Comparative study of stirred and fluidized tank reactor for hydroxyl-kojic acid derivatives synthesis and their biological activities

Ahmad Firdaus B. Lajis*, Muhajir Hamid, Syahida Ahmad and Arbakariya B. Ariff

Abstract

Background: Study on the synthesis of kojic acid derivatives (KADs) in solvent-free system using scalable reactors and their biological activities is still lacking.

Methods: In this study, two types of KADs, were synthesized using saturated-fatty acid [lauric acid (LA)] and unsaturated-fatty acid [oleic acid (OA)] in stirred tank reactor (STR) and fluidized tank reactor (FTR). The yield and biological activities of the synthesized KADs were evaluated and compared.

Results: The highest yield of KADs (42.95%) was obtained in the synthesis using OA, with molar ratio of 1:1, enzyme loading of 5% (w/v), temperature of 70°C, using immobilized lipase N435 in STR. However, FTR may provide biocatalyst protection and reusability with reduced loss of KADs yield up to three cycles. In antioxidant assay, the hydroxyl-unsaturated-fatty acid of kojic acid (HUFA-KA) showed better activity as compared to hydroxyl-saturated-fatty acid of kojic acid (HSFA-KA) at concentrations ranging from 125 to 2000 μg/mL. In contrast, HSFA-KA showed better cytotoxicity effect against G361 melanoma cell as compared to HUFA-KA.

Conclusion: The yield of KADs obtained in STR was higher than that obtained in FTR. HUFA-KA could be used as potential lipophilic antioxidant while HSFA-KA has the potential to be used to treat melanoma skin disorder.

Keywords: Biochemical reactor; Flow behavior; Immobilized lipase; Lipophilic antioxidant; Melanoma; Reusable biocatalyst; Solvent-free.

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Kojic acid (KA) is an aerobic fermentative metabolite naturally synthesized by Aspergillus and Penicillium species using simple carbon sources such as glucose as a precursor [1]. The prominent biological activity of KA is centered to its hypopigmenting action which gives to its commercial importance in cosmetics and pharmaceutics industry to lighten and treat hyper-pigmentation skin problems [1–4]. However, limited application, low stability and sensitive towards change in temperature, radiation, pH, and moisture content led to its low usage and decreased commercial value. In order to improve KA characteristics and biological functions, various KA derivatives (KADs) have been designed and synthesized via multiple steps of conversion and combination of other side chain like fatty acid, saccharides, metal and other aliphatic and aromatic heterocyclic organic compounds [5]. In industry, very high yield KADs (up to 98% conversion) can be produced via chemical process but the process is not environmentally friendly [1–3]. A green process using biocatalyst via enzymatic approach can be an alternative to the use of hazardous chemicals in organic synthesis [6]. As to date, only a few types of KADs could be synthesized via enzymatic process. For examples, KA esters and KA glucosides [1, 6].

Most research in the enzymatic synthesis of KA derivatives (KADs) was conducted using shake flasks. It is well known that shake flask is not scalable. The mixing mechanism and vessel geometric measurement of shake flask are different compared to the typical reactor vessel. This means that the optimal reaction conditions obtained in shake flask cannot be transferred into reactor system due to the differences in mixing pattern and capacity as well as the mode of operation. Therefore, the synthesis data in a suitable reactor design, with effective by-product removal and immobilized lipase reusability, are required for scaling-up and yield optimization in large scale production. Stirred tank reactor (STR) is a typical mixing type reactor which is a commonly used in laboratory and industrial scales due to ease of fabrication, construction, maintenance and operation [6]. On the other hand, fluidized tank reactor (FTR) may provide advantages such as less shear rate which facilitate in enzyme stability over long term usage [7]. Moreover, instead of synthesizing KADs in organic solvent, a solvent-free system has been used to eliminate possible solvent-biocatalyst incompatibility and the removal of by-product, water from the system that may enhance KADs synthesis. Study on KADs synthesis in batch reactor by comparing stirring and fluidization in solvent-free system is still lacking [8]. The rheological behaviors of the reactants (fatty acid and KA) and products (KADs) need to be determined where the information are needed in designing the process equipment such as reactor and its operation for the enzymatic process. The differences in viscosity of unsaturated fatty acid and saturated fatty acid is an essential parameter in estimating the efficiency of distillation purification column for separation of impurity like unreacted fatty acid. Knowledge on flow behavior also required for the design of heat exchanger, process piping, mixing tank, stripping tank and liquid extractors in industry.

The biological activity study of KADs, as reported in the literature, is solely focused on improving the depigmenting activity. The other biological activities such as antioxidant and cytotoxic activity, especially the variation with different KADs molecules having unsaturated and saturated fatty acid as its side chain, are rarely evaluated. KADs molecules can be a potential lipophilic antioxidant suitable for oil-based food applications. Several types of assay have been proposed for the determination of antioxidant activity. For example, phosphomolybdenum assay provides total estimation of antioxidant activity while FRAP assay is a simple and suitable method for lipophilic antioxidant molecules [9–11].

