RESEARCH

Induction optimization and scale-up of the fermentative process for the p-41 protein expressed in Escherichia coli

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ABSTRACT

A representative region of the gp41 transmembrane protein from the Human Immunodeficiency Virus (HIV) was expressed in *Escherichia coli* under control of the tryptophan promoter. Upon analysis of the influence of induction conditions on the production of the p-41 protein, it was noticed that while the expression levels were independent from the amount of the inducer at concentrations higher than 20 μg/mL of 3-β indoleacrylic acid, both the expression levels and the production showed a marked dependence on the concentration of tryptophan, used as a co-repressor. A fermentative process for production of the p-41 protein was established, based on derepression of the tryptophan promoter by the addition of 10 μg/mL of tryptophan to the culture medium. This process was scaled-up to 200 L, obtaining high levels of expression (25%) and production (321 mg/L) of the p-41 recombinant protein.

Key words: p-41 (HIV), tryptophan, 3-β indoleacrylic acid, scaled-up

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RESUMEN

Optimización de la inducción y escalado del proceso fermentativo de la proteína p-41 expresada en *Escherichia coli*. La región representativa de la proteína transmembránica gp-41 del virus de la inmunodeficiencia humana (VIH) fue expresada en *Escherichia coli* bajo el control del promotor triptófano. Al analizar la influencia de las condiciones de inducción sobre la producción de la proteína p-41, se observó que el nivel de expresión es independiente de la concentración del inductor, lo cual se evidenció a partir de 20 μg/mL de ácido 3-β indolacrílico. Además, se manifestó un efecto marcado en cuanto a la concentración del represor triptófano sobre el nivel de expresión y la producción de la proteína p-41. Finalmente, se estableció la producción de la proteína p-41 por derepresión del sistema de expresión, al añadir 10 μg/mL de triptófano en el medio de cultivo. El proceso fermentativo desarrollado se escaló a 200 L, y se obtuvieron altos niveles de expresión (25%) y de producción (321 mg/L) de la proteína recombinante p-41.

Palabras clave: p-41 (VIH), triptófano, ácido 3-β indolacrílico, escalado

*I*ntroduction

The Human Immunodeficiency Virus (HIV) is a retrovirus which constitutes the causative agent for the Acquired Immunodeficiency Syndrome (AIDS). During the first years after the discovery of HIV, the diagnostic systems to detect antibodies to the virus were based on the use of viral antigens purified from extracts of HIV-infected cells. This method did not yield representative amounts of each viral protein, affecting consequently the sensitivity and specificity of the early assays. The unraveling of the genomic structure of HIV made possible the development of new diagnostic systems based on the use of recombinant antigens, where the proportion of each viral protein could be adjusted; and thus led to the appearance of serologic assays that showed higher sensitivity and specificity than those based on viral lysates.

It is well known that essentially all the structural proteins from this virus are immunogenic during the natural course of the human HIV infection. However, most efforts in the development of diagnostic systems for HIV have concentrated on the cloning or synthesis of regions from the gp-41 protein, whose N-terminal region is highly conserved and immunogenic. We have cloned and expressed in *Escherichia coli*, under control of the tryptophan promoter, a representative region of the gene coding for the gp-41 protein from HIV-1 for its inclusion in diagnostic systems together with other recombinant portions from *gag* and *env* isolated from HIV-1 and HIV-2, respectively.

One of the most important stages during process development for the obtention of recombinant proteins is the reproduction in fermentors, at a manufacturing scale, of the results obtained earlier in flasks in the laboratory. However, there are almost no reports in the literature of the correlations for the scale up of the production of recombinant proteins.

This work describes the development of the fermentative process for the production of the p-41 protein from HIV-1 in *Escherichia coli*. The influence of the conditions selected for induction on the production of this protein was evaluated, and taken into account for establishing the final culture conditions at a laboratory scale. Finally, this process was scaled up to the level of 200 L cultures.

