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Effect of lipase hydrolysis on the antibacterial activity of coconut oil, palm mesocarp oil and selected seed oils against several pathogenic bacteria

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Abstract

The antibacterial activity of solvent-extracted oil of noni (*Morinda citrifolia* L.), spinach (*Spinacia oleracea* L.), lady's finger (*Abelmoschus esculentus* (L.) Moench), bitter gourd (*Momordica charantia* Linn.), and mustard (*Brassica nigra* L.) seed oils, and coconut (*Cocos nucifera* L.) oil, palm (*Elaeis guineensis* L.) mesocarp in hydrolyzed and unhydrolyzed form were determined in order to explore their potential usage as antibacterial agent. The hydrolysis process that was catalyzed by immobilized lipase of *Rhizomucor miehei* (RMIM) showed highest hydrolytic activity with 1.0 ml of added water volume except bitter gourd seed oil and palm mesocarp oil which has maximum hydrolytic activity with added water volume of 5 ml and 2.5 ml respectively. Before hydrolysis, all oil samples did not show inhibition ring zones (IRZ) on any of the tested bacteria strains (*Salmonella typhimurium*, *Listeria monocytogenes* and *Escherichia coli* O157:H7). Hydrolyzed lady's finger and bitter gourd seed oil showed IRZ on all tested bacteria strains; hydrolyzed mustard seed oil on *S. typhimurium* and *L. monocytogenes*; hydrolyzed spinach seed oil and coconut oil on *L. monocytogenes*; hydrolyzed noni seed oil and palm mesocarp oil did not exhibit IRZ on any of the tested bacteria strains. Most of the hydrolyzed oil exhibit an inhibition activity that was different from their respective dominant fatty acids except noni seed oil and palm mesocarp oil.

Introduction

An antibacterial agent is a compound that interferes with the growth and reproduction of bacteria. It was originally developed as a drug that fight against bacterial infection in human and its path of action usually involved compounds or structure that can only be found in bacteria. Thus, antibacterial agent can act very selectively against bacterial cell and their mode of action can be generally categorized into five main groups: action that target on the bacteria cell metabolism pathway, the bacteria cell wall synthesis pathway, the bacteria cell permeability, the bacteria enzyme and the bacteria nucleic acid (Bryskier, 2005). Phytochemicals such as thymol, carvacrol, geraniol, eugenol and many others are found to possess antibacterial activity (Moleyar and Narasimham, 1992; Dorman and Deans, 2000; Cavar *et al*., 2008). Allyl isothiocyanate that are found generally in mustard seed oil (Turgis *et al*., 2009) and allicin in garlic (Gonzalez-Fandos *et al*., 1994; Cellini *et al*., 1996) are also known to have antibacterial property. However, these phytochemicals exist only in minute amounts in their respective sources and intensive extraction of these compounds would certainly incur high cost. In order to face this challenge, new sources of antibacterial agents that exist abundantly in nature and could be obtained relatively easier are crucially needed. In this respect, a variety of short, medium and long chain fatty acids can serve as potential candidates as they had been found to possess antibacterial property (Wang and Johnson, 1992; Dilika *et al.*, 2000; Bergsson *et al.*, 2001; McGaw et al., 2002; Nair et al., 2005; Kitahara *et al*., 2006; Kamdem *et al*., 2008; Sado-Kamdem *et al*., 2009; Skrivanova *et al.*, 2009; Yang *et al.*, 2009; Huang *et al*., 2011). For example, the growth of *Escherichia coli* serogroup O157:H7, where cattle and chickens have been identified as reservoir of the bacterium that causes hemorrhagic colitis and hemolytic uremic syndrome in children (Martin *et al*., 1986), is reported to be inhibited by caprylic acid (Nair *et al*., 2004) and linolenic acid (McGaw *et al*., 2002). Ground beef and chicken meats that are contaminated by *Salmonella typhimurium* cause human salmonellosis when the meats are consumed (Combs *et al*., 2006; McLaughlin et al., 2006), and therefore, it is important that their growth is inhibited. Thus, it is of interest when the growth of *S. typhimurium* could be inhibited by medium chain

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fatty acids (Messens *et al*., 2010), α-linolenic acid (ALA) and docosahexanoic acid (DHA) (Babu *et al*., 2009). *Listeria monocytogenes*, which is commonly found in ready-to-eat foods and dairy products and has been associated with the outbreaks of listeriosis (Kleter *et al*., 2009) is inhibited by caprylic acid (Nair *et al*., 2004). Wang and Johnson (1992) showed that lauric acid could also do the same to the bacterium. Unsaturated fatty acids like oleic, linoleic, linolenic and arachidonic acids have exhibited inhibitory activity against bacterial enoyl-acyl carrier protein reductase (FabI), an enzyme that catalyzes fatty acid synthesis in bacteria, and can serve, therefore, as a potential antibacterial agent that is effective against many bacteria strains (Zheng *et al*., 2005). Since numerous fatty acids are inhibitory towards *E. coli*, *S. typhimurium* and *L. monocytogenes*, the triacylglycerol (oil/fat) form of these fatty acids may be potential inhibitors as well. Thus, plant-based oils that naturally possess a high level of these fatty acids are potential novel antimicrobial agents as well.

