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Optimization of cocoa fermentation parameters for high soluble solids content by using *candida tropicalis*

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Abstract
Spontaneous cocoa fermentation is inefficient, as the pectin in the pulp is structurally tough and difficult to be broken, which results in prolonged fermentation reduced soluble solids content (SSC). Due to the drawback of conventional methods, treatment with starter culture containing pectinases is preferred. The objective of this study was to optimize SSC in cocoa beans fermented by *Candida tropicalis* starter. The method used was Response Surface Methodology (RSM). Three fermentation variables including starter culture ratio, fermentation temperature and fermentation time were focused. The model reveals that optimum SSC was up to 2.80°Brix after 4 days of fermentation with starter ratio of 5% at 33.5°C. The findings demonstrate that addition of *C. tropicalis* starter improved the cocoa bean fermentation process and SSC extraction and its contribution to cocoa factory.

Keywords: Box-Behnken, *Candida tropicalis*, cocoa, fermentation, optimization, RSM, SSC

1. Introduction
Cocoa beans (*Theobroma cacao L.*) represent the seed of the tropical cocoa tree, growing in Central America, West Indian islands, South America, Africa, etc. Three important varieties of cocoa are commonly employed: *Forastero*, *Criollo* and *Trinitario*. Raw cocoa beans are inedible because of their bitter and astringent flavour and unpalatable and unpleasant taste; they must be cured before they can be processed into good-tasting and full-flavoured cocoa and chocolates. Therefore, the process of cocoa bean fermentation plays a significant role in determining the composition and flavour of chocolate and other cocoa-based products and it hence lays at the basis of the entire chocolate-making process.
At the onset of the fermentation, the pulp has low oxygen availability due to the tightly packed structure of the cocoa beans mass. The high contents of pectin (1 – 5%) [1] and other polysaccharides (cellulose, hemicellulose, lignin) make the pulp viscous and prevent air ingress into the fermenting cocoa pulp-bean mass, resulting in prolonged fermentation time. When the fermentation lasts more than 4 days, bacilli and filamentous fungi – which is often not desirable – may participate in the cocoa bean fermentation process [2, 3, 4, 5]. Also, the concentration of soluble solids may have a decrease when the fermentation time increases. Thus a rapid degradation of cocoa pulp using microorganisms with pectinolytic enzyme activity to speed up the fermentation process and to improve the soluble solids content (SSC) of the final products will be desirable.
To solve the problem, pectinases may be added to the pulp or starter culture strains overproducing pectinolytic enzymes may be used to enhance sweating production and obtain consistent, predictable, and high quality fermented dry cocoa beans [3, 6, 7]. *Candida tropicalis* is an ethanol and heat-tolerant yeast species which was reported to have the ability to produce pectinolytic enzymes [8, 9]. Thus, *C. tropicalis* is a highly potential candidate starter culture strain for cocoa fermentation.
After implementing the starter culture from *C. tropicalis*, it is necessary to optimize fermentative conditions to attain the maximum yield of SS extract. Traditionally, optimization has been carried out by one-variable-at-a-time technique, which is time consuming and does not include the interactive influences among the variables studied. An alternative and more efficient approach is Response Surface Methodology (RSM). In RSM it is possible to observe the interaction effect of the independent parameters on the response. With respect to these, RSM is a useful tool for the optimization process.
To rapidly remove the mucus layer, speed up fermentation and increase the SSC in cocoa beans, this study focused on implementing a pectinolytic starter culture composed of *C. tropicalis*. RSM were employed to optimize fermentative conditions for maximal SSC extraction.

2. Materials and methods
2.1 Materials
Microbial strain used in the starter was *Candida tropicalis* (VTCC-γ-1458) obtained from Institute of Microbiology and Biotechnology, Vietnam National University Hanoi.
Cocoa pods (*Trinitario* variety) were purchased from Kimmy's chocolatier located in Tien Giang, Mekong Delta, Vietnam. The pods were washed and disinfected with ethanol solution (70°), then opened under a clean bench and the seeds were removed from placenta. The fermenting mass weighed about 100 g based on the fresh beans weight using

sterile plastic fermentation boxes (diameter ~ 10 cm) with drilled holes on the sides and the base to facilitate juice drainage and aeration.

