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Production of Ethyl Valerate from *Burkholderia cepacia* Lipase Immobilized in Alginate

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Ethyl valerate (green apple flavor) production was investigated using immobilized *Burkhoderia cepacia* lipase by esterification of valeric acid with ethyl alcohol in a solvent system. Since the mechanisms of obtaining flavor esters using *Bukholderia cepacia* lipase are not well known, the synthesis of esters were studied in systems that contain organic solvent. The choice of the solvent was based on optimum result in the studies of lipase activity and stability in the presence of different solvents: alcohol, chloroform, toluene, hexane and heptane. In this case, the solvent which maintained increased activity and stability in the immobilized enzyme was heptane. For the experiments, the enzymatic extract was lyophilized and immobilized in solution of sodium alginate. The effect of acid to alcohol molar rate (0.1, 0.3 and 0.5 M) and the concentration of lipase (10, 20 and 30 % w/v) on the response esterification reaction were detected on the response of ester production yield. Aliquots were taken 0, 3, 6, 9, 12, 24, 48, 72 and 120 h and titrated with 0.02 M KOH and phenolphthalein used as indicator. The best result for obtaining ethyl valerate with yields almost 90 % was in 0.5 M and 20 % w/v.

1. Introduction

Esters of carboxylic acids are important components of natural flavors used in several industrial applications. Low molecular mass ester is responsible for the aroma of fruits and mainly composed of short-chain fatty acid derivatives such as acetate, propionate and butyrate. The worldwide market for natural "green notes" is estimated to be 5-10 mts (Chiang et al. 2003). The ethyl valerate, with typical apple green fragrance compound is widely used in the food, cosmetics and pharmaceutical industries (Karra-Châabouni et al., 2006).

Most available flavour compounds are now produced via chemical synthesis or extraction. Drawbacks of such chemical processes are the formation of undesirable racemic mixtures and the growing aversion of the consumer towards chemicals added to his food, cosmetics and other household products. This has caused flavour companies to direct their attention towards flavour compounds of biological origin, so called natural or bio-flavours (Kempler, 1983). Up to now, plants were also an important source of essential oils and flavours: however, active components are often present in minor quantities or in bound form or are only found in exotic plants, making isolation difficult and the flavour products expensive. Apart from plant cell and tissue culture techniques (which still need further development) a directly viable alternative route for flavour synthesis is based on microbial processes. The microbial processes can offer an alternative method for production of natural flavor as present possibilities for rapid production of compounds, and offer a simple system that is involved in the formation of volatile compounds (Janssens et al., 1992).

Studies have demonstrated obtaining esters synthesized in high yields by using lipases of microbial origin (Aragão et al. 2009). The esters produced by enzymatic synthesis are predominantly obtained in organic media with low maintaining the active conformation and stability of the enzyme (Pires Cabral et al., 2009). Furthermore, the production of esters from microbial lipases source has gained importance because it is considered a natural product, and the processes are not affected by unwanted by products and guarantees a desired specificity for obtaining flavor (Romero et al., 2007).

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The use of lipase in immobilized form ensures that the enzyme remains stable in the organic medium, facilitating its subsequent recovery and reuse. Thus, there is still a great field to be explored, related to the enzymatic synthesis of esters in systems employing low cost materials as support, generating results to verify the technical feasibility of producing these compounds. The purpose of this study was to evaluate the effect of the crude enzyme extract immobilized in alginate gel in esterification reactions to produce ethyl valerate; using as parameters molar ratio acid to alcohol and the amount of immobilized lipase in the ethyl valerate synthesis. The tests were carried out with the immobilized enzyme in the presence of organic solvents in order to test the behaviour for subsequent production of ester. Although the lipase from *Burkholderia cepacia* may be used in esterification reactions for biodiesel production, little is known about the behaviour in immobilized form in dried alginate gel for obtaining esters.

2. Material and Methods

2.1 Material

The strain of *Burkholderia cepacia* was obtained from the André Tosello Foundation (Brazil) and kept in test tubes at 4 °C on nutrient agar. Acetone, ethyl alcohol, chloroform, toluene, heptane, hexane, monobasic sodium phosphate, dibasic sodium phosphate, monobasic potassium phosphate, dibasic potassium phosphate, magnesium sulfate, sodium and phosphate hydroxide and calcium chloride were obtained from Synth (Diadema, SP, Brazil). Sodium alginate and valeric acid were provided by Sigma-Aldrich (São Paulo, Brazil). Gum Arabic, agar-agar, yeast extract and bacteriological peptone were obtained from Oxoid (London, UK). Soybean oil (Liza) and olive oil (Gallo) were obtained from the local market, with a maximal acidity of 0.3 % and 0.5 %, respectively.

