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Isolation of Cellulolytic Bacteria from Intestine of Termites and Their Utility in Saccharification and Fermentation of Lignocellulosic Biomass

Fermentation of Lignocellulosic Biomass from Cellulolytic Bacteria

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***Abstract* - Among the various sources (organisms) of cellulolytic enzymes, termites are one of the potential sources from which the cellulolytic bacteria can be isolated from its gut. These cellulolytic bacteria produce the cellulolytic enzymes which are used for maximum break down of cellulose, lingo-cellulose and hemicelluloses into glucose molecules and this glucose is next converted to ethanol by fermentation using co-cultured Saccharomyces cerevisiae. The isolated and screened bacteria in the Congo-red agar media is subjected to Hydrolysis capacity test, which is observed a 7-7.2 range. The process variables like pH, temperature and volume are optimized for maximum growth of cellulolytic bacteria. Flask level fermentation after five days incubation of 10% cellulose taken as the substrate under optimized conditions yielded an average of 33.6 % ethanol as measured using potassium dichromate method. 2.5g/l of crude samples (Sugarcane bagasse and corn cobs) were subjected to pre-treatment with 4% H_2SO_4, for optimum cellulose, 100gm of pre-treated samples of sugarcane bagasse and corncobs yielded 74ml and 73.5ml of ethanol at 96 hours incubation, when co-cultured with Saccharomyces cerevisiae.**

Keywords: Cellulolytic enzymes, ethanol, sugarcane bagasse, corncobs.

I. INTRODUCTION

Cellulolytic bacteria can be isolated from many sources like gut of termite, snails and other organisms [11][4]. Of the numerous aerobic and anaerobic microbes are present in the gut of termites, an emphasis is laid to separatecellulolytic bacteria from the termite's hindgut which have the capacity to breakdown long chains of cellulose into monomeric glucose molecules [3][6]. Termites are the insects that belong to the class *Insecta* of phylum *Arthropoda* [10] and classified at taxonomic rank of order Isoptera and are one of the abundant and important vertebrates which degrade significant amount of cellulose in nature [8][9].

In this study, Corn cobs and Sugarcane bagasse were chosen as the substrate to extract cellulose from. The association of lignin with cellulose and hemicellulose in most dry plants, effects the digestion of the lingo-cellulose, hence their separation is essential for enzymatic degradation [2][13]. Therefore the purpose of this study is also to separate and prepare the cellulose for digestion by pre-treatment of the cellulosic waste with H_2SO_4 [5]. Also, cellulolytic bacteria can be engineered for the improvement of ethanol production [1][7][12]. An overview of the ethanol production process by using bacteria is described in figure 1.

Lignocelluloses are the key complex structural constituent of the plants in which cellulose is bounded and surrounded in a matrix of hemicellulose and lignin which create a barrier and thick protective sheath around cellulose making it insoluble in water and creating a barrier for enzymatic attack. Therefore processing of lignocellulose is crucial for the conversion of lignocellulosic biomass to biofuel such as bio-ethanol [12]. Cellulases are the hydrolytic enzymes that break down the cellulose molecule into monosaccharide's ("simple sugars") or shorter polysaccharides and oligosaccharides. The reaction entails the breakdown of the 1, 4- beta-D-glycosidic bonds in cellulose.The diagrammatic representation is given in figure 2.

Bioethanol (C_2H_6O or C_2H_5OH), the fermented product of carbohydrate-rich crops, is a high octane fuel, currently being used as an additive for petrol in the fuel industry. Ethanol is extracted from cellulose following two methods, namely (1) Separate Hydrolysis and Fermentation (SHF) which further include Acid hydrolysis and fermentation and enzymatic hydrolysis and fermentation. (2) Simultaneous Saccharification and Fermentation (SSF).In the present study, it was mainly concentrated on the isolation of cellulose degrading bacteria from termite gut and their cellulolytic activity evaluation. The process of simultaneous saccharification and also fermentation occurs by co-culturing of the isolates (bacteria) and the yeast for ethanol production.