*M*aterials and methods

Bacterial strains and plasmids

The *E. coli* host strain used for expression was W3110 [1]. A 269 bp-long synthetic gene coding for a fragment of the gp41 transmembrane protein that contains its immunodominant epitope was ligated to a DNA fragment coding for the first 58 aminoacids from human interleukin-2 as fusion partner, and cloned into a plasmid under control of the tryptophan promoter

1. Sambrook J, Fritsch EF, Maniatis T, Molecular Cloning. A Laboratory Manual. Sec. Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor (NY); 1989.

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and the T4 phage gene 32 transcriptional terminator. The resulting expression vector, named pHIVTA-1 [2], codes for a 16.6 kDa fusion protein which will be referred to in this work by the name of p-41.

Preparation of the inoculum

A 0.1 mL aliquot from a Working Cell Bank (WCB) of the host strain transformed with the expression plasmid was used to inoculate a 5 mL culture of LB supplemented with 50 µg/mL ampicillin and 100 µg/mL tryptophan, which was then grown for 8 hours at 37 ºC on an orbital shaker (G-25 New Brunswick Scientific Co. Inc. EE.UU). These cells, collected at the early logarithmic growth phase, were then transferred to 100 mL of M9 medium [3] supplemented with 1% acid casein hydrolysate (Oxoid, UK), 2% glucose (BDH, UK), 50 µg/mL ampicillin and 100 µg/mL of tryptophan. This culture was grown again on an orbital shaker for 8 hours at 37 ºC and 250 r.p.m., and used as inoculum for all subsequent experiments. In all cases the starting optical density (O.D.) of the cultures was adjusted to a value from 0.05 to 0.1 units, at a wavelength of 530 nm.

Methodology for the study of the influence of the concentration of 3-β indoleacrylic acid as inducer

For small-scale cultures performed in shakers, 250 mL Erlenmeyers flasks containing 50 mL of M9 medium supplemented with 1% acid casein hydrolysate, 0.5% glucose, 0.1% yeast extract, 50 µg/mL ampicillin (production medium) and 20 µg/mL of tryptophan were used. After culturing for 1 hour, the tryptophan promoter was induced by the addition of different amounts of the inducer (3-β indoleacrylic acid) to achieve a final concentration of 0, 20, 40 or 60 µg/mL, and the culture was further grown at 37 ºC and 250 r.p.m. for 12 hours. For the measurement of growth kinetics in fermentors, culture samples were taken every 4 hours.

Methodology for the study of the influence of the concentration of tryptophan as a repressor

For small-scale cultures performed in shakers, 250 mL Erlenmeyer flasks containing 50 mL of production medium (see above) were used, supplemented with different starting concentrations of tryptophan (0, 10, 20, 40, 60 and 100 µg/mL) without the addition of 3-β indoleacrylic acid as an inducer. In these conditions, the expression system is induced upon depletion of the tryptophan present on the culture medium. The cultures were grown at 37 °C and 250 r.p.m., for a period of 12 hours. For the measurement of growth kinetics in fermentors, culture samples were taken every 4 hours.

Evaluation of the fermentative process for the p-41 protein at a scale of 50 L

The fermentative process for the p-41 protein was scaled to a volume of 50 L cultures by trial and error, on the basis of previous experiences from earlier work [4, 5]. The inoculum was transferred to a 7 L fermentor (5 L of effective volume) and cultured at 37 °C, 1 v.v.m. and 350 r.p.m. for 8 h, after which it was transferred to a 70 L fermentor containing 50 L of production medium. The fermentation was carried out at 37 °C, pH 7.0, 1 v.v.m. and 350 r.p.m. The expression system was induced under these conditions by the depletion, by cellular metabolism, of the tryptophan added to the culture media at a starting concentration of 10 µg/mL. The culture was grown for 12 h, taking samples every 4 h for following growth kinetics.

Scale-up to 200 L of the fermentative process for the p-41 protein

In order to scale up the fermentative process for the p-41 protein to a volume of 200 L, a correlation between the oxygen transfer coefficient and the operating variables of the system that takes into account the conditions established for 50 L was developed, based on the equation of Wang *et al.* [6]. By using this correlation on the parameters established for 50 L cultures, the optimal parameters for 200 L cultures were calculated to be 0.75 v.v.m. and 240 r.p.m., and a 200 L fermentation was set up to validate the effectiveness of the calculated conditions.

