Shear rate as scale-up criterion of the protein production with enhanced proteolytic activity by phosphate addition in the *Jacaratia mexicana* cell culture

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**ABSTRACT**

The main challenge in bioreactor scale-up is maintaining the bioprocess productivity. The aim of the present work was to scale-up the protein production with enhanced proteolytic activity by phosphate addition in *Jacaratia mexicana* cell culture, by using the shear rate as scale-up criterion. The volumetric proteolytic activity in the *J. mexicana* cell culture in shake flask increased 1.9 times with increasing phosphate concentration from 1.25 to 2.5 mmol L\(^{-1}\), while the protein concentration was similar (~55 mg L\(^{-1}\)). This culture was scaled-up from 0.4-L to 4-L in stirred tank bioreactors at average shear rate of 200 s\(^{-1}\) and 234 s\(^{-1}\), respectively, increasing the phosphate concentration in the 4-L bioreactor (2.5 mmol L\(^{-1}\)) with respect to the 0.4-L bioreactor (1.25 mmol L\(^{-1}\)). There was also an increase in the volumetric proteolytic activity (2 times), without statistically significant difference in the protein concentration. Scale-up strategy and improvement of the culture medium exhibited a similar behaviour maintaining the protein productivity and increasing the proteolytic activity. These results indicate that the average shear rate is a useful hydrodynamic parameter in the scale-up of protein production of *J. mexicana* cell culture. Additional phosphate and bioreactor hydrodynamics could be related to unusual lengthening and thinning of most cells in the 4-L stirred tank bioreactor. Finally, the power input per unit volume in the scaled-up bioreactor decreased by 53%, improving the volumetric proteolytic activity and maintaining protein productivity. These results encourage proceeding with the scale-up process using the shear rate as a scale-up criterion.

**Introduction**

Bioprocesses development has as a recurrent step the bioreactor scale-up; i.e. to carry out the culture in a bioreactor of greater operation volume than the bioreactor that reached the target productivity [1]. The main objective of bioreactor scale-up is to achieve at least the same productivity in the large bioreactor as in the small bioreactor. There are several scale-up strategies, which depend on the parameter that has the greatest influence on productivity. This parameter is called the scale-up criterion [2].

Plant cells can be sensitive to the mixing of culture medium, which is done to achieve a homogeneous distribution in bioreactors. Shear rate and shear stress are parameters used for describing the bioreactor hydrodynamics. In Newtonian liquids, the shear stress is a linear function of the shear rate, where the slope is the viscosity. Shear rate can decrease or increase metabolite production, associated or not with changes in morphology and cell size, as described below.

The coumarins and sphaeralcic acid produced by *Sphaerulcea angustifolia* cells cultured in stirred tank bioreactor (STB) decreased (10%) when the shear rate increased from 33 s\(^{-1}\) to 66 s\(^{-1}\) [3]. Ajmalicine and serpentine accumulation in *Catharanthus roseus* cells cultured in unbaffled 12-L STB decreased (87 and 50% respectively) when the agitation increased from 100 to 700 rpm [4]. Equal Ginseng saponin production (15 g L\(^{-1}\)) and productivity (77 mg L\(^{-1}\) d\(^{-1}\)) in *Panax notoginseng* cells in 3-L STB were observed when the agitation increased from 80 to 210 rpm [5]. The taxol production...
in *Taxus cuspidate* cells suspension culture in rotating wall vessel, decreased at shear stress values above 2.3 \( \times 10^{-3} \) N m\(^{-2} \) [6].

Some examples of improve metabolite production at increasing shear rate are as follows: the phenols accumulation in *Centaurea calcitrapa* cells culture was 74 and 140 µg L\(^{-1} \), at shear rates of 5 and 42 s\(^{-1} \), respectively [7]. Higher protein productivity was achieved in transgenic rice cell culture at 160 rpm [8]. The anthraquinones concentration in the *Rubia tinctorum* cell culture increased 233% in a 1.5-L STB at 450 rpm, when it scaled-up from shake flask at 100 rpm [9]. In a 7-L stirred tank bioreactor cell culture of *Thevetia peruviana*, the increase in agitation from 300 to 800 rpm favoured the metabolic activity (Reactive Oxygen Species); from 300 to 550 rpm, biomass concentration (11.92 g DW L\(^{-1} \)) and intracellular phenolic compounds production (57.78 mg EGA/100 g FW). The scale-up to 20-L stirred tank bioreactor for cell suspension of *Vitis labrusca* gave similar growth rates to those observed in shake flask culture and showed high production of resveratrol (4.23 g L\(^{-1} \)) and δ-viniferin (0.76 g L\(^{-1} \)) [10].

