

Original Research Article

Physical and chemical aspects of *Yarrowia lipolytica* lipase immobilization by adsorption

Abstract

In the present investigation, an attempt has been made to explain lipase immobilization by adsorption on three minerals matrix, i.e. celite 545, silica gel (60G) and avicel (PH 101). A methodical order was developed whereby the influences of water content, surface potentials and pH, on immobilization were explored. Maximum immobilisation yield was obtained with celite (70 %) and the lowest with silica gel (29 %). Total water content of free and immobilized lipase was determined by volumetric Karl Fischer titration. The water content of silica gel was higher than the one of other supports. Water content of silica gel could prevent the enzyme fixation. These results could be explained by the adsorption being governed mainly by electrostatic interactions between the enzyme and matrix. This hypothesis was further reinforced by measurements of electrical potential. They showed a lowest negative potential of silica gel after enzyme adsorption comparatively with celite. From these results celite was designated as an efficient matrix to immobilize *Yarrowia lipolytica* lipase (YLL) by adsorption.

Adsorbed YLL was used to realize an interesterification reaction between rapeseed oil and milk fat in comparison with a commercial silica-granulated *Thermomyces lanuginosus* lipase (Lipozyme TL IM).

Keywords: *Yarrowia lipolytica* lipase, celite, silica gel, avicel, immobilization, water content, electrostatic interaction, interesterification.

26

27

28

29 **1. Introduction**

30 Lipases (triacylglycerol ester hydrolases, E.C.3.1.1.3) belong to the class of enzymes that hydrolyze fats
31 and oils with subsequent release of free fatty acids, diacylglycerols, monoacylglycerols and glycerol [1,2].

32 During the last decade, lipases have gained a great interest in biotechnology applications. Thanks to their
33 commercial availability, low cost, high stereoselectivity and the possibility of use within a large range of
34 pH and temperature, lipases are among the most used biocatalysts in organic synthesis [3]. In addition to
35 their classical use in hydrolysis reactions, lipases have been more and more employed in esterification
36 and transesterification reactions; notably to produce esters having potential applications in fine chemical,
37 pharmaceutical and agrochemical industries. For instance numerous works reported the aptitude of lipases
38 to catalyze the synthesis of short-chain fatty acids and alcohols used as additives for a variety of perfumes
39 and flavours [4–6], biosurfactants [7, 8] and biofuels [9,10].

40 Immobilization of lipases protects the enzyme from solvent denaturation and facilitates ~~their~~ use in
41 continuous processes [11]. Another advantage of immobilization is the enhancement of enzyme
42 thermostability. Thus enzymatic reactions at higher temperatures have resulted in: higher conversion
43 rates, higher substrate solubility and lower viscosity of the reaction medium, there by favouring mass
44 transfer [12]. Several approaches have been reported for the immobilization of lipases: they consisted
45 either on physical adsorption of the enzyme on a carrier material [13, 14], its entrapment or
46 microencapsulation in a solid support [15, 16] or on the covalent binding to a solid matrix [17, 18].
47 Natural kaolin was used as support for the immobilization of *Candida rugosa* lipase [19]; celite and silica
48 gel were used to immobilize *Yarrowia lipolytica* lipase (YLL) [20]. These authors immobilized YLL by
49 three different methods: inclusion, adsorption and covalent bond. Catalytic activities of immobilized and
50 free enzymes in the three systems were compared and stability of the immobilized enzyme was tested in

several hydrolysis cycles. Lipase activity at alkaline pH, thermo-stability and resistance to solvents were enhanced by immobilization. In addition, lipase immobilization by inclusion did not allow multiple reuses of immobilized enzymes. However, adsorption and covalent bound allowed multiple reuses of the immobilized enzymes during a long period. Immobilization by adsorption is the easiest and least expensive technique to prepare solid-support biocatalysts. It is based on the physical adsorption or ionic binding, or both, of the lipase to the surface of the support. The weak linkages established between enzymes and supports (mainly van der Waals, hydrogen bonds and hydrophobic interactions) have little effect on catalytic activity [21]. The physical structure and chemical composition of the support can also influence the microenvironment of the immobilized lipases and consequently their properties. The aim of the present study was to determine some physico-chemical characteristics of YLL immobilization by adsorption in order to evaluate electrical potential as a potential tool for selecting the optimum matrix for maximal binding.

In this paper adsorption technology is used because it shows to be the easiest and less expensive method. According to our previous search, immobilization by adsorption on minerals matrix does not destroy enzyme [20]. In order to experiment and enhance YLL immobilization by adsorption, the performed system (lipase immobilized on celite) was used to realize an interesterification reaction between rapeseed oil and milk fat in comparison with a commercial *Thermomyces lanuginosus* lipase (Lipozyme TL IM)

2 Materials and methods

2.1 Materials

Lipase from *Y. lipolytica* LgX6481 was produced in 2000 L bioreactor (LSL Biolafitte, Poissy, France) in the same conditions as previously described [22,23]. The culture broth was centrifuged on a BTPX205 continuous centrifuge (Alfa Laval, Sweden) at 12,000 x g, at a flow rate of 500 L.h⁻¹ and the supernatant was freeze-dried for further studies. Celite 545, Silica gel and Avicel (PH 101) were obtained from Fluka Biochemika (Switzerland).

