ENHANCEMENT OF CELLULOSIC ETHANOL PRODUCTION THROUGH *ASPERGILLUS NIGER* MODIFICATION

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DECLARATION

I hereby declare that the work in this thesis titled "Enhancement of cellulosic ethanol production through *Aspergillus niger* modification" was performed by me in Department of Chemical Engineering, under the supervision of Dr. I.A. Mohammed Dabo, and Prof. J.B. Ameh.

The information derived from the literature has been duly acknowledgement in the thesis and a list of references provided. No part of this work has been presented for other degrees or diploma at any institution.

_____ _____

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CERTIFICATION

This thesis "Enhancement of cellulosic ethanol by simultaneous saccharification and fermentation through *Apergillus niger* modification" meets the regulation governing the award of the degree of masters of Science in Chemical Engineering of Ahmau Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to my beloved wife and children:

Mrs. Charity James Shiradiyi

Miss Mercy James Shiradiyi

Miss Justina James Shiradiyi

Miss Blessing James Shiradiyi

Mr. Isaac James Shiradiyi

Miss. Cecillia James Shiradiyi

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ABSTRACT

Simultaneous Saccharification and Fermentation (SSF) were carried out to produce ethanol from maize stalk in 500ml conical flask. *Aspergillus niger strains were isolated from four difference sources, maize stalk, soil, bambaranut, rotten wood. Cellulose degrading ability was screened by zone of clearance carried out by simple agar method.*. Apergillus strain from rotten wood (ANRW) produced the largest zone of clearance of 6.5mm; hence it was selected for further studies. The effect of pH, temperature, substrate particle size, and substrate concentration were studies and optimized to be 5.0, 3^{o}C, 300um, 3% respectively.Aspergillus niger was modified using UV irradiation technique by varying the exposure timings. The strain expose at 30 minutes gave largest zone of clearance and hence was selected. The ethanol yield by simultaneous saccharification and fermentation of modified and unmodified strain of A. niger and saccharomyces cerevisae isolate from burkutu by Debo in Micro biology department ABU Zaria was compared at optimum condition pH 5.0,temperature 30^{o}C, 3%, substrate concentration and 300um substrate particle respectively. The mutant strain from the UV irradiation gave the maximum ethanol yield of 9.3g/100ml which is higher than that of parent strain 3.4g/100ml.

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CHAPTER ONE

1.0 INTRODUCTION

In view of continuously rising petroleum cost and dependence upon fossil fuel resources, considerable attention has been focused on alternative energy resources. Production of ethanol or ethyl alcohol [$CH_3CH_2CH_2OH$] from biomass is one way to reduce both the cost of consumption of crude oil and environmental pollution. Ethanol represents an important, renewable liquid fuel for motor vehicles (Lewis, 1996). The use of bioethanol as an alternative motor fuel has been steadily increasing around the world for a number of reasons. Domestic production and use of ethanol for fuel can decrease dependence on foreign oil, reduce trade deficits, create jobs in rural area, reduce air pollution, and reduce global climate change due to carbon dioxide buildup. Ethanol unlike gasoline is an oxygenated fuel that contains 35% oxygen, which reduces particulate and NO_2 emission from combustion. When burned, ethanol derived from fermentation produces no net increase in carbon dioxide in the atmosphere. It is an octane enhancing additive and removes free water which can plug fuel lines in cold climates (Lang et al; 2001).

Ethanol is the most widely used liquid biofuel. It is an alcohol and is produced from sugars, starches or from cellulosic biomass. Most commercial production of ethanol is from sugar cane or sugar beet, as starches and cellulosic biomass usually require expensive pretreatment. Bioethanol is used as a renewable energy fuel source as well as for manufacture of cosmetics, pharmaceuticals and also for the production of alcoholic beverages. Being abundant and outside the human food chain makes cellulosic materials relatively inexpensive inputs for ethanol production. Vast quantities of agricultural and agro- industrial residues that are generated as a result of diverse agricultural practices represent the most important energy rich resources.

Accumulation of this biomass in large quantities every year results not only in pollution of the environmental but in a loss of valuable materials which can be processed to yield food, fuel, feed and variety of chemicals. Some examples of these agricultural wastes are, maize stalk, corn bran, rice bran, cotton linten, sugar cane, bagasse, wheat straw, corn cob, saw dust among others. The major components of these residues are cellulose and hemicelluloses (75 – 80%) while lignin comprises only 14% [Bowen and Harper, 1989]. Cellulose is the basic component of plant material and is produced in greater quantity than any other substance. It makes up about 50% of the total organic carbon in the biosphere while lignin is a polymeric substance that is quantitatively the most important component of plant after cellulose and hemicelluloses.

These agricultural residues which are dumped indiscriminately in the environment have constituted environmental pollution problem, but various studies and research have shown that these residues can be biologically exploited for the synthesis of chemicals and fuels [Zadrazil, 1985; Buswell and Odier, 1987, Raid, 1989]. The presence of lignin polysaccharide bonds in these ligno-cellulosic tissues severally limits the efficient bioconversion of these residues into valuable agro-industrial product [Smith et al., 1986]. However, some form of treatment had been employed to maximize these high fibre plant residues Chemical and physical delignification techniques had been used with limited success [Milestein et al., 1987] while the use of biological treatment methods had proved widely favorable [Reid, 1985; Zadrazil et al., 1990]. Aerobic microorganisms such as fungi, mycobacterial, eubacteria and few anaerobic organisms (fungi, protozoa, and bacteria) had been discovered to be able to degrade cellulose [Barton, 1979]. Fungi play a significant role in the degrading or bioconversion of cellulose under aerobic conditions.

1.1 RESEARCH PROBLEM

This research was designed to address the following problems:

i. Waste cellulose materials are being burnt, buried or otherwise discarded indiscriminately, thus causing environmental pollution.

ii. The conversion of cellulose biomass to useful substances such as liquid fuels through the enzymatic hydrolysis of *polysaccharide* into fermentable sugar is still too low.

1.2 AIM

To modify *Aspergillus niger* for enhancing bioethanol production from maize stalk using simultaneous saccharification and fermentation (SSF) by *Aspergillus niger and Sacchromyses Cerevisiae*

1.3 RESEARCH OBJECTIVES

a. To isolate and screen strains of *Aspergillus niger* for cellulose degrading ability.

b. To determine the optimum fermentation conditions that favor production of bioethanol from maize stalks using simultaneous saccharification and fermentation.

c. To modify *Aspergillus niger* using ultraviolent (UV) light to enhance hydrolysis.

d. To compare the ethanol yield of UV irradiated Apergillus niger and treated *Apergillus niger* by simultaneous saccharification and fermentation (SSF). (Aspergillus niger *and* saccharonyces cerevisiae*).*

e. To analyze the produced bioethanol by qualitative infrared spectrum test.

1.5 JUSTIFICATION

Maize stalks are renewable resources that are inexpensive, readily available and present in large quantities in Nigeria. In addition, converting maize stalks into valuable product (bioethanol) provides a potential alternative fuel to the fossil-based fuels, which has long drained our national resources. Conversion of these waste materials equally helps in converting their environmental pollution to wealth. This will also reduce the sources of Green House Gases which contribute to Global Warming.

1.5 SCOPE

This work covers the following:

- Screening for degrading ability of *Aspergillus niger* isolates
- Optimizing the fermentation culture conditions such as temperature, pH, substrate particle size and concentration.
- Analysis of the produce bio-ethanol from maize stalk by quantitative infrared spectrum test.

1.6 LIMITATION

Certain factors that limit this research are: erratic power supply, inefficient/poor equipment used.

CHAPTER TWO

2.0 Literature Review.

2.1 Ethanol

Ethanol is a clear, colorless chemical compound made from the sugars found in crops such as maize, sugar beets and sugar cane. In the 1850s, nearly 90 million gallons of ethanol were produced every year in the United States (US). At that time it was used as a fuel for lamps. It was also consumed as an alcoholic beverage. In 1862 the Union Congress put $ 2 per gallon excise tax on alcoholic beverages to help finance the civil war. The tax made ethanol too expensive to be used for lighting, so people started using kerosene and methanol instead (Keith, 2009.)

In 1896, Herry Ford built his first automobile, the quadricycle, to run on pure ethanol. In 1906, the liquor tax was repeated, and Ford declared ethanol the future. Ford designed his model T to run on a mixture of gasoline and ethanol. During World War 1, ethanol use increased rapidly, not only as a fuel but in manufacturing of war materials also. The year 1919 brought prohibition and a denaturing process was developed which made ethanol poisonous and undrinkable. In the 1920s ethanol was replaced as a booster to gasoline by other products (Keith, 2009.)

The prohibition ended in 1933, and ethanol production rose to 600 million gallons a year to meet the need of World War 11. After the war, production once again declined because there were no more government contracts. Farmers began exporting grains formerly used to make ethanol to help feed countries whose agriculture had been destroyed by the war. Large supplies of cheap foreign oil made gasoline less expensive than ethanol.

In the 1970s, the US placed embargoes on gasoline suppliers from foreign sources, and interest in ethanol as an alternative fuel rose again. Concerns about global warming and dependence on foreign oil had caused interest in ethanol as an alternative fuel source to grow in recent years. In 2006, 112 ethanol plants, mostly in the Midwest, produced about 5 billion gallons of ethanol (Wikipedia, 2008).

2.1.1 Uses of ethanol

Ethanol has found wide use both in industry and in the laboratory as a solvent and extracting agent. It is useful as fuel and in the preparation of pharmaceuticals and perfumes. It is used for the production of various liquors, acid, vanishes and dyes, cosmetics, printing inks and plastics. The largest market of ethanol is as a beverage and partly in form of liquors (Cheremisinoff, 1979).

2.1.2 Ethanol as disinfectants

Like many chemical disinfectants, alcohols are generally, considered to be non specific antimicrobials because of a multiplicity of toxic effect mechanism (Hugo and Russell, 1996). This has important implication for the spectrum, speed, and ultimately, overall effectiveness of ethanol as a disinfectant. The predominant mode of action appears to stem from protein coagulation/denaturation, with associated interference with cellular metabolism, disruption of cytoplasmic integrity and cell lysis (McDonnel and Russell, 1999).

2.1.3 Ethanol as domestic lighting agent

There is also extensive use of kerosene for lighting and cooking in less developed countries. Ethanol can have a role in reducing petroleum dependency in this too. There is a potential for bioethanol from feed stocks grown locally replacing kerosene use in domestic lighting. A 50%

(w/w) ethanol – water mixture has been reported in specially designed stoves and lanterns for rural areas (Wikipedia 2008).

2.1.4 Ethanol as transportation fuel

Ethanol is a very high octane fuel, replacing lead as an octane enhancer in gasoline and increases the antiknock capacity of the fuel (Chereminisinoff, 1979). Unlike gasoline, ethanol is biodegradable. It is used as alternative fuel when blended with gasoline. As a fuel, ethanol is the most commonly used additive to increase the octane and improve the emissions quality of gasoline (Addison and Hiragu, 2004). Because ethanol is made from crops that absorb carbon (iv) oxide and give off oxygen, it helps reduce the total volume of greenhouse gas emission (Farabee, 2009).

Ethanol fuel is the same type of alcohol found in alcoholic beverages. It can be used as a fuel, mainly as a biofuel alternative to gasoline and is widely used in cars in Brazil. It is easy to manufacture and process and can be made from very common crops such as sugar came maize grain, and cellulosic materials, It is therefore an increasingly common alternative to gasoline in some parts of the world (Wikipedia, 2008,)

Ethanol can be mass – produced by fermentation of sugar or by hydration of ethynlene ($CH_2 = CH_2$) from petroleum and other sources. (Wikipedia, 2008). Currently interest in ethanol mainly lies in bioethanol, produced from the starch, cellulose or sugar in a wide variety of crops, but there has been considerable debate about how useful bio – ethanol will be in replacing fossil fuels in vehicles. Recent developments with cellulosic ethanol production commercialization may allay some of these concerns.

According to the International Energy Agency, cellulosic ethanol could allow ethanol fuels to play a much bigger role in the future than previously thought. Cellulosic ethanol offers promises as resistant cellulose in plant cell walls can be used to generate ethanol. Crops such as switch grass are also promising cellulose sources that can be produced in many regions of the United State (Mick, 2008).

2.1.5 Other uses of ethanol

Ethanol is used in manufacturing of surface coating, yeast growth medium, solvent for resins, fats fatty acids, oils, hydrocarbons.It is also used in direct food additives like preparation of acetic acids, acetalde- hyde, and cleaning preparations,and in dyes and explosives (Robert, 1990).

Mono –, di –, and tri – ethyl amines, produced by catalytic reaction of ethanol with ammonia, are a significant outlet for ethanol. In the synthesis of ethyl acrylate, the esterification of acrylic acid is a major use for ethanol. The addition of ethanol to acetylene gives ethyl vingl (Wikipedia, 2008).

2.1.6 Performance of ethanol in Engines

Ethanol is a very high octane fuel, replacing lead as an octane enhancer capacity of the fuel (Chereminisinoff, 1979). Fuels that burn too quickly make the engine "knock". The higher the octane rating, the slower the fuel burns and the less likely the engines will knock. When ethanol is blended with gasoline, the octane rating of the petrol goes up by three full points without harmful additives (Addison and Huraga, 2004).

Adding ethanol to gasoline "oxygenates" the fuel, adding oxygen to the fuel mixture so that it burns completely and reduces pollution of the environment by carbon monoxide. Car manufacturers had testified that engines on ethanol last longer. (Robert, 1990).

Problems associated with the use of ethanol gasoline blends include starting performance problem and phase separation of ethanol and gasoline because of water contamination. Problems of corrosion may also occur (Chereminisinoff, 1979).

2.2 Chemistry of bioethanol

Simple sugar (Glucose) is created in plant by photosynthesis (Robert, 1990)

$6CO_2 + 6H_2O + light \rightarrow C_6H_{12}O_6 + 6O_2$ - - - - - - 2.1

During ethanol fermentation, glucose is decomposed into ethanol and carbon dioxide.

$C_6H_{12}O_{16} \rightarrow 2C_2H6O + 2CO_2 + heat$- - - - - - - 2.2

During combustion ethanol reacts with oxygen to produce carbon dioxide water and heat.

$C_2H_6O + 3O_2 \rightarrow 2CO_2 + 3H_2O + heat$ - - - - - - 2.3

Glucose itself is not the only substrate in the plant that is fermented. The simple sugar fructose also undergoes fermentation. Three other compounds in the plant can be ferme nted often breaking them up by hydrolysis into the glucose or fructose molecules that compose them. Starches and cellulose are molecules that are strings of glucose molecules, and sucrose is a molecule of glucose bonded to a molecule of fructose. Ethanol may also be produced industrially from ethane (ethylene).Addition of water to the double bond coverts ethane to ethanol.

$CH_2 = CH_2 + H_2O \rightarrow CH_3CH_2OH$ - - - - - - - 2.4

This is done in the presence of an acid which catalyzes the reaction, but is not consumed (Robert, 1990).

2.2.1 Properties of ethanol

The physicochemical properties of alcohols are associated with their chemical structures. Factors such as water solubility or miscibility lipohilicity, solvency, surface tension, vapor pressure, and protein denaturing vary with chemical structuEres and help explain corresponding variation in biological activity (Morrioson,and Boyad et al, 1972). The properties are shown in Table 2.1

Table 2.1. Physiochemical properties of ethanol

Molecular formula	C_2H_5OH
Molar mass	$46.07gmol^{-1}$
Appearance	**colorless liquid**
Density	$0.789g/cm^3$
Melting point	$- 114.3^0C$
Boiling point	78.4^0C
Solubility in water	Miscible
Acidity (pka)	15.9
Viscosity	$1.2000cp (20^0C)$
Dipode moment	1.69D (gas)

Source: Wikipedia, (2008)

2.2.2 Bioethanol production process

Bioethanol from entirely microbial source is the most economical and widely used way of producing ethanol (Kiran et al, 2000). The biological way of producing ethanol from renewable biomass had received considerable attention in recent years (Seeman et al, 2007).

The basic steps for large scale production of ethanol are microbial (yeast) fermentation of sugar, distillation, and dehydration. Prior to fermentation, some crops require saccharification or hydrolysis of carbohydrates such as cellulose and starch into sugars. Saccharification of cellulose is called cellulolysis. Enzymes are used to convert starch into sugar (Verma et al., 2000).

2.2.3 *Process Description* of simultaneaous saccharification and fermentation (SSF).

New bioprocess technologies had been developed that have the potential to lower the cost of ethanol production and improve the competitiveness of ethanol as a fuel or fuel additive (Bothas and Saha., 1997). One of these technologies is the simultaneous saccharification and fermentation (SSF) technique.

This process has been found to be superior to saccharification and subsequent fermentation (Kristna et al., 1999).due to the rapid assimilation of sugar by the yeast. Several methods had been proposed to overcome the end-product inhibition resulting from the rapid accumulation of sugars during hydrolysis. These include the use of SSF by co-culturing two organisms with synergistic relationship (Abouzied and Reddy, 1986; Coa et al., 1996).

In simultaneous sacchctrification and fermentation (SSF). Rapid fermentation of sugar to ethanol by S. cerevisiae had been found to keep the sugar concentration low enough to prevent feedback inhibition of cellulose activity observed in monoculture of *Aspergillus's niger* (Abouzied and Reddy, 1986). In their studies, they observed that monoculture of A. Niger tended to favor higher cell mass production, whereas in the co-culture, most of the substrate carbon is utilized for ethanol production. The process involves the enzymatic hydrolysis of cellulose to fermentable sugars and their conversion to ethanol in the same fermenter, thereby preventing the inhibiting effect of sugar on cellulose activity (Abouzied and Reddy, 1986). The conversion of cellulose

biomass to useful substances such as liquid fuels through the enzymatic hydrolysis of polysaccharides, into fermentable sugar, still suffers severe problems that are economical and technical in nature. The economic problems consist exclusively of the cost of production and purification of hydrolytic enzymes. It has been suggested therefore that any means that is capable of reducing this cost significantly would have a dramatic effect on the cost of production of useful substance, such as ethanol from renewable resources (Taniguchi et al., 1983).

The process was found to increase yield of ethanol not only by eliminating inhibition effects, but also by eliminating the need for separate reactors for saccharification and fermentation (Deshpande et al., 1983). Hydrolysis is the chemical reaction that converts the complex polysaccharides in the raw feedstock to simple sugars. In the biomass-to-bioethanol process, acids and enzymes are used to catalyze this reaction.

2.2.4 Fermentation

Fermentation is a series of chemical reactions that convert sugar to ethanol. The fermentation reaction is caused by yeast or bacteria, which utilize or breakdown certain sugar to ethanol and carbon (IV) oxide. The simplified fermentation reaction equation for the 6 – carbon sugar, glucose is

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \quad - \quad - \quad - \quad - \quad - \quad - \quad - \quad 2.5$$

Ethanol toxicity to yeast limits the ethanol concentration obtainable by brewing. The most ethanol tolerant strains of yeast can survive up to approximately 15% ethanol by volume [Mills and Eckluand, 1987].

Ethanol is produced by microbial fermentations of the sugar. Microbial fermentation work directly with sugars. Two major components of plants, starch and cellulose are both made up of sugars, and can in principle be converted to sugars during saccharification. Currently, only the sugar and starch portions can be economically converted. However, there is much activity in the area of cellulosic ethanol where the cellulose part of a plant is broken down to sugar and subsequently converted to ethanol [Krishna et al., 2001]

2.2.5 Distillation

Water must be removed for the ethanol to be usable as a fuel active disinfectant, solvent and extract Most of the water is removed by distillation, but the purity is limited to 95 – 96% due to formation of low boiling water ethanol azeotrope [Synder et al., 1994].

In another method to obtain absolute alcohol, a small quantity of benzene is added to rectified spirit and mixture is then distilled. Absolute alcohol is obtained in third fraction, which distills over at 78.3°C [Morrison and Boyad, 1972].

Because a small amount of the benzene used remains in the solution, absolute alcohol produced by this method is not suitable for consumption as benzene is carcinogenic [Synder et al., 1994].

2.2.6 Dehydration

There are basically five dehydration processes to remove the water from an azeotropic ethanol/water mixture. The first process used in many early fuel ethanol plants, is called azeotropic distillation and consists of adding benzene or cyclohexane to the mixture. When these components are added to the mixture, they form an heterogeneous azeotropic mixture which when distilled produces anhydrous ethanol in the column bottom, and a vapour mixture of water and cyclohexane/benzene [Synder, et al., 1994].

When condensed, this becomes a two phase liquid mixture. Another early method called extractive distillation consists of adding a ternary component which will increase ethanol relative volatility, when the ternary mixture is distilled, it will produce anhydrous ethanol on the top stream of the column [Synder et al., 1994].

New process uses molecular sieves to remove water from fuel ethanol. In this process, ethanol vapor under pressure passes through a bed of molecular sieve beads. The beads pores are sized to allow absorption of water while excluding ethanol. After a period of time, the bed is regenerated under vacuum to remove the absorbed water. Two beds are used so that one is available to absorb water while the other is being regenerated. (Synder et al, 1994).

2.3 Cellulose

Cellulose is the most abundant organic compound of natural origin on the face of the earth. The digestive systems of man and most other animals (except ruminants) do not contain the necessary enzymes (cellulase) for hydrolyzing β-glucosidic linkages. However, cellulases are found in ruminants, various insects, fungi, algae, bacteria (Lee, 1983).

Cellulosic materials are the most abundant renewable carbon source in the world (Liming and Xueling, 2004). Tremendous quantities of waste cellulose materials exist which are classified as municipal wastes, industrial wastes and agricultural wastes. The agricultural wastes represent the largest class of cellulosic waste. While a few of these agro-wastes are fed to some farm animals, most are being burnt, buried or otherwise discarded indiscriminately, thus, causing environmental pollution. Proper treatments could change them from liabilities to assets (Vlasenko et al., 1997).

Statistics from agricultural programmed in Nigeria shows that increase in prices of farm produce such as maize, wheat, rice and sorghum have risen by 30% over the price prevailing a few years ago. This has encouraged increase in crop production over the years, with the attendant increase in the straws and other by-products from these cereals (Abu et al., 2005).

2.3.1 Chemistry of Cellulose

Cellulose Chemistry has remained a difficult ground for many workers who have successfully applied science in other fields. Despite its simple chemical composition, cellulose exists in a number of crystalline and amorphous topologies. Its insolubility and heterogeneity make native cellulose a recalcitrant substrate for enzymatic hydrolysis. Microorganisms meet this challenge with the aid of a multi-enzymatic system. Specific enzymes act in synergy to elicit effective hydrolysis (Lee and Fan, 1982).