2.2 Methods

Preparation of inoculate and fermentation system: *Burkhoderia cepacia* was grown in medium containing yeast extract (3 g/L), peptone (3 g/L), K_2PO_4 (4 g/L), $MgSO_4$ (0.2 g/L) and 3 % soybean oil, liquid fermentation in bioreactor Bioflow III. The fermentations were performed at 150 rpm and 30 ºC. After 96 h, the supernatant was separated from the cells by centrifugation and it was used as enzyme extract, since the *Burkholderia cepacia* produces extracellular lipase (Kordel et al., 1991)

Enzymes assays: Substratum solution was made by mixing 75 mL of olive oil and 25 mL of a 7 % (w/v) arabic-gum emulsion, according to Macedo et al. (1997). An amount of 5 mL of substratum solution was added to 2 mL of 0.1 M phosphate buffer at pH 8.0 and 1 mL of enzyme solution. A control sample without enzyme was also made. After 30 min at 37 °C the acidity was measured by tittering using 0.05 M of NaOH solution. Phenolphthalein was used as indicator. One unit of enzyme activity was defined as µmol of acid released per min under the assay conditions.

Production of microcapsules by gelation: From Padilha's studies (2012) the polymeric solution (4 % m/v) was shaken in a magnetic shaker at 70 ºC, with slow agitation until the complete dissolution of the sodium alginate. At room temperature, calcium chloride was dissolved in deionized water to obtain concentrations of 4% (w/v). After cooling to 25 ºC, the sodium alginate solution was blended with the crude extract at a proportion of 1:1. The mixture was homogenized in Turrax for 2 min. The solution was atomized in the $CaCl_2$ solution at 25 ºC with a two-fluid atomizer with air pressure in constant slow agitation. The height between the atomizer and the bath was 12 cm. Following atomization, the microcapsules were kept in an ionic bath for 30 min, separated in sieves (25 µm) and washed with 700 mL of distilled water. The microcapsules were dried in an oven at 30 ºC during 24 h so that the enzyme activity was maintained.

Effect of solvents: Enzyme activity and stability were studied with the solvents: acetone, chloroform, toluene, hexane and heptane. The solvents were added to the tubes containing the immobilized enzyme. These solvents have log P values - 0.23, 2.0, 2.5, 3.5 and 4.0, respectively. The tubes were stirred in a homogenizer at room temperature. In one of the tubes only the immobilized enzyme was used as control considering 100 % activity. The free enzyme was kept in the other tube in order to evaluate its stability during the day of analysis. The enzymatic activity during the 30 days of storage was determined with the titration method described by Macedo et al. (1997). All solvents were previously dried over molecular sieve.

Ethyl valerate synthesis: The esterification reactions were performed at 37 °C in 50 mL screw-capped flasks in shaken incubator at 150 rpm. The molar ratio effect of acid and alcohol (0.1, 0.3 and 0.5 M) and the immobilized enzyme concentration (10, 20 and 30 % w/v) using heptane as the solvent were investigated. The flasks were incubated with the substrates for 5 min before adding the enzyme. To control and monitor the water level in the reaction media synthesis was carried out in the presence of molecular sieves. Aliquots of the reaction mixture were withdrawing time intervals: 0, 3, 6, 9, 12, 24, 48, 72 and 120 h, quantifying the fatty acid consumption. The analysis was determined by titration with 0.02 M sodium

phosphate using phenolphthalein as indicator and 10 mL of acetone:ethanol mixture (1:1, v/v) as quenching agent. The fatty acids percentage was estimated according Equation 1.

$$AGL(\%) = \frac{V_{KOH}.M.PM}{10.m} \quad (1)$$

Where AGL is the fatty acids percentage, V is the volume of the KOH titration at time t (mL), M is the KOH molarity; PM is the molecular weight of the fatty acid titrated and m is the mass of the aliquot titrated (g).