The objective of this study was to enzymatically synthesis two types of KADs, hydroxy unsaturated fatty acid of kojic acid (HUFA-KA) and hydroxy saturated fatty acid of kojic acid (HSFA-KA) using oleic acid (OA) (C18:1) and lauric acid (LA) (C12:0), respectively. The performance of the synthesis in two types of reactor, STR and FTR, with different mixing patterns were compared. The flow behaviors of the reaction mixture in the reactor were evaluated and
compared using rheological models. The antioxidant activity of the synthesized KADs was evaluated using phosphomolybdenum assay (MoV), FRAP and superoxide radical anion (·O2−). Superoxide anion plays an important role in the formation of cell-damaging free radicals. The cytotoxic activity of KADs was also investigated against G361 human melanoma cell lines for possible anti-melanoma treatment.

Materials and methods

Materials

OA, LA, acetonitrile (MeCN), dimethyl sulfoxide (DMSO), hexane and ethyl acetate (EtOAc) were purchased from Merck (Darmstadt, Hesse, Germany). Hypoxanthine (HX), hydroxylamine hydrochloride (HAH), xanthine oxidase (XO), N-1-naphthyl ethylene diamine (NNED), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), hydrochloric acid (HCl), sulfuric acid (H2SO4), ammonium molybdate, potassium hexacyanoferrate (K3[Fe(CN)6]) and iron(III) chloride hexahydrate (FeCl3·6H2O) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Source of biocatalysts

The synthesis of KADs were performed using three types of commercially available heterogeneous immobilized lipases; (i) triacylglycerol lipase B from Candida antarctica (Novozym® 435), (ii) Thermomyces lanuginosus lipase (Lipozyme® TLIM), and (iii) Rhizomucor miehei lipase (Lipozyme® RMIM). All enzymes were purchased from Novozymes A/S (Bagsværd, Hovedstaden, Denmark). Lipase of Novozym® 435 (N435) was immobilized on hydrophobic macroporous acrylic resin (poly-(methyl acrylate) with particle size ranging from 35 to 560–710 μm with strong affinity for the matrix and strong protein to protein interactions [12, 13]. The pore diameter, surface area and surface thickness of N435 were 250 Å, 500 m2/g and 80–100 mm, respectively. Lipases of Lipozyme® TLIM and Lipozyme® RMIM were immobilized using low-particle size silica granulated resin (300–1000 μm particle size) via ionic adsorption and macroporous Duolite ES 562 (a weak anion-exchange resin) based on phenol-formaldehyde copolymers, respectively [14, 15].

Enzymatic reaction of KADs synthesis

The synthesis of KADs was biocatalyzed using commercial immobilized lipases in two type of reactor systems, STR and FTR. The synthesis was performed using OA and LA to give unsaturated fatty acid and saturated fatty acid of KA, respectively. The reaction involve in the synthesis is illustrated in Figure 1. The reaction was conducted in solvent-free environment and the by-product of this reaction, water was removed using vacuum pump which was connected to the reactor.

STR

The STR with a working volume of 100 mL was equipped with magnetic bar (diameter and width size of 2.5 cm and 0.5 cm, respectively) to act as agitator. The STR vessel was placed on magnetic stirring hotplate RCT basic IKAMAG® (IKA® Works Inc, Wilmington, NC, USA) for stirring. The geometrical configuration of STR is illustrated in Figure 2. The homogenous mixing of the reactants (fatty acid and KA) and biocatalyst was achieved by the magnetic bar agitated at 400 rpm. At this agitation speed, the sedimentation of the immobilized enzyme particles at the bottom of the vessel was prevented. The temperature within the reactor vessel was kept controlled with hot water circulated through the vessel from the connected water bath. In STR, the experiments were conducted using different immobilized lipases (N435, RMIM and TLIM) and temperatures (ranged from 50° C to 70° C), where the yield obtained from each run was compared. The fatty acids (OA and LA) were initially liquefied and kept at 80° C for 30 min prior to the addition of enzyme at the loading size of 5% (w/v) into the reaction mixture.

FTR

Instead of using magnetic bar for agitation, the same vessel configuration used for STR (as described in Section “STR”) was sparged with air at fluidization rate of 5 L/min to allow proper circulation of the entire immobilized lipase element in the reaction mixture. The volumetric fluidization rate was measured using a rotameter. The temperature and molar mass ratio (KA to fatty acid) in the mixture was controlled at 70° C and fixed at 1:1, respectively.

Recycling of immobilized lipase in STR and FTR

In order to remove the immobilized lipase from reaction mixture, the reactants and products were dissolved and washed in 30 mL DMSO (ratio of washing solvent to mixture of 1 to 1) with stirring at 400 rpm at 70° C. This procedure was repeated twice before the washed reaction mixture was filtered using filter paper (Whatman
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no.1) and the recovered immobilized lipase was dried in an oven at 70°C for 12 h for reused in subsequent cycle. The yields of KADs were calculated and compared among the three cycles carried out in this study. This method was repeated using MeCN instead of DMSO as washing solvent to compare the yields of KADs.

**Figure 1:** Enzymatic synthesis of hydroxy unsaturated fatty acid of kojic acid (HUFA-KA) and hydroxy saturated fatty acid of kojic acid (HSFA-KA) using oleic acid and lauric acid as acyl donors, respectively.