*Buddleja cordata* cells cultured in a 2-L stirred tank bioreactor enhance the biomass production (13.62 g L\(^{-1} \)) and phenolic secondary metabolites (total phenolic (64.63 mg gallic acid equivalents g\(^{-1} \)), phenylethanoid glycoside (119.24 mg verbascoside equivalents g\(^{-1} \)) and flavonoid (5.02 mg quercetin equivalents g\(^{-1} \)) at 400 rpm [11]. The highest production of biomass (13.62 g L\(^{-1} \)) and PSMs [PeC of 64.63 mg gallic acid equivalents g\(^{-1} \) (mg GAE g\(^{-1} \)); PeGC of 119.24 mg VB equivalents g\(^{-1} \) (mg VBE g\(^{-1} \)); and FC of 5.02 mg quercetin equivalents g\(^{-1} \) (mg QE g\(^{-1} \))] occurred in B2RD at 400 rpm [12]. In addition, the alkaloids concentration in *Uncaria tomentosa* culture in a 2-L STB increased 10-fold at 400 rpm compared with shake flask at 110 rpm. Furthermore, an enlargement (\( f_e \) 1.4 to 2.55) of *U. tomentosa* cells was demonstrated [13].

One of our previous works [14] describes the protein production (proteases) of *J. mexicana* cells suspension culture in three different types of bioreactors. Higher proteolytic activity (170000 TU L\(^{-1} \)) and higher protein accumulation (56 mg L\(^{-1} \)) arisen in a 0.4-L STB at shear rate of 270 s\(^{-1} \). Under this hydrodynamic condition, the cells were smaller and thinner compared to cell culture in bubble column and airlift bioreactors (40 s\(^{-1} \)). The phosphate was 1.25 mmol L\(^{-1} \) (the basal phosphate concentration reported in Murashige & Skoog (MS) [15]) in the experiments mentioned above; however, few reports have mentioned that increasing phosphate concentration could improve the protein concentration [16–18].

The phosphorus is one of the six essential macronutrients required for plants, by its participation in photosynthesis, as building blocks of genes and phospholipids required in cellular division [19–21]. The role of phosphorus is evident in the maximum content of ginsenoside Rg1 achieved at 1.1 mmol L\(^{-1} \) phosphate, as well as the higher concentration of ginsenoside Rd at 2.21 mmol L\(^{-1} \) phosphate, in the *Panax quinquefolium* cells culture [22]. The *Astragalus membranaceus* roots suspension culture in shake flasks and bioreactor evidenced high yield (2-fold) of Calycosin-7-O-β-D-glucoside when the phosphate concentration was 2.5 mmol L\(^{-1} \) [23]. Also, in the adventitious root culture of *Panax ginseng*, a lineal correlation between the total saponin and phosphate concentration was significant [24]. Azadirachtin production from *Azadirachta indica* cell culture and biomass increased when the phosphate increased from 1.25 to 2.0 mmol L\(^{-1} \) [25]. In addition, callus initiation of *Agropyron repens* improved when the phosphate concentration increased from 1.25 to 8.5 mmol L\(^{-1} \) [26]. Some plant species increase the metabolite synthesis at low phosphate concentration, like camptothecin production from *Nothapodytes nimmoniana* cells culture optimized at 0.5 mmol L\(^{-1} \) [27] and ginseng saponin production from *Panax ginseng* cells at 0.25 mmol L\(^{-1} \) [18]. However, conessine production of *Holarrbena antidysenterica* cells decreased at 0.25 mmol L\(^{-1} \) phosphate [28]. In addition, high (12.5 mmol L\(^{-1} \)) and low (0.125 mmol L\(^{-1} \)) phosphate concentration inhibited the solasodine production from *Solanum khasianum* cells culture, the negative effect being greater at high concentration (12.5 mmol L\(^{-1} \)) [29].

