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Scaling-up fermentation of *Escherichia coli* for production of recombinant P64k protein from *Neisseria meningitidis*

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ABSTRACT

Background: P64k is a *Neisseria meningitidis* high molecular weight protein present in meningococcal vaccine preparations. The *lpdA* gene, which encodes for this protein, was cloned in *Escherichia coli* and the P64k recombinant protein was expressed in *E. coli* K12 GC366 cells under the control of a tryptophan promoter. P64k was expressed as an intracellular soluble protein about 28% of the total cellular protein. Several scale-up criteria of fermentation processes were studied to obtain the recombinant P64k protein at the pilot production scale.
Results: The best operational conditions at a larger scale production of P64k recombinant protein were studied and compared using the four following criteria: Constant Reynold's number (Re constant), Constant impeller tip speed (n di constant), Constant power consumption per unit liquid volume (P/V constant) and Constant volumetric oxygen transfer coefficients (KLa/k constant). The highest production of the recombinant protein was achieved based on the constant KLa/k scale-up fermentation criterion, calculating the aeration rate (Q) and the impeller agitation speed (n) by iterative process, keeping constant the KLa/k value from bench scale. The P64k protein total production at the 50 l culture scale was 546 mg l^{-1} in comparison with the 284 mg l^{-1} obtained at 1.5 l bench scale.
Conclusions: The methodology described herein, for the KLa/k scale-up fermentation criterion, allowed us to obtain the P64k protein at 50 l scale. A fermentation process for the production of P64k protein from *N. meningitidis* was established, a protein to be used in future vaccine formulations in humans.

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1. Introduction

Neisseria meningitidis, a gram negative diplococcus and an obligate human pathogen, causes significant morbidity and mortality in children and young adults worldwide through epidemic or sporadic meningitis and/or septicemia [1].

Immunization is the best and most efficacious preventive approach against meningococcal disease [2]. This is the reason why, first polysaccharide vaccines and later on conjugate vaccines against serogroups A, C, Y and W-135 meningococci were developed. However, most work on development of group B meningococcal vaccines has focused upon use of noncapsular surface components (i.e. lipooligosaccharides (LOS) and outer membrane proteins, either individually- or as in proteoliposome vaccine preparations).

Several outer membrane proteins (OMPs) have been examined as vaccine candidates. Besides major OMPs, other surface proteins are under investigation in several laboratories, including highly conserved OMPs that could potentially protect against meningococcal disease in humans [3].

Among proteins present in outer membrane vesicles vaccine preparations, a high-molecular mass protein (P64k) was identified. This protein is common to many meningococcal isolates, and the *lpdA* gene encoding for this proteins has been isolated and cloned

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in *Escherichia coli* for the further expression of the recombinant protein [4].

An extensive biochemical and biophysical characterization of the P64k protein has been carried out [5]. The protein has been described as a dihydrilopoamide dehydrogenase, based in its high degree of homology with two enzymes of the pyruvate dehydrogenase complex of several species. The first 110 amino acids are similar to those in the lipoyl-binding domain present in the enzyme dihydrolipoamide acetyl-transferase (E2), while the rest of the protein has homology with the enzyme lipoamide dehydrogenase (E3) [6,7]. The gene and protein have been sequenced and its 3D structure determined [8].

From the immunological point of view, the protein was first considered as a vaccine candidate against *Neisseria meningitidis* infection. The antigenicity of this protein was studied and sera from vaccines and convalescent patients were able to recognize the recombinant protein. At the same time, antibodies elicited in several animal models by the recombinant P64k protein recognized the natural protein, present in the whole cell meningococci and in outer membrane vesicle vaccine preparations [9,10,11]. However, the functional activity of antibodies elicited in several animal models and in human by the recombinant protein alone was not enough in terms of conferring protection against meningococcal disease to consider the protein as a unique vaccine candidate. On the other hand, taking into account that the recombinant protein was highly immunogenic [12,13], its function as an immunological carrier for peptides [14,15] and antigens [16,17] was evaluated. Recently, the P64k protein is employed as a recipient of anti-cancer vaccine candidate [18,19].

In order to satisfy the required quantities of P64k protein for the accomplishment of preclinical and clinical studies first, and subsequently for the use in these conjugate vaccines, it was necessary to scale up the fermentation process of the recombinant *E. coli* from 1.5 l [20] up to 50 l scale. Based on several scale up fermentation criteria, a comparative analysis was performed and the results are presented herein.

2. Materials and methods

2.1. E. coli strain and expression plasmid

E. coli K12 GC366 [21] strain was transformed with the plasmid *pM*-152 [10], which contains the entire *lpdA* gene encoding for the P64k protein under the control of the tryptophan (*trp*) promoter [22].

