PAPER • OPEN ACCESS

Modeling of Clavulanic Acid Production from *Streptomyces clavuligerus* using a Continuous Operation Mode

To cite this article: L Ariza *et al* 2021 *J. Phys.: Conf. Ser.* **2049** 012073

View the article online for updates and enhancements.

Modeling of Clavulanic Acid Production from *Streptomyces clavuligerus* using a Continuous Operation Mode

L Ariza[1], J Rubio[1], V Moreno[1], L Niño[2] and G Gelves[1]

[1]. Universidad Francisco de Paula Santander, Cúcuta, Colombia
[2]. Universidad de Antioquia, Medellín, Colombia

germanricardogz@ufps.edu.co

Abstract. Clavulanic acid is a β-lactam inhibitor produced by fermentation with *Streptomyces clavuligerus* cells, and it is usually used to prevent resistance to certain antibiotics. However, CA production is limited at the bioreactor level due to its low performance. The latter generates expensive processes and challenging to operate on a large scale. In this research, a mathematical model is proposed to simulate the clavulanic acid production from an operation strategy based on continuous mode. The preceding, to identify trends allowing to improve the productivity of the mentioned metabolite. Results are compared to the traditional operating batch mode. According to the results found, the final concentration of the β-lactam inhibitor could be increased by up to 60% regarding the simulated data in batch mode. Results obtained demonstrate the importance of computational techniques in bioprocess engineering since bioprocess simulation focuses on identifying critical operating parameters as a starting point in antibiotic production optimization.

1. Introduction

Antibiotics are chemicals produced by organisms or by synthetic processes. Generally, they are used to treat bacterial infections. Antibiotics were used first to describe formulations antagonistic to microorganisms' growth [1]. Bacterial resistance occurs when antibiotics are disproportionately used or misused. That is why there are more and more cases where resistance to antibiotics occurs, increasing medical costs. Therefore, inhibitors are necessary to avoid bacterial resistance. E. Coli was the first bacteria discovered with a resistant capacity to B-lactam antibiotics. Latter, new strains were found: *Klebsiella pneumoiae*, *Neisseria gonorrhoeae* and *Haemophilus influenzae*, among others [2]. In some cases, resistance to antibiotics is produced naturally through combinations of mechanisms in new bacterial phenotypes. These mechanisms are usually influenced by the environment, which plays an essential role in the maintenance and selection of these phenotypes [3].

B-lactam antibiotics have a B-lactam ring in their molecular structure as penicillin derivatives, monolactams, cephalosporins, and carbapenems [4]. B-lactamase is an enzyme produced by some bacteria, mainly from the *Enterobacteriaceae* family. The mentioned enzyme has resistance to B-lactam antibiotics since it hydrolyzes the B-lactam ring, inactivating the antibacterial properties of the molecule. To avoid this resistance, they are usually administered in combination B-lactamase inhibitor that prevents B-lactam antibiotics' bacterial breakdown and broadens the spectrum of antimicrobial activity.

The genus Streptomyces is a producer of 80% of the most widely used antibiotics in the world [5]. They are also generators of many extracellular enzymes of industrial interest, among which stand out: proteases, nucleases, cellulases, amylases, lipases, chitinases and xylanases [6].

Clavulanic acid is a crucial B-lactamase inhibitor in the pharmaceutical industry used to prevent certain antibiotics resistance. Clavulanic acid (CA) is produced by *Streptomyces clavuligerus,* which is a gram-positive bacterium. It is capable of metabolizing various carbon sources, including oils and carbohydrates. However, the primary carbon substrate for clavulanic acid biosynthesis is glycerol, given its ease of entry into cells and its rapid uptake rate and transport [7]. It also produces secondary metabolites such as cefamycin C, several clavama structures, and antifungal and antitumor activities [8].

