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HETEROLOGOUS EXPRESSION OF *Trichoderma atroviride* ENDOCHITINASE *ech42* IN *Pichia pastoris* AT LOW AND HIGH DISSOLVED OXYGEN TENSIONS

EXPRESIÓN HETERÓLOGA DE LA ENDOQUITINASA *ech42* DE *Trichoderma atroviride* EN *Pichia pastoris* A BAJAS Y ELEVADAS TENSIONES DE OXÍGENO DISUELTO

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Abstract

The expression of recombinant *Trichoderma atroviride* endochitinase *ech42* (r*ech42*) in *Pichia pastoris* driven by the constitutive P_{GAP} promoter was tested at low (3%) and high (40%) dissolved oxygen tension (DOT) conditions. Maximum volumetric enzyme activities of 18,600±1053 and 12,330±1000 U l^{-1} were attained at 3% and 40% DOT, respectively. Whereas maximum specific enzyme activities were 89.4±4.6 y 88.8±4.0 U $mg^{-1}_{protein}$. We found that conjunction of low DOT and the overexpression of r*ech42* affected the cell size of the *P. pastoris* but no the rech42 specific enzyme activity.

Keywords: chitin, cell size, recombinant endochitinase, oxygen-limited fermentation.

Resumen

La expresión de la Endoquitinasa de 42 kDa de *Trichoderma atroviride* recombinante (r*ech42*) en *Pichia pastoris* bajo la dirección del promotor constitutivo, P_{GAP}, fue analizada a baja (3%) y alta (40%) tensión de oxígeno disuelto (TOD). Las actividades enzimáticas volumétricas máximas logradas en cada condición fueron 18,600±1053 y 12,330±1000 U l^{-1}, a TOD de 3% y 40%, respectivamente. Mientras que las actividades específicas máximas fueron 89.4±4.6 y 88.8±4.0 U $mg^{-1}_{proteína}$. Se encontró que la conjunción de una baja TOD y la sobreexpresión de r*ech42* afectó el tamaño de la células de *Pichia pastoris* pero no afectó la actividad enzimática específica de rech42.

Palabras clave: quitina, tamaño celular, endoquitinasa recombinante, fermentación limitada por oxígeno.

1 Introduction

Methylotrophic yeast *Pichia pastoris* has been used as host for the production of a wide variety of recombinant proteins, due to its ability to grow at high cell densities in minimal medium and the capacity to use methanol as sole carbon source, as well as its well-understood genetics. The strong AOX1 promoter (P_{AOX1}) is currently used for the production of recombinant proteins in *P. pastoris* (Cereghino and Cregg, 2000; Canales, *et al*., 2008; Apte-Deshpande, *et al*., 2009; Weidner, *et al*., 2010; Uchima and Arioka, 2012). However, methanol is a flammable solvent, hazardous to handle at industrial scale, and its metabolism constitutes a highly oxygen demanding process which becomes a problem when scaling up the recombinant proteins production (Li, *et al*., 2007). The glyceraldehyde-3 phosphate dehydrogenase constitutive promoter (P_{GAP}) is an attractive alternative since expression of foreign gene occurs simultaneously with biomass generation using glucose as carbon source and no inducer is needed (Goodrick, *et al*., 2001).

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P. pastoris keeps an aerobic metabolism under microaerophilic conditions and it accumulates by-products such as acetate and ethanol below of the growth-inhibitory concentrations, this fact makes less challenging scaling-up the production processes (Sola, *et al*., 2004; Baumann, *et al*., 2008). Since, oxygen cross the cell wall by simple diffusion, it could be limiting for the cell-growth (Porro, *et al*., 2005). It has been reported that dissolved oxygen tension (DOT) above of 30% is needed for an efficient biotransformation of methanol to CO_2 and H_2O by *P. pastoris* (Wolff, *et al*., 2001; Ren and Yuan, 2005). Despite *P. pastoris* is an excellent host for the expression and secretion of heterologous proteins, to our knowledge there are few reports on the effect of DOT on the expression of proteins (Macauley-Patrick, *et al*., 2005).

