Production of lignocellulosic ethanol from *Lantana camara* by bacterial cellulase of termite symbionts

DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF

MASTER OF SCIENCE IN LIFE SCIENCE

BY:
PRAJNA MISHRA
ROLL NO. 411LS2052

UNDER THE SUPERVISION OF
Dr. R. JAYABALAN
ASST. PROFESSOR
DEPARTMENT OF LIFE SCIENCE
NATIONAL INSTITUTE OF TECHNOLOGY, ROURKELA

DEPARTMENT OF LIFE SCIENCE
NATIONAL INSTITUTE OF TECHNOLOGY
ROURKELA-769008, ORISSA
MAY, 2013

DEPARTMENT OF LIFESCIENCE

NATIONAL INSTITUTE OF TECHNOLOGY

ROURKELA-769008

CERTIFICATE

This is to certify that the thesis entitled "Production of lignocellulosic ethanol from *Lantana camara* by bacterial cellulase of termite symbionts" which is being submitted by Prajna Mishra, Roll No. 411LS2052, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafied research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

Dr. R.Jayabalan
Assistant Professor,
Department of Life Science
National Institute of Technology
Rourkela

ACKNOWLEDGEMNT

It is with a deep sense of gratitude that I wish to thank my guide and supervisor Dr. R. Jayabalan, Assistant Professor, Department of Life Science, National Institute of Technology, Rourkela. His continuous encouragement, unstinted support, love and affection have been the driving forces for me. I also gratefully acknowledge to Dr. Bismita Nayak, Dr. Sujit K Bhutia, Dr. Surajit Das, Dr. Suman Jha, Dr. Bibekanand Mallick and Dr. Sameer Kumar Patra (HOD), Department of Life Science, National Institute of Technology, Rourkela, for their wholehearted help and cooperation. Many bouquets, for the merit and gratitude to Ms. Indira Dash (JRF) , FBT lab (NIT Rkl) for her immense help and advice throughout my project work. I also wish to thank Ms. Moumita sahoo and Mr. Ajay Dethose for their good wishes, ceaseless encouragement, prudent suggestions and timely advice during my work. I express my heartiest devotion and deep sense of gratitude to my beloved parents for their encouragement, moral support, love and blessings bestowed on me without which the present investigation would not have been successful. Last but not the least; I bow my head before Almighty for his blessings.

Date: Prajna Mishra
Place: Rourkela

DECLARATION

I hereby declare that the thesis entitled "Production of lignocellulosic ethanol from *Lantana camara* by bacterial cellulase of termite symbionts" submitted to the Department of LIFE SCIENCE, National Institute of Technology, Rourkela for the partial fulfilment of the Master of Science in Life Science is a faithful record of original research work carried out by me under the guidance and supervision of Dr. R. Jayabalan, Department of Life Science, NIT, Rourkela. No part of this thesis has been submitted by any other research persons or any students.

Date:
Place: Rourkela Prajna Mishra

CONTENTS

List of Figures

Abbreviation

gm	Gram
mol	Mole
lit	Liter
mL	Millilitre
%	Percentage
^{0}C	Degree Centigrade
CMC	Carboxy methyl cellulose
TSA	Trypticase soy agar
YE	Yeast extract
TCPB	Termite cellulose producing bacteria
Rpm	Rotation per Minute
SHF	Separate saccharification and fermentation
DNS	Dinitro salicylic acid
SG	Specific gravity

Abstract

Due to the rapid growth in population and industrialization, worldwide ethanol demand is increasing continuously. Lignocellulosic biomasses are most abundant and renewable sources of the world and they can act as a promising source for bioethanol production. The major objective of the work was to evaluate the effect of acid and steam pretreatment on *Lantana camara* for improved yield of bioethanol production by using cellulase production by bacteria isolated from termite gut and optimization of conditions required for maximum activity of cellulase enzyme. Cellulase producing bacteria isolated from termite's gut screened by congored test. Cellulase activity was measured by DNS method. From the present study it is concluded that bacteria isolated from termite gut was producing maximum amount of cellulase enzyme after 40 hours (TCDB1, 2) and after 60 hours (TCDB 3). Cellulase enzyme produced by bacteria isolated from termite gut was found to have pH around 5, temperature 50°C for TCDB 1 and 70°C for TCDB 2 and 3 as optimum conditions .Activity of Cellulase enzyme produced by bacteria isolated from termite gut was found to be increased by the addition of 5 mM $MnSO_4$ (all three TCDB) and $MgSO_4$ (only TCDB3). It Possible to produce lignocellulosic bioethanol (11.66%) from *Lantana camara* after steam and acid pretreatment by using Cellulase for Saccharification (72 hours) and *Saccharomyces cerevisiae* for fermentation (72 hours). Bioethanol from lignocellulosic biomass is a globally accepted alternative fuel. The production of ethanol from *Lantana camara* would have the dual advantage of producing energy and serving as an effective method of weed management.

Key words: *Lantana camara*, Lignocellulosic biomass, Termite symbionts, Bioethanol

1. INTRODUCTION

The world has been confronted with an energy crisis due to depletion of finite resources of fossil fuel, difficulties in their extraction and processing, leading to an increase of its cost. Also fossil fuels contribute an important role in accumulation of greenhouse gases (GHG) which can ultimately pollute the environment.

