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Optimizing Process Conditions for Stingless Bee (*Melipona irridipennis*) Mead Fermentation Using Plackett-Burman Design and Response Surface Methodology

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In present investigation, mead (honey wine) fermentation parameters were optimized by Placket-Burman design and response surface methodology (RSM). Eleven nutritional and fermentation parameters (TSS, pH, temperature, inoculum size, fermentation time, tartaric acid, di ammonium phosphate, potassium sodium tartrate, magnesium sulphate, calcium sulphate and potassium meta bisulphate) were screened using Plackett-Burman experimental design were further optimized by central composite design of response surface methodology for maximizing the yield of ethanol in mead. Among the eleven independent variables TSS, temperature and fermentation time had high influence on the alcohol content. The maximum production of stingless bee mead obtained experimentally using the optimized parameters was 15 °Brix at 28 °C for 6 days which are in correlation with the predicted value. The model showed that the value of R^2 (0.9991) was high and p-value of interaction of variance was < 0.0001. Hence the model can be said to be of highly significant.

Keywords: Ethanol, Fermentation time, Mead, Plackett-Burman design, Response surface methodology, Temperature, TSS.

INTRODUCTION

Honey and bee keeping have a long history in India. Honey is a natural product, a highly concentrated solution of a complex mixture of sugars. It was the first sweet food tasted by the ancient Indian inhabiting rock shelters and forests. Honey is used extensively in Ayurveda, Siddha, Unani and Naturopathy systems of medicine. It has been reported to contain about 200 substances (complex mixture of sugars, but also small amounts of other constituents such as minerals, proteins, vitamins, organic acids, flavonoids, phenolic acids, enzymes and other phytochemicals) and is considered to be an important part of traditional medicine [1]. Honey serves as a source of natural antioxidants, which are effective in reducing the risk of heart disease, cancer, immune system decline and the autism disease. Additionally, the presence of hydrogen peroxide, as well as minerals in honey, may lead to the generation of highly reactive hydroxyl radicals as part of the antibacterial system [2,3] thus, it is evident that mechanisms must be available in honey to control the formation and removal of these reactive oxygen species.

Mead is a traditional drink, which results from the alcoholic fermentation of diluted honey carried out by yeasts. Mead has many advantages over grape wine because of the flavour, purity, ecological and health benefits of honey over grapes. Grape wines are prepared from limited number of variety, but in case of honey assimilated from the awesome number of flowers that bees visit. Honey wine is better for the environment than grape wine. Bottle for bottle, honey wine uses many times less water, land and other natural resources.

Plackett-Burman design is a "screening design" traditionally used for identifying important factors among many potential factors. It is a screening technique used to examine the effects of several variables in one experiment and avoid multiple runs of the same basic test. This method allows checking the main effect of various compounds [4].

Optimization of process condition is one of the most critical stages in the development of an efficient and economic bioprocess [5]. The conventional one-factor-at-a-time approach of optimization is not only tire some but also ignores to merge interaction of each factor. One of the most common optimization used in last two decades is the response surface methodology. Response surface methodology is a powerful mathematical model with a collection of statistical techniques by which interaction between multiple processes variables can be identified with fewer experimental trials. It is widely used to examine and optimize the operational variables for experimental design, model developing and test variable and condition optimization. There are various advantages in using statistical methodologies in terms of rapid and reliable short listing of process conditions, understanding interaction among them and a tremendous reduction in total number of experiments, resulting in saving time, glassware, chemicals and manpower.

In spite of various advantages, statistical designs have been applied to only limited number of aerobic submerged and solid state fermentation and anaerobic submerged fermentation processes deal with a large number of variables and there are several reports on the application of response surface methodology for the production of primary and secondary metabolites through microbial fermentation [5]. Although many advances in the developments of mead have been made over the last few years, particularly in terms of optimizing ethanol concentration, there is still scope for future development. Hence, the present study was aimed to find out the optimum fermentation condition for producing mead as a health drink. Response surface methodology was used for optimization of ethanol concentration less than five.

EXPERIMENTAL

In the present study, stingless bee honey was obtained from NBHM (Nagaland Bee Board and Honey Mission), Nagaland, India.