2.2. Growth conditions

The recombinant *E. coli* strain was grown at 37°C in 500 ml shake flasks (New Brunswick Scientific Co., USA) containing 100 ml Luria Bertani medium (LB) [21], supplemented with 10 g glucose l^{-1}, 100 μg tryptophan ml^{-1} (Merck, Germany) and 50 μg kanamycin ml^{-1} (Sigma Co. Ltd., USA) for 8 h and 150 rpm.

For growth in fermenters the minimal growth medium (MM) [21] supplemented with 10 g casein hydrolysate l^{-1}, 10 g tryptone l^{-1}, 10 g glucose l^{-1}, 0.015 g $CaCl_2 \cdot 2H_2O$ l^{-1}, 0.246 g $MgSO_4 \cdot 7H_2O$ l^{-1} and 50 μg kanamycin ml^{-1}, was used.

The bench scale was carried out in 2.5 l fermenters (B.H Marubishi Co., Ldt., Japan) with 1.5 l working volume and 37°C, 700 rpm, aeration rate 1.5 l min^{-1}, as fermentation parameters. The pH was controlled at 7.0 with 40% (*v*/v) NaOH or H_3PO_4 [20].

The scale-up batch process was optimized in 70 l fermenters (B.H. Marubishi Co, Ldt., Japan) with 50 l working volume, at 37°C and pH 7.0, as fermentation parameters. The aeration rate and agitation speed varied depending of the studied scale-up criteria. The fermenter geometries for both working scales are shown in Table 1.

Table 1
Geometry of fermenters employed in the scale-up processes.

Parameters	1.5 l scale	50 l scale
Total fermentation volume [m^3]	0.0025	0.070
Working fermentation volume [m^3]	0.0015	0.050
Vessel diameter (D) [m]	0.129	0.375
Impeller diameter (di) [m]	0.067	0.150
Static height of both (H) [m]	0.115	0.453
Number of impellers (N)	1	2
Geometric factor (fc)	0.61	0.92
Power number (Np)	6	6
Density (ρ) [kg m^{-3}]	1000	1000

2.3. Analysis

Four scale-up fermentation criteria were employed: (1) Constant Reynold's number (Re constant), (2) constant impeller tip speed (n di constant), (3) constant power consumption per unit liquid volume (P/V constant) and (4) constant volumetric oxygen transfer coefficients (K_La/k constant).

The cell dry weight, as cell growth correlation parameter, was determined using the Sartorius MA 30 moisture balance (Sartorius, Germany). The total protein concentration was measured by Lowry's method [23]. The recombinant P64k expression levels were analyzed by densitometry, on 12.5% SDS-PAGE of the recombinant *E. coli* whole cells.

3. Results and discussion

3.1. Bench scale growth, expression and production of P64k recombinant protein

The typical schematic fermentation process at 1.5 l scale, for the production of the P64k recombinant protein is shown in Fig. 1.

During the first 8 h of growth at 1.5 l bench scale, an increase in the optical density (cell dry weight) was observed. The maximum value of the specific growth rate was determined during the first 2 h of growth ($\mu = 1.2$ h^{-1}), being stabilized during the rest of the fermentation process at $\mu = 0.34$ h^{-1}, when an increase in the expression of the recombinant protein was detected. After 8 h of fermentation, the specific growth rate began to decline, reaching values of $\mu = 0.06$ h^{-1} at 10 h of growth, when the fermentation process finished.

The P64k expression level and the protein production increased through the fermentation process, reaching at 10 h growth the highest productivity level.

At this scale, 2.6 g cell dry wt l^{-1}, 22% of P64k recombinant protein expression levels, 284 mg 1^{-1} of volumetric production and 28.4 mg l^{-1} h^{-1} productivity, were obtained. The biomass yield on initial glucose as substrate ($Y_{x/s}$) and the product yield on this substrate ($Y_{p/s}$) values were 0.26 g biomass/g substrate and 0.028 g product/g substrate, respectively.

The P64k expression level is comparable with those values obtained for other recombinant proteins produced in *E. coli* with a similar expression system, such as p36 (20%) [24] and gag24 (25%) from HIV [25] and Opc from *N. meningitidis* (32%) [26]. Likewise, the productivity levels, biomass yield on substrate and the product yield on substrate of P64k protein are in the same order as for the above mentioned recombinant proteins.