The 200 L culture was performed by transferring the inoculum to a 7 L fermentor (5 L of effective volume) and growing it at 37 °C, pH 7.0, 1 v.v.m. and 350 r.p.m. for 8 h. This culture was then transferred to a 300 L fermentor (B.E. Marubishi, Japan) containing 200 L of production medium. The expression system is induced under these conditions by the depletion, through cellular metabolism, of the tryptophan added to the culture at a starting concentration of 10 µg/mL. This culture was grown at 37 °C and pH 7.0 for 12 h, taking samples every 4 h for following growth kinetics.

Analytical assays

Measurement of cellular growth

Cellular growth was followed by turbidimetry. The O.D. of the cultures was measured at a wavelength of 530 nm, using a photocolorimeter (ERMA, Japan).

Measurement of expression levels

The levels of expression of the recombinant protein in whole-cell extracts were estimated by densitometry of stained electrophoretic polyacrylamide gels, using a laser densitometer (Pharmacia, Sweden).

Measurement of glucose concentrations

The concentration of glucose was assayed by the dinitrosalicylic acid method [7].

Statistical analysis of the data

Each reported value is the mean from at least 3 independent fermentative processes, and 3 independent replicates were made for each measurement.

Results

Influence of inducer concentration on the production of the p-41 protein

The fermentative process for the production of the p-41 protein was initially set up using 20 µg/mL of 3-β indoleacrylic acid as an inducer, as is most commonly reported in the literature for the expression of recombinant proteins under control of the tryptophan promoter [8]. However, this inducer is expensive and

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is therefore rarely used at pilot or industrial scales. This work studied the effect of the concentration of the inducer on the production of the p-41 protein, with the aim of reducing or eliminating its use on the large-scale fermentative process.

The studies of the influence of the inducer concentration were performed on cultures in flasks, grown in shakers, supplemented with 20 µg/mL of tryptophan. After a growth period of 1 h, the expression system was induced by the addition of different concentrations (0, 20, 40 and 60 µg/mL) of 3-β indoleacrylic acid.

There was only minimal expression of the p-41 protein in the absence of the inducer, since a tryptophan concentration of 20 µg/mL is enough for repressing the tryptophan promoter almost completely. At 3-β indoleacrylic acid concentrations higher than 20 µg/mL, the expression levels plateaued at around 20% of the total protein (Figure 1). A similar effect was seen after analyzing the effect of 3-β indoleacrylic acid on the volumetric production and the productivity of the process. In all cases, cellular growth was essentially the same.

The above results show that in order to eliminate the inducer from the fermentative process, it is necessary to evaluate the influence of tryptophan concentration on the production of the p-41 protein.

