MICROBIAL ENZYMES FOR THE DEGRADATION OF LIGNOCELLULOSE

BY

Ahmed Abd El-Khalek Salem

B.Sc. Agric. Botany (plant pathology) (1999), M.Sc. Agric. Botany (Agric. Microbiology) (2004), Fac. of Agric. Moshtohor, Benha University.

Dissertation

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

Agricultural Microbiology

Agricultural Botany Department
Faculty of Agriculture,
Moshtohor, Benha University

2010

SUPERVISION SHEET

Name: **Ahmed Abd El-Khalek Salem**

Title: **Microbial enzymes for the degradation of lignocellulose**

Supervision committee:

Prof. Dr.: **Naseem Abd El-Aziz Neweigy**

Professor of Agricultural Microbiology

Fac. Agric., Moshtohor, Benha University.

Prof. Dr.: **Talaat Mohamed. El-Husseiny**

Professor of Agricultural Microbiology

Fac. Agric., Moshtohor, Benha University.

Prof. Dr.: **Hamed El-Sayed Abou-Aly**

Professor of Agricultural Microbiology

Fac. Agric., Moshtohor, Benha Univ.

Prof. Dr.: **Holger Zorn**

Professor of biotechnology and food chemistry,

Institute of Food Chemistry and Food Biotechnology, Giessen, Germany

APPROVAL SHEET

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This thesis for PH.D. degree in agricultural microbiology has been approved by:

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Date: / / 2010

ACKNOWLEDGEMENT

ACKNOWLEDGEMENT

Firstly, my obedience and deepest thanks to **Allah**. Moreover, praise to Allah, who have created us and have not left us without guidance. May Allah's peace and blessings be upon his messenger, our **lord Mohammed**, and upon his family, his companions and his followers.

Deeply indebted to **Prof. Naseem Abd El-Aziz Neweigy** Professor of Agricultural Microbiology Fac. Agric., Moshtohor, Benha University, for his supervision; his kind help; his advise and encouragement during the study and preparation of the manuscript.

Sincer appreciation to **Prof. Dr. Talaat Mohamed EL-Husseiny** Professor of Agricultural Microbiology Fac. Agric., Moshtohor, Benha University, for his supervision; his kind suggestions and helpful criticism.

I would like to thank **Prof. Dr. Hamed El-Sayed Abou-Aly** Professor of Agricultural Microbiology Fac. Agric., Moshtohor, Benha University, for his helpful guidance for offering all scientific and physical facilities required and constructive criticism throughout the work of this study.

I would like to express sincere appreciation to **Prof. Dr. Holger Zorn** Professor of biotechnology and food chemistry, Institute of Food Chemistry and Food Biotechnology, Giessen, Germany for his supervision; his kind help; his advise and encouragement, facilities and resolving the ultimately problems, for his time freely gave throughout the work.

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INTRODUCTION

1. INTRODUCTION

Cellulase, a family of enzymes that breaks down cellulose into glucose, catalyzes the cellulose hydrolysis, ultimately yielding cellobiose and glucose as available carbon and microbial energy sources. Although all cellulolytic enzymes share the same chemical specificity for β-1,4-glycosidic linkages, they show difference in their specificities towards macroscopic properties of substrate **(Dorland's Illustrated Medical Dictionary, 2003)**.

Endoglucanase (Endo-β-1,4-glucanase, E.C. 3.2.1.4) plays an important role in the cellulose hydrolysis by cleaving cellulose chains randomly and thus encouraging strong degradation **(Cao and Huimin, 2002)**. Generally, the indiscriminate action of endoglucanase progressively increases the accessibility of cellulose chain ends, in this manner increasing the specific surface area of the substrate for exocellulase activity. Endoglucanase attacks the β-1,4 glycosidic bonds within the amorphous regions of cellulose chains. The products of this attack are oligosaccharides of various lengths and subsequently new chain reducing ends **(Lynd *et al.*, 2002)**.

Fungi are the main cellulase producing microorganisms, although a few bacteria and actinomycetes have also been reported to yield cellulase activity. Microorganisms of the genera *Trichoderma*, *Aspergillus* and *Chaetomium* are thought to be cellulase producers, and crude enzymes produced by these microorganisms are commercially available for industrial use **(Szengyel *et al.*, 2000)**.

The use of microorganisms or their enzymes for the conversion of lignocellulose into simple organic carbon compounds is receiving increased attention. This is the result of growing concern over the accumulation of wastes, and our awareness of the vast quantities of residues, rich in lignocellulose, which result from agricultural operations and the manufacture of wood products.

Lignocellulose is converted to glucose by a multistep process that includes pretreatment and enzymatic hydrolysis **(Wayman *et al.*, 2005)**.

The aim of this work is to study the production conditions and characterization of cellulase. In addition, the effect of cellulase and some other lignocellulose degrading enzymes on corn stover degradation was investigated.

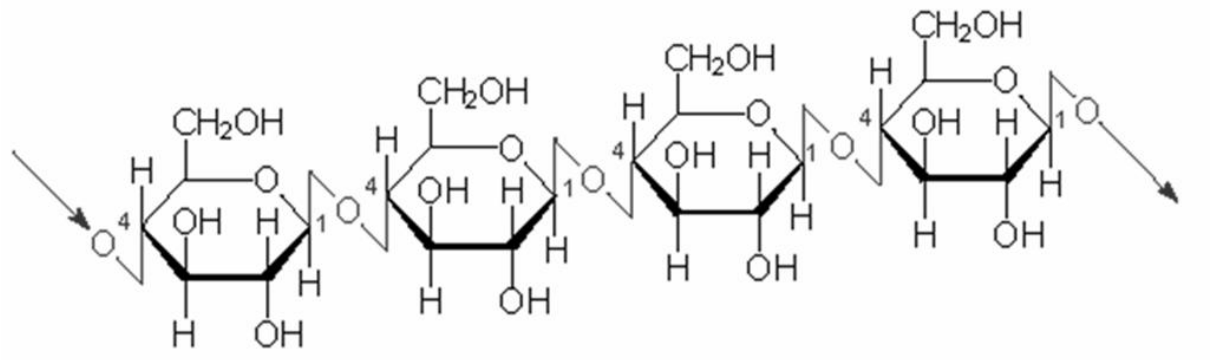
REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. Cellulose and its structure

Cellulose, a principle component of all plant materials, is considered one of the most abundant renewable resources in the world. An estimated synthesis rate of cellulose is approximately 4 X 10^{10} tones per year. Although it is the most promising renewable energy source to overcome the problems of energy resource, chemicals and food in the future, its enormous potential was recognized only after the cellulose degrading enzymes or cellulases had been identified **(Cen and Xia, 1999)**.

Cellulose is made of linked glucose molecules connected by β-1, 4 bonds (Fig.1.). Cellulose is regarded as a valuable resource largely because it can be decomposed into soluble cellobiose and glucose sugars when β bonds are broken **(Dorland's Illustrated Medical Dictionary, 2003)**. This process is called cellulose hydrolysis. Cellulose hydrolysis occurs naturally in soils, sediments, aquatic environments, and in the digestive tracts of animals by microorganisms capable of producing cellulase enzymes **(Leschine, 1995)**.



Non-reducing end **Reducing end**

Fig.1. Diagram showing the structural formula and β–1,4–linkages in a cellulose chain (Samejima *et al*.,1998)

2.2. Cellulase enzyme system

Cellulase, a family of enzymes that breaks down cellulose into glucose molecules, catalyzes the cellulose hydrolysis, ultimately yielding cellobiose and glucose as available carbon and microbial energy sources **(Dorland's Illustrated Medical Dictionary, 2003)**. Although all cellulolytic enzymes share the same chemical specificity for β-1,4-glycosidic bonds, they show difference in their specificities towards macroscopic properties of substrate. Generally, a typical cellulolytic complex includes a variety of hydrolytic and oxidative enzymes such as:

2.2.1. Endoglucanases (β-1,4- D glucan glucanohydrolase,) EC 3.2.1.4**:** These cellulases play an important role in the cellulose hydrolysis by cleaving cellulose chains randomly and thus encouraging strong degradation **(Cao and Huimin, 2002)**. Generally, the indiscriminate action of endoglucanases progressively increases the accessibility of cellulose chain ends, in this manner increasing the specific surface area of the substrate for exocellulase activity. Endoglucanase attacks the β-1,4 glycosidic bonds within the amorphous regions of cellulose chains. The products of this attack are oligosaccharides of various lengths and subsequently new chain reducing ends **(Lynd *et al.*, 2002)**.

2.2.2. Exoglucanases (β-1,4- D glucan cellobiohydrolase**)** EC 3.2.1.91**:** These degrade crystalline cellulose most efficiently and act in a processive mode on the reducing or non–reducing ends of cellulose polysaccharide chains, releasing either glucose (glucohydrolases) or cellobiose (cellobiohydrolases) as major products **(Lynd *et al.*, 2002)**.

2.2.3. β-glucosidases (1,4-β-D glucoside glucohydrolase**)** EC 3.2.1.21**:** These complete the hydrolysis of cellulose. They hydrolyse cellobiose, a potential inhibitor of cellobiohydrolases **(Lemos *et al.*, 2003)**. The catalytic activity of β-glucosidase is inversely proportional to the degree of substrate polymerisation. Though a cellulase, it does not attack cellulose.

These enzymes can be grouped as aryl β-D-glucosidases (hydrolysing exclusively aryl-β-glycosides), cellobiases (hydrolysing diglycosides and cellooligosaccharides) or β-glucosidases with wide range of substrate specificities **(Bhat and Hazlewood, 2001)**. The organization of native cellulose and its hydrolysis by different endoglucanases and cellobiohydrolases is demonstrated schematically in Fig.2.

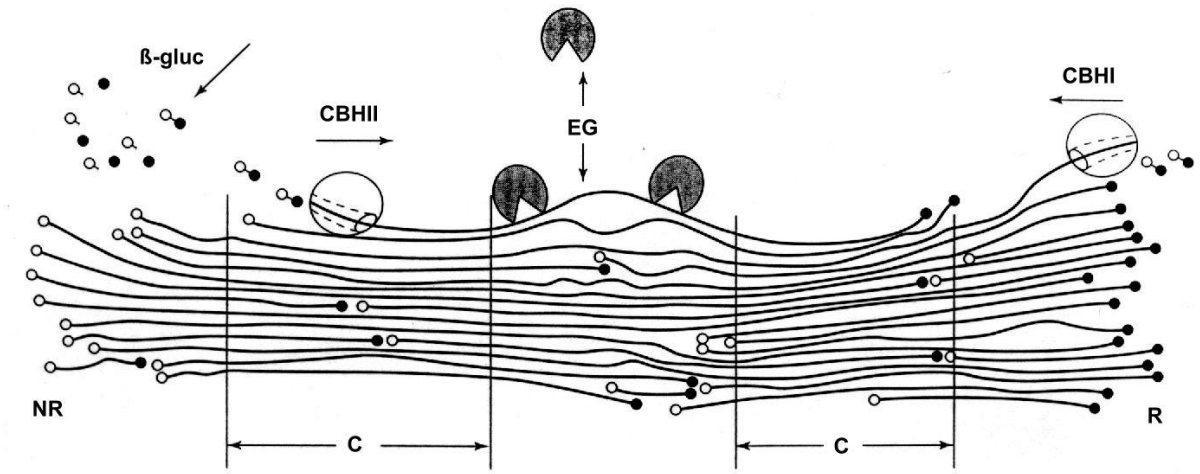


Fig. 2. A schematic view of the cellulose structure and action of the CBHs, EGs, and β-glucosidases (β-gluc)

C: Defines the highly ordered crystalline region.

R: The reducing ends (filled circles).

NR: The nonreducing ends (open circles).

CBH: Cellobiohydrolases or exoglucanases, thought to attack the crystalline areas at the opposite chain ends.

EG: Endoglucanases, thought to attack the middle of the more disordered regions of cellulose.

β-gluc: β -glucosidase action produces glucose **(Teeri, 1997)**

2.3. Importance of cellulase

A cellulase complex containing all three major cellulolytic components are involved in the process of saccharification where agricultural waste residues can be converted to glucose, single cell proteins and biofuels like ethanol. Despite of last 30 years of research, this application has not yet been economically feasible due to cost of enzymes and lack of efficiency of cellulases. Recently, cellulases gained significant commercial importance due to their potential applications in food, animal feed, detergents, paper and pulp, and textile industries.

Still, the growing concerns about the potential consequences of a worldwide shortage of fossil fuels, the emission of green house gases and air pollution by incomplete combustion of fossil fuel has also resulted in an increased focus on the production of bioethanol from lignocellulose **(Zaldivar *et al.*, 2001)** and especially the possibility of using cellulases and hemicellulases to perform enzymatic hydrolysis of the lignocellulosic material **(Sun and Cheng, 2002)**. However, in bioethanol production, it is necessary to reduce the costs of the enzymes used to hydrolyse the raw material and to increase their efficiency in order to render the process economically feasible **(Sheehan and Himmel, 1999)**. In addition, there is a general interest in obtaining new, more specific, stable enzymes **(Jorgensen *et al.*, 2003)** and to use a cheap source of inducer, such as sugar cane bagasse, and recycle all or part of the enzymes. Some of the uses of cellulases are discussed below:

2.3.1. Pulp and paper industry: Cellulases have several uses in this industry as the raw materials used are lignocellulosics in which cellulose is sequestered within a matrix of other components **(Tengerdy and Szakacs, 2003)**. Cellulases therefore are used at both the pre–production and post-production stages in pulp and paper making, and these include:

2.3.1.1. Treatment of waste paper: Detachment of ink (bio–deinking) is improved by using cellulases in waste paper treatment and this waste paper can be subsequently biotransformed to fermentable sugars **(Van Wyk and Mohulatsi, 2003)**. Glucose released from cellulose is fermented into ethanol, which shows a potential alternative for fossil fuels due to its low net emission of CO_2 upon combustion **(Levy *et al.*, 2002)**. The main advantage of enzymatic deinking is the prevention of the use of an alkali, it is therefore environmentally friendly **(Bhat, 2000)**.

2.3.1.2. Treatment of recycled pulps: Endoglucanase facilitates pulp drainage by removing amorphous cellulosic materials such as fines and surface elements **(Ramos *et al.*, 1999)**.

2.3.2. Feed and food industry: Cellulases hydrolyse plant cell wall, thus finding usage in animal feeds and the food industry. Applications of cellulases in this area are, 1- increase in juice production by decreasing the viscosity of the raw juice slurry from fruits during the production of fruit drinks **(Levy *et al.*, 2002)**. 2- Cellulases in combination with hemicellulases are added to animal feeds to supplement the animal's own digestive enzymes, thus increasing the feed digestibility and vitamin assimilation in the animal's gut as a result of the partial hydrolysis of the lignocellulosic materials **(Gilbert and Hazlewood, 1993).**

2.3.3. Textile and laundry industry: Cellulases have been widely used in textile and laundry due to their ability to alter cellulosic fibres in a controlled and mild manner, thus increasing the quality of the fabrics **(Bhat, 2000)**. Cellulases are used in the textile industry for removing excessive dye without damaging the fibre and defuzzing (depiling) fabrics **(Levy *et al.*, 2002)**. Moreover, cellulase preparations are used as additives to laundry detergents to improve the colour brightness, texture and dirt removal from cotton and cotton garments **(Bhat, 2000)**.

2.3.4. Biological control of plant pathogens: Cellulases and related enzymes (hemicellulases and pectinases) are used as biological control of plant pathogens and infections as a result of their ability to degrade the cell wall of plant pathogens, inhibit spore germination, germ tube elongation, and fungal growth. Thus, a combination of fungal strains and their enzymes could be useful as bio-control agents to protect the seeds and plants from plant pathogens. Many cellulolytic fungi including *Trichoderma* sp., *Geocladium* sp., *Chaetomium* sp., and *Penicillium* sp. are known to play a key role in agriculture by facilitating enhanced seed germination, rapid plant growth and flowering, improved root system as well as increased crop yields **(Bhat, 2000)**..

2.4. Cellulase producing microorganisms

In nature there are many microorganisms, fungal and bacterial, that produce enzymes that are capable of catalyzing the hydrolysis of cellulose. These microorganisms can be found in plant debris and soil, *i.e.,* where degradation of plant material takes place. The cellulolytic organisms can be sorted into two different subcategories depending on how the cellulolytic microorganism organizes its enzymes. The first class of cellulolytic microorganisms has cellulolytic enzymes that are organized into multi-enzyme complexes called cellulosomes. In these complexes the individual enzyme molecules are anchored onto a common scaffold. Several different types of enzymes, with different types of catalytic specificity's, *e.g.,* endoglucanases, and cellobiohydrolases CBH. An example of a cellulolytic organism in this class is the bacterium *Clostridium thermocellum*. In addition, the second class of cellulolytic organisms produces enzymes that are not attached to one another, and act individually on cellulose. But the different types of enzymes work cooperatively when hydrolyzing cellulose, and by doing this gain strong synergy effects. Examples of fungi from this class are *Trichoderma reesei* and *Humicola grisea,* and of bacteria, *Streptomyces lividans* and *Cellulomonas fimi* **(Bayer *et al.,* 1998).**

Harchand and Singh (1997) isolated cellulolytic actinomycetes from such sources as composts and soil and evaluated their enzymes. Of several isolates, the most attractive was thermotolerant *Streptomyces albaduncus* as its cellulases were found to be superior in terms of quantity and quality than others. Their report described the properties of cellulase complex of this streptomycete which determine its usefulness in the saccharification of cellulose. Both exoglucanase and endoglucanase enzymes of *S. albaduncus* were found to be thermostable and resistant to end product inhibition.

Cellulolytic enzymes are produced by variety of bacteria, fungi, aerobes and anaerobes. They are detected in mesophiles and thermophiles. Each of these microorganisms can produce different kinds of cellulases that differ in their mode of action as well as properties like activity towards crystalline cellulose, activity and stability in acidic or alkaline pH **(Bhat and Bhat, 1997)**.

The most favoured sources for research and production of cellulases are fungi such as *Aspergillus niger* **(Okada, 1985)**, *Trichoderma reesi* **(Kubicek *et al.*, 1993)**, *Sclerotium rolfsii* & *Fusarium sp* **(Christakopoulos *et al.*, 1995)** and *Penicillium sp.* **(Jorgensen *et al.*, 2004)**.

Moreover**, Ito *et al.* (1998)** mentioned that, although some aerobic *Bacillus* spp. can produce a number of endoglucanases to hydrolyse amorphous cellulose like carboxymethylcellulose (CMC), most of them cannot hydrolyse crystalline cellulose effectively.

In addition, cellulase enzymes production by bacteria with very low activity may indicate that they play a yet unknown role in the breakdown of cellulosic substrates in nature, acting probably as key components of the cellulolytic system of certain cellulase-producing bacteria by means of their synergistic action with other enzymes **(Zhang *et al.*, 2000)**.

There is a large number of microorganisms, including fungi (*Trichoderma, Aspergillus, Penicillium, Fusarium, Sclerotium, Schizophyllum, Monilia*, etc) and bacteria (*Clostridium, Cellulomonas, Pseudomonas, Streptomyces, Actinomycetes*, etc), which can form a cellulase enzyme complex. Among these microorganisms, *Trichoderma* strains are in the center of attention in enzyme production, because they excrete high amounts of cellobiohydrolyses and endoglucanases, however, their enzyme complex is deficient in the ß-glucosidase component. In addition, the most common cellulase enzyme is produced by an aerobic and mesophilic bacteria. However, anaerobic, thermophilic bacteria, such as *Clostridium thermocellum* have also become the subject of research studies recently, partially due to the development of the ethanol production industry **(Bollok and Reczey, 2000)**.

Fungi are the main cellulase-producing microorganisms, although a few bacteria and actinomycetes have also been reported to yield cellulase activity. Micro-organisms of the genera *Trichoderma* and *Aspergillus* are thought to be cellulase producers, and crude enzymes produced by these micro-organisms are commercially available for agricultural use **(Szengyel *et al.*, 2000)**.

Muzariri *et al.* (2001) isolated white-rot fungi from woodlands in two different temperate regions of Zimbabwe. These are namely the dry, hot region and the wet, cool, humid region. The thermophiles were isolated from Zimbabwean hot water springs. The springs are at temperatures of 65 °C, 70 °C, 75 °C, 80 °C and 100 °C and are located in different regions of the country. All the fungi and bacteria so collected were screened for cellulase, laccase, lignin peroxidase, manganese peroxidase and xylanase activities. From the enzyme activities, it is observed that of the dry, hot region collections, 48.3% of the microorganisms produced laccase, 70.7% produced lignin peroxidase, 100% produced xylanase in extremely low or very high amounts, 24% produced manganese peroxidase and 98.3% produced cellulase also in low or high amounts depending on species or strain.

Whereas of the wet, cool region collections, 31.3% produced laccase, 26.9% produced lignin peroxidase, 99.2% produced xylanase, 81.3%produced manganeseperoxidase and 31.3% produced cellulase. Of the presumptive thermophiles, 34.3% produced laccase, 78.1% produced lignin peroxidase, 100% produced xylanase, 28.1% produced manganese peroxidase and 78.1% produced cellulase. The five enzymes listed here are important lignocellulose biodegrading enzymes for the bioremediation of the pulp and paper industries' effluent water towards decontamination and re-cycling.

Arora *et al.* (2002) mentioned that, white-rot fungi such as *Daedalea flavida*, *Phlebia fascicularia*, *P. floridensis* and *P. radiate* have been found to selectively degrade lignin in wheat straw and hold out prospects for bioconversion biotechnology were the aim is just to remove the lignin leaving the other components almost intact. In addition, less prolific lignindegraders among bacteria such as those belonging to the genera *Cellulomonas*, *Pseudomonas* and the actinomycetes *Thermomonospora* and *Microbispora* and bacteria with surface-bound cellulase-complexes such as *Clostridium thermocellum* and *Ruminococcus* are beginning to receive attention as representing a gene pool with possible unique lignocellulolytic genes that could be used in lignocellulase engineering.

Al- Manoliu *et al.* (2002) studied the utilization of *Chaetomium globosum* in some cellulose biotechnological projects. The influence of a petroleum ferrofluid on the biology of *Chaetomium globosum* and on cellulase enzymes complex was also investigated. Cellulase production was determined in the presence of five ferrofluid concentrations (20, 40, 60, 80 and 100 µL/L) supplied in the liquid culture medium. The enzyme activity was spectrophotometrically assayed. They noticed that ferrofluid concentration range is important as well as the culture age, small concentration values led to stimulatory effects in younger cultures while higher concentration values had mainly inhibitory effects, especially in older cultures.

Jorgensen *et al.* (2003) separated and quantificated cellulases and hemicellulases from *Penicillium brasilianum.* Moreover, they mentioned that cellulases and hemicellulases (glycosylhydrolases) are produced by a range of microorganisms, including bacteria, actinomycetes, fungi, and yeast, but fungi appear to be the most efficient producers of extracellular enzymes. The complexity of the enzyme system is increased further by the fact that the microorganisms tend to produce several enzymes within each of the classes. *Trichoderma* sp. is one of the most well studied producers of cellulases and hemicellulases, produces at least two cellobiohydrolases, five endoglucanases, and three endoxylanases.

Kotchoni *et al.* (2003) isolated a mutant strain *Bacillus pumilus* (*BpCRI6*) for its ability to produce significant amounts of cellulase on agar plates. The isolation was after chemical mutagenesis. The selection of this cellulase-producing strain was based on the diameter of the clear zone surrounding the colonies on the plate-screening medium. This screening step was found to give fairly reliable indication of exhibited cellulolytic activities. The *Bacillus pumilus* (*BpCRI6*) clone and the wild type were further assessed in shaking flask cultures under conditions of catabolite repression. In addition, they mentioned that the enhancement of cellulase production in *Bacillus pumilus (BpCRI6)* is not due to an increase in cell growth but is only due to the chemical mutagenesis affecting the synthesis of cellulase within the strain.

Murashima *et al.* (2003) summarized that carbohydrate utilizing microorganisms living in soil or in animal guts can contribute to close the carbon cycle in the biosphere as a result of the combined action of several extracellular enzymes bearing complementary hydrolytic activities. Among these enzymes, cellulases and hemicellulases of bacterial or fungal origins play an essential role in vegetal biomass degradation.

