UNIVERSITY OF WESTMINSTER

MSc Applied Microbiology

Investigation of Extracellular Glucosidases From *Ganoderma Applanatum*

Master Thesis

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CONTENTS

1. INTRODUCTION

1.1 Fungi:

Fungi are important, useful and also widely used organisms in biotechnology and in industry. They have been used in many areas, such as the production and flavouring of foods, production of biochemicals in the pharmaceutical industry, as antitumour agents and immunomodulators. They also play an important role in agricultural and environmental biotechnology to treat hazardous wastes such as cyanide and in the bioremediation of soils polluted by pesticides and other chemical compounds. A variety of fungi find application in traditional Chinese herbal medicine (Yang, *et al* 1998), where they are ground and used as tea, which is reported to cure everything from colds to impotence to AIDS.

Currently, 70.000 species of fungi are accepted and new species are being described. (Gorin and Spencer, 1968; Williams and Kirk, 1988). Especially research on the production of cellulolytic enzymes has been carried out with fungi known to degrade cellulose. They produce a wide variety of extracellular enzymes to break down soluble and insoluble polysaccharides into small oligosaccharides and sugars, which can be taken easily into the cell for metabolism. Many more applications of fungal enzymes are still being investigated for the future. The great advantages of enzymes are their specificities and the main disadvantages relate to their costs. Since the introduction of technology which enables these organisms to be genetically engineered, the practical applications of fungi have increased enormously.

1.2 *Ganoderma applanatum*

Kingdom	Fungi
Phylum	Basidiomycota
Class	Basidiomycetes
Subclass	Holobasidiomycetidae, Hymenomycetes
Order	Aphyllophorales
Family	Ganodermataceae

Basidiomycetes are a large group of fungi. Their members range from the familiar edible button mushroom to the rusts and smuts may be enormous pathogens of crops. The characteristsic uniting all these fungi is that they bear their spores externally, on a structure called a basidium. The basidiomycete fungi are toxic and edible species decay wood or attack living trees. Decay caused by *Ganoderma* species, which are widespread polypore fungi, is influenced by both the *Ganoderma* species and type of wood, and can range from simultaneous decay of all wood components to selective delignification. (Hseu *et al.* 1996)

Wood rot fungi are important degraders of the major plant polymers lignin, cellulose, and hemicellulose in the biosphere. (Kirk *et al.* 1987; Reddy *et al.* 1994; D`Souza *et al.* 1996). Wood decaying fungi are categorised in different groups and are classified according to the mode of attack of wood into white-rot, brown rot and soft-rot. The white-rot group are able to degrade lignin, cellulose and hemicellulose. This group of fungi are the only microorganisms shown to be able to degrade lignin totally. Most white-rot fungi belong to the Basidiomycetes. Brown-rot fungi and soft-rot fungi degrade mainly cellulose and hemicellulose of wood but only limited degradation of lignin is observed (Winkelman, 1992). *Ganoderma* species are such as *G. applanatum, G .lucidum, G.japonicum, G. tsugae, G. boninense, G. capenc, G. browni, G. lipsiense, G. valesiacum, G. graminis* produced on a large scale for pharmacological and clinical studies. (Jong and Birmingham, 1992). *Ganoderma applanatum* has white pores on the under-surface of the fruiting body. (See figure1.1). It is also known as a "shelf fungus" because the fruiting body forms a stalkless shelf on the sides of trees and logs. It is perennial, woody, typically sessile, 6-60 cm broad, and 5-10 cm thick. *G. applanatum* is the best known shelf fungus which is distinguished from other woody polypores by grey-brown, bumpy, usually zonate cap characteristics. It is often powdered brown from released spores, and a white pore surface, which instantly darkens when injured. (Wood, 1999). Also *G. applanatum* is a very good enzyme producer. But relatively there is a little information about the potential of *G.applanatum* as an organism for commercial use.

Figure 1.1 *Ganoderma applanatum*

1.3 POLYSACCHARIDES

Polysaccharides constitute a structurally diverse class of biological macromolecules with a wide range of physicochemical properties. Several hundred natural polysaccharides currently known provide a reservoir of structurally and functionally diverse polymers which are the basis for the different applications in a broad field of industry and medicine such as cellulose, starch, hemicelluloses, inulin, locust bean gum, xanthan gum etc. (Franz, 1989). Gums in the form of natural, modified, or synthetic polysaccharides are consumed in very great quantities in industry. Although, the some polysaccharides used commercially are of plant derivation, such as seed extracts, tree extracts, starch derivatives etc., interest in polysaccharides from microbial sources is increasing. Besides the bacteria, yeast and plant, fungi are being investigated as potential producers of polysaccharides.

Sugars are crystalline substances with a sweet taste and soluble in water. Polysaccharides are more complex than sugars. Most are non-crystalline substances and either insoluble or much less soluble in water than the sugars. Both classes of compounds have similar structures, which are represented by a chemical formula, $(C_6H_{10}O_5)_n$ where $_n$ can be almost any number from one upwards. Besides the chemical classification, most polysaccharides can also be classified according to their biological roles. For example, starch and glycogen are storage polysaccharides, and cellulose and

chitin are structural polysaccharides. The most common storage homoglycan of glucose in plants and fungi is starch and in animals, glycogen. (Horton, *et al.*, 1996).

