P-3165

UTILIZATION OF BACTERIAL CELLULASE FOR THE BIOCONVERSION OF CELLULOSE INTO ETHANOL

A PROJECT REPORT

Submitted by

SAMEENA.M

in partial fulfillment for the award of the degree

of

BACHELOR OF TECHNOLOGY

in

BIOTECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY, COIMBATORE

ANNA UNIVERSITY :: CHENNAI 600 025

APRIL 2010

ANNA UNIVERSITY: CHENNAI 600 025

BONAFIDE CERTIFICATE

Certified that this project report "**UTILIZATION OF BACTERIAL CELLULASE FOR THE BIOCONVERSION OF CELLULOSE INTO ETHANOL**", is the bonafide work of SAMEENA.M who carried out the project work under my supervision.

SIGNATURE

Dr.S.SADASIVAM

DEAN

Department of Biotechnology
Kumaraguru College of Technology
Coimbatore – 641 006

SIGNATURE

Mr.T.SATHISH KUMAR

SENIOR LECTURER

Department of Biotechnology
Kumaraguru College of Technology
Coimbatore - 641006

Focusing the Science for the future

AIBI
Akshaya Institute of
Biotechnology & Informatics

April 07, 2010

CERTIFICATE

This is to certify that the dissertation entitled "Utilization of bacterial cellulase for the bioconversion of cellulose into ethanol" , is a bonafide record of the original research work done by Miss.SAMEENA.M of Kumaraguru College of Technology, Coimbatore (affiliated to the Anna University, Chennai) in partial fulfillment of the requirement for the award of degree of B.Tech in Biotechnology during the peroid of her study in the Department of Biotechnology, under my direct supervision and guidance in our center and the reported work has not formed the basis for the award of any other Degree/ Diploma / Assoiciateship / Fellowship or other similar title to any candidate of any university.

AKSHAYA INSTITUTE OF BIOTECHNOLOGY & INFORMATICS

K.S.Rishad
(Supervising guide)

Thaikkattukara p.o, Ernakulam Dt., Kerala-683106. Ph: 0484-2621361/62/63. Fax: 0484-2626639/83
email:info@akshayainstitutions.com, web: www.akshayainstitutions.com

CERTIFICATE OF EVALUATION

COLLEGE : Kumaraguru College of Technology

BRANCH : Biotechnology

SEMESTER : Eighth Semester

NAME OF THE STUDENT	TITLE OF THE PROJECT	NAME OF THE GUIDE WITH DESIGNATION
SAMEENA.M	**UTILIZATION OF BACTERIAL CELLULASE FOR THE BIOCONVERSION OF CELLULOSE INTO ETHANOL**	**Mr.T.SATHISH KUMAR SENIOR LECTURER**

The report of the project work submitted by the above students in partial fulfillment for the award of Bachelor of Technology degree in Biotechnology of Anna University was evaluated and confirmed.

(INTERNAL EXAMINER)

(EXTERNAL EXAMINER)

Acknowledgement

ACKNOWLEDGEMENT:

First and foremost, we thank the Almighty for giving us the courage and strength to carry out the project work successfully.

I wish to express my sincere gratitude to **Dr. S. Ramachandran** Principal and the college management for their help and support during the period of this investigation.

I wish to place on record our gratitude and heartfelt thanks to **Dr. S. Sadasivam**, The Head of the Department, for his constant motivation and support throughout the period of this investigation

I wish to express my deep sense of gratitude and profound heartfelt thanks to **Mr.T. Sathish Kumar** Senior Lecturer and **Mr. k.s Rishad** ,project supervisor, Akshaya Institute of Biotechnology and Informatics, for their valuable guidance, constant encouragement, constructive criticisms and enthusiastic suggestions in each and every step of this investigation.

I am highly thankful to **Dr. N.Saraswathy**, Assistant Professor for her valuable input of ideas and suggestions during this investigation as Members of the Review Panel.

I wish to place on record our sincere thanks to **Mr. Ramalingam**, Assistant Proffesor and all my staff members for their constant support and valuable suggestions during the course of this study.

I am highly thankful to all the non-teaching staff members of the Department of Biotechnology for their kind and patient help in all respects of this project work.

I wish to thank my friends for providing me with the moral support throughout my course of the project. Last but not the least, we express my gratitude to my parents for their motivation, unflinching support and encouragement which has enabled me to climb the ladder of success.

SAMEENA.M

Abstract

ABSTRACT

Production of ethanol from cellulose has the advantage of abundant and diverse raw material compared to sources like corn and cane sugars, but requires a greater amount of processing to make the sugar monomers available to the microorganisms that are typically used to produce ethanol by fermentation. The collected samples were serially diluted and plated on nutrient agar plates; well isolated colonies were selected from master plates and inoculated in to Congo red agar plates. Bacteria which have potential capacity to degrade cellulose will produce good zones on Congo red agar media. That bacteria was identified according to the gram characteristics and biochemical tests. Here the bacteria which identified as bacillus species. After identification they were inoculated in to carboxy methyl cellulose broth. The banana agro waste (Pseudo stem) substrate was prepared and the isolates from carboxy methyl cellulose broth were inoculated then kept it for saccharification. The amount of glucose produced was 4.99 mg/ml, % saccharification was 42% . The saccharified sample was filtred and inoculated the yeast *saccharomyces cereviseae* and kept for fermentation for 14 days. The concentration of ethanol was 83.75µg/ml.

Bioethanol production from cellulose is economical. It is useful for environment and also have much more commercial importance.

Keywords: Cellulose, Cellulase, Banana (Pseudostem), Ethanol

Table of Contents

TABLE OF CONTENTS

S.NO.	TITLE	PAGE NO
	ABSTRACT	**vi**
	LIST OF TABLES	**xi**
	LIST OF FIGURES	**x**
1	**INTRODUCTION**	**1**
	1.1 Cellulose	1
	1.1.1 Chemical Structure of Cellulose	2
	1.2 Cellulose	3
	1.2.1 Novel Cellulase Producing Bacteria	4
	1.3 Ethanol Production	8
	1.3.1 Ethanol Fermentation	12
	1.3.2 Combined Hydrolysis and Fermentation	13
	1.3.3 Biological Properties	13
	Objectives	17
2	**LITERATURE REVIEW**	18
	2.1 Cellulose Degradation by Bacteria	18
	2.2 Saccharification	22
	2.3 Ethanol Production	27
3	**MATERIALS AND METHODS**	31
	3.1 Isolation of cellulose-degrading bacteria	31
	3.1.1 Materials required	31
	3.1.2 Media preparation	31
	3.1.2.1 Nutrient Agar Medium	31
	3.1.2.2 Congo Red Agar Medium	31
	3.1.2.3 Carboxy Methyl Cellulose Broth	32
	3.1.3 Procedure	32

	3.1.4 Assay For Detecting Activity	34
	3.1.4.1 Filter Paper Test	34
	3.1.4.2 Plate Test	36
	3.1.5 Selection Of Strains For Saccharification	36
	3.1.5.1 Microscopical Characterization	37
	3.1.5.2 Biochemical Test	37
	3.2 Saccharification	38
	3.2.1 Materials Required	38
	3.2.2 Reagent Preparation	38
	3.2.3 Substrate Used	38
	3.2.4 Conditions Provided For Saccharification	39
	3.2.5 Saccharification Of Substrate	39
	3.2.6 Procedure for O-Toludine Test	40
	3.2.7 Formula	40
	3.3 Ethanol Production	41
	3.3.1 Determination Of Ethanol Concentration	41
4	**RESULTS AND DISCUSSION**	**43**
	4.1 Cellulase Activity Test Of the Isolates	43
	4.1.1 Filter Paper Test	43
	4.1.2 Plate Test	43
	4.2 Selection of Strain for Saccharification	46
	4.2.1 Identification of bacterial strain II	46
	4.2.2 Biochemical Characterization of the Strain II	47
	4.2.3 Carbohydrate Fermentation Test Result	49
	4.3 Calculation	50
5.	**CONCLUSION**	**56**
6.	**REFERENCES**	**57**

List of Tables

LIST OF TABLES

TABLE NO	TITLE	PAGE NO
1.1	Properties of ethanol	10
3.1	List of samples isolated	34
4.1	Diameter of zone of inhibition	43
4.2	Carbohydrate fermentation	47
4.3	Ortho-toludine test for sugar confirmation	49
4.4	OD values for O-toludine test	50
4.5	Determination of ethanol concentration.	51

List of Figures

LIST OF FIGURES

FIG NO	TITLE	PAGE NO
4.1	Filter Paper Test	44
4.2	Zone of Inhibition of S V Strain	44
4.3	Zone of Inhibition of S II Strain	45
4.4	Zone of Inhibition of S I Strain	45
4.5	Morphology of *Bacillus sp*	46
4.6	Microscopic View of *Bacillus sp*	47
4.7	Sucrose Fermentation	48
4.8	Glucose Fermentation	48
4.9	Banana Agro Waste (Substrate)	52
4.10	Saccharification	52
4.11	Fermentation	53
4.12	Concentration of Ethanol	54

Introduction

1. INTRODUCTION

1.1 CELLULOSE

Cellulose was discovered in 1838 by the French chemist Anselme Payen, who isolated it from plant matter and determined its chemical formula. (Crawford *et al.*, 1981, and Young *et al.* ,1986).Cellulose was used to produce the first successful thermoplastic polymer, celluloid, by Hyatt Manufacturing Company in 1870. Hermann Staudinger determined the polymer structure of cellulose in 1920. The compound was first chemically synthesized (without the use of any biologically-derived enzymes) in 1992, by Kobayashi and Shoda (Klemm *et al.*, 2005).

Cellulose is an organic compound with the formula $(C_6H_{10}O_5)n$, a polysaccharide consisting of a linear chain of several hundred to over ten thousand $\beta(1\rightarrow4)$ linked D-glucose units (Crawford *et al.*,1981 and Updegraff *et al.*,1969). Cellulose is the structural component of the primary cell wall of green plants, many forms of algae and the oomycetes. Some species of bacteria secrete it to form biofilms. Cellulose is the most common organic compound on Earth .