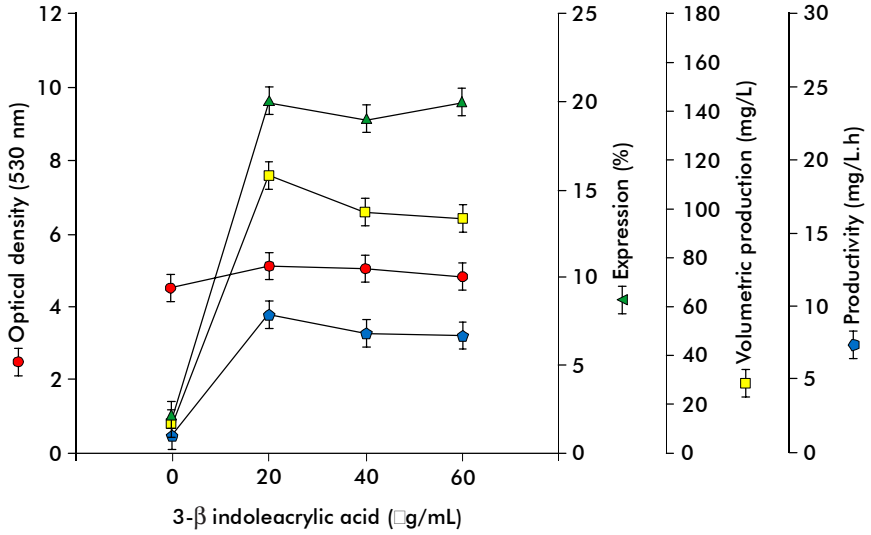

Figure 1. Influence of the concentration of the inducer (3-b indoleacrylic acid) on the production of the p-41 protein. Cultures were performed in 100 mL Erlenmeyer flasks containing the culture medium supplemented with tryptophan at 20 µg/mL. After 1 h of growth, the expression system was induced by the addition of different inducer concentrations (0, 20, 40 and 60 µg/mL of 3-β indoleacrylic acid). Growth conditions were 37 °C and 250 r.p.m.. The results charted correspond to 12 hours of growth.

Influence of tryptophan concentration on the production of the p-41 protein

The studies of the influence of tryptophan concentration were performed on cultures in flasks, grown in shakers, using production medium supplemented with different starting concentrations (0, 10, 20, 40, 60 and 100 µg/mL) of tryptophan, with no 3-β indoleacrylic acid added as an inducer. In these conditions the tryptophan promoter is induced through depletion, by the cellular metabolism, of the tryptophan initially present in the culture medium.

Figure 2 shows that in the absence or at low tryptophan concentrations in the culture medium (from 0 to 10 µg/mL) it is possible to achieve high expression levels (from 23 to 24%) of p-41. Tryptophan concentrations higher than 10 µg/mL decreased drastically the expression levels and therefore the production of the recombinant protein; however, the levels of cellular growth were not affected. These results prompted the establishment of culture conditions in which the expression system is induced by derepression, using 10 µg/mL of tryptophan in the culture medium in the absence of 3-β indoleacrylic acid.

Evaluation of the fermentative process for the p-41 protein at a culture scale of 50 L

The conditions established for the expression of p-41 in cultures in flasks were evaluated in 50 L cultures in fermentors. Figure 3A shows the results from a typical fermentation in which cellular growth increases with time until the O.D. reaches values of 11 after 12 hours of culture, with a growth rate in the logarithmic phase of 1.01 h^{-1}. However, the expression levels of the p-41 protein increased linearly with culture time, reaching 25% after 12 hours of growth.

The analysis of the volumetric production and the productivity of the fermentative process (Figure 3B) showed that both parameters increased with culture time until reaching values of 207.5 mg/L and 17.29 mg/L.h respectively at the end of the process. Furthermore, in these conditions the biomass/glucose and product/glucose yields reached values of 0.67 and 0.041, respectively.

Scale-up of the fermentative process for the p-41 protein to 200 L

In order to scale up the productive process to 200 L cultures, a correlation was developed between the oxygen transfer coefficient and the operating variables

