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ARTÍCULO ORIGINAL

Fermentación en lote de Escherichia coli G-01 para la producción de la enzima penicilino acilasa

Batch fermentation of Escherichia coli G-01 for production of penicillin acylase enzyme

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† In memory of Dr. Jorge Martínez Silva, professor, researcher, colleague and sincere friend (08/10/1944-15/09/2008)

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Recibido: 2023-09-23

Aceptado: 2024-04-16

RESUMEN

La enzima penicilino acilasa es altamente demandada en la actualidad en la industria farmacéutica para la obtención de antibióticos. Por ello, su producción a partir de cultivos bacterianos mediante fermentación en gran escala constituye una línea de investigación relevante en esta área. En el presente trabajo, se describe la obtención de penicilino acilasa mediante la fermentación en lote de la cepa G-01 de Escherichia coli en un medio sintético. Se utilizó ácido fenilacético como fuente de carbono e inductor de la biosíntesis de la enzima. La fermentación se realizó inicialmente en 1,5 L de volumen efectivo (VE), y posteriormente se escaló hasta 30 L de VE. La producción de la enzima tuvo lugar desde la fase exponencial media hasta la exponencial tardía del crecimiento bacteriano, en ambas escalas. Igualmente, en las dos escalas estudiadas, la cinética de solubilización de O2 es similar, con los valores mínimos observados a las 10 h de fermentación durante 4 h. Posteriormente, se incrementó el gas soluble, acompañado de un aumento de pH desde 7,5-7,6 hasta 8,0. Estos últimos eventos se tomaron como indicadores del final de la fermentación. No se detectaron diferencias en la densidad y viscosidad del cultivo bacteriano al inicio y al final de la fermentación en 1,5 L. Los parámetros agitación y flujo de aire son críticos para lograr, en volúmenes grandes, resultados similares a los obtenidos en las fermentaciones a menor escala. Una agitación de 350 rpm y una aireación de 1,0 vvm en 30 L de VE permitieron obtener valores de actividad enzimática, crecimiento del cultivo y productividad volumétrica iguales a los obtenidos con el fermentador de 1,5 L (500 rpm y 1,0 vvm).

Palabras clave: escalado, *Escherichia coli*, fermentación en lote, penicilino acilasa

ABSTRACT

At present, penicillin acylase enzyme is highly demanded by the pharmaceutical industry for the antibiotics obtainment. For this reason, its production from bacterial cultures by high-scale fermentation is a relevant research line in this field. In this work, the obtainment of penicillin acylase by batch fermentation of *Escherichia coli* G-01 strain in a synthetic medium is described. Phenylacetic acid was used as carbon source and inductor of the enzyme biosynthesis. Fermentation was initially performed in 1.5 L of effective volume (EV), and later was scaled-up to 30 L of EV. The enzyme was produced from the medium to the late exponential phase of bacterial growth, at both scales. Equally, at both studied scales, the variation of the dissolved O_2 was similar, with minimal values observed at 10 h of fermentation for 4 h. Afterward, the soluble gas was increased, accompanied of a pH increase from 7.5-7.6 to 8.0. These last events were taken as indicators of the fermentation end. It was not detected differences in density and viscosity of the bacterial culture at the beginning and the end of the 1.5 L-fermentation. The parameters stirrer speed and air flow rate are critical to achieve, in high volumes, similar results to those obtained in lower-scale fermentations. A stirrer speed of 350 rpm and an air flow rate of 1.0 vvm in 30 L of EV allowed obtaining values of enzymatic activity, culture growth and volumetric yield equal to those obtained with the 1.5 L-fermentor (500 rpm and 1.0 vvm).

Keywords*: batch fermentation, Escherichia coli, penicillin acylase, scale-up*

INTRODUCTION

Penicillin amidase or penicillin G acylase (E.C. 3.5.1.11) is an enzyme very abundant in nature. It has been identified in bacteria such as *Escherichia coli* (Erarslan *et al*., 1990; Dai *et al*., 2001), *Alcaligenes faecalis* (Kasche *et al*., 2003), *Arthrobacter viscosus* (Ohashi *et al.,* 1989), *Bacillus badius* (Rajendran *et al.,* 2011) and *Thermus thermophilus* (Torres *et al*., 2012). A similar enzyme, known as penicillin V acylase, is present in *Bacillus subtilis* (Olsson *et al.*, 1985) and *Fusarium oxysporium* (Lowe *et al*., 1986), among other species. A third enzyme, related with the previous ones, is ampicillin acylase, found in bacteria such as *Streptomyces lavendulae* (Torres *et al.*, 1999) and *Pseudomonas melangenum* (Kim and Byun, 1990).

