PRODUCTION OF XYLANASE ENZYME FROM *ASPERGILLUS NIGER* USING SUGARCANE BAGASSE; THE EFFECT OF SUBSTRATE CONCENTRATION

NORHAMLY B. MOHD NOR

A thesis submitted in fulfillment of the
requirement for the award or degree of
Bachelor of Chemical Engineering

Faculty of Chemical and Natural Resources Engineering
University College of Engineering & Technology Malaysia

NOVEMBER 2006

DECLARATION

"I declare that this thesis is the result of my own research expect as cited references. The thesis has not been accepted for any degree and is concurrently submitted in candidature of any degree."

Signature : ……

Name of Candidate : NORHAMLY B. MOHD NOR

Date : 20 NOVEMBER 2006

DEDICATION

Special Dedication to my family members that always love me,
My friends, my fellow colleague
and all faculty members

For all your Care, Support and Believe in me.

Sincerely
Norhamly b. Mohd Nor

ACKNOWLEDGEMENT

In preparing this thesis, I was In contact with many people, researchers, academicians and practitioners. They have contributed towards my understanding and thought during this project development. First of all praise and gratitude to Allah S.W.T for giving me strength went thought so many difficulties to successfully finishing up my project.

In particular, I would like to express my appreciation to my main thesis supervisor, Mr. Wan Mohd Hafizuddin b. Wan Yussof and my co-supervisor, Mr. Rozaimi b. Abu Samah for giving me guidance, advice and motivation. Without their continuous support and guideline, this thesis would not be success.

For my parent especially my beloved mother and father, Mrs. Jahara bte. Hj. Ali and Mr Mohd Nor b. Hassan for their love and understanding throughout this research process.

I am also would like to express my appreciation and thanks to all lecturer from Chemical Engineering & Natural Resources (FKKSA) for their support and knowledge in finishing this thesis.

My fellow undergraduate friends should also be recognized for their support and for their time doing discussion to share the knowledge.

ABSTRACT

The use of waste as raw material is important for government economy and natural balance. The purpose of this work is to study the production of xylanase and CMCase enzyme by *Aspergillus niger* in solid state fermentation (SSF) using agricultural residues sugar cane bagasse as substrates. The study is focus on the effect of substrate concentration which is 1 g/L, 3g/L and 5 g/L. All the enzymes were incubating in temperature 37 ^{o}C and pH 5.0 for 30 minutes. The reaction was quenched by cooling on ice and the amount of reducing sugar was measured by dinitrosalicyclic (DNS) acid procedure with slight modification (Miller, 1965). The mixture was then incubated at 100 ^{o}C for 5 minutes. Subsequently, the reaction was stopped by cooling on ice. Distilled water was added into the final volume of 16 ml and the color intensity was measured at wavelength of 575 nm. The concentration of SCB affected the xylanase and CMCase activity. Xylanase and carboxymethylcellulase activity was higher at lower concentration when using 3 g of SCB

ABSTRAK

Penggunaan bahan buangan sebagai sumber mentah adalah penting untuk ekonomi kerajaan dan keseimbanagan alam. Tujuan pembelajaran ini adalah untuk penghasilan enzim xylanase dan carboxymethylcellulase oleh *Aspergillus niger* di dalam penapaian dalam keadaan pepejal menggunakan baki dari sector pertanian; hampas tebu sebagai substrates. Pembelajaran kesan kepekatan substrates tertumpu kepada 1 g/L, 3 g/L dan 5 g/L. Kesemua enzim dieram pada suhu 37 ^{o}C dan nilai pH 5.0 selama 30 minit. Kemudian tindakbalas dihentikan dengan menggunakan ais dan jumlah gula yang dihasilkan akan dikira menggunakan prosedur dinitrosalicyclic acid (DNS) dengan perubahan (Miller, 1965). Campuran tadi kemudiannya dieram pada 100 ^{o}C selama 5 minit Berikutnya, tindakbalas dihentikan dengan menggunakan ais. Air suling ditambah sehingga jumlah isipadu bertambah kepada 16 ml dan perubahan warna diukur pada panjang gelombang 575 nm. Kepekatan SCB mempunyai kesan kepada xylanase dan CMCase aktiviti. Xylanase and carboxymethylcellulase aktiviti adalah tinggi pada kepekatan yang lebih rendah apabila menggunakan 3 g SCB..

TABLES OF CONTENTS

CHAPTER	TITLE	PAGE
	DECLARATION	ii
	DEDICATION	iii
	ACKNOWLEDGEMENT	iv
	ABSTRACT	v
	ABSTRAK	vi
	TABLE OF CONTENT	vii
	LIST OF TABLE	xi
	LIST OF FIGURES	xii
	LIST OF SYMBOL	xiii
1.0	**INTRODUCTION**	**1**
	1.1 Introduction	1
	1.2 Objectives	2
	1.3 Scope	3
	1.4 Problem Statement	3
2.0	**LITERATURE RIVIEW**	**4**
	2.1 Cellulase enzyme	4
	2.1.1 Application of Cellulase	6
	2.1.2 Physical and Chemical Properties	7
	2.1.3 Stability and Storage	7
	2.2 Hemicellulase	9
	2.3 Xylanase	10

	2.3.1 Applications	10
2.4	Carboxymethylcellulase enzyme (CMCase)	11
2.5	*Aspergillus species*	12
	2.5.1 Morphology	12
	2.5.2 Health Effect	13
2.6	Fermentation Basics	14
	2.6.1 Classification of Microorganisms	15
	2.6.2 Microbial Activity	16
2.7	Batch fermentation	17
	2.7.1 Lag Phase	17
	2.7.2 Log Phase	18
	2.7.3 Stationary Phase	18
	2.7.4 Death Phase	18
2.8	Fed Batch Fermentation	19
2.9	Continuous Fermentation	19
2.10	Solid State Fermentation (SSF)	20
2.11	Submerged Fermentation (SmF)	20
2.12	Nutrient Requirements	20
	2.12.1 Carbohydrates	20
	2.12.2 Lipides	21
	2.12.3 Purines and Pyrimidines	21
	2.12.4 Vitamins and Growth Factors	21
	2.12.5 Amino Acids	21
	2.12.6 Nitrogen Sources	22
	2.12.7 Sulfur Sources	22
	2.12.8 Chemical Elements and Inorganic Ions	22
2.13	Sugar cane Waste	22
2.14	Enzyme Inhibition	23
3.0	**MATERIALS AND METHODS**	**27**
3.1	Materials	27
3.2	Methods	28

