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Production of medium chain saturated fatty acids with enhanced antimicrobial activity from crude coconut fat by solid state cultivation of *Yarrowia lipolytica*

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ABSTRACT

Fatty acids profiles and antimicrobial activity of crude coconut fat hydrolysates obtained in solid-state cultivation system with a selected yeast strain *Yarrowia lipolytica* RO13 were performed. A preliminary step regarding extracellular lipase production and solid state enzymatic hydrolysis of crude fat at different water activity and time intervals up to 7 days was also applied. Gas chromatography–mass spectrometry analysis was used for quantification of medium chain saturated fatty acids (MCSFAs) and the results revealed a higher concentration of about 70% lauric acid from total fatty acids. Further, antimicrobial activity of fatty acids against some food-borne pathogens (*Salmonella enteritidis, Escherichia coli, Listeria monocytogenes* and *Bacillus cereus*) was evaluated. The minimum inhibitory concentration of the obtained hydrolysates varied from 12.5 to 1.56 ppm, significantly lower than values reported in literature. The results provide substantial evidence for obtaining biopreservative effects by coconut fat enzymatic hydrolysis.

1. Introduction

Cocos nucifera (family Aracaceae) is mainly used as a staple food crop, and a source of wood and handicrafts, among many other uses, and is thought by many to be the world's most useful medicinal plant in tropical and subtropical countries (Chen & Elevitch, 2006). For thousands of years, coconut oil products used in traditional medicine are believed to be antiblennorrhagic, antibronchitis, antifebrile and antigingivitic (Mandal & Mandal, 2011). Esquenazi et al. (2002) reported that in the traditional medicine of north-eastern Brazil, coconut husks were used for the treatment of diarrhoea and arthritis. Nowadays, coconut fat obtained from the fruit of coconut palm has been relegated mainly to non-food uses in developed countries but retains its importance for traditional uses in producing countries (Chen & Elevitch, 2006).

Coconut oil is very stable as it has a low oxidation point and the oxidation begins after 2 years of storage. This stability is due to the higher content of saturated fat. Moreover, this oil is rich in medium chain fatty acids and exhibits good digestibility (Che Man & Marina, 2006). The three valuable medium chain fatty acids in coconut fat are (C12:0) lauric acid, (C10:0) capric acid and (C8:0) caprylic acid (Obi, Oyi, & Onaolapo, 2010). Production of fatty acids and glycerol from fats and oils is important especially for oleochemical industries. Glycerol and fatty acids are widely used as raw materials in food, cosmetics and pharmaceutical industries (Mun, Rahman, Abd-Aziz, Sabaratnam, & Hassan, 2008). Vegetable fats are very important source of fatty acids which have high antimicrobial activity. The antimicrobial effect of fatty acids against bacteria, fungi, viruses and protozoa are suggested (Isaacs, Litov, & Thormar, 1995; Ogbolu, Oni, Daini, & Oloko, 2007; Ouattara, Simardb, Holley, Piettea, & Begin, 1997), with a similar action as other biopreservatives. Antibacterial potential of the husk of *C. nucifera* has been reported (Esquenazi et al., 2002).

The current techniques for production of fatty acids are based on chemical and physical methods which operate at higher temperature and pressure (Bahruddin, Cheng, & Jaba, 2007). The advantages of the enzymatic hydrolysis technique include the use of bio-route technology that operates at mild temperature, simple operational procedure, low cost as well as energy consumption (Destain, Roblain, & Thonart, 1997). Enzymatic hydrolysis of coconut fat with active microbial lipases and inactivation of harmful microorganisms by resulting fatty acids have been studied (Isaacs & Thormar, 1991). The use of highly active lipase from *Candida rugosa* has been widely studied for hydrolysis of vegetable fat and oil (Ting, Tung, Giridhar, & Wi, 2006). Few reports concerning the lipolytic activity of *Yarrowia lipolytica* are reported in the literature (Ota et al., 1968). In addition, the enzymatic hydrolysis of fats by yeast in solid-state cultivation system has not been extensively studied.

