



# Effect of impeller type on cellular morphology and production of clavulanic acid by *Streptomyces clavuligerus*

M. C. C. Bustamante<sup>1</sup> · C. L. L. Costa<sup>1</sup> · M. N. Esperança<sup>2</sup> · V. T. Mazziero<sup>3</sup> · M. O. Cerri<sup>3</sup> · Alberto C. Badino<sup>1</sup>

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## Abstract

It is essential to evaluate the effects of operating conditions in submerged cultures of filamentous microorganisms. In particular, the impeller type influences the flow pattern, power consumption, and energy dissipation, leading to differences in the hydrodynamic environment that affect the morphology of the microorganism. This work investigated the effect of different impeller types, namely the Rushton turbine (RT-RT) and Elephant Ear impellers in up-pumping (EEUP) and down-pumping (EEDP) modes, on cellular morphology and clavulanic acid (CA) production by *Streptomyces clavuligerus* in a stirred-tank bioreactor. At 800 rpm and 0.5 vvm, the cultivations performed using RT-RT and EEUP impellers provided higher shear conditions and oxygen transfer rates than those observed with EEDP. These conditions resulted in higher clavulanic acid production using RT-RT (380.7 mg/L) and EEUP (453.3 mg/L) impellers, compared to EEDP (196.6 mg/L). Although the maximum CA concentration exhibited the same order of magnitude for RT-RT and EEUP impellers, the latter presented 40% of the specific power consumption (4.9 kW/m<sup>3</sup>) compared to the classical RT-RT (12.0 kW/m<sup>3</sup>). The specific energy for CA production ( $E_{CA}$ ), defined as the energy cost to produce 1 mg of CA, was 3.5 times lower using the EEUP impeller (1.91 kJ/mg<sub>CA</sub>) when compared to RT-RT (5.91 kJ/mg<sub>CA</sub>). Besides, the specific energy for O<sub>2</sub> transfer ( $E_{O_2}$ ), the energy required to transfer 1 mmol of O<sub>2</sub>, was 2.3 times lower comparing the EEUP impeller (3.28 kJ/mmol<sub>O2</sub>) to RT-RT (7.65 kJ/mmol<sub>O2</sub>). The results demonstrated the importance of choosing the most suitable impeller configuration in conventional bioreactors to manufacture bioproducts.

**Keywords** Clavulanic acid · *Streptomyces clavuligerus* · Stirred-tank bioreactor · Shear rate · Rushton turbine · Elephant ear impeller

## Introduction

Many biotechnological products in the pharmaceutical and food industries are produced by submerged cultivation of strictly aerobic filamentous microorganisms in stirred-tank

bioreactors. *Streptomyces clavuligerus* is an important producer of secondary metabolites, among them medical products such as cephamycin C and clavulanic acid (CA) [1–3]. The clinical importance of CA has resulted in extensive research focused on increasing its production using a variety of strategies involving aspects such as operating conditions [1, 2, 4], medium composition [5–7], fed-batch operation mode [8, 9], extractive fermentation [10], new microbial strains [11], and temperature-shift techniques [12, 13].

In submerged culture, *S. clavuligerus* presents variable patterns of growth, with its morphology including compact pellets, clumps, and branched and unbranched free filaments, which influence the rheology of the culture broth. The shapes and sizes of the filamentous growth forms are affected by many factors, including medium composition, dissolved oxygen concentration, reactor model, and power input [14], consequently influencing the production of the

Responsible Editor: Luis Henrique Souza Guimaraes

✉ Alberto C. Badino  
badinojr@ufscar.br

<sup>1</sup> Department of Chemical Engineering, Federal University of São Carlos, C.P. 676, São Carlos, SP 13565-905, Brazil

<sup>2</sup> Federal Institute of Education, Science and Technology of São Paulo, Campus Capivari, Capivari, SP 13360-000, Brazil

<sup>3</sup> Department of Bioprocesses Engineering and Biotechnology, Faculty of Pharmaceutical Sciences, São Paulo State University, Araraquara, SP 14801-902, Brazil

bioproducts of interest. Therefore, the investigation of bioprocessing strategies can greatly contribute to further improvements in *S. clavuligerus* submerged cultivations.

Although the effects of cell morphology on process productivity are widely studied for some submerged cultures of filamentous microorganisms, *Aspergillus niger* e.g. [14–17], few studies have evaluated the relationships among *S. clavuligerus* morphology, bioreactor hydrodynamics, and clavulanic acid production [4, 18]. *S. clavuligerus* submerged cultivation presents a complex rheology, with high broth viscosity [19], requiring a suitable agitation level to provide adequate mixing, oxygen mass transfer [1], and bioproduct formation. Although bioreactor operating conditions and impeller type determine oxygen transfer in stirred tanks, high shear conditions and hydrodynamic stress can affect microorganism growth and production in certain situations [20]. Therefore, the effect of operating conditions (agitation and aeration) in submerged culture must be carefully evaluated, since the impeller type selected will determine the hydrodynamic forces affecting the morphology of the microorganism [21, 22]. The hydrodynamic conditions define the flow pattern and are related to power consumption, energy dissipation, broth rheology, and bioreactor/impeller geometric parameters [20, 23]. Hence, the correct choice of impeller can improve the cultivation performance in terms of cell growth, and morphology, and biosynthesis of the product of interest.

The Rushton turbine (RT) is the most common impeller type used in microbial cultivations. Despite its advantages such as elevated oxygen transfer and good mixing, the Rushton turbine has high energy consumption [24, 25]. In this sense, it becomes interesting to evaluate the behavior of other impeller geometries, such as the so-called Elephant ear (EE) design, whose inclined blades promote a mixed flow pattern (axial/radial) in the culture broth, being indicated as suitable for the cultivation of shear-sensitive cells [26].

Bustamante et al. [27, 28] evaluated the in vitro performance of Rushton turbine (RT) and Elephant Ear (EE) impellers, with the latter in down-pumping (EEDP) and up-pumping (EEUP) configurations, in a conventional bench-scale stirred-tank bioreactor. It was observed that the EEDP impellers generated lower average shear rate ( $\dot{\gamma}_{av}$ ) values, while RT-RT and EEUP impellers exhibited similar orders of magnitude for shear conditions. Also, the EEUP impeller combined good oxygen mass transfer and adequate shear conditions.

Considering the earlier discoveries, the current study sought to assess how different impeller types (RT, EEDP, and EEUP), and their respective shear rate and oxygen transfer conditions, influence both cellular morphology and clavulanic acid production by *Streptomyces clavuligerus* in a conventional (stirred and aerated) tank bioreactor.

