

Comparisons between Continuous and Batch Processing to Produce Clavulanic Acid by *Streptomyces clavuligerus*

Álvaro Baptista-Neto, Juliana Conceição Teodoro, Luiz Claudio Macedo Cassiano Filho, Alberto Colli Badino and Carlos Osamu Hokka*

Departamento de Engenharia Química; Universidade Federal de São Carlos; C. P. 676; 13565-905; São Carlos - SP - Brasil

ABSTRACT

The aim of the present work was to compare CA production in continuous culture with and without cell recycling and in batch process by *Streptomyces clavuligerus*. Continuous cultivations with high cell concentration using cell recycling were performed utilizing a hollow fiber ultrafiltration module to separate cells from the filtrate broth. The continuous cultures without cell recycling and the batch cultivations were performed conventionally. The highest productivity was attained in the continuous cultivation with cell recycling ($22.2 \text{ mg.L}^{-1}.\text{h}^{-1}$). The highest CA concentration was obtained in the batch process ($470 \text{ mg.L}^{-1}.\text{h}^{-1}$)

Key words: Clavulanic acid, *Streptomyces clavuligerus* and continuous culture

INTRODUCTION

The increase of the resistance mechanism is based on their ability to produce β -lactamases, which deactivate the antibiotic's β -lactam by hydrolyzing their β -lactam ring. To contour this problem, many strategies have been proposed for the use of a β -lactamase inhibitor combined with a sensitive antibiotic. Clavulanic acid (CA) is a β -lactam antibiotic with low antibacterial activity; it is, however, a potent inhibitor of β -lactamases (Baggaley et al., 1997). The combinations of clavulanic acid and amoxicillin or ticarcillin are used in the treatment of disease caused by pathogenic bacteria and producer β -lactamase enzymes.

Clavulanic acid could be produced industrially by cultivating many species of *Streptomyces*, mainly *S. clavuligerus* (Butterworth, 1984). This author

did not mention information about the industrial process, basing his work on patents and results on a 1500 L fermentor. It is possible to find many works showing increased productivity in batch (Gouveia et al. 1999) and fed-batch (Teodoro et al., 2004) production of clavulanic acid by *S. clavuligerus*.

Continuous cultivation permits to control the cell growth rate through the conditions of the reactor operation. Therefore, continuous cultivation is an important tool to study cell cycling, metabolic regulation and microbial product formation (Blanch and Clark, 1997).

Continuous operation is the most efficient compared to others operations conditions because the process does not have any down time (time to clean, load, unload, prepare, etc.). However, the maintenance of high cellular activity is often prejudicial due to genetic variation that occurs in continuous cultivation. It is important to consider

* Author for correspondence

that problems exist in maintaining asepsis in a long process. In spite of continuous cultivation being an important operation mode, there about clavulanic acid production by *S. clavuligerus* in a continuous process. There is only one study about continuous cultivation using *S. clavuligerus*. Kirk et al. (2000) studied the metabolic process of clavulanic acid in cultivation with limitation of sources of carbon, nitrogen or phosphate. In the medium with limitation of nitrogen and phosphate, the specific productivity obtained was 0.32 and 3.65 $\text{mg}_{\text{AC}} \cdot \text{g}_x^{-1} \cdot \text{h}^{-1}$, respectively. From the metabolic flux analysis, the authors concluded that the arginine formation was affected under any limiting condition studied; the arginine is precursor of clavulanic acid. No clavulanic acid was produced when C-limitation occurred. This was due to piruvate, which was another clavulanic acid precursor not to being formed.

Continuous cultivation with cell recycling is used to obtain high cell concentration (Shuler and Kargi, 1992). Knowing that secondary metabolite formation rate is proportional to cell concentration, continuous cultivations with cell recycling is an alternative to increase the productivity of clavulanic acid.

Maneaus and Holst (1997) studied alcohol dehydrogenase production by *Thermoanaerobium brockii*, in continuous culture with complete cell recycling. Since the cell growth is associated to production, low hydraulic residence time was used to improve enzyme production. The authors realized cultivation with high cell concentration to avoid the substrate inhibiting in the cell growth. A tangential filter was used to separate the cells. Maximum cell concentration and productivity was obtained in the continuous culture with cell recycling.