Yeast strain and culture growth conditions: *Saccharomyces cerevisiae* (KF233529) has been isolated from honey and standardized in the Department of Agricultural Microbiology was used for this study. The yeast cells were grown in Yeast Peptone Dextrose agar (YPD), containing glucose 20 g, peptone 10 g and yeast extract 5 g and agar 20 g/L. The culture was routinely maintained at 4 ºC on slants. Before use, the culture was transferred to yeast peptone dextrose broth and incubated for 24 h at 27 ºC.

Honey-must preparation and fermentation condition for mead fermentation: Stingless bee honey was diluted with tap water (35 g: 85 mL) and mixed to homogeneity. The insoluble solids were removed by filtering to obtain a clarified honey-must. Sulphur dioxide, in the form of potassium metabisulfite, was added up to a concentration of 100 mg/L of free SO_2 to inhibit the bacterial growth. Starter culture was prepared by pre-growing the yeast culture in yeast peptone dextrose broth for 24 h. Incubation was done at 27 ºC with gentle orbital shaker at 120 rpm. Above honey must was inoculated with 4 % inoculum with an initial population of 10^5 colony-forming units (cfu/mL). Fermentation was carried out in 250 mL Erlenmeyer flasks with 100 mL honey must. After 2 days, aerobic fermentation, the Erlenmeyer flasks were water sealed.

Estimation of ethanol: Ethanol content was estimated using refractrometer (%).

Plackett-Burman experimental design: The purpose of this optimization step was to identify which ingredients of the honey-must had a significant effect on alcohol content. The Plackett-Burman [6], statistical experimental design is a versatile method for screening the important variables. The total number of experiments to be carried out is n + 1, where n is the number of variables. For each variable, a high (+1) and low (-1) level was tested. All trials were performed in triplicate. The statistical software package Design-Expert software 8.04 (Stat-Ease Inc.) was used for analyzing the experimental data. The ingredients studied by Placket-Burman design were TSS, pH, temperature, inoculum size, fermentation time, tartaric acid, di ammonium phosphate (DAP), potassium sodium tartrate (PST), magnesium sulphate, calcium sulphate and potassium metabi sulphate (PMS).

These eleven independent variables in twelve combinations were organized according to the Placket-Burman design matrix. High and low concentration of all 11 ingredients was shown in Table-1.

Honey must (Stingless bee honey, May 2011) brought to a sugar concentration of 20 °B by diluted with tap water (35 g: 105 mL), then the above 11 ingredients were added and steam sterilized at 60 °C for 20 min was used for optimization of honey must. All the micro-fermentations were carried out in 250 mL Erlenmeyer flasks containing 150 mL of honey must and inoculated with 5 mL of a 48 h old culture (2×10^6 cells/mL) at 28 ± 1 °C. The anaerobic condition was fully maintained by using cork and water sealed special valve on the mouth of the flasks. The response for the design was measured in the terms of alcohol content. The experimental design based on Plackett-Burman design for the optimization of honey must for stingless bee mead production is given in the Table-2.

Experimental design and response surface methodology: Design Expert Software version (8.0) was used to optimize the fermentation condition for Indian stingless bee mead production. This software applies the principle of response surface methodology to determine the optimal response. Three important factors, namely temperature (A), fermentation time (B) and TSS (C), considered as operating (independent) parameters, were selected to study their effect on ethanol production. Table-3 states the actual values and the coded values of

TABLE-1
NATURAL LEVELS, TYPE AND INTERVALS OF VARIATION OF THE INDEPENDENT VARIABLES IN PLACKETT-BURMAN DESIGN OF EXPERIMENTS

Components	Codes	Type	Levels			Interval of variation
			-1	0	+1	
TSS (°Brix)	A	Numeric	20	22.5	25	2.5
pH	B	Numeric	3.0	4.25	5.5	1.25
Temperature (ºC)	C	Numeric	23	26.5	30	3.5
Inoculum size (%)	D	Numeric	5	6.5	8	1.5
Fermentation time (days)	E	Numeric	10	20	30	10
Tartaric acid (g/L)	F	Numeric	0.5	1.5	2.5	1
Di ammonium phosphate (mg/L)	G	Numeric	200	300	400	100
Potassium sodium tartrate (g/L)	H	Numeric	3	4	5	1
$MgSO_4$ (g/L)	I	Numeric	0.05	0.07	0.09	0.02
$CaSO_4$ (g/L)	J	Numeric	0.1	0.25	0.4	0.15
Potassium metabisulphate (mg/L)	K	Numeric	25	87.5	150	62.5