We previously reported the expression and characterization of recombinant *Trichoderma atroviride* endochitinase *ech42* (rech42) in *P. pastoris* under the control of the P_{GAP} promoter (Perez-Martinez, *et al*., 2007). Chitin-degrading enzymes such as rech42 are used for the hydrolysis of chitin and the production of chito-oligosaccharides (Rao, *et al*., 2000; Ramirez-Coutino, *et al*., 2006). The aim of this work was determine the effect of low and high DOT on the expression of rech42 driven by the P_{GAP} in *P. pastoris*, and its effect on the cell size is also discussed.

2 Materials and methods

2.1 Strains and culture conditions

P. pastoris SMD1168H (pepA-) referred as wild-type strain was maintained in YPD (10 g l^{-1} yeast extract, 20 g l^{-1} dextrose and 20 g l^{-1} peptone, 10 g l^{-1} bactoagar) plates. The transforming strain was obtained by electroporation of *P. pastoris* with the plasmid P_{GAP-}*ech42* and after it was grown on YPD plus 100 μg ml^{-1} zeocin (Invitrogen, Grand Island, NY, USA). A complete description of P_{GAP-}*ech42* has been reported elsewhere (Perez-Martinez, *et al*., 2007).

2.2 Batch cultures

Batch cultures were done in YPD liquid medium (Invitrogen) and using a 1 l work volume stirred tank bioreactor (Applikon, Schiedam, The Netherlands) connected to a Bioconsole ADI 1030 (Applikon) and controlled by a Biocontroller ADI 1035 (Applikon). Bioreactors were inoculated with an initial biomass of optical density at 600 nm (OD600nm) 0.2. Preinocula with 50 ml of each strain were grown overnight on YPD at 28°C with shaking at 150 rpm. Process variables were set as follow: 28°C, pH 5.5, 40% or 3% DOT. DOT was controlled by mixture of O_2/N_2 gasses. Foam formation was controlled by addition of anti-foam 405 (Sigma) 1:100 in water. The pH was controlled by addition of 8.5 M NH_4OH or 4 M H_3PO_4 when was necessary by peristaltic pumping. Samples were taken during the time course of fermentation for following growth kinetics. Aliquots of 1 ml were withdrawn with a syringe. Samples were centrifuged 2 min at 13,000 rpm and 4°C. Culture medium was recovered and stored at -20°C and cells were resuspended on phosphate buffer (100 mM K_2HPO_4 pH 6.0) for analysis. Batch cultures were carried out in duplicates.

2.3 Analytical methods

Biomass concentration was measured by OD600nm using a spectrophotometer (Agilent Technologies, Wilmington, DE) and converted onto dry-cell weight by a standard curve. Specific growth rate (μ) for each growth curve was calculated as the slope value from the Ln of biomass vs. time plot. Ethanol was measured by Gas Chromatography, following the procedure and conditions reported elsewhere (De Leon-Rodriguez, *et al*., 2006), as follow: 0.5 ml culture medium aliquot from each time point were mixed with equal volume of 1-buthanol on microtube. Samples were vortexed for 1 min at maximum speed and centrifuged 1 min at 13,000 rpm. Organic phase was recovered on fresh tubes and injected on gas chromatograph 6890N (Agilent Technologies) coupled to autosampler 7863 (Agilent Technologies, Whilmington, DE). Ethanol and acetic acid were separated using capillary column HP-Innowax 30 cm x 0.25 mm i.d., 0.25 μm film thickness (Agilent Technologies) and helium as carrier gas at 1.5 ml min^{-1}. All analytical determinations were done by triplicate.

