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Fed-batch cultivation and operational conditions for the production of a recombinant anti-amoebic vaccine in *Pichia pastoris* system

Cultivo en lote alimentado y condiciones de operación para la producción de una vacuna recombinante anti-amebiana con el sistema *Pichia pastoris*

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Abstract

It was developed a fed-batch bioprocess to produce a recombinant vaccine against *Entamoeba histolytica* under operational conditions attainable to large scale bioprocesses. We have produced this recombinant protein in shake flask and stirred tank bioreactor. Initial results in shake flask cultures under different methanol concentration of 0.5, 1.5 and 3% (v/v) produced extracellular protein at quantities of 10, 22 and 33 μg/mL, respectively. Then a scale-up process was performed from shake flask to stirred tank bioreactor by keeping similar volumetric power supply. The operational conditions were set up in bioreactor as those used at commercial scale and supply of pure oxygen was avoided to keep the scalability of the bioprocess. After the scale-up process, the production of the recombinant protein reached 0.43 mg/mL, an improvement in production of 12 times, although the methanol and oxygen limited conditions observed. Maximum volumetric productivity of 3.75 mg/L h was achieved in bioreactor against 0.26 mg/L h reached in shake flask. Besides the limited conditions in methanol and oxygen, the yields obtained from the bioprocess were comparable to those observed in Mut$^+$ strains previously reported, then saturated methanol conditions are not necessary to compensate limited oxygen conditions.

Keywords: methanol limited feed; recombinant vaccine; alcohol oxidase promoter; stirred tank bioreactor; shake flask.

Resumen

Fue desarrollado un proceso en lote alimentado bajo condiciones de operación limitadas a las encontradas en los bioprocesos de gran escala, para producir una vacuna recombinante contra *Entamoeba histolytica*. Los cultivos con matraz agitado y empleando diferentes concentraciones de metanol de 0.5, 1.5 y 3% (v/v) produjeron 10, 22 y 33 μg/mL de proteínas extracelulares, respectivamente. Posteriormente, se realizó un escalamiento del cultivo desde el matraz hasta biorreactor manteniendo constante el suministro de potencia. De igual forma, para mantener la escalabilidad comercial del bioproceso se evitó el suministro de oxígeno puro. Después del proceso de escalamiento, la producción de proteína recombinante alcanzó hasta 0.43 mg/mL, una mejora en la producción de hasta 12 veces, a pesar de las condiciones limitadas en oxígeno y metanol. La productividad volumétrica máxima alcanzada en el biorreactor fue de 3.75 contra 0.26 mg/L h obtenida en los cultivos con matraz agitado. Aun bajo las condiciones limitadas de oxígeno y metanol alcanzadas en los cultivos, los rendimientos obtenidos de los bioprocesos fueron comparables a aquellos observados en cepas Mut$^+$ previamente reportados; por lo tanto, condiciones saturadas de metanol no son necesarias para compensar las condiciones limitadas de oxígeno.

Palabras clave: alimentación limitada de metanol; vacuna recombinante; promotor alcohol oxidasa; biorreactor tipo tanque mezclado; matraz agitado.

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1 Introduction

The production of recombinant proteins and bioproducts is a cornerstone of microbiological and biochemical research which represents a high profit global market (Lara, 2011; Potvin *et al*., 2012). Yields and quality of produced proteins have a tremendous impact on structural and enzymology studies, development of new biopharmaceuticals and establishing new biocatalytic processes (Chrast *et al*., 2018). Initial stages of bioprocesses are developed at laboratory scale with low volumes of production by using small bioreactors as the shake flasks, to select properly culture conditions for the biological system used. However, production of biotechnological products are carried out in fermenters (stirred tank bioreactors, STB), these have considerably differences in geometrical and operational aspects to shake flask (Seletzky *et al*., 2007). Due to the hydrodynamic differences between shake flask and STB; molecular transfer processes (mass, momentum and heat) are better performed in fermenters (STB) than in shake flasks (Büchs, 2001). The mixing mode differences between these bioreactors do the scale-up process from shake flasks to fermenters troublesome and poorly understood. Nevertheless, most of the time product yields are improved in the fermenters after the scale-up process (Reyes *et al*., 2003).

The aim to scale-up a bioprocess is to obtain a large quantity of the desired bioproduct at commercial scale production, with at least the same yields obtained at laboratory scale under optimal conditions selected. However, this is not an easy task mainly due to changes in vessel geometries which lead to an increase in mass and heat gradients in the bioreactor bulk (Trujillo-Roldán *et al*., 2013). However, in the recent two decades there have been an increase in number of works to understand from an engineering point of view the process to scale-up bioprocesses from shake flasks to STB. As well for STB are used scale-up criteria to take bioprocesses from laboratory to pilot and large scale, the same occurs to transfer bioprocesses from shake flask to fermenters (Gamboa-Suasnavart *et al*., 2019).

The volumetric power input (in this work *P/V*, ungassed for shake flask; *Pg/V*, gassed for the STB) (Reyes *et al*., 2003; Rocha-Valadez *et al*., 2006; Gamboa-Suasnavart *et al*., 2013) and the volumetric oxygen transfer coefficient (k_La) (Seletzky *et al*., 2007; Gómez-Sánchez *et al*., 2012; Trujillo-Roldán *et al*., 2013), have been used to transfer bioprocesses cultures from shake flask to stirred tanks bioreactors. It is well known that oxygen transfer rate (OTR) in bioreactors is characterized by the k_La, at the same time, k_La has a big dependence on the *P/V* in both shake and stirred bioreactors (Maier *et al*., 2004; Garcia-Ochoa *et al*., 2009). Both scale-up criteria (k_La and *P/V*) are largely used for aerobic bioprocesses, mainly due to oxygen transfer from gas to liquid is the main limiting phenomenon in such kind of bioprocesses (Büchs, 2001; Pérez-Martínez *et al*., 2014).

The methylotrophic yeast *Pichia pastoris* has been widely used to produce recombinant proteins across a broad spectrum of functional types including (i) enzymes, (ii) antigens, (iii) engineered antibody fragments, and (iv) next gen protein scaffolds (Vieira-Gomes *et al*., 2018). More than 5, 000 proteins have been recombinant expressed using *P. pastoris* system and over 70 produced protein products have reached the market (Cos *et al*., 2006; Ahmad *et al*., 2014). This yeast is of particular industrial interest due to: (i) its tightly regulated methanol-inducible path for methanol consumption as source of carbon and energy (Cos *et al*., 2006), (ii) its capacity for foreign protein secretion, (iii) its ability to perform post-translational modifications (Potvin *et al*., 2012), (iv) the ability to grow on defined media at high cell densities and (v) its strong preference for respiratory growth, without production of by-products (López-Pérez and Viniegra-Gózalez, 2017).

