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DESIGN AND OPTIMIZATION OF BIOETHANOL PRODUCTION FROM FRUIT AND VEGETABLE WASTES USING *SACCHAROMYCES CEREVISIAE*

Suganya Ramar* and Gayatri M.[1]

P.S.R. Engineering College, Sivakasi – 626140.

***Corresponding Author: Prof. Suganya Ramar**

Assistant Professor, Department of Biotechnology, P.S.R. Engineering College, Sivakasi – 626140.

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ABSTRACT

The universal energy demand is contentiously increasing day by day because of rapid rise in world population and industrialization. Here is the main objective is to overcome this energy crisis by using biofuel as an alternative energy source. Bio ethanol is most often used as a biofuel additive for gasoline and also used as an alternative to conventional fossil fuels. Now-a-days bioethanol production from fruit and vegetable wastes are very easy process and also has low cost. In this study, raw materials are subjected to acid hydrolysis, which convert the wastes into fermentable sugar and *Saccharomyces cerevisiae* commonly known as baker's yeast used for fermentation process. The focus of this present study is to investigate the best carbon and nitrogen source and optimization of medium composition for maximum production of bioethanol. The best nitrogen source was selected by one variable at a time approach. The greatest nitrogen source was yeast extract used to maximize the bioethanol production. The Plackett-Burman design was used for identification of significant variables that are pH, temperature and inoculum's size. The composition level for each significant variable was determined using Response Surface Methodology. From central composite design, inoculum's size (4 ml/100ml), pH (6), temperature (42.5 ^{o} C) are the optimize values for bioethanol production and that confirmation location gives 95% confidence level in point prediction. Based on the optimization process 90% of ethanol yielded from wastes. This was analyzed using dichromate assay.

KEYWORDS: *S. cerevisiae*, fermentation, bioethanol, PB design, CCD.

1. INTRODUCTION

1.1 BIOETHANOL

With the rapid rise in world population and industrialization, the universal energy demand is contentiously increasing. The standard universal energy sources are fossil fuels such as petroleum, natural gas, hydropower, and nuclear coal. International Energy Agency (IEA) reported 80% of the energy utilization based on oil and coal. According to this agency, world oil demand is increasing by 1.6% each year.[26]

The global economy has no longer depends on fossil fuels and non-renewable energy resources. The researchers developed renewable and sustainable fuels that have recognized biofuel as a viable alternative to conventional fossil fuels. The depletion of fossil fuels, increasing energy demand, and accumulation of atmospheric CO_2 are the main reasons bringing about biofuel as alternative resources to fossil fuels. Bioethanol is widely investigated as a more important renewable fuel source. Ethanol gives energy and has less carbon-intensive than oil. Bioethanol produced from biomass via biochemical procedures. Effectively bioethanol produced by a fermentation process and that requires four important components: Fermentable carbohydrates, yeast strain, a few nutrients and culture conditions.[1,27]

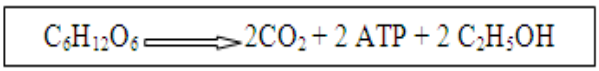
1.2 APPLICATIONS OF BIOETHANOL

Ethanol plays a major role in the industrial ingredient. It can be used as a base chemical for organic compounds and it can be utilized as a part of restorative wipes and most commonly it used as antimicrobial hand sanitizer gels and as an antiseptic. Biomass-based such as waste materials ethanol production is most often used as a biofuel additive for gasoline.[5,9] As liquid ethanol is very easily transported and it can be blended with gasoline to raise the octane booster of the fuel. Due to fluctuations in the price of petroleum have made commercial production of fermentation ethanol a more attractive.[3,11]

1.3 FERMENTATION PROCESS

Ethanol production from yeast fermentation consists of 3 major steps. Its starts from making a fermentable sugar solution and then conversion of fermentable sugar into ethanol done under microbial conditions. Final stage is to separation and purification of ethanol from the solution achieved by distillation technique.

The general conversion of ethanol production through anaerobic fermentation is,



$$C_6H_{12}O_6 \longrightarrow 2CO_2 + 2\ ATP + 2\ C_2H_5OH$$

1.4 FRUIT AND VEGETABLE WASTES

Fruit and vegetable wastes were rich in carbohydrates. Starch converted into fermentable sugars that process done by either acid hydrolysis or enzymatic hydrolysis. These pretreatment methods have both advantages and disadvantages. The most commonly used microorganisms for fermentation in industrial processes are *Saccharomyces cerevisiae*, *Hanseniaspora uvarum* and *starmerella bacillaris*. The fermentation process is carried out by a variety of microorganisms such as bacteria, fungi, and yeast. Production of bioethanol from yeast strain has low cost and high capacity to yield a large amount of ethanol.[18,23]

1.5 *Saccharomyces cerevisiae*

The most common and commercially important yeasts that been primarily used for the production of bioethanol are the related species and strains of *S. cerevisiae.* These microorganisms have long been utilized to ferment the sugars of wheat, barley, rice and corn to produce alcoholic beverages in large amounts and also used in the baking industry.[13,29] Approximately one yeast cell can ferment its weight of glucose per hour. Sugars derived from sugar cane, sugar beets and fruits can be directly converted into ethanol.[23] Immobilized yeast cells have been considered as a promising alternative for increasing and enhancing bioethanol productivity because it reduces the risk of contamination, retains the stability of cell growth, reduces the cost of production and minimizes the fermentation costs.[3]

1.5.1 Scientific Classification

Kingdom : Fungi
Division : Ascomycota
Class : Saccharomycetes
Order : Saccharomycetales
Family : Saccharomycetaceae
Genus : Saccharomyces
Species : *Saccharomyces cerevisiae*

1.6 OPTIMIZATION PROCESS

1.6.1 Plackett-Burman design

Ethanol production from fruit and vegetable wastes are a relatively new topic and limited research conducted. To increase large quantities of ethanol production with low costs, the optimization of various physicochemical parameters like pH, temperature, inoculum size, incubation time, etc., were important. This study aimed to produce ethyl alcohol under optimal conditions by *Saccharomyces cerevisiae* yeast commonly known as Baker's yeast from very cheap carbon source fruit and vegetable wastes.[15,30]

Several factors like high temperature, variation in pH, sugar tolerance and inoculum's size of the yeast limit the industrial ethanol production at low production costs. The use of concentrated sugar substrate derived from fruit and vegetable waste is one of the ways to obtain high ethanol yield during fermentation. Due to osmatic stress, high substrate concentrations are inhibitory to fermentation.[8,32]

1.6.2 Response surface methodology

The traditional method used for optimizing a multivariable fermentation process was Plackett-Burman design. Recently, many statistical experimental design methods have employed in bioprocess optimization. In the fermentation process, among these design methods the central composite design (CCD) suitable for identifying the variables to optimization.[20] Plackett Burman's design was used to identifying the most important variables in the fermentation system. CCD method used to optimize the fermentation process such as acids, antibiotics, enzymes, biomass and ethanol production by various microorganisms. Furthermore, CCD has used in analysis and unit operations. The pros of this method are reducing the number of experiments, reagents, time, financial input and energy.[21,33]

