Original Research Article

Electrostatic and water content effects on *Yarrowia lipolytica* lipase immobilization by adsorption

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ABSTRACT

Aims: In the present investigation, an attempt has been made to explain lipase immobilization by adsorption on three minerals matrixes, i.e. Celite 545, Silica gel (60G) and Avicel (PH 101).

Study design: immobilization by absorption on minerals matrixes, water content by volumetric karl Fischer titration and surface potentials using a particle charge detector Mutek PCD 03 were used.

Place and Duration of Study: Walloon Centre of Industrial Biology (CWBI) Unit of Bio-Industries, University of Liege, Gembloux Agro-Bio Tech, Passage des Deportes 2, B-5030 Gembloux, Belgium between Juin 2012 and juin 2013.

Methodology: A methodical order was developed whereby the influences of water content, surface potentials and pH, on immobilization by adsorption were explored. Adsorbed YLL was used to understand an interesterification reaction between rapeseed oil and milk fat in comparison with a commercial silica-granulated *Thermomyces lanuginosus* lipase (Lipozyme TL IM).

Results: Maximum immobilization 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 in comparison to Celite.

Conclusion: From these results celite was designated as an efficient matrix to immobilize *Yarrowia lipolytica* lipase (YLL) by adsorption. This performed system 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.*

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1. INTRODUCTION

Lipases (triacylglycerol ester hydrolases, E.C.3.1.1.3) belong to the class of enzymes that hydrolyze fats and oils with subsequent release of free fatty acids, diacylglycerols, monoacylglycerols and glycerol [1,2]. During the last decade, lipases have gained a great interest in biotechnology applications. Thanks to their commercial availability, low cost, high stereoselectivity and the possibility of use within a large range of pH and temperature, lipases are among the most used biocatalysts in organic synthesis [3]. In addition to their classical use in hydrolysis reactions, lipases have been more and more employed in esterification and transesterification reactions; notably to produce esters having potential applications in fine chemical, pharmaceutical and agrochemical industries. For instance numerous works reported the aptitude of lipases to catalyze the synthesis of short-chain fatty acids and alcohols used as additives for a variety of perfumes and flavours [4–6], biosurfactants [7, 8] and biofuels [9,10].

Immobilization of lipases protects the enzyme from solvent denaturation and facilitates their use in continuous processes [11-12]. Another advantage of immobilization is the enhancement of enzyme thermostability. Thus enzymatic reactions at higher temperatures have resulted in: higher conversion rates, higher substrate solubility and lower viscosity of the reaction medium, there by favouring mass transfer [13]. Several approaches have been reported for the immobilization of lipases: they consisted either on physical adsorption of the enzyme on a carrier material [14, 15], its entrapment or microencapsulation in a solid support [16, 17] or on the covalent binding to a solid matrix [18, 19]. Natural kaolin was used as support for the immobilization of *Candida rugosa* lipase [20]; Celite and Silica gel were used to immobilize *Yarrowia lipolytica* lipase (YLL) [21]. These authors immobilized YLL by three different methods: inclusion, adsorption and covalent bond. Catalytic activities of immobilized and 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 bounding allowed multiple reuses of the immobilized enzymes during a long period. Immobilization by adsorption is the easiest the 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 [22]. 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 [21]. In order to experiment and enhance YLL immobilization by adsorption, the performed system (lipase immobilized on Celite) was used to understand 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 [23,24]. 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 60G and Avicel (PH 101) were obtained from Fluka Biochemika (Switzerland).

2.2 Methods

2.2.1 Immobilization by adsorption

YLL was immobilized on Celite, Silica gel and Avicel by adsorption following the method previously described [20]. Briefly 20g of support was mixed thoroughly with 1.2 g of lipase in 100 mL phosphate buffer (pH 7.5), stirred for 4 hours and then freeze-dried. The freeze dried preparation was stored at 4 ℃ for further use. The immobilization essay onto supports was repeated three times.

