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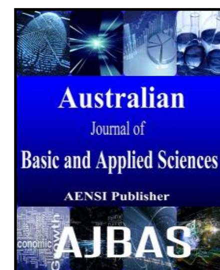

Biotransformation of Benzyl Acetone from *Aquilaria Malaccensis* Using Microorganisms

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ABSTRACT

The oil of *A. malaccensis* was extracted by microwave assisted extraction (MAE) technique. Extraction of agarwood oil from 100 g of sample using MAE in this study has yielded 0.209% (w/w); the extraction was carried out for 5 h at 300 W and the analysis was done using gas chromatography mass spectrometry (GCMS). Benzyl acetone (11.82%), agarospirol (1.28%), δ-guaiene (1.89%), γ-eudesmol (11.89%), guaiol (4.86%), β-guaiene (0.78%), α-guaiene (3.22%), α-humulene (4.67%) and aristolone (1.81%) were among the major constituents extracted in this study. Benzyl acetone was isolated using prep-GC and further verified by GCMS. *Pseudomonas aeruginosa, Escherichia coli, Enterococcus faecalis, Bacillus subtilis, Bacillus cereus*, *Staphylococcus aureus, Saccharomyces cerevisiae, Aspergillus niger, Candida albicans* and *Trichoderma reesei* were tested for biotransformation of benzyl acetone. Among all microorganisms tested, benzyl acetone was successfully reduced to its analog, 4-phenyl-2-butanol, by *S. cerevisiae*, *A. niger*, *P. aeruginosa, E. faecalis* and *B. cereus.* The range of working concentrations for biotransformation of benzyl acetone was determined for each of the selected microorganisms through minimal inhibitory concentration (MIC) test. The growth of *S. cerevisiae* and *A. niger,* were completely inhibited at 0.85 and 0.45 g/L benzyl acetone, respectively; the growth of *P. aeruginosa*, *E. faecalis* and *B. cereus* were completely inhibited at 0.4 g/L benzyl acetone.

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INTRODUCTION

Agarwood or commonly known as Gaharu, has been widely used since the early time by Arab, Chinese, Indian and Southeast Asian community as incense, perfume and medicinal ingredients (Barden *et al*., 2000). Extraction of essential oils from *Aquilaria* sp has traditionally been done using hydrodistillation (HD) method. Increase in market demand of essential oils is one of the chief factors that drive the industrial revolution in a way that development of an alternative technique that is rapid, sensitive, cost-effective and energy-efficient is highly desirable; these are the advantages that MAE has come to offer. There was no significant difference observed between the constituents of essential oils extracted using both methods despite the advantages conferred by MAE over HD (Asghari *et al*., 2012; Wang *et al*., 2010). MAE has been proven powerful as a tool in determining the accurate optimum values of experimental parameters since the interaction between variables is almost possible to evaluate with reduced number of experiments (Maran *et al*., 2013).

Monoterpenes with the structure of 10 carbon atoms are found in most of essential oils (Hunter, 2011). Previous studies have suggested monoterpenoids being one of the most important precursors used in biotransformation especially in the fields of fine chemical such as pharmaceutical, food, and fragrance industries (Arifin *et al*., 2011; King & Dickinson, 2000).

The toxicity test carried out on benzyl acetone, one of the major volatile constituents of agarwood, demonstrated that this compound exhibited pronounced effect against stored product insects (Yang *et al*., 2011). It was also found to be one of the most abundant repellent and attractant in *Nicotiana attenuata* (Kessler & Baldwin, 2006). There was limited study done on 4-phenyl-2-butanol except that it was found to be a common precursor to the synthesis of antihypertensive agents such as labetalol

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and bufeniode and antiepileptic such as emepronium bromide (Liese *et al*., 2006).

