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Synthesis of short chain alkyl esters using cutinase from *Burkholderia cepacia* NRRL B2320

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ABSTRACT

Short chain alkyl esters are well appreciated for fruity flavors they provide. These are mainly applied to the fruit-flavored products like jam, jelly, beverages, wine and dairy. Cutinase from *Burkholderia cepacia* NRRL B 2320 was found to be active in catalyzing the synthesis of alkyl esters in organic solvent. The optimal temperature range for the enzyme catalyzed synthesis was found to be from 35 °C to 40 °C. The maximum conversion (%) during synthesis of ester was obtained for butyric acid (C4) and valeric acid (C5) with butanol reflecting the specificity of the enzyme for short-chain length fatty acids. In case of alcohol specificity, butanol was found to be most preferred substrate by the enzyme and conversion (%) decreased with increasing carbon chain length of alcohol used in the esterification reaction. The kinetic analysis for the synthesis of butyl butyrate by varying concentration of one substrate at a time (butanol or butyric acid), showed that Ping–Pong Bi Bi model with acid inhibition and influence of initial water is most suitable model for the prediction of the reaction kinetics.

1. Introduction

Esters of short chain fatty acids and alcohols are important components of natural aromas used in the food industry. They are employed in fruit-flavored products like beverages, candies, jam, jelly, baked food, wines, butter, cream, yoghurt, cheese etc. Among the various esters butyl butyrate, ethyl butyrate significantly contribute the aroma of pineapple, apple, banana while butyl octanoate used for herbal flavor. Hexyl ester (hexyl butyrate) is an ingredient of fruit, beer and citrus aromas. Octyl ester (octyl butyrate) imparts flavor of peach and strawberry [1]. Although these important products are currently synthesized by traditional means of chemical method, which is still the most economical method, but it uses aggressive chemical catalysis and generates by-products during high temperature reaction [2,3]. Natural aromas obtained from plant extract consist of a mixture of different flavor compounds. These natural aromas are very expensive due to their low concentration in the natural product and very low extraction yields [4]. In contrast, the chemical synthesis of these fatty acid esters is economical but these are not classified as natural products as their synthesis requires strong acids or alkali as catalyst and high temperature and pressure. In comparison to the direct synthesis of esters from fatty acids and alcohols, enzymatic means has been suggested as a good alternative due to an energy-saving procedure with high selectivity [4]. The enzymatic approach allows mild reaction conditions and the resulting products are classified as natural by food regulatory agencies, a feature that increased their public acceptance as ingredients for food industry when compared to those synthesized by chemical processes. The synthesis of esters by enzymes can be carried out in a native enzyme suspension in an organic solvent or in a solvent-free system [5].

Until now, lipases and esterases are used for the production of a wide range of ester products in non-conventional media. But most of the lipases have higher affinity for longer-chain length substrates, and low molecular weight substrate may have some inhibitory effect on enzymes [6,7]. In case of esterases, except few, most esterases were lost their reactivity when the chain length of substrate exceeds two [3,8]. Recently, few reports also available where cutinases (EC 3.1.1.74), the hydrolytic enzymes and the smallest member of α/β hydrolase family were employed for synthesis of some alkyl esters [4,9–15]. Cutinases are believed to be a group of enzyme intermediate between lipase and esterase, which are able to hydrolyze cutin polymer, soluble esters and emulsified triacylglycerol [16]. Cutinases mostly belong to the group of serine-hydrolase family, which contains serine group in its active site. As the reaction is reversible, the enzymes can also catalyze the formation of alcohols and fatty acids from esters. In aqueous solutions, the equilibrium is strongly shifted towards the starting reagents and esters cannot be synthesized. To overcome this difficulty, reaction media containing very small amounts of water or comprised of organic solvents can be used to bring about a chemical equilibrium

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shift towards ester. This reversible reaction was previously shown to follow a Ping–Pong Bi Bi mechanism [14,17].

Cutinase was previously isolated and purified from *Burkholderia cepacia* NRRL B 2320. In this investigation, we have studied the *B. cepacia* cutinase catalyzed synthesis of alkyl esters in isooctane solvents under various conditions. Here, we have also studied the kinetics for the synthesis of butyl butyrate from butyric acid and butanol in isooctane.

2. Materials and methods

Butanol, pentanol, hexanol, octanol, butyric acid, valeric acid, caproic acid and octanoic acid purchased from sigma. Isooctane (Merck) was dried over 4 Å molecular sieve. Tris (hydroxymethyl) amino methane (Tris) was also purchased from Sigma. The cutinase enzyme used for reaction was isolated from *B. cepacia* NRRL B 2320 (previously known as *Pseudomonas cepacia*).

2.1. Production and purification of enzyme

The following medium was used for the production of cutinase ($g\,l^{-1}$): beef extract, 4.0; peptone, 17.8; urea, 5.0; KH_2PO_4, 3.0; KCl, 0.64; $MgSO_4{\cdot}7H_2O$, 5.55 and cutin 10.1. Initial pH of the medium was adjusted at 7.0. A 2% of inoculum from the seed culture was added to 200 ml of the production medium in 1000 ml Erlenmeyer flasks. The flasks were then incubated in a shaking incubator at 28 °C and 180 rpm. After 96 h of fermentation, cells were centrifuged at 8000 rpm for 10 min at 4 ± 1 °C to obtain the cell free broth containing extracellular cutinase. All purification steps were carried out at 0–4 °C unless otherwise indicated. All chromatographic runs were monitored for protein at 280 nm. The enzyme was purified in four steps. The crude enzyme was precipitated with finely powdered ammonium sulphate at 80% saturation. The precipitate was collected by centrifugation at 8000 rpm for 30 min and dissolved in minimal amount of 20 mM Tris–HCl buffer (pH 8.0) and dialyzed against the same buffer for 24 h. Dialyzed ammonium sulphate fraction was applied to a 2 cm × 50 cm CM-650 TOYOPEARL® column (Tosho Corporation, Tokyo, Japan) and eluted using linear gradient of NaCl (0–500 mM). After ion exchange chromatography two steps of gel filtration chromatography was used (Sephadex G100 and Sephacryl S-300 (Sigma)) to get purified homogenous cutinase enzyme. This purified enzyme was used for further study.