TABLE-2
PLACKETT-BURMAN EXPERIMENTAL DESIGN USED FOR OPTIMIZATION OF HONEY MUST COMPONENTS FOR STINGLESS BEE MEAD PRODUCTION

E. No.	Independent variable levels: Natural values										
	A	B	C	D	E	F	G	H	I	J	K
1	20.00	3.00	23.00	8.00	10.00	2.50	400.00	3.00	0.09	0.40	150.00
2	20.00	3.00	30.00	5.00	30.00	2.50	200.00	5.00	0.09	0.40	25.00
3	20.00	5.50	30.00	5.00	30.00	2.50	400.00	3.00	0.06	0.10	150.00
4	20.00	5.50	23.00	8.00	30.00	0.50	400.00	5.00	0.09	0.10	25.00
5	25.00	5.50	23.00	5.00	10.00	2.50	200.00	5.00	0.09	0.10	150.00
6	25.00	3.00	30.00	8.00	30.00	0.50	200.00	3.00	0.09	0.10	150.00
7	20.00	3.00	23.00	5.00	10.00	0.50	200.00	3.00	0.06	0.10	25.00
8	25.00	3.00	30.00	8.00	10.00	2.50	400.00	5.00	0.06	0.10	25.00
9	25.00	3.00	23.00	5.00	30.00	0.50	400.00	5.00	0.06	0.40	150.00
10	25.00	5.50	30.00	5.00	10.00	0.50	400.00	3.00	0.09	0.40	25.00
11	25.00	5.50	23.00	8.00	30.00	2.50	200.00	3.00	0.06	0.40	25.00
12	20.00	5.50	30.00	8.00	10.00	0.50	200.00	5.00	0.06	0.40	150.00
E. No.	Independent variable levels: Coded values										
	a	b	c	d	e	f	g	h	i	j	k
1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1
2	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1
3	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1
4	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1
5	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1
6	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1
7	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
8	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1
9	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1
10	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1
11	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1
12	-1	+1	1	+1	-1	-1	-1	+1	-1	+1	+1

TABLE-3
NATURAL LEVELS, CODES AND INTERVALS OF VARIATION OF THE INDEPENDENT VARIABLES IN THE DESIGN OF EXPERIMENTS

Process parameters	Codes	Levels					Interval of variation
		-1.682	-1	0	+1	+1.682	
Temperature (ºC)	A	21.27	24	28	32	34.72	4
Fermentation time (days)	B	-0.72	2	6	10	12.72	4
TSS (°Brix)	C	-1.81	5	15	25	31.81	10

the variables employed. Coded values of +1, 0 and -1 correspond to high, medium and low values of variables, respectively. Ethanol percentage was regarded as the response or output variable (r). The central composite design (CCD) was used to access the effects of the three input independent parameters on the desired responses and build a second order (quadratic) model for the response variable (r). The statistically designed experiments comprised 8 factorial points, 6 axial points and 6 replicates at the centre points resulting in a total of 20 experiments. The ethanol percentage was observed from each of the 20 experiments were analyzed by Analysis of Variance (ANOVA) to determine the optimum conditions. The regression analysis was performed to fit the response.

RESULTS AND DISCUSSION

Optimization of honey must components using Plackett-Burman experimental design: The application of a complete factorial design would require 2^n experiments if 'n' factors have to be investigated. Thus, eleven variables would lead to 256 trials, a huge number. However, the use of the factorial design considerably reduces the number of experiments without losing information about the main effect of variables. Eleven levels of culture variables were examined in the Plackett-Burman design matrix with 12 different trials.

The eleven independent variables (TSS, pH, temperature, inoculum size, fermentation time, tartaric acid, di ammonium phosphate, potassium sodium tartrate, magnesium sulphate, calcium sulphate and potassium meta bisulphate) and their concentrations at different coded and actual levels of the variables employed in the design matrix are shown in Table-2. The design matrix and the experimental responses of the dependent variable (alcohol per cent for SLB mead) are listed in Table-5.

