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Monoolein production by triglycerides hydrolysis using immobilized *Rhizopus oryzae* lipase

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ABSTRACT

Lipase extracted from *Rhizopus oryzae* was immobilized in alginate gel beads. The effects of the immobilization conditions, such as, alginate concentration, $CaCl_2$ concentration and amount of initial enzyme on retained activity (specific activity ratio of entrapped active lipase to free lipase) were investigated. The optimal conditions for lipase entrapment were determined: 2% (*w*/*v*) alginate concentration, 100 mM $CaCl_2$ and enzyme ratio of 2000 IU/mL.In such conditions, immobilized lipase by inclusion in alginate showed a highest stability and activity, on olive oil hydrolysis reaction where it could be reused for 10 cycles. After 15 min of hydrolysis reaction, the mass composition of monoolein, diolein and triolein were about 78%, 10% and 12%.

Hydrolysis' products purification by column chromatography lead to a successful separation of reaction compounds and provide a pure fraction of monoolein which is considered as the widest used emulsifier in food and pharmaceutical industries.

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1. Introduction

Monoacylglycerols (MG) of saturated or unsaturated fatty acids are the most commonly used surfactants in the food industry [1]. They are widely employed also in preparation of cosmetic and pharmaceutical products, manufacture of alkyd resins and detergents [1]. They are normally produced by alcoholysis of the corresponding triacylglycerols with glycerol in the presence of metal catalysts at temperatures of 210–240 °C [2]. However, yields of the desired compounds are usually low, and the resulting products are frequently collared and odiferous. Other disadvantages of the chemical processes include the polymerization of unsaturated fatty acids at high temperatures and the laborious purification steps to produce regioisomeric pure products [3].

Monoacylglycerols can be produced by enzymatic methods as lipase catalysis instead of inorganic catalysts which can be accomplished at low temperature, prevents side's products formation and save energy [4]. Different approaches have been reported in the literature described lipase catalysis for MAG production by esterification of free fatty acids (FFA) with glycerol [5], glycerolizes of oils [6] and hydrolysis or alcoholysis of triglycerides [7].

However, the use of free lipases in industrial practice was limited, mainly due to high cost of lipases, their instability and irrecoverability. Immobilization is one of the strategies broadens the scope for using these enzymes which lead to greater product quality, cleaner processes, economic operational costs, and so allows to circumvents limitations of the use of free lipases. Several immobilization techniques have been studied and utilized to contribute to the development of continuous processes, and immobilized enzymes are adaptable to a variety of configurations and specific processes carried out in reactors [8]. Multiple techniques used for lipase immobilization have been applied in previous works such as physical adsorption [8], covalent bonding to a solid support [9] and physical entrapment within a polymer matrix support [10].

Entrapment presents the advantage of being simple, carried out at room temperature and in the absence of organic solvents.

Alginate has been reported to be non-toxic, biocompatible and amenable to chemical modification and highly affinitive to protein due to its hydrophilic nature [11]. Due to all this useful proprieties, this support has been used in many biotechnology applications such as vehicles for cells and molecules [12] scaffolds for tissue engineering, [13] and modified with RGD-containing peptide sequences [14].

In this work, we performed the enzymatic production of MG by olive oil hydrolysis, this reaction occurs in aqueous medium in

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absence of organic solvents which presents an advantage for the production of MG for cosmetic and pharmaceutical industry.

Rhizopus oryzae lipase (ROL) has been immobilized by entrapment in alginate gel beads subsequently used for the hydrolysis of triolein in order to produce the monoolein. These products are highly sought emulsifiers in the agro-food, cosmetic and pharmaceutical industry.

2. Material and methods

2.1. Material

Calcium carbonate ($CaCO_3$), calcium chloride ($CaCl_2$) and alginate gel were obtained from Pharmacia (Uppsala, Sweden). Hexane, chloroform, acetone, diethyl ether, oleic acid, dioleine, monooleine and the florisil gel were obtained from Sigma Aldrich (Sigma Chemical Co, St Louis, MO, USA). Virgin olive oil was purchased from the local market. All organic solvents were obtained from Sigma Aldrich: CHROMASOLV Plus for HPLC ≥ 95%.

2.2. Production of Rhizopus oryzae lipase

Rhizopus oryzae lipase was produced as described by Ben Salah et al. [15]. After 72 h of growth, cells are removed by filtration from the broth medium. The lipase in the supernatant was then precipitated by the addition of ammonium sulphate up to 60% of saturation, and subsequently centrifuged at 8000 rpm at 4 °C for 30 min. The lipase pellets were dissolved in 20 mM sodium acetate buffer pH 5.2 containing 20 mM NaCl and 2 mM benzamidine. Then, the solution was centrifuged at 10,000 rpm for 10 min and the supernatant containing the lipase was used for the enzyme immobilization.

