ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 5 (1998) 277–281

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Synthesis of benzoates by enzymatic catalysis in heterogeneous medium

Jean-Pierre Leszczak, Canh Tran-Minh *

Ecole des Mines de Saint-Etienne, Centre SPIN, Laboratoire de Génie Enzymatique, 158 Cours Fauriel, 42023 Saint-Etienne, France

Received 26 September 1997; accepted 14 January 1998

Abstract

The enzyme-catalyzed synthesis of methyl and 1-butyl benzoates is reported. Direct esterification is performed with good yields by lipase from *Candida rugosa* suspended in a hexane/toluene mixture. Influence of substrates concentration, water content and toluene ratio are examined. Methanol and 1-butanol are lipase-inhibitors: they interact with the water adsorbed on lipase and on the enzyme as well. Increasing benzoic acid concentration in the system partially eliminates the alcohol inhibition. Water content greatly affects reaction kinetics and must be optimized to both activate the lipase and reach good conversion yields. Toluene, used to solubilize benzoic acid, negatively acts on reaction kinetics, mostly by increasing the partition of benzoic acid for the organic phase. The kinetic modelisation of 1-butyl benzoate synthesis agrees with a ping-pong Bi–Bi mechanism with 1-butanol inhibition. Apparent reaction coefficients are then determined.

Keywords: Candida rugosa lipase; Benzoates; Organic media; Esterification; Kinetics

---

1. Introduction

Esters produced from natural acids and alcohols by enzymatic catalysis can be considered as natural compounds [1,2]. Non-aliphatic esters, especially benzoates, are part of some fruits and berries flavors. Only two reports in the literature concern benzoic acid esterification by lipase catalysis, but esters are obtained in low yields [3,4]. In fact, benzoic acid is usually considered as a lipase inhibitor [4].

In this paper, operating conditions for benzoates biosynthesis are discussed to obtain those esters in good yields. Some influencing factors are pointed out and optimized. A mechanism model for the kinetics of 1-butyl benzoate synthesis is suggested.

---

* Corresponding author.

PII: S1381-1177(98)00093-9

2. Results and discussion

2.1. Operating conditions

It was necessary to find a reaction medium, an enzyme and reaction conditions to obtain benzoates in good yields. Methyl benzoate (MeOBz) synthesis was used as model. Solubilization of benzoic acid (BzOH) was the main problem. From solvent and lipase screenings, it appeared that esterification took place in a water-saturated mixture of hexane and toluene and was catalyzed by lipase from *Candida rugosa* (CRL). The presence of a minimum water content of 500 mg/l was needed to fully activate the CRL and obtain the Michaelis–Menten kinetics. Temperature and agitation speed were optimized at 37°C and 220 rpm, respectively.

2.2. Methyl benzoate

Conclusions obtained with methyl and 1-butyl benzoates are quite similar. However, reaction kinetics is slower and alcohol inhibition is higher when methanol is used.

2.2.1. Initial water content

In this heterogeneous catalytic system, water content is the key factor of the reaction [5]. Addition of water up to 1000 mg/l greatly activates the esterification. Then a maximal initial velocity V_i(sp) is reached (Fig. 1). Below 500 mg/l, the water produced at the beginning of the reaction provides the CRL with the required water to be active. An auto-catalytic kinetics is then observed and is all the more pronounced as the initial water content becomes low.

As the reaction produces water, the optimal initial water content decreases with conversion time from 1000 after 12 h to 500 mg of water/l after 72 h (Fig. 2). This phenomenon is mainly due to mass transfer limitations to active sites: powder agglomeration around water droplets reducing access to active sites and diffusion of benzoic acid through the increasing water layer protecting the lipase.

In order to reach good conversion yields and maintain the catalytic powder in an reusable form, the optimal water content is therefore 500 mg/l even if the initial velocity is not optimized. In this case, the lipase remains a fine powder.

