STUDIES ON BIOETHANOL PRODUCTION FROM PEEL AND STEM OF CASSAVA PLANT

PROJECT REPORT

Submitted by

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Register No: 1020203015

in partial fulfilment for the award of the degree

of

MASTER OF TECHNOLOGY

in

BIOTECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY
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DEPARTMENT OF BIOTECHNOLOGY

PROJECT WORK
PHASE II
APRIL 2012

This is to certify that the project entitiled

STUDIES ON BIOETHANOL PRODUCTION FROM PEEL AND STEM OF CASSAVA PLANT

is the bonafide record of project work done by

SUGANYA C

Register No: 1020203015

of M. Tech. (Biotechnology) during the year 2011-2012

....

Project Guide Head of the Department

Submitted for the Project Viva-Voce examination held on.....

....

Internal Examiner External Examiner

DECLARATION

I affirm that the project work titled "**STUDIES ON BIOETHANOL PRODUCTION FROM PEEL AND STEM OF CASSAVA**" being submitted in partial fulfilment for the award of M.Tech. (Biotechnology) is the original work carried out by me. It has not formed the part of any other project work submitted for award of any degree or diploma, either in this or any other university.

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(SUGANYA, C)

TABLE OF CONTENTS

ABSTRACT

The state of the art of starch hydrolysis and fermentation technologies to produce ethanol from cassava by-products was evaluated. Cassava stem, cassava peel, cassava waste, cassava twig and cassava leaves are samples collected from sago industry and tested for total carbohydrate and crude protein. Two samples from sago factory, namely cassava stem and cassava peel, with similar total carbohydrate content, were used as substrates. The samples were liquefied using various concentrations of α-amylase, in order to optimize the production of fermentable sugars, the enzyme α-amylase of 12.18% (w/w of Substrate) for cassava peel and 24.55% (w/w of Substrate) for cassava stem revealed higher performance. After liquefaction, the simultaneous saccharification and fermentation was conducted in shaker flask. Glucoamylase was used for saccharification, and *Zymomonas mobilis* MTCC 2427 for fermentation simultaneously. Sterile samples were withdrawn regularly for analysis. Glucose consumed promptly in both cases, cassava peel and cassava stem; meanwhile, the ethanol production was considerably higher in cassava stem (6.143 g/mL), compared to cassava peel (5.793 g/mL). The substrate containing cassava stem revealed higher potential as substrate for ethanol production.

Keywords: Cassava peel, cassava stem, pretreatment, liquefication, SSF, RSM.

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

ΔH^o	Standard enthalpy change
ΔS^o	Standard entropy change
Ad	Alcohol dehydrogenase
AFEX	Ammonia fiber / freeze explosion
AMG	Amyloglucosidase
ANOVA	Analysis of variance
BBD	Box-Behken design

C_{Ao}	Initial concentration of substrate
CBP	Consolidated bioprocessing
CCD	Central composite design
CLB	Circulating loop bioreactor
C_S	Concentration of substrate
CSH	Cassava starch hydrolysate
DNSA	Dinitrosalicylic acid
DO	Dissolved oxygen
E_a	Activation energy
ED	Entner-Duodoroff
EMP	Embden-Meyerhoff-Parnas
EtOH	Ethanol
FAO	Food and Agricultural Organization
F_{Ao}	Feed rate
FU	Functional unit
H	High value
HDS	Hemicellulose-derived sugars
HMF	Hydroxymethylfurfural
HPLC	High pressure liquid chromatography
IMTECH	Institute of microbial technology
k_B	Boltzman constant
k_m	Michaelis Menten constant
k_o	Frequency factor

L	Low value
LLD	Linear long-chain dextrin
M_c	Mass of the catalyst
MIECC	Microwave irradiation-enzyme coupling catalysis
MTBE	Methyl tertiary butyl ether
MTCC	Microbial type culture collection
OD	Optical density
PBD	Plackett-Burman Design
PBR	Packed bed reactor
Pd	Pyruvate decarboxylase
PFR	Plug flow reactor
PP	Pentose phosphate
q_p	Specific rate of product formation
r_A	Rate of the reaction
RSM	Responce surface methodology
RSSDE	Raw sago starch degrading enzyme
SD	Standard deviation
SEM	Scanning electron microscope
SHF	Separate hydrolysis and fermentation
SSCF	Simultaneous saccharification and co-fermentation
SSF	Simultaneous saccharification and fermentation
SSSF	Simultaneous starch saccharification and ethanol fermentation
UV	Ultra violet

V	Volume of reactor
V_c	Volume of catalyst
V_{max}	Maximum velocity
V_o	Volumetric flow rate
X_A and X_s	Conversion of reactant and substrate respectively
Xd	Xylose dehydrogenase
Xi	Xylose isomerise
Xk	Xylulokinase
Xr	Xylose reductase
P	Density
ρ_c	Density of catalyst

CHAPTER 1
INTRODUCTION

1.1 GENERAL

Ethanol and ethanol-gasoline mixtures have been considered for fuel since the early days of the automobile. In the past, abundant and less expensive petroleum supply prevented the extensive use of ethanol as fuel, but in the last few decades the general public has become aware of and concerned about the expensive petroleum supply. Interest in extending supplies with ethanol-gasoline mixtures has increased greatly (Klass, 1981).

Overutilization of energy is being a major problem of the present and future world community. The use of energy derived from the renewable sources like fossil fuels increases the level of toxic gases in the environment. Therefore, attention has currently dedicated in

production of biomass and bioenergy with the recognition that the global crude oil reserve is finite and its depletion is much faster than previously predicted (Betiku and Alade, 2010).

Biofuel production from the renewable sources (biomass) is advantageous for global energy security and environmental solutions because it poses a potential solution to energy shortage and climatic change (Hyun-Woo Kang *et al*., 2011). Bioethanol is an eco-friendly and efficient alternative for petroleum products. It can be used as automobile fuel additive and helps in decreasing greenhouse gases emission.

Currently, the ethanol is produced mainly from sugar, cellulose and starchy feedstocks for the global supply (Bai *et al*., 2008). A major drawback for ethanol production is high cost of raw materials for large scale expansion. Using agro – industrial residues (like cotton gin waste, paper industrial waste, sago waste etc) as substrate in bioethanol production will reduce cost of the production, since they are abundant, cheap and easily available. Ethanol can be used in automobiles as a fuel additive at the rate of 10%, which can decrease emission of carbon monoxide, and greenhouse gases. Ethanol can also replace methyl tertiary butyl ether (MTBE), an additive of gasoline, which is potentially toxic to human health. Furthermore, ethanol produced from lignocellulosic material is a domestic and renewable energy, which will reduce petroleum imports.

Industrial process generally involves gelatinization of starch-containing raw materials with steam, and subsequent liquefaction with α-amylase to dissolve and dextrinize starchy carbohydrates; this treatment is referred as cooking. Then, the resulting crude mash is saccharified with glucoamylase, and fermented with bacteria/yeast. Finally, the fermented mash is separated into alcohol and mash. Ethanol is concentrated using conventional distillation and then dehydrated using molecular sieves. Anhydrous ethanol is blended and is ready for shipment into fuel market (Olsen, 2001).

1.2 CASSAVA PLANT

Cassava plant is abundantly grown in the tropics as a supplementary staple food; but much of the produce is unutilized or underutilized due to its cyanide content. Utilization of cassava starch for ethanol production is one way of generating added economic return from this resource (Roble *et al.,* 2003).

Figure 1.1 Cassava plant

In South India, cassava (*Manihot esculenta*) is one of the major food crops, which is rich in starch. In Tamilnadu and Kerala, this crop cultivation is extensive (Figure 1.1). Cassava produces tuber called tapioca, which is used for the production of Sago, a starchy food substance. Sago production process releases huge waste such as knots, peels, fibrous waste and washings etc. which are rich sources of starch. These solid wastes are difficult to manage as they produce bad odour, high BOD and COD. As these wastes are rich in starch, an organic material, they can be intensively utilized for the bioethanol production (Sathya Geetha and Navaneetha Gopala Krishnan, 2009).

Cassava, a woody shrub of the Euphorbiaceae (spurge family) and native to South America, is extensively cultivated as an annual crop in tropical and subtropical regions for its edible starchy tuberous root, a major source of carbohydrates. It differs from the similarly-spelled yucca, an unrelated fruit-bearing shrub in the Asparagaceae family.

Cassava is the third largest source of food carbohydrates in the tropics (Claude and Denis, 1990). Cassava is a major staple food in the developing world, providing a basic diet for around 500 million people. Cassava is one of the most drought-tolerant crops, capable of growing on marginal soils. Nigeria is the world's largest producer of cassava. Cassava root is a good source of carbohydrates, but a poor source of protein. A predominantly cassava root diet can cause protein-energy malnutrition (FAO, 1995).

Cassava is classified as sweet or bitter. Like other roots and tubers, Cassava contains anti-nutrition factors and toxins (FAO, 1990). It must be properly prepared before consumption. Improper preparation of cassava can leave enough residual cyanide to cause acute cyanide intoxication and goiters, and may even cause ataxia or partial paralysis. Nevertheless, farmers often prefer the bitter varieties because they deter pests, animals, and thieves (Linley *et al.,* 2002). The more-toxic varieties of cassava are a fall-back resource in times of famine in some places (Ravindran and Velmerugu, 1992).

The cassava root is long and tapered, with firm and homogeneous flesh encased in a detachable rind, about 1mm thick, rough and brown on the outside. Commercial varieties can be 5 to 10 cm in diameter at the top, and around 15 cm to 30 cm long. A woody cordon runs along the root's axis. The flesh can be chalk-white or yellowish. Cassava roots are rich in starch, and contain significant amounts of calcium (50 mg/100 g), phosphorus (40 mg/100 g) and vitamin C (25 mg/100 g). However, they are poor in protein and other nutrients. In contrast, cassava leaves are a good source of protein (rich in lysine), but deficient in the amino acid methionine and possibly tryptophan (Olsen and Schaal, 1999).

1.3 ETHANOL PRODUCTION PROCESS

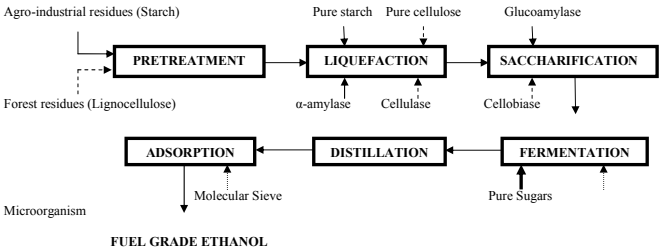

Figure 1.2 Ethanol production process

The ethanol production process from biomass includes four major stages (Figure 1.2). They are pretreatment, hydrolysis (liquefaction and saccharification), fermentation and purification (distillation and adsorption). The first stage is pretreatment, which helps in maximum utilization of the substrate. The second stage is hydrolysis, which converts the carbohydrates in the raw materials into sugars. The complication of conversion process depends on the carbohydrate types in the raw materials. The sucrose conversion is the easiest, while the lignocellulose is the most difficult among the three carbohydrates. The third stage is to convert the reducing sugars into ethanol by microbial fermentation, i.e. the monomeric sugars released from polysaccharides are used to produce ethanol in microbial fermentation through metabolic pathways. Many types of yeast and a few bacteria can convert glucose to ethanol. The most common yeast used in industrial processes is the genus *Saccharomyces*. Although yeasts have many of the attributes as an ideal ethanol producer, they have significant limitations, such as narrow substrate range (not taking up pentoses) and limited tolerance to ethanol. The final stage is to recover the ethanol by distillation and adsorption to make fuel grade ethanol for use.

1.3.1 AMYLOLYTIC MICROORGANISMS / ENZYMES

At present, there are about 30 different amylolytic and related enzymes (Janecek, 1997). Degradation of starch is essentially performed by the four groups of enzymes

(Guzman-Maldonado and Paredes-López, 1995): endoamylases, exoamylases, debranching enzymes and cyclodextrin glycosyl transferases. Endoamylases and exoamylases act primarily on α-1,4-linkages, debranching enzymes attack mainly the α-1,6-linkages, and cyclodextrin glycosyl transferases degrade starch by catalysing mainly cyclisation and disproportionate reactions (Figure 1.3).

Endoamylases cleave only the α-1,4-bonds in starch in the inner regions of the starch molecule by passing the α-1,6-branching points of amylopectin. The α-amylase (EC 3.2.1.1) is the best known endoamylase. It causes a rapid loss of viscosity of the starch solution. These enzymes are often divided, according to degree of hydrolysis of substrate, into two categories: liquefying (30 to 40%) and saccharifying (50 to 60%). This division is widely used to describe the properties of α-amylases (Vihinen and Mantsala, 1989). Thus the products of endoamylases are oligosaccharides of varying lengths.

Exoamylases also cleave the α-1,4-bonds, e.g. β-amylase (EC 3.2.1.2), but some of them are able to attack the α-1,6-bonds, e.g. glucoamylase (EC 3.2.1.3). These enzymes act externally on substrate bonds from the non-reducing end of starch and hence produce only low molecular weight products from starch, e.g. maltose and glucose, respectively (Wind, 1997).

Pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68) are the examples of debranching enzymes. Both are specific for α-1,6-bonds in starch (amylopectin) and related polysaccharides and branched limit dextrins. According to the inability or ability to degrade the α-1,4- glucosidic bonds, pullulanases are classified into two categories: pullulanase I and pullulanase II, respectively (Wind, 1997). Pullulanase type II is usually referred to as α-amylase, pullulanase or amylopullulanase. However, to make it clear the specificity should be proved which enzyme it refers to.

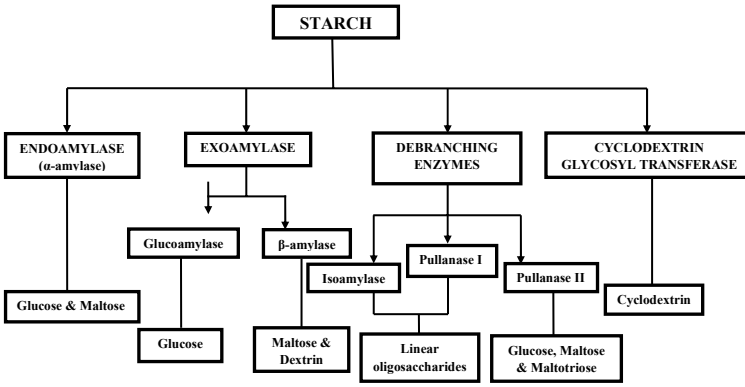

Figure 1.3 Action of starch degrading enzymes

Table 1.1 Characteristics of various amylolytic microorganisms used for starch hydrolysis

ENZYME	TYPE	SOURCE	AMOUNT	ACTION
α-amylase	Liquefying	*B.subtilis*	0.06% (w/w) of starch	Decreases viscosity (cleaves α-1,4, pH 5.5, 70°C)
		B.licheniformis	0.06% of starch	Decreases viscosity (92°C)
Glucoamylase	Saccharifying	*A.niger*	0.18% of starch (1.7 L/ton)	Generates glucose (cleaves α-1,6, pH 5.0, 60°C)

The fourth group of starch-degrading enzymes is cyclodextrin glycosyl transferases (CGTases, EC 2.4.1.19). They produce cyclodextrins from starch, the rings which are composed of 6, 7 or 8 glucose units bound by α-1,4- bonds (Pócsi, 1999). The CGTases

catalyse intra and intermolecular reaction of glycosyl transfer (Svensson & Søgaard, 1993). Table 1.1 shows some characteristics of the amylolytic microorganisms most commonly used for starch hydrolysis (Fiechter, 1992; Olsen, 2001)

1.3.2 CELLULOLYTIC MICROORGANISMS / ENZYMES

The important parameters in the hydrolysis (the yield and concentration of sugars, the duration of hydrolysis and the enzyme loading) are all interrelated, and obviously depend on both the quantity of cellulose and those of the enzyme cellulase. In the economic exploitation of cellulose to ethanol technology, effort is required to increase the activities and productivities of the cellulase enzyme thus play a significant role. The process of cellulose breakdown to sugars involves participation of three major classes of cellulases: (i) endoglucanases, which randomly split cellulose polymer, (ii) exoglucanases, which include glucan hydrolases that preferentially liberate glucose or glucose dimer units (cellobiose) from cellulose chain ends and cellobiohydrolases that preferentially liberate cellobiose from the end of the cellulose chain, and (iii) β-glucosidase, which catalyzes the hydrolysis of cellobiose and soluble cellodextrins to glucose.

Trichoderma reesei, the most studied fungus for cellulases, has been shown to possess two genetically distinct endoglucanases, two cellobiohydrolases and one β-glucosidase. These enzymes work synergistically, which results in efficient decrystallization and hydrolysis of native cellulose. *T. reesei* cellulase has been found to contain two distinct domains: a catalytic domain and a cellulose binding domain. These domains are joined by an extended flexible region. The cellulose binding domain contributes to maintaining a high concentration of cellulose on the solid cellulosic surface and is believed to interact preferentially with the crystalline region in cellulase. The catalytic domain, on the other hand, has a high affinity for the amorphous region. The flexible hinge enables the cellulose binding domain to attach the enzyme to the cellulose fiber with little restriction on the interaction of the catalytic domain with the cellulose (Glazer and Nikaido, 1995). The catalytic domain without the cellulose binding domain has a very limited overall action on cellulose.

The cellulolytic microorganisms produce a family of different cellulases with different specificities. Cellulases differ not only in the action mode, but also in the way they bind to the crystalline surface of the substrate. Because of the complexity of multiple and simultaneous actions of cellulase enzyme system, particularly those of fungal origins, the

Commission of Biotechnology, IUPAC took the initiative to propose a Unified Assay System of the most important enzymes of the cellulase complex. Methods recommended in the Commission Report (Ghose, 1987) form the basis for the comparison of cellulase activities reported by a large number of academic and industrial laboratories engaged in studies on cellulose saccharification. These are considered as global reference methods.

Cellulases are generally produced by submerged fermentation using the fungi *Trichoderma, Humicola*, *Aspergillus* and *Penicillium*. Cellulase synthesis in *Trichoderma* is governed by both induction as well as catabolite repression. While soluble sugars (cellobiose, sophorose, lactose) induce cellulase synthesis, the insoluble substrate cellulose has been found to be the best inducer to produce cellulases capable of degrading crystalline cellulose. Various approaches that have been suggested as a possible way of lowering the enzyme-associated costs include genetic improvement through mutagenesis, optimization of fermentation conditions, use of mixed fungal cultures and addition of surfactants and charged colloid materials. In the past 3 decades, a more than 200-fold increase in cellulase productivity has been reported (Tolan and Foody, 1999). It is generally believed that the enzyme hydrolysis process does not currently provide adequate profit margins for suppliers at an enzyme cost that the selling price of ethanol can support.

An assessment of cellulase enzyme availability for cellulose hydrolysis leads to the following conclusions: (i) a cellulase enzyme for cost effective bioethanol production from cellulose is not available, (ii) cost effective cellulase enzyme could be developed with R & D efforts of enzyme manufacturers and ethanol manufacturers and (iii) industry does not appear likely to take the lead in developing a cost effective enzyme unless catalyzed to do so. The current cost of producing the enzyme at US $ 0.25–0.45 per gallon of ethanol is considered not economical (http://www.ceassist.com/pdf/ cellulase.pdf).

The most desirable attributes of cellulases include the ability to produce a complete cellulase system with high specific activity and high catalytic activity against crystalline cellulose, thermal stability and decreased susceptibility to enzyme inhibition by cellobiose and glucose, selective adsorption on cellulose and ability to withstand shear forces. Suggested strategies to improve cellulases include discovering new enzymes through bioprospecting, creating new/better mixtures of enzymes and developing greatly improved expression systems through protein engineering (Sheehan and Himmel, 1999). Creating a more effective

cellulose binding domain in the enzyme molecule could be another approach to increase enzyme efficiency. There are reports on cloning and expression of cellulose genes from *T. reesei* and other cellulolytic organisms in *E. coli* and *S. cerevisiae* as well as other hosts. Protein engineering of cellulases may enable alteration of catalytic sites to produce hypercellulolytic mutants with greatly enhanced activities (Godbole *et al.,* 1999; Himmel *et al.,* 1999).

Improvements in specific activity of the enzyme are being attempted using the following strategies: increased thermal stability, decrease in non-specific binding, decrease in feedback inhibition, increase in enzyme turn-over, and enzymatic decrystallization enhancement. Among these options, thermostability enhancement holds the greatest potential with 3 to 5 times improvement in specific activity. The United States Department of Energy has launched programmes for low-cost cellulase development by Genencor and Novozyme. An important consideration in this regard is the possibility of producing transgenic plant cellulases (http://bioenergy. ornl.gov/99summaries/cellulase.html).