2.4 Electrophoresis and activity assay

Extracellular proteins in the culture medium samples were analyzed by SDS-PAGE. Samples of 10 μl were mixed with 5 μl of Laemmli buffer and electrophoresis was carried out in a MiniProtean III (BioRad, Hercules CA, USA). Gels were stained with Coomasie Brilliant Blue G250 (USB, Cleveland, Ohio, USA). Chitinolytic activity of r*ech42* of the

culture medium was carried out on microtiter plate as follow: 10 ng of proteins were poured on each well and mixed with 1 μM 4-methyl-umbelliferyl-N,N',N"-acetylchitotrioside (4-MUChT, Sigma) on activity buffer (5 mM sodium acetate, 10 mM EDTA, pH 5.5) reported previously (O'Brien and Colwell, 1987). Plate was incubated for 10 min at 40°C. Reaction was stopped by adding 150 μl of 500 mM Na_2CO_3. Reaction was transferred to 4-ml fluorometer cell and samples were read on VersaFluor Fluorometer (BioRad) at 360 and 460 nm as excitation and emission wavelengths, respectively. Fluorescence data were converted onto concentration of 4-methyl-umbelliferone (4-MU) using a standard curve. One unit of enzymatic activity was defined as the amount of protein that produces 1 nmol of 4-MU per minute.

2.5 *Atomic Force Microscopy*

Cell samples were diluted until reaching an OD_{600nm} of 0.5 on phosphate buffer (100 mM KH_2PO_4, 100 mM K_2HPO_4, 200 mM NaCl at pH 6). Samples of 50 μl were spread on mica slide and let them to be adsorbed on the surface. Slides were subjected to atomic force microscopy (AFM) using a tip scattering Scanning Probe Microscope JSPM-5200 (JEOL Peabody, MA, USA). The tip used for analysis was an NSC16/Al BS (MikroMasch, Lady's Island, SC, USA). Surface scanning was carried out by intermittent contact mode (tapping). Topography images of 100 cells were acquired and processed with WinSPM System Processing Software (JEOL).

2.6 *Fluorescence microscopy*

For analysis by fluorescence microscopy, cells from exponential phase culture grown under each condition were stained with fluorescent dye calcofluor white (Sigma-Aldrich, St. Louis, MO, USA), which binds specifically to structural carbohydrates, such as cellulose and chitin in the cell wall. One hundred microliters of 1:50 dilution of cells resuspended on phosphate buffer were mixed with one drop of dye and 1 drop of 1 g l^{-1} KOH, after mixed samples were kept in the dark for 3 min. One drop of stained cells was poured on glass slide and covered with cover slips and observed on fluorescence microscope Leica DMLII (Leica MicroSystems GmbH, Wetzlar, DE).

2.7 *Statistical analysis*

For assessing celular dimension under different culture conditions, groups of 25 cells of each condition were measured after AFM.. Statistical analysis was performed and Tukey's test was used to determine significance of differences among major diameter, minor diameter and perimeter means. When the means differed at α=0.05, they were significant.

3 Results and discussion

3.1 *Low oxygen tension improves the volumetric production of rech42*

In this work, the production of r*ech42* by *P. pastoris* cultured at low and high DOT was evaluated. Batch cultures of *P. pastoris* P_{GAP}-*ech42* at 3 and 40 % DOT, are shown in the Fig. 1 and the kinetic and stoichiometric data are summarized on table 1. For the culture at 3% DOT, the biomass concentration increased exponentially at a specific growth rate of 0.34 h^{-1} to reach a maximum concentration of 11.9 g l^{-1}, and thereafter it remained constant (Fig. 1A). The extracellular proteins in the culture medium increased following the biomass trend. For this culture, the maximum extracellular protein concentration was 208±1.05 mg l^{-1} (Fig. 1B). The recombinant enzyme represents more than 98% of the total proteins secreted to the culture medium, as it can be observed on Fig. 2, as a single band under both culture conditions tested. A maximum volumetric enzyme activity of 18,600±1053 and 12,330±1000 U l^{-1} was attained at 3% and 40% DOT, respectively (Fig. 1C). Whereas maximum specific enzyme activities were 89.4±4.6 y 88.8±4.0 U $mg^{-1}_{protein}$ at 3% and 40% DOT, respectively (Table 1). Previously, it was reported that an hypoxic fed batch allowed a better production of recombinant proteins, driven by P_{GAP} constitutive promoter (Baumann *et al.*, 2008), as done in the present work, where the limiting 3 % DOT yielded 1.5-fold higher than that attained at 40% DOT. They concluded that cultures at the limit of aerobic to fermentative metabolism could be beneficial for the production of heterologous proteins, at least in the configuration of a constitutive promoter such as the P_{GAP} on glucose. This promoter has shown a better performance for the overexpression of recombinant proteins, for instance Zhang *et al.* (2007) concluded that P_{GAP} is more efficient than P_{AOX1} for the expression of angiostatin in *P. pastoris* by shake flask culture or high-density cell fermentation, notion that is reinforced by the fact that systems

Table 1. Parameters measured in the batch cultures and cell dimensions of the wild-type and transforming *P. Pastoris* pGAP-*ech42* cells at 3 and 40% DOT.

