

Optimization of pyroligneous acid production from palm kernel shell and its potential antibacterial and antibiofilm activities

Suzami Junaidah Ariffin¹, Maizatulkamal Yahayu¹, Hesham El-Enshasy^{1,2}, Roslinda Abd. Malek¹, Astimar Abd. Aziz³, Najihah Mohd. Hashim⁴ & Zainul Akmar Zakaria^{1,2*}

¹Institute of Bioproduct Development, Universiti Teknologi Malaysia, 81310, Johor Bahru, Johor, Malaysia

²Department of Bioprocess Engineering and Polymer, Faculty of Chemical and Energy Engineering, Universiti Teknologi Malaysia, 81310, Johor Bahru, Johor, Malaysia

³Biomass Technology Unit, Engineering and Processing Division, Malaysian Palm Oil Board, Persiaran Institusi, 43000 Bandar Baru Bangi, Selangor, Malaysia

⁴Department of Pharmacy, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

Received 31 January 2017; revised 17 March 2017

Oil palm plantation generates huge income to Malaysia. At the same time, huge amount of oil palm biomass is also generated which needs to be properly managed. Pyrolysis is one of the approaches available to utilize the oil palm biomass to produce biocharcoal, pyroligneous acid (PA) and bio-oil. PA was produced by condensing the pyrolysis gas emitted and have wide range of potential application including antibacterial activity and antibiofilm activity. In this study, we tried to optimize the operating pyrolysis condition for palm kernel shell to produce maximum yield of PA with highest total phenolic contents (TPC). The optimized PA fraction was evaluated for its antibacterial and antibiofilm properties. Optimum pyrolysis condition resulted in the production of PA containing highest TPC was determined to be at 526°C, heating rate of 10°C/min and nitrogen flow rate of 0.43 L/min. The inhibition zone of concentrated PA extracted by ethyl acetate (CPAE) was determined within 29.3-32.7 mm while minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were in the range of 1.95-3.91 and 62.5-125 mg/mL, respectively. CPAE showed the capability to reduce biofilm formation by *B. cereus*, *S. aureus*, *E. coli* and *P. aeruginosa* up to 80-93% at 64 MIC within 24 h. The biofilm's metabolite activities were also reduced to 77-93% within 24 h. Results of this study suggest that PA produced from palm kernel shell at optimum condition i.e. containing highest total phenolic contents, has good potential to be used for antibacterial and antibiofilm applications.

Keywords: Biofilm, Pyrolysis, Wood vinegar

Palm industry is the main contributor to biomass sources in Malaysia with up to 65 metric tonnes per year produced per hectare of oil palm plantation¹. Typical oil palm biomass includes empty fruit bunch (EFB), palm kernel shell (PKS), palm oil mill effluent (POME), mesocarp fibre (MF), oil palm fronds (OPF) and oil palm trunk (OPT). Traditionally, the oil palm biomass would be disposed in the plantation and left to putrefy to be used as fertilizer. Disposal of EFB into oil palm plantation without recovering remnant oil in the EFB has been reported to contribute to oil spills².

Pyrolysis is one of the best ways to convert biomass into high value products by thermal decomposition of biomass in the absence of oxygen.

Pyrolysis process produced solid (char), liquid (bio oil, tar and pyroligneous acid) and gaseous products (CO, CO₂, H₂, CH₄). Pyroligneous acid (also known as wood vinegar) is an aqueous fraction generated through the condensation of vapour released during pyrolysis process and categorized as byproduct from charcoal production. Pyroligneous acid has been reported to be used as plant enhancer, antioxidant, antibacterial, anti-inflammatory, and antifungus³. These properties can be attributed to the presence of organic compounds such as organic acids and phenol⁴⁻⁶.

Biofilms have negative impact on various industries such as dairy, food and production of hospital equipment, in terms of maintenance cost and production quality. It is difficult to remove the already formed biofilm but it is always possible to reduce or prevent the formation of biofilm. Kainthola *et al.*⁷ have used electrical stimulations to remove biofilms

*Correspondence:

Phone: +60 7 5531655; Fax: +60 7 5569760

E-mail: zainul@ibd.utm.my, Phone:

formed by community associated methicillin resistant *Staphylococcus aureus* (CA-MRSA). Currently, disinfection using chemical solutions such as acids, aldehyde-based biocides, phenolic and surfactants, are used to prevent the formation of biofilm on materials surface. Phenolic compounds present in pyroligneous acid can be used to disinfect biofilm based on its high oxidizing properties. In addition, phenol and phenolic compounds have been claimed as antibacterial as well as antibiofilm agents^{4,5,8,9}. Most of natural compounds in plants have been reported to have an antibiofilm effect, thus the possibility to be used to counter the formation of biofilm⁹. To date, no characterization on the antibiofilm properties of pyroligneous acid has been reported.

Materials and Methods

Approximately, 10 kg of palm kernel shell was collected from the nut cracking waste section of the oil palm processing plant located at Felda Kulai, Johor, Malaysia. Basically, sulphuric acid (H₂SO₄) and deionized water were used in proximate and ultimate analysis. While, ethyl acetate was used as solvent extraction and Follin Ciocalteu reagents, sodium carbonate and gallic acid were used to determine total phenolic contents of pyroligneous acid. Palm kernel shell collected from oil palm processing plant was washed using tap water to eliminate oil debris and sundried until it fully dried. Then, 500 g of palm kernel shell was grounded to coarse particle, approximately 1-5 mm using commercial waring blender (Model 8010s, Waring, USA).