Penicillin acylase catalyzes the hydrolysis of penicillin G to produce 6-aminopenicillanic acid (6-APA) and phenylacetic acid (Martínez-Hernández *et al*., 2010). This enzyme also catalyzes the inverse reaction: penicillin G synthesis. This property is exploited in the semi-synthesis of penicillins and cephalosporins (Spence and Ramsden, 2007; Volpato *et al.*, 2010; Cecchini *et al*., 2012; Srirangan *et al*., 2013). In addition, penicillin acylase hydrolyzes certain cephalosporins (Erarslan, 1993) and different acyl-amino acids, amides and esters (Cole, 1969). These properties determine the relevant practical applications and the industrial value of penicillin acylase, a protein included in the group of enzymes currently produced at great scale by pharmaceutical and food industries (Polaina and MacCabe, 2007; Srirangan *et al*., 2013). Since 60s years, several antibiotic-producer companies have developed and improved the process of penicillin acylase obtainment by fermentation. For this, they have increased the productivity of mutant strains obtained by genetic engineering (Spence and Ramsden 2007; Nucci *et al.*, 2010; Akkaya *et al*., 2012; Orr *et al*., 2012).

To optimize the production processes by fermentative way of industrially interesting enzymes, it is necessary take into account different factors, such as: regulation mechanisms for the biosynthesis of these molecules, temperature, pH, aeration conditions and formulation of the culture medium (Dutta, 2008; Nucci *et al.*, 2010; Orr *et al*., 2012). Although other bacteria and fungi able to produce penicillin acylase are known, the most used strains for the study and obtainment of this hydrolase belong to the *E. coli* species (Srirangan *et al*., 2013).

The biosynthesis regulation of E. coli penicillin acylase was first studied in the ATCC 9637 strain and its auxotrophic derivative for methionine, ATCC 11105 (Erarslan et al., 1990). The gene expression for this protein is induced by phenylacetic acid and is repressed by catabolite in the presence of glucose (Babu and Panda, 1991a; Chen et al., 2012; Srirangan et al., 2013). However, repression is also produced due to high concentrations of phenylacetic acid in the culture medium (Babu and Panda, 1991a,b,c; Dai et al., 2001). On

the other hand, it has been observed as regularity that the highest levels of enzyme production are achieved at temperatures lower than 30ºC (Dai et al., 2001; Srirangan et al., 2013). Distinct authors have reported the decrease in the enzyme synthesis, until a 23 %, in the presence of high values of dissolved O2 in the culture medium (Babu and Panda, 1991a,b; Nucci et al., 2010; Orr et al., 2012).

The G-01 strain of E. coli, obtained by the Genín Company in Mexico (derived from E. coli ATCC 9637), has the attractive genetic potential to produce acceptable levels of penicillin acylase in synthetic medium, using phenylacetic acid as carbon source and inductor, and without b-lactamases (Ramírez et al., 1994a,b; Ospina et al., 1995, 1996). Therefore, the objective of this work was to establish the appropriate experimental conditions and operational parameters to produce the penicillin acylase enzyme by batch fermentation of E. coli G-01 in 1.5 and 30 L of effective volume (EV).

MATERIALS AND METHODS

MATERIALS

The *E. coli* G-01 strain was obtained from the Genín Company (Mexico). All used reagents were of reactive quality and, if other source is not indicated, were purchased in the Merck Company (Germany). The fermentor electrodes were supplied by the Ingold Company (Switzerland).

CULTURE MEDIUM

A synthetic medium (4.61 g/L NH_4Cl, 4.07 g/L KH_2PO_4, 0.277 g/L K_2SO_4, 0.057 g/L $CaCl_2$, 0.077 g/L $FeSO_4 \bullet 7H_2O$, 0.213 g/L $MgSO_4 \bullet 7H_2O$) with 5 g/L phenylacetic acid as carbon source and inductor was used.

FERMENTATION AND SCALE-UP

Aliquots of 150 mL culture medium were inoculated with colonies of E. coli G-01 grown over night at 37ºC in the same medium supplemented with 2 % agar. The resultant cultures were incubated overnight at 29ºC and 220 rpm in orbital shaking (shaker INFORS TR 125, Switzerland). Fermentations were performed in 1.5 and 30 L of EV in the synthetic culture medium for 20 h at 29ºC, using as inoculum the 10 % of fermentation EV of the previous cultures. Fermentor geometrical relationships are shown in Table 1.