This study aimed at extraction of essential oil from *A. malaccensis* using MAE, isolation of benzyl acetone using preparative GC and biotransformation of benzyl acetone using *Pseudomonas aeruginosa, Escherichia coli, Enterococcus faecalis, Bacillus subtilis, Bacillus cereus*, *Staphylococcus aureus, Saccharomyces cerevisiae, Aspergillus niger, Candida albicans* and *Trichoderma reesei*. The range of working concentration for biotransformation would also be determined.

MATERIALS AND METHODS

Chemicals:

n-Hexane (Chromatography grade), Ethanol (Analytical grade), Benzyl acetone (98%), 4-phenyl-2-butanol (98%) and Anhydrous sulphate were purchased from Merck. Potato dextrose agar (PDA), Nutrient agar (NA), Potato dextrose broth (PDB) and Nutrient broth (NB) were purchased from Sigma Aldrich.

MAE:

A sample of 100 g of ground wood was soaked in 1 L of pure water for 1 week at room temperature (RT). The sample was transferred into 2 L round bottom flask and subjected to microwave extraction using Clevenger type-apparatus. Hexane was used to contain the extracted oil and the extraction was carried out at 300 W for 5 hours. The solution was treated with Na_2SO_4 to eliminate the water content and carefully filtered afterwards. The extract was air dried in fume hood to remove hexane. The concentrated oil was weighed to determine the yield of essential oil per 100 g sample. The oil was stored at 4°C until further use.

Isolation of benzyl acetone using preparative GC:

Isolation of Benzyl acetone from the essential oil of *A. malaccensis* was carried out using prep-GC (GERSTEL, USA) equipped with flame ionization detector (FID) and DB5 column (15 m x 0.32 mm x 1µm film thickness). This system is endowed with three channels electronic pressure control (EPC) module which purpose was to provide sufficient pressure to the Deans switch (DS). The peak of interest eluted from the separation column outlet was isolated by micro-fluidic DS, which also works to direct the flow to FID for chromatogram development and to the trapping capillary. The purity of the isolated peak and its identification was confirmed and verified against a standard solution using GCMS.

Microorganisms and culture conditions:

P. aeruginosa, E. coli, E. faecalis, B. subtilis, B. cereus, *S. aureus, S. cerevisiae, A. niger, C. albicans* and *T. reesei,* were obtained from Culture Collection of Microbiology Laboratory, Universiti Malaysia Pahang. *S. cerevisiae* was propagated on PDA at 30°C while *A. niger, C. albicans* and *T. reesei* at 27°C respectively and transferred every two weeks; *P. aeruginosa, E. coli, E. faecalis, B. subtilis, B. cereus*, *S. aureus* were propagated on NA at 37°C and transferred monthly. Agar slants were stored at 4°C for short-term storage. Stock culture of *P. aeruginosa, E. coli, E. faecalis, B. subtilis, B. cereus*, *S. aureus* and *S. cerevisiae* were maintained in 25% glycerol at -20°C; spore suspension of *A. niger, C. albicans* and *T. reesei* was stored in the same manner. Inoculum of *S. cerevisiae* were prepared by growing the culture for 48 h at 30°C in 50 ml of fresh PDB; inocula of *P. aeruginosa, E. coli, E. faecalis, B. subtilis, B. cereus* and *S. aureus* were prepared by growing the culture for 24 h at 37°C in 50 ml of fresh NB; inocula of *A. niger, C. albicans* and *T. reesei* was prepared by growing the culture for 72 h at 27°C on fresh PDA and a spore suspension of 1 x 10^8 spores/ml was prepared.

Screening for biotransformation:

The screening was conducted in 7 ml vial containing a total of 5 ml working volume of NB (*P. aeruginosa, E. coli, E. faecalis, B. subtilis, B. cereus*, *S. aureus*) and PDB (*S. cerevisiae, A. niger, C. albicans* and *T. reesei*). The experiment was carried in incubator shaker at 37°C (*P. aeruginosa, E. coli, E. faecalis, B. subtilis, B. cereus*, *S. aureus*), 30°C (*S. cerevisiae*) and 27°C, 150 rpm. The reaction using bacteria was left for 4 days while the reaction using yeast and fungi were left for 7 days.

