ARTICLE IN PRESS

Process Biochemistry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Enzymatic synthesis of geranyl acetate in packed bed reactor in supercritical carbon dioxide under various pressure-temperature conditions and reactor configurations

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ARTICLE INFO

Keywords:
Supercritical CO_2
Candida Antarctica lipase B
Packed bed reactor
Solvent free geranyl acetate
Green synthesis
Catalytic efficiency and stability

ABSTRACT

As an alternative to the chemical synthesis of geranyl acetate, the aim of the present study is to better understand its enzymatic synthesis in a 1 mL packed bed reactor (PBR) using supercritical CO_2 as a green solvent and immobilized lipase B from *Candida antarctica* as a catalyst. Using a full factorial design in the range 150–300 bar and 45–65 °C, the conversion of geraniol to geranyl acetate versus pressure and temperature could be accurately modeled. The best conversion rate (73.8 ± 0.9%) was observed at 65 °C and 150 bar. Lipase was highly stable at 55 °C–200 bar. In these conditions and due to water accumulation along the PBR, the use of a five-fold longer PBR only led to 73.0 ± 0.1% conversion. The same % conversion could be achieved using 2.5 less enzyme in a two-parallel 1 mL PBR configuration. The positive impact of an increased residence time (Rt) in the PBR was investigated. Conversions of 83% and 98% were achieved with 36 and 27 s Rt at 55 °C–200 bar and 65 °C–150 bar, respectively. These results show that an efficient, green, enzymatic and continuous production of high-purity and solvent-free geranyl acetate is possible with supercritical CO_2.

1. Introduction

Monoterpene esters of short-chain fatty acids such as geraniol, linalool, citronellol or menthol contribute to the good smell of many essential oils. For this reason, they are widely used for the reconstitution of essential oils, as well as in fragrances and flavor industry [1,2]. These esters are hence largely used in cosmetics, pharmaceutics, perfumes and also food industry as flavoring agent and for their biological activities like antioxidant, antibacterial or anti-inflammatory activities [3–6]. According to a recent BCC Research report from 2016, the global market for flavors and fragrances including these terpene esters was evaluated at $26.0 billion in 2015. This market is expected to increase from $27.1 billion in 2016 to $37.0 billion in 2021 at a compound annual growth rate (CAGR) of 6.4% for 2016–2021 [6]. Geranyl acetate, is one of the most produced terpene esters with linalyl acetate. This ester can be found in essential oils from *Callitris* or *Eucalyptus* species and a smaller amount also occurs in geranium, citronella and lavender oils [1]. But extraction and purification steps are cost and time consuming and sometimes require organic solvents. Instead, chemical esterification of geraniol can be performed by using acetic acid, acetyl chloride (Schotten-Bauman fast reaction) or acetic anhydride (slower reaction). But these three approaches show all or part of the significant following drawbacks: (i) extreme basic pH (Schotten Bauman requires large amount of NaOH) or acidic pH (the use of acetic acid requires large amount of sulfuric acid as catalyst), (ii) massive chlorine salt production (NaCl), (iii) generation of HCl fumes, (iv) use of organic flammable and toxic co-solvents, (v) necessity to use expensive acetyl chloride, (vi) use of moderate to strong heating, (vii) residual unreacted acyl chloride, (viii) residual acetic acids due to the competitive hydrolysis [7]. In any cases, chemical reactions lead to large volumes of industrial effluent representing environmental issue and a significant cost for industrials. For these reasons, the enzymatic flavor synthesis appears to be a promising alternative. Researchers and industries have switched to enzymatic flavor synthesis instead of chemical one. Actually, the enzymatic synthesized flavor and fragrance esters can be labeled as "natural" [8]. Indeed, these reactions are carried out in mild operating conditions with high specificity and reduced side reaction [9].

