

Comparative Study on Production of Cellulase in Fresh and Sea Water by *Fursarium subglutinans* MTCC 11891

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CERTIFICATE

This is to certify that the thesis entitled “**Comparative Study on production of cellulase in fresh and sea water by *Fursarium subglutinans* MTCC 11891** by **SHARMILA D (212BM2004)**, in partial fulfilment of the requirements for the award of the degree of Master of Technology in Biotechnology during the session 2012-2014 in the Department of Biotechnology and Medical Engineering, National Institute of Technology Rourkela, is an authentic work carried out by her under my supervision and guidance. To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University/Institute for the award of any Degree or Diploma.

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ABBREVIATIONS

AAS - Atomic absorption spectroscopy

CMC - Carboxyl methyl cellulose

DNS - Di-nitro salicylic acid

mg - Milligram

µg - Microgram

ml - Millilitre

SEM - Scanning electron microscope

Units/ ml/ min- International unit of enzyme

nm – Nanometre

CCD - Charge-coupled device

CMOS – Complementary metal–oxide–semiconductor

STR - Stirred tank reactor

UV – Ultra violet

FPA – Filter paper assay

CBH - Cellobiohydrolases

EG – Endo glucanase

DPX – Di-butyl phthalate and Xylene

MTCC - Microbial Type Culture Collection

PDA – Potato dextrose agar

rpm – Revolutions per minute

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ABSTRACT

Cellulase is one of the most required industrial enzymes and it is the costliest process in bio fuel production. The present research work is based on the production of cellulase enzyme from agricultural waste like rice straw. Sea water was used as a media with the help of *Fusarium subglutinans*. The fungal strain was isolated from harvested rice straw and cellulose was produced by DNS method. The pre-treatment of rice straw was done using alkali treatment and minced well to make the cellulose structure to be simple. The morphology of the pre-treated rice straw was observed using scanning electron microscopy (SEM). The sea water was characterized using atomic adsorption spectroscopy to find the presence of metal ions like iron, cobalt, zinc which is essential for the growth of fungus. The enzyme production was carried out by submerged fermentation in a shaker incubator at 37°C for 21 days. The production was estimated at regular interval of time by reducing sugar analysis of cellulose to glucose. The enzyme produced was characterized using filter paper assay and analysed for its activity in different environments like pH, temperature and metal ions (copper, zinc, cobalt, magnesium, manganese, iron). The produced enzyme was then purified using anion exchange chromatography and checked for its activity. The production and activity of the enzyme was found to be better when compared to normal strains. When 5 mM metal ions like iron and manganese was added, the activity was even more than normal strain. The purified enzyme can be used for the hydrolysis of cellulose in juice production, as animal feed and the unprocessed enzyme can be used for bio fuel production and paper pulp industry.

Keywords: Cellulose, DNS assay, *Fusarium subglutinans*, chromatography, bio-fuel production.

Chapter-1

Introduction

1.1 Cellulase

Cellulases are a group of enzymes produced primarily by fungi, bacteria, and protozoans that hydrolyze cellulose [1]. Termites and microbial intestinal symbionts of some termites also produce cellulase. Several kinds of cellulases are structurally and mechanistically different [2].

Five different types of cellulases are known based on catalytic action they undergo. They are:

- Endocellulase – It randomly cleaves internal bonds at amorphous sites and creates new chain ends.
- Exocellulase – It cleaves two to four units from the ends of the exposed chains produced by endocellulase, resulting in formation of cellobiose.
- Cellobiase or beta-glucosidase – It breaks down the product of exocellulase enzyme into individual monosaccharides.
- Oxidative Cellulases – It depolymerizes cellulose by radical reactions, as for instance cellobiose dehydrogenase.
- Cellulose Phosphorylases – It depolymerize cellulose using phosphates instead of water.

Primary activity of cellulase is to breakdown cellulose to beta-glucose. This kind of cellulase is produced in symbiotic intestinal bacteria of herbivores. Besides ruminants, most animals don't produce cellulase intrinsically and are able to partially break down cellulose through fermentation. Thus, they cannot use energy from fibrous plant material [3]. Enzymes that hydrolyze hemicellulose are said to be hemicellulase and are usually classified under cellulase. Enzymes cleaving lignin are occasionally classified as cellulase, but this is considered incorrect [4].

1.2 Cellulose

Cellulose is a main renewable form of carbohydrates, with about 10^{11} tons synthesized annually. It is an unbranched β -1, 4-linked homopolymer of glucose, but cellulose have lots of variation in their chain lengths according to origin and in the degrees of interaction between chains [4, 5]. Cellulose is an important component of the primary cell wall of angiosperms and algae. Some species of bacteria secrete it into biofilms. Cellulose is the most abundant organic polymer on earth. The cellulose content of cotton fiber is 90%, wood is 40–50% and dried hemp is about 45%. It has the greatest potential for bioethanol production [5]. However, cellulose must be hydrolyzed to obtain fermentable sugars and the cellulolytic enzymes are central to the processing of biomass for bioethanol production [6].

1.3 Cellulose degradation

The two major ways to degrade cellulose to glucose are: Chemical and Enzymatic. Each molecule of cellulose is an unbranched polymer of 1 to 1000 million D-glucose units, linked together with beta-1, 4 glycosidic bonds. However, they differ in the crystalline structures and bindings by other biochemicals [7].

Two types of hydrogen bonds are present in cellulose molecules: one kind that forms between the CH₃OH group and the oxygen in the pyranose ring within the same molecule and the other one that forms between the C₆H₅OH group of one molecule and the oxygen of the glucosidic bond of another molecule. Ordinarily, the beta-1, 4 glycoside bonds easy to break. However, because of these hydrogen bonds, cellulose forms very tightly packed crystallites. These crystals are sometimes so tight that fluids (such as water, cellulase) cannot enter and break or dissolve; only *exoglucanase*, a subgroup of cellulase that attacks the terminal glucosidic bond, is able to effectively degrade it [8]. Water's inability to penetrate cellulose explains why cellulose is insoluble. On the other hand, amorphous cellulose allows the penetration of *endoglucanase*, another subgroup of cellulase that catalyzes the hydrolysis of internal bonds. This difference in the crystalline structure is that the hydrolysis rate is much faster for amorphous cellulose than crystalline cellulose [9]. The process of breaking the glucosidic bonds that hold the glucose basic units together to form a large cellulose molecule is called *hydrolysis* because a water molecule must be supplied to render each broken bond inactive. The chemical compounds surrounding the cellulose in plants, (for example lignin) also limit the diffusion of the enzyme into the reaction sites and thus play an important role in determination of hydrolysis rate. Sometimes, wood chips are pretreated with acid at approximately 160°C to strip hemicellulose and lignin before they are treated with an enzyme or a mixture of enzymes. Generally, 20 to 70% glucose yield can be expected after 24 hours [9, 10]. The kinetics of cellulose hydrolysis has been widely studied, and Michealis-Menten types of rate expressions with substrate or product inhibition terms have been proposed to describe the observed reaction kinetics [11].

Many fungi and bacteria produce cellulase and do it extracellularly. Although it is common to refer to a mixture of compounds that can degrade cellulose as cellulase, it is composed of many distinctive enzymes [12]. Recent research has shown that one of the components is relatively inert with the ability of recognizing and attaching itself to the surface of the cellulose mass, in addition to the ability of recognizing and holding onto another protein component that exhibits enzymatic activities. Thus, the chance of reaction is significantly

enhanced by a proximity effect, because the active enzyme is held onto the surface of a solid substrate by an inert protein which acts as a glue [13].

1.4 Fungal source of cellulase enzyme

The conversion of cellulose into glucose is a two-step process in *Trichoderma viride*.

In the first step, beta-1, 4 glucanase breaks the glucosidic linkage to *cellobiose*, which is a glucose dimer with a beta-1, 4 bond as opposed to maltose, a counterpart with an alpha-1, 4 bond. Subsequently, this beta-1, 4 glucosidic linkages is broken by beta-glucosidase.

The species most often used to study the production of cellulase are white-rot fungal cultures of *Trichoderma reesei* and *Trichoderma viride*. Other fungal species often used are *Fusarium solani*, *Aspergillus niger*, *Penicillium funicolsum*, and *Cellulomonas sp* [14]. The bacterial species *Clostridium thermocellum* and *Clostridium thermosaccharolyticum* also represent promising candidates for cellulase production since they are thermophilic (less contamination problem and faster rate at a high temperature), anaerobic (no oxygen transfer limitation), and ethanologenic (conversion of cellulose to ethanol via glucose with a single culture) [14, 15].

Aim

To produce, characterize and purify cellulase using fresh and sea water media by *Fusarium subglutinans* (MTCC 11891) and also to use it in bioethanol production.

Objectives

- To isolate and characterize the cellulase producing fungus from soil.
- Comparative study on the production of cellulase using fresh and sea water media
- To optimize the environmental conditions for maximum cellulase activity.
- To use the produced cellulase in ethanol production.

Chapter -2
Literature review

2.1 Fungi

Fungi preferentially metabolize wood polysaccharides and produce an array of cellulolytic and hemicellulolytic activities that may contribute to the degradation of plant cell wall material. They are also capable of some direct transformation of lignin from the outer layers of the cell walls, however they leave the middle lamella intact [16]. In addition to the important role of fungi in carbon cycling, they are also involved in many biotechnological processes. These processes include: brewing, winemaking, baking, cheese making and the preparation of other fermented food (e.g. tempe, miso, angkak, soy sauce) together with edible mushroom production are the most important fungal applications. Production of enzymes (amylase, cellulase, invertase, lipase, pectinase, proteinase, rennin and xylanase), organic acids (citric, itaconic and lactic acids), antibiotics and other pharmaceuticals (penicillin, mevivolin, cephalosporin, griseofulvin and cyclosporine) by fungi are common processes that have been reviewed [17]. Fungi are able to survive in intense environmental conditions. They can tolerate a broad range of temperature, pH, humidity, salinity, oxygen levels and ultraviolet radiation. Hence, their habitat varies from tropics to poles [18]. They prefer to be in an acidic environment but their metabolism occurs conveniently in pH range of 3.7 and 8.6 [19].

