



Flux dynamics of C-5 amino acid precursors in fed-batch cultures of *Streptomyces clavuligerus* during clavulanic acid biosynthesis

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ABSTRACT

Clavulanic acid (CA) is a well-known β -lactamase inhibitor that is mainly obtained from submerged cultures of *Streptomyces clavuligerus*. Dynamic genome-scale *in silico* studies were performed to gain insights into the intracellular metabolic fluxes of C-5 precursors during the cultivation of wild-type *S. clavuligerus* in batch and fed-batch operation. A preliminary literature screening was conducted for media selection and determination of culture conditions for further cultivation in a 5 L bioreactor. Simulations provided insights into the metabolism of C-5 precursors in CA biosynthesis. In addition, carbon utilization for CA biosynthesis was assessed in terms of the ratio of total carbon used in CA biosynthesis to the total carbon influx in batch and feed media. Based on simulation results, we proposed a modification of the glycerol-sucrose-proline-glutamate medium for fed-batch cultivation to improve the carbon utilization for CA biosynthesis. The proposed fed-batch scenario achieved higher specific CA concentration at lower biomass production, indicating better carbon utilization for its synthesis. The dynamic *in silico* fluxes suggest that metabolic fluxes in this scenario would be stable, favoring a longer stage of continued antibiotic secretion.

INTRODUCTION

Streptomyces species are among the most industrially relevant producers of antibiotics and other bioactive compounds [1]. Some of these compounds, like tetracyclines, have been used due to their efficacy against a wide range of pathogens; and others have also contributed to the development of new medications, as the case of Lipstatin [2,3]. Clavulanic acid (CA) is a C-8 β -lactam antibiotic well-known for its β -lactamase inhibition capacity which is mainly produced by submerged cultures of *Streptomyces clavuligerus* [4]. It is synthesized through a complex biosynthetic pathway that condenses arginine (C-5) and glyceraldehyde 3-phosphate (C-3) molecules [5,6]. A series of studies in the 1980s identified glycerol as the C-3 precursor through ¹³C labeling experiments [7–9]. Only by the mid-1990s L-arginine was confirmed as the C-5 precursor

over other candidates such as L-glutamic acid and L-glycine [10,11]. L-arginine, initially a C-6 molecule, is condensed with glyceraldehyde 3-phosphate to synthesize N²-(2-carboxyethyl)-arginine (a C-9 molecule). This C-9 structure is conserved in two reaction steps, but the ureido group of the arginine is lost in the formation of the intermediate proclavaminc acid (a C-8 molecule), thus five carbons of L-arginine are retained [5,6]. This C-8 structure is conserved up to the formation of CA. The chemical structure of CA and the β -lactam carbon precedence is shown in Figure 1.

Despite several studies on the optimization of nutritional conditions and metabolic and genomic perturbations, low CA yields persist during fermentation, motivating the application of metabolic engineering strategies like fluxomic and strain engineering, as alternatives for determination of feasible scenarios of production and improving the CA accumulation levels [5,12,13]. In addition, CA is temperature sensitive, which leads to its degradation during fermentation. The latter phenomenon further contributes to the low yields of CA [14,15].

Given the importance of the C-3 and C-5 precursors on CA synthesis, several organic nitrogen sources have exhibited a positive impact on CA production [16,17]. Suitable

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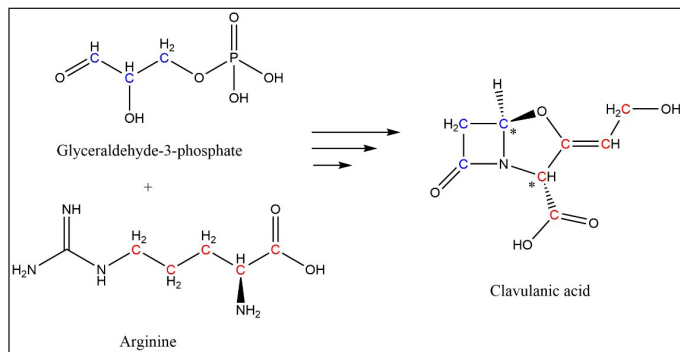


Figure 1. Clavulanic acid chemical structure. Labeled red and blue atoms correspond to those coming from L-arginine and D-glyceraldehyde-3-phosphate, respectively. *Stereochemical centers on CA structure.

nitrogen sources could enhance the metabolic activity of the urea and tricarboxylic acid cycle (TCA) cycles, and therefore the CA biosynthesis [18,19]. Furthermore, understanding the assimilation of these types of nitrogen sources, including amino acids, as well as maintaining high and stable C-3 and C-5 fluxes, is a key factor in increasing CA production levels.

Over the last decade, diverse systems biology approaches have been applied for further understanding of *Streptomyces* metabolic pathways for metabolite production. In fact, some genome-scale constraint-based modeling and *in silico* simulations have been used to understand the characteristic low CA titers observed in wild-type strains, as well as for the rational selection of potential metabolic targets under different bioprocessing conditions [4,5]. Nevertheless, the knowledge about the role of C-5 amino acid precursors in the regulation of CA production is still low. Some studies have pointed out that amino acids connected with 2-oxoglutarate may contribute to enhancing CA biosynthesis and secretion [20–22].

Specifically, flux balance analysis (FBA) helped to understand *S. clavuligerus* physiology concerning nutritional conditions [23,24]. In addition, dynamic FBA (dFBA) has been used recently for this purpose [12,25], as dFBA is able to estimate numerically time-dependent concentrations and reactions [26,27]. Therefore, fed-batch and batch operation modes can be evaluated and analyzed employing this approach in contrast to the classical FBA.

In this work, a preliminary literature screening was performed to identify nutritional conditions leading to high CA secretion rates in wild-type *S. clavuligerus* cultivated in the bioreactor scale. After this initial screening, cultivations were performed at the shake flask scale to confirm the optimal conditions of production referred in the literature and further scaled up to the 5 l bioreactor scale. A genome-scale *in silico* study of the nutritional conditions attained in each experimental scenario of CA production was performed to get insights into the genome-scale dynamic distribution of the metabolic fluxes during the cultivation, focusing on the metabolism of C-5 precursors for further enhancement of CA biosynthesis in cultivations using with a wild-type *S. clavuligerus*.