## Materials and methods

### Bioreactor and impellers

Cultivations were performed in a commercial stirred-tank bioreactor (Bioflo IIC fermenter, New Brunswick Scientific, USA) with a 4-L working volume, internal diameter ( $D_i$ ) of 0.17 m, and four baffles. The dissolved oxygen concentration was measured using an amperometric probe (model InPro 6800, Mettler-Toledo). The bioreactor was equipped with pH and temperature control systems and was fitted with a stainless-steel ring gas sparger with 4 holes of 1 mm diameter.

Three impeller configurations were used in this study. The first consisted of two conventional six-flat-blade Rushton turbines (RT-RT), spaced one impeller diameter ( $d_i = 0.076$  m) apart, with impeller blade width ( $W$ ) of 0.016 m, length ( $L$ ) of 0.019 m, disk diameter ( $d_0$ ) of 0.063 m, and thickness ( $T$ ) of 0.0015 m (Fig. 1a). The second and third impeller configurations consisted of a single three-blade Elephant Ear (EE) impeller (New Brunswick Scientific, USA) with a diameter of 0.08 m and blade width ( $W$ ) of 0.073 m. The second configuration was operated in the “up-pumping” mode (EEUP, Fig. 1b) while the third was in the “down-pumping” mode (EEDP, Fig. 1c).

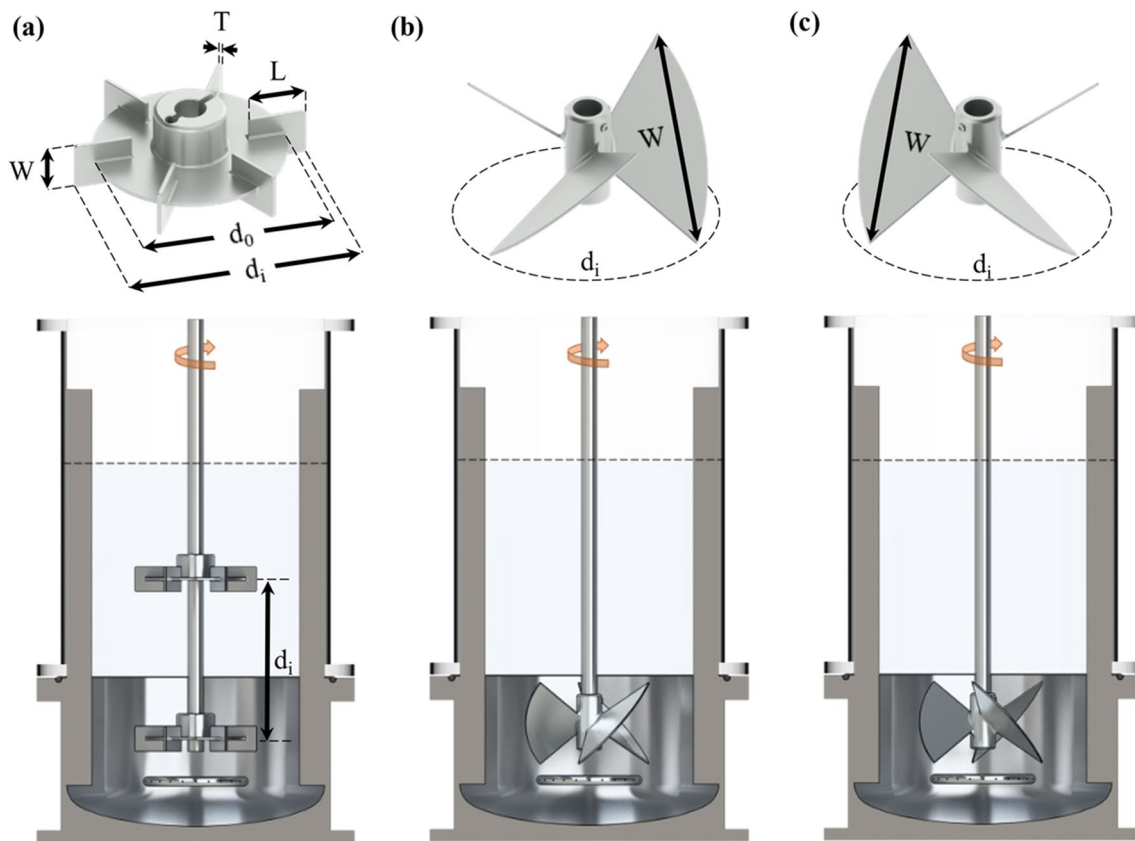
### Microorganism and culture media

The microorganism used in this work was *Streptomyces clavuligerus* ATCC 27064, stored as vegetative cells (5 g/L dry weight) at  $-70$  °C in 4 mL cryotubes containing glycerol (10% v.v<sup>-1</sup>).

The seed medium, proposed by Rosa et al. [1], had the following composition (g.L<sup>-1</sup>): glycerol (15.0); bacto peptone (10.0); malt extract (1.0); K<sub>2</sub>HPO<sub>4</sub> (0.8); MgSO<sub>4</sub>·7H<sub>2</sub>O (0.75); MnCl<sub>2</sub>·4H<sub>2</sub>O (0.0001); FeSO<sub>4</sub>·7H<sub>2</sub>O (0.001); ZnSO<sub>4</sub>·7H<sub>2</sub>O (0.001); and 3-(N-morpholino) propane sulfonic acid (MOPS) buffer (21.0) at pH 6.8. The inoculum medium was based on that used by Teodoro et al. [9] and contained (g.L<sup>-1</sup>): glycerol (15.0); soybean protein isolate (25.0); K<sub>2</sub>HPO<sub>4</sub> (0.8); MgSO<sub>4</sub>·7H<sub>2</sub>O (0.75); MnCl<sub>2</sub>·4H<sub>2</sub>O (0.0001); FeSO<sub>4</sub>·7H<sub>2</sub>O (0.001); ZnSO<sub>4</sub>·7H<sub>2</sub>O (0.001); and 3-(N-morpholino) propane sulfonic acid buffer (21.0) at pH 6.8. The production medium had the same composition as the inoculum medium, except that no MOPS buffer was used. All the media were autoclaved at 121 °C for 15 min.

### Batch cultivations

Vegetative cell suspensions were transferred from the cryotubes to 500 mL Erlenmeyer flasks containing 50 mL of seed medium, followed by incubation for 24 h in a rotary shaker at 250 rpm and 30 °C. Erlenmeyer flasks (500 mL)



**Fig. 1** Geometric characteristics of the 4-L stirred-tank bioreactor equipped with different impellers: **(a)** Rushton turbines (RT-RT), **(b)** Elephant Ear in “up-pumping” operating mode (EEUP), and **(c)** Elephant Ear in “down-pumping” operating mode (EEDP)

containing 45 mL of the inoculum medium were inoculated with 5 mL of cultivated seed broth and incubated for 24 h at 250 rpm and 30 °C.

