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Research Article
Optimization of Culture Medium for the Growth of *Candida* sp. and *Blastobotrys* sp. as Starter Culture in Fermentation of Cocoa Beans (*Theobroma cacao*) Using Response Surface Methodology (RSM)

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Abstract

Background and Objective: Inoculation of starter culture in cocoa bean fermentation produces consistent, predictable and high quality of fermented cocoa beans. It is important to produce healthy inoculum in cocoa bean fermentation for better fermented products. Inoculum could minimize the length of the lag phase in fermentation. The purpose of this study was to optimize the component of culture medium for the maximum cultivation of *Candida* sp. and *Blastobotrys* sp. **Materials and Methods:** Molasses and yeast extract were chosen as medium composition and Response Surface Methodology (RSM) was then employed to optimize the molasses and yeast extract. **Results:** Maximum growth of *Candida* sp. (7.63 log CFU mL^{-1}) and *Blastobotrys* sp. (8.30 log CFU mL^{-1}) were obtained from the fermentation. Optimum culture media for the growth of *Candida* sp., consist of 10% (w/v) molasses and 2% (w/v) yeast extract, while for *Blastobotrys* sp., were 1.94% (w/v) molasses and 2% (w/v) yeast extract. **Conclusion:** This study shows that culture medium consists of molasses and yeast extract were able to produce maximum growth of *Candida* sp. and *Blastobotrys* sp., as a starter culture for cocoa bean fermentation.

Key words: Response surface methodology, *Candida* sp., *Blastobotrys* sp., molasses, yeast extract

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Traditionally, fermentation technologies used molasses for ethanol and animal feed production[1]. Molasses is a byproduct from sugar refining. It is thick and dark syrup resulting from crystallization and removal of sucrose from original juice[2]. Molasses contain approximately 50% (w/w) of fermentable sugars including glucose, sucrose and fructose. It has been widely applied in food and non-food industries due to high nitrogen and sugar content. Other than that, molasses was used as a substrate for yeast production as it is highly available, low cost and does not contain substance that can inhibit fermentation[3,4].

Molasses as described is a source of carbon essential for the growth of microorgansims. Other than that, nitrogen which is essential for the growth of yeast is one of the main elements playing important role in structure and function that can be found in the cells[5]. Yeasts obtained nitrogen source from a wide variety of compounds. Yeast extract was made by extracting the cell content by exogenous or endogenous enzyme. Yeast extract are rich in amino acids, vitamins, peptides and growth simulating compounds. It is commonly used as food additives, flavoring and important component in the medium as supplement for the growth of microorganisms[3,6,7]. Therefore, addition of yeast extract into the culture medium would be able to promote the growth of yeast for inoculum development in cocoa bean fermentation.

Inoculum is important factor in fermentation in order to achieve optimal production of microorganisms. Healthy inoculum would be able to minimize the length of the lag phase in fermentation[8]. This could be beneficial in fermentation of cocoa beans. Use of starter culture in cocoa bean fermentation was suggested to control the fermentation process producing consistent, predictable and high quality of fermented cocoa[9]. Thus, the aim of this study was to optimize culture medium for the maximum production of *Candida* sp. and *Blastobotrys* sp. The cells obtained were used as starter cultures in fermentation of cocoa beans.

MATERIALS AND METHODS

Yeast cultures and maintenance: *Candida* sp. and *Blastobotrys* sp., used throughout this study were provided by Barry Callebaut Malaysia Sdn. Bhd, Kuala Lipis, Pahang. Stock cultures of this strain were prepared in 1 mL aliquots by adding 0.5 mL of overnight culture with an appropriate media to 0.5 mL of 20% (w/v) glycerol. The cultures were mix thoroughly with glycerol[10] by vortex and kept at -80°C. The cultures were maintained on Yeast Peptone Dextrose (YPD) agar containing 1% yeast extract (Oxoid, Basingstoke, UK), 2% peptone (Oxoid, Hampshire, England), 2% glucose (Merck, Darmstadt, Germany) and 2% agar (Oxoid, Hampshire, England).

