International Journal of Engineering

Journal Homepage: www.ije.ir

Synthesis and Characteristics of Mesoporous Sol-gels for Lipase Immobilization

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PAPER INFO

Paper history:
Received 22 February 2014
Received in revised form 07 June 2014
Accepted 26 June 2014

Keywords:
Enzymes
Sol-gel Process
Hydrolytic activity
Immobilization
Kinetics
Mesoporous

ABSTRACT

Enzyme cost is the major problem for industrial scale application. Immobilization is a promising approach to moderate the enzyme cost factor and increase its stability and activity. In this study, sol-gel method was used to prepare the immobilization platform and entrapped lipase as one of the most used enzyme in dairy processing, cosmetics and pharmaceutical industries. Lipase from *Candida rugosa* was immobilized onto glycidoxypropyltrimethoxysilane(GPTMS) and tetramethoxysilane (TMOS) derived sol-gels and its characteristics and hyrdrolytic activity were investigated. Michaelis-Menten kinetic properties reveal that although free enzyme can catalyze the reaction faster, but it has lower affinity for substrate molecules compared to sol-gel immobilized lipase. Entrapped lipase retained 67 % of its initial activity after six reaction cycles. It showed 100% activity compared to free lipase powder at 40-45°C. In pH 9, as free enzyme lost 90 % of its initial activity, immobilized lipase lost only 29% of its activity. Immobilized enzyme was more stable toward different pHs (100% activity at pH 7.5 compared to free form). Morphological characteristics of the immobilized enzyme were investigated by SEM images and BET. The sample had specific surface area and mean pore diameter of 2.599 m^2/g and 46.13 nm, respectively.
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***doi**: 10.5829/idosi.ije.2014.27.10a.02*

1. INTRODUCTION

Lipases are capable of catalyzing both hydrolysis and esterification reactions. They are categorized as glycerol ester hydrolyses (E.C.3.1.1.3) and can be found in plants, animals and microorganisms. Microbial lipases are more stable, and therefore used more frequently in industrial scale. They are hydrophobic protein molecules acting on carboxylic esters like triglycerides at the oil/water interface. They consist about 4% of total $600 million enzyme market [1].

One of the most important applications of lipase is free fatty acids and glycerol production. These are the raw materials in food, pharmaceutical and cosmetics industries. Conventional methods to produce free fatty acids and glycerol utilize high temperature and pressure (250°C and 50 bar) for two hours which consume high amounts of energy (high energy intensive) and require complicated reactor design and extra processes to separate the products [2]. With lipase as the hydrolytic catalyst, there would be no need for all these complicated processes. Free fatty acids can be synthesized under mild conditions with less waste materials. As lipases can partially hydrolyze triglycerides, it can be used in food industry for flavor enhancement of dairy products or speeding up the cheese ripening process, the product is called enzyme modified cheese (EMC). Lipase is used in detergent industry to lower the washing temperature and remove oily stains, or in paper industry to remove fatty pitches from wood chips. However, free lipases are unstable in alkaline environment. Therefore, it would be more applicable if its pH stability increases in alkaline conditions. Lipase, together with protease is used for leather manufacture to remove fats from hides. Lipases through inter esterification produce monoacylglycerol. It is one of the most famous emulsifiers used in drug, food and cosmetics industries (60,000 tons annually) [3-5].

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Please cite this article as: M. Nickpour, M. Pazouki, Synthesis and Characteristics of Mesoporous Sol-gels for Lipase Immobilization,International Journal of Engineering (IJE),TRANSACTIONSA: Basics Vol. 27, No. 10, (October 2014)1495-1502

Economic issues make some enzymatic reactions hard to apply in industrial sectors; moreover, the separation process is complex due to smallness of enzyme molecules (separation through dialysis) [6]. To overcome these problems, immobilization seems an effective way to moderate enzyme cost, solve reusability issues as it is easily separated by simple filtration at any specific level of reaction and have more stable and higher activity against harsh reaction conditions (pH, temperature and solvents). Enzyme immobilization defines as the attachment of the enzyme onto a solid support to limit its movement in space. Based on the enzyme type and its application, immobilization technique is different [7, 8].