**Calculation of KADs yield**

The KADs were analyzed using gas chromatography (Agilent Technologies Inc, Wilmington, NC, USA) using a non-polar gas chromatography (GC) column (15 m × 0.53 mm × 0.15 µm) Zebron™ ZB-5HT Inferno™ (Phenomenex Inc, Torrance, CA, USA) with nitrogen (N₂) and compressed air as carrier gas. The temperature for GC oven, injection port and flame ionization detector (FID) were programed at respective temperature and time, where the details as previously reported [4]. The KADs yield was calculated using equations (1) and (2):

\[
C\text{co} = \frac{A\text{co}}{A\text{iS}} \times \frac{C\text{iS}}{D\text{RF}}
\]  

(1)

Yield (%) = \(C\text{co} / \text{mole of KA}) \times \text{dilution factor} \times 100
\]

(2)

where,

\(A\text{co}\), peak area for each constituent; \(A\text{iS}\), peak area for internal standard; \(C\text{iS}\), molarity for internal standard; \(D\text{RF}\),
Purification of KADs

KADs were purified via column chromatography using silica gel [particles mesh size of 60–120 and pore diameter of approximately 60 angstroms (Å)] as solid stationary phase and a mixture of hexane:EtOAc (70:30, v/v) as liquid mobile phase. The eluted fractions were collected at every 5 min and analyzed using GC for a single KADs peak area, which was subsequently used for further analyses using GC/MS (PerkinElmer Inc., Waltham, MA, USA), NMR-HSQC and HMBC (Varian Inc, Palo Alto, CA, USA) [4].

Determination of rheological behavior

Thermo Scientific Rheometer HAAKE™ MARS III (Thermo Fisher Scientific Inc, Waltham, MA, USA) with parallel cone-plate diameter of 0.2 cm and gap size of 0.5 mm in a rotational mode was used for rheological measurement. The samples were equilibrated for 3 min at respective reaction temperature (50°C, 70°C and 90°C) prior to each measurement. The measurements of viscosity versus shear rate and shear stress versus shear rate were collected for reactants and products. The instrument was programmed at various shear rates, ranging from 50 to 1000 reciprocal seconds (s⁻¹). The measurement for each sample was performed in triplicate. The consistency coefficient and flow behavior of the samples was determined using Power Law equation (3) and Herschel-Bulkley equation (4) models

\[
\tau = K \cdot \gamma^n \tag{3}
\]

\[
\tau = (\tau_0) + K \cdot (\gamma)^n \tag{4}
\]

where:
- \( \tau \) is the shear stress (Pa), \( K \) is the flow consistency coefficient (Pa·sⁿ), \( \gamma \) is the shear rate (s⁻¹), \( n \) is the flow behavior coefficient (dimensionless) and \( \tau_0 \) is yield stress (Pa). \( K \) represents an apparent or effective viscosity as a function of the shear rate.

Antioxidant assay

Phosphomolybdenum assay

In this method, 100 μL of KADs were taken into 2 mL microcentrifuge tubes and mixed with 1 mL of reagent solution containing 28 mM sodium phosphate, 0.6 M H₂SO₄, and 4 mM ammonium molybdate before incubated at 95°C for 90 min [9]. Ascorbic acid and 1% DMSO was utilized as a positive and negative control, respectively. The optical density (OD) of the mixture was then measured at 695 nm with DMSO blank using spectrophotometer UV-U-1800 (Hitachi High-Technologies Corporation, Minato-ku, Tokyo, Japan). The activity was illustrated as the amount of ascorbic acid equivalents per mL (AAE/mL).
FRAP assay

In this assay, freshly prepared FRAP reagent containing 300 mM acetate buffer, pH 3.6, 10 mM TPTZ and 20 mM FeCl₃·6H₂O were mixed at a ratio of 10:1:1. KADs (10 μL) at concentration ranging from 125 to 2000 μg/mL were mixed with FRAP reagent (250 μL) and incubated at 37°C for 10 min [10]. The activity was measured at OD of 630 nm using MR-96A microplate reader (Mindray, Shenzhen, Guangdong, China). Ascorbic acid and 1% DMSO was used as a positive and negative control, respectively. The activity is illustrated as AAE/mL.

Superoxide (SO) scavenging activity

Reagent A solution (20 μL) containing of 0.5 mM HX, 10 mM HAH were mixed with 80 μL of EDTA–phosphate buffered saline (pH 8.2) solution and KADs samples, pre-incubated at 37°C for 10 min [11]. Twenty microliter XO solution (5 μM) was added to the mixture and allowed for 30 min incubation. Reagent B solution (180 μL) containing 2 mM sulfanilic acid, 20 mM NNED and 16.7% acetic acid was then added to the mixture. The final mixture was further incubated at 25°C for 30 min. The OD was measured at 492 nm using MR-96A microplate reader. The results were expressed as a relative superoxide anion activity in percentage (%) to untreated control. Ascorbic acid was used as a positive control.

Cytotoxic assay

G361 human Caucasian malignant melanoma cell lines (ECACC, Salisbury, Wiltshire, UK) were proposed a chosen model for evaluation of cytotoxic assay of KADs on human skin melanoma. The cell lines were cultured in McCoy’s 5A medium containing FBS (10%, v/v), glutamine (2 mM) and 1% (v/v) antibiotic [penicillin (100 IU)/streptomycin (50 μg/mL)] and incubated at 37°C in humidified air containing CO₂ (5%, v/v). In tetrazolium salt (MTT) assay, the cell culture (1 x 10⁵ cells/well) was seeded and allowed to adhere completely overnight onto 96-well microtiter plate. Then, the medium was discarded and a fresh medium containing various KADs concentrations (62.5–1000 μg/mL) was added into the well plate and incubated for 48 h. Subsequently, the medium was discarded and 50 μL of MTT solutions (1.0 mg/mL) was pipetted into each well. After 4 h incubation, DMSO was simultaneously pipetted into the well using eight-channel micropipette and measured at OD of 450 nm using microplate reader. Cell viability was calculated as percentage (%) in comparison with the untreated control.