76

77

78

79

80 2.2 Methods

81 2.2.1 Immobilization by adsorption

82 YLL was immobilized on Celite, Silica gel and Avicel by adsorption following the method previously
83 described [20]. 6 g of support was mixed thoroughly with 1.2 g of lipase in 100 mL phosphate buffer (pH
84 7.5), stirred for 4 hours and then freeze-dried. The freeze dried preparation was stored at 4 °C for further
85 use.

86 2.2.2 Immobilization yield

87 Immobilization yield was defined as follows:

$$88 \text{ Immobilization yield (\%)} = \left(\frac{a_{imm}}{a_{free}} \right) \times 100 \quad [24]$$

89 where, a_{imm} : total activity of immobilized enzyme (U/g) and a_{free} : total activity of the initial enzyme
90 preparation (U/g).

91

92 2.2.3 Enzyme assay and dry matter measurements

93 The hydrolytic activities of free and immobilized lipases were measured by a titrimetric method
94 previously described by [22,23] using olive oil (Extra Vierge Bertolli, Italy) as substrate. Activities are
95 expressed in international units (IU), where one unit of lipase is the amount of enzyme able to catalyse the
96 release of 1 µmol of fatty acid per min at pH 7 and at 37 °C.

97 Dry matter in liquid and solid phases was measured after desiccation at 105 °C over a period of 48 h until
98 reaching a stable mass.

99

100 2.2.4 Water activity and Volumetric Karl Fischer titration

Water activity (a_w) of the immobilized lipase was determined after freeze-drying. A sample of immobilized lipase in a plastic cup was loaded into an osmometer (FA-STlab GBX, Romans, France) and a reading was taken after equilibration.

The water content of free and immobilized lipase was determined with a Karl Fischer titrator DL31 (Mettler Toledo, Zurich, Switzerland) using a methodology described previously [25].

2.2.5 Particle charge detector

The electrical potential of support-lipase was determined using a particle charge detector Mutek PCD 03 (Mutek, Germany) in presence of phosphate buffer at different pH (4 to 8). The values represent a mean of two measurements.

2.2.6 Interesterification reaction

The interesterification protocol was previously described [26], however in the present case the volume was 200ml and the separation of the enzyme was done on filter Whatman paper number 1 (Fluka Biochemika, Switzerland).

3 Results and discussion

3.1 Immobilization yields

Lipase immobilization by adsorption was carried out on three supports: Celite, silica gel and Avicel. The highest immobilization yield of 70% was obtained with Celite and the lowest of 29% with Silica gel, (Table 1). The higher activity with Celite could be attributed to greater affinity between lipase and Celite than silica gel. Wang *et al.* reported 22.3% of *Candida rugosa* lipase activity immobilized on celite compared to 14.8% for silica gel [27].

3.2 Water content measurement

The total water content of samples was determined by volumetric Karl Fischer titration. Results of Table 2 show that the water content of the immobilized enzyme ranged from 2.95 to 7.73% and those of matrix without lipase ranged from 0.08 to 6.84%. The silica gel had the highest moisture content (6.84%). The presence of the water molecule could be the cause of low-fixing enzyme on this support. When lipase was in higher water content system, lipase may become inactivity or the conformation was changed [28]. In according this author the elimination of water helps to immobilize the fluorescent molecule. Therefore Celite with low water content (0.08%) could be suitable for the lipase fixation.

3.3 Measurement of particle charge

The electrical potential between lipase and matrix was studied for measuring particle charge in presence of phosphate buffer at pH 4, 5, 6, 7 and 8. The electrical charge of matrix and enzyme were measured progressively adding the lipase solution in order to obtain saturation.

Figure 1 shows that the electrical potential resulting from the enzyme-support interaction is lower negative (-100 and -450mV) in phosphate buffer pH 4, 5 to 6, and that the saturation is not obtained.

At these pH values lipase from *Y. Lipolytica* is almost around of its isoelectric points (5.1 to 5.3) [22]. Therefore it would explain why the potential is less negative. In contrast at pH 7 to 8, the electrical potential between -200 and -600 mV and saturation is obtained after 4 to 7 mL of lipase solution added.

According to authors [28] the surface saturation effect on the activity of immobilized enzyme is explained and ascribed to the surface diffusion effect of electric double layers. On the other hand at these pH values (7 and 8) the interaction between enzyme-support is strongly negative. Therefore, this pH (7 to 8) was used to immobilize YLL. In addition our experimentation shows that electrostatic attraction dominated lipase interactions with matrix, and when the pH increases the electrical potential decrease.

Figure 2 and 3 show the evolution of the electrical charge for free lipase dissolute in different pH (4 to 8) and immobilized lipase in phosphate buffer pH (7).

