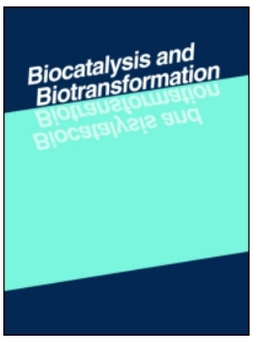

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SHORT COMMUNICATION

Biocatalyzed esterification of oleic acid using cell suspension and dried biomass of *Aspergillus* sp. RBD01

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Abstract

Esterification is an industrially important reaction in the field of food and fuel industries. In biofuel and allied industries, long-chain alkyl esters are generally produced from different fat rich feedstocks including non-edible oils, acid oils, and tallow, using a variety of catalysts. Amongst these, whole cell systems have prominently been explored in recent past. The present study focused on the use of *Aspergillus* sp. RBD01 as a whole cell catalyst, in dry and whole cell suspension, to esterify oleic acid with different alcohols as acyl acceptors. Esterification with dried biomass resulted in better conversion of oleic acid to its respective ester as compared to cell suspension. Further, increase in chain length of alcohol resulted in decrease in the yield from ethyl oleate (98% EO) to decyl oleate (77% DO) with alcohols having an even number of carbon atoms giving better yield of esters over alcohols with odd numbers.

Keywords: Aspergillus *sp.*, *esterification*, *alcohol*, *biocatalyst*, *alkyl ester*

Introduction

Biodiesel is emerging as one of the major alternatives expected to provide answers to the growing demand for fuel by being an eco-friendly and renewable substitute to diesel (Rottig et al. 2010). Biodiesel consists of long chain of fatty acid esters of vegetable oils and animal fat, primarily generated through a transesterification reaction in the presence of alcohols as acyl acceptors (Fukuda et al. 2001). Transesterification necessitates the presence of a catalyst which can either be chemical (acid/alkali/heterogenous) or biological (enzyme/whole cell) in nature. In the recent past, greater emphasis has been towards greener chemical synthesis that uses enzyme or whole cells as biological catalysts (Fukuda et al. 2008).

Use of pure lipases as catalysts in the transesterification reaction is limited by loss of activity in oil-rich medium in addition to the cost-intensive nature of the reaction. In contrast, whole cells capable of producing lipase in specific culture conditions are being explored as catalysts for transesterification since recent past. In general, different alcohols are being used as acyl acceptors in transesterification, with methanol and ethanol being more commonly used (Fukuda et al. 2001).

This study outlines the observations on potential use of an oil-tolerant fungus strain, *Aspergillus* sp. (RBD01) for esterification of a long chain fatty acid (oleic acid) with focus on generating different alkyl esters with different alcohols as acyl acceptors. Our earlier reports demonstrated the potential of this strain to facilitate near complete tranesterification of oil to ethyl ester at 70:30; oil to medium ratio (Prakash and Aulakh 2011). The study, further, compared the efficacy of suspended culture and dried biomass in catalyzing the esterification reaction.

Materials and methods

Different alcohols, hexane, ethyl acetate, and oleic acid were sourced from SD FineChem., India.

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Nitrogen sources included in the study were di-ammonium hydrogen ortho-phosphate ($(NH_4)_2HPO_4$) and mycological peptone (HiMedia, India). Culture media used were mineral salt medium, potato dextrose agar (PDA), potato dextrose broth (PDB) (HiMedia).

Freshly grown *Aspergillus* sp. RBD01 (MTCC5436), earlier isolated from biocontaminated clarified butter (Prakash and Aulakh 2011), was inoculated under sterile conditions into 500 mL Erlenmeyer flask containing 200 mL of potato dextrose broth and incubated at 28 °C, 120 rpm for 72 h. The fresh biomass thus obtained was further used for experimentation. The mineral medium comprising magnesium sulphate ($0.20\,g\,L^{-1}$), calcium chloride ($0.02\,g\,L^{-1}$), mono-potassium phosphate ($1.0\,g\,L^{-1}$), di-potassium phosphate ($1.0\,g\,L^{-1}$), and ferric chloride ($0.05\,g\,L^{-1}$), was used for the growth of fungal strain. The medium was supplemented (0.5% w/v) with mycological peptone or/and di-ammonium hydrogen ortho-phosphate as nitrogen source.