Cellulose is mainly obtained from wood pulp and cotton. It is mainly used to produce fiber cardboard and paper to a smaller extent it is converted into a wide variety of derivative products such as cellophane and rayon. Converting cellulose from energy crops into biofuels such as cellulosic ethanol is under investigation as an alternative fuel source. Some animals, particularly ruminants and termites, can digest cellulose with the help of

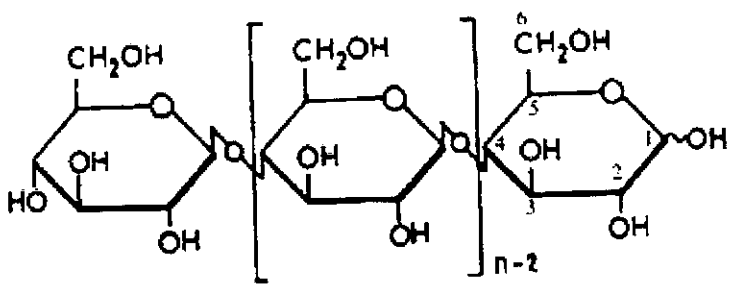
symbiotic micro-organisms that live in their guts. Humans can digest cellulose to some extent (Slavin *et al.*, 1980 and Joshi *et al.*, 1995). However it is often referred to as 'dietary ' or 'roughage' (e.g. outer shell of Maize) and acts as a hydrophilic bulking agent for feces.

Cellulose can be converted into cellophane, a thin transparent film, and into rayon, an important fiber that has been used for textiles since the beginning of the 20th century. Both cellophane and rayon are known as "regenerated cellulose fibers" they are identical to cellulose in chemical structure and are usually made from dissolving pulp via viscose. A more recent and environmentally friendly method to produce rayon is the Lyocell process.

1.1.1 Chemical structure of cellulose

A strand of cellulose (conformation I α), showing the hydrogen bonds (dashed) within and between cellulose molecule. Cellulose is odourless, hydrophilic, insoluble in water and most organic solvents, and also tasteless, chiral and biodegradable. It can be broken down chemically into its glucose units by treating it with concentrated acids at high temperature. Cellulose is derived from D-glucose units, which condense through β(1→4) glycosidic bonds. This linkage motif contrasts with that for α (1→4) glycosidic bonds present in starch, glycogen, and other carbohydrates. Cellulose is a straight chain polymer unlike starch, no coiling or branching occurs, and the molecule adopts an extended and rather stiff rod-like conformation, aided by the equatorial conformation of the glucose residues. The multiple hydroxyl groups on the glucose from one chain form

hydrogen bonds with oxygen molecules on the same or on a neighbor chain, holding the chains firmly together side-by-side and forming microfibrils with high tensile strength. This strength is important in cell walls, where the microfibrils are meshed into a carbohydrate matrix, conferring rigidity to plant cells. Cellulose is soluble in cupriethylenediamine (CED), cadmiumethylenediamine (Cadoxe), N-methylmorpholine N-oxide and lithium chloride / di methyl formamide (Stenius *et al.,* 2000). This is used in the production of regenerated celluloses (as viscose and cellophane) from dissolving pulp.



Cellobiose Unit

1.2 CELLULASE

Cellulase refers to a class of enzymes produced chiefly by fungi, bacteria protozoans. that catalyze the cellulolysis or hydrolysis of cellulose. However, there are also cellulases produced by other types of microorganisms such as plant and animals. Several different kind of cellulases are known, which differ structurally and mechanistically. In the most familiar case of cellulose activity, the enzyme complex breaks down

cellulose to beta glucose. This type of cellulase is produced mainly by symbiotic bacteria in the ruminating chambers of herbivores. The three types of reaction catalyzed by cellulases. 1. Breakage of the non-covalent interactions present in the crystalline structure of cellulose (endo-cellulase) 2. Hydrolysis of the individual cellulose fibers to break it into smaller sugars (exo-cellulase) 3. Hydrolysis of disaccharides and tetrasaccharides into glucose (beta-glucosidase).

Cellulase is used for commercial food processing in coffee. It performs hydrolysis of cellulose during drying of beans. Furthermore, cellulases are widely used in textile industry and in laundry detergents. They have also been used in the pulp and paper industry for various purposes, and they are even used for pharmaceutical applications. Cellulase is used in the fermentation of biomass into biofuels, although this process is relatively experimental at present. Cellulase is used as a treatment for phytobezoars, a form of cellulose bezoars found in the human stomach (Chapin *et al.*, 2002).

1.2.1 Novel cellulase producing bacteria

Isolation, screening and selection have favoured the discovery of several novel cellulase-producing bacteria from a wide variety of environments as previously discussed. Due to the vast diversity among bacteria the identification of novel cellulases remains a currently explored route to the improvement of biorefining industries. Here will be discussed briefly some of the new bacterial isolates and characterized cellulases, with potential use in the biorefining industry.

Recently, the bacterial strain B39, previously isolated from poultry manure compost in Taichung, Taiwan, was identified through 16S rRNA gene sequencing and phylogentic analysis to be a novel cellulose-degrading *Paenibacillus* sp. strain. A high-molecular weight (148 kDa) cellulase, possessing both CMCase and Avicelase activities, was found to be secreted by this isolate into the media. The CMCase activity of the newly isolated cellulase was much higher than the activity on Avicel or filter paper and this cellulase was found to have maximum CMCase activity at 60°C, pH 6.5. Due to the promising thermostability and slight acidic tolerance of this enzyme, it has good potential for industrial use in the hydrolysis of soluble cellulose as well as activity on microcrystalline sources of cellulose (Wang *et al.*, 2008).

Furthermore a novel cellulase-producing *Paenibacillus campinasensis* BL11 was isolated in 2006, from black liquor of brownstock at washing stage of the Kraft pulping process. This black liquor environment is strongly alkaline and therefore highly unfavorable to bacterial growth, isolation of a cellulase-degrading species from this environment provides possibility that the enzymes produced by such a species could be tolerant to some of the harsh conditions used in the different pretreatments of lignocellulosic biomass. *Paenibacillus campinasensis* BL11 is a hemophilic, spore-forming bacterium which was found to grow between 25 - 60°C over a wide range of pH. Optimal growth is around neutral pH, at 55°C. This isolate used a variety of saccharides and polysaccharides and produced multiple extracellular saccharide-degrading enzymes including a xylanase, two cellulases, pectinase and cyclodextrin glucanotransferase. The physiological properties of this strain and the vast number of free glycosyl

hydrolases produced give this strain potential for use in the biorefining industry (Wang *et al.*, 2008).

More recently, a thermostable cellulase was found in newly isolated *Bacillus subtilis* DR, extracted from a hot spring. The high temperature environment allowed for the production of a thermostable endocellulase CelDR with an optimum temperature at 50°C. It was found to retain 70% of its maximum activity (CMCase) at 75°C after incubation for 30 minutes. This strain offers a potentially more valuable thermostable enzyme for the biorefining industry due to extreme heat tolerance (LiW *et al.*, 2008).Cultivation of thermophiles offers several advantages, it reduces the risk of contamination, reduces viscosity thus making mixing easier, and leads to a high degree of substrate solubility while reducing the cost of cooling. This is a greatly sot after property for cellulases in industrial applications like the bioconversion of lignocellulose. Also recently, a novel hemophilic, cellulolytic bacterium was isolated from swine waste and identified as *Brevibacillus* sp. strain JXL. It was found to use a broad spectrum of substrates such as crystalline cellulose, CMC, xylan, cellobiose, glucose and xylose. The enzymes appeared to retain 50% of their activity after 1h at 100°C, making them highly thermostable (Liang *et al.*,2009). Furthermore, a salt-activated endoglucanase was recently isolated from another *Bacillus* strain, alkaliphilic *Bacillus agaradhaerens* JAM-KU023 which was shown to have increased optimal thermostability from 50°C to 60°C with the addition of 0.2M NaCl and optimal pH range from 7-9.4 (Hirasawa *et al.*, 2006).

In addition, bacteria are capable of producing more complex protein structures supporting enzymes for the hydrolysis of cellulose, such as the cellulosome, xylosome and bifunctional or multifunctional enzymes which are currently gaining a lot of attention. If these enzymes can be recombinantly produced on mass or produced *in situ* by the bacterial strains naturally encoding them, then they may have great potential in improving the cost of hydrolysis for the production of biofuels by reducing the need for production of multiple enzymes for efficient hydrolysis. For example, a bifunctional endoglucanase/endoxylanase was isolated from *Cellulomonas flavigena* providing potential for use in different industrial processes such as biofuel production. This bifunctional enzyme was found to have optimum cellulase and xylanase activity at pH 6 and 9, respectively, with a general optimum temperature at 50°C (Perez-Alvos *et al.*,2008). Similarly, in 2007, a multifunctional enzyme was found to be produced by *Terendinibacter turnerae* T7902, which is a bacterial symbiont isolated from the wood-boring marine bivalve *Lydrodus pedicellatus*. This CelAB was found to have two catalytic and two carbohydrate-binding domains. It binds both cellulose and chitin and possesses cellobiohydrolase and beta-1, 4 (3) endoglucanase activity allowing it to degrade multiple complex polysaccharides. This enzyme is marginally acid-tolerant at an optimum pH of 6 and mesophilic with a temperature optimum of 42°C. Additionally, this enzyme was able to reduce viscosity of CMC approximately 40% after 25 minutes, displaying promising characteristics for the biofuel industry (Ekborg *et al.*, 2007). All of these recently isolated enzymes and many more provide the framework needed to characterize and build highly efficient hydrolysis systems to be used in the biorefining industry. Isolation and characterisation of cellulase-

producing bacteria will continue to be an important aspect of biofuel research.