The results were evaluated by calculating the ethyl valerate conversion rates, as Equation 2.

$$\%Molar = \frac{(C_0.-C)}{C_0} * 100 \quad (2)$$

Where Co = initial concentration of the reactant and C = concentration of reactant at a given time.

3. Results and discussion

3.1 Effect of organic solvents

The logarithm of the partition coefficient (log P) of a compound between n-octanol and water has been widely used to predict the yield of a reaction, in the presence of a solvent. Laane et al. (1997) found that the enzymatic reaction occurs to a greater extent in the presence of solvents hydrophobic (log P > 3.0) and lower in the presence of hydrophilic solvents (log P < 2.0). The solvents can also act on the enzyme inhibiting its activity, altering hydrogen bonding with hydrophobic interactions, competing for the active site and removing water molecules from the outer surface of the enzyme molecules, a crucial factor for maintaining catalytic activity. Table 1 shows the effect of organic solvents on the activity of the immobilized lipase during 30 d. Without the addition of solvents, the activity of the immobilized enzyme as compared to free enzyme on the first day was 2.70 times higher, proving the efficiency of the immobilization method. Among the tested solvents, heptane is the solvent that yielded better results. It is suggested that solvents used in esterification reactions have log P > 4.0 (Krishna et al., 2001). The effect of immobilized enzyme in heptane was higher than the control (solvent-free immobilized enzyme) during the six days. After this period, the immobilized enzyme activity with and without heptane showed no change until the 13th day. After this period, the immobilized enzyme activity with and without heptane showed no change until the 13th day and then there was a decrease of the enzyme with the solvent, where on the 30th, the fall in immobilized enzyme activity in organic solvent was about 30 % compared with the control (Figure 1). As compared with chloroform, hexane and toluene, the use of acetone showed unless significant activity in the immobilized enzyme. The probable explanation of the activity of the immobilized enzyme is affected by the use of acetone (lowest value of log P) which can be attributed to the variation of the quantity of water removed from catalytic region, which is necessary to maintain the dynamic properties of the enzyme (Wu et al., 2002). Aragão et al. (2009) showed that the use of acetone as solvent in the esterification reaction to yield isoamyl butyrate was not effective. Similar results were observed by Castro et al. (1997) in the synthesis of butyl butyrate with Lipozyme TM, where the percentage of esterification using acetone as solvent was lower than with chloroform, hexane or heptane. After heptane, chloroform was the solvent that showed 0.09 units higher than control on the first day of analysis. On the 6th day there was a significant drop activity and from 1st to 17th days remained stable. The enzyme activity in toluene and hexane were stable during the 10 days, comparing with control activity from the 6th to 10th day. However, the immobilized enzyme in toluene had more gradual drop in activity up to the 30th day compared to hexane. The immobilized lipase in chloroform, toluene and hexane showed activity 62 % lower than the control on 30th day.

Table 1: Effects of organic solvents on activity (U.mL^{-1}) of immobilized Burkholderia cepacia lipase.

Solvents / Days	1	6	10	13	17	20	23	30
Acetone	2.13	2.04	2.04	2.04	2.04	2.04	1.11	1.11
Chloroform	3.61	2.87	2.59	2.59	2.59	2.00	1.94	1.57
Toluene	3.15	3.15	3.15	2.50	2.50	2.50	1.57	1.57
Hexane	3.15	3.15	3.15	2.78	2.78	2.78	1.57	1.57
Heptane	3.89	3.24	3.15	3.05	2.78	2.78	1.85	1.76
Immobilized	3.52	3.15	3.15	3.05	3.05	3.05	2.50	2.50
Free enzyme	1.30	1.30	0.74	0.74	0.74	0.74	0.74	0.74