Fossil fuels are being used for the production of fuel, electricity and other goods. Excessive consumption of these fossil fuels has resulted in high levels of pollution during the last few decades. The level of greenhouse gasses in the earth's atmosphere has drastically increased. In this scenario, renewable sources might serve as an alternative. Hence it is necessary to look forward for alternative fuels.

Wind, water, sun, biomass and geothermal heat can be the renewable sources for the energy industry, where as fuel production and the chemical industry may depend on biomass as an alternative source in the near future. This includes bioethanol, biohydrogen and biodiesel. Ethanol, which is a oxygenated fuel having high octane value has worldwide attention because of its multi field potential use

The traditional feed stocks like molasses, sugarcane juice, corn etc. are used for ethanol production but have social and economical barriers. Apart from these feed stocks, lignocellulosic biomass, which is the most abundant on earth (Purwadi et al., 2004), is an alternative feed stock for bioethanol production. This biomass including forest residues such as wood, agricultural residues such as sugarcane, industrial residues such as pulp and paper processing waste and most potential feed stocks for fuel ethanol (Mielenz et al., 2011).

Ethanol is considered the most potential next generation automotive fuel because it is carbon-neutral and could be produced from renewable resources like lignocellulosic biomass. Production of bioethanol from lignocellulosic biomass is known as second generation fuel. Lignocellulosic biomass has several advantages having better efficiency. In recent years, research and development efforts directed toward commercial production of ethanol as biofuel from renewable resources have increased.

Lignocellulosic biomass has been recognized as a promising resource for the production of bioethanol due to its abundance, low cost and non-competitiveness with foodstuffs. Lignocellulose is the most plentiful renewable biomass produced from photosynthesis. Cellulose, hemicelluloses and lignin are major components of the lignocellulosic biomass.

Lignocellulosic biomasses comprise cellulose (20–50 %), hemicelluloses (20–35 %), polyphenolic lignin (10–35 %), and other components (Knauf et al., 2004). Cellulose (linear polymer of several β -(1, 4) linked D-glucose units) binds tightly with lignin and hemicelluloses. For efficient hydrolysis of cellulose, lignin component must be separated in order to make cellulose more accessible to the enzymes (Hussain et al., 2009)

The biological process for converting lignocellulose to fuel ethanol requires three major steps; these are (1) delignification to liberate cellulose and hemicellulose; (2) depolymerisation of

carbohydrate polymers to produce free sugars; and (3) fermentation of mixed hexose and pentose sugars to produce ethanol. Pretreatment for delignification is necessary to liberate cellulose and hemicellulose before hydrolysis; hydrolysis of cellulose and hemicellulose to produce fermentable sugars including glucose, xylose, arabinose, galactose, mannose and fermentation of reducing sugars. The non-carbohydrate components of lignin also have value added applications (Balat et al., 2008). All these process accomplished with acid and enzymatic pretreatment. Pretreatment is one of the key strategies to access enzymatic saccharification of lignocellulosic biomass. The enzymatic hydrolysis of cellulose is affected by several factors, i.e., degree of polymerization, degree of crystallinity, structural composition and availability of surface area, etc (Gupta et al., 2009).

The most important criterion for selecting a suitable biomass for bioethanol production is its availability in a particular locality. The composition of the particular biomass is also very important before it can be considered for biofuel production. A particular biomass containing more than 70% cellulose and hemicelluloses can be considered as a good candidate. The lignin content should be less than 20% (Sindhu et al., 2010). *Lantana camara* fulfil all the above criteria. So it can be used as a potent lignocellulosic biomass for bioethanol production.

Lantana camara is an important non edible lignocellulosic biomass, which grows widely throughout India. It is nonedible due to its high toxin content (lantanedeneA , B and triterpene acid) and has been found to be toxic to animals, too But several important characteristics of *Lantana camara* such as easy availability, high cellulose content and no competition with the food chain makes it an ideal substrate for bioethanol production (Sharma et al.,2010).

The present study aimed to produce bioethanol using *Lantana camara* as substrate. In this work, enzymatic pretreatment was carried out by using cellulase enzyme, obtained from bacterial symbionts of termite gut. Fermentation was carried out using yeast isolated from Handia (a fermented rice product of Odisha). Considering the future needs, it is believed that Lantana camara could be one of the most promising source of lignocellulosic biomass for bioethanol production because of its ready availability, high cellulose content, and no competition with food chain having high feasibility.

2. REVIEW OF LITERATURE:

2.1. Ethanol

Ethanol (C_2H_5OH) is a colourless volatile flammable liquid act as an intoxicating agent in liquors and alcoholic beverages, also used as a solvent and as fuel. It is an oxygenated fuel with high octane value having a flash point of 16.6c. Ethanol for industrial purposes is made by either fermentation or chemical synthesis, and then purified by distillation. It is used in the synthesis of other organic chemicals, and as an additive to automotive gasoline. Ethanol, which is an oxygenated fuel have great importance because of its multi field use.

2.2. Bioethanol

Bioethanol is a high-octane, water-free alcohol produced from the fermentation of sugar or converted starch. In its purest form it is a colourless clear liquid with mild characteristic odour that boils at 78°C and freezes at -112°C. These are exclusively derived from plant origin. Bioethanol has all most all characteristics same as that of synthetic ethanol.

