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OPTIMIZATION OF THE PRECIPITATION OF CLAVULANIC ACID FROM FERMENTED BROTH USING T-OCTYLAMINE AS INTERMEDIATE

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Abstract - This work describes the use of clavulanic acid (CA) precipitation as the final step in the process of purification of CA from fermentation broth as an alternative to conventional methods employed traditionally. The purpose of this study was to use a stable intermediate (t-octylamine) between the conversion of CA to its salt form (potassium clavulanate), thereby enabling the resulting intermediate (amine salt of clavulanic acid) to improve the purification process and maintain the stability of the resulting potassium clavulanate. To this end, response surface methodology was employed to optimize the precipitation step. For the first reaction, five temperatures (6.6 to 23.4 °C), concentrations of clavulanic acid in organic solvent (6.6 to 23.4 mg/mL) and t-octylamine inflow rates (0.33 to 1.17 drop/min) were selected based on a central composite rotatable design (CCRD). For the second reaction, five temperatures (11.6 to 28.4 °C), concentrations of clavulanic acid amine salt in organic solvent (8.2 to 41.8 mg/mL) and concentrations of potassium 2-ethylhexanoate (0.2 to 1.2 molar) were also selected using CCRD. From these results, precipitation conditions were selected and applied to the purification of CA from the fermentation broth, obtaining a yield of 72.37%.
Keywords: Clavulanic acid; Precipitation reaction; Potassium 2-ethylhexanoate; T-octylamine.

INTRODUCTION

Clavulanic acid (CA) is a potent inhibitor of certain β-lactamases and its combined use with β-lactam antibiotics has enabled the cure of a variety of infections resistant to conventional therapies without requiring medications with strong side effects. Clavulanic acid is a highly unstable and hygroscopic oil whose use in drugs is only feasible in its salt form, specifically in the form of potassium clavulanate, which is more stable.

The purification of clavulanic acid (CA) from fermentation broth involves a series of steps such as filtration and centrifugation to separate its cells, as

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well as extraction techniques that are sometimes followed by an adsorption step for further purification of the antibiotic (Mayer *et al*., 1996).

Although the literature describes different ways of purifying clavulanic acid, its high instability in aqueous solutions always reduces its yield during the steps involved in the purification process (Bersanetti *et al*., 2005). Therefore, to obtain a higher final yield of clavulanic acid salt, several patents describe clavulanic acid purification processes that include chemical reactions such as esterification and precipitation.

The patents filed by Kim *et al*. (1995), Cook and Wilkins (1995) and Cardoso (1998) describe CA precipitation with potassium 2-ethylhexanoate (salt or sodium) as one of the steps in the purification process of CA from the fermentation medium. An extraction step is performed with organic solvent and the concentrated organic phase is subjected to a precipitation reaction which causes CA to react with potassium 2-ethylhexanoate (salt or sodium) to form the CA salt (potassium or sodium clavulanate).

In a previous work we studied the precipitation of clavulanic acid with potassium 2-ethylhexanoate and demonstrated that different combinations of CA and potassium 2-ethylhexanoate concentrations affect the yield, purification and stability of the resulting salt (Hirata *et al*., 2009). We also found that some of the conditions tested resulted in the formation of oil rather than CA salt, indicating that the direct precipitation of CA with potassium 2-ethylhexanoate only occurred satisfactorily within a limited range, making this technique difficult to employ on an industrial scale.

The preliminary conversion of CA into esters or amines (which are called stable intermediates) for subsequent conversion into potassium or sodium clavulanate can be employed to reduce the instability of CA, thereby increasing the yield of clavulanate salt. However, some amines are unsuitable for the manufacture of clavulanate salt or for use as intermediates because the resulting amine salts are toxic or hygroscopic. Moreover, the dissolution of certain salts for subsequent reactions requires large amounts of solvents. Thus, in addition to their economic unfeasibility, they may contaminate industrial effluents and intoxicate workers (Kim *et al*., 1995). Therefore, it is extremely important to choose the correct amine to produce the stable intermediate.

To increase the stability of CA during purification and thus increase the range in which the precipitation reaction occurs successfully and with a high yield, CA was subjected to a preliminary reaction with t-octylamine to form the stable intermediate. This amine was selected because it does not form toxic salts and because it renders CA more stable during handling, thereby reducing its degradation (Yang *et al*., 1994).

Considering that the performance of the precipitation reaction is directly influenced by the concentration of reagents, the temperature and the rate at which the reagents are added, a response surface model was developed to optimize the conditions employed to precipitate potassium clavulanate into a stable intermediate. The best values found in the factorial design were then applied to the purification of CA from the fermentation broth.

MATERIALS AND METHODS

Source of CA

The experiments were carried out using two distinct sources of CA:

- Potassium clavulanate from the pharmaceutical product Clavulin® produced by SmithKline Beecham, consisting of 625 mg of amoxicillin and 125 mg of potassium clavulanate; and
- CA produced by fermentation with *Streptomyces clavuligerus* ATCC 27064. The culture medium presents the following composition (in gL^{-1} distilled water): glycerol, 10.0; soybean flour, 20.0; soybean oil, 23.0; K_2HPO_4, 1.2; $MnC_{l2}4H_2O$, 0.001; $FeSO_47H_2O$, 0.001; $ZnSO_47H_2O$, 0.001, pH 6.8 (Ortiz *et al*., 2007). The fermentation was carried out in a Bioflo III model bioreactor (New Brunswick Sci.), with 3.6 L of production medium, making up 4 L of fermentation broth. The cultivation was conducted batchwise at 28 °C, 800 rpm and 0.5 vvm, and the pH was automatically controlled at 6.8±0.1 by adding 2M HCl or 2M NaOH solution. Dissolved oxygen concentration was monitored by a sterilized galvanic electrode (Mettler-Toledo InPro6000 Series), (Ortiz *et al*., 2007; Teodoro *et al*., 2010).

At the end of the fermentation, the pH of the fermentation broth was adjusted to 6.2 with 18N phosphoric acid, cooled between 11 ºC and 20 ºC, and filtered through a tubular polysulfone microfiltration membrane with 0.2 μm diameter pores supplied by Amersham Biosciences (CFP-2-E-8A). The resulting permeate was ultrafiltered through polysulfone membranes with 3 kDA (UFP-3-E-3MA) and 50 kDa pores (UFP-50-E-3MA) supplied by Amersham Biosciences.

Analytical Methods

The concentration of CA in the fermentation broth was determined by high performance liquid

chromatography (HPLC), as described by Foulstone and Reading (1982), by imidazole reaction. The samples were analyzed using a HPLC system equipped with a photodiode array detector (Waters 996 PDA) and a 3.9 x 300 mm C18-μ Bondapak analytical column. The HPLC device was operated at 28 °C and a flow rate of 2.5 mL/min, and standard solutions were prepared from the pharmaceutical product Clavulin®.

The NMR data were recorded on a Bruker ARX-400 9.4 T spectrometer operating at 400.35 MHz for ^{1}H and at 100.10 MHz for ^{13}C. All the NMR data were obtained at 25 ºC, using tetramethylsilane (TMS) as internal reference and deuterium oxide as solvent.

The morphology of the crystals of potassium clavulanate was analyzed by scanning electron microscopy (SEM) (Philips XL30 FEG-SEM), an ISIS microanalysis system (Oxford Instruments) and BSE (backscattered electrons). The BSE system shows the image by the difference in atomic weights, while the common system (SE-secondary electrons) shows the topographic image of the sample. The advantage of the BSE system is that it offers a better view of the crystals containing atoms of higher molecular weight than carbon, especially when these crystals are very small and fine, like those obtained for potassium clavulanate. The crystals with the larger atoms exhibited a shimmering white color, facilitating their identification in the sample. This method is suitable for potassium clavulanate due to the presence of a potassium atom in its molecule.

