EVALUATION OF BIOETHANOL PRODUCTION POTENTIALS OF SELECTED LIGNOCELLULOSIC WASTES

BY

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CERTIFICATION

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DEDICATION

This dissertation is dedicated to the Impeccable, Omnipotent, Omniscient and Almighty God under whose auspices and favour, I have been able to complete this course.

This dissertation is also dedicated to the memory of my (Late) father, Yinka Sokan-Adeaga and my caring and pertinacious Mother, Sayo Sokan-Adeaga and my darling siblings Ayodeji and Eniola Sokan-Adeaga who have been indelible pillars of strength in all my endeavours. Appropriate word of love and appreciation I have none, I love you all. May God grant you all longlife and prosperity to reap the fruit of your labour, Amen!

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Sokan –Adeaga Adawale

ABSTRACT

In the last three decades most countries, particularly the developing nations, have been experiencing energy deficit because of overdependence on fossil-based fuels. This development has led to the search for alternative energy sources. Nigeria is rich in biomass and waste materials that are suitable precursors for biofuels, yet these have not been fully explored. This study was therefore designed to evaluate the bioethanol production potentials of multi-substrate lignocelluloses-based wastes.

Four lignocellulose-based wastes – Cassava Peels (CP), Yam Peels (YP), Plantain Peels (PP) and Sawdust (SD) were purposively selected. They were subjected to pre-treatment, chemical hydrolysis, microbial fermentation and confirmatory biochemical tests following the methods described by the Association of Official Analytical Chemists (AOAC). Grab samples of the wastes were air dried and pulverized. Twenty grammes each of the powdery biomass was treated separately with 100ml 5.6M, 9.4M and 13.1M of H_2SO_4 in a two stage hydrolysis. The first hydrolysis was done at 100°C for 60mins, after which the residue was hydrolysed at 100°C for 50mins. The mixed hydrolysates were analysed for glucose and Total Reducing Sugars (TRS). The 13.1M H_2SO_4 gave the best yield of glucose and TRS hence was fermented with *Saccharomyces cerevisiae* at 30°C for 72 hours. Samples were taken from the fermenting broths every 24 hours for ethanol yield determination. Data were analysed using descriptive statistics, t-test, ANOVA and Spearman-rank correlation at $p=0.05$.

Mean glucose yield and TRS obtained from the 5.6M H_2SO_4 hydrolysis were: CP (50.5±5.0mg/kg, 91.8±3.0mg/kg); YP (231.0±3.6mg/kg, 388.8±6.9mg/kg); PP (255.5±5.4mg/kg, 314.7±5.1mg/kg) and SD (285.7±5.0mg/kg, 374.5±7.3mg/kg). At 9.4M H_2SO_4 hydrolysis, the mean glucose yield and TRS were: CP (71.5±3.0mg/kg, 123.2±5.0mg/kg); YP (240.0±5.0mg/kg, 460.2±4.7mg/kg); PP (278.1±6.5mg/kg, 396.4±6.0mg/kg) and SD (300.7±8.6mg/kg, 453.2±6.6mg/kg). The mean glucose yield and TRS obtained from the 13.1M H_2SO_4 Hydrolysis were: CP (85.1±5.7mg/kg, 209.8±3.7mg/kg), YP (269.2±11.2mg/kg, 541.3±7.8mg/kg), PP (304.0±6.1mg/kg, 461.2±3.6mg/kg) and SD (343.2±4.8mg/kg, 535.9±5.0mg/kg). The mean glucose yield and TRS obtained from the 13.1M H_2SO_4 were significantly higher than those

obtained from the 9.4M and 5.6M H_2SO_4 hydrolysis. The 13.1M hydrolysate was used for the ethanol production and the mean ethanol yield at 24 hours of fermentation were: CP (123.3±11.1ml/kg), YP (172.0±17.5mL/kg). PP (217.7±13.5mL/kg) and SD (240.3±14.0mL/kg) respectively. The maximum ethanol production was obtained at 48 hours, the mean ethanol yield being: CP – 160.0±15.1mL/kg, YP – 211.7±15.3mL/kg, PP – 265.0±20.5mL/kg and SD – 280.0±11.5mL/kg. Mean ethanol yield obtained at 48 hours of fermentation were significantly different from those obtained at 24 hours. A significant correlation exists between the ethanol yield of the substrates and the time of fermentation (r=0.95).

Sawdust produced the highest glucose and ethanol yields among the substrates. Therefore ethanol production from sawdust should be fully optimized.

Keywords: Lignocellulosic wastes, Biofuels, Bioethanol production.

Word Count: 471

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GLOSSARY OF TECHNICAL TERMS AND ABBREVIATIONS

ADH	Alcoholdehydrogenase
AOAC	Association of Official Analytical Chemists
ATSR	Automotive Technology Systems Research
BOD	Biochemical Oxygen Demand
CDM	Clean Development Mechanism
CGW	Cassava Grated Waste
CHP	Combined Heat and Power
CI	Combustion Ignition
CRIN	Cocoa Research Institute of Nigeria
CP	Cassava Peels
DDG	Distiller's Dried Grain
DM	Dry Matter
DME	Dimethyl Ether
EDP	Entner - Doudoroff Pathway
EIA	Environmental Impact Assessment
EMP	Embden – Meyerhof Pathway
EPA	Environmental Impact Assessment
FEPA	Federal Environmental Protection Agency
FTL	Fischer Tropsch Liquids
GHG	Green House Gas

IES	International Energy Statistics
IITA	International Institute of Tropical Agriculture
IMRAT	Institute of Medical Research and Advanced Training
NDF	Non Detergent Fibre
NIHORT	National Institute for Horticultural Research and Training
NMHC	Non – Methane Hydrocarbon
OD	Optical Density
PDC	Pyruvate Decarboxylase
PP	Plantain Peels
PPM	Parts Per Million
R&D	Research and Development
SD	Sawdust
SHF	Separate Hydrolysis and Fermentation
SSF	Solid State Fermentation
TOC	Total Organic Carbon
TRS	Total Reducing Sugars
UCH	University College Hospital
UI	University of Ibadan
UNEP	United Nations Environmental Programme
UNWUP	United Nations World Urbanization Prospects
YP	Yam Peels

CHAPTER ONE
INTRODUCTION

1.1 Background Information

Waste generation is increasing rapidly with urbanization and industrialization. The quantum of waste generated varies from place to place depending upon the population density and demand from society. Compared to other places, waste generation is more where the population density is maximum. Waste is generated from various sources including domestic and industrial sources, which cover mainly municipal solid and liquid waste; chemical, pharmaceutical, and agro industrial waste; plastic waste; waste water effluent; and so on (Lal and Reddy, 2005).

Wastes in the environment pose many harmful effects both on the ecology and public health. It also has adverse effect on non humans including those in phylogenetic kingdoms, including moncra, protista, fungi, plantae, and animalia. This informs the need for proper waste management in order to mitigate its, detrimental impact. Management of waste is o key element in the protection of public health and failure to manage and dispose waste properly may lead to severe consequences (Lucas and Gilles, 2003). Although nature has already created the process of converting one type of waste to another form by its natural biological cycle, however with growing demands of humans, these natural efforts have become insufficient in maintaining this biogeochemical cycle. On the contrary, sometimes, the natural biological processes also generate some form of waste that has a direct impact on the environment. For example, annually almost 250 million tones of methane gas is generated by anaerobic digestion by methanogenic bacteria, world over: methane traps 30 times more heat than carbon dioxide and contributes to 18% of the global warming (Lal and Reddy, 2005). Also the polluting gases produced by human beings especially by fossil fuels (carbon, methane, nitrous oxide, hydrofluoro carbon, perfluoro carbon, hexafluoro azide) have been degrading our ozone layer provoking green house effects.

This has led to the development of some alternatives for managing the hazardous wastes and making the environment free from the detrimental impacts of the said wastes. Various scientists and industrialists have made efforts to solve the challenging problem faced by the environment due to hazardous wastes. Today, various methods of treatment of solid and liquid waste have been successfully developed and implemented globally. However, the waste management practice is not sufficient to make the environment completely free from the detrimental impact of the wastes (Lal and Reddy, 2005). But, with continuous improvement in the fields of biotechnology, scientist have developed new techniques by which some of these wastes can be harnessed into some alternate products which can be highly useful to the society. Today, examples of such efforts are seen in the production of biofuels from wastes of organic origin, often known as Biomass.

Cellulose, the most abundant component of plant biomass, is found in nature almost exclusively in plant cell walls, although it is produced by some animals (e.g., tunicates) and a few bacteria. Despite great differences in composition and in the anatomical structure of cell walls across plant taxonomy, high cellulose content typically in the range of approximately 35 to 50% of plant dry weight is a unifying feature. In a few cases (notably cotton bolls), cellulose is present in a nearly pure state. In most cases, however, the cellulose fibers are embedded in a matrix of other structural bipolymers, primarily hemicellulose and lignin, which comprise 20 to 35% and 5 to 30% of plant dry weight. Although these matrix interactions vary with plant cell type and with maturity, they are a dominant structural feature limiting the rate and extent of utilization of whole, untreated biomass materials (Dunlap and Chaing, 1980).

The term "biomass" is generally referred to as renewable organic matter generated by plants through photosynthesis wherein solar energy combines with CO_2 (carbon dioxide) and moisture to form carbonhydrates and oxygen. Materials having combustible organic matter are reffered to as biomass. It contains Carbon, Hydrogen and Oxygen, (oxygenated hydrocarbon), with high level of moisture and volatile matter, low bulk density and

calorific value (Lal and Reddy, 2005). A lignocellulosic biomass is composed primarily of plant fibres that are inedible by humans and have cellulose as a prominent component.

Lignocellulosic biomass may be available as either:

Residues: these are biomass resulting from activities or processes undertaken for some purpose other than ethanol production. Examples of such residues include corn stalks and other non-edible parts of plants used to produce food, municipal solid waste, and pulp and paper industry wastes.

Dedicated crops: crops grown for the primary purpose of energy production. Examples of potential dedicated crops for producing cellulosic biomass include grass and short rotation tree. These fibres may be hydrolyzed to yield a variety of sugars that can be fermented by microorganisms (Lynd *et al*, 2003).

The term "bio-fuel" on the other hand, means any liquid fuel made from plant material that can be used as a substitute for petroleum-derieved fuel. It is a renewable energy, unlike other natural resources such as petroleum, coal and nuclear fuels because its carbon was extracted from atmospheric carbon dioxide by growing plants and as such does not result in a net increase of carbon dioxide in the earth' s surface (Agarwal, 2005). Biofuels include relatively familiar ones, such as ethanol made from sugar cane or diesel-like fuel made from soyabean oil, to less familiar fuels such as dimethyl ether (DME) or Fischer-Tropsch Liquids (FTL) made from lignocellulosic biomass. Biofuels are commonly used throughout the world. The most common use for biofuels is automotive transport.

Essentially a biofuel can be produced from any short term carbon cycle organic compound; due to this, there is a high variety of resources and therefore many types of biofuels. They include: Vegetable oil, Biodiesel, Ethanol, Methanol, Butanol, Biogas, Biohydrogen and Biodimethyl ether.The use of waste derieved biofuels has many benefits. Cellulose is present in every plant, in the form of straw, grass, and wood. Most of these "biomass" products are currently discarded (Lynd *et al*, 2003). It is estimated that 233 million tons of cellulose containing raw materials that could be used to produce ethanol are thrown away

each year in US alone. This includes 36.8 million dry tons of urban wood wastes, 90.5 million dry tons of corn stalks and wheat straws. Transforming them into ethanol using efficient and cost effective lignocellulolytic enzymes or other processes might provide as much as 30% of the current fuel consumption in the United States and probably similar figures in other oil-importing regions like China or Europe.

One of the major reasons for increasing the use of biofuels is to reduce greenhouse gas emissions. Cellulosic ethanol contributes little to the greenhouse effect and has a five times better net energy balance than corn-based ethanol. Reduction of the disposal of solid waste through cellulosic ethanol conversion would reduce solid waste disposal costs by local and state government.

1.2 Problem statement

Solid waste disposal is of enormous concern in developing countries across the world, as poverty and urbanization combined with poor government support prevent efficient management of wastes generated from domestic and industrial activities (UNEP, 2002). In developing countries, there is a much higher proportion of organics, and considerably less plastics (Cointreau, 1982). The large amount of organic material makes the waste more dense, with greater moisture and smaller particle size (Cointreau, 1982).

Nigeria as a developing nation exemplifies chronic solid waste management problems in conjunction with population growth. It is the most populous county in Africa, with over 162.5 million people in 2011 from 45.2 million in 1960, changing by 251 percent during the last 50 years. It has 2.29 percent of the world's population (CIA. World Factbook, 2012). and over the past 50 years, has had the third largest urban growth rate in the world at 5.51% annually (UNWUP, 1999).

Consequently, the environmental and health impacts of solid wastes are enormous, in the absence of proper management and consist of a large number of components. Health impacts includes: exposure to toxic chemicals through air, water and solid media; exposure to infection and biological contaminants; stress related to odour, noise, vermin and visual

amenity; risk of fires, explosions and subsidence; spills, accidents and transport emissions (Dolk, 2002).

Environmental impacts can be clustered into six categories: global warming, photochemical oxidant creation, abiotic resource depletion, acidification, eutrophication and ecotoxicity to water (Seo *et al.* 2004).

The enormous growth in the world populations, during the last few decades has led to a difficult situation in the field of energy supply and demand. At present, the world is confronted with the twin crises of fossil fuel depletion and environmental degradation. Indiscriminate extraction and consumption of fossil fuels have led to a reduction in the underground carbon sources. The global reserves of primary energy and raw materials are obviously limited. According to an estimate, the reserves will last for 218 years for coal, 41 years for oil, and 63 years for natural gas under a business-as-usual scenario coupled with their inherent environmental impact (Agarwal, 2005). This has made the search for alternative and renewable sources of energy inevitable.

Studies have shown that indiscriminate use of fossil fuels has led to increased carbondioxide levels in the atmosphere from 280 ppm in the pre-industrial era to 350 ppm now (Agarwal, 2005). The CO_2 levels are still climbing and the resulting environmental implications are being felt in our day-to-day lives. Excessive use of fossil fuels has led to global environmental degradation effects such as the green house effects, acid rain, ozone depletion and wide spread climate change. This scenario has generated a lot of interest on renewable energy sources, the need to reduce the use of fossil fuels and potential of biomass derived biofuels in order to prevent global warming.

1.3 Rationale for the study

Many industrialized countries are pursuing the development of expanded or new biofuels industries for the transport sector, and there is growing interest in many developing countries for similarly "modernizing" the use of biomass in their countries and developing greater access to clean liquid fuels while helping to address energy costs, energy security and global warming concerns associated with fossil fuels (Green, 2006). The Federal

Environmental Protection Agency (FEPA) in an attempt to address environmental problems came up with vision 2010. The report proposed goals to be accomplished by the year 2010 that would lead to sustainable development. As regards to solid waste management; the report says the goal is to "achieve not less than 80% effective management of the volume of municipal solid waste generated at all levels and ensure environmentally sound management" (vision 2010, 2003). Therefore, this research is a welcome development, revealing the bio-ethanol potential of cellulosic Biomass from wastes, thus contributing to the realization of the aforementioned goal.

Kyoto's protocol is one of the environmental agreements signed by the firstline countries to reduce green house gas production. This commitment established that every country has to reduce from 25% to 40% green house gas emission by 2020 in relation to its 1990 rate. According to Kyoto's protocol commitment the need for an alternative energy production appears, (being biofuels) a viable answer. So most countries are evolving new technologies of producing and using biofuels; biofuels have two main goals: to substitute fossil fuels and to reduce green house gases which are the main culprit in climate change. In the energy production cycle, the best known biofuels is ethanol.

Furthermore, Nigeria recently adopted an ethanol production policy with cassava as its main feedstock, in response to the global initiative (bio-fuel production), which promises a harmonious correlation with sustainable development, efficient and energy conservation. Although fuel ethanol is currently produced from sugarcane and other starch rich grains, ethanol also can be made from cellulosic materials such as wood, grass and agro-residue (Lynd *et al.*, 2003). This would reduce the pressure on food security due to excessive use of food crops for bio-fuel produce and reduce dependence on imported petroleum for vehicle, ensure environmental sustainability, sound public health and create wealth and employment opportunities.

The long term benefits of this research will be to introduce a sustainable solid waste management strategy for a number of livestock manure and other lignocelluloses waste materials, contribute towards the mitigation of greenhouse gases emissions through

sustained carbon and nutrient recycling; reduce the potential for water, air, and soil contamination associated with land disposal of organic waste materials; and to broaden the feedstock source of raw materials for the ethanol production industry.

1.4 Objectives

1.4.1 Broad Objective

The main objective is to evaluate the bio-ethanol production potential of different agro-based cellulosic wastes.

1.4.2 Specific Objectives

The specific objectives are to:

1. Identify the viable biomass substrates.
2. Characterize the quantity of agro-based cellulosic wastes from different sources.
3. Process each of the selected cellulosic wastes into ethanolic by product /ethanol.
4. Optimize bioethanol production from the selected cellulosic wastes.
5. Evaluate the bioethanol yield of the selected agro-based cellulosic wastes.

CHAPTER TWO

LITERATURE REVIEW

2.1 Lignocellulose Biomass

The term "biomass" generally refers to renewable organic matter generated by plants through photosynthesis in which solar energy combines with CO_2 (carbon dioxide) and moisture to form carbohydrates and oxygen materials having combustible organic matter are referred to as biomass. Biomass contains carbon, Hydrogen and Oxygen (oxygenated hydrocarbon, with high level of moisture and volatile matter, low bulk density and calorific value (Lal and Reddy, 2005). Lignocellulose biomass refer to the major structural component of woody and non-woody plants such as grass and represents a major source of renewable organic matter. A lignocellulosic biomass composed primary of plant fibres that are inedible by humans and have cellulose as a prominent component. Lignocellulose biomass consists of Lignin, hemicelluloses and cellulose. The composition is in the following proportion: cellulose (30 – 50%), hemicelluloses (20 – 35%) and lignin (5 – 30%) of plant dry matter. Lignocellulose biomass is a renewable resource that is virtually inexhaustible and is a potential feedstock for alternate fuel production. It may be available as either (a) residues corn stalks or other non-edible parts of plants used to produce food, municipal solid waste, pulp and paper industry wastes; (b) dedicated crops grown for the primary purpose of energy production (Lynd *et al*, 2003).

2.2 Components of Lignocellulose

Lignocellulose consists of lignin, hemicellulose and cellulose and Table 2.1 shows the typical compositions of lignocellulosic materials.

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Table 2.1 Lignocellulose contents of common agricultural residues and wastes.

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood stems	40-55	24-40	18-25
Softwood stems	45-50	25-30	25-35
Nut shells	25-30	25-30	30-40
Paper	85-99	0	15
Wheat straw	30	50	15
Rice straw	32.1	24	18
Sorted refuse	60	20	20
Leaves	15-20	80-85	0
Cotton seeds hairs	80-95	5-20	20
Newspaper	40-55	25-40	18-30
Waste paper from chemical pulps	60-70	10-20	5-10
Primary wastewater solids	8-15	NA	24-29
Fresh bagasse	33.4	30	18.9
Swine waste	6	28	NA
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12.0
S32 rye grass (early leaf)	21.3	15.8	2.7
S32 rye grass (seed setting)	26.7	25.7	7.3

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Orchard grass (medium maturity)	32	40	4.7
Grasses (average values for grasses)	25-40	25-50	10-30
Bagasse	41.4	21.9	25.5
Forest residue	51	13	26.5

Source: Compiled from Betts *et al.* (1991); Sun and Cheng, (2002)

2.2.1 Lignin

In general lignin contains three aromatic alcohols (coniferyl alcohol, s-inapyl and p-coumaryl). In addition, grass and dicot lignin contain large amounts of phenolic acids such as p-coumaric and ferulic acid, which are esterified to alcohol groups of each other and to other alcohols such as s-inapyl and p-coumaryl alcohols. Because of the difficulty in dissolving lignin without destroying it and some of its subunits, its exact chemical structure is difficult to ascertain.

Lignin is further linked to both hemicellulose and cellulose forming a physical seal around the latter two components that is an impenetrable barrier preventing penetration of solutions and enzymes. Of the three components, lignin is the most recalcitrant to degradation whereas cellulose, because of its highly ordered crystalline structure, is more resistant to hydrolysis than hemicellulose.

Indentifying lignin degrading microorganisms has been hampered because of the lack of reliable assays, but significant progress has been made through the use of a ^{14}C-labelled lignin assay (Freer and Detroy, 1982). Fungi breakdown lignin aerobically through the use of a family of extracellular enzymes collectively termed "lignases". Two families of

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lignolytic enzymes are widely considered to play a key role in the enzymatic degradation: phenol oxidase (LiP) and manganese peroxidase (MnP) (Krause et al., 2003; Malherbe and Cloete, 2003). Other enzymes whose roles have not been fully elucidated include H producing enzymes; glyoxal oxidase (Kersten and Kirk, 1987), glucose oxidase (Kelly and Reddy, 1986), veratryl alcohol oxidase (Bourbonnais and Paice, 1988), methanol oxidase (Nishida and Eriksson, 1987) and oxido-reductase (Bao and Renganathan, 1991). Enzymes involved in lignin breakdown are too large to penetrate the unaltered cell wall of plants, so the question arises, how do lignases affect lignin biodegradation. Suggestions are that lignases employ low- molecular, diffusible reactive compounds to affect initial changes to the lignin substrate (Call and Mucke, 1997).

2.2.2 Hemicellulose

Hemicellulose is a collective term referring to those polysaccharides soluble in alkali, associated with cellulose of the plant cell wall, and these would include non-cellulose $\beta - D -$ glucans, pectic substances (polygalacturonans), and several heteropolysaccharides such as those mainly consisting of galactose (arabinogalactans), mannose (galactogluco-and glucomannans) and xylose (arabinoglucuro- and glucuronoxylans). However, only the heteropolysaccharides, those with a much lower degree of polymerisation (100-200 units) as compared to that of cellulose (10000-14000 units) are referred to as hemicelluloses. The principal sugar components of these hemicellulose heteropolysaccharides are: D-xylose, D-mannose, D-glucose, D-galactose, L-arabinose, D-glucuronic acid, 4-O-methyl-D-glucuronic acid, D-galacturonic acid, and to a lesser extent, L-rhamnose, L-fucose, and various O-methylated sugars.

Rabinovich *et al*. (2002a) and Shallom and Shoham (2003) present recent reviews covering the types, structure, function, classification of microbial hemicellulases. The details of catalytic mechanism and structure of glycoside hydrolases, are also reported in (Withers, 2001; Rabinovich *et al*., 2002b). Hemicellulases like most other enzymes which hydrolyse plant cell polysaccharides are multi-domain proteins (Henrissat and Davies, 2000; Prates *et al*., 2001). These proteins generally contain structurally discrete catalytic and non-catalytic

modules. The most important non-catalytic modules consist of carbonhydrate binding domains (CBD) which facilitate the targeting of the enzyme to the polysaccharide, interdomain linkers, and dockerin modules that mediate the binding of the catalytic domain via cohesion-dockerin interactions, either to the microbial cell surface or to enzymatic complexes such as the cellulosome (Shallom and Shoham, 2003; Prates *et al.*, 2001). Based on the amino acid or nucleic acid sequence of their catalytic modules hemicellulases are either glycoside hydrolases (GHs) which hydrolyse glycosidic bonds, or carbonhydrate esterases (CEs), which hydrolyse ester linkages of acetate or ferulic acid side groups and according to their primary sequence homology they have been grouped into various families (Henrissat and Davies, 2000; Rabinovich *et al.*, 2002a,b).

Xylan is the most abundant hemicellulose and xylanases are one of the major hemicellulases which hydrolyse the β-1,4 bond in the xylan backbone yielding short xylooligomers which are further hydrolysed into single xylose units by β-xylosidase. Most known xylanases belong to the GH10 and 11 families 3, 39, 43, 52 and 54. Bifunctional xylosidase-arabinosidase (Lee *et al.*, 2003) enzymes are found mainly in families 3, 43 and 54. B- mannanases hydrolyse mannan-based hemicellulose and liberate short β-1,4-manno-oligomers which can further be hydrolyseed to mannose by β-mannosidases. About 50 mannases are found in GH families 5 and 26, and about 15 β- mannosidase in families 1, 2 and 5. α–L-Arabinofuranosidases and α -L-arabinanases hydrolyse arabinofuranosyl-containing hemicellulose, and are distributed in GH families 3, 43, 51, 54 and 62. Some of these enzymes exhibit broad substrate specificity, acting on arabinofuranoside moieties at O-5, O-2 and / or O-3 bonds as a single substituent, as well as from O-2 and O-3 doubly substituted xylans, xylooligomers and arabinans (Saha, 2000). Other xylanases are α-D-glucuronidases which hydrolyse the α-1,2- glycosidic bond of the 4-O-methyl-D-glucuronic acid side chain of xylans and are found in family 67. Hemicellulolytic esterases include acetyl esterases which hydrolyse the acetyl substitutions on xylose moieties, and feruloyl esterase which hydrolyse the ester bond between the arabinose substitutions and ferulic acid. Feruloyl esterases aid the release of hemicellulose from lignin and render the

free polysaccharide product more amenable to degradation by the other hemicellulases (Prates *et al.*, 2001).

2.2.3 Cellulose

In most lignocellulosic materials cellulose forms the major part of the three components. Cellulose is composed of insoluble, linear chains of β-(1→4)-linked glucose units with an average degree of polymerisation of about 10,000 units but could be as low as 15 units (Eveleigh, 1987). It is composed of highly crystalline regions and amorphous (non-crystalline) regions forming a structure with high tensile strength that is generally resistant to enzymatic hydrolysis, especially the crystalline regions (Walker and Wilson, 1991).

Cellulases, responsible for the hydrolysis of cellulose, are composed of a complex mixture of enzyme proteins with different specificities to hydrolyse glycosidic bonds. Cellulases can be divided into three major enzyme activity classes (Goyal *et al.*, 1991; Rabinovich *et al.*, 2002a, b). These are endoglucanases or endo-1,4-β-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC3.2.1.21). Endoglucanases, often called carboxymethylcellulose (CM)-cellulases, are proposed to initiate attack randomly at multiple internal sites in the amorphous regions of the cellulose fibre opening- up sites for subsequent attack by the cellobiohydrolases (Wood, 1991). Cellobiohydrolase, often called an exoglucanase, is the major component of the fungal cellulase system accounting for 40-70% of the total cellulase proteins and can hydrolyse highly crystalline cellulose (Esterbauer *et al.*, 1991)

Cellobiohydrolases remove mono- and dimer from the end of the glucose chain. B-glucosidase hydrolyse glucose dimers and in some cases cello-oligosaccharides to glucose. Generally, the endoglucanases and cellobiohydrolases work synergistically in the hydrolysis of cellulose but the details of the mechanism involved are still unclear (Rabinovich *et al*, 2002b). Microorganisms generally appear to have multiple distinct variants of endo- and exo glucanases (Beldman *et al.*, 1987; Shen *et al.*, 1995). Similar to hemicellulases most cellulase are multi-domain protein. There is still uncertainty in the current definition and classification of "true" cellulase families (Rabinovich *et al.*, 2002a).

2.3 Potential Sources of Agro- based Wastes (Residues)

Billions of tons of agricultural waste are generated each year in the developing and developed countries. Agricultural residues includes all leaves, straw and husks left in the field after harvest hulls and shells removed during processing of crop at the mills, as well as animal dung. The types of crop residue which play a significant role as biomass fuel are relatively few (Dhingra, Mande, Kishore, *et al.*, 1996). The quantity of agricultural residues produced differs from crop to crop and is affected by seasons, soil types, and irrigation conditions. Production of agricultural residues is directly related to the corresponding crop production and ratio between the main crop produce and the residues, which varies from crop to crop and, at times, with the variety of the seeds in one crop itself. Thus, for known amounts of crop production, it may be possible to estimate the amounts of agricultural residues produced using the residue to crop ratio (vimal and Tyagi 1984).

Iyer *et al.* (2002), reported, that agro-residue does suffer two major constraints: high moisture content and relatively low bulk density. These constraints inhibit their economical transportation over long distances, thereby necessitating their utilization near the sources of production. Unlike fossil fuels, which are concentrated sources of energy and chemicals the management strategy for agro-residues utilization has to be different. These are, therefore, most appropriate for decentralized technological applications in rural environments. The processing of the agricultural produce and utilization of agro-residues, therefore, can contribute their maximum share to rural development.

A wide variety of biomass resources are available on our planet for conversion into bioproducts. These may include whole plants, plant parts (e.g seeds, stalks), plant constituents (e.g. starch, lipids, protein and fibre), processing byproducts (distiller's grains, corn solubles), materials of marine origin and animal byproducts, municipal and industrial wastes (Smith *et al.*, 1987). These resources can be used to create new biomaterials and this will require an intimate understanding of the composition of the raw material whether it is whole plant or constituents, so that the desired functional elements can be obtained for bioproduct production. Several literature (Bhat, 2000; Sun and Cheng,

2002; Wong and Saddler, 1992a,b; Beauchemin *et al.*, 2001, 2003; Subbramaniyan and Prema, 2002; Beg *et al.*, 2001) have also reported on the applications.

2.3.1 Chemicals

Bioconversion of lignocellulosic wastes could make a significant contribution to the production of organic chemicals. Over 75% of organic chemicals are produced from five primary base- chemicals: ethylene, propylene, benzene, toulene and xylene which are used to synthesize other organic compounds. which in turn are used to produce various chemical products including polymers and resins (Coombs, 1987). The aromatic compounds might be produced from lignin whereas the low molecular mass aliphatic compounds can be derieved from ethanol produced by fermentation of sugar generated from the different potential bioproducts and their many cellulose and hemicelluulose. Table 2.2 shows the estimations of totals demands for chemicals which could be made by fermentation.

Table 2.2 Annual production of chemicals which potentially be made from fermentation.

Products	World demand (thousand of tonnnes)
Ethanol	16,000
Acetone	1659
Butanol	1400
Glycerol	414
Acetic acid	2539
Citric acid	300
Fumaric acid	60

Source: Modified from Coombs, 1987

2.3.2 Enzymes

Cellulases and hemicellulases have numerous applications and biotechnological potential for various industries including chemicals, fuel, food, brewery and wine, animal feed, textile and laundry, pulp and paper and agriculture (Bhat, 2000; Sun and Cheng, 2002; Wong and Saddler, 1992a,b; Beauchemin *et al.*, 2001, 2003). It is estimated that approximately 20% of the >1billion US dollars of the world's sale of industrial enzymes consists of cellulases, hemicellulases and pectinases and that the world market for industrial enzymes will increase in the range of 1.7 – 2.0 billion US dollars by the year 2005 (Bhat, 2000).

In the baking industry xylanases are used for improving desirable texture, loaf volume and shelf- life of bread. A xylanase, Novozyme 867, has shown excellent performance in the wheat seperation process (Christopherson *et al.*, 1997). Hemicellulases are used for pulping and bleaching in the pulp and paper industry where they are used to modify the structure of xylan and glucomannan in pulp fibres to enhance chemical delignation (Suurnakki *et al.*, 1997). A patented Lignozyme process is effective in delignifying wood in a pilot pulp- and paper process (Call and Mucke, 1997). In bio- pulping where lignocellulytic enzymes were used the following was achieved: tensile, tear and burst indexes of the resultant paper improved, brightness of the pulp was increased and an improved energy saving of 30- 38% was realised (Scott *et al.*, 1998). Laccases can degrade a wide variety of synthetic dyes making them suitable for the treatment of wastewater from the textile industry (Rosales *et al.*, 2002). Organisms such as the white rot fungi producing lignases could be used for the degradation of persistent aromatic pollutants such as dichlorophenol, dinitrotoulene and anthracene (Gold and Alic, 1993).

There is a huge potential market for fibre- degrading enzymes for the animal feed industry and over the years a number of commercial preparations have been produced (Beauchemin *et al.*, 2001, 2003). The use of fibre- degrading enzymes for ruminants such as cattle and sheep for improving feed utilsation, milk yield and body weight gain have attracted considerable interest. Steers fed with an enzyme mixture containing xylanase and cellulase shown an increased live-weight gain of approximately 30-36% (Beauchemin *et al.*, 1995).

In diary cows the milk yield increased in the range of 4 to 16% on various commercial fibrolytic enzyme treated forages (Beauchemin *et al.*, 2001).

2.3.3 Other high-value bioproducts

Currently a number of products such as organic acids, amino acids, vitamins and a number of bacterial and fungal polysaccharides such as xanthan are produced by fermentation using glucose as the base substrate but theoretically these same products could be manufactured from "lignocellulose waste". Based upon the predicted catabolic pathway metabolism and the known metabolism of *Phanerachaete chrysosporium* of lignin, Ribbbons (1987) presented a detailed discussion of the potential value adddcd products which could be dericved from lignin. Vanillin and gallic acid are the two most frequently discussed monomeric potential products which have attracted interest. Vanillin extraction from Vanilla pods costs between $1200 to $4000 per kilogram,whereas synthetic vanillin costs less than $15 per kilogram (Walton *et al.*, 2003). Vanillin is used for various purposes including being an intermediate in the chemical and pharmaceutical industries for the production of herbicides, anti-foaming agents or drugs such as papavcrine, L-dopa and the anti microbial agent, trimethoprim. It is also used in household products such as air-fresheners and floor polishes (Walton *et al.*, 2003). The high price and limited supply of natural vanillin have necessitated a shift towards its production from other sources (Priefert *et al.*, 2001).