2.3. Need for Bioethanol

The world has been confronted with an energy crisis due to depletion of finite resources of fossil fuel, difficulties in their extraction and processing, leading to an increase of its cost. Also fossil fuels contribute an important role in accumulation of greenhouse gases (GHG) which can ultimately pollute the environment. These are being used for the production of fuel, electricity and other goods. Excessive consumption of these fossil fuels had resulted in high levels of pollution during the last few decades. The level of greenhouse gasses in the earth's atmosphere has drastically increased. In this scenario, renewable sources might serve as an alternative. Hence it is necessary to look forward for alternative fuels. Wind, water, sun, biomass and geothermal heat can be the renewable sources for the energy industry, where as fuel production and the chemical industry may depend on biomass as an alternative source in the near future. This includes bioethanol, biohydrogen and biodiesel. Looking for future needs bioethanol is considered as the promising source, which completely replace fossil fuel.

2.4. Generations of Bioethanol

2.4.1. First generation bioethanol

These are mainly extracted from food crops like sugarcane, sugar beets, maize, and molasses using conventional technology. Its growth and development is limited due to competition with food and fibre production for the use of arable land, regionally constrained market structures, lack of well managed agricultural practices in emerging economies, high water and fertilizer requirements, and a need for conservation of bio-diversity.

2.4.2. Second generation bioethanol

This is intended to produce ethanol from the whole plant matter or agricultural residues, forest harvesting residues or wood processing waste, weeds rather than from food crops. To reduce the dependency on food crops, alternative biofuel sources, such as non-food feedstocks, have been developed to produce bioethanol. Lignocellulosic biomass fall under this category are promising source for second generation bioethanol production (Kim et al., 2002).

2.4.3. Third generation bioethanol

The third generation bioethanol, which are derived from microalgae, have emerged as one of the most promising source of bioethanol production because of their high photosynthetic efficiency to produce biomass and their higher growth rates and great productivity compared to conventional crops.

2.5. Lignocellulose

Lignocelluloses are the main structural component of the plants. The main constituents of lignocellulosic biomass comprise lignin, hemicellulose and cellulose (Fig.1). This is a complex structure in which the cellulose is surrounded by a monolayer of hemicellulose and embedded in a matrix of hemicellulose and lignin. Furthermore lignin specifically creates a barrier to enzymatic attack while the highly crystalline structure of cellulose is insoluble in water while the hemicellulose and lignin create a protective sheath around the cellulose. (Carillo et al., 2005) .This structure of lignocellulose therefore plays a huge role in inhibiting degradation of the hemicellulose and cellulose structure to monomeric sugars which is necessary to effectively convert biomass into ethanol. Processing of lignocellulose is therefore essential for the conversion of lignocellulosic biomass to biofuel such as bio-ethanol (Dillion et al., 2004). Lignocellulose is the most plentiful renewable biomass produced from photosynthesis. Cellulose, hemicelluloses and lignin are major components of the lignocellulosic biomass. Lignocellulosic biomasses comprise cellulose (20-50%), hemicelluloses (20–35%), polyphenolic lignin (10–35%), and other components (Knauf et al., 2004).

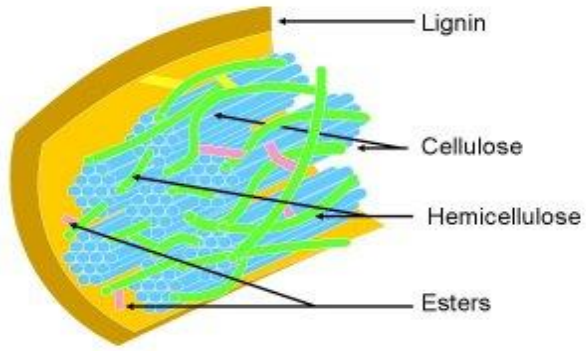

Fig.1: Major composition of lignocellulosic biomass (Ref: www.chem alignment.com)

Cellulose consists of repeating unit of cellobiose. The D- glucopyranose units are lineally linked together by β- (1, 4) glycosidic linkage. These are supporting material of cell wall. Cellulose molecule aggregate with each other due to hydrogen bonding and form micro fibrils, which are building block of cellulose molecule. Consist of two region namely crystalline region and amorphous region (Moiser et al., 2005). Hemicellulose is a polymer that composed of several different sugars like D-glucose, D-mannose, D-Xylose and D- arabinose and also different uronic acids. The degree of polymerization of hemicellulose is about 100-200 and molecule can highly branched (Wana et al., 2011). Lignins are complex polymer consists of phenylpropanre units linked together by ether or carbon-carbon bonds. Its classification based on their structural elements and differs from species to species. Besides these three main components lignocellulosic biomass contain a group of chemical called as extractives which include terpinoids, steroids and phenolic constituents. Some of them are important for plant's defence system.

