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Potential of corn cobs a lignocellulosic waste as an emerging source for bioethanol production

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Abstract : *In the present study simultaneous saccharification and fermentation is carried out in which the fixed and complex carbohydrate present in the corn cobs are first hydrolysed to fermentable sugars by acidic and enzymatic methods and then sugars are subsequently converted to ethanol. The locally isolated strains as compared to Saccharomyces cereviseae were found to be efficient in producing bioethanol from waste materials. As the overall process utilizes locally isolated strains and waste as starting material, therefore, it may be helpful in economical production of bioethanol.*

Key words : *Renewable, lignocellulosic, 'corn cobs', bioethanol.*

Introduction :

Ethanol made from biomass provides unique environmental, economic, strategic benefits and can be considered as a safe and cleanest liquid fuel alternative to fossil fuels. There is a copious amount of lignocellulosic biomass worldwide that can be exploited for fuel ethanol production. Significant advances have been made at bench scale towards the fuel ethanol generation from lignocellulosics. However, there are still technical and economical hurdles, which make the bio ethanol program unsuccessful at commercial scale.

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The cost of ethanol production from ligno-cellulosic waste is relatively high. Based on the current technologies, the main challenges are the low yield and high cost of the hydrolysis process. Considerable research efforts have been made to improve the hydrolysis of lignocellulosic waste by various pre treatment processes, one of which is ammonia steeping to release lignin and hemicelluloses and enhance hydrolysis efficiently to a significant level. Further, among all the available technologies, dilute acid hydrolysis followed by enzymatic hydrolysis by less expensive and more efficient cellulases has been found more promising towards the potential economic and environmental impact. Simultaneous saccharification & fermentation effectively removes the glucose which is an inhibitor to the cellulase activity thus increasing the yield and rate of cellulose hydrolysis. Simultaneous research studies have been carried out on the production of bioethanol from corn cobs through simultaneous saccharification & fermentation of lignocellulosic agricultural waste by *Kluveromyces marxianus 6556* (Zheng et al., 2009) using *Aspergillus niger* and *Saccharomyces cereviseae* in simultaneous saccharification and fermentation (Zakpaa et al., 2009) and from lignocellulosic biomass (Kumar et al., 2009). Furthermore, apart from being used as a bio fuel the bioethanol can serve for a wide variety of applications such as an industrial solvent, chemical sterilant, industrial reagent and is environmentally friendly.

This study aims to isolate local strains from easily accessible agricultural and market waste and includes both cellulose degrading bacteria & fermenting yeasts. Consequently, the analysis of efficiency of locally isolated strains from cheaper and easily accessible wastes against that of the dignified i.e, commercial brewer's yeast strain namely *Saccharomyces cerevisae*.

Materials & Methods :

Isolation and screening of cellulose degrading bacteria from soil associated with vegetable waste:

One cellulose degrading strain of actinomycete was selected as the working strain from the strains isolated from different wastes. Soil sample was collected from an area associated with vegetable waste. Serial Dilution was done upto 10^{-7} dilution. Normal saline (0.875g NaCl + 100ml distilled water) was prepared, autoclaved (9.9ml in one vial + 9ml in six vials each) 0.1g of the soil waste was added into vial with 9.9ml of saline. Further, under aseptic conditions serial dilutions were prepared by sterile pipette transfer of 1ml into subsequent vials from each preceeding vial (9ml each). After each transfer the vials were properly vortexed to ensure adequate mixing of suspension. The diluted waste soil sample was inoculated on Carboxy Methyl Cellulose Agar medium by using spread plate technique under aseptic conditions and incubated at 37°C temperature for optimum growth. Colony characteristics were analyzed and gram staining was done for their characterization as bacteria. Apart from bacteria certain mixed fungal contaminants were observed which could not be characterized. The suspected isolated colonies identified as bacteria were transferred to Nutrient Agar Slants. Isolated strains were tested for cellulose degrading activity by Congo-red test.

Isolation of Strains and screening subsequent from fruit market waste:

The fruit pulp & peel wastes were collected from various fruit juice vendors. Normal saline was

prepared. 0.1g of the waste sample was weighed and added to 9.9ml of autoclaved normal saline & vortexed. Further, Serial dilutions were prepared upto 10^{-6} dilution. The diluted waste soil sample was inoculated on Yeast Extract Potato Dextrose media by using spread plate technique under aseptic conditions and incubated at 26°C temperature for 24-48 hours until growth was observed. Colonies were observed by staining technique. The isolated colonies were transferred to YEPD Slants. Isolated strains were tested for fermenting activity & gas production by carbohydrate fementation broth in test tubes with Durham tubes.