MATERIALS AND METHODS

Dynamic genome-scale simulations

The static optimization approach was used in the dFBA formulation [12,26,27]. In this approach, each time interval assumes a pseudo-steady state, then, the FBA optimization problem is solved according to a dynamic set of hard constraints applied to the uptake of nutrients. The instantaneous uptake flux vector is obtained by solving a nonstructured model comprised of the set of ordinary differential equations presented in the following equations (Equations 1 to 4):

$$\frac{dv}{dt} = F \quad (1)$$

$$\frac{d\gamma_x}{dt} = \gamma_x (\mu_{ns} - k_d) - \frac{F}{V} \gamma_x \quad (2)$$

$$\frac{dc_i}{dt} = X \frac{\mu_{ns}}{Y_{x/i}} - \frac{F}{V} (c_{i,f} - c_i) \quad (3)$$

$$\mu_{ns} = \frac{\mu_{max} c_s}{k_s x + c_s} \quad (4)$$

where F is the feed rate (when applicable, during fed-batch), V is the volume, C_i is the instantaneous concentration of species i at time t , $C_{i,f}$ is the concentration of species i in the feeding, γ_x is the dry cell weight (DCW) biomass concentration, μ_{ns} is the growth rate in the nonstructured model, μ_{max} is the maximum experimentally observed growth rate, k_d is the death constant, k_s is the Monod constant for the substrate, and C_s is the substrate concentration.

The instantaneous FBA problem was solved by accounting for sub-optimal growth by means of a weighted objective function of maximum enzymatic efficiency and growth maximization. The rate of biomass production was conciliated by minimizing the error between the specific growth rates (μ) estimated by both, the unstructured dynamic model and the steady-state genome-scale model. The formulation of the optimization problem is shown as follows (Equation 5):

$$\min \sum (\mu_{ns} - \mu_f)^2 \quad (5)$$

$$\begin{aligned} & s. t \\ & \left[\begin{array}{l} \min [-\alpha \mu_f + (1 - \alpha) \sum_{i=1}^n v_i^2] \\ s. t. S \cdot v_n = 0 \\ lb \leq v_{n-m} \leq ub \\ v_m = \frac{1}{\gamma_x} \left(\frac{dc_m}{dt} \right) \\ 0 \leq \alpha \leq 1 \end{array} \right] \quad (6) \end{aligned}$$

where μ_f is the growth rate estimated in the instantaneous optimization for solving the FBA problem, and v_i is a vector of instantaneous intracellular fluxes. Similarly, v_m is the constrained set of uptake fluxes calculated by solving the unstructured dynamic model system, and v_n is the set of fluxes calculated by solving the FBA problem. The dFBA was implemented in MATLAB R2022b with Gurobi 10.0 optimization API.

Simulations were performed according to the concentration of the substrates, operation volume, feeding rate, and cultivation time reported in the literature for each set of conditions. The carbon utilization to synthesize the product of interest was assessed in terms of the ratio of the total carbon used in CA biosynthesis to the total carbon influx in batch and feed media. In addition, time profiles of reaction fluxes toward key pathways and enzymes impacting CA biosynthesis were analyzed to adjust further fed-batch operation for the media under evaluation. Finally, a medium modification for enhanced fed-batch production of CA was proposed based on experimental data and *in silico* fluxes prediction.

Strain and media compositions

The literature search was carried out in ScienceDirect, Scopus, and PubMed databases using predetermined terms such as “*S. clavuligerus* fermentation” and “CA production,” exclusively focusing on research articles that used a wild-type strain. The scenarios yielding the best carbon utilization for its biosynthesis *in silico* were tested after the initial screening of fed-batch and batch cultivations available in the literature. Fed-batch operation was adjusted according to the observed *in silico* distribution of metabolic fluxes to improve the carbon utilization in CA biosynthesis. According to this rationale, a complex medium and two synthetic media were selected to promote the highest CA production.

Streptomyces clavuligerus DSM 41826, cryopreserved, was activated in seed medium (50 ml) and pre-cultured in 250 ml as indicated in previous works [28]. All cultivations were carried out in a 5.0 l stirred-tank bioreactor (Labfors 5, Infors AG, Bottmingen, Switzerland). Batch cultivations were performed in a volume of 2.0 l. Fed-batch cultivations were operated up to a final volume of 4.5 l. Inoculum of 10% v/v from the preculture was used. The pH was maintained at 6.8 by using 4 M solutions of either hydrochloric acid or sodium hydroxide. Air was supplied at 0.6 vvm and temperature was controlled at 28°C. Agitation was controlled in the range of 300–600 rpm, assuring dissolved oxygen ranging between 40% and 75%. Sampling was performed as described elsewhere [28].

After the screening, the media for experimental testing were selected. The complex medium, referred to here as isolated soy protein (ISP), was used and prepared as specified previously [21,29,30]. Similarly, a synthetic medium named here as glycerol-sucrose-proline-glutamate (GSPG), optimized by Saudagar and Singhal was prepared [31,32]. Likewise, the so-called restricted medium modified with ornithine (RMO), previously used by Gómez-Ríos [23], was also evaluated. All reactants were from Merck-Sigma-Aldrich (Darmstadt, Germany).

Preliminary simulations were performed to set up the feeding start and flow rate to avoid substrate accumulation during the culture (data not shown). All the fed-batch cultures were operated at 120 hours with the same basal media as that used for batch operation. The carbon and nitrogen feeding composition corresponded to those reported by the authors who previously used those media in CA production with remarkable results in wild-type cultivations [21,25,31]. The fed-batch

cultivation with the ISP medium had the following composition in g L⁻¹: glycerol, 180.04; L-ornithine HCl, 4.72; K₂HPO₄, 2.0; and (NH₄)₂SO₄, 8.0; feed rate 0.01 L⁻¹, feeding time 19 hours. For the GSPG medium, the fed-batch composition was the following in g L⁻¹: L-arginine HCl, 21.07; L-threonine, 119.12; K₂HPO₄, 2.0; and (NH₄)₂SO₄, 8.0; feed rate 0.02 L⁻¹, feeding time 18 hours. In the case of the RMO cultivation, the feed was prepared as follows (concentration in g L⁻¹): glycerol, 27.6; K₂HPO₄, 2.0; (NH₄)₂SO₄, 8.0 and mono-sodium glutamate, 25.4; feed rate 0.035 L⁻¹, feeding time 25 hours [28].