Precipitation Procedure

The precipitation of potassium clavulanate by passing through a stable intermediate involves two reactions. In the first precipitation reaction, CA reacts with t-octylamine to form the amine salt of CA (stable intermediate). In the second reaction, this stable intermediate reacts with potassium 2-ethylhexanoate to precipitate potassium clavulanate. Figure 1 illustrates the precipitation step to potassium clavulanate through these two reactions. A separate experimental design was devised for each reaction to better evaluate the variables involved in each reaction.

The experimental designs were devised with Clavulin®. The CA in the product Clavulin® was chosen for use in the factorial design assays to ensure the reproducibility of results, since they show a standard behavior in terms of CA composition and amount. This behavior cannot always be ensured when working with different fermented broths. The conditions that yielded good values in the experiments with Clavulin® were applied to the CA from the fermentation broth.

For the first precipitation reaction, the concentrated solution of clavulanic acid in ethyl acetate was transferred to a jacketed glass reactor and kept at a specific temperature in a thermostatic bath under continuous stirring (at 250 rpm) by a propeller stirrer connected to a speed control. T-octylamine was added dropwise and the solution was stirred for one hour. The induction time of 1.5 hours is defined in the Cook and Wilkins patent (1995). However, preliminary assays indicated that 1 hour was sufficient to obtain the highest yield of amine salts of clavulanic acid (CA). The precipitated product was filtered and washed with acetone. The resulting crystals were vacuum-dried for 24h at room temperature and then weighed.

The yield of crystals (Y) was calculated by Equation (1).

$$Y\% = \frac{m_{exp}}{m_t} \times 100 \quad (1)$$

where m_{exp} is the mass obtained experimentally and m_t is the theoretical mass for 100% conversion.

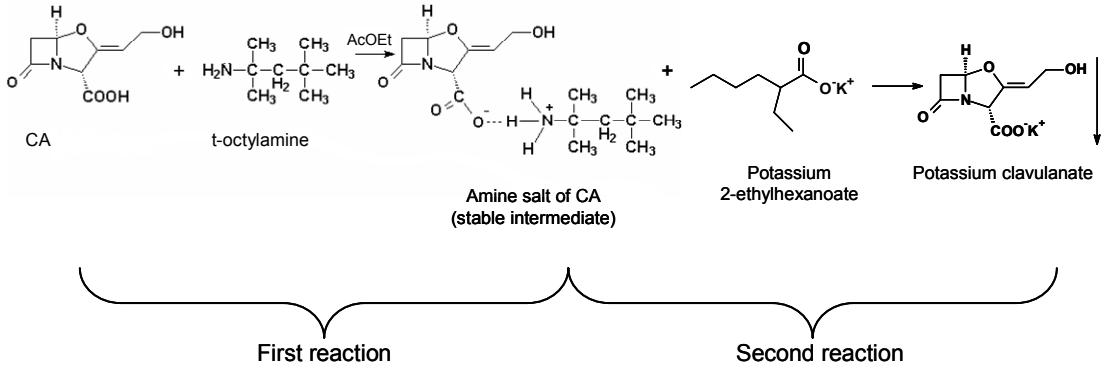


Figure 1: Precipitation of potassium clavulanate by passage through a stable intermediate.

In the second reaction, the CA amine salt was dissolved in 10 mL of isopropanol and 0.12 mL of water. The crystals of CA amine salts must be dissolved in isopropanol to begin the second reaction. However, salt crystals do not dissolve readily, thus requiring the addition of water. The volume of 10 mL was selected based on preliminary assays performed with different volumes to determine the most suitable volume for use in the jacketed reactor. The 0.12 mL of water used here corresponded to the minimum quantity that would allow the total dissolution of the highest concentration of amine salt of CA in isopropanol, defined by the CCRD. The amount of added water should be minimal so as not to interfere in the precipitation reaction for the formation of potassium clavulanate.

This solution was transferred to a jacketed glass reactor similar to the one used for the first reaction (with controlled temperature and stirring speed). Potassium 2-ethylhexanoate was added dropwise at a constant rate of 0.75 drop/min. The rate at which the reagent is added is undoubtedly one of the factors that affect the formation of precipitates (Söhnel and Garside, 1992). The rate of 0.75 drop/min was chosen based on a preliminary study of the direct precipitation reaction of AC with 2-ethyl hexanoate, for which a fractional factorial design was used in which the additional flows were variables to be studied. This preliminary study indicated that the best flow rate was 0.75 drop/min.

In the factorial design used here, after the addition of 2-ethylhexanoate salt was completed, the stock solution was left at the same temperature and under agitation for 45 minutes. This was the period corresponding to crystal growth, called the induction time, determined from preliminary assays.

The potassium clavulanate precipitate was filtered and washed with isopropanol and acetone. The resulting crystals were vacuum-dried for 24h at room temperature and then weighed. The crystal yield (Y) was also calculated by Equation (1).

For the CA fermentation broth, the 3kDa membrane permeate was extracted with ethyl acetate at pH 2.0 using sulfuric acid. The experimental procedure of the precipitation step was the same as that employed in the Clavulin® experiments.

Experimental Design

For the first reaction, five temperatures (6.6 to 23.4 °C), concentrations of clavulanic acid in organic solvent (6.6 to 23.4 mg/mL) and t-octylamine inflow rates (0.33 to 1.17 drop/min) were selected based on a central composite rotatable design (CCRD), as shown in Table 1 (Rodrigues and Iemma, 2005). The central point of the CCRD was performed in triplicate to estimate the error due to random experimental variability.

Because of the high rate of CA degradation at temperatures above 35 °C (Bersanetti *et al*., 2005), these were kept below 30 °C, thus limiting the temperature interval studied here.

For the second reaction, five temperatures (11.6 to 28.4 °C), concentrations of clavulanic acid amine salt in organic solvent (8.2 to 41.8 mg/mL) and concentrations of potassium 2-ethylhexanoate (0.2 to 1.2 molar) were also selected using CCRD, as shown in Table 2. The central point in the CCRD was performed in triplicate.

The results were analyzed using the Statistica version 5.1 software package (StatSoft).

Statistical Analyses

The results of the experiments were analyzed using Statistical Analysis System (SAS, 1996) software. A general second-order polynomial was used to correlate the yield of the precipitation reactions to the process variables.

The experimental factors and levels are also shown in Tables 1 and 2.

Table 1: Independent variables and their levels in the first precipitation reaction.

	Symbol	Levels				
Independent variables	**Coded**	**-1.682**	**-1**	**0**	**1**	**1.682**
Temperature (°C)	x_1	6.6	10	15	20	23.4
Concentration of CA (mg/mL)	x_2	6.6	10	15	20	23.4
T-octylamine inflow rate (drop/min) [a]	x_3	0.33	0.5	0.75	1.0	1.17

[a] This is equivalent to the addition of one drop at intervals of 140; 120; 90; 60 and 40 seconds, respectively.

Table 2: Independent variables and their levels in the second precipitation reaction.

Independent variables	Symbol Coded	Levels -1.682	-1	0	1	1.682
Temperature (°C)	z_1	11.6	15	20	25	28.4
Concentration of CA amine salt (mg/mL)	z_2	8.2	15	25	35	41.8
Concentration of potassium 2-ethylhexanoate (molar)	z_3	0.2	0.4	0.7	1.0	1.2

RESULTS AND DISCUSSION

Regression Models for Responses

Table 3 lists the values of the first experimental design used for the first reaction and the responses obtained for the yield of CA amine salt.