In view of the results in Figure 2, free lipase dissolved in buffers included an electric charge between -80 and -100mV and when it is immobilized on the matrix in the presence of phosphate buffer pH 7 (figure 3), its charge ranged from -100 to -180mV. Results confirmed that it was an interaction between the enzyme and support which could be governed by electrical charges. Lipase immobilized on silica gel shows the least negative electric charge compared to Celite and Avicel as shown above.

3.4 Interesterification of a fat blend

As the water content of Celite was extremely low and the immobilization yields of YLL on this support were high, the lipase immobilized on Celite was tested in a reaction occurring in micro-aqueous conditions. Thus, a blend composed of 70% milk fat and 30% rapeseed oil was interesterified. This reaction was compared to the interesterification of the same blend by a commercial silica-granulated *Thermomyces lanuginosus* lipase (Lipozyme TL IM) which was previously shown to be efficient in micro-aqueous and solvent-free reaction conditions [26, 29].

Table 3 shows low values of IA (interesterification activity) for YLL immobilized on Celite than the lipozyme TL IM. We noted that when the enzyme is immobilized, its IA increases.

Figure 4 shows the profiles of triglyceride species of the initial blend and after interesterification of it by both enzymes. The profiles of triglyceride species of the initial blend show the same evolution. The value of the ID (interesterification degree) with YLL on celite after 6 h of reaction were equal to respectively 78% and 100% of the value obtained with Lipozyme TL IM at 6 h (fig. 5).

This result was encouraged because YLL immobilized by simple adsorption has a good value of ID and can be compared to a commercial enzyme.

4 Conclusions

In this work, we described an easy method to immobilize lipase on different supports such as Celite 545, silica gel (60G) and avicel (PH 101). The immobilization showed that Celite 545 exhibited high lipase loading capacity due to high surface area and strong interactions between lipase and the matrix. The immobilized lipase on celite showed high immobilization yield and low water content. Celite 545 was shown to be a promising matrix for obtaining an active and stable immobilized enzyme. This system was then tested in a low-water containing media to perform an interesterification reaction.

References

- [1] Jaeger KE, Dijkstra BW, Reetz MT. Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications of lipases. *Annu. Rev. Microbiol.* 1999; 53: 315-351.
- [2] Woolley P, Petersen SB. *Lipases: Their Structure Biochemistry and Application*, Cambridge University Press, New York/Cambridge, 1994; pp. 77.
- [3] Dalla-Vecchia R, Sebrão D, Da Garc MA, Nascimento A, Soldi V. Carboxymethylcellulose and poly (vinyl alcohol) used as a film support for lipases immobilization. *Process Biochem.* 2005; 40: 2677-2682.
- [4] Abbas H, Comeau L. Aroma synthesis by immobilized lipase from *Mucor* sp. *Enzyme Microbiol. Technol.* 2003; 32:589-595.
- [5] Karra-Châabouni M, Ghamgui H, Bezzine S, Rekik A., Gargouri Y. Production of flavour esters by immobilized *Staphylococcus simulans* lipase in a solvent-free system. *Process Biochem.* 2006; 41:1692-1698.
- [6] Bezbradica D, Mijin D , Siler-Marinkovic S, Knezevic Z. The effect of substrate polarity on the lipase-catalyzed synthesis of aroma esters in solvent-free systems. *J Mol Catal B: Enzyme.* 2007; 45: 97-101.
- [7] Sabeder S, Habulin M, Knez Z. Lipase-catalyzed synthesis of fatty acid fructose esters *J. Food Eng.* 2006;77: 880-886.

- 200 [8] Chen J, Kimura Y, Adachi S. Synthesis of linoleoyl disaccharides through lipase-catalyzed
201 condensation and their surface activities. J Biosci Bioeng. 2005; 100 : 274-279
- 202 [9] Salis A, Pinna M, Monduzzi M, Solinas VJ. Biodiesel production from triolein and short chain
203 alcohols through biocatalysis. J Biotechnol. 2005;119:291-299.
- 204 [10] Royon D, Daz M, Ellenrieder G, Locatelli S. Enzymatic production of biodiesel from cotton seed oil
205 using t-butanol as a solvent. Biosource Technol. 2007; 98: 648-653.
- 206 [11] Mateo C, Palomo JM, Fernandez-Lorente G, Guisan JM, Fernandez-Lafuente R. Improvement of
207 enzyme activity, stability and selectivity via immobilization techniques. Enzyme Microbiol Technol.
208 2007; 40:1451-1463.
- 209 [12] Matsumoto M, Ohashi K. Effect of immobilization on thermostability of lipase from *Candida*
210 *rugosa*. Biochem Eng. J. 2003;14:75-77.
- 211 [13] Zhen-Xing T, Lu-E S, Jun-Qing Q Neutral lipase from aqueous solutions on chitosan nano-particles
212 Biochem. Eng. J. 2007; 34: 217-227.
- 213 [14] Zhen-Gang W, Jian-Qin W, Zhi-Kang Xu X. Immobilization of lipase from *Candida rugosa* on
214 electrospun polysulfone nanofibrous membranes by adsorption. J Mol Catal B: Enzyme. 2006; 42: 45-51.
- 215 [15] Antczak T, Mrowiec Bialon J, Bielecki S, Jarzebski AB, Malinowskim JJ, Lachowski AI,
216 Galas E. Thermostability and esterification activity of *Mucor javanicus* lipase entrapped in silica aerogel
217 matrix and in organic solvents, Biotechnol Technol. 1997; 11: 9-11.
- 218 [16] El Rassy H, Perrard A, Pierre AC. Application of lipase encapsulated in silica aerogels to a
219 transesterification reaction in hydrophobic and hydrophilic solvents: Bi-Bi Ping-Pong kinetics. J Mol
220 Catal B: Enzyme. 2004; 30:137-150.
- 221 [17] Yong-Xiao B, Yan-Feng L, Yong Y, Liu-Xiang Y. Covalent immobilization of triacylglycerol lipase
222 onto functionalized novel mesoporous silica supports. J Biotechnol. 2006; 125: 574-582.
- 223 [18] Hong J, Xu D, Gong P, Yu J, Ma H, Yao S. Covalent-bonded immobilization of enzyme on
224 hydrophilic polymer covering magnetic nanogels. Micropor. Mesopor. Mater. 2008; 109:470-477.