Hemicelluloses are of particular industrial interest since these are a readily available bulk source of xylose from which xylitol and furfural can be dericved. Xylitol used instead of sucrose in food as asweetner, has odontological applicat.ons such as teeth hardening, remineralisation, and as an antimicrobial agent, it is used in chewing gum and toothpaste formulations (Roberto *et al.*, 2003; Parajo *et al.*, 1998). The yield of xylans as xylitol by chemical means is only about 50-60% making xylitol production expensive. Various bioconversion methods, therefore, have been explored for the production of xylitol from hemicellulose using microorganisms or their enzymes (Nigam and Singh, 1995). Furfural is used in the manufacture of furfural- phenol plastics, varnishes and pesticides (Montane

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et al., 2002). Over 200,000 tones of furfural with a market price of about $1700 per ton (Montane *et al.*, 2002) is annually produced (Zeitch. 2000).

2.1 Microorganisms and their Lignocellulolytic Enzymes

A diverse spectrum of lignocellulolytic microorganisms, mainly fungi (Baldrian and Gabriel, 2003; Falcon *et al.*, 1995) and bacteria (Mc Carthy, 1987; Zimmermann, 1990; Vicuna, 1988) have been isolated and identified over the years and this list still continues to grow rapidly. Already by 1976 an impressive collection of more than 14,000 fungi which were active against cellulose and other and other insoluble fibres were collected (Mandels and Sternberg, 1976).

Despite the impressive collection of lignocellulolytic microorganisms only a few have been studied extensively and mostly *Trichoderma reesei* and its mutants are widely employed for the commercial production of hemicellulases and cellulases (Esterbauer *et al.*, 1991; Jorgensen et al., 2003; Nieves *et al.*, 1998). This is so, partly because *T. reesei* was one of the first cellulolytic organisms isolated in 1950's and because extensive strain improvement and screening programs, and cellulose industrial production processes, which are extremely costly, have been developed over the years in several countries.

T. reesei might be a good producer of hemi-and cellulolytic enzymes but is unable to degrade lignin. The white-rot fungi belonging to the basidiomycetes are the most efficient and extensive lignin degraders (Akin *et al.*, 1995; Gold and Alic, 1993) with *P. chrysosporium* being the best-studied lignin-degrading fungus producing copious amounts of a unique set of lignocellulytic enzymes. *P. chrysosporium* has drawn considerable attention as an appropriate host for the production of lignin-degrading enzymes or direct application in lignocellulose bioconversion processes (Ruggeri and Sassi, 2003; Bosco *et al.*, 1999). Less known, white-rot fungi such as *Daedalea flavida*, *Phlebia fascicularia*, *P. floridensis* and *P. radiate* have been found to selectively degrade lignin in wheat straw and hold out prospects for bioconversion biotechnology where the aim is just to remove the lignin leaving the other components almost intact (Arora *et al.*, 2002). Less prolific lignin-

degraders among bacteria such as those belonging to the genera *Cellulomonas*, *Pseudomonas* and actinomycetes *Thermomonospora* and *Microbispora* and bacteria with surface-bound cellulase-complexes such as *Clostridium thermocellum* and *Ruminococcus* are beginning to receive attention as representing a gene pool with possible unique lignocellulolytic genes that could be used in lignocellulase engineering (Vicuna, 1988; McCarthy, 1987; Miller (Jr) et al., 1996; Shen *et al.*, 1995; Eveleigh, 1987).

It is conventional to consider lignocellulose-degrading enzymes according to the three component of lignocellulose (lignin, cellulose and hemicellulose) which they attack but bearing in mind such divisions are convenient classifications since some cross activity for these enzymes have been reported (Kumar and Deobagkar, 1996). The exact mechanism by which lignocellulose is degraded enzymatically is still not fully understood but significant advances have been made to gain insight into the microorganisms, their lignocellulolytic genes and various enzymes involved in the process.

2.5 Biofuel: Production from Biomass

Biofuel, on the other hand, is any fuel that is derived from biomass. It is a renewable energy, unlike other natural resources such as petroleum, coal and nuclear fuels because its carbon was recently extracted from atmospheric carbon dioxide by growing plants and as such does not result in a net increase of carbon dioxide in the earth's surface (Agarwal, 2005). Biofuels are commonly used throughout the world. The most common use for biofuels is automotive transport (Rossillo and Cortez, 1998). Essentially biofuels can be produced from any short term carbon cycle organic compound; due to this there is a high variety of resources and therefore many types of biofuels (Licht, 2003).

According to Agarwal (2005) the various biofuels energy resources explored include biogass and biogas energy, primary alcohols, vegetable oils and bio-diesel, among others. These alternative energy resources are by and large environment-friendly. Some of these fuels can be used directly while others need to be formulated to bring the relevant properties close to the conventional fuels. The choice of biofuels consumed depends on

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availability, local customs, and seasons. Generally, the sub-Sahara African population depends mainly on wood, as does the rural population in Latin American. While the population in Asia uses all biofuels (ATSR, 2000). In the developed world biofuels are important as they serve also, as the major energy sources in the urban areas, but provide a smaller fraction of total energy consumed (Licht, 2003).

2.6 Types of Biofuel

2.6.1 Biodiesel: Bio-diesel is a clean burning, mono-alky ester-based oxygenated fuel made from natural renewal resources such as new and used vegetable oils and animal fats. Bio-diesel is quite similar to conventional diesel fuel in its main characteristics. It contains no petroleum, but it is compatible with conventional diesel and can be blended in any proportion with petroleum diesel to create a bio-diesel blend (Agarwal, 2005).

2.6.2 Woodfuel: Wood fuel is the principal source of domestic energy in developing countries. Wood fuel includes charcoal as well as firewood, brushwood, twinges, branches, and cut branches. Where available, fuel wood is generally the biofuels of choice. Climate and terrain are the two strongest natural influences on the growth and abundance of the forest resources and these vary significantly throughout the developing world. The carbonization process of wood usually emities volatiles including CO_2, CO, CH_4, and non-methane hydrocarbons (NMHC) estimated at 60% by weight of the original wood (Andrae and Merlet, 2000).

2.6.3 Vegetable oils: are available from various sources such as cultivated plants, forests, and agricultural by-products. They are liquid fuels from renewable sources and do not over-burden the environment with emission. Vegetable oils can be successfully used in the CI (combustion ignition) engine through engine modifications and fuel modifications (Agarwal, 2005).

2.6.4 Biomass gasification: Biomass-gasfier-based system converts solid biomass into gaseous fuel (producer gas) by a series of thermo-chemical processes such as drying, pyrolysis, oxidation, and reduction, which is a more user-friendly gaseous form, which can

be used directly in IC engines to generate power (Jain, 2001).

2.7 Bio-Ethanol

The world's energy supply is mainly dependent on non-renewable, crude oil-derieved (fossil) liquid fuels, of which almost 90% are consumed for energy generation and transportation. The problem of rapidly increasing population has caused many developing countries to expand their industrial base, resulting in increased energy demands. The world's energy needs have been estimated to be about 10^{21} Joules per year, which equals a continuous average per capita consumption of about 4-6 KW (Kilowatt) (Tripetchkul, Hillary, and Ishizaki, 1998). It is inevitable that fossil fuels such as oil, coal, and natural gas will be exhausted with time. Hence, there is a need to explore the possibilities of using alternative energy sources, which are as efficient as oil: ethanol fermentation is one such option (Nomuro, Bin, and Nakae, 2002).

A few major drawbacks of non-renewable fuels are that emissions of CO_2 (carbon dioxide), CO (carbon monoxide), nitrogen oxide, and sulphur-containing residues from the combustion of these fuels causes global warming. Also, dependence on imported petroleum for vehicle fuel is a fiscal burden as a concern for the national energy security. Studies show that vehicular emissions cause 40%-50% of the urban air pollution, which reduces the atmospheric or environmental quality. The development of alternative transportation fuels made from biomass resources, such as plant material and biodegradable waste, may contribute to reduced CO emissions and smog-forming compounds that cause air pollution. The other group of fuels comprises liquefied natural gas, compressed natural gas, liquefied petroleum gas, ethanol, methanol, and bio diesel. Among these, ethanol is the oldest of the few alternative energy fuels; fermentation of biomass converts it to pure ethanol, a useful energy source.

Ethanol produced from cellulosic biomass materials instead of traditional feedstock is known as bio- ethanol: a carbon- neutral compound. The traditional process of ethanol production is through fermentation of sugars with a species of yeast called *Saccharomyces*

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cerevisiae. However, the changing needs, energy demands, and technologic fermentations have led to exploration of different methods, using a broad range of substrates and novel organisms, indigenous or genetically modified. New technologies are being developed that convert the fibrous portion of plant material to ethanol. These feedstock materials are abundant and inexpensive.

Bio-ethanol is a fuel derived from renewable resources like locally grown crops and even waste products/waste paper or grass and tree trimmings, etc). Although fuel ethanol is currently produced from sugarcane and other starch rich grains, ethanol can also be made from cellulosic materials such as wood, grass and wastes (Lynd *et al*, 2003). These materials contain "cellulose", indentified as the simplest of the polymers: cellulose, hemicelluloses and lignin, it is made up of continuous chain of D-glucose molecules linked in the β -1, 4 configurations. These chains or micelles may contain more than 10^4 anhydroglucopyranose units (Shitole and Neimo, 1975) giving a molecular mass of greater than 1.5MDa. Cellulose micelles are bunched together to form a thread-like micro fibrils. The individual cellulosic polymer strands are hydrogen-bonded between the ring oxygen of glucose molecules and the hydroxyl group at position 3. The cellulosic fibrils are composed of highly ordered micelles possessing crystalline structure interspersed with disorderly areas of so-called amorphous cellulose (Dunlap and Chiang, 1980). Crystalline material is sometimes referred to as $^{\alpha}$ cellulose, which is that material that will not dissolve in a solution of 17.5% sodium Hydroxide.

Celluloses are the most abundant natural organic compounds on Earth and are less expensive than carbohydrate sources. The lignocellulosic structure is more resistant to decay by organism and is not perishable like soluble sugar and starch. The complex substrates may be broken down into sugars by either acid treatment at various temperatures or by enzymatic treatment. It has been known for over 100 years that acids act as catalysts to hydrolyze cellulose and hemicelluloses into simple sugars. Acid treatment includes mild acid hydrolysis and concentrated acid hydrolysis.

Ethanol is a two carbon alcohol (C_2H_5OH) ic a monohydric primary alcohol. It melts at-1173^{0}C and boils at 78.5°C. Except for alcoholic beverages, nearly all ethanol is a mixture of 95% alcohol and 5% water. It is high performance fuel, which is used in spark ignited internal combustion engines (Lynd *et al*, 2003). It is considerably cleaner, less toxic, also less Corrosive (Lal and Reddy, 2005; Risch, 1998). Alcohols have a higher octane number than gasoline. Fuel with a higher octane number can endure a higher compression ratio before the engine knocking takes place. This gives the engine an ability to deliver more power, and thus became more powerful, efficient, and economical (Agarwal, 2005). According to Agarwal (2005), Alcohol fuel burns cleaner than the regular gasoline and produces less CO, HC, and NO. It has a high heat of vapourization therefore; it reduces the NOx emissions and increases the engine power. However, the aldehyde emission goes up significantly.

In comparison to gasoline, ethanol burns cleaner with a greater efficiency, thus putting less carbondioxide and overall pollution in the air. Additionally, only low levels of smog are produced from combustion. According to the U.S Department of Energy, ethanol from cellulose reduces greenhouse gas emission by 90 percent, when compared to gasoline and in comparison to corn-based ethanol which decreases emissions by 10 to 20 percent. Carbondioxide gas emissions are shown to be 85% lower than those from gasoline.

Bata and Vermen (1989) studied the effect of ethanol addition to gasoline on the exhaust emissions from 1978 Ford 2.3 litre engine operating under fixed conditions. They observed lower HC emissions and a 40% - 50% decrease in the CO concentration as compared to the based fuel. This was corroborated by stump, kaapp and Racy (1996) in a similar investigation. Ethanol is one of the possible fuels for diesel replacement in the CI (Combustion Ignition) engines also. Application of ethanol as a supplementary CI engine fuel may reduce the environmental pollution, strengthen the agricultural economy, create job opportunities and reduce diesel fuel requirements, thus contributing to conserving a major commercial energy source (Agarwal, 2005).

Steps involved in producing ethanol from cellulosic biomass involve an activation step, biological conversion, product recovery (typically via distillation), and residue processing and utilities. Activation involves converting recalcitrant cellulosic biomass into reactive intermediates, and may be accomplished via pretrentment & hydrolysis (acid and enzymatic) or gasification. Of these alternatives, the lowest future cost has been projected pretreatment and enzymatic hydrolysis. Process design studies consistently indicate that steps associated with overcoming the recalcitmnce of lignocellulose biomass are the most costly, involve the greatest technical risk, and have the largest potential for R & D driven cost reduction.

2.8 Microorganisms and Ethanologenic Processes

The simplest method of ethanol production is fermentation and distillation of sugar cane molasses and sugar beet. These materials contain high amounts of sugar, available for direct conversion to ethanol. Crops containing sugars and starch are usually expensive to produce and are perishable. Ethanol from these crops is affordable only if they are waste by – products of other processes. Ethanol produced from cellulosic biomass materials instead of traditional feedstock is known as bio-ethanol: a carbon – neutral compound. The traditional process of ethanol production is through fermentation of sugars with a species of yeast called *Saccharomyces cerevisiae*.

Alcohol fermentation was done by using the mash of dried sweet potato with its dregs as substrate (Yu zhang, and Zheng, *et al.* 1994). In another study, cellolosic pyrolysate – containing levo – glucosan was chemically hydrolysed and n maximum glucose yield of 17.35% was obtained through hydrolysis with 2 mol/litre H_2S04 (Sulphuric acid) at 121^0c for 20minute. The total initial glucose level was maintaincd at 41.9g/litre by diluting the hydrolysate. Then, neutralization methods were employed, including either the addition of solid $Ca(OH)_2$ (to bring to about pH 6.0 or 10.4) or its combination with the absorbents. The neutralization and diatomite – shaking method gave the hydrolysate, which was completely fermented by *S cerevisiae* and *Pichia sp.* Yz – 1. A maximum ethanol yield of 0.45/g glucose was obtained by *S. cerevisiae* (Yu and Zhang 2002).

Another substrate. liquelied cassava starch, was used for ethanol production by co – immobilized cells of *Z. mobilis* and *S. diastaticus*. The co – immobilized cells produce 46.7 g/ litre ethanol from 150 g/ litre liquelied cassva starch, while the immobilized cells of yeast *S. diastaticus* alone produced 37.5g/litre ethanol. Thus, co-immobilized cells of *S.diastaticus* and *Zmobilis* produced a high ethanol concentration as compared to the immobilized cells of *S. diastaticus* during batch fermentation of liquelied cassava starch (Amutha and Gunasekaran 2001). Pretreatment of sun- flower with *Trichoderma reesei* Rut-C30 cellulase resulted in 57.8% saccharification (Sharma, Kalra, and Grewal 2002). Enzyme hydrolysate concentrated to 40g/litre, reducing sugars fermented with *Zmobilis* under optimum fermentation conditions like an incubation time of 24 hours, pH 5.0, temperature 30°C, and innoculum size 3% v/v showed a maximum ethanol yield of 0.444g/g. Ethanol production scaled up in a 1-litre and 15-litre fermenter under optimum conditions, revealing a maximum ethanol yield of 0.439g/g and 0.437g/g, respectively.

For direct and efficient ethanol production from cellulosic materials, a novel cellulose – degrading yeast strain was developed by genetically modifying two cellulolytic enzymes on the surface of *S.cerevisiae*. This could grow in a synthetic medium containing glucan as the sole source of carbon and could directly ferment 45g of glucan per litre to produce 16.5g of ethanol per litre within 50 hours. Thus, 0.48g of ethanol was produced per gram of carbohydrate utilized, which corresponded to 93.3% of the theoretical yield. This result indicates that efficient and simultaneous sacharification and fermentation of cellulose to ethanol was carried out by recombinant yeast cells displaying cellulolytic enzymes (Fujita, Takahashi, Ueda, *et al.* 2002). In another study Jirku (1999) used immobilized *S. cerevisiae* cells and found that the maximum fermentation capacity of the system was at 30°c and was relatively pH – sensitive. A packed column reactor was used to test this biocatalyst's operational sensitivity to key fermentation variables. Results of this study as well as characteristics of the polymer, prepared by an epoxy resin and diamino polyethylene oxide polymerization establish the suitability of this method for ethanol production.

There are several bottlenecks in the alcoholic fermentation process and they must be overcome to achieve a high and competitive performance of bio ethanol production by the yeast *S.cerevisiae*. In the process of conversion of lignocelluloses to ethanol, the capital and process cost may well exceed that of the feedstock. Although compositions vary, the lignocellulosic material comprised 50% cellulose, 25% hemicelluloses, and 25% extractable lignin. For bioconversion, the carbohydrate portion must be solubilized while the lignin and residues are used to provide energy for ethanol production (Ingram and Doran 1995).

Alfenore, Jouve, and Guillouent, *et al.* (2002) described a nutritional strategy that allowed *S.cerevisiae* to produce a final ethanol litre of 19% (V/V) ethanol in 45hours in a fed – batch culture at 30^{0}C. This performance was achieved by implementing exponential feeding of vitamins throughout the fermentation process. A maximum instantaneous productivity of 9.5g/litre/hour was reached in the best fermentation. These performances resulted from improvements in growth, ethanol production rate, and concentration of viable cells in response to the nutritional strategy.

Lignocellulosic biomass is an abundant renewable resource that can serve as a substrate for ethanol. Extensive research has demonstrated that alcoholic fermentation of xylose, the major hemicelluloses derived sugar, improves the economics of biomass conversion as 70% of the raw material can be expected to convert into ethanol. A complete conversion of glucose and xylose mixture was obtained by a respiratory deficient mutant of *S.diastaticus* co-cultivated with *Pichia stipitis* in continuous culture (Delgenes, Laplace, and Moletta, *et al.* 1996). Stee, Sridhar, Rao *et al.* (1998) evolved a novel solid substrate fermentation system to produce fuel ethanol from sweet sorghum and sweet potato using a thermo-tolerant yeast *S.cerevisiae* strain, VS 3, and a local isolate of amylolytic *Bacillus sps*, VB9. The maximum amount of ethanol produced in co-culture with a mixed substrate was 5g/100g of substrate at 37°C and 3.5g/100g of substrate at 42°C.

The glycosidic linkage in hemicelluloses are readily hydrolysed by diluting acid at elevated temperature to yield a syrup containing xylose and arabinose for agricultural by-products and hardwoods or xylose, arabinose, and glucose for softwoods (Puls,1993). Saccharification of cellulose is a complex and poorly understood process, in which three enzymes- exoglucanases, endoglucanases, and glucosidases –that cleave cellulose into cellobiose and then into glucose are involved (Enari,1993; Eriksson, Blan Chetter, and Ander (1990). The products of saccharification induce non-competitive and non-productive inhibition of the enzyme system.

In the first case where xylose, a pentose becomes a non-amenable substrate for efficient conversion of the same into ethanol, it requires a microbial system, which can use the xylose or pentoses as efficiently as it uses glucose, fructose, and sucrose. For avoiding the end-product inhibition, simultaneous saccharification and fermentation was developed by the Gulf oil company (Blotkamp, Takagi, Pemberton, *et al* 1978), using *S.cerevisia*. The juice of rotten or discarded pineapples and the waste material generated during the production of pineapple juice serve as low-cost substrates for ethanol production by *Z. mobilis*. *Z. mobilis* ATTC 10988 produced 59 g/litre ethanol in undiluted pineapple juice without nutritional supplementation and without regulation of the pH, while 42.5 g/litre ethanol was obtained using a 125 g/litre sucrose medium supplemented with 10 g/litre yeast extract and mineral salts. These results suggest that pineapple juice and the waste material can serve as useful low-cost substrates for ethanol production by Z. mobilis without the use of expensive organic nitrogen complex supplements such as yeast extract, and without regulation of pH during cultivation, leading to a reduction in production costs (Tanaka *et al.*, 1999).

The fermentation process is a traditional, well-known method throughout the world: it includes various studies. A comparison of the rates of growth and ethanol production was by studying 11 different strains of *Zymomonas* that revealed a wide range of characteristics (Skotnicki, Lee, and Tribe, *et al.* 1981). From these studies it was found that some strains were more tolerant to high sugar or ethanol concentrations and high incubation temperatures than others. Some strains were unable to utilize sucrose: some were able to

produce large amount of levan; and one strain grew well on the carbon source but there was no production of levan. One strain, CP4, was found to be superior in all respects to most other strains and was finally chosen as a starting strain to be genetically modified for enhanced ethanol production. One more anaerobic bacterium Thermoanerobium brockii was used for ethanol fermentation. The study showed that it followed the Embden – Meyerhof Pathway (EMP) pathway and converted glucose into ethanol, acetic acid, carbonic acid, and lactic acid. This species has actively fermented saccharides and starch into ethanol (Lamed and Zeikus, 1980). The ethanol yield gets reduced with molasses, and the most probable reason for this may be the impurities present in raw molasses that are inhibitory to yeast growth as well as to fermentation. For that, 22 different yeast strains were studied in isolation from the natural resources. The highest yield of ethanol by a strain from molasses and grapes, respectively, was 7.8% and 2.8% (with 16% dextrose medium) and 7.4% and 8.0% (with molasses medium). The optimum temperature for maximum ethanol production was 30-35°C while the optimum pH was 5-5.5. Any variation in these conditions severely affected the ethanol production, possibly due to excessive enzyme degradation and loss of activity at a high temperature, reduced activity of glycolytic/fermentative enzymes at a low temperature, and inactivation of enzymes due to pH variation (Bajaj *et al.*, 2001).

One more substrate, whey, a by-product generated in large quantities from cheese production, was used for ethanol fuel. Whey poses a major waste-disposal problem due to its high BOD (biochemical oxygen demand). When dumped untreated, whey ends up in rivers and oceans, consuming the oxygen needed to support aquatic life. Pre-dispoal treatment of whey to reduce its BOD to the EPA (Environmental Protection Agency) – approved levels is extremely expensive. This study presents an economically feasible solution to the whey-disposal problem (Ahmed and Morris, 1991). The availability of corn stover as a sustainable feedstock for bioethanol production was studied by Kadam and McMillan (2003). According to them, 60-80 million dry tones/year of corn stover should be available to fermentative routes. To achieved an ethanol production potential of 11 billion litres (3 billion gallons) per year, about 40% of the harvestable corn stover is

needed. This amount should be available as long as the diversion of corn stover to non-ethanol fermentative products remains limited.

In fermentation process, ADH and PDC are the wellstudied key enzymes. According to this study, it was found that the enzyme ADH (isolated from *Z. mobilis*) is activated by ferrous ions but not by zinc ions after inactivation with the metal-complexing agents; cobalt ions also reactivated to a lesser extent. This study suggested that in this species, ADH naturally contain iron. Kinetic studies on the iron-treated enzyme indicate an alcohol-activation phenomenon that may have physiological relevance in overcoming product inhibition during fermentation (Scopes, 1983). Tamarit, Cabiscol, and Aguilar, *et al.* (1997) studied the tolerance of the metal binding of ADH in *Z. mobilis* endowed with two isoenzymes – a zinc-containing enzyme (ADH I) and an iron-containing enzyme (ADH II). The activity of ADH I remained fully conserved, while the ADH II activity decayed when the culture conditions were shifted from anaerobiosis to aerobiosis. This differential response was due to the metal present on each isoenzyme because pure preparations of the ADH I are resistant to oxidative inactivation and preparation of zinc-containing ADH II, obtained by incubation of pure ADH with $ZnCl_2$, showed no modification of the target for oxidative damage (His^{297}-containing peptide).

It was consistently found that activity of the zinc-containing ADH II, once submitted to oxidative treatment, was fully restored when iron was reintroduced into the enzyme structure. These results indicate that zinc bound to these proteins plays an important role in the production role in the protection of their active centres against oxidative damage and may have relevant biochemical and physiological consequences in this species. After that three enzymes, glucokinase (EC 2.7.1.2), fructokinase (EC 2.7.1.4), and glucose-6-phospate dehydrogenase (EC 1.1.1.49), were isolated in high yield from extracts of Z.mobilis (Scopes, Testolin, and Stoter, et al. 1985). The principal steps in the isolation procedures involved the use of selected dye-ligand adsorbent columns, with affinity elution of two of the three enzymes. Neale, Scopes, Wettenhall, *et al.* (1986) found that the PDC is the key enzyme, which directs the production of CO_2 and ethanol as the end products of

sugar fermentation by *Z. mobilis*. They studied isolation, properties, and genetic expression of this enzyme in *E.coli* and were purified to homogeneity using dye-ligand and ion exchange chromatography. The genomic fragment encoding the encoding the enzyme expressed high levels of PDC in *E. coli*.

Osman. Conway. Bonetti. (1987) analysed the rate at which *Z. mobilis* converts glucose to ethanol and CO_2, and found that in the Entner – Doudoroff (ED) pathway, glycolytic flux did not remain constant during batch fermentation of 20% glucose. The initial rate of conversion resulted in high ethanol accumulation in the surrounding broth. As a result, glycolytic activity was repressed due to the inhibitory effects of ethanol and the activity could be reversed immediately its removal. Fermentation activity enhanced (58mol of CO_2 evolved per milligram of cell protein per hour) after accumulation of 1.1% ethanol in 18 hours and declined to one-half this rate after 6.2% accumulation of ethanol in 30hours. They also identified three possible reasons for this behavior of *Z. mobilis*, namely reduced number of nucleotides. reduction in internal pH from 6.3 to 5.3, and reduced specific activity of pyruvate kinase and glyceraldehydes-3-phosphate dehydrogenase of the glycolytic pathway.

Loos, Kramer, Salun, *et al.* (1994) studied the basis of osmotolerance in *Z.mobilis* when grown in a media containing high concentrations of glucose or other sugars. They described sorbitol as a comparative solute in bacteria, which enhances the growth of *Z. mobils* at higher glucose concentrations, exceeding 0.83M (15%). Sorbitol can protect proteins during dehydration of osmotic or thennal stress and is, therefore, used to preserve proteins during storage. The sorbitol transport system followed the Michaelis-Menten Kinetics. with an apparent K_m of 34Mm and a V_{max} of 11.2 nmol (nano moles)/ minute/mg (dry mass). Sorbitol was produced by the cells themselves and was accumulated when grown on sucrose (1M or 36%) by the action of the periplasmic enzyme glucose-fructose oxidoreductase, which converts glucose and fructose ioto gluconolactone and then into sorbitol. Thus, *Z. mobilis* can form and accumulate the compatible solute sorbitol from a natural carbon source, sucrose, in order to overcome the osmotic stress in a high-sugar

media. No other major compatible solute (betaine, proline, glutamate, or trehalose) was detected.

Sprenger (1996) discussed the carbon flux and its regulation, and how it branches into anabolic pathways together, with the recent approaches to broaden the substrate range of the bacterium. Sucrose, glucose, and fructose are degraded by *Z. mobilis* via an anaerobic version of the ED pathway. Sucrose splits extracellularly into glucose and fructose (or levan). The two sugars are transported into the cell via facilitated diffusion (uniport). A periplasmic enzyme glucose-fructose oxidoreductase provides the novel compatible solute sorbitol to counteract the detrimental osmotic stress.

In further studies, Deng and Coleman (1999) introduced new genes into a cyanobacterium in order to create a novel pathway for fixed carbon utilization, which results in the synthesis of ethanol. The coding sequences of the PDC and ADH II from the bacterium *Z. mobilis* were cloned into the shuttle vector pCB4 and were then used to transform the cyanobacterium *Synechococcus* sp strain PCC 7942. The PDC and ADH genes were expressed at high levels, as demonstrated by Western blotting and enzyme activity analyses. The transformed cyanobacterium synthesized ethanol, which diffused from cells into the culture medium. As cyanobacteria have simple growth requirements and use light, CO_2, and inorganic elements efficiently, production of ethanol by cyanobacteria is a potential system for bioconversion of solar energy and CO_2 into a valuable resource. Metabolic engineering of *Z. mobilis* strains was tried to maximize the ethanol production from mixtures of hexose and pentose sugars through the application of metabolic flux control techniques (Kompala, Ramkrishna, Jansen, *et al.* 1986).

The successful engineering of ethanol-fermenting *Z. mobilis* to broaden its substrate utilization range from hexoses, glucose, and fructose, to include the additional pentose sugars, xylose, and arabinose found in the renewable biomass feedstock and agricultural residues was done. Pentose fermentation was accomplished by introducing two gene encodings for key enzymes in the xylose assimilation pathway, three gene encodings for

key enzymes in the xylose assimilation pathway, and two gene encodings for key enzymes in the pentose phosphate pathway into the recombinant ethanol producer *Z. mobilis*. An important study was done in which transcript levels of several enzymes of glycolytic and alcoholic fermentation pathways were examined in the shoot and root tissues of rice seedlings subjected to different abiotic stresses by northern blot-analysis employing homologous DNA probes. This work includes ADH, PDC, phosphate isomerase, aldolase, glyceraldehydes phosphate isomerase, and pyruvate kinase enzymes. Cold, desiccation, salt (NaCl), high temperature, and oxygen-deprivation stresses are used to see their effect on pathway regulatory enzymes. Evidently, glycolytic and alcoholic fermentation enzymes have a sufficient degree of flexibility to adjust to the increased energy demand and supply of intermediates for acclimatization to stress conditions.

Though *K. oxytoca* and *Erwina chrysanthemi* transformed with PET operon did not perform better than *Z. mobilis* in getting transformed with the same operon, their performance in converting cellulose into ethanol was good, particulary in case of *K. oxytoca* (Wood and Ingram. 1992). The strain *K. oxytoca* utilizes cellobiose and cellotriose and, thus, is a good choice for ethanol fermentation with cellulose as the inhibitory effects of certain cellulose preparations can be avoided. The PET operon on low and high copy number plasmids has had an influence on the ethanol production with the low copy number plasmid pLO1555, giving a higher ethanol production. However, in both cases, the ethanol yield was 92%-98% of the theoretical values (Ohta, Beall, Mejia, et al. 1991b). The best strains of the transformants converted 10% glucose and 10% cellobiose into 44-45 g/litre of ethanol within 48 hours. Different substrates were tried to assess the efficiency of the strain viz., mixed office paper (Brooks and Ingram, 1995), sugar cane bagasse (Doran, Aldrich, and Ingram, 1994), corn fibre (Moniruzzaman, Dien, Ferrer, *et al.*, 1996), and sugar beet pulp (Doran, Cripe, Sutton, *et al.*, 2000). Integrating cellulose components like extracellular endoglucanase can reduce the ethanol production costs (Dien, Cotta, and Jeffries, 2003). Two extracellular endoglucanase genes (*cel Z* and *cel Y*) from *E. chrysanthemi* were cloned into the recombinant *K. oxytoca* strain P2 along with auxillary transporter gene (out) on a plasmid pCPP2006. The recombinant strain was named SZ21

(Zhou, Davis, and Ingram, 2001). Though its cellulose activity was 99% less than the commercial preparations of cellulose, the ethanol productivity was 7%-16% higher than the parental strain when commercial cellulose was added. When a comparative study was done, in which galacturonic acid-rich sugar beet pulp was fermented, KO11 produced significantly higher quantities of ethanol due to *E.coli* KO11 affinity for the substrate. Dien, Hospell, Wyckoff, *et al.*, (1998) developed a novel hexose and pentose utilizing the ethanologenic *E. coli* strain FBR 3 by incorporating the plasmid pLO1297. An ethanol yield of 4.38%-4.66% (w/v) with 90%-91% theoretical conversion in 70-80 hours was achieved.At the National Chemical Laboratories, Pune, India; the D N Deobagkar Group utilized a protoplast fusion technology to develop a hybrid microbe.

2.9 Fossil Fuels

Fossil fuels are fuels formed by natural resources such as anaerobic decomposition of buried dead organisms. The age of the organisms and their resulting fossil fuels is typically millions of years, and sometimes exceeds 650 million years. The fossil fuels include coal, petroleum, and natural gas which contain high percentages of carbon. Fossil fuels range from volatile materials with low carbon:hydrogen ratios like methane, to liquid petroleum to nonvolatile materials composed of almost pure carbon, like anthracite coal. Methane can be found in hydrocarbon fields, alone, associated with oil, or in the form of methane clathrates. It is generally accepted that they formed from the fossilized remains of dead plants and animals by exposure to heat and pressure in the Earth's crust over millions of years. This biogenic theory was first introduced by Georg Agricola in 1556 and later by Mikhail Lomonosov in the 18th century.

It was estimated by the Energy Information Administration that in 2007 primary sources of energy consisted of petroleum 36.0%, coal 27.4%, natural gas 23.0%, amounting to an 86.4% share for fossil fuels in primary energy consumption in the world (US, EIA International Energy Statistics, 2010). Non-fossil sources in 2006 included hydroelectric 6.3%, nuclear 8.5%, and (geothermal, solar, tide, wind, wood, waste) amounting 0.9 percent (IES, 2006). World energy consumption was growing about 2.3% per year. Fossil

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fuels are non-reoewable resources because they take millions of years to form, and reserves are being depleted much faster than new ones are being formed. The production and use of fossil fuels raise environmental concerns. A global movement toward the generation of renewable energy is therefore under way to help meet increased energy needs.

The burning of fossil fuels produces around 21.3 billion tonnes (21.3 gigatonnes) of carbon dioxide per year, but it is estimated that natural processes can only absorb about half of that amount. so there is a net increase of 10.65 billion tonnes of atmospheric carbon dioxide per year (one tonne of atmospheric carbon is equivalent to 44/12 or 3.7 tonnes of carbon dioxide). Carbon dioxide is one of the greenhouse gases that enhances radiative forcing and contributes to global warming, causing the average surface temperature of the Earth to rise in response, which climate scientists agree will cause major adverse effects.

2.9.1 Advantnges of Fossil Fuels

1. **Industry:** Fossil fuels are of great importance because they can be burned (oxidized to carbon dioxide and water), producing significant amounts of energy. Prior to the latter half of the eighteenth century, windmills or watermills provided the energy needed for industry such as milling flour, sawing wood or pumping water, and burning wood or peat provided domestic heat. The wide-scale use of fossil fuels, coal at first and petroleum later, to fire steam engines, enabled the Industrial Revolution. The use of coal as a fuel predates recorded history. Coal was used to run furnaces for the melting of metal ore. Semi-solid hydrocarbons from seeps were also burned in ancient times, but these materials were mostly used for waterproofing and embalming. Steam locomotives, the quintessential machine of the Industrial Revolution, used coal as a fuel source from early on to compensate for lack of firewood and charcoal. Not only was a seemingly inexhaustible supply of coal available from easily exploited seams near the surface, but it could be used in its natural form.