	3.2.1	Substrate Preparation	28
	3.2.2	Preparation of Broth	28
	3.2.3	Preparation of Agar	28
	3.2.4	Sub Culturing of *Aspergillus niger*	29
	3.2.5	Preparation of Spore Suspension	29
	3.2.6	Preparation of Basic Mandel Media	30
	3.2.7	Preparation of Inoculums	30
	3.2.8	Cultivating in Erlenmeyer flask	31
	3.2.9	Research on Growth and Yield of Cellulase	31
3.3		Enzyme assay	31
	3.3.1	Preparation of DNS reagent	31
	3.3.2	Standard Calibration Curve	31
	3.3.3	DNS Reagent Test	32
	3.3.4	Substrate Preparation	32
	3.3.5	Preparation of Buffer Solution	33
	3.3.6	Xylanase Assay	33
	3.3.7	Carboxymethylcellulase Assay	33
4.0		**RESULT AND DISCUSSION**	**35**
4.1		Standard Calibration Curve	35
	4.1.1	Standard Calibration Curve for Xylanase Enzyme	35
	4.1.2	Standard Calibration Curve for carboxymethylcellulose enzyme	36
4.2		Effect of Different Concentration	37
	4.2.1	Enzyme Activity of Xylanase	37
	4.2.2	Enzyme Activity of CMCase	39
4.3		Influence in varying SCB concentration	40
5.0		**CONCLUSION AND RECOMMENDATIONS**	**42**
5.1		Conclusion	42
5.2		Recommendations	43

REFERENCES **44-46**

APPENDIX **47-50**

LIST OF TABLES

TABLE	TITLE	PAGE
2.1	Example of commercial available enzymes produced in recombinant filamentous fungi.	8
2.2	Product produces by microbial activity	14
2.3	Products being addressed by recombinant technology	14
2.4	Thermal characterization of microorganisms	16
3.1	Chemicals for preparation of Mandel Media	30
3.2	Concentration diluted procedures	32
4.1	Xylose Absorbance at Different Concentration	35
4.2	Glucose Absorbance at Difference Concentration	36
4.3	Xylanase enzyme for different concentration	37
4.4	Carboxymethylcellulase enzyme for different concentration	38

LIST OF FIGURES

FIGURE	TITLE	PAGE
2.1	Cellulase types and action	5
2.2	Cellulase mechanism	5
2.3	General classification of microorganism	15
2.4	Growth curve of a bacterial culture	17
2.5	Lock Key Theory Competitive Inhibitors	24
2.6	Non-Competitive Inhibition	25
2.7	Substrate Inhibition	26
4.1	Standard Calibration curve for xylose	36
4.2	Standard calibration curve for glucose	37
4.3	Enzyme activity of xylanase at different concentration of substrate	38
4.4	Enzyme activity of carboxymethylcellulase at different concentration of substrate	40

LIST OF SYMBOLS

^{o}C	degree Celsius
pH	potential hydrogen
m	Micro
ml	milliliters
U	amount of enzyme release 1 mol xylose /minutes/milliliters
%	percentage
rpm	radius per minutes
w	weight
v	volume
h	hours
min	minutes
g	gram
M	molar
L	liter

CHAPTER 1

INTRODUCTION

1.1 Introduction

The ability of some microorganism to metabolize lignin and hemicellulase make them potentially important to take advantage of vegetable residue. Agricultural and agro-industrial waste, like sugarcane bagasse (a fibrous residue of cane stalks left over after the crushing and extraction of the juice from the sugar cane), wheat bran, rice peel, corn straw, corncob, fruit peels and seeds, effluents from paper industry and orange bagasse, have increased as results of industrialization, becoming a problem regarding space for disposal and environmental pollution. However, those residues represent and alternatives source for the microbial growth aiming the production of biomass or enzymes.

Hemicelluloses and cellulose represent more than 50% of the dry weight of agricultural residues. They can be converted into soluble sugars either by acid or enzymatic hydrolysis, so they can be used as a plentiful and cheap source of renewable energy in the world. For many residues, xylan is the main component of the hemicellulose fraction. It is degraded by xylanase produced by fungus, bacteria, seaweed, protozoa, gastropod and arthropods. These enzymes also have application in maceration of vegetables, in clarification of juices and wines, in extraction of juices, scent and pigments, in biobleaching of pulp.

Amongst xylanolytic microorganism, filamentous fungi have been more extensively studied, and the genus *Aspergillus* has been shown to be an efficient producer of xylanases on an industrial scale (Haltrich et al., 1996). Several different species of *Aspergillus* have been reported to produce xylanase, including *A. niger*, *A. ochraceus, A. oryzae, A. awamori A. tamari* and *A. fumigatus* (Bailey and Poutanen, 1989; Haltrich et al., 1996; Kadowaki et al., 1997; Siedenberg et al., 1998)

The complete degrading of cellulose by fungi is made by a cellulotic enzyme system. The role and the action mechanism of the component of the system have been the center of many studies for the last 3 decades. It has been established that there are three main types of enzymes found in the cellulases system that can degrade the cellulose: exo-β-1,4-glucanase, endo-β-1,4-glucanase and β-glucosidase. Studies have shown that the endoglucanase act internally on the chain of cellulose cleaving β-linked bonds liberating non-reducing ends, and exoglucanase act removing cellobiose from this non-reducing end of cellulose chain. Finally, β-glucosidue completes the saccharification by splitting cellobiose and small cello-oligossaccharification into glucose molecule.

The purpose of this work was to study the production of xylanase and the endoglucanase CMCase (carboxymethylcellulase) from *Aspergillus niger* strains in solid fermentation (SSF) using agricultural residues which is sugarcane bagasse as substrates in different concentrations.

1.2 Objectives

To produce xylanase enzyme and endoglucanase CMCase (carboxymethylcellulase) from *Aspergillus niger* using sugarcane bagasse as substrate.