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II. MATERIALS AND METHODS

A. Isolation and Screening:

Cellulolytic bacteria is isolated in 0.9% saline solution (Gupta, P, 2012) from the macerated hindgut of the termites collected from the old wooden storage cupboards through serial dilution and inoculated firstly on Basal agar media ($NaNO_3$ 2.5 g; KH_2PO_4 2 g; $MgSO_4$ 0.2 g; NaCl 0.2 g; $CaCl_2 \cdot 6H_2O$ 0.1 g, Agar 15 g, per 1000ml) with only cellulose as the available carbon source. The culture obtained after 5 days incubation the culture is then subjected to determinative screening by inoculation on Congo-red agar media (KH_2PO_4 0.5 g, $MgSO_4$ 0.25 g, Cellulose 2.0 g, Agar 15 g, Gelatin 2 g and Congo Red 0.2 g per 1000ml at pH 6.8–7.2). Biochemical characterization is done to characterize the isolated cellulolytic bacteria on their reaction towards certainreagent.

Cellulose degrading ability is measured quantitatively by Hydrolysis capacity (HC), in which the Congo red agar plates with the zone of clearance are washed with acid indicating blue color making the zones more clear for measurement

$$HC = \frac{zone\ of\ clearance}{Diameter\ of\ well}$$

a. Enzyme production optimization:

Flask level optimization was done to find optimal pH, optimal temperature, optimal volume and optimal time for analyzing the activity of the enzyme in the culture broth.

For optimal volume different volumes of the culture broth was inoculated in the cellulose agar Congo red media then HC zone was measured after incubation. The obtained optimal volume is set as constant and the optimal pH for enzyme production is calculated by incubating culture broth at varying pH (6.8-7.2). The broth supernatant from the aforementioned cultures is inoculated in the wells of Cellulose Congo red agar medium and incubated for 48 hours. Optimum pH was determined by observing the maximum HC Zone inplates.

For determining optimal temperature, optimal volume of the broth and optimal pH were fixed and the broth was added to the Cellulose Congo red agar plate and incubated for 48hrs. Optimal temp was found by observing the maximum HC zone. For determining optimal time, flask level fermentation was performed with the cellulolytic bacteria by maintaining optimal pH and optimal temperature and then measuring OD every 4 hours for 48hours. The graph was plotted which gives growth curve of bacteria.

b. Enzyme assay:

Initially, total protein concentration is estimated by Lowry method, later the enzyme assay is performed for exoglucanase (total cellulose) and endoglucanase activity.

Total cellulase activity of fresh cellulolytic bacterial strains was determined and described and expressed in filter paper unit (FPU) per ml of undiluted culture filtrate. One FPU is defined as the amount (mg) of reducing sugar liberated per hour under the standard assay conditions (540nm). Exoglucanase activity was calculated using the formula,

$$FPU\ (\frac{mg}{ml}) = (mg\ of glucose\ released) * 0.185 - [13]$$

The Endoglucanase activity was determined by using the colorimetric method as described by Miller, (1959) by DNS reagent method.

$$CMC\ (\frac{mg}{ml}) = (mg\ of\ glucose\ released) * 0.37 - [13]$$

The principle is based on the determination of the color developed after the reaction between the reducing sugars (glucose) by DNS reagent. Endoglucanase unit was calculated using theformula,

The enzymatic hydrolysis of cellulose results in its breakage into monomeric glucose subunits; 1:10 (w/v) ratio of 1% acid treated biomass and 50mM molar of Citrate buffer (pH-4.8) along with the enzyme (5%) are added and incubated at 50ºC for 24-72 hours resulting in cellulose degradation to glucose.

The OD of glucose is estimated by DNS method at 540nm, against the glucose standards.

$$IU = \frac{[(Amt\ of\ reducing\ sugar\ in\ \mu mol\ per\ ml) * (Total\ assay\ vol\ in\ ml)]}{[(Mol.\ wt\ in\ g\ per\ mol) * (Timeofincuba(\min)]} * 1000$$

c. Alcohol assay:

Cellulolytic bacterial Isolates were grown in 1% cellulose broth medium along with salts and incubated at 37^{O}C which results in cellulolytic enzyme production by saccharification procedure. After the incubation, the above culture broth was co-cultured with *Saccharomyces cerevisiae* for 5 days to facilitate fermentation for ethanol production, with regular ethanol estimation by $K_2Cr_2O_7$ reagent method for every 24hrs.

$$Alcohol\ yeild(\%) = \frac{\%\ of\ Alcohol * Broth\ volume}{(weight\ of\ sample\ in\ gm)}$$

Sugarcane bagasse and corn cobs were pre-treated with acid and subjected to bioethanol production by co-culturing with *Saccharomyces cerevisiae* which simultaneously undergoes saccharification and fermentation.

