



Research article

Rational selection of culture medium for clavulanic acid production by *Streptomyces clavuligerus* based on a metabolic modeling approach

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Abstract

Importance of the work: Clavulanic acid (CA) is a β -lactam antibiotic produced by *Streptomyces clavuligerus* and is an inhibitor of β -lactamase enzymes responsible for antibiotics resistance in several pathogenic bacteria. CA productivity in wild-type *S. clavuligerus* is low and determination of favorable environmental condition for CA biosynthesis is required.

Objectives: To determine the culture conditions that promote secondary metabolism activity in *S. clavuligerus* and redirection of metabolic fluxes.

Materials & Methods: An *in silico* approach was implemented to select amino acids supplementation to culture media as a rational strategy to promote CA biosynthesis during cultivation. Submerged cultures of *S. clavuligerus* were investigated in synthetic and complex media selected according to the *in silico* study.

Results: Amino acids supplementation favored CA production over the complex media. Fed-batch operation favored both biomass maintenance and CA accumulation compared with the batch operation. The increase in the agitation velocity had a positive effect on biomass and CA accumulations.

Main finding: The defined media lead to specific nutrients starvation that act as stress factors potentially triggering the CA secretion. The *in silico* analysis showed that during phosphate-limited conditions, the high concentration of glutamate boosts the urea cycle from aspartate, increasing the carbon flux toward arginine and CA.

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Introduction

Antimicrobial natural products have been the core of the pharmacological fight against infectious diseases in humans and animals. Actinobacteria is a phylum of Gram-positive bacteria with a high guanine-cytosine content (Gómez-Ríos et al., 2020). In this phylum, the genus *Streptomyces* is recognized as the most important source of bioactive compounds due to its diverse secondary metabolism (Phongsopitanun et al., 2021). Most *Streptomyces* species have been isolated from terrestrial habitat, especially soil, and many of their antibiotic metabolites were reported before the 1980s (Phongsopitanun et al., 2021). Despite this, *Streptomyces* are still the richest sources of antibiotics, with several of their secondary metabolites currently being used in human and veterinary medicine, agriculture and the fishing industry (Apichaisataienchote et al., 2006). This genus remains one of the most important producers of new and effective antibiotics to combat the emergence of resistant pathogenic bacteria (Gómez-Ríos and Ramírez-Malule, 2019).

Streptomyces clavuligerus was isolated first by Higgins and Kastner (1971) from a soil sample from South America. *S. clavuligerus* can produce several β -lactam compounds with antibiotic activity. Among them, clavulanic acid (CA), reported initially by Reading and Cole (1977), is considered the most important due to its inhibitory effect on β -lactamase enzymes. CA is commercialized in its sodium or potassium salt form and it is formulated with wide spectrum β -lactam antibiotics to enhance its effectivity. CA is synthesized via the clavam pathway, usually in submerged cultures under nutritional restriction. However, production of CA has a high cost given the characteristic low yields of both cultivation and downstream processes (López-Agudelo et al., 2021). Several strategies have been explored aimed to improve the productivity of the strain, including the effects of nutrient concentration, environmental conditions and genetic modifications (López-Agudelo et al., 2021).

Different nutritional and environmental conditions have been explored to improve the understanding of the biosynthesis of clavam metabolites. As reviewed by Ser et al. (2016), media containing glycerol have been reported as favoring CA biosynthesis based on the direct utilization of glyceraldehyde-3-phosphate as an early precursor of its biosynthetic pathway. Some studies pointed out that CA production is enhanced by amino-acids supplementation and complex nitrogen sources, such as soy protein and soy flour (Bushell et al., 2006; Saudagar

and Singhal, 2007a; Sánchez et al., 2012). A complex culture medium can be a source of important amounts of free or hydrolyzed amino acids, which act not only as nitrogen sources but also as secondary carbon sources.

The pharmaceutical industry typically operates in batch mode, but cultivation using this technique is, in general, less efficient in terms of CA accumulation, since this kind of operation is limited by substrate starvation, biomass decline and product degradation (Neto et al., 2005; Ser et al., 2016; Gómez-Ríos et al., 2019a). The CA molecule shows susceptibility to temperature and ionic strength in aqueous solutions, which compromise the CA titers attained during cultivation (Brethauer et al., 2008; Santos-Ebinuma et al., 2009; Gómez-Ríos et al., 2019b). The pH culture conditions seem to affect CA yield, possibly more linked to molecule degradation than biosynthesis inhibition (Marques et al., 2009). The maximum concentration of CA has been reported at neutral or slightly acidic pH conditions (6.8), regardless of the substrates used during cultivation (Ser et al., 2016). Low temperatures favor molecular stability; in this regard, higher titers have been reported during operating fermentations at 20°C but the growth rate at such temperature is lower than that observed at 28°C (Costa and Badino, 2012). Thus, optimal *S. clavuligerus* cultivation must consider media that can promote CA accumulation in addition to adequate operating conditions. The aim of the current study was to apply a metabolic modeling approach for the rational selection of amino acids supplementation for *S. clavuligerus* cultures using biomass and CA as response variables, to establish suitable conditions for further upscaling of the process.