Response surface methodology (RSM) is an experimental model widely used to study the effect of several variables and identifying optimum conditions for a multivariable system. Factors combination generating a certain optimization response. It can be identified through factorial designs as well as RSM. According to our knowledge, a combination of RSM with CCD used for optimization conditions of fuel ethanol production using fruit and vegetable wastes.[32,33]

In contrast with other optimization designs, RSM needs fewer trails to calculate numerous variables and their interactions. The RSM experimental design has been adopted to upgrade the production of antibacterial compounds in various *Streptomyces* species, including *Streptomyces* sp. HJC-D1, *Streptomyces nogalater* NIIST A30, *Streptomyces* sp. SY-BS5, and *Streptomyces* sp. SYYLWHS-1-4. Therefore, RSM only one strategy to maximize ethanol production at a low cost.[30]

2. MATERIALS AND METHOD

2.1 MATERIALS

2.1.1 Chemicals

Sodium hydroxide, Potassium dihydrogen phosphate, Sodium chloride, Magnesium Sulfate, Ammonium sulfate, Phenol, Sulfuric Acid, Glucose, Dinitro salicylic acid, Sodium potassium tartrate, Hydro chloric acid, Sodium dichromate, Potassium dichromate and Potassium iodide.

2.1.2 Sample collection

The various fruit wastes like orange, banana, papaya, watermelon, etc., and vegetable wastes like cabbage, potato peel etc., were collected from the local market in Virudhunagar area.

2.1.3 Microorganism

Saccharomyces cerevisiae (Baker's yeast) obtained from the public market in Virudhunagar area. This yeast was commercially available in that market.

2.1.4 Medium for culture and maintenance

2-gram yeast was inoculated in 100 ml of Yeast Extract Peptone Dextrose (YEPD) medium under sterilized conditions. This culture was incubated at 30^{o}C for 24 hours. Composition[17] of YEPD medium (for one liter) are Yeast extract-5g, Glucose-10g, KH_2PO_4-1g, $MgSO_4.7H_2O$-0.5g and Peptone-10g.

2.1.5 Preparation of ethanol fermentation medium

The programmed protocol of production medium for ethanol under fed-batch condition contained[26] (g/l):KH_2PO_4-0.1%, NaCl- 0.1%, $MgSO_4$-0.07%, $(NH_4)_2SO_4$-0.4%, Yeast extract-0.2% and hydrolyzed sample was principle solution for the medium. The pH of the medium adjusted to 7 and then neutralize with 1M NaOH. 30ml of this medium was distributed in screw cap bottles and then autoclaved at 121^{o}C for 15 minutes. After sterilization, the yeast was inoculated in the ethanol fermentation medium. The fermentation period was carried out for 5 to 7 days under anaerobic conditions.

2.2 METHODS

2.2.1 Drying method

Initially, moisture content of the sample was analyzed. It can be done through measuring the mass of wastes before and after water content removed by evaporation. In this evaporation technique, we used a hot air oven for analyzing moisture. Finally, the initial and final moisture content analyzed by the drying method (Hot air oven) based on a dry basis at 105^{o}C for 2 hours using given formula.

$$\% \text{ Moisture} = M_{Initial} - M_{Dried} / M_{Initial} * 100$$

2.2.2 PRETREATMENT METHOD

2.2.2.1 Acid hydrolysis of sample

The substrate was prepared by using the acid hydrolysis method. Acid hydrolysis pretreatment method used to convert the disaccharides into monosaccharide present in the sample. Wastes were collected from local areas. These wastes dried at 80^{o}C and powdered using a blender. 0.5% HCl or sulphuric acid was added to the waste powder and then the mixtures were autoclaved at 121^{o}C for 15 minutes. Finally, the samples were cooled down and then analyzed for glucose concentration. The final pH value of the filtrate was adjusted to approximately 6-7.[18]

2.2.3 ANALYTICAL METHODS

2.2.3.1 Estimation of total carbohydrate

Phenol sulfuric acid method

Reagents

5% Phenol, 96% sulfuric acid, 2.5 N HCl and NaOH.

Standard Glucose-1mg in 1ml of distilled water.

Procedure

100mg of sample was taken in 100ml beaker and 5ml of 2.5 N HCl was added. The beaker was covered with aluminum foil and that content was boiled for 3 hours on a hot plate. After, the sample was cooled down at room temperature and neutralized with NaOH. The volume was made up to 100ml and then the content was centrifuged. 0.1 ml was pipped out and made up to 1ml with sterile distilled water. After that, 1ml of phenol and 5ml of 96% sulfuric acid were added in that test tube. The solution was kept in a water bath for 20 minutes at 30^{o}C. Next, the solution was taken and read out in colorimeter at 490nm. The unknown concentration will be calculated using standard values.[18,26]

2.2.3.2 Estimation of reducing sugar

Dinitro salicylic acid method

Reagents

DNSA was added in 20ml of 2 N NaOH and the solution is mixed. 30g of Rochelle salt was dissolved in 50ml of distilled water. Next, Rochelle salt was slowly poured into DNSA and NaOH solution. The volume was made up to 100ml and stored in a brown bottle.

Standard Glucose-1mg in 1ml of distilled water.

Procedure

100mg of sample was washed with hot 80% ethanol for three times. The supernatant was collected and the content was evaporated using a boiling water bath at 80^{o}C. After that, 10ml of distilled water was added and 0.1ml of sample was pipped out. Next, the solution was made up to 3ml with sterile distilled water. Then, 3ml of DNSA reagent was added. Finally, the solution was kept in a boiling water bath for 15 minutes and the absorbance was taken in colorimeter at 540nm. The unknown concentration will be calculated using standard values.[18,26]

2.3 PURIFICATION AND ANALYSIS OF ETHANOL

2.3.1 Distillation

Distillation was a separation technique used to separate ethanol from sample. After fermentation, the fermented sample 10ml was collected. Then, the sample was taken in the round bottom flask. After that, it was loaded in a simple distillation unit. The process temperature was maintained at 78.2^{0}C. Finally, the distillate was collected in a conical flask. 1ml of distillate was taken out in a fresh test tube.[15,17]

Fig. 1: Simple distillation process setup.

2.3.2 Ethanol estimation

Dichromate assay

Reagents

Chromic acid preparation: 100 g of sodium dichromate or potassium dichromate was taken and a small amount of water was added to form a paste consistency. 300ml of concentrated sulfuric acid was added and the solution was made up to 1000ml with distilled water. Finally, the solution was stored in a glass bottle.

Standard- Ethanol

Procedure

1 ml of distillate was collected and then 5 ml of chromic acid was added. After that, the solution was boiled at 80^{o}C for 15 minutes. The sample was cooled down and the content was made up to 10ml with sterilized distilled water. Finally, the absorbance was taken at 590nm using colorimeter. Thus, the obtained results were plotted with standard values.[17]

3.4 MEDIUM OPTIMIZATION

3.4.1 Plackett-Burman design

Initially, the suitable nitrogen source was selected using one variable at a time approach. The various parameters such as source, nitrogen source, buffer salt, trace element, pH, temperature, inoculum's size and incubation time were chosen for the optimization process of ethanol production. Based on that result, the most important variables were obtained using Minitab software. File description: Minitab 19 statistical software and File version: 19.2.0.0. For PB design, low values (per 100ml) are C1-1%, C2-0.1%, C3-1%, C4-5, C5-30^{o}C, C6-0.05g, C7-0.01g and high values (per 100ml) are C1-5%, C2-0.5%, C3-5%, C4-7, C5-37^{0} C, C6-0.1g, C7-0.05g.

