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DEVELOPMENT OF MEDIA COMPOSITIONS OF *Lactobacillus plantarum* FOR IMPROVEMENT OF SCAVENGING ABILITY OF *Curcuma caesia*

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Among the lesser-known species of Curcuma, is *Curcuma caesia* (Trivedi, 2003) which is commonly known as *Kunyit hitam in Malaysia*. Its medicinal value is due to the presence of natural bioactive compounds.

Lactic acid bacteria (LAB) have been included in the daily diet of health-conscious individuals due to their significant roles as health-promoting probiotics which can encourage a healthy digestive tract that could prevent the onset of many illnesses (Bothe *et al*., 2017). Lactic acid bacteria, *Lactobacillus plantarum* is frequently used in the fermentation of plant materials for improvement of bioactive compounds like phenolic compounds, which are considered as the main dietary antioxidants in addition of various healing properties. These compounds have shown biological activities in vivo and it is expected that they may contribute to prevent some diseases which are due to the formation of excess oxygen radical which affects the defence capacity of human body are mainly due to their ability as reducing agent, hydrogen donators, and singlet and triplet oxygen quenchers and metal chelation properties (Saura-Calixto & Goñi, 2006; Idris *et al*., 2015; Morelló *et al*., 2005).

Thus, this study focuses on increasing DPPH scavenging ability of *C. caesia* crude extract by incorporating it into the growth media of *L. plantarum* after screening and optimizing selected growth factors of the media through Plackett Burman experimental design and face centered central composite design (FCCCD).

A commercial strain *of L. plantarum* bacteria were collected from Department of Biotechnology Engineering, IIUM. *C. caesia* rhizomes were collected from Kota Tinggi, Johor. It was dried and ground to powder form before stored at -20°C.

The *L. plantarum* in MRS broth was incubated in sterilised shake flask at 37°C, 120 rpm for 48 hr. The inoculum concentration was kept constant at 2% (1 mL) in every experiment. Thus, the total volume of the broth and the inoculum will always be kept at 50 mL. A 1.60×10^8 cells/mL based on the OD600 reading was maintained for every inoculum at the start of each experimental run.

Plackett-Burman design (PBD) was used to determine the most influential factors out of 11 variables which includes, yeast extract, lactose, glucose, peptone, dipotassium hydrogen phosphate, sodium acetate, sodium sulphate, potassium dihydrogen phosphate, sucrose, sodium chloride and lentil flour. A total of 12 runs were generated by the design software (Table 1). Then, 5.3 mL of each factor was put into 150 mL shake flask, followed by adding 2% (v/v) of *C. caesia* extract. The flask was autoclaved and 2% (v/v) of the inoculum was added to 48 mL of the sterile media. Thus maintaining 50 mL as the working volume for the fermentation process. The control used contained only MRS broth and the inoculum. The inoculated media were cultured for 8 hr. at 35°C with agitation speed set to 120 r.p.m. After that, all the samples were centrifuged with a speed of 2000 ×g and 4°C for 20 min. The supernatant of each sample was kept for DPPH scavenging analysis.

DPPH scavenging assay was carried out using the method (Khoo, 2009), adding 100 µL of the supernatant to 3.9 mL of 60 µM ethanolic DPPH and then allowed to stand in the dark for 30 min. Then the absorbance of sample was measured at 517 nm by using UV-VIS spectrophotometer. The DPPH free radical scavenging activity (%) was calculated by using the formula given in equation 1. The control used was ethanolic DPPH.

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Table 1. The DPPH scavenging activity with selected media components based on the Plackett-Burman Design

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8	Factor 9	Factor 10	Factor 11	Scavenging activity
	A: yeast extract (g/L)	B: Peptone (g/L)	C: Glucose (g/L)	D: K_2HPO_4 (g/L)	E: Lactose (g/L)	F: Sodium acetate (g/L)	G: Sodium sulphate (g/L)	H: KH_2PO_4 (g/L)	J: Sucrose (g/L)	K: NaCl (g/L)	L: Lentil flour (g/L)	% DPPH
1	0.5	10	5	0.2	5	0.5	0.2	0	15	5	1	71.12
2	0.5	1	5	2	5	0.5	2	0.25	10	5	0.5	71.85
3	0.5	1	1	2	5	5	0.2	0.25	15	0	1	67.92
4	5	1	5	2	0	5	0.2	0	10	5	1	72.05
5	0.5	10	1	0.2	0	5	2	0.25	10	5	1	75.21
6	5	10	5	0.2	5	5	0.2	0.25	10	0	0.5	74.78
7	5	10	1	2	0	0.5	0.2	0.25	15	5	0.5	76.19
8	5	1	5	0.2	0	0.5	2	0.25	15	0	1	76.51
9	0.5	1	1	0.2	0	0.5	0.2	0	10	0	0.5	72.99
10	5	10	1	2	5	0.5	2	0	10	0	1	72.18
11	0.5	10	5	2	0	5	2	0	15	0	0.5	75.33
12	5	1	1	0.2	5	5	2	0	15	5	0.5	76.45