2.6. General outline of the lignocellulose to bioethanol production process (Fig. 2)

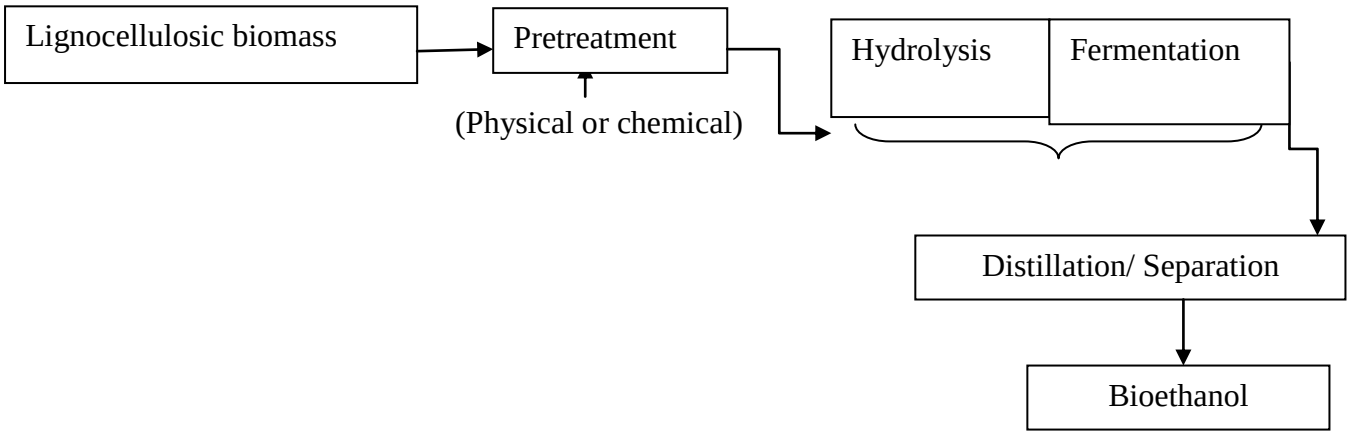

Fig.2: General process of bioethanol production from lignocellulosic biomass.

2.7. *Lantana camara* as a source of lignocellulosic biomass

Lantana camara, commonly known as red sage, is one of the world's top 100 worst invasive species. A particular biomass containing more than 70% cellulose and hemicelluloses can be considered as a good candidate for bioethanol production. The lignin content should be less than 20% (Sindhu et al., 2010). *Lantana camara* fulfill all the above criteria. So it can be used as a potent source as lignocellulosic biomass for bioethanol production (Fig. 3). *Lantana camara* is an important non edible lignocellulosic biomass, which grows widely throughout India. It is nonedible due to its high toxin content (Lantanedene A, B and triterpene acid) and has been found to be toxic to animals too. But several important characteristics of *Lantana camara* such as easy availability, high cellulose content and no competition with the food chain makes it an ideal substrate for bioethanol production (Sharma et al.,2010)

Taxonomic Position

Division: Magnoliophyta

Class: Magnoliopsida

Order: Lamiales

Family: Verbenaceae

Scientific name: *Lantana camara* L.

Common names: Sleeper weed, lantana, wild sage

Fig .3: A twig of *Lantana camara,* source of lignocellulosic biomass

Lantana camara has also a strong industrial importance, as a source of oleanolic acid and carboxymethylcellulose (CMC). *Lantana camara* biomass can be implicated as a substrate for bioethanol and biogas production (Gamez et al., 2005).

2.8. Cellulase enzyme

Cellulase is an enzyme complex which breaks down cellulose to beta-glucose (Fig.4). It is produced mainly by symbiotic bacteria in the ruminating chambers of herbivores. Cellulase refers to a family of enzymes which act in concert to hydrolyze cellulose. Cellulase is widely distributed throughout the biosphere and are most manifest in fungal and microbial organisms.

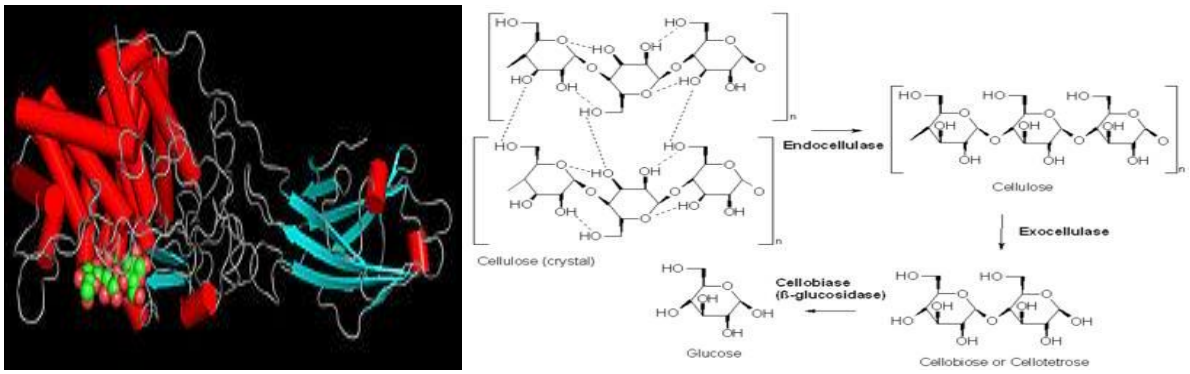

Fig.4: Cellulase enzyme complex (Ref: www. Chem alignment.com)

Three general types of enzymes make up the cellulase enzyme complex. Endocellulase breaks internal bonds to disrupt the crystalline structure of cellulose and expose individual cellulase polysaccharide chains. Exocellulase cleaves 2-4 units from the ends of the exposed chains produced by endocellulase, resulting in the tetrasaccharides or disaccharide such as cellobiose. Cellobiase or beta-glucosidase hydrolyses the endocellulase product into individual monosaccharides. It has been found that enzyme preparations containing only endocellulases have little effect on native cellulose. On the other hand those containing both endo and exocellulases will cause significant degradation of native cellulose. Thus, the endo and exocellulases appear to work in a synergistically or cooperative manner on native cellulose (Karamakar and Ray., 2010).