1.3.3 ETHANOLOGENIC MICROORGANISMS

The yeast *Saccharomyces cerevisiae*, due to its large size, thick cell wall, resistance to bacterial and viral infection and its ability to produce a high ethanol yield, has found greater acceptance for and use in industrial ethanol production. The bacteria *Zymomonas mobilis* produces ethanol at a much faster rate than the yeast and has a higher osmotolerance and alcohol tolerance. Both *Saccharomyces cerevisiae* and *Zymomonas mobilis* can ferment only glucose, fructose and sucrose. On the other hand, *E. coli* utilizes all the sugars (glucose, mannose, xylose, arabinose and galactose), but lacks two key enzymes, namely, pyruvate decarboxylase and alcohol dehydrogenase, required for ethanol production. It is economically important that all sugars, hexoses and pentoses, obtained from biomass are used as substrate for ethanol production.

Microorganisms that ferment both hexoses and pentoses have not been found in nature. Efforts are therefore being made by researchers to construct organisms that can meet these requirements. The two main approaches to genetically engineer a microorganism for ethanol production are (i) insertion of genes into a potent ethanol producing organism, such as *Saccharomyces* and *Zymomonas*, to enlarge its substrate utilization range and (ii) insertion of ethanologenic traits into an organism capable of multiple substrate utilization, e.g. *E. coli*.

A number of yeasts (e.g. *Pichia stipitis*, *Pachysolen tannophilus*, *Candida shehate*) ferment xylose to ethanol. *P. stipitis* is known to be the best naturally occurring xylose fermenting organism. Specific ethanol productivities of xylose fermenting organisms are relatively much lower than those obtained with *Saccharomyces* and *Zymomonas* with glucose. Xylose fermenting yeasts also have low ethanol tolerance and xylose conversion is affected in the presence of glucose due to catabolite repression.

S. cerevisiae and *Z. mobilis* can utilize xylulose but not xylose. *Saccharomyces* and *Zymomonas*, however, follow different pathways to convert xylose to xylulose. *Saccharomyces* converts xylose to xylulose via xylitol through xylose reductase (Xr) and xylose dehydrogenase (Xd), whereas *Zymomonas* uses xylose isomerase (Xi) for the catabolism of xylose. The metabolism of xylulose to xylulose-5-P is catalyzed by xylulokinase (Xk). Xylulose-5-P is further metabolized to glyceraldehyde-3-P and fructose-6-P by the pentose phosphate (PP) pathway. These intermediates are converted to pyruvate in the Embden-Meyerhoff-Parnas (EMP) or Entner-Duodoroff (ED) pathway. Pyruvate is converted to acetaldehyde by pyruvate decarboxylase (Pd) which is further reduced to ethanol by alcohol dehydrogenase (Ad) (Chandrakant and Bisaria, 1998).

E. coli utilizes all the major sugars and is believed to be least burdened with genetic modification. This makes it a potential organism for ethanol production. Ingram's group (Ingram and Conway, 1988) has done extensive work on the development of recombinant *E. coli* for ethanol production. *E. coli*, genetically engineered to contain the PET operon (*Z. mobilis* PD and AD genes), produced high levels of ethanol. *E. coli* strains were constructed by transforming conditionally lethal *E. coli* with PET operon plasmid to overcome the requirement of antibiotics in the media and to improve genetic stability (Hespell *et al.,* 1996). The strains were capable of anaerobic growth and displayed no apparent plasmid losses after 60 generations. The PD gene from *Z. mobilis* and the AD gene from *Bacillus stereothermophiles* were identified to construct a thermophilic (60°C), gram-positive ethanologen (Ingram, 2000).

Ideally, for an ethanol fermenting organism, the following characteristics are desirable: high ethanol yielding capacity and productivity, high ethanol tolerance, capability to ferment a broad range of sugars, resistance to inhibitory compounds (e.g. acetic acid, furfural, hydroxymethylfurfural, lignin degradation products etc. present in the pretreatment

product stream), production of a low level of byproducts (e.g. acids and glycerol), ability to withstand high osmotic pressure (due to high sugar concentration), high temperature and low pH tolerance, high cell viability for a repeated cell recycling and appropriate flocculation and sedimentation characteristics to facilitate cell recycle (Picataggio and Zhang, 1996). Serious research efforts are needed in India to design genetically engineered ethanologens for their use in conventional fermentation as well as for future application in cellulose-based bioethanol technologies.

1.4 MOTIVATION

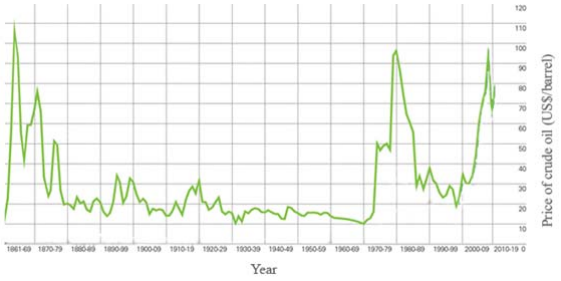

Figure 1.4 History of crude oil price from 1860 to 2012

In many countries, significant research has begun to evaluate the use of cassava as an ethanol feedstock. Under the Development Plan for Renewable Energy in the Eleventh Five-Year Plan in the People's Republic of China, the target is to increase the application of ethanol fuel by non-grain feedstock to 2 million tonnes, and that of biodiesel to 200 thousand tonnes by 2010. This will be equivalent to a substitute of 10 million tonnes of petroleum. As a result, cassava (tapioca) chips have gradually become a major source for ethanol production. On December 22, 2007, the largest cassava ethanol fuel production facility was completed in Beihai, with annual output of 200 thousand tons, which would need an average of 1.5 million tons of cassava. In November 2008, China-based Hainan Yedao Group reportedly invested $51.5m in a new biofuel facility that is expected to produce 33 million US gallons (120,000 m^3) a year of bioethanol from cassava plants (http://biowealthresourceltd.com/cassava.html).

1.5 OBJECTIVES

The major objectives of this work are

1. To characterize plant and industrial residues of cassava (cassava stem, cassava twig, cassava leaves, cassava peel and cassava waste) to test their potential to convert to bioethanol, a petrol substitute
2. To carry out preliminary experiments for screening parameters like particle size of substrate, pretreatment methods, hydrolytic methods and fermentative organisms
3. To study the growth curve of *Zymomonas mobilis* to determine its doubling time
4. To screen the process parameters by Plackett-Burman method for investigating the dependence of response on a number of factors, each taking two levels
5. To optimize ethanol production by Box-Behnken method to study the quadratic effect of factors after identifying the significant factors using Plackett-Burman method
6. To perform kinetic and thermodynamic studies of ethanol production
7. To design a packed bed reactor for continuous ethanol production

CHAPTER 2
LITERATURE REVIEW

2.1 GENERAL

Adelekan (2010) investigated the ethanol productivity of cassava crop in a laboratory experiment by correlating volumes and masses of ethanol produced to the masses of samples used. Cassava tubers (variety TMS 30555) were peeled, cut and washed. 5, 15, 25 and 35 kg samples of the tubers were weighed in three replicates, soaked in water for a period of a day, after which each sample was dried, crushed and the mash mixed with 500 ml of n-hexane (C_6H_{14}). This crushed mash was then allowed to ferment for a period of 8 days and afterwards pressed on a 0.6 mm aperture size and sieved to yield the alcohol present in it. The alcohol was heated at 79°C for 5 h at intervals of 1 h followed by 1 h cooling. Ethanol yield was at average volumes of 0.31, 0.96, 1.61 and 2.21 litres, respectively, for the selected masses of cassava samples. Quantitative relationships were obtained to relate the masses of cassava used to the masses and volumes of ethanol produced. These were used to relate known production values of cassava from tropical countries to ethanol that can be potentially produced. The ethanol had boiling point of 78.5°C and relative density of 0.791. The dried mash was found to contain 61.8 calories of food energy per 100 g. This study found that a total of 6.77 million tonnes or 1338.77 million gallons of ethanol are available from total cassava production from tropical countries.

Ado *et al. (*2009) studied the production of ethanol by co-cultures of *A. niger* (GS4) and *S.cerevisiae* (BK6) using cassava starch as substrate. At 1% substrate concentration ethanol yield was 0.35 g/100 mL while the ethanol concentration increased to a maximum of 3.60 g/100 mL at 8% substrate concentration. When the culture conditions were optimized, the ethanol yield further increased to 4.30 g/100 mL at a temperature of 35°C, pH 5.0, 300 rpm agitation rate and reduced fermentation period of 4 days.

Aggarwal *et al. (*2001) prepared α-amylase commercially and obtained Biotempase from Biocon India Pvt. Ltd., and crude glucoamylase produced from *Aspergillus sp.* NA21 were used to hydrolyse tapioca powder, a non-conventional starchy substrate. Among various

concentrations of starch (15–35%, dry weight/volume) studied for maximum liquefaction; slurry made with 25% substrate concentration proved optimal. An economical process of liquefaction was carried out using steam under pressure (0.2–0.3 bar, 104–105°C) to liquefy 25% slurry in just 45 min, contrary to a slower process carried out at 95°C in a water bath. For liquefaction of starch, pH of 5.0 proved to be optimum. The dose of Biotempase, as prescribed by the supplier, could be reduced by 33% achieving the same degree of liquefaction, by addition of $CaCl_2$ to the starch slurry at the concentration of 120 mg/L. The conditions for the saccharification of liquefied starch were optimized to be 60°C and pH 5.0, producing 90% saccharification in 24 h. Supplementation of divalent ions Ca^{2+}, Mg^{2+} and Zn^{2+} in the process of saccharification showed no effect. Finally, glucose was found to be the main hydrolysis product in the saccharification of tapioca starch.

Amutha and Gunasekaran (2001) produced a high ethanol concentration with coimmobilized cells of *Saccharomyces diastaticus* and *Zymomonas mobilis* and compared to immobilized cells of *S. diastaticus* during batch fermentation of liquefied cassava starch. The co-immobilized cells produced 46.7 g/L ethanol from 150 g/L liquefied cassava starch, while immobilized cells of yeast *S. diastaticus* produced 37.5 g/L ethanol. The concentration of ethanol produced by immobilized cells was higher than that by free cells of *S. diastaticus* and *Z. mobilis* in mixed-culture fermentation. In repeated-batch fermentation using coimmobilized cells, the ethanol concentration increased to 53.5 g/L. The co-immobilized gel beads were stable up to seven successive batches. Continuous fermentation using coimmobilized cells in a packed bed reactor operated at a flow rate of 15 mL/h (residence time, 4 h) exhibited a maximum ethanol productivity of 8.9 g/L.h.

Azlin *et al. (*2009) conducted preliminary experiment to test the hypothesis that coculturing commercialised ragi tapai with *S. cerevisiae* might have improved the ethanol production and reduce the accumulation of inhibitory concentration of reducing sugar and this would enhance the amylotic activity. Coculture of a commercialized ragi tapai with *S. cerevisiae* using unhydrolysed raw starch in single step fermentation produced a high ethanol concentration. 35.26 g/L of ethanol was produced when the starch inoculated with ragi tapai and cocultured it with *S. cerevisiae* after 1 h. This was 48% higher than the yield obtained when the starch was inoculated with only ragi tapai (23.79 g/L). The glucose concentration was maintained at low concentration in the coculture medium compared to the

medium with ragi tapai only. However, stachyose concentration profile was higher in cocultured to one with ragi tapai only. In conclusion, cocultured ragi with *S.cerevisiae* enhanced the ethanol production and prevented the inhibitory effect of reducing sugar on amylase activity.

Betiku and Alade (2010) evaluated different fermentation media for bioethanol production using *Saccharomyces cerevisiae*. Three different nitrogen sources, concentration of carbon source (cassava starch hydrolysate, CSH) and the pH were combined to generate nine different fermentation media which were run in shaker flasks. Analysis of variance of the results from shake flask experiments showed that there was no significant difference ($P > 0.05$) in the maximum concentration of ethanol produced from the nine media after 24 h fermentation. From the bioreactor studies using minimal medium containing only ammonium sulphate as nitrogen source, final ethanol concentration of 55.28 g/L (process product yield coefficient ($Y_{P/S}$) of 0.49 g ethanol/g substrate and 96% (Y_{EtOH}) ethanol yield (% theoretical), specific productivity (Q) of 1.00 g/g.h, volumetric productivity of ethanol (Q_P) of 1.94 g/L.h, volumetric substrate consumption rate (Q_S) of 4.98 g/L.h and biomass yield coefficient ($Y_{X/S}$') of 0.11 g cell/g substrate (specific growth rate, μ, of 0.11 h^{-1}) were obtained. The possibility of substituting ammonium sulphate for yeast extract in fermentation medium for bioethanol production from cassava starch hydrolysate (CSH) was confirmed.

Bundit *et al. (*2010) utilized cassava waste as a potential source to produce ethanol due to its containing cellulose, hemi-cellulose and starch at levels of 24.99, 6.67 and 61% (w/w), respectively. The bioconversion of cassava waste into sugar by using microbial enzyme was carried out. The experiment using of two fungal strains, *Aspergillus niger* TISTR 3352 which produce alpha-amylase for conversion of starch into sugar and *Trichoderma reesei* TISTR 3080 which produce cellulase for conversion of cellulose into sugar. The conversion of cassava waste starch by *A. niger* was conducted in submerge cultivation. *A. niger* at 10% (v/v) inoculum (10^7 spore/mL) was inoculated in cultivation medium containing of 45% (w/v) cassava waste, supplemented with 0.02% (w/v) ammonium sulfate, adjusted initial pH to 5.5. After cultivation at 30°C with shaking speed of 200 rpm for 72 h, the maximum yield of reducing sugar was 56.2 g/L. In the same way, conversion of cassava waste-lignocellulose into sugar by *T. reesei* was conducted in similar system. *T. reesei* at 10% (v/v) inoculum (108 spore/mL) was inoculated in cultivation medium

containing of 15% (w/v) lignocellulose, supplemented with 0.02% (w/v) ammonium sulfate, 0.04% (w/v) potassium dihydrogen phosphate, 0.025% (w/v) magnesium sulfate and 0.05% (w/v) yeast extract, adjusted initial pH to 6.0. After cultivation at 35°C with shaking speed of 150 rpm for 72 h, the maximum yield of cellulase was 0.3 U/mL. The fungal cellulase properties were investigated. The optimum temperature, pH and conversion period for bioconversion were 60°C, 6.0, and 6 h, respectively.

Chul-Ho *et al. (*1992) studied a simultaneous saccharification of sago starch and ethanol fermentation in batch and semi-batch modes, by an enzyme, amyloglucosidase, and a bacterium, *Zymomonas mobilis* on pilot-scale. In the batch SSF process using a 72 litre stirred-tank fermentor, the final ethanol concentration obtained from 20% (w/w) sago starch was 92.6 g/L with 97.4% of the theoretical ethanol yield. In 100 and 500 litre airlift fermentors equipped with systems for self-mixing by CO_2 recycle, the fermentation kinetics were similar to those obtained from the stirred-tank fermentor. Semi-batch SSF processes using a cell-recycle system by ultrafiltration gave about 80% increase of ethanol productivity compared with batch SSF processes.

Eneojo *et al. (*2010) identified yeast strain isolated from rotten Irish potato, as *S. cerevisiae*. It was characterized biochemically and morphologically and found to be identical with the standard strain of *S. cerevisiae*. The isolate potential for ethanol production from cassava starch was analyzed and found to produce ethanol from starch under natural environmental conditions. The optimal pH was found to 3.5-3.7 with a directly proportional increase in ethanol and glucose concentration (Mean ± SD). The wild strain of *S. cerevisiae* possessed ability to degrade starch, thus salvaging the cost of purchasing starch degrading enzymes in ethanol production. The present study hypothesized that certain wild strain of *S. cerevisiae* could posses the ability to produce starch degrading enzymes in their natural environment and as such could produce both starch and ethanol.

Gi-Wook *et al. (*2008) investigated the optimal conditions of ethanol fermentation process by *Zymomonas mobilis* CHZ2501. Brown rice, naked barley, and cassava were selected as representatives of the starch-based raw material commercially available for ethanol production. Considering enzyme used for saccharification of starch, the ethanol productivity with complex enzyme was higher than glucoamylase. With regards to the

conditions of saccharification, the final ethanol productions of simultaneous saccharification and pre-saccharified process for 1 h were not significantly different. The result suggested that it is possible for simultaneous saccharification and fermentation as a cost-effective process for ethanol production by eliminating the separate saccharification. Additionally, the fermentation rate in early fermentation stage was generally increased with increase of inoculum volume. As the result, optimal condition for ethanol production was simultaneous saccharification and fermentation with complex enzyme and 5% inoculation. Under the same condition, the volumetric productivities and ethanol yields were attained to be 3.26 g/L.h and 93.5% for brown rice, 2.62 g/L.h and 90.4% for naked barley, and 3.28 g/L.h and 93.7% for cassava, respectively.

Kim and Rhee (1993) developed various simultaneous starch sacchariflcation and ethanol fermentation (SSSF) processes and evaluated on batch, semibatch and continuous modes of fermentation in laboratory and pilot scale fermentors using *Zymomonas mobilis*. Compared with a two-step process involving separate saccharification and fermentation stages, the SSSF reduced the total process time by half. In order to produce ethanol from saga starch economically, the immobilization of amyloglucosidase and *Z. mobilis* cells was studied. Among the various immobilization methods tested, a co-immobilized system using chitin and sodium alginate appeared most promising with respect to ethanol productivity and operational stability. In order to scale-up the SSSF process, large scale SSSF processes were studied in batch and semibatch fermentation modes. In pilot scale SSSFs, the kinetic results were found to be similar to those from laboratory scale fermentation. Finally, a mathematical model was described for the SSSF process. Simulations of glucose concentration and other fermentation parameters agreed closely with experimental results.

Kim *et al. (*1988) studied immobilization of amyloglucosidase (AMG) and *Zymomonas mobilis* cells in order to produce ethanol from sago starch economically. Among various immobilization methods tested, a coimmobilized system using chitin and sodium alginate appeared to be most promising with respect to ethanol productivity and operational stability. When the system was run in the continuous simultaneous saccharification and fermentation (SSF) mode, the maximum ethanol productivity was found to be 72.2 g/L.h at a dilution rate of 3.28 h^{-1}. This system could be run in the stable manner over 40 days with a steady-state ethanol concentration of 44 g/L and an ethanol conversion yield of 93%.

Ku *et al. (*2008) discussed the thermo-enzymatic hydrolysis of *Manihot esculenta* (cassava) starch in order to produce sufficient glucose for ethanol production. One of the greatest challenges for the 21st century society is to meet the growing demand for energy for transportation in a sustainable way. Petroleum based products are in critical state and alternatives for fuel is needed. One of the alternatives is ethanol, which adds octane value to the gasoline blend and provides a clean burning. In this study, the favourable starch concentration for the hydrolysis was found to be 30% with the addition of 0.25% α-amylase for liquefaction and 0.15% amyloglucosidase for saccharification. This combination resulted in producing 204.5 g/L glucose. For fermentation condition, a 30% slurry resulted better glucose consumption by *S. cerevisiae* than the 20% slurry. However, the 10% inoculum was considered inadequate for the conversion of glucose to ethanol.

Kumoro *et al. (*2008) studied the hydrolysis of dried-powdered fibrous sago waste by sulphuric acid and glucoamylase. Both studies were carried out in Erlenmeyer flask placed in a controlled temperature water bath. Samples were taken from the reaction flask at every 30 min interval for reducing sugar determination. The optimum condition for acid hydrolysis was found to be at 90°C, using 1.5 M acid concentration and reaction time of 120 min yielding 0.6234 g glucose/g waste. The kinetic parameters of acid hydrolysis in the Saeman's model, were the rate constant (k_1 = 0.01405 l/min), activation energy (E_a = 120.40 KJ/mol) and pre-exponential factor (A = 9.52×10^{16} l/min). The optimum condition for enzymatic hydrolysis using glucoamylase was found to be at enzyme concentration of 6 AGU m/L and reaction time of 30 min, yielding 0.5646 g Glucose/g Waste. The kinetic parameters in the competitive inhibition model corresponding to the optimum condition, namely the equilibrium constant for enzyme-inhibitor complex, Michaelis-Menten constant and maximum velocity, are 1.4727, 0.24175 and 1.35460 g min/L, respectively.

Lee *et al. (*1986) studied Simultaneous Saccharification and ethanol Fermentation (SSF) of sago starch using amyloglucosidase (AMG) and immobilized *Zymomonas mobilis* ZM4 on sodium alginate. The immobilized *Zymomonas* cells were more thermo-stable than free *Zymomonas* cells in this system. The optimum temperature in the SSF system was 40°C, and 0.5% (v/w) AMG concentration was adopted for the economical operation of the system. The final ethanol concentration obtained was 68.3 g/L and the ethanol yield, Y was 0.49 g/g (96% of the theoretical yield). After 6 cycles of reuse at 40°C with 15% sago starch

hydrolysate, the immobilized *Z. mobilis* retained was about 50% of its ethanol fermenting ability.