The enteric parasite *E. histolytica* can cause amoebiasis after infecting the human large intestine, with symptoms including dysentery and amoebic liver abscesses (Bruckner, 1992). Drug therapies such as metronidazole and other nitroimidazole derived compounds are effective for treating invasive parasites. However, these drugs display adverse side effects and are expensive and not easily available in certain countries and areas; thus, the application of new immunogenic schemes as vaccines are of high interest to solve such issues (Bansal *et al*., 2006). Recently it has been developed a new recombinant *P. pastoris* Mut^+ strain to produce a recombinant *Entamoeba histolytica* (*E. histolytica*) LC3 protein with a potential application as an immunogen (Martínez-Hernández *et al*., 2017). The candidate for a vaccine against *E. histolytica* (VA*Eh*) produced, is a chimeric construction between *E. histolytica* LC3 fragment; the retrograde delivery domains (I-II) from *Pseudomonas aeruginosa* exotoxin A and the carboxy-terminal signal KDEL3 (Martínez-Hernández *et al*., 2017).

The implementation of bioprocesses to supply the expected demand of this therapeutic recombinant protein is of high interest for public health. The pharmaceutical production of recombinant proteins by *P. pastoris* requires proper bioprocess development, for example the obtaining of high-cell-density cultivation, proper methanol concentration on broths and enough oxygen supply (Trentmann *et al*., 2004; Markošová *et al*., 2015; López-Pérez and Viniegra-Gózalez, 2017). In spite the available cultivation protocols based on the commercial Invitrogen® expression kit; the recent trend is to move away from standard protocols towards a conceptual approach that allows the development of a specific process strategy that is tailored to both particular recombinant strain and the characteristics of specific bioreactor equipment (Looser *et al*., 2015). Different approaches have been implemented in *P. pastoris* bioprocesses; as oxygen limited conditions (Charoenrat *et al*., 2005); methanol saturated conditions (Khatri *et al*., 2006) and simple fed-batch regimes, with an improvement in the production of the recombinant proteins with respect to those standard protocols (Gurramkonda *et al*., 2009).

Accordingly, it is essential to develop appropriate and technologically feasible bioprocesses to be scaled-up, which can allow a high and efficient production of new recombinant proteins at commercial scale. The aim of this work is to scale-up the production of a recombinant vaccine against *E. histolytica* from shake flask to stirred tank bioreactor by setting up suitable culture conditions of the bioprocess to improve the availability of the vaccine and propose scaling-up strategies for *P. pastoris* bioprocesses. We have established the operational conditions and a simplified methanol feeding protocol that can be used in feasible technological and economical fed-batch bioprocesses which may be performed at commercial scale.

2 Materials and methods

2.1 Strains

A recombinant strain *P. pastoris* GS115 containing the LC3 gene from *E. histolytica* was used. The characteristics of chimeric gene for the vaccine (VA*Eh*) is described in previous work (Martínez-Hernández *et al*., 2017).

2.2 Media

The inoculum for the flasks and the fermenter cultures were grown in YPG medium (composition per liter: 10 g yeast extract; 20 g peptone; 20 g glycerol) and incubated at 30 °C in an orbital shaker at 200 rpm for 48 h. The fermentation media used to production was the defined media FM22 supplemented with 1.0 mL of sterile PTM4 trace salt solution, the media culture was prepared according to Stratton *et al*., (1998). This medium except for trace salts was sterilized at 120 °C for 20 min, and the PTM4 salt solution was filter sterilized and stored.

2.3 Production of VAEh and estimation of oxygen transfer rate and volumetric power consumption in baffled shake flask

Glass baffled shake flasks with a nominal volume of 500 mL; an inside diameter (d) of 0.1 m, three 120 degrees spaced baffles with 0.015 m of deep and 0.025 m of height were used to produce VA*Eh*. For cultures in baffled shake flasks, 100 mL of FM22 media culture closed with cotton-plugs were poured into the flasks and then inoculated with 5 mL of previous growth inoculum. In order to avoid oxygen limitation in flask production, glycerol was limited to 10 g/L. Shake flasks cultures were incubated at 30 °C and 200 rpm in an orbital incubator with a shaking diameter of 2.0 cm.

To estimate the volumetric power input in our baffled shake flasks, the correlation reported by Büchs *et al*. (2001) was used (Eq. 1); where, n is the shaking frequency and V is the liquid filling volume given in min^{-1} and mL, respectively.

$$\frac{P}{V} = 1.17 \times 10^{-6} n^{2.95} V^{-0.24} \tag{1}$$

In baffled shake flask, the volumetric oxygen transfer coefficient is equivalent to the sum of resistances at the sterile plug and the gas-liquid interface as shown in Eq. (2) (Gupta and Rao 2003).

$$(k_L a)eq = \frac{1}{V}\left[\frac{1}{Mk} + \frac{1}{Vk_L a}\right]^{-1} \tag{2}$$

$$k_L a = n^{1.4} \tag{3}$$

The Eq. (2) was also proposed for baffled shake flasks under similar conditions used in this study (water like liquid at 37 °C). For this equation, M corresponds to dimensionless Henry's law constant

with a value of 41 and k is the plug transfer coefficient of 2.7×10^{-4} m^3/h according to Gupta and Rao (2003). The k_La is the gas-liquid interface transfer coefficient in h^{-1} and V is the working volume in m^3. The k_La coefficient in Eq. (2), was estimated according to Van Suijdam *et al*., (1978) by Eq. (3), where n is given in s^{-1}, as well the resulted k_La coefficient. This correlation was also developed for baffled shake flasks and for water like viscosity liquids, as those founded in yeast cell cultures (Montes *et al*., 1999).

Equations above described for the *P/V* and k_La estimation are given for those called "in-phase conditions", which are operating conditions at which the bulk of the liquid within the flask circulates in synchrony with the shaking table (Büchs *et al*., 2000b; Peter *et al*., 2006). At the operation conditions above described, the "in-phase" conditions were always observed during the time of fermentation, which indicates that, the estimation of the *P/V* and k_La have good certainty. Also, viscosity did not change significative during the fermentation time in the shake flask cultures (data not shown), which is characteristic of yeast broths (Montes *et al*., 1999). For baffled shake flasks the *P/V* usually is around five times greater than that found in the similar unbaffled shake flask. The *P/V* value estimated by Eq. (1) was of 2.38 W/L, which is similar to that previously reported for 500 mL baffled shake flasks of 2.0 W/L (Peter *et al*., 2006). This range of *P/V* is in the limits of the scalable conditions up to commercial scale operating conditions (Neubauer *et al*., 2016). Meanwhile, the k_La estimated by the equations 2 y 3 was of 110.07 h^{-1} for the operating conditions used in the 500 mL baffled flasks, above described. To investigate the methanol concentration effect on VA*Eh* production, different concentrations of methanol were used during the induction phase in shake flasks cultures. After 30 h of incubation on glycerol and then each 24 h, were added methanol pulses of 0.5, 1.5 and 3.0% (v/v) to the medium. Methanol concentration expressed as percentage in the text length always corresponds to % (v/v). Methanol used to induce VA*Eh* production was supplemented with 5 mL of PTM4 per liter of pure methanol.