	Wild-type strain		Transforming strain	
Parameter	3% DOT	40% DOT	3% DOT	40 % DOT
Biomass maximum (g l^{-1})	9.5±1.7[b]	10.2±1.1[b]	11.9±0.5[a]	10.5±0.5[b]
Specific growth rate (h^{-1})	0.15±0.02[b]	0.32±0.012[a]	0.34±0.003[a]	0.32±0.017[a]
Maximum extracellular protein concentration (mg l^{-1})	8.3±0.78[c]	10.6±0.53[c]	208±1.05[a]	138.5±4.92[b]
Volumetric enzyme activity (U l^{-1})	ND	ND	18,600±1053[a]	12,330±1000[b]
Specific enzyme activity (U $mg^{-1}_{protein}$)	ND	ND	89.4±4.6[a]	88.8±4.0[a]
Average major diameter (μm)	2.86±0.47[a]	2.65±0.61[a]	1.29±0.14[b]	2.67±0.22[a]
Average minor diameter (μm)	2.43±0.39[a]	2.33±0.82[a]	1.13±0.13[b]	2.21±0.6[a]
Average perimeter (μm)	8.42 ± 0.97[a]	8.15 ± 0.55[a]	3.82 ± 0.35[b]	7.8 ± 0.86[a]

[a,b,c]Means in the same row with different superscript are significantly different at α =0.05, ND: not detected.

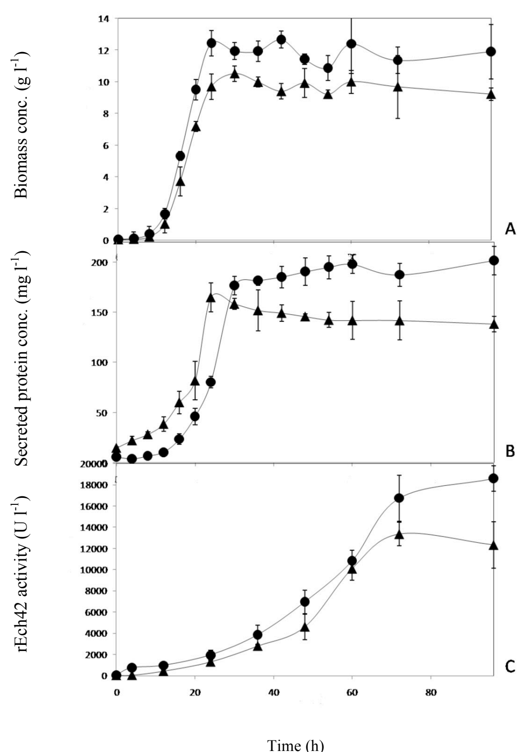

Fig. 1. Typical growth kinetics of P. pastoris pGAP-*ech42* cultured at 3% (•) and 40% DOT (▲). A) Biomass concentration. B) Protein concentration in the culture medium. C) Volumetric rEch42 activity.

