Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(1): 1386-1398
Received: 21-11-2018
Accepted: 25-12-2018

Poonam Sharma
PhD scholar, Microbiology laboratory, Department of Basic sciences, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni Solan, Himachal Pradesh, India

Nivedita Sharma
Professor, Microbiology laboratory, Department of Basic sciences, Dr. YS Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India

Nisha Sharma
Research Associate, Microbiology laboratory, Department of Basic sciences, Dr. YS Parmar University of Horticulture and Forestry Nauni, Solan, Himachal Pradesh, India

Correspondence
Nisha Sharma
Research Associate, Microbiology laboratory, Department of Basic sciences, Dr. YS Parmar University of Horticulture and Forestry Nauni, Solan, Himachal, Pradesh, India

Scale up and optimization of process parameters for high gravity ethanol fermentation from a fresh water algae *Rhizoclonium* sp. of Trans Himalayas using Taguchi orthogonal array design, kinetics and modeling

Poonam Sharma, Nivedita Sharma and Nisha Sharma

Abstract
The optimization of agitation rate, aeration timing, substrate concentration for ethanol production from fresh water *Rhizoclonium* sp. algal biomass of Trans Himalayas by *Saccharomyces cerevisiae* was designed using an L9 (3^4) orthogonal array design. The results showed that the optimum condition for ethanol fermentation were $A_2B_3C_2$ corresponding to agitation rate, 200 rpm, aeration timing 8 h and substrate concentration 40 g/lt. The maximum ethanol found in Run 7 at 48 h with 37.34 g/lt ethanol with fermentation efficiency of 71.15%. The modified Monod and Luedeking-Piret models provided a precise way for mathematical modeling of experimental data for optimum ethanol production.

Keywords: Bioethanol; orthogonal array design; *Saccharomyces cerevisiae; Rhizoclonium* sp

1. Introduction
The demand for energy is growing globally due to increase in population and industrialization (Sharma and Sharma, 2018) [20]. Generally, it is a common thing to know that fossil fuels are non-renewable, finite resources and could harm the environment because of its gas emission. Recently, research and development in finding and improving the production of alternative energy to fossil fuels has been increasing rapidly, one of the most known product is bioethanol. The biorefinery industry is looking for cheaper, abundant and more accessible feedstock for producing bioethanol (Wang *et al.* 2012) [23]. Most of the industrial processes are currently based on hexose carbohydrates from starch or sucrose-containing biomass (Kumari and Pramanik, 2012; Duhan *et al.* 2013) [13, 7]. Among these substrates, algal biomass has received a major interest being a carbohydrate rich biomass for its bioconversion to ethanol. The performance of multiple experiments by analyzing one variable at a time (OVAT) approach is time consuming and laborious for identifying various independent variables with their effects (Vishwanatha *et al.* 2010) [22]. Statistically based experimental designs, namely, Plackett-Burman design, Box Behnken design and Taguchi- orthogonal array design is important for the collection and sorting of variables to be taken for consideration, determine the variable amount and analyze the variable at different parameters and finally the effect of variable error for enhancing the yield of ethanol production. Better quality at low cost is the main aim for generation of Taguchi design of experiments (DOE) approaches to maximize robustness of products and processes (Antony *et al.* 1998). Taguchi method, also known as the Orthogonal Array Design (OAD) incorporates the advantages of the simplex method and factorial design (Taguchi, 1990) [21]. OAD notably reduces the number of tests and obtains the optimum value. It also arranges different factors for effective optimization of the experimental conditions (Ke *et al.* 2009; Yang *et al.* 2011) [11, 24]. With increasing interest in the industrial application of batch alcoholic fermentation, various kinetic models have been examined for microbial growth, product formation and substrate consumption. Kinetic modeling may be regarded as an important step in developing a fermentation process, since models help in process control, reducing process costs and increasing product quality. The aim of this study was to optimize the three main parameters, namely agitation rate (100, 200 and 300 rpm), aeration time (2, 4 and 6 h) and substrate concentration (30, 40, 50 g/lt) with one factor as blank for error estimation for ethanol production from pretreated *Rhizoclonium* sp. algal hydrolysate by *Saccharomyces cerevisiae* using the L9 (3^4) orthogonal array design. This work symbolizes a step towards interpretation of this process to industrial scale and the various kinetic parameters have been explained for this technological process that

provide insight to meet out the final objective of the present study.

2. Material and Methods

2.1 Biomass collection and identification

The fresh water green algae had been collected from different districts Trans Himalayan belts of Himachal Pradesh, India. The algae was identified with the help of Algae Identification Field Guide as *Rhizoclonium* sp.

2.2 Ethanologens used

Fermentation of the algal hydrolysate was carried out using yeast strain *Saccharomyces cerevisiae* I (MTCC 3089).

2.3 Scale up and optimization of fermentation parameters by using Taguchi orthogonal array design under Separate Hydrolysis and Fermentation (SHF)

2.3.1 Batch Fermentation in a stirred tank bioreactor

Batch cultivation for bioethanol production was carried out in a 7.5lt stirred tank bioreactor (New Brunswick Scientific, New Jersey USA) with a 3 lt working volume at 25°C and pH 5.5, utilizing the conditions optimized in the shake flask experiment by performing 9 runs. The enzymatic hydrolysate was seeded with 10% inoculum of *S. cerevisiae*-I (MTCC-3089) under aseptic conditions. The fermentation was carried out under anaerobic conditions. The samples were withdrawn regularly at different intervals ranging from 6, 12, 24……..54 h and ethanol yield as well as reducing sugars were estimated.

2.3.2 Orthogonal Experimental Design

Different conditions for bioethanol production from pretreated biomass under separate hydrolysis and fermentation (SHF) mode were optimized in batch mode by employing Taguchi method of Orthogonal Array Design. The independent variables selected were agitation rate, aeration time, aeration rate, substrate concentration and the dependent variables were ethanol (g/lt), ethanol (g/g) and fermentation efficiency (%). The L_9 (3^4) orthogonal table was designed to investigate the influence of four main parameters: agitation rate (A), aeration time (B), blank (C) and substrate concentration (D). Each factor was set at three levels (A; 100, 200, 300 rpm, B; 4, 6, 8 h, C; blank, D; 30, 40, 50 g/lt. There were 9 runs in total. In Taguchi method, orthogonal arrays and analysis of variance (ANOVA) were used for analysis.

Table: The values for independent variables used in the L_9 (3^4) orthogonal design

Run	Factor A Agitation rate (rpm)	Factor B Aeration time (h)	Factor C Blank	Factor D Substrate Concentration (g/lt)
1	100	6	3	40
2	100	8	1	50
3	300	4	3	40
4	100	4	2	30
5	300	8	2	30
6	200	4	3	50
7	200	8	1	40
8	200	6	2	30
9	300	6	1	50

2.3.3 Statistical analysis

The statistical software package Design Expert® version 7.0 (Stat Ease, Inc, Minneapolis, USA) was used for regression analysis of the experimental data to obtain working parameters and to generate polynomials and the contour plots. A second-order polynomial equation was established based on analysis of variance and the optimum ratio of the medium components was found using the Design-Expert 7.1 software optimization toolbox. Standard deviation, PRESS, R^2 values were also analyzed.

2.3.4 Model validation

The mathematical model generated during Taguchi orthogonal array implementation was validated by conducting check point studies. The experimentally obtained data were compared with the predicted one and the prediction error was calculated.

2.4 Mathematical and kinetic modeling for ethanol production

Microbial fermentation does not essentially follow the conventional kinetic model of substrate-limiting biomass growth and product formation proposed by Monod Eq. (1). Therefore, the logistic equation is used as an alternative empirical function (Luedeking and Piret 1959) [14] for microbial cell growth.

Monod equation= $\mu = \mu_m S/(Ks+S)$----------(1)

Where μ = specific growth rate, / μ_{max} = maximum specific growth rate, S = substrate concentration, Ks = substrate, saturation constant (i.e. substrate concentration at half μ_{max}). In Monod's model, the growth rate is related to the concentration of a single growth-limiting substrate though the parameters μ_{max} and Ks.

$Y_{x/s} = dx/ds$---------- (2)

$Y_{x/s} = X_m\text{-}X_o/S_o\text{-}S_m$ $\mu = dx/dt.1/x$----------(3)

In addition to this, Monod also related the yield coefficient (Yx/s) (equation 2) to the specific rate of biomass growth (m) and the specific rate of substrate utilization (q) (equation 3).

$q_p = \alpha\mu+\beta$ $q_p = dp/dt = \alpha dx/dt + \beta x$----------(4)

Where α = growth associated constant, β = non-growth associated constant.

Luedeking-Piret equation (Eq. 4) was applied for comparison between experimental (actual) results and the model prediction for ethanol production. During this modeling, rate of ethanol production is linearly proportional to the biomass growth rate.

