BIOCONVERSION OF AGRICULTURAL RESIDUES FOR THE PRODUCTION OF ALKALINE CELLULASE, PECTINASE AND XYLANASE FOR INDUSTRIAL APPLICATION BY SOLID STATE FERMENTATION

A Project Report
Submitted to the

University Grants Commission

By

Dr. Samir C.Parikh
(Principle Investigator)
&
Rajendrasinh N. Solanki
(Co-Investigator)

Department of Microbiology
Smt. S.M.Panchal Science college,
Talod-383215 (Gujarat)

CHAPTER 1

INTRODUCTION

Enzymes are highly efficient catalysts from biological sources which catalyse all synthetic and degradative reactions of living organisms. Enzyme was first reported in the second half of the nineteenth century. Since then its usage has increased many folds in various industries. In the last three decades with rapid strides in the field of biotechnology especially in the fields of genetic and protein engineering there has been many exerting research works involving enzymes with the development of new commercially important industrial processes. Enzymes are used because of their high catalytic power, specific mode of action, stereo specificity eco-friendly use, reduced energy requirements etc.

All types of living organisms, where metabolic reactions occur produce enzymes. A whole range of sources is used for the production of commercial enzymes. Out of the total enzymes being used industrially, over half are extracted from fungi and yeast, one third are obtained from bacterial systems, and the remaining from animal (8%) and plant (4%) sources. (Marwaha and Kaur. 2000)

Microorganisms are generally preferred to plant and animal as source of industrial enzymes because their production cost are low. Enzyme contents are more predictable and controllable and more so because of the easy availability of raw materials with constant composition for their cultivation. The presence of potential / harmful materials like phenolic compounds (in plants). proteases and enzyme inhibitors in plant and animal tissues limit the use of plant and animal sources in enzyme production.

There is an ever increasing demand to replace traditional chemical processes with advanced biotechnological processes involving microorganisms and enzymes, such as pectinases (Bajpai, 1999; Bruhlman *et*

al., 2000). xylanases, (Beg et al., 2000 a and b), celluloses (Bajpai. 1999). All these not only provides an economically viable alternative, but also are more eco friendly.

In rural areas, of agro-based countries, where farming is main occupation, leads to deposit lignocellulosic wastes. So scientist always aims to develop new techniques to reuse lignocellulosic wastes. Bioconversion of waste material into valuable product is best way of balancing components on earth. The use of hemicellulases has been promising in converting lignocellulosic waste into simple form.

Bioconversion of Agricultural residues

Bioconversion is the process of conversion of material in to valuable products by the microorganisms, enzymes etc. Bioconversions of agricultural residues into other products involve the following steps:

- Pretreatment: Delignification to liberate the cellulose and hemicellulose fibres from the lignin complex.
- Hydrolysis: Depolymerisation of the Carbohydrate polymers to produce the free sugar residuals.
- Fermentation: Microbial conversion of the sugars to produce the value –added products.

Agriculture residues

All the agricultural resides are lignocellulosic material. Lignocellulosic biomass reserve is the most abundant organic matter (1.8×10^{12} tons/year) available on this terrestrial globe fixed through different biological process. It mainly composed of following: cellulose, hemicelluloses and lignin in 4:3:3 ratio (**Tsao, 1978)**.

Lignocellulose contains five major sugars, the abundance of which varies with the feedstock. They are the hexoses D-glucose, D-mannose, and D-galactose, and the pentoses D-xylose and L-arabinose. Fructose is not normally found in lignocelluloses and lignocellulosic waste. Currently, the second generation bio-products such as bioethanol, biodiesel, biohydrogen and methane from lignocelluloses biomass are increasingly been produced from wastes.

The use of food crops such as corn and sugarcane to produce biofuels is increasingly being discouraged due to the current worldwide rise in food prices. In order to minimize food-feed-fuel conflicts, it is necessary to integrate all kinds of biowaste into a biomass economy **(Mahro, 2007).**

The lignocellulosic biomass, represent the largest renewable reservoir of potentially fermentable carbohydrates on earth **(Mtui and Nakamura, 2005),** is mostly wasted in the form of pre-harvest and post-harvest agricultural losses and wastes of food processing industries.

Table 1.1 Examples of lignocellulosic material and their current uses.(Howard et al., 2003)

Lignocellulosic material	Residues	Competing use
Grain harvesting		
wheat, rice, oats barely and com Processed grains	Straw, cobs, stalks, hunks	Animal feed, burnt as fuel, compost, soil conditioner
Com, wheat, rice	Waste wter, bran	Animal feed

Fruit and vegetable harvesting	Seeds, peels, husks, stones, rejected whole fruit and juice	Animal and fish feed, some seeds for oil extraction
Fruit and vegetable processing	Seeds, peels, waste water, husks, shells, stones, rejected whole fruit and juice	Animal and fish feed, some seeds for oil extraction
Sugar cane other sugar products	Bagasse	Burnt as fuel
Oils and oilsees plants, Nuts, cotton seeds, oilves, soybean etc.	Shells, husks, lint, fibre, sludge, presscake, wastewater	Animal feed, fertilser, burnt fuel
Animal waste	Manure, other waste	Soil conditioners
Forestry-paper and pulp		
Harvesting of logs	Wood residuals, barks, leaves etc.	Soil conditioners, burnt
Saw and plywood waste	Woodchips, wood shavings, saw dust	Pulp and paper industries, chip and fibre board
Pulp & paper mills	Fibre waste, sulphite liquor	Reused in pulp and board industry as fuel
Lignocellulose waste from communities	Old newspapers, paper, cardboard, old boards, disused furniture	Small percentage recycled, other burnt
Grass	Unutilised grass	Burnt

Lignocellulose is cellulose, hemicelluloses and lignin. Hemicelluloses form a coat around the underlying cellulose fibre bound by hydrogen bonding.

The main polysaccharides that cell walls of plants are composed of are shown in Table 1.2.it is found that structurally different carbohydrates can perform analogous functions in plants of different taxonomic groups,hence the difference in the types of hemicelluloses occur in nature.

Table 1.2 The composition of lignocellulose for various biomass source (Marais, 2008)

Biomass source	Lignin %	Cellulose %	Hemicellulose %
Softwood	27-30	35-40	25-30
Hardwood	20-25	45-50	20-25
Wheat straw/bran	15-20	33-40	20-25
Paper	0-15	85-99	0
Leaves	0	15-20	80-85
Newspaper	18-30	40-45	25-40
Rice straw	18	32.1	24
Fresh bagasse	18	33.4	30
Solid cattle manure	2.7-5.7	1.6-4.7	1.4-3.3
Grasses(average values for grasses)	10-30	25-40	25-50
Nut shells	30-40	25-30	25-30

Note: Value expressed wt %

Lignocelluloses are highly recalcitrant substrate and its delignification is the most difficult step to accomplish **(Lee,1997)**. Various methods are employed to fractionate, solubilise, hydrolyse and separate the cellulose-hemicellulose-lignin matrix. During pretreatment the biomass is reduced in size and the structure is physically opened to allow for easier access to the fibers for subsequent treatment processes.

Currently, the second generation bio-products such as bio-ethanol, biodiesel, bio-hydrogen and methane from lignocellulose biomass are increasingly been produced from wastesrather than from energy crops. The use of food crops such as corn and sugarcane to produce biofuels is increasingly being discouraged due to the current worldwide rise in food prices.

The barrier to the production and recovery of valuable materials from LCW is the structure of lignocellulose which has evolved to resist degradation due to crosslinking between the polysaccharides (cellulose and hemicellulose) and the lignin via ester and ether linkages **(Yan and Shuya, 2006; Xiao,et al., 2007)**.

Cellulose, hemicelluloseand lignin form structures called microfibrils, which are organized into microfibrils that mediate structural stability in the plant cell **(Rubin, 2008**). These methods cause mechanical, physical chemical or biological changes in the plant biomass in order to achieve the desired products.

Mechanical treatment:

Mechanically based pretreatment technologies are aimed at reducing the size of LCW to facilitate subsequent treatments. Reduction of biomass

size below #20 sieves shows the best mechanical performance **(de Sousa et al.,2004)**. Mechanical pretreatment technologies increase the digestibility of cellulose and hemicellulose in the lignocellulosic biomass.

Physical treatment:

Elevated temperatures and irradiation are the most successful physical treatments in the processing of LCW. Thermogravimetric treatment of wood waste under both inert and oxidant atmospheres from room temperature upto 1100 K leads to moisture loss; hemicellulose, cellulose and lignin decomposition **(Lapuerta, 2004)**.

Irradiation can cause significant breakdown of the structure of LSW. Microwave irradiation at a power of upto 700 W at various exposure times resulted to weight loss due to degradation of cellulose, hemicellulose and lignin, and the degradation rates are significantly enhancedby the presence of alkali **(Zhu, et al., 2005a)**.

Physicochemical treatment:

The combinations of chemical and physical treatment are most powerful teachnique.The most successful physicochemical preatments include thermochemical treatments such as steam explosion or (steam disruption), liquid hot water (LHW), ammonia fiber explosion (AFEX) and CO_2 explosion.

The processes cause hemicellulose degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis. Such pretreatments also lead to higher digestion efficiencies during production of monosaccharides, oligosaccharides, lactic acid, antibacterial violet pigments and methane gas. Liquid hot water

pretreatment utilizes pressurized hot water at pressure less than 5 Mpa and temperature range of 170 - 230°C for several minutes followed by decompression up to atmospheric pressure.

Chemical treatment:

Chemicals ranging from oxidizing agents, alkali, acids and salts can be used to degrade lignin, hemicellulose and cellulose from LCW. Poweful oxidizing agents such as ozone and H_2O_2 effectively remove lignin; does not produce toxic residues for the downstream processes; and the reactions are carried out at room temperature and pressure **(Sun and Cheng, 2002).** More concentrated H_2SO_4 (up to 2.5 M) has been shown to be able not only to hydrolyse cellulose and hemicellulose, but also in separating lignin and other organic components.

Recent studies have shown that when acids are combined with alkali, they play a more effective role in LCW pretreatment than acids and alkalis alone.

Biological treatment

Biological treatments are either given to microorganism or enzyme to improve bioconversion process. Both fungi and bacteria are used for biotreatment of LCW. Commercial preparations of fungal and bacterial hydrolytic and oxidative enzymes are also widely used instead of these microorganisms. Bacterial pretreatment of LCW involves both anaerobic aerobic systems. Anaerobic degradation utilizes mainly mesophillic, rumen derived bacteria. Enzymatic pretreatment of LCW utilize hydrolytic and oxidative enzymes which are mainly derived from fungiand bacteria. Cellulases are usually a mixture of several enzymes.

During the enzymatic hydrolysis, cellulose is degraded by cellulases to reducing sugars that can be fermented by yeasts or bacteria to ethanol.

Products from lignocellulosic wastes:

A range of valuable products are produce from LCW such as,

Reducing sugars:

Fermentable sugars comes first in the value chain of processed LCW with glucose, xylose, xylitol, cellobiose, arabinose, pentose and galactose being the main reduced sugars produced.

Biofuels:

Today, world is facing problem of fuel shortage. One of the objective is to find alternative for fuel. Conversion of LCW to bio-fuels provides the best economically feasible and conflict-free second generation renewable alternatives **(Rubin, 2008).** Bioconversion of plant biomass wastes into bio-ethanol, biodiesel, bio-hydrogen, biogas (methane). Production of ethanol from sugars or starch from sugarcane and cereals, respectively, impacts negatively on the economics of the process, thus making ethanol more expensive compared with fossil fuels.

Currently, research and development of saccharification and fermentation technologies that convert LCW to reducing sugars and ethanol, respectively, in eco-friendly and profitable manner have picked tempo with breakthrough results being reported **(Prasad, et al., 2007**). Ethanol yield of 6 - 21% has been obtained through fermentation of agricultural and municipal residues.

Since the current supplies from LCW based oil crops and animal fats account for only approximately 0.3%, biodiesel from algae is widely used as one of the most efficient ways of generating biofuels and also appears to represent the only current renewable source of oil that could meet the global demand for transport fuels **(Schenk, et al., 2008).**

Hydrogen has been considered a potential fuel for the future since it is carbon-free and oxidized to water as a combustion product. Conversion of wheat straw wastes into bio-hydrogen gas.

Enzymes:

They include hydrolytic enzymes such as cellulases; hemicellulases and pectinases; degradative enzymes like amylases, proteases; and ligninolytic enzymes like laccases, peroxidases and oxidases. Lignocellulosic enzymes, mainly from fungi and bacteria, are important commercial products of LCW bio-processing used in many industrial applications including chemicals, fuel, food, brewery and wine, animal feed, textile and laundry, pulp and paper and agriculture.

Hemicellulolytic enzymes, mainly xylanases, are produced from a wide range of LCW biomass. Pectinases such as endopolygalacturonase (endo-PG), exo-polygalacturonase(exo-PG) and pectin ligase are mainly produced from solid state fermentation processes utilizing agricultural residues.

Organic acids:

Volatile fatty acids including acetic acid, propionic acids and butyric acid are produced from a wide range of LSW such as cereal hulls ; bagasse residues**,** food wastes. Furthermore formic acid, levulinic acid, citric acid,

valeric acid, caproic acid and vanillinic acid are obtainable from bioprocessing of LCW.

Microorganism involved into Bioconversion

Microorganisms are used extensively to provide a vast range of products and services. Their ability to readily undergo genetic manipulation has also opened up almost limitless further possibilities for new products and services from the fermentation industries. Traditional fermentations were originally performed (and still are in some cases) by a mixture of wild microorganisms emanating from the raw materials or the local environment, e.g. some food and alcoholic beverage fermentations.

