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Highly diastereoselective acylation of L-menthol by a lipase from *Stenotrophomonas maltophilia* CGMCC 4254

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ABSTRACT

l-menthol, one of the world's largest flavor components, is widely utilized in food and cosmetics industries. Disappointingly, menthol which was synthesized by thymol hydrogenation route consisted of diastereomeric menthol mixture, containing several isomers leading to undesirable taste. Therefore, there is a need for efficient and economical way of preparing optically pure L-menthol from diastereomeric mixture. In our work, *Stenotrophomonas maltophilia* CGMCC 4254 lipase (SML) was chosen as a potential biocatalyst for diastereoselective acylation of *l*-menthol. Subsequently, different reaction factors were investigated, such as organic solvent, acyl donors, temperature, menthol concentration and molar ratio of donor to L-menthol. SML-catalyzed L-menthol acetylation reaction at 641 mM concentration and 35 °C resulted in 95.1% conversion (24 h) and 93.4% diastereomeric excess (d.e.$_p$), which were much better than commercially available lipases tested. Finally, the acetylation reaction was scaled up to 0.9 L, resulting in an average L-menthyl acetate volumetric productivity 1.33 g/L/h with 93.4% d.e.$_p$ and 95.1% conversion. Therefore, SML is a potentially promising biocatalyst for preparation of optically pure L-menthol from diastereomeric mixture in an industrial scale.

1. Introduction

L-menthol, which is one of the world's largest flavor components, is extensively utilized in a range of food, cosmetics and pharmaceuticals industries, due to its cooling effect and refreshing flavor [1,2]. However, the yield and quality of natural L-menthol, which is extracted from mint plant (*Mentha arvensis var. piperascens*), are affected seriously by weather, region and so on [3–5]. In an alternate way, L-menthol was produced by chemical synthesis such as Haarmann & Reimer/Symrise process (hydrogenation of thymol). Although that was well known to be a real industrial production process of L-menthol [5,6], the menthol product was a mixture of diastereomeric isomers. Unfortunately, several diastereomers are responsible for undesirable taste [2,6,7]. Therefore, preparation of optically pure L-menthol from the diastereomeric mixture is necessary.

In the last few decades, chemocatalysis was the main method for the production of L-menthol [5,8,9]. However, high energy consumption and environmental pollution from chemical process are becoming more and more serious. Recently, biocatalysis is attracting great interest for its environmental friendliness, lower energy consumption, and lower costs [10]. Lipases (E.C. 3.1.1.3) are a class of enzymes that preferentially act on the carboxylic ester bonds [12]. Lipases were versatile biocatalysts utilized in resolution of chiral compounds because they are stable in organic solvent, do not require cofactors, have a broad substrate spectrum and frequently exhibit good stereoselectivity [13]. Moreover, stereoselective acylation catalyzed by lipase, as an environment friendly and economical way for resolution of chiral alcohol, is of great interest recently [11].

Although the diastereoselective acylation of L-menthol from diastereomeric mixture by lipases is a high efficient method (Scheme 1), only very few examples of the preparation of L-menthol in this manner have been reported so far [5,14]. Because the lipases displayed moderate activity and low diastereoselectivity towards L-menthol, they did not satisfy the demands of industry [15,16]. Physical isolation of L-menthyl acetate from other menthol isomers, such as by distillation, followed by hydrolysis yielded the desired

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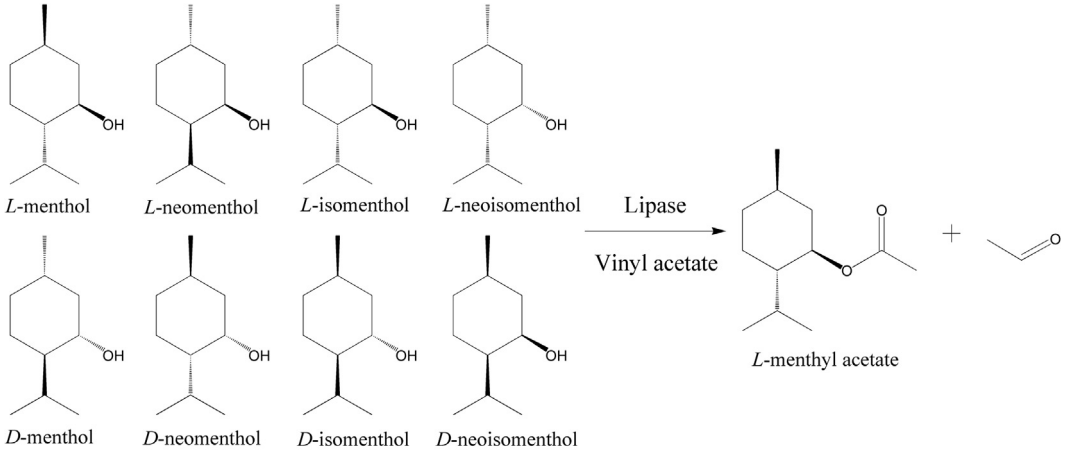

Scheme 1. Diastereoselective acylation of L-menthol from diastereomeric mixture by lipase.

pure optically active L-menthol [5,6]. Thus, screening and selecting of novel lipases with high activity and excellent diastereoselectivity for L-menthol acylation is of great importance.

