Scholars Academic Journal of Biosciences (SAJB) **ISSN 2321-6883 (Online)**
Sch. Acad. J. Biosci., 2013; 1(7):363-373 **ISSN 2347-9515 (Print)**

(An International Publisher for Academic and Scientific Resources)
www.saspublishers.com **DOI:** 10.36347/sajb.2013.v01i07.004

Research Article

Process Optimization for Batch Culture of *Saccharomyces cerevisiae*

Zarifa M. Raceme*, Dr. Babar Sheikh, Md. Enayetul, Akbor Md. Ahedul
Analytical Research Division, Bangladesh Council of Scientific & Industrial Research, Dhanmondi, Dhaka-1205, Bangladesh.

***Corresponding author**
Zarifa M. Raceme
Email: g_mat_zmr@yahoo.com

Abstract: Optimization of the batch culture of Saccharomyces cerevisiae for cell growth and ethanol production via anaerobic condition, an analysis using a three level, three factorial Box–Behnken designs was performed. Intact dry yeast was used as it is relatively easily obtained. The Box–Behnken design can be a useful approach to determine the optimum conditions for maximum production. Three separation parameters, Glucose % (X1), pH (X2), and Incubation temperature 0C (X3), were chosen to observe the effect. The responses were detected via spectrophotometer and gas-chromatography. Standard curve of concentration vs. OD of yeast cell was done to help further study. The optimum conditions and process validation were determined using statistical regression analysis, sigma and contour plot diagrams. Under low pH, high glucose concentration and temperatures around 30°C, ethanol production was highest. The optimum conditions were established to be 3% glucose media of pH 6.0 at an incubation period of 30 hour at 33ºC. Maximum cell growth found OD630 1.45 and ethanol production 2%. The fact behind this distribution of efficiencies was due to glucose concentration and incubation temperature manly. The method was validated by randomly selecting values from combinations. It was also observed that R2 values were over 0.99 which refers the result was almost accurate. By using the analysis technique, the prediction of responses was satisfactory and process verification yielded values within the ±5% range of the predicted efficiency. The relationship between coded variables and responses are better understood by examining the series of 3D line plots i.e. contour plot and Sigma plot 12.0.
Keywords: Saccharomyces cerevisiae, Bath Culture, Box–Behnken design, contour plot, Sigma plot 12.0, Standard Curve, Growth Curve, GC, GC-MS..

INTRODUCTION

Saccharomyces cerevisiae is better known as baker's yeast. The batch culture with the intermittent addition of glucose and without the removal of fermentation broth is one of the most common methods for the production of ethanol. A high concentration of sugar in fermentation medium inhibits growth and ethanol production. Other advantages of this process are higher productivity, higher dissolved oxygen in the medium, decreased fermentation time and reduced toxic effects of the medium components, which are present at high concentrations.

Though there exist many parameters like optical density, pH, incubation time, incubation temperature, etc., the important separation parameters are considered to be optical density, incubation temperature, and pH, all of which can have important effects. As an optimizing approach, the Box–Behnken design has so far been employed with moderate method optimizations. Response surface methodology is a very useful statistical tool to optimize multiple variables for predicting the best performing conditions by using a minimum number of experiments [1, 8].

Growth parameters: lag time (time to adapt to the environmental change), maximum relative growth rate (kinetics of growth), and stationary phase OD increment (related to the efficiency of growth), developments are important initial steps towards large-scale analysis of mutants based on rigorous statistical grounds. However, more analytical tools need to be put in place before the methodology becomes fully operational [2].

The specific objectives were as follows to study batch growth of *Saccharomyces cerevisiae* and to optimize growth parameters for batch growth using Box-Behnken design.

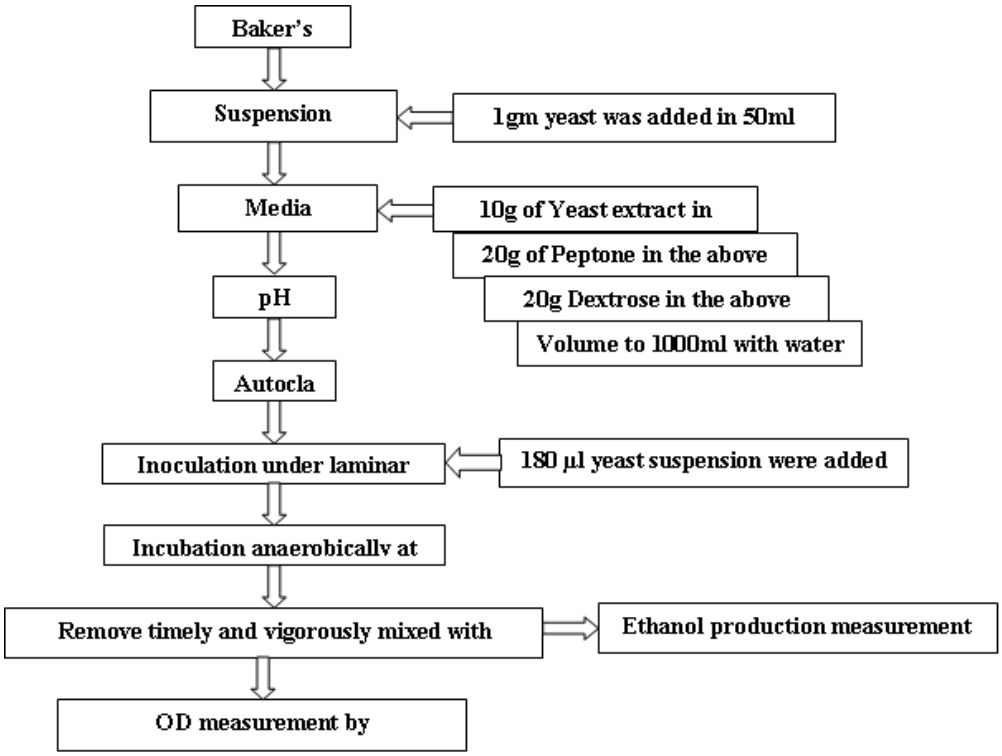
MATERIALS AND METHODS

Materials

All the chemicals that were used in the process running were good in quality and instruments were operated in proper way. Baker's yeast (Grocery Shop, Khulna), YPD media (Dextrose, Yeast Extract, Peptone) from *Sigma Aldrich*, USA, Distill Water-prepared in laboratory, Absolute ethanol from TEDIA, USA, Electric Balance, Measuring cylinder, pH meter, Autoclave, Micropipette, GC-MS, GC, HT3, Glass road, biker, screw cap bottle, etc from Pyrex were used.