Table 1: Plackett-Burman design.

Std order	Run order	C1	C2	C3	C4	C5	C6	C7
3	1	-	+	+	-	+	-	-
4	2	+	-	+	+	-	+	-
9	3	-	-	-	+	+	+	-
5	4	+	+	-	+	+	-	+
8	5	-	-	+	+	+	-	+
12	6	-	-	-	-	-	-	-
7	7	-	+	+	+	-	+	+
2	8	+	+	-	+	-	-	-
11	9	-	+	-	-	-	+	+
6	10	+	+	+	-	+	+	-
1	11	+	-	+	-	-	-	+
10	12	+	-	-	-	+	+	+

From **Table 1**: - Low value, + High value, C1-Carbon, C2-Nitrogen, C3-Inoculum's size, C4-pH, C5-Temperature, C6-Buffer salt and C7-Trace element.

3.4.2 Response Surface Methodology

From Plackett- Burman design, the three important variables chosen for Central Composite Design (CCD). A rotatable CCD with three factors were used for studying response patterns. Design Expert 2018 software was used to determine the optimal combination of variables and the design model was quadratic.

Table 2: Central Composite Design.

Std	Run	Factor 1 A.Inoculum's Size(ml)	Factor 2 B.pH	Factor 3 C.Temperature(^{O}C)
14	1	3	6	36.7045
20	2	3	6	32.5
4	3	5	7	30
2	4	5	5	30
11	5	3	4.31821	32.5
3	6	1	7	30
5	7	1	5	35
16	8	3	6	32.5
19	9	3	6	32.5
13	10	3	6	28.2955
10	11	6.36359	6	32.5
6	12	5	5	35
17	13	3	6	32.5
18	14	3	6	32.5
1	15	1	5	30
7	16	1	7	35
9	17	-0.363586	6	32.5
12	18	3	7.68179	32.5
8	19	5	7	35
15	20	3	6	32.5

2.5 APPLICATION STUIDES OF BIO ETHANOL

2.5.1 Combustion test

5ml of bioethanol was transfer into clean test tube and it can be hold with a test tube holder. Then, the content was heated until the liquid was evaporated. Next, the test tube was cooled down. Finally, ethanol was ignited.[22]

2.5.2 Comparison of bioethanol and petrol

The properties of bioethanol were compared to the properties of fossil petrol. The properties were density, viscosity, flash point, caloric value, octane number and fuel equivalence.[6]

3. RESULTS AND DISCUSSION

3.1 COMPARATIVE ANALYSIS

Distilled water, sucrose and sample were hydrolyzed and analyzed for comparative studies. OD was noted at 590nm. The result was shown in fig 2. Distilled water yielded no ethanol. 60% ethanol derived from sucrose and 82% ethanol derived from fruit and vegetable wastes. From the results, high concentration of ethanol derived from our sample.

Fig 2 Comparative analysis.

From **Fig 2,** 0-Blank, 1-Distilled water, 2-Sucrose, 3-Hydrolysis sample

3.2 MOISTURE ANALYSIS

200g of fruit and vegetable wastes dried over 2 hours at 105 ^{o}C. Applying the %moisture formulae,
Initial moisture content= 200 g
Final moisture content= 48. 47g
% Moisture= 200-48.47/ 200 * 100
Total % Moisture= 75.77%

3.3 ONE VARIABLE AT A TIME APPROACH

There were nine nitrogen sources selected for further process. From those sources, yeast had high concentration and chosen for further optimization process. It can be selected through the process of one variable at a time approach (OVAT). The result was shown in fig 3.

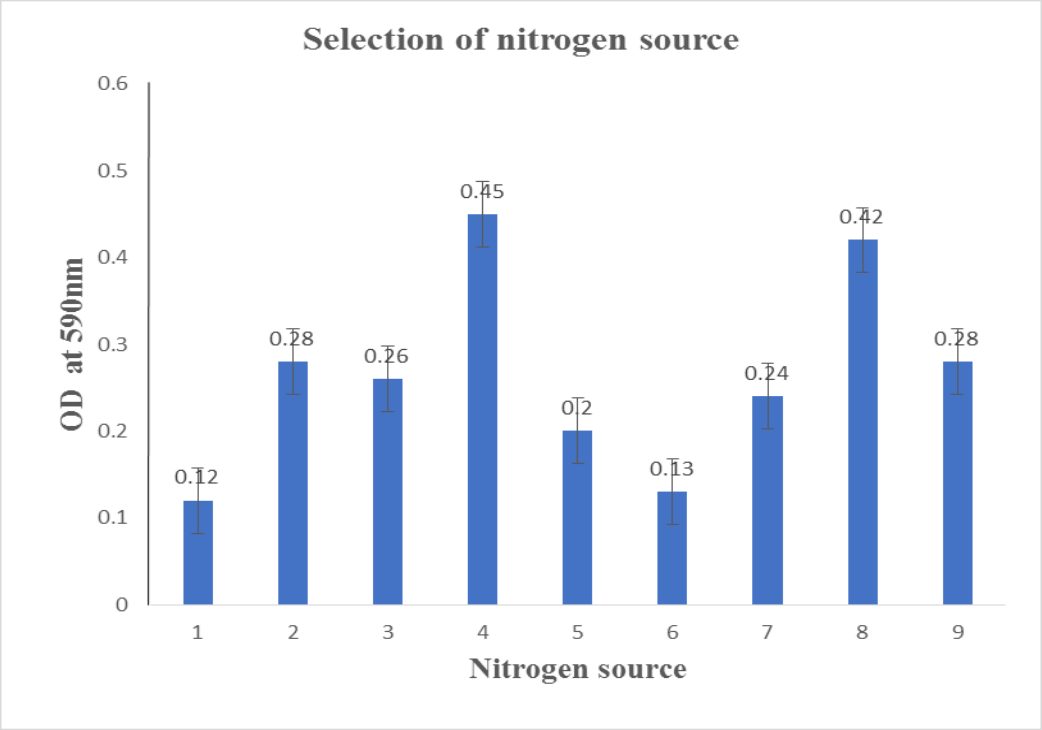


Fig. 3: Selection of nitrogen source.

From **Fig 3,** 1-Ammonium nitrate, 2-Urea, 3-Ammonium sulfate, 4-Yeast, 5-Ammonium chloride, 6-Ammonium ferrous sulfate, 7-Ammonium phosphate, 8-Peptone and 9-Ammonium oxalate.

3.4 ANALYTICAL RESPONSES FOR FRUIT AND VEGETABLE WASTES

3.4.1 Estimation of total carbohydrate

The total carbohydrate analyzed from fruit and vegetable wastes using phenol sulfuric acid method. Estimation of total carbohydrate in the sample using glucose as a standard solution was shown in fig 4. Based on the results, 0.1ml of sample contained 80% of carbohydrate.

