

Esterification of oleic acid catalyzed by an *Aspergillus niger* strain. Influence of water activity and alcohol concentration

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Abstract

Effects of water activity and 1-propanol concentration on synthesis of propyl oleate from oleic acid using *Aspergillus niger* cell-bound lipases in isoctane are described. *A. niger* produces lipases (EC 3.1.1.3) which partly bind to the mycelium during growth. Ester production was monitored for 72 hours at different 1-propanol concentrations and water activities. Aliquots were sequentially withdrawn and propyl esters were quantified using GC and methyl palmitate as an internal standard. In all assayed conditions *A. niger* cell-bound lipases catalyzed propyl oleate synthesis, but at different degrees.

Resumen

Los efectos de la actividad de agua y la concentración de 1-propanol en la síntesis de oleato de propilo usando lipasas “bound-Cell” de *Aspergillus niger* en isoctano, son descritos en este artículo. *A. niger* produce lipasas (EC 3.1.1.3) que en parte están unidas al micelio durante el crecimiento. La producción de éster fue monitoreada por 72 horas en concentraciones diferentes de 1-propanol y actividades de agua. Alícuotas de las muestras fueron secuencialmente tomadas y los ésteres propílicos fueron cuantificados por GC usando palmitato de metilo como estándar interno. En todas las condiciones analizadas las lipasa “Bound –Cells” de *A. niger* catalizaron la síntesis de oleato de propilo en diferentes grados.

Key words: *Aspergillus niger*, biocatalysis, esterification, water activity

Palabras clave: *Aspergillus niger*, biocatalisis, esterificación, actividad de agua

1. INTRODUCTION

Enzymatic inter-esterification is a potentially attractive route for the modification of the physical and chemical properties of edible fats and oils (Ujand y Vaidya 1998). Lipases (EC 3.1.1.3) catalyze both the hydrolysis and the synthesis of esters formed from glycerol and

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long-chain fatty acids. These reactions usually proceed with high regio- and/or enantioselectivity, making lipases an important group of biocatalysts in organic chemistry.

Industrial applications of lipases include the production of non-esterified fatty acids, inter-esterification of oils and fats and synthesis of esters and peptides (Schmidt 1996). A survey of the literature shows that most studies on the application of lipases involve the immobilisation of the enzyme. Moreover, enzymatic transformations must be carried out with precise water activity control to achieve an economically viable process (Ujand y Vaidya 1998). The importance of this control was suggested over a decade ago by (Goderis 1987). It should be noted that small amounts of water are needed to maintain the enzyme activity and that this activity is widely dependent on the source of lipases used (Valivety 1992) (Valivety 1994).

A number of lipases have been produced commercially, the majority of them originating from fungi and bacteria (Jaeger 1998). Many of these lipases derived from *Aspergillus niger*, *Mucor javanicus*, *M. miehei*, *Rhizopus arrhizus*, *R. delemar*, and *R. niveus* and show useful specificities (Gunstone 1998). Cell-bound lipases from micro-organisms such as *A. niger* (Mahmoud 1986), *Rhizopus chinensis* (Kyotani 1988), *R. arrhizus* (Bell 1981), *Pseudomonas mephitica* var. *lipolytica* (Kosugi 1973) and *Penicillium cyclopium* (Legier 1992) have also been described as biocatalysts. The use of naturally-bound lipases can be cost effective because the biomass can be used directly, thus eliminating the isolation, purification and immobilisation procedures, and so minimising the loss of enzyme activity. Furthermore, naturally bound-lipases offer advantages such as increased stability to organic solvents, high temperature and pH values (Long 1996).

For the present study, a strain of *Aspergillus niger* isolated from sunflower seeds was selected as a source of mycelium-bound lipase. This paper describes the effect of a_w and 1-propanol concentration in the synthesis of propyl oleate from oleic acid and 1-propanol in isooctane.

2. EXPERIMENTAL

2.1 General

The strain of *Aspergillus niger* used in this work was isolated from sunflower seeds. Oleic acid was purchased from Merck. Methyl palmitate and 1-propanol were obtained from Fluka. Isooctane was purchased from Panreac.