Methods

Working Procedure:
The laboratory working procedure was as follows:



The laboratory working procedure

Desirable media preparation:

For 3% Glucose contained media 30gm dextrose dissolved. Likewise, 2% Glucose contained media means 20gm dextrose. Thus 1% Glucose contained media has 10gm dextrose with others.

According to Table- 1 these three factors were Glucose%(X_1), pH(X_2), Incubation temperature ^{o}C (X_3) and the three levels are high, middle, and low which are designated as +1, 0, and –1, respectively. The variables (factors) and their levels are presented in Table 1.

Table-1: Levels of variables chosen for Box–Behnken study

	Level		
variables	+1	0	-1
Glucose% (X_1)	3%	2%	1%
pH (X_2)	7.0	6.0	5.0
Incubation temperature ^{0}C (X_3)	36	30	24

Two responses namely, optical density Y(c), ethanol production Y(e) migration time (t), and resolution (R), were tested in this study. The quadratic response equation was used as follows:

$Y=A_0A_1X_1 + A_2X_2 + A_3X_3 + A_4X_1X_2 + A_5X_1X_3 + A_6X_2X_3 + A_7X_1^2 + A_8X_2^2 + A_9X_3^2$

Where A0 the regression coefficient, A1–A3 are the linear coefficients, A4–A6 the crossproduct coefficients, and A7–A9 are the quadratic coefficients. The regression analysis and statistical significance were carried out using Microsoft Excel software. Surface plots and contour plot were developed using the same software along with SigmaPlot 12.

Optical density:

By calculating the amount of light that a solution is able to absorb and applying Beer's Law, the spectrophotometer can determine the concentration of a colored solution. For yeast cell count absorbance was at 630nm.

There is a way to measure the concentration of some unknown solution using the spectrophotometer by generating a standard curve: a graph of absorbance vs. concentration for standard solutions whose yeast concentrations are known.

Program of GC:

Conditions which can be varied to accommodate a required analysis include inlet temperature, detector temperature, column temperature and temperature program, carrier gas and carrier gas flow rates, the column's stationary phase, diameter and length, inlet type and flow rates, sample size and injection technique. Used program of GC is given here where analysis time was 60 minutes.

SPL1: Temperature 280℃; Injection mode Splt; Sampling time 1 minute; Pressure 35.0 kPa; Holding time 0; Totals program time 0; Carrier gas He; Row control mode: pressure; Pressure: 35.5 kPa; Total flow

39.0 ml/min; Linear velocity 18.4 cm/sec; Purge flow: 3.0 ml/min; Split ratio 35.0; Column information: RTX-5; Length 35.0 m; Inner diameter: 0.32 mmID; Film thickness: 0.250 m.

Column: Temperature 40℃; Equilibration time 3.0 min; Total program time 45min; Temperature 40 ℃ holding 20 min; Increase 10 ℃ till 240℃ holding 5.0 min; Column information: RTX-5; Serial number 1044406; Installation date 12/02/27; Column maximum temperature 350℃.

FID1: Temperature 280℃ ; Sampling rate 40msec; Stop time 60.0 min; Flow 30; Holding time 0; Total program time 0.

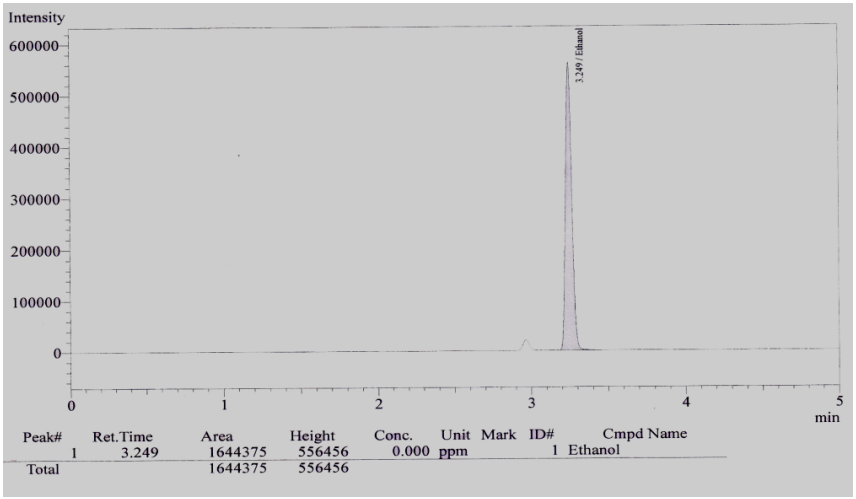
Program of HT3: In the static set up, a sample is placed in a vial and then delivered to the autosampler. Once in the auto sampler, the vial is loaded into a platen for heating, upon reaching the final heat time it is then mixed for a set period of time. Using an electronic mass flow controller the static vial pressure is recorded and the sample is pressurized to a user-defined set point. Next, the sample is passed through a fixed volume loop to another user-defined final pressure set point. The loop containing the sample is then placed in line with the GC column for separation and detection.

