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Screening and Optimization of Fermentation Media for Increased Production of Ethanol from *Gracilaria* sp. using Response Surface Methodology

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The study is aimed at identifying and optimizing physical parameters and composition of fermentation media for enhanced ethanol production from *Gracilaria* sp. (red macroalgae) by *Saccharomyces cerevisiae* using response surface methodology. The sea weed was first undergone for acid hydrolysis and enzyme saccharification for releasing of reducing sugar. These pretreated algal hydrolysates were used for optimization study. The variables such as algal hydrolysate, incubation period, Na_2HPO_4 and pH were found to be significant variables to influence the ethanol production. The identified variables were optimized by Box Benhen design (BBD). The optimum conditions are: algal hydrolysates - 8.5%, incubation period - 1.7 days, Na_2HPO_4 - 0.3gL-1 and pH - 6.1. These conditions were validated by scale up study which revealed an increased production of ethanol (23.612 gL^{-1}).

Key words: Biofuel, Ethanol, Seaweeds, Response surface methodology, Optimization and Saccharification.

The increasing of industries and motors in the world has led to a steep rise for the demand of petroleum-based fuels (Agrawal, 2007). Progressive exhaustion of conventional fossil fuels with increasing energy consumption and green house gas emissions have led to a shift towards alternative, renewable, sustainable, efficient and cost-effective energy sources with lesser emissions (Singh *et al*., 2010; Prasad *et al*., 2007) . Among many energy alternatives, biofuels, hydrogen, natural gas and synthetic gas might come forth as the four strategically important sustainable fuel sources in the foreseeable future. Of these four, biofuels are the most environment friendly energy source. Biofuels are considered an important way of progress for limiting greenhouse gas emissions, improving air quality and finding new energetic resources (Delfort *et al*., 2008).

It is very important to find a substrate suitable for continuous processing which produces a high sugar and low inhibitor concentration. Since they are known to contain a low content of lignin or nonlignin at all they (macroalgae) are attaining massive interests as a potential source for producing bioethanol (Yun *et al*., 2011). Seaweed can grow faster with higher CO2 fixation ability than land plants (Luning and Pang, 2003). Generally, it can be cultivated in the vast expanse of the ocean with free sunlight and no need for nitrogen-based fertilizers (Buck and

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Buchholz, 2004). Seaweeds are classified into three groups such as green, brown, and red, and they contain various types of glucans, polysaccharides composed of glucose (Percival, 1979). While having low lignin content, macroalgae contain significant amount of sugars (50%) that could be used during fermentation for bioethanol production (Wi *et al*., 2009). Among the different types of seaweeds, red seaweed are known for high carbohydrate content and one of the most available seaweed appearing along the warm and shallow coastal area of many sub-tropical countries (Park *et al*., 2012). These features make them an ideal candidate for the production of bioethanol as carbohydrates from macroalgae can be extracted to produce fermentable sugars.

In this aspect, determining the optimum condition and estimation of bioethanol production from macroalgae can be very valuable in industrial applications as the main goals in the present work. Because any kind of raw materials as carbon sources at first, must be converted to glucose and then ethanol fermentation is performed. Modelling such process is difficult and demanding (Kwiatkowski *et al*., 2006). Considerable studies have been done to propose a methodology based on mathematical models (Albiol *et al*., 1993; Schlosser and Bailey, 1990). Major disadvantage of this is that they need a large number of experiments and often the trials are very complicated to describe the experimental observation (Albiol *et al*., 1995). To overcome such problem is to use simple and efficient model like response surface methodology (RSM).

RSM is a potential mathematical model with a collection of statistical techniques where in, interactions between different variables can be identified with fewer experimental trials. It is extensively used to screen and optimize the operational variables for experiment designing and factors and conditions optimization (Lee and Rogers, 1983; Cheynier *et al*., 1983). In recent years, fermentative production of bioethanol from renewable resources has received much attention due to increasing petroleum scarcity. Hence there is a need to extend and implement viable technologies for the production of alternative renewable energy and feedstock. Till date, however, many of the technologies for the production of alternative fuels such as bioethanol are not competitive with the cheap fossils fuels available (Karuppaiya *et al*., 2009). Since, macroalgae is evolutionary diverse and abundant in the world's ocean and coastal waters and their easiest harvesting process, this work has focused the bioethanol production from macro algae. The present study is carried out to optimize the process conditions (physical factors like agitation speed, inoculum level, incubation period, temperature and pH and nutritional factors like algal hydrolysates, yeast extract, urea,(NH_4) $2SO_4$, KH_2PO_4, Na_2HPO_4 and $MgSO_4$. $7H_2O$) using RSM for enhanced production of ethanol from red seaweed (*Gracilaria* sp) by *S. cerevisiae* MTCC174. Their influence of process variables on ethanol production is well studied by central composite design (CCD).