1.3 ETHANOL PRODUCTION

Cellulase is an enzyme produced by several micro organisms. There are only two modes of action for the hydrolysis of cellulose by cellulase, either conversion or retension of the configuration of the anomeric carbon.Cellulase- producing bacteria have been isolated from a wide variety of sourses such as composting heaps, decaying plant material from forestry or agricultural waste, the feces of ruminants such as cows, soil and organic matter, and extreme environments like hot-spring (Doi *et al.*,2008). Screening for bacterial cellulase activity in microbial isolates is typically performed on carboxy methyl cellulose (CMC) containing plates (Hankin *et al.*,1977).This method can be timely and zones of hydrolysis are not easily discernable. Recently ,Kassana and colleagues found that Gram's iodine for plate flooding in place of hexadecyltrimethyl ammonium bromide or Congo red, gave a more rapid and highly discernable result (Kasana *et al.*,2008).The main cellulose degrading bacteria are *Paenibacillus* sp.,*Brevibacillus* sp.strain JXL,*Bacillus agaradhaerens* ,*Cellulomonas flavigena, Lydrodus pedicellatus, Clostridium thermocellum, Bacillus subtilis etc...*

Ethanol has been used by humans since prehistory as the intoxicating ingredient of alcoholic beverages. Dried residues on 9,000-year-old pottery found in China imply that alcoholic beverages were used even among Neolithic people (Roach, *et al.*2005). Its isolation as a

relatively pure compound was first achieved by the Persian alchemist, Muhammad bin Zachariah Raze (Razes, 865–92Antoine Lavisher described ethanol as a compound of carbon, hydrogen, and oxygen, and in 1808 Nicolas Theodore de Assure determined ethanol's chemical formula (Couper,1858). Fifty years later, Archibald Scott Cooper published the structural formula of ethanol, which placed ethanol among the first compounds whose chemical structure had been determined (Fennel *et al.*, 1828). Ethanol was first prepared synthetically in 1826 through the independent efforts of Henry Hennel in Great Britain and S.G. Sérullas in France. In 1828, Michael Faraday prepared ethanol by acid-catalyzed hydration of ethylene, a process similar to that which is used today for industrial ethanol synthesis (Siegel *et al.*,2007). Ethanol was used as lamp fuel in the United States as early as 1840, but a tax levied on industrial alcohol during the Civil War . Ethanol is a volatile, colorless liquid that has a strong characteristic odor. It burns with a smokeless blue flame that is not always visible in normal light. The physical properties of ethanol stem primarily from the presence of its hydroxyl group and the shortness of its carbon chain. Ethanol's hydroxyl group is able to participate in hydrogen bonding, rendering it more viscous and less volatile than less polar organic compounds of similar molecular weight. Ethanol, also called ethyl alcohol, pure alcohol, grain alcohol, or drinking alcohol. The largest single use of ethanol is as a motor fuel and fuel additive. Brazil has the largest national fuel ethanol industry. Gasoline sold in Brazil contains at least 25% anhydrous ethanol. Hydrous ethanol (about 95% ethanol and 5% water) can be used as fuel in more than 90% of new cars sold in the country. Brazilian ethanol production is praised for the high carbon sequestration capabilities

of the sugar cane plantations, thus making it a real option to combat climate change (Jones *et al.*,2008).

Table 1.1 Ethanol properties

Properties	
Molecular formula	C_2H_6O
Molar mass	46.07 g mol^{-1}
Appearance	colorless liquid
Density	0.789 g/cm^3
Melting point	−114.3 °C, 159 K, -174 °F
Boiling point	78.4 °C, 352 K, 173 °F
Solubility in water	miscible

Henry Ford designed the first mass-produced automobile, the famed Model T Ford, to run on pure anhydrous (ethanol) alcohol - he said it was "the fuel of the future". Today, however, 100% pure ethanol is not approved as a motor vehicle fuel in the U.S. Added to gasoline, ethanol reduces volatile organic compound and hydrocarbon emissions, carcinogenic benzene and butadiene emissions, and particulate matter emissions from gasoline combustion (Horn *et al.*, 2006). Recent research suggests that

cellulosic crops such as switchgrass and Miscanthus grass provide a much better net energy production than corn, producing over five times as much energy as the total used to produce the crop and convert it to fuel (Boggan *et al.*,2007). If this research is confirmed, cellulosic crops will most likely displace corn as the main fuel crop for producing bioethanol.

Ethanol is also used as a cooking and lighting fuel. In India, ethanol stoves and lanterns has been developed that can run on 50% by weight ethanol/water mixture. This mixture (hooch or illicit liquor) is easy to distill, safer to handle and use than 100% ethanol, can be produced by small local producers, and uses less energy in its production (John *et al.*, 2007).

Alcoholic beverages vary considerably in their ethanol content and in the foodstuffs from which they are produced. Most alcoholic beverages can be broadly classified as fermented beverages, made by the action of yeast on sugary foodstuffs, or as distilled beverages, whose preparation involves concentrating the ethanol in fermented beverages by distillation. The ethanol content of a beverage is usually measured in terms of the volume fraction of ethanol in the beverage, expressed either as a percentage or in alcoholic proof units. (Boggan *et al.*, 2007) in medical wipes and in most common antibacterial hand sanitizer gels at a concentration of about 62% (percentage by volume, not weight) as an antiseptic. Ethanol kills organisms by denaturing their proteins and dissolving their lipids and is effective against most bacteria and fungi, and many viruses including SARS (Myers *et al.*,2007). But is ineffective against bacterial spores.

1.3.1 Ethanol fermentation

Traditionally, baker's yeast (*Saccharomyces cerevisiae*), has long been used in the brewery industry to produce ethanol from hexoses (6-carbon sugar). Due to the complex nature of the carbohydrates present in lignocellulosic biomass, a significant amount of xylose and arabinose (5-carbon sugars derived from the hemicellulose portion of the lignocellulose) is also present in the hydrolysate. For example, in the hydrolysate of corn stover, approximately 30% of the total fermentable sugars is xylose. As a result, the ability of the fermenting microorganisms to use the whole range of sugars available from the hydrolysate is vital to increase the economic competitiveness of cellulosic ethanol and potentially bio-based chemicals.

In recent years, metabolic engineering for microorganisms used in fuel ethanol production has shown significant progress (Jeriff *et al.*, 2004). Besides *Saccharomyces cerevisiae*, microorganisms such as *Zymomonas mobilis* and *Escherichia coli* have been targeted through metabolic engineering for cellulosic ethanol production.

Recently, engineered yeasts have been described efficiently fermenting xylose (Bat, 2009 and Ohgren *et a*l.,2006) and arabinose, (Becker *et al.*,2003) and even both together. (Karhumaa *et al.*,2006). Yeast cells are especially attractive for cellulosic ethanol processes as they have been used in biotechnology for hundreds of years, as they are tolerant to

high ethanol and inhibitor concentrations and as they can grow at low pH values which avoids bacterial contaminations.

1.3.2 Combined hydrolysis and fermentation

Some species of bacteria have been found capable of direct conversion of a cellulose substrate into ethanol. One example is *Clostridium thermocellum,* which uses a complex cellulosome to break down cellulose and synthesize ethanol. However, *Clostridium thermocellum* also produces other products during cellulose metabolism, including acetate and lactate, in addition to ethanol, lowering the efficiency of the process. Some research efforts are directed to optimizing ethanol production by genetically engineering bacteria that focus on the ethanol-producing pathway.

1.3.3 Biological properties

Ethanol can be used as an antidote for poisoning by other more toxic alcohols, in particular methanol (McDonnell *et al.*, 1999) and ethylene glycol. Ethanol competes with other alcohols for the alcohol dehydrogenase enzyme, preventing metabolism into toxic aldehyde and carboxylic acid derivatives, and it reduces the glycols' tendency to crystallize in the kidneys (which is one of its more serious toxic effects). Ethanol is easily miscible in water and is a good solvent. Ethanol is lesspolar than water and is used in perfumes, paints and tinctures. Ethanol is also used in design and sketch art markers, such as Copic, and Tria. Ethanol is also found in certain kinds of deodorants. Before the development of modern medicines, ethanol was used for a variety of medical purposes. It has been known to be used as a truth

drug (as hinted at by the maxim "in vino veritas"), as medicine for depression and as an anesthetic.

Pure ethanol will irritate the skin and eyes. Nausea, vomiting and intoxication are symptoms of ingestion. Long term use can result in serious liver damage (Barceloux *et al.*, 2002). Ethanol is a central nervous system depressant and has significant psychoactive effects in sublethal doses for specifics, see effects of alcohol on the body by dose. Based on its abilities to change the human consciousness, ethanol is considered a psychoactive drug (Pohorecky *et al.*, 1988). Death from ethyl alcohol consumption is possible when blood alcohol level reaches 0.4%. A blood level of 0.5% or more is commonly fatal. Levels of even less than 0.1% can cause intoxication, with unconsciousness often occurring at 0.3–0.4%.

The amount of ethanol in the body is typically quantified by blood alcohol content (BAC), the milligrams of ethanol per 100 milliliters of blood. Small doses of ethanol generally produce euphoria and relaxation; people experiencing these symptoms tend to become talkative and less inhibited, and may exhibit poor judgment. At higher dosages (BAC > 100 mg/dl), ethanol acts as a central nervous system depressant, producing at progressively higher dosages, impaired sensory and motor function, slowed cognition, stupefaction, unconsciousness, and possible death. More specifically, ethanol acts in the central nervous system by binding to the GABA-A receptor, increasing the effects of the inhibitory neurotransmitter GABA , it is a positive allosteric modulator (David *et al.*,2002).

Frequent drinking of alcoholic beverages has been shown to be a major contributing factor in cases of elevated blood levels of triglycerides (Agarwal *et al.*, 1998). Ethanol is not a carcinogen (Burdick *et al.*, 2007). However, the first metabolic product of ethanol, acetaldehyde is toxic, mutagenic, and carcinogenic.

Sugars for ethanol fermentation can be obtained from cellulose (Taherzadeh *et al.*, 2007). Until recently, however, the cost of the cellulase enzymes capable of hydrolyzing cellulose has been prohibitive. The Canadian firm Iogen brought the first cellulose-based ethanol plant on-stream in 2004 (Ritter *et al.*, 2004). Its primary consumer so far has been the Canadian government, which, along with the United States Department of Energy, has invested heavily in the commercialization of cellulosic ethanol. Deployment of this technology could turn a number of cellulose-containing agricultural by-products, such as corncobs straw, and sawdust, into renewable energy resources. Other enzyme companies are developing genetically engineered fungi that produce large volumes of cellulase, xylanase, and hemicellulase enzymes. These would convert agricultural residues such as corn stover, wheat straw, and sugar cane bagasse and energy crops such as switchgrass into fermentable sugars (Tom, 2006).

Cellulose-bearing materials typically also contain other polysaccharides, including hemicellulose. When undergoing hydrolysis, hemicellulose decomposes into mostly five-carbon sugars such as xylose. *Saccharomyces cerevisiae*, the yeast most commonly used for ethanol production, cannot metabolize xylose. Other yeasts and bacteria are under investigation to ferment xylose and other pentoses into ethanol. On January

14, 2008, General Motors announced a partnership with Coskata, Inc. The goal is to produce cellulosic ethanol cheaply, with an eventual goal of US$1 per U.S. gallon ($0.30/L) for the fuel. The partnership plans to begin producing the fuel in large quantity by the end of 2008. In June 2009, this goal is still ahead of the firm. By 2011 a full-scale plant will come on line, capable of producing 50 to 100 million gallons of ethanol .

OBJECTIVES

- Isolation of cellulose degrading bacteria using plate screening method (Soil & Cow dung).

- Saccharification of cellulose by the isolated bacteria.