2.5 Recovery of ethanol as fuel grade alcohol (Nuwamanya *et.al.* 2012) [17]

Ethanol was separated from the fermentation broth by using

three phase distillation procedure in which first distillation was carried out at a temperature range of 20–94ºC to recover the first distillate. The distillate was then redistilled at 90ºC twice consecutively, to produce ethanol for further evaluation. The amount of ethanol produced from each 500 ml batch was used to calculate the ethanol concentration by using back titration method. The pH of the distillate was measured with the help of pH meter. The different attributes i.e. ethanol concentration, titrable acidity, presence of chlorides and sulphates, copper content were assessed.

3. Results and Discussion

3.1 Collection and identification of algal sample

In the present study, the algae samples were collected from different water bodies of Trans Himalayas Himachal Pradesh i.e. from Mandh and Khabli khad at Kangra, Sunail Khad at Bilaspur and Sheer Khad at Hamirpur districts. The alga was identified as *Rhizoclonium* sp. based upon its morphological characteristics with the help of algal monographs (Anand, 1998).

3.2 Scale up and optimization of process parameters under SHF in a batch mode using stirred tank bioreactor

3.2.1 Scale up of ethanol in a stirred tank bioreactor

Batch ethanol fermentations of Run 1 to Run 9 were carried out and compared in terms of ethanol yield and fermentation efficiency as shown in table 1. With increase in fermentation time, an increase in ethanol production was noted till fermentation period of 48 h, afterwards ethanol production reduced significantly at 54 h. In run 1, the maximum ethanol yield observed was 31.97 g/lt with fermentation efficiency of 64.59%. Aeration for the first 6 h had caused oxygen to be soluble in the aqueous broth and the initial aerobic growth of yeast cells was extremely important to increase yeast cells and to dictate the fermentation. Hammond (2012) [10] reported that the oxygen present earlier in the fermentation was rapidly used up for the synthesis of membrane components which were essential for growth. In run 2 the highest ethanol production was noted at 48 h with 30.81 g/lt ethanol and 64.60% fermentation efficiency. As compared to run 1, a slight decrease in ethanol production was observed even at high aeration time and substrate concentration. The decrease may be attributed due to substrate inhibition caused by high substrate concentration of 50 g/lt. In run 3, the highest ethanol yield noticed was 33.97 g/lt with fermentation efficiency of 66.34%. Both the permeation intensity of nutrition materials from the fermentation broth to the inside of yeast cells and that of ethanol from the inside of yeast cells to the fermentation broth was improved by increasing the agitation rate (from 100 to 300 rpm). These processes enhance the sugar utilization and weaken the inhibition of the ethanol to the yeast cells. Run 4 gave a significantly lower ethanol production in comparison to previous runs. Even highest ethanol yield observed in this run was 23.70 g/lt which was comparatively lower than previous runs. The decrease was due to low substrate concentration as lesser amount of fermentable sugars was present in fermentation broth for utilization by yeast cells. The run 5 consisted of Agitation rate: 300 rpm, Aeration time: 8 h, Substrate concentration: 30 g/lt. A low substrate concentration of 30 g/lt resulted in low ethanol productivity as 27.65 g/lt with fermentation efficiency of 58.70, but ethanol productivity in run 5 was higher than run 4 with substrate concentration 30 g/lt, the increment in ethanol fermentation was found to be due to increase in agitation rate and prolonged aeration time as these play a significant role in ethanol fermentation. The higher ethanol production in run 6 was reported at 48 h of 32.39 g/lt ethanol with 0.34 g/g ethanol yield and fermentation efficiency of 66.53%. Run 7 showed the profile of ethanol production over increase in fermentation time. As noticed in previous runs, increase in ethanol yield was observed with increase in fermentation period. The maximum ethanol formation was shown at 48 h with 37.34 g/lt ethanol and efficiency of 71.15%. The ethanol productivity reported in run 7 found highest over all other runs. The high ethanol productivity was due to a process balance between agitation rate, aeration time and substrate concentration as these conditions proved optimal for production of maximum ethanol. Further Run 8, comprised of different parameters as agitation rate: 200 rpm, aeration time: 6 h, substrate concentration: 30 g/lt had shown a dip in amount of ethanol i.e 25.28 g/lt at 48 h with ethanol yield 0.29 g/g. The ethanol production was low due to low substrate concentration leading to fewer amounts of sugars in fermentation broth. In case of run 9, maximum ethanol production reported was 33.18 g/lt, ethanol yield 0.35 g/g and fermentation efficiency 68.49% at 48 h of fermentation time. As depicted in table 1, at high substrate concentration other than optimized level, the total sugars had not been completely consumed by *S. cerevisiae*. This might be due to the osmotic stress occurred under these conditions. The profiles of parameters measured during the batch ethanol fermentation were similar in all 9 runs. The fermentation period of 48 h which was shown to produce the maximum ethanol, was further selected for orthogonal array analysis of 9 runs.

Table 1: Optimization of scale up parameters for bioethanol fermentation from pretreated *Rhizoclonium* sp. algal biomass in stirred tank bioreactor by using Taguchi's orthogonal array design

Std	Run	Block	Factor A agitation rate (rpm)	Factor B aeration time (h)	Factor C Substrate concentration (g/lt)	Response 1 Ethanol conc. (%)		Response 2 Ethanol yield (g/lt)		Response 4 Fermentation efficiency (%)	
						Experimental value	Predicted value	Experimental value	Predicted value	Experimental value	Predicted value
2	1	Block 1	100	6	40	4.10	4.15	31.97	31.44	64.59	64.37
3	2	Block 1	100	8	50	3.90	3.92	30.81	30.98	64.60	64.75
7	3	Block 1	300	4	40	4.30	4.32	33.97	34.14	66.34	66.49
1	4	Block 1	100	4	30	3.00	2.69	23.70	23.48	54.79	54.76
9	5	Block 1	300	8	30	3.50	3.49	27.65	27.70	58.70	58.57
4	6	Block 1	200	4	50	4.10	4.09	32.39	32.44	64.53	64.40
6	7	Block 1	200	8	40	4.60	4.59	37.34	37.12	71.15	71.12
5	8	Block 1	200	6	30	3.20	3.22	25.28	25.45	55.96	56.11
8	9	Block 1	300	6	50	4.20	4.19	33.18	32.96	65.49	65.46

3.2.2 Optimization of process parameters by Regression model of response in Taguchi orthogonal array design

Taguchi design based on orthogonal array, used for the optimization of different fermentation variables as 3^4 factorial design. The experiment comprised of total 9 runs with different combinations of four factors was carried out. The parameters which were taken into consideration for analysis were agitation rate (A), aeration time (B) and substrate concentration (C), whereas dependable response variables chosen were ethanol g/lt (Y_{11}) and fermentation efficiency (Y_{12}). The complete orthogonal design with actual factors and values of response variables has been shown in table 1, which shows considerable variation in the amount of ethanol produced depending upon the interaction of independent variables for batch fermentation. By applying multiple regression analysis on the experimental data obtained from batch fermentation of pretreated algal biomass, a quadratic model was generated for different responses of ethanol fermentation. The significant model terms were evaluated by annotated analysis of variance (ANOVA) in the optimization study (p<0.05) and were identified as A, B, C.

Ethanol (g/lt) (Y_{11}) = +30.19 -2.89* A [1] +1.48* A[2] -1.17* B[1] -0.57* B[2] -5.65* C[1] +3.71* C[2] (Eqn. 1.1)

Ethanol fermentation efficiency (%) (Y_{12}) = +62.45 -2.49 * A [1] +1.43* A[2] -1.90* B[1] 0.47* B[2] -7.30* C[1] +4.88* C [2] (Eqn. 1.2)

Where ethanol g/lt (Y_{11}) and fermentation efficiency (Y_{12}) were response variables, A is agitation rate, B is aeration rate, C is substrate concentration. By applying Taguchi orthogonal array, maximum ethanol production noticed was 37.34 g/lt ethanol and fermentation efficiency of 71.15% in run 7 comprised of different parameters 200 rpm agitation rate, 8 h aeration time and substrate concentration 40 g/lt (Table 1). The statistical significance of different equations Eq. 1.1, 1.2 were checked by *F*-test, and the annotated analysis of variance (ANOVA) of quadratic model for batch fermentation of pretreated algal biomass have been given in tables 2, 3 respectively. The coefficient of determination (R^2) was calculated for all the models as 0.998 for ethanol g/lt, 0.999 for ethanol fermentation efficiency in batch fermentation of pretreated *Rhizoclonium* sp. algal biomass, indicating that the statistical models can explain respective variabilities of 99.8% and 99.9%. Normally, a regression model having an R^2 value > 0.9 is considered to have a very high correlation. The closer the R^2 (correlation coefficient) is to 1.0, the stronger the model and the better it predicts the response (Haaland, 1989). The values of R= 0.995 for ethanol g/lt, 0.998 for ethanol fermentation efficiency indicates a close agreement between the experimental results and the theoretical values predicted by the model equations. The adequate precision values for different responses observed were 55.15 and 95.14. Usually the higher value of CV, the lower the reliability of the experiment is (Gangadharan *et al*. 2008) [8]. A lower value of CV i.e. 1.13 and 0.39 were noticed for different ethanol responses. The *P*-values are used as a tool to check the significance of each of the coefficients which are necessary to understand the pattern of the mutual interactions between the variables. The smaller the *P*-values, the bigger the significance of the corresponding coefficient. In batch fermentation, the parameter estimates and the corresponding *P*-values suggested that all the independent variables A (agitation rate), B (aeration time), C (substrate concentration) have significant effect on enhancement of ethanol yield for all responses. According to F-value, the order of influences was $F_{\text{substrate concentration}}$, $F_{\text{agitation rate}}$, $F_{\text{aeration time.}}$