- 225 [19] Panzavolta F, Soro S, D'Amato R, Palocci C, Cernia E, Russo MV. Acetylenic polymers as new
226 immobilization matrices for lipolytic enzymes J. Mol. Catal.B: Enzyme. 2005; 32: 67-77.
- 227 [20] Alloue WAM, Destain J, Thami El M, Ghalfi H, Kabran P, Thonart P. Comparison of *Yarrowia*
228 *lipolytica* lipase immobilization yield of entrapment, adsorption and covalent bond techniques. Applied
229 Biochem. Biotechnol. Part A: Enzyme Engineering and Biotechnology 2008; 150: 51-63.
- 230 [21] Andrade JD, Hlady V A. tutorial review and suggested hypotheses. Adv. Pol. Sci. 1986; 79:1-63.
- 231 [22] Destain J. Roblain D., Thonart P. Improvement of lipase production from *Yarrowia lipolytica*
232 Biotechnol. Lett. 1997; 19:105-107.
- 233 [23] Fickers P, Ongena M, Destain J, Weekers F, Thonart P. Production and down-stream processing of
234 an extracellular lipase from the yeast *Yarrowia lipolytica*. Enzyme Microb Technol. 2006; 38: 756-759.
- 235 [24] Won K, Kim S, Kim K-Je, Park HW, Moon S-Jin Optimization of lipase entrapment in Ca-alginate
236 gel beads. Process Biochem. 2005; 40: 2149-2150.
- 237 [25] Ronkart SN, Paquot M, Fougnyes C, Deroanne C, Van Herck J-C, Blecker C. Determination of total
238 water content in inulin using the volumetric Karl Fischer titration. Talanta 2006b; 70:1006-1010.
- 239 [26] Aguedo M, Hanon E, Danthine S, Paquot M, Lognay G, Thomas A, Vandenbol M, Thonart P,
240 Wathelet J.-P, Blecker C.. Enrichment of Anhydrous Milk Fat in Polyunsaturated Fatty Acid Residues from
241 Linseed and Rapeseed Oil through Enzymatic Interesterification. J. Agric. Food Chem. 2008; 56 (5):
242 1757-1765.
- 243 [27] Wang H, Hou W, Chi-Tang H, Weng X. Cocoa butter equivalent from enzymatic interesterification
244 of tea seed oil and fatty acid methyl esters. Food Chem. 2006; 97 (4):661-665.
- 245 [28] Chao, C-H., Li, K-L., Wu, C-S., Lee, C.C., Chiang, H-P., Yang, Y-S. Pan, T-M., Ko F-H. Surface
246 Effect of Assembling Enzyme and Modulation of Surface Enzyme Activity with Electric Potential Stress.
247 Int. J. Electrochem. Sci. 2012; 7:5100-5114.

248 [29] Zhang H., Smith P., Adler-Nissen J. Effects of degree of enzymatic interesterification on the physical
 249 properties of margarine fats: solid fat content, crystallization behaviour, crystal morphology, and crystal
 250 network. J. Agric. Food Chem. 2004; 52:4423-443.

251

Legends

Table 1: Immobilization yield of lipase onto supports

Table 2: Determination of water content, dry matter and water activity of supports, free and immobilized lipase

Table 3: Hydrolytic and interesterification activities of free and immobilized lipases, expressed in international units (IU).

Fig. 1: Electrical charge of supports at different pH (4 to 8) depending of lipase solution added.

Fig. 2: Electrical charges of free lipase dissolved in solutions with different pH (4 to 8)

Fig 3: Electrical charges of immobilized enzymes in phosphate buffer at pH 7.

Fig. 4: Triglycerides composition of the fat blend (AMF/Rapeseed 70/30) by carbon number.

