Lipase of *Aspergillus niger* NCIM 1207: A Potential Biocatalyst for Synthesis of Isoamyl Acetate

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Received: 10 February 2009 / Accepted: 8 December 2009 / Published online: 25 January 2011
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Abstract Commercial lipase preparations and mycelium bound lipase from *Aspergillus niger* NCIM 1207 were used for esterification of acetic acid with isoamyl alcohol to obtain isoamyl acetate. The esterification reaction was carried out at 30°C in *n*-hexane with shaking at 120 rpm. Initial reaction rates, conversion efficiency and isoamyl acetate concentration obtained using Novozyme 435 were the highest. Mycelium bound lipase of *A. niger* NCIM 1207 produced maximal isoamyl acetate formation at an alcohol/acid ratio of 1.6. Acetic acid at higher concentrations than required for the critical alcohol/acid ratio lower than 1.3 and higher than 1.6 resulted in decreased yields of isoamyl acetate probably owing to lowering of micro-aqueous environmental pH around the enzyme leading to inhibition of enzyme activity. Mycelium bound *A. niger* lipase produced 80 g/l of isoamyl acetate within 96 h even though extremely less amount of enzyme activity was used for esterification. The presence of sodium sulphate during esterification reaction at higher substrate concentration resulted in increased conversion efficiency when we used mycelium bound enzyme preparations of *A. niger* NCIM 1207. This could be due to removal of excess water released during esterification reaction by sodium sulphate. High ester concentration (286.5 g/l) and conversion (73.5%) were obtained within 24 h using Novozyme 435 under these conditions.

Keywords Isoamyl acetate formation · Fusel oil · Mycelium bound lipase · Novozyme 435 · *Aspergillus niger* NCIM 1207

Introduction

The industrial alcohol produced by fermentation contains 0.1–0.2% of fusel oil which is recovered as byproduct during the process of rectification of alcohol. With 300 distilleries in India and annual ethanol production more than 14,000, 68 million liters of fusel oil was anticipated. The current selling price of fusel oil is Rs. 30–35/l which is mainly used in paint, shoe polish industries and also as fuel for energy source. The fusel oil contains mainly isoamyl alcohol (55–60%) followed by *n*-propyl alcohol (15–20%), isobutyl alcohol (6–8%) and traces of *n*-butyl alcohol and ethanol. Esters obtained from alcohols of fusel oil can be used as solvents, flavoring agents, extractants, and plasticizers [1]. The acetates of most alcohols are also commercially available. Ethyl, isopropyl, butyl, isobutyl, amyl, and isoamyl acetates are used in cellulose nitrate and other lacquer type coatings due to their high solvent power. Butyl and hexyl acetates have excellent solvent properties useful for polyurethane coating systems. Ethyl, isobutyl, amyl, and isoamyl acetates are frequently used as flavors. Among all these esters, isoamyl acetate is of commercial importance in the food industry (75 tons per year) because of its strong banana flavor [2–4].

Most of the simple esters used commercially are produced synthetically by chemical reaction of an alcohol with an acid in presence of acid catalysts or from extraction from natural sources [2]. Natural flavor esters extracted from plant materials are often in short supply and are most expensive for commercial exploitation [5]. The chemically synthesized product is cheap but not natural. Hence biotechnological production of flavor esters through enzymatic synthesis can be attractive/alternative route to the traditional chemical synthetic methods, particularly in the production of natural flavor and fragrances. This is due to

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selectivity of the enzymes, milder operation conditions, the degree of purity of the product and their acceptability in the food industry [6]. Esters obtained through bio-catalytic route are considered to be “Natural” satisfying the consumer’s demand [7]. Flavor esters are generally produced by free and immobilized lipases in organic solvents [8–10]. Recently, lipase catalyzed production of various esters such as isoamyl butyrate [11], isoamyl propionate [12], hexyl butyrate and laurate [13, 14], citronellyl flavor ester [15] has been investigated.

