Optimization of Bioethanol Synthesis from Sugarcane Bagasse using *Saccharomyces Cerevisiae*

*** Offor-Emenike, I.U., [1] Ibekwe, V.I., [2] Akujobi, C., [2] and Braide, W.[2]**
1 Department of Biology, Alvan Ikoku Federal College of Education, Owerri, Nigeria.
2 Department of Microbiology Federal University of Technology Owerri, Nigeria.
* Corresponding author: ezeamakababe@gmail.com

Abstract: This study investigated the production of bioethanol from sugarcane bagasse in an optimized condition. Optimization of production medium helps to maximise metabolite yield. The capacity of *Saccharomyces cerevisiae* to ferment wort derived from sugarcane bagasse, an agricultural waste, in optimized conditions to produce bioethanol, was studied. A box-behnken design of five factors (substrate weight, temperature, inoculum size, pH, incubation time) and three levels was adopted to improve production efficiency. The substrate was subjected to physical and biological pretreatments to obtain simple sugars. Cellulase enzyme was used to breakdown the substrate to simpler sugars. Alcoholic fermentation was done using *S. cerevisiae* for six days. Brix content was measured before and during the fermentation process, as well as alcohol content after fermentation. Response surface plots of the factors were plotted. The results showed that brix value ranged from 2.3 ^{o}Bx to 3.9 ^{o}Bx while bioethanol production ranged from 1.38g/l to 2.35g/l. At optimal conditions of pH 6, temperature of 40^{o}C, inoculum size of 4, substrate weight of 10g and fermentation time of 72h, predicted ethanol yield will be 4.23g/l. Sugarcane bagasse is a good substrate for bioethanol production. 4.23g/l of bioethanol was realised with optimization of the fermentation medium.

Key words: Sugarcane bagasse, Bioethanol, Brix, Optimization, Response Surface Methodology.

INTRODUCTION

The impact of continuously burning non-renewable fossil fuel has been reduced rapidly by the use of alternative fuel. Bioethanol is generally used blended with gasoline, to reduce the usage of conventional fuel. This is currently used in existing motor engines (Liguori *et al*., 2015). Bioethanol is a liquid obtained from fermentation of sugar, which is gotten from plants containing carbohydrates (starch) (Renó *et al*., 2014). During the fermentation process, microorganisms are used as enzymes (Pejin *et al*., 2015). The following reasons make bioethanol widely used as a biofuel: bioethanol has high oxygen content and octane number; bioethanol is non-toxic; and bioethanol is environmentally friendly since it decreases pollutant emissions such as carbon monoxide, sulphur and nitrogen oxides (Hansdah *et al*., 2013 ; Wang *et al*., 2013). Although bioethanol is widely used as a biofuel, it has a number of disadvantages, one of them being that it has low vapour pressure compared to gasoline, making engine starts difficult at low temperatures. Bioethanol can also cause corrosion in engines (Balat, 2011; Küüt *et al*., 2011).

Bioethanol production from starch-based biomass depends on the type of feedstock and the steps involved in the bioethanol production process (Mahalaxmi and Williford, 2012). The production of bioethanol consists of five main steps namely, pre-treatment, hydrolysis, fermentation, distillation and dehydration (Sebayang *et al*., 2016). The hydrolysis process breaks down the carbohydrates in the feedstock into sugar by using enzymes (Yangcheng *et al*., 2013), and hydrolysis is often taken as the parameter of interest in the effort to improve bioethanol production. Due to global food security, first-generation bioethanol feedstocks such as corn, sweet potatoes, cassava and sugar cane have raised serious concerns, and this hinders worldwide acceptance of using bioethanol as fuel for compression-ignition engines. Hence, much effort is being made to produce bioethanol from non-edible feedstocks (Brunschwig *et al*., 2012; Ahmed *et al*., 2013; Yu *et al*., 2013).

Sugarcane bagasse is the waste that remains after the recovery of sugar juice through

crushing and extraction. Due to its well-known energy properties, it has been the principal fuel used around the world in the sugarcane agro-industry (Jenkins *et al*., 1998; Dermibas, 2004). It is an agricultural waste used to produce bioethanol using fermentation process. In Brazil, ethanol produced from the sugar in sugarcane is a popular fuel. Sugarcane bagasse is also used for the production of pulp, paper, board etc.