2.2.2 Enzyme assay and dry matter measurements

The hydrolytic activities of free and immobilized lipases were measured by a titrimetric method previously described by [23, 24] using olive oil (Extra Vierge Bertolli, Italy) as substrate. Activities are expressed in international units (IU), where one unit of lipase is the amount of enzyme able to catalyse the release of 1 µmol of fatty acid per min at pH 7 and at 37 °C.

Dry matter in liquid and solid phases was measured after desiccation at 105 °C over a period of 48 h u ntil reaching a constant mass.

2.2.3 Immobilization yield

Immobilization yield was defined as follows:

$$\text{Immobilization yield (\%)} = \left(\frac{a_{imm}}{a_{free}}\right) \times 100 \qquad [25]$$

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

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 [26].

2.2.5 Particle charge detector

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

2.2.6 Interesterification reaction

Lipase immobilized on Celite (2g) was used to understand an interesterification reaction between 70% milk fat and 30% rapeseed oil in micro-aqueous conditions in comparison to a commercial *Thermomyces lanuginosus* lipase Lipozyme® TL IM (2g). The mixture was incubated for 24 h in a double jacketed 200-mL glass vessel. Water was circulated through the jacket from a constant temperature bath (60 ℃). The mixture was stirred magnet ically at 300 rpm throughout the reaction. After the reaction, the mixtures were filtered on Whatman paper number 1(Fluka Biochemika, Switzerland) to remove the enzyme. The products were used in subsequent analysis. Interesterification protocol was previously described [27].

- Determination of triglycerides (TAG) species

Isolated TAG was also analyzed by high-temperature GC for the TAG species according to the CN (carbon number). For analysis of TAG, 0.5 mL of a solution diluted in hexane (6 mg/mL) was injected into HP 6890 GC (Hewlett-Packard Company, Wilmington, DE, USA) equipped with FID. Runs were performed from 180 to 360 ℃ at a rate of 3 ℃/min and then isothermally at adequat e time. A capillary column CP-Sil5 CB (10 m´0.32 mm i.d.) with a film thickness of 0.12 mm (Chrompack, Middleburg, and The Netherlands) was used. Injection and detector temperatures were 183 and 350 ℃, respectively. Hel ium was used as a carrier gas at a flow rate of 0.9 mL/min. Identification of the TAGs was made by comparison of retention times with those of TAG standards. Results were expressed as area% without any corrections. Analyses were performed in duplicate and mean values are reported [27].

3. RESULTS AND DISCUSSION

3.1 Immobilization yields

The immobilization of YY Lipase was carried out on three matrixes such as Celite, Silica gel and Avicel. Immobilized lipase enzymatic activity was determinate by tritimetric assay. The highest immobilization yield (70%) was obtained with Celite and the lowest (29%) with Silica gel, (Table 1). The higher activity with Celite could be attributed to greater affinity between lipase and Celite, than Silica gel. Several studies reported 76% of *Yarrowia lipolytica* lipase immobilized on celite and 35 % onto Silica gel [21]. 22.3% of *Candida rugosa* lipase activity immobilized on celite compared to 14.8% for silica gel [28].

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%, those of matrixes without lipase ranged from 0.08 to 6.84% and water activity was ranged from 0.4 to 0.6. The Silica gel had and Avicel had the highest moisture content (6.84% and 6.55%). The presence of the water molecule could be the cause of low-fixing enzyme on this support. In presence of high water content, lipase may become inactive or its conformation can change [29]. In according to 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 enzyme fixation. In spite of Avicel (microcrystalline cellulose) has high water content, the enzyme fixation on this matrix is strong, because of its characteristic topography.

3.3 Measurement of particle charge

The electrical potential between lipase and matrix was studied by 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 (1ml to 7ml) in order to obtain saturation.

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

At these pHs values lipase from *Y. Lipolytica* is around to its isoelectric points (5.1 to 5.3) [23]. 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 [29] 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.

Figures 2 and 3 show the evolution of the electrical charges for free lipase dissolved 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 (0.08) 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 [27, 30].

