Microbioz Jouranls, Journal of Microbiology and Biomedical Research
ISSN 2395-5678, Volume: 2 Issue: 1st
Access online: www.microbiozjournals.com

Statistical Optimizations of Fermentation Factors on Bioethanol Production from Mahua Flower (*Madhuca indica*) with *Saccharomyces cerevisiae* by Response Surface Methodology in Batch Bioreactor

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Abstract

The aim of the present investigation is to enhance the bioethanol production from agricultural feed stocks through fermentation process. To bring out facts, experiments were conducted on the biochemical analysis of Mahua flower (*Madhuca indica*) for its suitability as a raw material and optimizations of fermentation conditions using response surface methodology. The results of fermentation processes on bioethanol productions were compared and tabulated. From the data, it was observed that *Saccharomyces cerevisiae*-3190 NCIM is high bioethanol tolerant, acid resistant, and is able to produce high yields of bioethanol in high gravity medium. The optimum conditions such as pH 4.9, temperature 31.43 ^{0}C, agitation 117.28 RPM, and ammonium sulphate $(NH_4)_2SO_4$) of 0.629 mg.l^{-1} were obtained on bioethanol production with statistical optimizations. The yeast strain could produce 150.562g.l^{-1} and 195.284 g.l^{-1} with the substrate concentration of 360 g.l^{-1} and 409 g.l^{-1} after 48 hours by medium-I and medium-II respectively.

Date of Submission : Oct5, 2015
Date of Acceptance : March 02, 2016
Date of Publication : March 29, 2016
Type of article : Research article

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Key words: Bioethanol, Fermentation, RSM, Bioethanol Method, Gas Chromatography.

Introduction

Ethanol is an ancient organic solvent which next to the water and is named as "Bioethanol", after its production through fermentation process using microorganisms. Conversion of sugars into ethanol is one of the earliest anaerobic organic reactions employed by humanity. Several authors reported that the attention has been devoted to the conversion of various substrates such as molasses, sugar cane, sorghum, potato, cassava, cashew apple juice, fruit juices and corn, wheat, pearl millet, rice to fuel bioethanol using bacterial and yeast cells. The industrial production of ethanol and commercial use of yeasts started at the end of the 19th century after their identification and isolation by Louis Pasteur. In 1908, Henry Ford has designed a fuel with a mixture of Gasoline and Alcohol and further it was referred as "The fuel of the future" in the year 1925 (Ravindra, 2007). Ethanol (C_2H_5OH) is pure in color, volatile flammable and non toxic. Worldwide fuel prices are rising due to increase in demand by the population (Dake, et al, (2010). It has a molecular weight of 46.07, melting point of -115 ^{0}C, boiling point of 78 ^{0}C and specific gravity of 0.79 GM/ml at 20 ^{0}C. The oxygenated bio-fuels like biodiesel and bioethanol are an effective substitute for renewable fuels and reduce particulate matter from in-use diesel vehicles (Krishna swamy, et al, 2012).

Substrate Mahua flower (*Madhuca indica*) belongs to the family Sapotaceae. It is a medium sized to large deciduous tree, usually with a short bole and rounded crown and found throughout the greater part of India upto an altitude of 1200m. It is usually found in mixed deciduous forest, usually of dry type, and grows on rocky and sandy soil and flourishes on the Deccan plateau. It is common throughout the deciduous forest in central India, Madhya Pradesh, Maharashtra, Gujarat, Orissa, Chota Nagpur, and Andhra Pradesh (Wealth of India, 1962). Mahua flower contains total fermentable sugars of 731.343µg/ml, Moisture content of 17 %, Reducing sugars of 18%, Protein of 4.6 mg and Fat of 0.5 %. A gram of glucose can be converted to 0.511 grams of ethanol (Maiorella, et al, 1981). The stoichiometric glucose conversion to bioethanol in the presence of yeast cells can be represented as follows:

Yeast cells

$C_6H_{12}O_6$ (180 g.mol) $\rightarrow\rightarrow\rightarrow\rightarrow$ $2C_2H_5OH$ (92 g.mol) + $2CO_2$ (88 g.mole)

Optimization of medium constituents by laboratory method is a single–dimensional search involving change of one variable while fixing the others at a certain level is laborious and time consuming, especially when the number of variables is in large. Therefore, an alternative and potential method in microbial system is the function of statistical methods. Hence, the present optimization studies were carried out with Box-Wilson (Box and Wilson, 1951) response surface methodology using software Statistica8.