In order to examine whether an oil possesses antimicrobial property or otherwise, the fatty acid moieties that are esterified to the glycerol backbone of triacylglycerol must first be hydrolyzed into their free forms (free fatty acids). Such a process is made possible either through the presence of a chemical catalyst such as sodium hydroxide (NaOH) or a biocatalyst, namely lipase. The advantages of a biocatalyst are that it is substrate specific and has low toxicity when compared with a chemical catalyst. Lipase is specific in its action as it is regio-specific (sn), with a preference for the hydrolysis of TAG in the following sequence: sn-1 > sn-3 >> sn-2 (Eigtved, 1992). When compared between immobilized and free RM lipase, immobilized RM lipase is more stable, more reactive, and more regio-selective (Bastida *et al*., 1998; Fernandez-Lafuente *et al*., 1998; Soumanou *et al*., 1998; Xu *et al*., 1998). Thus, immobilized RM lipase (under the trade name of *Lipozyme IM*, Novozymes, Bagsvaerd, Denmark) was used in this study to hydrolyse the oils extracted from the seeds of bitter gourd (*Momordica charantia* Linn.), spinach (*Spinacia oleracea* L.), noni (*Morinda citrifolia* L.), mustard (*Brassica nigra* (L.) Koch) and lady's finger (*Abelmoschus esculentus* (L.) Moench), coconut (*Cocos nucifera* L.) kernel and oil palm (*Elaeis guineensis* L.) fruit mesocarp. The antibacterial property of the unhydrolysed oils and hydrolysates were then tested against *S. typhimurium*, *L. monocytogenes*, and *E. coli* O157:H7. Fatty acids that are dominant in these oils and known to possess antibacterial property were also tested for their antibacterial property against the three bacteria strains.

Materials and Methods

Oil samples and chemicals

The oils used in the study were sovent-extracted (petroleum ether, 40-60 ºC boiling point, from Merck, Darmstadt, Germany) from bitter gourd, lady's finger, and spinach seeds that were purchased from the Malaysian Agricultural Research and Development Institute, MARDI (Selangor, Malaysia). Other sample like mustard seed and coconut were bought from a local store in Serdang, Selangor, Malaysia; noni seed and palm mesocarp were collected from Bukit Expo and Persiaran Golf respectively (Universiti Putra Malaysia, Selangor). Pure fatty acids, namely capric/decanoic acid (C10:0), lauric/dodecanoic acid (C12:0), oleic/octadecenoic acid (C18:1), linoleic/octadecadienoic acid (C18:2) and linolenic/octadecatrienoic acid (C18:3) were purchased from Sigma-Aldrich, USA at 99.9% purity. Immobilized *R. miehei* lipase (Lipozyme RMIM) was purchased from Novozymes, Denmark.

Bacterial culture

Strains of *E. coli* O157:H7, S. typhimurium (ATCC 14028), *L. monocytogenes* (ATCC 19155) (ATCC, Manassas, VA, USA) were acquired from the Laboratory of Food Safety and Bacteriology, Faculty of Food Science and Technology, Universiti Putra Malaysia. *E. coli* O157:H7 was a strain that was previously isolated from chicken meat and was identified by plating on sorbitol MacConkey agar and PCR assay according to the method of Radu *et al*. (2001).

Preparation of hydrolyzed oil samples

The process used to hydrolyze the oil samples were carried in two parts. The first part focused on finding the percentage of water that would maximize the degree of hydrolysis of the oils while the second part was focused on obtaining the similar degree of hydrolysis for each of the oil. The hydrolytic process in the first part of the experiment was carried out by first adding 5 g of oil into a 250 ml screw-capped conical flask, followed by 50 ml of hexane and 0.5 g of Lipozyme RMIM. The effect of water content on the degree of hydrolysis of oil was determined by the addition of 0 ml (Control), 1.0 ml, 2.5 ml, or 5.0 ml of distilled water. The reaction mixture was then incubated with agitation at 200 rpm and 50ºC for 24 hours. The reaction was subsequently terminated by quickly filtering out the immobilized enzyme through a sieve. Water and hexane in the filtrate was

then removed by rotary evaporation (Model N-1, Eyela, Tokyo Rikakikal Co., Ltd., Japan) at 77ºC. The residue was either used immediately for analysis or stored at -18oC when not in use.

In the second part of the hydrolysis protocol, the same procedure as described above was used except that the volume of water added was dependent on results obtained in the first part of the experiment and the incubation time was varied between 0 to 6 hours. In both cases, the experiments were carried out in triplicate. After determination of degree of hydrolysis, all samples were then stored at -18ºC prior to further analysis.