III. RESULTS AND DISCUSSION

A. Screening and characterisation:

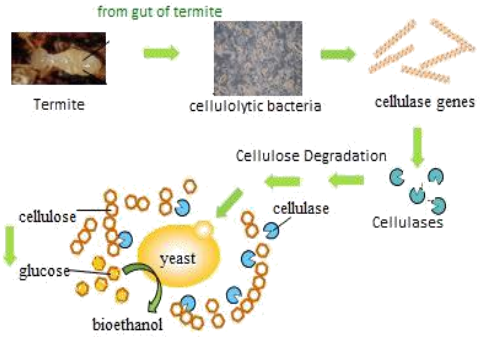

Fig 1: An overview of isolation of bacteria and their mode of action in the process of ethanol production

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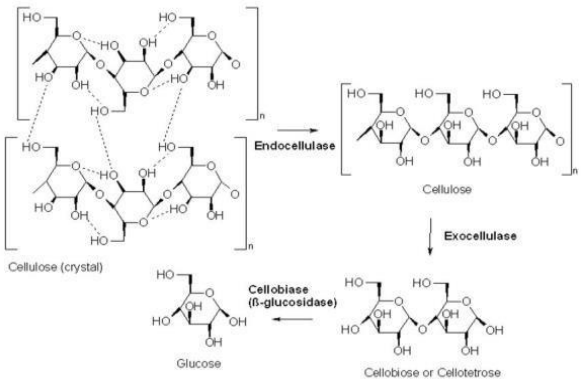

Fig 2: The three forms of cellulase catalyzed reactions: (a) Endocellulase cleave the non- covalent interactions in the amorphous structure of cellulose (b) Exocellulase hydrolyze the chain ends to break the polymer into simple sugars (c) Beta-glucosidase results in hydrolysis of disaccharides and tetrasaccharides into monomeric glucose units

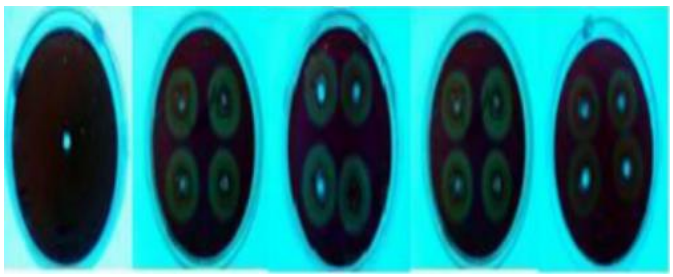

Fig 3: Isolation, Screening, Characterization of Cellulolytic bacteria isolated indicating the clearance zone on the Congo-red media

The cellulolytic bacteria are isolated, screened and sbiochemically characterized indicating as gram-positive bacteria and the cellulase enzyme screening resulted in the formation of clearance zones form figure 3 on the Congo-red agar media.

Table 1: Biochemical Characterization of Bacteria

TEST	RESULT
Gram staining	Positive
KOH Test	Positive
Catalase Test	Positive
Starch Hydrolysis	Positive
Amylase	Positive
H-L Test	Aerobic
Methyl red Test	Negative
Urease Test	Negative
Carbohydrate fermentation Glucose test	Positive
Carbohydrate fermentation Dextrose test	Positive

B. HC Estimation:

Hydrolysis capacity was estimated by acidifying the Congo red media plate with 1M H2SO4 so that it gives blue color and the zones are measured and result as seen in Figure 4