Statistical analysis

All experiments were performed in triplicate (n = 3) and data were expressed as means ± standard error of means (SEM). Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software Inc, La Jolla, CA, USA) where p-values <0.05 (p < 0.05) were considered significant using one way analysis of variance (ANOVA) with Bonferroni’s multiple comparison posteriori tests.

Results and discussion

Immobilized lipases for KADs synthesis in STR

The selection of biocatalyst is important for successful biomanufacturing in large scale biochemical reactors. In general, biocatalyst immobilization often leads to the loss to about 50% of its native activity due to incorrect selection of immobilization method for a specific application. Correct lipase immobilization strategy usually enhanced the thermostability of lipases and mechanical strength, chemical-physical stability, lipophobic-lipophilic nature, amount of lipase, renewal, and lipase remaining functionality [16, 17]. Immobilization can be in the form of binding to a prefabricated support (carrier), entrapment in organic or inorganic polymer matrices, cross-linking of enzyme crystal (CLEC) and aggregate (CLEA).

Immobilized lipase as biocatalyst served industrial important in bioconversion due to ease of separation and reusable for the following batch. In this study, the highest yields of HUFA-KA and HSFA-KA synthesized using RMIM in STR were 34.76% and 30.51% at 70°C, respectively (Figures 3A and 4A). At the same reaction temperature (70°C) and molar ratio (1:1), slightly higher yield of HUFA-KA (42.95%) and HSFA-KA (30.62%) were noted when synthesized with N435 (Figures 3C and 4C) as compared to RMIM. A very low yield of HUFA-KA and HSFA-KA were synthesized using TLIM in STR, which was 0.04% and 4.82%, respectively (Figures 3B and 4B). Low yield of KADs synthesis using TLIM may be due to reactant-biocatalyst selectivity as compared to N435 and RMIM. The lipase from Thermomyces lanuginosus (TLIM) preferably selective for only one hydroxyl group in the
Acylation of sucrose, maltose, leucrose and maltotriose, as compared with lipase from *Candida antarctica* [18]. Low yield using TLIM can be explained due to abrasion where inorganic supports (silica) of TLIM were weak to stirring-shear effect [19]. Immobilized TLIM had also different characteristics and specificity depending on the reaction systems (solvent or solvent-free) and the hydrophobicity of its carriers [16]. Lipase from *Rhizomucor miehei* (RMIM) and *Candida antarctica* (N435) were selective to short-medium fatty acids as reactants. Lipases from different sources may also lead to different molecular structures of KADs. In previous study, it has been proposed that the use of lipases from *Penicillium camemberti*, *Pseudomonas*

Figure 3: The experimental data of HUFA-KA synthesis in solvent-free system up to 240 min using commercial immobilized lipases RMIM (A), TLIM (B) and N435 (C) at 50°C, 60°C and 70°C in stirred tank reactor (STR). Lipase loading, agitation speed and molar ratio were fixed at 5% (w/w), 400 rpm and 1:1. Data are expressed as means ± SEM, n = 3.

Figure 4: The experimental data of HSFA-KA synthesis in solvent-free system up to 240 min using commercial immobilized lipases RMIM (A), TLIM (B) and N435 (C) at 50°C, 60°C and 70°C in stirred tank reactor (STR). Lipase loading, agitation speed and molar ratio were fixed at 5% (w/w), 400 rpm and 1:1. Data are expressed as means ± SEM, n = 3.
cepacia and Rhizomucor miehei as biocatalyst led to the formation of KADs having phenolic OH group esterified to fatty acid [1, 4]. However, in a new study showed that lipase from Candida antarctica led to the formation of hydroxyl-fatty acid of KA [2].

**Effect of temperature on KADs synthesis**

The yield of HUFA-KA using RMIM at reaction temperature of 50°C, 60°C and 70°C was 0.78%, 1.02% and 34.76%, respectively. However, the yield of HUFA-KA using TLIM at 50°C, 60°C and 70°C was very low (<0.05%). The yield of HUFA-KA using N435 also was increased (0.48, 0.73 and 42.95%) with increasing temperature (50°C, 60°C to 70°C), respectively. On the other hand, the yield of HSFA-KA using RMIM at reaction temperature of 50°C, 60°C and 70°C was 11.00%, 26.92% and 30.51%, respectively. Even though, low yield of HSFA-KA using TLIM at 50°C, 60°C and 70°C but the pattern of yield was increased respective to increasing reaction temperature (3.13%, 3.89% and 4.82%). The same pattern was also observed where yield of HSFA-KA using N435 at 50°C, 60°C and 70°C was 11.08%, 20.93% and 30.62%, respectively. In general, the yield of KADs obtained at 70°C was higher than at 50°C and 60°C. The water molecule, which is the by-product of esterification, can be efficiently removed via evaporation as proved by the high yield at high temperatures. There are several factors that determine the evaporation rate of water as the water can evaporate at any temperature between the melting point and boiling point. Evaporation is the process where water molecule is change from liquid to vapor. Under vacuum condition, water vapor can be removed out from the reaction mixture in the reaction vessel. As temperature increased, the kinetic energy of water molecule is increased and allowed the formation of water vapor, which in turn, increased the rate of evaporation. Nevertheless, the water from the process of esterification can be successfully removed from the system by the addition of molecular sieves [1, 14, 20]. The removal of water from the reaction mixture is also important to prevent leaches of enzymes from their support due to the presence of water [13].

