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Review Article

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Effects of Process Variables on the Fermentation of Corn Stover: A Review

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Abstract Bioethanol production involves the fermentation of feedstock from raw lignocellulosic biomass to chemical fuel via biological routes. Response Surface Methodology (RSM) was considered as a tool to represent the optimization of ethanol production as a function of the fermentation independent variables *i.e.* fermentation temperature and time, pH of the hydrolysate and yeast concentration (*Saccharomyces cerevisiae*), in a batch fermentation. The effect of fermentation independent variables and their combined interactions on the production of bioethanol by *Saccharomyces cerevisiae* was evaluated to improve the bioethanol fermentation performance. The interactions of the fermentation independent variables using RSM indicated that the highest yield could be reached near the center point of the operating conditions. The range of temperatures, time and pH were established to optimize the fermentation condition by RSM which could save experiment times and cost.

Keywords Bioethanol, Fermentation, Hydrolysate, Biomass, *Saccharomyces cerevisiae*, Response Surface Methodology (RSM)

Introduction

Bioethanol production by fermentation is one of the popular subjects in the world with regards to the biological environment and economic challenges. It involves fermentation, a complex process intensely studied in many bioprocesses [1]. In fact, bioethanol feedstock is produced from biomass which depends on solar energy for converting simple raw lignocellulosic materials to chemical fuel via biological routes [2]. In this observation, knowing the optimum condition and estimation of bioethanol production from glucose can be very useful in industrial applications as the main goals in the present work. Since any kind of raw materials as carbon sources at first, must be converted to glucose and then ethanol fermentation is performed. Fermentation process has both the nonlinear and dynamic properties. Modeling such process is difficult and challenging [1]. Considerable attempts have been made by several researchers to propose a methodology based on mathematical models [3]. A way of dealing with such problem is to use simple and efficient models like response surface methodology (RSM). Modeling and optimization to enhance the efficiency of a process are the most significant stages in a biochemical process [4]. The conventional one-factor-at-a-time approach of optimization is not only tedious but as well ignores the combined interaction of each factor [5]. One of the most common optimization methods used in the last two decades is the Response Surface Methodology (RSM). RSM is a statistical technique based on the essential principles of statistics, randomization and duplication, which makes the optimization easier by studying the reciprocal interactions among the variables over a wide range of values in a statistically logical manner [6]. Thus, RSM is an effective approach to deal with a large number of variables and there are various reports on the application of RSM for fermentation [7].

This review, considers RSM as a tool to represent the optimization of ethanol production as a function of the fermentation independent variables i.e. fermentation temperature and time, pH of the hydrolysate and yeast

concentration (*Saccharomyces cerevisiae*), in a batch fermentation. The effect of temperature, time, pH value and yeast concentration and their combined interactions on the production of ethanol by *Saccharomyces cerevisiae* was evaluated to improve the ethanol fermentation performance.

Response Surface Methodology (RSM): Response Surface and Contour Plots of Interactions

One of the best models to describe the biological processes in analytical methods is quadratic model in comparison with linear and cubic models. RSM is a frequently useful technique for modeling and determining the optimal process conditions. In order to determine the optimal levels of each variable for maximum ethanol production, response contour plots, were constructed by plotting the responses (bioethanol concentration) on the Z-axis versus the two independent variables on the X-axis and Y-axis respectively, while other variables were kept at their optimal levels, which was useful for understanding both the main and the interaction effects of these two factors. The response surface can be used to predict the optimum range for the different experimental variables, and the main interactions between the experimental variables can also be identified from the circular or elliptical nature of the contours. The circular nature of the contours imply that the interactive effects between the experimental variables are not significant and optimum values of the experimental variables can be easily obtained with regards to the center point of the contour plots [8].