2.2 Methods

2.2.1 Preparation of Starter Culture

Candida tropicalis was grown in yeast extract peptone dextrose (YEPD) medium supplemented with 0.5% pectin, incubated at 30ºC for 2 days under shaking conditions (120 rpm). Cell growth (expressed as CFU per ml of starter culture) was evaluated by plate count method. The starter culture was then centrifuged (5,000 rpm, 10 min, 4ºC) and the cell-free supernatant was assayed for pectinase activity by Miller method [10]. One unit of enzyme was defined as the quantity of enzyme required to liberate of 1 µmol of galacturonic acid per minute under standard assay condition.

2.2.2 Optimization of fermentation parameters for high soluble solids content

2.2.2.1 Selection of fermentation parameters

The effects and delimitation of the experimental region of three independent parameters: the ratio of starter culture, fermentation temperature, and fermentation time on the yield of SSC were investigated by varying one factor at a time while keeping the others constant. An appropriate range for each factor was determined for RSM.

2.2.2.2 Box-Behnken experimental design

Based on preliminary selections, a three-factor and three-level BBD with 15 individual design points was adopted in this study. The optimal value of each parameter was chosen as middle "0" level (Table 2.1). The independent factors were the ratio of starter culture (%; A), fermentation temperature (ºC; B) and fermentation time (day; C); (Table 2.1). Response or dependent variable (Y) studied was soluble solids extracted from cocoa bean (ºBrix). Duplicate experiments were carried out at all design points.

The second order polynomial model predicted for optimization of SSC extracted from cocoa bean (Y) was:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC$$

where Y was the predicted response; β_0 was the offset term; $\beta_1, \beta_2, \beta_3$ were the linear coefficients, $\beta_{11}, \beta_{22}, \beta_{33}$ were the quadratic coefficients, and $\beta_{12}, \beta_{13}, \beta_{23}$ were cross-product coefficients.

2.2.2.3 Determination of soluble solids content

All mucilage was removed by washing. Subsequently, the beans were dried in an air-ventilated oven for 3 days at temperature of 60ºC and subjected to roasting at 150ºC for 15 mins. Roasted beans were manually shelled to obtain the nibs which were then ground in a blender, then sifted through a mesh sieve (diameter ~ 0.50 mm).

The extracts were obtained by dissolving 20 g of cocoa grounds with 100 ml of water at 90ºC, then mixed well using vortex for 1 min. The mixtures were then centrifuged at 3,500 rpm for 15 mins at 4ºC. The mixtures were filtered through filter paper and the filtrates were considered as cocoa extract. Brix degrees were measured by refractometer at 25°C.

2.2.3 Data analysis

All the experiments (except for BBD) were carried out independently in triplicates. Statistical analysis was carried out using the SPSS 22 for analysis of variance (ANOVA) and significant differences between means for all treatments (Duncan's multiple range test) at a level of $p < 0.05$. The statistical software Design Expert 11 was used for all the response surface regression and the response surface plotting.

3. Results and Discussions

3.1 Microbial population and pectinase activity of starter culture

Starter culture was made from *C. tropicalis* – a yeast species reported in cocoa beans fermentation. The starter culture showed a population of $1.32 \pm 0.45 \times 10^7$ CFU/ml and pectinase activity of 74.42 ± 1.206 (U/ml) after 48 h of incubation at 30ºC, which is suitable for removing the mucilaginous layer of cocoa bean as the yeast and enzyme respectively degrade the sugar and pectin present in the pulp.

3.2 Selection of fermentation parameters

One-variable-at-a-time design was employed to analyze the effects of starter culture ratio, fermentation temperature and fermentation time on SSC extraction. ANOVA confirmed highly significant effects of three variables on SSC.