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In the present study, coconut fat hydrolysates were obtained by solid-state fermentation with extracellular lipase biosynthesised by a yeast strain *Y. lipolytica* RO13. These hydrolysates were analysed by gas chromatography–mass spectrometry analysis (GC–MS). The antimicrobial activity of hydrolysates was tested against some pathogenic bacterial strains in order to establish the use of the crude coconut hydrolysates as food preservatives.

2. Materials and methods

2.1. Chemicals and microbial strains

The crude coconut fat obtained by traditional extraction method was purchased from a local market from Cameroun, Africa. Analytical grade chemical reagents (sodium sulphate, sulphuric acid, ethyl ether, heptane, diazomethane and methanolic potassium hydroxide) used for extraction of fatty acids from hydrolysed oil were purchased from Sigma–Aldrich GmbH (Germany). For antimicrobial activity, the solid samples were dissolved in dimethylsulphoxide (DMSO) (Sigma–Aldrich GmbH, Germany). For yeast and bacterial cultivation, Sabouraud (SAB) medium, Spirit blue agar (SBA) (Merck KgaA Darmstad, Germany), and brain heart infusion (BHI) medium (Sigma–Aldrich, Germany) were used.

The yeast strain (*Y. lipolytica* RO13), and bacterial strains (*Listeria monocytogenes* 56 LY, *Bacillus cereus* DSM 10, *Escherichia coli* 555 and *Salmonella enteritidis* 15S) used as test microorganisms for antimicrobial activity testing were collected from the Central Microbial Cultures Collection, Department of Microbiology, Facolta di Scienze degli Alimenti, Cesena, Universita di Bologna, Italy.

2.2. Yeast inoculum preparation

For inoculum preparation, *Y. lipolytica* RO13 strain was first cultivated on SAB agar medium in Petri dishes at 28 °C for 72 h. A small quantity of activated yeast biomass was further transferred in SAB broth and cultivated at 28 °C, for 72 h. Cells were harvested by centrifugation, suspended in sterile distilled water and used immediately. The number of viable cells of inoculum was established by plate count method, except where viable cell numbers were less than 10^3 CFU ml^{-1}, when the MPN (Most Probable Number) technique was used (Olitzkia, 1952).

2.3. Effect of water activity on coconut fat hydrolysis potential of Y. lipolytica

Sterilized and homogenised crude fat was mixed in concentration of 30 ml l^{-1} with SBA medium and several plates of different water activity (0.98, 0.96 and 0.93) were prepared by dissolving 3%, 6% and 10.1% sodium chloride (NaCl), respectively. These SBA plates with homogenised crude coconut fat were spot inoculated with 30 μl yeast culture suspension (10^6 CFU ml^{-1}) and incubated at 25 °C for 216 h (9 days). After incubation, all plates were examined for yeast colony growth diameter and the diameter of fat hydrolysis zone developed around the yeast colonies. The diameters of these zones were measured at every 24 h up to 9 days. Yeast growth diameters were also evaluated in control samples, without added coconut fat in SBA medium, for all water activity levels.

2.4. Solid-state enzymatic hydrolysis of crude coconut fat

SBA medium was prepared with 200 ppm chloramphenicol and 5 g l^{-1} of $(NH_4)_2SO_4$ and after sterilization, 30 g l^{-1} crude coconut fat was added and mixed properly. These SBA plates with homogenised crude coconut fat were inoculated with 100 μl yeast culture suspension (10^6 CFU ml^{-1}) and incubated at 25 °C for 7 days. Immediately after inoculation (T0) and after 3 days (T3) and 7 days (T7) of incubation, the samples were collected for fatty acids determination and antimicrobial activity evaluation of hydrolysates.

2.5. Fatty acids composition of hydrolysed coconut fat

2.5.1. Fatty acids extraction

For fatty acids extraction, 2 g solid medium containing hydrolysed fat was mixed with Na_2SO_4 (6 g), 2.5 M H_2SO_4 (0.6 ml), and 3 ml solution of ethyl ether and heptane (1:1) and agitated it by vortex mixture for 5 min in Falcon tubes (Ukeda, Wagner, Bilitewski, & Schmid, 1992). After the centrifugation at 3000 rpm for 4 min, supernatant was filtered through filter paper coated with 3 g Na_2SO_4. The filtrate was subjected to derivatisation before the GC–MS analysis.