The investigation of the hydrolysis mechanism opens up a new way of looking at enzymatic activity. The current research efforts on cellulose are focused at elucidating the enzymatic reaction mechanisms at the surface of the insoluble substrate and to study the extent to biomass conversion into valuable products, using isolated enzymes or cellulolytic microorganism. Based on intensive research on cellulose – Trichoderma reesei system, it is accepted for sometimes as a general concept that cellulolysis is performed by the synergistic action of three types of enzymatic activities namely: Endoglucanases, Exoglucanasses and Glucosidases. (Lee, 1983).

No single enzymes were able to hydrolyze crystalline cellulose substantially. However, significant increase in the rate and extent of cellulose degradation were achieved by mixing several components (Lee, 1992).

2.3.2 Cellulases

Many fungi are capable of producing extra cellular enzymes that can degrade cellulose. They are Trichoderma reesei; Trichoderma viride, Aspergillus niger, etc. Bacteria such as *Cellulomonas along* with Clostridium thermacellum can also produce cellulases. Typical proportions of the endoglucanase can hydrolyse cellulose molecule randomly within the molecule and are more effective in combination with cellobiohydrolases. Cellobiohydrolases cleave cellobiose and glucose from the non-reducing end of cellulose molecule. B-glucosidase converts cellobiose to glucose, with some indication of the ability to degrade oligosaccharides. Endoglucocanases and cellobiodydrolases must adsorb to the cellulose before a reaction can occur. B-glucosidase, however, remains in solution and hydrolyses the cellobiose in solution (Lee, 1992)

2.2.3 Cellulose conversion

Interest in cellulose degradation has centered on production of products, usually via glucose and as an alternative method of disposal of cellulosic wastes, e.g. domestic refuse. Also, it has been found that cellulosic wastes have a great potential as a feedstock for producing fuels and chemicals (Wen et al., 2004).

Kaur et al., (1998) made some efforts to produce glucose from rice straw and sawdust, employing cellulose from Trichoderma viride. The enzyme has high thermal stability and is not easily inhibited by impurities in the substrate material. However, the conversion of cellulosic biomass to useful substances such as liquid fuels through the enzymatic hydrolysis of polysaccharides, into fermentable sugars, still suffers severe problem, that are economic and technical in nature. The economic problems consist exclusively of the cost of the production of cellulose and of the pretreatment of native cellulose substances to enhance their susceptibility to

enzymatic attack. Enzyme production is the most expensive factor, which has been estimated to be 50% of the total cost of the glucose production from cellulosic materials. Any means that is capable of reducing this cost significantly would have a dramatic effect on the cost of production of useful substances from renewable resources (Taniguchi et al, 1983).

2.4 Genus Aspergillus

Aspergillus was first defined by Micheli in 1729 as microscopic fungus possessing erect stalks and spore heads. Its tendency to radiate from the central stalks created a pattern not unlike the Apsergillus used by priests and this led Micheli to give the generic name to this group of fungi (Bartnicki, 1973; Smith and Anderson, 1973).

In 1965, Raper and Jennel listed eighteen groups of Aspergilli and recognize 132 species. Information regarding pigmentation of the aerial parts of Aspergillus colony is one of the most striking characters used to identify Aspergillus as there is a characteristic range of colors in the aerial parts of each group of Aspergilli and a much narrower range for particular species. (Smith and Anderson, 1973).

Many produced colony described as velvet that consist of a submerged mycelium from which only fruiting structure rise above the surface, suggesting a turf or a field of ripening grain. Colony margin may be heavy and sharply delimited or thin and diffused, smooth and entered or irregularly lobed, submerged or aerial. The basal mycelium may be thin or dense, brittle, fragile or tough (Thom and Raper, 1944).

The distribution of *Aspergilli* is vast from the artic region to the tropics. The spores are found contained in air as well as the soil (Alexopoulos and Mims, 1979). They are very ubiquitous

organism found both in field and storage such as on fruits, vegetable, cereals cotton seeds and meat (Jay,.1986 and Woloshuck et al, 1995).

2.4.1 Aspergillus niger group

Aspergillus niger also commonly called the black Aspergilli are one of the most common of all saprophytic fungi – molds that are widely in distribution and capable of growing upon the widest possible organic matter. (Raper and Fennel, 1965).

The colonies rapidly grow on mold extract 6 – 7 cm in diameter in one week at room temperature,with large conidial heads and in some shade of black, greenish – black, globose, radiate, or split into thin many irregular or well defined columns of conidial chains.

A conidiophores, hyaline to brown, are typically smooth but in a few species, slightly granular as punctuate, usually heavy welled, brittle, splitting, longitudinally when cured (Raper and Fennel, 1965).

They are worldwide in distribution and occur in and upon the greatest variety of substrata including grains, forage products, spoiled fruits and vegetables, exposed cotton, textiles and fabrics, leather, dairy products and protein rich substrates and decaying vegetation in the field. They are abundant in all soils with the exception of the A. flavus group which is of great economic importance in the orient. The black *Aspergill*i are undoubtedly more widely used in industries than any other group of molds (Raper and Fennel, 1965).

2.4.2 Saccharomyces cerevisiae

This organism belongs to a group of unicellular fungi, the yeast. They are mostly used to leaven bread and ferment alcohol beverages. Most yeast belongs to the division Ascommycotta. Yeast

fermentation comprises the oldest and the largest application of microbial technology (Richards et al., 1989). In 1837, Theodore Schwann and others had earlier proposed that yeast cells were responsible for the conversion of sugar to alcohol (Wikipedia et al., 2008).Saccharomyces cerevisiae associated with palm wine and burkutu (BKT), a local wine brewed from sorghum has been found to produce wines of appropriate alcohol content of about 18-29% since it is also alcohol tolerant (Oke and Isebor, 1997).

Most yeast is found associated with sugar –rich materias (Sun and Cheng et al., 2005) .They grows over a temperature range of 10-37^{o} C, with optimum temperature in range of 30-37^{o} C depending on the species. *S. cerevisiae* works best at about 30^{o} C. Most cells die about 50^{o} C (Wikipedia, 2008).

Saccharomyces cerevisiae is generally grown in the laboratory on solid growth media or broths. Common media are Potato Dextrose Agar (PDA), Wallerstien Laboratories Nutrient agar (WLN), Yeast Peptone Dextrose Agar (YPDA) and Yeast Mould Agar or broth (Sun and cheng, 2005).

Industrial ethanol production had been studied to depend on microbial activity, particularly that of yeast. In this process, high quality cellulose substrates that are susceptible to enzyme activities are used (Kiran et al., 2000). Yeasts which ferment sugar rapidly and which show appreciable ability to flocculate, to produce high levels of ethanol and to tolerate relatively high temperature and high carbohydrate concentration have been suggested to be potentially useful for industrial ethanol production. The study concluded that sugar fermentation is a pre-requisite, while other attributes enhance productivity and minimize cost of production (Ameh et al., 1989). It has been remarked that during yeast alcohol fermentation, a small concentration of oxygen is usually

provided as it is necessary for the biosynthesis of certain polysaturated fats and lipids (Wikipedia, 2008).

2.4.3 Macroscopic morphology of Yeasts

Colonies on solid media are usually white or cream, domed, smooth or semi-matt up to 5mm in diameter and of a butyrous consistency. In liquid media, sediment is formed, sometime with ring growth at the air –liquid glass junction but pellicle is not formed (Wikipedia. 2008).

2.4.4 Enzyme production by Fungi

In the production of industrial enzymes, filamentous fungi are well used. This includes lipases, celluloses and amylases which are produced by fermentation.

2.4.5 Cellulolytic enzymes from Fungi

The susceptibility of cellulosic substrates to enzymatic hydrolysis is determined largely by their accessibility to cellulolytic enzymes (Tanaka et al, 1988). Celluloses are synthesized by a large number of micro organisms which include fungi, actionomy celes, gliding bacteria and true bacteria. The fungal cellulose act in strong synergy where cooperative action is greater than the sum of the action of the individual enzyme components (Bisaria, 1991).

Extracellular celluloses with a microbial origin have importance for the conversion of the cellulose component of lignocellulosic waste to fermentable sugars such as glucose (Dijkaerman et al, 1996). Some of the *Aspergillus* species capable of producing cellulose include Aspergillus niger, and Aspergillus furnigatus. The celluloses produced catalyse the enzymatic cleavage of cellulose.

The cellulose system consists of;

(i) Endo – beta – 1, 4 – glucanases which attack the beta – 1, 4 – glucosidic bonds producing long chain fragments with free ends.

(ii) Exo – beta – 1, 4 – glucanases which remove the disaccharide cellobiose from the ends of the cellulose chains.

(iii) Beta – glucosidose which hydrolyse cellobiose with formation of glucose (Schlegel, 1996).

2.4.6 Kinetics of Ideal Enzyme Reactor Systems

The Michaelis – Menten expression for a single substrate uninhibited irreversible reaction is of the form (Daniel et al, 1979).

$$r = -\frac{ds}{dt} = \frac{KE}{V} \cdot \frac{1}{1 + Km/s} \quad \text{2.6}$$

Where, r is the reaction rate in mol/sec/1s is the substrate concentration in mol/1. KE is the maximal activity of total enzyme in reactor in mol/sec, Km is Michaelis constant, and v is the volume of aliquot in liters.

For a batch reactor under isothermal conditions equation (2.6) may be written as:

$$XS_o - K_m \ln(1 - \times)\frac{KE.t}{V} \quad \text{2.7}$$

Where, x is defined as the proportion of substrate converted.

In a similar way for plug flow reactor equation (2.6) may be written as

$$XS_o - K_m \ln(1 - \times) = \frac{KE}{q} = \frac{KE}{V} \quad \text{2.8}$$

Where q is the volumetric feed of substrate to reactor in litres/sec, and y is the space time in sec.

For a well – mixed continuous flow reactor, a substrate mass balance gives

$q(S_O - S)$ rv _____2,9

Where S is the substrate concentration in the reactor. Substituting r from equation (2.6) and writing in terms of x.

$$XS_O + K_m \cdot \frac{X}{1-X} = \frac{KE}{q} = \frac{KE}{V} \quad \text{_____} \quad 2.10$$

2.5 Biodegradation

The genuse *Aspergillus* is involved in several biodegradation and biodeterioration of organic substances some of which are of ecological importance. The ability to degrade plant tissue is an important characteristic for many plant pathogens and saprophytic organism; hence micro organisms produce better plant cell wall degrading enzymes (Whitehead et al, 1995). Aspergillus niger is a common contaminant of food , before or after canning and result to loss of the spoilage that occur (Fujikwa and Itoh, 1996). Members of genus Aspergillus are common contaminants of corn, cereals, nuts, peanuts, cotton seeds. Some of these are field organisms and some are storage fungi that cause deterioration of these plant products. (Jay 1986, Woloshuk et al, 1995).

However among the fungi found in grains, Aspergillus species do not invade grains at any appreciable extent before harvest or until in storage (Christeinsen and Kaufman, 1965, Mishira et al, 1969). Tripatti (1974) showed that Aspergillus flavus, constituted 15% of the total fungi isolated from sorghum grain directly collected from the field in India. Some osmophilic fungi

like Aspergillus glancus cause spoilage of confectionary products and raw materials (Frazier and Westhoff, 1987).

Fawcette and Lec, (1926), reported that *Aspergillus ngier* causes rot of citrus plants. This rot is a minor form of soft decay due to infection with *Aspergillus niger*. An important biodeterioration cuased by *Aspergillus* is the production of mycotoxin in food and feeds. Such toxin include aflatoxin, ocharotoxin, polyketides which present both health and economic threat to public (Keller et al, 1995). The most important toxin from the view point of hazard to human health is the aflatoxin produced by Aspergillus parasiticus, Aspergillus flavuns (Raper and Fennel, 1965).

On the other hand, it has become evident that fungi such as Aspergilli have broad metabolic capabilities that enable them deal with many industrial pollutants as well as natural metabolites such as phenolic and the degradation of their compound is important to carbon cycle, *Aspergillus fumigalus* species has such ability. Aspergillus sydowri and other *Aspergillus* species produce xylanose which is the key enzyme in degradation of xylan (Ghosh and Nanda, 1994).

2.5.1 Bioconversion of agricultural residues (waste) to glucose

Members of *Aspergillus* genus are able to form fermentable sugar by their direct growth on different cellulosic waste materials as source of carbon and energy. The resulting sugar act as substrates for production of chemicals and biofuels (Zayed and Mayer, 1996).

All *Aspergillus niger* are able to secrete cellulose enzymes, are able to produce glucose from their residues. (Schlegel, 1996). The agricultural residues are made up of complex macro molecule held by β – 1, 4 glucosidic bonds. The cellulose system of *Aspergillis* consist of three enzymes, endobeta – 1, 4 glucanoses which attack the β – 1, 4 glucosidic bonds in the center of the macro molecule and produce long chain fragment with free ends, exo beta – 1, 4 glucanoses

which removes the disaccharide cellobiose from the ends of the cellulose chains and the Beta – glucosidose which hydrolyses cellobiose with the production of glucose (Schlegel, 1996).

2.5.2 Pretreatment of lignocelluloses materials

Lignocellulosic biomass is a complex mixture of cellulose, hemicelluloses, lignin along with extractives, (Cao et al., 1996). The effect of pretreatment of lignocellulosic materials had been recognized for a long time (Ryu et al., 1982). The purpose of the pretreatment is to remove lignin and hemicellulose, reduce cellulose crystallinity, and increase the porosity of the materials. Pretreatment must meet the following requirement: (i) improve the formation of sugars or the ability to subsequently form sugar by enzymatic hydrolysis (ii) avoid the degradation or loss of carbohydrates (iii) avoid the formation of byproducts inhibitory to the subsequent hydrolysis process; (iv) be cost effective. Physical, chemical and biological processes had been used for pretreatment of lignocellulosic materials (Sun and Cheng, 2005).

Grinding and milling are the primary physical pretreatment operations currently employed. Chemical pretreatment can be accomplished by using strong acids or bases (Esteghlalain et al., 1997; Chosdu et al., 1993; Taniguchi, et al.,(1983) and Weimer et al., 1995). However, these processed options are also expensive and compounded with the necessity of chemical recovery. In addition, sugar sulphonate a byproduct of acid pretreatment will inhibit microbial activity.

Steam explosion of biomass is another pretreatment method that is effective, but involves considerable thermal energy in the form of steam. Moreover, the high temperature involved is capable of degrading the xylose that is formed by the hydrolysis of hemicellulose (Grous et al., 1986), leading to formation of furfurals and hydroxymethylfurfurals that are inhibitory to microorganisms.

In biological pretreatment processes, microorganisms such as brown-white and soft-rot fungi are used to degrade the lignin and hemicellulose in waste materials. White-rot fungi are the most effective basiodimycetes for biological pretreatment of lignocellulosic materials (Hattaka, 1983, Fan et al., 1987 and Akin et al., 1995). The advantages of biological pretreatment include low energy requirements and mild environmental conditions. However, the rate of hydrolysis in most biological pretreatment process is very slow (Sun and Cheng, 2002). Cao et al., (1996) reported a new pretreatment method that involves steeping of the lignocellulosic biomass (using corn cob as a model feedstock) in dilute NH_4OH at ambient temperature to remove lignin, acetate and extractives. This is followed by a dilute acid treatment that readily hydrolyses the hemicelluloses fraction to simple sugars, primary xylose. The residual cellulosic fraction of the biomass can then be enzymatically hydrolyzed to glucose. Hence, by using this pretreatment method in biomass processing, the lignin, hemicelluloses, and cellulose can be easily separated and processed separately.

2.5.3 Factors affecting enzymatic hydrolysis of cellulose

Past techno-economic modeling work had identified the relatively large contribution that enzymatic hydrolysis adds to the cost of producing ethanol from lignocellulosic substrates. This cost was primarily due to high concentration of enzymes and long incubation time that was required to obtained a complete hydrolysis. Hydrolysis of cellulose in a batch fashion is generally characterized by a limited logarithmic phase, associated with the rapid release of soluble sugars, followed by a declining rate of sugar production as the reaction proceeds. Several studies have shown that the specific hydrolysis rates declines rapidly with increased conversion of substrate. There are several proposed explanations for the diminishing rate of hydrolysis. One proposal is that as hydrolysis proceeds, the substrates become enriched in the more recalcitrant

cellulose as the less recalcitrant amorphous cellulose is hydrolyzed. Other possible contributing factors to the declining hydrolysis rate include enzyme denaturation through thermal, mechanical, and chemical actions (Lee and Fan, 1982 and Sharma et al., 2001 ;). However, the complete mechanism of cellulose hydrolysis has not been determined, primarily due to the complexity of both the substrate and the enzymatic system required to hydrolyze crystalline cellulose. Consequently, the characteristics of a typical batch hydrolysis reaction, such as denaturation or inhibition, the intrinsic structural features of the substrate as the reaction processes are yet to be properly understood. Only some of these factors are readily available for manipulation within the design or operation of the enzymatic hydrolysis step. [David et al., 1978].

2.6.0 Optimization of culture condition

2.6.1 Enzyme Related Factors Affecting Hydrolysis

Improvement in the hydrolysis rates of lignocellosic materials can be obtained by increasing to a certain extent the amount of cellulose used in the conversion process. Cellulose dosage of filter paper unit (10FPU/g) cellulose is often used in the laboratory studies because it provides a hydrolysis profile with high levels of glucose yield in a reasonable time (48 – 72h) at a minimal enzyme cost [Gregg and Saddler, 1996]. However, it would be desirable if considerable higher hydrolysis rates and glucose yield could be obtained using even lower enzyme concentration [Sun and Cheng, 2002]. Attempts to enhance the rate of cellulose hydrolysis have met with some success. Several methods have been proposed to overcome the end product inhibition resulting from the rapid accumulation of sugar during hydrolysis. These include the use of simultaneous saccanification and fermentation, the supplementation of cellulose with oxygenous β-glucosidase activity. It has been shown that the endoglucanases of the cellobiohydroloses are inhibited by

increase concentration of cellobiose, whereas β-glucosidases are more sensitive to glucose accumulation. Although the glucanases are also inhibited by glucose the drop in the overall enzyme activity of the system due to glucose accumulation is relatively small compared to that associated with cellobiose accumulation. Also, it was found that periodic removal of product sugar was effective at initially reducing the end-product inhibition. However, it was found that extended incubation (≥48hours) was still required before complete hydrolysis could be obtained. End product inhibition appeared to greatly affect the initial rate of hydrolysis while other factors, such as increasing substrate recalcitrance reduced the overall rate and yield of hydrolysis [James and David, 1992, Duff and Murray, 1996].

2.6.2 Effect of Substrate Particle Size

Low substrate levels normally results in an increase of the yield and reaction rate of the hydrolysis [Cheng and Anderson, 1907]. However, high substrate concentration can cause substrate inhibition and consequently lower the rate of the hydrolysis. Kademi and Baratti, (1996) found that the substrate inhibition occurred when the ratio of the microcrystalline substrate (Avicel) to the cellulose from Trichoderma risei (grains of cellulose per filter paper unit) was greater than 5. Their reports show that the optimum substrate to enzyme ratio was 1.25 for the microcrystalline substrate.

Two independent experiments were conducted in the investigation of the effect of substrate concentration on enzymatic hydrolysis. One was at fixed enzymes to substrate ratio. For each experimental run. glucose concentration and yield showed an opposite trend, with glucose concentration increasing and yield decreasing with increasing substrate concentration. (Wen et al 2004 and Tengborg etal 2001). When enzyme concentration was fixed, increasing substrate concentration resulted in more cellulose available for hydrolysis. However, the amount of

enzyme was not proportionally increased and as a result, more intact, cellulose was present in the system. When the ratio of enzyme to s substrate was fixed, sufficient enzymes could be supplied with increased substrate concentration ranging from 10 to 50g/L. However, further increases in substrate concentration (50 – 100/L) resulted in the lower glucose yield. This may be due to the end-product inhibition caused by high concentration of glucose [Wen et al., 2004]. However, in simultaneous sacharification and fermentation system, this is eliminated as products of hydrolysis are simultaneously and spontaneous fermented by the yeast to ethanol

2.6.3 Optimal substrate concentration.

Substrate concentration has been recorded as one of the main factor that affects the yield and initial rate of enzymatic hydrolysis of many substrates. Low substrate concentration normally results in an increase in the yield and reaction rate of hydrolysis, (Cheng and Anderson, 1997). However, high substrate concentration causes substrate inhibition and consequently lowers the rate of hydrolysis. However, in simultaneous saccharification and fermentation system of ethanol production, this inhibition is eliminated as products of hydrolysis are spontaneously converted by the yeast to ethanol and carbon (iv) oxide (Abouzied and Reddy,1986).Banerjee et al.,(2004) reported that by increasing starch concentration, there was an increase in ethanol yield and maximum ethanol production at 12.5g/100ml.

2.6.4 Optimal fermentation pH

pH is the measurement of the grams of hydrogen ions per liter of a solution. Its scale varies from 0 for extreme acidity (1 gram – ion of hydrogen ions per liter) to 14 for extreme alkalinity (1 gram – ion of hydroxyl ion per liter). It is expressed as:

$$PH = \log\frac{1}{(H^{+})} \quad \text{2.11}$$

Where (H^{+}) is the hydrogen ion concentration in the solution.

Medium pH is an important parameter affecting growth and product formation. Most organisms will function over a pH range of 3 – 4 pH units. Their maximum growth rate frequency falls over $1 - 1^{1}/_{2}$ pH unit range. Because pH is so important, it is controlled in most fermentation either by means of a buffer or a pH control system.

Various factors have been attributed to decrease ethanol fermentation. This includes pH of the fermentation medium and loss of sugar transport ability at high initial sugar concentration. (Salmon and Maurico 1994).

Ekumankama et al., 1997). earlier recorded an optimum pH of 4.5-5.0 for ethanol production by S.cerevisiae. This is in agreement with those of Adams and Flynn, (1982), and Morris and Sarad,(1990).In a region outside the optimum pH, the cells of S. cerevisiae were observed to be less tolerant to the environment and hence less active and less efficient in substrate utilization (Ekumankama et al,1997). Enzymes are very sensitive to pH, therefore, the selection of optimum pH has been recommended for the production of alpha and gloamyases ,(Alva et al.,2007).

2.6.5 Optimal fermentation temperature.

Microbial growth and product formation are the result of a complex. Series of chemical reactions. Like all chemical reactions, they are influenced by temperature. Growth way be described by;

$$\frac{dx}{dt} = \mu x - \alpha x \quad or \quad \frac{1}{x}\frac{dx}{dt} = \mu - \alpha \qquad \text{2.12}$$

Where μ is the specific growth rate of the microorganism, α is dilution rate, $\frac{dx}{dt}$ is growth rate-death rate and x is the concentration of biomass.