Table 1: Variable factors used for Box-Behnken design

No.	Independent variables	Symbols	Coded-variable levels		
			-1	0	+1
1	Ratio of starter culture (%)	A	4	5	6
2	Fermentation temperature (º C)	B	30	35	40
3	Fermentation time (day)	C	3	4	5

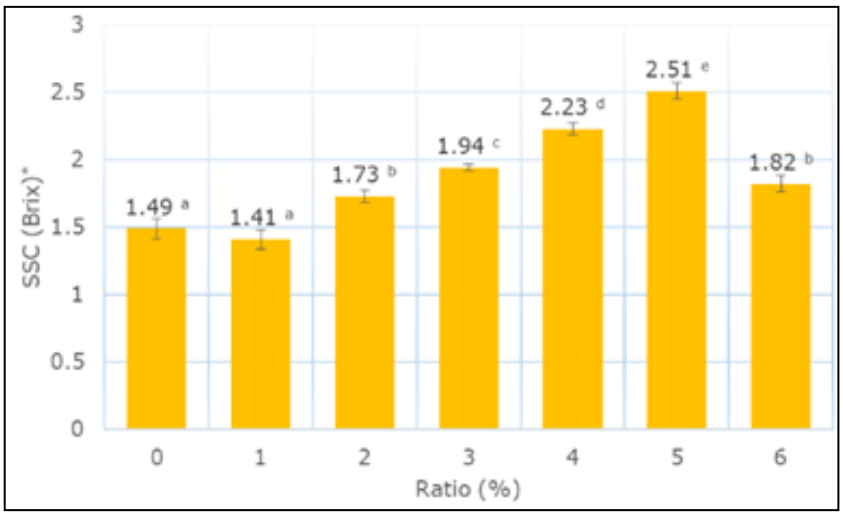
3.2.1 Effect of starter culture ratio on soluble solids content

The highest SSC extraction (2.51ºBrix) was observed at 5% of starter culture. Upon increasing or decreasing the ratio the SSC yield was decreased (Fig. 3.1). During cocoa fermentation, the bean death caused cellular membranes breakdown, facilitating different constituents which were kept in the living tissue to diffuse from the beans to the environment during extraction. As the starter culture helps hydrolyze the mucilage, it facilitates fermentation. The more starter is added, the faster beans are broken down. The measure demonstrated structural breakdown caused by 5% of starter culture might be sufficient for the extraction of SSC; while more than 5% starter would over-break the cocoa beans, resulting in SSC diffusion from the bean into the environment along fermentation, and thus the SSC remained inside the cocoa beans would be decreased.

3.2.2 Effect of fermentation temperature on soluble solids content

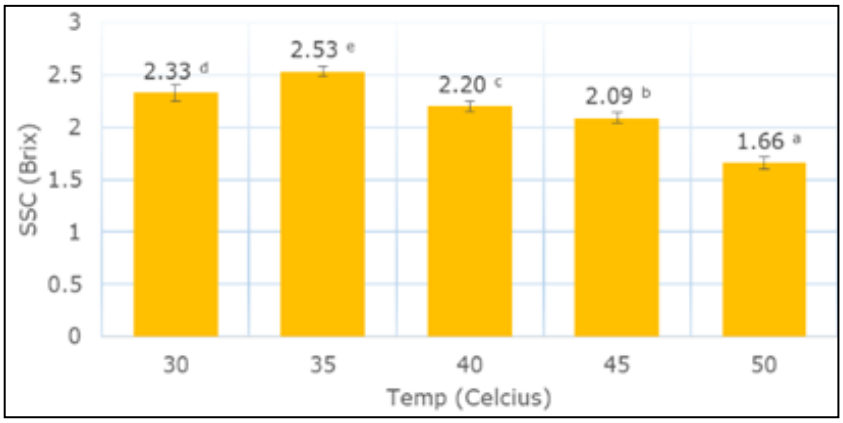
Effect of temperature on SSC yield can be seen from Fig. 3.2. Sufficient SSC was achieved when fermentation was carried out between temperature ranges of 30ºC and 40ºC, whereas extraction efficiency decreased drastically as temperature of fermentation raised to 50ºC. Temperature is directly related to the metabolic activities of the microorganism, and it affects the proper growth and product formation of the organism [11]. Under optimal temperatures, the growth rate of *C. tropicalis* was increased, as the growth

