ENZYMATIC SYNTHESIS AND CHARACTERIZATION OF PALM-BASED KOJIC ACID ESTER

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ABSTRACT

Kojic acid ester was synthesized from acyl donor (fatty acid/palm oil) and kojic acid by esterification using lipase as a biocatalyst in an organic medium. Analysis of the product using GC and FTIR showed the presence of kojic acid ester. The gas chromatography-mass spectrometry (GC-MS) analysis and ^{1}H-NMR and ^{13}C-NMR spectral data confirmed the molecular structure of the kojic acid ester. Among the enzymes tested, lipase from Pseudomonas cepacia *gave the highest synthetic specific activity. Oleic acid was found to be the best substrate with which to produce the ester in acetonitrile. The optimum conditions for the synthesis of kojic acid derivatives using* Pseudomonas cepacia *lipase were time, 24 hr, temperature, 50°C, amount of enzyme, 0.15 g, solvent of log P = -0.33, mole ratio of 4 (kojic acid/oleic acid), with no added water, no control of water activity and oleic acid as substrate.*

Keywords: esterification, lipase, *Pseudomonas cepacia*, synthesize, kojic acid ester.

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INTRODUCTION

Kojic acid [5-hydroxy-2-(hydroxymethyl)-1,4-pyrone], a fungal metabolite, is produced by many species of *Aspergillus, Acetobacter* and *Penicillium* (Kitada *et al*., 1967). The biochemical properties of kojic acid show it to be one of the safest food products. The anti-bacterial and fungicidal properties of kojic acid contribute to its use as a food additive (Chen *et al.,* 1991). It is also widely used as an antioxidant or anti-browning agent. Due to its melatonin production inhibition property (Cabanes *et al.,* 1994), it is also an important ingredient in cosmetics. Recent research has shown genotoxicity and toxicity risk of kojic acid, as a skin lightening agent, to be negligible (Chen *et al*., 2002).

It has been reported that enzymatic glycosylation improves the stability of kojic acid (Hassan *et al*., 1995; Ichimoto and Tatsumi, 1962; Liu and Shaw, 1998). However, since kojic acid and its saccharide derivatives are water-soluble, the application of kojic acid to oily foods or cosmetics is restricted. However, its oil solubility can be improved by chemical- or enzyme-catalyzed acylation with fatty acids or triglycerides. Chemical synthesis of kojic acid ester in the presence of an acid or alkali may be more economical, but usually produces a complex mixture, difficult to purify. In general, an enzymatic reaction is more natural and the product is easy to be purified (Posorske, 1984). Enzymatic synthesis of kojic acid esters has already been reported by Liu and Shaw (1998) but with unsatisfactory yield. Therefore, this work is an attempt to improve the yield of the reaction as well as to identify the ideal reaction parameters for esterification of kojic acid and fatty acids. The optimized conditions were then used for reaction with palm oil.

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MATERIALS AND METHODS

Materials

Pseudomonas cepacia (Amano PS) lipase was purchased from Amano International Enzyme Co. (Japan), Lipozyme IM and Novozym 435 from Novozyme A/S (Bagsvaerd, Denmark), *Candida rugosa* lipase from Sigma Chemicals (USA). *Aspergillus niger* lipase from Fluka Chemika (Switzerland), kojic acid (99%) from Acros (Japan) and fatty acids (lauric acid, 98%; myristic acid, 95%; palmitic acid, 95%; oleic acid, 75% and stearic acid, 95%) from an oleochemicals company in Malaysia. Palm oil (MW=3 x average of saponification equivalent of palm oil) (Cocks and Rede, 1976) was obtained from Southern Edible Oils Sdn Bhd (Malaysia). The fatty acids composition of the palm oil was 0.1%-0.3% lauric acid, 0.9%-1.5% myristic acid, 39.2%-45.2% palmitic acid, 3.7%-5.1% stearic acid, 37.5%-44.1% oleic acid and 8.7%-12.5% linoleic acid. Methanol, acetonitrile, ethyl acetate, chloroform, toluene, hexane, heptane and isooctane were obtained from J T Baker (USA), all of analytical grade.

Methods

Esterification reaction. The reaction mixture consisted of 90 mM fatty acid/palm oil, 360 mM kojic acid and enzyme (1.15 mg protein) (unless otherwise stated) in 2 ml acetonitrile. The reaction is as shown in *Figure 1*. The reaction mixture was incubated in a horizontal water bath shaker at 50°C with stirrer speed of 150 rpm for 24 hr. All experiments were carried out in triplicates. The reaction was terminated by removing the enzyme from the mixture by filtration through filter paper. The control experiments were carried out without enzyme. The amounts of substrates, lipase concentration and the effects of various reaction parameters on the lipase activity were identified for later use.

