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FULL FACTORIAL DESIGN FOR PRODUCTION OF METHYL 3-(3,5-DI-TERT-BUTYL-4-HYDROXYPHENYL) PROPIONATE USING OIL PALM FROND JUICE AS A SOLE SUBSTRATE BY *Ceratocystis fimbriata*

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ABSTRACT

Oil palm frond (OPF) juice has been known as a good source of sugars to replace commercial sugars to produce methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate. The aim of this study was to investigate the effect of temperature (°C), initial pH medium, agitation speed (rpm) and concentration of glucose (g/L) on the production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate from OPF juice by using *Ceratocystis fimbriata (C. fimbriata).* The design of experiment (DOE) method was used to screen the best parameters affecting the production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate. The Gas Chromatography-Mass Spectroscopy (GC-MS) with Solid Phase Micro Extraction (SPME) was used to analyze and separate the peak of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate. Based on the GC-MS analysis, results showed that the most favorable condition for methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production was achieved at an initial pH medium (8), agitation speed (100 rpm), temperature (25°C) and 30 g/L of glucose in OPF juice. Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate were produced when the retention time was 32.80 min, the relative peak area was 0.24 % of chromatogram area. This result showed the great potential usage of OPF juice as a substrate to produce methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate by *C. fimbriata.*

Key words: Oil palm frond juice, Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate, *Ceratocystis fimbriata*

INTRODUCTION

Oil palm frond (OPF) juice has been elucidated as a good source of sugar to replace commercial sugar as it contained renewable sugars such as glucose, sucrose and fructose (Zahari *et al.*, 2012). OPF juice is expected to reduce the environmental issues to produce ester since OPF is abundantly available as a biomass from palm oil industry and easily obtainable around Malaysia. Microorganism plays an important role in the production of natural compounds, especially in the field of food aromas. Filamentous fungi are interesting because they can produce flavouring compounds and flavor related enzymes (Bigelis, 1992). *C. fimbriata* has the potential to synthesize esters, it grows quickly and produces a variety of aromas (peach, pineapple, banana, citrus and rose), and depending on the strain and culture conditions (Medeiros *et al.*, 2003). Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate is a key starting material for the preparation of many other antioxidants through transesterification reactions with other alcohols (Fung *et al.*, 2014; Gatto *et al.*, 2011). In this study, the design of experiments (DOE) methodology was used as it is a powerful technique for discovering a set of process variables which are most important to the process and then to determine at what levels these factors must be kept optimizing the process performance. From previous study, several types of

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volatile compounds such as aldehydes, alcohols, ketones, and esters can be produced from OPF juice supplemented with a mineral salt medium.

Therefore, in this study the ability of *C. fimbriata* to produce methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate by using OPF juice as a sole carbon source was investigated in shake flask experiment. The experimental work was carried out using a 2^4 full factorial design to examine the main effects and the interactions between the agitation speed, pH of the initial medium, temperature and also the concentration of glucose. The head space-solid phase micro extraction (HS-SPME) technique combined with gas chromatography-mass spectroscopy (GC–MS) was used to analyze methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate produced during the fermentation. So far, researches on the production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate by *C. fimbriata* from OPF juice as substrates has not been reported yet. Therefore, it is hoped that this research will contribute to further investigation on the production of produce methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate utilizing this *C. fimbriata* at the commercial scale.

MATERIALS AND METHODS

Microorganism and culture medium

Fungal strain *C. fimbriata* (ATCC 12866) was obtained from a freeze-dried microorganism purchased from American Type Culture Collection (ATCC), USA. The fungal was grown and transferred periodically onto Potato Dextrose Agar (PDA). The seed culture was prepared after 7 days of growth at 30°C. After 7 days, the spores were collected from the surface of the plate by adding distilled water containing a few drops of Tween 80 and some glass beads. The basal growth medium consisting of glucose, 20 g/L; urea, 0.75 g/L; ammonium sulfate ($(NH_4)_2SO_4$) 2.25 g/L; monopotassium phosphate (KH_2PO_4) 1 g/L; calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$) 0.5 g/L; magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) 0.5 g/L; trace element solution, 2 ml/L. The trace element solution contained: Iron(III) nitrate nonahydrate ($Fe(NO_3)_3.9H_2O$) 723.8 mg/L; zinc sulfate heptahydrate ($ZnSO_4.7H_2O$) 439.8 mg/L; manganese(II) sulfate tetrahydrate ($MnSO_4.4H_2O$) 203 mg/L. The medium was supplemented with chloramphenicol 0.5 g/L to inhibit bacterial contamination and the pH was adjusted to 6.0 with sodium hydroxide (NaOH) 0.5 N before autoclaved at 121°C for 20 minutes (Christen & Raimbault, 1991). For the seed culture preparation, 1 mL of spores were taken aseptically and transferred into 50 mL centrifuge tube containing 9 mL of basal growth medium. The seed cultures were then incubated at 27°C for 9 days on a rotary shaker at (150 rpm).