Lee *et al. (*1987) produced ethanol more economically with high productivity and low production cost. To this end, a continuous ethanol production from sago starch using immobilized amyloglucosidase (AMG) and *Zymomonas mobilis* cells was studied. Chitin was used for immobilization of AMG and *Z. Mobilis* cells were immobilized in the form of sodium alginate beads. Ethanol was produced continuously in a simultaneous saccharification and ethanol fermentation (SSF) mode in a packed bed reactor. The maximum ethanol productivity based on the void volume was 37 g/L.h with ethanol yield of 0.43 g/g (84% of theoretical ethanol yield) in this system. The steady-state concentration of ethanol (46 g/L) could be maintained in a stable manner over two weeks at the dilution rate of 0.46 h^{-1}.

Leticia *et al. (*2010) optimized the process for producing ethanol from cassava starch based on 22 experimental designs with three central points, using statistical software. This methodology was applied to the stage of saccharification of cassava starch by acid hydrolysis as well as to the stage of fermentation using *Saccharomyces cerevisiae*. From the experimental data of acid hydrolysis, they proposed a first-order kinetic model which presented an average error of 1.87% compared to the quadratic regression obtained. The development of a semi-continuous process showed 89.84% conversion of starch initially considered, yielding an ethanol concentration of 49.76%.

Madihah *et al. (*2001) studied direct fermentation of gelatinized sago starch into solvent (acetone-butanol-ethanol) by *Clostridium acetobutylicum* P262 using a 250 mL Schott bottle anaerobic fermentation system. Total solvent production from fermentation using 30 g sago starch/L (11.03 g/L) was compared to fermentation using corn starch and about 2-fold higher than fermentation using potato or tapioca starch. At the range of sago starch concentration investigated (10-80 g/L), the highest total solvent production (18.82 g/L) was obtained at 50 g/L. The use of a mixture of organic and inorganic nitrogen source (yeast extract + NH_4NO_3) enhanced growth of *C. acetobutylicum*, starch hydrolysis and solvent production (24.47 g/L) compared to the use of yeast extract alone. This gave the yield based on sugar consumed of 0.45 g/g. Result from this study also showed that the individual concentration of nitrogen and carbon influenced solvent production to a greater extent than carbon to nitrogen (C/N) ratio.

Mariamma and Muraleedhara (1997) studied the saccharification of pretreated tapioca waste and water hyacinth under two different conditions: using microbial enzymes (cellulase from *Myrothecium verrucaria, Coprinus comatus, Pleurotus florida,* and *Cellulomonas* sp.) and solid-state fermentation. The rate of saccharification was determined at different temperatures, pH, substrate concentration, and incubation period. It was found that as the source of the enzyme varies, the optimal temperature and pH for the saccharification varies. Among the two different treatments, enzymatic saccharification was found to be the most efficient. Among the various cellulase sources tested, *M. verrucaria* cellulase was found to be the most efficient one followed by *C. comatus, P. florida,* and finally *Cellulomonas* sp.

Muhamad (2009) produced bioethanol from tapioca starch by involving two processes which are enzymatic hydrolysis and microbial fermentation. The objective of the study was to investigate the influence of temperature and agitation speed on the production of bioethanol from tapioca starch using *Saccharomyces cerevisiae*. The fermentation was conducted under various temperatures (30, 35 and 37°C) and agitation speeds (100, 200 and 300 rpm) in 250 mL shake flask. The cell density, glucose consumption and ethanol concentration were analyzed. The ethanol concentration in the fermentation broth increased rapidly with the increase in temperature and agitation speed. The high temperature resulted in the higher cell density and higher glucose consumption. The high agitation speeds also preferred for both cell density and glucose consumption. The maximum ethanol concentration of 57.8 mg/L was obtained at a temperature of 35°C and 200 rpm of agitation speed.

Raman and Pothiraj (2008) efficiently produced ethanol using *Zymomonas mobilis* (NRRL B808) and *Saccaromyces cerevisiae* (NRRL Y898) by utilizing the sago industry waste (Tippi), was studied in the liquid state fermentation process. The fermentation parameters for ethanol production were optimized for various pH and temperature ranges. Both strains of *Z. mobilis* and *S. cerevisiae* were selected for ethanol production potential at optimized conditions because *Z. mobilis* can tolerate in higher sugar concentration (more than 15%) and also *S. cerevisiae* is higher ethanol tolerant. Ethanol production at pH 6.0 and 36 h (residence time) of fermentation was the highest in the *Z. mobilis* mediated fermentation (5% more) than the ethanol production rate of *S. cerevisiae*.

Rhee *et al* (1984) evaluated the feasibility of substrate, cassava starch, for ethanol production using *Zymomonas mobilis* ZM4 strain. Before fermentation, the starch materials were pretreated employing two commercial enzymes, Termamyl (thermostable α-amylase) and AMG (amyloglucosidase). Using 2 l/g of Termamyl and 4 l/g of AMG, effective conversion of both cassava and sago starch into glucose was found with substrate concentration up to 30% (w/v) dry substances. Fermentation study was performed using these starch hydrolysates as substrates resulted in ethanol yield at an average of 0.48 g/g by *Z. mobilis* ZM4.

Roble *et al. (*2003) constructed a Circulating Loop Bioreactor (CLB) with cells immobilized in loofa sponge for simultaneous aerobic and anaerobic processes. The CLB consists of an aerated riser and a non-aerated downcomer column connected at the top and bottom by cylindrical pipes. Ethanol production from raw cassava starch was investigated in the CLB. *Aspergillus awamori* IAM 2389 and *Saccharomyces cerevisiae* IR2 immobilized on loofa sponge were placed, respectively, in the aerated riser column and non-aerated downcomer column. Both α-amylase and glucoamylase activities increased as the aeration rate was increased. Ethanol yield and productivity increased with an increase in the aeration rate up to 0.5 vvm, but decreased at higher aeration rates. The CLB was operated at an aeration rate of 0.5 vvm for more than 600 h, resulting in an average ethanol productivity and yield from raw cassava starch of 0.5 g.Ethanol/L.h and 0.45 g.Ethanol/g.Starch, respectively. In order to increase ethanol productivity, it was necessary to increase the Dissolved Oxygen (DO) concentration in the riser column and decrease the DO concentration in the downcomer column. However, increasing the aeration rate resulted in increase in the DO concentration in both the riser and the downcomer columns. At high aeration rate, there was no significant difference in the DO concentration in the riser and downcomer columns. The aeration rate was therefore uncoupled from the liquid circulation by attaching a time controlled valve in the upper connecting pipe. By optimizing the time and frequency of valve opening, and operation at high aeration rate, it was possible to maintain a high DO concentration in the riser column and a low DO concentration in the downcomer column. Under these conditions, ethanol productivity increased by more than 100%, to 1.17 g/L.h.

Saifuddin and Refal (2011) investigated enzymatic hydrolysis of sago starch by various amylolytic enzymes to reveal the potential coupling mechanism of Microwave Irradiation-Enzyme Coupling Catalysis (MIECC). Environmental issues such as global

warming and recent events throughout the world, including the shortage of petroleum crude oil, the sharp increase in the cost of oil and the political instability of some crude oil producing countries, have demonstrated the vulnerability of the present sources for liquid fuel. These situations have created great demand for ethanol from fermentation process as green fuel. A main challenge in producing the ethanol is the production cost. A rapid and economical single step fermentation process for reliable production of bioethanol was studied by co-culturing commercialized ragi tapai with *Saccharomyces cerevisae* using raw sago starch. It was shown that enzymatic hydrolysis of starch using typical enzymes may successfully be carried out at microwave condition. The MIECC resulted in increasing initial reaction rate by about 2 times. The results testify on specific activation of enzymes by microwave and prove the existence of non-thermal effect in microwave assisted reactions. Low power microwave irradiation (80 W) does not increase the temperature beyond 40°C and hence denaturation of the enzyme is avoided. The maximum ethanol fermentation efficiency was achieved (97.7% of the theoretical value) using 100 g/L sago starch concentration. The microwave assisted process improved the yield of ethanol by 45.5% compared to the non-microwave process. Among the other advantages of co-culturing of ragi tapai with *S. cerevisiae* is the enhancement of ethanol production and prevention of the inhibitory effect of reducing sugars on amylolytic activity and the reaction could be completed within 32±1 h. The present study have demonstrated the ability of using cheaply and readily available ragi tapai, for conversion of starch to glucose and the utilization of sago starch as a feed stock, which is cheaper than other starches like corn and potato. The present study highlighted the importance of well controlled microwave assisted enzymatic reaction to enhance the overall reaction rate of the process.

Teerapatr *et al*. (2004) used cassava residue, starch-processing waste from cassava starch plant, as a raw material in ethanol production. The experiment was performed into two steps: (1) enzymatic hydrolysis, the step in conversion of cellulosic materials and starch to fermentable sugar, and (2) ethanol fermentation, the step in conversion of fermentable sugar to ethanol by *Saccharomyces cerevisiae* TISTR 5596. Cassava residues were hydrolyzed by mixed-enzyme of cellulase and pectinase at 28°C for 1 h then by α-amylase at 100°C for 2 h and finally glucoamylase at 60°C for 4 h. It was found that cassava residue with initial concentration of 11% (w/v) non-water-soluble carbohydrate could yield 122.4 g/L of reducing sugars. The increased amount of produced reducing sugars resulted from synergistic

action of cellulase, α-amylase and glucoamylase. Pectinase did not directly promote the hydrolysis but its mixer with cellulase helped to reduce viscosity and eased for the process of solid filtration. The optimum ethanol concentration obtained was 3.62% (w/v), correspondent to 91% of theoretical yield, after 24 hours fermentation at initial reducing sugars concentration 89.2 g/L. The production cost of one liter of ethanol using cassava residues as a raw material was 1.5 fold higher than the one from cassava root. Considering the expense of cassava waste management either due to land seeking/purchasing or landfill operation could be decreased up to 81%. Moreover, over 1.5 million tons of cassava residues produced every year in Thailand could use to produce 97.6 million liters of fuel ethanol.

Thalisa (2010) investigated the ethanol production from cassava starch by co-culture of selected fungi and *S. cerevisiae*. Firstly starch hydrolysis fungi were isolated and screened from tan-Koji (loog-pang). Enzymatic activities of 10 isolated *Rhizopus sp*. strains were determined on 0.1% starch agar plate at various pH (3-10) as primary screening/ clear zone diameter was occurred at pH ranging from 3 to 8 and no response at pH 10. The highest clear zone diameter that were found from 2 strains of *Rhizopus sp.* #2Bu and *Rhizopus sp.* #3Su at pH 4 were nearly equal. The results showed that *Rhizopus sp.* #3Su has the maximum efficiency. The highest reducing sugar yield was 25.9% from 6% cassava starch medium after 72 h. Ethanol was produced by SSF process, using co-culture of *Rhizopus sp.* #3Su and *Saccharomyces cerevisiae* 5088. After 24, 48 and 72 h of saccharification period by the fungal strain, the fermentation process was begun by adding yeast inoculums. The highest ethanol production was achieved at 14.36 g/L after 24 h of saccharification process on 6% cassava starch medium.

Thanapoom *et al. (*2009) investigated feasibility study on ethanol production from cassava pulp by simultaneous saccharification and fermentation (SSF) with co-culture of *Rhizopus oryzae* and *Sacharomyces cerervisiae* in solid state culture. The ethanol production performed on solid substrate at initial moisture content of 80% consisted of 16 g cassava pulp and 4 g corncob by each of four strains of *R. oryzae*. The results indicated that each culture was able to produce ethanol, particularly *R. oryzae* TISTR 3165 and TISTR 3523 gave higher ethanol production. Addition of $MgSO_4.7H_2O$ and KH_2PO_4 increased the ethanol production. In contrast, no ethanol production was found with addition of nitrogen source. An increase of cassava pulp increased ethanol production. Co-culture of *R. oryzae* and *S. cerervisiae*

increased ethanol concentration and production rate. Inoculation of *S. cerevisiae* after 3 days culture of *R. oryzae* in SSF enhanced the yield and productivity of ethanol 2 folds as compared with the culture inoculated with single culture of *R. oryzae.*

Thu *et al. (*2006) carried out life cycle cost analysis to assess the economics of cassava-based fuel ethanol for transport in Thailand. Its relative high cost over gasoline has put an economic barrier to commercial application. So far, there are different opinions about government support for ethanol in the forms of tax incentives and subsidies. The scope of the study includes the cassava cultivation/processing, the conversion to ethanol, the distribution of the fuel, and all transportation activities taking place within the system boundary. A distance of one kilometer driven by new passenger cars was used as the functional unit (FU) to compare ethanol (in the form of gasohol E10) with gasoline. The results of the analysis show that gasohol has the potential to be competitive with gasoline in terms of cost per FU if cassava farmers can raise their crop yield but lower chemical (fertilizer, herbicide) consumption for crop maintenance.

Veera *et al. (*2005) optimized the conditions of simultaneous saccharification and fermentation (SSF), viz. temperature, pH and time of fermentation of ethanol from sago starch with co-immobilized amyloglucosidase (AMG) and *Zymomonas mobilis* MTCC 92 by submerged fermentation using statistical experimental design. Maximum ethanol concentration of 55.3 g/L was obtained using a starch concentration of 150 g/L. The optimum conditions were found to be a temperature of 32.4°C, pH of 4.93 and time of fermentation of 17.24 h. Thus, by using SSF process with co-immobilized AMG and *Z. mobilis* cells MTCC 92, the Central Composite Design (CCD) was found to be the most favourable strategy investigated with respect to ethanol production and enzyme recovery.

Wang *et al. (*1995) studied hydrolysis patterns of five batches of sago starch by using Novo Nordisk and sigma α-amylase and glucoamylase. Native sago starch was a poor substrate to the enzymes and the hydrolysis patterns were surface erosion, pitting and crevassing. After incubation with acetate buffer of pH 3.5 at 60°C for 2 h, the hydrolysis pattern was different: a single deep round hole developed regardless of the batch or enzyme(s) used. This step also significantly increased the degree of hydrolysis. Granule size distribution results indicated that at about 67% hydrolysis, treated granule residues were the

same mean size as native granules while untreated granule residues had two major size populations. DSC results suggested that amorphous regions of the untreated granule were preferentially hydrolysed, however, upon pretreatment regions within the granule were more uniform towards the action of enzymes.

Wong *et al. (*2007) generated more linear-chain dextrin from sago starch (24.9% amylose) using pullulanase (EC.3.2.1.41) such that the resulting product could act as a high amylose starch. A starch suspension of 5.0% (w/v) sago starch was heated at 100°C for 45 min and after cooling, the gelatinized sago starch was hydrolyzed with 2.0% (V/dry weight starch) pullulanase (Promozyme 400L, Novozymes A/S, Denmark) for 24 h. The Linear Long-chain Dextrin (LLD) content of the hydrolysate after drying was then compared with the initial LLD content. The surface morphology of the starch granules was observed with SEM. Raw sago starch was resistant to the action of pullulanase, but caused an increase in the LLD of that sago starch from an initial concentration of 24.9–33.2% following gelatinization. The best conditions to maximize the amount of LLD were 5.0% (w/v) sago starch, 2.0% (v/w) enzyme and 12 h reaction time. Acid pretreatment of the sago starch did not cause greater improvement in the accessibility and susceptibility of pullulanase as the LLD content, following pullulanase action did not change significantly. Shrinkage on the surface of the starch granules was observed with the SEM.

Yetti Marlid *et al. (*2000) attempted to improve the growth of endophytic fungus *Acremonium sp*. and its raw sago starch degrading enzyme (RSSDE) production using different nitrogen and carbon sources at varying pH values and temperatures. It was observed that growth and enzyme activity levels were highest with peptone and sodium nitrate as the nitrogen sources and raw sago starch as the carbon source of which the optimum concentrations were 0.5 g/L, 3 g/L, and 20 g/L respectively. Cell growth and RSSDE production reached their optimum at pH 5.0 and incubation temperature of 30°C. Under these conditions, the enzyme production was significantly increased by 19 to 22-folds compared to the activity obtained in the original basal medium.

Yusaku *et al. (*1984) characterized the ethanol fermentation of uncooked cassava starch with *Rhizopus koji*. Studies have been conducted in a gas circulation type fermentor. Results showed that ethanol concentration reached 13-14% (v/v) in 4 day broth, and the

maximum productivity of ethanol was 2.3 g ethanol/L h. This productivity was about 50% compared to the productivity of a glucose-yeast system. Ethanol yield reached 72.3% of the theoretical yield for the cassava starch used. The fermentor used in the present work has been proven by experiment to be suitable for ethanol fermentation of the broth with solid substrate.

2.2 ETHANOL PRODUCTION FROM CASSAVA PEEL

Adesanya *et al.* (2009) identified and screened for amylolytic activity in fungal isolates from rotten Cassava wastes. *Aspergillus niger* (isolate A1), displaying the highest amylase activity on starch agar, was used to degrade a liquid suspension of milled Cassava peel under sterile, closed conditions. *Saccharomyces cerevisiae* (isolate Y1) was subsequently used to ferment the degraded medium for ethanol production. The liquid suspension of milled cassava peel was inoculated with *A. niger* and the production of simple sugar was monitored using the dinitrosalicylic method. The highest concentration of simple sugar (0.88 mg/mL) was produced by the seventh day, while the ethanol produced after seeding the cell-free extracts with *S. cerevisae* was 1.05% after three days.

Akponah and Akpomie (2011) investigated the feasibility of bioethanol production from yam, potato and cassava root peels. Slurry of each peel was saccharified using acid, commercially available α-amylase and *Aspergillus niger* which was screened and certified for amylase production. Subsequent fermentation by *Saccharomyces cerevisiae* followed at room temperature for 72 h. Composition analysis revealed that yam, potato and cassava root peels contained 46, 96 and 114.5 (mg/g) starch respectively. Glucose yield obtained after hydrolysis of yam peels using amylolytic fungi, enzyme and acid were 51.6, 48.7 and 78.1 (mg/g) respectively. Similarly, glucose levels of the respective potato peel hydrolysates were 160.8, 90.7 and 248.3 (mg/g). Saccharification of cassava root peels resulted into glucose concentrations of 210.4 mg/g, 110.4 mg/g and 250.0 mg/g in amylolytic fungi, enzyme, and acid hydrolysates respectively. The ethanol yield after fermentation of yam peels hydrolysed using amylolytic fungi, enzyme and acid were 1.68, 0.56 and 2.7% v/w respectively. Ethanol yield from potato peels were 4.02% v/w (amylolytic fungi hydrolysate), 1.94% v/w (enzyme hydrolysate) and 9.38% v/w (acid hydrolysate). Fermentation of the respective cassava peel hydrolysates resulted in 10.5% v/w, 4.07% v/w and 17.52% v/w ethanol. Results obtained suggested that increasing the fermentation duration did not result in a corresponding increase in ethanol production hence maximum ethanol yield in various hydrolysates was at the 24^{th} h of fermentation following the order acid hydrolysates > amylolytic fungi hydrolysates > enzyme hydrolysates. Also, in terms of substrate yield, highest ethanol production was from

cassava root peels, followed by potato peels while yam peels yielded the least ethanol concentration. This research therefore, indicates that these peels could serve as cheap sources of glucose which can be fermented locally for bio-ethanol production especially in areas where they are in abundance.

Bagus and Bondan (2008) explored Cassava Peel Waste (CPW), as substrate and sweet corn, as enzyme source, to produce bioethanol for the determination of optimum conditions in hydrolysis process and fermentation process. The results show that, by using 30 mg/ml enzyme concentration, optimum conditions for hydrolysis is 90 mg/ml CPW concentration utilized in 90 minutes to produce 80.13 mg/ml glucose concentration and the optimum conditions for fermentation process is 5% (v/v) of starter utilized in 24 hours to produce 5.7 % (v/v) of ethanol.

Buddhiporn and Jirasak (2011) studied and screened on feasibility of ethanol production from cassava peels which pretreated with diluted sulfuric acid, diluted sodium hydroxide and distilled water (control) by Simultaneous Saccharification and Fermentation (SSF) with mono-culture of *Saccharomyces cerevisiae* 1195 and *Saccharomyces cerevisiae* 7532 and co-culture of *Saccharomyces cerevisiae* 1195 and *Candida tropicalis* 5045. The results indicated that each strain of mono-culture was able to produce ethanol with high yield. *Saccharomyces cerevisiae* 1195 could fermented the cassava peels pretreated with distilled water at 135°C under pressure of 15 lb/inch^2 to produce ethanol yield as high as the cassava peels pretreated with diluted sulfuric acid under the same condition. The cassava peels pretreated with diluted sulfuric acid and fermented by co-culture of *Saccharomyces cerevisiae* 1195 and *Candida tropicalis* 5045 produce ethanol higher than that of by *Saccharomyces cerevisiae* 1195 alone.