The specific microorganisms employed were often isolated from the natural environment, which involved the random screening of a large number of isolates. Alternatively, suitable microorganisms were acquired from culture Collections.

ENZYMES

Enzymes are natural catalysts, which permit endogenous biological reactions to occur rapidly through well-defined pathways. They accelerate the rate of reactions, without being lost in the process. Enzymes are from different sources like animals, plants and microorganisms with wide applications in different industries. In compare to plant and animal enzymes microbial enzymes are more used today.

Present study is concentrated on microbial enzyme and their application for bioconversion of waste materials into renewable products. Enzymes are an important class of globular proteins of biological origin that act as biochemical catalysts. The most distinguishing property of an

enzyme in its catalytic action is its specificity and selectivity, each enzyme catalysis only a specific reaction involving a specific substrate.

To get an idea of the potency of enzymes, consider the following facts:

- It would take our digestive tract about 50 years to digest a single meal without enzymes.
- The hydrolysis of a peptide which would require very drastic conditions such as prolonged heating with a base, in the absence of enzymes, occurs under rather moderate conditions (as prevalent in a living system) in their presence.
- Enzymes can speed up an uncatalysed reaction by as much as 10 million times.

Table 1.3 Common types of enzymes: (Michael, et al 2001)

ENZYME	REACTION CATALYZED
HYDROLASES	General term for enzymes that catalyze a hydrolytic cleavage reaction
NUCLEASES	Break down nucleic acids by hydrolyzing bonds between nucleotides.
PROTEASES	Break down proteins by hydrolyzing bonds between amino acids.
SYNTHESES	General term for enzymes that synthesize molecules in anabolic reactions by condensing
ISOMERASES	Catalyze the rearrangement of bonds within a single molecule.
POLYMERASES	Catalyze polymerization reactions such as the sythesis of DNA and RNA.

KINASES	Catalyze the addition of phosphate groups to molecules. Protein kinases are an important group of kinases that attach phosphate groups to proteins.
PHOSPHATASES	Catalyze the hydrolytic removal of a phosphate group form a molecule/
OXIDO-REDUCTASES	General name for enzymes that catalyze reactions in which one molecule is oxidized and the other is reduced. They are also called *oxidases, reductases and dehydrogenases.*
ATPASES	Hydrolyze ATP. Many proteins with a wide range of roles have an energy-harnessing ATPase activity as part of their function, for example, motor proteins such as *myosin* and membrane transport proteins such as the *sodium-potassium pump.*

Enzymes have several advantages over chemical catalysts, including the ability to function under relatively mild conditions of temperature, pH and pressure. Enzymes are specific, often stereoselective, catalysts, which do not produce unwanted byproducts. Consequently, there is less need for extensive refining and purification of the target product. Also, compared with chemical processes, enzyme-based processes are '**environmentally friendly'** as enzymes are biodegradable and there are fewer associated waste disposal problems.

Most of the reactions in living organisms are catalysed by protein molecules called enzymes. Enzymes can rightly be called the catalytic

machinery of living systems. Man has indirectly used enzymes almost since the beginning of human history. Enzymes are responsible for the biocatalytic fermentation of sugar to ethanol by yeasts, a reaction that forms the bases of beer and wine manufacturing.

Naturally occurring enzymes are quite often not readily available in sufficient quantities for food applications or industrial use. Commercial sources of enzymes are obtained from three primary sources, i.e., animal tissue, plants and microbes. However, by isolating microbial strains that produce the desired enzyme and optimizing the conditions for growth, commercial quantities can be obtained. This technique, well known for more than 3,000 years, is called fermentation.

Today, this fermentation process is carried out in a container vessel. Once fermentation is completed, the microorganisms are destroyed; the enzymes are isolated, and further processed for commercial uses. The manipulation of biotechnological techniques have played an important role in the recent advances occurred in baking industry. India is an agricultural country; resultantly agro-industrial wastes and by-products are in abundance here. Wheat bran, sugar cane bagasse, corn cobs, rice bran etc. are some of the prominent waste materials from the allied food industries.

Probably the first application of cell free enzymes was the use of rennin isolated from calf or lamb stomach in cheese making. Rennin is an aspartic protease which coagulates milk protein and has been used for hundreds of years by cheese makers. Röhm in Germany prepared the first commercial enzyme preparation in 1914.

This trypsin enzyme isolated from animals degraded proteins and was used as a detergent. It proved to be so powerful compared to traditional washing powders that the original small package size made the German housewives suspicious so that the product had to be reformulated and sold in larger packages. Some of the typical applications include enzyme use in the production of sweeteners, chocolate, syrups, bakery products, alcoholic beverages, precooked cereals, infant foods, fish meal, cheese and dairy products, egg products, fruit juice, soft drinks, vegetable oil and puree, candy, spice and flavor extracts, and liquid coffee.

Starch industry became the second largest user of enzymes after detergent industry. Producers and stack holders are taking interest to utilize even a bit of resources to cope with the economic cost of finished products. The processing units generating agro-waste materials/by-products are struggling hard for their conversion into value added products.

The agricultural waste materials if manipulated properly can play a significant role in the economic uplift of a state. Enzymes are also indirectly used in biocatalytic processes involving living or dead and permeabilised microorganisms.

The use of microorganisms as biocatalysts in chemical production is, however, an interesting and growing field. The techniques of genetic, protein and pathway engineering are making chemical production by living cells an interesting green alternative to replace traditional chemical processes.

Table 1.4 Large scale enzyme application:

INDUSTRY	ENZYME	APPLICATION
Pulp and paper	Xylanase	Bioleaching
Textile	Cellulose, Laccase	Microfibril removal Color brightening
Animal feed	Xylanase, Phytase	Fiber solubility Release of phosphate
Starch	Amylases Glucose isomerise	Glucose formation Fructose formation
Fruit juice	Pectinase, cellulose, xylanase	juice clarification, juice extraction
Baking	Xylanase	Dough quality
	alpha-amylase glucose oxidase	Loaf volume;shelf-lifeDough quality
Detergent	Proteinase Lipase cellulase	Protein degradation Fat removal Color brightening
Brewing	GlucanasePapain	filter aid haze control
Dairy	Rennin Lactase	Protein coagulation Lactose hydrolysis

Lignocellulolytic enzymes

Alkaline Cellulase

The term cellulases refers to a complicated enzymatic system. This enzymatic system is a catalyst for the enzymatic reaction by which cellulose and similar polysaccharides are decomposed into glucose, cellobiose or celloligosaccharides.

The term cellulases is considered to be a general name for enzymes which are called, depending upon their mechanism of activity, C_1 enzyme, Cx enzyme and beta-glucosidase, or exo-beta-glucanase, endobeta-glucanase, cellobiase and the like.

Cellulases have been studied mainly for the purpose of effectively utilizing biomass resources. For instance, the main source of cellulase supply has been fungi belonging to the genera *Trichoderma, Aspergillus, Acremonium*, and *Humicola*. However, cellulases deriving their origin from microorganisms including fungi involve a diversity of enzymes having differing working specificities and physiocochemical properties. The different enzymes which make up this enzymatic system have not yet been completely identified or studied.

Of these cellulases, those which have a high action on carboxymethyl cellulose (CMC) or Cx enzymatic action are generally called CMCases. In recent years, novel industrial utility for cellulases, including the CMCases, have been developed, particularly, as an additive for use in detergent compositions for clothing.

However, so far as cellulases produced by microorganism in nature and, particularly, the abovementioned cellulases originating from microorganism are concerned, they are, in most cases, so unstable that their activity is lost in an alkaline pH. These are so-called acidic and neutral cellulases (whose optimum working pH is in the range of 4 to 6). So-called alkaline cellulases which meet the requirement for detergent compositions for clothing, i.e. those which have a maximum activity and are resistant in an alkaline range, are very small in number.

For instance, with regard to alkaline cellulases which are usable in detergent compositions for clothing, there are only several known methods for producing alkaline cellulases originating from alkalophilic microorganisms. These methods include a method in which microorganisms belonging to the genus *Bacillus* are cultivated and cellulase A is collected from the medium.

They also include a method in which alkalophilic bacteria belonging to the genus Cellulomonas are cultivated to produce alkaline cellulase 301-A, a method for producing CMCase by cultivation of alkalophilic Bacillus No. 1139, and a method of producing an alkaline cellulase using a strain belonging to the genus Streptomyces.

There is thus a strong demand and need for cellulases having an optimum working alkaline pH and possessing enzymatic activity making them suitable for use in detergents for clothing in an alkaline range. The ever-increasing human population and the lack of corresponding increase in food production have raised the importance of microbial sources of food/feed rich in proteins. The technology for the by conversion of crop and forest residues

into microbial products may solve the food/feed problems and also spare substrate for the production of fuel ethanol.

Cellulose is an abundant and renewable resource with a world production of 17^{12} tones per year. Biochemical or chemical conversion is required to transform this insoluble, polymeric form of glucose into useful material. Enzymatic hydrolysis of cellulose for sugar production offers advantages like higher conversion, minimal byproduct formation, low energy requirement and mild operating conditions over other chemical conversions.

Enzymatic hydrolysis of cellulose leads to the formation of reducing sugars, which can be used as a source of food as a substrate for single cell protein and as a raw material for industrial fermentation.

By the use of microorganism utilizing lignocellulose for developing unconventional source of proteins as food/feed, because of the following characteristics of microorganisms.

1. Microorganisms have a very fast growth rate.
2. They can, be easily modified genetically for growth on a given substrate and under favorable cultural conditions .
3. Their protein content is quite high.
4. They can be grown in liquid or solid state
5. Their nutritional values are as good as those of other conventional foods rich in proteins.

India being an agricultural country, a large amount of waste is generated from agro-industries and it is perennial in nature. Over 60% of any agricultural product is going as waste. Most of such crop residues are composed of mainly 30-45% cellulose, 16-25% hemicellulose and 3-13%

lignin. Wood harvesting and wood processing residues are Composed of 45-56% cellulose and 10-25% hemicllulose. Celluloses and hemicelluloses from decomposing plant materials are a readily available source of energy and carbon for various fungi, bacteria and actinomycetes.

The production of 1ignocellulolytic enzymes has received importance in recent years because of their possible role in achieving effective bioconversion of lignocellulosic wastes into simple sugars, chemicals, alcohols,, etc. Research and development efforts in the last decade have led to the formulation of technologies for the production of cellulase.

Various physical, chemical, and biological methods are suggested for improving the nutritive value of crop residues. Biological treatments employ fermentation of agricultural wastes with 1ignocellulolytic fungi.

In the food industries, cellulose can be used for the production of glucose syrup, fructose syrup and alcohol. Glucose is used extensively as an additive in a wide range of products like soft drinks, health drinks, packed cake mixes, confectioneries, whipped cream, pickled onions and chutney. Cellulose and alcohol are required for the production of a variety of chemicals, such as cellulose triacetate, vinyl acetate, polyvinyl chloride, styrene, and polystyrene. In the non-food industries, cellulose is widely used in paper and textile industries.

Fermentation is one of the oldest methods of food preparation. Generally traditional methods of preparing fermented foods are simple and inexpensive. However, the ancient methods of making such foods are changing rapidly through modern microbial technology. Fermentation processes have been highly developed in recent years for the production of

alcohol, biomass, enzymes, sugar syrups, secondary metabolites like amino acids, vitamins, pharmaceutical compounds, etc. employing both aerobic and anaerobic microorganisms in an extensive manner all over the world . It is believed by many that the biotechnological processes may even substitute the chemical processes in future. Consequently an intensive search for cheaper as well as renewable raw materials have drawn the attention of many scientists towards economic preparation of products.

Pectinase

Pectic enzymes are widely distributed in nature. They mainly occur in plants, bacteria, fungi, yeasts, insects, nematodes and protozoa. Many plant-pathogenic bacteria and fungi are known to produce pectolytic enzymes useful for invading host tissues. Moreover, theses enzymes are essential in the decay of dead plant material by nonpathogenic microorganisms and thus assist in recycling carbon compounds in the biosphere.

The biotechnological potential of pectinolytic enzymes from microorganisms has drawn a great deal of attention from various researchers worldwide. They are widely used as biological catalysts in the industrial process.

The commercial application of pectic enzymes was noted in 1930 for the preparation of wines and fruit juices. But the chemical nature of the plant tissues was apparent only in 1960s. After this, the scientists began in search the commercial utility of these enzymes. Now pectinases are an integral part of food industry and in the field of biotechnology.

Acidic pectinases are mainly used in the fruit industry and wine making, most commonly theses are isolated from fungal sources especially from *Aspergillus niger*.

Alkaline pectinases are used mainly in the degumming and retting of fiber crops, pretreatment of pectic wastewater from fruit juice industry, production of paper and pulp, oil extraction and coffee and tea fermentation.. Pectic enzymes are also involved in wood preservation here enzyme preparations or specific bacteria trial produce these enzymes are used.

In view of the diverse applications of these acidic and alkaline pectinases, they form the backbone of biotechnology industry. Due to the wide range of applications of pectinases in food industry, the industrial production of pectinases has drawn worldwide attention. Suitable organisms include strains of *Aspergillus niger*, *Aspergillus wentti, Aspergillus oryzee* etc.

Pectinases include depolymerizing and demethoxylating enzymes. Depolymerizing enzymes are polygalacturonase (EC 3.2.1), which cleaves the a-1,4 glycosidic bonds between two galacturonic acid residues, and pectin-lyase (EC 4.2.2), which catalyses a b-elimination reaction between two methylated residues. De-esterifying enzymes include pectin-esterase (EC 3.1.1), which catalyses the demethoxylation of methylated pectin, producing methanol and pectin.