Data from 12 experiments were used for the following equations, where the coded values were obtained from regression analysis for the SLB mead production. The application of Placket-Burman gave rise to the following regression equation for alcohol percentage. Applying the multiple regression analysis on the experiment, the response variables and the test variables are related by following second order polynomial equation:

TABLE-4
CENTRAL COMPOSITE DESIGN EXPERIMENTAL DESIGN USED FOR OPTIMIZATION OF FERMENTATION PARAMETERS FOR STINGLESS BEE MEAD PRODUCTION

Experiment No.	Independent variable levels						
	Natural values			Coded values			
	T	F	TS	t	f	ts	
1	24.00	2.00	5.00	-1	-1	-1	2^k type factorial experiments
2	32.00	2.00	5.00	+1	-1	-1	
3	24.00	10.00	5.00	-1	+1	-1	
4	32.00	10.00	5.00	+1	+1	-1	
5	24.00	2.00	25.00	-1	-1	+1	
6	32.00	2.00	25.00	+1	-1	+1	
7	24.00	10.00	25.00	-1	+1	+1	
8	32.00	10.00	25.00	+1	+1	+1	
9	21.27	6.00	15.00	-1.682	0	0	2k extra experiments with star points
10	34.73	6.00	15.00	+1.682	0	0	
11	28.00	-0.73	15.00	0	-1.682	0	
12	28.00	12.73	15.00	0	+1.682	0	
13	28.00	6.00	-1.82	0	0	-1.682	
14	28.00	6.00	31.82	0	0	+1.682	
15	28.00	6.00	15.00	0	0	0	n_0 base level experiments
16	28.00	6.00	15.00	0	0	0	
17	28.00	6.00	15.00	0	0	0	
18	28.00	6.00	15.00	0	0	0	
19	28.00	6.00	15.00	0	0	0	
20	28.00	6.00	15.00	0	0	0	

TABLE-5
PLACKETT-BURMAN EXPERIMENTAL DESIGN OF INDEPENDENT VARIABLES AND THEIR CORRESPONDING EXPERIMENTAL YIELD OF SLB MEAD ALCOHOL CONTENT

Run No.	Independent variable levels											Response ethanol (%)
	A	B	C	D	E	F	G	H	I	J	K	
1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	3.0
2	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	6.8
3	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	6.3
4	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	9.0
5	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	3.3
6	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	6.0
7	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	2.8
8	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	3.7
9	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	3.4
10	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	9.6
11	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	7.0
12	-1	+1	1	+1	-1	-1	-1	+1	-1	+1	+1	3.6

Final equation in terms of coded factors = + 5.38 + 0.16 * A-0.74 * B + 0.57 * C-0.34 * D + 2.64 * E-0.02 * F-0.66 * G + 0.85 * J

Final equation in terms of actual factors = -3.92 + 0.06 * TSS -0.59 * pH +0.16 * temperature + 0.22 * inoculums size + 0.26 * fermentation time-0.02 * tartaric acid-6.58E-003 * DAP + 56.66 * $MgSO_4$

Screening of honey must components for maximizing alcohol content: The ANOVA results of quadratic regression model for SLB mead alcohol content is described in Table-6. ANOVA of the regression model for alcohol content demonstrated that the model was significant due to an F-value of 19.04 (SLB mead alcohol content) and very low probability value (p models > F-0.0001). The fit of the model was also expressed by the coefficient of determination R^2, which was found to be 0.9807 (SLB mead alcohol content) indicating that 90 % of the variability in the response could be explained by the model. The regression co-efficient along with the corresponding p values for the models of stingless bee mead is presented in Table-7.

TSS, $MgSO_4$, temperature and fermentation time showed positive effects on SLB alcohol content (Table-7). It showed that the regression coefficient of all the quadratic coefficient of above positive components were significant at 0.05 per cent level. The half normal plot and pareto chart as shown in Fig. 1 offers a convenient way to view the results obtained by Placket-Burman design. The pareto chart illustrates the order of significance of variables affecting the alcohol content of SLB mead. TSS, temperature and fermentation time had high influence on the alcohol content. Based on the result the above parameters were chosen for optimizing the mead production using response surface methodology.