Materials and Methods

In silico analysis of amino acids supplementation in culture media

A metabolic modeling approach was applied to determine nutritional conditions potentially favoring CA production in wild type *S. clavuligerus* cultures. A high-quality stoichiometric model for the organism *S. clavuligerus* (iDG1237) was recently reported by Gómez-Ríos et al. (2020) as the most recent genome-scale reconstruction of *S. clavuligerus* metabolism. This model was validated and curated topologically and thermodynamically; it contains 1,518 metabolites, 2,177 reactions and 1,237 genes.

Based on a literature screening of experimental studies, all the amino acids with some enhancer effects were identified and those with significant improving levels were selected for the *in silico* study. A sensitivity analysis (Equation S1) was performed as proposed by Ramirez-Malule et al. (2021) for a first analysis of the effect of the amino acid in-flux on the objective functions of biomass and CA biosynthesis (and secretion). Once the sensitivity of the metabolic network to the amino acids in-fluxes had been evaluated, an *in silico* design of experiments (DOE), using a central composite design with two central points, was applied in a two-stage flux balance analysis (FBA) approach (Equation 1). The DOE considered combinations of the amino acids in-fluxes with a potential positive effect on CA biosynthesis in addition to the *in silico* basal medium (Table 1). As the physiological objective of a cell is growth, the FBA informs the probable carbon fluxes distribution (v) along the network under different nutritional conditions while maintaining biomass maximization (Z_{max}) along with maximum enzymatic efficiency (v^2) as the objective function, having l_b and u_b as the flux boundaries.

$$\begin{aligned} & \text{Min } \sum v^2 \\ & \left[\begin{array}{l} s. t. Sv = 0 \\ l_b \leq v \leq l_u \\ f^T v = Z_{max} \end{array} \right] \quad (1) \end{aligned}$$

In the DOE, the in-flux (lower bound) was set at -0.1 mmol/(g dry cell weight [DCW].hr) for each amino acid considered as supplement to the basal medium, since its uptake

is considerably lower than for the primary carbon source and oxygen. This value coincided with the maximum instantaneous uptake observed experimentally for an individual amino acid in *S. clavuligerus* cultures in synthetic media (Ramirez-Malule et al., 2018; Gómez-Ríos et al., 2019a). All FBA simulations were carried out in the COBRA Toolbox v3.0 (Heirendt et al., 2019) under MATLAB 2020b (<https://www.mathworks.com/products/matlab.html>) with Gurobi 7.0 (<https://www.gurobi.com>) as the optimization solver.

S. clavuligerus cultivations for clavulanic acid production

Activation of the strain and media composition

A cryostock was used of *S. clavuligerus* ATCC 27064 mycelial suspension (1.2 mL) stored in glycerol sterile solution (16.7%) at -80°C and activated in seed medium as described by Ramirez-Malule et al. (2018). Based on the simulation study of amino acids effects on the *S. clavuligerus* metabolism, three production media containing those amino acids screened *in silico* were finally tested. The media were selected from experimental reports of *S. clavuligerus* cultivations and modified if necessary. Two synthetic media with amino acids addition were used in addition to a complex medium. The first synthetic medium considered was glycerol-sucrose-proline-glutamate (GSPG) (Romero et al., 1984) prepared as follows, (with concentrations in distilled water): glycerol, 15 g/L; sucrose, 20 g/L; NaCl, 5.0 g/L; L-proline, 2.5 g/L; L-glutamate 1.5 g/L; K₂HPO₄, 2.0 g/L; MgSO₄·7H₂O, 1.0 g/L; CaCl₂, 0.4 g/L; MnCl₂·4H₂O, 0.1 g/L; FeCl₃·6H₂O, 0.1 g/L; ZnCl₂, 0.05 g/L; and 3-(N-morpholino) propanesulfonic acid (MOPS),