In case of cell suspension, the esterification reaction was carried out with cell suspension in 500 ml Erlenmeyer flask, containing 200 ml of oleic acid: mineral medium (50:50) with oleic acid acting as carbon source, peptone, and bi-ammonium hydrogen ortho phosphate (0.5%) as nitrogen sources, and pH of the medium set at 7.0 ± 0.2. Inoculum from fresh biomass of *Aspergillus* sp. was added and incubated for 72 h at 28 °C and 120 rpm. Following incubation, the influence of chain length of alcohols on the esterification process was studied by addition of various alcohols (methanol, ethanol, propanol, butanol, pentanol, hexanol, heptanol, octanol, nonanol, and decanol) in the ratio of 1:2 (oleic acid:alcohol). Marginal additional amounts of alcohols were supplemented to avoid reversible reaction. The addition of alcohols was carried out stepwise at an interval of 12 h. After completion of the reactions, biomass was recovered by simple filtration. The upper layer of ester was separated by using separation funnel and used further for analysis.

Similarly, to examine the esterification process with powdered biomass of *Aspergillus* sp. freshly cultivated biomass generated as discussed previously, was separated from reaction mixture using Whatman filter paper and washed with n-hexane followed by water to remove adhering oil. This was then air-dried overnight to remove excess water and crushed to powder in liquid nitrogen. Esterification of oleic acid was carried out with varying chain length of alcohol (methanol, ethanol, propanol, butanol, pentanol, hexanol, heptanol, octanol, nonanol, and decanol) for generation of ethyl oleate. To examine the esterification process, 2 g of biomass and 10 g oleic acid were taken in round bottom flask and kept at 35 °C with constant stirring. Alcohols (propanol, butanol, pentanol, hexanol, heptanol, octanol, nonanol, or decanol) were added stepwise at an interval of 6 h, with total reaction time of 36 h. The reactions were carried out in triplicate and the data are represented as mean (±SD). The samples obtained from above experiments with each of the different acyl acceptors were pooled and independently analyzed using TLC and proton NMR.

The alkyl oleate separated from reaction was analysed using thin layer chromatography with silica gel G as stationery phase and hexane:ethyl acetate:acetic acid (90:10:1) as a mobile phase and the chromatogram was developed in the iodine chamber (Samukawa et al. 2000). The ester was further quantified using proton nuclear magnetic resonance spectroscopy (^{1}H NMR) (400 MHz, Bruker-Advance II-400 with 5mm BBO probes). $CDCl_3$ (deuterated chloroform) was used as solvent and tetra methyl silane as internal standard. ^{1}H NMR spectra were recorded with pulse duration of 2.72 s with a relaxation delay of 1 s and 16 scans. Alkyl ester produced by esterification was quantified by using the derivation given by Satyarthi et al. (2009).

$$\begin{aligned}&\%\text{ of FFA (free fatty acids)}\\&= ([4 \times \text{area of unmerged peak of} \propto CH_2\\&\quad \text{of fatty acid}] \div [\text{total area of} \propto CH_2\\&\quad \text{of fatty acid and ester}]) \times 100\end{aligned}$$

To draw a comparison between the activities of wet and dry forms of biomass, lipase activity was determined following method outlined by Sigurgisladottir et al. (1993). The enzyme activity was determined by adding 1 g of biomass to a reaction mixture containing 0.9 ml of 0.05 M phosphate buffer (pH 7.0) and 0.1 ml of 0.005 M pNP (p-nitrophenol laurate) in ethanol. The mixture was incubated at 60 °C for 30 min, followed by addition of 0.25 ml of 0.1 M Na_2CO_3 on cooling to room temperature. The activity was determined at 420 nm. One unit of lipase activity is defined as the amount of enzyme that liberates 1 μg p-nitrophenol (molar extinction coefficient 1.336×10^7 cm^2/mol at 420 nm) with pNP-laurate as substrate under standard assay conditions in 30 min.

Result and discussion

The study compared the potential of the culture suspension and dry biomass of *Aspergillus* sp. RBD01 to esterify oleic acid in the presence of alcohols of varying chain length and polarity.