Due to the clinical and industrial importance of clavulanic acid, current research has focused on improving its productivity by evaluating the effect of nutrient concentration, environmental conditions and the metabolic capacities of *S. clavuligerus* for acid biosynthesis. The most studied traditional way to obtain CA has been the batch strategy. Its application is based on the versatility of operating the bioreactor to avoid any risk of contamination since no material is added or extracted during the fermentation process [9]. The synthesis of CA has been established as a great difficulty when implementing strategies to improve its production. The latter is due to low yields during batch production and high processing costs. In addition to the above, some reports identify the CA degradation once the fermentation is finished due to changes in pH, temperature, and contaminating compounds, which prevent the inhibitor recovery in high percentages [10,11].

Contrary to batch mode, the substrate is added during the continuous operating mode, and the product is simultaneously released. The preceding makes the biomass remain metabolically active so that production can be higher than the batch mode. For this reason, the mathematical modeling of CA production using a continuous mode is proposed in this research. The previous, to identify trends allowing to improve the productivity of the mentioned metabolite. The results are compared to the traditional batch mode of operation.

2. Methodology

The batch mode of operation is proposed in this research as a starting point to identify the growth of *Streptomyces clavuligerus*, substrate consumption and formation of CA from a glycerol-based culture medium [12]. Once the batch fermentation is simulated, CA production modeling in continuous mode is proposed as a second production strategy. Finally, to know the effects of cellular recirculation in CA production continuous mode, two bioreactors' simulation was presented. The second equipment recirculates biomass to the first production tank. In a batch bioreactor operating mode, *Streptomyces clavuligerus* cell concentration evolution X can be modeled according to Eq. (1):

$$\frac{dX}{dt} = \mu X \tag{1}$$

Considering previous studies [12], *Streptomyces clavuligerus* growth is regulated by the glycerol and biomass concentration. Thus, the growth rate μ is calculated using the Contois model exposed in Eq (2):

$$\mu = \mu_{max}\left(\frac{S}{k_s X + S}\right) \tag{2}$$

The substrate consumption profile in batch mode depends on cell concentration at the dynamic state and the glycerol uptake rate, and it is simulated by Eq. (3):

$$\frac{dS}{dt} = -q_s X \tag{3}$$

q_s is the specific substrate uptake and is determined using Eq. (4):

$$qs = \frac{\mu}{Y_{XS}} \tag{4}$$

CA production also depends on cell concentration at the dynamic state, and it can be simulated using Eq. (5):

$$\frac{dP}{dt} = q_p X \tag{5}$$

Rate of CA production q_p from *Streptomyces clavuligerus* cells is considered to be indirectly associated with microbial growth. The above is argued that once microbial growth ceases, CA production continues to take place, in such a way that Eq. (6) captures this phenomenon:

$$q_p = \left[\alpha + \frac{\mu}{\beta}\right] w \tag{6}$$

Where α and β are kinetic constants of the mathematical model. w is a constrain parameter with an initial value equal to 0. According to experimental data [12], clavulanic acid starts its production after 48 hours of fermentation. That is why from this moment (t> = 48 h), the CA production rate is activated in the mathematical model, in such a way that $w = 1.0$. Eqs (1) - (6) constitute the mathematical basis for simulating AC production in batch mode. The continuous mode is considered in this research as a second operating strategy to propose strategies to increase AC production.

For cell growing in continuous mode, it is considered that all the culture medium entering the fermenter of volume V is previously sterilized. In such a way that no cells enter with the fresh medium and only the cell extraction that leave with the medium is considered. The steady-state concentration in continuous mode depends on the growth and extraction cell rates based on the above. These phenomena are considered in Eq. (7):

$$\frac{dX}{dt} = \mu X - \frac{F}{V} X \tag{7}$$

The rate of substrate change ds⁄dt in continuous mode depends on the fresh substrate feeding at a concentration S_i and extraction medium depleted. This is taken into account by adding the second and third terms in Eq. (8):

$$\frac{dS}{dt} = \frac{F}{V} S_i - \frac{F}{V} S - q_s X \tag{8}$$

It is also considered that there is no product in the fresh medium fed in continuous mode. In such a way that CA does not enter with the medium fed, and only the product extraction is considered at the bioreactor's outlet. The above is simulated with the third term of Eq. (9):