Three typical growth temperature curves are illustrated in Fig. 2.1 below. Most microorganisms fall into one of these patterns. Those with a temperature for maximum growth rate below 20^{o}C are psychrophilic, those around 30 – 35^{o} C are mesophilic and those above 50^{o}C are thermophilic. Special enrichment and isolation programmers are often used to produce thermo tolerant strains that can grow at temperature above 40^{o}C (Kiran et al.,2000).In a typical ethanol fermentation where exothermic metabolic reaction increase to 40^{o}C above had been reported to have a consequential reduction in ethanol productivities(Kiran etal.,2000). Where cooling is affordable, it adds to overhead cost of the ethanol production (Gera *et al., 1997).*

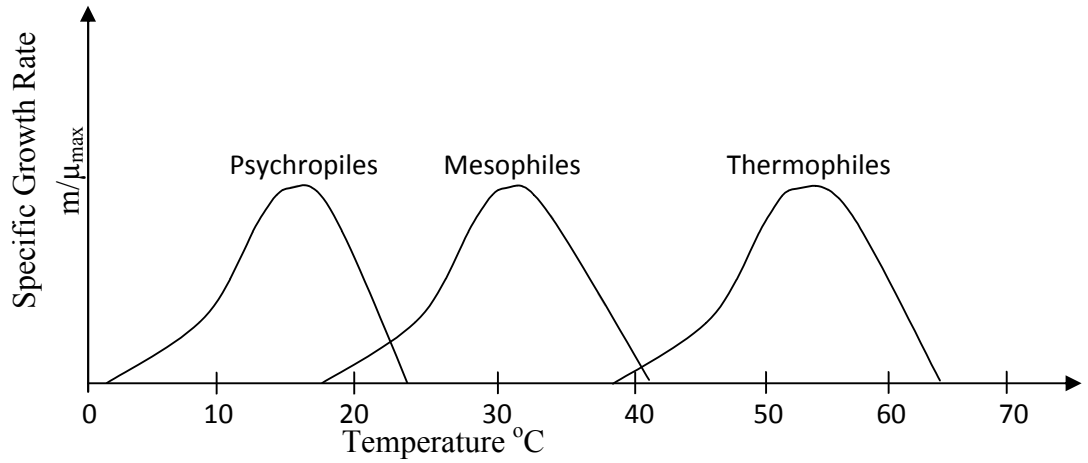


Figure 2.1: Typical growth relationships for psychrophiles mesophiles and thermophiles.

The temperature of fermentation medium is one of the critical factors that have direct effect on many industrial processes. Low temperature was observed to reduce activities of enzymes, giving no significant impact on yield. In the same study when the temperature of the medium was increased above 30^{o}C, biosythesis decreased. This was attributed to the fact that high temperature can cause denaturation of enzymes such as cellulose or acumination of other by-products and enzyme catabolism repressing (Pandey et al., 2000). In another study, optimum temperature for maximum ethanol production using cellulose in co-cultures was observed to be 30^{o}C (Verma et al., 2000).

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2.6.6 Substrates

In most developing countries of the tropics, carbohydrates based agricultural products like cellulose ,starch tubers and cereals occur abundantly (Okolo et al.,1996).Sweet potato and maize stalk can be saccharified and fermented to produce ethanol.(sin-yie et al.,1983). Many cellulose processing wastes of the biomass like maize stalk could also be converted by fermentation into ethanol (Abouzied and Reddy. 1986). For a high alcohol yield, a high cellulose containing sugar within the plant affect ethanol yield (Sin-yie and Chum-ling , 1983).

2.6.7 Substrate Inhibition

The equation for a substrate – inhibited enzyme is

$$V = \frac{V_{mas}.S}{S + K_m + S/ks} \quad \text{2.13}$$

Hence, equations for each of the three ideal reactors are as blow:

For Batch and Plug – flow

$$XS_o - K_m \ln(1-\times) + \frac{S}{2ks}(2\times - \times^2) = \frac{KE.t}{V} \; or \; \frac{KE}{q} \quad \text{2.14}$$

For CST Reactor

$$XS_o + K_m.\frac{\times}{1-\times} + \frac{S}{ks}(\times - \times^2) = \frac{KE}{V} \quad \text{2.15}$$

2.6.8 Product Inhibition

The equations for the three ideal reactor systems when the enzyme is subject to competitive inhibition by product are:

For Batch and plug – flow (Daniel et al. 1979).

$$\left(1-\frac{K}{KIP}\right)\times S_o-\left(1+\frac{S}{KIP}\right)K_m\ln(1-x)=\frac{KE.t}{V}=\frac{KE}{q} \quad \text{---------- 2.16}$$

Where KIP is the product inhibition constant in mol/l, for CST reactor.

2.7 Strain improvement

Strain improvement is an essential part of process development for microbial fermentation products since by developing strains with increased productivity or ability to use cheaper raw material, cost reduction can be achieved in the industries (Viswanathan and Kulkarni, 1995). The industrially useful strain must be amenable to genetic process, non – pathogen tic, non toxic and non antibiotic producing (Pariza and Foster, 1983). The knowledge of genetics has given a tremendous contribution in the process of strain improvement. Fower et al, (1990) reported that synthesis of gene products (glucoamylose) in Aspergillus niger and Aspergillus oryzae is induced by the presence of starch and maltose and is controlled primarily at transcriptional level, hence alternation of nucleotide base sequence of the gene (DNA) can improve the strain ability to produce certain products. The techniques used in strain improvement include mutation gene cloning, protoplast fusion, hybridization, transformation and parasexual recombination.

2.7.1 Mutation

Physical and chemical agents are used to induce mutation for strain improvement. The agents are thus referred to as mutagens. These agents alter the character of the genetic material which specify a certain specific amino acid (Daeschel et al, 1984; Vishwanathan and Kulkarni, 1995). The foremost mutagenic agent are ultraviolet (UV), and high energy ionizing radiations.The primary effect of UV, light on DNA is the production of pyrimidine dimmers cause by linking the 4, 5 unsatirated bonds of adjacent pyrimidines to form cyclobutane ring.The primary mutagenic effect produced by UV light is thought to be a consequence of the replication errors caused by the primidine dime – induced distortion of the DNA helix. The effectiveness of UV light as a lethal and mutagenic agent is closely related to its wavelength, the more effective bactericidal wave length is in the range 240 – 280nm with optimum at 260nm which corresponds with maximum absorption of DNA (Joklic and Smith, 1972).

The chemical mutagens are of different categories. These, include base analogues, base modifiers and alkylating agents, 2 – amino purine, 5 – bromo uracil, diethyl sulphate, diepoxy butanes, nitrous acid, N – methyl – N – Nitro – N – Nitro – N – Nitrosoquanitine (NTG) and ethyl sulphonate (Harwood et al, 1992; Burnet, 1975). The effect of light (UV and visible) on fungi are quite diverse.They respond by change in growth, spore production, pigment formation and enzymes production (Osman et al, 1989). Vishwanathan and Kulkarmi, (1995) reported that *Aspergillus niger* Van Teigham, a soil isolate was mutagenised by UV light. A mutan strain with three times increased productivity was generated on exposure of the parent strain to UV light for 15 minutes.

2.7.2 Gene cloning

This process is referred to as molecular cloning or genetic engineering. The process provides avenue for genetic manipulation of microorganism in other to change their characteristics and produce certain products in large quantity (Voet and Voet, 1990). The process involves transferring whole gene from one organism to another (Herman and Mc Kay, 1986, Wood et al, 1986; Shingo et al 1995). The process start with purification of desired DNA and extraction of autonomously replicating DNA called plasmid.This then followed by subjecting the purified DNA and plasmid to enzymatic action which binds them together with recipient cell in the same medium (Woods et al, 1986; Satoh et al, 1993).

2.7.3 Protoplast fusion

Protoplast Fusion is the fusion of the cells of microorganisms with desired genes in an appropriate medium. The cells are subjected to certain treatment such as osmotic shock to make the cells susceptible to fusion. The cell wall – less cells are then grown together in a medium containing salt and sugar in an be specific quantity, and then plated out in a regeneration medium (Iwata et al, 1986, Tamaki, 1986, Gupta and Gautam, 1995). The technique of protoplast formation and regeneration for enhanced amylase production had been described by (Iwata et al 1986).

Protoplasts are formed from bacteria fungi and Actinomycetes when dividing cells are forced to lose their cell – walls. Protoplast fusion is a system with great potential since it permits recombination in strain with no efficient means of conjugation. The technique involve the formation of stable protoplast by using cell wall lysing enzymes and chitinase and then fusing the protoplast electrically in fusion chamber and subsequent regeneration of viable cells from the

protoplasts. Fusion from mixed population of protoplasts is greatly enhanced by the use of polyethylene glycol (PEG) and Ca^{++} ion.

2.7.4 Transformation

This is a change in genetic properties of an organism which is brought about when foreign DNA is absorbed by and integrated into the genome of the recipient cell. The mechanism involves naked or free DNA. Cells in which transformation can occur. These cells are called competent cells. In some cases, competence is artificially induced by treatment with calcium Ca^{++}. The transforming DNA must have a certain minimum length before it can transform. It is cut by the enzymes called endonuclease produced by the host organism before it is absorbed. The recipient cell adsorbs on to their cell surface pieces of the DNA which are then transported across the cell membrane into the cytoplasm and incorporated by recombination into the recipient cells

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 List of Equipment and Materials

1. Spectrophotometer CECL instrument Cambridge England NO. 91745 obtained from microbiology department ABU Zaria.
2. 500ml, 250ml, Erlenmeyer flasks obtained from chemical Engineering department ABU Zaria.
3. Bent glass rod obtained from microbiology department ABU Zaria.
4. Refrigerator made in England NO. 54761 obtained from microbiology department ABU Zaria.
5. Specific density bottle obtained from Steve shop opposite VIO office Kwangila Zaria.
6. Rotary shaker incubator LAB-LINE ORBIT ENVIRON- shaker 18 NO. 3527-1/34 obtained from microbiology Department ABU Zaria.
7. Petri dishe obtained from Steve shop opposite VIO office Kwangila Zaria.
8. Dilute Sodium hydroxide solution (analar grade, 0.1 M), BDH industries London obtained from chemical Engineering department ABU Zaria.
9. Potato destrose agar (PDA) (analar grade) obtained from microbiology department ABU Zaria.

10. Ampiclin, Magnesium sulphate, Carboxylmethylcellulose (CMC), Calcium carbonate, Congo red, Polyoxyethene, monoaleate (Tween-80), Sodium trioxonitrate (iv), Potassium dihydrogenphosphate, and Dinitrosalicyclic acid solution (DNS) all analar grade obtained from Steve shop opposite VIO office Kwangila Zaria.

11. Glucose (analar grade 0.1 M), BDH Industries London obtained from Steve shop opposite VIO office Kwangila Zaria.

12. Dilute hydrochloric acids (analar grade 0.1 M) BDH Industries London obtained from Steve shop opposite VIO office Kwangila Zaria.

13. Immersion Thermometer. Temperature range 0-110 oc

14. Density bottle 50ml obtained from Steve shop opposite VIO office Kwangila Zaria.

15. Distillation colum improvised by me in microbiology.

16. Autoclave (Dixon's surgical instrument LTD) obtained from microbiology department ABU Zaria.

17. Analytical weighing balance(mettle instrument) obtained from microbiology department ABU

18. Yeast (*saccharomyces cerevisiae*)obtained from Debo microbiology department.

19. Distilled water obtained from chemical Engineering department ABU Zaria.

20. Absolute ethanol (analar grade 98%-100%) BOH Industries, UK obtained from chemical Engineering department ABU Zaria

3.2.0 **Sample collection**

Samples of maize stalk were collected from a farm near Zaria dam Junshi Waje Sabon *Gari Zara*. Soil and rotten wood collected in-between Biochemistry and Chemistry Departments ABU Zaria. Bambaranut ane milk Tin was purchase from Samaru Market.

3.2.1 Sample preparation

The leaves and stem of the maize stalk were mixed and chopped into small pieces using a pair of scissors and then sun dried. The maize stalks were pounded into smaller pieces using a mortar and pestle. The pounded pieces of the sample were dried in an oven at 70°C for 1 hour and then ground with milling machine, sieved with a mesh of different sizes 100µm, 200 µm, 300 µm, 400 µm and 500 µm respectively. The different sizes of the maize stalk were pretreated with 1M NaOH for one hour, washed thoroughly and neutralized by 1M HCl. The samples were then washed thoroughly with distilled water and dried in an oven at 70°C for one hour. The treated samples were kept in bottles for further use.

3.3.0 Media and reagent preparation

3.3.1 Potato dextrose agar preparation

An aliquot of 3.3g of potato dextrose agar (PDA) was weighed and dissolved in 100ml distilled water, and autoclaved at 121°C for 15 minutes. The medium was allowed to cool to about 45^0C, 5µg/ml ampicillin was added to suppress bacterial contamination and maintained at 45°C in water bath until used.

3.3.2 Preparation of reagents

All reagents purchased were of analytical grade and prepared for use according to manufacturer's instructions.

3.4.0 Enrichment for mould growth

10 grams of the maize stalk, soil, bambara nut and rotten wood sample each were weighed in a 250ml beaker. The sample was moistened with 5ml sterile distilled water and kept at room temperature (29±3°C) to grow mouldy for four days.

3.4.1 Isolation of Aspergillus niger

5 grams of each stalk samples mould were suspended in 90ml of distilled water. Then 0.1ml of the resulting mixture was spread on the surface of solid potato dextrose agar plate using a bent glass rod and incubated at room temperature (29±3°C) for 5 days observing daily for fungal growth. The distinct fungal colonies formed were further sub cultured on fresh potato dextrose agar to obtain pure cultures. The pure cultures were maintained on potato dextrose agar slants and stored in a refrigerator at 4^0 C for further use.

3.4.2 Screening for cellulose degrading Aspergillus niger

The screening for cellulose degrading ability was carried out by simple agar plate method. An enrichment culture technique using minimum basal medium containing cellulose as the only source of carbon described by Akpan et al, (1999) was abdopted. Composition of the selective medium (g/100ml) 0.75g of $MgSO_4.7H_2O$, 2.5g $NaNO_3$, 1.75g KH_2PO_4, 2g $CaCl_2.H_2O$, 10 grams of carboxymethyl cellulose (CMC) and 1.5% agar-agar. The above medium was prepared and autoclaved at 121°C for 15 minutes and was allowed to cool to room temperature. The medium was dispensed using flame inoculation needle. The *Aspergillus niger* isolates were point inoculated on solidified media agar plates and incubated at 30°C for 72 hours.

The diameter (mm) of the colonies and zone of clearance were measured at 24 hours interval. Strains which presented largest colony (mm) diameter after 72 hours were subcultured and maintained on potato dextrose agar slants at 4°C in a refrigerator for further use.

3.4.3 Preparation of inoculum

Inoculum suspensions were prepared from fully sporulated (5 -7 day old) cultures grown on potato destrose agar slants. The colonies were covered with 10ml of sterile distilled water containing 2.5% Tween 80 (polyoxyethlene sorbitan monooleate), added to facilitate the uniform distribution of Aspergillus niger inocula as adopted by Petrikkou et al., (2001). The spores were dislodged by aseptic gentle shaking for 15 minutes and transferred to a sterile test-tube (Sabo et al., 2005). Then the inoculums concentration was microscopically enumerated using the cell-counting hemacytometer (Neubauer Chamber, Merck, S.A, Madrid, Spain as decribed by Petrikkou et al., (2001). The spore suspension was prepared by proper mixing and approximately 9 micro liters of the cell suspension was pipette into one of the two counting chambers. And all

of the spores in each of the 4 0.1mm3 corner squares were counted. Average cell count was calculated as:

Spore/ml = (n) × $10^{4.}$

Where n = the average cell count per square of the four corner squares counted.

3.4.3.1 Aspergillus niger inoculum

Aspergillus niger strain inoculum was prepared from slant cultures and used to inoculate 20ml of sterile growth medium.The medium is composed of (1g glucose, o.1g peptone, 0.1g malt extract 0.7g yeast, 0.1 magnesium sulphate 0.2g calcium carbonate and 0.001g ferrous sulphate in 100ml distilled water) contained in 50ml Erlenmeyer flasks. The flasks were incubated with shaking (150/ rpm) at 30^0C for 5 days.

3.4.3.2 *Saccharomyces cerevisiae* inoculum

Saccharomyces cerevisiae incoculum was prepared the same way as the Aspergillus niger inoculum. The pH was however adjusted to 5.5 and incublation was for 72 hours.

3.4.4 Substrate preparation

Banbara nut, soil, maize stalks and rotten wood used in this study were collected from a Farm near Anguwa Maigwodo Sabon Gari Zaria of Kaduna State. They were prepared into soluble cellulose in the laboratory as outlined below.

The raw biomass was cut into small size using a table knife and ground into different sizes 100um, 200um, 300 um, 400µm and 500 µm particles. The stock samples were pretreated by refluxing the powder with 0.1M sodium hydroxide (NaOH) for 2hours and then neutralized with hydrochloric acid for 5 minute and finally washed with distilled water. The pre – treated samples

were dried at 65^0C in an oven. These were then pulverized and stored until needed (Abouzend and Reddy, 1986).

3.5.0 Experimental set – up and fermentation procedure

One percent, two percent, three percent, four percent and five percent of maize stalk was weighted into 500ml Erlenmeyer flasks respectively containing 10.0g KH_2PO_4 10.5g $(NH_4)_2.7H_2O$ 0.5g $MgSO_4.7H_2O$, 0.5g $CaCl_2$, 0.004g $FeSO_4.7H_2O$, 0.5g yeast extract, 0.004 $MnSO_4.H_2O$, 0.004g $ZnSO_4.H_2O$ 0.0067g $CaCl_2.6H_2O$, in 100mls of sterile distilled water.

The flasks with the medium were plugged with cotton wool to avoid contamination and autoclaved at 121^0 for 15 minutes. The pH of the media was adjusted to 6.5 with O.1M NAOH and 0.1M HCl prior to sterilization, and then sterilyed. After sterilization,the media was allowed to cool. All the experiments were performed in duplicate. After cooling, the sterile media at room temperatures, each flask was inoculated with 5% of Aspergillus niger inoculum which contained 3.5 $x10^6$ spores/ml, determined using haemocytometer. The 5% of *Sacccharomyces cerevisiae* inoculums, which contained 4 x 10^8 cells/ml determined using spectrophotometer at 560nm, was added to each flask. These flasks were incubated at 30^0C for 5 to 7 days on a shaker at 100rpm.

3.5.1 Mutagenic treatment of isolated Aspergillus niger

Mutation with UV irradiation at 254nm was carried out by using a modification of the procedure. The organism was grown on nutrient broth far 18 – 24hours and their microbial counts were determined. 10 milliliters of each organism was aseptically transferred into separate sterile Petri-dishes and placed at 6cm from the source of UV light hight for 5, 10,15,20,25 and 30 minis in a dark room. The UV irradiated organism were then transferred into a sterile 20 milliliter test tube in a dark room and treated with 0.2%(w/v) caffeine and allowed to stand at room temperature in

the dark for 5 hours. The irradiated cell was then centrifuged at 1500rpm for 3 to 5 minutes re-suspended in normal saline and re – centrifuged. The supernatant was discarded.The treated organism was then incubated at 18^0C for 12 – 16hours and their microbial count were determind.The one that has lower organisms or least number was tested for cellulose production. The one that gave wide zone of clearance was selected for further use and was stored on agar slant bottle.

3.5.2 Fungal mycelia dry weight

10 milliliters of the fermentation medium was removed at 24 hours interval in a test tube after vigorous shaking, using sterile 10ml syringes as adopted by Ooijkaas et al., (1998).

The samples were centrifuged at 150 rpm for 2 minutes, decanted and then recentrifuged with 10ml sterile distilled water. The fungal biomass was separated by filtration. The filters were then dried at room temperature to constant weights and the weight difference gives the mycelias dry weight.

3.5.3 Determination of ethanol concentration.

Ethanol was determined by measuring its specific gravity after distillation using specific gravity bottle. 30 ml aliquots of the fermentation stock solution was taken every 24 hours and centrifuged at 1500rpm, for 3 minutes. The cell – free supernatant recovered was distilled using a $25cm^3$ conical flask at 78^0C over 20 minutes for all the samples. The distillate was used to determine the ethanol concentration at 27 to 30^0C as follows.

A clean and dry specific gravity bottle of capacity 50ml was weighed with its stopper. It was filled with distilled water equal to the volume of the distillate to be measured. The excess water

was wiped with a cloth, and then reweighed. The bottle was emptied and dried. It was then filled with the ethanol sample and reweighed and the specific gravity was calculated as;

$$\text{Specific gravity} = \frac{\text{unit of volume of sample}}{\text{unit of on equal volume of water}} \quad \text{----------3.1}$$

The specific gravity values were used to determine ethanol concentration from a standard curve prepared using known concentrations of ethanol as reported by Emeka, (`2001).



Fig. 3.1: Laboratory Bioethanol Distillation unit.

3.5.4 Preparation of ethanol standard curve

6 stocks of varying concentrations of ethanol, 5%, 10%, 15%, 20%, 25% and 30% were prepared. Aliquots of 5ml of each of these concentrations was taken into the specific gravity bottle and weighed against equal weight of de-ionized distilled water. The values obtained were then used to plot a standard curve against the six known concentrations previously determined. (Emeka, 2001).

3.5.5 Preparation of DNS reagent

Dinitrosalicyclic acid solution was prepared by dissolving 10g 3,5 Dinitrosalicyclic acid in 200ml, 0.2M NaOH. 300 grams of Rachelle salt was dissolved in 500ml distilled water. The 2 solutions were mixed with constant stirring and diluted with 100ml of distilled water

3.5.6 Determination of residual sugar in the fermentation medium.

The liberated glucose in the fermentation, medium was analyses by the Dinitrosalicyclic (DNS) acid method of Miller et al (1959). Aliquot of 0.1ml DNS solution in a test tube was heated in a water bath for three minutes for color development.

After cooling at room temperature, 6ml of distilled water was added and about 5ml of the sample was put in the corvette and absorbance measured against reagent blank using spectrophotometer value read from a glucose standard curve which was previously calibrated.

3.5.7 Calibration for the qualitative determination of glucose concentration

1 millitre of glucose solution from concentrations, 0.5, 1.0, 1.5, 2.0 and 2.5 g/l was added to 1ml of DNS reagent and heated for 3 – 5 minute in a water bath. It was allowed to coal and finally diluted to 12ml final volume with distilled water. The color absorbance was determined using spectrophotometer at 540nm against reagent blank. The graph of absorbance against glucose concentrations was plotted and used to determine glucose concentration in subsequent sample.

3.6.0 Optimization of the culture condition

3.6.1 Optimization of substrate concentration

This was investigated by carrying out fermentation experiment with concentration in the range of 1% to 5% in ten 500ml Erlenmeyer flasks. The initial pH was 6.5, and the fermentation was done at 30^0C and maintained at 1500rpm agitation rate. (Papagianni, 1999).