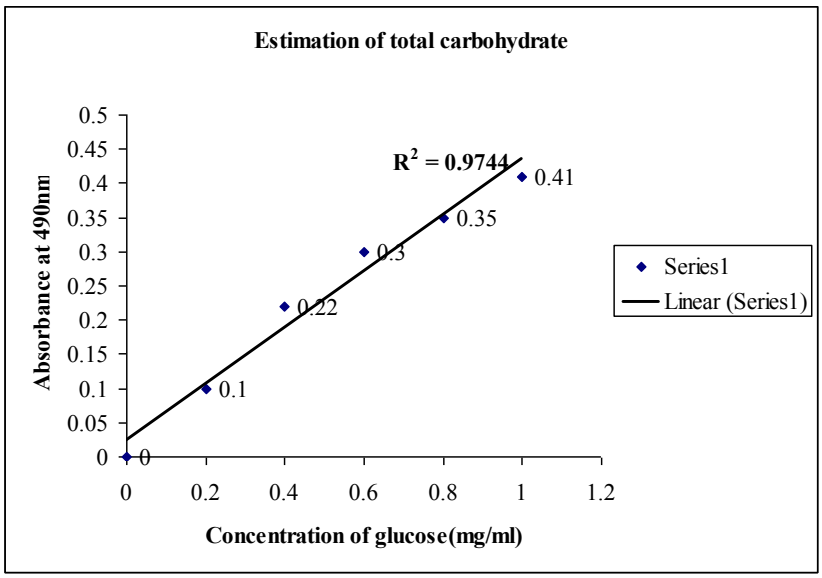


Fig. 4: Estimation of total carbohydrate.

3.4.2 Estimation of reducing sugar

Reducing sugar analyzed from the sample. Sample concentration was calculated using glucose as a standard solution. 0.1 ml of sample contained 90% of reducing sugar. That result was shown in fig 5.

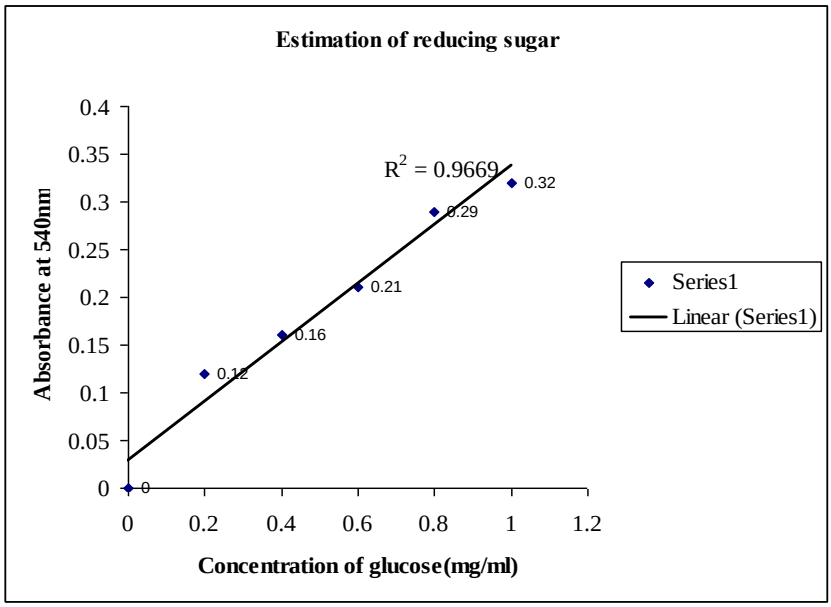


Fig. 5: Estimation of reducing sugar.

3.5 ETHANOL ANALYSIS

Ethanol was analyzed by dichromate assay. 1ml of distillated sample contains 90% of ethanol using ethanol as a standard. The result was calculated from fig 6.

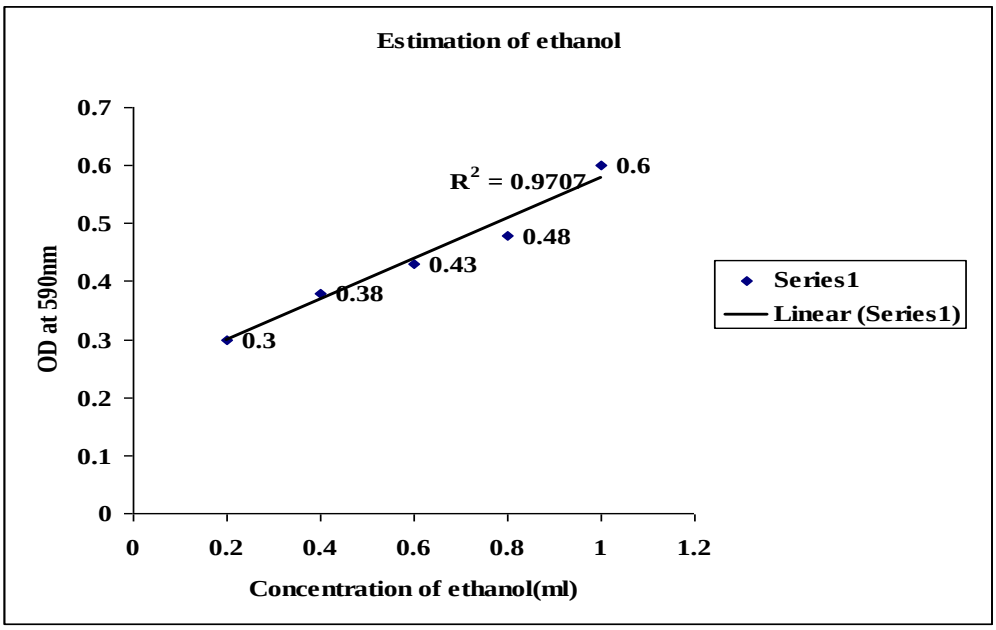


Fig. 6: Estimation of ethanol.

3.6 MEDIUM OPTIMIZATION RESPONSES

3.6.1 PB design

The PB design values were showed in table 3 and the variances were analyzed based on factorial regression.[20] Most important variables among a set of factors were identified using Pareto chart shown in fig 7. On the pareto chart of the standardized effects, bars that pass over the reference line were statistically significant. Based on the results, Inoculum's size, pH and temperature were chosen as important factors.

Table 3: PB responses.

Std order	Run order	C1	C2	C3	C4	C5	C6	C7	Responses
3	1	-	+	+	-	+	-	-	0.49
4	2	+	-	+	+	-	+	-	0.09
9	3	-	-	-	+	+	+	-	0.42
5	4	+	+	-	+	+	-	+	0.49
8	5	-	-	+	+	+	-	+	0.40
12	6	-	-	-	-	-	-	-	0.40
7	7	-	+	+	+	-	+	+	0.10
2	8	+	+	-	+	-	-	-	0.40
11	9	-	+	-	-	-	+	+	0.42
6	10	+	+	+	-	+	+	-	0.52
1	11	+	-	+	-	-	-	+	0.20
10	12	+	-	-	-	+	+	+	0.50

From **Table 3**: - Low value, + High value, C1-Carbon, C2-Nitrogen, C3-Inoculum's size, C4-pH, C5-Temperature, C6-Buffer salt and C7-Trace element.