Response surface methodology (RSM) is a technique that is based on design of experiments. RSM is used to analyse the changes of the dependent variable (i.e. response variabes to changes in the independent variables, and it is also used to optimize the dependent variable. RSM has been widely used to optimize the process parameters for bioethanol and biodiesel production (Zhang *et al*., 2013; Ali *et al*., 2015; Dharma *et al*., 2016). RSM has been shown to be an efficient method to optimize every stage of the bioethanol production process, which significantly reduces time, costs and effort associated with conventional experimental techniques (Cheng *et al*., 2015).

Starch-to-glucose hydrolysis process have been widely studied and performed. The enzymatic and the acid hydrolysis process use conventional methods (Tasi´c *et al*., 2009; Naguleswaran *et al*., 2012; Meinita *et al*., 2015; Gumienna *et al*., 2016) Therefore, RSM optimization methods are used in the starch hydrolysis process into reducing sugar. This helps to obtain optimum conditions globally.

In this study, sugarcane bagasse was used as the feedstock to produce bioethanol using *Saccharomyces cerevisiae.* In addition, optimization of brix/reducing sugar was performed, with expectation of yielding the optimum quality of the produced reducing sugar. Response surface methodology based on the Box-Behnken experimental design was used to optimize the operating parameters of the hydrolysis processes for bioethanol production. Crude Fibre, ash, fat, crude protein, and carbohydrate content of the sugarcane bagasse was determined in triplicate according to the method of Copersucar (1989).

MATERIALS AND METHODS

Sample Collection and Processing

Large quantity of sugarcane was given to people to consume and the bagasse which served as substrate was collected. Processing of substrate was done according to the method of (Offor-Emenike *et al*., 2020). The substrate was dried for weeks and was separately grinded using a grinding machine and sieved using a mesh of 350mm to obtain fine powdered stock. This was labelled and stored at room temperature in transparent polyethylene bags. Proximate analysis of the sugarcane bagasse was done and the crude fibre, ash, fat, crude protein, and carbohydrate content were determined (Copersucar, 1989).

Isolation and preparation of Inoculum

S*accharomyces cerevisiae* was obtained from 33 Consolidated Breweries, Awo-Omanma, Imo State, Nigeria. The strain obtained was characterized to ascertain their microscopic and biochemical characteristics (Scholar and Benedikte, 1999; Suh *et* al., 2007). It was standardized using a spectrophotometer at wave length 600 (A_{600}) and MacFarland standards 3(9.0×10^8cfu/ml), 4(1.2×10^9cfu/ml) and 5(1.5×10^9cfu/ml) respectively.

Pretreatment of Sugarcane bagasse

Two stages of pretreatments were used:

(i) Heat treatment

Different weights (10g, 15g and 20g) of the substrate (sugarcane bagassse) were dissolved in 150 ml of deionized water in 46 separate Erlenmeyer flasks according to the design of experiment. After capping, the flasks were sterilized in batches in an autoclave at 121^{o}C for 15 mins, to convert the cellulose sources into sugary liquid. The samples were filtered using a filter bag (Yu and Zhang, 2004).

(ii) Enzymatic Hydrolysis

Starzyme (cellulose) enzyme with an activity of 5000u/g was dissolved in buffer solution and added to the flasks. The enzyme breaks down cellulose into dissolved sugars. This was allowed to stand for 24 hours. (Martin *et al*., 2002; Braide *et al*., 2016). After 48 hours of addition of enzyme, the contents of the flasks were autoclaved to stop the action of the enzymes (Yu and Zhang, 2004).

Design of Experiment for Optimization of Fermentation of Wort

Box-Behnken design was adopted for optimization dissolved sugars in a 5×3 design (that is, five factors in three levels) using Minitab 17. Substrate weight (10g, 15g and 20g); pH (6, 7 and 8); inoculum size (3, 4 and 5); temperature (30^oC, 35^oC and 40^oC) and incubation time (72h, 96h and 120h) were factored. This produced 46 runs, each comprising of different combinations of the factors as shown in Table 1.