Table 2: Analysis of variance (ANOVA) for quadratic model obtained from orthogonal array design (Ethanol g/lt)

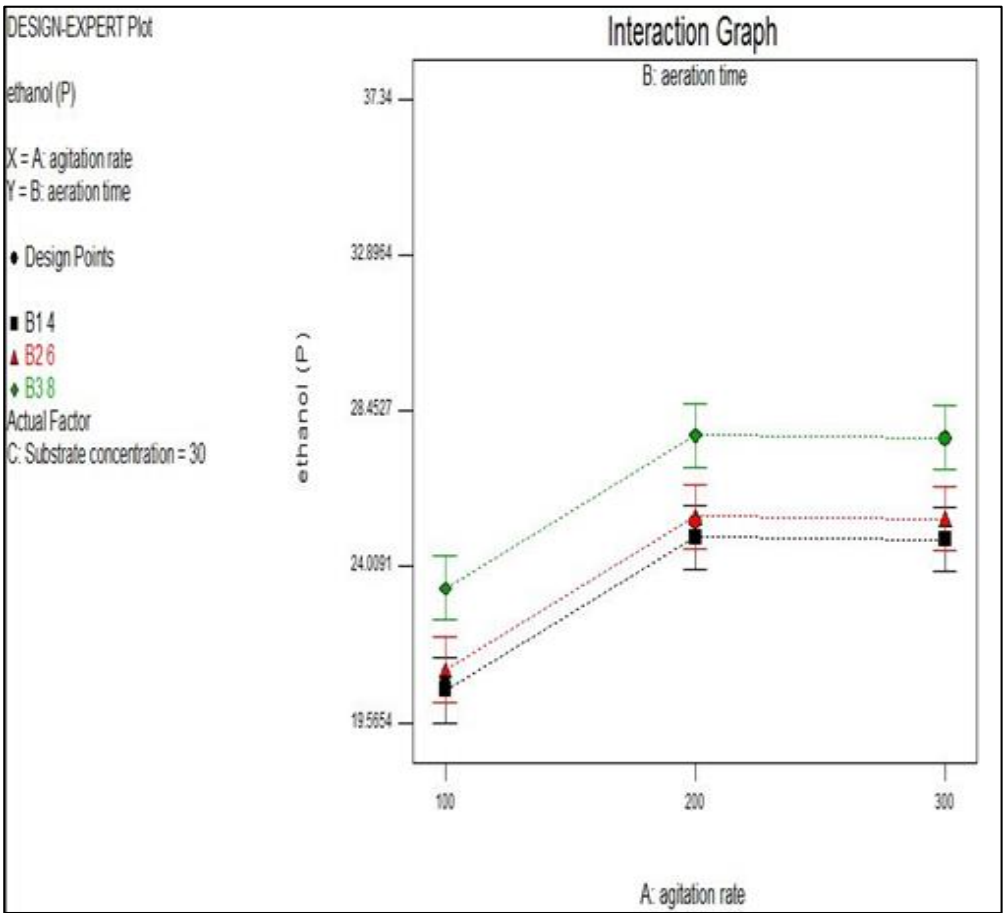
Source	Sum of square	Df	Mean square	F value	Prob>F	
Model	200.00	6	33.33	284.82	0.0035	Significant
A	37.59	2	18.80	160.60	0.0062	
B	14.21	2	7.11	60.71	0.0162	
C	148.20	2	74.10	633.15	0.0016	
Residual	0.23	2	0.12			
Cor Total	200.24	8				

Table 3: Analysis of variance (ANOVA) for quadratic model obtained from orthogonal array design (Ethanol fermentation efficiency %)

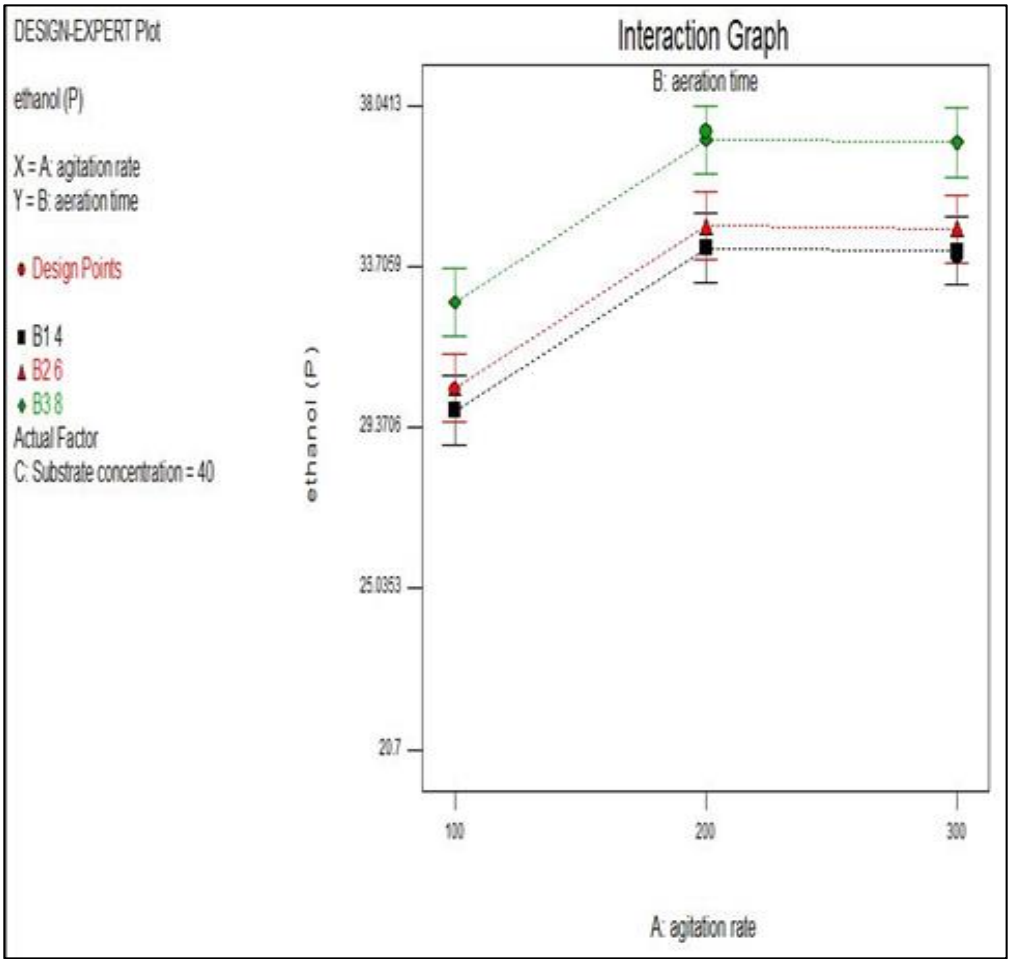
Source	Sum of square	Df	Mean square	F value	Prob>F	
Model	305.24	6	50.87	864.04	0.0012	Significant
A	28.06	2	14.03	238.26	0.0042	
B	28.25	2	14.12	239.89	0.0042	
C	248.93	2	124.47	2113.96	0.0005	
Residual	0.12	2	0.059			
Cor Total	305.35	8				

3.2.3 Localization of optimum conditions

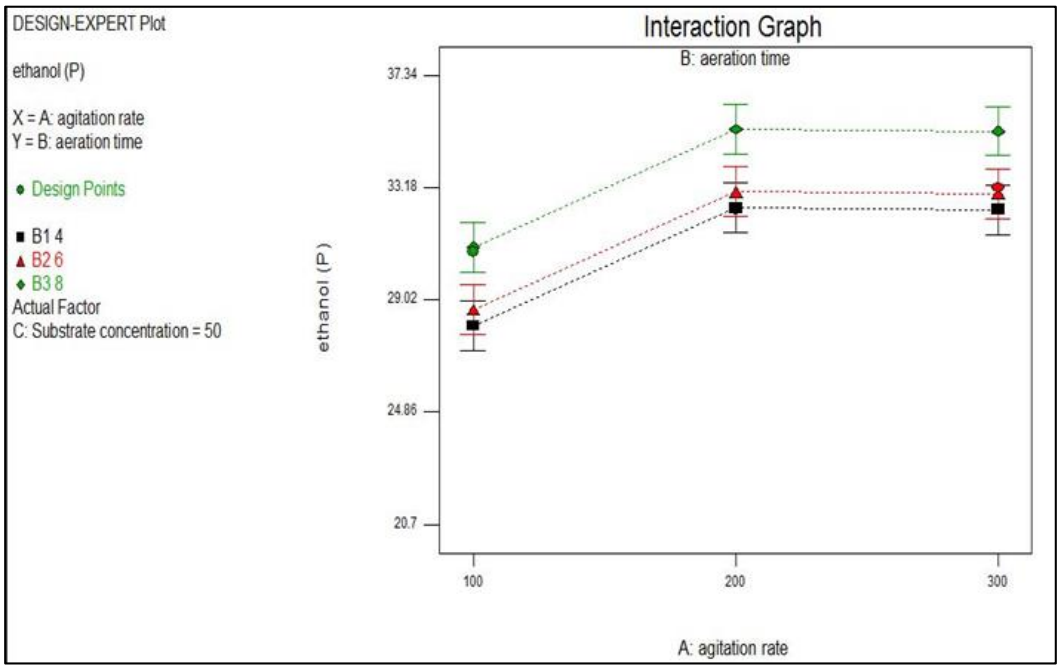
The 2D interaction graphs described by the regression model were drawn to illustrate the effects of the independent variables on the response variable. Fig. 1a, b and c shows the expected responses for ethanol g/lt and correlation between the independent variables. Fig. 2a, 2b and 2c shows the interaction between agitation rate at X-axis and aeration time at Y- axis respectively while keeping third variable substrate concentration at only one level shows the expected responses for fermentation efficiency (%) and correlation between the independent variables. In all the responses, an increase was observed with increase in agitation rate and aeration time irrespective of substrate concentration. But among all substrate concentrations, higher ethanol production was observed at substrate concentration of 40 g/lt. As shown in interaction graphs fig. 1 and 2 the maximum ethanol yield for all responses was observed at 200 rpm and 8 h aeration time with all substrate concentration.