Jirasak and Buddhiporn (2011) produced ethanol from pretreated cassava peels by simultaneous saccharification and fermentation (SSF) with a monoculture of *Saccharomyces diastaticus* 2047 and *S. cerevisiae* 7532 and a co-culture of *S. diastaticus* 2047 and *Candida tropicalis* 5045 was studied. The results indicated that each strain of yeast was able to produce ethanol. From the cassava peels pretreated with distilled water at 135°C for 30 min under pressure of 1.03 bar, *S. diastaticus* 2047 could produce ethanol yields as high as that of pretreatment with diluted sulfuric acid under the same conditions. The cassava peels pretreated with diluted sulfuric acid and fermented by co-culture of *S. diastaticus* 2047 and

C. tropicalis 5045 produce greater amounts of ethanol than those fermented by *S. diastaticus* 2047.

Jirasak and Preyarat (2007) optimized production of ethanol by Simultaneous Saccharification and Fermentation (SSF) which required knowledge about the influence of substrate and enzyme concentration on yield and productivity. Although SSF has been investigated extensively, the optimal conditions for SSF of cassava peel have yet not been determined. In this study, cassava peel was used as substrate for the production of ethanol by SSF. Commercial enzymes were used in combination with the yeast *Saccharomyces cerevisiae*. The effects of the concentration of substrate (2% to 10% w/w) and of amylase and amyloglucosidase (1.4 U and 2.7 U/g cassava peel) were investigated. SSF was found to be sensitive to contamination because lactic acid was produced. The ethanol yield increased with increasing cassava peel loading. The highest ethanol yield, 68% of the theoretical based on the glucose and reducing sugars present in the original cassava peel, was obtained at 5% substrate concentration. This yield corresponds to 82% of the theoretical based on the cassava peel and soluble glucose and reducing sugars present at the start of SSF. A higher substrate concentration caused inefficient fermentation, whereas a lower substrate concentration, 2%, resulted in increased formation of lactic acid, which lowered the yield. Compared with separate hydrolysis and fermentation, SSF gave a higher yield and doubled the productivity.

Kanlaya *et al. (*2007) produced ethanol from cassava peel hydrolysate prepared by dilute-acid hydrolysis. Sulfuric acid was found to be more effective acid for the degradation of cassava peels to fermentable sugars, compared to hydrochloric acid and acetic acid. Acid hydrolysate of cassava peels, comprised mainly of glucose, was obtained after dilute-acid hydrolysis under optimum condition at 135°C for 90 min. Neutralized hydrolysate containing reducing sugars and glucose ca 60.74 g/100 g and 37.09 g/100 g cassava peels, respectively, were used as substrates for ethanol production. Cassava peel hydrolysates with high sugar concentration were used as the substrates in the fermentation medium to evaluate the kinetic behavior of *Saccharomyces cerevisiae* (5019) during the fermentation. Experiments were conducted with using glucose semi-synthetic medium in shaking flasks. Glucose was consumed within 18 h of fermentation. The volumetric ethanol productivity of 0.51 g/L.h and ethanol yield of 0.43 g/g were achieved. When reducing sugars from hydrolysates were used the fermentable sugars were consumed within 10 h. The volumetric ethanol productivity of 0.29 g/L.h and ethanol yield of 0.27 g/g were obtained. These results showed that diluted-acid hydrolysis of cassava peels are promising substrates for use in ethanol production.

Kanlaya *et al. (*2011) used cassava peel as raw material for reducing sugars production. Reducing sugar was maximal produced by 1.5% cassava peel hydrolysed by 0.1M sulfuric acid (w/v) at 135°C under pressure 15 lb/inch^2 for 90 min producing 66.28% yield. For α-amylase and amyloglucosidase hydrolysis producing 49.14% yield. HPLC analysis demostrated that the sugar produced from the hydrolysate were 37.09, 4.79 and 4.05% yield for glucose, xylose and rhamnose, respectively. However, the hydrolysate contained 0.14% yield of 5-hydroxymethylfurfural. HPLC analysis found that the sugar in the enzymatic hydrolysate 16.23 and 2.04% yield for glucose and xylose, respectively. Batch fermentation by *Saccharomyces cerevisiae* showed dramatic substrate utilization at 30°C with 150 rpm in shaking incubator and resulted in the formation of 5.54 g/L ethanol after 20 h. This study suggests that the cassava peel could be used for the carbon source for yeast fermentation to produce ethanol.

Oyeleke *et al. (*2012) examined the enzymatic production of bioethanol from cassava and sweet potato peels using two groups of organisms. *Gloeophyllum sepiarium* and *Pleurotus ostreatus were* used to hydrolyse 20 g, 35 g and 50 g of substrates at 28°C for 7 days. *Zymomonas mobilis* and *Saccharomyces cerevisiae* were further used to ferment the substrates at 28°C for 5 days. The fermented liquid was distilled at 78°C and quantity of ethanol produced determined. When both *G. sepiarium* and *P. ostreatus* were used for hydrolysis and both *Z. mobilis* and *S. cerevisiae* were used for fermentation, 50 g of cassava peel and 50 g of sweet potato peel yield 11.97 g/cm^3 (26%) and 6.5 g/ cm^3 (12%) of ethanol respectively. When only *Z. mobilis* was used for fermentation, the mass of bioethanol produced from cassava peels and sweet potato peels were 10.6 g/cm^3 (23%) and 5.9 g/cm^3 (12%) respectively and when only *S. cerevisiae* was used for fermentation, the mass of bioethanol produced from cassava peels and sweet potato peels were 10.36 g/cm^3 (22%) and 5.68 g/cm^3 (12%) respectively. When 35g of substrate was used, cassava peel had a yield of 9.64 g/cm^3 (20%) while sweet potato peel had a yield of 5.3 g/cm^3 (10%). When 20 g of substrate was used, cassava peel had a yield of 7.8 g/cm^3 (14%) while sweet potato peel had a yield of 4.66 g/cm^3 (9%). The study revealed that bioethanol can be produced from cassava and sweet potato peels with maximum yield obtained using *Gloeophyllum sepiarium* and *Pleurotus ostreatus* for hydrolysis and *Zymomonas mobilis* and *Saccharomyces cerevisiae* for fermentation.

Table 2.1 Consolidated literatures for ethanol production from cassava peel

S. No.	Substrate(s)	Microbe(s)	Pre-treatment	Hydrolysis	Process conditions	Reactor configuration	Cell type	Result	Reference
1.	Cassava peel	*A.niger* & *S.cerevisae*	Steam explosion	Enzyme: amylase	-	Mc Cartney bottles	Free cell	0.88 mg/mL of simple sugars	Adesanya *et al. (*2009)
2.	Cassava peel	-	Steam explosion	Acid: sulphuric acid	T=100-120˚C, pH b/w 6.5 &7.0	500ml conical flask	-	Yield: 80.76 g (w/w) of sugars	Sathya Geetha and Navaneetha Gopala Krishnan (2009)
3.	Cassava peel	*S.diastaticus*, *S.cerevisae* & Candida tropicalis	Milling (sized 63-425 µm)	[E]= 0.1M, T=135°C, P=1.03 bar, t=30mins	T=30 °C, t=48hr	Rotary shaker	Free	Co-culture produces greater amount of ethanol	Jirasak and Buddhiporn (2011)
4.	Cassava peel	*S.cerevisae*	-	Enzyme: Amylase & amyloglucosidase	[S]=2-10%	-	Free	Higher yield can be obtained with SSF than SHF	Jirasak and Preyarat (2007)
5.	Cassava peel	*S.cerevisae*	Milling	Acid: [A]=0.1M, [S]=1.5%V, T=135˚C, P=15lb/inch2, t=90min	pH=7.0, T=30˚C, t=20h	Rotary shaker at 150 rpm	Free	5.54 g/L ethanol after 20 h	Kanlaya *et al. (*2011)

Sathya Geetha and Navaneetha Gopala Krishnan (2009) produced fuel ethanol from cassava peel through hydrolysis in an enzymatic process. Pretreatment is necessary, prior to enzymatic hydrolysis of the peeling waste. In their study, steam pretreatment along with dilute sulphuric acid impregnation was investigated to improve the overall sugar yield. A wide range of pretreatment conditions were used to determine the most favourable treatment condition. The temperatures investigated were between 100°C to 120°C, the residence time were 5, 10, 15, 20 and 25 minutes and the dilution of concentrations of H_2SO_4 were 0%, 0.25%, 0.5%, 0.75% and 1%. The effects of pretreatment were assessed for the whole slurry at various time intervals. For each set of pretreatment conditions the liquid fraction was determined for any inhibiting factors. 5-HMF (hydroxymethylfurfural) an inhibitory compound's concentration during the various pretreatment processes for the sugar conversion from the cassava peel was also determined. Maximum yield of 80.76 g (w/w) was obtained when the pretreatment step was performed at 105°C for 10 min with 0.5% H_2SO_4. Steam pretreatment accompanied with dilute acid hydrolysis is a promising method of increasing the overall yield in the Cassava peel to ethanol process.

The different literatures on the production of ethanol from cassava peel were consolidated (Table2.1).

2.3 ETHANOL PRODUCTION FROM CASSAVE STEM

Magesh *et al. (*2011) studied the direct fermentation of 226 white rose tapioca stem to ethanol by Fusarium oxysporum in a batch reactor. Fermentation of ethanol can be achieved by sequential pretreatment using dilute acid and dilute alkali solutions using 100 mesh tapioca stem particles. The quantitative effects of substrate concentration, pH and temperature on ethanol concentration were optimized using a full factorial central composite design experiment. The optimum process conditions were then obtained using response surface methodology. The quadratic model indicated that substrate concentration of 33 g/L, pH 5.52 and a temperature of 30.13°C were found to be optimum for maximum ethanol concentration of 8.64 g/L. The predicted optimum process conditions obtained using response surface methodology was verified through confirmatory experiments. Leudeking-piret model was used to study the product

formation kinetics for the production of ethanol and the model parameters were evaluated using experimental data.

Magesh *et al. (*2011) studied the Simultaneous Saccharification and Fermentation (SSF) of tapioca stem var. 226 white rose to ethanol using cellulase enzyme and *Saccharomyces cerevisiae* in a fermentor. The fermentation conditions were optimized by studying the effect of particle size, substrate concentration, pH and temperature. In this study, the sequential pretreated of tapioca stem using dilute acid and alkali showed significant hemicelluloses and lignin degradation when compared with untreated tapioca stem. The optimum values of particle size, substrate concentration, pH and temperature were found to be 100 mesh size, 50 g/L, 5 and 35°C respectively with the maximum ethanol concentration of 13.6 g/L. Logistic model for growth and Leudeking-Piret model for Substrate utilization kinetics were used for the production of ethanol and the model parameters were evaluated using experimental data.

Minhee *et al. (*2011) established the ethanol production processes using crops such as corn and sugar cane. However, the utilization of cheaper biomasses such as lignocellulose could make bioethanol more competitive with fossil fuels, without the ethical concerns associated with the use of potential food resources. A cassava stem, a lignocellulosic biomass, was pretreated using dilute acid to produce bioethanol. The pretreatment conditions were evaluated using response surface methodology (RSM). As a result, the optimal conditions were 177°C, 10 min and 0.14 M for the temperature, reaction time and acid concentration, respectively. The enzymatic digestibility of the pretreated cassava stem was examined at various enzyme loadings (10-40 FPU/g cellulose of cellulase and 30 CbU/g of β-glucosidase). With respect to economic feasibility, 20 FPU/g cellulose of cellulase and 30 CbU/g of β-glucosidase were selected for the test concentration and led to a saccharification yield of 70%. The fermentation of the hydrolyzed cassava stem using *Saccharomyces cerevisiae* resulted in an ethanol concentration of 7.55 g/L and a theoretical fermentation yield of 89.6%. This study made a significant contribution to the production of bioethanol from a cassava stem. Although the maximum ethanol concentration was low, an economically efficient overall process was carried out to convert a lignocellulosic biomass to bioethanol.

CHAPTER 3

CHARACTERIZATION OF SUBSTRATES AND GROWTH CURVE OF *Zymomonas mobilis*

3.1. MATERIALS

Muffle furnace and Hot air oven, procured from Inlab equipments (Madras Private Limited), were used for the purpose of incineration. Analytical weighing balance from Shimadzu Coorporation (Model: AUY 120) was used for weighing the materials. Water bath, purchased from Technico Limited, was used for maintaining the temperature. Kjeldhal flask of 500 mL capacity was purchased from Borosil Glass Works Limited, Chennai for measuring the total nitrogen. UV – Visible spectrophotometer of Shimadzu (Model: UV-1800) was used for measuring the absorbance of solutions. Incubator shaker, purchased from Scigenics (India) Private Limited (Model: Orbitek), Chennai and autoclave were also used.

3.2 METHODS

3.2.1 CHARACTERIZATION OF SUBSTRATES

Cassava plant and industrial residues (cassava stem, cassava twig, cassava leaves, cassava peel and cassava waste) were collected from agricultural fields and sago factory of Namakkal district. The materials were grinded and mixed completely and then stored in incubator maintained at 4°C. Each material was characterized quantitatively for starch, cellulose, hemicelluloses, total reducing sugars, total carbohydrate, protein, moisture, amylose, amylopectin, total organic carbon, fat and total nitrogen.

The starch was estimated using iodine method (Walter, 1916), cellulose was estimated using acetic/nitric reagent followed by anthrone (Updegroff, 1969), hemicellulose was determined by estimating xylose by Orcinol method (Goering and Vansoest, 1975), total reducing sugars was estimated by dinitrosalicylic acid method (Miller, 1959), total carbohydrate present in the sample was determined by phenol sulphuric acid method (Dubois *et al.,* 1956; Krishnaveni *et al.,* 1984), protein estimation was done by Lowry's method (Lowry *et al.,* 1951),

moisture content was determined by analyzing the dry matter at 105°C (ISI 05-2e, 2010), amylose and amylopectin were estimated by using iodine reagent after extraction by phenolphthalein (Mc Cready *et al.,* 1950; Juliano, 1971), total organic carbon was estimated by oven drying method (Brain, 2012), fat content was determined using 20% sodium carbonate (Shanmugam, 2010), and total nitrogen present in the sample was analysed by micro-kjeldhal method (Pellett and Young, 1980).

3.2.2 GROWTH CURVE OF *Zymomonas mobilis*

Zymomonas mobilis MTCC 2427 is procured from Institute of Microbial Technology (IMTECH), Chandigarh. The composition of medium used for bacterial growth is tabulated (Table 3.1).

Table 3.1 Composition of growth medium

COMPONENT	QUANTITY
Glucose	20.0 g
Yeast extract	10.0 g
KH_2PO_4	2.0 g
Agar	15.0 g
Distilled water	1.0 L
pH	6.0

Add 1 mL of overnight *Zymomonas mobilis* culture to the flask containing 100 mL of medium mentioned above. Swirl the flask so that there is an even suspension of bacteria. Take the initial absorbance of this culture using the spectrophotometer at 600 nm using 3 mL of inoculated broth. Place the culture flask in the incubator shaker at 120 rpm at 37°C. Every 60 minutes, aseptically transfer 3 mL of the culture to a cuvette and determine its absorbance. Bacterial cells absorb light well at the wavelength of 600 nm when grown in standard media. The growth curve of *Z. mobilis* was recorded in UV-Visible spectrophotometer for 18 hours (Cappuccino and Sherman, 2011).

3.3 RESULTS AND DISCUSSION

3.3.1 CHARACTERIZATION OF SUBSTRATES

The starch to cellulose ratio of cassava stem, cassava peel, cassava waste, cassava twig and cassava leaves were 12.56, 8.44, 2.46, 0.23 and 0.56 respectively. The substrates, cassava stem and cassava peel, are rich in starch (whose starch to cellulose ratio is greater than 5) and are called as first generation substrates for bioethanol production. The cassava waste and cassava twig (whose starch to cellulose ratio is less than 5) are lignocellulosic substrates and are called as second generation substrates for bioethanol production. The cassava leaves are rich in crude protein but not in starch and cellulose (carbohydrates) when compared to other substrates.

Table 3.2 Characterization of substrate

S. No.	Parameters	Cassava stem	Cassava peel	Cassava waste	Cassava leaves	Cassava twig
1.	Starch (% of total carbohydrate)	61.15	61.08	42.15	3.66	4.05
2.	Cellulose (% of total carbohydrate)	4.87	7.24	17.11	6.58	17.37
3.	Hemicellulose (% of total carbohydrate)	7	5.5	11	6	8
4.	Total Reducing Sugars (% of total carbohydrate)	3.44	2.65	2.22	0.3	3.7
5.	Total Carbohydrate (%)	76.47	76.47	72.35	30	33.53
6.	Crude Protein (%)	2.7	2.98	4.72	8.6	3.72
7.	Moisture (%)	7.93	2.0	8.78	6.5	7.0
8.	Amylose (% of starch)	17.98	25.57	35.56	1.8	2.4
9.	Amylopectin (% of starch)	43.17	35.51	6.59	1.86	1.65
10.	Total Organic Carbon (%)	33.52	33.62	31.63	7.08	14.2

11.	Fat (%)	0	0	0	0	0
12.	Total Nitrogen (%)	0.432	0.4768	0.7552	2.35	0.5952

Total carbohydrate content of cassava twig and cassava leaves are less comparatively. Even though the carbohydrate content of cassava waste is high, the starch and cellulose interference affects the ethanol production process. Thus, cassava peel and cassava stem are selected as substrates for ethanol production for further studies.

3.3.2 GROWTH CURVE OF *Zymomonas mobilis*

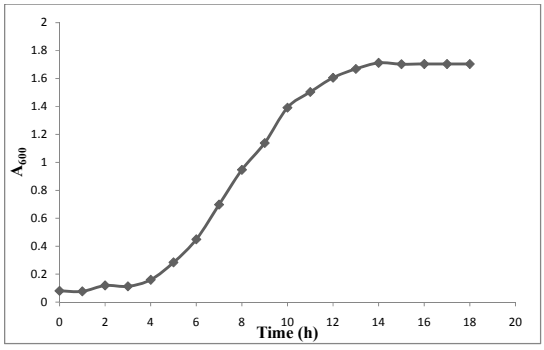

Figure 3.1 Growth curve of *Zymomonas mobilis*

The generation time of the *Z. mobilis* can be determined by indirect spectrophotometric method by using absorbance of data from growth curve (Figure 3.1). The generation time can be calculated as follows:

$$t_d = t_{(A=0.4)} - t_{(A=0.2)} = 5.8 - 4.5 = 1.3 \text{ h} = 78 \text{ min.}$$

The doubling time is the period of time required for the microorganism to double in number. In batch culture the cells have different physiological states during incubation. The doubling time changes from time to time during growth, except in the exponential phase (Horvath, 1969). The doubling time of *Zymomonas mobilis* is 78 min. For ethanol fermentation, the mid log phase culture inoculum is required, for which the growth curve is plotted.

CHAPTER 4
PROCESS OPTIMIZATION OF ETHANOL PRODUCTION FROM CASSAVA PEEL AND CASSAVA STEM

4.1. MATERIALS

Jaw crusher, purchased from Almech Enterprise, Coimbatore was used for milling the substrates. Lawrence and Mayo sieve shaker was used to segregate the substrates of different particle sizes. Analytical weighing balance from Shimadzu Corporation (Model: AUY 120) was used for weighing the materials. Water bath, purchased from Technico Limited, was used for maintaining the temperature. UV – Visible spectrophotometer of Shimadzu (Model: UV-1800) was used for measuring the absorbance of the solutions. Incubator shaker, purchased from Scigenics (India) Private Limited (Model: Orbitek), Chennai and autoclave were also used. pH meter of Elico (Model: LI 120) was used for adjusting the pH. Centrifuge from Remi (Model: C-23BL) was used for removing the suspended solids from the substrate after gelatinizing the starch. A trial version of Design Expert 8.0.7 was used for Response Surface Methodology (RSM) experiments.

4.2 METHODS

4.2.1 PRODUCTION OF ETHANOL FROM CASSAVA PEEL AND CASSAVA STEM

The materials are milled in jaw crusher and sieved using Lawrence and Mayo sieve shaker from 850 µm sieve to pan. Slurries of materials at various concentrations of different particle sizes were prepared in phosphate buffer [pH 7]. The gelatinization process was done on a heating mantle by cooking at 100°C until it becomes transparent. 5 to 10% [w/w of substrate] of α-amylase was added to the gelatinized starch and the optimum pH was adjusted. For a liquefaction to occur, the mixture was placed in a water bath maintained at optimum conditions of temperature and time. The liqueified material was centrifuged at 10000 rpm for 12 min to remove the suspended solids present in it. The liquefication process was completed by autoclaving the mixture at 121°C for 15 min. After being liquefied, saccharification was carried

out by the addition of amyloglucosidase at the rate of 2.7 U/g substrate. The hydrolysis was assumed to be complete by testing starch-iodine method.

Fermentation was performed under aerobic/anaerobic condition. The optimum pH was adjusted for hydrolysed starch before addition of 5% (v/v) inoculum to the hydrolysate. Then the mixture was incubated at optimum conditions of temperature and time (Anita and Narsi, 2012). Finally the ethanol concentration was determined by dichromate method (Caputi *et al.,* 1968).