2.4 *Scale-up of the production of VAEh from shake flask to a simplified methanol fed-batch in stirred tank bioreactor*

Production of VA*Eh* was performed in a 3 L fermenter (STB) attached to the SCADA system myControl (Applikon, Delft, The Netherlands). The vessel has a diameter and height of 13 and 25 cm, respectively. Agitation was provided by two Rushton turbines with a diameter of 4.5 cm with a separation distance between turbines of 6.5 cm. Bioprocesses were performed under a fed-batch regime at 30 °C and a pH of 5.5, which was controlled by adding ammonium hydroxide (28%, w/v).

To maintain bioprocess feasible to be scaled up, operational conditions in bioreactor were attainable to those found in commercial scale bioprocesses (Neubauer *et al*., 2016); and the use of pure oxygen to control dissolved oxygen (DO) level was avoided. Agitation (1100 rpm) and aeration (1 vvm) were set for a maximal volumetric gassed power consumption (*Pg/V*) of 2.9 W/L; at these conditions a k_La of 152 h^{-1} was achieved. The *Pg/V* in the STB was determined by measuring current intensity and voltage supplied to the stirring motor accordingly to Marín-Muñoz *et al*., (2019). Meanwhile, k_La was measured by the dynamic method with the gassing out technique (García-Ochoa and Gomez, 2009). These operating conditions are similar to those estimated before in the shake flasks cultures. Operating conditions (agitation and aeration rate) were constant through bioprocess time.

In the initial batch stage (growth on glycerol), 1.8 L of FM22 medium were inoculated with 200 mL inoculum. Depletion of glycerol after inoculation was correlated with an instantaneous DO increase. After glycerol depletion, the fed-batch methanol phase (production phase) was started by adding a pulse of 1.0% of methanol. After a period of cell adaptation to methanol, indicated by a second instantaneous DO increase, pulses of methanol were fed by a DO control loop as described in Marín-Muñoz *et al*. (2019). When DO was above 30% (indicating methanol limitation) the methanol pump turned on until oxygen demand increased (decreasing DO below 30%), then the methanol pump turned off. With this configuration for methanol supply, it was no reached inhibitory concentration, because the volume of methanol in each pulse was less than one mL. Foam level was controlled by addition of antifoam (SAG-710, Momentive, NY).

2.5 *Determination of biomass, glycerol and proteins*

Samples collected from shake flask and stirred bioreactor cultures were centrifuged at 9000 × g for 10 min. Recovered biomass was dried at 70 °C for 48 h to obtain dry cell weight biomass concentration (DCW). Supernatants were used to measure soluble

protein content and glycerol concentration. Glycerol concentration was measured by Malaprade and Hantzsch consecutive reactions. The intensity of the resulting dye (3,5-diacetyl-1,4-dihydrolutidine) was directly proportional to glycerol concentration, and it could be measured at 410 nm in the range of 0-10 mg/mL (Kuhn *et al*., 2015). Total protein concentration in broth was measured according to Bradford method using bovine serum albumin (BSA) as standard protein.

For the SDS-PAGE analysis of secreted proteins, 1 mL of culture medium for each sample was taken and precipitated with trichloroacetic acid (TCA) or acetone, following the protocol described by Santana *et al*. (2016) and was resuspended in 25 μL of Tris-base 1 M and 25 μL of sample buffer (Sigma Aldrich, USA), 25 μL were loaded. The precipitated proteins were separated on a polyacrylamide at 12% (w/v) for 2 h at 100 V. The gel was silver stained using the ProteoSilver^{TM} Silver Stain Kit (Sigma Aldrich, USA). The proteins were transferred to a polyvinylidene difluoride membrane Sequi-Blot^{TM} PVDF 0.2 μm (Bio-Rad, USA). After transfer, the membrane was washed with TBS 1X-Tween (0.5 g/L Tween) buffer and blocked overnight. The recombinant proteins were visualized by immunostaining using as primary antibody rabbit anti-6X His tag-HRP (ab1187, Abcam) diluted 1:5000.

2.6 Cell viability

The density of viable cells was determined by the serial decimal dilution method and counting the colony forming unit (CFU) on YPG-agar plates incubated at 30 °C during 48 h.

2.7 Statistical analysis

Analysis of variance (ANOVA, p = 0.05) was performed using R software (R Foundation for Statistical Computing, Vienna, Austria) to assess significant differences. Cultures for VA*Eh* production in shake flask and stirred tank bioreactor were conducted by triplicate.

3 Results and discussion

The establishment of bioprocesses with technical and economic feasibility to be scaled up to produce therapeutic recombinant proteins with appropriate yields, are of high interest to cover the demand of this kind of biotechnological products. We have developed a scale-up process from shake flask to stirred tank bioreactor by keeping constant the volumetric power input between bioreactor systems, due to *P/V* (or *Pg/V*) is the main affecting the oxygen-mass transport phenomena in aerobic bioprocesses as those performed in *P. pastoris* cultures (García-Ochoa and Gomez 2009; Potvin *et al*., 2012). As it is known, the limiting transport phenomenon in *P. pastoris* bioprocesses is described by the k_La (Pérez-Martínez *et al*., 2014, Marín-Muñoz *et al*., 2019). Performing a scale-up process under a constant *Pg/V*, may guarantee the same k_La between scales, which ensures a successful scale-up. Also, *Pg/V* is a parameter well associated with the physical phenomena of mass, heat and momentum transport; and is the only scale-up criteria free of heterogeneities in dynamic bioreactors (Gamboa-Suasnavart *et al*., 2019). Based on these assumptions, we made the scale-up from shake flask to stirred bioreactor by coupling the *Pg/V* between bioreactors.

3.1 Production of VAEh in shake flask cultures

Recombinant expression of proteins by *P. pastoris* under the control of the alcohol oxidase promoter (p*AOX*) requires a tightly control on the methanol feed to avoid build up or lack of methanol in media culture (Potvin *et al*., 2012; López-Pérez and Viniegra-González 2017). For this reason, shake flask cultures were carried out to evaluate the effect of methanol concentration on VA*Eh* production, since a rapid way to evaluate culture conditions is through cultures in such kind of bioreactors (Klöckner and Büchs 2012).