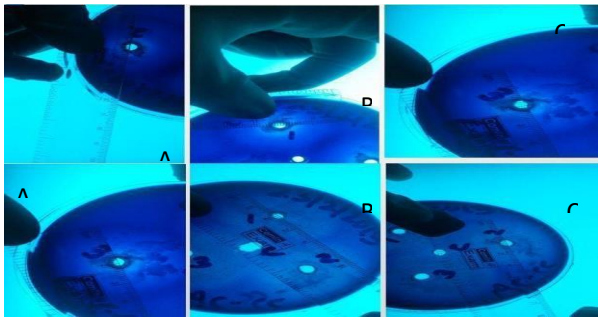

Fig 4: Measurement of HC (Hydrolysis Capacity) of the bacteria after treatment of Congo-red medium with 1M H_2SO_4 for 15 minutes

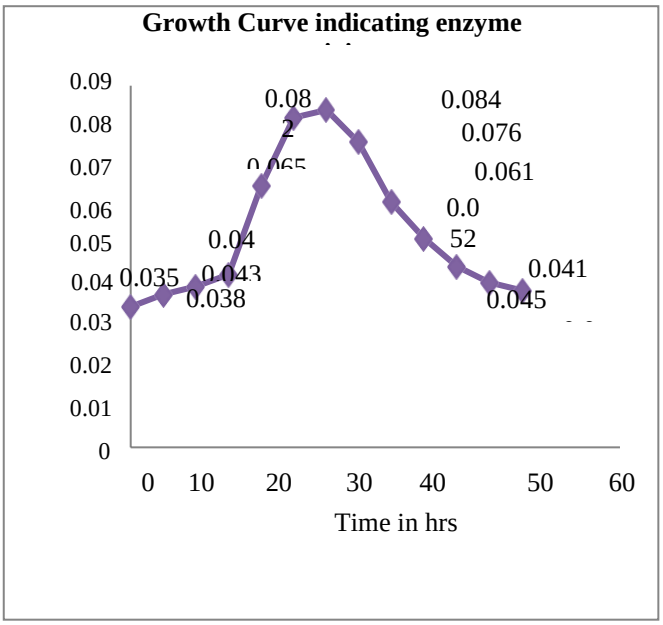

Fig 5: Growth curve of bacteria plot by analyzing enzyme activity indicating high enzyme production at 24 hr incubation time.

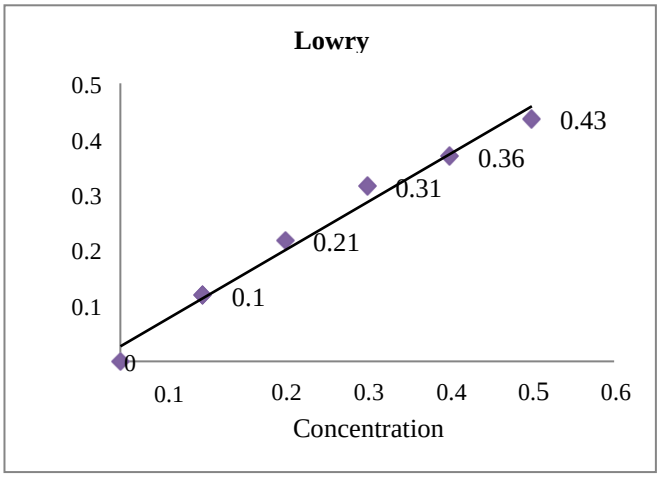

Fig 6: Absorbance vs. Concentration (mg/ml) graph plotted indicates the, 0.0633 mg/ml total protein concentration against BSA standard

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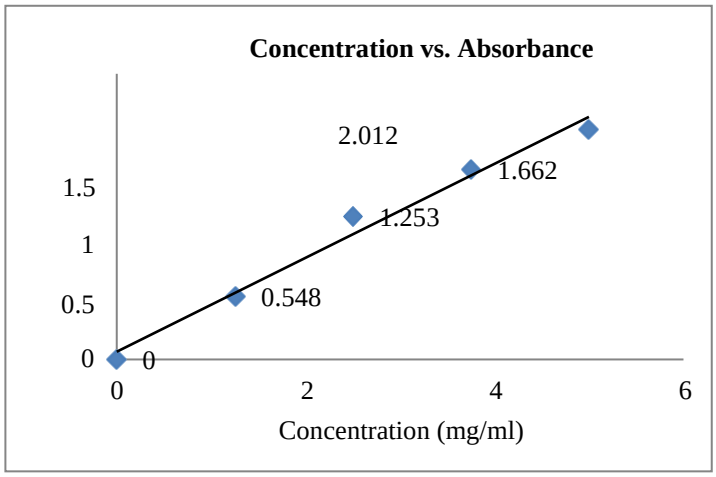