2.9. Termite gut as a source of cellulase enzyme

Termites play an important role in the turnover and mineralization of complex bio polymers, such as wood and other cellulose and hemicelluloses containing materials (Wenzel et al., 2002).

Termites play a great role in terrestrial ecosystem by recycling lignocellulosic biomass, which refers to a mixture of cellulose, hemicellulose and lignin. Termites are one of the most important soil insects that efficiently decompose lignocelluloses with the aid of their associated microbial symbionts to simpler form of sugars, which later can be fermented to ethanol using yeasts. Termites are said to dissimilate a significant proportion of cellulose (74-99%) and hemicellulose (65-87%) components of lignocellulose they ingest (Ohkuma., 2003).

There are mainly three types of termites found namely Soldier termite, queen termite and general worker termite. Termites gut is composed of three main regions: foregut, midgut and hindgut. Foregut region consists of the oesophagus, crop and salivary gland. Salivary gland secretes endogenous glucanase and other relevant enzymes into the digestive tract. The midgut is slender tubular region that secretes a peritrophic membrane around food material and presumably is a location where some lignocellulosic degradation occurs. Midguts of higher termites are also known to secrete endoglucanase. The hindgut consists of a fermentation chamber or paunch that is generally anaerobic but does possess a micro-oxic zone around its periphery. Hindgut houses the majority of gut symbionts and is the location where most cellulose degradation as well as fermentation occurs. Analogous to kidney, malpighian tubules connect at junction of midgut and hindgut and participate in waste excretion and nitrogen recycling. So choosing termite as a source to isolate cellulose digesting bacteria is good choice.

3. Objectives

1. To isolate cellulase producing bacteria from termite gut

2. To study the effect of time on production of cellulase by bacteria

3. To study the effect of pH, temperature and metals on cellulase activity

4. To produce bioethanol from steam and acid pretreated lignocellulosic biomass of *Lantana camara*

4. Materials and Methods

4.1. Collection of termites

Termites were collected from institute campus National Institute of Technology Rourkela, Odisha on the month of January. General worker termites were selected for isolation of cellulose producing bacteria (Fig.5).

Fig 5. Different types of termites

4.2. Preparation of gut extract from termites

Termites were surface sterilized in 70% ethanol for 2 min followed by three washes in sterile distilled water. The posterior paunch and colon regions were dissected from the termite abdomen and placed in separate reaction vessels containing 2 ml sterile phosphate buffer (10 mM $Na_2HPO_4\_2H_2O$, 1.8 mM KH_2PO_4; pH 7.0). The contents were vigorously vortexed for 5 min. A standard serial dilution series was performed using sterile phosphate buffer (same as above). 100 µl of each dilution was plated on to Trypticase Soy Agar (TSA). Cultures will be incubated at 30°C until maximum colony development occurs. Each colony type will be restreaked several times in sequence of TSA to obtain pure cultures (Tartar et al., 2008).

4.3. Isolation of cellulase producing bacteria from termite's gut extract

The macerated gut of the collected organisms was inoculate in Trypticase soy agar (TSA) media (15 g Tryptone, 5 g Soytone ,5 g Sodium Chloride ,15 g agar/ 1L Distilled water). Individual colony was obtained by streaking the culture in same media. Confirmation of cellulose-degrading ability of bacterial isolates was performed by streaking on the Carboxy methyl cellulose (CMC) media with the following composition: 15 g CMC, 3 g $NaNO_3$, 3 g K_2HPO_4, 3 g KCl, 0.5 g $MgSO_4$, 0.5 g yeast extract , 1g glucose, 17g agar. Congored (1mg/ 1 ml of distilled water) was used as staining solution and 1M NaCl as a de staining solution. The use of Congo-Red as an indicator for cellulose degradation in an agar medium provides the basis for a rapid and sensitive screening test for cellulolytic bacteria. Colonies showing discoloration of Congo-Red was taken as positive cellulose-degrading bacterial colonies (Wang et al., 2004). And only these were taken for further study.

4.4. Cellulase Enzyme Production

The selected CDB isolates were cultured at 37℃ at 150 rpm in an enzyme production media composed of 1g $NaNO_3$, 1g KH_2PO_4, 1g KCl,0.5 $MgSO_4$, 0.5g yeast extract , 1g glucose and at pH 6.8–7.2. Broth culture after three days of incubation period was subjected to centrifugation at 5000 rpm for 15 min at. Supernatant was collected and stored as crude enzyme preparation at 4℃ for further enzyme assays (Girard et al., 1989).

4.5. Cellulase Assay

Total cellulose activity was determined by measuring the amount of reducing sugar formed from CMC. Endoglucanase (*β*1-4 endoglucanase-EC 3.2.1.4).Activity was assayed by measuring the amount of reducing sugar from amorphous cellulose. In these tests, reducing sugars were estimated spectrophotometrically with3, 5-dinitrosalicylic acid using glucose as standards (Ghose 1987). Then enzymatic activities of total endoglucanase were defined in units. One unit of enzymatic activity is defined as the amount of enzyme that releases one micromole reducing sugars (measured as glucose) per minute.

4.6. Effect of time period on cellulase activity

Different incubation times (24, 30, 36, 42, 48, 54, 60, 66, 72 hours) were employed to study their effect on the cellulase activity. The culture filtrates were collected at respective time interval and assayed (Gamez et al., 2005).

4.7. Effect of temperature on cellulase activity

The experiment was carried out at different temperatures such as 30, 40, 50, 60, 70°C to study their effect on cellulase activity. The culture filtrates were then collected and assayed (Gamez et al., 2005).

