EVALUATION OF UNDER UTILIZED PLANT RHIZOMES AND TUBERS FOR ETHANOL PRODUCTION

A PROJECT REPORT

Submitted by

MUTHU DHARANI R

(Reg. No.1120203006)

in partial fulfillment for the requirement of award of the degree

of

M.TECH. BIOTECHNOLOGY

FACULTY OF TECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY, COIMBATORE 641 049

(An Autonomous Institution Affiliated to Anna University, Chennai)

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BONAFIDE CERTIFICATE

Certified that this project work titled '**EVALUATION OF UNDER UTILIZED PLANT RHIZOMES AND TUBERS FOR ETHANOL PRODUCTION'** is a bonafide work of **Ms. MUTHU DHARANI, R. (Reg. No.1120203006)** who carried out the research under my supervision. Certified further that to the best of my knowledge, the work reported herein does not form part of any other thesis or dissertation, on the basis of which, a degree or award was conferred on an earlier occasion on this or any other students.

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ABSTRACT

The current global energy needs are met by the fossil fuels. Due to modernization and rapid industrialization there is an increase in demand for the fossil fuels globally and they release green house gases that cause irreversible damage to the environment. Hence it is necessary to find an alternative fuel whicsh is sustainable and eco-friendly. Alcohol fermentation is a process of transformation of starchy material and lignocellulosic biomass to alcohol by the action of microorganisms. The economics of ethanol production by fermentation is greatly influenced by the usage of raw materials and the pretreatment used. Ethanol can produced from agricultural residues, animal wastes and other renewable sources. Banana rhizome and Cocoyam tuber are considered as underutilized substrates for ethanol production. It was anaylsed for its chemical composition and various pretreatment like autoclaving, steam and alkali treatment, Enzyme treatment and combined steam and enzyme treatment, dilute acid pretreatment, amylase treatment, amylase and glucoamylase treatment were performed for both the substrates. *Z.mobilis* and *S.cerevesiae* was used in the study. Among the pretreatment methods used, autoclaving method resulted in high alcohol yield. *Z.mobilis* showed high volume of ethanol production for banana rhizome and *S.cerevesiae* showed high volume of ethanol from Cocoyam tuber. Hence, banana

rhizome and Cocoyam tuber could be exploited as potential source for ethanol production.

Keywords: Banana rhizome, Cocoyam tuber, Pretreatment, Autoclaving, Ethanol production.

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(MUTHU DHARANI R)

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LIST OF ABBREVATIONS

min	Minutes
h	Hours
nm	Nano meter
°C	Celsius
mg	Milligrams
g	Grams
N	Normality
rpm	Revolutions per minute
%	Percentage
M	Molar
ml	Milli litre
OD	Optical density
RSM	Response Surface Methodology
SLR	Solid Liquid Ratio
SAC	Sulphuric Acid Concentration
ANOVA	Analysis of Variance
L	Litre
w/v	Weight/ Volume

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

The world is currently facing two environmental problems one of which is energy crises mainly due to advancement in technology and rapid growth in industrial sector and the other is emission of green house gases like Carbondioxide, Methane, Nitrous oxide etc, which causes irreversible damage to the environment and results in global warming. An increase in population and technological advancement resulted in rapid depletion of naturally occurring conventional fossil fuels reserves like coal, petrol etc. The fossil fuel reserves are considered as one of the non-renewable energy resources but they are utilized in a much faster rate than being restored in the environment. Most of the non- renewable energy resources are consumed for transportation purpose. It accounts for nearly 50% of the total energy consumed. In order to meet the demand, importing has become a regular practice which accounts to major part of our requirement of petroleum. Therefore, it is necessary for us to look for an alternate energy sources like solar, wind, biomass, etc.which are not only renewable but also non-petroleum based resources. Since 1970s, considerable amount of research has been carried out to find sources of energy alternative to fossil fuels. One such source is ethanol, which is produced from fermenting sugars present in sugar rich biomass. Ethanol is commercially produced by fermentation of cereal grains, molasses or other materials with high starch and/or sugar contents. The fermentation process involves conversion of sugars to alcohol and carbon dioxide by microorganisms like yeast *Saccharomyces cerevisiae, Zymomonas mobilis*, etc. Raghavendra., (2006)

1.2 ETHANOL PRODUCTION SCENERIO WORLD WIDE

Ethanol is the world's largest and fastest growing source of renewable energy. Besides ecofreindly, it can be manufactured easily and can be blended with petrol. Hence, it is readily accepted by the government, manufactures and consumers. Initially in 1975 ethanol is commercially produced to satisfy the industrial needs and as a beverage for consumption. But as the time progressed all the industries is focused in producing ethanol as a transportation fuel which is shown in the Figure 1.1. By 2010 the usage of ethanol as a fuel has been increased tremendously Trever., (2006).

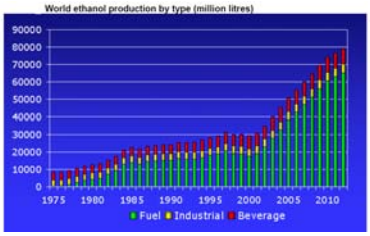

Figure 1.1: Total ethanol production in the world

1.3 INDIAN SCENERIO OF ETHANOL PRODUCTION 2012

In a recent survey report, the US Department of Agriculture (USDA) has said that India's ethanol production has been increased to 2,170 million litres in 2012, compared to against 1,681 million litres in 2011. It was also stated that domestic consumption of ethanol is estimated to rise marginally by 4.5 per cent to 2085 million litres in 2012 from 1995 million litres in 2011. About 880 million litres of ethanol is likely to be used in manufacturing of portable liquor, 720 million litres for industrial use and 400 million litres for blended gasoline in 2012. The ethanol production in India mainly depends on the availability of sugar molasses, a byproduct of sugar production. Out of 330 distilleries in India, the USDA analysed that about 140 distilleries have the capacity to distill around 2 billion litres of conventional ethanol per year(www.economictimes.com).

1.4 ETHANOL PRODUCING STATES IN INDIA

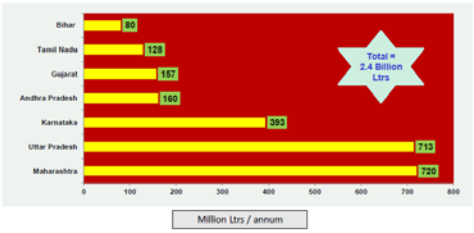

Figure 1.2: Total amount of ethanol produced in India annually.

These 7 states are the major sugar producing states and only ethanol suppliers in India. Ethanol Purchase Price as on 2011= INR 29.78 / L Abhay Chaudhari., (2012).

1.5 DEMAND FOR ETHANOL

The demand for ethanol is classified into three different types namely potable sector, industrial sector and fuel ethanol. The Table 1.1 shows these various demand for ethanol in the forthcoming years.

Table 1.1: Various demand for ethanol.

Figures in billion ltrs

Year	Potable sector demand	Industrial sector demand	Fuel Ethanol Demand @ 5% blending	Total Ethanol demand	Ethanol Demand growth rate (%) CAGR
2011	1.6	0.6	0.9	3.2	10%
2013	2	0.8	1	3.8	
2015	2.6	1	1.2	4.7	
2017	3.1	1.2	1.4	5.7	

As per 20% target, demand = 8.7 Billion Ltrs

Denatured spirit demand is estimated to rise by 10% and Gasoline consumption is estimated to grow by 7-8% per annum.

1.6 TYPES OF ETHANOL

Ethanol can be generally produced in two forms hydrous and anhydrous. Hydrous ethanol has a purity of about 95% and 5% water. Hydrous ethanol can be used as a pure form of fuel in specially modified vehicles. Only Brazil produces vehicles that run on this form of ethanol. Anhydrous alcohol, is also called as absolute alcohol and is produced when the last traces of water are removed. Anhydrous ethanol requires a second-stage process to produce high purity ethanol resulting in 99% purity. This can then be blended with petrol. Anhydrous ethanol is usually blended with 10-25% petrol and is used in most unmodified or slightly modified engines. Trever., (2006).

1.7 ETHANOL BLENDING PROGRAM (EBP)

EBP was initiated with the objective of energy security and reduced green house gas emission.The Ministry of Petroleum and Natural Gas (MoPNG) issued a notification in September 2002 for mandatory blending of 5% ethanol in 9 major sugar producing states and four union territories. However, due to reduced availability of fuel ethanol the policy of mandatory ethanol-blending was made optional. In October 2007, the Group of Ministers recommended to blend 5% Ethanol in petrol across the country, with the exception of J&K, the Northeast and island territories. The blending level of bio-ethanol at 5% with petrol was proposed from October 2008, leading to a target of 20% blending of bio-ethanol by 2017 Abhay Chaudhari., (2012).

1.8 OBSTACLES IN IMPLEMENTATION OF EBP

Molasses is the only feedstock used for fuel ethanol and it is available in 9 states only. While demand for fuel ethanol is spread all over India each state has its own policies and duty structure for ethanol. This puts limitation on movement of fuel ethanol across states. Competitive users of ethanol were not taken into account while going ahead with blending policy & deciding fuel ethanol price. This policy de-motivated the fuel ethanol producers Abhay Chaudhari., (2012).

1.9 BIOFUEL AND REDUCING CARBON FOOTPRINT

The uses of biofuels as a substitute for fossil fuel will result in reduction of green house gas emission. Sugarcane juice, Molasses, Sweet sorghum and other Lignocellulosic materials are considered as a eco-friendly substitute for ethanol production. The Table 1.2 shows the effect of these different biomass in reducing carbon emission.

Table 1.2: Different feedstocks that can be utilized for Bioethanol production.