Identification of the product. The products of the reactions were examined by thin layer chromatography (TLC) on pre-coated silica gel plate ($60F_{254}$) and developed in hexane/ethyl acetate (70:30, vol/vol). Iodine vapour was used as the detection system.

Gas chromatography analysis. The solvent from the reaction mixture was removed by rotary evaporation. The products formed were analysed on a Hewlett Packard HP6890 plus gas chromatograph after being silylated to TMS derivatives using a non-polar column HT-5 (12 m x 0.53 mm x 0.15 μm) with nitrogen as carrier gas. The oven temperature was programmed to rise from 120°C to 340°C at 6°C min^{-1}, and then held at the final temperature for 10 min. The injector and flame ionization detectors were set at 360°C. The product compositions were quantitated by an integrator with 1, 2, 3-tributyrylglycerol as internal standard.

Determination of percentage yield of product. The yields of ester synthesized in the optimization studies were calculated based on the concentration of product in the reaction mixture using the following equation:

$$\text{Yield (\%)} = (C_{comp}/\text{mole of kojic acid}) \times \text{dilution factor} \times 100$$

$$C_{comp} = (A_{comp}/A_{IS}) \times (C_{IS}/D_{RF})$$

where,

A_{comp} = area for each component;
A_{IS} = area for internal standard;
C_{IS} = concentration for internal standard;
D_{RF} = D_{RF} standard / D_{RF} internal standard; and
C_{comp} = concentration for each component.

GC-mass spectrometry analysis. The GC-mass spectrometry (GC-MS) analysis of the product isolated using preparative TLC was performed on a Perkin Elmer Instrument model Q-mass 910 spectrometer. The GC was equipped with a non-polar column, SGE BPX-5 (30 m x 0.32 mm x 0.25 μm). The carrier gas was helium at a flow rate of 1 ml min^{-1}.

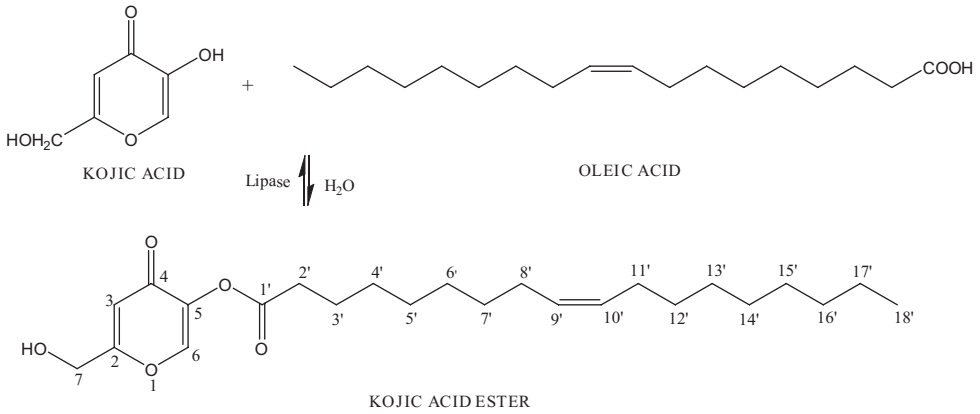

Figure 1. Esterification of kojic acid and oleic acid to kojic acid oleate.

NMR analysis. Proton NMR (^{1}H-NMR) and carbon NMR (^{13}C-NMR) spectra were obtained using a JEOL instrument model JNM-ECA 400 spectrometer. The samples were dissolved in deuterated chloroform with tetramethylsilane as internal standard.

FTIR analysis. FTIR spectra were recorded on a Nicolet Magna-I650 spectrophotometer using potassium bromide (KBr) pellets for solid and paste samples and a sodium chloride (NaCl) cell for liquid samples. The IR spectra were to suggest the possible molecular structures for the pure components. They were also used to determine the chemical changes in the reaction.

Screening of enzyme. The reaction mixtures were incubated with different lipases, the amounts used was based on the same amount of protein (1.15 mg protein). The percentage of yield was determined as described earlier.

Effect of fatty acids. The samples of fatty acids of different chain lengths ($C_{12.0}$, $C_{14.0}$, $C_{16.0}$, $C_{18.0}$, $C_{18.1}$, $C_{18.2}$) were incubated in a water bath shaker at 50°C for 24 hr. The yield was determined as described earlier.

Effect of reaction time. The reaction catalyzed by *Pseudomonas cepacia* was carried out in triplicate. Each set of experiments at different reaction times (8, 16, 24, 32, 40 and 48 hr) were used. The yield was determined as described earlier.

Effect of temperature. The samples were incubated for 24 hr in a water bath shaker at 150 rpm at different reaction temperatures (30°C, 40°C, 50°C and 60°C). The yield was determined as described earlier.