Table 1: Interpretation from Experimental Design Table (Uncoded)

StdOrder	pH	Temp (^oC)	Time (hours)	Inoculum size (OD)	Substrate (grams)
1	6	30	96	4	15
2	8	30	96	4	15
3	6	40	96	4	15
4	8	40	96	4	15
5	7	35	72	3	15
6	7	35	120	3	15
7	7	35	72	5	15
8	7	35	120	5	15
9	7	30	96	4	10
10	7	40	96	4	10
11	7	30	96	4	20
12	7	40	96	4	20
13	6	35	72	4	15
14	8	35	72	4	15
15	6	35	120	4	15
16	8	35	120	4	15
17	7	35	96	3	10
18	7	35	96	5	10
19	7	35	96	3	20
20	7	35	96	5	20
21	7	30	72	4	15
22	7	40	72	4	15
23	7	30	120	4	15
24	7	40	120	4	15
25	6	35	96	3	15
26	8	35	96	3	15
27	6	35	96	5	15
28	8	35	96	5	15
29	7	35	72	4	10
30	7	35	120	4	10
31	7	35	72	4	20
32	7	35	120	4	20
33	6	35	96	4	10
34	8	35	96	4	10
35	6	35	96	4	20
36	8	35	96	4	20
37	7	30	96	3	15
38	7	40	96	3	15
39	7	30	96	5	15
40	7	40	96	5	15
41	7	35	96	4	15
42	7	35	96	4	15
43	7	35	96	4	15
44	7	35	96	4	15
45	7	35	96	4	15
46	7	35	96	4	15

Alcoholic Fermentation Process

One-tenth normality (0.1 N) of NaOH and 0.1 N H_2SO_4 were prepared (Haynes, 2011) and used to adjust the pH of the contents of the flasks to pH 6, 7 and 8 respectively to conform to design of experiment. Buffer solution was introduced to the flasks to maintain the respective pH. The contents of all the flasks were made up to a volume of 100ml each, to ensure uniform fermentation volume. According to the design of the experiment, 3, 4 and 5 MacFalands standards of the yeast (*Saccharomyces cerevisiae*) were aseptically introduced into the flasks. The content of the 46 flasks was allowed to ferment according to the parameters in the table of design (Abouzeid and Reddy, 1986). Fermentation was stopped after 72h, 96h or 120h respectively as defined by the design and brix/liquid mixture of the samples in the flasks was measured using the refractometer. The alcohol was determined by distillation method. This entire process was done in triplicate to avoid error.

Optimization of Parameters for Alcohol Production

Various concentrations of bioethanol produced under the specified conditions by each run were fed into Response Optimizer (Minitab 17) and used to derive optimal factors for maximum bioethanol production. Using the optimum, fermentation of substrate was carried out and resulting concentration of bioethanol was compared to the predicted value (Offor-Emenike *et al*., 2020).

RESULTS

Chemical composition of sugarcane bagasse

Sugarcane bagasse is a by-product of sugarcane industry composed of approximately 40-46% cellulose, 25-28% hemicullulose, 20-23% lignin, 2-6% ash, 1-2% protein, 1.4% glucose, 2.5% fat.

Table 2: Sugarcane bagasse composition

Component	Chemical composition (wt %)
Cellulose	46
Hemicelluloses (pentoses)	27
Lignin	23
Pectin	0
Protein	1.8
Fat	2.5
Glucose	1.4
Ash	4

Determination of brix and alcohol content

The highest yield of ethanol was 2.35g/l with brix value of 3.9 from flask 14 at conditions of pH 8, temperature 35^{o}C, fermentation time of 72h, inoculum size of 5 and substrate weight of 15g while the lowest yield was 1.37g/l with brix value of 2.3 from flask 19 at conditions of pH 7, temperature 35^{o}C, fermentation time of 96h, inoculum size of 6 and substrate weight of 20g. At optimal conditions, the predicted ethanol yield will be 4.23g/l. This is quite higher than the alcohol content of the other set up operated under different combination of parameter. These are shown in Figures 1 and 2.