Equation (2) expresses the model with the coded values that represent the yield (Y) of clavulanic acid amine salt, which was produced using statistically significant parameters ($p < 0.1$). The terms that were not statistically significant were incorporated into the lack-of-fit to calculate the R-squared value. The coefficient of determination of 0.95 was considered excellent for this type of process.

Table 4 shows the ANOVA results for the CA amine salt yield, considering only the statistically significant terms. Based on the F-test, the model is more predictive ($p < 0.0001$) and higher than the listed F (2.51).

Figure 2 shows that the experimental points were distributed around the diagonal, indicating the model's excellent performance. The variation of the relative deviations is small (less than 10%), indicating the good fit of the model to the experimental points. Therefore, the coded model expressed by Equation (2) was used to generate the response surfaces (Figure 3) for yield of clavulanic acid amine salt.

$$\begin{aligned} &\text{CA amine salt yield}(\%) = \\ &71.03 - 1.73\,x_1 + 4.65(x_1)^2 \\ &-7.61\,x_2 + 4.34(x_2)^2 - 1.83(x_3)^2 \\ &+4.78\,x_1.x_3 + 2.86\,x_2.x_3 \end{aligned} \quad (2)$$

Table 3: Matrix of the experimental design used to investigate the influence of temperature, CA concentration and t-octylamine inflow rate on the yield of CA amine salt (%).

Runs	Independent variables			Response
	Temperature	CA concentration	T-octylamine inflow rate	CA amine salt yield (%)
	Coded Actual (°C)	Coded Actual (mg/mL)	Coded Actual (drop/min)	
1	-1 (10)	-1 (10)	-1 (0.5)	94,49
2	1 (20)	-1 (10)	-1 (0.5)	84.45
3	-1 (10)	1 (20)	-1 (0.5)	76.69
4	1 (20)	1 (20)	-1 (0.5)	59.07
5	-1 (10)	-1 (10)	1 (1)	81.32
6	1 (20)	-1 (10)	1 (1)	89.25
7	-1 (10)	1 (20)	1 (1)	73.81
8	1 (20)	1 (20)	1 (1)	76.43
9	-1.68 (6.6)	0 (15)	0 (0.75)	84.38
10	1.68 (23.4)	0 (15)	0 (0.75)	80.50
11	0 (15)	-1.68 (6.6)	0 (0.75)	93.57
12	0 (15)	1.68 (23.4)	0 (0.75)	69.52
13	0 (15)	0 (15)	-1.68 (0.33)	67.30
14	0 (15)	0 (15)	1.68 (1.17)	60.90
15	0 (15)	0 (15)	0 (0.75)	71.55
16	0 (15)	0 (15)	0 (0.75)	72.63
17	0 (15)	0 (15)	0 (0.75)	69.53

Table 4: ANOVA of CA amine salt yield as response.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F-test	p-value
Regression	1628.960	7	232.71	23.86	0.00004
Residual	87.770	9	9.75		
Lack of fit	82.845				
Pure error	4.926				
Total	1716.730	16			
R^2 =0.95% ; $F_{7;9;0.1}$=2.51.					

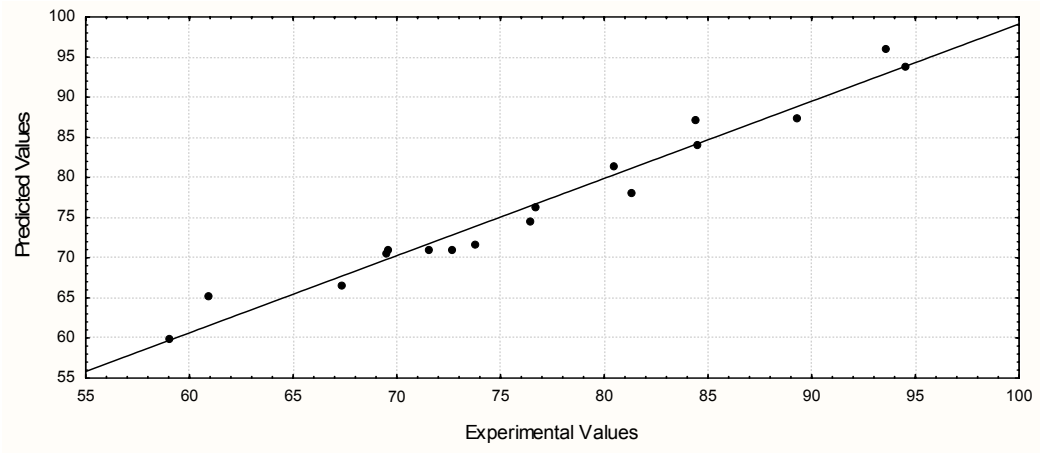


Figure 2: Experimental versus predicted yield (%) of clavulanic acid amine salt.

An analysis of the response surfaces and contour curves (Figure 3) indicated that the region of the highest yield of clavulanic acid salt is associated with the low CA concentrations studied here. The maximum values obtained for Y (%) were 94.49% and 93.57%, which corresponded to levels -1 and -1.68, i.e., concentrations of 10 and 6.6mg/mL, respectively.

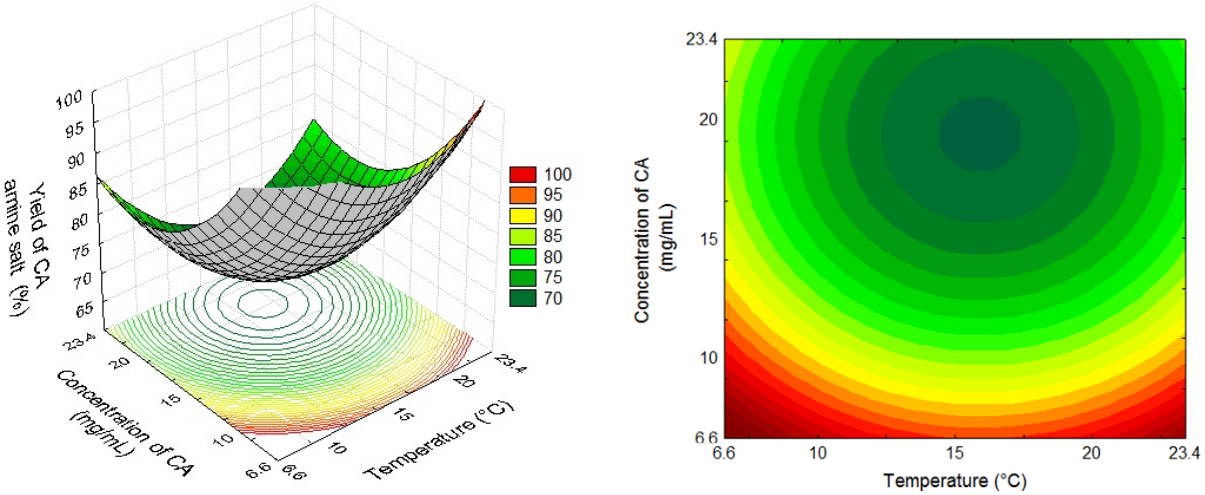
Although the inflow rate of t-octylamine proved statistically significant, its effect on yield was almost negligible when compared to the effect of the CA concentration. The t-octylamine inflow rate had a more marked effect when associated with temperature, i.e., in the lowest range of temperatures (6.6 to 10 ºC), the highest CA salt yield was obtained with an inflow of around 1 to -1.68 (1.0 to 0.33 drop/min) (Figure 3(b)). At high temperatures, a higher t-octylamine inflow rate (0.75 to 1.17 drop/min) was found to produce better results.