Aspergillus niger NCIM 1207 produces extracellular and mycelium bound lipase which is active at extremely acidic pH [16, 17]. The purified extracellular lipase was found to be unique since it cleaved triolein at only 3-position releasing 1,2-diolein [18]. The purpose of this study is to evaluate these enzyme preparations for bio-conversion of isoamyl alcohol to isoamyl acetate. For comparison, the commercial lipase enzyme preparations were also tested for isoamyl acetate formation. The optimization studies on the effect of various reaction parameters alcohol/acid molar ratio, substrate concentration, reaction time on isoamyl acetate formation were carried out using mycelium bound lipase.

Materials and Methods

Chemicals and Enzymes

Novozyme 435 (lipase from *Candida antarctica* 10,000 U/g), Lipase from *Candida rugosa* (1140 U/mg), lipase from *Rhizomucor miehei* (20,000 U/g), Lipase from *Mucor miehei* (5770 U/mg) were obtained from Sigma Chemical Co., USA. Lipolase Ultra 50T 50 KULU/g, (kilo ultra lipase units), is a protein engineered lipase produced by genetically modified *Aspergillus*. Fusel oil (25 ml) was obtained from local sugar factory. All the other chemicals used were of analytical grade.

Growth of *A. niger* NCIM 1207 for Lipase Production

Aspergillus niger strains, which are known as lipase producers acting on various substrates were tested for bio-conversion to isoamyl acetate. The culture was grown in synthetic oil based medium (NaNO₃ 0.05%, KCl 0.05%, MgSO₄·7H₂O 0.05%, KH₂PO₄ 0.2%, yeast extract 0.1%, bacto-peptone 0.5%, olive oil 1.0%, glucose 1.0%), pH 5.5 by inoculating the flasks with spores (10⁶/ml) and incubating the flasks at 28°C with shaking (180 rpm). The culture was harvested after 120 h and the dry mycelium was separated by filtration, which was used as a source of intracellular enzyme. The culture broth was used as a source of extra-cellular enzyme.

Preparation of Dry Mycelium

The mycelium harvested by filtration was washed twice with distilled water to remove traces of medium followed by rapid washing with chilled acetone. The acetone treated mycelium was vacuum dried for 6 h to remove acetone and water. This vacuum dried mycelium was used for bioconversion.

Celite Adsorption of Extracellular Enzyme

A standard procedure based on Colman and Macrae (1973) was used to immobilize the extracellular lipase. Celite 545 (1.0 g) was added to 20 ml of the culture filtrate with mixing. Ice-cold acetone (25 ml) was then added over a period of 5 min while stirring with magnetic stirrer and the suspension was stirred for an additional 30 min at 0°C, then filtered and air dried. The celite-adsorbed preparation (1.2 g) contained approximately 200 mg (±25) of water. This celite-adsorbed enzyme preparation was used as a source of extracellular enzyme in *trans*-esterification experiments.

Esterification Reaction Using Isoamyl Alcohol

Esterification reaction was carried out in *n*-hexane with a working volume of 10 ml in 25 ml stopper flask. An appropriate amount of enzyme was added into a reaction mixture containing a solution of isoamyl alcohol and acetic acid and sodium sulphate pre-equilibrated at the conditions of experiment. The reaction mixture was incubated at 30°C with shaking at 120 rpm and the samples were withdrawn at different time intervals to analyze the isoamyl acetate content in the reaction mixture.

Bioconversion of Fusel Oil

The bioconversion reaction was carried out in 25 ml stoppered flasks with 10 ml *n*-hexane containing fusel oil (1.9 ml), acetic acid (360 µl) and known amount of enzyme source. The fusel oil contained 55% isoamyl alcohol along with other alcohols such as *n*-butanol, isobutanol, *n*-propanol etc. The reaction mixture was kept shaking at 120 rpm at room temperature and samples were removed at different time intervals for analysis of product (isoamyl acetate) in the reaction mixture. The analysis was performed using Gas Chromatography (Varian GC Model 3800) using CP-Wax 52 CB column. The above-mentioned commercial enzymes were used.