MATERIALS AND METHODS

Collection and Processing of Seaweed Substrate

Red seaweed (*Gracilaria* sp.) was collected from Mandapam coastal region, Southest coast of India. The sample was washed with distilled water to remove the sand particles and epiphytes. Then the seaweeds were dried to remove excess water. The dried samples were cut into small pieces to prepare powder form of seaweeds and stored in precleaned polythene containers. The composition of the Gracilaria sp. was given in Table 1.

Microorganism

Saccharomyces cerevisiae MTCC174 obtained from Microbial Type Culture Collection Centre, Chandigarh, India was used throughout this study. The strain was maintained in yeast extract peptone dextrose (YPD) agar slants containing 2% glucose, 2% peptone, 1% yeast extract and 2% agar and were stored at 4°C by periodic transfer.

Growth conditions

Yeast strains were maintained in Yeast extract peptone dextrose (YPD) agar slants having a composition: 2% glucose, 2% peptone, 1% yeast extract and 2% agar. pH of the medium is maintained at 7.0, and the slants were incubated at 30 °C for 24h. Subculturing was carried out once in a month and culture was stored at 4 °C.

Inoculum preparation

The yeast culture is inoculated into

medium containing same components as in the maintenance medium except agar-agar. A loopful of culture was inoculated on to 25ml of the medium and was incubated in an orbital shaker at 30 °C and 120 rpm for 24h. This culture was then used as inoculum for fermentation process.

Hydrolysis of *Gracilaria* sp

Acid Hydrolysis and Enzyme Saccharification

About two gram of powder of Gracilaria sp. was added separately to 4% H_2SO_4 (80 ml) and heated in an autoclave at 121° C for 30 minutes for high pressure sterilization. Afterwards, each sample was stirred at 150 rpm for 1 hour at 30° C and neutralized with sodium bicarbonate to adjust the pH to 6.5 to 7. The hydrolysate obtained from acid hydrolysis was used for enzymatic saccharification by using cellulase and β- galactosidase (1U) (Sigma Aldrich). The enzymatic saccharification was performed at pH 5.0, 50°C and at 150 rpm for 4h. The supernatant obtained by centrifuging at 8,000 rpm for 10 min was used for ethanol production. Hydrolysate of the sample was used for estimation of reducing sugar by DNS method using glucose as standard (Miller, 1959).

Optimization of physical and chemical parameters

The parameters that were optimized were algal hydrolysates, yeast extract, urea, $(NH_4)\ 2SO_4$, KH_2PO_4, Na_2HPO_4, $MgSO_4.\ 7H_2O$, agitation speed, inoculum level, incubation period, temperature and pH.

Experimental design

RSM is of a group of experimental techniques used for estimating the relationship between experimental factors and for determining their response. The significant variables of ethanol production were screened by Plackett- Burman design. The design was done by Minitab version 15 with the risk factor (α) value of 0.05 (95% level of confidence) for PBD. Criterion of the predicted model acceptance was based on their adjusted coefficient of regression (R2adj) with value of above 0.95. Variables having P values lower than 0.05 and 0.01for PBD and BBD respectively were considered as significant effect on the response.

Plackett Burman Design and Box Benhen Design

In the present study, 12 variables were screened in 27 experimental runs (Table 3). Their low (-1) and high (+1) levels were given in table 4. Based on Pareto chart results, Box Benhen Design matrix was constructed with four significant factors (algal hydrolysate, incubation period, Na_2HPO_4 concentration and pH) each having 3 levels (β1, 0 and 1) with 27 runs as shown in table 5. Rest non-significant factors namely KH_2HPO_4, agitation level, $(NH_4)_2SO_4$ and KH_2PO_4, $MgSO_4.\ 7H_2O$, urea, inoculum level and temperature and yeast extract were maintained at their respective low level values. The dependent variable selected for this study was ethanol (g/l) yield.

