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Lipase Selective Entrapment in Alginate from *Burkholderia Cepacia* Crude Extract

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Considered one of the most important enzymes for the biotechnology industries, lipase is a very versatile biocatalyst been target of several researches and review papers and used in many different industrial applications, such as household detergent additive, chemicals and pharmaceutical compounds synthesis, biodiesel production and others. Lipase produced by *Burkholderia cepacia* was entrapped by addition of the crude extract and alginate solution in Ca^{++} hardening solution. Using factorial design of experiments, the procedure conditions were studied: alginate solution concentration, Ca^{++} concentration, ph of solution, bead size and crude extract/alginate solution proportions. In a second step of the work, the alginate solution concentration, calcium concentration and Ph were evaluated against responses relating to entrapment of the lipase and purification of the biocatalyst using a surface response methodology. In a single-step procedure it was possible to entrap lipase directly from crude achieving purification factors of 1.68 (168.07 % immobilization yield) with activity recoveries around 99%.

1. Introduction

Triacylglycerol acyl hydrolases (EC 3.1.1.3), more commonly known as lipases, are biocatalysts which promote esterification, and transesterification reactions with wide industrial applications (Dalla-Vecchia et al., 2004). Ubiquitous in nature, these enzymes are produced from bacteria to animals. In 1958, Sarda and Danuelle determined the difference between a lipase from other esterases as enzymes unable to act lipolyticaly in a real monomer ester solution, i.e. they are carboxilesterases that act above the solubility limit, on emulsified substrates (Jaeger and Reetz, 1998, Verger, 1997). *Burkholderia cepacia* lipase was previously classified as an alkaline lipase, with an optimal pH of 9.0 and temperature of 40 °C (Dalal et al., 2008).
Regardless the fact that the use of enzymes in industrial or laboratorial processes reveals several advantages over the chemical ones, the costs involving its use can still be a barrier. One possible solution is immobilization, which would allow the sequential use of an enzyme several times, for it is now a heterogeneous catalyst. Since the 70's, researchers have been able to immobilize lipases in diverse supports. More recently the enzyme has been successfully covalently immobilized in sepharose, alumina and styrene–divinylbenzene copolymer (Arroyo et al., 1999, Aybastier and Demir, 2010).

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Immobilization by entrapment is another used technique, where the lipase can be confined in different materials, such as sodium alginate (Won et al., 2005), kaolinite (Iso et al., 2001), silicates (Kawakami et al., 2009) and others.
Although the immobilization processes are a great achievement towards the viability of the enzyme in the industry, in most of the analyzed papers, the lipase was either purified prior to immobilization of bought commercially available purified grade. The main objective of this paper was to make use of the already known affinity between the alginate and lipases (Wingender et al., 1987), immobilizing the target molecule directly from a crude extract, skipping the purification processes.

2. Materials and Methods

2.1 Lipase obtaining

The lipase rich crude extract was obtained through *Burkholderia cepacia* fermentation. The growing media was composed by peptone (0.5%), yeast extract (0.3%), meat extract (0.1%), NaCl (0.5%) and 1% olive oil as inducer (Dalal et al., 2008). The fermentation process lasted 48 hours at 30 °C and 150 revolutions per minute. The extract was centrifuged for 10 minutes at 6000 g in order to remove bacterial cells.

2.2 Encapsulation

Lipase extract and alginate solution were mixed thoroughly in graduated tubes ensuring total mixture of the components in a total volume of 8 ml. With the help of a peristaltic pump the enzyme/alginate solution was poured dropwise in 50 ml $CaCl_2$ solution under constant rotation. The drops were kept in the calcium hardening solution for twenty minutes to assure its complete hardening. The drop sizes were controlled using different or adapted pipette tips or needles. The alginate spheres were collected and submitted to a double wash in 50 ml of glycine/NaOH buffer 0.05M, pH 9.0. Both calcium solution and washes were collected to protein and enzyme content analysis (Won et al., 2005).