Determination of degree of hydrolysis

Briefly, 0.5-2 g of hydolysed sample was measured into a 250 ml conical flask and 5.0 ml of warm (77 ºC) neutralized 95% ethanol was added. The extent of hydrolysis (percent free fatty acid) was determined by titrating the mixture with 0.1 N NaOH to a phenolphthalein end point (AOAC, 1984). Percent free fatty acid (FFA) was calculated as shown below. Results obtained were reported as the mean of triplicate analysis. Non-enzyme treated oil samples (control) were also analyzed to obtain the initial fatty acid content.

$$\text{Percent FFA} = \frac{\text{V x 0.1 N NaOH x M x100\%}}{\text{Weight of sample (g)}}$$

Where

V = Volume of NaOH used to titrate the oil sample

M = Molecular weight of fatty acid that is dominant in the oil sample

Preparation of pure fatty acids for antibacterial test

Solutions of capric acid (C10:0), lauric acid (C12:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) were prepared by diluting the pure fatty acids in 95% ethanol to give working concentrations of 5, 10, 25 and 60% (v/v). Fatty acids of C10:0 and C12:0 that were originally in the solid form were heated in an oven at 60ºC for 60 minutes to liquid them, and then diluted with ethanol as described above. Due to its high viscosity, pure liquid form of C12:0 was not included in this study.

Dispersion of oil samples as emulsions

Prior to antibacterial screening test, 0.5 g of each oil sample (hydrolysed and control) that had been thawed in an oven at 60ºC for 90 minutes were added to a glass tube containing 500 µl of polyoxyethylene sorbitan monooleate (Tween® 80, Sigma-Aldrich, USA), an emulsifier which had a hydrophilic and lipophilic balance (HLB) of 15 (CHEMMUNIQUE, 1976). The ratio of sample and emulsifier was 1:1 (v/v), which give an accumulated HLB of 11.5 that is within the HLB interval (8-18) required for oil in water phase emulsion (Griffin, 1949; Griffin, 1954). The mixture was then vortexed for 1 minute to obtain a uniform emulsion. The same procedure was used when pure fatty acid solutions were used to give working emulsions of HLB of 14.6-15.0%.

Preparation of McFarland standard suspension

In order to standardize bacterial inoculum density that would be used in the antibacterial screening test, a 0.5 McFarland standard was prepared according to the method of National Committee for Clinical Laboratory Standards (NCCLS, M7-A7, 2006, Section 8.1). In this method, 0.5 ml of 0.048 M $BaCl_2$ (1.17% w/v $BaCl_2 \cdot 2H_2O$) was added slowly with constant stirring into 99.5 ml of 0.18 M H_2SO_4 (1% v/v) to make an approximately 100 ml suspension. Verification of the standard suspension was carried out by measuring the absorbance with a spectrophotometer (Spectronic Genesys 20, Thermo Electronic Corporation, Madison, USA) at 625 nm. The absorbance value should be within the range of 0.08-0.13 for a 0.5 McFarland standard. The suspension was transferred then into a screw-capped Universal bottle which was then sealed with paraffin, capped and wrapped with aluminum foil to prevent light penetration. Before each usage, vigorous agitation of the bottle and inspection of agglutination was carried out. The standard solution was kept at room temperature and replaced monthly with newly made suspension.

Preparation of bacteria inoculum

Pure bacteria cultures were inoculated on their respective agar medium as follows: *E. coli* O157:H7 on sorbitol MacConkey agar (CT-SMAC) for 24 hours at 35ºC, *L. monocytogenes* on PALCAM agar for 48 hours at 35ºC, and *S. typhimurium* on Bismuth sulfite agar for 48 hours at 35ºC (Wilson and Blair, 1927; Van-netten *et al*., 1989; Weagant *et al*., 1995). The agar preparation steps are shown in Appendix B-D. A single colony from each agar plate was then inoculated separately into 10 ml Tryptic Soy Broth (TSB; Merck, Darmstadt, Germany) and incubated at 37°C for 24 hours. The broth cultures were then diluted with sterile saline solution (0.85% of sodium chloride (w/v) in water) to approximately 1.5 x 10^8 CFU/ml by visually comparing the inoculum with the 0.5 McFarland standard against a white background card with black line on it [NCCLS, M7-A7, 2006, Section 8.2 (2)]. This working culture was then used for the antibacterial activity screening method.

Glycerol stock of each bacteria strain was prepared according to the method of Guthrie and Fink (2002). Briefly, a single bacterial colony from an agar was re-suspended in 1.5 ml capped EZ micro test tube containing 1 ml of glycerol stock solutions [TSB that contained 10% glycerol (v/v)] and stored at -80ºC until used.