3.2. Scale-up fermentation process of P64k recombinant protein

The scale-up parameters to determine the best operational conditions at a larger scale production of P64k recombinant protein were studied and compared using the four following criteria:

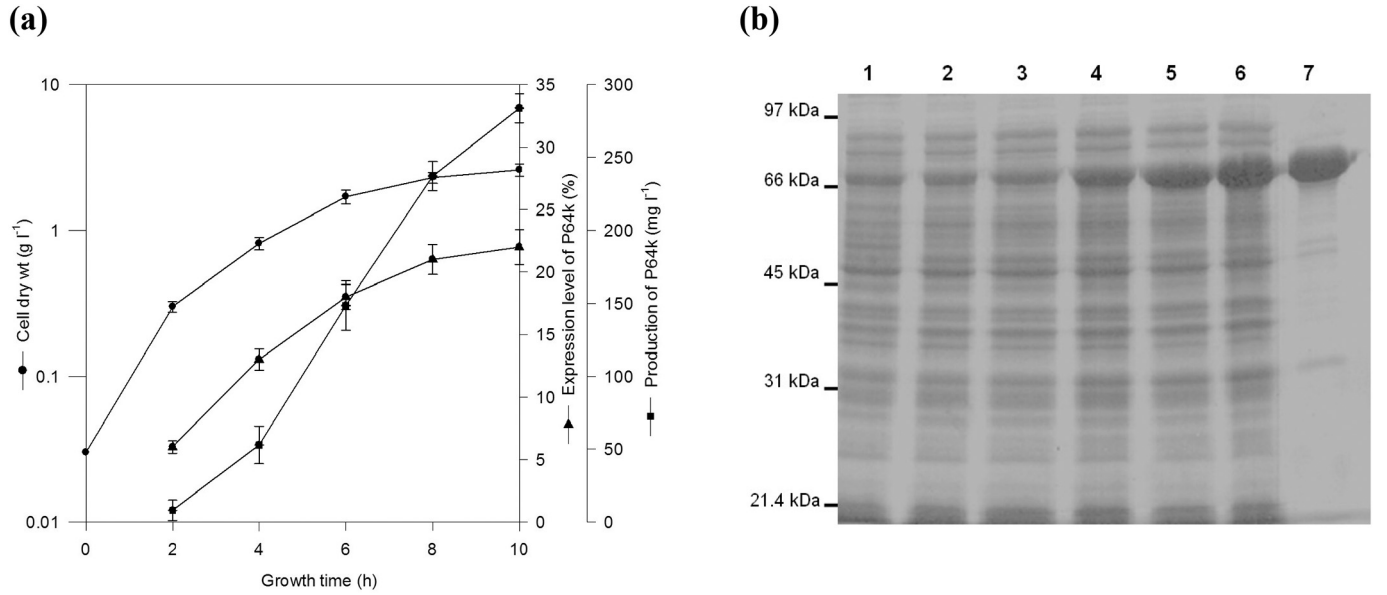

Fig. 1. P64k protein production at 1.5 l bench scale (a) Culture kinetics of *E. coli* expressing the recombinant protein grown during 10 h at 37°C, 700 rpm, 1.5 l min^{-1}. The cell dry weight (●) is expressed in [g l^{-1}]. The expression level of the recombinant P64k protein (▲) is expressed in percent. The production of the recombinant P64k protein (■) is expressed in mg l^{-1}. (b) SDS-PAGE (12.5%) of samples taken during the fermentation process: lane 1: at 0 h, lane 2: at 2 h, lane 3: at 4 h, lane 4: at 6 h, lane 5: at 8 h and lane 6: at 10 h. Lane 7: purified recombinant P64k protein.

(1) Constant Reynold's number (Re constant), (2) Constant impeller tip speed (n di constant), (3) Constant power consumption per unit liquid volume (P/V constant) and (4) Constant volumetric oxygen transfer coefficients (K_La/k constant).

3.2.1. Scale-up under constant Reynold's number criterion

This scale-up procedure was performed using the Reynold's equation, $Re = \rho\, n\, di^2/\mu$ [27,28,29,30,31], where ρ is the fluid density, μ is the viscosity, n represents the impeller agitation speed, and di the impeller diameter. For this scale-up criterion, then:

$$n_{50\ l} = n_{1.5\ l}(di_{1.5\ l}/di_{50\ l})^2 \quad \text{[Equation 1]}$$

The aeration rate at the larger scale, considering a constant aeration number ($Na = Q/n\, di^3$), was calculated as [27,29,32]:

$$Q_{50l} = Q_{1.5\ l}(n_{50\ l}/n_{1.5\ l})\,(di_{50\ l}/di_{1.5\ l})^3 \quad \text{[Equation 2]}$$

3.2.2. Scale-up under constant impeller tip speed criterion

The scale-up procedure using the constant impeller tip speed criterion [27,28,29,30,31] was performed according [Equation 3]:

$$n_{50\ l} = n_{1.5\ l}(di_{1.5\ l}/di_{50\ l}) \quad \text{[Equation 3]}$$

where, n represents impeller agitation speeds and di impeller diameters.

