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**ORIGINAL RESEARCH PAPER**

# CONTINUOUS SYNTHESIS OF CINNAMATE ESTER USING MEMBRANE REACTOR IN A SOLVENT-FREE SYSTEM

AZIS BOING SITANGGANG[1]*, RAHMA AULIARISYA HIDAYATI[1], LILIS NURAIDA[1,2], NURI ANDARWULAN[1,2], SLAMET BUDIJANTO[1]

[1]*Department of Food Science and Technology, IPB University, Kampus IPB Darmaga Bogor, 16680, Indonesia*
[2]*South-East Asia Food and Agricultural Science and Technology (SEAFAST) Center, IPB University, Kampus IPB Darmaga Bogor, 16680, Indonesia*
* Corresponding author: boing.lipan@apps.ipb.ac.id

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**Abstract**

Cinnamic acid is a phenolic compound with the potential to act as a natural antioxidant. Cinnamic acid esterification can be performed by adding a long-chain alcohol to one of its hydroxyl groups to improve its antioxidant activity. In this study, cinnamic esters were synthesized in an enzymatic membrane reactor (EMR) and were carried out continuously. This study aimed to determine the optimum conditions for the synthesis of cinnamic esters using various types of alcohols (*e.g.*, butanol, hexanol, and octanol) as alkyl group donors and the impact of the esterification on the antioxidant activity. The continuous synthesis of cinnamic esters with TL IM lipozyme as the biocatalyst had the highest conversion (20.91%) with 1-butanol as the alkyl donor, in a synthesis strategy of a 12-h batch followed by continuous operation with a residence time of 9 h. The resulting ester increased the antioxidant activities by 2.18 and 3.85-fold, respectively based on the DPPH and FRAP assays.

**Keywords:** antioxidant activity, cinnamic acid, enzymatic membrane reactor (EMR), esterification, lipozym TL IM, membrane reactor

## Introduction

Antioxidants inhibit oxidation by binding free radicals and reactive molecules. The presence of free radicals or reactive molecules can disrupt cell function, damage cell structure, and cause mutations (Amin *et al*., 2016). In addition to the human body, oxidation reactions can occur in food. In food, antioxidants are used to inhibit fat/oil oxidation reactions caused by the presence of oxygen, light, or heat. Oxidation reactions occuring in food can cause rancidity or enzymatic browning. Therefore,

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antioxidants play a role in maintaining product quality before consumption (Lourenço *et al*., 2019).

The addition of antioxidants to food products is usually in form of synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tert-butylhydroquinone (TBHQ). Some studies have shown that synthetic antioxidants can cause allergies, carcinogenesis (Stamatis et al., 1999), and digestive problems (Lourenço *et al*., 2019). Therefore, there is an increasing interest in the use of natural antioxidants. Cinnamic acid and its derivatives are phenolic compounds that can be used as antioxidants. Several studies have shown that cinnamic acid has the potential as an anti-inflammatory, antioxidant, anticancer, and antimicrobial agent (Pontiki and Hadjipavlou-Litina, 2018).

The esterification reaction of phenolic compounds has been reported to increase the antioxidant capacity of the esters produced (Guyot *et al*., 1997; Lee *et al*., 2006; Widjaja *et al*., 2008; Pontiki and Hadjipavlou-Litina, 2018; Garrido *et al*., 2012). The cinnamic acid can be esterified using acid as a catalyst or by enzymatic reactions. Esterification via enzymatic reactions requires mild reaction conditions. In addition to the use of lower temperature compared to the use of acid catalysts, enzymatic esterification is also an environmentally friendly method (Bezbradica *et al*., 2016; Baek *et al*., 2020). One of the routes of cinnamic acid esterification is the use of an alcohol as an alkyl donor. Cinnamic acid esterification can be performed using batch or continuous processes. Continuous synthesis can avoid back-reactions caused by the presence of water molecules produced during the esterification reaction. The back-reaction (also referred to as secondary hydrolysis) typically occurs in a batch process (Sitanggang *et al*., 2014a; Sitanggang *et al*., 2016). Therefore, continuous synthesis is expected to increase the reaction yield.