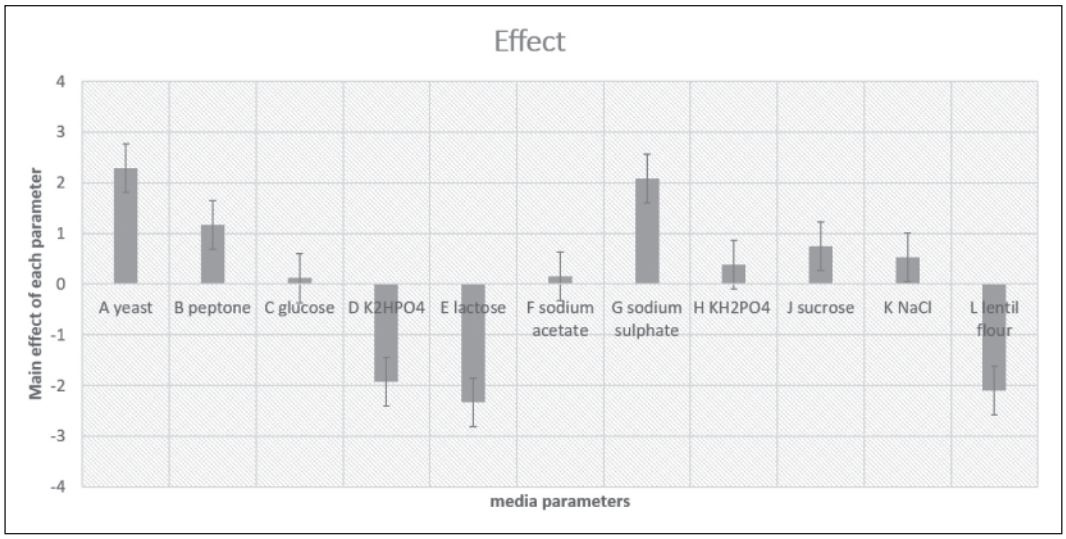

Fig. 1. Main effects of the selected media components based on the Plackett-Burman Design.

$$Scavenging\ activity\ (\%) = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100\% \quad (1)$$

Face Centered Central Composite Design (FCCCD) under Response Surface Methodology (RSM) was used to determine the optimum concentration of the three significant factors selected that would exhibit the maximum DPPH scavenging activity. The software proposed 20 experiments. All parameters of process conditions along with concentration of *C. caesia* and inoculum during fermentation were maintained. Each experiment was performed three times and the average of test analysis was measured. Then, ANOVA was employed to analyse the measured and predicted responses and experiment validation was performed to verify the equation accuracy and the optimum results.

Main effects of each investigated media components are shown in Figure 1. Results show that yeast extract, peptone, sucrose, sodium sulphate, sodium chloride and potassium dihydrogen phosphate contributed positively to scavenging activity. After careful deliberation, yeast extract, peptone and sucrose were chosen based on their ability to increase DPPH scavenging ability of the media supernatant.

Second-order polynomial regression function based on DPPH scavenging activity analysis is shown below:

$$Y = 79 - 2.86A - 1.33B + 2.23C - 4.38A^2 - 4.78B^2 - 11.08C^2 - 10.04AB + 4.04\ AC + 3.27BC \quad (2)$$

where Y is response variable (predicted DPPH scavenging activity); A, B, C are independent variables (yeast extract, peptone and sucrose, respectively) that are significant to Y. The highest yield of DPPH scavenging activity was observed to be 84.25%, which was obtained when concentration of yeast extract, peptone and sucrose at 7, 8 and 10 g/L, respectively (Table 2).

According to ANOVA (Table 3) the regression function based on DPPH scavenging activity is significant in which model F-value of 28.33 is shown as evidence and it has a very small probability value ($Prob_{model}$ ˆ F is 0.0001). Also, the $Prob_{LOF}$ ˆ F of 0.4093 implies that lack-of-fit (LOF) is not significant. In addition to that, P-value of less than 0.100 is an indication that the coefficient is

Table 2. Design summary of media optimization using FCCCD

Run	Yeast (g/L)	Peptone (g/L)	Sucrose (g/L)	%DPPH Scavenging
1	11	12	5	35
2	7	12	10	69.05
3	7	8	10	77.15
4	3	12	5	71.05
5	7	8	10	80.05
6	11	4	15	68.55
7	11	8	10	74.25
8	3	4	15	48.25
9	7	8	15	69.75
10	7	8	10	84.25
11	11	4	5	63.25
12	3	12	15	73.25
13	7	4	10	77.55
14	3	4	5	58.25
15	7	8	10	77.75
16	3	8	10	73.15
17	7	8	10	82.09
18	7	8	10	76.38
19	11	12	15	54.25
20	7	8	5	64.25