Studies of Gamborg’s (BS) [30] and Murashige & Skoog (MS) [15] on vitamins indicated that BS improved the production of asodine of *S. khasianum* cells culture [29]. Also, favoured the regeneration from the proximal ends of the cotyledons of *Vigna radiata* [31] and significantly increased the levels of hypericins in shoot cultures of *Hypericum rumeliacum* and *Hypericum tetrapterum* [32]. The use of vitamin BS in the *J. mexicana* culture could improve the protein production.

Phosphate and the vitamin type affect the metabolite synthesis and cell growth in different ways, according to the plant cell species, as mentioned above. Therefore, the objective of this work was to optimize the culture medium for protein production and scale up the improved process for the protein production of *J. mexicana*, using the shear rate as the scale-up criterion.
Materials and methods

J. mexicana seeds germination

J. mexicana seeds were washed with extran for 15 min, later were sterilized in 70% (v/v) ethanol for 1 min followed by immersion in a 1.2% (v/v) sodium hypo-chlorite solution for 15 min, after were rinsed four times in sterile water. The sterile seeds were germinated on 6 g L⁻¹ agar noble, MS/₄ [15] basal salts at pH 5.7. The cultures ran at 25 ± 2 °C and 16:8 h photoperiod (50 µmol photons m⁻² s⁻¹) for 14 days, when the plantlets reach size 10-15 cm.

Callus and cell cultures of J. mexicana in shaken flask

The J. mexicana callus cultures began with stem cuttings (1 cm) placed on MS medium supplemented with 30 g L⁻¹ sucrose, 0.5 mg L⁻¹ 6-Benzylaminopurine (BA), 0.25 mg L⁻¹ 2,4-Dichlorophenoxyacetic acid (2, 4-D) and 6 g L⁻¹ agar noble at pH 5.7. The phosphate concentration and vitamin type are mentioned in the experimental design section. Cultures were at 25 ± 2 °C and 16:8 h photoperiod (50 µmol m⁻² s⁻¹). The calluses were transferred to fresh media every two weeks. Three independent experiments with ten repetitions were carried out.

The J. mexicana cell cultures in 250 mL shaken-flask containing 50 mL MS medium supplemented with 40 g L⁻¹ sucrose, 0.5 mg L⁻¹ BA and 0.25 mg L⁻¹ 2, 4-D at pH 5.7, began with 3.0 g of calluses, at 150 rpm, 25 ± 2°C and 16:8h photoperiod (50 µmol photons m⁻² s⁻¹). The phosphate concentration and vitamin type Gamborg’s (BS) [30] or Murashige & Skoog (MS) [15] were conducted as mentioned in the experimental design. Three independent experiments were carried out.

Experimental design

As a first step in the improvement of protein productivity in the shake flask cultures, a two-level fractional factorial design 2² (FFD) [33] was used to analyze the medium components influencing the protein production. The FFD had two factors with two coded levels (-1 and +1) and was run to evaluate the linear effects and possible interactions between the two factors tested on the proteolytic activity, protein, and biomass. The factors were phosphate concentration, which were 0.125 to 0.250 mmol L⁻¹ and vitamin type, MS and BS. The results were fitted with a first-order model.

Scale-up strategy

The J. mexicana cells culture grown in a 0.4-L STB was scaled-up to a 4-L STB. The scale-up strategy selected the average shear rate as the scale-up criterion, because in the 0.4-L STB (using 1.25 mol L⁻¹ phosphate in the MS basal salt mixture), the protein concentration and the proteolytic activity correlated linearly with the average shear rate (r² > 0.96). The highest average shear rate was 274 s⁻¹, with which the highest protein concentration (56 mg L⁻¹) and the highest proteolytic activity (170000 TU L⁻¹) were obtained [14]. The 4-L bioreactor operated at 229 rpm achieved an average shear rate of 274 s⁻¹. This average shear rate was in the scale-up zone, from 200 s⁻¹ to 274 s⁻¹, as it is observed in Figures 3A and 4A in Oliver-Salvador et al. [14].