- Production of ethanol using saccharified sugar by *Saccharomyces cerevisia.*

Literature Review

2. LITERATURE REVIEW

2.1 CELLULOSE DEGRADATION BY BACTERIA

Plant life on earth depends on photosynthesis, which results in production of plant biomass having cellulose as the major component. The carbon cycle is closed primarily as a result of the action of cellulose-utilizing microorganisms present in the soil and guts of animals. Thus, microbial cellulose utilization is responsible for one of the largest materials flows in the biosphere and is of interest in relation to analysis of carbon flux at both local and global scales. The importance of microbial cellulose utilization in natural environments is further enhanced by the status of ruminants as a major source of dietary protein. Finally, microbial cellulose utilization is an integral part of widely used processes such as digestion and composting (Lynd *et al.* ,2004)

Smirnova *et al.,(* 2001) studied the use of cellulose degrading nitrogen-fixing bacteria in the enrichment of roughage with protein. a new strain of acid tolerant facultative anaerobic cellulose degrading bacteria *Bacillus cytaseus* 21, which is capable to fixing atmospheric nitrogen, was isolated and reported. This strain is intended for solid-phase fermentation and enrichment with protein of cellulose containing waste of plant cultivation.

Studies on the cellulolytic bacteria and cellulose degradation in a cattle waste-fed biogas digester was conducted by (Singh *et al.,* 1985). production of acetate is the key substrate for methane formation in anaerobic digesters, is limiting in cattle waste-fed biogas digesters due to the lignocellulose

nature of the substrate . Since the rate of cellulose hydrolysis affects acetate production, and the number and the type of cellulolytic bacteria that exist in a cattle waste biogas digester is not known,110 cellulolytic bacterial isolates belonging to the *Bacillaceae, Propionibacteriaceae, Lactobacillaceae* and *Corneyform* bacteria like *Cellulomonas,* were isolated. Some bacterial isolates exihibited higher cellulose activity than others, with acetate as a major end product. In general, acetate, propionate and butyrate were the common end products but one isolate was homoacetogenicn and a few others produced propionate or butyrate or isobutyrate in small amounts with acetate. Cellulose degradation rate in the digester was dependent upon the cellulose: lignin ratio

Studies on the cellulose degradation and ethanol production by thermophilic bacteria using mineral growth medium was done (Ahn *et al.,* 1996). Growth of thermophilic cellulase utilizing bacteria in vitamin free growth medium is reported for both a previously described strain, *Clostridium thermocellum* 31549, and now isolates HJA1 and HJA2 was maintained in continuous culture on a vitamin-free mineral medium with Avicel as the carbon source. At a 38 hr residence time, Avicel conversion was higher (81%) at Ph 6.42 than pH6.97 (73%) or at 6.10(58%). Ethanol and acetate were produced in significant amounts by strain JHA2at all pH values tested (6.97,6.42&6.01). Lactic aid was the primary fermentation product at pH 6.97, but was not a significant product at both the lower values.Efforts to grow thermophilic,cellulose-utilizing becteria at pH<6.0 were unsuccessful for described strains, new isolates, and enrichment cultures.

degradation by *Ruminococcus Flavefaciens*. Highly methylated, long-chain celluloses strongly inhibited cellulose degradation by several species of

cellulolytic bacteria of ruminal origin. Specifically, the inhibitory effects of methylcellulose on the growth of *Ruminococcus Flavefaciens* FDI were concentration dependent, with complete inhibition at 0.1%(wt/volt). However, methylcellulose did not inhibit growth on cellobiose or cellulooligosaccharides. Mixtures of methylated cellulooligosaccharides having an average degree of polymerization of 6.7 to 9.5 inhibited cellulose degradation, but those with an average degree of polymerization of 1.0 to 4.5 did not. Similar inhibitory effects by methylcellulose and, to a lesser extent, by methyl cellulooligosaccharides were observed on cellulose activity, as measured by hydrolysis of *p*-nitrophenyl-β-D-cellobioside. *R.Flavefeciens* cultures hydrolyzed cellulooligosaccharides to cellobiose and cellotriose as final end products. Cellopentanose and Cellohexanose. Methylcellulose did not inhibit hydrolysis of cellulooligosaccharides. These data are consistent with the presence of separate cellulose(β-1,4-glucanase) and cellulodextrinase activities in *R.flavefaciens.*

Despite its simple chemical composition cellulose exists in a number of crystalline and amorphous topologies (Schwarz et al., 2001) . Its insolubility and heterogenicity makes native cellulose a recalcitrant substrate for enzymatic hydrolysis. Microorganisms meet this challenge with the aid of a multienzyme system. Aerobic bacteria produce numerous individual, extra cellular enzymes with binding modules for different cellulose confirmations. Specific enzymes act in synergy to elicit effective hydrolysis. In contrast anaerobic bacteria posses a unique extra cellular multi-enzyme complex, called cellulosome. These multi-enzyme complexes attach both to the cell envelope and to the substrate, mediating the proximity of the cells to the cellulose. Binding to the scaffolding stimulates the activity of each

individual component towards the crystalline substrate. The most complex and best investigated cellulosome is that of the thermophilic bacterium *Clostridium thermocellum*, but a scheme for the cellulosomes of mesophilic *Clostridia* and the *ruminococci* emerges.

Higher levels of aggregation encompassing the structure and composition of cellulosic biomass, taxonomic diversity, cellulose enzyme systems, molecular biology of cellulase enzymes, physiology of cellulolytic microorganism, ecological aspects of cellulose degrading communities, and rate limiting factors in nature (Lynd et al., 2002) . The methodological basis for studying microbial cellulose utilization is considered relative to quantification of cells and enzymes in the presence of solid substrate as well as apparatus and analysis for cellulose grown continuous cultures. Quantitative description of cellulose hydrolysis is addressed with respect to adsorption of cellulose enzymes, rates of enzymatic hydrolysis, bioenergitics of microbial cellulose utilization, kinetics of microbial cellulose utilization, and contrasting features compared to soluble substrate kinetics. A biological perspective on processing cellulosic biomass is presented, including the features of pretreated substrates and alternative process configurations. Organism development is considered for "consolidated bioprocessing"(CBP), in which the production of cellulolytic enzymes, hydrolysis of biomass, and fermentation of sugars to desired products occur in one step. Two organism development strategies for CBP are examined.

1. Improve product yield and tolerance in microorganism able to utilize cellulose, or
2. Express a heterologus system for cellulose hydrolysis and utilization in microorganism that exhibit high product yield

2.2 SACCHARIFICATION

The process of breaking a complex carbohydrate (as starch or cellulose) into its monosaccharide components is called saccharification. Chemical reaction in which the action of water or its ions breaks down a substance into smaller molecules. Hydrolysis occurs in certain inorganic salts in solution, in nearly all non-metallic chlorides, in esters, and in other organic substances. It is one of the mechanisms for the breakdown of food by body, as the conversion of starch to glucose.

Shigenori *et al.*,(2006) studied on the saccharification of cellulose by dry pyrolysis. Pyrolysis was studied for the purpose of practical production of 1,6-anhydro- β-D-glucopyranoside (levoglucosan LG). To minimize secondary degradation of levoglucosan, two methods were examined: 1.conductive heating by glass bottle 2.radiation heating from the surface by CO_2 laser beam, both under vacuum and in nitrogen atmosphere. Glass bottle pyrolysis under vacuum gave levoglucosan yield of 50%-55% in the optimum temperature range of 350°-410°C, where placing the cold trap in the vicinity of heated area was effective in improving the yield. In contrast glass bottle pyrolysis under nitrogen gave low yields of 17%-20% probably due to slower diffusion of pyrolysis from hot region. The CO_2 laser pyrolysis under vacuum gave the product aerosol(White smoke), causing difficulty in recovery of the product, And the maximum yield was 5%-17%. In this case the treatment under nitrogen flow was effective for recovery of aerosol, and the maximum yield reached approximately25%.

A method for effecting saccharification of raw cellulosic material taken from any available source (Pilipski *et al.,* 1981). The cellulosic material is hydrolyzed by immersing it in a bath of anhydrous liquid hydrogen chloride to yield usable glucose and other products. The cellulosic material may be processed through one or more pre-treatment steps, each acting to enhance the yield of glucose and other products when the pre-treated material is subjected to hydrolyzation by anhydrous liquid hydrogen chloride. The raw material may first be immersed in a caustic solution which swells the cellular structure to render it more reactive and acts to solubilize the lignins and other compounds contained there in, these being washed away to provide a prepared cellulosic material. In another or second preparatory step, the material may be further prepared by subjecting it to a viscose process to produce a viscous solution of sodium cellulose xanthate in sodium hydroxide, from which solution aqueous phase is separated, leaving a viscose intermediate. In final step, the viscose intermediate is hydrolyzed by immersing it in a bath of anhydrous liquid hydrogen chloride.

Saccharification of cellulose from waste water sludge and subsequent conversion to organic acids occurs with the help of *Corneybacteria* (Katsumi *et al.,* 2004). Waste water sludge containing cellulose from a paper manufacturing factory was saccharised using meicelase (cellulose from *Trichoderma viride*). As a result of process optimization, maximum saccharification was obtained at pH 4.0 and 0.1% enzyme concentration. After neutralization of hydrolyzed products by acid, a *Corneybacterium glutamicium* inoculum and cultivatation medium were added to the solution. Lacitc acid and succinic acid, as major metabolite products were synthesized in this reaction under anaerobic conditions by bubbling of carbondioxide

gas. Lactic acid was identified to be of L-form, which is useful as a starting material for the manufacture of biodegradable polymers such as poly lactic acid. Both these organic acid can also be converted to a wide variety of organic products. This production of organic acids was thus accompanied by CO_2 fixation. Paper sludge saccharification and organic acid production were done in the same reactor to reduce the reaction time and simplify the overall process by using celluzyme (cellulose from *Humicola* microorganism), whose optimum activity is at around pH7.

According to (Ueda *et al.*, 2004), synergistic saccharification, and direct fermentation to ethanol, of amorphous cellulose by use of three types of cellulolytic enzyme from an engineered yeast strain. A whole-cell biocatalyst with the ability to induce synergistic and sequential cellulose-degrading reaction was constructed through codisplay of three types of cellulolytic enzyme on the cell surface of the yeast *Saccharomyces cerevisiae.* When a cell surface display system based on α-agglutinin was used, *Trichoderma reesei* endoglucanase II and cellobiohydrolase II and Aspregillus aculeatus β-glucosidase I were simultaneously co displayed as individual fusion proteins with the C-terminal-half region of α-agglutinin. Codisplay of three enzymes on the cell surface was confirmed by observation of immunoflourescence-labelled cells with a fluorescence microscope. A yeast strain codisplaying endoglucanase II and cellobiohydrolase II showed significantly higher hydrolytic activity amorphous cellulose (phosphoric acid-swollen cellulose) than one displaying only endoglucanase II, and its main product was cellobiose; codisplay of β-glucosidase I, endoglucanase II, and cellobiohydrolase II enabled the yeast strain to directly produce ethanol from the amorphous cellulose (which a

yeast strain codisplaying β-glucosidase I and endoglucanase II could not),with a yield of approximately 3g per gram from 10g per liter within 40 hr. The yield (in grams of ethanol produced per gram of carbohydrate consumed) was 0.45 g/g, which corresponds to 88.5% of theoretical yield. This indicates that simultaneously and synergistic saccharification and fermentation of amorphous cellulose to ethanol can be efficiently accomplished using a yeast strain codisplaying the three cellulolytic enzymes.