2.5.2. Transesterifications of fatty acids

Diazomethane is the quickest and cleanest method available for the preparation of analytical quantities of methyl esters (Hartman & Lago, 1973). For methyl esterification, fatty acid samples with hexane were dried with liquid nitrogen and 4–5 drops of diazomethane were added. The reaction of diazomethane with a carboxylic acid was quantitative and essentially instantaneous in ether solutions. Methyl esters were prepared by titration of ether solution with diazomethane until the yellow colour persists. After that 1 ml 2 N methanolic KOH and 1 ml hexane were mixed with vortex mixture properly and separated with separating funnels. After separation, the upper phase was collected and stored at −20 °C until GC–MS analysis.

2.5.3. Gas chromatographic–mass spectrometry analysis

The GC–MS analysis conditions, selected on the basis of different experiments, were the following: after extraction and/or bromination, fatty acid methyl ester analysis was performed both in SCAN and SIM mode using an Agilent Technologies gas chromatograph 6890N (Palo Alto, CA, USA), equipped with an Agilent Network Mass Selective detector HP 5973 (Palo Alto, CA, USA) and a capillary column SPB-5; 30 m × 0.25 mm × 0.25 mm (Supelco Inc., Bellefonte, PA, USA). The injector and the detector were both held at 250 °C. The temperature was programmed from 120 °C (held for 5 min) to 215 °C at a rate of 3 °C min^{-1}, then from 215 to 225 °C at a rate of 0.5 °C min^{-1} and the final temperature held for 5 min. The carrier gas was helium with a flow rate of 1 ml min^{-1} and a split ratio of 1:10. Fatty acids were identified by comparing their retention time and mass fragmentation profiles with those of the standards mix FAME 37 (Sigma Aldrich, Milan, Italy). Results were expressed as relative percentage of each fatty acid.

2.6. Antimicrobial activity evaluation

The minimum inhibitory concentration (MIC) was defined as the lowest concentration of hydrolysed coconut fatty acids that completely inhibit the growth of test bacteria in a micro-dilution well plate (Oke, Aslim Belma, Sahlan, & Senol, 2009). The 96-well plates were prepared by dispensing into each well 100 μl of BHI, 100 μl of fatty acids extract (25 ppm in 10% DMSO) and 100 μl of the test bacteria cell suspension. The inoculum of test bacteria was prepared using 24 h growing cultures and suspensions were adjusted to 5.0 McFarland standard turbidity. Since a slight turbidity may be due often to the inoculum itself, the inoculated tube kept in the refrigerator overnight was used as the standard for the determination of complete inhibition. A positive control (containing 100 μl of bacterial suspension and 100 μl of BHI) and negative control (containing 100 μl of fatty acids extract and 100 μl of BHI) were prepared for studying the effect of bacterial culture age

and DMSO on MIC. The contents of the wells were mixed and the microplates were incubated at 37 °C for 24 h.

2.7. Statistical analysis

All the experiments were done in triplicate and the data presented here represents the mean of these replicates. Data related to the zone of inhibition due to fatty acids were subjected to analysis of variance (one way ANOVA) in Duncan multiple range test using SPSS (version 10) statistical software. The differences with $p < 0.05$ were considered significant.

3. Results and discussion

3.1. Evaluation of lipolytic potential of Y. lipolytica RO13 in solid state cultivation system

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are well known biocatalysts for the hydrolysis of water-insoluble fatty-acid esters, triacylglycerol of fatty acids being their natural substrates. Lipids are generally required as inductors for a high lipase production yield.

Lipolytic efficiency of *Y. lipolytica* was evaluated at different water activity levels and time of hydrolysis (Fig. 1). It was observed that at higher water activity of 0.98, the extracellular lipase production by the *Y. lipolytica* was higher as indicated by 6 mm hydrolytic zone on coconut fat within 24 h. In contrast, at lower water activity of 0.93, the coconut fat hydrolysis started after 144 h and hydrolytic zone of 20 mm was observed after 168 h. After 216 h cultivation at 0.98 water activity, the hydrolytic potential of *Y. lipolytica* were 2.36 and 1.6 times higher than the fat hydrolysis at 0.96 and 0.93 water activity, respectively (Fig. 1a). Accordingly, the qualitative analysis of the hydrolytic zone of inhibition also showed that with decreasing water activity, the lipolytic activity decreases (Fig. 1b).