DESIGN-EXPERT Plot
ethanol (P)
X = A: agitation rate
Y = B: aeration time
Design Points
B1 4
B2 6
B3 8
Actual Factor
C: Substrate concentration = 30
Interaction Graph
B: aeration time
37.34
32.8964
28.4527
24.0091
19.5654
ethanol (P)
100
200
300
A: agitation rate

1(a)

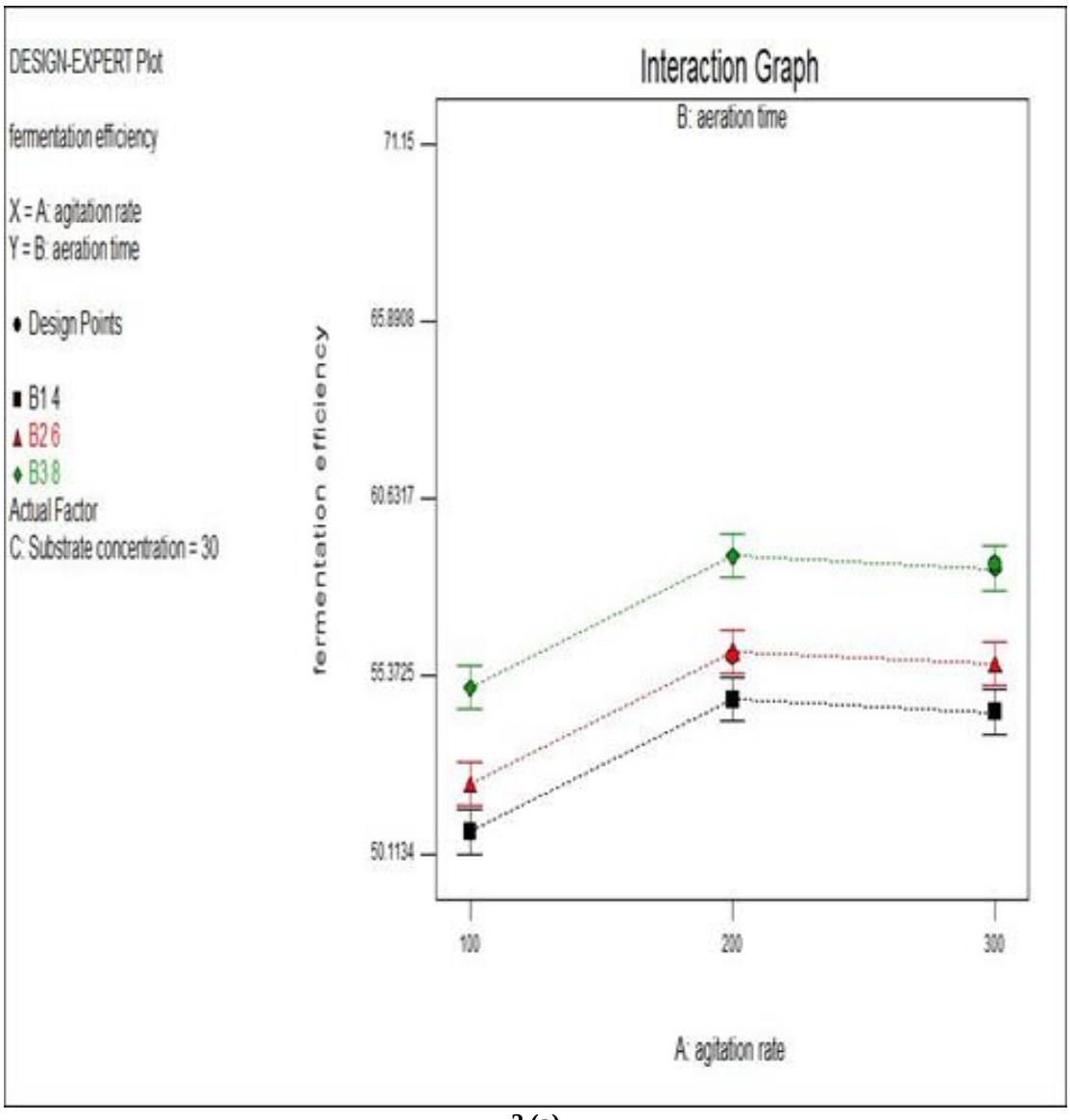

DESIGN-EXPERT Plot
ethanol (P)
X = A: agitation rate
Y = B: aeration time
Design Points
B1 4
B2 6
B3 8
Actual Factor
C: Substrate concentration = 40
Interaction Graph
B: aeration time
38.0413
33.7059
29.3706
25.0353
20.7
ethanol (P)
100
200
300
A: agitation rate

1(b)

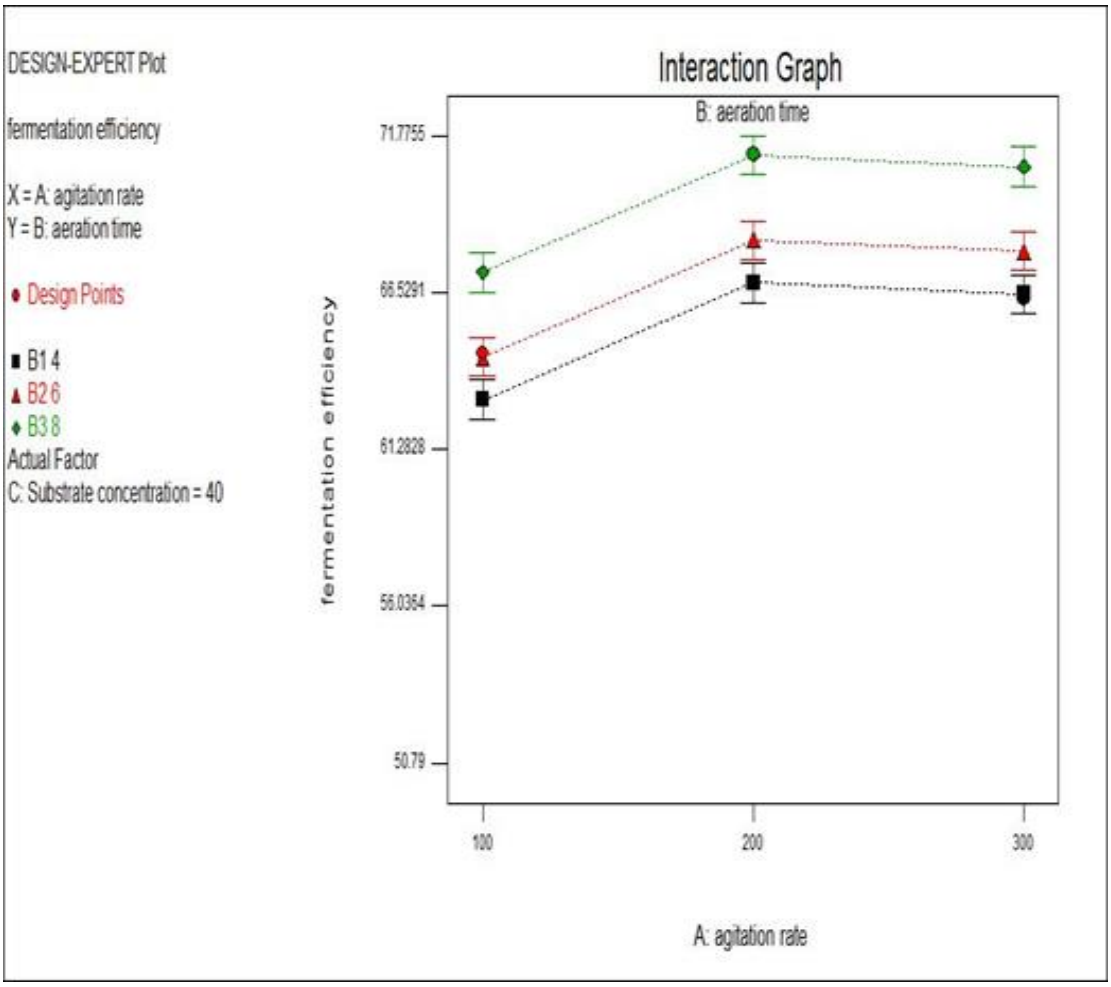


1(c)