The objective of the present work was to compare clavulanic acid productivity in continuous processing with and without cell recycling and batch cultivation.

MATERIALS AND METHODS

2.1 Microorganism

The microorganism used was *Streptomyces clavuligerus* ATCC 27064 stored in cryotubes (glycerol 10% v/v) at -70°C .

2.2 Culture Medium

Reactivation Medium containing ($\text{g} \cdot \text{L}^{-1}$): glycerol (15), malt extract (10), bacto-peptone (10), yeast extract (1), $\text{Mg} \cdot \text{SO}_4 \cdot 7\text{H}_2\text{O}$ (0.75), K_2HPO_4 (2.5), MOPS (21) and a solution of salts ($1 \text{ mL} \cdot \text{L}^{-1}$) composed by $1 \text{ g} \cdot \text{L}^{-1}$ $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, $1 \text{ g} \cdot \text{L}^{-1}$ $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and $1 \text{ g} \cdot \text{L}^{-1}$ $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$.

Growth, Production and Feed Medium containing ($\text{g} \cdot \text{L}^{-1}$): glycerol (15), Samprosoy 90NB (10), yeast extract (1), $\text{Mg} \cdot \text{SO}_4 \cdot 7\text{H}_2\text{O}$ (0.75), K_2HPO_4 (0.8), MOPS (21), used only in growth medium and a solution of salts ($1 \text{ mL} \cdot \text{L}^{-1}$) composed by $1 \text{ g} \cdot \text{L}^{-1}$ $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, $1 \text{ g} \cdot \text{L}^{-1}$ $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and $1 \text{ g} \cdot \text{L}^{-1}$ $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$.

2.3 Analytical methods

Clavulanic acid concentration: Clavulanic acid concentration was assayed according to the method of Foulstone and Reading (1982). The imidazole derivative of clavulanic acid was injected into a high performance liquid chromatography (HPLC) equipped with a C-18 Bondapack (Waters) column. The mobile phase was composed of 94% KH_2PO_4 0,1M (pH=3.2) and 6% methanol. The detection was at 311 nm.

Glycerol concentration: Glycerol concentration was determined by HPLC. The column utilized was the Shodex KS series made by Waters at 80°C . The mobile phase was MILLI-Q water. The flow rate was $1 \text{ mL} \cdot \text{min}^{-1}$, the detector being an RI detector.

Rheological parameters: Rheological measurements were made using BROOKFIELD viscometers, mol LVT DVIII+, with concentric cylinders. Shear stress and shear rate were obtained. The rheological parameters, consistency index (K) and flow behavior index (n), from the power law model were calculated by non-linear regression of the experimental value of Shear stress and shear rate.

Cell Concentration: was evaluated in continuous cultivation with or without cell recycling taking the dry cell weight at 110°C for 24 h, after centrifuging (11180g for 10 min). The values of cell concentration in batch cultivation were

obtained using equation 1, proposed by Barsi *et al.*, 2003.

$$C_x = 3.760.K^{0.333}.n^{-0.079} \quad (1)$$

Residence time determination: Considering Fig. 1, equation 2 defines the residence time, in the continuous culture without cell recycling and hydraulic residence time, in the continuous culture with cell recycling.

$$\tau = \tau_h = \frac{V}{F_1} \quad (2)$$

Where: τ is residence time (h)

τ_h is hydraulic residence time (h)

V is culture volume (L)

F_1 is feed flow rate (L.h⁻¹)

Considering Fig. 2, the cell residence time is defined by equation 3:

$$\tau_c = \frac{V}{F_2} \quad (3)$$

Where: τ_c is the cell residence time (h)

F_2 is the flow rate of the broth with cells retired from the bioreactor (L.h⁻¹)

Productivity Determination: The clavulanic acid productivity (P_{AC}) in the batch cultivations was defined by equation 4:

$$P_{AC} = \frac{C_p}{t} \quad (4)$$

Where: t is the cultivation time (h)

To calculate the microorganism-specific productivity, it was necessary to determine the global production velocity (v_p), parameter defined by equation 5. The microorganism-specific productivity (Pe_{AC}) was determined through equation 6.

$$(v_p)_i = \left(\frac{dC_p}{dt} \right)_i \quad (5)$$

$$Pe_{AC} = \frac{v_p}{C_x} \quad (6)$$

The derived value was calculated using Microcal Origin 6.0 software.