Effect of amount of enzyme. *Pseudomonas cepacia* lipase was added to separate mixtures at 0.01, 0.05, 0.1, 0.15, 0.2, 0.3 and 0.4 g. The yield was determined as described earlier.

Effect of organic solvents. The effect of using different organic solvents in the reaction mixtures was studied. The solvents used were methanol (Log P -0.76), acetonitrile (Log P -0.33), ethyl acetate (Log P 0.68), chloroform (Log P 2.0), toluene (Log P 2.5), *n*-hexane (Log P 3.5), *n*-heptane (Log P 4.0) and isooctane (Log P 4.5). The yield was determined as described earlier.

Effect of mole ratio. Samples were prepared at different mole ratios of the reactants, keeping the concentration of the enzyme and volume of the solvent constant. The moles of oleic acid were kept constant while moles of kojic acid varied (*n* mM kojic acid/ 90 mM oleic acid) so that the ratios between the two were from 0.5:1, 1:1, 2:1, 3:1, 4:1 to 5:1. The yield was determined as described earlier.

Effect of initial water activity (a_w). The lipase and substrates were pre-equilibrated overnight in bottles containing different saturated salts at room temperature. The saturated solutions of six salts were used with the following water activity values: (0.1) LiCl, (0.328) $MgCl_2.6\ H_2O$, (0.529) $Mg(NO_3)_2.6H_2O$, (0.75) NaCl, (0.8) KCl and (0.936) KNO_3. The yield was determined as described before.

Synthesis of kojic acid derivatives from kojic acid and palm oil. The reaction system consisted of 90 mM palm oil (saponification value = 197), 360 mM kojic acid, 2 ml acetonitrile and 0.15 g *Pseudomonas cepacia* lipase. The reaction mixture was incubated in a horizontal water bath shaker for 24 hr at a speed of 150 rpm and 50°C. The yield was determined as described earlier.

RESULTS AND DISCUSSION

Identification of the Product

The chromatogram of the product from the reaction of kojic acid and oleic acid detected by TLC (R_f = 0.28) showed a retention time of 31.752 min when analysed by gas chromatography. The mass spectrum for the silylated ester product from kojic acid and oleic acid showed a molecular ion peak at m/z 478 and two major fragment peaks at m/z 214 and 463. These fragments may have arisen from cleavage via paths *a* and *b* as shown in *Figure 2*. The fragment ion peak at m/z 461 resulted from the cleavage of OTMS from the molecule. Fragmentation at the bend between positions 1′ and 2′ gave two peaks at m/z 281 and 198. The position of a hydroxyl group at C-7 and an ester link at C-5 of the pyrone ring were established.

The NMR data for kojic acid ester are shown in *Table 1*. The ^{1}H-NMR spectrum of the ester gave a 3-hydrogen triplet at δ 0.88, indicating a terminal methyl group. Two hydrogen methylene signals observed at δ 1.28 and δ 1.30. Two other methylene multiplet signals were seen at δ 1.65 and δ 2.00. They were assigned to H3′-H8′ and H11′-H17′ (*Table 1*). A doublet at δ 5.33, which integrated to two protons, was assigned to H-10′ and H-9′. The downfield methylene signal at δ 2.39 was due to the CH_2 group, next to the ester linkage. The oleic acid chain was thus established to be present in the product. The kojic acid portion of the molecule was confirmed by the presence of two singlet signals at δ 6.50 and δ 7.85 which were assigned to H-3 and H-6. H-7 gave a singlet signal at δ 4.92. The ^{13}C-NMR spectrum gave a total carbon count of 24. The C-1′ (ester group) peak appeared at δ 162.97. Two very low field signals were observed at δ 172.64 and δ 173.98 which were due to C-2 and C-4 of the pyrone ring. The spectrum also indicated 1 methyl group

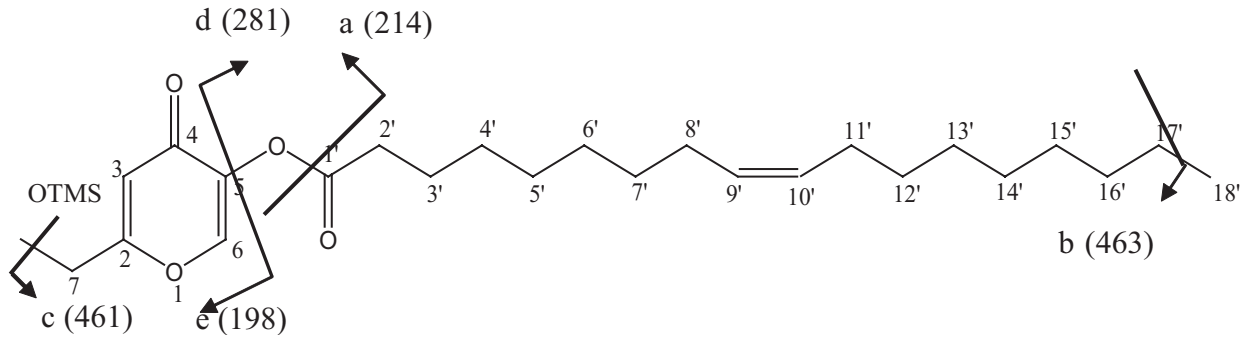

Figure 2. General MS fragmentation pattern.