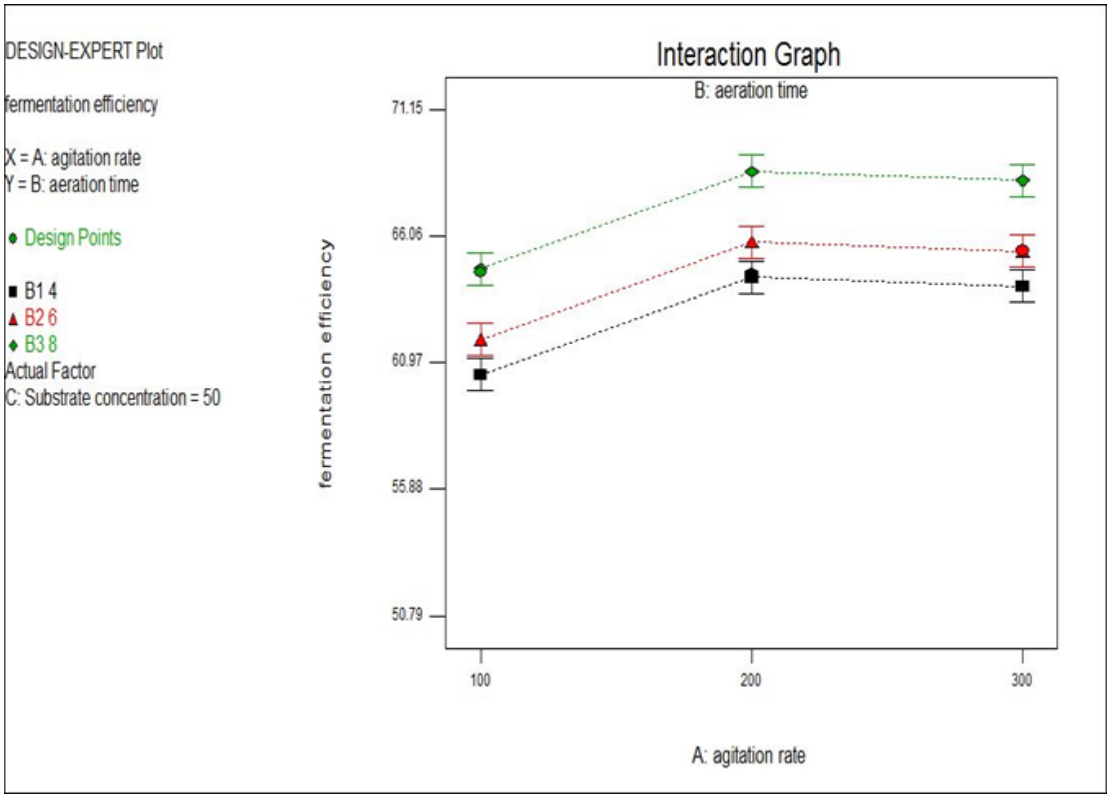
Fig 1: Interaction graph showing interaction of agitation rate and aeration time with substrate concentration 30 g/lt (1a), 40 g/lt (1b) and 50 g/lt (1c) for response ethanol yield (g/lt)



2 (a)



2(b)

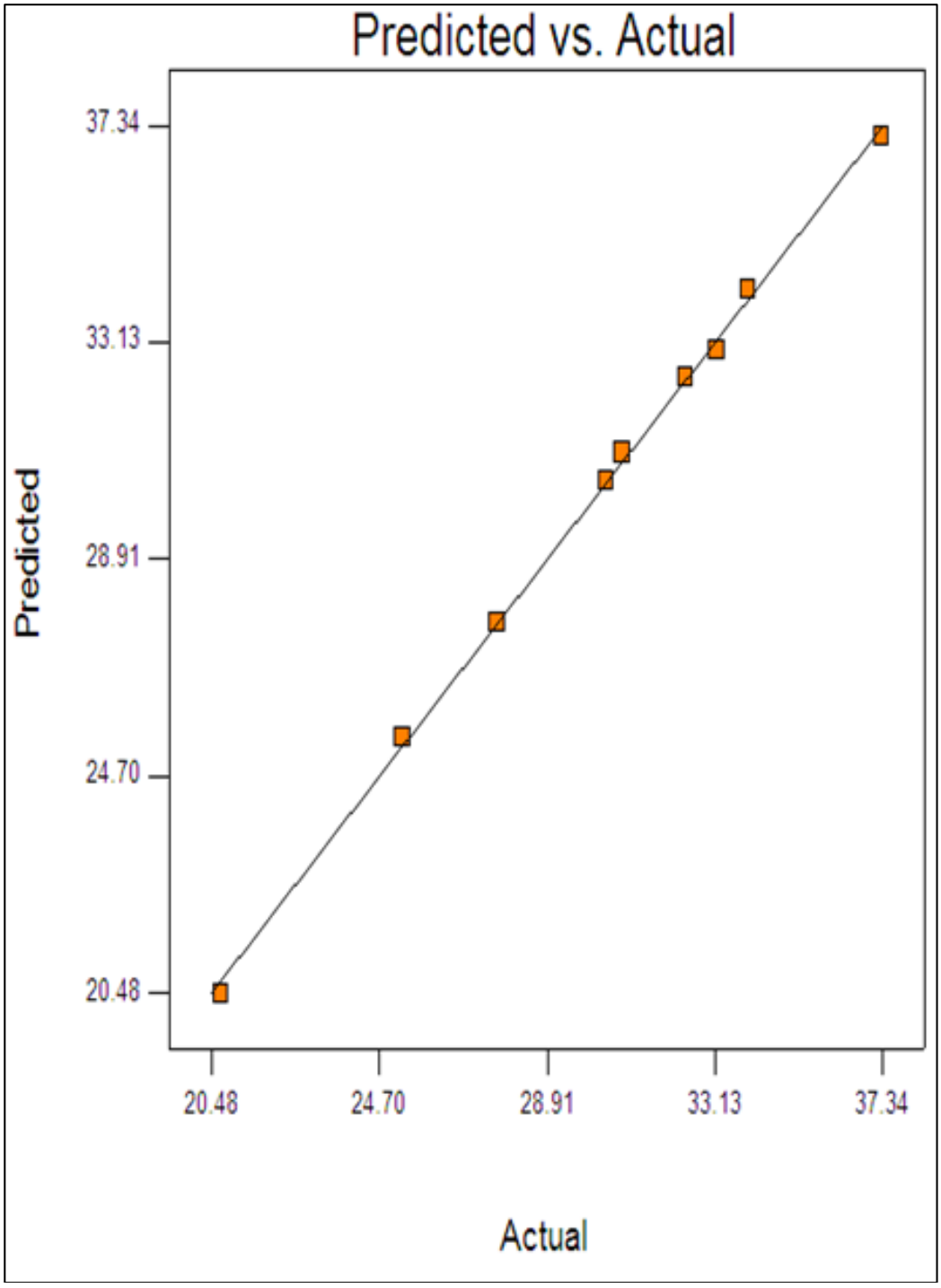


2(c)

Fig 2: Interaction graph showing interaction of agitation rate and aeration time with substrate concentration 30 g/lt (a), 40 g/lt (b) and 50 g/lt (c) for response ethanol fermentation efficiency (%)

3.2.4 Comparison of observed and predicted reducing sugars

A regression model has been used to predict future observations on the response Y (reducing sugars) corresponding to the particular values of the regression variables. Fig. 3a and b shows observed values of different ethanol responses versus those from the empirical models given in equations 1.1 and 1.2 as given earlier. The predicted R^2 for ethanol g/lt, ethanol fermentation efficiency of 0.976, 0.992 were in reasonable agreement with the adjusted R^2 of 0.995, 0.998. This indicated good agreement between the experimental and predicted values for reducing sugars. The adjusted R^2 corrects the R^2 value for the sample size and for the number of terms in the model. The model F-values for different responses were 284.62, 864.04 and values of prob. > F (<0.05) indicated that the model terms are significant. The figures prove the predicted data for different ethanol production responses from the empirical models were in agreement with the observed ones in the range of the operating variables.