For the continuous cultivations the P_{AC} and Pe_{AC} were calculated using equations 7 and 8:

$$P_{AC} = \frac{C_p}{\tau} \quad (7)$$

$$Pe_{AC} = \frac{P_{AC}}{C_x} \quad (8)$$

In the continuous cultivation with cell recycling, the hydraulic cell residence was utilized to calculate P_{AC} .

2.4 Experimental Procedure

The cultivation was carried out in a Bioflo III fermentor (New Brunswick Scientific) at 28°C, 800 rpm and air flux at 2 L/min. The pH was controlled at 6.8 by adding 1M NaOH and 4M HCl solution. The cultivation procedure was the following: initially, 50 mL seed medium in a 500 mL Erlenmeyer flask was inoculated with 3.5 mL of cell suspension from cryotubes. This was incubated at 28°C, 250 rpm for 24 h. After that, 45 ml of production medium in 500 ml Erlenmeyer flasks was inoculated with 5 mL of the cultivate seed broth under the same conditions as described before. After 24 h, the broth was transferred to the fermentor. 10% from the initial volume was inoculum. Fig. 1 illustrates the experimental procedure used in the cultivation.

For the batch cultivation, the initial volume in the reactor was 4 L and samples were withdrawn every 3 h over the first 24 h and every 4 h over the subsequent period. In cultivations with and without cell recycling, samples were withdrawn periodically to verify stationary state. For the continuous cultivation without cell recycling the system was operated in 5 residence periods between 5.6 and 30.6 hours.

The volume was maintained at 2.5 L by adding fresh medium and retired culture broth. For the continuous culture with cell recycling, the reactor was operated with fixed hydraulic residence time (τ_h), of 11.8 h with different cell residence times (τ_c), varying from 15 to 28.2 h.

The tangential filtration module utilized to separate the cells from the culture broth. The microfiltration module is composed of several membranes of polysulfona with a pore diameter

of 0.22 micrometers and a filtration area of 3600 cm². The Fig. 2 shows a system used to perform continuous cultivation with cell recycling.

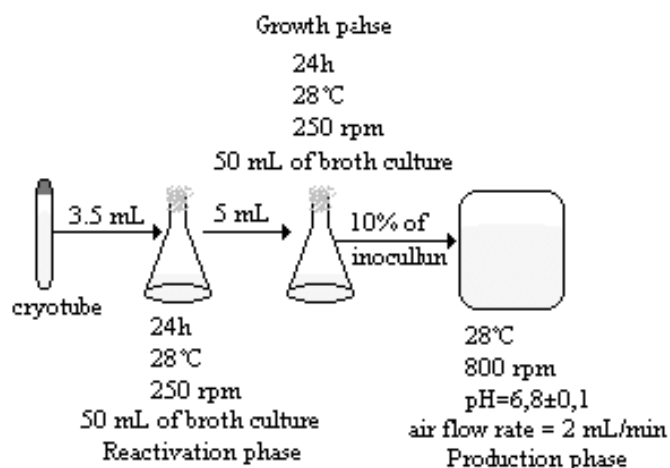


Figure 1 - Illustration of experimental procedure utilized in the experiments.

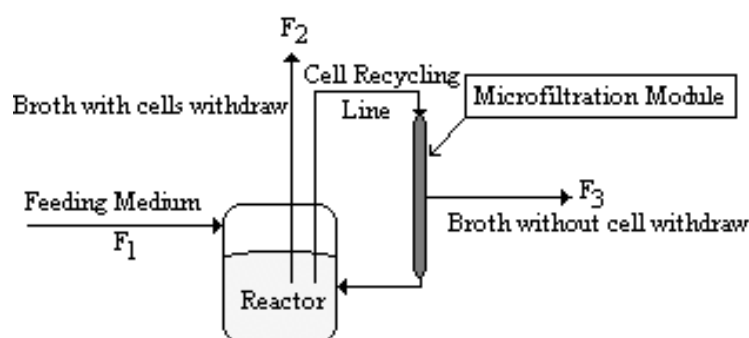


Figure 2 - Procedure utilized to perform the continuous culture

RESULTS AND DISCUSSION

Fig. 3 shows clavulanic acid, glycerol and cell concentration as well as rheological parameters (K and n) in the batch cultivation.