Optimization of Bioethanol Fermentation by RSM from Various Biomass Sources and Microorganisms

Literatures about optimization and modeling of bioethanol fermentation by RSM from various biomass sources and microorganisms as function of different parameters such as temperature, pH, and fermentation time and inoculums size were discussed. Ezhumalai and Thangavelu [9] had investigated optimization of incubation temperature (25 –45 °C), pH (5–7) and fermentation time (24–120 h) using RSM and ANN in bioconversion of steam pretreated sugarcane bagasse into ethanol by cellulase and thermotolerant yeast *Kluyveromyces marxianus var. marxianus* MTCC 3013. They have reported that the optimum values of temperature, pH and fermentation time were 39 °C, 5.7 and 110 h, respectively. At optimum conditions, they have achieved a maximum ethanol concentration of 5.89 g/l from 50 g/l pretreated sugarcane bagasse in aerobic batch fermentation. Also, in another investigation Ezhumalai and Thangavelu [9], studied on bioconversion of lignocellulosic material such as pretreated sugarcane bagasse into ethanol by cellulase and *Candida wickerhamii* MTCC 3013 based on CCD experiments. Optimum condition were obtained at temperature of 33^{o}C, pH of 5.7 and fermentation time of 104 h. Maximum bioethanol concentration at optimum condition was 4.28 g/l from 50 g/l pretreated sugarcane bagasse in aerobic batch fermentation. Beside, Yan *et al.* [10] have assessed the optimization of the alcoholic fermentation of blueberry juice by AS 2.316 *S. cerevisiae* wine yeast. Through statistically designed optimization, the optimal condition of alcoholic fermentation were found to be temperature of 22.65 °C, pH value of 3.53 and inoculums size of 7.37 %. At the optimal condition, the production of ethanol and volatile acid of blueberry wine had achieved up to 7.63 % and 0.34 g/l, respectively. High ethanol concentration (47 g/l) for an immobilized cell reactor (ICR), using high substrate concentration (150 g/l) has been reported in the literature as the enhanced ICR system was continuous [11].

After hydrolysis, the hydrolysates at optimized conditions from previous study were used for fermentation using *Saccharomyces cerevisiae*. The effects of fermentation time, fermentation temperature, and pH on the ethanol yield were studied below.

Effects of Optimize Fermentation Conditions on Bioethanol Yield

Effect of pH on Fermentation and Ethanol Yield

pH is one of the important factors that affect the bioethanol production through SHF (separate hydrolysis and fermentation). The rate of ethanol production by yeast cells is highly affected by the pH of the fermentation medium. The acidic condition hinders the growth of harmful bacteria and enhances yeast growth [12-13]. However, more acidic and basic conditions retard the yeast metabolic pathways and the growth of the cells [12]. So, optimum pH is required for growth of the yeast and ethanol yield. Lin *et* al., [14] report shows the results of the batch test used to investigate the effect of pH on ethanol production. When the pH was lower than 4.0, the incubation time for maximum ethanol concentration was prolonged, but the maximum concentration was not

very low. When the pH value was above 5.0, the quantity of ethanol produced substantially decreased. Therefore a pH range of 4.0-5.0 may be regarded as the operational limit for the anaerobic ethanol production process.

Previous studies showed that high ethanol production was obtained using pH of 5.0 to 6.0. It was also shown that no ethanol production exists lower than pH of 4.0 [15]. Optimum pH for *S. cerevisiae* BY4742 was in the range of 4.0–5.0 [14]; when the pH was lower than 4.0, the incubation period was prolonged though the ethanol concentration was not reduced significantly and when the pH was above 5.0, the concentration of ethanol diminished substantially. Unlikely, pH of 3.5 was optimal for ethanol production by *S. cerevisiae* ITV-01 at 30^{o}C with initial glucose concentration of 150 g/L [16]. A wide range of optimum pH (4.0–8.0) was reported for *S. cerevisiae* JZ1C isolated from rhizosphere of Jerusalem artichoke using inulin and Jerusalem artichoke tuber as substrate at 35 ^{o}C [17].

Currently, stillage (a waste after ethanol production) is commonly reused for yeast substrate to make the ethanol production more efficient; however, stillage contains more organic acids than expected. The organic acids present in the stillage elongated the ethanol fermentation time [18]; ethanol fermentation from cassava mash using *S. cerevisiae* was more inhibited by propionic acid as medium pH decreased, undissociated acid being the effective inhibitory form, whereas glycerol production decreased as propionic acid increased irrespective of solids in cassava mash and pH condition. The plasma membrane allows the easy entrance of undissociated acids, dissociating intracellular and thus cytoplasm could be acidified. At the same time, the proton must be transported by membrane ATPase to maintain intracellular pH and thus it results in increased ATP consumption and decreased biomass yield [18]. The above discussion shows that different acids produced by the yeast or added exogenously created optimum pH or unfavourable pH range for the *S. cerevisiae*. On the other hand, different investigations proved that yeast uses organic acids as a substrate. *S. cerevisiae* NAM34-4C grew rapidly and produced ethanol (2.7 g/L) in YPDL (10, yeast extract; 20, peptone; and 20, D-lactic acid g/L) medium at pH of 3.5 and temperature 35 ^{o}C [19]. Similarly, the volatile acidity from acidic white wine was efficiently reduced by *S. cerevisiae* S26 when the acetic acid and ethanol concentration were kept below 1.0 g/L and 11% (v/v), respectively [20].