3.6.1.1 Factorial Regression

Responses versus carbon, Nitrogen, Inoculum's size, pH, Temperature, Buffer salt, Trace element. Fig 8 shown the residual plots for PB responses. Estimation process improvements and interpretation of results were shown as coded terms in table 4.

From table 4, unknown values were estimated using standard error of the co efficient. T value used for calculating difference in units of standard error and the probability of obtained results were analysed by using P value. VIF value less than 10 indicate good design.

Model summary for PB design shown in table 5. Analysis of variance for PB design shown in table 6.

Table 4: Coded coefficient for PB design.

Coded Coefficients						
Term	**Effect**	**Coef**	**SE Coef**	**T-Value**	**P-Value**	**VIF**
Constant		0.3692	0.0187	19.75	0.000	
Carbon	-0.0050	-0.0025	0.0187	-0.13	0.900	1.00
Nitrogen	0.0683	0.0342	0.0187	1.83	0.142	1.00
Inoculum's Size	-0.1383	-0.0692	0.0187	-3.70	0.021	1.00
pH	-0.1050	-0.0525	0.0187	-2.81	0.048	1.00
Temperature	0.2017	0.1008	0.0187	5.40	0.006	1.00
Buffer Salt	-0.0550	-0.0275	0.0187	-1.47	0.215	1.00
Trace element	-0.0350	-0.0175	0.0187	-0.94	0.402	1.00

Table 5: Model summary for PB design.

MODEL SUMMARY			
S	**R-sq**	**R-sq(adj)**	**R-sq(pred)**
0.0647431	93.45%	82.00%	41.08%

Table 6: ANOVA for PB design.

ANALYSIS OF VARIANCE					
SOURCE	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-value**
Model	7	0.239325	0.034189	8.16	0.030
Linear	7	0.239325	0.034189	8.16	0.030
Carbon	1	0.000075	0.000075	0.02	0.900
Nitrogen	1	0.014008	0.014008	3.34	0.142
Inoculum's Size	1	0.057408	0.057408	13.70	0.021
pH	1	0.033075	0.033075	7.89	0.048
Temperature	1	0.122008	0.122008	29.11	0.006
Buffer salt	1	0.009075	0.009075	2.17	0.215
Trace element	1	0.003675	0.003675	0.88	0.402
Error	4	0.016767	0.004192		
Total	11	0.256092			

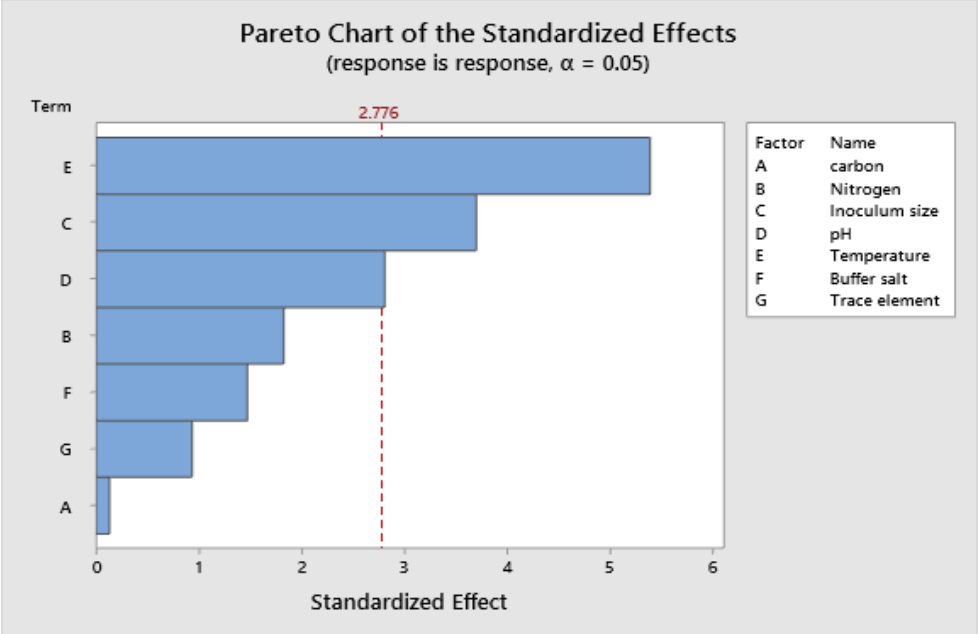


Fig. 7: Pareto chart of the standardized effects.

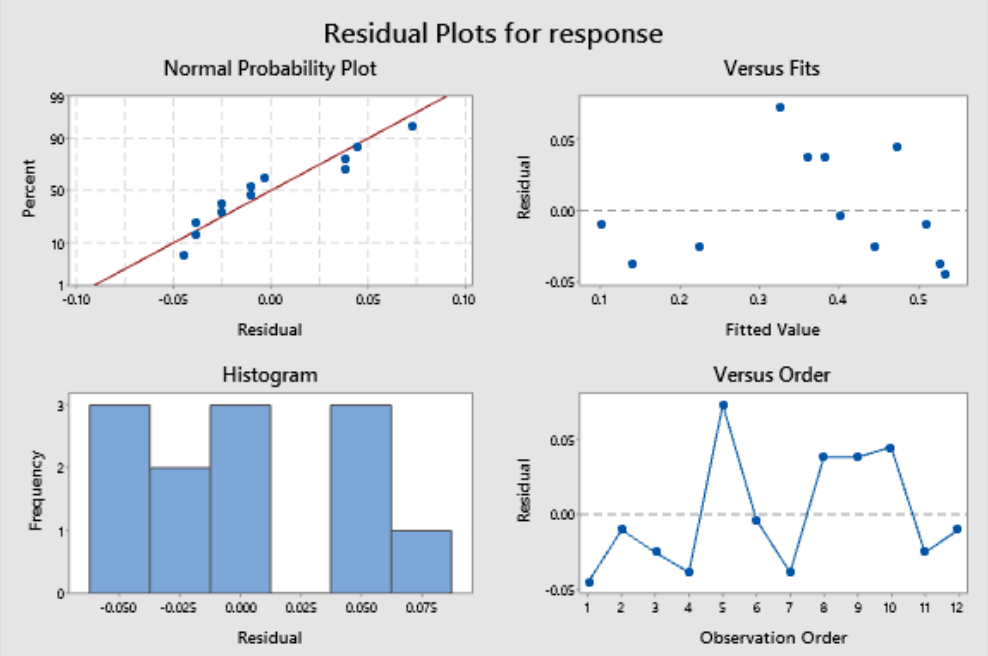


Fig. 8: Residual plots for response.

Regression equation in un coded units

From PB design temperature, pH and inoculum's size parameters were considering as significant factors that involved in the process of enhancing ethanol production from fruit and vegetable wastes.

response= -0.116 - 0.00125 carbon + 0.1708 Nitrogen - 0.04458 Inoculum size - 0.0525 pH+ 0.02881 Temperature - 1.100 Buffer salt - 0.875 Trace element

3.6.2 RSM

From PB design, the 3 most important variables are chosen for central composite design.[28] Temperature, pH and inoculum's size are chosen for CCD. The responses are shown in table 7 and the analysis of variance (ANOVA) are shown in table 8.[28]

Table 7: CCD responses.

