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Bioethanol production by mangrove-derived marine yeast, *Sacchromyces cerevisiae*

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Abstract The present work tested the bioethanol production by two strains of yeast, *Saccharomyces cerevisiae*. The marine strain displayed higher bioethanol production than the terrestrial strain did and hence, it was selected for optimizing the fermentation conditions for ethanol production by using the center composite design of response surface methodology. The R^2 value of 0.92 of statistical model revealed its acceptability. The factors such as temperature, incubation period, acid processed sawdust and agitation were optimized on bioethanol production by *S. cerevisiae*. The marine strain showed the maximum ethanol production of 69.58% of total distillate under the optimal conditions: temperature 30 °C, sawdust concentration of 6.84 mg/l under the agitation speed of 360 rpm at 89 h of incubation. This work revealed that mangrove-derived marine yeast strain of *S. cerevisiae* was potential for bioethanol production over the terrestrial strain.

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1. Introduction

Bioethanol production has gained prominence as an alternative source of energy due to the predictable exhaustion of fuel energy supply (Zaldivar et al., 2001; Ariyajaroenwong et al.,

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2012). The bioethanol production through microbial fermentation provides an economically competitive source of energy (Mcaloon et al., 2000; Yasuyuki et al., 2011). Plant-derived lignocellulose can be used for the production of biofuels after undertaking some pretreatments (Zhang et al., 2007; Minhee et al., 2011). Biological and acid treatments of the lignocellulosic waste materials improve the bioethanol production by *Saccharomyces* sp. (Saritha et al., 2011; Yasuyuki et al., 2011; Lei et al., 2012). Cellulose can be effectively hydrolyzed and depolymerized into sugars which are then fermented to produce ethanol (Mitsunori et al., 2011). Crude enzymes are also used for biological conversion of plant biomass into fuels and high value chemicals, which find a wide range of applications in food, animal feed, textile, fuel and chemical industries (Mitsunori et al., 2011). The pretreatment of lignocellulosic waste materials produces cellulose, glucose and other mono sugars which favor the high yield of ethanol (Jorgensen et al., 2007; Claudio et al., 2011).

Cellulosic materials such as sawdust are renewable natural biological resources and generation of bio-based products and bioenergy from such substances is highly necessary. Various industries across the world generate huge volumes of cellulosic wastes which have an immense potential to be utilized for the production of several bio-products (Das and Singh, 2004). These substances provide a low-cost and uniquely sustainable resource for production of many organic fuels and chemicals that can reduce greenhouse gas emissions, enhance energy security, improve the economy, dispose problematic solid wastes, and improve air quality (Das and Singh, 2004).

The yeast *Saccharomyces cerevisiae* is the potential yeast for the ethanol production from lignocellulose against toxic chemicals present in the substrate (Tomas et al., 2010). Many yeasts are also known to be the potential source of extracellular enzymes to produce ethanol and they are *Pichia stipitis*, *Candida shehatae* and *Pachysolan tannophilus* (Kirk et al., 2002), *P. salicaria* (Kathiresan and Saravanakumar, 2011), *Zymomonas mobilis* (Laura et al., 2011), *S. cerevisiae* (Tomas et al., 2010). Similarly marine algae such as *Undaria pinnatifida*, *Chlorella vulgaris*, *Chlamydomonas reinhardtii* and *Kappaphycus alvarezii* have also been reported as the efficient substrate for ethanol production with bacterial fermentation (Maria et al., 2011, 2012; Soojin et al., 2011; Lin Tanaka, 2006; Tian et al., 2009; Bing et al., 2010; Jin et al., 2011; Maria et al., 2012). However, marine strains of the yeast are less known for their potential in ethanol production. Hence, the present study was conducted to compare the efficacy of marine and terrestrial strains of *S. cerevisiae* on ethanol production and to optimize the fermentation conditions, required for the ethanol production.

2. Materials and methods

2.1. Yeast cultures

Marine strain of *S. cerevisiae* (JN387604) was isolated from mangrove soil. Mangrove soil samples collected from Pichavaram mangrove forest, southeast coast of India were transferred at 4 °C and analyzed for marine yeasts within 4–6 h of sampling. The soil samples were serially diluted and Yeast Malt Agar medium was used for isolation of yeasts (Yeast malt Agar) (Fell, 2005). In this method, sterile media were poured into Petri dishes aseptically and allowed to solidify. One milliliter of the serially diluted sample was pipetted out into sterile Petri-dishes. It was spread in the plate first by rotating it in clockwise and then in anti-clockwise direction for three times and then spread with the help of a "L" rod. The plates were incubated in an inverted position at 28 ± 2 °C. After 7 to 10 days of incubation the yeasts were identified up to the molecular level as *S. cerevisiae* and deposited in National Center for Biotechnology Information (NCBI). Another terrestrial strain of *S. cerevisiae* was obtained from the commercially available baker's yeast. Both strains were maintained at below 4 °C in agar slants and sub cultured for further experimental studies.