3.6.2 Optimization of pH

The experiment was carried out with maize stalk of the most ethanol yielding substrate concentration which was 3% (w/v) of maize stalk. Using this concentration in 100ml basal medium, the initial pH was adjusted to 3, 4, 5, 6, 7 and incubated for 5 days at 30^0C and 1500 rpm [Omemu et al, 2005].

3.6.3 Optimization of temperature

The effect of temperature on ethanol yield was determined with other fermentation parameters set at pH 5, 150rpm agitation rate, 3% (w/v) substrate concentration and at different temperature 25^0C, 30^0C, 35^0C, 40^0C and 45^0C using a Laboratory adjustable water bath for five days [Omemu et al, 2005].

CHAPTER FOUR: Results and Discussion.

4.0 RESULTS AND DISCUSSION

The results of this work: Isolation, Degrading performance, Optimizing culture condition, modification simultaneous saccharification and fermentation are presented and discuss below;

4.1 Macroscopic observation

The growth of Aspergillus niger were monitored on PDA at 30^0C. The colonies initially observed as white turned to yellow which later become dense dark brown to carbon black mass as shown in plate 4.1.

4.2 Microscopic observation

The microscopic characterization of Aspergillus niger isolate ANRW was observed as shown in Table 4.1, and Plate 4.2

The result was compared with that of an already characterized *Aspergillus niger* isolate (V.An) obtained from the Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Ahmadu Bello University Zaria

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Table 4.1 Microscopic characterization of Aspergillus niger isolate ANRW

Feature	Description
Septation	Non septate
Hypha	Blue –black with transparent tubular appearance growing from a substratum.
Head	Black, globular head on hyphae growing from substratum

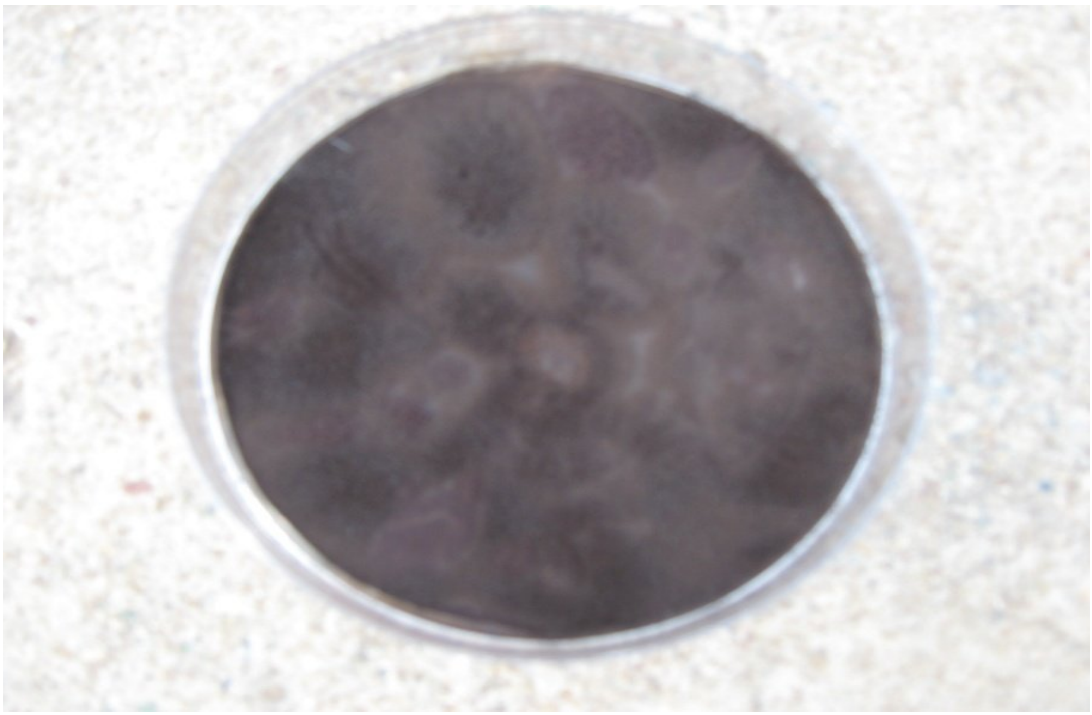


Plate 4.1: colonies of Aspergellus niger isolate

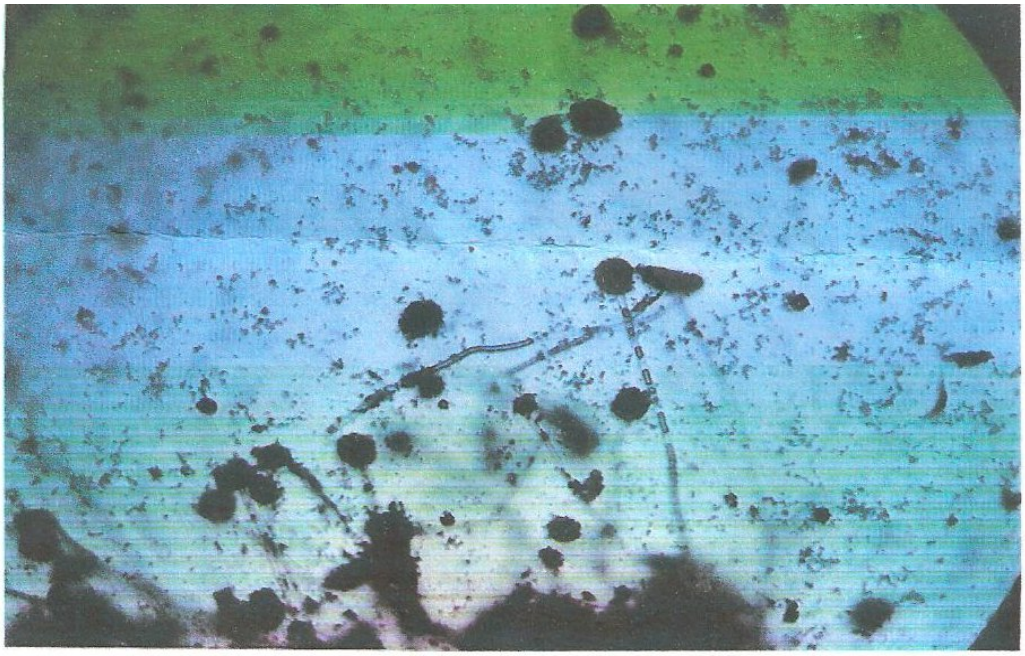


Plate: 4.2 Portion of young culture of Aspergellus niger isolate as viewed under microscope.



Plate 4.3: Aspergillus niger from maize stalks

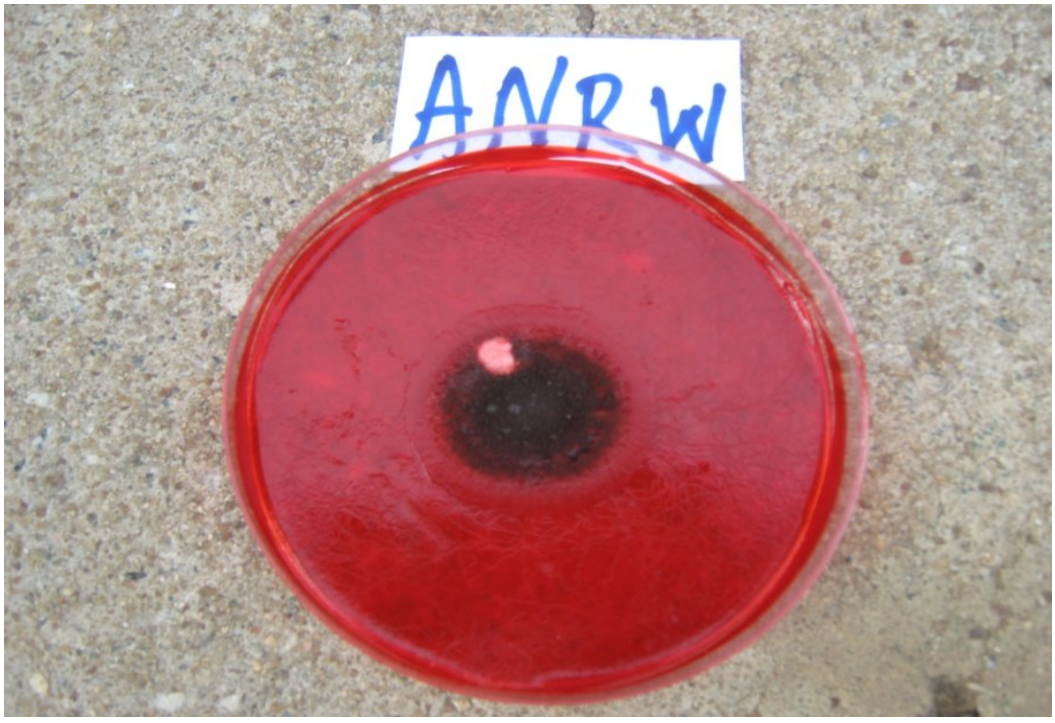


Plate 4.4: Aspergillus niger from Rotten Wood



Plate 4.5: *Aspergillus niger* from Bambara nut



Plate 4.6 Aspergillus niger from soil

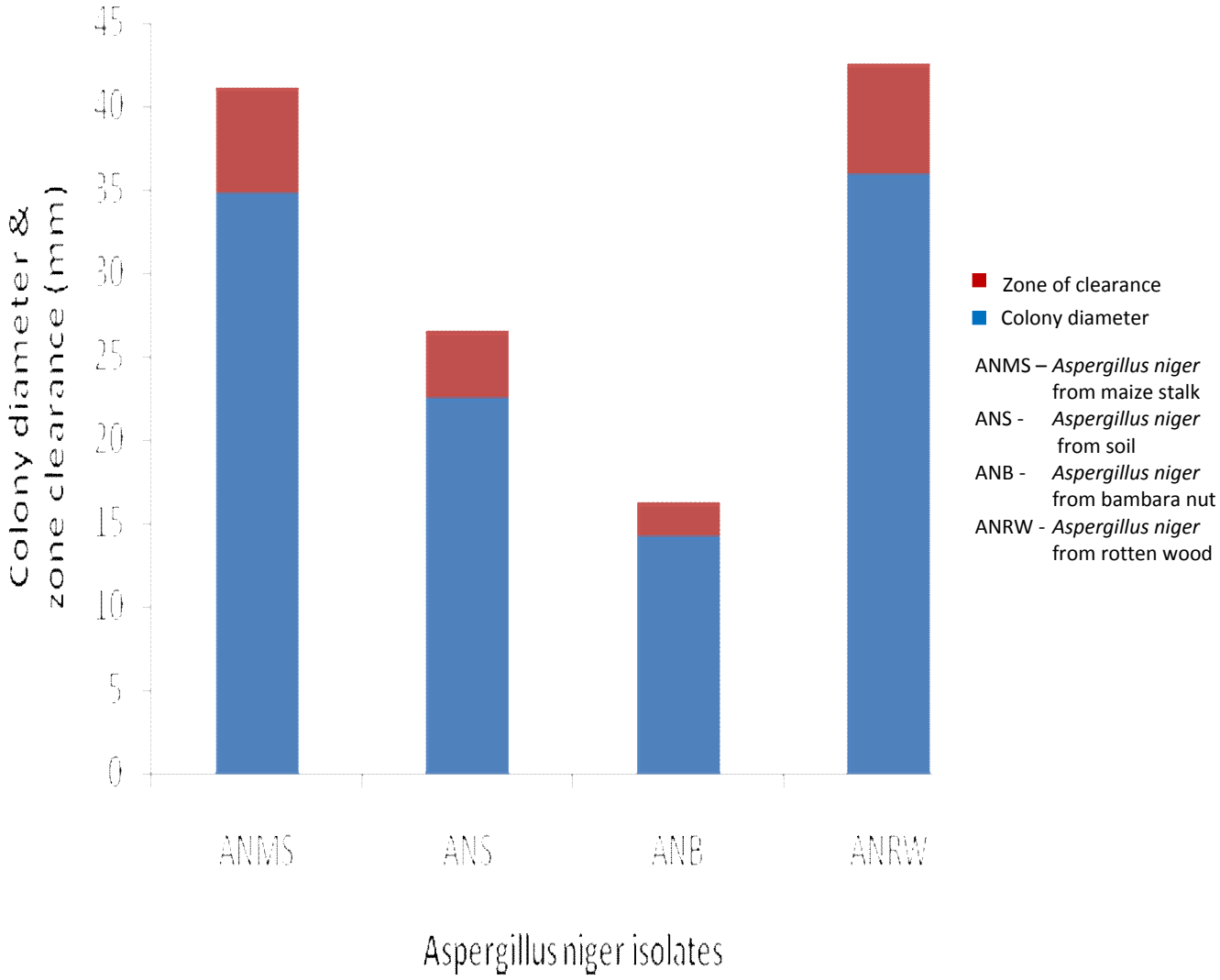


Figure 4.1 Growth of Aspergillus niger colony diameter and zone of clearance on cellulose agar plates

4.3 Screening for cellulose degrading performance of *Aspergillus nigre*

. Cellulose degrading ability of Aspergillus niger isolates from four sources, soil, bambara nut, maize stalk and rotten wood are show in plates I-VI and Figure 4.1. The isolate obtained from rotten wood showed an average colony diameter of 36.0mm with a zone of clearance of 6.35mm on cellulose agar plate after 48hrs. The least colony diameter 14.23 mm was observed with a zone of clearance of 2.0mm for *Aspergillus niger* strain isolated from Bambara nut after 48 hours incubation.

These results. Showed that Aspergillus niger from rotten wood had the highest zone of clearance. This could be as a result of availability of cellulo lytic materials in the rotten woods. The result agreed with Nam (1979) who reported that, cellulose from various sources all the same at the molecular level. However, they differ in their crystalline structure and binding by other biochemicals.

Also, it could be that the index of relative enzyme activity which compares the width of the clearing zone with the width of fungi growth was producing cellulase as reported by Bradner et al., (1999).

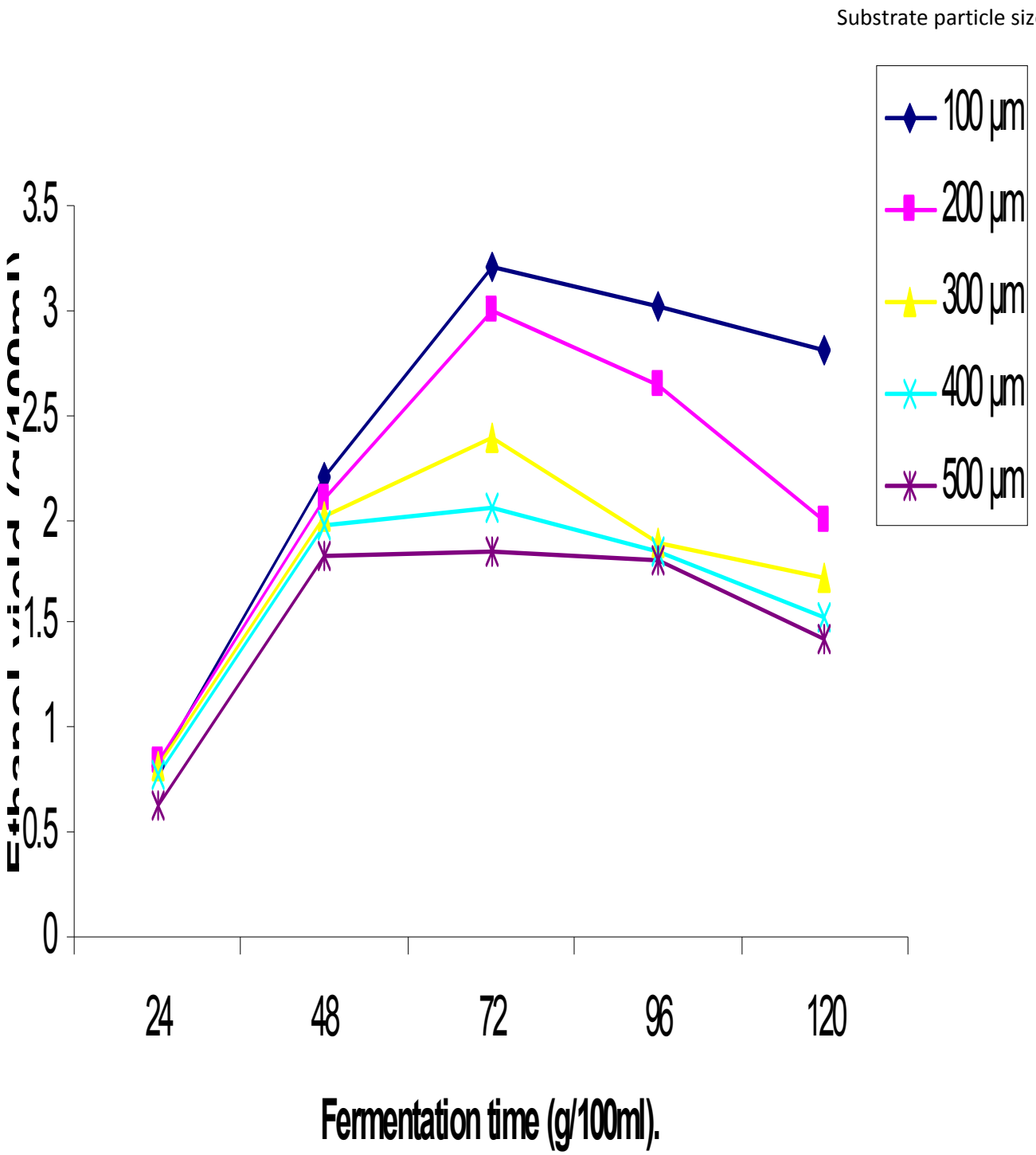


Fig 4.2: Effect of particle size on ethanol yield

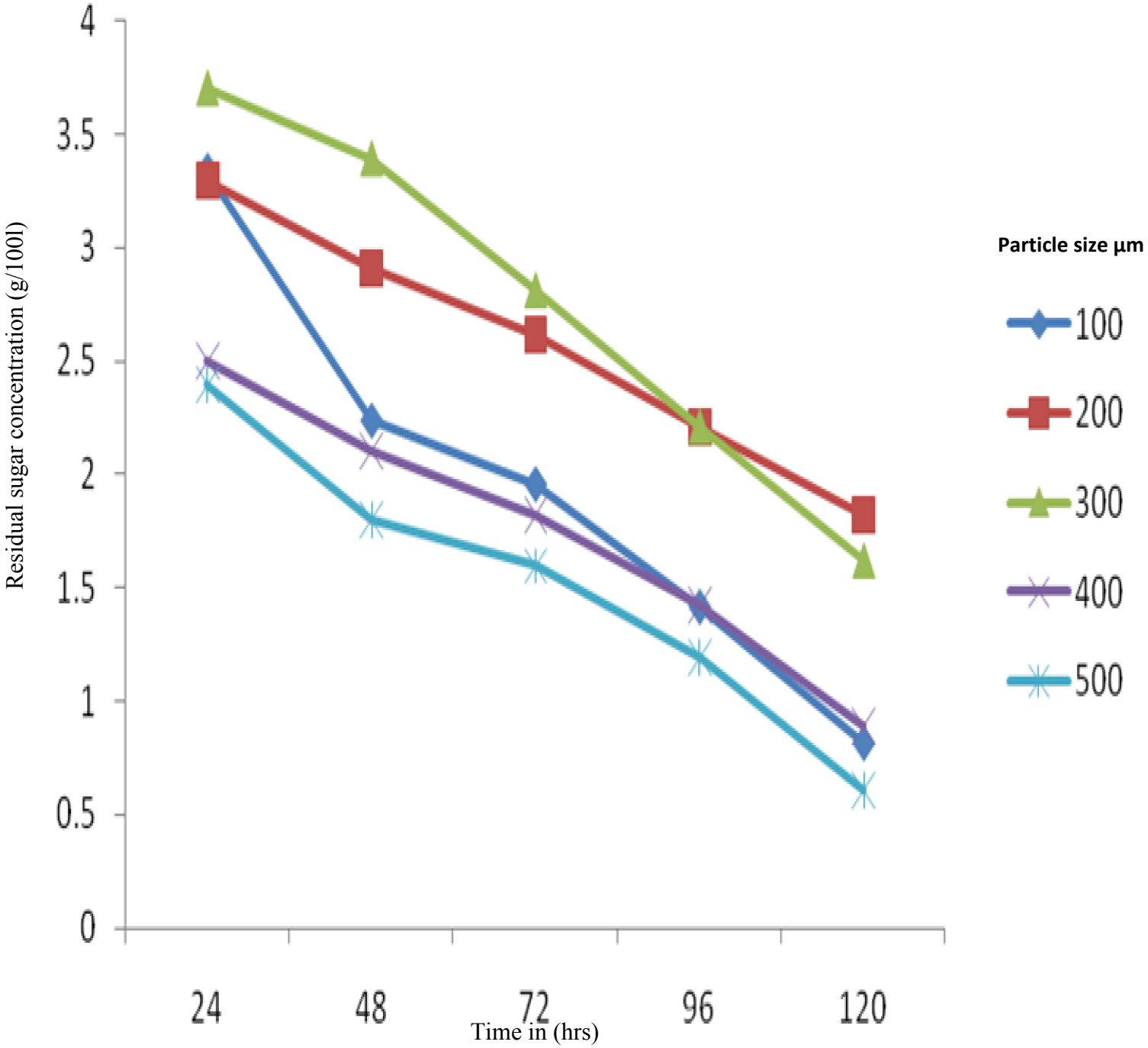


Fig 4.3: Effect of particle size and fermentation Time on residual sugar

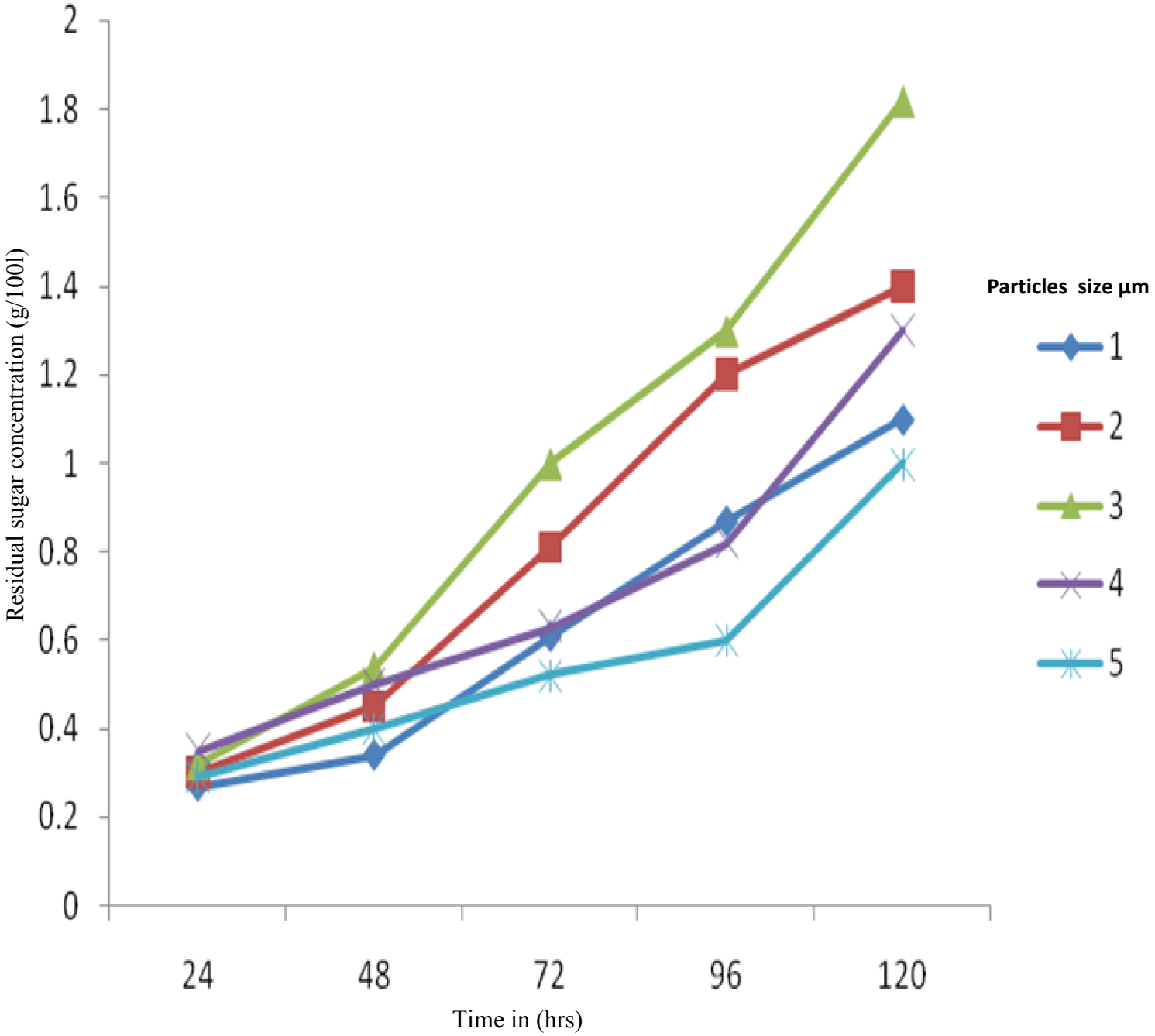


Fig. 4.4: Effect of particle size on cell dry weight

4.4 Effect of Substrate particle size on Ethanol Yield

The effect of Substrate particle particles size on ethanol yield was studied using maize stalk as a substrate, by carrying out the experiment at different initial Substrate particle size of 100um, 200um, 300um, 400um, 500um, Keeping all other fermentation condition constant, temperature 30^{o}C pH 6.5, and 3% substrate concentration. The results are shown in Table.4.3 and Fig.4.2. The rate of ethanol production increases with the initial Substrate particle size of 100um to 300um.