The factorial design applied here successfully optimized the first reaction, resulting in higher CA amine salt yields.

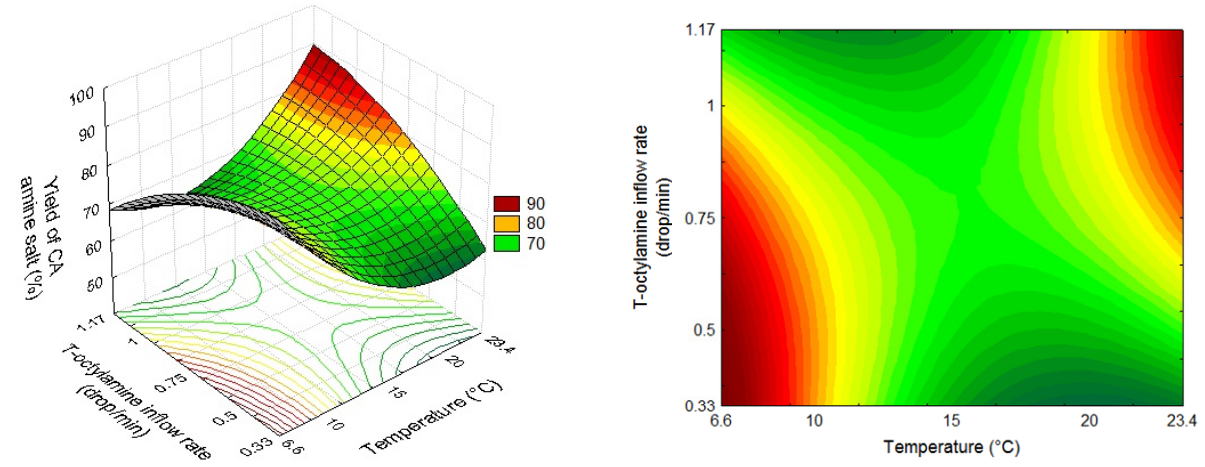
Moreover, no oil was formed in any of the experiments, demonstrating that the passage through the stable intermediate (amine salt) really increased the stability of the potassium clavulanate precipitation reaction in the range of conditions studied here.

In all these experiments, nucleation only started after the addition of at least half the amount of amine required for a theoretical conversion of 100%. This may render the reaction stable because it indicates that the initial supersaturation in this system was lower than in the reaction performed without passing through the stable intermediate, in which nucleation has most often occurred in response to the addition of the first drop of potassium 2-ethylhexanoate (Hirata *et al*., 2009). Thus it is believed that the introduction of t-octylamine as an intermediate increased the metastable zone of supersaturation in comparison to the direct reaction of CA with potassium 2-ethylhexanoate. This increase enabled the precipitation reaction to occur satisfactorily in a wider operating range, which can be considered an advantage in terms of industrial processing.

Furthermore, the CA amine salt formed was highly stable and non-hygroscopic, showing no change in its crystals even when left at room temperature for more than a month without controlled air humidity.



(a)



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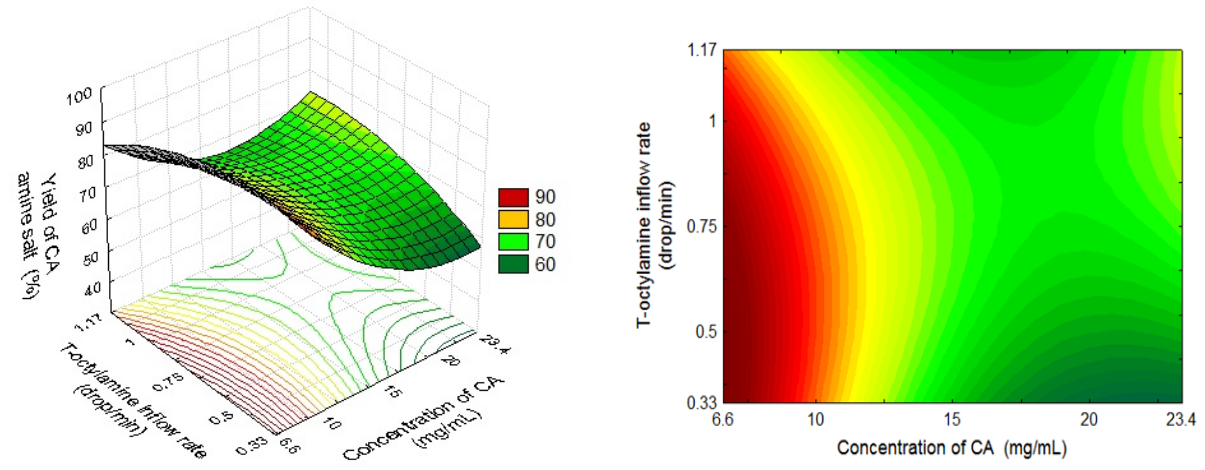


(c)

Figure 3: Response surfaces and contour diagrams of CA amine salt yield (%): (a) Concentration of CA and temperature. (b) t-octylamine inflow rate and temperature. (c) t-octylamine inflow rate and Concentration of CA.

The photomicrographs in Figure 4 were obtained by SEM (scanning electron microscopy), using 800X magnification.

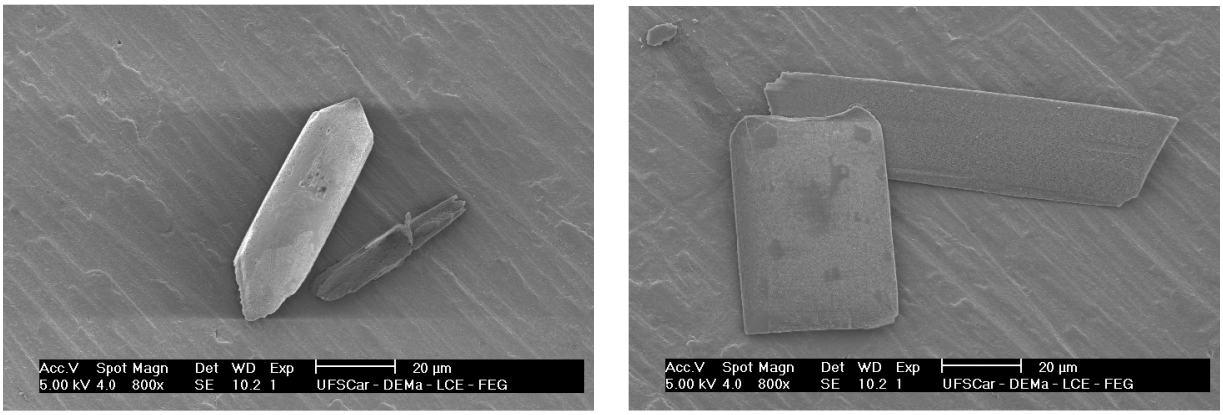
In Figure 4, note that the crystals of CA amine salt obtained in experiment 11 of the factorial design (CCRD) are well formed, with no sign of agglomeration.

Table 5 lists the coded values of the second experimental design and the real values (in parentheses) utilized for the second reaction, as well as the responses obtained for potassium clavulanate.