Sol-gel method can be applied for entrapment, encapsulation and adsorption and covalent immobilization method, based on the preparation formula and process. Sol-gel involves the capture of lipase (or any other biomolecules) within the matrix of a polymer. Since the process is conducted at room temperature, lipase can retain its initial activity and is dispersed evenly due to gel phase nature. Sol-gel has been reported to be the most successful immobilization technique [9-11].

Hydrolysis:

$\equiv\equiv Si–OR + H_2O \Rightarrow \equiv\equiv Si–OH + ROH$

Condensation:

$\equiv\equiv Si–OH + HO–Si\equiv\equiv \Rightarrow \equiv\equiv Si–O–Si\equiv\equiv + H_2O$

Sol-gel entrapment of enzymes was first introduced by Avnir in 1995. TMOS ($Si(OCH_3)_4$) was used as a gel precursor in the described procedure to immobilize biomolecules in general [12]. Reetz immobilized lipase from *Ps. cepacia* (Amano PS) using TMOS, results showed only 5% activity compared to free enzyme. Tetraethoxysilane (TEOS) and polyvinyl alcohol (PVA) used for immobilization yielded 28% activity compared to free enzyme. But, when another precursor with an alkyl group like methyltrimethoxysilane (MTMS) was investigated, the activity increased up to 52% compared to the free form; and as the chain length of this alkyl group increased (up to 20 carbon atoms), the activity of immobilized lipase improved [13, 14]. This phenomenon is caused by the nature of lipase that is an interphase-active enzyme, it means that there should be hydrophobic and hydrophilic phases at the same time to open the enzyme lid and change into active catalyst. SiO_2 matrix provides the hydrophilic phase due to the remaining silanol groups and the alkyl group and silicon dioxide would provide hydrophobic phase. Therefore, sol-gels have the advantage of spatial orientation besides providing a suitable environment for the enzyme [11].

Persson et al. prepared sol-gels using propyl-trimethoxysilan (PTMS) and TMOS precursors and *Humicolalanuginosa* lipase and the results showed 22.5% activity compared to crude lipase [15]. Chen and Lin explained PVA effects on enzyme activity as it protected lipase against environmental conditions [16]. Reetz et al. immobilized different lipases in different alkyl modified silanes and additives, according to enzyme activity assay each lipase has its own optimal silane precursor and additive and there is no strict and general procedure for all enzymes [17]. Noureddini et al. studied *Candida rugosa* immobilized ontoiso-butyltrimethoxysilane (iso-BTMS) and TMOS derived sol-gels. Results indicated that as the specific surface area of sol-gel increased, the catalytic activity decreased [18]. Meunier and Legge studied double immobilization of lipase (NS44035), first onto PTMS/ TMOS sol-gels and then on Celite® support. Lipase activity was investigated through transesterification of triolein; 60% conversion was reported in six hour reaction [19, 20].

To improve enzyme activity, other alkoxysilane derivatives like $RSi(OMe)_3$ (R could be an alkoxy or an aryl group instead of an alkyl one) can be used instead [14]. In this study, lipase from *Candida rugosa* was first immobilized onto GPTMS/TMOS sol-gels and then the characteristics were studied using SEM and BET. The hydrolytic activity of immobilized lipase was also studied at different temperatures and pHs to find the optimal conditions for further applications. Reusability of the enzyme was also determined to see how the activity was affected after each use. The scientific research conducted so far has not reported the effect of alkoxy modified sol-gels on the morphology and activity of the enzyme.

$$RSi(OCH_3)_3 + Si(OCH_3)_4 \xrightarrow{\text{Lipase}} \text{Immobilized Lipase}$$

2. MATERIALS AND METHODS

3-(2,3-epoxypropoxy) propyltrimethoxysilane(GPTMS), tetramethylorthosilicate (TMOS), polyvinyl alcohol (PVA) were purchased from Merck Millipore (Darmstadt, Germany), Lipase from *Candida rugosa* was purchased from Sigmaaldrich Company. All phosphate buffers were prepared using the sodium salt. Other chemicals used for the synthesis were of analytical grade.