Experimental testing of the proposed scenario of improved CA yield was performed at the same bioreactor scale, using a modified GSPG medium prepared as follows (concentrations in g L⁻¹): sucrose, 12.09; glycerol, 9.3; NaCl, 5.0; L-proline, 11.36; monosodium glutamate, 9.8; K₂HPO₄, 2.0; CaCl₂, 0.4; MnCl₂·4H₂O, 0.1; MgSO₄·7H₂O, 1.0; ZnCl₂, 0.05; FeCl₃·6H₂O, 0.1; and trace elements solution (1.0 ml). The feeding conditions were the same as those used for the RMO fed-batch culture, delaying the feeding time up to 34 hours to promote a precise substrate addition and optimal carbon utilization according to the *in silico* study of the production scenario.

RESULTS AND DISCUSSION

The impact of several complex nitrogen sources on CA production has been studied in batch and fed-batch operations. Rich nitrogen sources such as peptone, isolate soybean protein, yeast extract, and soybean meal, are commonly used for CA production, usually leading to high concentrations in bioreactor scale (over 600 mg/l) with wild-type strains [17,29,33]. The highest CA concentrations in the bioreactor scale for both batch and fed-batch operations have been reported for glycerol and isolate soy protein-based media [21] while the highest concentration for a synthetic media was reported by Saudagar and Singhal for a medium based on glycerol, sucrose, proline, and glutamate [31]. In this study, we examined the effect of fed-batch operation with amino acid supplementation, from a dynamic-fluxomic standpoint as an approach for tuning of fed-batch *S. clavuligerus* cultures.

Batch and fed-batch operation mode for CA production

Figure 2 displays the specific CA concentrations (b_{CA}) and the biomass (DCW) in batch and fed-batch operations with the three media tested. Batch production is still the most used operation mode in the pharmaceutical industry [5,34]. In the case of *S. clavuligerus*, the low specific growth rate constitutes a challenge for continuous operation. In batch conditions, the complex ISP and synthetic GSPG showed higher biomass accumulation, 17.0 and 18.9 gDCW/l, respectively, than the RMO medium (10.8 gDCW/l). The GSPG medium contains a high amount of free amino acids that promoted more growth than the complex medium ISP did. As has been extensively reported, phosphate limitation is key in the activation of secondary metabolism and for achieving high CA secretion rates [24,31]; this condition is attained rapidly in the RMO medium, inducing CA biosynthesis since the first hours of cultivation. In the case of the rich media, CA biosynthesis was detectable from 10 hours onward. The exponential phase lasted

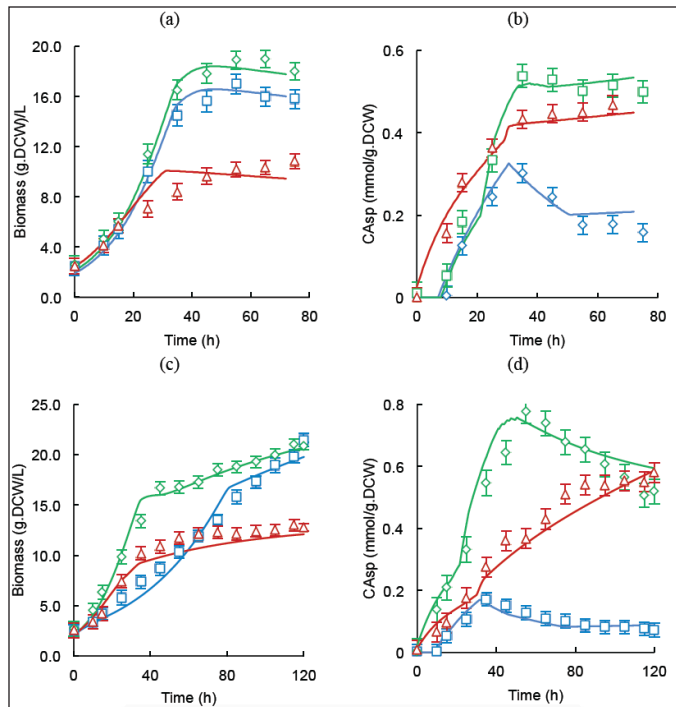


Figure 2. Experimental and simulated time courses of batch and fed-batch cultivations of *S. clavuligerus* in ISP (blue), GSPG (green) and RMO (red) media. (a) Biomass concentration (γ_x) in batch cultures. (b) CA specific concentration (b_{CA}) in batch cultures. (c) Biomass concentration (γ_x) in fed-batch cultures. (d) CA specific concentration (b_{CA}) in fed-batch cultures.

between 35 and 45 hours in both the GSPG and the ISP medium with a $\mu_{max} = 0.09$ /hours. More limited RMO medium led to a longer growth phase and lower biomass level, with an up to the 55th hour with $\mu_{max} = 0.07$ /hours. In terms of CA secretion, both synthetic media yielded higher mean specific production rates (0.016 mmol CA/gDCW.h and 0.012 mmol CA/gDCW.h for the GSPG and RMO media, respectively), which are higher than that obtained in the rich nitrogen medium ISP (0.010 mmol CA/gDCW.h). This synthetic medium was selected to promote high uptake of glycerol (C-3) and amino acids (C-5), as CA precursors and hence, for better carbon channeling via glycolysis and urea cycle toward the CA pathway. The RMO medium provides less carbon and total nitrogen than the ISP and GSPG media (Table 1), hence lower maximum biomass and specific growth rate were expected. The final specific CA concentration in RMO cultivations was lower than that reached in the GSPG cultures, but it was expected given the lower initial concentration of C-3 and C-5 precursors. Figure 2a and b shows the biomass and specific CA time courses for batch cultures of *S. clavuligerus* showing the absence of a lag phase, possibly as a consequence of the high volume of inoculum and the high concentration of cells in the seed culture.