For the production stage, 400 mL of the inoculum suspension were transferred to the bioreactor containing the production medium, resulting in a 4-L working volume. Three batch cultivations, each with a different impeller, were performed in duplicate, at 800 rpm and 0.50 vvm. The operating conditions were kept constant to assess only the impact of impeller design in the cultivations. The temperature was kept at 30 °C and the pH was controlled at  $6.8 \pm 0.1$  by the addition of 1 M NaOH and 2 M HCl solutions. An antifoaming agent (30% m.m<sup>-1</sup>, Dow Corning Co.) was used to avoid foam formation. Samples (25 mL) were withdrawn every 6 h for analysis of cell morphological parameters, cell growth, and the concentrations of clavulanic acid (CA) and glycerol.

### Analytical methods

Cell growth was evaluated indirectly by measuring the broth consistency index ( $K$ ) of the power law model (Eq. 1) which relates the rheological variables shear stress ( $\tau$ ) and shear

rate ( $\dot{\gamma}$ ). In cultivations using filamentous microorganisms,  $K$  is mainly affected by biomass concentration, growth rate, and cellular morphology [2, 29, 30].

$$\tau = K \cdot \dot{\gamma}^n \quad (1)$$

where  $\tau$  is the shear stress (Pa),  $K$  is the broth consistency index (Pa.s <sup>$n$</sup> ),  $\dot{\gamma}$  is the shear rate (s<sup>-1</sup>), and  $n$  is the broth flow index (-).

The broth rheological parameters  $K$  and  $n$  were determined from rheograms ( $\tau$  vs  $\dot{\gamma}$ ) of samples of the cultivation broths using a digital concentric-cylinders rheometer (model LV-DVIII+, Brookfield Engineering Laboratories Inc., USA).

The concentrations of clavulanic acid and glycerol were determined in the supernatants of broth samples centrifuged at 17,500-g for 5 min, at 4 °C, and filtered through a 0.22  $\mu$ m membrane.

The clavulanic acid concentrations ( $C_{CA}$ ) were determined by UV spectrophotometric analysis [31], which consisted of reading at the 311 nm wavelength of the product of the derivation of clavulanic acid with the imidazole reagent. The standard used was CA contained in

the pharmaceutical product Clavulin® (Glaxo-SmithKline Farmacêutica, Rio de Janeiro, Brazil).

The glycerol concentrations ( $C_{\text{Gly}}$ ) were determined by an enzymatic method, using a triglycerides GPO-PAP test kit (Laborlab, Brazil).

### Image analysis

The cultivation broth samples were diluted using distilled water, fixed, and stained with methylene blue, as described by Pamboukian et al. [32]. The dilution resulted in a suitable cell concentration for observation using an optical microscope. The cell morphology parameters were measured using Image-Pro Plus 6.0 software and an optical microscope (Olympus BX-50) operated at magnifications of 10× and 20× [32]. A total of 50 images with approximately 200 and 300 elements were captured for each sample, including pellets (P), clumps (C), branched hyphae (BH), and unbranched hyphae (UH). The images were recorded in JPG format, with a resolution of 2048 × 1536 pixels.

### Volumetric oxygen transfer coefficient ( $k_L a$ ) and average shear rate ( $\dot{\gamma}_{\text{av}}$ )

The  $k_L a$  and  $\dot{\gamma}_{\text{av}}$  values for the stirred-tank bioreactor equipped with different impellers (RT-RT, EEDP, and EEUP) were determined using the correlations proposed by Bustamante et al. [27, 28]:

$$k_{L a(\text{RT})} = 1.13 \cdot 10^{-5} \cdot N^{1.431} \cdot \phi_{\text{air}}^{0.488} \cdot K^{0.131} \cdot n^{1.193} \quad R^2 = 0.98 \quad (2)$$

$$k_{L a(\text{EEDP})} = 1.92 \cdot 10^{-7} \cdot N^{1.522} \cdot \phi_{\text{air}}^{0.409} \cdot K^{-0.535} \cdot n^{-0.686} \quad R^2 = 0.98 \quad (3)$$

$$k_{L a(\text{EEUP})} = 4.45 \cdot 10^{-6} \cdot N^{1.512} \cdot \phi_{\text{air}}^{0.462} \cdot K^{0.124} \cdot n^{1.338} \quad R^2 = 0.98 \quad (4)$$

$$\dot{\gamma}_{\text{av}(\text{RT})} = (1.482 \cdot N^{0.653} \cdot \phi_{\text{air}}^{-0.200} \cdot K^{0.751} \cdot n^{1.193})^{\frac{1}{0.620 \cdot (1-n)}} \quad R^2 = 0.98 \quad (5)$$

$$\dot{\gamma}_{\text{av}(\text{EEDP})} = (0.389 \cdot N^{0.326} \cdot \phi_{\text{air}}^{-0.176} \cdot K^{-0.014} \cdot n^{-0.686})^{\frac{1}{0.521 \cdot (1-n)}} \quad R^2 = 0.98 \quad (6)$$

$$\dot{\gamma}_{\text{av}(\text{EEUP})} = (739.8 \cdot N^{-0.194} \cdot \phi_{\text{air}}^{-0.156} \cdot K^{0.840} \cdot n^{1.338})^{\frac{1}{0.716 \cdot (1-n)}} \quad R^2 = 0.98 \quad (7)$$

where  $N$  is the impeller speed ( $N = 800$  rpm) and  $\phi_{\text{air}}$  is the specific air flow rate ( $\phi_{\text{air}} = 0.50$  vvm).

Knowing the profiles for  $k_L a$  and the dissolved oxygen concentration in the broth ( $C_{O_2}$ ) along the *S. clavuligerus* cultivations, the oxygen transfer rate (OTR) could be calculated using Eq. 8:

$$\text{OTR} = k_L a \cdot (C_{O_2}^* - C_{O_2}) \quad (8)$$

where,  $C_{O_2}^*$  and  $C_{O_2}$  are the oxygen solubility in the medium ( $\text{mol.L}^{-1}$ ) and the oxygen concentration in the medium ( $\text{mol.L}^{-1}$ ), respectively.

### Gassed power consumption ( $P_g$ )

The gassed power consumption ( $P_g$ , in W), at 800 rpm and 0.50 vvm, was calculated as the product of the torque required for the agitation of the gas-liquid dispersion ( $T$ , in N.m) and the angular velocity of the agitator shaft ( $\omega = 2 \cdot \pi \cdot N$ , in  $\text{rad.s}^{-1}$ ). The torque was calculated as the product of the force ( $F$ , in N) and the length of the arm ( $b = 0.175$  m) connecting to the motor shaft ( $T = F \cdot b$ ) [33]. The gassed power consumption was then calculated using Eq. (9):

$$P_g = T \cdot \omega = (F \cdot b) \cdot (2 \cdot \pi \cdot N) \quad (9)$$

The motor was placed over a vertical ball bearing allowing torque measurement. The force ( $F$ ) was measured in triplicate using a digital dynamometer (FG 6005SD, Lutron, accuracy of 0.4% full scale) attached to the arm coupled to the motor shaft.