Analysis

The samples (for isoamyl acetate) were analyzed on Varian (Varian Analytical Instruments, USA) gas chromatograph

(Model CP-3800 GC) equipped with CP-Wax 52 CB column (30 m length, 0.32 mm internal diameter) and a flame-ionization detector. Nitrogen gas was used as a carrier gas with a flow rate of 30 ml/min. Column oven, injection port, and detector temperatures were 220, 230, and 240°C, respectively. The ester formed was calculated as being equivalent to acid consumed. The conversion of acetic acid to isoamyl acetate was calculated as the ratio of ester concentration to initial acid concentration [(mmol of ester formed/mmol of ester calculated for initial acid concentration) × 100]. Lipase assay was performed as described earlier [16].

Results

Effect of Enzyme Source

The effect of lipase source on isoamyl production was investigated by comparing different commercial lipases and the results are given in Table 1. Mycelium bound lipase of *A. niger* NCIM 1207 was also tested for isoamyl acetate formation. It was observed that extent of esterification was higher for Novozyme 435 lipase yielding 100% in 4 h. Mycelium bound lipase of *A. niger* NCIM 1207 also gave high esterification but the rate of reaction was slower. No other commercial preparations produced isoamyl acetate except *C. rugosa* lipase which gave only 2.5 g/l of isoamyl acetate with 3.1% esterification. Although the conversion was almost 100% using both the enzymes for esterification, the reaction reached equilibrium for Novozyme 435 in 4 h where as lipase from *A. niger* took almost 96 h to reach equilibrium.

The effect of alcohol to acid molar ratio on the esterification yield was investigated by fixing alcohol concentration at 0.8 M and by varying acid concentration (0.32–1.3 M). All the experiments were performed at

mycelium bound *A. niger* NCIM 1207 lipase 100 g/l. Reaction mixtures were incubated at 30°C with shaking at 120 rpm for 96 h. The rate of reaction increased with increase in alcohol to acid ratio, because the equilibrium of the reaction was pushed toward the product formation as the alcohol concentration increased. At isoamyl alcohol to acetic acid molar ratios more than 1.6 did not change the reaction rate significantly. A maximum yield of isoamyl acetate (100% in 96 h) was achieved when alcohol to acid ratio of 1.3–1.6 was employed (Fig. 1).

Effect of Substrate (Acetic Acid) Concentration on Activity of Mycelium Bound Lipase of *A. niger* NCIM 1207

We also evaluated the effect of different substrate (acetic acid) concentration (0.15–0.62 M) on esterification using mycelium bound lipase of *A. niger* NCIM 1207 with fixed alcohol to acid ratio at 1.6. We found that the conversion was 98% up to 0.3 M acetic acid concentration followed by sudden drop to 60% with higher substrate level of 0.6 M (Table 2). This reduction in the conversion could be due to the acid inactivation of the enzyme or to the excess water generated during the esterification reaction, which might shift the equilibrium of the reaction towards hydrolysis. The first possibility could be ruled out since the mycelium bound lipase was found to be active and stable even at pH 2.0 for 24 h. The second possibility was confirmed by carrying out the esterification reactions in presence of sodium sulphate, which removes water generated during esterification reaction. The presence of sodium sulphate (5%) resulted in 99% conversion of acetic acid (0.62 M) yielding 80 g/l of isoamyl acetate. The results showed that the drop in conversion was due to excess water formed during esterification (Table 2).

Table 1 Bioconversion using different commercial enzyme sources

Enzyme source	Product (g/l)	Conversion (%)
Novozyme 435 (10 g/l)	81.0 ± 3.5	100.0 (4 h)
<i>A. niger</i> NCIM 1207, dry mycelium (100 g/l)	80.0 ± 4.0	99.0 (96 h)
Celite bound lipase (10 g/l)	–	–
<i>M. miehei</i> lipase (10 g/l)	–	–
Lipolase ultra 50T (10 g/l)	–	–
<i>C. rugosa</i> lipase (10 g/l)	2.5 ± 0.5	3.1 (96 h)
<i>R. miehei</i> lipase (10 g/l)	–	–

The esterification reaction was carried out at 30°C using alcohol/acid molar ratio of 1.6 with 37.7 g/l acetic acid and 130 g/l of isoamyl alcohol. The numbers in parenthesis denote the time of incubation of esterification reaction