Optimum ethanol yield 3.4g/100ml was observed on day 3 with substrate particle size of 300µm. Minimum yield was observed at 0.5g/100ml for the particle size of 500µm. Ethanol yield generally increases from 0.58/100ml to 3.4g/100ml and then decreased.

Smaller particle size of cellulosic substrates offers larger surface area for enzyme adsorption and hence preferred for improved degradation of ling cellulosic's. It has been shown that the smaller sized fractions within the pulp are hydrolyzed preferentially in the initial stages of the hydrolysis reaction (Jackson and Grossmann.et al, 2001).

4.5 Effect of substrate particle size on reducing sugar

The results of particle size effect on the hydrolysis of maize stalk are shown in Fig.4.3

It is observed that as the particle size of the maize stalk was reduced from 500µm to 300µm, the glucose concentration increases from 2.4g/100ml 3.7g/100ml presumable, due to increase in the surface area available for enzyme attach. However, further decreasing in particle size below 300µm resulted in no significant change in glucose yield. These could be due to depletion of the more amorphous substrates product inhibition and enzyme inactivation. Ghose,(1987) Caritas and Humphrey (2006) and Narasimha et al (2006) also gave similar report of maximum glucose yield on 5th day of fermentation using Aspergillus niger

Recent works reported by Wen, (2004) shows that hydrolysis of animal manure (lignocellulosics) for reducing sugar production using particle size ranging from 590- 350 enhanced the residual sugar by 29%. However, further decrease in particle size resulted in no significant change in glucose yield.

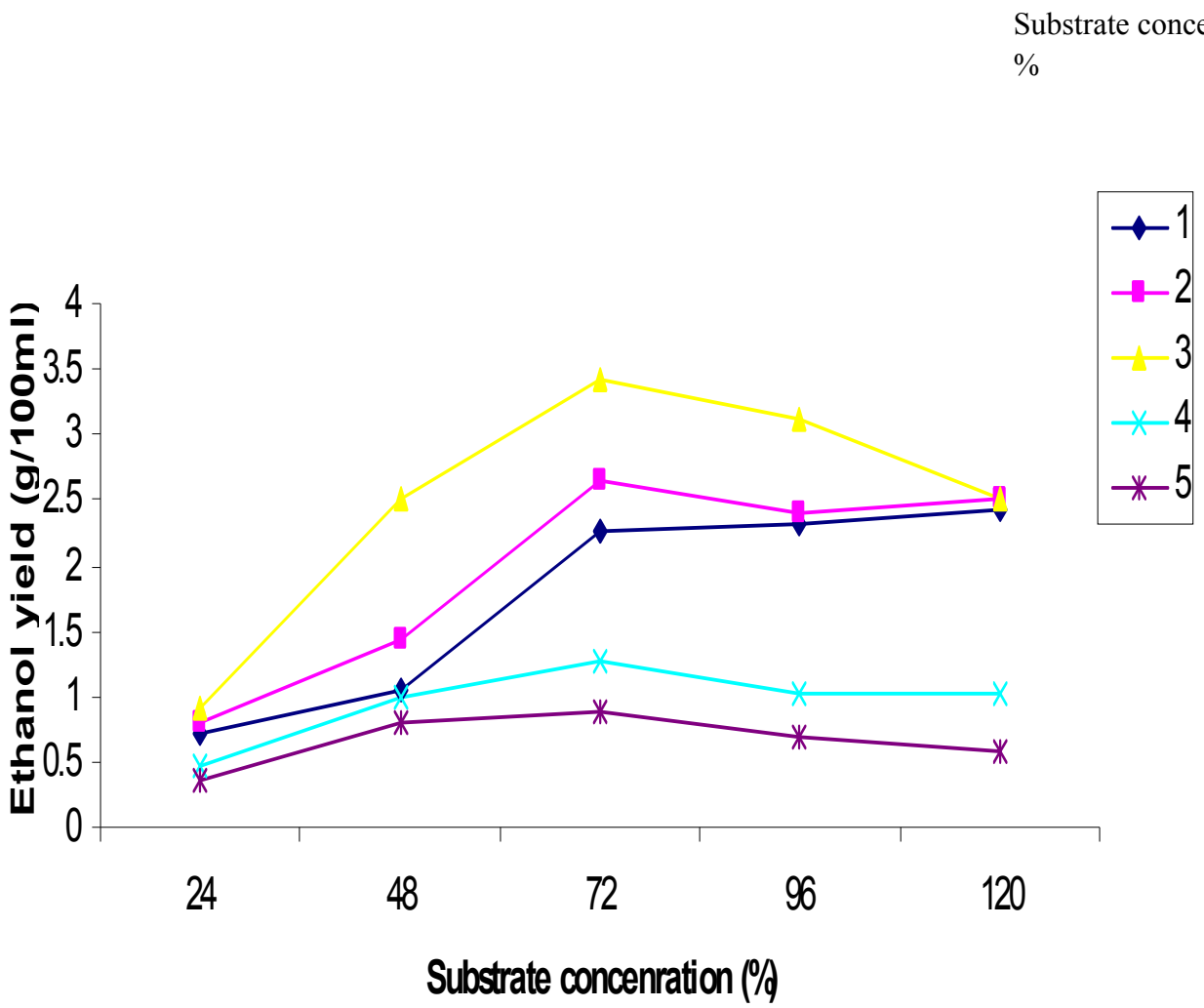


Fig.4.5: Effect of substrate concentration on ethanol yeild druing co cultrue fermentation of maize stalk

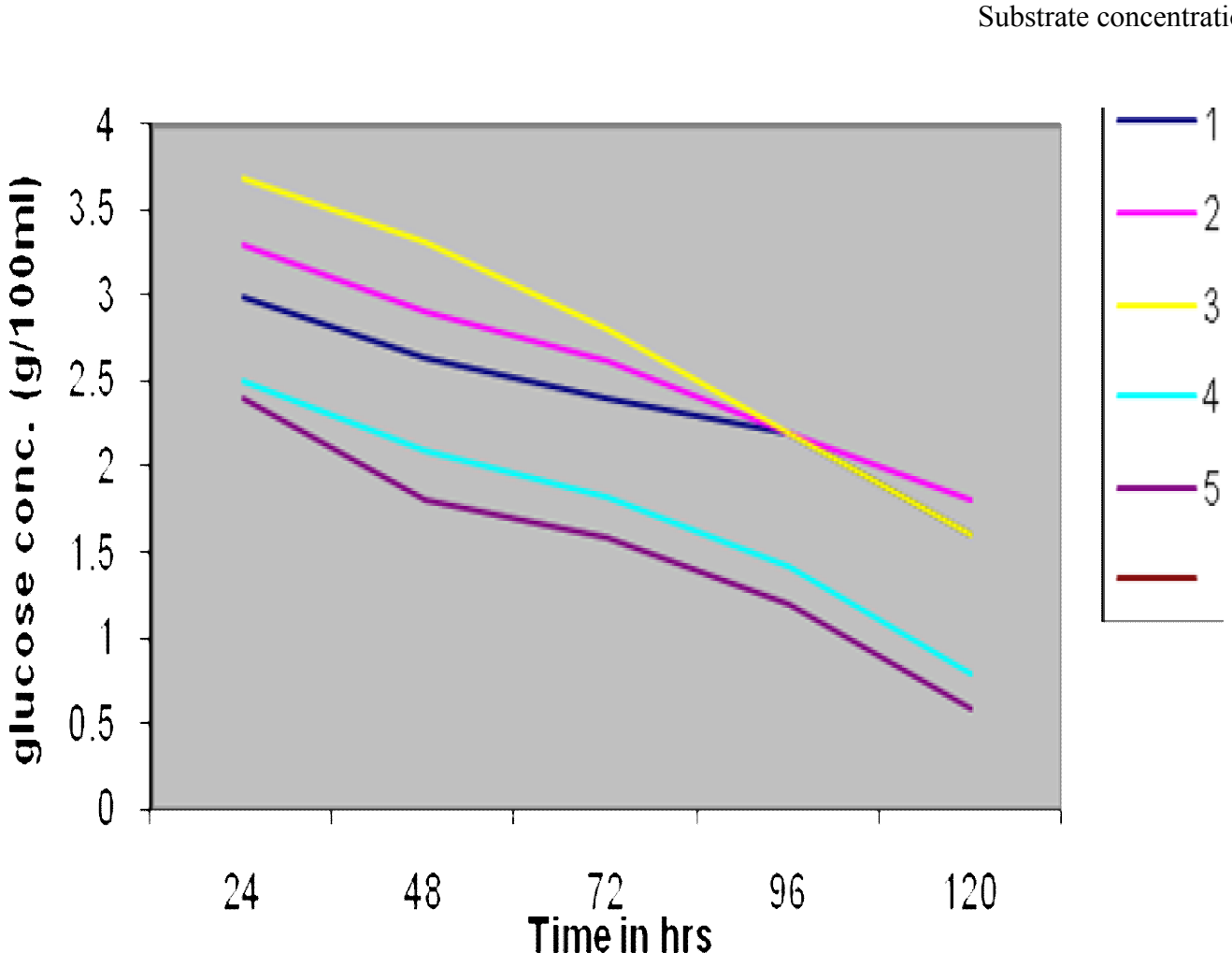


Figure 4.6: Effect of Maize stalk substrate concentration and fermentation on residual sugar

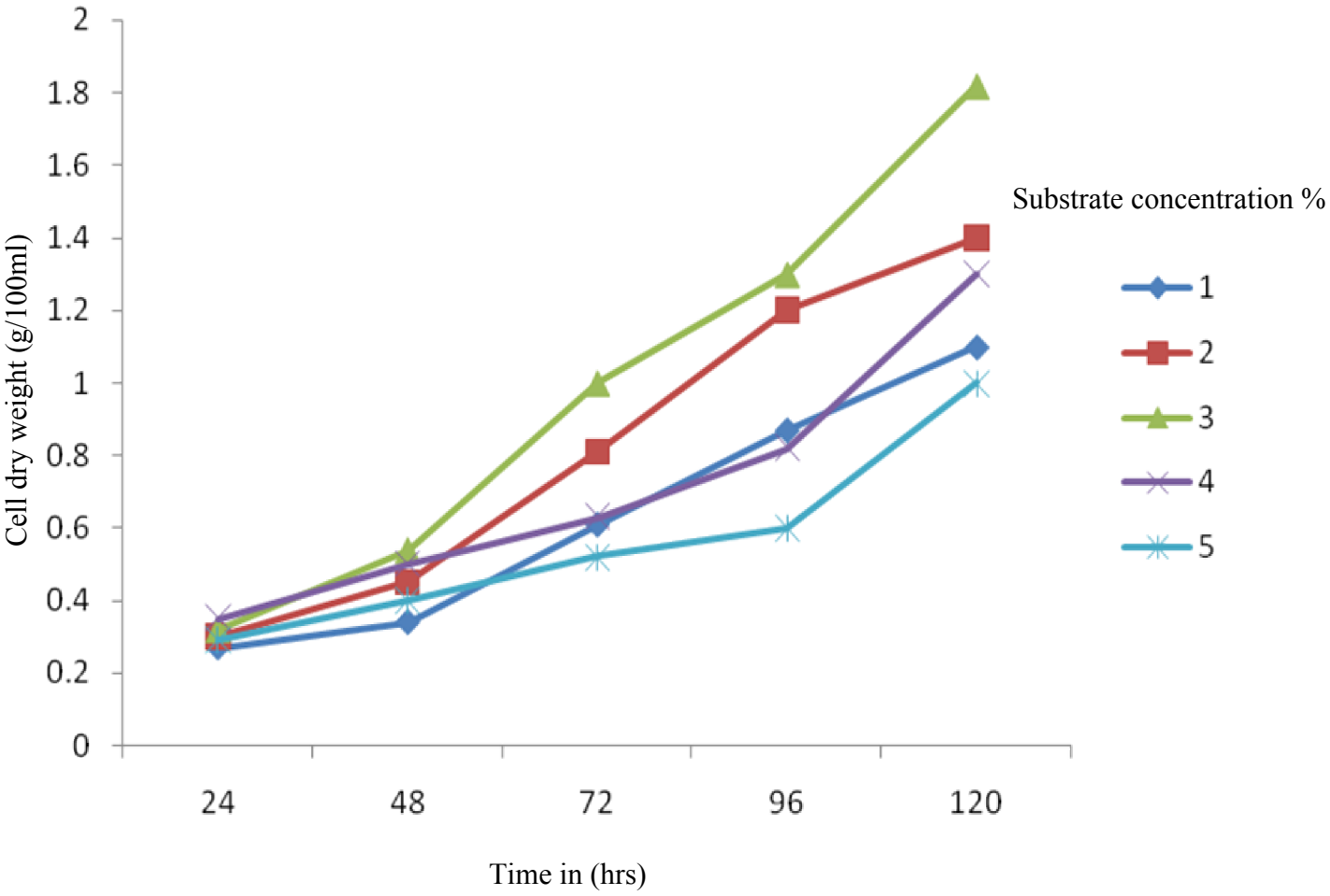


Fig 4.7: Effect of substrate concentration on cell dry weight.

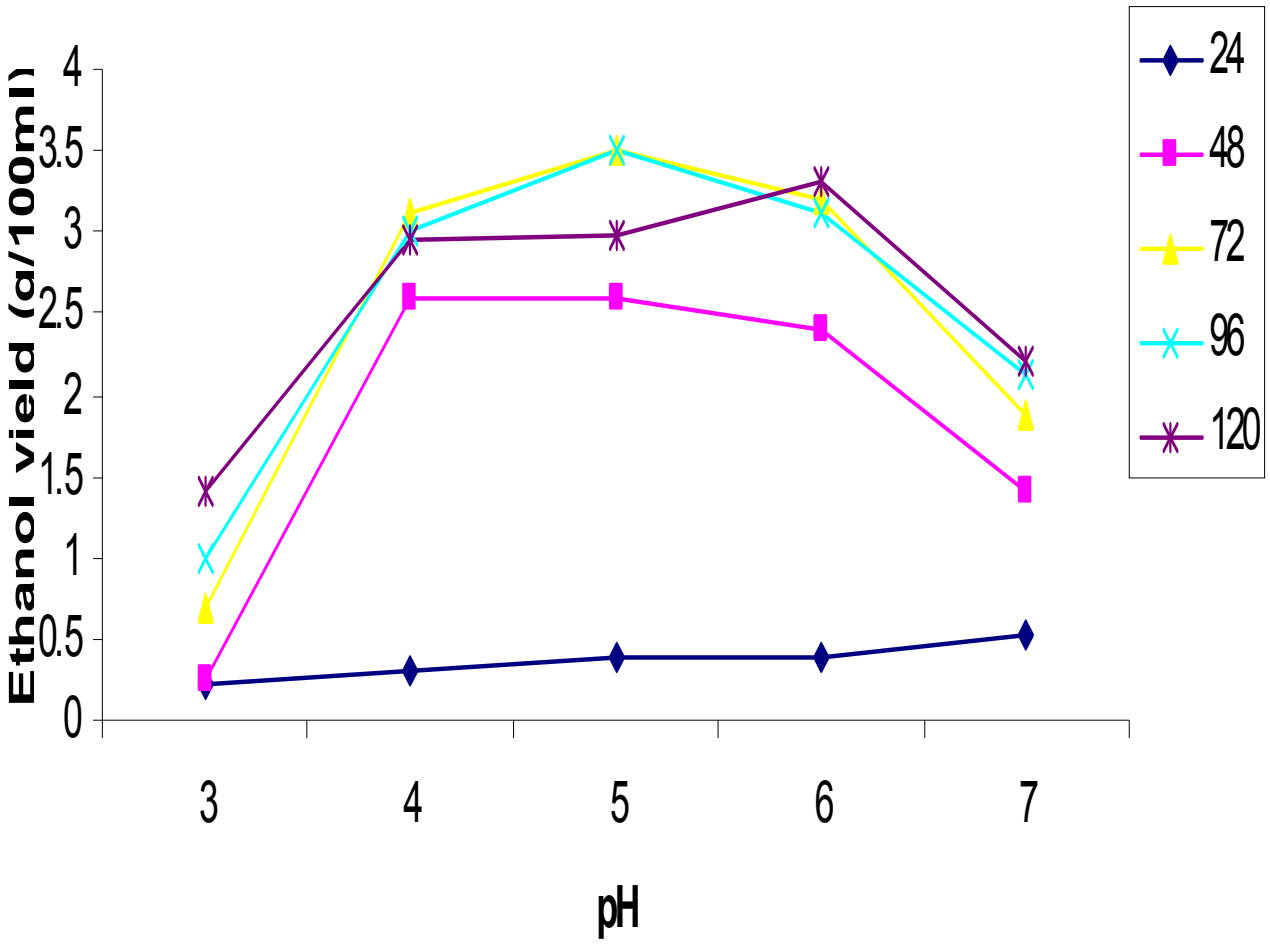


Figure 4.8: Effect of pH on ethanol concentration during co-culture fermentation of Maize stalk