The aeration rate at the larger scale was calculated as previously define in [Equation 2].

3.2.3. Scale-up under constant power consumption per unit liquid volume criterion

This scale-up procedure, using the constant power per unit of volume criteria [27,28,29,30,31], is performed according the following equations:

$$(P/V)_{1.5\ l} = (P/V)_{50\ l} \quad \text{[Equation 4]}$$

$$P = Np\, \rho\, n^3\, di^5\, f_c\, N \quad \text{[Equation 5]}$$

where, P corresponds to the non-gassed power, Np the power number, ρ the fluid density, n the impeller agitation speed, di the impellent diameter, N the number of impellents and fc is the geometric factor. The geometric factor was calculated as $fc = ((D\,H)^{1/2}/(3\,di))$ and the volume as $V = \pi\, D^2\, H/4$, where D is the vessel diameter and H is the static height of broth. Substituting [Equation 5] in [Equation 4],

$$n_{50\ l} = n_{1.5\ l}(di_{1.5\ l}/di_{50\ l})^{5/3}(D_{50\ l}/D_{1.5\ l})^{2/3}\,[(H_{50\ l}/H_{1.5\ l})(N_{1.5\ l}/N_{50\ l})(fc_{1.5\ l}/fc_{50\ l})]^{1/3} \quad \text{[Equation 6]}$$

The aeration rate at the larger scale was calculated as previously define in [Equation 2].

3.2.4. Scale-up under constant volumetric oxygen transfer coefficients K_La/k criterion

The implementation of constant K_La/k scale-up criterion was performed using the most successful correlations with dimensional equations of the form: $K_La = k\,(Pg/V)^{\alpha}(Vs)^{\beta}$ [33], where k, α and β are constants, Pg is the gassed power, V is the liquid volume and Vs is the superficial gas velocity. Taking into account that $V = \pi\, D^2\, H/4$, where D is the vessel diameter and H is the static height of broth, and $Vs = (4Q/\pi\, D^2)$, where Q the is the aeration rate, then.

$$(K_La/k) = \left(4\,Pg/\pi\, D^2H\right)^{0.4}\left(4\,Q/\pi\, D^2\right)^{0.2} \quad \text{[Equation 7]}$$

The gassed power factor fp ($fp = Pg/P$), where P corresponds to the non-gassed power, is determined from the power requirements for agitation in a gassed system [27,28,29,31,32].

The value for the gassed power factor *fp* is taken from the experimental correlations between Pg and operating variables presented as curves of Pg/P vs aeration number (Na), where $Na = (Q/n\, di^3)$, being Q the aeration rate, n the rotation speed of impeller and di the impeller diameter.

Therefore, from the gassed power factor,

$$Pg = fpP \quad \text{[Equation 8]}$$

and the non-gassed power calculated as:

$$P = N_p \rho n^3 d i^5 N f c \quad \text{[Equation 9]}$$

where N_p is the power number, ρ is the fluid density, n is the rotation speed of impeller, N is the number of impellers, and *fc* is the geometric factor, calculated as $fc = (D\ H)^{1/2}/(3di)$, and substituting [Equation 9] and [Equation 8] in [Equation 7],

$$K_L a/k = \left(4/\pi\ D^2\right)^{0.6} \left(N \rho r d i^5 N\ fc\ fp/H\right)^{0.4} n^{1.2} Q^{0.2} \quad \text{[Equation 10]}$$

According to this scale-up criterion, $(K_L a/k)_{1.5l} = (K_L a/k)_{50l}$. The $(K_L a/k)_{1.5l}$ value of 8.7 was calculated from experimental conditions having the aeration rate (Q) of 1.5 l min^{-1} and the impeller agitation speed (n) of 700 rpm at 1.5 l fermentation scale. The n and Q values for the 50 l fermenter conditions, were calculated by iterative process using the [Equation 10], keeping a constant factor of 8.7 for $(K_L a/k)_{1.5l}$. The calculated values for the scale-up processes are shown in Table 2.

Using the calculated fermentation parameter combinations of aeration rate (Q) and the impeller agitation speed (n), three fermentation processes were performed for each condition. The average of P64k recombinant protein production is shown in Table 3. According to these results, the best combination of aeration rate and the rotation speed of impeller parameters to keep a constant $K_L a/k$ value of 8.7 was Q of 50 l min^{-1} and 320 rpm. This set of values was used to perform the comparison among the four scaling up fermentation processes.

