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Aspergillus niger whole-cell catalyzed synthesis of caffeic acid phenethyl ester in ionic liquids

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ABSTRACT

Synthesis of caffeic acid ester essentially requires an efficient esterification process to produce various kinds of medicinally important ester derivatives. In the present study, a comprehensive and comparative analysis of whole-cell catalyzed caffeic acid esters production in ionic liquids (ILs) media was performed. Olive oil induced mycelial mass of halotolerant *Aspergillus niger (A.niger)* EXF 4321 was freeze dried and used as a catalyst. To ensure maximum solubilization of caffeic acid for highest substrate loading several ILs were screened and 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ([Emim][Tf_2N]) was found to have the maximum solubility and favoured for enzymatic activity of freeze dried mycelia. The whole-cell catalyzed synthesis of caffeic acid phenethyl ester (CAPE) conditions were optimized and bioconversion up to 84% was achieved at a substrate molar ratio of 1:20 (caffeic acid:2-phenyl ethanol), 30 °C for 12 h. Results obtained during this study were encouraging and helpful to design a bioreactor system to produce caffeic acid derived esters.

1. Introduction

Caffeic acid (3,4-dihydroxycinnamic) (CA) and its derivatives are members of the hydroxycinnamate and phenylpropanoid metabolites, and widely distributed in plant tissues as a common source of phenolics [1]. These phenolics are widely used as folkloric medicine especially as carcinogenic inhibitor, antioxidant and antimicrobial etc. In addition to that new findings revealed their potentials in prevention of cancer and other cardiovascular diseases, which is another area of interest in this molecules [2]. Due to the low solubility of CA in hydrophobic medium, its applications in oil based foods and cosmetics are limited. In order to maintain its hydrophobicity and activity, it is essential to esterify with fatty alcohol [3].

Among these phenolics, the caffeic acid phenethyl ester (CAPE) an ester of CA is the most important active ingredient of propolis based cosmetic products. In addition to skin bioactive properties of CAPE, it has been reported for a wide range of bioactivities such as antioxidant [4], anti-inflammatory [5], anticancer activities [6,7], neuroprotective, hepatoprotective, and cardioprotective capacities [8], cytoprotective, protective against ischemia–reperfusion (I/R) injury, and protective effects [9]. Therefore, a huge and continuous demand of the CAPE and their derivatives in industries are maintained.

However, isolation and purification of CAPE from natural resources is expensive, low yielding and time consuming. Naturally CAPE coexists with several catechol ring containing phenolic which are major hurdle for isolation [10]. A chemical synthesis also has similar challenges due to use of toxic chemicals, low yields and product purification [11]. Therefore, a greener alternative is being required to meet the industrial demand.

To overcome these environmental constraints, use of biocatalyst such as lipase (either in pure or cell bound) enzyme becomes an increasingly attractive alternative to traditional chemical methods, due to the high selectivity, mild reaction conditions and environment-friendly process. In recent decade, using of whole cell as a biocatalyst was paid great attention in bio-catalyzed reaction, to reduce the overall cost of production. Whole-cell microbe mediated bio-conversion is relatively cost effective and easy to handle than the immobilized enzyme system [12]. Among them, filamentous fungi have been arisen as a most robust whole cell biocatalyst for industrial applications [13].

Ionic liquids (ILs) are molten salt at room temperature, which have a wide range of tunable physio-chemical properties [14]. Unlike organic solvents, ILs possess wide range of temperature, able to dissolve many compounds, good biological compatibility and negligible vapor pressure [15]. Over the past decades, the use of ILs in biotacalysis for organic synthesis has been extensively studied. In the presence of ILs, the biocatalysts (isolated enzyme and whole-cell) showed enhanced catalytic activity, entioselectivity and operational stability [16,17]. However, the major drawbacks of enzyme catalysts are their very

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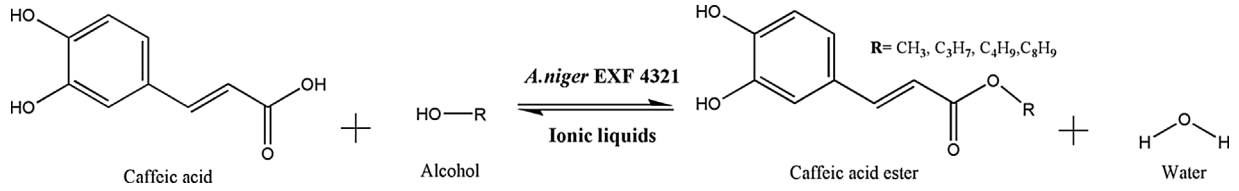

Fig. 1. Halotolerant *A.niger* EXF 4321 mycelia catalyzed esterification of caffeic acid with alcohol as a model reaction.

higher costs, since they need multiple time-consuming purification processes and in some cases, removal of an enzyme from its natural cell environment may lead to partial or even complete loss of the enzyme activity [18]. Therefore, direct use of microbial whole-cells as biocatalysts instead of isolated enzymes is considered as a potential way to reduce the cost of industrial process, since they could avoid the tedious preparation procedures of the enzymes and maintain the enzyme activity by protecting the cells [19].