Sample collection & processing:

The 'corn cobs' were collected as a fresh by product after use. The corn cobs were sun-dried and grounded to powder form with the help of 'Willey grinder' (Courtesy: Animal Nutrition Laboratory, Patna Veterinary College).

Pre treatment of ligno-cellulosic waste (Ammonia steeping):

20g dried and milled corn cobs were taken in a 250ml Erlenmeyer flask and 100ml 3M NH_4OH solution was mixed with it. The mouth of the flask was covered and sealed with an aluminium foil so as to prevent escape of ammonia. This mixture was then incubated for 24 hours in a shaker-incubator at 30°C. The content was then filtered using a Whatman no.1 filter-paper into another 250ml Erlenmeyer flask. This was further rinsed twice with distilled water. The corn cobs were then dried at a temperature of 35°C in an oven overnight.

Acid hydrolysis:

The dried corn cobs were then weighed again on an electronic balance and delignified by the process of acid hydrolysis. This was done by treating the corn cobs with 0.3M HCl solution in a 500ml Erlenmeyer flask at 121°C for 1hr in an autoclave. The amount of HCl added to the dried biomass was in the ratio of 1:10 w/v. The acidic hemi-cellulosic hydrolyzate (filtrate) was then obtained by re-filtration with a Whatman no.1 filter paper which was neutralized by using 0.5M NaOH solution and tested with the help of a pH indicator strip for proper neutralization. The pre-treated cellulosic residue was rinsed & washed thoroughly with distilled water to remove residual acid.

Enzymatic hydrolysis:

The washed cellulosic residue was taken in a 250ml Erlenmeyer flask and to it 100ml of sterile distilled water was added and this mixture was inoculated with 5ml of nutrient broth culture of cellulose degrading bacterial strain under aseptic conditions. This was then incubated at optimum temperature of 37°C for about a week in an incubator. The growth & cellulose degrading activity was tested by visual analysis of changes in the incubated flask and confirmed by testing a small amount of the filtrate with Congo red and sodium chloride treatment. The samples from the flasks with positive results were filtered with a Whatman no.1 filter paper. The cellulosic hydrolyzate was obtained as the filtrate and transferred into a clean and autoclaved 500ml Erlenmeyer flask with autoclaved cotton plugs.

Bioethanol fermentation :

25 ml each of hemicellulose and cellulose hydrolyzate was mixed , inoculated with 5ml of yeast broth culture in a 500ml flask covered with cotton

plug to allow fermentation aerobically. 10 such flasks were set up each with 8 different strains including one flask with *Saccharomyces cereviseae* and one uninoculated kept as a control. Samples were then incubated at ambient temperature for 48-72 hours (*Cao et al, 1996).* After the incubation at optimum conditions for 24hours, the tests for detection of ethanol were performed after every 24hours interval, and finally a residual sugar test was also performed by spectrophotometric analysis initially, after 24hours and 48hours.

Tests for detection of bio ethanol:

These included sensorial analysis of characteristic smell of ethanol, sodium metal test, esterification reaction tests.In the Sodium metal test, in a test tube a little amount of fermentation broth to be tested was taken and to it a pinch of sodium metal was added. The production of effervescence was indicative of positive test. In case of an esterification reaction, a little of glacial acetic acid and concentrated H_2SO_4 was taken -and to it a little of the test sample was added in a clean test tube. This was covered properly with an aluminium foil and gently warmed on a water bath. The production of a fruity smell after a while is indicative of ester formation.ie, presence of ethanol (positive test).

Initial & Residual sugar test:

The residual sugar tests were performed by spectrophotometric analysis by anthrone method. Standards of glucose were prepared in the concentrations ranging from 100µg/ml to 1000µg/ml. The absorbance was taken at 620nm. A standard curve was plotted for concentration against the optical density (O.D.) or absorbance. The absorbance of the hydrolyzates were taken after hydrolysis and sample O.D. were then taken after fermentation and the corresponding concentration values were obtained from the standard graph. This gave the residual sugar in the fermented sample. The difference between initial and final sugar concentrations gave the amount of sugar fermented which gives an estimate of ethanol produced.

Standards of glucose concentrations in the range from 100 µg/ml-1000 µg/ml were prepared and using the dilution formula for each concentration as under:

$S_1V_1 = S_2V_2$ Where; S_1 = Stock concentration

S_2 = Required standard concentration

V_1 = Vol. of stock

Results and Discussion:

All the strains except strain II were found to give a positive result for ethanol production.