Since the discovery by Zaks and Klibanov [10] that enzymes such as hydrolases and more specifically lipases could perform various synthesis reactions in anhydrous media such as organic solvent, many efforts have been put in studying these reactions not only in organic solvent or

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https://doi.org/10.1016/j.procbio.2018.05.008
Received 6 February 2018; Received in revised form 11 May 2018; Accepted 13 May 2018
1359-5113/ © 2018 Published by Elsevier Ltd.

solvent free media but also in more eco-compatible solvent such as supercritical fluids. Supercritical carbon dioxide ($scCO_2$) exhibits many advantages such as (i) its critical point (73.8 bar and 31 °C) that is quite easy to reach and compatible with mild process conditions and enzymes thermal stability, (ii) its properties (viscosity, density, diffusivity, dielectric constant) that can easily be tuned by playing on pressure and/or temperature, (iii) its low-cost, no flammability and non-toxicity that are compatible with industrial scale applications with reduced associated hazards. Last but not least, CO_2 is easily recyclable under pressurized forms making possible to recycle it after appropriate re-pressurization and heating. Finally, the key advantage of using $scCO_2$ as a solvent is the possibility to develop bioprocesses that can be claimed as "green labelled" because totally free of any trace of organic solvents such as hexane or toluene.

Despite its interest in terms of green and sustainable chemistry, only few studies on geranyl acetate synthesis based on enzymatic esterification or transesterification in supercritical fluids have been published [11–13]. Chulalaksananukul et al. [11] were the first to describe the biosynthesis of geranyl acetate by transesterification between ethyl acetate (200 mM) and geraniol (60 mM) in a batch stirred tank reactor (BSTR) using a $scCO_2$ at 40 °C and 100 bar and 100 mg of macroporous anionic resins beads supporting lipase from *Mucor miehei* as a catalyst. By appropriately controlling the water content of the reaction mixture (10%, w:w) about 30% conversion after 24 h was reached.

Couto et al. [13] investigated the synthesis of geranyl acetate catalyzed by the Novozym ® 435, the lipase B from *Candida antarctica* non-covalently immobilized in macroporous acrylic beads, both in batch stirred reactor (BSTR) and packed bed reactor (PBR). In PBR and using equimolar concentrations of geraniol and acetic acid (100 mM) in a 0.25 mL min^{-1} CO_2 flow rate at 35 °C and 100 bar with 900 mg of Novozym®435 beads pre-equilibrated at a water activity of 0.23, these authors reached a good and stable steady-state conversion of about 87% over 14 days. One key advantage of the PBR versus the BSTR, when using esterification instead of transesterification, is that the water released by the reaction is continuously removed from the reactor by the CO_2 flow in which water is very slightly soluble, thus limiting the risk of hydrolysis. PBR are continuous reactors widely used in the chemical and biochemical industry due to their relative simplicity and their convenience. As an illustration, Dos Santos et al. [14] recently demonstrated that PBR was the best option, as compared to batch reactors, for the enzymatic synthesis of isoamyl-acetate in supercritical CO_2. To date, besides the information achieved by [13] at one single pressure-temperature couple, there is a true lack of data on the enzymatic production of geranyl acetate by esterification in a continuous process (packed bed reactor) with supercritical CO_2 as a green solvent.

In the framework of a process intensification, the aim of this study was therefore mainly to investigate the potential of the immobilized *Candida antarctica* lipase B (Lipozyme ® 435) in such operating conditions but using different conditions of pressure and temperature in order to achieve a better knowledge on the best pressure-temperature conditions for the geranyl acetate production. Other aspects such as the time necessary to achieve steady state conditions with various PBR lengths and the influence of the PBR length on the conversion rate were investigated and will be discussed together with results on the influence of the CO_2 flow-rate (i.e. residence time of CO_2 and substrates in the PBR) on the conversion rate. The operational stability of the catalyst, which is as key factor in the perspective of an industrial production of a "green labelled" geranyl acetate of interest for cosmetic, pharmaceutic and food Industry, will be also described.