Screening for antibacterial activity

The agar well diffusion method was employed to determine the antibacterial activity of the oil samples (control and hydrolysed) and pure fatty acids based on the method described by Deans and Ritchie (1987). The Mueller-Hinton agar (Sigma-Aldrich, USA) was used and was prepared as described previously. One milliliter of 1.5 x 10^8 CFU/ml working culture (*S. typhimurium*, *L. monocytogenes* and *E. coli*) was aseptically pipetted onto the agar surface and then cotton-swapped evenly. Three wells (4 mm diameter each) were made on each agar plate using sterilized yellow pipette tips and 50 µl of an emulsified oil/fatty acid sample were pipetted into each of the well. Each agar plate contained a same oil sample for the purpose of replication. Plates that contained only Tween-80 (emulsifier) inside the three wells served as the negative control. The plates were then incubated for 24 hours at 37ºC. After incubation, inhibition ring zones (IRZ) were observed visually to determine whether there is any inhibition activity. The agar plates were separated into two groups: the control group using un-hydrolyzed emulsified oil samples and the test group which used hydrolyzed emulsified oil samples. Agar plates that contained emulsified 100% pure fatty acids (except C12:0 as the emulsion was too viscous), and 60%, 25%, 10% and 5% fatty acid emulsion were also incubated for 24 hours at 37ºC. Plates that contained ethanol solution and emulsifier in 1:1 ratio served as the negative control. After incubation, inhibition ring zones (IRZ) were observed visually to determine whether there is any inhibition activity from the respective oils sample against the cultured bacteria.

Statistical analysis

All measurements with replication were statistically analysed by using Minitab 14 software. Two-way ANOVA (Analysis of variance) test was carried out to determine the interaction effect of different factors on a single measured average value. Measured average value with a single factor was analysed with one-way ANOVA test and compared for significance difference using Turkey's test. Statistical significance differences were considered at the level of $p< 0.05$.

Results and Discussion

Hydrolytic activity

Hydrolytic activity of the respective oil samples with varied water volume was shown in Table 1. TAG hydrolysis (by RMIM) without additional water volume is less effective when compared to hydrolysis with additional water volume (Table 1). When oil samples are hydrolyzed without additional water, degree of hydrolysis is low as the sample grand mean (sum up all the hydrolysis values of all oil samples with 0 ml water volume and divided by 21) is just around 9.3%. This indicates that hydrolyzing TAG by RMIM without additional water cannot even achieve 10% of hydrolytic activity. In other words, less than 10% of the substrates (TAG) are being converted into products. However, hydrolysis with 1 ml of water volume increases the hydrolytic activity greatly. Extent of increment (for the hydrolytic activity) is not the same but depends on the type of oils sample and amount of water that is added. In order to verity implication of different factors (sample type and added water volume) on response values (FFA%), two-way Analysis of Variance (ANOVA) test is run by using minitab 14 software (Minitab Inc, USA).

Result from the analysis test shows significant interaction effect of different factors on the response values ($p< 0.05$). The plot of factors interaction on the response values (FFA %) is shown in Figure 1.

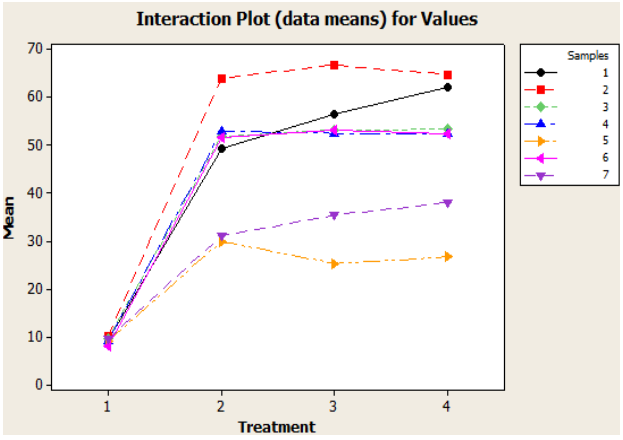

Figure 1. Plot of sample type and water volume factors interaction on the FFA% values

*Label on the y-axis, "Mean"= Mean values of FFA%; Label on the x-axis, "Treatment"= Different water volume, 1= 0 ml, 2= 1 ml, 3= 2.5 ml, 4= 5 ml

*Samples, 1= Bitter gourd seed oil, 2= Mustard seed oil, 3= Spinach seed oil, 4= Noni seed oil, 5= Lady's finger seed oil, 6= Coconut oil, 7= palm mesocarp oil

Parallel lines would be shown on the plotted graph if no interaction among the factors. Lines plotted on the graph that shown in Figure 1 is not parallel and lines crossing are observed in several sample lines (1, 3, 4, and 6). This indicates that not all sample oils

Table 1. Degree of hydrolysis (FFA %) with varied water volume (%) for respective oil samples