2.2. Preparation of sawdust

Sawdust containing cellulose, hemicelluloses and polysugars, was used for the bioethanol production as a substrate after pretreatment. Sawdust was obtained from local wood mills,

sieved through a 1.5 mm sieve for maintaining uniform particle size, and washed with tap water for removing the impurities from sawdust and finally dried at 60 °C overnight. The sawdust was pre-hydrolyzed by using 0.8% phosphoric acid by using the method of Kathiresan and Saravanakumar (2011). The processed sawdust was incorporated at different concentrations into the medium based on the experimental design given in Table 1.

2.3. Selection of potential strain for production of bioethanol

In order to select the potential strain for bioethanol production, a primary experiment was done by using two different strains of yeasts following the method of Caputi et al. (1968). In this method, an inoculum of 1 ml of the yeast (23×10^3 CFU ml^{-1}) was enriched in Yeast Malt Broth production medium (Dextrose-5.0 g, Peptone-5.0 g, Yeast extract-3.0 g and Malt extract-3.0 g in 1000 mL distilled water added with 50% seawater for marine derived strain and distilled water for terrestrial strain). The fermentation was carried out in 500 mL Erlenmeyer flasks using 100 mL of medium. It was kept for fermentation at 28 °C on a shaker at 120 rpm in triplicate. The level of ethanol in the distilled culture filtrate was tested in a gas chromatographically after 120 h of incubation.

2.4. Determination of bioethanol

Concentration of ethanol in the distillate of culture filtrate was estimated by using a Hewlett Packard 5890 Series II gas chromatography with chromosorb 105 column and nitrogen as a carrier gas. The temperature of the injection port, oven and detection port was 250, 120, and 250 °C, respectively. For the analysis, 1 µl of liquid samples was injected into gas chromatography. The ethanol concentration was determined by using an ethanol standard plot and is expressed in percentage.

2.5. Experimental setup by response surface methodology (RSM) for bioethanol production

In this experiment the potential strain of *S. cerevisiae* (JN387604) was selected for optimization. The testing of individual and interaction effect of the temperature (20 to 40 °C), incubation period (h) (0 to 120 h), sawdust (1–10 mg l^{-1}) and agitation (100–500 rpm) on ethanol production was carried out. The maximum yield of ethanol production was tested by using 30 experimental setups derived from a statistical model – central composite design of response surface methodology. The coded values of the fermentation factors were determined by the following Eq. (1).

$$Y = \beta_0 + \Sigma_i \beta_i X_i + \Sigma_i \beta_{ii} X_i^2 + \Sigma_{ij} \beta_{ij} X_i X_j \tag{1}$$

where Y is the predicted response, X_i, X_j are independent variables, β_0 is the offset term, β_i is the ith linear coefficient, β_{ii} is the ith quadratic coefficient and β_{ij} is the ijth interaction coefficient. The experiment design in coded and uncoded value is presented in Table 1. In this study, the independent variables were coded as X_1, X_2, X_3 and X_4. Thus, the second order polynomial equation can be presented as follows in the Eq. (2).

Table 1 Center composite model experimental design along with experimental and predicted values of bioethanol production.