Effect of Fermentation Time on Ethanol Yield

Previous works revealed that, the ethanol yield increased gradually by increasing the incubation time and reaching its maximum after 60-72 hrs and dramatically decreased with further extension of time [21-22]. For general ethanol production by yeast, the maximum fermentation time in a batch process was 72 hrs [23].

Verma *et al*., [24] studied the effects of four different fermentation periods *viz*., 24, 48, 72 and 96 hours on ethanol production from starch medium. A maximum ethanol concentration of 24.8 g L at 48 hours was achieved as compared to 13.7 and 21.6 g/L at 24 and 96 hours respectively. Marakis and Marakis [25] studied the effects of 6 different fermentation period *viz*., 0, 24, 48, 72, 90 and 100 hours on ethanol production from aqueous carob extract and achieved maximum alcohol concentration of 4.75% (v/v) at 100 hours of fermentation period. Hence, the optimum fermentation time for bioethanol production is usually based on the biomass and kind of substrate used.

Effect of Temperature on Fermentation and Ethanol Yield

Competition during ethanol fermentation carried out at different temperatures may be a way of testing the endurance of the strain used in this system. This could then be used as a method for determining the optimal condition for ethanol fermentation and also a criterion for rapidly selecting one of several strains while at the same time studying resistance to temperature in a controlled situation, i.e. under laboratory conditions. It is commonly believed that 20-35 ^{o}C is the ideal range for fermentation and at higher temperatures almost all fermentation would be problematic [23, 26-27].

Nimbkar *et al*., [28] also studied the effect of three different incubation temperatures *viz*., 25, 30 and 35 °C, on the ethanol production from unsterilized juice of sweet sorghum with *Saccharomyces cerevisiae* and obtained maximum alcohol of 12.45 % (v/v) at 30 °C. Araquue *et al*., [29] studied the bioethanol production at higher temperatures, wherein yeast cells dies, resulting in a decrease in alcohol yield when the pulp was concentrated,

while optimal temperatures for maximal productivity occurs at 32 °C. It was therefore, necessary to select the optimal temperature at which yeast strains can ferment the sugars from lignocellulosic material. It can also be that between 25 °C and 30 °C, the sugars were used up faster than at 20 °C and 40 °C.

The effect of temperature on bioethanol production was also studied by Duhan *et al.*, [30] and obtained the maximum bioethanol production at 35 ^{o}C. Temperatures between 30-35 ^{o}C has been usually employed for culturing of yeast and temperatures above 35 ^{o}C has been found inhibitory to ethanol fermentation due to yeast growth inhibition at higher temperatures [13]. When the temperature is too high the yeast is destroyed, on the other hand, yeast activity decreases at lower temperatures [31]. Further, the increasing temperature reduced the percentage of ethanol production and it is mainly due to denaturation of the yeast cells [32]. Temperature greatly affects the enzymatic activity and membrane turgidity of yeast cells and yeasts which are active and tolerant at high temperature are ideal for industrial bioethanol production. *S. cerevisiae* ITV-01 yeast, isolated from sugar cane molasses, was found to produce more ethanol (58.4 g/L) optimally at 30 ^{o}C with pH of 3.5 [16].

In the other study, 30–40 ^{o}C was optimal for *S. cerevisiae* BY4742; higher temperature shortened the exponential phase of the yeast cell [14]. Ethanol production reduced considerably at 50 ^{o}C and this might be due to change in transport system which might increase accumulation of toxin including ethanol in the cell [14]. In addition, enzymes and ribosome denaturation and membrane fluidity problems might be brought in by higher temperatures. Though 30–35 ^{o}C was best for yeast strain fermentation, *S. cerevisiae* JZ1C inulinases function efficiently at the temperature range between 40 and 50 ^{o}C [17]. Therefore, the yeast should be active and tolerant at higher temperature to produce ethanol using inulin as a carbon source. In another study, ethanol production decreased when the temperature was raised to 30 ^{o}C using alkali pretreated palm fruit bench fiber under fed-batch SSF condition [33]; uneconomical ethanol was produced at 37 ^{o}C and above.

