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Media Optimization And Saccharification of Lignocellulosic Waste By Cellulolytic Bacteria Isolated From Gut of A Termite

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Abstract **-** *Cellulase creation from bacteria can be a favourable position as the enzyme generation rate is typically higher because of the higher bacterial development rate when contrasted with fungi. Screening of bacteria, advancement of fermentation conditions and choice of substrates are imperative for the fruitful creation of cellulase.*

This study was conducted from isolation of cellulolytic bacteria from Damp wood termite. They were similar like Citrobacter, Enterobacter and Cellulomonas and using media optimization method for highest production of cellulase and using this cellulase for saccharification of lignocellulosic waste. The highest cellulase producing ability of isolate from this study was found to be 51.9 IU/ml with 48 hours of incubation and as carboxymethyl cellulose as substrate. Treated and untreated corncob was subjected to saccharification. Highest saccharification was observed with 15gm of substrate treated with 3% NaOH at 50ºC for 30min.

Keywords **–** *Termites, Cellulolytic Bacteria, Gram Staining, Media Optimization, Placket – Burman Method, Saccharification, Corn cob*

I. Introduction

TERMITES assume as a splendidwork in earthly biological community by reusing lignocellulosic biomass that contains a blend of lignin, hemicellulose and cellulose. In addition, Termites are a standout amongst the most imperative soil bugs that productively disintegrate lignocelluloses with the assistance of their related microbial symbionts to less difficult type of sugars, which later can be matured to ethanol utilizing yeasts. They are said to dissimilate a huge extent of hemicellulose (65-87%) and cellulose (74-99%) parts of lignocellulose they ingest. Besides, they assume a critical job in the turnover and mineralization of complex biopolymers, for example, wood and other cellulose and hemicelluloses containing materials[1].Lignocellulose is the most real part of the woody and dead plant materials, and in addition it is in overabundance extent of biomass on earth, particularly in earthbound biological systems. Then again, to cellulose, lignin debasement does not seem, by all accounts, to be fundamental in the gut of wood bolstering termites[2].

Depending upon their nourishing natural surroundings, termites are separate into two gatherings: the lower termites and higher termites. The lower termites predominately feed on wood. As wood is hard to process, termites like to devour growth contaminated wood since it is simpler to process, and the organisms are high in protein. In the meantime, the higher termites devour a wide assortment of materials, including dung, humus, grass, leaves and roots. The gut in the lower termites contains numerous types of microscopic organisms alongside protozoa, while the higher termites just have a couple of types of microbes with no protozoa[3].

Endogenous cellulase, endo β-1,4 glucanase and β-glucosidase which are excreted from the salivary glands or the gut have been identified and characterized in both higher and lower termites. Molecular analysis reveals these endogenous enzymes are members of glycosyl hydrolase family 9 (GHF9). In higher termites the endogenous cellulolytic activity meets the metabolic needs. In lower termites, most of cellulolytic activity is found in the hindgut. Thus, the ingested cellulose can be partially degraded by the endoglucanase of termite origin, and then unhydrolyzed cellulose goes to the hind gut, where it can be endocytosed and is fermented by the symbiotic protists in lower termites. Termites grind and crunch the ingested material, which may improve digestion by increasing the exposed surface area[4].

Termite gut microorganisms can be ordered into *Citrobacter*, *Enterobacter* and *Cellulomonas bacteriodales, clostridiales, cyanobacteria, Mycoplasmatales, firmicutes, actinobacteria, proteobacteria and bacillales*. Some gut microscopic organisms from terminidae have been distinguished. Those microscopic organisms have closeness with clostridium variety, *anaerovorax odorimutans, erysipelothrix rhusiopathie, eubacterium seraeum, sporobacter termitidis*[5]. Plackett Burman designs goal was to find experimental designs for investigating the dependence of

some measured quantity on several independent variables. Low level and high level minimize the variance and estimate their dependencies using experiments. As a result of the high cost of cellulase production with its low enzyme activities owing to the use of chemicals in its industrial production, efforts are needed to economize cellulase production through media (cultural) optimization and use of supplements and/or additives[6].