TABLE 1. ^{1}H-NMR AND ^{13}C-NMR DATA FOR KOJIC ACID OLEATE

Carbon	$\delta^{1}H$	$\delta^{13}C$
C-2	-	172.64
C-3	6.50 (*s*)	111.16
C-4	-	173.98
C-5	-	129.64
C-6	7.85 (*s*)	145.89
C-7	4.92 (*s*)	61.10
C-1′ ester group	-	162.97
C-2′	2.39 (*t*)	33.82
C-3′	1.65 (*m*)	24.71
C-4′	1.28	29.01
C-5′	1.28	29.28
C-6′	1.28	29.62
C-7′	1.30	29.72
C-8′	2.00	27.17
C-9′	5.33 (*d*)	138.12
C-10′	5.33 (*d*)	138.12
C-11′	2.00	27.17
C-12′	1.30	29.72
C-13′	1.28	29.62
C-14′	1.28	29.62
C-15′	1.28	29.06
C-16′	1.28	31.85
C-17′	1.30	22.64
C-18′ (Me)	0.88 (*t*)	14.08

and 15 methylenes. The other carbon assignments are also shown in *Table 1*.

The product formation and reactant disappearance were monitored by IR spectroscopy. The IR spectrum of pure oleic acid, used as one of the basic substrates in esterification showed a C=C absorption peak at 3004 cm^{-1} and CH_2 and CH_3 stretching at 2852 cm^{-1} and 2924 cm^{-1}. The absorption peak at 1710 cm^{-1} indicated the presence of the carboxyl group (C=O). The infrared spectrum of the product showed the stretchings of CH_2 and CH_3 which gave absorption peaks at 2856 cm^{-1} and 2924 cm^{-1}. The C=O stretching for the expected ester carbonyl gave an absorption peak at 1746 cm^{-1}. The ester compound has a characteristic strong absorption band arising from the C=O stretching at 1740 cm^{-1} (Silverstein *et al*., 1991). Thus, the carbonyl group absorption at 1710 cm^{-1} for the carboxyl group shifted to 1746 cm^{-1} in the ester. The electron attracting nature of the adjacent oxygen atom (inductive effect) increased the force constant of the carbonyl bond. The CH_2 and CH_3 bends showed absorptions at 1456 cm^{-1} and 1392 cm^{-1}. Meanwhile, the O=C-O stretching absorbed in the range 1160-1214 cm^{-1}. The differences between the two spectra indicated that the kojic acid ester was formed in the lipase-catalysed esterification reaction.

Screening of Enzyme

The screening of the various lipases showed that *Pseudomonas cepacia* lipase, Lipozyme IM and Novozym 435, and *Candida rugosa* lipase were able to catalyze the esterification reaction (*Table 2*). However, the *Pseudomonas cepacia* lipase gave the highest specific activity- 3.738 x 10^{-6} mmol ester min^{-1} mg^{-1} protein content in the enzymatic synthesis of kojic acid ester. This lipase was selected for use in the subsequent experiments.

TABLE 2. SCREENING OF LIPASES FOR SYNTHESIS OF KOJIC ACID ESTER

Lipase	Specific Activity (mmol ester/min/ mg protein)
Pseudomonas cepacia lipase	3.738 x 10^{-6}
Lipozyme IM	1.678 x 10^{-6}
Novozym 435	4.407 x 10^{-7}
Candida rugosa lipase	2.597 x 10^{-7}
Aspergillus niger lipase	0

Notes: Reaction conditions: temperature= 50°C; mole ratio of kojic acid to oleic acid =4, reaction time = 24 hr; shaking speed = 150 rpm.

Effect of Fatty Acids

The reaction was carried out at 50°C for 24 hr. Among the linear chain saturated fatty acids used, C_{12} produced a high yield. However, the highest yield was from oleic acid (*Table 3*). Thus, the synthesis conditions for kojic acid oleate were further studied. The results showed an improvement in maximum yield of kojic acid oleate produced as compared to work done by Liu and Shaw (1998).