Figure 2c and d displays the biomass and specific CA time courses for the fed-batch operation of *S. clavuligerus* cultures. In the case of fed-batch cultures, the results agreed with those of the batch operation, yielding higher biomass and CA concentrations, as a consequence of more extended growth and stationary phases with maintenance of the nutritional conditions

Table 1. CA carbon utilization (r_{CAC}), carbon to nitrogen (r_{CN}) and C-3/C-5 precursors ($r_{C-3/C-5}$) ratios in *S. clavuligerus* cultivations.

Mode	Medium	r_{CAC}	r_{CN}	$r_{C-3/C-5}$
Batch	ISP	0.97	10.05	9.17
	GSPG	3.00	4.98	0.44
	RMO	2.31	10.20	1.73
Fed-batch	ISP	0.75	107.23	69.82
	GSPG	1.96	1.56	-
	RMO	3.62	10.20	1.73

that promoted continuity of the secondary metabolism and CA accumulation. The biomass concentrations reached 21.5, 20.9, and 13 g L⁻¹ for the ISP, GSPG, and RMO media, respectively. An earlier feeding than the reported for those media allowed to avoid drops in biomass concentration and CA secretion, as well as a sustained growth considering the dilution rate due to feed addition. The addition of phosphate in small amounts in the feed media regulated growth while maintaining CA biosynthesis. The CA concentrations attained in the fed-batch cultures were 0.17, 0.77, and 0.58 mmol CA/gDCW of biomass for the ISP, GSPG, and RMO media, respectively. The high synthesis rate of biomass in the ISP medium did not reflect a high CA production, while the synthetic media supplying a high concentration of C-3 and C-5 precursors favored the CA accumulation concomitantly with biomass. The RMO medium promoted considerably lower biomass, indicating a strong limitation of nutrients, but this favored high synthesis rates of CA considering the low biomass concentrations.

Table 1 presents the percentage of carbon dedicated to CA biosynthesis regarding the total carbon in the media, the carbon to organic nitrogen, and the C-3 to C-5 precursors ratio. The ISP medium has a high glycerol concentration, and it is expected that most of the carbon uptake proceeds via the glycolytic pathway. The medium is rich in biomass precursors but not only C-5 amino acids, which promote biomass synthesis but comparatively lower CA production, leading to a ~1.0% of carbon effectively dedicated to synthesizing CA: The GSPG medium has a more balanced C/N and C-3/C-5 composition, which presumably give a better cellular response to phosphate limitation, increasing the carbon usage for CA biosynthesis (3.0%). The RMO medium showed an intermediate carbon utilization in CA biosynthesis (2.3%) in batch operation. However, in fed-batch operation, the C/N and C-3/C-5 ratio was maintained in the feeding medium, yielding a closer carbon utilization to the batch culture, while the feeding composition for the ISP and GSPG media reduced the carbon diverted to secondary metabolism, possibly due to lack of one precursor, either C-3 or C-5. This suggests a longer and more stable production phase when providing both precursors in the feed.

Genome-scale dynamic flux-distribution of *S. clavuligerus* cultures in batch and fed-batch operation

Figures 3 and 4 summarize the dynamic distribution of metabolic fluxes for the main pathways related to CA biosynthesis in batch and fed-batch operation modes for the three