The regression coefficients of a second-order model using the coded variables were obtained from the results of the potassium clavulanate yield. The parameters with $p < 0.1$ were considered to be significant for the precipitation reaction under study. The statistically non-significant terms were incorporated into the lack-of-fit to calculate the R-squared value. The value of the F-test obtained for the regression (24.71) was highly significant ($p < 0.00001$).

Based on the ANOVA (Table 6), a second-order model (Equation (3)) was obtained to describe the potassium clavulanate yield using the coded variables.

$$\begin{aligned}&\text{Potassium clavulanate yield (\%)} = \\ &87.48 + 1.75\,(z_1) \\ &-3.02\,(z_1)^2 + 3.49\,(z_2) \\ &+2.39(z3)^2 + 1.29\,z_1.z_3\end{aligned} \tag{3}$$



(a) (b)

Figure 4: Photomicrographs of clavulanic acid amine salt obtained in experiment 11 using the BSE System with 800X magnification.

Table 5: Matrix of the experimental design used to investigate the influence of temperature, concentration of CA amine salt and potassium 2-ethyhexanoate concentration on the yield of potassium clavulanate (%).

Runs	Independent variables			Response
	Temperature	Concentration of CA amine salt	Potassium 2-ethylhexanoate concentration	Potassium clavulanate yield (%)
	Coded Actual (°C)	Coded Actual (mg/mL)	Coded Actual (molar)	
1	-1 (15)	-1 (15)	-1 (0.4)	83.68
2	1 (25)	-1 (15)	-1 (0.4)	85.33
3	-1 (15)	1 (35)	-1 (0.4)	87.80
4	1 (25)	1 (35)	-1 (0.4)	89.42
5	-1 (15)	-1 (15)	1 (1)	80.30
6	1 (25)	-1 (15)	1 (1)	87.23
7	-1 (15)	1 (35)	1 (1)	91.81
8	1 (25)	1 (35)	1 (1)	94.25
9	-1.68 (11.6)	0 (25)	0 (0.7)	74.70
10	1.68 (28.4)	0 (25)	0 (0.7)	81.42
11	0 (20)	-1.68 (8.2)	0 (0.7)	80.09
12	0 (20)	1.68 (41.8)	0 (0.7)	92.55
13	0 (20)	0 (25)	-1.68 (0.2)	91.99
14	0 (20)	0 (25)	1.68 (1.2)	94.72
15	0 (20)	0 (25)	0 (0.7)	85.16
16	0 (20)	0 (25)	0 (0.7)	89.40
17	0 (20)	0 (25)	0 (0.7)	88.72

Table 6: ANOVA of potassium clavulanate yield as response.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F test	p-value
Regression	457.76	5	91.55	24.71	< 0.00001
Residual	40.76	11	3.71		
Lack of fit	30.39				
Pure error	10.37				
Total	498.52	16			
$R^2 = 0.92\%$; $F_{5;11;0,1} = 2.45$					

Figure 5 presents the experimental versus predicted values, showing they are in good agreement.

The variation in relative deviations was lower than 5%, demonstrating that the differences between the experimental responses and those predicted by the model were minimal, indicating the model's excellent fit.

An analysis of the response surfaces and contour curves generated by the model (Eq. (3)) indicated that high concentrations of CA amine salt increase the yield of potassium clavulanate (Figure 6 (a)). The maximum potassium clavulanate yields were 94.72, 94.25 and 92.55%, which corresponded to levels 0, 1 and 1.68, i.e., concentrations of 25, 35 and 41.8 mg/mL, respectively.

A higher yield is associated with a range of temperatures between 15 and 25 °C, i.e., about 20 ºC. For a high concentration of CA amine salt, a higher concentration of potassium 2-ethylhexanoate is desirable. The maximum yield (94.72%) was obtained in experiment 14, in which the concentration of 2-ethylhexanoate was the highest (1.2 molar) (Figure 6 (c)).

Temperatures below 15 ºC combined with low concentrations of amine salt (less than 15mg/mL) decreased the yield. An analysis of Figure 6(b) indicated that the yield of the precipitation reaction was favored in a temperature range of 15-25 °C, reaching 91.99 and 94.72% at the most extreme levels of 2-ethylhexanoate (-1.68 and 1.68 for runs 13 and 14, respectively), which represent the lowest and highest concentrations of potassium 2-ethylhexanoate (0.2 and 1.2 molar, respectively).

From an analysis of the response surfaces it was found that the best temperature for the precipitation reaction under study was about 20 ºC. This temperature was maintained throughout the addition of potassium 2-ethyl (which varied in each experiment due to the variation of the initial concentration of CA amine salt), and also during the induction time of 45 minutes after completing the addition of potassium 2-ethylhexanoate. This information provided by the factorial design is very important because it means less energy spent (industrially) in maintaining a high final yield of potassium clavulanate. In their patent, Cook *et al*. (1984) stated that, for a similar reaction to obtain potassium clavulanate, after the addition of potassium 2-ethyl, the temperature of the stock solution was kept at 5 ºC for 1.5 h, resulting in a yield of 91.72%. Our experiment 14, which was performed at 20 ºC with an induction time of only 45 minutes and held at that temperature throughout the experiment, yielded 94.72% of potassium clavulanate, which was also the highest yield observed in the factorial design.