The results of total P64k production related to the influence of aeration rate and the impeller agitation speed are shown in Fig. 2. The Fig. 2a represents the row data from the fermentation processes according to Table 3. The highest level of P64k production is obtained in a range of 315–340 rpm of impeller agitation speed and 35–55 l min^{-1} aeration rates, having the maximum P64k production of 546 mg l^{-1}, with 320 rpm agitation and 50 l min^{-1} aeration rates. A surface response graph for the P64k production is presented in Fig. 2b. This graph is characterized by a none-linear regression model, with Q (aeration rate) and n (impeller agitation speed) as independent variables [34]. The P64k production is represented in [Equation 11]:

$$\text{Production} = -76,352.6315 + (904.934\ Q) + (359.0292\ n) - \left(2.2763\ Q^2\right) - (2.172\ Q\ n) - (0.4187\ n^2) \quad \text{[Equation 11]}$$

A positive influence of the aeration rate and the impeller speed, as independent variable parameters, over the theoretical total protein production is observed. This means, that at certain value of aeration rate with an increase of the impeller agitation speed, an optimal protein production might be expected. In concordance, at certain value of impeller speed, when the aeration rate increased a final protein production might also be expected. However, analyzing the combination of these parameters over the final production, a negative influence is observed. This means, that to keep a certain value of production if the impeller agitation speed increased the aeration rate has to decrease. This is characterized by the negative signal in the term (Q n). Nevertheless, the input of this term to the total production value in the equation is negligible. From this equation, the maximum value for protein production of 548 mg l^{-1} falls in 315 rpm and 48.7 l min^{-1} aeration speed. These values are similar to those obtained experimentally, and when the $K_L a/k$ coefficient was recalculated with these parameters a value of 8.4 was obtained, compared with the 8.7 used for this scale-up criterion.

Table 2
Calculated theoretical relation between the aeration rate (Q) and the impeller agitation speed (n) to keep constant a $K_L a/k$ value of 8.7, for the studied scale-up fermentation process.

Variant no.	Q [l min^{-1}/l s^{-1}/vvm]	n [rpm/rps]	Pg/V [(W m^{-3}]	Vs [m s^{-1}]	$K_L a/k$ [$W^{0.4}$ m^{-1} $s^{-0.2}$]
1	10/0.17/0.2	420/7.00	5574.87	0.0015	8.7
2	15/0.25/0.3	390/6.50	4463.54	0.0023	8.7
3	20/0.33/0.4	370/6.17	3811.45	0.003	8.7
4	25/0.42/0.5	360/6.00	3510.68	0.0038	8.7
5	30/0.50/0.6	350/5.83	3226.16	0.0045	8.7
6	35/0.58/0.7	340/5.67	2957.45	0.0053	8.7
7	40/0.67/0.8	330/5.50	2704.09	0.006	8.7
8	45/0.75/0.9	325/5.42	2583.02	0.0068	8.7
9	50/0.83/1.0	320/5.33	2465.62	0.0075	8.7
10	55/0.92/1.1	315/5.25	2351.84	0.0083	8.7

Table 3
Average of P64k recombinant protein production as a result of triplicate fermentation processes from each variant.

Variant no.	Q [l min^{-1}/l s^{-1}/vvm]	n [rpm/rps]	$K_L a/k$ [$W^{0.4}$ m^{-1} $s^{-0.2}$]	Production [mg l^{-1}]
1	10/0.17/0.2	420/7.00	8.7	277 ± 15
2	15/0.25/0.3	390/6.50	8.7	310 ± 23
3	20/0.33/0.4	370/6.17	8.7	305 ± 38
4	25/0.42/0.5	360/6.00	8.7	293 ± 26
5	30/0.50/0.6	350/5.83	8.7	301 ± 30
6	35/0.58/0.7	340/5.67	8.7	325 ± 29
7	40/0.67/0.8	330/5.50	8.7	398 ± 49
8	45/0.75/0.9	325/5.42	8.7	430 ± 48
9	50/0.83/1.0	320/5.33	8.7	546 ± 51
10	55/0.92/1.1	315/5.25	8.7	405 ± 42

3.3. Comparison of all scale-up criteria

The scale-up process is one of the key points, not only in fermentation but also in the industry as a whole. In the real practice, several scale-up criteria have been studied and applied, but all of them based in empirical results [35,36].

The constant power input per unit volume of liquid (P/V) and constant volumetric oxygen transfer coefficient ($K_L a$) are among the most scale-up fermentation criteria used in the industry [37,38,39]. No scale-up strategy as such is generally established, so for each process a suitable approach needs to be determined [27,32,40]. In practice, often combinations of methods are used for adequate scale-up of a bioreactor [41,42].