Inoculum development and growth curve of yeasts: Inoculum for optimization of culture media was prepared by growing the culture in YPD broth. One to two loops of the culture colony from the YPD agar was inoculated into 10 mL of YPD broth by means of an inoculating loop. Incubation was carried out with constant shaking at 180 rpm at 30°C for 24 h in orbital shaker (Jeio-Tech orbital shaking incubator, model SI-600 R, Seoul, Korea). Growth curve of yeasts was determined by inoculation of 1% (v/v) culture into 100 mL of YPD broth. The cultures were shaken using rotary shaker incubator at 30°C with 180 rpm. At 0, 2, 4, 8 and every 4 h until reached 60 h, sampling was done by pipetting out of 10 mL culture from each flask for optical density and cell count analysis. About 2 mL aliquot of culture was transferred into cuvette. The absorbance was read at 600 nm. The cell count was performed by serially diluted 0.1 mL of culture into 0.9 mL Phosphate Buffer Solution (PBS). About 0.1 mL from each dilution was spread onto YPD agar. The plates were then incubated at 30°C for 48 h and the numbers of colonies were counted. The analysis was done three times with three replicates ($n = 3 \times 3$).

Optimization of Molasses Yeast Extract (MYE) media: Yeast extract (Merck, Darmstadt, Germany) and molasses (Liqueur Agency Sdn. Bhd., Malaysia) were used in fermentation media preparation. The RSM was used to determine optimum yeast growth affected by two factors (molasses and yeast extract). A 2^2 Central Composite Design (CCD) was employed in this study. A total of 13 runs containing different levels of molasses and yeast extract are shown in Table 1. Fermentation media containing molasses and yeast extract with 100 mL working

Table 1: Experimental design of Response Surface Methodology (RSM)

Run	Molasses (g/100 mL)	Yeast extract (g/100 mL)
1	5.25	1.05
2	1.89	1.72
3	0.50	1.05
4	5.25	1.05
5	10.00	1.05
6	5.25	1.05
7	5.25	2.00
8	5.25	0.10
9	5.25	1.05
10	8.60	0.37
11	1.89	0.37
12	5.25	1.05
13	8.60	1.72

volume were prepared in 500 mL conical flask. Flasks were plugged with cotton stoppers and sterilized at 121°C for 15 min. Fresh prepared inoculum was added 1% (v/v) aseptically into the fermentation. The runs were conducted for 32 and 36 h in a temperature controlled orbital shaker at 180 rpm, which enabled adequate homogenization for better contact between substrates and the yeast culture[11]. At the end of fermentation, 1 mL samples were aseptically taken out from the media for determination of cell counts. About 0.1 mL sample was placed into an eppendorf tube containing 0.9 mL PBS solution and mixed thoroughly using vortex. This study suspension was designated as 10^{-1}. About 0.1 mL of diluted samples was transferred into another tube containing 0.9 mL sterile PBS and thoroughly mixed. The dilution was made sequentially from tube to tube to increase diluted cell suspensions. About 0.1 mL from each dilution was pipetted and transferred onto YPD agar plate[12]. Inverted plates were incubated at 30°C for 48 h and viable colonies grown on agar plates were counted. The analysis was done 3 times with 3 replicates ($n = 3 \times 3$).

Statistical analysis: Minitab software (Version 14.0, Minitab Co., PA, USA) was used for statistical analysis. Analysis of variance (ANOVA) was carried out for its statistical significance. The $p < 0.05$ was considered as significant.

RESULTS

Inoculum development and growth curve of yeasts: Determination of growth curve requires inoculation of viable cells into broth medium and incubated at optimum conditions. Under these conditions, the cells produce rapidly and dynamic of growth charted by plotting number of cells versus incubation time. In this study, yeasts (*Candida* sp. and *Blastobotrys* sp.) were inoculated separately into enrich medium, YPD broth to determine the growth curve of these yeasts. Time of incubation period which gave the maximum number of yeast cells was used for harvesting the cell in optimization of molasses-yeast extract media for the cultivation of *Blastobotrys* sp. and *Candida* sp. Result shows *Blastobotrys* sp., lag phase was approximately 6 h before enter the log phase (Fig. 1). The log phase of *Blastobotrys* sp., was from 12 h until 32 h. *Blastobotrys* cell number increased to maximum number from 10^5 CFU mL^{-1} until 10^8 CFU mL^{-1} after incubated for 32 h. From this experiment, *Blastobotrys* sp., achieved maximum number of cells after 32 h of incubation after no further increased was observed in yeast cells over incubation time. Therefore, the time of incubation was used for harvesting the yeast cell in next experiment. Meanwhile, *Candida* sp., showed lag phase occurred less than 10 h (Fig. 2). Growth of *Candida* sp., started to increase from 10^4-10^8 CFU mL^{-1}. *Candida* sp., achieved maximum number of cells after incubation of 36 h. From this experiment, *Blastobotrys* sp. and *Candida* sp., achieved 8.28 and 7.55 log CFU mL^{-1} after 32 and 36 h of incubation, respectively. Thus, these times of incubation were used for next experiment.

