Esterification reactions catalysed by surfactant-coated *Rhizopus arrhizus* lipase

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The esterification activity of microbial lipase (triacyl glycerol hydrolase, E.C. 3.1.1.3) in water-organic biphasic solvent system has been investigated. The lipase produced from *Rhizopus arrhizus* NCM997 strain by solid-state fermentation, using rice bran as substrate, exhibited novel capability of catalyzing esterification reaction. The partially purified lipase was coated with nonionic surfactant (Span 60). The esterification activity of the enzyme was found to be optimum at 30°C and pH 6.5, and isoctane was the best solvent for esterification. Surfactant-coated lipase showed enhanced esterification activity compared to native enzyme. Among the substrates tested, palmitic acid and glycerol gave maximum conversion of 74.02%. Effect of initial water activity has been investigated using different salts of varying water activity. The maximum conversion (85.75 %) of ester was obtained with salts having less water activity (LiCl; a_w 0.12). Thus, this surfactant-coated microbial lipase in organic solvent (isoctane) has a good potential of biocatalysis.

**Keywords:** esterification, lipase, solvent, surfactant coating, water activity

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**Introduction**

Lipases (triacylglycerol ester hydrolase; E.C. 3.1.1.3) catalyse wide range of reactions that include hydrolysis, alcoholysis, esterification, and inter-esterification. Lipases have been successfully used as a catalyst for synthesis of esters. The esters produced from short chain fatty acids have application as flavouring agents in food agents. Recently, the enzymatic reactions in biphasic system have been noted as a novel production method for nutrients, pharmaceuticals and basic materials for a wide range of applications. Large-scale industrial use of lipases occurs in food industry for the production of emulsifying agents and flavouring agents. They have also been widely used in pharmaceutical and laundry detergent industries. The ability of lipases to catalyze trans- and inter-esterification reactions has been utilized for the production of a glucoside ester used as a biosurfactant, as well as for certain anti-inflammatory and analgesic drugs, namely naproxen and ibuprofen. These enzymes have also been applied for the production of certain agrochemicals.

Coating enzymes with surfactants has been extensively studied over the past decade for making enzymes active in organic solvents. The most significant advantages of the surfactant-coated enzymes are their simple preparation procedure and good solubility in a wide range of organic solvents. Therefore, surfactant-coated enzymes have been regarded as very competitive and promising biocatalyst in organic media in comparison with enzymes modified with polyethylene glycol. In esterification reactions, it is desirable to shift the position of thermodynamic equilibrium by removal of water produced by the reaction. Several approaches, e.g. evaporation, pervaporation, use of molecular sieves and metal salts, have been adopted to remove this water.

In this work, lipase produced from *Rhizopus arrhizus* was coated with a non-ionic surfactant and used for catalysis of esterification reactions in organic solvent. The effects of various operational conditions, such as pH of buffer for enzyme coating, reaction temperature, organic solvents and water activity, as well as substrate specificity of the coated lipase were investigated on a model esterification reaction.

**Materials and Methods**

**Enzyme Production**

*R. arrhizus* NCIM 997 was used for lipase production. The extra cellular lipases were produced by solid-state fermentation by employing rice bran as solid substrate inoculated with 10% w/v spore...
suspension. After fermentation, the enzyme was extracted using phosphate buffer (pH 6.5) and partially purified using solid ammonium sulphate (50-70% fraction). The partially purified enzyme was used for nonionic surfactant coating. Surfactant-coated lipase was prepared with a slight modification of dissolving the surfactant in warm ethanol (40°C) rather than in a buffer solution. Partially purified lipase (250 mg) was dissolved in 50 mL of 0.05 M phosphate buffer, pH 6.5 (unless otherwise specified) and stirred for 20 min at 4°C. A solution containing 500 mg surfactant (Span 60) in 10 mL warm alcohol (ethanol) was added dropwise to the enzyme solution and mixed constantly using magnetic stirrer. The solution was then incubated in refrigerator for 24 h. The precipitate was collected by centrifugation and dried in desiccators. The surfactant-coated lipase was obtained as an off-white powder and used for esterification studies.

**Determination of Lipase Esterification Activity and Stability**

The reaction system consisted of fatty acid (0.12 mol/L of palmitic acid), alcohol (0.12 mol/L of glycerol) and surfactant coated lipases (5 mg) in isooctane. The reaction mixture (20 mL) was incubated at constant temperature for 3 h with continuous shaking at 200 rpm. Then, 1 mL sample was withdrawn and mixed with 10 mL ethanol-acetone (1:1, v/v) to stop the reaction. The remaining acid was determined by titration with 0.01 M NaOH, using phenolphthalein as indicator.