2. 1. Sol-gel Immobilization Procedure Sol-gels were prepared according to Reetz and Hara with some modifications; 552 µl of GPTMS and 148 µl of TMOS as precursors were mixed with acidic water (HCl (0.1 M) and water, pH 1.25) to obtain water to silane molar ratio of 1:4. The mixture was sonicated for one hour to have a clear, homogeneous solution using Ultrasonic bath (Wiseclean® model WUC-D10H) until a clear single phase solution was formed and no alcohol smell was detected anymore. Then, the partially hydrolyzed sol was put in an ice bath to cool down and complete the hydrolysis stage. Then, 1.2 ml of the lipase solution

(100 mg/ml phosphate buffer 0.1 M, pH 7.0) was added. The mixture was vigorously shaken until gelation occurred and then it was moved to a Petri dish and dried at room temperature for 24 hours. The immobilized lipase was then broken up using a spatula. The immobilized enzyme was washed three times with isopropanol and water and centrifuged at 27,167 x g (9,000 rpm) for 15 minutes (Hettich ZENTRIFUGEN-Rotina 380). The supernatant was collected to be analyzed for potential free enzyme content. The immobilized enzyme was dried at room temperature, then it was crushed in a mortar and pestle and stored at 4 °C [21, 22].

2. 2. Sol-gel Characterization

2. 2. 1 Morphological Characteristics General morphological characterization was investigated by scanning electron microscopy (SEM) (S360 Cambridge 1990) at an accelerating voltage of 20 kV. BET method was used to estimate morphology, specific surface area, mean pore diameter and total pore volume of the sol-gels. The nitrogen adsorption experiments were carried out at 77 (K) in Microtrac BELSORP-MINI II, USA. The adsorption/desorption data were processed through the BELSORP analysis program.

This software provides the plot of the adsorption isotherms, fits the adsorption data into the Brunauer-Emmett-Teller (BET) model and estimate specific surface area and porosity of the tested material. Manipulation of the desorption isotherms with this software provides a fit of the data to other models such as Barrett, Joyner and Halenda (BJH) model, which ultimately renders information about the pore size and the pore size distribution of the tested material. To evacuate the residual water, prior to BET measurements the sample was heated at 200°C under high vacuum for 90 minutes.

2. 2. 2. Hydrolytic Activity Assay Lipase activity was determined by assessing the amount of free fatty acids liberated during the hydrolysis process which can be determined through titration of olive oil emulsion with 0.1 M potassium hydroxide (using phenolphthalein as the indicator). 10 mg of enzyme (immobilized or free) was placed into a 15 ml Falcon tube and 100 µl of phosphate buffer (different pHs based on the experiment type) plus 1 ml of substrate (olive oil and gum arabic) were added.

The reaction mixture was placed into a shaker incubator (200 rpm agitation speed) for 30 minutes, then the reaction was stopped by addition of 0.1 ml acid (concentrated sulfuric acid 1 ml and hexane/isopropanol [1:5] 10 ml). The mixture was titrated with potassium hydroxide, and a blank titration is done as control sample and all the experiments were replicated three times [14].

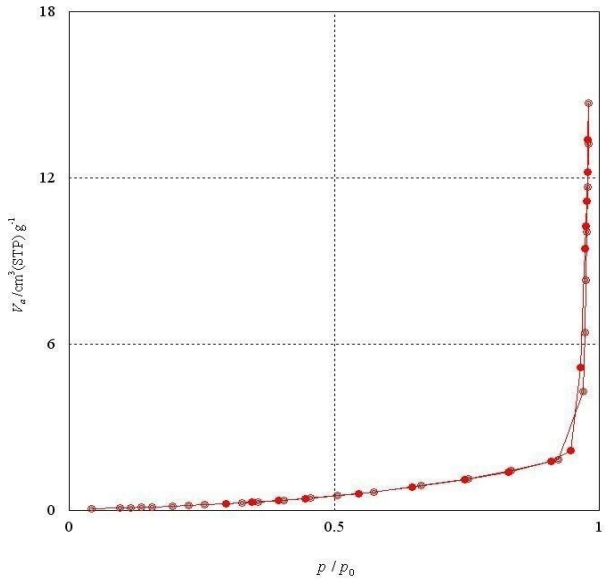

Figure 1. Adsorption/desorption isotherm curve (isotherm Type III)