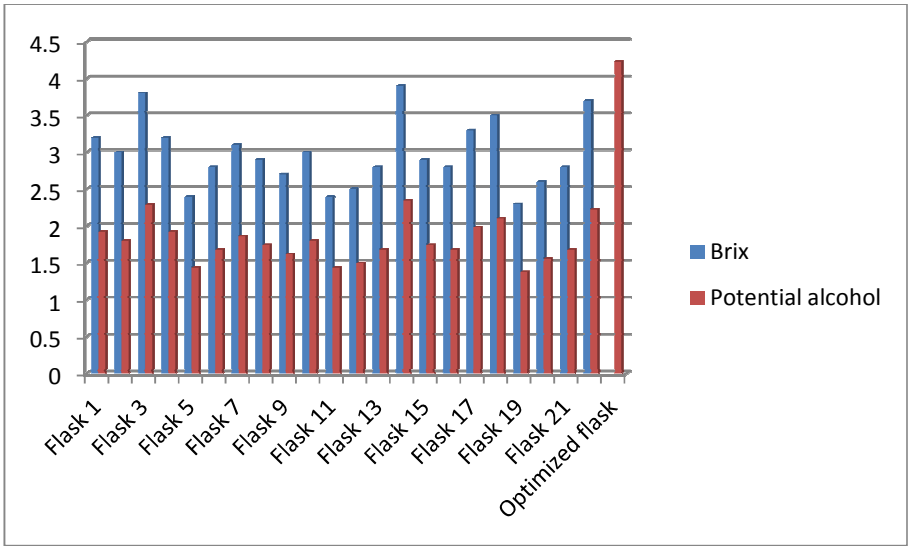


Fig 1: Brix and ethanol content of each flask

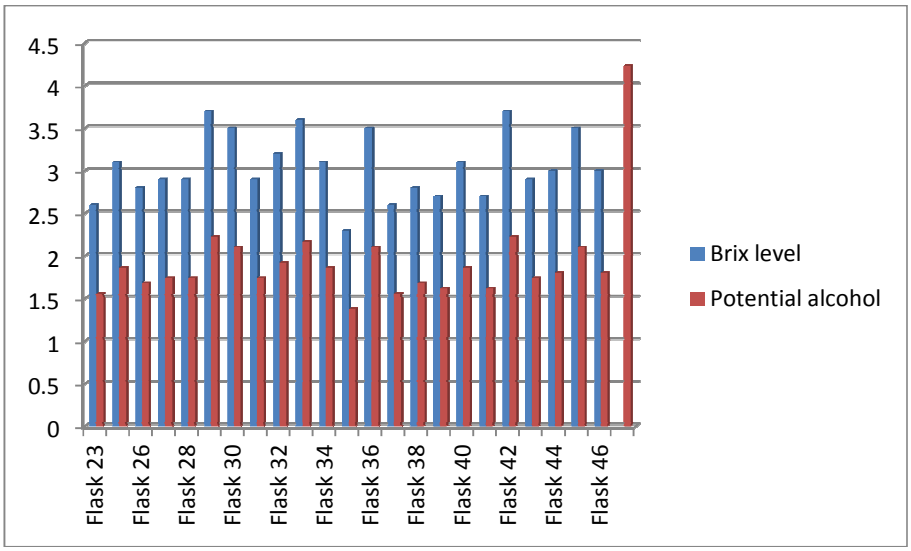


Fig 2: Brix and ethanol content of each flask

Main effect plot of the five factors indicated that at pH 6, response was almost 3.2. The yield dropped as the pH was increased to 7 but gave its maximum yield of 3.28 at pH 8. Temperature of 40^{o}C was seen as the best temperature for highest yield. Fermentation time of 72days, inoculum size of 4 and substrate weight of 10, gave the highest yield as shown in Figure 3.