2.3 Protein and enzyme content

The protein content was estimated using dye-binding method, using bovine serum albumin as a standard (Bradford, 1976). Lipase activity was determined according to the Winkler and Stuckmann method modified (Jain et al., 2005). One enzyme unit was defined as the amount of enzyme that released 1 μmol of p-nitrophenol per minute using p-nitrophenyl palmitate (pNPP) as substrate under assay conditions.

2.4 Responses calculations

Two parameters were used as responses: Immobilization yield and enzyme recovery, according to Equations (1) and (2):

$$Immobilization\ yield\ (\%) = \left(\frac{a_{e(imm)}}{a_{e(free)}}\right) x100 \quad (1)$$

$$Recovery = \left(\frac{a_{imm}}{a_{free}}\right) \quad (2)$$

The specific activity (a_e) is the relation between lipase activity and total protein content.

2.5 Experimental design

The experimental designs were composed and analyzed using Statsoft Statistica 7.0. The response surface methodology was applied to evaluate the influences of already previously known variables that could possibly modify the enzyme entrapment, so a Central Composite Rotatable Design (CCRD) was designed. The influence and interaction of the factors were studied in five different levels, as can be seen in Table 1.

Table 1: Variables and its levels used in the 2^3 a Central a Central Composite Rotatable Design

Variables	levels				
	-α	-1	0	+1	+α
Alginate concentration - %	0.32	1	2	3	3.68
$CaCl_2$ solution - mM	32	100	200	300	368
pH	3.64	5	7	9	10.36

3. Results and Discussion

In order to evaluate the effect of parameters in the immobilization it was used a statistical design of experiments (DOE). Contrarily to the One-Variable-At-a-Time (OVAT) method which involves single factor variation keeping the other factors constant, DOE is more suitable for multifactor optimization not only because it is less time-consuming, requiring a relatively small number of runs per factor (Giovanni, 1983), but also such designs permit the identification of the most important variables and its optimum ranges indicates a promising direction for further experimentation (Weuster-Botz, 2000).

A fractional experimental design was used as a first approach for a latter optimization using a CCRD (results not shown). The results obtained in this first step indicated what levels of the variables should be tested and what variables would be kept fixed. All the experiments in this set where performed using a proportion of enzyme extract/alginate solution of 0.4 and alginate beads diameters of 2 mm.

The analysis of the CCRD yielded the Equation 3:

$$\text{Immobilization yield} = (2{,}58441 - 0{,}372404*A + 0{,}00220011*B - 0{,}0725566*C + 0{,}0164179*A^2 - 0{,}00000512709*B^2 - 0{,}0586075*C^2 + 0{,}00000757451*A*B + 0{,}038474*A*C - 0{,}0000414533*B*C) \times 100 \quad (3)$$

Where A= pH; B= $CaCl_2$ concentration (mM); C= alginate concentration (%).

The obtained coefficient of correlation was 87%. This explains 87 % R^2 of the total variation in the lipase immobilization yield, which is a good result, considering the experimental difficulties in reproducibility associated with the handling a so viscous substance as alginate solution.

Won (2005) studied the influence of calcium hardening solution and alginate solution concentrations in the yield using an OVAT approach and only the alginate showed to be statistically significant to the process. Similarly alginate was found to exert a negative influence in the same range tested and the linear effect of the calcium concentration

showed no linear influence at all. However when associated with other factors, calcium concentration in the hardening solution exhibited a negative influence over the yield. Such effect could only be perceived with the use of DOE. Variations in the response by a factor may not be perceptible at fixed points of others variables. However, these same changes in the factor levels can display huge differences when exposed to different ranges of the other controllable experimental characteristics. The pH exhibited large negative effect over the immobilization yield, i.e. the response grows significantly as the pH is reduced. The mean value of the Immobilization yield obtained was 113.69 and a maximum of 168.07 in the central points of the alginate and $CaCl_2$ concentrations with the lower pH level.