Std	Run	Factor 1 A.Inoculum's Size(ml)	Factor 2 B.pH	Factor 3 C.Temperature(^{O}C)	Responses
14	1	3	6	36.7045	0.522
20	2	3	6	32.5	0.525
4	3	5	7	30	0.415
2	4	5	5	30	0.466
11	5	3	4.31821	32.5	0.424
3	6	1	7	30	0.412
5	7	1	5	35	0.495
16	8	3	6	32.5	0.529
19	9	3	6	32.5	0.544
13	10	3	6	28.2955	0.469
10	11	6.36359	6	32.5	0.411
6	12	5	5	35	0.416
17	13	3	6	32.5	0.544
18	14	3	6	32.5	0.496
1	15	1	5	30	0.458
7	16	1	7	35	0.527
9	17	-0.363586	6	32.5	0.427
12	18	3	7.68179	32.5	0.42
8	19	5	7	35	0.499
15	20	3	6	32.5	0.485

Table 8: ANOVA for Quadratic Model Response: Ethanol Estimation.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.0456	9	0.0051	11.48	0.0004	significant
A-Inoculum's Size	0.0000	1	0.0000	0.0864	0.7749	
B-pH	0.0009	1	0.0009	2.04	0.1840	
C-Temperature	0.0022	1	0.0022	5.05	0.0485	
AB	0.0004	1	0.0004	0.8188	0.4868	
AC	0.0000	1	0.0000	0.0910	0.7691	
BC	0.0122	1	0.0122	27.44	0.0004	
A^2	0.0169	1	0.0169	47.95	0.0001	
B^2	0.0159	1	0.0159	45.64	0.0001	
C^2	0.0007	1	0.0007	1.67	0.2249	
Residual	0.0045	10	0.0004			
Lack of Fit	0.0014	5	0.0004	0.4782	0.7814	not significant
Pure Error	0.0040	5	0.0006			
Cor Total	0.0500	19				

Factor coding is **Coded**.
Sum of squares -**Type III - Partial**

The **Model F-value** of 11.48 implies the model was significant. There was only a 0.04% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms were significant. In this case C, BC, A^2, B^2 were significant model terms. Values greater than 0.1000 indicate the model terms were not significant. If there were many insignificant model terms (not counting those required to support hierarchy), model reduction may improve our model.

The **Lack of Fit F-value** of 0.48 implies the Lack of Fit was not significant relative to the pure error. There was a 78.14% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit was good.

Table 9: Fit statistics.

Std. Dev.	0.0211	**R^2**	0.9110
Mean	0.4692	**Adjusted R^2**	0.8409
C.V. %	4.50	**Predicted R^2**	0.6874
		Adeq Precision	8.4248

From **Table 9**, the **Predicted R^2** of 0.6874 was in reasonable agreement with the **Adjusted R^2** of 0.8409; i.e. the difference was less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 was desirable. Our ratio of 8.425 indicates an adequate signal. This model could be used to navigate the design space.

Table 10: Coefficients in Terms of Coded Factors.

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	0.5201	1	0.0086	0.5009	0.5494	
A-Inoculum Size	-0.0017	1	0.0057	-0.0144	0.0110	1.0000
B-pH	0.0081	1	0.0057	-0.0046	0.0209	1.0000
C-Temperature	0.0128	1	0.0057	0.0001	0.0255	1.0000
AB	-0.0068	1	0.0075	-0.0244	0.0099	1.0000
AC	-0.0024	1	0.0075	-0.0189	0.0144	1.0000
BC	0.0490	1	0.0075	0.0224	0.0556	1.0000
A^2	-0.0442	1	0.0056	-0.0466	-0.0219	1.02
B^2	-0.0442	1	0.0056	-0.0456	-0.0208	1.02
C^2	-0.0072	1	0.0056	-0.0196	0.0052	1.02

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors were held constant. The intercept in an orthogonal design was the overall average response of all the runs. The coefficients were adjustments around that average based on the factor settings. When the factors were orthogonal the VIFs=1; VIFs greater than 1 indicate multi-colinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 were tolerable.

3.6.2.1 Final Equation in Terms of Coded Factors

Ethanol estimaton=0.52009 + -0.00167745 * A + 0.00814776 * B + 0.012824 * C + -0.00675 * AB + -0.00225 * AC + 0.049 * BC + -0.0442457 * A^2 + -0.0441751 * B^2 + -0.00718891 * C^2

The equation in terms of coded factors could be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors were coded as +1 and the low levels were coded as -1. The coded equation was useful for identifying the relative impact of the factors by comparing the factor coefficients.

3.6.2.2 Final Equation in Terms of Actual Factors

Ethanol estimation= 0.758124 + 0.0854899 * Inoculum Size + -0.0906262 * pH + -0.0124557 * Temperature + -0.004475 * Inoculum Size * pH + -0.00045 * Inoculum Size * Temperature + 0.0156 * pH * Temperature + -0.00855894 * Inoculum Size^2 + -0.0441751 * pH^2 + -0.00115024 * Temperature^2

The equation in terms of actual factors could be used to make predictions about the response for given levels of each factor. Here, the levels should be specified in the original units for each factor. This equation should not be used to determine the relative impact of each factor because the coefficients are scaled to accommodate the units of each factor and the intercept is not at the center of the design space.

3.6.2.3 Contour plot

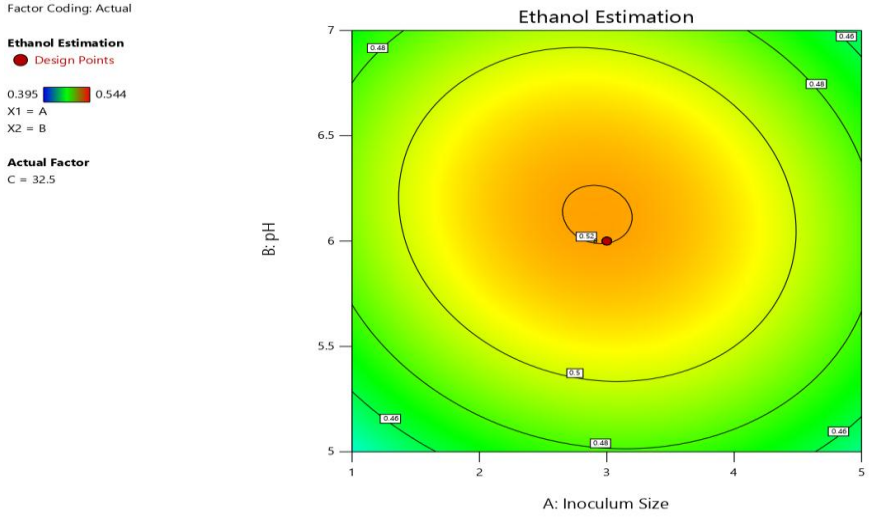


Fig. 9: A Ethanol estimation- Inoculum's size Vs pH.