One way to synthesize cinnamate esters continuously is by using an enzymatic membrane reactor (EMR). The use of EMR for cinnamate ester synthesis yields highly productive products (Eisele *et al*., 2013). Continuous synthesis using EMR with immobilized enzymes dispersed in alcohol resulted in a solvent-free synthesis system. In EMR, the immobilized enzymes can be kept on the reactor side, while the substrate can be continuously supplied to the reactor, and the reaction products can be withdrawn by membrane filtration. Hence, the purpose of this study was to determine the optimum reaction conditions for cinnamate ester synthesis using continuous EMR, and to investigate the enhancement of the antioxidant capacity of the esterification product as influenced by the length of the alcohol chain.

## Materials and methods

### *Materials*

Polyethersulfone (PES) membrane with a molecular weight cut-off (MWCO) of 10 kDa was purchased from Mann+Hummel Water and Fluid Solutions GmbH (Wiesbaden, Germany). Cinnamic acid, alcohols (1-octanol, 1-hexanol, and 1-butanol), lipozyme TL IM (lipase from *Thermomyces lanuginosus* immobilized on silica gel, 0.25 U/mg, Novozyme Denmark), 2,2-5 diphenyl-1-picrylhydrazyl

(DPPH), sodium phosphate buffer 0.2 M (pH 6.6), potassium ferricyanide ($K_3Fe(CN_6)$ 1%), trichloroacetic acid (TCA) 10%, methanol pro-liquid chromatography, ascorbic acid, and $FeCl_3$ 0.1% were obtained from Merck KGaA (Darmstadt, Germany). All other reagents were of analytical grade.

***Design of EMR***

The EMR design used in this study was the same as that reported elsewhere (Sitanggang *et al*., 2021). The EMR was operated either at constant transmembrane pressure (TMP) or at constant flux operation. PES with an MWCO of 10 kDa was placed at the bottom of the reactor to facilitate the dead-end mode filtration. The permeate was collected in an analytical balance, and the information was retrieved continuously through a personal computer. The constant flux operation was facilitated by a proportional-integral-derivative (PID) controller, where the controller gain, integral,- and derivative time were 0.020 (-), 0.536 (min), and 0.136 (min), respectively. The automation of the EMR allows the monitoring and saving of the actual flux and TMP in real-time mode. To evaluate the performance of the reactor operating under constant flux, a series of constant flux filtrations were conducted. Before filtration, the membrane was submerged in a designated alcohol (1-octanol, 1-hexanol, or 1-butanol) for 15 min. Filtration of the corresponding alcohol was conducted at three flux values, that is 10, 15, and 20 L/($m^2$.h). The actual flux $J_{PV}$ and transmembrane pressure (TMP) $\Delta P$ were observed and recorded, and the control error $e$ was analyzed according to Equation 1 as follows:

$$e = \left|1 - \left(\frac{J_{SV}}{J_{PV}}\right)\right| \times 100\% \qquad (1)$$

where $e$ = control error (%), $J_{SV}$ and $J_{PV}$ = set flux and actual (process variable) flux (L/($m^2$.h) = LMH), respectively.

***Membrane compacting phenomenon***

PES membranes were soaked for 15 min and 24 h at 25 °C. Filtration of the corresponding alcohol was performed at six TMP values (0.5- 2.5 bar) for 5 min. The relationship between TMP $\Delta P$ and the corresponding flux value $J_{PV}$ was established and compared for the two soaking times. Because filtration was performed for pure alcohol, the membrane resistance could be calculated using Equation 2:

$$J_{PV} = \frac{\Delta P}{\eta_{25^oC} \times R_m} \qquad (2)$$

where $\eta$ = dynamic viscosity of the alcohol (Pa. s) and $R_m$ = membrane resistance ($m^{-1}$).

