Original Research Article

Optimisation for production of Bioethanol by zymomonas mobilis and saccharomyces cerevisiae using starch substrate from corn

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Abstract

The current hike in the gas prices, oil prices and depletion of fossil fuels leads bioethanol to become a new attractive arrival. Compare to diesel and petrol Bioethanol is Biodegradable, Less Toxic and Less Explosive. The main agenda is optimization of Temperature, pH, Inoculum level and fermentation period for bioethanol production from corn kernels using substrate starch.This study shows that agricultural waste that contains fermentable sugar can no longer be thrown off but is converted into useful products like bioethanol. Microorganisms are able to convert into ethanol and ample attempts are there to genetically engineer good ethanol producing microorganisms. Bioethanol from corn cob waste were produced according to the following stages pretreatment, hydrolysis, and fermentation. Other experimental variables were Temperature, inoculum, fermentation duration's sugar, ethanol and pH values. This experiment has led to the optimization of fermentation parameters for maximum yield from fermentating agent *S.Cerevisiae* and *Z.mobilis*.

Key Words: Bioethanol, corn cob waste, starch, optimization.

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INTRODUCTION

Fermentation is the process by which large organic molecules are broken down into simpler molecules as the result of the activity of microorganisms. Biofuel are a wide range of fuels which are derived from biomass. Energy requirement and environmental pollution are the two major challenges today. Ethanol is a clear liquid alcohol that is made by the fermentation of different biological materials. Fermentation ethanol process based on starch or sugar based feedstock such as corn, potato and sugarcane was being used to meet the demand for ethanol as a fuel. Additionally, the ethanol from biomass based waste materials was considered as bioethanol (sivasakthivelan *et al*-2014). Currently, there is growing interest for ecologically sustainable biofuels. Bioethanol is one of the famous biofuels. Ethanol fermentated from renewable sources for fuel or fuel additives are known as bioethanol. Additionally the ethanol from biomass based waste material is considered as bioethanol. Bioethanol produced from corn uses only a small part of the plant material, whereby only the starch from the kernel is transformed into bioethanol (cao *et al*-1986). The selection of microorganism is one of the important factors for the production as it should be able to withstand the osmotic pressure and tolerance to ethanol. Bioethanol production is by fermentation using the *saccharomyces cerevisiae* with high ethanol yields from starch based substrates and with *Zymomonas mobilis* with lower risk of contamination, increased bioconversion rates and product recovery. The present investigation was undertaken to optimize various fermentation parameters and finally to study ethanol production from the hydrolysate of substrate from corn kernel. Bioethanol is renewable source of energy and in addition ethanol

derived from biomass is the only liquid transportation fuel that does not contribute to the greenhouse gas effect. This reduction of greenhouse gas emission is the main advantage of utilizing biomass conversion into ethanol.

MATERIALS AND METHODS

There are a number of advanced technologies of bioethanol production in the world presently, depending on the raw material subjected to fermentation. Starchy materials required reaction of starch with water (hydrolysis) to break down the starch into fermentable sugars (saccharification). Bioconversion of biomass to ethanol requires four main stages, pretreatment, hydrolysis (saccharification), fermentation and product separation/ distillation.

A] Milling: Thoroughly dried corn kernels were milled in a flour to obtain a coarsely milled powder. The
initial step in ethanol production using dry-grind technology is to reduce the particle size of corn by grinding it. Particle size of the grain can affect ethanol yield (Kelsall and Lyons, 1999), and therefore, ethanol producers tend to use finelyground corn to maximize ethanol yield.

B] Liquefaction: In a 250 ml conical flask, 20 g of corn flour of mill is added along with 100 ml of distilled water. This slurry is cooked, also known as "liquefaction." It is cooked at 121 0C and 15 psi for 30 min in an autoclave. The heat and mechanical shear of the cooking process break apart the starch granules present in the kernel endosperm, and the enzymes break down the starch polymer into small fragments. The gelatinized corn is then allowed to cool down followed by the addition of alpha amylase (1:2000 and pH-5.5-6.0). The flask is maintained at the stirrer for 2 hours at 90 0C and at 150 rpm. (A. Meenakshi *et al*-2014). Sample was withdrawn from the flask at 1 hr interval and analyzed for reducing sugar by the DNSA method to determine the enzyme concentration on gelatinization and liquefaction.