2.2 Cellulose and hemicellulose

Cellulose is commonly found as a crystal and is enclosed by amorphous cellulose hemicellulose and lignin. Crystalline cellulose is resistant to the action of individual cellulases due to structural rigidity. Hence, efficient conversion of cellulose to monosaccharides needs combination of many cellulases. Amorphous regions occur near the crystal surface and are prone to enzymatic attack [20]. The rate of cellulose degradation directly depends on crystallinity index and substances with which the cellulose is associated e.g. Lignin [21].

Hemicelluloses, the third most abundant constituents of plant cell walls found in nature, represent about 20 to 35% of the lignocellulose dry mass. They are heterogeneous polymers of pentoses (D-xylose, L-arabinose), hexoses (D-mannose, D-glucose, and D-galactose) and sugar acids. Links are formed by β -1,4- glycosidic bonds, but β -1,3-, β -1,6-, α -1,2-, α -1,3- and α -1,6- glycosidic bonds have also been reported [reference needed]. Hemicelluloses are chemically bound to or cross-linked to other polysaccharides, proteins and lignin. They are linked to pectin in primary cell walls and to lignin in secondary cell walls. This linked matrix has protective role for the plant cell [22]. The major hemicelluloses of hardwood and annual

plants are xylans (15–30%), which probably interact with lignin and cellulose more than any other hemicellulose. The main hemicelluloses of softwood are galactoglucomannans (15–20%), arabinoglucoroxylan and arabinogalactan. Hemicelluloses are degraded readily due to their amorphous nature and low degree of polymerization (approximately 70–200) as compared to cellulose [23].

2.3 Cellulose and hemicellulose biodegradation

The interaction between both populations leads to a complete degradation of cellulose, which releases CO₂ and water under aerobic conditions, or carbon dioxide, methane and water under anaerobic conditions. The hydrolysis of cellulose in fungi is carried out by cascade of cellulolytic enzymes which are of many kinds like, extracellular endoglucanases, cellobiohydrolases and β -glucosidases, which synergistically degrade cellulose [24]. Endoglucanases (EGs) represent less than 20% of the total protein in *Hypocrea jecorina* (anamorph *Trichoderma reesei*) and preferentially hydrolyse cellulose microfibrils in the amorphous parts of the fibril, releasing new terminal ends. Cellobiohydrolases (CBHs), which may account for 20% to 60 % of the total cellulase proteins of fungi act on the existing or endoglucanase-generated chain ends. In *H. jecorina*, CBH I attack reducing ends and CBH II the non-reducing ends of the fiber. CBHs and EGs can degrade amorphous cellulose, but CBHs are the only enzymes that efficiently degrade the crystalline form of cellulose. Both enzymes release cellobiose molecules. The effective hydrolysis of cellulose also requires β -glucosidases (BGL), which break down cellobiose and release two glucose molecules [25]. Products of cellulose degradation are made available as carbon and nitrogen sources for either cellulolytic organisms or other microbes living in the environment containing cellulosic materials [26]. The ability to produce cellulolytic enzymes is widespread among microorganisms, but only a limited number of species are actually able to degrade native cellulose in its crystalline form. Cellulolytic microorganisms (fungi and bacteria) can establish synergistic relationships with non-cellulolytic species in cellulosic wastes [27]. The hydrolysis of hemicellulose requires similar types of enzymes to those required for cellulose hydrolysis. However, more enzymes are required for the complete degradation of hemicellulose, because of the greater complexity of hemicellulose compared to cellulose [28]. Hemicellulases hydrolyze glycosidic linkages in hemicelluloses and are classified according to their substrate specificities [29]. The total enzymatic degradation of hemicellulose polymers requires the action of “endo-type” enzymes that liberate short oligosaccharides, which are subsequently degraded by sidegroup cleaving enzymes and “exo-type” enzymes and finally monomeric sugars and acetic acid are formed. Similar to the

cellulases, hemicellulases act synergistically [30]. In fungi the production of cellulases may also be regulated by factors other than induction and repression by sugars. Several lignin-related aromatic compounds, which are found in association with cellulose in nature, stimulate or inhibit cellulase production. This feature is common to some wood-rotting white and brown rot basidiomycetes such as *Phlebi aradiata*, *Trametes gibbosa* and *Trametes coriolus* [31].

2.4 Cellulase enzyme

Usually for production of cellulase enzyme filamentous fungi are widely used. Figure 1 shows the multi enzyme have the combination of three enzymes used for cleavage at amorphous site in cellulose. These enzymes have various production levels when it comes to fermentation process and it also depends upon the reactor shape and design [32].

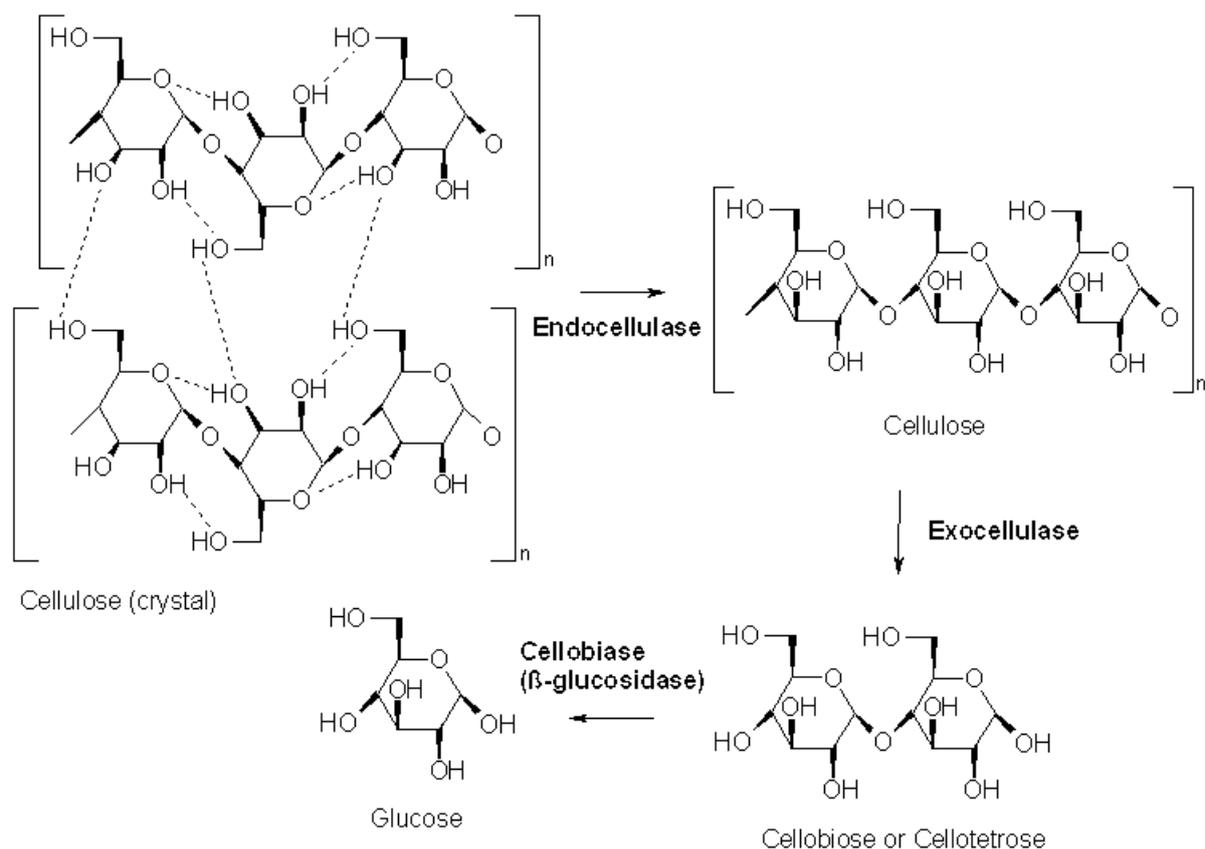


Figure 1 - Conversion of cellulose to glucose by cellulase enzyme [36]

Cellulase activity is good at 50°C in pH 5.8. Cellulase production from *Pencillium funiculosum* displayed better stability over six days of incubation [33]. Cellulase production depends upon biomass and cellulose concentration but it is not only an essential source but it acts as inducer. mRNA quantities of cellulase increased because of gene silencing and down regulation of cre 1 [34]. Maximum production of cellulase was produced by plant pathogenic

fungi, which produce degrading enzymes to break the cell wall polymers of plant and structural complex of substrate (example : If paper or fruit peels were used, it is easy but with kraft pulp was bit tough, because breaking the complex structure is highly complicated, so pretreatment is necessary) [35].

2.5 Pretreatment of ligno cellulosic materials

Pretreatment process is most important to get the easily accessible with cellulose by removing lignin, xylan and other molecules which are main ingredients in lingo cellulosic materials. Physiochemical and structural factors decides the degradation of native lignocellulosic biomass. Pretreatment step is necessary to improve the accessibility of cellulose when we use enzyme as a catalyst. This step changes the degree of polymerization, crystallinity index, lignin-carbohydrate linkages and increase the porosity of lignocelluloses [37, 38]. Selection of pretreatment depends on how much the accessibility to cellulose is obtained. This step is most eminent step in the total cost and time reduction of fermentation process. In this process at high solid loading, when there is release of high amount of sugars then the bottlenecks we have to deal with are mass transfer limitations, increased medium viscosity and generation of high concentration of inhibitors. Cellulosic materials have low density and are highly hygroscopic, thus slurries will become paste like material after pretreatment [39, 40]. To avoid high viscosity issues, caused by pretreatment we have to reduce particle size of slurry [40].