The most abundant organic material in the world is cellulose. It is the major constituent of plant material. The basic molecular structure of cellulose is a linear polymer of 8000-12 000 glucose units linked together by 1,4 -β glucosidic linkages. (Fogarty, 1983). (See figure1.2). Also hemicelluloses are a group of substances, they associated with cellulose, and are usually a mixture of xylan, which its backbone is based on the pentose D-xylose unit linked by β(1-4) bonds or xyloglucans, arabinogalactans, glucomannans and galactoglucomannans. (Coughlan and Hazlewood, 1993).

Starches are plant reserve food polysaccharides.They are mixture of two polysaccharides, amylose and amylopectin. Amylose is a glucan with linear chains of glucose linked α(1-4) and chain length up to 3000 residues. Amylopectin is also a glucan with α(1-4) residues on the main chain. It contains branches linked α(1-6), and occur about once every 12-25 residues along the chain. Hydrolysis of the chains into fragments yields maltose, glucose usually done by enzymes such as α-amylase. (Dyke 1960; Florkin and Stotz 1963; Tombs and Harding, 1998).

In this investigation range of polysaccharides, which are starch, cellulose, xylan, xanthan gum, inulin and locust bean gum has been studied.

Figure 1.2 cellulose structure

1.4 EXTRACELLULAR ENZYMES

Enzymes are a specific group of proteins that are synthesised by living cells to function as catalysts for the many thousands of biochemical reactions that constitute the metabolism of a cell. Extracellular enzymes are usually defined as enzymes that have been exported across the cytoplasmic membranes and are also the most commonly used

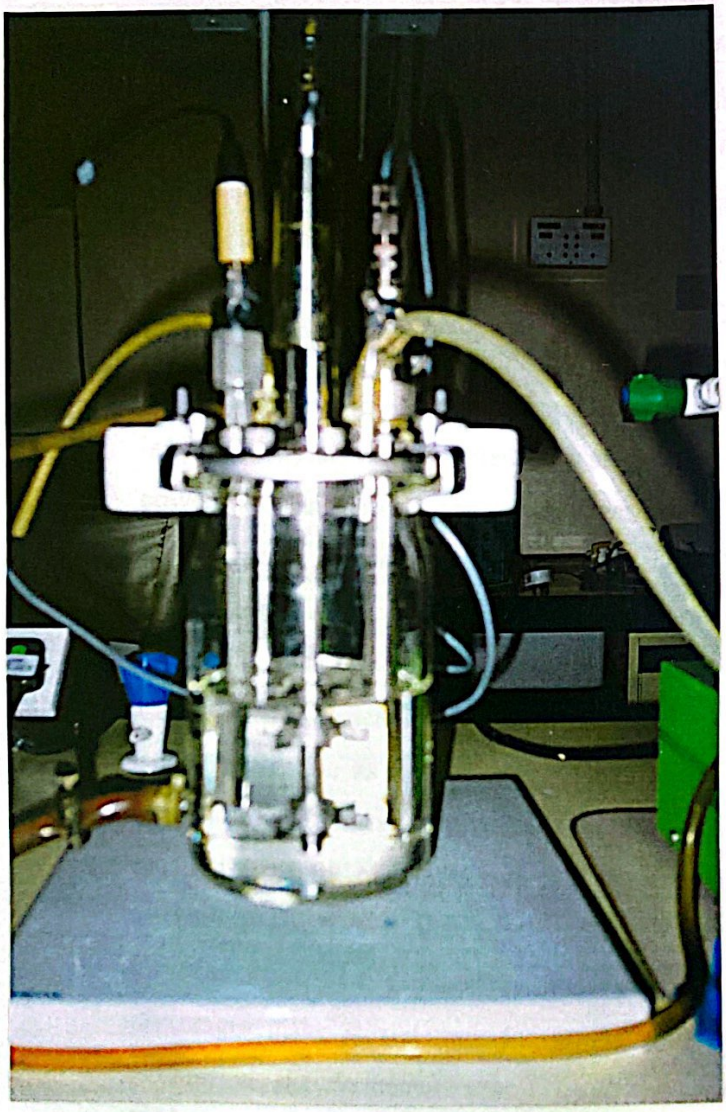
types. They are usually more stable than intracellular enzymes due to the containing of disulphide bonds. Many enzymes are produced by fungi and more than 50 % are used in large-scale processes in industry. (Bucke, 1998). Most polysaccharides are too large to enter the cell and extracellular enzymes are secreted as scavenger enzymes which hydrolyse macromolecules in the environment to provide low molecular nutrients for the microorganism. Enzymes are induced which can hydrolyse polysaccharides that may be transported into the cell. (Winkelman, 1992). And enzyme induction is defined as an increase in the rate of a specific enzyme synthesis by adding an inducer to the fungal culture. Some enzymes are synthesized in the absence of inducer. For example starch or dextrins can induce amylase production, furthermore β-galactoside is an inducer to produce high level of β-galactosidase.

1.5 MEDIA

For the most suitable medium and for each fermentation process there are some basic requirements, which must be met. Because all microorganisms need water, carbon, nitrogen, sources of energy, mineral elements, vitamins and oxygen if they are aerobic.
Components of a production medium cost can have a profound effect on the overall cost of a fermentation process. Therefore, the types of media might be chosen which are marked as complex and defined. In complex media, there are variable concentrations of the component parts and it causes the unpredictable biomass and (or) yield. In defined media, known components are used, therefore more predictable yields are obtained. Although defined media components are more expensive, many manufacturers prefer this type of media because of the having fewer recovery steps. (Stanbury, *et al*, 1995).