Materials and Methods

Microorganisms

Yeast and Bacterial strains such as *Saccharomyces cerevisiae*-3190 NCIM, *S.cerevisiae-171 MTCC, K.thermotolerance-30 MTCC, S.cerevisiae-3288 NCIM*, K.marxianus-1389 MTCC, *Z.mobilis*-92 MTCC, *E.coli* and *S.cerevisiae*-463 MTCC, which are obtained from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratories, Pune and (MTCC) Microbial Type Culture Collection, Chandigarh, India) cultures were tested for bioethanol production.

Methods

Total sugars in Mahua flowers extract (MFE) estimated by Anthrone method (Yemm and Willis, 1954), Moisture content by Association of Official Agricultural Chemists (AOAC, 2000), Method No: 930.15, reducing sugars by Miller method (1959), protein by Lowry's method (1951), and Fat by American association of cereal chemists (AACC, Method: 30-25, 1983). Determination of total cell count by American public health association (APHA Method, 1967). Estimation of total viable cell count was determined by methylene blue reagent (Bonara and Mares (1982).

Preparation of bacterial culture medium

Bacterial culture medium was prepared using beef extract of 3 g.l^{-1}; peptone, 5 g.l^{-1}; sodium chloride of 8 g.l^{-1} and 15 grams of Agar were added to 1000 ml of distilled water in 2 litre Erlenmeyer flask. The pH of the medium was adjusted to 7.5 using 1 N HCL and 1 N NaOH with the aid of pH Meter (Systronics). Then, the medium was allowed to sterilize at 121 ^{0}C for 30 minutes. Then, 10 ml of sterilized medium was aseptically transferred to the culture tubes and rotated at 450 for 25 minutes to develop agar slopes. After the solidification completed, one loopfull of original culture of bacterial strains were aseptically streaked on agar slopes and tightly capped with non-adsorbent cotton. Then, these agar slants were incubated at 25 ^{0}C for 48 hours of growth period.

Preparation of yeast culture medium

The yeast culture medium was prepared in 2 litre Erlenmeyer flasks containing glucose, yeast extract, malt extract and peptone (GYMP) in 1 litre of distilled water and pH was adjusted to 6 using 1 N HCL and 1 N NaOH .The medium was autoclaved at 1210C for about 30 minutes.

After autoclave was completed, 10 ml of medium was aseptically transferred to petri plates and 30 ml tubes. Then, the original cultures of yeast were aseptically inoculated with loop on agar slopes. Using this medium composition, cultures were incubated at 300C for 48 hours. For every 30 days, yeast culture was freshly prepared for maintaining cell viability and the total experiments were carried out with freshly prepared nutrient agar medium and nutrient broth medium.

Response surface methodology

Response surface methodology (RSM) is one of the suitable methods for identifying the effect of individual variables and optimizing the conditions for a multivariable system efficiently. Multiple regression and correlation analysis are used as tools to assess the effects of two or more independent factors on the dependent variables (Ratnam, et al., 2003). The central composite design (CCD) with 3k factorial design was applied for the optimization of fermentation conditions on bioethanol production. Three different levels such as low concentration level (-1), Middle level (0) and High level (+1) and three independent variables represented as X1, X2, X3 and dependent variable is the production of bioethanol ($g.l^{-1}$) were applied to CCD. Recently, many statistical experimental design methods have been employed in bioprocess optimization. Two axial points on the axis of each design variable at a distance of α from the design centre were applied. Thus the total number of design points in central composite design (CCD) consisting of k variables are given as follows

$$N=2k+2k+no$$

$$\gamma i = \beta_{\circ}+\beta 1x1+ \beta 2x2+ \beta 2x3+ \beta 11\ x12+ \beta 22\ x22+ \beta 33x32+ \beta 12x1x2+ \beta 13x1x3+\beta 23x2x3$$

