditions

Inoculum was prepared in Erlenmeyer flasks containing 50 ml of culture medium in a New Brunswich G-24 rotatory shaker (200 rpm, 30°C) and growth 16-18 h. Fermentations were conducted in a New Brunswick BioFlo IIc fermenter with 1 l of medium at 30°C during 9 h, using 50 ml of inoculum with an optical density of 1.8 (measured at 650 nm). Aerobic fermentations were performed with an air flow of 2 vvm; the dissolved oxygen saturation was automatically controlled at 60% by adjustments in the stirring rate. Anaerobic fermentations were performed without air flow and a stirring rate of 50 rpm. Samples of 15 ml were taken at 0, 3, 6 and 9 h; biomass was determined by turbidity at 650 nm and reported as dry weight basis biomass using a standard curve as reported by Garcia-Garibay *et al*., (1987); samples were centrifuged at 3020 g for 5 min, and enzymatic activity was measured in the supernatant; cells were diluted in 1 ml of sterile DEPC-water (diethyl pyrocarbonate, Sigma) and maintained at -70°C for RNA extraction.

2.4 Adjustment of yeast growth

Experimental data of yeast growth were adjusted by Ec. (1) according to the logistic model of Velhurst-Pearl using the Solver tool of Excel (Microsoft Office 2007).

$$X(t) = \frac{X_{max}}{1 + \frac{X_{max} - X_o}{X_o} \exp^{-\mu t}} \quad (1)$$

Where:
X_o = initial biomass concentration g l^{-1}
X_{max} = maximal biomass concentration g l^{-1}
μ = specific growth rate h^{-1}
t = time h

2.5 *Endo-PG activity*

It was measured by the increase in reducing groups release from a polygalacturonic acid solution (Sigma) (García-Garibay *et al*., 1987). One unit of endo-PG was defined as the amount of enzyme which releases 1 μmol of reducing groups per minute (uPG ml^{-1}). Specific activity was defined as the enzymatic activity per mg of yeast biomass (uPG mg^{-1}).

2.6 *RNA isolation and reverse transcription*

RNA was isolated from 9 h aerobic and anaerobic culture samples using Purelink RNA minikit (Ambion), following manufacturer´s instructions. Cell disruption was done using 400-600 μm acid washed glass beads (Sigma). RNA quality was evaluated through 1% agarose gel electrophoresis performed in a Thermo EC Minicell chamber (USA) using constant voltage of 60 V, 90 min; the running buffer was TAE 1X prepared with DEPEC-water; gels were stained with GelRed (Biotium, USA) and the bands were visualized by means of an image analyzer (MiniLumi DNR, Bio-Imaging Systems Ltd, Jerusalem, Israel). RNA purity was also confirmed measuring the absorbance ratio at 260/280, with NanoDrop 2000/2000c (Thermo Scientific); absorbance at 260 nm was used to determine RNA concentration.

RNA samples were treated with RQ1 RNase-Free DNase (Promega) to remove any residual DNA contamination; total mixture reaction (20 μl) contained 100 ng of total RNA, DNase buffer and DEPC-water; it was incubated at 30°C, 1 h; then buffer DNAsa stop was added and was incubated at 65°C, 10 min. Conventional PCR was performed to check the absence of genomic DNA (gDNA). When necessary, the DNAse treatment was repeated. RNA was converted into cDNA (complementary DNA) using Quantitec Reverse Transcription Kit (Qiagen Hilden, Germany) incubating at 42°C, 30 min and then at 95°C for 3 min.

2.7 *Optimization of reaction conditions for quantitative real time PCR (qPCR)*

The oligonucleotide primers were designed by Integrated DNA technologies (IDT https://www .idtdna.com/). The following sequences were considered for primer design: GenBank accession numbers AY426825, the *K. marxianus EPG*1-2 gene; and as normalizing genes: AB054675, the *K. marxianus* 18S gene, and AJ389078, the *K. marxianus* actin gene (ACT1).