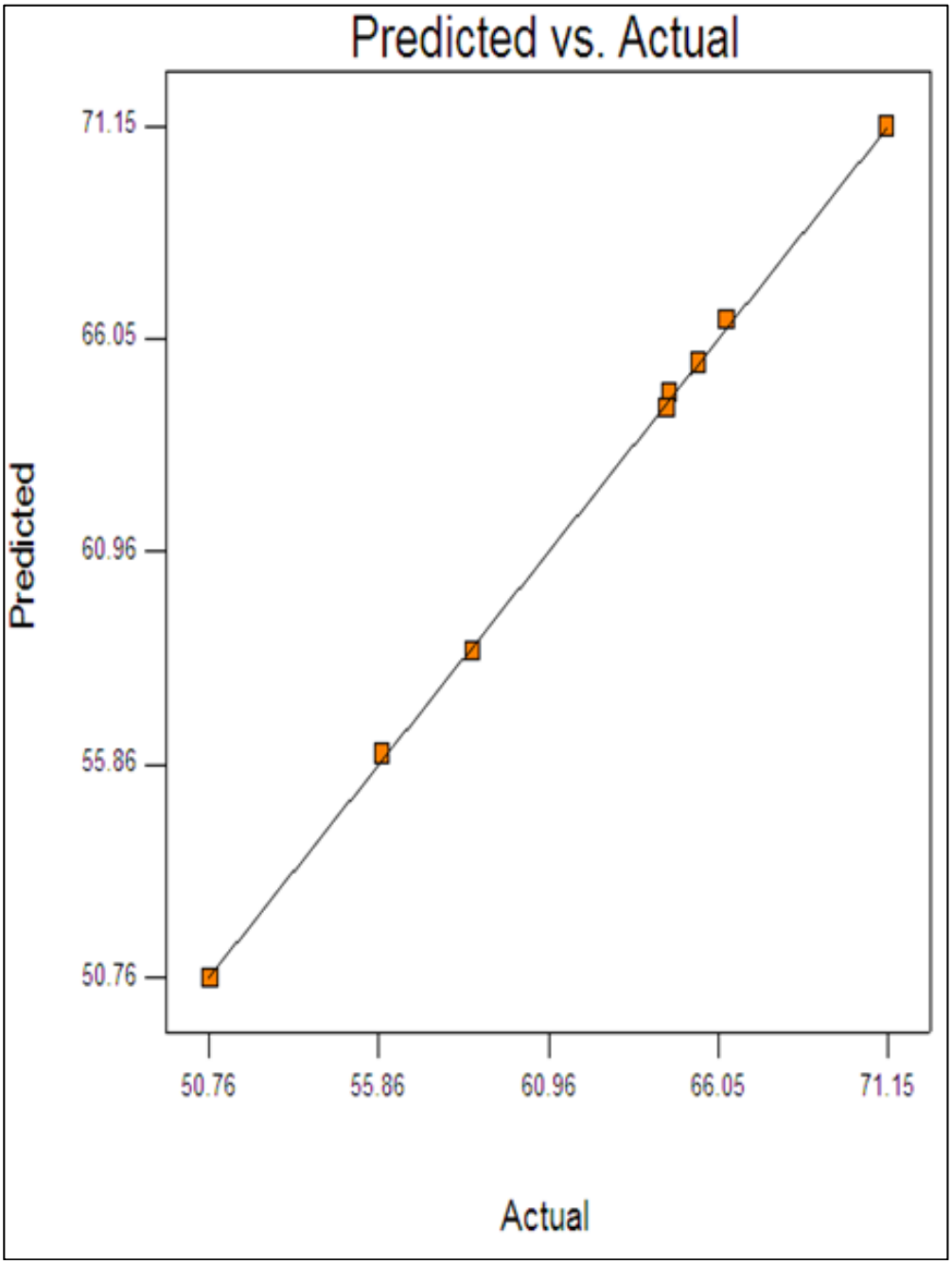


Fig: 3a and b Parity plot showing distribution of experimental and predicted values of ethanol (g/lt) and Fermentation efficiency (%)

3.2.5 Model adequacy checking

The residuals from the least squares fit play an important role in judging model adequacy. By constructing a normal probability plot for the residuals, a check was made for the normality assumption, as given in Fig. 4a, b for different ethanol responses. The normality assumption was satisfied as the residual plot approximated along a straight line. The general impression is that the residuals scatter randomly on the display, suggesting that the variance of the original observation is constant for all values of predicated response (Y). Fig. 4a, b are satisfactory, so we conclude that the empirical models were adequate to describe the ethanol fermentation responses by orthogonal array design.

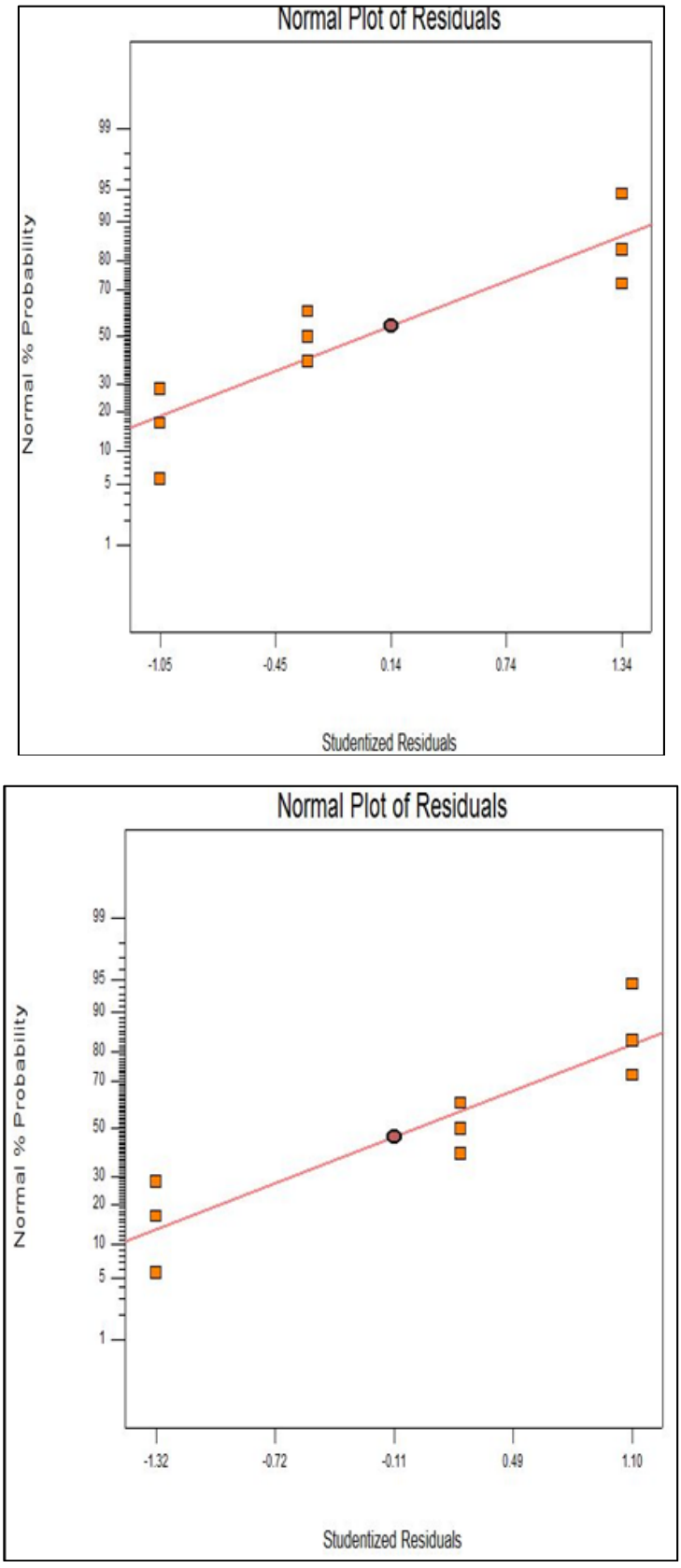


Fig: 4a and b Normal probability of internally studentized residuals for batch fermentation of pretreated algal biomass for ethanol g/lt and fermentation efficiency %

3.2.6 Validation of the model

The statically data point presented maximum batch ethanol fermentation from pretreated algal biomass 37.34 g/lt ethanol with fermentation efficiency of 71.15%. Numerical optimization was carried out to obtain the best combination for maximum ethanol production. Table 4 shows software generated three optimum conditions of independent variables with the predicted values of responses for ethanol production. The optimum condition for improving ethanol fermentation was determined as $A_2B_3C_2$. Solution number 1 having the maximum desirability value of 0.996 was selected for ethanol fermentation. Chan-u-tit *et al.* (2015) carried out the optimization of nutrient supplements *i.e.*, yeast extract (1, 3 and 5 g/lt), dried spent yeast (DSY: 4, 12 and 20 g/lt) and