Fermentors MBR AG (Switzerland) were used, with agitators of kind Rushton turbine, equipped with modules for the control of stirrer, temperature, air flow, dissolved oxygen and pH. In this manner, the temporal course of dissolved O2 concentration and pH was followed. The pH was kept between 7.5 and 7.6 by addition of 10 % ammonium hydroxide or phosphoric acid (Merck, Germany). Liquid density (r) and viscosity (m) were assessed at the beginning and end of the 1.5 L -fermentation, using a densitometer and a rotatory viscosimeter RN MLW (Germany) at 29ºC.

Tabla 1. Parámetros de las relaciones geométricas de los fermentadores utilizados

Table 1. Parámetros de las relaciones geométricas de los fermentadores utilizados

Parameters	1.5 L scale	30 L scale
H/D	3	9.4
H_L/T	1.27	3.46
W/D	0.26	0.24
# impellers	2	3
W	0.012	0.02
# blades	6	6

H: total height. D: impeller diameter. H_L: liquid height. T: total vessel diameter. W: impeller blade width (m).

For 1.5 L-fermentation a stirrer speed (N) of 500 rpm and an air flow rate (Q) of 1.0 vvm were used. Due to the 1.5 L-fermentation comply with restrictions reported by Rushton et al. (1950), ungassed power consumption (P) was estimated by calculating the Reynold number (Re; allows characterizing the fluid movement; Schmidt, 2005):

$$Re = ND^2\rho/m \quad (1)$$

P was fitted using a geometrical correction factor and the number of impellers for used turbines, according to Aiba et al. (1973). Gassed power consumption ($Pg_{1.5}$) was estimated using the model reported by Joshi et al. (1982), substituting the P previously calculated:

$$Pg_{1.5}/P = 0.1\ (NV/Q)¼\ x\ (N^2D^4\ /\ (gWV^{2/3}))^{-1/5} \quad (2)$$

Where V is working tank volume, Q is the air flow rate necessary to supply the oxygen needed by fermentation, g is gravitational acceleration.

For 30 L-fermentation, N was calculated using the criteria expressed in the equation 3:

$$(Pg/V)_{1.5}=(Pg/V)_{30}=N^3D^2 \quad (3)$$

Pg for 30 L-fermentation (Pg30) was calculated according to equation 4:

$$Pg_{30}=(Pg/V)_{1.5}V_{30} \quad (4)$$

Q to apply in this scale was calculated using equation 2. In this manner, it was determined that the recommendable values of N and Q for 30 L-fermentation are 326 rpm and 1.4 vvm, respectively.

Fermentation was performed with the N and Q parameters selected for both scales, considering the conditions distant from the flooding phenomenon. In addition, it was decided to test a second condition for 30 L scale, with a higher N (350 rpm) and a lower Q (1.0 vvm), according to the report of Biesecker (1972). Assays were replicated nine times for 1.5 L-fermentation and three times for 30 L-fermentation.

ASSESSMENT OF GROWTH OF BACTERIAL CULTURE

The bacterial culture growth was assessed by the turbidimetric method reported by Harley and Prescott (2002), performing lectures at 560 nm in a spectrophotometer Ultrospect UV-VIS (Pharmacia-LKB, Sweden).

ASSESSMENT OF ENZYMATIC ACTIVITY

The acylase enzymatic activity was determined by the method reported by Balasingham *et al.* (1972), using *p*-dimethylaminobenzaldehyde and 6-APA as substrates. 6 -APA (Sigma Chemical Co., USA) was used as standard. The enzymatic activity unit (U) was defined as the enzyme amount required to consume 1 mmol 6APA per min at 37ºC and pH 7.8 in 100 mM potassium phosphate buffer. Each assay was performed nine times, for 1.5 L-fermentation, and three times, for 30 L-fermentation.

PROTEIN CONCENTRATION ASSAY

Protein concentration was assessed by the Bradford method (Bradford, 1976), using bovine serum albumin as standard protein.