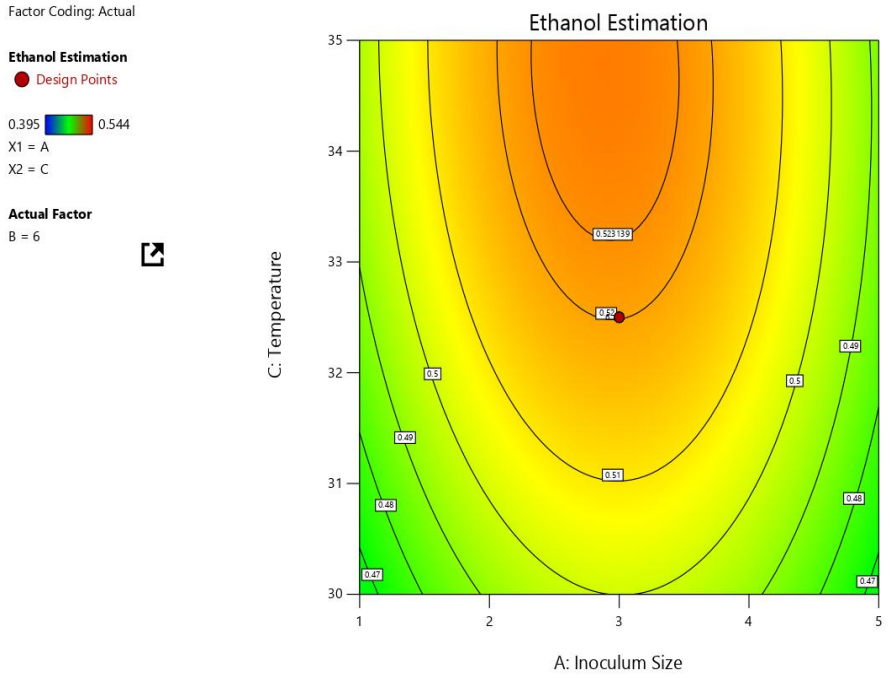


Fig. 9: B Ethanol estimation- Inoculum's size Vs temperature.

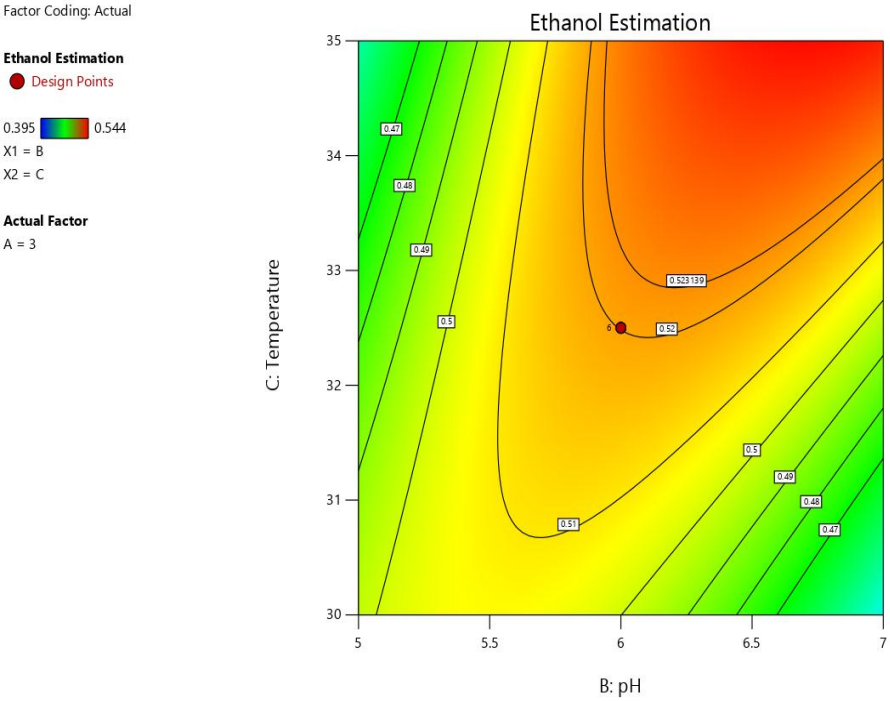


Fig. 9: C Ethanol estimation-pH VS temperature.

Fig 9A, 9B, 9C shows that ethanol estimation. Where A is Inoculum's size, B is pH and C is Temperature.

From the contour plots, Fig.9A show that there was a significant interaction between the inoculum's size and pH. Fig 9B shows that there was a significant interaction between inoculum's size and temperature. Fig 9C showed that important interaction between pH and temperature. The statistical optimal values of each important variable were obtained. In each contour plots, the response at the center point yield for maximum ethanol production. From the study of the contour plots, the predicted values are obtained as follows; inoculum's size, pH and temperature.

3.6.2.4 Three-dimensional Surface plots

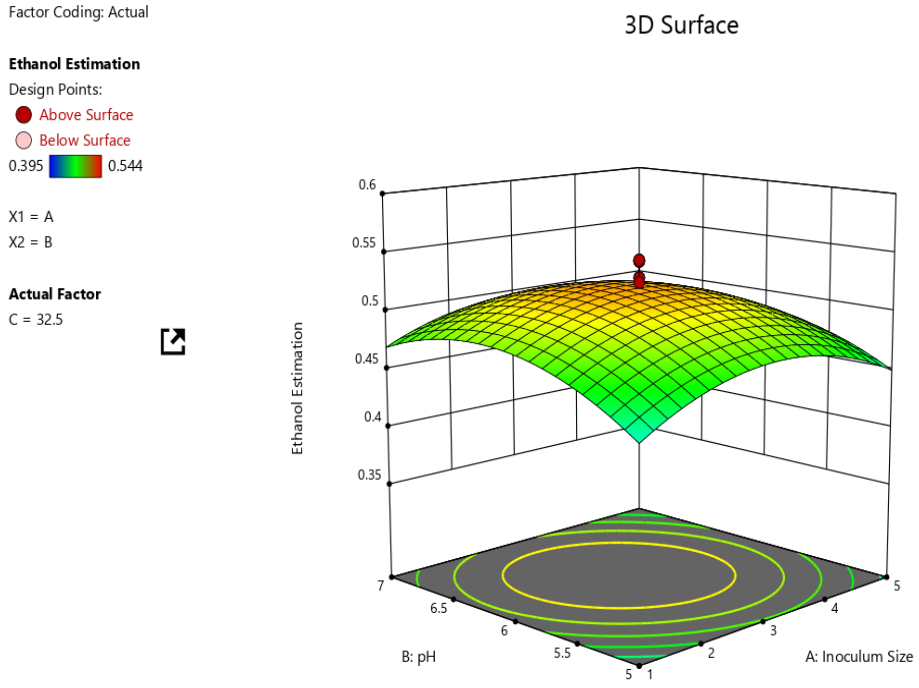


Fig. 10: A Effect of inoculum's size and pH.