Table 1.1: Tests for Ethanol production (fermentation)

Strain	Esterfication	Sodium metal test
I.	+	+
II.	–	–
III.	+	+
IV.	+	+
V.	+	+
VI.	+	+
VII.	+	+
VIII.	+	+
Saccharomyces cerevisae	+	+

\+ presence of ethanol

– absence of ethanol

Comparative analysis of ethanol production through calculation based on residual sugar concentration (sugar utilization) :

Table 1.2: Standard glucose concentrations and respective absorbance

Glucose standard Concentrations (µg/ml)	(O.D.) Absorbance (620nm)
100	0.038
200	0.090
300	0.136
400	0.105
500	0.230
600	0.083
700	0.078
800	0.085
900	0.204
1000	0.052

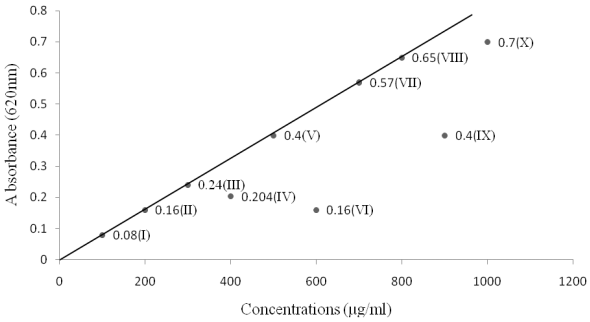

Fig 1.1 : Standard graph for glucose concentrations (Anthronmethod)

The absorbances for the respective standard concentration given in the table 1.2 were plotted against the concentrations and a standard linear curve was prepared by joining the points on the curve as shown in figure 1.1 above.

Table 1.3 : Residual sugar concentrations of positive and negative control after 48h of incubation

Negative control	(O.D.) Absorbance	Concentration
Cellulose + Hemi cellulose hydrolysate	0.62	760
Positive control		
Saccharomyces cerevisae	**24hours** 0.44 **48hours** 0.26	540 320

Table 1.4 : Residual sugar concentrations after 24h and 48h of incubation

Strains	Concentration(µg/ml)	
	24hours	48hours
I.	535	340
II.	740	320
III.	690	245
IV.	530	500
V.	500	290
VI.	620	280
VII.	540	440
VIII.	720	520

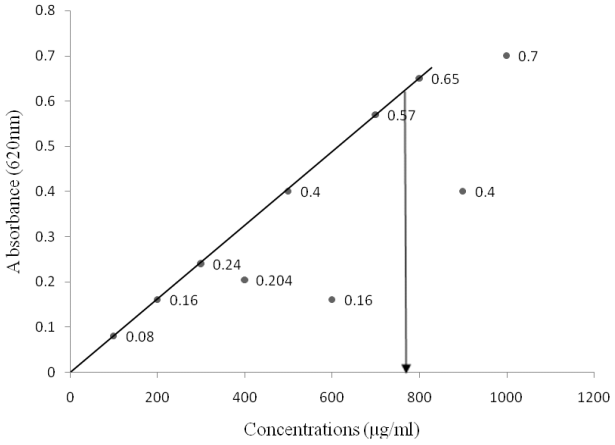

Fig 1.2: Residual sugar concentrations after 24h of incubation

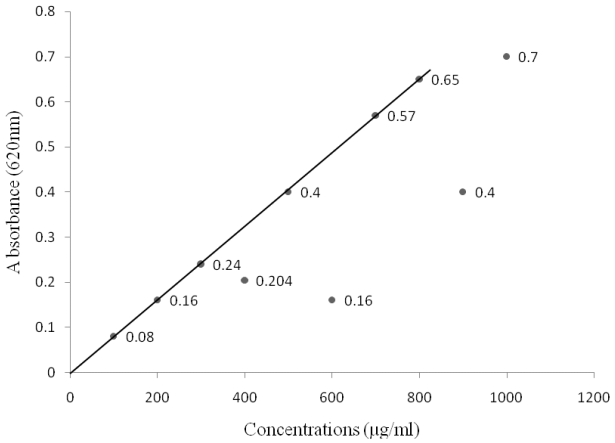

Fig 1.3: Residual sugar concentrations after 48h of incubation

Table 1.5 : Sugar utilization by *Saccharomyces cerevisae*

Incubation Period (Hrs.)	Initial sugar concentration in the corn cob hydrolysate before inoculation (µg/ml)	Residual sugar (µg/ml)	Difference (µg/ml)
24	760	560	200
48	760	320	440

Table 1.6 : Average Sugar utilization by all inoculated strains

Incubation Period (Hrs.)	Initial sugar concentration in the corn cob hydrolysate before inoculation (µg/ml)	Average Residual sugar (µg/ml)	Difference (µg/ml)
24	760	609.375	150.625
48	760	363.125	396.875

An estimation of the difference in the initial and residual sugar in the fermenting media provides a basic understanding of the fermentation i.e. ethanol production potential of a microbe. A lower value of residual sugars in a sample is an indicative of higher value of sugar utilized and consequently ethanol produced. The average sugar utilization in 24 and 48 hours of inoculation by the eight locally isolated strains were found to be 150.625µg/ml and 363.125µg/ml respectively, whereas by the dignified commercial Brewer's Yeast i.e. *Saccharomyces cerevisae* were 440.0 µg/ml and 200.0 µg/ml respectively. The analysis of sugar utilization by the fermenting microbe in both cases gives a comparative idea of ethanol fermentation potential of locally isolated yeast strains to that of the dignified yeast strain.