Table 3. ANOVA result for media optimization

Source model	Sum of squares	DF	Mean square	F value	Prob>F	
	2732.24	9	303.58	28.33	<0.0001	significant
A	82.08	1	82.08	7.66	0.0199	
B	17.56	1	17.56	1.64	0.2294	
C	49.51	1	49.51	4.62	0.0571	
A^2	52.82	1	52.82	4.93	0.0507	
B^2	62.9	1	62.9	5.87	0.0359	
C^2	337.77	1	337.77	31.52	0.0002	
AB	807.02	1	807.02	75.32	<0.0001	
AC	130.82	1	130.82	12.21	0.0058	
BC	85.48	1	85.48	7.98	0.018	
Residual fit	107.15	10	10.71			
Lack of fit	59.33	5	11.87	1.24	0.4093	Not significant
Pure error	47.82	5	9.56			
Cor total	2839.39	19				

Table 4. Validation of the quadratic model and optimised media constituents

Yeast (g/L)	Peptone (g/L)	Sucrose (g/L)	Actual value	Predicted value	RSE%
11	8	10	73.25	71.79	1.993174
7	8	10	74.25	71.75	3.367003
7	8	10	80.05	79	1.31168

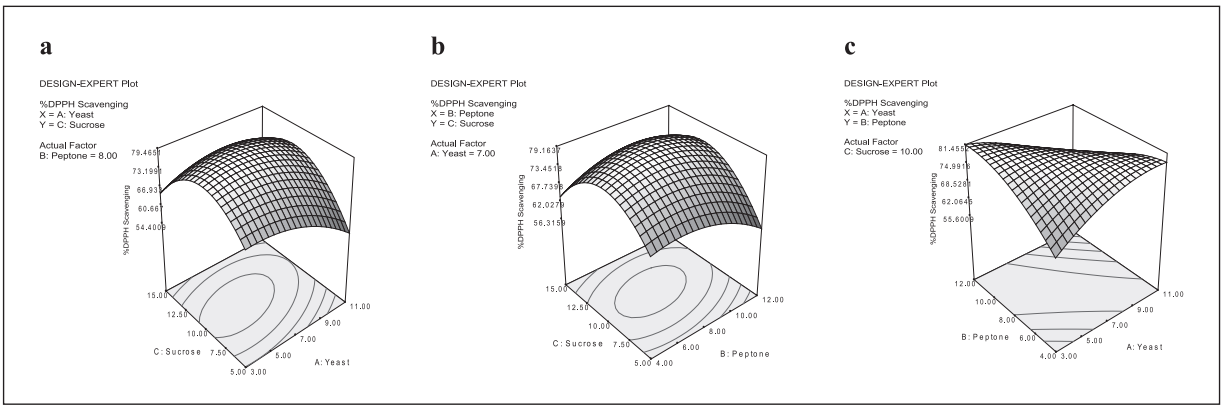

Fig. 2. 3D surface curve showing the interaction between (a) yeast extract, (b) sucrose and (c) peptone towards DPPH scavenging activity.

significant. From the analysis, it appears that square term of peptone (B^2), sucrose (C^2) linear effect of yeast extract (A) as well as interactive term between yeast extract and sucrose (AC), yeast extract and peptone (AB) and peptone and sucrose (BC) were significant. The quality of the data obtained by the proposed model was evaluated by considering the correlation coefficient, R^2 between the experimental and modelled data. A high determination coefficient ($R^2 = 0.9623$) was demonstrated by this model which indicates that there is a 96.23% correlation between the experimentally observed and predicted values. The high correlation also indicates that about 96.23% of the variables were considered in the response. The RSE values obtained (Table 4) is an indication that there are no significant differences between the actual and predicted values, proving that the models were adequate.

Three-dimensional (3D) response surface plot (Figure 2a, b, c) showed the interaction between yeast extract and sucrose; peptone and sucrose; and yeast extract and peptone respectively on DPPH scavenging activity. It is observed that as the concentration of sucrose, peptone and yeast extract increases, the DPPH scavenging activity also increases. The OD 600 reading of the media was also

recorded at the end of the fermentation period and it was observed that the number of cells per mL increased to 2.85×10^8 cells/mL and when it is plated on an MRS agar plate, there were 600 cfu per millilitre on the plate. Malaysian *C. caesia* deionised water crude extract had been shown to contain 592.86 mg/L GAE of phenolics content with a DPPH scavenging activity of 67.03% (Jemain *et al*., 2017). The evaluation of the fermentation media supernatant showed that by using *L. plantarum* during fermentation there was an increase in their DPPH scavenging activity to 84.25%. This increase was expected due to the ability of *L. plantarum* to convert the phenolic compounds into volatile phenols that could help in increasing the antioxidant activity. This observation is similar to a research done on olives (Kachouri *et al*., 2015), palm oil mill effluent (Jamal *et al*., 2011) grape pomace extracts (Gil-Sánchez *et al*., 2018), and grape fermentation (Devi & Anu-Appaiah, 2018). The increase in DPPH scavenging activity through fermentation is also an indication that the *L. plantarum* used is able to utilise the carbohydrates and protein contents in order to elicit higher DPPH scavenging activity by liberating or synthesising more of the DPPH scavengers. *C. caesia* contains significant amount of protein, minerals and carbohydrates as well (Rannema & Reddy, 2017) thus the fermentation process is able to synergistically enhance and increase DPPH scavenging activity, as on its own the *L. plantarum* strain could only have 62.64% DPPH scavenging activity.

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