1.3 Scope

To study the effect of substrates concentration in 1 g/L, 3 g/L and 5 g/L to find which concentration that can produce highest enzyme activity.

1.4 Problem Statement

The plantation area is 9,215 acres in Perlis and 25,245 acres in Kedah. That gives the total plantation area in Malaysia to be nearly 34,500 acres. On average, about 32 % of bagasse is produced from every tonne of sugar cane processed. The amount of sugar cane processed in 2002 is about 1,111,500 tonnes [1]. Thus, the amount of bagasse produced is 355,680 tonnes. Sugar cane bagasse was one of the largest cellulosic agro-industry by-products wastes that had been increased in Malaysia even in another country now. Otherwise, it can make air pollution if the bagasse were burn to the environment.

CHAPTER 2

LITERATURE REVIEW

2.1 Cellulase enzyme

Cellulase is an enzyme complex which breaks down cellulose to beta-glucose. It is produced mainly by symbiotic bacteria in the ruminating chambers of herbivores. Aside from ruminants, most animals (including humans) do not produce cellulase, and are therefore unable to use most of the energy contained in plant material

Cellulase refers to family of enzymes which act in concert hydrolyze cellulose. Cellulose is widely distributed throughout the biosphere and are most manifest in fungal and microbial organisms.

Three general types of enzymes make up the cellulose complex. Endocellulase breaks internal bonds to disrupt the crystalline structure of cellulose and expose individual cellulose polysaccharides chains. Exocellulase cleaves 2-4 units from the ends of the exposed chains. Cellobiase or beta-glucosidase hydrolyses the endocellulase product into individual monosaccharides. Figure 2.1 show the cellulase types and action.

Within the above types there are also progressive and non-progressive cellulose will interact once then disengage and engage another polysaccharide strand

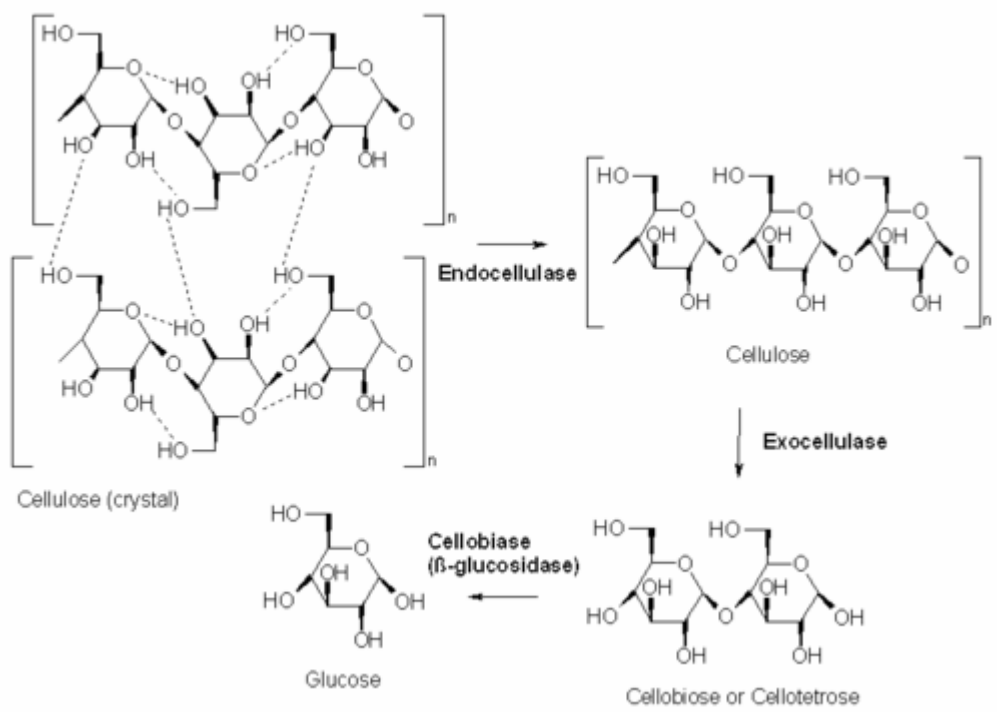

Figure 2.1: Cellulase types and action

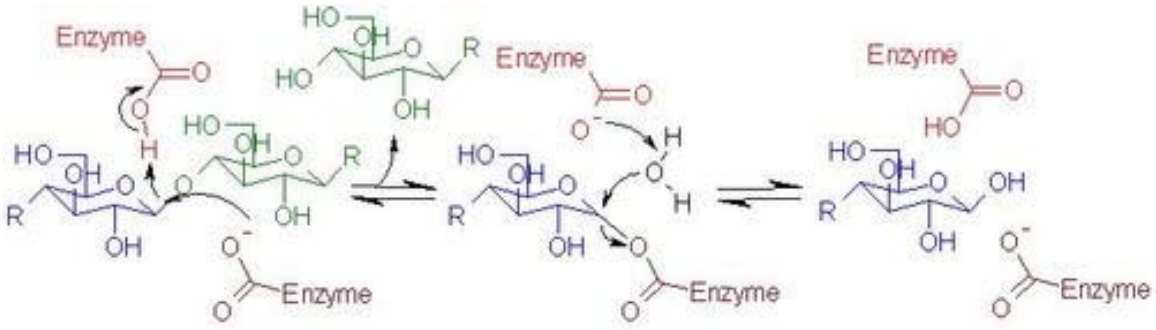

Figure 2.2: Cellulase mechanism

a) Endocellulase

These are capable of hydrolyzing the β(1,4) bonds randomly along the cellulose chain. Source: *Tricoderma reesei, T viride, Aspergillus niger*

b) Exocellulase

These enzymes cleave off glucose molecule from one end of the cellulose strand. Source: *Tricoderma reesei*

It has been found that enzyme preparations containing only endocellulases have little effect on native cellulose. On the other hand those containing both endo and exocellulases will cause significant degradation of native cellulose. Thus, the endo and exocellulases appear to work in a synergestic or cooperative manner on native cellulose.

c) Exo-Cellobiohydrolase

It hydrolyze β(1,4) bonds in cellulose to release cellobiose from the non reducing end of the chains. Source: *Tricoderma reesei, T. viride*

d) Cellobiase: It hydrolyzes the β(1,4) bonds in cellubiose, giving two molecules of glucose. Source: *Aspergillus niger, T. viride, S. cerevisae*

Tricoderma reesei has an extensively studied cellulase enzyme complex. This complex converts crystalline, amorphous and chemically derived celluloses quantitatively to glucose. The vital characteristics of this cellulase complex are the system is multienzymatic, at least three enzyme components are both physically and chemically distinct. Also all three components play essential roles in the hydrolysis of cellulose to glucose.