For cultures in shake flasks we have used an own fed-batch protocol in order to emulate similar fermentation regime as that we have run in the fermenter. Traditional protocols for culture in shake flask suggest the growth of yeast biomass and the expression of the recombinant protein (induction) in separate complex media culture using either glycerol or methanol (Markošová *et al*., 2015). We can approach a better similitude in culture conditions between shake flask and fermenter to produce VA*Eh*, by using similar media culture and methanol feeding strategy. Nevertheless, there are considerable hydrodynamic differences between bioreactors. With this consideration, we attempted to keep as possible the culture conditions and regime between both scales used.

Table 1. Production and kinetics of VA*Eh* in baffled shake flask cultures with different methanol volume supply (P/V = 2.38 W/L and k_La = 110 h^{-1}).

Methanol (% v/v)	**VA*Eh* concentration (mg/mL)**	***YP/S* (mg/g)**	μ_{MeOH} **(h^{-1})**	**qP (mg/g_{DCW} h)**	**Volumetric productivity (mg/L h) ***
0.5	0.01 (0.004)^c	0.60 (0.25)^a	0.005 (0.001)^a	0.10 (0.005)^b	0.12 (0.03)^c
1.5	0.02 (0.005)^b	0.46 (0.10)^a	0.005 (0.002)^a	0.10 (0.004)^b	0.19 (0.04)^b
3	0.03 (0.003)^a	0.38 (0.04)^a	0.005 (0.002)^a	0.13 (0.004)^a	0.26 (0.03)^a

* Volumetric productivity at the end of the time of fermentation. ** Total methanol added per treatment in 0.5, 1.5 and 3.0% (v/v) was 2.0, 6.0 and 12.0 mL, respectively. ***Mean values are presented (±SD), n = 3. Different exponent script indicates a significant statistical difference among treatments $p \leq 0.05$.

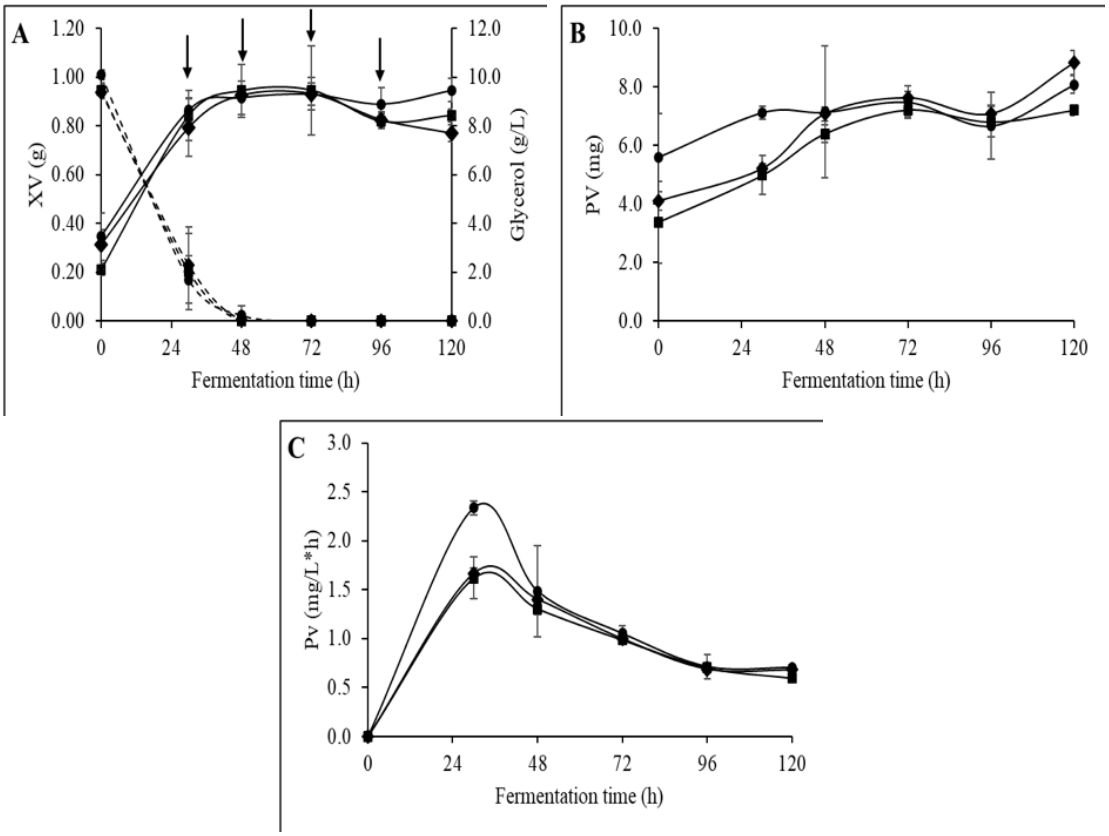

Fig. 1. Production of VA*Eh* and dry cell biomass in baffled shake flask with different methanol supplies. (A) Dry biomass production (lines); glycerol consumption (dashed lines); each arrow indicates the time for addition of the corresponding volume of methanol. (B) Total protein in broth during the incubation time. (C) Volumetric productivity of the produced recombinant protein. Concentration of methanol used: • 0.5%, ■ 1.5%, ♦ 3.0% (v/v).

Table 1 shows the production of the VA*Eh* with different volumes of methanol supply to shake flask cultures. Cultures in shake flasks have disadvantages as the lack of control on critical fermentation parameters and limited oxygen transfer capacity in submerged fermentations leading to low yield bioprocesses (Klöckner and Büchs 2012). In this case, we reduced the glycerol availability in shake flask cultures to avoid oxygen limitation associated to high cell densities, as contrary when higher glycerol concentration than 40 g/L is used which results in oxygen limitation (Gurramkonda, *et al*., 2009). The average biomass produced at the end of the glycerol phase was of 5.3 g $_{DCW}$/L after 30 h of incubation with an average specific growth rate (μ) of 0.04 h^{-1} (Fig. 1(A)).