2. Material and methods

2.1. Chemicals

Lipozyme®435, which consists of *Candida antarctica* Lipase B (CALB) physically immobilized on macroporous poly(methyl metacrylate-*co*-divinynbenzene) beads [13], was kindly supplied by Novozymes. Pure industrial carbon dioxide (class 4.5, 99.995%) was collected in a bottle equipped with a dip tube from Messer Group GmbH (Krefeld, Germany). Geraniol (purity ≥ 97%), acetic acid (purity ≥ 99.8%), geranyl acetate (purity > 98%), methyl decanoate (purity ≥ 99.5%) were all from Sigma-Aldrich (Schnelldorf, Germany). Acetone (purity 99.8%) was from Carlo Erba (Val de Reuil, France).

2.2. Water activity and water content measurements

During storage, the water activity of Lipozyme®435 was adjusted to 0.23 at 10 °C thanks to the microclimate method using a saturated salt solution of potassium acetate [15]. The water activity was then controlled with a Novasina Lab-Master aw-meter (Basel, Switzerland) at 10 °C and was observed as remaining 0.23 when measured at 25 °C. The water content of the Lipozyme®435 adjusted at $a_w = 0.23$ was found to be 0.41% (w/w) based on a triplicate measurement with a Mettler Toledo Karl-Fisher C20 coulometric titrimeter equipped with a DO-308 oven using nitrogen gas.

2.3. Analysis

Products analysis was performed by gas chromatography using an Agilent 7890 A gas chromatograph equipped with a FID detector for quantification and coupled with an Agilent 7000 A MS triple quad detector (Agilent technologies, Santa Clara, USA) for ensuring qualitative analysis. 1 µL volume samples were injected with a split ratio of 100:1 using an Agilent 7683B auto-sampler (Agilent technologies, Santa Clara, USA) with an injection temperature of 240 °C. We used an Agilent HP-INNOWax 30 m × 0.32 mm internal diameter × 0.25 µm column. Oven temperature was 60 °C during 2 min. Temperature was then increased up to 200 °C at 10 °C/min and from 200 °C to 220 °C at 5 °C/min. The temperature of the FID detector was set at 250 °C. Pure helium was used as a mobile phase at 1.5 mL/min flow. Methyl decanoate was used as an internal standard. The calibration curves of the acetic acid, geraniol and geranyl acetate presented R^2 values of 0.99. The limits of quantification (detection) were found to be 20.7 (6.2) mg/L for acetic acid, 48.6 (14.6) mg/L for geraniol, and 54.8 (16.4) mg/L for geranyl acetate.

2.4. Geranyl acetate synthesis in supercritical carbon dioxide

Geranyl acetate enzymatic synthesis was conducted in triplicate, for each tested process condition, through a packed bed reactor (PBR) by using a continuous automated high-pressure supercritical fluid equipment ($SFC-CO_2$) from JASCO (Tokyo, Japan). This equipment consisted in a CO_2 pump (Pu-2080 − CO2 Plus unit, JASCO, Japan) and a semi-micro HPLC pump for the two reaction substrates mixed together (Pu-2085 Plus unit, JASCO, Japan). The liquid CO_2 pump was equipped with an internal Peltier cooling system for cooling at −4 °C. In order to be sure that all CO_2 coming from the CO_2 bottle was in the liquid form, before pumping by the CO_2 pump, an additional cooling of CO_2 at −4 °C was performed with a Lauda RM6 cryostat (Lauda-Königshofen, Germany). The pressure was controlled with an automated back pressure regulator (BP-2085 Plus unit, JASCO, Japan) at a precision of ± 0.2 MPa of the setting value. This back pressure regulator (BPR) is based on the use of a CO_2 flow-switching valve (FSV) mechanism that enables stable system pressure control together with efficient collection of the SFE extract in a glass tube. Because CO_2 decompression is highly endothermic (Joule-Thomson effect), especially when using "high" CO_2 mass flow, and can generate CO_2 dry ice at the exit of the BPR, the latter was equipped with an internal Peltier heating system maintained at 60 °C in order to avoid CO_2 solidification during decompression. A 30 mm lengths × 4.6 mm diameter Sulzer SMX™ stainless steel static mixer (Winterhur, Switzerland) was used for mixing $scCO_2$ and substrates before their entrance in the packed bed reactor (PBR). This PBR

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