Preliminary experimental studies were conducted by varying the particle size of the substrate. The materials passed through 850 µm screen and retained in 212 µm screen were used as particle-sized substrate. The materials passed through 150 µm screen and retained on pan were used as fine-sized substrate. Pretreatment methods (Milling, Cooking and both combined), hydrolytic methods (acid using glacial acetic acid at the rate of 40% (v/v) and enzyme using α-amylase), and fermentative organisms (*Zymomonas mobilis* and *Saccharomyces cerevisiae*) were also varied. The preliminary parameter screening was done by calculating the concentration, yield and productivity of the ethanol.

The optimization of ethanol production involves two steps – (i) Screening of parameters by Plackett-Burman method to minimize the parameters and (ii) Optimization of parameters by Box-Behnken method for maximum ethanol production.

The kinetic study was performed by varying the following parameters while maintaining all other parameters at optimum value

(i) Time of hydrolysis
(ii) Substrate concentration
(iii) Enzyme (α-amylase) concentration

Thermodynamic study was done by varying the fermentation temperature while keeping all other parameters at optimum value.

4.2.2 PLACKETT-BURMAN DESIGN

The Plackett-Burman method was used to identify which variables have significant effects on ethanol production by *Zymomonas mobilis* MTCC 2427. The 12 run Plackett-Burman design included the process parameters of hydrolysis and fermentation. Among the process parameters, substrate concentration, α-amylase concentration, pH, time and temperature of hydrolysis, amyloglucosidase activity, agitation speed during fermentation, pH, time and temperature of fermentation and inoculum size were tested for their effects on the ethanol production. Low levels and high levels were assigned for each variable (Table 4.3). The experimental designs for ethanol production from cassava peel and cassava stem are shown in Tables 4.1 and 4.2 respectively. The ethanol concentration was used as the response in this design. The significance of variables was determined by calculating their effects on ethanol production (Ma *et al.,* 2008).

4.2.3 BOX-BEHNKEN DESIGN

Box-Behnken design was used for the optimization of ethanol production. Three factors namely substrate concentration, enzyme concentration and fermentation recipe [mixture of amyloglucosidase and *Z. mobilis*] for cassava peel and substrate concentration, enzyme concentration and hydrolysis time for cassava stem were chosen after identifying significant factors using Plackett-Burman method. Ethanol concentration was used as dependent output variable. 15 experiments were performed according to Box-Behnken design table to optimize the parameters. Among them three replications were at centre points, while the actual points were determined to be √(3/2). A coefficient of the quadratic model were calculated using the following equation,

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ij} {X_i}^2 + \sum_{i<j}^{k} \sum_{j}^{k} b_{ij} X_i X_j \quad (4.1)$$

where Y is predicted response, and i, j are linear, quadratic coefficients, respectively. b and k are regression coefficients and the number of factors studied in the experiment respectively (Ma *et al.,* 2008).

The significance of each coefficient was determined and the results were analysed by Design Expert 8.0.7. Three dimensional contour plots were obtained to study the interaction of

one parameter with another. The optimum concentration was identified based on the humps in the three dimensional plots.

4.2.4 KINETICS FOR CONSUMPTION OF SUBSTRATE

According to the mechanism proposed by Michaelis and Menten (1913), enzyme reacts with substrate to form enzyme-substrate complex, which further reacts to form enzyme and product (Equation 4.2).

$$E + S \leftrightarrow ES \rightarrow E + P \quad (4.2)$$

The reaction between enzyme and substrate is reversible and the reactions are represented by the equations (4.3) and (4.4).

$$E + S \overset{k_1}{\rightarrow} ES \quad (4.3)$$

$$ES \overset{k_{-1}}{\longrightarrow} E + S \quad (4.4)$$

$$ES \overset{k_2}{\rightarrow} E + P \quad (4.5)$$

The rate of consumption of substrate is given by equation (4.6)

$$-\frac{d[S]}{dt} = k_1[E][S] - k_{-1}[ES] \quad (4.6)$$

After steady state approximation,

$$-\frac{d[S]}{dt} = \frac{d[P]}{dt} \quad (4.7)$$

$$k_1[E][S] - k_{-1}[ES] = k_2[ES] \quad (4.8)$$

$$k_1[E][S] = [ES]\,(k_{-1} + k_2) \quad (4.9)$$

Rearranging the above equation,

$$[E][S] = [ES]\frac{(k_{-1}+k_2)}{k_1} \quad (4.10)$$

Replacing the term $\frac{(k_{-1}+k_2)}{k_1}$ by k_m(Michaelis Menten constant),

$$[E][S] = [ES]k_m \quad (4.11)$$

Rearranging the above equation,

$$[ES] = \frac{[E][S]}{k_m} \quad (4.12)$$

It is known fact that the total enzyme concentration is decomposed into enzyme concentration at any time t and concentration of enzyme-substrate complex at any time t.

$$[E_o] = [E] + [ES] \quad (4.13)$$

Substituting equation (4.12) in equation (4.13),

$$[E_o] = [E] + \frac{[E][S]}{k_m} \quad (4.14)$$

Rearranging the above equation,

$$[E_o] = [E](1 + \frac{[S]}{k_m}) \quad (4.15)$$

$$[E] = \frac{[E_o]}{(1+\frac{[S]}{k_m})} = \frac{k_m E_o}{k_m+[S]} \quad (4.16)$$

Substituting equations (4.13) and (4.16) in (4.6),

$$-\frac{d[S]}{dt} = k_1 \frac{[E_o]}{(1+\frac{[S]}{k_m})}[S] - k_{-1}([E_o] - [E]) \quad (4.17)$$

Rearranging the above equation,

$$-\frac{d[S]}{dt} = \frac{k_1[E_o]k_m[S]}{k_m+[S]} - k_{-1}\left([E_o] - \frac{k_m[E_o]}{k_m+[S]}\right) \quad (4.18)$$

$$-\frac{d[S]}{dt} = \frac{k_1[E_o]k_m[S]}{k_m+[S]} - \frac{k_{-1}[E_o](k_m+[S])}{k_m+[S]} + \frac{k_{-1}k_m[E_o]}{k_m+[S]} \quad (4.19)$$

$$-\frac{d[S]}{dt} = \frac{(k_1k_m[E_o]-k_{-1}[E_o])[S]}{k_m+[S]} \quad (4.20)$$

$$-\frac{d[S]}{dt} = \frac{k[S]}{k_m+[S]} \quad (4.21)$$

where $k = (k_1k_m[E_o] - k_{-1}[E_o]) = k_2[E_o] = V_{max}$

Taking reciprocal of equation (4.21)

$$-\frac{dt}{d[S]} = \frac{k_m+[S]}{V_{max}[S]} \quad (4.22)$$

Rearranging the above equation,

$$-\frac{dt}{d[S]} = \frac{k_m}{V_{max}[S]} + \frac{1}{V_{max}} \quad (4.23)$$

Integrating the above equation by applying the boundary conditions: at time t=0, S=S_0 and at time t=t, S=S. where S_0=initial substrate concentration

$$-\int_0^t dt = \int_{S_o}^{S} \frac{k_m}{V_{max}[S]} dS + \int_{S_o}^{S} \frac{1}{V_{max}} dS \quad (4.24)$$

$$-t = \frac{k_m}{V_{max}} \ln (\frac{S}{S_o}) + \frac{(S-S_o)}{V_{max}} \quad (4.25)$$

Rearranging the above equation,

$$t = \frac{k_m}{V_{max}} \ln (\frac{S_o}{S}) + \frac{(S_o-S)}{V_{max}} \quad (4.26)$$

$$\frac{t}{(S_o-S)} = \frac{k_m}{V_{max}} \frac{\ln (\frac{S_o}{S})}{(S_o-S)} + \frac{1}{V_{max}} \quad (4.27)$$

Equation (4.27) is the reaction kinetic equation for substrate consumption in enzyme catalysed reactions.

4.2.5 THERMODYNAMICS FOR ETHANOL FERMENTATION

Empirical approach of the proposed equation (Aiba *et al.* 1973) was used to describe the relationship between temperature-dependent reversible and irreversible inactivation of ethanol production for wide temperature range. For this purpose, specific rate of product formation (q_p, g/mL.h) was used to calculate these variables using equation (4.28):

$$q_p = T\left(\frac{k_B}{h}\right) e^{\Delta S^\circ/R} e^{-\Delta H^\circ/RT} \quad (4.28)$$

Plot of ln (q_p/T) against 1/T gave a straight line whose slope was $-\Delta H°/R$ and intercept was $\Delta S°/R$ + ln (k_B/h), where h (Planck's constant) = 6.63 × 10^{-34} J.s and k_B (Boltzman constant) = 1.38 × 10^{-23} J/K.

For many reactions, and particularly elementary reactions, the rate expression can be written as a product of a temperature-dependent term and a concentration dependent term. For such reactions, the temperature-dependent term, the reaction rate or rate constant, has been found in practically all cases to be well represented by Arrhenius law (1889):

$$r = k_o e^{-E/RT} \quad (4.29)$$

where k_o is called the frequency or pre-exponential factor and E is called the activation energy of the reaction. This expression fits experiment well over wide temperature ranges and is strongly suggested from various standpoints as being a good approximation to the true temperature dependency. At the same concentration, but at two different temperatures, Arrhenius' law indicates that provided that E remains constant.

4.3 RESULTS AND DISCUSSION

4.3.1 PRELIMINARY EXPERIMENTAL RESULTS

Starting from pretreatment to fermentation in the production of bioethanol from cassava peel and stem, 3 process methods and 15 process parameters are involved. The preliminary experiment helps to design the process for the further studies. The trials are conducted to screen particle size of the substrate, pretreatment method, hydrolytic method and fermentative organism by analysing the yield, concentration and productivity of ethanol (Table 4.1). The concentration of ethanol increases to a certain level with respect to time. As the concentration varies, the yield also varies with respect to time.

$$\text{Yield of Ethanol} = (Moles\ of\ Ethanol)/(Moles\ of\ Starch\ in\ Substrate) \quad (4.30)$$

The productivity is calculated by the formula,

$$\text{Productivity of Ethanol} = (Yield\ of\ Ethanol)/(Time\ taken\ for\ Ethanol\ Production) \quad (4.31)$$

The productivity depends on the yield of ethanol produced at a particular time, thus it is high at a lesser time and suddenly decreases as the time increases.

Table 4.1 Preliminary experimental results for screening parameters

PARTICLE SIZE	PRE TREATMENT	HYDROLYSIS	FERMENTATION		ETHANOL		
			Microorganism	Time (h)	Concentration	Yield	Productivity
Fine Powder	Milling	Acid	*Zymomonas mobilis*	0	0	0	0
				48	2.881	0.091	1.495
				60	5.189	0.164	2.154
				84	6.727	0.212	1.994
				144	9.007	0.284	1.558
			Saccharomyces cerevisiae	0	0	0	0
				48	3.55	0.112	1.842
				60	5.858	0.185	2.431
				84	6.627	0.209	1.965
				144	5.858	0.185	1.013
		Enzyme	*Zymomonas mobilis*	0	0.00	0	0
				48	8.354	0.264	4.334
				60	8.414	0.266	3.492
				84	8.38	0.264	2.484
				144	7.494	0.237	1.296
			Saccharomyces cerevisiae	0	0.00	0	0
				48	7.404	0.234	3.841
				60	7.284	0.230	3.023
				84	6.504	0.205	1.928
				144	2.044	0.065	0.353
	Cooking	Acid	*Zymomonas mobilis*	0	0	0	0
				48	2.979	0.094	1.545

		60	5.287	0.167	2.194
		84	6.825	0.215	2.023
		144	9.105	0.287	1.575
		0	0	0	0
		48	3.698	0.117	1.918
	Saccharomyces cerevisiae	60	6.006	0.190	2.493
		84	6.775	0.214	2.008
		144	6.006	0.190	1.039
		0	0	0	0
		48	8.452	0.267	4.385
	Zymomonas mobilis	60	8.512	0.269	3.533
		84	8.478	0.268	2.513
		144	7.592	0.240	1.313
Enzyme		0	0	0	0
		48	7.552	0.238	3.918
	Saccharomyces cerevisiae	60	7.432	0.235	3.084
		84	6.652	0.210	1.972
		144	2.192	0.069	0.379

PARTICLE SIZE	PRE TREATMENT	HYDROLYSIS	FERMENTATION		ETHANOL		
			Microorganism	Time (h)	Concentration	Yield	Productivity
				0	0	0	0
				48	3.077	0.097	1.596
			Zymomonas mobilis	60	5.385	0.170	2.235
				84	6.923	0.218	2.052
				144	9.203	0.290	1.591
		Acid		0	0	0	0
				48	3.846	0.121	1.995
			Saccharomyces cerevisiae	60	6.154	0.194	2.554
				84	6.923	0.218	2.052
				144	6.154	0.194	1.064
Fine Powder	Milling and cooking			0	0	0	0
				48	8.550	0.270	4.435
			Zymomonas mobilis	60	8.610	0.272	3.573
				84	8.576	0.271	2.542
				144	7.690	0.243	1.330
		Enzyme		0	0	0	0
				48	7.700	0.243	3.995
			Saccharomyces cerevisiae	60	7.580	0.239	3.146
				84	6.800	0.215	2.016
				144	2.340	0.074	0.405
				0	0	0	0
212 µm	Milling	Acid	*Zymomonas mobilis*	48	0.288	0.009	0.149
				60	0.673	0.021	0.279

		84	3.75	0.118	1.112
		144	7.596	0.240	1.314
		0	0	0	0
		48	1.393	0.044	0.723
	Saccharomyces cerevisiae	60	5.239	0.165	2.174
		84	6.008	0.190	1.781
		144	6.008	0.190	1.039
		0	0.00	0	0
		48	8.105	0.256	4.204
	Zymomonas mobilis	60	7.666	0.242	3.182
		84	6.618	0.209	1.962
		144	5.204	0.164	0.900
Enzyme		0	0.00	0	0
		48	7.484	0.236	3.882
	Saccharomyces cerevisiae	60	7.854	0.248	3.260
		84	7.866	0.248	2.332
		144	1.834	0.058	0.317

PARTICLE SIZE	PRE TREATMENT	HYDROLYSIS	FERMENTATION		ETHANOL		
			Microorganism	Time (h)	Concentration	Yield	Productivity
				0	0	0	0
				48	0.336	0.011	0.174
			Zymomonas mobilis	60	0.721	0.023	0.299
				84	3.798	0.120	1.126
				144	7.644	0.241	1.322
		Acid		0	0	0	0
				48	1.466	0.046	0.761
			Saccharomyces cerevisiae	60	5.312	0.168	2.205
				84	6.081	0.192	1.803
				144	6.081	0.192	1.052
	Cooking			0	0	0	0
212 µm				48	8.153	0.257	4.230
			Zymomonas mobilis	60	7.714	0.243	3.201
				84	6.666	0.210	1.976
				144	5.252	0.166	0.908
		Enzyme		0	0	0	0
				48	7.557	0.238	3.920
			Saccharomyces cerevisiae	60	7.927	0.250	3.290
				84	7.939	0.251	2.353
				144	1.907	0.060	0.330
				0	0	0	0
	Milling and cooking	Acid	*Zymomonas mobilis*	48	0.384	0.012	0.199
				60	0.769	0.024	0.319

		84	3.846	0.121	1.140
		144	7.692	0.243	1.330
		0	0	0	0
		48	1.539	0.049	0.798
	Saccharomyces cerevisiae	60	5.385	0.170	2.235
		84	6.154	0.194	1.824
		144	6.154	0.194	1.064
		0	0	0	0
		48	8.201	0.259	4.254
	Zymomonas mobilis	60	7.762	0.245	3.221
		84	6.714	0.212	1.990
		144	5.300	0.167	0.916
Enzyme		0	0	0	0
		48	7.630	0.241	3.958
	Saccharomyces cerevisiae	60	8.000	0.252	3.320
		84	8.012	0.253	2.375
		144	1.980	0.062	0.342

Pretreatment is viewed as a critical step in processing of agro wastes and forest residues since it makes the substrate more accessible to hydrolytic breakdown and cause solubilisation of hemicelluloses-derived sugars (HDS). It is considered one of the most expensive processing steps, accounting for up to 33% of the total cost (Pérez *et al.,* 2008).

Physical pretreatment (milling) reduces the substrate size to increase its surface area to enhance the hydrolysis rate. Pretreatments employing chemical reagents, and particularly those using dilute acid, have demonstrated to be effective in the hydrolysis of hemicelluloses while producing highly digestible residues (Ballesteros *et al.,* 2006; Wyman *et al.,* 2005). Notwithstanding, high costs associated to the use of acid resistant materials and treatment of effluents in neutralization process.

Liquid hot water pretreatment (cooking) is a hydrothermal treatment and does not employ any catalyst (Negro *et al.,* 2003). It used liquid hot water under pressure, with temperatures between 180ºC and 270ºC, which penetrates into fibers and hydrates of cellulose, also removing part of hemicellulose and lignin. This creates fibers with an enlarged accessible and susceptible surface, which are more accessible to hydrolytic attack. Thus, the process of cooking yields more when comparing to the milling process.

The method which includes pretreatment that combines both physical and chemical methods is termed as physicochemical method. Process of milling followed by cooking helps to utilize the maximum amount of substrate for ethanol production. Fine powder, as substrate, pretreated with combined milling and cooking increase the yield of ethanol.

The acid or enzymatic hydrolysis of cassava substrate produces two fractions: the liquid composed of soluble sugars from the starch hydrolysis (basically glucose), and one solid fraction composed of insoluble fibers (Adenise *et al.,* 2002). On comparison of acid and enzymatic hydrolysis, enzymatic hydrolysis yields more ethanol than acid hydrolysis in short duration of time.

In order to evaluate the potential of *Zymomonas mobilis* and *Saccharomyces cerevisiae* for ethanol production, both the microbes were used for fermentation. *S. cerevisiae* yields more

ethanol but it is not as tolerant as *Z. mobilis*. *Zymomonas* is superior to *Saccharomyces* because it gains its maximum efficiency after 48 h, whereas the maximal production in *Saccharomyces* requires 72 h. The biomass value of *Z. mobilis* is smaller and its efficiency is 6% higher than that of *S. cerevisiae* (Thomas *et al.,* 1983).

Thus, by the analysis, hydrolysis by using enzyme and *Zymomonas mobilis* as fermentative organism produces high ethanol concentration thereby increases the yield and productivity of ethanol (Table 4.1).

4.3.2 PLACKETT-BURMAN DESIGN

Process optimization by the classical method changing one independent variable (time, pH, temperature, etc.) while fixing all the others at certain level can be time consuming and expensive for a large number of variables. To make full factorial search which would examine each combination of independent variable at approximate levels could require a large number of experiments n^n, where x is the number of levels and n is the number of variables. This may be quite appropriate for three nutrients at two concentrations (2^3 trials) but not for six nutrients at three concentrations. In this instance, 3^6 (729) trials would be needed.

Industrially, the aim is to perform the minimum number of experiments to determine optimal conditions. Other alternative strategies must therefore be considered which allow more than one variable to be changed at a time. These methods have been discussed (Hicks, 1993; Bull *et at.,* 1990; Stowe and Mayer, 1966; McDaniel *et al.,* 1976; Hendrix, 1980; Nelson, 1982 and Greasham and Inamine, 1986).

When more than five independent variables are to be investigated, the Plackett-Burman design may be used to find the most important variables in system, which is then optimized in further studies (Plackett and Burman, 1946). This technique allows for the evaluation of (X-1) variables by X experiments. X must be a multiple of 4, e.g. 8, 12, 16, 20, 24, etc. Normally one determines how many experimental variables need to be included in an investigation and then selects the Plackett-Burman design which meets the requirement most closely in multiples of 4. Any factors not assigned to a variable can be designated as a dummy variable. Alternatively, factors known to not have any effect may be included and designated as dummy variables.