2.2 Liquid culture conditions

Aspergillus niger was cultivated in a synthetic liquid medium that contained 2.0 g asparagine, 1.0 g K_2HPO_4 , 0.5 g $MgSO_4$, 2.0 g glucose, 5.0 g thiamine hydrochloride, 1.45 mg $Fe(NO)_3 \cdot 9H_2O$, 0.88 mg $ZnSO_4 \cdot 7H_2O$ and 0.24 mg $MnSO_4 \cdot 4H_2O$ /litre distilled water. The pH was adjusted to 5.5-6.0 using 1 mol. dm^{-3} NaOH or 1 mol. dm^{-3} HCl. 250 ml of liquid medium were sterilised in 1 l Erlenmeyer flask at 121 °C for 15 minutes, and 2% of refined sunflower oil was added. Two and a half millilitres of a spore suspension ($4 \cdot 10^6$ spores/ml) of *A. niger* was inoculated to the medium. Cultivation was carried out

at 200 rpm on a orbital shaker at 28 °C for five days. Mycelium obtained from the culture medium was recovered using a Buchner funnel, washed with distilled water followed by acetone and dried under vacuum for 48 hours. It was then milled to powder consistency and the water activity of the powder was determined using a Novasina apparatus.

2.3 Pre-equilibration of water activity

The powdered cells were equilibrated with glycerol solutions at 25 °C in separate containers. The solutions were 5.52 g glycerol/100 ml water ($a_w=0.99$) and 50.6 g glycerol/100 ml water ($a_w=0.90$) (Dallyn 1978).

2.4 Esterification reaction

The esterification of oleic acid and 1-propanol in isooctane catalysed by an *Aspergillus niger* strain was assayed at different water activities (0.11, 0.90 and 0.99) and at different 1-propanol concentrations (0.67 M, 0.33 M and 0.10 M). In a typical procedure 0.5 g (1.77 mmol) of oleic acid and 0.8 g (13.3 mmol) of 1-propanol were dissolved in 20 ml isooctane in a capped 50 ml Erlenmeyer flask. Six hundred milligrams of powdered cells at the desired water activity were then added and the flask was incubated in an orbital shaker at 28 °C and 200 rpm until the end of the experiment. A sample of 100 µl reaction mixture was removed at intervals and 100 µl of methyl palmitate solution was added as an internal standard for gas chromatography analysis. The reaction was carried out in triplicate. The yield was calculated based on the conversion of initial oleic acid to the corresponding oleate.

2.5 Blank Reactions

Reaction without oleic acid. Blank experiments without oleic acid were carried out using the same 1-propanol concentrations and water activities described above. In a typical procedure 0.8 g (13.3 mmol) of 1-propanol was dissolved in 20 ml isooctane in a capped 50 ml Erlenmeyer flask. Six hundred milligrams of powdered cells at the desired water activity was then added and the flask was incubated in a orbital shaker at 28 °C and 200 rpm until the end of the experiment. A sample of 100 µl reaction mixture was removed at intervals and 100 µl of methyl palmitate solution was added as an internal standard for gas chromatography analysis. The reaction was carried out in triplicate. The yield was calculated considering the highest concentration of propyl oleate found.

Reaction without resting cells. Blank experiments without resting cells were carried out using the same 1-propanol and oleic concentrations described above. In a typical procedure 0.5 g (1.77 mmol) of oleic acid and 0.8 g (13.3 mmol) of 1-propanol were dissolved in 20 ml isooctane in a capped 50 ml Erlenmeyer flask. The flask was incubated in a orbital shaker at 28 °C and 200 rpm for 24 h. Samples were analysed by thin layer chromatography using silica gel 60F₂₅₄ supported on aluminium sheets and hexane/diethyl ether (9+1) as a mobile phase.

2.6 Gas chromatography

Samples were analysed in a Fisons instrument (GC 8000) with flame ionisation detection. A glass column (id. 2 mm, length 2 m) packed with FFAP 10% on 80/100 WAW was used. Separation was accomplished using nitrogen as a carrier gas (35 ml/min) with a gradient temperature program between 200 and 230 °C. Before analysis of samples, a standard curve of propyl oleate was obtained to calculate the response factor.

3. RESULTS AND DISCUSSION

Figure 1 shows that mycelium-bound lipase catalysed propyl oleate synthesis in all assayed conditions. Yields were calculated based on the conversion of initial oleic acid to the corresponding oleate. The highest yield was obtained when water activity (a_w) of mycelium was 0.90 in any 1-propanol concentration. When a_w of mycelium was 0.11 and 0.99, lower propyl oleate concentrations were obtained. Both a_w at initial 0.67 M 1-propanol concentration presented the same yield.