A mathematical model, explaining the relationship among the process dependent variable and the independent variables in a second-order polynomial equation, was developed (Giovanni, 1983). Design-based experimental data was matched according to the following second-order polynomial equation

$$Y=\beta_0 +\beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4+ \beta_5X_5+\beta_{11}X_1^{\ 2} + \beta_{22}X_2^{\ 2} + \beta_{33}X_3^{\ 2} +\beta_{434}X_4^{\ 2}+ \beta_{55}X_5^{\ 2} + \beta_{12}X_1X_2+ \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \quad ...(1)$$

Where Y is the measured response, β_0 is the intercept term, β_1, β_2, β_3 are linear coefficient, β_{11}, β_{22}, β_{33} are quadratic coefficient, β_{12}, β_{13}, β_{23} are interaction coefficient and X1, X2, X3 are coded independent variables.

The fitness of the second order polynomial equation was expressed by the regression coefficient (R2), and its statistical significance was determined by F-test. The significance of each regression coefficient was determined using Student's t-test. The coefficients of the equation and analysis of variance (ANOVA) for the final predictive equation was done using MINITAB version 15. The response surface equation was optimized for increased yield in the range of process variables using the MINITAB software version 15 (Minitab Ltd., Coventry CV3 2TE, UK). The respective contour plots were obtained based on the effect of the levels of two parameters (at five different levels each) and their interactions on the yield of ethanol by keeping the other three parameters at their optimal concentrations. From these contour plots, the interaction of one parameter with another parameter was studied. The optimum concentration of each parameter was identified based on the hump in the contour plots.

Estimation of Ethanol

The product obtained from the fermentation medium was a mixture of ethanol, cell mass and water. The water content of the product

was removed by distillation remained with the solid part. It was concentrated in a rectifying column and their ethanol content was estimated by dichromate oxidation method (Neish, 1952).

RESULTS AND DISCUSSION

The major components of the red seaweeds are 56% carbohydrate that can be hydrolyzed to fermentable sugars before transforming into ethanol, and 30.5% moisture that provide good growth of microorganisms and save water material used in the fermentation process. The low lipid content (0.7%) of *Gracilaria* sp. is expected to depend on conversion of carbohydrate feedstock during ethanol production, rather than the lipids conversion (Mcderrmid and Stuercke, 2003).

Hydrolysis of *Gracilaria* sp

The seaweeds are hydrolysed by acid and enzyme and the product was analyzed for their reducing sugar. It was found to be 141± 1.7 and 110± 1.6mg/g biomass of Gracilaria sp treated with acid alone and acid and enzyme (Table 2). This result indicates that acid pretreatment significantly play important role during enzyme saccharification ($P< 0.05$). The similar result was also obtained by previous works (Kim *et al*., 2011; Kumar *et al*., 2013 and Borines *et al*., 2013) and proved the effectiveness of combining acid hydrolysis and enzymatic hydrolysis for saccharification of seaweed.

Optimization of Process Parameters for Ethanol Production

Screening of Parameters Affecting Ethanol Production

The data on ethanol production according to Plackett-Burman design shown in table 3 was subjected to multiple linear regression analysis to estimate t-value, p-value and confidence level of each component. The results indicated that there was a variation in each ethanol production in response to the twenty seven trials employed (ethanol production: 14 to 22 gL^{-1}) with predicted ethanol production. These variations reflected the importance of medium optimization to obtain higher ethanol yield.

PBD screened the key variables among 12 variables via Pareto chart shown in Figure 1. Variables such as algal hydrolysates (%), incubation period, Na_2HPO_4 and pH with confidence level above 95 as represented by regression analysis (Table 6) had a substantial effect on ethanol and were considered for further evaluation by BBD, while remaining variables did not have considerable contribution to ethanol production. This result is correlated to Dasgupta *et al*. (2013) who found that substrate concentration, pH, fermentation time and Na_2HPO_4 are significant variables to influence ethanol production. Ethanol production requires various micro and macro elements along with fermentable sugar and nitrogen which showed best result in optimum production yield where a controlled environment is again a prerequisite (Anupama *et al* 2010).