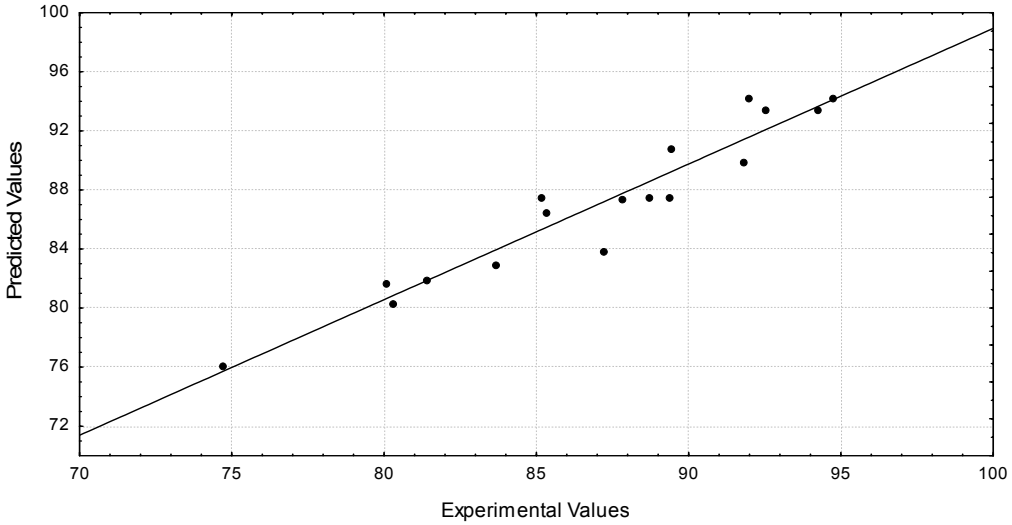
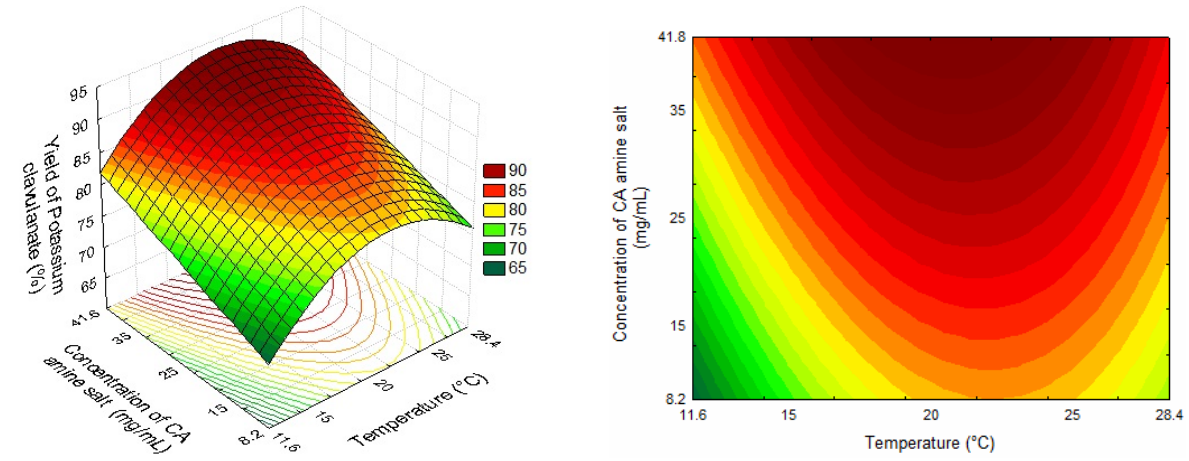
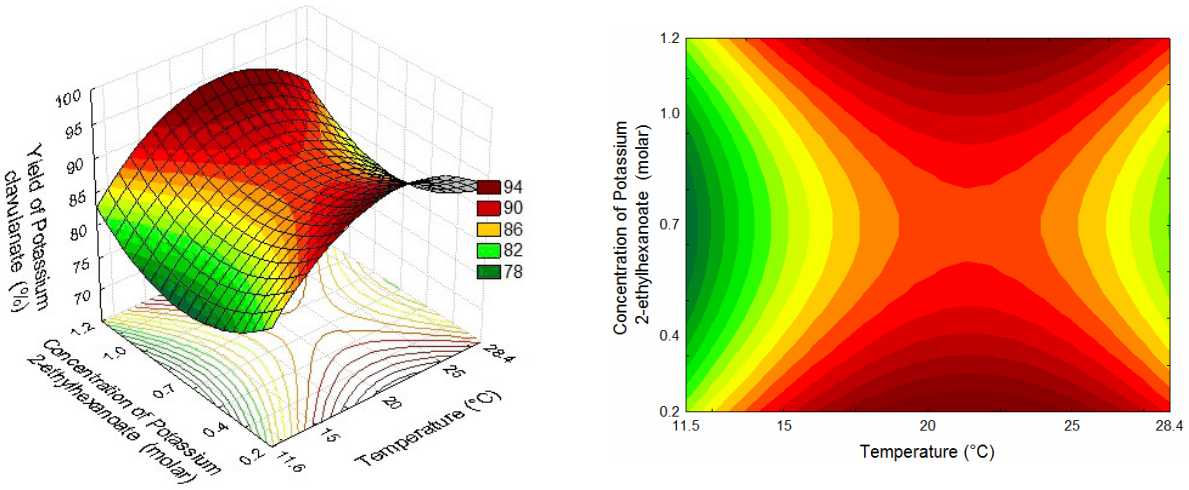


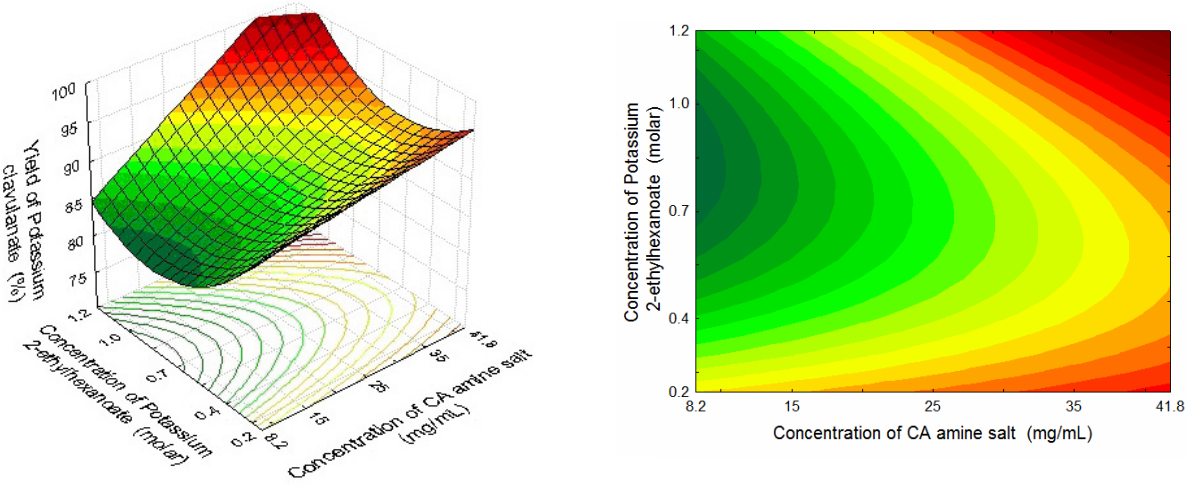
Figure 5: Experimental versus predicted yield (%) of potassium clavulanate



(a)



(b)

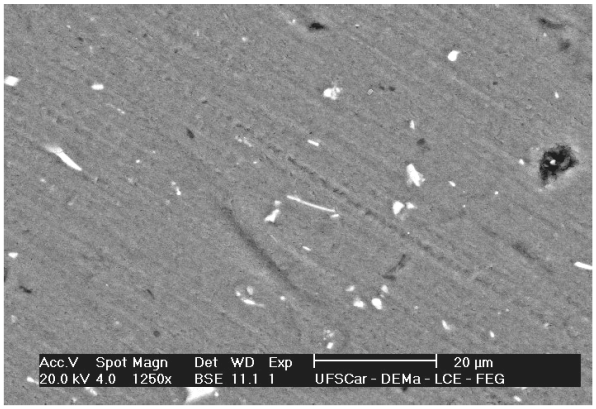


(c)

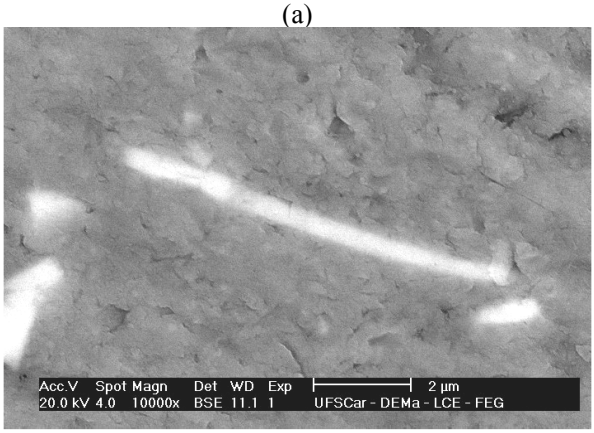
Figure 6: Response surfaces and contour diagrams for potassium clavulanate yield (%): (a) Concentration of CA amine salt (mg/mL) and Temperature. (b) Concentration of Potassium 2-ethylhexanoate (molar) and Temperature. (c) Concentration of Potassium 2-ethylhexanoate (molar) and Concentration of CA amine salt (mg/mL).

All the potassium clavulanate precipitation reactions resulted in a slightly yellow solid crystalline salt.

Figure 7 shows the SEM photomicrographs of the potassium clavulanate precipitate obtained in experiment 17 (central point).



(a)



(b)

Figure 7: Photomicrographs of potassium clavulanate crystals obtained in experiment 17 (CCRD) using the BSE System: (a) 1250X magnification, (b) 10000X magnification.