4.8. Effect of pH on cellulase activity

The pH of the test solution was adjusted to different pH range of 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.5, and 8.8 with phosphate buffer (10 mM Na_2HPO_4_$2H_2O$, 1.8 mM KH_2PO_4). The production was carried out to study their effect on enzyme activity (Gamez et al., 2005).

4.9. Effect of metals on cellulase activity

After getting optimum temperature and pH value different metal ions such as $MgSO_4$, $ZnSO_4$, $MnSO_4$, $FeCl_2$ and CoCl2, were subjected to study their effect on enzyme activity. They all were taken in a concentration of (5 mM) and assayed (Sommer et al., 2004).

4.10. Collection of *Lantana camara*

Lantana camara was collected locally from National Institute of Technology Rourkela, Odisha campus on the month of December. It was sun dried for one month followed by making it powder by using mixer grinder. Stored under dry condition for further use.

4.11. Pretreatment of Lantana *camara* stems

Lantana *camara* stems were pretreated by steam treatment and acid treatment.Steam treatment (autoclaving the slurry (5%, w/v) for 15 mins. Supernatant discarded and pellet was obtained).Acid treatment (5% H_2SO_4, 140°C for 45 min incubation. Supernatant discarded and pellet was obtained) (Ghose et al., 2003).

4.12 Saccharification

Enzymatic hydrolysis performed separately from fermentation step is known as separate hydrolysis and fermentation (SHF) (Wingren et al., 2003). In saccharification process 10ml of mixed enzyme from TCPB1, TCPB2, and TCPB3 mixed with 10g of steam and acid pretreated biomass incubated in 55℃ for 72 hrs. After 3 days Saccharified biomass was heated up to 90°C and cooled. Centrifuged at 5000 rpm for 10 mins and supernatant was obtained. Then supernatant which was obtained subjected for fermentation process.

4.13. Fermentation

Saccharomyces cerevisiae grown in glucose yeast extract broth medium for 48 hrs.10% inoculum was inoculated into 50 mL fermentation medium containing Saccharified solution of Lantana *camara* from the previous step and kept for 3 days at room temperature.Ethanol was estimated by gravimetric analysis (Ghose et al ., 2003).

4.14. Estimation off Ethanol

Bioethanol produced by separate saccharification and fermentation process was estimated by gravimetric analysis by using following formula.

$$\% \text{ of alcohol} = \frac{1.50 * (SG1 - SG2) * 100}{SG2}$$

Where

SG1= Initial specific gravity SG2= Final specific gravity

5. Result and discussion

5.1. Isolation and Screening of Cellulose producing Bacteria

Cellulose producing bacteria were enriched and isolated by inoculating termite gut extract on TSA medium and by restreaked on CMC agar medium. A total of three bacterial isolates found to be positive on screening media (CMC agar) producing clear zone with Congo red stain (as shown in Fig. 6).

Fig.6: Zone of clearance on CMC agar plates for isolate cellulose producing bacteria after 48 hrs of incubation. The formation of clearing zone around the colonies confirms the secretion of extracellular cellulase.

5.2 Effect of time period on cellulase activity

Enzyme activity recorded at different time period revealed that all the three TCPB yielded maximum cellulase production at 72 hrs of incubation (Figure 7). The time period was found to influence extracellular enzyme secretion, possibly by changing the physical properties of the cell membrane.

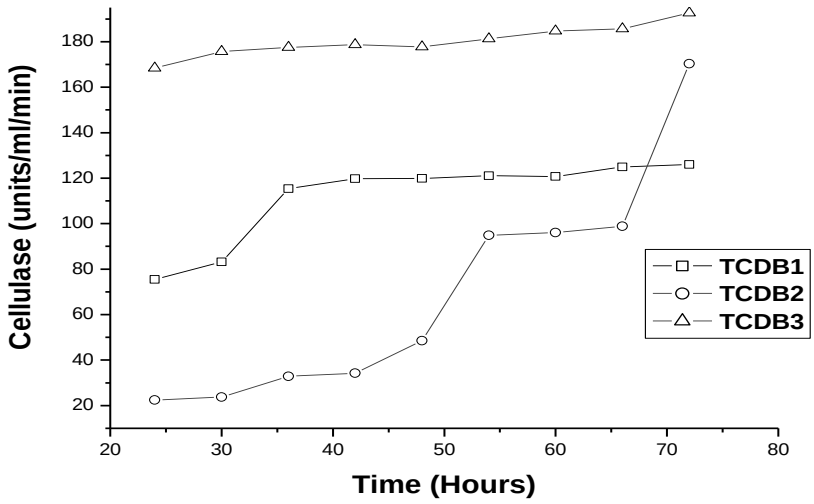

Fig. 7: Effect of time period on cellulase by termite symbionts

5.3. Effect of temperature on cellulase activity

Enzyme activity recorded at different temperature revealed that the optimum temperature for TCPB1 is 50̊ c and for bacteria TCPB2 and TCPB3 is 70̊ c (Fig 8), where maximum enzyme production occurs. Like most chemical reactions, the rate of an enzyme-catalyzed reaction increases as the temperature is raised. In every ten degree centigrade rise in temperature will increase the activity of most enzymes by 50 to 100%. Here the enzyme may infer as therostable enzyme as it can withstand temperature up to 50̊c to 70̊c and shows its maximum activity.