Substrate preparation

The oil palm frond (OPF) juice used in this research were obtained from Lembaga Kemajuaan Pertubuhan Peladang (LKPP) Oil Palm Plantation, Lepar Hilir, Gambang Pahang, Malaysia. The OPF juice was extracted by pressing the fresh frond using a conventional sugarcane press machine. The OPF juice was centrifuged at 10,000 rpm for 10 min at 4°C. The sugars content in the OPF juice were determined by using High Performance Liquid Chromatography (HPLC) (Agilent Series 1200, USA) using the Supercoil LH-NH_2 column (Sigma Aldrich) (25 cm x 4.6 mm ID, 5 µm particles) with RI detector operated at 30°C. The mobile phase comprised of acetonitrile: water (75%: 25%) at a flow rate of 1.0 mL/min (Zahari *et al.*, 2012).

Fermentation procedure

The experiment was done by using OPF juice containing 20 g/L and 30 g/L of glucose using 250 mL flasks and mixed with a mineral salt medium. Both medium had the following composition: urea, 0.75 g/L; $(NH_4)_2SO_4$, 2.25 g/L; KH_2PO_4, 1 g/L; $Ca(NO_3)_2.4H_2O$, 0.5 g/L; $MgSO_4.7H_2O$, 0.5 g/L; trace element solution, 2 ml/L; chloramphenicol 0.5 g/L and the pH was adjusted to pH 4 and pH 8 with NaOH 0.5 M and hydrochloric acid (HCL) 1M before autoclaved. The flasks were covered with cotton and sterilized at 121°C for 20 minutes (Christen *et al.*, 1997). One mycelium cell from seed cultures was taken aseptically and introduced into a 250 mL Erlenmeyer flask containing 100 mL of the synthetic medium. The fermentation was carried out at 25°C and 35°C on a rotary shaker for 100 rpm and 150 rpm for 4 days.

Design of experiments

A total of sixteen (16) experiments were done according to a 2^4 full factorial design. Table 1 presents the variable factors with the coded and actual values for each set of parameters for the experiment. The experimental design and analysis of data were done using Design Expert version 7.1 (State-Ease, Inc., Minneapolis, MN). All experiments were done in triplicates and the results were recorded as mean values of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production.

Analytical procedure

The volatile compounds were detected by gas chromatography-mass spectroscopy solid phase micro extraction (GC-MS SPME). In this study, fiber DVB-CAR-PDMS (50/30 µm), which have been frequently employed for the extraction of the volatile fraction from natural products, was tested

to analyse the present of volatile compounds. The coating was 1 cm long for the fiber. Before GC–MS analysis, the fiber was conditioned in the injector of the GC system, according to the instructions provided by the manufacturer. 1 mL amount of sample was placed in a 4 mL at-bottom headspace vial sealed with screw cap with PTFE/silicone septum (Agilent). The sample was heated for 45 min on a hot plate at 60°C. The SPME device was then inserted into the sealed vial by manually penetrating the septum and the fiber was exposed to the headspace for 45 min during the extraction time. After sampling, the SPME fiber was immediately inserted into the GC injector and thermally desorbed. A desorption time of 1 min at 230°C was used in the splitless mode. Before sampling, the fiber was reconditioned for 5 min in the GC injector port at 230°C (Pellati *et al.*, 2013).

RESULTS AND DISCUSSION

To study the variables that defined the experimental process, full factorial (2^4) experimentations were carried out, in two levels, which is at high level and low level. The experimental effects were designed in accordance to Table 1 which shows the values of the factors selected in this study. This factorial design has resulted in sixteen tests with all possible combination of X_1, X_2, X_3 and X_4. The production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate (Y) was measured for each of these tests as shown in Table 2. A first-order model with all possible interactions was chosen to fit the experiment:

$$Y = X_0 + X_1 - X_2 - X_3 - X_4 - X_1X_3 + X_2X_4 + X_3X_4 \quad (1)$$

The production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate by *C. fimbriata* from OPF juice were done according to the parameters (factors) as shown in Table 2. With reference to Table 2, 16 fermentation runs, were carried out with different levels of initial pH medium, temperature, agitation speed and total glucose in OPF. As observed, run 10 and 2 showed the highest methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production with the value of 0.24% and 0.21% respectively. Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production at 0.24%

Table 1. Factors and levels used in the 2^4 factorial design study

Parameter name	Code	Units	Low	High
pH	X_1	–	4	8
Temperature	X_2	°C	25	35
Agitation speed	X_3	rpm	100	150
Glucose concentration	X_4	g/L	20	30

Table 2. Experimental result for production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate

Run	Factor 1: pH	Factor 2: Temperature °C	Factor 3: Agitation speed (rpm)	Factor 4: Glucose concentration (g/L)	Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production (%)
1	4	25	100	20	0.19
2	8	25	100	20	0.21
3	4	35	100	20	0.07
4	8	35	100	20	0.14
5	4	25	150	20	0.05
6	8	25	150	20	0.07
7	4	35	150	20	0.03
8	8	35	150	20	0.04
9	4	25	100	30	0.11
10	8	25	100	30	0.24
11	4	35	100	30	0.15
12	8	35	100	30	0.19
13	4	25	150	30	0.10
14	8	25	150	30	0.13
15	4	35	150	30	0.08
16	8	35	150	30	0.09

Table 3. Analysis of variance (ANOVA) analysis for 2^4 full factorial design (FFD)

Source	Sum of square	Df	Mean square	F value	p-value prob > F	
Model	0.055	7	7.813E-003	9.84	0.0022	Significant
X_1-pH	6.806E-003	1	6.806E-003	8.57	0.0190	
X_2-Temperature	6.006E-003	1	6.006E-003	7.57	0.0250	
X_3-agitation speed	0.032	1	0.032	39.69	0.0002	
X_4-glucose in OPF	5.256E-003	1	5.256E-003	6.62	0.0330	
X_1X_3	2.256E-003	1	2.256E-003	2.84	0.1303	
X_2X_4	1.806E-003	1	1.806E-003	2.28	0.1699	
X_3X_4	1.056E-003	1	1.056E-003	1.33	0.2820	
Residual	6.350E-003	8	7.938E-004			
Cor total	0.061	15				

was done at a condition where the pH value of was 8, temperature at 25°C, total glucose in OPF at 30 g/L and the agitation speed of 100 rpm. Meanwhile for methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production at 0.21%, it was done at the condition where the pH value was 8, temperature at 25°C, total glucose in OPF at 20 g/L and agitation speed of 100 rpm. The lowest methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate was at run 7 where it comprised of higher agitation speed of 150 rpm, higher temperature at 35°C, and the acidity of the pH medium was at pH 4. Thus, it can be said that *C. fimbriata* cannot grow and tolerate at higher agitation speed, higher temperature and high acidic of medium.

The final equation, after putting values of all coefficients, is as follow:

$$Y = 0.64750 + 0.04X_1 - 0.0145X_2 - 1.97500E\text{-}003X_3 - 0.017250X_4 - 2.37500E\text{-}004X_1X_3 + 4.25000E\text{-}004X_2X_4 + 6.50000E\text{-}005X_3X_4 \quad (2)$$

Where X_1 was the pH, X_2 was the temperature, X_3 was the agitation speed, X_4 was the total glucose in OPF, respectively. X_1, X_2, X_3 and X_4 were the main effect while X_1X_3, X_2X_4 and X_3X_4 were the interaction effect.

Table 3 showed the statistical significance calculated using ANOVA. From Table 3, the F value of 9.84 with a probability value (Prob>F) of 0.0022 suggests that the model was significant and fitted well to the experimental data ($P<0.05$) hence the model was valid for further studies. The statistically significant variables at 95% level of confidence were: R squared = 0.8960, where R squared is the correlation coefficient. Equation (2) shows the effect of individual variables and interactional effects for methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate.

With reference to Table 4, it was shown that X_3 (Agitation speed) contributes the most to the production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate as much as 51.61%. The rate of the agitation speed influenced the extent of mixing in the shake flasks system and affected the nutrient availability as well (Venugopal *et al.*, 2007). Agitation speed has affected many enzymes activities in different strains of bacteria and fungi (Darah *et al.*, 2011; Kalaichelvan, 2012) as well as microalgae (Sobczuk *et al.*, 2006). At lower agitation speed, resulting in insufficient oxygen in the culture medium affects the microbial growth, whereas higher agitation speeds sometimes also lowered the production of microorganism (Seth & Chand, 2000). Initial pH medium is the second factor that contributed to the production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate with the percentage of 11.15%. In this study, the fermentation was done at different initial pH medium. The values of pH studied in this experiment were 4 and 8. Fungus generally grows maximally over a certain range of the initial pH of the medium and will not grow at high and low extremes under given conditions. Previous reports have shown that the *C. fimbriata* grow very well at pH 6 (Soares *et al.*, 2000).

Table 4. The percentage contribution of each main factors and their interaction

Effect list	Contribution (%)
X_1 pH	11.15
X_2 Temperature	9.84
X_3 Agitation speed	51.61
X_4 Total glucose in OPF	8.61
X_1X_2	0.50
X_1X_3	3.70
X_1X_4	0.83
X_2X_3	0.83
X_2X_4	2.96
X_3X_4	1.73

CONCLUSION

Based on the GC-SPME analysis, it showed that, the most favorable condition for methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production were achieved at an initial pH medium of 8, agitation speed (100 rpm), temperature (25°C) and 30 g/L of glucose present in the OPF juice. Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate were produced at the retention time of 32.80 min and the relative peak area was 0.24% of chromatogram area by using GC-SPME. Therefore, it can be concluded that OPF juice has the potential as a carbon source for the production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate by *C. fimbriata*.

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