Natural gas has broad use, for business, manufacturing, petrochemical production and power generation. Chemical constituents – known as natuml gas liquids or NGLs are exuacted from natural gas to provide chemical building blocks for the manufacturing of

consumer products. NGLs are converted into core ingredients for manufacturing bottles, clothing, electronics, detergents, paint, fertilizer, adbensives, carpet, furniture, diapers, tires and toys.

2. Economic Growth: Ever since the Industrial Revolution took off in the the 18th century, vast quantities of fossil fuels have been used to power the economy and deliver unprecendental affluence to many countries across the world and also to huge numbers of people. The usuage of fossil fuels were prerequisites for the birth of a new industrial civilization that transformed our world.

3. Electricity: A major advantage of fossil fuels is their capacity to generate huge amounts of electricity in just a single location. Natural gas, once flared-off as un-needed byproduct of petroleum production, is now considered a very valuable resource. Natural gas are use as fuel in electrical power. Natural gas –fired power plants are the least expensive source of new power supply; almost 40 percent less costly than coal, 45 percent less than wind and 50 percent less than nuclear power. In addition, natural-fired power plants produce low emissions and use less water and land than most alternatives.

4. Transportation: The invention of the internal combustion engine and its use in automobiles and trunks greatly increased the demand for gasoline and diesel oil, both made from fossil fuels. Other forms of transportation, railways and aircraft also required fossil fuels. Increasingly natural gas is also being utilized to power motor vehicles. Some companies and countries are moving to convert fleet of buses, trunks and some automobiles to run on natural gas as a way to reduce emissions form vehicle exhausts. Direct use of natural gas as an engine fuel in vehicles is limited by costly infrastructure requirements and short driving distances.

5. Domestic Light: Commercial exploitation of petroleum, largely as a replacement for oils from animal sources (notably whale oil) for use in oil lamps began in the nineteenth century. At the same time, gas lights using natural gas or coal gas were coming into wide use. Natural gas is used for heating and cooling homes and cooking food. The dramatic increase in available supply brought on by the surge in shale gas production, as well as

significant recent infrastructure investment in interstate pipelines and near storage capacity, has reduced the price and made gas a dependable energy source for homes and business. Kerosene, used for lighting and heating, is a principal product derived from petroleum.

6. Road Construction: Heavy crude oil, which is much more viscous than conventional crude oil, and tar sands, where bitumen is found mixed with sand and clay, are becoming more important as sources of fossil fuel. Oil shale and similar materials are sedimentary rocks containing kerogen, a complex mixture of high-molecular weight organic compounds, which yield synthetic crude oil when heated (pyrolyzed). These materials have yet to be exploited commercially. Tar, a leftover of petroleum extraction, is used in construction of roads.

2.9.2 World Proved Reserves of Fossil fuels

Levels of primary energy sources are the reserves in the ground and flows are production. The most important part of primary energy sources are the carbon based fossil energy sources. Coal, oil, and natural gas stood at 79.6% of primary energy production during 2002 (in million tonnes of oil equivalent. According to the World Recoverable coal and Reserves of oil and natural gas the estimate were report:

Table 2.3: World Recoverable Primary Energy Sources and Reserves

Types of Fossil fuels	Levels (proved reserves) during 2005-2007	Flows (daily production) during 2006.	Years of production left in the ground with current proved reserves and flows above
Coal	997,748 million short tonnes (905 billion metric tones), 4,416 billion barrels of oil equivalent	18,476,127 short tonnes (16,761,260 metric tonnes), 52 million barrels of oil equivalent per day	148 years
Oil	1,119-1,317 billion barrels (178-209 billion kilolitres).	84 million barrels per day (13 million kilolitres).	43 years
Natural gas	6,183-6,381 trillion cubic feet (175-181 trillion cubic metres), 1,161 billion barrels of oil equivalent.	104,435 billion cubic feet (2,960 billion cubic metres), 19 million barrels of oil equivalent per day.	61 years

Source: International Energy Annual (IEA) Statistics, 2006.

Years of production left in the ground with the most optimistic proved reserve estimates (Oil & Gas Journal, World Oil)

- Coal: 417 years
- Oil: 43 years
- Natural gas: 167 years

Note that this calculation assumes that the product could be produced at a constant level for that number of years and that all of the proved reserves could be recovered. In reality, consumption of all three resources has been increasing. While this suggests that the resource will be used up more quickly, in reality, the production curve is much more akin to a bell curve. At some point in time, the production of each resource within an area, country, or globally will reach a maximum value, after which, the production will decline until it reaches a point where is no longer economically feasible or physically possible to produce.

2.10 Bioethanol and the Environment

The idea behind the introduction of bioethanol as a replacement fuel is not one of cost reduction but environmental gains. The ethanol process was assessed on a lifecycle basis, taking into account the whole life cycle from growing crops to burning the fuel, and compared with that of petrol. The main positive environmental benefits of the whole system are as follows:

- Reduction in consumption of fossil fuels
- Greener emissions when burnt in comparison to petrol
- Greener process, less waste and harmful emissions during production
- Less pollution to water, air and land
- Production of useful by-products
- Biodegradable fuel
- Cleaner power generation from Combined Heat and Power (CHP) replacing electricity from the grid.

2.10.1 Reduction in Carbon Dioxide Emission

Bioethanol is made from plants, which absorb CO_2 as they grow. This means their 'lifecycle' carbon emission performance is much better than petrol (Agarwal, 2005). For this reason, bioethanol is being promoted by the European Union and the British government. According to the Renewable Fuel Association (2007), replacing gasoline with ethanol blends will help to reduces greenhouse gas emissions by up to 40% with current production technology, and by about 86% once production of cellulosic ethanol becomes viable.

2.10.2 Reduction in Carbon Monoxide Emission

When fuels such as petrol burn they often experience 'incomplete combustion'. This happens when insufficient air gets into the combustion chamber to react with all of the hydrocarbons in the fuel. Ethanol is an oxygenated fuel, meaning it carries its own air. This reduces instances of incomplete combustion, and hence engines running on ethanol or ethanol blends produce less carbon monoxide than conventional engines (Lynd *et al.*, 2003). Ethanol reduces tailpipe carbon monoxide emissions by as much as 30% toxics content by 13% (mass) and 21% (potency). This is a particular advantage in heavy traffic, as the cumulative effect of the un-burnt hydrocarbons can be hazardous to health.

2.10.3 Reduction in Particulate Matter

As an oxygenate, ethanol also displaces high – octane aromatics in conventional gasoline, resulting in a reduction in soot and particulate emissions. Ethanol can reduced tailpipe soot and particulate emissions by as much as 50% overall, with the greatest reductions being achieved in the highest emitting vehicles (Brett and Brook, 2006) Ethanol also reduces secondary PM formation by diluting aromatic content in gasoline.

2.10.4 Reduction in Smog Formation

Ethanol reduces smog pollution. Blending ethanol in gasoline dramatically reduces carbon

monoxide tailpipe emissions. According to the National Research Council (2006), carbon monoxide is responsible for as much as 20% of smog formation. Additionally, ethanol-blended fuels reduce tailpipe emissions of Volatile Organic Compounds which readily form ozone in the atmosphere. These reductions more than offset any slight increases of evaporative emissions due to high volatility of ethanol – blended fuel. Thus, the use of ethanol of ethanol plays an important role in smog reduction. These ethanol blends have added benefit of providing reduced tailpipe carbon monoxide emissions and therefore, further emissions reductions of smog. Brooke (2007) reported that smog formation depends heavily on local weather conditions and atmospheric composition, making it difficult to estaiblish a clear connection between the emissions profile of ethanol and deterioration of air quantity.

2.10.5 Reduction in Acid Producing gases (SO_x and NO_x)

SO_x and NO_x are Sulphur Oxides and Nitrogen Oxides respectively. These are very dangerous chemicals in the environment and can lead to acid rain. Bioethanol does not produce Sulphur Oxides when it burns, and has also been found to reduce Nitrous Oxide emissions by 10% (Risch, 1998).

CHAPTER THREE

METHODOLOGY

3.1 Study Design

The pilot study was experimental and laboratory based involving chemical hydrolysis, microbiological fermentation and biochemical tests. Different types of agro-based cellulosic wastes such as cassavn peels, yam peels, plantain peels and sawdust were utilized in the experiment. The experiment employed a complete randomized design with three (3) replicates of each of the biomass samples. An Evaluation (appraisal) of the ethanol yielding capacity of the different biomass was carried out.

3.2 Description of Study Area

The study area of this research was in Ibadan, the capital city of Oyo State of Nigeria. It is the third largest city in Nigeria by population and geographical area. It is located in south-west Nigeria and according to 2006 census results; Ibadan has a total population of 2,258,625 inhabitants, made up of 1,125,843 urban and 1,132,728 rural populations (Omonijo *et al*, 2007). It is located along the rainforest belt in the humid tropical region with an annual rainfall of about 2,500mm and temperature below 53^{0}F. The major occupation of the inhabitants are mostly farming and trading.

The choice of Ibadan as a study area is because of large scale agricultural activities, which is evident by the presence of Research Institute viz International Institute of Tropical Agriculture (IITA), Cocoa Research Institute of Nigeria (CRIN), National Institute for Horticultural Research and Training (NIHORT); Agricultural plantations (Government and private owned); Bodija Timber processing centre; Eleyele cassava processing unit; and it also serves as a market nerve centre for agricultural produce such as grains (rice, millet, maize), yam and other tuber crops brought from the Northern part of Nigeria. This has led to the generation of enormous agro-based wastes. This is of great concern to the government because of the poor waste management facility and infrastructure in the state.

3.3 Sample Source and Morphological description of Material (Samples)

The source of the different agro-based cellulosic wastes utilize in this study with their morpbological description is given below:

3.3.1a International Institute of Tropical Agriculture (IITA): The cassava peels was obtained from IITA. IITA is located at Ido-Ose along Ibadan-Oyo Road in Akinyele Local Government Area of Oyo State. It occupies a land area of about 1000 acres. It was established in 1967 as a non-profit organization to food solutions for hunger and povetty through research for development activities. The Institute has a cassava plantation which covers about one (1) hectare of land; beside it is a cassava processing unit with modernized processing machine for making garri. Large quantity of cassava peels are generated from this processing unit while the waste water is subjected to anacrobic digestion in a sedimentation tank to produce biogas.

Plate 3.1: Cassava Processing Unit at IITA where samples of the cassava peels were collected

3.3.1b Cassava Peels

Overview: Cassava peels are a major by-product of the cassava tuberous root processing industry. In parts of Nigeria where cassava is grown and the tubers processed, the peel is largely underutilized as a livestock feed. In Nigeria, the average annual yield of cassava tuberous roots is 21.1 t/ha (Hahn and Chukwuma, 1986). Since the peel constitutes 20.1 percent of the tuber (Hahn, Chukwuma and Almazan 1986). It follows that about 4.2 t of cassava peels per ha are available annually for feeding ruminants especially goats. The following composition has been reported for cassava peel: residual DM 86 – 594.5%, OM 89.0 – 93.9%, CF 10.0 – 31.8%, CP 4.2 – 6.5% (i.e N 0.7 – 1.0%) by Onwuka (1983), Carew (1982) and Adegbola (1985).

Processing: Cassava by- products are generally found in the vicinity of factories where cassava tubers are processed into starch or flour. Fresh cassava peels have 3 main

deficiencies: they spoil very quickly and they contain high amounts of cyanogenic glycosides. They should thus be processed in order to reduce cyanogenic potential and phytate content and to preserve their nutritive quality (Adegbola, 1985). Different processes are effective in reducing cyanogenic glycosides: sun-drying, ensiling and soaking + sundrying have ben assessed and have yielded satisfactorily result (Hahn *et al.*, 1986, and Onwuka, 1983). Good quality silage can obtained after chopping the peels to equal lengths of about 2cm for easy compaction, and wilting for 2 days to reduce moisture content from 70 – 75% to about 405. Under these conditions cassava peel silage after 21 days was light brown in colour, firm in texture and had a pleasant odour. The Ph was 4.4, and no fungal growth was observed (Onwuka, 1983).

Environmental and Health effect: Cassava peels contain significant levels of cyanogenic conpounds and can contribute to cyanide poisoning (Adegbola *et al.*, 1985). This foodstuff has the tendency to increase its cyanide content if the soil on which it is cultivated is contaminated with cyanide. Cyanide is a fast acting poison because it binds to key iron-containing enzymes required for cells to use oxygen and as a result tissues are unable to take up oxygen from the blood (Onwuka, 1983). Leachate from cassava peel caused a lot of death to plants and aquatic living organisms (Adegbola *et al.*, 1985).

Plate 3.2: Cassava Peels

3.3.2a Bodija Timber Processing Unit: The sawdust was obtained from the Bodija timber processing unit which is located in Ibadan North Local Government Area of Ibadan, Oyo State. It is located beside the Bodija market. It was established in 1974. Various types of Timber such as Mansonia, Omon, Manhogamy, Iroko, Afara, Ofon etc were brought from different forest reserve in Akure, Ondo and Osun. It occupies a land area of about 25acres.

Plate 3.3: Bodija Timber Processing Unit where samples of the saw dust were collected

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Plate 3.3: Bodija Timber Processing Unit where samples of the saw dust were collected

3.3.2b Sawdust

Overview: Sawdust or wood dust is a byc product of cutting, grinding, drilling or otherwise pulverizing wood with a saw or other tool. It is composed of finc particles of wood. It is also the by-product of ccrtain animals, birds and insects which live in wood such as wood pecker and carpenter ant. It can present hazard in manufacturing industries especially in terms of its inflammability. Sawdust is the main component of particle board.

Practical Uses: A major usc of sawdust is for particle board. Coarsc sawdust may be used for wood pulp. Sawdust is used in the manufacture of charcoal briquettes. The claim for invention of the first commercial charcoal briquettes goes to Henry Ford, who created them from the wood scraps and sawdust produced by this automobile factory (Green, 2006).

Health and Safety: Airborne sawdust and sawdust nccumulations present a number of health and safety hazards. Wood dust becomes a potential health product whcn, for example, the wood particles from the processes such as sanding, becomes airborne and are inhaled. Wood dust is a known human carcinogen. Certain woods and their dust contain toxins that cnn produce severe allergic reactions (Meier, 2003). Sawdust is flammable and accumulations provide a ready source of fuel. Airborne dust can be ignited by sparks or even heat accumulation and result in explosions.

Environmental Effects: At sawmills unless reprocessed into particleboard, burned in a sawdust burner or used to make heat for other milling operations, sawdust may collect in piles and add harmful leachates into local water systems creating an environmental hazard. This has place small sawyers and environmental agencies in a deadlock.

Plate 3.4: Saw Dust

3.3.3a **Abadina Quarters:** The yam peels were collected in Abadina quarters. It is a residential community within the main campus of the University of Ibadan. It is adjacent to works and maintenance Department near UI postgraduate school. The inhabitants of this area are mostly farmers who engaged in subsistence farming with yam being the major crop of interest. These yams are used for personal consumption and also sold to canteens, road side food vendors who also use it to make pounded yam, yam powder, fry yam etc within the community.

Plate 3.5: One acre of yam farm at Abadina Quarter, UI

3.3.3b Yam Peels

Overview: Yam peels is a major by – product of yam processing centres, where yam is grown and the tuber is process into various foodstuff. In Nigeria, yam peels are also generated from household consumption. They can be found in all Tropical and subtropical regions of America, and Africa where yams are grown. The peels are rarely utilized as livestock feed.

Environmental Effects: In Nigeria, yam peels are indiscriminately disposed in the environment, which attract flies and other arthropod-borne diseases. Large piles can cause clogging of drainage channels creating breeding sites for mosquitoes. When dumped into the environment, this waste ends up in rivers and other water bodies, consuming the oxygen needed to support aquatic life (Dolk, 2002).

Plate 3.5: One acre of yam farm at Abadina Quarter, UI

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Plate 3.6: Yam Peels

3.3.4a Ajose Building Canteen: The plantain peels was obtained from the Ajose building canteen which is located within the Oladele Ajose main building, Faculty of Public Health, UCH, Ibadan. This canteen processes large quantities of plantain everyday due to the large population comprising majorly students who patronize it.

3.3.4b Plantain Peels

Overview: Plantain peels are the outer envelope of plantain fruits and by – product of household consumption and plantain processing. Plantain peels are available in populated areas or in the vicinity of plantain processing plants . They can be found in all Tropical and subtropical regions of Asia, America, Africa and Australia where plantains are grown.

Nutritional Attributes: Plantain peels contain 6 – 9% Dry Matter (DM) of protein, 20 – 30%, Nutritional Detergent Fiber (NDF) and variable quantities of starch and soluble sugars, depending on the stage of maturity. Green plantain peels contain 40% starch that is fully transformed into sugars after ripening, while banana peels contain much less starch (about 15%) when green and end up with 30% free sugars when ripe. Lignin content also increases with ripening (from 7 to 15% DM). Plantain peels contain less fibre than bananas (Happi Emaga *et al.*, 2011). Plantain peels are widely used by small holders as complementary feeds for cattle and small ruminant in the tropics (Onwuka et al., 1997; Happi Emaga *et al.*, 2011). Their nutritive value for ruminants is estimated to be similar to that of cassava peels or citrus peels. Green peels contain more metabolizable energy than ripe peels. The study of these variation and of the potential effects of the changes in tannin content during ripening requires further research (Happi Emaga *et al.*, 2011).

Potential Concerns:

- Tannins: are the main antinutritional factors of plantains and are mostly contained in the peels. Ripening causes those tannins to migrate to the pulp of to be degraded by polyphenol oxidases and peroxidases (Emaga *et al.*, 2011). In addition to their antinutritional effects, in monogastrics, plantain tannins are supposed to be responsible for the astringent taste of immature fruits, making them less palatable than mature fruits (Ly, 2004).
- Mycotoxins: Cases of mycotoxin contamination due to fusarium were reported in plantain fruits in 1986 (Swammy, 2009).
- Pesticides: As plantains are subjected to heavy pesticide applications, feeding animals with plantain fruits or stalks may result in toxic residues in animal tissues. An Australian survey assessing 28 plantain pesticides reported that carbonyl chlorothanlonil, dicofol and prochloraz could be responsible for residu violations in meat and edible offal (Mac Lachlan, 2006).

Plate 3.7: Plaintain Peels

3.3.5 *S. cerevisiae*

Baker yeast (*Saccharomyces cerevisiae*) is an industrial yeast belonging to the (Family Endomycetecease), those group of yeast reproducing sexually by production of ascospores. They are unicellular, some appear cyclindrical or elongated. Their cells range from 2 to 6 microns in width and from 10 to 30 nticrons in length. This yeast play an important role in food industry because they produce enzymes that are useful in the leaven of bread, the production of alcohol, glycerol etc.

3.4 Feasibility Study on Sample Collection Areas

A feasibility test was carried out on the sample collection areas to estimate the sample population and to determine the amount of wastes being generated from the parent food materials as shown below:

PHASE ONE: Estimation of Sample Population

The various sample population was determined using the formular below:

$$\text{Plant population} = \frac{\text{Area of land used}}{\text{Feeding area (spacings)}}$$

PHASE TWO: Quantification of Agro-based Cellulosic Wastes from the Parent Sources

Thus for any known amounts of crop production, it is possible to estimate the amounts of agro by-product generated using the method of Vimal and Tyagi (1984) which utilizes the residue to crop ratio approach.

(i) The weight of the waste of the sample population was determined using the Top-Load Weigh Balance.

(ii) The volume of the waste of the sample population was determined using the Hirschmann Measuring Cyclinder.

(iii) The density of the waste of the sample population was calculated using the formula:

$$\text{Density} = \frac{\text{Mass (in Kg)}}{\text{Volume (in m}^3\text{)}}$$

3.5 Sample Collection and Transport

A considerable quantity was packed in polythene bags and then transported directly to the laboratory for drying, milling and other processes. The wet and dry weight of the sample to be utilized in the experiment was recorded.

3.6 Sample Preparation

- **Drying:** The samples were sun dried under the sun or placed in an oven for about 3-5days to reduce the moisture content to about 10%.

- **Milling:** The dried samples were pulverized using a mortar and pestle to a size of about 15mm. This allowed for a large surface area of the substrate to facilitate chemical hydrolysis.

- **Weighing of the Substrate**

Each of the substrate utilized in the experiment was weighed using a Toledo Mettle weighing Balance (± 0.001g) at IMRAT, College of Medicine, UCH, Ibadan.

Twenty grammes each of the powdery biomass was weighed as shown below:

Wt of Aluminum foil (W_1) =2.4809

Wt of Aluminium + substrate (W_2) =22.4809

Wt of Substrate= $W_2 - W_1$

$$= 22.4809 - 2.4809 = 20g$$

Hence 20g each of the substrate (saw dust, cassava peels, plantain, peels and yam peels) was utilized for the chemical hydrolysis.

3.7 Materials and Methods

3.7.1 Materials

The following materials were utilized in this study: Conical flasks, Beakers, Test tubes, Measuring cyclinders, Bunsen burner / Lighter, Cotton wool, Incubator, Oven, Foil-paper, Analytical balance (± 0.001g), pH meter, Spatula, Polythene bag, Siever, Whatman No1 filter paper, Glass funnel, Burette, Pipette, Glass bottles, Reagent bottles, Tripod stand e.t.c

3.7.2 Consumables

The following consumables were utilized in the course of the experiment: Lime [$(Ca(OH)_2$], Cone H_2SO_4, Distilled water, Fehling solution, Baker yeast (*Saccharomyces cerevisiae*), Methylated spirit, Detergent and Acidified $KMnO_4$.

3.7.3 Collection of Materials

The materials used in this study were obtained from the PSM Laboratory, Faculty of Public health and IMRAT both in the College of Medicine, UCH, Ibadan. The Baker yeast (*S. cerevisiae*), Lime, Fehling solution and Acidified KMn04 were purchased.

3.7.4 Quality Control and Quality Assurance

All the glass-wares used for this study was thoroughly washed with detergent, rinsed with distilled water and then allowed to dry in a hot- air oven. The process of sterilization of the equipment was to safeguard against possible contamination of the sample under study.

Disinfection was carried out by cleaning the whole surface of the working bench with cotton wool soaked in methylated spirit before and after each process.

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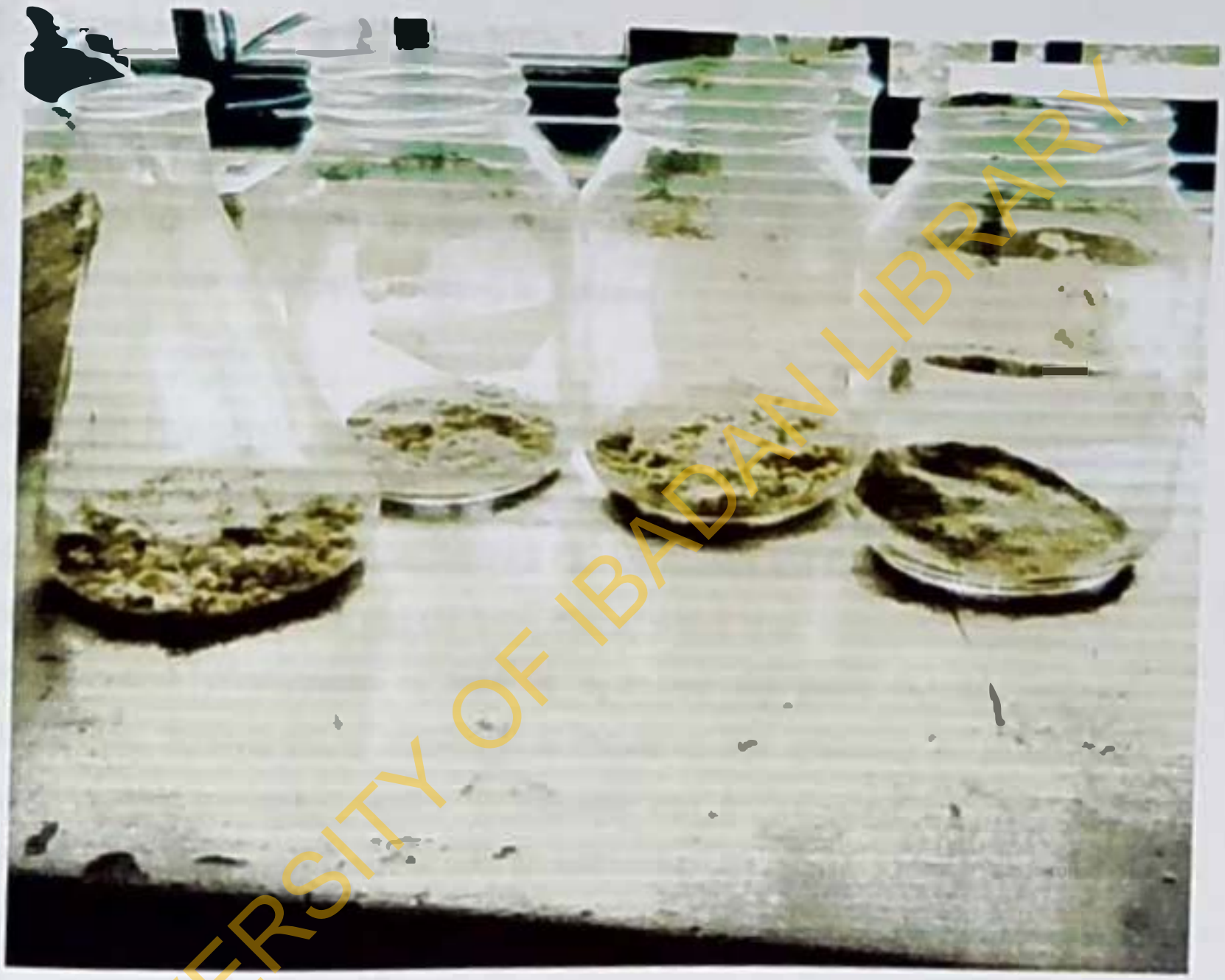

Plate 3.8: Powdery form of biomass (20g each) ready for acid hydrolysis

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3.8 Laboratory Methods

The procedure that was adopted in this study was the concentrated sulphuric acid process (Separated Hydrolysis and Co-fermentation process configuration). This acid-based technology is a generic process that consists of five basic steps:

1. Pre-treatment to hydrolyze the sample
2. Chemical hydrolysis of lignocellulose to produce sugars.
3. Biochemical test: For Total Organic Carbon (T.O.C), Nitrogen, Phosphorus, Glucose, Total Reducing Sugar (T.R.S).
4. Neutralization process (to separate the sugars from acid).
5. Fermentation of Sugars (Glucose) to Ethanol.

The Seperated Hydrolysis and Co-fermentation (SHCF) process is a non-enzyme-based approached, acid is used for both hemicelluloses and cellulose hydrolysis. The other configuration is called the SSF (Simultaneous Saccharification and Fermentation) or SSCF (Simultaneous Saccharification and Co-fermentation) which is an enzymatic approach, in which dilute-acid pretreatment is used to hydrolyze the hemicelluloses portion. Saccharification (hydrolysis) of celluolose to cellobiose and eventually to glucose is catalysed by the synergistic action of cellulase and b-glucosidase enzymes. Co-fermentation refers to fermentation of both six-carbon hexoses (that is, glucose, mannose, and galactose) and five-carbon pentoses (that is, xylose and arabinose) sugars to ethanol.

Benefits of the SHCF processing mode are as follows:

- Enables each step to be carried out at its respective optimal conditions (temperature, pH, etc)
- Large benefits if significantly different optima exist
- Produce intermediate sugar product
- Sugars released by cellulose hydrolysis are decoupled from fermentation (unlike in SSF), hence, not captive to any specific fermentation/products.

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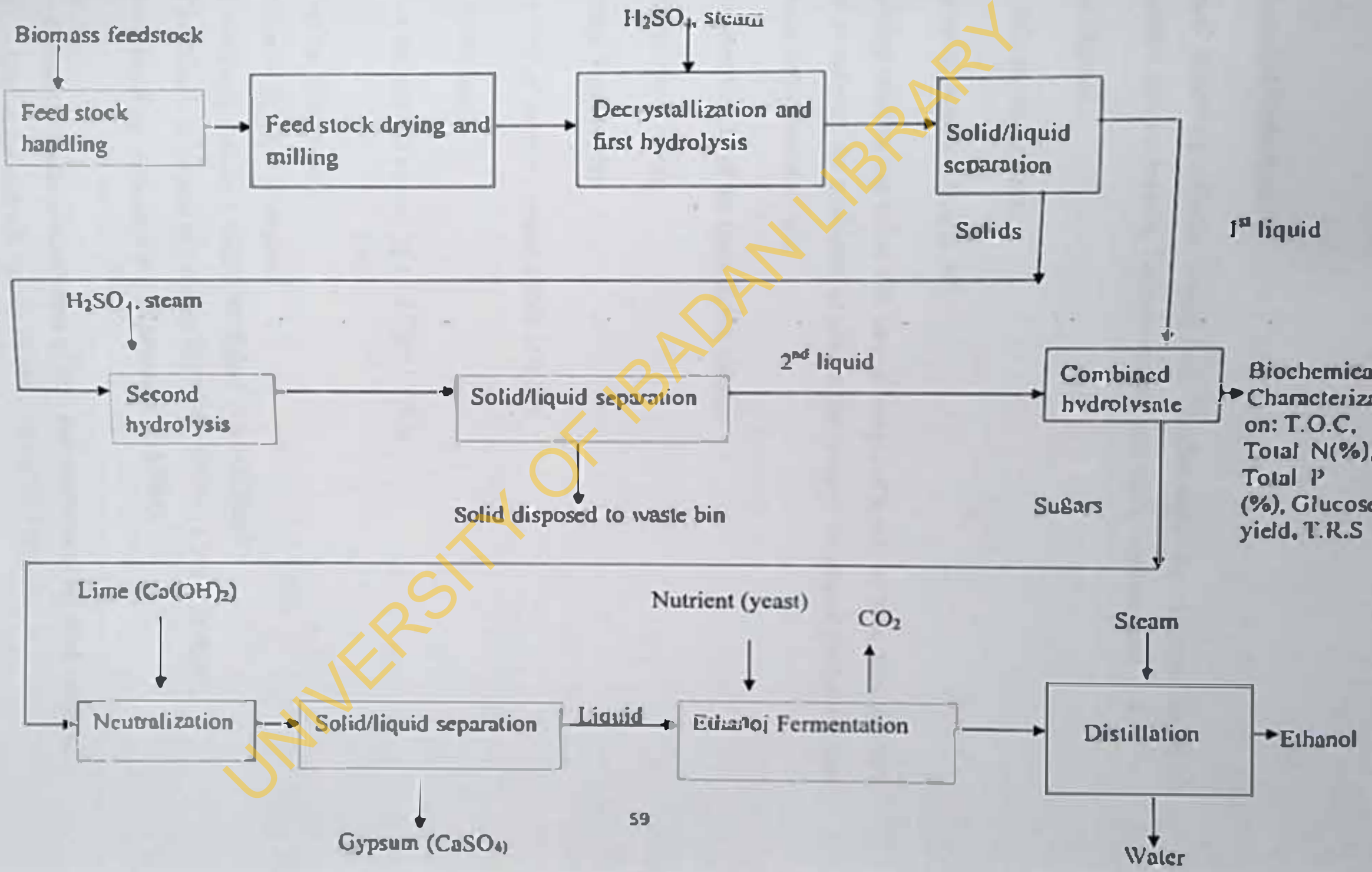
- Large benefits if significantly different optima exist
- Produce intermediate sugar product
- Sugars released by cellulose hydrolysis are decoupled from fermentation (unlike in SSF), hence, not captive to any specific fermentation/products.

- Less insoluble solids in fermentation, which reduce fermentation mixing requirements (or absence of solids if solid-liquid separation is used prior to fermentation.

Figure 3.1: EXPERINMENTAL FLOW CHART



3.8.1 Chemical Hydrolysis

Materials: Measuring cylinder, conical flask, Distilled water, funnel, stove, sieving net metal, presser (spoon), beakers, Thermometer, Reagent bottle, sulphuric, acid, A.R.

H_2SO_4= 98g/mol

Assay: 98% minimum purity

Weight per ml Approx 1.835g at 20°C

The powdery biomass was mixed with various strenght of H_2SO_4 viz 30%, 50% and 70% in order to optimize the production of glucose (the precusor for ethanol production) from the various concentration of acid.

Molar concentration of the stock H_2SO_4 solution

Specific gravity = 1.835g

Percentage Purity = 98%

: Since $1cm^3$ of the acid contains 1.835g of H_2SO_4.

If it is 98% pure

: $1cm^3$ of the acid will contain $\frac{98}{100} \times 1.835g = 1.7983g$

Convert the mass to mole

Molar mass of $H_2SO_4 = 98.08gmol^{-1}$

: $1cm^3$ of the acid contains $1.79835g/98.08gmol^{-1} = 0.018335mol$

: $1dm^3$ ($1000cm^3$) of the acid will contain $0.018335 \times 1000 = 18.34mol$ of H_2SO_4

Concentration of the stock reagent is **$18.34moldm^{-3}$ (18.34M).**

a. To find the molar concentration of 30% concentration of the stock solution.

$1cm^3$ of the acid will contain $30/100 \times 1.835g = 0.5505g$.

Convert mass to mole

: $1cm^3$ of the acid contains $0.5505g/98.08gmol^{-1} = 0.005613mol$.

: $1dm^3$ ($1000cm^3$) of the acid will contain = $0.005613 \times 1000 = 5.613$ mol of H_2SO_4

Molar concentration is $5.6moldm^{-3}$ (5.6M).

