Optimization of Ethanol Production by Response Surface Methodology from Enzymatically Hydrolyzed Sugarcane Bagasse

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In

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Submitted by

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CERTIFICATE

This is to certify that the M. Tech. dissertation entitled **"Optimization on Ethanol Production Using Response Surface Methodology from Enzymatically Hydrolysed Sugarcane Bagasse"**, submitted by **Anand Kumar Gupta (DTU/13/MTECH/381)** in partial fulfilment of the requirement for the award of the degree of Master of Engineering, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by him under my guidance.

The information and data enclosed in this dissertation is original and has not been submitted elsewhere for awarding of any other degree.

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DECLARATION

I hereby declare that the details furnished above are true and correct to the best of my knowledge and belief and I undertake to inform you of any changes therein, immediately. In case any of the above information is found to be false or untrue or misleading or misrepresenting, I am aware that I may be held liable for it.

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Place: Signature of candidate

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Optimization of Ethanol Production by Response Surface Methodology from Enzymatically Hydrolyzed Sugarcane Bagasse

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ABSTRACT

With industrialization there has been a sharp increase in the energy consumption. Since emergence of bioethanol as a potential fuel, the world scenario shows that large share of research in past thirty years have been focussed on technological know-how development for bioethanol. Sugarcane juice has been used as an alternative fuel in few countries like Brazil and United states since 2006. Sugarcane bagasse has the potential to be the second generation bioethanol. Use of modern computer based technology can increase the yield of ethanol production .This can be done by optimization of various parameters involved during fermentation. The aim of the study was to use sugarcane bagasse for alcohol production. Fuel produced through bagasse is eco-friendly, cheap, easily renewable. The MTCC 178 strain of yeast has been used for fermentation to produce alcohol. For optimizing the production of alcohol from sugarcane bagasse different conditions with respect to temperature, pH and inoculums concentration were used via Response Surface Methodology (RSM). The result obtained shows that the production of alcohol from the sugarcane bagasse can be optimized at pH 6.54, 23^{o} C temperature and at 6% inoculum concentration with 6.27% alcohol production.

INTRODUCTION

With industrialization of many more countries and growing population, the consumption of energy has increased over last century. There has been prediction that yearly global oil production will fall from the current 25 million barrels to around 5 million in 2050 (http://www.bloomberg.com/news/2013-03-05/russia-s-oil-lead-challenged-as-taxes-strangle drilling.html). Economy of many nations depend on oil, the inadequate supply of oil can lead to severe consequences. Therefore there is a great interest and need for search of alternative energy source. The development of biologically derived and environmentally sustainable fuel such as bioethanol is recognized as an important alternative to reduce our reliance on fossil fuel. Lignocellulose can be utilized for the manufacture of ethanol which can form an alternative energy source for the limited amount of crude oil (Isaias 2008). Since the ethanol is manufactured by fragmenting grains, plant biomass and others organic material using micro-organism such as yeast cells ethanol is referred to as a biofuel (Pimentel & Patzek, 2005). Bioethanol is already making significant contribution towards energy needs. Canada and Brazil are major producers of this energy source and much of it is derived from grains such as corn and wheat (Rerto et al, 1991).

Review of literature

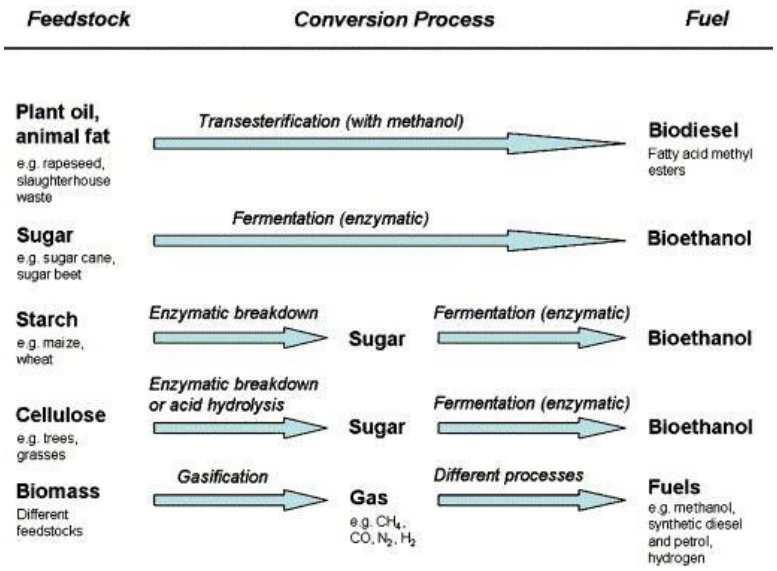


FIGURE 1: Different feedstock and their conversion process into fuel

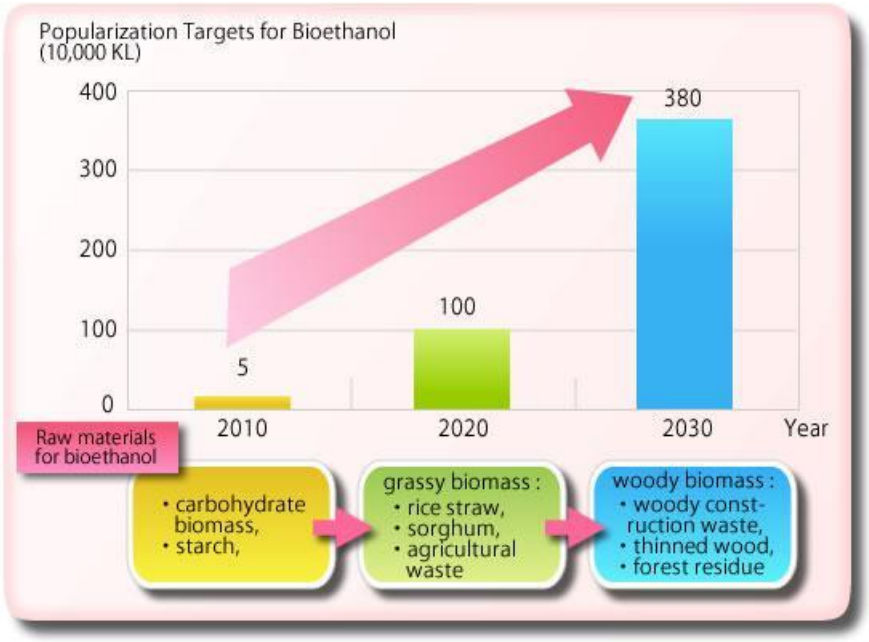


FIGURE 2: Estimated increase of bioethanol production by 2030

Mainly there are two processes involved in the conversion of lignocellulosic biomass to ethanol. First is the hydrolysis of cellulose in the lignocelluloses of bio-mass to produce reducing sugar followed by fermentation of sugar to ethanol (Ye Sun et al 2002) (Taherzadeh et al 2007). Sugarcane bagasse can form a substrate for production of ethanol (Betancur & Pereira Jr. 2010). To extract ethanol from sugarcane bagasse, the bagasse is first dried, ground and then treated with concentrated sulfuric acid at high temperature to open crystalline structures of cellulose and hemicellulose. Components are enzymatically broken down to release smaller sugar entities that can be readily fermented by micro-organism to produce ethanol (Taherzadeh et al 2007) (Neubeck, 1975). The ethanol released during fermented process is later distilled from the liquid and further purified. Yeast is commonly used for the fermentation of biomass into ethanol.

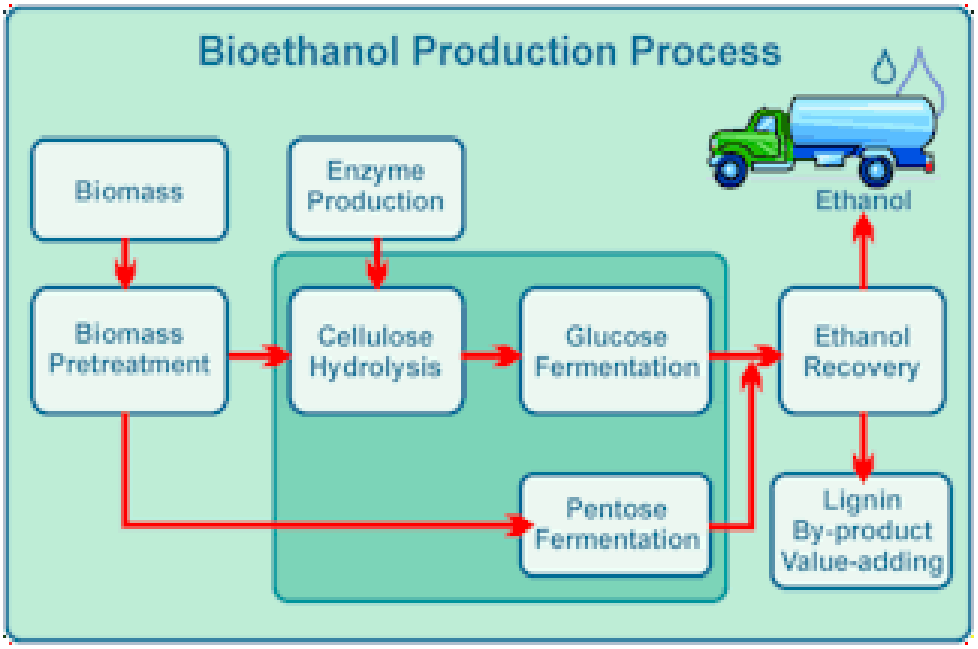


FIGURE 3: Bioethanol Production Process

Fermentation is a part of cellular respiration that is shared by essentially all cells. Based on current technologies available the cost of production of ethanol from lignocellulosic biomass is relatively high. Considerable research work are being done in order to optimize the process and pre-treatment of lignocelluloses for removal of lignin and hemicelluloses so that the amount of cellulose available for fermentation can be enhanced.

SUGARCANE

Sugarcane is one of the important agriculture cash crop cultivated in tropical countries. Sugarcane is grown more successfully in such regions where the climatic condition is tropical but it can also grow in sub tropics too as in north India. Sugarcane is cultivated in the world from 35° N latitude to 35°S latitude, from sea level to 1000m of altitude.

FIGURE 4: Sugarcane

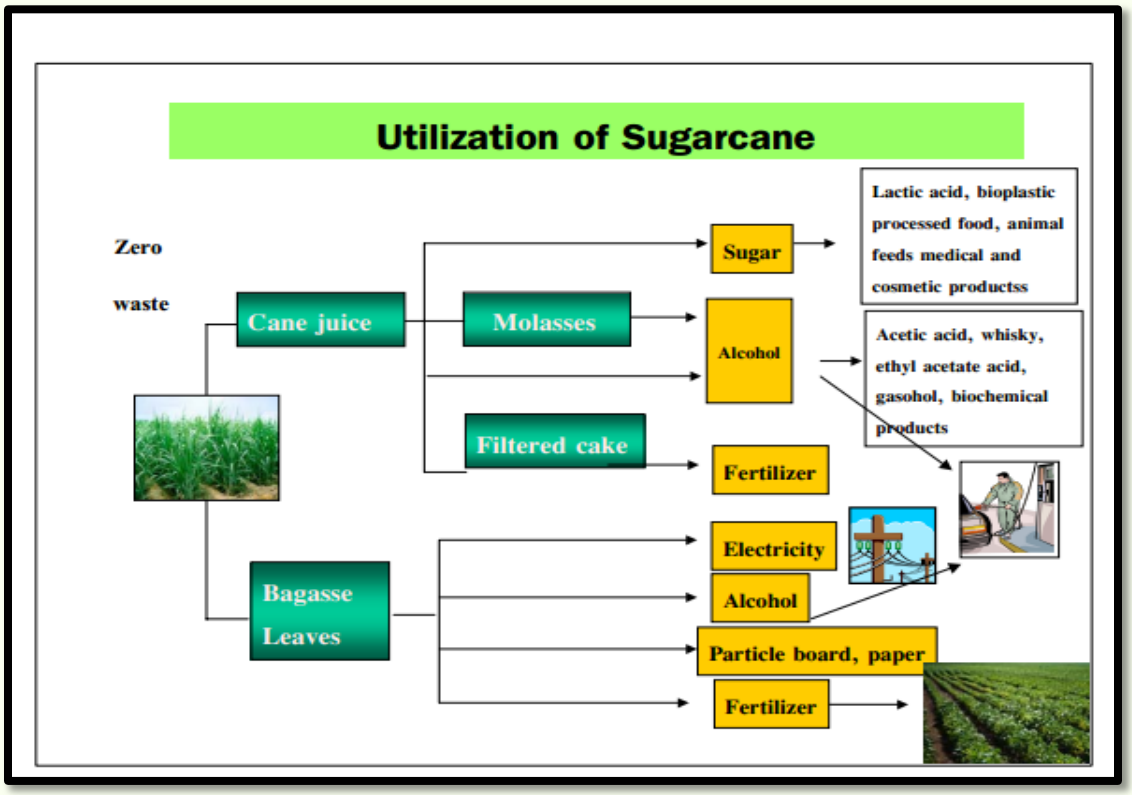


FIGURE 5: Showing utilization of sugarcane juice and bagasse

The yearly world production of sugarcane is nearly 1.6 billion tons and it generates 279 million metric tons of biomass residue.