Where, Yi is Predicted Response; X1, X2 and X3 are Independent Variables; $\beta_{\circ}$ is Offset term; β1, β2, β3 are Linear effects; β11, β22 and β33 are Squared effects and β12, β13, β23 are Interaction terms.

Fermentation with 5 litre bioreactor

Bioreactor (B-Lite, Sartorious Private Limited, Mumbai, India) is batch scale bioreactor that can ferment up to 5 Litres of fermentative medium. In the present study, 2 litres of Mahua fermentative medium was used for bioethanol production under controlled conditions like pH, temperature, and agitation. During the batch fermentation process, bioreactor was sterilized at 121 ^{0}C for 15 minutes.

Bioethanol determination

Total content of bioethanol in fermented sample were determined by gas chromatograph (GC). Gas chromatography method for bioethanol production was developed based on the dilutions used in British pharmacopeia (2007).

Bioethanol (%)=(Peak Area of Biothanol)/(Peak Area of n-Butanol) x (Wt of Std)/(Std Volume) x (Sample Volume)/(wt.of sample) x Std Potency

Therefore,

Peak Area of Bioethanol = peak hight of chromatogram of bioethanol

Peak Area of n- Butanol = peak hight of chromatogram of n-butanol

Sample Volume = sample dilution

Wt of Std = weight of the standard

Std Volume = standard volume

Wt of sample = weight of sample

Std Potency = standard concentration of bioethanol

Results & Discussion

During the process of fermentations, screening of various microorganisms was carried on bioethanol productions. The microorganisms such as *Saccharomyces cerevisiae-171* MTCC, *Kluyveromyces thermotolerance-30* MTCC, *S.cerevisiae-3288* NCIM, *S.cerevisiae-3190* NCIM, *K.marxianus-1389* MTCC, *Zymomonas mobilis-92* MTCC, *Escherichia coli* and *S.cerevisiae-463* produced bioethanol concentrations of 36.437$g.l^{-1}$, 35.573 $g.l^{-1}$, 41.241 $g.l^{-1}$, 43.865 $g.l^{-1}$, 40.531 $g.l^{-1}$, 39.657 $g.l^{-1}$, 37.559 $g.l^{-1}$, and 41.327 $g.l^{-1}$ respectively. Amongst, *S.cerevisiae-3190* (NCIM) produced maximum bioethanol yield was 43.865 $g.l^{-1}$ after 48 hours of fermentation than other yeast and bacterial cells. From the results, it was found that the yeast strain *S.cerevisiae-3190* (NCIM) is efficient and used throughout the optimizations of fermentative conditions on bioethanol productions. The result shown in figure: 1.

The initial standardization of fermentative conditions such as physico-chemical and nutritional factors were carried out using *S.cerevisiae-3190* (NCIM) with 5l bioreactor (B-Lite Sartorious limited, Mumbai).