Proximate analysis for the PKS sample was carried out using the American Society for Testing and Materials (ASTM) International methods as follows; moisture contents (ASTM E1756), ash contents (ASTM E1755), volatile matter contents and fixed carbon contents (ASTM D3174). The experiment was carried out in triplicate and results are expressed as means of three experiments. Lignin, hemicellulose and cellulose contents were determined using laboratory analytical procedure (LAP-2008, LAP-001 and LAP-004). The concentrations of hemicellulose and cellulose were determined using the High Performance Liquid Chromatography (HPLC) system (Milford, MA, USA) connected with refractive index indicator. The ultimate analysis was analyzed using Vario Macro Cube (Elementar, Germany). Dumas method was used to estimate the amounts of C, H, N and S contents of PKS.

Pyrolysis

Slow pyrolysis system as used in this study consists of electrical furnace, temperature controller, quartz glass reactor, thermocouple, glass tube connector, condenser and water cooling circulator system (Fig. 1). PKS, 200 g were loaded into quartz glass reactor (length 46 cm, i.d. 12 cm, and 8 cm bore opening diameter) prior to external heating by an electric furnace. Thermocouple was placed inside the glass reactor to monitor the temperature inside the bed using data logger system (PicoLog Recorder software, version 5.23.0). Nitrogen gas connected to the pyrolysis system was allowed to pass through the system. Temperature, heating rate and holding time of pyrolysis process was set using temperature controller, while nitrogen flow rate and condensing temperature were controlled using nitrogen flow rate controller and water cooling circulator system. Liquid and gaseous products of pyrolysis process passed through glass tube connector and condenser before liquid products (pyroligneous acid and bio oil) were collected using round bottom flask (receiving flask) and excess gases was released to the environment through the laminar flow. Biochar remained inside the glass reactor. Upon completion pyrolysis process, liquid and solid products were collected and weighted to determine the percentage of solid, liquid and gases product of pyrolysis process.

Optimization of pyroligneous acid production

The production of pyroligneous acid (PA) from 200g of PKS was optimized by varying the final



Fig. 1 — Pyrolysis diagram for setup of a slow pyrolysis process. [(1) Electric furnace and Quartz glass reactor; (2) Temperature controller; (3) Thermocouple, connected to data logger system; (4) Nitrogen gas; (5) Glass tube connector; (6) Condenser; (7) Receiving flask; (8) Water cooling circulator; and (9) Safety liquid collector]

reactor temperature between 200 and 600°C, heating rate (1-10°C/min) and nitrogen flowrate (0.2-1 L/min) through pyrolysis process. The condenser temperature and holding time were maintained at 5°C and 1 h respectively to ensure complete pyrolysis process and the entire liquid product has been collected. The optimization was carried out using Central Composite Design (Design Expert software Version 6) with 17 runs including replicated of 3 central points, 1 factorial point and 1 axial point respectively with alpha value of 1.2. The responses for the optimization of PA were PA yields and total phenolic contents. Liquid products collected were transferred into falcon tube (50 mL) and stood for two weeks in refrigerator (0°C) to allow good separation between the tarry (bio-oil) and liquid fraction (PA). After that, the percentage of biochar, PA, bio oil and gaseous product of pyrolysis process were calculated as Equation 1.

$$\text{PA yield (\%w/w)} = \text{Pyrolysis product} / \text{Initial PKS load} \quad (\text{Eq. 1})$$

Pyroligneous acid extraction

PA obtained from the pyrolysis process was collected and filtered using Whatman No. 1 filter paper. It was then extracted for phenolic compounds using Ethyl Acetate, EA (99.5% purity, QREC) as follows¹⁰; 25 mL of PA was extracted with 25 mL of EA using a 100 mL separator funnel and let to stand for 10 min for phase separation. The organic layer or top layer (fraction soluble in EA) was collected. The bottom layer was extracted again using EA and concentrated under reduced pressure (120 mBar) at 40°C using rotary evaporator (Heidolph, Germany) until small volume was obtained to give concentrated EA-extracted pyroligneous acid (CPAE)⁴. Then, CPAE was placed inside desiccator for 2 days to remove remaining moisture.

Antibacterial activity

Bacillus subtilis (WICC IBD – b69), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 35218) and *Pseudomonas aeruginosa* (ATCC 27853) were used throughout the study. It was obtained from the culture collection of Institute of Bioproduct Development, Universiti Teknologi Malaysia, Johor. The bacteria were cultivated in incubator at 35°C for 24 h on nutrient agar slants (20 g/L, Merck, Germany) prior to use. Nutrient agar was used for disk diffusion method and nutrient broth was used for broth dilution method. 0.5 McFarland standard solution was used as