Effect of Reaction Time

Figure 3 shows the progress of kojic acid ester synthesis. The yield increased rapidly with the reaction time from 8 hr to 24 hr, after which the reaction slowed down, probably due to the equilibrium state being approached (Miller *et al*., 1988). The optimum reaction time was 24 hr, an improvement over the 48 hr by Liu and Shaw (1998).

TABLE 3. SYNTHESIS OF KOJIC ACID ESTER FROM FATTY ACIDS OF VARIOUS CHAIN LENGTHS

Fatty acid chain length	Yield (%)
$C_{12:0}$	25.39 ± 1.50
$C_{14:0}$	2.34 ± 0.24
$C_{16:0}$	11.87 ± 0.97
$C_{18:0}$	6.99 ± 0.59
$C_{18:1}$	40.12 ± 0.46
$C_{18:2}$	9.65 ± 1.92

Notes: Reaction conditions: temperature = 50°C; mole ratio of kojic acid to fatty acid =4; lipase concentration = 0.15 g; shaking speed = 150 rpm.

Effect of Temperature

The effect of reaction temperature on the percentage yield of ester is shown in *Figure 4*. The yield increased with temperature until a maximum of 44.01% at 50°C. Beyond 50°C, the yield decreased to 24.05%, probably due to denaturing of the enzyme by higher temperature.

Effect of Amount of Enzyme

Figure 5 depicts the results from using different amounts of enzyme. The yield of ester produced increased with the lipase concentration up to 0.15 g, beyond which it decreased. These results indicate that excess enzyme did not further increase the yield. The lower yield may also be due to the steric hinderance produced by an excessive enzyme. The active sites of the enzyme molecules present in excess would not be exposed to the substrates and remain inside the bulk enzyme particles without contributing significantly to the reaction.

Effect of Organic Solvents

Figure 6 shows that the yields of kojic acid ester were highest in organic solvents with log P values of -0.33 (acetonitrile) and 2 (chloroform). The good ester conversion in acetonitrile may be due to the good solubility of the substrates in it, which would have improved the contact between enzyme and substrates. Conversely, the low yields at log P > 2 could be due to the low solubility of product in high hydrophobic solvents.

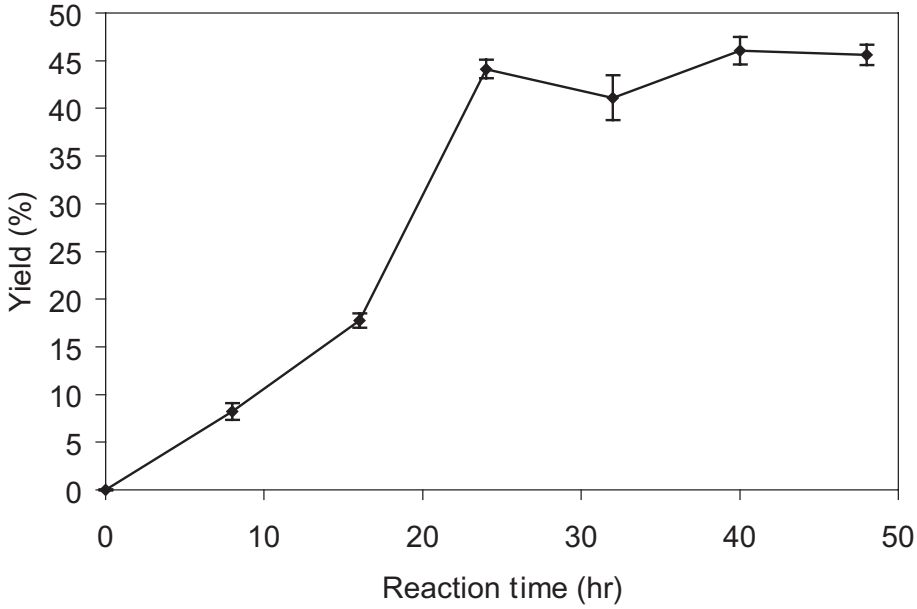

Figure 3. Effect of reaction time on enzymatic synthesis of kojic acid oleate by lipase from Pseudomonas cepacia *(Amano PS). Reaction conditions: temperature = 50°C, mole ratio of kojic acid to oleic acid = 4, lipase concentration = 0.15 g, shaking speed = 150 rpm.*

