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Statistical Screening of Medium Components on Ethanol Production from Cashew Apple Juice using *Saccharomyces Diasticus*

Karuppaiya Maruthai, Viruthagiri Thangavelu, and Manikandan Kanagasabai

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Abstract**—**In the present study, effect of critical medium components (a total of fifteen components) on ethanol production from waste cashew apple juice (CAJ) using yeast *Saccharomyces diasticus* was studied. A statistical response surface methodology (RSM) based Plackett-Burman Design (PBD) was used for the design of experiments. The design contains a total of 32 experimental trails. The effect of medium components on ethanol was studied at two different levels such as low concentration level (-) and high concentration levels (+). The dependent variables selected in this study were ethanol concentration (g/L) and cellmass concentration (g/L). Data obtained from RSM on ethanol production were subjected to analysis of variance (ANOVA). In general, initial substrate concentration significantly influenced the microbial growth and product formation. Of the medium components evaluated, CAJ concentration, yeast extract, $(NH_4)_2SO_4$, and malt extract showed significant effect on ethanol fermentation. A second-order polynomial model was used to predict the experimental data and the model fitted the data with a high correlation coefficient ($R^2 > 0.98$). Maximum ethanol (15.3 g/L) and biomass (6.4 g/L) concentrations were obtained at the optimum medium composition and at optimum condition (temperature-30°C; initial pH-6.8) after 72 h fermentation using *S.diasticus.*

Keywords**—**cashew apple juice; ethanol; fermentation; yeast; response surface methodology

I. Introduction

GROWING attention has been devoted to the conversion of biomass into fuel ethanol as the promising liquid fuel alternative to fossil fuels in recent years [1-5]. Bioethanol has the advantage of reducing greenhouse gas emissions because it has about 10-14% oxygen content [6]. Major technical hurdles in converting lignocelluloses to ethanol include the lack of low-cost efficient enzymes for saccharification of biomass to fermentable sugars and the development of microorganisms for the fermentation of these mixed sugars [7]. Inexpensive waste products from the forestry industry as well as agricultural residues can be utilized as raw material for biofuels [8].

M. Karuppaiya is with the Department of Chemical Engineering, Annamalai University, Annamalai Nagar 608 002 India (phone: 4144-239-737; e-mail: karuppaiya1962@ yahoo.co.in).

T. Viruthagiri is with the Department of Chemical Engineering, Annamalai University, Annamalai Nagar 608 002 India (e-mail: drtvgiri@rediffmail.com).

K. Manikandan is with the Department of Chemical Engineering, Annamalai University, Annamalai Nagar 608 002 India (e-mail: kmchemical_27@yahoo.com).

Forestry industry wastes such as cashew apple or false fruit (*Anacardium occidentale* L.) contains approximately 30% fermentable sugars (fructose and glucose), which can be utilized for fermentation of ethanol [9]. It can also be squeezed for fresh juice, and then fermented into cashew wine, which is a very popular drink in West Africa. In parts of India, it is used to distill cashew liquor, referred to as *fenni* (alcoholic drink) [10]. Cashew apple juice normally has sufficient organic nutrients and minerals (vitamin C, calcium, iron, phosphorus, sodium, and potassium) to make it suitable for ethanol production by fermentation with microorganisms [11, 12]. Furthermore, cashew apple has no commercial value, except for its use by rural inhabitants in the production of homemade alcoholic beverages [13].Screening of appropriate carbon, nitrogen and other nutrients is one of the most critical stages in the development of an efficient and economic bioprocess [14]. Response surface methodology (RSM) is a powerful mathematical model with a collection of statistical techniques by which interactions between multiple process variables can be identified with fewer experimental trials [15, 16]. It is widely used to examine and optimize the operational variables for experimental design, model developing, and factors and conditions optimization [17]. There are various advantages in using statistical methodologies in terms of rapid and reliable shortlisting of process conditions, understanding interactions among them, and a tremendous reduction in total number of experiments. Thus, in the present study, critical medium components (a total of fifteen components) on the production of ethanol from waste cashew apple juice using yeast *Saccharomyces diasticus* were evaluated. Response surface methodology (RSM) adopted Plackett-Burman Design (PBD) was used for the design of experiments and optimization, and a second order polynomial was used for the prediction of experimental data.