Dotted line: non-interesterified initial blend. After interesterification with YLL immobilized on celite 21 h (●) and with Lipozyme 6 h (○)

Fig: 5 Interesterification of a blend milk fat/rapeseed oil 70/30 (% w/w) illustrated through the ratio CN 50/CN 42. YLL on celite 21 (◇) and with Lipozyme 6 h (◆).

1 Table 1

2

Immobilized lipase	Immobilization yield %
Lipase + Celite	70 ± 10
Lipase + Silica gel	29 ± 6
Lipase +Avicel	66 ± 2

16

17

18 Table 2

Samples (immobilized and free lipase)	Water content (%)	Water activity	Dry matter (%)
Lipase + Celite	2.95 ± 0.10	0.421 ± 0.00	96.88 ± 0.18
Lipase + Silice	7.73 ± 0.21	0.533 ± 0.00	95.01 ± 0.26
Lipase + Avicel	6.97 ± 0.05	0.461 ± 0.00	95.15 ± 0.23
Celite	0.08 ± 0.10	0.567 ± 0.02	99.92 ± 0.05
Avicel	6.55 ± 0.07	0.564 ± 0.00	94.37 ± 0.10
Silica gel	6.84 ± 0.04	0.597 ± 0.01	94.74 ± 0.10
free lipase	4.94 ± 0.04	0.612 ± 0.00	95.35 ± 0.05

19

1 Table 3

	Hydrolytic activity	Interesterification activity
	IU/mg/min	IUN/g/min
Lipozyme TL IM	47142.9 ± 6890.5	1.741 ± 0.048
YYL on celite	5476.2 ± 628.6	0.186 ± 0.011
Lyophilized YYL	17552.1 ± 1815.4	0.077 ± 0.015

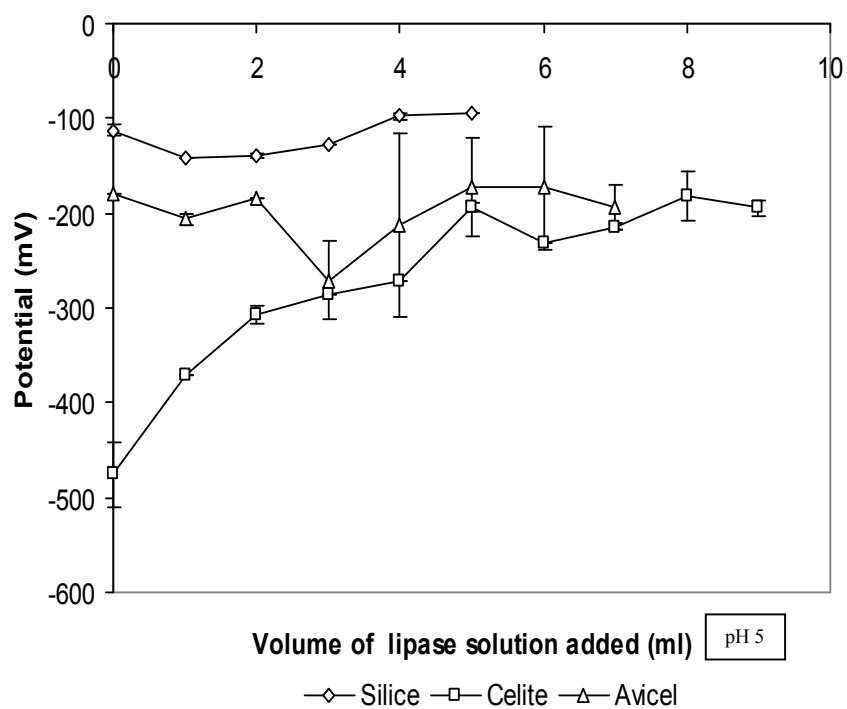
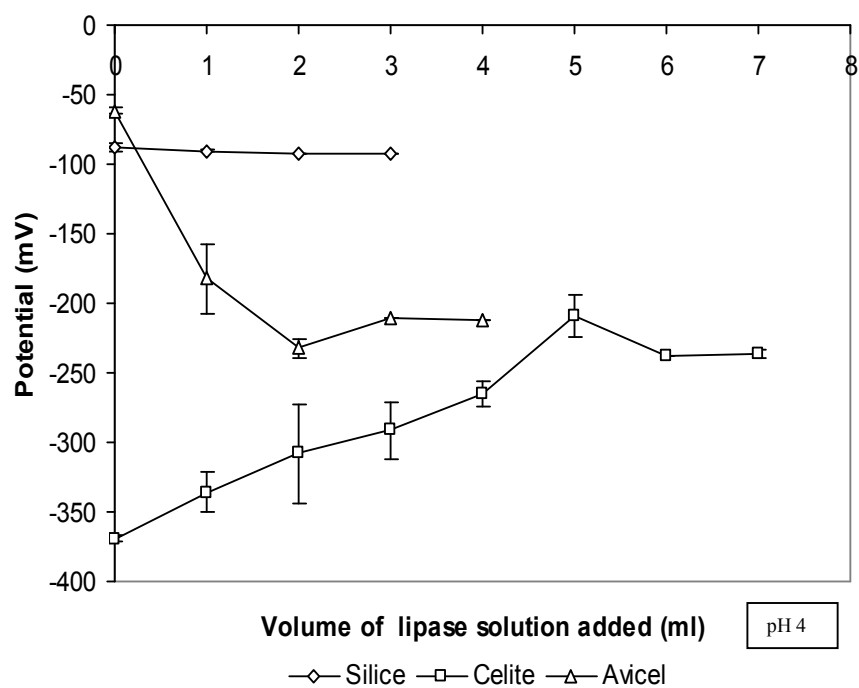
2