Moreover, *Paenibacillus* sp. BP-23 strain (formerly *Bacillus* sp. BP-23) shows a multienzymatic glycanase system, including endoglucanases **(Pastor *et al*., 2001)** cellobiohydrolase **(Sanchez *et al*., 2003)**, several xylanases **(Blanco *et al*., 1999)**, and pectinases **(Soriano *et al*., 2000)**, acting on vegetal biomass.

Chand *et al*. (2005) isolated cellulase producing fungi and studied how to increase cellulase production using novel mutations. Cellulase-producing fungi were isolated from different soil samples using enriched Mandels cellulose agar, which is a selective media and seven different fungi were selected in the screening programme. These organisms were tested for cellulase production and two potent strains were identified. Two methods of mutations for strain improvement were employed to these strains. Hypercellulolytic mutants were selected on the basis of the diameter of the hydrolysis zone surrounding the colonies and used for the fermentation and for checking their cellulase production abilities. They found that, many isolates were able to grow on Mandels medium. Judging from the ratio between the clearing zone diameter and colony diameter, only seven best isolates (CMV1 to CMV7) were chosen for enzyme production studies. CMV1, CMV3, CMV4 were identified as *Trichoderma* species and CMV2, CMV5, CMV6, CMV7 were identified as *Aspergillus* species.

Camassola and Dillon (2007a) used *Penicillium echinulatum* to produce cellulase and hemicellulases during the solid-state fermentation using a cheap medium containing pretreated sugar cane bagasse and wheat bran. They mentioned that, the highest amounts of cellulase and hemicellulases could be measured on mixtures of pretreated sugar cane bagasse and wheat bran.

Nonetheless, few *Bacillus* and *Paenibacillus* spp. possess endoglucanases with avicelase activity, a catalytic activity towards the commercial microcrystalline cellulose **(Ogawa *et al*., 2007)**.

Wang *et al*. (2008) identified and characterized a novel thermophilic *Paenibacillus* sp. strain B39 which produced a high-molecular weight cellulase with both CMCase and avicelase activities. Members of the *Paenibacillus* genus are facultatively anaerobic organisms, from which many industrial enzymes have been produced widely. In addition, they summarized that strain B39 was closely related to *Paenibacillus cookii* in 16S rRNA gene sequence. A cellulase with both CMCase and avicelase activities was secreted from strain B39 and purified by ion-exchange chromatography. By sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis, the molecular weight of B39 cellulase was also determined.

Also, a wide spectrum of fungi can secrete cellulases to hydrolyse cellulose. For example, *Trichoderma* spp. and *Aspergillus* spp. can produce all three types of cellulases to degrade cellulose efficiently **(Mach and Zeilinger, 2003; Villena and Gutierrez-Correa, 2006)**. Apart from fungi, anaerobic bacteria *Clostridium thermocellum* and *Clostridium cellulolyticum* are among the few bacteria that can hydrolyse cellulose, in particular crystalline cellulose, with their complex cellulase system called cellulosome **(Gal *et al*., 1997 and Kataeva *et al*., 1999)**.

2.5. Optimization of submerged fermentation conditions for maximum cellulase production

2.5.1. Nutritional factors

2.5.1.1. Effect of media

Bollok and Reczey (2000) produced cellulase enzyme from *Trichoderma* sp. and studied the effect of pH on the production process. Concerning the fermentation, the fungus had been grown at 30°C for 2-3 weeks, and afterwards the shake flasks were inoculated by two loops of conidia. 750 ml shake flasks were used with 10 g/l Solka Floc cellulose in 150 ml Mandels medium. The pH was adjusted to 5.5-6.0 before sterilization.

Garcia-Kirchner *et al.* (2002) used fermentation media containing sugarcane bagasse pith for the production of cellulase enzymes. The production was by two filamentous fungi strains, *Penicillium* sp. and *Aspergillus terreus.* Inoculum was prepared and flasks of 500 ml with 180 ml of the fermentation culture medium were incubated in a rotary shaker at 180 rpm and 29°C for 6 days. Samples were periodically removed (after 2, 4, 5, and 6 days) and analyzed for pH, filter paper (FPase), carboxymethylcellulase (CMCase), β-glucosidase, xylanase activities, and soluble protein.

Jorgensen *et al.* (2003) separated and quantificaed cellulases and hemicellulases from *Penicillium brasilianum*. The fermentation was in a 5-L bioreactor with a working volume of 4 L. The carbon source was either 20 gl^{-1} cellulose or 10 g l^{-1} cellulose and 10 g l^{-1} oat spelt xylan. In addition, the medium contained (g l^{-1}) ammonium sulphate (2.8), potassium sulphate (4.0), magnesium sulphate (0.6), calcium chloride (0.6), urea (0.6), yeast extract (0.5), bacto peptone (1.5) and Tween 80 0. (2 ml l^{-1}). The fermentor was inoculated then, the pH and the temperature were maintained at 5.0 and 30°C, respectively. The fermentor was aerated and agitated at 300– 500 rpm. Samples were filtered through lowprotein binding filter and stored until analysis.

Rao *et al*. (2003) studied the development of cellulase from extremophilic actinomycetes for application in textile industry. *Thermomonospora* sp. was grown for 120 hours under constant shaking at 50°C in a modified media, containing 1% yeast extract, 4% cellulose paper powder and 0.1% Tween 80. The pH of the medium was adjusted after autoclaving, to 9. Moreover, various fermentation conditions were studied in order to improve the cellulase productivity.

Chand *et al*. (2005) used enriched Mandels cellulose agar, which is a selective media. During screening programme, seven different fungi were selected as cellulase producers. For cellulase enzyme production, the fungi were grown in normal medium of **Mandels and Weber (1969)** which containing (in g l^{-1} of distilled water) KH_2PO_4 (2.0); $(NH_4)_2$ SO_4 (1.4); $CaCl_2$ (0.4); $MgSO_4.7H_2O$ (0.3); urea (0.3); Proteose peptone (1.0); Cellulose (10.0) and trace metal solution, 1ml [$FeSO_4.7H_2O$, 5 mg/ l ; $MnSO_4.7H_2O$, 5.6 mg/ l ; $ZnSO_4.7H_2O$, 3.34 mg/l; $CoCl_2.2H_2O$, 2 mg/ l], Tween 80 (1ml).

Vyas (2004) produced cellulase enzyme from alkalotolerant fungal strain was selected and identified as *Fusarium* sp. He mentioned that the enzyme can be produced on agricultural residues like bagasse pith, corncob, and wheat bran. For cellulase enzyme production, the fungus was grown in normal medium of **Reese and Mandels (1963)** which containing (in g l^{-1} distilled water) KH_2PO_4 (2.0); $(NH_4)_2$ SO_4 (1.4); $CaCl_2$. $2H_2O$ (0.3); $MgSO_4.7H_2O$ (0.3); urea (0.3); Proteose peptone (0.25); Cellulose (10.0) and trace metal solution 1ml [$FeSO_4.7H_2O$, 5 mg l^{-1} ; $MnSO_4.7H_2O$, 5.6 mg l^{-1} ; $ZnSO_4.7H_2O$, 3.34 mg l^{-1}; $CoCl_2.2H_2O$, 2 mg l^{-1}], Tween 80, 1ml. The crude culture filtrate showed following extracellular cellulolytic and xylanolytic activities: CMCase, Filter Paper degrading (FPase), β-D-glucosidase, β-D-xylanase and β-D-xylosidase.Thus the concentrations of the key variables in the optimized production medium obtained from the factorial design were as follows (g l^{-1}): cellulose powder (25); ammonium sulphate (0.1); yeast extract (1.0); urea (1.0) and Tween 80 0.05 % (v/v). The maximum CMCase activity obtained on the 8^{th} day was 28-30 IU/ml that is four fold higher as compared with enzyme yields obtained in basal medium.

The optimization of fermentation conditions is an important problem in the development of economically feasible bioprocesses. Therefore, response surface methodology, which is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions, has successfully been used in the optimization of bioprocesses. The objective of this work was to apply response surface methodology to evaluate the effects of the medium components on cellulase production by the mutant *T. reesei* WX-112 and to search for the optimal medium composition for attaining a higher cellulase yield. The maximum production of cellulase by *T. reesei* WX-112 was obtained in the optimized medium when the initial concentration of Avicel and soybean cake flour was 36.4 and 24.7 g l^{-1}, respectively. The final concentration of the medium optimized with response surface methodology was (in g l^{-1}): wheat bran (30), Avicel (36.4), soybean cake flour (24.7), KH_2PO_4 (4.0) and corn steep flour (5.0). Compared with the original medium, the cellulase activity increased from 7.2 to 10.6 IU/ml **(Hao *et al*., 2006).**

Narasimha *et al*. (2006) studied the effects of nutrients on production of cellulolytic enzymes (exo-glucanase, endo-glucanase and β-glucosidase activities) by *Aspergillus niger*. The production was compared on three different liquid media (minimal, basal and Czapek-Dox) each amended with 1% cellulose as carbon source. The culture filtrate of this organism exhibited relatively highest activity of all three enzymes and extracellular protein content at 7-day interval during the course of its growth on Czapek-Dox medium supplemented with 1.0% (w/v) cellulose. The extracellular protein content in the culture filtrate of 3 media followed the same pattern of growth with maximal values in the respect of Czapek-Dox medium. Thus, it is clearly evident from the results of the present study that Czapek-Dox medium appeared to be superior for growth and cellulase production by *A. niger*.

Camassola and Dillon (2007a) used the pretreated sugar cane bagasse and wheat bran as the support and main carbon sources for the production of cellulases and hemicellulases by *Penicillium echinulatum*. The culture media consisted of mixtures of different ratios of sugar cane bagasse and wheat bran, ammonium phosphate and ammonium sulphate as nitrogen sources and some metal ions were also included. The flasks were autoclaved and then inoculated with sufficient conidial suspension. The medium moisture was adjusted to 67% by the addition of distilled water. The flasks were incubated at 28°C and 90% humidity for 5 days. After incubation, cellulase enzymes were extracted for further studies.

Camassola and Dillon (2007b) studied the effect of supplementing liquid cellulase production media (CPM) with methylxanthines (aminophylline, caffeine and theophylline), with and without the addition of glucose, on the synthesis of cellulases by *Penicillium echinulatum* strain 2HH (wild-type) and the derived mutant strain 9A02S1. Stock cultures for inocula and production culture were carried out by culturing the strains on cellulase production agar (CPM) containing (g l^{-1}): cellulose (10.0); proteose peptone (1.0); KH_2PO_4 (0.2); $(NH_4)_2SO_2$ (0.13); CO $(NH_2)_2$ (0.03); $MgSO_4.7H_2O$ (0.03); $CaCl_2$ (0.03); $FeSO_4.7H_2O$ (0.005); $MnSO_4$.H2O (0.0156); $ZnSO_4.7H_2O$ (0.0014); $CoCl_2.6H_2O$ (0.002); agar (15.0).

2.5.1.2. Effect of carbon source and its concentrations

Many cellulolytic organisms do not express their cellulases constitutively. The production of the cellulases is only turned on when these are needed, *i.e.,* the cellulases are induced **(Kubicek and Penttila, 1998)**. When *T. reesei* is grown with cellulose as the only carbon source, the genes for the cellulases are induced, and the enzymes are expressed. However if the fungus is grown on glucose there is no cellulase expression, *i.e.,* the cellulases are glucose repressed.

It has been shown that this glucose repression of the *T. reesei* cellulases is on a transcriptional level. It has also been shown that the expression of most *T. reesei* cellulases is coregulated, and that they always are expressed at same relative amounts **(Ilmen *et al.*, 1997)**.

There are many known molecules that induce cellulase expression in *T. reesei, e.g.*, cellobiose, cellotriose, cellotertraose, lactose, and sophorose **(Kubicek *et al.*, 1993)**. The exact mechanism by which the cellulase expression is induced in *T. reesei* is not fully understood.

As soluble carbon sources (lactose, glucose, fructose, etc.) can be metabolized easily, their use results in rapid cell growth with lower enzyme yields than those achieved on purified cellulosic substrates (Avicel, Solka-Floc, cotton) **(Chen and Wayman, 1991)**.

Moreover, an increase in enzyme titers was observed with the increase in substrate concentrations **(San-Martin *et al.*, 1986)**, whereas the productivity was lower at higher concentrations.

Refined cellulosic substrates such as Solka-Floc **(Hayward *et al.*, 2000)**, Avicel (trade name for microcrystalline cellulose) **(Aiello *et al.*, 1996)** are better substrates for cellulase production than agricultural residues. This may be attributed to higher lignin content in agricultural residues affecting the cellulase production **(Bigelow and Wayman, 2002)**.

The substrate used for enzyme production exerts a strong influence on enzyme activity and the composition of cellulase enzymes. Cellulase is an inducible enzyme system, thus cellulose, cellobiose, and lactose effectively induce the enzyme at high concentrations, whereas others such as sophorose and sorbose are effective at low concentrations. Sorbose inhibits the glucan synthetase, thus the glucan, which is the main component of fungal cell walls, is not synthesized, resulting in an enhanced excretion of enzyme components **(Chen, 1996)**.

Mohagheghi *et al.* (1988) achieved higher enzyme yields with *T reesei Rut C-30* growing on a mixture of xylose and cellulose than that obtained on a pure cellulose carbon source. Xylose, as a soluble sugar, is necessary for the initial growth, while the cellulose is used as an inducer and an additional carbon source, for enzyme production after consumption of soluble sugars. This is of great importance since the xylose content in hardwood is significant, and this is not fermented to ethanol with *S. cerevisiae*, so it can be utilized for enzyme production**.**

Maj *et al.* (2002) found that the strain of *Trichoderma hamatum* C-1 is able to produce extracellular lytic enzymes *i.e.* β-1,3- glucanases, chitinases, β-1,6-glucanases in the shaked cultures in the base medium enriched with fodder yeast supplemented with Avicel cellulose, as well as with glucose. The enzymes were precipitated from liquid culture medium by ethanol and then optimal conditions for the enzymatic activity and stability of β-1,3-glucanases were determined. They mentioned that glucose had a repressive effect on β-1,3-glucanase activity only when the fungus was cultivated in a high quantity (3%) of glucose medium.

Kotchoni *et al.* (2003) produced cellulase enzyme from a mutant strain *Bacillus pumilus* (*BpCRI6*). The *Bacillus pumilus* (*BpCRI6*) clone and the wild type were further assessed in shaking flask cultures under conditions of catabolite repression. The cultures were incubated for 24 h. to obtain an optimum production of the enzyme. They investigated that at 10 mM glucose concentration, the mutant strain *BpCRI 6* exhibited approximately ten times higher activity on CMC than the wild type, and approximately 6 times higher on cellobiose than the wild type. These represent approximatively 80 % of the mutant's ability to produce cellulase in the absence of glucose, which means that the mutant loses only 20 % of its cellulase production ability under catabolite repression. On the other hand, at 20 mM glucose concentration, no cellulase activity was detected in the wild type, indicating a complete repression of the

enzyme synthesis, but *BpCRI 6* strain still produced significant amount of cellulase (0.9 U/ml on CMC, and 0.5 U/ml on cellobiose) under the same condition. Interestingly, a higher cellulase activity was detected under condition of catabolite repression (40 mM glucose) when the wild type and *BpCRI 6* strain were combined in the same culture.

Rao *et al.* (2003) produced cellulase from extremophilic actinomycetes, *Thermomonospora* sp., for application in textile industry. The growth was for 120 hours under constant shaking at 50°C in a modified media, containing 1% yeast extract, 4% cellulose paper powder and 0.1% Tween 80. The pH of the medium was adjusted after autoclaving, to 9. Various fermentation conditions were studied in order to improve the cellulase productivity. The microorganism was grown in a medium containing different carbon sources. The maximum CMCase production of 5 IU/ml was obtained at 120 hours, when 4% cellulose powder (CPP) was used as the carbon source. Agricultural wastes such as wheat bran and corncob were also suitable for CMCase production. However, very low activity was obtained whey xylan, sawdust and coconut stalks were used as carbon sources.

Vyas (2004) produced cellulase enzyme from alkalotolerant fungal strain was selected and identified as *Fusarium* sp. The inhibition of endoglucanase by cellobiose and glucose was examined using viscometric method. The plots of fluidity of 1% CMC solution as a function of incubation time for endoglucanase in the absence and presence of cellobiose (0.1%, 1%, 5% W/V) or glucose (0.1%, 1%, 5%) were determined. Both cellobiose and glucose have shown to inhibit cellulose hydrolysis. Cellobiose at 5 % concentration inhibited Endoglucanase A activity by 82% while 60% inhibition of endoglucanase A activity was observed by glucose at 5% concentration. Indicating that the inhibition of cellulase due to glucose is weaker than cellobiose. In addition, maximum CMCase activity obtained with using 25 g/l cellulose powder.

Similarly, induction of cellulase production by *Humicola fuscoatra* in presence of cellulosic substrates has been reported by **Rajendran *et al*. (1994)**.

In addition, Low level of cellulolytic enzymes in the presence of glucose could be attributed to repression of synthesis of cellulolytic enzymes involved in the utilization of cellulose by easily metabolisable carbon, glucose that was demonstrated in many organisms **(Ruijter and Visser, 1997 and Suto and Tomita, 2001)**. However, insensitization of this repression by mutations resulted in higher production of cellulase even in the presence of glucose **(Kotchoni and Shonukan, 2002)**.

2.5.1.3. Effect of nitrogen source and its concentrations

Various researchers have shown that different organic and inorganic nitrogen sources such as yeast extract **(Ganguly and Mukherjee, 1995)**, soyameal **(Gomes *et al*., 2000)** and corn steep liquor **(Hayward *et al*., 2000)** influence the cellulase production.

Abu and Ado (2004) studied the effect of nitrogen source and supplementation of millet and sorghum pomace on the production of cellulase by *Aspergillus niger* SL.1 using a submerged culture fermentation. The nitrogen sources (ammonium sulphate, yeast extract groundnut cake and neem seed cake) were supplemented at 5 – 20% level in the pomace medium and fermentation was carried out for 72 hours. They investigated that, supplementation of both millet and sorghum pomace with 10 – 20% groundnut cake and also 5 – 20% neem seed cake in sorghum pomace media significantly (0.05) recorded higher level of cellulase production than the control.

Vyas (2004) used alkalotolerant fungal strain which was screened and identified as *Fusarium* sp. for cellulase enzyme production. The crude culture filtrate showed following extracellular cellulolytic and xylanolytic activities: CMCase, Filter Paper degrading (FPase), β-D-glucosidase, β-D-xylanase and β-D-xylosidase. The effect of inorganic nitrogen source was studied similarly by

replacing ammonium sulphate from the medium by different inorganic nitrogen sources. They found that there is substantial increase in the enzyme activity when the medium is supplemented with complex nitrogen sources like yeast extract and urea. Moreover, ammonium sulphate (0.14%), yeast extracts (0.05%), urea (0.06%) were found to be the best inorganic, organic and complex nitrogen sources for cellulase production by the fungus.

Narasimha *et al*. (2006) studied the effects of nutrients on production of cellulolytic enzymes (exo-glucanase, endo-glucanase and β-glucosidase activities) by *Aspergillus niger*. The production was compared on three different media in liquid shake culture. In addition, different nitrogen sources at concentration of 0.03% nitrogen were compared. The culture filtrate of this organism exhibited relatively highest activity of all three enzymes and extracellular protein content at 7-day interval during the course of its growth on Czapek-Dox medium supplemented with 1.0% (w/v) cellulose. They investigated that, the effectiveness of nitrogen source in supporting cellulase production along with growth, secretion of extracellular protein by *A. niger* decreased in the following order: Urea>peptone>NaNO3. Data indicating that, urea as a nitrogen source is optimal for growth and cellulase production by *A.niger*.

Similarly, increase of urea concentration from 2 to 6 g/L and reduction of yeast extract from 6 to 4 g L^{-1} in the medium improved endocellulase production of *Clostridium thermocopriae* **(Jin and Toda, 1989)**.

Moreover, amino acids, irrespective of carbon sources used, highly stimulated extracellular production of cellulase enzyme by *Termitimyces clypeatus* **(Khowala and Sengupta, 1992)**. In contrast, the growth of *Trichoderma reesei* on production medium without nitrogen source increased cellulase production **(Turker and Mavituna, 1987).**

2.5.2. Environmental factors

2.5.2.1. Effect of initial pH

Bollok and Reczey (2000) studied the effect of pH on cellulase enzyme production by *Trichoderma reesei Rut C-30.* The fermentation was on Solka-Floc and fibrous pretreated willow. The effect of pH on cellulase production was studied for both substrates. The pH was adjusted daily to 6.5, 6.0, and 5.0. For each experimental condition three parallel fermentations were run, and the mean values of the enzyme activity and the reducing sugar concentration were calculated.They investigated that, cellulase enzyme production showed a distinct increase with increase of the pH. Consequently, the highest enzyme activity was achieved at pH 6.5 with a maximum of 0.663 U/ml, which was 96% of that obtained for Solka-Floc. In addition, the pH 5.0 was unfavorable for cellulase enzyme production.

Vyas (2004) used alkalotolerant fungal strain was screened and identified as *Fusarium* sp. for cellulase enzyme production. The crude culture filtrate showed following extracellular cellulolytic and xylanolytic activities: CMCase, Filter Paper degrading (FPase), β-D-glucosidase, β-Dxylanase and β-D-xylosidase. The effect of initial pH on the production medium showed that there is a difference in the growth and yield of enzyme. The fungus grew well around pH 7 and produced high amounts of enzyme in neutral to alkaline medium confirming alkalotolerant nature of the fungal isolate.

2.5.2.2. Effect of fermentation period

Bollok and Reczey (2000) studied the effect of pH on cellulase enzyme production by *Trichoderma reesei Rut C-30.* The fermentation was on Solka Floc and fibrous pretreated willow. The effect of pH on cellulase enzyme production was studied for both substrates. For each experimental condition three parallel fermentations were run, and the mean values of the enzyme activity and the reducing sugar concentration were calculated. They investigated

that, cellulase enzyme production reached maximum values after five days fermentation.

El-Baz (2003) studied the time course of cellulolytic activities production by different local fungal cultures using the same standard conditions. For achieving this purpose, seven different fungal cultures of *Trichodernia viride* and *Aspergillus niger* were grown in the basal culture medium containing pure cellulose as sole source for carbon and energy. The fermentation was carried out for different fermentation periods under shaking conditions. It was recorded that the most fungal strain and its culture filtrate of seven days old sllowed the highest CMCase activity.

Vyas (2004) produced cellulase enzyme from alkalotolerant fungal strain was selected and identified as *Fusarium* sp. The crude culture filtrate showed following extracellular cellulolytic and xylanolytic activities: CMCase, Filter Paper degrading (FPase), β-D-glucosidase, β-Dxylanase and β-D-xylosidase. He investigated that onset of activity in the fermentation broth starts after two days fermentation onwards. In addition, the maximum enzyme production obtained on the 8^{th} day while it remains constant thereafter with slight decrease after the 10^{th} day. Moreover, with a shortened culture period of 12 days, elevated activities of 530 U/ml for CMCase with an elevated protein content of 40 mg/ml in the culture broth.

Narasimha *et al.* (2006) investigated the effects of nutrients on production of cellulolytic enzymes (exo-glucanase, endo-glucanase and β-glucosidase activities) by *Aspergillus niger*. The culture filtrate of this organism exhibited relatively highest activity of all three enzymes and extracellular protein content at 7-day interval during the course of its growth on Czapek-Dox medium supplemented with 1.0% (w/v) cellulose.

2.5.2.3. Effect of inoculum volume

El-Baz (2003) designed an experiment to study the effect of inoculum volume on the capacity of cellulase production by *Trichodernia viride* and *Aspergillus niger*. Fermentation was then performed with different volumes of inoculum ranging from 2.0 - 10.0 %. The obtained results showed that the inoculum size greatly affected the cellulase production by test fungi. 5.0% inoculum volume was sufficient for maximum cellulase formation. Moreover, higher proportion than 5.0% of inoculum led to low cellulase enzyme formation. Increasing the inoculum volume was associated by gradual increase in protein content of culture filtrate. The protein content in the culture filtrate was increased by the increase in the volume size from 2.0-10.0%.