The maximum bioethanol yields obtained by the standard optimizations of fermentative conditions (Medium-I) were 98.14 $g.l^{-1}$ at 400 $g.l^{-1}$ of substrate concentration , 103.15 $g.l^{-1}$ at 30 ^0C of temperature, 108.69$g.l^{-1}$ at pH 5, 110.63 $g.l^{-1}$ at 120 RPM of agitation 111.19 $g.l^{-1}$ at 8v/v of inoculum volume,112.98$g.l^{-1}$ at 0.6 $g.l^{-1}$ of ammonium sulphate $(NH_4)_2SO_4$,110.189 $g.l^{-1}$ at 0.5 $g.l^{-1}$ of copper chloride ($CuCl_2$),116.64 $g.l^{-1}$ at 0.06 $g.l^{-1}$ of manganese chloride ($MnCl_2.4H_2O$), 118.0 $g.l^{-1}$at 0.4 $g.l^{-1}$ of magnesium chloride ($MgCl_2.6H_2O$), 114.75 $g.l^{-1}$ at 50 $mg.l^{-1}$ of zinc sulphate ($ZnSO_4.7H_2O$), 115.68 $g.l^{-1}$ at 24 $mg.l^{-1}$ of biotin, 114.36 $g.l^{-1}$ at 0.150 of $g.l^{-1}$ of proline, 119.342 $g.l^{-1}$ at 5.0 $g.l^{-1}$sodium di hydrogen phosphate (NaH_2PO_4), 117.653 $g.l^{-1}$ at 5 $g.l^{-1}$ of ethylene di-amine tetraacetic acid (EDTA), 116.981 $g.l^{-1}$ 2.0 $g.l^{-1}$ of potassium phosphate (K_2HPO_4), 115.947 $g.l^{-1}$ at 0.06 $g.l^{-1}$ of calcium chloride ($CaCl_2$), 118.635 $g.l^{-1}$ at 80 $mg.l^{-1}$ of cobalt chloride ($CoCl_2$), 102.721 $g.l^{-1}$ at 0.5 $g.l^{-1}$, oxygen (O_2) at 0.3 $mg.l^{-1}$ of ferrous sulphate ($Fe_2(SO_4)_3.H_2O$), 93.641 $g.l^{-1}$ at 0.10 $g.l^{-1}$ of sodium chloride ($NaCl_2$), 100.634 $g.l^{-1}$ at 3.0 $g.l^{-1}$ of peptone, 114.735 $g.l^{-1}$ at 2.5 $g.l^{-1}$ of urea and 118.462 $g.l^{-1}$ at 1.5 $g.l^{-1}$ of yeast extract. Under these optimum concentrations of medium-I, bioethanol yield obtained was 150.562$g.l^{-1}$ at the fermentation efficiency of 3.1367$g.l^{-1}.h^{-1}$ and the percentage of yield of bioethanol was 73.66 %. Elena Patrascu, *et al.*, (2009) who have been reported that some selected yeast strains can produce bioethanol yields up to 15 % or higher. The viable yeast cells were found to be 98 % after 48 hours of fermentation time. The experimental results are shown in figure. no. 2.

Statistical optimizations of fermentative conditions (Medium-II) were conducted in order to enhance the bioethanol yields with *S.cerevisiae*-3190 (NCIM) response surface methodology (RSM) with software Statistica8. The Three factors at a time was applied using central composite design and eight central composite designs were developed based on the standard optimization of fermentative factors of medium-I. The maximum bioethanol concentrations were produced with statistical optimum fermentative conditions: the experiments on fermentation conditions optimum with substrate concentration of 409.916 $g.l^{-1}$, temperature of 31.43 ^0C and pH of 4.9 of central composite design-I produced bioethanol yield was 113.570 $g.l^{-1}$. The surface plot bioethanol production was shown in figure no. 3. The optimum concentrations of central composite design-II produced bioethanol yield was 121.878 $g.l^{-1}$ with inoculum volume of 9.000 v/v, agitation of 117.28 RPM and inoculum age of 53.66 hours (figure.no. 4).

The statistical optimization of fermentation conditions with ammonium sulphate $(NH_4)_2SO_4$) of 0.629 $mg.l^{-1}$, copper chloride ($CuCl_2$) of 0.522 $mg.l^{-1}$ and manganese chloride ($MnCl_2.4H_2O$) of 0.061 $mg.l^{-1}$ produced bioethanol yield was 128.763 $g.l^{-1}$ (figure. no. 5). The optimum concentrations of central composite design-IV with magnesium chloride ($MgCl_2.6H_2O$) of 0.430 $g.l^{-1}$, zinc sulphate ($ZnSO_4.7H_2O$) of 54.021 $mg.l^{-1}$, biotin of 22.453 $mg.l^{-1}$ produced bioethanol yield was 131.281 $g.l^{-1}$(figure. no. 6).