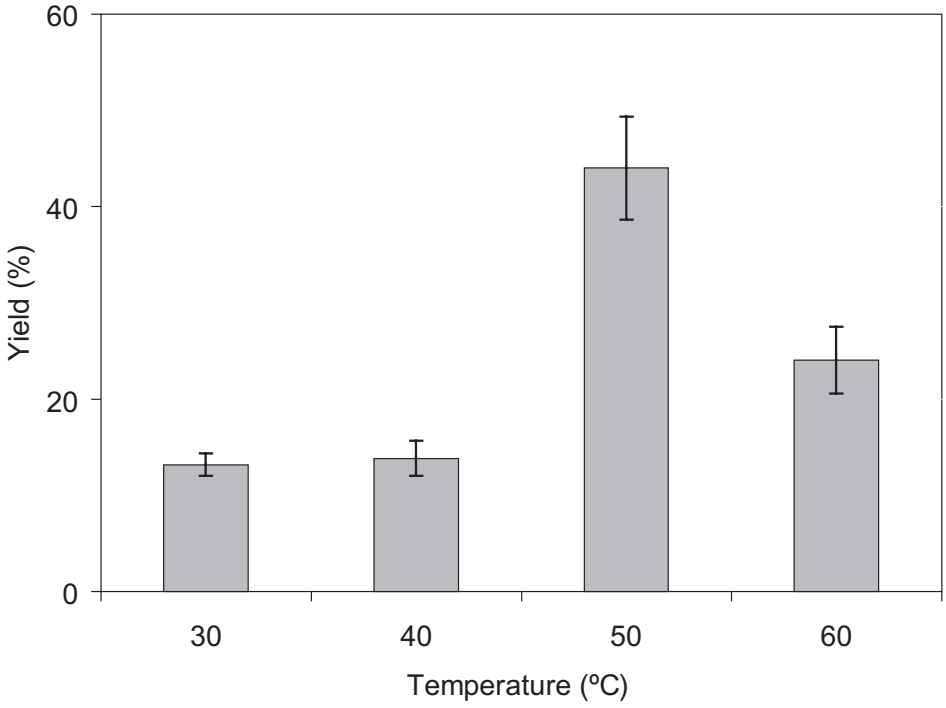

Figure 4. Effect of reaction temperature on enzymatic synthesis of kojic acid oleate by lipase from Pseudomonas cepacia *(Amano PS). Reaction conditions: reaction time = 24 hr, mole ratio of kojic acid to oleic acid = 4, lipase concentration = 0.15 g, shaking speed = 150 rpm.*

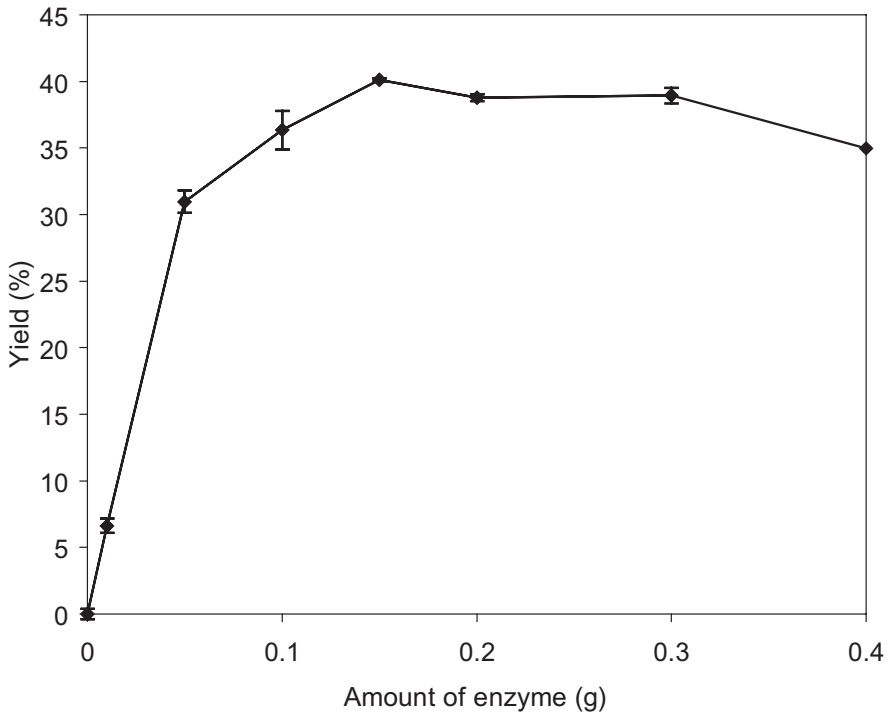

Figure 5. Effect of amount of enzyme on enzymatic synthesis of kojic acid oleate by lipase from Pseudomonas cepacia *(Amano PS). Reaction conditions: reaction time = 24 hr, mole ratio of kojic acid to oleic acid = 4, temperature = 50ºC, shaking speed =150 rpm.*