Fig. 1 Effect of alcohol to acid molar ratio on synthesis of isoamyl acetate. The esterification reaction was carried out in *n*-hexane with different alcohol to acid molar ratios at fixed concentration (0.8 M) of isoamyl alcohol using *A. niger* NCIM 1207 lipase (100 g/l)

Table 2 Effect of substrate (acetic acid) concentration on isoamyl acetate formation using mycelium bound lipase of *A. niger* NCIM 1207

Acetic acid (g/l)	Isoamyl acetate (g/l)		Conversion (%)	
	Without sodium sulphate	With sodium sulphate (5%)	Without sodium sulphate	With sodium sulphate (5%)
9.0 (0.15 M)	20 ± 1.2	20.4 ± 1.3	98	100
18.6 (0.31 M)	40 ± 2.5	40.9 ± 2.8	98	100
28.2 (0.47 M)	52 ± 3.5	54.5 ± 3.6	85	89
37.2 (0.62 M)	50 ± 3.5	80.0 ± 4.0	61	99

The esterification reaction was carried out using alcohol/acid molar ratio of 1.6 with 100 g/l of mycelium bound lipase. The samples were analyzed for isoamyl acetate content after 96 h

Table 3 Effect of acetic acid (substrate) concentration on isoamyl acetate formation using Novozyme 435

	Enzyme (g/l)	Acetic acid (g/l)	Sodium sulphate (g/l)	Product (g/l)	Conversion (%)
The esterification reaction was carried out using alcohol/acid molar ratio of 1.6 with Novozyme 435. The samples were analyzed for isoamyl acetate content after 24 h	10	48.0 (0.8 M)	Nil	103.0 ± 7.5	99.3
	10	60.0 (1.0 M)	Nil	128.0 ± 9.5	98.4
	10	75.5 (1.25 M)	Nil	148.0 ± 10.0	90.7
	10	75.5 (1.25 M)	50	152.0 ± 10.0	93.2
	10	105.0 (1.74 M)	50	187.5 ± 11.0	82.4
	10	143.7 (2.37 M)	50	219.0 ± 13.5	70.3
	20	143.7 (2.37 M)	50	220.5 ± 13.0	70.5
	20	143.7 (2.37 M)	100	265.5 ± 15.5	85.2
	20	180.0 (3.0 M)	100	259.5 ± 14.0	66.5
	20	180.0 (3.0 M)	150	286.5 ± 14.5	73.5

Effect of Substrate Concentration on Novozyme 435

The effect of varying concentrations of acetic acid (0.8–3.0 M) on extent of esterification is presented in Table 3. The esterification of about 100% within 8 h was observed up to 1.0 M acid concentration and it was reduced to 90% at 1.25 M acid concentration. The presence of sodium sulphate in the reaction mixture did not help to enhance the conversion indicating water generated has no effect on synthetic reaction probably at these concentrations of the substrate. The further increase in substrate concentration (acetic acid) resulted in less conversion possibly due to water produced during esterification. Therefore the experiment was planned to see the effect of addition of sodium sulphate during esterification at higher (>1.25 M) acetic acid concentration. It was observed that the addition of sodium sulphate increased the esterification of isoamyl alcohol to isoamyl acetate (Table 3). Maximum isoamyl acetate concentration of 286 g/l was obtained using 20 g/l of Novozyme 435 with 73% esterification.

Bioconversion of Fusel Oil

We evaluated the commercially available enzymes and also mycelium bound lipase of *A. niger* NCIM 1207 for synthesis of isoamyl acetate using fusel oil which contained 55% isoamyl alcohol. It was observed that bioconversion

Table 4 Bioconversion of fusel oil by different enzymes

Enzyme source	Enzyme amount	Product (g/l)	Conversion (%)
<i>C. antarctica</i>	100 mg (1000 U)	57.5 ± 3.5	71 (4 h)
<i>A. niger</i> NCIM 1207, dry mycelium	1 g (14 U)	50.0 ± 2.8	61 (96 h)
<i>M. miehei</i> lipase (sigma)	5 mg (28850 U)	–	–
<i>A. niger</i> lipase (fluka)	1 g (194 U)	51.0 ± 3.2	62 (96 h)

The bioconversion reaction was carried out at 30°C with shaking at 120 rpm in 25 ml stoppered flasks with 10 ml *n*-hexane containing fusel oil (1.9 ml), acetic acid (360 µl), and known amount of enzyme source

was maximum using both Novozyme 435 and mycelium bound lipase of *A. niger* NCIM 1207 yielding 57.5 and 50 g/l of isoamyl acetate respectively (Table 4). Novozyme 435 gave isoamyl acetate within 4 h since the activity used was much higher.