Std run	Temperature	Incubation period (h)	Sawdust (mg/l)	Agitation (rpm)	Bioethanol production (%)	
					Actual	Predicted
1	20	0	1	100	0.60	5.35
2	40	0	1	100	0.50	5.66
3	20	120	1	100	29.35	20.54
4	40	120	1	100	16.59	12.56
5	20	0	10	100	0.20	2.19
6	40	0	10	100	0.30	0.37
7	20	120	10	100	56.59	48.87
8	40	120	10	100	42.26	38.76
9	20	0	1	500	0.30	6.40
10	40	0	1	500	0.56	12.16
11	20	120	1	500	25.69	29.49
12	40	120	1	500	26.35	26.96
13	20	0	10	500	0.56	8.47
14	40	0	10	500	0.69	12.10
15	20	120	10	500	65.60	63.04
16	40	120	10	500	59.26	58.38
17	10	60	5.5	300	32.56	33.06
18	50	60	5.5	300	35.69	28.71
19	30	60	5.5	300	0.26	21.00
20	30	180	5.5	300	25.69	40.47
21	30	60	3.5	300	0.23	6.12
22	30	60	14.5	300	22.27	22.14
23	30	60	5.5	100	19.56	28.85
24	30	60	5.5	700	65.29	49.53
25	30	60	5.5	300	69.25	69.42
26	30	60	5.5	300	69.20	69.42
27	30	60	5.5	300	69.56	69.42
28	30	60	5.5	300	69.58	69.42
29	30	60	5.5	300	69.58	69.42
30	30	60	5.5	300	69.36	69.42

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + B_{11} X_1^2 + \beta_{22} X_2^2$$
$$+ \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4$$
$$+ \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_4 \qquad (2)$$

A statistical program package Design Expert 8.0.6, was used for regression and model fit analysis of the data obtained and to estimate the coefficient of the regression equation, and analyzed the variance of selected factors and model significance.

3. Results

3.1. Selection of the potential strain for the ethanol production

The terrestrial and marine strains of *S. cerevisiae* were tested for their efficiency on ethanol production. The percentage of the ethanol production was calculated with instrumental default standard value of $65 \, g \, l^{-1}$ which is equivalent to 100%. Among the two strains the mangrove-derived marine strain of *S. cerevisiae* produced 38.56% yield as against 21.25% by the terrestrial strain. In other words, the marine strain produced 17.31% higher ethanol than the terrestrial strain did. The marine strain was then selected for further optimization of fermentation conditions for bioethanol production.

3.2. Optimization of bioethanol production by marine strain of S. cerevisiae

The experimental design fitness was tested by the quadratic model along with the contour error plot that are presented in Fig. 1a,b. The bioethanol production for each cycle was performed as per the experimental design. Experimental responses along with predicted response are given in Table 1. The application of the response surface methodology based on the estimates of the parameters indicated an experimental relationship between the response and input variables expressed by the following quadratic model Eq. (3)

$$\text{Ethanol yield } (\%) = 69.42 - 1.09A + 15.37B + 7.07C$$
$$+ 5.17D - 2.07AB - 0.53AC$$
$$+ 1.36AD + 7.87BC + 1.97BD$$
$$+ 1.31CD - 9.63A^2 - 14.92B^2$$
$$- 15.32C^2 - 7.56D^2 \qquad (3)$$