II. Materials and Methods

A. Materials

Waste cashew apple juice (CAJ) was exploited as a feed stock for ethanol production, since it contains appreciable amount of sugars (total reducing sugar, TRS 28.5 g/L). Cashew apples were cut into slices and were crushed in a mixer cum grinder. The juice was extracted by a juice squeezer and clarified by adding 1 % gelatin to remove tannin and suspended solids [18, 19]. The treated juice was filtered and treated with sodium or potassium meta-bisulphite to prevent the growth of microorganisms. The juice sample was collected in a jar and preserved at 4°C.

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B. Microorganism and culture maintenance

Yeast *Saccharomyces diasticus* MTCC 251 was obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India. The yeast stock culture was maintained on potato dextrose agar slants of following composition in grams per liter dextrose-20.0; peptone-1.0; and agar-20.0. The culture was periodically subcultured to maintain the cultures active and suitable for fermentation.

C. Cellmass estimation

Centrifuge tubes were well washed and dried in an oven to remove all the moisture content. About 10 ml of the cell broth was centrifuged (10, 000 rpm) for 20 min using high speed centrifuge. The settled biomass was dried in the oven to remove all moisture content. The weight of the cell mass was found from the difference in measured weights [20].

D. Analysis

The ethanol concentration was quantified using NUCON 5765 gas chromatography (GC) with a flame ionization detector (FID) and chromatopak column (10% Carbowax 20 M) using N_2 as the carrier gas. Hydrogen and compressed air were used as fuel gas [21] and the oven temperature was held at 80°C. The injector and detector temperature were maintained at 250°C. Absolute ethanol (0.760 g/ml) was used as the internal standard (injection volume 1.0 µl). The peak eluted was noted (using WINACDS 6.2 software) and by knowing the area of peak, the concentration of ethanol was calculated using calibration chart. Total reducing sugar was measured by the dinitrosalicylic acid (DNS) method [22].

E. Batch fermentation

Ethanol fermentation experiments were conducted in an online monitored modular fermentor (BIOFLO 110 New Brunswick Scientific Co., INC, USA) 3-Litre capacity having provision to measure and control agitation, temperature, pH, dissolved oxygen and antifoam. Experiments were conducted at various initial substrate (cashew apple) concentration keeping the temperature, pH, agitation and dissolved oxygen (DO) as constant using microbes for 72 hours. Samples are drawn at regular intervals of 6 h and centrifuged in a laboratory desktop centrifuge at 1200 rpm. The collected supernatants were analyzed for ethanol and residual sugar concentrations [19]. Finally, residue was used for determination of cell mass.

F. Design of experiments and statistical analysis

RSM based Plackett-Burman Design (PBD) was used for the design of experiments for the screening of 15 critical medium components, as independent variables. The variables were cashew apple juice, yeast extract, malt extract, NaCl, KCl, $ZnSO_4{\cdot}3H_2O$, $NaNO_3$, $(NH_4)_2SO_4$, $MgS0_4{\cdot}7H_2O$, Peptone, $CuSO_4$, Tween80, $CaCl_2$, $MnSO_4$ and KH_2PO_4. Ethanol concentration (g/L) and cellmass concentrations (g/L) were selected as the dependent variables. The design consists of 32 experimental trials and the experiments were conducted in a randomized fashion at two levels of concentrations (high level and low level) using *S.diasticus*, as shown in Table 1. A second order-polynomial equation was used for describing the relationships among the process-dependent variable and the independent variables. The second order-polynomial model is given by equation (1)

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ij} X_i^2 + \sum_{i_i<j}^{k} \sum_{j}^{k} b_{ij} X_i X_j + e \quad (1)$$

where i and j are linear and quadratic coefficients, respectively, b is a regression coefficient, k is the number of factors studied and optimized in the experiment, and e is random error. The quality of fit of the second-order equation was expressed by the coefficient of determination R^2, and its statistical significance was determined by the F-test. The significance of each coefficient was determined using Student's t-test. The coefficients of the equation were determined by employing MINITAB 11 software. Analysis of variance (ANOVA) for the final predictive equation was done using MINITAB 11 software, as presented in Table II.