$$\frac{dP}{dt} = q_p X - \frac{F}{V} P \tag{9}$$

Two cascade bioreactors were proposed to simulate the cellular recirculation effects on CA continuous mode. Also, the second equipment recirculates Xr biomass to the first production tank. A recirculated flow rate r is assumed equivalent to 20% of flow F that enters the entire plant composed of two bioreactors. The flow fed to each of the bioreactors is considered equal to 50% of the total flow F fed to the plant. Therefore, the two equipment operate at the same dilution rate. The simulated biomass in the first and second tanks is calculated in this case using Eqs. (10)-(11):

$$\frac{dX_1}{dt} = \frac{Fr}{V_1}Xr - \frac{F_1}{V_1}X_1 + \mu_1 X_1 \tag{10}$$

$$\frac{dX_2}{dt} = \frac{F_1}{V_2}X_1 - \frac{F_2}{V_2}X_2 + \mu_2 X_2 \tag{11}$$

Where X_1 and X_2 are the cell concentration at bioreactors of volume V_1 and V_2, respectively. μ_1 and μ_2 are the microbial growth specific rates, and they are calculated with Eq. (2). The dynamics in continuous mode with recirculation is simulated by Eqs (12) - (13):

$$\frac{dS_1}{dt} = \frac{Fr}{V_1}Sr + \frac{F_a}{V_1}S_i - \frac{F_1}{V_1}S_1 - q_{s_1}X_1 \tag{12}$$

$$\frac{dS_2}{dt} = \frac{F_1}{V_2}S_1 + \frac{F_b}{V_2}S_{i2} - \frac{F_2}{V_2}S_2 - q_{s_2}X_2 \tag{13}$$

Where F_a and F_b are the fresh medium feed rates to tanks one and two, respectively. Sr is the concentration of substrate recirculated to tank one; in this case, it is assumed to be equal to the glycerol concentration leaving the second bioreactor. q_{s_1} and q_{s_2} are glycerol uptake rates determined with Eq. (4). S_{i2} is the glycerol concentration fed at the second bioreactor, and it is assumed to be increased at 50 % the substrate concentration fed at the first tank. The Eqs. (14)-(15) model the CA concentration produced in the fermenters:

$$\frac{dP_1}{dt} = \frac{Fr}{V_1}Pr - \frac{F_1}{V_1}P_1 + q_{p1}X_1 \tag{14}$$

$$\frac{dP_2}{dt} = \frac{F_1}{V_2}P_1 - \frac{F_2}{V_2}P_2 + q_{p2}X_2 \tag{15}$$

Where P_r is the product recirculated to the first bioreactor. In this case, it is assumed that P_r presents the same output value of the second tank P_2. q_{p1} and q_{p2} are the kinetic rates of clavulanic acid formation calculated by Eq. (6). Kinetic parameters were taken from previous references [12], and they are shown in Table 1.

**Table 1.** Constants used for CA kinetic modeling

Parameter	Value
μ_{max} (h^{-1})	0.0461
k_s (g/L)	0.4500
Y_{XS} (g/g)	0.2103
β (g/mg)	0.00803
α (mg/g)	0.25

The batch simulations, continuous and continuous with recirculation mode, were implemented in the Matlab R2017b software. The fourth-order Runge-Kutta numerical method was used to solve the differential equations. The initial conditions in all cases were programmed with a value of 0.625 g/L for biomass, 52 g/L for glycerol and 0.0 g/L for AC. F=0.1 L/h; V=5 L; V_1=5 L; S_i=52 g/L.