Preparatioo of 5.6M of $1dm^3$ H_2SO_4 solution

No of moles before dilution = No of moles after dilution

$$C_1(moldm^{-3}) \times V_1(dm^3) = C_2(moldm^{-3}) \times V_2(dm^3)$$

$$18.34 \times V_1 = 5.61 \times 1$$

$$V_1 = 5.61/18.34$$

$$V_1 = 0.306dm^3 = 306cm^3.$$

5.61M of $1000cm^3$ solution was prepared by taking $306cm^3$ of the stock solution and diluted with distilled water up to $1000cm^3$ ($1dm^3$) mark.

b. To find the molar concentration of 50% concentration of the stock solution.

: $1cm^3$ of the acid will contain $50/100 \times 1.835g = 0.9175g$.

Convert mass to mole

: $1cm^3$ of the acid contains $0.9175g/98.08gmol^{-1} = 0.009355mol$.

: $1dm^3$($1000cm^3$) of the acid will contain = $0.009355 \times 1000 = 9.355mol$ of H_2SO_4.

Molar concentration is $9.4moldm^{-3}$ (9.4M).

Preparation of 9.4M of $1dm^3$ H_2SO_4 solution

No of moles before dilution = No of moles after dilution

$$C_1(moldm^{-3}) \times V_1(dm^3) = C_2(moldm^{-3}) \times V_2(dm^3)$$

$$18.34 \times V_1 = 9.36 \times 1$$

$$V_1 = 9.36/18.34$$

$$V_1 = 0.510\,dm^3 = 510cm^3.$$

9.36 M of $1dm^3$ H_2SO_4 solution was prepared by taking $510cm^3$ of the stock solution and diluted with distilled water up to $1000cm^3$ ($1dm^3$) mark.

e. To find the molar concentration of 70% concentration of the stock solution.

: $1cm^3$ of the acid will contain $70/100 \times 1.835g = 1.2845g$.

Convert mass to mole

: $1cm^3$ of the acid contains $1.2845g/98.08gmol^{-1} = 0.0131mol$.

: $1dm^3(1000cm^3)$ of the acid will contain $= 0.0131 \times 1000 = 13.10mol$ of H_2SO_4.

Molar concentration is $13.1 mohlm^{-3}$ (13.1M).

Preparation of 13.1M of $1dm^3$ H_2SO_4 solution

No of moles before dilution = No of moles after dilution

$C_1 (moldm^{-3}) \times V_1 (dm^3) = C_2 (moldm^{-3}) \times V_2 (dm^3)$

$18.34 \times V_1 = 13.10 \times 1$

$V_1 = 13.10/18.34$

$V_1 = 0.714\ dm^3 = 71.4cm^3$.

13.10M of $1dm^3$ H_2SO_4 solution was prepared by taking $71.4cm^3$ of the stock solution and diluted with distilled water up to $1000cm^3$ ($1dm^3$) mark.

i. **Pre-Treatment Phase:** This is to make the lignocellulose material amenable to hydrolysis. Prior to acid hydrolysis, the biomass was dried to a moisture content of approximately 10% and milled to an average size of 15mm with the aid of a grinding machine or miller.

ii. **Hydrolysis:** The milled biomass was mixed with various concentration of H_2SO_4 of 5.6M, 9.4M and 13.1M respectively. The ratio of the biomass to the concentrated acid was in 1:5 (w/v). Thus 20g of each substrate was mixed with 100ml of the various acid solution. This results in the disruption of the bonds between the crystalline cellulose chains/crystalline structure making the long cellulose chain accessible for hydrolysis. In the first hydrolysis, the mixture of acid and biomass was heated to 100°C for about 60 minutes to hydrolyse the lignocellulose. This resulted in the formation of a thick gel, which was pressed

on a sieving metal (siever) to obtain an acid- sugar stream. The volume of the hydrolysate obtained was measured using a measuring cylinder and recorded. The second hydrolysis step was similar to the first one. The solids remaining after the first hydrolysis and the solid-liquid seperation are mixed with 100ml concentrated H_2SO_4. The mixture was heated for 50minutes at 100°C to hydrolyze the lignocelluloses materials. The resulting gel was again pressed to obtain a second acid- sugar stream and the volume also recorded. The stream from the two hydrolysis steps was combined together.

Equation of reaction:

$$(C_6H_{10}O_5)n \xrightarrow{+H_2O} C_6H_{12}O_6$$

Lignocellulose Conc.H_2SO_4 reducing sugar

The left over solid which is lignin, the most recalcitrant to degradation out of the 3 component of liginocellulose material (Lignin, hemicellulose and cellulose) was disposed in a waste basket.

The acid-sugar stream (hydrolysate) obtained from the various acid concentrations at 5.61M, 9.36M and 13.10M respectively were subjected to biochemical analysis. 50ml from each of the hydrolysate were taken for the following bio-chemical analysis:

- Total Organic Carbon (T.O.C)
- Total Nitrogen
- Total Phosphorus
- Total Reducing Sugar
- Glucose (Precursor for ethanol production)

The important of this step was to determine which of the acid concentration hydrolysis produces the best yield of Glucose and other Fermentable sugars.

Plate 3.9: Hydrolysates of each of the substrate obtained from the 13.1M H_2SO_4 hydrolysis performed in triplicate

Plate 3.9: Hydrolysates of each of the substrate obtained from the 13.1M H_2SO_4 hydrolysis performed in triplicate

3.8.2 Proximate Analysis

Samples were analyzed chemically according to the official methods of analysis described by the Association of Official Analytical Chemists (A.O.A.C.1984, 1990, 1998). All analysis was carried out in triplicate.

3.8.2.1 Determination of Total Organic Carbon

The Total Organic Carbon was determined by using the Walkey Method.

Reagents and Methods of Preparation

1. Standard Normal Potassium Dichromate

K_2CrO_7 was oven dry at 130-150^{o}C for 2 to 3 hours. It was cooled in a desiccator, weighed at exactly 49.035g of the dried salt, dissolved in about 950ml of the distilled water, and placed in a cool place or room overnight. When cool, it was made up to 1000ml with distilled water (cold).

2. Standard Normal Ferrous Ammonium Sulphate.

156.86g of $Fe(NH_4)(SO_4)_2$ was weighed out and dissolved in about 900ml of distilled water. 25ml Con. H_2SO_4 was added and allowed to cooled. It was made up to the mark with distilled water and standardizes using the Normal Potassium dichromate

3. Diphenylamine Indicator

1g of diphenylamine was dissolved in 200ml of 1 to 1 solution of H_20 to H_2SO_4.

Procedure

1. Between 0.1- 3.0gm of the sample was weighed; depending on how dark the colour of the analyte is.

2. 10ml of the 1N $K_2Cr_2O_7$ was added from an automatic burette, then added to this very carefully was appropriately 20M Conc. H_2SO_4 from an acid dispensing burette. Shake gently and left to cool.

3. Distilled water was added to make up to approximately 150ml mark on the conical flask

4. Added to it was 8-10 drops of diphenylamine- indicators; the colour was now dark violet.

5. It was titrated with 0.4N Ferrous Ammonium Sulphate until the Voilet colour changed to Green.

6. A duplicate blank determination was carried out on 10ml of the Normal $K_2Cr_2O_7$ using all the reagents each time a set of determination was done.

Calculation

Let y be the vol. in millimeters of 0.4N Ferrous ammonium sulphate used to react with the remaining in $K_2Cr_2O_7$ is 0.4y e.g since 10ml of $K_2Cr_2O_7$ were used in the first place, then the amount used to oxidize any carbon in the sample will be (10.0 – 0.4y). 1ml of $K_2Cr_2O_7$ = 0.003g carbon. However, the reaction is only approximately 75% complete.

Therefore, 1ml of $K_2Cr_2O_7$ = 0.003×100/75= 0.004gC.

That is % Total organic carbon in the sample (hydrolysate)

$$= \frac{(10.0-0.4y) \times 0.004 \times 100}{\text{Wt of sample taken}}$$

Since y is Titre value used for the titration (T.V)

Hence

$$\% \text{ Total Organic carbon} = \frac{(10.0 - 0.4 \times T.V) \times 0.004 \times 100}{W1 \text{ of sample taken}}$$

Where T.V= Titre Value.

3.8.2.2 Determinntion of Total Nitrogen (%)

The total nitrogen (%) in the samples was determined by the routine semi- micro Kjeldahl procedure/ technique. This consists of three techniques namely Digestion, Distillation and Titration.

Apparatus: Analytical balance, Digestion tubes, Digestion Block Heater, 50ml Burette, 5ml Pipette, 10ml pipette, 10ml Measuring Cyclinder, 100ml Beakers, Fume cupboard

Reagents: Cone H_2SO_4, 0.01N HCl, 40% (w/v) NaOH, 2% Boric Acid Solution, Methyl Red-Bromocresol green mixed indicator, Kjeldahl catalyst tablet.

Digestion

0.5g of each sample was weighed carefully into the kjeldahl digestion tubes to ensure that all sample materials got to the bottom of the tubes. To this were added 1 Kjeldahl catalyst tablet and 10ml of Conc H_2SO_4. These were set in the appropriate hole of the Digestion Block Heaters in a fume cupboard. The digestion was left on for 4 hours, after which a clear colourless solution was left in the tube. The digest wns cooled and transferred into 100ml volumetric flask, thoroughly rinsing the digestion tube with distilled water and the flask was made up to mark with distilled water.

Distillation

The distillation was done with Markham Distillation Apparatus which allows volatile substances such as ammonia to be steam distilled with complete collection of the distillate. The apparatus was steamed out for about ten minutes. The steam generator was then removed from the heat source to the developing vacuum to removed condensed water. The

Hence

$$\% \text{ Total Organic carbon} = \frac{(10.0 - 0.4 \times T.V) \times 0.004 \times 100}{\text{Wt of sample taken}}$$

Where T.V= Titre Value.

3.8.2.2 Determinnation of Totnl Nitrogen (%)

The total nitrogen (%) in the samples was determined by the routine semi- micro Kjeldahl, procedure/ technique. This consists of three techniques namely Digestion, Distillation and Titration.

Apparatus: Analyticnl balance, Digestion tubes, Digestion Block Heater, 50ml Burette, 5ml Pipette, 10ml pipette, 10ml Measuring Cyclinder, 100ml Beakers, Fume cupboard

Reagents: Conc H_2SO_4, 0.01N HCl, 40% (w/v) NnOH, 2% Boric Acid Solution, Methyl Red-Bromocresol green mixed indicator, Kjeldahl catalyst tablet.

Digestion

0.5g of each sample was weighed carefully into the kjeldahl digestion tubes to ensure that all sample materials got to the bottom of the tubes. To this were added 1 Kjeldahl catalyst tablet and 10ml of Conc H_2SO_4. These were set in the appropriate hole of the Digestion Block Heaters in a fume cupboard. The digestion was left on for 4 hours, after which a clear colourless solution was left in the tube. The digest was cooled and transferred into 100ml volumetric flask, thoroughly rinsing the digestion tube with distilled water and the flask was made up to mark with distilled water.

Distillation

The distillation was done with Markham Distillation Apparatus which allows volatile substances such as ammonia to be steam distilled with complete collection of the distillate. The apparatus was steamed out for about ten minutes. The steam generator was then removed from the heat source to the developing vacuum to removed condensed water. The

steam generator was then placed on the heat source (i.e heating mantle) and each component of the apparatus was fixed up appropriately.

Determination: 5ml portion of the digest obove was pipetted into the body of the apparatus via the small funnel aperture. To this was added 5ml of 40% (w/v) NaOH through the same opening with the 5ml pipette.

The mixture wns steam- distilled for 2 minutes into a 50ml conical flask containing 10ml of 2% Boric Acid plus mixed indicator solution placed at the receiving tip of the condenser. The Boric Acid plus indicator solution changed colour from red to green showing that all the nmmonia liberated had been trapped.

Titration

The green colour solution obtained was then titrated against 0.01N HCl contained in a 50ml Burette. At the end point or equivalent point, the green colour turned to wine colour which indicated that all the Nitrogen trapped as Ammonium Borate [$(NH_4)_2 BO_3$] was removed as Ammonium chloride (NH_4Cl).

Calculation

The percentage nitrogen in this analysis was calculated using the formula:

$$\% N = \frac{\text{Titre value} \times \text{Normality of HCl used} \times \text{Atomic mass of N} \times \text{Volume of Flask containing the digest} \times 100}{2000}$$

3.8.2.3 Determination of Total Phosphorus (%)

Phosphorus was determined routinely by the Vanado-Molybdate colourimeter or spectrophotometric method.

Apparatus: Spectrophotometer or colourimeter, 50ml volumetric flask, 10ml Pipette, Whatman filter paper, Funnel, Wash bottle, Glass rod, Heating mantle, Crucibles, Flame photometer, Analytical balance.

Reagents: Vanadate- molybdate yellow solution, 2M HCl.

Preparation of Standard Phosphate Solution: 219.5mg anhydrous KH_2PO_4 was dissolved in distilled water and diluted to 1000ml; 1.00ml = 50.0ug PO_4^{3-} P.

Preparation of Calibration Curve: 10ml of the standard phosphate solution was placed in a 50ml volumetric flask. 10ml vanadate- molybdate yellow solution was added and diluted to the mark with distilled water, stoppered and left for 10mins for full yellow development. After 10mins or more, the absorbance was measured versus a blank solution (using 15ml, 20ml, 25ml 30ml). A graph of Absorbance against Concentration was drawn and the slope was calculated.

Procedure: 20mg (0.02g) of each sample was digested by adding 5ml of 2 M HCl solution to the hydrolysate in the crucible and heated to dryness on a heating mantle. 5ml of 2M HCl was added again, heated to boil, and filtered through a whatman No 1 filter paper. 10ml of the filtrate solution was pipetted into 50ml standard flask and 10ml of vanadate yellow solution was added and the flask was made up to mark with distilled water, stoppered and left for 10minutes for full yellow development. The concentration of phosphorus was obtained by taking the optical density (OD) or absorbance of the solution on a Spectronic 20 spectrophotometer or colourimeter at wavelenght of 470nm.

NOTE: A wavelenght of 470nm is usually used because ferric ion causes interference at low wavelenghts, particularly at 400nm.

Calculation

The percentage phosphorus was calculated from using the formula:

$$\% P = \frac{\text{Absorbance reading} \times \text{Slope} \times \text{Dilution factor}}{1000}$$

Apparatus: Spectrophotometer or colourimeter, 50ml volumetric flask, 10ml Pipette, Whatman filter paper, Funnel, Wash bottle, Glass rod, Heating mantle, Crucibles, Flame photometer, Analytical balance.

Reagents: Vanadate- molybdate yellow solution, 2M HCl.

Preparation of Standard Phosphate Solution: 219.5mg anhydrous KH_2PO_4 was dissolved in distilled water and diluted to 1000ml; 1.00ml = 50.0ug PO_4^{3-} P.

Preparation of Calibration Curve: 10ml of the standard phosphate solution was placed in a 50ml volumetric flask. 10ml vanadate- molybdate yellow solution was added and diluted to the mark with distilled water, stoppered and left for 10mins for full yellow development. After 10mins or more, the absorbance was measured versus a blank solution (using 15ml, 20ml, 25ml 30ml). A graph of Absorbance against Concentration was drawn and the slope was calculated.

Procedure: 20mg (0.02g) of each sample was digested by adding 5ml of 2 M HCl solution to the hydrolysote in the crucible and heated to dryness on a heating mantle. 5ml of 2M HCl was added again, heated to boil, and filtered through a whatman No 1 filter paper. 10ml of the filterate solution was pipetted into 50ml standard flask and 10ml of vanadate yellow solution was added and the flask was made up to mark with distilled water, stoppered and left for 10minutes for full yellow development. The concentration of phosphorus was obtained by taking the optical density (OD) or absorbance of the solution on a Spectronic 20 spectrophotometer or colourimeter at wavelenght of 470nm.

NOTE: A wavelenght of 470nm is usually used because ferric ion causes interference at low wavelenghts, particularly at 400nm.

Calculation

The percentage phosphorus was calculated from using the formula:

$$\% P = \frac{\text{Absorbance reading} \times \text{Slope} \times \text{Dilution factor}}{1000}$$

Where

Absorbance× Slope× Dilution faction=PPM/10,000

Hence

% P = PPM/10,000

Where

Absorbance = Reading obtained from the spectrophotometer.

Slope = Result of the Standard curve

Dilution factor = Volume of the extract/ weight of the sample.

3.8.2.4 Determination of Glucose Yield.

The ferric cyanide method described by the Association of Official Analytical Chemists (1984) was used to determine the glucose yield

Principle

Unlike alkaline conditions, glucose reduced ferric cyanide to ferrocyanide, which reacted with ferric ions to form a yellow colour. This reaction was then utilized in a colorimetric assay.

Preparation of Glucose standard

The stock glucose standard was made by dissolving 0.1g of standard D- glucose in distilled water and made up to the mark in 100ml volumetric flask. 10ml of this was then diluted with distilled water and made up to the mark in a 100ml volumetric flask. 1ml of the diluted solution contained 100mg of glucose.

Calibration curve

Different concentration was prepared from the stock solution of D- glucose i.e 1ppm, 2ppm, 3ppm, 4ppm and 5ppm respectivcly. The absorbance was measured at 380nm. with

distilled water as the reference/zero. A graph of concentration against absorbance was plotted and the gradient was calculated.

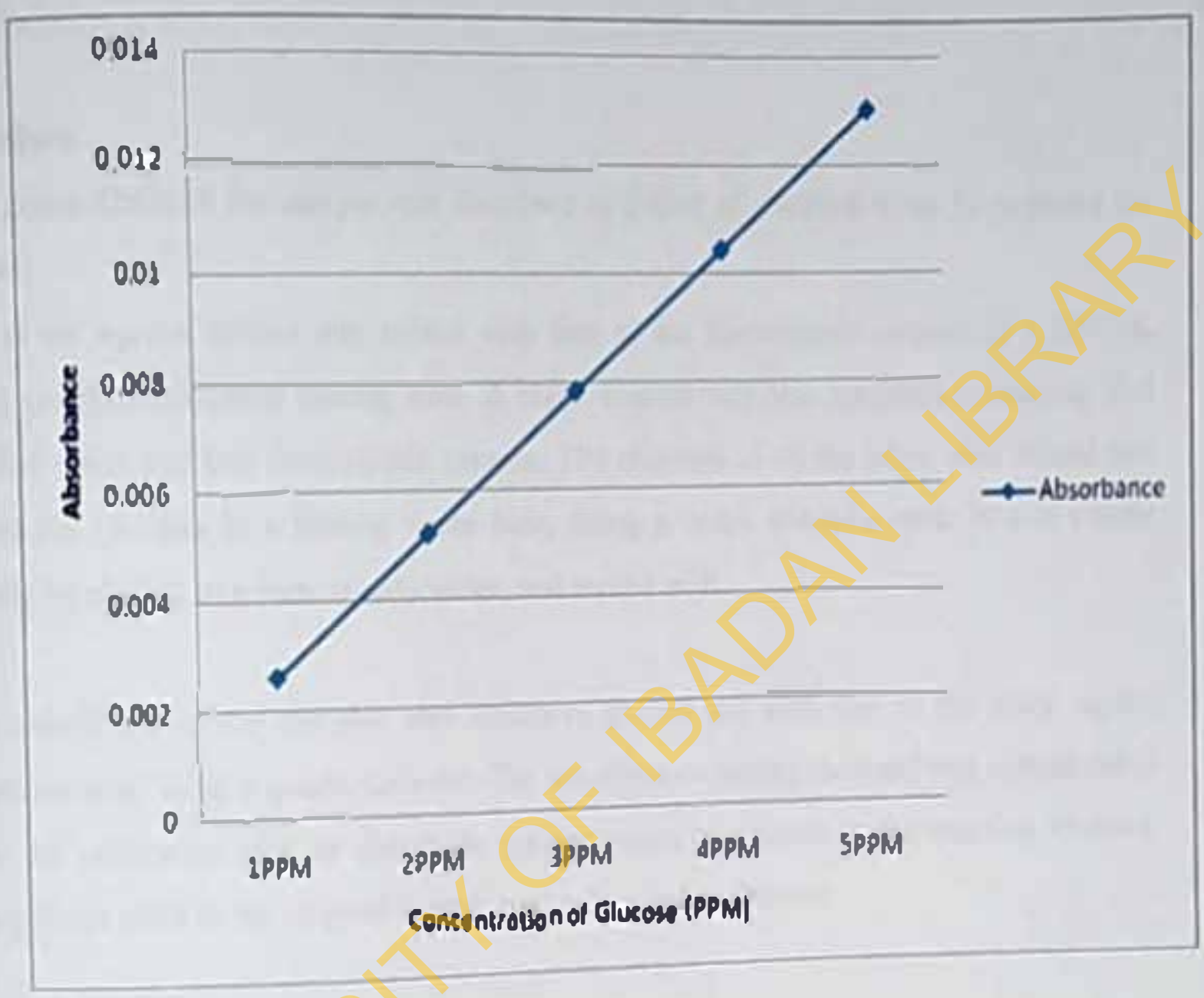


Fig 3.2: Glucose Standard Curve Slope = 0.384

Preparation of reagents

Solution A: 12.5g potassium ferricyanide (potassium hexacyanoferrate (iii)) and 10.0g anhydrous sodium carbonate was poured into a 100ml beaker, distilled water was added to dissolved it; after which the solution was poured into a 250ml labeled, Grade A volumetric flask and make up to volume with distilled water.

Solution B: 87.5g anhydrous sodium carbonate was poured into a 500ml beaker, distilled water was added to dissolve it; after which it was transferred into a 100ml labeled, Grade A volumetric flask, and made up to volume with distilled water.

Ferricyanide reagent: 25ml of solution A and 100ml of solution B was pipetted into a 1000ml labeled, Grade A volumetric flask, and was made up to volume with distilled water.

Procedure

Two grams (2ml) of the sample was dissolved in 250ml of distilled water to prepared the extract.

2ml of the aqeous extract was mixed with 8ml of the ferricyanide reagent in a labeled, 50ml, Quickfit stoppered boiling tube. A blank control was also prepared containing 2ml distilled water nnd 8ml ferricyanide reagent. The contents of all the tubes were mixed and boiled for 15 mins in a boiling water bath, using a metal test-tube rack. It was cooled rapidly by placing in a bath of cold water, and mixed well.

The absorbance of the samples was measured at 380 nm with that of the blank as the reference/zero, using a quartz curvette. The absorbance reading obtained was extraplolated from the calibration plot, to obtain the concentration of glucose in the reaction mixture. The glucose yield in the original sample was calculated as follows:

Calculation

$$\text{Glucose yield} = \frac{(C_1 \times 125)}{D_1} \times 1000 \text{ mg/Kg dry weight}$$

Where

C_1 = Concentration of glucose in the 10ml reaction mixture read from the calibration plot.

125 = Conversion factor since 2ml of the sample was dissolved in 250ml aqeuous extract, and the absorbance was measured.

1000 = Conversion factor to convert final value from mg/g to mg/Kg.

D_1 = Dry weight of the original sample, which is 20g.

Ferricyanide reagent: 25ml of solution A and 100ml of solution B was pipetted into a 1000ml labeled, Grade A volumetric flask, and was made up to volume with distilled water.

Procedure

Two grams (2ml) of the sample was dissolved in 250ml of distilled water to prepared the extract.

2ml of the aqeous extract was mixed with 8ml of the ferricyanide reagent in a labeled, 50ml, Quickfit stoppered boiling tube. A blank control was also prepared containing 2ml distilled water and 8ml ferricyanide reagent. The contents of all the tubes were mixed and boiled for 15 mins in a boiling water bath, using a metal test-tube rack. It was cooled rapidly by placing in a bath of cold water, and mixed well.

The absorbance of the samples was measured at 380 nm with that of the blank as the reference/zero, using a quartz curvette. The absorbance reading obtained was extraplolated from the calibration plot, to obtain the concentration of glucose in the reaction mixture. The glucose yield in the original sample was calculated as follows:

Calculation

$$\text{Glucose yield} = \frac{(C_1 \times 125)}{D_1} \times 1000 \text{ mg/Kg dry weight}$$

Where

C_1 = Concentration of glucose in the 10ml reaction mixture read from the calibration plot.

125 = Conversion factor since 2ml of the sample was dissolved in 250ml aqeuous extract, and the absorbance was measured.

1000 = Conversion factor to convert final value from mg/g to mg/Kg.

D_1 = Dry weight of the original sample, which is 20g.

3.8.2.5 Determination of Total Reducing Sugars (T.R.S)

The Phenol- Sulphuric Acid Method according to Dubois *et al*, (1956) was followed.

Preparation of Solutions

A. **52% Phenol solutions**

52ml (52g) of phenol liquid / oil crystals i.e v/v or w/v was dissolved in 100ml volumetric flask and made up to the mark with distilled water.

B. **96% Sulphuric Acid**

96ml of Sulphuric Acid (H_2SO_4) pure Analar Reagent (v/v) was measured into 100ml volumetric flask and made up to the mark with distilled water and allowed to cool before being used.

Preparation of Glucose Standard

The stock glucose standard was made by dissolving 0.1g standard glucose in distilled water and made up to the mark in 100ml volumetric flask. Ten (10ml) of this was then diluted with distilled water and made up to the mark in a 100ml volumetric flask. One (1ml) of the diluted solution contained 100mg of glucose.

Different concentrations were prepared from the stock solution (i.e 1ppm, 2ppm, 3ppm, 4ppm, 5ppm). A graph of Absorbance against concentration was plotted and the slope was determined.

Procedure

Two grams (2ml) of sample was dissolved in 250ml of distilled water or ethanolic extract can also be used (80%) ethanol) by adding 6- 8ml of 80% ethanol and centrifuged to get the supernatant for the analysis.

One (1ml) of the diluted solution was pipetted into test- tubes and 1ml of 52% phenol was added to each test- tube, 5ml of 96% H_2SO_4 was also added drop by drop. The test- tubes were allowed to stand for 10minutes before their contents were transferred into clean, grease- free cuvettes and the absorbance was read with a UV spectrophotometer at a

wavelenght of 490nm. A blank was also prepared as above but distilled water took the place of sample being analyzed. The blank was used to set the equipment to the zero mark. D- Glucose was used as the standard.

Calculation

$$TRS = \frac{(C_2 \times 125)}{D_1} \times 1000 \text{ mg/Kg dry weight}$$

Where

C_1= Concentration of reducing sugars read from the appropriate calibration graph.

TRS= Total Reducing Sugars.

125= Dilution factor, since 2ml of the analyte was dissolved in 250ml of the distilled water.

D_f= Dry weight of the original sample, which is 20g.

1000 = Conversion factor, to convert final value from mg/g to mg/Kg.

3.8.3 Neutralization Process

Apparatus : Analytical balance, pH meter, Whatman No1 filter paper, 250ml Conical flask, Bunsen burner / stove, 10ml Test tubes, 50ml Burette, 5ml Pipette, 500ml Conical flask.

Reagents: $Ca(OH)_2$ solution / Lime, Fehling solution, Distilled water.

Preparation of Calcium hydroxide solution / Lime

One hundred grams (100g) $Ca(OH)_2$ powder was weighed into a reagent bottle, from this a substantial quantity was dissolved in distilled water contained in a 500ml conical flask with continuous stirring which ensured that all the solute were dissolved in the distilled water. The $Ca(OH)_2$ powder was continuously added with stirring until a saturated solution was obtained. The saturated solution was filtered through a Whatman No1 filter paper to obtain a pure solution of $Ca(OH)_2$

Procedure

Lime [$Ca(OH)_2$] contained in a 50ml burette was titrated against each of the hydrolysate in a 250ml conical flask to neutralize the sulphuric acid and this raised the pH of the hydrolysate to about 5.5. The titration was stopped when $CaSO_4$ (gysum) precipitated out of solution accompanied by an exothermic reaction (evolution of heat to the surrounding). The $CaSO_4$ was removed by filtration using a Whatman No1 filter paper and sent to the disposal (waste bin). The filtrate which is a free sugar stream was tested for the presence of reducing sugar before being subjected to ethanol fermentation.

Equation of Reaction:

$$C_6H_{12}O_6\text{-------}H_2SO_4 + Ca(OH)_2 \longrightarrow C_6H_{12}O_6 + CaSO_{4(PPT)} + 2H_2O$$

Hydrolysate Lime Free sugar

3.8.4 Test for the presence of Reducing/ Simple sugar

About 1ml of Fehling solution was added to 5ml of the sugar solution, in a 10ml test tube and heated to boil. The Fehling solution changed from blue to orange- red colour, confirming the presence of reducing sugars.

3.8.5 Sugar (Glucose) Fermentation

Apparatus: Sterile cotton wool, Incubator, Analytical balance, 250ml conical flask, Bunsen burner.

Consumables: *Saccharomyces cerevisiae* (Dry Baker yeast), Distilled water, Detergent, Methylated spirit.

Sterilization of materials and disinfection

All the glass-wares used for this process was thoroughly washed with detergent, rinsed properly with distilled water and then allow to dry in a hot-air oven. The process of

sterilization of the equipment was to safeguard against possible contamination of the sugar solution.

Disinfection wns carried out by cleaning the whole surface of the working bench with cotton wool sonked in methyloted spirit before the fennenting process.

Weighing of *Saccharomyces cerevisiae*

The weight of the dry yeast to be ndded to each of the sugar solution was determined by using the method described by (Barnett, 1975): Using 2 grams of dry yeast per gallon (4 litres) of sugar solution.

Procedure (The fermenting process was done under an aseptic condition)

Xgrams of dry ycnst wns weighed, added and thoroughly mixed with each of the sugar solution obtained from the various substuate based on the above proportion in a 250ml conical flask flamed around the mouth region with a lighter after which it was sealed at the mouth with a sterile cotton wool to make it nirtight and to prevent air entry. The fermenting broth was placed in the laboratory at an optimal temperature of 30°C for 72hrs (3day's) to ensure maximum ethanol production. The effectiveness of fermentation was checked by taking sample from the fermented broth every 24 hrs by testing for the presence of ethanol. The ethanol yield was also determined quantitatively and qualitatively.

Equation of the reaction:

$$C_6H_{12}O_6 \xrightarrow{\text{yeast}} 2C_2H_5OH + 2CO_2\uparrow(g)$$

Glucose — Ethanol — Carbon dioxide

Note: Any variation in these conditions severely affects the ethanol production, possibly due to excessive enzymes degradation and loss of activity at a high temperature, reduced activity of glycolytic / fermentative enzyme at a low temperature, and inactivation of enzymes due to pH variation (Bajaj, Yousuf and Thakur, 2001)

3.8.6 Confirmatory Test for Ethanol

About 1ml of acidified $KMnO_4$ solution was added to 5ml of the fermented broth and heated to boil. The formation of a colourless liquid with an unpleasant smell of ethanal confirmed the presence of ethanol.

The Ethanol Concentration and Yield was determined by using the Official Methods of Analysis described by the Association of Official Analytical Chemists (1984).

3.8.7 Determination of Ethanol Yield (v/w)

Principle

Alcohol was distilled from sample and collected in an acid solution of potassium dichromate where it is oxidized to acetic acid at 60°C. The residual dichromate was determined by back titration with ferrous sulphate in a strong acid solution using ferroin indicator (1,10 – phenanthroline ferroussulphate complex)

Equipment

- Quicklit 500ml flask, round-bottomed, uniform height of 205mm with 70mm neck and 24/29 socket FR500/30.
- Quickfit 150ml conical flask with 24/29 conical flask with 24/29 socket FE 150/3.
- Quickfit stopper, 24/29 cone.
- Heating mantle for 500ml flask.
- 25ml burette, Grade A
- 25ml pipettes, bulb type Grade A
- 500ml, 1L and 2L volumetric flasks, Grade A

Consumables

- Ferrous ammonium sulphate
- Ferroin indicator BDH 21056 (1,10-phenanthroline-ferrous sulphate complex solution 0.025mol/l redox indicator.

- Potassium dichromate, AR.
- Sulphuric acid, concentrated.
- Anti-bumping granules.

Procedure

Preparation of reagents

Potassium dichromate (0.25N): 6.129g of potassium dichromate was dissolved in water and made up to 500ml and kept in an amber glass-stoppered bottle.

Sulphuric acid (approximately 25N): 300ml water was placed in a 2L conical flask and cautiously added 600ml of sulphuric acid AR while cooling was done in a bath of tap-water during addition. The solution was then kept in a glass bottle.

Ferrous ammonium sulphate (approximately 0.1N): 40g of ferrous ammonium sulphate was dissolved in water, after which 30ml of sulphuric acid (concentrated) was added to it. The solution was cooled and then made up to1 L with water. Note: this was best made up as required as the strength slowly diminishes due to oxidation by atmospheric oxygen.

Ferroin indicator (1. 10-phenanthroline-ferrous sulphate complex solution 0.025mol/l redox indicator, BDH 21056).

Method

0.8ml of the sample was added with anti-bumping granules to 50ml of distilled water and distilled from a 500ml round-bottomed Quickfit flask. The distillate was collected in a 150ml Quickfit conical flask marked at 35ml and containing 10ml of 0.25N potassium dichromate (i.e. 25ml of distillate was collected).

Twenty five (25ml) of 25N sulphuric acid (using measuring cyclinder) was added to the flask, and promptly stopper it. The volumes were chosen so that the temperature after mixing was equal to 60°C. After 30min interval the contents of the flask was then titrated. A blank was run at the same time. Ten (10ml) of 0.25N potassium dichromate, 25ml 25N sulphuric acid and 25ml water was added to a 150ml flask, and cooled and then titrated.

Titration: Two (2) drops of ferroin indicator was added to the contents of the flask, and then titrated against 0.1N ferrous ammonium sulphate from a 25ml burette. The colour gradually changed from yellow to blue-green, then changes in one drop through grey to pinkish-brown: this is the end-point. The volume titrated was noted.

