ENANTIOSELECTIVE SYNTHESIS OF (-)-MENTHYL ACETATE IN A BATCH STIRRED TANK REACTOR: EFFECTS OF FED BATCH FEEDING STRATEGY

Mohamad Roji bin Sarmidi; Jinny Lim Lay Poh; Ng Tit Soo
Department of Bioprocess Engineering
Faculty of Chemical and Natural Resources Engineering
Universiti Teknologi Malaysia
81310 Skudai
Johor Bahru.

Tel: 607-5540209, Fax: 607-5581463
mroji@fkkksa.utm.my; jinny_923@hotmail.com; tsng230@tm.net.my

ABSTRACT

Investigation involving enantioselective synthesis of chiral compounds catalysed by immobilised enzyme lipase is a viable option. In this study, acetic anhydride as an acylating agent was employed with (±)-menthol, a secondary alcohol in enantioselective synthesis of (-)-menthyl acetate in a fed batch reactor. (-)-menthyl acetate is an optically active flavour and fragrance compound. Molar ratio concentration of acetic anhydride to (±)-menthol is 1:4. The ratio of alcohol to acyl donor, acid anhydride to (±)-menthol was prepared at 15 mmole/L : 60 mmole/L in 25 ml *n*-heptane. The reaction was catalysed by commercially available immobilised enzyme lipase, Chirazyme®L-3, c. -f., C2 lyo from *Candida rugosa*. The incorporation of a fed batch feeding system to the batch reactor configuration was investigated. The system was evaluated in terms of molar percentage yield and productivity. The reactions were carried out at 30°C in *n*-heptane and molar yield as high as 8.72% - 29.57% were attained after 48 hours of reaction. This fed batch feeding system was thought able to minimise the hydrolysis of acetic anhydride. A high concentration of acetic anhydride in a batch reaction system is not a viable option since water present in the reaction mixture would promote the hydrolysis of acetic anhydride catalysed by the same enzyme. A fed batch feeding strategy of 12µl, 10µl, 8µl and 6µl produced the highest molar percentage yield with 29.57% of (-)-menthyl acetate formation and productivity of 0.00154mM/min. The enzyme loading used was only 40% from the actual equivalent amount.

Keywords: Chiral compound, fed batch feeding strategy, immobilised enzyme lipase, (-)-menthyl acetate

INTRODUCTION

Lipases (EC 3.1.1.3, triacylglycerol acyl hydrolases) have been shown to be active in organic media [1,2,3]. It catalyses esterification reactions in the organic media and is more enantioselective than the corresponding hydrolytic reaction in the aqueous media. Thus, lipases have been utilised in the resolution of racemic mixtures for the preparation of optically pure compounds [1,3]. It is seen as an alternative method to the existing chemical synthesis. This has opened up novel applications in the area of aroma and flavour chemistry to synthesise "natural" flavour esters. Short chain carboxylic esters of secondary terpene alcohol, menthol are among the important flavour and fragrance compounds used in food, beverage and pharmaceutical industries [4,5]. This paper highlighted the usage commercially available immobilised enzyme lipase, Chirazyme ®L-3, c.-f., C2 lyo from *Candida rugosa* to catalyse the synthesis of (-)-menthyl acetate in a batch reactor mode. (-)-menthyl acetate is an optically active flavour and fragrance compound. Racemic menthol, a secondary terpene alcohol was esterified with acetic anhydride as the acylating agent in *n*-heptane. Acetic anhydride was used as the acyl donor because it would make the acyl transfer irreversible [6, 7]. Ester synthesis with free fatty acids as acyl donors led to problems caused by the water produced during the reaction was reported [8]. However, enantioselective ester synthesis carried out in batch mode utilising acetic anhydrides has its setback too. A high concentration of acetic anhydride in a batch reaction system is not a viable option since water present in the reaction mixture would promote the hydrolysis of acetic anhydride catalysed by the same enzyme. Thus, this paper investigated the possibility of a fed batch feeding strategy of acetic anhydride into the reaction system to overcome this setback. The molar percentage yield and productivity for (-)-

menthyl acetate by the immobilised enzyme lipase was evaluated via Gas Chromatograph and a chiral column.