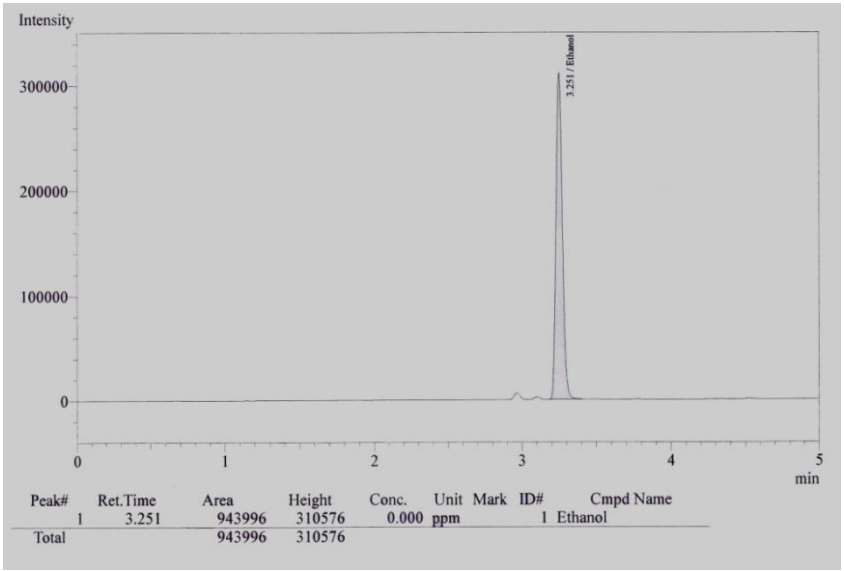
Used method: Residual ethanol method; Constant heat time ON; GC cycle time 50.0 min; Valve oven temperature 105℃; Transfer line temperature 105℃; Standby flow rate 50ml/min; Platen/sample temperature: 80℃; Platen temperature equilibrium time 1.0min; Sample equilibrium time 45.0min; Mixer : ON; Mixing level: level 5; Mixing time: 5.0 min; Mixer stabilize time: 0.50 min; Pressurize: 10 PSIG; Pressurize time: 0.50min; Pressurize equilibrium time: 0.20min; Loop fill pressure: 5 PSIG; Loop fill time 2.0min; Inject time: 1.0min [5,6,9].

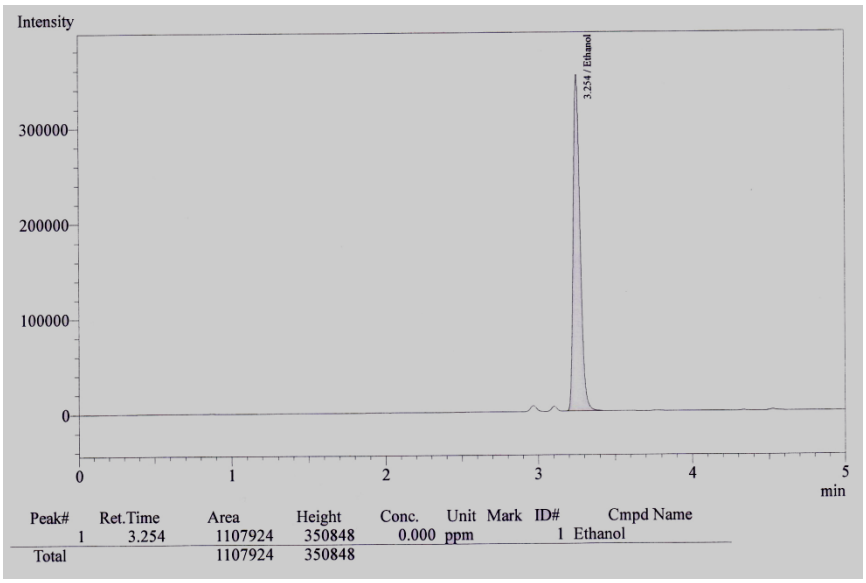
RESULT AND DISCUSSION

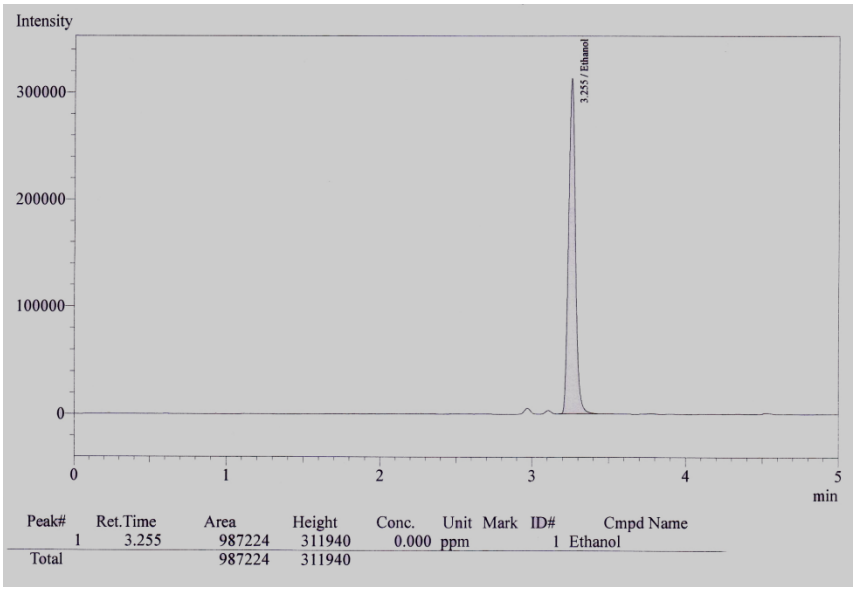
Calculation of ethanol production

Ethanol production was calculated in %. GC gave the print out result of ethanol in area.