III. Results and Discussion

A. Optimization of medium components on ethanol production

The effect of critical medium components (total of fifteen components) on ethanol production using yeast *S. diasticus* was studied using PBD experimental design. Table 1 shows the experimental design of 15 independent variables with corresponding ethanol and cellmass production, as dependent variables. The variables were evaluated at different concentrations levels i.e. high level (+) and low level (-), as presented in Table 2. The predicted ethanol yield by the model equation is also shown in Table. 1. It was apparent that the second-order polynomial model well predicted the experimental yields with a high correlation coefficient (R^2 > 0.98). The multiple coefficients of correlation (R) and the determination coefficient of correlation (R^2) were calculated to evaluate the adequacy of the model, data not shown. The minimum (6.43 g/L) and maximum ethanol yield (15.30 g/L) was obtained at run #28 and #23, respectively after 48 h fermentation. The low ethanol yield obtained at run#28 was apparently due to the low supplementation of substrate (cashew apple juice). Thus, substrate concentration significantly influences the ethanol production and microbial growth. It has been demonstrated that high substrate concentration could adversely affect the microbial growth and product formation due to substrate inhibition [23-25]. In addition, presence of other chemicals that are partially inhibitory to the yeast fermentation may also affect the production rate [26, 27]. Among the medium components evaluated, CAJ, yeast extract, $(NH_4)_2SO_4$, KCl, $MgSO_4{\cdot}7H_2O$, peptone, $ZnSO_4{\cdot}3H_2O$, $MnSO_4$, and Tween 80 showed increased ethanol production (Table 1 and Table 2). Whereas, the remaining components such as malt extract, $CuSO_4$, $CaCl_2$, KH_2PO_4, NaCl, and $NaNO_3$ showed decrease in ethanol production (~5 points).

ANOVA result of the quadratic regression model for ethanol yield is described in Table II. ANOVA of the regression model for ethanol yield demonstrated that the model was significant due to a very low probability value (P > F). In general, P-values used as a tool to check the significance of each of the coefficients in turn indicate the pattern of interactions between the variables. A smaller of P-value was more significant to the corresponding coefficient.

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Student's *t*-test was employed to determine the statistical significance of the regression coefficients, and Fischer's test (F) was employed to determine the second-order model equation. The significant factors and their interactions were identified. It can be seen from the degree of significance that the linear terms of concentrations of CAJ and yeast extract have greatest effect, followed by the concentrations of remaining components. Fig. 1 shows the effect of medium components on ethanol production using *S.diasticus*. The above statement is consistent with the effect of components on ethanol production, as shown in Fig. 1. It was also observed that CAJ and malt extract showed the highest (~99%) and lowest (<10%) effect on ethanol production among the components after 72 h fermentation.

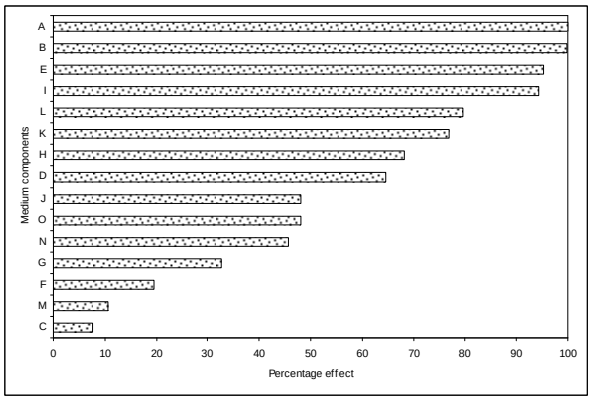


Fig. 1 Pareto plot for the effect of medium components on ethanol production using *S.diasticus* after 72 h fermentation.