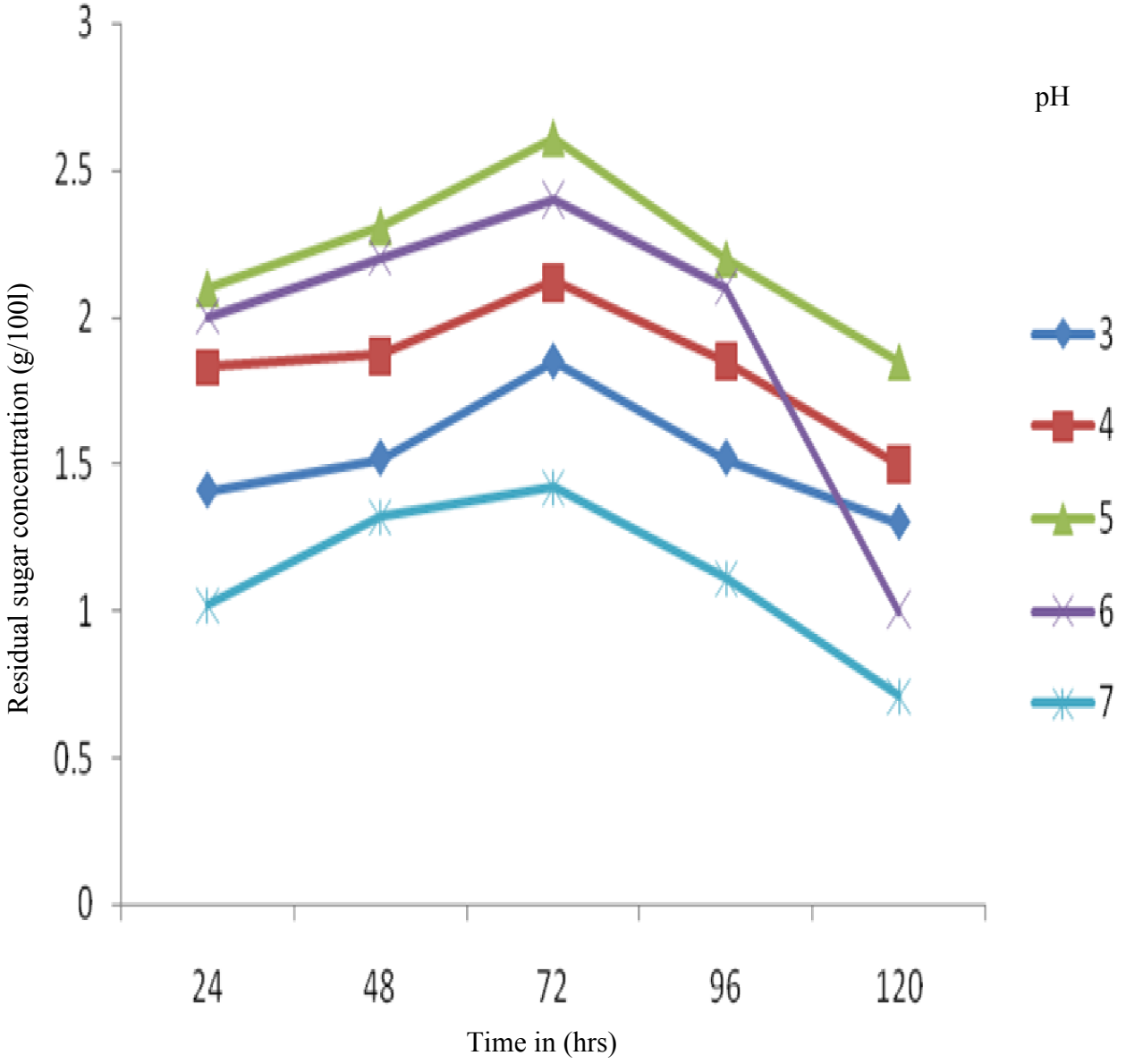


Fig.4.9: Effect of pH and fermentation time on residual sugar

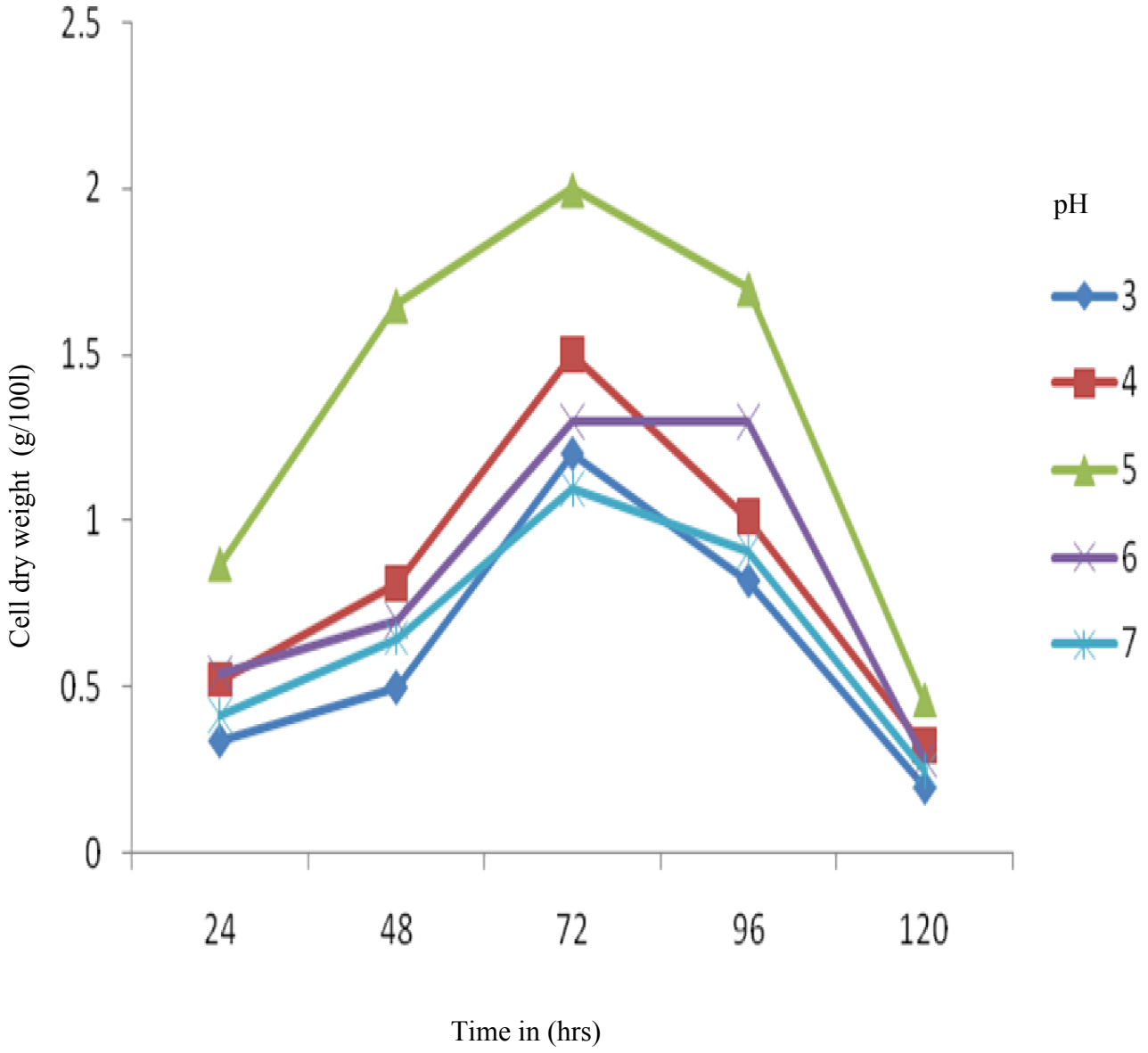


Fig. 4.10: Effect of pH and fermentation time on cell dry weight

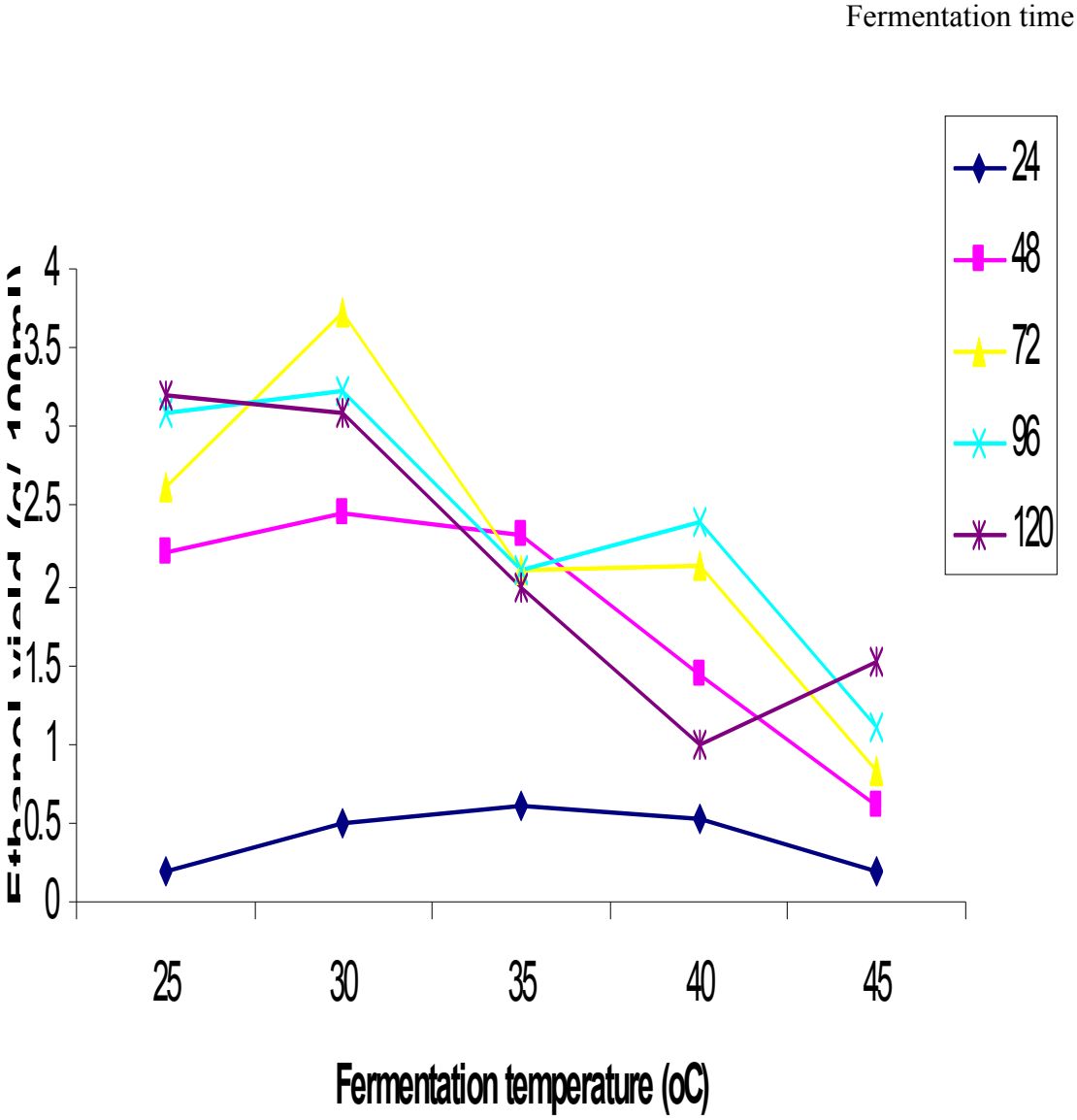


Fig 4.11 Effect of Temperature on Ethanol Yield during Coculture Fermentation

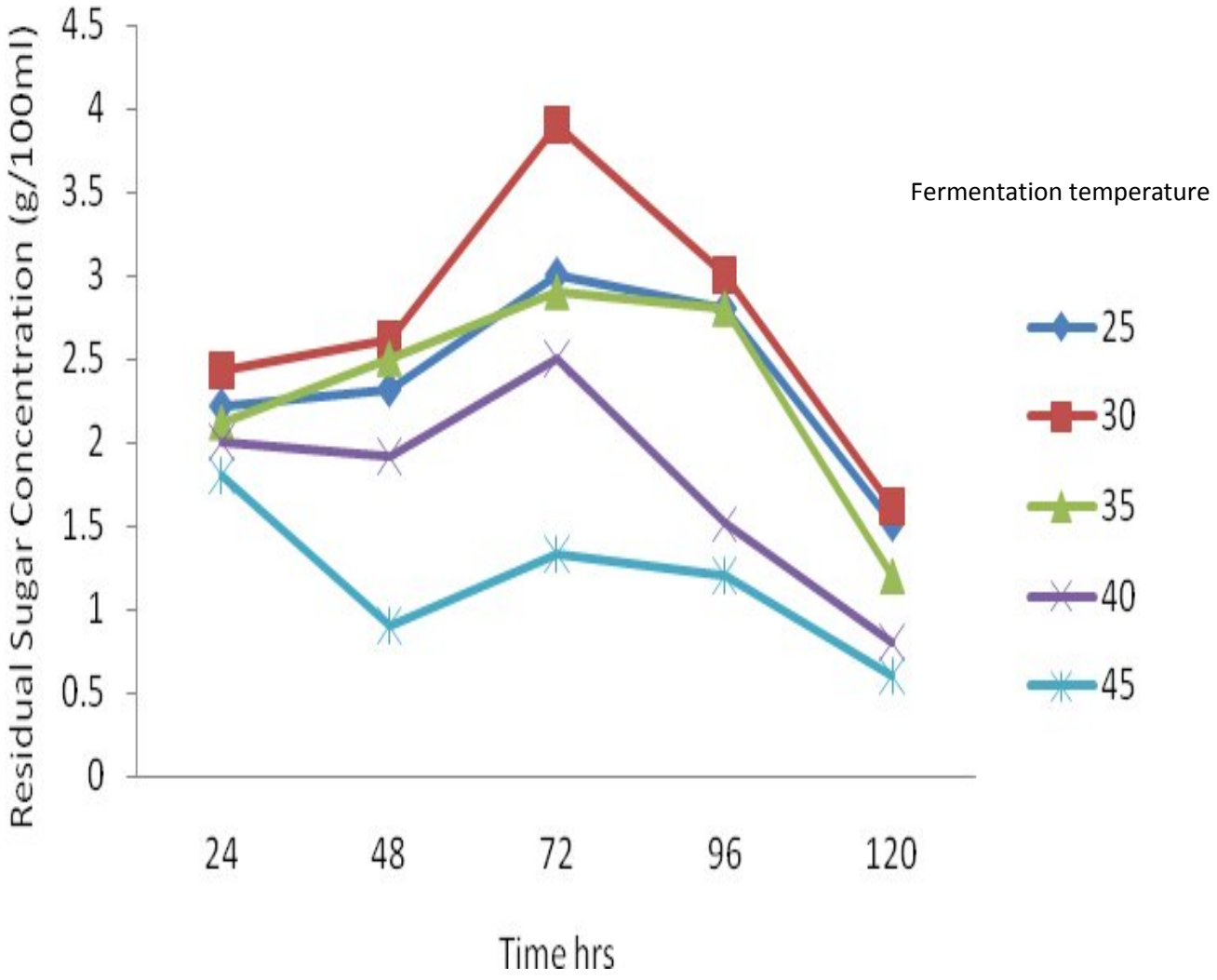


Fig. 4.12: Effect of temperature and Fermentation on residual sugar.

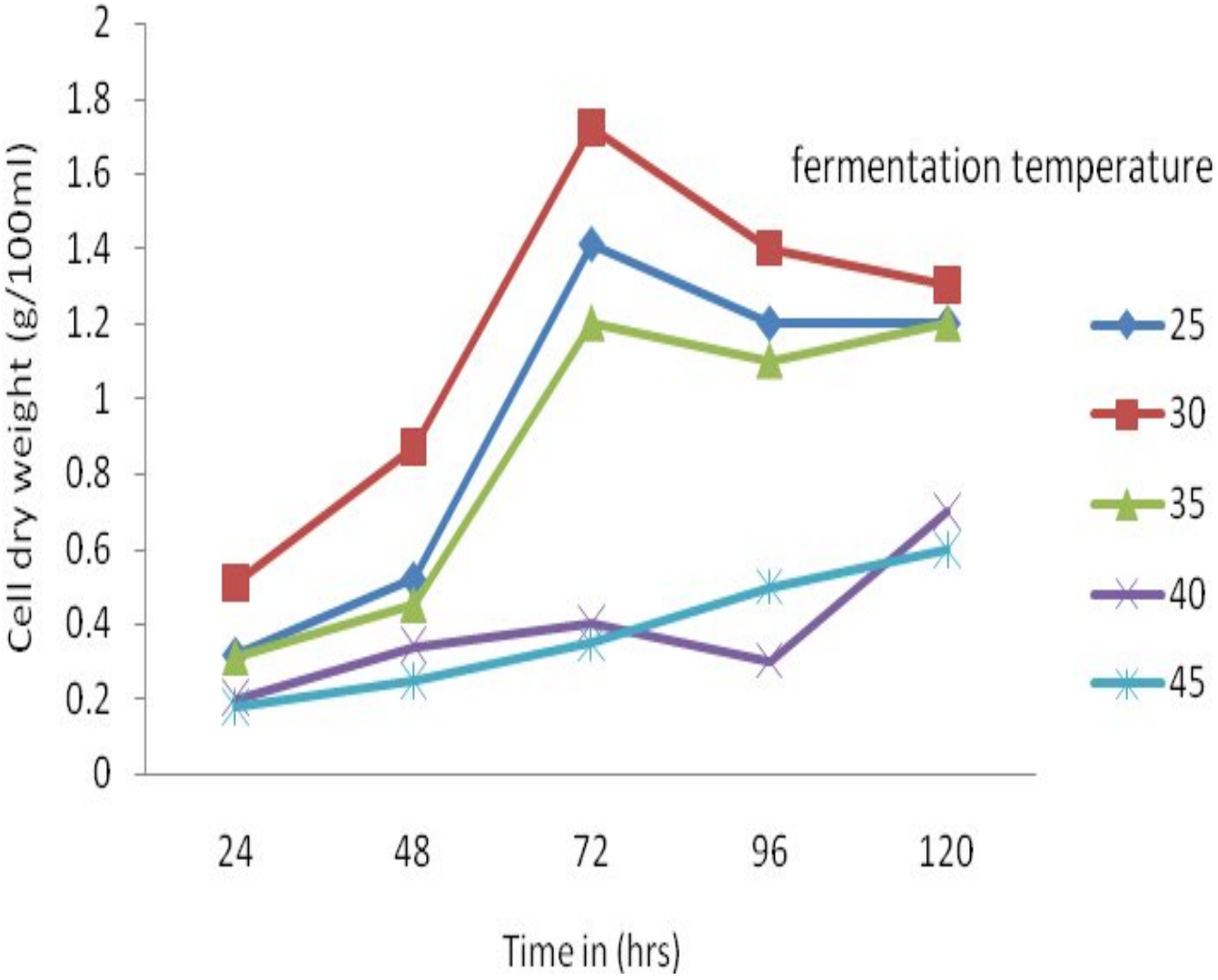


Fig 4.13: Effect of Temperature on cell dry weight

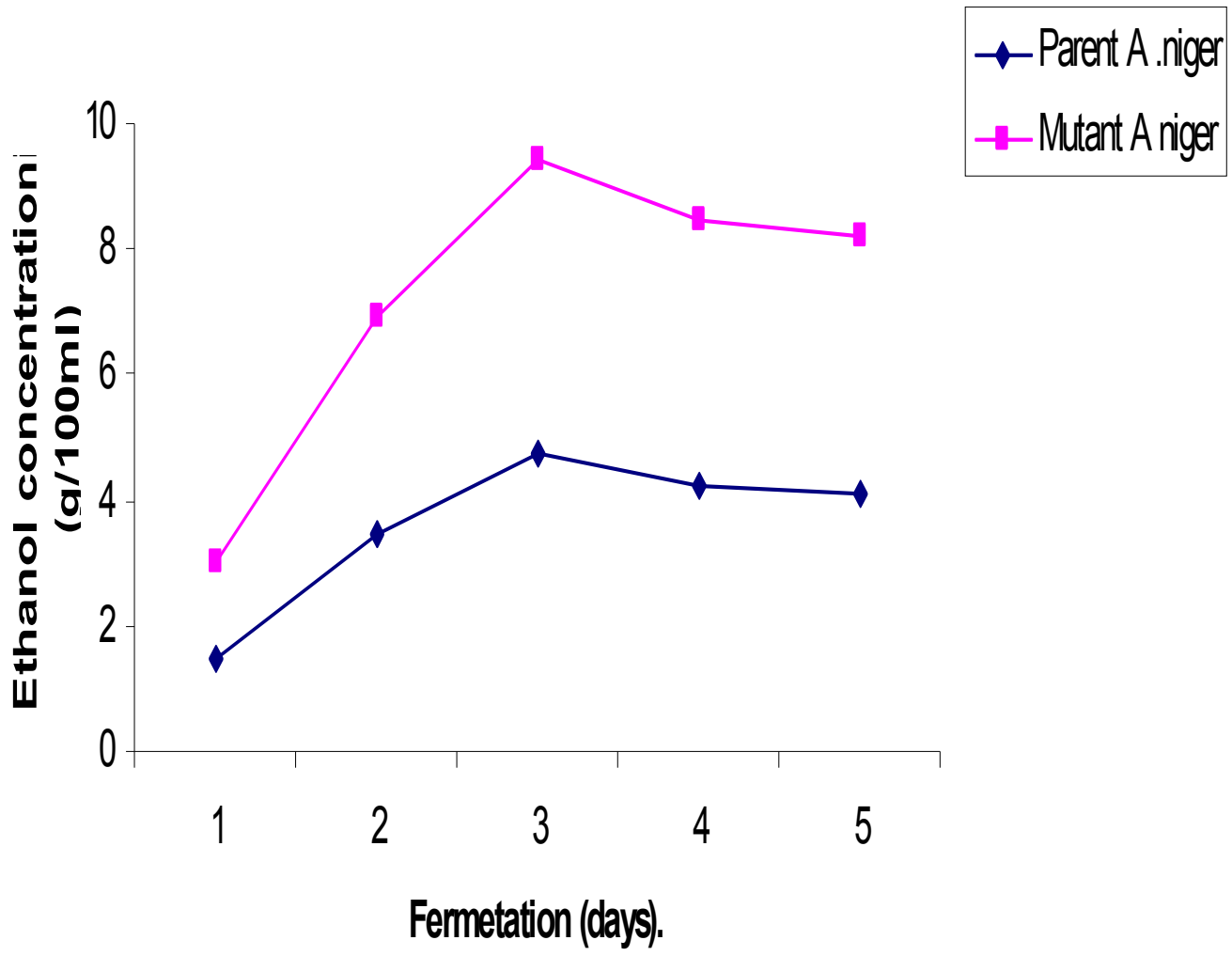


Figure 5.14: Effect of UV on *Aspergillus niger* and ethanol concentration during co-culture fermentation of maize stalk

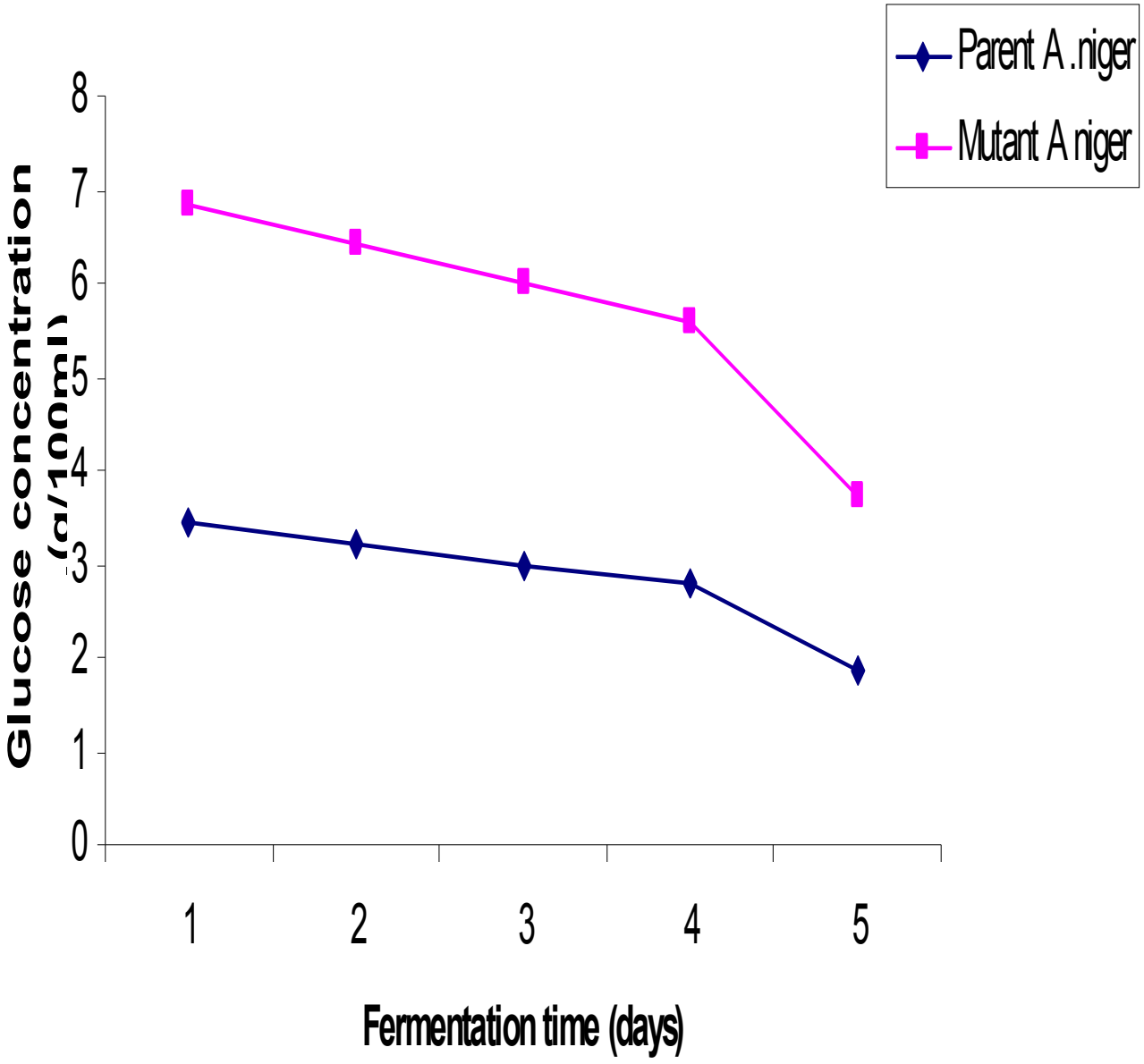


Fig 4.15: Effect of UV on *Aspergilluse niger* and glucose concentration during co culture fermentation of maize stalk