Optimization of Molasses Yeast Extract (MYE) media: In this study, high production of *Candida* sp. and *Blastobotrys* sp., were evaluated in 13 different concentration of medium

Table 2: Analysis of variance (ANOVA) for the fitted regression model of *Candida* sp., production

Source	DF	SS	MS	F-value	p-value
Model	3	35.714	11.904	65.25	0.00
Residual (error)	9	1.641	0.182		
Lack of fit	5	1.590	0.318	24.50	0.40
Pure error	4	0.051	0.013		

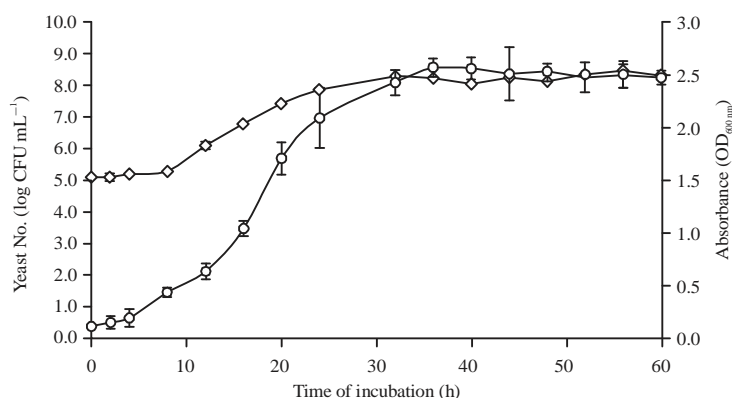DF: Degree of freedom, SS: Sum of square, MS: Mean of square

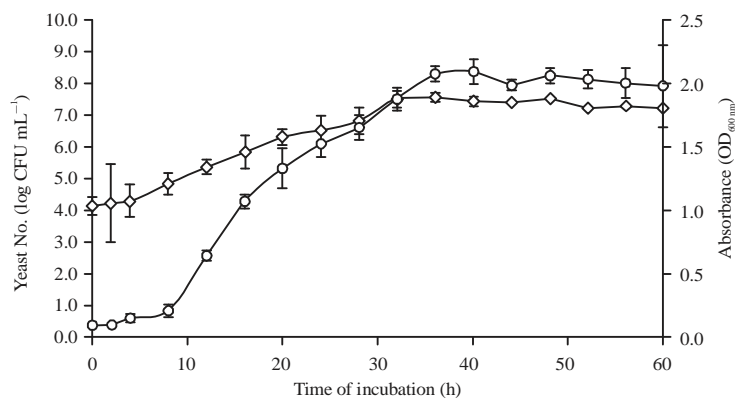Fig. 1: *Blastobotrys* sp., growth in YPD medium. Right axis: ○: Absorbance at 600 nm. Left axis: ◇: Yeast number (log CFU mL^{-1})

Fig. 2: *Candida* sp. growth in YPD medium. Right axis: ○: Absorbance at 600 nm. Left axis: ◇: Yeast number (log CFU mL^{-1})

Table 3: Analysis of variance (ANOVA) for the fitted regression model of *Blastobotrys* sp., production

Source	DF	SS	MS	F-value	p-value
Model	3	24.323	8.107	33.23	0.00
Residual (error)	9	2.196	0.244		
Lack of fit	5	1.253	0.250	1.06	0.48
Pure error	4	0.942	0.235		

DF: Degree of freedom, SS: Sum of square, MS: Mean of square

Table 4: Validated yeast concentration of optimized molasses-yeast extract media

	Yeast count (log CFU mL^{-1})	
Yeast strains	Predicted	Experimental
Candida sp.	8.95	7.63
Blastobotrys sp.	9.90	8.30

component using RSM. Table 2 and 3 show the results on analysis of variance (ANOVA) to indicate the adequacy of fitted model. The p<0.05 indicates that the equation obtained was appropriate and suitable after model reduction. The experimental results for both of *Candida* sp. and *Blastobotrys* sp., were fitted in the second order polynomial model as follow:

$$Y_{Candida\ sp.,\ yield} = 0.98x_1 + 2.93x_2 - 0.039x_1^2 - 0.58x_2^2 - 0.407 \quad (1)$$

$$Y_{Blastobotrys\ sp.,\ yield} = -0.86x_1 + 1.39x_2 + 0.04x_1^2 + 7.057 \quad (2)$$

where, Y is the response (i.e., *Candida* sp. and *Blastobotrys* sp., yield), x_1 is molasses and x_2 is yeast extract.