Box Benhen Design (BBD)

BBD with response is shown in table 5. The coefficients t and p values for linear, quadratic and combined effects are given in Tables 8 at 95% significance level. The individual effect of algal hydrolysate ($p=0.000$) and incubation period

Table 1. Biochemical Composition of *Gracilaria* sp

Composition	*Gracilaria* sp. (Red sea weed) % Mean ± SD*
Moisture	30.5± 1.0
Protein	9.5± 0.3
Lipid	0.7± 0.4
Ash	3.0± 0.4
Carbohydrate	56.3± 1.3

* Standard Deviation

Table 2. Reducing sugar of the biomass after hydrolysis processes of *Gracilaria* sp

Composition	*Gracilaria* sp.(Red sea weed) Mean± SD*
Total Carbohydrate content (%)	56.3± 1.3
Reducing sugar of the biomass after acid hydrolysis (mg/g biomass)	71± 1.6
Reducing sugar of the biomass after two stage hydrolysis (acid and enzyme) (mg/g biomass)	140.6± 1.7

*Standard Deviation

Table 3. Plackett Burman Design for screening of variables affecting ethanol production

Trails	Algal Hydrolysates (%)	Yeast Extract (g/L)	Urea (g/L)	$(NH_4)_2SO_4$ (g/L)	KH_2PO_4 (g/L)	Na_2HPO_4 (g/L) (Days)	$MgSO_4.7H_2O$ (g/L)	Incubation Periods	Temp (°C)	pH Level (rpm)	Agitation level (%)	Inoculum	DV-I	DV-II	DV-III uction (gL^{-1})	Ethanol production (gL^{-1})	Predicted Ethanol	Residue
1	10	1	2.5	5	0.2	0.2	0.05	1	40	5	300	1	1	1	1	22.43	20.78	1.65
2	10	5	0.5	5	0.6	0.2	0.05	1	20	8	100	7	-1	1	1	19.82	18.482	1.338
3	2	5	2.5	1	0.6	0.6	0.05	1	20	5	300	1	1	-1	1	18.76	18.54	0.22
4	2	1	2.5	5	0.2	0.6	0.2	1	20	5	100	7	-1	1	-1	14.22	13.708	0.512
5	10	1	0.5	5	0.6	0.2	0.2	3	20	5	100	1	1	-1	1	21.2	22.194	-0.994
6	10	5	0.5	1	0.6	0.6	0.05	3	40	5	100	1	-1	1	-1	23.24	22.42	0.82
7	10	5	2.5	1	0.2	0.6	0.2	1	40	8	100	1	-1	-1	1	15.3	15.86	-0.56
8	10	5	2.5	5	0.2	0.2	0.2	3	20	8	300	1	-1	-1	-1	19.2	19.196	0.004
9	2	5	2.5	5	0.6	0.2	0.05	3	40	5	300	7	-1	-1	-1	19.7	20.912	-1.212
10	10	1	2.5	5	0.6	0.6	0.05	1	40	8	100	7	1	-1	-1	15.24	16.71	-1.47
11	2	5	0.5	5	0.6	0.6	0.2	1	20	8	300	1	1	1	-1	15.2	15.548	-0.348
12	10	1	2.5	1	0.6	0.6	0.2	3	20	5	300	7	-1	1	1	21.7	21.4	0.3
13	2	5	0.5	5	0.2	0.6	0.2	3	40	5	100	7	1	-1	1	17.2	16.366	0.834
14	2	1	2.5	1	0.6	0.2	0.2	3	40	8	100	1	1	1	-1	19.72	19.162	0.558
15	2	1	0.5	5	0.2	0.6	0.05	3	40	8	300	1	-1	1	1	17.36	17.674	-0.314
16	2	1	0.5	1	0.6	0.2	0.2	1	40	8	300	7	-1	-1	1	19.52	18.732	0.788
17	10	1	0.5	1	0.2	0.6	0.05	3	20	8	300	7	1	-1	-1	19.88	19.874	0.006
18	10	5	0.5	1	0.2	0.2	0.2	1	40	5	300	7	1	1	-1	19.76	20.854	-1.094
19	2	5	2.5	1	0.2	0.2	0.05	3	20	8	100	7	1	1	1	17.23	17.232	-0.002
20	2	1	0.5	1	0.2	0.2	0.05	1	20	5	100	1	-1	-1	-1	17.84	18.876	-1.036

(p=0.021) and pH and the quadratic effect of algal hydrolysate (p=0.000), incubation period (p=0.000), Na2HPO4 (p=0.01), and pH (p=0.00) and the interactive effect of algal hydrolysate and incubation period (p=0.01) and incubation period and pH (p=0.01) were found to be most significant factors on ethanol production from *Gracilaria* sp. Based on the response from BBD, the value for correlation coefficients were determined using regression analysis and was found to be (R2) 0.9772 ethanol production (Table 8). A second order polynomial model fit to the experimental data for optimizing the media for ethanol production by RSM predicts response by 4 variables and their interactions in terms of their coded values