Country	Area (million ha)	Production (million tons)	Productivity (Tons/ha)
Brazil	5.343	386.2	72.3
India	4.608	289.6	62.8
China	1.328	92.3	65.5
Thailand	0.970	64.4	66.4
Pakistan	1.086	52.0	47.9
Mexico	0.639	45.1	70.6
Colombia	0.435	36.6	84.1
Australia	0.423	36.0	85.1
USA	0.404	31.3	77.5
Philippines	0.385	25.8	67.1
Indonesia	0.350	25.6	73.1
Cuba	0.654	22.9	35.0
South Africa	0.325	20.6	63.4
Argentina	0.295	19.2	65.2
Myanmar	0.165	7.5	45.4
Bangladesh	0.166	6.8	41.2

TABLE 1: Sugarcane production in different countries

SUGARCANE BAGASSE

Sugarcane bagasse is a byproduct of sugarcane which is left after the juice has been extracted from the sugarcane. It is the fibrous residue of sugarcane . Because of the fibrous nature sugarcane bagasse has been widely used in fuel, paper and pulp, structural material and agricultural uses (Han et al, 1983). In recent times due to shortage of food and energy all over the world sugarcane bagasse is being considered as a substrate for renewable energy production animal feed and single cell protein.. The conversion of sugarcane into ethanol gives rise to two main byproducts which are mainly thermal energy and bagasse. The first is responsible for all of the energy (thermal and electricity) used in ethanol and sugar production during the distillation and refining processes (Smeets et al., 2008). Bagasse on the other hand, is agro waste product and is comprised of cellulose , hemicellulose, lignin, ash and other components in decreasing order respectively. (Luz et al., 2007).

FIGURE 6: Sugarcane Bagasse

YEAST

Yeasts are eukaryotic microorganisms classified in the kingdom Fungi with 1500 species identified (Kurtzman & Cletus , 2005). Yeasts are unicellular organisms, although some species with yeast forms may become multicellular. This is achieved through the formation of strings of connected budding cells known as pseudohyphae which is seen in most molds (Fell and Kirtzman 2006). By fermentation, the yeast species Saccharomyces cerevisiae converts carbohydrates to carbon-dioxide and alcohols; for thousands of years the carbon dioxide has been used in baking and alcohol in alcoholic beverages (Legras, 2007).

Fermentation

Microorganisms are capable of using a wide range of substrates and can produce a diversified spectrum of products. Because of this commercial exploitation of the biochemical diversity of microorganisms, it has resulted in the development of the fermentation industry. Fermentation depends of many different extrinsic and intrinsic variables, which are microbiologically mediated (Pramanik, 2003). A large diversity of microbes is inherent to fermentation which includes various bacteria, yeasts and fungi. Prominent in this process are *Saccharomyces* species (predominantly *S. cerevisiae*), which dominate the alcoholic fermentation. Fermentation is an anaerobic process carried out by micro-organism to produce energy & others secondary metabolite by oxidising carbohydrate and similar compound.

For alcohol production batch fermentation is commonly employed. Batch fermentation can be considered to be a closed system. At time t=0 the nutrient solution which is sterilized and placed in the fermentor is inoculated with yeast microorganisms and incubation is allowed to happen. During the entire fermentation period, nothing is added, except an antifoam agent, acid or base for pH control and oxygen (for aerobic microorganisms). The culture medium's composition, the concentration of biomass, and concentration of the metabolite generally change constantly as a result of the metabolism of the cells.

Yeast Reproduction and Pattern of Growth

Cell division in yeast occurs through budding. During budding, a daughter cell is formed as an outgrowth from mother cell following nuclear division and cell wall formation and cell separation. The sizes of cell vary with the phase of growth and from strain to strain (Sherman, 1997). After the inoculation of a nutrient solution with yeast microorganisms and cultivation under appropriate physiological conditions, three phases of growth are seen: lag phase, exponential phase and stationary phase. When freshly cultured yeast cells are inoculated in a growth medium, it initially enters the lag phase in which the cells are metabolically active but do not divide. During this phase the cells adapt themselves to the new medium and hence called the adaption phase. The cells actually metabolize in preparation for cell division. The cells activate the metabolic pathways to make sufficient components available for cell division. Duration of this phase depends upon initial population size and various environmental conditions such as temperature, pH, alcohol content, oxygen concentration, salt concentration and nutrients availability. Once all the metabolic pathways are activated DNA replication begins and shortly the cell division follows. The second phase of growth called exponential phase begins after this. Exponential phase depends upon factors such as growth medium, organism, and temperature. These factors have an important role in the determination of the generation time. The final phase i.e. the third phase is called stationary phase wherein the cell metabolism slows down and cells stop dividing rapidly. Change in environment is a major cause that makes the cell enters the stationary phase.

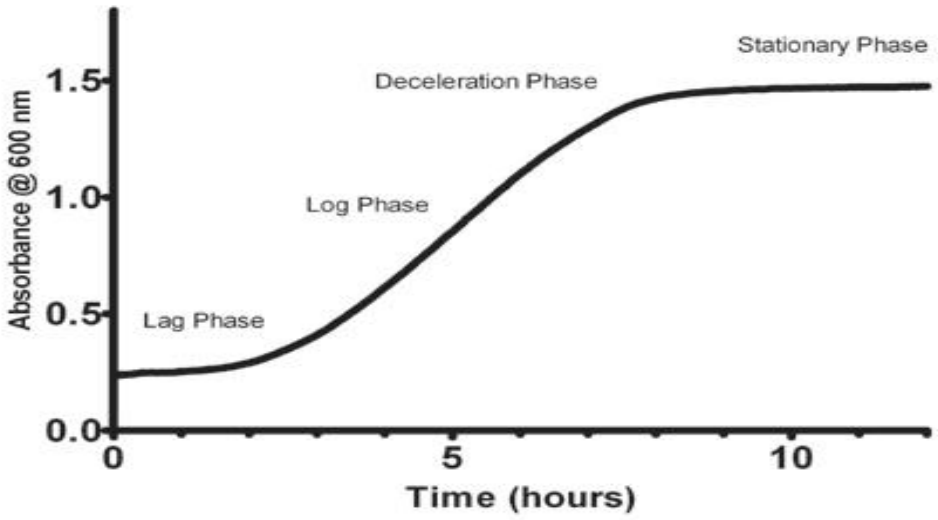


FIGURE 7:Pattern of growth of Yeast

FACTORS EFFECTING YEAST PERFORMANCE DURING FERMENTATION:

Effect of pH:

The pH of the growth medium is an important parameter for the successful progress of fermentation because it influences yeast growth as well as ethanol formation. It has been observed that a beverage with a pH of less than 3.4 presents a notable resistance to bacterial attack. However, in a beverage with a pH more than 3.6, the development of harmful microbial flora may occur. Also it has been seen that, fermentations carried out in excessively acidic media become too slow due to the low growth rate of the yeast (Ough et al, 1991. The pH of the medium may also change in response to metabolic activities of microorganisms.

Effect of temperature:

As yeast strains differ in their response to temperature, the optimum temperature for carrying out vinification can vary widely. Torija and team, in 2001, obtained a mixed response to fermentation temperature on mixed strain of *S. cerevisiae*. The yield of alcohol was more at higher temperature while secondary metabolites such as volatiles, esters, glycerol etc. increased at lower temperature (Robinson, 2006).

Effect of initial sugar level:

Initial sugars concentration is an important parameter in the final production of ethanol and its sensory quality. It has been seen that initial sugar level in bagasse greatly affects the rate of fermentation. Using concentrated sugar substrate has been one of the ways to obtain high ethanol yield during fermentation. However, it is observed that high substrate concentrations are inhibitory to fermentation due to osmotic stress (Jones, 1981).

Carbon Sources:

Fructose and glucose make 15 to 25% of the sugar content of bagasse. These compounds are excellent carbon source for yeast growth. Sugar content of the substrate above 25% stops fermentation due to osmotic effect. Also it has been observed that higher sugar content enhances the fermentation time with decreased production of alcohol. Yeast can also grow on many of other carbon sources.

Detoxification of Bagasse

On acid hydrolysis of bagasse formation of large number of inhibitory components of fermentation are formed. These factors cause toxicity which is a major limitation in the fermentation procedure. Hydrolysis of bagasse depends upon time period, temperature, acid conc., HCL and H_2SO_4 (2–5 %). A temperature under 16^0C is enough for hemicelluloses hydrolysis (Mussattto, 2003). A temperature of above 16^0C favours cellulose hydrolysis which generates a high quantity of sugar and lignin decomposed product. Cellulose on hydrolysis degrades to glucose and hemicelluloses degrades to xylose, mannose, acetic acid and galactose. At high temperature glucose and xylose degrades to furfural and hydroxymethyl furfural respectively. Further degradation of furfural and hyroxymethyl furfural results in formation of formic acid and levulinic acid (Cardona, 2010). Apart from them a large no. other compounds are also produced. These components are toxic to fermenting micro- organism (Mart´ın et al, 2002). Hence in order to make conditions suitable for micro-organism detoxification of lignocellulosic substance is essential. Various detoxification methods are available to transform inhibitory compounds to inactive compounds. Chemical method includes making use of citric acid (Martinl, 2002).

RESPONSE SURFACE METHODOLOGY:

Response surface methodology helps in determining the relationship between several explanatory variable and one or more response variables. It is used to design experiments and then obtained an optimal response. The aim is to optimize a response (output variable) which is influenced by several independent variables (input variables). When the experiments are carried out changes are made in the input variablesso that the reasons for changes in the output response can be identified(Ratman et al, 2003) (Myers, 1989). Response is represented graphically in 3D space or as counter plots that help visualize shape of response surface. Contours are curves of constant response drawn in Xi and Xj plane keeping all other variables fixed each contour corresponds to a particular height surface. Response surface methodology is used in designing, formulating, developing and analyzing new scientific studying and products in industrial, biological, clinical sciences, food sciences and engineering sciences (Kalil et al, 2000). RSM has been applied successfully to optimize alcoholic fermentation from sugarcane bagasse (Sasikumar & Viruthagiri, 2008).

MATERIALS AND METHOD

MATERIALS USED:

1. **Sugarcane bagasse**

Order : Poales

Family : Poacelae

Genus : Saccharum

2. **Enzymes**- Pectinase and cellulase

3. **Chemicals**- Hydrochloric acid (HCl) 1N, Sodium Hydroxide (NaOH) 2N, 95% Ethanol, Monobasic Potassium Phosphate (KH_2PO_4), Dibasic Potassium Phosphate (K_2HPO_4), Dextrose, Peptone, Yeast extract and Agar, Citric acid 1N,magnesium sulfate heptahydrate ,Ammonium dihydrogen phosphate, Diammonium phosphate

4. **Yeast Strain-** Saccharomyces cerevisiae MTCC178.

5. **Glass wares/Plastic wares-** Testubes, Testube stand, Falcon tubes, Ochridge tubes, conical flasks, Beaker, Petri plate, Autoclave bags, Plastic tray.