Fig 7: Absorbance vs. standard concentration (stock 5mg/ml) and test sample (FPU) is 0.12062mg/ml with OD 0.311 (conc. 0.652mg/ml)

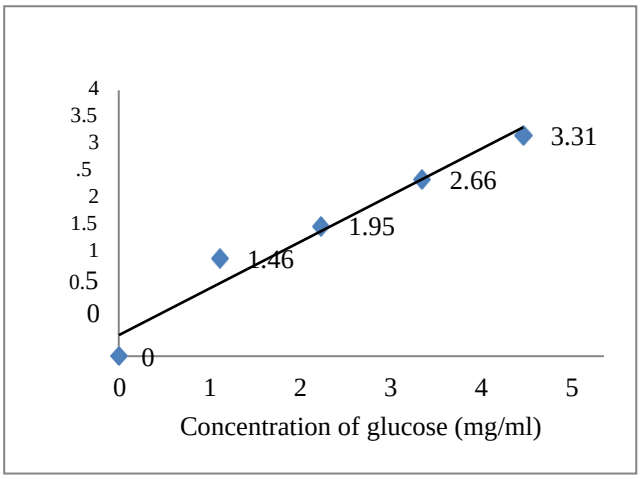

Fig 8: Absorbance vs. standard concentration (stock 5mg/ml) and test sample (CMC) is 0.93mg/ml (for supernatant) with OD 2.284 (conc. 2.52mg/ml)

Table 2: Hydrolysis capacity is done for different cellulose sources showing almost similar zones regardless bacteria where the diameter of the well is 5mm

Cellulose source	Zone of clearance	Hydrolysis capacity
Crystalline cellulose(A)	36.33 mm	7.26
Amorphous cellulose(B)	35.11 mm	7.02
Carboxymethyl cellulose (C)	36.22 mm	7.24

a. Optimization & Enzyme Assay:

The process variables (volume, pH, temperature and time) are set as optimized for the growth and activity of cellulolytic bacteria. Optimal volume for the culture broth (bacteria) is 10µl and the clearance zone of 28mm and optimal pH is 8, where a largest distinct zone is observed with 27mm diameter and optimal temperature is 35ºC with a clearing zone of 29mm is obtained and finally, the bacteria activity is highest at optimal time 24 hours from the bacterial curve.

Table 3: pH optimization, with 8 as optimal pH

pH	Clearance zone diameter (mm)	Remarks
4	0	No distinct zone observed
6	7	No distinct zone observed
8	25	Large distinct zone observed
10	7	No distinct zone observed

Table 4: Temperature optimization, with 35ºC as optimal temperature

Temperature (ºC)	Clearing zone diameter(mm)	Remark
15	2	No distinct zone observed
25	24	Distinct zone observed
35	29	Largest distinct zone observed
45	28	Distinct zone observed

Table 5: Volume optimization, with 10µL as optimal volume

Volume (µL)	Clearing zone Diameter (mm)	Remarks
2	25	Distinct zone observed
4	26	Distinct zone observed
6	27	Distinct zone observed
8	27	Distinct zone observed
10	28	Largest Distinct zone observed
12	27	Distinct zone observed

A graph is plot against the time optimization indicating the maximum enzyme production and activity. Hence all the process parameters are optimized.

Total protein concentration is estimated by Lowry method, by plotting the graph against the unknown sample and BSA as the standard. The sample indicated 0.0633mg/ml protein and it is further subjected to exoglucanase and endoglucanase enzyme activity.

The activity of endoglucanase, exoglucanase was found to be at 0.0155 IU from CMC (Carboxymethyl Cellulose) and 0.004 IU Whatmann filter paper (W) for all using formula.

