

Artigo original

ESTERIFICATION OF ACETIC ACID WITH ALCOHOL ISOAMYL IN THE PRESENCE OF ENZYMATIC CATALYST

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Abstract: The *Candida antarctica* Lipase B immobilized on polyurethane catalysed esterification of acetic acid with isoamyl alcohol in mechanical and ultrasonic system in a system solvent-free, was studied. The maximum esterification of isoamyl acetate used immobilized catalyst was the highest esterification, 666.05 U/g with 19 cycles of reuse after 360 minutes of reaction time in mechanical system and 1319.48 U/g was observed and 14 cycles de reuse after 60 minutes of reaction time in ultrasound system. Thus, the process was considered efficient with significant reduction of the reaction time, low instrumental requirements and improve the bioprocess performance. Until now, there were no studies available in the open literature in relation to the ester synthesis catalyzed by immobilized Lipase in polyurethane as support in the ultrasound system. In addition, considered an environmentally correct and economically viable technology, it can be used in cosmetics, pharmaceuticals and food industry.

Keywords: *Candida antarctica* Lipase B. Éster synthesis. Isoamyl acetate. Biocatalysis.

Esterificação do ácido acético com álcool isoamílico na presença do catalisador enzimático

Resumo: A Lipase B da *Candida antarctica* imobilizada em esterificação catalisada por poliuretano de ácido acético com álcool isoamílico em sistema mecânico e ultrassônico em sistema isento de solventes foi estudada. A esterificação máxima do acetato de isoamila catalisador imobilizado utilizado foi a maior esterificação, 666,05 U/g com 19 ciclos de reuso após 360 minutos de tempo de reação no sistema mecânico e 1319,48 U/g foi observado, e 14 ciclos de reuso após 60 minutos de reação no sistema de ultrassom. Assim, o processo foi considerado eficiente com redução significativa do tempo de reação, baixos requisitos instrumentais e melhora no desempenho dos bioprocessos. Até o momento não havia estudos disponíveis na literatura aberta com relação à síntese de ésteres catalisada pela Lipase imobilizada em poliuretano como suporte no sistema de ultrassom. Além disso, considerada uma tecnologia ambientalmente correta e economicamente viável, pode ser usada em cosméticos, produtos farmacêuticos e indústria alimentícia.

Palavras-chave: *Candida antarctica* Lipase B. Síntese de éster. Acetato de isoamila. Biocatálise.

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1 INTRODUCTION

Short-chain esters are fragrance and flavours widely used in food, cosmetic and pharmaceutical industries. Among these esters, isoamyl acetate is one of the most employed in food industries (74.000 kg/year) because of its characteristic banana flavour. The demand for flavors and fragrances, including essential oils, other natural aromatic extracts and their blends is forecasted to increase 4.4% per year and \$26.5 billion in 2016.¹⁻⁴

According to Markets and Markets⁵ the food flavor market is projected to grow at a CAGR (Compound Annual Growing Rate) of 5.4% from 2015 to 2020. The production of this kind of compounds has been traditionally achieved by chemical synthesis, since extraction from plant materials or the production by fermentation is at present too expensive for commercial exploitation.

Natural flavors have attracted the attention of manufacturers of food ingredients due to the increasing consumer demand for fresh and natural products. With increasing orientation toward 'natural' production, employment of enzymes has been gaining importance. Thus, lipases have been widely investigated for ester synthesis.¹⁻³

Furthermore, conventional chemical synthesis leads to the formation of undesirable products for food and pharmaceutical industries. Therefore, biocatalyzed chemical synthesis has become attractive due to the high chemo-regio-and stereo-selectivity of the enzymes.^{6,7}

One of the cheap and commercially available nonmicrobial lipases is *Candida antarctica* Lipase B which has high thermostability, such as high selectivity and specificity, mild reaction conditions, wide pH range, activity in anhydrous reaction mixtures as demonstrated for esterification and transesterification reactions, allowing to obtain products with high purity, reduction of co-products and/or toxic waste, consequently reducing the environmental impact.⁷⁻¹⁰

To be economically viable and an efficient process, the biocatalyst immobilization is an alternative for the use of a free enzyme, mainly for its reuse and for the possibility of performing the continuous process.^{4, 11-13} Preliminary results demonstrated the potential use of polyurethane as support for CALB Lipase immobilization, increasing significantly the reaction yield and the enzyme thermal stability.¹⁴

The ultrasonic system is considered a green technology, little explored yet, is an alternative technology for the conventional mechanical agitation that provides significant reductions in the processing time and can increase esterification yields. These characteristics can be explained by the better mass transfer between substrates and enzyme.^{15,16}

In this context, the present study aims to maximize the esterification of isoamyl acetate using CALB immobilized in polyurethane, in mechanical and ultrasonic agitation in a solvent-free system, in addition to analyze the reusability in repeated cycles.