RESULTADOS

As is shown in figure 1 (green curve), in the 1.5 L-fermentor and the experimental conditions tested in this work, the *E. coli* culture grew exponentially for 12 h, moment in which the stationary phase was reached. The production of the penicillin acylase enzyme (Fig. 1, blue curve) began in the medium exponential phase (approximately at 5 h fermentation) and it was extended until the late exponential phase (around 11 h) of the bacterial growth. A similar result was obtained in the 30 L-fermentation (data not shown).

Equally, the variation of the dissolved O_2 in 1.5 L of EV is similar to that of the 30 L-fermentation, at N = 500 or 350 rpm, respectively, and Q = 1.0 vvm (Fig. 2). After 2 h fermentation, the dissolved O_2 concentrations began to decrease, reaching their minimal levels at 10 h approximately. These minimal levels were maintained for around 4 h. At this moment, the dissolved O2 concentrations increased abruptly, matching a fast pH increase since neutral-nearly values to approximately 8.0. These last events, commons for the fermentative processes at both studied scales, were selected as indicators of the fermentation end (Fig. 2). As is shown in table 2, there were not detected differences in ρ and μ parameters of the bacterial culture at the beginning and the end of fermentation performed in 1.5 L EV.

Table 2. Assessment of physical parameters density (ρ) and viscosity (μ) in the *Escherichia coli* G-01 culture at the beginning and the end of 1.5 L-fermentation

Tabla 2. Evaluación de los parámetros físicos densidad (ρ) y viscosidad (μ) en el cultivo de *Escherichia coli* G-01 al comienzo y al final de la fermentación en 1,5 L

Fermentation stage	ρ (kg/cm^3)	μ (Pa×s)
Initial	1009	0.002
End	1011	0.002

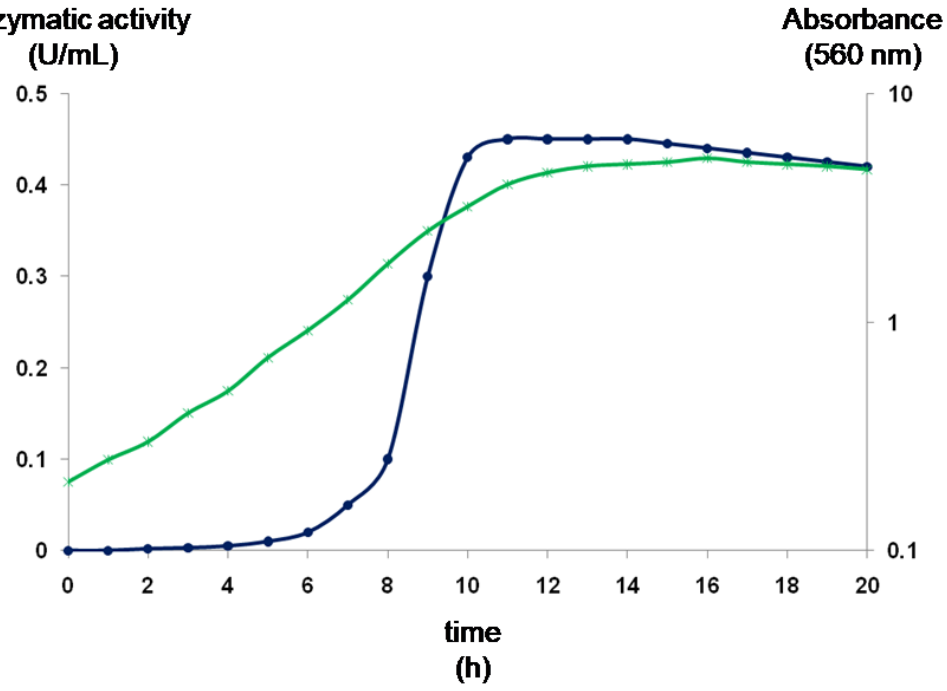

Figure 1. Production of the penicillin acylase enzyme from *Escherichia coli* G-01 during 20 h fermentation in 1.5 L effective volume. In green, the growth of bacterial culture is shown, at logarithmic scale. In blue, the temporal course of enzymatic activity is shown. Data are presented as the means of nine replicates (the standard deviations were lower than 10 % of the means in all cases).