In HUFA-KA synthesis, the yield and rate were also linearly increased over time, showing that by-product water was successfully removed from the reaction mixture, where the equilibrium was shifted to HUFA-KA synthesis. In HSFA-KA synthesis, the rate of conversion was initially high at initial reaction time but slowly decreased at the end of experimental reaction time which may be due to its equilibrium state. As for comparison, the rate of HUFA-KA synthesis at 70°C was higher than HSFA-KA synthesis at the same reaction temperature (70°C). The rate of heterogeneous catalytic reaction was usually decreased in batch mode of operation over time due to the catalyst deactivation. For example, the components in the reaction system may block the catalyst pores, thus, restrict the mass transfer rate to the internal catalyst surface from the external pore opening [13, 14].

**Free and immobilized lipases**

The comparative stability study of lipases in their immobilized form using respective immobilization supports as compared to free lipases have been reported by several researchers [13, 16–18]. For example, the hydrolytic activity and stability in pH and temperature of poly(N-methylolacrylamide)-immobilized C. rugosa lipases were improved as compared to free lipases [13]. For the immobilized T. lanuginosa lipase on resin support, the highest activity was obtained at the optimal temperature, which was 10°C higher than that of the free lipase [17]. Immobilized Lipase B from Candida antarctica (Novozym® 435) was also more thermostable (relative activity of 60%) at high temperature (90°C) as compared to lipase in free form (relative activity of 0%) [12]. Free Rhizomucor miehei lipase tends to form bimolecular aggregates even at very low concentrations or to be adsorbed to any hydrophobic reaction mixtures (i.e. containing fatty acid) [14]. Aggregation of lipase led to different properties when compared to the monomeric form of the immobilized lipases [14]. In immobilized form, lipase is protected from interactions with any external interfaces, and may not longer suffer interfacial activation by the external interface [14].

**Mass transfer**

In homogeneous reaction using free enzyme, the effect of mass transfer between phases is low. On the other hand, the mass transfer in heterogeneous reaction using physically immobilized enzyme on carrier was greatly affected, where the interphase diffusion and intraparticle diffusion interaction occurs between reactants and biocatalyst [21]. In addition, the use of immobilized enzyme greatly affects the intraphase mass transfer resistance due to catalytic reaction presence in the heterogeneous form [13]. A high reaction temperature affected the sorption process on the biocatalyst surface, subsequently affected the reaction rate. At high temperatures,
the presence of total pressure difference across the pore, generates a forced flow in pores [21]. This can be another possible explanation for high KADs yield at high temperature.

The particle and pore size of immobilized lipase also influence catalytic activity and the yield of conversion. The activity of immobilized lipase using porous particle involve diffusion of reactants into pores which may become rate limiting [22]. It has been observed that smaller particle size increased the reaction rate and thus the percentage of conversion [13, 22]. For instance, nanoPSG- *Candida antarctica* Lipase B (CALB) with nanoparticles (nanoPSG, diameter 68 nm) with a poly(glycidyl methacrylate) outer region gave higher product conversion as compared to the same amount of enzyme catalyzed by Amberzym-CALB covalently immobilized onto epoxy-activated macroporous poly(methyl methacrylate) Amberzyme beads (235 μm particle size, 220 Å pore size) and Novozym® 435 [22]. The use of immobilization on nanoparticles reduced the diffusion and mass transfer problems and even possibly used for solid reactants [15]. The catalytic efficiency was also increased with an increasing pore size up to 100 nm although the size of lipase molecule (i.e. *Candida antarctica* and *Thermomyces lanuginosus* lipase) was around 4–5 nm (~50 Å) [12, 15, 22]. In general, large surface area also increased rate of product conversion where more active site is available for reaction [22]. However, porous supports with high specific surface areas can increase the amount of immobilized enzyme which also increase the yield of conversion.

N435 and RMIM which were immobilized on macroporous carrier (pore diameter >50 nm) may have some limitations as compared to mesoporous carrier with narrow pore size distributions, where the size is almost similar to the target lipase with high specific surface area. Thus, the total amount of active lipase on the carrier could be increased, which in turn, the catalytic conversion could also be increased. Mesoporous carrier may also better at preventing the removal of lipases from the support surface, aggregation and degradation of their three-dimensional (3D) molecular structure than macroporous. If the pores are considerably larger than the lipase molecules, leaching and low loading may occur; while the enzyme penetration will be prevented and will be located only at the surface of carrier if the pores are too small [23]. Lipase of *Candida antarctica* can be desorbed from the surface of N435 with organic solvents and detergents at different temperatures and times, indicating that the lipase was probably absorbed physically onto the support mostly via hydrophobic interactions [12].