3. Results and Discussions
This research's primary goal is to propose a mathematical model to simulate clavulanic acid production from a continuous operating mode. The preceding, to simulate trends for identifying

productivity improvements of CA from *Streptomyces clavuligerus* cells. The results are compared to the traditional batch operating mode. Results of batch kinetics are shown in Figure 1
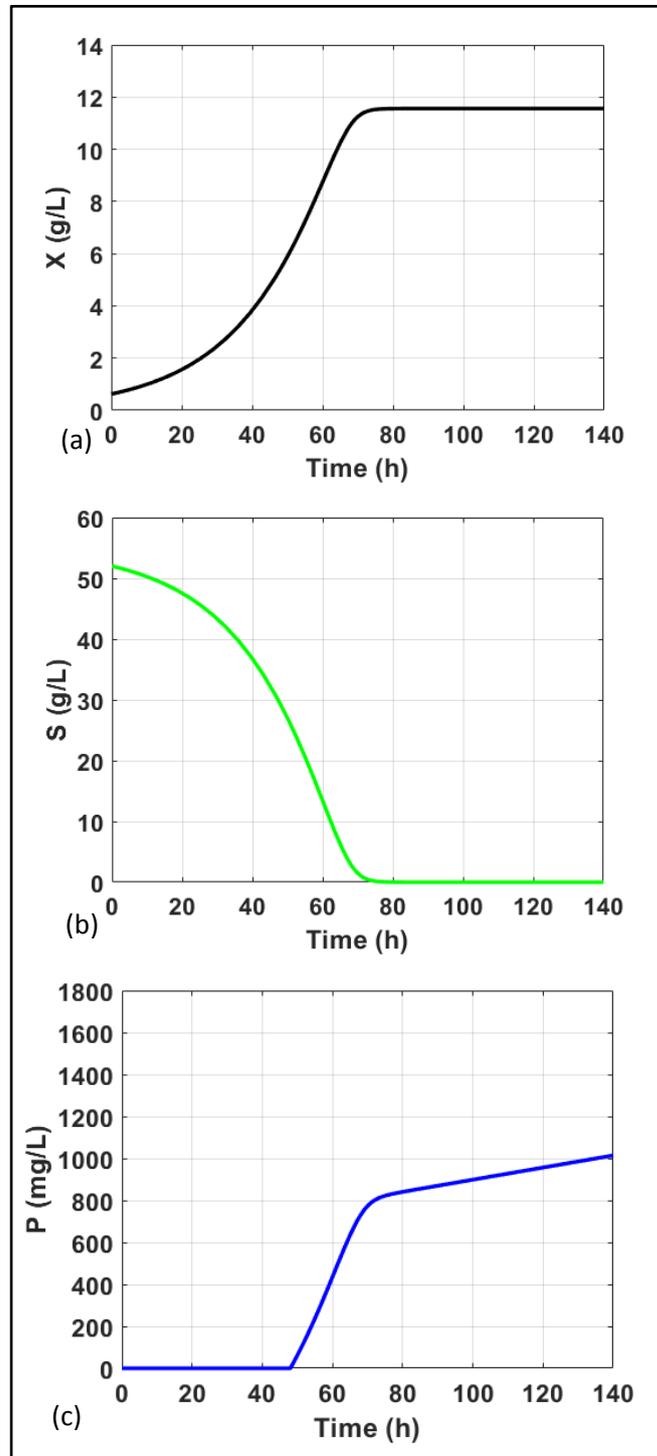
Figure 1. AC production simulated at batch operating mode (a) Biomass, (b) Glycerol and (c) AC.

According to the CA production results, it is observed that clavulanic acid begins its exponential production phase after 48 hours of fermentation in batch mode (Figure 1). After 81 hours, the CA production rate experienced a substantial reduction due to the low glycerol concentration during this phase.

According to the simulated results, the maximum clavulanic acid production was achieved at 1000 mg/L at 136 hours and maximum biomass production of 11.56 g/L. These results are in agreement with those reported in the bibliography [12]. The model accuracy explains the above reached to simulate CA's production in a bioreactor operated in batch mode since its values are close to those reported from the mentioned reference.