2.5.2.4. Effect of Tween 80 concentration

Kaar and Holtzapple (1998) focused on improving cellulase efficiency using Tween 20 and Tween 80. They recorded that Tween 20 is slightly more effective than Tween 80. The recommended Tween loading was 0.15 g Tween/g dry biomass. Addition of Tween improved the cellulose, xylan, and total polysaccharide conversions by 42, 40, and 42%, respectively. Kinetic analyses showed that Tween improved the enzymic absorption constants, which increased the effective hydrolysis rate compared to hydrolysis without Tween. Furthermore, Tween prevented thermal deactivation of the enzymes, which allows for the kinetic advantage of higher temperature hydrolysis.

It appears that Tween improves the hydrolysis through three effects: enzyme stabilizer, lignocellulose disrupter, and enzyme effector.

According to study carried out by **Domingues *et al.*, 2000**, Tween 80 influences the morphology of *Trichoderma reesei Rut* C-30 as well as the enzyme production. Moreover, emulsification with Tween 80 led to higher cellulase activities presumably by causing increased permeability of cell membranes and/or by promoting the release of cell-bound enzymes.

The effect of the agent added to the culture medium depends on the microorganism itself rather on the synthesized enzyme. Therefore, the effect of Tween 80 concentration on the production of cellulases by *Trichodernia viride* and *Aspergillus niger* was investigated. Experimentally, different amounts of Tween 80, ranging for1.0 – 4.0 ml/L were added to the culture medium. It is seen from the obtained results that addition of increasing amounts of Tween 80 up to 2.0 ml/L in the culture medium led to a gradual improvement in cellulolytic enzyme production whereby maximal cellulase production was attained. Moreover, addition of 1.5 ml/L of Tween 80 to the culture medium led to 2.4 and 1.5 fold increase in activities of CMCase of *Trichodernia viride* and *Aspergillus niger*, respectively **(El-Baz, 2003)**.

Vyas (2004) studied the effect of surfactant addition to the culture medium on cellulase production by alkalotolerent *Fusarium* sp. It was suggested that addition of Tween 80 (0.1 %, v/v) was essential in order to facilitate the release of cellulases in the medium. Tween 80 was found to be the best surfactant in this regard.

2.6. Characterization of cellulase and xylanase enzymes

2.6.1. The optimum temperature and heat stability

The loss of enzyme activity depends both on time and temperature, it is therefore important to investigate the stability of the enzyme with respect to these factors **(Roe, 2001)**.

Li *et al.* (1998) purified a novel endocellulase, designated as endoglucanase S, from culture supernatant fluids of the newly isolated *Streptomyces* sp. LX. Temperature optima and stability were studied. In all the determinations, endocellulase activity was measured using CMC as a substrate. For estimation of the temperature optimum, the activity was determined by carrying out standard assays at several temperatures. To estimate temperature stability, the residual activity, after the incubation of a liquor of endoglucanase S

for 30 min at different temperatures, was measured under standard conditions. They investigated that, the optimum temperature is 50 °C and enzyme activity in the range between 30 and 100 °C revealed quite stability to temperature up to 60°C. The relative activity of endoglucanase S dropped only 2% at 60 °C, whereas it showed a 70% loss of activity at 70 °C.

Fadel (2001) studied the effect of temperature on xylanase activity and stability. The crude xylanase was incubated with the substrate at different temperatures ranging from 30 to 100 °C for a period of 10 min followed by the estimation of the enzyme activities. In an attempt to determine the heat stability, the enzyme dissolved in buffer solution pH 5.5 was incubated for 4 h at different temperatures and then cooled prior to measure the residual activity under optimum temperature resulted from the above determination. He investigated an increase in activity with the rise in temperature. The optimum activity was reached at 55-60 °C. Moreover, it was found that the enzyme expressed nearly its original activity after incubation at 55 °C and 52% of its original activity after incubation at 100°C.

Coral *et al*. (2002) prepared carboxymethyl cellulase enzyme from a wild type strain of *Aspergillus niger*. For the determination of optimal enzyme temperature, the enzyme was incubated with the substrate for 30 min at various temperatures between 30 and 90°C. The thermostability of carboxymethyl cellulase was studied by incubation in the standard buffer at 40, 50, 60, 70, 80 and 90 °C for 15 min and reaction tubes were placed in ice. Then the activity was assayed under the standard conditions. They investigated that, **t**he optimum temperature was around 40°C and 41.2% of the original activity was retained after heat treatment at 90°C for 15 min.

Oyekola (2003) determined the temperature profile for endoglucanase (immobilized and soluble form) at the optimum pH. The soluble enzyme extract was incubated with the substrate (CMC) at the varying temperatures ranging from 10 to 70°C. The endoglucanase activity was determined. Moreover, in an

attempt to determine the thermal stability, enzyme extracts (immobilized and soluble) were heated at 30, 50, and 70 °C for 1 h at the optimum pH. Samples were prepared in triplicate and residual enzyme activity was measured by starting the reaction with the addition of the substrate to the samples. Estimation of enzyme activity in the immobilized extract was also determined, but the reaction mixture was centrifuged after assay so as to remove the suspended solids that could cause interference during the absorbance reading. It was found that, the optimal temperature for endoglucanases at pH 6 was 50°C. Concerning the thermal stability, the enzyme remained 100 ± 1.29 % active at 30 °C for 1 h both in its soluble and immobilized forms. At the optimum temperature (50 °C) however, the enzyme retained full activity for 1 h only when immobilized to the floc matrix. In the soluble form, the enzyme lost 50 ± 0.84 % of this activity after 10 min, maintaining this for the next 50 min. Moreover, at 70 °C, there was a complete loss of activity in both soluble and immobilized forms after 10 min.

Rao *et al*. (2003) studied the effect of temperature on the activity and stability of CMCase enzyme. Estimations of CMCase activity at different temperature (30-90°C) values were carried out under standard assay conditions to determine temperature for enzyme activity. The temperature stability was determined by incubating 5 IU of enzyme, at varying temperatures for different time intervals, and then estimating the residual activity under standard assay conditions. It was found that, the CMCase showed maximum activity at 50°C. On either side of this temperature, there was a decline in activity. 50% activity was found at 40 and 60°C. The CMCase was highly stable at 50°C. It showed 100% activity up to 72 h and had half lives of 7 h and 3 h at 60 and 70°C, respectively.

The conditions of temperature for maximum xylanase activity and thermal stability were studied. Thermal stability was investigated by incubating the enzyme at 30, 55, 60 and 65°C for different times. Immediately afterwards the enzyme was immersed in an ice bath and then the residual activity was

determined under standard conditions. The optimum temperature was 50-55°C. The enzyme was stable at temperature up to 55°C, and it retained 50% and 23% of its initial activity when heated for 1 h at 60 and 65°C, respectively **(Reis *et al*., 2003)**.

Temperature is crucial environmental factor influencing enzyme activity and the rate of solubilization in reactors. Most reactors are mesophilic and increasing the temperature should increase enzyme activity. However, in increasing the reactor temperature and maintaining the pH of the bioreactors for optimal enzyme production, the optimum temperature of the enzyme as well as its thermal stability should be considered. The rates of enzyme-catalysed reactions are usually directly proportional to temperature. On the other hand, at temperature above 50 to 60°C, some mesophilic enzymes characteristically show a decrease in activity. The loss of enzyme activity at elevated temperatures is a consequence of thermal denaturation of protein **(Whiteley *et al*., 2003).**

Chen *et al*. (2004) purified and characterized carboxymethyl cellulase from *Sinorhizobium fredii*. In an attempt to determine the effect of temperature on activity and stability, the enzyme was assayed at various temperatures in 50 mM phosphate buffer (pH 7.0). They indicated that, although it had maximal activity at 35°C, it retained 96% of its activity at 40°C. The thermal stability of the enzyme indicated that the activity was stable at 30 to 45°C, kinetic was low at 20 to 25°C.

Vyas (2004) purified endoglucanase from the culture filtrate of alkalotolerent *Fusarium* sp. The effect of temperature on endoglucanase activities was estimated in a temperature range of 30-80°C. The temperature stability of the enzyme was detected by incubating 100µg enzyme at 50-70°C for different time intervals. The samples were removed periodically and assayed for residual CMCase activity under standard assay conditions. It was investigated that, the enzyme showed maximum activity at 60°C and retained

80% of its maximum activity after 4h of incubation at 50°C. The enzyme was rapidly inactivated at 70°C and shows half-life of 1.5 h at this temperature.

Wang *et al.* (2008) identified and characterized a novel thermophilic, cellulose-degrading bacterium *Paenibacillus* sp. strain B39, which produced a high-molecular weight cellulase with both CMCase and avicelase activities. The effect of temperature on the activity and thermostability of cellulase enzyme was investigated. They found that, the enzyme showed a maximum CMCase activity at 60°C. Considering its stability under high temperature, after 30 min of incubation at 70°C, it retained over 80% of the activity indicating that B39 cellulase was thermoactive and may be exploited for industrial usage.

2.6.2. The optimum pH and pH stability

The ionization state of amino-acid residues in the active site of an enzyme is dependent on pH. Since catalytic activity is dependent on the state of ionization of these residues, enzyme activity is consequently pH dependent. Usually, enzymes are active only over a narrow range of pH with a particular pH (pH optimum) at which their catalytic activity is maximal **(Wilson, 2000).**

Fadel (2001) produced xylanase enzyme from *Trichoderma harzianum* strain and studied the effect of pH on xylanase activity and stability. The activity of the crude xylanase was measured in pH range of 2.5 to 10.5 using four different 0.1 M buffers (Sodium citrate for pH 3.0-6.0, sodium phosphate for pH 6.0-8.0, tris-HCI for pH 8.0-9.0, and glycine-NaOH for pH 9-10.5). It was investigated that the enzyme exhibited high activity in a pH range of 3.0-10.0 and optimum was recorded at pH 4.5-5.5. The enzyme also exhibited remarkable stability at pH values ranged from 3.0 to 10.0 for 20 h by retaining its original activity at these pH values before incubating the enzyme solution with different buffers.

Coral *et al.* (2002) studied the optimum pH of carboxymethyl cellulase enzyme prepared from a wild type strain of *Aspergillus niger*. Analyses of the enzyme preparation by SDS-PAGE revealed two protein bands showing cellulolytic activity. For the estimation of optimum pH, the enzyme was mixed with substrates at different pH levels (pH 3.0 to 9.0). These substrates were prepared in two buffer solutions: citrate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4, pH 3.0 to 7.0), and tris buffer (0.08 M tris, 0.1 M HCl, pH 7.5 to 9.0). Then the activity was assayed by DNS method under the standard conditions. It was found that the CMCase activity of *A. niger* has a broad pH range between 3.0 and 9.0. Moreover, the enzyme shows two major activity peaks at pH 4.5 and 7.5. This result is probably due to the presence of two isoenzymes or subunits in the enzyme preparation.

In an attempt to investigate the effect of pH on activity of carboxymethyl cellulase enzyme, the substrate and the soluble enzyme extract were prepared in the different buffers. The different buffers used at varying pH were: 0.05 M sodium acetate (pH 3.0-4.5), 0.05 M sodium citrate (pH 5.0 - 5.5) and 0.05 M sodium phosphate buffer (pH 6.0-8.0). Concerning the substrate, since carboxymethyl cellulose is an ionic substrate, its properties would change with pH, therefore a non-ionic substrate, hydroxyethylcellulose, was used in this study. The activity of the endoglucanase was determined and the pH optimum of endoglucanases was found to be pH 6.0 **(Oyekola, 2003).**

Reis *et al.* (2003) investigated the effect of pH on xylanase (produced by a wild strain of *Aspergillus nidulans*) activity and stability. The optimal pH was determined assaying the enzyme at various pH degrees in McIlvaine buffer (pH 2.8-8.1). The pH stability was investigated by incubating the enzyme at different pH for 4 h at 40°C. The remaining activities were measured under standard conditions. To pH superior than 8.0, the experiments were done by using 0.1 M glycine buffer. It was found that crude xylanase was most active between pH

5.0-6.0 and the enzyme was stable in pH range 3.5 to 10 after 4 hours of incubation at 40°C.

Chen *et al*. (2004) studied the effect of pH on activity and atability of endoglucanase enzyme. Considering the optimum pH, they investigated that the enzyme hydrolyzed CMC in the pH range of 4.0 to pH 9.0, with maximum activity occurring at pH 7.0 in 50 mM phosphate buffer and retained 75% of its activity at pH 4.0 in 50 mM potassium phosphate buffer. In addition, the enzyme's stability was investigated in buffer solutions of various pH values. Purified enzyme solution (30 µg in 100 µl) was mixed with 100 µl of the appropriate buffer and incubated at 4°C for 24 h, after that the residual activities were examined. The enzyme was stable over the pH range of 6.0 to 9.0 within these conditions, but alkaline pH value (pH 12) affected the CMCase activity.

Vyas (2004) observed the stability of alkaline active cellulase enzyme under different conditions. Endoglucanase was purified to homogeneity from the culture filtrate of alkalotolerent *Fusarium* sp. The optimum pH of the Endoglucanase was determined by measuring its activity by incubating it at different pH values of the reaction mixtures for 30 min. The various buffers used for observing effect of pH on the enzyme activity were 50 mM citrate buffer (pH 3.0-6.0), sodium phosphate buffer (pH 7.0-8.0) and glycine-NaOH buffer (pH 8.5 to 10.0). For the determination of pH stability of the enzyme, it was pretreated in buffers of different pH at 50°C, 60°C for 2 h and assayed at 60°C at pH 7.0. It was found that purified endoglucanase enzyme was active in a broad pH range of 4.0 to 9.0 with pH optima 5.0. The enzyme showed 60% of its maximum activity at pH 8.0. In addition, the enzyme was stable in alkaline pH range of 7.0-9.0 at 50°C. The endoglucanase retains around 75% activity after incubation for 2h at pH 8.0 while 50% residual activity was detected at pH 9.0.

Wang *et al.* (2008) identified and characterized a novel thermophilic, cellulose-degrading bacterium *Paenibacillus* sp. strain B39, which produced a high-molecular weight B39 cellulase with both CMCase and avicelase activities. In an attempt to determine the effect of pH on activity and stability, it was found that maximum CMCase activity was at pH 6.5, and maintained a high activity (over 80% of maximum activity) at pH 7–8. Moreover, CMCase enzyme was very acid-labile below pH 6, but was quite stable under mild alkaline condition in the pH range of 7.5–9.0.

2.7. Application of some lignocellulose degrading enzymes in corn stover degradation

The use of microorganisms or their enzymes for the conversion of cellulose into simple carbohydrates is receiving increased attention. This is the result of growing concern over the accumulation of wastes, and our awareness of the vast quantities of residues, rich in cellulose, which result from agricultural operations and the manufacture of wood products. Lignocellulose is converted to glucose and biofuels by a multistep process that includes pretreatment, enzymatic hydrolysis, and fermentation **(Wayman *et al.*, 2005)**.

However, it is now recognized that the hydrolytic efficiency of fungal cellulas complexes determined using a model cellulosic substrate does not provide a reliable indication of its performance on pretreated lignocellulose **(Kabel *et al.*, 2005).**

Plant biomass contains large amounts of cellulose and other polysaccharides that can be hydrolyzed to glucose and various other simple sugars for subsequent fermentation to fuel ethanol or used in the production of other industrial chemicals. In nature, the biodegradation of plant biomass is a slow process because lignin and substrate crystallinity greatly restrict the access of hydrolytic enzymes to the polysaccharide components. However, raw biomass can be pretreated and partially fractionated, using processes that typically involve elevated temperature and pressure combined with acid or base

catalysis, to yield lignocellulosic materials that are much more susceptible to enzyme attack **(Palonen *et al*., 2004** and **Kim and Lee, 2005)**.

Corn stover is an abundant agricultural residue that is left behind after corn grain harvest. Corn stover is concederd as a promising substrate for fuel production with an annual estimated availability in the USA of 170 million dry tons. The sample is a heterogeneous substrate containing stalks, leaves, etc. Leaf includes leaf midrib, leaf blade, and leaf sheath; stem (stalk) includes outer fiber and inner pith **(Sheehan *et al.*, 2004)**.

Eggeman and Elander (2005) stated that corn stover or cereal stalks, purpose-grown crops like switchgrass or hybrid poplar, and softwoods such as spruce and pine from boreal forests show significant quantitative and qualitative differences in their non-cellulosic polysaccharide components. Further differences in composition are introduced according to the pretreatment technology employed. Therefore, it has been suggested that enzyme mixtures could be customized for particular feedstocks and pretreatments in order to optimize hydrolysis.

Pretreatment effects on the enzymatic hydrolysis of lignocellulose producing reducing sugars as glucose and xylose. Hot water pretreatment enhances the enzyme digestibility of corn stover with about a 4 folds increase in glucose conversion over the same time period. Moreover, pretreatment is an important and necessary step that opens up the tightly structured cell wall, thereby, allowing carbohydrolytic enzymes access to hemicellulose **(Wayman *et al.*, 2005 and Zeng *et al.*, 2007)**.

2.7.1. Lignocellulose structure

2.7.1.1. Cellulose and its structure

Cellulose is chemically simple because it contains simple repeating units of glucose, but has a complex structure because of the long chains of glucose subunits joined together by β–1,4 linkages. Cellulose is stabilized by some

interactions, these stabilizing factors are weak individually but collectively form strong bonds. The chains are in layers held jointly by van der Waals forces and hydrogen–bonds (intramolecular and intermolecular). About thirty individual cellulose molecules are arranged into units called protofibrils, which are further arranged into larger units called microfibrils **(Gan *et al*., 2003)**.

2.7.1.2. Hemicellulose and its structure

Hemicellulose consists of several different sugar units and substituted side chains in the form of a low molecular weight linear or branched polymer. This polymer is more soluble than cellulose with a degree of polymerization of less than 200. Hemicelluloses are named according to their main sugar residues in the backbone. Xylans, consisting of D-xylose units, and glucomannans, consisting of D-glucose and D-mannose units contribute to the main hemicelluloses in hardwoods and softwoods, respectively. Branched polymers contain neutral and/or acidic side groups. These groups render hemicelluloses noncrystalline or poorly crystalline, so that they exist more like a gel than as oriented fibres. Hemicelluloses form a matrix together with pectins and proteins in primary plant cell walls and with lignin in secondary cell walls. Covalent hemicellulose-lignin bonds involving ester or ether linkages form lignin-carbohydrate-complexes (LCCs) **(Jeffries, 1990)**.

Hemicelluloses can be included within and between crystalline cellulose domains during the synthesis of cellulose microfibrils. The relationship between cellulose and hemicellulose in the cell walls of higher plants may be extremely intimate, so it is possible that molecules at the cellulosehemicellulose boundaries and those within the domains of crystalline cellulose require different enzymes for efficient hydrolysis **(Atalla *et al*., 1993)**.

Subramaniyan and Prema (2002) mentioned that xylan, the second most abundant polysaccharide and a major component in plant cell wall consists of β-1,4 linked xylopyranosyl residues. The plant cell wall is a composite

material in which cellulose, hemicellulose (mainly xylan) and lignin are closely associated. Three major constituents of wood are cellulose (35-50%), hemicellulose (20-30%), a group of carbohydrates in which xylan forms the major class, and lignin (20-30%).

In addition, xylan is a heteropolysaccharide containing substituent groups of acetyl, 4-O-methyl-D-glucuronosyl and β-arabinofuranosyl residues linked to the backbone of β-1,4-linked xylopyranose units and has binding properties mediated by covalent and non-covalent interactions with lignin, cellulose and other polymers. Lignin is bound to xylans by an ester linkage to 4-O-methyl-D-glucuronic acid residues **(Bissoon *et al*., 2002).**

2.7.1.3. Lignin and its structure

Lignin is a branched polymer of substituted phenylpropane units joined by carbon and ether linkages. Biosynthesis of lignin formation proceeds *via* polymerisation of the free radical forms of precursors, i.e. the monolignols *p*-coumaryl, coniferyl and sinapyl alcohol. In the final polymer they form *p*-hydroxyphenyl-, guaiacyl-, and syringyl type units, respectively. Plant laccases and peroxidases catalyse the generation of radical formations. It seems plausible that lignin polymerization pattern and assembly is guided by the orientation of cellulose and the structure of hemicelluloses. The major linkage in lignin, the arylglycerolß- aryl ether substructure, comprises about half of the total interunit linkages **(Atalla, 1995)**.

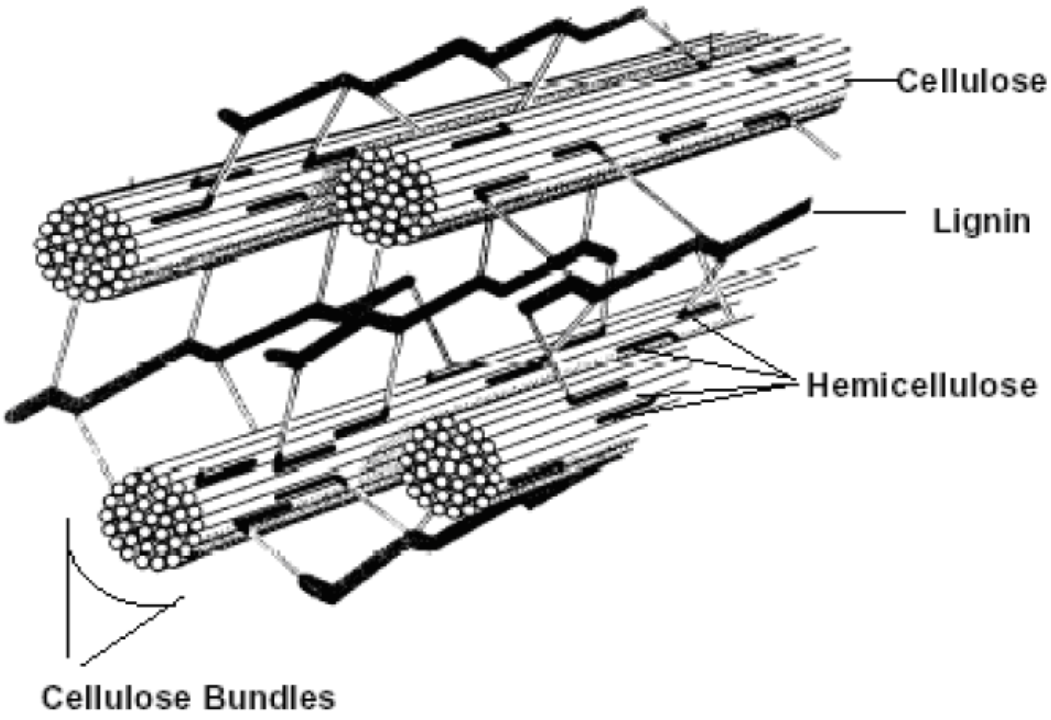


Fig.3. Generalized view of plant cell wall composition (Shleser, 1994)

2.7.2. Enzymes required for lignocellulose degradation

2.7.2.1. Enzymes required for cellulose degradation

As a result of the insolubility and heterogeneity of native cellulose, it is recalcitrant to enzymatic hydrolysis. The degradation of crystalline cellulose is a complex process requiring the participation of many enzymes. Cellulases are the enzymes responsible for the cleavage of the β-1,4-glycosidic linkages in cellulose. They are members of the glycoside hydrolase families of enzymes that hydrolyse oligosaccharides and/or polysaccharides **(Schwarz, 2001).**

Sandgren (2003) stated that cellulase enzymes perform the major part of cellulose degradation. Cellulases can be produced by a great number of organisms such as plants, plant pathogens and cellulolytic microorganisms, both bacterial and fungal, and have the capability of hydrolyzing highly ordered crystalline cellulose into shorter cellooligomers and glucose. The ability of a commercial *Trichoderma reesei* cellulase preparation, to hydrolyze the cellulose

and xylan components of pretreated corn stover was significantly improved by supplementation with three types of crude commercial enzyme preparations nominally enriched in xylanase, pectinase, and β-glucosidase activity. Although the well-documented relief of product inhibition by β-glucosidase contributed to the observed improvement in cellulase performance, significant benefits could also be attributed to enzymes components that hydrolyze non-cellulosic polysaccharides. It is suggested that socalled 'accessory' enzymes such as xylanase and pectinase stimulate cellulose hydrolysis by removing non-cellulosic polysaccharides that coat cellulose fibers. A high-throughput microassay, in combination with response surface methodology, enabled production of an optimally supplemented enzyme mixture.