bacterial suspension standard. The antibacterial activities of CPAE were determined as follows⁴; several loopful of pathogenic bacterial species cultured on nutrient agar plates (35°C, 24 h) were transferred into universal bottle (28 × 85 mm) containing 5 mL of sterilized distilled water until the turbidity of the bacterial suspension equals to 0.5 McFarland standard solution. Pre-sterilized cotton swab was then used to inoculate the agar plates with the bacterial suspension. Then, 6.0 mm diameter of sterilized commercial disks (Whatman, England) each were impregnated with 20 µL of the CPAE, corresponding to 1000 mg/mL. Chloramphenicol disk (10 µg/disk, Merck, USA) and commercial disks impregnated with methanol were used as positive and negative control, respectively. The CPAE-impregnated disks and negative control were air dried in a laminar flow before aseptically transferred onto surface of the agar plates followed by incubation at 35°C for 24 h. The antibacterial activity of CPAE is determined by measuring the diameter of the inhibition zones formed around the disks. The experiment was carried out in triplicate and results are expressed as means of three experiments. McFarland standard solution was prepared by mixing 0.05 mL of 1.175 % barium chloride dihydrate (BaCl₂•2H₂O) with 9.95 mL of 1 % (v/v) H₂SO₄. Similar sets of experiments were repeated using disk impregnated with methanol, acting as control.

Microbial minimum inhibitory concentration

The microbial minimum inhibitory concentration (MIC) for CPAE was evaluated as follows¹¹; 100 µL of nutrient broth was transferred into each of the 96-wells present on the microtiter plate (Kartell, Italy). Then, 100 µL of CPAE solution was pipetted from the CPAE stock solution (1000 mg of CPAE in 1 mL methanol) and mixed into the well containing NB located in the first column of the plate. After well mixing, 100 µL of the mixture was transferred into the second column to make it two folds dilution. The procedure is repeated until the tenth column. The eleventh and twelfth columns were left for sterility control and blank for the plate scanner. Then, 10 µL of bacterial suspension of *B. cereus* (equivalent to 0.5 McFarland standard solution) was pipetted into each of the wells from column first until eleventh followed by incubation at 35°C for 24 h. The MIC value of CPAE was determined both visually as well as using the Elisa plate reader (Infinite M200 pro, Tecan). The lowest CPAE concentration that retained

its inhibitory effect was recorded as the MIC value (relative to turbidity of mixture in the wells). All of the experimental procedures were repeated for *S. aureus*, *E. coli* and *P. aeruginosa*. The evaluation was carried out in triplicates and the result is expressed as means of three experiments.

Microbial minimum bactericidal concentration

Minimum bactericidal concentration (MBC) is the lowest concentration of CPAE capable of killing the bacterium. MBC is performed immediately after the MIC test. Mixture without visible growth in the wells was inoculated onto nutrient agar using cotton swabs followed by incubation at 35°C for 24 h. The presence of bacterial growth indicates that CPAE was bacteriostatic but not bactericidal at that dilution while the absence of bacterial growth means CPAE is bactericidal at that dilution. The test was performed in triplicate and the result is expressed as means of three experiments.

Antibiofilm activity

Biofilm formation

Biofilm was developed according to method proposed earlier⁸. Sterile 96-wells microtiter plate, each containing 100 µL nutrient broth, was filled with 100 µL of bacterial suspension of either *B. cereus*, *S. aureus*, *E. coli* or *P. aeruginosa* followed by incubation at 35°C for 24 h. Negative control wells only contain nutrient broth without any bacterial suspension. The tendency for biofilm formation by bacteria was determined as follows¹²:

Non-biofilm producer (0) : $OD \leq OD_c$; Weak biofilm producer (+) : $OD_c < OD \leq 2 \times OD_c$; Moderate biofilm producer (++) : $2 \times OD_c < OD \leq 4 \times OD_c$; and Strong biofilm producer (+++) : $4 \times OD_c < OD$.

where OD_c is cut-off of the optical density (OD_c) values and OD is negative control¹³.

Biofilm prevention and control

The biofilm forming prevention and control capability of CPAE was evaluated by mixing 100 µL of CPAE (from stock solution of 500 mg/mL) with 100 µL nutrient broth in the first column of 96-well microtiter plate. Then, the mixture of broth and CPAE was mixed gently using pipette. About 100 µL of the mixture was transferred into column second containing 100 µL nutrient broth to make two fold dilutions. The same procedure was repeated until tenth column. After preparing the mixture of broth and CPAE, 100 µL of bacterial suspension (either *B. cereus*, *S. aureus*, *E. coli* or *P. aeruginosa*) was dispersed into each column of 96-well microtiter

plate. Column eleventh and twelfth was left for negative control and blank which is contained mixture of bacteria suspension and broth without CPAE and only broth, respectively. The plate was covered and incubated aerobically at 35°C for 24 h to produce matured biofilm⁸.

Quantification of biomass in biofilm using crystal violet staining

Mature biofilm was aspirated from each well and washed with physiological saline (0.9%w/v) three times. After that, 200 µL of pure methanol (99.8% purity, QREC) was added into each well and stood for 15 min before decanted from each well using pipette and left to dry under laminar flow. 200 µL of crystal violet reagent (Fluka Analytical, Switzerland) were added into each well and left to stand for 5 min to stain the bacteria followed by washing using tap water. Crystal violet molecules bound to the biofilm formed was dissolved with 200 µL of glacial acetic acid (33%v/v). The absorbance was then measured at 600 nm using a Microplate Reader (Infinite M200 pro, Tecan). All tests were performed in triplicate. The percentage of biofilm removal was calculated according to Equation 2.

$$\% \text{ BR} = [(OD_c - OD_w) / OD_c] \times 100 \quad (\text{Eq. 2})$$

where % BR is the percentage of biomass removal, OD_c is the OD_{600} value of biofilms non-exposed to phenolic acids and OD_w is the OD_{600} value for biofilm exposed to CPAE⁸.