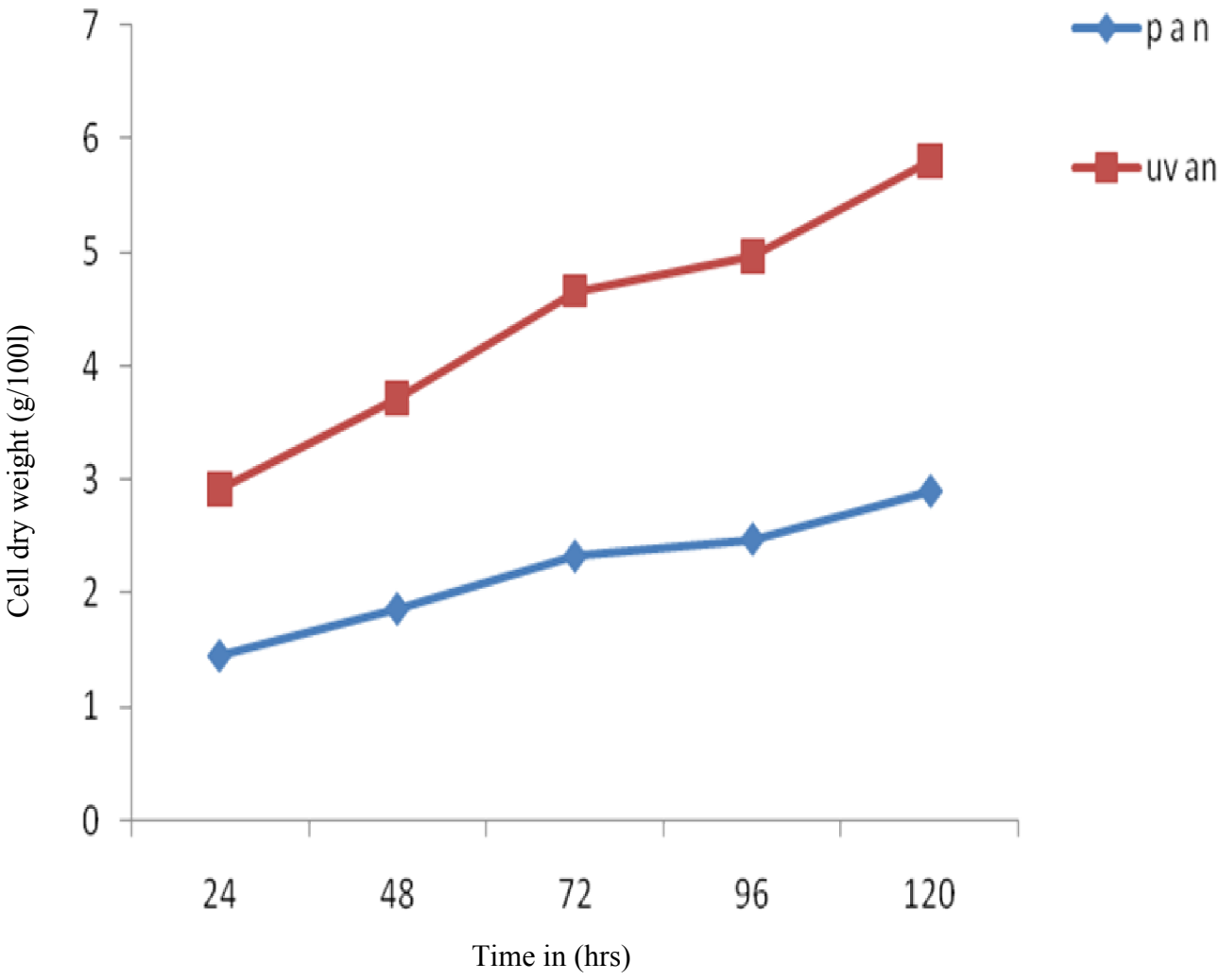


Fig 4.16: Effect of UV irradiation and fermentation time and cell dry weight

4.6. Effect of substrate concentration on ethanol yield

The effect of substrate concentration on ethanol yield was studied using maize stalk as a substrate by carrying out the experiment at different initial substrate concentration 1%, 2%, 3%, 4%, 5%. Keeping all other fermentation condition constant, temperature 30^{o}C pH 6.5, and substrate particle size 300µm. The results are shown in Table 4.6 and Fig.4.5. The result showed that as substrate concentration increases from 1% to 3% ethanol yield increased to a maximum of 3.5g/100ml. Further increase in substrate concentration up to 5% resulted in low ethanol yield. The increase in ethanol yield may be due to continuous excretion of enzymes by the micro organism in the solution. The decrease in ethanol yield beyond the optimum concentration could be that at the high substrate concentration, the active sites of the enzyme molecules at any given moment were virtually saturated with substrate. Thus any extra substrate has to wait until the enzyme complex has released the products before it may itself enter the active size of the enzyme. Wen, (2004)

The decrease in ethanol yield could also result in low saccharification yield due to difficulty in stirring and product inhibition. The maximum ethanol yield 3.58g/100n obtained at the 72 hours is agreement with most of the researchers (Takagi et al, 1977, Saddler and Chang et al, 1982b, Destipande et al, 1983, Spanger and Emerrt, 1986) reported the conversion (up to 2% wt) ethanol in 3 – 6 day. Krishna et al, (1996) reported ethanol yields of 2.2% (w/v) in 72 hours and 2.1% (w/v) in 48hours using celluloses from sugar cane. In co cu cultured of *S. fibuigera* and S. cerevisiae the highest ethanol yield was obtained in 50g/L starch medium. With increasing concentration of starch in the fermentation medium the ethanol yield was reduced (Abouzied and Reddy, 1987). Similar results have also been observed for other materials such as animal

manure, soft wood, weeds and bagasses (Wen et al., 2004; Manomani and Streekantiah, 1987; Krishna et al., 1997 and Tengborg et al., (2001).

Ethanol yield decrease as the substrate concentration increased beyond the optimum substrate concentration could be due to fact that increasing substrate concentration resulted in a more cellulose available for hydrolysis and since the amount of cell was not proportionally increased, it means cellulose quantities in excess of the cells digestive capacity are left inactive, result in the low ethanol yield observed. (Verma et al,.2000) reported that by increasing the substrate concentration fermentation efficiency decreases from 93% to 26.6% Nagodawithana et al,.(1974) also observed that at higher concentration of sugar , substrate inhibition may affect the rate of glycolysis. Similar observations were made in co culture ofE.capsularis and S. cerevisiea where a maximum ethanol produced was 16g/l at 60g/l starch concentration.

4.7. Effect of substrate concentration on reducing sugar

The effect of substrate concentration on residual sugar of maize stalk was investigated at a fixed cell concentration and the results are presented in Table 4.7 and Fig 4.6. The results show that, as the substrate concentration increases, there was an increase in glucose concentration. . Similar results had also been observed for other materials such as animal manure, softwood, weeds and also bagasse (Wen et al., 2004; Manomani and Streekantiah, 1987, Krishna et al, 1996).

4.8. Effect of substrate concentration on cell dry weight.

The effect of substrate concentratio on cell dry weigth is shown in Table 4.8 and Fig.4.7. From the result, cell dry weight increases as substrate concentration increased from 1% to 3% and decreases from 4% to 5%, this could be that substrate concentration has no direct effect on cell dry weight.

4.9. Effect of pH on ethanol yield

The effect of pH on ethanol yield during co culture fermentation of maize stalk was studied by conducting the fermentation at different controlled pH (3, 4, 5.6 and 7) keeping all fermentation condition constant as shown in Table.4.9 and Figure 4.8. As the pH increased from 3 to 5, the ethanol yield also increased and reached a maximum of 3.53g/100ml the ethanol yield then decreases with further increase in pH to 7. The decreases in ethanol yield due to the fact that the organism was more active in acidic medium than basic medium. This result is in agreement with that reported by Tengbore et al.,(2001) who obtained the optimum glugose yields at a pH of 4.8. When pH is altered above the optimum value, the rate of enzyme activity diminishes

This could be due to the fact that metabolic processes are generally temperature and pH dependent. The Optimum ethanol yield obtained in this study 3.53g/100ml at pH 5 agree with (Alva et al, .2007). Omemu et al. (2005) who reported that amylase activity was optimum at pH 4 and then decreases beyond the optimum pH.

4.10. The effect of pH on reducing sugar

The effect of pH on the residual sugar of maize stalk was investigated.and the result are shown in Table 4.10 and Fig 4.9. The pH of 5 gives the optimum yield of glucose 2.61g/100ml which is in agreement with the result obtained by Tengborg et al,.(2001) who reported that the optimum glucose yield obtained at pH of 4.8. The rate of enzyme reaction is influenced by pH because, enzyme is a protein which consists of amino acid residues. These residues possed basic, neutral, or acidic side groups which can be positively or negatively charge at any given pH. Also, an enzyme is catalytically active when the amino acid residues at the active site each posses a

particular charge. Therefore, the fraction of the catalytically active enzyme depends on the pH (Lee,1992).

4.11. The effect of pH on cell dry weight

Fig 4.10 and Table 4.11 Show the effect of pH on cell dry weight. The result shows that cell dry weight increased from pH of 3 to optimum pH of 5 and then decreased.This could be that the ogarnism grows better at the optimum pH.But as it grows it releases some byproduct which are harmful to the cells and thus render them less active. If extremes pH are encountered the organism died.

4.12. Effect of temperature on ethanol yield

Effect of temperature on ethanol yield was studies by conducting the experiments at different incubation temperature of 25^{o}C, 30^{o}C, 35^{o}C, 40^{o}C and 45^{o}C using maize stalk as the substrate. Keeping all other fermentation parameters constant. The results are shown in Table 4.12 and Figure 4.11. The result showed an optimum ethanol yield of 3.7g/100ml at 30^{0}C after 72 hours of fermentation. Lowest ethanol yield 1.0g/10ml was observed when the temperature increased to 45^{0}C.

Optimum temperature for maximum ethanol yield using maize stalk in co culture was observed to be 30^{0}C. The increase in the ethanol yield at temperature up to 30^{0}C might be due to the fact that enzyme activity and other chemical reaction in the cells are favored at that temperature. Increasing the temperature beyond the optimun might dispel the available oxygen or increase the toxic effect of the ethanol in the medium due to dissociation of molecules. Alva et al, (2007) reported maximum amylase activity at 30^{o}C and that further increase in temperature resulted in decrease in the activity of enzyme. The high temperature inactivation may be due to increased

hydrolysis of the peptid chain destruction of amino acid, or aggregation according to Snyder et al, (1999).This may affect both enzyme activity as well as the enzyme of their organism producing them as it was observed in this study.The change in temperature from 30^0C to 45^0C could be that the temperature is not favorable to the organism. (Tengborg, 2001).

4.13. Effect of temperature on reducing sugar

Effect of temperature on the hydrolysis of maize stalk are shown in Fig 4.11.The result shows that optimum glucose yield obtained was 2.4g/100ml at 30^0C. At temperature above 30^0 C there was a rapid decrease in glucose yield, most likely due to enzyme denaturing. At temperature below 30^0C also there was low glucose yield. This confirms the extra sensitivity of biological systems to changes in temperatures..

4.14. Effect of temperature on cell dry weigth

Growth of the organism seemed not favored by increase in temperature as cell dry weight decreased from 0.4g/100ml at 30^0C to 0.18g/100ml at 45^0C. The growths of organism are very sensitive to temperature and at temperature above the optimum temperature the cells died. This is in agreement with the result obtained from the hydrolysis of steam pretreated softwood reported by Tengbord et al (2001).

4.15 The effect of UV on ethanol yield

Table 4.14 and Figure 4.13 shows the ethanol yield of the parent *Aspergillus niger* and the UV exposed Aspergillus niger during a coculture fermentation using Aspergillus niger and Sacchoramyres cerevisiae at 30^0C 3% substrate concentration, pH 5 and particle size of 300µm.

The mutant strain gave the optimum ethanol yield of 8.5g/100ml on day three (3) as shown in Fig 4.14. The optimum ethanol yield from parent Aspergillus niger was observed to be 4.2g/100ml on the third day. Ethanol concentration decreased after the third day for the parent and mutant strain.

The high ethanol yield could be due to the fact that more of substrates were highly hydrolyze and more reducing sugar was produced and the high yield could also be that the Sacchoronyres convisiae were high ethanol tolerant

4.16. The effect of UV on reducing sugar

Glucose concentration of maize stalk using coculture and fermentation was determined and the result was as shown in Figure 4.15 and Table 4.16. The improved Aspergillus niger strain gave twice glucose yield of 7.2g/100ml as compared to the parent strain 3.6g/100ml. This is in agreement with Viswanathan and Kulkarmi, (1995) who reported that when Aspergillus niger Van teigham the soil isolate was matagenised by ultraviolet light, the mutant strain increased by three (3) time in enzyme productivity was generated on exposed of the parent strain to ultraviolet light for 15 minutes. The increase in residual sugar yield in the mutant could be as the result of the organism producing a high quality strain than the parent Aspergillus niger when expose to UV irradiation.

4.17. The effect of UV on cell dry weight

Fig 4.16 show the effect of UV on cell dry weight . From the result the cell dry weight of the exposed Aspergillus is hiher than the unexpose *Aspergillus niger*.This could be that exposed A .niger has high ability to hydrolyse maize stalk and as more glucose was produced the organism had more food to feed and grow whicch also resulted in high cell dry weight.

The result of infra red spectrum obtained showed that the ethanol obtained from UV A. niger was more than that of unexposed *A. niger.* This could be that the exposed *A. niger* has high ability to hydrolyse maize stalk and in coculture product inhibition is eliminated.

CHAPTER FIVE

5.1 CONCLUSIONS RECOMMENDATION.

From the results obtained in this work, the following conclusion can be made.

1. Aspergillus niger isolated from rotten wood have the highest degrading ability with the zone of clearance of 3.6mm
2. The optimum fermentation condition obtained were pH 5, temperature 30^{o}C, substrate concentration 3%, and particle size 300um, respectively.
3. The optimum exposure time was 30 minutes.
4. This study has shown the effect of UV Ultraviolet irradiation in the improvement of strain by inducing mutation on parent *Aspergillus niger* strain. The mutant strain could be exploited for large industrial production.
5. The bioethanol yield obtained at optimum fermentation condition of the mutant strain was 9.5g/100ml which is higher than that of the parent strain 4.5g/100 respectively.

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5.2 RECOMMENDATIONS

With regard to the results obtained and the problems encountered in this work, the following recommendations are made:

a. Government research institutes should award grants to students so as to encourage then to proceed up studies in production of biofuels.

b. Government at all levels should encourage small scale ethanol production for domestic use.

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APPENDIX I

Growth response of Aspergillus niger

Table4.2. Comparative growth response of Aspergillus niger from difference substrates

Substrate	Colony\ diameters (mm)	Zone of clearance (mm)
ANMS	34,83	6.33
ANS	22.50	4.00
ANB	14.23	2.00
ANDS	36.00	6.53

APPENDIX 11

Effect of particle size on ethanol, residual surgar, and cell dry weight

Table 4.3 Effect of maize stalk particles size on ethanol yield at pH 6.5, 35C, 4% substrate conc. (g/100ml)

	24	48	72	96	120
100	0.81	2	2.39	1.89	1.72
200	0.82	2.2	3	2.65	2.21
300	0.83	2.1	3.2	3.1	2.82
400	0.62	1.82	1.84	1.8	1.42
500	0.54	1.52	1.69	1.63	1.4

Table 4.4 Effect of maize stalk particle size and fermentation on residual sugar

	24	48	72	96	120
100	3.34	2.24	1.96	1.42	0.82
200	3.3	2.91	2.62	2.21	1.82
300	3.7	3.39	2.81	2.21	1.62
400	2.5	2.1	1.82	1.421	0.89
500	2.4	1.8	1.6	1.2	0.61

Table 4.5 Effect of maize stalk and fermentation on cell dry weight

	24	48	72	96	120
100	0.28	0.43	0.81	1.2	1.53
200	0.32	0.45	0.62	0.87	1.42
300	0.3	0.5	1	1.3	1.82
400	0.32	0.45	0.63	0.87	1.2
500	0.29	0.32	0.5	0.72	1

APPENDIX 111

Effect of substrate Concentration on ethanol, residual sugar, and cell dry weight-

Table 4.6 Effect of maize stalk substrate concentration on ethanol at pH 6.5 35C 300 particle size

Days Substrate	24	48	72	96	120
1	0.72	2	2.41	2.2	1.71
2	0.81	2	2.82	2.72	2.22
3	0.92	2.51	3.41	3.11	2.52
4	0.62	1.8	2.51	2.32	2.2
5	0.5	0.91	1.7	1.62	1

Table 4.7 Effect of maize stalk substrate conc. and fermentation on residual sugar

Substrate day%	24	48	72	96	120
1	3	2.64	2.4	2.21	1.61
2	3.3	2.91	2.62	2.21	1.82
3	3.7	3.33	2.81	2.21	1.61
4	2.5	2.1	1.82	1.42	0.8
5	2.4	1.81	1.6	1.2	0.6

Table 4.8: Effect of substrate concentration and fermentation time on cell dry weight

	24	48	72	96	120
1	0.27	0.34	0.61	0.87	1.1
2	0.3	0.45	0.81	1.2	1.4
3	0.32	0.54	1	1.3	1.82
4	0.35	0.5	0.63	0.82	1.3
5	0.29	0.4	0.52	0.6	1

APPENDIX 1V

Effect of pH on ethanol, residual sugar and cell dry weight

Table 4.9 Effect of pH and fermentation time on ethanol

	24	48	72	96	120
3	0.21	0.25	0.7	1	1.4
4	0.29	2.6	3.12	3	2.65
5	0.2	2.58	3.72	3.3	2.98
6	0.4	2.4	3.2	3.06	2.82
7	0.52	1.4	1.87	2.13	2.21

Table 4.10 Effect of pH and fermentation time on residual sugar concentration

	24	48	72	96	120
3	1.41	1.52	1.85	1.52	1.3
4	1.83	1.87	2.12	1.85	1.5
5	2.1	2.31	2.61	2.2	1.85
6	2	2.2	2.4	2.1	1
7	1.02	1.32	1.42	1.11	0.71

Table 4.11 Effect of pH and fermentation time on cell dry weight

	24	48	72	96	120
3	0.34	0.5	1.2	0.82	0.2
4	0.52	0.81	1.5	1.01	0.32
5	0.87	1.65	2	1.7	0.46
6	0.54	0.7	1.3	1.3	0.28
7	0.42	0.65	1.1	0.91	0.25

APPENDIX V

Effect of temperature on ethanol, residual, and cell dry weight

Table 4.12 Effect of temperature and fermentation time on ethanol at pH 5, 3% conc. 300

	24	48	72	96	120
25	0.18	2.21	2.62	3.1	3.2
30	0.5	2.45	3.72	3.24	2.1
35	0.6	2.32	2.1	2.4	2
40	0.52	1.42	2.12	2.2	1.5
45	0.2	0.61	0.82	1.2	1

Table 4.13 Effect of temperature and fermentation on residual sugar

	24	48	72	96	120
25	2.22	2.32	3	2.8	1.52
30	2.43	2.62	3.9	3	1.62
35	2.12	2.5	2.9	2.8	1.2
40	2	1.91	2.5	1.51	0.8
45	1.8	0.9	1.33	1.2	0.6

Table 4.14 Effect of temperature and fermentation on cell dry weight

	24	48	72	96	120
25	0.32	0.52	1.41	1.2	1.2
30	0.51	0.87	1.72	1.4	1.3
35	0.31	0.45	1.2	1.1	1.2
40	0.2	0.34	0.4	0.3	0.7
45	0.18	0.25	0.35	0.5	0.6

APPENDIX V1

Effect of UV irradiation on ethanol, residual sugar, and cell dry weight

Table 4.15 Effect of UV irradiation and fermentation time on *Aspergillus niger* ethanol concentration

	24	48	72	96	120
p a n	1.5	3.45	4.72	4.24	4.1
uv an	3	6.9	9.44	8.48	8.2

Table 4.16 Effect of UV irradiation on *Aspergillus niger* and fermentation time on residual sugar

	24	48	72	96	120
p a n	3.42	3.22	3	2.9	1.57
uv an	6.86	6.4	6	5.6	3.74

Table 4.17 Effect of UV irradiation and fermentation time on cell dry weight

	24	**48**	**72**	**96**	**120**
p a n	1.46	1.87	2.33	2.48	2.9
uv an	2.92	3.72	4.66	4.96	5.8

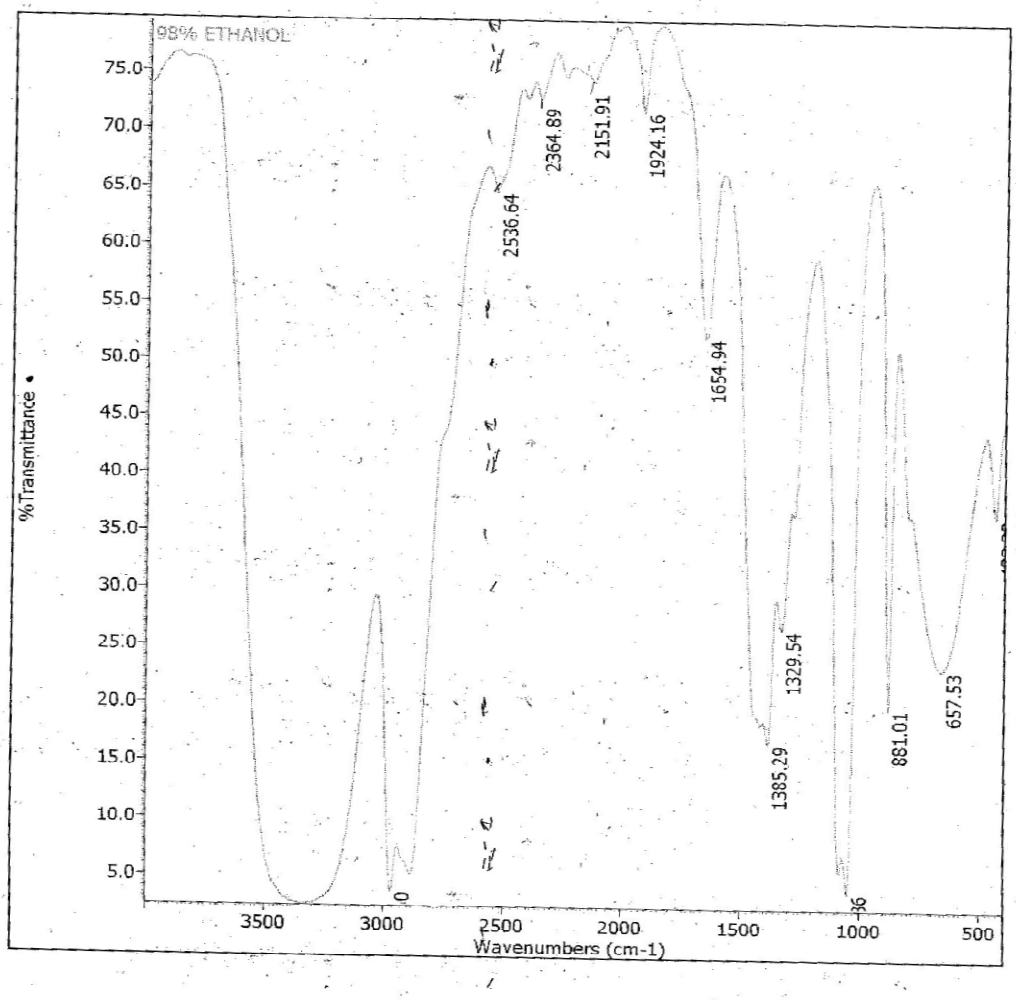
APPENDIX XIII

QUALITATIVE INFRARED SPECTRUM TEST FOR ETHANOL PRODUCTION FROM STANDARD ETHANOL (98%).