The gassed power consumption was measured using a solution of glycerol (67% v.v<sup>-1</sup>) as a model fluid. This solution was chosen because it had a viscosity (0.0015 Pa.s) similar to the average apparent viscosity observed for the culture broths during the *S. clavuligerus* cultivations (data not shown).

## Results and discussion

### Effect of impeller type on clavulanic acid production

The influence of the impeller configuration on clavulanic acid production was evaluated by performing three cultivations at 800 rpm and 0.50 vvm in the stirred-tank bioreactor.

Figure 2 shows the experimental values of the clavulanic acid concentration ( $C_{CA}$ ), broth consistency index ( $K$ ), and dissolved oxygen in the broth (DO) during the cultivations. In the cultivation performed with the RT-RT impeller (Fig. 2a), the clavulanic acid concentration reached a maximum value ( $380.7 \text{ mg.L}^{-1}$ ) at 54 h. The cultivation performed with the EEUP impeller (Fig. 2b) exhibited the highest  $C_{CA}$  value among the three assays ( $453.3 \text{ mg.L}^{-1}$ ), at 45 h of cultivation. In these two assays (RT-RT and EEUP), no oxygen limitation was observed, since the dissolved oxygen levels were higher than 42% of air saturation throughout the cultivations. The EEUP cultivation exhibited higher and faster clavulanic acid production, compared to the RT-RT cultivation, which also indicated higher clavulanic acid productivity (Table 1).

In the cultivation performed with the EEDP impeller, dissolved oxygen reached a critical value of about 10% at 21 h (Fig. 2c), which probably explained the low clavulanic acid production, with a maximum value of 196.6 mg.L<sup>-1</sup> at 45 h. Low clavulanic acid productivity was also observed for this impeller configuration (Table 1). Rosa et al. [1], who performed *S. clavuligerus* cultivations at low agitation speeds (300 and 600 rpm) and 0.50 vvm, also observed that oxygen limitation led to low (or no) clavulanic acid production.

Based on the maximum clavulanic acid production values ( $C_{CA}^{max}$ ) and the time required to achieve that condition, the clavulanic acid productivity ( $P_{CA}$ , in mg.L<sup>-1</sup>.h<sup>-1</sup>) was calculated for the three cultivations (RT-RT, EEUP, and EEDP) and compared to literature data, as shown in Table 1. All the values were obtained for the same operating conditions (800 rpm and 0.50 vvm). For the RT-RT and EEUP configurations, clavulanic acid production and productivity were of similar orders of magnitude as values reported previously.

It is important to highlight that the only difference among the cultivations in the present study was in the utilization of different impeller configurations (RT-RT, EEUP, and EEDP), which led to differences in flow patterns and oxygen transfer (Fig. 3) [26, 34].

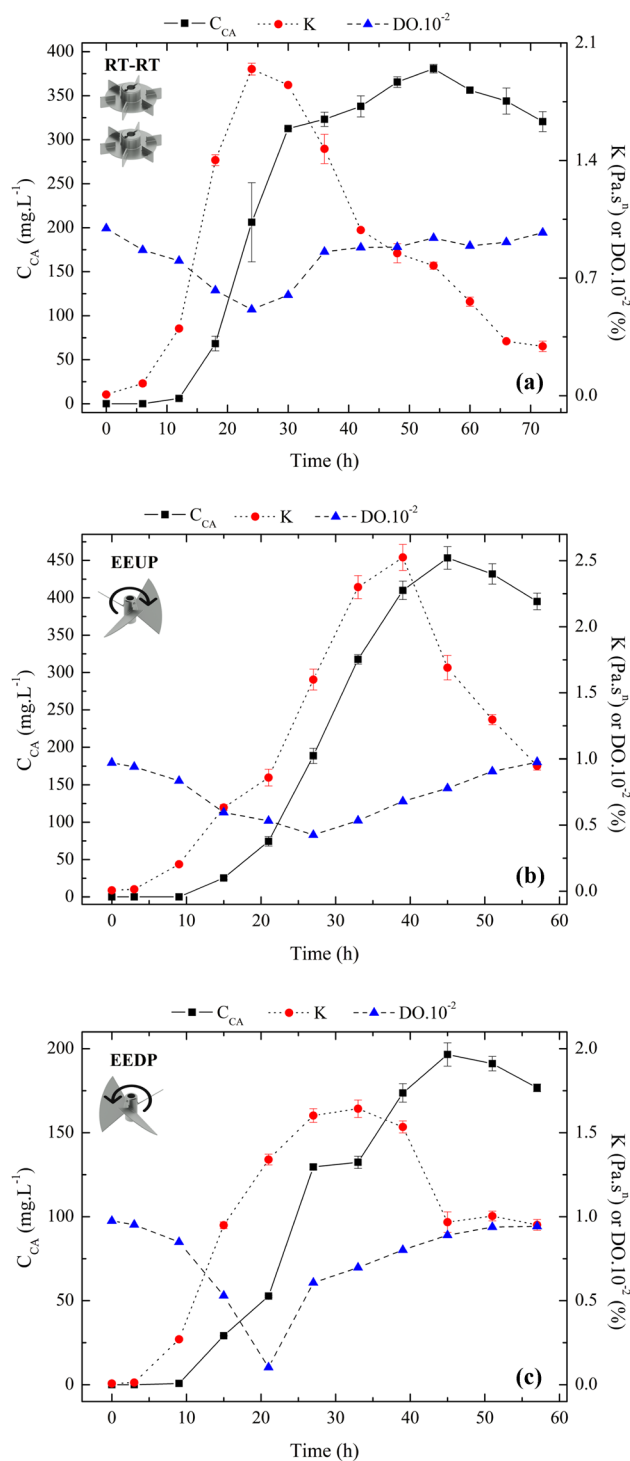
### Effect of impeller type on *S. clavuligerus* morphology

In submerged cultures, the mycelial cell morphology of the microorganism can significantly affect cultivation performance [35]. The effect of impeller configuration on *S. clavuligerus* morphology was investigated in three cultivations using the RT-RT, EEUP, and EEDP impeller configurations. Four morphological classes, namely pellets (P), clumps (C), branched hyphae (BH), and unbranched hyphae (UH), were characterized and quantified by image analysis. These morphologies are illustrated in Fig. 4.

Based on the quantification of each morphological class (P, C, BH, and UH) and its contribution to the total quantity (sum of all the classes), a morphological class distribution (in %) was obtained during each cultivation (Fig. 5). The initial morphological class distribution was similar for the three cultivations, consisting of about 12.3% of pellets, 56.2% of clumps, and 31.5% of branched hyphae. No unbranched hyphae were observed at the beginning of the cultivations.