This mixture allowed for about twofold reduction in the total protein required to reach glucan to glucose and xylan to xylose hydrolysis targets (99% and 88% conversion, respectively), thereby validating this approach towards enzyme improvement and process cost reduction for lignocellulose hydrolysis**(Berlin *et al.*, 2007).**

2.7.2.2. Enzymes required for hemicellulose degradation

Viikari *et al*., (1994) mentioned that hemicellulose degrading enzymes are hydrolytic, and specifically degrade those glycans that make up the backbone of the hemicelluloses. Typical hemicellulases are therefore endo-1,4-β-D-xylanases that catalyse the random hydrolysis of β-1,4-glycosidic bonds in xylans. Xylanases are probably the most widely studied group of hemicellulases in bacteria and fungi due to their numerous biotechnological applications.

The enzymatic degradation of hemicelluloses requires a complex set of different enzymes reflecting the variability of the hemicellulose structure. Hemicellulose hydrolysis proceeds through the action of endo-type enzymes that liberate shorter fragments of substituted oligosaccharides, which are further degraded by side-group cleaving enzymes and exotype enzymes. Alternatively,

side-branches may be cleaved first. Similarly to cellulose hydrolysis, the hydrolases act synergistically to convert hemicellulose polymer into soluble units **(Tenkanen *et al*., 1996)**.

The complex structure of xylan needs different enzymes for its complete hydrolysis. The depolymerisation action of endo-xylanase results in the conversion of the polymeric substance into xylooligosaccharides and xylose. Endo- β-1,4- xylanases that depolymerise xylan by the random hydrolysis of xylan backbone and β-1,4-D-xylosidases which split off small oligosaccharides. The side groups present in xylan are liberated by β-L-arabinofuranosidase, β-Dglucuronidase, galactosidase and acetyl xylan esterase **(Subramaniyan and Prema, 2002).**

It is probable that hemicelluloses restrict the access of cellulolytic enzymes by coating cellulose fibers. In some lignocelluloses, pectin could exert a similar effect. Consequently, enzyme mixtures with similar cellulase activity may show differences in performance on lignocellulose if they differ in hemicellulase composition **(Berlin *et al.*, 2006)**.

Ohgrena *et al.* (2007) recorded that a near-theoretical glucose yield (96–104%) from acid-catalysed steam pretreated corn stover can be obtained if xylanases are used to supplement cellulases during hydrolysis. Xylanases hydrolyse residual hemicellulose, thereby improving the access of enzymes to cellulose. Under these conditions, xylose yields reached 70–74%. When pre-treatment severity was reduced by using autocatalysis instead of acid-catalysed steam pretreatment, xylose yields were increased to 80–86%. Partial delignification of pretreated material was also evaluated as a way to increase the overall sugar yield. The overall glucose yield increased slightly due to delignification but the overall xylose yield decreased due to hemicellulose loss in the delignification step.

2.7.2.3. Enzymes required for lignin degradation

Lignin is the most recalcitrant to degradation whereas cellulose, because of its highly ordered crystalline structure, is more resistant to hydrolysis than hemicellulose. Alkaline and acid hydrolysis methods have been used to degrade lignocellulose. Weak acids tend to remove lignin but result in poor hydrolysis of cellulose whereas strong acid treatment occurs under relatively extreme corrosive conditions of high temperature and pH which necessitate the use of expensive equipment. Also, unspecific side reactions occur which yield non-specific by-products other than glucose, promote glucose degradation and therefore reduce its yield. Some of the unspecific products can be deleterious to subsequent fermentation unless removed. There are also environmental concerns associated with the disposal of spent acid and alkaline.

For lignin degradation, enzymes are preferred than acid or alkaline processes since they are specific biocatalysts, can operate under much milder reaction conditions, do not produce undesirable products and are environmentally friendly **(Howard *et al.*, 2003)**.

Evidently, other components in pretreated biomass, particularly hemicellulose and lignin, exert significant restraints on cellulose hydrolysis. For example, one mechanism whereby lignin seems to reduce hydrolytic performance is by binding enzyme components nonproductively. Consequently enzyme mixtures with similar cellulase activity may show differences in performance on lignocellulose if they differ in affinity for lignin **(Berlin *et al.*, 2007)**.

Most cellulolytic microorganisms lack efficient ligninase systems, and cannot or have problems degrading lignin. It is only some basidomycetes (white-rot fungi) that have such efficient systems. The complex nature of lignin makes its direct degradation by enzymes a difficult task. The degradation of lignin is

less well characterized, and there are conflicting opinions on how the different lignin-degrading enzymes act and cooperate **(Sandgren, 2003)**.

Two families of lignolytic enzymes are widely considered to play a key role in the enzymatic degradation: phenol oxidase (laccase) and peroxidases (lignin peroxidase (LiP) and manganese peroxidase (MnP) **(Krause *et al*., 2003; Malherbe and Cloete, 2003)**. Enzymes involved in lignin breakdown are too large to penetrate the unaltered cell wall of plants so the question arises, how lignases affect lignin biodegradation. Suggestions are that lignases employ low-molecular, diffusible reactive compounds to affect initial changes to the lignin substrate **(Call and Mücke, 1997)**.

Peroxidases are enzymes that catalyze the oxidation of a variety of organic and some inorganic compounds by hydrogen peroxide and other peroxides. Oxidation of an electron donating substrate by hydrogen peroxide involves the overall transfer of electrons. One electron oxidation of phenols results in the formation of phenoxy radicals, which may diffuse from the enzyme active site and undergo a variety of post-enzymatic reactions in aqueous solution **(Griffin, 1991)**.

Martinez *et al.* (2005) stated that lignin peroxidase (LiP) and manganese peroxidase (MnP) were described as true ligninases because of their high redox potential. LiP degrades non-phenolic lignin units (up to 90% of the polymer), whereas MnP generates Mn^{3+}, which acts as a diffusible oxidizer on phenolic or non-phenolic lignin units via lipid peroxidation reactions.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

I- Materials

1. Microorganisms

Ten microbial strains, previously used for the degradation of organic compounds, were tested for the production of cellulases (Table 1). The most potent strains for cellulase activity were chosen.

Moreover, in an effort to obtain novel microbial strains capable of degrading cellulose, fifty cellulolytic microbial isolates were collected from different environments rich in cellulosic materials. Isolation was carried out on cellulose-congo red agar medium. The most active cellulase producers were selected for the succeeding experiments.

Table 1. Microbial strains used and tested for cellulase activity

Strain	Source	Reference
1. *Chaetomium globosum*	Agric. Research Centre	A.R.C.
2. *Trichoderma viride*	Agric. Research Centre	A.R.C.
3. *Gliocladium roseum*	Agric. Bot. Dept. (Plant pathology), Fac. of Agric. at Moshtohor, Benha Univ.	
4. *Trichoderma harzianum*	Agric. Bot. Dept. (Plant pathology), Fac. of Agric. at Moshtohor, Benha Univ.	
5. *Aspergillus niger* (A)	Agric. Bot. Dept. (Microbiology), Fac. of Agric. at Moshtohor, Benha Univ.	Shaban (2004)
6. *Aspergillus niger* (B)	Agric. Bot. Dept. (Microbiology), Fac. of Agric. at Moshtohor, Benha Univ.	Shaban (2004)
7. *Bacillus subtilis*	Agric. Bot. Dept. (Microbiology), Fac. of Agric. at Moshtohor, Benha Univ.	Neweigy *et al.* (2003)
8. *Bacillus coagulans*	Agric. Bot. Dept. (Microbiology), Fac. of Agric. at Moshtohor, Benha Univ.	Neweigy *et al.* (2003)
9. *Bacillus megaterium*	Agric. Bot. Dept. (Microbiology), Fac. of Agric. at Moshtohor, Benha Univ.	Abou-Aly *et al.* (2005)
10. *Streptomyces aureofaciens*	Agric. Bot. Dept. (Microbiology), Fac. of Agric. at Moshtohor, Benha Univ.	Zaghloul *et al.* (2007)

2. Maintenance of stock cultures

The original cultures of the bacterial and fungal strains were maintained on nutrient agar and potato dextrose agar (PDA) slants, respectively. Maintenance media were supplied with 0.5% cellulose. Stock cultures were kept at 5˚C and monthly subcultered.

3. Lignocellulose degrading enzymes and lignocellulose materials

Cellulase produced in this work and seven commercial lignocellulose degrading enzymes were applied for the degradation of agricultural residues such as corn stover. Lignocellulose degrading enzymes (Table 2) and corn stover were obtained from Dortmund University, Faculty of Biochemical and Chemical Engineering, Technical Biochemistry.

Table 2. Lignocellulose degraging enzymes tested for corn stover degradation

Enzymes	The source
1. Cellulase	Produced from *T. reesei* ATCC 26921 (Sigma-Aldrich, C8546), EC 3.2.1.4
2. Xylanase	Produced from *Aspergillus niger*, EC 3.2.1.8
3. Xylanase (ultra-filtrated)	Produced from *Disporotrichum sp.*, EC 3.2.1.8
4. Laminex	Mixture of cellulase and xylanase (DSM, Batch:4900673869)
5. CWDEC	Cell wall degrading enzyme complex of cellulase and xylanase (Novozyme, Batch:085k1587)
6. MsP1	Recombinant peroxidase enzyme produced from *Marasmius scorodonius*
7. Mangan-Peroxidase (Mn-P)	Produced from *Nematoloma frowardii* (lyophilized), EC 1.11.1.13

4. Culture media

4.1. Maintenance media of stock cultures

4.1.1. Nutrient agar medium, (Atlas, 1995) was used for the maintenance of the bacterial strains. The medium has the following composition:

Component	g/L
Proteose peptone	5.0
Beef extract	3.0
Agar	20.0
pH	7.0

4.1.2. Potato dextrose agar (PDA), (Atlas, 1995) was used for the maintenance of the fungal strains. The medium has the following composition:

Component	g/L
Potatoes	200.0
Glucose	20.0
Agar	15.0
pH	7.0

4.2. Inocula preparation media

4.2.1. Basal Mineral Salt broth, (Chen and Wayman, 1991) was used for inoculum preparation. The medium has the following composition:

Component	g/L
Glucose	10.0
Proteose peptone	1.0
KH_2PO_4	2.0
$(NH_4)_2SO_4$	2.0

$MgSO_4$. $7H_2O$	0.3
$CaCl_2$	0.3
pH	7.0

4.3. Isolation media

4.3.1. Cellulose-congo red agar medium, (Hendricks *et al.*, 1995) was used for isolation of cellulose decomposers. The medium has the following composition:

Component	**g/L**
Cellulose powder	1.88
K_2HPO_4	0.50
$MgSO_4$. $7H_2O$	0.25
Congo red	0.20
Gelatin	2.00
Agar	5.00
Soil extract	100 ml
pH	7.0

4.4. Identification media

4.4.1. Nutrient gelatin medium, (Lelliott and Stead, 1987) was used for gelatin liquefaction test. The medium has the following composition:

Component	**g/L**
Beef extract	3.0
Proteose peptone	5.0
Gelatin	120
pH	7.0

4.4.2. Skim milk agar medium, (Lelliott and Stead, 1987) was used for testing casein decomposition. The medium has the following constitution:

Component	**g/L**
Yeast extract	2.5
Casein	5.0
Glucose	1.0
Skim milk solution (10%)	100 ml
Agar	15
pH	7.0

4.4.3. Esculin hydrolysis medium, (Qadri *et al.*, 1981) was used for esculin decomposition test. The medium has the following composition:

Component	**g/L**
Esculin	5.0
Ferric citrate	0.5
NaCl	0.8
K_2HPO_4	0.4
KH_2PO_4	0.1
pH	5.6

4.4.4. Urease medium, (Lelliott and Stead, 1987): It was used for urea decomposition test. The medium has the following composition:

Component	g/L
Peptone	1.0
Glucose	1.0
NaCl	5.0
KH_2PO_4	2.0
Aqueous phenol red solution (1.2%)	1.0 ml
Agar	12.0
pH	6.8

20 % aqueous urea solution was individually sterilized by filtration and added to the molten urease medium to a final concentration of 2 % urea.

4.4.5. Voges-Proskauer broth, (Lelliott and Stead, 1987) was used for Voges-Proskauer test. The medium has the following composition:

Component	g/L
Peptone	7.0
Glucose	5.0
K_2HPO_4	5.0
pH	6.5

4.4.6. Koser medium, (Lelliott and Stead, 1987) was used for carbon source utilization test. The medium has the following composition:

Component	g/L
NaCl	1.0
$MgSO_4.7H_2O$	0.2
$NH_4H_2PO_4$	1.0

KH_2PO_4	0.5
Agar	15.0
pH	7.0

Individually, 2.0 g of benzoate, citrate, succinate, tartrate, mucate, lactate and oxalate as a sole carbon source were added to Koser's medium.

4.4.7. Basal medium, (Lelliott and Stead, 1987) was used for acid formation test from different sugars. The medium has the following composition:

Component	**g/L**
$NH_4H_2PO_4$	1.0
KCl	0.2
$MgSO_4.7H_2O$	0.2
Peptone	1.0
Aqueous bromothymol blue (1.0%)	0.3 ml
pH	7.0

Individually, 10% aqueous solutions of glucose, xylose, mannitol, glycerol, arabinose, cellobiose, erythritol, inositol, lactose, maltose, mannose, melibiose, raffinose, rhamnose and sorbitol, were prepared, autoclaved at 115 °C for 15 minutes and added to the basal medium to a final concentration of 1 %.

4.4.8. Nitrogen-free medium, (Lelliott and Stead, 1987) was used for testing nitrogen fixation capability. It has the following composition.

Component	**g/L**
Glucose	20.0
K_2HPO_4	0.2

$MgSO_4.7H_2O$	0.2
NaCl	0.2
$CaCO_3$	5.0
pH	7.0

4.4.9. Tryptic soy agar medium supplemented with yeast extract (TSAYE, Difco, 1998) was used to test the antimicrobial effect. It has the following composition.

Component	**g/L**
Pancreatic digest of casein	15.0
Enzymatic digest of soybean meal	5.0
NaCl	5.0
Yeast extract	6.0
Agar	15.0
pH	7.0

4.4.10. Indole production medium, (Lelliott and Stead, 1987) was used for indole production test. The medium has the following composition:

Component	**g/L**
Tryptone	10.0
Tryptophane	0.1
K_2HPO_4	3.13
NaCl	1.0
KH_2PO_4	0.27
pH	7.0

4.5. Cellulase production media

To study the influence of medium composition on cellulase production by the selected microorganisms, the following media were used:

4.5.1. Cellulase production medium (CPM), (Camassola and Dillon, 2007b)

Component	g/L
Cellulose	10.0
Proteose peptone	1.0
KH_2PO_4	0.2
$(NH_4)_2SO_4$	0.13
$MgSO_4.7H_2O$	0.03
$CaCl_2$	0.03
$FeSO_4.7H_2O$	0.005
$MnSO_4.7H_2O$	0.016
$ZnSO_4.7H_2O$	0.0014
$CoCl_2.2H_2O$	0.002

4.5.2. Basal Mineral Salt medium, (Chen and Wayman, 1991)

Component	g/L
Cellulose	10.0
Proteose peptone	1.0
KH_2PO_4	2.0
$(NH_4)_2SO_4$	2.0
$MgSO_4.7H_2O$	0.3
$CaCl_2$	0.3

4.5.3. Cellulose broth, (Bagga *et al.*, 1990)

Component	g/L
Cellulose	5.0
Casein hydrolysate	0.5
Yeast extract	0.5
$NaNO_3$	1.0
KH_2PO_4	0.9
$Na_2HPO_4.2H_2O$	1.2
$MgSO_4.7H_2O$	0.5
KCl	0.5

4.5.4. Czapek-Dox liquid medium containing 1% CMC, (Coral *et al*., 2002)

Component	g/L
Carboxymethyl cellulose	10.0
$NaNO_3$	3.0
K_2HPO_4	1.0
$MgSO_4$	0.5
KCl	0.5
$FeSO_4$	0.01

4.5.5. Reese and Mandels basal medium, (Reese and Mandels, 1963)

Component	g/L
Cellulose powder	10.0
Proteose peptone	0.25
$(NH_4)_2SO_4$	1.4
Urea	0.3
KH_2PO_4	2.0
$CaCl_2.\ 2H_2O$	0.3
$MgSO_4.7H_2O$	0.3

Tween 80	1 ml
Trace element solution	1 ml

Trace element solution

Component	**mg/L**
$FeSO_4.7H_2O$	5.0
$MnSO_4.7H_2O$	5.6
$ZnSO_4.7H_2O$	3.34
$CoCl_2.2\ H_2O$	2.0

II. Methods

1. Microorganisms isolation and screening

After collection of samples from different environments rich in cellulose, isolation of cellulase-producing microorganisms was carried out by using pouring plate and streaking plate methods on cellulose-congo red agar. The activity of the cellulolytic enzymes was indicated by the clear zone surrounding the colonies according to **Hendricks *et al.* (1995)**.

Diameters of the clear zone were measured in millimeters and colonies producing great clear zones were selected. Moreover, the selected isolates, which found to clearly hydrolyze cellulose, were tested for their cellulase activity by fermentation on cellulase production medium.

2. Identification of the selected two cellulase producing isolates

2.1. Identification of isolate A7

Some morphological observations, staining properties, biochemical characteristics, growth conditions and physiological characteristics were applied for the tentative identification of isolate A7.

Cultural characteristics of isolate A7 was observed after fermentation at 30°C on Czapek-Dox agar **(Waksman, 1961)**. The growth on the agar plates was examined after 5, 10, and 15 days for the appearance of the colonies and formation of aerial hyphae and spores **(Gordon and Horan, 1968)**. Motility, acid fastness, gram staining, temperature range for growth and the survival at 50°C for 8 hours were determined using the methods described by **Mishra *et al.*, 1980**.

Physiological and biochemical reactions such as acid formation from different sugars and decomposition of casein, starch, esculin, urea, and gelatin were investigated by the methods described by **Lelliott and Stead, 1987**.

Utilization of benzoate, citrate, succinate, tartrate, mucate, lactate and oxalate as a sole carbon source was tested according to the modified protocol of **Koser (1924)**.

2.2. Identification of isolate B25

Based on some morphological observations, growth conditions, staining properties, physiological and biochemical reactions, the taxonomic properties of isolate B25 were investigated. The morphology, motility, staining properties and spore formation were tested by the methods described by **Ding *et al.*, 2005** and **He *et al.*, 2007.**

Nitrogen fixation capability, Voges-Proskauer test, catalase activity, indole formation, oxidase test and salt tolerance were analysed using the methods described by **Collins *et al.*, 1995.** The paper disc diffusion method described by **Wu (1984)** was used to test the culture broth of isolate B25 for antimicrobial activity against some gram positive (*Bacillus subtilis, Lactococcus lactis, Lactobacillus plantarum* and *Pediococcus cerevisiae*) and gram negative (*Pseudomonas putida*) bacteria.

3. Fermentation process

About 95 milliliter of the medium which proved to be the best medium for cellulase production were dispended into 250 ml Erlenmeyer flasks, sterilized and inoculated with 5 ml of a 3-days-old culture for each of the four experimental strains. The inoculated flasks were incubated under shaking at 150 rpm for 6 days at 30˚C and 25˚C for bacterial and fungal strains, respectively. Afterwards, the cultures were centrifuged at 4000 X g for 30 min at 4˚C to remove the cells and obtain a clear crude broth. The supernatant was used for determination of cellulase activity and protein concentration.

4. Optimization of submerged fermentation conditions for maximum cellulase production by the selected four strains

Suitable nutritional factors, environmental conditions, and process control strategies were investigated and optimized.

The present work deals with some external factors that influence the productivity of cellulase by *Chaetomium globosum, Trichoderma viride, Paenibacillus polymyxa* and *Streptomyces griseus*. Different media, carbon and nitrogen sources, the suitable concentrations of carbon and nitrogen were investigated as nutritional factors. In addition, the initial pH, fermentation period and the inoculum volume have been studied.

4.1. Effect of some nutritional factors

4.1.1. Effect of different culture media

The aforementioned five production media were tested to their suitability for cellulase production. Individually, all media were dispended in 250 ml flasks (95 ml/ flask). After sterilization, inoculation and incubation, the supernatant was used for measurement of enzyme activity and protein concentration to identify the most suitable medium for maximum cellulase production.

4.1.2. Effect of supplementation with glucose

The best medium for maximum enzyme production (Reese and Mandels basal medium) was supplied with glucose concentrations from 0.1 to 0.3%. After sterilization, inoculation and incubation, the supernatant was used for determination of enzyme activity and protein concentration.

4.1.3. Effect of cellulose concentration

To investigate the effect of the cellulose concentration in the culture medium on cellulase secretion, cellulose concentrations of 0.5 to 2.5% were used.

Flasks were sterilized and inoculated with standard inoculum of the four experimental strains and incubated. The culture supernatant was used for determination of cellulase activity and protein concentration.

4.1.4. Effect of nitrogen source

The best medium for the enzyme production was modified by replacing ammonium sulphate with different other nitrogen sources, namely yeast extract, urea, proteose peptone, and sodium nitrate. The above mentioned nitrogen sources were calculated to give equal final nitrogen concentration, irrespective of the chemical constitution. The medium was sterilized and inoculated with standard inoculum of the four experimental strains and incubated. Cellulase activity and protein concentration were determined.

4.1.5. Effect of nitrogen source concentration

Since urea and yeast extract were found to be the most suitable nitrogen sources for cellulase production by the tested strains, it was of interest to investigate the effect of different concentrations of urea and yeast extract on cellulase production. Concentrations from 0.1 to 0.4% were added to the optimized medium. After sterilization, inoculation and incubation, the supernatant was used for measurement of enzyme activity and protein

concentration to find out the most suitable nitrogen concentration for maximum cellulase production.

4.2. Effect of some environmental factors

4.2.1. Effect of initial pH

The most suitable medium for cellulase production was adjusted to pH values from 4.0 to 8.0. The flasks were sterilized and inoculated with standard inoculum of the four experimental strains and incubated. Cellulase activity and protein concentration were determined to investigate the most suitable initial pH for maximum cellulase production.

4.2.2. Effect of the fermentation period

The optimized medium was sterilized and inoculated with standard inoculum of the four experimental strains. The optimized factors already concluded were considered. Fermentation process was performed for different peroids (2, 4, 6, 8, 10 and 12 day). The culture supernatant was used for determination of enzyme activity and protein concentration.

4.2.3. Effect of the inoculum volume

Inoculation of the main cultures was performed with inoculum volumes of 2.0 - 12.0%. Cellulase activity and protein concentration were determined.

4.2.4. Effect of Tween 80 concentration

Concentrations of Tween 80 from 0,5 – 2,5 ml/L were tested to induce the cellulase production. The flasks were sterilized, inoculated and incubated, under all optimized factors which already concluded. Cellulase activity and protein concentration were determined to find out the most suitable concentration of Tween 80 for the highest enzyme production.

5. Determination of cellulase and xylanase activities

The activity of endo-β-1,4-glucanase (the most important type of cellulases) and endo-β-1,4-xylanase (the most important type of xylanases) were determined in the culture supernatant by measuring the released amount of reducing sugars (glucose and xylose). One international unit of enzyme activity is defined as the amount of enzyme that liberates one micromole reducing sugars (glucose and xylose)/min under the standard assay conditions.

5.1. Endo-β-1,4-glucanase activity

Endo-β-1,4-glucanase activity was determined according to the method of **Bailey (1981)**; 100 µl of culture supernatant was added to 300 µl of 1.0% hydroxyethylcellulose (HEC) in 0.05 M sodium-acetate buffer (pH 3.5 and 6.5 for fungal and bacterial cellulases, respectively).

The reaction mixture was incubated at 50°C for 25 min. Finally, the concentration of reducing sugars was determined and calculated as glucose.