The pareto and half normal chart shows the order of significance of variables affecting the alcohol content of SLB mead. Abdulaziz *et al.* [7] isolated fungal strain *Monascus*

TABLE-6
ANALYSIS OF VARIANCE (ANOVA) FOR ALCOHOL CONTENT OF STINGLESS BEE MEAD PRODUCTION USING PLACKETT-BURMAN DESIGN

Source	Sum of squares	df	Mean square	F value	p-value Prob >F
Model	62.12	8	7.76	19.04	0.0170
TSS (A)	0.30	1	0.30	0.74	0.4535
pH (B)	4.40	1	4.40	10.79	0.0462
Temperature (C)	3.97	1	3.97	9.73	0.0525
Inoculum size (D)	0.93	1	0.93	2.29	0.2274
Fermentation time (E)	27.91	1	27.91	68.45	0.0037
Tartaric acid (F)	7.500E-003	1	7.500E-003	0.018	0.9007
Di ammonium phosphate (G)	3.47	1	3.47	8.50	0.0617
Magesium sulphate (J)	1.93	1	1.93	4.72	0.1180
Residual	1.22	3	0.41	–	–
Corrected total	63.34	11	–	–	–
R^2 = 0.9807		Adjusted R^2 = 0.9292		C.V (%) = 11.88	

TABLE-7
REGRESSION COEFFICIENTS FOR OPTIMIZATION OF HONEY MUST FOR ALCOHOL PERCENTAGE IN SLB MEAD PRODUCTION USING PLACKETT-BURMAN DESIGN

Factor	Coefficient estimate	df	Standard error
Model	5.38	1	0.18
TSS (A)	0.16	1	0.18
pH (B)	-0.74	1	0.23
Temperature (C)	0.57	1	0.18
Inoculum size (D)	-0.34	1	0.23
Fermentation time (E)	2.64	1	0.32
Tartaric acid (F)	-0.025	1	0.18
Di ammonium phosphate (G)	-0.66	1	0.23
Magesium Sulphate (J)	0.85	1	0.39

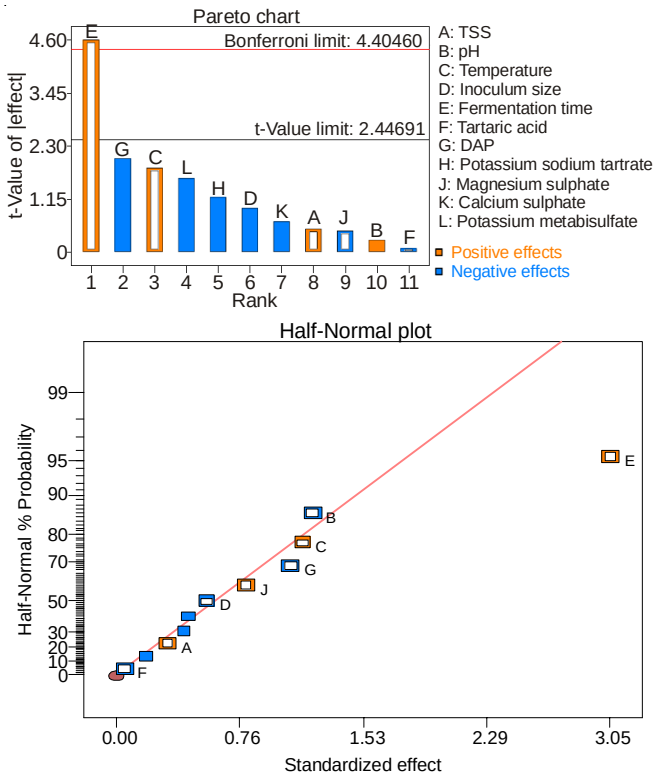

Fig. 1. Pareto chart and Half-Normal plot of Plackett-Burman experimental design for stingless bee mead

ruber which produced an antibacterial substance citrinin using batch cultures. They used Plackett-Burman experimental design to optimize the components of medium to improve citrinin production for non-food applications. They observed 1.75-fold improvement of the antibacterial activity using Plackett-Burman. Imandi and Rao [8] observed maximum lipase activity of 18.58 units per gram of dry fermented substrate in four days of fermentation by *Yarrowia lipolytica* in solid state fermentation (SSF) using Plackett-Burman design.