rate increases the pectinase production was also increased. The optimal temperatures of *C. tropicalis* growth and pectinase enzyme are 30ºC and 40ºC respectively, so a temperature between this range is expected to be ideal for SSC enhancement. Too cool or hot environment could slow down the growth of microorganism, which leads to the decrease in production of enzyme and the change in enzyme activity.



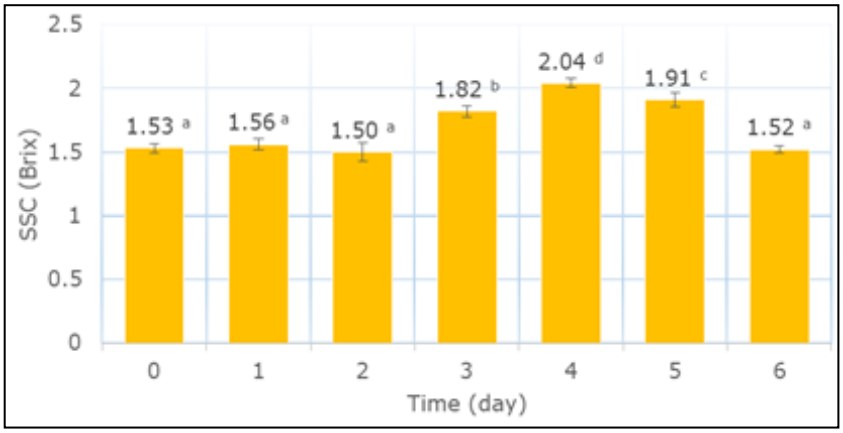
* Means that do not share a letter are significantly different.

Fig 1: Effect of starter culture ratio on SSC when fermentation temperature and fermentation time were fixed at 40ºC and 4 days respectively



* Means that do not share a letter are significantly different.

Fig 2: Effect of fermentation temperature on SSC when starter culture ratio and fermentation time were fixed at 4% and 4 days respectively



* Means that do not share a letter are significantly different.

Fig 3: Effect of fermentation time on SSC when starter culture ratio and fermentation temperature were fixed at 4% and 40ºC respectively

3.2.3 Effect of fermentation time on soluble solids content

Results for the study of fermentation time showed that, as time increased, SSC extraction increased, reaching a maximum, and then declining with increasing time. Initially, the SSC yield (1.53ºBrix) was observed at the beginning (0 day) of fermentation. It was increased to 2.04ºBrix at 4 days of treatment and then decreased to 1.52ºBrix on day 6 (Fig.

3.3). Jinap [12] reported that the volatile acids (acetic, propionic, butyric, isobutyric and isovaleric) and nonvolatile acids (citric, lactic, malic, succinic, oxalic and tartaric) are produced in the pulp through sugar degradation by the metabolism of microorganisms absorbed into the cotyledon during fermentation. In other words, the fermentation resulted in enhancement of SSC in cocoa beans. However, when the treatment happens too long, SSC is dissolved into the environment, which consequently decreases the SSC extracted from cocoa beans.