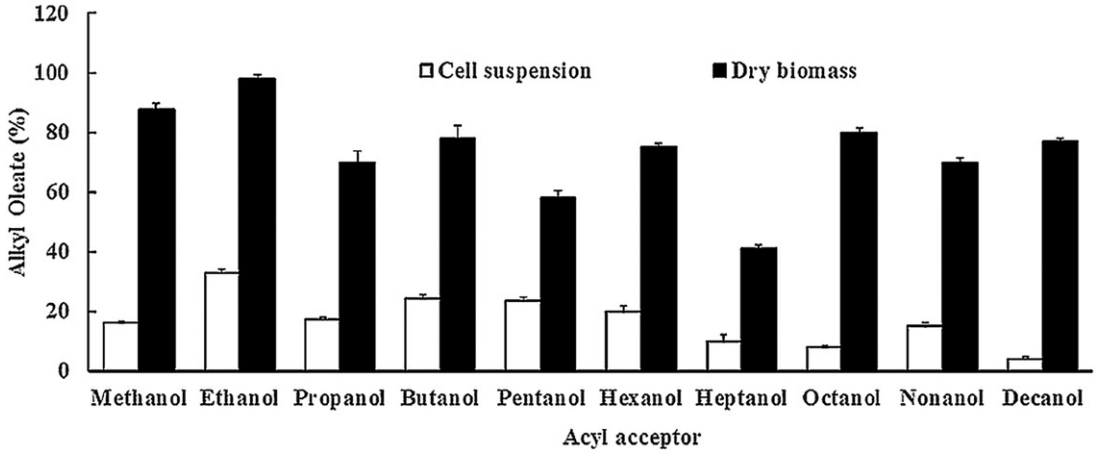

Figure 1. Transesterification (alkyl ester %) of oleic acid with different alcohols using cell suspension (AC) and dried biomass (DB) of *Aspergillus* sp. RBD01.

Observations between cell suspension and dry biomass indicated much higher yield of esters in case of dry biomass when compared to the cell suspension (Supplementary Information). The yield of ethyl oleate (32.8%) was maximum when compared to the rest of the alcohols, with trend of odd and even carbon chains remaining nearly same. In the earlier studies, carried out by the group with oil as carbon source and ethanol as acyl acceptor, the transesterification reaction catalyzed by the test strain resulted in nearly complete conversion of oleic acid (Aulakh et al. 2011). The lower potential of culture suspension, towards catalyzing the esterification reaction, is expected to be due to absence of any ester bond in oleic acid required for inducing lipolytic activity. Correspondingly, in the case of dry biomass, due to presence of optimal concentration of biomass preinduced with lipolytic activity, the extent of esterification was significantly higher (Figure 1). This was also represented by the lipase activity obtained wherein the culture inoculated in 50% oleic acid medium and the dried biomass in 100% oleic acid showed 15.3 U/g and 195 U/g, respectively.

With reference to the esterification of oleic acid in the presence of alcohols with varying chain lengths, methanol or ethanol could effectively be converted to corresponding esters methyl (>98%) and ethyl oleate (87.5%). However, further increase in chain length of alcohol, resulted in varying yield of esters i.e., from propyl oleate (70% PO) to decyl oleate (77% DO). In addition, it was observed that alcohols with even carbons resulted in better yield as compared to alcohols with odd carbons. The variations in yield of ester with increasing chain length of alcohols from ethanol to decanol, is expected to be due to decrease in the polarity of alcohol, as esterification process occurs dominantly at the polar-nonpolar interfacial region. As the length of the carbon chain of the alkoxide anion increases, a corresponding decrease in nucleophilicity occurs, resulting in reduced reactivity (Sridharan and Mathai 1974). As reported by other research groups and also observed in this study, long-chain alcohols, propanol, and butanol have less negative effect on lipase stability (Salis et al. 2005; Nie et al. 2006) and there was no noticeable influence on the esterification when propanol, butanol, hexanol, and octanol were used as acyl acceptors (Hsu et al. 2001; Issariyakul et al. 2007; Jin et al. 2008).

Conclusions

Esterification of oleic acid with different alcohols was successfully carried out using dried biomass as well as cell suspension of *Aspergillus* sp. (RBD01) as whole cell catalyst. Dried biomass resulted in higher yield of esters as compared to cell suspension, however, among different alcohols ethyl esters resulted in better yield as compared to rest of the alcohols. It was observed that alcohols with even carbons resulted in better yield as compared to alcohols with odd carbons. The present study, thus, demonstrates the potential use of a whole cell catalyst for generation of different alkyl esters under different culture conditions, a process that can potentially be exploited for biocatalyzed production of alkyl esters for biodiesel and other industrial applications.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Supplementary material available online