No substrate is added during the batch operating mode, and a constant volume is maintained in these processes. Likewise, the culture media concentrations define the course of the process. As seen in Figure 1, The kinetics are divided into several growth phases. The first stage consists of the exponential phase, which ends around 65 hours of fermentation. The deceleration phase then leads the process since the impacts of substrate deficiency on reducing the microbial growth rate and CA formation is evidenced. The substrate is almost wholly exhausted at 81 has observed in Fig 1. (b). Even so, the last phase of the fermentation, called the "stationary phase," is characterized by an appreciation of clavulanic acid production from 75 hours of fermentation with an average value of 800 mg/L of CA. Also, a maximum level is reached at the end of the process with 1000 mg/L. This batch operating mode strategy's choice is due to the ease of set up equipment to avoid any risk of contamination [9,13-16].

The product and depleted medium are extracted, and simultaneously, the bioreactor is fed with a fresh culture medium during the continuous operating mode. Therefore, cells can keep growing continuously. The latter allows a more significant amount of metabolites. Likewise, it must be taken into account that the feed flow rate must be in balance with the growth since a culture washout is produced if the flow level is increased to a value higher than microbial growth. Contrarily, a bacterial death is expected if its feed is decreased due to lack of nutrients.

For this reason, the mathematical modeling of CA production from the continuous mode was proposed in this research. The preceding, to identify trends allowing to improve CA production. Results found are compared to the traditional batch operating mode. According to the equations proposed to simulate the CA production, the results are shown in Figure 2 (c), which define a clavulanic acid production of 1300 mg/L. Regarding biomass, an average value of 10 g/L is seen in Figure 2 and glycerol is reached as steady state after 130 hours of fermentation. Interestingly, the findings suggest an increase in clavulanic acid production using the continuous operating mode with a value 30 % higher than the batch mode levels. The permanent fresh medium feeding could explain the latter since *Streptomyces clavuligerus* cells would have a higher degree of metabolic activity concerning batch mode.

Based on the results obtained, operating two cascade bioreactors and biomass recycling was proposed with the primary goal of maintaining the culture concentration at higher levels avoiding its washout.

The results obtained demonstrate the superiority of this fermentation strategy in comparison to batch mode. The latter is supported by the finding of 1300 and 1600 mg/L of clavulanic acid simulated as shown in Figure (3). The above means that clavulanic acid production from *Streptomyces clavuligerus* could be increased by up to 60 % concerning the traditional batch mode.