Effect of Inoculum Size (Yeast Concentration) on Fermentation and Ethanol Yield

Lower inoculum size reduces cost of production in ethanol fermentation. For instance, 5 % (v/v) and 12 hrs old inoculum sizes yielded almost the same result with 10 % using *S. cerevisiae* Y5 in enzymatic hydrolysate of non-detoxified steam-exploded corn stover supplemented with corn steep liquor (CSL) [34]. Ethanol productivity by baker yeast decreased as yeast concentration increased from 3 to 4 and 5 g/L in coffee husk based substrate [35]. However, 10% (v/v) *S. cerevisiae* TISTR 5596 was used to produce high ethanol using waste from cassava starch production without nitrogen source supplementation [36]. Nimbkar *et al*., [28] studied the effect of different sunflower head waste inoculum size *viz*., 2, 4, 6, 8 and 10 % on the ethanol production from unspecialized juice of sweet sorghum and obtained maximum alcohol concentration of 12.45 and 12.23 % (v/v) at inoculum sizes of 6 and 2 % respectively.

The effect of inoculum size on ethanol yield was studied by Laluce, *et al.*, [37] using response surface methodology and it was found that raised ethanol yields were obtained with high inoculum size. The ethanol production was raised from 1.29 to 2.35 g/L/h when the yeast load increased from 0.5 to 5 g/L by shortening the lag phase in fed-batch separate saccharification and fermentation (SSF) process, though the study did not report on the effect of yeast loading greater than 5 g/L yeast [33].

Interactions of the Various Combined Fermentation Variable Factors versus Bioethanol Yield

According to a research carried out by Ohimor *et al.*, [38] on modeling and optimization of bioethanol production process from cornstover, using the RSM, the interactions of any two of the the various fermentation variables versus bioethanol yield can be shown and interpreted by the 3-D response surface and 2-D contour plots below:

The response surface and contour plots (Fig. 1) shows the interactions between fermentation temperature and fermentation time on bioethanol yield. Bioethanol yield increased with increase in fermentation temperature and fermentation time respectively. Thus, the bioethanol yield is favoured by the interactions between fermentation temperature and fermentation time. Lin *et al.*, [14] demonstrated that when temperature increased, the maximum fermentation time was shortened, but a much higher temperature inhibited the growth of cells and then the fermentation process significantly declined. In this study, cell growth and ethanol production declined considerably at 50^{o}C, which showed the inhibitory effect on cell growth at higher temperatures. This

phenomenon may be explained thus; the higher temperature results in changing the transport activity or saturation level of soluble compounds and solvents in the cells, which might increase the accumulation of toxins including ethanol inside the cells. Moreover, the indirect effect of high temperature might also be ascribed to the denaturation of ribosomes and enzymes and problems with the fluidity of membranes [23, 39].

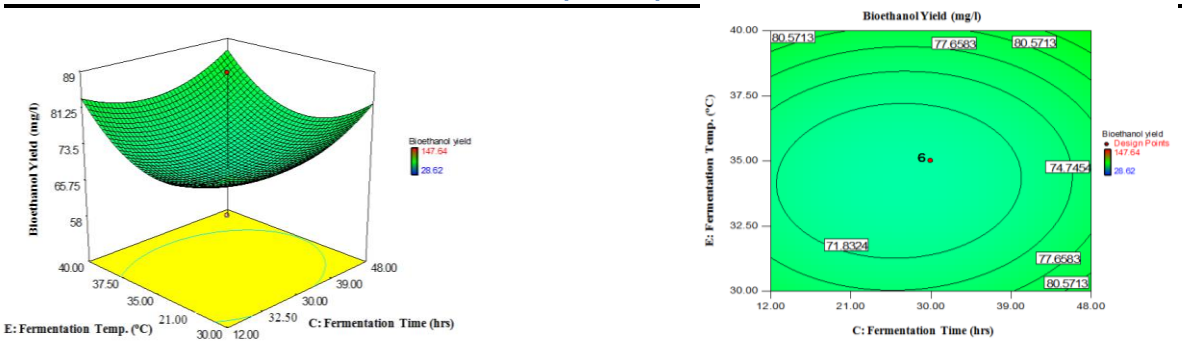

Figure 1: 3-D response surface and 2-D contour plots of bioethanol yield showing interactions with fermentation temperature and fermentation time for concentration of yeast and pH of 6.00g/l and 6.50 respectively