2.6 Submerged fermentation for cellulase production

Submerged Fermentation is a type of fermentation which is used for most of the industrial enzyme production because of its ease of process and separation. It utilizes the liquid substance such as molasses and broth. The biomolecules are secreted into fermentation broth and after the process it's easy to purify it. This process is highly suitable for the microbes that require high moisture conditions e.g. bacteria and Fungus. The yield of product in this fermentation differs for each substrate so it is important to select the correct substrate and conditions for fermentation should be optimized [41]. Cellulase production is carried out in aerobic aseptic culture. The growth medium contains salts, nutrients, surfactant and inducers which are required for the fungus to survive and grow in medium. The important metal ions which are required for cellulose production are iron, cobalt, copper, magnesium, calcium, potassium, manganese and ammonium. The microbial contamination is avoided using antibiotics and the sterilization of equipments before inoculation [42]. Steam sterilization is done at 121°C for 20 minutes. The quantitative aspects of cellulose production were

improved between the years of 1972-1989. The maximum fold increase in enzyme production happened in this period. The progress in cellulose production was averaged doubling every two years due to the strain improvement and optimization of fermentation process. Genencor International, Novo Nordisk, Rohm Finland are few of the companies producing cellulose using submerged fermentation [43].

2.7 Instruments

2.7.1 Optical microscope

The optical microscope, commonly known as the "light microscope", is a type of microscope which uses visible light and a system of lenses to magnify images of small samples. Optical microscopes are the oldest types of microscope and were in compound form in the 17th century. Basic optical microscopes can be very simple, although there are many complex designs which aim to improve resolution and sample contrast [44].

The image from an optical microscope can be captured by normal light-sensitive cameras to generate a micrograph. Originally images were captured by photographic film but modern developments in CMOS and charge-coupled device (CCD) cameras allow the capture of digital images. Purely digital microscopes are now available which use a CCD camera to examine a sample, showing the resulting image directly on a computer screen without the need for eyepieces [45, 46].

2.7.2 Scanning electron microscope (SEM)

A scanning electron microscope (SEM) is a type of electron microscope that produces images of a sample by scanning it with a focused beam of electrons. The electrons interact with atoms in the sample and are used to study sample's surface topography and composition [47]. The electron beam is generally scanned in a raster scan pattern, and the beam's position is combined with the detected signal to produce an image. SEM can achieve resolution better than 1 nanometer. Specimens can be observed in high vacuum, in low vacuum, and (in environmental SEM) in wet conditions [48].

The most common mode of detection is by secondary electrons emitted by atoms excited by the electron beam. The number of secondary electrons is a function of the angle between the surface and the beam [49]. On a flat surface, the plume of secondary electrons is mostly contained by the sample, but on a tilted surface, the plume is partially exposed and more

electrons are emitted. By scanning the sample and detecting the secondary electrons, an image displaying the tilt of the surface is created [50].

2.7.3 UV spectroscopy

UV Spectroscopy is an absorption spectroscopy or reflectance spectroscopy in the ultraviolet-visible spectral region. This means it uses light in the visible and adjacent (near-UV and near-infrared (NIR)) ranges [51]. The absorption or reflectance in the visible range directly affects the perceived color of the chemicals involved. In this region of the electromagnetic spectrum, molecules undergo electronic transitions.

If the compound is more concentrated more light will be absorbed by the sample; within small ranges, the Beer-Lambert law holds and the absorbance between samples vary with concentration linearly. Beer-Lambert law is a law which states that the amount of light absorbed by a body is proportional to the amount of absorbing particles in it. This means that it will be proportional to the concentration of that additive multiplied by the thickness of that body (the path length of the light through the body). Samples are usually prepared in cuvettes; depending on the region of interest, they may be constructed of glass, plastic (visible spectrum region of interest), or quartz (Far UV spectrum region of interest) [51, 52].

2.8 Techniques

2.8.1 Glucose estimation by DNS

This method is used to test the presence of free carbonyl group (C=O), the so-called reducing sugars. Figure 2 reveals the oxidation of the aldehyde functional group present in glucose and the ketone functional group in fructose. Simultaneously, 3, 5-dinitrosalicylic acid (DNS) is reduced to 3-amino, 5-nitrosalicylic acid under alkaline conditions:

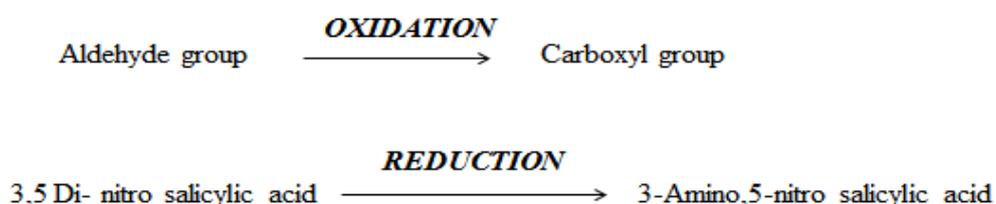


Figure 2 - Oxidation and reduction reactions in reducing sugar analysis

Since dissolved oxygen can interfere with glucose oxidation, sulfite, which itself is not necessary for the color reaction, is added in the reagent to absorb the dissolved oxygen [53].

The above reaction scheme shows that one mole of sugar will react with one mole of 3, 5-dinitrosalicylic acid. However, it is suspected that there are many side reactions, and the actual reaction stoichiometry is more complicated than that previously described. The type of side reaction depends on the exact nature of the reducing sugars [54]. Different reducing sugars generally yield different color intensities; thus, it is necessary to calibrate for each sugar. In addition to the oxidation of the carbonyl groups in the sugar, other side reaction such as the decomposition of sugar also competes for the availability of 3, 5-dinitrosalicylic acid [55].

2.8.2 Filter paper assay

Filter Paper Activity/Assay (FPA) is used to determine the quantity of cellulolytic enzymes required to hydrolyze lignocellulosic biomass. The FPA was initially proposed as a simple, reproducible and quantitative method that predicts enzyme action under practical saccharification conditions. This measures the ability of the cellulose to hydrolyze both crystalline and amorphous regions of cellulose [56]. Therefore, it is generally believed that FPase assay is the best measure of the activities of both endo- and exo- type in a cellulase with a strip of filter paper (Whatman #1, 50mg, 1 X 6 cm) for 1hour reaction at the given temperature [57].

2.8.3 Atomic absorption spectroscopy

This instrumentation is a setup which is used to detect metals and metalloids in sample present in environment. The principle depends upon the fact that ground state metals to absorb light of particular wavelength was sourced and amount of light absorbed can be obtained against a standard curve.

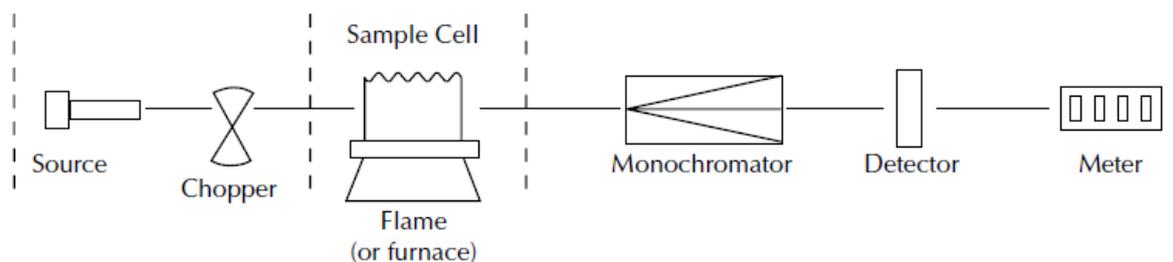


Figure 3 - Basic instrumentation of flame atomic absorption [59]

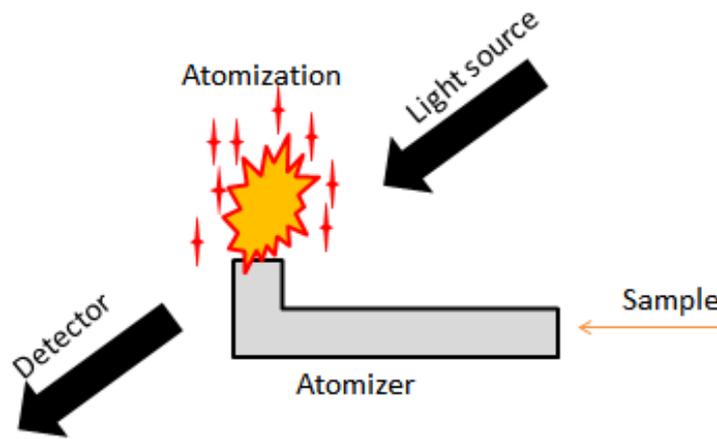


Figure 4 – Schematic of atomization of flame in an absorption spectroscopy

Figure 3 and 4 the basic instrumentation setup has seven fundamental parts (1) Light source (2) Atom cell (3) monochromator (4) Detector (5) Amplifier (6) Signal display (7) Data station.

Hallow cathode lamp is a common light source which has glass tube with an inert gas (neon or argon) at $1\text{Nm}^{-2} - 5\text{Nm}^{-2}$. To produce atoms from the sample two systems are commonly used (1) Sucking a sample solution into the flame (2) Dropping of sample through electro thermal atomization, which is placed inside a heated graphite tube [59, 60].