osmoprotectant (glycine: 1, 3 and 5 g/lt) to improve the efficiency of ethanol production from a synthetic medium under very high gravity (VHG) fermentation by *Saccharomyces cerevisiae* NP 01 was performed using a statistical method, an L9 (3^4) orthogonal array design. According to the orthogonal results, the order of influence on the *P* and *Qp* values were yeast extract > glycine > DSY, and the optimum nutrient concentrations were yeast extract, 3; DSY, 4 and glycine, 5 g/lt, respectively. The verification experiment using these parameters found that the *P*, *Yp/s* and *Qp* values were 119.9 g/lt, 0.49 g/g and 2.14 g/lt/h, respectively. The statistical optimization of different fermentation process parameters in SSF of mixed MAA and organosolv pretreated 1% (w/v) wild grass by Taguchi orthogonal array design was assessed in a study (Das *et al.*, 2014**)** **[19]**. The ethanol titre obtained in Taguchi optimized shake flask SSF was 2.0 g/lt implying a 1.3-fold increase as compared to ethanol titre of 1.5 g/lt in unoptimized shake flask SSF. A 1.5-fold gain in ethanol titre (3.1 g/lt) was obtained with the same substrate concentration in lab scale bioreactor on scaling up the shake flask SSF with Taguchi optimized process parameters. Optimization of three parameters: agitation rate (A; 100, 200 and 300 rpm), aeration rate (B; 0.5, 1.5 and 2.5 vvm) and aeration timing (C; 2, 4 and 6 h), for ethanol production from sweet sorghum juice under very high gravity (VHG, 290 g/lt of total sugar) conditions by *Saccharomyces cerevisiae* NP 01 was attempted using an L9 (3^4) orthogonal array design. The results showed that the optimum condition for ethanol fermentation should be $A_2B_3C_2$ corresponding to agitation rate, 200 rpm; aeration rate, 2.5 vvm and aeration timing, 4 h. The verification experiments under the optimum condition clearly indicated that the aeration and agitation strategies improved ethanol production. The ethanol concentration (*P*), productivity (*Qp*) and ethanol yield (*Yp/s*) were 132.82 ± 1.06 g/lt, 2.55 ± 0.00 g/lt/h and 0.50 ± 0.00, respectively (Khongsay *et al.* 2012**)** **[12].**

Table 4: Software generated three optimum conditions of independent variables with the desirability values for optimum batch ethanol production from pretreated *Rhizoclonium* sp. algal biomass

Agitation rate (rpm)	Aeration rate (h)	Substrate concentration (g/L)	Desirability
200	8	40	0.996
200	6	40	0.865
200	4	40	0.812

3.3 Mathematical modelling for cell growth in best optimized condition of orthogonal array design

Most of the growth processes are explained in terms of Monod or logistic equations (unstructured models). The idea of microbial growth kinetics has been dominated by an empirical model originally proposed by Monod (1942). Fig. 5 revealed the physiological behaviour of *S. cerevisiae* in a stirred tank bioreactor under SHF in best optimized condition by orthogonal array design in Run 7 (Agitation rate 200 rpm; aeration time 8 h, substrate concentration 40 g/lt). According to Musatto *et al.* (2010) the carbon source consumption is divided by the yeast into biomass and ethanol production. This behaviour of yeast is strongly influenced by available oxygen. This metabolic behaviour can be observed clearly with a comparison between $Y_{(P/S)}$ and $Y_{(X/S)}$ values. In the present work, $Y_{(P/S)}$ was significantly higher than $Y_{(X/S)}$ which reflected the amount of distributed oxygen was satisfactory for the yeast metabolism. The sigmoidal growth pattern of *S. cerevisiae* (MTCC-3089) had been analyzed using logistic equation for its variation against time during log phase. A conventional growth pattern was observed during batch fermentation in separate hydrolysis and fermentation where exponential phase lasted for 48 h and was followed by stationary phase up to 54 h. The production of ethanol had started at 6 h and obtained maximum of 37.34 g/lt at 48 h (Fig. 5). In Monod's model, the growth rate is related to the concentration of a single growth-limiting substrate though the parameters μmax and Ks. In addition to this, Monod also related the yield coefficient ($Y_{X/S}$) to the specific rate of biomass growth (μ) and the specific rate of substrate utilization (Q) (Fig. 6).

$$Y_{X/S} = dx/ds$$

$$Y_{X/S} = X_m - X_0/S_0\text{-}S_m$$

In, model prediction for cell growth was followed with the experimental results with coefficient of determination, which revealed that this kinetic model was appropriate for predicting the experimental cell growth i.e.

$Y_{X/S}$ = 1.19
$Y_{P/S}$ = 4.21
μ_{max} = 3.67 h^{-1}
Q_{pmax} = 2.46 g/l/h
Ks = 108.99 mg/ml

The interpretation of μ_{max} as the maximum specific growth rate is straight forward. The empirical constant K_S is the substrate concentration at which organisms are substrate limited to a growth rate of half the prevailing maximum value. This constant is closely related to the mechanism of transport of the substrate over the cell membrane, so it depends on cell membrane properties and intracellular conditions, on the type of transporter proteins in the cell and on the substrate properties. For this reason, the value of K_S can be interpreted as a reflection of the affinity of the cell towards the substrate (S) (Luederking and Piret, 1959). Because of the adaptive ability of organisms, both μ_{max} and K_S can vary with the environmental conditions, for example with the nature of the growth medium and the duration of exposure to it, with temperature and pressure. For these reasons the values of μ_{max} and K_S have to be estimated for each specific couple cell-substrate and under constant conditions of temperature, pressure and medium composition. Daneal and Kana (2017) examined the kinetics of bioethanol production from waste sorghum leaves using *Saccharomyces cerevisiae* BY4743 and maximum specific growth and Monod constant (KS) of 0.176 h^{-1} and 10.11 g/lt, respectively, were obtained. The bioethanol production data fit the modified Gompertz model with an R^2 value of 0.98. A maximum bioethanol production rate of 0.52 g/lt/h, maximum potential bioethanol concentration (Pm) of 17.15 g/lt, and a bioethanol production lag time (tL) of 6.31 h were observed. Caldeirão *et al.* (2016) studied the modeling and kinetic study of bio-ethanol production from soy protein concentrate by-product. The maximum ethanol concentration was in 44 hours, the ethanol productivity was 0.946 g/lt/h, the yield over total initial sugars (Y_1) was 47.87%, over consumed sugars (Y_2) was 88.08% and specific cells production rate was 0.006 h-1.

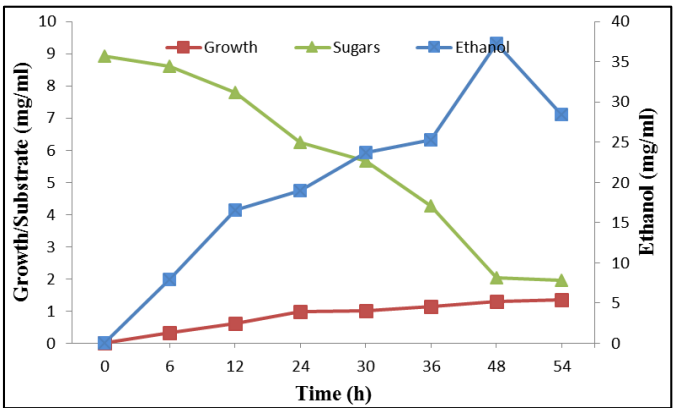


Fig 5: Growth kinetics for ethanol production under best optimized condition in a stirred tank bioreactor

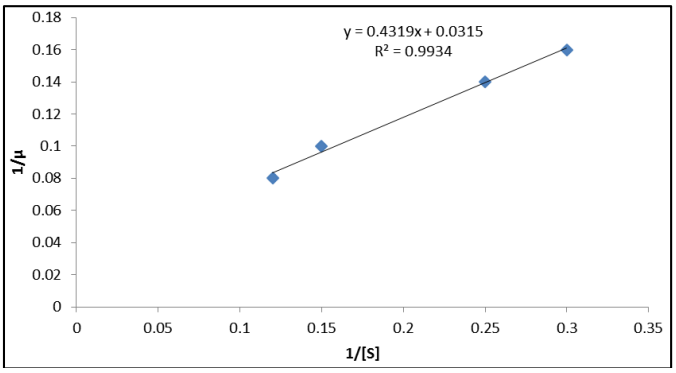


Fig 6: Comparison between experimental results of specific growth rate and substrate utilization and model prediction for best optimized condition

3.4 Kinetic analysis for ethanol production

The Luedeking-Piret equation (Luedeking and Piret, 1959) [14] was applied for the kinetic modeling of ethanol production. The Luedeking-Piret model indicates that the rate of ethanol production (Q_p) is linearly proportional to cell growth rate and cell biomass in best optimized conditions obtained in orthogonal array design Run 7 (Fig. 7). The results indicated that this model evidently was very appropriate for describing the product formation rate. The coefficient of determination, R^2 was 0.984. Ali *et al.* (2017) [1] evaluated the kinetic models and parameters estimation study of biomass and ethanol production from inulin by *Pichia caribbica* (KC977491). Unstructured models by logistic equation for growth, and kinetic parameters (X_0, µm, m, n, p and q) were determined by nonlinear regression. Since the production of ethanol was associated with *P. caribbica* cell growth, a good agreement between model predictions and experimental data was obtained. Indeed, significant R^2 values of 0.91, 0.96, and 0.95 were observed for biomass, ethanol production and substrate consumption, respectively. Dodic´*et al.* (2012) [6] examined the
kinetic modelling of batch ethanol production from sugar beet raw juice. The results show a good agreement with experimental data (R^2 = 0.99), thus, the logistic equation was found to be an appropriate kinetic model for successfully describing yeast cell growth in batch fermentation of raw juice system.