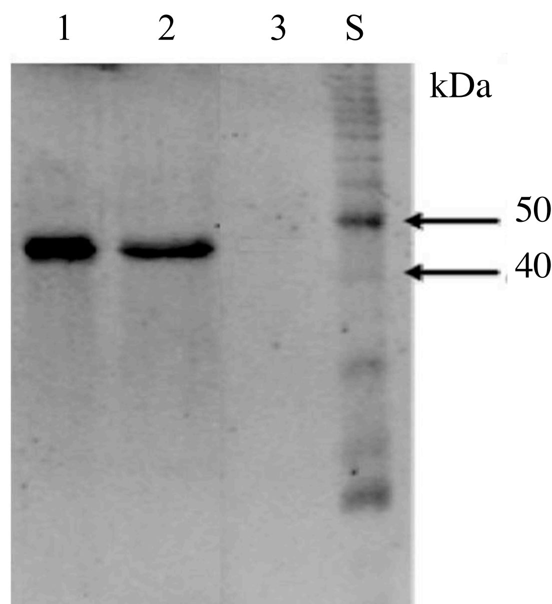

Fig. 2. SDS-PAGE of extracellular proteins produced in cultures at two DOT conditions. Lane 1: *P. pastoris* pGAP-*ech42* at 3% DOT; lane 2: *P. pastoris* pGAP-ech42 at 40% DOT; lane 3: *P. pastoris* at 40% DOT; lane S: Molecular weight standard (Invitrogen).