Y= 22.29+2.05*A-0.48*B-0.25*C-2.07*D-2.21*A*A-1.61*B*B-0.91*C*C-3.93*D*D-0.56*A*B-1.10*A*C-0.21*A*D-0.44*B*C+0.91*B*D-0.31*C*D ...(2)

Table 4. Factors with their coded levels

S. No.	Variables	Low point (-1)	Centre Point (0)	High point (+1)
1	Algal Hydrolysate	2	6	10
2	Yeast extract	1	3	5
3	Urea	0.5	1.5	2.5
4	(NH4)2 SO4	1	3	5
5	KH2PO4	0.2	0.4	0.6
6	Na2HPO4	0.2	0.4	0.6
7	MgSO4	0.05	0.13	0.2
8	Incubation period	1	2	3
9	Temperature	20	30	40
10	pH	5	6.5	8
11	Agitation level	100	200	300
12	Inoculum level	1	4	7

Table 5. Regression Analysis of variables used in Plackett Burman Design

Term	Effect	Coefficients	T	P	Confidence interval
Intercept		18.7260	57.92	0.000	100
Algal Hydrolysates (g/L)	2.1020	1.0510	3.25	0.014	98.6*
Yeast Extract (g/L)	-0.3700	-0.1850	-0.57	0.585	41.5
Urea (g/L)	-0.7520	-0.3760	-1.16	0.283	71.7
$(NH_4)2SO_4$ (g/L)	-1.1380	-0.5690	-1.76	0.122	87.8
KH_2PO_4(g/L)	1.3680	0.6840	2.12	0.072	92.8
$Na2HPO_4$(g/L)	-1.8320	-0.9160	-2.83	0.025	97.5*
$MgSO_4.7H_2O$ (g/L)	-0.8480	-0.4240	-1.31	0.231	76.9
Incubation Periods	1.8340	0.9170	2.84	0.025	97.5*
Temperature (°C)	0.4420	0.2210	0.68	0.516	48.4
pH	-1.7580	-0.8790	-2.72	0.030	97.0*
Agitation level	1.2500	0.6250	1.93	0.094	90.6
Inoculum level	-0.5980	-0.2990	-0.92	0.386	61.4

R^2 = 87.73% R^2(adjusted) = 66.70%

Table 6. ANOVA for Plackett Burman Design for ethanol production from *Gracilaria* sp

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	12	104.66	104.66	8.722	4.17	0.034
Residual Error	7	14.63	14.63	2.091	-	-
Total	19	119.30	-	-	-	-

ANOVA was used to test the significance and adequacy of the second order polynomial model. The ANOVA of the model is shown in table 9 for ethanol production at 95% confidence level to evaluate the adequacy of the fitted model. ANOVA of the regression model demonstrated that the model was highly significant and this was also evident from the calculated F-value (F-model=36.71) and probability value (p=0.00). It is also evident that the linear (p=0.000) quadratic effect (p=0.000) and interaction effect (p=0.013) of the variables had greater influence (p=0.05) on ethanol production from *Gracilaria* sp.

Interactive Effect of Variables on Ethanol Production

The interaction between the algal hydrolysate, incubation period, Na_2HPO_4 and pH and their effects on ethanol production were plotted in 3D graphs using Sigma plot- 10 (Fig. 2-7). It showed an increased yield of ethanol was obtained as the concentration of algal hydrolysate and incubation period reached the optimum level beyond which a decline could be observed (Fig 2). Likewise, the effect of algal hydrolysate and Na_2HPO_4 is shown in Fig. 3. The ethanol yield increased with the increase in algal hydrolysate and Na_2HPO_4. In contrast, further increase in algal hydrolysate and Na_2HPO_4 from the optimum value caused reduction in ethanol yield. Effect of pH and algal hydrolysate at hold values for Na_2HPO_4 ($0.4gL^{-1}$) and incubation period (2 days) demonstrates that both variables should be maintained at their optimum level to increase ethanol yield, beyond their optimum level they reduced the ethanol content (Fig 4).