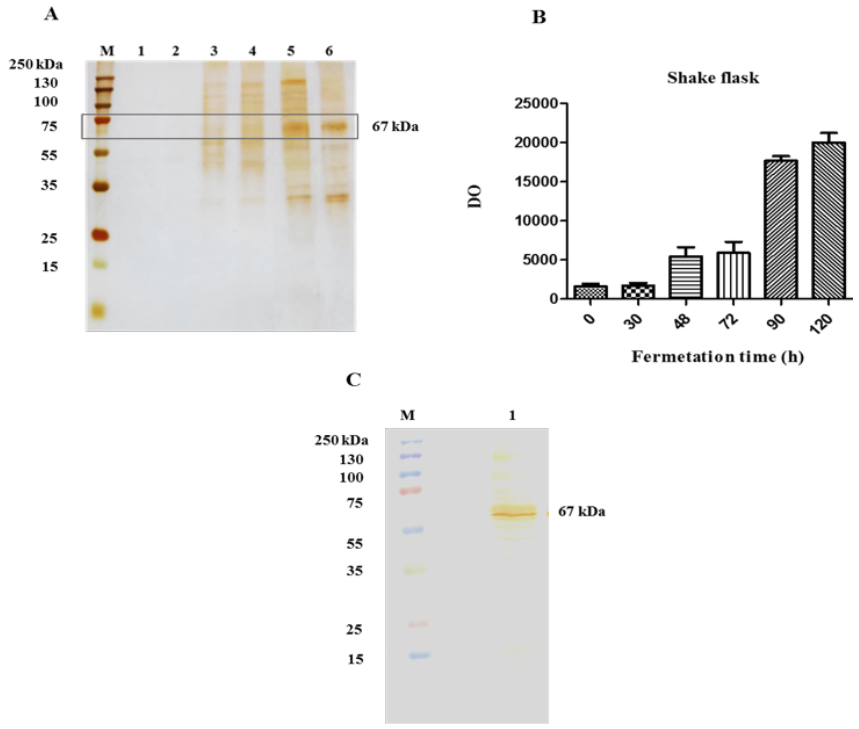

Fig. 2. Recombinant protein analysis by electrophoresis and western blot from protein produced in baffled shake flask cultures with 3% (v/v) of methanol. (A) SDS-PAGE from a cell-free broth taken at different times during the fermentation. Lane M protein molecular weight ladder (in each pattern contains the same molecular markers); lane 1-2 are samples during the batch stage on glycerol (0 and 30 h of fermentation time, respectively); lanes 3-6, samples during fed-batch stage with methanol (samples from 48, 72, 96 and 120 h of fermentation time). (B) Densitometric analysis of the band corresponding to the molecular weight of the VA*Eh* from the SDS-PAGE. (C) Western blot analysis of VA*Eh* with the rabbit anti-6X His (lane 1) with a molecular weight of 67 kDa.

This allowed to start methanol induction with same biomass in all treatments with different methanol supply. However, the specific growth rate achieved on glycerol growth is up to 80% lower than those values reported for *P. pastoris* growing in glycerol (Looser *et al*., 2015). This lower specific growth rate could be associated to a lack of oxygenation when cells were growing on glycerol.

Biomass production during methanol growth almost still constant in the three methanol concentration supplied (Fig. 1(A)). Then, the biomass production achieved an average specific growth rate on methanol (μ_{MeOH}) of 0.005 h^{-1} for the three methanol concentrations (Table 1), which is up to 20 times lower than those values recorded for Mut^+ recombinant strains (Looser *et al*., 2015). Meanwhile, biomass to methanol yield averaged between 0.04 to 0.23 g_{DCW}/g for methanol concentration from 0.5% to 3.0%, respectively. Such biomass yields values are in the range for *P. pastoris* cultures (Looser *et al*., 2015). Besides the fact that *P. pastoris* does not produce anaerobic by-products even under oxygen limited conditions (Marín-Muñoz *et al*., 2019).

Besides the possible oxygen limitation on the shake flask cultures during growth on glycerol; the low specific growth rate achieved during the methanol induction phase may be due to the lack of methanol between intervals of supply. With the k_La used in the shake flask cultures the maximum OTR for these cultures is of 22 mmol/L h ($OTR_{max} = k_La \cdot C^*$; where $C^* = 0.2$ mmol/L for the conditions used). The specific rate of oxygen consumption (q_O) for *P. pastoris* cells growing on methanol is around 1.1 mmol/g_{DCW} h (Charoenrat *et al*., 2005). For the biomass produced at the end of the glycerol batch phase corresponds an oxygen uptake rate (OUR) of 6 mmol/L h; which is lower than the OTR that can be supply by the shake flasks. During the methanol induction, biomass achieved up to 8.5 g_{DCW}/L which corresponds an OUR of 9.35 mmol/L h. It seems, there was enough oxygen

supply to the cultures for methanol assimilation (Charoenrat *et al*., 2005). Nevertheless, it is possible that the lack of methanol depleted the production of VA*Eh*, as well as biomass, which were almost static during the methanol induction phase (Fig. 1). This also lead that the volumetric productivity decreased inversely proportional to the fermentation time (Fig. 1(C)).

The results indicated, there is necessary an adequate supply of methanol to keep on the production of biomass and recombinant protein under the control of the p*AOX1* (Cos *et al*., 2006). As can been seen in Table 1, a greater methanol supply produced more VA*Eh*. For an optimal induction of the p*AOX* it has been reported that proper levels of methanol between 0.25 to 0.5% are required; due to an excessive methanol concentration between 0.5 to 2.5%, can be cytotoxic and lead to growth inhibition (Potvin *et al*., 2012). In spite to the low rate of growth of the cells in shake flask cultures probable caused by lack of methanol; with concentrations beyond 0.5%, the improvement in VA*Eh* production can be linked to shorter periods of substrate starvation. When more inducer substrate was supplied, it was kept up-regulated the p*AOX* between pulses, allowing a larger expression of the recombinant gen.

Despite the decrease in biomass production as methanol supplied increased, production of the VA*Eh* protein increased as the volume of methanol added (Fig 1(B)). Both VA*Eh* production and volumetric productivity increased close of three times with the use of 3% of methanol in comparison with the supply of 0.5%. However, product-substrate yield was the same between treatments around 0.5 mg/g (Table 1). The specific production rate (q_P) increased 35% when it was used 3% of methanol in comparison with the use of 0.5 and 1%, methanol, which reached similar q_P around 0.1 mg/g_{DCW} h in both methanol concentrations. In comparison, production of VA*Eh* in the shake flask cultures was improved with those previous reported results of 3.8 μg/mL (Martínez-Hernández *et al*., 2017). The higher methanol concentration supplied in shake flask cultures is in the range for the Mut^+ strain at which it can be assimilated adequately without causing cell intoxication or lysis (Cos *et al*., 2006). Also, as was discussed above, there were more posibilities for lack of methanol in shake flask cultures than an excces which can cause cell damage (Jahic *et al*., 2003). Then, the increasing in the VA*Eh* concentration is asociated with the increase in the production as well (Fig. 2).

The enhancement in VA*Eh* production reported here may be associated with a higher methanol supply for the induction as that used by those authors, and by the improve in *P/V* and k_La achieved in the baffled shake flask against that achieved in smooth shake flasks, improving homogeneity in broth and oxygen supply. The results obtained, indicated that the yeast strain used is able to produce VA*Eh* with methanol concentration in broth up to 3% without inhibition of protein expression with a positive effect by the methanol supply. The results also show that the increase in methanol supply beyond 3% may did not duplicate the VA*Eh* production as it was observed between the use of 0.5 and 1.0% treatments for methanol supply.