Preparations containing pectin-degrading enzymes have been extensively used to improve the stability of fruit and vegetable nectars and in the clarification of fruit juices and wines. Currently, they are widely used in

industry for retting of natural fibers and extraction of oils from vegetable and citrus peels.

The enzymes preparations used in the food industry are of fungal origin because fungi are potent producers of pectic enzymes and the optimal pH of fungal enzymes is very close to the pH of many fruit juices, which range from pH 3,0 to 5,5. Such preparations are not suited for production of vegetable purées or other preparations in which pH values are close to neutral.

Furthermore, due to the relatively low temperature stability of the fungal enzyme preparations, maceration needs to be carried out at temperatures not exceeding 45ºC, necessitating the incorporation of a pasteurization step to limit the growth of mesophilic microorganisms.

The present investigation was on pectinolytic activities of bacteria strains isolated from Brazilian soil and samples of vegetable in decomposition.

Xylanase

Xylanolytic enzymes from microorganisms have attracted the attention for the last few decades, particularly because of their biotechnological potential in various industrial processes such as food, feed and pulp and paper industries. Considering the future prospects of xylanases in biotechnology, especially in the field of biopulping and bleaching, a search for microorganisms which can produce considerable amount of xylanases with desirable characteristics for the applications in various industries are encouragable.

The xylanases are used in fruits and vegetable juices for improving juice clarification and thereby to reduce the viscosity. In baking, they improve elasticity and strength of the dough thereby increasing loaf volumes and texture of bread. In feed industry, incorporation of xylanase into the rye-based diet of broiler chickens results in the reduction in intestinal viscosity, thereby improving both weight gain of chicks and their feed conversion efficiency.

In pulp and paper industry, xylanases are used in biobleaching thereby reduces chlorine consumption and toxic discharges. In biomechanical pulping, xylanases facilitates the pulping process and reduces the use of mechanical pulping method, thereby reducing energy consumption. Xylanases improves fibrillation and drainage properties of pulp, resulting in the improvement of the process efficiency and the paper strength.

Xylan is a major component of plant hemicellulose. After cellulose it is the next most abundant renewable polysaccharide in nature. Xylan represent upto 30-35% of the total dry weight of land plants. Xylan is typically located in the secondary cell wall of plants, but it is found in the primary cell wall in monocots. Due to the heterogeneity and complex chemical nature of plant xylan, its complete breakdown requires the action of a complex of several hydrolytic enzymes with diverse specificity and modes of action. The xylanolytic enzyme carrying out the xylan hydrolysis is usually composed of a repertoire of hydrolytic enzymes.

In addition to the production of a variety of xylanolytic enzymes, many organisms produce multiple xylanases. These may have diverse physico-chemical properties, structures, specific activities and yields, as well as

overlapping but dissimilar specificities, thereby increasing the efficiency and extent of hydrolysis, but also the diversity and complexity of the enzymes.

Hemicellulose is an important biomass reservoir in the plant cell wall. Agricultural wastes that contain hemicellulose were globally generated. With reference to biomass regeneration, hemicellulose degradation has been intensively studied in the last decade. Xylan, after cellulose, is the most abundant polysaccharide present in wood, agricultural and several agro-industrial wastes.

This complex heteropolysaccharide consists of a main chain of 1,4-b-D-xylose monomers containing different substituents or ramifications. The substituents including arabinofuranosyl, glucuronyl and acetyl groups show a pronounced influence on its chemical and structural properties and also on the enzymatic degradability of xylan in lignocelluloses.

Xylanases (E.C. 3.2.1.8) show great potential for industrial applications mainly for the bioconversion of lignocelluloses to sugar, ethanol, and other useful substances, clarification of juices and wines, improving the nutritional quality of silage and green feed and the de-inking processes of waste papers. Enzyme can be produced by a number of microorganisms including bacteria, yeasts and fungi such as *Trichoderma, Bacillus, Cryptococcus, Aspergillus, Penicillium, Aureobasidium, Fusarium, Chaetomium, Phanerochaete, Rhizomucor, Humicola, Talaromyces* and many more. These fungi produced xylanase enzymes extracellularly using various substrates both in submerged and solid state fermentation (SSF) processes.

Extracellular enzymes are considered important from the industrial viewpoint as they ease the extraction procedure. Solid state fermentation was found to be more economical mainly due to the cheap and abundant availability of agricultural wastes which can be used as substrates. Submerged method has been used for the production of fine chemicals of commercial value from microbial sources such as enzymes, antibiotics, flavouring compounds and also microbial biomass which was used as animal feeds. Our study is particularly interested in xylanase enzymes producing using local raw materials from indigenous isolates.

Xylanolytic enzymes appear to be inducible. Xylanases and xylosidases are produced in high amounts during growth on xylan, and synthesis of the enzyme is catabolitly repressed by easily metabolizable carbon sources such as glucose or xylose. Since xylan cannot enter the cells, the oligosaccharides formed by the hydrolysis of xylan in the medium by tiny amount of xylanases produced constitutively.

Xylan is a potential significant resource for renewable biomass, which can be utilized as a substrate for the preparation of many products such as fuels, solvents and pharmaceuticals. Agriculture residues of Corn, wheat stem,ricehusks,soymeal barley shells and similar material contain xylan**.**

Solid-state fermentation (SSF) presents some advantages over submerged fermentation concerning the readiness of recuperation of bioproduct, besides the use of natural substrates such as nutritional support. Several agricultural or agro-industrial residues, cellulosic or amilaceous, have been used in solid-state fermentation such as sugarcane bagasse, cassava bagasse, rice and wheat bran, coffee husks, and others.

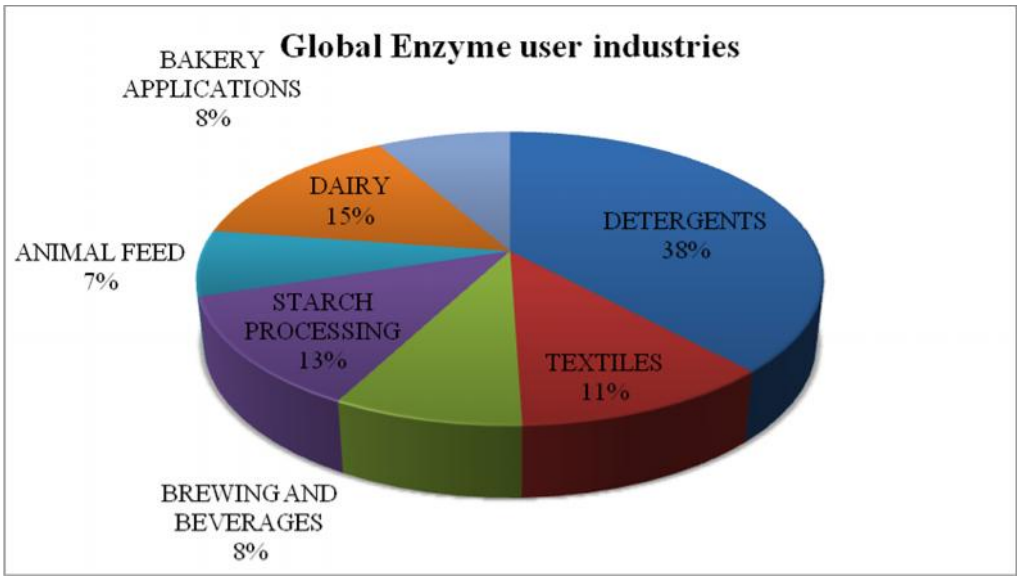
SSF can be carried out directly with abundant low-cost biomaterials (including starch, cellulose, lignin, hemicellulose, and chitin) with minimal or no pre-treatment, and is therefore relatively simple, uses less energy, and can provide unique microenvironments conducive to microbial growth and metabolic activities.

SSF also has advantages over submerged cultivation and has been used for the production of cellulolytic and xylanolytic enzymes. At the end of the fermentation, the enzymes can be extracted from the substrate easily and inexpensively by percolating the bioreactor with the appropriate buffers .The industrial application of xylanase may be limited by its high cost of production. The use of cost-effective agroresidues as substrates in solid state fermentation may reduce the cost of enzyme production substantially. Solid state fermentation offers several advantages over submerged fermentation including simplicity of media, greater product yield, easier scale up of process, economy of space, no complex machinery, equipment and control systems.

Global scenario of microbial enzymes

Presently the industrial enzyme companies sell enzymes for a wide variety of applications. The estimated value of world enzyme market will rise to grow to almost 8 billion US $ by 2015 according to the survey of freedonia group published in 2011 .Growth will be led by enzymes used in the diagnostic, research and biotechnology and other uses. Detergents (37%), textiles (12%), starch (11%), baking (8%) and animal feed (7%) are the main industries, which use about 75% of industrially, produced enzymes.

Figure 1.1 Application of bulk microbial enzyme. (Michael, et al 2001)



Furthermore today , many improved recombinant forms of this enzyme are in use in molecular biology. Although these enzymes have been isolated from nature, the culturing and mass production of these organisms or their enzymes is very difficult due to their growth requirements or conditions.

Another major application of enzymes in an industrial scale is the production of biofuel. In order for this to be achievable, enzyme application is needed for the conversion of this biomass to bio-fuels.

FERMENTATION TECHNOLOGY:

Microbiologists use the term fermentation in two different ways.

Fermentation refers to energy-generating processes where organic compounds act as both electron donor and acceptor. Second, in the context of industrial microbiology, the term also refers to the growth of large quantities of cells under aerobic or anaerobic conditions, within a vessel referred to as a fermenter or bioreactor.

Fermentations are also broadly classified according to the organization of the biological phase. Whether, it is in suspension or in the form of a supported film.

Types of Fermentation

Submerged liquid Fermentation(SmF)

In this case, microorganisms are cultivated in a liquid medium in which contains the required nutrients in the required concentrations. Nutrients are generally supplied in the form of cheap and readily available materials such as rice bran and wheat bran (sources of carbohydrates) or in pure and concentrated forms such as bacteriological peptone (source of proteins) which are either dissolved or suspended in water to form the growth medium. The advantage of this process is the ease with which various parameters can be monitored (by periodic sampling of broth) and controlled if necessary by the addition of further nutrients or reagents.

Solid Substrate Fermentation(SSF)

Solid-state fermentation (SSF) processes can be defined as "the growth of microorganisms (mainly fungi) on moist solid materials in the absence of free-flowing water". These processes have been used for the production of food, animal feed, and both pharmaceutical and agricultural products.

Substrates that have been traditionally fermented by solid-state include a variety of agricultural products such as rice, wheat, millet barley, grains, beans, corn and soybeans. Microorganisms are cultivated on a solid substrate with a high content of nutrients and a large surface area,

such as wheat germ, wheat bran, rice bran and cereal meal, with the addition of mineral salts. Solid state fermentation [SSF] has been recently considered as the most cheapest and more environmentally friendly relative to submerged liquid fermentation [SmF] in the production of value added industrial based products such as enzymes, bio fuels and the likes

In contrast to Submerged (liquid state) Fermentation, Solid State Fermentation (SSF) is the growth and/or Cultivation of micro organisms under controlled conditions in the absence of free water for the production of desired products of interest. Examples of products of Solid State Fermentation include industrial enzymes, fuels and nutrient enriched animal feeds. In SSF, two types of process can be distinguished depending on the nature of the solid phase.

In the first and the most used, the solid serves both as a support and a nutrient source. These substrates are heterogeneous water insoluble materials from agriculture or by-products from food industry, which have an amylaceous or ligno-cellulosic nature (grains and grain by-products, cassava, potato, beans and sugar beet pulp).

Although solid-state fermentation (SSF) has been practiced for many centuries in the preparation of traditional fermented foods, its application to newer products within the framework of modern biotechnology is relatively restricted. For the majority of fermentation products, it gives better yields and is easier to apply.

It is notoriously difficult to control the fermentation conditions in SSF; these difficulties are already apparent at small scale in the laboratory and are exacerbated with increase in scale.

However, there are particular circumstances and products for which SSF technology is appropriate. For example, a desire to reuse solid organic wastes from agriculture and food processing rather than simply discarding them leads naturally to the use of SSF. Further, some microbial products,such as fungal enzymes and spores, amongst others, are produced in higher yields or with better properties in the environment provided by SSF systems.

Basic parameters for Fermentation process development

Once a microorganism has been selected as the producer organism for a particular process, research is initially performed under laboratory-scale conditions using 1–10 L fermenters. Optimization of product yield in the laboratory is followed by process **scale-up**; first to pilot scaleof 10–100L and finally to industrial scale of 1000–100000L, or more, depending upon the specific process.

However, during scale-up, decreased product yields are often experienced; the reason is that the conditions in the larger-scale fermenters are not identical to those experienced in the smaller-scale laboratory or pilot plant systems.

The wide variety of solid substrates employed in SSF, which have important differences such as composition, size, mechanical resistance, porosity and water holding capacity.

The SSF bioreactors must be constructed with a strong material, which must be anticorrosive and non-toxic to the process organism. It should also have a low cost.

The entry of contaminants into the process as well as the uncontrolled release of the process organism into the environment must be avoided by using filters on outlet air stream and by a careful design of seals and filtration of the inlet air stream.

Application of SSF:

It has been reported that in many bioproductions, the amounts of products obtained by solid state fermentation are many-fold higher than those obtained in submerged cultivations. SSF plays a significant role at laboratory level than SLF and it is often considered a cost effective process than its counterpart for the production of wide arrays of bioproducts. Such as animal feed, enzymes, organic acids, biopulp, aroma compounds, antibiotics, compost, biopesticide, biofertilizer etc. In addition, the products obtained have slightly different properties (e.g. more thermotolerance) when produced in SSF and SLF. Therefore, if SSF variables are well controlled and the purity of the product is defined, this technology may be a more competitive process than is commonly thought.