**Water Activity Studies**

The reaction mixture and the enzyme were equilibrated separately for at least 16 h in salt hydrate environment before being mixed to initiate the reaction. Supersaturated solutions of these salts were placed in airtight bottles. Reaction mixtures and enzyme were pre-equilibrated separately in these bottles for at least 16 h at room temperature. Karl-Fischer analysis was made to find out the water activity of the enzyme. To study the effect of water activity on the esterification, molecular sieves (type 4 Å) were added at the concentration of 10% (w/v) of the reaction mixture and carried out the esterification reaction at specified conditions.

**Results and Discussion**

**Effect of Buffer pH on Esterification Reaction**

The effect of buffer pH on esterification activity is shown in Fig. 1. The surfactant-coated lipase showed high conversion of ester at pH 6.5, which is in agreement with optimum pH of the same native enzyme. However, the enzyme activity decreased rapidly at higher pH, indicating a more severe denaturation of the enzyme under alkaline condition. It was observed that surfactant-coated lipase gave maximum conversion rate than native lipase. These results indicate that the native lipase have some deactivation in organic solvent (isooctane), possibly due to reduced conformational stability or even a conformational change as a result of the direct exposure to the solvent.

**Effect of Temperature on Esterification Reaction**

The optimum reaction temperature for esterification was observed around 30°C, which is also in agreement with that of native lipase in organic solvent (Fig. 2). Beyond this temperature, the activity declined due to denaturation of the enzyme. It has been recognized that the coating of enzyme with surfactants does not affect the enzyme active site. It is worth mentioning that, even beyond the optimum temperature, surfactant-coated enzyme maintained some amount of activity compared to the native lipase. It may be due to the less deactivation of the enzyme by the organic solvent.

**Effect of Solvents on Esterification**

The surfactant-coated lipase showed higher conversion of esters in more hydrophobic solvents (having high log P value) and the optimum conversion was found with isooctane (log P=4.5). Lowest ester conversion was found with chloroform (log P=2.0; Fig. 3). The lower enzyme activity in polar solvents might be attributed to the stronger ability of polar solvents to strip the essential water off the enzyme surface. Although the polarities of cyclohexane and hexane are very close, the enzyme showed higher conversion in the former than in the latter. It shows that molecular structure of solvents also plays an important role in affecting enzyme activity in addition to polarity and solvents with a ring structure are more favourable compared with those having a straight chain. The ester conversion in isooctane was slightly higher than in octane, although the two solvents are of same log P. This further suggests that the solvents with a branched structure may be more suitable for the enzymatic reaction compared with those having a straight chain.
Effect of Surfactant Coating on Esterification

Surfactant-coated lipases are generally more stable than native lipases. The surfactant coating seems to protect the enzyme from the denaturation caused by the presence of organic solvent (isooctane). The maximum ester conversion was observed at 3 h, beyond which the conversion started to decrease (Fig. 4). The surfactant coating may induce the surrounding water to be tightly bound with the enzyme, which is necessary for the enzyme activity, and thus cannot be easily stripped by the solvent. It may be concluded that enzymes tend to be stable in apolar solvents, as the apolar solvents do not strip water from within the protein molecule. Among the substrates tested, palmitic acid and glycerol gave maximum conversion of ester, i.e. 74.02% (data not shown).

Effect of Water Activity

In 1994, Halling proposed that the thermodynamic water activity ($a_w$) of the reaction system is the parameter that can be used to quantify the water level associated with the enzymes. The water released during the esterification reaction may disturb the equilibrium and hydrolyze the esters formed. However, the use of salt hydrates help to control the water activity of the system. The effect of varying initial water activity (using salts hydrates) of the reaction system was studied on the esterification. It showed that lower water activity of the reaction system favoured the higher percentage of conversion (Fig. 5). The low initial $a_w$ of 0.12 and 0.33 (obtained with LiCl and MgCl₂.6H₂O, respectively) gave the optimal ester conversion (77.75 and 73.81%). However, high initial $a_w$ of 0.97(using K₂SO₄) gave the conversion of only 45.18%.

Conclusion

The esterification activity of fungal lipase in water-organic biphasic solvent system has been investigated.
The optimum esterification was found in isooctane at 30°C and pH 6.5. Surfactant-coated lipase showed enhanced esterification activity as compared to native enzyme. Among the fatty acids tested, palmitic acid and glycerol gave maximum conversion of 74.02%. Effect of initial water activity of the reaction system has been studied on the esterification, using different salts of varying water activity. Maximum conversion (85.75%) was obtained with salts having less water activity (LiCl, aw 0.12). Thus, the surfactant-coating makes this enzyme a good candidate for potential biocatalysts.

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References