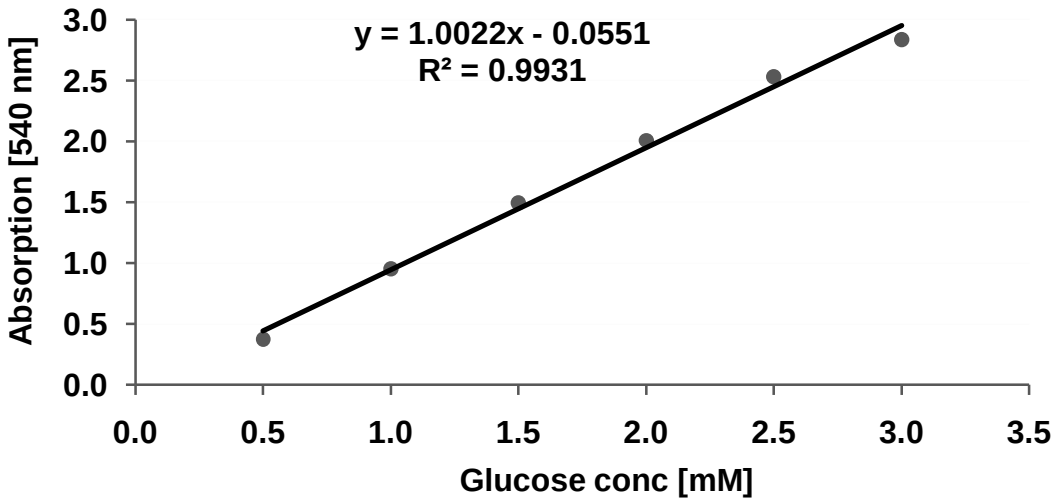


Fig. 4. Calibration curve of glucose

5.2. Endo-β-1,4-xylanase activity

Endo-β-1,4-xylanase activity was determined according to the method of **Bailey (1981)** by adding 100 µl of enzyme solution to 300 µl of 1.0% xylan in 0.05 M sodium-acetate buffer (pH 3.5). The reaction mixture was incubated at

50°C for 25 min. Finally, the released reducing sugars were quantified and calculated as xylose.

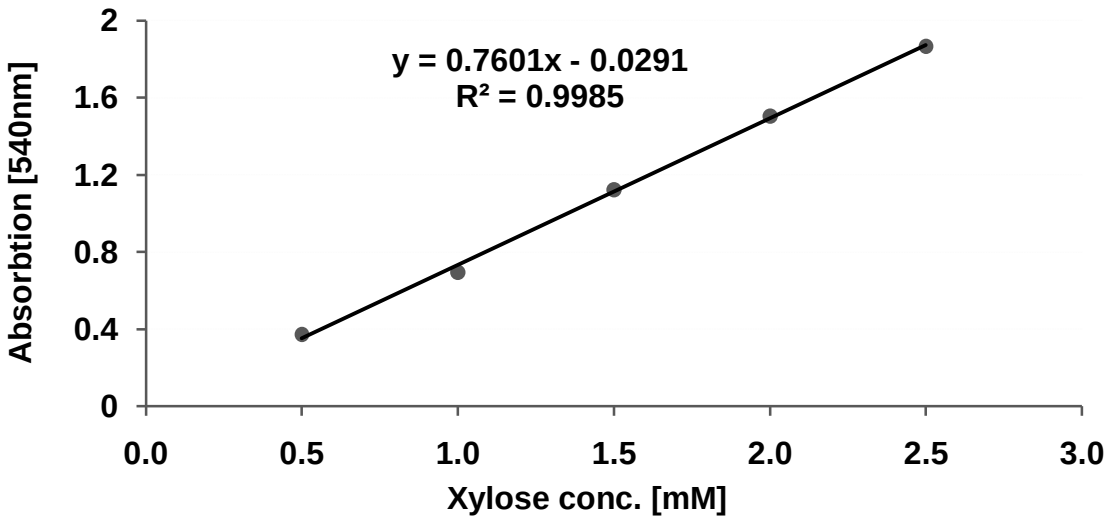


Fig. 5. Calibration curve of xylose

6. Determination of reducing sugars (glucose and xylose)

Determination of reducing sugars (glucose and xylose) was carried out using 3,5 dinitrosalicylic acid method **(Miller *et al*., 1960)**.

6.1. Preparation of 3,5 dinitrosalicylic acid reagent

Dinitrosalicylic acid reagent was prepared as follows: 5.0 g of dinitrosalicylic acid were dissolved in 300 ml water at 50 °C. Dropping of 50 ml sodium-potassium hydroxide solution (4 mol/L). 150g sodium- potassium tartrate (Rochelle salt) were added and the solution was filled up to a total volume of 500 ml with distilled water. The solulion was stored in darkness at room temperature.

6.2. Procedure

Dinitrosalicylic acid reagent (600 µl) was added to the enzyme reaction. The solution was boiled for 5 min, and the absorbance was recorded at 540 nm after cooling down for 5 min. After subtraction of enzyme and substrate blank (dinitrosalicylic acid reagent was added to the reaction mixture before

incubation), the absorbance values were translated using the standard curves into micromoles of reducing sugars produced during the enzyme reaction.

7. Determination of protein concentration

Protein concentration was determined according to **Lowry *et al.*, (1951)**. Four reagents were prepared, Reagent **A:** 2 % Na_2CO_3 in 0.1 N NaOH; Reagent **B:** 1% $CuSO_4$.5 H_2O in 2% sodium potassium tartrate; Reagent **C:** Immediately prepared before using by adding 1 ml of reagent B to 50 ml of reagent A; Reagent **D:** Folin Ciocalteu reagent diluted to be 1 ml folin to 3 ml distilled water.

7.1. Procedure

One ml of culture filtrate and 5 ml of reagent C were mixed in a test tube and left for 10 min at room temperature. Afterwards, 0.5 ml folin reagent was added and left for 30 min at room temperature. The absorbance of the colored solution was measured at 750 nm. The protein concentrations were calculated by comparison to a protein standard curve set up with bovine serum albumin.

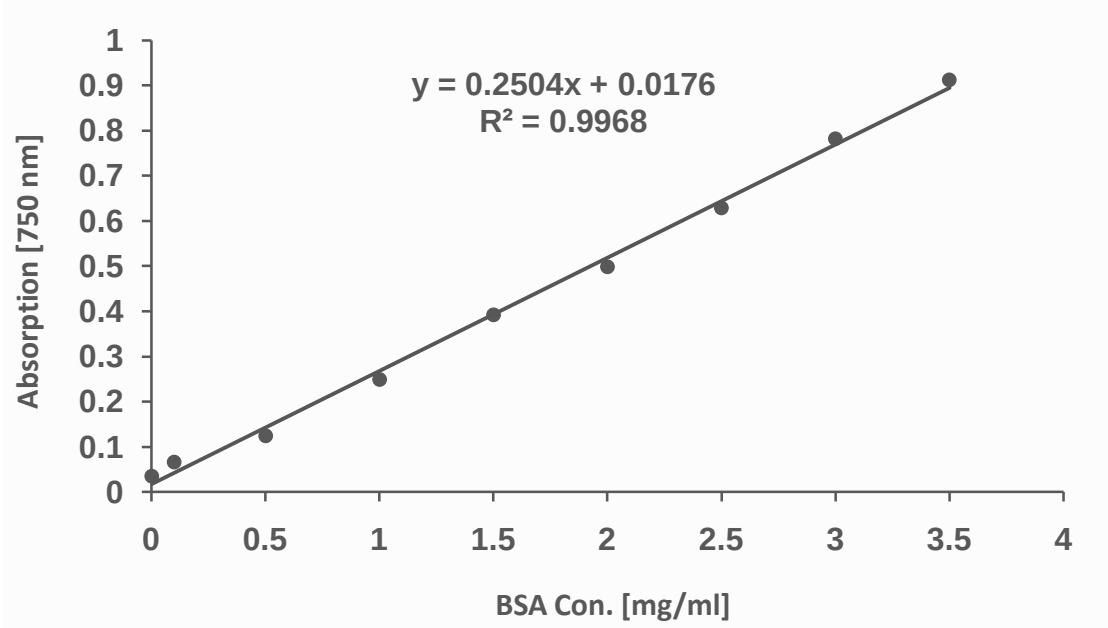


Fig. 6. Calibration curve of bovine serum albumin

8. Determination of peroxidase activity

8.1. Peroxidase (MsP1) activity

The activity was determined as described by **Zorn *et al.*, (2003).** Shortly, the time-dependent decrease of absorbance of an aqueous β-carotene emulsion was monitored at 450 nm using a tempered spectral photometer. Applicability of the test was checked by diluting aliquots of the enzyme sample with buffer solution (50 mM sodium acetate buffer, pH 3.5, 27°C). A linear correlation between activity and sample amount was found.

8.2. Manganese-peroxidase activity

Mn-P activity was estimated from the formation of Mn^{3+}-tartrate complex (ε_{238} 6,500 $M^{-1}cm^{-1}$) from 0.1mM $MnSO_4$ in 0.1M sodium tartrate buffer (pH 5) in the presence of 0.1 mM H_2O_2 . One unit (U) was defined as the amount of enzyme that oxidizes 1 µmol Mn^{2+} per min at 25°C and pH 5, **(Martinez *et al.*, 1996)**.

9. Concentration of cellulase produced by *C. globosum*

The modified medium was prepared, and the optimized parameters were considered. After inoculation and incubation, the culture was centrifuged at 4000 Xg for 30 min at 4°C and the clear supernatant was used as source of crude enzyme. The protein fraction was concentrated by ultrafiltration (Jumbosep™ Centrifugal Devices, exclusion limit 10 kDa) at 3000 Xg and 4 °C. The protein concentration and the cellulase activity were determined in the retentate and the filtrate .

10. Characterization of cellulases and xylanases

Concentrated cellulase produced from *C. globosum* as well as five cellulases and xylanases from different fungal strains were characterized. The physicochemical characteristics of these cellulose and hemicellulose degrading enzymes, such as the molecular weight, isoelectric point, optimum pH, optimum temperature, kinetic constnats and their pH and temperature stabilities were investigated.

10.1. Activity and Kinetic constants

The activity was measured during the reaction at 30°C for incubation periods from 5 – 35 min. The kinetic parameters such as K_m and v_{max} of the enzymes were determined under steady state conditions by Lineweaver-Burk double reciprocal plot of enzyme activity against substrate concentration.

Various substrate concentrations from 0.5 to 21.0 mg/ml were used. Graphs were drawn using the Enzyme Kinetic Module. The enzyme assay was carried out at pH 3.5 and 30°C. The formation of reducing sugars in the reaction mixtures was determined.

10.2. Effect of temperature on cellulase and xylanase activity and thermal stability

For determining the effect of temperature on cellulase and xylanase activity, the activities were determined in a temperature range of 30-75 °C for 25 min. The temperature stability of enzymes was analyzed by incubating the enzymes at their optimum temperature for different time intervals from 2 to 12 h. Samples were withdrawn periodically every 2 h. and assayed for residual activity under standard assay conditions.

10.3. Effect of pH on cellulase and xylanase activity and pH stability

The optimum pH of the cellulases and xylanases was tested by measuring the enzymes' activity after incubating the reaction mixtures for 25 min at different pH values from pH 3 to 6. The various pH values were adjusted using 50 mM sodium acetate buffer. For the determination of pH stability, enzymes were incubated at their optimum pH for different time intervals from 2 to 12 hours. Samples were withdrawn periodically every 2 hours and assayed for residual activity under standard assay conditions.

10.4. Isoelectric point of cellulase produced by *C. globosum*

The Isoelectric point (pI) of the *C. globosum* cellulase was determined by isoelectric focusing (IEF).

Polyacrylamide gels (IEF-gel, Serva) with immobilized pH gradient from 3.0 – 10.0 were used. The IEF gel was prepared according to the manufacturer's instructions. The protein content of the sample was 6.76 mg ml^{-1} and cellulase activity remained high as possible.

To determine the pl, 5 µl of marker (Servalyt®, precote® 3-10) and 6 µl of the cellulase sample were individually applied to a flatbed electrophoresis apparatus. In parallel, 8 µl of the cellulase sample were used for the activity staining. After the electrophoresis, the separated proteins were visualized by staining for 1 h with 0.2% Coomassie Brilliant Blue CBB-R250 dye in methanol-acetic acid-water solution (4:1:5 by volume). The same solution without dye was used for the destaining process.

For the activity staining, the gel was placed on 1% agarose plate containing 0.1% hydroxyl ethyl cellulose and incubated at 40 °C for 4-5 h. The agarose plate was then stained with 0.1% congo red solution for 30 min and destained in 1 M NaCl for 15 min. A clear (unstained) zone on the agarose plate indicates the presence of cellulase activity **(Kluepfel, 1988)**.

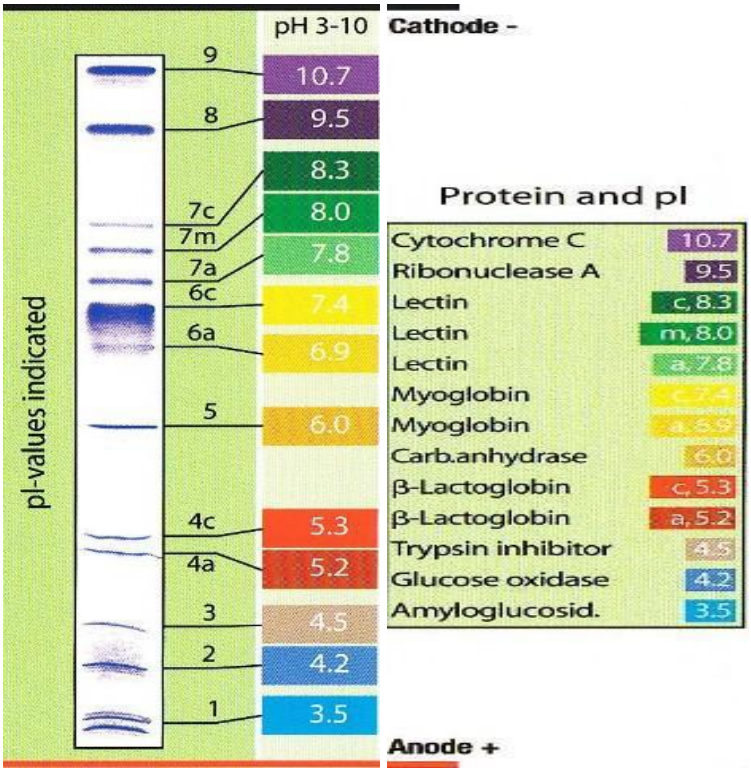


Fig. 7. Protein marker (IEF) for isoelectric point detection, pH 3 – 10

10.5. Molecular weight determination of cellulase produced by *C. globosum* on sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS –PAGE)

The molecular weight was determined according to the method of **Laemmli (1970)**.

10.5.1. Preparation of SDS separating and collecting gels

Separating gel (10%)

Ultrapure water	4.1 ml

1.5M Tris-HCl pH 8.8	2.5 ml
Acrylamide	3.3 ml
40% APS (Ammonium Persulphate)	50 µl
10% SDS	100 µl
TEMED	5 µl

Collecting gel (6%)

Ultrapure water	6.1 ml
0.5M Tris-HCl pH 6.8	2.5 ml
Acrylamide	1.3 ml
40% APS (Ammonium Persulphate)	50 µl
10% SDS	100 µl
TEMED	10 µl

10.5.2. Preparation of buffers

10.5.2.1. Loading buffer

Tris-HCl	0.1 M
DTT	0.2 M
SDS	40.0 gL^{-1}
Bromophenolblue	2.0 gL^{-1}
Glycerol	200 gL^{-1}
Ultrapure water	

10.5.2.2. Running buffer

Tris-Base	30.3 g L^{-1}
Glycine	144.0 g L^{-1}
SDS	10.0 g L^{-1}
Ultrapure water	

The running buffer was diluted before use at dilution factor of 1:10 with ultrapure water

10.5.3. Gel plates preparation

The separating gel was poured between the gel plates up to about 6.5 cm below the rim plate and covered with isopropanol to obtain a straight dividing line. After hardening of the separation gel, isopropanol was removed and the gel surface was dried with a paper towel.

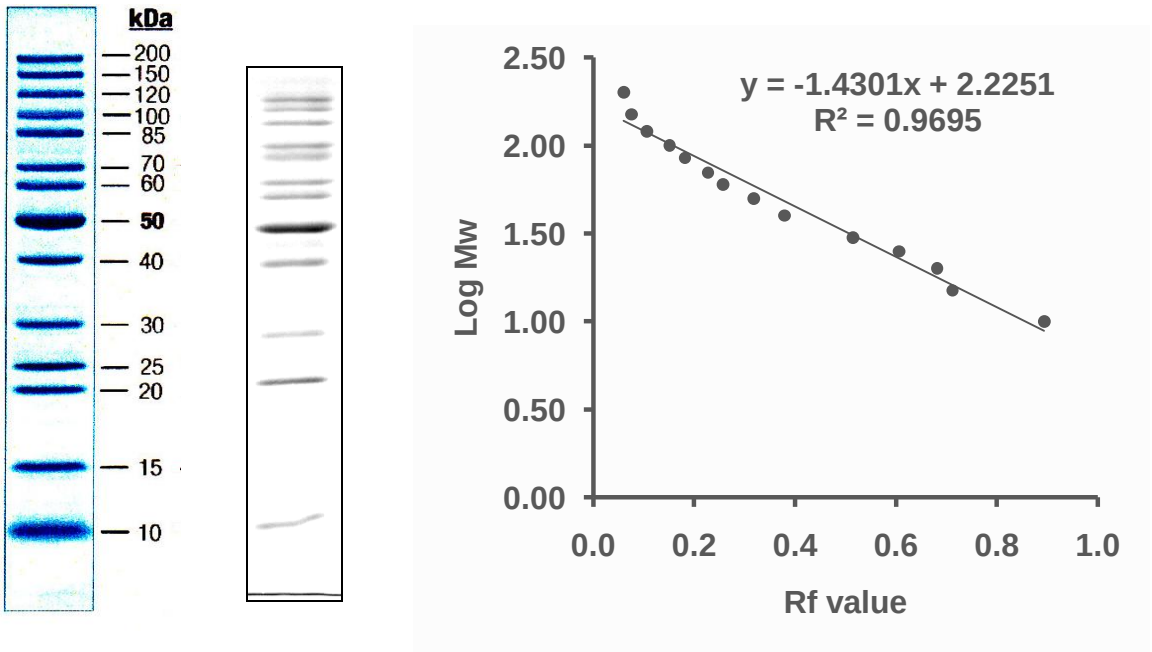
The collecting gel was then poured onto the separating gel and the sample comb was set up. After polymerization of the collecting gel, the comb was removed.

10.5.4. Sample preparation and application

The active IEF band was excised from the gel, ground in 60 µl ultrapure water and mixed with 10 µl loading buffer. 50 µl were applied to

polyacrylamide gel electrophoresis. Moreover, 5 µl protein marker for SDS-PAGE (Roti®-Mark STANDARD Roth) were used (80V, 22mA and 3W). When the samples were transferred into the separating gel, the voltage was increased to 140V until the end of electrophoresis.

Protein bands were visualized on the gel by staining for 1 h. with 0.2 % Coomassie Brilliant Blue CBB-R250 dye in methanol-acetic acid-water solution (4:1:5 v/v). The same solution without dye was used for the destaining process. A plot of log molecular weight versus relative mobility (Rf) of standard proteins was used to estimate molecular weight.



(a) (b) (c)

Fig. 8. Original protein marker for SDS-PAGE (Roti®-Mark STANDARD Roth) (a), Prepared protein marker (b) and Plot of log molecular weight versus relative mobility (Rf) of prepared protein marker (c)

11. Application of some lignocellulose-degrading enzymes for corn stover degradation

Corn stover consists of about 38% cellulose, 32% hemicellulose, 17% lignin, and 13% other components **(Sheehan *et al.*, 2004)**. This experiment was performed in two steps, the first is to discover the effect of peroxidase enzymes (peroxidase and mangan-peroxidase) on lignin degradation. The second is to

find out the effect of cellulase and xylanase enzymes (five units of cellulase (*C. globosum*), cellulase (*T. reesei*), xylanase (*A. niger*), xylanase (UF), laminex and Cwdec) on the degradation of cellulose and hemicellulose, respectively.

Pretreatment of lignocellulose may affect the enzymatic hydrolysis of lignocellulose to reducing sugars. Hot water pretreatment enhanced the enzymatic digestibility of corn stover with about 4 folds increase in glucose conversion over the same time period. Corn stover was pretreated by milling (5 mm length), soaking in water (1: 10 w/v) for one hour followed by hot water pretreatment **(Wayman *et al.*, 2005** and **Zeng *et al.*, 2007)**.

Samples were weighted (about 50 mg for every sample) and dispersed in 10 ml of 50 mM Na-acetate buffer. Individually or combined, peroxidase enzymes (MsP1 and Mn-P) were applied.

Concerning the blank sample, Na-acetate buffer was used instead of peroxidases. All treatments were incubated at 30 ºC for 24 h. under shaking. For the activity of peroxidase enzymes, 20 mM hydrogen peroxide was added periodically after complete consumption of hydrogen peroxide. After that this pretreatment, all samples were centrifuged and the residual corn stover was washed three times with the same buffer. In the last washing step one unit of catalase was added to remove the remaining hydrogen peroxide.

To investigate the effect of cellulase and xylanase enzymes on peroxidase treated or untreated corn stover, 10 ml of 50 mM Na-acetate buffer were added followed by 5 Units of each enzyme, individually. All samples were incubated at 30 ºC for 24 or 30 h. under shaking.

Samples were taken after 0, 6, 12, 24 and 30 h. to determine the catalytic activity of cellulase and xylanase enzymes on corn stover by measuring the amounts of released reducing sugars, glucose and xylose, as abovementioned.

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

1. Isolation, screening and identification of cellulase producing microorganisms

1.1. Isolation and screening of cellulase producing microorganisms

In nature there are many microorganisms (fungi and bacteria) produce enzymes capable of catalyzing the hydrolysis of cellulose. These microorganisms can be found in plant debris and soil, *i.e.*, where degradation of plant material takes place.

This work was focused on the identification of potent cellulase producers. Accordingly, samples were collected from different environments rich in cellulose. A total of fifty microbial isolates, as cellulolytic organisms, were isolated on cellulose-congo red agar medium which contained cellulose as a sole carbon source, Table 3 and illustrated in Fig. 9.

Moreover, the fifty isolates were screened for their cellulase activity by cultivating on cellulase production medium, Table 4 and illustrated in Fig. 10.

The obtained data show that from fifty isolates, two isolates showed maximum zones of clearance around their colonies and were thus found to have the highest cellulase activity. These two isolates which designated as A7 and B25 were selected and identified as described in the materials and methods section.

