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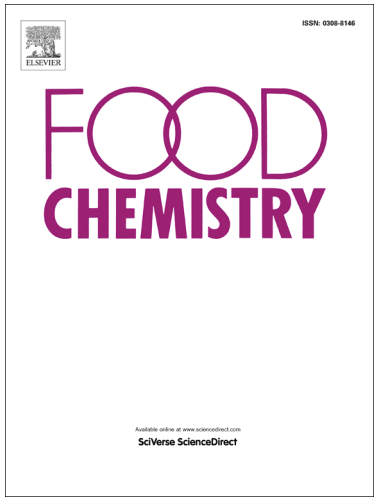

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Synthesis of medium-chain length capsinoids from coconut oil catalyzed by *Candida rugosa* lipases

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ABSTRACT

A commercial preparation of *Candida rugosa* lipases (CRL) was tested for the production of capsinoids by esterification of vanillyl alcohol (VA) with free fatty acids (FA) and coconut oil (CO) as acyl donors. Screening of FA chain length indicated that C8-C12 FA (the most common FA found in CO triglycerides) are the best acyl-donors, yielding 80-85 % of their specific capsinoids. Hence, when CO, which is rich in these FA, was used as the substrate, a mixture of capsinoids (vanillyl caprylate, vanillyl decanoate and vanillyl laurate) was obtained. The findings presented here suggest that our experimental method can be applied for the enrichment of CO with capsinoids, thus giving it additional health promoting properties.

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Key Words:

Capsinoids, Vanillyl alcohol, Coconut oil, Candida rugosa lipases, Esterification

1. Introduction

The effects on health of capsaicin, a pungent active species from peppers, have been known for a long time and are upheld by numerous scientific studies(Chinn, Sharma-Shivappa, & Cotter, 2011). However, capsaicin is highly irritating on skin or eye contact. Additionally, high-dose or long term exposure to capsaicin has a detrimental effect on the gastric mucosa and in extreme cases can be lethal (Luo, Li, et al., 2011). The search for a non-pungent and less irritant species with similar health benefits gave rise to capsinoids. In structural terms, capsinoids are lipophilic esters of vanillyl alcohol (VA) and fatty acids (FA) (Roby et al., 2015). Three functional groups contribute to their physiological activity: aromatic ring, ester bond and FA moiety. Capsinoids are equally pharmacologically potent to capsaicin, exerting a wide scope of health benefits, including weight management, hypocholesterolemic effect, chemopreventive and anticancer effect, antioxidant properties and gastroprotective properties (Dimitrijevic, Velickovic, Milosavic, & Bezbradica, 2012; Luo, Li, et al., 2011; Luo, Peng, & Li, 2011; Tremblay, Arguin, & Panahi, 2016; Velickovic et al., 2012; Whiting, Derbyshire, & Tiwari, 2012; Zhang, Fang, Zheng, Chen, & Liu, 2013). All of these effects are mediated through activation of transient receptor potential vanilloid subfamily member 1 - TRPV1, widely distributed throughout tissues (Yoneshiro, Aita, Kawai, Iwanaga, & Saito, 2012).

Even though capsinoids are naturally present in pepper fruit, the traditional way of production by isolation from the natural source has been abandoned, since it is laborious and ineffective in the terms of product yield. Chemical synthesis is another traditional way of obtaining capsinoids (Anderson, Afewerki, Berglund, & Cordova, 2014; Macho et al., 2003). Although satisfactory product yields can be achieved, this approach is controversial, from an environmental perspective. A large body of evidence speaks in favour of enzyme-mediated production of capsinoids. So far, Novozyme 435 has been most extensively used (Ishihara, Kwon, Masuoka, Nakajima, & Hamada, 2010; Kobata et al., 1999). However, the high

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cost of this catalyst imposes the necessity for alternative approaches (Chang et al., 2014; Zhao, Herbst, Niemeyer, & He, 2015).