Maruthai *et al.* [9] statistically screened the medium components on ethanol production from cashew apple juice using Plackett-Burman experimental design. They observed cashew apple juice concentration, yeast extract; diammonium sulphate and malt extract showed significant effect on ethanol production. Padhiar and Modi [10] also screened eight various nutritional parameters using Plackett-Burman experimental design was further optimized by central composite design of response surface methodology for lipase production in submerged fermentation. Among the different carbon sources supplemented, castor oil was most suitable for lipase production while in nitrogen source, meat extract was most suitable.

Optimization by response surface methodology (RSM): The mead fermentation was carried out by controlling various fermentation parameters which were important for production of alcohol. The three independent variables (temperature, fermentation time and TSS) and their concentrations at different coded and actual levels of the variables employed in the design matrix are shown in Table-4. Three level central composite design matrix and the experimental responses of the dependent variable (alcohol per cent SLB mead) are listed in Table-8. The data obtained were used to develop models in which each depended variable was obtained as the sum of the contributions of the independent variables through second order and interaction terms.

Data from 20 experiments were used for the following equations, where the coded values were obtained from regression analysis for the wine production. The application of response surface methodology gave rise to the following regression equation for alcohol percentage.

Applying the multiple regression analysis on the experiment, the response variables and the test variables are related by following second order polynomial equation:

Final equation in terms of coded factors = + 4.66 + 0.07 * A + 0.79 * B + 0.83 * C + 0.06 * A * B-0.06 * A * C-0.15 * B * C-0.63 * A^2-0.88 * B^2 -0.98 * C^2

TABLE-8
CENTRAL COMPOSITE DESIGN MATRIX OF INDEPENDENT VARIABLES AND THEIR CORRESPONDING EXPERIMENTAL AND PREDICTED YIELDS OF SLB MEAD ALCOHOL CONTENT

Run No.	Independent variables			Alcohol (%)	
	Temp. (°C)	Fermentation time (days)	TSS (°Brix)	Observed	Predicted
1	-1	-1	-1	0.40	0.32
2	+1	-1	-1	0.55	0.50
3	-1	+1	-1	2.10	2.07
4	+1	+1	-1	2.50	2.48
5	-1	-1	+1	2.45	2.41
6	+1	-1	+1	2.34	2.31
7	-1	+1	+1	3.57	3.57
8	+1	+1	+1	3.69	3.71
9	-α	0	0	2.69	2.75
10	+α	0	0	2.99	3.01
11	0	-α	0	0.76	0.85
12	0	+α	0	3.50	3.49
13	0	0	-α	0.42	0.50
14	0	0	+α	3.29	3.29
15	0	0	0	4.70	4.66
16	0	0	0	4.65	4.66
17	0	0	0	4.60	4.66
18	0	0	0	4.67	4.66
19	0	0	0	4.71	4.66
20	0	0	0	4.63	4.66

Final equation in terms of actual factors = -33.70 + 2.22 * temperature + 0.80 * fermentation time + 0.44 * TSS + 3.75E-003 * temperature * fermentation time-1.68E-003 * temeprature * TSS-3.68 * fermentation time * TSS-0.03 * temperature^2-0.05 * Fermentation time^2-9.77E-003 * TSS^2

Optimization of fermentation parameters for optimum alcohol content: Based on the experimental response, the alcohol content of stingless bee mead ranged from 0.40 to 4.71 run 1 and 19 had the minimum and maximum mead production respectively. The ANOVA results of quadratic regression model for SLB mead alcohol content is described in Table-9. ANOVA of the regression model for alcohol content demonstrated that the model was significant due to an F-value of 1244.35 (SLB mead) and very low probability value (p models > F-0.0001).

ANOVA for the model explained the response of the depends variable. Table-9 showed that the experimental yields fitted the second order polynomial equation as well as indicated by R^2 value (0.9991). The P values were used as a tool to check the significance of each of the coefficients, which in turn, are necessary to understand the pattern of the mutual interactions between the test variables. The smaller the magnitude of the P, the more significant was the corresponding coefficient. Values of P less than 0.05 indicate model terms were significant.

The regression co-efficient along with the corresponding p values for the models of stingless bee mead is presented in Table-10. It showed that the regression coefficient of all the quadratic coefficient of A, B, C were significant at 0.05 % level.