Table 3. The clearing zone diameter test of fifty microbial isolates grown on cellulose-congo red agar medium

No.	Clearing zone diameter [mm]	No.	Clearing zone diameter [mm]
1F	1.0	26F	2.0
2B	1.0	27F	2.5
3B	1.3	28F	1.3
4B	1.0	29B	1.0
5B	0.9	30A	1.9
6B	1.7	31A	1.7
7A	9.0	32A	2.0
8F	1.0	33B	1.5
9F	1.3	34B	1.5
10F	2.0	35F	3.3
11F	2.0	36F	4.7
12F	2.0	37F	1.6
13B	1.0	38F	3.5
14B	2.5	39F	1.5
15B	2.5	40F	1.5
16A	1.2	41F	1.8
17A	1.2	42A	4.4
18F	1.0	43B	1.5
19B	1.6	44B	2.0
20A	1.0	45B	1.0
21F	1.0	46A	1.7
22B	1.5	47A	1.5
23B	5.5	48A	3.0
24F	1.0	49B	2.2
25B	10.0	50F	2.0

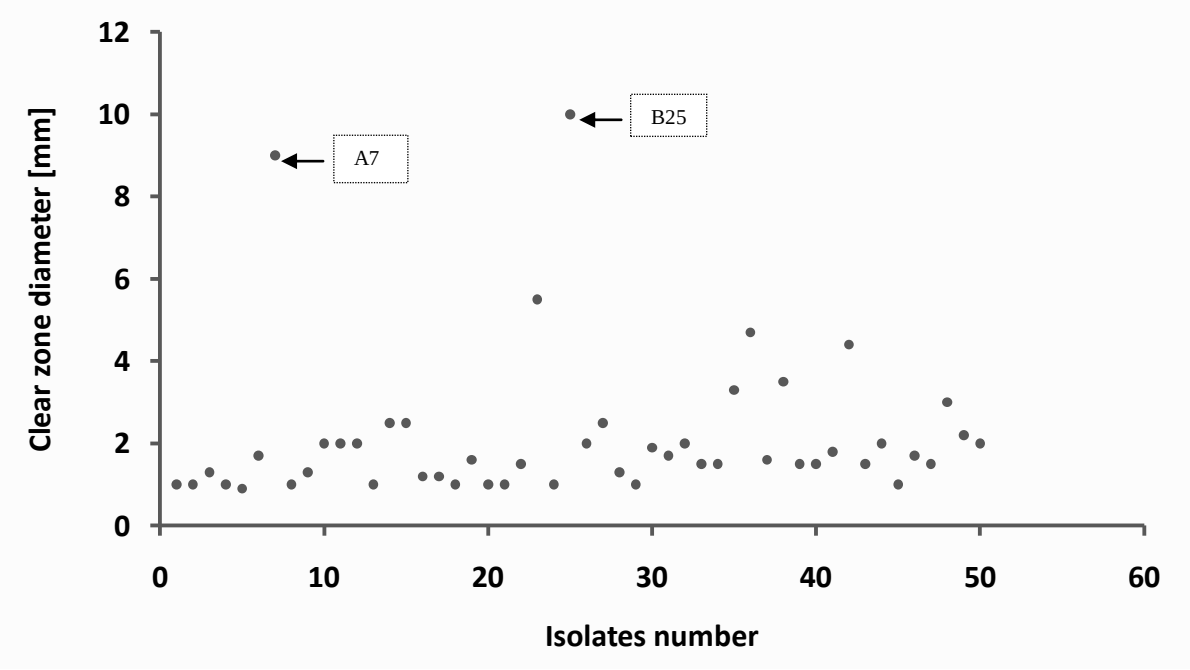


Fig. 9. The clearing zone diameter test of fifty microbial isolates grown on cellulose-congo red agar medium

Table 4. Cellulase activity of fifty microbial isolates on cellulase production medium

No.	Cellulase activity [U/ml]	No.	Cellulase activity [U/ml]
1F	0.09	26F	0.06
2B	0.06	27F	0.14
3B	0.14	28F	0.10
4B	0.08	29B	0.10
5B	0.10	30A	0.07
6B	0.14	31A	0.14
7A	0.33	32A	0.07
8F	0.10	33B	0.15
9F	0.11	34B	0.12
10F	0.06	35F	0.14
11F	0.09	36F	0.08
12F	0.16	37F	0.12
13B	0.07	38F	0.17
14B	0.14	39F	0.15
15B	0.11	40F	0.15
16A	0.07	41F	0.13
17A	0.12	42A	0.16
18F	0.11	43B	0.11
19B	0.07	44B	0.14
20A	0.14	45B	0.14
21F	0.09	46A	0.13
22B	0.07	47A	0.05
23B	0.13	48A	0.15
24F	0.06	49B	0.11
25B	0.24	50F	0.10

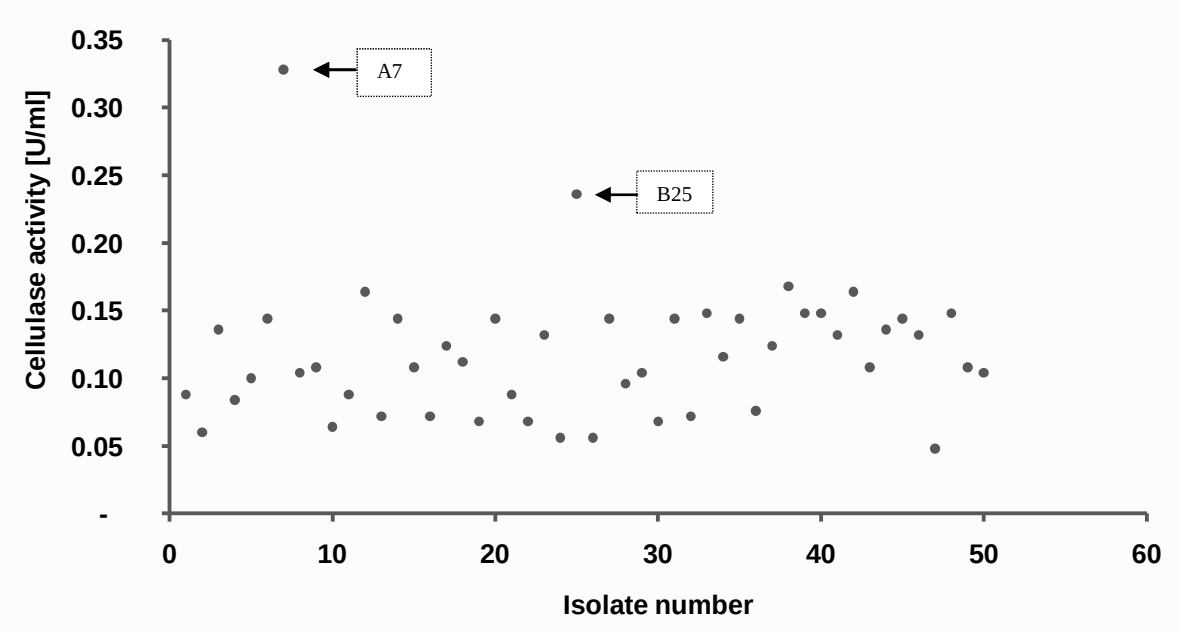


Fig. 10. Cellulase activity of fifty microbial isolates on cellulase production medium

In addition, ten different microbial strains were tested for their cellulase and protein production capability on CPM.

All of the investigated strains produced cellulase activity (Fig. 11). The lowest cellulase activity was obtained by *Bacillus subtilis*, while, *Trichoderma viride* and *Chaetomium globosum* were the most active cellulase producers. The protein concentration of the culture filtrates depended on the microbial strain. Slight relation was observed between the protein concentration of the culture filtrates and the enzyme activity.

Generally, from the previous ten strians, *T. viride* and *C. globosum* were selected for further cellulase production studies beside two unidentified isolates (A7 and B25) obtained from the previous isolation and screening.

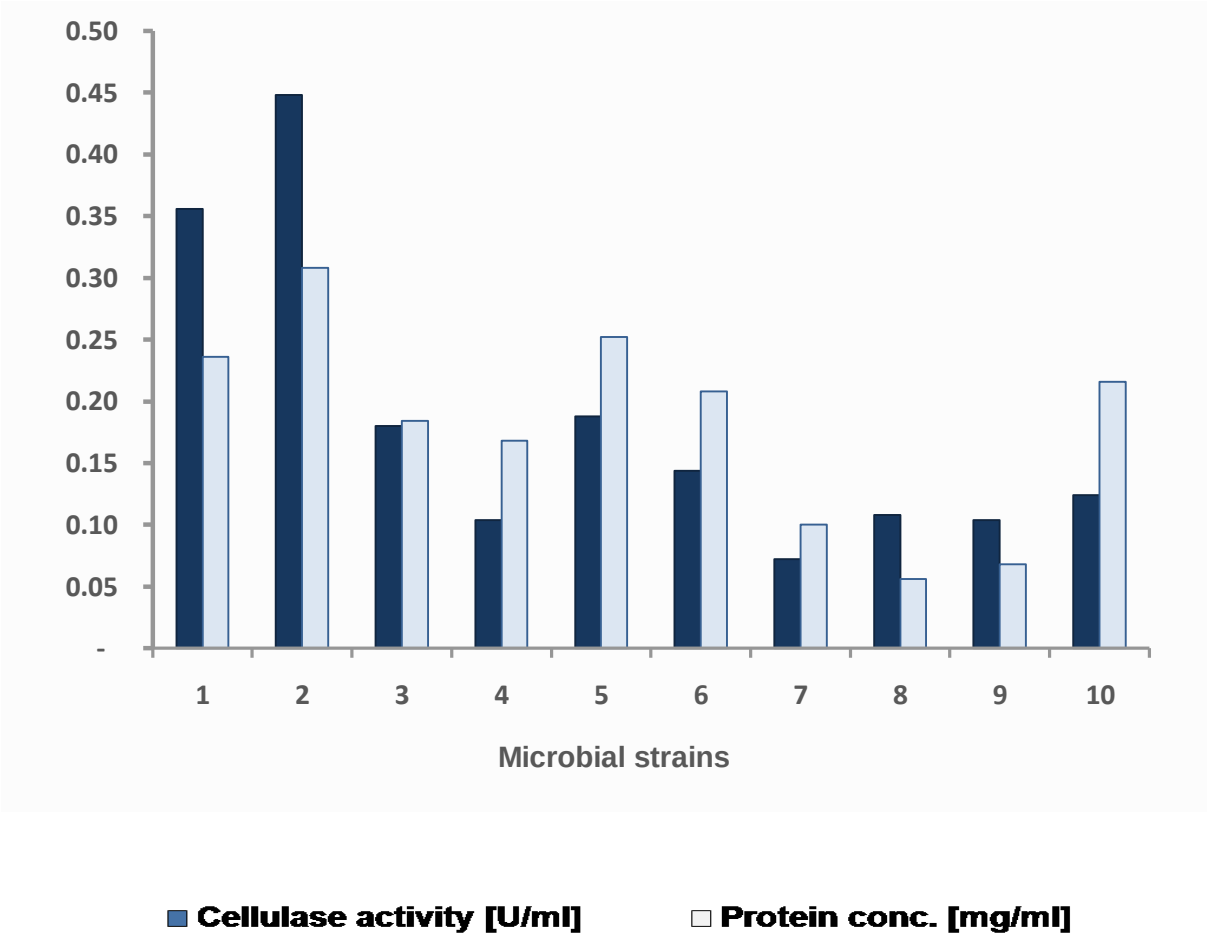


Fig. 11. Cellulase activity and protein concentration of ten microbial strains on cellulase production medium

1- *Chaetomium globosum*

2- *Trichoderma viride*

3- *Gliocladium roseum*

4- *Trichoderma harzianum*

5- *Aspergillus niger* (A)

6- *Aspergillus niger* (B)

7- *Bacillus subtilis*

8- *Bacillus coagulans*

9- *Bacillus megaterium*

10- *Streptomyces aureofaciens*

1.2. Identification

1.2.1. Isolate A7

By means of morphological characteristics, analysis of the growth conditions, staining properties, physiological and biochemical reactions (**Waksman, 1961; Gordon and Horan, 1968** and **Mishra *et al.*, 1980)**, isolate A7 was tentatively identified as *Streptomyces griseus* (Table 5).

Typically, the colonies were elevated, spreaded, and covered with grey aerial mycelia and spores. No clear diffusible pigments were observed. Isolate A7 was gram positive but, negative for acid fastness staining.

Physiologically, isolate A7 was capable of casein, starch, cellulose, urea and esculin degradation. Out of benzoate, citrate, succinate, tartrate, mucate, lactate, and oxalate as sole carbon source, citrate, succinate and lactate were well utilized. Acid formation from various carbohydrates by isolate A7 was investigated after inoculation and incubation at 30°C for 10 days.

Isolate A7 formed acid from glucose, xylose, mannitol, glycerol, arabinose, cellobiose, lactose, maltose, mannose and rhamnose.

No acid was formed from erythritol, inositol, melibiose, raffinose, and sorbitol. The growth temperature range was examined from 10 to 50°C on Czapek-Dox agar for 10 days of fermentation. Isolate A7 grew well when incubated at 30 and 40°C, but only moderate growth was obtained at 10°C. No growth was observed at 45 and 50°C.

Isolate A7 showed a good survival and growth on Czapek-Dox broth after heating the inoculum at 50°C for 8 h.

Table 5. Properties of the cellulase producing bacterial isolate (A7)

Cultural Characteristics		**Acid formation from**	
Aerial mycelium	Grey	Glucose	+
Spores	Grey	Xylose	+
Diffusible pigment	-	Mannitol	+
Motility	Non-motile	Glycerol	+
Gram stain	G+	Arabinose	+
Acid fastness	-	Cellobiose	+
Biochemical characteristics		Erythritol	-
Degradation of		Inositol	-
Casein	+	Lactose	+
Starch	+	Maltose	+
Cellulose	+	Mannose	+
Gelatin	-	Melibiose	-
Esculin	+	Raffinose	-
Urea (urease activity)	+	Rhamnose	+
Utilization of		Sorbitol	-
Benzoate	-	**Growth at**	
Citrate	+	10 °C	+
Succinate	+	30 °C	+
Tartrate	-	40 °C	+
Mucate	-	45 °C	-
Lactate	+	50 °C	-
Oxalate	-	Survival at 50 °C for 8 hr.	+

1.2.2. Isolate B25

From the morphological characteristics, staining properties, biochemical characteristics, growth conditions and physiological characteristics (**Collins *et al*., 1995; Ding *et al*., 2005** and **He *et al*., 2007)**, the microbial isolate B25 was tentatively identified as *Paenibacillus polymyxa* (Table 6).

After incubation at 30°C for 1 day, the colonies were white, circular, mucous and convex. Morphologically, B25 was found to be gram-positive. Upon prolonged incubation on agar medium, cells produced central endospores.

During fermentation on nitrogen-free medium, isolate B25 grew well when incubated at 30°C for 10 days, indicating nitrogen fixation capability. The strain grew well at pH 5.7 and 6.8, while the growth was inhibited in the presence of 5 and 10% NaCl. Isolate B25 was positive for catalase, urease, voges-proskauer test, gelatin liquefaction, starch & casein hydrolysis and esculin test.

Negative reactions were found for oxidase, indole production, and citrate utilization. The bacterium grew well under aerobic conditions.

When examined for acid formation from different sugars, isolate B25 showed positive results for acid formation from all tested substrates including glycerol, glucose, mannitol, arabinose, xylose, cellobiose, lactose, sucrose, sorbitol and fructose.

Cell-free culture supernatant of isolate B25 exhibited a broad spectrum of antimicrobial activity against a panel of gram-positive (*Bacillus subtilis, Lactococcus lactis*, *Lactobacillus plantarum* and *Pediococcus cerevisiae*) and gram-negative (*Pseudomonas putida*) bacteria.

Table 6. Properties of the cellulase producing bacterial isolate (B25)

Characteristic		**Hydrolysis of**	
Form (shape)	Rod shape	Casein	+
Motility	+	Gelatin	+
Gram staining	+	Starch	+
Spore formation	+	**Growth at NaCl**	
Growth on nitrogen-free medium	+	5%	-
V.P. test	+	10%	-
Indole production	-	**Growth at pH**	
Catalase production	+	6.8	+
Citrate utilization	-	5.7	+
Oxidase	-	**Acid formation from**	
Urease	+	Glycerol	+
Esculin hydrolysis	+	Glucose	+
Antimicrobial activity against		Mannitol	+
1- Gram positive bacteria		Arabinose	+
Bacillus subtilis	+	Xylose	+
Lactococcus lactis	+	Cellobiose	+
Lactobacillus plantarum	+	Lactose	+
Pediococcus cerevisiae	+	Sucrose	+
2- Gram negative bacteria		Sorbitol	+
Pseudomonas putida	+	Fructose	+

2. Optimization of submerged fermentation conditions for maximum cellulase production by the selected strains

After the screening, *Chaetomium globosum, Trichoderma viride, Paenibacillus polymyxa* and *Streptomyces griseus* were identified to be the most potent strains for cellulase production.

The identification of an efficient cellulase producing microorganism is only the first step towards the development of an enzyme production process. Therefore, suitable nutritional factors, environmental conditions, and process control strategies should be investigated and optimized.

Various media, carbon and nitrogen sources, as well as the suitable concentrations of carbon and nitrogen were investigated. In addition, the initial pH, fermentation period and the inoculum volume were studied.

2.1. Effect of medium composition

2.1.1. Effect of different media on cellulase enzyme production

The influence of the production medium on cellulase production by *C. globosum, T. viride, P. polymyxa* and *S. griseus* was investigated. The aforementioned five production media were tested for their ability to support growth and enzyme production (Fig. 12).

Reese and Mandels basal medium was the best medium for growth, protein synthesis, and cellulase production for all of the investigated strains. This may be attributed to the presence of Tween 80 within the conistituents of the medium, which supports the release of the enzyme complex into the medium.

The obtained results are in accordance with those reported by **Vyas (2004)** who demonstrated that the addition of Tween 80 as surfactant led to higher cellulase activities.

Moreover, Tween 80 facilitates the release of cellulases into the medium by causing an increased permeability of cell membranes and/or by promoting the release of cell-bound enzymes.

Reese and Mandels basal medium was followed by basal mineral salt medium for *T. viride*, and cellulase production medium (CPM) for *C. globosum, P. polymyxa* and *S. griseus*.

On the other hand, Czapek-Dox liquid medium containing 1% carboxymethyl cellulose (CMC) gave the lowest cellulase activities and extracellular protein concentrations of *C. globosum* and *S. griseus*. While, cellulose broth medium showed the lowest cellulase activity when used for the fermentation of *T. viride* and *P. polymyxa.*

Consequently, Reese and Mandels basal medium was used for the succeeding experiments.

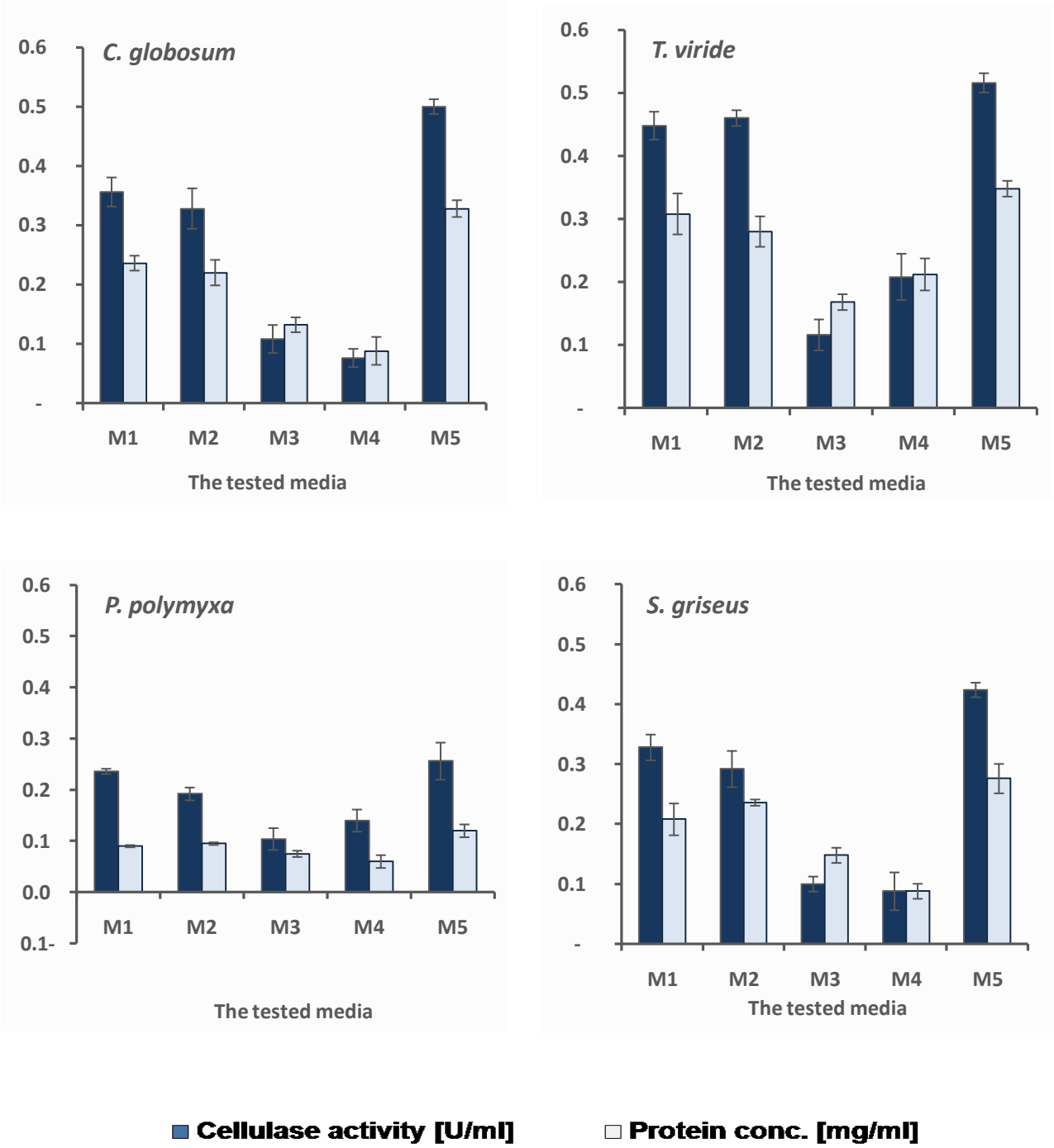


Fig. 12. Effect of different media on cellulase activity and protein concentrations

M1: Cellulase production medium (CPM)

M2: Basal mineral salt medium

M3: Cellulose broth

M4: Czapek-Dox liquid medium containing 1% CMC

M5: Reese and Mandels basal medium

2.1.2. Effect of supplementation with glucose

The influence of fermentation medium supplementation with glucose was studied. *C. globosum*, *T. viride*, *P. polymyxa* and *S. griseus* were grown in Reese and Mandels basal medium supplied with various glucose concentrations from 0.1 to 0.3% (w/v). Cellulase production and protein concentrations were investigated (Fig. 13).

Surprisingly, glucose was found to induce cellulase formation and protein synthesis of *C. globosum* and *T. viride* when used at concentrations of 0.10 and 0.15%, respectively. Cellulase activity was increased by 14.4 and 19.4% after six days of fermentation for *C. globosum* and *T. viride*, respectively. With increasing glucose concentrations, cellulase activities gradually decreased by 44 and 28.7% at 0.3% (w/v) glucose concentration for *C. globosum* and *T. viride*, respectively.

Most probably, glucose, as an easily metabolized sugar, is initially consumed to form high biomass necessary for the production of cellulose degrading enzymes. In addition, cellulolytic activity may not be subject to catabolite repression by glucose at 0.10 and 0.15% for *C. globosum* and *T. viride*, respectively. These results are in good agreement with those obtained by **Robson and Chambliss, (1984).** They reported that the presence of metabolized carbohydrates in the growth medium stimulated the production of cellulolytic activity. Similarly, **Mohagheghi *et al.*, (1988)** achieved higher enzyme yields with *T. reesei Rut C-30* growing on a mixture of xylose and cellulose than those obtained on pure cellulose. This could indicate that xylose as a soluble sugar is necessary for the initial growth, while cellulose is used as an inducer and an additional carbon source for enzyme production after consumption of the soluble sugars. **Sutherland, (1999)** stated that microbes produce enzymes such as endoglucanases in response to their carbon and energy needs as the soluble hydrolysis products are needed for further metabolic activities. **Kotchoni *et al.*, (2003)** reported that the mutant strain *Bacillus pumilus BpCRI 6* exhibited

approximately ten times higher endoglucanase activity than the wild type at 10 mM glucose concentration. Interestingly, a higher cellulase activity was detected under condition of catabolite repression (40 mM glucose) when the wild type and *Bacillus pumilus BpCRI 6* strain were combined in the same culture. Moreover, the highest cellulase activity of *Streptomyces* sp. (J2) was observed after fermentation in carboxymethyl cellulose broth that was supplemented with 0.5% glucose and 0.2% starch **(Jaradat *et al.*, 2008).**

Liang *et al.*, (2010) stated that different carbohydrates can induce cellulase secretion by *Anoxybacillus* sp. to various extents. Cellobiose, glucose, lactose, and sucrose were more effective inducers compared with cellulose. Growth of *Anoxybacillus* sp. in medium containing glucose resulted in the greatest production of cellulolytic activity.

In contrast, glucose caused an inhibition of cellulolytic activity of *P. polymyxa* and *S. griseus* when supplied at a concentration of 0.1%. The inhibition effect gradually increased with the increase of glucose concentration up to 0.3 %with inhibition of 45.3 and 38.7% of *P. polymyxa* and *S. griseus,* respectively. This observation is well in agreement with the results of **Gan *et al.*, (2003)** and **Zaldivar *et al.*, (2001)** who reported that cellulases are inhibited by glucose. Glucose repressed not only enzyme production, but also inhibited the enzyme activity. Similarly, **Suto and Tomita, (2001)** reported that low levels of cellulolytic enzymes in the presence of glucose could be attributed to repression of synthesis of cellulolytic enzymes by readily available glucose. **Chen *et al.*, (2004)** showed that cultures containing 0.5% (w/v) glucose as carbon source produced the minimum endoglucanase activity from *Sinorhizobium fredii.*

Moreover, **Du *et al.*, (2010)** mentioned that there are three groups of enzymes (cellobiohydrolases, endoglucanases, and β-glucosidases) found in cellulases that work synergistically to degrade cellulose. It is known that all three groups can be inhibited by intermediate (cellobiose) or final hydrolysis product (glucose).