Figura 1. *Producción de la enzima penicilino acilasa de Escherichia coli G-01 durante 20 h de fermentación en 1,5 L de volumen efectivo. En verde se muestra el crecimiento del cultivo bacteriano, a escala logarítmica. En azul se muestra el curso temporal de la actividad enzimática. Los datos se presentan como las medias de nueve réplicas (las desviaciones estándar fueron menores que el 10 % de las medias en todos los casos).*

In table 3, the operational parameters and obtained results in both studied fermentation scales are shown. Between the two tested conditions at the 30 L-scale, fermentation performed with an N = 350 rpm and a Q = 1.0 vvm allowed obtaining a value of enzymatic activity equal to the small-scale fermentation value. Culture growth and volumetric yield were similar in the two conditions of stirrer speed and aeration tested with 30 L EV and in 1.5 L-fermentation.

DISCUSSION

Penicillin acylase enzyme can be used as biocatalyst to condensate an acyl group with the b-lactam nucleus (6-APA or 7-aminocepha-losporanic acid). This reaction is used to produce semi-synthetic penicillins or cephalosporins (Cecchini et al., 2012; Srirangan et al., 2013). These drugs account for the largest fraction of global sales of antibiotics (Elander, 2003; Srirangan et al., 2013).

The industrial production of penicillin acylase, by fermentation of E. coli strains, has reached the scale of 250,000 L fermentors (Spence and Ramsden, 2007). The study of this fermentative process is very important, principally when high volumes of raw materials are used at industrial scale, and losses due to contamination or low yields should be avoided. In this sense, the exhaustive knowledge of the biology of this microorganism and its relationship with penicillin acylase biosynthesis is a

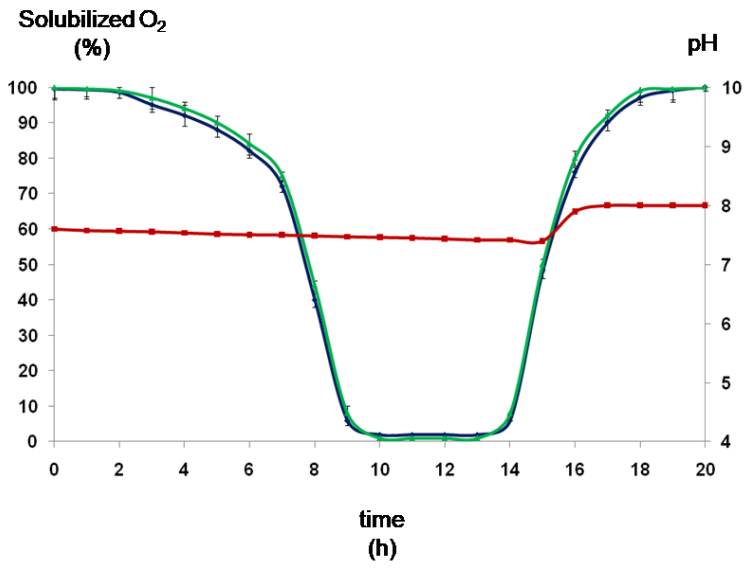

Figure 2. Temporal course of the dissolved O_2 and pH during *Escherichia coli* G-01 fermentation for production of penicillin acylase enzyme. A shaking of 500 rpm (for 1.5 L scale) and 350 rpm (for 30 L scale) and an air flow rate of 1.0 vvm were used. Dissolved O_2 concentrations in 1.5 L- (blue) and 30 L-fermentation (green) are shown. pH variation at both scales is shown in red. Data are presented as the means ± standard deviations (blue and green curves) and means (red curve) of nine replicates, for 1.5 L-fermentation, and three replicates, for 30 L-fermentation. The standard deviations were lower than 10 % of the means in the red curve.
Figura 2. Curso temporal de la disolución de O_2 y el pH durante la fermentación de *Escherichia coli* G-01 para la producción de la enzima penicilino acilasa. Se utilizaron una agitación de 500 rpm (para la escala de 1,5 L) y 350 rpm (para la escala de 30 L) y un flujo de aire de 1,0 vvm. Se muestran las concentraciones de O_2 disuelto en las fermentaciones en 1,5 L (azul) y 30 L (verde). La variación de pH en ambas escalas se muetra en rojo. Los datos se presentan como las medias ± las desviaciones estándar (curvas azul y verde) y las medias (curva roja) de nueve réplicas, para la fermentación en 1,5 L, y tres réplicas, para la fermentación en 30 L. Las desviaciones estándar fueron menores que el 10 % de las medias en la curva roja.