Average shear rate, microscale of turbulence and power input were calculated as indicated elsewhere [14]. Maximum shear rate was calculated from impeller diameter (D_i), agitation rate (N), liquid viscosity (µ) and density (ρ) by Equation 1 (Robertson and Ulbrecht, 1987 [34]).

\[
\gamma_{max} = 3.3 \cdot N \cdot D_i \cdot \left(\frac{\rho}{\mu}\right) \quad (1)
\]

The pumping number (NQ) was evaluated employing a stirred tank with two Rushton impellers of standard geometry, in two-phase flow operating at 225, 300 and 400 rpm (Moghaddas et al., 2008 [35]). Equation 2 evaluated the pumping capacity (Q).

\[
Q = NQ \cdot ND_i^3 \quad (2)
\]

and the residence time in the high shear rate zone (t_r) can be expressed as Equation (3)

\[
t_r = \frac{V_o}{NQ \cdot N \cdot D_i} \quad (3)
\]

where V_o is the operating liquid volume.

J. mexicana cell culture in stirred tank bioreactor

Experiments were performed in a 4-L STB of standard design (New Brunswick, New Jersey USA) with two Rushton turbines. The culture began with 200 g fresh weight of J. mexicana cells in MS medium supplemented with 40 g L⁻¹ sucrose, 0.5 mg L⁻¹ BA and 0.25 mg 2, 4-D at pH 5.7; 25 ± 2°C, and 16:8h photoperiod (50 µmol m⁻² s⁻¹). Sodium phosphate and MS salt concentrations, as well as the vitamin type added
were determined as optimal, based on results of the FFD. The bioreactor operated at 229 rpm with an aeration rate of 0.5 vvm.

**J. mexicana cell size and morphology**

Cell images were obtained using a microscope Leica CTR5000. Magnification of the image was 100X. The image analysis from photographs of cell culture samples was realized using ImageJ software. The length, width and cell area were calculated using Excel (Microsoft, Inc. USA) and the statistical analysis was performed using SAS (Statistical Analysis System Version 9.0, Trial Version). The length-to-width ratio ($f_e$) and the areas ratio ($f_a$), defined as the area of a cell relative to the area of an ellipse with the same length and width of the cell, was determined. At least 700 cells were measured for each sampling.

**Analytical methods**

For biomass analysis reported as dry weight (DW), 1.0 mL aliquots were filtered through 0.22 µm (Millipore) nitrocellulose membrane to separate from broth. The cells were weighed after drying at 60 °C to constant weight for 24 h.

Protein concentration from filtrated broth was determined by the Bradford method [36] (this extracellular protein concentration henceforth is referred to as protein concentration) and cell viability was evaluated by the Trypan Blue dye exclusion test, as described elsewhere [14].

Proteolytic activity of filtrated broth was determined according to [37] as modified by [38] employing as substrate 1% casein solution in 0.05 mol L$^{-1}$ sodium phosphate at pH 7.6 and 35 °C. The proteolytic activity expressed as Tyrosine Units per litre (TU L$^{-1}$) means the activity that liberates the equivalent of one microgram of tyrosine per hour.

Viscosity was determined using a Haake viscometer (model RV20 Germany) at 25 ± 0.1 °C and the oxygen uptake and oxygen transfer rates were measured using the dynamic method. The response time of the dissolved oxygen probe (Cole Parmer Instruments Co., USA) was 19 s.

Molecular weight of *J. mexicana* protease produced in the 4-L STB was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% SDS-containing polyacrylamide. Protein samples were stained with Coomassie blue and visualized in a ChemiDoc system (Bio-Rad).

Immunoblot analysis of *J. mexicana* protease produced in the 4-L STB was performed by transferring the proteins separated by electrophoresis, to polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The protease was detected with a rabbit anti-mexican (MX-4) polyclonal antibody (produced in our laboratory, unpublished data). Immunobiochemical detection was performed using the Western ECL Blotting Substrates (Bio-Rad) following the manufacturer’s instructions.