Type	Feedstock Types	Reduction in Carbon Emission
Bioethanol	Sugarcane Juice	80-90%
	Molasses	70-75%
	Sweet sorghum	80-90%
	Grains (Maize, cereals)	15-30%
	Ligno-Cellulosic Feedstock	70-80%

1.10 VARIOUS SUBSTRATES FOR ETHANOL PRODUCTION

Ethanol obtained from biomass is an attractive and sustainable energy source for transportation fuel to substitute gasoline. The three main raw materials for ethanol production are sugars (from sugarcane, sugar beet, molasses and fruits), starch (from corn, cassava, potatoes and root crops) and cellulosics. First generation of ethanol production uses crops such as sugar cane and corn is well established, whereas second generation ethanol production utilises cheaper and non-food feedstocks like lignocelluloses or municipal solid waste. Production of ethanol from lignocelluloses requires a more complex process compared to first generation ethanol production. These lignocellulosic materials have to be processed in such a way that it is suitable for ethanol production. The lignocellulose is one of the most abundant renewable natural resource and substrate available for conversion into fuels. It meets about two-thirds of the world's energy requirement. The benefits of using biomass is that they are easily available and they do not require high capital investment. The biomass used for ethanol production includes agricultural residues such as corn stover, wheat straw, paddy straw, sugarcane bagasse, forest residues and potential energy crops like switch grass. In Brazil, ethanol is mainly produced from sugarcane (sucrose). Brazil is total energy independent, while in US, ethanol is mainly produced from corn grain (starch). In India, ethanol is primarily produced from molasses which is a by product from sugar cane industry. This is not sufficient to meet all the energy requirement of current scenario. Hence, it is essential to produce ethanol from various feed stocks.

1.11 LIGNOCELLULOSIC ETHANOL PRODUCTION

Lignocellulosic biomass, is the most abundant and low-cost raw material for production of biofuels. The steps involved in transformation of lignocellulosic biomass to bioethanol involves procurement of substrate, pretreatment of substrate, hydrolysis to produce monomeric sugars followed by fermentation. Lignocellulosic biomass such as wheat straw and bagasse are in expensive, and widely available resources that contains 75-80% polysaccharides (cellulose and hemicelluloses). These can be hydrolysed to monomeric sugars such as glucose and xylose. Over the last few years, researches have been focused on converting lignocellulosic materials to bioethanol. Lignocellulosics are composed of heterogeneous complex of carbohydrate polymers like cellulose, hemicelluloses and lignin). Cellulose consists of high molecular weight polymers of glucose rigidly held together as bundles of fibers. Hemicellulose is shorter polymers of various sugars that bind cellulose bundles together. Lignin consists of a tri-dimensional polymer of propyl-phenol that is imbedded in and bound to hemicellulose to provide rigidity. Cellulose and hemicellulose are densely packed by layers of lignin, that offer protect in against enzymatic hydrolysis. So it is necessary to open the lignin content and expose cellulose and hemicellulose to enzymatic action. Various pretreatment techniques are available for processing of the lignocellulosic substrates Amrita verma *et al*., (2011).

1.12 COCOYAM

Cocoyam grow as underground corms. Annual global production of Cocoyam is 10 million tonnes. Major countries producing cocoyam includes Nigeria, Ghana, Kenya, Malawi, India and Indonesia. Cocoyam corm is an excellent source of carbohydrate, the majority being starch of which 17- 28% is amylose, and the remainder is amylopectin. The Cocoyam corm is low in fat and protein content. The percentage content of starch in cocoyam is 72% which makes it an excellent raw material for alcohol production.The high carbohydrate (10% w/v) level in cocoyam and the presence of other sugar like sucrose, maltose, glucose and fructose makes it a convenient source for the production of bio ethanol Braide *et al*., (2011). The Figure 1.3 shows the underground corm of Cocoyam that can be used for ethanol production.

Figure 1.3: Corm of Cocoyam that can be used for ethanol production.

1.13 BANANA

Banana is one of the most important major fruit crops grown in India. India stands first in banana production next to China and Philippines. Chen *et al.,* (2007). According to 2010-11 statistics 29,780 MT of banana was produced in 830 ha with the yield of 35.9 MT/ha. Totally 56.3 thousand tonnes of banana are exported from India to various countries all over the world. Tamil Nadu, Maharashtra, Gujarat, Andhra and Madhya Pradesh are the major producers of banana in India.

Figure 1.4: Banana plantation

1.14 BANANA RHIZOME

Banana grows from rhizome which is a large underground stem. Banana rhizome is a readily available agricultural waste and available throughout the year, but they were untapped potential source for ethanol production. Typical analyses of banana rhizome showed (www.greenstone.org) water-72%, protein-2.2%, fat-0.1%, starch 19.4%, fibre-0.6% and ash-1.3%. Nearly 1200 acres of banana is cultivated in Tamil Nadu alone with the output of 18-20 tonnes per acre. Normally banana plantation is retained for about 3-5 years and thus the rhizome removed from the soil can be used for alcohol production. It could be an effective waste management process.

To best of our knowledge there are no reports in the literature on the use of banana rhizome as potential source for ethanol production.

Figure 1.5: Banana rhizomes

Banana rhizomes and other agricultural residues are abundant non-food valued materials that can be used for production of ethanol. The major obstacle for bioconversion of the agricultural residues is the utilization of insoluble hemicellulose by microorganisms. Various methods for the pretreatment of the materials have been developed, but problems appear because of toxicity and pollution among many others. It is highly desirable to use minimal pretreatment of the raw materials and achieve maximum bioconversion to renewable energy sources.

1.15 OBJECTIVES

i) To analyze the chemical composition of banana rhizome and Cocoyam tuber (starch, protein, cellulose and lignin content)

ii) To study the suitability of different pretreatment techniques to process banana rhizome and Cocoyam tuber.

iii) To optimize the parameters for ethanol production and to scale-up the ethanol production

1.1.1 LITERATURE REVIEW

Braide *et al.,* (2011) reported that cocoyam can be exploited for ethanol production due to its high starch content. It was subjected to gelatinization in a cooker and treated with two enzymes like bacterial and fungal alpha amylases. The hydrolysed sample was inoculated with *Saccharomyces uvarum* and subjected to fermentation for about 7 days. The pH and the brix level was carefully studied by them and they also noted a decrease in sugar level. They determined the percentage ethanol produced using simple distillation process.

Osuji *et al.,* (2011) reported that yam tubers and cocoyam corms can be processed separately and used for ethanol production. These yam tubers and cocoyam corms were subjected to milling and treated with dextrinase and fungal alpha-amylases. This is then subjected to autoclaving and inoculated with yeast. The fermentation was carried out for 5 days during which pH, specific gravity, total titratable acidity and % sugar content were measured. The sugar content was reduced and the ethanol production was increased. They analysed that raw cocoyam offer a greater potential for flour and ethanol production than yams.

Arrendondo *et al.,* (2009) studied that banana fruit and its lignocellulosic residues can be exploited for ethanol production. The banana fruit was either directly used or peeled pulp exposed to acid hydrolysis. This is then followed by enzyme hydrolysis. The obtained syrup is subjected to fermentation after being neutralized with NaOH. During fermentation ethanol and residual biomass were produced. Thus banana peels and its lignocellulosic residues can be used for ethanol production after fermentation in case of separate hydrolysis and fermentation over Simultaneous Saccarification and Fermentation.

Almgren *et al.,* (2010) reported that starch can serve as an excellent source for ethanol production. The important step in utilizing starch as a feedstock is efficient pretreatment. Gelatinization of the starch can be obtained by cooking or heating of the hydrated starch. When the temperature is increased the starchy material contain amylose and amylopection will loose their structure and hence it is accessible by the enzymes. The cooking followed by enzyme treatment using alpha-amylase released monomeric sugar units which is then subjected to ethanol production.

Gunasekaren *et al.,* (1986) studied the fermentation ability of ethanol production of four strain of *Z.mobilis* on various substrates. Three different substrates like synthetic medium, cane juice and molasses were used. They studied the effect of pH, effect of sugar concentration and fermentation pattern of four strains of *Z.mobilis* on ethanol production.

Amrita Verma *et al.,* (2011) studied that cellulosic plant materials were mainly composed of cellulose, hemicelluloses and lignin and they are considered as one of the cheapest and easily available source for fermentable sugars. The presence of lignin affects the acid and enzyme pretreatment of these materials. Optimization of enzyme parameters like temperature, pH and selection of suitable fermentation techniques based on biomass, may further improve ethanol yield. They also focused on various pretreatments based on composition of lignocellulosic biomass and also the simultaneous saccharification and co-fermentation for cellulosic ethanol production. proper delignification process. The ethanol produced was estimated by simple distillation.

Pia Maren *et al.,* (2010) reported that wheat straw can be efficiently used for the production of bioethanol and biogas. Three different pretreatment of wheat straw was followed. Steam pretreatment along with water, acetic acid and phosphoric acid was performed on wheat straw and the reducing sugars was estimated. They adopted Simultaneous Saccarification and Fermentation for ethanol production.

Chongkhong *et al.,* (2012) reported that fresh jackfruit seeds consist of 36% carbohydrates and it can used for ethanol production. Ethanol fermentation conditions of jackfruit seeds using microorganisms from rice cake starter were optimized by the response surface methodology (RSM). The central composite design (CCD) was used to investigate optimum parameter levels in ethanol production like temperature, pH and time. The high ethanol yield was obtained under optimum fermentation conditions at a temperature of 32.2°C, pH of 5.2 and time of 124.5 h.

Badal *et al.,* (2005) studied that wheat straw consist of cellulose, hemicelluloses and hence it has a potential to serve as source for ethanol production. They adopted dilute acid pretreatment at varying temperatures and enzyme saccarification to evaluate the yield of monomeric sugars. The optimum pH and temperature of the enzyme was identified, When 0.75% sulphuric acid was used, no measurable amount of furfural and hydroxyl methyl furfural was produced. Recombinant *E.coli* was used for fermentation process. Detoxification of the acid by overliming reduced the time needed for

Senthil kumar *et al.,* (2005) studied that agricultural and forest residues, waste paper and industrial waste can be used as an ideal and inexpensive source of sugars for the production of ethanol fuel. Generally *Saccharomyces cerevisiae* and *Zymomonas mobilis* are considered as potential candidates for ethanol production. They analysed that *Saccharomyces cerevisiae* and *Zymomonas mobilis* were not able to hydrolyse sugars from lignocellulosic materials. They used various engineered organisms like *Lactobacillus casei, Klebsilla oxytoca* and *Clostridum cellulolyticum* for bioethanol production from cellulosic substrates. These organisms were able to produce ethanol from a wide range of sugars. They reported various strategies for ethanol production from these lignocellulosic substrates.