The parameters for the studied scale-up criteria and the influence over the P64k production are shown in Table 4. Scaling up with the constant Reynold's number criterion, very low values for impeller agitation speed (20% regarding 1.5 l scale), P/V (4% regarding 1.5 l scale) and $K_L a/k$ (20% regarding 1.5 l scale) were obtained at 50 l scale. This might provoke an inappropriate mixing, resulting in around 50% lower values for cell dry weight and P64k production obtained at 1.5 l scale. This is in agreement that Reynold's scaling up criterion is not a good one to use for such a fermentation process, since the degree of agitation decreases very strongly with the increase of the production scale [42,43,44,45,46].

The impeller tip speed scale-up criterion has some advantages in the case of bioprocesses with shear sensitive microorganisms or the absolute value of the impeller tip speed [42,47,48], but it is also argued that it is not a good enough criterion for scaling [45,49]. In our case, the cell dry weight and the final P64k production increased in 50%, compared to values obtained for Reynold's scale-up criterion. To do so, an increase in the impeller agitation speed and the aeration rate of around 50% had to be implemented.

Maintaining a constant power per unit volume is another popular scale-up method, and has been successfully applied as a primary scale-up parameter for many antibiotic fermentations [40,42].

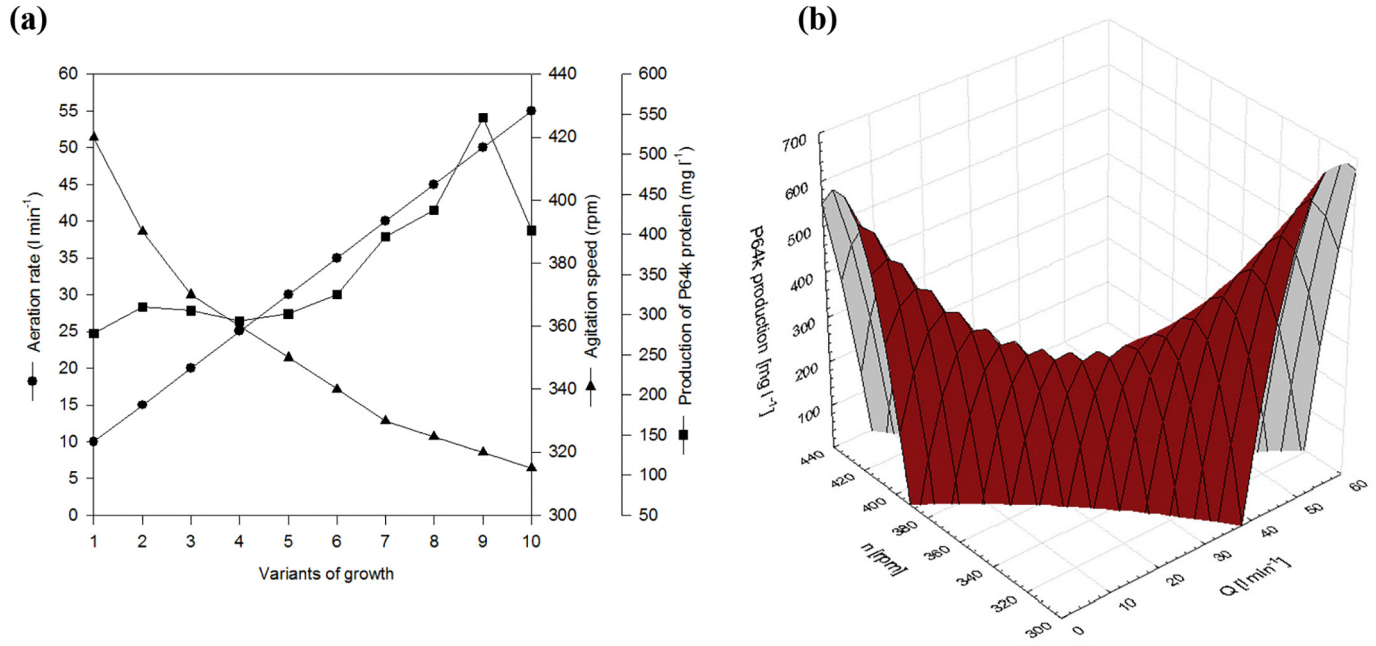

Fig. 2. Schematic representation of results from the scale up variants using K_La/k criteria. (a) Influence of aeration rate and agitation speed on the final production of P64k protein. The production of the recombinant P64k protein (■) is expressed in [mg l^{-1}], the aeration (●) is expressed in [l min^{-1}] and the agitation speed (▲) is expressed in [rpm], (b) Surface response graph from this analysis (n: agitation speed, Q: aeration rate).