The adequacy for 2 regression models (*Candida* sp. and *Blastobotrys* sp.) production were determined by ($R^2 = 0.956$ and 0.917), which means 95.6 and 91.7% are the response variability of the model. The adjusted R^2 (0.941 and 0.889) further supported the model accuracy. Based on the regression model a maximum production of *Candida* sp., was predicted to produce log 8 CFU mL^{-1} using 10 g/100 mL

molasses and 2 g/100 mL yeast extract. *Blastobotrys* sp., production was predicted to give log 9 CFU mL^{-1} using 1.92 g/100 mL molasses and 2 g/100 mL yeast extract. Validation of the predicted models was done experimentally based on optimum concentration of independent variables. Under optimized culture medium, *Candida* sp. and *Blastobotrys* sp., produced 7.63 and 8.30 log CFU mL^{-1} (Table 4). The good correlation between predicted and experimental values verifies the validity of the response models. Molasses seem to affect the production of yeasts.

DISCUSSION

Microbial growth curve has sigmoid curve consist of lag, log and stationary phase. Lag phase referred as adaptation time with growth rate is close to zero. The cells are biochemically active but not increase in cell number. During this period, metabolic pathway of the cells is initiated prior to start the cell division. Length of lag phase varies might be because of initial inoculum size and environmental factor such as nutrient, incubation temperature and pH of culture medium[13]. When cells are adapted, they started to divide. This phase called as exponential or log phase[14]. Exponential or log phase characterized by high energy consumption with constant growth rate[15]. The cells continue to grow until the total number of viable cell constant in stationary phase[12,13]. During exponential growth phase, yeast cells used sugar via glycolysis produced ethanol and acetic acid into the medium. As sugar depleted, yeast cells start to switch from consumption of sugar to ethanol until completely utilized[16]. Kusch *et al.*[15] reported *Candida albican* made energy from glucose in medium through substrate phosphorylation or oxidative phosphorylation. In a medium with rich glucose medium based, the mitochondrial structure were disrupted but it back to normal when aerobic metabolism replaces

alcoholic fermentation[17]. When the cells lack of essential nutrient, they enter resting stage known as stationary phase[18]. Stationary phase occurred when the cells stop to divide[19]. In a study by Abadias *et al*[8], maximum growth of *Candida sake* was observed after 30 h grown in molasses medium. Maximum number achieved in short period was due to agitation-aeration combination in fermenter. The best agitation speed allows the oxygen distributed well in the culture medium to be used by yeast cells for respiration.

The MYE medium consist of molasses as carbon source. Molasses contain fermentable sugar compose of fructose, glucose and sucrose. The membrane of yeast cell was not freely permeable for all sugar[20]. The sugars were transported into the cell through protein transport embedded in the cell membrane either in their intact form (glucose and fructose) or they were firstly hydrolysed to monosaccharide components[21]. Hydrolysis of disaccharide sugar depends on the yeast strain and the nature of sugar may occur inside or outside the cell membrane. In most cases, hydrolysis of sucrose occurred outside the cell membrane by the action of enzyme invertase into glucose and fructose[22,23]. Molasses was found to be the most preferred carbon source compared to glucose, sucrose and rice water by marine yeasts (*Debaryomyces hansenii*, *Debaryomyces hansenii*, *Candida sake* and *Candida tropicalis*) in a study by Sarlin and Philip[24]. Molasses are rich in nutrients mostly required by the microorganism, such as nitrogen, sodium, phosphorus and potassium[25]. Optimal sugar concentration for the yeast growth was found to be 17.5% (w/v) of commercial sucrose and average 8.75% (w/v) of cane molasses[26]. Different in concentrations between these 2 carbon sources probably because of the impurities in cane molasses such as SO_2, clorets and Al_2O_3. High impurities concentration may inhibit the yeast growth[26]. Sarlin and Philip[24] stated that molasses served as a good carbon source with addition of growth promoting components which one of it found to be amino acid[27].

CONCLUSION

This study demonstrated that cultivation of *Candida* sp. and *Blastobotrys* sp., could be produced in liquid medium. Maximum growth of these 2 yeasts was produced in medium consist of molasses and yeast extract. Under optimized culture media, yeasts were able to produce about 7.63 log CFU mL^{-1} of *Candida* sp. and 8.30 log CFU mL^{-1} of *Blastobotrys* sp. The optimum concentration of medium compositions for *Candida* sp., was 10% (w/v) molasses and

2% (w/v) yeast extract, while for *Blastobotrys* sp., was 1.94% (w/v) molasses and 2% (w/v) yeast extract, respectively.

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