***Continuous cinnamate ester synthesis using EMR***

The EMR was equipped with an ultrafiltration (UF) membrane made of PES with an MWCO of 10 kDa. When using the EMR, automatic control was required to run the system in a flux condition. To determine the values of the residence time $\tau$ and flux $J_{PV}$, the following equations were used (Equation 3 and 4):

$$\tau = \frac{V_r}{\dot{V}} \quad (3)$$

$$J_{SV} = \frac{\dot{V}}{A_m} \quad (4)$$

where : residence time (h): $V_r$ : reactor volume (L); $\dot{V}$ : volumetric flow rate (mL/h); and $A_m$ : membrane area ($m^2$). The reactor volume and the membrane area were invariant. Consequently, different values of volumetric flow rates can be obtained from different flux values. Therefore, different flux values could result in different residence times (Sitanggang *et al*., 2014b).

The synthesis of octyl cinnamate was carried out according to the reaction conditions in the studies performed by Stamatis *et al*. (1999) and Khor *et al*. (2010) with modifications of the temperature of 40 °C and stirring velocity of 350 rpm. To determine the optimum conditions for the esterification of cinnamic acid (0.005 M), the influence of reaction time and the type of alcohol (*e.g*, 1-octanol, 1-hexanol, and 1-butanol) used with the Lipozyme (2% w/v) TL IM was investigated. Four reaction strategies were performed, such as (a) batch reaction for seven hours (b) continuous reaction for seven hours, (c) batch reaction for three (hours followed by continuous reaction for seven hours, and (d) batch reaction for 12 h followed by continuous reaction for seven hours. For each continuous operation, a residence time $\tau$ of nine hours was used. Optimum conditions were determined by observing the highest yield of cinnamate esters produced after treatment. For each reaction strategy, samples were withdrawn every hour. In addition, for reactions where the continuous flow was performed, the total conversion was observed by the cumulative permeate collected in the balance after seven hours of reaction.

***Cinnamic acid analysis***

The cinnamate ester was analyzed using a UV-Vis HPLC detector (Shimadzu SPD-10A, Japan) at 280 nm according to Wang et al. (2015) with modifications. A C18 column (SIU columns packed by PT Semesta Inti Usaha, Indonesia) was used. Pro-analysis methanol was used as the mobile phase, with a flow rate of 1.0 mL/min (Shimadzu LC-10ATVP, Japan) and a retention time of six minutes. Before being injected into the HPLC system (Shimadzu SCL-10AVP, Japan), 0.02 mL of the sample was diluted to 10 mL using methanol. Subsequently, 20 µL of the sample was injected into the HPLC system. The cinnamic acid conversion was calculated by observing the peak area (Equation 5):

$$X(\%) = \left|1 - \frac{C}{C_0}\right| \times 100\% \quad (5)$$

where $C, C_0$ : concentrations of cinnamic acid at time $t$ and initial (M).

***Antioxidant activity with the DPPH method***

In a lidded test tube, a 0.3 mL of sample was placed and then added with 0.7 mL of pure water dan 3.0 mL 2,2-diphenyl-1-picrylhydrazil (DPPH) 120 µM solution. The mixture was vortexed for 10 s and incubated in the dark for 30 min. After incubation, the absorbance was measured using a UV-Vis spectrophotometer (Thermo Scientific Genesys 150, USA) at a 515 nm wavelength (Brand-Williams *et al.*, 1995). Absolute methanol was used as the blank. A standard curve (y = 0.8794 – 0.0111x; $R^2$ = 0.990) was constructed using a series of ascorbic acid concentrations (0-70 ppm). The absorbance of the sample was compared to that of the ascorbic acid standard, and the antioxidant activity of the sample was expressed as ascorbic acid equivalent antioxidant capacity/mL sample (mg AEAC/mL sample).

***Antioxidant activity using FRAP assay***

A volume of 1 mL sample was placed in a dark test tube and added to 1.25 mL of 0.2 M $NaH_2PO_4$ buffer at pH 6.6. After the mixture was homogenized by vortexing, the sample was then added with 1.25 mL of potassium ferricyanide and vortexed again. The samples were then incubated in a water bath at 50 °C for 20 min. The sample was added to 1.25 mL trichloroacetic acid (TCA) 10% and then vortexed again. The sample was then centrifuged at 3000 rpm, at4 °C for 10 min. After centrifugation, the reaction mixture was suplemented with 0.5 mL $FeCl_3$ (0.1%). The samples were incubated again for 10 min at room temperature. After incubation, the absorbance of the samples was measured at a wavelength of 700 nm (Oyaizu, 1986). The antioxidant capacity of the sample was calculated using a standard curve (y = 0.0111x + 0.0458; $R^2$ = 0.9927) constructed from a series of ascorbic acid concentrations (0-80 ppm). Both antioxidant analyses (DPPH and FRAP methods) were performed only on the cumulative permeate collected on balance. The antioxidant activity enhancement was expressed as the ratio of between the initial and the final antioxidant activity obtained after the reaction for each alkyl donor.