B. Optimum concentrations and verification

A quantitative evaluation of ethanol production using *S.diasticus* was performed at the optimum medium concentrations and verified. The critical medium components and their optimum concentration were identified as CAJ-10.0 g/L, yeast extract-2.5 g/L, ammonium sulphate-1.0 g/L, and malt extract-2.5 g/L. It was evident that maximum ethanol (15.3 g/L) and biomass concentration (6.4 g/L) were achieved when the fermentation was conducted at the optimum medium concentrations. It was calculated that about 30-40% relative increase in ethanol concentration was achieved when the fermentation conducted without critical medium components. Hence, medium components play a vital role in ethanol fermentation and microorganism growth.

TABLE I
THE EXPERIMENTAL DESIGN (CODED VALUES) FOR THE OPTIMIZATION OF MEDIUM COMPONENTS ON ETHANOL PRODUCTION USING *S.DIASTICUS*

Trial	Medium components (coded level)*															Ethanol (g/L)		Cell mass (g/L)
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	Exp	Theo	
1	-	-	+	+	-	+	+	+	-	+	-	+	-	-	-	2.2	2.4	1.3
2	+	+	-	-	-	+	+	-	+	+	+	-	+	-	+	12.4	12.5	5.8
3	+	-	+	+	+	-	+	-	+	-	-	-	-	+	-	4.5	4.3	3.0
4	+	-	-	+	+	+	+	+	-	-	-	+	+	-	+	6.4	6.3	3.1
5	-	+	+	+	-	+	-	+	-	-	-	-	+	-	-	1.5	1.5	0.9
6	+	-	-	+	-	+	+	-	-	+	+	+	+	+	-	8.6	8.4	3.2
7	+	+	-	-	+	+	+	+	+	-	-	-	+	+	-	10.7	10.2	4.0
8	-	+	+	-	-	+	+	+	+	+	-	-	-	+	+	4.5	4.0	2.8
9	-	-	-	+	-	-	+	-	+	+	-	-	+	+	+	3.8	3.7	1.9
10	-	+	-	+	+	-	-	+	+	+	+	+	-	-	-	2.6	2.1	1.9
11	+	+	+	+	+	+	-	+	-	+	-	-	-	-	+	13.4	13.5	6.0
12	+	-	+	-	-	-	-	+	-	-	+	-	+	+	-	3.6	3.3	1.8
13	-	-	+	-	-	+	-	+	+	-	-	+	+	+	+	1.5	1.5	1.1
14	+	-	-	-	+	+	-	+	+	+	-	+	-	+	-	4.3	4.5	2.9
15	-	+	-	-	-	-	+	-	-	+	-	+	+	-	-	1.7	1.4	1.0
16	+	-	+	+	-	-	+	+	+	+	+	-	-	-	+	4.5	4.7	2.6
17	-	-	+	+	+	+	+	-	-	-	+	+	-	+	+	2.1	3.4	1.2
18	-	+	+	-	+	+	+	-	+	-	+	-	-	-	-	1.3	1.9	1.0
19	+	+	+	+	-	-	-	+	+	-	+	+	+	-	+	7.6	8.1	4.0
20	+	+	+	-	-	-	+	+	-	+	+	+	-	+	-	10.3	10.3	4.0
21	+	-	-	-	-	+	-	-	+	-	+	+	-	-	+	4.7	4.6	2.9
22	-	-	-	-	+	-	-	+	-	+	+	-	-	+	+	1.6	1.4	1.1
23	+	+	-	+	-	+	-	-	-	-	+	-	-	+	-	15.3	15.8	6.4
24	+	-	+	-	+	-	-	-	-	+	-	-	+	-	+	5.6	5.1	3.3
25	-	-	-	+	+	-	+	+	+	-	+	-	+	-	-	2.5	2.7	1.3
26	-	+	+	+	+	+	-	-	-	+	+	-	+	+	+	2.1	1.8	1.2
27	+	+	+	+	+	-	-	-	+	+	-	+	+	+	-	5.8	5.3	2.9
28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.1	1.5	1.0
29	-	+	-	-	+	-	+	+	-	-	+	+	+	+	+	1.6	1.5	0.9
30	-	+	-	+	-	-	-	-	+	-	-	+	-	+	+	2.4	2.6	1.4
31	+	+	+	-	+	-	+	-	-	-	-	+	-	-	+	6.1	6.2	3.1
32	-	-	+	-	+	+	-	-	+	+	+	+	+	-	-	2.2	2.7	1.5