In term of Saccharification using termite gut cellulolytic bacteria responsible for producing cellulase was used to saccharified a substrate. Cellulases are a group of hydrolytic enzymes capable of hydrolyzing the most abundant organic polymer i.e. cellulose to smaller sugar components including glucose subunits. Cellulases are inducible enzyme complex involving synergistic action of endoglucanase, exoglucanase and cellobiose have gained a huge economic importance due to their wide range of application in industries like starch processing, animal food production, grain alcohol fermentation, malting and brewing, extraction of fruits and vegetable juices, pulp and paper industry and textiles industry[3,7].

II. Materials and Methods

A. Sample Preparation

Drywood termites were collected from local area of Vallabh Vidyanagar district of Anand.

B. Extraction from termites

The termites were taken out of their nests and placed in sterilizedPetri plates. They were then surface sterilized by washing with 70% alcohol. Each termite was separated into its head and body. After removing the heads with forceps, the bodies were crushed with the help of glass rods. The paste obtained from the termites' gut was used for isolation of the bacteria with the help of inoculating loops.

C. Isolation of cellulase producing microorganism

The macerated gut of the collected organisms was inoculated in a media (CMC 1.5g; KH_2PO_4 0.1g; K_2HPO_4 0.1g; $MgSO_4$ 0.04g; NaCl 0.005g; $FeCl_3$ 0.02g; agar 3g; /100ml) for isolation of cellulolytic bacteria. Nutrient agar media used for isolation of general bacteria. Plates were incubated for 24 hours 37°C and after incubation the isolates obtained were screened for cellulase production.

D. Test for Cellulolytic Activity

Gram's Iodine Stain was prepared by dissolving 0.665 g KI and 0.335 g Iodine in 100 ml distilled water. Petri plates were flooded with Gram's iodine stain and washed with distilled water. The Cellulolytic activity was indicated by the appearance of halo zone around bacterial isolate. The magnitude of the activity was calculated by measuring the diameter of the zones.

E. Gram Staining

Gram staining was performed to identify bacterial species group.

F. Fermentation process of Cellulase production

To 100 ml of the optimized culture medium, a loopful of cell culture from respective species was inoculated under controlled conditions. Then it was kept in incubator at 37°C for a 3days and after every 24 hours of incubation 5ml of broth from the flask was withdrawn aseptically and centrifuged at 5000rpm at 40ºC and the supernatant used for cellulase activity estimation. The composition of fermentation medium used for cellulase production at high level (+) contained (g/100ml):

Carboxymethyl cellulose,2.5; Urea,0.05; Protease Peptone, 0.5; $(NH_4)_2SO_4$,0.5; KH_2PO_4, 0.5; $CaCl_2$, 0.5; $MgSO_4$, 0.05; $FeSO_4$, 0.5mg; $MnSO_4$, 0.16mg; $ZnSO_4$,0.14mg; $CoCl_2$, 0.2mg; pH 7.0.

A ten time's lower concentration of each component is maintained at low level (-) for cellulase production.

G. Fermentation media optimization for Cellulase production using Plackett Burman Design

Plackett-Burman experimental design assumes that there are no interactions between the different variables in the range under consideration[8]. A linear approach is enough for screening. Plackett-Burman experimental design is a fractional factorial design and the main effects of such a design may be simply calculated as the difference between the average of measurements made at the high level (+) of the factor and the average of measurements at the low level (−). To determine the variables that significantly affect cellulase activity, Plackett-Burman design is used. Eight variables (Table II) are screened in 12 experimental runs (Table III) and insignificant ones are eliminated in order to obtain a smaller, manageable set of factors. The low level (−) and high level (+) of each factor are listed in (Table II). The statistical software package Design-Expert software (version 6.1.5, Stat-Ease, Inc., Minneapolis, USA) is used for analysing the experimental data. Once the critical factors are identified through the screening, the central composite design can be used to obtain a quadratic model.

The Plackett-Burman design was used for screening of the factors (media components) that significantly influenced on cellulase production. The design considers the main effect of the variables but not their interaction effects. It can represent by the first order polynomial equation:

$$Y = ßo + \Sigma n\ i=1\ ß\ i\ Xi \ldots\ldots\ldots (1)$$

Where Y represents the response, ßo is the model coefficient, i is the linear coefficient, Xi is the variables, and n is the number of the parameters (variables).