Primer specificity was checked by end point PCR with 10-25 ng of gDNA as template amplifying a 163 bp fragment, sequence fw primer (cac ttc ttg tcc aac act atc t) and rw primer (ctg taa gcg gaa gag tca ac) for the *EPG* target gene; a 123 bp fragment, sequence fw primer (aat tcc agc tcc agt agc gt) and rw primer (gaa gga aag atc cgg ttg aa) for the 18S normalized gene; and a 125 bp fragment, sequence fw primer (aag aaa tgc aaa ccg ctt ct) and rw primer (aac aga tgg tgg aac aaa gc) for the ACT1 gene. Reaction conditions were 94°C, 2 min for denaturation step; 30 cycles at 94°C, 1 min for melting; temperature gradient for annealing step 52-60°C, 1 min for each primer; 72°C, 5 min for extension step. After the annealing temperature gradient testing, only at 54°C a single amplification product with the expected size was detected; hence, this temperature was selected to perform RT-PCR.

In order to check the specificity of the TaqMan probes (Applied Biosystems) amplification reactions were performed by triplicate RT-PCR (Rotor gene 5, Corbett Life Science) using both the specific primers and the designed probes (*EPG* gene FAM-agg tag caa ggt ccc aat cac caa-TAMRA; 18S gene FAM-acc ggc caa cca gac cca aa-TAMRA; ACT1 gene FAM-cca atg gtg ata act tga cca tct ggc-TAMRA). Reaction volume was 25 μl with the following reagents: TaqMan PCR Universal master mix 1X, primers 0.15 μM, probe 0.05 μM and cDNA 4 μl. Reaction conditions were 50°C, 2 min; 95°C, 10 min and 40 cycles at 95°C, 15 s and 54°C, 1 min.

The efficiency of *EPG*, 18S and ACT1 genes was determined in aerobic growth conditions using four dilutions of cDNA of known concentration. The amplification efficiency, the correlation coefficient and the slope were calculated by the Rotor-gene 2000 software version 4.7 (Corbett Life Technology).

2.8 *Relative quantification of EPG gene*

Quantification was done from the cDNA; samples were amplified by triplicate, and a negative control of reactives (without cDNA) was done. Fluorescence threshold for all the samples was 0.05 RFU. The threshold reaction cycle of the product was defined as Ct. Relative expression rate of the target gene was calculated according to Hellemans *et al*., (2007).

2.9 Statistical analysis

A t-Student test was performed (P <0.05) to compare the total and specific enzymatic activity between the aerobic and anaerobic fermentations using Excel (Microsoft Office 2007).

3 Results and discussion

3.1 Growth and endo-PG activity under aerobic and anaerobic conditions

Fig. 1 shows that maximal growth of *K. marxianus* CDBB-L-278 under aerobic conditions (60% DO saturation) was 2.56 mg ml^{-1}, while under anaerobic conditions was 0.71 mg ml^{-1}. The microbial growth adjusted to the logistic model lead to a correlation of 0.97 for aerobic conditions and 0.99 for anaerobic conditions. Additionally, specific growth rate (μ) was 0.85 h^{-1} and 0.53 h^{-1} for aerobic and anaerobic conditions respectively, suggesting that aerobic conditions led to a more favorable production and rate of biomass. In spite that Fig. 2A does not show difference for total enzymatic activity between aerobic and anaerobic cultures, considering the remarkable difference in growth, the specific enzymatic activity was five times higher under anaerobic conditions (0.233 vs. 0.048 uPG mg $biomass^{-1}$) (Fig. 2B). These results demonstrated that under anaerobic growth the synthesis of endo-PG was promoted as reported previously (Wimborne and Rickard 1978; García-Garibay *et al*., 1987; Cruz-Guerrero *et al*., 1999).

3.2 Quantitative real time PCR (qPCR)

Amplification efficiency for *EPG* gene was 0.98 (r=0.9948) and a slope of -3.34; amplification efficiency data and the slope for 18S and ACT1 genes were 0.99 (r=0.997) and -3.34; and 1.01 (r=0.999) and -3.29 respectively, suggesting a good amplification efficiency (Sellars *et al*., 2007) in all cases. The 18S gene and the ACT1 gene were chosen as normalized genes because they did not show any differences in expression under the studied growth conditions.