C] Saccharification: After liquefaction, the slurry, is called "mash," is cooled to around 30°C, and a second enzyme
Glucoamylase (38.5 U/mg) is added. Glucoamylase completes the breakdown of the starch into simple sugar (glucose). This step, called "saccharification," often occurs while the mash is filling the fermentor in preparation for the next step (fermentation) and continues throughout the next step. Glucoamylase activity was determined by using glucose oxidase/peroxidase method.

D] Fermentation: The fermentation broth (100ml) comprising of (% w/v), Peptone, 8g; yeast extract, 8g; and the product of the saccharification of corn starch as the fermentating sugar (Orji Jerry *et al*-2016).

The bottles was filled into a 100ml sealable bottle, sterilized in an autoclave and inoculated S. cerevisiae and Z. mobilis. The bottles were sealed with the aid of an adhesive tape and incubated at 30 0C for 96 –120 Hrs. At intermittent intervals of 0, 24, 48, 96 and 120 hrs., the amount of ethanol produced and the residual sugar in the medium was determined.

E] Distillation: Ethanol boils at lower temperature than water does, the ethanol can be seperated by the process of distillation. The entire content is distilled using Fractional Distillation Assembly for the recovery of ethanol produced. Ethanol which evaporated at 78 0C was condensed and collected and the measured. The distillate is collected and quantity of ethanol concentration is determined.

Optimization of Fermentation process: Parameter optimisation is important in fermentation process carried out by Saccharomyces cerevisiae and Zymomonas mobilis and is known to vary with respect to pH, temperature, substrates, its concentrations and cell density etc. It is therefore imperative to optimize the fermentation conditions for starch hydrolysate was used as substrate and yeast and bacterial cells as fermentating microorganisms so that the production efficiency is increased. (Udhayraj *et al*-2012).

Effect of Fermentation Period: Fermentation time was optimized by putting various tests, which contain the fermentation medium, at 30^{o}C from 1 day to 5 days and was further analyzed for ethanol production using corn kernels as substrate with *S. Cerevisiae and Z. mobilis*.

Effect of Temperature on Ethanol Production: *S. Cerevisiae and Z. mobilis* was inoculated to fermentation medium, pH was adjusted to 5.5 for S. Cerevisiae and pH 6.0 was adjusted for Z. mobilis. It is incubated at different temperatures
viz., 25˚, 30˚, 35˚, 40˚ C. for 5 days. After fermentation, the samples were analyzed for ethanol yield.

Effect Of pH on Ethanol Production: The pH of the hydrolysate was adjusted to 4.5, 5.0, 5.5, 6.0 and 6.5 was adjusted by using 1 N
HCl or 1 N NaOH. The yeast culture of *Saccharomyces cerevisiae and Zymomonas mobilis* was inoculated and fermentation was carried up to 5 days at 30ºC for both *saccharomyces* and *Zymomonas* respectively. The sample was analyzed for ethanol yield. The pH has profound effect on fermentation. The increase in pH inhibits the growth of *S. Cerevisiae and Z. mobilis* as they grow well on acidic pH. Risk of bacterial contamination is lessen at low pH. Yeast has tendency to produce acids at high pH rather than alcohols.

Effect of inoculum size on Ethanol Production: Microorganisms are the major agents aiding in the field of fermentation, these microbes utilize the sugar content of

the substrate and thus produces the ethanol. Lower inoculum size reduces the cost of production in ethanol fermentation. These microbes when taken in a varying quantity (%) play a vital role in this process. The starch hydrolysate extract (100ml of pH 5.5 and 6.0) was inoculated with 0.2, 0.4, 0.6, 8.0 and 10.0 % of inoculums levels of yeast *Saccharomyces cerevisiae* and bacterial *Zymomonas mobilis* cultures and keeping factor temperature and pH stable for 5 days and thereafter samples were analyzed for unfermented residual sugar and ethanol yield.

RESULT AND DISCUSSION

An important issue for the efficient ethanol production is to optimize the fermentation step regarding following main parameters temperature, pH, media composition, inoculum level, Elimination of infection etc. In the present study, corn cobs and corn kernels was selected to find out its suitability for alcohol production to get maximum yield of ethanol, a viable technology has to be developed.