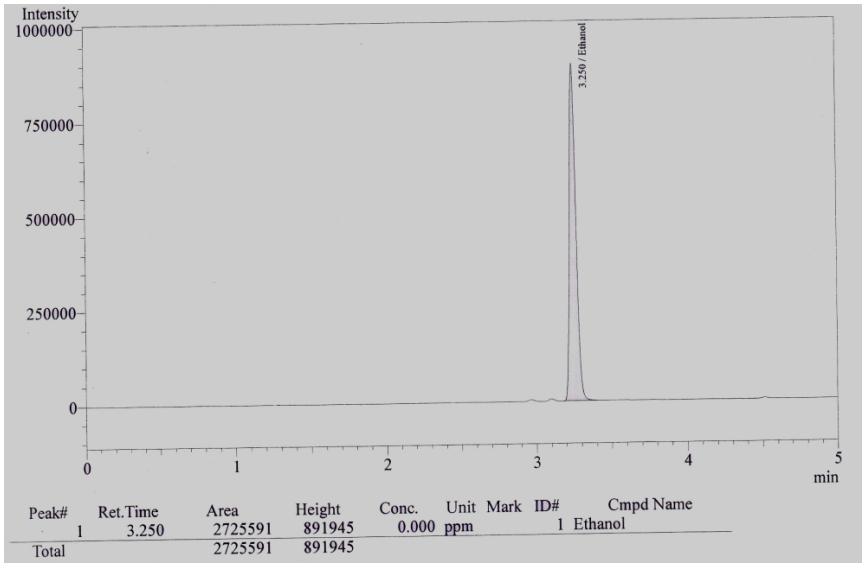


Figure-1: Some GC result of ethanol production.

From the above picture (Figure-1) ethanol retention time was 3.25 [10, 11, 12]. They show the ethanol production rate through area. These were raw data from which blank area had to reduce. Final area was multiply with dilution time to get actual ethanol amount.

$$\text{Ethanol \%} = \frac{\text{Actual area of sample} \times \text{Standard Value}}{\text{Area of standard}}$$

Table -2: Records from GC

Actual Area of Sample	Area of Standard	Ethanol %
7670770	1644375	0.46415302
14305760	1693524	0.84050956
27222330	1644375	1.64720446
27262710	1644375	1.64964783
9711600	1693524	0.57058784
18019660	641662	1.39009817
35842440	1693524	2.10585901
29950920	1693524	1.7597132
5313260	641662	0.40988304
18812760	850372	1.09508735
22091320	850372	1.28593173
13021240	850372	0.75796402
19677320	850372	1.14541323
19411980	850372	1.12996784
14650200	641662	1.13016651

Actual area of sample = Sample area-Blank area
Area of standard varies with time and ethanol concentration.

Standard value measured regularly. Here used two values are as follows:

$$\text{99.5\% 0.05ml used to make 50ml standard of values} = \frac{0.05 \times 99.5}{50} = 0.0995\%$$

$$\text{99.5\% 0.05ml used to make 100ml standard of values} = \frac{0.05 \times 99.5}{50} = 0.0495\%$$

Blank area was 3358.

Yeast cell concentration: Firstly OD was measured from known Concentration. Then an equation (y=21.965x+0.0211) was developed by using these data. Using this equation, concentrations were calculated by measured OD.

Table-3: Concentration vs OD

Known Concentration (mg/ml)	OD	Concentration from Equation (mg/ml)	OD
0.0009	0.025	0.008383	0.205
0.001	0.053	0.001321	0.05
0.003	0.084	0.008337	0.204
0.006	0.16	0.000592	0.034
0.009	0.229	0.003736	0.103
0.01	0.249	0.012301	0.291
0.02	0.43	0.004738	0.125
0.03	0.706	0.010661	0.255
0.04	0.885	0.008519	0.208
0.05	1.124	0.022005	0.504
		0.009704	0.234
		0.007654	0.189
		0.001549	0.055
		0.001686	0.058
		0.001412	0.052

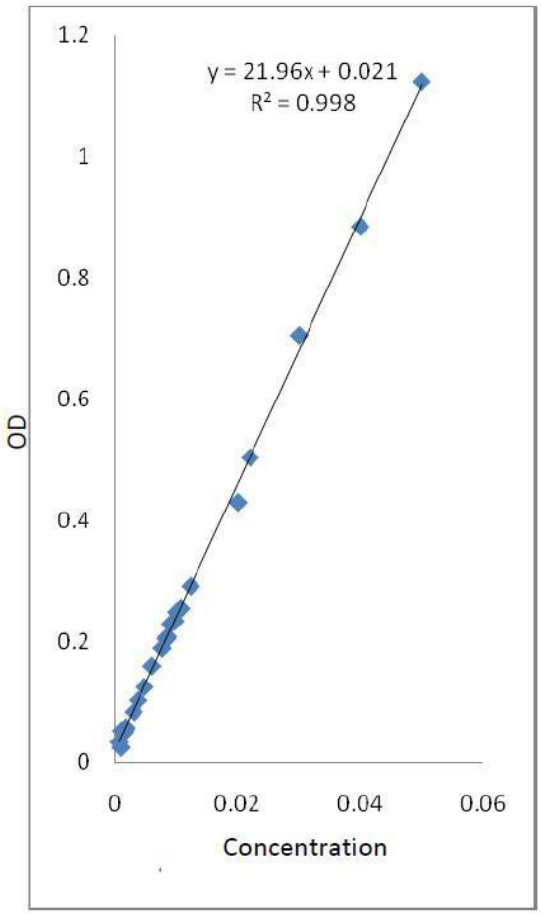


Figure-2: Concentration vs OD

Finding favorable parameters

Yeast growth pattern observed on different pH, glucose amount, and incubation temperature. Best result giving parameters were examined finally [3, 10]. Here are some growth patterns of yeast:

Table-4: Parameters Calculations

Incubation time (hour)	A		B		C	
	pH 7, Glucose 3% at 24^0C		pH 5, Glucose 1% at 36^0C		pH 6, Glucose 2% at 30^0C	
	OD	Ethanol	OD	Ethanol	OD	Ethanol
1	0.1	1.05436206	0.1	1.00436206	0.1	1.08436206
5	0.13	1.069013729	0.13	1.089013729	0.13	1.129013729
10	0.18	1.12781206	0.15	1.11751326	0.15	1.12983016
15	0.2	1.132604237	0.2	1.122705252	0.2	1.130664254
20	0.25	1.139856777	0.23	1.141836234	0.23	1.142846287
25	0.33	1.167735059	0.33	1.163415542	0.33	1.165536099
30	0.4	1.180685099	0.4	1.185145289	0.4	1.182355802
35	0.46	1.25008235	0.46	1.25388624	0.46	1.25226154
40	0.5	1.295316457	0.5	1.292561475	0.5	1.293516626
50	0.55	1.30882774	0.55	1.32842624	0.55	1.35852872
60	0.6	1.383074589	0.6	1.383573144	0.6	1.583033879
70	0.7	1.474867169	0.77	1.472892329	0.77	1.674845269
80	0.8	1.487446967	0.8	1.484562664	0.8	1.687267478
90	0.85	1.493773511	0.75	1.495672615	0.75	1.88691566
100	0.88	1.44691566	0.75	1.48882774	0.75	1.893773511

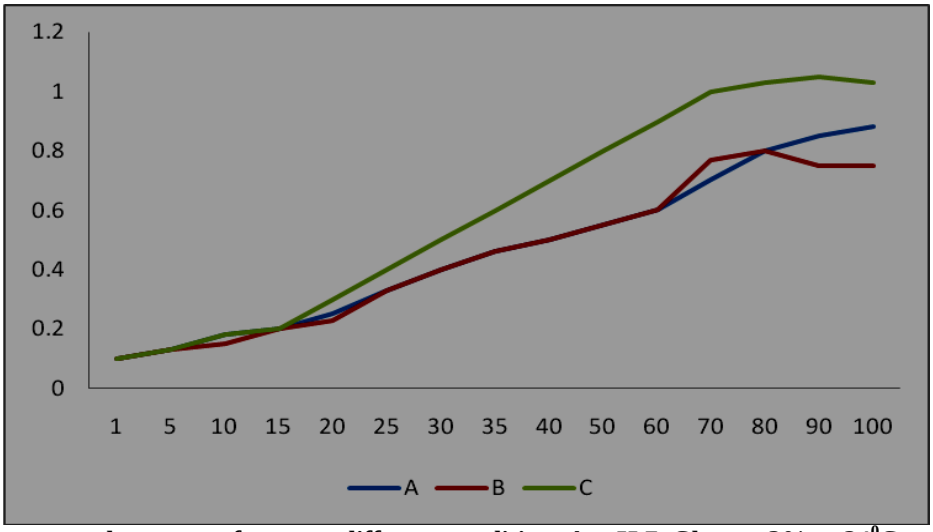


Figure-3: Different growth pattern of yeast at different condition. A: pH 7, Glucose 3% at 24^{0}C, B: pH 5, Glucose 1% at 36^{0}C, C: pH 6, Glucose 2% at 30^{0}C.

After examine all the patterns; most suitable is found in pH 5 to 7, Glucose 1 to 3% at 24^{0}C to 36^{0}C. Low glucose was primary obstacle of yeast cell growth. pH was minor factor here. As acidic condition prohibits microbial growth, basic pH initiates other organisms' growth that will inhibit yeast cell growth and interfere in ethanol production. Moreover, low incubation produce low ethanol and excess incubation initiate cell decline phase first rather than ethanol production.

Table- 5: The Box-Behnken design matrix employed for three independent variables (Glucose%, pH, Incubation temperature) with observed values (Optical density and Ethanol Production).

Run No	Glucose%	pH	Incubation temperature (^{0}C)	OD	Ethanol (%)
1	1	5	30	1.025	0.464153
2	1	7	30	0.25	0.84051
3	3	5	30	1.02	1.647204
4	3	7	30	0.17	1.649648
5	1	6	24	0.515	0.570588
6	1	6	36	1.455	1.390098
7	3	6	24	0.625	2.105859
8	3	6	36	1.275	1.759713
9	2	5	24	1.04	0.409883
10	2	5	36	2.52	1.095087
11	2	7	24	1.17	1.285932
12	2	7	36	0.945	0.757964
13	2	6	30	0.275	1.145413
14	2	6	30	0.29	1.129968
15	2	6	30	0.26	1.130167

From regression analysis all nine coefficients are used in making the response equation.

Table-6: Regression coefficient and corresponding probability values (*p*-values) for specific responses (Optical density of Yeast cell and Ethanol Production)

Parameter (coefficient)	OD		Ethanol Production	
	coefficient	*p*-value	coefficient	p-value
Constant(A_0)	19.83313	9.91E-05*	0.179755	0.000102*
X_1(A_1)	0.675625	0.054613	0.006156	0.054613
X_2(A_2)	-2.97	0.000827*	-0.02706	0.000827*
X_3(A_3)	-0.73615	7.43E-05*	-0.00671	7.43E-05*
X_1X_2(A_4)	-0.01875	0.565121	-0.00017	0.565121
X_1X_3(A_5)	-0.01208	0.063152	-0.00011	0.063152
X_2X_3(A_6)	-0.07104	3.35E-05*	-0.00065	3.35E-05*
X_1X_1(A_7)	-0.055	0.143301	-0.0005	0.143301
X_2X_2(A_8)	0.39625	5.82E-05*	0.00361	5.82E-05*
X_3X_3(A_9)	0.020764	2.56E-06*	0.000189	2.56E-06*

*Most significant factors and interaction effects (*p*-value <0.05)

The calculation of regression analysis also gives the value of the determination coefficient R^2 represented in table 4.7. All the values are over 0.90, which indicates that all factors are well related to the response.