1.6.1 FERMENTATION

Fermentation techniques have been applied for many years. The first large-scale process was the production of alcohol by the action of yeast on malt or fruit extract. By the development of the biotechnology various products were obtained such as food (milk, cheese. etc), pharmaceuticals, textiles, production of some chemicals e.g. citric acid, ethanol, etc. Commercially important fermentations are divided into some groups, such as the production of microbial cells, microbial enzymes, microbial metabolites, recombinant products and modifying a compound, which is added to the fermentation to get a more financially valuable compound. (Stanbury and Whitaker, 1995).



Fermentation parameters are optimised to maximize the growth of the microorganism by controlling the nutrients, temperature, pH, and oxygen transfer.

1.6.2 STIRRED TANK REACTOR

Stirred tank fermenters are the most commonly used type for aerobic fermentation. They have so many advantages such as being well-documented and versatile characters, also to be used as growth medium reservoirs for oxygenation. Rapid dispersion of injected gases and liquids and transfer of heat for temperature control cause efficient mechanical agitation. As a result of these effects, maintenance of the culture volume homogeneity is provided, and these functions are related to the power dissipated by the agitator into the liquid that has to be removed by the cooling system. Rotating impellers agitate the contents of the vessel, which are mounted on a shaft suspended from a thrust bearing outside the vessel. A major contamination risk point in STR is that the entry point of the rotating agitator shaft into the vessel and is usually closed by a mechanical seal. Also, the fermenter is held above atmospheric pressure so the place where the shaft enters should not be placed where air escapes. (Spang, 1999). The various types of impellers may also be mounted on a single shaft. For example, turbine impellers deliver power, well and assist aeration by shearing bubbles. Propellers provide predominantly axial mixing. Turbines are most often used, but the may be in combination with propellers. However, the space of the impellers on the shaft is important because, if they are too far apart, there may be stagnant regions of liquid between well-mixed regions around each impeller. In contrast if the impellers are mounted too closely together, the liquid may rotate as a single mass. Baffles which are fitted to the inside walls of the vessel, prevent vortex formation and promote turbulence. The width of baffles is usually 10-12 % of the vessel. The advantages of stirred tank reactor over non-mechanical reactors is that mechanical agitation could provide the well mixing and homogeneity to be attained without gas flow through the liquid, therefore this advantages make stirred tank fermenters suitable for *in situ* heat sterilization of the medium in the fermenter vessel itself, without the need for a separate cooker. (Wiseman, 1995). Although the stirred tank reactor is not the most economic type to install or maintain, it is still the most widely used design because of its versatility and reliability. (Wainwright, 1992).

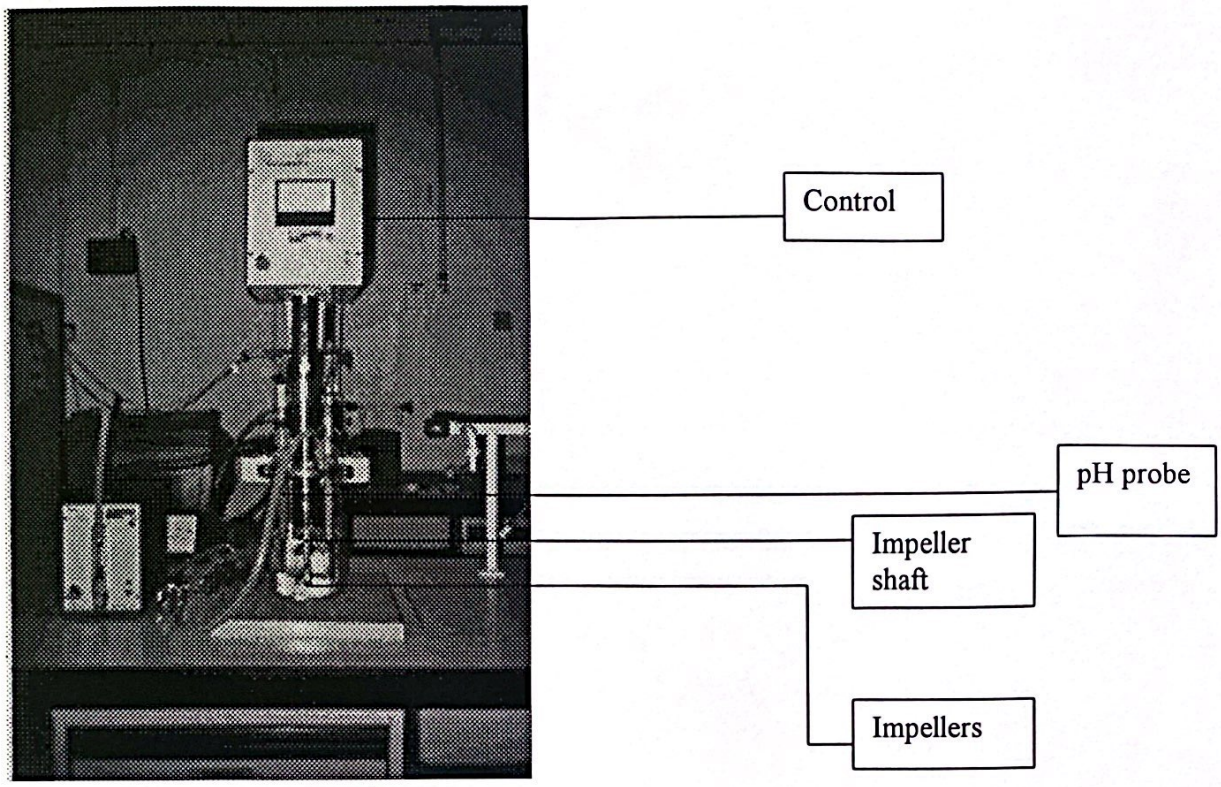


Figure 1.2 Photo taken from the stirred tank reactor (STR)