MATERIALS AND METHODS

Chemicals

The racemic secondary alcohol, (±)-menthol (purity ≥ 99%) was purchased from Fluka Chemie AG (Switzerland) and acetic anhydride (≥ 97%) from Fisher Scientific (USA) while the solvent used, *n*-heptane was of HPLC grade, and procured from J.T. Baker (USA). Commercially available standard solution of product (-)-menthyl acetate and (+)-menthyl acetate (≥ 99%) were purchased from Fluka Chemie AG (Switzerland).

Enzymes

Immobilised enzyme lipase from *Candida rugosa*, Chirazyme ®L-3, c. -f., C2 lyo (specific activity 130 U/g) was purchased from Roche Diagnostics (Mannheim, Germany).

Lipase activity assay

Hydrolytic activity of lipase was evaluated using tributyrin as substrate. The activity assay measures the liberation of butyric acid by titrating with 100-mM sodium hydroxide. 1 micromole sodium hydroxide consumed per minute corresponds to one unit of lipase activity in the assay. The specific activity of the lipase was measured from the linear portion of the base consumption curves. A pH-Stat method using a Metrohm autotitrator, model 718 Stat Titrino at 25°C and pH 7 was used.

Batch mode experiments

Batch mode esterification synthesis was carried out in 50-ml Erlenmeyer flasks at 30°C in a water bath. The flasks were attached to a mixer shaft that rotated at 150 rpm. The ratio of alcohol to acyl donor, acid anhydride to (±)-menthol was prepared at 1:4 molar ratio or 15 mmole/L : 60 mmole/L in 25 ml of *n*-heptane. Immobilised enzyme lipase, Chirazyme ®L-3, c. -f., C2 lyo amounted to a 170 U were utilised as biocatalysts. It was added immediately before the reaction started. Four fed batch feeding strategy was adopted. Acetic anhydride was the limiting substrate in this study and (±)-menthol was in excess. Acetic anhydride was fed into the vessels at four different intervals with varying volume via a digital micropipettor.

Identification Of (-)-Menthyl Acetate

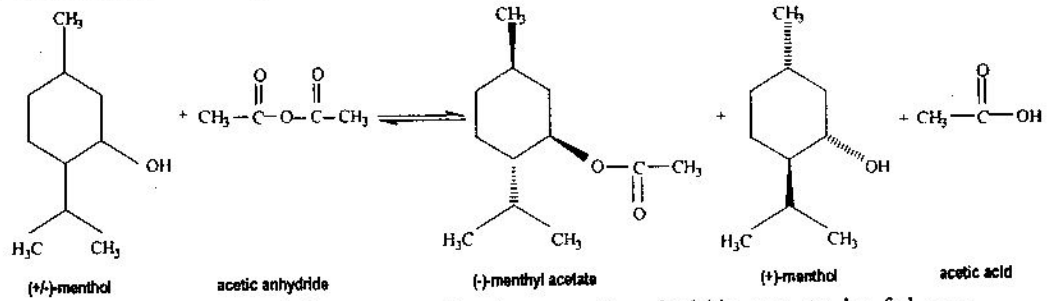
The identification of (-)-menthyl acetate produced in the lipase-catalysed esterification was carried out using a Shimadzu GC-17A Ver.3 Gas chromatography (Kyoto, Japan). It was equipped with a flame-ionisation detector (FID). The column used was a chiral Beta-DEX™ 120 fused-silica capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness (Supelco, USA). The injector and detector temperatures were 200°C and 250°C respectively. The temperature program used for the analysis of (+) and (-)-menthyl acetates was held isothermally at 140°C for 35 minutes. The flow rate of the carrier gas, nitrogen was 0.2 ml per minute. The amount of each enantiomer was determined by peak area recorded and integrated by a Shimadzu CLASS-VP™ Chromatography Data System (Columbia, USA) software. Periodically, an aliquot of 1μl was pipetted and injected into the Beta-DEX™ 120 column. Commercially available (-)-menthyl acetate and (+)-menthyl acetate were used as standard for capillary GC identification of the optically active menthyl acetates synthesised by the lipase-catalysed reactions. The retention times for (-)-menthyl acetate and (+)-menthyl acetate were 29 47 and 29.91 minutes respectively.