2.1.1 Application of cellulase

Cellulase digests fiber. It helps remedy digestive problems such as malabsorption. It is a very important enzyme because the human body cannot produce it on its own. Also helps in the breakdown of plant wall (cellulose, and increase the overall efficiency of binding excess cholesterol and toxins in the intestines for removal. It may be beneficial

for food and environmental allergies, drug withdrawal, cell detox, colon cleaning and pain syndromes, yeast infections, gas, bloating, acute food allergies, facial pain or paralysis

Cellulase is used for commercial food processing in coffee. It performs hydrolysis of cellulose during drying of beans. It is used in the fermentation of biomass into biofuels, although this process is relatively experimental at present. Cellulase is used as a treatment for Phytobezoars, a form of cellulose bezoar found in the human stomach. Otherwise, cellulase is used in animal healthcare as a feed supplement for better FCR and Milk yeild Inhancer in Poultry and Cattle industry. Cellulase is used in textile industry as a fading agent.

2.1.2 Physical and Chemical properties

Most cellulase studied has similar pH optima, solubility and amino acid composition. Thermal stability and exact substrate specificity may vary. However, it should be remembered that cellulase preparations generally contain other enzymatic activates besides cellulase, and these may effect the properties of the preparations. The optimum pH for cellulase preparations are effective between pH 3 and but generally lay between pH 4 and 5. The optimum temperature is 30 to 50 ^{o}C (Potrykus, 1986)

2.1.3 Stability and Storage

The activity of cellulase preparation has been found to be completely destroyed after 10 to 15 minutes at 80^{o}C. Solutions of cellulase at pH 5 to 7 are stable for 24 hours at 4 ^{o}C. These products should be stored at 4 ^{o}C, in dry place in tightly closed containers. If stored in this manner, lyophilized preparation is stable for several months without significant loss of activity (Potrykus, 1986) [2]

Table 2.1: Examples of commercially available enzymes produced in recombinant filamentous fungi. Examples of specific applications are listed below the table. (Data derived from the Association of Manufactures of Fermentation Enzyme Products, 2001)

Enzyme	Production (host) organism	Source organism	Industry in which applied
Aminopeptidase	*T. reesei* or *T. longibrachiatum*	*Aspergillus* sp.	Food [a] and feed [b]
Catalase	*A. niger*	*Aspergillus* sp.	Food [c, d]
Cellulase	*T. reesei* or *T. longibrachiatum*	*Trichoderma* sp.	Textile [e] and feed [f]
	A. oryzae	*Humicola* sp., *Thielavia* sp., *Myceliopthora* sp.	Textile [d], detergent and pulp and paper
α-Galactosidase	*A. oryzae*	*Aspergillus* sp.	Feed [e]
ß-Glucanase	*T. reesei* or *T. longibrachiatum*	*Trichoderma* sp.	Textile [e, g] and feed [f]
Glucoamylase	*A. niger*	*Aspergillus* sp.	Food [h, i]
Glucose oxidase	*A. niger*	*Aspergillus* sp.	Food [d, j]
Laccase	*A. oryzae*	*Myceliopthora* sp. *Polyporus* sp.	Textile [k]
Lactase	*A. oryzae*	*Aspergillus* sp.	Food [l]
Lipase	*A. oryzae*	*Candida* sp., *Fusarium* sp., *Rhizomucor* sp. or *Thermomyces* sp.	Food [d, m], textile [n], detergent [o], leather [p], pulp and paper [q]
Mannanase	*T. reesei* or *T. longibrachiatum*	*Trichoderma* sp.	Feed [f]
Pectin lyase	*A. niger*, *T. reesei* or *T. longibrachiatum*	*Aspergillus* sp.	Food [r], feed [f] and textile [s]
Pectinase	*T. reesei* or *T. longibrachiatum*	*Aspergillus* sp.	Food [r], feed [f] and textile [s]
Pectinesterase	*A. niger*, *A. oryzae* or *T. reesei*	*Aspergillus* sp.	Food [r], feed [f] and textile [s]
Phospholipase A	*T. reesei* or *T. longibrachiatum*	*Aspergillus* sp.	Food [d, t], feed [f] and textile
Phospholipase B	*T. reesei* or *T. longibrachiatum*	*Aspergillus* sp.	Food [d, t] and feed [f]
Phytase	*A. niger*, *A. oryzae*, *T. reesei* or *T. longibrachiatum*	*Aspergillus* sp. or *Peniophora* sp.	Feed [f]
Protease	*A. niger*, *A. oryzae*	calf stomach, *Aspergillus* sp. or *Rhizomucor* sp.	Food [m] and leather [p, u]
Pullulanase	*T. reesei* or *T. longibrachiatum*	*Hormoconis* sp.	Food [d] and feed [f]
Xylanase	*A. niger*, *A. oryzae* *T. reesei* or *T. longibrachiatum*	*Aspergillus* sp. *Thermomyces* sp. *Actinomadura* sp.	Food [d] and feed [f]

[a] reduce bitterness in protein digests, cheese, egg meat and milk industries
[b] pet food (to increase palatability)
[c] cheese, egg, starch, fat, sugar, industries, cold sterilisation of milk
[d] baking applications
[e] stone-washing of denim
[f] increasing the nutritional value of pig/chicken feed (e.g. cellulases and ß-glucanase for barley and oats, xylanase for rye, α-galactosidase & mannanase for legumes, phytase and phospholipases for phosphate release)
[g] treatment of hemp, jute, flax, etc. for textile and rope manufacture
[h] high fructose/glucose syrup production
[i] clarifying juices, wine or beer
[j] stabilizing foods (e.g. egg whites, mayonaise) and beverages (e.g. fruit juice, beer), improving flour
[k] bleaching of dyes such as indigo
[l] dairy products (e.g. yoghurt) and production of lactose syrup
[m] cheese ripening and flavour
[n] increase wettability
[o] stain removal
[p] during soaking and degreasing
[q] removal of pitch
[r] fruit processing
[s] bioscouring (removal of non-cellulose matter from cottons)
[t] production of lyso-lecithin (e.g. for margarine)
[u] liming, deliming and bating, softening

2.2 Hemicellulase

Hemicellulase is an enzyme that breaks down hemicellulose. Hemicellulose catagorizes a variety of polysaccharides that are more complex than sugars and less complex than cellulose, that are found in plant walls.