where A is temperature, B is incubation period (h), C is sawdust and D is agitation and these are independent variables. Significance of each coefficient is presented in Eq. (3), determined by Student's *t*-test and *p*-values (Montgomery, 2001) The results of the quadratic model for bioethanol production in the form of analysis of variance (ANOVA) are given in Table 2. The value of R^2 and adjusted R^2 was close to 0.92 and it revealed a
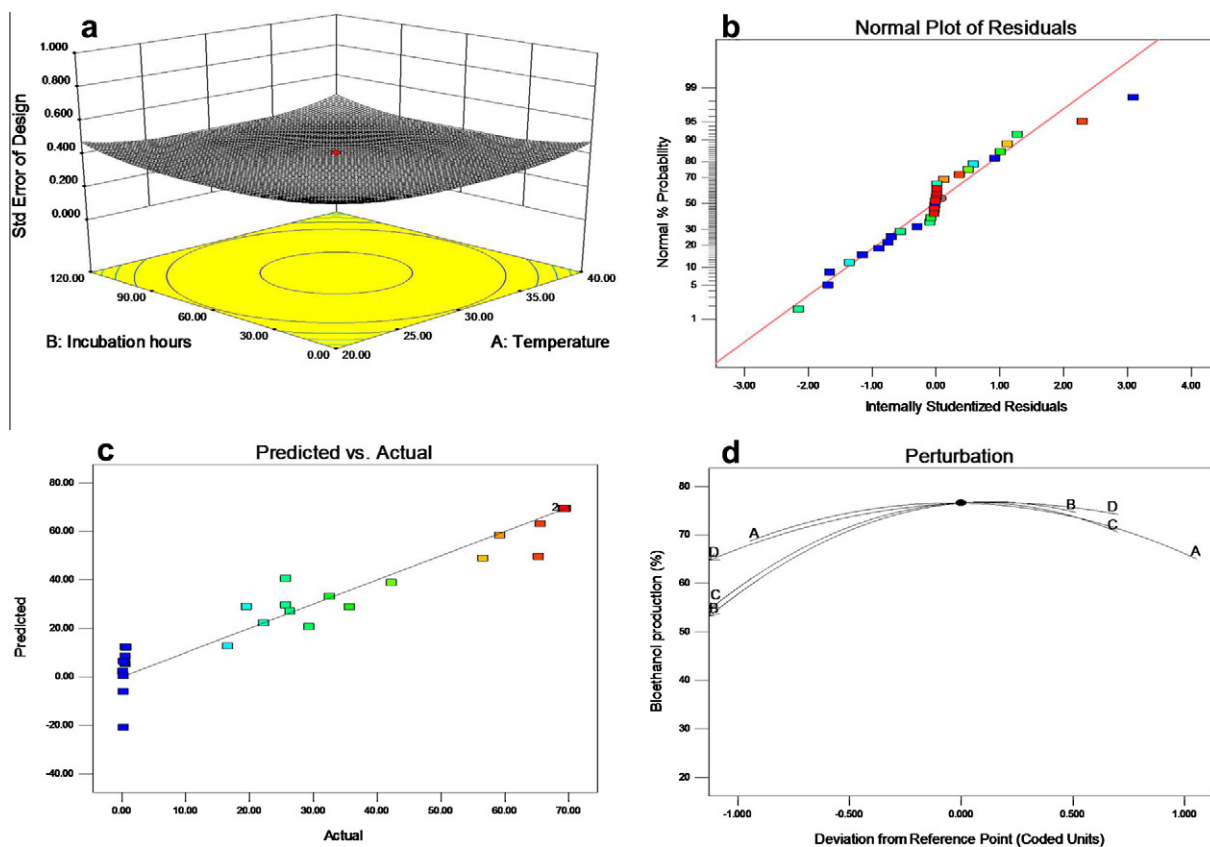
Figure 1 (a) Three-dimensional standard error plot, (b) Normal plot for the residuals and normal percentage of probability for the response of predicted and experimental values, (c) Predicted and actual experimental response for the bioethanol production and (d) perturbation plot for bioethanol production by mangrove derived *S. cerevisiae*.

Table 2 Analysis of variance table (ANOVA) for response surface methodology of main effects and interacting effects of parameters in quadratic model.

Source	Sum of squares	D_f	Mean square	F Value	p-Value Prob > F
Model	20975.75	14	1498.26	13.21	<0.0001***
A-Temperature	28.42	1	28.42	0.25	0.6238NS
B-Incubation period (h)	5668.45	1	5668.45	50.00	<0.0001***
C-Sawdust (mg/l)	1198.27	1	1198.27	10.57	0.0054**
D-Agitation (rpm)	641.49	1	641.49	5.65	0.0311*
AB	68.72	1	68.72	0.60	0.4483NS
AC	4.51	1	4.51	0.03	0.8445NS
AD	29.70	1	29.70	0.26	0.6162NS
BC	991.30	1	991.30	8.74	0.0098**
BD	62.41	1	62.41	0.55	0.4695NS
CD	27.30	1	27.30	0.24	0.6307NS
A^2	2545.49	1	2545.49	22.45	0.0003***
B^2	6106.59	1	6106.59	53.87	<0.0001***
C^2	6464.87	1	6464.87	57.03	<0.0001***
D^2	1567.02	1	1567.02	13.82	0.0021**
Residual	1700.36	15	113.35		
Lack of fit	1700.21	10	70.02	4.492	0.23NS
Pure error	0.15	5	0.03		
Core total	22676.12	29			

Statistically significant, NS Non-significant.
* ($p < 0.05$).
*** ($p < 0.0001$).

high correlation between the observed values and the predicted values. This means that regression model provides an excellent explanation of the relationship between the independent variables (factors) and the response (bioethanol production). The lack-of-fit term was non-significant as it was desired. The non-significant value of lack of fit observed was 0.23 which was more than the probability of 0.05 and this revealed that the quadratic model was valid for the present study. Standard error plot was also predicted and is presented in Fig. 1a–d.

The individual and interaction effects of incubation period and temperature on the bioethanol production were tested. Incubation period was significant but temperature and its interactions were not significant on bioethanol production (Table 2). The maximum bioethanol yield of 69.58% was obtained at a temperature of 30 °C in 89 h of incubation (Fig. 2a).