2.2. Cutinase assay

The cutinase hydrolytic activity was measured by following the hydrolysis of p-nitrophenyl butyrate (p-NPB) (Sigma) as substrate. An aliquot of (0.02 ml) culture supernatant was added to 0.98 ml of reaction mixture, which was prepared by adding 1 ml 23 mM pNPB in tetrahydrofuran to 40 ml of 50 mM potassium phosphate buffer containing 11.5 mM sodium deoxycholate. The reaction was monitored for 15 min at 37 °C and absorbance of released p-nitrophenol was measured at 410 nm. One enzyme unit is defined as the amount of enzyme required to release 1 μM of p-nitrophenol per min under assay conditions. The cutinase production from *B. cepacia* NRRL B 2320 was confirmed previously (data not shown) using cutinase specific substrate, p-nitrophenyl (16 methyl sulphone ester) hexadecanoate (p-NMSH).

2.3. Enzymatic synthesis of alkyl esters

In the standard protocol, ester synthesis was carried out in screw-capped test tubes as bioreactors. Unless otherwise specified, 0.1 mM of enzyme in lyophilized powder form (calculated on the basis of molecular mass of enzyme as obtained from SDS PAGE 26.5 kDa) was added to 2.5 ml of isooctane containing 0.25 M fatty acid and 0.25 M alcohol. All reagents were previously dried over 4 Å molecular sieves. The tubes were kept in a horizontal shaker at 37 °C. At regular intervals, 300 μl of the mixture (for butyric acid) or 100 μl (for all other acids) were transferred to 2 ml micro centrifuge tube and centrifuged at 10,000 rpm for 1 min to remove the suspended enzyme particles. The progress of reaction was determined by measuring the decreasing profile of acids by titration with 0.01 N NaOH using phenolphthalein as indicator and also with Lowry and Tinsley method [18]. According to Lowry and Tinsley method 700 μl (for butyric acid) or 900 μl (for all other acids) isooctane was added to the samples. Cupric acetate aqueous solution (0.2 ml) containing pyridine (5%, w/v, pH 6.0) was then added into the tube and the solutions were vigorously mixed for 1 min using a vortex mixture. After centrifugation at 2500 rpm for 5 min, the upper organic phase was measured by a UV/visible spectrophotometer (Cary 100, Varian) at 715 nm. The formation of ester was also confirmed by gas chromatography. Water percentage in the reaction mixture was determined by Karl Fisher titration method [2,4]. All reactions and analysis were performed in duplicate.

2.3.1. GC analysis of esters

Synthesis of fatty acid ester was analyzed by gas chromatograph (Varian 390). The diluted aliquots of the reaction mixture were injected into CPSIL 8CB column and compounds were detected by FID. The injector and detector temperature were set at 250 °C. The program (temperature and time) and retention time (t_R) used for different esters are given below.

- Butyl butyrate: 150 °C for (0.5 min) −15 °C/min-250 °C (10 min); t_R 2.42 min
- Butyl valerate: 150 °C for (0.5 min) −15 °C/min-250 °C (10 min); t_R 3.14 min
- Butyl hexanoate: 150 °C for (0.5 min) −15 °C/min-250 °C (10 min); t_R 4.38 min
- Butyl octanoate: 150 °C for (0.5 min) −30 °C/min-250 °C (10 min); t_R 6.65 min
- Butyl decanoate: 150 °C for (0.5 min) −30 °C/min-270 °C (10 min); t_R 15.5 min
- Butyl Palmitate: 150 °C for (0.5 min) −30 °C/min-300 °C (10 min); t_R 20.3 min
- Ethyl butyrate: 100 °C for (0.5 min) −15 °C/min-150 °C (10 min); t_R 1.36 min
- Ethyl valerate: 100 °C for (0.5 min) −15 °C/min-150 °C (10 min); t_R 2.55 min
- Ethyl hexanoate: 50 °C for (0.5 min) −30 °C/min-160 °C (10 min); t_R 2.15 min
- Pentyl butyrate: 150 °C for (0.5 min) −30 °C/min-250 °C (10 min); t_R 3.58 min
- Hexyl butyrate: 150 °C for (0.5 min) −30 °C/min-250 °C (10 min); t_R 4.18 min
- Octyl Butyrate: 150 °C for (0.5 min) −30 °C/min-250 °C (10 min); t_R 5.43
- Decyl butyrate: 100 °C for (0.5 min) −30 °C/min-150 °C (10 min); t_R 2.20 min

2.4. Kinetic study for butyl butyrate synthesis

Originally Michaelis–Menten equation was derived for kinetics of single substrate reaction. However, a reaction involving two substrates may also thought to obey the Michaelis–Menten kinetics, if the reaction rate depends on the concentration of both the substrates, so that if one substrate concentration varied while other maintaining constant, the reaction behaves like a single substrate reaction obeying Michaelis–Menten kinetics.