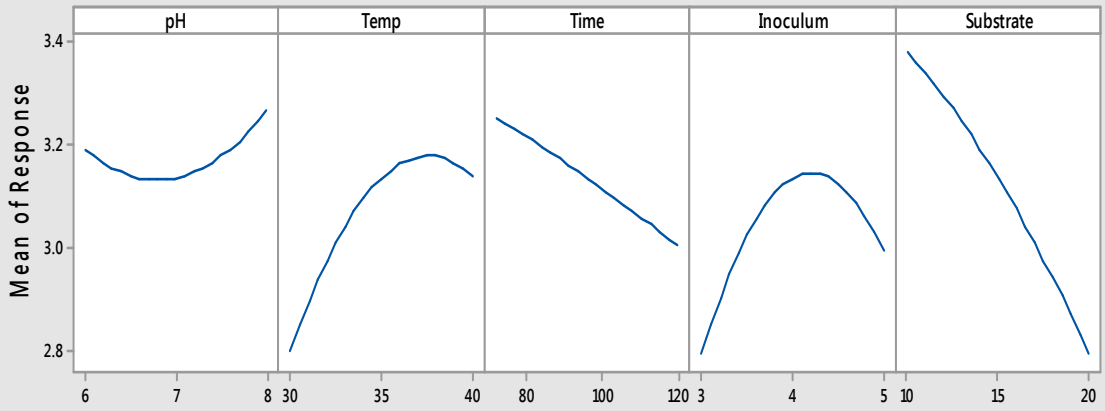
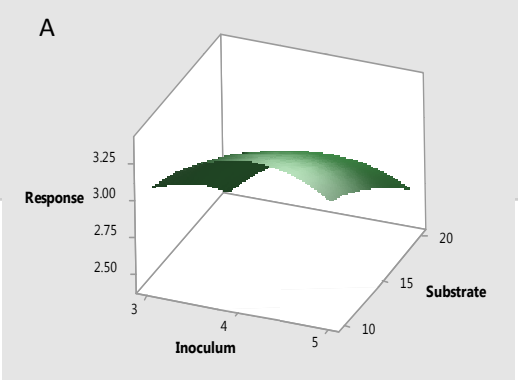
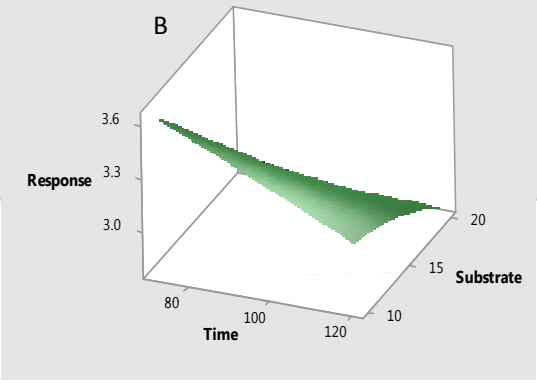
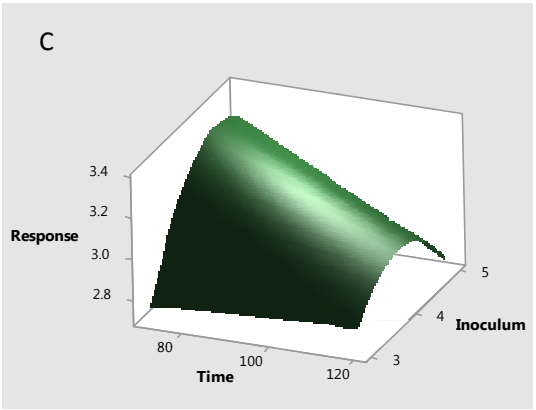


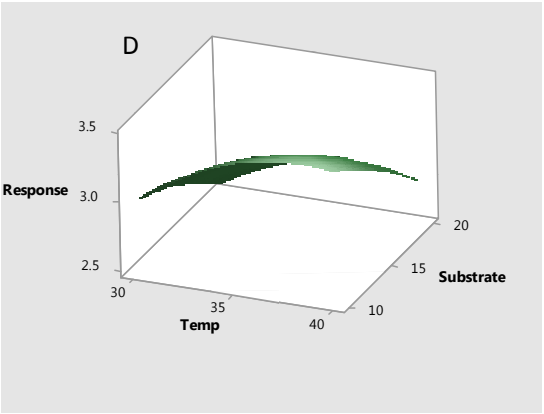
Fig 3: Main effect plot for carbohydrate (sugar) converted to ethanol

Response surface plots which showed the interactions between the factors that affected the production of bioethanol are shown in Figure 4.