Chapter - 3

Materials and methods

3.1 Collection of sample

The soil samples were collected from the agricultural fields located on the outskirts of Rourkela city (22.2492° N, 84.8828° E), Sundergarh district, Odisha, India. Rourkela city is strategically located in the northwestern border of the Indian state of Odisha. Over a period of year, the temperature varies from 7°C to 47°C. All soil samples were collected in the month of June, 2013 from six randomly chosen agricultural fields (paddy fields). The collected samples were kept in plastic bags. Samples were stored at 4°C until use for culturing fungi (not more than 48 h).

3.2 Preparation of media

Potato Dextrose Agar (PDA, Himedia) medium was used for the isolation of fungi from soil. 3.9 ml of PDA medium was added to 100 ml of distilled water and autoclaved at 121°C, 15 psi for 15 min.

3.3 Isolation of fungi

Serial dilution method was followed to isolate the fungal strains from soil sample.

3.3.1 Stock Preparation

20 g of soil sample was diluted in 100 ml of sterile distilled water.

3.3.2 Serial dilution

Eight test tubes each containing 9 ml of sterile distilled water was taken. To the first test tube, 1 ml stock was added and mixed well. From the first test tube, 1 ml was transferred to second test tube and mixed well. This was continued till the eight test tubes. From eighth test tube 1 ml of stock solution was discarded. Hence, the dilution factor from first to eighth test tube was 10^{-1} to 10^{-8} . From each test tube three different samples of volume 5 μ l, 10 μ l and 15 μ l were added to PDA plates and were incubated at 30°C for three days [4].

3.4 Preparation of permanent slides

For the preparation of permanent slides, lactophenol and lactophenol with cotton blue stains were used. The slides were sealed with cover slip.

3.4.1 Stain Used

3.4.1.1 Preparation of lactophenol cotton blue

Table 1: Composition for preparation of Lacto-phenol cotton blue

Components	Contents
Cotton Blue	0.5 g
Phenol	25 g
Lactic Acid	25 g
Glycerol (pure)	50 g
Distilled water	100 ml

Lactophenol was readily prepared by heating the phenol in a water bath until it dissolved and then lactic acid and glycerol was added. The volume was then made up to 100 ml with distilled water after which cotton blue was added.

3.4.2 Preparation of permanent slide

A drop of lactophenol blue solution was placed on a slide. Using an inoculating needle, the fungal culture was spread carefully to make a thin layer. An edge of the coverslip was placed on the drop and slowly spread over it. Air bubbles should be avoided under the coverslip. The slide with the fungal culture was heat fixed by passing it carefully over a flame. The slide was then observed under a microscope.

3.4.3 Phenotypic characterization of fungi

Sporulating isolates were identified with the help of standard manuals. Further confirmation of the fungal species was done after observation under a light microscope and pictures were taken using a Sony 8.1 MP digital camera. The fungi samples were sent to Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, Punjab, India for identification.

3.5 Slant culture

The culture made on the slanting surface of a solidified PDA medium in a test tube was tilted to provide a greater area for growth. The microorganisms that showed effective cellulose degrading capacity were maintained as slant culture.

3.6 Identification of cellulase producing fungi using congo red staining method

Congo red staining was done using 0.1% congo red stain and 1% sodium hydroxide solution. The fungal culture was grown in petri plate using PDA medium with 1% carboxyl methyl cellulose (Himedia) for seven days at 37°C in an incubator. Then 15 ml of congo red solution was poured, left for 5 min and destained using sodium hydroxide [5].

3.7 Estimation of reducing sugars using DNS assay

DNS assay was done using DNS reagent (3, 5-dinitrosalicylic acid (DNS) - 10 g, sodium sulfite- 0.5 g, and sodium hydroxide-10 g in 1litre of distilled water) and 40% potassium sodium tartarate. The fungal culture was grown in Mandel's media as a substrate of carboxyl methyl cellulose (CMC) for 21 days. The culture was taken and centrifuged (REMI centrifuge / C- 24BL) at 4°C and 15,000 rpm for 10 min to separate the fungal biomass. Then the crude extract was filtered with silica gel 'G' (Rankem) packed column for removal of large agglomerates in processed broth. Again it was centrifuged under the same condition. After that 3 ml of extract was taken and added to 3ml of DNS reagent and incubated at 90°C for 5 min in water bath [7]. When it was warm, 1 ml of 40 % Rochelle's salt solution was added and observed under UV-visible spectrometer (Double beam spectrop C2203, Systronics) at 510 nm. Reducing sugar present in the sample was calculated using the standard graph plotted by known glucose concentration.

3.7 Pretreatment of rice straw

Pretreatment was done to remove the lignin, hemicellulose and xylan from the rice straw. It was done by alkali treatment using 1 M sodium hydroxide (4 g of sodium hydroxide in 100 ml of distilled water) at 121°C, 15 psi for 20 min in autoclave. The processed rice straw was then filtered using nylon cloth and the liquid was discarded. Then the pretreated rice straw residue was used for cellulase production.

3.8 Collection of sea water

Sea water was collected from Gopalpur, Orissa (19.27°N, 84.92°E) on the Bay of Bengal coast during the month of July, 2013 using 50 ml ficol tube (Tarson) and stored at 4°C. The stored water was filtered using whatman filter paper No. 1 (F1000-C, Himedia) and used for cellulase production process.

3.8.1 Determination of concentration of metal ions in sea water using atomic absorption spectroscopy

Atomic absorption spectroscopy (AANALYST200, PerkinElmer, figure 5) was used to determine concentration of metal ions in sea water. The standard solution for required metal ions was prepared in three different concentrations (1 ppm, 2 ppm, 3 ppm). The sea water was diluted in 5X concentration and passed through the sample holder. The atomization of flame happened through oxygen-nitrous oxide gas. Then according to flame the concentration of metal ions were found.



Figure 5 – Instrumentation of flame atomic absorption spectroscopy

3.9 Production of cellulase using CMC as substrate in Mandel's media and sea water

Enzyme production assay was done using CMC in Mandel's media. The media was prepared according to the mentioned components (Table 2). Then 2 g of substrate (CMC) was added to the media followed by addition of 1 g of fungus as inoculum. The broth was then fermented for 21 days at pH 5.8, 37°C in a shaker set at 300 rpm. After fermentation process, the broth was centrifuged at 4°C, 10000 rpm, following which further analysis was done by DNS method, total filter paper assay [3].

Table 2 – Composition of Mandel’s Media

Ingredients	Contents
KH ₂ PO ₄	2 g/l
CaCl ₂ .H ₂ O	0.4 g/l
MgSO ₄ .7H ₂ O	0.3 g/l
FeSO ₄ .7H ₂ O	5 g/l
MnSO ₄ .4H ₂ O	1.6 g/l
CoCl ₂ .6H ₂ O	1.4 g/l
Distilled water	1 litre

Table 3 - Elemental composition of sea water (%)

Element	Percent	Element	Percent
Oxygen	85.84	Sulphur	0.091
Hydrogen	10.82	Calcium	0.04
Chloride	1.94	Potassium	0.04
Sodium	1.08	Bromine	0.0067
Magnesium	0.1292	Carbon	0.0028

3.10 Enzyme production using pretreated rice straw in Mandel’s media and sea water

Enzyme production assay was done using pretreated rice straw in Mandel’s media. The media was prepared according to the mentioned components in table 2, then 2 g of substrate (Rice Straw) was added to the media. It was followed by addition of 1g of fungus as inoculum. The

broth was then fermented for 21 days at pH 5.8, 37°C in a shaker set at 300 rpm. After fermentation process, the broth was centrifuged at 4°C, 10000 rpm. Further analysis was done by DNS method and total filter paper assay. Similar process was carried out using sea water as media. The composition of both media and sea water is same as shown in Table (2 and 3).

3.11 Effect of time on cellulase production

Cellulase production was assessed at regular intervals to check the production rate. It was done by monitoring the glucose production by breakdown of cellulose. For this, 3 ml of fermentation broth was taken and centrifuged at 4°C and 10000 rpm. DNS reagent was freshly prepared and 3 ml of it was added to the sample along with 1 ml of Rochelle's salt. Then sample's absorbance was taken at 560 nm using a U-V spectrophotometer [61].

3.12 Effect of pH on cellulase activity

The pH of reaction is affected by the enzyme activity. The pH of crude enzyme samples were adjusted to 4, 5, 6, 7, 8 and 9 in different flasks using 0.1 M NaCl and 0.1 M HCl. The samples were later collected and assayed to find the maximum activity [61].

3.13 Effect of temperature on cellulase activity

The temperature of reaction affects the enzyme activity. Beyond a critical temperature, the enzyme activity will decrease. Enzyme activity was assayed in flasks maintained at different temperatures of 30°C, 40°C, 50°C, 60°C, 70°C in a water bath. The cultured filtrates were later collected and assayed to find the maximum activity [61].

3.14 Effect of metal ions on cellulase activity

The effect of different metal ions on enzyme activity was studied. Different metal salts, Mn(II)SO₄, Fe(II)SO₄, Cu(II)SO₄, Mg(II)SO₄ and Zn(II)SO₄ were added to the fermentation broth in concentration of 5mM on to separate flasks and its cellulase activity was studied [61].

3.15 Purification of cellulase

Purification of crude enzyme was done using ion exchange chromatography. Crude enzyme was poured to DEAE sepharose (Sigma Aldrich) packed syringe column which was equilibrated using a buffer (5 mM EDTA, 20 mM tris base (Himedia) at pH 8) shown in figure 6. Elution was done by passing linear gradient of 0.2 M – 1.2 M NaCl through the

column. The eluted enzyme was collected as a fraction of 10 ml and tested for cellulase activity [62].