Table 1 *In silico* basal medium in iDG1237 biomodel

Exchange reaction	Reaction ID	Model reaction	Lower bound	Upper bound
Cl ⁻	R_EX_cl_e	cl[e] <=>	-1	1
NH ₄ ⁺	R_EX_nh4_e	nh4[e] <=>	-1	1
K ⁺	R_EX_k_e	k[e] <=>	-1	1
Ca ²⁺	R_EX_ca2_e	ca2[e] <=>	-1	1
Mg ²⁺	R_EX_mg2_e	mg2[e] <=>	-1	1
Mn ²⁺	R_EX_mn2_e	mn2[e] <=>	-1	1
Co ²⁺	R_EX_cobalt2_e	cobalt2[e] <=>	-1	1
Zn ²⁺	R_EX_zn2_e	zn2[e] <=>	-1	1
Cu ²⁺	R_EX_cu2_e	cu2[e] <=>	-1	1
O ₂	R_EX_o2_e	o2[e] <=>	-10	10
Fe ²⁺	R_EX_fe2_e	fe2[e] <=>	-1	1
MnO ₄ ²⁻	R_EX_mobd_e	mobd[e] <=>	-1	1
SO ₄ ²⁻	R_EX_so4_e	so4[e] <=>	-1	1
PO ₄ ³⁻	R_EX_pi_e	pi[e] <=>	-0.01	1
Citrate	R_EX_cit_e	cit[e] <=>	-0.01	10
Glycerol	R_EX_glyc_e	glyc[e] <=>	-1	10

20.9 g/L. The second synthetic medium used was the one formulated by Roubos et al. (2002) and adapted by Ramirez-Malule et al. (2018); modified in this work with ornithine addition (RMO), (concentrations in distilled water): glycerol, 9.3 g/L; K_2HPO_4 , 0.8; $(NH_4)_2SO_4$, 1.26 g/L; monosodium glutamate, 9.8 g/L; ornithine, 2.5 g/L; $FeSO_4 \cdot 7H_2O$, 0.18 g/L; $MgSO_4 \cdot 7H_2O$, 0.72 g/L; MOPS, 10.5 g/L and trace elements solution (1.44 mL). The trace elements solution contained: H_2SO_4 , 20.4 g/L; monosodium citrate $\cdot 1H_2O$, 50 g/L; $ZnSO_4 \cdot 7H_2O$, 16.75 g/L; $CuSO_4 \cdot 5H_2O$, 2.5 g/L; $MnCl_2 \cdot 4H_2O$, 1.5 g/L; H_3BO_3 , 2 g/L; and $Na_2MoO_4 \cdot 2H_2O$, 2 g/L. Finally, the complex medium referred to as isolated soy protein (ISP) was also used, prepared as follows (concentrations in distilled water): glycerol, 15.0 g/L; soy protein isolate, 10.0 g/L; malt extract, 10.0 g/L; yeast extract, 1.0 g/L; K_2HPO_4 , 2.5 g/L; $MgSO_4 \cdot 7H_2O$, 0.75 g/L; $MnCl_2 \cdot 4H_2O$, 0.001 g/L; $FeSO_4 \cdot 7H_2O$, 0.001 g/L; $ZnSO_4 \cdot 7H_2O$, 0.001 g/L; MOPS, 21.0 g/L (Pinilla et al., 2018). In all cases, the feed medium used in the fed-batch operation using distilled water was composed of: glycerol, 120 g/L; K_2HPO_4 , 2 g/L; and $(NH_4)_2SO_4$, 8 g/L.

Operational conditions

Pre-cultures were performed in 45 mL of production media after inoculation of 5 mL of mycelial suspension from seed cultures for a total operating volume of 50 mL. The pre-cultures were performed in 250 mL baffled shake flasks during 20 hr at 200 rpm in an orbital shaker. Initially, batch and fed-batch cultivations were performed in duplicates maintaining agitation at 200 rpm for selecting the culture medium yielding the highest CA accumulation. Batch cultivations were performed during 160 hr with 50 mL operation volume, inoculated with 10% volume per volume of mycelial suspension from pre-culture in identical medium. For the case of fed-batch operation, the starting volume was 50 mL; the initial batch operation lasted up to 33 hr. Then, addition of 50 mL of feed media was accomplished in the subsequent 104 hr. Finally, there was a starvation phase of 23 hr prior to the end of the cultivation at 160 hr. Once the more suitable medium had been selected, additional experiments were performed at 200 rpm, 220 rpm and 250 rpm to explore the effect of agitation on *S. clavuligerus* growth and CA production.

Analytical methods

Culture samples (1 mL) were taken at intervals of approximately 12 hr and then centrifuged at 15,000 rpm and 4°C for 10 min; wet biomass was washed with 0.9% NaCl and centrifuged. Test tubes were dried overnight at 75°C for

dry cell weight (DCW) determination. CA was determined using high performance liquid chromatography (HPLC) with an Agilent 1200 Series equipped with a Diode Array Detector, using a reverse phase column ZORBAX Eclipse XDB-C18 (4.6×150 mm, internal diameter 18 µm) following the method described by Ramirez-Malule et al. (2016).