The interactive effect of incubation

Table 7. Box Benhen Design for ethanol production from *Gracilaria* sp

Trails	Algal Hydrolysates (A) (%)	Incubation Periods (B) (Days)	Na_2HPO_4 (C) (g/L)	pH (D)	Ethanol Production (gL^{-1})	Predicted Ethanol production (gL^{-1})	Residue
1	-1	-1	0	0	16.03	16.3425	-0.3125
2	+1	-1	0	0	20.98	21.55583	-0.57583
3	-1	+1	0	0	16.62	16.48917	0.130833
4	+1	+1	0	0	19.34	19.4725	-0.1325
5	0	0	-1	-1	19.38	19.4775	-0.0975
6	0	0	+1	-1	19.12	19.58417	-0.46417
7	0	0	-1	+1	15.96	15.94083	0.019167
8	0	0	+1	+1	14.48	14.8275	-0.3475
9	-1	0	0	-1	16.89	15.96542	0.924583
10	+1	0	0	-1	21.06	20.48875	0.57125
11	-1	0	0	+1	12.56	12.24375	0.31625
12	+1	0	0	+1	15.88	15.91708	-0.03708
13	0	-1	-1	0	20.84	20.06708	0.772917
14	0	+1	-1	0	20.07	19.97375	0.09625
15	0	-1	+1	0	21.23	20.43875	0.79125
16	0	+1	+1	0	18.71	18.59542	0.114583
17	-1	0	-1	0	15.57	16.27375	-0.70375
18	+1	0	-1	0	22.49	22.57708	-0.08708
19	-1	0	+1	0	17.62	17.97542	-0.35542
20	+1	0	+1	0	20.13	19.86875	0.26125
21	0	-1	0	-1	19.63	20.21375	-0.58375
22	0	+1	0	-1	17.08	17.43042	-0.35042
23	6	1	0.4	8	14.16	14.25208	-0.09208
24	6	3	0.4	8	15.24	15.09875	0.14125
25	6	2	0.4	6.5	22.64	22.28667	0.353333
26	6	2	0.4	6.5	21.89	22.28667	-0.39667
27	6	2	0.4	6.5	22.33	22.28667	0.043333

period and Na_2HPO_4 is displayed in figure 5 and it showed that as incubation period and Na_2HPO_4 values increased, the ethanol concentration also increased but this was only upto the midpoint of variables and thereafter the ethanol concentration decreased though the two variables increased. Figure 6 showed the effect of incubation period and pH. The ethanol productivity was increased with increasing incubation period and pH till their optimum level (1.7 and 6.1 days respectively). However, the yield was reduced for a further increase in incubation period and pH value. The similar interactive result was produced for the variables, Na_2HPO_4 and pH (Fig. 7).

Validation of Predicted Model

The response optimizer in Minitab Version 15.0 software was used to obtain optimum value of the variables for maximum ethanol production by *Gracilaria* sp. The optimum value of the variables in actual unit was predicted as algal hydrolysate (8.5%), incubation period (1.7days), Na_2HPO_4 (0.3 gL^{-1}) and pH (6.1) with the predicted maximum ethanol production of 23.3424 gL^{-1}. The data was further evaluated by shake flask study where the experiments were performed under optimized condition. A mean value 23.612 $gL^{-1}$of ethanol production was acquired from experiments which are marginally identical to the predicted

Table 8. Regression Analysis of variables used in BBD for ethanol production from *Gracilaria* sp

Term	Coefficient	Standard Error	T value	P value	Confidence interval
Constant	22.29	0.37	60.96	0.000	100*
A	2.05	0.18	11.21	0.00	100*
A	-0.48	0.18	-2.65	0.021	97.9*
B (days)	-0.25	0.18	-1.38	0.19	81
C	-2.07	0.18	-11.34	0.00	100*
D	-2.21	0.27	-8.05	0.00	100*
A*A	-1.61	0.27	-5.88	0.00	100*
B*B	-0.91	-.27	-3.30	0.01	99*
C*C	-3.93	0.27	-14.31	0.00	100*
D*D	-0.56	0.32	-1.38	0.20	80
A*C	-1.10	0.32	-3.48	0.01	99*
A*D	-0.21	0.32	-0.67	0.52	48
B*C	-0.44	0.32	-1.38	0.19	81
B*D	0.91	0.32	2.87	0.01	99*
C*D	-0.31	0.32	-0.96	0.35	65

R^2=97.72%; R^2(adj) = 95.06 %
A: Algal Hydrolysates (%); B: Incubation period (days); C: Na_2HPO_4 (g/L), D: pH
t – student's test, p – corresponding level of significance, * Significant