The volume titrated was used to obtain the ethanol concentration in the reaction mixture; the ethanol yield in the original sample was calculated as shown below:

Calculation

Ethanol Concentration in mg/ml calculated as follows:

$$\text{Ethanol (in mg) per ml of sample} = \frac{1.152\times(B-T)\times 25/B}{\text{Sample volume (ml)}}$$

Where T= ml titration of sample

B= ml titration of Blank

$$\text{Ethanol Yield} = \frac{C \times V \times 50 \times 10^6}{De} \text{ ml/kg dry weight.}$$

Where

C = Concentration of ethanol (mg/ml)

V = Volume of sugar (ml)

50 = Multiplication factor used to extrapolate the result from the original sample of 20 to kg.

De = Density of ethanol (789kg/m^3).

10^6 = Conversion factor to convert final answer from m^3/kg to cm^3/kg (ml/kg).

3.9 Data Management & Statistical Analysis

Data was recorded at every given step in the process. This was achieved by measurement of weight, volumes, specific gravity, fermentation time etc.

Titration: Two (2) drops of ferroin indicator was added to the contents of the flask, and then titrated against 0.1N ferrous ammonium sulphate from a 25ml burette. The colour gradually changed from yellow to blue-green, then changes in one drop through grey to pinkish-brown: this is the end-point. The volume titrated was noted.

The volume titrated was used to obtain the ethanol concentration in the reaction mixture; the ethanol yield in the original sample was calculated as shown below:

Calculation

Ethanol Concentration in mg/ml calculated as follows:

$$\text{Ethanol (in mg) per ml of sample} = \frac{1.152 \times (B-T) \times 25/B}{\text{Sample volume (ml)}}$$

Where T= ml titration of sample

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$$\text{Ethanol Yield} = \frac{C \times V \times 50 \times 10^6}{De} \text{ ml/kg dry weight.}$$

Where

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50 = Multiplication factor used to extrapolate the result from the original sample of 20 to kg.

De = Density of ethanol ($789kg/m^3$).

10^6 = Conversion factor to convert final answer from m^3/kg to cm^3/kg (ml/kg).

3.9 Data Management & Statistical Analysis

Data was recorded at every given step in the process. This was achieved by measurement of weight, volumes, specific gravity, fermentation time etc.

- All data was summarized using descriptive statistics such as proportions, means and standard deviation.
- The results collected on the proximate analysis and ethanol yield from the various substrates were subjected to Student t-test, One-Way Analysis of Variance (ANOVA) at 5% level of Precision (α=5%) to compare the mean ethanol yield.
- Spearman-rank correlation was used to check if a relationship exists between the ethanol yield of the substrates and the time of fermentation.

CHAPTER FOUR

RESULTS

This chapter presents the results of the pilot/feasibility study which includes the estimation of quantity of sample from source, quantification of the agro-based cellulosic wastes (weight, volume, density) as well as results of the proximate analysis (% Total Organic Carbon (T.O.C), Total Nitrogen (%), Total Phosphorus (%), Glucose yield, Total Reducing Sugar (TRS) and Ethanol yield of the substrates).

4.1 Exploration/Fensibilityof Sample from Parent Source

A feasibility study was carried out on the sample collection areas to determine the sample populations and the quantity of agro by-product generated from the parent food materials. Table 4.1 shows the estimation of the quantity of sample generated from the various sources. The 1hectare (1ha) of cassava plantation at International Institute of Tropical Agriculture (IITA) produces between 20,000 to 70,000 cassava roots at harvest. The 1acre of yam farm at Abadina quarters in the University of Ibadan produces between 800 to 2000 yam tubers per harvest. The Bodija Timber Processing Unit which occupied about 25acres of land contains between 44,080 and 88,160 planks from which sawdust is generated. The Ajose Building Canteen in UCH processes between 3,000 to 5,000 plantain fingers per week.

4.2 Quantification of Agro-based Wastes

Table 4.2b shows the quantity of agro-based cellulosic wastes generated from the sample sources. The 1 hectare (1ha) of cassava plantation in IITA generates cassava peels (CP) of mean weight ranging from 6660 ± 616.0 Kg to 23310 ± 2156.8 Kg and a mean volume ranging from 19.466 ± 2.203m^3 to 68.133 ± 7.710m^3 per harvest. The mean density was estimated as 342.69 ± 8.0755kg/m^3. The 1acre of yam farm in Abadina quarter of U.I generates yam peels (YP) with a mean weight ranging from 183.3±7.0 Kg to 458.2 ± 1.2 Kg and a mean volume ranging from 0.555 ± 0.036074m^3 to 1.387 ± 0.090185m^3 per harvest. Also the mean density was estimated as 330.99 ± 12.86050kg/m^3.The Timber processing centre in Bodija which covers about 25 acres of land produce sawdust with a

weight capacity ranging from 3051.8±52.0 Kg to 6.103.61±103.5 Kg and a mean volume ranging from 25.42 ± 3.0065m^3 to 50.08 ± 6.00130m^3 with mean density of 121.09 ± 13.20465kg/m^3. The Ajose Building Canteen in UCH generates plantain peels every week with a mean weight ranging from 253.2 ± 41.0 Kg to 422 ± 67.60 Kg and a mean volume ranging from 0.84 ± 0.06m^3 to 1.4 ± 0.1m^3 with mean density of 300.50 ± 30.1917762kg/m^3.

Table 4.1: Shows the location, Parent source and population of the various samples

SAMPLE	LOCATION	PARENT SOURCE	POPULATION
Cassava peels	IITA	1hectare (1ha) of cassava plantation.	20,000 to 70,000 cassava r per harvest.
Yam peels	ABADINA QUARTERS.	1acre of yam farm.	800 to 2,000 yam tubers p harvest.
Sawdust	BODIJA TIMBER PROCESSING UNIT.	25 acres of land	44,080 to 88,160 planks.
Plantain peels	AJOSE BUILDING CANTEEN, UCH.	Plantain farm	3,000 to 5,000 plantain finge processed per week.

Table 4.2a: Shows the weight, volume and density of the various agro – based cellulosic wastes from the parent source.

	Sample	Cassava Peels		Yam Peels		Plantain Peels		Saw Dust	
		Min	Max	Min	Max	Min	Max	Min	Max
Weight of waste (Kg)	1	6640	23240	178.6	446.6	300	500	3001.85	6003.70
	2	7286	25501	183.04	457.6	229.5	382.5	3050.34	6100.67
	3	6054	21189	188.16	470.4	230.1	383.5	3103.23	6206.46
Volume of Waste (m^3)	1	19.6	68.6	0.52	1.3	0.9	1.5	22.04	44.08
	2	21.6	75.6	0.552	1.38	0.78	1.3	26.45	52.90
	3	17.2	60.2	0.592	1.48	0.84	1.4	27.77	55.54
Density of Waste (Kg/m^3)	1	338.78		343.54		333.33		136.20	
	2	337.32		331.59		294.23		115.33	
	3	351.98		317.84		273.93		111.75	

Table 4.2b: Estimation of the quantity of agro-based wastes from parent source

Sample	Mean Weight (Kg) Mean ± S.D		Mean Volume (m^3) Mean ± S.D		Density (Kg/m^3) Mean ± S.D
	Min. Limit	Max. Limit	Min. Limit	Max. Limit	
Cassava peels	6660 ± 616.0	23310 ± 2156.8	19.466 ± 2.203	68.133 ± 7.710	342.69 ± 8.0755
Yam peels	183.3±7.0	458.2 ± 1.2	0.555±0.0361	1.387 ± 0.0902	330.99 ± 12.8605
Plantain peels	253.2 ± 41.0	422 ± 67.6	0.84 ± 0.06	1.4 ± 0.1	300.50 ± 30.1918
Sawdust	3051.8±52.0	6.103.61±103.5	25.42±3.0065	50.84±6.0013	121.09 ± 13.2047

4.3 Characteristics of Wastes Substrates (Proximate Analysis)

Fig 4.1 – 4.5 shows the mean value results obtained from the proximate analysis of the different sample hydrolysate at various acid concentrations of 5.6M, 9.4M and 13.1M. The mean glucose yield and TRS obtained from the 5.6M H_2SO_4 hydrolysis were : CP (50.5 ± 12.8mg/kg , 91.8 ± 3.0mg/kg) ; YP (231.0 ± 3.6mg/kg , 388.8 ± 6.9mg/kg) ; PP(255.5 ± 5.4mg/kg , 314.7 ± 5.1mg/kg) and SD (285.7 ± 5.0mg/kg , 374.5 ± 7.3mg/kg) ($p<0.05$). At 9.4M H_2SO_4 hydrolysis, the mean glucose yield and TRS were : CP (71.5 ± 3.0mg/kg, 123.2 ± 5.0mg/kg) ; YP (240.0 ± 5.0mg/kg, 460.2 ± 4.7mg/kg) ; PP (278.1 ± 6.5mg/kg , 396.4 ± 6.0mg/kg) and SD (300.7 ± 8.6mg/kg , 453.2 ± 6.6mg/kg) ($p<0.05$).The mean glucose yields and TRS obtained from the 13.10M H_2SO_4 hydrolysis of the substrates were as follows : CP (85.1 ± 5.7mg/kg, 209.8 ± 3.7mg/kg); YP (269.2 ± 11.2mg/kg, 541.3 ± 7.8mg/kg); PP (304.0 ± 6.1mg/kg, 461.2 ± 3.6mg/kg) and SD (343.2 ± 4.8mg/kg, 535.9 ± 5.0mg/kg) ($p<0.05$). The mean glucose yield and TRS obtained from the 13.1M H_2SO_4 were significantly higher than those obtained from the 9.4M and 5.6M H_2SO_4 hydrolysis ($p<0.05$). The 13.1M hydrosylate was used for ethanol production, since glucose and other reducing sugars serve as precursor for ethanol production.

From Table 4.3 – 4.5 and Figure 4.6, the mean T.O.C (%) decreased as the concentration of the acid increased and vice versa. Among the substrates, PP recorded the highest mean T.O.C (%) at different acid concentrations while the least mean T.O.C (%) was found in CP. At 13.1M hydrolysis, the mean T.O.C (%) of PP was significantly higher than those of CP, YP and SD ($p<0.05$). For the Total Nitrogen (%); it was evident from Figure 4.7, that the mean Total nitrogen (%) increased as the concentration of acid decreased and vice versa. The mean Total nitrogen (%) was found to be greatest for the CP throughout the various acid concentrations and least for the SD. This indicated that SD recorded the least amount of Total (organic) nitrogen than the other wastes (PP, CP and YP). At 13.1M, the mean Total nitrogen (%) of the various substrates were significantly different from each other ($p<0.05$).

4.3 Characteristics of Wastes Substrates (Proximate Analysis)

Fig 4.1 – 4.5 shows the mean value results obtained from the proximate analysis of the different sample hydrolysate at various acid concentrations of 5.6M, 9.4M and 13.1M.The mean glucose yield and TRS obtained from the 5.6M H_2SO_4 hydrolysis were : CP (50.5 ± 12.8mg/kg , 91.8 ± 3.0mg/kg) ; YP (231.0 ± 3.6mg/kg , 388.8 ± 6.9mg/kg) ; PP(255.5 ± 5.4mg/kg , 314.7 ± 5.1mg/kg) and SD (285.7 ± 5.0mg/kg , 374.5 ± 7.3mg/kg) ($p<0.05$). At 9.4M H_2SO_4 hydrolysis,the mean glucose yield and TRS were : CP (71.5 ± 3.0mg/kg, 123.2 ± 5.0mg/kg) ; YP (240.0 ± 5.0mg/kg , 460.2 ± 4.7mg/kg) ; PP (278.1 ± 6.5mg/kg , 396.4 ± 6.0mg/kg) and SD (300.7 ± 8.6mg/kg , 453.2 ± 6.6mg/kg) ($p<0.05$).The mean glucose yields ond TRS obtained from the 13.10M H_2SO_4 hydrolysis of the substrates were as follows : CP (85.1 ± 5.7mg/kg, 209.8 ± 3.7mg/kg); YP (269.2 ± 11.2mg/kg, 541.3 ± 7.8mg/kg); PP (304.0 ± 6.1mg/kg, 461.2 ± 3.6mg/kg) and SD (343.2 ± 4.8mg/kg, 535.9 ± 5.0mg/kg) ($p<0.05$). The mean glucose yield and TRS obtained from the 13.1M H_2SO_4 were significantly higher than those obtained from the 9.4M and 5.6M H_2SO_4 hydrolysis ($p<0.05$). The 13.1M hydrosylate was used for ethanol production, since glucose and other reducing sugars serve as precursor for ethanol production.

From Table 4.3 – 4.5 and Figure 4.6, the mean T.O.C (%) decreased as the concentration of the acid increased and vice versa. Among the substrates, PP recorded the highest mean T.O.C (%) at different acid concentrations while the least mean T.O.C (%) was found in CP. At 13.1M hydrolysis, the mean T.O.C (%) of PP was significantly higher than those of CP, YP and SD ($p<0.05$). For the Total Nitrogen (%); it was evident from Figure 4.7, that the mean Total nitrogen (%) increased as the concentration of ocid decreased and vice versa. The mean Total nitrogen (%) was found to be greatest for the CP throughout the various acid concentrations and least for the SD. This indicated that SD recorded the least amount of Total (organic) nitrogen than the other wastes (PP, CP and YP). At 13.1M, the mean Total nitrogen (%) of the various substrates were significantly different from each other ($p<0.05$).

From Figure 4.8 and Table 4.3 – 4.5, the "acid-hydrolyzable phosphate" increased as the concentration of the acid decreased thus impling an inverse relationship between the percentage phosphorus and acid concentration. Comparing the different substrates, throughout the various acid concentrations, the PP had the highest mean Total phosphorus (%), followed by YP, then CP with SD recorded the least mean Total phosphorus (%). However at 13.1M hydrolysis, the mean Total Phosphorus (%) of the various substrates were not statistically different from each other ($p>0.05$).

EXPERIMENT 1

Table 4.3 Pattern of Proximate Analysis of Substrate Hydrolysates at Different Acid Concentrations

	5.6M H_2SO_4 HYDROLYSIS					9.4 M H_2SO_4 HYDROLYSIS					13.1 M H_2SO_4 HYDROLYSIS *				
Sample Description	T.O.C (%)	T.N (%)	T.P (%)	G.Y (mg/kg)	T.R.S (mg/kg)	T.O.C (%)	T.N (%)	T.P (%)	G.Y (mg/kg)	T.R.S (mg/kg)	T.O.C (%)	T.N (%)	T.P (%)	G.Y (mg/kg)	T.R.S (mg/kg)
Sawdust hydrolysate	23.8	2.3	0.22	286.0	375.0	18.6	1.8	0.20	300.0	450.0	13.2	1.2	0.19	343.0	537.5
Plantain hydrolysate	28.5	2.5	0.27	257.0	315.0	24.2	1.9	0.26	275.0	395.0	17.3	1.4	0.24	305.0	460.5
Yam hydrolysate	24.8	2.7	0.25	230.0	390.0	19.2	2.1	0.23	240.0	460.0	13.6	1.6	0.22	271.5	544.5
Cassava hydrolysate	19.2	2.8	0.24	65.0	91.5	13.8	2.2	0.21	70.0	125.0	8.7	1.8	0.20	84.0	209.0

* The hydrolysate obtained from the 13.1M hydrolysis gave the highewst yield or mean value of Glucose and other fermentable sugars throughout the three (3) trials than the 5.6M and 9.4M hydrolysis. Hence it was used for the ethanol production.

EXPERIMENT 2

Table 4.4 Pattern of Proximate Analysis of Substrate Hydrolysates at Different Acid Concentrations

	5.6M H_2SO_4 HYDROLYSIS					9.4M H_2SO_4 HYDROLYSIS					13.1M H_2SO_4 HYDROLYSIS *				
Sample Description	T.O.C (%)	T.N (%)	T.P (%)	G.Y (mg/kg)	T.R.S (mg/kg)	T.O.C (%)	T.N (%)	T.P (%)	G.Y (mg/kg)	T.R.S (mg/kg)	T.O.C (%)	T.N (%)	T.P (%)	G.Y (mg/kg)	T.R.S (mg/kg)
Sawdust hydrolysate	25.0	2.5	0.27	280.6	367.0	19.8	1.8	0.24	292.5	448.8	14.2	1.3	0.23	338.5	530.3
Plantain hydrolysate	30.2	2.6	0.31	249.5	309.5	25.8	1.9	0.29	273.8	391.3	18.9	1.5	0.27	297.5	458.0
Yam hydrolysate	26.6	2.9	0.29	228.0	381.3	20.3	2.4	0.27	235.0	455.6	14.8	1.8	0.25	257.0	532.5
Cassava hydrolysate	21.0	3.1	0.28	41.0	89.0	14.2	2.4	0.23	69.5	117.5	9.4	1.9	0.22	80.0	206.5

* The hydrolysate obtained from the 13.1M hydrolysis gave the highest yield or mean value of Glucose and other fermentable sugars throughout the three (3) trials than the 5.6M and 9.4M hydrolysis. Hence it was used for the ethanol production.

EXPERIMENT 2

Table 4.4 Pattern of Proximate Analysis of Substrate Hydrolysates at Different Acid Concentrations

	5.6M H_2SO_4 HYDROLYSIS					9.4M H_2SO_4 HYDROLYSIS					13.1M H_2SO_4 HYDROLYSIS *				
Sample Description	T.O.C (%)	T.N (%)	T.P (%)	G.Y (mg/kg)	T.R.S (mg/kg)	T.O.C (%)	T.N (%)	T.P (%)	G.Y (mg/kg)	T.R.S (mg/kg)	T.O.C (%)	T.N (%)	T.P (%)	G.Y (mg/kg)	T.R.S (mg/kg)
Sawdust hydrolysate	25.0	2.5	0.27	280.6	367.0	19.8	1.8	0.24	292.5	448.8	14.2	1.3	0.23	338.5	530.3
Plantain hydrolysate	30.2	2.6	0.31	249.5	309.5	25.8	1.9	0.29	273.8	391.3	18.9	1.5	0.27	297.5	458.0
Yam hydrolysate	26.6	2.9	0.29	228.0	381.3	20.3	2.4	0.27	235.0	455.6	14.8	1.8	0.25	257.0	532.5
Cassava hydrolysate	21.0	3.1	0.28	41.0	89.0	14.2	2.4	0.23	69.5	117.5	9.4	1.9	0.22	80.0	206.5

* The hydrolysate obtained from the 13.1M hydrolysis gave the highest yield or mean value of Glucose and other fermentable sugars throughout the three (3) trials than the 5.6M and 9.4M hydrolysis. Hence it was used for the ethanol production

EXPERIMENT 3

Table 4.5 Pattern of Proximate Analysis of Substrate Hydrolysates at Different Acid Concentrations

	5.6M H_2SO_4 HYDROLYSIS					9.4M H_2SO_4 HYDROLYSIS					13.1M H_2SO_4 HYDROLYSIS *				
Sample Description	T.O.C (%)	T.N (%)	T.P (%)	G.Y (%)	T.R.S (%)	T.O.C (%)	T.N (%)	T.P (%)	G.Y (%)	T.R.S (%)	T.O.C (%)	T.N (%)	T.P (%)	G.Y (%)	T.R.S (%)
Sawdust hydrolysate	22.8	2.2	0.18	290.5	381.5	17.2	1.6	0.17	309.5	460.8	12.8	1.1	0.14	348.0	540.0
Plantain hydrolysate	27.3	2.4	0.23	260.0	319.5	23.0	1.7	0.23	285.5	403.0	16.9	1.4	0.20	309.6	465.0
Yam hydrolysate	23.7	2.5	0.21	235.0	395.0	18.5	2.0	0.20	245.0	465.0	12.6	1.6	0.18	279.0	547.0
Cassava hydrolysate	18.3	2.6	0.20	45.5	95.0	12.3	2.2	0.19	75.0	127.0	8.0	1.8	0.15	91.2	213.8

* The hydrolysate obtained from the 13.1M hydrolysis gave the highest yield or mean value of Glucose and other fermentable sugars throughout the three (3) trials than the 5.6M and 9.4M hydrolysis. Hence it was used for the ethanol production.

EXPERIMENT 3

Table 4.5 Pattern of Proximate Analysis of Substrate Hydrolysates at Different Acid Concentrations

	5.6M H_2SO_4 HYDROLYSIS					9.4M H_2SO_4 HYDROLYSIS					13.1M H_2SO_4 HYDROLYSIS *				
Sample Description	T.O.C (%)	T.N (%)	T.P (%)	G.Y (%)	T.R.S (%)	T.O.C (%)	T.N (%)	T.P (%)	G.Y (%)	T.R.S (%)	T.O.C (%)	T.N (%)	T.P (%)	G.Y (%)	T.R.S (%)
Sawdust hydrolysate	22.8	2.2	0.18	290.5	381.5	17.2	1.6	0.17	309.5	460.8	12.8	1.1	0.14	348.0	540.0
Plantain hydrolysate	27.3	2.4	0.23	260.0	319.5	23.0	1.7	0.23	285.5	403.0	16.9	1.4	0.20	309.6	465.0
Yam hydrolysate	23.7	2.5	0.21	235.0	395.0	18.5	2.0	0.20	245.0	465.0	12.6	1.6	0.18	279.0	547.0
Cassava hydrolysate	18.3	2.6	0.20	45.5	95.0	12.3	2.2	0.19	75.0	127.0	8.0	1.8	0.15	91.2	213.8

* The hydrolysate obtained from the 13.1M hydrolysis gave the highest yield or mean value of Glucose and other fermentable sugars throughout the three (3) visits than the 5.6M and 9.4M hydrolysis. Hence it was used for the ethanol production.