Wang *et al.,* (2008) reported ethanol production from poplar wood through enzymatic saccarification and fermentation. They used dilute sulphuric acid pretreatment and sulfite pretreatment to overcome recalcitrance of lignocellulosics. These pretreatment were directly applied to wood chips of four poplar wood samples of various genotypes to evaluate the bioconversion potential. It was found that sulphite pretreatment of the samples resulted in high sugar and ethanol yield compared to dilute acid pretreatment.

Sipos *et al.,* (2010) studied that lignocellulosic substrates like spruce, williow, sweet sorghum, bagasse, wheat straw and corn stover can be used as carbon source for ethanol production. Different pretreatment methods were used and it was analysed that the treatment should be efficient enough to break the walls of the lignocellulosic materials. Enzymes obtain from *Trichoderma ressei* can be used for enzyme

pretreatment.Various pretreatments like steam and enzyme treatment was performed and the yield of sugar was estimated. Optimal conditions were maintained and it resulted in high sugar yield. They adopted Simultaneous Saccharification and Fermentation for ethanol production process.

Ragavendra *et al.,* (2006) reported the efficiency of paddy straw, wheat straw and sugarcane bagasse for bioethanol production. The effect of commercially available enzyme cellulase on wheat straw was studied.Various yeast strains were screened for ethanol yield from hydrolysed paddy straw and sugarcane bagasse. They also studied the concentration of substrates, sulphuric acid concentration, effect of NaOH at different incunbation time in varying particle size of all the substrates. Fungal enzymes was isolated from *Trichoderma ressei* and treated with all these substrates. These five different parameters were analysed for varying particle size of the substrate. The amount of reducing sugars released was estimated and all the pretreated substrates were subjected to ethanol production and the ethanol yield was estimated.

Badal *et al.,* (1998) reported that corn fiber consist of 20% starch, 14% cellulose and 35% hemicelluloses and it has the potential to serve as a low cost feedstock for the production of bioethanol. Various ecofreindly pretreatment have been followed to hydrolyse the corn fibers. Ammonia fiber steam explosion, dilute sulphuric acid pretreatment, enzyme treatment and steam pretreatment were studied and the yield of fermentable sugars was analysed. Genetically modified yeast and bacterial strains were used for the experiments.

Diane et al., (1991) reported that four different woody crops were pretreated by dilute sulfuric acid and evaluated in the simultaneous saccharification and fermentation (SSF) process for ethanol production. The yeast *Saccharomyces cerevisiae* was used in the fermentations alone, and in mixed cultures with glucosidase producing *Brettanomyces clausenii***.** Cellulase enyme was either employed alone or supplemented with glucosidase. *S. cerevisiae* alone achieved the highest ethanol yields .There were some differences in SSF performance, all these pretreated woody crops show promise as substrates for ethanol production.

CHAPTER 2

MATERIALS AND METHODS

2.1 COLLECTION OF SUBSTRATES

Banana rhizomes were collected from various regions of Tirupur and Cocoyam tuber was collected from local markets in Coimbatore and were checked as substrates for alcohol production.

2.2 PREPARATION OF SUBSTRATES

Banana rhizome and Cocoyam tuber used for ethanol production were thoroughly washed with tap water to remove soil and dust particles. Banana rhizome and Cocoyam tubers were peeled, then cut into small pieces and mashed thoroughly using pestle and mortar.

2.3 CHEMICAL ANALYSIS OF SUBSTRATES

2.3.1 Estimation of starch by Anthrone reagent

About 0.5 g of banana rhizome and Cocoyam tuber were homogenized separately with 5 ml hot 80% (v/v) ethanol and contents were centrifuged at 10000 rpm for 10 min. The residue was washed twice with hot 80% ethanol and dried thoroughly. Five ml of distilled water and 6.5 ml of 52% (v/v) perchloric acid was added to the residue. The mixture was kept at 0ºC for 20 min., centrifuged and the supernatant was collected. The extraction was repeated twice with perchloric acid and the supernatants were collected. The volume of supernatant was then made up to 100 ml with distilled water. About 0.2 ml of the supernatant (in duplicate) was transferred to test tubes and the volume made to 1 ml with distilled water. Standard stock glucose solution was prepared by dissolving 100 mg of glucose in 100 ml of distilled water, and 10 ml of this standard stock solution was diluted to 100 ml to make the working standard. About 0.2, 0.4, 0.6, 0.8, and 1 ml of working standard glucose solution was added to a series of test tubes and the reaction volume was made up to 1 ml with distilled water. Four ml of Anthrone reagent was added to all the tubes. The tubes were kept in a boiling water bath exactly for 8 min and then cooled rapidly. The absorbance of orange-red was measured using spectrophotometer at 630 nm. The glucose content in the Banana rhizome and Cocoyam was estimated from standard graph was followed by the method as described by Hodge *et al*., (1962)

2.3.2 Estimation of protein by Lowry's method

A standard graph was constructed by the method described by Lowry *et al*., (1951). Bovine serum albumin [(BSA) 20 mg/100 ml] was used as standard protein. Different concentrations (0.2, 0.4, 0.6, 0.8, and 1 ml) were added to a series of test tubes and the reaction volume was made up to 1 ml with distilled water. The sample was prepared by grinding 0.5 g of banana rhizome and Cocoyam tuber separately with acid washed sand using pestle and mortar. The resultant mixture was centrifuged and the supernatant was used for protein estimation. About 0.2 ml of the supernatant added in duplicate in test tubes and the reaction volume was made up to 1 ml. About 5 ml of alkaline copper reagent (Reagent C) was added to all the tubes and was kept for 10 min. About 0.5 ml of Folin Ciocalteau reagent (FCR) (Reagent D) was added to all the tubes and incubated in at dark for 20 min. The absorbance was measured using spectrophotometer at 660 nm. The amount of protein in the banana rhizome and Cocoyam tuber was estimated from standard graph.

2.3.3 Estimation of Cellulose

About 3 ml of acetic/nitric reagent was added to the test tubes containing 0.5 g of banana rhizome and Cocoyam tuber separately. The tubes were kept in a water bath at 100°C for 30 min. Then the content was cooled, centrifuged and the supernatant was discarded. The residue was washed with distilled water and 10 ml of 67% (v/v) sulphuric acid was added. About 10 ml of 67% sulphuric acid was added to a test tube containing 100 mg of authentic cellulose. The mixture was then allowed to stand aside for 1 h. At the end of 1 h, distilled water was added to make the final volume to 100 ml and 1 ml was used to estimate the cellulose content. The cellulose mixture was taken as 0.4 ml, 0.8 ml, 1.2 ml, 1.6 ml, and 2 ml in separate tubes and the reaction volume was made up to 2 ml. About 10 ml of Anthrone reagent was added to all the tubes. The tubes were kept in a boiling water bath exactly for 10 min. The test tubes were cooled and absorbance was measured using spectrophotometer at 630 nm. The amount of cellulose in the banana rhizome and Cocoyam tuber was estimated from standard graph by the methods as described by Updegroff (1969).

2.3.4 Estimation of lignin

About 0.3 g of banana rhizome and Cocoyam (W_1) was taken in a boiling tube seperately. 3 ml of 72% sulphuric acid was added to the boiling tubes and mixed thoroughly for 1 min. This boiling tubes were placed in water bath at 30ºC for 2 h. This tubes were stirred every 15 min. The contents were transferred to a conical flask and 84 ml of water was added to this mixture. It was autoclaved at 121ºC for 1 h and cooled to room temperature. The content of the flask was filtered and the residue was transferred to a watch glass and dried in oven at 105 ºC for 2 h. Absorbance was measured using Spectrophotometer at 205 nm for the filtrate obtained in above step. Now the watch glass is taken from oven and weight of the dried material is noted as (W_2). Now the watch glass is transferred to muffle furnace at 600 ºC for 3 h. the weight after removing from muffle furnace is noted as (W_3). David *et al.*,(1995).

Total lignin content was calculated using the following formula:

% Acid insoluble lignin = $(W_2 - W_3 / W_1 * T_{final} / 100) * 100$ %

Where

W_1 = Initial weight of sample

W_2 = Weight of the crucible, acid insoluble lignin and acid insoluble ash

W_3 = Weight of the crucible acid insoluble ash

% T = Total solids content in the sample determined at 105º C

% Acid soluble lignin = $((A / b * a) * df * V * (L/100) / (W_1 * T_{final}/100) * 100)$

Where

A = Absorbance at 205 nm

df = dilution factor

b = path length

a =110 L/g-cm

V = filtrate volume 87 ml

Total lignin content = Acid soluble lignin + Acid insoluble lignin

2.3.5 Estimation of total carbohydrate by phenol-sulphuric acid

Five ml of 2.5 N HCl was added to the tubes containing 0.1 g of banana rhizome and Cocoyam tuber separately and they were kept in a boiling water bath for 3 h. At the end of 3 h, the mixture was neutralized with solid sodium carbonate until the effervescence ceases. The total volume was made up to 100 ml and centrifuged. 0.5 ml of supernatant was added in duplicate to test tubes and the volume was made up to 1 ml with distilled water. Standard stock was prepared by dissolving 100 mg of glucose in 100 ml of distilled water. Ten ml of the standard stock solution was diluted to 100 ml to make the working standard. About 0.2, 0.4, 0.6, 0.8, and 1 ml of working standard solution was taken in a series of test tubes and the reaction volume was made up to 1 ml with distilled water. One ml of 5% (w/v) phenol solution was added to all the tubes. Then 5 ml of 96% sulphuric acid was added to all the tubes and mixed thoroughly. Then the tubes were placed in a water bath at 30ºC for 20 min. The absorbance was measured using spectrophotometer at 540 nm. Total reducing sugar content in the banana rhizome and Cocoyam tuber was estimated from standard graph was followed by method as described by Dubois *et al.,* (1956).

2.4 PRETREATMENT METHODS USED FOR BANANA RHIZOME

2.4.1 Autoclaving

Five grams of banana rhizome was weighed separately and added to 3 Erlenmeyer flasks each containing 50 ml of YPD medium. The flasks were autoclaved at 121°C for 20 min. and the flasks were cooled to room temperature. One flask was inoculated with log phase S. *cerevesiae*, the other was with *Z. mobilis* and the third flask was used as control.