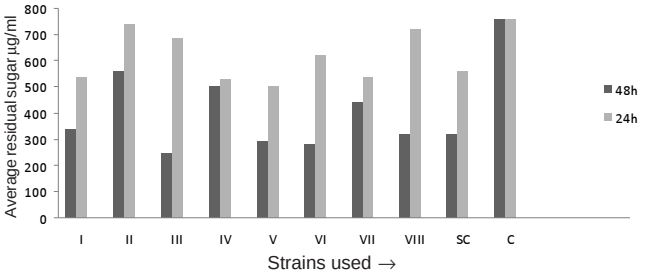

Fig 1.4 : Comparative analysis of residual sugar after 24h and 48h of incubation

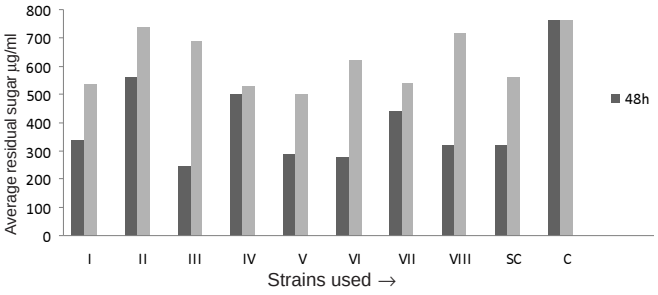

Fig 1.5 : Comparative analysis of sugar utilized after 24h and 48h of incubation

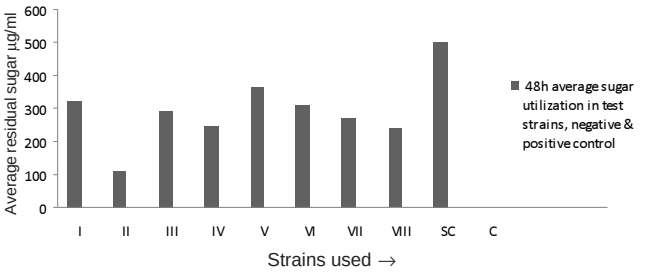

Fig 1.6 : Comparative analysis of average sugar utilized in each case after 48h of incubation

All strains except II were found to give positive result for carbon dioxide production at normal conditions of incubation, at high temperature (42°C) and at high dextrose sugar concentration (40%dextrose) and also ethanol production tests. On spectro photometric analysis of sugar concentration by Anthrone method , it was found that the uninoculated cellulose and hemicelluloses hydrolysate in the ratio 1:1 contained 760 µg/ml of glucose. Further, samples that were inoculated with *Saccharomyces cerevisae* and locally isolated yeast strains from agricultural and fruit market wastes were analyzed for residual sugars. Etha-

nol production tests were also performed. Moreover, an estimation of difference in the sugar concentrations after every 24 & 48 hours gave an approximate idea of the sugar utilization by the strain i.e, the fermentation activity and ethanol production potential. The strains were found to give a good result nearly at par when compared to the result shown by *Saccharomyces cerevisae*. The maximum sugar utilized and thus ethanol produced among all locally isolated strains was found to be highest in case of strain V while lowest in case of strain VIII and no ethanol was detected in case of strain II but its residual sugar was higher than all other strains. This indicated that sugar was utilized by strain II which may be for its metabolic energy requirements, though no ethanol was detected as such indicating its poor fermentation activity.

Conclusion :

The above analysis of sugar utilization and ethanol production by dignified and locally isolated strains using lignocellulosic wast i.e, corn cobs emphasizes the fact that a significant amount of sugars are present in the corn cob waste which can be released by easily accessible and cheap processes like pretreatments, acid and enzymatic hydrolysis. The use of cellulase enzyme is an indispensible step in bioethanol production which is a very expensive one. This research study was designed on a low cost in order to assess the potential of wastes as starting material as well as potential of local strains isolated from wastes itself. The cellulose degrader isolated from waste proved to be highly efficient in making the fixed sugars accessible to the yeast strains for the production of bioethanol. Moreover, the corn cobs were found to have a significant amount of sugars for the bioethanol production.The chemicals and methods used were simple and cheap for a good scale up. Thus, all the above facts emphasize and contribute to an overall reduction in the cost of bioethanol production

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