3.2 Production of VAEh by a simplified methanol fed-batch fermentation in stirred tank bioreactor

As well, *Pg/V* is one of the main technical factors to maintain the feasibility to scale-up a bioprocess, we have considered this parameter as our scale-up criterion. Following this approach, we have made the scale-up process from shake flask to fermenter by matching volumetric power supply. We proposed to use *Pg/V* as the scale-up criterion, due to *Pg/V* does not create heterogeneities as k_La does. Thus, we can make an approach where we maintain the main aspects of the mixing in culture, and at the same time we can fix a parameter to consider whether or not feasible to develop a scale-up process according to the maximum *Pg/V* available at commercial scale. By keeping similar volumetric power input between shake flask and the STB we achieved k_La values for shake flask and STB of 110 and 152 h^{-1}, respectively. The improvement in the k_La under similar volumetric power supply is due to the improvement in mixing and gas supply in the stirred fermenter in comparison with the baffled shake flask (Büchs, 2001).

To match both *P/V* and *Pg/V* values between shake flask and the STB, it was necessary to use a stirring rate up to 1100 min^{-1}; which is a rate commonly used in *P. pastoris* bioprocesses in stirred fermenter with impellers whit a geometric relation impeller diameter to vessel diameter between 1/3 to 1/2 (Lee *et al*., 2003; Charoenrat *et al*., 2005; Gurramkonda *et al*., 2009; Potvin *et al*., 2012). A simple fed-batch bioprocess with methanol was developed under proper and feasible conditions for the production of VA*Eh* in STB, as main equipment used at commercial scale for such kinds of bioprocesses.

Table 2. Production and kinetics of VA*Eh* in the scaled up bioprocess to 3 L stirred tank bioreactor (*Pg/V* = 2.9 W/L and k_La = 152 h^{-1}).

VA*Eh* concentration (mg/mL)	$Y_{P/S}$ (mg/g)	μ_{MeOH} (h^{-1})	q_P (mg/g_{DCW} h)	Volumetric productivity (mg/L h) *
0.43 (0.19)	2.93 (0.79)	0.01 (0.00)	0.07 (0.03)	3.75 (1.53)

* Volumetric productivity at the end of the fermentation time.

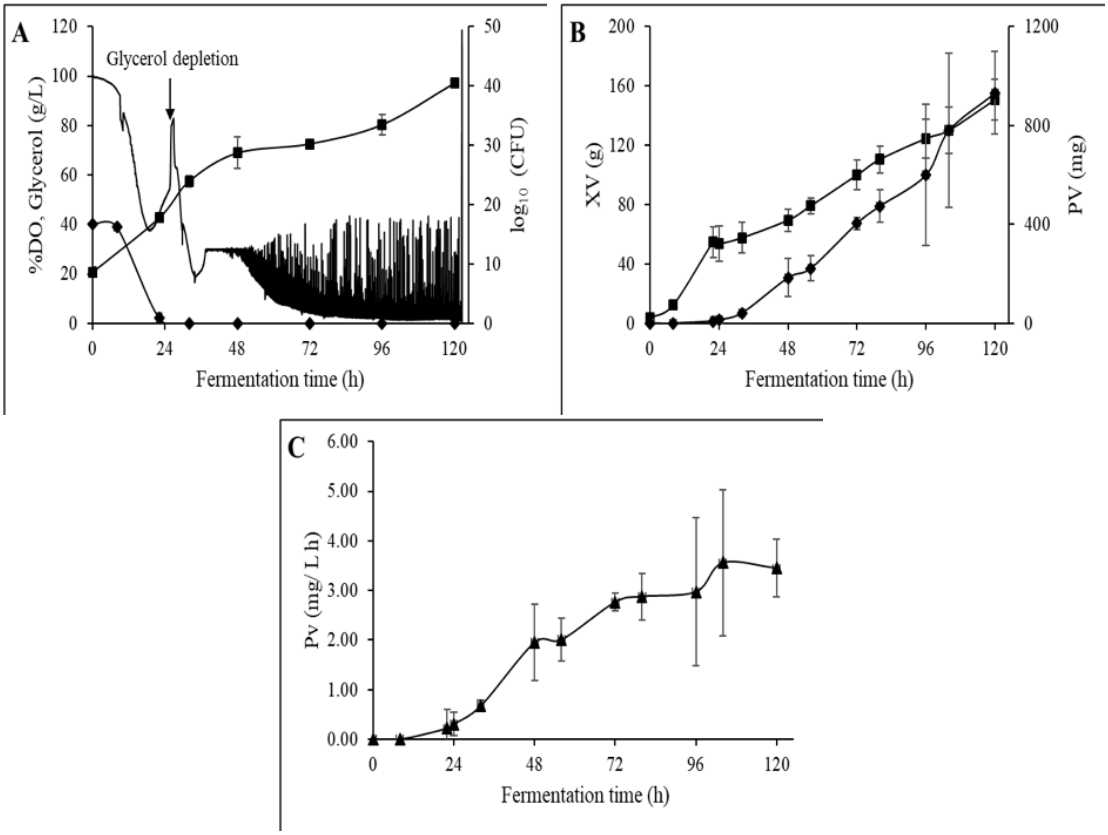

Fig. 3. Bioprocess kinetics from the fed-batch cultures in stirred tank bioreactor at *Pg/V* and k_La of 2.9 W/L and 152 h^{-1}, respectively. (A) The DO profile (−) in both batch glycerol and fed-batch methanol phases, which also was used as an indirect response for the metabolic state of the cultures as glycerol consumption (♦) and cell culture viability (■). (B) Dry biomass production (■) and total protein production on broth from the fed-batch bioprocesses (●). (C) Volumetric productivity of VA*Eh* production in fed-batch bioprocesses. *In Fig. 1(A) DO profile corresponds only to one replica, other determinations are the mean ± DE valued from n=3.

Table 2 contains the results obtained from the production of VA*Eh* in fermenter, meanwhile in Fig. 3 is described the kinetics of the production of the biomass and the recombinant protein.

Cultures carried out with the operational conditions used in the fed-batch fermentations showed good reproducibility and robustness during growth in both glycerol and methanol stages, with low control of parameters due to the automatization in methanol supply. To produce enough yeast cell biomass for the methanol induction phase, it was only used a simple batch glycerol phase which produced up to 26 g_{DCW}/L after 22 h of culture. The specific growth rate reached also during growth on glycerol was up to 0.12 h^{-1}, which is three times larger than those achieved in shake flask cultures. The improvement in the growth rate is associated to the enhancement on the k_La achieved in the fermenter, which denotes the

importance of this scale-up criterion for *P. pastoris* bioprocesses.