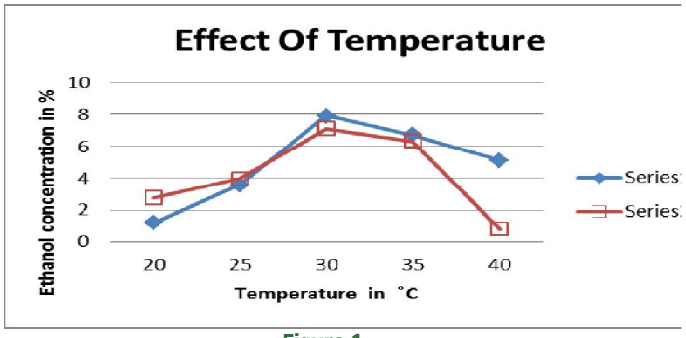
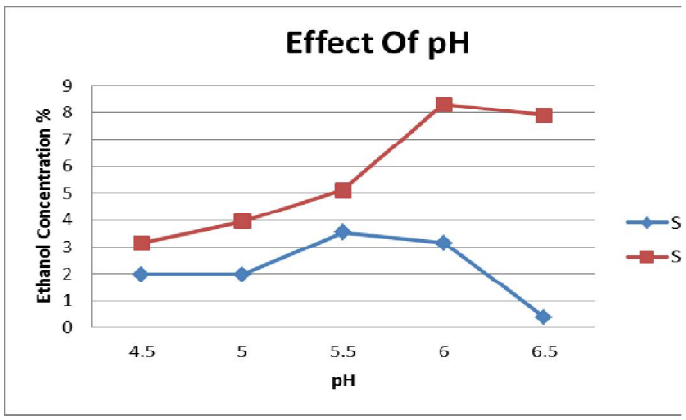


Figure 1:



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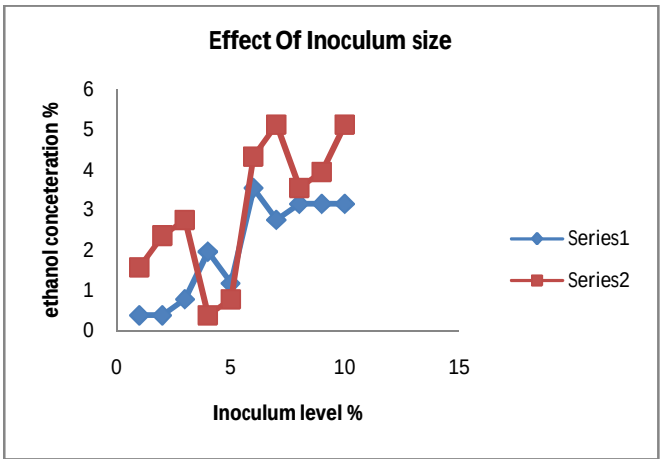

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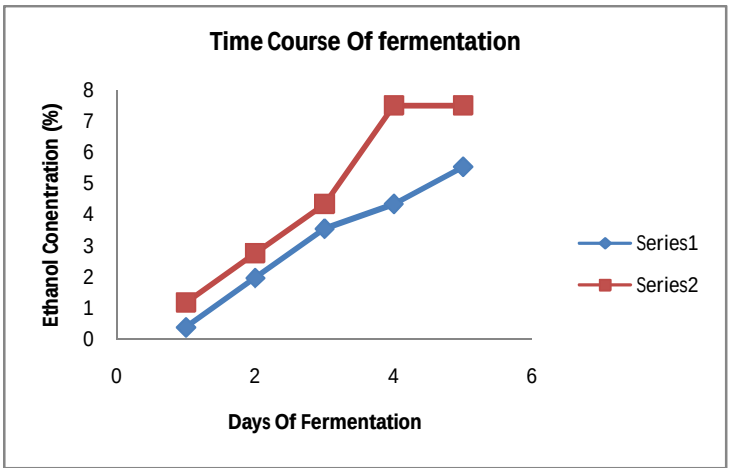

Figure 4: Saccharomyces cerevisiae – Series1, Zymomonas mobilis – Series2

Optimisation of temperature Fig (A) was carried out by incubating the fermentation flask at 20, 25, 30, 35 and 40˚ C. The Ethanol yield increased due to the increasing temperature from 20-30˚C with the inoculation of both *Z. mobilis and S. cerevisiae*. Beyond this temperature, the ethanol content decreased significantly for 35 and 40˚ C. In *Z. mobilis* and *S. cerevisiae* there was initial increase in ethanol concentration with temperature increase from 20˚ C to 30˚ C and beyond 30˚ C increasing temperature became inhibitory to ethanol production, The decreased was more at the 40˚ C. *Z. mobilis* and *S. cerevisiae* produced maximum amount of ethanol at 30˚ C and further increase in temperature was inhibitory to its ethanol production ability. The highest concentration of ethanol was produced at temperature of 30˚ C for *S. cerevisiae (7.90%) and Z. mobilis* (7.11%). The lowest concentration is at 20˚C for S. cerevisiae (1.18%) and 40˚ C for *Zymomonas mobilis*. The concentration of ethanol produced is 7. 90% v/v and 7.11% v/v for S. *cerevisiae and Z. mobilis* respectively is same at 30˚C for both the