Water volume (ml)	Value of Free Fatty Acids (%) for different source of oils						
	Noni seed	Spinach seed	Lady's finger seed	Bitter gourd seed	Mustard seed	Copra	Palm mesocarp
0.0	9.2 ± 1.1^{a1}	9.7 ± 0.5^{a1}	9.1 ± 0.9^{a1}	9.4 ± 0.8^{a1}	$10.2\pm0.3^{(a1)b1}$	$8.0\pm0.5^{(a1)f1}$	9.6 ± 0.5^{a1}
1.0	52.9 ± 1.2^{d2a2}	51.8 ± 1.6^{c2a2}	29.9 ± 1.6^{e2g2}	49.3 ± 0.9^{a2}	64.1 ± 2.0^{b2}	51.6 ± 1.3^{f2a2}	31.1 ± 2.0^{g2}
2.5	52.6 ± 1.3^{d2a3}	53.3 ± 1.7^{c2a3}	25.3 ± 2.9^{e2}	56.6 ± 1.7^{a3}	66.8 ± 2.0^{b2}	53.3 ± 0.9^{f2a3}	35.4 ± 0.6^{g3}
5.0	52.4 ± 0.7^{d2c3}	53.4 ± 0.8^{c2c3}	26.7 ± 2.3^{e2}	62.3 ± 1.1^{a4}	64.8 ± 3.0^{b2a4}	52.5 ± 0.9^{f2c3}	38.1 ± 0.7^{g3}

have the same changes in hydrolytic activity when the water volume is increased. Different degree of hydrolysis enhancement due to the factor of sample type is obviously shown by sample 1 line as it crosses the lines of sample 3, 4, and 6 when water volume is increased from 1 ml to 2.5 ml. This crossing indicates that sample 1 shows higher hydrolytic activity as the added water volume increased from 1 ml to 2.5 ml when compared with sample 3, 4 and 6. Existence of significant interaction effect impedes any conclusion that states the variation in hydrolytic values is either due to different treatment (water volume) or sample type. Instead, the only justifiable conclusion is, hydrolytic values depend on both of these two factors. In order to know the direct effect of the respective factors on the hydrolytic values, the obtained data is reanalysed with a slightly different method.

Effect of treatment factor alone is analysed by collapsing the sample factor into seven groups with four different levels (0 ml, 1 ml, 2.5 ml, and 5 ml) so that one-way ANOVA test can be conducted. On the other hand, effect of sample factor alone is analysed by collapsing the treatment factor into four groups with seven different levels (palm mesocarp oil, coconut kernel oil, bitter gourd, mustard, spinach, noni and lady's finger seed oils). Result from the one-way ANOVA test (treatment factor) shows that hydrolysis without added water is significantly different ($p<0.05$) from hydrolysis with added water for all seven groups of oil (Table 1). However, hydrolysis with 2.5 ml of water volume is not significantly different from 1 ml of water volume for all groups of oil except bitter gourd seed oil and palm mesocarp oil. In other words, the increment on hydrolytic value of bitter gourd seed oil from 49.3±0.9% to 56.6±1.7% and palm mesocarp oil from 31.1±2.0% to 35.4±0.6% is statistically significant ($p<0.05$). When the water volume is further increased to 5 ml from 2.5 ml, only bitter gourd seed oil shows increment in hydrolytic activity that is statistically significant ($p<0.05$).

Result from the one-way ANOVA test that analyse the effect of sample factor reveals that oil samples do not have hydrolytic values that are significantly different ($p>0.05$) from each other at 0 ml of water volume except mustard seed oil and coconut oil. This indicates that hydrolytic value of mustard seed oil is statistically higher than the hydrolytic value of coconut oil with 0 ml of added water volume. When added water volume is increased from 0 ml to 1 ml, mustard seed oil obtains the highest hydrolytic value which is statistically different from the other oil values ($p<0.05$). Hydrolytic value of lady's finger seed oil and palm mesorap oil is not significantly different from each other ($p>0.05$) and is relatively lower than the other oil sample values. Hydrolytic value for coconut oil, bitter gourd, spinach and noni seed oils range from 49% to 53% and are not significantly different from each other ($p>0.05$). Grand mean of hydrolytic value for sample that hydrolysed with 1 ml of added water volume is 47.3%, which is approximately five times of the hydrolytic value (9.3%) for sample that hydrolysed with 0 ml of added water volume. When hydrolysed with 2.5 ml of added water volume, mustard seed oil has the highest hydrolytic value which is significantly different from the other oils hydrolytic values. Hydrolytic value for coconut oil, bitter gourd, spinach and noni seed oils are not significantly different from each other. However, palm mesocarp oil has hydrolytic value that is higher than lady's finger seed oil ($p<0.05$). Thus, when hydrolysed with 2.5 ml of added water volume, lady's finger seed oil has the lowest hydrolytic value when compared with other oils sample.

When hydrolysed with 5 ml of added water volume, hydrolytic values of bitter gourd and mustard seed oil are not significantly different ($p>0.05$) from each other. However, both of these samples hydrolytic values are statistically higher from the other oils hydrolytic values ($p<0.05$). Coconut oil, spinach and noni seed oils have hydrolytic values that are within the range of 52-54% and are not significantly different from each other ($p>0.05$). Among all oil samples, lady's finger seed oil has the lowest hydrolytic value. Due to the low hydrolytic activity of lady's finger seed oil (26-31%), other oils samples hydrolytic activity is reduced to within the range of 26-31% so as to maintain approximately equal hydrolysis extent for all oil samples. Added water volume for obtaining similar degree of hydrolysis in all oil samples is 1 ml because most of the oil samples do not show significant increment of hydrolytic activity with higher added water volume. In the subsequent