3. RESULTS AND DISCUSSION

3. 1. Morphological Characterization The Brunauer–Emmett–Teller (BET) theory to determine the surface area and the Barret–Joyner–Halenda (BJH) theory for the size distribution was based on the fact that: A porous solid is capable of adsorbing a large volume of condensable gas after the gas molecules have penetrated the porous material, which would result in a gradual decline of the gas pressure (which is equivalent to the amount of gas adsorbed).

According to Figure 1, the synthesized powder belongs to adsorption isotherm type III. This type is characterized by heats of adsorption of less than the adsorbate heat, meaning that adsorption proceeds as the adsorbate interaction with an adsorbed layer is greater than the interaction with the adsorbent surface. Isotherm type III is formed due to the presence of enzyme molecules that covers most of the pores [23].

The average pore diameter is about 46.13 nm. Low specific surface area can be due to high enzyme loading which would result smaller amounts of empty pores. The degree of immobilization test [17] also confirms this as it revealed that 83.3 % the enzymes were entrapped inside the mesoporous structure of the sol-gels.

3. 2. SEM SEM micrographs of the sample from two different angles are shown in Figure 2. These micrographs reveal the disordered and porous structure of the immobilization platform. Lipases are bonded by PVA (cross-linked), which would not only protect the enzyme against denaturation during the immobilization process, it also acts as a bridge to connect the enzyme

molecules together and form a mass that would fit into the support pores. This would somehow count as "double immobilization", it increases substrate and enzyme interface which would result higher activity.

3. 3. Hydrolysis Kinetics Study The hydrolysis kinetic was studied by finding the initial rate when the substrate concentration in the reaction mixture varied. The Michaelis-Menten kinetic equation was used to find kinetic parameters. The double reciprocal plot (Lineweaver-Burk plot) of reaction rate was used to evaluate the Michaelis-Menten constant (K_m) and maximum velocity (V_{max}). A plot at $1/V_0$ against 1/[S] yielded a straight line (Figure 3).

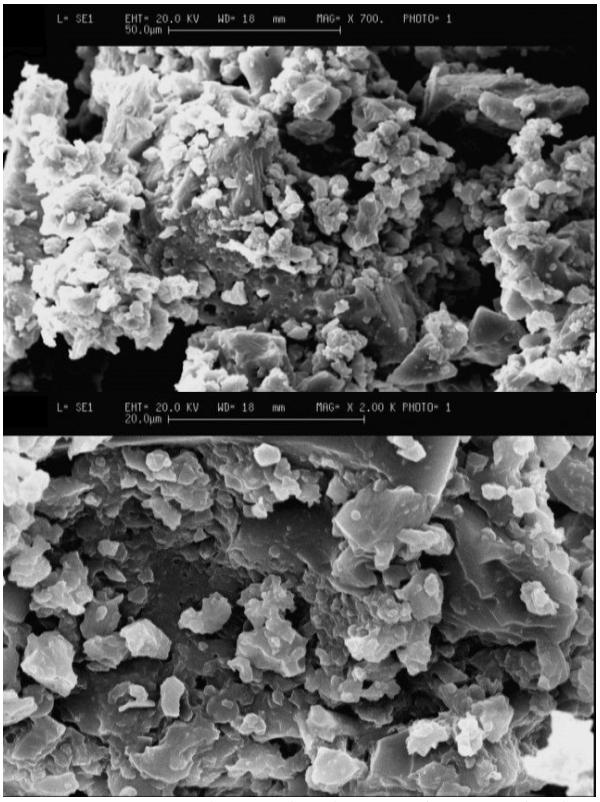

Figure 2. SEM micrographs of GPTMS/TMOS sol-gel

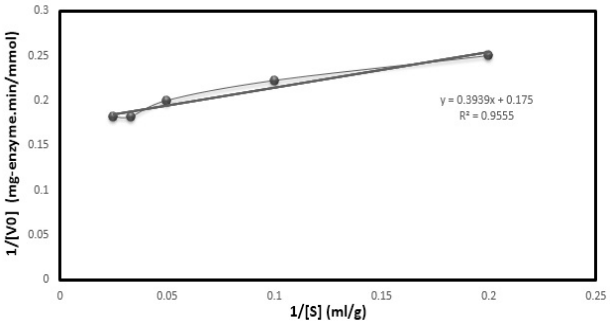

Figure3. Lineweaver-Burk plot of reaction rate and olive oil concentration (Temperature: 40°C, pH: 7.5)