Table 4.2 Plackett-Burman Design matrix for the screening of variables influencing ethanol production from cassava peel

Trials	A	B	C	D	E	F	G	H	I	J	K	EtOH
1	L	L	H	L	H	H	L	H	H	H	L	1.692
2	L	L	L	H	L	H	H	L	H	H	H	3.300
3	H	L	L	L	H	L	H	H	L	H	H	3.789
4	H	H	L	L	L	H	L	H	H	L	H	4.281
5	H	H	H	L	H	L	H	L	H	H	L	4.732
6	**L**	**H**	**H**	**H**	**L**	**L**	**L**	**H**	**L**	**H**	**H**	**5.632**
7	H	L	H	H	H	L	L	L	H	L	H	4.590
8	H	H	L	H	H	H	L	L	L	H	L	4.005
9	L	H	H	L	H	H	H	L	L	L	H	1.683
10	H	L	H	H	L	H	H	H	L	L	L	1.432
11	L	H	L	H	H	L	H	H	H	L	L	2.341
12	L	L	L	L	L	L	L	L	L	L	L	1.157

Table 4.3 Plackett-Burman Design matrix for the screening of variables influencing ethanol production from cassava stem

Trials	A	B	C	D	E	F	G	H	I	J	K	EtOH
1	L	L	H	L	H	H	L	H	H	H	L	1.834
2	L	L	L	H	L	H	H	L	H	H	H	0.999
3	H	L	L	L	H	L	H	H	L	H	H	3.582
4	H	H	L	L	L	H	L	H	H	L	H	3.585
5	**H**	**H**	**H**	**L**	**H**	**L**	**H**	**L**	**H**	**H**	**L**	**4.274**
6	L	H	H	H	L	L	L	H	L	H	H	1.744
7	H	L	H	H	H	L	L	L	H	L	H	3.841
8	H	H	L	H	H	H	L	L	L	H	L	3.690
9	L	H	H	L	H	H	H	L	L	L	H	1.423
10	H	L	H	H	L	H	H	H	L	L	L	3.350
11	L	H	L	H	H	L	H	H	H	L	L	1.757
12	L	L	L	L	L	L	L	L	L	L	L	1.126

Table 4.4 Actual level of variable tested with the Plackett-Burman Design and their effect and rank on ethanol production

VARIABLE		UNIT	L	H	CASSAVA PEEL		CASSAVA STEM	
					EFFECT	RANK	EFFECT	RANK
A	Substrate concentration	%w/v	10	40	1.17	3	2.24	1
B	α-amylase concentration	%w/w	1	5	1.12	4	0.29	3
C	pH of hydrolysis	-	4	7	0.15	9	0.288	4
D	Temperature of hydrolysis	°C	40	60	0.66	7	0.074	10
E	Time of hydrolysis	min	15	240	0.1	10	0.754	2
F	Agitation speed	rpm	0	100	0.97	5	0.241	5
G	pH of fermentation	-	4	6	0.68	6	0.073	11
H	Temperature of fermentation	°C	27	37	0.05	11	0.083	9
I	Time of fermentation	h	60	108	0.54	8	0.229	6
J	AMG activity	U/g	27	108	1.28	2	0.174	7
K	Inoculum size	%v/v	5	15	1.32	1	0.142	8

Table 4.4 shows a Plackett-Burman design for 11 variables (A - K) at high and low levels. These can then be used in the design to obtain an estimate of error. Each horizontal row represents a trial and each vertical column represents the H (high) and L (low) values of one variable in all the trials. This design requires that the frequency of each level of a variable in a given column should be equal and that in each test (horizontal row) the number of high and low variables should be equal.

Consider the variable A; for the trials in which A is high, B is high in three of the trials and low in the other three. Similarly, C will be high in three trials and low in three, as will all the remaining variables. For those trials in which A is low, B will be high three times and low three times. This will also apply to all the other variables. Thus, the effects of changing the other variables cancel out when determining the effect of A. The same logic then applies to each variable.

Greasham and Inamine (1986) state that although the difference between the levels of each variable must be large enough to ensure that the optimum response will be included, caution must be taken when setting the level differential for sensitive variables, since a differential that is too large could mask the other variables. The trials are carried out in a randomized sequence.

The procedure of Plackett-Burman method will identify the important variables and allow them to be ranked in order of importance to decide which to investigate in a more detailed study to determine the optimum values to use. The steps in analyzing the data (Tables 4.4) are as follows (Nelson, 1982):

(i) Determine the difference between the average of the H (high) and L (low) responses for each independent and dummy variable. Therefore the difference = ∑A (H) - ∑A (L). The effect of an independent variable on the response is the difference between the average response for the six experiments at the high level and the average value for six experiments at the low level. Thus the effect of A is given by

$$A = \frac{\sum A\,(H) - \sum A\,(L)}{6} \quad (4.32)$$

(ii) When probability tables are examined, it is found that factors A, B, J and K show large effects which are significant for ethanol production from cassava peel and factors A, B and E showed significant effect for ethanol production from cassava stem. The next stage would then be the optimization of each factor.

Nelson (1982) has also referred to the probability of two factor interactions which might occur when designing Tables 4.2 and 4.3. This technique has also been discussed (McDaniel *et al.,* 1976; Greasham and Inamine, 1986; Bull *et at.,* 1990 and Hicks, 1993).

4.3.3 BOX-BEHNKEN DESIGN

After the screening of parameters affecting ethanol production by Plackett-Burman design, Box-Behnken design (BBD) was adopted to know the optimum response region of ethanol production and optimize the variables (Box and Behnken, 1960): substrate concentration, enzyme concentration and hydrolysis time for cassava stem and substrate concentration, enzyme concentration and fermentation recipe for cassava peel.

The results of Box–Behnken design experiments for studying the effect of three independent variables (optimized by Placket-Burman design) were presented along with the observed responses in Tables 4.5 and 4.6. In this study, Box–Behnken design was used to evaluate the main and interaction effects of the factors determined from Plackett-Burman design on ethanol production (Anita and Narsi, 2012). The equation that relates the ethanol production as the dependent variable (Y, g/mL) to other significant terms, as listed in Tables 4.5 and 4.6 can be expressed as follows:

Ethanol production from cassava peel Y (g/mL) $= -4.855 + 0.329 * FR + 0.169 * S - 0.600 * E - 0.012 * FR^2 - 0.001 * S^2 - 0.020 * E^2 + 4.859 * 10^{-6} * FR* S - 0.003 * FR * E - 0.001 * E * S$ (4.31)

Ethanol production from cassava stem Y (g/mL) $= -5.204 + 0.359 * E + 0.387 * S - 0.007 * t - 0.005 * E^2 - 0.004 * S^2 - 0.093 * t^2 - 0.003 * E * S + 3.093 * 10^{-5} * E* t + 0.001 * S * t$ (4.32)

Table 4.5 Box-Behnken Design matrix for optimization of ethanol production from cassava peel identified by Plackett-Burman design

StdOrder	RunOrder	PtType	Blocks	FR	S	E	Ethanol (g/mL)
14	1	0	1	15.075	40	15	5.068
12	2	2	1	15.075	70	25	1.611
1	3	2	1	5.025	10	15	2.127
9	4	2	1	15.075	10	5	1.398
13	5	0	1	15.075	40	15	5.068
10	6	2	1	15.075	70	5	4.446
8	7	2	1	25.125	40	25	0.009
3	8	2	1	5.025	70	15	5.339
2	9	2	1	25.125	10	15	0.003
6	10	2	1	25.125	40	5	2.580
4	11	2	1	25.125	70	15	3.221
5	12	2	1	5.025	40	5	3.032
11	13	2	1	15.075	10	25	0.006
15	14	0	1	15.075	40	15	5.068
7	15	2	1	5.025	40	25	1.700

Table 4.6 Box-Behnken Design matrix for optimization of ethanol production from cassava stem identified by Plackett-Burman design

StdOrder	RunOrder	PtType	Blocks	E	S	t	Ethanol (g/mL)
2	1	2	1	40	20	2	4.623
15	2	0	1	25	40	2	6.385
9	3	2	1	25	20	0.5	5.281
14	4	0	1	25	40	2	6.385
5	5	2	1	10	40	0.5	5.680
10	6	2	1	25	60	0.5	4.813
4	7	2	1	40	60	2	2.429
13	8	0	1	25	40	2	6.385
6	9	2	1	40	40	0.5	5.428
1	10	2	1	10	20	2	3.073
12	11	2	1	25	60	3.5	3.905
3	12	2	1	10	60	2	4.480
8	13	2	1	40	40	3.5	4.447
11	14	2	1	25	20	3.5	4.224
7	15	2	1	10	40	3.5	4.697

Table 4.7 Analysis of variance for selected Box-Behnken Design (cassava peel)

Source	Sum of Squares	DF	Mean square	F value	p-value Prob>F	
Model	52.242	9	5.805	30.252	0.0008	Significant
A-FR	5.112	1	5.112	26.642	0.0036	
B-S	15.326	1	15.326	79.876	0.0003	
C-E	8.262	1	8.262	43.060	0.0012	
AB	4E-06	1	4E-06	2.08E-05	0.9965	
AC	0.384	1	0.384	2.000	0.2164	
BC	0.521	1	0.521	2.713	0.1605	
A^2	5.464	1	5.464	28.477	0.0031	
B^2	5.154	1	5.154	26.862	0.0035	
C^2	15.084	1	15.085	78.616	0.0003	
Residual	0.959	5	0.192			
Lack of Fit	0.959	3	0.320			
Pure Error	0	2	0			
Cor Total	53.201	14				
R^2	0.982					
Adj R^2	0.950					

Analysis of variance (ANOVA) of the quadratic equation for ethanol production are summarized in Tables 4.7 and 4.8. ANOVA of regression model demonstrates that the model is highly significant, as is evident from the Fisher's F-test with a very low probability value [(p model > F)]. The p-value denoting the significance of the coefficients was also important in understanding the pattern of the mutual interactions between the variables. The goodness of the fit of the model can be checked by the determination coefficient R^2, the value of R^2 for cassava peel and cassava stem are 0.982 and 0.999 respectively. The values of adjusted R^2 for cassava peel and cassava stem are 0.950 and 0.998 respectively. The goodness of fit shows a

high correlation between the observed values and the predicted values. This means that regression model provides an excellent explanation of the relationship between the independent variables (factors) and the response (ethanol production). No abnormality was observed from the diagnoses of residuals. Thus, it can be concluded that the model was statistically sound. The interactions between the three factors were significant (Tables 4.7 and 4.8). The exception in the interaction between inoculum level and temperature was non-significant (Tables 4.7 and 4.8).

Table 4.8 Analysis of variance for selected Box-Behnken Design (cassava stem)

Source	Sum of Squares	DF	Mean square	F value	p-value Prob>F	
Model	18.93	9	2.10	3.24E+06	<0.001	Significant
A-E	0.13	1	0.13	1.94E+05	<0.001	
B-S	0.31	1	0.31	4.79E+05	<0.001	
C-t	1.93	1	1.93	2.96E+05	<0.001	
AB	3.24	1	3.24	4.90E+06	<0.001	
AC	1.00E-06	1	1.00E-06	1.54	0.2699	
BC	5.26E-03	1	5.26E-03	8086.54	<0.001	
A^2	4.58	1	4.58	7.05E+06	<0.001	
B^2	9.69	1	9.69	1.49E+07	<0.001	
C^2	0.16	1	0.16	2.46E+05	<0.001	
Residual	3.25E-06	5	6.50E-07			
Lack of fit	3.25E-06	3	1.08E-06			
Pure error	0.000	2	0.000			
Cor total	18.93	14				

R^2	0.999				
Adj R^2	0.998				

Tables 4.7 and 4.8 summarize regression parameters used in the ethanol production model. As in Tables 4.7 and 4.8 showed, linear terms (A, B and C), square terms (A^2, B^2 and C^2), and two-way interaction terms (AB, AC and BC) are the major factors, with p-values of under $\alpha = 0.05$, significantly affecting ethanol production. Tables 4.9 and 4.10 summarize the predicted and actual value table of ethanol production from cassava peel and cassava stem.

Table 4.9 Predicted and actual value table of ethanol production from cassava peel

Standard order	Actual value	Predicted value	Difference
1	2.127	2.084	0.043
2	0.003	0.004	-0.001
3	5.339	5.355	-0.015
4	3.211	3.254	-0.043
5	3.032	3.036	-0.004
6	2.58	2.569	0.011
7	1.7	1.923	-0.223
8	0.009	0.010	-0.001
9	1.398	1.366	0.032
10	4.446	4.463	-0.017
11	0.006	0.007	-0.001
12	1.611	1.672	-0.061
13	5.068	5.068	0.000
14	5.068	5.068	0.000
15	5.068	5.068	0.000

Table 4.10 Predicted and actual value table of ethanol production from cassava stem

Standard order	Actual value	Predicted value	Difference
1	3.07	3.07	0.00
2	4.62	4.62	0.00
3	4.48	4.48	0.00
4	2.43	2.43	0.00
5	5.68	5.68	0.00
6	5.43	5.43	0.00
7	4.70	4.70	0.00
8	4.45	4.45	0.00
9	5.28	5.28	0.00
10	4.81	4.81	0.00
11	4.23	4.23	0.00
12	3.90	3.91	-0.01
13	6.38	6.39	-0.01
14	6.38	6.39	-0.01
15	6.38	6.39	-0.01

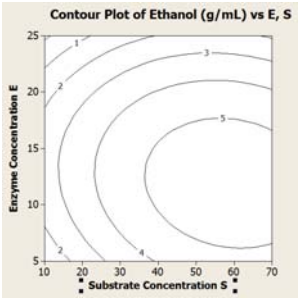

Figure 4.1 Contour plot showing the effect of enzyme and substrate concentrations on ethanol production from cassava peel

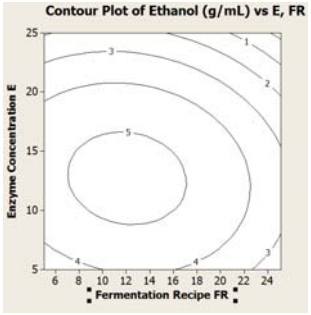

Figure 4.2 Contour plot showing the effect of enzyme concentration and fermentation recipe on ethanol production from cassava peel

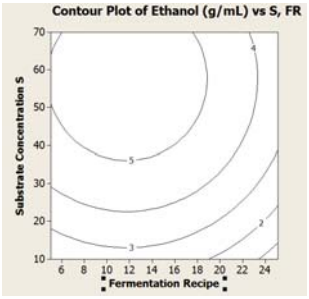

Figure 4.3 Contour plot showing the effect of substrate concentration and fermentation recipe on ethanol production from cassava peel

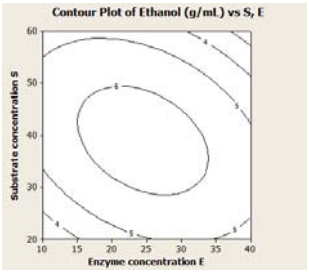

Figure 4.4 Contour plot showing the effect of enzyme and substrate concentrations on ethanol production from cassava stem

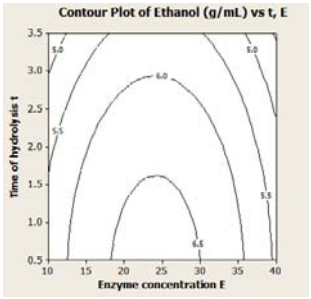

Figure 4.5 Contour plot showing the effect of enzyme concentration and time of hydrolysis on ethanol production from cassava stem

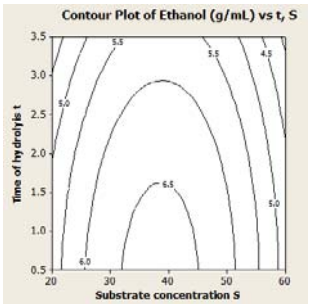

Figure 4.6 Contour plot showing the effect of time of hydrolysis and substrate concentration on ethanol production from cassava stem

The graphical representation of the regression equations 4.33 and 4.34 are presented in Figures 4.1 – 4.6. The response surface model was used to predict the result by isoresponse contour plots. Figure 4.1 showed a plot at varying substrate and enzyme concentrations at fixed value for fermentation recipe. Enzyme concentration has a profound effect on ethanol production, since enzyme is a biocatalyst. Ethanol production is low at enzyme concentration

of low value of enzyme concentration and increases with increase in enzyme concentration up to 12.18% (w/w) and further increases in enzyme concentration decreases ethanol production for cassava peel. But for cassava stem (Figure 4.4), ethanol production is low at low value of enzyme concentration and increases with increase in substrate concentration up to 25% (w/w) and further increases in enzyme concentration decreases ethanol production.

Figure 4.2 represents a plot at varying enzyme concentration and fermentation recipe, at fixed substrate concentration. Ethanol production increases with increase in fermentation recipe level up to a certain extent after, ethanol production rate decreases. The ethanol concentration reached a peak value at the mid-value of fermentation recipe. Powchinda *et al.,* (1999) stated that up to a critical amount, the increase in fermentation recipe increases ethanol yield due to better utilization of the sugars. However, a high amount of fermentation recipe can adversely affect ethanol production due to the fact that high increase in fermentation recipe level decreases the viability of bacterial population and causes inadequate development of biomass and ethanol production (Powchinda *et al*., 1999). Figure 4.5 represents a plot at varying enzyme concentration and hydrolysis time, at fixed substrate concentration. Ethanol production increases with increase in hydrolysis time to a certain extent and after, ethanol production rate decreases. The ethanol concentration reached a peak value at the intermediate value of fermentation recipe.

Figure 4.3 illustrates graphically a plot at varying fermentation recipe and substrate concentration with fixed enzyme concentration. Substrate concentration is an important factor in fermentation process; ethanol production is low at substrate concentration of 10% (w/v) and increases with increase in substrate concentration up to 60% (w/v) and further increases in substrate concentration decreases ethanol production for cassava peel because of product inhibition. But for cassava stem (Figure 4.6), ethanol production is low at substrate concentration of 10% (w/v) and increases with increase in substrate concentration up to 40% (w/v) and further increases in substrate concentration decreases ethanol production.

The developed model was verified by performing trial under optimum conditions (Table 4.11). The results of the experiments at optimum conditions were presented in Table 4.11 in terms of predicted and experimental ethanol concentrations. A high value of coefficient of determination (R^2 = 0.999) showed that the model was successful in predicting ethanol concentration.

Table 4.11 Validation of the model for bioethanol production from cassava peel and cassava stem

VARIABLE		UNIT	OPTIMUM VALUE	
			CASSAVA PEEL	CASSAVA STEM
A	Substrate concentration	%w/v	59.10	38.59
B	α-amylase concentration	%w/w	12.18	24.55
C	pH of hydrolysis	-	4	7
D	Temperature of hydrolysis	°C	60	40
E	Time of hydrolysis	min	15	30
F	Agitation speed	rpm	0	0
G	pH of fermentation	-	4	6
H	Temperature of fermentation	°C	37	27
I	Time of fermentation	h	60	108
J	AMG activity	%v/v	0.13	1.5
K	Inoculum size	%v/v	12	5
Ethanol concentration (Predicted result by BBD)		g/mL	5.766	6.677
Ethanol concentration (Experimental result)			5.793	6.143

4.3.4 KINETICS FOR CONSUMPTION OF SUBSTRATE

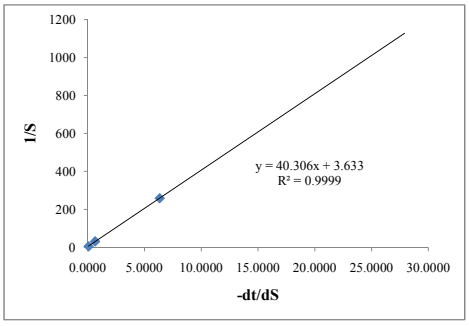

Figure 4.7 Kinetic plot for substrate consumption of cassava peel

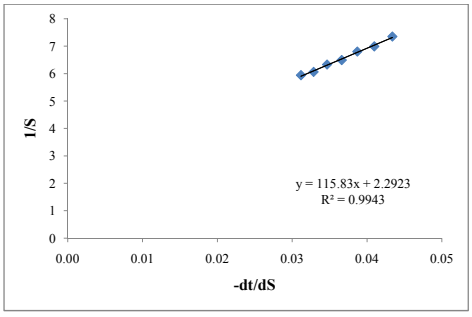

Figure 4.8 Kinetic plot for substrate consumption of cassava stem

In general, a factor that increases the number of collisions between particles will increase the reaction rate and a factor that decreases the number of collisions between particles will decrease the chemical reaction rate.

The rate of a chemical reaction is defined as number of moles of reactant consumed or number of moles of product formed per unit time. Enzymes lower the activation energy of a chemical reaction and increase the rate of a chemical reaction without being consumed in the process. Enzymes work by increasing the frequency of collisions between reactants, altering the orientation of reactants so that more collisions are effective, reducing intramolecular bonding within reactant molecules, or donating electron density to the reactants. The presence of an enzyme helps a reaction to proceed more quickly to equilibrium. Aside from enzymes, other parameters can affect a reaction rate.

Reaction time is one of the important factors that affects the reaction rate. As the reaction time increases i.e., reaction progresses, the concentration of substrate decreases and the concentration of product increases. After a certain period of time, concentrations of the substrates and products remain constant. The time at which the concentration of substrates and product remains constant is called equilibrium time. Equilibrium time may vary for the consumption of substrate and also for the product.