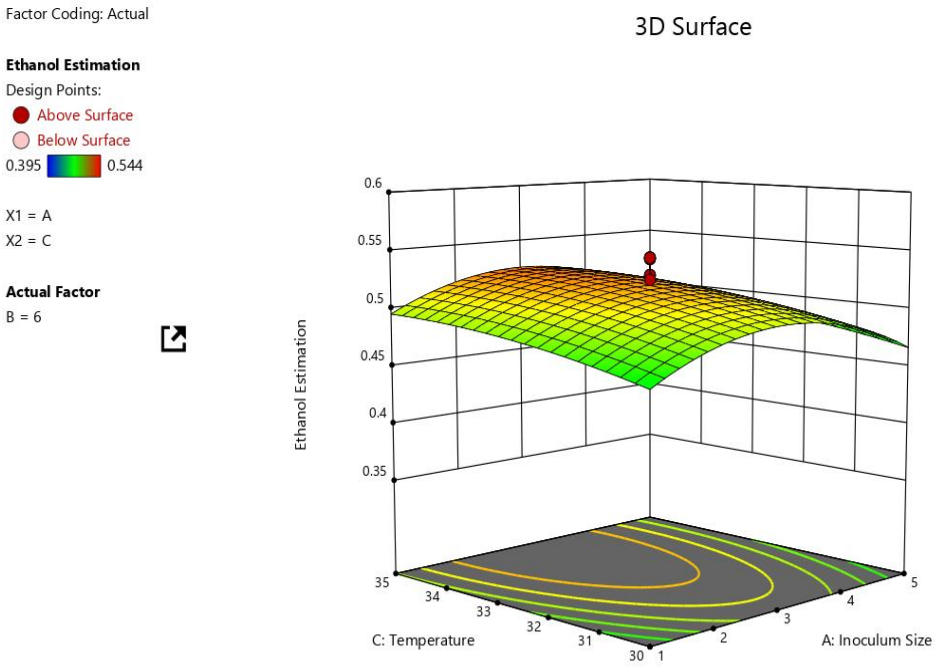


Fig. 10: B Effect of inoculum's size and temperature.

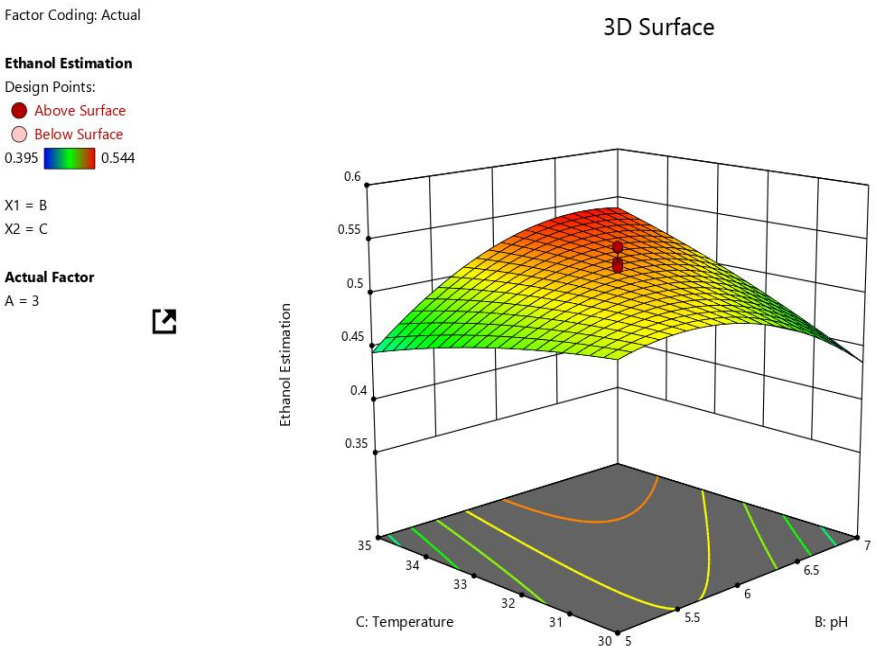


Fig. 10: C Effect of pH and temperature.

Fig 10 A, 10 B, 10 C were shown the ethanol estimation. Where A- Inoculum's size, B - pH and C - Temperature. The 3D response surface (Fig.10 A, B, C) were generally the graphical representations of the regression equation.

3.6.2.5 Point Prediction

Positions of the points are used to determine the quality of an estimation. The detailed estimation process shown in table 11.

Table 11: Point prediction.

Response	Predicted Mean	Predicted Median	Observed	Std Dev	SE Mean	95% CI low for Mean	95% CI high for Mean	95% TI low for 99% Pop	95% TI high for 99% Pop
Ethanol Estimation	0.52009	0.52009		0.0210984	0.00860492	0.500917	0.549264	0.417917	0.622264

Two-sided Confidence = 95% Population = 99%

3.6.2.6 Confirmation Location

Inoculum Size	pH	Temperature
4	6	42.5

Confirmation

Two-sided Confidence = 95%

Table 12: Confirmation.

Run 9 Response	Predicted Mean	Predicted Median	Observed	Std Dev	n	SE Pred	95% PI low	Data Mean	95% PI high
Ethanol Estimation	0.52009	0.52009	0.544	0.0210984	9.00	0.0111144	0.495428		0.544852

3.7 APPLICATIONS STUDIES

3.7.1 Combustion test

Commonly ethanol was used as a fuel additive in gasoline industry. Ethanol was blend with gasoline and used to run motor vehicle. This composition increase octane boost level in motor engine so it can be experimented by combustion test. Fig 11 shown the burning test for ethanol.

Fig. 11: Combustion test.

3.7.2 Comparative analysis of bioethanol and petrol

The comparative analysis of bioethanol and fossil petrol results were shown table 13.[31] compared to this article our properties of bioethanol were mostly equal. In this comparison bioethanol was added as fuel additive. 15% bioethanol (E15) was blend with gasoline.

Table 13: Comparative analysis of bioethanol Vs petrol.

S. No.	PROPERTIES	PETROL	BIOETHANOL
1	Density (kg/m^3)	**0.75**	**0.79**
2	Viscosity (mm^2/s)	0.6	1.5
3	Flash point (^{o}C)	<21	<21
4	Caloric value (MJ/I)	30.40	20.15
5	Octane number (RON)	85	87
6	Fuel equivalence (%)	98	63

4. CONCLUSION

The new technological development for processing of bioethanol is steadily increasing. Bioethanol is becoming more and more competitive to conventional fossil fuels. The use of all biofuel attributes a number of advantages suitable for achieving environmental, energy, agricultural and trade strategies. As a result, bioethanol is emanating as a fashionable step towards a most long-lasting transportation sector. Generally, bioethanol reduced greenhouse gas emissions, decreased air pollution and additionally decrease dependency from crude oil imports. In future bioethanol is becoming even more superior to conventional fossil fuels mostly due to development of feedstock manufacture and conversion processes.

LIST OF ABBREVIATIONS

ANOVA	Analysis of varience
CCD	Central Composite Design
CS	Cotton stalks
LB	Luria broth
OD	Optical density
OVAT	One variable at a time
PB	Plackett Burman
PPW	Potato peel wastes
PSSF	Pre hydrolysis and simultaneous saccharification and fermentation
RSM	Response surface methodology
S.cerevisiae	*Saccharomyces cerevisiae*
YEPD	Yeast Extract Peptone Dextrose

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