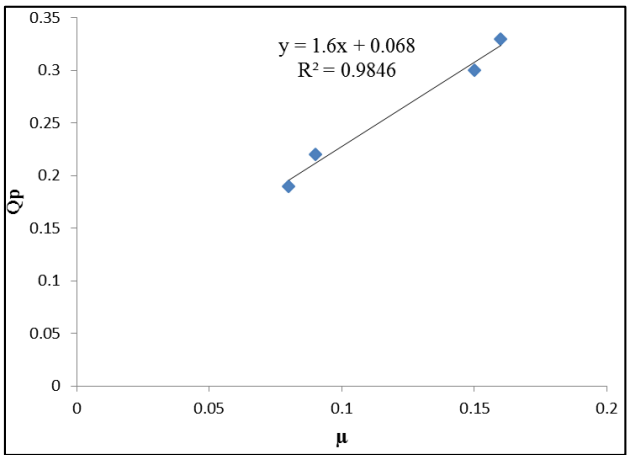


Fig 7: Comparison between experimental results of specific rate of product formation and model prediction for separate hydrolysis and fermentation (SHF)

3.5 Recovery of ethanol as fuel grade alcohol

The ethanol initially produced after 48 h of fermentation was further distilled. Three successive distillations turned it to a concentrate of 70.56% of the azeotropic mixture to label it as a fuel grade ethanol. Different properties of thus concentrated ethanol are presented in Table 5. The pH levels of the ethanol produced did not show a variation as it remained 6.0. There was no precipitation formed when $BaSO_4$ was added to ethanol, thus indicating the absence of sulphates. But there was a precipitate formation when $AgCl_2$ was added to ethanol, which confirmed the presence of chloride ions in ethanol. The quantitative estimation of chloride content was done after every successive distillation which increased with every distillation. After final distillation, chloride content was found to be 17.02 mg/lt. Copper content was also measured quantitatively to check its concentration in ethanol which was reported as 0.0159 mg/lt after final distillation. The titrable acidity of produced ethanol decreased after every distillation and was 0.015% after final distillation. American Society for testing and materials (ASTM) has industry standard for fuel grade ethanol. The standard specification for denatured fuel alcohol needs to contain approx. 92.1% ethanol content whereas ethanol produced in current study was of low grade up to 70%. Nuwamanya *et al.* (2012) [17] carried out the bioethanol production from non-food parts of Cassava (*Manihot esculenta* Crantz). The ethanol produced after 6 days of fermentation was of a very low concentration, the first distillate held a concentration of 11%. Three successive distillations increased the yield to a maximum concentration of 60% of the azeotropic mixture with percentages of ethanol produced ranging from 59.5% acquired from the leaves to 61.4% from the roots. The pH levels of the ethanol produced did not show a large variation between the plant parts (roots and peels 2.87 and stems 2.82). No precipitation was formed when adding barium (Ba^{2+}) or silver (Ag^{+}) ions, indicating absence of chloride or sulfate.

Table 5: Estimation of different attributes of ethanol recovered after distillation

Different attributes of fuel ethanol	After 1st distillation	After 2nd distillation	After 3rd distillation
Ethanol concentration (%)	41.78	52.54	70.56
pH	6.0	6.0	6.0
Titrable acidity (%)	0.025	0.020	0.015
Sulphates	-	-	-

Chlorides (mg/lt)	13.16	15.52	17.02
Copper (mg/lt)	-	0.0143	0.0159

4. Conclusion

This study has reported for the first time the significance of statistical optimization and validation of different fermentation process parameters for bioethanol production from *Rhizoclonium* sp. algal biomass by *S. cerevisiae* I by using Taguchi orthogonal array design and product recovery. In order to attain higher ethanol concentrations and productivity, the main parameters of ethanol fermentation, i.e., agitation rate, aeration time and substrate concentration were optimized. Under the optimum condition (agitation rate, 200 rpm; aeration timing, 8 h, substrate concentration 40 g/lt), the ethanol concentration and efficiency were significantly improved. These findings were further confirmed by kinetic analysis and mathematical modeling of the optimum ethanol production. The final concentration of ethanol was increased by three phase distillation up to 70%. In essence, the statistical optimization of fermentation process is a cost effective and time saving stepping stone to transform successfully the *Rhizoclonium* sp. algal biomass into the fuel of tomorrow, bioethanol.

5. References

1. Ali MKS, Hiligsmann N, Outili R, Cherfia, Chaouche NK. Kinetic models and parameters estimation study of biomass and ethanol production from inulin by *Pichia caribbica* (KC977491). African Journal of Biotechnology 2017; 16:124-131.
2. Anand, N. Indian freshwater microalgae. 1st ed. Bishen Singh Mahendra Pal Singh, Dehradun, 1998, 94.
3. Antony JFJ, Antony, Ross PJ. Reducing manufacturing process variability using experimental design technique: A case study. Integrated Manufacturing Systems. 1998; 10(3):162-169.
4. Caldeirao L, Tanaka, C, Ida E, Spinosa W. Modeling and kinetic study of bio-ethanol production from soy protein concentrate by-product. Food Science and Technology 2016; 98:2415-2425.
5. Daneal C, Rorke S, Evariste B, Gueguim K. Kinetics of Bioethanol Production from Waste Sorghum Leaves Using *Saccharomyces cerevisiae* BY4743. *Fermentation* 2017; 3:19-25.
6. Dodic JM, Vucurovic DG, Dodic SN, Grahovac JA, Popov SD, Nedeljkovic NM. Kinetic modelling of batch ethanol production from sugar beet raw juice. Applied Energy. 2012; 99:192-197.
7. Duhan JS, Kumar A, Tanwar SK. Bioethanol production from starchy part of tuberous plant (potato) using *Saccharomyces cerevisiae* MTCC-170 1. African Journal of Microbiology Research. 2013; 7:5253-5260.
8. Gangadharan D, Sivaramakrishnan S, Nampoothiri KM, Sukumaran RK, Pandey A. Response surface methodology for the optimization of alpha amylase production by *Bacillus amyloliquefaciens.* Bioresource Technology. 2008; 99:4597–4602.
9. Haaland PD (ed). Experimental design in biotechnology. Marcel Dekker, Inc., New York, 1989.
10. Hammond J. Yeast growth and nutrition. In: Brewing Yeast Fermentation Performance; Smart, K., Ed.; Oxford Brookes University Press: Oxford, UK, 2012.
11. Ke W, Jiuyu H, Lei W Huimin L. Phase factor sequences algorithm in partial transmit sequence. Transactions of Tianjin University. 2009; 15:23–26.
12. Khongsay N, Laopaiboon L, Jaisil P, Laopaiboon P. Optimization of agitation and aeration for very high gravity ethanol fermentation from sweet sorghum juice by *Saccharomyces cerevisiae* using an orthogonal array design. Energies. 2012; 5:561–576.
13. Kumari R, Pramanik K. Improved bioethanol production using fusants of *Saccharomyces cerevisiae* and xylose-fermenting yeasts. Applied biochemistry and biotechnology. 2012; 167:873-84.
14. Luedeking R, Piert EL. A kinetic study of lactic acid fermentation: batch process at controlled pH. Journal of Biochemical and Microbiological Technology and Engineering. 1959; 1(12):393-431.
15. Monod J. Recherches sur la croissance des cultures bacteriennes. Ed. Hermann and Cie, 1942.
16. Mussatto SI, Teixeira JA. Lignocellulose as raw material in fermentation processes. Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology. 2010; 2:887-907.
17. Nuwamanya E, Chiwona-Karltun L, Kawuki RS, Baguma Y. Bioethanol production from non-food Parts of Cassava (*Manihot esculenta* Crantz). Ambio. 2012; 41:262-70.
18. Pachaya C, Laopaiboon L, Jaisil P, Laopaiboon P. High Level Ethanol Production by Nitrogen and Osmoprotectant Supplementation under Very High Gravity Fermentation Conditions. Energies. 2015; 6:884-899.
19. Saprativ P, Das D, Goyal A. Statistical Optimization of Fermentation Process Parameters by Taguchi Orthogonal Array Design for Improved Bioethanol Production. Journal of Fuels. 2014:1-11.
20. Sharma N, Sharma N. Second generation biofuel production from lignocellulosic waste and future perspectives: a review. International Journal of current Microbiology and Applied Sciences. 2018; 7(5):1285-1290.
21. Taguchi G. System of Experimental Design; Krus International Press: New York, NY, USA, 1990.
22. Vishwanatha T, Jain SN, Reena V, Divyashree BC, Siddalingeshwara KG, Karthic J, Sudipta KM. Screening of substrates for protease production from Bacillus licheniformis. International Journal of Engineering, Science and Technology. 2010; 2:6550-6554.
23. Wang L, Sharifzadeh M, Templer R, Murphy RJ. Technology performance and economic feasibility of bioethanol production from various waste papers. Energy and Environmental Sciences. 2012; 5:5717–5730.
24. Yang LJ, Qi YM, Dang, XN. Design and optimization of technology and structure parameters for sheet metal drawing by orthogonal experiment. Advanced Materials Research. 2011; 295:1714–1717.