Table 2 displays the results of the experiments having immobilization yield as response along with the results predicted by the model generated. A response surface was designed demonstrating the optimal levels of the alginate and $CaCl_2$ concentrations. Figure 1-A displays the yield values for alginate and calcium concentrations at pH 7.0 and Figure 1-B the same variables at the lowest pH level, in which the best results were achieved (3.64). It is easy to notice that the point where the best immobilization yield was achieved is at the central point of both alginate solution and calcium chloride solution concentrations at neutral pH. The decrease of the pH, although relocated the optimal value of alginate concentration to 1%. Among the variables levels tested, in the factorial design and the central composite rotatable design, it was not possible to observe any statistical significance in the enzyme recovery results. Therefore no model could be constructed to the response. Nonetheless in all the experiments the lipase recovery was high, with a mean value of 0.99531 and maximum obtained 0.99949. These results clearly show that in all the cases the enzyme, independently of other proteins, interacted strongly with the polymer (Wingender et al., 1987). In order to validate the model generated (Equation 3) a random set of experiments was performed and the results are exhibited in Table 3.

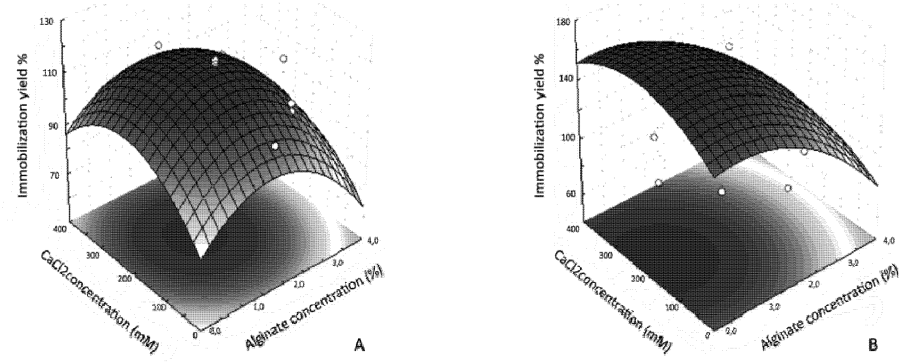

Figure 1- Results of immobilization yield having alginate concentration and calcium chloride concentration as variables. 1-A contains the results for neutral pH; 1-B the results for pH 3,64.

Table 2: Results of the CCRD with the observed and predicted responses for each run with Immobilization yield as response.

Run	Alginate (%)	$CaCl_2$ solution (mM)	pH	Imm. yield	
				Actual	Predicted
1	-1	-1	-1	136.11	136.24
2	-1	-1	+1	101.73	94.92
3	-1	+1	-1	137.51	139.15
4	-1	+1	+1	104.13	98.44
5	+1	-1	-1	103.29	112.50
6	+1	-1	+1	100.08	101.96
7	+1	+1	-1	103.42	113.75
8	+1	+1	+1	100.43	103.82
9	0	0	-α	168.07	157.10
10	0	0	+α	107.99	113.99
11	0	- α	0	101.45	100.53
12	0	+ α	0	108.60	104.55
13	-α	0	0	100.12	108.19
14	+ α	0	0	105.79	92.75
15	0	0	0	118.23	117.00
16	0	0	0	117.94	117.00
17	0	0	0	116.31	117.00
18	0	0	0	115.23	117.00

Table 3:Results of the experiments for validation of the model(Equation 3) with the observed and predicted responses with Immobilization yield as response.

Run	Alginate (%)	CaCl2 solution (mM)	pH	Imm. Yield	
				Actual	Predicted
1	2	368	7	101.04	104.54
2	2	100	5	128.12	130.22
3	1	300	9	99.26	98.43
4	3.68	200	5	97.38	99.20
5	3	200	7	108.76	106.55

The observed values of this new experiment set are very close to the predicted ones, hence, successfully validating the model.

4. Conclusions

Through these experiments it was possible to build a suitable model to describe the behavior of lipase when submitted to immobilization in sodium alginate. The enzyme recovery was very high (more than 99% in all experiment set) and the best immobilization yield achieved was predicted to be 168.74%, corresponding to a purification factor of 1.68 with alginate concentration of 1% (at pH 3.64) and calcium hardening solution concentration of 200 mM. These are very good results considering

the fact that the lipase was selectively entrapped directly from its crude extract, in a single step procedure.

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