The second order polynomial equations for each response were found as follows:

$$Y(c)=19.83313+0.675625X1-2.97X2-0.73615X3-0.01875X1X2-0.01208X1X3-0.07104X2X3-0.055X1X1+0.39625X2X2+0.020764X3X3\ldots\ldots\ldots\ldots (1)$$

$$Y(e)=0.179755+0.006156X1-0.02706X2-0.00671X3-0.00017X1X2-0.00011X1X3-0.00065X2X3-0.0005X1X1+0.00361X2x2+0.000189X3X3\ldots\ldots\ldots\ldots(2)$$

Where Y (c) and Y (e) = Optical density of Yeast cell and Ethanol Production.X_1, X_2 and X_3 are coded values for Glucose%, pH, Incubation temperature respectively.

Some general characteristics of ethanol based on which HT3 was used with GC to separate ethanol from sample: Ethanol C_2H_6O, also called ethyl alcohol, pure alcohol, grain alcohol, or drinking alcohol, is a volatile, flammable, colorless liquid. Boiling point: 78.37 °C, Density: 789.00 kg/m³, Melting point: -114 °C. It was examined through that the collected samples were ethanol.

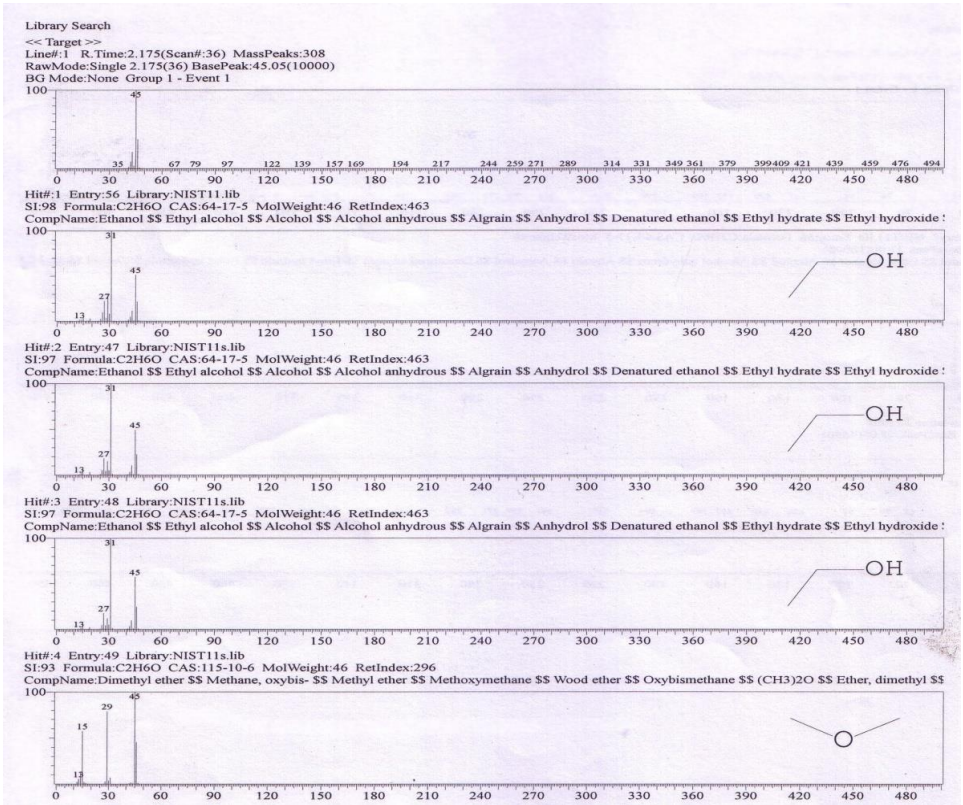
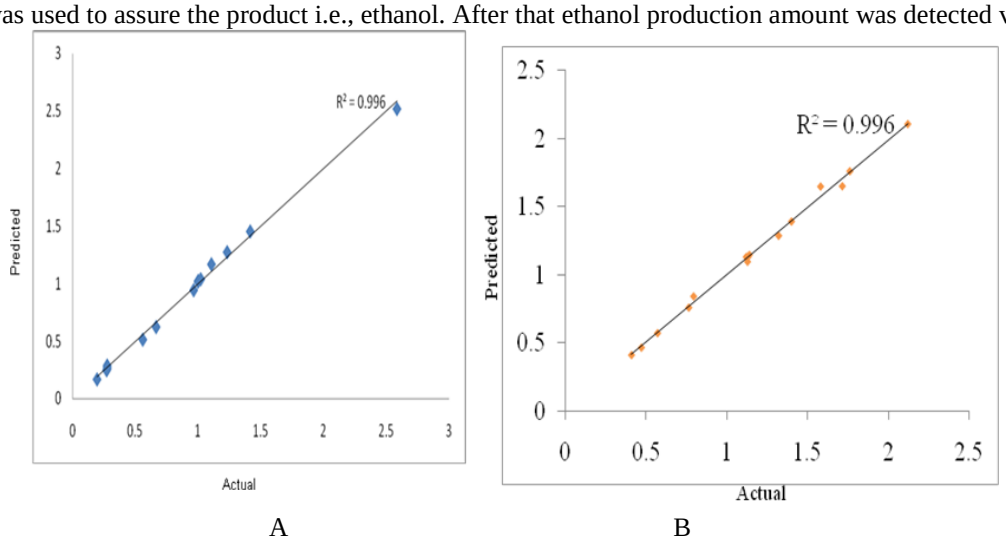


Figure -4: Ethanol Peak detected by GC-MS

GC-MS was used to assure the product i.e., ethanol. After that ethanol production amount was detected via GC.



A B

Figure-5: Linear relationship between the experimental and the predicted responses (A) OD (B) Ethanol concentration.

Table -7: R^2 values for the ANOVA analysis of the three response output

R^2 value	OD	Ethanol Production
	0.996	0.996

From equation (1) - (2) predicted values of the three responses were obtained.

3D line plot analysis: The relationship between coded variables and responses can be better understood by examining the series of 3D line plots. These 3D lines display the effect of variation of two factors while the third is kept constant. The plots were created with the aim to observe optimum condition from predicted values.