Table 2. Degree of hydrolysis (FFA %) with varied incubation period (hours) of noni, spinach, bitter gourd and mustard seed oil and coconut oil

Incubation period (hour)	Value of Free Fatty Acids (%) for different source of oils				
	Noni seed	Spinach seed	Bitter gourd seed	Mustard seed	Copra
1	11.1 ± 1.4^{a1}	13.4 ± 1.0^{a1}	12.4 ± 0.8^{a1}	17.7 ± 0.6^{b1}	10.3 ± 2.4^{a1}
2	20.4 ± 1.0^{a2b2}	23.2 ± 0.8^{b2}	18.7 ± 0.7^{a2}	22.2 ± 1.2^{b2}	18.8 ± 1.6^{a2}
3	25.1 ± 1.9^{a3}	25.4 ± 1.3^{a3}	24.1 ± 1.4^{a3}	25.4 ± 0.8^{b3a3}	23.2 ± 1.1^{a3}
4	28.9 ± 0.8^{a4}	29.7 ± 1.9^{a4}	28.9 ± 0.6^{a4a4}	28.6 ± 1.3^{b4a4}	27.5 ± 1.0^{a4}
5	32.5 ± 1.1^{a5e4}	33.9 ± 0.5^{c4a5}	$32.3\pm2.1^{a5a/a5}$	$35.2\pm1.3^{b5(a5)}$	$31.5\pm0.7^{e4(a5)}$
6	32.4 ± 1.1^{a6e5e4}	33.6 ± 0.9^{a6c5c4}	$34.9\pm1.4^{a6a/}$	34.6 ± 1.1^{b5a6}	31.4 ± 1.0^{e5e4c5}

*All of the standard deviation is calculated by triplicate
*Value of FFA (%) with different superscript is significantly different ($p<0.05$)
*Value of FFA (%) with only one superscript that is same as the superscript the other values have is not significantly different ($p>0.05$) from that values regardless of the presence of other different superscripts
*Value of FFA (%) with same superscript but both in the form of bracket is significantly different ($p<0.05$)
*Value of FFA (%) with same superscript but one in the form of bracket while another not, are not significantly different ($p>0.05$)

hydrolysis process, palm mesocarp oil and lady's finger seed oil are excluded because both of these two oil samples had already obtained hydrolytic value that is within the range required range (26-31%). For other oil samples that had their hydrolytic values out of the required range were adjusted by manipulating the incubation period.

Hydrolytic activity of noni, spinach, bitter gourd and mustard seed oil and coconut oil with varied incubation period is shown in Table 2. Two-way ANOVA test that analysed the effect of different factors (sample type and incubation period) on the response value (FFA%) is carried out and interaction plot of different factors on response value is shown in Figure 2.

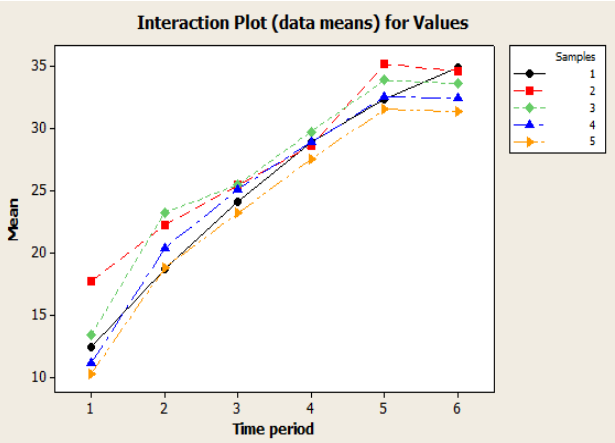

Figure 2. Plot of sample type and incubation period factors interaction on the FFA% values
*Label on the y-axis, "Mean"= Mean values of FFA%; Label on the x-axis, "Time period"= Different incubation period, 1= 1 hour, 2= 2 hours, 3= 3 hours, 4= 4 hours
*Samples, 1= Bitter gourd seed oil, 2= Mustard seed oil, 3= Spinach seed oil, 4= Noni seed oil, 5= Coconut kernel oil

Result from two-way ANOVA test shows significant interaction effect of different factors on the response values ($p<0.05$). Interaction plot that exhibits nonparallel lines in Figure 2 indicates that different oils sample react differently toward varying incubation period. Presence of an interaction effect between two different factors required data reorganization before one-way ANOVA test is carried out to analyse the effect of each respective factors on the response values.

Effect of incubation period factor alone is analysed by collapsing the sample factor into five groups of six levels (1 hour, 2 hours, 3 hours, 4 hours, 5 hours, and 6 hours) while effect of sample type factor is analysed by collapsing the incubation period factor into six groups of five levels. Result from the one-way ANOVA test shows that mustard seed oil has statistically higher hydrolytic value than the other oils ($p<0.05$) at the 1 hour of incubation. When incubation period is elongated to 2 hours, hydrolytic values of the oil samples can be separated into high, middle and low groups. The high group consists of mustard and spinach seed oil, middle group is noni seed oil while low group are bitter gourd seed oil and coconut oil.