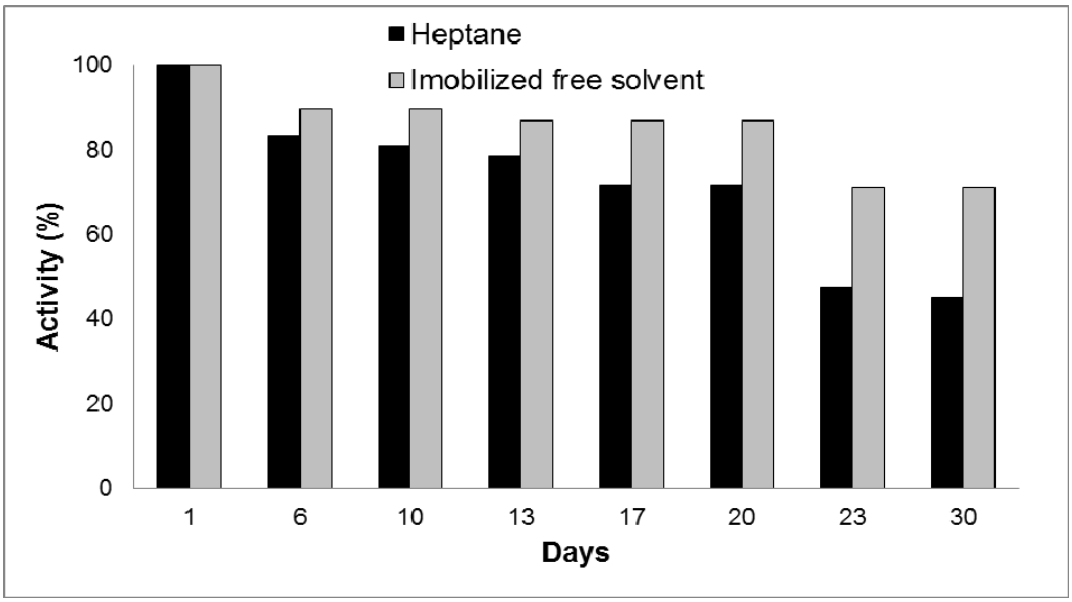

Figure 1: Relationship of the activity of the enzyme immobilized with and without heptane

3.2 Effect of the lipase immobilized amount and acid-alcohol molar ratio

Tables 2, 3 and 4 showed different molar ratios between the substrates ethyl alcohol and valeric acid in different enzyme concentrations. Table 2 showed that at 48 h, the conversion of ethyl valerate was approximately 83 % and after this reaction time, using lipase concentration of 10 % (w/v), there was a decrease in the molar percentage, behaviour was not observed at 20 and 30 % (w/v). Table 3 with 20 % (w/v) after 48 h the yield conversion molar was 80 % and with 30 % (w/v) after 24 h reaction was 77 %. After this time, for the two enzyme amounts there was a decrease of molar conversion. However, in Table 4 there was a gradual increase of molar conversion during 120 h, with the best results using immobilized lipase to 20 % (w/v). From the results, we note that in 24 h of reaction it is possible to obtain quite significant results, contributing to the optimization of the process. Karra-Châabouni et al. (2006) observed 78 % conversion in the production of ethyl valerate after 72 h of reaction using Staphylococcus simulans lipase immobilized in CaCO3 and heptane as organic solvent. Xu et al. (2002) using Ryzopus chinesis lipase showed yield of around 93 % at ethyl valerate conversion with heptane. This shows that the present study presents quite satisfactory conversions using sodium alginate as the immobilizing agent. Our study shows that efficient synthesis of ethyl valerate can be due to the use of heptane in the system displaced the equilibrium towards synthesis of ester over a total transfer in the organic phase.

Table 2: The effect of the amount enzyme on the molar rate 0.1M at the synthesis of the ethyl valerate

Time h)	Fatty acids (mM)	Rate Molar (%)	Fatty acids (mM)	Rate Molar (%)	Fatty acids (mM)	Rate Molar (%)
lipase (w/v)	**10 %**		**20 %**		**30 %**	
0	1387.45	0.0	1304.31	0.0	1324.29	0.0
3	849.33	38.8	793.78	39.1	709.19	46.4
6	587.32	57.7	581.66	55.4	658.26	50.3
9	516.22	62.8	499.03	61.7	548.09	58.6
12	499.03	64.0	475.86	63.5	497.95	62.4
24	270.09	80.5	355.04	72.8	441.59	66.7
48	236.84	82.9	331.69	74.6	399.05	69.9
72	276.96	80.0	286.21	78.1	363.82	72.5
120	285.03	79.5	238.51	81.7	339.27	74.4

Table 3: The effect of the amount enzyme on the molar rate 0.3M at the synthesis of the ethyl valerate