Results and Discussion

In silico analysis of amino acids supplementation in *S. clavuligerus* cultures

The amino acids exerting a potential beneficial effect on CA biosynthesis were selected from experimental studies addressing amino acids addition to *S. clavuligerus* cultures (Bushell et al., 2006; Saudagar and Singhal, 2007a, b; Rodrigues et al., 2018). From those studies it was determined that the following amino acids influenced CA production (in descending order): 1) glutamic acid; 2) ornithine; 3) threonine; 4) arginine; and 5) proline. The use of complex nutrient sources in the medium such as hydrolyzed protein extracts results in the indirect addition of amino acids and dipeptides, so the use of these complex sources is generally associated with an increase in the productivity of the specialized metabolite compared to non-supplemented media (Ser et al., 2016). Studies *in silico* with experimental validation might confirm the effects of these nutrients and facilitate the rational design of culture media and feeding for batch, fed batch and continuous cultures. The sensitivity of growth and CA secretion objectives to in-fluxes of those amino acids was preliminarily evaluated using a robustness analysis with the iDG1237 metabolic model and modifying the default restrictions of the *in silico* medium to -0.1 to allow the extracellular uptake of the amino acids under study. The robustness analysis (Fig. S1) confirmed that arginine and ornithine could increase the carbon fluxes toward biomass and CA biosynthesis in a slightly higher proportion than threonine, proline and glutamic acid (data not shown). However, in all cases it was plausible that amino acids addition could increase the synthesis rate of biomass almost proportionally with the increase in the uptake. In the case of CA, this effect could not be as direct as in the case of biomass production, since CA maximization is not a physiological objective of the organism at the sacrifice of growth. Both amino acids (arginine and ornithine) are directly incorporated in the urea cycle, and it is expected that both favor amino acids and protein biosynthesis, while CA synthesis implies the synthesis of L-carboxyethyl

arginine from glyceraldehyde 3-phosphate (C-3 precursor) and arginine (C-5 precursor). The L-threonine metabolism could yield glutamate via isoleucine through transamination, but this path is considerably larger and leads to several byproducts to be considered as an important C-5 precursor of CA biosynthesis.

CA is secreted during cultivation under phosphate limitation, coexisting its biosynthesis at low rates with growth, while carbon and nitrogen sources are available in the medium (Gómez-Ríos et al., 2019a). Therefore, growth is not changed as the metabolic objective of the organism and low carbon fluxes are directed to the clavam pathway as part of the response to nutritional restrictions. Simulations must reflect those scenarios of CA production while maximizing biomass. An *in silico* central composite DOE with the five amino acids as experimental factors (28 FBA runs) varied the uptake between -0.1 (maximum uptake) and 0 mmol/(g DCW.hr). The amino acids combination and levels entailing positive effects on CA production *in silico* are presented in Table 2.

The first three results were compatible with the formulation of synthetic media, since the single combination of two amino acids would favor CA production if they were added to the glycerol based medium. The in-flux of glutamate that promotes aspartate in the urea cycle favored the flux toward arginine as a CA precursor, while the uptake of ornithine decreased the degradation rate of arginine toward ornithine in the cycle, also increasing the flux of the C-5 precursor of CA. L-Proline degradation ended in L-glutamate production via the proline and glutamate- γ -semialdehyde dehydrogenases, which would increase the fluxes toward aspartate and arginine, enhancing CA biosynthesis. Runs 8–26 listed in Table 2, show that a complex amino acid profile providing in-fluxes of all five amino acids favored CA production by increasing the carbon fluxes in the urea cycle and the C-5 precursor while coexisting with phosphate limitation and the high uptake of C-3 precursor.

The latter was assured via the carbon source availability during the cultivation.

Clavulanic acid production in synthetic and complex media

The growth and CA concentrations were followed in all cultivations, using the procedures previously described in the Materials and Methods section. In batch conditions, the highest biomass accumulation (23.2 g DCW/L) was observed in cultures with the ISP medium, given the high concentration of carbon and nitrogen sources (glycerol, proteins and hydrolyzed amino acids). This was approximately 2-fold the maximum biomass observed in the cultivations with the RMO synthetic medium. Similarly, the mean ($\pm$ SD) maximum specific growth rate was observed in the ISP medium with 0.110 ± 0.004 hr⁻¹, followed by the GSPG medium 0.082 ± 0.005 hr⁻¹ and the RMO medium 0.071 ± 0.006 hr⁻¹. An accelerated growth phase was observed in the ISP medium compared to the other media tested. Nevertheless, the accelerated growth led to early starvation conditions and, therefore, a pronounced decline in biomass was observed after reaching the maximum concentration at 46 hr. For the GSPG, the concentrations of carbon and nitrogen sources were lower than those of the ISP medium, leading to less accelerated growth and lower nutrients uptake. The maximum biomass accumulated was 17.0 g DCW/L and the decline in biomass was less pronounced. The RMO medium had a lower glycerol concentration; since this is the most assimilable carbon source for *S. clavuligerus*, the strain had a lower specific growth rate and the exponential phase lasted up to 45 hr. Notably, the biomass decline was less pronounced after finishing the exponential phase, with the maximum biomass of 10.4 g DCW/L. This was probably due to the high concentration of amino acids that could supply carbon and nitrogen once the glycerol became scarce in the medium. The biomass and CA time courses for batch cultures