***Statistical analysis***

Statistical analysis was performed using SPSS version 22.0 (IBM, USA). One-way ANOVA and Duncan's tests were used to determine significant differences among treatments ($P < 0.05$).

## Results and discussion

***Evaluation of reactor performance***

The performance of an automated EMR equipped with a PID controller was evaluated to carry out constant-flux filtration. Filtration was performed using several types of alcohols at a constant level of flux changed in a series (10 →15→20 LMH). The flux and TMP profiles are shown in Figure 1. The TMP increased with

increasing length of carbon in the alcohol used, where the highest TMP was obtained from 1-octanol filtration. 1-octanol has the highest density compared to other alcohols, which contributes to the highest filtration pressure (Compostizo *et al*., 2005). In addition, the PES membranes have been reported to have a hydrophilic surface (Alenazi *et al*., 2017). Thus, the increase in the non-polar characteristics of the alcohol with increasing number of carbon atoms could cause the highest rejection of 1-octanol, which led to the highest TMP. It is also worth mentioning that the TMP also increased with increasing value of the set flux $J_{SV}$. A higher convective transport is required at a higher flux value, which consequently requires a higher differential pressure within the system. The control errors during the constant flux filtration of the three alcohols were below 2%. There was no significant difference in control errors between the tested alcohols ($P>0.05$). According to Sitanggang *et al*. (2014b), a sufficiently automated process during bulk filtration has a control error of less than 5%. This indicates that the reactor can proceed with a small difference between the set flux J_SV and the process variable flux J_PV (Sitanggang *et al*., 2016).

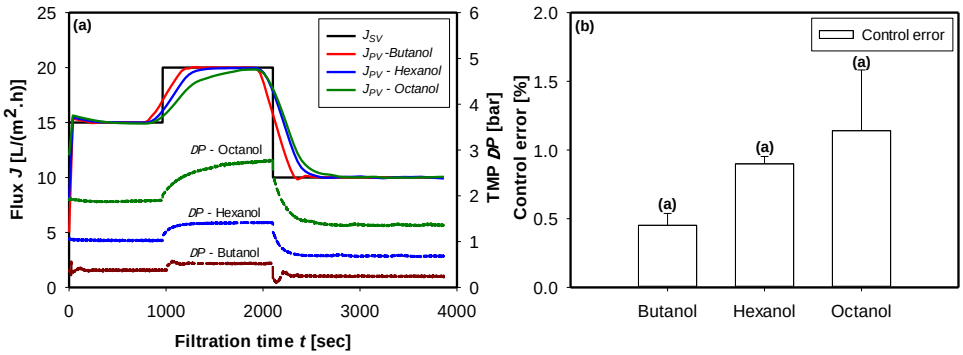


**Figure 1.** Flux and TMP profiles during alcohol filtrations (a) and the corresponding control errors (b); the bars with the same letter indicate insignificant difference ($P>0.005$).

### *Membrane compaction phenomenon*

Membrane compaction is a phenomenon in which a membrane experiences compression when it is subjected to pressure (Davenport *et al.*, 2020). It is observed when the flux declines under multiple filtrations at the same pressure value. As shown in Figure 2a, no membrane compaction was observed during filtration of the three alcohols. From the three alcohol filtrations, the flux of 1-butanol filtration after 24-h soaking was slightly lower than 15-min soaking. However, this difference was not statistically significant ($P>0.05$). Additionally, the membrane resistance for each alcohol after 15-min and 24-h soaking was not significantly different ($P>0.05$) (Figure 2b).