2 MATERIAL AND METHODS

2.1 MATERIALS

Lipase B de *Candida antarctica* – CALB (Novozyme NZL-102), acetic acid (Vetec, 99%), isoamyl alcohol (Merck, P.A) and dichloromethane (Vetec, 95%). Toluene diisocyanate (TDI) and polyether polyol were kindly donated by Manes Industry (Santa Catarina, Brazil).

2.2 ESTERIFICATION OF ISOAMYL ACETATE

The esterification of acetic acid and isoamyl alcohol to isoamyl acetate ester was carried out in triplicate (n=3) in 50 mL glass flask keeping constant mass catalyst in 0.5 g and mass substrate in 5 g. After each reaction, the reaction medium was filtered to separate the immobilized biocatalyst to the reactional medium. 500 μ L aliquots, performed in triplicate, were taken from the reaction mixture. 15 mL of acetone-ethanol solution was added in each sample. Titration with NaOH 0.05 mol L⁻¹ was the method used to determine the amount of acetic acid that have reacted until the system reach the pH 11. The blank samples were made by mixing 500 μ L of standard mixture and 15 mL of acetone-ethanol solution.

The immobilization methodology of Lipase CALB in polyurethane (PU) support was performed according to described by Nyari, Fernandes, Bustamante-Varga, Steffens, Zeni and Dallago.¹⁴ Enzyme activity unit was defined as the amount of catalyst that is able to convert 1 μ mol of fatty acid per minute, calculated by the Equation 1:

$$AE = \frac{(V_b - V_a) \times M \times 1000 \times V_f}{t \cdot M_{EL} \times V_c} \quad (1)$$

Where: Esterification ($U \text{ g}^{-1}$); V_a : Volume of NaOH consumed during the sample titration (mL); V_b : Volume of NaOH consumed during the blank sample titration (mL); M : Molarity of NaOH solution; V_f : Final Volume of the reaction medium; t : time (min); m : free mass or immobilized catalyst mass (g); V_c : Aliquot Volume of the reaction medium with drawal from the titration (mL).

2.3 MECHANICAL AND ULTRASONIC SYSTEMS

The kinetic study was conducted to evaluate the effect of the reaction time (0 to 400 min) in terms of esterification to isoamyl acetate. For the system with mechanical agitation, the variables studied were molar ratio acetic acid and isoamyl alcohol (1:4.18-1:9.82) and reaction temperature (35.9-64.1 °C), keeping mechanical stirring at 160 rpm and reaction time in 400 min. For the ultrasonic system, the variables studied were molar ratio acetic acid and isoamyl alcohol (1:3.64-1:10.36) and reaction temperature (24.8-75.2 °C) and ultrasonic power (26.4-93.6%), relative to maximum power 1800 A, US 40 KHz, US 132 W), the reaction time was fixed in 60 min.

2.4 OPERATIONAL STABILITY

The study of the operating cycles number for the immobilized catalyst used in the isoamyl acetate synthesis was evaluated using optimized condition from DCCR. After each reaction, the catalyst was filtered to remove the reaction medium and reused in a new reaction. This process was successively repeated until esterification less than 50% of initial activity esterification. The results were expressed in terms of esterification, considering the initial esterification to 100%.

3 RESULTS AND DISCUSSION

3.1 MECHANICAL SYSTEM

Figure 1 shows the evolution of isoamyl acetate esterification of acetic acid and isoamyl alcohol (400 minutes), for the free and immobilized catalyst according to the condition designed by the full DCCR 2² (Table 1).