**Data analysis**

Statistical analysis was conducted by means of analysis of variance (ANOVA) using the Microsoft Excel version 365. All experiments were repeated three times.

**Results and discussion**

The success of the bioprocesses scale-up occurs when the productivity in the large bioreactor is equal or greater than that obtained in the small bioreactor. In this work, we calculated the productivity, expressed in grams of the product per litre per day, with the concentration of extracellular protein, as well as with the cellular concentration obtained at 21 days of the culture. Once a better medium for protein production was obtained in shake flask experiments, subsequently the optimized medium was employed for the scale-up the protease production from a 0.4-L STB to a 4-L STB. We describe below the procedure to improve the culture medium.

**Experimental design for improving protein productivity**

Preliminary studies showed that the sodium phosphate and the vitamin type affected callus growth and its friability, as well as the cell growth and metabolite production (data not shown). These components have been reported to have an important effect on metabolite production and cell growth [26, 32]. Therefore, a factorial design of experiments $2^2$ was performed for determining the phosphate concentration and the vitamin type, influencing on protein production and proteolytic activity. Table 1 shows the coded values and actual values of those factors and the responses measured. The ANOVA showed that the factor that significantly increased the proteolytic activity and biomass production was phosphate ($p<0.05$) as shown in Table 2 and Figure 1 for proteolytic activity, and Table 3 and Figure 2 for biomass. The protein content was neither affected by the phosphate concentration nor by the vitamin type (data not shown). This behaviour was similar in an alfalfa cell culture, where
the soluble protein concentration in media supplemented with phosphorus was not significantly different when the phosphorus concentration increased from 0.5 mmol L$^{-1}$ to 5 mmol L$^{-1}$ [17].

Here the data showed similar protein concentration, and enhanced proteolytic specific activity was observed at 2.5 mmol L$^{-1}$ phosphate. One possible explanation is that the presence of phosphate affects gene expression, as demonstrated before [39, 40] thus, increasing de novo protease synthesis in a non-specific manner or, perhaps enhancing proteolytic isoforms. There is also a possibility that phosphate itself stimulates the proteolytic activity of these proteolytic enzymes. Additional experiments are required to confirm the phosphate effect. Meanwhile, as the vitamin type did not influence the proteolytic activity or the protein concentration, Gamborg’s vitamins (B5) were chosen in the subsequent scale-up experiments.

Shear rate as the scale-up criterion

The selection of the scale-up criterion is based on choosing a process parameter that most influences on productivity and finding the range of values of said...
parameter in which productivity remains high, with the purpose of replicating it in the process scale-up. Productivity is obtained by dividing the product concentration by process time. In this work, the average shear rate was chosen as the scale-up criterion because in *J. mexicana* cultures grown in various types of bioreactors, the protein concentration and proteolytic activity had a linear correlation with the average shear rate ($r^2 > 0.96$), not so with the power input, nor with the microturbulence, nor with the volumetric oxygen transfer coefficient, since the oxygen uptake rate of the cells was always lower than the oxygen transfer rate of the bioreactor, as mentioned at the end of this section. Furthermore, the cells had changes in shape and size when they were at an average shear rate from $15\,\text{s}^{-1}$ to $270\,\text{s}^{-1}$. However, at average shear rate between $200\,\text{s}^{-1}$ and $270\,\text{s}^{-1}$ there was a greater proteolytic activity and a greater accumulation of extracellular proteins.

The average shear rate in the scale-up of the *J. mexicana* cell culture has the advantage that the culture medium, even with the maximum cell concentration reaches ($4.15\,\text{g DW L}^{-1}$), exhibits a Newtonian behaviour, with a viscosity of $1.03\,\text{mPa s}$, which is expected to
remain constant throughout the growth. Also, Newtonian behaviour in B. vulgaris cell cultures with a biomass of 4 g DW L\(^{-1}\) has been reported [41]. The low viscosity of the culture medium is explained by the fact that the cells of J. mexicana grow in a unicellular manner and do not produce extracellular polymers that can increase the viscosity. In Newtonian liquids, by varying the shear stress, the viscosity remains constant, in such a way that it is possible to know the average and maximum shear rate to which the cells are subjected, using the equations described in this work.