Table 2 Results of two-stage flux balance analysis simulations of amino acids combination in biomass and clavulanic acid (CA) production

Run	v_{AA} (mmol/g DCW.hr)					μ (L/hr)	(v_{CA}) (g DCW.hr)	Ratio (v_{CA}/μ)
	Arg	Glu	Orn	Thr	Pro			
3	0	-0.100	0	0	-0.100	0.065	0.0042	0.065
4	0	-0.100	-0.100	0	0	0.065	0.0042	0.065
5	0	0	-0.100	0	-0.100	0.066	0.0041	0.062
8	-0.050	-0.050	-0.050	-0.050	-0.050	0.070	0.0025	0.036
18	-0.050	-0.050	-0.050	-0.050	-0.064	0.071	0.0045	0.063
22	-0.050	-0.050	-0.050	-0.050	-0.050	0.070	0.0025	0.036
23	-0.064	-0.050	-0.050	-0.050	-0.050	0.071	0.0045	0.063
25	-0.050	-0.050	-0.064	-0.050	-0.050	0.071	0.0045	0.063
26	-0.050	-0.050	-0.050	-0.064	-0.050	0.071	0.0015	0.021
Base	0	0	0	0	0	0.051	0	0

v_{AA} = amino acid uptake flux; v_{CA} = CA secretion flux; DCW = dry cell weight; μ = growth rate

of *S. clavuligerus* are presented in Figs. 1A and 1B. In all cases no lag phase was observed, most likely due to high biomass concentration in the inoculum from seed culture.

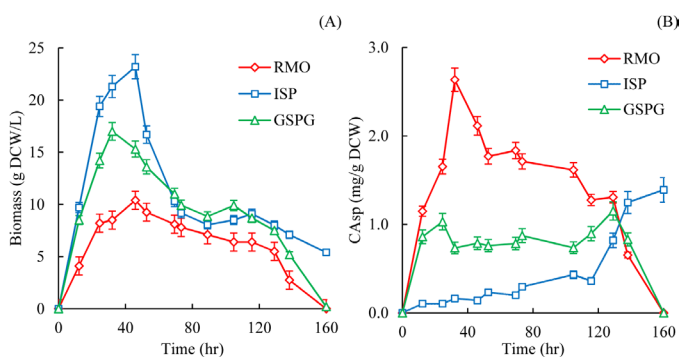


Fig. 1 *Streptomyces clavuligerus* batch cultures in glycerol-sucrose-proline-glutamate (GSPG) medium, isolated soy protein (ISP) medium and formulation with ornithine addition (RMO) medium: (A) biomass concentration; (B) specific clavulanic acid concentration (CA_{sp}), where DCW = dry cell weight; error bars indicate $\pm$ SD

The RMO medium led to a specific CA production of (0.240 ± 0.026) mg/(g DCW.hr), coincident with a lower biomass accumulation. The maximum CA concentration in batch cultures in the RMO medium was 28.5 mg/L. Cultures in the GSPG and ISP media reached CA concentrations of 14.6 mg/L and 8.8 mg/L, respectively. The productivity of CA was 0.140 ± 0.010 mg/(g DCW.hr) and 0.101 ± 0.016 mg/(g DCW.hr) in the ISP and the GSPG media, respectively. These results suggested that CA biosynthesis was favored under nutritional restrictions, mainly phosphate limitation. The RMO medium had the lowest phosphate concentration which was coincident with the highest CA production and lowest biomass synthesis.

The FBA simulations under constraints approximately equivalent to the nutritional conditions of cultivation showed that the high availability of carbon and nitrogen of the ISP and GSPG media activated the central metabolism in the oxidative direction, favoring energy production and increasing the synthesis of biomass precursors (Fig. 2). Under such favorable conditions, growth was preferential over secondary metabolism, as indicated by the high fluxes along the glycolysis and tricarboxylic acid (TCA) cycles. Notably, arginine synthesis by the arginine-succinate lyase (ARGSL) behaved as a bottleneck. The simulations showed that although the glycerol and amino acids uptakes were considerably higher for the ISP medium, for the GSPG medium, the flux along the ARGSL was almost the same, leading to similar CA secretion rates, as were experimentally observed. The in-flux of

L-proline for the GSPG medium would influence the aspartate transaminase (ASPTA) flux, increasing the reaction rate toward aspartate compared to the scenario of the RMO medium.