Although the clavulanic acid production began during the cell growth phase, it was not associated with cell growth because clavulanic acid production still occurred during the stationary phase. This is a secondary metabolite behavior. Hence, the clavulanic acid production rate was

related to cell concentration as expected due to the secondary metabolite (Shuler and Kargi, 1992).

The highest value of clavulanic acid concentration was 475 mg.L⁻¹ and the productivity was 11.7 mg.L⁻¹.h⁻¹. The major global clavulanic acid production rate was 23.9 mg.L⁻¹.h⁻¹ and the specific productivity was 2.04 mg_p.g_x⁻¹.h⁻¹.

The experimental values of product, cell concentration and rheological parameters for continuous cultivation without recycling are presented in Fig. 4. The highest clavulanic acid concentration at the continuous cultivation was

obtained for high cellular residence times. When the reactor was operated at a low residence time, clavulanic acid was not detected in the broth.

This effect could be related to the delay observed in clavulanic acid production in batch cultivation too.

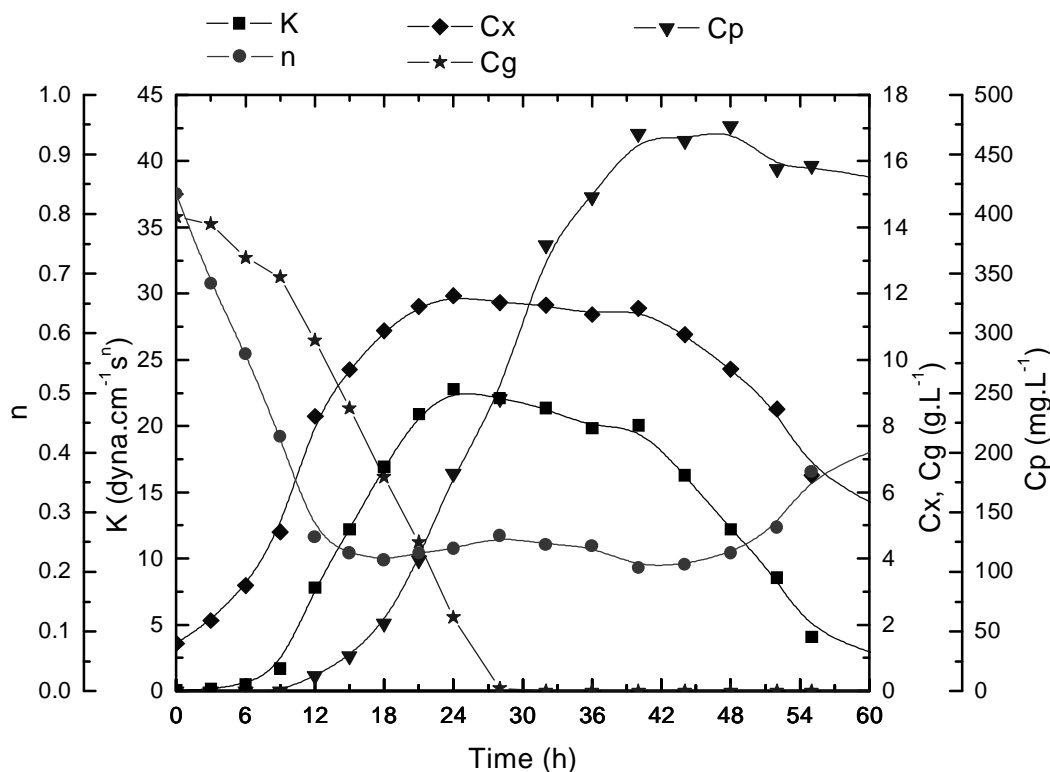


Figure 3 - Profile of clavulanic acid, glycerol and cell concentration as well as rheological parameters (K and n) in the batch cultivation.