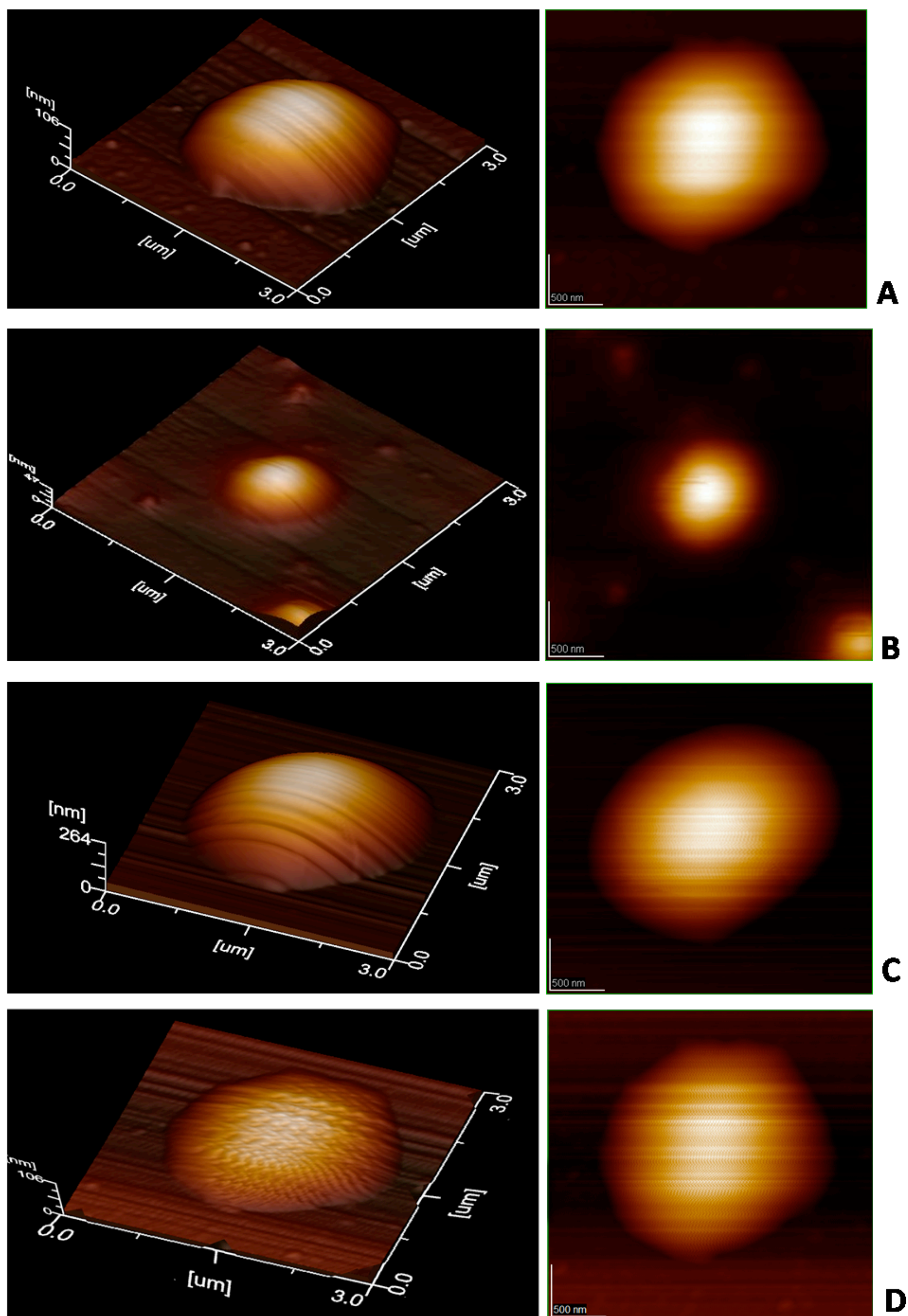

Fig. 3. Topographic images 3D by AFM of wild type and recombinant cells cultured under two DOT conditions: A) *P. pastoris* pGAP-*ech42* at 40% DOT, B) *P. pastoris* pGAP-*ech42* at 3% DOT, C) *P. pastoris* at 40% DOT and D) *P. pastoris* at 3% DOT.

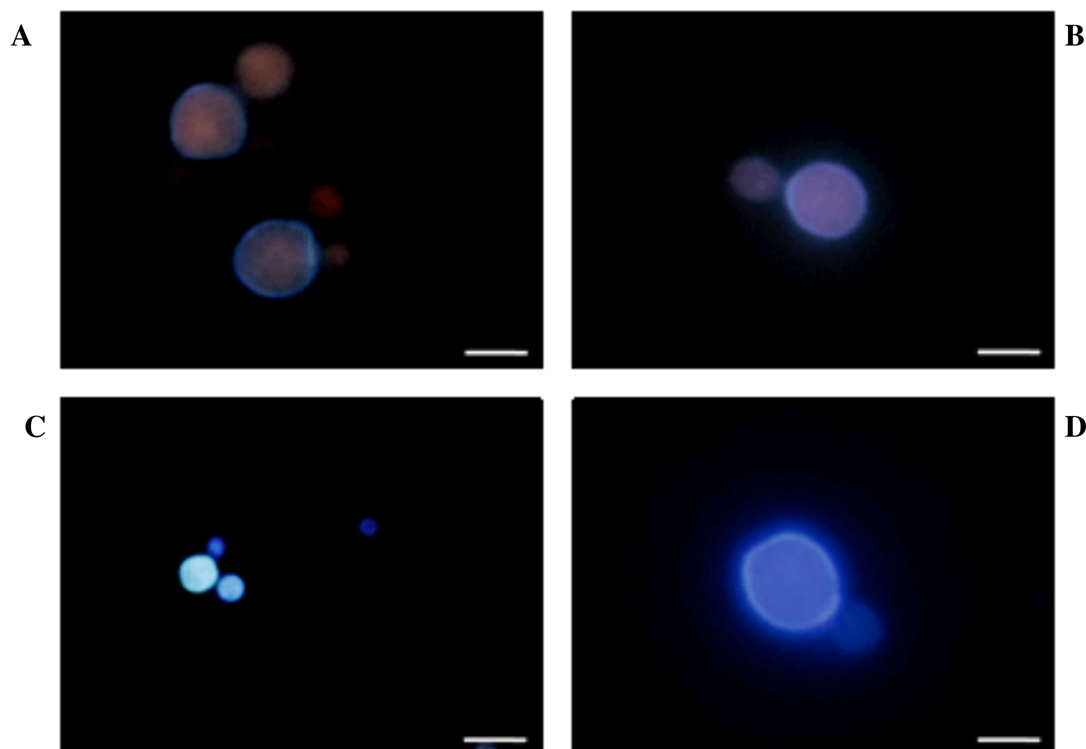

Fig. 4. Fluorescence microscopy of cells stained with calcofluor white cultured at different DOT conditions. A) *P. pastoris* at 3% DOT, B) *P. pastoris* at 40% DOT, C) *P. pastoris* pGAP-*ech42* at 3% DOT and D) *P. pastoris* pGAP-*ech42* at 40% DOT. White bar: 2 μm.

based on P_{AOX1} promoter have to be unrepressed prior to recombinant protein to be produced, whereas constitutive P_{GAP} promoter is active all the yeast lifespan. Shake flask-scale experiments comparing both systems, constitutive *pGAP-ech42* and methanol-inducible one, pPIC-*ech42* carried out by our research group (Pérez-Martínez, 2007) showed clearly that better production is achieved with constitutive system, 202 mg l^{-1} vs. 80 mg l^{-1} produced with the inducible system.