Objective of research:

The major objective of the research was to explore a novel Alkaline cellulose, pectinase and xylanase secreting bacteria and develop a production technique suitable for Alkaline cellulose, pectinase and xylanase production. Alkaline cellulose, pectinase and xylanase production should be highly potential and competent for industrial applications. Main focus of research was to optimize culture condition for maximum enzyme production and medium optimization suitable for lab level and directly useful for pilot level.

CHAPTER 2

MATERIALS AND METHODS

Source:

Different locations were selected for the search of alkaline cellulolytic, pectinolytic and xylanolytic organisms from local area of Sabarkantha district. Mainly soil samples were collected from agricultural farms, gardens, cow-dungs, hay, paper mill's soil and non agricultural region soil. More than 77 different soil samples were collected. Even certain samples were also collected from decayed woody materials.

Collection of lignocellulosic samples and processing:

All selected sources were used for sampling. Lignocellulosic wastes containing soil include Agriculture soil, garden soil and other non agricultural region soil samples were taken, after removing approximate 3-4 cm of soil surface.

All samples were collected in sterilized vials & brought to the laboratory. Each sample was crushed, mixed thoroughly and sieved through a 2 mm sieve to get rid of large debris and the sieved sample was used to evaluate on the same day for the screening of potential xylanase producer.

Primary Screening

Samples of 1g soil were suspended in 100ml distilled water with 1% Tween-80 for dispersion of soil clumps, then incubated in an orbital shaker incubator at 28°C with shaking at 140 rpm for 30 min. Mixtures were allowed to settle and then serial dilutions up to 10^{-6} were prepared.

Form this test, 0.1 ml of suspension was transferred to the growth medium for specific screened medium.

Following medium used for the screening for the alkaline cellulase, pectinase and xylanase producing microorganism.

A. Alkaline Cellulase

CMC (Carboxy Metyl Cellulase) agar medium

Ingredients	**g/l**
CMC	10.0
KH_2PO_4	1.0
$MgSO_4.7H_2O$	0.5
NaCl	0.5
$FeSO_4.7H_2O$	0.01
$MnSO_4.H_2O$	0.01
NH_4NO_3	0.03
pH	7.0*
Agar	30.0

Same ingredients were used in the medium to screened out for alkaline cellulase producing bacteria but the pH value was varies from pH 8 to 10.

B. Pctinase

Pectin Agar Medium

Ingredients	**g/l**
Pectin	5.0
KH_2PO_4	0.5
$MgSO_4.7H_2O$	0.1
$CaCl_2.2H_2O$	0.2
$FeCl.36H_2O$	0.01
Yeast Extract	1.0
pH	7.0
Agar	30.0

C. Xylanase

Xylan Agar Medium

Ingredients	**g/l**
Oat-spelt xylan,	5.0
Peptone,	3.0
KH_2PO_4,	2.0
$CaCl_2$,	0.3
Tween80	2.0
$MgSO_4 \cdot 7H_2O$	0.3
$FeSO_4 \cdot 7H_2O$	0.005
$ZnSO_4 \cdot 7H_2O$	0.0014
$MnSO_4 \cdot H_2O$	0.0016
$CoCl_2$	0.002
pH	7.0
Agar	30.0

The agar powder in this medium was supplied with 25g /L but on basis of environmental condition it was used 30g/L.

SCREENING

All plates were incubated at 37 °C for 48 to 72 hrs on the basis of growth in incubator. After incubation, the screening process was done.

For the screening of cellulase producing microorganism the plates were filled with the 1.0% congo red solution for 15 minutes and than treated by flooding 1M NaCl solution for 15 minutes. The formation of clear zone was

observed surrounded the colony which was a cellulase producing microorganism. All the colony were transferred the same medium having alkaline condition for the screening of alkaline cellulose producing microorganism. All the plates were incubated at 37 °C for 48 to 72 hrs in incubator.

After the incubation the same process was used to screen of alkaline cellulase microorganism. The colonies having notable clear zone surrounded colonies were transferred to new medium plates. The selected isolates which was transferred to new medium were labeled as AC_1, AC_2, AC_3, AC_4, AC_5, AC_6 ……

Alkaline cellulase producing isolates were inoculated into 100 ml of Xylanase broth in a 250 mm Erlenmeyer flask and incubated on a rotary shaker at 200 rpm at 37°C for 48 hrs. The crude enzyme was obtain from each isolated colonies by applying centrifugation at 10,000 rpm for 10 minutes and the supernatant was removed. The enzyme was obtained as dry powder form.

The crude enzyme was analysed by DNSA method.

For the isolation of all theses three isolates, the pour plate technique was used. For pectinase plates were incubated in an inverted position at 37°C for 72 - 120 hrs. After the incubation period over the plates were flooded with 0.5% aqueous solution of cetavion (cetyl trimethyl ammonium bromide) and allowed it was stand for 20 - 30 minutes.

Cetavion is a polysaccharide precipitant, and thus microorganisms, which decompose pectic acid, are surrounded by a clear zone in an otherwise

white and opaque medium. The diameter of the clear zone generally corelated with the pectin decomposing potential of the organism. The colonies, which showed pectinolytic activities, were sub-cultured separately on Sabouraud's dextrose agar (SDA) plates. The colonies having notable clear zone surrounded colony was transferred to new medium plates. The selected isolates which were transferred to new medium were labeled as PE_1, PE_2, PE_3, PE_4, PE_5, PE_6, …… etc.

For the screening of the xylanase producing microorganism cultures were repeated to cultivate in fresh xylanase broth medium. The diluted enriched cultures (0.1 ml) were dropped and spreaded on the Xylanase agar plate and incubated at 37°C for 48 days. Their colonies grown on Xylanase agar plate were flooded with 0.1% (w/v) Congo red solution and kept for 1min, then were washed by 0.1 M NaCl. The colonies surrounded by a clearance zone were selected as xylanase producing isolates. The colonies having notable clear zone surrounded colony was transferred to new medium plates. The selected isolates which were transferred to new medium were labeled as XY_1, XY_2, XY_3, XY_4, XY_5, XY_6 ……

Xylanase-producing isolates were inoculated into 100 ml of Xylanase broth in a 250 ml Erlenmeyer flask and incubated on a rotary shaker at 200 rpm at 37°C for 48 hrs.

Xylanase producing isolates were inoculated into 100 ml of Xylanase broth in a 250 ml Erlenmeyer flask and incubated on a rotary shaker at 200 rpm at 37°C for 48 hrs. The crude enzyme was obtains from each isolates by applying centrifugation at 10,000 rpm for 10 minutes and the supernatant was removed. The enzymes were obtained in dry powder form. The crude enzyme was analysed by DNSA method.

Fermentation process:

100ml of fermentation medium was used for study, each flask was cotton plugged and then sterilized in the autoclave at 121°C for 15 min. After cooling the medium at room temperature, 4 ml of the culture suspension was transferred to each fermentation flask and the flasks were placed in the rotary incubator shaker (200 rpm) at 30°C, pH 7 for 72 h. After 12h equal time intervals, filtrate was withdrawn. Enzyme activity was estimated by DNS method.

Bacterial strain identification by Gram staining

All screened five isolates were evaluated for purity by gram staining. Staining is used to differentiate isolates into gram positive or gram negative bacteria. The microscopic morphological characters were also studied.

Colony characteristics.

All screened isolates were characterized by colony appearances. Colonies are classified according to their size, shape margin, elevation, texture and pigmentation etc.

Physiological and Biochemical characteristics

Among all, maximum enzyme activity showing strain was evaluated for physiological and biochemical characteristics. such as oxidase test, catalase-test, fermentation test, citrate-utilization test, lactose fermentation and Indole test.

Selection of agricultural wastes/residues as Substrate:

Agricultural wastes and byproducts (lignocellulosic waste) like wheat straw, bajra straw, rice straw, wheat bran, dry grass, sugarcane bagasse, saw-dust, soybean bran and corn cobs were obtains form local farms and used as media components.

Waste material chopped in small pieces, dried in oven at 60°Cfor 72 hrs and ground well using mortar and pestle. These ground materials were then separated by sieves into particles to provide equal surface area for growth of organism. It provides equal oxygen diffusion and nutrient absorption for the assimilation by organisms. The prepared substrates were stored in air tight container, for further utilization as fermentation media for xylanase synthesis. (**Sanghi, et al 2008)** Solid-state fermentation was initially carried out using all the substrates individually.

Pretreatment of substrate:

The substrate was pretreated with 0.5%, 1% and 2% NaOH at room temperature for two hours followed by washing several times with water until neutral and then dried in an oven at 50°C to obtain a constant weight. Pretreatment was given to increase conversion of hemicellulose (xylan) to simple sugar (xylose). (**Goyal, et al., 2008**)

Effect of fermentation time

To study the effect of fermentation time on alkaline cellulase, Pectinase and xylanase production at various time interval alkaline cellulase, Pectinase and xylanase activities were checked. All other parameters were kept constant. During fermentation after equal intervals of

12hours samples were withdrawn from trays and enzyme activities were checked by DNSA method.

Optimization of pH

Selection of initial pH is necessary for microbial metabolism and enzyme production. Effect of media pH on alkaline cellulase, Pectinase and xylanase production was estimated by culturing the strain in fermentation media with different buffers ranging between pH 5 and 11(sodium phosphate buffer pH 5 to 10) at 50mM buffer solution. Cell free supernatant was collected and used as crude enzyme to check alkaline cellulase, Pectinase and xylanase activity.

Optimization of temperature

To determine optimum temperature for alkaline cellulase, Pectinase and xylanase production by isolates, a range of temperature from 25° to 55°C were selected. Alkaline cellulase, Pectinase and Xylanase production was allowed at different temperature with other constant parameters such as initial pH, moisture content 1:2.5, inoculum size etc. While fermentation was carried out for 48 hours alkaline cellulase, Pectinase and xylanase activity was checked by DNS method.

Effect of different agro- residues

To study, influence of different agro-residues on alkaline cellulase, Pectinase and xylanase production, different agriculture residues such as wheat bran, rice straw, wood husk, dry grass and corn meal, sugarcane bagassae, soybean flour/bran were collected from local farms and flour meals. All collected agro-residues were used as substrate individually with mineral salt solution for fermentation. Fermentation was carried out at

37°C with initial pH 10 for alkaline cellulase and pH 7 for Pectinase and Xylanase for 48 hours. Alkaline cellulase, Pectinase and Xylanase activity was checked by DNS method.

Recovery and partial purification

Extraction of crude Enzymes

Approximately 2-3 gram of crude enzyme sample was collected and mixed with 0.1% Tween-80 containing 50ml of 50mM phosphate buffer (pH 7).the reaction mixture was incubated was for 2h on Environ-Rotary shaker at 200 rpm. After incubation period the suspended slurry was centrifuged at 10,000 rpm for 20 min at 4°C slurry was filtrated through whatman no.2 filter paper, the clear supernatant (cell-free filtrate) used as crude enzyme. For submerged fermentation, the enzyme extraction was performed by directly centrifuging the culture without adding any buffer. The rest of the procedure was the same as for SSF.

Partial purification of Enzymes

Treatment by ammonium-sulphate:

The supernatant obtained from SSF was subjected to different percent of ammonium sulphate saturation in order to precipitate enzyme ranging from 20-60% at 4°C. This was carried out by adding solid ammonium sulphate into the culture supernatant with constant stirring on magnetic stirrer at 4°C. After each of saturation, the precipitate was collected by centrifugation at 10,000 rpm for 10 min at 4°C and pellet was dissolved in minimum volume of 50mM Tris-HCl (pH 7) and enzyme activity was studies by DNSA method and protein concentration by Lowry's method (**Kitsada, et al 2000).**

Treatment by chilled acetone

The cold filtrate of the semi purified alkaline cellulose, pectinase and xylanase solution was treated with different concentration of chilled acetone (10% to 90%) and kept in the refrigerator overnight. The resulting precipitate was separated by cold centrifugation, dissolved in 50mM sodium phosphate buffer pH 7.0. The fractions solutions were designated as partially purified enzyme preparation. (**Nadia, et al 2010)**

ASSAY METHOD

Measurement of Alkaline Cellulase, Pectinase and Xylanase Activity

1. For soluble enzyme, filter or centrifuge culture sample to remove solids and analyze supernatant or filtrate. For cell bound enzymes, homogenize cells in appropriate buffer such as 0.05 M citrate, Dissolve enzyme powders at 1-5 mg per ml in buffer. Dilute enzyme solutions in buffer.
2. Blanks of enzyme without substrate and substrate without enzyme are included with all enzyme assays and sample values are corrected for any blank value.
3. For quantitative results, enzyme must be diluted or assay reaction time decreased until the amount of product plotted against enzyme concentration is reasonably linear. For the assay procedures described here, this would be when about 0.5 mg (or less) of glucose is produced from carboxymethyl cellulose or 2.0 mg of glucose (or less) is produced from filter paper, or other insoluble substrates. For insoluble cellulose, initial rates are of little value since there is always some amorphous cellulose which is readily hydrolyzed, but rates fall off rapidly to zero if the cellulase is incomplete. For quantitative results enzyme preparations should be compared on the basis of significant and equal conversion. Twice as much enzyme will give equal sugar in half the time, but it will not give twice as much sugar in equal time. An arbitrary value of 2.0 mg

of reducing sugar as glucose from 50 mg of filter paper (4% conversion) in 60 minutes has been designated as the intercept for calculating filter paper cellulase units.