As indicated in the previous section, the flux decreased with an increase in the chain length of the alcohol. This was due to the hydrophilic properties of the PES membrane surface (Alenazi *et al*., 2017) that rejected the nonpolar substance, which

is owned by the longer alcohol chain. In this observation, the total resistance was considered the membrane resistance because the feed was pure alcohol (Burghardt *et al*., 2019; Di Bella and Di Trapani, 2019). The lowest membrane resistance was obtained from 1-butanol filtration, while the highest was obtained from 1-octanol filtration, which corresponded to the flux values obtained for each alcohol tested. The results of reactor performance and membrane compaction showed the suitability of EMR coupled with PES 10 kDa to facilitate the continuous esterification of cinnamic acid with different types of alcohols.

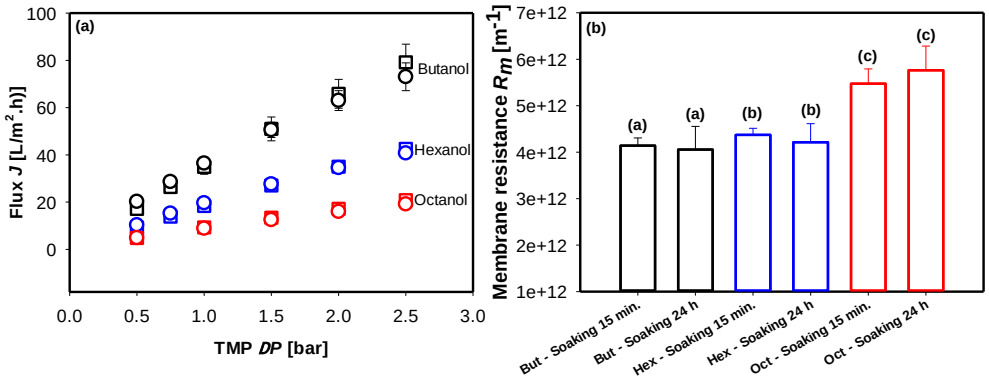


**Figure 2**. Flux profiles of the alcohol filtrations after 15-min and 24-h soaking (a), and (b) their corresponding membrane resistances (b); the same color bars with the same letter indicate insignificant difference (*P>0.005*).

### *Esterification of cinnamic acid: Conversion*

Cinnamic acid conversion during the esterification reactions with the four different operation strategies exhibited a similar trend. According to Stamatis *et al.* (1999), cinnamate ester synthesis occurred slowly. In this study, this trend was observed within 8 h of reaction where the batch synthesis had conversion lower than 20%. The influence of the synthesis strategies on the conversion of cinnamic acid to the same alcohol used was significantly different (*P<0.05*). In addition, the carbon length of the alcohols significantly contributed to the amount of cinnamate ester produced (*P<0.005*) (Table 1). Esterification with 1-butanol resulted in the highest conversion (lowest $\frac{C}{C_0}$) for all the synthesis strategies. The second-highest cinnamate conversion was obtained using 1-hexanol as the donor, followed by 1-octanol. Based on this, the suitable alkyl donor for the esterification of cinnamic acid is an alcohol with a short length of carbon (*e.g*., 1-butanol). This trend was also observed for the apparent conversions (Figure 3). The highest apparent conversion was achieved by the combination of a batch reaction held for 12 h followed by a continuous reaction at a residence time of 9 h. The cinnamate conversion obtained was 25.67%, corresponding to 20.91% cumulative conversion. The amount of cinnamate

converted is influenced by the reactivity of the alcohol. In the esterification reaction, the reactivity of the alcohol is influenced by the presence of a methyl group and the number of carbon atoms. The presence of the methyl group lowered the reactivity of the alcohol, and the alcohol was more reactive in the esterification reaction if the carbon chain length decreased (Liu *et al*., 2006).