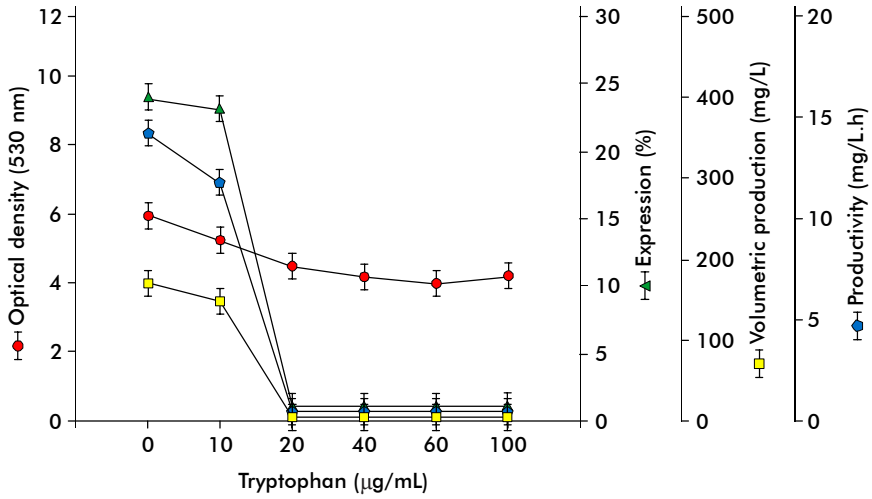

Figure 2. Influence of the concentration of tryptophan (used as a co-repressor) on the production of the p-41 protein. The cultures were performed in shakers, with 100 mL Erlenmeyer flasks containing the production medium supplemented with different starting concentrations of tryptophan (0, 10, 20, 40, 60 and 100 µg/mL), and in the absence of 3-β indoleacrylic acid as inducer. The tryptophan promoter was derepressed through the depletion of the tryptophan present on the culture media by the cellular metabolism. The growth conditions were 37 °C and 250 r.p.m. The results shown in the chart correspond to 12 hours of growth.

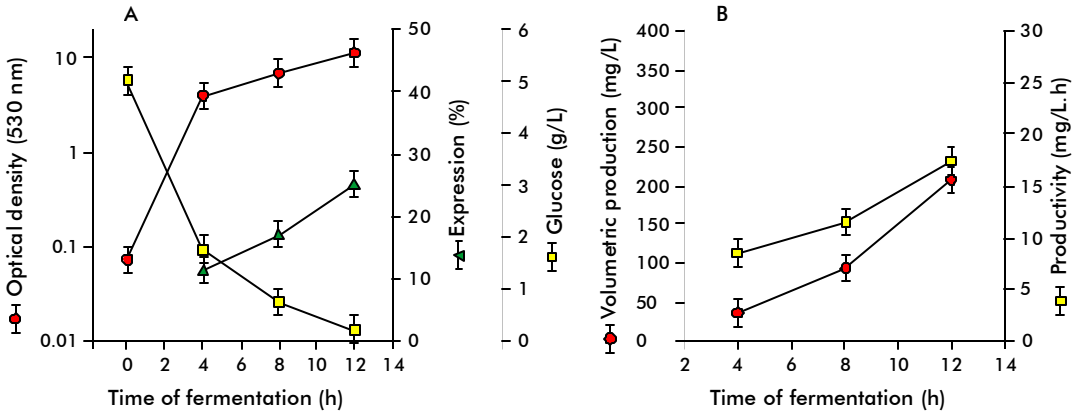

Figure 3. Evaluation of the productive process at 50 L scale. The culture was performed on a 70 L fermentor with 50 L of production medium, at 37 °C, pH 7, 1 v.v.m. and 350 r.p.m.. The promoter was derepressed after depletion of the starting concentration of tryptophan on the culture medium (10 μg/mL) by cellular metabolism. The length of the fermentation was 12 h, with samples taken for analysis every 4 hours.
A) Typical curves of the fermentative process, B) Production of the p-41 recombinant protein.

of the system, using as a starting point the conditions established for the 50 L cultures.

Starting from the equation of Wang *et al*. [6]:

$$kla / H = (\alpha + \beta\, Ni)\, (Pg / V)^{0.77}\, (Vs)^{0.67}$$

kla: oxygen transfer coefficient
H: Henry constant (Depends on the culture medium. It is assumed to behave like a Newtonian fluid).
α and β: constants (Depend on the number ant type of the impeller).
Pg: Power consumed by the gassed system
V: Tank volume
Vs: Superficial speed of the air
Ni: Number of impellers.

and knowing that:

$$Pg\ \alpha\ (P^2\, N\, Di^3 / Q^{0.56})^{0.45}$$

and therefore:

$$P = Np\, N^3\, Di^5\, \rho / gc$$

Considering the turbulent flow, the geometrical similarity and the use of the same culture media, it then results that:

$$P\ \alpha\ N^3\, Di^5$$

Since, furthermore:

$$Di\ \alpha\ Dt\ ;\ A\ \alpha\ Di^2\ ;\ Hi\ \alpha\ Di\ ;\ V\ \alpha\ Di^3$$

where:

Di: Impeller diameter
Dt: Tank diameter
Hi: Impeller height

For fermentors with the same number of impellers and the same culture medium:

$$kla\ \alpha\ (Pg / V)^{0.77}\, (Vs)^{0.67}$$

Substituting (2, 4, 5 in 6):

$$kla\ \alpha\ N^{2.43}\, Q^{0.48}\, V^{0.28}$$

where:

N: r.p.m..
Q: Air flow
V: Useful volume

In order to scale up, considering kla constant:

$$(kla)_1 / (kla)_2 = (N_1 / N_2)^{2.43}\, (Q_1 / Q_2)^{0.48}\, (V_1 / V_2)^{0.28}$$

So, for scaling up from 50 L to 200 L

$$V_2 = 4\, V_1 \qquad (kla)_1 = (kla)_2$$
$$1 = (N_1 / N_2)^{2.43}\, (Q_1 / Q_2)^{0.48}\, (V_1 / V_2)^{0.28}$$
$$1 = (N_1 / N_2)^{2.43}\, (Q_1 / Q_2)^{0.48}\, (1 / 4)^{0.28}$$
$$1 = 0.678\, (N_1 / N_2)^{2.43}\, (Q_1 / Q_2)^{0.48}$$

we obtain:

$$N_2 = 0.65\, N_1\, (vvm_1 / vvm_2)^{0.20} = 0.852\, N_1\, (Q_1 / Q_2)^{0.20}$$

Using this relationship and the fermentation parameters for 50 L cultures, the parameters for 200 L cultures were calculated (0.75 v.v.m. and 240 r.p.m.) and used for setting up a fermentative process to validate the effectiveness of these ratios.

Figure 4A shows the results from a typical 200 L culture. The curves for cellular growth, glucose consumption and p-41 protein expression were similar to those obtained for 50 L cultures. The specific growth rate was μ=0.97 h-1, whereas the saturation level of dissolved oxygen decreased to 85%.

The analysis of the volumetric production and the productivity of the fermentative process (Figure 4B) showed that both parameters increased with culture time until reaching values of 321 mg/L and 26.83 mg/L.h, respectively. These values are 1.55-fold higher than those obtained at the 50 L scale. Likewise, the biomass/glucose and product/glucose yields also

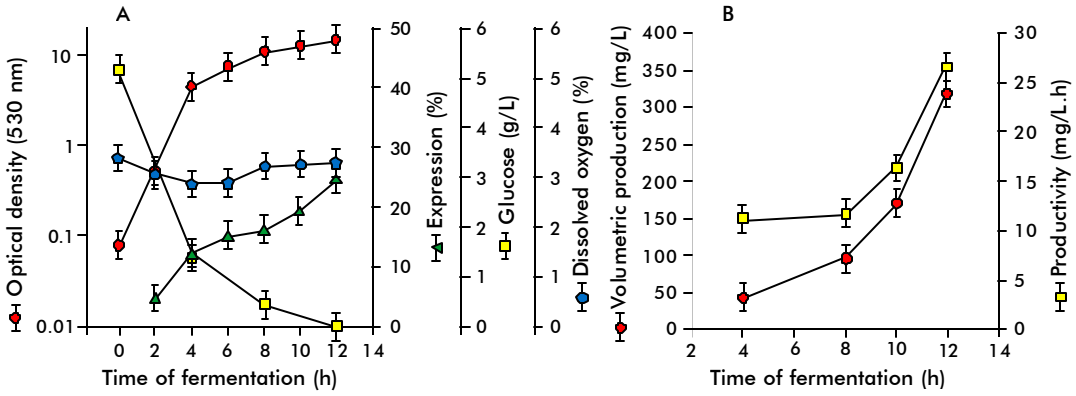

Figure 4. Scale up of the productive process to 200 L cultures. The culture was performed on a 300 L fermentor containing 200 L of production medium, at 37 °C, pH 7, 0.75 v.v.m. and 240 r.p.m., for a total time of 12 hours, with samples taken every 4 hours. The promoter was derepressed after depletion of the starting concentration of tryptophan on the culture medium (10 μg/mL) by cellular metabolism.
A) Typical curves of the fermentative process, B) Production of the recombinant protein p-41.

increased to 0.72 and 0.064, respectively, when compared to the values reported for the 50 L cultures.