Table 9. ANOVA of Regression model for Ethanol production from *Gracilaria* sp

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	206.083	206.083	14.7202	36.71	0.00
Linear	4	105.547	105.547	26.3866	65.80	0.00
Square	4	89.819	89.819	22.4548	56.00	0.00
Interaction	6	10.718	10.718	1.7863	4.45	0.013
Residual Error	12	4.812	4.812	0.4010	-	-
Lack-of-Fit	10	4.528	4.528	0.4528	3.19	0.262
Pure Error	2	0.284	0.284	0.1420	-	-
Total	26	210.895				

F - Fischers's function, p - corresponding level of significance; * significant

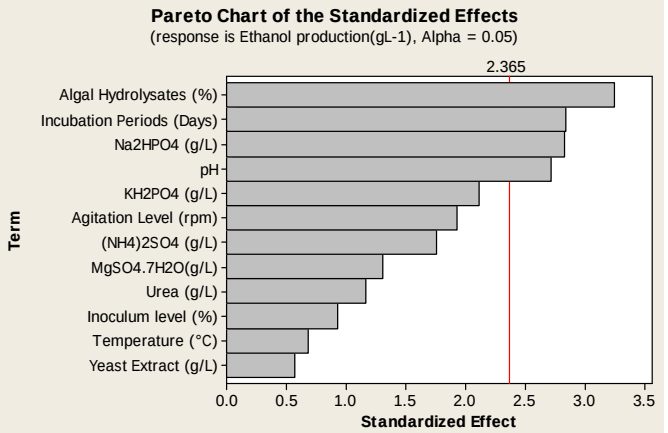


Fig. 1. Pareto Chart of Plackett Burman Design for screening of ethanol production from *Gracilaria* sp

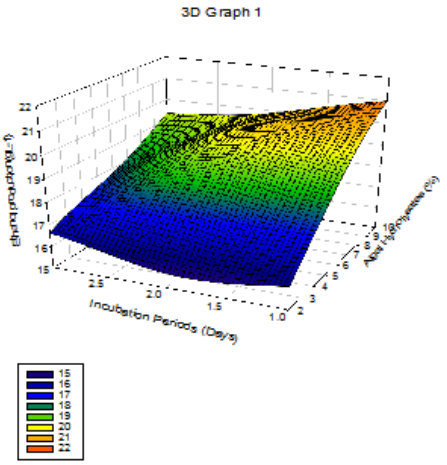


Fig. 2. Interactive Effect of Algal hydrolysate (%) and incubation period (days) on ethanol production (gL^{-1})

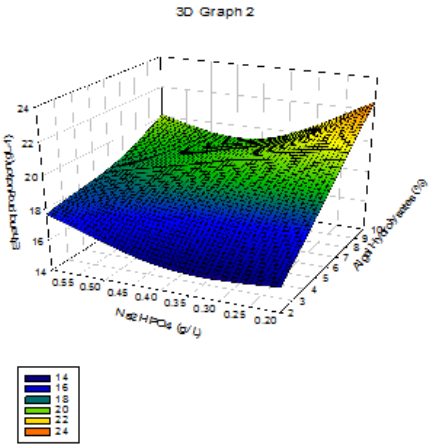


Fig. 3. Interactive Effect of Algal hydrolysate (%) and Na_2HPO_4 (gL^{-1}) on ethanol production (gL^{-1})

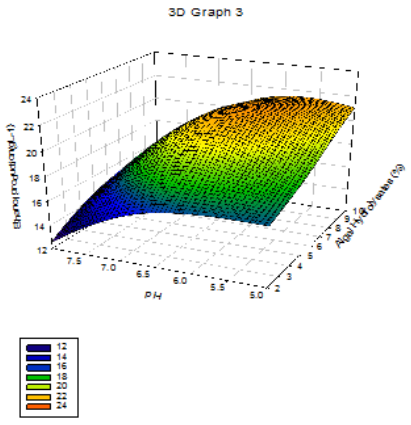


Fig. 4. Interactive Effect of Algal hydrolysate (%) and incubation pH on ethanol production (gL^{-1})

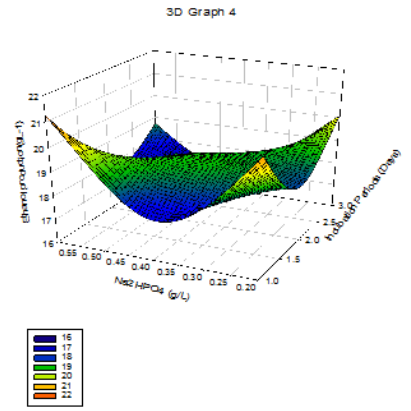


Fig. 5. Interactive Effect of Algal hydrolysate (%) and Na_2HPO_4 (gL^{-1}) on ethanol production (gL^{-1})