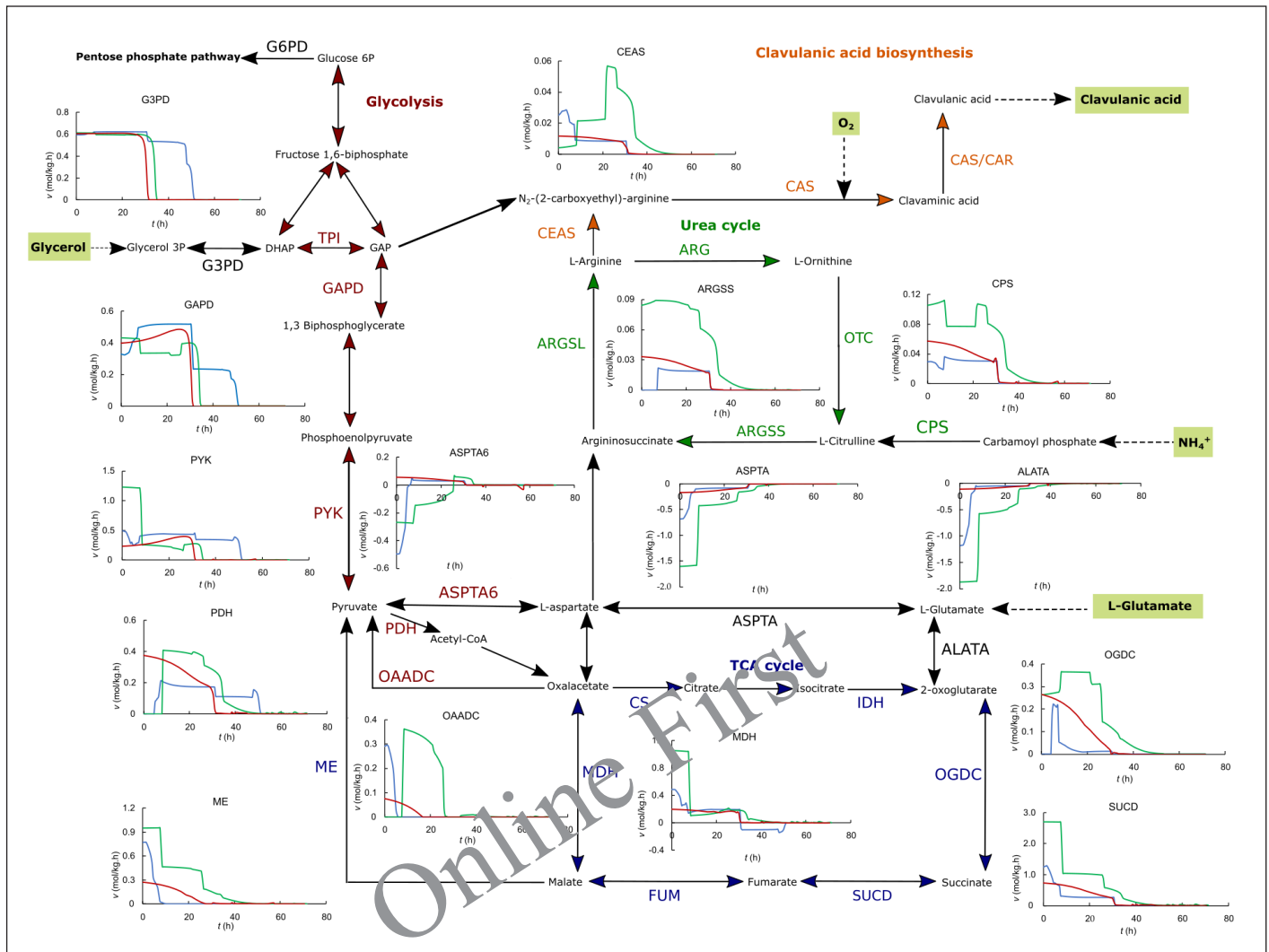


Figure 3. *In silico* dynamic flux distribution in the central metabolism and CA biosynthesis for the ISP (blue), GSPG (green), and RMO (red) media, in batch operation.

media tested, *i.e.*, ISP, GSPG, and RMO media. As mentioned, CA accumulation depends on reaching the phosphate limitation but having precursors available. Although the availability of the C-3 precursor sustains the CA biosynthesis even after a C-5 precursor starvation, the CA secretion rate is lower in this condition, as shown in previous experimental reports [28,33].

Streptomyces clavuligerus has a deficient transportation of glucose, thus glycerol is often used as the carbon source for CA production [5,22]. The dynamic flux distribution shows that the flux through the glyceraldehyde-3-phosphate dehydrogenase (GAPD) is highly dependent on the extracellular glycerol concentration, and hence the glycerol uptake. In all cases, and more pronounced in the batch operation, glycerol depletion leads to a strong decrease in the glycolysis flux, also shown by the trend of pyruvate dehydrogenase (PDH). However, in the case of the GSPG cultivations, the C/N ratio is ~5, and the intracellular fluxes *in silico* show that the activity in central metabolism is higher in the direction of the urea cycle and upstream of the TCA cycle, as a consequence of high uptake of amino acids. Proline and

glutamate are direct biomass precursors, the high availability of those free amino acids in the medium leads to a decrease in the demand for glycerol, as suggested by the lower fluxes through GAPD. The effect of proline and glutamate uptake during the cultivation also occurs in reactions that use 2-oxoglutarate as a reactant, as is the case of reactions catalyzed by 2-oxoglutarate dehydrogenase and succinyl-CoA synthetase. 2-oxoglutarate is produced by various amino acid degradation reactions, as well as pyruvate, which would stimulate the flux through PDH.

In batch operation, the highest fluxes in the urea cycle and through carboxyethyl arginine synthase (CEAS) as the first reaction of the CA pathway occur for the GSPG medium since proline and glutamate incorporate directly via aspartate toward arginine and later, CA biosynthesis. Proline, the major component of the medium, is degraded via glutamate semialdehyde, which also yields glutamate. Thus, glutamate and proline increase substantially the fluxes of aspartate transaminase (ASPTA), argininesuccinate synthase (ARGSS) in the urea cycle, as well as CEAS. The mix of amino acids in the ISP medium provides free C-5 precursors to a lesser extent