6. **Instruments-** pH meter, Mixer, Centrifuge, Incubator shaker, Weighing balance, Hot water bath, Hot plate, Thermometer, Autoclave, Alcohol and Brix Refractometer, Spectrophotometer, Soil digester, Laminar flow hood.

7. **Others-** Non absorbent cotton, Muslin cloth, Gloves, Aluminum foil, Para film, Wash bottle, Distilled water, Spirit lamp, Inoculating loop, Tissue roll, Pipette and tips, Dropper, Butter paper, Surgical blades.

FIGURE 8: Alcohol and Brix Refractometer

METHODS:

Revival of yeast strain:

A strain of Saccharomyces cerevisiae MTCC 178 was bought from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. The Yeast strain Saccharomyces cerevisiae MTCC 178 was stored at minus $4^{o}C$ in a freezer. The contents of the ampoule were first dissolved in $1.8cm^3$ of physiological solution. For activating, the test tube containing the cells were warmed in a thermostat maintained at $30^{0}C$ for nearly 30 minutes which is sufficient time to restore the viability of the yeast cells. The next step after restoring yeast viability to undertake culturing and sub culturing of the cells on media petriplates.

Sub-culturing of yeast:

Preparation of media plates:

Yeast extract - 1 g/100ml

Dextrose - 2 g/100ml

Peptone - 2 g/100ml

Agar - 2 g/100ml

All the chemicals were weighed individually and put into the 200 ml flask. After that volume was made up to 100ml by using distilled water. The growth media was then autoclaved at 121^{o}C and 15 psi. The autoclaved media then poured into Petri-plates in the laminar flow and allowed them to gel. After preparation of media plates, these were then inoculated with the yeast strain (revived in the above method) through streaking. After plating, plates were then sealed using parafilm and then kept in the incubator (30^{o}C) for 24 hours. After 24 hours some of the cells from the previous culture are transferred to a new perti-plate using inoculation loop. This is called sub-culturing it is done to prolong life and increase the number of cell of microorganisms. Sub-culturing was performed thrice.

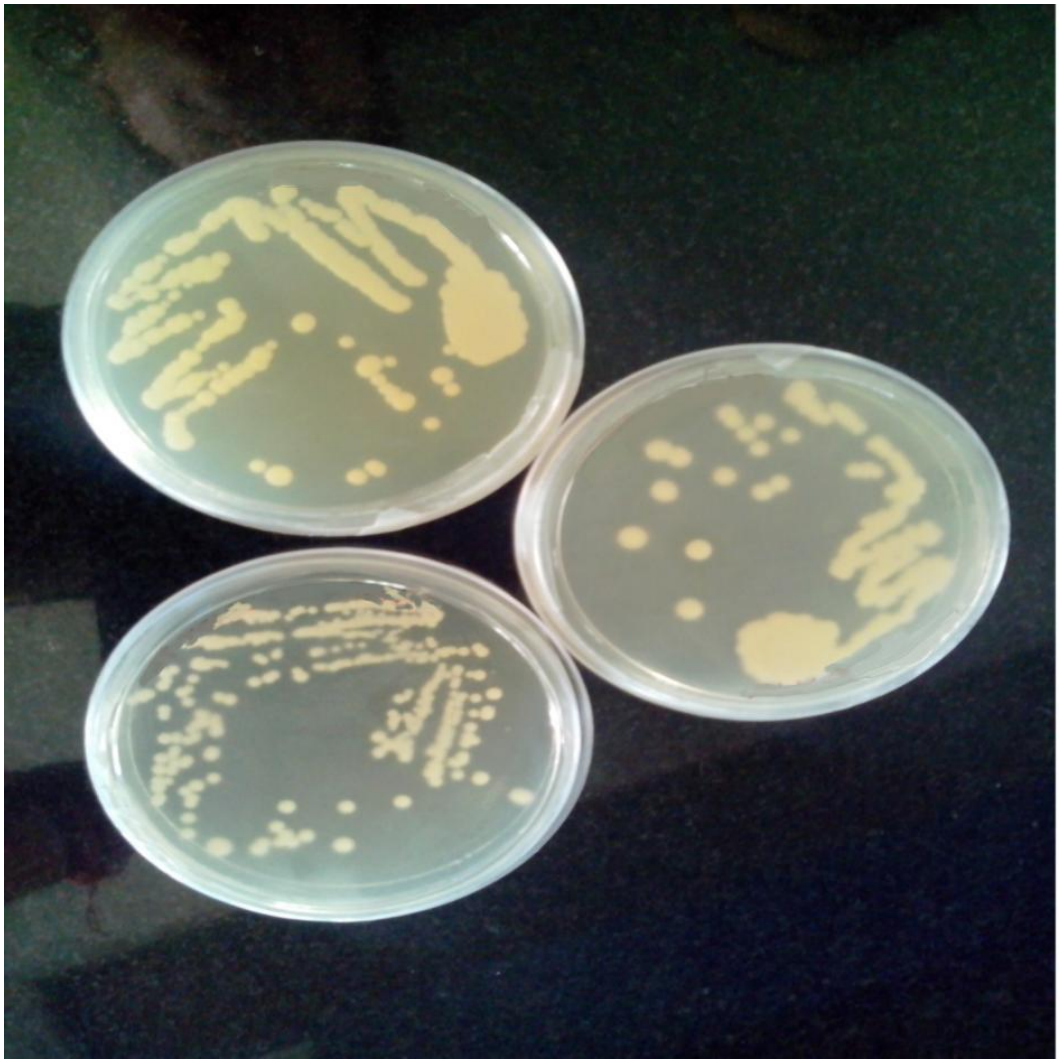


FIGURE 9: Plates having colonies of MTCC 178 strain obtained through streaking method

Growth curve:

The growth medium was prepared initially by mixing peptone, dextrose and yeast extract in 100ml of distilled water**.** After that the growth medium was autoclaved (121^{o}C, 15psi). The plates which were streaked and incubated before were used and a single colony from the plate was picked up through the help of loop and inoculated in a 100 ml autoclaved growth media. After that flask was then placed in incubator-shaker (30^{o}C, 200 rpm). After that O.D was taken at 600nm through spectrophotometer for an interval of every 1 hour.

Alcohol Tolerance

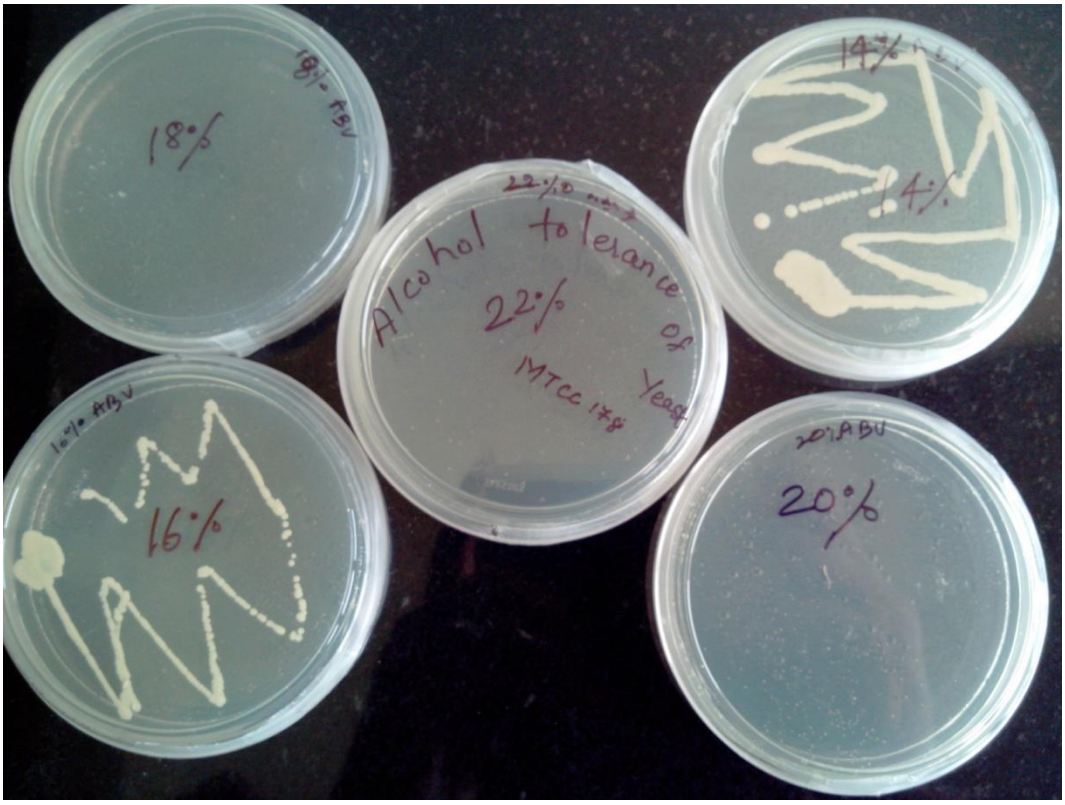


FIGURE 10: Alcohol tolerance of Yeast strain MTCC 178

The production of alcohol through fermentation cannot go indefinitely using a yeast strain, there is a limit beyond which there is no alcohol production despite the amount of sugar content in the media It is generally observed that thr fermentation process stops when the same alcohol concentration is obtained in case the same type of yeast strain is used, it is suitable to make an assumption that there is a limit beyond which the yeast strain cannot tolerate alcohol concentration. So it is of utmost importance that we determine the tolerance of the yeast to alcohol concentration. This is done by plating the yeast cells on mediaplates which contain alcohol in different percentages. So the streaking of the cells was done on media which contained 14, 16, 18, 20 & 22% of alcohol. The MTCC 178 strain showed the alcohol tolerance level to be 16%.

Bagasse slurry preparation

Fresh Bagasse was collected from a sugarcane juice shop. 204gm of bagasse was taken and spread on a blotting sheet. The blotting sheet was put inside the hot air oven at 103C for overnight drying. Next day dried bagasse was weighed again and the weight of the bagasse was estimated to be74.74gm. The dried bagasse was then converted into the fine powder form using grinder. 0.5% dilute sulphuric acid was prepared and 10% by mass bagasse was soaked into it. The bagasse and sulphuric acid slurry were put into the soil digester and the temperature was raised by 20°C starting from 60°C. Each temperature was maintained for an interval of 15 min. The temperature was raised till 180°C and then brought to room temperature. Treatment with sulphuric acid is done to open up the crystalline structure of baggase. The slurry was then put on to a two layered muslin cloth and then centrifuged to completely remove sulphuric acid. The bagasse was then collected into a glass beaker. It was covered with aluminum foil and autoclaved.

Preparation of Pectinase enzyme stock:

Pectinase enzyme was present in powder form so prior to use, it was first dissolved in the phosphate buffer. As pectinase works best at pH 3.4 so the pH of the phosphate buffer was set by mixing monobasic and dibasic in a particular ratio.

Monobasic (KH_2PO_4) - 13.795 g/100ml

Dibasic (K_2HPO_4) - 0.008g/100ml

} (http://www.egr.msu.edu/biofuelcell/tools/phosphate/phosphate.html)

Preparation of Cellulase enzyme stock:

Cellulase enzyme is present in powder form so prior to use it is first dissolved in phosphate buffer. As cellulase works best at pH 5 so the pH of the phosphate buffer was set by mixing monobasic and dibasic in a particular ratio.

Monobasic (KH_2PO_4) – 136.1448 g/l

Dibasic (K_2HPO_4) – 3.6029g/l

(http://www.egr.msu.edu/biofuelcell/tools/phosphate/phosphate.html)

Preparation of hydrolysate

0.5 gm of diammonium phosphate, 1 gm of yeast extract, 0.025 gm of magnesium sulfate heptahydrate and 1.38 gm of ammonium dihydrogen phosphate were weighed on weighing balance and put in a conical flask. 1 L of distilled water was then added to the conical flask. The mouth of the flask was covered with aluminum foil and the hydrolysate was autoclaved.