It is a mixture of enzymes which can hydrolyze the indigestible components of plant fibers. Since humans lack the endogenous enzymes required to digest plant fibers, the supplementation of hemicellulase provides humans with an additional source of nutrition and reduces the bulking effect of fibrous foods. Scientific evidence suggests that carbohydrolytic enzymes, such as hemicellulase, can be useful supplements for digestive support and general nutritional support.

The hemicellulase will attack on the hemicellulose fiber to liberate smaller fragments of cellulose which is further attacked by exo-cellulase to liberate glucose.

The influence of saccharification with added hemicellulases on non-starch carbohydrates of regular rye as well as on some quality factors of mashes and stillages were examined. Laboratory experiments showed the significant influence of the type of pretreatment and hemicellulases enrichment on the dynamic of saccharification and efficiency of ethanol fermentation. It was concluded that the increase of ethanol yield as a result of hemicellulase supplementation is not connected with pentosans fermentation but with the increased availability of hexose (starch) bound with pentose chain for amylolytic enzymes. Because of the high yield of rye hybrids, this grain might be a potential source of raw materials in the distillery industry.

Hemicellulase is widely used in animal feed supplement, baking industry and dietary supplement. [3]

2.3 Xylanase

The enzyme named xylanase deconstructs plant structural material by breaking down hemicellulose, a major component of the plant cell wall. Plant cell walls are necessary to prevent dehydration and maintain physical integrity. They also act as a physical barrier to attack by plant pathogens. In nature, some plant consumers or pathogens use xylanase to digest or attack plants. Many microorganisms produce xylanase, but mammals do not. Some herbivorous insects and crustaceans also produce xylanase.

The xylanase will attack on the xylan or pentose polysaccharide from plant fiber to liberate xylose which is readily absorbed in the intestine.

2.3.1 Applications

The poultry feed mainly consist of mainly non starch polysaccharides (NSP) from plant origin. The digestive system of poultry birds has insufficient fiber digesting enzymes hence the absorption of all the nutrients from feed is not observed. The enzymes present in SEBfeed xylanase will degrade this NSP material and will liberate ready source of energy in the form of reducing sugars which is absorbed easily from the intestine of the birds and helps in increasing the weight of the bird.

The paper industry uses vast amounts of naturally occurring raw materials, the most important of which are cellulose fiber, china clay or chalk and starch. Cellulose fiber and starch need to be modified by mechanical, chemical or biochemical techniques for the paper making process which can be achieved with xylanase.

Xylanase used in wet-processing treatments of cotton, such as scouring and stone-washing in the textile industry and the possibilities of enzyme treatment for other fibers, especially wool, are under extensive research.

The enzyme xylanase has long been used by the cereal industry to standardise and improve flour performance. Until now, the performance of microbial xylanases has varied from flour to flour, due to the natural content of xylanase inhibitors in the wheat. It enhances the raising of the bread dough when baked. Also useful for clarification of non citrus fruit juice.

Xylanase is produced by many microorganisms but not mammals. Xylanase is used to break down plants as well as the sugar xylose. It has been found in many different fungi and bacteria. The enzyme is from *Trichoderma sp* and consists of 190 amino acids. Xylanase belongs to the glucanase enzyme family, which is characterized by their ability to break down various xylans to produce short-chain xylo-oligosaccharides. Enzymes are important to proper nutrition and proper digestion. Without the proper levels of enzymes from foods or supplements, you are susceptible to excessive gas and bloating, diarrhea, constipation, heartburn, low energy, acne, arthritis, allergies, insomnia, high cholesterol and many other discomforts. [4]

2.4 Carboxymethylcellulase enzyme (CMCase)

Carboxymethylcellulase is a derivative of cellulose formed by its reaction with alkali and chloroacetic acid. The CMCase structure was based on the β-(1,4)-D-glucopyranose polymer of cellulose (Chaplin, 2004). Different preparations may have different degrees of substitution, but it is generally in the range 0.6 to 0.95 derivatives per monomer unit.

CMCase molecules are somewhat shorter, on average, than native cellulose with uneven derivatization giving areas of high and low substitution. This substitution is mostly 2-O- and 6-O-linked, followed in order of importance by 2,6-di-O- then 3-O-, 3,6-

di-O-, 2,3-di-O- lastly 2,3,6-tri-O-linked (Chaplin, 2004). It appears that the substitution process is a slightly cooperative (within residues) rather than random process giving slightly higher than expected unsubstituted and trisubstituted areas. CMCase molecules are most extended (rod-like) at low concentrations but at higher concentrations, the molecules overlap and coil up and then, at high concentrations, entangle to become a thermo reversible gel. Increasing ionic strength and reducing pH both decrease the viscocity as they cause the polymer to become more coiled (Chaplin, 2004) [5]

2.5 A*spergillus species*

The genus *aspergillus* is important economically, ecologically and medically. It is cosmopolitan and ubiquitous in nature with over 185 species. The fungus owes its name from the Latin word *aspergillum*, to the resemblance of its conidiophores to a device used to sprinkle holy water. Members of this genus have been recovered from a variety of habitats but are especially common as saprobes on decaying vegetation, soil, stored food, feed products and a variety of building materials found in indoor environments (e.g. wallboard, carpet, ceiling tiles etc.).