organism and was optimized for further study. Optimization of pH Fig (B) was done at pH values 4.5, 5, 5.5, 6, 6.5 with both *S. cerevisiae* and *Z. mobilis*. The Ethanol yield significantly influenced by different pH as shown in the above fig B. There is an increase in the concentration of ethanol with increase in pH from 4.5 to 6 for Z. *mobilis* and 4.5 to 5.5 for *S. cerevisiae* and there found a decrease in the production with the increase in pH. In the case of *Z. mobilis* ethanol concentration was highest at the pH 6 and lowest at pH 4.5 and for the *S. cerevisiae* concentration was highest at pH5.5 and lowest at pH 6.5. Considering all the experimental cases for pH *Z. mobilis* was higher compared to *S. cerevisiae* for ethanol production. Hence pH5.5 for *S. cerevisiae* and pH 6 for *Z. mobilis* was optimized for this studies of ethanol production. The effect of inoculum level (from 1%-10%) Fig (C) for the maximization of ethanol yield was studied in the present study and the results were showed in the figure above. The ethanol yield was maximum at 6% inoculum level for *S. cerevisiae* with 3.55% (v/v) of ethanol production followed by 8%, 9%, 10%. Minimum ethanol yield was observed in 1% inoculum level. Whereas for *Z. mobilis* maximum ethanol production was seen at 7% and 10% of the inoculum level with 5.13 % v/v which was followed by 6%, 8% and 9% and minimum ethanol yield was observed at 4% of inoculum level with 0.39 % v/v of ethanol produced. Hence inoculum level of 6% was chosen for *S. cerevisiae* and 7% for *Z. mobilis* was optimized. The flasks were incubated for fermentation for 1, 2, 3, 4, 5 days. Fig (D) *S. Cerevisiae* was observed to give maximum ethanol production on 5th day with 5.53% and *Z. mobilis* gives on 4th day of fermentation period with 7.50 % of ethanol production. The concentration of ethanol produced at each fermentation time analysed with *Z. mobilis* was higher compared to that of S. *cerevisiae*. There was slight increase in each consecutive days in the production of ethanol. Hence 4th day for *Z. mobilis* and 5th day for *S. cerevisiae* is optimized for production of bioethanol. P. Sivasktivelan in his studies showed that optimum temperature was 30℃ and pH was 6.5, inoculum size was 10% for 48 hours. for maximum bioethanol production for *Zymomonas mobilis*. The optimum pH 5.5 temperature 30˚C, inoculum size was 8% for bioethanol production from *S. cerevisiae*. Umamaheshwari and Jayakumari in their research showed pH 5.5, and temperature at 35℃ for ethanol production. Clarence s yah in his studies of temperature optimization from corn cobs using mixed yeast strains found the optimum time and temperature for bioethanol production *S. Cerevisiae* and p. stipites strains found to be at 50 hours and 25℃. Meenakshi and Kumaresan in there research of ethanol production from corn potato peel waste has optimized pH 5.5 and inoculum level 10 % for bioethanol production. Above analysis is found to be significant where ($p<0.05$) with one way ANOVA.

Table 1:

Groups	Count	Sum	Average	Variance
Temperature	10	0.62	0.062	0.006262
pH	10	0.28	0.028	0.001307
Inoculum size	10	20.51	2.051	1.590277

Table 2:

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	26.83269	2	13.41634	25.18956	0.0000007	3.354131
Within Groups	14.38061	27	0.532615			
Total	**41.2133**	**29**				

CONCLUSION

Bioethanol is produced from corn kernels using *Saccharomyces cerevisiae* and *Zymomonas mobilis*. Studies reveal that corn starch is good substrate for this Ethanogeneic microrganisms for utilizing substrate with enzyme activity of amylase and Glucoamylase and thereafter optimizing the parameters for ethanol production for *Saccharomyces cerevisiae* was found to be temperature 30℃, pH5.5 6%of inoculum level with 5 days of fermentation period. Optimum parameters for *Zymomonas mobilis* are pH 6, temperature 30˚ C, inoculum level 7 % with 4 days of fermentation period and was found to be significant($p<0.05$). *Zymomonas mobilis* was found to produce higher ethanol concentration and greater efficiency as compared to *saccharomyces cerevisiae.*

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