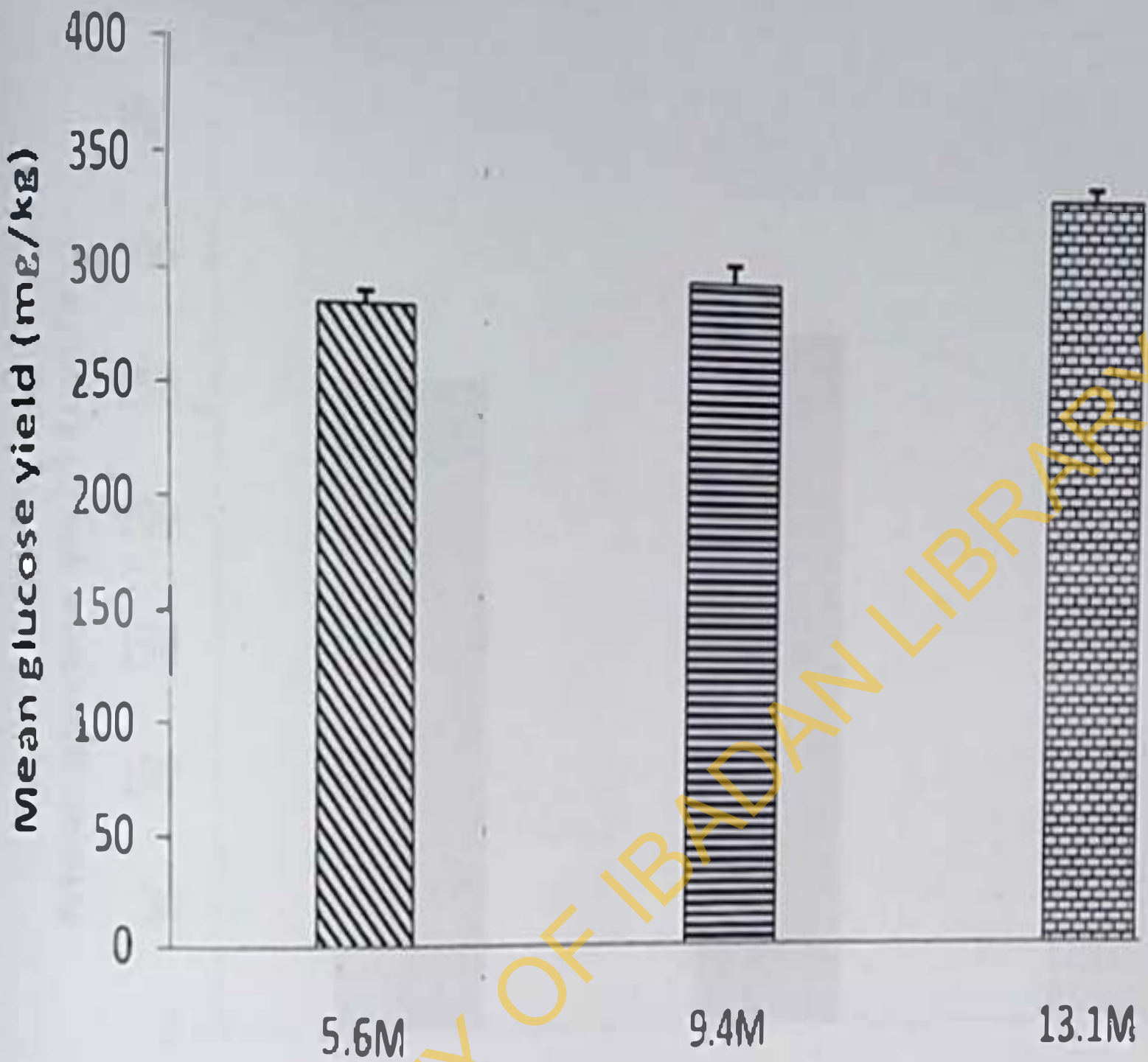

Fig 4.1: Mean Glucose Yield of SD at different Acid Hydrolysis

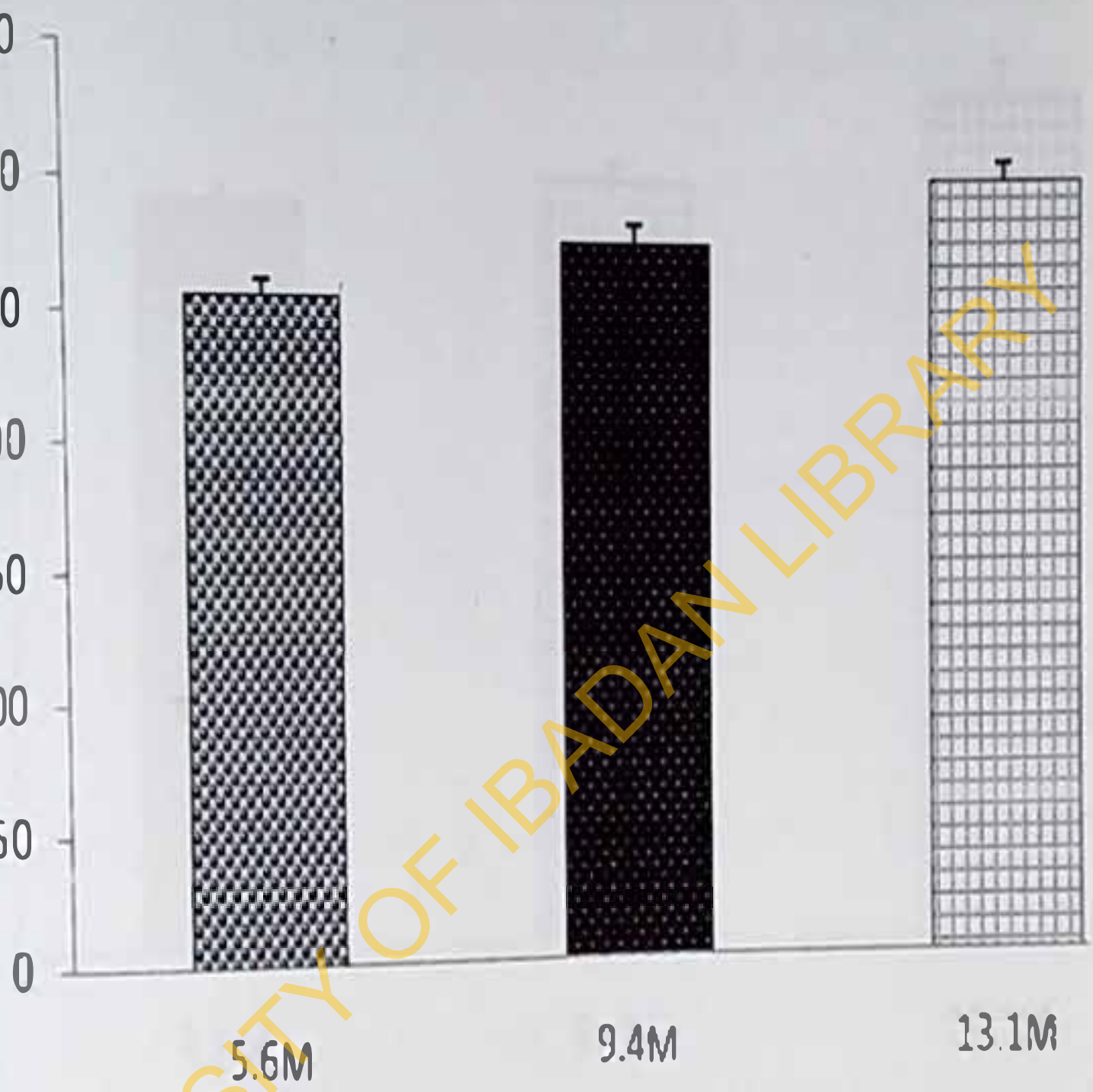

Fig 4.2: Mean Glucose Yield of PP at different Acid Hydrolysis

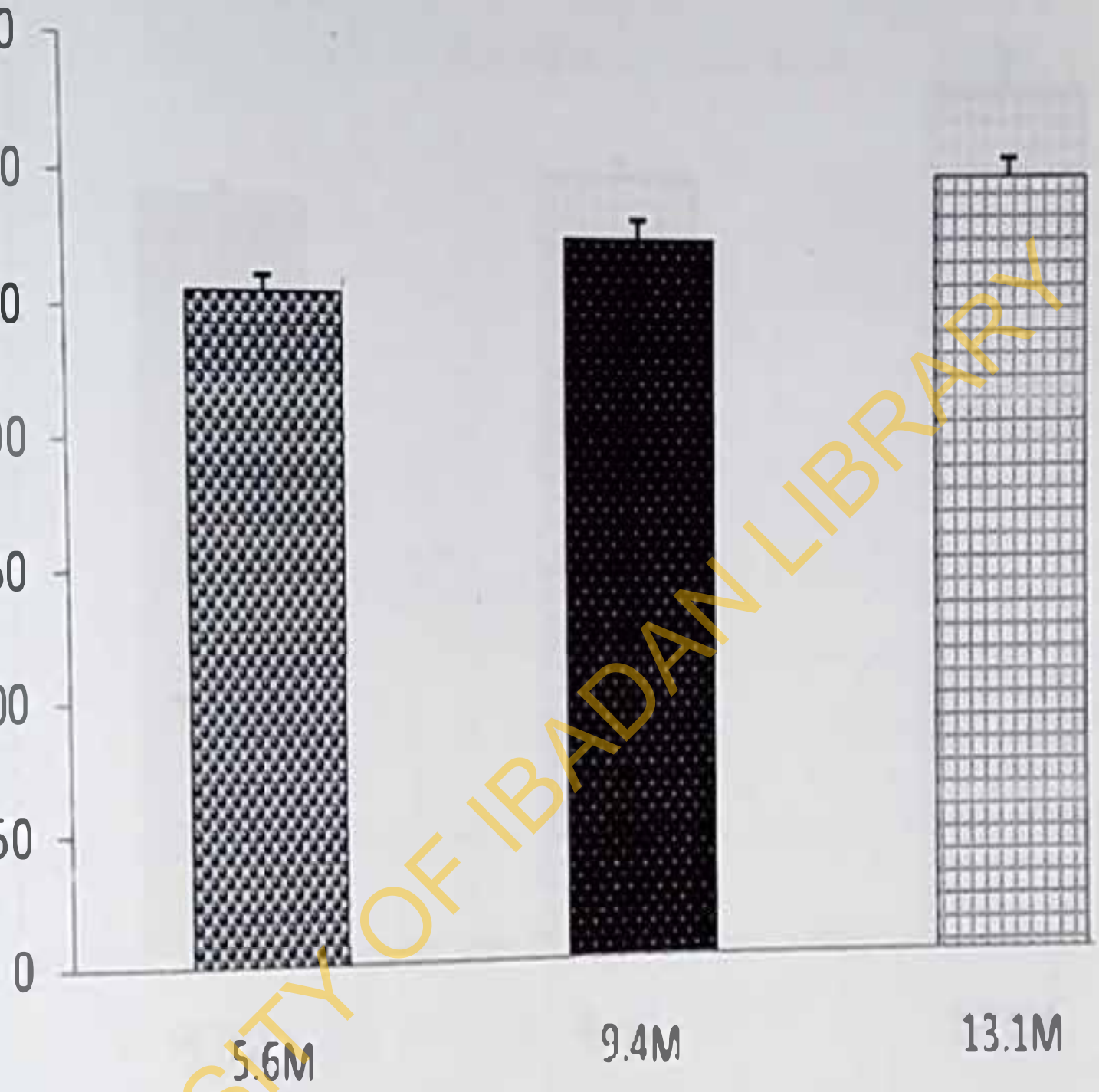

Fig 4.2: Mean Glucose Yield of PP at different Acid Hydrolysis

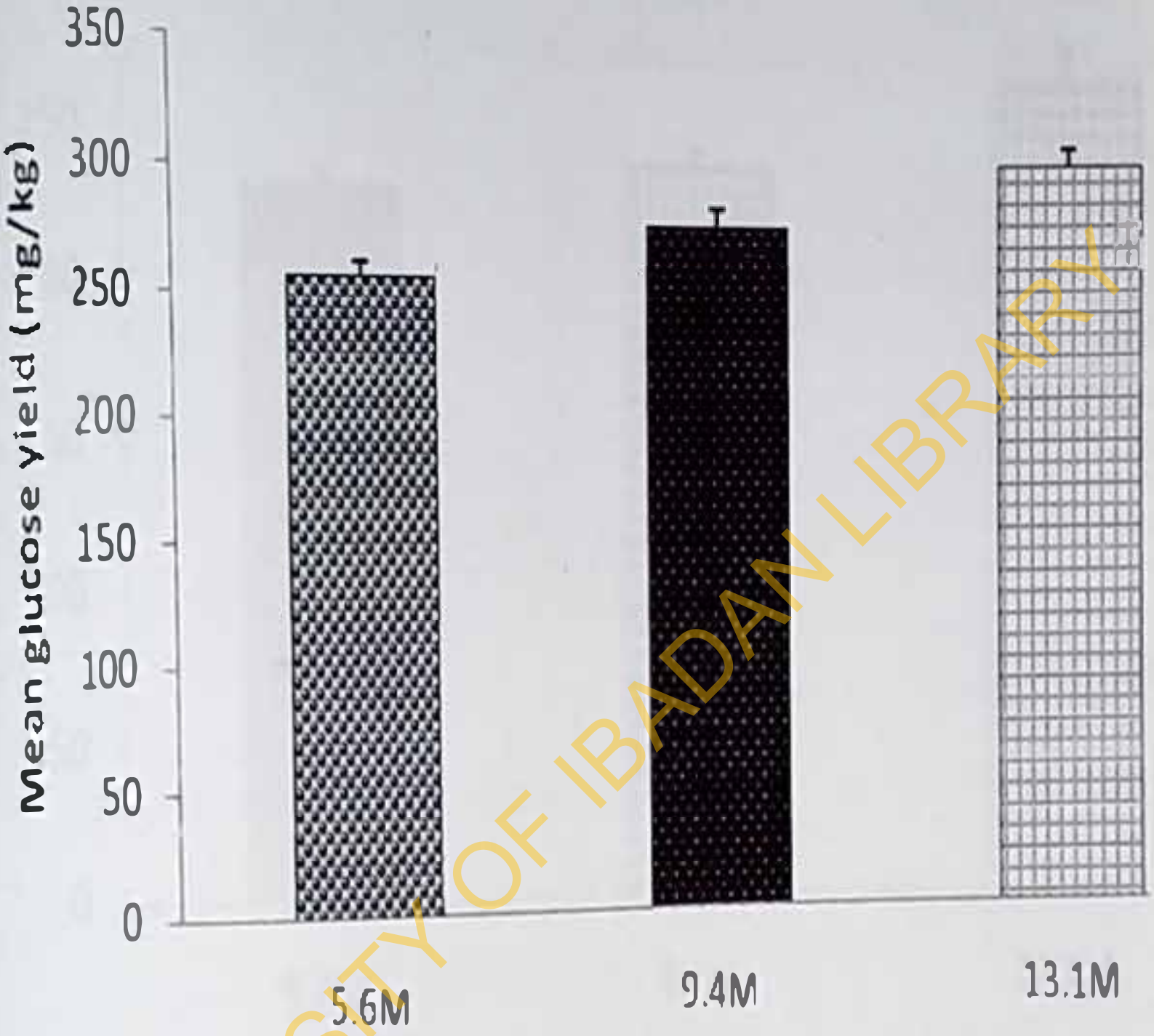

Fig 4.2: Mean Glucose Yield of PP at different Acid Hydrolysis

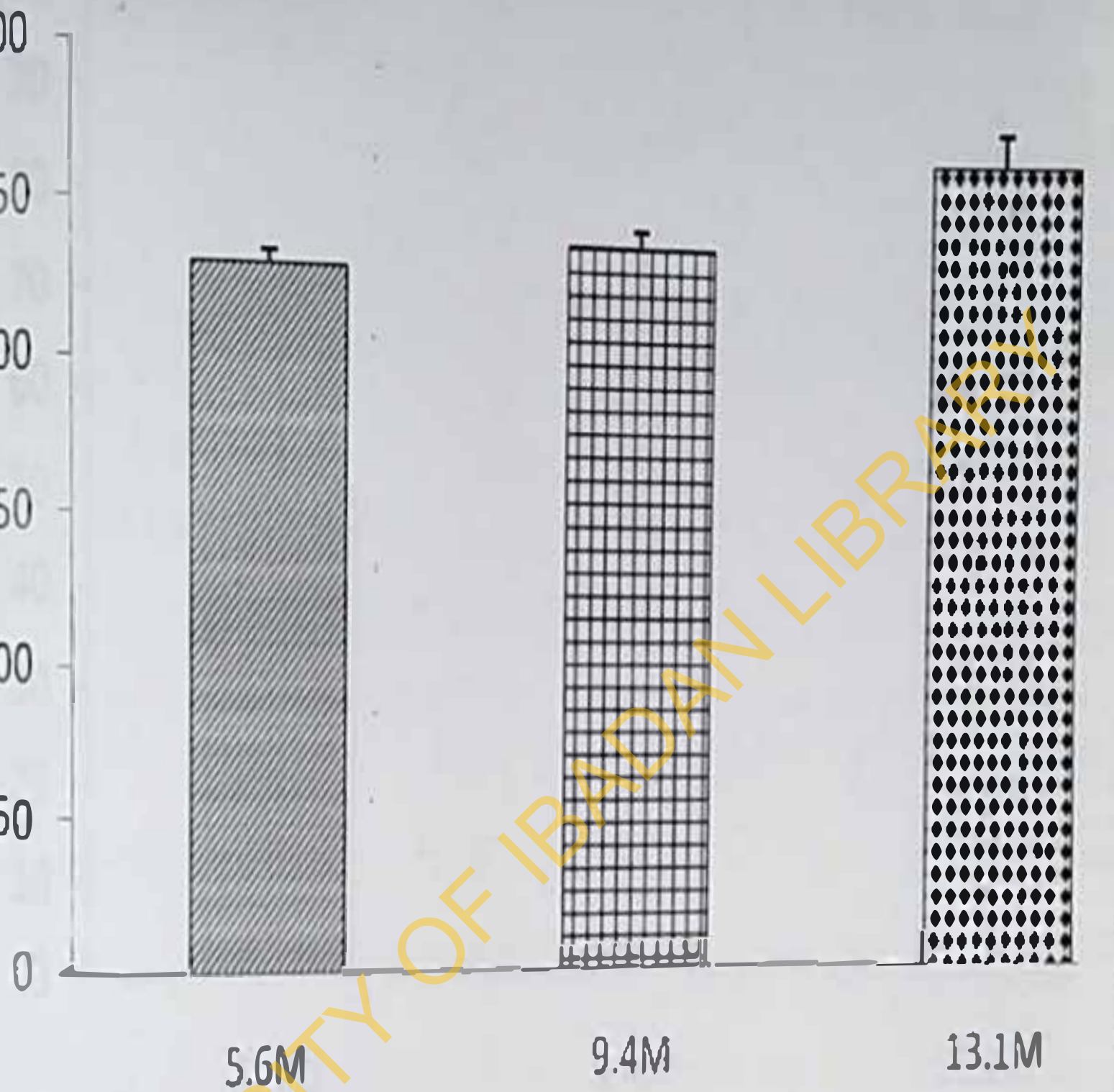

Fig 4.3: Mean Glucose Yield of VP at different Acid Hydrolysis

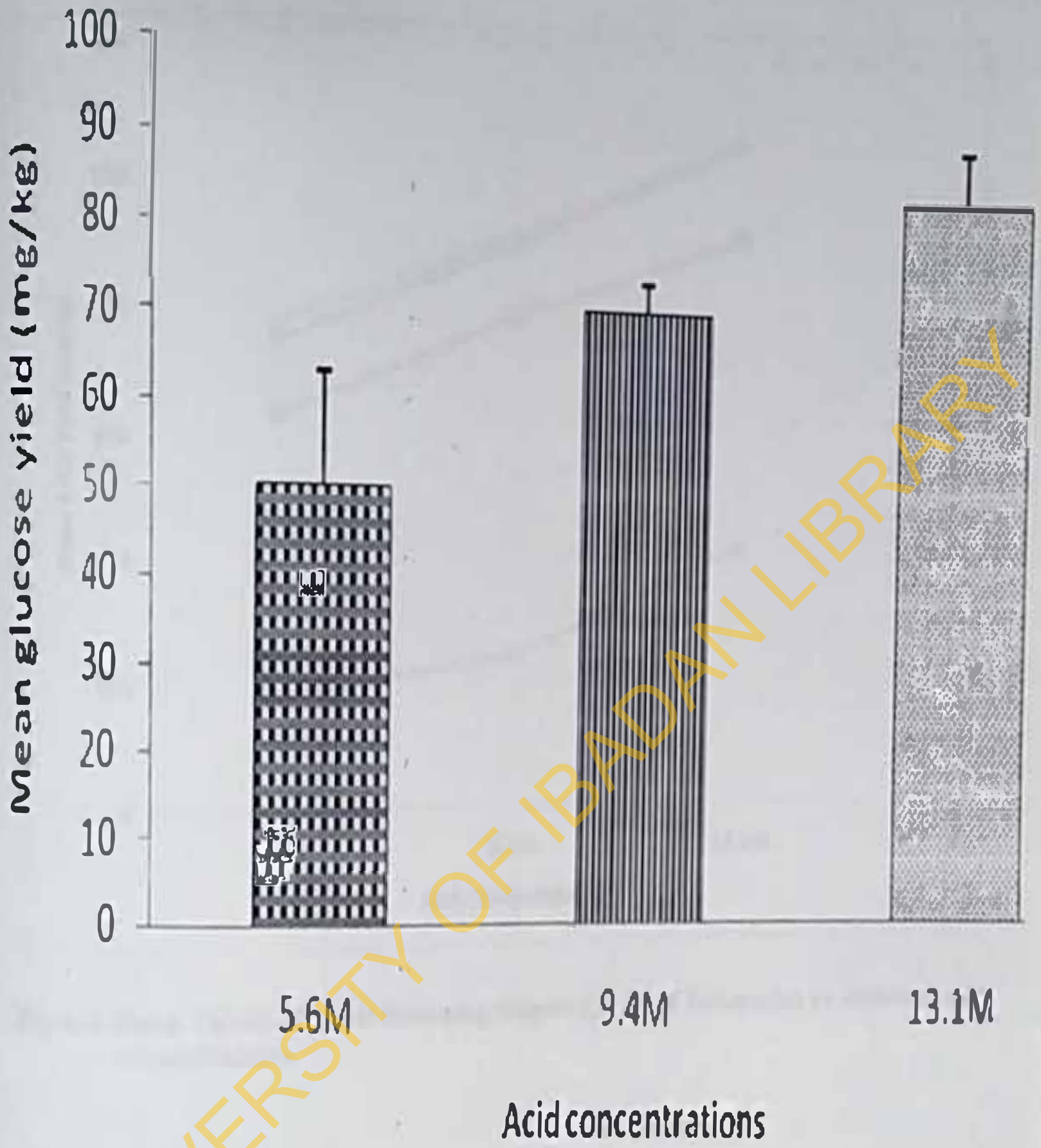

Fig 4.4: Mean Glucose Yield of CP at different Acid Hydrolysis

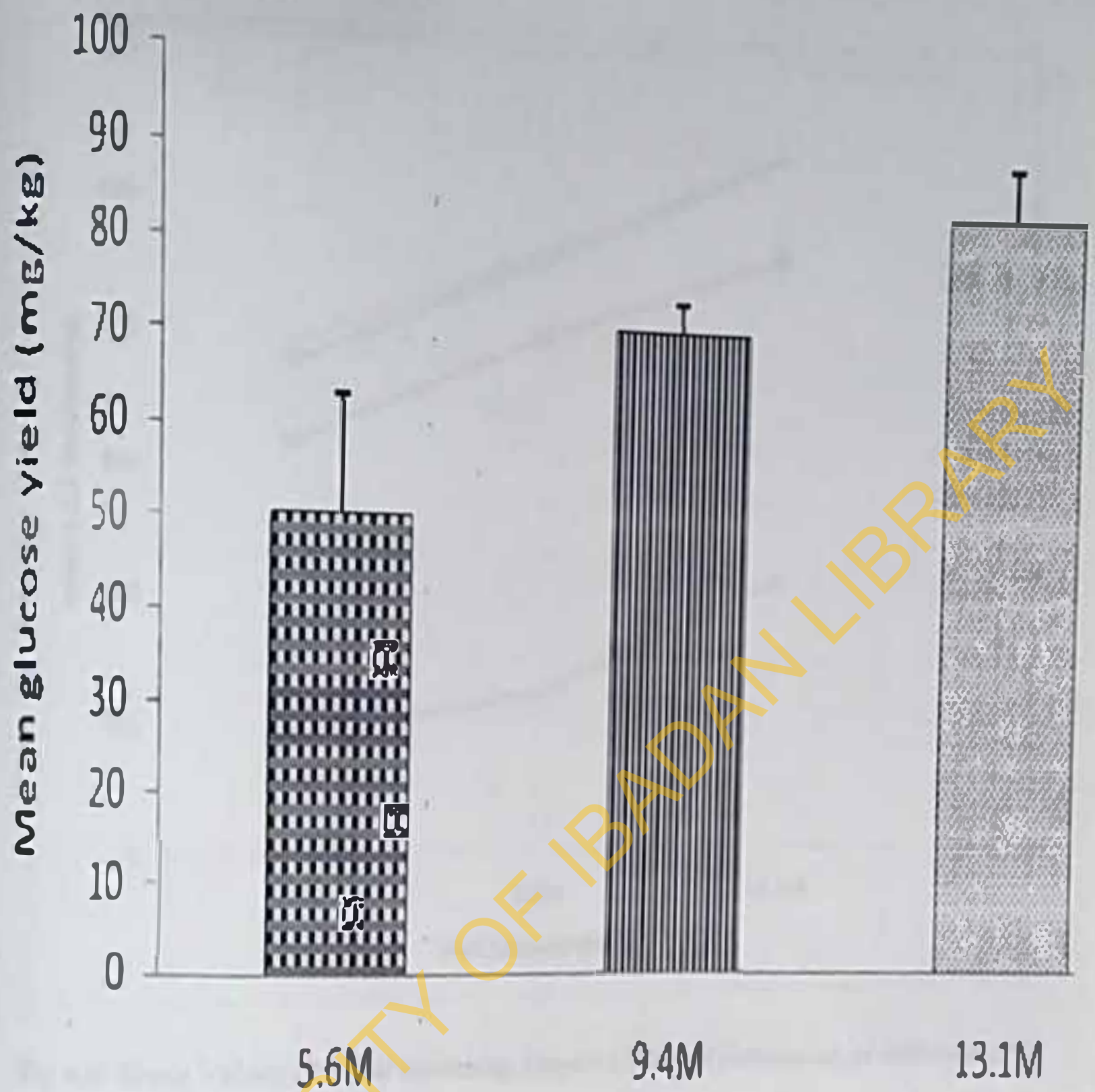

Fig 4.4: Mean Glucose Yield of CP at different Acid Hydrolysis

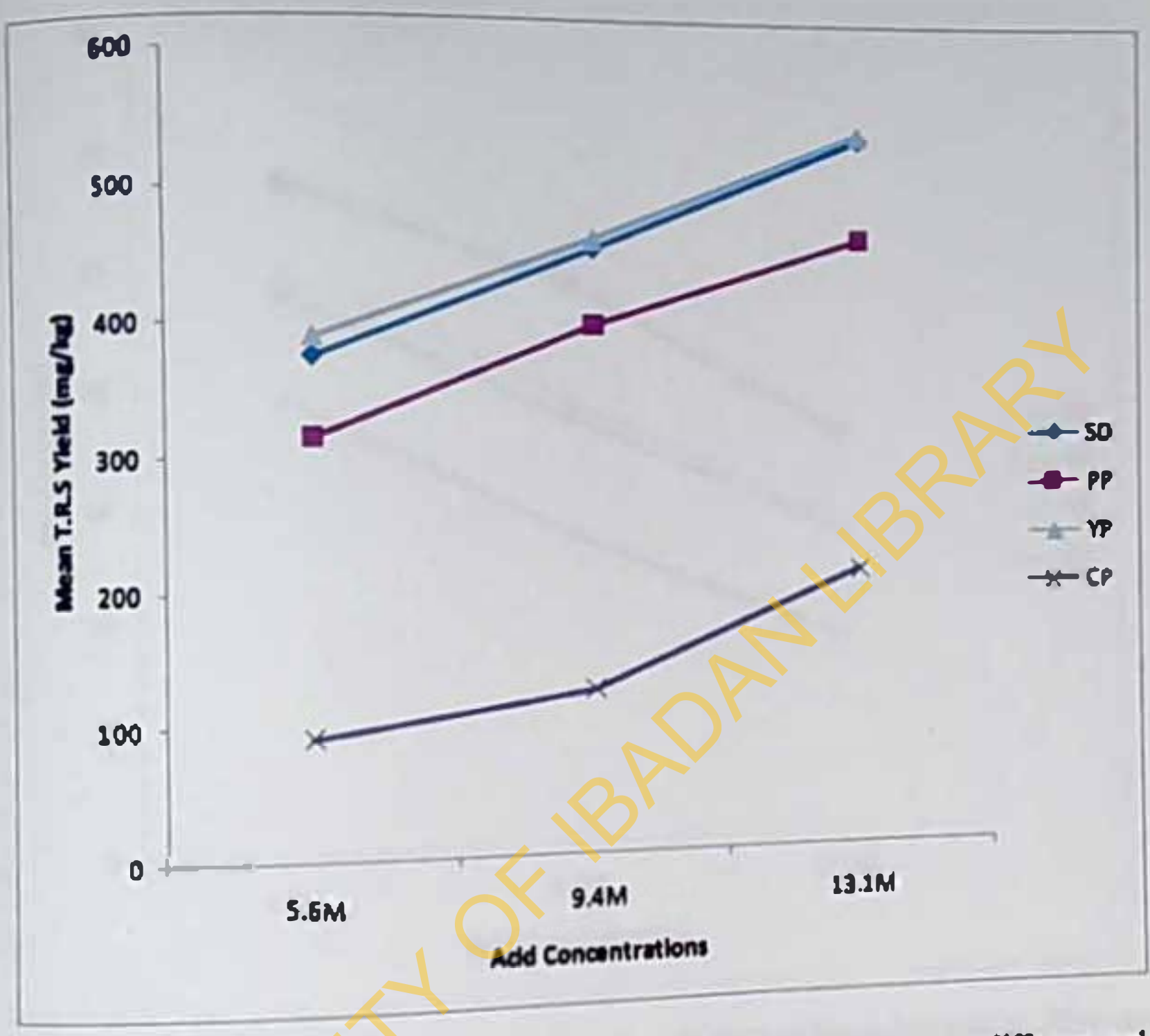

Fig 4.5: Mean Values of Total Reducing Sugars (TRS) of Substrates at different acid concentrations

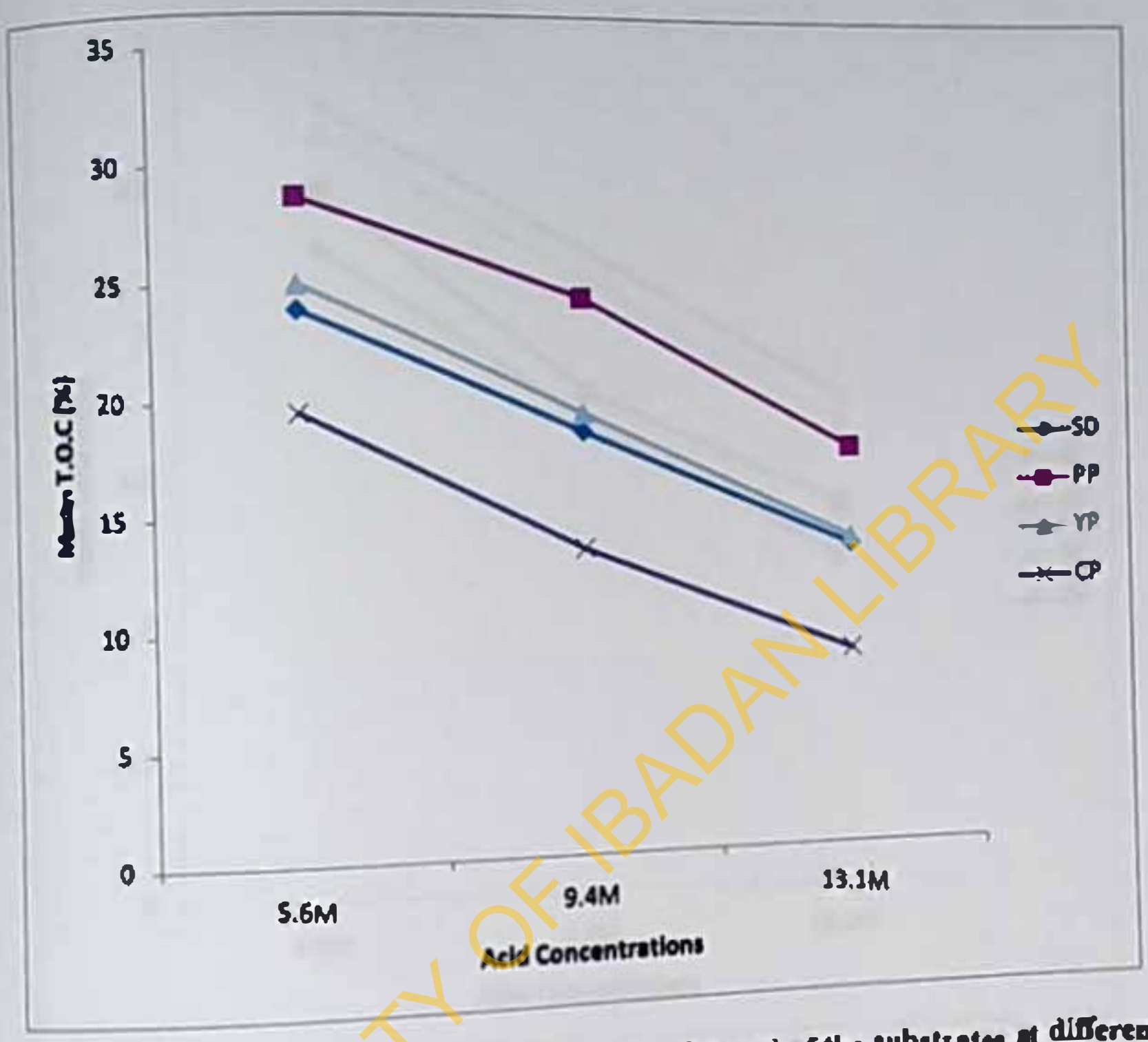

Fig 4.6: Mean Values of Total Organic Carbon (T.O.C) of the substrates at different acid concentrations

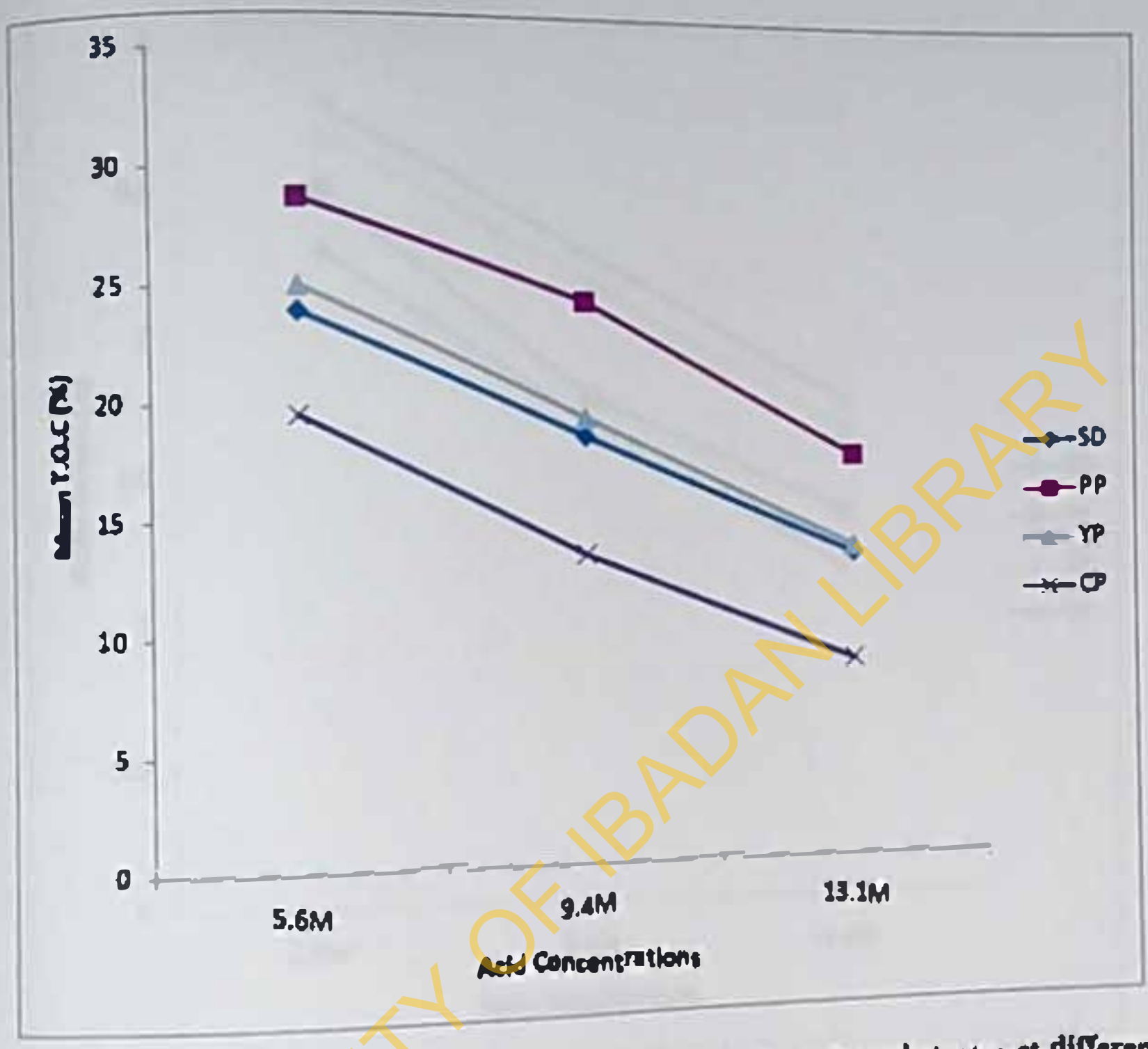

Fig 4.6: Mean Values of Total Organic Carbon (T.O.C) of the substrates at different acid concentrations

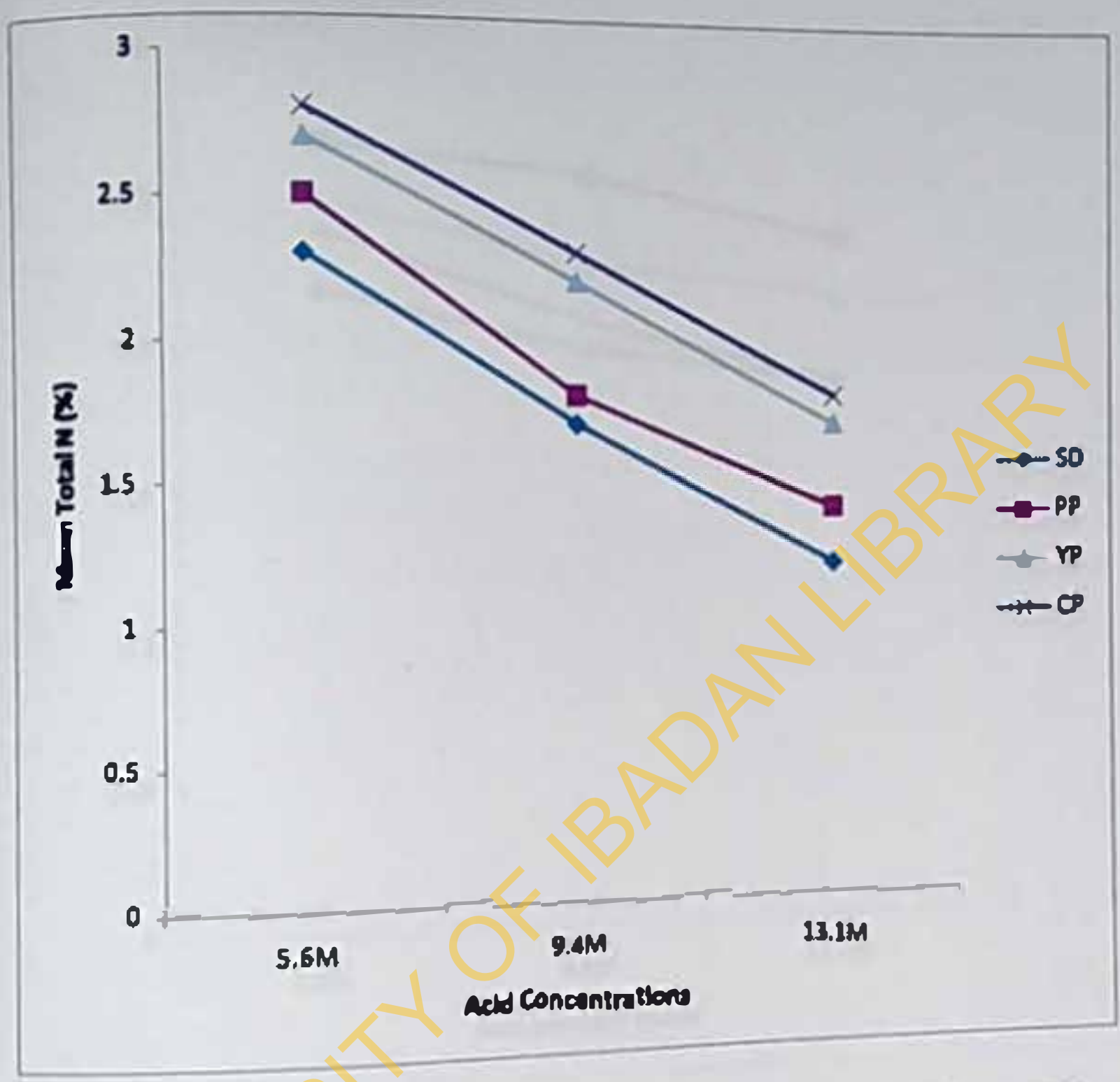

Fig 4.7: Mean Values of Total Nitrogen (%) of the Substrates at different acid concentrations

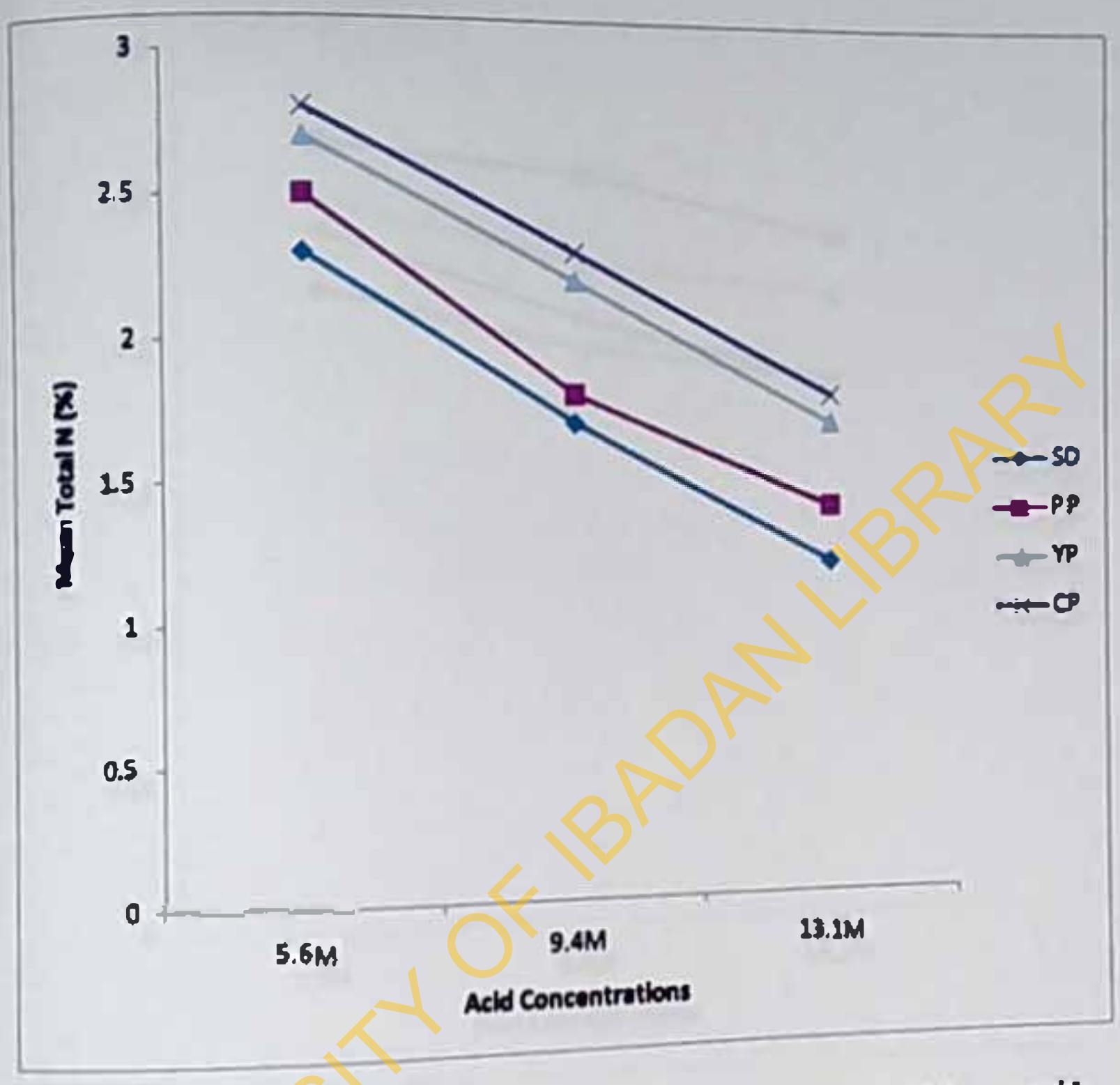

Fig 4.7: Mean Values of Total Nitrogen (%) of the Substrates at different acid concentrations