Each variable was represented in the two levels, i.e. high (+), and low (-). The effect of each variable was determined by the following equation:

$$E(Xi) = \frac{\sum Mi+ - \sum Mi-}{N} \ldots\ldots\ldots\ldots (2)$$

Where E(xi) represents is the response value effect of the tested variable; ΣMi- is the summation of the response value at low level, and N is the number of experiments.

Table I represented the selected variables to be evaluated at High and low levels, Table II showed the design matrix; eight assigned variables were screened in the 12 experimental runs. The cellulase production was carried out in the triplicate. The fraction significant at 95% level (p-value < 0.05) was considered reliable.

H. Enzyme Assay

Cellulase activity measured as CMC hydrolysing activity was assayed according to the method recommended by Ghose (1987) and expressed as international units (IU). One international unit of cellulase activity is the amount of enzyme that forms 1 μmol glucose (reducing sugars as glucose) per minute during the hydrolysis reaction. Reducing sugar was determined by the di-nitro salicylic acid (DNS) method[9].

I. Saccharification source

Corn cob is a waste material obtained after removing the seed from corn which was collected from local fields. They were sun dried for three days to remove the remaining moisture. The dried samples were stored in airtight bag at room temperature and stored in moisture free environment.

J. Pre – Treatment of lignocellulosic waste

Mechanical treatment: the previously dried corncob samples were mechanically disintegrated into small pieces and again dried in hot air oven to remove the excess moisture if present and further crushed into 20 mesh size particles. The crushed samples were again stored in airtight zip-lock bag.

Chemical treatment: corn cob was dried, weighted (5,10 and 15 grams) and each flask of corn cob were treated with different concentration of sulphuric acid (1,3 and 5g%) and sodium hydroxide (1,3 and 5g%).

The treated samples were socked in chemicals for few minutes and incubated at desired temperatures for time interval by design expert tool.

K. Cellulose Estimation

Cellulose content of treated and untreated samples was determined by weighing 1g (W1) of each treated and untreated corncob sample (oven dried) which is collected in flask. The substrate was digested using 15ml of 80% acetic acid and 1.5ml of concentrated HNO_3.Reflux was carried out for 20mins on hot plate.

After refluxing, it was filtered using Whatman filter paper#1. The residue was washed thrice using hot water. The solid residue after washing was dried and weighted as W2. Further the solid is incinerated in furnace at 200°C for 20 minutes. The ash weighted is noted as W3.

Cellulose% can be calculated by following equation:

$$\text{Cellulose\%} = \frac{\text{weight of the digested material (W2)} - \text{weight of the ash (W3)} \times 100}{\text{Weight of the dry material (W1)}}$$

L. Filter Paper Assay

Place a rolled filter paper strip into each test tube.1.0 mL 0.05 M Na-citrate, pH 4.8 added to the tube; thebuffer should saturate the filter paper strip. Equilibrate tubes with buffer and substrate to 50° C. following this, 0.5 mL enzyme added and diluted appropriately in citrate buffer. At least two dilutions must be made of each enzyme sample, with one dilution releasing slightly more than 2.0 mg of glucose (absolute amount) and one slightly less than 2.0 mg of glucose. Then incubated at 50º C for exactly 60 min.After end of incubation reaction was stopped by added 3.0 ml DNS reagent.Samples were cool down at room temperature and measure against a reagent blank at 540 nm in spectrophotometer.

III. TABLE I. Experimental range and levels of independent variables in the Plackett Burman experiment

Variable	H (+)	L (-)	Trace Elements	mg%	Stock Solution
Substrate CMC	2.5	0.25	$FeSO_4$ $7H_2O$	0.5	50mg%
Urea	0.05	0.005	$MnSO_4$ $4H_2O$	0.16	16mg%
Peptone	0.5	0.05	$ZnSO_4$ $7H_2O$	0.14	14mg%
$(NH_4)_2SO4$	0.5	0.05	$CoCl_2$ $6H_2O$	0.2	20mg%
K_2HPO_4	0.5	0.05			
$MgSO_4$ $7H_2O$	0.05	0.01			
$CaCl_2H_2O$	0.5	0.05			
Trace elements	1ml	0.1ml			