2.5.1 Morphology

Aspergilli colonies are downy to powdery in texture. The surface color varies depending on the species. Microscopically, the asexual fruiting structure of *Aspergillus* species includes a long stipe (conidiophore), a rounded head (vesicle), and flask shape structures (phialides) from which spores (conidia) are formed. The conidia (2-5 μm in diameter) may be spherical to elongate and form chains which may radiate (e.g., in *Aspergillus versicolor*) or form themselves into compact columns (e.g., *Aspergillus fumigatus* and *Aspergillus nidulans*). Some species may form masses of thick-walled cells called "hülle cells". These cells are often visible on tape lift samples and are especially useful as an additional character to confirm the presence of *Aspergillus*

growth. The morphological characters of *Aspergillus* species are more distinguishable in culture, which makes identification much easier.

On spore trap samples, *Aspergillus* spores are very similar to *Penicillium* spores. This is why they are clumped together as "*Penicillium/Aspergillus* types". We do the same reference on tape lifts because, sometimes, we only see masses of spores. This may indicate that growth is old and the underlying structures have disintegrated, or that the tape lift has been collected with inadequate pressure or with tape that has lost its stickiness

2.5.2 Health Effect

Approximately 20 species of *Aspergillus* have been reported as causative agents of opportunitic infections in human. *Aspergillus fumigatus* is the most important opportunist and is commonly encountered in hospitals as well as other environments. Other species, such as *A. flavus, A. Terreus, A. niger* and *A. nidulan* can also cause human infections. *A. fumigatus* prefers high temperature and its optimum temperature for growth is about 37oC (normal human body temperature). However, it can grow at temperature from 20 to 50oC. In a susceptible host, conidia germinate into hyphae, the invasive form of the disease. Invasive *aspergillosis* rarely occurs in people with competent immune system.

Aspergillus can also grow in the mucous that accumulates in the lungs of asthmatic and children with cystic fibrosis. This disease is called allergic bronchpulmonary *aspergillosis*. People who have this condition produce antibodies against proteins in airbone *aspergillus* spores.

Another noteworthy species is *aspergillus nidulans*. Its rapid growth on defined media, compact colony morphology, uninucleate conidia, and several other complex genetic traits make this fungus a suitable experimental system for the genetic analysis of gene regulation.

2.6 Fermentation Basics

Fermentation is the term used by microbiologists to describe any process for the production of a product by means of the mass culture of a microorganism.

The product can either be the cell itself: referred to as biomass production. A microorganisms own metabolite: referred to as a product from a natural or genetically improved strain. See Table 2.2. A microorganism's foreign product: referred to as a product from recombinant DNA technology or genetically engineered strain, i.e. recombinant strain. See Table 2.3

Table 2.2: Product produces by microbial activity

Amino acids	Lipids
Antibacterial agents	Nucleotides and precursors
Antifungal agents	Organic synthesis intermediates
Antiprotozoal agents	Pharmaceutical significant compounds
Carbohydrates	Plant growth factors
Dyes and cosmetics	Steroids
Enzymes	Toxins
Foods	Vitamins and coenzymes

Table 2.3: Products being addressed by recombinant technology

Human therapeutics
Enzymes
Amino acids

2.6.1 Classification of Microorganisms

The kingdom Protista comprises unicellular organisms capable of self duplication or of directing their own replication. Prokaryotes do not possess a true nucleus or a nuclear membrane, whereas eukaryotes have a nucleus enclosed within a distinct nuclear membrane. The non-cellular protists do not undergo self-replication; instead they direct their reproduction within another cell temed the host.

The criteria used for the classification of microorganisms include morphology, reproductive mechanisms, pigment presence, means of motility, physiology and structural features. (Figure 2.3)

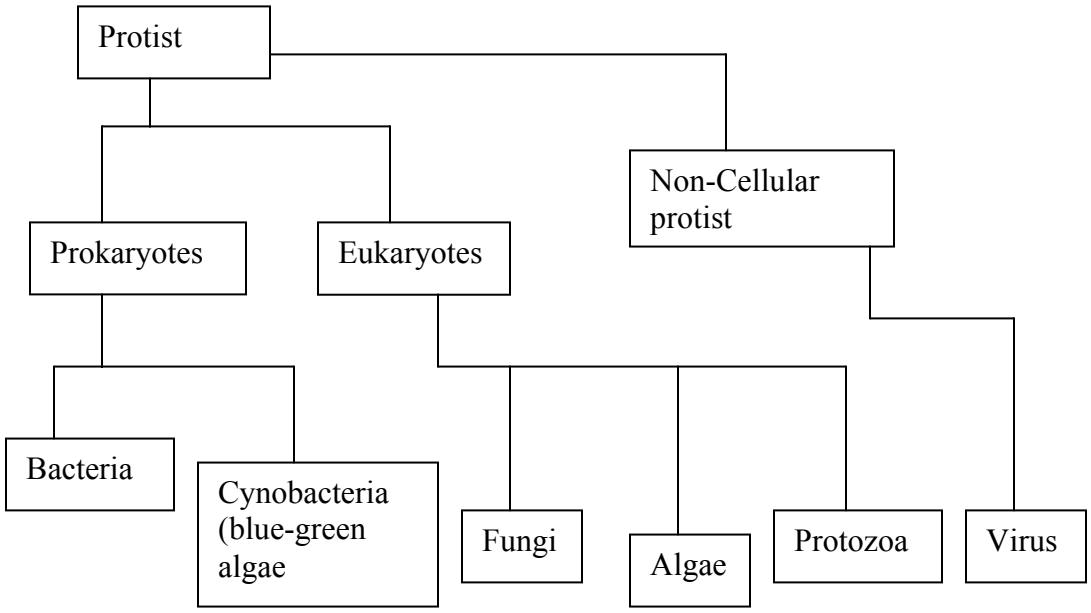

Figure 2.3: General classification of microorganism

2.6.2 Microbial Activity

Microorganisms in the process of self replication produce numerous complex macromolecules from about 100 different monomer units. In the biochemical pathways to achieve a bacterial cell uses well over 1000 different enzymes and a eukaryotic cell may employ twice as many.

The biochemical metabolism can be divided into two broad classes. First is the anabolic pathways (anabolism) synthesize the complex molecules and their intermediate precursors, another one is the catabolic pathways (catabolism) that supply the energy needed for the anabolic processes. These two divergent activities are closely linked.