1.7 Aims

The major aims of this project were to:

1. Achieve a growth of *Ganoderma applanatum* in liquid culture (with different carbon sources) using shake-flask or stirred tank reactor. (STR)
2. Investigate extracellular glycosidase activity from *G. applanatum* grown in various carbon sources.
3. Measurement of protein from shake flask studies using polysaccharides as the main carbon source.

2.MATERIALS AND METHODS

2.1 Materials:

All the experiment materials were provided by Sigma.

2.2 Micro organism:

Ganoderma applanatum was used during this experiment. *G. applanatum* strains were provided from two different sources. One of them was cultivated from an apple tree by Professor Christine Evans, and the other strain was obtained from the International Mycological Institute (IMI strain No° 15618). The IMI strain was used throughout this investigation.

2.3 Medium:

Defined media was used for *G. applanatum* maintenance. (Bland, 1998).

5.00g Carbon source
0.2g Glucose
1.25g L-asparagine
0.75g DL-Phenylalanine
0.13g Adenine
25 µg Thiamine HCL
0.25g KH_2PO_4
0.025g Na_2HPO_4
0.0025g $CaCl_2$
0.0025g $FeSO_4$
0.0025g $MnSO_4$
0.0025g $ZnSO_4$
0.0025g $CuSO_4$
0.1250g $MgSO_4$

Technical agar No° 3 was added (15g per litre) to the medium to make the defined agar on which *G. applanatum* was cultivated.

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0.0025g $MnSO_4$
0.0025g $ZnSO_4$
0.0025g $CuSO_4$
0.1250g $MgSO_4$

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2.4 Shake flask studies:

Preparation: The component of medium that is given in 2.3 was added to a 1-litre shake flask with distilled water. It was divided into 5, 200 ml shake flasks.

Sterilization: The shake flasks were sterilised for 15 minutes at 121 °C, 15psi.

Inoculation: Plugs were cut from the growing edge and removed aseptically into the universal bottle (U.B), which contains sterile water and glass beads (2.5-3.5 mm), it was shaken until cloudy appearance was obtained.

Incubation: The shake flasks were placed inside an orbital shaker at 200 rpm and incubated at 26 °C for 7 days.

Calculation of final biomass: The culture was filtered through a whatman number 1 paper (of known weight) and than placed in an oven (at 60 °C). After 24 hours the dry biomass was weighed.

Testing of broth: After getting growth, culture broth was tested to measure enzyme activity by using 3,5-dinitrosalicylate method. Protein determination was also measured by Lowry's method.

2.5 Stirred tank reactor:

The stirred tank reactor used had a working volume of 1.7 litres. The stirred tank reactor has a height of 18.5 cm, a diameter inside the reactor was that 11.4 cm.

Preparation: 1.5 litres of defined media was prepared and added to the reactor with 200 ml growth culture.

Sterilization: The reactor was autoclaved for 15 minutes at 121 °C, 15psi.

Inoculum for STR: 200 ml shake flask was incubated at 200 rpm and 26 °C for 6-7 days. The broth in the shake flask was aseptically kept for the inoculum of reactor.

Incubation: The temperature of the reactor was maintained at 26 °C. Temperature, pH and dissolved oxygen were accordingly monitored.

Biomass: The culture was filtered with the whatman number 1 paper (of known weight) and then placed in an oven (at 60 °C). After 24 hours the dry biomass was weighed. The dry weight was recorded. The medium was kept in fridge to test enzyme activity.

2.6 3,5-DINITROSALICYLATE METHOD FOR REDUCING SUGAR ASSAY

The method was provided by Prof. Chris Bucke from the University of Westminster that used as an assay for reducing sugar.

Solutions:

- 3,5 Dinitrosalicylic acid reagent (DNS reagent)

The DNS reagent was prepared by dissolving 5 gm 3,5-dinitrosalicylic acid and 150 gm of sodium potassium tartrate (Rochelle salt) in 100 ml 2 M sodium hydroxide (NaOH) which was then diluted to a final volume of 500 ml with distilled water.

Method

3 ml of dinitrosalicylic acid reagent (DNS reagent) was added to 3 ml of samples (Sensitivity 0-10 µmol), standards and controls. The mixtures were heated to 100 °C in a boiling water-bath for 5 minutes. After this time the solutions were cooled and absorbance at 540 nm was determined in a spectrophotometer. Standard curves were prepared for each carbohydrate solution to measure concentration of reducing sugar in glucose, fructose, maltose, arabinose, rhamnose (obtained by using stirred tank reactor); starch, cellulose, xylan, xanthan gum and locust bean gum (obtained by shake flask studies) media.