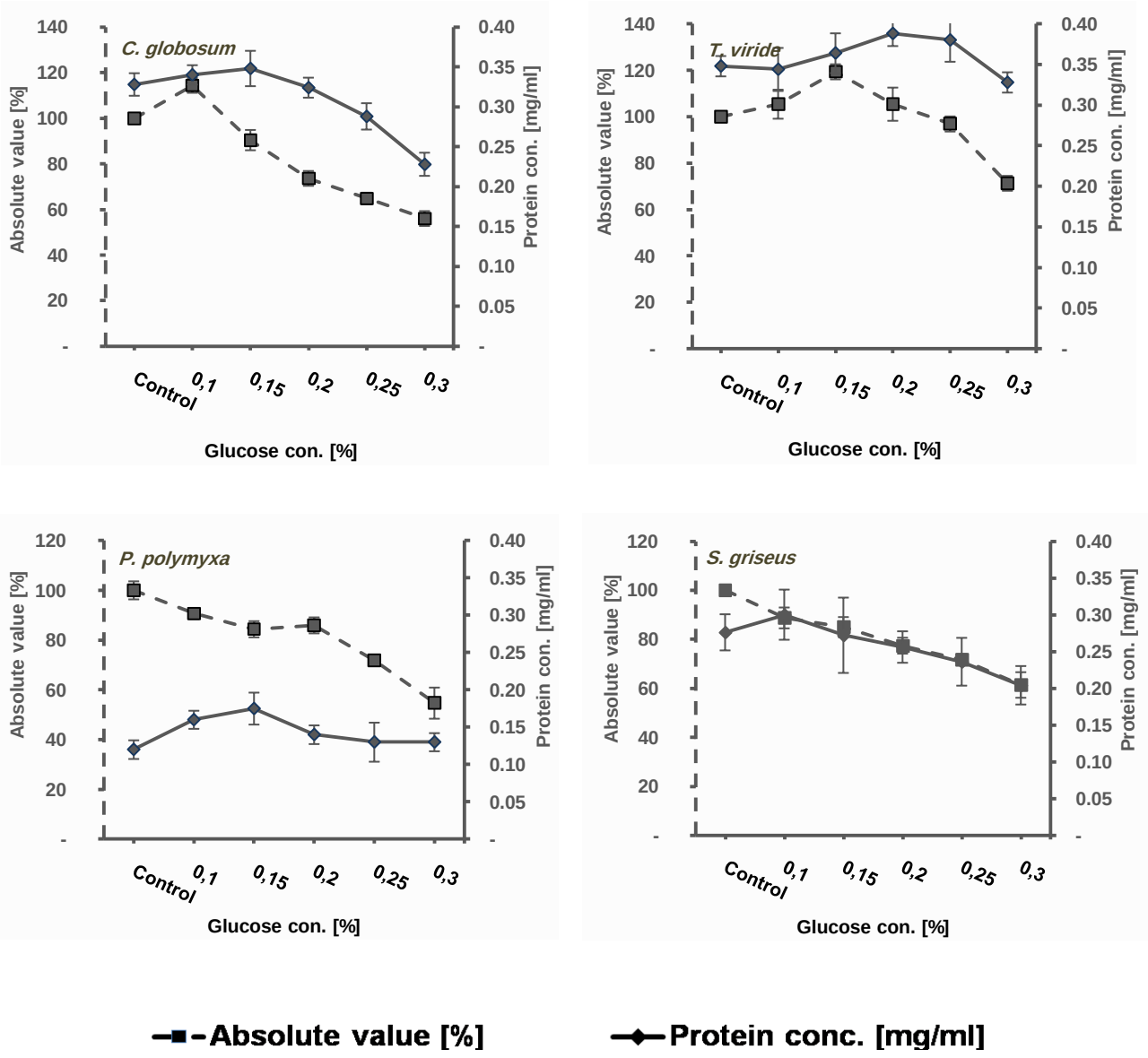


Fig. 13. Effect of glucose on cellulase activity and protein concentration

2.1.3. Effect of cellulose concentration

The production of hydrolytic enzymes is directly related to the available substrate (**Nybroe *et al.*, 1992**). Cellulase is an inducible enzyme system. Therefore, an increase in the concentration of a particular substrate may stimulate the specific enzyme production by microorganisms.

In the present study, the effect of different concentration of cellulose on cellulase production by *C. globosum*, *T. viride*, *P. polymyxa* and *S. griseus* was investigated. 0.5 - 2.5% of cellulose were added to Reese and Mandel's basal medium. Cellulase production and protein concentration were determined and the results were graphically illustrated in Fig. 14.

As expected, cellulase activity and extra-cellular protein concentrations increased with an increase of cellulose concentration. 2.0% cellulose was the optimum concentration for enzyme activity and protein synthesis of *T. viride* and *S. griseus*.

The maximum cellulase activity of *P. polymyxa* (0.26 U/ml) was found at 1.0% cellulose. A further increase in cellulose concentration beyond 1.0% did not result in a further increase in cellulase activity. The decrease in cellulolytic productivity at higher cellulose concentration may be attributed to substrate inhibition as described by **Guedon *et al.*, (2002)**. They reported complex media with high substrate concentrations to be unfavorable for *Clostridium cellulolyticum,* as the bacterium was unable to utilize excessive substrate. Similarly, **Narasimha *et al.*, (2006)** stated that supplementation of cellulose at 1.0% to the culture medium was optimal for growth and cellulase production by *A. niger*.

Data illustrated in Fig. 14 also show that *C. globosum* exhibited the highest capacity of cellulose decomposition. A gradual increase of cellulase activity and protein synthesis was found, reaching a maximum activity of 0.94 U/ml at 2.5% cellulose. These results are in harmony with those obtained by **Rao *et al.*, (2003)** who recorded maximum endocellulase production of the extremophilic actinomycete *Thermomonospora* sp. when 4% cellulose powder was used as the carbon source. Moreover, **Vyas, (2004)** stated that maximum CMCase activity of *Fusarium* sp. was obtained with 2.5% cellulose powder.

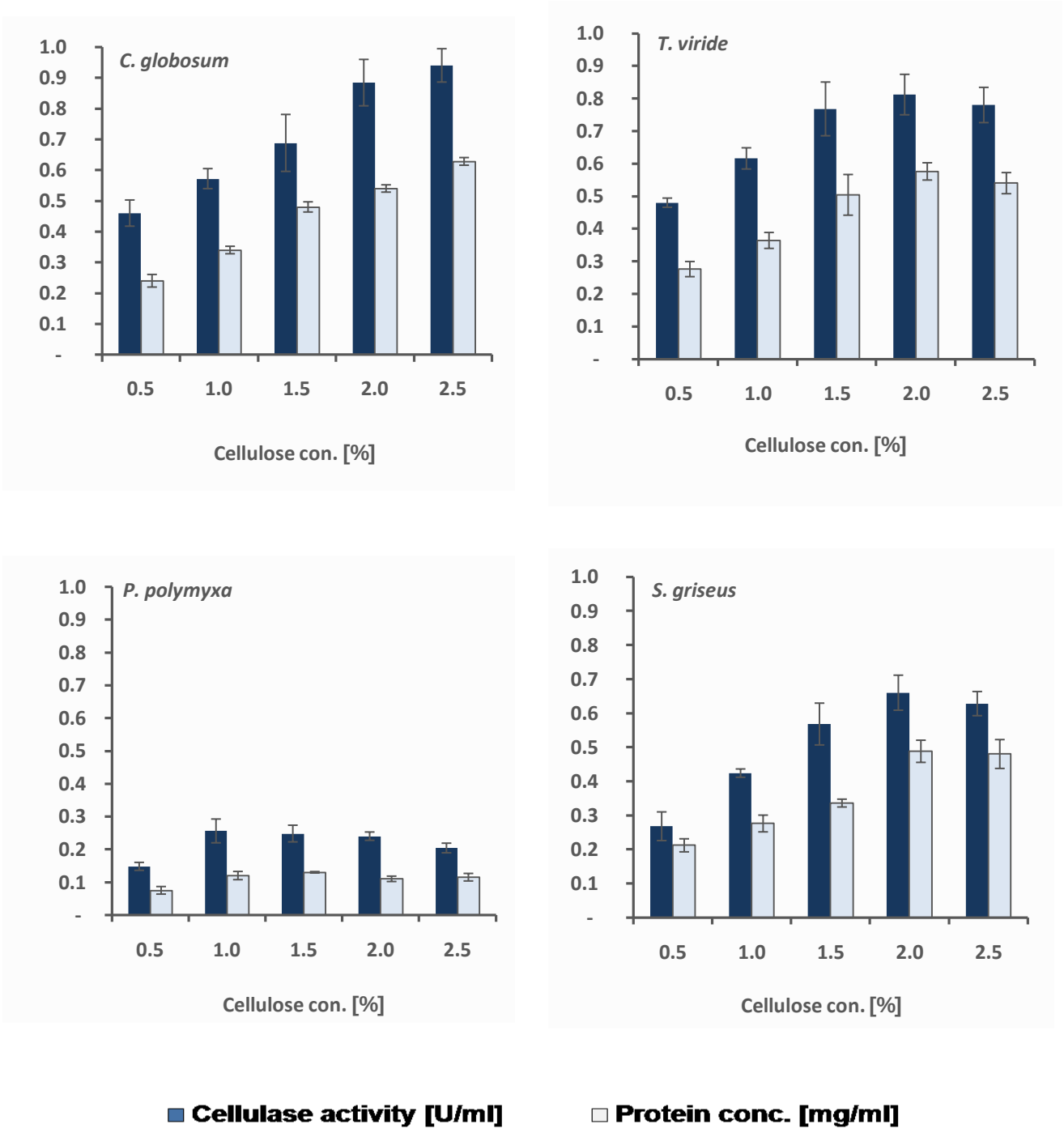


Fig. 14. Effect of cellulose concentration on cellulase activity and protein synthesis

2.1.4. Effect of nitrogen source

The effect of various nitrogen sources on cellulase production was investigated by *C. globosum*, *T. viride*, *P. polymyxa,* and *S. griseus,* using Reese and Mandels basal medium which was modified for glucose and cellulose concentrations according to the previous experiments. Ammonium sulfate (the nitrogen source in the basal medium) was substituted by yeast extract, urea, proteose peptone and sodium nitrate. Cellulase production and protein concentrations were determined (Fig. 15).

The obtained data revealed that, using of organic nitrogen sources responsed in the positive cellulase activity more than the inorganic ones. Among the tested organic and inorganic nitrogen sources, urea was found to be optimal for growth, protein synthesis, and cellulase production by *C. globosum* and *T. viride.* Yeast extract was shown to be the most suitable nitrogen source for *Paenibacillus polymyxa* and *Streptomyces griseus*. On the other hand, sodium nitrate yielded the lowest cellulolytic activity and protein concentrations with all of the used microbial strains.

Nitrogen source has got profound influence on enzymes production as it is the ultimate precursor for protein biosynthesis. Yeast extract and proteose peptone have been reported to induce maximum cellulase production by *Bacillus* sp. **Nizamudeen and Bajaj, (2009).** Previous experiments on the effect of various nitrogen sources on cellulase production demonstrated a substantial increase in the enzyme activity when the media were supplemented with yeast extract and urea **(Vyas, 2004)**. Cellulase production and secretion of extracellular proteins by *A. niger* increased in the presence of urea. While the lowest cellulase production was obtained when sodium nitrate was used as nitrogen source **(Narasimha *et al*., 2006).**

Data also are in accordance with the results of **Abou-Taleb *et al.*, (2009)** who reported that organic nitrogen sources were found to be more suitable for optimizing cellulase production by *Bacillus* strains than inorganic sources.The presence of yeast extract or peptone in the culture medium enhanced cellulase activities more compared with inorganic nitrogen sources. This enhancement may be due to other nutrients and growth stimulators in the organic nitrogen **(Liang *et al*., (2010).**

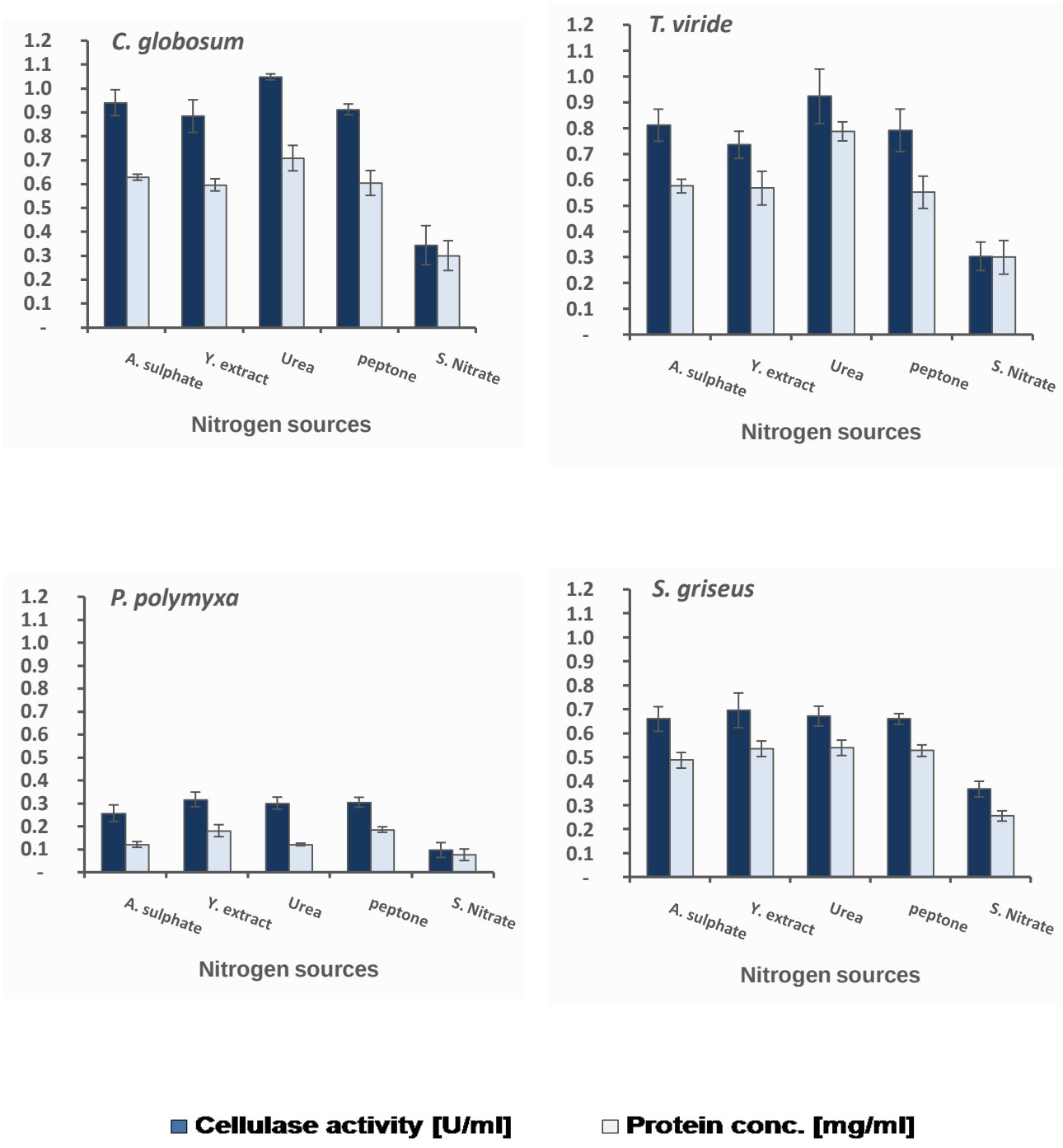


Fig. 15. Effect of different nitrogen sources on cellulase activity and protein concentration

2.1.5. Effect of different concentrations of urea and yeast extract

As shown in Fig. 16, urea was found to be the most suitable nitrogen source for growth, protein synthesis, and cellulase production of *C. globosum* and *T. viride* while, yeast extract was the best nitrogen source for *P. polymyxa* and *S. griseus*. It was thus of interest to investigate the most suitable concentrations for maximal cellulase activity.

Reese and Mandels basal medium was supplemented with 0.1 – 0.4% urea or yeast extract. Cellulase activities and protein concentrations were determined.

Data illustrated in Fig. (16) obviously indicate that nitrogen concentration greatly affected cellulase activity and protein synthesis. A nitrogen concentration of 0.3% either of urea or yeast extract gave maximum cellulase activities and protein concentrations with *C. globosum* and *P. polymyxa*. With regard to *T. viride*, 0.2% urea resulted in maximum cellulase activity of 1.0 U/ml, while maximum cellulase activity of *Streptomyces griseus* (0.70 U/ml) was obtained when 0.14% yeast extract was used. In this respect **Jaradat *et al.*, (2008)** investigated that the highest level of endocellulase production by *Streptomyces* sp. (J2) was achieved when the carboxymethyl cellulose broth contained 0.2 % nitrogen.

Obtained data confirmed the findings reported by **Jin and Toda, (1989)** who reported that an increase of urea concentration from 0.2 to 0.6 % improved endocellulase production of the bacterium *Clostridium thermocopriae*. In contrast, the growth of *Trichoderma reesei* on production medium without nitrogen source increased cellulase production **(Turker and Mavituna, 1987).** Moreover, **Maeda *et al.*, (2010)** investigated that peptone and ammonium sulfate did not present significant effect on cellulase production by *Penicillium funiculosum*. and the optimal concentrations of urea and yeast extract for the maximum cellulase production were 0.97 and 0.36 %, respectively.

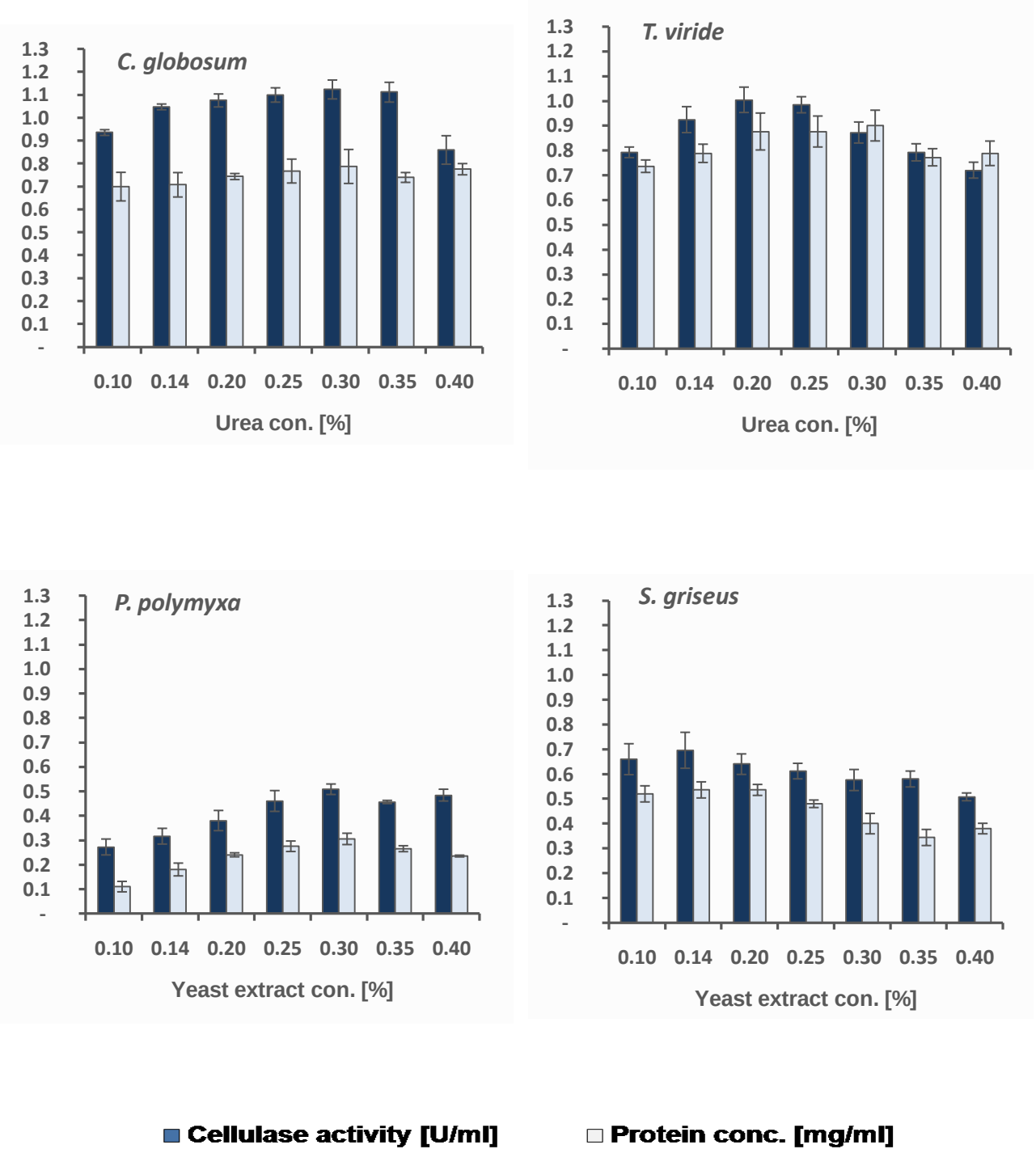


Fig.16. Effect of nitrogen concentration on cellulase activity and protein synthesis

2.2. Effect of further culture parameters

2.2.1. Effect of initial pH

Proper pH control is necessary for optimum enzyme yield influencing much enzymatic system and the transport of several species of enzymes across the cell membrane **(Agnihotri *et al.*, 2010).** So, the initial pH of Reese and Mandels basal medium was adjusted at pH 4.0 to 8.0, to study their effect on cellulase activity and protein synthesis (Fig. 17).

Data illustrated by Fig. (17) cleary show that cellulase formation strongly depended on the pH value. Cellulase activity followed the same trend as observed for protein synthesis. For *C. globosum* and *S. griseus*, the optimum pH was 6, while pH 5 was found to be optimal for growth, protein synthesis, and cellulase production by *T. viride.* Extracellular cellulase activity of *P. polymyxa* reached a maximum of 0.51 U/ml at pH 7. Similarly, **Jaradat *et al.*, (2008)** found pH 6 as optimum for the maximum endocellulase production from *Streptomyces* sp. (J2) when grown in carboxymethyl cellulose broth.