As shown in Fig. 3(A), during glycerol consumption the bioreactor capacity for oxygen transport was enough for the yeast cells, since DO level did not drop beyond 40% of saturation. Even with the use of operational conditions as those found at large scale, without pure oxygen enrichment or increased internal pressure in vessel (Neubauer *et al*., 2016). After depletion of glycerol, methanol induction for VA*Eh* expression was immediately started. The DO outline showed in Fig. 3(A) is due to the methanol addition strategy used by automated-pulses, where each DO sharp indicates an instantaneous methanol addition. It may be recognized that under this strategy for methanol supply, the carbon source stayed limited in the broth to the yeast cells without methanol build up. Also, with this methanol feed strategy, it was avoided cell intoxication and growth inhibition, as can be seen in the colony forming unit (CFU) count Fig. 3(A). As biomass accumulated in bioreactor, after 72 h of cultivation the oxygen demand increased which can be observed by the approximation of DO lectures near to 0% of saturation. However, accumulation of biomass and VA*Eh* did not stop until the end of the bioprocess (Fig. 3), which in this case denotes the robustness of the fermentation.

On the other hand, volumetric productivity (Pv) of the VA*Eh* reached a maximum at 104 h (82 h of induction time) of 3.75 mg/L h (Fig. 3(C)). As well the tendency in VA*Eh* accumulation seems to increase as the induction time, volumetric productivity did not show the same tendency. For the growth of the yeast cell in methanol specific growth rate of 0.01 h^{-1} was achieved with a biomass methanol yield of 0.33 g_{DCW}/L. The improvement in the VA*Eh* yield and specific growth rate in fermenter compared to those in in shake flask cultures, indicates a successful scale-up process from shake flask to fermenter operated under common commercial conditions.

To follow the formation of extracellular VA*Eh* during the induction phase with methanol in the scaled-up cultures, SDS-PAGE analysis with cell-free supernatants were performed. As shown in Fig. 3 and 4 the increase of the protein content in the broth is attributed to an increase in the production of VA*Eh*. As it is observed, the main protein fraction in the cell-free cultivation broth is constituted for the recombinant product VA*Eh* (Fig. 4(A)). From densitometric analysis the band density associated to the VA*Eh* in the SDS-PAGE gel, increased his density up only from 90 to 120 h in in shake flask in comparation with 21 to 120 h in fermenter cultures (Fig. 2(B) and 4(B)). Which means that the recombinant protein production was kept during the induction time in both scales.

At the same time, other bands started to appear in the samples from the induction stage, specially in fermenter cultures (Fig. 4(A)). These bands are associated to the liberation of other proteins from the yeast due to cellular lysis phenomenon associated to the oxygen limitation during growth on methanol (Jahic *et al*., 2003; López-Pérez and Viniegra-González 2017). This oxygen limitation was more remarkable in fermenter than in shake flask cultures. The SDS-PAGE from VA*Eh* produced in shake flask cultures contains less bands from other proteins (Fig. 2(A)) as that obtained from the fermenter culture (Fig. 4(A)). As previous discussed, is reasonable there were not limited oxygen conditions in shake flask as in fermenter cultures.

Nevertheless, production of VA*Eh* in STB was improved up to 12 times in comparison to the highest obtained in shake flask cultures with 3% of methanol addition (Table 1 and 2). In this way, an important improvement in VA*Eh* production was reached with the process scale-up from shake flask to STB, despite the limited oxygen conditions achieved in this last. This means an improvement in overall availability of the vaccine in case of high demand, at least for research purposes. The yield of product substrate and volumetric productivity did increase up to 6 and 14 times with respect to shake flask production, respectively. In spite to the overall improvement in bioprocess due to the scale-up, q_P did not increase in the fed-batch bioprocesses as it was expected; instead, this parameter decreased up to 100% with respect to the obtained in shake flask cultures with the 3% of methanol supply. The reduction in q_P can be associated to the lysis phenomenon associated to the oxygen limitation in fermenter cultures, which increases the biomass as well not the production of the VA*Eh*. Still, due to the improvement in biomass production in the stirred tank bioreactor the production of VA*Eh* also was enhanced.

The q_P and μ_{MeOH} obtained from the fed-batch fermentations (Table 2) are in the range of those obtained for Mut^+ recombinant strains (Looser *et al*., 2015). Also, the lower q_P achieved in fed-batch cultures could be associated to the methanol limitation observed in the fed-batch cultures in the STB. The substrate utilization to produce the recombinant vaccine during the induction phase was also enhanced, related to an improvement in bioprocess control and the oxygen transfer rate in the STB.

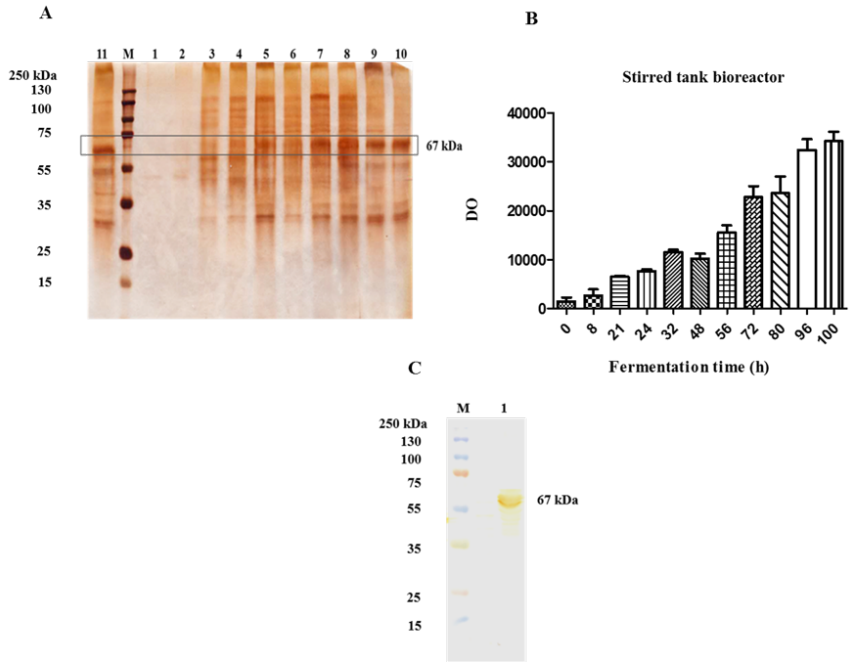

Fig. 4. Recombinant protein analysis by electrophoresis and western blot from protein produced in STB. (A) SDS-PAGE from cell-free broth taken at different times during the fermentation. Lane M protein molecular weight ladder (in each pattern contains the same molecular markers); lane 1-3 are samples during the batch stage on glycerol (0, 8 and 21 h of fermentation time, respectively); lanes 4-11, samples during fed-batch stage with methanol (samples from 24, 32, 48, 56, 72, 80, 96 and 100 h of fermentation time). (B) Densitometric analysis of the band obtained corresponding to molecular weight of 67 kDa. (C) Western blot analysis of the produced VA*Eh* with the rabbit anti-6X His (lane 1) with a molecular weight of 67 kDa.