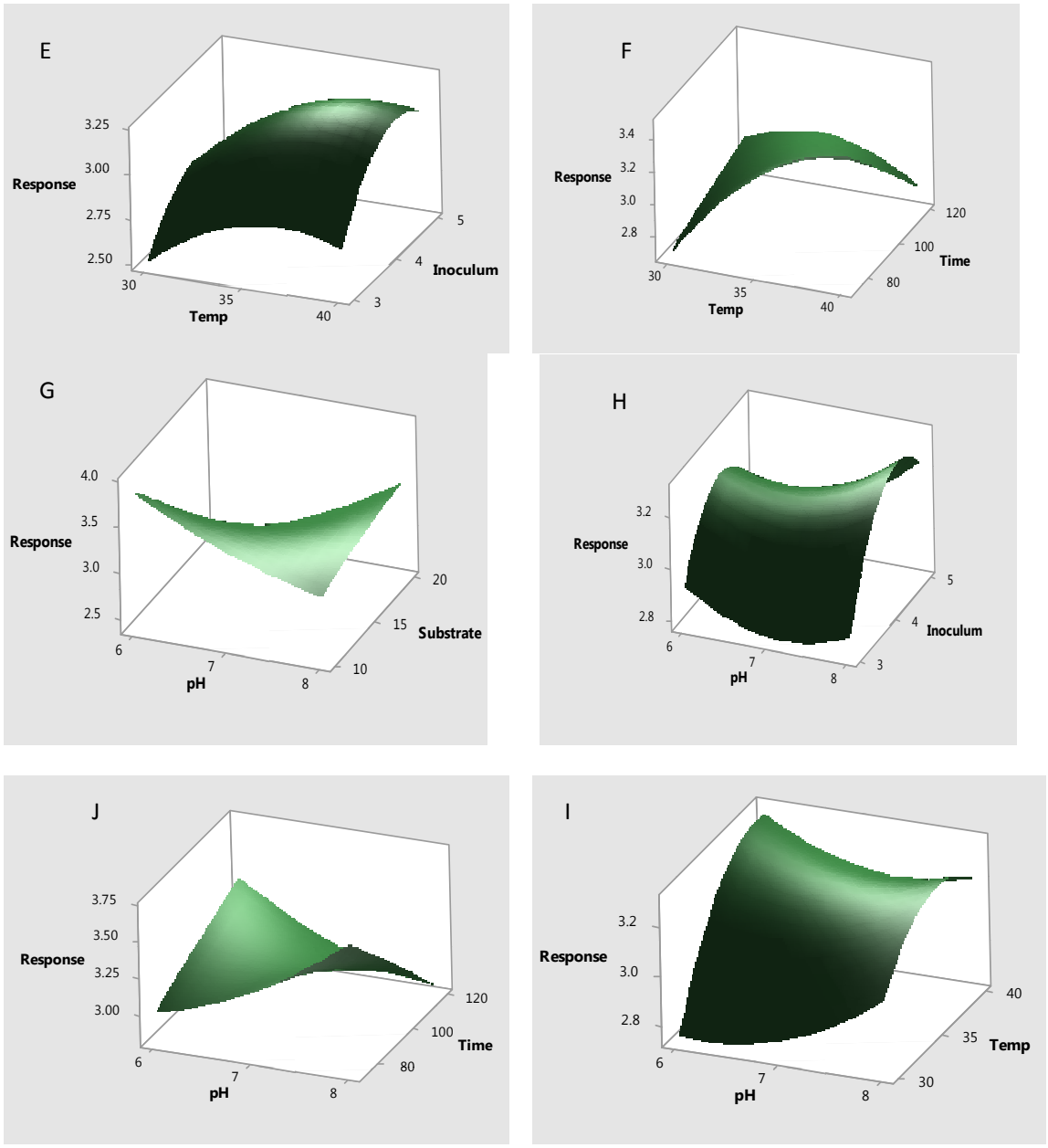


Fig 4 : Surface plots of carbohydrate converted.