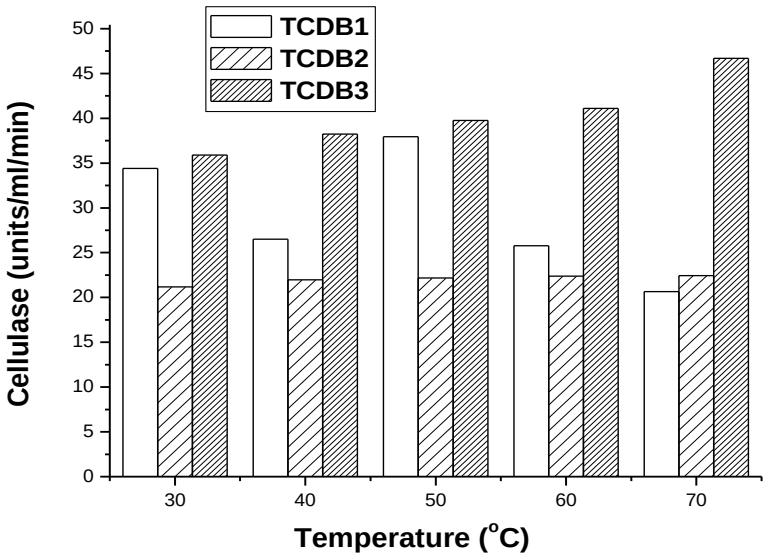

Fig .8: Effect of temperature on cellulase by termite symbionts

5.4. Effect of pH on cellulase

Enzymes are affected by changes in pH. The most favourable pH value is the point where the enzyme is most active -is known as the optimum pH. The three TCPB enzymes were allowed to test in different pH ranging from 3.0 to 8.8. Maximum enzyme activity was observed around pH 5.0 in case of all three TCPB (Figure 9). As TCPB were isolated from termite gut it shows acidic pH value.

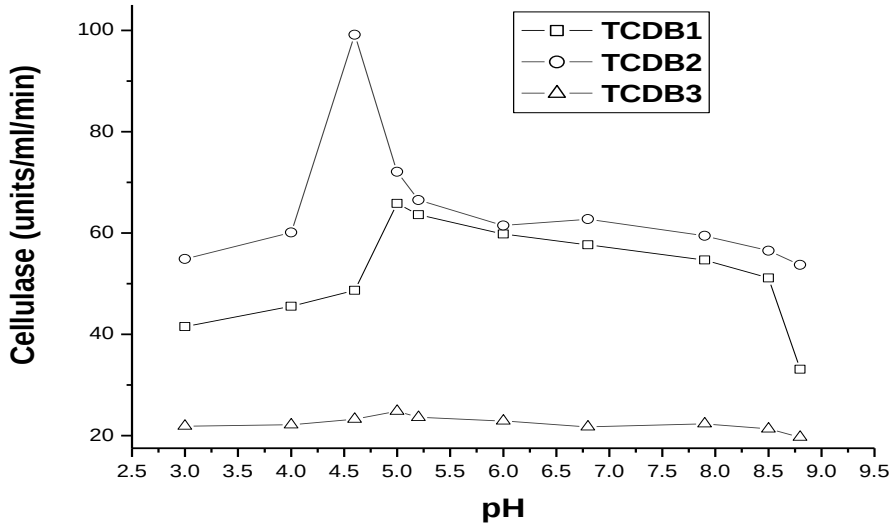

Fig. 9: Effect of pH on cellulase production by termite symbionts

5.5. Effect of metals on cellulase activity

After getting optimum temperature and pH value different metal ions such as MgSO4, ZnSO4, MnSO4, FeCl2, and CoCl2 were subjected to study their effect on enzyme activity. Activity of Cellulase enzyme produced by bacteria isolated from termite gut was found to be increased by the addition of 5 mM $MnSO_4$ (all three TCPB) and $MgSO_4$ (only TCPB1). It can be inferred that $MnSO_4$ (all three TCPB) and $MgSO_4$ (only TCPB 3) may act as a cofactor or enhancer of enzyme productivity (Fig .10

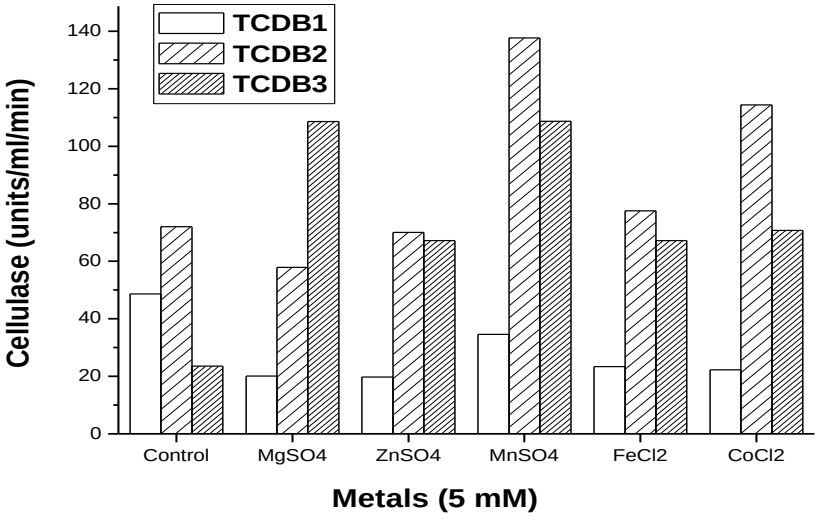

Fig .10: Effect of metal ions on cellulase activity

5.6. Bioethanol production

The experiment setup for separate saccharification and fermentation of mixed bacterial culture (TCPB 1, 2, 3) with *Saccharomyces cerevisiae* resulted in production of ethanol, estimated by gravimetric analysis and it was 11.66%.The less amount of ethanol production is due to there may be some inhibitor for cellulase enzyme from lignin and hemicellulose even after saccharification and to maintain yeast growth in its optimal temperature is very difficult at room temperature in summer season.