Extraction of biotransformation product:

One vial of was taken out every day and the cells were removed by centrifugation (6000 *g*, 20 min). The supernatant was extracted with n-hexane (3 x 2.5 ml). The sample was treated with anhydrous sulphate and filtered using Whatman No. 1 filter paper. The filtrate was air-dried to remove solvent. The extract was further subjected to GCMS analysis.

Minimal inhibitory concentration (MIC):

The concentration Benzyl acetone used was (g/L): 0.05, 0.1, 0.2, 0.4, 0.8, and 1.6. The bacterial culture was subjected to incubation at 37°C for 1 day while the fungal culture was subjected to incubation at 30°C for 4 days. The control for each experiment was conducted using a broth media in the absence of microorganisms

Analysis using GCMS:

Analysis of the compound was carried out using Gas Chromatograph equipped with mass detector (GCMS) (Agilent, USA) and fused silica capillary column (60 mm x 0.25 mm, film thickness 0.25 µm). The system ran on the injection volume of 0.5 µl with split ratio 1:60 and helium as a carrier gas at 1.0 ml/min constant flow mode. The column temperature

was programmed from 60 to 120°C at 3°C/min, with injector temperature set at 230°C. The mass detector operated in electron impact (EI+) mode at 70 eV, with detector temperature 250°C. The mass spectra recorded in the range of 50-500 a.m.u. Concentration of biotransformation product was calculated based on the calibration curve generated using internal standard method.

RESULTS AND DISCUSSION

Yield of essential oil:

The extraction of essential oil of *A. malaccensis* in 100 g sample at 300 W of microwave power for 5 h yielded 0.209% (w/w) compared to a study by Yoswathana (2012), who reported that HD yielded only 0.075 % (w/w) essential oil after 5 days of extraction period with high energy consumption. It was apparent that extraction time and energy consumption was greatly reduced by MAE compared to HD and thus, the former allowed a cost-effective extraction process.

Woody plant including *A. malaccensis* possesses complex cellular structure and arrangement which includes primary cell walls- embedding the growing and dividing cells as well as providing mechanical strength to the plant- and layers of secondary cell walls- encapsulating the mature cells and account for most of the carbohydrate in biomass (Keegstra *et al.,* 1973); extraction of essential oil from *A. malaccensis* is therefore achieved only by disrupting these cell walls which are typically protected by incorporated lignin. Yield of extraction is thus greatly affected by the level of cell walls disruption ensued during the extraction process.

MAE operated under the influence of electric and magnetic field that worked perpendicular to each other. While heat conduction and convection in traditional HD could not afford a complete extraction of essential oil (Golmakani and Rezaei, 2008), the use of MAE on the other hand, granted a localized superheating by dipolar rotation and ionic conduction which effectively heated the entire sample simultaneously (Kauffman and Christen, 2002). The mode of heat transfer in MAE allowed a complete disruption of the plant cells which eventually resulted in the release of the essential oil from the oil glands; the same result could not be produced using HD since the disruption of the plant cells were usually incomplete due to the limitation in transferring the heat to the whole sample.

Composition of essential oil:

The essential oil extracted from *A. malaccensis* by MAE contained benzyl acetone (11.82%), agarospirol (1.28%), δ-guaiene (1.89%), γ-eudesmol (11.89%), guaiol (4.86%), β-guaiene (0.78%), α-guaiene (3.22%), α-humulene (4.67%) and aristolone (1.81%) as its major constituents (Figure 4.1). The result concurred with the previous analyses on the chemical composition of essential oil extracted from *Aquilaria* sp. (Tajuddin and Yusoff, 2010; Bhuiyan *et al*., 2009; Nor Azah *et al*., 2008). The fact that HD was employed in these precedent studies is something worth a note since it is a solid proof that the quality of essential oil extracted by MAE was not compromised despite advantages offered by MAE over HD. According to the GCMS analysis, a total of 26 compounds were detected in the essential oil extracted from *A. malaccensis* using MAE.