Estimation of metabolism activity for biofilm using resazurin

Mature biofilm was aspirated from each well and washed twice using 200 mL of phosphate buffer saline (pH 7.3). Then, 200 µL of nutrient broth were added into each of the wells followed by 10 µL of resazurin reagent (Invitrogen, US). The mixture was then incubated at 35°C for 90 min prior to measurement at 560 nm and 590 nm for $\lambda_{\text{excretion}}$ and $\lambda_{\text{emission}}$, respectively to determine the metabolite activity of biofilm present in each well. All tests were performed in triplicate. The reduction in metabolism activity of biofilm was estimated using Equation 3.

$$\% \text{ MR} = [(F_c - F_w) - F_c] \times 100 \quad (\text{Eq. 3})$$

where % MR is the percentage of reduction in biofilm's metabolism activity, F_c is the fluorescence absorbance value at 560 nm (F_{560}) for biofilms not exposed to CPAE and F_w is the F_{560} value for biofilm exposed to CPAE^{14,15}.

Morphology biofilm using scanning electron microscopy

The morphology of matured biofilm was recorded using Scanning Electron Microscopy (SEM)

technique. Matured biofilm was removed from the well and washed with 200 μ L of pure methanol (99% purity) for 15 min. After that, methanol was removed from the well and left to dry under laminar flow before dried at 40°C until fully dried. The dried biofilm was then viewed under SEM (Zeiss Supra 35 VP SEM) at the Materials Science Laboratory, Universiti Teknologi Malaysia.

Results and Discussion

The physical and lignocellulosic characteristics of PKS are as follows (in wt.%); Proximate analysis - Moisture contents (7.56 ± 0.08), Volatile matter contents (77.30 ± 0.03), Ash contents (1.20 ± 0.01), Fixed carbon contents (13.94 ± 0.03); Lignocellulosic contents - Lignin (48.09 ± 0.11), Cellulose (30.97 ± 0.17), Hemicellulose (16.68 ± 0.02), Extractive (ethanol, 4.26 ± 0.04); Ultimate analysis - N (0.72), C (47.52), H (6.90), S (0.34) and O (44.52).

Characteristics of pyroligneous acid

In the present study, PA-PKS produced in optimized condition contained 47.24% of water contents that is lower than commercial PA i.e. 64.35% (Commercial PA 1/ MPOB) and 61.50% (Commercial PA 2/ Maju Sakti), respectively. This is because the production of PA was carried out at high temperature (above 500°C) where the water contents and acidic compounds will be decreasing due to high proton concentration^{16,17}. Minimum hemicellulose contents produced small amounts of water contents as dehydration reaction of hemicellulose produced water, char and carbon dioxide¹⁸. The water contents in PA are highly dependent on the amounts of lignocellulosic contents, namely hemicellulosic component. One example is the high water contents for the Bark free heartwood birch (74.7 %) and *C. oleifera* (84 %) plants that has high hemicellulose content^{2,19}. In addition, Rakmai²⁰ reported that bamboo, white popinac, *eucalyptus* and rubber wood PA have 78-88 % of water contents. GC-MS analysis of PA-PKS extracted using ethyl acetate (termed as PAE) showed the presence of 29 major compounds (i.e. >80% similarities) consisting of phenol and its derivatives (71.34%, 18 compounds), ketones (3.84%, 3 compounds), organic acid (11.29%, 2 compounds) as well as alcohol and ester groups. Phenol was the main constituent present in PAE with 23.20% followed by its derivatives as follows; 2,6-dimethoxyphenol i.e. syringol (10.31%), 3-methoxy-1,2-benzenediol i.e. pyrocatechol (6.59%), 2-methoxyphenol i.e. *o*-guaiacol (5.92 %), 2-methoxy-4-methylphenol i.e. creosol (4.54%)

and 4-ethyl-2-methoxy phenol i.e. 4-ethylguaiacol (4.08%). Meanwhile, 4-hydroxybenzoic acid, and n-Hexadecanoic acid are classified under organic acid with concentrations of 10.54 and 0.75%, respectively. 1-(4-hydroxy-3,5-dimethoxyphenyl) ethanone; 2-cyclopenten-1-one, 2-hydroxy-3-methyl-; and 1-(3-hydroxy-4-methoxyphenyl)-ethanone are presents in PAE under ketones group with total concentration of 1.5%.