The kinetic equation for the consumption of substrate is given by equation (4.35).

$$-\frac{dt}{dS} = \frac{k_m}{V_{max}[S]} + \frac{1}{V_{max}} \quad (4.35)$$

A plot of –dt/dS versus 1/S gives a slope of k_m/V_{max} and intercept of 1/ V_{max}. The values of k_m and V_{max} determined for cassava peel and cassava stem are tabulated as follows

Table 4.12 Kinetic constants for ethanol production from cassava peel and cassava stem

S.No.	PARAMETER	UNIT	CASSAVA PEEL	CASSAVA STEM
1.	k_m	g/mL	11.09	50.53
2.	V_{max}	g/mL.s	0.275	10.436

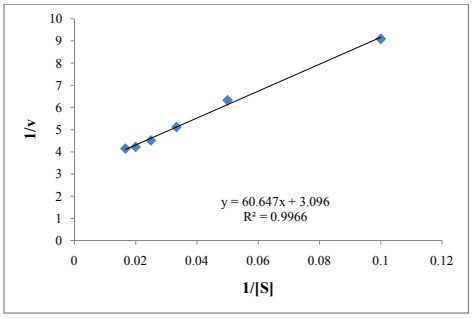

Figure 4.9 Michaelis-Menten plot for cassava peel

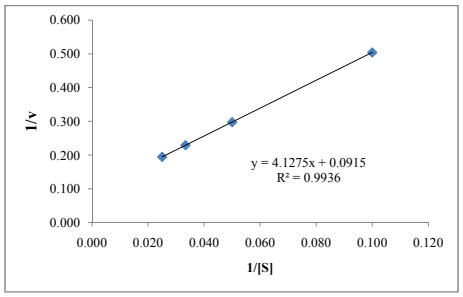

Figure 4.10 Michaelis-Menten plot for cassava stem

It has been shown experimentally that, if the concentration of enzyme is kept constant and the substrate concentration is gradually increased, the reaction rate will increase until it reaches a maximum. After this point, increase in substrate concentration will not increase the reaction rate. It is theorized that, when this maximum velocity had been reached, all of available enzyme has been converted to the enzyme-substrate complex. This point is designated as V_{max} by Michaelis and Menten. Michaelis and Menten developed a set of

mathematical expressions to calculate the rate of formation of product in terms of substrate concentration from measurable experimental data. Michaelis-Menten equation is given by

$$v = \frac{v_{max}[S]}{k_m + [S]} \quad (4.37)$$

where v is the rate of product formation, [S] is substrate concentration and k_m is Michaelis - Menten constant. Michaelis-Menten constant have been determined to know the requirement for substrate to reach maximum reaction rate. A plot of 1/v versus 1/[S] gives a slope of k_m/V_{max} and intercept of $1/V_{max}$. The values of k_m and V_{max} determined for cassava peel and cassava stem are tabulated as follows:

Table 4.13 Maximum velocity and Michaelis-Menten constant for cassava peel and cassava stem

S.No.	PARAMETER	UNIT	CASSAVA PEEL	CASSAVA STEM
1.	k_m	g/mL	19.589	45.109
2.	V_{max}	g/mL.s	0.323	10.929

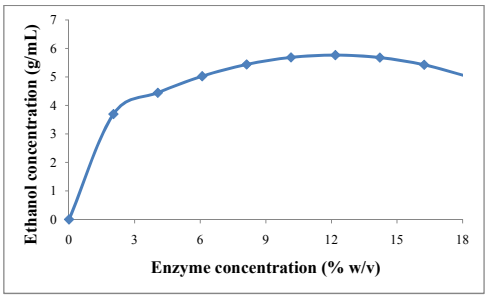

Figure 4.11 Effect of enzyme concentration on ethanol concentration for cassava peel

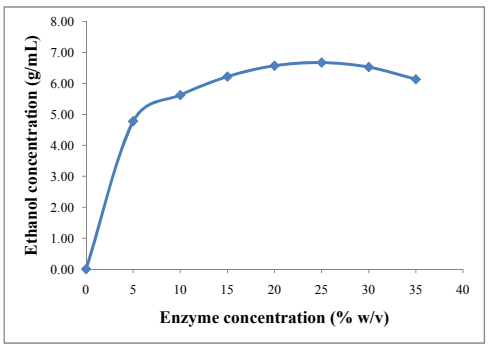

Figure 4.12 Effect of enzyme concentration on ethanol concentration for cassava stem

In order to study the effect of increasing the enzyme concentration on ethanol concentration, substrate concentration must be maintained at a constant value i.e. the reaction must be independent of the substrate concentration. Any change in the concentration of ethanol formed over a specified period of time depends only on the concentration of enzyme.

In Figures 4.11 and 4.12, ethanol concentration is directly proportional to the enzyme concentration from 0% (w/w) to 5% (w/w), but not beyond 5% (w/w). Between 0% (w/w) and 5% (w/w) of enzyme concentration the curve represents a zeroth order reaction i.e. one in which the ethanol concentration is constant with time. As substrate is utilized, the active sites of enzymes are no longer saturated, substrate concentration becomes rate limiting and the reaction order changes from zeroth order beyond 5% (w/w).

There are three types of reactions which might be encountered while studying the effect of enzyme concentration on product concentration. They are leading, lagging and linear reactions. The reaction is zero order initially and then reaction rate slows, presumably due to substrate utilization or product inhibition. This type of reaction is called as leading reaction. Ethanol production is an example of leading reaction.

4.3.5 Thermodynamics of ethanol production

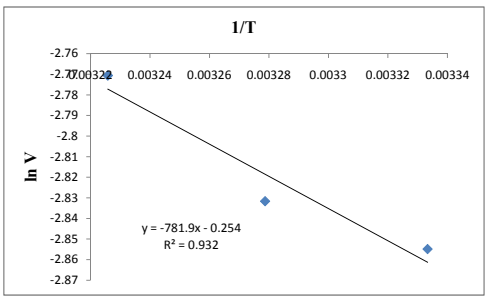

Figure 4.13 Arrhenius plot for ethanol production from cassava peel

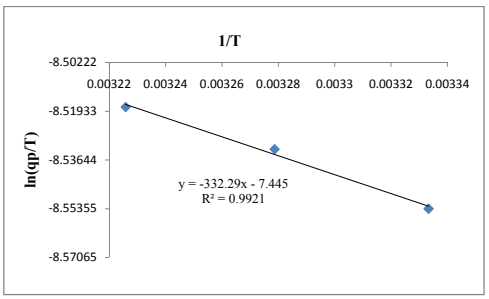

Figure 4.14 Calculation of enthalpy and entropy of ethanol production from cassava peel

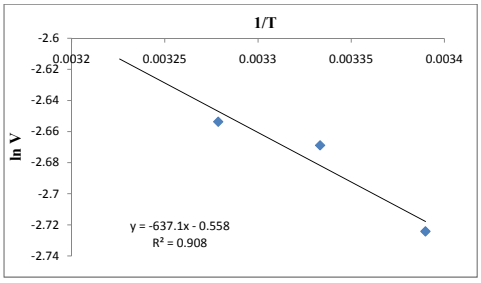

Figure 4.15 Arrhenius plot for ethanol production from cassava peel

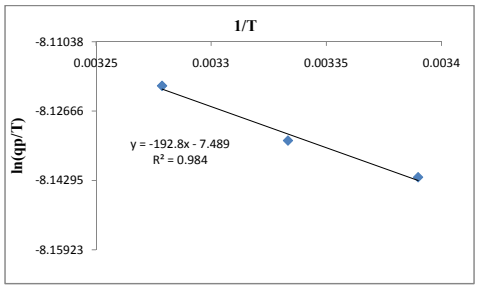

Figure 4.16 Calculation of enthalpy and entropy of ethanol production from cassava stem

Table 4.14 Thermodynamic constants for ethanol production from cassava peel and cassava stem

S. No.	PARAMETER	UNIT	CASSAVA PEEL	CASSAVA STEM
1.	Activation Energy (E_a)	J/mol	6501.5	5297.1
2.	Frequency factor (k_0)	g/mL.s	0.775	0.572
3.	Entropy (ΔS°)	J/mol.K	-259.44	-259.8

4.	Enthalpy (ΔH°)	J/mol	2762.65	1603.43

Requirement of lower energy of activation (E_a) for growth and lower need of enthalpy and entropy of activation for product formation, may be considered as potential indices for thermal stability of cultures during production processes, as they (parameters) are considered for thermal stable enzymes (Declerck *et al.,* 2003). The cassava peel (E_a = 6501.5 J/mol) was 1.22-fold more stable than cassava stem (E_a = 5297.1 J/mol) as it required lower energy of activation for growth (Figures 4.13 and 4.15) (Aiba *et al.* 1973).

The values of the thermodynamic parameters (Table 3) indicated that the activation enthalpy of ethanol formation by cassava stem (ΔH° = 40 kJ/mol) is lower than that of ethanol production by cassava peel (10–30 kJ/mol) (Converti and Dominguez 2001), but compares favorably with those estimated for many different whole-cell bioprocesses, such as cell growth (34±74 kJ/mol) (Aiba *et al.* 1973).

The activation entropy of ethanol formation by cassava stem (−259.8 J/mol.K) is low and compares favourably with that of ethanol formation reactions (Converti and Dominguez 2001). The activation entropy value of thermal inactivation by cassava peel (−259.4 J/mol.K) is also low and is comparable with that for ethanol production by a *Zymomonas mobilis* (Rajoka *et al.,* 2003) (and has negative symbol) which reflects that this phenomenon implies a little disorderliness during growth. Practically this value is lower than those estimated for alcohol production by other systems (Converti and Dominguez 2001). This suggests more protection exerted by cassava stem compared with cassava peel.

CHAPTER 5
DESIGN OF PACKED BED REACTOR FOR CONTINUOUS ETHANOL PRODUCTION

5.1 DESIGN OF PACKED BED REACTOR

Performance equation of ideal PFR is given by

$$\frac{V}{F_{Ao}} = \int_0^{X_A} \frac{dX_A}{-r_A} \quad (5.1)$$

where,

V is volume of reactor

F_{Ao} is feed rate = V_oC_{Ao}

V_o is volumetric flow rate

C_{Ao} is initial concentration of substrate

X_A is conversion

$-r_A$ is rate of the reaction

The plug flow reactor can be used as the packed bed reactor since the construction and the reactor forms the same reaction dynamics. The difference between PFR and PBR are as follows:

- Only homogenous reaction can be carried out in the PFR where as heterogenous reactions can be carried out in PBR.
- In PFR, the reaction rate depends on the volume of the reactor (V_r) but in PBR the reaction rate depends on mass of the catalyst (M_c).

Since the rate of reaction in PBR depends on mass of catalyst, equation (5.1) should be rewritten in terms of mass of the catalyst. Therefore equation (5.1) becomes

$$\frac{V}{V_oC_{Ao}} = \int_0^{X_A} \frac{dX_A}{-r_A} \quad (5.2)$$

$$Density\ of\ catalyst\ \rho_c = (Mass\ of\ catalyst\ M_c)/(Volume\ of\ catalyst\ V_c)$$

$$V = \frac{\rho_c}{M_c} \quad (5.3)$$

Assuming that volume of catalyst is equal to volume of reactor and by substituting equation (5.3) in (5.2) we get,

$$\frac{M_c}{\rho_c V_o C_{Ao}} = \int_0^{X_A} \frac{dX_A}{-r_A} \quad (5.4)$$

The rate of enzymatic reaction is given by Michaelis Menten equation

$$-r_s = \frac{V_{max}[S]}{K_m + [S]} = \frac{V_{max}C_s}{K_m + C_s} \quad (5.5)$$

Expressing equations (5.4) in terms of substrate S instead of reactant A, equation (4) becomes

$$\frac{M_c}{\rho_c V_o C_{So}} = \int_0^{X_s} \frac{dX_s}{-r_s} \quad (5.6)$$

By substituting equation (5.5) in (5.6) we get,

$$\frac{M_c}{\rho_c V_o C_{So}} = \int_0^{X_s} \frac{dX_s}{\frac{V_{max}C_s}{K_m + C_s}}$$

$$\frac{M_c}{\rho_c V_o C_{So}} = \int_0^{X_s} \frac{(K_m + C_s)dX_s}{V_{max}C_s} \quad (5.7)$$

Reaction conversion X_s is given by

$$X_s = \frac{C_{So} - C_s}{C_{So}}$$

$$C_S = (1 - X_s)C_{So} \quad (5.8)$$

By substituting equation (5.8) in (5.7) we get,

$$\frac{M_c}{\rho_c V_o C_{So}} = \int_0^{X_s} \frac{[K_m + (1 - X_s)C_{So}]dX_s}{V_{max}(1 - X_s)C_{so}}$$

$$\frac{M_c}{\rho_c V_o} = \int_0^{X_s} \frac{K_m dX_s}{V_{max}(1 - X_s)} + \int_0^{X_s} \frac{(1 - X_s)C_{so}dX_s}{V_{max}(1 - X_s)}$$

$$\frac{M_c}{\rho_c V_o} = \int_0^{X_s} \frac{K_m dX_s}{V_{max}(1- X_s)} + \int_0^{X_s} \frac{C_{so} dX_s}{V_{max}}$$

$$\frac{M_c}{\rho_c V_o} = -\frac{K_m}{V_{max}} \ln (1 - X_s) + \frac{C_{so} X_s}{V_{max}}$$

(5.9)

By rearranging the above equation (5.9) we get,

$$M_c = \frac{\rho_c V_o}{V_{max}} [C_{so} X_s - K_m \ln(1 - X_s)]$$

(5.10)

The above equation is performance equation of packed bed reactor.

CHAPTER 6
CONCLUSION

The state of the art of starch hydrolysis and fermentation technologies to produce ethanol from cassava by-products was evaluated. Cassava stem, cassava peel, cassava waste, cassava twig and cassava leaves are samples collected from sago industry and tested for total carbohydrate and crude protein. Two samples from sago factory, namely cassava stem and cassava peel, with similar total carbohydrate content (76.47%), were used as substrates. The samples were liquefied using various concentrations of α-amylase, in order to optimize the production of fermentable sugars, the enzyme α-amylase of 12.18% (w/w of Substrate) for cassava peel and 24.55% (w/w of Substrate) for cassava stem revealed higher performance. After liquefaction, the simultaneous saccharification and fermentation was conducted in shaker flask. Glucoamylase was used for saccharification, and *Zymomonas mobilis* MTCC 2427 for fermentation simultaneously. Sterile samples were withdrawn regularly for analysis. Glucose consumed promptly in both cases, cassava peel and cassava stem; meanwhile, the ethanol production was considerably higher in cassava stem (6.143 g/mL), compared to cassava peel (5.793 g/mL). The substrate containing cassava stem revealed higher potential as substrate for ethanol production.

REFERENCES

Adelekan, B. A. (2012) 'Cassava as a potent energy crop for the production of ethanol and methane in tropical countries', International Journal of Thermal & Environmental Engineering, Vol. 4, No. 1, pp. 25-32

Adenise Lorenci Woiciechowsk, Saul Nitsche, Ashok Pandey and Carlos Ricardo Soccol (2002) 'Acid and enzymatic hydrolysis to recover reducing sugars from cassava bagasse: An economic study', Brazilian Archives of Biology and Technology, Vol. 45, No. 3, pp. 393-400.

Adesanya, O. A., Oluyemi, K. A., Josiah, S. J., Adesanya, R. A., Shittu L. A. J., Ofusori D.A., Bankole, M. A. and Babalola, G.B. (2008) 'Ethanol production by *Saccharomyces cerevisiae* from cassava peel hydrolysate', Internet Journal of Microbiology, Vol. 5, pp. 1-6.

Ado, S. A., Olukotun, G. B., Ameh, J. B. and Yabaya, A. (2009) 'Bioconversion of cassava starch to ethanol in a simultaneous saccharification and fermentation process by co-cultures of *Aspergillus niger* and *Saccharomyces cerevisiae*', Science World Journal, Vol. 4, pp. 19-22.

Aggarwal, N. K., Nigam, P., Singh, D. and Yadav, B.S. (2001) 'Process optimization for the production of sugar for the bioethanol industry from Tapioca, a non-conventional source of starch', World Journal of Microbiology and Biotechnology, Vol. 17, pp. 783-787.

Aiba, S., Humphery, A.E. and Mullis, N.F. (1973) 'Scale up in biochemical engineering', 2nd edition, Academic Press, New York, 195-217.

Akponah, E. and Akpomie, O.O. (2011) 'Analysis of the suitability of yam, potato and cassava root peels for bioethanol production using *Saccharomyces cerevisae*', International Research Journal of Microbiology, Vol. 2, No. 10, pp. 393-398.

Arrhenius, S. (1889) 'On the Reaction Velocity of the Inversion of Cane Sugar by Acids', Zeitschrift für physikalische Chemie, Vol. 4, pp. 226.

Azlin Azmi, Masitah Hasan, Maiziwan Mel, and Cheng Ngoh (2009) 'Single-step bioconversion of starch to bioethanol by the coculture of ragi tapai and *Saccharomyces cerevisiae*', Chemical Engineering Transactions, Vol. 18, pp. 557-562.

Bagus Wahyu Murtianto and Bondan Aria Chandra (2008) 'Production of bioethanol from cassava peel waste by using sweet corn enzyme powder', B.Tech. Thesis, Semesta Bilingual Boarding School, Semarang, Indonesia

Bai F.W., Anderson W.A. and Moo-Young M. (2008) 'Ethanol fermentation technologies from sugar and starch feedstocks', *Biotechnology Advances*, Vol. 26, pp. 89-105.

Ballesteros, I., Negro, M., Oliva, J. M., Catanás, A., Manzanares, P. and Ballesteros, M. (2006) 'Ethanol production from steam-explosion pretreated wheat straw', Applied Biochemistry and Biotechnology, Vol. 130, No. 3, pp. 495-508.

Betiku, E. and Alade, O. S. (2010) 'Media evaluation for bioethanol production from cassava starch hydrolysate using *Saccharomyces cerevisiae*', Proceedings of Third International Symposium on Energy from Biomass and Waste, pp. 26-40.

Betiku, O. S., and Alade, E. (2010) 'Design of a continuous bioreactor for laboratory scale production of ethanol from cassava starch hydrolysate using *Saccharomyces cerevisiae*', IFE Journal of Technology, Vol. 19, No. 2, pp. 15-20.

Bio-Wealth Resource Limited, http://biowealthresourceltd.com/cassava.html

Box, G.E.P. and Behnken, D.W. (1960) 'Some new three level designs for study of quantitative variables', Technometrics, Vol. 2, pp. 455-475.

Brian A. Schumacher (2002) 'Methods for the determination of total organic carbon in soils and sediments', Ph.D. Thesis, National Exposure Research Laboratory, Environmental Sciences Division, United States Environmental Protection Agency, Las Vegas.

Buddhiporn Sornvoraweat and Jirasak Kongkiattikajorn (2011) 'Bioethanol production from cassava peels by simultaneous saccharification and fermentation by mono-culture and co-culture of yeast', 35th Congress on Science and Technology of Thailand, pp. 1-7.

Bull, A. T., Huck, T. A. and Bushell, M. E. (1990) 'Optimization strategies in microbial process development and operation', Microbial growth dynamics, IRL Press, Oxford, pp. 145-168.

Bundit Fungsin, Suthkamol Suttikul, Ancharida Akaracharany, and Teerapatr Srinorakutara. (2010) 'Conversion of cassava waste into sugar using *Aspergillus niger* and *Trichoderma reesei* for ethanol production', Proceedings of Thailand Institute of Scientific and Technological Research, pp. 42-43.

Caputi, A.J., Ueda, M. and Brown, T. (1968) 'Spectrophotometric determination of ethanol in wine', American Journal of Enology and Viticulture, Vol. 19, pp. 160–165.

Cellulase assessment for biomass hydrolysis. http://www.ceassist.com/pdf/ cellulase.pdf

Chandrakant, P. and Bisaria, V. S. (1998) 'Simultaneous bioconversion of cellulose and hemicellulose to ethanol', Critical Reviews in Biotechnology, USA, Vol. 18, No. 4, pp. 295-331.

Chul-Ho Kim, Zainal Abidin, Chong Chok Ngee and Sang-Ki Rhee. (1992) 'Pilot-scale ethanol fermentation by *Zymomonas mobilis* from simultaneously saccharified sago starch', Bioresource Technology, Vol. 40, pp. 1-6.

Claude Fauquet and Denis Fargette. (1990) 'African cassava mosaic virus: Etiology, epidemiology, and control', Plant Disease, Vol. 74, No. 6, pp. 404–11.

Converti, A. and Dominguez, J.M. (2001) 'Influence of temperature and pH on xylitol production from xylose by *Debarryomyces hansenii*', Biotechnology and Bioengineering, Vol. 75, pp. 39–45.

Declerck, N., Machius, M., Joyet, P., Wiegand, G., Huber, R. and Gaillardin, C. (2003) 'Hyperthermo stabilization of *Bacillus licheniformis* α-amylase and modulation of its stability over a 50°C temperature range', Protein Engineering, Vol. 16, pp. 287–293.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) 'Colorimetric method for determination of sugars and related substances', Analytical Chemistry, Vol. 28, No. 3, pp. 350-356.

Eneojo, A. S., Shugaba Aliyu and Bukbuk, D. N. (2010) 'Potential of wild strain *Saccharomyces cerevisiae* in ethanol production', American-Eurasian Journal of Scientific Research, Vol. 5, No. 3, pp. 187-191.

FAO report (1995) 'Dimensions of need: An atlas of food and agriculture', Food and Agriculture Organization (United Nation)

Fiechter, A. (1992) 'Enzymes and products from bacteria fungi and plant cells', Advances in Biochemical Engineering / Biotechnology, Vol. **45**, pp. 144.