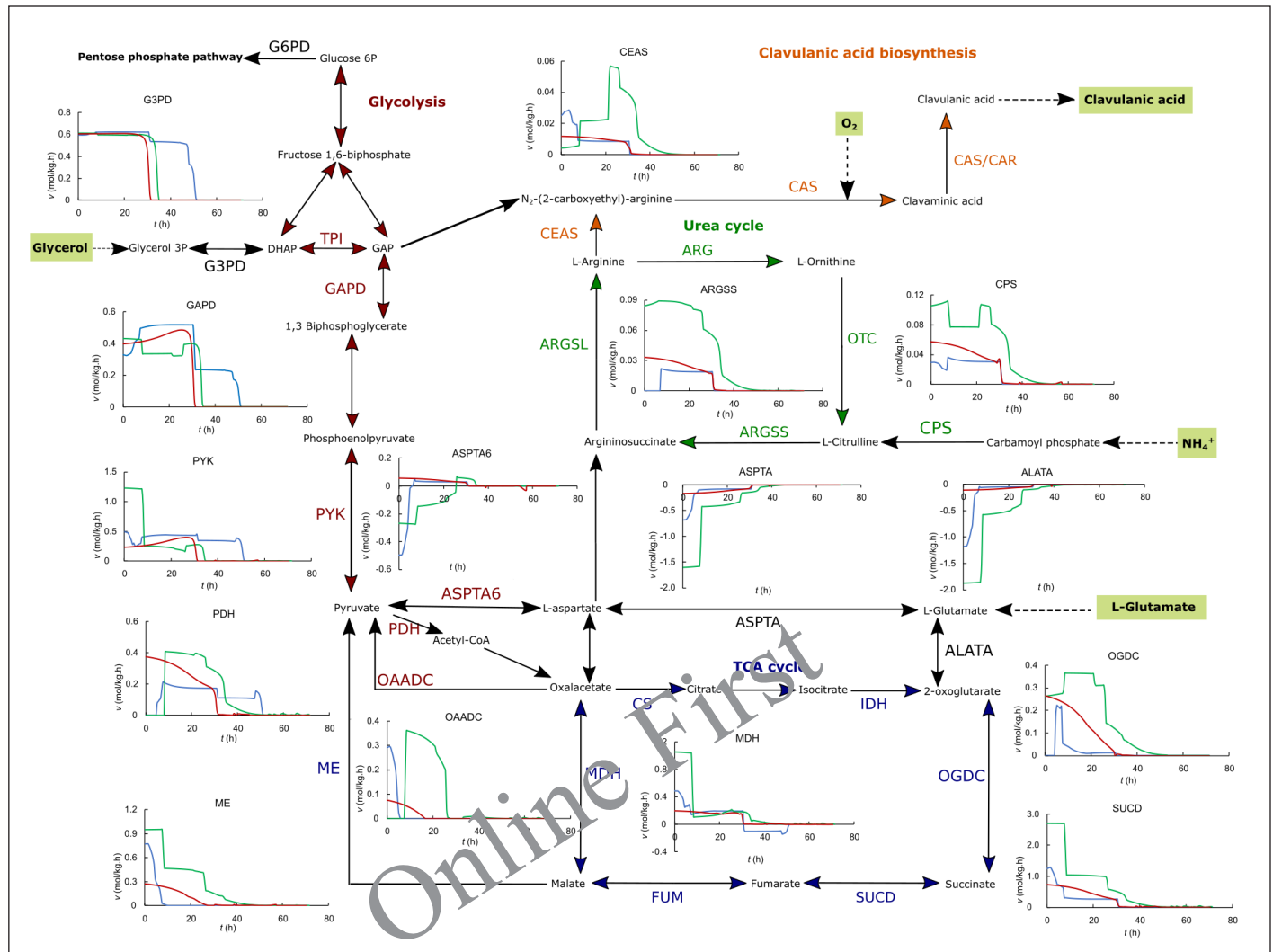


Figure 4. *In silico* dynamic flux distribution in the central metabolism and CA biosynthesis for the ISP (blue), GSPG (green), RMO (red), and modified GSPG (yellow) media, in fed-batch operation.

than those added directly to the GSPG medium, leading to lower fluxes in the arginine direction of the urea cycle and CA biosynthesis. The fluxes in the CA biosynthesis direction from the urea cycle were marginally higher for the RMO medium than for the ISP, given the higher concentration of glutamate, which is a direct C-5 precursor; while the major contribution of C-5 from ISP comes from arginine and proline in lower concentrations. In both synthetic media, the flux results confirm the importance of adding high concentrations of C-5 precursors in the glutamate-aspartate direction for promoting high CA biosynthetic fluxes in batch operation.

The effects of amino acid supplementation on CA production are still under discussion since a wide range of conditions and supplements have been shown to increase CA secretion rate [5,22]. However, several experimental studies have concluded that the addition of urea cycle intermediates such as arginine and ornithine, although it has a positive effect, it has not been as high as expected in boosting CA production, considering that arginine is the precursor of the

first product in the CA pathway [21,31,35]. *In silico* fluxes suggest that supplementation with C-5 precursors in the line of glutamate/aspartate degradation has a more significant effect on increasing CA secretion rates. This is likely due to the role of 2-oxoglutarate in CA biosynthesis and TCA cycle regulation. Previously, it was verified experimentally that 2-oxoglutarate supplementation may favor CA secretion, but not as much as C-5 amino acids [31,32]. This experimental test was supported by the fact of the requirement of 2-oxoglutarate for CA production since three molecules of 2-oxoglutarate are consumed per molecule of CA synthesized; specifically, in the oxidizing steps catalyzed by clavamate synthase, which yields succinate [5]. The *in silico* fluxes suggest that the addition of either glutamate or glutamate precursors might increase the cataplerotic flux of 2-oxoglutarate from the glutamate and aspartate transaminations by ASPTA and alanine transaminase (ALATA) as observed in simulations of cultures with high organic nitrogen content (Fig. 3). This would increase the fluxes in the urea cycle toward arginine and thus, toward

carboxyethyl arginine in the CA pathway (see CEAS in Fig. 3), under an adequate C-3 uptake via glyceraldehyde 3-phosphate. The increasing anaplerotic flux toward 2-oxoglutarate also stimulates the further oxidative steps of the TCA cycle, such as those catalyzed by 2-oxoglutarate dehydrogenase (OGDC) and succinyl-CoA synthetase, as shown in Figure 3, while increasing the cataplerotic disposal of malate and oxalacetate via the malic enzyme (ME) and oxalacetate decarboxylase (OAADC), both to pyruvate (see ME and OAADC in Fig. 3).

In all cases, the initial high fluxes in the anaplerotic reactions of amino acid synthesis/degradation catalyzed by L-alanine-alpha-keto acid aminotransferase (ASPTA6 and ALATA, in Fig. 3) decrease as the concentration of C-5 precursors in the media depletes. The higher the concentration of C-5 precursors in the basal media, the longer those fluxes remain during the exponential growth as displayed in Figure 3 for the batch operation. Given the lower initial concentration of the glutamate family precursors in the ISP medium, the fluxes in the anaplerotic metabolism of amino acids drop in the first 10 hours of cultivation, turning into the lowest among the three media tested. The time course of the fluxes during the batch culture suggests that most of the carbon proceeds via glycolysis-TCA toward the urea cycle, given the high C-3/C-5 ratio (9.17). Although this would stimulate fluxes in the TCA, it would do so to a lesser extent in the urea cycle and CA biosynthesis, compared to those media with a narrower C-3/C-5 balance. The CA biosynthetic flux for the ISP medium approaches that estimated for the RMO medium after 10 hours, despite the considerably lower C-3 and amino acid concentrations of the latter. In batch operation, the simulations show that the high concentration of proline and glutamate in the GSPG medium manages to maintain high fluxes through reactions related to CA biosynthesis, such as ALATA, ASPTA, ARGSS, and CEAS, for much longer; even in the face of the high demand for C-5 precursors. This is not so in the ISP medium, in which the uptake of C-5 precursors leads to a rapid depletion that negatively impacts fluxes in CA synthesis.

Interestingly, pyruvate shows a relationship with CA synthesis despite not being a direct precursor as previously hypothesized [36]. The fluxes through PDH estimated for the ISP medium are approximately half of those estimated for GAPD, considering the various bifurcations of the glycolytic pathway and that the assimilable carbon source is glycerol, given its high initial concentration in the medium. Conversely, in the case of both synthetic media, the fluxes through PDH are almost the same or even higher than those in GAPD, which is probably connected to the high uptake of C-5 precursors downstream of the TCA cycle and the conversion of oxalacetate and malate into pyruvate. This behavior was observed *in silico* only for the scenarios of high concentration of C-5 precursors in the basal media, *i.e.*, for GSPG and, to a lesser extent, for RMO media. The high flux through PDH drops once the C-5 is depleted in the culture and the flux in the anaplerotic reactions of glutamate and aspartate decreases (see ASPTA6 and ALATA in Fig. 3). This scenario would cause a small accumulation of pyruvate, especially at the end of the culture, since the demand for pyruvate decreases substantially in the stationary phase. Recently, Fu *et al.* reported metabolomic

and transcriptomic data comparing rich organic nitrogen media [16]. Media enhancing CA accumulation upregulated intracellular glutamate, pyruvate, and succinate, concomitantly with decreasing pools of 2-oxoglutarate, ornithine, citrulline, aspartate, argininosuccinate, and arginine, compared to a complex medium producing a lower CA secretion in batch operation [16].