*Exp- experimental data; Theo- theoretical data; A- cashew apple juice; B-Yeast extract; C-Malt extract; D-$MgSO_4.7H_2O$; E -$CaCl_2$; F-$(NH_4)_2SO_4$; G-$ZnSO_4.3H_2O$; H-$CuSO_4$; I-KH_2PO_4; J-MnSO4; K-Peptone; L-NaCl; M-$NaNO_3$;N-KCl; O-Tween-80;

TABLE II
ANALYSIS OF VARIANCE (ANOVA) OF THE ETHANOL PRODUCTION USING *S.DIASTICUS*

Variables (g/L)	Low level (-1)	High level (+1)	Main Effect	β-coefficient	F-value	*P*-value	Confidence Level (%)
A- cashew apple juice	50	100	5.6313	2.8156	7.16	0.000	100
B-Yeast extract	1	5	2.4813	1.2406	3.16	0.006	99.4
C-Malt extract	1	3	-1.6687	-3.8344	-2.12	0.050	95.0
D-$MgSO_4.7H_2O$	0.1	1	0.6937	0.3469	0.88	0.391	60.9
E -$CaCl_2$	0.1	1	- 0.8438	- 0.4219	- 1.07	0.299	70.1
F-$(NH_4)_2SO_4$	0.1	1	1.8063	0.9031	2.30	0.035	96.5
G-$ZnSO_4.3H_2O$	0.1	1	0.5187	0.2594	0.66	0.519	48.1
H-$CuSO_4$	0.1	1	- 0.0938	- 0.0469	- 0.12	0.907	9.3
I-KH_2PO_4	0.1	1	- 0.5563	- 0.2781	- 0.71	0.490	51.0
J-$MnSO_4$	0.05	1	0.7937	0.3969	1.01	0.328	67.2
K-Peptone	0.5	2	0.4312	0.2156	0.55	0.591	40.9
L-NaCl	0.5	3	- 1.1687	- 0.5844	- 1.49	0.157	84.3
M-$NaNO_3$	0.1	1	- 0.1813	- 0.0906	- 0.23	0.821	17.9
N-KCl	0.1	2	0.4688	0.2344	0.60	0.559	44.1
O-Tween-80	1	2	0.1688	0.0844	0.21	0.833	16.7

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IV. Conclusion

The effect of critical medium components on the fermentation of ethanol from waste cashew apple juice using yeast *S.diasticus* was studied. Only 32 experiments were used to find out the most influential components on ethanol fermentation and the obtained model was adequate ($P < 0.001$). Cashew apple juice (CAJ), yeast extract, $(NH_4)_2SO_4$ and malt extract are found to be very essential for the maximum growth of *S. diasticus* and ethanol production. By solving the regression equation, the optimum components and their concentrations were determined: CAJ-10.0 g/L, yeast extract-2.5 g/L, ammonium sulphate-1.2 g/L, and malt extract-2.5 g/L. Maximum ethanol (15.3 g/L) and biomass (6.4 g/L) concentrations were obtained at the optimum medium composition and at optimum condition after 72 h fermentation using *S.diasticus.* Results indicate that RSM not only helps us locate the optimum concentrations of the process variables in order to enhance the maximum ethanol production, but also proves to be well suited in evaluating the main and interaction effects of the process variables on ethanol production from cashew apple juice.

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