In addition to the above, a continuous operating mode has significant advantages over batch mode, thus avoiding downtime for processing (loading and unloading of biomass, preparation of the bioreactor and sterilization of the medium, etc.) [17,18]. Therefore, overall productivity tends to be higher using this production strategy.
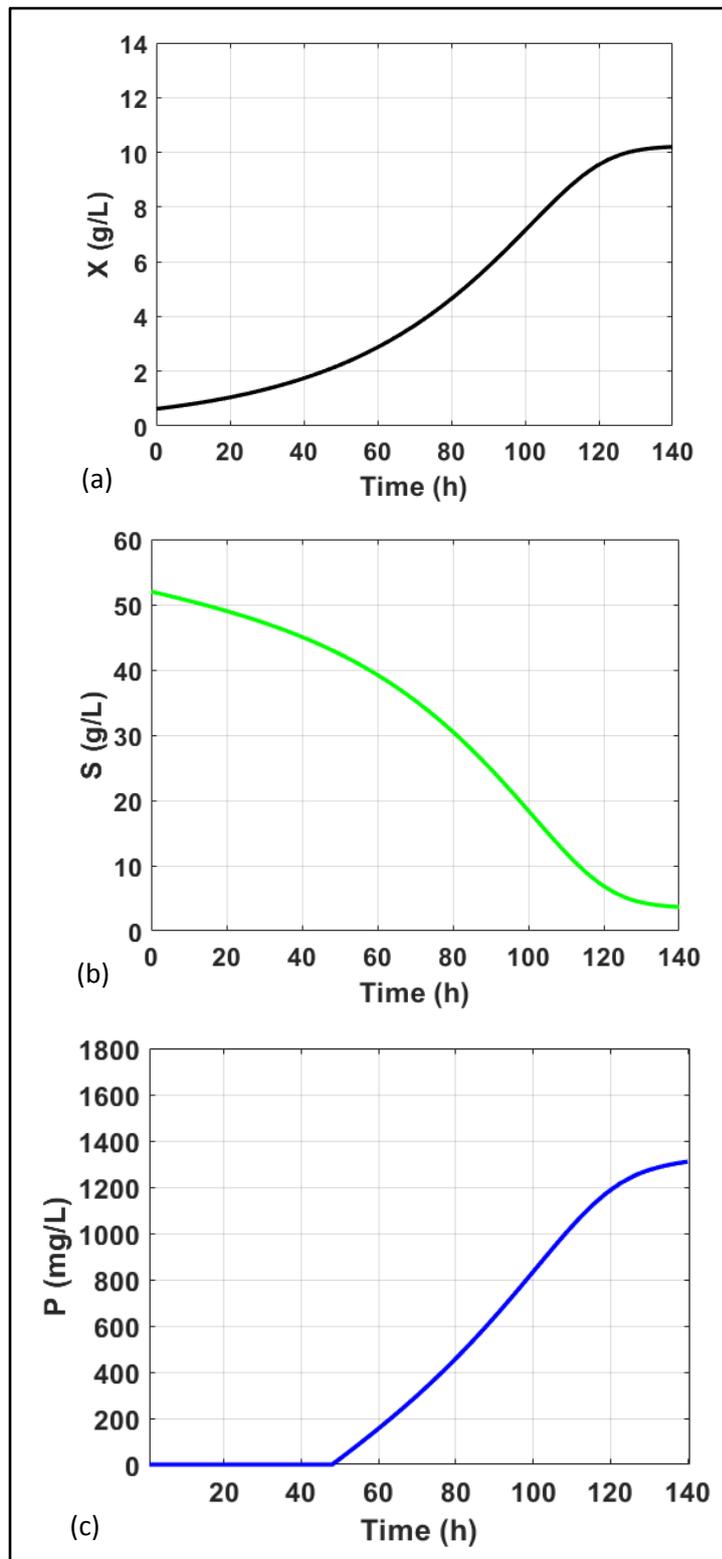
Figure 2. AC production simulated at continuous operating mode (a) Biomass, (b) Glycerol and (c) AC.
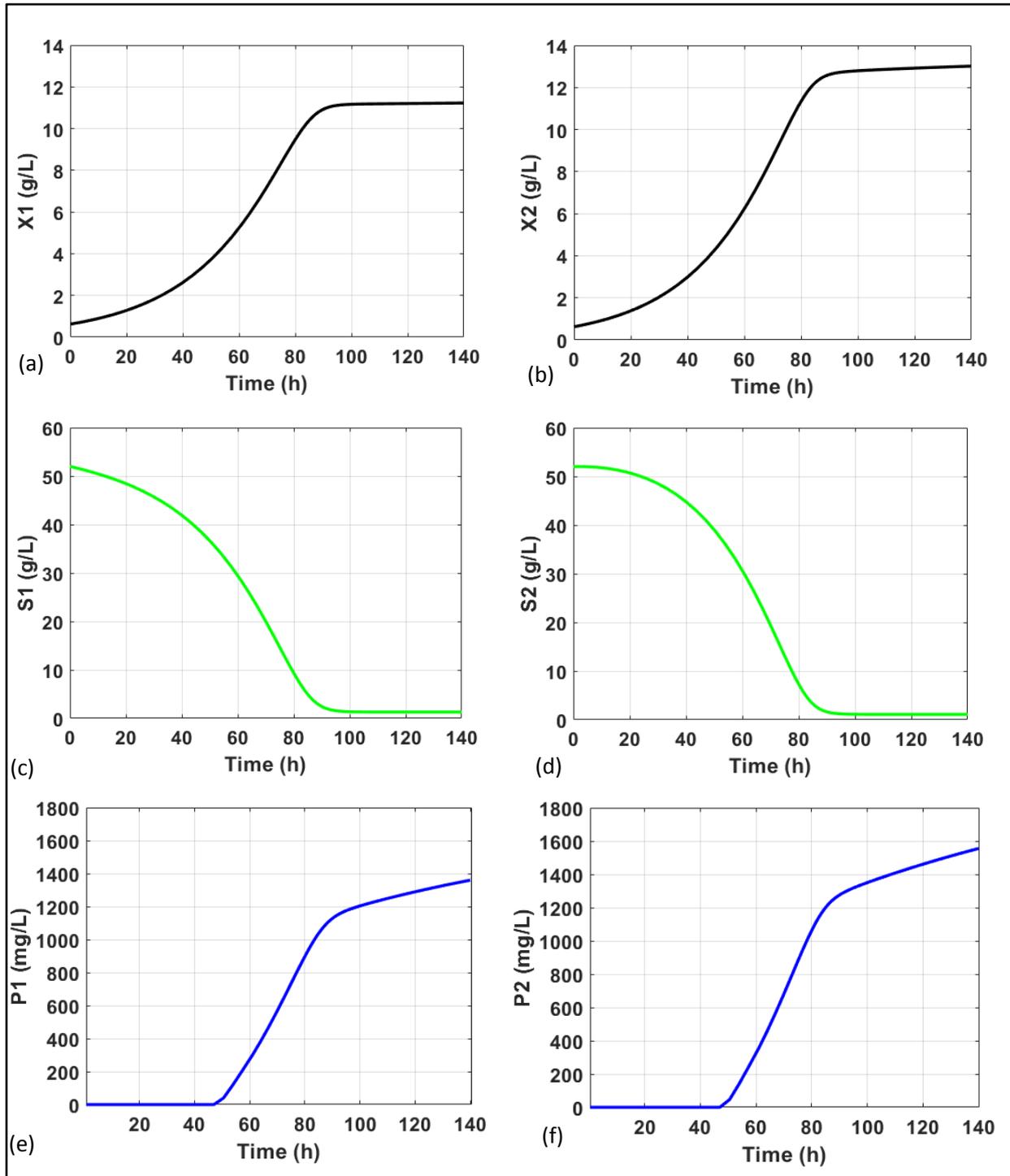
Figure 3. AC production simulated at continuous recycling operating mode (a) and (b) Biomass; (c) and (d) Glycerol; (e) and (f) clavulanic acid.