Dadi *et al.*, (2005) studied on an efficient approach for saccharification of cellulose from biomass for ethanol production. The production of sugars from lignocellulosic biomass is, at, present not cost competitive with the corn starch-based approach due to difficulty associated with hydrolyzing the cellulose fraction to glucose. Cellulose, a highly complex carbohydrate exists in a number of crystalline and amorphous form. Insolubility and heterogenecity due to highly organized hydrogen bonding makes native cellulose a recalcitrant substrate for present enzymatic hydrolysis. The alternative acid hydrolysis accompanied by undesirable side products. This study intends to overcome these limitations and enhance the rate of cellulose hydrolysis and improve the saccharification kinetics by using solvents capable of dissolving cellulose. Various solvents capable of dissolving cellulose are ionic liquids, aqueous non derivatizing solvents and non aqueous, non dervatizing solvents. As a first step in this direction, the dissolution and hydrolysis kinetics of cellobiose(an oligomer of cellulose) is reported. Work is in progress for cellulose hydrolysis in various solvents and development of different solvents and enzyme mimics. A systematic

approach combing and optimizing all these proceses can lead to an efficient saccharification of cellulosic biomass.

Studies on the enhancement of enzymatic saccharification of cellulose by cellulose dissolution pretreatments were carried by (Kuo *et al.*, 2008). Attempts were made to enhance cellulose saccharification by cellulose using cellulose dissolution as a pretreatment step. Four cellulose dissolution agents, NaOH/Urea solution, *N*-methylmorpholine-*N*-oxide (NMMO), ionic liquid (1-butyl-3-methylimidazolium chloride) [BMIMCI] and 85% phosphoric acid were employed to dissolve cotton cellulose. In comparison with conventional cellulose pretreatment process, the dissolution pretreatments were operated under a milder condition with temperature< 130°C and ambient pressure. The dissolved cellulose was easily regenerated in water. The regenerated celluloses exhibited a significant improvement (about 2.7 to 4.6-fold enhancement) on saccharification yield ranged from 87% to 96% for the regenerated celluloses while around 23% could be achieved for the untreated cellulose. Even with high crystallinity, cellulose regenerated from phosphoric acid dissolution achieved the highest saccharification rates and yield probably due to its highest specific surface area and lowest degree of polymerization (DP).

2.3 ETHANOL PRODUCTION

Ethanol from cellulosic biomass resources were experimented by (Ayhan *et al.,* 2004). Cellulosic materials can be used to produce ethanol. Ethanol represents an important , renewable liquid fuel for motor vehicles. Production of ethanol from biomass is one way to reduce both the consumption of crude oil and environmental pollution. Conversion technologies for producing ethanol from cellulosic biomass resources such as forest materials, agricultural residues and urban wastes are under development and have not yet been demonstrated commercially. In order to produce ethanol from cellulosic biomass, a pretreatment process is used to reduce the sample size, break down the hemicelluloses to sugars, and open up the structure of the cellulose component. The celluolose portion is hydrolyzed by acids or enzymes into glucose sugar that is fermented to ethanol. The sugars from the hemicelluloses are also fermented to ethanol.

Ethanol production in simultaneous saccharification and fermentation of cellulose with temperature profiling was performed by yow,2001. The effects of temperature on enzymatic saccharification of cellulose and simultaneous saccharification and fermentation (SSF) were investigated with *Trichoderma reesei* cellulose, and *Zymomonas mobilis* ATTC 29191. The following results were obtained: 1) Ethanol fermentation under glucose deficient conditions can proceed for more than 100 hr at 30°C but gradually ceases after 50hr of operation at 40°C. 2) Equivalent glucose yield based on cellulose for SSF operated at its optimum temperature (37°C) is higher than that for enzymatic saccharification of cellulose at the same temperature by

32%. However, the same equivalent glucose yields were obtained for both processes if they were operated at their respective optimum temperature. 3) SSF with temperature cycling increased the ethanol productivity but gave similar ethanol yield to SSF at 37°C.

Lynd *et al.,* (1991) studied on the fuel ethanol from cellulosic biomass. Ethanol produced from cellulosic biomass is examined as a large scale transportation fuel. Desirable features include ethanol's fuel properties as well as benefits with respect to urban air quality, global climate change, balance of trade, and energy security. Energy balance, feedstock supply, and environmental impact considerations are not seen as significant barriers to the widespread use of fuel ethanol derived from cellulosic biomass. Conversion economics is the key obstacle to be overcome.

Overview and evaluation of fuel ethanol from cellulosic biomass was studied by (Lynd *et al.,* 1996). Ethanol is high performance fuel in internal combustion engines. It is a liquid, which is advantageous in terms of storage, delivery, and infrastructural compatibility. Ethanol burns relatively cleanly, especially as the amount of gasoline with which it is blended decreases. Evaporative and toxicity-weighed air toxics emissions are consistently lower for ethanol than for gasoline. It is likely that vehicles can be configured so that exhaust emissions of priority pollutants are very low for ethanol-burning engines, although the same can probably be said for most other fuels under considerations. Research and development---driven advances have clear potential to lower the price of cellulosic ethanol to a level competitive with bulk fuels. Process areas with particular potential for large cost reductions include biological processing (with consolidated bioprocessing particularly

notable in this context), pretreatment, and incorporation of an advanced power cycle for cogeneration of electricity from process residues. The cellulosic ethanol fuel cycle has a high thermodynamic efficiency and had a decidedly positive net energy balance. Cellulosic ethanol is one of the most promising technological options available to reduce transportation sector greenhouse gas emissions. To do so however will require responsible management and increased understanding of relevant technological and natural systems. The potential biomass resource is large, but so is demand for transportation fuels as well as other uses. It is suggested than an appropriate policy objective is to foster a transition to cellulosic feed stocks at a pace such that opportunities for ethanol producers and the farmers that supply them are expanded rather than contracted.

Ethanol production by coupled saccharification and fermentation of sugar cane bagasse was experimented by (Blanco *et al.*, 2004). As initial studies showed that enzymatic saccharification of sugar cane bagasse in columns with recycling of elute was slightly more efficient than in agitated flask, ethanol production by fermentation of the elutes with fast-desiccating yeast and recycling of the ferment ate through the bagasse columns was studied. The alcohol yield from these coupled after 24 or 48 hr was more than that in a simultaneous saccharification and fermentation in agitated flask at 40°C.

Ultrasound stimulates ethanol production during the simultaneous saccharification and fermentation of mixed waste.(Wood *et al.*,1997). The commercial production of ethanol from cellulose by simultaneous saccharification and fermentation (SSF) is prevented in part by the high cost of fungal cellulose enzymes. Intermittent exposure of SSF processes to

ultrasonic energy under selected conditions (5 FPU of cellulose/g of substrate; 15 min of exposure/240 min cycle during the latter half of SSF) was found to increase ethanol production from mixed waste by approximately 20%, producing 36.6 g/L ethanol after 96 hr (70% of the maximum theoretical yield). Without ultrasound, 10 FPU of cellulose/g of substrate was required to achieve similar results. Conditions exposure of the organism to ultrasonic energy was bacteiostatic and decreased ethanol production but may be useful for the controlling growth in other processes.

Materials & Methods

3. MATERIALS AND METHODS

3.1 Isolation of cellulose-degrading bacteria

3.1.1 Materials required

- Petri plates
- Flask
- Cellulose filter paper

3.1.2 Media preparation

3.1.2.1 Nutrient agar medium:

- Peptone -1gm
- Yeast extract - 0.3gm
- Sodium chloride - 0.3gm
- Agar - 4gm
- pH - 7.0 ± 0.2

3.1.2.2 Congo red agar medium:

- Di-potassium phosphate - 0.05gm
- Magnesium sulfate - 0.025gm
- Carboxyl methyl cellulose - 0.188gm

- Congo red - pinch
- Agar - 1gm
- Gelatin - 0.20gm

3.1.2.3 Carboxyl methyl cellulose broth:

- Cellulose powder - 5gm
- Sodium nitrate -2.5gm
- Di-hydrogen potassium phosphate -1gm
- Magnesium sulfate - 0.6gm
- Sodium chloride - 0.1gm
- Calcium chloride - 0.1gm
- Ferric chloride - 0.01gm
- Gelatin - 2gm
- Yeast extract - 0.1gm
- D.H2O - 1L

3.1.3 Procedure

The cellulose degrading microbes were isolated from the different source of the environment. A total of six samples were collected for the isolation of cellulose degrading bacteria. The samples include cowdung, soil from paddy field and coir mill.

1. Serially diluted the given samples (cow dung, soil from paddy field and coir mill).
2. Prepared the nutrient agar medium (100 ml) and pour it in the sterile Petri plates.
3. The Serially diluted samples were inoculated in the agar plates and kept for incubation at 38 °C for one day.
4. Prepared the Congo red agar medium (100 ml) and pour it in the sterile Petri plates.
5. Well isolated colonies were chosen from the agar Petri plates and were inoculated in the Congo red agar plate and kept for incubation at 38°C or three to four days to see the zone of inhibition.
6. The bacteria which had good zone of inhibition were confirmed by the cellulose filter paper test and plate test
7. This chosen bacterium was sub cultured in agar plates to get single isolated colonies.
8. Prepared the CMC broth (1L).
9. This single isolated colony were taken and inoculated in this CMC broth and kept for incubation at 38°C for three to four days.
10. Out of six samples five strains were selected and subjected to cellulose activity test.
11. From the five strains selected three strains had a good zone of Inhibition.