(a) Against substrate weight and inoculum size
(b) Against substrate weight and time of fermentation
(c) Against inoculum size and time of fermentation
(d) Against substrate weight and temperature of fermentation
(e) Against inoculum size and temperature of fermentation
(f) Against time of fermentation and temperature of fermentation
(g) Against substrate weight and pH of fermentation
(h) Against inoculum size and pH of fermentation
(i) Against time of fermentation and pH of fermentation
(j) Against temperature of fermentation and pH of fermentation.

In (a), Inoculum size and substrate weight interacted, while pH, temperature and time were the hold values. Inoculum size of 4 and substrate weight of 10 gave the highest yield. Fermentation time and substrate weight interacted in (b) while inoculum size, pH and temperature were kept on hold. Highest yield was seen at fermentation time of 80h and substrate weight of 10. In (c), fermentation time and inoculum size interacted while substrate weight, pH and temperature were kept on hold. There was no significant increase in yield with increase in fermentation time; inoculum size of 4 gave the best yield. Substrate weight of 10 and temperature of 35^{o}C gave the highest yield in (d), while the inoculum size, fermentation time and pH kept as hold values. In (e) temperature and inoculum size interacted. As the temperature was increased from 30^{o}C to 35^{o}C, increased yield was seen. The inoculum size of 5 gave the highest yield. Inoculum size, pH and substrate weight were the factors kept on hold while fermentation time and temperature interacted in (f). Best yield was seen at fermentation time of 80h and temperature of 40^{o}C. In (g), Substrate weight of 20 and pH of 6 gave the highest yield when the two factors interacted, while keeping inoculum size, time and temperature on hold. The inoculum size and pH interaction in (h) showed that at pH of 6 and inoculum size of 5, maximum yield was realised, with temperature, time and substrate weight kept as hold values. In (i), Substrate weight, temperature and inoculum size were the hold values. The interaction between pH and fermentation time gave the best yield with pH of 8 and fermentation time at 80h. Temperature and pH were the interacting factors in (j) while the inoculum size, fermetation time and substrate weight were the hold values. At pH of 8 and temperature of 40^{o}C, the highest yield was realised.

There was increase in yield as the pH increased from 6 to 8. As the temperature was increased from 35^{o}C to 37^{o}C, the yield increased but dropped with further increase of the temperature to 40^{o}C. The yield decreased as the time of fermentation increased. Increase in the inoculum size from optical density 3 to optical density of 4 gave a high yield which dropped as the inoculum size was increased to optical density 5. Highest yield was seen at substrate weight of 10g. As the substrate weight was increased, the yield decreased.

The optimization plot shows that at pH 6, temperature of 40^{o}C, fermentation time of 72h, inoculum size of 4 and substrate weight of 10g, predicted ethanol yield will be 4.23g/l, as shown in Figure 5.