When incubation period is extended to 3 hours, all oil samples have hydrolytic values (23-26%) that are not significantly different from each other ($p>0.05$). Hydrolytic activity of oil samples after 4 hours of incubation are in the range of 27-30% and not significantly different from each other. After 5 hours of incubation, hydrolytic value of mustard seed oil is significantly different ($p<0.05$) from the hydrolytic value of coconut oil, while the remaining oil are not significantly different from each other. After 6 hours

of incubation, spinach, bitter gourd and mustard seed oil has hydrolytic value that are not significantly different from each other ($p>0.05$). Their values are different from noni seed oil and coconut oil that are also significantly different each other.

Noni seed oil, spinach seed oil, mustard seed oil and coconut oil have similar hydrolysis pattern as each other. All these mentioned oil samples when being hydrolysed for different duration shows hydrolytic values that are statistically different ($p<0.05$), except between incubation duration of 5 hours and 6 hours. Incubation of 4 hours is deemed as the most suitable duration for the noni, spinach, bitter gourd and mustard seed oil in order to obtain the required hydrolytic value. On the other hand, incubation of 5 hours is deemed as the most suitable duration for coconut oil as hydrolytic value for incubation of 4 hours is slightly lower than the required value.

Bitter gourd seed oil when being hydrolysed for different duration shows hydrolytic values that are statistically different ($p<0.05$), except between incubation duration of 4 hours and 5 hours, and also between 5 hours and 6 hours. Hydrolytic values for incubation of 4 hours and 5 hours are both within the required range (27-31%). However, due to the reason that hydrolytic value for incubation after 5 hours is not significantly different from the hydrolytic value of 6 hours which is out of the range, incubation of 4 hours is deemed as the more suitable duration for bitter gourd seed oil to achieve the required hydrolysis range.

Screening for antibacterial activity

Result of the agar well-diffusion test for the respective oil samples are shown in Table 3. Before enzymatic treatment, all oil samples do not exhibit any inhibition ring zone (IRZ) on any tested bacteria strains (*E. coli*, *S. typhimurium*, and *L. monocytogenes*). After enzymatic treatment, all hydrolyzed oil samples exhibit IRZ on at least one type of bacteria strains except noni seed oil and palm mesocarp oil. Among oil samples, lady's finger and bitter gourd seed oil are the most potential hydrolyzed oil that can be used as antibacterial agent as both of them show positive result on all the tested bacteria strains (*S. typhimurium*, *L. monocytogenes*, and *E. coli*). Hydrolyzed mustard seed oil exhibit IRZ on *S. typhimurium* and *L. monocytogenes*, but not on *E. coli*. Reason for the disappearance of IRZ on *E. coli* may due to its acid adaptation ability which can occur in a slightly higher pH, 5.0 when compared with *S. typhimurium* and *L. monocytogenes* (pH, 4.5) (Leyer *et al*., 1995; Koutsoumanis and Sofos, 2003).

Hydrolyzed coconut oil and spinach seed oil exhibit IRZ on *L. monocytogenes* only. Among the bacteria strains that used in this study, *L. monocytogenes* is the bacterium that most of the oil sample (except noni seed oil and palm mesocarp oil) exhibit IRZ on it because it is also the only Gram-positive bacterium that is known to be more susceptible to the action of polyunsaturated fatty acids (Knapp and Melly, 1987).

Table 3. Antibacterial screening of respective oil sample

Sources of oil	Presence of Inhibition Zone					
	SA (BH)	SA (AH)	LM (BH)	LM (AH)	EC (BH)	EC (AH)
Noni seed	-	-	-	-	-	-
Spinach seed	-	-	-	+	-	-
Lady's finger seed	-	+	-	+	-	+
Bitter gourd seed	-	+	-	+	-	+
Mustard seed	-	+	-	+	-	-
Copra	-	-	-	+	-	-
Palm mesocarp	-	-	-	-	-	-

Antibacterial screening for pure fatty acids that exist in oil sample in high proportion is shown in Table 4. C10:0 at 5% and 10% does not have any inhibition activity and IRZ does not exist on any bacteria strains that are being tested. However, at higher concentration (25%, 60% and 100%) C10:0 shows positive result with IRZ appears on all tested bacteria strains. At 5%, C12:0 only shows IRZ on *S. typhimurium*, and not on *E. coli* and *L. monocytogenes*. However, at higher concentration of C12:0 (10%, 25% and 60%), all tested bacteria strains show IRZ around the wells. Fatty acid of C18:1 and C18:2 are ineffective as antibacterial agent as no IRZ is shown in any of the tested bacteria strains at any level of concentration. Fatty acid of C18:3 at 5% and 10% concentration do not exhibit IRZ on any tested bacteria strains. However, C18:3 at 25% exhibits IRZ on *L. monocytogenes*. According to Knapp and Melly (1987), Gram-positive bacteria such as *L. monocytogenes* is more susceptible to polyunsaturated fatty acid like C18:3 when compared with Gram-negative bacteria (such as *E. coli* and *S. typhimurium* used in this study). At subsequent higher concentration of 60% and 100%, C18:3 show IRZ on both *L. monocytogenes* and *E. coli*. Among the pure fatty acids that used in this study, C10:0 and C12:0 are fatty acids that appear in high proportion in coconut oil and known to have antibacterial property (Skrivanova *et al*., 2008; Sado-Kamdem., 2009). However, antibacterial screening results for fatty acids of C10:0 and C12:0 is different from the coconut oil. This indicates that fatty acids in a mixture can exhibit an inhibition activity that is different from single pure fatty acid. This different inhibition activity may arise from simple interaction effect among different type fatty acids or complex inhibition mechanism