Table 3 presents the values of IA (interesterification activity) and hydrolytic activity of free YYL, YYL immobilized on Celite and the lipozyme TL IM. The results show that free YY lipase hydrolytic activity was high, but its interesterification activity was low than the others. We noted that when enzyme is immobilized, its IA increases because immobilization of lipases protects the enzyme from solvent denaturation, enhance the enzyme thermostability and facilitates their use in continuous processes [11].

Figure 4 presents the profiles of triglyceride (TAG) species of the initial blend and after its interesterification by both enzymes. The profiles of triglyceride species of the initial blend show the same evolution. The TAG can be grouped according to their CN, which is the number of carbons in the constituent fat Acid. TAG species were determined at the end of 2, 4, 6, 8, 12 and 24 h of the reaction. As a result of these changes, the relative percentage of several TAG species (CN42-50) increased, while others (CN 30, 32, 34, 36, 38, 40 and 52) decreased. However, a significant increase was observed between 6 and 8 h of reaction.

The value of the ID (interesterification degree) which represents the ratio CN50/CN42 with YYL 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 YYL 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.

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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 (◆).

Table 1

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

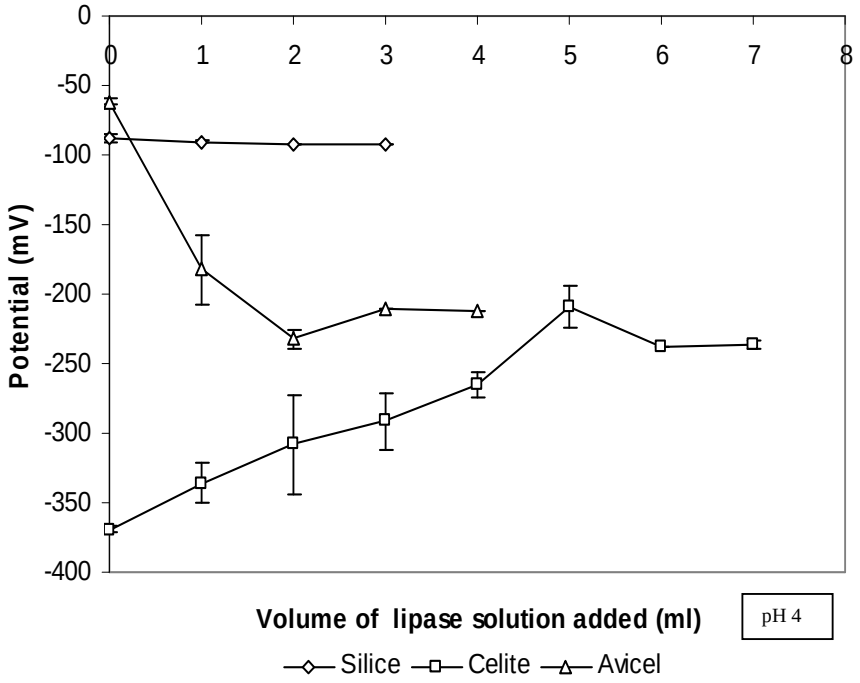
Table 2

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 + Silica gel	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

Table 3

	Hydrolytic activity IU/mg/min	**Interesterification activity IU/mg/min**
Lipozyme TL IM	47142.9 ± 6890.5	1.741 ± 0.048
YYL on celite	5476.2 ± 628.6	0.186 ± 0.011
Free YY Lipase	17552.1 ± 1815.4	0.077 ± 0.015