Figure 6 – Anion exchange chromatography set-up for purification of cellulase.

3.16 Application of crude enzyme extract in ethanol production

Crude enzyme was applied through the silica gel packed column and further subjected to centrifugation at 10000 rpm, 4°C. Saw dust was pretreated using 1 M NaCl and 10 ml of crude enzyme was added along with 2 g of saw dust. It was incubated in the water bath with the following optimized parameters (80°C, pH 5.8, MnSO₄·7H₂O (5 mM) as catalyst) for 1 hr. Then 2 ml of *Saccharomyces cerevisiae* culture was inoculated to the broth. After 5 days, production of ethanol was estimated by gravimetric analysis using the following formula [63].

Chapter - 4

Results and discussion

This chapter is organized in to three sections. Section 4.1 discusses about the results obtained in the isolation, characterization and screening of various fungal cultures for the potential production of cellulases. Section 4.2 deals with the results obtained in various batches for optimizing the environmental conditions such as pH, temperature, time and metal ions to maximize the cellulase production. In addition, evaluating the possibility of growing fungal cultures on sea water rather than the basal medium were also carried out and reported in this section. 4.3 Cellulase activities checked with saw dust was reported. Section 4.4 deals with its purification using anion exchange chromatography technique results.

4.1 Isolation, screening and characterization of fungi

4.1.1 Isolation of fungal cultures

The collected soil samples (100 g of each from the five random locations) on agricultural fields at the depth of 8-10 cm were mixed well and homogenized. The homogenized soil samples were subjected to two different techniques to isolate the fungal cultures present in the soil. One is direct plating technique and the other is serial dilution method. 100 mg of homogenized soil samples were directly sprinkled to the medium and incubated at room temperature for a week in case of direct plating technique. Since the fungal diversity was quite high in the inoculated soil sample and each species occupied a specific niche, merged colonies were observed in this method. Either the inoculated soil volume or the medium level has to be reduced to achieve the better isolation of fungal colonies since the area available for the fungal cultures to spread and occupy their niches will be high in this situation.

The direct plating technique involves the sprinkling of a known amount of soil to the medium. On contrary, the serial dilution technique involves the systematic dispersion of the soil fungi out of which the very minimal amount used to inoculate and incubated further. Since the serial dilution was done up to 10^{-8} times, the fungal cultures could be easily isolated in the respective plates. Three predominant fungal cultures were observed in this petriplate as shown in Figure 7. These fungal colonies were well distinct it was isolated further through sub culturing techniques. These colonies were named as F1, F2 and F3. Figure 8 shows the three distinct predominant fungal cultures isolated from the soils collected on agricultural fields.



Figure 7 - Three different colonies of fungus isolated from paddy field



F1

F2

F3

Figure 8 - Pure isolates of three fungal cultures (F1, F2, F3) isolated from paddy field

4.1.2. Identification of cellulase producing fungi using Congo red assay

As a preliminary qualitative study, the isolated fungal cultures were screened for its cellulose activity by Congo red assay. Out of the three fungal isolates, F3 showed the significant zone

of clearance. The zone of clearance in Congo red assay clearly depicts the cellulase activity of F3 culture as shown in Figure 9.

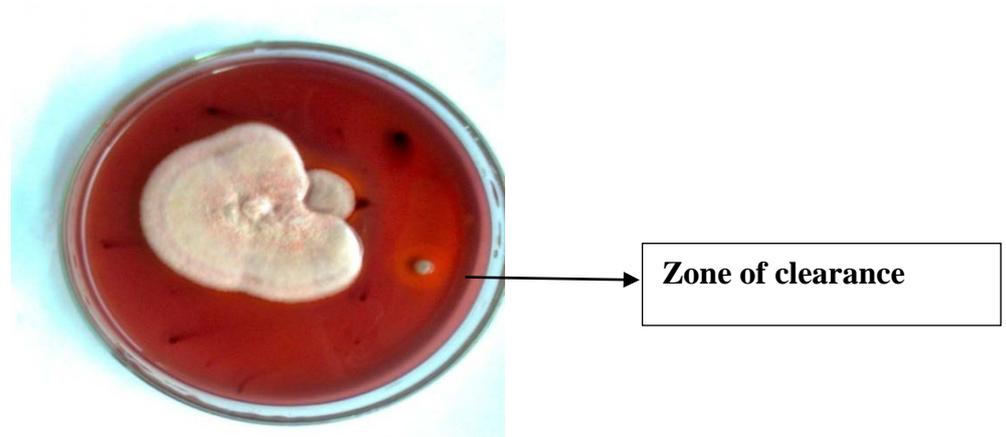


Figure 9 - Identification of cellulase activity by zone of clearance

4.1.2.2 Estimation of reducing sugar using DNS assay

Cellulase activity was measured by the DNS method through the determination of the liberated amount of reducing sugars. Glucose was used as a standard in this assay and all the experiments were done in triplicates. One unit of enzyme activity was defined as the amount of enzyme that releases 1 mol of glucose per minute per ml. All the three fungal isolates were able to degrade the cellulose present in the medium confirmed that all the fungal isolates have expressed its cellulase activity. Figure 10 revealed that F1 has lowest cellulase activity of 5.86 $\mu\text{g/ml}$. F2 and F3 isolates have 10 and 30% greater cellulase activity than the isolate F1. Among the three fungal isolates, F3 have greatest cellulase activity of 7.67 $\mu\text{g/ml}$ as shown in Figure 9. Since F3 could be the promising candidate for high cellulase production, it was concluded to recheck the cellulase activity.

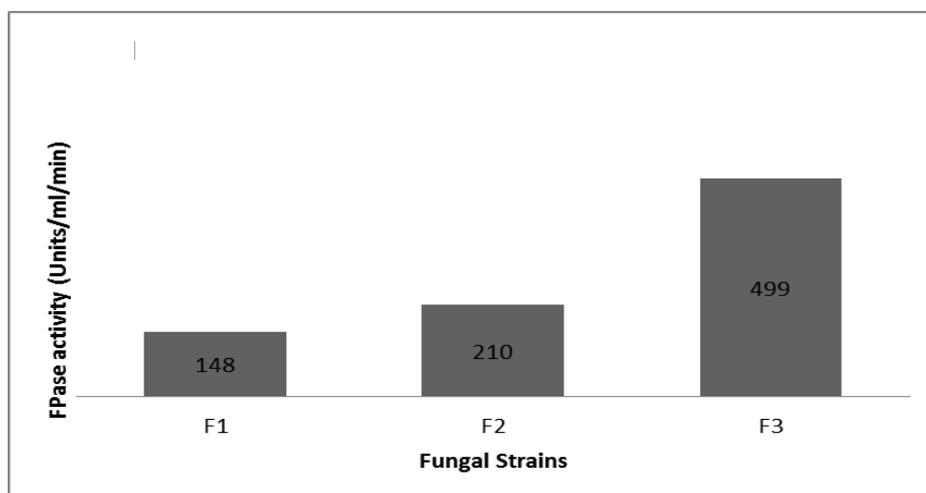


Figure 10 - Production of cellulase by fungal isolates

4.1.3 Screening of fungal cultures for its growth in sea water

The novel attempt has been made to explore the possibilities of cultivating fungal cultures on CMC broth that was already adjusted for pH with sea water. Three fungal strains previously isolated from the paddy agricultural fields were inoculated on the CMC broth adjusted for pH with sea water and incubated further for 14 days.

Visual observation of growth of fungal isolates on CMC broth adjusted for pH with sea water revealed that F1 fungal isolate could not sustain in the newly saline environment. The growth rate of F1 was almost hindered as it might be highly sensitive to the saline conditions, no significant growth was observed. In case of F2 isolate growth on CMC broth adjusted for pH with sea water, the growth rate was relatively higher as compared than that of F1. Yet within the incubation time of seven days, notable cell death was observed. At the end of tenth day the F2 fungal isolate was completely death.

On contrary, significant glucose concentrations were observed in CMC broth adjusted for pH with sea water inoculated with F3 fungal isolate. It showed faster growth by depleting the cellulose and converting it to glucose. Substantial spreading of the mycelium was also observed with this fungal strain. This particular characteristic reveals that the strain could have acclimatized to the saline conditions and it might have aided in sustaining the salinity environment. This further reaffirmed that the F3 fungal isolate could be the promising candidate for cellulase productions even with sea water.

4.1.4 Characterization of fungal cultures

The isolated fungal cultures were identified by performing traditional methods of classification and identification of the organisms based on morphological, physiological, biochemical, and nutritional characteristics performed at the Microbial Type Culture Collection Center and Gene Bank (MTCC & GB), Institute of Microbial Technology (IMTECH), Chandigarh, India.

Various biochemical tests such as assimilation of sugars, utilization of starch, urea, tyrosine, asparagine and citrate components, and nitrate reduction, tolerance range of pH, NaCl and temperature were performed. Cultural characteristics were studied on different media regarding their growth intensity, growth pattern, colour of aerial mycelia and formation of soluble pigments. F3 fungal isolate was identified as *Fusarium subglutinans* (MTCC 11891). This fungal strain was deposited in the MTCC & GB of IMTECH, India. White aerial

mycelium growing rapidly, tinged with purple sometimes, microconidia abundant, oval, usually single celled, may become 1-3 septate, produced in false heads, polyphialidic, macroconidia abundant, slightly sickle shaped to almost straight with delicate walls and basal cells foot shaped are the salient features of this fungus. This was abundant source in plants as plant pathogen but have the ability to deplete the cellulose into glucose for its own growth.