The response surface and contour plots (Fig. 2) shows the interactions between concentration of yeast and fermentation time on bioethanol yield. Bioethanol yield increased with increase in concentration of yeast and fermentation time respectively. Thus, the bioethanol yield is favoured by the interactions between concentration of yeast and fermentation time such that a clear maximum point for bioethanol yield can be attained at higher values of concentration of yeast and fermentation time.

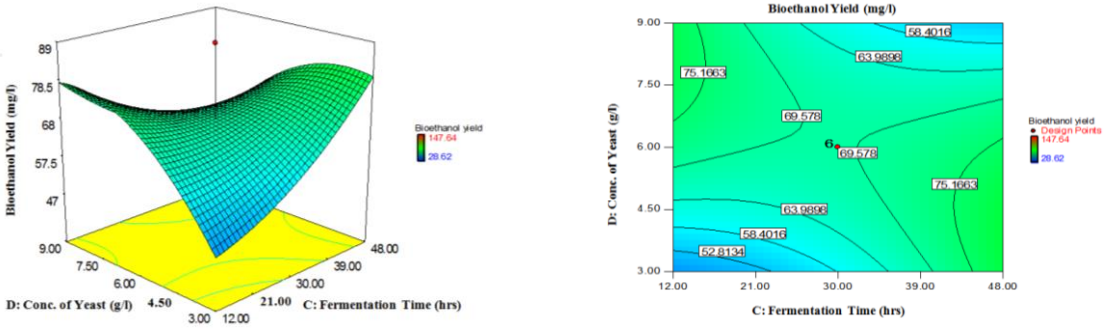

Figure 2: 3-D response surface and 2-D contour plots of bioethanol yield showing interactions with concentration of yeast and fermentation time for fermentation temperature and pH of 35.00 ^{o}C and 6.50 respectively

The response surface and contour plots of pH of hydrolysate sample and fermentation time versus bioethanol yield (Fig. 3) show the dependency of bioethanol yield on pH of hydrolysate sample and fermentation time. Bioethanol yield increased with increasing pH of hydrolysate sample but the corresponding increase is small for increasing fermentation time. Thus, the bioethanol yield is favoured by the interactions between pH of hydrolysate sample and fermentation time such that a clear maximum point for bioethanol yield can be attained at higher values of pH of hydrolysate sample and fermentation time. The effects of fermentation time and pH on ethanol content were studied by Chongkhong *et al*, [40] where the ethanol yield increased with an increase in pH and fermentation time but, a higher pH from 5.9 to 6.7 caused a reduction in the yield.

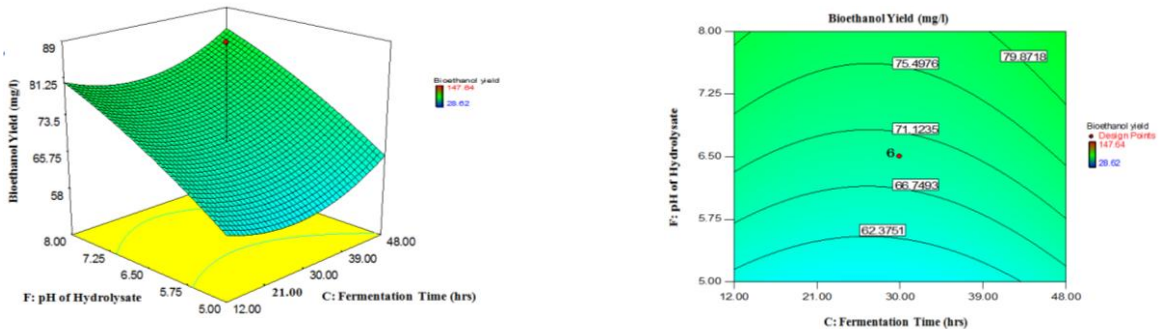

Figure 3: 3-D response surface and 2-D contour plots of bioethanol yield showing interactions with pH of hydolysate and fermentation time for concentration of yeast and fermentation temperature of 6.00g/l and 35.00 ^{o}C respectively