Table 3. Operational parameters and obtained results with both tested fermentation scales for production of penicillin acylase enzyme from *Escherichia coli* G-01
Tabla 3. Parámetros operacionales y resultados obtenidos con ambas escalas de fermentación evaluadas para la producción de la enzima penicilino acilasa a partir de *Escherichia coli* G-01

Effective volume Fermentor (L)	N (rpm)	Q (vvm)	Final OD 560 nm	Enzymatic activity (U/L)	Volumetric yield (U/L/h)
1.5	500	1.0	6.2 ± 0.6	423 ± 12	21 ± 2
30	350	1.0	5.8 ± 0.4	403 ± 17	20 ± 2
	326	1.4	6.1 ± 0.6	373 ± 14	19 ± 2

Initial pH was adjusted at 7.6 and temperature was maintained at 29ºC. Data are presented as the means ± standard deviations of nine replicates, for 1.5 L-fermentation, and three replicates, for 30 L-fermentation.

decisive factor to accomplish the objective of all fermentative process.

Penicillin acylases are classified into constitutive and inducible. The first ones are enzymes whose expression is associated to the culture growth. The production of the second is related with the microorganism secondary metabolism (Tishkov *et al*., 2010; Srirangan *et al*., 2013). Inside of the first group are, among others, the majority of the enzymes specific for penicillin V, such as those from *B. sphaericus* and *B. plumbea* (Mahajan, 1984). The greater part of penicillin acylases specific for penicillin G, such as those from *E. coli, Kluyvera citrophila* and *B. megaterium*, are inducible enzymes (Vandamme and Voets, 1974; Dai *et al*., 2001; Srirangan *et al*., 2013). The specific inductor for inducible penicillin acylases, showing the highest capacity, is phenylacetic acid (Dai *et al*., 2001).

In this work, the fermentation conditions in 1.5 L EV were defined on the basis of results previously reported for the same *E. coli* strain (Ramírez *et al*., 1994a; Ospina *et al*., 1996). The growth of *E. coli* G-01 cultures (Fig. 1, green), in the tested experimental conditions and culture medium, match the classic model of growth previously reported for this microorganism (Vandamme, 1988; Ramírez *et al*., 1994a). The variation of the dissolved O_2 during fermentations at both scales (Fig. 2, blue and green) is also similar to that observed by these authors. In optimal experimental conditions for bacterial growth, the synthesis of *E. coli* penicillin acylase is inhibited by the increase of the dissolved O_2 concentration (De Leon *et al*., 2003; Supartono *et al*., 2008), by the presence of glucose, glycerol or maltose (catabolite repression) or increasing the temperature at 37ºC (the enzyme is produced at temperatures lower than 30ºC) (Dai *et al*., 2001). In fact, the highest increases in penicillin acylase activity, between 8 and 10 h (Fig. 1, blue), match (with a 1 h-delay) the highest decreases of the dissolved O_2 concentrations, between 7 and 9 h (Fig. 2, blue and green). In this manner, the increments of the dissolved O_2 and pH (Fig. 2) were selected as practical criteria to stop fermentation.

The research in the fermentations field not only takes into account biological aspects, inherent to the microorganism of interest, but also it is related with process engineering and scale-up (Fig. 3), mainly when industrial objectives are pursued.

The invariability of culture ρ and μ parameters, between the beginning and the end of 1.5 L-fermentation (table 2), could be due to the low biomass levels obtained in the used synthetic medium (table 3), as well as the absence of compounds in the culture that produce changes in these parameters. Since N and Q used in the 1.5 L-fermentation (table 3) allowed obtaining similar results to those previously reported for *E. coli* G-01 strain (Ramírez *et al.*, 1994a), these conditions are suitable for this process at the indicated scale. Further, N = 350 rpm and Q = 1.0 vvm are the most appropriated conditions, between the studied ones in this work for 30 L-fermentation, since them allow obtaining similar results to those of the small-scale process (table 3).

For the scale-up of fermentative processes, generally are performed correlations that define specific conditions, and that can be used as theoretical tools to select the process dominant criterion. For example, the volumetric oxygen transfer coefficient (K_La) depends on operational parameters, P and the air superficial velocity (v_s) (Schmidt, 2005). On the other hand, in the fermentations scale-up it is also possible to use distinct criteria and/or combinations of these (Aiba *et al*., 1973). This depends on the system features, as in aerated fermentations, where K_La can be the dominant criterion. Since this has not been demonstrated for the fermentation performed in this work, it is very attractive to perform evaluations with other criteria and combine them with the based one on K_La. According to Aiba *et al*. (1973), the most used criteria are: (1) K_La (Kim and Jagannadha, 2003; Losada-Nerey *et al*., 2017). (2) Power per volume unit, equation 3 (Zhi-Hua and Jian-Ping, 2004). (3) Reynold number, equation 1 (Schmidt, 2005). According to Rushton *et al*. (1950), it is possible to calculate P using the Re (equation 1). Equally, other researchers have reported the possibility of calculate Pg from the volumetric oxygen transference rate (Na) (Oyama and Endoh, 1955; Michel and Miller, 1962).