Microorganisms that carry out their metabolism using oxygen are referred to as aerobic microorganisms. Some microorganisms can substitute nitrate, others sulfate or ferric ion, for oxygen and thus grow in the absence of oxygen. These microorganisms are referred to as anaerobic.

Microorganisms can be classified according to the lowest temperatures at which significant growth occurs. See Table 2.4

Table 2.4: Thermal characterization of microorganisms

Classification (^{o}C)	Minimum Growth Temperature
Psychrophiles	< 20
Mesophiles	20 – 45
Thermophiles	45 - 60

2.7 Batch Fermentation

A batch fermentation can be considered to be a closed system. At time t=0 the sterilized nutrient solution in the fermentor is inoculated with microorganisms and incubation is allowed to proceed. In the course of the entire fermentation, nothing is added, except oxygen (in case of aerobic microorganisms), an antifoam agent, and acid or base to control the pH. The composition of the culture medium, the biomass concentration, and the metabolite concentration generally change constantly as a result of the metabolism of the cells.

After the inoculation of a sterile nutrient solution with microorganisms and cultivation under physiological conditions, four typical phases of growth are observed as indicated in Figure 2.4.

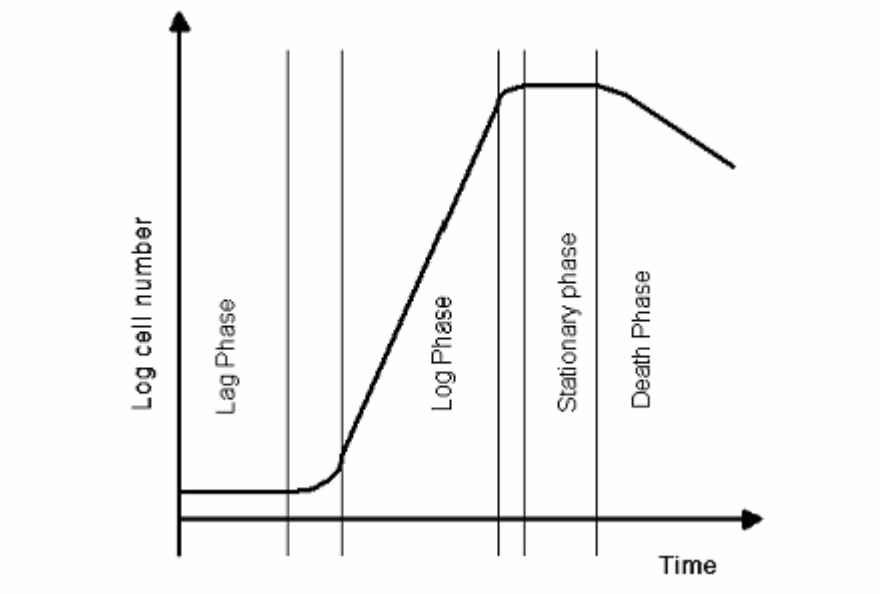

Figure 2.4: Growth curve of a bacterial culture.

2.7.1 Lag phase

Physicochemical equilibration between microorganism and the environment following inoculation with very little growth

2.7.2 Log phase

By the end of the lag phase cells have adapted to the new conditions of growth. Growth of the cell mass can now be described quantitatively as a doubling of cell number per unit time for bacteria and yeast's, or a doubling of biomass per unit time for filamentous organisms as fungi. By plotting the number of cells or biomass against time on a semi logarithmic graph, a straight line results, hence the term log phase. Although the cells alter the medium through uptake of substrates and excretion of metabolic products, the growth rate remains constant during the log phase. Growth rate is independent of substrate concentration as long as excess substrate is present.

2.7.3 Stationary phase

As soon as the substrate is metabolized or toxic substances have been formed, growth slows down or is completely stopped. The biomass increases only gradually or remains constant during this stationary phase, although the composition of the cells may change. Due to lysis, new substrates are released which then may serve as energy sources for the slow growth of survivors. The various metabolites formed in the stationary phase are often of great biotechnological interest.

2.7.4 Death phase

In this phase the energy reserves of the cells are exhausted. A straight line may be obtained when a semi logarithmic plot is made of survivors versus time, indicating that the cells are dying at an exponential rate. The length of time between the stationary phase and the death phase is dependent on the microorganism and the process used. The fermentation is usually interrupted at the end of the log phase or before the death phase begins.

2.8 Fed batch fermentation

In the conventional batch process just described, all of the substrate is added at the beginning of the fermentation. An enhancement of the closed batch process is the fed-batch fermentation. In the fed-batch process, substrate is added in increments as the fermentation progresses. In the fed-batch method the critical elements of the nutrient solution are added in small concentrations at the beginning of the fermentation and these substances continue to be added in small doses during the production phase.

2.9 Continuous Fermentation

In continuous fermentation, an open system is set up. Sterile nutrient solution is added to the bioreactor continuously and an equivalent amount of converted nutrient solution with microorganisms is simultaneously taken out of the system. In the case of a homogeneously mixed bioreactor we refer to a chemostat or a turbidistat. In the chemostat in the steady state, cell growth is controlled by adjusting the concentration of one substrate. In the turbidistat, cell growth is kept constant by using turbidity to monitor the biomass concentration and the rate of feed of nutrient solution is appropriately adjusted.

2.10 Solid state fermentation (SSF)

The fermentation involved solid in absence (or near absence) of free water; however, substrate must possess enough moisture to support growth and metabolism of microorganism (Pandey, 1992). SSF offers numerous opportunities in processing of agro-industrial residues. This is partly because solid-state processes have lower energy requirements, produce lesser waste water and environmental-friendly as they resolve the problem of solid wastes disposal

2.11 Submerged Fermentation (SmF)

The fermentation involving solids in presence of liquid. Principle of SmF is use by dissolved substrates or solid substrates suspended in large amount of water. In liquid environments, fungi grow as pellets or free mycelia, depending n the genotype of the strain and culture conditions. Each form has its own characteristics, which will greatly affect the process yields, and attempts have been made to manipulate morphology in order to achieve increased productivity.

2.12 Nutrient Requirements

All microorganisms need for their microbial activity the presence of several nutrients.