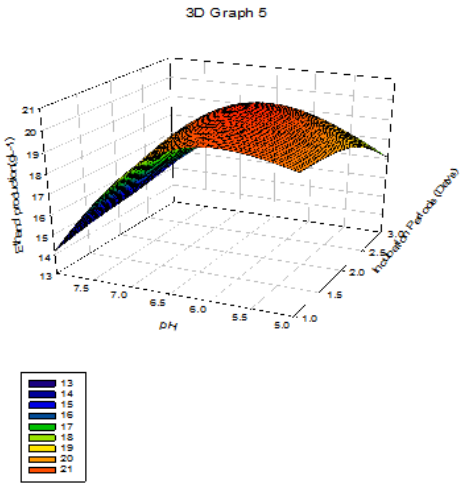


Fig. 6. Interactive Effect of incubation period (days) and pH on ethanol production (gL^{-1})

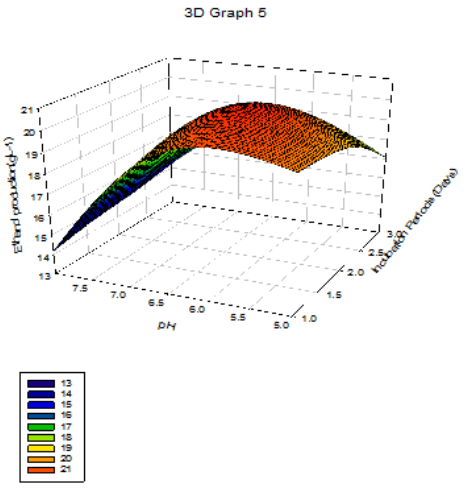


Fig. 7. Interactive Effect of Na_2HPO_4 (gL^{-1}) and pH on ethanol production (gL^{-1})

value. It showed the accuracy of the predicted model and confirmed the optimum point within the system for attaining increased ethanol production. From this study, it is evident that the use of statistical optimization approach, RSM has helped to find out the most significant conditions with minimum effort and time. In addition, it has also proved to be useful in increasing ethanol concentration. Only 27 experiments were necessary and the obtained model was adequate ($P < 0.001$). A similar attribute of result was obtained by Dasgupta *et al.* (2013) to optimize the fermentation media for ethanol production from bagasse pith hydrolysate by *Kluyveromyces* sp. IIPE453. The ethanol yield obtained from red sea weed (*Gracilaria* sp.) is found to be significantly high in comparison to other processes utilizing different feedstock like bagasse pith hydrolysate- 17.44g/L (Dasgupta *et al.*, 2013); tapioca stem- 8.64g/L (Man *et al.*, 2010); cashew apple juice- 15.64g/L (Karuppaiya *et al.*, 2010). Some other feed stocks (soft wood- 27.40g/L (Wan *et al.*, 2012); kinnow waste and banana peels- 26.84g/L (Han *et al.*, 2011)) also showed marginally increased ethanol production than the seaweeds. But seaweeds have been considered as an excellent feedstock for bioethanol production due to their cheap and easy availability and their high cellulosic content.

CONCLUSION

Due to the falling of fossil fuel resources, microbial production of biofuel from marine sea weeds has acquired significance as a fuel for the future. This study looks at the possibility of marine sea weed for ethanol production. Medium optimization by conventional methods may lead to unreliable and wrong conclusions and also time consuming and expensive. In this study, screening and optimization of significant variables for increased ethanol production was effectively attained by response surface methodology. The algal hydrolysate used in this study was pretreated with acid and enzymes for releasing of reducing sugars. The yeast used in the present study is Saccharomyces cerevisiae MTCC. Among 12 variables, 4 variables such as algal hydrolysate, incubation period, Na_2HPO_4 and pH were identified as most significant variables affecting ethanol production by PBD. The optimization of each variable was done by BBD. Final ethanol yield under optimized conditions was 23.612 $gL^{-1}$and ....% which was correlated to the model predicted value (23.3424 gL^{-1}). This work would pave a way for utilizing a novel renewable feedstock for ethanol production. Further studies are required for scaling up to industrial level.

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