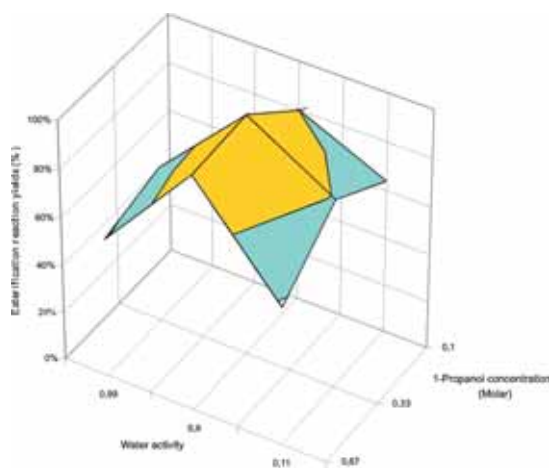


Figure 1. Effect of water activity and 1-propanol concentration on propyl oleate yields. The esterification reaction of oleic acid was catalysed by a mycelium from an *Aspergillus niger* strain.

However, when in the mixture there was a lower concentration of 1-propanol (0.33 M or 0.11 M), more propyl oleate was obtained at $a_w=0.11$.

As shown in Figure 2, the highest initial conversion rate was observed when a_w of mycelium was 0.90 in any concentration of 1-propanol. At $a_w=0.11$, the oleic acid conversion is faster than when a_w was 0.99. As several authors (Valivety 1992) (Ujang 1998) have observed, the control of the precise a_w is very important in order to maintain the activity of the enzyme. This behaviour is not related to the nature of the supports (Arroyo 1995).

The conversion rate is 1-propanol dependent. Thus, the conversion rate for the 0.10 M 1-propanol concentration was higher than the conversion rate for the 0.33 M 1-propanol concentration, and the latter was higher than that for the 0.67 M 1-propanol concentration. However, for the 0.10 M 1-propanol concentration the reaction reached equilibrium before the whole conversion of oleic acid. This is probably due to an accumulation of water (Colombié 1998). Several authors have already described the inhibitory effect on lipase activity at high alcohol concentrations (Coulon 1996).

As shown in the figures, the best ratio of conversion rate to yield was obtained when a_w of mycelium was 0.90 and the initial 1-propanol concentration was 0.33 M. As Boutur *et al.* (Boutur 1995) have described, ester synthesis catalysed in aqueous media by *Candida deformans* was dependent on the nature and the concentrations of the accepting alcohol. The optimal concentration depended on the nature of the alcohol, being lower when the carbon chain of the alcohol was longer.

Thin-layer chromatography analyses of samples from reaction mixtures containing oleic acid and different 1-propanol concentrations (0.67 M, 0.33 M and 0.10 M) without any mycelium show no synthesis of propyl oleate. Thus, observed esterification reactions are catalysed by the *A. niger* mycelium.

As shown in Table 1, some oleic acid was retained by mycelium from culture medium during its growth. It should be noted that a maximum of almost 10% of the propyl oleate produced could come from the retained oleic acid.

Table 1. Average propyl oleate concentrations obtained from three replicates. The reaction mixture consisted of 0.6 g of powdered mycelium at the desired a_w and 1-propanol at different concentrations dissolved in 20 ml isoctane. Reactions were carried out at 28°C and 200 rpm for 72 hours in an orbital shaker.

Reaction Conditions		
a_w	1-Propanol concentration (Molar)	Propyl oleate concentration (Molar) \pm SD
0.90	0.67	0.007 \pm 0.001
0.11	0.33	0.006 \pm 0.001
0.99	0.10	0.010 \pm 0.001

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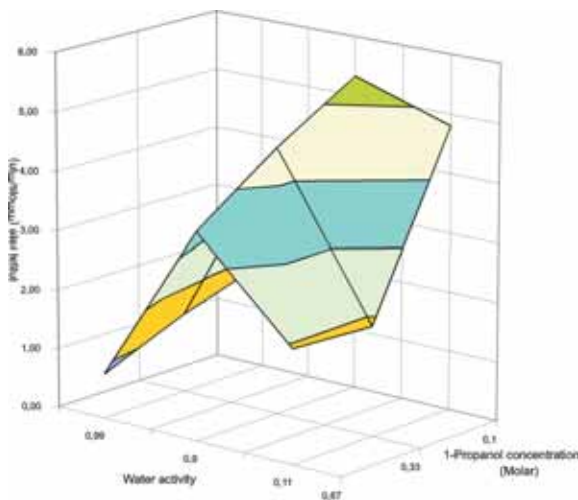



Figure 2. Effect of water activity and 1-propanol concentration on the initial esterification rate of oleic acid catalysed by a mycelium from an *Aspergillus niger* strain.

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