2.4.2 Combined steam and alkali treatment

Five grams of banana rhizome was weighed separately and added to 3 Erlenmeyer flasks each containing 50 ml distilled water. About 0.5 g of NaOH was added to all the flasks and stirred thoroughly. The flasks were autoclaved at 121°C for 20 min. The flasks were cooled and then each flask was washed thoroughly with water until the filtrate shows pH 7. About 50 ml of YDP medium was added to the residue left out and pH was adjusted suitably for the growth of microorganisms. The flasks were autoclaved and then cooled to room temperature. One flask with pH 5 was inoculated with *S. cerevesiae* and the second flask with pH 6 was inoculated *Z. mobilis* and the third flask was used as control.

2.4.3 Enzyme treatment (solid state fermentation, SSF)

Ten grams of rice bran was added to Erlenmeyer flask, moistened with distilled water and was sterilized. An autoclaved flask was inoculated with *P. chrysogenum.* The flask was kept statically condition at 28ºC with intermittent shaking. After 4 days, 25 ml of citrate buffer (pH 5) was added to the flask and kept on an orbital shaker for 2 h. Then the contents in the flask (rice bran + buffer) were filtered through muslin cloth and the filtrate thus obtained was again filtered through Whattman No.1 filter paper. The filtrate obtained was as source tested for xylanase and cellulase enzymes.

The filtrate obtained from *P. chrysogenum* showed high enzyme (cellulase and xylanase) activity. About 20 ml of the filtrate was added separately to the 3 separate flasks each containing 5 g of banana rhizome. The pH was adjusted to 5.5. The flasks were kept in a water bath maintained at 40ºC for 3 h. The flasks were autoclaved and cooled to room temperature. One flask was inoculated with *S. cerevesiae* and the other with *Z. mobilis* and the remaining one was kept as blank.

2.4.3.1 Xylanase assay

Xylanase assay was carried out with 1% (w/v) birchwood xylan as substrate. The reaction mixture contains 1.5 ml of 0.2 M citrate buffer, 0.5 ml of culture filtrate as enzyme source, and 0.5 ml of substrate. The reaction mixture was incubated at 40ºC for 15 min. For blank experiment 3 ml of DNS was added to the tube prior to the

addition of substrate. Three ml of DNS reagent was added to the test and the test tubes were kept in a boiling water bath exactly for 5 min. The test tubes were cooled and absorbance was measured using spectrophotometer at 540 nm. Xylanae enzyme activity was carried out at 4 pH (5, 6, 7 and 8) by the methods as described by Alexander *et al*., (2011).

One unit of xylanase enzyme activity is defined as the amount enzyme required to produce one micromole of xylose per ml per minute.

Enzyme activity = C*2*6/ 15*150.13 U

Where

C = Concentration of xylose from the standard graph (µg/ml)
2 = Dilution factor (ml)
6 = Reaction volume (ml)
15 = Incubation time (min)
150.13 = Molecular weight of xylose g/mol)

2.4.3.2 Cellulase assay

Cellulase assay was carried out with 1% (w/v) cellulose as substrate. The reaction mixture consist of 1.5 ml of 0.2 M citrate buffer, 0.5 ml of culture filtrate,3 ml of DNS reagent in blank, 0.5 ml of substrate the reaction mixture was incubated at 40ºC for 15 min. Three ml of DNS reagent was added in the test and the tubes kept in a boiling water bath exactly for 5 min. The test tubes were cooled and absorbance was measured using spectrophotometer at 540 nm. The enzyme activity was carried out at pH 5, 6, 7 and 8 followed by the methods of Charitha Devi *et al*., (2012).

One unit of cellulase enzyme activity is defined as the amount enzyme required to produce one micromole of glucose per ml per minute.

Enzyme activity = C*2*6/ 15*180.16 U

Where

C = Concentration of cellulose from the standard graph (µg/ml)
2 = Dilution factor (ml)
6 = Reaction volume (ml)
15 = Incubation time (min)
180.16 = Molecular weight of glucose (g/mol)

2.4.4 Combined steam and enzyme treatment

About 10 grams of rice bran and 3 g of banana rhizome was added to 250 ml capacity Erlenmeyer flasks and sterilized. Autoclaved flasks were inoculated with *P.chrysogenum*. Batch fermentation was carried out by keeping the flasks statically at 28ºC with intermittent shaking. After 4 days 25 ml of citrate buffer (pH 5) was added to the flask and kept on an orbital shaker maintained at 125 rpm for 2 h. Then the content in the flask was filtered through muslin cloth and the filtrate obtained was again filtered with Whattman No.1 filter paper. The filtrate thus obtained was tested for xylanase and cellulase enzymes activities. The filtrate showed high cellulase and xylanase enzyme titres.

Fifteen grams of banana rhizome was added separately to 3 separate Erlenmeyer flasks and autoclaved. Ten ml of filtrate was added to all the flasks. The pH was adjusted to 5.5. The flasks were kept in a water bath at 40ºC for 6 h. The flasks were autoclaved and cooled to room temperature. The first flask was inoculated with *S. cerevesiae* and the second with *Z. mobilis* while the third one was used as blank.

2.4.5 Amylase enzyme treatment on banana rhizome

About 50 g of banana rhizome was peeled, cleaned and ground to fine paste by adding 100 ml of water. 10 ml of the sample was taken separately in two conical flasks and pH was adjusted to 5. These flasks were then autoclaved at 120ºC for 15 min. Forty ml of amylase was added to both the flasks and were incubated in a water bath at 40ºC for 4 h. At the end of 4th h, one flask was inoculated with *Saccharomyces cerevesiae* and the other with *Zymomonas mobilis*. Batch, submerged fermentation was carried out for 3 days at room temperature under static condition. Ethanol estimation was performed after 3 days.

2.4.6 Amylase and glucoamylase enzyme treatment on banana rhizome

About 50 g of banana rhizome was peeled, cleaned and ground to fine paste by adding 100 ml of water. 10 ml of the sample was taken separately in a two conical flasks. The pH was adjusted to 5. These flasks were then autoclaved at 120ºC for 15 min. About 20 ml of amylase and 20 ml of glucoamylase was added to both the flasks and the flasks were incubated in a water bath at 50ºC for 4 h. At the end of 4th h, one flask was inoculated with *Saccharomyces cerevesiae* and the other with *Zymomonas mobilis*. Ethanol estimation was performed after 3 days.

2.4.6.1Estimation of optimum pH and temperature for Amylase enzyme

Amylase assay was carried out with 1 (%, w/v) starch as substrate. The reaction mixture consisted of 1.5 ml of 0.2 M acetate buffer, 0.1 ml of enzyme and 0.5 ml of substrate the reaction mixture was incubated at 40ºC for 15 min. Three ml of DNS reagent was added to all the tubes and the tubes were kept in a boiling water (100°C) bath exactly for 5 min. The test tubes were cooled and absorbance was measured at 540 nm using SPINCO Spectrophotometer. The enzyme activity was carried out at different pH 5,6,7 and 8 and the maximum enzyme activity was observed at pH 5. The incubation was carried out at 30, 40, 50 and 60ºC and the maximum enzyme activity was observed at 40ºC.

Amylase enzyme activity was calculated using the following formula:
Enzyme activity = C*2*6/ 15*180.13 U
C = Concentration of glucose from the standard graph (µg/ml)
2 = Dilution factor (ml)
6 = Reaction volume (ml)
15 = Incubation time (min)
180.13 = Molecular weight of glucose, g/mol

2.4.6.2 Estimation of optimum pH and temperature for glucoamylase enzyme

Glucomylase assay was carried out with 1 (w/v%) starch as substrate. The reaction mixture consists of 1.5 ml of 0.2 M acetate buffer, 0.1 ml of enzyme and 0.5 ml of substrate. The reaction mixture was incubated for 15 min. Three ml of DNS reagent was added to all the tubes and kept in a boiling water bath for 5 min. The test tubes were cooled to room temperature and absorbance was measured at 540 nm using SPINCO Spectrophotometer at 540 nm. The enzyme activity was carried out at 4 different pH (5, 6, 7 and 8) and the maximum enzyme activity was observed at pH 5. In a similar fashion, enzyme solution at pH 5 was used incubated at 30, 40, 50 and 60ºC to find the optimum temperature and the maximum enzyme activity was observed at 50ºC.

Glucoamylase enzyme activity was calculated using the following formula:

Enzyme activity = C*2*6/ 15*180.13 U

C = Concentration of glucose from the standard graph (µg/ml)

2 = Dilution factor (ml)

6 = Reaction volume (ml)

15 = Incubation time (min)

180.13 = Molecular weight of glucose g/mol

2.4.7 Dilute Sulphuric acid pretreatment of banana rhizome

Dilute sulphuric acid pretreatment of banana rhizome was performed using Response Surface Methodology. Sulphuric acid concentration (SAC), autoclaving time and Solid-Liquid ratio (SLR) were taken as factors. The minimum and the maximum values used for these factors are shown below:

Table 2.1: Parameters optimized for dilute acid treatment of Banana Rhizome

PARAMETERS	MINIMUM VALUE	MAXIMUM VALUE
Sulphuric acid concentration (v/v %)	0.3	1
Solid-Liquid ratio (w/v %)	0.05	0.25
Time (min)	16	30

Box-Behken design was used and 15 experimental designs were obtained in Table 2.2. 5 g of banana rhizome was taken in all the flasks and they were mixed with varied concentration sulphuric acid (0.30, 0.65 and 1%) and varied Concentration of SLR (0.05,0.15 and 0.25%) as obtained from Box-Behken design. These flasks were then autoclaved at 120ºC at appropriate time period (16, 23 and 30 min). The flasks were then cooled to room temperature and the contents were filtered through Whatman filter paper No.1. The filtrate thus obtained was collected separately and used to estimate the total reducing sugars produced.