**Immovilized lipases for KADs synthesis in FTR**

In FTR where the reaction mixture was fluidized, the yield of HUFA-KA and HSFA-KA was 28.88% and 22.20%, respectively, which was slightly lower as compared to HUFA-KA (42.95%) and HSFA-KA (30.62%) synthesis in STR at similar reaction temperature of 70°C, molar ratio 1:1 using immobilized lipase N435 (Figure 5). Higher yield of KADs in STR may be due to sufficient mixing and better contact between reactants and catalytic site of lipases that increase esterification process. In FTR, the yield of HUFA-KA (13.25%) was higher than HSFA-KA (9.57%) using RMIM as biocatalyst at 4 h reaction time. However, the yield of HUFA-KA (22.20%) was lower than HSFA-KA (28.88%) using N435 as biocatalyst at 4 h reaction time. Even though lower yield of KADs was synthesized in FTR.
as compared to STR, the yield of KADs was increased with reaction time which indicates that possible further bioconversion of KADs after 4 h reaction time until it reached its equilibrium state. For example, the conversion rate of HSFA-KA in FTR was still increased linearly after 4 h of reaction time as compared to conversion rate of HSFA-KA in STR which almost reached its maximum yield of conversion. This might be the reason of better stability of biocatalyst in fluidization mode of FTR as compared to stirring mode of STR. The mechanical shear stress of stirring can affect biocatalyst stability where very high shear rate may damage the carrier of immobilized biocatalyst and may affect overall enzymatic bioconversion of KADs especially after several batches. Only the support material that resistant to abrasion is suitable for stirring mode of STR.

In this study, the use of compressed air as compared to inert gas was demonstrated due to its inexpensive cost. Oxygen may be used as an alternative to inert gas where the yield of product was significantly increased over reaction time. Moreover, lipase inactivation could be prevented in microporous carrier due to the presence of gas bubbles [24]. Very often, water (H₂O) is formed as the only by-product of aerobic oxidation reactions [25]. The presence of water caused equilibrium shift to reactant than the product (KADs), led to low yield. Nevertheless, the effect of oxygen and possible presence of other oxidation products are an interesting topic for further evaluation. Moreover, the product (HSFA-KA and HUFA-KA) were stable (especially at respective reaction temperature, i.e. giving decomposition temperature is higher than reaction temperature) and the stability of such product was also mentioned in other literature [26]. The presence of oxygen, even in trace amount dispersed in the reaction mixture is critical, at very high reaction temperatures (260–280°C) where it causes a rapid oxidation of the product with subsequent darkening and degradation of immobilization support [26].

Biocatalyst reusability in biochemical reactor

Immobilization of lipases on a solid support, including sol–gel silica, polymer beads, and glasses allows easy separation and reuse of the biocatalyst, makes product recovery easier and is able to enhance resistance against inactivation by different denaturants [13]. The use of DMSO is advantage as universal solvent, efficiently remove reactants and biocatalyst. Most biological catalysts are inactivated by the polar solvents such as DMSO and dimethylformamide (DMF) [18]. However, Thermomyces lanuginosus lipase immobilized via sol–gel method using silanes to catalyze transesterification reaction in DMF retained more than 80% of the activity after five cycles of 96 h reaction [27]. The effect of N345 lipase reusability on the yield of KADs was investigated where the immobilized lipase was reused for another two cycles using two polar aprotic solvents (DMSO and MeCN) as washing solvents. Figure 6A summarizes the yields of KADs during the lipase recycling reactions. The yields of KADs in solvent-free system were significantly reduced after the second cycle using DMSO as the washing solvent. The yield of HUFA-KA synthesized using fresh N435 which was initially 43.0% was greatly declined to 9.79% and 6.71% after the second and third cycle, respectively. The yield of HSFA-KA synthesized using fresh N435 was 30.62% and decreased to 21.66% and 6.20% after second cycle.

![Figure 6](image-url)
and third cycle, respectively. In contrast, the yields of KADs were significantly improved when MeCN was used as the washing solvent in about 2–3 times higher than DMSO. The yield of HUFA-KA synthesized using N435 at the second cycle (24.94%) was significantly higher than the third cycle (11.97%). The yield of HSFA-KA synthesized using the reused lipase N435 was 41.03% and 31.11% for the second and third cycle, respectively.

The yield of HUFA-KA synthesized using fresh RMIM was 22.20% and decreased to 12.19% and 2.16% after second and third cycle, respectively. In contrast, the yield of HUFA-KA synthesized using the reused lipase RMIM and MeCN was used as the washing solvent was 10.48% and 7.50% for the second and third cycle, respectively. In contrast, the yield of HSFA-KA synthesized using fresh RMIM was 30.51% and decreased to 2.68% and 2.16% after second and third cycle, respectively. The yield of HSFA-KA synthesized using RMIM at the second cycle (16.16%) was significantly higher than the third cycle (14.66%). However, the yield of KADs synthesis in comparison to N435 was higher than RMIM even after several cycles. The use of an appropriate washing solvent may reduce the enzyme deactivation problems due to the presence of product or by-product. The deactivation of lipase which reduces the catalytic activity may be due to sintering, accumulation of unwanted material on lipase surfaces and degradation [21].

In FTR, the yield of KADs after N435 and RMIM reusability were improved as compared to STR (Figure 7). In general, there was a smaller yield difference between first, second and third cycles for KADs synthesis in FTR when compared to STR. The yield of HSFA-KA synthesized using fresh N435 in FTR which was initially 28.88% was slightly declined to 24.22% and 17.02% after the second and third cycle, respectively. The yield of HUFA-KA synthesized using fresh N435 was 22.20% and decreased to 12.19% and 7.57% after second and third cycle, respectively. For comparison, the yields of HSFA-KA using fresh RMIM in FTR which was initially 9.57% was slightly declined to 8.17% and 7.40% after the second and third cycle, respectively. The yield of HUFA-KA synthesized using fresh RMIM was 13.24% and decreased to 5.44% and 4.22% after second and third cycle, respectively. The use of N435 probably would be the preferred selection due to higher yield and reusable stability at high temperature as compared to RMIM and TLIM [14, 20].