In this figure, note that the potassium clavulanate crystals obtained in experiment 17 are very small, thin and needle-shaped. Highly supersaturated solutions usually nucleate rapidly, producing numerous small crystals and resulting in the formation of needles or platelets. This was the case of the precipitation reactions of this study.

The experimental design also allowed for optimization of the second reaction, resulting in higher potassium clavulanate yields. Like in the first reaction, no oil was formed in any of the experiments, demonstrating that this reaction is more stable than the direct reaction of potassium clavulanate (Hirata *et al*., 2009). This stability can be attributed to the passage through the stable intermediate, t-octylamine. Nucleation in the second reaction did not occur in response to the addition of the first few drops of potassium 2-ethylhexanoate – as was the case in the reaction without passing through the stable intermediate (Hirata *et al*., 2009), but only after this addition within a range of 10 to 30 minutes, depending on the initial concentration of CA amine salt and the concentration of potassium 2-ethylhexanoate. This indicates that the solution had a lower initial supersaturation than that studied by Hirata *et al*. (2009), which favored the formation of potassium clavulanate. It is known that the problems commonly found in precipitation reactions (agglomeration, formation of colloidal solutions and crystal incrustations) are due to the very high initial supersaturation of these solutions (Mullin, 1993).

Fermentation Broth

After optimizing the first and second precipitation reactions of potassium clavulanate through the factorial designs, one experiment of each reaction was performed using the fermentation broth.

The same conditions as those used in experiment 11 for the first reaction were chosen, because that experiment presented one of the best responses for CA amine salt yield (93.57%) and lowest requirement of CA (6.6 mg/mL). Moreover, fermentation broths that produce a high concentration of CA are not easily obtained and the literature shows that many studies have focused on increasing its production. Therefore, a precipitation reaction that can produce high yields with a low initial concentration of CA is very advantageous, justifying the choice of this experiment to reproduce the conditions used in the factorial design for the fermentation broth.

The yield of CA amine salt obtained in this step was 80.21%. It is believed that other substances also present in the fermentation broth, such as pigments, may have interfered slightly in the precipitation reaction of the CA amine salt, but without precipitating along with it.

The signals that indicate the presence of CA hydrogen amine salt are described in Hirata *et al*. (2007) and were compared here with the signals observed in the ^{1}H-NMR spectrum (Figure 8), indicating that practically no substance other than the amine salt of clavulanic acid was present. Therefore, this intermediate salt may be used for the preparation of highly pure non-toxic clavulanic acid and its pharmaceutically acceptable salts. This demonstrates that the reaction of CA with t-octylamine is even more selective than the direct reaction of CA with potassium 2-ethylhexanoate (Hirata *et al*., 2009), promoting high purification of CA from the fermentation broth through the formation of CA amine salt.

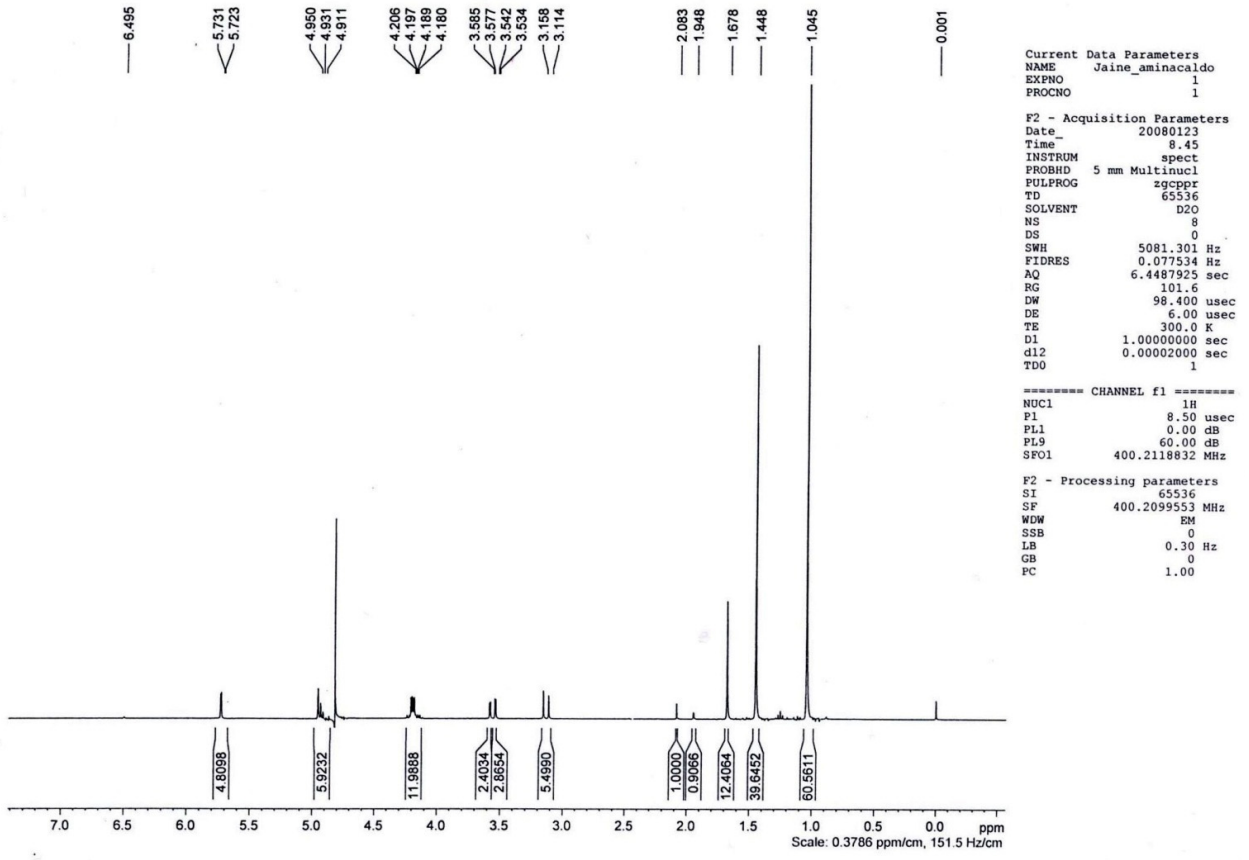


Figure 8: ^{1}H-NMR spectrum of clavulanic acid amine salt precipitated from the fermentation broth for CA reaction with t-octylamine, using the same conditions as those of experiment 11 (first factorial design).

The CA amine salt obtained in the first reaction from fermentation broth was used in the second reaction. The conditions used in the second reaction were chosen based on stability tests (not shown here), in which experiment 17 presented the best responses to the stability of the resulting crystals.

The yield obtained in this step was 72.37%. Figure 9 shows the ^{1}H-NMR spectrum of the precipitate (potassium clavulanate) formed in the second reaction from fermentation broth.

An analysis of the ^{1}H-NMR spectrum (Figure 9) confirmed the high purity of the precipitated potassium clavulanate. The signals that indicate the presence of potassium clavulanate are described in Hirata *et al*. (2009) and were compared here with the signals observed in the ^{1}H-NMR spectrum (Figure 9). No signal of aminoketone (one of its degradation products) (Finn *et al*., 1984) was detected, thus demonstrating that this reaction offers the advantage of yielding potassium clavulanate with no subsequent degradation, unlike other CA purification processes in which solvent or water is evaporated, leading to marked degradation of CA and therefore low yields.