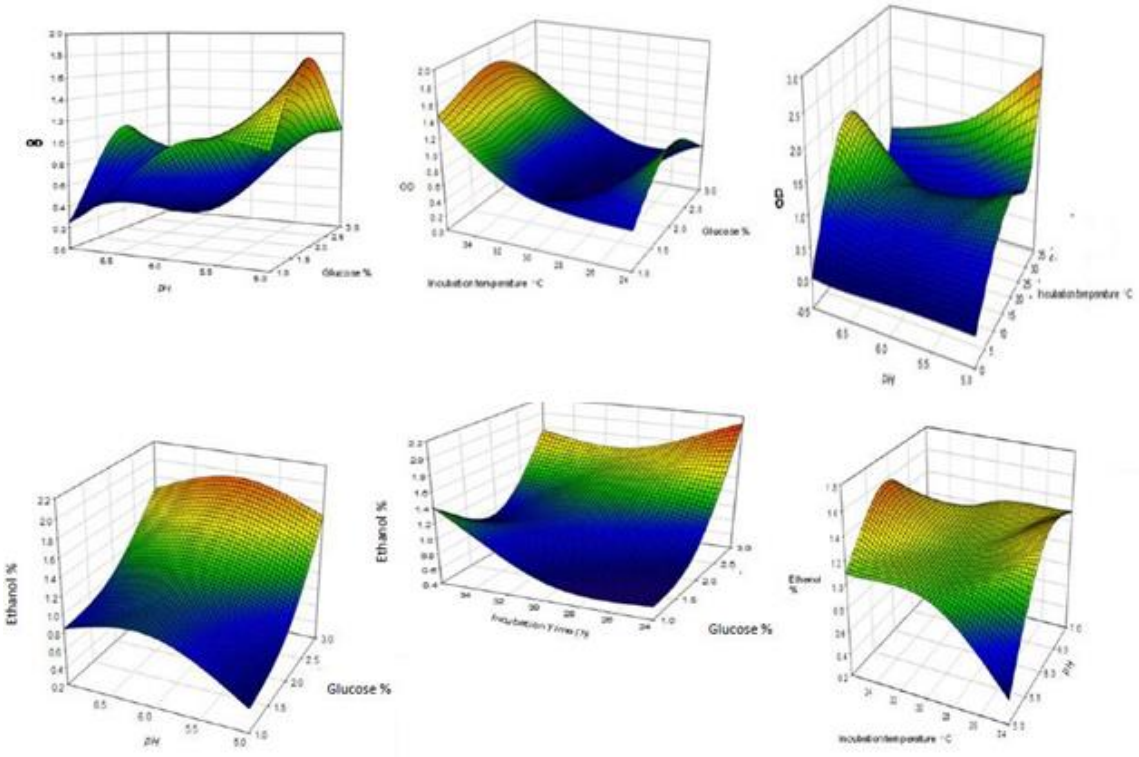


Figure-6: Representing the effect of Glucose%, pH, Incubation temperature on cell density and ethanol production.

Figure-6 represents the effect of glucose amount. pH and incubation temperature on cell density and ethanol production. When incubation time was constant maximum OD was at 3% glucose and pH6. If pH is constant, then maximum cell growth is in 3% glucose at 30^0C incubation zone. Likewise, for pH and incubation temperature variation highest cell density is at 6pH and 32^0C incubation. In case of ethanol production, peak area is in pH 5.5 and 3% glucose where incubation temperature was constant. When pH is constant, maximum ethanol will be produced in 3% glucose and 32^0C incubation. For pH 5.5 and 28^0C incubation time this production is also in top [7, 12].

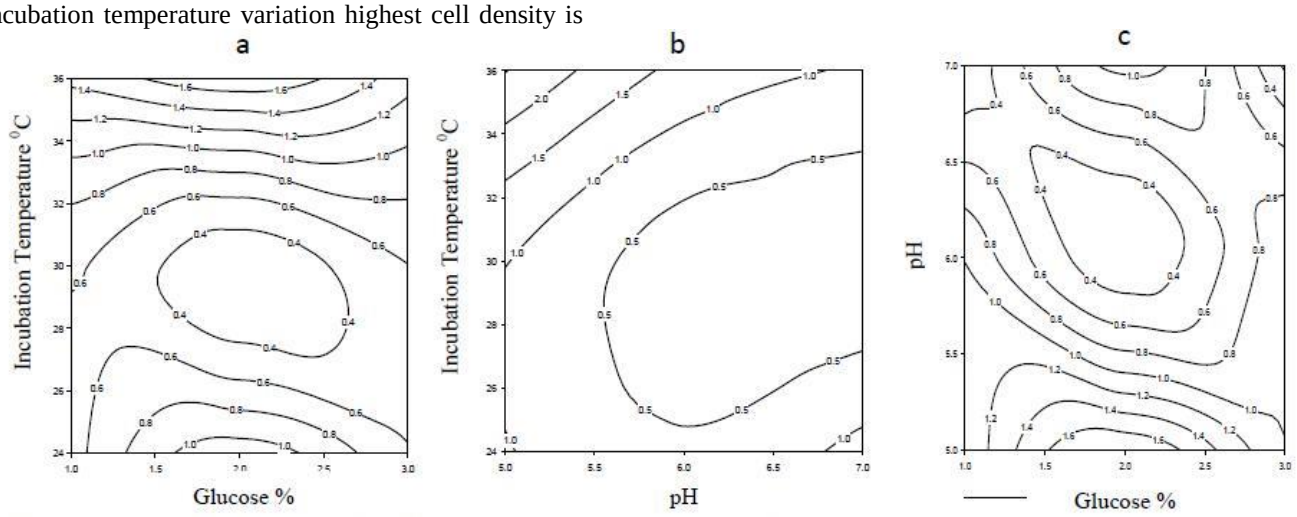


Figure-7: a. Effects of Glucose concentration and Incubation temperature on ethanol production at constant pH. b. Effect of Incubation temperature and pH on ethanol production at constant glucose concentration. c. Effect of Glucose concentration and pH on ethanol production at constant incubation temperature.