Fig. 1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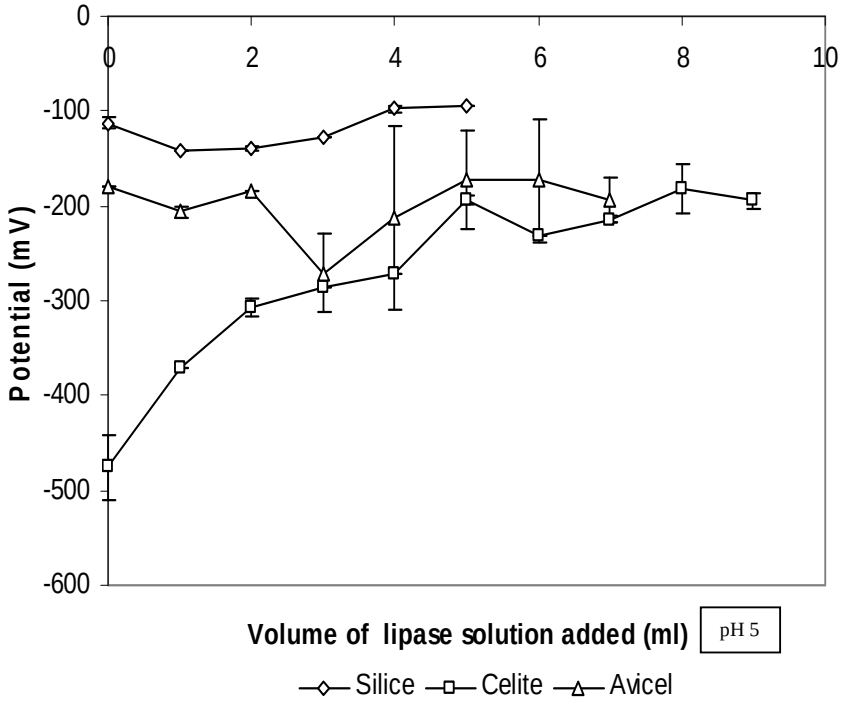
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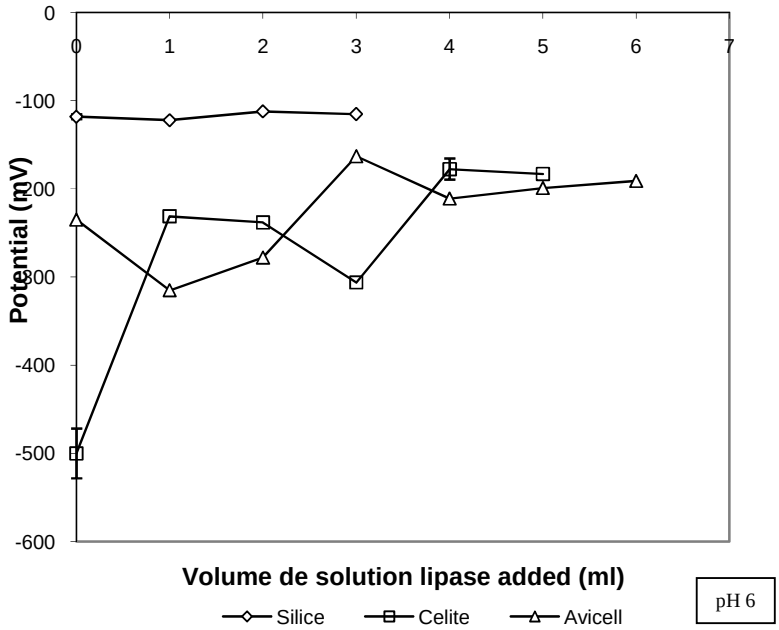
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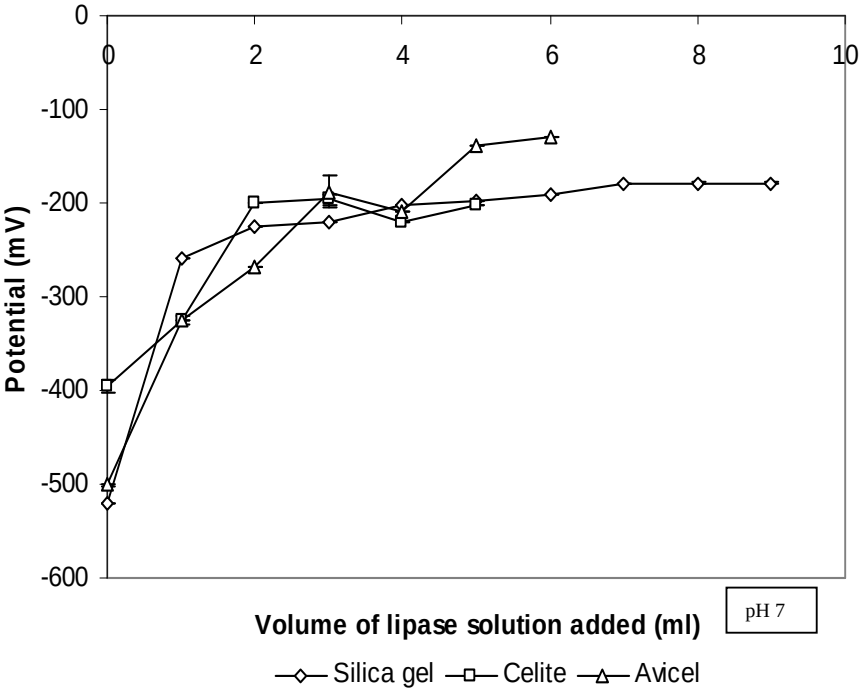