Optimization of pyroligneous acid

In this study, optimized condition of PA production from PKS was obtained at temperature 526°C, heating rate at 10°C/min and nitrogen flow rate at 0.4 L/min. This finding is comparable with other studies using other types of biomass including oil palm trunks, *Cynara cardunculus* L, oil palm shell, pistachio shell, grape bagasse and stalk of rapeseed plant, as the maximum PA yield was produced at temperature 500-600°C^{21,22}. Highest yield of PA (32.25%w/w) was obtained at high final pyrolysis temperature (600°C) and low heating rate (1°C/min) and low nitrogen flow rate (0.20 L/min). Low final pyrolysis temperature of 200°C, high heating rate (10°C/min) and low nitrogen flow rate (0.20 L/min) resulted in the production of PA with the lowest yield (19.64%w/w). These results clearly indicated that high yield of PA can be produced from PKS using higher final pyrolysis temperature, lower heating rate as well as lower nitrogen flow rate. This is supported from the low PA yield obtained (21%w/w) when pyrolysis was carried out at high temperature with fast heating rate and fast nitrogen flow or low temperature, low heating rate and low nitrogen flow rate (yield of 24.24%w/w). The fact that moderate PA yield were produced when pyrolysis was carried out at medium points for all factors involved, this further strengthens the earlier conclusion that high PA yield can be obtained at high final temperature and low heating rate as well as low nitrogen flow rate. The application of pyrolysis below than 200°C did not produced any PA. This can be explained from the high moisture content for PKS (as deduced earlier using thermogravimetric analysis) where moisture content would be removed between 110 and 200°C. No pyrolysis run was carried out for very low heating rate i.e. 0.1°C/min due to the limitation of the pyrolysis equipment. One interesting observation was that high TPC was not directly proportional to PA yield. For instance, highest PA yield of 32.25%w/w only resulted in 506.56 mg/mL TPC. Phenol and its derivative were produced by degradation of

lignocellulosic content especially lignin and cellulose. Thus, it can be concluded that final pyrolysis temperature, heating rate and nitrogen flow rate have a significant effects toward PA yield and TPC value of the sample. Slow pyrolysis process not only produced pyrolygneous acid but also other products such as biochar, bio-oil and gases.

Antibacterial activity

CPAE was slightly more effective towards foodborne pathogen compared to raw PA with growth inhibition of 44.04% (*B. subtilis*), 31.74% (*S. aureus*), 40.25% (*P. aeruginosa*) and 33.79% for *E. coli*. These values are slightly higher than that obtained using raw PA i.e. 33.23, 30.00, 27.59 and 26.86 % for *B. subtilis*, *S. aureus*, *P. aeruginosa* and *E. coli*, respectively. CPAE showed higher inhibition effect towards *B. subtilis* and *E. coli* compared to *P. aeruginosa* and *S. aureus*. CPAE contains higher concentration percentage of phenolic compounds compared to raw PA. The amounts of organic acids, phenolic compounds and carbonyls in PA has been reported to be responsible for the strong biological activities^{6,17,23-25}. Combinations of organic acid and phenolic result in strong bactericidal inhibition⁴. Phenolic derivatives and compounds such as 4-ethyl-2-methoxyphenol and 4-propyl-2-methoxyphenol have been identified as being primarily responsible for any antimicrobial activity²¹. Phenolic acid with low boiling points have been demonstrated to be good bacteriostatic agents. Disruption of bacterial cell membranes and inhibition of synthesis of cell wall, proteins also nucleic acid caused the inhibition of bacteria growth by an antibacterial agents²⁶. Inhibition by organic acid is caused by the undissociated acid parts (anion moieties) which penetrates the bacterial cell wall into the cytoplasmic region, hence disrupting normal cells physiological function²⁷. For phenolic compounds, inhibition effect has been suggested through the disruption of cell wall followed by protein precipitation²⁸. Acetic acid acetifies the interior of bacterial cell resulting in degeneration and loss of bacterial components while phenol is involved in the inactivation of bacterial enzymes and depletion of metabolites from bacterial cells⁵.

Minimum inhibitory concentration

Both Gram positive and Gram negative bacteria showed identical MIC values i.e. 1.95 and 3.91 mg/mL, respectively. CPAE showed a higher efficiency to inhibit the growth of *E. coli* (MBC of 62.5 mg/mL) compared to the growth of *B. subtilis*, *S. aureus* and

P. aeruginosa (MBC of 125 mg/mL). Gram negative bacteria required higher concentration of CPAE to inhibit the bacteria growth compared to Gram positive bacteria. MBC value is higher than MIC value because higher concentration is required to kill the bacterium instead of inhibiting the bacterial growth. Different MIC and MBC values between Gram negative and Gram positive bacteria can be attributed to the difference in cell wall compositions⁴. Gram-negative bacteria are more resistant to CPAE due to the presence of high lipopolysaccharide (LPS) content in their outer membrane section. LPS has been reported to act as barrier from the penetration of various environmental substances (including antibiotics) into the bacterial cytoplasmic region^{4,29}. Gram positive bacteria do not have the LPS content as well as having the lipoteichoic acid (LTA) component, which is a good target for the bioactive compounds present in the extract⁴. Harada *et al.*⁵ studied the minimum inhibition dilution (MID) of bamboo PA produced at 100-400°C against *E. coli*, *P. aeruginosa* and *S. aureus*. The results indicated that the bamboo PA was more effective to *P. aeruginosa* and *E. coli* compared to *S. aureus* with MID of 140-150, 110-120 and 70-folds, respectively.