When scaled up with constant P/V criterion, similar values of cell dry weight and P64k production were obtained, as compared to those for the constant impeller tip speed criterion. This was observed in spite of the 30% increase of the impeller agitation speed and the aeration rates used for the constant impeller tip speed scale up criterion.

For instance, scale-up based on constant P/V resulted in an increase of the shear rate (calculated as: n di) of 30%, as compared with the constant impeller tip speed criterion and the K_La/k scale up criterion. This was the highest value among all the studied criteria. With this criterion, a P/V value of 5.2 kW m^{-3} was obtained, slightly higher than the range of values reported as a general rule (1 to 3 kW m^{-3}) [27,43]. This increase in the shear stress may correlate with an increase of the possible cell damage and therefore with the decrease of the final production of the protein of interest [45,50].

The volumetric mass transfer coefficient is the most often applied physical scale-up variable. It includes relevant parameters that influence oxygen supply such as agitation and aeration [36,40,51,52]. Therefore, a strong emphasis has been put on maintaining a constant K_La in scale-up of process to ensure the same oxygen supply rate and to satisfy the oxygen demand of the desired cultivation. The same K_La values can be achieved in different sized vessels by adjusting the operating conditions, i.e. stirrer speed and aeration rates on the larger scale to match the optimum K_La conditions determined in the small-scale studies [53]. Several correlations estimating K_La have been developed over the years for a range of different vessel sizes [33,37,54,55] and successfully scaled-up of processes from bench scale to pilot scale based on this criterion or in conjunction with another scale-up parameter have been established [56,57,57,58].

In this study, when the K_La/k scale up criterion was used, assuming a constant value of 8.7 (calculated for the 1.5 l scale), the aeration rate increased in 5–6 times (50 l min^{-1}) as compared with values for the impeller tip speed (7.5 l min^{-1}) and P/V (9.8 l min^{-1}) criteria. However, a similar impeller agitation speed value was obtained when compared theses three scale-up criteria. Using the parameters calculated for the K_La/k scale up criterion, no shear increase occurred and the highest cell dry weight and P64k production was achieved.

Using the estimated parameters for the K_La/k scale up criterion (n = 320 rpm, Q = 50 l min^{-1}), a total of 10 fermentation processes were

Table 4
Parameters for comparison of scale-up fermentation criteria.

Parameters	1.5 l scale	50 l scale			
		Re[a]	n di[b]	P/V[c]	K_La/k[d]
n [rpm/rps]	700/11.67	139.7/2.33	312.7/5.21	406.7/6.78	320/5.33
P [W]	7.85	10.57	118.64	261	127.18
Re	5.20E + 04	5.20E + 04	1.10E + 05	1.50E + 05	1.20E + 05
n di	46.9	20.95	46.95	61	48
P/V [W m^{-3}]	5223.34	211.4	2372.4	5223.34	2543.6
Pg/V [W m^{-3}]	5064.01	204.97	2300.02	5060.01	2465.62
Q [l min^{-1}/l s^{-1}/vvm]	1.5/0.03/1.00	3.4/0.06/0.07	7.5/0.13/0.15	9.8/0.16/0.20	50.0/0.83/1.00
Vs [m s^{-1}]	0.0019	0.0005	0.0011	0.0015	0.0076
K_La/k [$W^{0.4}$ m^{-1} $s^{-0.2}$]	8.7	1.8	5.7	8.2	8.7
Dry weight [g l^{-1}]	2.6 ± 0.11	1.4 ± 0.06	2.7 ± 0.13	3.0 ± 0.15	3.9 ± 0.19
P64k production [mg l^{-1}]	284 ± 12	168 ± 7.0	338 ± 15	383 ± 19	546 ± 27

[a] Constant Reynold's number scale-up criterion.
[b] Constant impeller tip speed scale-up criterion.
[c] Constant power consumption per unit liquid volume scale-up criterion.
[d] Constant volumetric oxygen transfer coefficients scale-up criterion.