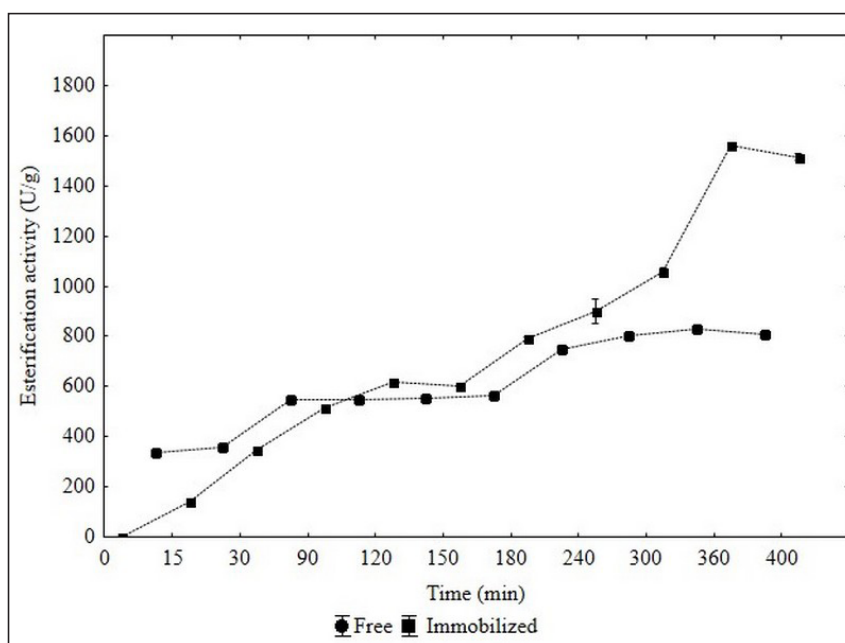


Figure 1 – Kinetics of isoamyl acetate esterification for mechanical system immobilized and free catalyst

From the results, it was observed the maximal esterification of 826.43 U/g and 1571.12 U/g, for the catalyst free and immobilized, respectively after 40 minutes of reaction time. Moreover, both assays show the same trend, increasing the acid precursor (esterification) esterification, besides the ester, water up to a certain time reaction. Followed by the reduction (hydrolysis), increasing the water concentration in the reaction system, through ester consumption, bound to lipases.¹⁷

In a general context, it is noted that in an esterification process were the reaction is reversible, it is extremely important to optimize reaction times, since it interferes significantly in the energy costs and in the economic feasibility of the process.¹⁸ Table 1 shows the full

DCCR 2² for the esterification of acetic acid and isoamyl alcohol, as a function of the studied variables, molar ratio acid alcohol and temperature (°C).

Table 1 – Matrix of full 2 DCCR experimental design (real and coded values) using a free and immobilized catalyst in terms of isoamyl acetate esterification by mechanical system

| Run | Temperature (°C) | Molar ratio acid alcohol | Free | Immobilized |
|-----|------------------|--------------------------|----------------|----------------|
| 1 | -1 (40) | -1 (1:5) | 196.71 ± 33.01 | 238.97 ± 38.21 |
| 2 | 1 (60) | -1 (1:5) | 284.38 ± 24.35 | 249.01 ± 41.99 |
| 3 | -1 (40) | 1 (1:9) | 275.88 ± 16.56 | 271.62 ± 19.22 |
| 4 | 1 (60) | 1 (1:9) | 305.27 ± 20.88 | 426.26 ± 23.88 |
| 5 | -1.41 (35.9) | 0 (1:7) | 553.54 ± 11.56 | 259.40 ± 28.76 |
| 6 | 1.41 (64.1) | 0 (1:7) | 505.66 ± 22.09 | 819.10 ± 21.87 |
| 7 | 0 (50) | -1,41 (1:4.18) | 167.43 ± 21.55 | 100.12 ± 25.98 |
| 8 | 0 (50) | 1,41 (1:9.82) | 194.26 ± 25.33 | 814.47 ± 32.55 |
| 9 | 0 (50) | 0 (1:7) | 471.84 ± 10.90 | 661.87 ± 35.77 |
| 10 | 0 (50) | 0 (1:7) | 464.53 ± 11.61 | 660.97 ± 36.65 |
| 11 | 0 (50) | 0 (1:7) | 466.25 ± 10.24 | 666.05 ± 37.99 |

Source: Romero, Calvo, Alba, Daneshfar and Ghaziaskar.²

Note: Fixed parameters: substrate mass 5 g, catalyst mass 0.5 g, reaction time 360 min and 160 rpm of mechanical agitation.