This study is focusing on microbe mediated greener synthesis of CAPE in ILs using a halotolerant filamentous fungus *A.niger* EXF 4321, isolated from solar saltern [20]. *A. niger* EXF 4321 was selected as catalyst due to their highly tolerance to ILs containing media in our previous screening study (data not shown). In this study, an organic solvent free green CAPE biosynthesis was performed in ILs and to meet the goal, freeze dried mycelia was used as whole cell catalyst while caffeic acid and 2 -phenyl ethanol was used as substrates (Fig. 1). This study was the first report on such kind of phenolic acid ester synthesis using microbial whole-cell catalyst and ILs system.

2. Materials and methods

2.1. Materials

Ionic liquids 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl) imide ([Emim][Tf_2N]), 1-Butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ([Bmim][Tf_2N], 1-Hexyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ([Hmim][Tf_2N]), 1-octyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ([Omim][Tf_2N]), 1-Benzyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ([Bzmim][Tf_2N]) 1-Ethyl-2,3-dimethylimidazolium bis(trifluoromethylsulfonyl) imide ([Edmim][Tf_2N]) used in this study were purchased from C-TRI (South Korea) and had residual chloride content of less than 30 ppm. All ILs were dried in an oven with vacuum pressure of 90 kPa at 80 °C for 48 h before use. 2-Phenyl ethanol, isooctane, CA, CAPE, methanol, 1-propanol and n-butanol were purchased from Sigma-Aldrich. All other chemicals were of analytical grade and used without further purification. *A.niger* EXF 4321 was received from EX Culture Collection (University of Ljubljana, Slovenia).

2.2. Inoculum preparation

The cultures of *A.niger* EXF 4321 were incubated on potato dextrose agar slants at 30 °C for 5 days. The spores obtained were suspended in sterile distilled water containing 0.1% (v/v) Tween 80 for the preparation of inoculums. The spore concentration was determined by using hemocytometer. The spore suspension of 10^7 spores/mL inoculated in 250 mL flasks containing 100 mL of the Czapek dox (CZ) consist of K_2HPO_4 (1.0 g L^{-1}), KCl (0.5 g L^{-1}), $NaNO_3$ (3.0 g L^{-1}), $MgSO_4$ (0.5 g L^{-1}), $FeSO_4$ (0.1 g L^{-1}) and sucrose (30.0 g L^{-1}) in distilled water. To induce lipase activity, 3% olive oil was added to 24 h grown culture at 30 °C with shaking speed of 200 rpm. Further cultures were continuously incubated in shaker for 96 h. After incubation, mycelia was filtered and washed thoroughly with chilled acetone and dried in freeze drier at −55 °C for 24 h and kept at 4 °C for further use.

2.3. Lipase assay

The rhodamine B-olive oil agar (ROA) plate assay was used for identification of lipase production from organism [21]. This primary plate assay method is used to determine the lipase secretion, based on the interaction of rhodamine B with fatty acid released from olive oil by the lipase hydrolysis. The mycelia were inoculated with the plate containing ROA medium and incubated for 48 h at 30 °C. After incubation, the plates were observed under UV light (350 nm). The hydrolysis of substrate causes the formation of orange fluorescent halos which are visible upon UV irradiation. Lipase activity was also quantified by standard protocol, in brief, the absorbance at 400 nm due to the release of *p*-nitrophenol during the hydrolysis of 50 mM *p*-nitro phenyl palmitate at pH 7.2 and temperature at 37 °C [22]. To initialize the reaction, freeze dried *A.niger* EXF4321 mycelia was added and absorbance was measured spectrophotometrically at 400 nm. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the production of 1 nmol of p-nitrophenol per minute at 30 °C.

2.4. Solubility of caffeic acid

The solubility experiment was performed as conventional method to quantify the maximum CA solubility in various ILs. In brief, an excess amount of CA was added to a 5 mL screw-capped vial containing ILs (1 mL) and the samples were vortexed for few seconds to ensure the solubilization of CA [23]. The suspension was stirred for 24 h at 30 °C. Then, the samples were centrifuged at 10,000 rpm for 5 min. The supernatant of ILs phase was sampled and diluted with methanol. The concentration of CA was determined by HPLC analysis.

2.5. Caffeic acid esters synthesis

Caffeic acid ester synthesis was carried out in 5 mL screw-capped vials containing caffeic acid and fatty alcohol in a molar ratio of 1:20 placed in magnetic reaction block at 30 °C with shaking speed of 200 rpm. Reaction was initiated by the addition of freeze dried *A. niger* EXF 4321 mycelia. From the reaction mixture, sample was carefully withdrawn at specified time intervals and centrifuged at 8000 rpm for 5 min to remove the mycelia from the reaction mixture. The samples were diluted with methanol and quantity of the ester synthesis was analyzed by HPLC. All reactions were duplicated and mean values were presented.

2.6. HPLC analysis

Quantitative analysis of CA and esters were performed by HPLC analytical method. Separation was accomplished using a Shimadzu HPLC system (Model LC-10A, Japan) equipped with a reverse-phase C18 column (4.6 × 250 mm, Waters, USA) with 5 µm particle size. The mobile phase consists of methanol and distilled water (70:30% v/v) with flow rate of 1 mL min^{-1}. The absorbance was detected using UV detector at 325 nm.