3.1. Table 1: List of samples isolated

Sl no	Sample no	Sample	Location of isolation	Date of isolation
1	SI	Garbage soil	Calicut	04-01-10
2	SII	Paddy field soil	Calicut	12-01-10
3	SIII	Coir mill soil 30cm	Aleppy	17-01-10
4	SIV	Coir mill soil 20cm	Aleppy	22-01-10
5	SV	Cow dung	Trissur	27-01-10
6	SVI	Coir mill hypersoil	Aleppy	29-12-10

3.1.4 Assay for detecting cellulolytic activity

3.1.4.1 filter paper test

The ability of organism to hydrolyze cellulose was routinely investigated by this test. For determining the cellulolytic capacity of the bacteria, a mineral based medium was prepared which contains:

5g-NH_4Cl

1g-NH_4NO_3

2g-Na_2SO_4

3g-K_2HPO_4

1g-KH_2PO_4

3g-NaCl

100 mg yeast extract

1mg-Thiamine

5ml-Trace metal mix

1000 ml Distilled water

The distilled water was autoclaved at 15 lbs for 20 minutes in a screw capped saline bottle. After adjusting the pH to 7 with 1 M NaOH, the required quantity of mineral based medium was autoclaved along with the basal medium, Culture tubes containing Whatman No.1 filter paper strips of 2-3 cm long which act as a polymer was also sterilized at 10 lbs for 15 minutes, devoid of Thiamine. After autoclaving, the required quantity of thiamine was added to the medium. The medium dispensing in 5 ml aliquots into sterile tubes (Woodward *et al.*,1983).

The test organism were inoculated and incubated at 28°C until growth appeared in the medium. After seven days the residual cellulose of filter paper was gravimetrically determined against an un-inoculated control (Ryu *et al.,* 1983). Turbidity or growth indicated that the test organism were able to utilize cellulose as sole source of carbon and hydrolyze it by producing cellulase.

3.1.4.2 Plate test

After the tube test, plate test was done to confirm the cellulolytic bacterial population and the congo red agar media was used. To check the cellulolytic activity, these cultures were again spread plated to cellulose congo red agar. Bacterial colonies with a cellulose degrading zone were observed in these plates and this confirms the bacteria is cellulose degrading. Among th 5 strains isolated by these methods, only three strains with high potency of cellulose degradation were observed based on the diameter of the zone and were selected for experiments. These strains are labeled as 1.S I, 2. S II and 3. S V

3.1.5 Selection of strains for saccharification

Only one strain with high potency of cellulose degradation was selected for saccharification. The strain based on the diameter of zone was produced by the bacteria when spread plated on to congo red cellulose agar medium. All the strains were preserved using standard methods.

The morphological characterization of each isolate was performed, including color, size, and colony characteristics. Physiological and biochemical tests were processed based on Bergey's Manual of systematic bacteriology (Sandhu *et al* .,1992).

3.1.5.1Microscopical characterization.

Gram staining

A thin uniform of smear was prepared and the smear was air dried and heat fixed and cooled. 2 drops of crystal violet was applied and kept it for 1 minute. The crystal violet stain was discarded and washed off with distilled water. The smear was covered with gram's iodine solution and was left for 30 seconds. The iodine was discarded and washed with water. The smear was rinsed ethanol for 5-10 seconds and washed off. The the secondary stain, saffranin was applied on the slide and left for 45 seconds then washed off. The slide is air dried then it is observed under microscopic field first under low power then under high power.

3.1.5.2 Biochemical test

Biochemical testrs were done for identification and characterization of the bacteria. pure culture of bacteria was maintained after isolation from selective media in broth nutrient agar slants and stab culture at room temperature. This pure culture helps in performing the following biochemical test as follows

Carbohydrate fermentation test

Culture was inoculated in media having different sugar content and incubated at 37° C for 24 hours with inverted Durham's tube inside it. Observed for gas and color production.

3.2 Saccharification

3.2.1 Materials required.

- Banana agro waste
- Phosphate Buffer(pH 5.8)
- Carboxy methyl cellulose broth(cmc broth)
- Culture sample
- Incubator
- Pipette, conical flask, beaker etc...

3.2.2 Reagent preparation.

Phosphate buffer [50mM pH-5.8 volume – 100 ml]

- Volume of 1M K_2PO_4 - 4.25 ml
- Volume of 1M KH_2PO_4 - 45.75ml
- Volume of water - 50ml

3.2.3 Substrate used.

Banana agro waste (pseudostem)

3.2.4 Conditions provided for saccharification

The following conditions were given for saccharification and the glucose value was estimated at regular intervals. The data was recorded and analysis was performed using charts.

- Time course (7 days to 15 days)
- pH (4.5)
- Tmperature (50° C)

3.2.5 Saccharification of substrate.

The banana agro waste (pseudostem) used for saccharification was freshly collected from local farm following harvest. The waste was washed thoroughly with water and air dried. It was ground to powder and sieved through mesh.

- Substrate was prepared by adding 1gm of agro waste in 100ml of phosphate buffer and distributed in 6 bottles at 15 ml of substrate each.
- This substrate was inoculated with 5 ml of our isolated bacterial culture.
- Each bottle was incubated for 12 days.

- After 12 days they were taken. The amount of glucose present and the % of Saccharification were calculated using O-Tholidine method.

3.2.6 Procedure for O-Toludine test.

- Three test tubes were labeled as Blank (B), Test (T) and Standard (S).
- 5ml of Ortho toluidine colour reagent (reagent1) was added to each.
- And 0.1ml of distilled water was added into the blank, Glucose reagent into the stanrdard into the test and saccharised sugar was added to each tubes.
- Then mixed well and heated in boiling water bath for 10min.
- After that cooled it in cold water for 5min.
- The OD Value was measured at 620-660 nm (red filter) within 30min against blank.

3.2.7 Formula used

For calculating glucose level:

(Test O.D/Std O.D)*Amount of Glucose Present in STD

For calculating the % saccharification:

(Amount of glucose (mg/ml) / amount of substrate (mg/ml))*100

3.3 ETHANOL PRODUCTION

- For ethanol production the saccharified sugar was filtered and the filtrate was taken in culture flask under aseptic conditions.

- Yeast culture is prepared (put 3 pellet of yeast in the sugar solution containing 1gm sugar in 100ml water) and kept for one day to grow.

- Two ml of yeast culture is added to the saccharified solution and kept for 14 days for complete Fermentation.

- After fermentation ethanol is extracted by distillation process at 78° C.

- The concentration of ethanol is estimated by potassium dichromate assay.

3.3.1 Determination of ethanol concentration.

- For the determination of ethanol concentration of sample, different dilutions of absolute ethanol was taken in 8 test tubes

- 5 ml potassium dichromate was added to each test tube including test.

- The total volume was made upto 10 ml in each test tube using distilled water

- Then the tubes were incubated at 60° C for 30 minutes, the absorbance was measured at 600 nm
- The standard graph was plotted from the graph , the concentration of sample was determined from the standard ethanol curve.

Results & Discussion

4. RESULTS & DISCUSSION

4.1 Cellulase activity test of the isolates

The activity was measured based on the diameter of the zone on cellulose Congo red agar plates and filter paper degradation.

4.1.1 Filter paper test

The filter paper test indicated that the test organism were able degrade cellulose. The turbidity was observed and it was due to the hydrolysis of cellulose, since the filter paper was a rich source of cellulose, the organism were able to utilize cellulose as sole source of carbon and hydrolyze it by producing cellulase. (Ryu *et al.,* 1983).

Table 4.1. : Diameter of the zone of inhibition

SAMPLE NO	SAMPLE	DILUTION	ZONE DIAMETER cm
S-II	FIELD SOIL	1O*-5	2.8 cm
S-V	COW DUNG	10*-4	2.1 cm
S-I	GARBAGE SOIL	10*-4 B	1.6 cm

4.1.2 Plate test

In the plate test for cellulase activity, a media containing Carboxy methyl cellulose was plated, Congo red was acting as indicator and it sticks to the agar giving red color. The bacterial culture is inoculated in the Congo red agar and the organism were able to degrade the cellulose present in the media, the red color disappears around the circular zones, thus indicating the cellulase activity by the zones of inhibition observed.

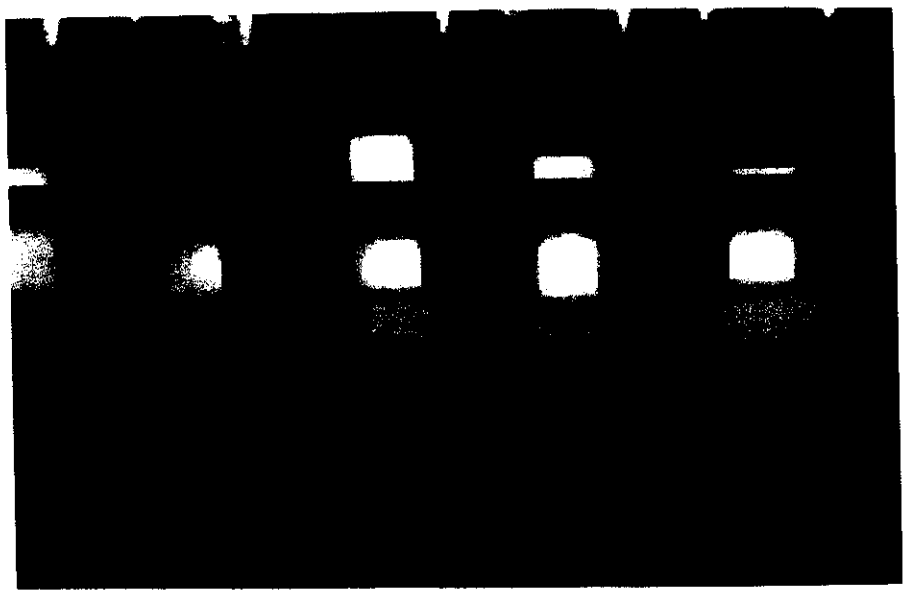


Figure 4.1 Filter paper test revealing the cellulase activity of the isolated strain

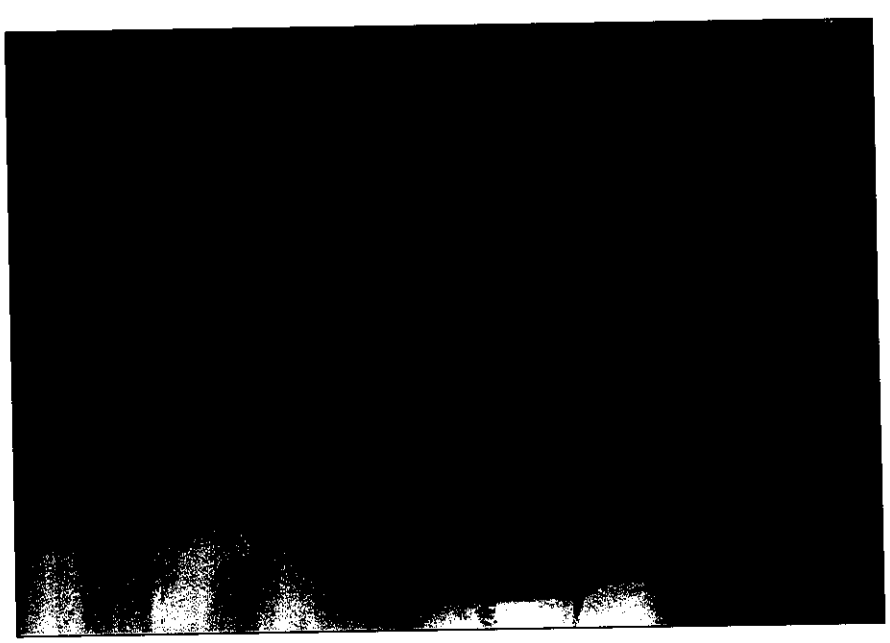


Figure 4.2 Zone of inhibition of SV strain on Congo red agar Indicating cellulase activity.