The sawdust concentration was significant but the interaction effect of the temperature and sawdust was not significant on bioethanol production (Table 2). The maximum yield of 69.58% bioethanol was obtained with the sawdust concentration of 6.84 mg l^{-1} at 30 °C (Fig. 2b).

The individual and interaction effects of the agitation and temperature on the bioethanol production were tested. The individual effect of the agitation was significant but its interaction was not significant on the bioethanol production. Statistically, bioethanol production was significantly increased with increasing rotations per minute (Table 2; Fig. 2c).

Bioethanol production varied significantly between the sawdust concentrations or incubation period (h) and also their interaction and combined effects (Table 2). The maximum yield of 69.58% bioethanol was obtained with the sawdust concentration of 6.84 mg l^{-1} at 89 h of incubation (Fig. 2d).
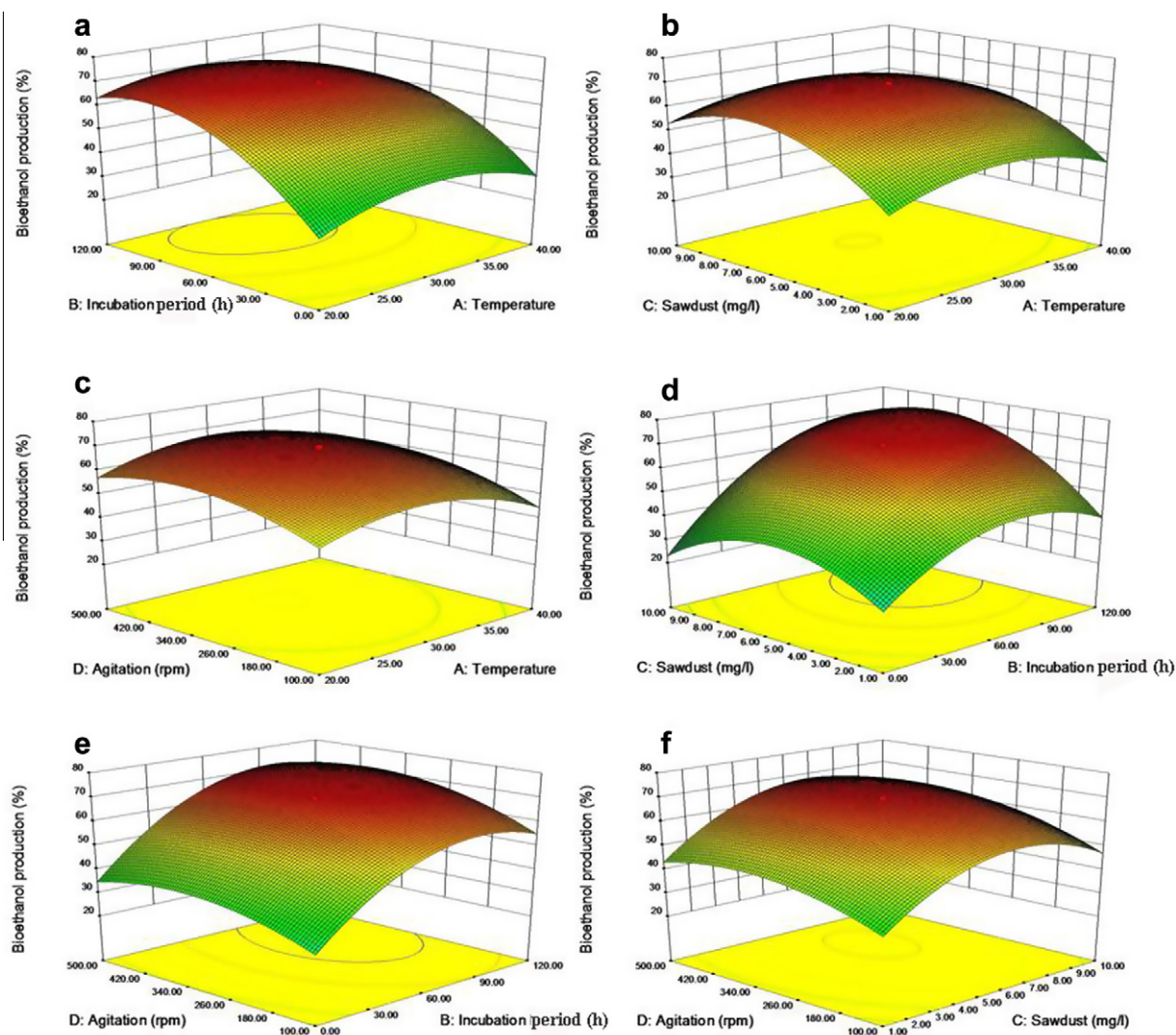