According to the results, the maximum esterification in terms of oleic acid was obtained in the assay 5 (553.544 U/g) to free and assay 9, 10 and 11 (666.05 U/g) to immobilized catalyst after 360 minutes of reaction time.

From the assays 1 and 2, 3 and 4, it was observed that esterification was directly proportional to the reaction temperature and molar ratio in the assays 1 and 3, 2 and 4, 7 and 8, indicating a positive effect of the temperature and molar ratio (free or immobilized) in the acetic acid esterification.

This positive effect of temperature is consistent with the endothermic ($\Delta H +$) nature of the esterification reactions, which is characterized by the reversibility, that is, it presents a chemical equilibrium, indicates that it occurs with heat absorption, and that the increase of temperature provides an equilibrium in the reaction system, shifting the reaction for the products side, increasing reaction yield.

Another variable of extreme importance, combined with temperature was the molar ratio acid and alcohol, according to the Table 1, positive effect of concentration molar ratio acetic acid: isoamyl alcohol can be linked to two factors: displacement of the chemical

equilibrium and reduction of the medium acidity. It is a reversible reaction (esterification), where the excess of alcohol (nucleophile/acyl receptor) leads to high conversion levels due to the excess of nucleophile to substrate transfer. Also positively affects the conversion by equilibrium displacement to the products, besides the isoamyl alcohol being of branched structure, being able to hinder the micelles formation around the immobilized enzyme, thus facilitating the product solubility and the mass transfer in the reaction system.¹⁸

In general, all tests using the immobilized catalyst showed higher esterifications of acetic acid in relation to the free catalyst, which is the main advantage through an efficient immobilization method, as presented in our study, besides the possibility of reuse and reduction of inactivation by distortion of its native structure by the influence of temperature, pH and solvents.

According to Nyari, Fernandes, Bustamante-Varga, Steffens, Zeni and Dallago.¹⁴ This performance involves factors such as: any enzyme added in the immobilization process is adhered to the support, there is no leaching caused by the reaction medium and the interaction of the support material with the active center of the enzyme, leading to the opening of the hydrophobic lid and leaving the exposed site, Providing an increase in the activity/esterification of the esterification reaction. According to Orellana-Coca, Törnvall, Adlercreutz, Mattiasson, Hatti-Kaul¹⁹ and Colombo, Mazutti, Di Luccio, De Oliveira, Olivera,²⁰ an excess in the catalyst content is necessary to keep the enzyme activity during the reaction time.

Equations 2 and 3 presents the second-order coded model, which describes the isoamyl acetate esterification as a function of the independent variables (factors) analyzed (molar ratio acetic acid:isoamyl alcohol and temperature) within the studied range. The correlation coefficient obtained (0.94) with $F_{cal} (8.93) > F_{tab} (4.01)$ and $F_{cal} (2.20) > F_{tab} (5.05)$ allowed the construction of the contour plot presented in Figure 2 for free and immobilized catalyst.

$$\text{Esterification activity (U/g)} = 461.35 + 8.60R^2 + 19.56T - 166.68T^2 - 14.50R.T \quad (2)$$

$$\text{Esterification activity (U/g)} = 663.12 + 124.79R - 113.55R^2 + 147.03T - 154.04T^2 - 36.00R.T \quad (3)$$

Where: R = Molar ratio acid:alcohol and T = temperature (°C).