Discussion

Regulated promoters are often used for the expression of cloned genes, since they allow the control of expression levels through the manipulation of environmental variables. The specialized literature contains examples of different schemes for the induction of the expression of heterologous proteins placed under control of the tryptophan promoter [8-11]. The most used method involves the use of tryptophan analogs such as 3-β indoleacrylic acid or indolyl-3-propionic acid, acting in a competitive manner [12, 13].

According to the literature, the behavior of the expression of recombinant proteins under the tryptophan promoter is dependent on factors such as the composition of the culture medium, culture conditions, and, mainly, induction conditions [6, 12, 14, 15].

Upon analysis of the influence of the inducer concentration on the production of the p-41 protein it was observed that the expression levels are independent from the inducer at concentrations above 20 μg/mL (Figure 1). This behavior does not match previous reports in the literature stating that inducer concentrations higher than 25 μg/mL actually reduce the productivity and levels of expression of recombinant proteins [16]. Probably, there are other factors that influence the process such as the specific characteristics of the protein under study, the host strain, and culture conditions [15].

When the cultures were grown in the absence of the inducer, the expression levels decreased drastically, due to the repression of the promoter by the starting tryptophan concentration of 20 μg/mL. This coincides with the results reported by other authors [12, 15].

The results obtained in this study evidence that when tryptophan is not present or used at low concentrations, high levels of expression and productivity can be achieved that are, in addition, higher than those obtained by induction with 3-β indoleacrylic acid (Figure 2). With this result it is possible to completely suppress the use of this inducer at higher culture scales, thus lowering the cost of the fermentative process. This is essentially in agreement with the results published by different authors who report that the expression of recombinant proteins under the tryptophan promoter can be achieved once the starting amounts of tryptophan are depleted from the culture medium due to cellular metabolism [10-12, 15].

The use of high tryptophan concentrations resulted on low or no expression of the p-41 protein (Figure 2). This is due to the formation of the tryptophanrepressor protein complex, which inhibits binding of the RNA polymerase to the *trp* promoter and therefore ultimately blocks the synthesis of the polypeptide under study [10-12]. The condition finally chosen for the production of the p-41 protein by derepression of the expression system was a starting tryptophan concentration of 10 μg/mL in the culture medium, which achieves expression levels higher than those previously reported for the production of different fragments of the gp41 protein of HIV-1 for diagnostic means [17-19].

The results of this study also coincide with previous reports from the literature, evidencing the multifactorial influence of environmental conditions on the expression of recombinant proteins, among which the composition of the culture medium plays a major role [20, 21].

After evaluation of the productive process for the p-41 protein at 50 L scale, a 2.4-fold increase in cellular growth was observed when compared with that reached in shaker cultures. However, the expression levels were similar to those obtained at smaller scales (Figure 3A). The volumetric production and the productivity of the fermentative process (Figure 3B) increased 1.4- and 1.1-fold, respectively, when compared to the values reached in shaker cultures. This behavior is due to the better conditions for aeration and mixing than can be obtained in 50 L fermentors, which have a positive effect on cellular growth and the production of recombinant proteins [22].

The development of a correlation between the oxygen transfer coefficient and the operating variables of the system allowed the scaling up of the fermentative process from 50 to 200 L cultures. The curves for the different parameters under analysis were similar for both scales (Figure 4); however, there is an up to 1.55-fold consistent increase in the final values

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for the 200 L scale due to a 1.66-fold increase in the level of synthesis of total protein, keeping constant at the same time the expression level of the p-41 protein. This is the result of the improvement in culture conditions achieved at the 200 L scale, reflected in plasmid stability values higher than 95% and a saturation level for dissolved oxygen higher than 85% throughout the exponential growth phase. These values are above the threshold figures reported as limiting for growth and expression of heterologous genes [23].

During the scale-up process, it was observed that the behavior of the parameters under analysis was not only similar to that observed for the 50 L cultures, but was in fact better. Therefore, it can be concluded that the scale-up process was successful. The methodology described here can be used for the scale up of the fermentative processes for other recombinant proteins expressed in *E. coli* under a variety of promoters, and specially under control of the tryptophan promoter.

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