4. Reducing Sugar Estimation by Dinitrosalicylic Acid (DNS) Method

   DNS Reagent

Mix:	Distilled Water	1416 ml
	3,5-Dinitrosalicylic acid	10.6 g
	NaOH	19.8 g
Dissolve above, then add:		
	Rochelle salts (Na-K tartarate)	306 g
	Phenol (melt at 50°C)	7.6 ml
	Na metabisulfite	8.3 g

Glucose Standards: 0.2— 5.0 mg of glucose per ml or per 0.5 ml as appropriate.

Procedure: Place 1-2 ml sample in a test tube and add 3 ml DNS Reagent. Place in boiling water for 5 minutes. Cool to room temperature. Dilute samples if necessary so That light transmittance in the colorimeter will be between 3% and 80%. Include glucose standard made up and diluted like samples.

Comments:

Color develops only under alkaline conditions, so acidic samples should be neutralized.

This method is non-specific and measures any reducing compound. If glucose is used as the standard, values for cellobiose will be 15% low and values for xylose will be 15% high on a weight basis.

Boiled samples may be left a reasonable time before reading. Unboiled samples gradually deteriorate.

Measurement of Pactinase Activity

Pactinase activity was measured by determining the amount of reducing groups released according to the method described by Nelson and modified by Somogyi

Definition of unit

One unit of pectinase activity has been defined as the amount of enzyme that releases one m mol of galacturonic acid /ml/minute under the assay conditions

Reagents

1) 0.2 M Tris acetate buffer pH 4 5
2) 0 01 M $CaCl_2$;
3) Polygalacturonic acid (Sigma Aldrich) 1%
4) Alkaline copper tartrate reagent

Solution A: Dissolved 2.5 gm anhydrous sodium carbonate 2 gm sodium bicarbonate. 2 5 gm potassium sodium tartarate and 20 gm anhydrous sodium sulphate in 80 ml water and made upto 100 ml.

Solution B. Dissolved 15 gm copper sulfate in a small volume of distilled water Added one drop of sulfuric acid and made up to 100 ml

→Mixed 4 ml of solution B and 96 ml of solution A before use.

Arsenomolybdate solution

Dissolved 2 5gm ammonium molybdale in 45 ml water. Added 2.5 ml sulfuric acid and mixed well. Then added 0.3-gm disodium hydrogen arsenate

dissolved In 25 ml water Mixed well and incubated at 37°C for 24 to 48 hours

Standard solution

Galacturonic; acid monohydrate (Fluka)- 1.0%

Procedure

1) The assay mixture was prepared with the following components.
   - 0.2 ml enzyme
   - 0.2 ml 0.2 M Tris-acetate buffer pH 4 5
   - 0.1 ml 0.01 M $CaCl_2$
   - 0.5 ml of 1 0% solution of polygalacturonic acid (PGA)
2) Prepared a Blank for each sample by boiling the reaction mixture before the addition of substrate.
3) Incubated at 37°C for 1 hour
4) Stopped the reaction by heating at 100°C for 3 minutes
5) 0.5 ml of the solution mixture was taken and analyzed for reducing sugars by Nelson-Somogyi's method.
6) Pipetted out 2.0 ml distilled water in a separate tube to set up a blank.
7) Added 1.0 ml of alkaline copper tartarate and kept for 10 minutes.
8) Cooled the tubes and added 10 ml of arsenomolybdate reagent to each of the tubes.
9) Made up the volume m each tube to 10 mi with distilled water.
10) Read the absorbance of blue colour at 620 nm after 10 minutes.

In the present study, enzyme activity was measured by the reaction between unsaturated end products of pectin degradation.

Application of Partially Purified Alkaline Cellulase, Pectinase and Xylanase

Hydrolysis of agro-industrial residue

In order to assess the enzyme ability to hydrolyze several polymeric substrate, comparative studies were done respectively with partially purified alkaline cellulase, pectinase and xylanase and commercially available cellulase, pectinase and xylanase. 10ml of enzyme solution and 50mg dry weight of selected agro industrial residues such as rice straw, wheat bran, wheat straw, and Carboxymethyl Cellulose, Pectin and brichwood xylan, and carboxymethyl cellulose suspended in 100mM Sodium phosphate buffer pH 7.0(100ml) were incubated for 1 h at 60°C with periodic shaking flasks. The reaction was stopped by placing the mixture in boiling water for 5 min, and then centrifuged at 8000 rpm for 10min. Reducing sugars liberated by hydrolysis of these substrates were quantified by the DNSA method. (**Poorna. 2011)**

CHAPTER 3
RESULTS AND DISCUSSION

Isolation of alkaline cellulase, pectinase and xylanase producing microorganisms:

Source:

By exploring sources more than 77 soil samples were collected. Samples were collected in sterile vials.

alkaline cellulase, pectinase and xylanase producing microorganisms:

After incubation out of 77 soil sample 11 soil sample showing cellulolytic activates from that plates 14 isolated colonies were selected for alkaline cellulase, 7 soil sample showing pectinolytic activates from that plates 6 isolates for pectinase and 11 soil sample showing cellulolytic activates from that plates 9 isolated colonies for xylanase producing microorganisms. Colonies were checked for purity by Gram staining.

Secondary Screening

On CMC agar plate for Alkaline Cellulase with pH 10, Pectin agar Plate for Pectinase and xylan agar plates were used to obtain xylanolytic Organism. Selected isolates were spread on CMC agar plate, Pectin agar and xylan agar plates to obtain clear zone as shown in Figure 4:1.

Table 3. 1 Screening of Alkaline cellulolytic, Pectinolytic and xylanolytic isolates by zone of hydrolysis.

Isolates	**Diameter of Clear zone (mm)**
Alkaline Cellulase	

AC4	**26**
AC11	**23**
AC14	**27**
Pectinas	
PE3	**17**
Xylanase	
XY5	**9**
XY7	**10.5**

Figure 3.1 Secondary Screening Alkaline Cellulase

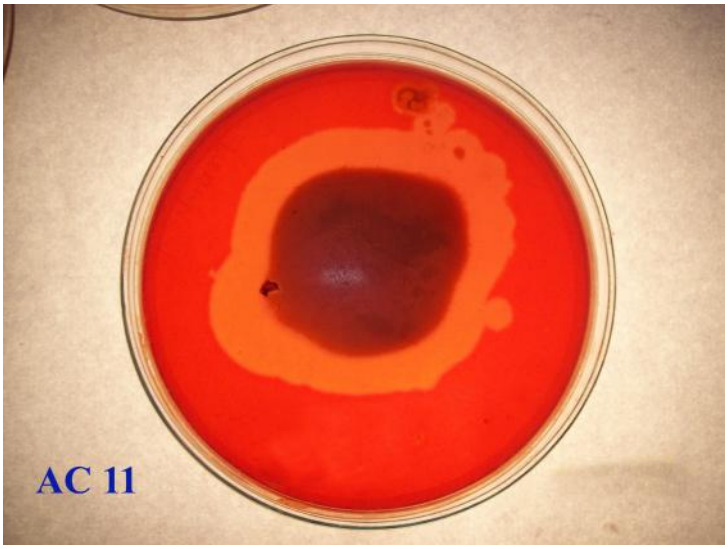


Figure 3.2 Secondary Screening Pectinase

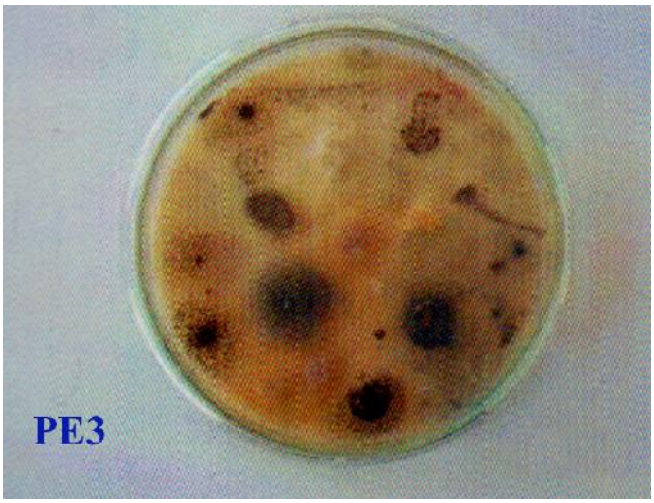


Figure 3.3 Secondary Screening Xylanase

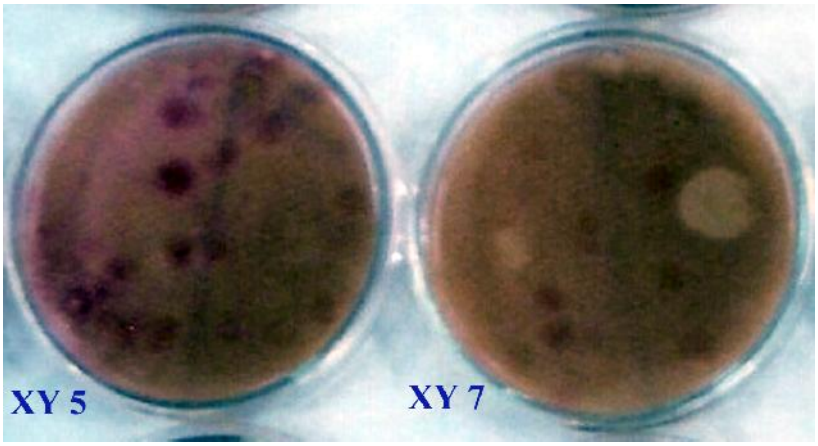


Figure 3.4 Gram staining of Alkaline Cellulase producing isolates.

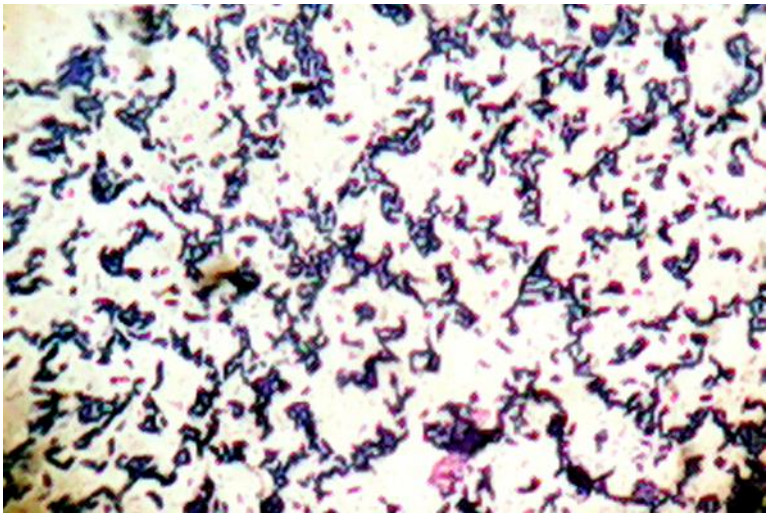
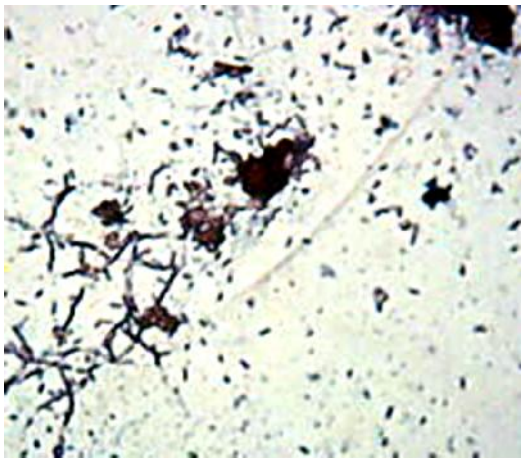


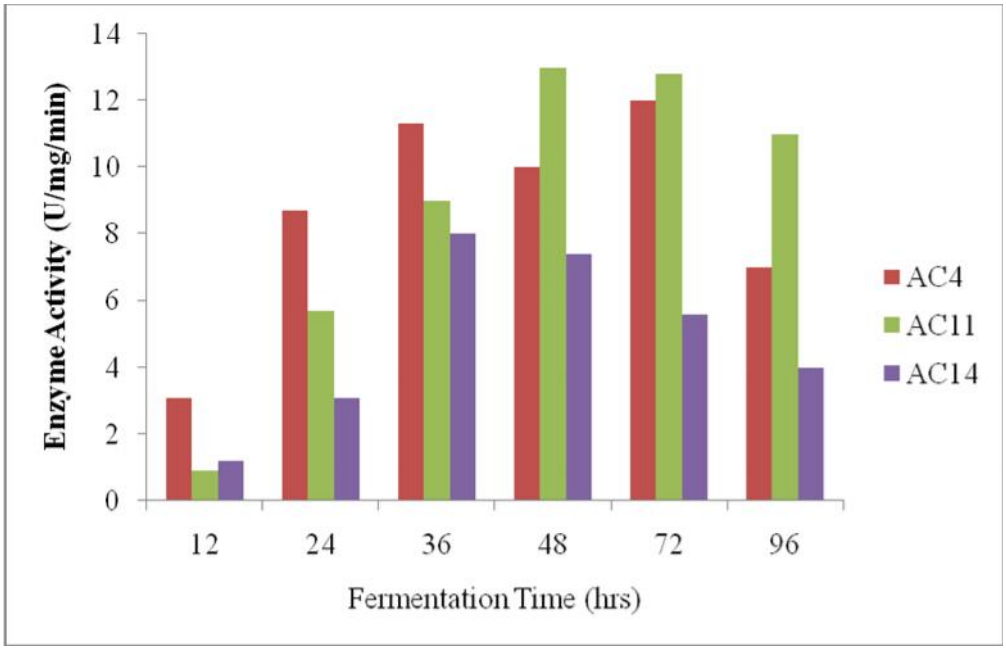
Figure 3.5 Gram staining of Xylanase producing isolates.