The response surface and contour plots of fermentation temperature and concentration of yeast versus bioethanol yield (Fig.4) show the dependency of bioethanol yield on fermentation temperature and concentration of yeast. Two regions of inflexions were noticeable, resulting in bioethanol yield increase with decreasing fermentation temperature and concentration of yeast respectively in one region. However, at the other region, the bioethanol yield increased with increasing fermentation temperature and concentration of yeast respectively. Lin *et al,* [14] reported that ethanol concentration rose steadily at low temperatures and won't decline within 168 h, possibly because at these lower temperatures the yeast was not active which is because of the low tolerance to ethanol. However, at lower temperatures the cells showed lower specific growth rates which may be attributed to their low tolerance to ethanol at lower temperatures [41-42].

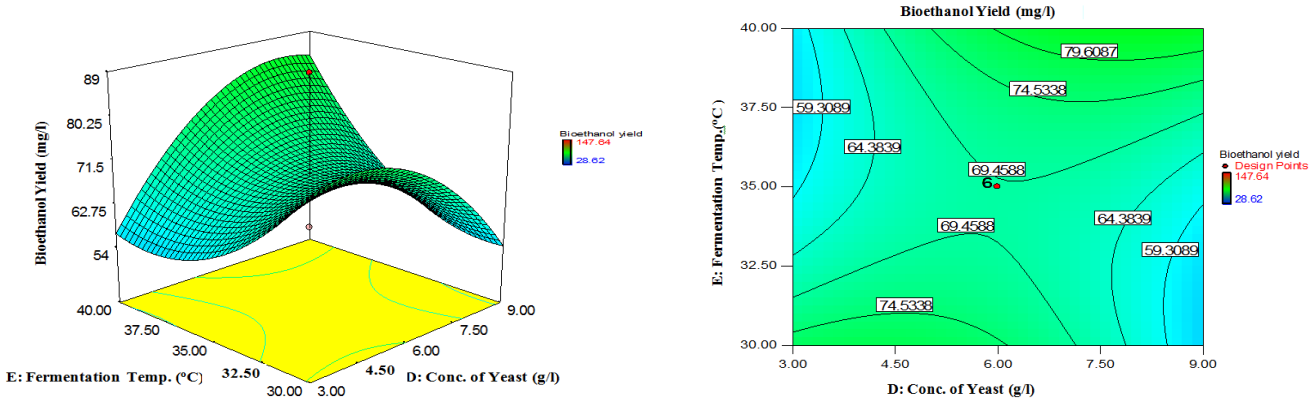

Figure 4: 3-D response surface and 2-D contour plots of bioethanol yield showing interactions with fermentation temperature and concentration for fermentation time and pH of 30.00hrs and 6.50 respectively

The response surface and contour plots of pH of hydrolysate sample and concentration of yeast versus bioethanol yield (Fig.5) show the dependency of bioethanol yield on pH of hydrolysate sample and concentration of yeast. Bioethanol yield increased with increasing pH of hydrolysate sample but the corresponding increase is small for increasing concentration of yeast. Thus, the bioethanol yield is favoured by the interactions between pH of hydrolysate sample and concentration of yeast such that a clear maximum point for bioethanol yield can be attained at pH of 7.0 - 8.0 of hydrolysate sample and concentration of yeast of 4.5 – 6.5 g/l.