These delays as well as the absence of product when the reactor was operated at low residence time characterize an effect of inhibition and/or repression of clavulanic acid production caused by glycerol, because in this experimental stage, glycerol concentration was high. In continuous cultivation without cell recycling, the maximum clavulanic acid concentration occurred when the system was operating at the lowest residence time (25 h) showing a value of 430 mg.L⁻¹. The highest clavulanic acid productivity (18.3 mg.L⁻¹.h⁻¹) was obtained when the reactor was operated for a residence time of 14.3 h and the highest specific productivity was 1.93 mg.L⁻¹.h⁻¹.

Fig. 5 illustrates the concentration of cells, glycerol and product and rheological parameter values (K and n) for continuous cultivation with cell recycle. The experiment was performed with a hydraulic residence time (11.8h). Highest value of clavulanic acid obtained was 230 mg.L⁻¹ and

the productivity was 22.1 g.L⁻¹.h⁻¹ for the reactor operating at a high cell residence time (28.2 h). The specific productivity for the process was 1.13 g.h⁻¹.g⁻¹.

When the continuous culture with cell recycling was operated at a low cell residence time (15 h), clavulanic acid concentration was zero. One hypothesis for this was based on the cultivation time, which could favor genetic variation in microorganism and consequently cause the loss of production capacity.

Figs. 3, 4 and 5 showed that the cell concentration in the continuous culture with cell recycle was the highest. For the batch cultivation, the cell concentration was 12 g.L⁻¹. For the continuous culture without cell recycling it was 9 g.L⁻¹ and for the continuous cultivation with cell recycling, it was 20 g.L⁻¹. However, continuous cultivation with cell recycling could be utilized to obtain a

high cell concentration in *S. clavuligerus* cultivations.

The experimental values showed that a lower clavulanic acid concentration was obtained in the continuous culture with cell recycling, which was twice as much as batch operation. However, the specific productivity obtained for continuous cultivation without cell recycling and batch

cultivation was similar. The specific productivity obtained for the continuous culture with cell recycling was lower than the value obtained for the continuous culture without cell recycling. The specific productivity decrease, this fact could be related to cell injury caused by the operating mode of the tangential filter which may cause oxygen limiting and shear stress.

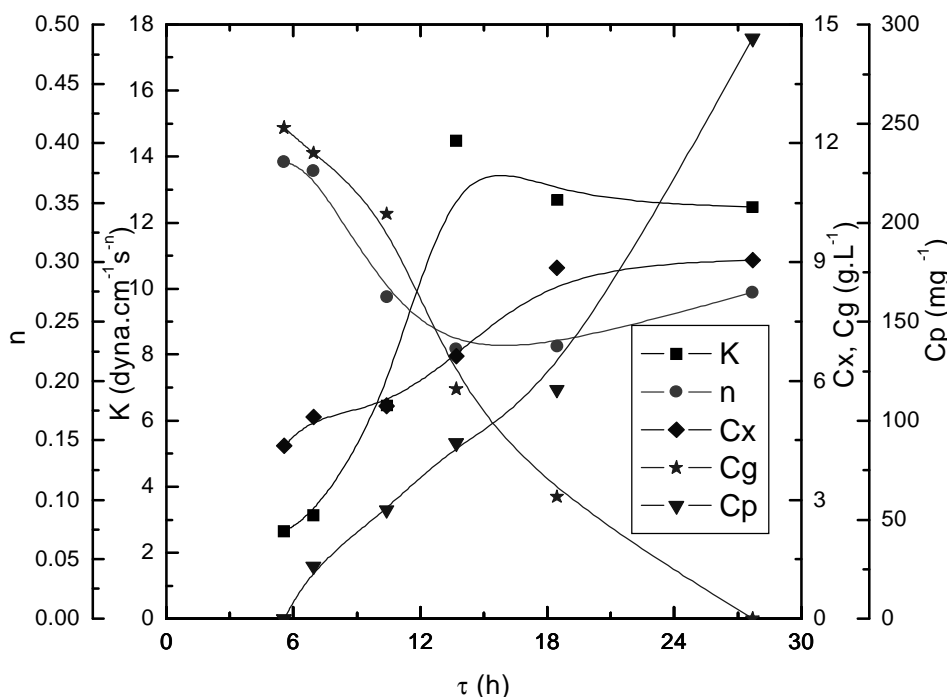


Figure 4 - Profile of cell (Cx), glycerol (Cg) and clavulanic acid (Cp) concentration as well as rheological parameters (K and n) with residence time.