TABLE 1. Kinetic studies of the immobilized enzyme-experimental data and SigmaPlot® prediction

Substrate concentration (g/ml)	Experimental Initial velocity(mmol/min mg-enzyme)	SigmaPlot® Predicted Initial velocity
0.05	4	3.8829
0.10	4.5	4.6483
0.20	5	5.1565
0.30	5.5	5.3515
0.40	5.5	5.4547

TABLE 2. Enzyme Kinetics Module - SigmaPlot® data sheet according to experimental data

Parameter	Value	±Std. Error	95% Confidence Interval
V_{max}(mmol/min mg-enzyme)	5.7895	0.1580	5.2866 -6.2924
K_m(mM)	27.726	4.7762	12.5363 -42.9283

From the values at slope and intercept, the values of K_m and V_{max} were determined. These values especially K_m varies for different lipase sources or for different substrates of the same enzyme. Substrate concentration varies from 5 to 40% (w/v). The analysis results were double-checked by Enzyme Kinetics Module - SigmaPlot® software (Table 1 and 2). Results are in conformity with the values obtained from Figure 3. The Michaelis-Menten Equation:

$$\frac{1}{V} = \frac{K_m + [S]}{V_{max}[S]} = \frac{K_m}{V_{max}}\left[\frac{1}{[S]}\right] + \frac{1}{V_{max}}$$

where;
V_{max}= Initial velocity (mmol/min.mg-enzyme)
K_m= the Michaelis-Menten constant (g/ml)
S= Substrate concentration (g/ml)

The same procedure has been conducted for free enzyme and K_m and V_{max} values were 31.7 mM and 6.37 (mmol/min.mg of free enzyme),respectively. V_{max} reflects how fast an enzyme can catalyze a reaction and K_m value indicates the enzyme affinity for the substrate. In this case, V_{max} and K_m are higher for free enzyme which means that it can catalyze the reaction faster (it is natural because the enzyme content in the reaction media is lower after immobilization) and it has a lower enzyme affinity. Hence, more substrate molecules must be present to saturate free enzyme.

3. 4. The Effect of Temperature on Hydrolytic Activity of Lipase The temperature was varied from 20°C to 60°C to observe the changes in fatty acid formation (Figure 4). Free *Candida rugosa* lipase showed 83% and 92% activity loss at 50°C and 60°C, but the immobilized lipase only lost 9% and 12% of its activity at these temperatures. There is a shift of the

optimum temperature from 35°C to 40°C after immobilization. The activity of the immobilized lipase remained at the maximum level in the range of 40-45°C and then decreased afterward with a slight trend. As free lipase lost its activity sharply after 40°C. Immobilization seems to protect the enzyme in temperature over 45°C. That is because immobilization fixes the enzyme in a particular orientation, so restricted movement can reduce the degree of unfolding and denaturation.

Temperature can increase the hydrolytic activity, but at very high temperatures, enzyme structure is disrupted. The shape of the enzyme can be influenced by temperature as it relies on relatively weak non-covalent bonds (e.g., hydrogen bonds, van der Waals forces, and ionic bonds) to link different regions of the enzyme together. Increasing temperature causes more random movements in different regions of the enzyme, thus these weak bonds are destabilized and the shape of enzyme protein changes (denaturation). If these weak bonds break, the shape of the active site will begin to distort too, and the enzyme will lose its ability to bind substrate and catalyze the reaction.

3. 5. The Effect of pH on Lipase Activity

Experiments were conducted in the pH range of 6.5-9. A change in pH can alter the ionization states of enzyme, which affects its activity and selectivity. According to Figure 5, the optimum pH range for both free and immobilized enzyme was 7.5. Immobilized lipase was stable in the pH range of 6.5-8.