Production of alcohol from sugarcane bagasse:

Sugarcane bagasse was used as a raw material for alcohol production. Through response surface methodology (design expert software) run sheet was prepared. A run sheet contains pH values (3.86, 4.54, 5.54, 6.54, 7.22,), temperature values (18.23^{o}C ,23^{o}C, 30^{o}C, 37^{o}C & 41.77^{o}C) and inoculum concentration values (3.95%, 6%, 9%, 12%, 14.05%) in different combination according to the data procured from the software. Before initiating the fermentation process a YEPD broth of 100 ml was prepared and autoclaved. A single colony of yeast strain was picked from the petriplate and was added into the YEPD broth and kept it into the incubator-shaker (30^{o}C, 200rpm) for 8-9 hours.

The fermentation was carried out in 50 ml falcon tube. All the falcon tubes were labeled with different combination of conditions. The sugarcane bagasse was put into falcon tubes and the pH was set individually in all falcon tubes according to the marked conditions. After that the yeast strain was added into the falcon tubes as per the marked conditions. After adding yeast sample in all the falcon tubes they were then kept in incubator-shakers with different temperature conditions as marked on the falcons. The alcohol concentration and brix was measured through Refractometer from 1-7 days and the readings were carefully noted.

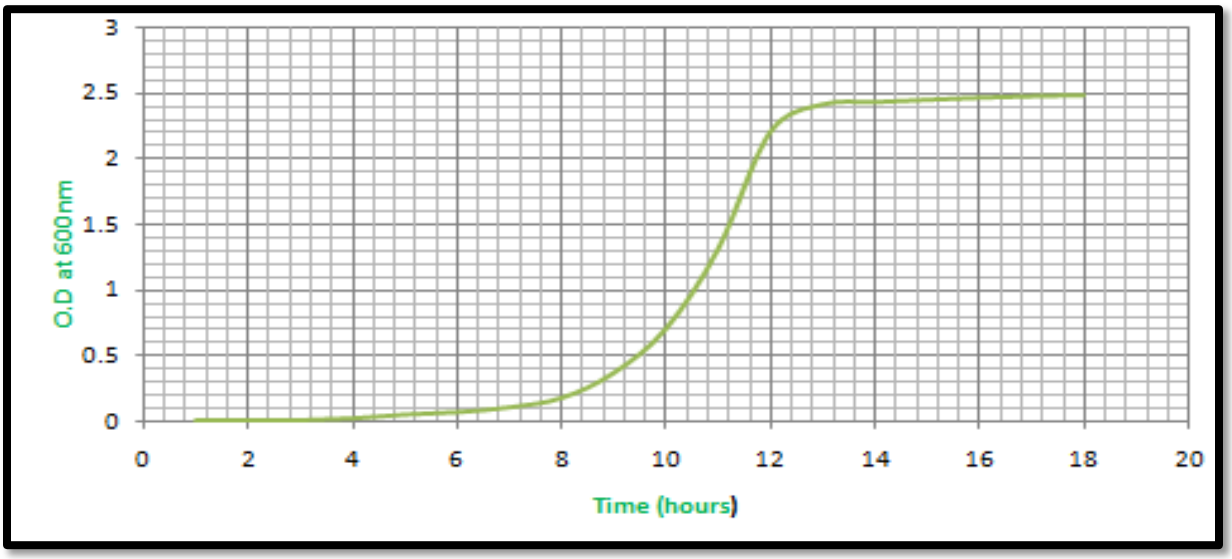
FIGURE 11: Fermentation in falcon tubes with bagasse

RESULTS & DISCUSSION

MTCC178 yeast strain grwth curve in medium (M1):

S. No.	Time (hours)	O.D (600nm)
1	**1**	**0.006**
2	**2**	**0.007**
3	**3**	**0.009**
4	**4**	**0.023**
5	**5**	**0.053**
6	**6**	**0.071**
7	**7**	**0.108**
8	**8**	**0.179**
9	**9**	**0.371**
10	**10**	**0.710**
11	**11**	**1.319**
12	**12**	**2.215**
13	**13**	**2.426**
14	**14**	**2.443**
15	**15**	**2.461**
16	**16**	**2.476**
17	**17**	**2.485**
18	**18**	**2.497**

TABLE 2: Data of Growth Curve

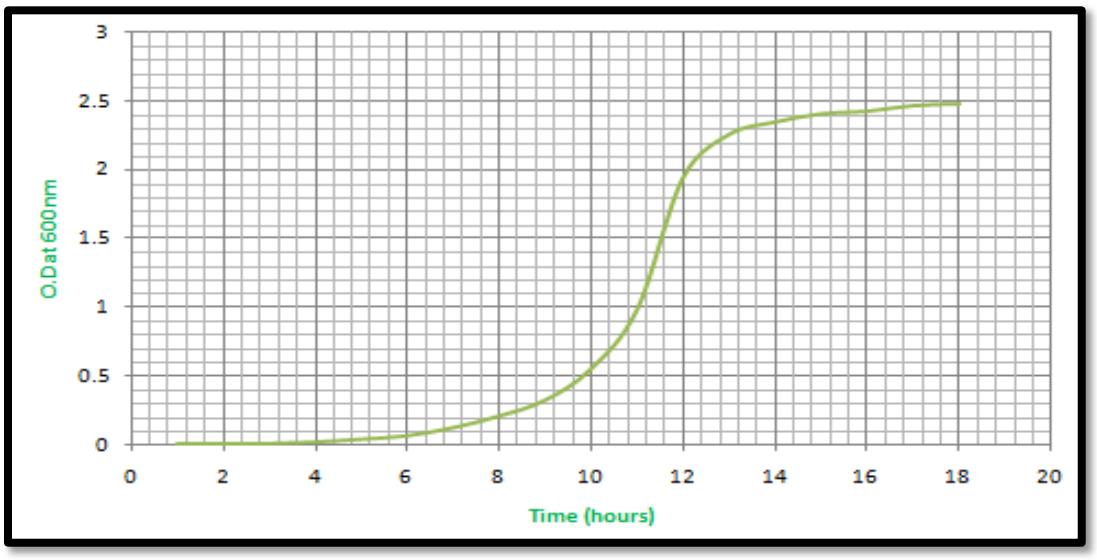


GRAPH 1: MTCC 178 strain growth curve

MTCC178 yeast strain in medium (M2)

S.No	Time (hours)	OD (600nm)
1	1	0.012
2	2	0.013
3	3	0.015
4	4	0.025
5	5	0.046
6	6	0.072
7	7	0.130
8	8	0.215
9	9	0.330
10	10	0.559
11	11	0.991
12	12	1.950
13	13	2.260
14	14	2.350
15	15	2.409
16	16	2.428
17	17	2.469
18	18	2.481

TABLE 3: Data of M2



GRAPH 2: MTCC 178 strain Growth curve

FIGURE 12: Optical Density reading is taken from samples M1, M2 and R

Experimental data generated through Design Expert software and the corresponding response obtained:

Select	Std	Run	Factor 1 A:pH No	Factor 2 B:Temp C	Factor 3 C:Yeast Conc. %	Response 1 alcohol %	Response 2 brix B
	12	1	5.54	41.77	9.00	6.5	3.2
	10	2	7.22	30.00	9.00	4.5	2.5
	16	3	5.54	30.00	9.00	5	2.2
	8	4	6.54	37.00	12.00	6	2.4
	17	5	5.54	30.00	9.00	5	2.2
	20	6	5.54	30.00	9.00	5	2.2
	2	7	6.54	23.00	6.00	7	3.1
	11	8	5.54	18.23	9.00	4.5	2.4
	1	9	4.54	23.00	6.00	6	2.9
	7	10	4.54	37.00	12.00	6	2.5
	3	11	4.54	37.00	6.00	5	2.1
	9	12	3.86	30.00	9.00	4.5	2.5
	6	13	6.54	23.00	12.00	6	2.9
	5	14	4.54	23.00	12.00	5	2.8
	18	15	5.54	30.00	9.00	5	2.2
	4	16	6.54	37.00	6.00	6	3
	14	17	5.54	30.00	14.05	5	2.2
	15	18	5.54	30.00	9.00	5	2.2
	13	19	5.54	30.00	3.95	5	2.2
	19	20	5.54	30.00	9.00	5	2.2

FIGURE 13: Run sheet prepared design software based on RSM

Central composite design of response surface methodology was used for optimizing the procuction of alcohol from sugarcane bagasse as raw material. This is best suited for quadratic surfaces and works fine for optimization processes.

Firstly various parameters to be optimized are chosen. Next range of these parameters was set. A run sheet was generated having 20 set of run. All 20 setups were experimentally performed and response that is alcohol concentration and brix were noted.

FIGURE 14: Alcohol response on an alcohol refractrometer

When the value of response was put into a run sheet a design summery was prepared showing standard deviation and design model for the response.

Design Summary

File Version	8.0.7.0		
Study Type	Response Surface	**Runs**	20
Design Type	Central Composite	**Blocks**	No Blocks
Design Mode	Quadratic	**Build Time (r**	74.55

Factor	Name	Units	Type	Subtype	Minimum	Maximum	Coded	Values	Mean	Std. Dev.
A	yeast conc.	g/l	Numeric	Continuous	3.95	14.05	-1.000=6.00	1.000=12.00	9.00	2.48
B	temp.	c	Numeric	Continuous	18.23	41.77	-1.000=23.00	1.000=37.00	30.00	5.78
C	ph		Numeric	Continuous	3.32	6.68	-1.000=4.00	1.000=6.00	5.00	0.83

Response	Name	Units	Obs	Analysis	Minimum	Maximum	Mean	Std. Dev.	Ratio	Trans	Model
Y1	brix		0	Polynomial	No Data	No Data	No Data	No Data	N/A	None	No model chos
Y2	alcohol		0	Polynomial	No Data	No Data	No Data	No Data	N/A	None	No model chos

FIGURE 15: Design summary prepared by software

Analysis of the result:

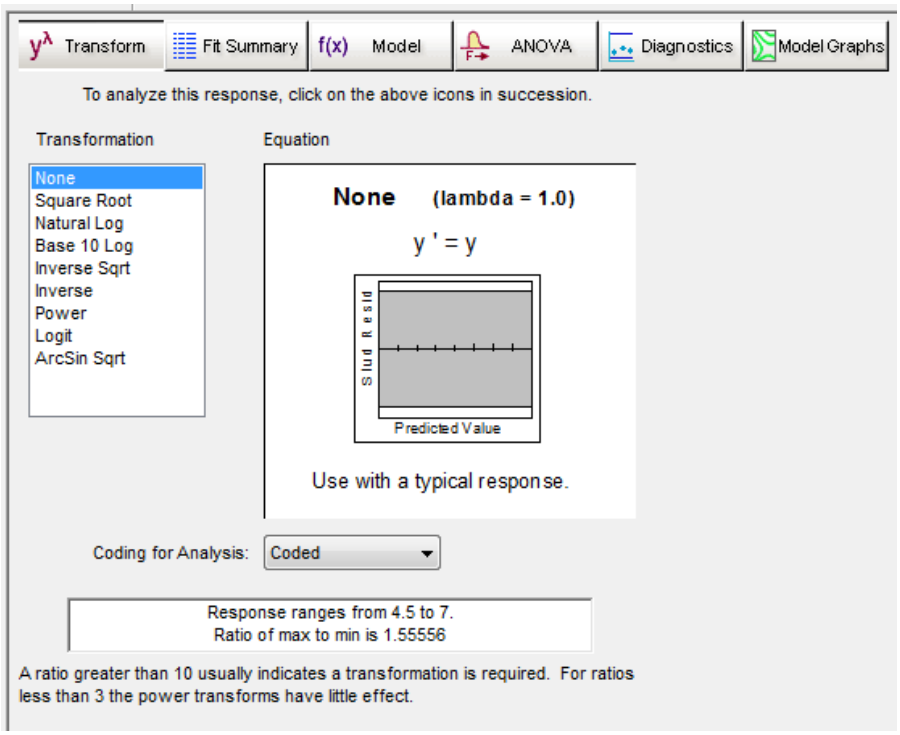
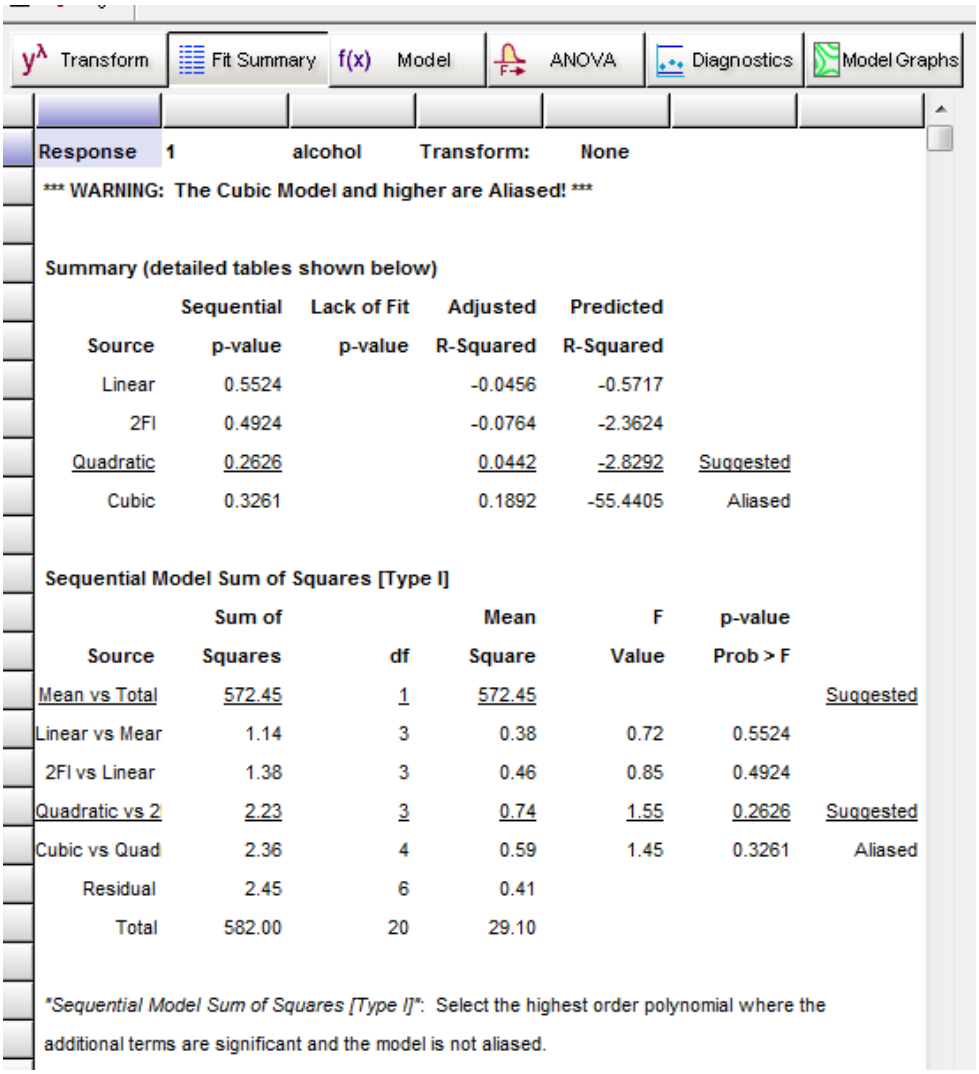


FIGURE 16: Analysis of response of alcohol.

On the left corner of the screen we click on the analysis button and the two parameters of response under study appears that is alcohol and brix.

Now, for specifically studying any one of the parameters of the response a response node is selected. A click on the response results in appearance of various terms such as transformation, fit summery, model, ANOVA, diagnoses, and model graph.

Response	Maximum	Minimum	Ratio
Alcohol	7	4.5	1.55
Brix	3.2	2.0	1.5



Transform | Fit Summary | f(x) Model | ANOVA | Diagnostics | Model Graphs

Response 1 alcohol Transform: None

*** WARNING: The Cubic Model and higher are Aliased! ***

Summary (detailed tables shown below)

Source	Sequential p-value	Lack of Fit p-value	Adjusted R-Squared	Predicted R-Squared	
Linear	0.5524		-0.0456	-0.5717	
2FI	0.4924		-0.0764	-2.3624	
Quadratic	0.2626		0.0442	-2.8292	Suggested
Cubic	0.3261		0.1892	-55.4405	Aliased

Sequential Model Sum of Squares [Type I]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Mean vs Total	572.45	1	572.45			Suggested
Linear vs Mear	1.14	3	0.38	0.72	0.5524	
2FI vs Linear	1.38	3	0.46	0.85	0.4924	
Quadratic vs 2	2.23	3	0.74	1.55	0.2626	Suggested
Cubic vs Quad	2.36	4	0.59	1.45	0.3261	Aliased
Residual	2.45	6	0.41			
Total	582.00	20	29.10			

*"Sequential Model Sum of Squares [Type I]": Select the highest order polynomial where the additional terms are significant and the model is not aliased.

FIGURE 17: Fit summary data for alcohol response

Clicking on the fit summary button starts the regression calculation to fit all the polynomial models to the selected response. Effect of all model terms is calculated such as f value, lack of fit test and R-squared value for comparing the model.

Statistically significant model is detected by the programme. The model becomes the default model.

TYPE 1- Sequential model sum of square.

It shows the terms of increasing complexity contribute to the total model. In the table for each source of terms the probability (prob >F) has been calculated the software and one which fall below 0.05 will be the next model.

The suggested model by the software is quadratic model even though the value of cube value is below 0.5 but the central composite matrix too provides some unique design points to determine all the terms in cubic model, so the cubic model is aliased.

Lack of Fit Tests

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Linear	8.41	11	0.76		
2FI	7.03	8	0.88		
Quadratic	4.80	5	0.96		
Cubic	2.45	1	2.45		
Pure Error	0.000	5	0.000		

*"Lack of Fit Tests": Want the selected model to have insignificant lack-of-fit.

FIGURE 18: Lack of Fit Test of alcohol response

The Lack of Fit Tests table compares the residual error with the pure errors from the replicated design points. If we have significant lack of fit which is shown by a low probability value , then we must be careful in using the model as a response predictor. In such type of case, the linear model can be ruled out, because its prob > F falls below 0.05. The quadratic model, identified earlier as the likely model, does not show significant lack of fit. As the cubic model is aliased, hence it is not be chosen.

Model Summary Statistics

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	0.72	0.1195	-0.0456	-0.5717	15.01	
2FI	0.74	0.2635	-0.0764	-2.3624	32.11	
Quadratic	0.69	0.4970	0.0442	-2.8292	36.57	Suggested
Cubic	0.64	0.7440	0.1892	-55.4405	539.01	Aliased

"Model Summary Statistics": Focus on the model maximizing the "Adjusted R-Squared" and the "Predicted R-Squared".

FIGURE 19: Showing Model Summary Statistics table for alcohol response node

The quadratic model is suggested by the program as it shows a low a low press value, high R squared values and a low standard deviation.

The program on itself chooses at least one model as suggested model.

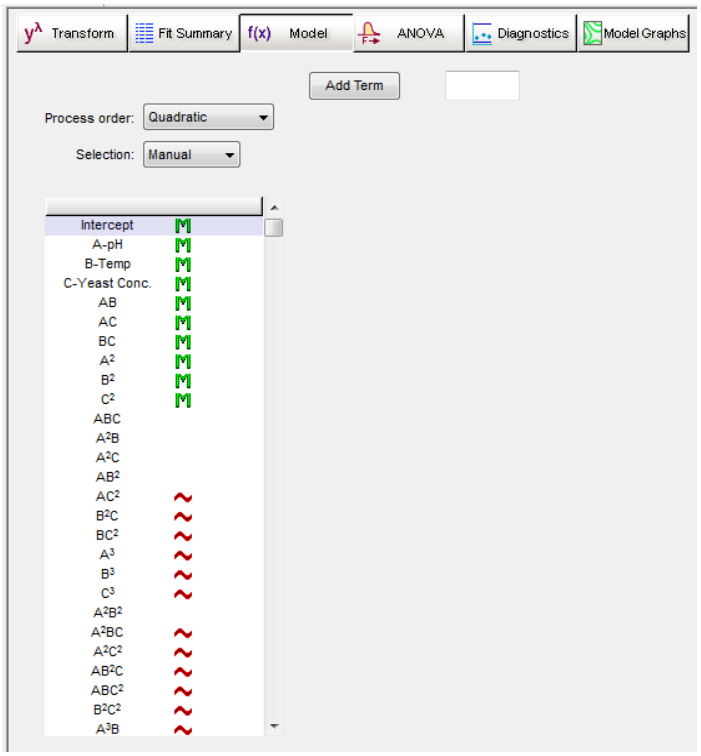


FIGURE 20: Shows the Model information for the alcohol response

By default the program proceeds with the –Suggested‖ model detected in the earlier Fit Summary table. We also have the option to choose an alternative model from the Process Order pull down list.

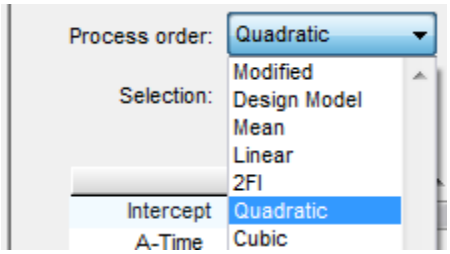
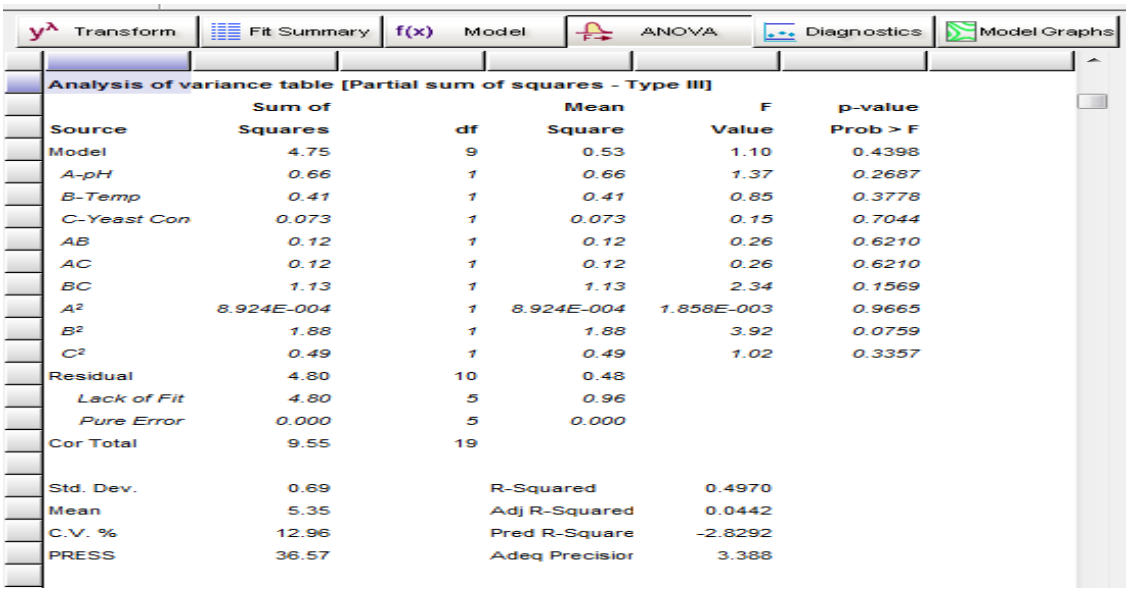


FIGURE 21: Various options of modeling provided by design expert

Analysis of variance (**ANOVA**): It is a collection of statistical models which are used to analyze the differences between group means and their associated procedures.



Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	4.75	9	0.53	1.10	0.4398
A-pH	*0.66*	*1*	*0.66*	*1.37*	*0.2687*
B-Temp	*0.41*	*1*	*0.41*	*0.85*	*0.3778*
C-Yeast Con	*0.073*	*1*	*0.073*	*0.15*	*0.7044*
AB	*0.12*	*1*	*0.12*	*0.26*	*0.6210*
AC	*0.12*	*1*	*0.12*	*0.26*	*0.6210*
BC	*1.13*	*1*	*1.13*	*2.34*	*0.1569*
A²	*8.924E-004*	*1*	*8.924E-004*	*1.858E-003*	*0.9665*
B²	*1.88*	*1*	*1.88*	*3.92*	*0.0759*
C²	*0.49*	*1*	*0.49*	*1.02*	*0.3357*
Residual	4.80	10	0.48		
Lack of Fit	*4.80*	*5*	*0.96*		
Pure Error	*0.000*	*5*	*0.000*		
Cor Total	9.55	19			

Std. Dev.	0.69	R-Squared	0.4970
Mean	5.35	Adj R-Squared	0.0442
C.V. %	12.96	Pred R-Square	-2.8292
PRESS	36.57	Adeq Precisior	3.388

FIGURE 22: Analysis for alcohol response by ANOVA

By clicking **ANOVA** button,the analysis of variance for the selected model is shown.