- Preparation of acetate buffer

0.1 M acetate buffer pH 6.0 was prepared in 1litre. When preparing the acetate buffer solution, 4.102-g of sodium acetate was dissolved in 500 ml distilled water. Then acetic acid was added until pH6.0 was reached and made up to 1 litre. They were kept in the fridge (in two 500 ml medical bottles).

- polysaccharide solutions

1% solutions of the polysaccharide (starch, cellulose, xylan, xantan gum, inulin and locust bean gum) were prepared in distilled water. When preparing solutions of polysaccharides (each 40 gr), the correct volume of water was taken which was 40 ml, and then small amounts of the polysaccharide were added gradually, because one large mass of the polysaccharide addition does not dissolved easily. Also water was heated to aid solution. They were kept frozen in plastic vessels to measure enzyme activity by using 3,5 dinitrosalicylate method.

- Extracellular fluids by using different carbon sources (glucose, fructose, maltose, rhamnose, arabinose, which were produced by using stirred tank reactor; starch, cellulose, xylan, xanthan gum, locust bean gum, which were produced by using shake flask).

Control 1

3 ml DNS +3ml Extracellular fluid

Control 2

1 ml polysaccharide + 2 ml Acetate buffer pH6.0

For setting

3 ml DNS + 3 ml Distilled water

Enzyme activity assay

1 ml Extracellular fluid + 1 ml polysaccharide + 1 ml acetate buffer pH6.0

* Control 1 and 2 readings were measured without being incubation.
* The enzyme assay measuring for production of reducing sugar was observed after 1-hour incubation at room temperature.
* In the first experiment, solutions were incubated overnight, but readings were too high, therefore 1-hour incubation was preferred not to get highly concentrated solutions.
* The absorbance of the solutions was determined using a spectrophotometer at 540 nm,
* This experiment was repeated a further 3 times.

2.7 PROTEIN DETERMINATION BY LOWRY`S METHOD

This method was provided by Prof. C. Bucke from University of Westminster and from the journal searches (Tan, *et al.*, 1984) to assay the protein content of extracellular fluids.

Reagents

Reagent A: 2 % Na_2CO_3 (Sodium carbonate) in 0.1M NaOH (Sodium hydroxide).

Reagent B: 0.5 % $CuSO_4.5H_2O$ in 1 % NaKTartrate (Rochelle Salt)

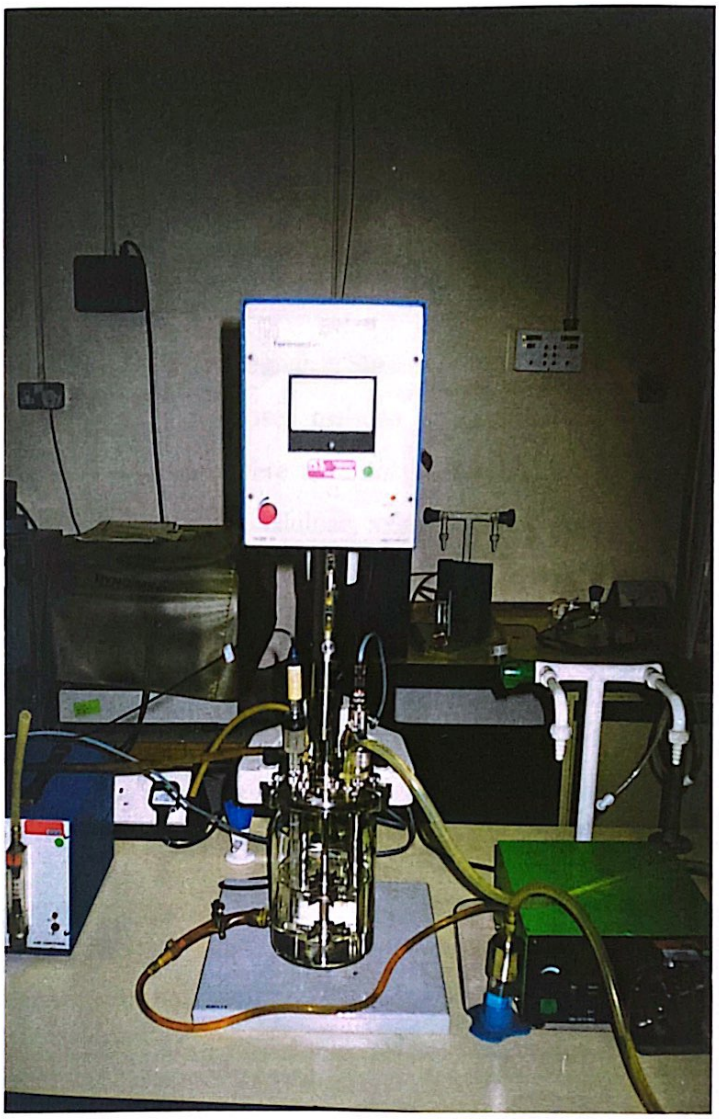
Reagent C: Alkaline copper solution: 50 ml solution A+ 1 ml solution B, and it was discarded after one day.

Reagent D: Folin and Ciocalteu`s Phenol Reagent. It was diluted from the Sigma "2.0 Normal" solution 1 part plus 2 parts water.