3

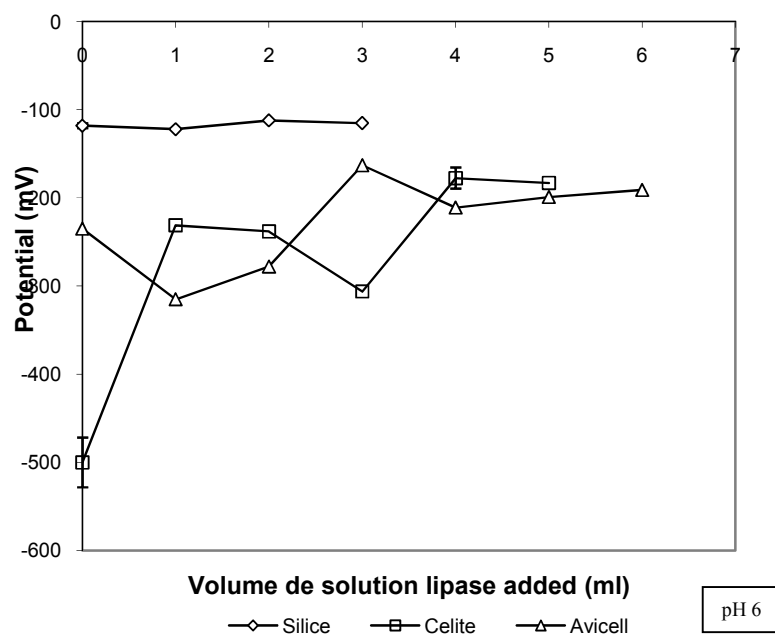
4

5

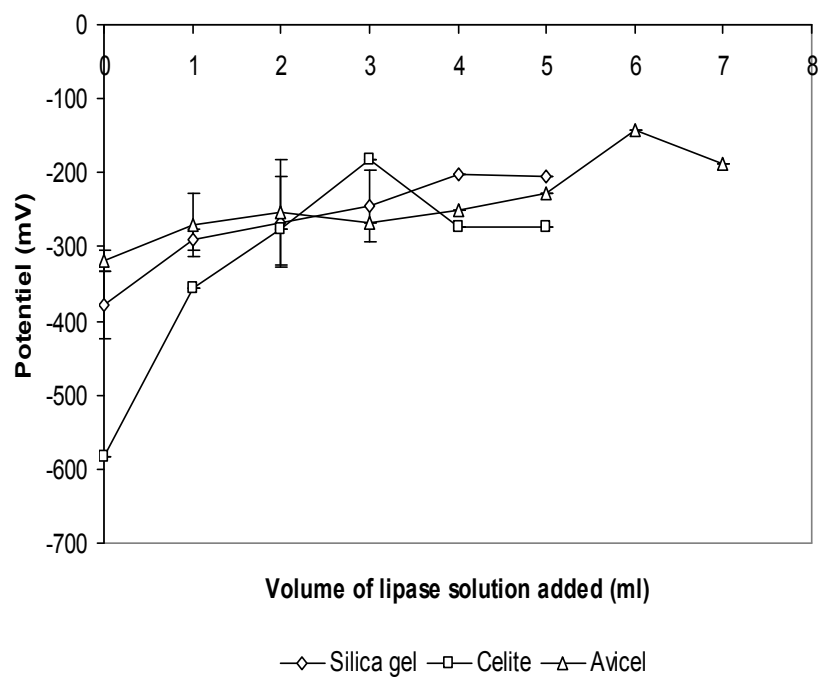
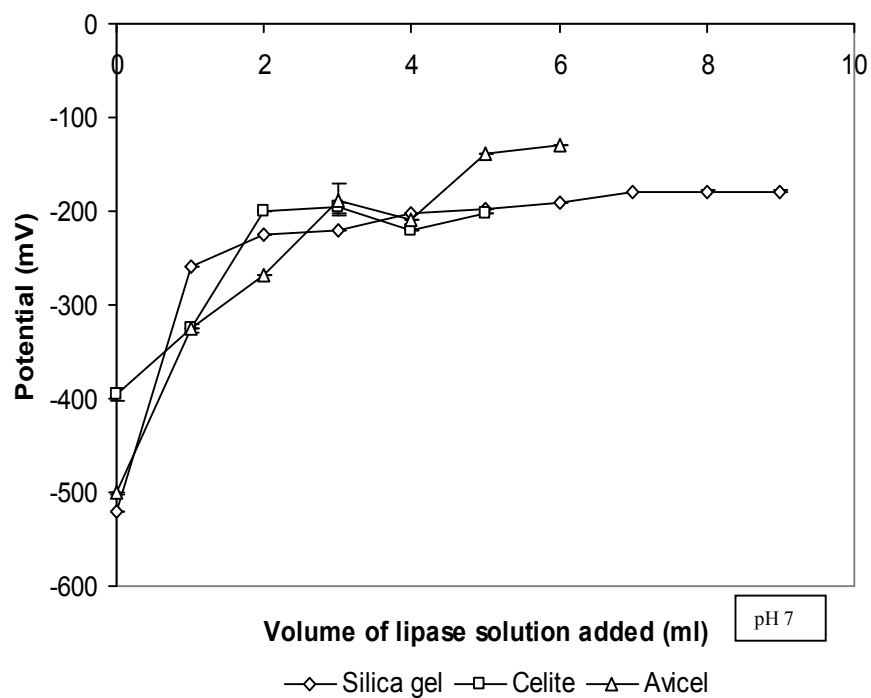
Fig. 1