The ANOVA in this case confirms the adequacy of the quadratic model (the Model Prob>F is less than 0.05.). Probability values for each individual term in the model can also be seen.

The "Model F-value" of 1.10 implies the model is not significant relative to the noise. There is a 43.98 % chance that a "Model F-value" this large could occur due to noise.

Std. Dev.	0.69	R-Squared	0.4970
Mean	5.35	Adj R-Squared	0.0442
C.V. %	12.96	Pred R-Square	-2.8292
PRESS	36.57	Adeq Precisior	3.388

FIGURE 23: ANOVA analysis showing R-square terms for alcohol response node

Adequate precision measures the signal to noise ratio. A ratio greater than 4 was desirable. The alcohol response shows noise ratio as 3.338 indicates an adequate signal

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	4.97	1	0.28	4.34	5.60	
A-pH	0.22	1	0.19	-0.20	0.64	1.00
B-Temp	0.17	1	0.19	-0.24	0.59	1.00
C-Yeast Conc.	-0.073	1	0.19	-0.49	0.34	1.00
AB	-0.12	1	0.25	-0.67	0.42	1.00
AC	-0.12	1	0.25	-0.67	0.42	1.00
BC	0.38	1	0.25	-0.17	0.92	1.00
A^2	7.869E-003	1	0.18	-0.40	0.41	1.02
B^2	0.36	1	0.18	-0.045	0.77	1.02
C^2	0.18	1	0.18	-0.22	0.59	1.02

FIGURE 24: ANOVA analysis showing coefficient term for alcohol response node

ANOVA provides the details on model coefficients on the screen and also gives the information about the actual and coded factors.

The details diagnostic provided by the software can be obtained by observing plots which is available through the Diagnostics button.

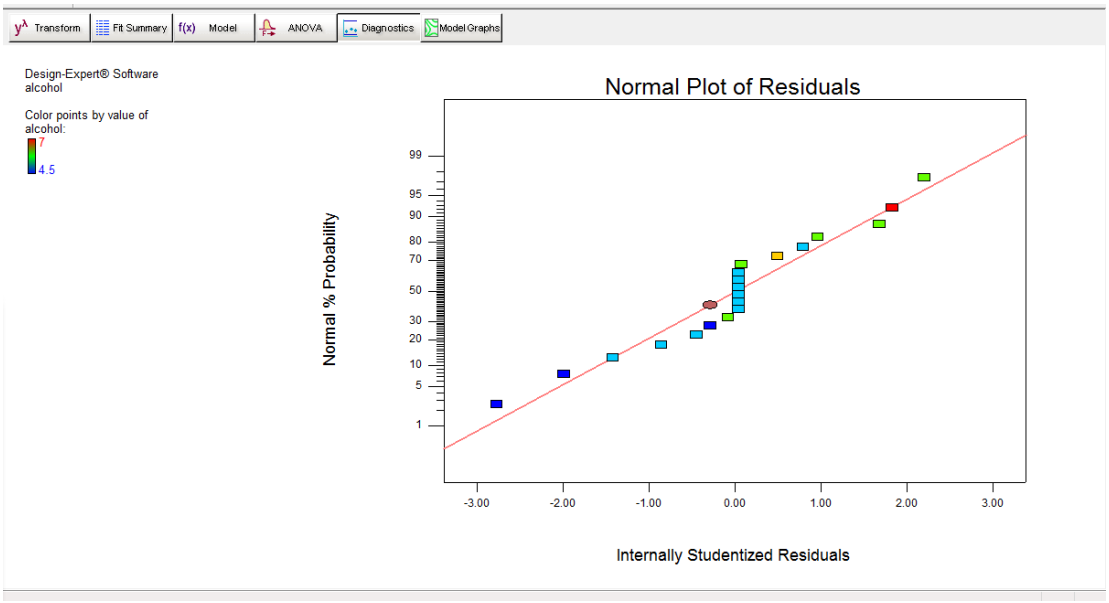


FIGURE 25: Normal Plot of Residuals of alcohol response

By default we have the most important diagnostic, normal probability plot of the residuals. Data points are nearly linear. A non-linear pattern shows non-normality in the error term, which can be corrected by a transformation.

In case of alcohol normal probability plot was linear and the points lie very near to the line whereas in the case of brix the normal probability plot was linear but the points were away from the line as compare to the alcohol response.

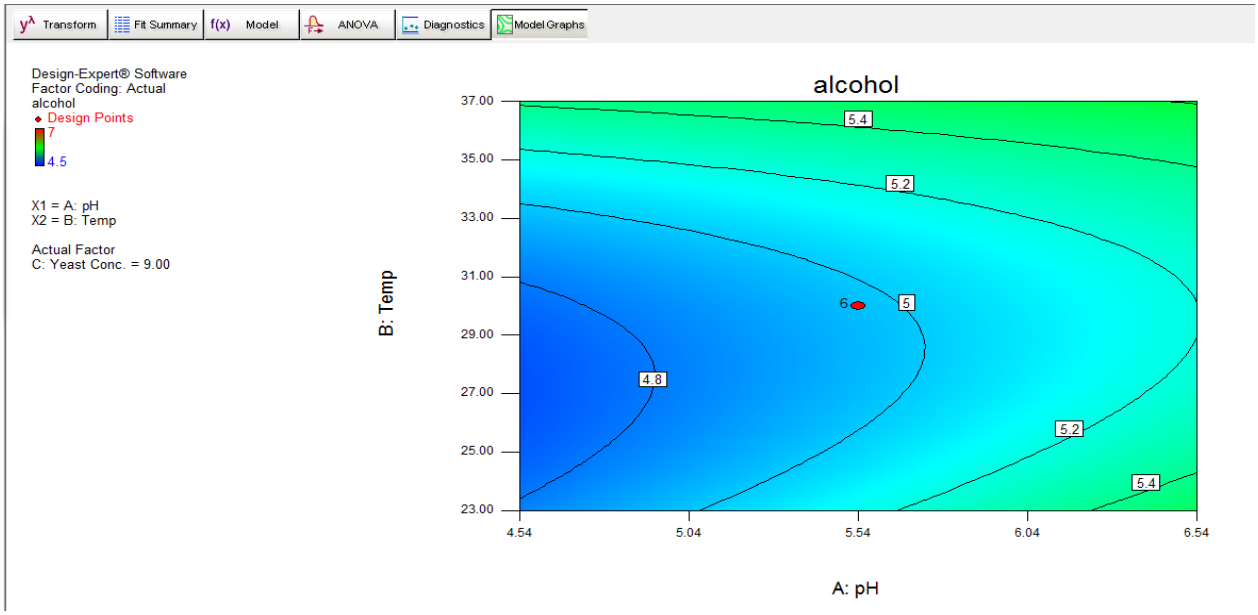


FIGURE 26: Two dimensional contour plot between temperature and pH for alcohol response

In contour plot the response was drawn as a function of 2 parameters and the mid level slice of the third factor was chosen. The colour gradation in the plot shows the response range. The color key on the left shows that the surface becomes red when the response levels are and blue at the lower response level.

The contour plot in the above figure shows the colour gradation from blue to green i.e. value varies from 4.8-5.4% of alcohol response as a function of pH and temperature with 9 as the actual inoculum concentration.

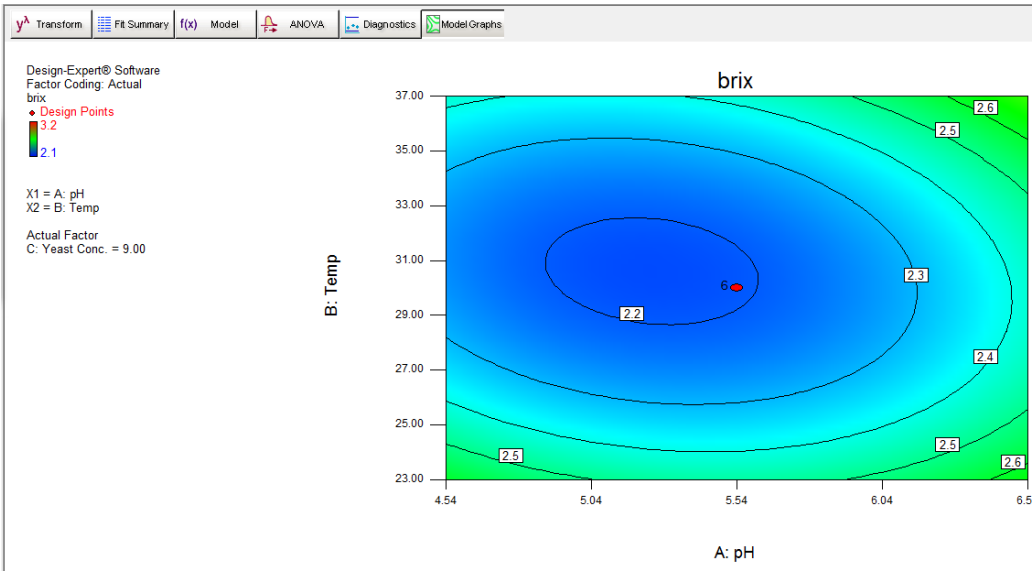


FIGURE 27: 2 Dimensional contour plot between pH and temperature for Brix response

. Similarly for brix response a color gradation from blue to green was observed with value varies from 2-2.6 Brix.

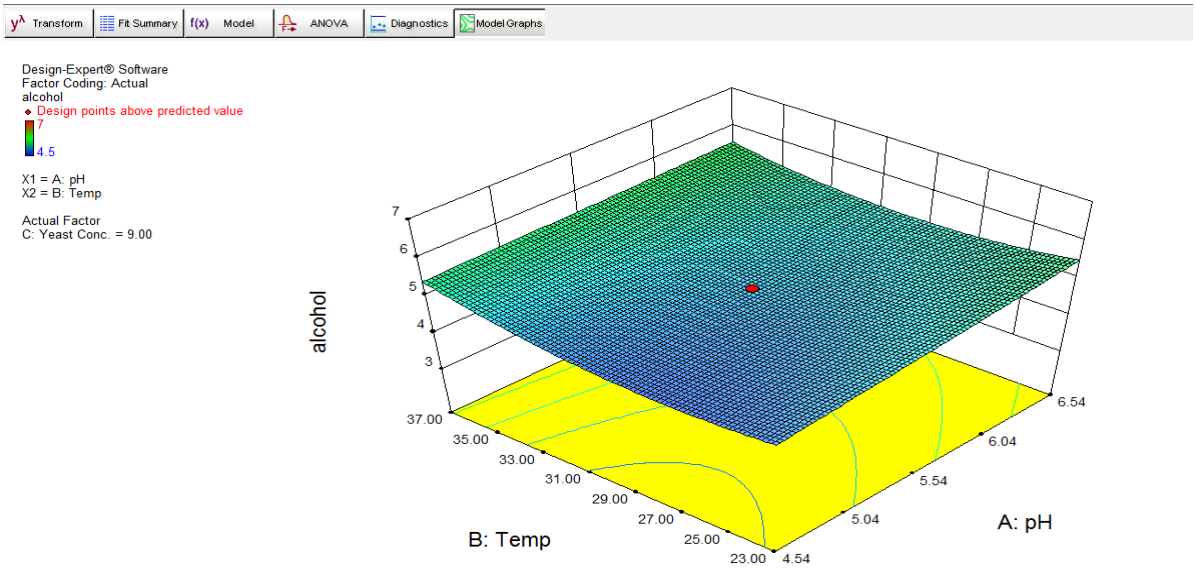


FIGURE 28: 3D surface plot for the alcohol response node at constant yeast concentration.