Table 4. Antibacterial screening for pure fatty acids that exist in oil sample in high proportion

Fatty acids	Presence of inhibition ring zone		
	SA	LM	EC
Capric/Decanoic acid (C10:0, 5%)	-	-	-
Capric/Decanoic acid (C10:0, 10%)	-	-	-
Capric/Decanoic acid (C10:0, 25%)	+	+	+
Capric/Decanoic acid (C10:0, 60%)	+	+	+
Capric/Decanoic acid (C10:0, 100%)	+	+	+
Lauric/Dodecanoic acid (C12:0, 5%)	+	-	-
Lauric/Dodecanoic acid (C12:0, 10%)	+	+	+
Lauric/Dodecanoic acid (C12:0, 25%)	+	+	+
Lauric/Dodecanoic acid (C12:0, 60%)	+	+	+
Oleic/Octadecenoic acid (C18:1, 5%)	-	-	-
Oleic/Octadecenoic acid (C18:1, 10%)	-	-	-
Oleic/Octadecenoic acid (C18:1, 25%)	-	-	-
Oleic/Octadecenoic acid (C18:1, 60%)	-	-	-
Oleic/Octadecenoic acid (C18:1,100%)	-	-	-
Linoleic/Octadecadienoic acid (C18:2, 5%)	-	-	-
Linoleic/Octadecadienoic acid (C18:2, 10%)	-	-	-
Linoleic/Octadecadienoic acid (C18:2, 25%)	-	-	-
Linoleic/Octadecadienoic acid (C18:2, 60%)	-	-	-
Linoleic/Octadecadienoic acid (C18:2, 100%)	-	-	-
Linolenic/Octadecatrienoic acid (C18:3, 5%)	-	-	-
Linolenic/Octadecatrienoic acid (C18:3, 10%)	-	-	-
Linolenic/Octadecatrienoic acid (C18:3, 25%)	-	+	-
Linolenic/Octadecatrienoic acid (C18:3, 60%)	-	+	+
Linolenic/Octadecatrienoic acid (C18:3, 100%)	-	+	+

EA stands for *Escherichia coli*
SA stands for *Salmonella typhimurium*
LA stands for *Listeria monocytogenes*
"-" stands for No IRZ detected
"+" stands for IRZ detected
BH stands for before hydrolyzed
AH stands for after hydrolyzed

that involve fatty acids metabolism pathway of bacteria. Nevertheless, without further investigation this different inhibition activity that portray between single fatty acid and mixture fatty acids cannot be explained by any definitive reason. C18:1 and C18:2 are the fatty acids that exist in high proportion in lady's finger, spinach and noni seed oil and are known to have antibacterial effect against some bacteria strains (Dilika *et al.*, 2000). However, both of these fatty acids do not exhibit antibacterial effect against any tested bacteria strains that used in this study. Result of antibacterial screening for noni seed oil is accordance with the result of these two fatty acids (C18:1 and C18:2) but the result of antibacterial screening for lady's finger and spinach seed oil are dissimilar from the result of these two fatty acids. Such contradict scenario cannot be explained without any further investigation. However, there is a great possibility that certain specific fatty acid that does not possess antibacterial property itself may play role as cofactor in the bacteria inhibition pathway and thus explaining why oils with similar dominant fatty acids but different minor fatty acids have different antibacterial activity. Among the oils sample, mustard seed oil is the only sample that is abundant in C18:3. However, the antibacterial screening result of mustard seed oil deviates from C18:3 as mustard seed oil shows IRZ on *S. typhimurium* instead on *E. coli*. Such scenario is most probably due to the different environment pH as pure C18:3 (an organic acid) would create an environment of lower pH than hydrolysed mustard seed oil and *S. typhimurium* is having better resistance at lower pH when compared with *E. coli* and *L. monocytogenes* (Koutsoumanis and Sofos, 2004).

Conclusion

Hydrolytic activity of enzyme RMIM can be used to increase the antibacterial activity of oil. The increment of antibacterial activity of the oil samples are depending both on the oil type and the hydrolytic condition. Among the oil samples, bitter gourd and lady's finger seed oil serve as a potential source that can be turned into antibacterial agent solely through the lipase enzymatic hydrolysis process.

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