The results found in this research are in agreement with previous studies [10] that experimentally compared batch and continuous modes for the CA production, and they discovered that CA concentration was 60 % higher than that obtained in batch mode, being the process in continuous mode the most effective to produce clavulanic acid [10].

The continuous operation mode with biomass recycling allows controlling the microbial growth rate through the operating conditions. That is why the continuous mode is essential to capture the main kinetic regulation for biomass and product formation. The ability to control and manipulate set up conditions is crucial in allowing a cost reduction. The proposed recycling procedure increases productivity since the washout phenomenon is avoided by recycling cells (biomass). Therefore, biomass growth rate and CA production velocity are linked to cell concentration. Once the stationary phase is reached, a more significant product accumulation can be promoted in a continuous operating mode with biomass recycling. Consequently, the substrate uptake rate is used for biomass generation and product formation. Results found here agree with previous reports [19] who showed a higher CA concentration using a continuous operating mode with cell recycling.

4. Conclusions

In this research, a mathematical model was evaluated to simulate the clavulanic acid production in continuous mode. The previous focused on improving CA concentration. It was identified that the operating mode significantly influences the CA production. In addition to the above, cell recycling avoids washout and should be considered the starting point to optimizing the industrial clavulanic acid production process. Finally, simulation software is of great importance for research in industrial applications since physical, financial, and labor resources are reduced.