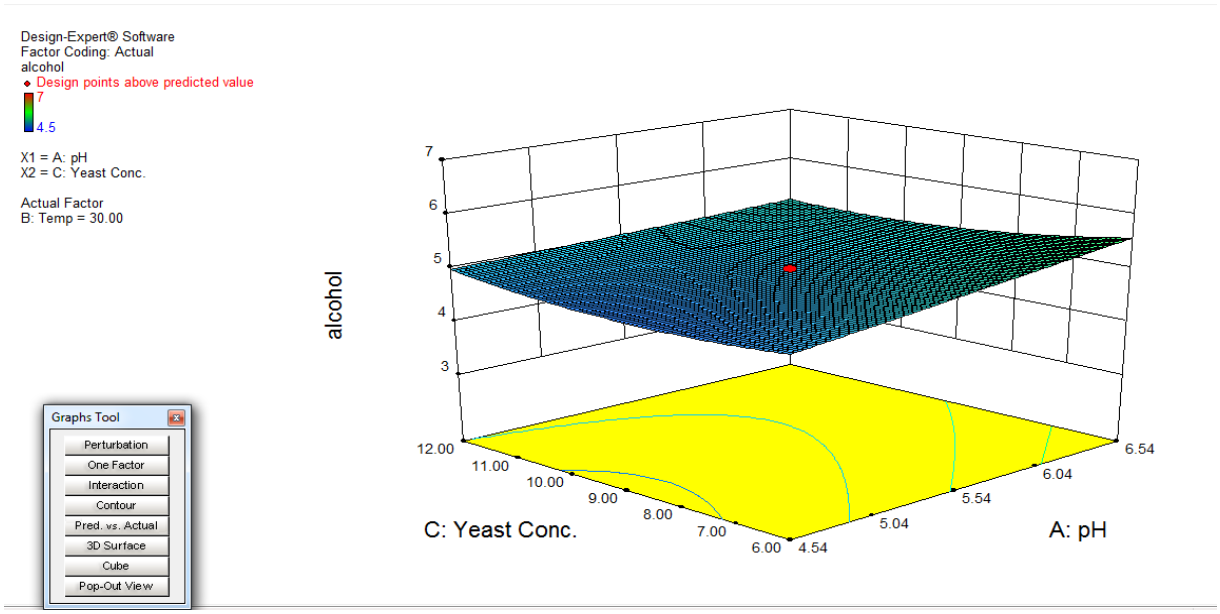


FIGURE 29: 3D surface plot for the alcohol response node at constant temperature

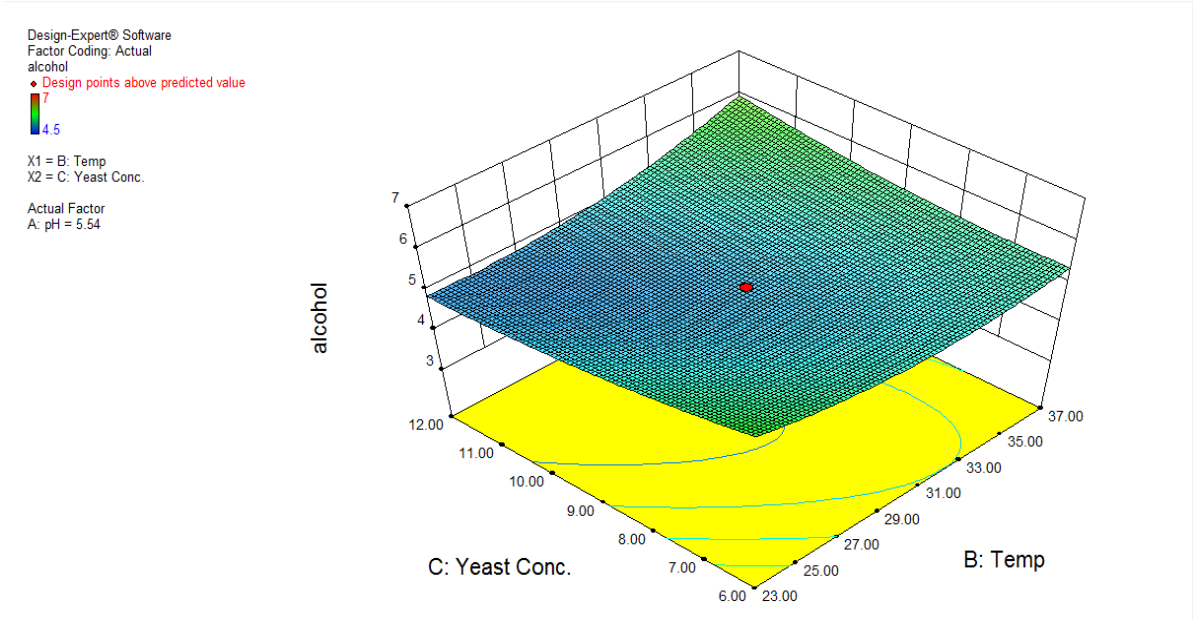


FIGURE 30: 3D surface plot for the alcohol response node at constant pH

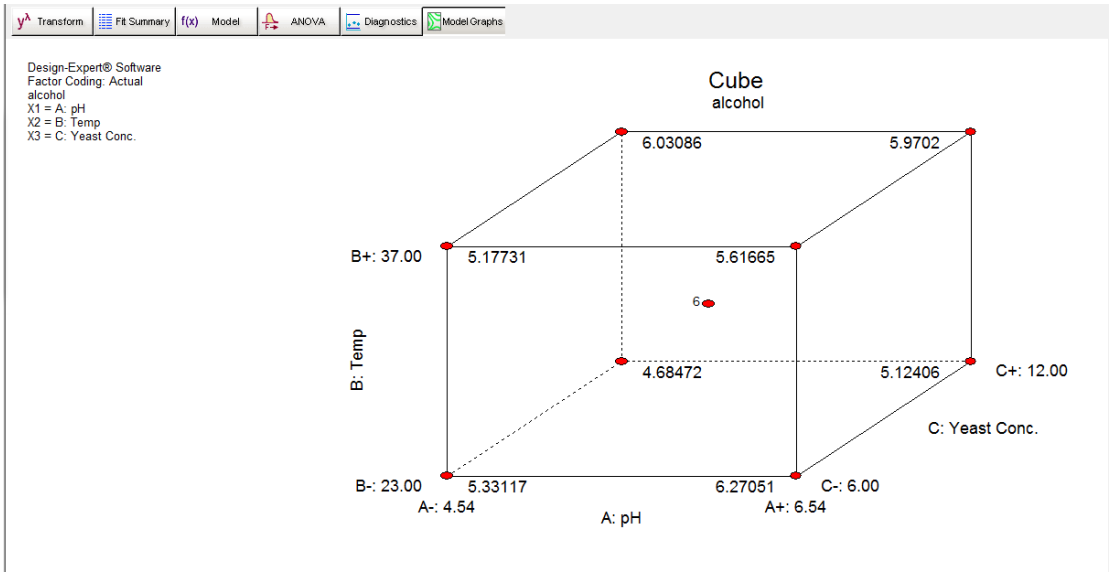


FIGURE 31: Cubical representation of various parameters and response

Optimization Results:

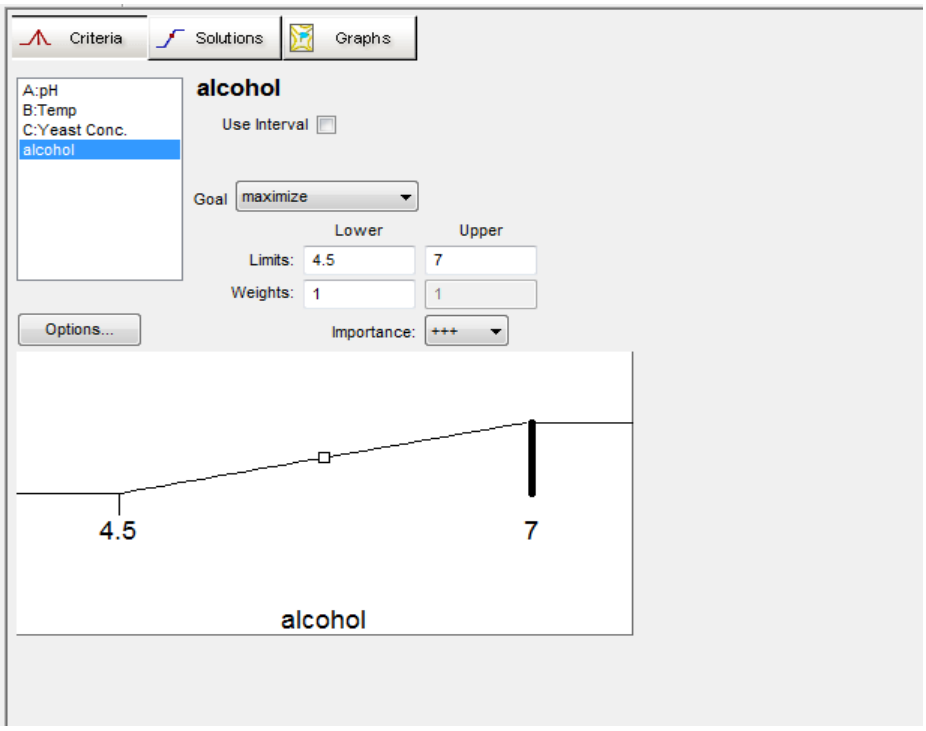


FIGURE 32: Optimization of alcohol production

The optimization looks out for a combination of factor levels which simultaneously satisfy the requirements placed on each of the responses and factors. Optimization of one response or optimization of multiple responses can be performed graphically or numerically.

Numerical optimization

In numerical optimization a goal was set for each factor as well as for response. The goal set for each factor was in range and the goal set for the response was to maximize as we want the alcohol percentage from sugarcane bagasse to be maximum under the given set of conditions. The goals for factors and response are then combined into an overall desirability function. Desirability ranges from zero outside of the limits to one at the goal which is the objective function. The numerical optimization searches for a point which maximizes the desirability function. After setting up the criteria a solution sheet was generated showing the best response generated in function with other factors.

Criteria | Solutions | Graphs

Solutions 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Constraints

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A:pH	maximize	4.54	6.54	1	1	3
B:Temp	is in range	23	37	1	1	3
C:Yeast Conc.	is in range	6	12	1	1	3
alcohol	maximize	4.5	7	1	1	3

Solutions

Number	pH	Temp	Yeast Conc.	alcohol	Desirability	
1	6.54	23.00	6.00	6.27051	0.842	Selected
2	6.54	23.00	6.04	6.25732	0.838	
3	6.54	23.00	6.22	6.20361	0.825	
4	6.54	23.00	6.43	6.14031	0.810	
5	6.43	23.00	6.00	6.21679	0.805	
6	6.54	23.00	6.50	6.11803	0.804	
7	6.42	23.00	6.00	6.21134	0.802	
8	6.34	23.00	6.00	6.17519	0.777	
9	6.54	37.00	12.00	5.9702	0.767	
10	6.54	36.94	12.00	5.96081	0.764	
11	6.53	37.00	12.00	5.97038	0.764	
12	6.54	37.00	11.93	5.95752	0.764	
13	6.54	36.85	12.00	5.94545	0.760	
14	6.50	37.00	12.00	5.97064	0.760	

FIGURE 33: Solution sheet of numerical optimization for alcohol response

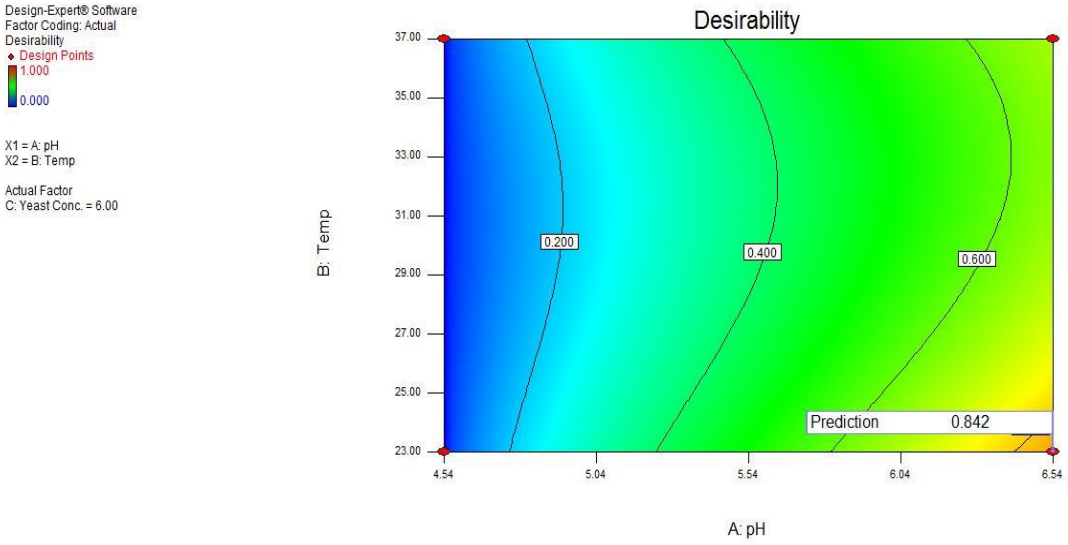


FIGURE 34: Contour plot of desirability

According to the solution sheet generated for alcohol response the best solution was selected i.e. at 23^{o}C temperature, 6.54 pH and at 6% inoculum concentration 6.270% alcohol was produced with the desirability of 0.842.