3.3 Box-Behnken design analysis

The results of BBD experiments for studying the effect of three independent variables are presented along with the mean predicted and observed responses in Table 3.1. The coefficients of starter culture ratio (A), fermentation temperature (B) and fermentation time (C), determined for the quadratic polynomial model for SSC extracted from cocoa beans (Y) were as follows:

$$Y = -15.3525 + 2.25625A + 0.50075B + 2.42C - 0.0115AB + 0.03AC - 0.013BC - 0.19875A^2 - 0.00615B^2 - 0.29125C^2$$

The model F-value of 30.97 and the p-value < 0.05 implied the model was significant. The lack of fit F-value of 1.16 implied the lack of fit was insignificant relative to the pure error and therefore the fitted model was appropriate for the description of the response surface. p-values less than 0.05 indicated model terms were significant. In this case, two linear coefficients of fermentation temperature (B) and fermentation time (C) plus three quadratic terms of starter culture ratio (A^2), temperature (B^2) and duration (C^2) were significant for SSC extraction. The extraction of SSC was not affected by the interaction model terms (Table 3.2).

Table 2: BBD design and observations of response

Run	Independent variables			Response *		
	Ratio (%)	Temp (°C)	Time (day)	Actual		Predicted
1	6	40	4	2.16	±0.04	2.12
2	5	35	4	2.76	±0.04	2.74
3	4	40	4	2.26	±0.05	2.26
4	5	35	4	2.79	±0.12	2.74
5	6	30	4	2.63	±0.08	2.63
6	5	40	5	1.83	±0.08	1.82
7	5	30	3	2.63	±0.05	2.64
8	6	35	5	2.00	±0.10	2.05
9	4	30	4	2.50	±0.03	2.54
10	5	40	3	2.33	±0.15	2.38
11	4	35	5	2.01	±0.05	2.02
12	4	35	3	2.56	±0.05	2.51
13	6	35	3	2.43	±0.03	2.42
14	5	30	5	2.39	±0.04	2.34
15	5	35	4	2.67	±0.12	2.74

*Averages of duplicate experiments.

Table 3: Analysis of variance (ANOVA) for quadratic polynomial model fitted to response surface

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	1.19	9	0.1327	30.97	0.0007
A-Ratio	0.0015	1	0.0015	0.3530	0.5783
B-Temp	0.3081	1	0.3081	71.90	0.0004
C-Time	0.3698	1	0.3698	86.30	0.0002
AB	0.0132	1	0.0132	3.09	0.1393
AC	0.0036	1	0.0036	0.8401	0.4014
BC	0.0169	1	0.0169	3.94	0.1038
A^2	0.1459	1	0.1459	34.04	0.0021
B^2	0.0873	1	0.0873	20.37	0.0063
C^2	0.3132	1	0.3132	73.09	0.0004
Residual	0.0214	5	0.0043		
Lack of Fit	0.0136	3	0.0045	1.16	0.4929
Pure Error	0.0078	2	0.0039		
Cor Total	1.22	14			

Table 4: Fit statistics for quadratic polynomial model fitted to response surface

Std. Dev.	0.0655	R^2	0.9824
Mean	2.40	Adjusted R^2	0.9507
C.V. %	2.73	Predicted R^2	0.8063
		Adeq Precision	17.2364

The coefficient of determination (R^2 = 0.9824) implied that 98.24% of the variations could be explained by the fitted model. A coefficient of variation (C.V%) of less than 5% indicated that the model was reproducible (Table 4). The Predicted R^2 of 0.8063 is in reasonable agreement with the Adjusted R^2 of 0.9507 (the difference is less than 0.2). Adeq precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 17.236 indicated an adequate signal (Table 3.3).

The linear, quadratic and cross-product terms in the second order polynomial were used to generate a three dimensional response surface graph and a two-dimensional contour plot (Fig. 3.4) of SSC yield.