Table 2.2: Fifteen experiments for banana rhizome obtained from RSM

SLR	time	sac
0.25	16	0.65
0.15	30	1.00
0.25	30	0.65
0.15	16	1.00
0.25	23	1.00
0.05	16	0.65
0.25	23	0.30
0.15	30	0.30
0.05	23	0.30
0.15	23	0.65
0.15	16	0.30
0.15	23	0.65
0.05	23	1.00
0.05	30	0.65
0.15	23	0.65

2.5 ULTRAFILTRATION

Czepak dox broth (400 ml) was prepared and transferred to 5 Erlenmeyer flasks. All the flasks were inoculated with *P.chrysogenum* mycelia and incubated the flasks were kept on an orbital shaker at room temperature for 4 days. At the end of the fourth day, fermented broth was filtered through Whatman No.1 filter paper and the filtrate (270 ml) thus obtained was used was assayed for amylase, cellulase and xylanase enzymes. The filtrate was then subjected to Ultrafiltration, 70 Kda molecular weight membrane catridge cut off was used. The permeate and retentate was collected separately. About 100 ml of permeate and 150 ml of retentate was collected and assayed separately for amylase enzyme activity.

2.5.1 Ultrafiltered enzyme treatment on banana rhizome

About 25 g of banana rhizome was taken in 6 separate Erlenmeyer flasks (with 250 ml capacity). To each flask 25 ml of ultrafiltered enzyme (permeate which showed maximum activity for amylase) was added and kept for incubation at 40ºC for varied time intervals. Two flasks were incubated for 1 h, other two flasks for 2 h and the remaining two flasks for 5 h. After enzyme treatment, three flasks one from each incubation time were inoculated with *Saccharomyces cerevesiae* and the rest were inoculated with *Zymomonas mobilis*. Ethanol estimation was performed after 3 days.

2.6 PRETREATMENT METHODS USED FOR COCOYAM TUBER

2.6.1 Autoclaving

About 5 g of Cocoyam tuber was weighed separately and added to 2 Erlenmeyer flasks each containing 50 ml of YPD medium. They were autoclaved at 121°C for 15 min. The flasks were cooled to room temperature. One flask was inoculated with *Saccharomyces cerevesiae* and the other with *Zymomonas mobilis*. Ethanol estimation was performed after 3 days.

2.6.2 Combined steam and alkali treatment

About 5 g of Cocoyam tuber was weighed separately and added to 2 Erlenmeyer flasks containing 50 ml of distilled water. NaOH (0.5%, w/v) was added to both the flasks. They were autoclaved at 121°C for 15 min. The flasks were cooled and then each flask was washed thoroughly with water until the filtrate shows neutral pH. About 50 ml of YDP medium was added to the flask containing the residue and pH was adjusted suitably for the growth of microorganisms. The flasks were autoclaved and then cooled to room temperature. One flask with pH 5 was inoculated with *Saccharomyces cerevesiae* and the other with pH 6 was inoculated *Zymomonas mobilis*. Ethanol estimation was performed after three days.

2.6.3 Amylase enzyme treatment on Cocoyam tuber

About 50 g of Cocoyam tuber was peeled, cleaned and ground to fine paste by adding 100 ml of water. 10 ml of the sample was taken separately in a two conical flasks and pH was adjusted to 5. These flasks were then autoclaved at 120ºC for 15 min. 40 ml of amylase was added to both the flasks and incubated in water bath at 40ºC for 4 h. One flask was inoculated with *Saccharomyces cerevesiae* and the other with *Zymomonas mobilis*. Batch, Submerged fermentation was carried out for 3 days at room temperature in static condition. Ethanol estimation was performed after 3 days.

2.6.4 Amylase and Glucoamylase enzyme treatment on Cocoyam tuber

About 50 g of Cocoyam tuber was peeled, cleaned and ground to a fine paste by adding 100 ml of water. 10 ml of the sample was taken separately in a two conical

flasks. The pH was adjusted to 5. These flasks were then autoclaved at 120ºC for 15 min. 20 ml of amylase and 20 ml of glucoamylase was added to both the flasks and incubated in water bath at 50ºC for 4 h. One flask was inoculated with *Saccharomyces cerevesiae* and the other with *Zymomonas mobilis*. Ethanol estimation was performed after 3 days.

2.6.5 Ultrafiltered enzyme treatment on Cocoyam tuber

About 50 g of Cocoyam tuber was peeled, cleaned and ground to fine paste adding 100 ml of water. 10 ml of the sample was taken separately in a two conical flasks and 50 ml of permeate was added to each flasks. The pH was adjusted to 5.6. The flasks were incubated in a water bath at 50ºC for 4 h. One flask was inoculated with *Saccharomyces cerevesiae* and the other with *Zymomonas mobilis*. Ethanol estimation was performed after 3 days.

2.6.6 Dilute sulphuric acid pretreatment of Cocoyam tuber

Dilute sulphuric acid pretreatment of Cocoyam tuber was performed using Response Surface Methodology. Sulphuric acid concentration (SAC), autoclaving time and Solid-Liquid ratio (SLR) were choosen as factors. The minimum and the maximum values used for the experiments are shown below:

Table 2.3: Parameters optimized for dilute acid treatment of Cocoyam tuber

PARAMETERS	MINIMUM VALUE	MAXIMUM VALUE
Sulphuric acid concentration (%)	0.50	2.5
Solid-Liquid ratio (%)	0.010	0.1
Time (min)	20	120

Box-Behken design was used and 15 experimental designs were obtained as shown in Table 2.4. 5 g of Cocoyam was taken in all the flasks and they were mixed with varied concentration sulphuric acid (0.5, 1.5 and 2.5) and varied % of SLR (0.010,0.055 and 0.100) as obtained from Box-Behken design. These flasks were then autoclaved at 120ºC at appropriate time periods (20, 70 and120 min). The flasks were then cooled to room temperature and the contents were filtered through Whatman filter paper No.1. The filtrate thus obtained was collected separately and used to estimate the total reducing sugars produced. Lecticia *et al.*,(2010)

Table 2.4: Fifteen experiments for Cocoyam tuber obtained from RSM

SAC	TIME	SLR
2.5	20	0.055
1.5	70	0.055
0.5	120	0.055
2.5	120	0.055
1.5	20	0.010
1.5	120	0.100
0.5	70	0.010
2.5	70	0.010
1.5	20	0.100
0.5	20	0.055
2.5	70	0.100
1.5	70	0.055
1.5	70	0.055
0.5	70	0.100
1.5	120	0.010

2.6.6.1 Overliming of the filtrate obtained by acid treatment

The filtrate obtained from Cocoyam tuber after dilute acid pretreatment was subjected to overliming. Overliming is generally performed to alter the pH of filtrate suitable for the growth of microorganisms. $CaCO_3$, Na_2CO_3 and $Ca(OH)_2$ are separately used for overliming. 50 ml of filtrate was dispensed separately in 5 flasks. One flask was subjected to overliming by 2 M $CaCO_3$. The pH was adjusted to 5.5 and inoculated with *Saccharomyces cerevesiae*. Two flasks were overlimed with 2 M Na_2CO_3. Of the two flasks one flask was inoculated with *Saccharomyces cerevesiae* and the other with *Zymomonas mobilis*. The remaining two flasks were overlimed with $Ca(OH)_2$ and inoculated separately with *Saccharomyces cerevesiae* and *Zymomonas mobilis*. Batch, submerged fermentation was carried out for 3 days at room temperature in static condition. At the end of third day all the flask were estimated for ethanol.

2.7 FERMENTATION

Batch, submerged fermentation was carried out at room temperature and static conditions. All flasks were inoculated with 5% (v/v) log phase cells of *Z. mobilis* and *S. cerevesiae* and fermentation was carried out for 5 days. After 5 days, 5 ml of fermented broth was collected aseptically from all flasks. Five ml of medium was also taken from the flask kept 4°C was used as blank. Fermented broth from each flask was centrifuged at 10000 rpm for 10 min. The supernatant was removed and estimated for ethanol.

2.8 ESTIMATION OF ETHANOL BY DICHROMATE OXIDATION METHOD

Ethanol estimation was performed by the methods of Crowell *et al.,* (1979). 1% (v/v) ethanol standard was prepared. Different percentage solution (0.1-1%) of ethanol was taken in test tubes and the total volume was made up to 1 ml with distilled water. 1 ml of the supernatant from the fermented sample was taken in test tubes separately. 5 ml of 0.25 M acid dichromate solution was taken added to all the tubes and incubated in boiling water bath at 60ºC for 20 min. The test tubes were cooled and absorbance was measured at 600 nm using Spectrophotometer. The volume of ethanol obtained from different pretreatment of the substrates was estimated using standard graph.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 ESTIMATION OF CHEMICAL COMPOSITION OF BANANA RHIZOME

The chemical composition of banana rhizome and Cocoyam tuber is shown in Table 3.1.

Table 3.1: Chemical composition of banana rhizome

S. No.	Chemicals	Banana rhizome (%)	Cocoyam (%)
1	Starch	25.2	27
2	Cellulose	14	43
3	Total Carbohydrate	24.0	39.67
4	Lignin	3.0	4.3
5	Protein	5.1	12

It is evident from Table 3.1 that local banana cultivar rhizome contain 25.2 (%, w/v) starch, 10.2 (%, w/v) cellulose, 24 % (%, w/v) total carbohydrate, 3 (%, w/v) lignin and 5.1 (%, w/v) protein. It is also shown in Table 3.1 that Cocoyam tuber contain 27 (%, w/v) starch, 43 (%, w/v) cellulose, 39.67 (%, w/v) total carbohydrate, 4.3 (%, w/v) lignin and 12 (%, w/v) protein. Sweet potato residue contains 50.1 (%, w/v) starch, 8.9 (%, w/v) cellulose, 11.7 (%, w/v) hemicelluloses 2.2 (%, w/v) lignin and 10.5 (%, w/v) water (Wu *et al.,* 2012).

pretreatment method, NaOH (1%, w/v) was added to Cocoyam tuber paste and autoclaved. The culture filtrate obtained from *P.chrysogenum* was subjected to ultrafiltration and the maximum amylase activity was observed in the permeate. Cocoyam tuber was treated with the permeate from ultrafiltration and this served as source of enzyme for the enzymatic pretreatment of Cocoyam tuber.