The behavior observed for the variation of the morphological classes, shown in Fig. 5, could be summarized as follows:

- The number of pellets decreased rapidly, with no pellets observed at 12, 3, and 15 h in the cultivations using the RT-RT, EEUP, and EEDP impellers, respectively.



**Fig. 2** Clavulanic acid concentration ( $C_{CA}$ ), broth consistency index ( $K$ ), and dissolved oxygen ( $DO$ ) during the *S. clavuligerus* cultivations at 800 rpm and 0.50 vvm in the stirred-tank bioreactor equipped with different impellers: **(a)** Rushton turbines (RT-RT), **(b)** Elephant Ear in “up-pumping” operating mode (EEUP), and **(c)** Elephant Ear in “down-pumping” operating mode (EEDP)

**Table 1** Comparison among results of maximum clavulanic acid concentration ( $C_{CA}^{max}$ ), cultivation time to reach the maximum clavulanic acid production ( $t$  for  $C_{CA}^{max}$ ) and maximum clavulanic productivity ( $P_{CA}^{max}$ ) by *S. clavuligerus* cultivations performed in 4-L stirred-tank bioreactors, at 800 rpm and 0.5 vvm

Reference	Impeller	T (°C)	$C_{CA}^{max}$ (mg.L <sup>-1</sup> )	$t$ for $C_{CA}^{max}$ (h)	$P_{CA}^{max}$ (mg.L <sup>-1</sup> .h <sup>-1</sup> )
Rosa et al. [1]	RT-RT	28	475.0	45	10.6
			482.0*	45*	10.7*
Cerri and Badino [2]	RT-RT	30	402.0	54	7.4
Ribeiro et al. [4]	RT-RT	28	871.5	54	16.1
Present study	RT-RT	30	380.7	54	7.1
	EEDP		196.6	45	4.4
	EEUP		453.3	45	10.1

\*Cultivation supplied with air enriched with oxygen

*RT-RT* Rushton turbines, *EEUP* Elephant Ear in “up-pumping” operating mode, *EEDP* Elephant Ear in “down-pumping” operating mode

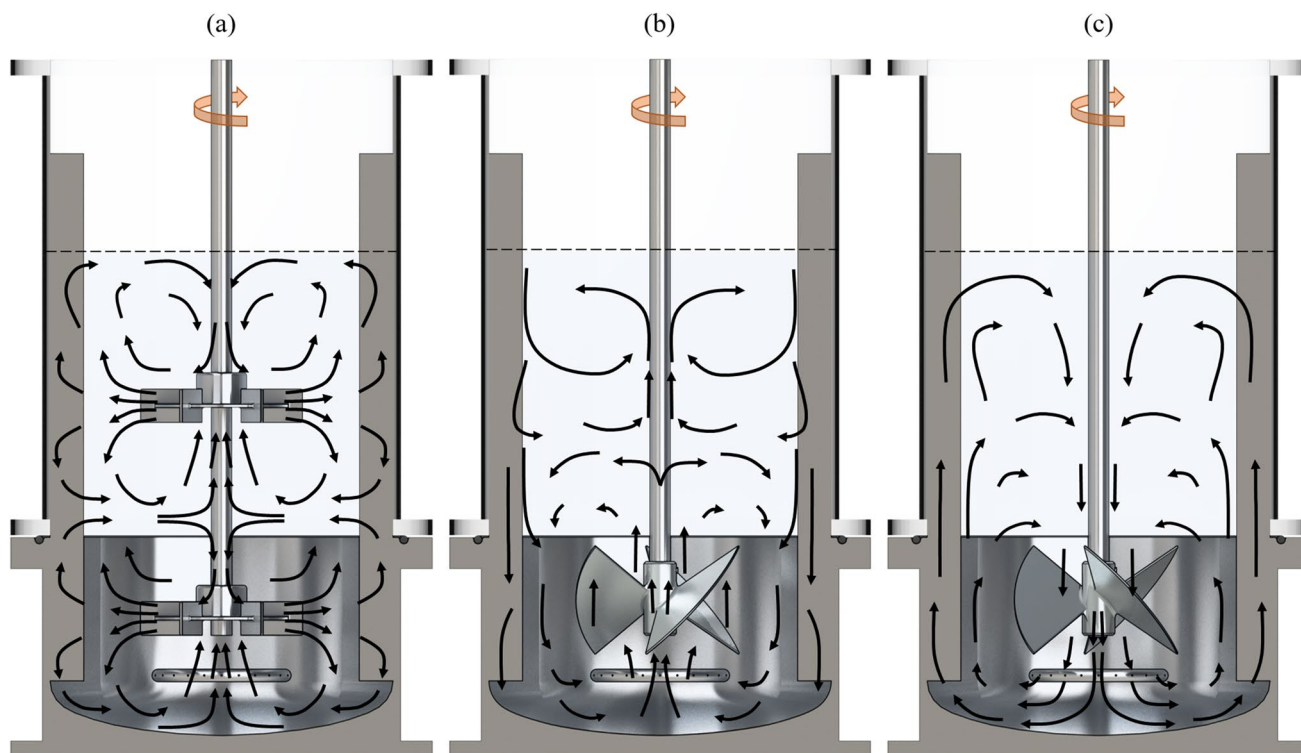
- The number of clumps decreased until reaching stable values around 7.3% (at 15 h), 12.0% (at 39 h), and 19.2% (at 21 h) in the cultivations using the RT-RT, EEUP, and EEDP impellers, respectively. These dynamics resulted

from a complex relationship among pellet fragmentation (leading to increased clumps), hyphae formation (leading to decreased clumps), and cell multiplication.

- The branched and unbranched hyphae distributions increased in all three cultivations, exhibiting different dynamics and reaching fairly stable values for the different impellers.

Despite the complex relationship among morphology, broth rheology, and clavulanic acid production, the cultivation broth exhibited higher shear rates in the cultivations using the RT-RT and EEUP impellers, compared to the EEDP cultivations. This was supported by the following observations:

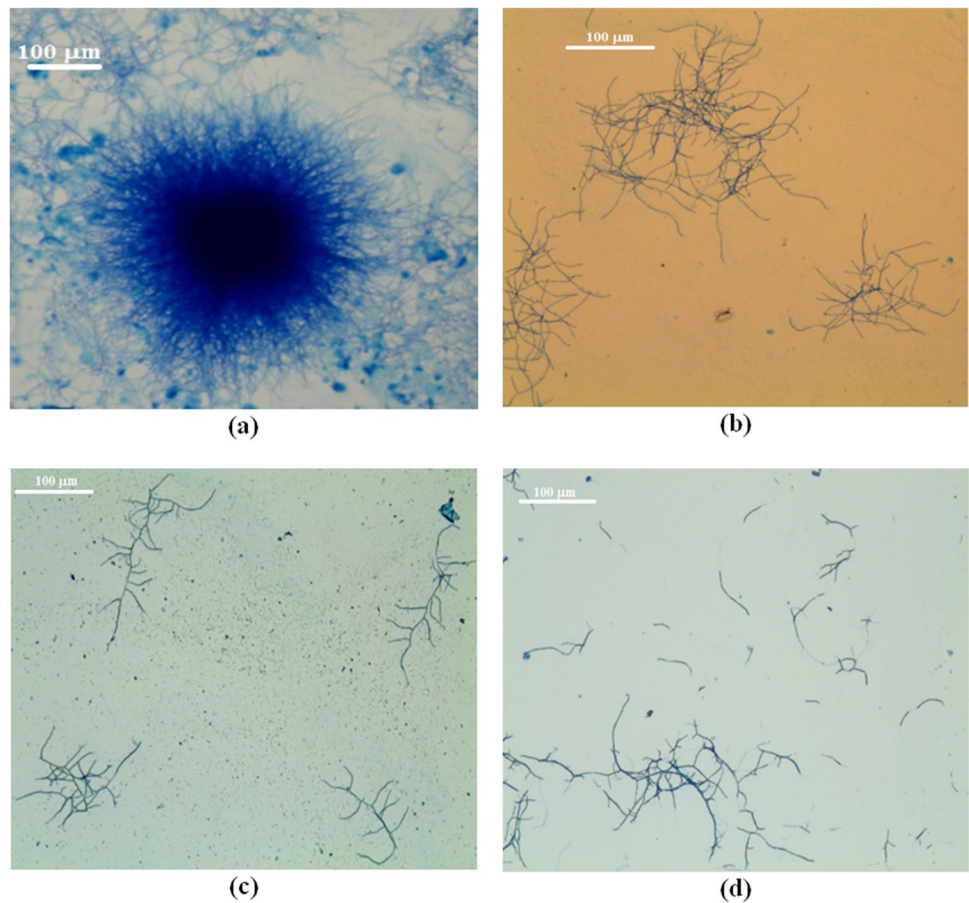
- Higher clavulanic acid production was obtained in the RT-RT and EEUP cultivations, compared to the EEDP cultivation. This could be expected since clavulanic acid production is positively affected by the shear rate [4]. As reported by Rosa et al. [1], a high shear rate leads to greater cell wall disruption, releasing clavulanic acid from the periplasmic space between the membrane and the cell wall, consequently increasing the CA concentration in the medium.



**Fig. 3** Expected fluid flow fields in the 4-L stirred-tank bioreactor equipped with different impellers: **(a)** Rushton turbines (RT-RT), **(b)** Elephant Ear in “up-pumping” operating mode (EEUP),

and **(c)** Elephant Ear in “down-pumping” operating mode (EEDP). Adapted from Doran (2013) [34] and Zhu et al (2009) [26]

**Fig. 4** Representative images of morphological classes of the *S. clavuligerus* extracted from the fermentation broths: (a) pellet ( $\times 40$ ), (b) clumps ( $\times 100$ ), (c) branched hyphae ( $\times 100$ ), and (d) unbranched hyphae ( $\times 100$ )



- The stable value for clumps in the EEDP cultivation, of around 19.2%, was higher than in the other cultivations (RT-RT and EEUP).
- The hyphae distribution (branched and unbranched) in the EEDP cultivation, of about 80.8%, was lower than in the RT-RT (92.7%) and EEUP (88.0%) cultivations.

Finally, the dynamics of the number of hyphae (branched and unbranched, BH+UH) were similar in the RT-RT and EEUP cultivations, with the hyphae numbers becoming almost constant after a certain cultivation time (Fig. 6). The BH+UH values became almost stable at 24 h and 39 h in the RT-RT and EEUP cultivations, respectively.

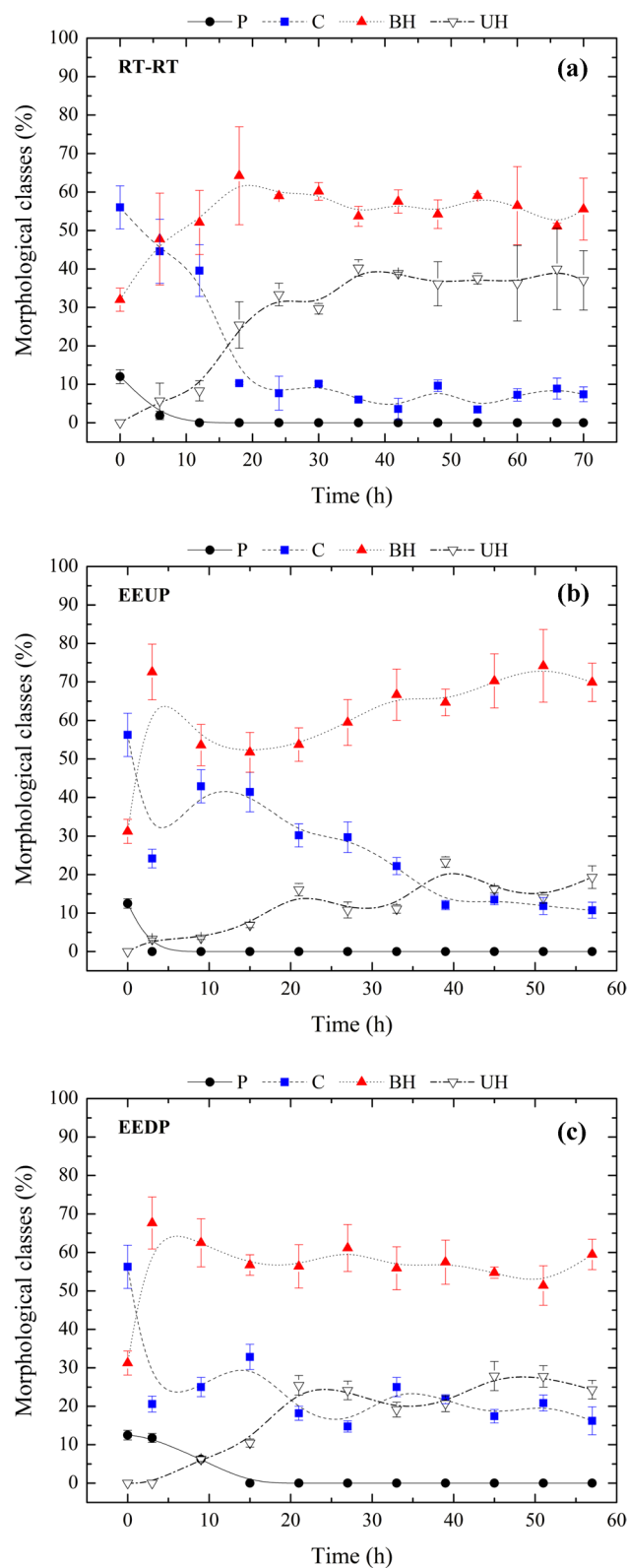
A comparison of the BH+UH profiles with the corresponding broth consistency index ( $K$ ) profiles showed that there was a direct relationship between the stabilization of the hyphae distribution and the maximum consistency index value (Fig. 6). This behavior could explain the higher clavulanic acid production observed in the EEUP cultivation ( $453.3 \text{ mg.L}^{-1}$ ), compared to the RT-RT cultivation ( $380.7 \text{ mg.L}^{-1}$ ). After stabilization, both EEUP and RT-RT achieved similar BH+UH values (88.0 and 92.7%, respectively). However, distinct dynamics were observed. While RT-RT provided faster fragmentation kinetics (stable