Effect of fermentation time

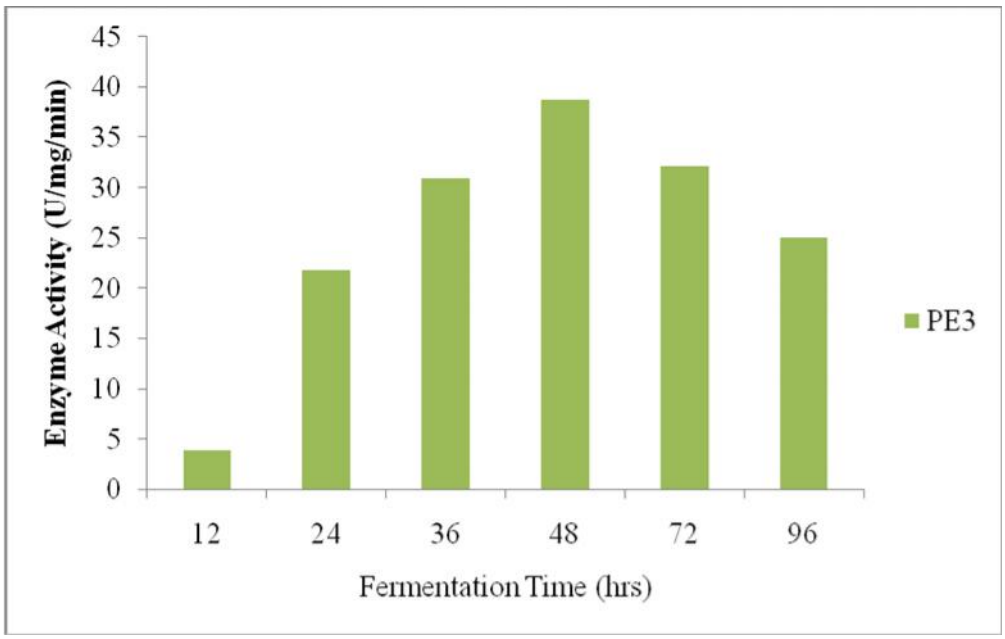
The effect of fermentation time on alkaline cellulae, pectinase and xylanase production by isolates shown in following graph 3.1, 3.2 And 3.3 respectively.

Graph 3.1: Effect of fermentation time of Alkaline Cellulase.



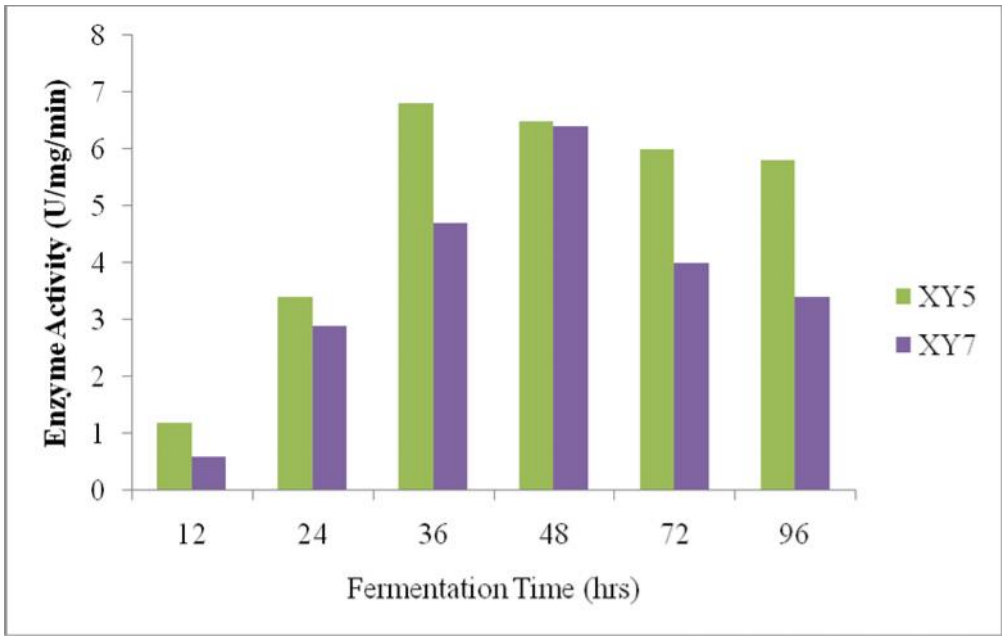
From the above result for the production of alkaline cellulase enzyme AC4 having high enzyme activity (12 U/mg/min) at 72 of fermentation time, AC11 having high enzyme activity (13 U/mg/min) at 48 hrs and (12.8 U/mg/min) at 72 hrs fermentation time and AC14 having high enzyme activity (8 U/mg/min) at 36 of fermentation time.

Graph 3.2: Effect of fermentation time of Pectinase.



PE3 showing high enzyme activity (38.8 U/mg/min) at 48 of fermentation time.

Graph 3.3: Effect of fermentation time of xylanase.



According to result XY5 having high enzyme activity (6.8 U/mg/min) at 36 hrs of fermentation time while XY7 having high enzyme activity (6.4 U/mg/min) at 48 hrs.

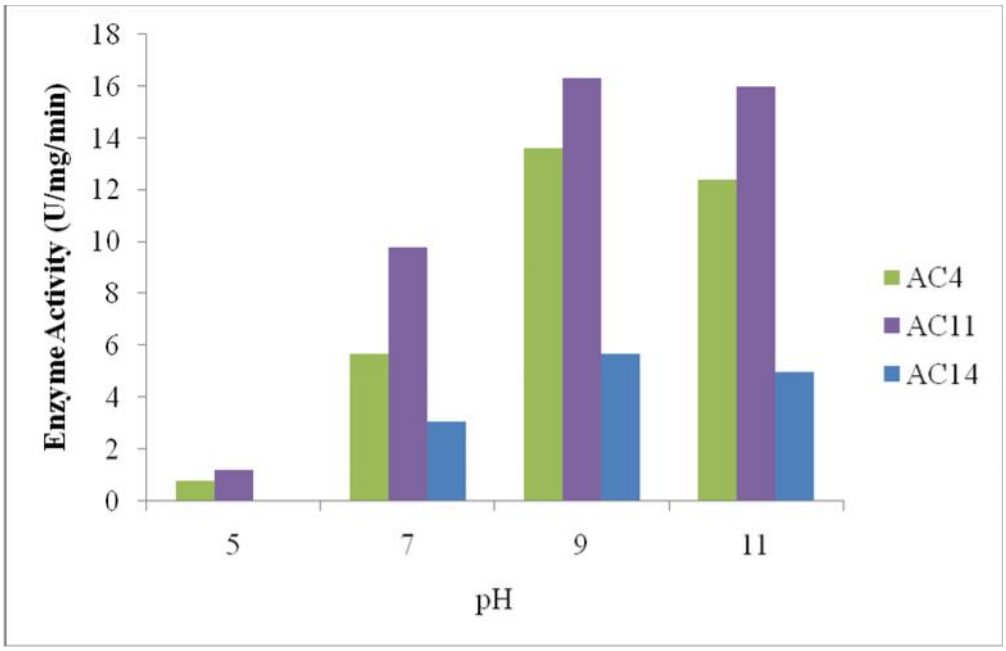
Overall form above result the 48 hrs and 72 hrs preferred for Alkaline cellulose, 48 hrs for pectinase and 48 hrs for xylanase production.

Optimization of pH

Selection of initial pH is necessary for microbial metabolism and enzyme production. Effect of media pH on alkaline cellulose, Pectinase and xylanase production was estimated by culturing the strain in fermentation media with different buffers ranging between pH 5 and 11(pH 5, pH 7, pH 9 and pH 11).

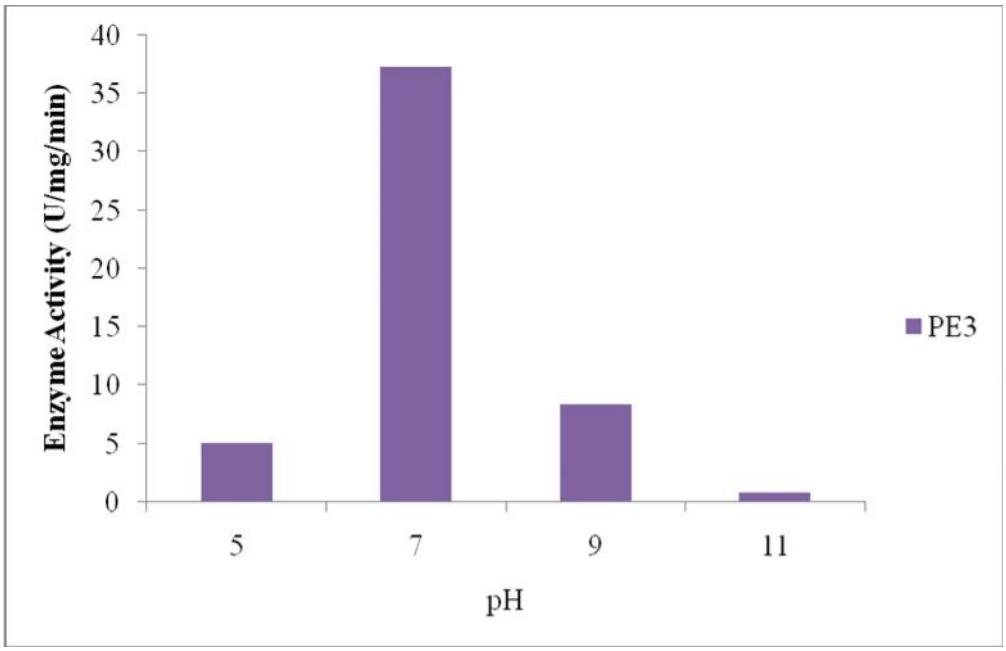
The effect of pH on alkaline cellulae, pectinase and xylanase production by isolates shown in following graph 3.4, 3.5 And 3.6 respectively.

Graph 3.4: Effect of pH on Alkaline Cellulase.



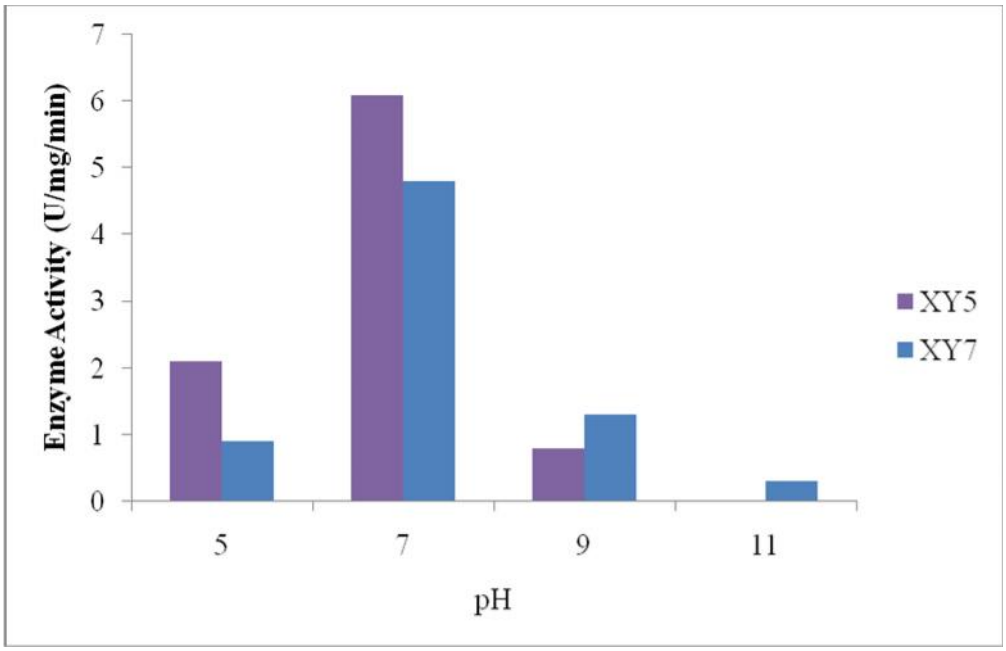
From the above result for the production of alkaline cellulase enzyme AC4 (13.6 U/mg/min), AC11 (16.3 U/mg/min) and AC14 (5.7 U/mg/min) showed maximum enzyme activity at 9 while moderate activity showed at pH 11 with 12.4 U/mg/min, 16 U/mg/min and 5 U/mg/min respectively.

Graph 3.5: Effect of pH on Pectinase production.



pH 7 preffered for PE3 with enzyme activty 37.3 U/mg/min for the fermentation process.

Graph 3.6: Effect pH on xylanase production.