Antibiofilm activity

Biofilm formed by *B. cereus* is more susceptible to CPAE based from the increase in the inhibition of biofilm formation from 8.66% (MIC) to 15.64% (4-times the dose of MIC), after 24 h of contact period as shown in Fig. 2. The inhibition of biofilm formation continues to increase with the increase in CPAE concentration as evident from the increase to 92.93% (using 64-times dose of MIC) from 60.77 % (16-time MIC) previously. Similar situation was observed for *S. aureus*, *P. aeruginosa* and *E. coli* where the formation of biofilm was highly reduced from the use of 16MIC onwards. CPAE was determined to be less effective towards the inhibition

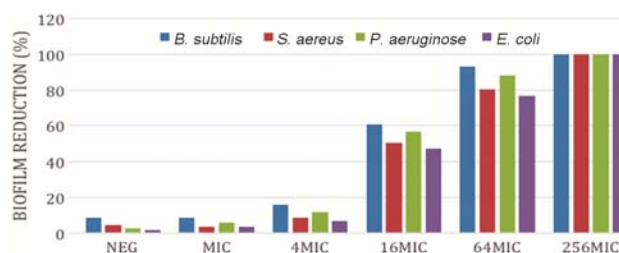


Fig. 2 — Preventive action (24 h aged biofilm formed in the presence of CPAE) on biomass formation of *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa*

of biofilm formation by *E. coli* with 76.77% inhibition even a dose of 64 MIC was used. The percentage of biofilm reduction by *B. subtilis*, *S. aureus*, *P. aeruginosa* and *E.coli* against CPAE for 24 and 48 h is shown in Table 1. High absorbance values indicate more biofilm being developed by bacteria⁷. With the results of the present study (Table 1), *S. aureus* can be considered as a weaker biofilm producer relative to *B. subtilis*, *P. aeruginosa* and *E. coli*. This can be substantiated from its lower absorbance value of 0.94 (without the presence of CPAE) after incubation period of 24 h relative to *B. subtilis* (1.13), *P. aeruginosa* (1.23) and *E. coli* (1.18). *E. coli* is considered as a strong biofilm producer based on the highest amount of biofilm produced within 24 h followed by *P. aeruginosa* and *B. cereus* (both classified as moderate biofilm producer) and *S. aureus* (weak biofilm producer)¹³. Lower percentage of biofilm reduction by CPAE after 48 h compared to 24 h may be due to higher amount of bacterial accumulation after 48 h. The effect of different dosage of CPAE (based on MIC) on biofilm's metabolic activity was also evaluated. *B. subtilis* biofilm is most susceptible to CPAE compared to other bacteria while *E. coli* biofilm is most resistance to CPAE. The biofilm metabolic activity increased gradually from MIC to 4MIC and from 16MIC to 64MIC. This could be due to the increase in total phenolic contents present in CPAE when a more concentrated CPAE was used.

From Table 2 it is clear that without the addition of CPAE i.e. control set, the biofilm metabolic activity at 24 h was decreased when the contact time was prolonged to 48 h. This might be due to reduction of active bacterial cells in the biofilm community from the depletion of nutrient supply. *E. coli* was most susceptible to CPAE with highest reduction in biofilm metabolic activity of 29.92% followed

by *S. aureus* (13.71%), *B. subtilis* (8.64%) and *P. aeruginosa* (8.44%). It was noted that the bacterial metabolite activity reduction was higher than the biofilm reduction.

The micrographs of *B. subtilis*, *S. aureus*, *P. aeruginosa* and *E. coli* cells before and after treated with CPAE as shown in Fig. 3. It can be seen that prior to contact with CPAE, all cells appear in slimy,

Table 1 — Biofilm reduction of various bacteria against 64-MIC of CPAE for 24 and 48 h

Bacterial strain	Time (h)	Absorbance		% Biofilm reduction	% Biofilm deduction
		Control	CPAE		
<i>B. subtilis</i>	24	1.13±0.3	0.08±0.1	92.93	-
<i>S. aureus</i>		0.94±0.6	0.18±0.1	80.28	-
<i>E. coli</i>		1.23±0.2	0.29±0.1	76.77	-
<i>P. aeruginosa</i>		1.18±0.1	0.14±0.1	87.99	-
<i>B. subtilis</i>	48	1.66±0.7	0.20±0.1	87.95	5.36
<i>S. aureus</i>		1.37±0.3	0.35±0.1	74.47	7.24
<i>E. coli</i>		1.94±0.5	0.80±0.1	58.54	23.75
<i>P. aeruginosa</i>		1.70±0.6	0.35±0.1	79.51	9.64

Table 2 — Reduction in biofilm metabolic activity from various bacteria after 24 and 48 hours contact with CPAE

Bacterial Strain	Time (h)	Absorbance		% Reduction	% Deduction
		Control	CPAE		
<i>B. subtilis</i>	24	35212.33	387.34	98.90	-
		±0.2	±0.4		
<i>S. aureus</i>		44531.00	2609.52	94.14	-
		±0.3	±0.2		
<i>E. coli</i>		41450.00	3548.00	91.44	-
		±0.2	±0.3		
<i>P. aeruginosa</i>		14444.33	544.55	96.23	-
		±0.5	±0.2		
<i>B. subtilis</i>	48	30544.00	2974.99	90.26	8.64
		±0.3	±0.3		
<i>S. aureus</i>		11795.33	2213.98	81.23	13.71
		±0.3	±0.1		
<i>E. coli</i>		5512.67	1979.60	64.09	29.91
		±0.4	±0.2		
<i>P. aeruginosa</i>		4602.33	547.22	88.11	8.44
		±0.2	±0.2		