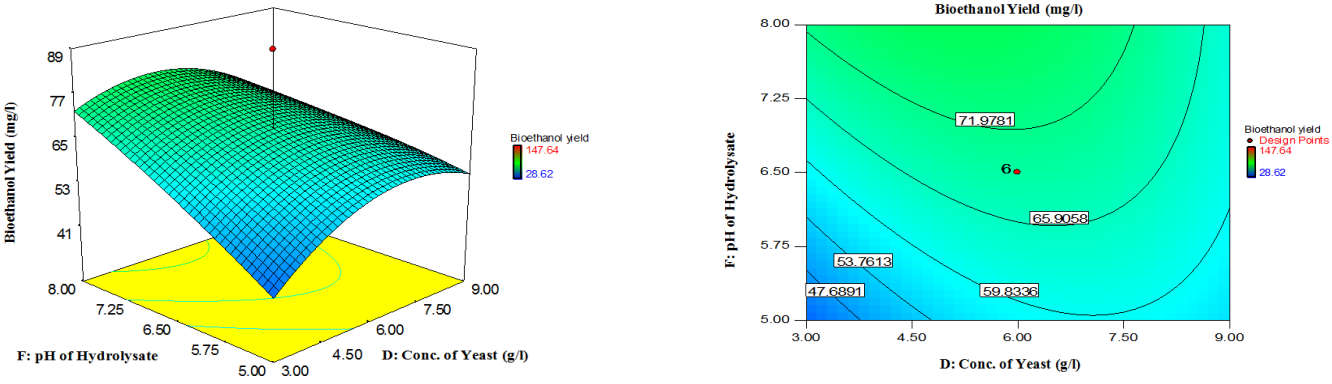

Figure 5: 3-D response surface and 2-D contour plots of bioethanol yield showing interactions with pH and concentration of yeast for fermentation time and fermentation temperature of 30.00hrs and 35.00 ^{o}C respectively

The response surface and contour plots of pH of hydrolysate sample and fermentation temperature versus bioethanol yield (Fig. 6) show the dependency of bioethanol yield on pH of hydrolysate sample and fermentation temperature. Bioethanol yield increased with increasing pH of hydrolysate sample and fermentation temperature respectively. Thus, the bioethanol yield is favoured by the interactions between pH of hydrolysate sample and fermentation temperature such that a clear maximum point for bioethanol yield can be attained at pH of 7.0-8.0 of hydrolysate sample and fermentation temperature of 35-40 °C. The effects of pH and temperature on ethanol content was studied by Chongkhong *et al.*, [40] and obtained an increased ethanol production with increasing temperature and pH in the range of 27 to 36 °C and pH of 4.4-5.9 but, the conversion rates were reduced for a further increase in temperature and pH value.

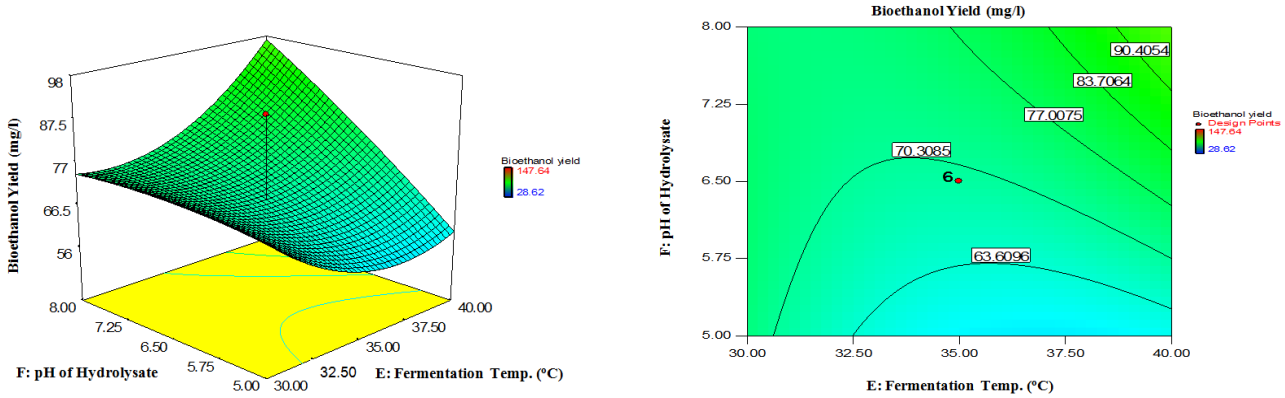

Figure 6: 3-D response surface and 2-D contour plots of bioethanol yield showing interactions with pH and fermentation temperature for fermentation time and concentration of yeast of 30.00hrs and 6.00g/l respectively

Conclusion

The figures from the RSM study, show that the four independent fermentation variables; fermentation temperature, fermentation time, yeast concentration and pH of the hydrolysate has effects on the response variable, bioethanol yield. Thus, the study of the interactions between the fermentation variables is to establish the optimum fermentation condition for effective bioethanol production in order to save experimental time and cost.

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