IV. TABLE II. The Plackett-Burman design for 11 parameters

substrate CMC	Urea	Peptone	$(NH_4)_2 SO_4$	K_2HPO_4	$MgSO_4.7H_2O$	$CaCl_2.2H_2O$	Trace elements	D1*	D2*	D3*	Response (IU/ml)
1	1	-1	1	1	1	-1	-1	-1	1	-1	40.2
-1	1	1	-1	1	1	1	-1	-1	-1	1	30.4
1	-1	1	1	-1	1	1	1	-1	-1	-1	33.5
-1	1	-1	1	1	-1	1	1	1	-1	-1	21.9
-1	-1	1	-1	1	1	-1	1	1	1	-1	15.9
-1	-1	-1	1	-1	1	1	-1	1	1	1	13.5
1	-1	-1	-1	1	-1	1	1	-1	1	1	51.9
1	1	-1	-1	-1	1	-1	1	1	-1	1	38.8
1	1	1	-1	-1	-1	1	-1	1	1	-1	45.8
-1	1	1	1	-1	-1	-1	1	1	1	1	8.4
1	-1	1	1	1	-1	-1	-1	1	-1	1	32.7
-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	22.6

M. Saccharification of corncob

Pretreated corncob along with untreated samples was subjected to saccharification using cellulase enzyme. Treated and untreated lignocellulosic substrates was taken in test-tube at 0.5 gm% concentration and citrate buffer to it cellulase loading was carried out at 1FPU/gm of substrate. Saccharification of lignocellulosic substrate was carried outat 50º C and the samples are withdrawn after every 4 hours to check the saccharified sugar released which was estimated using DNS reagent. Saccharification of lignocellulosic was monitored up to 72 hours.

Saccharification can be calculated by following equation:

$$\% \text{ saccharification} = \frac{\text{sugar produced at T0} - \text{sugar produced at T1} \times 0.92 \times 100}{\text{Substrate concentration (mg)}}$$

* Dummy variables

V. RESULT AND DISCUSSION

Isolation and screening of cellulolytic bacteria

The diverse diet of termites is rich in cellulose, hemicellulose and lignin[10]. Previous investigations have shown that aromatic compounds, but not polymeric lignin, seem to be degraded by bacteria during the passage of wood through the termite gut. Cellulose and hemicellulose undergo substantial digestion in the termite gut. Hemicellulose-degrading bacteria and yeasts were described[11].

Bacteria present an attractive potential for the exploitation of cellulases due to their rapid growth rate, enzyme complexity and extreme habitat variability[12]. The high cost of cellulase production (due to use of pure chemical in production) coupled with low enzyme activities, limits its industrial use. Therefore, efforts are needed to economize cellulase production by media optimization and hence, isolation and characterization of cellulase producing bacteria will continue to be an important aspect of biofuel research[6][8].

Termites were selected for isolation of cellulolytic bacteria. Total ten different types of isolates were obtained from gut; among them six isolates were shown cellulolytic activities. From these six, isolate no.1, 2 and 3 was produced zone of cellulose hydrolysis and maximum zone of cellulose hydrolysis observed with isolate 3 (YJV 3) when compared with other isolates. Isolate YJV 3 magnitude of activity was found to be 1.3cm (Figure 2).

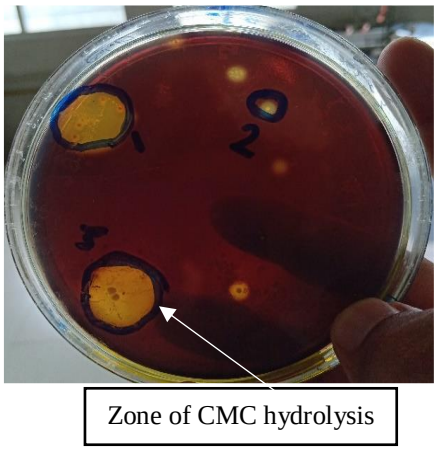

Fig2: Screening of Cellulase producing Bacteria from termite Gut

Morphological characterization

Identification is the practical application of the taxonomy. The process of determining that an isolate belongs to recognized group. The colony morphology was analyzed, and the results were validated by standard protocol as shown in table III isolated cellulolytic bacteria form gut of worker termite and isolate was an aerobic Gram-negative bacterium. Its bacterial colony was rounded, smooth, and convex[5].