From figure-7 ethanol productions increased with higher incubation temperature and higher glucose amount, and also vice versa. But, it is constant at 2% glucose and 30^0C incubation. Again, ethanol rate also rose with incubation time where pH was 4.5. In spite of that, from figure 4.10, 2% glucose and pH6 create a zone of constant ethanol production and it decreases with pH lower than 5.0, like as glucose amount of 1.5%. Higher pH levels may well have aided in the denaturing of the enzyme that help yeast to ferment. The optimal pH would have a faster rate of reaction. Under low pH, high glucose concentration and temperatures around 30°C, ethanol production was highest.

Statistical Validation : The method was validated by randomly selecting a few values from among the combinations. Validation results are presented in Table-8.

Table-8: Predicted and experimental values

Run No.	Glucose %	pH	Incubation Temperature (^{0}C)	OD		Ethanol Production	
				Predicted	Experimental	Predicted	Experimental
1	1.5	6.0	33	1.0830	1.089114	0.007778	0.00814
2	2.5	5.5	30	1.0448	1.038443	0.007467	0.007824
3	3.0	6.5	28	0.3118	0.466759	0.002544	0.002602
4	2.5	7.0	35	0.0209	0.013918	0.009484	0.009128
5.	2.0	6.5	24	1.0352	1.053337	0.007928	0.008054

The fact behind this distribution of efficiencies was due to glucose concentration and incubation temperature manly. The method was validated by randomly selecting a few values from among the combinations. Validation results are presented in Table 4.8. It was found that the experimental efficiency was almost close to predicted efficiency value, within about ±5% limit. It was also observed that R^2 values were over 0.99. It can thus be inferred that efficiency varies if any of the factors are varied [4, 12, 13].

CONCLUSION

Using ethanol as an alternative to gasoline provides several key benefits which is relatively low-cost. Ethanol production supports farmers and creates domestic jobs. Ethanol is widely considered a way to reduce greenhouse gases from fossil fuel use and thereby reduce human caused global warming.

The Box–Behnken design can be a useful approach to determine the optimum conditions for maximum production. This study developed coefficients of a quadratic equation for each kind of

responses and used them in designing a valid predictive. The optimum condition was found when a 3% glucose media of pH 6.0 at 30^0C of an incubation period over 100 hours was used. These parameters produced maximum efficiency as well as good resolution. Using various random conditions, the ethanol production rate was also validated, resulting in experimental efficiency values varied within ±5% of the predicted values.

Intact dry yeast is relatively easily obtained. As ethanol can inhibit yeast cell growth thus fermentation, further study can be done on producing more ATP with ethanol and for these continuous formation and pure culture of yeast could be effective.

Reference

1. Al-Judaibi AA; Effect of some fermentation parameters on ethanol production from beet molasses by *Saccharomyces cerevisiae*, American Journal of Agricultural and Biological Sciences, 2011;6 (2):301-306.
2. Asaduzzaman,Md; Standardization of Yeast Growth Curves from Several Curves with Different Initial Sizes, Chalmers, 2007; 1-57.
3. Arroyo-López FN, Orlić S, Querol A, Barrio E; Effects of temperature, pH and sugar concentration on the growth parameters of *Saccharomyces cerevisiae, S. kudriavzevii* and their interspecific hybrid, International Journal of Food Microbiology, 2009; 131:120–127.
4. Babar SE, Song EJ, Hasan Md.N, Yoo YS; Experimental design optimization of the capillary electrophoresis separation of leucine enkephalin and its immune complex, J.Sep. Sci., 2007; 30:2311-2319.
5. Cheng NG, Masitah Hasan M, Kumoro AC, Ling CF, Tham M; Production of Ethanol by Fed-Batch Fermentation, Pertanika J. Sci. & Technol., 2009; 17 (2): 399 – 408.
6. Jana Hajšlová J, Cajka T; Gas chromatography–mass spectrometry (GC–MS), Food Toxicants Analysis, 2007; 1-55.
7. Mirsky N, Chinkov N; A simple rapid gas-chromatography flame-ionization-detector (GC-FID) method for the determination of ethanol from fermentation processes, African Journal of Biotechnology, 2012; 11(15):3612-3616.
8. Prasad S, Anoop Singh A, Joshi HC; Ethanol as an alternative fuel from agricultural, industrial and urban residues, Resources, Conservation and Recycling, 2007;50:1–39.
9. Reeves EGM, Louisiana BS; Kinetic analysis of *Kluyveomyces Marxlanusv y*east strain, The Department of Biological and Agricultural Engineering, State University, 2001, May 2004, 40-60.
10. Suresh Babu CV, Chung B C, Lho DS, Yoo YS,; J. Chromatogr. A 2006; 1111:133 – 138.
11. Serra A, Strehaiano P, Taillandier P; Influence of temperature and pH on *Saccharomyces bayanus* var. uvarum growth; impact of a wine yeast interspecific hybridization on these parameters. International Journal of Food Microbiology, 2005; 104:257–265.
12. Sorensen BJ, Jakobsen M; The combined effects of temperature, pH and NaCl on growth of Debaryomyces hansenii analyzed by flow cytometry and predictive microbiology. International Journal of Food Microbiology, 1997; 34:209–220.
13. Swinnen IAM, Bernaerts, Kdens EJJ, Geeraerd AH, Van Impe JF; Predictive modelling of the microbial lag phase: a review. International Journal of Food Microbiology, 2004; 94:137–159.
14. Wen-jun G, Yu-ping Z, Yi-Jun Z, Guang-ri XU, Xin-jun W, Kwang-pill L; Optimization strategies for separation of sulfadiazines using Box-Behnken design by liquid chromatography and capillary electrophoresis , J. Cent. South Univ. Technol, 2007; 2:196−206.