4.2 Production of cellulase enzyme and optimization of environmental conditions

4.2.1 Pretreatment of rice straw

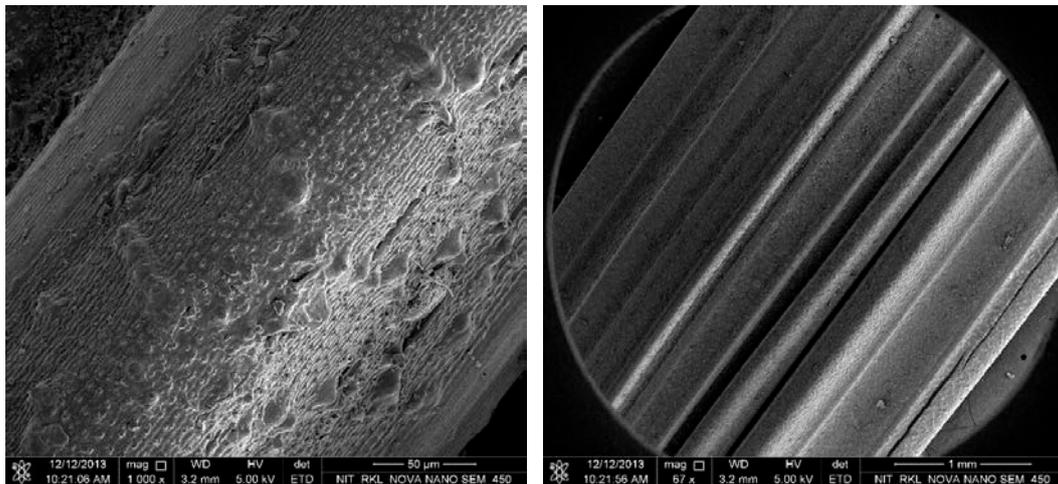


Figure 11 - Scanning electron micrographs of rice straw before pretreatment

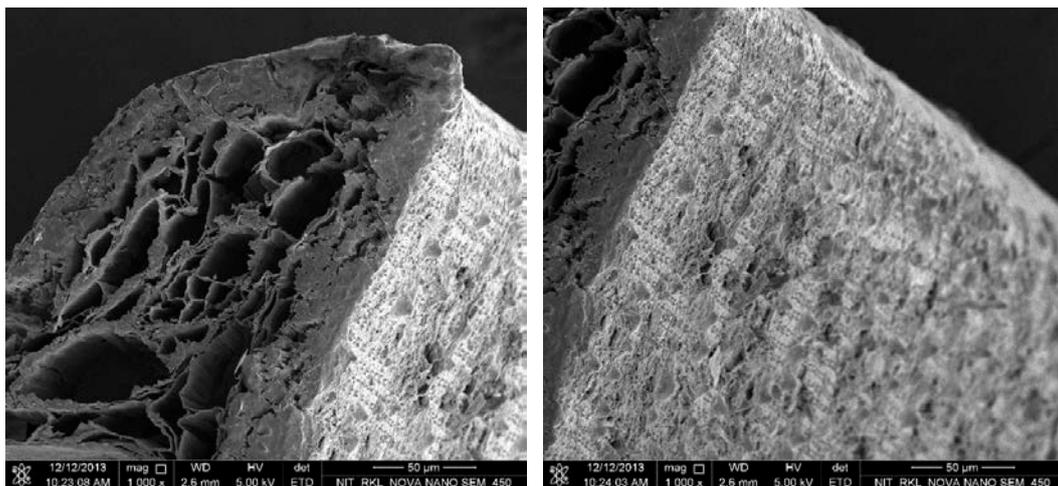


Figure 12 - Scanning electron micrographs cross sectional area of rice straw before pretreatment

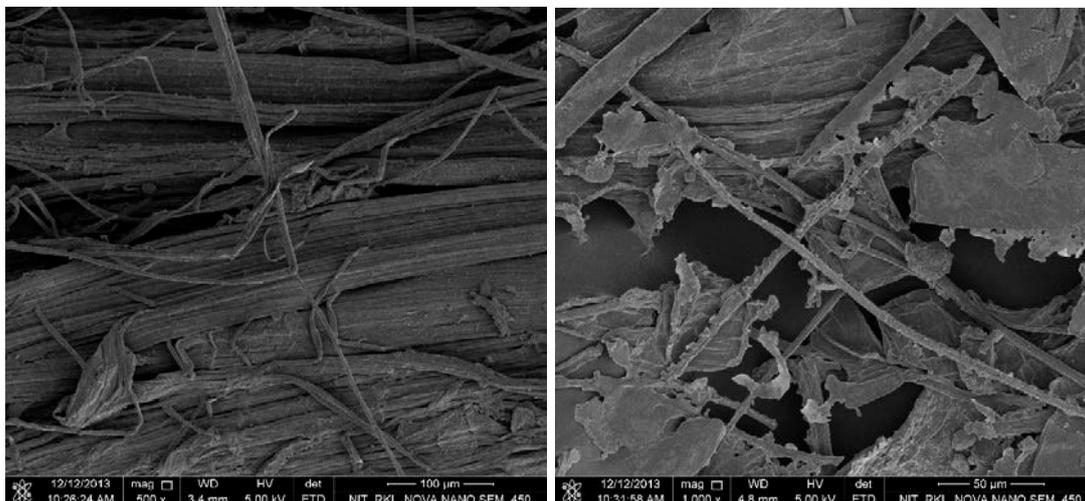


Figure 13 – Scanning electron micrographs of rice straw after pretreatment

Sodium hydroxide pretreatment for rice straw was reported as an effective method. Lignin was removed by breaking the ester bonding between lignin and it enabled availability of cellulose to fungus. Figure 11 and 12 show the fibre and the cross sectional area of rice straw in which the arrangement of xylan, lignin and cellulose fiber. The SEM image in figure 13 showed the maximum removal of xylan, lignin and the cellulose fibers which act as substrate for cellulase production. The optimum concentration used for pretreatment was 1 M at 121°C for 15 min. The rate of increase in depletion of ester bond was dependent on rate of increase in various factors like temperature, concentration of base and time. The SEM image in figure 11 shows the single rice straw before pretreatment fulfilled with lignin and xylan crosslinking.

4.2.2 Determination of metal ions in sea water

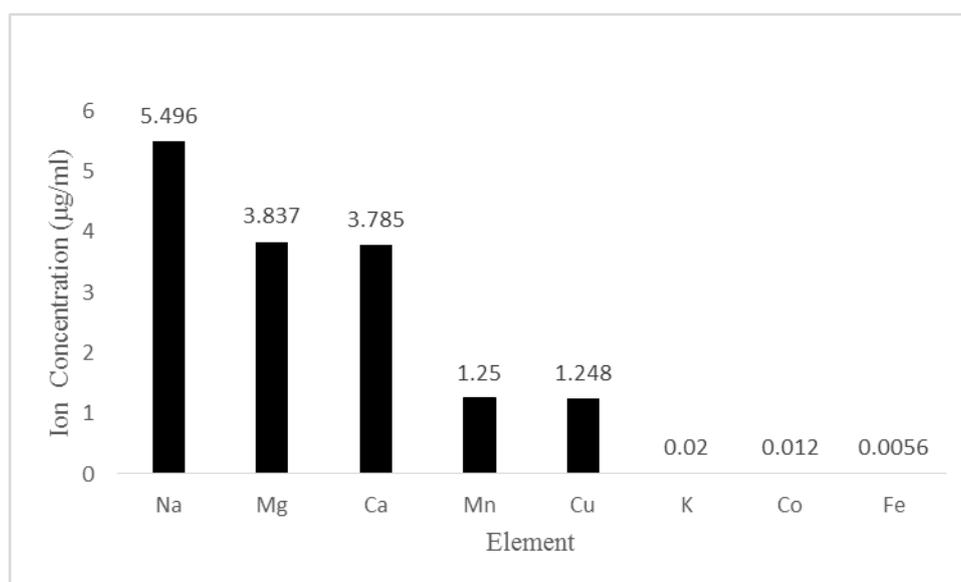


Figure 14 – Elemental analysis of sea water by atomic absorption spectroscopy

Growth media was supplemented with metal ions which are essential for the cellular and subcellular functions of fungus. Similarly, when sea water was used as basal medium, it is important to determine the adequate metal ions. So, a screening test was performed and all required ions were found to be in appropriate concentrations which were given in Figure 14.

4.2.3 Enzyme production using pretreated rice straw in Mandel's media and sea water

The production process was extended up to 21 days while the growth was initiated at 5th day. The fungus was grown on the surface of the cellulose fiber. At a regular interval of time the hydrolysis of cellulose to glucose was analyzed by DNS assay and the cellulase activity was analyzed using filter paper assay, at the end of the process, it was seen that the cotton used as stopper was also depleted because of its cellulose content. Fungus was found to be grown on the surface of the cellulose fiber. Cotton which was used as the stopper for the flask found to be hydrolyzed due to fungal action which may be replaced by a non-cellulosic material. The fungal growth was observed half the time in sea water then that in media. Even the activity was less compared to basal media cellulase but it was not that much less compared to *Trichoderma viride* in free cell formation in Stirred tank reactor [64]. The production of enzyme could be increased by giving alternate stress, nutrient deprivation stress. The fast depletion of cellulose was due to the pretreatment because it decreased the crystallinity and increased the porosity in rice straw cellulose fiber. A steady increase in FPase activity was significant due to the improved cellulase production due to extracellular protein secretion. At the end of fermentation (21 days), enzyme production was observed to be increased. Diminished production volume aids the availability of an increased surfaced area a factor which is difficult to achieve using conical flasks. Hence tray type reactors could be considered to enhance the production of enzyme. Paddle type agitators may be implemented to solve aeration related issues.