Capsinoids, such as vanillyl nonanoate, can be synthesized directly from precursor alcohol and FA derivatives, however this strategy is more suitable for laboratory scale production. When it comes to scale-up, more economical substrates must be employed. In that sense, coconut oil (CO) could be an excellent alternative, since it contains medium-chain fatty acid residues. It is characterized by high saturated fat content, thus it is resistant to rancidification and can last up to six months at room temperature (RT), without spoiling (Kempton, 2006). Apart from its importance in the food and cosmetics industry, CO is also a precursor of different products (Be Lan & Hoa, 2015; J. Sun, Chin, Yu, Curran, & Liu, 2012). Its popularity lies in its wide availability and affordable price. It consists of 92 % saturated fats, mainly in the form of medium-chain length triglycerides (MCT).

The guiding principle of this research was to obtain capsinoids in satisfactory yield, by applying an environmentally and industrially favourable reaction system composed of an economical catalyst – CRL and an affordable substrate – CO.

2. Experimental

2.1. Materials

A commercial preparation of *Candida rugosa* lipases (type VII) and other chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich, USA). Coconut oil was purchased at the local supermarket (Lučar, SRB). Solvents used as mobile phases in detection and quantification of reaction products were of HPLC grade. All other chemicals were of analytical grade.

2.2. Lipase-mediated synthesis of capsinoids

Reactions were carried out in screw-capped glass vials. The reaction mixture contained: 25 mM substrates VA and saturated C8-C16 FA and 5 mg of commercial CRL preparation in 1 ml of *n*-hexane. Mixtures were incubated 48 h at 45°C on a PST-60HL thermoshaker (Biosan, LT) with constant agitation at 600 shakes/min. 100 µl aliquots of reaction mixtures were withdrawn and centrifuged in a Minispin

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minifuge (Eppendorf, D) for 3 min at 6700 x g. Supernatants (SN) were evaporated in a Concentrator 5301 (Eppendorf, D) and the remaining solid was dissolved in 100 µl of HPLC grade methanol. All reactions were carried out in duplicate.

Prepared samples were analyzed on a Dionex Ultimate 3000 HPLC system (Thermoscientific, USA) by reverse-phase chromatography (RPC) on a Symmetry C18 column (4.6 x 150 mm; particle size 5 µm) (Waters, USA). 10 µl of sample was loaded onto the column, under 1 ml/min flow at 25℃. Mixture components were separated in isocratic mode with 85 % methanol with 0.1 % (v/v) formic acid for C8 and C10 samples and the mobile phase was then changed to 100 % methanol with 0.1% (v/v) formic acid for C12-C18 samples. Components were identified by the absorbance change at 235 nm (A_{235}) during a 5 min run. Data were analyzed using Chromeleon 7.0 software. Vanillyl esters were quantified according to Equation1:

$$Yield = \frac{A}{(A+B)} * 100,$$

where A is the peak area of an individual vanillyl ester (mAU*min) and B is the peak area of vanillyl alcohol (mAU*min).

2.3. Enzymatic transformation of coconut oil

Transformation of CO was based on a procedure by Mbatia *et al.* , with slight modifications: 4 mg of VA and 3 mg of CO were dissolved in 1 ml of *n*-hexane (VA : FA = 1.5 : 1 (mol/mol)) (Mbatia, Kaki, Mattiasson, Mulaa, & Adlercreutz, 2011). 5 mg of commercial CRL preparation was added to initiate reaction and the mixture was treated as described above. A 100µL aliquot of reaction mixture was removed and centrifuged. Enzyme-free supernatant was analyzed by HPLC.

RPC quantitative analysis was performed in the same chromatographic system as in section 2.2.2, on a Hypersil gold C 18 column (150 mm x 4.6 mm; 5 µm) (Thermoscientific, USA). The sample was diluted 10 x with methanol, and 30µl was loaded on the column, under the flow of 1ml/min, pressure of 46 bar at 30℃. The mobile phase consisted of 85 % methanol (A) and 100 % *i*-propanol with 0.1 % (v/v) formic acid (B) in isocratic elution mode (15 min with solvent A and 30 min with solvent B). Product formation

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was monitored by the absorbance change at 210 nm (A_{210}) using Chromeleon 7.0 software. Individual peaks were identified by comparison with standards vanillyl octanoate (VO), vanillyl decanoate (VD) and vanillyl laurate (VL). These esters were prepared according to the the procedure described in 2.2.1. Reaction yield was calculated according to Equation 1.