The fermentation experiments on optimization fermentative factors of central composite design-V produced bioethanol yield was 129.936 $g.l^{-1}$ with proline of 0.163 $g.l^{-1}$, sodium di-hydrogen phosphate (NaH_2PO_4) of 5.385 $g.l^{-1}$, ethylene di-amine tetraacetic acid (EDTA) of 5.197 $g.l^{-1}$ (figure. No.7).

As well, the optimum concentrations of central composite design-VI produced bioethanol yield was 132.515 $g.l^{-1}$ with potassium phosphate (K_2HPO_4) of 2.170 $g.l^{-1}$, calcium chloride ($CaCl_2$) of 0.064 $g.l^{-1}$ and cobalt chloride ($CoCl_2$) of 99.48 $mg.l^{-1}$ (figure no. 8). The production of bioethanol by central composite design-VII on fermentative factors was 125.929 $g.l^{-1}$ with ferrous sulphate ($Fe_2(SO_4)_3.H_2O$) of 0.533 $g.l^{-1}$, oxygen (O_2) of 0.330 $g.l^{-1}$, sodium chloride (NaCl) of 1.105 $g.l^{-1}$ (figure no. 9). The optimum concentrations of central composite design-VIII produced bioethanol yield was 135.164 $g.l^{-1}$ with peptone of 3.038 $g.l^{-1}$, urea of 2.566 $g.l^{-1}$ and yeast extract of 1.572 $g.l^{-1}$ (figure no.10).

Under these optimized fermentative conditions (Medium-II), the highest yield of bioethanol achieved was 195.284 $g.l^{-1}$ at the rate of productivity of 4.068 $g.l^{-1}.h^{-1}$ and the percentage of bioethanol was 93.22 % with *S.cerevisiae*-3190 after 48 hours of fermentation. After the fermentation process completed, 1.9 gm of sugars are present fermentation medium. Infact, the yeast growth inhibited by 6% of ethanol, even though some selected brewing yeast strains can tolerate higher bioethanol concentrations over 10 % or even 20 % in the case of sake yeast strains (Shimoi, *et al.,* (2011a). The optimum conditions of fermentative condition, bioethanol productions and P-value and F-value are shown in Table no. 11. It can be conclude from the figure no.12.