4.2.4 Effect of time on cellulase production

The flasks were incubated in shaker for 21 days, the degradation of biomass started at 3rd day approximately. The cellulase activity was analyzed from 4th day by taking out 3 ml of sample and replaced by media. The decrease in biomass concentration was present on day 14 due to the increase in cellulase activity of basal media and sea water media alike. After 21 days cellulase activity was recorded as 277.5 Units/ ml/ min and 126.72 Units/ ml/ min in basal media and sea water showed in figure 15 and 16 respectively. To avoid the contamination the

process was terminated on 21 days and moved on further studies and moved on to further studies.

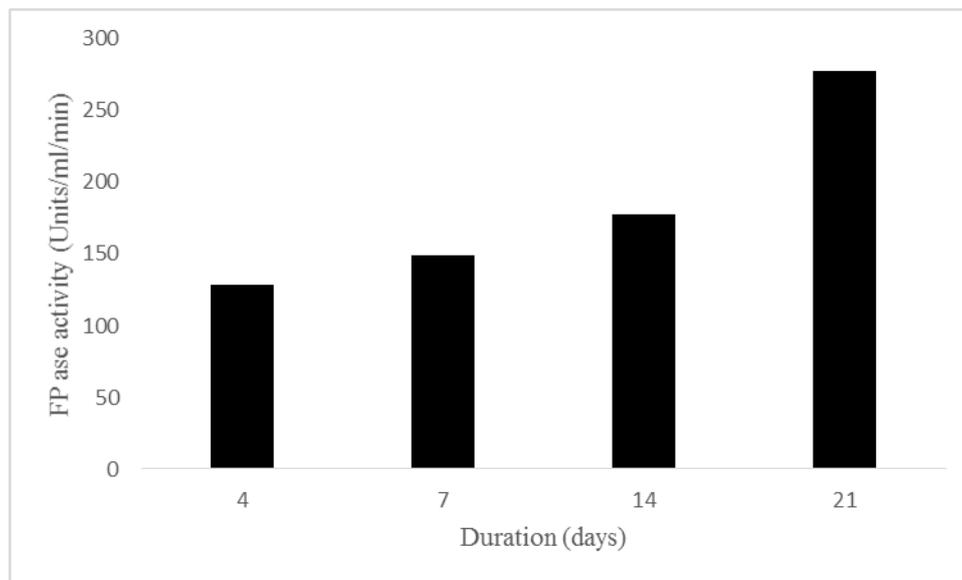


Figure 15 – Production of cellulase by *Fusarium subglutinans* MTCC 11891 in shake flask using Mandel's media

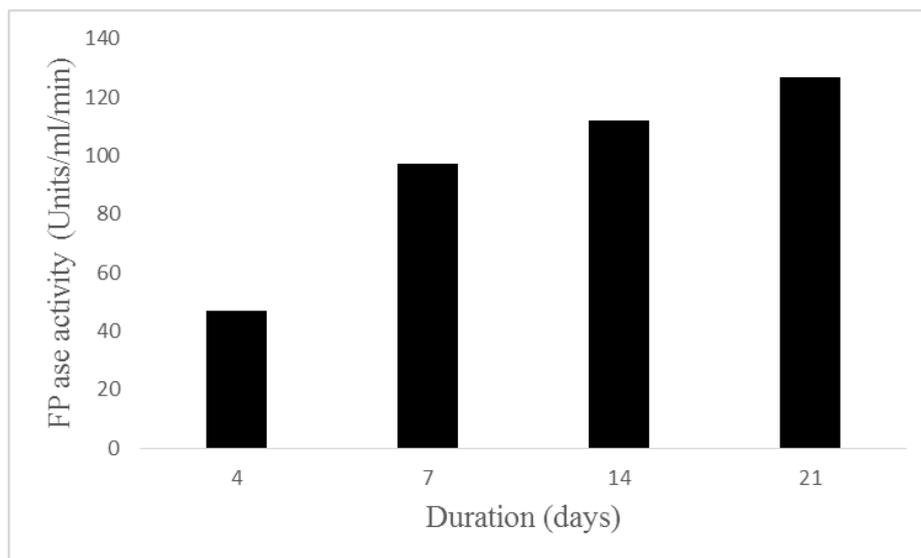


Figure 16 – Production of cellulase production by *Fusarium subglutinans* MTCC 11891 in shake flask using sea water media

4.2.5 Effect of pH on cellulase activity

Effect of pH was analyzed from range of pH 4 to pH 9. There was no much variation in FPase activity of crude enzyme from basal media and sea water produced cellulase in different pH. Figure 17 and 18 shown that at pH 9 cellulase activity started decreasing and reached 275.4 Units/ ml/ min for basal media produced cellulase and 177 Units/ ml/ min for

sea water produced cellulase. The maximum activity was observed at pH 5 as 292.53 Units/ml/ min and 184 Units/ ml/ min for basal media and sea water produced cellulase. From the above results it was obtained that the enzyme was pH independent. These crude enzymes could be used in many industrial processes where the pH fluctuations are dominant.

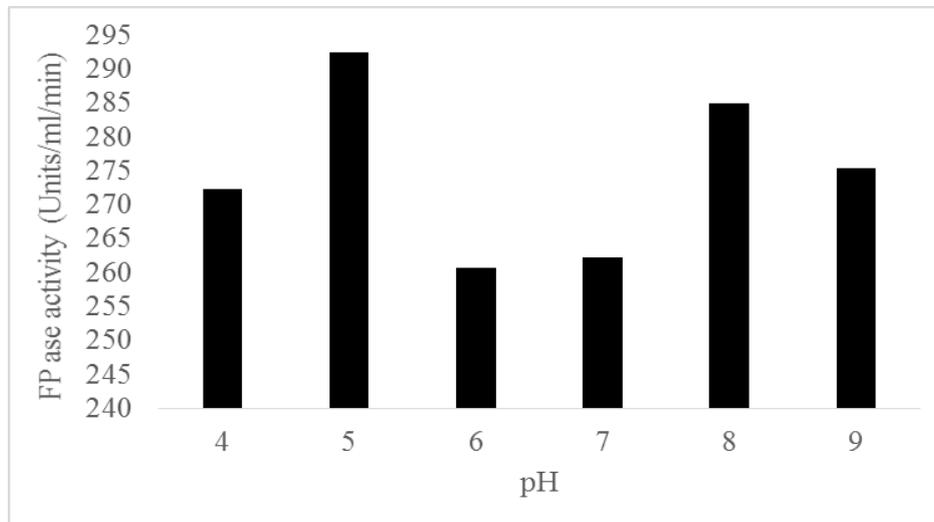


Figure 17 – Effect of pH on cellulase activity in fresh water

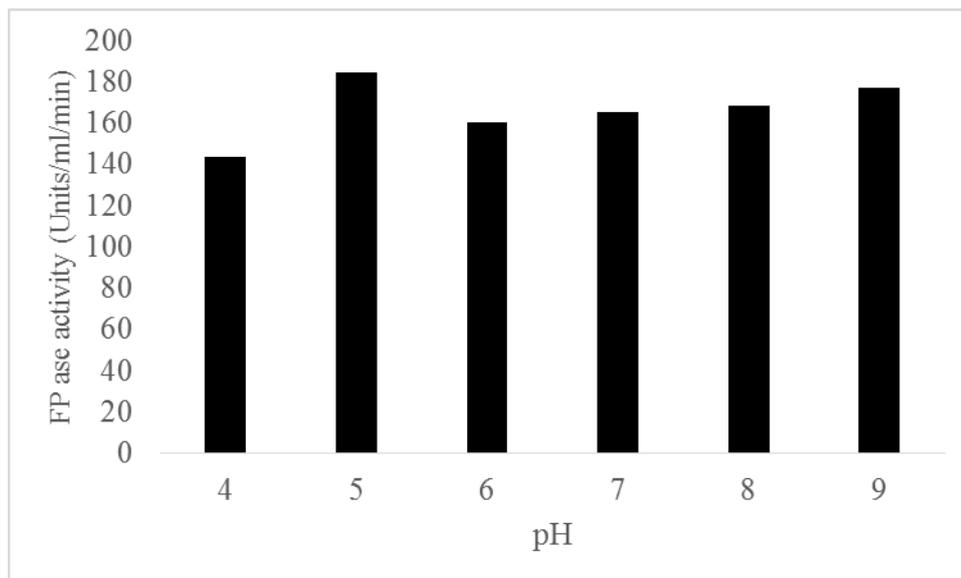


Figure 18 – Effect of pH on cellulase activity in sea water

4.2.6 Effect of temperature on cellulase activity

Effect of temperature was analyzed from a range of 37°C to 80°C showed in figure 19 and 20. It was revealed that there was gradual increase in the activity of crude enzyme from 37°C [260 Units/ ml/ min for basal media produced cellulase and 143.28 Units/ ml/ min for sea water produced cellulase] to 80°C [347.43 Units/ ml/ min for basal media produced cellulase and 232 Units/ ml/ min for sea water produced cellulase]. Therefore it is highly possible that enzyme is thermo stable and showed its maximum activity up to 80°C. When FPase assay was done at 80°C for both enzymes, the hydrolysis was performed very rapidly.