Method

0.5 ml protein sample of standards or control solutions (10-200 µg) was mixed with 5 ml. Reagent C, and the mixture was mixed thoroughly on a vortex mixer and allowed for 10 minutes. After this time 0.5 ml. Reagent D (Folin-Ciocalteau's phenol reagent) was added. The reaction mixture was thoroughly mixed again and after 10 minutes absorbance at 600 nm was determined. Standard curves were prepared for protein determination of starch, cellulose, xylan, xanthan gum and locust bean gum.

3.RESULTS



A Series of stirred tank reactor fermentations and shake flasks studies were carried out in defined media to investigate extracellular glycosidases using *Ganoderma applanatum* . This was followed by extracellular enzyme activity was measured from the using fermentation for reducing sugar assay. In fermentation studies different type of carbon sources were used such as glucose, fructose, maltose, arabinose and rhamnose. In shake flask studies, only polysaccharides were used to get a growth by using *G. applanatum.* These polysaccharides were starch, cellulose, xylan, xanthan gum and locust bean gum. The amount of protein in polysaccharide solutions was measured.

4.1 Shake Flask Results:

First trials were carried out using the shake flask. This was the one of the most time consuming parts of this experiment because, initially growth was not rapid in the liquid culture. The slow growth was probably due to the fact that the organism used had been kept in cold storage for a long time therefore strain might be lost its metabolic activity. Following tries were carried out by new culturing of the *G. applanatum* on defined agar. Then plugs were transferred aseptically from defined media into the shake flasks. Growth occurred on these plugs in shake flasks and in stirred tank reactor.Plugs were cut from the growing edge and removed aseptically into the universal bottle (U.B), which contains sterile water and glass beads (2.5-3.5 mm) and it was shaken until cloudy solution was obtained.

4.2 Fermentation Results:

The fermentation run was another time consuming part of this study, because in the beginning rapid growth did not happen. The reason was probably due to the fact that the same like in shake-flask studies which is long storage of the organism in fridge and some activity losted from its metabolism. This was followed by the culturing of *G. applanatum* on defined agar. Then plugs were transferred into shake flasks. After growth occurred, fermenter was used for the next growths. Initially, both stirred tank reactor and airlift reactors were used. However in the airlift reactor *G. applanatum* did not grow well. Therefore the stirred tank reactor was used. For this trial, plugs were taken and bits of mycelium removed by using glass balls. Because whilst the airlift reactor was used inoculation with *G. applanatum* was not achieved by using gas balls. This was thought as a reason for unsuccessful growth, which is having of pelleted form.

After that, in stirred tank reactor the growth was obtained successfully. Dissolved oxygen tension versus time was the parameter to understand clearly the growth of organism. The results obtained stirred tank reactor was carried out showed that *G. applanatum* was grown better by using the fructose, glucose arabinose as a carbon sources then using maltose and rhamnose.

While fructose is used as the main carbon source (figure 3.10) the dissolved oxygen tensions (% D.O.T) decreased from 99.6 to 55,6 in 80 hours and from 55,6 to 29 within a further 70 hours.

With glucose as the main carbon source (figure. 3.11) the % D.O.T decreased from 100.7 to 92.5 in 80 hours and from 92.5 to 74.9 within a further 70 hours.

With a maltose as the main carbon source (figure 3.12) the % D.O.T decreased from 100.7 to 94.5 in 80 hours and from 94.5 to 90.3 within a further 70 hours.

Using arabinose as the main carbon source (figure 3.13) the % D.O.T decreased from 99.1 to 76.5 in 80 hours and from 76.5 to 55.8 within a further 70 hours.

Using arabinose as the main carbon source (figure 3.14) the % D.O.T decreased from 100 to 86.0 in 80 hours and from 89.0 to 87.0 in 70 hours.

The biomass collected from the arabinose fermentation (see table 5.8) was the largest collected at 0.93 grams the fructose stirred tank reactor feremantation (see table 5.4) produced 0.44 grams the glucose fermantation produced 0.28 grams (see table 5.2) maltose stirred tank reactor produced 0.25 grams (see table 5-10) only produced 0.24 grams.

Biomass collected from the starch shake flask (see table 5.11) was 0.15 grams, xylan shake flask produced 0.15 grams locust bean gum shake flask produced 0.11 grams.

In xanthan gum, there was not any growth. This might be caused due to the fact that the having of high viscosity. The % D.O.T was the very good parameter to understand the growth of organism besides the collected biomass from each end of fermentation run.

4.3 Enzyme Activity Results:

Enzyme activity studies were carried out by using the 3.5 dinitrosalicylic method. The reason of using this method due to the fact that is not to be too sensitive, and to be easy to use. From the journal searches this method appears very common and inexpensive. Using fructose as the main carbon source some sugar inhibition was observed. Absorbance of starch at 540 nm was 1.3 µmol (see table 3.2) and the concentration of starch from the fructose standard curve (see figure 3.2) was 14.5 by using 3,5 dinitrosalicylic method. Also cellulose, xanthan gum and locust bean gum

showed high amount of concentration like starch. Concentration of xylan (7.8) and inulin (5.5) results were showed some degradation of polysaccharides happened. According to the xylan reading, D-xylose might be produced from the break down of the sugar.