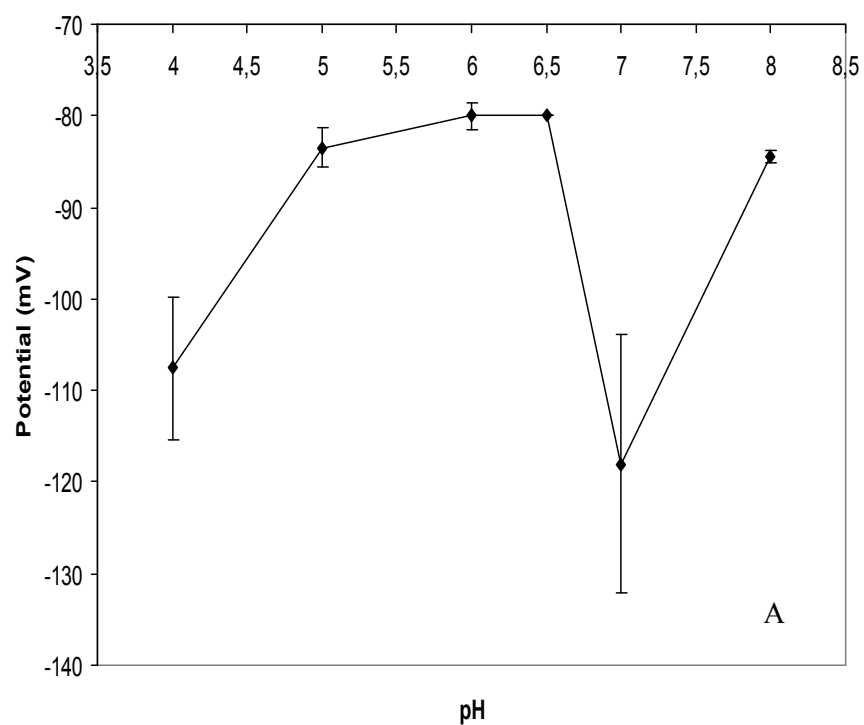
1
2
3
4



5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26



1 Fig. 2
2



3
4
5 Fig. 3