At the highest growth rate, the GSPG and ISP media maintained higher phosphate uptakes since these media had higher phosphate concentrations than the RMO medium; therefore, the metabolic scenarios might have differed slightly in the phosphate balance for maintaining adenosine triphosphate synthesis under phosphate limitation. The mechanism of antibiotics biosynthesis repression due to phosphate availability is not completely understood. In *S. clavuligerus* cultures it has been hypothesized that the antibiotics synthesis would contribute to regulate cellular energy by supplying phosphate under scarcity conditions (Gómez-Ríos et al., 2020; Virolle, 2020). In the current study, the CA secretion rates were still low in all scenarios; the cultivation conditions limited oxygen transfer at high biomass concentrations, which is an important operating variable in CA production. Oxygen-limited conditions repressed CA biosynthesis even under carbon and nitrogen sources availability. High activity in the TCA cycle at high oxygen uptake favored CA production, given the requirements of 2-oxoglutarate and molecular oxygen during CA biosynthesis (Gómez-Ríos et al., 2019a; Ramirez-Malule et al., 2021). Hence, a high volumetric mass transfer coefficient (k_La) is necessary to maintain the oxygenation and metabolite production when the culture is dense and viscous (at high biomass concentrations). Although baffled Erlenmeyer flasks are expected to provide high k_La values at early stages of cultivation, the dense and viscous broth characteristic of the *S. clavuligerus* cultures could make oxygen transfer difficult at high biomass values.

Fed-batch cultivation of S. clavuligerus with synthetic and complex media

Fed-batch has been reported as an operational mode that might enhance CA production in *S. clavuligerus* cultures when provided with carbon and nitrogen sources (Ser et al., 2016; López-Agudelo et al., 2021). Since the current experimental and simulation evidence suggested that phosphate depletion was one of the main factors triggering CA secretion, the fed-batch operation was tested in cultivations with the RMO, ISP and GSPG media, once the phosphate limitation condition started between 32 hr and 46 hr. A feed medium containing glycerol, ammonium and phosphate was used for all the cultivations operated in fed-batch. The feeding medium was designed to maintain the glycerol and ammonium supply based on the C/N ratio of 21.5 like the culture medium, with a minimum amount of phosphate for maintenance purposes (C/P ratio of 113.5).

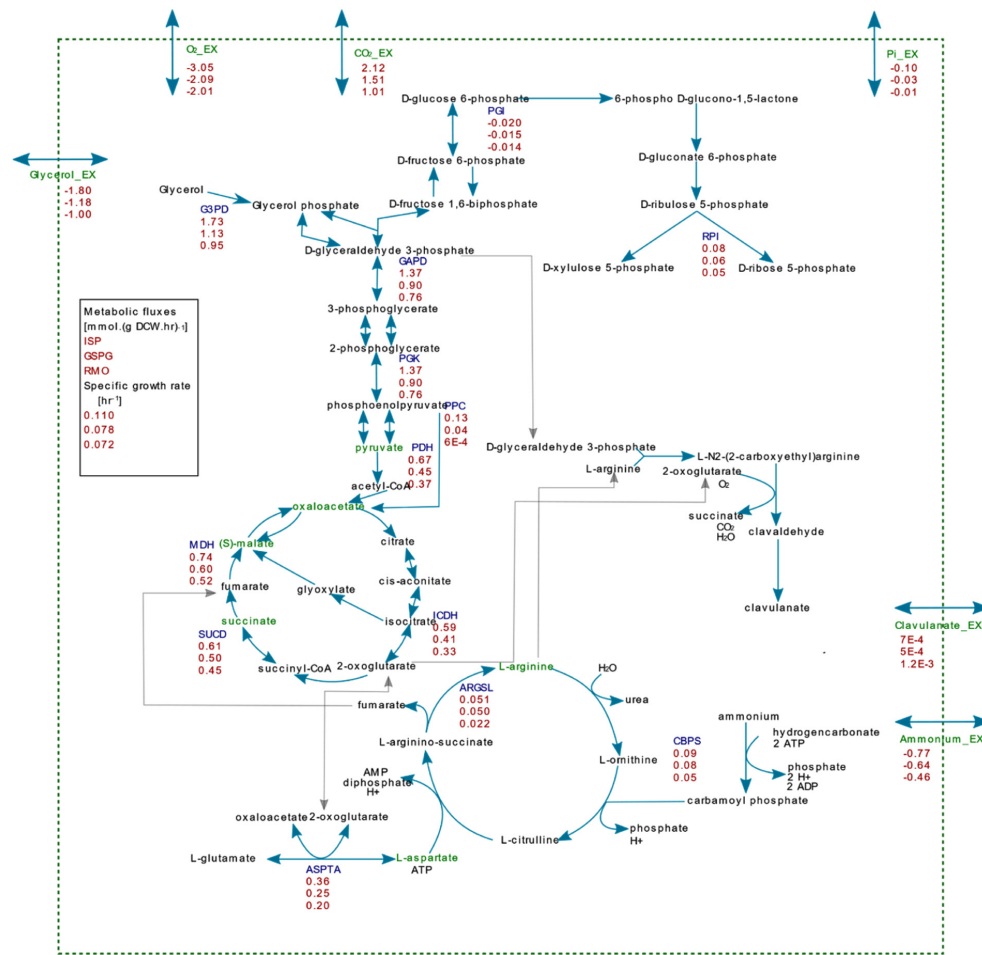


Fig. 2 *In silico* carbon flux distribution throughout central metabolism and clavulanic acid biosynthesis for *Streptomyces clavuligerus* cultivations in synthetic and complex media