Since the side chains of lipase amino acids have net charges, they can form ionic interactions with one another, where like charged side chains repel one another and opposite charged side chains attract one another. Under acidic conditions (high [H+]), hydrogen ions tend to bind to negatively charged side of amino acid chains (COO^-), so the side chain loses its negative charge. Under alkaline conditions (low [H+]), hydrogen ions tend to dissociate from positively charged side of amino acid chains (NH_3^+), so the side chain loses its positive charge. This would change the structure of the enzyme because the catalytic ability of an enzyme is so tightly linked to the specific shape and chemical properties of its active site, alteration of normal ionic bonding patterns within the enzyme tends to reduce catalytic function.

Immobilization protected the enzyme in different pHs, as in pH 9, free enzyme lost 90 % of its initial activity, while immobilized enzyme lost 29%. This is an important property for lipase, especially for detergent production because free lipase loses its activity sharply in the washing alkaline conditions. Immobilized enzyme was almost stable in different pHs. This was due to the nature of sol-gel support which surrounded the enzyme so that the lipase was restricted and its structure did not change.

3. 6. Reusability of the Immobilized Enzyme

This procedure is designed to determine the stability and reusability of the immobilized enzyme. Recyclability is one of the main benefits of an immobilized enzyme. While the free form cannot be used for another reaction cycle. After each hydrolysis reaction, the substrate and lipase are separated and subsequently reused.

According to Figure 6, immobilized enzyme still retains 67% of its activity after six runs. No activity loss was observed between runs one and two, but afterwards immobilized enzyme lost 16.66% of its activity between runs 2 and 3, 5 and 6, which could be due to the inactivation of the enzyme, denaturation of protein and leakage of protein from the supports after multiple uses.

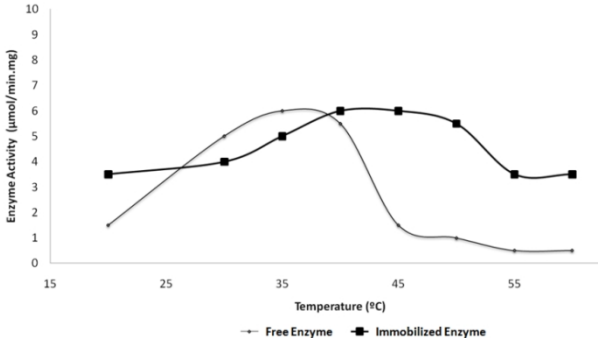

Figure 4. The effect of temperature on hydrolytic activity of lipase – comparison between free and immobilized form (pH: 7.5)

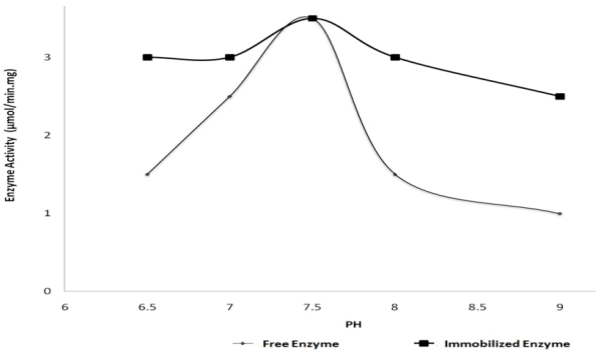

Figure 5. The effect of pH onlipase activity – comparison between free and immobilized form (Temperature: 40°C)

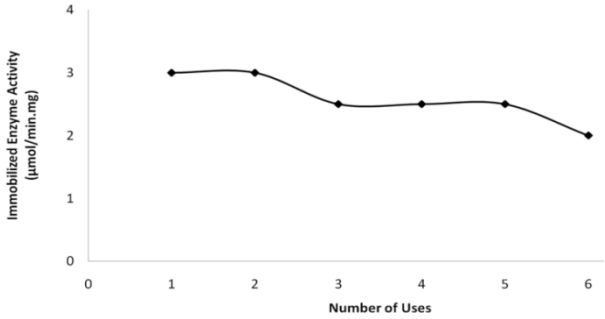

Figure 6. Reusability of immobilized enzyme (Temperature: 40°C, pH: 7.5)