When scaling-up bioprocesses, all hydrodynamic and oxygen transfer parameters change, even the scale-up criterion, always within the scale-up zone values. Then, an analysis of the parameters in the bioreactor being scaled-up must be carefully done to avoid adverse effects on cell growth and metabolite production [42].

In bioreactors containing Newtonian liquids, there is shear stress distribution in the bioreactor determined for the viscosity and the shear rate distribution. There are maximum and minimum shear rates to which the plant cells are subjected; notwithstanding, the average shear rate is used as a parameter to relate growth rate and metabolite production with the bioreactor hydrodynamics.

The culture medium of the J. mexicana cell culture is a Newtonian liquid; its viscosity is 1.03 cP, approximately like the water viscosity. Table 4 presents the hydrodynamic parameters, power input and volumetric oxygen transfer coefficient of the 0.4-L STB and 4-L STB used in the scale-up strategy, operated at 400 rpm and 229 rpm, respectively. In the 4-L STB operated at 229 rpm, the average shear rate was in the scale-up zone (200 s\(^{-1}\) – 274 s\(^{-1}\)). The average shear rate describes the bulk behaviour of the flow, while the zones of highest turbulent flow are in the impellers discharge zones [43], where the cells can be affected by the high shear rate and by the time they stay in these zones; which is related to the hydraulic residence time in the above-mentioned zones [1, 2, 44].

In this work, the maximum shear rate in the 4-L STB was 15% lower than that in the 0.4-L STB and in both bioreactors was 6.2-fold higher than the average shear rate. The residence time in the high shear rate zone of the 4-L STB was 83% higher than that in the 0.4-L STB (Table 4). Therefore, cells in the 4-L STB would be subjected for a longer time to a lower maximum shear rate than in the 0.4-L STB. In addition, the 0.4-L STB and the 4-L STB operated at 6000 Re number and 13200 Re number, respectively. In such hydrodynamic conditions the movement of liquid in all directions is random and unsteady [45].

The power input is related to the energy transferred from the movement of the impellers to the liquid and therefore, to the energy dissipated per unit mass of liquid, which is implicit in the Kolmogorov microturbulence scale [46], which is responsible for causing damage to fragile cells smaller than the mentioned microturbulence. The microscale of turbulence was 15% higher in the 4-L STB (54 µm vs 47 µm in the 0.4-L STB); and therefore, the microturbulence was potentially less harmful to cells than that in the small reactor, because greater microturbulence causes lesser energy dissipation in the culture medium [47]. Maximum shear rate and microturbulence could have several implications in cell growth and metabolite production, as it is described in sections below.

The power input in the 4-L STB was 53% lower than in the 0.4-L STB; even so, the cell distribution in the 4-L STB was homogenous, avoiding its sedimentation. In addition, the volumetric oxygen transfer coefficient in the 4-L STB was 18% lower than that in the 0.4-L STB; however, there was no oxygen limitation for cell growth in the 4-L STB because, the oxygen uptake rate (5.4 × 10\(^{-4}\) g oxygen/L h) of the maximum cell concentration (4.15 g DW/L) was lower than the oxygen transfer rate (6 × 10\(^{-2}\) g oxygen/L h).

**Productivities**

Volumetric proteolytic activity and cell productivity in the 4-L STB, using culture medium with 2.5 mmol L\(^{-1}\) phosphate, were higher (3.2 times and 1.5 times, respectively) than those obtained in the 0.4-L STB (Figure 3B, C, respectively), using 1.25 mmol L\(^{-1}\) phosphate, while the protein productivity reached in the 4-L STB was like the one obtained in the 0.4-L STB (Figure 3A). These results were most probably due to the use of the culture medium with 2.5 mmol L\(^{-1}\) phosphate in the 4-L STB (as it was demonstrated in the

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**Table 4.** Hydrodynamic parameters, power input and volumetric oxygen transfer coefficient of the stirred tank bioreactors used in the scaling-up strategy of Jacaratia mexicana cell culture.