Food and Agriculture Organization of the United Nations, 'Roots, tubers, plantains and bananas in human nutrition', Rome, 1990, Ch. 7 "Toxic substances and antinutritional factors", third paragraph.

Ghose, T.K. (1987) 'Measurement of cellulase activities', International *Journal* of *Pure* and *Applied Chemistry*, Vol. 59, pp. 257-268.

Gi-Wook Choi, Hyun-Woo Kang, Young-Ran Kim, and Bong-Woo Chung. (2008) 'Ethanol production by Zymomonas mobilis CHZ2501 from industrial starch feedstocks', Biotechnology and Bioprocess Engineering, Vol. 13, pp. 765-771.

Glazer, A. N. and Nikaido, H. (1995) 'Microbial Biotechnology: Fundamentals of Applied Microbiology', W.H. Freeman and Company, New York.

Godbole, S., Decker, S. R., Nieves, R. A., Adney, W. S., Vinzant, T. B., Baker, J. O., Thomas, S. R. and Himmel, M. E. (1999) 'Cloning and expression of *Trichoderma reesei* cellobiohydrolase I in *Pichia pastoris*', Biotechnology Progress, Vol. 15, No. 5, pp. 828-833.

Goering, H. D. and Vansoest, P. J. (1975) 'Forage fibre analysis US dept of agricultural research service', Washington.

Greasham, R. and Inamine, E. (1986) 'Nutritional improvement of processes', In Manual of Industrial Microbiology and Biotechnology, (Editors Demain, A. L. and Solomon, N. A.), American Society for Microbiology, Washington, pp. 41-48.

Guzman-Maldonado and Paredes-López. (1995) 'Amylolytic enzymes and products derived from starch: A review', *Critical* Reviews in *Food Science* and *Nutrition,* Vol. 35, No. 5, pp. 373-403.

Hendrix, C. (1980) 'Through the response surface with test tube and pipe wrench'. Chemtech, Vol. 10, pp. 488-497

Hespell, R. B., Wyckoff, H., Dien, B. S. and Bothast, R. J. (1996) 'Stabilization of pet operon plasmids and ethanol production in Escherichia coli strains lacking lactate dehydrogenase and pyruvate formate-lyase activities', Applied and Environmental Microbiology, Vol. 62, pp. 4594.

Hicks, C. R. (1993) 'Fundamental concepts in the design of experiments', 4th edition, Saunders, New York.

Himmel, M. E., Ruth, M. F. and Wyman, C. E. (1999) 'Cellulase for commodity products from cellulosic biomass', Current Opinion in Biotechnology, Vol. 10, pp. 358.

Horváth, S. (1969) 'Importance of the generation time in microbiological experiments', Folia Microbiologica, Vol. 15, No. 4, pp. 259-266.

Hyun-Woo Kang, Yule Kim, Seung-Wook Kim and Gi-Wook Choi. (2012) 'Cellulosic ethanol production on temperature-shift simultaneous saccharification and fermentation using the thermostable yeast *Kluyveromyces marxianus* CHY1612', *Bioprocess* and *Biosystems Engineering*, Vol. 35, pp. 115–122.

Ingram, L. (2000) 'Development of portable ethanol-producing operons that can be expressed in gram-positive bacteria'. Project Summaries 1998–1999, US Department of Energy, http://bioenergy.ornl.gov/99summaries/fermentation.html

Ingram, L. O. and Conway, J. (1988) 'Expression of different levels of ethanologenic enzymes from *Zymomonas mobilis* in recombinant strains of *Escherichia coli'*, Applied and Environmental Microbiology, Vol. 54, No. 2, pp. 397-404.

International standard: ISO 1741, (2010) Determination of Dry matter in oven at 105˚C.

International standard: ISO 3593, (2010) Determination of Ash at 900˚C.

James Cappuccino and **Natalie Sherman. (2011) '**Microbiology: A laboratory manual', 9th edition, Benjamin Cummings Publisher.

Janecek, S. (1997) 'α-amylase family: Molecular biology and evolution', Progress in Biophysics and Molecular *Biology,* Vol. 67, pp. 67-97.

Jirasak Kongkiattikajorn and Buddhiporn Sornvoraweat. (2011) 'Comparative study of bioethanol production from cassava peels by monoculture and co-culture of yeast', Kasetsart Journal (Nat. Sci.), Vol. 45, pp. 268-274.

Jirasak Kongkiattikajorn and Preyarat Yowapui. (2007) 'Effect of substrate and amylolytic enzyme concentration on simultaneous saccharification and fermentation of

cassava peel for ethanol production', Proceedings of the 2nd International Conference on Fermentation Technology for Value Added Agricultural Products, pp. 2-8.

Juliano, B. O. (1971) 'A simplified assay for milled rice amylose', Cereal Science Today, Vol. 16, pp. 334-338.

Kanlaya Yoonan, Jirasak Kongkiattikajorn and Kanok Rattanakanokchai. (2009) 'Batch fermentation of ethanol production by *Saccharomyces cerevisiae* on cassava peel hydrolysate', Proceedings of Thailand Institute of Scientific and Technological Research, pp. 1-3.

Kanlaya Yoonan, Jirasak Kongkiattikajorn and KanokRattanakanokchai (2011) 'Fermentation of *Saccharomyces cerevisiae* yeast during ethanol production from highly concentrated hydrolysate of cassava peel', Proceedings of 1st International Conference on Fermentation Technology for Value Added Agricultural Products, pp. 26.

Kanlaya Yoonan, Preyarat Yowapui and Jirasak Kongkiattikajorn (2007) 'Ethanol production from acid hydrolysate of cassava peels using *Saccharomyces cerevisiae*', KMUTT Reseach and Development Journal, Vol. 30, No. 3, pp. 405-417.

Kim, C. H. and Rhee, S. K. (1993) 'Process development for simultaneous starch saccharification and ethanol fermentation by *Zymomonas mobilis*', Process Biochemistry, Vol. 28, pp. 331-339.

Kim, C. H., Lee, G. M., Zainal Abidin, Han, M. H. and Rhee, S. K. (1988) 'Immobilization of *Zymomonas mobilis* and amyloglucosidase for ethanol production from sago starch', *Enzyme* and *Microbial Technology,* Vol. 10, pp. 426-430.

Klass, D. L., and Emert, G. H. (1981) 'Fuels from biomass and wastes', pp. 307.

Krishnaveni, S., Theymoli Balasubramanian and Sadasivam, S. (1984) 'Sugar distribution in sweet stalk sorghum', Food chemistry, Vol. 15, No. 3, pp. 229-232.
Ku Ismail, Ahmad, A. A., Inba, T., Kasim, K. F. and Daud, M. Z. M. (2008) 'Thermo-enzymatic hydrolysis of cassava starch from α-amylase and amyloglucosidase', Proceedings of Malaysian Technical Universities Conference on Engineering and Technology, pp. 19-21.

Kumoro, A. C., Ngoh, G. C., Hasan, M., Ong, C. H. and Teoh, E. C. (2008) 'Conversion of fibrous sago (*Metroxylon sagu*) waste into fermentable sugar via acid and enzymatic hydrolysis', Asian Journal of Scientific Research, Vol. 1, pp. 412-420.

Lee, G. M., Kim, C. H., Zainal Abidin, Han, M. H. and Rhee, S. K. (1987) 'Continuous ethanol production from sago starch using immobilized amyloglucosidase and *Zymomonas mobilis*', Journal of Fermentation Technology, Vol. 65, pp. 531-535.

Lee, G. M., Kim, C. H., Zainal Abidin, Han, M. H. and Rhee, S. K. (1986) 'Simultaneous saccharification and ethanol fermentation of sago starch using immobilized *Zymomonas mobilis*', Fermentation Technology, Vol. 64, pp. 293-297.

Leticia Lopez Zamora, Jose Amir Gonzalez, Calderon Evangelina, Trujillo Vazquez, and Eusebio Bolanos. (2010) 'Optimization of ethanol production process from cassava starch by surface response', Journal of the Mexican Chemical Society, Vol. 54, pp. 198-203.

Linley Chiwona-Karltun, Chrissie Katundu, James Ngoma, Felistus Chipungu, Jonathan Mkumbira, Sidney Simukoko and Janice Jiggins. (2002) 'Bitter cassava and women: an intriguing response to food security', LEISA Magazine, Vol. 18, No. 4.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) 'Protein measurement with the Folin phenol reagent', Journal of Biological Chemistry, Vol. 193, pp. 265.

Ma, H., Wang, Q., Gong, L., Wang, X., Wang, X. and Yin, W. (2008) 'Ethanol production from kitchen garbage by *Zymomonas mobilis*: Optimization of parameters through statistical experimental designs', Chemical and *Biochemical Engineering* Quarterly, Vol. 22, No. 3, pp. 369-375.

Magesh, A., Preetha, B. and Viruthagiri, T. (2011) 'Simultaneous saccharification and fermentation of tapioca stem var. 226 white rose to ethanol by cellulase enzyme and *Saccharomyces cerevisiae'*, International Journal of ChemTech Research, Vol. 3, No. 4, pp. 1821-1829.

Magesh, A., Preetha, B. and Viruthagiri, T. (2011) 'Statistical optimization of process variables for direct fermentation of 226 white rose tapioca stem to ethanol by *Fusarium oxysporum*', World Academy of Science, Engineering and Technology, Vol. 75, pp. 786-791.

Maldihah, M. S., Ariff, A.B., Sahaid, K.M., Suraini, A.A. and Karim, M.I.A. (2001) 'Direct fermentation of gelatinized sago starch to acetone-butanol-ethanol by *Clostridium acetobutylicum*', World Journal of Microbiology and Biotechnology, Vol. 17, pp. 567-576.

Mariamma Abraham and Muraleedhara Kurup, G. (1997) 'Kinetics of the enzymatic saccharification of pretreated tapioca waste (*Manihot esculenta*) and water hyacinth (*Eichhornia crassipes*)', Applied Biochemistry and Biotechnology, Vol. 66, pp. 133-1142.

Maynard, A. J. (1970) 'Methods in Food analysis', Academic Press New York, pp. 176.

Mc Cready, R. M., Guggolz, J., Siliviera, V. and Owens, H. S. (1950) 'Determination of starch and amylose in vegetables', Analytical Chemistry, Vol. 22, No. 9, pp. 1156-1158

McDaniel, L. E., Bailey, E. G., Ethiraj, S. and Andrew, H. (1976) 'Application of response surface optimization techniques to polyene macrolide fermentation studies in shake flasks', Developments in Industrial Microbiology, Vol. 17, pp. 91-98.

Michaelis, M.I. and Menten, L. (1913), 'Die Kinetik der Invertinwirkung', Biochemistry, Vol. 49, pp. 333–369.

Miller, G.L. (1959) 'Use of dinitrosalicylic acid reagent for determination of reducing sugar', Analytical Chemistry, Vol. 31, pp. 426–428.

Minhee Han, Yule Kim, Youngran Kim, Bongwoo Chung, and Gi-Wook Choi. (2011) 'Bioethanol production from optimized pretreatment of cassava stem', Korean Journal of Chemical Engineering, Vol. 28, No. 1, pp. 119-125.

Muhamad Fauzi Bin Ibrahim. (2009) 'Production of Bioethanol from Tapioca Starch using *Saccharomyces Cerevisiae*: Effects of Temperature and Agitation Speed', B.Tech dissertation, Universiti Malaysia Pahang.

Negro, M. J., Paloma Manzanares, Ignacio Ballesteros, Jose Miguel Oliva, Araceli Cabañas and Mercedes Ballesteros. (2003) 'Hydrothermal pretreatment conditions to enhance ethanol production from poplar biomass', Applied Biochemistry and Biotechnology, Vol. 105, No. 1-3, pp. 87-100

Nelson, L. S. (1982) 'Technical aids', Journal of Quality *Technology*, Vol. 14, No. 2, pp. 99-100.

Olsen, H. S. and Andersen, E. (2001) 'Utilization of enzymes in agricultural product processing', Novozymes A/S, Catalogue A-6901

Olsen, K. M. and Schaal, B. A. (1999) 'Evidence on the origin of cassava: phylogeography of *Manihot esculenta*', Proceedings of the National Academy of Sciences of the United States of America, Vol. 96, No. 10, pp. 5586-91.

Oyeleke, S.B., Dauda, B.E.N., Oyewole, O.A., Okoliegbe, I.N. and Ojebode, T. (2012) 'Production of bioethanol from cassava and sweet potato peels', Advances in Environmental Biology, Vol. 6, No. 1, pp. 241-245.

Pellett and Young. (1980) 'Nutritional evaluation of protein foods', UN Universal Publications

Pérez, J. A., Ballesteros, I., Ballesteros, M., Sáez, F., Negro, M. J. and Mazanares. P. (2008) 'Optimizing liquid hot water pretreatment conditions to enhance sugar recovery from wheat straw for fuel-ethanol production', Fuel, Vol. 87, pp. 3640-3647.

Picataggio, S. K. and Zhang, M. (1996) 'Biocatalyst development for bioethanol production from hydrolysates', In: Wyman CE, Handbook on Bioethanol: Production and Utilization, Taylor and Francis, Washington DC

Plackett, R. L. and Burman, J. P. (1946) 'The design of multifactorial experiments', Biometrika, Vol. 33, pp. 305-325.

Pócsi, I. (1999) 'Physiological and ecological evaluation of bacterial cyclodextrin glycosyltransferases', Biologia, Bratislava, Vol. 54, No. 6, pp. 603-616.

Powchinda, O., Delia-Dupuy, M.L. and Strehaiano, P. (1999) 'Alcoholic fermentation from sweet sorghum: some operating problems', Journal of KMUTNB, Vol. 9, pp. 1–6.

Project Summaries US Department of Energy. (1998–99) 'Production of cellulases in tobacco and potato plant bioreactors', http://bioenergy.ornl.gov/99summaries/cellulase.html.

Rajoka, M.I., Khan, S. and Shahid, R. (2003) 'Kinetics and regulation of the production of β-galactosidase from *Kluyveromyces marxianus* grown on different substrates', *Food Technology* and *Biotechnology,* Vol. 41, pp. 315–320.

Raman N. and Pothiraj C. (2008) 'Screening of *Zymomonas mobilis* and *Saccharomyces cerevisiae* strains for ethanol production from cassava waste', Rasayan Journal of Chemistry, Vol. 1, pp. 537-541.

Ramasamy Amutha and Paramasamy Gunasekaran. (2001) 'Production of ethanol from liquefied cassava starch using co-immobilized cells of *Zymomonas mobilis* and *Saccharomyces diastaticus*', Journal of Bioscience and Bioengineering, Vol. 92, pp. 560-564.

Ravindran and Velmerugu (1992) 'Preparation of cassava leaf products and their use as animal feeds', FAO animal production and health paper (Rome, Italy: Food and Agriculture Organization of the United Nations) Vol. 95, pp. 111–125.

Rhee, S. K., Lee, G. M., Han, Y. T., Zainal Abidin Mohd Yusof, Han, M. H. and Lee, K. J. (1984) 'Ethanol production from cassava and sago starch using *Zymomonas mobilis*', Biotechnology Letters, Vol. 6, pp. 615-620.

Roble N. D., Ogbonna J. C. and Tanaka H. (2003) 'A novel circulating loop bioreactor with cells immobilized in loofa (*Luffa cylindrica*) sponge for the bioconversion of raw cassava starch to ethanol', Applied Microbiology and Biotechnology, Vol. 60, pp. 671-678.

Saifuddin, N. and Refal Hussain. (2011) 'Microwave assisted bioethanol production from sago starch by coculturing of Ragi Tapai and *Saccharomyces cerevisiae*', Journal of Mathematics and Statistics, Vol. 7, pp. 198-206.

Sathya Geetha, G. and Navaneetha Gopala Krishnan, A. (2009) 'Dilute acid hydrolysis and steam explosion of *Manihot esculenta* peeling waste for bioethanol production', Proceedings of Annual Conference on National solid waste association of India, pp. 19-30.

Shanmugam, S., Sathish Kumar, T. and Paneer Selvam, K. (2010) 'Laboratory handbook on Biochemistry', PHI Learning Private Limited, New Delhi.

Sheehan, J. and Himmel, M. E. (1999) 'Enzymes, energy, and the environment: A strategic perspective on the U.S. department of energy's research and development activities for bioethanol', Biotechnology Progress, Vol. 15, No. 5, pp. 817-827.

Singh, A. and Bishnoi, N.R. (2012) 'Optimization of ethanol production from microwave alkali pretreated rice straw using statistical experimental designs by *Saccharomyces cerevisiae*', Industrial Crops and Products, Vol. 37, pp. 334– 341

Stowe, R. A. and Mayer, R. P. (1966) 'Efficient screening of process variables', Journal of Industrial and Engineering Chemistry, Vol. 56, pp. 36-40.

Svensson and Søgaard. (1993) 'Mutational analysis of glycosylase function', Journal of Biotechnology, Vol. 29, pp. 1-37.

Teerapatr Srinorakutara, Cholada Suesat, Bongotrat Pitiyont, Wichien Kitpreechavanit and Sirintip Cattithammanit. (2004) 'Utilization of waste from cassava starch plant for ethanol production', Proceedings of the Joint International Conference on "Sustainable Energy and Environment, pp. 344-349.

Thalisa Yuwa Amornpitak. (2010) 'Ethanol production from cassava starch by selected fungi from Tan-Koji and *Saccharomyces cerevisiae*', Biotechnology, Vol. 9, pp. 84-88.

Thanapoom Maneeboon, Ancharida Akaracharanya, Teerapatr Srinorakutara, and Vichien Kitpreechvanich. (2009) 'Simultaneous saccharification and fermentation of cassava pulp to ethanol by co-culture of *Rhizopus oryzae* and *Saccharomyces cerevisiae* in solid state culture', Proceedings of Thailand Institute of Scientific and Technological research, Vol. 35, pp. 14-21.

Thomas Karsch, Ulf Stahl and Karl Esser. (1983) 'Ethanol production by *Zymomonas* and *Saccharomyces*: Advantages and disadvantages', European Journal of Applied Microbiology and Biotechnology, Vol. 18, pp. 387-391.

Thu Lan T. Nguyen, Shabbir H. Gheewala and Savitri Garivait. (2006) 'Life cycle cost analysis of fuel ethanol produced from cassava in Thailand', Proceedings of the 2nd Joint International Conference on Sustainable Energy and Environment, pp. 1-7.

Tolan, J. S. and Foody, B. (1999) 'Cellulase from submerged fermentation', Advances in Biochemical Engineering/Biotechnology, Vol. 65, pp. 42.

Updegroff, D. M. (1969) 'Semimicro determination of cellulose in biological materials', Analytical Biochemistry, Vol. 32, pp. 420-424.

Veera Venkata Ratnam Bandaru, Subba Rao Somalanka, Damodara Rao Menduc, Narasimha Rao Madicherla, and Ayyanna Chityala. (2005) 'Optimization of fermentation conditions for the production of ethanol from sago starch by co-immobilized amyloglucosidase and cells of *Zymomonas mobilis* using response surface methodology', Enzyme and Microbial Technology, Vol. 38, pp. 209-214.

Vihinen, M. and Mantsala, P. (*1989*) 'Microbial amylolytic enzymes', *Critical Reviews in Biochemistry and Molecular Biology*, Vol. 24, pp. *329-418*.

Walter S. Long (1916) 'A method for determination of starch', Transactions of the Kansass Academy of Science, Vol. 28, pp. 172-174.

Wang W. J., Powelf A.D. and Oates C.G. (1995) 'Pattern of enzyme hydrolysis in raw sago starch: Effects of processing history', Carbohydrate Polymers, Vol. 26, pp. 91-93.

Wind (1997) 'Amylolytic enzymes: Their specificities, origin and properties', Ph.D. Thesis. Rijksuniversiteit Groningen, The Netherlands, pp. 159.

Wong, C. W., Hyun-Woo Kang, Young-Ran Kim, and Bong-Woo Chung. (2007) 'Enzymatic production of linear long-chain dextrin from sago (*Metroxylon sagu*) starch', Food Chemistry, Vol. 100, pp. 774-780.

Wyman, C. E., Dale, B. E., Elander, R. T., Holtzapple, M., Ladisch, M. R. and Lee, Y. Y. (2005) 'Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover', Bioresource Technology, Vol. 96, No. 18, pp. 2026-2032.

Yetti Marlida, Nazamid Saari, Zaiton Hassan and Son Radu. (2000) 'Improvement in raw sago starch degrading enzyme production from *Acremonium sp.* endophytic fungus using carbon and nitrogen sources', Enzyme and Microbial Technology, Vol. 27, pp. 511-515.

Yusaku Fujio, Masafumi Ogata and Seinosuke Ueda. (1985) 'Ethanol fermentation of raw cassava starch with *Rhizopus koji* in a gas circulation type fermentor', Biotechnology and Bioengineering, Vol. 27, pp. 1270-1273.