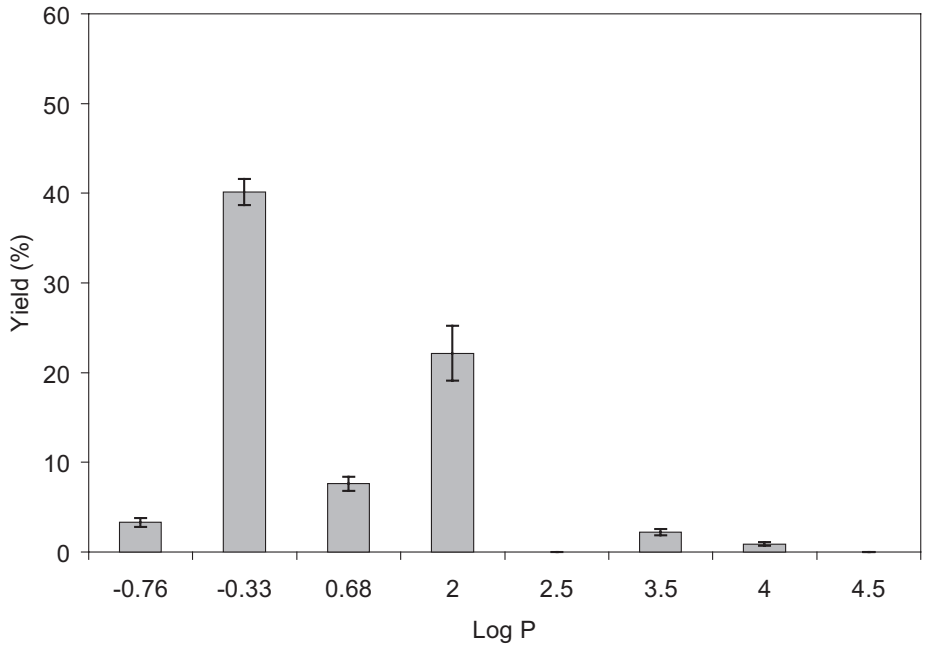

Figure 6. Effect of organic solvents on enzymatic synthesis of kojic acid oleate by lipase from Pseudomonas cepacia *(Amano PS). Reaction conditions: reaction time = 24 hr, mole ratio of kojic acid to oleic acid = 4, temperature = 50°C, lipase concentration = 0.15 g, shaking speed = 150 rpm.*

Effect of Mole Ratio

Figure 7 shows that increasing the mole ratio of kojic acid to oleic acid from 0.5 to 4.0 gradually increased the product formation. Mole ratio 4 produced the highest yield (44.01%), above which it decreased rapidly to as low as 25.82%. This observation may reflect the excess kojic acid distorting the essential water layer from *Pseudomonas cepacia*. At the same time, the excess acid will hinder contact between substrates and lipases (Kanasawud *et al*., 1992).

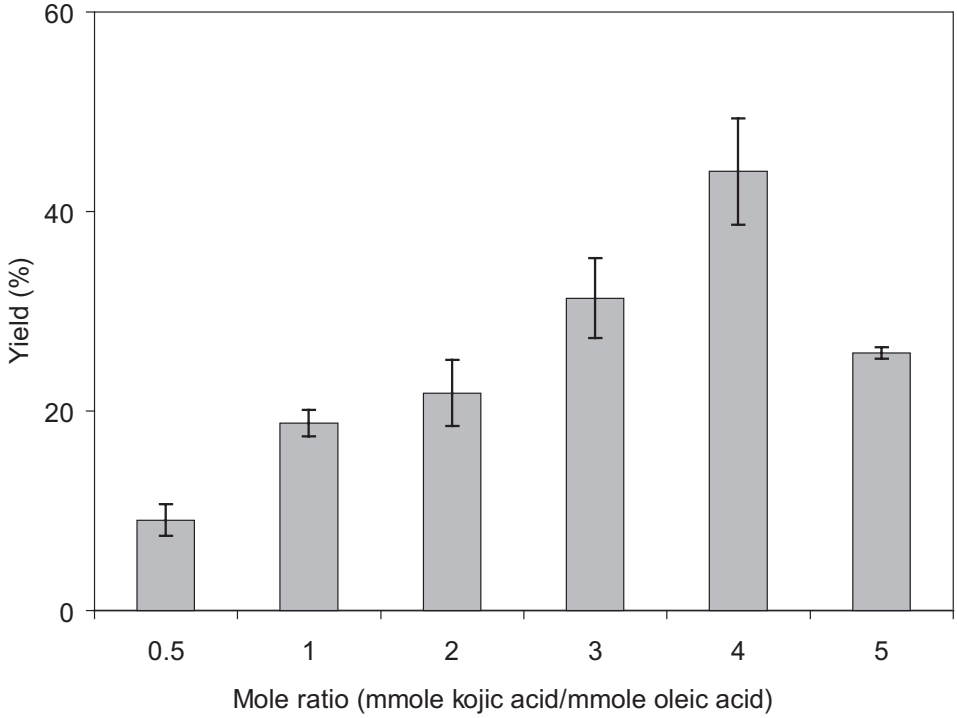

Figure 7. Effect of mole ratio on enzymatic synthesis of kojic acid oleate by lipase from Pseudomonas cepacia *(Amano PS). Reaction conditions: reaction time = 24 hr, temperature = 50°C, lipase concentration = 0.15 g, shaking speed = 150 rpm.*

Effect of Initial Water Activity (a_w)

The effect of initial a_w on the yield of ester was investigated and the results shown in *Figure 8*. In this study, the yield decreased with increasing a_w, probably due to enzyme inactivation during a_w equilibration (Svensson *et al*., 1994) or competition with reverse hydrolysis in the excess water.