| Hydrodynamic parameters, power input and volumetric oxygen transfer coefficient | Operating volume |
|---|---|
| | 0.4-L | 4-L |
| Agitation rate N (rpm) | 400 | 229 |
| Average shear rate \(\gamma_{ave}\) (s\(^{-1}\)) | 274 | 234 |
| Maximum shear rate \(\gamma_{max}\) (s\(^{-1}\)) | 1700 | 1450 |
| Pumping number \(N_p\) (–) | 0.72 | 0.90 |
| Pumping capacity Q (L s\(^{-1}\)) | 0.13 | 0.70 |
| Residence time in high shear zone \(t_h\) (s) | 3.1 | 5.7 |
| Reynolds number Re (–) | 6000 | 13,200 |
| Micromerobulance \(\lambda\) (µm) | 47 | 54 |
| Power input \(P_{g/V}\) (W/L) | 0.17 | 0.08 |
| Volumetric oxygen transfer coefficient \(k_{L}a\) (h\(^{-1}\)) | 38 | 31 |
protein production improvement experiments in shake flask with 2.5 mmol L\(^{-1}\) phosphate) and to the scale-up strategy employed, because the hydrodynamic conditions which the cells were subjected to in the scaled-up bioreactor, allowed them to have a similar metabolism to the one they had in the culture medium improvement experiments with 2.5 mmol L\(^{-1}\) phosphate, specifically in increasing the volumetric proteolytic activity while maintaining protein productivity, which also continues suggesting that a greater number of proteases are part of the extracellular proteins formed by cells in the 4-L STB.

**Proteolytic activity and protein kinetics**

The proteolytic activity in the 4-L STB, with 2.5 mmol L\(^{-1}\) phosphate, after 7 days was approximately 2 times higher than that obtained in the 0.4-L STB, with 1.25 mmol L\(^{-1}\) phosphate (Figure 4A). In addition, the kinetics of the protein concentration was similar in both bioreactors (Figure 4B). It is noticeable that both results, mentioned above for bioreactors, were like those found in the improved experiments of the culture medium in shake flask cultures, where the proteolytic activity obtained with 2.5 mmol L\(^{-1}\) phosphate (MSMB + P medium) was 1.96 times greater than that obtained using 1.25 mmol L\(^{-1}\) phosphate (MSMS medium), and the protein concentration was similar for both media, considering the standard deviation (Table 1).

Similar effects of the increase of phosphate concentrations on proteolytic activity and protein concentration in shake flasks and bioreactors were evidence that the main objective of the scale-up strategy was achieved. Therefore, the hydrodynamic conditions of the flasks and bioreactors, although they were different, allowed to obtain the same results.

**Proteinase identification**

*J. mexicana* produce an enzymatic complex of proteases. It was characterized by cation-exchange chromatography, finding at least five isoform proteases (P-I to P-V) and P-IV (MX-4), and was denominated mexicain owing to being present in the highest quantity in fruit latex [48]. To biochemically identify the mexicain produced in the 4-L STB, SDS-PAGE and Western Blot were carried out. Figure 5A shows a band with molecular weight of \(\sim24\) kDa in the latex (Lane 1); the same band can be seen in the samples of cell culture of *J. mexicana* in the 4-L STB (Lane 3 and 4) and the molecular weight marker proteins (Lane 2). For molecular identification of mexicain Figure 5B shows the immunodetection of mexicain. The enzyme of *J. mexicana* cell culture has equal molecular weight (\(\sim24\) kDa) and cross reacted with anti-mexicain antibodies, thus are immunochemically homologous with mexicain (MX-4). Our findings demonstrate that protease produced in the 4-L STB, was equal with the mexicain obtained from fruit latex of *J. mexicana*.

**J. mexicana cell size and morphology**

In a previous work, the *J. mexicana* cells changed in size (average length 4.5% smaller and average width...
13.1% smaller) and morphology (8.6% thinner) when were submitted to average shear rate from 20 s⁻¹ − 40 s⁻¹ to 200 s⁻¹ − 270 s⁻¹ [14]. In this work, although the average shear rate was 234 s⁻¹ in the 4-L STB, the cells were 2 times longer and 0.7 times thinner on average than the cells cultivated in the 0.4-L STB (Figure 6A,B). Also, the cell morphology significantly changed in the 4-L bioreactor with respect to the 0.4-L bioreactor, since the average $fe$ factor changed from 1.6 to 5.3, indicating that the cells were 3.3 times larger (Figure 6C); while the average $fa$ factor changed from 1.01 to 0.52 (Figure 6D), indicating that the cells were 49% thinner.