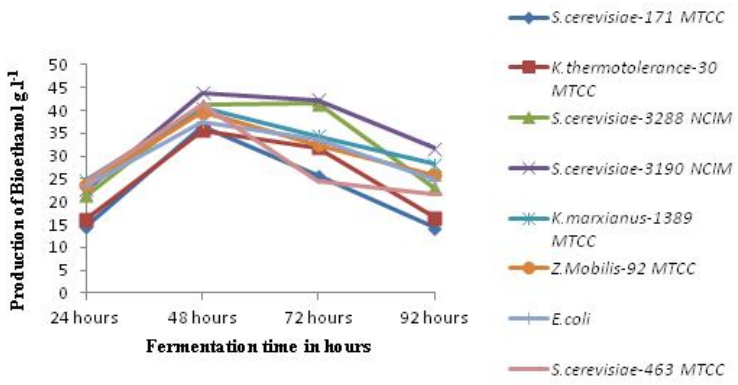

Figure no.1: Screening of microorganisms

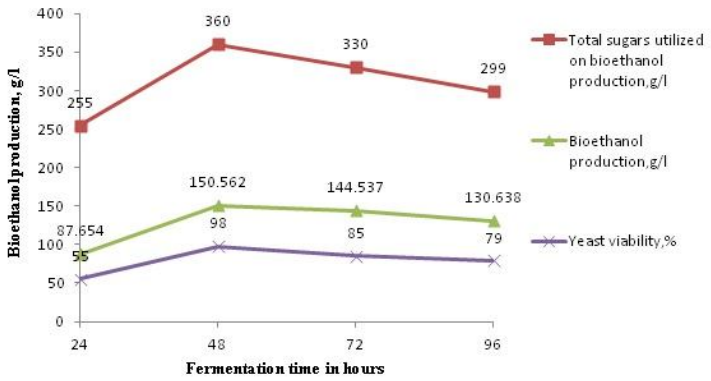

Figure no.2: Bioethanol production with medium-I

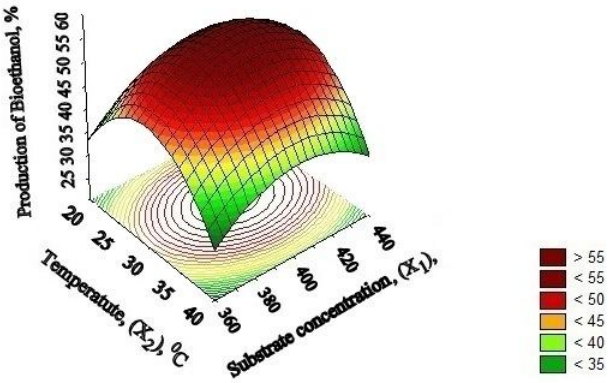

Figure no.3: statistical optimizations of substrate concentration and temperature with pH were kept constant on bioethanol production.

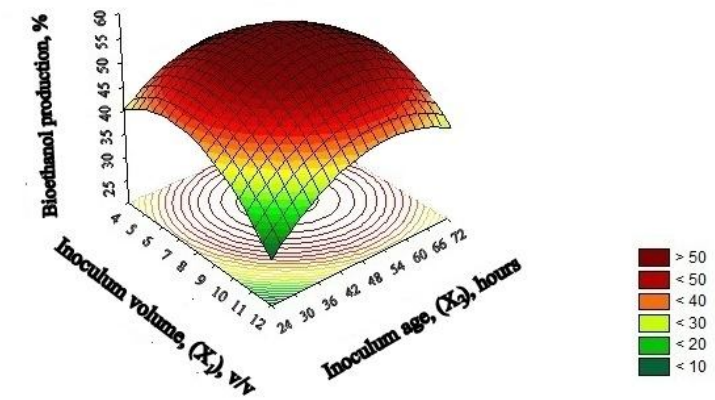

Figure no.4: statistical optimizations of inoculum volume and inoculum age with agitation were kept constant on bioethanol production.

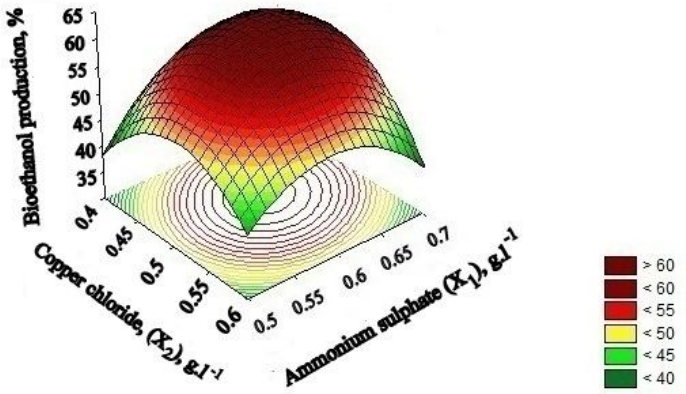

Figure no.5: statistical optimizations of copper chloride and ammonium sulphate with manganese were kept constant on bioethanol production.