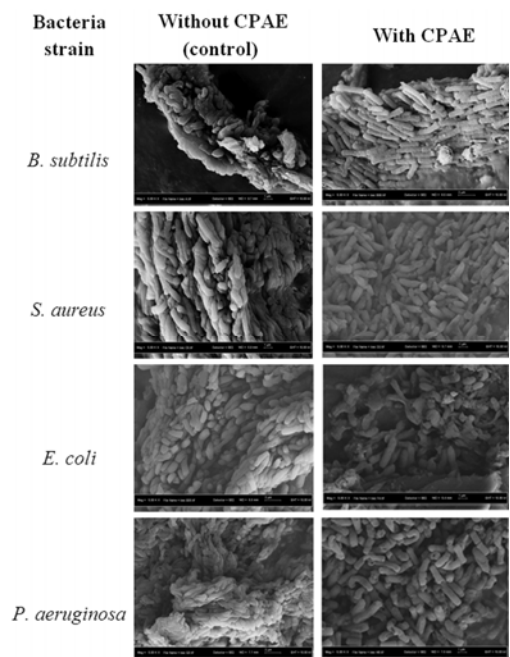


Fig. 3 — SEM micrographs of *B. subtilis*, *S. aureus*, *P. aeruginosa* and *E. coli* before and after treatment with CPAE at 5000x magnification

clumping and aggregated form with possible linkage by the extracellular polymeric substances (EPS). Upon contact with CPAE, the slimy layer seems to disappear with cell structure (rod-shaped for *B. subtilis*, *P. aeruginosa*, *E. coli* and spherical for *S. aureus*). EPS is resistance to antimicrobial agents as it forms a hydrated barrier between cells and their external environment³⁰. Individual bacterial strain produced higher EPS concentration compared to bacteria grown in a non-competitive environment³¹. Phenolic compounds from *Zingiber officinale* were reported to be able to function as quorum sensing inhibitors, thus reducing the formation of biofilm³². EPS production by *P. aeruginosa* was reduced after treated with piper betle extract which is known to have phenolic compounds³³. This result is comparable to the finding in this study. Carvacrol extracted from oregano herbs have also shown ability to reduce EPS production³⁴ where upon contact with carvacrol for 24 h, the EPS production of the bacteria was reduced as evident from the clearer appearance of bacterial cells as a whole unit.

Conclusion

This study demonstrated the potential of pyrolygneous acid produced from palm kernel shell as antibacterial and antibiofilm agent.

Acknowledgement

The authors are grateful to Universiti Teknologi Malaysia for the Research University grant (Q.J130000.2544.03G44) and Yang Di-Pertuan Agong scholarship to Suzami Junaidah binti Ariffin.

References

- Zakaria ZA, Mahmud KN, Yahayu M, Ariffin SJ, Shahrudin AR, Oil Palm Biomass: Management and Pretreatment Technologies for Biofuel and Bioproducts Generation, in Govil JN (ed.), *Env Sc and Eng Vol. 5: Sol Waste Manage*, Studium Press LLC, USA (2017) 156.
- Mathew S & Zakaria ZA, Pyrolygneous acid — the smoky acidic liquid from plant biomass. *Appl Microbiol Biotechnol*, 99 (2015) 611. DOI 101007/s00253-014-6242-1.
- Bilehal D, Li L & Kim YH, Gas Chromatography-Mass Spectrometry Analysis and Chemical Composition of the Bamboo-Carbonized Liquid. *Food Analyt Methods*, 5 (2012) 109–112 ISSN 19369751. Doi:101007/s12161-011-9194-4.
- Darah I, Kassim J, Lim S & Rusli W, Evaluation of antibacterial effects of *Rhizophora apiculata* pyrolygneous acid on pathogenic bacteria. *Malaysian J Microbiol*, 10 (2014) 197.
- Harada K, Iguchi A, Yamada M, Hasegawa K, Nakata T & Hikasa Y, Determination of maximum inhibitory dilutions of bamboo pyrolygneous acid against pathogenic bacteria from companion animals: An *in vitro* study. *J Vet Adv*, 3 (11) (2013) 300.
- Vitt S, Himelbloom B & Crapo C, Pyrolygneous acid. *J Food Saf*, 2 (2001) 111.
- Kainthola A, Uniyal A, Srivastava N & Bhatt AB, Electric stimulations mediated beta lactam resistance reversal and correlation with growth dynamics of community acquired methicillin resistant *Staphylococcus aureus*. *Indian J Exp Biol*, 53 (2015) 530.
- Jagani S, Chelikani R & Kim DS, Effects of phenol and natural phenolic compounds on biofilm formation by *Pseudomonas aeruginosa* *Biofouling*, 25 (2009) 321.
- Siddiqui MF, Sakinah M, Ismail AF, Matsuura T & Zularisam AW, The anti-biofouling effect of Piper betle extract against *Pseudomonas aeruginosa* and bacterial consortium. *Desalination*, 288 (2012) 24.
- Loo A, Jain K & Darah I, Antioxidant and radical scavenging activities of the pyrolygneous acid from a mangrove plant, *Rhizophora apiculata*. *Food Chem*, 104 (2007) 300.
- Wiegand I, Hilpert K & Hancock RE, Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat Protocol*, 3 (2008) 163. doi:101038/nprot2007521.
- Stepanovic S, Vukovic D, Dakic I, Savic B & S'vabic'-Vlahovic M, A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *J Microbiol Meth*, 40 (2000) 175.
- Borges A, Maria JS & Simoes M, The activity of ferulic and gallic acids in biofilm prevention and control of pathogenic bacteria. *Biofouling*, 28 (2012) 755. doi:101080/089270142012706751.
- Crement L, Corvec S, Batard E, Auger M, Lopez I, Pagniez F, Dauvergne S, & Caroff, N, Comparison of three methods to study biofilm formation by clinical strains of *Escherichia coli*. *Diag Microb and Infect Disease* 75 (2013) 252
- Sandberg ME, Schellmann D, Brunhofer G, Erker T, Busygin I & Leino R, Pros and cons of using resazurin staining for quantification of viable *Staphylococcus aureus* biofilms in a screening assay. *J Microbiol Methods* 78 (2009) 104
- Jiang EC, Zhao CX, Qin LY & Chen AH, Experiment of wood vinegar produced from pine nut shell continuous pyrolysis. *Transac of the CSAE* 30(5) (2014) 262
- Zhai M, Shi G, Wang Y, Mao G, Wang D & Wang Z, Chemical composition and biological activities of pyrolygneous acid from walnut shell. *Bioresources* 10 (1) (2015) 1715
- Collard FX, & Blin J, A review on pyrolysis of biomass constituents: mechanisms and composition of the products obtained from the conversion of cellulose, hemicelluloses and lignin. *Renew and Sust Ener Rev*, 38 (2014) 594.
- Hagner M, Penttinen OP, Tiilikkala K & Setälä H, The effects of biochar, wood vinegar and plants on glyphosate leaching and degradation. *Eur J Soil Biol* 58 (2013) 1.
- Rakmai J, Chemical determination, antimicrobial and antioxidant activities of Thai wood vinegars. *MSc Thesis*, Prince of Songkla University, Thailand (2009).
- Lee SH, Hong PSH, Lee AN, Sajap AS, Tey BT & Salmiah U, Production of pyrolygneous acid from lignocellulosic biomass and their effectiveness against bacterial attacks. *J Appl Sci* 10 (2010) 2441.
- Onay O, Influence of pyrolysis temperature and heating rate on the production of bio-oil and char from safflower seed by