It has been widely reported that the highest CA production in bioreactors for wild-type strains of *S. clavuligerus* is achieved by fed-batch operation. Different feeding media have been reported with good results in enhancing CA secretion rates and increasing maximum biomass and exponential growth phase. Threonine and arginine were added in fed-batch operation to the GSPG medium, based on the experimental results of Saudagar and Singhal [31]. However, several studies have highlighted the importance of adding a C-3 source, typically glycerol. The feeding medium consisting of threonine and arginine modulates the fluxes through glycolysis to maintain the flux of C-3 toward CA biosynthesis. The *in silico* flux distribution suggests that threonine degradation to glycine and further to serine contribute to supply pyruvate and, in consequence, a C-3 precursor in the absence of glycerol (see glycerol 3-phosphate dehydrogenase (G3PD), GAPD, PDH, and CEAS in Fig. 4). As a biomass precursor, glycine may be synthesized from serine, and this occurs during the batch operation. On the contrary, when threonine feeding starts, the conversion of glycine to serine, by hydroxymethyl transferase increases the flux in the direction of serine, which is further metabolized into pyruvate. Nevertheless, most of the relevant reports about fed-batch production of CA point to a glycerol-based feeding medium to support biomass synthesis and energetic metabolism as well as to provide a direct C-3 precursor of CA.

The purpose of the fed-batch operation is to extend the productive phase of the cultivation; the dynamic simulation suggests that this goal is better achieved by maintaining more stable fluxes along the pathways related to CA [25]. As observed in the simulation of batch cultures, the intracellular fluxes tend to drop drastically as the substrates deplete, and cells must adapt to the scarcity by decelerating growth and secreting metabolites that help energetic metabolism regulation [24,37–39]. As observed in the dynamic simulations, the fed-batch operation extends a stable flux toward central metabolism beyond 50 hours of cultivation, *i.e.*, during the stationary phase, even in the secondary metabolism favoring CA continued secretion under phosphate limitation. The fed-batch operation for the ISP medium, consisting of glycerol and a small amount of ornithine, would maintain the fluxes along glycolysis and the TCA cycle, but is less effective in promoting fluxes in the urea cycle and CA biosynthesis (Fig. 4). In the case of the GSPG medium with the feeding of threonine and arginine, it is plausible that the effect on maintaining the fluxes in the urea cycle and CA biosynthesis during the stationary phase but with low fluxes through glycolysis owing to the earlier depletion of C-3 and C-6 as the most assimilable carbon sources. Despite the lack of a direct C-3 precursor, the fluxes through CEAS are slightly higher than those in the RMO medium. However, the RMO medium has a lower content of total carbon, C-3 and C-5 precursors in batch and fed-batch (Table 1). This leads to

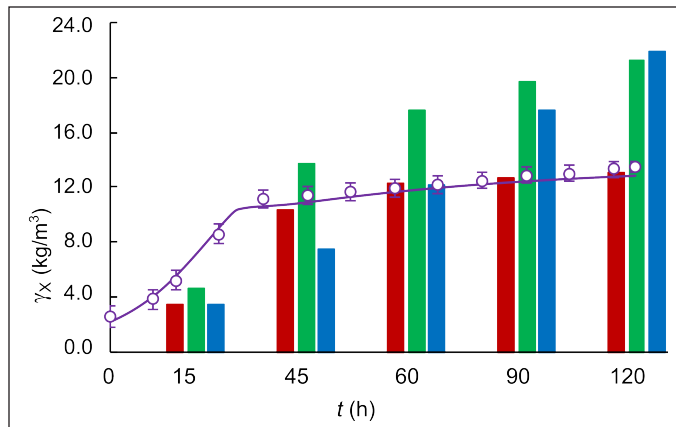


Figure 5. Experimental (circles) and simulated (solid line) time course of biomass concentration in fed-batch cultivations of *S. clavuligerus* in modified GSPG medium (purple). Comparison of experimental points of biomass concentration (bars) in cultures with RMO (red), GSPG (green), and ISP (blue) media.

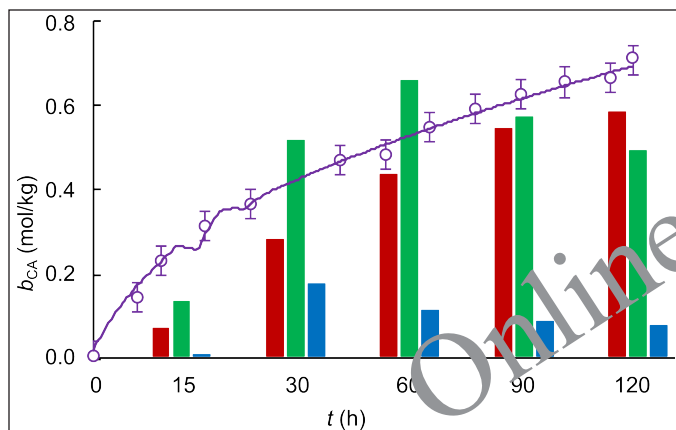


Figure 6. Experimental (circles) and simulated (solid line) time course of CA-specific concentration in fed-batch cultivations of *S. clavuligerus* in modified GSPG medium (purple). Comparison of experimental points of CA-specific concentration (bars) in cultures with RMO (red), GSPG (green), and ISP (blue) media.

more efficient carbon usage, considering the product of interest. The GSPG medium promotes both high biomass and high CA production in batch, but a feeding consisting mainly of highly concentrated threonine does not lead to better carbon utilization than the feeding medium consisting of glutamate and glycerol used for the RMO fed-batch cultivation.