C. Alcohol assay:

Table 6: Study of enzyme activity at different acid percentages indicates efficient value at 4% of H2SO4 measured at 540nm.

Sample	Treatment	OD (540nm)		
		0 hours	24 hours	48 hours
Corn Cobs	+ve Control	0.038	0.046	0.039
	4% treatment	0.038	0.069	0.079
	6% treatment	0.038	0.061	0.071
Sugarcane Bagasse	+ve Control	0.038	0.058	0.042
	4% treatment	0.038	0.068	0.075
	6% treatment	0.038	0.062	0.071

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Table 7: Alcohol Assay for 10% pure cellulose as test sample by using Potassium dichromate colorimetric method at 600nm

Sample	Percentage volume	OD (600nm)				
		0 hours	24 hours	48 hours	72 hours	96 hours
10% Cellulose		0.046	0.113	0.119	0.127	0.139
Ethanol Standards	2.5%	0.274	0.247	0.277	0.253	0.221
	5%	0.428	0.401	0.435	0.391	0.413
	7.5%	0.583	0.542	0.610	0.547	0.549
	10%	0.712	0.679	0.763	0.661	0.685

Table 8: Calculation of alcohol concentration from the OD value of Table 7

10% cellulose	Ethanol yield (ml per 100-grams of substrate)				
	At 0 hour	At 24 hours	At 48 hours	At 72 hours	At 96 hours
	1.06	24.6	26.7	28.8	33.6

Table 9: Alcohol Assay for different concentration 4% H_2SO_4 treated sample by using Potassium dichromate colorimetric method at 600nm

Sample	Weight (g/L)	OD at 24 hours	OD at 48 hours	OD at 72 hours	OD at 96 hours
Corn Cobs	5	0.116	0.118	0.120	0.149
	7.5	0.118	0.127	0.126	0.152
	10	0.143	0.153	0.159	0.175
Sugarcane bagasse	5	0.121	0.136	0.142	0.148
	7.5	0.123	0.138	0.150	0.156
	10	0.127	0.149	0.155	0.158
Ethanol standards	2.5%	0.27	0.288	0.268	0.271
	5%	0.415	0.402	0.459	0.439
	7.5%	0.578	0.567	0.563	0.605
	10%	0.715	0.706	0.720	0.779

Table 10: Calculation of alcohol concentration from the OD value of table 9

Sample	Weight (g/L)	Ethanol yield (ml per 100 gram substrate)			
		At 24hours	At 48hours	At 72hours	At 96 hours
Corn cobs	5	54	52.5	53	74
	7.5	37	38.33	37.67	50.33
	10	36.25	38	39.75	45.25
Sugarcane bagasse	5	57.5	66	67	73.5
	7.5	39.33	45	49.33	51
	10	30.5	37	38.25	39.25

IV. CONCLUSION

The overall result of this process concludes that the cellulolytic bacteria isolated from the termite gut produces the enzyme, cellulase, which decomposes the cellulose taken as the substrate for ethanol production. A combination of various chemical and biological methods led to the maximum conversion of cellulose to ethanol. The process parameters, volume, temperature, pH and the time were optimized for the cellulolytic bacteria. The hydrolysis capacity of the bacteria is estimated to be 7 - 7.2. Enzymatic hydrolysis involves the conversion of cellulose to glucose, which on co-culture with *Saccharomyces cerevisiae* yielded bioethanol. 2.5g/l of the substrate (sugarcane bagasse and corn cobs) which are pretreated with 4% H2SO4 gave highest cellulose yield. By performing flask level fermentation with optimized parameters, using 10% cellulose and by co-culture technique yielded an average of 33.5 % of ethanol, as estimated by potassium dichromate method. The pre-treated samples of corn cobs and sugarcane bagasse yielded a maximum of 74ml and 73.5ml of ethanol respectively per 100g of the substrate at 96 hours incubation. Many different genetic modification techniques can be applied to the rigorous study to increase the ethanol yield by this method. Through r-DNA technology, the gene responsible for the production of cellulase enzyme can be incorporated in yeast or both the gene responsible for saccharification and fermentation can be incorporated into third strain like *E.coli* for preparation of a single hybrid organism.

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