References

[1] Marques A, Santos V, Oliveira P, Lima G, Araújo J, Lima J, Converti A, Pessoa A and Porto A 2014 Screening of wild type Streptomyces isolates able to overproduce clavulanic acid *Brazilian Journal of Microbiology* **45(3)** 919

[2] Drawz S Bonomo R 2010 Three decades of B-lactamase inhibitors, *Clinical Microbiology Reviews* **23(1)** 160

[3] Bonomo J and Gill R 200 Antibiotic resistance as a model for strain engineering *Computers and Chemical Engineering* **29(3)** 509

[4] Holten B and Onusko E 2000 Appropriate prescribing of oral beta-lactam antibiotics *Am Fam Physician* **62(3)** 611

[5] Lima E, Procópico I, Reis da Silva M, Kassawara J and Araújo J 2012 Antibiotics produced by Streptomyces *Brazilian Journal of Infectious Diseases* **16(5)** 466

[6] Li R and Townsend C 2006 Rational strain improvement for enhanced clavulanic acid production by genetic engineering of the glycolytic pathway in *Streptomyces clavuligerus Metabolic engineering* **8(3)** 240

[7] Pinilla L, Toro F, Avignone A, Peñuela M and Ríos-Estepa R 2018 *Streptomyces clavuligerus* strain selection for clavulanic acid biosynthesis: a study based on culture composition effects and statistical analysis *Dyna* **85(205)** 111

[8] Liras R, Gomez J, Escribano E and Santamarta I 2008 Regulatory mechanisms controlling antibiotic production in *Streptomyces clavuligerus Journal of Industrial Microbiology & Biotechnology* **35(7)** 667

[9] Hernandez S, Niño L, Gelves L 2020 Simulating of Microbial Growth Scale-Up in a Stirred Tank Bioreactor for Aerobic Processes using Computational Fluid Dynamics *Journal of Physics: Conference Series* **1655** 012109

[10] Neto A, Hirata D, Cassiano L Bellao C, Badino A and Hokka C 2005 A study on clavulanic acid production by *Streptomyces clavuligerus* in batch, fed-batch and continuous processes *Brazilian Journal of Chemical Engineering* **22(4)** 557

[11] Brethaur S, Held M and Panke S 2008 High concentrations of clavulanic acid but not of its degradation products decrease glycerol consumption and oxygen uptake rates in cultures of Streptomyces clavuligerus *Biotechnology and Bioengineering* **100(3)** 439

[12] Sánchez C, Gómez N and Quintero J Producción de ácido clavulánico por fermentación de *Streptomyces clavuligerus*: evaluación de diferentes medios de cultivo y modelado matemático *Dyna* **79(175)** 158

[13] Pacheco S, Niño L and Gelves G 2020 Recombinant Anti-Thrombin Production from *Saccharomyces Cerevisiae*: Large Scale Trends Based on Computational Predictions *Journal of Physics: Conference Series* **1655** 012081

[14] Caicedo Y, Suarez C and Gelves G 2020 Evaluation of preliminary plant design for *Chlorella vulgaris* microalgae production focused on cosmetics purposes *Journal of Physics: Conference. Series* **1655** 012086

[15] Ibañez A, Rolon Y and Gelves G 2020 Evaluating Cost-Effective Culture Media for Nutraceutics Production from Microalgae Using Computer-Aided Large Scale Predictions *Journal of Physics: Conference. Series* **1655** 012082

[16] Nieto L, Rivera C and Gelves G 202 Economic Assessment of Itaconic Acid Production from *Aspergillus Terreus* using Superpro Designer *Journal of Physics: Conference Series* **1655** 012100

[17] Bello A, Morales K, Sánchez L, Lidueñez V, Leal A and Gelves G 2020 Computational Implementation of Required Industrial Unit Operations for Bio-Plastic Production From Starch Extracted from Banana Peels by Aerobic Fermentation using *Rizophus Oryzae* *Journal of Physics: Conference Series* **1655** 012078

[18] Araque J, Niño L and Gelves G 2020 Industrial Scale Bioprocess Simulation for *Ganoderma Lucidum* Production using Superpro Designer *Journal of Physics: Conference Series* **1655** 012077

[19] Neto A, Conceicao J, Macedo L, Colli A and Osamu C 2005 Comparisons between continuous and batch processing to produce clavulanic acid by *Streptomyces clavuligerus Brazilian archives of biology and technology* **48** 97