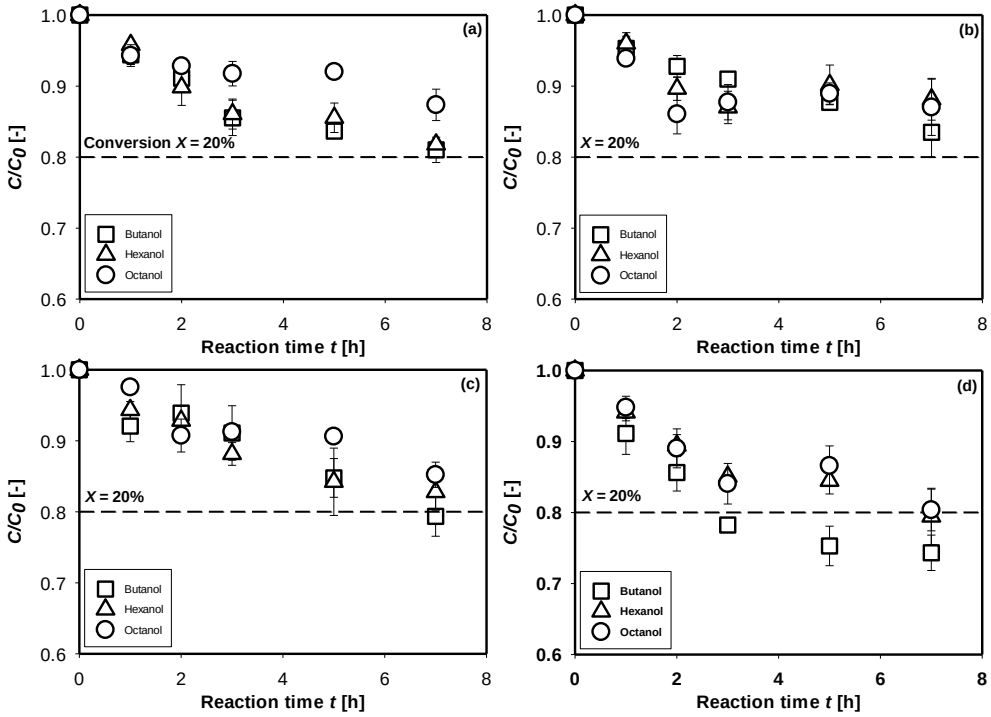


**Figure 3**. Apparent concentrations and conversion profiles of cinnamic acid that was esterified with different alcohols: (a) batch operation, (b) continuous operation with residence time of 9 h, (c) combination of 3 h – batch and continuous operation with a residence time of 9 h, and (d) combination of 12 h – batch and continuous operation with a residence time of 9 h.

**Table 1**. Influence of alcohol type on the conversion (%) of cinnamic acid collected on the balance as permeate.

| **Reaction conditions** | **Conversion (%)** | | |
|---|---|---|---|
| | **1-butanol** | **1-hexanol** | **1-octanol** |
| Batch | 18.98±1.78$^{b}$ | 18.21±0.45$^{b}$ | 12.64±2.21$^{a}$ |
| Continuous | 17.92±0.59$^{b}$ | 10.67±1.14$^{a}$ | 8.93±1.58$^{a}$ |
| 3-h Batch + continuous | 18.70±0.72$^{c}$ | 15.99±1.54$^{b}$ | 9.56±1.20$^{a}$ |
| 12-h Batch + continuous | 20.91±1.21$^{c}$ | 13.05±0.52$^{b}$ | 9.43±0.74$^{a}$ |

Numbers in the row followed by different superscripts are significantly different (P<0.05).

Evaluation of TMP during the esterification of cinnamic acid using different alcohols revealed that the increase in TMP corresponds to the number of carbon atoms in the alcohol structure (Compostizo *et al.*, 2005). Corresponding to the previous results, 1-octanol, owing to its nonpolar properties, yielded the highest TMP (Figure 4).