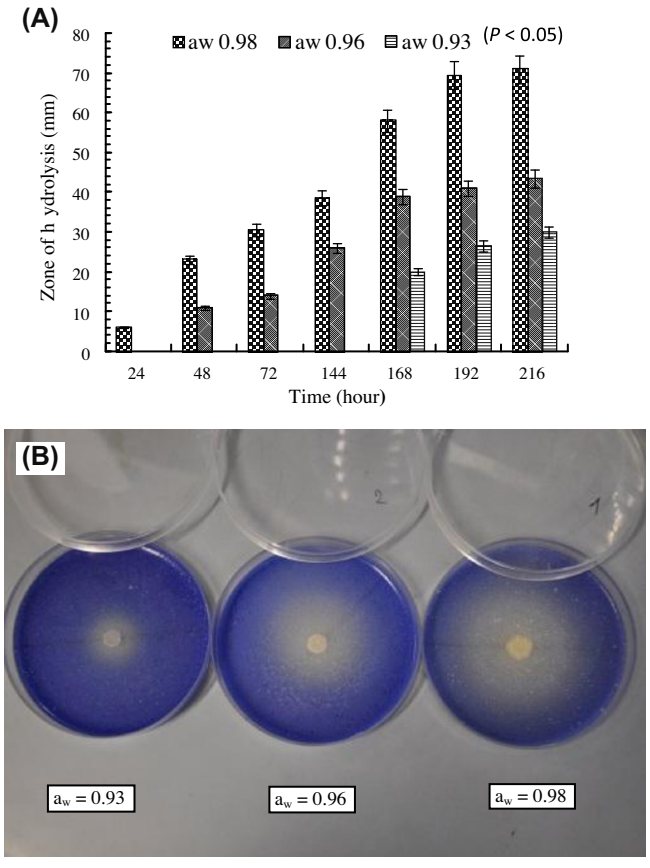

Fig. 1. Effect of water activity (0.98, 0.96 and 0.93) and time duration (24–216 h) upon coconut fat hydrolysis by *Y. lipolytica* RO13 by solid-state cultivation (column height represents the mean of triplicate results and error bar represents the range of the results at significant levels $p < 0.05$) (A); enzymatic hydrolysis of coconut fat by *Y. lipolitica* RO13 during solid-state cultivation at different water activity (lower to higher; left to right) after 168 h (B).

There are several reports dealing with extracellular lipase production by fungus such as *Aspergillus* spp., *Rizhopus* spp., *Penicillium* spp. on different solid substrates (Christen, Angeles, Corzo, Farres, & Revah, 1995; Cordova et al., 1998; Gombert, Pinto, Castilho, & Freire, 1999; Kamini, Mala, & Puvanakrishnan, 1998; Miranda et al., 1999) under submerged conditions. However, several studies have investigated the synthesis of lipase by yeasts using solid-state fermentation technique. Among them, Rao, Jayaraman, and Lakshmanan (1993) determined that the C/N ratio of the medium is an important parameter for lipase production by the yeast *Candida rugosa*. Dominguez, Costas, Longo, and Sanroman (2003) have reported a significant potential for lipase production in solid-state cultures of the yeast *Y. lipolytica*. Furthermore, several factors can affect extracellular lipase production such as pH, temperature, aeration, water activity and medium composition. The presence of triglycerides or fatty acids has been reported to increase lipolytic enzyme secretion by lipase producing microorganisms (Marek & Bednarski, 1996).

It is well known that several factors including water activity, temperature, cell inoculum and some growth parameters (i.e. oxygen pressure, pH, cell morphology) affect lipase biosynthesis and lipolytic activity of *Y. lipolytica*. Moreover, several authors reported the effects of temperature, water activity and inoculation level on the lipolytic and aroma production capabilities of *Y. lipolytica* (Kar, Delvigne, Masson, Destain, & Thonart, 2008; Patrigani, Vannini, Gardini, Guerzoni, & Lanciotti, 2011). Guerzoni et al. (2001), showed that variables such as NaCl, and fat concentration were able to generate differences in the fatty acids release during butter milk hydrolysis by *Y. lipolytica*. Also, Najjar, Robert, Guérin, Violet-Asther, and Carrière (2011) observed that lipase secretion, extracellular lipolysis, and fatty acid uptake varied when *Y. lipolytica* was grown in the presence of olive oil and/or glucose.