Acid pretreatment for Cocoyam tuber was performed using RSM. The amount of reducing sugars produced was estimated. The data indicated that maximum amount of reducing sugars will be produced when cocoyam was autoclaved at 121° C for 78.58 min with the SAC of 1.97% and SLR of 0.1%. The filtrate obtained from the above acid treatment was collected and overliming was performed using $Ca(OH)_2$. The mixture was then filtered, autoclaved and then inoculated with ethanol producing organisms. Cocoyam was treated with the commercially available enzyme amylase and the combination of amylase and glucoamylase for 4 h. This was then subjected to ethanol production. The amount of ethanol produced from cocoyum tuber after various pre-treatment methods was shown in Table 3.3

Table 3.3: Production of ethanol from Cocoyam tuber after different pre-treatment methods

Pre-treatment method	Ethanol yield (ml/g) (*Z.mobilis*)	Ethanol yield (ml/g) (*S.cerevesiae*)
Autoclaving	0.60	0.61
Steam and Alkali treatment	0.55	0.56
Enzyme treatment (Ultra-filtered)	0.11	0.51
Sulphuric acid treatment	0.347	0.461
Amylase treatment	0.298	0.447
Amylase and Glucoamylase treatment	0.271	0.535

3.2 AMYLASE ENZYME ACTIVITY- ULTRAFILTRATION

Cell filtrate (270 ml) of fungus *P.chrysogenum* was obtained by filtration through Whatman filter paper no.1. The filtrate thus obtained was subjected to ultrafiltration using 70 kDa membrane catridge. About 100 ml of permeate and 150 ml of retentate were obtained. Culture filtrate, permeate and retentate were checked for amylase enzyme activity and the results are shown in Table 3.2.

Table 3.2: Amylase enzyme activity after Ultra-filtration.

S.NO	Sample	Absorbance at 540 nm	Enzyme Activity (µmol / ml / min)
1	Culture filtrate	0.090	$4.281*10^{-3}$
2	Permeate	0.147	$6.994*10^{-3}$
3	Retentate	-0.050	No activity

Table 3.2 indicates that permeate showed amylase enzyme activity while there was no enzyme activity in the rententate. Therefore, permeate was used as amylase enzyme source to treat starch in banana rhizome and Cocoyam tuber.

3.3 EFFECT OF VARIOUS PRETREATMENTS ON ETHANOL PRODUCTION FROM COCOYAM TUBER

Cocoyam tuber was subjected to six different pre-treatment methods for the production of ethanol. Autoclaving was performed at 121°C for 15 min that serve the purpose of sterilization and also heat pretreatment of the substrate. In the alkali

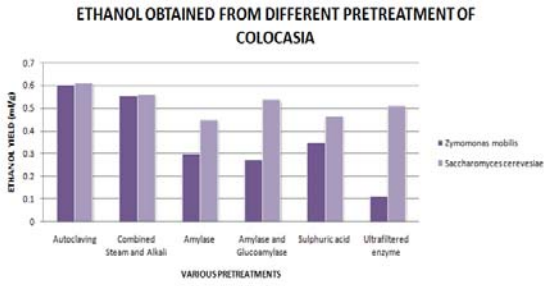

Figure 3.1: Effect of different pretreatment methods on ethanol production from Cocoyam tuber

Cocoyam tuber was subjected to six different pretreatment methods. After pretreatment, the pretreated samples were femented with *Zymomonas mobilis* and *Saccharomyces cerevisiae* for alcohol production. Batch, submerged fermentation was carried out at 28°C for 3 days. Ethanol produced was estimated by dichromate oxidation method.

Autoclaving was found to be an effective pretreatment method and ethanol yield was found to be nearly equal for both organisms (0.61 ml/g in *Z.mobilis* and 0.60 ml/g in *S.cerevesiae*). Combined steam and alkali pretreatment produced 0.55 ml/g and 0.56 ml/g ethanol by *Z.mobilis* and *S.cerevesiae*, respectively.

Ultrafiltered enzyme treatment produced 0.11 ml/g and 0.51 ml/g of ethanol by *Z.mobilis* and *S.cerevesiae*, respectively. It is clear from the results that ethanol yield was higher by *S.cerevesiae* than that of ethanol produced by *Z.mobilis*. Dilute sulphuric acid treatment produced 0.35 ml/g and 0.46 ml/g of ethanol by *Z.mobilis* and *S.cerevesiae*, respectively. Amylase enzyme treatment produced 0.30 ml/g and 0.45 ml/g ethanol by *Z.mobilis* and *S.cerevesiae*, respectively. It is clear from the results that ethanol yield was higher by *S.cerevesiae* when compared to ethanol produced by *Z.mobilis*.

Combined amylase and glucoamylase enzyme treatment produced 0.27 ml/g and 0.53 ml/g ethanol by *Z.mobilis* and *S.cerevesiae*, respectively. It evident from the results that alcohol yield was higher (nearly 50%) by *S.cerevesiae* than *Z.mobilis*. Among pretreatment methods employed, the amount of ethanol produced from *S.cerevesiae* was high when compared to *Z.mobilis* (Figure 3.1).

3.4 EFFECT OF VARIOUS PRETREATMENTS ON ETHANOL PRODUCTION FROM BANANA RHIZOME

Autoclaving was found to be an effective pretreatment method and ethanol yield was slightly higher using *Z.mobilis* when compared to *S.cerevesiae*. In the combined steam and alkali pretreatment, banana rhizome was autoclaved with 1% (w/v) NaOH. Combined steam and alkali pretreatment produced 0.14 ml/g ethanol by *Z.mobilis* and there was no traceable alcohol produced by *S.cerevesiae*.

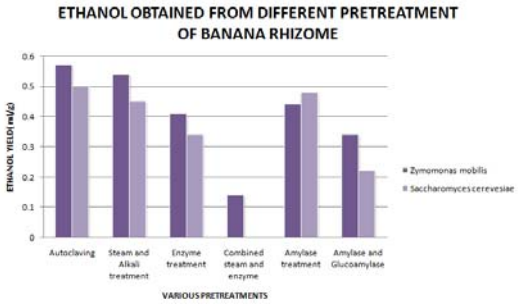

Figure 3.2: Ethanol obtained from different pretreatments of Banana rhizome

The banana rhizome was subjected to six different pretreatment methods and batch fermentation was carried out at 28ºC for 3 days. Ethanol produced was estimated by dichromate oxidation method.

Among the pretreatment methods used, (i) autoclaving, and steam and alkali methods produced high quantity of ethanol (Figure 3.2). Ethanol yield was higher in all pretreatment methods by *S.cerevesiae* except amylas enzyme treatment method.

Various agro-residues like pineapple, orange and sweet lime were used for ethanol production. Effect of different constant times, pH, temperature and autoclave pretreatment were studied to improve the ethanol yield. *S.cerevesiae* and *Candida albicans* were used for alcohol production. Fermentation was carried out by both solid state fermentation and submerged fermentation. From the studies it was found that *P.chrysogenum* was chosen in the present study because it was secreting high xylanase and cellulase enzyme titres. Ultrafiltered enzyme treatment produced 0.41 ml/g and 0.34 ethanol by *Z.mobilis* and *S.cerevesiae*, respectively. It is clear from the results that ethanol yield was higher by *Z.mobilis* than that of ethanol produced by *S.cerevesiae*. Amylase enzyme treatment produced 0.34 ml/g and 0.22 of ethanol by *Z.mobilis* and *S.cerevesiae*, respectively. It is clear from the results that ethanol yield was higher by *Z.mobilis* when compared to ethanol produced by *S.cerevesiae*.

Combined amylase and glucoamylase enzyme treatment produced 0.44 ml/g and 0.49 ethanol by *Z.mobilis* and *S.cerevesiae*, respectively. It evident from the results that alcohol yield was marginal by *S.cerevesiae* than *Z.mobilis*. The amount of ethanol produced from various pretreatment was shown in Table 3.4.

Table 3.4: Effect of various pretreatment methods on banana rhizome for ethanol production

Pretreatment method	**Ethanol yield (ml/g) (*Z.mobilis*)**	**Ethanol yield (ml/g) (*S.cerevesiae*)**
Autoclaving	0.57	0.50
Steam and Alkali treatment	0.54	0.45
Ultrafiltered Enzyme treatment	0.41	0.34
Combined steam and enzyme treatment	0.14	-
Amylase treatment	0.34	0.22
Combined amylase and Glucoamylase treatment	0.44	0.49

submerged fermentation showed substantial increase in ethanol production. Autoclave pretreatment method served protected the sample from contamination and increased the volume of ethanol production. (Mishra *et al.,* 2012).

Cocoyam tuber was exploited for ethanol production due to its high starch content. A two stage enzyme hydrolysis was employed using bacterial amylases and fungal α-amylases after gelatinisation of the substrate. *S. cerevesiae* was used for fermentation and the yield of ethanol was found to 12.9 % (Braide *et al.,* 2011). The dried leaves and pseudostem of banana plant was used as substrate for ethanol production. Saccarification of banana agrowaste by cellulase obtained from *Trichoderma lignorum* yielded 1.34 mml/g of reducing sugars. Maximum sugar release was obtained at pH 6 and temperature of 40ºC (Baig *et al.,* 2004).

Cassava residue is considered as one of the starch rich substrate that can be used for ethanol production. Enzymatic hydrolysis was employed for the pretreatment of the cassava waste. Enzymes such pectinase, alpha-amylase and glucoamylase were used to treat cassava waste for alcohol production (Teerapate *et al.,* 2004).

Sugarcane bagasse, saw dust and water hyacinth were subjected to alkali and enzyme pretreatment separately and the effect of these pretreatment methods on all the substrates and bioconversion rates were investigated. Alkali treatment [using 1% (w/v) NaOH] was also used treat all the substrates at varying time periods to find the optimum conditions (Ferdousi *et al.,* 2011).

3.5 DETERMINATION OF OPTIMUM pH AND TEMPERATURE FOR AMYLASE ENZYME ACTIVITY

The effect of pH on amylase enzyme was estimated and their results are shown in Table 3.5.