VI. TABLE. III. Colony characteristics of well-isolated colony of cellulase producer

Size	Medium
Shape	Circular
Margin	Entire
Elevation	Convex
Texture	Mucoid
Opacity	Opaque
Pigmentation	Yellowish Orange
Gram Group	Negative

Microscopic characterization of isolate by Gram's Staining

Gram's staining of pure culture was done, and it was found strain was gram negative, rod shaped bacteria. Three genera are suspected to have been isolated which includes Citrobacter, Enterobacter and Cellulomonas. Figure 4 shows gram staining of cellulolytic bacteria.

Fig3: Gram staining of Cellulase producing bacteria

Media optimization using Plackett Burman method

Plackett-Burman statistical design, a Verification experiment was applied to compare between the predicted optimum levels of independent variables, anti-optimum levels and the basal condition settings. The highest cellulase producing ability of isolate from this study was found to be 51.9 IU/ml with 48 hours of incubation (Table II). This was probably higher when compared to other report of Cellulomonas, as well as other bacteria. Sangbriba et al., reported during growth of *C. biazotea*, on kallar grass medium 37.5 IU/ml was observed. Total cellulase yield of 51.9 IU/ml as optimized by Plackett Burman design(Table II),this enzyme can used in crude and purified forms and saccharification of pretreated wastes converted into sugar then ethanol after that it can be biogas and methane[13]. Moreover, according to kazemi et. al., cellulase activity in optimized medium was 49.80 U/ml and high level of enzyme production (50.30 U/ml)was obtained by using fermenter system [14].

By performing Plackett-Burman design, the effect of components on maximum cellulase production by isolated bacteria were found to be Urea, di potassium phosphate and trace elements as observed in table IV. The problem of high cost enzyme can be solved by meticulously observing the optimal cultural growth conditions and production under appropriate variable.

VII. TABLE IV. Analysis of Plackett- Burman Design for Cellulase production

Variables	Σ H	Σ L	Difference	Effect	Mean Square	Mean Square for Error	F-value	p value	95% CI
substrate CMC	242.9	112.7	130.2	21.70	470.89	3.46	136.04	0.005	99.5
Urea	185.5	170.1	15.4	2.57	6.6		1.90	0.308	69.2
Peptone	166.7	188.9	-22.2	-3.70	13.69		3.95	0.158	84.2
$(NH_4)_2$ SO_4	150.2	205.4	-55.2	-9.20	84.64		24.45	0.026	97.4
K_2HPO_4	193.0	162.6	30.4	5.07	25.67		7.42	0.085	91.5
$MgSO_4.7H_2O$	172.3	183.3	-11	-1.83	3.36		0.97	0.509	49.1
$CaCl_2.2H_2O$	197.0	158.6	38.4	6.40	40.96		11.83	0.054	94.6
Trace elements	170.0	185.2	-15.2	-2.53	6.4		1.85	0.315	68.5

*** Statistically significant at 95% of probability level.**

VIII. TABLE V. Saccharification of corncob treated with NaOH at constant temperature

run	Substrate (gm%)	Time (min)	NaOH (gm%)	Temp (ºC)	4hr	24hr	48hr	72hr
1	15	60	5	50	1.5	13.1	24.2	38.9
2	10	60	1	50	3.7	13.9	23.1	33.6
3	10	90	3	50	3.3	14.2	22.8	32.9
4	5	30	1	50	9.8	15.3	28.6	31.2
5	15	30	3	50	9.4	21.2	23.7	**39.6**
6	5	60	3	50	9	18.2	24.6	31.6
7	10	30	5	50	8.1	17.8	23.5	30.7
8	15	90	1	50	7.1	18.2	20.6	36.7
9	5	90	5	50	10	10.5	27.6	33.9

IX. TABLE VI. Saccharification of corncob treated with H_2SO_4 at constant temperature

run	Substrate (gm%)	Time (min)	H_2SO_4 (gm%)	Temp (ºC)	4hr	24hr	48hr	72hr
1	15	60	5	50	2.5	12.3	13.5	15.6
2	10	60	1	50	1.2	11.2	13.6	18.6
3	10	90	3	50	1.3	10	11.3	21.3
4	5	30	1	50	2.2	11.3	13.9	28.6
5	15	30	3	50	1	13.3	19.5	20.2
6	5	60	3	50	1.3	19.6	19.6	22.3
7	10	30	5	50	2	17.6	18.2	**29.6**
8	15	90	1	50	1.1	16.4	17.1	20
9	5	90	5	50	1.6	14.6	16.2	21.3

Saccharification of chemically treated corncob

Saccharification of lignocellulosic waste was carried out in both alkali and acid. Saccharification was monitored up to 72hour. Their concentration was varied in all trails. Highest saccharification was achieved by substrate treated with different concentration of NaOH. Table V shows Saccharification of corncob treated with NaOH.