### Viscosity and flow behavior of KADs

The flow behavior (n) <1 for all fatty acid, mixture and KADs showed the behavior of pseudoplasticity (Table 1). The viscosity of a suspension depends on several factors such as of solid materials size and distribution, reactants concentration, pressure and reaction temperature. In this study, the consistency coefficient (K) is a direct measure of viscosity at a given shear rate. At the given shear rate of 500 s⁻¹, the K values for LA were decreased (declining pattern of 0.0172, 0.0148 and 0.0140 Pa · sⁿ) when the temperature increased (from 50°C to 90°C) during enzymatic reaction in STR which indicates that viscosity of LA was lower at higher temperature (70–90°C) as compared to low temperature (50°C). However, OA was slightly more viscous than LA which K values were 0.0194, 0.0194 and 0.0159 Pa · sⁿ at 50°C, 70°C and 90°C, respectively. The viscosity of fatty acids and reaction mixture can be reduced with increased shear rate at increasing agitation and fluidization rate in STR and FTR, respectively.

The yield stress τₜ (Pa) of the reaction mixture (ranging from 0.9142 to 6.6212) was significantly larger than yield stress of fatty acids (ranging from 0.2087 to 0.3792). High viscosity and yield stress indicated that significant stirring and fluidization rate were required.
Table 1: The rheological behavior of fatty acids, substrate mixture and hydroxyl-fatty acid of kojic acid derivatives.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Temperature (°C)</th>
<th>Ratio</th>
<th>Consistency coefficient K (Pa·s⁻¹)</th>
<th>Flow behavior coefficient n (–)</th>
<th>Herschel-Bulkley’s consistency coefficient K (Pa·s⁻¹)</th>
<th>Yield stress τₙ (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lauric acid</td>
<td>50</td>
<td>–</td>
<td>0.0172 ± 0.0002</td>
<td>0.8702 ± 0.0086</td>
<td>0.0158 ± 0.0001</td>
<td>3.0801 ± 0.0153</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>70</td>
<td>–</td>
<td>0.0194 ± 0.0015</td>
<td>0.8958 ± 0.0138</td>
<td>0.0181 ± 0.0071</td>
<td>3.1101 ± 0.0169</td>
</tr>
<tr>
<td>Lauric acid</td>
<td>90</td>
<td>–</td>
<td>0.0140 ± 0.0013</td>
<td>0.8006 ± 0.0147</td>
<td>0.0119 ± 0.0011</td>
<td>0.2919 ± 0.0054</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>–</td>
<td>1:1</td>
<td>0.0159 ± 0.0004</td>
<td>0.8485 ± 0.0053</td>
<td>0.0145 ± 0.0004</td>
<td>0.2720 ± 0.0069</td>
</tr>
<tr>
<td>KA:LA 90</td>
<td>1:1</td>
<td></td>
<td>0.4280 ± 0.0499</td>
<td>0.4846 ± 0.0400</td>
<td>0.2628 ± 0.0596</td>
<td>3.9503 ± 0.9274</td>
</tr>
<tr>
<td>KA:OA 90</td>
<td>1:3</td>
<td></td>
<td>0.1058 ± 0.0588</td>
<td>0.9979 ± 0.1669</td>
<td>0.0568 ± 0.0309</td>
<td>2.3064 ± 0.6133</td>
</tr>
<tr>
<td>KA:OA 90</td>
<td>1:1</td>
<td></td>
<td>0.1253 ± 0.0422</td>
<td>0.7959 ± 0.0322</td>
<td>0.0720 ± 0.0224</td>
<td>6.6212 ± 1.2686</td>
</tr>
<tr>
<td>HSFA-KA 90</td>
<td>–</td>
<td></td>
<td>0.0385 ± 0.0055</td>
<td>0.8430 ± 0.0013</td>
<td>0.0395 ± 0.0095</td>
<td>2.6159 ± 0.1655</td>
</tr>
<tr>
<td>HUFA-KA</td>
<td>1.1823 ± 0.4683</td>
<td></td>
<td>0.5101 ± 0.0338</td>
<td>0.6988 ± 0.2675</td>
<td>7.4769 ± 1.7379</td>
<td>0.0117 ± 0.0020</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM, n = 3. KA, kojic acid; OA, oleic acid; LA, lauric acid; HSFA-KA, hydroxyl-saturated fatty acid of kojic acid; HUFA-KA, hydroxyl-unsaturated fatty acid of kojic acid.

in STR and FTR to provide sufficient mixing quality to increase pressure and mass transfer rate between the reactants and biocatalyst. A viscous mixture may reduce the chance of interphase and intraphase diffusion of reactants and macroporous immobilized lipase which resulted in low yield although reactant such as OA is still available. Several Newtonian and viscous like mixture compounds were successfully synthesized in STR and required high agitation speed (>400 rpm) to achieve the highest yield where it may be related to their flow behavior and viscosity [22, 28].