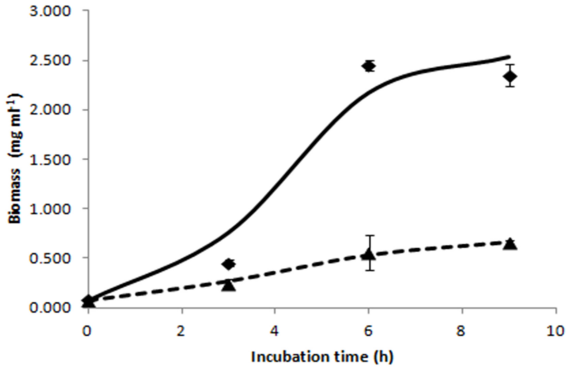

Fig. 1. Cell growth of *Kluyveromyces marxianus* CDBBL-278 in anaerobic (filled triangle) and aerobic (filled diamond) conditions. Dotted line represents anaerobic growth while solid line represents aerobic growth data adjusted to the logistic model.

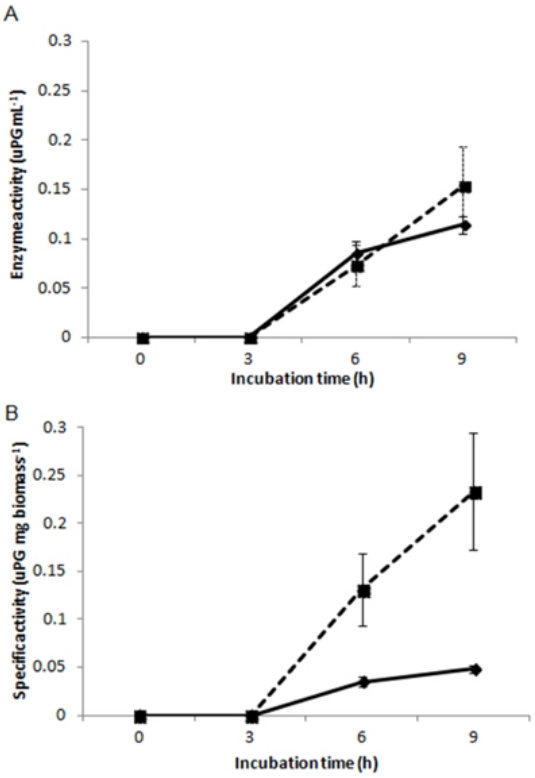

Fig. 2. Enzymatic activity (a) and specific enzymatic activity (b) from *K. marxianus* CDBB-L-278 in aerobic (filled diamond, solid line) and anaerobic growth (filled square, dotted line).

3.3 Quantification of EPG gene expression under aerobic and anaerobic growth conditions

The Ct value obtained at 9 h for anaerobic growth was 27.94±0.87, while that for the aerobic growth at the same time was 32.69±1.34; as the Ct value is inversely proportional to the initial value of cDNA, this means a higher expression of the *EPG* gene under anaerobic conditions. These results confirm that diminishing DO saturation in the growth medium leads to an induction of the gene expression, resulting in an increase of endo-PG activity. Relative quantification of *EPG* gene expression was calculated by the equation reported by Hellemans *et al*. (2007); results showed an increase of 21.19 times of *EPG* gene expression under anaerobic conditions with respect to the aerobic ones. Due to the increment in *EPG* gene expression of *K. marxianus* observed under anaerobic conditions, it could be supposed that this regulation takes place at transcriptional level; namely, it could be regulated by some factors related to the adaptation of the yeast to anaerobic growth conditions or stress factors, such as the case of the PGU1 gene in *Saccharomyces cerevisiae* which encodes an endo-PG, whose expression has been observed to be regulated by transcriptional factors that also regulate genes responsible for flocculation and pseudomycelium formation under starvation stress conditions (Louw *et al*., 2010).

It was also observed that the specific activity of endo-PG increased five times at 9 h under anaerobic conditions compared with aerobic conditions, which is related to the expression of the gene. The fact that gene expression level is higher than the increment in enzymatic activity under anaerobic conditions, could be due to translational aspects denoting that not all the mRNA is translated to protein (Shah *et al*., 2013).

Wimborne and Rickard (1978) established that the production of endo-PG by *K. marxianus* is partially constitutive, since it is repressed by aerobic conditions, while Schwan *et al*. (1996) pointed out that it is constitutive and not subject to carbone catabolite repression. The expression of the *EPG* gene is rather a complex question, since it is repressed by oxygen, but has been demonstrated to be induced by pectin under aerobic conditions (García-Garibay *et al*., 1987); therefore, it worth to continue studying the several aspects involved in the repression-activation of this gene, which seems to be unique and multifactorial.