2.12.1 Carbohydrates

Carbohydrates are capable of being used by all microorganisms, although in no case is there an absolute requirement for this group of organic compounds. Glucose is the most readily metabolized sugar. Most fungi can use disaccharides.

2.12.2 Lipides

Microbial requirements for steroids and long-chain fatty acids can be summarized as follows. Long-chain fatty acids like linoleic acid and oleic acid are required for bacteria and fungi. Generally, steroids, other than cholesterol, are not required or utilized by microorganisms. In all fungi, and including yeast, ergosterol is a nutritional requirement.

2.12.3 Purines and pyrimidines

It is generally only in bacteria that cases of purine and pyrimidine metabolism have been reported. Algae do not utilize these compounds at all.

2.12.4 Vitamins and growth factors

There is considerable species variation in the requirements of vitamins and related factors by other microorganisms. Generally, vitamins A, C, D, and K are not necessary for growth.

2.12.5 Amino acids

Amino acids are not generally required by algae, although several algae species are capable of utilizing them. Species of other microorganisms are capable of utilizing all amino acids, except for yeast's, where there is no evidence of critrulline being used. It is usually the L-form of the acids that are biologically active but, unlike higher animals, some bacteria can also utilize the D-amino acids.

2.12.6 Nitrogen sources

It should be stressed that not all species require or utilize these compounds but rather that some species have been identified that are able to utilize these compounds. Fungi require ammonia, nitrate and nitrite.

2.12.7 Sulfur sources

Some species of yeast's can utilize elemental sulfur and sulfate. Generally yeast's do not require or utilize sulfur containing organic compounds. Bacteria require glutathione and thio-acetic acid while yeast's require sulphonic acid amides, thioacetate, thiocarbonate, thioglycolate and glutathione.

2.12.8 Chemical elements and inorganic ions

Mineral nutrients required by microorganisms are species dependent but consists generally of Fe, K, Mg, Mn. Sometimes S, N, Ca, Co, Cu, P, Zn is required.

2.13 Sugar cane Waste

Commonly known as sugar cane bagasse (SCB), is a fibrous residue of cane stalks left over after the crushing and extracton of the juice from the sugar cane, is one of the largest cellulosic agro-industrial by-products (Pandey, 1992)

Bagasse composition consists of approximately 50% cellulose and 25% each of lignin and hemicelluloses. Chemically, bagasse contains about 50% a-cellulose, 30%

pentosans and 2.4% ash. Bagasse offer numerous advantages in comparison to other crop residue because of its low ash content such as rice straw and wheat straw, which have 17.5% and 11.0%, respectively, ash contents, for usage in bioconversion processes using microbial cultures (Pandey, 1992)

In recent years, there has been an increasing trend towards more efficient utilization of agro-industrial residues, including sugar cane bagasse. Sugar cane bagasse is been reported utilize as a raw material for several processes and products. These include products based on fermentation, which is what this research has chosen bagasse as the raw material. Enzyme production is one of the significant applications of bagasse. Bagasse can also use for the production of biofuel (ethanol). However, processes involving bagasse for ethanol production do require it in substantial quantity. Processes such as production of enzymes and other products (e.g drugs) utilizing bagasse as solid substrate or support would need relatively a small fraction of total bagasse (Pandey, 1992)

2.14 Enzyme Inhibiton

Enzyme inhibitors are substances, altar the catalytic action of the enzyme and consequently slow down or in some cases, stop catalysis. There are three common types of enzyme inhibition which are competitive, non-competitive and substrate inhibition. Most theories concerning inhibition mechanisms are based on the existence of the enzyme-subsrate complex ES.

Competitive inhibition occurs when the substrate and a substance resembling the substrate are both added to enzyme. A theory called the “lock-key theory” of enzyme catalysts can be used to explain why inhibiton occurs. The lock and key theory utilizes the concept of an “active site”. The concept holds that one particular portion of the enzyme surface has a strong affinity for the substrate. The substrate is held in such a way that its conversion to the reaction products is more favorable. If we consider the enzyme

as the lock and the substrate the key, the reaction that will be occur which the key is inserted in the lock and the door is opened. However, when an inhibitor, which resembles the substrate, is present, it will compete with other substrate for the position to open the door. When the inhibitor wins, it gains the lock position but is unable to open the lock. Hence, the observed reaction is slowed down because the inhibitor occupies some of the available enzyme sites. If a dissimilar substance which does not fit the site is present, the enzyme will reject it and accept the substrate, the reaction proceeds normally.

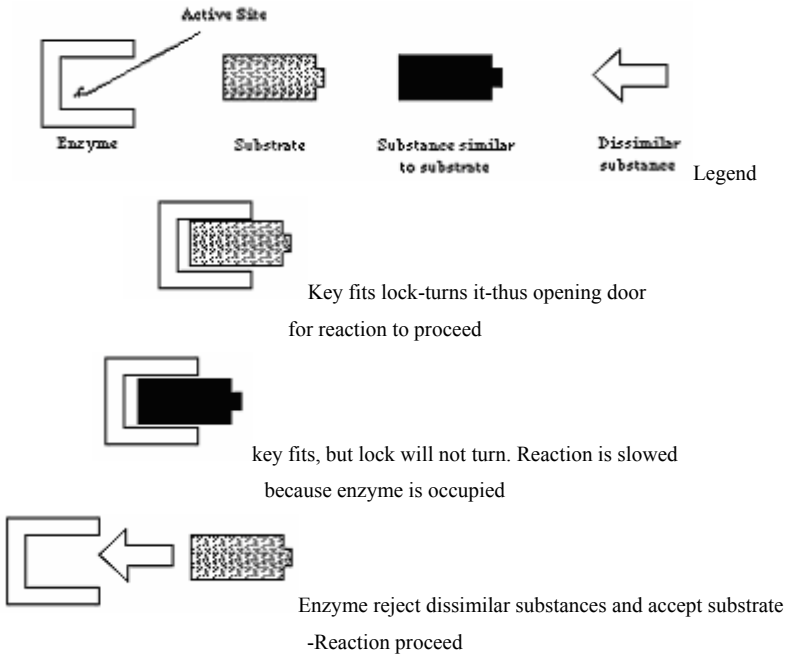

Figure 2.5: Lock key theory competitive inhibitors