BH+UH values at 24 h), EEUP exhibited a slower fragmentation with stabilization of BH+UH values at 39 h. This behavior allowed reaching a higher cell concentration in EEUP cultivation, indicated by the higher  $K$  values, when compared to RT-RT, resulting in higher CA production. After the maximum value of the consistency index ( $K$ ) is reached, a decrease in cultivation occurred due to cell lysis as reported by Cerri and Badino (2012) [2].

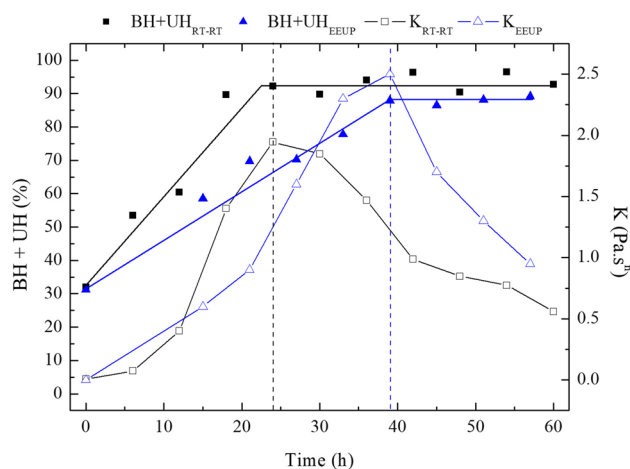
Therefore, it could be concluded that the use of different impellers led to differences in the morphological and rheological behaviors, which affected the production of clavulanic acid.

#### Effect of oxygen transfer and power consumption on clavulanic acid production

The utilization of different impellers (RT-RT, EEUP, and EEDP) led to differences in clavulanic acid production and productivity, as a result of different oxygen transfer rates and shear levels (Table 2). Ribeiro et al. [4] performed *S. clavuligerus* cultivations for evaluation of the isolated effects of average shear rate ( $\dot{\gamma}_{av}$ ) and initial oxygen transfer rate ( $OTR_{initial}$ ) on CA productivity ( $P_{CA}$ ), using a stirred-tank bioreactor equipped with RT-RT impellers. It was observed



**Fig. 5** Distribution of morphological classes (in %) during the *S. clavuligerus* cultivations at 800 rpm and 0.50 vvm in a 4-L working volume stirred-tank bioreactor equipped with different impellers: **(a)** Rushton turbines (RT-RT), **(b)** Elephant Ear in “up-pumping” operating mode (EEUP), and **(c)** Elephant Ear in “down-pumping” operating mode (EEDP). Legend: pellets (P), clumps (C), branched hyphae (BH), and unbranched hyphae (UH)



**Fig. 6** Branched and unbranched hyphae (BH+UH) distributions and broth consistency index (K) values during the *S. clavuligerus* cultivations conducted at 800 rpm and 0.50 vvm in a stirred-tank bioreactor with a working volume of 4-L, using Rushton turbines (RT-RT) and Elephant Ear “up-pumping” operating mode (EEUP) impeller. The solid lines indicate the general trends for variation of the BH+UH values

that both  $\dot{\gamma}_{av}$  and  $OTR_{initial}$  had a positive effect on  $P_{CA}$ , with higher initial  $OTR$  values resulting in faster clavulanic acid production, while higher shear rates increased the maximum CA concentration. It was found that  $\dot{\gamma}_{av}$  had the greatest influence on  $P_{CA}$ .

The shear and oxygen transfer rate values were similar in the cultivations performed with the RT-RT and EEUP impellers, while lower values were obtained in the cultivation using the EEDP impeller. These findings hold particular significance given the variation in the number of impellers installed in the stirred tank bioreactor. While RT-RT consisted of a dual-impeller system, EEUP and EEDP were operated in a single impeller mode. Despite of this, RT-RT and EEUP promoted quite similar variations in the morphological classes and shear rates. Buffo et al. [15] employed *A. niger* pellets to assess shear conditions in a stirred tank bioreactor equipped with different dual-impeller associations: a two Rushton turbines system (RT-RT) and a two Elephant Ear system (EEDP-EEUP). Through pellet fragmentation assays, the authors observed no significant differences between the evaluated configurations.

Waldherr et al. [36] assessed the fragmentation of *A. niger* pellets under non-growing conditions in a 3-L stirred tank bioreactor equipped with different impellers, including the Rushton turbine (RT), propeller (Prop), and wave-ribbon (WRI). By conducting the fragmentation assay at a specific power input equal to 0.59 W/kg, the authors verified the following trend in pellet size reduction:  $WRI > Prop > RT$ . This outcome highlights the Rushton turbines as the least shearing impeller among those examined.



**Table 2** Comparison of maximum clavulanic acid concentration ( $C_{CA}^{max}$ ) and productivity ( $P_{CA}^{max}$ ), shear rate, power consumption, and oxygen transfer rate for the *S. clavuligerus* cultivations (at 800 rpm and 0.50 vvm) in a stirred-tank bioreactor equipped with different impellers

Parameter	RT-RT	EEUP	EEDP
$C_{CA}^{max}$ (mg/L)	380.7	453.3	196.6
t for $C_{CA}^{max}$ (h)	54	45	45
$P_{CA}^{max}$ (mg.L <sup>-1</sup> .h <sup>-1</sup> )	7.05	10.07	4.37
$\dot{\gamma}_{av}$ (s <sup>-1</sup> )	3,025	3,015	368
$P_{g,av}$ (W)	48.08	19.42	11.65
$OTR_{av}$ (mmolO <sub>2</sub> .L <sup>-1</sup> .s <sup>-1</sup> )	$1.57 \cdot 10^{-3}$	$1.48 \cdot 10^{-3}$	$8.40 \cdot 10^{-4}$

$\dot{\gamma}_{av}$ : average shear rate during cultivations (calculated using Eqs. 5–7);  $P_{g,av}$ : average power consumption during cultivations;  $OTR_{av}$ : average oxygen transfer rate during cultivations (calculated by Eq. 8)

RT-RT Rushton turbines, EEUP Elephant Ear in “up-pumping” operating mode, EEDP Elephant Ear in “down-pumping” operating mode

Buffo et al. [37] employed morphology engineering to boost cellulolytic enzyme production by *A. niger*. By manipulating parameters like spore concentration and pH, diverse initial inoculum morphologies were achieved, ranging from pellets of various sizes to fully dispersed inoculum. Subsequent cultivations took place in a 4-L stirred tank bioreactor equipped with two impeller configurations: RT-RT or EEDP-EEUP. Irrespective of the inoculum morphology and the operating conditions, cultivations with the Elephant Ear impeller association consistently exhibited higher endoglucanase activity.