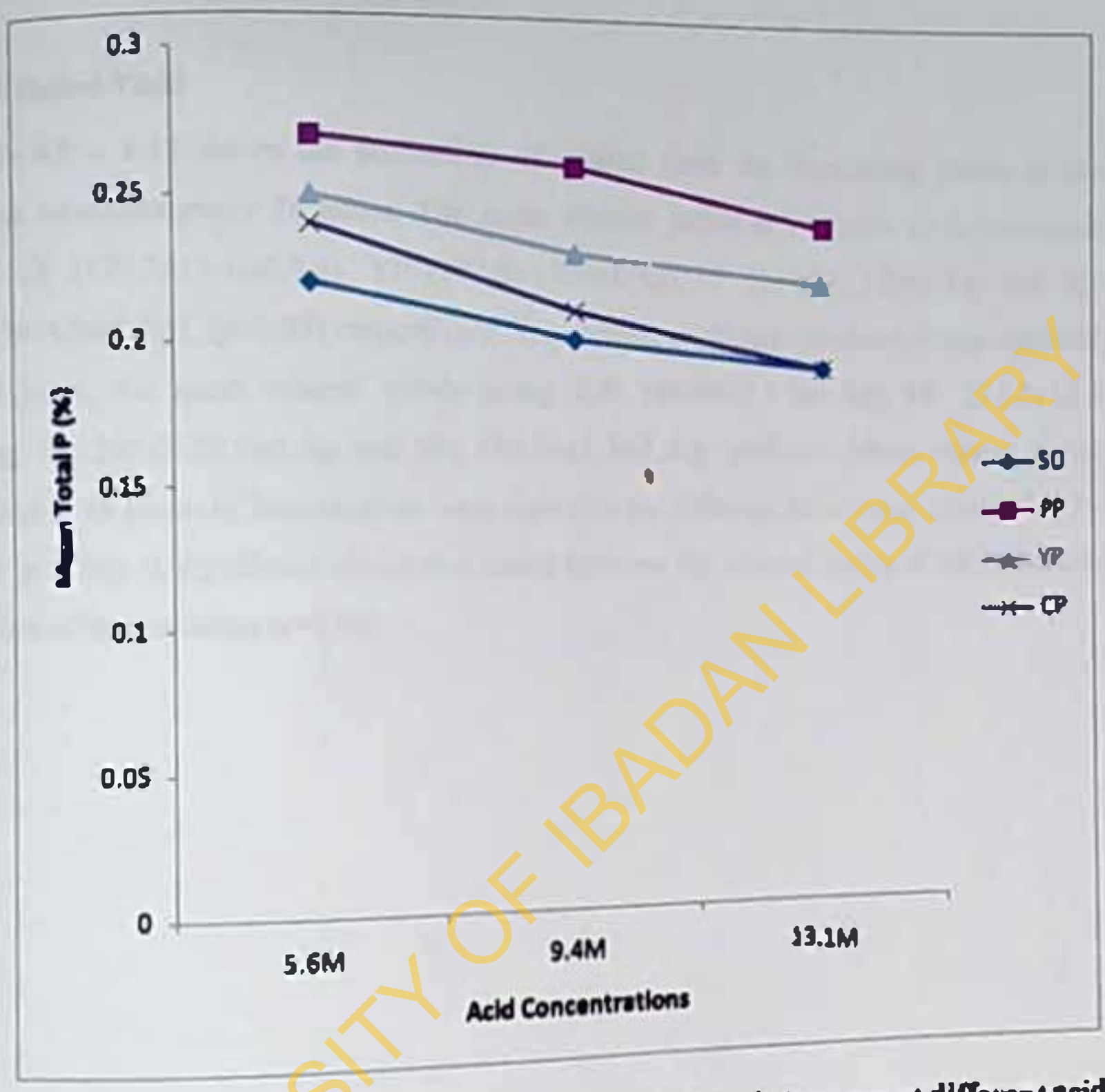

Fig 4.8: Mean Values of Total Phosphorus (%) in the Substrates at different acid concentrations

4.4 Ethanol Yield

Figure 4.9 – 4.12 shows the production of ethanol from the fermenting broths of the various substrates every 24 hours. The mean ethanol yields at 24 hours of fermentation were: CP (123.3±11.1mL/kg), YP (172.0±17.5mL/kg) PP (217.7±13.5mL/kg) and SD (240.3±14.0mL/kg), ($p<0.05$) respectively. The maximum ethanol production was obtained at 48 hours, the mean ethanol yields being: CP: 160.0±15.1 mL/kg; YP: 211.7±15.3 mL/kg; PP: 265.0±20.5mL/kg and SD: 280.0±11.5mL/kg, ($p<0.05$). Mean ethanol yields obtained at 48 hours of fermentation were significantly different from those obtained at 24 hours ($p<0.05$). A significant correlation exists between the ethanol yields of the substrates and time of fermentation ($r=0.95$)

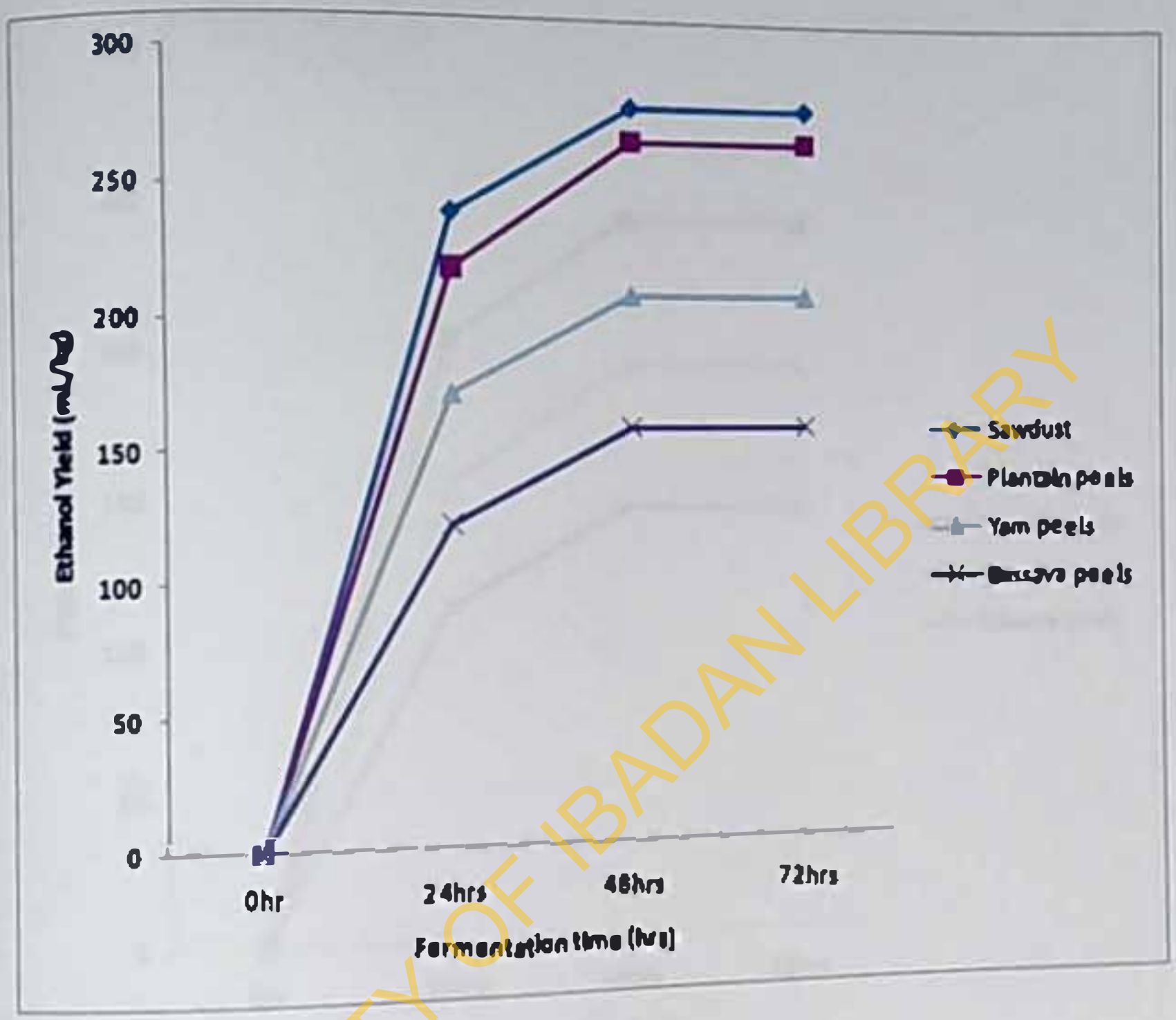

Figure 4.9: Ethanol Production from glucose fermentation during the first experiment.

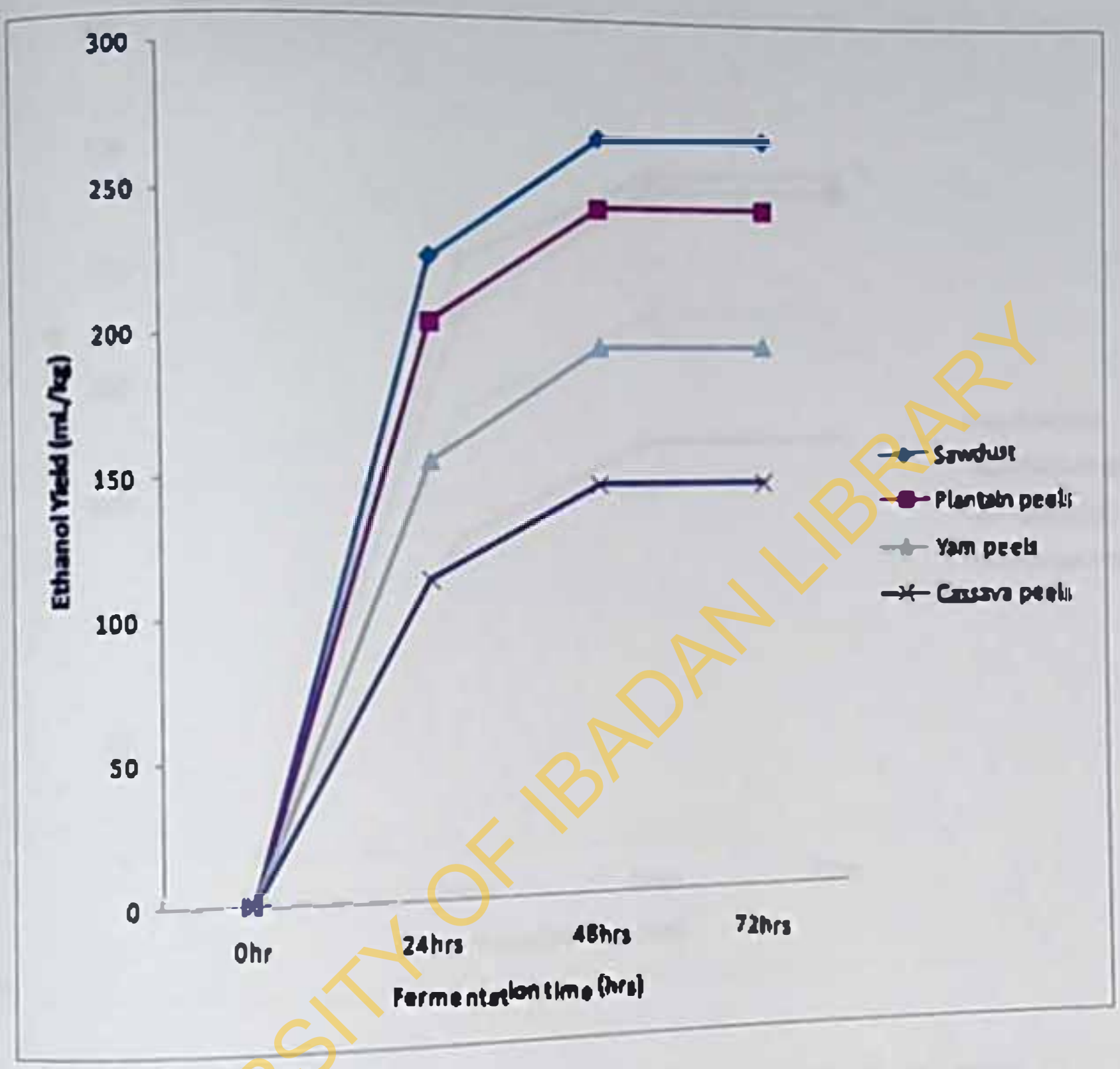

Figure 4.10: Ethanol Production from glucose fermentation during the Second experiment.

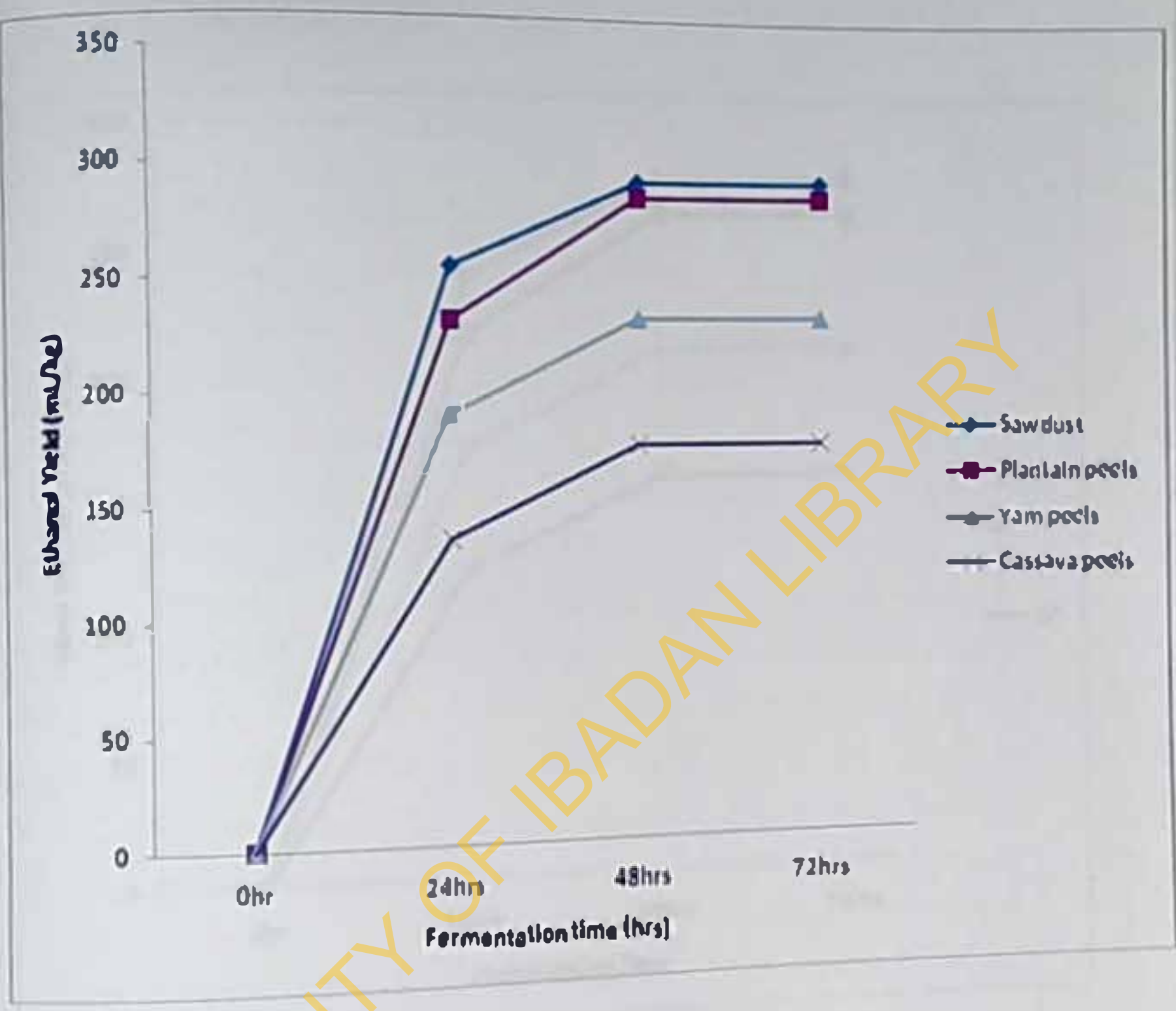

Figure 4.11: Ethanol Production from glucose fermentation during the third experiment.

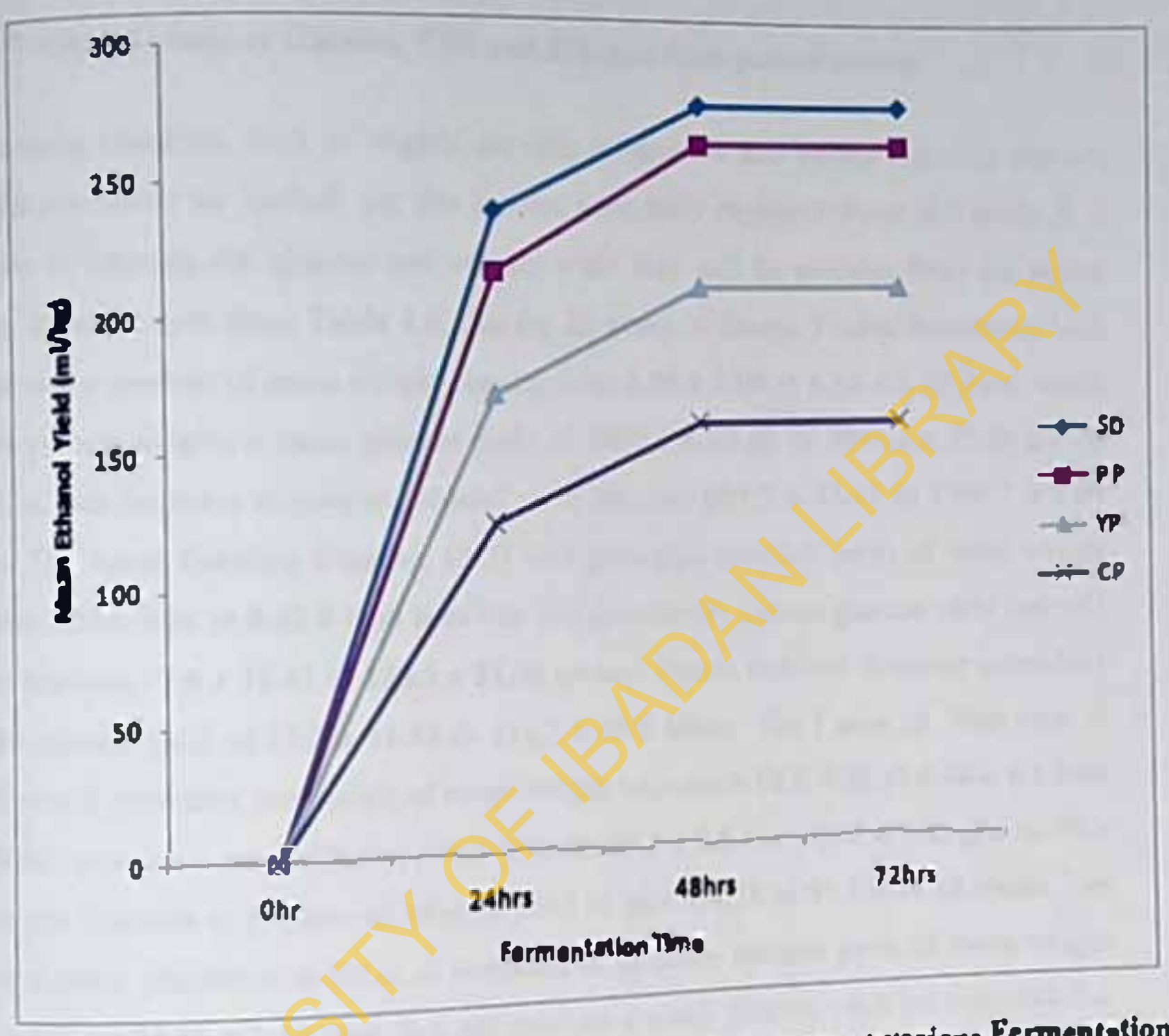

Figure 4.12: Mean Values of Ethanol Yield of the Substrates at various Fermentation Time

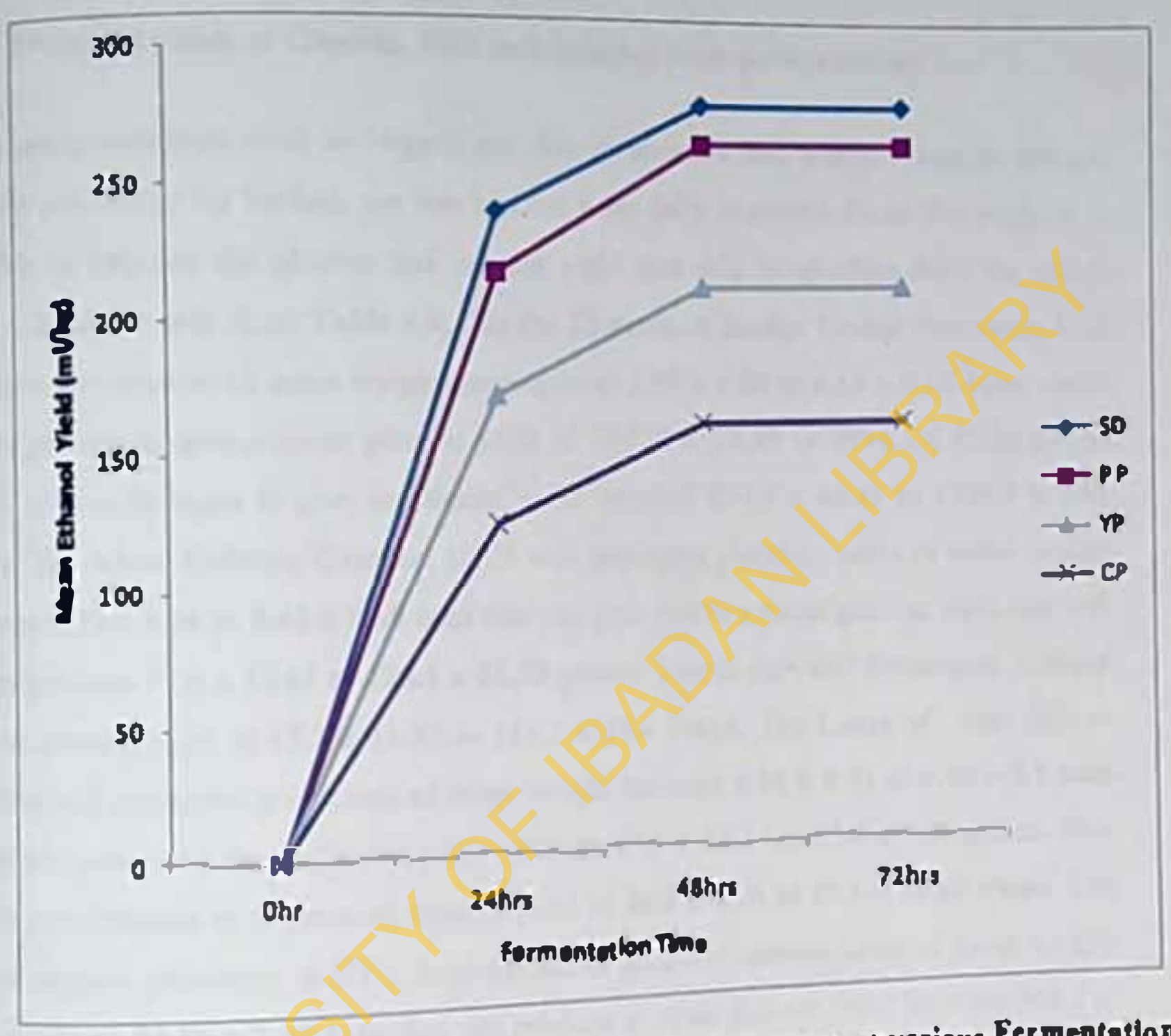

Figure 4.12: Mean Values of Ethanol Yield of the Substrates at various Fermentation Time

4.5 Projected yields of Glucose, TRS and Ethanol from parent source

Developing countries, such as Nigeria are rich in biomass and wastes mat crops that are suitable precursors for biofuel. yet this has not been fully explored. From this study, it is possible to estimate the glucose and ethanol yield that will be produce from the parent source. It can be seen from Table 4.6. that the 25 acres of Bodija Timber Processing Unit will generate sawdust of mean weight ranging from 3.05 ± 0.05 to 6.10 ± 0.10 Tons which can be process to give a mean glucose yield of 10.17.1 ± 28..10 to 209.1.3 ± 57.00 g/tons which in turn ferments to give on ethanol yield between 854.5 ± 43.41 to 1709.1 ± 9.00 l/tons. The Ajose Building Canteen, UCH will generates plaintain peels of mean weight between 0.25 ± 0.04 to 0.42 ± 0.68 tons that can give rise to a mean glucose yield that will ranges between 77.0 ± 12.61 to 128.1 ± 21.32 g/tons. This in turn will ferments to produce a mean ethanol yield of 67.2 ± 11.93 to 111.7 ± 28.6 l/tons. The 1 acre of Yam farm at Abadina will generates, yam peels of mean weight between 0.18 ± 0.01 to 0.46 ± 0.1 tons which will produce a mean glucose yield between 49.3 ± 2.84 to 123.4 ± 7.03 g/tons. This in turn can ferments to produce an ethanol yield of38.9 ± 4.25 to 97.1 ± 10.58 l/tons. The 1ha of cassava plantation nt IITA, is projected to generate cassava peels of mean weight 6.66 ± 0.62 to 23.31 ± 2.16 tons that can produce a mean glucose yield between 562.7 ± 13.95 to 1974.9 ± 57.23 g/tons and this can result in ethanol yield of 1056.5 ± 7.88 to 3708.1 ± 31.50 l/tons.

Table 4.6: Projected mean Value of Glucose yield, Total Reducing Sugar (TRS) yield and Ethanol yield that will be generated from the parent source.

Sample	Mean Weight (Tons) Mean ± SD		Mean Glucose yield (g/ton) Mean ± SD		Mean TRS yield (g/ton) Mean ± SD		Mean Ethanol yield (l/ton) Mean ± SD	
	Min. limit	Max. limit	Min. limit	Max. limit	Min. limit	Max. limit	Min. limit	Max. limit
SD	3.05 ± 0.05	6.10 ± 0.10	1047.1 ± 28.40	2094.3 ± 57.0	1635.2 ± 35.10	3270.5 ± 70.55	854.5 ± 45.41	1709.1 ± 91.0
PP	0.25 ± 0.01	0.42 ± 0.68	77.0 ± 12.61	128.1 ± 21.32	116.8 ± 18.52	177.5 ± 53.15	67.2 ± 11.93	111.7 ± 28.6
YP	0.18 ± 0.01	0.46 ± 0.01	49.3 ± 2.84	123.4 ± 7.03	99.3 ± 3.24	248.1 ± 8.0	38.9 ± 4.25	97.1 ± 10.58
CP	6.62 ± 0.62	23.31 ± 2.16	562.7 ± 13.95	1974.9 ± 57.23	1391.4 ± 99.22	4884.5 ± 368.46	1056.5 ± 7.88	3708.1 ± 31.50

CHAPTER FIVE

DISCUSSION

5.1 Source of Substrates

From the feasibility study carried out on some selected major agricultural centres in the state viz International Institute of Tropical Agriculture (IITA), Bodija Timber Processing Centre, Yam Processing Centre at Abadina Quarters in U.I and Ajose Building Canteen, UCH. It is evident that huge amount of wastes are generated from these production centres. A similar study conducted by Omonijo *et al*., (2007) reported that Ibadan is a state which is known for large scale agricultural activities where huge amount of lignocellulosic "wastes" are generated through forestry and agricultural practices, timber industries and many agro-industries. Due to poor waste management and inadequate infrastructures, these wastes pose serious environmental pollution problem in the state and also constitute great challenge to the state government.

Many sources of lignocellulosic biomass, such as agricultural and forestry residues, pulp and paper streams, and municipal solid waste are abundant and underutilized resources, which can be converted to ethanol. Woody and herbaceous energy crops, such as hybrid poplar and switchgrass can also be used as renewable resources for ethanol production. In the developing countries, biomass accounts for approximately 35% of the affordable source of energy (Mc Gowan 1991; Hall, Rosillo – calle, and de Groot 1992). Dedicated energy crops, grown on our nation's wastelands, could possibly represent one of the largest biomass sources. Advances in plant sciences and process technologies promise to revolutionize production of energy and other products from biomass. However it appears that the first materials used for ethanol production will most probably be waste materials and residues (Kuhad and Singh, 1993).

5.2 Physical Characteristics of Substrates

Lignocellulose is the major structural component of woody plants and non-woody plants such as grass and represents a major source of renewable organic matter. Lignocellulose consists of lignin, hemicelluloses and cellulose. The chemical properties of the components

of lignocellulosic wastes make them a substrate of enormous biotechnological value (Malberbe and Cloete, 2003). Cellulose is the major constituent of organic matter of plant origin. Lignocellulose materials are the most abundant and renewable resources on earth, which makes them attractive for production of ethanol (Zsolt, 2000). It can be seen that wastes left over from these production processes are abundant and still contain a high amount of oligomers and cellulosic materials, which can be used to produced ethanol. The use of lignocellulosic wastes as raw materials in ethanol production not only reduce waste material created from these various agro-based centres, but also lower the cost of ethanol production (Badger, 2002).

Sadly, much of the lignocelluloses waste is often disposed off by biomass burning, which is not restricted to developing countries alone, but it is considered a global phenomenon (Levine, 1996). However the huge amounts of residual plant biomass considered as "waste" can potentially be converted into various different biotechnological value and human nutrient. Biomass is a renewable resource that is virtually inexhaustible and is a potential for alternate fuel production. Worldwide, biomass ranks fourth as an energy resource providing approximately 14% of the world's energy needs.

5.3 Total Organic Carbon (T.O.C), Nitrogen and Phosphorus content of the various biomass

The Total Organic Carbon (T.O.C) refers to all carbon atoms, covalently bonded in organic molecules. From Figure 4.6, the mean T.O.C decreased as the concentration of the acid increased, meaning that at higher concentration of acid, the organic bonded carbon in the substrates/samples were oxidized to carbon dioxide (CO_2) and other inorganic Carbon (IC) such as carbonate, bicarbonate etc.

Among the various substrates, the mean T.O.C was greatest for the PP throughout the various acid concentrations while the least mean T.O.C was found in CP; with the implication that PP had a high quantity of organic bonded carbon in its composition than other wastes. Environmentally, this implies that the natural degradation of these wastes

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contributes a substantial amount of greenhouse gases such as CO_2, CH_4 etc to the environment. This was in agreement with Lal and Reddy (2005) who also reported that natural degradation of lignocellulosic wastes by anaerobic digestion of methanogenic bacteria, generate about 25 millions tons of methane gas annually worldwide.

In terms of Nitrogen (N); it is evident from Table 4.3 – 4.5 and Figure 4.7, that the mean Total Nitrogen (%) increases as the concentration of acid decreases. This implies that at high concentration of acid, most of the nitrogen in the substrates/samples was oxidized and liberated as Ammonia ($-NH_3$) while at low concentration of acid, oxidation of the nitrogen in the sample was less. The mean percentage (%) Nitrogen was found to be greatest for the CP throughout the various acid concentrations and least for the SD. This means that SD has the least amount of organic nitrogen in its content than the other wastes (PP, CP and YP).

Acid hydrolysis at boiling-water temperature converts dissolved and particulate condensed phosphates to dissolved orthosphosphate. The hydrolysis unavoidably releases some phosphate from organic compounds, but this may be reduced to a minimum by judicious selection of acid strength and hydrolysis time and temperature. The term "acid-hydrolyzable phosphate" is preferred over "condensed phosphate" for this fraction. From Figure 4.8 and Table 4.3 – 4.5, the "acid-hydrolyzable phosphate" increased as the concentration of the acid decreased. This showed that there is an inverse relationship between the total phosphorus (%) and acid concentration. The decrease in the Total phosphorus (%) in the form of "dissolved orthosphate" at 13.1M concentration of acid may be attributed to the fact that at high concentration of acid, the dissolved and particulate condensed phosphates, as well as the organic bound phosphate were converted to other products or by excess oxidation by the highly concentrated acid. Throughout the various acid hydrolysis, the mean total phosphorus (%) among the substrates was in the following order: PP > YP > CP > SD.

From the results obtained in the study, it is evident that all the samples had a very low amount of mean Total Nitrogen and Phosphorus (%), through the various acid concentrations. This agrees with Stantets (1993) which reported that lignocellulosic wastes are usually low in nitrogen and phosphorus content.

5.4 Glucose yield and Total Reducing Sugars (TRS) Content of Substrates

Production of glucose from biomass origin is a common process of saccharification employed in the industries. Several authors have studied the kinetics of the hydrolysis of cellulose and lignocelluloses by acid and enzymatic processes.

In our study, the various percentage acid concentration hydrolysis was investigated in order to optimize the sugar production. The results showed that hydrolysis at 13.1M (70%) provided the maximum sugar content in the substrates. This agrees with the concentrated acid technology of using 70% cone H_2SO_4 for sugar production from cellulosic materials developed from Farone and Cuzens (1996a). At higher percentage concentration of H_2SO_4 than 70%, a lot of charring or browning or dehydrating reactions occurred to a varying degree. Similar results at a high acid concentration was reported by Agu *et al.*, (1997) on cassava granted waste (CGW) biomass at 120°C for 30mins and using a high concentration of H_2SO_4 (1-5M) hydrolysis was achieved but with excessive charring or dehydrating reactions. Other chemical reactions reported in previous studies include the formation of furfural from xylose. Furfural was reported to inhibit activities of some glycolytic enzymes particularly dehydrogenase in *S. cerevisiae* for ethanol production (Banerjee *et al.*, 1981).