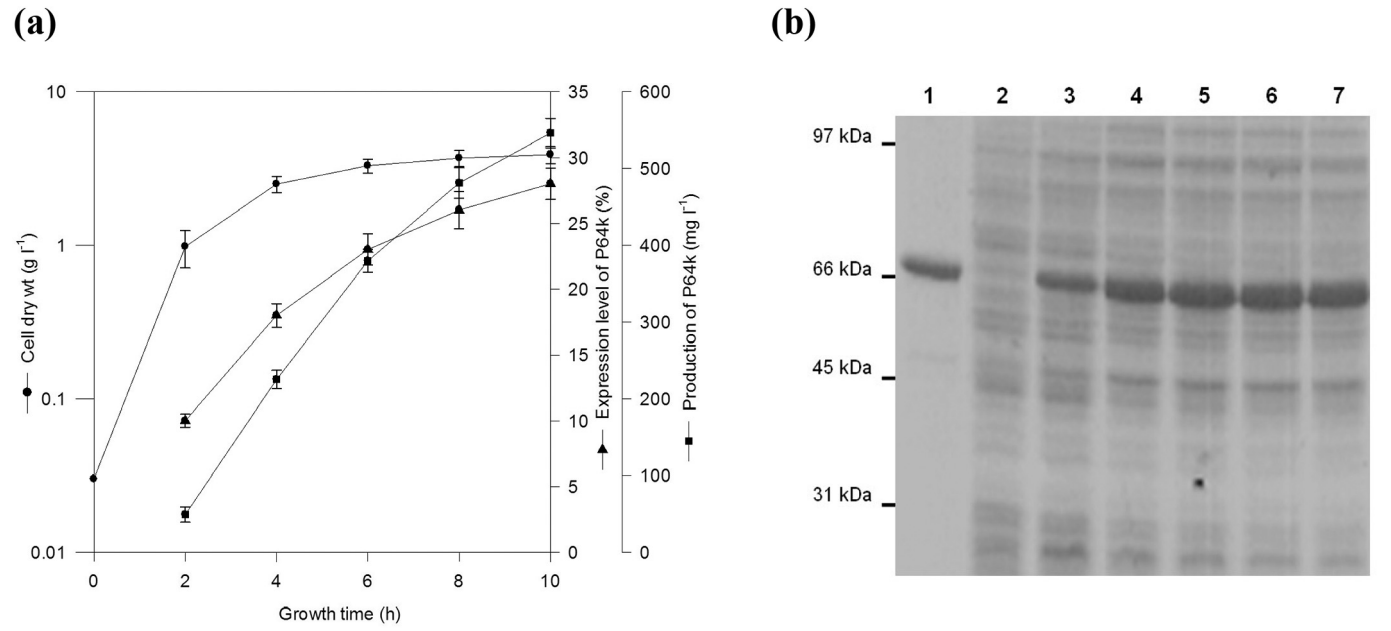

Fig. 3. P64k protein production at 50 l production scale (a) Culture kinetics of *E. coli* expressing the recombinant protein grown during 10 h at 37°C, 320 rpm, 50 l min^{-1}. The cell dry weight (●) is expressed in [g l^{-1}]. The expression level of the recombinant P64k protein (▲) is expressed in percent. The production of the recombinant P64k protein (■) is expressed in mg l^{-1}. (b) SDS-PAGE (12.5%) of samples taken during the fermentation process: lane 2: at 0 h, lane 3: at 2 h, lane 4: at 4 h, lane 5: at 6 h, lane 6: at 8 h and lane 7: at 10 h. Lane 1: purified recombinant P64k protein.

performed. At the 50 l scale, during the first 6 h of growth an increase in the optical density (and the corresponding total cell dry weight) was observed. The maximum value of the specific growth rate was obtained during the first 2 h of growth ($\mu = 1.4\ h^{-1}$), being stabilized during the rest of the fermentation process at $\mu = 0.33\ h^{-1}$. After 8 h of fermentation the specific growth rate began to decline, reaching values of $\mu = 0.03\ h^{-1}$ at 10 h of growth.

The P64k expression level and the protein production increased through the fermentation process, reaching at 10 h growth the highest productivity level. As an average, at 50 l scale (using the K_La/k scale up criterion), 3.9 g cell dry wt l^{-1}, 28% level of P64k carrier protein expression, 546 mg l^{-1} volumetric production and 54.6 mg l^{-1} h^{-1} productivity were obtained. The biomass yield on initial glucose as substrate and the product yield on this substrate values were 0.39 g biomass/g substrate and 0.054 g product/g substrate, respectively. A behavior of these fermentation processes is shown in Fig. 3.

The total P64k protein production increased 1.9 folds, when data from both scale were analyzed. This augment is influenced by the 6% slightly increase in P64k protein expression and the 1.5 fold increase in the final cell growth. These values are comparable with those obtained for other recombinant proteins produced in *E. coli* under the same expression system such as gag24, gp-41 and p36 from HIV [24,59,60], and the Opc from *N. meningitidis* [26], where the proteins in study become "toxic" for the cell.

In conclusion, the methodology employed, using the K_La/k scale-up fermentation criterion and calculating the aeration rate (Q) and the impeller agitation speed (n) by iterative process, keeping constant the K_La/k value from bench scale, allowed us to obtain the highest P64k protein production at 50 l scale, in comparison with the other studied scale-up fermentation criteria. As a result, we have established a fermentation process for the production of the P64k recombinant protein from *N. meningitidis*, a protein to be used in future vaccine formulations in humans.

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