- pyrolysis, using a well-swept fixed-bed reactor. *Fuel Proc Tech* 88 (2007) 523.
- 23 Mun SP, & Ku CS, Pyrolysis GC-MS analysis of tars formed during the aging of wood and bamboo crude vinegars. *J Wood Sci*, 56 (2010) 47.
- 24 Sameshima K, Sasaki M, & Sameshima I, Fundamental evaluation on termicidal activity of various vinegar liquids from charcoal making. *Proc of the 4th Internat Wood Sc Symp*, September 2-5, Serpong, Indonesia (2002) 134-138.
- 25 Wei Q, Ma X, & Dong J, Preparation, chemical constituents and antimicrobial activity of pyroligneous acids from walnut tree branches. *J Anal. Appl. Pyrolysis* 87 (2010), 24 .
- 26 Jawetz E, Melnick GE, & Adelberg CA, *Medical Microbiology* 25th edition, McGraw Hill, New York, (2010)
- 27 Theron MM & Lues JF, *Organic acid and food preservation* CRC Press, Boca Raton, USA (2011)
- 28 Panagan AT & Syarif N, Uji kaji hambat asap cair hasil pirolisis kayu pelawan (*Tristania abavata*) terhadap bakteri *Escherichia coli*. *J Penel Sains* 6 (2009) 30-32.
- 29 Narayan SS Antibacterial potential of crude methanolic extract of *Leonotis nepetifolia* (L) R Br. *Inter Res. J. of Pharmacy* 3 (2012) 277- 278.
- 30 Mohamed AR, Hamzah Z, Mohamed Daud MZ & Zakaria Z, The effects of holding time and the sweeping nitrogen gas flowrates on the pyrolysis of EFB using a fixed bed reactor. *Proc Engg*, 53 (2013) 185.
- 31 Subramanian, S, Yan S, Tyagi RD & Surampalli RY Extracellular polymeric substances (EPS) producing bacterial strains of municipal wastewater sludge: isolation, molecular identification, EPS characterization and performance for sludge settling and dewatering. *Water Res*, 44 (2010) 2253.
- 32 Nikolić M, Vasić S, Đurđević J, Stefanović O & Čomić L, Antibacterial and antibiofilm activity of Ginger *Zingiber officinale* (Roscoe) ethanolic extract Kragujevac. *J Sci*, 36 (2014) 129.
- 33 Siddiqui MF, Munaim MSA, Ismail AF, Matsuura T & Wahid ZA, The anti-biofouling effect of *Piper betle* extract against *Pseudomonas aeruginosa* and bacterial consortium. *Desalination*, 288 (2012) 24.
- 34 Neyret C, Herry JM, Meylheuc T & Dubois-Brissonnet F, Plant-derived compounds as natural antimicrobials to control paper mill biofilms. *J Ind Microb. Biotech*, 41 (2014) 87.