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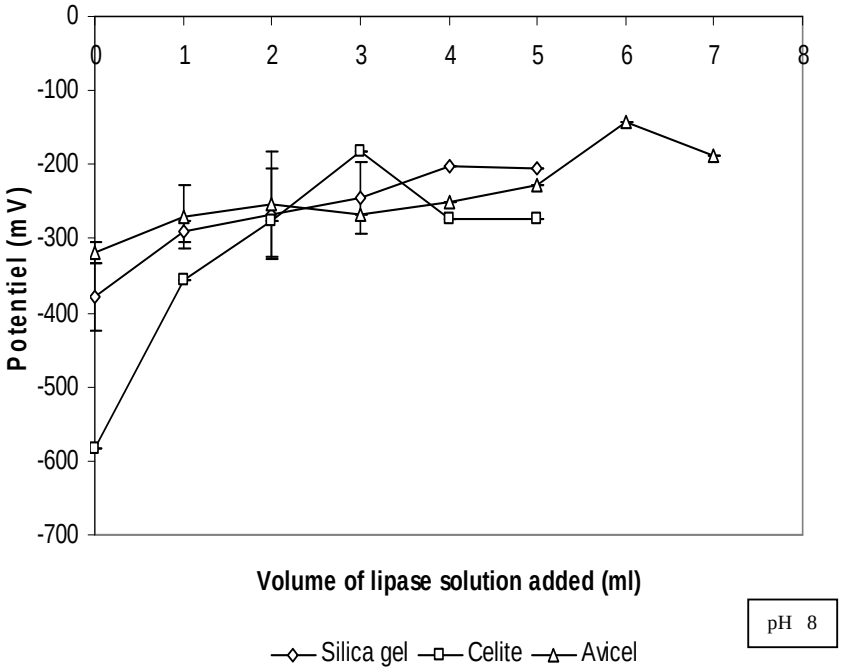
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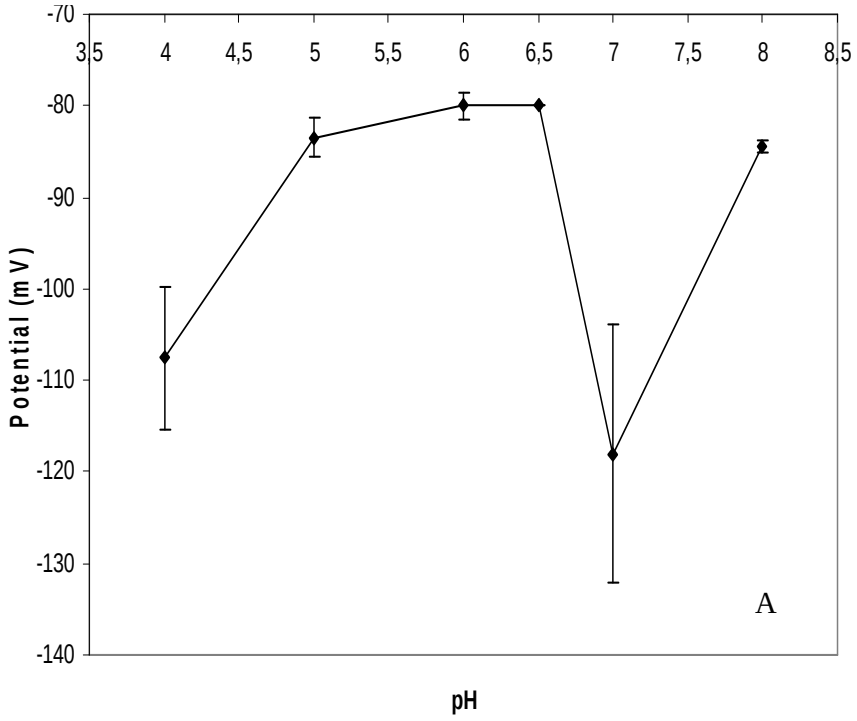
Fig. 2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pH