2.4. Identification of vanillyl laurate by nuclear magnetic resonance (NMR)

VL was prepared at 10-fold scale, following the procedure from subsection 2.2.1 and purified by preparative RPC on a Hypersil gold C18 column (250 x 10 mm; particle size 5 µl; pore size 175 Å) (Thermoscientific, USA). 1 ml of reaction mixture was loaded onto the column in each run. The general chromatographic separation procedure was identical to that described in subsection 2.2.2. VL was obtained as a clear liquid.

NMR spectra (^{1}H NMR at 200 MHz, ^{13}C NMR at 50MHz) of purified ester of VA and lauric acid were recorded on Varian Gemini 200, and compared with the spectra of chemically synthesized VL standard (Appendino, Minassi, Daddario, Bianchi, & Tron, 2002).

3. Results and discussion

3.1. Fatty acid specificity of CRL

Investigation into the substrate specificity of CRL is depicted by Figure 1. Figure 1 unambiguously demonstrates that all vanillyl esters were synthesized in significant yield, (70-85%), with the highest yields achieved for C8-C12. This observation is supported by literature data, according to which CRL operates best with medium-chain length FA (MCFA) (Benjamin & Pandey, 1998). Kobata and associates have employed a similar strategy for the synthesis of vanillyl nonanoate. Their approach resulted in a product yield of only 1.4 – 11.9%, with CRL as catalyst (Kobata, Kawaguchi, & Watanabe, 2002). Such a low yield could be attributed to inappropriate solvent choice: in Kobata's paper transformations were carried out in polar organic solvents dioxan and acetone. In contrast, our previous study revealed that *n*-hexane was the most suitable solvent when working with CRL and there are numerous studies confirming the higher esterification activity of CRL in non-polar solvents (Bezbradica, Mijin, Siler-Marinkovic, & Knezevic, 2006, 2007; Trbojević Ivić et al., 2016).

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Since VA has two potential acylation sites, structural analysis was necessary to determine regiospecificity of the enzyme. In order to reveal this, a chemoselective procedure, based on the Mitsunobu reaction, was applied (Appendino et al., 2002). Figure 2 shows the comparison of ^{1}H NMR spectra of chemically synthesized vanillyl laurate (VL) standard and purified C12-ester, obtained by the enzymatic transformation we have described in this paper. As can be seen from Figure 2, all signals in these two spectra are identical. Furthermore, analysis of purified C12-ester revealed that only the benzyl OH group was esterified since all chemical shifts were identical to the shifts of the vanillyl laurate standard synthesized using the classical chemical route. Thus, structures of CRL synthesized VA esters are consistent with a capsinoid structural pattern.

1H NMR (200 MHz, CDCl3): δ 6.90-6.85 (m, 3H), 5.03 (s, 2H), 3.89 (s, 3H), 2.33 (t, J = 7.5 Hz, 2H), 1.73 – 1.52 (m, 2H), 1.36-.20 (m, 16H) 0.88 (t, J = 6.4 Hz, 3H). 13C NMR (50 MHz, CDCl3) δ 173.84, 146.46, 145.74, 128.00, 121.97, 114.32, 111.22, 66.24, 55.85, 34.34, 31.84, 29.53, 29.40, 29.26, 29.20, 29.08, 24.91, 22.62, 14.03 (1 signal is missing due to overlap).

3.2. Production of capsinoids from coconut oil

Our findings, depicted in Figure 1 fit in well with the general composition of coconut oil; therefore we tested the performance of CRL with regards to esterification of this valuable stock. The results are summarized in Table 1 and are expressed as an average value of the two measurements.

According to Table 1, the highest product yield was achieved for VL, which is in complete agreement with the declared composition of the applied oil, hence this result confirms similar specificity of CRL toward MCFA (http://www.chempro.in/fattyacid.htm). Acylation of VA with CO is a beneficial one-pot reaction, which yields a mixture of capsinoids instead of individual compounds. Furthermore, our experimental strategy could also potentially be used for the enrichment of vegetable oils and similar foodstuffs with these non-pungent, physiologically active species.