With glucose as the main carbon source, enzyme activity was observed only using starch and locust bean gum polysaccharides (see table 3.1). Concentration of starch and locust bean gum was 2.9 µmol when the absorbance (at 540 nm) showed 0.4 µmol. Inulin absorbance was 2.2 µmol and concentration showed 2.2µmol from standard curve (see figure 3.1). With cellulose and xylan used as polysaccharide in glucose medium very poor sugar degradation was observed. Xanthan gum did not show any sugar inhibition.

With a monosaccharide, rhamnose as the main carbon source, starch and locust bean gum (at 540 nm) were 0.4 µmol (see table 3.3) and concentration from standard curve 6.8. Absorbance of the xanthan gum 0.5 and concentration from standard curve (see figure 3.3) was 8.8 µmol. There was no enzyme activity against inulin in rhamnose medium however cellulose and xylan were poorly degraded. Using extracellular rhamnose fluid induced starch and locust bean gum suitably.

Using maltose as the main carbon source absorbance (at 540 nm) of cellulose (see table 3.4) 0.4 and concentration from standard curve (see figure 3.4) was 3.2 µmol. Inulin was not reduced suitably and starch, xylan, xanthan gum and locust bean gum did not show any degradation.

With arabinose as the main carbon source, cellulose was the better-degraded polysaccharide than other starch, inulin and locust bean gum polysaccharides in extracellular arabinose medium. The absorbance of cellulose at 540 nm (see table 3.5) 0.3 µmol and calculated concentration from standard graph was 3.7 (see figure 3.5). Absorbance of both starch and inulin 0.2 and concentration from standard curve was 2.5 µmol. Although locust bean gum showed very less activity xylan and xanthan gum did not had any activity.

With a polysaccharide, starch, as the main carbon source the absorbance of starch (at 540 nm) was 0.21µmol (see table 3.6) and concentration from standard curve 3.2 µmol (see figure 3.6). Absorbance of xylan 0.1 and concentration 3.0 µmol. Xanthan gum and inulin was also degraded poorly in starch medium. However cellulose and locust bean gum did not showed any enzyme activity.

Using cellulose as the main carbon source very less degradation was observed with starch, xylan, inulin and locust bean gum. (See table and figure 3.7) Cellulose and xanthan gum did not showed any activity. The reason not to be shown any good enzyme activity in cellulose medium might be caused due to the fact that the les growth of *G. applanatum* in cellulose medium.

With xylan as the main carbon source, absorbance (540 nm) of xylan 0.5 µmol (see table 3.8) and concentration from standard curve (see figure 3.8) was 5.6 µmol. Absorbance of starch 0.46 µmol and concentration 5.2, absorbance of locust bean gum 0.4 and concentration 4.5 µmol was observed. Other cellulose xanthan gum and inulin showed less degradation of polysaccharides than starch, xylan and locust bean gum in xylan medium.

Using locust bean gum as the main carbon source absorbance of xylan 0.2 µmol (see table 3.9) and concentration from standard curve (see figure 3.9) 1.4 µmol. Xylan absorbance was 0.3 and concentration was 2.2 µmol. Inulin and locust bean gum showed very little sugar degradation although cellulose and xanthan gum not showed any activity locust bean gum medium.

4.4 Protein assay results:

Protein contents were determined in defined media using polysaccharides as the main carbon source, which are starch, cellulose, xylan and locust bean gum. Although there are several methods, which provide a relatively quick, simple and sensitive determination, from the journal searches the Lowry`s method appears currently, the most widely and successfully used procedure in determining soluble proteins besides its reliability. Bovine serum albumin (BSA) was taken as standard protein solution. Solutions containing 0-200 µg/ml protein was prepared. The absorbance (at 600 nm) was plotted against the protein concentration of the standard to obtain standard curve.

With starch used as the main carbon source, absorbance (at 600 nm) of the extracellular starch 0.391µg/ml (see table 3.10) and concentration from standard curve (see figure 3.15) was 74 µg/ml. Amount of protein was determined in starch media and this might be caused due to the fact that the starch showed better growth than other used polysaccharides.

With cellulose as the main carbon source, absorbance (at 600 nm) 0.124 µg/ml (see table 3.11) and concentration from standard curve was 23 µg/ml in cellulose medium. Using xylan as the main carbon source, absorbance (at 600 nm) 0.169 µg/ml and concentration from standard curve (see table 3.12) was 31.5µg/m

Absorbance (at 600 nm) of the locust bean gum, which is used as the main carbon source, 0.273 µg/ml and concentration from standard curve, was 52 µg/ml. High amount of protein was observed in starch than locust bean gum, xylan and cellulose. This might be happen due to the fact that the more biomass collected media had more amount of protein and also showed more enzyme activity than less biomass-collected media.

Many published reports show that the fungus *Ganoderma applanatum* produce extracellular enzymes when suitably induced. (Leal *et al.*, 1994). In this investigation some carbohydrates, which are xylan, arabinose, maltose etc., were not reduced suitably. For further experiments, some chromatographic techniques might be applied for reducing sugar. Because results from this work suggest that shaking may have and adverse effect on enzyme activity in shake flask and stirred tank reactor studies. Reduced enzyme activity associated with shake cultures might be caused by forces gathered through agitation damaging mycelium and disrupting enzyme synthesis.