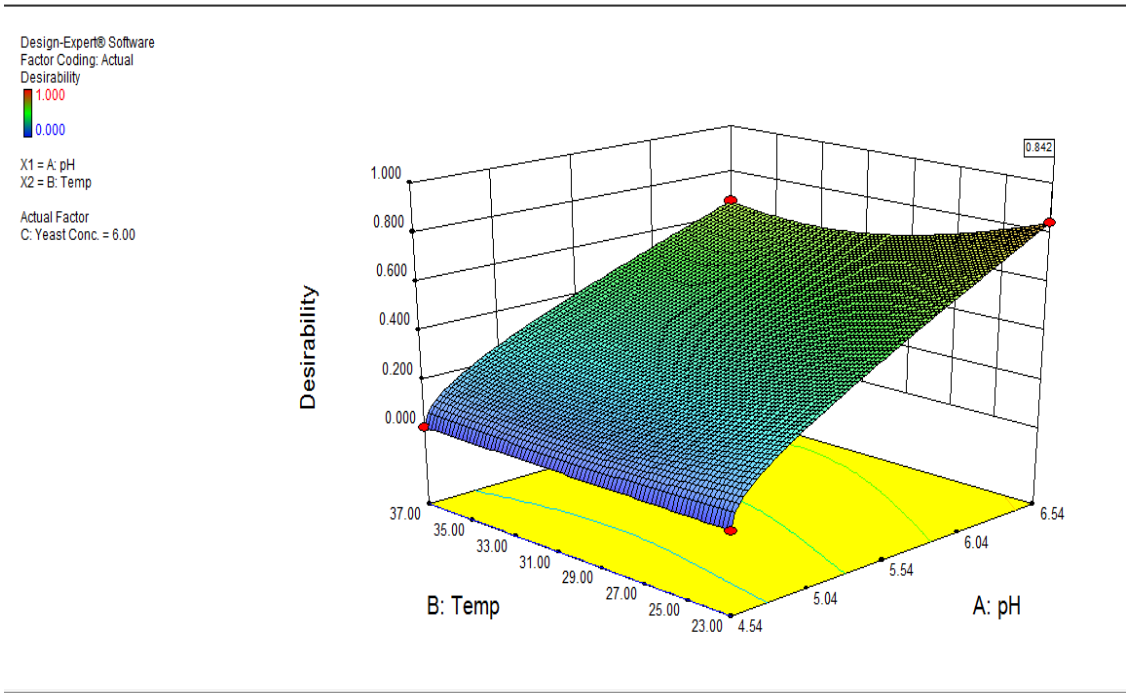


FIGURE 35: 3D plot showing desirability as a function of pH and temperature.

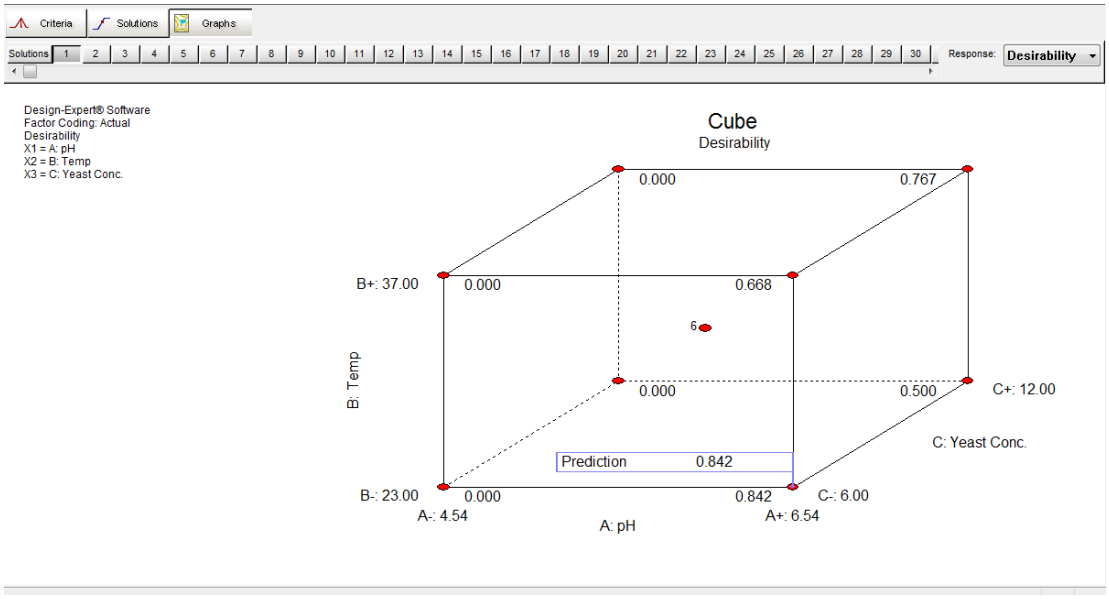


FIGURE 36: Cubical representation of desirability

Graphical Optimization:

This displays areas of feasible response values in the factor space. Regions that do not fit the optimization criteria are shaded grey. Any response window that is not gray shaded satisfies the goal for each response. On clicking the criteria tab in the graphical optimization node a screen appeared which showed responses. Next we clicked on a response in order to specify the response limits i.e. lower and upper, so that each response is included in the optimization. After the criteria were set a plot was generated by clicking on the graph tab.

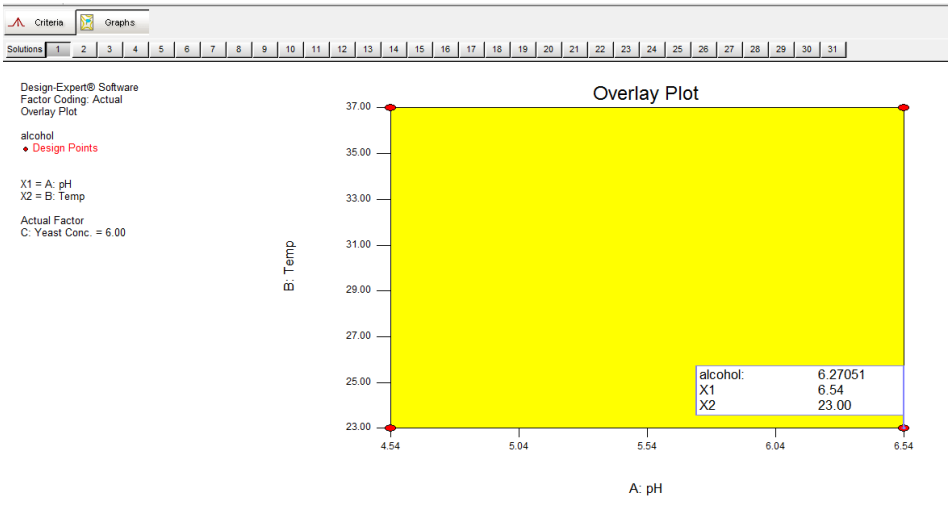


FIGURE 37: Overlay plots between temperature and pH showing alcohol response.

As no region appeared gray so it can be concluded that all the values were in the optimization criteria and no values outside of it.

CONCLUSION AND FUTURE PROSPECTIVE

It can be observed that using numerical optimization combined with graphical analysis provides powerful insights. We use numerical optimization when we want to study many factors which have many responses. In this project optimization of temperature, pH and inoculum concentration was performed for maximizing alcohol concentration and the conditions obtained were 23^{o}C temperature, 6.54 pH and at 6% inoculum concentration for 6.270% alcohol production.

The production can further be enhanced if yeast strain can be made to convert the whole substrate into product for which further research needs to be done. The production can be further enhanced when some other factors like substrate concentration,time which affect the process are optimized.

Brazil has been using sugarcane for ethanol production for more than 30 years and India has already started adding 5% ethanol in gasoline which is expected to rise to 10% in the years to come, so there is going to be an increase in its demand too.

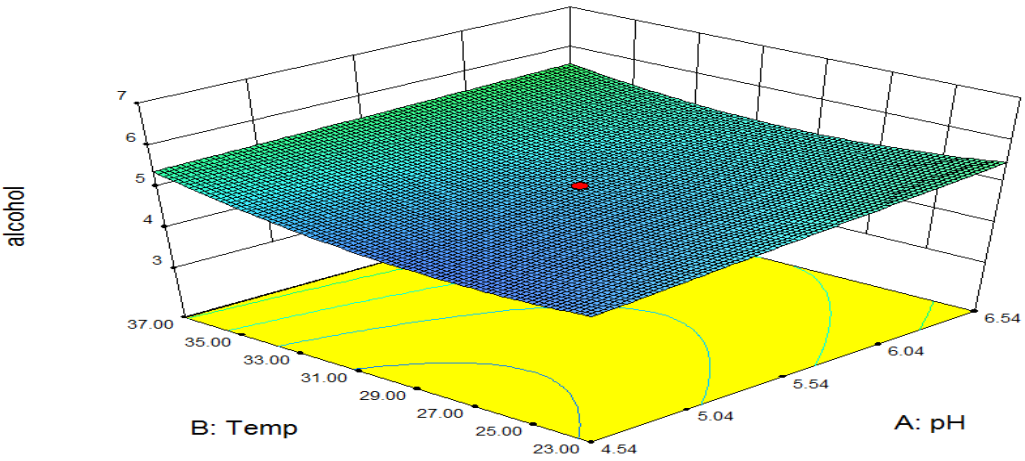


FIGURE 38: 3D plot showing computerized optimization

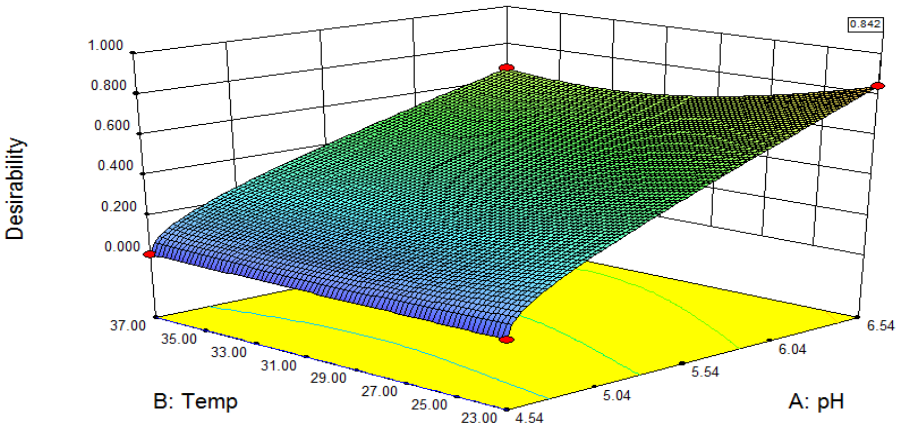


FIGURE 39: 3D plot showing numerical optimization

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