As specific rates (μ and q_P) and yields are in the range to those observed by Mut$^+$ strains (Looser *et al*., 2015), it is demonstrated that the fed-batch bioprocess here proposed may be feasible to be scaled up and used for other recombinant strains of *P. pastoris*. Since the operational conditions here used are in accordance to those attainable in large scale applications (Neubauer *et al*., 2016), it is expected to obtain similar yields of VA*Eh* at commercial scale as those obtained here at bench scale.

To increase the OTR in large scale fermenters it is common to increase the inner vessel pressure. However, increasing inner pressure carry out to different disadvantages in *P. pastoris* cultures like (i) increases the carbon dioxide solubility, which is a common antimicrobial agent; (ii) a higher oxygen equilibrium concentration may result in toxic side-effects of molecular oxygen, in particular when methanol is oxidized (Potvin *et al*., 2012); (iii) it has been proposed that high pressures can lead to some sort of stress in *P. pastoris* cells when growing on methanol, causing cell death and lysis reducing biomass yield and increasing the maintenance energy demand (wasting carbon substrate) (Charoenrat *et al*., 2006). Taking into account these issues, our bioprocess here proposed does not use high pressure to increase oxygen solubility; leading the bioprocess to operate under oxygen limitation, which may represent and advantage to improve cell productivity of *P. pastoris*. Besides, we attempt to keep constant the operational conditions used in the fermenter culture up to the commercial scale, which means do not increase the pressure or use pure oxygen streams.

As well there are different way to carry out the methanol feed to the culture these either need a direct methanol measurement in broth or are based on pre-stablished mass balance methanol feeding rates. When pre-stablished methanol feed is used, the methanol concentration can be susceptible to significant deviations from optimal methanol concentration and even periods of exhaustion and build-up can be observed, making difficult the process standardization

(Cos *et al*., 2006). The bioprocess here developed does not use pure oxygen streams to control DO level and neither expensive nor sophisticated equipment to control methanol concentration at adequate level for the p*AOX* induction. This bioprocess could simplify the production of recombinant proteins induced by methanol, since it is not necessary the implementation of on/off-line methods to track the methanol concentration in the broth. The fed-batch mode used in this work is more robustness and easy to control during the methanol induction than using a pre-stablished methanol feed mode based on μ-stat method (Marín-Muñoz *et al*., 2019). Simple and high yield bioprocesses are of high interest to produce new pharmaceutical recombinant proteins.

In contrast with shake flasks, the adequate control of culture parameters such substrates concentration, pH and temperature of bioprocesses are improved with the use of fermenter systems as the STB, this leads a major control of the culture conditions which regulate the cells biological activity to properly produce the desired bioproduct. Besides the previous results obtained in shake flask, the VA*Eh* production increased in spite the limited methanol conditions achieved with the methanol feed mode used in the fermenter. The improvement in VA*Eh* production could be associated whit a better oxygen supply in the fermenter in comparison to shake flasks, as the k_La estimation indicates, and also due to the methanol limitation achieved during the induction. Limited methanol conditions are preferable to induction of the p*AOX* (Potvin *et al*., 2012), as well, a greater oxygen availability has a positive effect on the production of recombinant proteins under the p*AOX* (Lee *et al*., 2003).

Limited methanol conditions have been previously described by similar DO profiles as in Fig. 3(A), where it was observed an enhancement in the production of biomass and in the recombinant protein during the induction time (Markošová *et al*., 2015). In fed-batch cultures preformed the cell density achieved was moderate (60 g_{DCW}/L) in comparison with high-cell densities cultures above 100 g_{DCW}/L (Looser *et al*., 2015). This moderate biomass production may represent certain advantages for large scale bioprocesses, among others: (i) decrease in heat production, (ii) lower oxygen demand and (iii) improvement in product downstream processing with less biomass to handle. This kind of features are also of high interest for commercial purposes.

As is shown in Fig. 3(A) and (B) as biomass increased, the oxygen transfer supply by the bioreactor was exceeded by the culture oxygen uptake which is a phenomenon associated to oxygen limited conditions (Gurramkonda *et al*., 2009). In this kind of oxygen limited bioprocesses is recommended to use saturated methanol concentration in broth to improve the production of recombinant proteins under the control the p*AOX* (Khatri *et al*., 2006). With our bioprocess, control for saturated methanol conditions are not necessary to produce the VA*Eh* protein, although DO level was not controlled at a given set point. However, volumetric productivity started to decline after 104 h of bioprocess, which is associated with a lower production rate and the length of the induction time in the bioprocess. In this case, periods of fermentation beyond 120 h are not necessary to achieve the maximum volumetric productivity, remaining the bioprocess in shorter periods of fermentation as those generally used to produce recombinant proteins by *P. pastoris* (Potvin *et al*., 2012; Looser *et al*., 2015).

Conclusions

It was successfully developed and transferred a feasible fed-batch bioprocess from shake flasks to stirred tank bioreactor to produce a recombinant vaccine against *E. histolytica*, by keeping similar volumetric power input even the geometrical differences between bioreactors. The simplified fed-batch methanol process performed in fermenter with operational conditions attainable to commercial scale bioprocesses may improve the production and availability of the vaccine to cover an expected demand for its use in public health schemes or at least for further research activities. The bioprocess here proposed simplifies the production of the recombinant proteins, since the use of pure oxygen and a tight regulation of methanol concentration is easily performed in culture through a DO feed-back control loop. DO probes are standard in all current fermenter systems which may be configurated in accordance to the bioprocess. Besides the methanol and oxygen limitation in bioprocesses, the yields obtained were comparable to those observed in Mut^+ strains, then saturated methanol conditions are not necessary to compensate limited oxygen conditions.

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Nomenclature

d	maximum inside diameter of shake flask, m
DO	dissolved oxygen concentration in broth, mmol/L
DCW	dry cell weight
k	plug transfer coefficient in shake flask, m^3/h
k_La	volumetric oxygen transfer coefficient, h^{-1}
M	dimensionless Henry's law constant
n	shaking frequency, min^{-1}
OTR	oxygen transfer rate; mmo/L h
P	product concentration, mg/mL
P/V	volumetric power input for shake flask, W/L
Pg/V	gassed volumetric power input for the stirred tank bioreactor, W/L
P_V	volumetric productivity, mg/L h
q_P	specific production rate, mg/g_{DCW}, h
q_O	specific oxygen consumption rate, $mmol/g_{DCW}$, h
V	liquid volume of work, mL or L

Greek symbols

μ	specific growth rate, h^{-1}

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