The findings of this study revealed that hydrolysis at 13.1M H_2SO_4 gave the best glucose and TRS yield for all the substrates when steamed at 100°C for 60mins and 50mins respectively. Jeffries and Lee (1999) also reported autohydrolysis (steam explosion) as an effective pretreatment method for lignocellulose materials for hydrolysis. In fact, Bousaid *et al.*, (1999) reported an increasing glucose concentration in hydrolysate at the severity of steam explosion increases.

Among the substrates, the highest glucose yield was obtained from sawdust. The high amount of glucose yield in sawdust is due to the lignocelluloses content of Hard and Soft wood stem. Cellulose (40-55%), Hemicellulose (24-40%) and Lignin (18-35%) as reported by Betts *et al* (1991); Sun and Cheng, (2002) from which it is produced. Ojumu *et al*.,(2003) also reported that sawdust obtained from the tree Triplochiton scleroxylon contained 69.5 – 80% cellulose and hemicelluloses and 25 – 30% lignin. The high cellulose content of SD is responsible for its high mean glucose yield; since cellulose is a homogenous polymer of glucose. Badmus (2002) also produce glucose from palm tree trunk using auto hydrolysis prior to the acid hydrolysis; he obtained 70% glucose yields from the substrate at 2.0% sulphuric acid concentration.

The lowest amount of mean glucose yield and TRS found in CP can be attributed to its containing cellulose and hemicelluloses at levels of 24.99% and 6.67% (w/w) respectively as reported by Teerapatr *et al*., (2006). This agrees with previous study done by Teerapatr *et al*., (2006) who reported that the maximum reducing sugar of 6.09% (w/v) was recovered from cassava waste after pretreatment with 0.6M H_2SO_4 at 120°C for 30mins. At higher concentrations of H_2SO_4 than 0.6M, the reducing sugar was lower than 6.09%. This suggests that less reducing sugar in the solution may be derived as a result of dehydration or oxidation by sulphuric acid on hydrolytic products.

The high value of mean TRS found in VP, SD and PP may be attributed to the high appreciable amount of hemicelluloses in their composition. Hemicellulose macromolecules are often polymers of pentoses (xylose and arabinose), hexoses (mostly mannose) and a number of sugar acids. Hemicelluloses are of particularly industrial interest since they are readily available bulk source of xylose from which xylitol and furfural can be derived (Roberto *et al*., 2003; Parajo *et al*., 1998). Extensive research has demonstrated that alcoholic fermentation of xylose, the major hemicelluloses derived sugar, unproves the economies of biomass conversion as 70% of the raw material can be expected to convert into ethanol.

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5.5 Ethanol production from substrates

Ethanol produced from cellulosic biomass materials instead of traditional feedstock is known as bioethanol: a carbon-neutral compound. The traditional process of ethanol production is through fermentation of sugars with a species of yeast called *Saccharomyces cerevisiae*. However, the changing needs, energy demands, and technological advances to overcome the general limitations in yeast-based ethanologenic fermentations have led to an exploration of different methods using a broad range of substrates and novel organisms, indigenous or genetically modified. New technologies are being developed that convert the fibrous portion of plant material to bioethanol. These feedstock materials are abundant and inexpensive (Lal, Reddy and Sonali, 2005).

In this present study, the Simultaneous Saccharification and Co-Fermentation (SSCF) was employed which involves the fermentation of both six-carbon hexoses (glucose, mannose, and galactose) and five–carbon pentoses (xylose and arabinose) sugars to ethanol. This is in line with several authors who reported that the Simultaneous Saccharification and Co-Fermentation (SSCF) is superior to the Simultaneous Saccharification and Fermentation (SSF) technology in terms of cost effectiveness, better yields, and shorter processing time (Lynd *et al.*, 2003; Chendel *et al.*, 2007). A complete conversion of glucose and xylose mixture was obtained by a respiratory deficient mutant of *S.diastaticus* co-cultivated with Pichia stipitis in continuous culture (Delgenes, Laplace, and Moletta, *et al.*, 1996).

In terms of ethanol yield, the result showed that SD gave the highest ethanol yield among all the substrates. This may be attributed to its high glucose yield and TRS, since glucose is a precursor for ethanol production. According to Kadam (2002), the total sugar content is important for the ethanol yield; a key economic parameter depending upon the sugar content. The maximum ethanol production was obtained at 48 hours of fermentation for all the substrates and after which the level remained constant. The study outcome of this corrobates previous studies in which different substrates were used to assess the efficiency of the strain *Klebsiella oxytoka* viz., mixed office paper (Brooks and Ingram 1995), sugar cane bagasse (Doran, Aldrich, and Ingram 1994), corn fibre (Moniruzzaman, Dien, Ferrer, *et al.* 1996), and sugar beet pulp (Doran, Cripe, Sutton, *et al.* 2000). The best strains of the

transformants converted 10% glucose and 10% cellobiose into 44.45g/litre of ethanol within 48 hours. Integrating cellulose components like extracellular endoglucanase can reduce the ethanol production costs (Dien, Cotta, and Jeffries 2003). When a comparative study was done, in which galacturonic acid-rich sugar beet pulp was fermented, KO11 produced significantly higher quantities of ethanol due to *E.coli* KO11 affinity for the substrate. Dien, Hospetl, Wyckoff, *et al.*, (1998) developed a novel hexose and pentose utilizing the ethanologenic *E.coli* strain FBR 3 by incorporating the plasmid pLOI297. An ethanol yield of 4.38%-4.66% (w/v) with 90%-91% theoretical conversion in 70-80 hours was achieved. Although several microorganisms, including *Clostridium sp.*, have been considered as ethanologenic microbes, the yeast *S. cerevisiae* and the facultative bacteria *Z .mobilis* have better candidates for the industrial alcohol production (Bothast, Nichols and Dien, 1991). The feedstock typically account for more than one-third of the production costs, thus maximizing the ethanol yield is imperative. A high ethanol yield means using those strains of bacteria that can produce fewer side products and metabolize all major sugars, which typically include glucose, xylose, arabinose, galactose, and mannose (Wiselogel *et al.*, 1996). Ethanologenic bacteria that currently show maximum promise for industrial exploitation are *E.coli, K. oxytoca,* and *Z.mobilis.*

Mixing has an important role in fermentation. The influence of mixing (from 100-110 rpm [revolutions per minute]) on the performance of *Z.mobilis* anaerobic continuous culture was studied. It was found that the biomass yield and ethanol productivity were improved at higher stirring intensities along with a decrease in the by-product formation. Vigorous mixing led to a better coupling between catabolism and anabolism (Toma *et al.*, 2002).

Ethanol production from corn and sugar cane (*Saccharum officinarum*) is a well-established technology, with several plants using these crops located in the mid-western United States and Brazil, respectively. There are upper limits however, to using corn as feedstock as the economics is tied to by-products such as the DDG (distiller's dried grain), the effective marketing of which is problematic due to potential market saturation. Use of lignocellulosic biomass as substrate to make ethanol is also a promising approach, which

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Ethanol production from corn and sugar cane (*Saccharum officinarum*) is a well-established technology, with several plants using these crops located in the mid-western United States and Brazil, respectively. There are upper limits however, to using corn as feedstock as the economics is tied to by-products such as the DDG (distiller's dried grain), the effective marketing of which is problematic due to potential market saturation. Use of lignocellulosic biomass as substrate to make ethanol is also a promising approach, which

does not suffer from these limitations. Although currently, a more expensive approach, it also offers concomitant environmental benefits. Many sources of lignocellulosic biomass, such as agricultural and forestry residues, pulp and paper waste streams, and municipal solid waste are abundant and underutilized resources, which can be converted to ethanol. Woody and herbaceous energy crops, such as hybrid poplar and switchgrass can also be used as renewable resources for ethanol production; researchers at the Oak Ridge National Laboratory (Oak Ridge, Tennessee, USA) studied the logistics and economics of energy crops (Turhollow 1994; Wright 1994; Perlack and Wright 1995; Perlack, Watsh, Wright, *et al.*, 1996). However, it appears that the first materials used for ethanol production will most probably be waste materials and residues (Wyman and Goodman, 1993).

Ethanol is a two carbon alcohol (C_2H_5OH) i.e n monohydric primary alcohol which is isomeric with DME (di-methyl ether). It melts at -117°C and boils at 78.5°C Except for alcohol beverages, nearly nll ethanol is n mixture of 95% nlcohol and 5% water. It is n high performance fuel, which is used in spark ignited internal combustion engines (Lynd *et al*, 2003). It is considerably cleaner, less toxic, also less corrosive (Lal and Reddy,2005; Risch,1998). Biocthanol produced from renewable biomass has received considerable attention in current years. Using ethanol as n gasoline fuel additive, as well as transportation fuel, helps to alleviate globnl warming and environmental pollution. In the last decade, most research has tended to focus on developing an economical and ecofriendly ethanol production process. Much emphasize is being given to the production of ethnnol from ngricultural and forestry residues and forms of lignocellulosic biomass (Kadam *et al.*, 2000).

5.6 Environmental and Health benefits of Biocthanol Production

5.6.1 Environmentnl benefits:

- Ethanol is good for the Environment: Overall, ethanol is considered to be better for the environment than gasoline. Ethanol-fueled vehicle produce lower carbon monoxide and carbon dioxide emissions and the same or lower levels of hydrocarbon and oxides of nitrogen emission. E85 a blend of 85 percent ethanol

and 15 percent gasoline, which means fewer emissions from evaporation. Adding ethanol to gasoline on lower percentages, such as 10 percent ethanol and 90 percent gasoline (E10) reduces carbon monoxide emissions from the gasoline and improves fuel octane rating.

- **Net Reduction in Ground-level Ozone forming Emissions:** Ground – level ozone causes human respiratory problems and damages many plants that but does nothing to increase ozone concentration in the stratosphere that protects the earth from the sun's ultraviolet radiation. There are many compounds that react with sunlight to form ground-level ozone, which in combination with moisture and particulate matter, creates 'smog' the most visible form of air pollution. These compounds include carbon monoxide, unburned hydrocarbons, benzene and nitrogen oxides (nitrous oxide and nitric oxide). Ethanol blended fuel burns with a greater reduction of all these gases.

- **Reduction in harmful Greenhouse Gases:** In comparison to gasoline, ethanols (alcohols) have a higher octane number than gasoline and burns cleaner with a greater efficiency, thus putting less carbondioxide and overall pollution in the air. Additionally, only low levels of smog are produced from its combustion. According to Agarwal (2005) fuels with a higher octane number can endure a higher compression ratio before the engine knocking takes place, this gives the engine an ability to deliver more power, and thus become more powerful, efficient and economical.

5.6.2 **Health benefits include:**

- The used of ethanol help to decreased emissions of benezene, a hydrocarbon classified by the Environmental Protection Agency (EPA) as a known human carcinogen. Benzene accounts for about 70% of the total toxic emissions from vehicles running on conventional gasoline (Gary, 2004). According to the EPA (2000) hazard summary, exposure to benezene can lead to blood disorders,

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including anaemia and higher instances of leukaemia, as well as short – term impacts such as headache and respiratory irritation. Studies have indicated that blending ethanol with gasoline at a 10% rate can reduce benzene emissions by as much as 25%.

- The America Lung Association links the emissions of tailpipe soot and particulate matter to cancer, asthma and heart attacks; thus ethanol blending plays an important role in improving Public Health (Jacobson, 2007). Replacing gasoline with ethanol also reduces emissions of butadiene, a probable human carcinogen and formaldehyde, a toxic contaminant.
- The conversion of cellulosic wastes into bioethanol helps to prevent leachates and runoff from these wastes to percolates through the soil thereby causing pollution of the underground waters. Pollution of underground water cause both acute and long-term effects on the human health. Acute effects includes GIT infections, cerebrospinal menegitis etc while chronic effects are carcinogenic which includes carcinoma of the liver and skin, foetal abnormalities. The bioconversion of these wastes into ethanol also helps to create a friendly environment by preventing stress related to odour, noise, vermin and visual amenity (Dolk, 2002).

5.7 Climate benefits

The term 'climate change' refers to a wide range of changes in weather patterns that result from global warming. A substantial increase in the Earth's average temperature could result in a change in agricultural patterns and melting of polar ice caps, raising sea levels and causing flooding of low lying coastal areas. Under current conditions, use of ethanol-blended fuels as E85 (85% ethanol and 15% gasoline) can reduce the net emissions of greenhouse gases (CH_4, CO, CO_2 & NOx) by as much as 37.1 %. Ethanol-blended fuel as E10 (10% ethanol and 90% gasoline) reduces greenhouse gases by up to 3.9 % (Gary, 2004). It is expected that once ethanol is made from cellulose, the greenhouse gas emissions reductions will further improve.

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5.8 Sustainability of the Production Process

In the late 1970's, the first major oil crisis occurred; the need for renewable liquid fuel such as ethanol was recognized (Lichts, 2006). Ethanol is nowadays an important product in the fuel market. Its market grew from less than a billion litre in 1975 to more than 39 billion litres in 2006 and is expected to reach 100 billion litres in 2015 (Lichts, 2006).

Lignocellulosic materials are renewable, largely unused and abundantly available sources of raw materials for the production of fuel ethanol. Lignocellulose materials can be obtained at low cost, from a variety of resources (Wyman, 1996). These materials contain sugar polymerized in form of cellulose and hemicelluloses which can be liberated by hydrolysis and subsequently fermented to ethanol by microorganisms (Millati *et al.*, 2002; Palmquist and Hahn-Hagerdal, 2000). Based on a review of literature, it is estimated that ethanol yields from lignocellulosics will range between 0.12 – 0.32 L/Kg undried feedstock depending upon the efficiency of the five carbon sugar conversion (Wingren *et al.*, 2003).

In countries with surplus of agricultural capacity, ethanol produced from biomass may represent a sensible substitute, extender or octane booster for traditional motor fuel. While sugar-based raw materials such as cane juice or molasses can be fermented directly, this is not possible for starch based raw materials. They have to be converted to fermentable sugar first. Although the equipment is different, the principle of using enzymes to produce fuel alcohol is similar to that for producing portable alcohol.

Many traditional chemical processes based on acid or base catalysed reactions for processing of agricultural products have inherent drawbacks from a commercial and environmental point of view. Non specific reactions may result in poor product yields. High temperatures and high pressures needed to drive reactions may lead to high cost and requirement of large volumes of cooling water downstream. Harsh, and hazardous processes involving high temperatures, pressures, acidity or alkalinity need high capital investment and specially designed equipment and control systems. Unwanted by-products

may prove difficult or costly to dispose off. High chemical and energy consumption and harmful by-products have a negative impact on the environment. The use of enzymes may virtually eliminate these drawbacks within the non – food as well as within the food area. Over the last 8–10yrs, new enzymes systems have been developed for the bioethanol industry (Sejrolsen and Schafer, 2006). To minimize investment and operating cost novel enzyme system have been developed.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The purpose of this study was to investigate the ethanol yielding capacity of some selected lignocellulosic based wastes. The results show that sawdust produced the highest glucose and ethanol yield among the substrates. Therefore ethanol production from sawdust should be fully optimized.

Bioconversion offers a cheap and safe method of not only disposing the agricultural residues. but also it has the potential to convert lignocellulosic waste into usable forms such as reducing sugars that could be used for ethanol production. Hence the conversion of lignocellulosic "wastes" into biofuel such as ethanol will help reduce environmental pollution, contribute toward the mitigation of greenhouse gases emissions and serve as a sustainable solid waste management strategy.

Incentives available across the globe for investments in ethanol research and production are lucrative enough to make ethanol available at a cheaper price. However, this may be a buiden economically for many countries. Hence, there is a need to put research efforts into more focused goal of ethanol production from cheaper and feasible agricultural wastes rather than from corn, sugar cane molasses, sugar beet, etc., which in fact, contribute greatly to inflating the cost of ethanol production apart from the cost of enzymes to be added for hydrolysis of the complex carbonhydrates.

Conclusively, the utilization of lignocellulosic wastes in biotechnological process has cut across wide range of product which has potentiol for industrial application and commercialization. However, the economic viability depends solely on efficient use of the agro-waste. The sensonal harvesting of the parent source of these wastes remained an impediment to constant availability of the feedstock for bio-ethanol production thereby damping its viability for industrial production of the renewable bio-fuel. In addition, the present environmental legislation in most developing countries is not efficient for effective collection of these agro-based residues from major producers. If such situation prevails for

long it may remain a lifetime challenge for commercialization of bio-ethanol from the waste.

Utilization of agro-based wastes for the production of bio-ethanol serves a dual benefit: wastes reduction strategy and mitigation of greenhouse gases emissions. The following recommendations are suggested at individual, researcher and government levels:

6.2 Recommendations

Individual level

Individuals should try to minimize waste generation and mitigate greenhouse gases emissions, which could be achieved through the following ways:

- Public enlightment and health education should be undertaken to educate individuals/community on environmental and health hazards associated with indiscriminate disposal of wastes.
- Dissuade people from the opening burning of biomass.
- Vehicles owners should be encouraged to use ethanol blended fuel in their automobiles like in most other countries such as Brazil, China, Canada etc.
- Waste minimization and segregation at point source should be encouraged by keeping all agro-based wastes in a specially coated container for easy collection and utilization by designated agencies and authorities.

Research Institutions/Stakeholders

Research institutions and other stakeholders in energy sectors can contribute towards the sustainability of bio-ethanol production in the following ways:

- Research should be focused in the direction of evolving microbes that can convert the complex lignocelluloses materials to simpler metabolizable sugars and then to ethanol.

- Develop engineering systems with improved activities suitable for industrial-scale application through specially designed equipment and control systems.
- Technological advancements in genomics and proteomics areas that will be able to overcome the feedstock inhibition of the hydrolyzed products, non-specific reactions and harmful by-products.
- Develop automobile engines that will be able to work efficiently using ethanol blended gasoline.

For Government

Based on the findings of this study, it is recommended that the Federal Government should as a matter of urgency properly address the problem of solid waste management and climate change, as follows:

- Encourage the suitable bioconversion processes of agro wastes on a wider scale.
- Promoting urban intensification to reduce the need to use private automobile.
- Government should make legislation that will promote the use of ethanol-blended gasoline in all automobiles.
- By facilitating the development and/or expansion of commercial intergrated energy systems.
- Government should give out incentive for waste minimization.
- Political will: Governments should show commitment to the actualization of the goals of the Kyoto's Protocol and other environmental agreements signed by the firstline countries to reduce greenhouse gas productions.
- By adopting ethanol production policy with lignocellulosic wastes as its main feedstocks in response to the global initiative (bio-fuel production).

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APPENDICES

Appendix 1: Experiment 1

Table 1.1: Volume of Hydrolysates obtained from the acid hydrolysis of the various substrates

Acid Hydrolysis	Substrate Type	1st hydrolysis	2nd hydrolysis	Total volume (ml)
5.6M	YP	30	125	155
	CP	50	128	178
	PP	75	110	185
	SD	25	98	123
9.4M	YP	52	128	180
	CP	76	100	176
	PP	92	98	190
	SD	50	90	140
13.1M	YP	50	122	172
	CP	110	125	235
	PP	15	100	115
	SD	5	85	95

Table 1.2: Determination of Total Organic Carbon (T.O.C)

Acid Concentration	Sample	Weight of sample taken (g)	Titre Value (cm^3)
5.6M	SD hydrolysate	0.1	10.10
	PP hydrolysate	0.1	7.20
	YP hydrolysate	0.1	9.50
	CP hydrolysate	0.1	13.0
9.4M	SD hydrolysate	0.1	13.40
	PP hydrolysate	0.1	9.90
	YP hydrolysate	0.1	13.00
	CP hydrolysate	0.2	16.40
13.1M	SD hydrolysate	0.1	8.56
	PP hydrolysate	0.2	14.19
	YP hydrolysate	0.2	7.95
	CP hydrolysate		14.08

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	CP	76	100	176
	PP	92	98	190
	SD	50	90	140
13.1M	YP	50	122	172
	CP	110	125	235
	PP	15	100	115
	SD	5	85	95

Table 1.2: Determination of Total Organic Carbon (T.O.C)

Acid Concentration	Sample	Weight of sample taken (g)	Titre Value (cm^3)
5.6M	SD hydrolysate	0.1	10.10
	PP hydrolysate	0.1	7.20
	YP hydrolysate	0.1	9.50
	CP hydrolysate	0.1	13.0
9.4M	SD hydrolysate	0.1	13.40
	PP hydrolysate	0.1	9.90
	YP hydrolysate	0.1	13.00
	CP hydrolysate	0.2	16.40
13.1M	SD hydrolysate	0.1	8.56
	PP hydrolysate	0.2	14.19
	YP hydrolysate	0.2	7.95
	CP hydrolysate		14.08

Table 1.3: Determination of Total Phosphorus (%)

Acid Concentration	Sample	Absorbance reading (470nm)	ppm P
5.6M	SD hydrolysate	0.097	2206.75
	PP hydrolysate	0.119	2707.25
	YP hydrolysate	0.110	2502.5
	CP hydrolysate	0.106	2411.5
9.4M	SD hydrolysate	0.089	2024.75
	PP hydrolysate	0.114	2593.50
	YP hydrolysate	0.101	2297.75
	CP hydrolysate	0.092	2093.00
13.1M	SD hydrolysate	0.082	1865.50
	PP hydrolysate	0.105	2388.75
	YP hydrolysate	0.095	1979.25
	CP hydrolysate	0.087	1979.25

Table 1.4: Determination of Total Nitrogen (%)

Acid Concentration	Sample	Titre Value (cm^3)
5.6M	SD hydrolysate	3.30
	PP hydolysate	3.50
	YP hydrolysate	3.80
	CP hydrolysate	4.00
9.4M	SD hydrolysate	2.50
	PP hydrolysate	2.70
	YP hydrolysate	3.00
	CP hydrolysate	3.20
13.1M	SD hydrolysate	1.71
	PP hydrolysate	2.03
	YP hydrolysate	2.33
	CP hydrolysate	2.61

Table 1.5: Determination of Glucose Yield (GY) and Total Reducing Sugars (TRS)

Acid Concentration	Sample	Absorbance GY(490nm)	Glucose concentration (mg/ml)	Absorbance TRS (490nm)	TRS Concentration (mg/ml)
5.6 M	SD	0.119	45.7	0.156	60
	PP	0.107	41.1	0.132	50.7
	YP	0.096	36.9	0.162	62.2
	CP	0.027	10.4	0.038	14.6
9.4M	SD	0.125	48.0	0.188	72.2
	PP	0.114	43.8	0.165	63.3
	YP	0.100	38.4	0.191	73.3
	CP	0.029	11.1	0.052	20
13.1M	SD	0.143	54.89	0.224	86
	PP	0.127	48.80	0.192	73.70
	YP	0.113	43.40	0.227	87.1
	CP	0.035	13.44	0.087	33.4

Table 1.6: Volume of Sugar solution obtained from the 13.1M acid hydrolysis and subjected to hydrolysis

Substrate	Volume of Sugar solution (ml)
SD	130
PP	140
YP	110
CP	190

Table 1.5: Determination of Glucose Yield (GY) and Total Reducing Sugars (TRS)

Acid Concentration	Sample	Absorbance GY(490nm)	Glucose concentration (mg/ml)	Absorbance TRS (490nm)	TRS Concentration (mg/ml)
5.6 M	SD	0.119	45.7	0.156	60
	PP	0.107	41.1	0.132	50.7
	YP	0.096	36.9	0.162	62.2
	CP	0.027	10.4	0.038	14.6
9.4M	SD	0.125	48.0	0.188	72.2
	PP	0.114	43.8	0.165	63.3
	YP	0.100	38.4	0.191	73.3
	CP	0.029	11.1	0.052	20
13.1M	SD	0.143	54.89	0.224	86
	PP	0.127	48.80	0.192	73.70
	YP	0.113	43.40	0.227	87.1
	CP	0.035	13.44	0.087	33.4

Table 1.6: Volume of Sugar solution obtained from the 13.1M acid hydrolysis and subjected to hydrolysis

Substrate	Volume of Sugar solution (ml)
SD	130
PP	140
YP	110
CP	190

Table 1.7: Determination of Ethanol yield

	24 hrs of Fermentation		48 hrs of Fermeotation	
Fermenting broth	Titre value (ml)	Ethanol Concentration (mg/ml)	Titre value (ml)	Ethanol Concentration (mg/ml)
SD	4.82	29	1.38	33.90
PP	7.90	24.60	4.12	30.00
YP	7.97	24.50	4.22	29.84
CP	18.05	10.13	15.93	13.14

Appendix 2: Experiment 2

Table2.1: Volume of Hydrolysates obtained from the acid hydrolysis of the various substrates

Acid Hydrolysis	Substrate Type	1st hydrolysis	2nd hydrolysis	Total volume (ml)
5.6M	YP	28	120	148
	CP	52	132	184
	PP	70	113	183
	SD	30	104	134
9.4M	YP	60	125	185
	CP	75	100	175
	PP	95	102	197
	SD	47	95	142
13.1M	YP	60	120	180
	CP	108	130	238
	PP	20	105	125
	SD	10	93	103

Table 2.2: Determination of Total Organic Carbon (T.O.C)

Acid Concentration	Sample	Weight of sample taken (g)	Titre Value (cm^3)
5.6M	SD hydrolysate	0.1	9.40
	PP hydrolysate	0.1	6.14
	YP hydrolysate	0.1	8.40
	CP hydrolysate	0.1	11.90
9.4M	SD hydrolysate	0.1	12.63
	PP hydrolysate	0.1	8.90
	YP hydrolysate	0.1	12.31
	CP hydrolysate	0.1	16.10
13.1M	SD hydrolysate	0.2	9.25
	PP hydrolysate	0.1	13.20
	YP hydrolysate	0.2	6.45
	CP hydrolysate	0.2	13.30

Table 2.3: Determination of Total Phosphorus (%)

Acid Concentration	Sample	Absorbance reading (470nm)	ppm P
5.6M	SD hydrolysate	0.1011	2300.25
	PP hydrolysate	0.1187	2700.425
	YP hydrolysate	0.1099	2500.225
	CP hydrolysate	0.097	2206.75
9.4M	SD hydrolysate	0.1055	2400.125
	PP hydrolysate	0.1275	2900.625
	YP hydrolysate	0.1187	2700.425
	CP hydrolysate	0.1011	2300.025
13.1M	SD hydrolysate	0.1187	2700.425
	PP hydrolysate	0.1363	2388.75
	YP hydrolysate	0.1275	1979.25
	CP hydrolysate	0.1231	1979.25

Table 2.4: Determination of Total Nitrogen (%)

Acid Concentration	Sample	Titre Value (cm^3)
5.6M	SD hydrolysate	3.56
	PP hydolysate	3.73
	YP hydrolysate	4.13
	CP hydrolysate	4.43
9.4M	SD hydrolysate	2.61
	PP hydrolysate	2.77
	YP hydrolysate	3.43
	CP hydrolysate	3.36
13.1M	SD hydrolysate	1.83
	PP hydrolysate	2.16
	YP hydrolysate	2.50
	CP hydrolysate	2.70

Table 2.5: Determination of Glucose Yield (GY) and Total Reducing Sugars (TRS)

Acid Concentration	Sample	Absorbance GY(490nm)	Glucose concentration (mg/ml)	Absorbance TRS (490nm)	TRS Concentration (mg/ml)
5.6 M	SD	0.117	44.9	0.153	58.7
	PP	0.104	39.9	0.129	49.5
	YP	0.095	36.5	0.159	61.0
	CP	0.017	6.53	0.037	14.2
9.4M	SD	0.122	46.80	0.187	71.8
	PP	0.114	43.8	0.163	62.6
	YP	0.098	37.6	0.190	72.9
	CP	0.029	11.1	0049	18.80
13.1M	SD	0.141	54.13	0.221	84.84
	PP	0.124	47.60	0.191	73.30
	YP	0.107	41.10	0.222	85.2
	CP	0.033	12.7	0.086	33.01

Table 2.6: Volume of Sugar solution obtained from the 13.1M acid hydrolysis and subjected to hydrolysis

Substrate	Volume of Sugar solution (ml)
SD	128
PP	135
YP	108
CP	179

Table 2.7: Determination of Ethanol yield

	24 hrs of Fermentation		48 hrs of Fermentation	
Fermenting broth	Titre value (ml)	Ethanol Concentration (mg/ml)	Titre value (ml)	Ethanol Concentration (mg/ml)
SD	5.52	28	1.91	33.13
PP	8.46	23.80	5.17	28.50
YP	9.30	22.60	5.19	28.46
CP	18.16	9.97	16.11	12.90

Appendix 3: Experiment 3

Table 3.1: Volume of Hydrolysates obtained from the acid hydrolysis of the various substrates

Acid Hydrolysis	Substrate Type	1st hydrolysis	2nd hydrolysis	Total volume (ml)
5.6M	YP	33	125	158
	CP	48	131	179
	PP	72	108	180
	SD	28	100	128
9.4M	YP	58	127	185
	CP	73	105	178
	PP	97	100	197
	SD	52	93	145
13.1M	YP	57	125	182
	CP	112	128	240
	PP	28	107	135
	SD	12	103	115

Table 2.6: Volume of Sugar solution obtained from the 13.1M acid hydrolysis and subjected to hydrolysis

Substrate	Volume of Sugar solution (ml)
SD	128
PP	135
YP	108
CP	179

Table 2.7: Determination of Ethanol yield

	24 hrs of Fermentation		48 hrs of Fermentation	
Fermenting broth	Titre value (ml)	Ethanol Concentration (mg/ml)	Titre value (ml)	Ethanol Concentration (mg/ml)
SD	5.52	28	1.91	33.13
PP	8.46	23.80	5.17	28.50
YP	9.30	22.60	5.19	28.46
CP	18.16	9.97	16.11	12.90

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5.6M	YP	33	125	158
	CP	48	131	179
	PP	72	108	180
	SD	28	100	128
9.4M	YP	58	127	185
	CP	73	105	178
	PP	97	100	197
	SD	52	93	145
13.1M	YP	57	125	182
	CP	112	128	240
	PP	28	107	135
	SD	12	103	115

Table 3.2: Determination of Total Organic Carbon (T.O.C)

Acid Concentration	Sample	Weight of sample taken (g)	Titre Value (cm^3)
5.6M	SD hydrolysate	0.1	10.7
	PP hydrolysate	0.1	7.93
	YP hydrolysate	0.1	10.19
	CP hydrolysate	0.1	13.58
9.4M	SD hydrolysate	0.1	14.23
	PP hydrolysate	0.1	10.62
	YP hydrolysate	0.1	13.46
	CP hydrolysate	0.1	17.32
13.1M	SD hydrolysate	0.2	9.03
	PP hydrolysate	0.1	14.4
	YP hydrolysate	0.2	9.30
	CP hydrolysate	0.2	15.06

Table 3.3: Determination of Total Phosphorus (%)

Acid Concentration	Sample	Absorbance reading (470nm)	ppm P
5.6M	SD hydrolysate	0.0791	1799.525
	PP hydrolysate	0.1011	2300.025
	YP hydrolysate	0.0923	2099.825
	CP hydrolysate	0.0879	1999.725
9.4M	SD hydrolysate	0.0747	1699.425
	PP hydrolysate	0.1011	2300.25
	YP hydrolysate	0.0879	1999.725
	CP hydrolysate	0.0835	1899.625
13.1M	SD hydrolysate	0.0615	1999.725
	PP hydrolysate	0.0879	1383.75
	YP hydrolysate	0.0791	1799.525
	CP hydrolysate	0.0659	1979.25

Table3.4: Determination of Total Nitrogen (%)

Acid Concentration	Sample	Titre Value (cm^3)
5.6M	SD hydrolysate	3.20
	PP hydolysate	3.37
	YP bydrolysate	3.54
	CP hydrolysate	3.73
9.4M	SD hydrolysate	2.3
	PP hydrolysate	2.47
	YP hydrolysate	2.80
	CP hydrolysate	3.10
13.1M	SD hydrolysate	1.59
	PP hydrolysate	1.96
	YP hydrolysate	2.26
	CP hydrolysate	2.50

Table 3.5: Determination of Glucose Yield (GY) and Total Reducing Sugars (TRS)

Acid Concentration	Sample	Absorbance GY(490nm)	Glucose concentration (mg/ml)	Absorbance TRS (490nm)	TRS Concentration (mg/ml)
5.6 M	SD	0.121	46.5	0.159	61.0
	PP	0.108	41.5	0.133	51.1
	YP	0.109	37.6	0.165	63.3
	CP	0.0109	7.29	0.039	15.0
9.4M	SD	0.129	49.52	0.192	73.7
	PP	0.119	45.70	0.168	54.5
	YP	0.102	39.2	0.194	74.5
	CP	0.031	11.9	0.053	20.35
13.1M	SD	0.145	55.70	0.225	86.4
	PP	0.129	49.52	0.194	74.5
	YP	0.115	44.15	0.228	87.5
	CP	0.038	14.6	0.089	34.2

Table 3.6: Volume of Sugar solution obtained from the 13.1M acid hydrolysis and subjected to hydrolysis

Substrate	Volume of Sugar solution (ml)
SD	134
PP	142
YP	115
CP	205

Table 3.7: Determination of Ethanol yield

	24 hrs of Fermentation		48 hrs of Fermeotation	
Fermenting broth	Titre value (ml)	Ethanol Concentration (mg/ml)	Titre value (ml)	Ethanol Concentration (mg/ml)
SD	4.12	30	1.00	34.13
PP	7.13	25.70	2.92	31.69
YP	6.88	26.05	2.83	31.82
CP	17.89	10.35	15.65	13.54

Table 3.6: Volume of Sugar solution obtained from the 13.1M acid hydrolysis and subjected to hydrolysis

Substrate	Volume of Sugar solution (ml)
SD	134
PP	142
YP	115
CP	205

Table 3.7: Determination of Ethanol yield

	24 hrs of Fermentation		48 hrs of Fermentation	
Fermenting broth	Titre value (ml)	Ethanol Concentration (mg/ml)	Titre value (ml)	Ethanol Concentration (mg/ml)
SD	4.12	30	1.00	34.13
PP	7.13	25.70	2.92	31.69
YP	6.88	26.05	2.83	31.82
CP	17.89	10.35	15.65	13.54