Figure 4.3 Zone of inhibition of S II strain on Congo red agar Indicating cellulase activity.

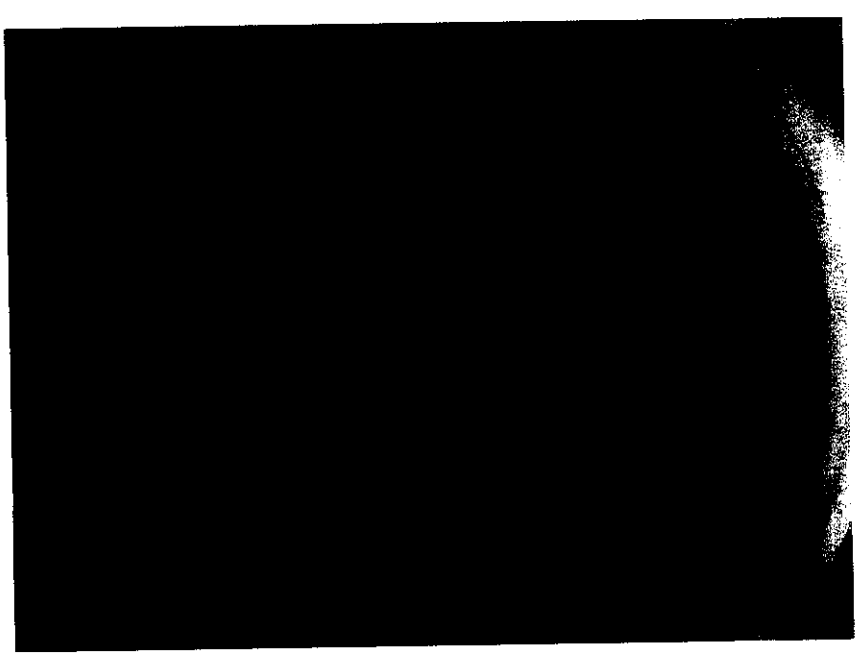


Figure 4.4 Zone of inhibition of S I strain on Congo red agar Indicating cellulase activity.

4.2. Selection of strains for saccharification

The selected strains are designated as S I, S II, S V. diameter of zone produced by S-I is 1.6cm, S-II is 2.8cm and S-V is 2.I. The largest zone produced strain S-II was selected for saccharification and ethanol production.

4.2.1 Identification of bacterial strain S-II

Morphological examination showed white, circular, dry, opaque and convex with an entire margin.

Figure 4.5 Morphology of *Bacillus sp.*

Figure 4.6 miscrospic view of *Bacillus sp.*

The microscopical examination revealed that the isolated bacteria was gram positive and rod shaped.

4.2.3 Biochemical characterization of the S-II strain

Table : 4.2 Carbohydrate fermentation

Sample	Glucose Acid/gas production .	Mannitol Acid/gas production.	Sucrose Acid/gas production.
Field soil	+/-	-/-	+/-

+ present, - Absent

Carbohydrate Fermentation Tests

Figure 4.7 Sucrose fermentation test for characterizing the isolated organism

Figure 4.8 Glucose fermentation for characterizing the Isolated organism

4.2.4 Carbohydrate fermentation test result:

The result of the biochemical identification confirmed that the strain S-II is *Bacillus sp.*

The yellow color indicates the production of acid, and the red colour indicates no gas production

Since the organism isolated is gram positive only glucose and sucrose are able to produce acid and gives positive result.

Table 4.3 : Ortho toludine test for sugar confirmation.

Component	Blank	Standard	Test
o-Toludine	5 ml	5 ml	5 ml
D.H_2O	0.1 ml	--------	--------
Glucose	--------	0.1	--------
Unknown sample	--------	--------	0.1

Table 4.4: OD values for the o-toludine test

Component	OD values
Blank	0.0
Standard	1.48
Test	0.79

4.3 CALCULATION:

- Amount of glucose present in the test per ml =

(Test O.D/Standard O.D)*Amount of Glucose Present in STD

= (0.79/1.48) * 9.36

=4.99 mg/ml

- % of scarification = (amount of glucose (mg/ml)/amount of substrate (mg/ml))*100

= (9.36/ 20) * 100

= 46.8 %

Cellulosic waste of banana plant left over otherwise for natural degradation was effectively used as component in the medium for the production of enzymes. Subsequently, these enzymes produced on the medium containing banana agro waste can be further implicated in the saccharification of the same agro waste. Amount of glucose produced is 4.99 mg/ml and % saccharification is 46.8%

Table:4.4

Determination of ethanol concentration by potassium dichromate assay

S.no	Component	Concentration of ethanol in %	Volume of ethanol added(ml)	Volume of water added(ml)	Volume of Potassium di-chromate added (ml)	O.D value
1	Blank	0	0	5	5	0.00
2	Std 1	80	4	1	5	1.50
3	Std 2	70	3.5	1.5	5	1.40
4	Std 3	60	3	2	5	1.11
5	Std 4	50	2.5	2.5	5	0.92
6	Std 5	40	2	3	5	0.74
7	Std 6	30	1.5	3.5	5	0.55
8	Std 7	20	1	4	5	0.36
9	Std8	10	0.5	4.5	5	0.18
10	Test	---	5	---	5	0.67

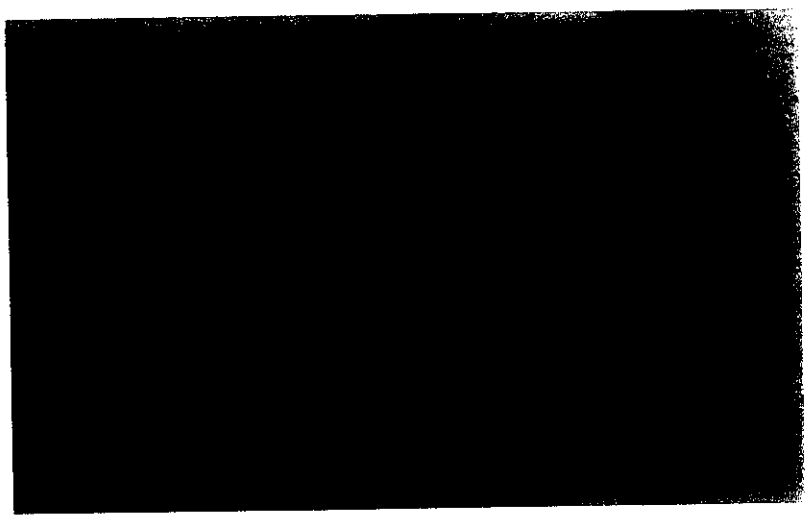


Figure 4.10 Banana Agro waste (Substrate)