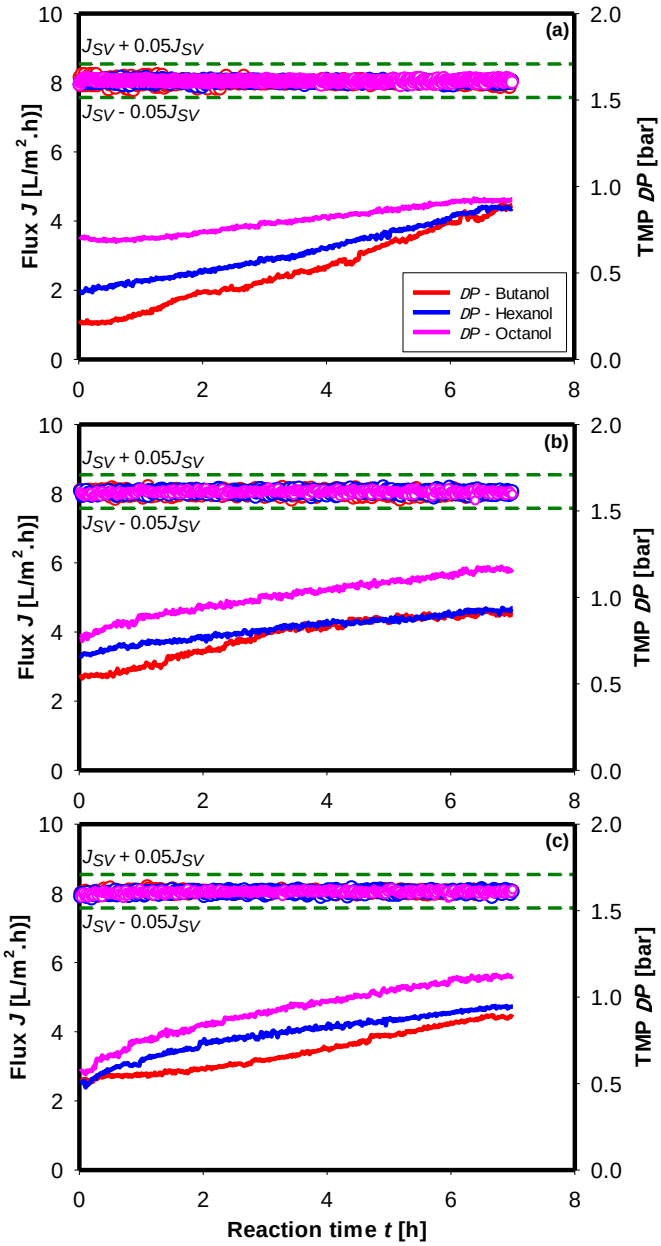

**Figure 4**. Flux and TMP profiles during the esterification of cinnamic acid with different alcohols: (a) continuous with a residence time of 9 h, (b) 3-h Batch + continuous with a residence time of 9 h, and (c) 12-h Batch + continuous with a residence time of 9 h.

In addition, the longer the cinnamate ester synthesis, the higher was the TMP generated to reach the set flux. This could be influenced by various factors such as the presence of fouling (Sitanggang *et al*., 2014a). Macromolecules such as enzymes could settle on the surface of the membrane pores so that the membrane pores were blocked and formed a layer on the surface of the membrane that would cause fouling (Sitanggang *et al*., 2016; Lim and Ghazali, 2020), thus to reach a predetermined value of set flux, a higher TMP was required.

***Cinnamate ester antioxidant capacity***

The enhancement of the antioxidant activity of cinnamic acid after esterification using different alcohols, as measured by both the DPPH and FRAP methods, is presented in Figure 5. Antioxidant capacities measured using the FRAP assay were higher than those measured using the DPPH method. Similar results have been reported elsewhere, where the antioxidant activity of caffeic acid, which is a derivative of cinnamic acid, was lower when assayed using the DPPH method compared to the FRAP method. This could be due to the lipophilic nature of the compound (Garrido *et al*., 2012).