Oliveira et al. [38] examined the impact of impeller type utilizing the same impeller associations as employed by Buffo et al. [15] (two Rushton turbines (RT-RT) and two Elephant Ear impellers (EEDP-EEUP)) on the production of red colorant by *Talaromyces amestolkiae* in a 4-L stirred-tank bioreactor. The authors assessed the oxygen transfer coefficient using a non-growing medium composed of monosodium glutamate-glucose (without fungi). At 100 rpm and a specific airflow rate ranging from 0.5 to 2.0 vvm, the EEDP-EEUP association demonstrated higher  $k_L a$  values. Following this, cultivations were carried out at 100 rpm and 2.0 vvm and the Elephant Ear association promoted the highest production of red colorant. The morphology of *T. amestolkiae* also exhibited variation based on the impeller association: dense and spherical pellets were observed with RT-RT, whereas the EEDP-EEUP association facilitated the formation of hairy pellets, predominantly characterized by an increased number of long hyphae.

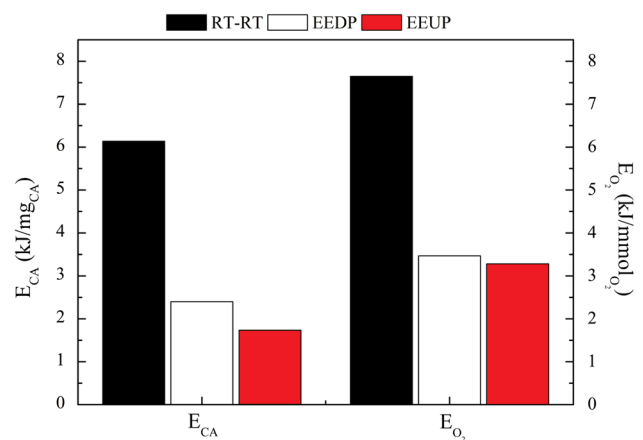
The higher  $\dot{\gamma}_{av}$  values for the RT-RT and EEUP cultivations resulted in higher CA production and productivity (Table 2), with the EEUP cultivation presenting the highest  $C_{CA}^{max}$  and  $P_{CA}^{max}$ . This result by itself showed the

suitability of using EEUP impellers for clavulanic acid production by *S. clavuligerus*. The results achieved using the EEUP configuration became even more impressive when the energy requirement for the agitation provided by the impellers was taken into consideration. The average power consumption ( $P_{g,av}$ ) during the *S. clavuligerus* cultivations was highest for RT-RT, with a value around 2.5 and 4 times higher than for EEUP and EEDP, respectively (Table 2). The lower power input for Elephant Ear impellers compared to the Rushton turbines was consistent with previous studies [39, 40].

The relation between power consumption and clavulanic acid productivity, defined by the specific energy for CA production ( $E_{CA} = P_{g,av}/(P_{CA}^{max} \cdot V)$ ), expresses the energy cost to produce 1 mg of clavulanic acid. Here, the EEUP configuration required 3.5 times less energy, compared to the use of the RT-RT impeller (Fig. 7).

Regarding oxygen transfer, the relation between power consumption and the average oxygen transfer rate, defined by the specific energy for O<sub>2</sub> transfer ( $E_{O_2} = P_{g,av}/(OTR_{av} \cdot V)$ ), expresses the energy cost to transfer 1 mmol of O<sub>2</sub> from the gas phase to the liquid phase. The RT-RT and EEUP configurations presented similar average oxygen transfer rates during the cultivations (Table 2), with the RT-RT impeller requiring more energy to transfer 1 mmol of oxygen to the culture media, compared to the EEUP impeller (Fig. 7).

The findings collectively suggest that employing the EEUP impeller yields superior performance in clavulanic acid production and energy efficiency, particularly when contrasted with the RT-RT impeller at 800 rpm and 0.5 vvm.



**Fig. 7** Values of specific energy for clavulanic acid production ( $E_{CA}$ ) and O<sub>2</sub> transfer ( $E_{O_2}$ ) in the *S. clavuligerus* cultivations at 800 rpm and 0.50 vvm in a stirred-tank bioreactor with 4-L working volume, equipped with different impellers. Legend: RT-RT: Rushton turbines; EEUP: Elephant Ear in “up-pumping” operating mode; EEDP: Elephant Ear in “down-pumping” operating mode

## Conclusions

Clavulanic acid production was higher using the RT-RT (380.7 mg.L<sup>-1</sup>) and EEUP (453.3 mg.L<sup>-1</sup>) impellers, compared to the EEDP impeller (196.6 mg.L<sup>-1</sup>), showing that a change in impeller configuration, on its own, can result in a different level of clavulanic acid production. The branched and unbranched hyphae distribution exhibited a direct relationship with broth consistency index variation during the cultivations using the RT-RT and EEUP configurations, demonstrating that the use of different impellers modified the morphological and rheological behaviors, consequently affecting clavulanic acid production. The single EEUP impeller required 3.5 times less energy to produce clavulanic acid, as well as half the energy to transfer oxygen, when compared to the dual RT-RT impeller, and exhibited the best relation between clavulanic acid production and energy cost, when compared to the RT-RT and EEDP impellers, at 800 rpm and 0.5 vvm. The combined clavulanic acid production and energy requirements criteria provide an important parameter for the proper impeller selection and will impact the scale-up strategy.

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**Author contributions** Maritza C. C. Bustamante: Conceptualization; Data curation; Formal analysis; Investigation; Methodology. Cecília L. L. Costa: Conceptualization; Data curation; Formal analysis; Investigation; Methodology. Mateus N. Esperança: Data Curation; Formal analysis; Investigation; Visualization; Roles/Writing—Original Draft; Writing—Review & Editing. Marcel O. Cerri: Data Curation; Formal analysis; Investigation; Visualization; Roles/Writing—Original Draft; Writing—Review & Editing. Vitor T. Mazziere: Visualization; Roles/Writing—Original Draft. Alberto C. Badino: Conceptualization; Formal analysis; Funding acquisition; Project administration; Resources; Supervision; Validation; Writing—Review & Editing.

**Data availability** The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

## Declarations

**Conflict of interest** The authors have no competing interests to declare that are relevant to the content of this article.

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