4. Conclusion

The quest for less-irritant and equally potent capsaicin analogues resulted in the discovery of capsinoids – lipophilic esters of VA and FA. The procedure described here unites substrate specificity of

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environmentally suitable biocatalyst CRL with a prominent industrial substrate – coconut oil, obtaining a capsinoid mixture with satisfactory product yield. Our experimental design meets industrial striving for "greener" and efficient solutions for the generation of useful products. Additionally, as it was shown in the example of CO, the method we developed has good potential for enrichment of foodstuff with capsinoids, thus giving it more than just a nutritive value.

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Conflict of Interest

None declared.

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Figure and table captions

Figure 1. Study of fatty acid specificity of *Candida rugosa* lipase in esterification of vanillyl alcohol. Results are presented as average values of two measurements (around 4% deviation between the two measurements as indicated by error bars).

Figure 2. NMR spectral analysis of vanillyl-laurate. A) ^{1}H NMR spectrum of chemically synthesized vanillyl laurate standard. B) ^{1}H NMR spectrum of vanillyl laurate, obtained by enzymatic transformation of vanillyl alcohol.

Table 1. Performance of CRL in synthesis of different capsinoids from coconut oil.

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Compound	VA conversion yield (%) (48 h)	Content of major FA in coconut oil (%)
Vanillyl caprylate	26	Caprilyc acid: 5-9 %
Vanillyl decanoate	13	Decanoic acid: 6-10 %
Vanillyl laurate	41	Lauric acid: 44-52 %

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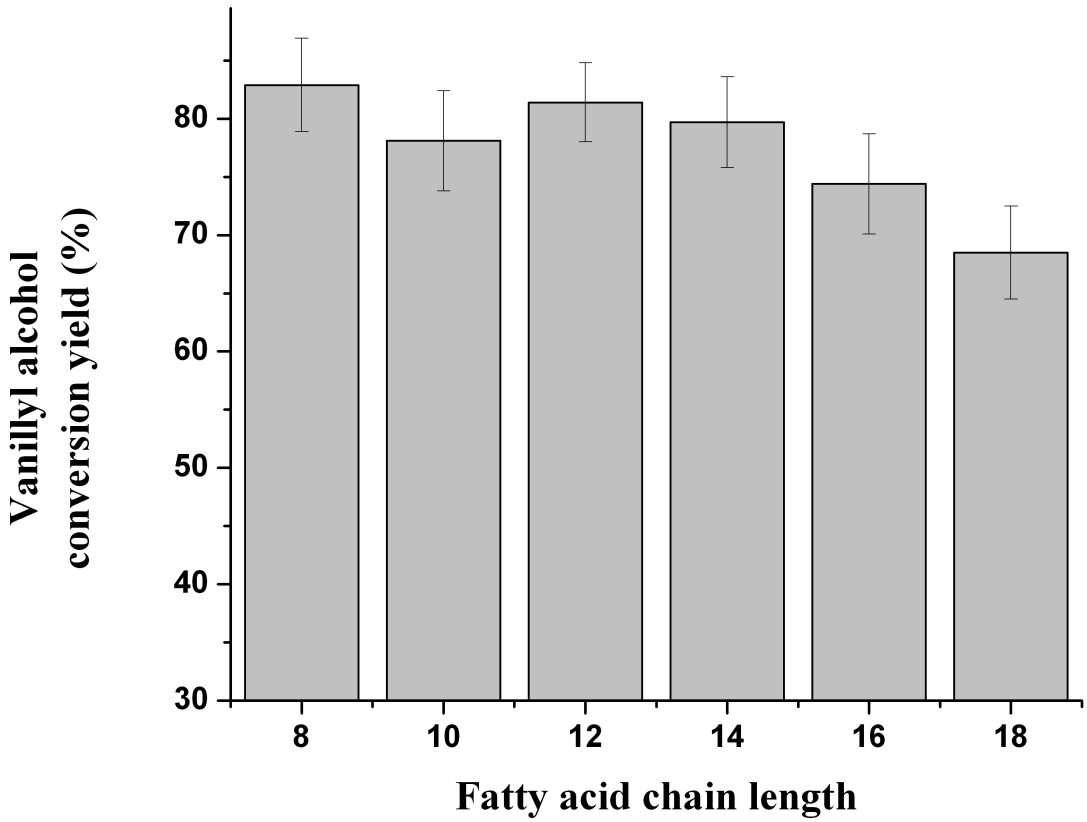
Vanillyl alcohol conversion yield (%)
80
70
60
50
40
30
8
10
12
14
16
18
Fatty acid chain length