When the substrate was subjected to enzymatic saccharification at constant temperature 50°C for time of incubation between 30 to 90 minutes, in 1 to 5% NaOH concentration, highest saccharification observed at 72hour. maximum saccharification was observed with the 15gm of substrate treated with 3% NaOH at 50ºC for 30minutes which denotes optimum condition. Sartori et al.,. **Error! Reference source not found.**depicts 26.787 U/mL for saccharification of corncob using commercial cellulase enzyme[15].

Annamalai et al., study shows of enzymatic saccharification pretreated rice straw by cellulase produced *Bacillus carboniphilus* utilizing lignocellulosic wastes found that the optimum temperature, pH, and NaCl for enzyme activity were determined as 50°C, 9, and 30% and more than 70% of its original activity was retained even at 80°C, 12, and 35%, respectively. In view of that, they suggest that higher temperature, pH, and halo stability of the purified cellulase could be useful for harsh industrial and various biotechnological applications[16].

In case of corncob treated with H_2SO_4, maximum saccharification was observed at 10gm of substrate treated with 5.0 gm% H_2SO_4 for 30 minutes of incubation time.

Whereas in case of untreated corncob, saccharification was obtained up to 20% only. According to Marangoni et al., enzymes present perfect ranges in determined temperature and pH conditions and they are characterized by their high specificity. Enzymatic activity most of the cases influenced by pH and temperature[17].

Saccharification of lignocellulosic waste treated by alkali, they show highest saccharification when compare to acid treated lignocellulosic waste. Saccharification, the reducing sugars arrangement demonstrated continuous increment, staying stable after some period. This might be clarified by the warm denaturation of the compounds and furthermore by the impact of substrate fixation on the enzymatic movement, which as per Michaelis and Menten predicts that the response rates increment in view of the substrate focus until the point that a cut off from which it goes to be steady[18].

Filter paper activity for cellulase shows 0.0016 units/ml activity. According to Sartori et al., maximum enzymatic activity of the cellulolytic complex on filter paper was shown (10.634 U/g) with temperature of 40°C and this results also reveal that enzymatic reaction was favored in the lower level of temperature (40°C)[15]. Dillon et., al showed, result of an FPA 38.6 FPU/mL using the commercial cellulase[19].

X. Conclusion

Termites may produce up to two liters of hydrogen from digesting a single sheet of paper, making them one of the planet's most efficient bioreactors. In the present investigation, a termite, which was used for Cellulase producing bacteria isolation and isolate, exhibited considerable cellulolytic activity. Effect of various substrates on cellulase enzyme production was studied by Plackett Burman design. The statistical design of experiment offers efficient methodology to identify the significant variables and to optimize the factors with minimum number of experiments for cellulose production by isolate from termite gut. In this experiment, the maximum saccharification rate achieved was up to 39% at 72hr for substrate treated with NaOH and untreated sample, which was found to be 20% only. Therefore, pre-treatment of waste is an important step along with its optimum pre-treatment parameter in the synthesis of bioethanol from lignocellulosic waste, and there is a critical need to understand the fundamentals of various processes, which can help in making a suitable choice depending on the structure and the hydrolysis agent.

Further optimization of cellulase production by central composite design would give the interactions of various substrates for better cellulase production as well the results would be further helpful for large scale production of cellulase under optimized environmental conditions.

REFERENCES

[1] M. Ohkuma, S. Noda, and T. Kudo., Phylogenetic Diversity of Nitrogen Fixation Genes in the Symbiotic Microbial Community in the Gut of Diverse Termites, Appl. Environ. Microbiol., 65(11) (1999) 4926–4934.

[2] Peristiwati, Y. S. Natamihardja, and H. Herlini., Isolation and identification of cellulolytic bacteria from termites gut (Cryptotermes sp .), J. Phys. Conf. Ser., 1013 (2018) 012173.