6. Conclusion

From the present study it is concluded that bacteria isolated from termite gut was producing maximum amount of cellulase enzyme after 40 hours (TCPB1, 2) and after 60 hours (TCPB 3). Cellulase enzyme produced by bacteria isolated from termite gut was found to have pH around 5, temperature 50°C for TCPB 1 and 70°C for TCPB 2 and 3 as optimum conditions .Activity of Cellulase enzyme produced by bacteria isolated from termite gut was found to be increased by the addition of 5 mM $MnSO_4$ (all three TCPB) and $MgSO_4$ (only TCPB3).It Possible to produce lignocellulosic bioethanol (11.66%) from *Lantana camara* after steam and acid pretreatment by using Cellulase for Saccharification (72 hours) and *Saccharomyces cerevisiae* for fermentation (72 hours). Bioethanol from lignocellulosic biomass is a globally accepted alternative fuel. The production of ethanol from *Lantana camara* would have the dual advantage of producing energy and serving as an effective method of weed management.

References

Balat M, Balat H, Oz C. Progress in bioethanol processing. Progress in Energy and Combustion Science 2008; 34:551e73.

Binod P, Sindhu R, Singhania RR, Vikram S, Devi L, Nagalakshmi S, Bioethanol production from rice straw: an overview. Bioresource Technology2010; 101:4767–74.

Carillo, F., Lis, M.J., Colon, X., Lo'pez-Mesas, M., Valladeperas, J., 2005. Effect of alkali Pretreatment on cellulose hydrolysis of wheat straw, kinetic study. Process Biochem. 40, 3360–3364

Dwivedi P, Vivekanand V, Pareek N,Sharma A, Singh RP. Bleach enhancement of mixed wood pulp by xylanase-laccaseconcoction derived through co-culture strategy.Appl Biochem Bioethanol 2010; 160:255–68.

Gupta R, Mehta G, Khasa YP, Kuhad RC.Fungal delignification of lignocellulosic biomass improves the saccharification of cellulosics. Biodegradation 2010 Aug 14.[Epub ahead of print]; doi: 10.1007/s10532-010-9404-6.

J. R. Mielenz, Curr. Opin. Microbiol. 2011, 4 (3), 324. DOI: 10.1016/S1369-5274 (00)00211-213

K. H. Kim, M. P. Tucker, Q. A. Nguyen, Bioethanol. Prog.2002, 18 (3), 489. DOI: 10.1021/bp025503i

M.Karamkar and R.R.Ray, (2010).Extracellular endoglucanase production by Rhizopus oryzae in solid and liquid state fermentation of agro waste. Asian journal of Biotechnology, 2(1):27-36.

M. Knauf, M. Moniruzzaman, Int. Sugar J. 2004, 106 (1263), 147.

Hussain MA, Huq ME, Rahman SM. Estimation of lignin in jute by titration method. Pak. J Biol Sci 2002; 5:521–2.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple,M. and Ladish, M. (2005) Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technology 96, 673–686.

Ohkuma M, Noda S, Kudo T (1999b) Phylogeny of symbiotic methanogenes in diverse termites. FEMS Microbiol Lett 171:147–153.

P. Ghosh, T. K. Ghose, in Advances in Biochemical Engineering/Biotechnology (Ed: T. Scheper), Vol. 85, Springer, New York **2003**. DOI: 10.1007/3-540-36466-8

R. J. Dillon and V. M. Dillon, "The gut bacteria of insects non-pathogenic interaction," Annual Review of Entomology, vol. 49, pp. 71–92, 2004.

R. Purwadi, C. Niklasson, M. J. Taherzadeh, J. Biotechnol.2004, 114, 187. DOI: 10.1016/j.jbiotec.2004.07.006

Scharf, M.E., Tartar, A. 2008. Termites digestomes as sources for novel lignocellulases. Biofuels. Bioproducts and Biorefining, 107 : 1-13

S. Gámez et al., J. Food Eng. 2006, 74, 78.DOI: 10.1016/j.jfoodeng.2005.02.005

T. K. Ghose, “Measurement of cellulase activity,” Pure and Applied Chemistry, vol. 59, pp. 257–268, 1987.

Wana C, Zhou Y, Li Y. Liquid hot water and alkaline pretreatment of soybean straw for improving cellulose digestibility. Bioresource Technology 2011; 102: 6254e9.

Wingren A, Galbe M, Zacchi G (2003). Techno-Economic evaluation of producing ethanol from softwood: comparison of SSF and SHF and identification of bottlenecks. Biotechnol. Prog. 19: 1109-1117.

W. J. Lu, H. T. Wang, Y. F. Nie et al., “Effect of inoculating flower stalks and vegetable waste with ligno-cellulolytic microorganisms on the composting process,” Journal of Environmental Science and Health, Part B, vol. 39, no. 5-6, pp. 871–887, 2004.