RESULTS AND DISCUSSION

Kinetic resolution of racemic substances particularly, (±)-menthol employed free acid as the acyl donor. However, the formation of water as a by-product makes this option unfavourable. Acid anhydrides were used as a replacement of the free acid because it is non-water producing acyl donors. Moreover, the reactivities of acid anhydrides were found to be higher than the corresponding free acids. Nevertheless, acid anhydrides were also found to be easily hydrolysed into free acids under the catalysis of the same enzyme [9,11]. A high concentration of acetic anhydride in a batch reaction system is not a viable option since water present in the reaction mixture would promote the hydrolysis

of acetic anhydride catalysed by the same enzyme and acetic acid is produced as depicted in Figure 1. Therefore, a fed batch feeding strategy was essential to control the concentration of acetic anhydride in the reaction system.

As mentioned in Materials and Methods, four feeding strategies were adopted. The molar ratio of the reaction mixture was at 60 mmole of racemic menthol and 15 mmole of acetic anhydride. Racemic menthol was in excess according to stoichiometic amount while acetic anhydride was the limiting



substrate in this study. The equivalence or total volume acetic anhydride was to be fed was approximately 36 µl. Thus, acetic anhydride was being injected by means of a digital micropipettor at four different intervals. The first involves a feeding strategy of equal amounts of acetic anhydride (9µl, 9µl, 9µl, and 9µl). The second involved an injection of 10µl, 10µl, 10µl, and 6µl while the third had a gradual-declining feeding pattern of 12µl, 10µl, 8µl, and 6µl respectively. The fourth adopted a 15µl, 7µl, 7µl, and 7µl strategy.

Figure 1: Chemical equation of enantioselective synthesis of (-)-menthyl acetate

From Table 1, it was observed that out of four fed batch feeding strategy, a feeding volume of 9µl, 9µl, 9µl and 9µl produced the least molar percentage yield of 24.85%. Molar percentage yield or better known as extent of conversion was calculated from Equation 1.

$$\xi = \frac{C_p}{C_{so}} \qquad (1)$$

where ξ denotes extent of conversion

c_p denotes concentration of product

c_{so} denotes the initial concentration of substrate

This was followed by the feeding volume of 10µl, 10µl, 10µl and 6µl which gave a 25.49% molar percentage yield. The next feeding strategy of 15µl, 7µl, 7µl and 7µl produced a 25.54% product formation while a 12µl, 10µl, 8µl and 6µl feeding strategy yield the highest molar percentage yield with 29.57%. This strategy was feasible as in the initial stage of the reaction: the rate of reaction was faster. Thus, a higher volume, 12µl of acetic anhydride was fed first. The preceeding volume gradually decreased as the enzyme activity decreased.

In comparison to previous work [10], an enzyme loading of 170 U was used when the reaction volume was 10 ml. In this study, a 25 ml reaction volume was used and the enzyme loading was scaled down to 170 U instead of a 425 U of immobilised Chirazyme. Fresh immobilised enzyme was used for each experiment. In other words, the results obtained for the present study was catalysed using an enzyme loading of only 40% from the actual supposed amount. This was a significant improvement in terms of performance of the immobilised Chirazyme with a comparable molar percentage yield when compared to results depicted in Table 2.

Table 1: Molar percentage yield of (-)-menthyl acetate with corresponding fed batch feeding strategy with 170 U of enzyme loading in 25 ml of reaction mixture.