Fig. 3

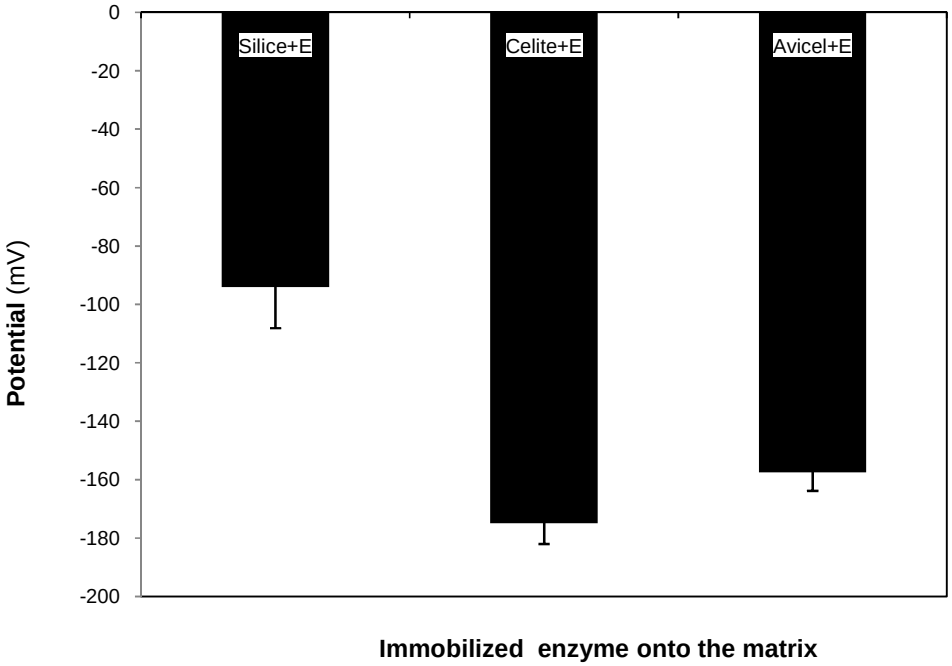

0
-20
-40
-60
-80
-100
-120
-140
-160
-180
-200
Silice+E
Celite+E
Avicel+E
Potential (mV)
Immobilized enzyme onto the matrix