Similar to batch cultivations, the highest biomass accumulation was observed in cultures with the ISP medium (25.6 g DCW/L). However, the extensive growth did not continue even under the feeding conditions. The maximum biomass attained in the GSPG medium was 17.6 g DCW/L during the batch phase and reached 18.6 g DCW/L during the fed-batch phase. The feeding conditions delayed the pronounced decline of biomass until 70 hr, maintaining the biomass concentration at over 11 g DCW/L, circumstances not observed in the batch cultivations. Feeding with the RMO medium supported the growth until 69 hr, reaching a higher biomass value (11.5 g DCW/L) and higher specific growth rate ($0.091 \pm 0.006 \text{ hr}^{-1}$) than those obtained in batch cultures. Additionally, the biomass decline was less pronounced than in batch cultures. For both synthetic media, stopping the feeding led to a rapid decline in biomass. The biomass and CA time courses for fed-batch cultivations of *S. clavuligerus* are presented in Fig. 3.

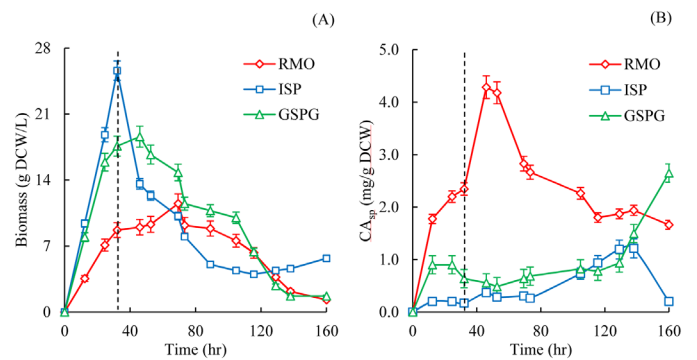


Fig. 3 *Streptomyces clavuligerus* fed-batch (50–100 mL) cultures in glycerol-sucrose-proline-glutamate (GSPG) medium, isolated soy protein (ISP) medium and formulation with ornithine addition (RMO) medium: (A) biomass concentration; (B) specific clavulanic acid concentration (CA_{sp}), where DCW = dry cell weight and error bars indicate $\pm$ SD

The CA production was enhanced in the fed-batch operation mode using the RMO medium. The maximum CA concentration was 36% higher than that obtained in the batch cultures. The CA accumulation rates for the GSPG and ISP media were similar to that observed in batch cultures, considering the dilution effect of the feeding. The specific CA production in the RMO medium was 0.390 ± 0.002 mg/(g DCW.hr); in the GSPG and ISP media, they were 0.070 ± 0.007 mg/(g DCW.hr) and 0.022 ± 0.005 mg/(g DCW.hr), respectively. The highest concentration attained in fed-batch operation using the defined RMO medium was 38.9 mg/L of CA. Notably, in batch operation, a higher CA concentration was reached with the RMO medium. Although, the synthetic GSPG medium promoted CA production, the concentrations attained were approximately half those obtained with the RMO medium. The lowest production of CA was observed in the complex media, probably due to a high availability of nutrients that did not allow limitation conditions to be reached that triggered CA production. In this regard, the RMO medium was designed to provide high glycerol and glutamate concentrations at low phosphate concentrations. Glycerol and glutamate are directly incorporated in the oxidative direction of glycolysis, TCA and urea cycles, favoring the synthesis of carboxyethyl arginine as the starting point of the clavam pathway.

The CA concentration was considerably more stable for the case of fed-batch cultivations, since continuous synthesis could occur in contrast to batch cultivations. Although high CA concentrations were reached in batch cultivations, a rapid degradation seemed to occur once the maximum concentration had been reached. Thus, the CA production with the RMO medium, was considerably higher than that observed with the GSPG and ISP media for the operating conditions considered in this study.

The biomass and CA accumulated productivities were calculated to compare the performance of batch and fed-batch operating modes with the RMO medium (Figs. 4A and 4B). As expected, biomass (Fig. 4A) and CA behaved rather the same in both cases before the start of the fed-batch operation at 33 hr. The biomass productivity was slightly higher in the fed-batch operation, since higher biomass concentration and growth rates were observed at the end of the exponential growth phase. The exponential growth under phosphate limitation favored CA productivity, as shown in Fig. 4B. The fed-batch operation caused a sharp increase in the CA productivity up to 2-fold the CA accumulated productivity of the batch cultures. During the stationary phase, the CA productivity remained almost constant

in batch and fed-batch, indicating a low CA synthesis rate after 73 hr when the amino acids concentrations were depleted. In fed-batch operation amino acids starvation caused a drop in the biomass production rate but the supply of nutrients in the feed helped to maintain the high cell density in the culture until the feeding ended. In fact, CA started to decline prior to biomass, suggesting that CA net accumulation was the result of both production and degradation, since the molecule was unstable in the broth and hydrolyzed following concentration dependent kinetics (Gómez-Ríos et al., 2019b,c). This would explain the total loss of CA at the end of the culture, as shown in Figs. 1 and 3.