5.APPENDIX

80	4.92	55.6
90	4.76	59.6
100	4.33	51.4
105	4.3	47.5
110	4.34	46.8
120	4.68	4.81
130	4.59	46.2
140	4.55	42.3
150	4.67	29

Table 5.4 Biomass collected from fructose fermentation

	Fructose
Biomass obtained from fermentation (gr)	0.44

Table 5.5 Maltose STR

Time (hours)	pH	% D.O.T
0	5.75	100.7
1	5.62	99.5
5	5.61	98.8
10	5.59	98.4
20	5.49	97.6
30	5.22	96.6
40	4.89	96.1
50	4.57	95.7
60	4.33	95.1
70	4.16	95.2
80	4.05	94.5
90	3.97	93.4
100	3.90	92.8
105	3.88	92
110	3.86	91.6
120	3.83	91.9
130	3.81	91.3
140	3.79	90.7
150	3.77	90.3

Table 5.6 Biomass collected from maltose fermentation

	Maltose
Biomass obtained from fermentation (gr)	0.25

6.REFERENCES

Bland, E., (1998). Immunomodulatory properties of polysaccharides extracted from *Ganoderma applanatum* grown in liquid culture. MSc project. University of Westminster.

Bucke, C., (1998). Biochemistry of Bioremediation by fungi. *Journal of Chemical Technology and Biotechnology*. **71**(4): 356-57

Coughlan, M.P., and Hazlewood, G.P., (1993). Hemicellulose and Hemicellulases. Pg. 1-6, 30-31

D`Souza, T.M., Boominathan, K., Reddy, C.A. (1996). Isolation of laccase gene-specific sequences from white rot and brown rot fungi by PCR. *Applied and Environmental Microbiology*. **62**(10): 3739-3744

Dyke, S.F., (1960).The Carbohydrates. The chemistry of natural products. Volume 5. Pg. 172-176

Florkin, M. and Stotz, E.H., (1963). Comprehensive biochemistry. Carbohydrates. Volume 5. Pg. 209-216

Fogarty, W.M., (1983). Microbial Enzymes and Biotechnology. Pg. 183-190

Franz, G. (1989). Polysaccharides in pharmacy : Current applications and future concepts. *Plant Medica*. **55**:493-497

Horton, H.R., Moran, L.A., Ochs, R.S., Rawn, J.D., Scrimgeour, K.G. (1996). Principles of Biochemistry. Pg. 230-234

Hseu, R.S., Wang, H.H., Wang, H.F., Moncalvo, J.M. (1996). Differentiation and grouping of isolates of the *Ganoderma lucidum* complex by random amplified polymorphic DNA-PCR compared with grouping on the basis of internal transcribed spacer sequences. *Applied and Environmental Microbiology*. **62**(4): 1354-1363

Jong, S. C. and Birmingham, J.M. (1992). Medicinal benefits of the mushroom *Ganoderma. Advances in Applied Microbiology*. **37**:101-129

Kirk, T.K., and Farrel, R.L., (1987). Enzymatic "combustion" : the microbial degradation of lignin. *Annual Review of Microbiology*. **41**: 465-505

Leal, D.P., Isla, M.I., Vattuone, M.A., Sampietro, A.R. (1994). Production of plant protoplast by enzymes from selected wood-destroying fungi. *Applied Microbiology and Biotechnology*. **41**:58-61

Reddy, C.A., and D`Souza, T..M. , (1994). Physiology and molecular biology of the lignin peroxidases of Phanaerochaete chrysosporium . *FEMS Microbiological Review*. **13**: 137-152

Spang, B., (1999). Chemical Engineering: Systems and Surroundings. http://www.Systems and Surroundings-Chemical Engineering-06-14-99.htm.

Stanbury, P.F., Whitaker, A., Hall, S.J., (1995). Principles of Fermentation Technology. Pg. 93-94; 339-340.

Tan, L.U.L., Chan, M.K.H., Saddler, J.N., (1984). A modification of the Lowry method for detecting protein in media containing lignocellulosic substrates. *Biotechnology Letters*. **6**(3):199-204

Tombs, M. and Harding, S.E., (1998). An introduction to polysaccharide biotechnology. Pg. 95-110

Wainwright, W., (1992). An introduction to fungal biotechnology. Pg. 28-31

Williams, M.A.J., and Kirk, P.M. (1988). Introduction : Characteristics of Industrial Fungi.

Winkelman, G., (1992). Microbial Degradation of Natural Products. Pg. 2-5; 28-29; 82-120; 178.

Wiseman, A., (1995). Handbook of Enzyme Biotechnology. Pg. 370-371

Wood, M., Stevens, F. (1998). Bay area fungi: *Ganoderma applanatum*. http://www.mycoweb.com/BAF/species/Ganoderma -applanatum.html

Yang, F.C., and Liau, C.B. (1998). The influence of environmental conditions on polysaccharide formation by *Ganoderma lucidum* in submerged cultures. *Process Biochemistry*. **33**(5):547-553

Winkelmann, G. (1992). Microbial Degradation of Natural Products. Pg. 2-3, 20-25, 83-120, 178.

Wiseman, A. (1985). Handbook of Enzyme Biotechnology. Pg. 170,175.

Wood, M., Stevens, F. (1998). Bay area fungi: *Ganoderma applanatum*. http://www.mykoweb.com/BAF/species/Ganoderma_applanatum.html

Yang, F.C. and Liau, C.B. (1998). The influence of environmental conditions on polysaccharide formation by *Ganoderma lucidum* in submerged cultures. *Process Biochemistry* 33(5):547-553.

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