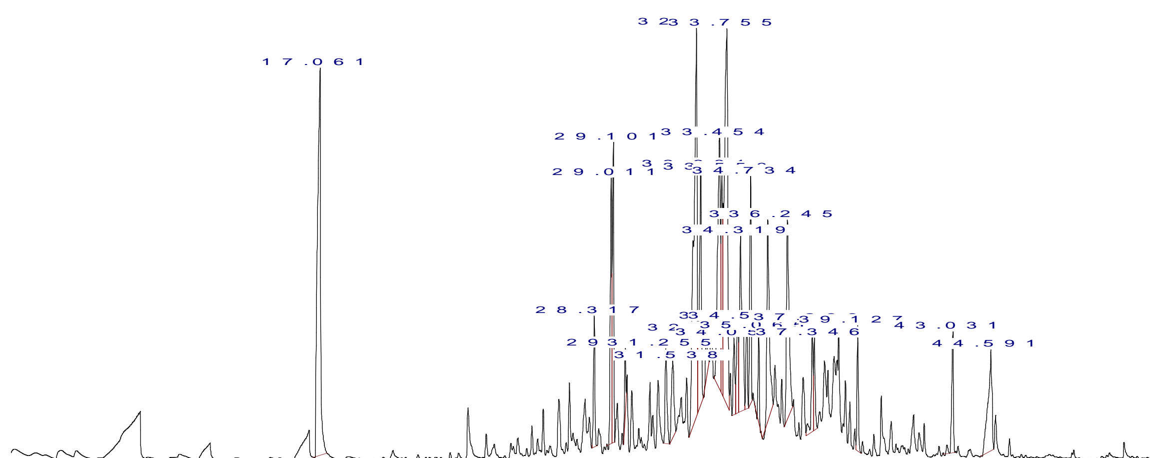

Fig. 1: Chromatogram of essential oil of *A. malaccensis* produced by GCMS.

Isolation of benzyl acetone using preparative GC:

The fraction collected from prep-GC was subjected to analysis using GCMS by which a single peak was produced, as shown figure 2a with a mass spectra illustrated in figure 3a. The peak was identified as benzyl acetone, in reference to the retention time and mass spectra of an external chemical standard, ran on the GCMS within the same parameter Figure 2b and 3b. The result has proven that application of prep-GC may allow a direct transfer of retention times given that both prep-GC and analytical GC uses the same type of column and GC condition. The molecular ion (*m/z* 148) and base peak (*m/z* 105) of this compound was consistent with

a $C1_{10}$ compound with a phenyl side loss. The result was also in consonance with a previous isolation of benzyl acetone carried out by Qi *et al*. (1998). The good quality of mass spectrum obtained in this study was most probably attributable to the good resolution of single compound produced by prep-GC. Previous studies reported on high rate of recovery permitted by prep-GC with a value more than 80% (Ball *et al*., 2012; Yang *et al*., 2011). Sutton *et al*. (2005) was of the opinion that a good quality mass spectrum could be produced with an adequate amount of target compound. Considering that benzyl acetone is a highly volatile compound, a good quality mass spectrum obtained in this work demonstrated that a considerable yield of resolved benzyl acetone was successfully isolated using prep-GC.