[3] S. Moriya, K. Tanaka, M. Ohkuma, S. Sugano, and T. Kudo., Diversification of the Microtubule System in the Early Stage of Eukaryote Evolution: Elongation Factor 1α and α-Tubulin Protein Phylogeny of Termite Symbiotic Oxymonad and Hypermastigote Protists," J. Mol. Evol., 52(1) (2001) 6–16.

[4] G. Tokuda and H. Watanabe., Hidden cellulases in termites: revision of an old hypothesis, Biol. Lett., 3(3) (2007) 336–339.

[5] A. Ferbiyanto, I. Rusmana, and R. Raffiudin., Characterization and Identification of Cellulolytic Bacteria from gut of Worker Macrotermes gilvus, HAYATI J. Biosci., 22(4) (2015) 197–200.

[6] Z. Barta, K. Kovacs, K. Reczey, and G. Zacchi., Process Design and Economics of On-Site Cellulase Production on Various Carbon Sources in a Softwood-Based Ethanol Plant, Enzyme Res., 2010 (2010) 1–8.

[7] C.-M. Wang, C.-L. Shyu, S.-P. Ho, and S. Chiou., Characterization of a novel thermophilic, cellulose-degrading bacterium Paenibacillus sp. strain B39, Lett. Appl. Microbiol., 47(1) (2008) 46–53.

[8] S. Chovatiya, S. Ingle, D. Patel, and B. Thakkar., Isolation of Bacteria Producing Cellulase from Tilapia Fish Gut and Media Optimization for Celluase Production using Plackett Burman Design, Int. J. Biotech Trends Technol. (IJBTT), 7(2) (2017)13–19.

[9] T. K. Ghose., Measurement of Cellulase Activities, Pure Appl. Chem., 59(2) (1987) 257–268.

[10] S. Sharma et al., Isolation and Characterization of Cellulose Degrading Bacteria of Termite Gut from North Eastern Region of India Special Issue on : Recent Advances in Isolation and Characterization of Cellulose Degrading Bacteria of Termite Gut from North Eastern Region, South Asian J. Exp. Biol., 5(6) (2015) 283–290.

[11] M. Wenzel, M. Berchtold, P. Kampfer, and H. Konig., Aerobic and facultatively anaerobic cellulolytic bacteria from the gut of the termite Zootermopsis angusticollis, J. Appl. Microbiol., 92 (2002) 32–40.

[12] M. Maki, K. T. Leung, and W. Qin., The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass, Int. J. Biol. Sci., 5(5) (2009) 500–516.

[13] G. M. Bohlmann., Process economic considerations for production of ethanol from biomass feedstocks, Ind. Biotechnol., 2(1) (2006) 14–20.

[14] A. Kazemi, S. Rasoul-Amini, M. Shahbazi, A. Safari, and Y. Ghasemi*, Isolation, Identification, And Media Optimization Of High-Level Cellulase Production By Bacillus Sp. Bccs A3, In A Fermentation System Using Response Surface Methodology, Prep. Biochem. Biotechnol., 44(2) (2014) 107–118.

[15] T. Sartori, H. Tibolla, E. Prigol, L. M. Colla, J. A. V. Costa, and T. E. Bertolin., Enzymatic Saccharification of Lignocellulosic Residues by Cellulases Obtained from Solid State Fermentation Using Trichoderma viride, Biomed Res. Int., (2015) 1–9.

[16] N. Annamalai, M. V. Rajeswari, and T. Balasubramanian., Enzymatic saccharification of pretreated rice straw by cellulase produced from Bacillus carboniphilus CAS 3 utilizing lignocellulosic wastes through statistical optimization, Biomass and Bioenergy, 68 (2014) 151–160.

[17] A. G. Marangoni., Enzyme Kinetics: A Modern Approach. , John Wiley & Sons, New York, NY, USA., (2003).

[18] D. Schell., A bioethanol process development unit: initial operating experiences and results with a corn fiber feedstock, Bioresour. Technol., 91(2) (2004) 179–188.

[19] M. Camassola and A. J.P. Dillon., Cellulase Determination: Modifications to Make the Filter Paper Assay Easy, Fast, Practical and Efficient, J. Anal. Bioanal. Tech., 1(S1) (2012) 1–5.