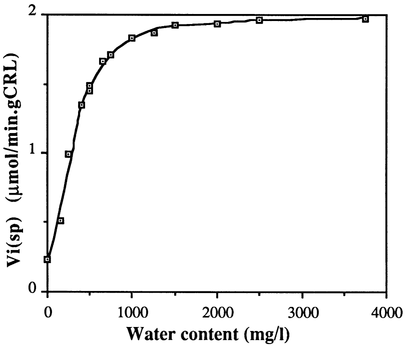

Fig. 1. Influence of initial water content on initial specific velocity V_i(sp). Conditions: [BzOH] 50 mM, [MeOH] 62 mM, CRL 10 g/l, water-saturated hexane/toluene 50/50.

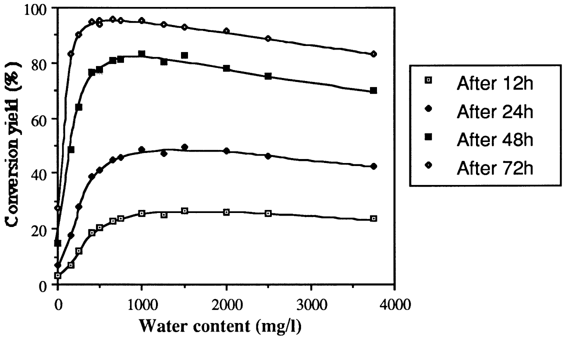

Fig. 2. Influence of water content on conversion yield at different time courses. Same conditions as Fig. 1.

2.2.2. Influence of substrates concentration

Whereas methanol (MeOH) is an inhibitor even for concentrations as low as 12 mM, no inhibition by BzOH is observed below 100 mM. For BzOH concentrations above 77 mM, BzOH even protects the CRL against MeOH inhibition and a maximal specific velocity V_i(sp) is then obtained for 37 mM of MeOH (Fig. 3).

2.2.3. Influence of toluene on reaction kinetics

Bioesterification of BzOH only occurs in the presence of toluene. Toluene is necessary to solubilize the BzOH powder. However, reaction rates are very slow in pure toluene. Addition of hexane greatly improves the kinetics until a toluene/hexane volumic ratio down to 35% (Fig. 4). V_i(sp) decreases again between 35 and 10% of toluene. For ratio less than 10%, no reliable result is obtained because of rapid recrystallisation of BzOH. In fact, the partition coefficient of BzOH in the organic phase to the aqueous layer surrounding the CRL powder increases with toluene volumic ratio. Therefore, the local BzOH concentration in the aqueous layer around the active sites and the formation of the acyl-enzyme intermediate decrease with high toluene concentration.

2.3. Butyl benzoate

Kinetic modelisation of MeOBz production was unsuccessful because of the high desorption of water adsorbed on CRL by MeOH even at small concentrations (data not shown): the high

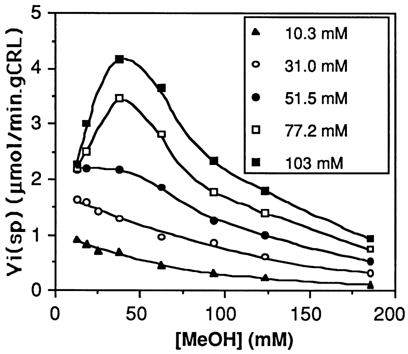

Fig. 3. Influence of MeOH concentration on reaction kinetics for different BzOH concentrations. Conditions: CRL 10 g/l, water-saturated hexane/toluene 50/50, water content 500 mg/l.

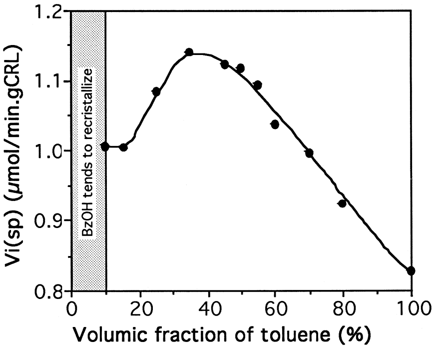

Fig. 4. Influence of volumic fraction of toluene on specific initial velocity. Conditions: [BzOH] 50 mM, [MeOH] 62 mM, CRL 10 g/l. CRL, toluene and hexane were pre-equilibrated at $a_w = 0.65$.

hydrophilicity of MeOH prevents the total system to be pre-equilibrated with water. However, 1-butanol (BuOH), which is less hydrophilic, can be used since it can easily be pre-equilibrated at a fixed water activity (a_w).