KADs as lipophilic antioxidant

The purified HUFA-KA and HSFA-KA was used for antioxidant study. Table 2 shows oxidation inhibition potential of KADs using phosphomolybdenum and FRAP assay. In phosphomolybdenum assay, HUFA-KA showed slightly higher antioxidant activity as compared to HSFA-KA at concentration ranging from 125 to 2000 μg/mL. In this study, the source of Mo(IV) is come from ammonium heptamolybdate, (NH₄)₆Mo₇O₂₄ · 4H₂O. In aqueous condition, (NH₄)₆Mo₇O₂₄ · 4H₂O form [MoO₄]⁶⁻, one of Mo(VI) species, but in the presence of acidic pH, [MoO₄]⁶⁻ form [Mo(H₂O)₆]⁴⁺. The ability of KADs to reduce [Mo(H₂O)₆]⁴⁺ to [Mo(H₂O)₆]⁰⁺, result in phosphate/Mo(V) formation. HUFA-KA at concentration of 2000 μg/mL also had significantly antioxidant activity (6794 AAE/mL) as determined in FRAP assay, which was higher than antioxidant activity of HSFA-KA (4234 AAE/mL). FRAP assay directly measured antioxidant in a sample that react with Fe²⁺-TPTZ complex and produce colored Fe³⁺-TPTZ. The assay is valid to quantify KADs as lipophilic antioxidants [9–11].

HUFA-KA also reduced the superoxide scavenging activity (68.85%), significantly lower than HSFA-KA (91.28%) and ascorbic acid (84.73%) at concentration of 125 μg/mL (Figure 8). Superoxide anion is known as the main precursor of most ROS and a mediator in oxidative chain reactions. The dismutation of superoxide anion

![Table 2: Antioxidant activity of HUFA-KA and HSFA-KA measured using phosphomolybdenum and FRAP assays.](image-url)
produces hydrogen peroxide which results in the production of hydroxyl radical. The formation of hydroxyl radical is catalyzed by reduced transition metals, which may be re-reduced by superoxide anion, disseminate this process [10, 29]. In XO/hypoxanthine system, XO containing molybdopterin catalyzes the oxidation of hypoxanthine to xanthine and from xanthine to uric acid, hydrogen peroxide and superoxide. Superoxide reacts with coloring solution to produce pink solution. Superoxide scavenging activity of HUFA-KA was effective at a very low concentration of 62.5 μg/mL but 500 μg/mL, no significant difference was noted. In this study, it is suggested that KADs have the potential to interact with molybdenum cofactor that reside within XO especially at high concentration. This is correlated with ability of KADs to reduce Mo(IV) to Mo(V).

Cytotoxicity

Melanoma has a very high mortality rate and the outmost dangerous type of skin cancer examined in clinical practice because of its well-known metastasis and survival against chemotherapy. Therefore, various anti-melanoma compounds have been developed to take care of this type of cancer [19]. The cytotoxicity of KADs were evaluated against G361 melanoma cells where the cell viability are shown in Figure 9. There was insignificant reduction in cell viability of G361 cells after incubated with KADs at a concentration of 62.5 μg/mL. However, HSFA-KA at concentration ranging from 125 μg/mL to 1000 μg/mL significantly reduced the G361 cell viability (ranging from 56.24% to 17.06%). On the other hand, more than 90% of G361 cells were still viable even after treatment with HUFA-KA at high concentration (1000 μg/mL). Other KADs such as 5-benzyloxy-2-selenocyanatomethyl-4-pyranone also showed anti-melanoma and anti-proliferative effect against melanoma and cancer cells [30, 31]. KADs such as HSFA-KA were lipophilic and short-small compounds which could pass through the membrane easily led to their increased cytotoxic activities on tumor cells. This study served as preliminary investigation of possible anti-melanoma activity of KADs. Further
evaluation shall be carried out in upcoming study in order to understand better using other assay such as lactate dehydrogenase (LDH) cell membrane damage assays.

In animal cell culture, DMSO at a suitable concentration and low temperature is used to prevent cell membranes breakdown under cryopreservation conditions and avoid intracellular ice crystal formation during freezing and thawing of phase transition. DMSO is non-toxic to cells at concentration <1% as reported in previous study, therefore it was used as a negative control [4]. DMSO concentrations more than 10% (v/v) lead to cellular toxicity through the pore formation of plasma membrane [32]. At concentrations of 2–4%, DMSO lead to the activation of poly-(ADP-ribose)-polymerase and apoptosis-inducing factor translocation from mitochondria to the nucleus which eventually caused caspase-3 independent neuronal death of the cells which reduce cell viability [32].

Conclusions

The highest performance of enzymatic esterification for the synthesis of KADs was obtained in STR. However, the yield of KADs in FTR was lower to that obtained in STR. The sufficient mixing in STR is one of the reasons for high yield of KADs in STR as compared to FTR. Rheology study showed that OA, LA and KADs displayed a pseudoplastic behavior. The different in rheological behavior of the reactants can be the reason of its suitability for KADs synthesis in STR. Moreover, HUFA-KA has better antioxidant activity as compared to HSFA-KA. On the other hand, HSFA-KA showed better anti-proliferation activity than HUFA-KA. Result of this study also showed that HUFA-KA may be used as antioxidant while HSFA-KA as a potential anti-proliferative agent.

Conflict of interest statement: Authors have no conflict of interest.

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References