Peak finding results for: 98% ETHANOL
Frequency: 406.75 - 4000.00, threshold: 78.830, sensitivity: 50.00
Peak finding result table:

Peak#	1	2	3	4	5	6	7	8	9
Position	3354.44	2972.60	1924.16	1654.94	1385.29	1329.54	1051.36	881.01	657.53
Height	2.414	3.416	71.680	52.122	16.787	26.755	3.725	19.934	23.318

Peak#	10
Position	433.33
Height	36.651



APPENDIX IX

QUALITATIVE CORRELATION COEFFICIENT INFRARED SPECTRUM TEST FOR ETHANOL PRODUCTION FROM STANDARD ETHANOL (98%).

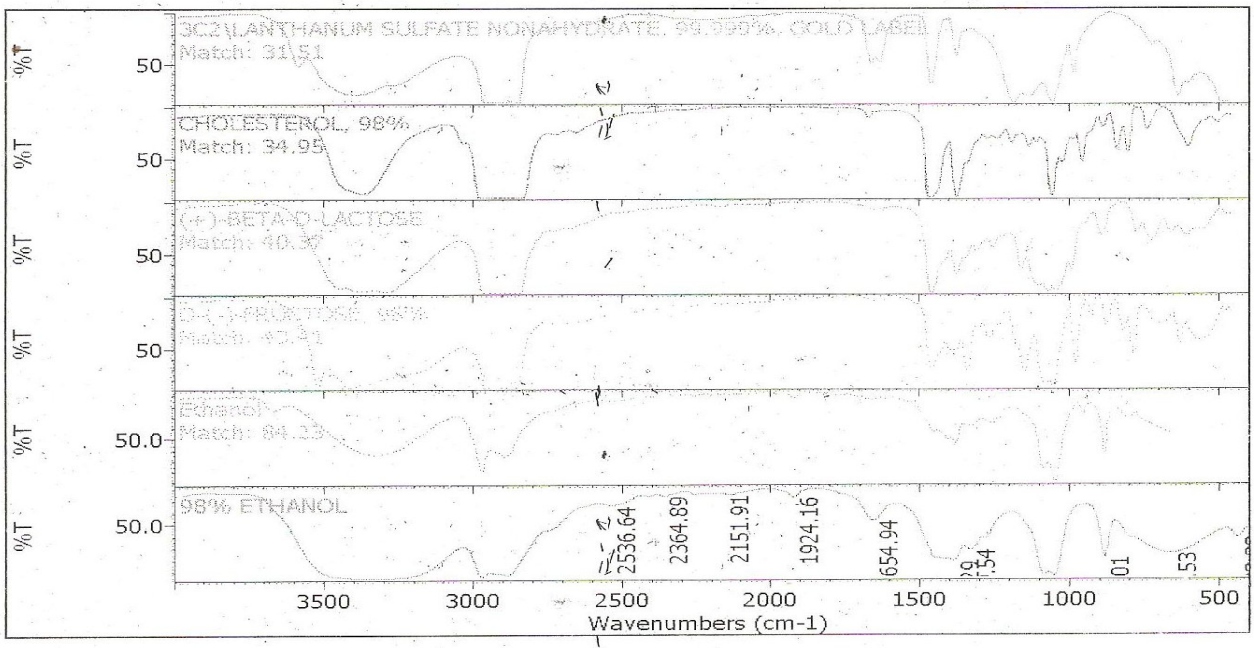
Peak finding results for: 98% ETHANOL
Frequency: 406.75 - 4000.00, threshold: 78.830, sensitivity: 50.00
Peak finding result table:

Peak#	1	2	3	4	5	6	7	8	9
Position	3354.44	2972.60	1924.16	1654.94	1385.29	1329.54	1051.36	881.01	657.53
Height	2.414	3.416	71.680	52.122	16.787	26.755	3.725	19.934	23.318

Peak#	10
Position	433.33
Height	36.651

Search results for: 98% ETHANOL
Search algorithm: Correlation coefficient
Search region (cm-1) : 4000.0000 - 400.0000
Table of matches:

#	Index	Match	Compound Name	Library Name
1	6	84.23	Ethanol	Example Library
2	6	43.41	D-(-)-FRUCTOSE, 98%	Aldrich Condensed Phase Sample Library
3	7	40.37	(+)-BETA-D-LACTOSE	Aldrich Condensed Phase Sample Library
4	90	34.95	CHOLESTEROL, 98%	Aldrich Condensed Phase Sample Library
5	100	31.51	3C2\LANTHANUM SULFATE NONAHYDRATE, 99.999%, GOLD LABEL	Aldrich Condensed Phase Sample Library

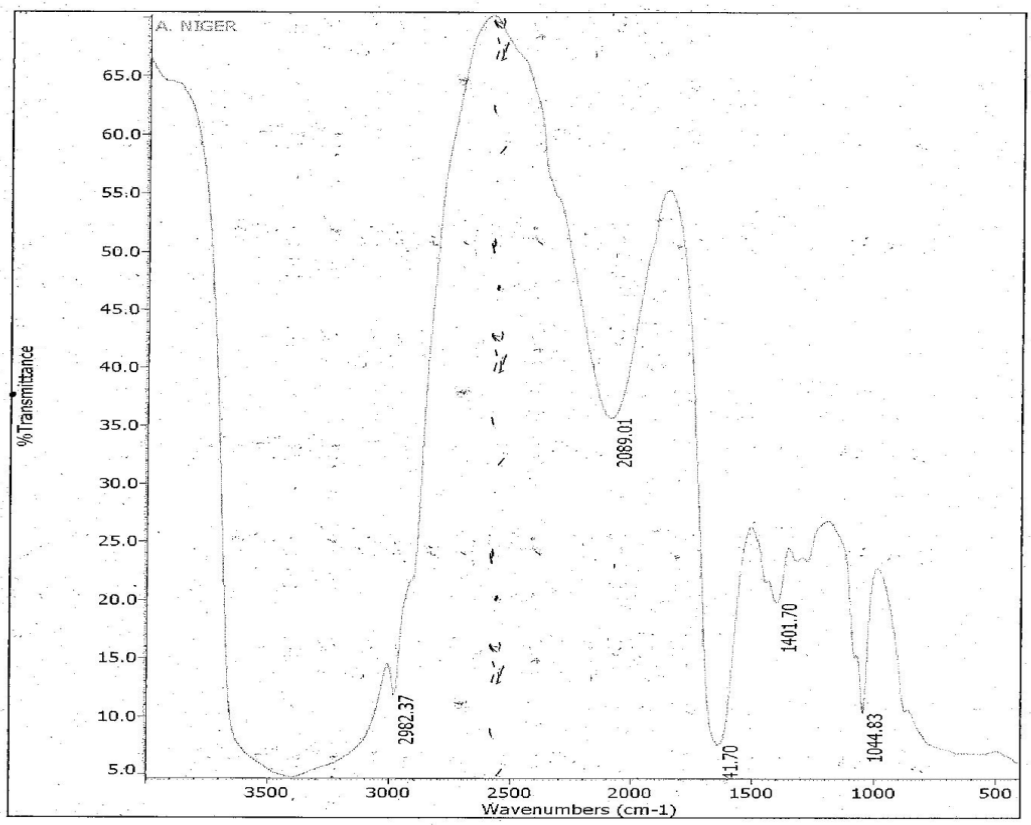


APPENDIX III

QUALITATIVE INFRARED SPECTRUM TEST FOR ETHANOL PRODUCTION FROM *Aspergillus niger*.

Peak finding results for: A. NIGER
Frequency: 406.75 - 4000.00, threshold: 66.997, sensitivity: 50.00
Peak finding result table:

Peak#	1	2	3	4	5	6			
Position	3406.54	2982.37	2089.01	1641.70	1401.70	1044.83			
Height	4.627	11.721	35.629	7.443	19.725	10.227			



APPENDIX IV

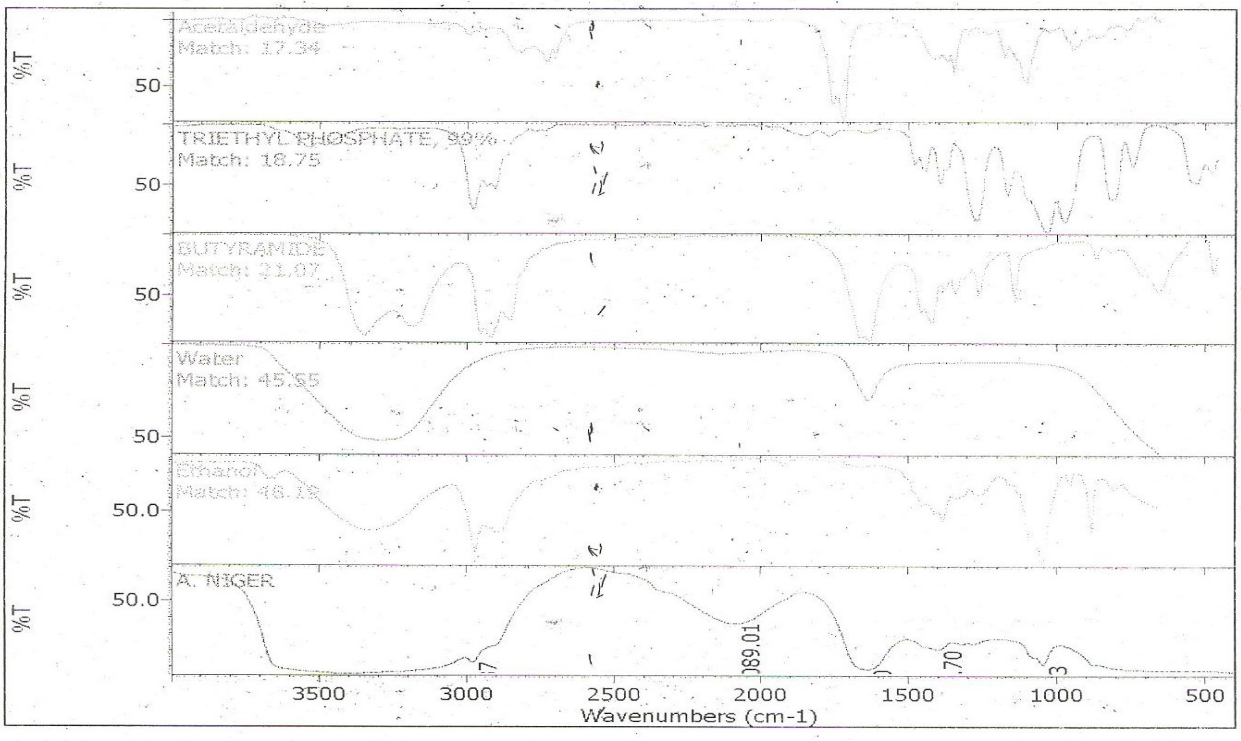
QUALITATIVE CORRELATION COEFFICIENT INFRARED SPECTRUM TEST FOR ETHANOL PRODUCTION FROM *Aspergillus niger*.

Peak finding results for: A. NIGER
Frequency: 406.75 - 4000.00, threshold: 66.997, sensitivity: 50.00
Peak finding result table:

Peak#	1	2	3	4	5	6			
Position	3406.54	2982.37	2089.01	1641.70	1401.70	1044.83			
Height	4.627	11.721	35.629	7.443	19.725	10.227			

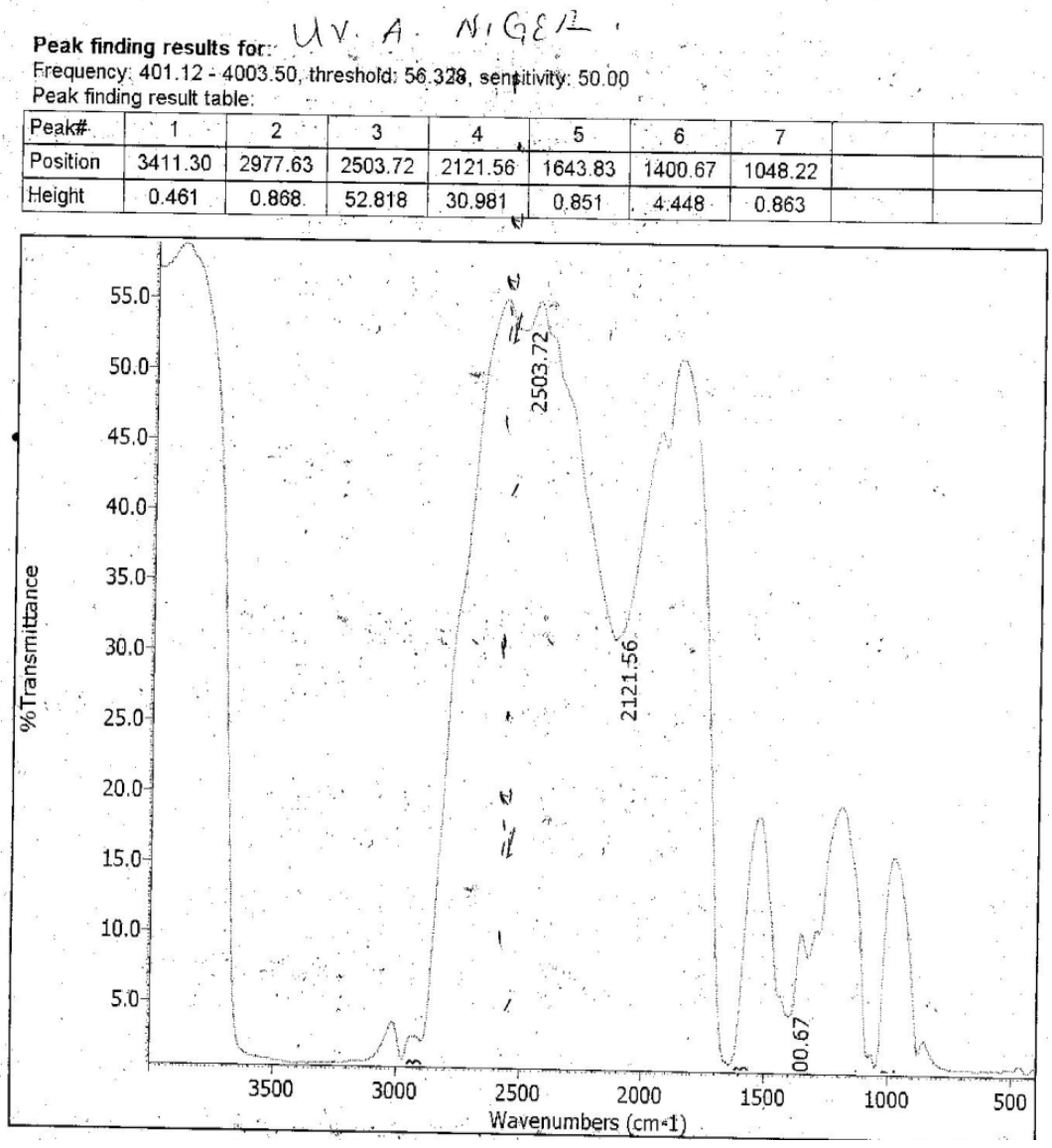
Search results for: A. NIGER
Search algorithm: Correlation coefficient
Search region (cm-1) : 4000.0000 - 400.0000
Table of matches:

#	Index	Match	Compound Name	Library Name
1	6	48.19	Ethanol	Example Library
2	15	45.55	Water	Example Library
3	19	21.07	BUTYRAMIDE	Aldrich Condensed Phase Sample Library
4	27	18.75	TRIETHYL PHOSPHATE, 99%	Aldrich Condensed Phase Sample Library
5	1	17.34	Acetaldehyde	Example Library



APPENDIX V

QUALITATIVE INFRARED SPECTRUM TEST FOR ETHANOL PRODUCTION FROM UV IRRADIATION OF *Aspergillus niger*.



APPENDIX VI

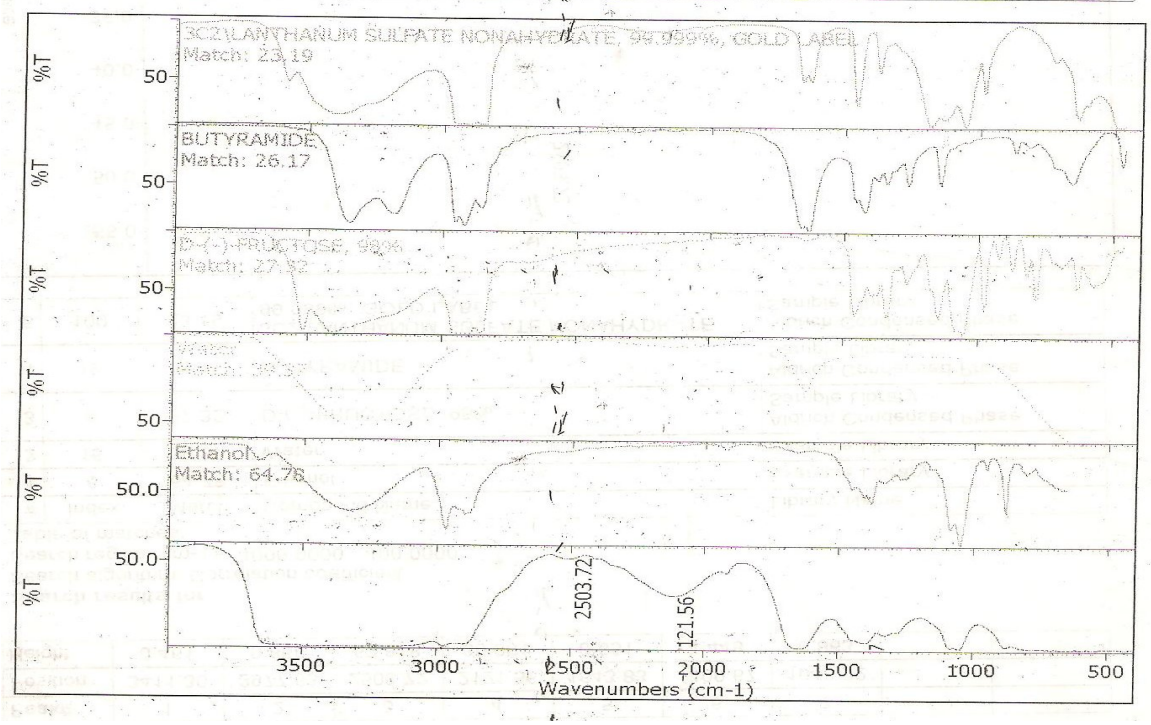
QUALITATIVE CORRELATION COEFFICIENT INFRARED SPECTRUM TEST FOR ETHANOL PRODUCTION FROM UV *Aspergillus niger*.

Peak finding results for: UV A. NIGER
Frequency: 401.12 - 4003.50, threshold: 56.328, sensitivity: 50.00
Peak finding result table:

Peak#	1	2	3	4	5	6	7		
Position	3411.30	2977.63	2503.72	2121.56	1643.83	1400.67	1048.22		
Height	0.461	0.868	52.818	30.981	0.851	4.448	0.863		

Search results for:
Search algorithm: Correlation coefficient
Search region (cm-1) : 4000.0000 - 400.0000
Table of matches:

#	Index	Match	Compound Name	Library Name
1	6	64.78	Ethanol	Example Library
2	15	39.37	Water	Example Library
3	6	27.32	D-(-)-FRUCTOSE, 98%	Aldrich Condensed Phase Sample Library
4	19	26.17	BUTYRAMIDE	Aldrich Condensed Phase Sample Library
5	100	23.19	3C2\LANTHANUM SULFATE NONAHYDRATE, 99.999%, GOLD LABEL	Aldrich Condensed Phase Sample Library



APPENDIX VII

ABBREVIATION

r = rate of reaction mol/sec

s= substrate concentration in mol/l

K.E= maximal enzyme activity mol/sec

Km= Michaelis constant

V= Volume in litres

X = proportion of substrate converted

q = Volumetric feed of substrate to reactor in l/sec

Y = space time (s)

μ = specific death rate/growth of organism