Conclusions

Anaerobic growth conditions resulted in the promotion of endo-PG activity as well as of the expression of *EPG* gene; these results point out that the higher production of endo-PG under anaerobic culture conditions is a consequence of the regulation of *EPG* gene by DO saturation in the growth medium.

Acknowledgement

Authors Alicia Rivera-Noriega acknowledge a doctoral grant and Aurea I. Morales-Estrada a postdoctoral fellowship from the National Council of Science and Technology (CONACyT), Mexico.

References

Alimardani-Theuil, P., Gainvors-Claisse, A., and Duchiron, F. (2011). Yeasts: An attractive source of pectinases-From gene expression to potential applications: A review. *Process Biochemistry 46*, 1525-1537.

Cruz-Guerrero, A., Bárzana, E., García-Garibay, M., and Gómez-Ruiz, L. (1999). Dissolved oxygen threshold for the repression of endo-polygalacturonase production by *Kluyveromyces marxianus. Process Biochemistry 34*, 621-624.

Espinoza, P., Bárzana, E., García-Garibay, M., and Gomez-Ruiz, L. (1992). Evaluation of *Kluyveromyces marxianus* for the production of lactase simultaneously to pectinase or inulinase. *Biotechnology Letters 14*, 1053-1058.

Fonseca, G.G., Heinzle, E., Wittmann, C., and Gombert, A.K. (2008). The yeast *Kluyveromyces marxianus* and its biotechnological potential. *Applied Microbiology and Biotechnology 79*, 339-354.

García-Garibay, M., Gómez-Ruiz, L., and Bárzana, E. (1987). Studies on the simultaneous production of single-cell protein and endo-polygalacturonaase from *Kluyveromyces fragilis. Biotechnology Letters 9*, 411-416.

Gómez-Ruiz, L., García-Garibay, M., and Bárzana, E. (1988). Utilization of endo-polygalacturonase from *Kluyveromyces fragilis* in the clarification of apple juice. *Journal of Food Science 53*, 1236-1237, 1240.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., and Vandesompele, J. (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biology 8*, R19.

Louw, C., Young, P.R., Van Rensburg, P., and Divol, B. (2010). Regulation of endo-polygalacturonase activity in *Saccharomyces cerevisiae. FEMS Yeast Research 10*, 44-57.

Luh, B.S., and Phaf, H.J. (1951). Studies on polygalacturonase of certain yeast. *Archives of Biochemistry and Biophysics 33*, 212- 227.

Luh, B.S., and Phaf, H.J. (1954). Properties of yeast polygalacturonase. *Archives of Biochemistry and Biophysics 48*, 23-37.

Martínez-Corona, R., González-Hernández, J.C., Radames-Trejo, V., Cortés-Penagos, C., Chávez-Parga, M.C., and Zamudio-Jaramillo, M.A. (2015). Effect of initial substrate concentration and agitation on xylitol production by fermentation of hydrolyzed tamarind seed media with *Kluyveromyces marxianus*. *Revista Mexicana de Ingeniería Química 14*, 393-403.

Pérez, E., González-Hernández, J.C., Chávez-Parga, M.C., and Cortés-Penagos, C. (2013). Caracterización fermentativa de levaduras productoras de etanol a partir de jugo de *Agave cupreata* en la elaboración de mezcal. *Revista Mexicana de Ingeniería Química 12*, 451-461.

Schwan, R.F., Cooper, R.M., and Wheals, A.E. (1996). Endopolygalacturonase of the yeast *Kluyveromyces marxianus* is constitutive, highly active on native pectin and is the main extacellular protein. In: *Pectins and Pectinases, Proceedings of an International Symposium*, (J. Visser and A.G.J. Voragen, eds.), Pp. 861-868. Elsevier Science Publisher, New York.

Sellars, M.J., Vuocolo, T., Leeton, L.A., Coman, G.J., Degnan, B.M., and Preston, N.P. (2007). Real time RT-PCR quantification of kuruma shrimp transcripts: A comparison of relative and absolute quantification procedures. *Journal of Biotechnology 129*, 391-399.

Shah, P., Ding, Y., Niemczyk, M., Kudla, G., and Plotkin, J.B. (2013). Rate-limiting steps in yeast protein translation. *Cell 13*, 1589-1601.

Wimborne, M.P., and Rickard, P.A.D. (1978). Pectinolytic activity of *Saccharomyces fragilis* cultured in controlled environments. *Biotechnology and Bioengineering 20*, 231-242.