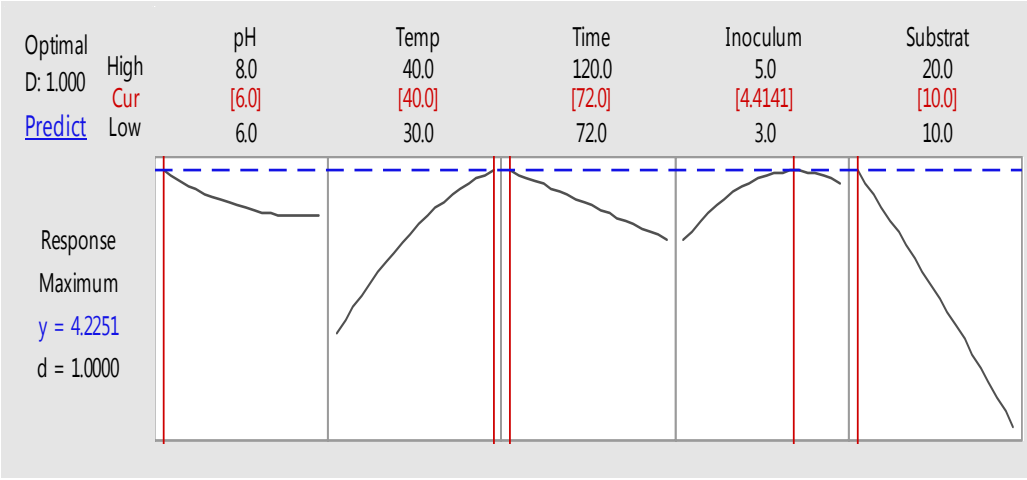


Fig 5: Optimization of Bioethanol Production

DISCUSSION

Optimization of Brix and production of bioethanol from agro wastes involve the pretreatment of the agro wastes to expose the simple sugars which the yeast can utilize to produce ethanol (Mahro and Timm, 2007; Sheoran *et al*., 1998). Fermentation is done by the yeast which converts the sugars in the substrates to ethanol (Wyman, 1996). *S. cerevisiae* have been used in alcohol production especially in wine making and in the brewing industries. Kasavi *et al*. (2012) reported that the microorganism gives a high ethanol yield at a low distillation cost and can withstand high ethanol concentration. Yeasts are used to generate fuel ethanol from renewable energy sources (Kosaric and Velikonja, 1995).

Results from the study showed that optimum temperature for alcohol production was 40^{o}C. Yah *et al*. (2010) reported that the optimum temperature of ethanol production is 25^{o}C. According to MarelneCot (2007), high temperature above 40^{o}C is not favourable for cells growth and it is a stress factor for microorganisms. Singh *et al*. (2013) recorded an ethanol concentration of 78.6 g/l at 30^{o}C. Ado *et al*. (2009) also observed a reduction in ethanol yield as temperature increased beyond 35^{o}C. Temperature is one of the major factors that determine ethanol production. Fermentation process requires a suitable temperature for the yeast to react (Rivera and Cardona, 2006). Temperature that is too high kills yeast, and low temperature slows down yeast activity.

Higher yield of ethanol was seen at fermentation time of almost 72h when compared with the yield at 96h and 120h. According to Zabed *et al*. (2014), when the fermentation time becomes too long, it gives toxic e□ect on microbial growth, especially in batch mode, due to the high concentration of ethanol in the fermented broth. Fermentation time has an effect on the growth of microorganisms. Brix conversion works with the fermentation time.

The result showed that 10g of the substrate gave the highest yield of brix conversion. The lowest yield was found with substrate weight of 20g. Zabed *et al*. (2014) stated that high ethanol productivity and yield in batch fermentation can be obtained by using higher initial sugar concentration; the maximum rate of ethanol production is achieved when using sugars at the concentration of 150 g/L. However, it needs longer fermentation time and higher recovery cost. High substrate loading for industrial fermentation is feasible and hence always desired (Nagodawithana *et al*., 1974).

Although inoculum size of 4 gave a higher yield compared with inoculum size of 5, increase in the inoculum size did not really have a great effect in the yield of brix converted. This result corroborates with the work of Laopaiboon (2007), which reported that inoculum concentration does not give significant effect on the final ethanol concentration but it affects the consumption rate of sugar and ethanol productivity.

From the result, highest yield of ethanol was obtained at an alkaline pH of 6. This agrees with the work of Ganigue *et al*. (2016) who reported that when ethanol is continuously produced from the glucose fermenting culture, other acids like carbonic acid and acetic acid are continuously generated making the system more acidic and low pH could trigger the production of ethanol.

CONCLUSION

In conclusion, sugarcane bagasse which causes nuisance to the environment, was converted to bioethanol. From the response surface plots, the maximum ethanol yield was 2.29g/l but with the optimal conditions of pH 6, temperature of 40^{o}C, inoculum size of 4 substrate weight of 10g and fermentation time of 72h, maximum ethanol yield of 4.23g/l was predicted.

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