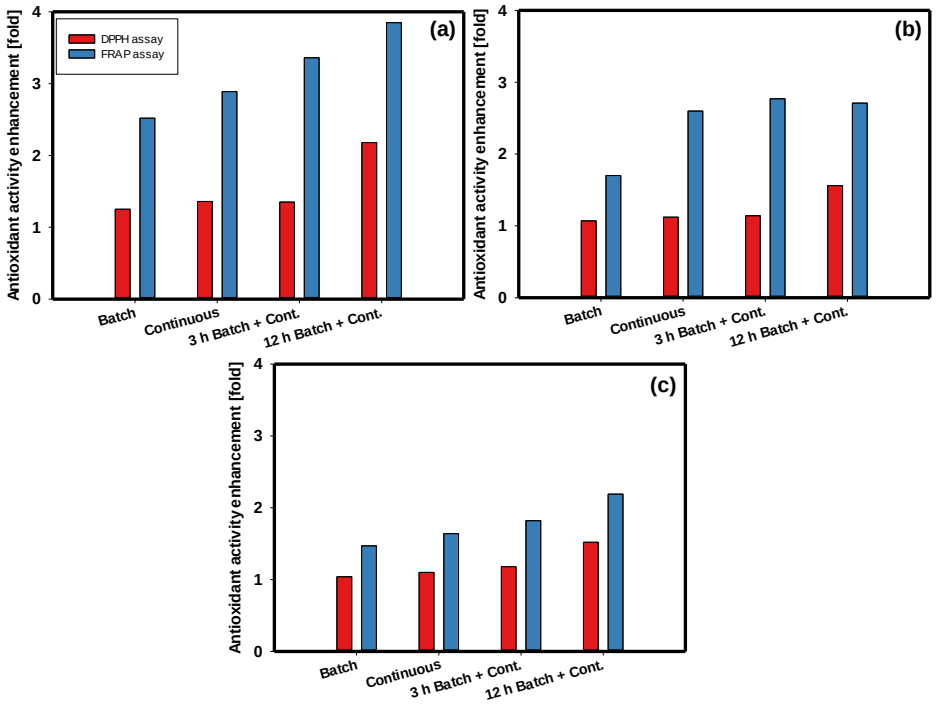


**Figure 5**. The enhancement of antioxidant activity monitored using the DPPH and FRAP methods with different alkyl donors: (a) 1-butanol, (b) 1-hexanol, and (c) 1-octanol.

Cinnamic acid esterified with different alcohols exhibited different antioxidant activities. In general, steric hindrance influences reactivity and slows the reaction of a certain molecule. The larger the steric bulk of an atom, the greater steric hindrance generated (Jowett *et al*., 2019). The good antioxidant capacity of a phenolic compound originates from a compound with low steric hindrance (Laguerre *et al*., 2009). Herein, the higher molecular weight of the alcohol used (*e.g*., 1-octanol) for esterification yielded higher steric hindrance for the obtained cinnamate ester (Hanh *et al*., 2009; Laguerre *et al*., 2009; Laguerre *et al*., 2011; Gallardo *et al*., 2016). Therefore, butyl cinnamate, as shown in Figure 5a, had the highest antioxidant activity in both the DPPH and FRAP assays. The hydrophobicity level of the alcohol could also influence the resulting antioxidant capacity; however, this influence was not always linear with the resulting antioxidant capacity (Gallardo *et al*., 2016). The synergistic effect between cinnamate ester and cinnamic acid might also contribute to its antioxidant activity. However, further studies are required to confirm this.

The highest antioxidant capacities, of 34.70 mg AEAC/mL sample and 61.98 mg AEAC/mL sample, respectively for the DPPH and FRAP methods, were obtained by cinnamate ester that was synthesized using 1-butanol as an alkyl donor in a synthesis strategy of 12-h batch reaction followed by continuous reaction (Figure 5a). The trends of the antioxidant activities measured by the DPPH and FRAP assays were similar, where the highest to the lowest antioxidant capacities were obtained from the synthesis using 1-butanol, 1-hexanol, and 1-octanol (Figure 5a-c). The resulting antioxidant capacity also increased as a result of the batch reaction, continuous reaction, 3-h batch reaction followed by continuous reaction, and 12-h batch reaction followed by continuous reaction. It has been reported that the longer the contact time between the enzyme molecules and the substrates, the more cinnamate ester is formed (Stamatis *et al*., 1999).

## Conclusions

Continuous cinnamate ester synthesis using the Lipozyme TL IM was facilitated by EMR application. The highest conversion was obtained with 1-butanol as the alkyl donor and with the synthesis strategy of a 12-h batch reaction followed by a continuous operation with a residence time of 9 h. The antioxidant capacity of the produced cinnamate esters increased compared to unesterified cinnamate esters. In this study, to obtain higher conversion and antioxidant capacity, the suitable alkyl donor for the esterification of cinnamic acid was the alcohol with the shortest length of carbon (*e.g*., 1-butanol).

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