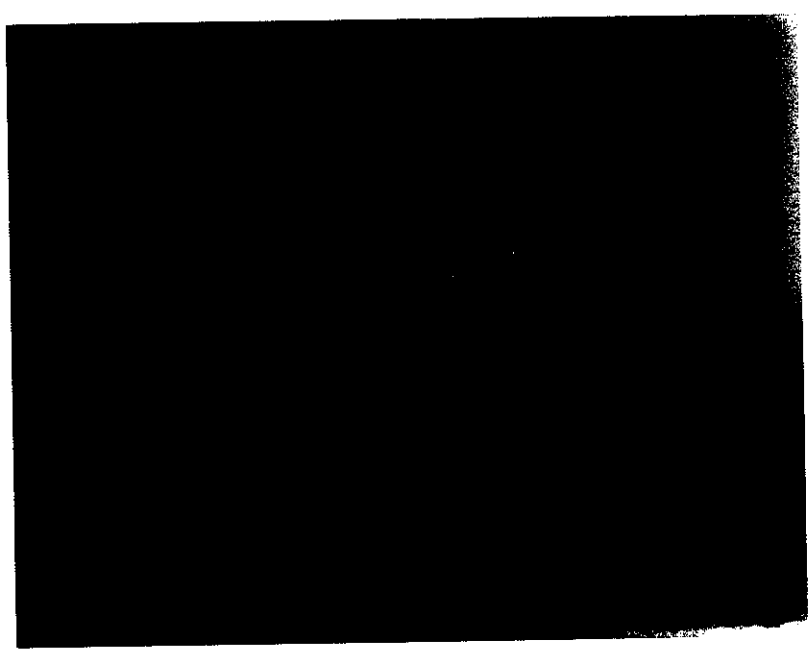


Figure 4.11 Saccharification

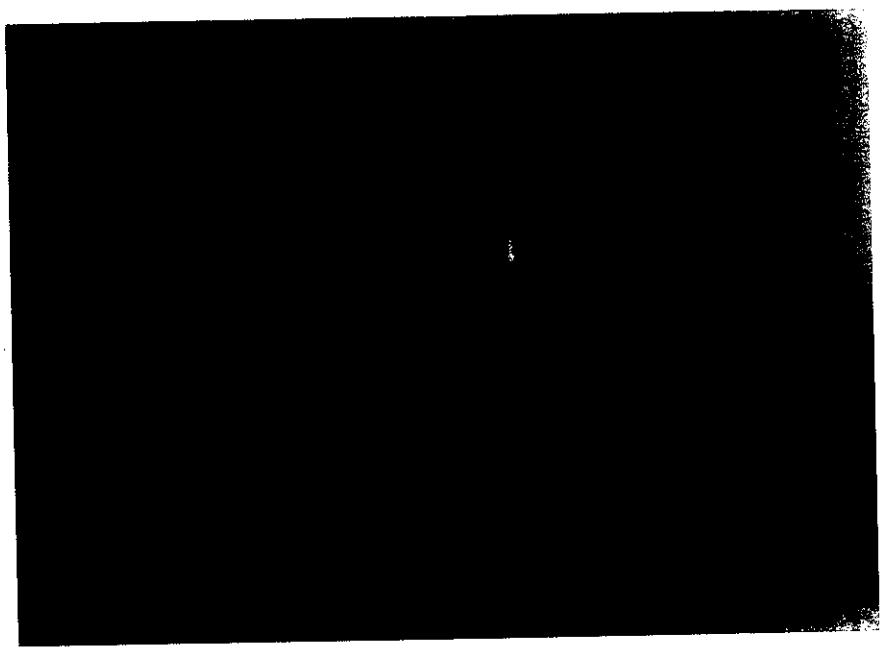


Figure 4.12 Fermentation

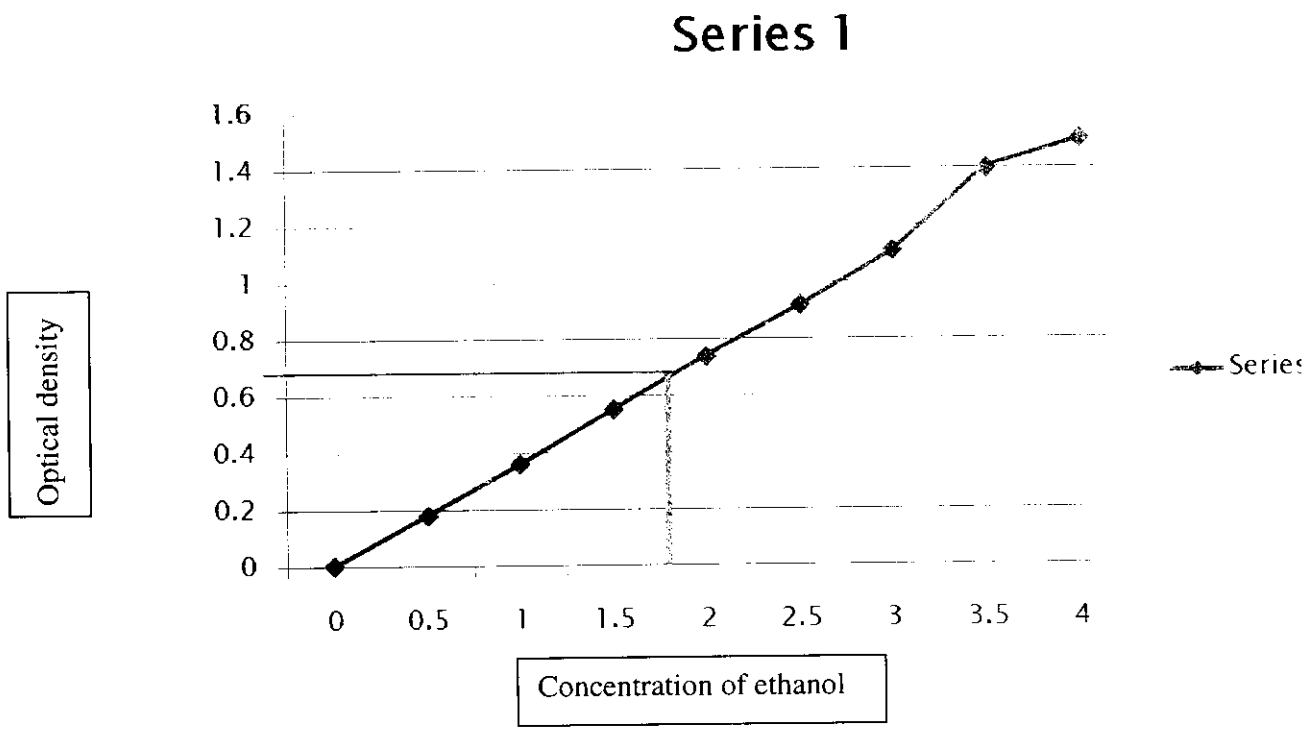


Figure 4.13 Concentration of ethanol

Calculation :

From the graph plotted slope m=0.008

y =0.67 (absorbance)

x =concentration

0.67=0.008x

x=0.67/0.008

=83.75μg/ml

The ethanol was produced from the saccharified product using *Saccharomyces cerevisiae* which yielded 35% ethanol production and the concentration is 83.75 µg/ml. More improved yield can be obtained by standardizing the above parameters and this may help our people to utilize the waste resources to make useful products having commercial importance .

Conclusion

5. CONCLUSION

Bio ethanol has a number of advantages over conventional fuels. It comes from a renewable resource i.e. crops. Another benefit over fossil fuel is the green house gas emissions. Also, blending bio ethanol with petrol will help extend the life of the diminishing oil supplies and ensure greater fuel security, avoiding heavy reliance on oil producing nations. By encouraging bio ethanol's use , the rural economy would also receive a boost from growing the necessary crops. Bio ethanol is also biodegradable and far less toxic that fossil fuels. In addition, by using bio ethanol in order engines can help reduce the amount of carbon monoxide produced by the vehicle thus improving air quality. Another advantage of bio ethanol is the ease with which it can be easily integrated in to the existing road transport fuel system. In quantities up to 5% bio ethanol can be blended with conventional fuel without the need of engine modifications. Bio ethanol is produced using familiar methods, such as fermentation, and it can be distributed using the same petrol forecourts and transportation systems as before.

Reference

REFERENCES

Agarwal, D.P., and H.W. Goedde. 1999. Pharmacogenetics of alcohol metabolism and alcoholism. Pharmacogenetics 2 (2): 48.

Ananthram P. Dadi, 2005. An Efficient Approach for Saccharification of Cellulose from Biomass for Ethanol production. *J. Appi Environ Micrabiol.* 20(3): 256-260

Barceloux, D.G., G.R. Bond, E.P. Krenzelok, H. Cooper and J.A.Vale.2002.American academy of clinical toxicology practice guidelines on the treatment of methanol poisoning. J. Toxicol. Clin. Toxicol. 40 (4): 415

Blanco S, A. Gamarra, C. Cuevas and Ellenrieder, 2004. Ethanol production by couple saccharification and fermentation of sugar cane bagasse. *J. Appl. Biotech* 4(10) :661-665.

Chia-Huang Kuo and Cheng-Kang, 2008. Enhancement of enzymatic saccharification of cellulose by cellulose dissolution pretreatments. *J. Appl. Environ Microbial* 77(1): 41-46.

Crawford, R. L.,1981. Lignin biodegradation and transformation. New York: John Wiley and Sons.

David , A.,and M.D . Yost. 2002. Acute care for alcohol intoxication. 112.

Du – Joong; Kuga, Shigenori,2006. Saccharification of cellulose by dry pyrolysis. *Wood science.* 52(5): 452-455.

Ekborg, N.A., and W.A. Morrill .2007.Multifunctional cellulase encoded by ***Teridinibacter*** *turnerae*, a culturable symbiont isolated from the wood-boring marine bivalve *Lyrodus pedicellatus*.Vol 73(23),p. 7785-7788.

Fujita, Y., Ito, J and M. Ueda, 2004. Synergistic Saccharification, and Direct Fermentation to Ethanol, of Amorphous Cellulose by Use of an Engineered Yeast strain Codisplaying Three Types of Cellulolytic Enzyme. *J. Appl Environ Microbial.* 70(2): 1207-1212

Henrissat, B., and T.T.Teeri. 1998. A scheme for designating enzymes that hydrolyse the polysaccharides in the cell wall of lands

Hirasawa, K., and M. Kashiwa. 2006.Salt activated endoglucanase of a strain of alkalophilic *Bacillus agaradhaerens.*Vol 89 (2): P. 211-219

Hyuang-Jun Ahn[1] and Lee R. Lynd, 1996. Cellulose degradation and ethanol production by thermophilic bacteria using mineral growth mediun. *J. Appl. Biochem and biotech.* 12(1): 76-80

Jones,T.T.M.,2008. The Clean Fuels Report: A quantitative comparison of motor fuels, related pollution and technologies.

Klemm., Dieter,Brigitte ,Heublein, Hans-Peter Fink, and Andreas Bohn. 2005. Cellulose: Fascinating biopolymer and sustainable raw material. ChemInform 36 (36).

Lee R. Lynd, Janeth , 1991 studied on the fuel ethanol from Cellulosic Biomass. *J.Appl. Environ Microbiol.*251(3): 1318-1323

Liang,Y., and J.Yesuf.2009.Study of cellulose from a newly isolated thermophilic and cellulolytic Brevibacillussp.strain JXL.

Li,W., and W.W.Zhang. 2008 .Cloning of the thermostable cellulase gene from newly isolated *Bacillus subtilis* and its expression in *Escherchia coli* .Vol 2:P. 195-201.

Lynd, L. R., J. W. Paul, W. H. Van Zyl, and I. S Pretorious. 2002. *Microbial cellulose utilization: Fundamentals and biotechnology. 166:506-577*

McDonnell, G., and A.D. Russell. 1999. Antiseptics and disinfectants: activity, action, andresistance. Clin. Microbiol. Rev. 12 (1): 147.

Miranda Maki., Kam Tin Leung, and Wensheng Qin.2009.The prospects of cellulase - producing bacteria for the bioconversion of lignocellulosic biomass.Vol 5(5): P.500-516.

Myers., L.Richard, and L.Rusty. 2007. The 100 most important chemical compounds. Westport, p. 122.

Perez-Avalos,O.,and L.M. anchez-Herrera.2000.A bifunctional endogluconase/endoxylanase from *Cellulomonas flavigena* with potential use in Industrial processes at different pH. Vol 57(1): P. 39-44.

Pilipski, Mark, 1981. Saccharification of cellulose. *J. Appl. Biochem and Biotech.* 34(5): 45-46.

Pohorecky, L,A., and J . Brick. 1988.Pharmacology of ethanol. Pharmacol. Ther. 36 (2-3): 335.

Prathiba. R Singh and M.K Jain, 1985. Studies on the cellulolytic bacteria and cellulose degradation in a cattle waste-fed biogas digester. *J. Appl. Biotech and microbio. 24(2): 46-50*

Ritter, S.K., 2004. Biomass or Bust. Chemical & Engineering News 82: 31.

Roach, J., 2005. 9,000-Year-old beer re-created from Chinese recipe. National Geographic News.

Shwarz. W. H.2001. The cellulosome and cellulose degradation by anaerobic bacteria. *Appl. Microbiol. Biotechnol.*. 56:634-649

Slavin, J.L., P.M. Brauer, and J.A. Marlett. 1980. Neutral detergent fiber, hemicellulose and cellulose digestibility in human subjects.Vol 111(2): P.287-297.

Stenius., and Per.2000. Forest products chemistry papermaking science and technology .Vol3: p. 35 Finland.

Smirnova E, and M.G Saubenova, 2001. Use of cellulose-degrading nitrogen-fixing bacteria in the enrichment of roughage with protein. *J. Appl. Biochem and microbio.* 37(1): 76-79