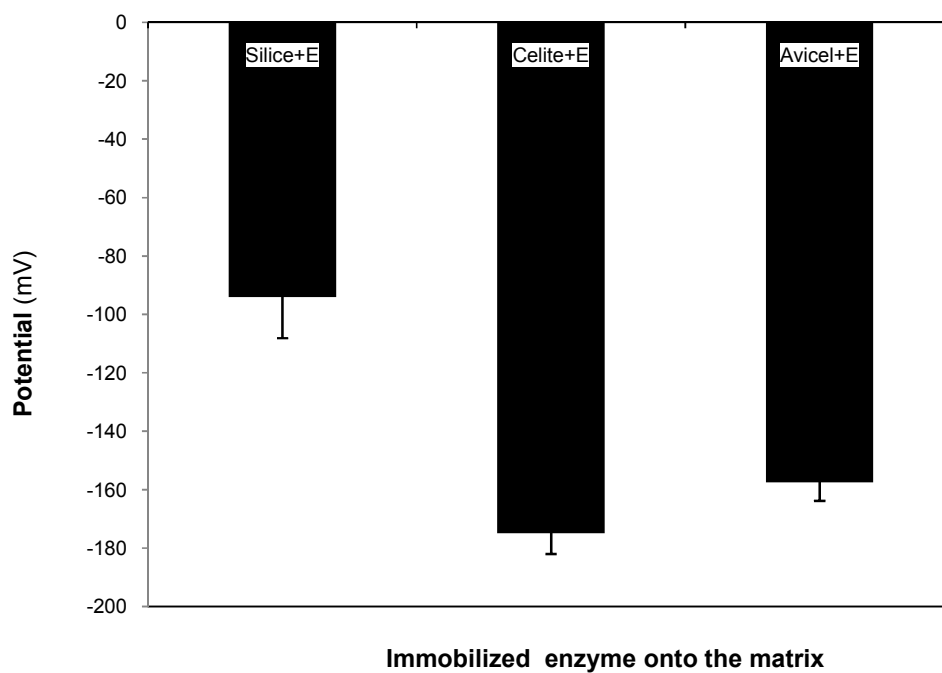


Fig. 4

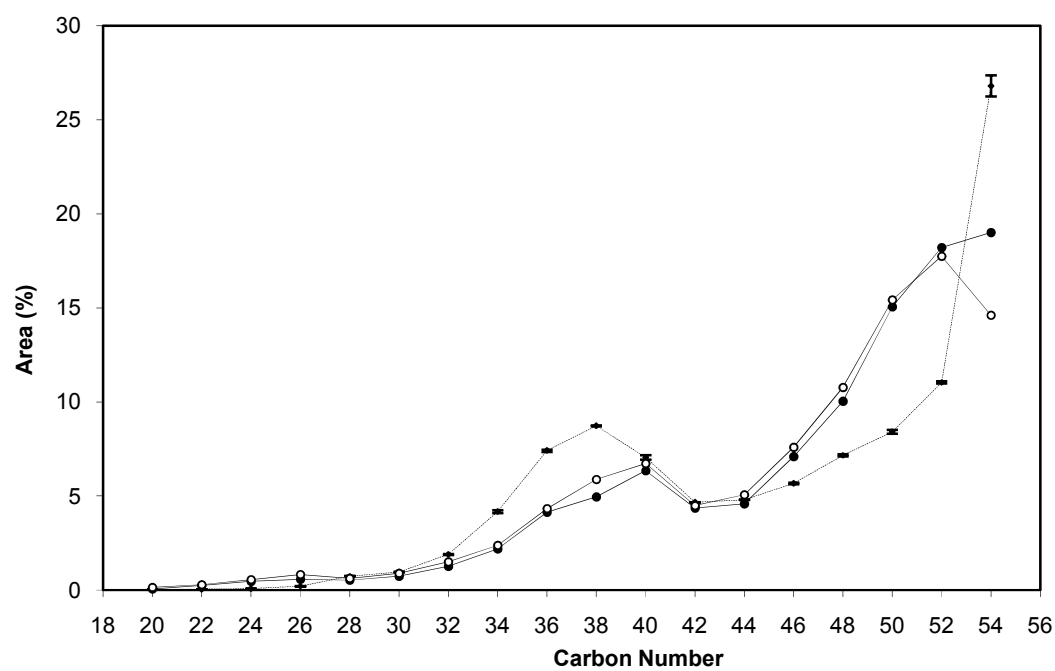
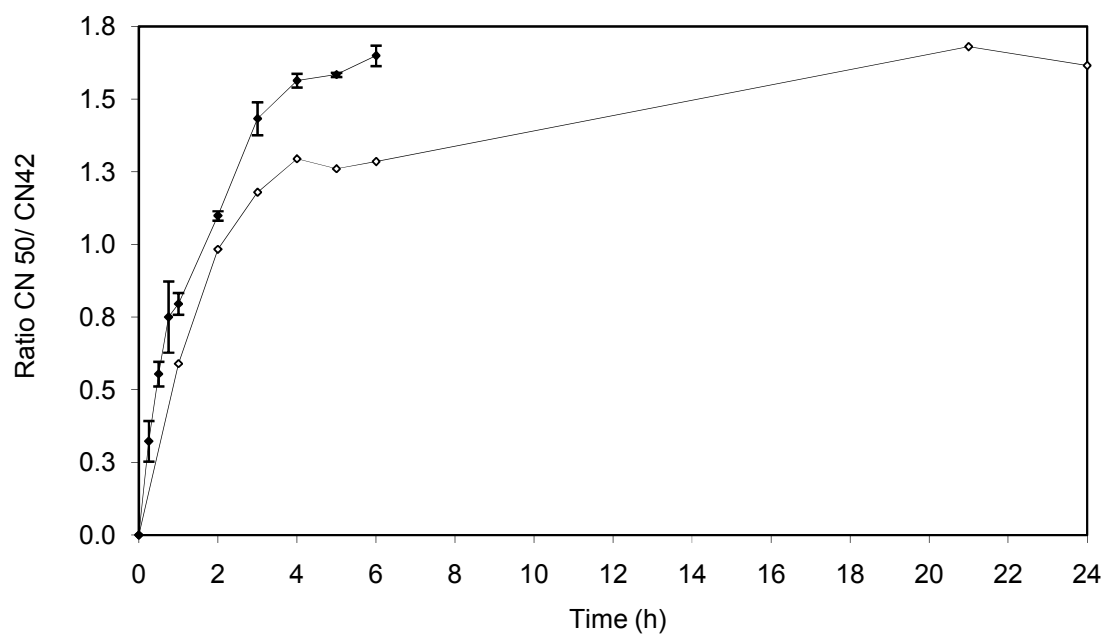


Fig 5



13

14

15

16