TABLE-10
REGRESSION COEFFICIENTS FOR OPTIMIZATION OF FERMENTATION PARAMETERS FOR ALCOHOL CONTENT OF STINGLESS BEE MEAD PRODUCTION USING CENTRAL COMPOSITE DESIGN

Factor	Coefficient estimate	Degrees of freedom	Standard error	p value
Intercept	4.66	1	0.026	< 0.0001
Temperature (A)	0.078	1	0.017	0.0010
Fermentation time (B)	0.79	1	0.017	< 0.0001
TSS (C)	0.83	1	0.017	< 0.0001
AB	0.060	1	0.022	0.0220
AC	-0.067	1	0.022	0.0123
BC	-0.15	1	0.022	< 0.0001
A^2	-0.63	1	0.016	< 0.0001
B^2	-0.88	1	0.016	< 0.0001
C^2	-0.98	1	0.016	< 0.0001

The 3D response surfaces based on independent variables were obtained using the same software package indicated that a local optimum exists in the area experimentally investigated. Fig. 2 shows three dimensional (3D response) surface plots of the effect of temperature, fermentation time and TSS on the alcohol content. The alcohol yield was significantly affected by temperature, fermentation time and TSS where temperature produced greater effect.

The results showed that as the values of process variables increased, the yield also increased but only upto the midpoint

TABLE-9
ANALYSIS OF VARIANCE (ANOVA) FOR ALCOHOL CONTENT OF STINGLESS BEE MEAD PRODUCTION USING CENTRAL COMPOSITE DESIGN

Source	Sum of squares	Degrees of freedom	Mean square	F value	P value
Model	43.94	9	4.88	1244.35	< 0.0001
Temperature (A)	0.083	1	0.083	21.15	0.0010
Fermentation time (B)	8.43	1	8.43	2148.04	< 0.0001
TSS (C)	9.39	1	9.39	2394.45	< 0.0001
AB	0.029	1	0.029	7.34	0.0220
AC	0.036	1	0.036	9.29	0.0123
BC	0.17	1	0.17	44.36	< 0.0001
A^2	5.70	1	5.70	1452.19	< 0.0001
B^2	11.15	1	11.15	2843.18	< 0.0001
C^2	13.76	1	13.76	3506.31	< 0.0001
Residual	0.039	10	3.923E-003	–	–
Lack of fit	0.030	5	6.087E-003	3.46	0.0997
Pure error	8.800E-003	5	1.760E-003	–	–
Corrected total	43.98	19	–	–	–
R^2 = 0.9991		Adjusted R^2 = 0.9983		C.V (%) = 2.12	