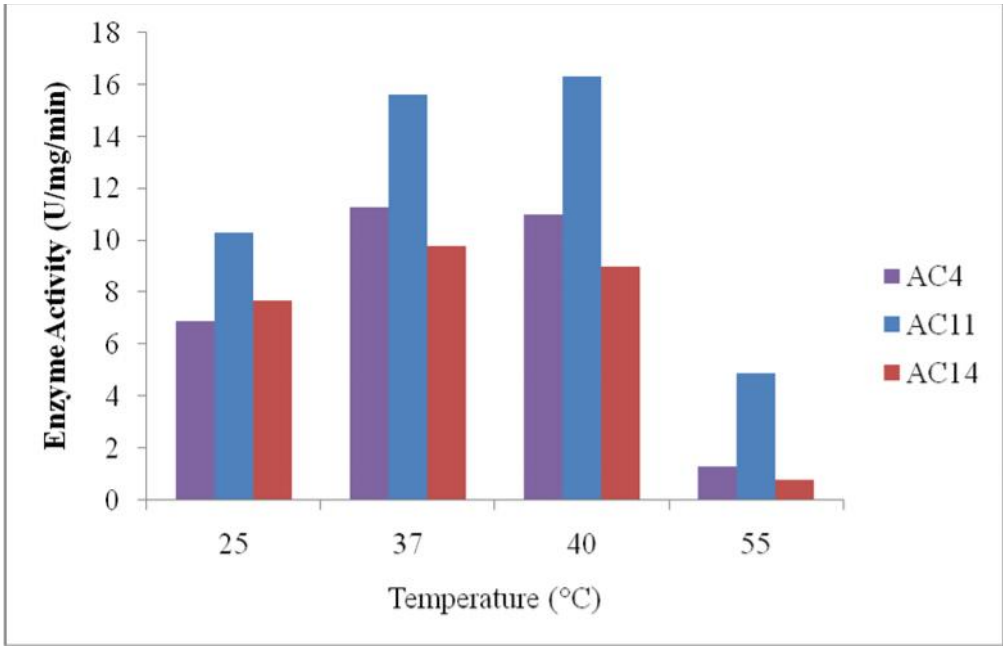


Above result of isolates XY5 (12 U/mg/min) and XY7 (13 U/mg/min) showed maximum activity at pH 7.

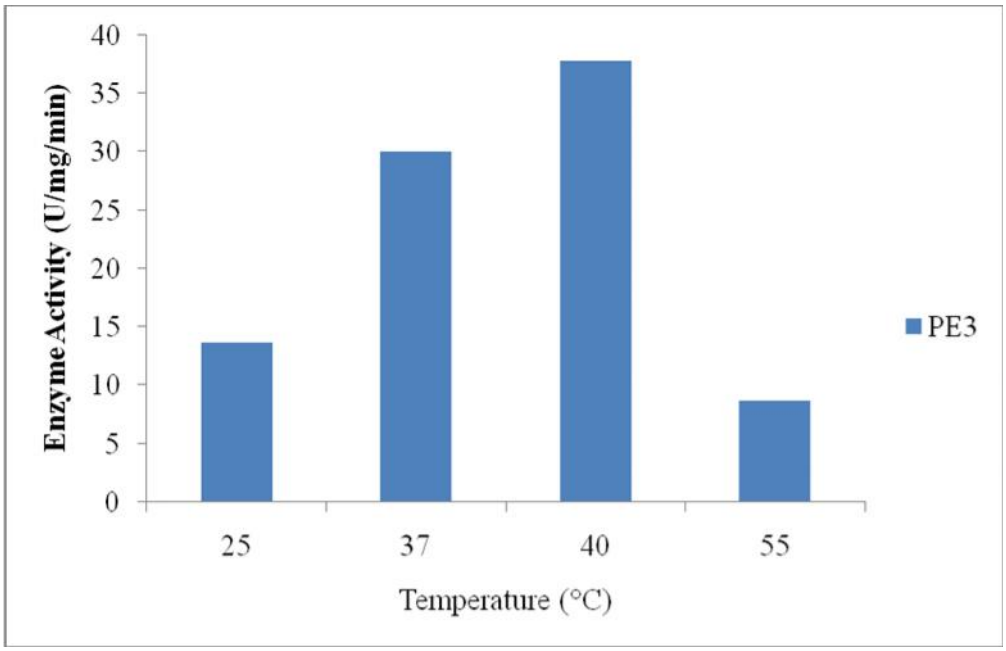
Optimization of temperature

Aiming at finding the best temperature for alkaline cellulose, pectinase and xylanase production, isolates were grown in various temperature range 25°C to 55°C. Graph 3.7, 3.8 and 3.9 showed the result of effect of various temperature range. At 37° the alkaline cellulase and xylanase isolates having showing maximum activity. While pectinase isolates shown maximum activity at 25°C.

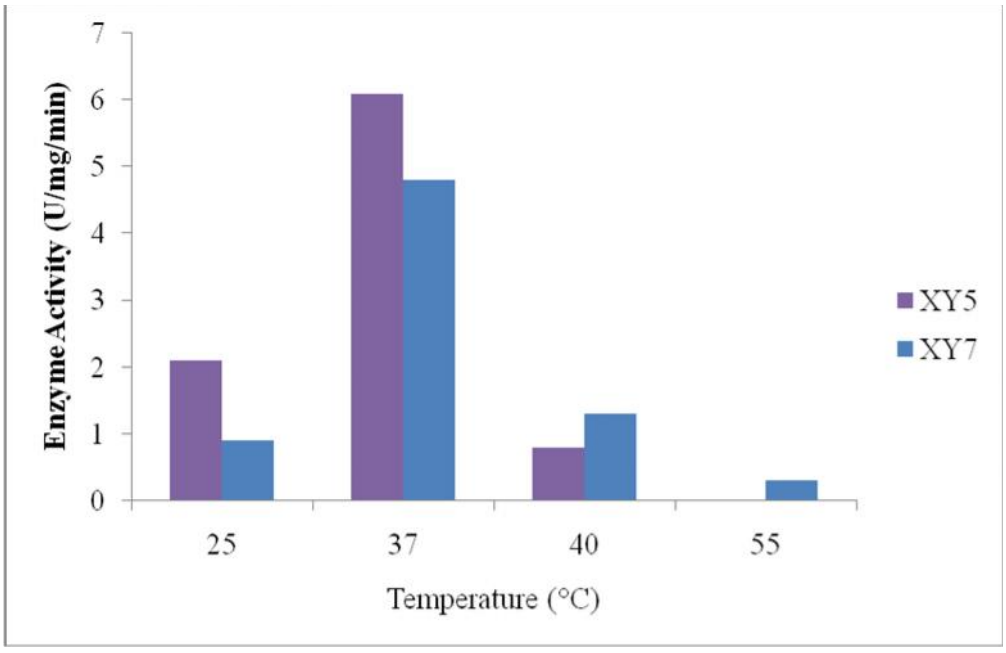
Graph 3.7: Effect temperature on Alkaline cellulase production.



Graph 3.8: Effect temperature on pectinase production.



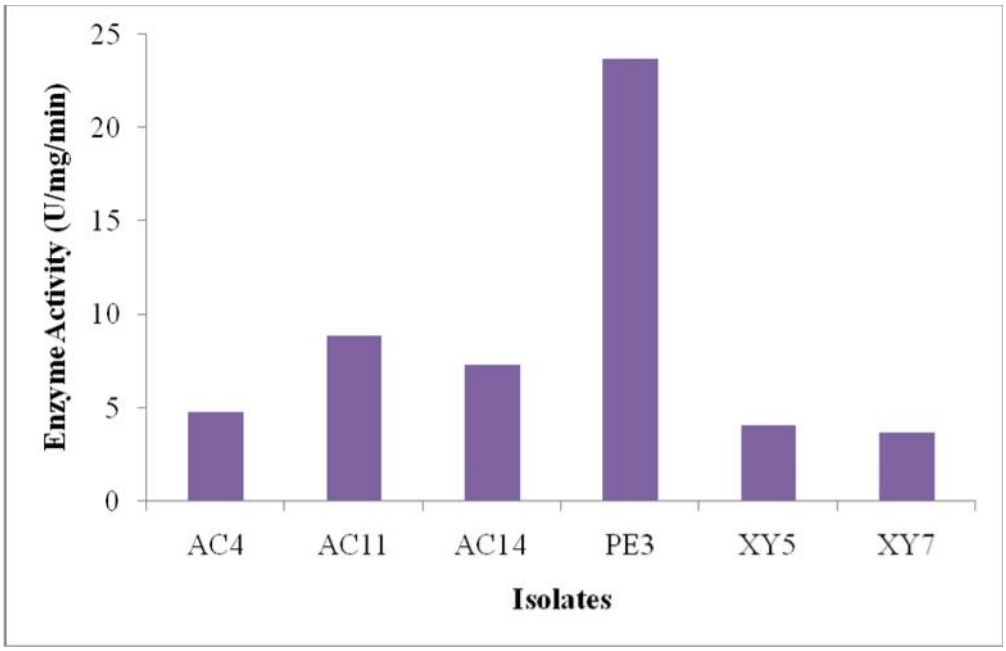
Graph 3.9: Effect temperature on xylanase production.



Selection of agricultural wastes/residues as Substrate:

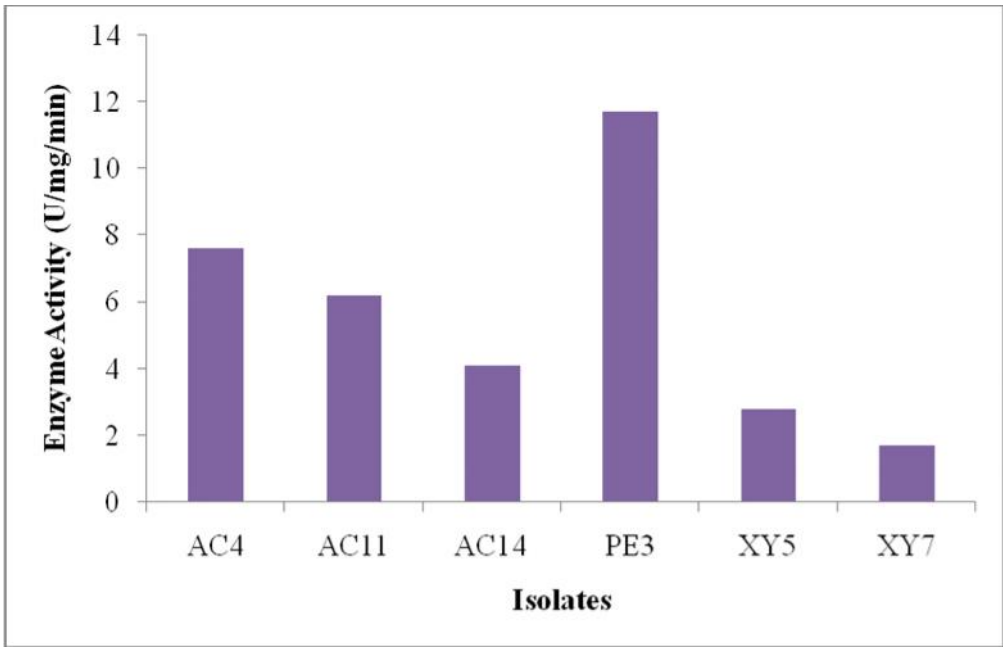
Various agricultural wastes were tested as substrate for solid state fermentation. The agro-residues were used as a sole carbon source with mineral salt solution for alkaline cellulose, pectinase and xylanase production.

Graph 3.10: Enzyme production from Wheat bran.



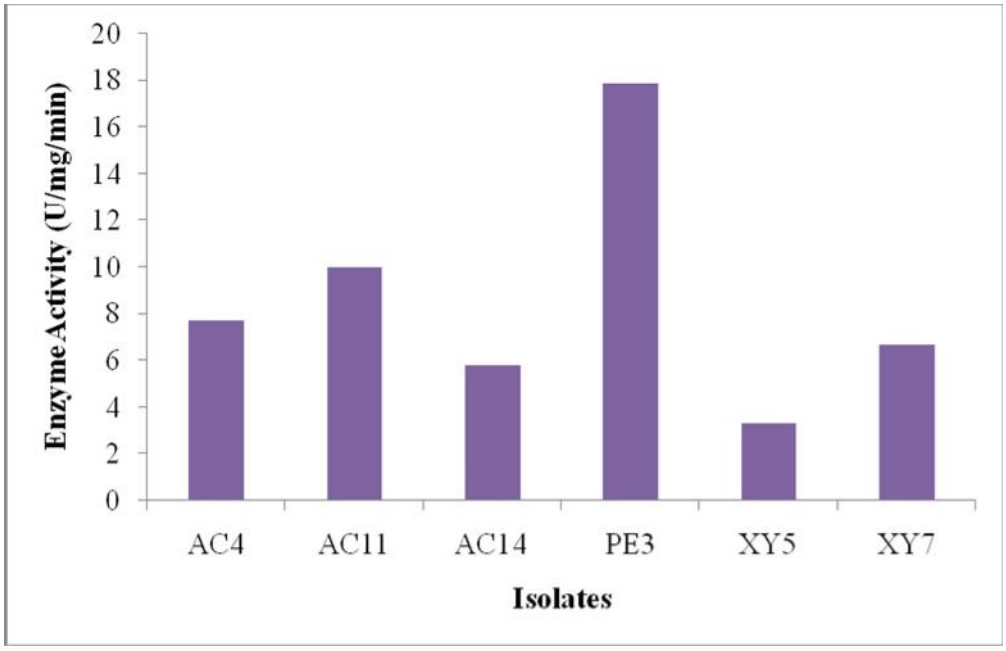
AC11 produced maximum alkaline cellulose (8.9 U/mg/min), PE3 produced pectinase production (8.9 U/mg/min) and XY5 (4.1 U/mg/min) and XY7 (3.7 U/mg/min) produced equal amount xylanase production form wheat barn.

Graph 3.11: Enzyme production from Rice husk.