It was expected that CA biosynthesis would increase the demand for oxygen, but oxygen limitation could be occurring at high biomass values. This fact is inherent to shake flask cultivations of filamentous organisms like *S. clavuligerus* that generate dense and viscous broths. Such an environment limits cell growth, even under substrate availability. For example, the extensive growth observed in the GSPG and ISP media (Fig. 3A) might have made oxygen diffusion difficult due to the high biomass concentration, leading to the subsequent pronounced biomass decline. For the RMO medium, the growth and biomass accumulations were lower than those attained for the GSPG and ISP media, suggesting that the oxygen limitation condition most likely did not occur. This was consistent with the less-pronounced biomass decline and larger CA biosynthesis phase.

The aeration and agitation rates were closely related to CA production and biomass accumulation, due to the strictly aerobic metabolism of *S. clavuligerus*. The increase in the agitation rate produced a positive effect on biomass concentration

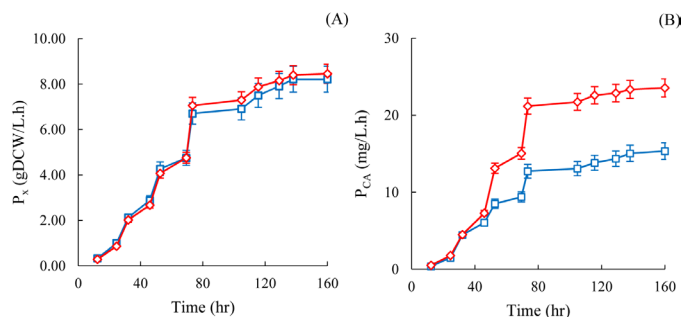


Fig. 4 Accumulated mass productivity of *Streptomyces clavuligerus* cultures: (A) biomass in batch (blue) and fed-batch (red) operations; (B) clavulanic acid in batch (blue) and fed-batch (red) operations, where DCW = dry cell weight, P_x = biomass productivity, P_{CA} = clavulanic acid productivity and error bars indicate ± SD

(see Fig. S2). The maximum biomass values observed when increasing the agitation speed by 10% and 25% were 12.8% and 20.9% higher, respectively. The rate of decline of biomass was more pronounced at high agitation rates, suggesting that shear stress favored fast biomass accumulation and consequently, higher nutrients uptake rates that led to earlier substrate starvation. The highest CA concentration (5.12 mg/g DCW.hr) was obtained at the highest agitation rate at 32 hr, as a consequence of mycelial fragmentation and enhanced $k_L a$. Nevertheless, the decline in CA was also more pronounced under this condition, more likely due to an increase in the degradation reaction rate (Gómez-Ríos et al., 2019a). In all cases, the CA production decreased along with the biomass decline. At the lowest agitation rate, the CA concentration was more stable, since the cell population was also more stable, considering the nutrients availability during the stationary phase (50–100 hr).

Three media formulations were selected based on a *in silico* study of the effect of amino acids on the metabolism of *S. clavuligerus*, with the aim of improving CA production in submerged cultures. The FBA simulations suggested that glutamate, ornithine and proline would improve the carbon fluxes toward the clavam pathway when using a chemically defined medium. Complex media supplying fluxes of glutamate, ornithine, threonine, arginine and proline promoted clavulanic acid production but at lower rates than adding individual amino acids related to arginine precursors. The defined media tested (GSPG and RMO) led to specific nutrients starvation that acted as stress factors potentially triggering the CA secretion. According to the *in silico* analysis, during such phosphate-limited conditions, the high concentration of glutamate boosted the urea cycle from aspartate increasing the carbon flux toward arginine and CA. This was also accompanied with higher demand for glyceraldehyde-3-phosphate from glycolysis, thus decreasing the flux toward pyruvate. Fed-batch operation contributed to maintaining more stable values of biomass and CA throughout the culture period, since this operation assured the supply of carbon and nitrogen sources to the central metabolism required for growth and maintenance, without eliminating the nutritional restrictions that activated the secondary metabolism. A moderate increase in the agitation rate had a positive effect on the oxygen transfer rate, which, in addition to the enhanced mycelial fragmentation, favored biomass and CA production.

Conflict of Interest

The authors declare that there are no conflicts of interest

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