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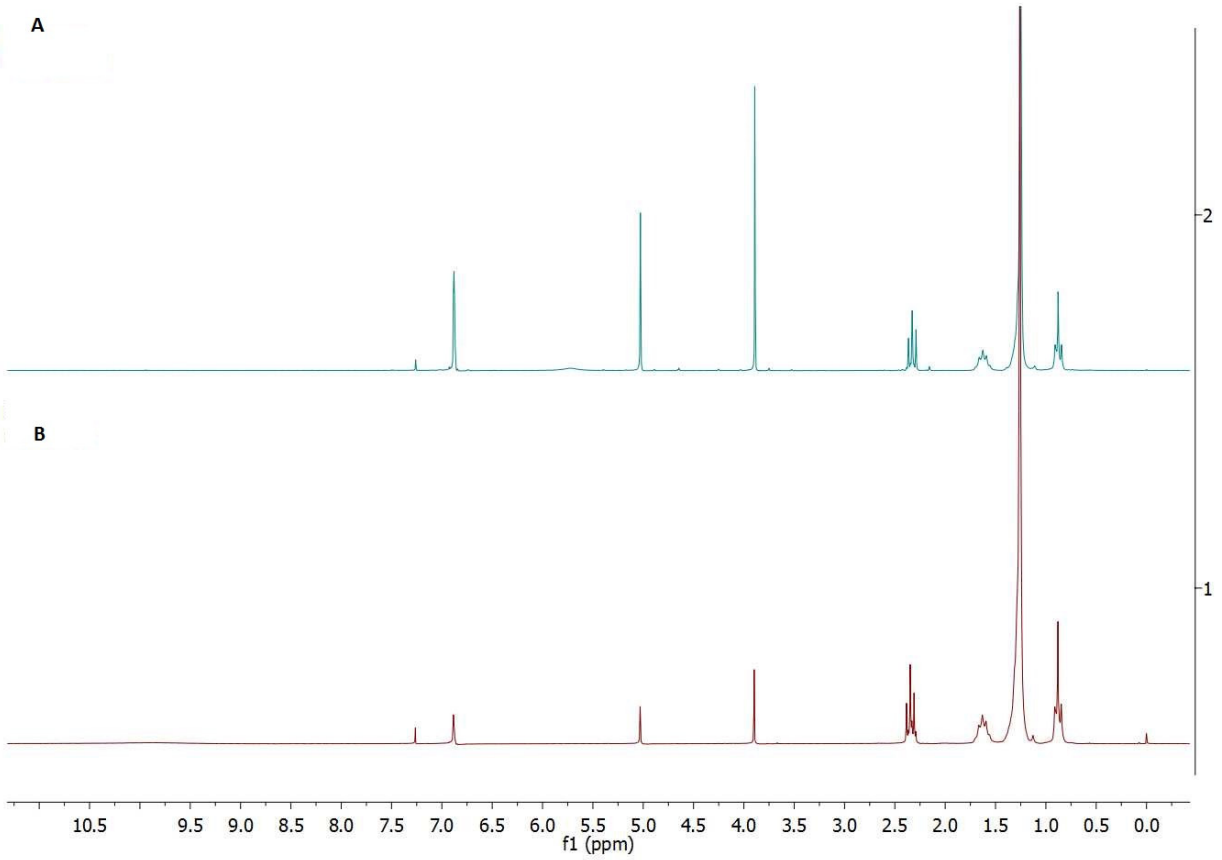

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