Figure 2 Three-dimensional response surface plot for (a) Effect of incubation period and temperature, (b) Effect of sawdust concentration and temperature, (c) Effect of agitation speed and temperature, (d) Effect of sawdust concentration and incubation period, (e) Effect of agitation and incubation period and (f) Effect of agitation and sawdust concentration, on bioethanol production by *S. cerevisiae*.

Bioethanol production varied significantly between the sawdust concentration and incubation period (h) and also their interaction effects (Table 2). The maximum yield of 69.58% bioethanol was recorded under the agitation speed of 360 rpm at 89 h of incubation (Fig. 2e).

Bioethanol production varied significantly with sawdust concentration and agitation speed but not significantly with interactive effect (Table 2). Statistically optimized condition for the maximum yield of bioethanol production was analyzed by the method of contour plotting (Fig. 2f). The maximum yield of the bioethanol production reached to 69.58% under the optimal conditions of temperature at 30 °C, sawdust concentration of 6.84 mg l^{-1} under the agitation speed of 360 rpm in 89 h of incubation (Fig. 1d). The bioethanol productions were experimentally performed by using statistically optimized fermentation conditions. In that 72.5% of bioethanol was produced by marine yeasts. It was 2.98% higher than that in the statistically optimized condition. It revealed that the statistical optimization was acceptable for the large scale production of the bioethanol.

4. Discussion

Researchers have analyzed bioethanol production by microorganisms of terrestrial or marine origin (Sun and Cheng, 2002; Lin Tanaka, 2006; Kathiresan and Saravanakumar, 2011; Senthilraja et al., 2011). However, the marine and terrestrial strains have not been compared experimentally, but only theoretically for their bioethanol production (Senthilraja et al., 2011). The present work perhaps for the first time compared experimentally the marine and terrestrial strains of S. cerevisiae on the bioethanol production. The present experiment proved that both strains of S. cerevisiae produced the bioethanol but the marine strain produced 17.31% higher bioethanol than the terrestrial strain, indicating the efficiency of marine yeast over terrestrial strain. This is in accordance with Kathiresan and Saravanakumar (2011) who theoretically compared terrestrial and marine yeasts.

Optimization of fermentation conditions on bioethanol production was carried out with the statistical model of response surface methodology. The important fermentation factors such as temperature, incubation period (h), phosphoric acid processed sawdust and agitation speed on bioethanol production were tested and also their individual and interaction effects. The experimental design fitness was tested by the quadratic model along with the contour error plot that are presented in Fig. 1a,b and the value of adjusted R^2 0.85 was close to predicted R^2 value 0.92 that revealed a high correlation between the observed values and the predicted values and also lack of the fit was not significant. It revealed that the present model was acceptable. Hence the present study used sawdust as a cellulose biomass. Among the various types of processing of the sawdust used for alcohol production, McCain (2003) suggested the use of corn for making huge profits from ethanol production. However, the wood cellulose system requires slightly more energy to produce the ethanol than using other sources (Arkenol, 2004). (Not clear). Arkenol (2004) has reported that 2 kg of wood produces 1 l of ethanol. The 2 kg wood materials produce 1 l of ethanol whereas 2.69 kg of corn grain is required to produce the same volume of ethanol (Kidd and Pimentel, 1992). Based on the previous

reports, the wood materials such as saw dust are a potential source for the fuel production than the corn grain and other glucose derivatives. Therefore the present study used sawdust as substrate for the bioethanol production.

Marine strain of S. cerevisiae showed the maximum bioethanol production of 69.58% under the optimal conditions of temperature (30 °C), sawdust concentration of (6.84 mg l^{-1}) under the agitation speed of (360 rpm) in 89 h of incubation (Fig. 1d). Thus the present study suggested that the statistical tool of central composite design of response surface methodology was suitable to find out the reliable values of fermentation conditions for enhanced ethanol production, as supported by the work of Ratnam et al. (2005). The present study concluded that mangrove-derived marine S. cerevisеae was promising over terrestrial strain for bioethanol production.

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