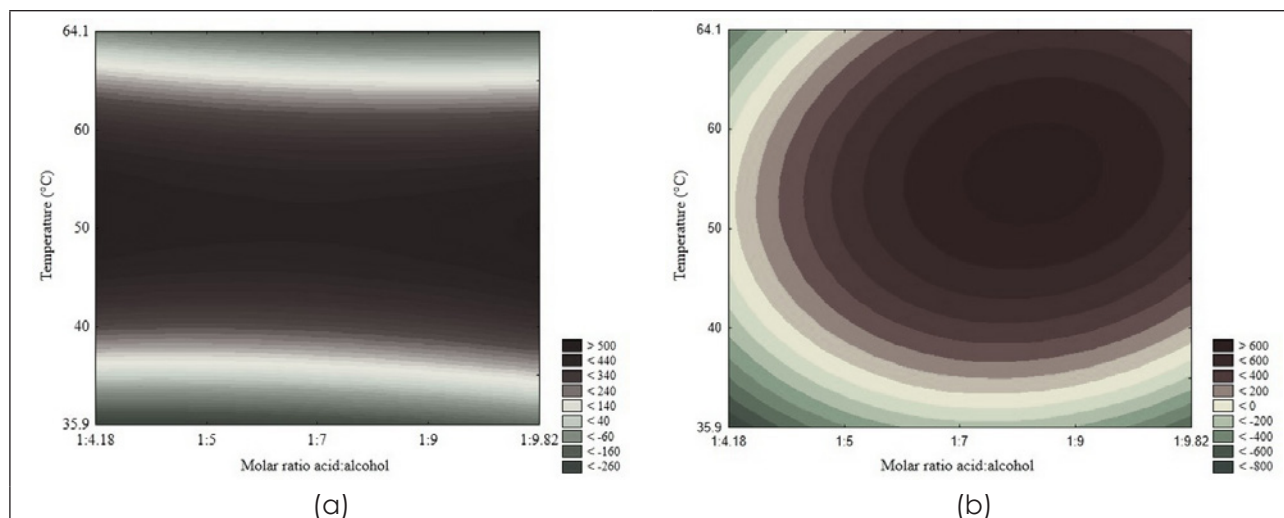


Figure 2 – Contour Curve using a free (a) and immobilized (b) catalyst in terms of isoamyl acetate esterification by mechanical system

Figure 2 shows the contour plot for the interactions between the variables: molar ratio acid:alcohol and temperature for the batch reaction under the mechanical stirring. It was observed that the higher esterifications for the isoamyl acetate were achieved in the temperature range of 50-60 °C and molar ratio acid:alcohol 1:7-1:9 (central point condition) to immobilized catalyst and temperature range 40-60 °C to free catalyst. In this sense for the ultrasonic system, only the immobilized catalyst was studied, due to the better results in terms of esterification of isoamyl acetate.

3.2 ULTRASONIC SYSTEM

Figure 3 shows the evolution of isoamyl acetate esterification of acetic acid and isoamyl alcohol (400 minutes), for the catalyst free and immobilized according to the condition designed by the full DCCR 2³ (Table 2).

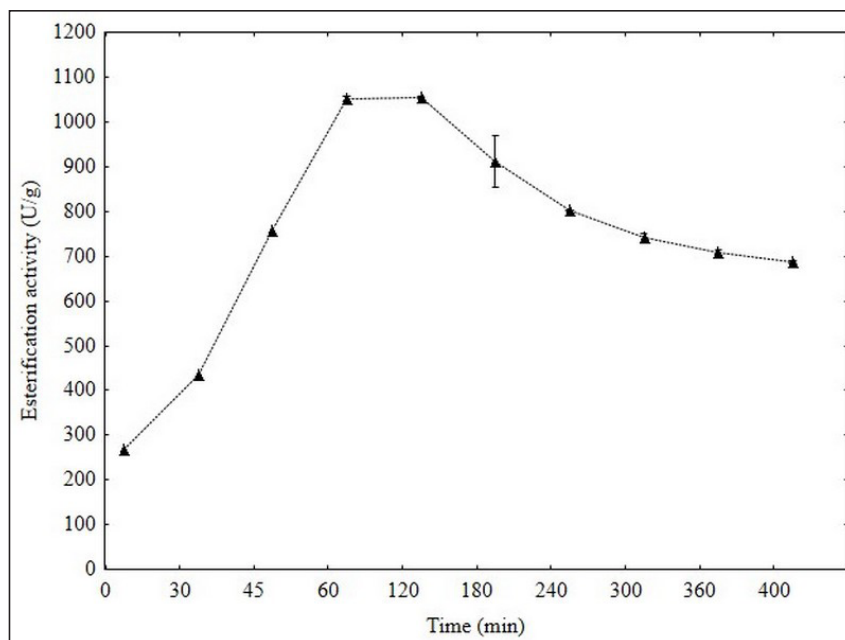


Figure 3 – Kinetics of isoamyl acetate esterification for ultrasonic system

From the results, it was observed the maximal esterification of 1058.78 U/g after 60 minutes of reaction time. From this results, it was possible to relate the initial reaction velocity with the agitation type. The reduction in the reaction time can be linked to the increase in the reaction rate, which can be obtained using ultrasonic agitation system, mainly due the formation of microscopic droplets in the system, increasing the interfacial area by increasing surface contact reducing mass transference limitations between substrate and catalyst.^{20,21}

Table 2 shows the full DCCR 2³ for the synthesis of isoamyl acetate ester in ultrasonic system, as a function of the studied variables, temperature (°C), molar ratio acid:alcohol and ultrasonic power (%).