As the microturbulence in the 4-L STB was 54 μm (Table 4) and the length of all cells was greater than 54 μm (Figure 6A) and the width of all cells was smaller than 54 μm (Figure 6B), the cells could undergo hydrodynamic stress at the large (longitudinal direction) and...
not at the width, as it is reported by Nienow et al. [49], where the hydrodynamics could elicit the proteases synthesis and/or its excretion toward the culture medium [14]. However, the results indicate that the protein production of the cells was not negatively affected by the hydrodynamic condition in the 4-L STB and was influenced mainly for 2.5 mmol L⁻¹ phosphate in the culture medium. The lengthening and thinning of most cells could be due to the combined effect of the 2.5 mmol L⁻¹ phosphate and the hydrodynamic conditions in the 4-L STB, as it is described below.

**Cell growth kinetics**

In the 4-L STB the initial cell concentration was lower than the initial cell concentration in the 0.4-L STB; however, the cell concentration at 21 days was practically similar in both bioreactors, approximately 4.15 g DW/L; consequently, the cell productivity obtained in the 4-L STB was 1.5 times greater than that obtained in the 0.4-L STB (Figure 3C). These results were due to the maximum specific growth rate in the 4-L STB (0.0169 d⁻¹), which was 1.7 times higher than that in the 0.4-L STB (0.0099 d⁻¹), and the fact that the maximum specific growth rate was maintained along the 21 days of the culture. Therefore, the effect of 2.5 mmol L⁻¹ phosphate was again demonstrated by causing a faster specific growth rate in the 4-L STB. The lengthening and thinning of most cells in the 4-L STB could be due to the higher maximum specific growth rate, compared with that obtained in the 0.4-L STB, because in the exponential growth phase it appears that the cells are more sensitive to the shear, because the cell wall strength may decrease during the increase in cell size [50]. Therefore, the hydrodynamic conditions in the 4-L bioreactor and the culture medium with 2.5 mmol L⁻¹ phosphate seem to be associated with more changes in cell size and morphology than those expected.

**Conclusions**

The volumetric proteolytic activity in the J. mexicana shake flask cell culture increased 1.9 times with increasing phosphate concentration from 1.25 to 2.5 mmol L⁻¹, while the protein concentration was similar. The J. mexicana cell culture was scaled-up from 0.4-L to 4-L in stirred tank bioreactors using the shear rate as scale-up criterion, with an average shear rate of 234 s⁻¹. In the 4-L bioreactor the phosphate concentration was 2.5 mmol L⁻¹, while in the 0.4-L STB was 1.25 mmol L⁻¹. In the scaled-up bioreactor, the volumetric proteolytic activity increased 2 times, while the protein concentration in both bioreactors was similar, approximately 55 mg/L. The results obtained in shake flask by phosphate addition were similar to those obtained in the scale-up strategy with phosphate addition, indicating that the average shear rate is a useful hydrodynamic parameter in the scale-up of J. mexicana cell culture. The lengthening and thinning of most cells in the 4-L stirred tank bioreactor were not expected. However, they may be related with a higher maximum specific growth rate due to 2.5 mmol L⁻¹ phosphate concentration and to the bioreactor hydrodynamics. The power input per unit volume decreased 53% in the scaled-up bioreactor, maintaining the volumetric proteolytic activity and the protein productivity.

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**Disclosure statement**

The authors report no conflict of interest.

**Data availability statement**

All data that support the findings reported in this study are available from the corresponding author upon reasonable request.

**Author contributions**

García-Salas: Conceptualization, Data analysis and interpretation, Writing – review & editing. Gómez-Montes: Experiment conducting, data collect, statistical analysis, drafting the manuscript. Ramírez-Sotelo: Data analysis, Drafting the manuscript and review the manuscript. Oliver-Salvador: Concept and design, Data analysis and interpretation, drafting the manuscript & review.

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