Table 3.5: Optimum pH for amylase enzyme activity

pH	Enzyme activity(µmol ml/min)
5	114.19* 10^{-3}
6	112.22* 10^{-3}
7	68.22* 10^{-3}
8	26.6* 10^{-3}

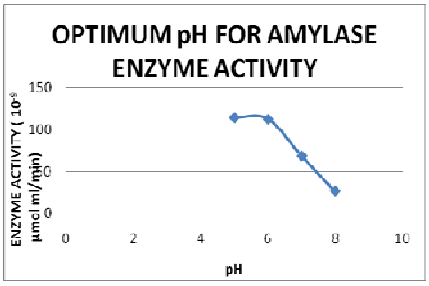

Figure 3.3: Optimum pH for amylase enzyme activity

Table 3.6: Optimum temperature for amylase enzyme activity

Temperature (°C)	Enzyme activity (µmol ml/min)
30	12.6* 10^{-3}
40	25.5* 10^{-3}
50	17.5* 10^{-3}
60	8.3* 10^{-3}
70	2.0* 10^{-3}

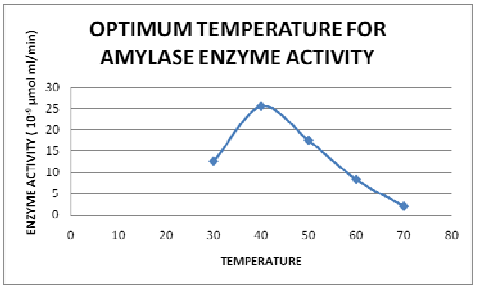

Figure 3.4: Optimum temperature for amylase enzyme activity

Amylase enzyme showed its optimal at pH 5.0 (Figure 3.3). The optimum temperature for the amylase enzyme was at 40ºC (Figure 3.4).

3.6 DETERMINATION OF OPTIMUM pH AND TEMPERATURE FOR GLUCOAMYLASE ENZYME ACTIVITY

The optimum pH and temperature of glucoamylase enzyme was also estimated.

Table 3.7: Optimum pH for glucoamylase enzyme activity

Ph	Enzyme activity(µmol ml/min)
4	0.47* 10^{-3}
5	6.08* 10^{-3}
6	2.33* 10^{-3}
7	1.09* 10^{-3}

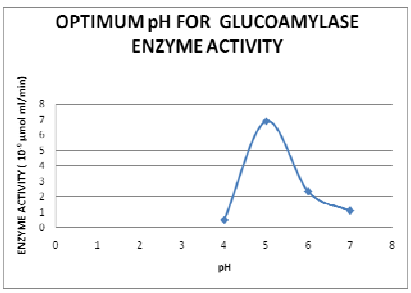

Figure 3.5: Optimum pH for glucoamylase enzyme activity

Table 3.8: Optimum temperature for glucoamylase enzyme activity

Temperature (°C)	Enzyme activity (µmol ml/min)
30	0.9* 10^{-3}
40	2.9* 10^{-3}
50	5.7* 10^{-3}
60	3.5* 10^{-3}
70	0.02* 10^{-3}

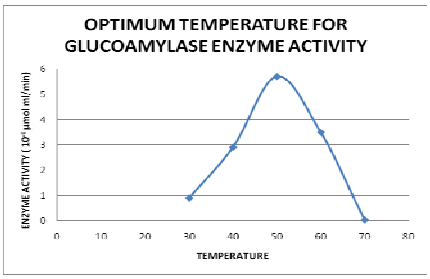

Figure 3.6: Optimum temperature for glucoamylase enzyme activity

The optimum pH for glucoamylase was found to be pH 5 (Figure 3.6). It is evident from Figure 3.7 that glucoamylase enzyme activity was observed at at 50ºC.

Kirti rani et al. (2012) performed optimum temperature and pH studies for bacterial and fungal amylases. Bacterial amylase from *Bacillus subtilis* and fungal amylase *Aspergillus niger* were studied. It was found that optimum pH for bacterial amylase is 6.6 and optimum temperature is 50ºC. The fungal amylase showed maximum enzyme activity at a pH of 6.6 and a temperature of 30 ºC.

Nahar et al. (2008) reported that *Rhizopus* can be used for glucoamylase production. They optimized the sutiable growth condition, temperature, pH , incubation time and nitrogen source for high yield of glucoamylase. The high percentage of enzyme activity was observed at a temperature of 45ºC and a pH of 4.5. Ladokun et al. (2000) reported the production of amylase from *Aspergillus fumigatus*. They studied the substrate concentration, incubation time, temperature stability and pH stability of the enzyme. The optimum temperature studies and pH studies were carried out and it was found that the enzyme has maximum activity at a temperature of 35ºC and a pH of 6. They also reported that enzyme activity got increased with the increase in substrate concentration

Mariana et al.,(2000) studied that the thermophilic fungus *Scytalidium thermophilum* is found to produce glucoamylase enzyme. Optimum pH and temperature studied were carried out and it was found that the enzyme has maximum activity at a temperature of 60ºC and a pH of 6.5. They further purified the enzyme by Ion- exchange chromatography.

3.7 DILUTE ACID PRETREATMENT OF BANANA RHIZOME AND COCOYAM TUBER

Banana rhizome and cocoyam tuber were treated with dilute sulphuric acid for the release of reducing sugars and the optimal conditions required for dilute sulphuric acid pretreatment were determined using response surface methodology (RSM). Three parameters like sulphuric acid concentration, solid-Liquid ratio and autoclaving time at 120ºC required for the release of maximum reducing sugars was studied and the results obtained were shown below.

3.7.1 Cocoyam tuber

Box-Behken design was used for sulphuric acid treatment of Cocoyam tuber. The maximum and the minimum values were set for the all the three parameters and the optimum values for each parameters obtained were shown in the Table 3.9.

Table 3.9: Maximum and minimum values set for RSM

PARAMETERS	MINIMUM VALUE	MAXIMUM VALUE	OPTIMUM VALUE OBTAINED AFTER EXPERIMENTS
Sulphuric acid concentration (%)	0.50	2.5	1.97
Solid-Liquid ratio (%)	0.010	0.10	0.10
Time (min)	20	12O	78.58

Fifteen experiments were performed as per the data obtained from the RSM. The total reducing sugars was estimated for all experiments and the results obtained was shown below.

TRS CONTENT IN COCOYAM

The Table 3.10 shows the total reducing sugars (TRS) released by treating cocoyam tuber with dilute sulphuric acid.

Table 3.10: TRS obtained for Cocoyam tuber by varying the 3 factors (SAC, Time & SLR).

SAC	TIME	SLR	TRS
2.5	20	0.055	10.4845
1.5	70	0.055	14.2799
0.5	120	0.055	0.5787
2.5	120	0.055	13.8358
1.5	20	0.010	1.8573
1.5	120	0.100	30.0538
0.5	70	0.010	0.8345
2.5	70	0.010	0.1211
1.5	20	0.100	26.9448
0.5	20	0.055	5.5855
2.5	70	0.100	33.6608
1.5	70	0.055	22.6514
1.5	70	0.055	23.3244
0.5	70	0.100	24.6837
1.5	120	0.010	0.8479

Response Surface Regression: TRS versus SAC, TIME, and SLR

The analysis was done using coded units.

Estimated Regression Coefficients for TRS

Term	Coef	SE Coef	T	P
Constant	20.0852	2.097	9.577	0.000
SAC	3.3025	1.284	2.572	0.050
TIME	0.0555	1.284	0.043	0.967
SLR	13.9603	1.284	10.870	0.000
SAC*SAC	-6.2825	1.890	-3.323	0.021
TIME*TIME	-6.1816	1.890	-3.270	0.022
SLR*SLR	1.0223	1.890	0.541	0.612
SAC*TIME	2.0895	1.816	1.150	0.302
SAC*SLR	2.4226	1.816	1.334	0.240
TIME*SLR	1.0296	1.816	0.567	0.595

S = 3.63239 PRESS = 357.334
R-Sq = 96.76% R-Sq(pred) = 82.46% R-Sq(adj) = 90.93%

Analysis of Variance for TRS

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	1971.57	1971.57	219.06	16.60	0.003
Linear	3	1646.40	1646.40	548.80	41.59	0.001
SAC	1	87.25	87.25	87.25	6.61	0.050
TIME	1	0.02	0.02	0.02	0.00	0.967
SLR	1	1559.12	1559.12	1559.12	118.17	0.000
Square	3	280.00	280.00	93.33	7.07	0.030
SAC*SAC	1	130.58	145.74	145.74	11.05	0.021
TIME*TIME	1	145.56	141.09	141.09	10.69	0.022

```
    SLR*SLR      1     3.86     3.86     3.86    0.29  0.612
  Interaction    3    45.18    45.18    15.06    1.14  0.417
    SAC*TIME     1    17.46    17.46    17.46    1.32  0.302
    SAC*SLR      1    23.48    23.48    23.48    1.78  0.240
    TIME*SLR     1     4.24     4.24     4.24    0.32  0.595
Residual Error   5    65.97    65.97    13.19
  Lack-of-Fit    3    15.19    15.19     5.06    0.20  0.889
  Pure Error     2    50.78    50.78    25.39
Total           14  2037.55
```

Global Solution

```
SAC    =   1.97475
TIME   =   78.5859
SLR    =       0.1
```

Predicted Responses

```
TRS    =   36.5442   ,    desirability =   1.000000
```

CONTOUR PLOTS OBTAINED FOR 1G COCOYAM TUBER

The contour and the surface plots obtained from above experiment was shown below (Figure 3.7 and Figure 3.8).

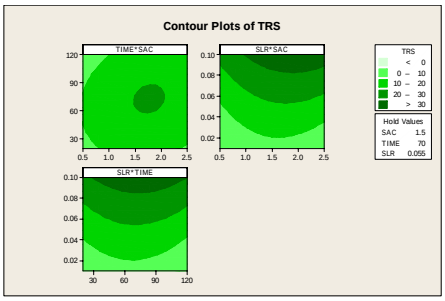

Figure 3.7: Contour plots obtained for sulphuric acid treatment of Cocoyam tuber

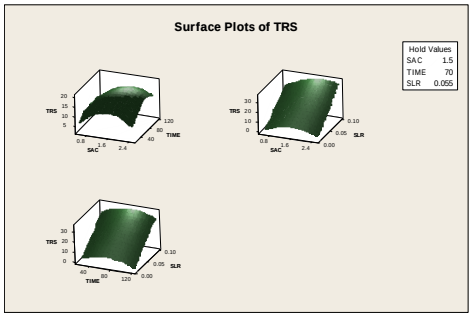

Figure 3.8: Surface plots obtained for sulphuric acid treatment of Cocoyam tuber

Inference

From the global solution obtained it was found that 1.97% SAC, 0.1% SLR and 78.58 min incubation of Cocoyam tuber released maximum reducing sugars (36.544 mg/ml). The R square value is more that 95 and is significant. In ANOVA the F value is greater than P value and the Lack of Fit is insignificant. The contour and the surface plots also showed significant results for Autoclaving time and SAC. TRS increases with an increase in ratio of SLR.