Time h)	Fatty acids (mM)	Rate Molar (%)	Fatty acids (mM)	Rate Molar (%)	Fatty acids (mM)	Rate Molar (%)
lipase (w/v)	10 %		20 %		30 %	
0	4485.50	0.0	4071.52	0.0	4145.13	0.0
3	3655.54	18.5	3065.93	24.7	2406.94	41.9
6	3427.50	23.6	2119.30	47.9	1718.92	58.5
9	3362.07	25.0	1776.84	56.4	1664.15	59.9
12	3177.26	29.2	1295.94	68.2	1262.38	69.5
24	1540.68	65.7	825.62	79.7	935.68	77.4
48	1206.73	73.1	804.12	80.3	949.13	77.1
72	1159.31	74.2	805.58	80.2	1028.30	75.2
120	1131.70	74.8	893.61	78.1	995.08	76.0

Table 4: The effect of the amount enzyme on the molar rate 0.5M at the synthesis of the ethyl valerate

Time h)	Fatty acids (mM)	Rate Molar (%)	Fatty acids (mM)	Rate Molar (%)	Fatty acids (mM)	Rate Molar (%)
lipase (w/v)	10 %		20 %		30 %	
0	7660.68	0.0	7507.18	0.0	6497.69	0.0
3	6696.81	12.6	6303.30	16.0	4051.74	37.6
6	6324.20	17.4	5320.42	29.1	2756.28	57.6
9	5567.01	27.3	4185.32	44.2	1600.24	75.4
12	5333.59	30.4	2929.31	61.0	1585.31	75.6
24	3747.49	51.1	1687.55	77.5	1419.74	78.2
48	2233.26	70.8	1132.00	84.9	1331.50	79.5
72	1635.96	78.6	1007.24	86.6	1235.95	81.0
120	1595.05	79.2	970.75	87.1	1097.28	83.1

Figure 2 shows the best conditions for ethyl valerate esterification versus the reaction time in each molar ratio between the substrates. It was possible to verify the percentage molar conversion of around 90 % (0.5 M) after 120 h of reaction.

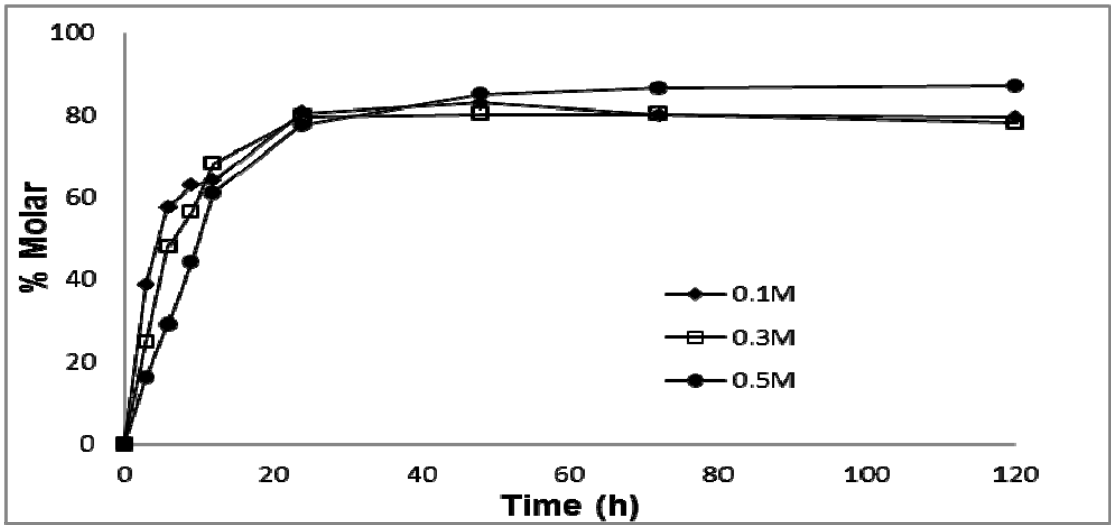

Figure 2: Effect of rate molar on the ester production for immobilized lipase in the conditions 0.1 M and 10 % (w/v), 0.3 M and 20 % (w/v) and 0.5 M and 20 % (w/v)

4. Conclusion

The results of this work showed that the *Burkholderia cepacia* lipase immobilized on sodium alginate has higher activity compared to free lipase. In the ethyl valerate synthesis, the best yield was in 0.5 M and 20 % w/v. As the alginate is a nontoxic polymer, the lipase-alginate mixture can be used as biocatalyst in the esterification reactions of aromatic esters for the food industry, which is one of the sectors that use these esters.

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