Feeding strategy	24 hr	36 hr	48 hr
S1 (9,9,9,9)	9.52%	21.90%	24.85%
S2 (10,10,10,6)	10.07%	20.64%	25.49%
S3 (12,10,8,6)	8.72%	22.44%	29.57%
S4 (15,7,7,7)	14.91%	23.55%	25.54%

Table 2: Molar percentage yield of (-)-menthyl acetate in batch mode with 170 U of enzyme loading in 10 ml of reaction mixture [10].

Time course	24 hr	36 hr	48 hr
Molar Percentage Yield	25.54%	35.20%	35.35%

In terms of productivity, fed batch feeding strategy S3 produced the highest product after 48 hours of reaction. The results were as illustrated in Figure 2. Productivity was calculated according to Equation 2.

$$\text{Productivity} = \frac{[\text{mM}]}{t} \qquad (2)$$

where [mM] denotes concentration of product, (-)-menthyl acetate in milliMolar
t denotes time course of reaction that is 48 hours

From Figure 2, productivity of fed batch feeding strategy S3 was 0.00154 mM/min which was the highest. This was followed by 0.001328 mM/min and 0.001333 for fed batch feeding strategy of S2 and S4 respectively. The productivity for this two feeding strategy was approximately the same while fed batch feeding strategy S1 had the least value of 0.00129 mM/min. This results was comparable to works of other researchers [10,11].

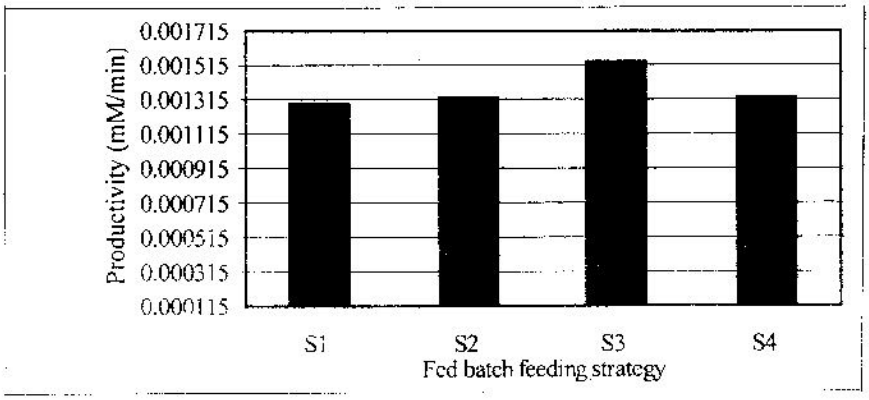


Figure 2: Productivity (mM/min) of (-)-menthyl acetate with corresponding fed batch feeding strategy after 48 hours of reaction

CONCLUSION

A fed batch feeding strategy of 12µl, 10µl, 8µl and 6µl produced the highest molar percentage yield with 29.57% of (-)-menthyl acetate formation. The corresponding productivity was 0.00154 mM/min. The enzyme loading used was only 40% from the actual equivalent amount. This indicated a fed batch feeding strategy is able to minimise the hydrolyse of acetic anhydride. The molar percentage yield and productivity would be higher if the actual amount of enzyme loading was use.

NOTATION

ξ	Molar Percentage Yield or Extent of Conversion
c_p	Concentration of Product, (-)-Menthyl Acetate
c_{so}	The Initial Concentration of Substrate, Acetic Anhydride
EC	Enzyme Commission
FID	Flame Ionisation Detector
GC	Gas Chromatograph
HPLC	High Performance Liquid Chromatograph
S1	Fed Batch Strategy of 9µl, 9µl, 9µl and 9µl Volume
S2	Fed Batch Strategy 10µl, 10µl, 10µl and 6µl Volume
S3	Fed Batch Strategy of 12µl, 10µl, 8µl and 6µl Volume

S4 Fed Batch Strategy of 15µl, 7µl, 7µl and 7µl Volume
U Units
[mM] Concentration of product, (-)-menthyl acetate in milliMolar
t Time course of reaction that is 48 hours or equivalent to 2880 minutes

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