Lecticia et al. (2010) studied that process of optimization of ethanol production from Cassava Starch based o experimental design. Sulphuric acid hydrolysis was performed using three factors. Concentration of Cassava starch (177 to 283 g/L), Agitation speed (510- 760 rpm) and Sulphuric acid (0.5- 4.5%) concentration was taken as factors. Optimum results were obtained at 190 g/L of Cassava starch, 600 rpm of agitation speed and 1% of Sulphuric acid concentration.

3.7.2 Banana rhizome

Dilute sulphuric acid pretreatment of the banana rhizome was performed using RSM. The maximum and the minimum values were set for the all the three parameters and the optimum values for each parameters obtained were shown in Table 3.11.

Table 3.11: Maximum and minimum value taken for RSM

PARAMETERS	MINIMUM VALUE	MAXIMUM VALUE	OPTIMUM VALUE OBTAINED AFTER EXPERIMENTS
Sulphuric acid concentration (%)	0.30	1	1
Solid-Liquid ratio (%)	0.05	0.25	0.1268
Time (min)	16	30	30

TRS CONTENT IN BANANA RHIZOME

The Table 3.12 shows total reducing sugars produced after pretreatment of cocoyam tuber with dilute sulphuric acid.

Table 3.12: TRS obtained for banana rhizome by varying the 3 factors (SAC, Time & SLR)

SLR	time	sac	TRS
0.25	16	0.65	0.9787
0.15	30	1.00	16.0000
0.25	30	0.65	1.0106
0.15	16	1.00	16.4894
0.25	23	1.00	0.9681
0.05	16	0.65	0.9362
0.25	23	0.30	0.9043
0.15	30	0.30	12.7766
0.05	23	0.30	4.5106
0.15	23	0.65	14.6596
0.15	16	0.30	12.9468
0.15	23	0.65	12.7553
0.05	23	1.00	7.0106
0.05	30	0.65	9.1170
0.15	23	0.65	12.4787

Response Surface Regression: TRS versus SLR, time, and SAC

The analysis was done using coded units.

Estimated Regression Coefficients for TRS

Term	Coef	SE Coef	T	P
Constant	13.2979	1.0060	13.218	0.000
SLR	-2.2141	0.6161	-3.594	0.016
time	0.9441	0.6161	1.533	0.186
sac	1.1662	0.6161	1.893	0.117
SLR*SLR	-10.7460	0.9068	-11.850	0.000
time*time	0.4588	0.9068	0.506	0.634
sac*sac	0.7965	0.9068	0.878	0.420
SLR*time	-2.0372	0.8712	-2.338	0.067
SLR*sac	-0.6090	0.8712	-0.699	0.516
time*sac	-0.0798	0.8712	-0.092	0.931

S = 1.74245 PRESS = 204.122
R-Sq = 97.15% R-Sq(pred) = 61.62% R-Sq(adj) = 92.01%

Analysis of Variance for TRS

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	516.607	516.607	57.401	18.91	0.002
Linear	3	57.230	57.230	19.077	6.28	0.038
Square	3	441.267	441.267	147.089	48.45	0.000
Interaction	3	18.110	18.110	6.037	1.99	0.234
Residual Error	5	15.181	15.181	3.036		
Lack-of-Fit	3	12.361	12.361	4.120	2.92	0.265
Pure Error	2	2.820	2.820	1.410		
Total	14	531.788				

Global Solution

SLR = 0.126768
time = 30
sac = 1

Predicted Responses

TRS = 17.1329 , desirability = 1.000000

The contour and the surface plots obtained from above experiment was shown below (Figure 3.9 and Figure 3.10)

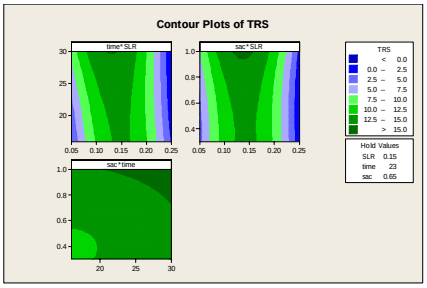

Figure 3.9: Contour plots obtained for sulphuric acid treatment of banana rhizome

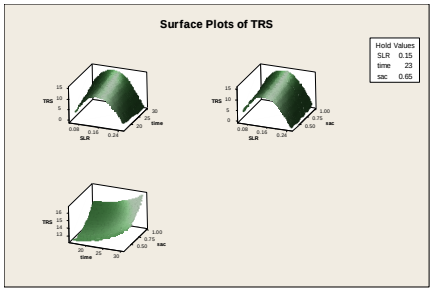

Figure 3.10: Surface plots obtained for sulphuric acid treatment of banana rhizome

Inference

From the global solution obtained it was found that 1% SAC , 0.126% SLR and 30 min incubation of Banana rhizome released maximum reducing sugars (17.132 mg/ml). The R square value is more that 95 and is significant. In ANOVA the F value is greater than P value. The contour and the surface plots also showed significant results. The SLR is found to be significant. TRS value increases with the increase in Autoclaving time and SAC.

Saw dust obtained from the hard wood tree *Prosopis nigra* can be exploited for ethanol production. Acid pretreatment was employed for the yield of reducing sugars from the substrate. Response surface methodology was studied considering two factors like concentration of sulphuric acid and heating time. This is then followed by enzyme hydrolysis. The concentration of sugars is found to be higher in the acid treated substrate than the untreated substrate (Dagnino *et al.*, 2013).

Zheng et al.,(2013) reported that Sugar beet pulp obtained as residue of beet sugar processing can serve as a feedstock for ethanol production. Response surface methodology was used to investigate the effects of temperature, acid concentration and solid loading on dilute sulfuric acid pretreatment a nd enzymatic hydrolysis of Sugar beet pulp. Acid pretreatment increased the enzymatic digestibility of Sugar beet pulp from 33% (raw) to 93% (treated). Pretreatment at optimum conditions temperature at 120°C, acid concentration of 0.66% and solid loading ratio 6% resulted in 62% total reducing sugar yield.

Muhammad et al. (2013) conducted a study for the optimization of pretreatment for lignocellulosic biomass -Water Hyacinth for the production of Bioethanol. Response surface methodology has been employed for the optimization of temperature , time and different concentrations of maleic acid, sulfuric acid and phosphoric acid which was found to be the significant variables. The pretreated biomass produced 39.96 g/l of reducing sugars during enzymatic hydrolysis with high conversion in the phosphoric acid treated sample .

3.8 CONCLUSION

Results from the present study indicated banana rhizome and Cocoyam tuber could be exploited for ethanol production.Both banana rhizome and Cocoyam tuber were processed by six different pretreatment techniques like Autoclaving, Steam and alkali treatment, Enzyme pretreatment, Steam and Enzyme pretreatment, Amylase treatment, Amylase and Glucoamylase treatment, using two different alcohol producing microorganisms such as *Z.mobilis* and *S.cerevesiae*. Appreciable results were obtained when autoclaving was used as pretreatment for both banana rhizome and Cocoyam tuber using *Z.mobilis* and *S.cerevesiae*. *Z.mobilis* showed high amount of ethanol production in all the pretreatments when compared to *S.cerevesiae* for banana rhizome. *S.cerevesiae* showed high amount of ethanol production in all the pretreatments when compared to *Z.mobilis* for Cocoyam tuber. Hence autoclaving is found to be effective pretreatment of banana rhizome and Cocoyam tuber.Autoclaving is cheap and scaled-up when compared to the other pretreatment methods. Optimization of process parameters like pH, inoculum size and substrate concentration will be performed and the process will be scaled up for the production of ethanol on large quantities. To the extent of our knowledge there are no reports on the use of banana rhizome and Cocoyam tuber as substrates for ethanol production. The results obtained from this study will be a boon to farmers to raise additional income by selling the rhizomes to companies for ethanol production as well as to save the environment.

APPENDIX –I

1. YPD MEDIUM (pH 5.6)

Composition for 100 ml

Yeast extract - 0.3g

Peptone -1g

Dextrose- 2g

2. RM MEDIUM (pH 6)

Composition for 100 ml (pH)

Yeast extract -1g

Glucose - 2g

KH_2PO_4 - 0.2g

3. ESTIMATION OF PROTEIN BY LOWRY'S METHOD

Reagents

Working standard solution

Dissolve 20 mg of BSA and make up to 100 ml with distilled water

2% Sodium carbonate in 0.1N sodium hydroxide (Reagent A)

0.5% Copper sulphate in 1% potassium sodium tartarate (Reagent B)

Alkaline Copper Solution

Mix 50 ml of A and 1 ml of B prior to use (Reagent C)

Folin Ciocalteau reagent (FCR).

Mix 1 ml of FCR and 1 ml of distilled water just before use.

4. ESTIMATION OF STARCH CONTENT BY ANTHRONE REAGENT

80% ethanol

52% perchloric acid

Glucose

Anthrone reagent

5. ESTIMATION OF CELLULOSE

Acetic / Nitric reagent

150 ml of 80% Acetic acid and 15 ml of Concentrated Nitric acid

67 % Sulphuric acid

Anthrone reagent

200 mg of Anthrone reagent 95% chilled Sulphuric acid. Prepare fresh and chill before use.

Cellulose

6. ESTIMATION OF LIGNIN

72% Sulphuric acid

7. ESTIMATION OF TOTAL CARBOHYDRATE

2.5 N HCl

Sodium carbonate

5% Phenol

96% Sulphuric acid

1% NaOH

8. ENZYME ASSAY

1% Xylan

1% Cellulose

0.2 M Citrate buffer

DNS reagent

Dissolve 1g of DNS in 100 ml of 1% NaOH. To this add 200 mg of crystalline phenol. 50 mg of sodium sulphite should be added just before use.

9. ETHANOL ESTIMATION

0.25 M Potassium dichromate

Sulphuric acid

Ethanol

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  Presented a paper entitled "Screening of edible mushrooms for L-ergothioniene a potent nutraceutical" organized by Department of Botany, Bharathiar University, Coimbatore.