Table 2 – Matrix of full 2 DCCR experimental design (real and coded values) in terms of isoamyl acetate esterification by ultrasonic system

| Run | Temperature (°C) | Molar ratio (acid:alcohol) | Ultrasonic power (%) | Esterification (U/g) |
|-----|------------------|----------------------------|----------------------|----------------------|
| 1 | -1 (35) | -1 (1:5) | -1 (40) | 238.38 ± 178.21 |
| 2 | -1 (35) | -1 (1:5) | 1 (80) | 229,16 ± 120.62 |
| 3 | -1 (35) | 1 (1:9) | -1 (40) | 265.23 ± 146.13 |
| 4 | -1 (35) | 1 (1:9) | 1 (80) | 351.38 ± 198.51 |
| 5 | 1 (65) | -1 (1:5) | -1 (40) | 752.18 ± 11.54 |
| 6 | 1 (65) | -1 (1:5) | 1 (80) | 758.49 ± 2.47 |

| Run | Temperature (°C) | Molar ratio (acid:alcohol) | Ultrasonic power (%) | Esterification (U/g) |
|-----|------------------|----------------------------|----------------------|----------------------|
| 7 | 1 (65) | 1 (1:9) | -1 (40) | 665.07 ± 13.59 |
| 8 | 1 (65) | 1 (1:9) | 1 (80) | 771.99 ± 22.13 |
| 9 | - 1.68 (24.8) | 0 (1:7) | 0 (60) | 156.03 ± 12.81 |
| 10 | 1.68 (75.2) | 0 (1:7) | 0 (60) | 999.90 ± 17.00 |
| 11 | 0 (50) | - 1.68 (1:3.64) | 0 (60) | 144.18 ± 12.65 |
| 12 | 0 (50) | 1.68 (1:10.36) | 0 (60) | 603.25 ± 12.30 |
| 13 | 0 (50) | 0 (1:7) | - 1.68 (26.4) | 175.06 ± 14.89 |
| 14 | 0 (50) | 0 (1:7) | 1.68 (93.6) | 825.49 ± 23.19 |
| 15 | 0 (50) | 0 (1:7) | 0 (60) | 1319.48 ± 21.58 |
| 16 | 0 (50) | 0 (1:7) | 0 (60) | 1315.84 ± 20.62 |
| 17 | 0 (50) | 0 (1:7) | 0 (60) | 1316.14 ± 27.48 |

Source: World Flavors & Fragrances: Industry Study with Forecasts for 2016 & 2021.³

Note: Fixed parameters: substrate mass 5 g, catalyst mass 0.5 g and reaction time 60 min.

The highest esterification, 1319.48 U/g was observed in the assay 15, 16 and 17 (central point – 50 °C, acid:alcohol 1:7 and ultrasonic power of 60%). In general, as in the study conducted with mechanical agitation, the variables evaluated, when analyzed independently, had a positive effect.

The increase in temperature (assays 1 and 5, 2 and 6, 3 and 7, 4 and 8, 9 and 10), molar ratio (assays 1 and 3, 2 and 4, 5 and 7, 6 and 8, 11 and 12) and ultrasonic power (assays 3 and 4, 5 and 6, 7 and 8, 13 and 14), they were favorable for increasing the synthesis of esters.

This positive effect of temperature is consistent with the endothermic ($\Delta H +$) nature of the esterification reactions, which is characterized by the reversibility, that is, it presents a chemical equilibrium, indicates that it occurs with heat absorption, and that the increase of temperature provides an equilibrium in the reaction system, shifting the reaction for the products side, increasing reaction yield. Apart from of the reduction in the system viscosity, reducing the mass transfer limitation.