2.3. Lipase hydrolytic activity

The activities of the free and the immobilized lipases were measured by titration, under the standard assay conditions, using olive oil emulsion as substrate [16]. The reaction mixture contains 10 mL of olive oil emulsion (1 mL of olive oil and 9 mL of Arabic gum at 10% v/v), 20 mL of distilled water and 100 μL of bovine serum albumin at 12.5% (w/v).

The activity was expressed as units per volume of enzymatic solution (mL). One international unit (IU) of lipase activity was defined as the amount of enzyme that catalyzes the liberation of 1 μmol of fatty acid from olive oil per min at pH 8.0 and at 37 °C.

2.4. Entrapment of ROL in alginate gel beads

A lipase solution (with different activity ranging from 1000 to 7000 IU/mL) was mixed with sodium alginate (concentration ranging from 1% to 3%). Then the mixture was stirred thoroughly to ensure complete mixing. The mixed solution was dripped into $CaCl_2$ solution (50–200 mM) with an injection to form Ca-alginate beads of lipase. After 20 min of hardening, the beads were separated from the $CaCl_2$ solution by vacuum filtration and subsequently washed on a filter twice with 50 mM Tris–HCl buffer solution pH 7.0.

Immobilized yield was calculated as mentioned below.

$$\text{Immobilized yield} = \frac{a_f}{a_i} \times 100$$

where a_i and a_f are respectively the lipase activity before and after immobilization.

2.5. Adsorption of ROL on calcium carbonate ($CaCO_3$)

A support powder of $CaCO_3$ (1 g) was added to 2 mL enzymatic solution (containing 3000 IU). The mixture was incubated 1 h at 4 °C under mild agitation. Afterwards, 10 mL of chilled acetone was added, and the suspension was filtered through a Buchner funnel. The preparation of immobilized lipase was washed twice with another 10 mL aliquot of chilled acetone, dried in vacuum desiccators at room temperature for 6 h and stored at 4 °C until use.

2.6. Hydrolysis of olive oil by free and immobilized lipases

The hydrolysis reaction of olive oil was performed using the free and the immobilized lipases as biocatalysts. The reaction was carried out in a reactor maintained at 37 °C by a water jacket with stirring at pH 8.0 and 7.0 for the free and the immobilized lipase, respectively. The reaction mixture contains 5 mL of olive oil emulsion (0.5 mL of olive oil and 4.5 mL of Arabic gum at 10% (v/v)), 10 mL of distilled water and 50 μL of bovine serum albumin 12.5% (w/v). Reaction was started by adding either free or immobilized lipase form.

2.7. Effect of immobilization on pH activity

The effect of pH on the activity of the free and the immobilized lipases were determined by measuring the hydrolytic activity using olive oil emulsion as substrate [16]. The essays were carried out by incubating the enzyme reaction in 50 mM of different buffer at pH values (3–11) for 30 min at 37 °C.

2.8. Thermal stability of free and immobilized lipase

Each free and immobilized lipase forms were incubated at 37 °C temperature for 24 h. The residual activity after incubation was determined using the standard assay method [16]. The hydrolytic activity of the initial enzyme was taken as 100%.

2.9. Qualitative analysis of reaction products

Lipids were extracted from the reaction media using 30 mL of chloroform/methanol solution (2/1, v/v). The organic phase was retained, the solvent was evaporated on a rotary evaporator and the samples were dissolved in 2 mL of chloroform solution. The different lipid classes were analyzed by thin layer chromatography (TLC) on Silica 60 F254 previously activated at 60 °C for 30 min. The developing solvent was a mixture of n-hexane/diethyl ether/methanol/acetic acid (75/20/2/3, v/v/v/v). The lipid spots were visualized with iodine vapor. The oil was separated into triolein (TO), diolein (DO), monoolein (MO) and oleic acid (OA) and quantified using MCID™ Analysis 7.0 software. The analysis was done and the area percentages for each component were calculated by standards while using molecular weights of 885.45, 620.99, 356.54 and 282.45 Da, respectively for TO, DO, MO, and OA.

2.10. Purification of monoolein

At the end of the reaction, the medium contains a mixture of triolein, diolein, monoolein and free oleic acid. The purification of MO was achieved by chromatography on Florisil using a glass column (18 cm × 2 cm). After the removal of the enzyme by centrifugation at 15,000 rpm for 15 min, the reaction mixture was dried under nitrogen and taken up in a minimal volume of hexane (3 mL). The sample was deposited at the top of the column previously equilibrated with hexane. Indeed, the column is washed first with hexane:diethyl ether (90/10, v/v) to elute triolein, and then with hexane:diethyl ether (75/25, v/v) to elute diolein. The fraction of

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