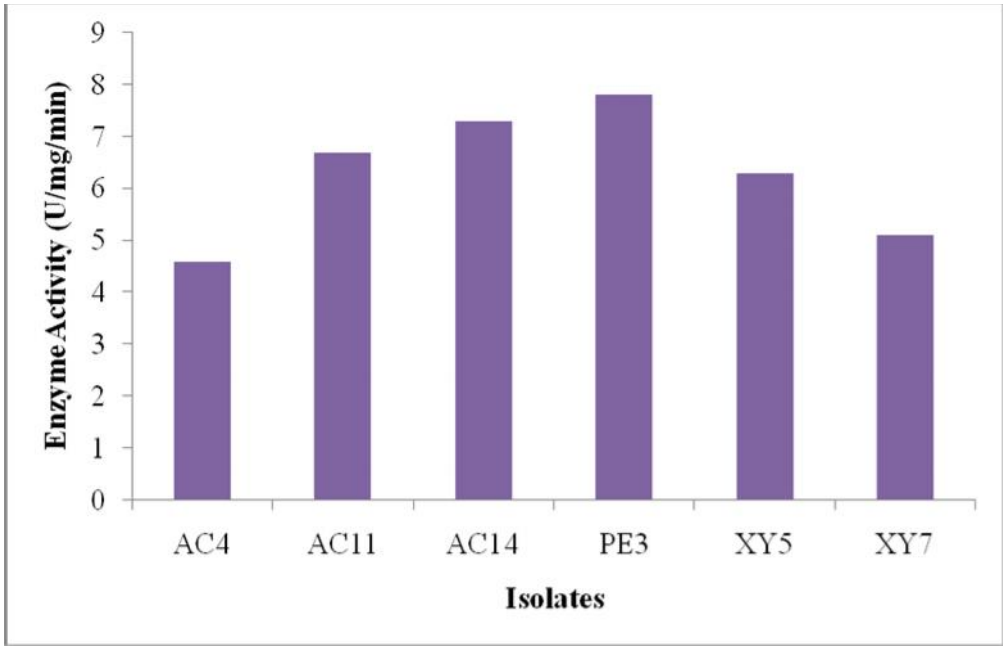
AC4 produced maximum alkaline cellulose (7.6 U/mg/min), PE3 produced pectinase production (11.7 U/mg/min) and XY5 (2.8 U/mg/min) produced xylanase form Rice Husk.

Graph 3.12: Enzyme production from Rice Straw.



AC11 produced maximum alkaline cellulose (10.0 U/mg/min), PE3 produced pectinase production (17.9 U/mg/min) and XY7 (6.7 U/mg/min) produced xylanase form Rice Straw.

Graph 3.12: Enzyme production from Groundnut cake.

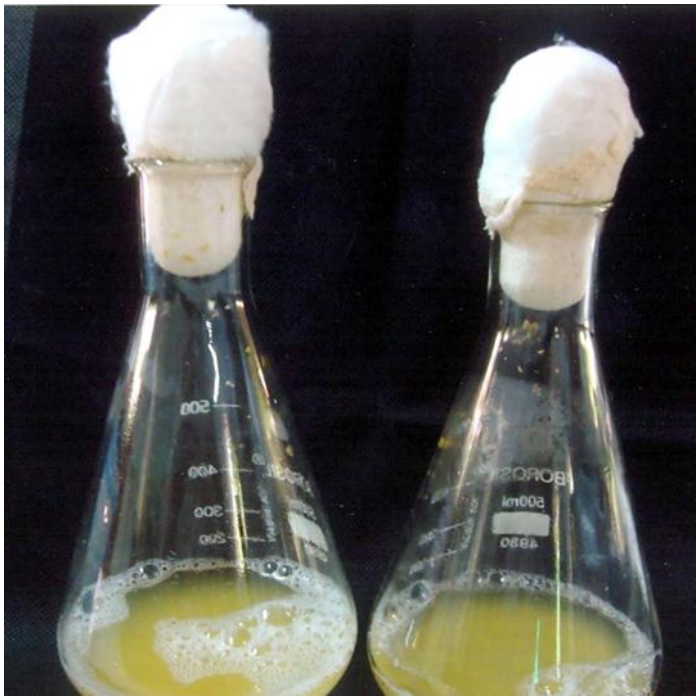


AC11 and AC14 produced maximum alkaline cellulose (6.7 U/mg/min and 7.3 U/mg/min), PE3 produced pectinase production (7.8 U/mg/min) and XY5 (6.3 U/mg/min) and XY7 (5.1 U/mg/min) produced xylanase production form Groundnut cake.

Pretreatment of substrate:

The agro residues used in the study as main substrate was pretreated with different concentration of NaOH. After pretreatment, substrate was used alkaline cellulose, pectinase and xylanase activity and results are presented in tabular form. Results indicated that alkali treatment was effective method for increasing alkaline cellulose, pectinase and xylanase activity. 1% NaOH was found optimum level for pretreatment of lignocellulosic wastes.

Figure 3.3 Inoculum flasks for SSF Medium



Recovery and Partial purification:

Extraction of crude enzyme

The culture was harvested using 50mM phosphate buffer pH 7 for SSF. Then cells were removed by centrifugation at 10,000 rpm for 20 min at 4°C. Extracted cell-free supernatant was used as crude enzyme. Crude enzyme was assayed for alkaline cellulose, pectinase and xylanase activity and protein content.

Parital purification by Ammonium sulphate

Freshly extracted crude enzyme was subjected to ammonium sulphate precipitation at different saturation levels (20-60%).Each fraction was tested for protein content and alkaline cellulose, pectinase and xylanase activity. In the present study, Ammonium sulpate precipitation of alkaline cellulose, pectinase and xylanase showed maximum activity.

Purification by chilled acetone:

Acetone was used for further purification of semi purified alkaline cellulose, pectinase and xylanase with different saturation levels (10-90%). Each fraction was tested for protein content and alkaline cellulose, pectinase and xylanase activity. Fractionation with 30% chilled acetone precipitation showed maximum alkaline cellulose, pectinase and xylanase activity .

Enzyme assay by DNS method:

According to the International Union of Biochemistry, one international unit of alkaline cellulose, pectinase and xylanase (1 IU) corresponds to the amount of enzyme required to release 1 micromole of reducing sugar in 1 min under standard condition. alkaline cellulose, pectinase and xylanase activity was calculated using following standard graph.

Protein estimation by Lowry's method

Protein contain of enzyme extracted was determined by Lowry's method. The intensity of blue color developed by the reduction of the phosphomolyb dicphosphotungstic components in the Folin-Ciocalteau reagent by the amino-acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartrate are measured.

PILOT PLANT PRODUCTION OF XYLANASE BY SSF

CHAPTER 4

SUMMARY AND CONCLUSION

Industrial enzymes find wide applications in the areas of textile industries, food and feed industries, pharmaceutical industries, bio-treatment of organic wastes, healthcare industries, etc. Since the production of enzymes by microbial fermentation is cheaper, faster and easily manageable, microbial fermentation technologies are widely followed worldwide for industrial enzyme production. Among the industrial enzymes used worldwide, proteolytic enzymes or proteases account for nearly 60% of the market for industrial enzymes in the world. They find application in a number of biotechnological processes, viz. food processing and pharmaceuticals, leather, detergent, baking, textile and brewing industries, animal feed, etc.

Agricultural residues are utilized by microbial fermentation technologies for the production of enzymes. Studied enzymes are mostly produced by using agricultural resides using microorganism, the fermentation process. It is always economical if a cheap and easily available material is used as a substrate for fermentation & study alkaline cellulose, pectinase and xylanase producing bacteria which can be exploited to carry out bioconversion of agricultural residues to produce industrial valuable enzymes.

Eventually, form the selected 77 soil samples from various areas, 14-isolates were selected for alkaline cellulose production, 6 isolates were selected for pectinase and 9 isolates were selected for xylanase producing microorganisms. The potent enzyme producers for each enzyme was selected specified code was given for alkaline cellulose isolates AC4, AC11 & AC14 were selected, for pectinase PE3 was selected, while for xylanase XY5 and XY7 isolates were selected.

For the maximizing the activity, different parameter were optimized and were found that alkaline cellulase maximum produced at 48 hrs of fermentation time by AC11 (13 U/mg/min), maximum pectinase produced at 48 hrs by PE3 (38.8 U/mg/min) while XY5 produced maximum xylanase (6.8 U/mg/min) production at 36 hrs of fermentation time.

The maximum alkaline cellulase activity obtained by isolates AC11 at pH 9 (16.3 U/mg/min) and pH 11(16.0 U/mg/min), maximum pectinase activity obtained by isolates PE3 at pH 9(37.3 U/mg/min) and maximum xylanase activity obtained at pH 9 (6.1 U/mg/min).

At 40 °C, alkaline cellulase activity observed by AC4 (11.0 U/mg/min), AC11 (16.3 U/mg/min), AC14 (9.0 U/mg/min), maximum pectinase activity observed by isolates PE3 (37.8 U/mg/min) and maximum xylanase activity obtained at XY(6.8 U/mg/min) and XY7(5.5 U/mg/min).

AC11 produced maximum alkaline cellulase by fermenting rice bran (10 U/mg/min) compared to other agro residues, PE3 produced maximum pectinase from what bran having activity 23.7 U/mg/min from while XY5 produced xylanas from groundnut cake with activity 6.3 U.mg/minand XY7 produced xylanase from rice straw with 6.7 U/mg/min activity.

Present findings indicate that the bacterial isolates can be exploited for production of alkaline cellulose, pectinase and xylanase by Solid State fermentation technology.

The isolates exploited in the present investing for the production of alkaline cellulose, pectinase and xylanase will be further investigated for 16s RNA analysis and species level identification.

It is also important to emphasize that in order for bioconversion biotechnology processes need to be developed on a much wider scale, also novel enzymes and engineering enzymes with improved activities suitable for industrial scale application.

REFERENCES

1. Bajpai, P., (1999). Application of enzymes in the pulp and study industry. Biotechnol. Prog., 15(2):147-157.
2. Beg QK, Kapoor M, Mahajan L, Hoondal GS. (2001). Microbial xylanases and their industrial applications a review. Appl Microbiol Biotechnol. 56: 326–338
3. Goyal, M., Kalra, K. L., Sareen, V. K., Soni, G.(2008), Xylanase production with xylan rich lignocelulósico wastes by a local soil isolate of Trichoderma viridae. Braz. J. Microb., 39, 535-541.
4. Howard, R. L, Abotsi, E., Jansen van Rensburg, E. L., and Howard, S. (2003). Lignocellulose biotechnology: Issues of bioconversion and enzyme production. Afr. J. Biotechnol. 2(12): 602-619.
5. Kitsada, W and Pairoh, P(2000) Purification and Characterization of Xylanases from Streptomyces sp. PC22 J. Sci. Res. Chula. Univ., Vol. 25.(2)245-258.
6. Lapuerta M, Hernández J. J., and Rodríguez J. (2004). Kinetics of devolatilisation of forestry wastes from thermogravimetric analysis. Biomass Bioenergy. 27(4):385391 doi:10.1016/j.biombioe.2003.11.010.
7. Lee B, Vasmatzis G. Stabilization of protein structures. Curr. Opin. Biotechnol. 8 (1997) 423-8 .
8. Mahro, B., Timm, M., (2007). Potential of Biowaste from the Food Industry as a Biomass Resource. Eng. Life Sci. 7: 457-468.doi :10. 1002/ elsc. 200620206.
9. Marais, S. (2008) Enzymatic Hydrolysis with Commerical enzymes of a xylan extracted from Hardwood pulp.thesis submitted to University of Pretoria.
10. Mtui, G. and Nakamura, Y. (2005): Bioconversion of lignocellulosic waste from selected dumping sites in Dares Salaam, Tanzania Biodegradation, 16(6): 493-499 doi: 10.1007/s10532-004-5826-3.

11. Nadia, H. Abd El-Nasser; Amal, M.Ali and Abeer, A. Keera(2010). XylanaseProduction by Streptomyces Lividans(NRC) and It's Application onWaste Paper Australian Journal of Basic and Applied Sciences, 4(6): 1358-1368.
12. Poorna, C.A. (2011) Purification and Biochemical Characterization of Xylanases from Bacillus Pumilus and their Potential for Hydrolysis of PolysaccharidesFermentat Technol1:1doi:10.4172/fmt.1000101.
13. Prasad S, Singh A, Joshi H (2007). Ethanol as an alternative fuel from agricultural, industrial and urban residues. Reasourc. Conserv. Recycling, 50(1): 1-39 doi: 10.1016/j.resconrec.2006.05.007.
14. Rubin EM (2008). Genomics of cellulosic biofuels. Nat. 454(14): 841-845. doi: 10.1038/nature07190.
15. Sanghi, A, Garg, N, Sharma, J, Kuhar, K Kuhad, RC.and Gupta, VK. (2008.) Optimization of xylanase production using inexpensive agro-residues by alkalophilicBacillussubtlisASH in solid state fermentation. World J. Microbiol. Biotechnol., 24:633-64.
16. Schenk PM, Thomas-Hall SR, Stephens E, Marx UC, Mussgnug PostenJHC, Kruse O, Ben Hankamer B (2008). Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production. J. BioEnergy Res. 1(1): 20-43. doi: 10.1007/s12155-008-9008-8.
17. Sun Y, Cheng J (2002). Hydrolysis of lignocellulosic materials for ethanol production: A review. Bioresour. Technol. 83(1): 1-11. doi:10.1016/S0960-8524(01)00212-7.
18. Tsao , G . T . (1 9 7 8) Cellulosic Material as a Renewable Resource. Proc. Biochem. (13): 12- 14
19. Xiao C, Bolton R, Pan WL (2007). Lignin from rice straw kraft pulping: Effects on soil aggregation and chemical properties. Bioresour. Technol. 98(7): 1482-1488. doi:10.1016/j.biortech.2005.11.014.

20. Yan L, Shuya T (2006). Ethanol fermentation from biomass resources: Current state and prospects. Appl. Microbiol. Biotechnol. 69(6): 627-642. doi: 10.1007/s00253-005-0229-x.
21. Zhu S, Yu Z, Wu Y, Zhang X, Li H, Gao M (2005a). Enhancing enzymatic hydrolysis of rice straw by microwave pretreatment. Chem. Eng. Communications, 192(12): 1559-1566(8). doi:10.1080/ 009864491007750.