CONCLUSIONS

The cell concentration was higher in continuous cultivation with cell recycle. In this process, glycerol was not observed. The high cell concentration increase glycerol rate consumption. The specific productivity value was similar in the continuous culture without cell recycling and the batch cultivation. Probably, the processes weren't influenced by metabolites generated by the microorganism in the first 42 h. In continuous culture with cell recycling, some factors could negatively influence the specific productivity. Possibly the shear stress of the medium during the tangential filtration could

negatively influence the specified productivity. Another hypothesis could be that the high cell concentration could favor the formation of some non-determined by-product from *S. clavuligerus*, which could negatively influence the clavulanic acid production.

In spite of the highest clavulanic acid concentration was obtained in batch cultivation, the highest clavulanic acid productivity was obtained in continuous cultivation. With the results shown in the present work it could be concluded that culture cultivation with cell recycling was the best operation mode to produce clavulanic acid from *S. clavuligerus*.

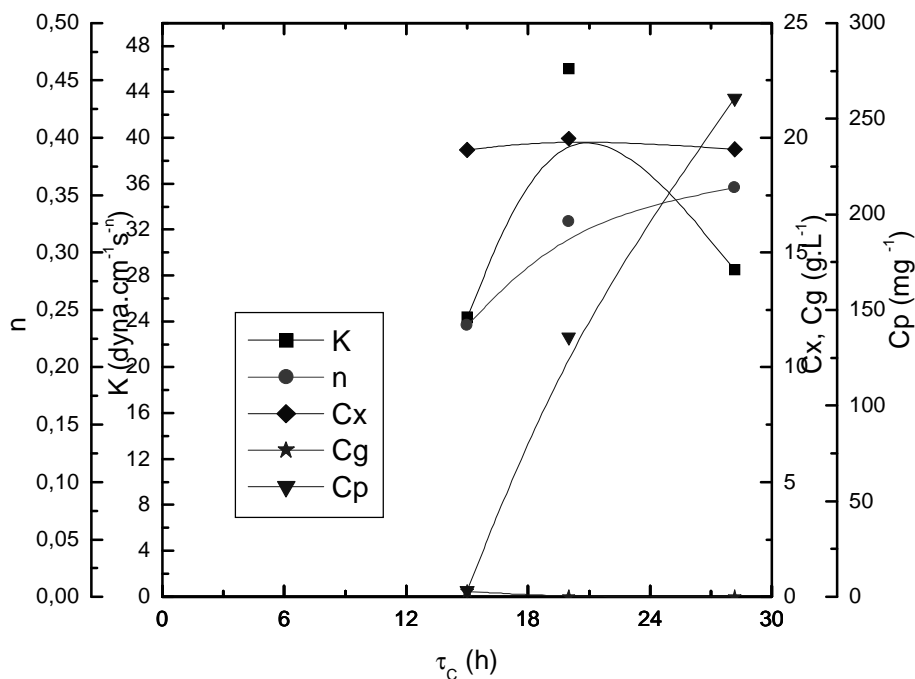


Figure 5 - Profile of cell (Cx), glycerol (Cg) and clavulanic acid (Cp) concentration as well as rheological parameters (K and n) versus the cell residence time.

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RESUMO

O ácido clavulânico (AC) é um importante inibidor de β -lactamases, enzimas que degradam a partir do metabolismo secundário do *Streptomyces clavuligerus*, bactéria filamentosa e estritamente aeróbia. Considerando que a velocidade de produção de metabólitos secundários está ligada à concentração celular, o presente trabalho teve como objetivo comparar a produção de AC nos processos contínuos com e sem reciclo celular e em batelada, realizando cultivos dessa bactéria com alta densidade celular. Para cumprir com o objetivo proposto, foram realizados experimentos em biorreator

operando na forma contínua com reciclo utilizando-se um módulo de filtração tangencial de fibra oca para a separação celular. Os processos contínuos sem reciclo e em batelada foram realizados de forma convencional. A produtividade em AC no cultivo contínuo com reciclo celular ($22,2 \text{ mg.L}^{-1}\text{h}^{-1}$) foi superior aos processos convencionais, apesar de obter-se maior concentração do produto (470 mg.L^{-1}) em batelada.

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