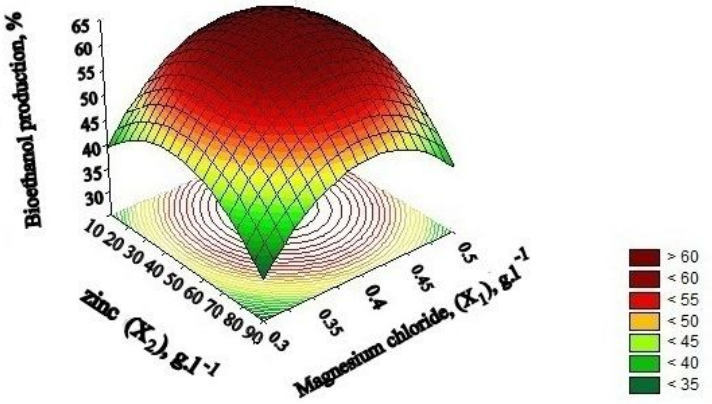

Figure no.6: Figure no.7: statistical optimizations of Zinc and magnesium with biotin were kept constant on bioethanol production.

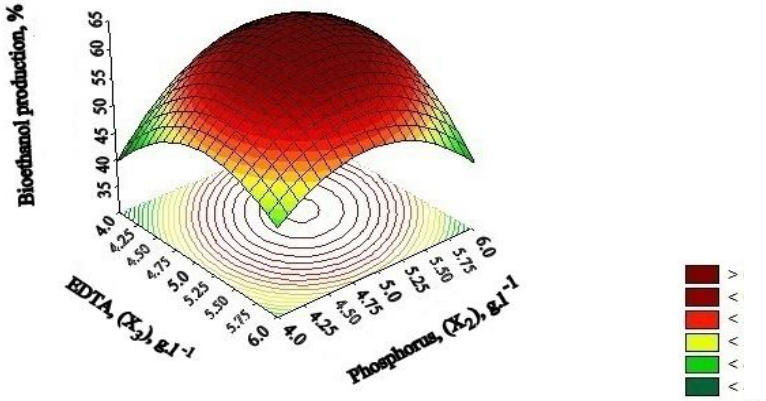

Figure no.8: statistical optimizations of EDTA and phosphorous with proline was kept constant on bioethanol production.

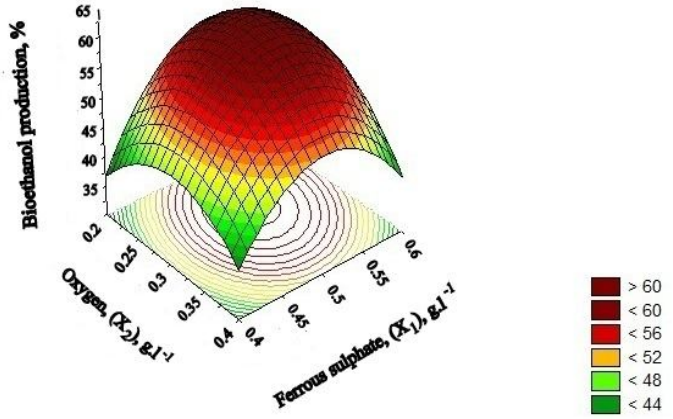

Figure no.9: statistical optimizations of oxygen and ferrous sulphate with cobalt were kept constant on bioethanol production.

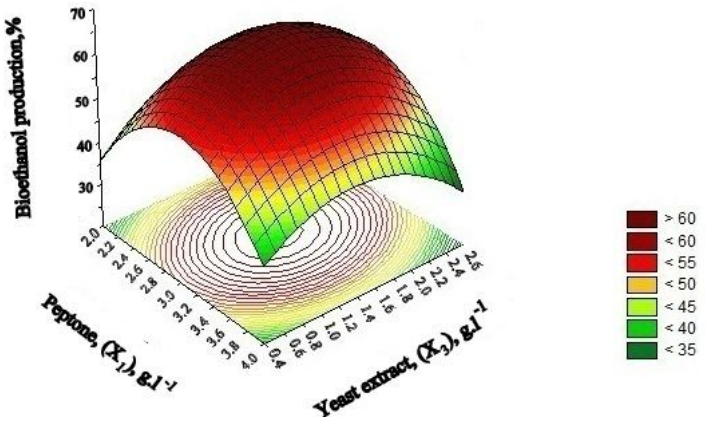

Figure no.10: statistical optimizations of peptone and yeast extract with urea were kept constant on bioethanol production.