Nevertheless, the effect of salinity on the extracellular lipase production has been less explored. This aspect is important in food conservation for combining the classical preservation techniques with biopreservation.

3.2. Fatty acids composition of coconut fat hydrolysates

Fatty acid profile of hydrolysates was analysed by GC–MS. The fatty acids profile of hydrolysed coconut fat samples at different time interval is presented in Fig. 2. It can be seen that MCSFA were found in higher concentrations. From this group, the fatty acid with the highest concentration (70%) was identified as lauric acid (C12:0).

At time 0, there were no significant differences ($p < 0.5$) in the concentrations of medium chain fatty acids. Both caprilic acid (C8:0) and caprinic acid (C10:0) represented 5% of the total fatty acids. Further, lauric acid (C12:0), myristic acid (C14:0) and palmitic acid (C16:0) represented 40%, 22% and 10% of total fatty acids, respectively. After 3 and 7 days of hydrolysis, the amount of medium chain fatty acids increased instead of decreasing of long chain fatty acids. After 7 days, the concentration of lauric acid (C12:0) increased up to 70% while linoleic acid (C18:2) and oleic acid (C18:0) were found in traces. Hence, this study revealed that enzymatic hydrolysis of coconut fat increased the percentage of MCSFA as compare with long chain saturated and unsaturated fatty acids. These results are in agreement with the Codex Alimentarius

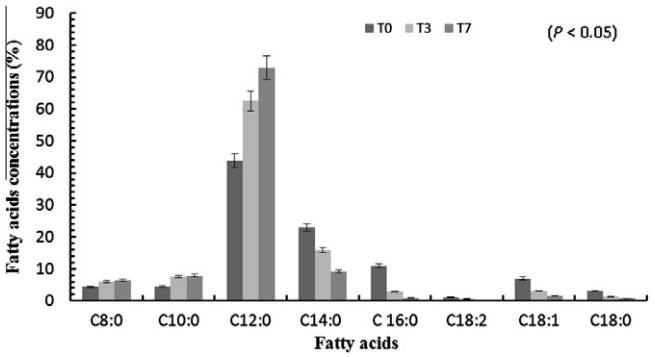

Fig. 2. Fatty acid profile of coconut fat hydrolysates after 3 days (T3) and after 7 days (T7) of enzymatic hydrolysis using *Y. lipolytica* RO13 by solid-state cultivation (column height represents the mean of triplicate results and error bar represents the range of the results at significant levels $p < 0.05$).

Commission International Standard value (Young, 1983). Our results are in good agreement with those reported by Gopala Krishna, Raj, Bhatnagar, Kumar, and Chandrashekar (2010), who suggested a concentration of 62% lauric acid in fatty acids obtained by enzymatic hydrolysis from coconut oil and some other vegetable oils.

3.3. Antimicrobial activity of coconut fat hydrolysates

Fatty acids extract obtained after hydrolysis of coconut fat had a high antimicrobial potential against Gram-positive (*B. cereus* and *L. monocytogenes*) and Gram-negative (*E. coli* and *S. enteritidis*) bacteria. At time T0, the MIC of extract against *E. coli* (25 ppm) was higher than for *B. cereus, L. monocytogenes* and *S. enteritidis* strains (12.5 ppm) (Fig. 3). The fatty acid extract obtained after 3 days of hydrolysis had higher antimicrobial potential against the tested bacterial strains. The MIC of this extract decreased significantly for *L. monocytogenes, S. enteritidis* and *B. cereus* from 12.5 to 3.12 ppm and from 25 to 6.24 ppm for *E. coli*, respectively. Moreover, after 7 days of reaction (T7), the MIC of the extract was significantly lower for all the tested bacterial strains (1.56 ppm). McKellar, Paquet, and Ma (1992) reported that Gram-negative bacteria were resistant to medium and long chain fatty acids. This resistivity had been attributed to the presence of cell wall lipopolysaccharides, which could screen out the fatty acids and accumulation on cell membrane (Russel, 1991). However, in the present study, considerable inhibition was observed for the Gram-negative bacteria, *E. coli* and *S. enteritidis*.