A fed-batch cultivation scenario for enhancing CA production

Dynamic simulations at the genome scale have provided insights into the stoichiometric effect of amino acid supplementation on *S. clavuligerus* batch and fed-batch cultivations. According to those observations and contrasting with the experimental results at the bioreactor scale, a fed-batch production scenario was proposed by modifying the GSPG basal medium and the respective feeding for better carbon utilization in terms of CA biosynthesis. Dynamic simulation of

CA production using the proposed medium modification was done to confirm the potential CA enhancement, adjustment of the feeding time according to the carbon sources uptake rate, and effect on intracellular fluxes through key enzymes related to CA. The reaction fluxes for this proposed fed-batch production scenario were included in Figure 4 for the sake of comparison with the other conditions previously tested. The composition of the GSPG medium followed the same C/N and C-3/C-5 ratio which led to a carbon usage of 3.0% toward CA. The feeding medium was the one used in the RMO medium, which gave a better carbon utilization during the fed-batch operation owing to the provision of both precursors, glycerol, and glutamate. The feeding time was delayed to 34 hours considering the higher amount of carbon sources in the medium, thus preventing substrate accumulation and overflow metabolism [24,28].

Moreover, the dynamic FBA applied to batch and fed-batch cultivations with the ISP, GSPG, and RMO media has shown the importance of 2-oxoglutarate and pyruvate in modulating CA biosynthesis in connection with C-5 amino acids metabolism. The experiments and *in silico* flux distribution suggest a fed-batch operation at a low dilution rate considering the specific growth rate of *S. clavuligerus* under phosphate limitation. Previously, it was shown that starting the fed-batch operation before phosphate and carbon limitation prevents sharp declines in carbon fluxes. As observed, a feed composed of C-3 and C-5 direct precursors might maintain higher and more stable fluxes through the urea cycle and CA biosynthesis, than when using glycerol or amino acids as the only assimilable substrate. The feed medium formulation for *S. clavuligerus* cultures for CA production should provide sufficient glycerol as a C-3 precursor and a C-5 precursor such as glutamate or proline, as well as ammonia and phosphate in narrow concentrations to maintain the limitation that releases the antibiotic synthesis, while maintaining the activity along central metabolism and avoiding substrate accumulation or strong starvation potentially hampering the antibiotic secretion.

A higher specific CA concentration was obtained in the fed-batch scenario with modified GSPG medium, 0.72 mmol CA/gDCW compared to 0.69 mmol CA/gDCW reached with the GSPG medium in the first fed-batch scenario (Fig. 6). Although closer, as observed in Figures 5 and 6, the highest CA-specific concentration was attained at a maximum biomass concentration of 13.5 g L⁻¹, which is 55% lower than that obtained in the standard GSPG medium (see Fig. 5). This suggests a better carbon utilization by the secondary metabolism, since less biomass is synthesizing more CA. Indeed, the ratio of total carbon used in CA biosynthesis was higher for the fed-batch cultivation with the modified GSPG medium (3.34%), suggesting a higher selectivity toward the product of interest. Although CA is not strictly a growth-coupled product, in a defined medium, the more carbon is channeled to a particular pathway, the less carbon is available for biomass synthesis; since the antibiotic's biosynthesis recruits precursors directly from the central carbon metabolism. By feeding carbon in a precise concentration and flow rate, overflow metabolism and deviations toward pathways synthesizing side products are avoided. Both the nutritional restriction and the cultivation conditions favored CA biosynthesis, allowing sustained production with a

quasi-stationary biomass production due to the dilution and cell death, as occurred in chemostat cultivations. Moreover, during bioreactor operation, it is desirable to have high CA-specific production rates at low biomass concentrations, since the *S. clavuligerus* mycelium confers to the broth a non-Newtonian behavior, increasing density and viscosity as biomass increases [28]. The dense and dispersed mycelia make it difficult to maintain high dissolved oxygen tension at moderate agitation rates, facilitating bioprocess control.

The dynamic flux distributions shown in Figures 4 and 4 suggest that metabolic fluxes in this scenario would be rather stable with this operation, avoiding the sharp fluctuations of the instantaneous fluxes that are estimated for the richer media used in this study, favoring the continued secretion of CA, without an apparent decline during all the fed-batch operation. As expected, the composition of the GSPG-modified medium allowed high and stable intracellular fluxes in the TCA and urea cycles (e.g., through ARGSS and ASPTA in Fig. 4), favoring the flux toward CA biosynthesis. Although the fluxes in central carbon metabolism were marginally lower than those estimated for the GSPG standard medium due to the reduced glycerol, glutamate, and proline concentrations, the higher carbon dedicated to CA production instead of biomass would result in lower operation costs for achieving comparable CA final concentrations. In addition, by producing CA in defined media, downstream processing might be simplified since fewer metabolites, biomass, and debris are present in the broth.

CONCLUSION

The dynamic flux distribution obtained from simulations with experimental constraints provided insights into the importance of the availability of C-5 precursors and highlighted the importance of 2-oxoglutarate and pyruvate in modulating CA biosynthesis in *S. clavuligerus*. The application of a dynamic modeling approach highlighted the importance of adding C-5 precursors in the line of glutamate and aspartate, in promoting high CA biosynthetic fluxes in both, fed-batch and batch operation. The addition of glutamate or glutamate precursors might increase the fluxes in the urea cycle toward arginine and thus, carboxyethyl arginine in the CA pathway, leading to enhanced CA production in wild-type *S. clavuligerus*. The use of a fed-batch cultivation scenario with a modified GSPG medium and adjusted feeding strategy enhanced CA production at lower biomass concentrations, and better carbon utilization in CA biosynthesis, which are desirable conditions for culture operation. The dynamic simulations suggest that metabolic fluxes in this scenario would be rather stable, favoring the continued secretion of CA. Overall, the proposed fed-batch cultivation scenario can contribute to the development of efficient CA production processes in *S. clavuligerus*.

LIST OF ABBREVIATIONS

ALATA: alanine transaminase; ARGSS: argininosuccinate synthase; ASPTA: aspartate dehydrogenase; ASPTA6: L-alanine-alpha-keto acid aminotransferase; CEAS: N²-(2-carboxyethyl)-arginine synthase; G3PD: glycerol 3-phosphate dehydrogenase; GAPD: glyceraldehyde

3-phosphate dehydrogenase; GSPG: glycerol-sucrose-proline-glutamate medium.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This article does not contain any studies with human participants or animals.

INFORMED CONSENT

For this type of study formal consent is not required.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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