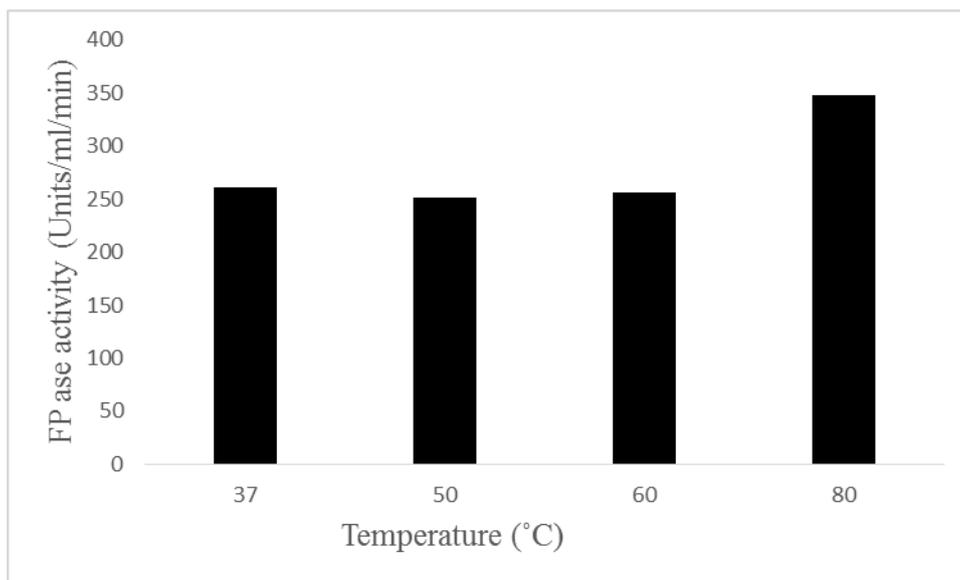


Figure 19 – Effect of temperature on cellulase activity in fresh water

The optimum temperature for both crude enzymes were 75°C – 80°C, because the increase in temperature would increase the kinetic energy of the enzyme system so the number of collisions per unit volume increases and enzyme would have collide with substrate and bind to active site.

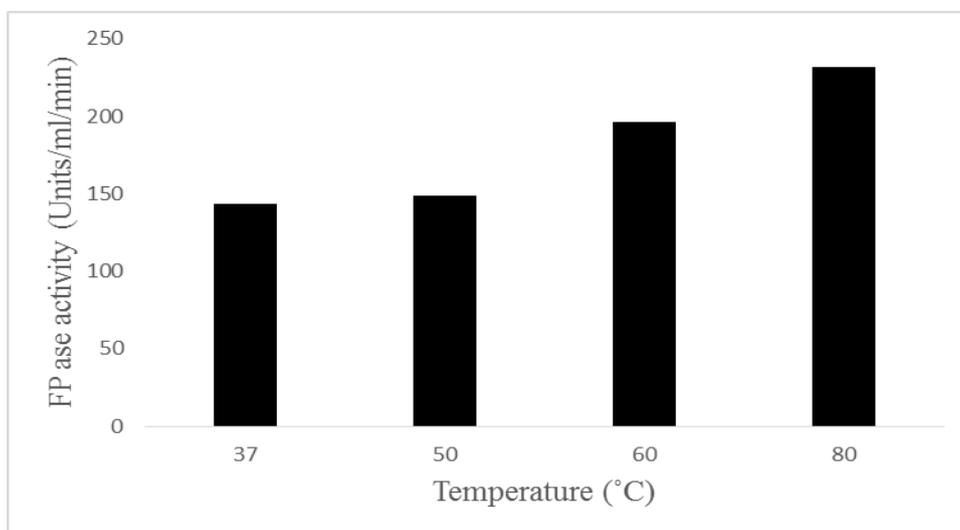


Figure 20 – Effect of temperature on cellulase activity in sea water`

Above results indicated that the enzyme extract can be used directly from the process and for the industries where high temperature plays vital role [e.g. Paper pulp industry].

4.2.7 Effect of different metal catalyst on cellulase activity

Effect of metal ions was analyzed using 5 metals ions which were regularly used as catalyst in many chemical reactions. Mn(II) and Fe(II) showed increased enzyme activity in both enzymes. Figure 21 and 22 revealed that Fe(II) increased 2 folds of enzyme activity in basal media produced cellulase and four folds in sea water media produced cellulase

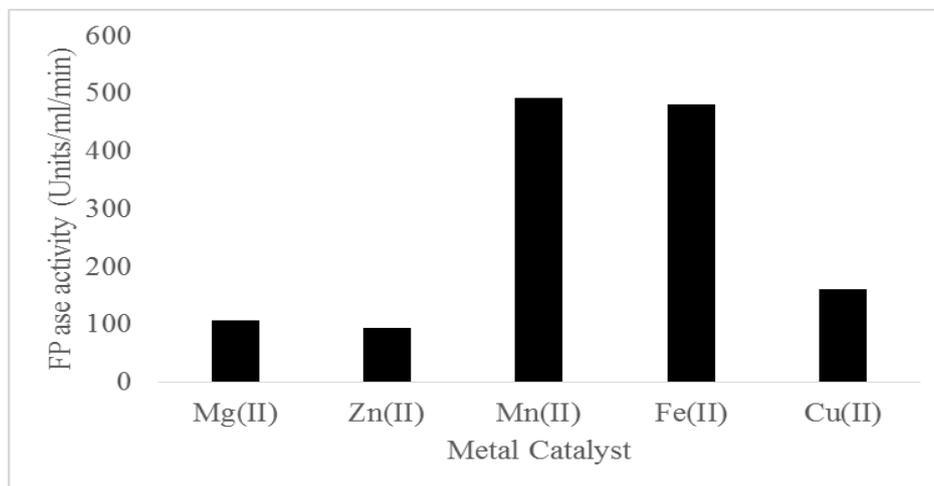


Figure 21 – Effect of metal ions on cellulase activity in fresh water

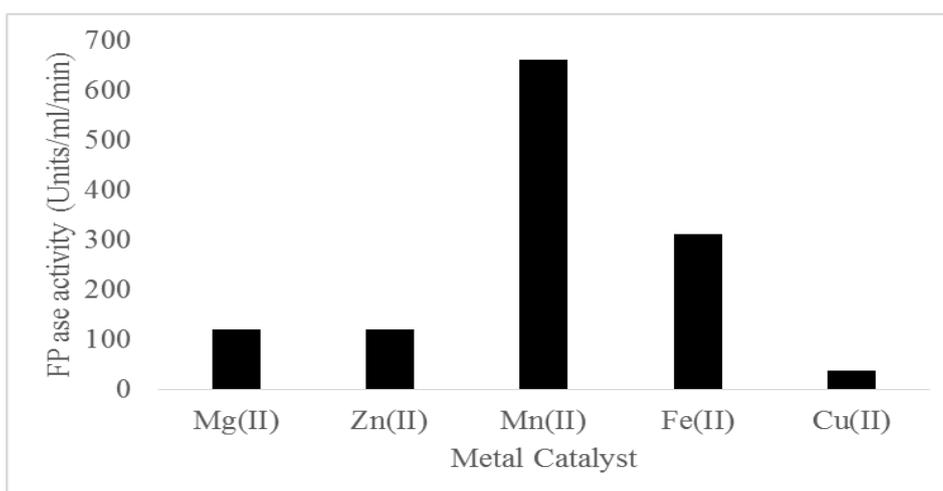


Figure 22 – Effect of metal ions on cellulase activity in sea water

Mn(II) 3 folds in basal media produced cellulase and 4 folds in sea water media produced cellulase. Mg(II) and Zn(II) showed significant level of activity. But Cu(II) showed very low activity this was due to its non-competitive inhibitor nature, was unable to bind to an active site act as catalyst or co-factor. Zn(II) and Mg(II) was not explained well versed in mechanistic or enzyme chemistry aspects. But it was believed that inhibition happened due to the redox active effect of metal ions or some compounds on cellulase during degradation of biomass by fungus. The usage of metal ions with sulphite was eliminating the distinguished

effects of other combinations. As stated earlier the metal ions have proved their effect of metal ions on cellulase activity.

4.3 Partial purification of cellulase using anion exchange chromatography

Partial purification was done using anion exchange chromatography. When the pH of protein was higher than pI value the charge of the protein would be negative. The pI of the cellulase enzyme was nearly 5.2. So that the pH of the enzyme crude extract was increased upto pH 8 and crude sample was applied for elution. After elution using various linear gradient of NaCl (0.2 to 1.2 M) followed by FPase activity, it was shown that at 0.8 and 1 M, cellulase was eluted.

Table 4 – Effect of concentration of NaCl on elution of cellulase in anion exchange chromatography

Concentration of NaCl (M)	FPase activity (Units/ml/min)
0.2	35.32
0.4	49.08
0.6	52.73
0.8	298.68
1	273.12
1.2	45.62
1.4	40.73
1.6	38.95

4.4 Application of crude enzyme extract in the ethanol production

Saw dust was chosen as major source of saccharification because of its availability and low cost. The production of ethanol at 7th day of fermentation was studied using gravimetric method and estimated as 15.76%. The reduced production of ethanol was due to the decreased hydrolysis of cellulose to glucose. This was due to its tightly packed arrangement of cellulose in saw dust.

CHAPTER - 5

Summary and conclusion

- ✓ Substantial research was carried out on the production and characterization of cellulase in fresh water and sea water.
- ✓ Rice straw is an important agricultural lignocellulose waste which can be used as renewable energy source. Pretreatment using alkali treatment improves the utilization of rice straw.
- ✓ *Fusarium subglutinans* (MTCC 11891) was found to be eminent filamentous fungi for the production of cellulase activity.
- ✓ Usage of sea water as an alternative media provides the cost reduction and utilization of saw dust increase the waste consumption.
- ✓ The cellulase showed activity at various pH and it is also capable to sustain at high temperature around 80°C (thermo stable).
- ✓ The estimation of ethanol revealed that this crude enzyme can be used for bio-ethanol production.

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