Optimal air dispersion strongly depends of stirring and system geometry, imposing conditions to Q. For this reason, there is a critical Q value, from which air dispersion does not increase and appears the phenomenon known as flooding (Biesecker, 1972). Taking into account these theoretical principles, it is possible to predict N and Q recommendable conditions to define the most promising levels in order to obtain an acceptable result in the higher scale.

One of the main objectives of fermentative processes scale-up is to maintain, and even to increase, the yield achieved in lower scales. On the basis of this criterion, it is possible to state that the scale-up methodology used in this work was successful (table 3), even when the geometrical relationships of the systems at both scales were different (table 1). However, it is possible to perform a further optimization of these results, in order to increase the yields and the production efficiency of this enzyme.

Notation

D impeller diameter (m)

H total height (m)

H_L liquid height (m)

g gravitational acceleration ($m.s^{-2}$)

N stirrer speed (s^{-1})

P ungassed power consumption (W)

P_g gassed power consumption (W)

Q air flow rate ($m^3.s^{-1}$)

Q_g volumetric gas flow rate

Re Reynold number (dimensionless)

T total vessel diameter (m)

V working tank volume (m3)

W impeller blade width (m)

ρ Density (kg.m−3)

μ Viscosity (Pa·s)

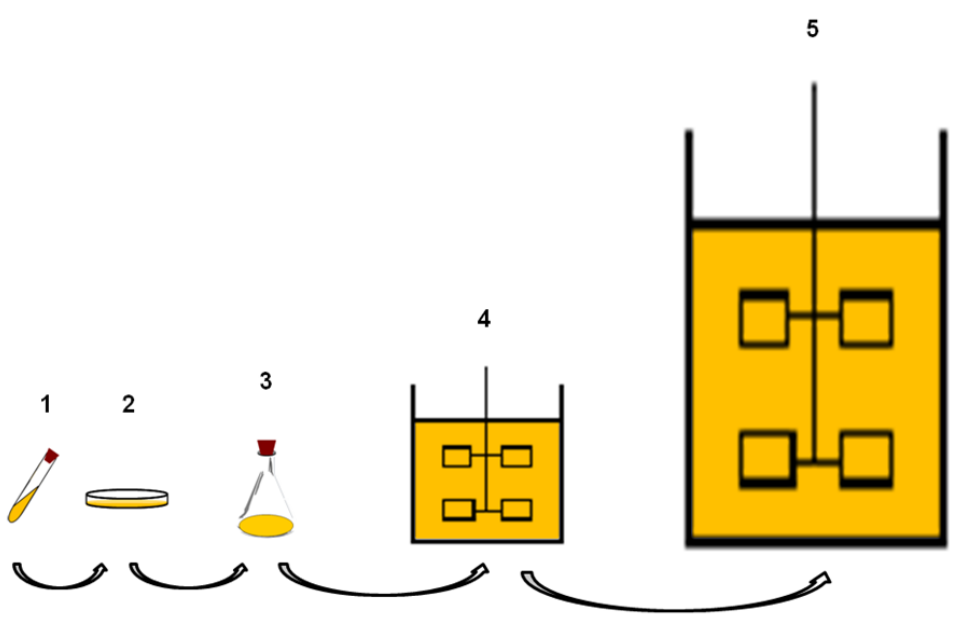

Figure 3. Scheme for a fermentative process since the strain to the highest scale. 1) Conserved strain. 2) Refreshment in Petri dish. 3) Fermentation in shaker. 4) Fermentation in 1.5 L-fermentor. 5) Fermentation in 30 L-fermentor.

Figura 3. Esquema de un proceso fermentativo desde la cepa hasta la mayor escala. 1) Cepa conservada. 2) Refrescamiento en placa de Petri. 3) Fermentación en zaranda. 4) Fermentación en fermentador de 1,5 L. 5) Fermentación en fermentador de 30 L.

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