This positive effect of concentration molar ratio acetic acid: isoamyl alcohol can be linked to two factors: displacement of the chemical equilibrium and reduction of the medium acidity.²² It is a reversible reaction (esterification), where the excess of alcohol (nucleophile/ acyl receptor) leads to high conversion levels due to the excess of nucleophile to substrate transfer. Also positively affects the conversion by equilibrium displacement to the products,

besides the isoamyl alcohol being of branched structure, being able to hinder the micelles formation around the immobilized enzyme, thus facilitating the product solubility and the mass transfer in the reaction system.¹⁸

In relation to ultrasonics power, was observed from the results that increasing ultrasonics power was possible to increase isoamyl acetate esterification. Choudhury, Malani and Moholkar¹³ and Khan and Rathod²¹ relate the higher esterification due to the cavitation bubbles increasing the solubility of the molecule consequently increasing the reaction rate, and providing a low energy use.^{22,23}

Equation 4 presents the second-order coded model, which describes the isoamyl acetate esterification as a function of the independent variables (factors) analyzed (molar ratio acid:alcohol), temperature and ultrasonic power) within the studied range. The correlation coefficient obtained (0.94) with $F_{cal} (3.34) > F_{tab} (5.05)$ allowed the construction of the contour plot presented in Figure 4.

$$\text{Esterification activity (U/g)} = 1189.05 + 254.47T - 212.45T^2 + 60.63R - 315.06R^2 + 92.64P - 251.43P^2 - 28.37T.R + 3.87T.P + 24.87T.P \quad (4)$$

Where: T = temperature (°C) R = molar ratio acid:alcohol, and ultrasonic power (%).

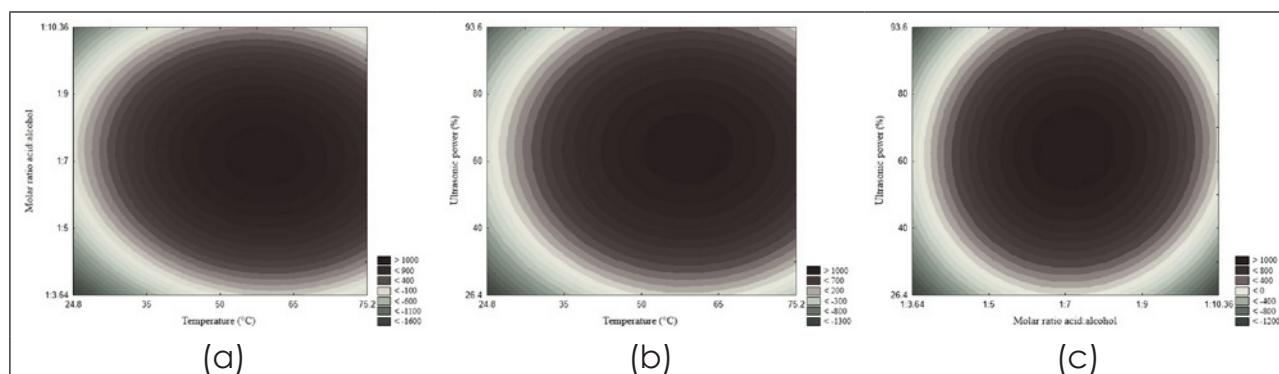


Figure 4 – Contour Curve in terms of isoamyl acetate esterification by ultrasonic system

The highest esterification for the synthesis of isoamyl acetate esters were achieved in the region corresponding to high temperature (45-75 °C), molar ratio acid:alcohol (1:6-

8.5) and ultrasonic power (50-80%). The literature reports several concerns regarding the application of the ultrasonic system as a tool in the reactions of ester synthesis.

Through the results obtained in this study, it was shown that by using ultrasonic system it was possible to obtain isoamyl acetate esterification in a relatively short reaction time (60 minutes), especially when compared to the mechanical agitation, presenting as an alternative process, simple, and promising to improve reaction conditions and esterification yield.

3.3 OPERATIONAL STABILITY

Figure 5 shows the operational stability (number of reuse cycles) for the esterification of isoamyl acetate (mechanical agitation and ultrasonic system). Considering 100% as residual activity with 4 cycles in mechanical agitation.