This study was conducted with a previous pre-equilibration of CRL, BuOH and solvents at an a_w of 0.65. Considering the experimental points, the CRL is inhibited by an excess of BuOH as observed for MeOH (Fig. 5). No inhibition effect of BzOH has been detected. When no diffusional limitation is present, the reaction mechanism agrees with a ping-pong Bi–Bi model with only BuOH inhibition of the free enzyme (Eq. (1)) [6].

$$\frac{V_i}{V_m} = \frac{[\mathrm{BzOH}][\mathrm{BuOH}]}{K_{\mathrm{M(BuOH)}}[\mathrm{BzOH}] + K_{\mathrm{M(BzOH)}}[\mathrm{BuOH}](1 + [\mathrm{BuOH}]/K_i) + [\mathrm{BzOH}][\mathrm{BuOH}]} \tag{1}$$

with V_i initial velocity, V_m maximal velocity, K_i inhibition constant of BuOH, $K_{\mathrm{M(BuOH)}}$ and $K_{\mathrm{M(BzOH)}}$ affinity constant for CRL of BuOH and BzOH, respectively.

These different constants were determined by successive interpolations and their values are 155 μmol/(min · g CRL), 121 mM, 1147 mM and 901 mM for V_m, K_i, $K_{\mathrm{M(BuOH)}}$ and $K_{\mathrm{M(BzOH)}}$ respectively. The mean deviation on 109 experimental points is 4.6%.

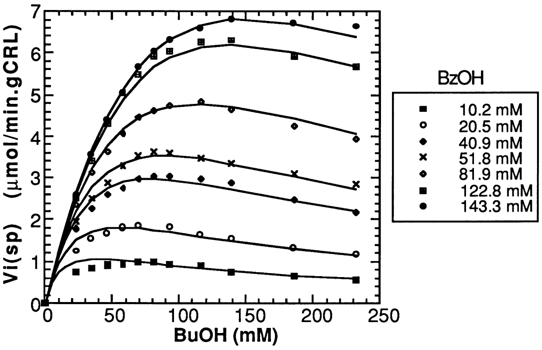

Fig. 5. Influence of BuOH concentration on kinetics. Conditions: CRL 10 g/l, toluene/hexane 50/50. CRL, toluene, hexane and 1-BuOH were pre-equilibrated at $a_w = 0.65$. The points represent the experimental values and the curve the calculated values.

3. Conclusion

Methyl and butyl benzoates can be synthetised with good yields by CRL suspended in a hexane/toluene mixture. The initial water content in the system acts both on initial velocity and reaction time course: for MeOBz synthesis, a water addition of 500 mg/l is a good compromise to activate the CRL and reach good conversion yields in a short reaction time. CRL inhibition by an excess of MeOH or 1-BuOH is observed. Toluene, for ratio higher than 35% in the hexane/toluene medium, negatively acts on V_i(sp). It is attributed to a diminution of BzOH concentration in the aqueous layer surrounding the CRL powder. In fact, the partition coefficient of BzOH between the organic medium and the aqueous layer increases when toluene ratio increases in the organic medium.

The esterification kinetics of BzOH by 1-BuOH agrees with a ping-pong Bi–Bi mechanism with a dead-end inhibition by 1-BuOH.

References

[1] I.L. Gatfield, Food Technol. 42 (1988) 110–121.
[2] J.S. Dordick, Enzyme Microbiol. Technol. 11 (1989) 194–211.
[3] Y. Tsujisaka, S. Okumura, M. Iwai, Biochim. Biophys. Acta 489 (1977) 415–422.
[4] Y. Kodera, K. Takahashi, H. Nishimura, A. Matshushima, Y. Saito, Y. Inada, Biotechnol. Lett. 8 (1986) 881–884.
[5] A. Zaks, A.M. Klibanov, J. Biol. Chem. 263 (1988) 3194–3201.
[6] A. Marty, W. Chulalaksananukul, R.M. Willemot, J.S. Condoret, Biotechnol. Bioeng. 39 (1992) 273–280.