In order to compare the growth kinetic of the wild-type strain with the transforming strain, batch cultures were performed at 3 and 40% DOT with the wild-type strain and the results are summarized in Table 1. Both wild-type and transforming strains at 40% DOT reached the same maximum biomass concentration and grown at the same specific grow rate, whereas at 3% DOT, the wild-type strain rendered less biomass and grew slower than the transforming strain (Table 1). In all cases, ethanol and acetic acid were not produced under any condition tested here. Several authors have discussed the advantage of the bad-fermentation capacity of *P. pastoris* in regard to recombinant protein production, because well-fermentative yeasts cause the accumulation of considerable amounts of ethanol inside the bioreactor, which has a deleterious effect on cell metabolism (Larsson *et al*, 2001).

3.2 *Overexpression of rech42 affects the cells size*

Chitin is a component in the cell wall of fungi and yeasts (Duran and Nombela, 2004). Despite chitin represents about 1-2%, this material is fundamental scaffold for the cell as well as the budding (Schmidt, *et al*., 2003; Schmidt, 2004). Wild-type and recombinant cells cultured under both DOT conditions were visualized by AFM technique (van der Mei, *et al*., 2000) in order to get their morphology and dimensions (Fig. 3). In all cases, the cells were taken at the middle of the exponential phase. Wild-type cells cultured at 3 or 40% DOT had similar size (Table 1). Since, cells had eliptical shape, major and minor diameter were measured. The major and minor diameters were 2.86±0.47 and 2.43±0.39 μm,

respectively and the average perimeter was 8.42 ± 0.97 μm for 3% DOT and 2.65 ± 0.61, 2.33 ± 0.82 and 8.15 ± 0.55 μm values for major and minor diameters and perimeter respectively under 40% DOT. The transforming strain cultured at 40% DOT did not show significant difference (Tukey α=0.05) with respect to the wild-type cells cultured at 3% or 40% DOT, and the major and minor diameters were 2.67±0.22 and 2.21±0.6 μm, respectively and the average perimeter was 7.8 ±0.86 μm for the transforming strain cultured at 40% DOT. However, when transforming strain was cultured at 3% DOT, the cell size was strongly affected (Fig. 3), and the major and minor diameters were 1.29±0.14 and 1.13±0.13 μm, respectively, and the perimeter was 3.82 ± 0.35 μm (Table 1). Yeast cells stained with calcofluor white are shown in the Fig. 4. Since, yeast cells divide by budding, large and small cells are currently called mother and dauther cells, respectivelly. It can be observed that mother wild-type cells grown at 3% and 40% DOT, as well as *P. pastoris pGAP-ech42* cells cultured at 40% DOT showed an intense fluorescence, whereas mother cells of the transformed strain under 3% DOT showed less fluorescence, suggesting a reduction in the amount of chitin in the cell wall. However, additional experiments to confirm this issue, are needed. Overexpression of r*ech42* could reduce the amount of chitin and therefore compromise the cell wall integrity of *P. pastoris*, resulting in a best nutrient diffusion (oxygen from medium to cytoplasm). It has been reported that cell wall integrity is critical for cell expansion during growth and morphogenesis in *Saccharomyces cerevisiae* (Levin, 2005). Since, the reduction of cell size was not observed on transforming strain growing on 40% DOT, the aforementioned phenomenon is the result of the conjunction of low dissolved oxygen tension and the overexpression of active rech42, which comprises a very big advantage since one of the principal issues when dealing with *P. pastoris* expression systems based on methanol metabolism, which is obligated aerobic, is to keep the high enough dissolved oxygen inside the bioreactor for supporting the high oxygen demand during the exponential growth phase, while our expression system allows excellent biomass production coupled with an excellent recombinant enzyme expression under more friendly conditions such at lower DOT value, it is now to determine if expression of other recombinant proteins which is not probable to comprise cell wall integrity could be produced as well as rech42 does.

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