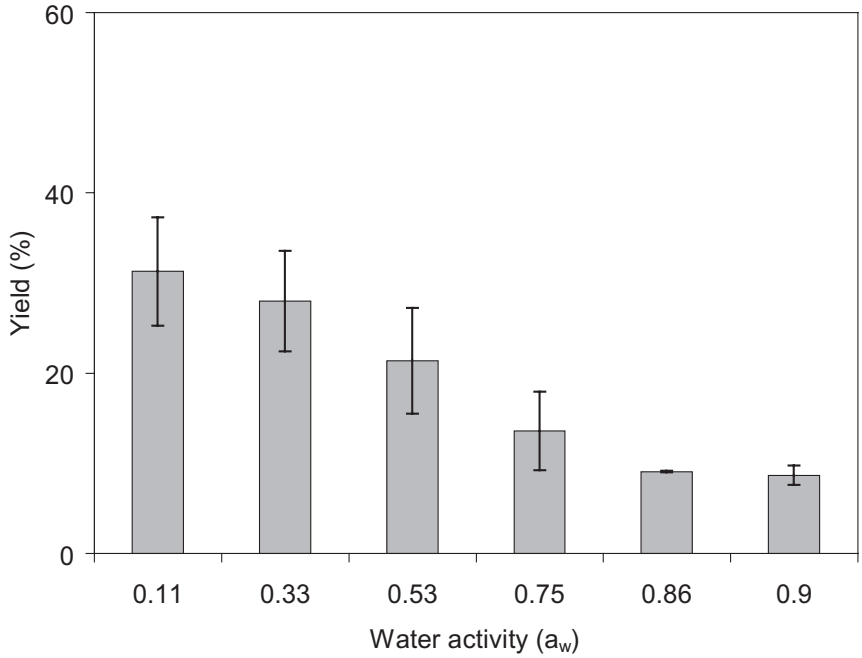

Figure 8. Effect of water activity (a_w) on enzymatic synthesis of kojic acid oleate by lipase from Pseudomonas cepacia *(Amano PS). Reaction conditions: reaction time = 24 hr, temperature = 50°C, lipase concentration = 0.15 g, shaking speed = 150 rpm, mole ratio of kojic acid to oleic acid = 4.*

Synthesis of Kojic Acid Derivatives from Kojic Acid and Palm Oil

Table 4 shows the syntheses of kojic acid derivatives from kojic acid and palm oil. *Pseudomonas cepacia* lipase gave the highest yield in 24 hr based on the optimization studies. As expected, the highest yield (8.88%) was kojic acid palmitate as palmitic acid is the largest component in palm oil. Kojic acid oleate constituted 7.56% and kojic acid linoleate 1.10% of kojic acid derivatives. Generally, the composition of the kojic acid derivatives produced coincided with the composition the respective fatty acids in palm oil. In the alcoholysis between palm oil and kojic acid, the products were not only kojic acid derivatives but also glycerol as well as monoglycerides and diglycerides of different fatty acids. The accumulation of glycerol may inhibit the reaction by limiting the contact between the substrates and the enzymes.

TABLE 4. SYNTHESIS OF KOJIC ACID DERIVATIVES FROM KOJIC ACID AND PALM OIL

Kojic acid derivative	Carbon number	Yield (%)
Kojic acid laurate	C_{12}	-
Kojic acid myristate	C_{14}	-
Kojic acid palmitate	C_{16}	8.88 ± 0.58
Kojic acid stearate	C_{18}	Trace
Kojic acid oleate	$C_{18:1}$	7.56 ± 0.17
Kojic acid linoleate	$C_{18:2}$	1.10 ± 0.10

CONCLUSION

This work suggests that kojic acid esters can be produced using the lipase from *Pseudomonas cepacia* as catalyst. Oleic acid was the best substrate for esterification among various fatty acids tested. Higher kojic acid oleate was obtained (44.01% in 24 hr) compared to in previous work (22.0% in 48 hr) using the same lipase. A mixture of kojic acid esters may be synthesized by reacting kojic acid and palm oil.

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