In our previous works, 21 organic solvent-tolerant lipases were selected as candidates for diastereoselective acylation of L-menthol [17,18]. In this study, *Stenotrophomonas maltophilia* CGMCC 4254 lipase (SML) was selected as an enzyme suited for acylation of L-menthol. The effects of different reaction parameters and acyl donors on SML activity and diastereoselectivity were investigated. Furthermore, the acylation reaction in a preparative scale was also investigated. SML displayed excellent diastereoselectivity and high acylation activity for the production of optically active L-menthol.

2. Materials and methods

2.1. Materials

2.1.1. Chemical compounds

DL-Menthol, L-menthol, D-menthol, L-menthyl acetate, D-menthyl acetate, D-isomenthol and D-neomenthol were purchased from Sigma–Aldrich Co., Ltd. (Shanghai, China). All acyl donors (vinyl acetate, vinyl propionate, vinyl butyrate, vinyl hexanoate, isopropenyl acetate and phenyl acetate) were also obtained from Sigma–Aldrich Co., Ltd. (Shanghai, China). All other chemicals were of analytical grade from Guoyao Co.Ltd. (Shanghai, China).

The diastereomeric mixture of menthol, which was generated by hydrogenation of thymol as previously described [19], contained diastereoisomers of menthol, namely DL-menthol (50.0%, w/w), DL-isomenthol (19.0%, w/w), DL-neomenthol (26.0%, w/w) and DL-neoisomenthol (3.0%, w/w), as well as 2.0% (w/w) menthones. The diasteroisomeric mixture was analyzed by chiral GC method.

2.1.2. Commercial lipases preparations

PS (from *Burkholderia cepacia*), RAL (from *Rhizopus arrhizus*), CLL (from *Candida lipolytica*), CRL (from *Candida rugosa*), PPL (from *Porcine pancreas*), TTL (from *Thermus thermophilus*), CALA (from *Candida antarctica*) and CALB (*C. antarctica*) were purchased from Sigma–Aldrich Co., Ltd. (Shanghai, China). Ultra was purchased from Meito Sangyo Co. Ltd (Kiyosu, Japan).

2.1.3. Strains

The strains used in this work were studied in our previous works and deposited in our laboratory [17,18]. The strain *S.maltophilia* CGMCC 4254 was deposited at China General Microbiology Collection Center, with an accession number of CGMCC No. 4254. The strains were cultivated in 50 mL of olive oil medium (olive oil, 10.0 g/L; tween-80 5.0 g/L; $(NH_4)_2SO_4$, 5.0 g/L; K_2HPO_4, 1.0 g/L; KH_2PO_4, 3.0 g/L; $MgSO_4$, 0.5 g/L; pH 7.2) in 250 mL Erlenmeyer flasks at 200 rpm and 30 °C for 48 h [17].

2.2. Selection of lipases for diastereoselective acylation of L-menthol

2.2.1. Lipase preparation

In general, cells and residual olive oil in liquid medium were removed by centrifugation at 4 °C and 12,000 × *g* for 15 min after cultivation. The resultant supernatant was ultra-filtrated through cutoff membrane filter (10 kDa, Millipore, USA) by 10-fold concentration. The resultant lipase solution was then mixed with 5% (m/v) lactose, and the mixture was subjected to lyophilization (Labconco Freezone 12, Kansas City, MO, USA) [17]. All lipase powders were stored at 4 °C.

2.2.2. Screening of lipases for diastereoselective acylation of L-menthol

The diastereomeric menthol mixture (0.1 mmol) and vinyl acetate (0.025 mmol) were mixed in toluene (2.0 mL), and then the reaction was started by adding lipase powder with equivalent activity (1550 U, tridecanoate as substrate). The reaction was carried out in a capped glass vessel thermostated at 40 °C and 200 rpm for 24 h. In the reference experiment, 0.1 mmol diastereomeric mixture, 0.025 mmol vinyl acetate and 2.0 mL toluene were added into a screw-capped vial in the absence of lipase, and the reaction was carried out at the same conditions. The menthyl ester products were separated from reaction mixture by silica chromatography. The conversion and diastereomeric excess were calculated by chiral GC method. The best lipase was further studied in the subsequent study. Diastereomeric mixture, vinyl acetate and toluene were dehydrated with anhydrous $CaCl_2$ at room temperature for 72 h before used.

2.3. Improvement procedure for SML-catalyzed diastereoselective acylation of L-menthol

To determine the optimal organic solvent, SML powder (40 mg; 1550 U, tridecanoate as substrate), diastereomeric mixture (0.2 mmoL) and vinyl acetate (0.05 mmol) were mixed in 2.0 mL of different organic solvents (acetone, acetonitrile, tetrahydrofuran, chloroform, toluene, cyclohexane, *n*-hexane and *n*-heptane). The reaction mixture was incubated in capped glass vials at 40 °C and 200 rpm for 24 h. The effect of acyl donor on SML was studied as follows: 2.0 mL *n*-hexane, 0.2 mmol diastereomeric mixture