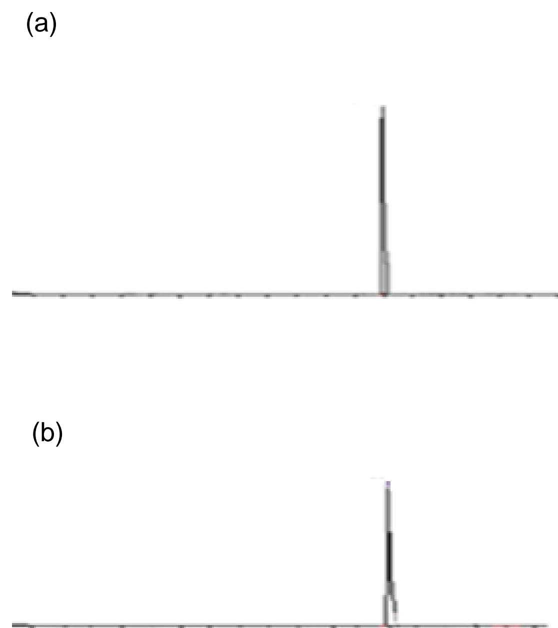

Fig. 2: Chromatogram of (a) benzyl acetone isolated from *A. malaccensis* and (b) standard peak of benzyl acetone.

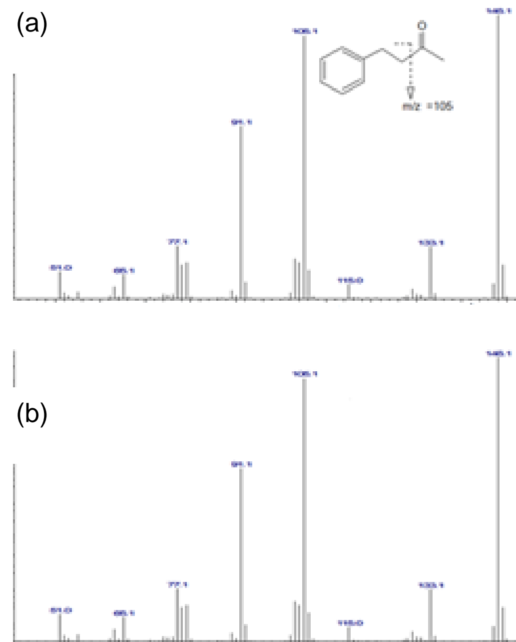

Fig. 3: Mass spectra of (a) benzyl acetone isolated from *A. malaccensis* and (b) standard peak of benzyl acetone.

Screening for biotransformation:

Based on the result obtained, *S. cerevisiae*, *A. Niger*, *P. Aeruginosa*, *E. Faecalis* and *B. cereus* have reduced benzyl acetone into 4-phenyl-2-butanol, a reaction which normally attributable to the activity of reductases. Faber (2011) demonstrated the possibility that the orientation and fit of the molecules in the catalytic site of the monooxygenases, hydrogenases and reductases correspond to the positions of methyl and isopropyl groups relative to carbonyls group; this argument might explain the same reaction observed in biotransformation of benzyl acetone using *S. cerevisiae*, *A. niger*, *P. Aeruginosa, E. Faecalis* and *B. Cereus. S. cerevisiae* exhibited relatively high tolerance towards the toxicity of benzyl acetone compared to other microorganisms tested. The growth of *S. Cerevisiae* was completely inhibited at 1.0 g/L benzyl acetone while that of *A. niger* at 0.8 g/L and *P. aeruginosa*, *E. faecalis* and *B. cereus* at 0.6 g/L.

Conclusion:

Extraction of essential oil using MAE was proven superior to HD since the former granted a cost-effective extraction without compromising the quality of the extract. Isolation of benzyl acetone, a highly volatile component was successfully accomplished using preparative GC. Biotransformation of benzyl acetone has been successfully carried out using *S. cerevisiae*, *A. niger*, *P. aeruginosa*, *E. faecalis* and *B. cereus*, forming 4-phenyl-2-butanol. Based on viability, biotransformation of benzyl acetone using *S. cerevisiae* and *A. niger* is possible at a substrate concentration less than 1.0 and 0.8 g/L, respectively while that of *P. aeruginosa*, *E. faecalis* and *B. cereus* at a substrate concentration less than 0.6 g/L.

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