4. CONCLUSION

Lipase from *Candida rugosa* was first entrapped in GPTMS/TMOS sol-gels. SEM micrographs revealed the disordered structure of the synthesized platform for lipase immobilization. According to adsorption /desorption isotherm curve, sol-gel belonged to the adsorption isotherm type III.The average pore diameter is about 46.13 nm according to the BJH method, so the sample is categorized as mesoporous material. Low specific surface area can be due to high enzyme loading which would result smaller amount of empty pores. The immobilized lipase showed higher activity at higher temperature (above 40°C). One of the main problems with crude lipase is its sensitivity in alkaline conditions, but immobilized enzyme seems to retain its activity in higher pHs. Immobilization improved enzyme activity in alkaline conditions.Kinetic properties K_m and V_{max} were lower for immobilized lipase, which reveals that although immobilized enzyme cannot catalyze the reaction as fast as free form, but it has a higher affinity for substrate. Immobilized lipase was reused for six times and thereafter lost 33% of its initial activity.

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P A P E R I N F O

Paper history:
Received 22 February 2014
Received in revised form 07 June 2014
Accepted 26 June 2014

Keywords:
Enzymes
Sol-gel Process
Hydrolytic activity
Immobilization
Kinetics
Mesoporous

چکیده

قیمت بالا به‌عنوان چالش اصلی برای استفاده ان در مقیاس صنعتی مطرح است. در سال‌های اخیر، تثبیت آنزیم به‌عنوان راهکاری برای متعادل‌سازی هزینه و بهبود فعالیت و پایداری آنزیم بررسی شده است. در این تحقیق، روش سل-ژل به‌منظورآماده‌سازی پایه و تثبیت و محبوس سازی آنزیم لیپاز به‌عنوان یکی از پرکاربرد ترین بیوکاتالیست‌ها در صنایع غذایی، داروسازی و تولید محصولات آرایشی بهداشتی بررسی شده است. آنزیم لیپاز تهیه‌شده از منبع میکروبی کندیداروگوسا ابتدا روی سل ژل سنتز شده از گلایسیدوکسیپروپیل تری متوکسی سیلان (GPTMS) و تترامتوکسی سیلان (TMOS) تثبیت‌شده و سپس فعالیت و مشخصات ساختاری آن مورد بررسی قرار گرفت. سینتیک واکنش هیدرولیز روغن توسط آنزیم لیپاز نیز بر اساس مکانیسم میکائیلیس – منتن مشخص شد، که بر اساس آن آنزیم آزاد سریع‌تر واکنش را کاتالیز می‌کند اما تمایل کمتری به مولکول سوبسترا دارد. آنزیم محبوس شده پس از شش دوره واکنش ۳۳ درصد فعالیت اولیه خود را از دست داد. فعالیت نسبی آنزیم تثبیت‌شده در بازه دمایی ٤٥-٤٠ درجه سانتی‌گراد، میزان ۱۰۰٪ بود. در pH=۹، آنزیم آزاد ۹۰ درصد فعالیت خود را از دست می‌دهد، در حالیکه در همان شرایط آنزیم تثبیت‌شده، تنها ۲۹٪ فعالیت اولیه خود را از دست می‌دهد. این ویژگی برای کاربرد آنزیم در تولید صنعتی شوینده‌ها با توجه به شرایط قلیایی شستشو حائز اهمیت است. آنزیم تثبیت‌شده در pH های مختلف دارای ثبات فعالیت بیشتری بوده (۱۰۰٪ فعالیت نسبی در pH =۷/۵). مورفولوژی و خصوصیات کاتالیست تثبیت شده توسط آنالیزهایSEM و BET بررسی شد. سل ژل سنتز شده به همراه آنزیم لیپاز دارای سطح ویژه ۲/۵۹۹ مترمربع بر گرم و میانگین قطر تخلخل ٤٦/۱۳ نانومتر است.

***doi**: 10.5829/idosi.ije.2014.27.10a.02*