The photomicrographs of potassium clavulanate crystals precipitated from the fermentation broth (second reaction) were obtained using the backscattered electron system with 1500X and 10000X magnifications (Figure 10). These photomicrographs reveal that the potassium clavulanate crystals precipitated from CA present in the fermentation broth were in the form of very small thin needles, but did not agglomerate.

The crystals were very similar to those obtained in experiment 17 (Figure 7), which was performed with the Clavulin® sample, thus confirming that the precipitation reaction from fermentation broth (performed in the same conditions as those of experiment 17) was very satisfactory. This reaction also showed no formation of oil or colloids, indicating that it is suitable for application on an industrial scale.

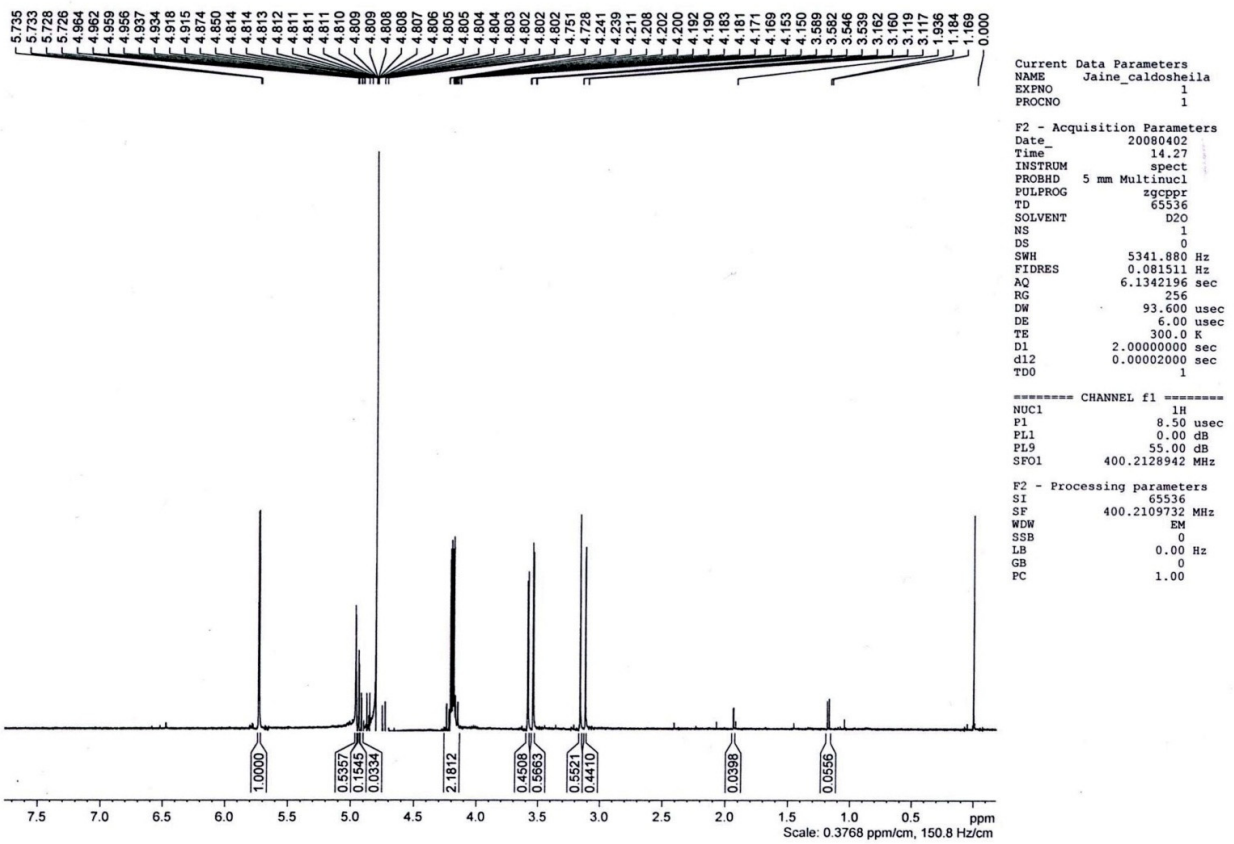
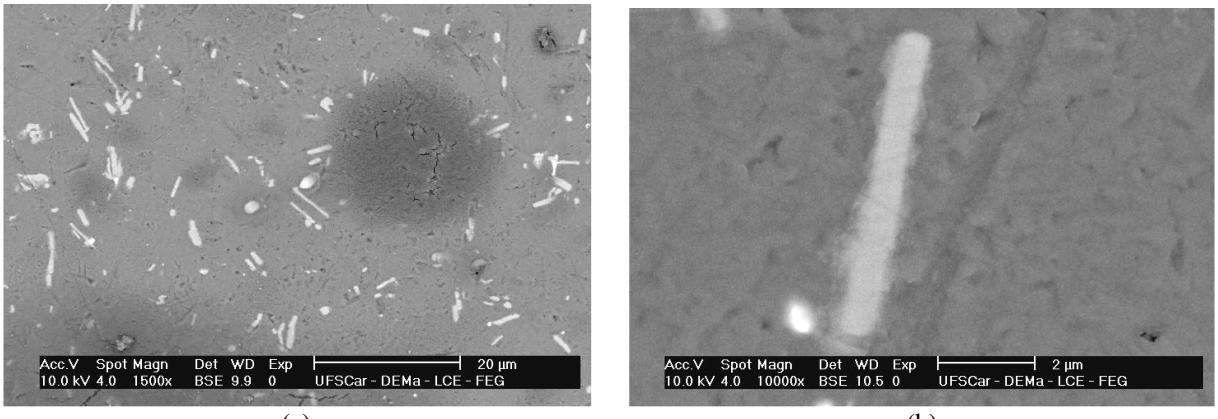


Figure 9: [1]H-NMR spectrum of potassium clavulanate precipitated from the fermentation broth for CA amine salt reaction with potassium 2-ethylhexanoate, using the same conditions as those of experiment 17 (second factorial design).



(a) (b)

Figure 10: Photomicrographs of potassium clavulanate crystals precipitated from fermentation broth obtained with the BSE System: (a) 1500X magnification; and (b) 10000X magnification.

The purification process described in the literature and traditionally used for purifying clavulanic acid is performed in a series of steps that usually involve the use of adsorption chromatography techniques. However, due to the instability of CA molecules, these techniques afford a very low CA recovery rate during purification (Butterworth 1984; Mayer *et al*., 1996; Barboza *et al*., 2002). In the present work, the use of precipitation reactions in place of the aforementioned chromatographic techniques was therefore very advantageous, since it resulted in higher yields of high purity potassium

clavulanate. Moreover, precipitation reactions imply lower energy costs because, at the end of the precipitation reaction, the crystals can be separated from the stock solution by vacuum filtration. In contrast, chromatographic purification involves lyophilizing the potassium clavulanate to obtain the end product – the salt, thus implying higher operational costs.

CONCLUSIONS

The use of factorial design and response surfaces proved to be very advantageous, allowing optimization of the reactions, which were then used to promote the purification of CA in the fermentation broth, producing a high yield (72.37%).

The results confirmed that the indirect reaction involving the passage through a stable intermediate (t-octylamine) is very suitable for use as the final step in the purification process of CA from fermentation broth. Its advantages over the other options described in the literature are that it promotes purification without causing the degradation of CA, increases the stability of the reaction without forming oils, incrustations or colloids, and allows for a broader operational range, thus favoring its use on an industrial scale.

The choice of t-octylamine proved suitable for commercial use since the intermediate formed was neither toxic nor hygroscopic. Moreover, the marked selectivity of the amine was essential to promote high purification of the CA in the fermentation broth.

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