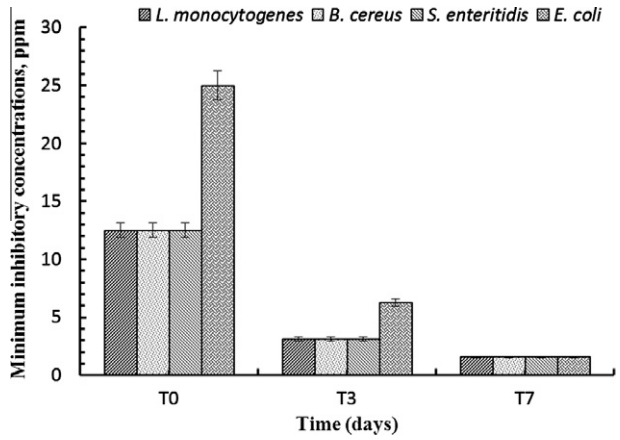

Fig. 3. Antimicrobial potential, expressed as MIC, of coconut fat hydrolysates against some pathogenic bacteria (column height represents the mean of triplicate results and error bar represents the range of the results at significant levels $p < 0.05$).

The antibacterial properties of FFAs are used by many organisms to defend against parasitic or pathogenic bacteria but antibacterial mechanism is still poorly understood. The prime target of FFA action is the cell membranes, where FFAs disrupt the electron transport chain and oxidative phosphorylation. Besides interfering with cellular energy production, FFA action may also result in the inhibition of enzyme activity, impairment of nutrient uptake, generation of peroxidation and auto-oxidation degradation products or direct lysis of bacterial cells (Desbois & Smith, 2010).

This antimicrobial potential of fatty acids is usually attributed to MCSFA including caprinic, capronic, caprilic, and lauric acids, while long-chain saturated fatty acids, including palmitic and stearic acids, are less active. The antibacterial activity of selected fatty acids and essential oils were evaluated against seven meat spoilage bacteria and it was found that *B. thermosphacta, P. fluorescens* and *S. liquefaciens* were not affected at any concentration of fatty acids (Ouattara et al., 1997). Among the fatty acids, lauric and palmitoleic acids exhibited the greatest inhibitory effect (MIC, 250–500 μg ml^{-1}), while myristic, palmitic, stearic and oleic acids were completely ineffective. The antimicrobial activity of fatty acids increased during hydrolysis due to the enhancement in the concentration of medium chain fatty acids. Therefore, high antimicrobial activity observed in the present study can be attributed to the presence of a high concentration of the lauric acid (70%). For saturated fatty acids, hydrophobic groups are known to have greatest influence on antibacterial activity (Branen, Davidson, & Katz, 1980), but hydrophobicity enhancement with long chain length reduces their solubility in aqueous systems. Thus hydrophobic groups could prevent the interaction with hydrophobic proteins or lipids on the bacterial cell membranes (Wang & Johnson, 1992). Lauric acid had been reported to have the best balance between hydrophilic and hydrophobic groups (Branen et al., 1980). Among the MCSFA, lauric acid exhibited the greatest inhibitory effect while other saturated fatty acids with chain length between C14 and C18 were completely ineffective (Babic, Nguyen, Amiot, & Aubert, 1994).

4. Conclusions

In the present study, the hydrolysis of crude coconut fat by *Y. lipolytica* RO13 in solid-state cultivation system, chemical characterisation of hydrolysates and their antimicrobial activity were studied. It was found that extracellular lipase production and fat hydrolysis increased with increasing water activity and time of yeast cultivation in solid state system. The variation in fatty acids profile of the coconut fat hydrolysates after 3 and 7 days was evaluated by the GC–MS analysis. The MCSFA concentration in hydrolysates, especially lauric acid was significantly higher than others. Further, antimicrobial activity of these fatty acids was evaluated against some pathogenic bacteria, an increase in inhibitory activity with increasing concentration of MCSFA being highlighted. Therefore, further studies in food systems are required to confirm the antimicrobial activity of these hydrolysates, which may be used for extension of the shelf life of raw and processed food and also for food safety assurance.

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