Discussion

Various enzyme sources either in the form of free lipases [19, 20] or immobilized lipases [9, 21] have been used for production of flavor acetates in organic solvents. The effect

of various commercial lipases and also mycelium bound lipase on isoamyl acetate production was investigated. We found that the reaction rate and esterification were higher using Novozyme 435 yielding 100% esterification in 4 h. Novozyme 435 was also found suitable in the synthesis of other acetate esters in hexane [22], in heptane [23] and also in solvent-free systems [3]. *A. niger* NCIM 1207 produces lipase active at acidic pH and it was also found to produce isoamyl acetate (80 g/l) indicating the new potential candidate for production of flavor esters in organic solvents. Guvenc et al. [24] carried out optimization studies on lipase-catalyzed production of industrially important isoamyl acetate by response surface methodology using Novozyme 435. They found that acid/alcohol molar ratio was the most effective parameter in the production of isoamyl acetate. The maximum esterification was obtained with acid/alcohol ratio of 0.8 with 12% enzyme concentration. In our studies, we found that alcohol/acid molar ratio of 1.3–1.6 was optimum for getting maximum esterification. The extent of esterification increased with increase in alcohol to acetic acid molar ratio because the equilibrium of the reaction was pushed toward the product formation as the nucleophile (alcohol) concentration is raised. The esterification leveled off at the alcohol to acid molar ratios higher than 1.6. The strong effect of acid inhibition on enzyme activity was observed at alcohol to acid ratios below 1.3 leading to decreased esterification. Such strong effect of acid inhibition was also reported by Guvenc et al. [3, 24] and Romero et al. [25, 26]. The acid inhibition of lipase could be due to either accumulation of water during reaction which favors hydrolysis or due to substrate (acid) inhibition. It was also asserted that alcohols are terminal inhibitors of lipases and acids may cause acidification of micro-aqueous interface leading to enzyme inactivation.

The isoamyl acetate concentration obtained so far in literature using Novozyme 435 are 150 g/l in 72 h in heptane [10], and 381 g/l in 6 h in solvent-free system [3], both with 80% conversion. Very recently, Novozyme 435-mediated synthesis of isoamyl acetate was carried out using isoamyl alcohol obtained from fusel oil and acetic acid in solvent-free system by RSM technique [24]. They obtained 490 g/l of isoamyl acetate with 75% esterification efficiency using 12% enzyme. In our study, we were able to produce 281 g/l of isoamyl acetate with 73.5% esterification using only 2% Novozyme 435 under the optimized conditions. We also carried out experiments using mycelium bound *A. niger* NCIM 1207 lipase which gave 80 g/l of isoamyl acetate with 99% esterification indicating that this could be potential candidate for production of isoamyl acetate. It is noteworthy to mention that the amount of *A. niger* enzyme used in terms of enzyme activity was approximately 70 times less (1400 IU/l) as compared to Novozyme 435 (1,00,000 PLU/l).

The attempt was made to make isoamyl acetate from fusel oil using different enzymes. It was found that Novozyme 435 and mycelium bound lipase of *A. niger* NCIM 1207 formed isoamyl acetate indicating the potentiality of *A. niger* NCIM 1207 lipase for isoamyl acetate formation from fusel oil. No other enzyme tested formed isoamyl acetate from either fusel oil or from isoamyl alcohol.

In conclusion, mycelium bound lipase of *A. niger* NCIM 1207 was able to produce isoamyl acetate indicating its potential use in bioconversion of isoamyl alcohol/fusel oil to isoamyl acetate. This mycelium bound lipase showed acidic pH optimum and also it exhibits significant stability at acidic pH. Therefore this could be a potential biocatalyst to be used for bio transformations at acidic environment where acetic acid is one of the substrates used in bio-transformation reaction.

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