Fig. 4

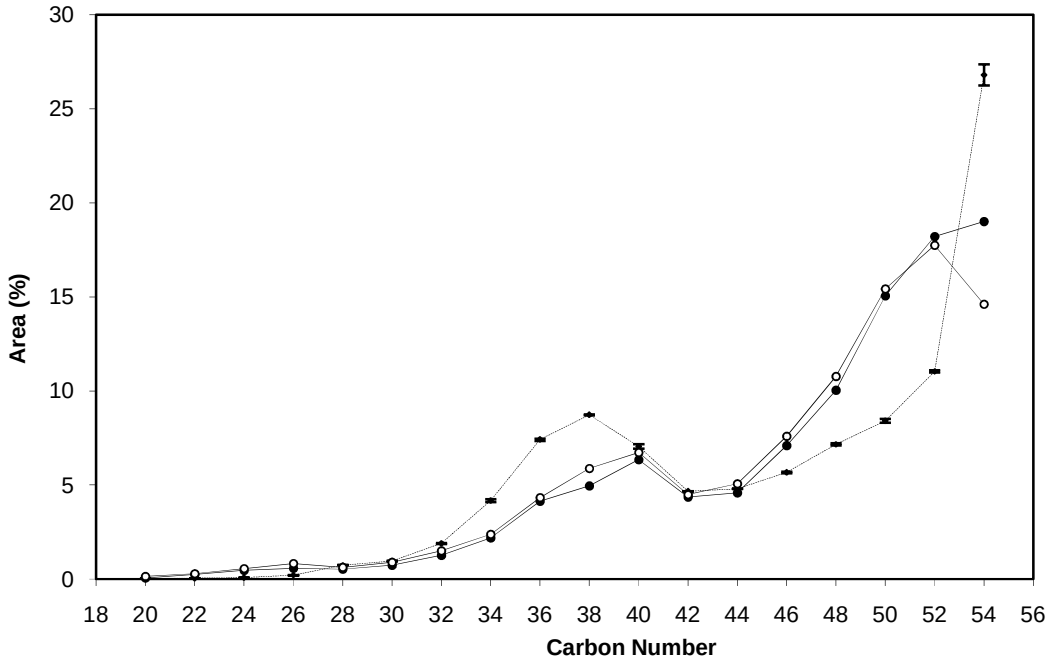
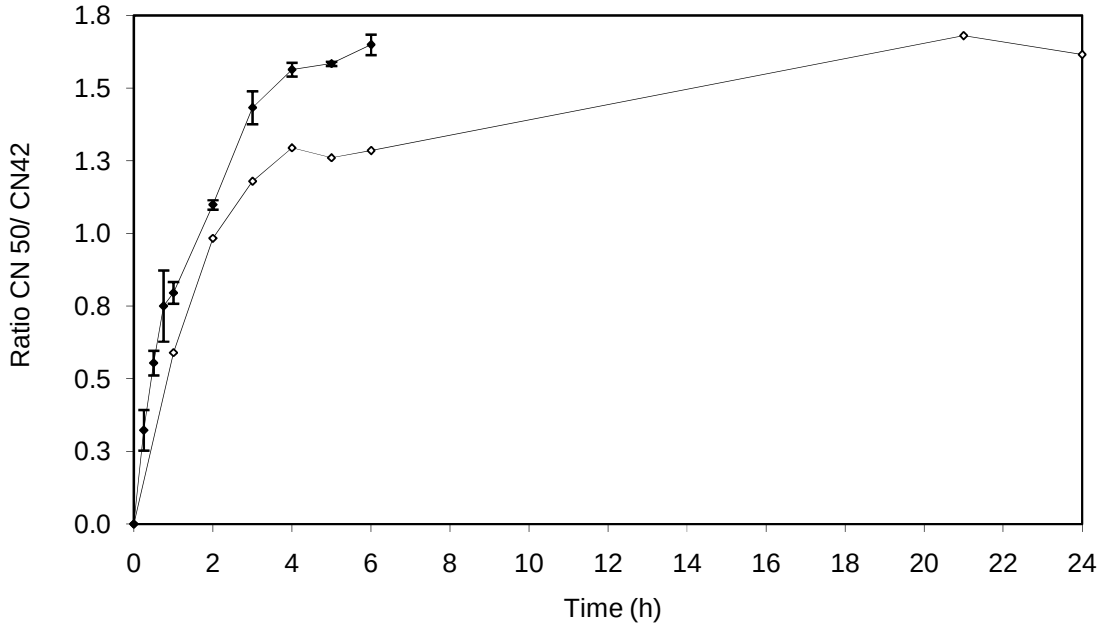

Area (%)
Carbon Number
0
5
10
15
20
25
30
18
20
22
24
26
28
30
32
34
36
38
40
42
44
46
48
50
52
54
56

Fig 5


Ratio CN 50/ CN42
Time (h)
0.0
0.3
0.5
0.8
1.0
1.3
1.5
1.8
0
2
4
6
8
10
12
14
16
18
20
22
24