Critical levels of three variables to give maximal SSC of 2.80°Brix were 5% of *C. tropicalis* starter culture, fermentation temperature of 33.5°C and duration of 4 days (Table 3.4). Results of independent experiments carried out under RSM optimized conditions was very close to predicted value of response Y (Table 3.4), which revealed that the model was reasonable and reproducible. As compared to cocoa fermentation without starter culture, a 30% increase in SSC was recorded in RSM optimized conditions. Previous studies on this aspect reveal different results for different strains and even with the same strain. The research on the addition of yeast (*Saccharomyces cerevisiae var. chevalieri*) to the cocoa bean fermentation indicated that the addition of yeast starter might fasten the fermentation [13]. *Aspergillus niger* and *Bacillus subtilis* [14] showed that the duration was lessened from 120 hours for spontaneous sample to 20 hours. Moreover, the total SSC values increased up to 30.56 – 37.22% compared to spontaneous sample. *Aspergillus unguis* and *Penicillium*

citrinum [15] increased SSC extraction about 17 – 20% in comparison to control fermentation. The quality of cocoa beans was also improved by increasing of polyphenol and caffeine contents about 6% and 11 – 32% compared to control fermentation.

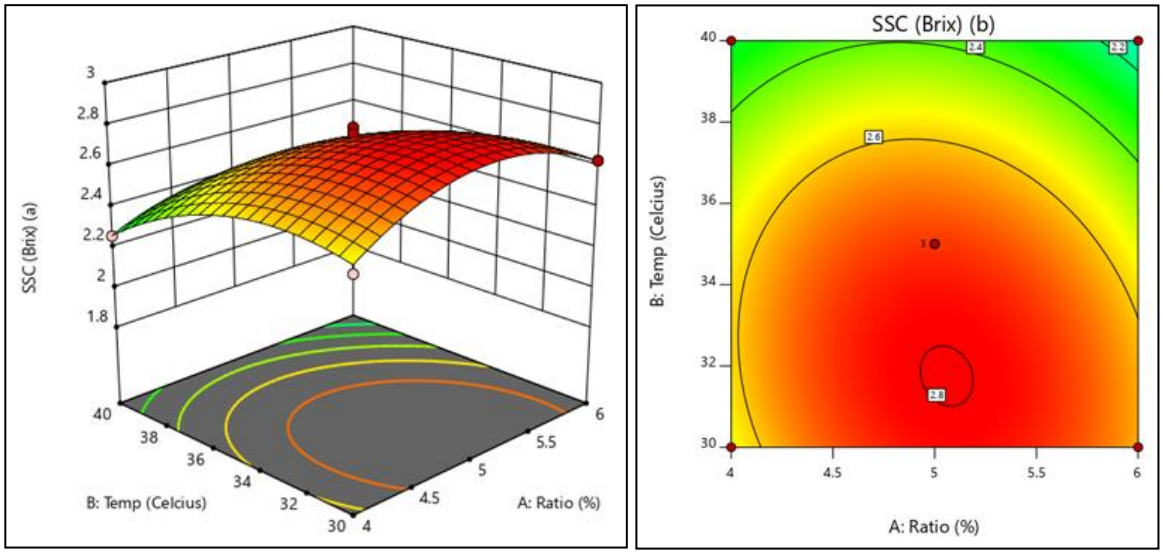


Fig 4: Three-dimensional response surface (a) and two-dimensional contour plot (b) illustrating SSC yield when fermentation time was fixed at 4 days

Table 5: Critical levels of three variables to give maximal response

Factor	Coded	Uncoded
Starter culture ratio (A)	-0.084	5
Fermentation temp (B)	-0.300	33.5
Fermentation time (C)	-0.085	4
Stationary point	Maximum	
Predicted value	2.80	
Observed value	2.79 0.09	

4. Conclusion and recommendation

A successful attempt to optimize SSC extraction from cocoa bean using starter culture from *C. tropicalis* was achieved by RSM. Addition of 5% starter culture on the weight of cocoa bean mass at 33.5ºC for 4 days enabled a 30% enhancement in SSC yield. This study showed possibility to use this starter cultures in cocoa bean fermentation to achieve high quality fermented dry cocoa beans.

More researches on the effects of factors involving in post-fermentation processes such as drying time and temp, grinding size, roasting time and temp, etc. are needed to better optimize the use of this starter culture. Also, scale-up studies are required to further evaluate applicability of this starter culture in cocoa industry.

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