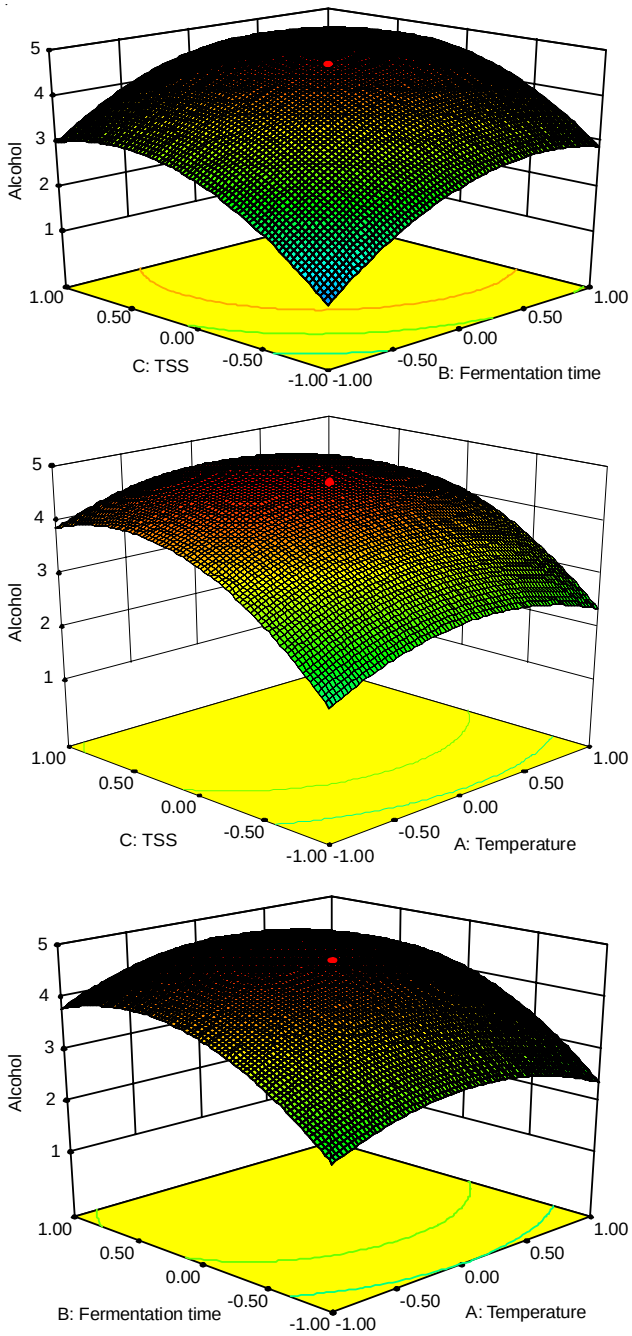

Fig. 2 Response surface curve showing the effects fermentation parameters on the ethanol yield of mead

range of variables and thereafter the yield decreased even though the values of variables increased. Based on the model, the optimal working conditions were obtained to attain optimum alcohol content.

Interaction of fermentation parameters for mead production: Fermentation is slow in a medium containing low sugar; whereas its speed increases in must which have 15 °Brix. More than 15 °Brix, the fermentation process slowed. Thus, an elevated amount of sugar hinders yeast growth and decreases the ethanol concentration [11]. It is known that the high substrate concentrations may cause osmotic shock of the yeast cells and slow down the mass and heat transfer. Glucose and fructose utilization was almost completed within 6 days of fermentation time. The glucose and fructose consumption was in accordance with the results of ethanol concentration since the glucose and fructose was consumed as a carbon source by the yeast. Substrate inhibition significantly effect ethanol yield and their results concerning the substrate inhibition were in agreement with the results in this study [12].

In mead fermentations, the fermentation process is influenced by the temperature. But temperature tolerance for growth of yeast and fermentation is strongly strain dependent [13]. The optimum yield for ethanol production was obtained at 28 ºC. Ethanol production was decreased with the increasing temperature. The reason was that after a period of time this high temperature (34.73 °C) inactivated the yeast cell. It was reported that ethanol producing yeast could grow rapidly at temperature 25-33 ºC [14]. At less than 25 ºC and more than 30 °C, it was not favourable temperature for our yeast strain therefore at these stress conditions ethanol production was lowest. Temperature controls the cell viability, growth rate, exponential phase, enzyme activity and membrane function [15].

The 3D response surface plots described by the regression model were drawn to illustrate the effects of interaction of each independent variable (temperature, fermentation time and TSS) on the response variable [16]. The alcohol yield was significantly affected by temperature, fermentation time and TSS where temperature produced greater effect. The point prediction tool of the software was used to determine the optimum values of the factors for maximum alcohol content: temperature 28 °C, fermentation time 6 days and TSS 15 °Brix. The result confirmed that the model was adequate for reflecting the expected optimization. Hajar *et al*. [17] also studied the fermentation parameters (pH, temperature, inoculum concentration, sugar concentration and time) for maximizing ethanol production. They reported that it could be achieved at the conditions when inoculum concentration 6-14 % (v/v), pH (4.0-6.0), sugar concentration (14-22 °Brix), temperature (24-32 °C) and time of incubation (30-54 h). Ghosh *et al*. [18] optimized the process condition for palm wine fermentation using response surface methodology. In this study temperature, TSS and fermentation time were considered as independent variable.

Conclusion

This study optimized the ethanol yield using Plackett-Burman and response surface methodology. The response surface methodology allowed a rapid screening of the important influence factors and development of a polynomial model to optimize the process parameters for enhancing ethanol yield. Data obtained from experiment were analyzed with response surface methodology software (Version 8) gave the optimum ethanol yield 4.66 % was determined at the optimum condition of temperature 28 °C, TSS 15 °Brix and 6 days after fermentation. The significant regression equation or model at the 5 % level with correlation value 99.96 % was also obtained.

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