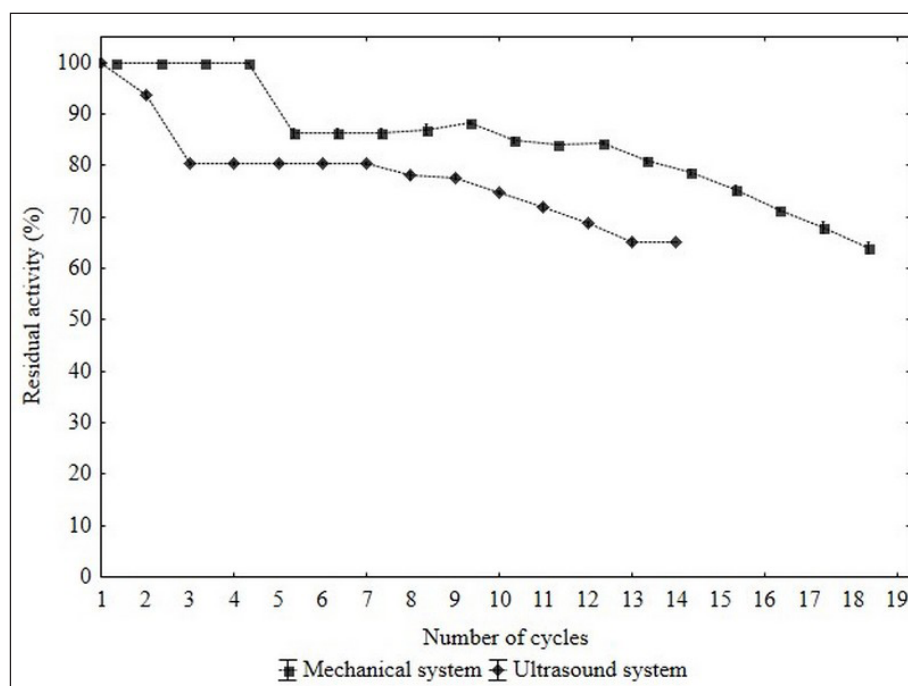


Figure 5 – Operational stability for the esterification of isoamyl acetate using mechanical system (360 minutes) and ultrasound system (60 minutes)

In a more extensive analysis, it was observed that the mechanical system showed 19 cycles with residual activity higher than 60% of residual activity and 14 cycles higher than 50% residual activity in ultrasound system. The observed reduction in the esterification during

the reuse cycles may be related to the biocatalyst loss of mass between cycles, by the leaching of the enzyme from the support and by the catalyst denaturation.²⁴⁻²⁶

Poppe, Fernandez-Lafuente, Rodrigues and Ayub²⁷ using mechanical agitation for the synthesis of methyl esters using immobilized Novozym 435 obtained 8 cycles of reuse keeping 70% of the initial, Khan, Jadhav and Rathod²⁸ in the synthesis of cetyl oleate ester by an ultrasonic-assisted system and mechanical agitation using commercial lipase *Candida antarctica* Lipase B CALB™ 10000, obtained only one reuse cycle with 80% of initial enzyme activity for both systems, Adewale, Dumont and Ngadi²⁹ reported the transesterification of biodiesel by an ultrasonic-assisted system using *Candida antarctica* B Lipase (CALB), the authors reported 3 and 5 cycles of reuse keeping 40% of the initial enzyme activity and Michelin, Penha, Sychoski, Scherer, Treichel, Valério, *et al.*³⁰ in the synthesis of ethyl esters using immobilized lipase (Novozym 435) reported the possibility of 5 cycles of reuse keeping enzyme activity up to 50% of the initial activity.

4 FINAL CONSIDERATIONS

The operating mechanical system the maximum esterification of isoamyl acetate used immobilized catalyst was 666.05 U/g with 19 cycles of reuse after 360 minutes of reaction time in mechanical system and 1319.48 U/g was observed and 14 cycles de reuse after 60 minutes of reaction time in ultrasound system used *Candida antarctica* Lipase B immobilized on polyurethane. Thus, the process was considered efficient with significant reduction of the reaction time, low instrumental requirements and improve the bioprocess performance. Until now, there were no studies available in the open literature in relation to the ester synthesis catalyzed by immobilized lipase in polyurethane as support in the ultrasound system. Thus, the results obtained in this work are promising in relation to the results observed in the literature for different lipases immobilized on different supports and applied in the synthesis of different esters.

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