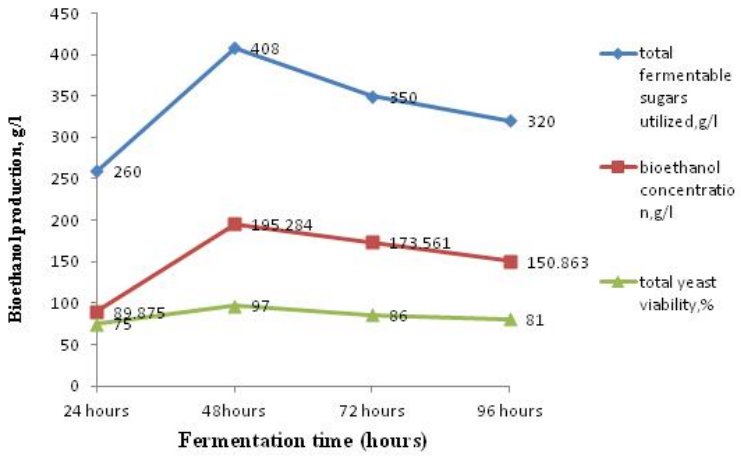

Figure no.12: bioethanol production with medium-II

S.No	CCD	Variables	Unit	Optimum concentrations	Experimental bioethanol Yields ($g.l^{-1}$)	P-value	F-value
1	Design I	Substrate Concentration	$g.l^{-1}$	409.916	113.570	0.004	28.604
		Temperature	^0C	31.430			
		pH	pH	4.9			
2	Design II	Inoculum volume	v/v	9.000	121.878	0.440	2.552
		Agitation	RPM	117.286			
		Inoculum age	Hours	53.660			
3	Design III	Ammonium Sulphate	$g.l^{-1}$	0.629	128.763	0.0001	30.568
		Copper	$g.l^{-1}$	0.522			
		Manganese	$g.l^{-1}$	0.061			
4	Design IV	Magnesium	$g.l^{-1}$	0.430	131.281	0.000061	61.275
		Zinc	$mg.l^{-1}$	54.021			
		Biotin	$mg.l^{-1}$	22.453			
5	Design V	Proline	$g.l^{-1}$	0.163	129.936	0.000597	63.553
		Phosphorous	$g.l^{-1}$	5.385			
		EDTA	$g.l^{-1}$	5.197			
6	Design VI	Potassium	$g.l^{-1}$	2.340	132.515	0.0009	56.679
		Calcium	$g.l^{-1}$	0.064			
		Cobalt	$mg.l^{-1}$	99.43			
7	Design VII	Ferrous	$g.l^{-1}$	0.533	125.929	0.003	26.791
		Oxygen	$mg.l^{-1}$	0.330			
		Sodium chloride	$g.l^{-1}$	1.105			
8	Design VIII	Peptone	$g.l^{-1}$	3.038	135.164	0.665	3.001
		Urea	$g.l^{-1}$	2.566			
		Yeast Extract	$g.l^{-1}$	0.572			

Table No.11: Statistical optimum
fermentation conditions of central composite designs on Bioethanol productions

Conclusion

From the data of experiments, it can be concluded that the yeast strain *Saccharomyces cerevisiae*-3190 is resistant to acidic conditions, osmotic stresses by very high gravity medium and high ethanol tolerant. Mahua flower (*Madhuca indica*) was proved as a suitable substrate for bioethanol production. One million tonns of Mahua flower could produce 3, 48,303 Litres of bioethanol. When bioethanol used as blend in petrol, thus replace 3,48,303 Litres of petrol in India. To meet the required fuel quantity, bioethanol

Production through fermentation process in industrial scale is the only alternative method to avoid fuel crisis. The statistical optimizations of fermentation conditions for bioethanol productions with Mahua flower by response surface methodology could be feasible economic bioprocess.

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