The cellulase activity and protein concentration were less in other tested pH levels, where endoglucose activity was minimal at pH 4.0 for *P. polymyxa*. Further, this activity was greatly reduced to reach the lowest at pH 8.0 for both fungal strains and actinomycetes. These results were approximately in correlation with the findings of many workers. **El-Baz, (2003)** found pH 5.0 to be the most suitable for cellulase formation with *Trichoderma viride* and *Aspergillus niger.* On the other hand, **Vyas, (2004)** reported that *Fusarium* sp. grew well in neutral to alkaline media and produced high amounts of cellulases. **Bollok and Reczey, (2000)** stated that pH 5.0 was unfavorable for cellulase production by *Trichoderma reesei.*

Generally, each microorganism possesses a pH range for its growth and activity with an optimum value within the range. Filamentous fungi have reasonably good growth over a broad range of pH, 2–9, with an optimal range of 3.8 to 6.0 **(Xin and Geng, 2009).**

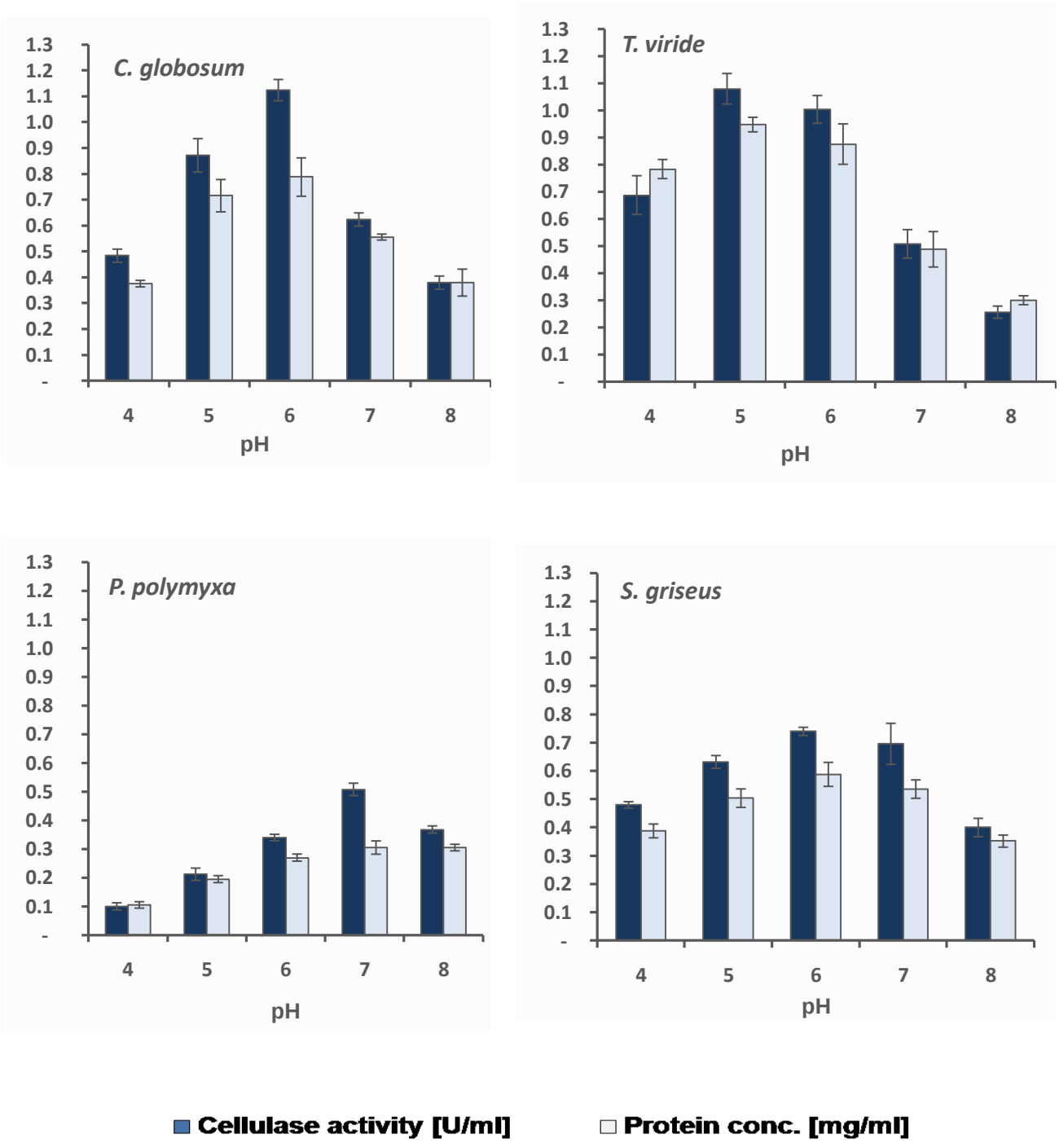


Fig.17. Effect of initial pH on cellulase activity and protein concenctration

2.2.2. Effect of the fermentation period

The time course of cellulase and protein production was studied to determine the point of time with maximum activity. Data illustrated in Fig (18) showed the effect of different incubation periods on cellulase productivity by the tested microorganisms.

From the recorded results, it was found that cellulase activity and extracellular protein concentration progressively increased with the incubation period from 2-8 days and reached the maximum cellulase activities of *C. globosum* and *T. viride* at 8 days of fermentation. After 8 days, the enzyme activity began to decrease. Similarly, **Vyas, (2004)** obtained maximum cellulase production from *Fusarium* sp. after 8 days of fermentation. Moreover, **Garcia-Kirchner *et al.*, (2002)** grew *Penicillium* sp. and *Aspergillus terreus* for 6 days for the maximum yield of cellulolytic and xylanolytic activities. While **Raza and Ur-Rehman, (2009)** found that the optimum time period for production of cellulase from *Chaetomium thermophile* is up to four days. In addition, **Camassola and Dillon, (2007a)** obtained the highest activity of an endoglucanase from *Penicillium echinulatum* after four days of fermentation.

The highest cellulase activities and protein concentrations of *Paenibacillus polymyxa* were obtained after 4 and 10 days, respectively. The further production of protein, reaching the maximum after 10 days fermentation, may be attributed to the secretion of other extracellular enzymes into the growth medium. These results are in good agreement with those reported by **Nizamudeen and Bajaj, (2009).** They reported that growth of the *Bacillus* sp. NZ and cellulase enzyme production displayed a precise relationship, showing maxima after four days of fermentation.

Generally, further culture resulted in the reduced enzyme activities. The decrease of the enzyme activity may be due to the denaturation of the enzymes, resulted from the variation of pH value and the cellular metabolism during fermentation **(Xin and Geng, 2009).**

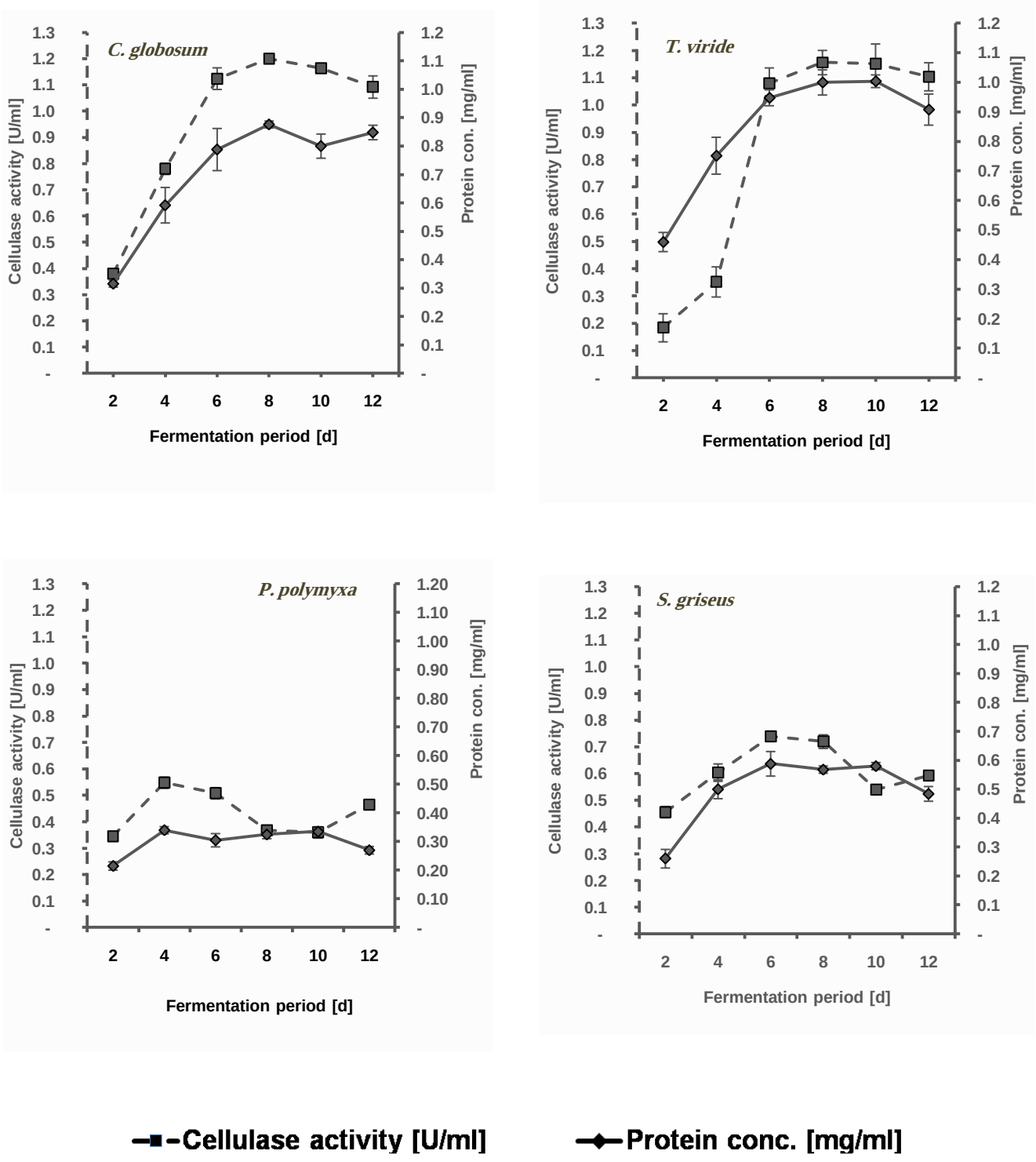


Fig.18. Effect of fermentation period on cellulase activity and protein concentration

2.2.3. Effect of the inoculum volume

The inoculum volume is one of the most important factors that affect the enzyme yield. Inoculation was performed with inoculum volumes from 2.0 - 12.0%. The cultures were grown under the previously optimized conditions.

It is seen from the obtained results, which graphically illustrated in Fig. (19) that protein concentrations of all strains gradually increased with the increase of inoculum volume up to 12% (v/v). Cellulase activities of *C. globosum* and *S. griseus* reached maximum activities of 1.45 and 1.06 U/ml, respectively, at 8% (v/v) inoculum volume. An inoculum volume of 5% was more favorable for formation of cellulase by *T. viride* and *P. polymyxa* (1.16 and 0.55 U/ml) respectively.

Similarly, **El-Baz, (2003)** found 5% inoculum volume to be sufficient for maximal cellulase production from *Trichoderma viride* and *Aspergillus niger*. While **Hao *et al.*, (2006)** used an inoculum volume of 10% for the production of cellulase by *Trichoderma reesei* WX-112.

Lower inoculation volume required longer time for the cells to multiply to reach sufficient number for substrate utilization and enzyme production. An increase in the number of spores in inoculum would ensure a rapid proliferation and biomass synthesis.

After a certain limit, enzyme production could decrease because of depletion of nutrients due to the enhanced biomass, which would result in decrease in metabolic activity. A balance beteewn the proliferating biomass and available nutrients would be maximum (**Sun *et al*, 2010**).

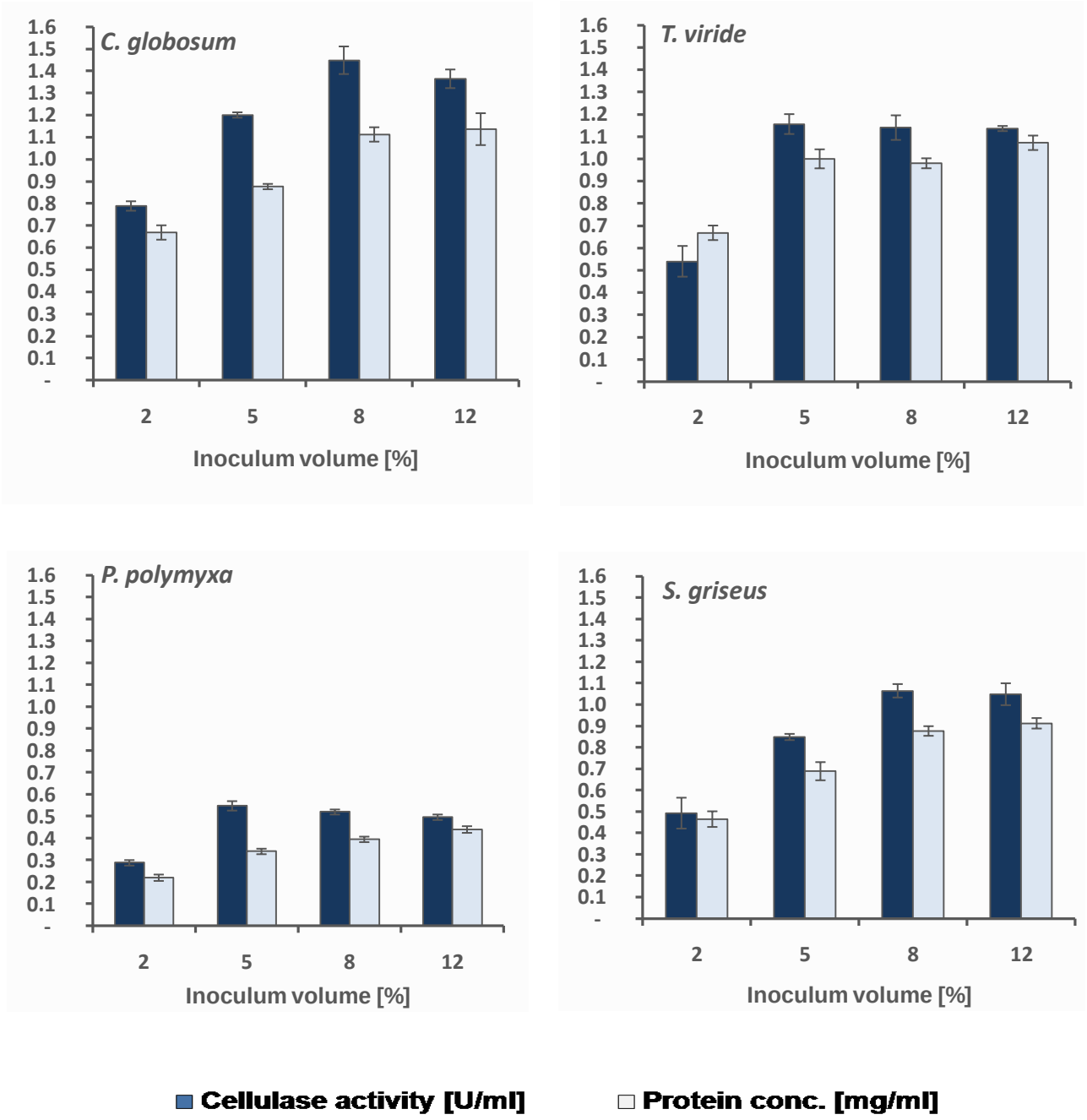


Fig.19. Effect of inoculum volume on cellulase activity and protein concentration

2.2.4. Effect of Tween 80

It was of interest to investigate the effect of Tween 80 as surfactant material on cellulase productivity by *C. globosum*, *T. viride*, *P. polymyxa* and *S. griseus*. Tween 80 at concentrations of 0.5 to 2.5 ml/L were added to Reese and Mandels basal medium, and cellulase production and protein concentration were determined and the data was illustrated in Fig. (20).

The obtained data show that addition of Tween 80 led to higher cellulase activities. The cellulase activity increased with increasing concentrations of Tween 80 up to 1.5 ml/L for *T. viride* and *P. polymyxa* with maximum activities of 1.29 and 0.64 U/ml, respectively. According to studies of **Domingues *et al.*, 2000**, Tween 80 influences the morphology of *Trichoderma reesei* Rut C-30 as well as the enzyme production by facilitating the release of enzymes into the medium. In addition, a concentration of 1.0 ml/L was found to be the most suitable for cellulase formation by *C. globosum* and *S. griseus*. Generally, the protein concentrations of the culture supernatants of all strains increased with increasing Tween 80 concentrations up to 2.0 ml/L.

If surfactant increase the amount of enzyme production and secreted into the medium by acting on membrane, they might also cause other internal components to be similarly released (**Gashe, 1992**). While **El-Hawary and Mostafa, (2001)** reported that detergents like Tween 80 have been reported to enhance cellulase activities by increasing availability of nutrients.

On the other hand, **Pie-Jun *et al.*, (2004)** reported negative effect of Tween 80 0n cellulase activities.The increase in cellulase production was also found by **El-Baz, (2003)** when Tween 80 was added to the culture medium of *Trichoderma viride* and *Aspergillus niger* in amounts of up to 2.0 ml/L. The activity increased 1.5 fold when 2.0 ml/L of Tween 80 was added to the culture medium. High concentrations of Tween 80 (3.0 ml/L) resulted in decreased cellulase activities. The increased enzymatic activity by non-ionic surfactant (Tween 80) is attributed to the unmasking of SH groups in the microbial enzymes and subsequently augmenting the interactions between enzymes and potential substrate **(Liang *et al.*, 2010).**

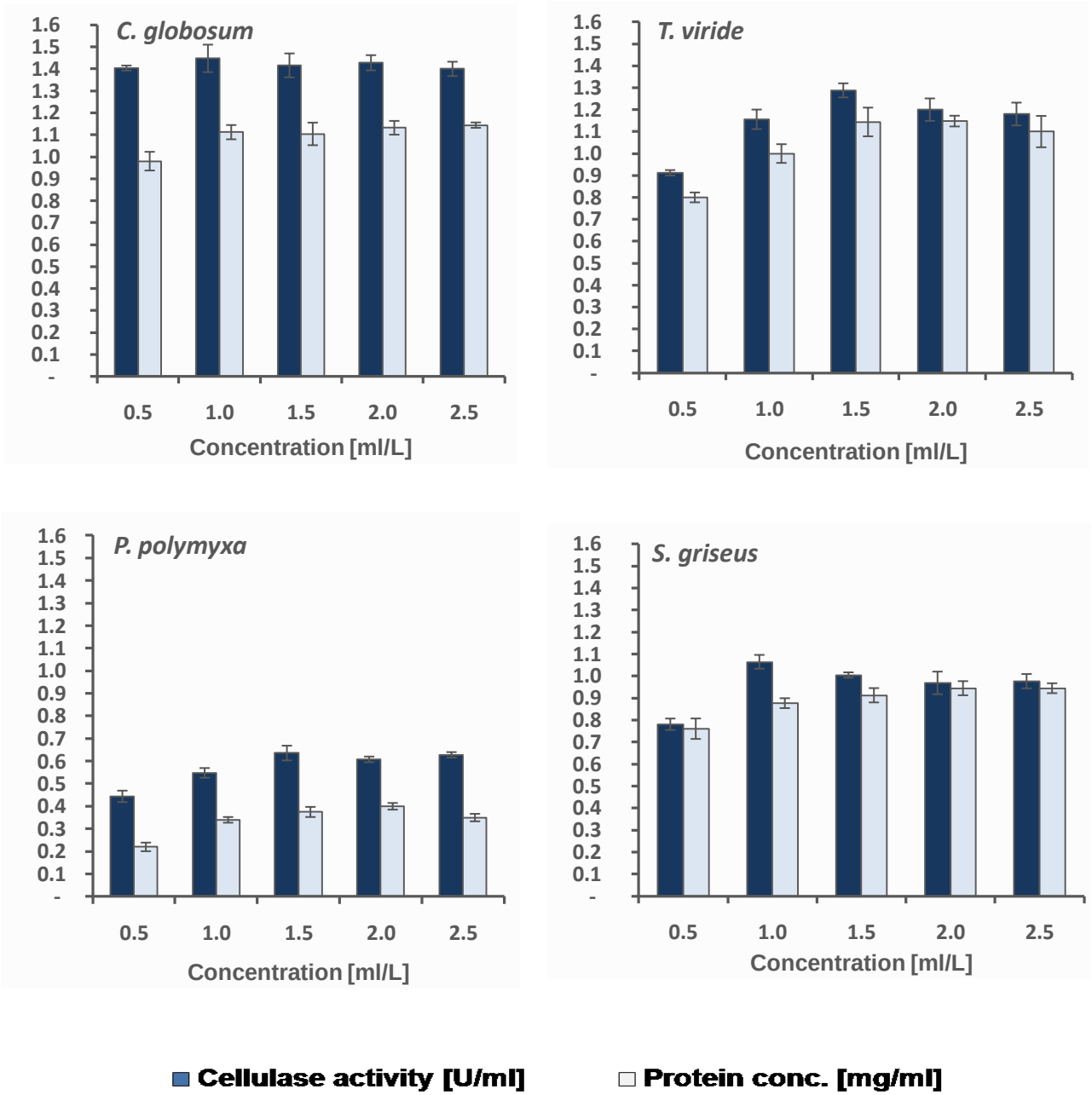


Fig.20. Effect of Tween 80 concentration on cellulase activity and protein concentration

3. The optimum conditions for cellulase production by *C. globosum, T. viride, P. polymyxa* and *S. griseus*

The optimum conditions for cellulase production by *C. globosum*, *T. viride*, *P. polymyxa* and *S. griseus* were concloded in Tables 7, 8, 9, and 10. *C. globosum* exhibited the highest cellulase activity which increased to be about three fold higher as compared with enzyme yields obtained in basal medium. Therefore, *C. globosum* was choosen to be used for cellulase production for the succeding work.

Table 7. Comparison of basal medium Vs. optimized medium composition for production of cellulase by *C. globosum*

Reese Basal medium	(g L^{-1})	Optimized production medium	(g L^{-1})
Cellulose powder	10.0	Cellulose powder	25.0
Proteose peptone	0.25	Proteose peptone	0.25
$(NH_4)_2SO_4$	1.4	Glucose	1.0
Urea	0.3	Urea	3.3
KH_2PO_4	2.0	KH_2PO_4	2.0
$CaCl_2$. $2H_2O$	0.3	$CaCl_2$. $2H_2O$	0.3
$MgSO_4.7H_2O$	0.3	$MgSO_4.7H_2O$	0.3
Tween 80	1 ml	Tween 80	1 ml
Trace element solution	1 ml	Trace element solution	1 ml
pH	6.0	pH	6.0
Inoculum size	5.0 %	Inoculum size	8.0 %
Fermentation period	6 days	Fermentation period	8 days
Cellulase activity	0.50 U/ml	Cellulase activity	1.45 U/ml

Table 8. Comparison of basal medium Vs. optimized medium composition for production of cellulase by *T. viride*

Reese Basal medium (g L^{-1})		Optimized production medium (g L^{-1})	
Cellulose powder	10.0	Cellulose powder	20.0
Proteose peptone	0.25	Proteose peptone	0.25
$(NH_4)_2SO_4$	1.4	Glucose	1.5
Urea	0.3	Urea	2.0
KH_2PO_4	2.0	KH_2PO_4	2.0
$CaCl_2$. $2H_2O$	0.3	$CaCl_2$. $2H_2O$	0.3
$MgSO_4.7H_2O$	0.3	$MgSO_4.7H_2O$	0.3
Tween 80	1 ml	Tween 80	1.5 ml
Trace element solution	1 ml	Trace element solution	1 ml
pH	6.0	pH	5.0
Inoculum size	5 %	Inoculum size	5.0 %
Fermentation period	6 days	Fermentation period	8 days
Cellulase activity	0.52 U/ml	Cellulase activity	1.29 U/ml

Table 9. Comparison of basal medium Vs. optimized medium composition for production of cellulase by *P. polymyxa*

Reese Basal medium	(g L^{-1})	Optimized production medium (g L^{-1})	
Cellulose powder	10.0	Cellulose powder	10.0
Proteose peptone	0.25	Proteose peptone	0.25
$(NH_4)_2SO_4$	1.4	Yeast extract	3.0
Urea	0.3	Urea	0.3
KH_2PO_4	2.0	KH_2PO_4	2.0
$CaCl_2$. $2H_2O$	0.3	$CaCl_2$. $2H_2O$	0.3
$MgSO_4.7H_2O$	0.3	$MgSO_4.7H_2O$	0.3
Tween 80	1 ml	Tween 80	1.5 ml
Trace element solution	1 ml	Trace element solution	1 ml
pH	7.0	pH	7.0
Inoculum size	5 %	Inoculum size	5.0 %
Fermentation period	6 days	Fermentation period	4 days
Cellulase activity	0.26 U/ml	Cellulase activity	0.64 U/ml

Table 10. Comparison of basal medium Vs. optimized medium composition for production of cellulase by *S. griseus*

Reese Basal medium	(g L^{-1})	Optimized production medium (g L^{-1})	
Cellulose powder	10.0	Cellulose powder	20.0
Proteose peptone	0.25	Proteose peptone	0.25
$(NH_4)_2SO_4$	1.4	Yeast extract	1.4
Urea	0.3	Urea	0.3
KH_2PO_4	2.0	KH_2PO_4	2.0
$CaCl_2$. $2H_2O$	0.3	$CaCl_2$. $2H_2O$	0.3
$MgSO_4.7H_2O$	0.3	$MgSO_4.7H_2O$	0.3
Tween 80	1 ml	Tween 80	1.0 ml
Trace element solution	1 ml	Trace element solution	1 ml
pH	7.0	pH	6.0
Inoculum size	5 %	Inoculum size	8.0 %
Fermentation period	6 days	Fermentation period	6 days
Cellulase activity	0.42 U/ml	Cellulase activity	1.06 U/ml

4. Concentration of cellulase produced by *C. globosum*

The culture supernatant of *C. globosum* was concentrated by ultrafiltration (Table 11). Cellulase and protein yields after concentration were 60.2 and 35.6%, respectively. The obtained specific activity was 2.17 U/mg. The protein concentration after ultrafiltration was 6.76 mg/ml which was sufficient for analysis by means of isoelectric focused electrophoresis.

Table 11. Concentration of cellulase produced by *C. globosum*

	Volume [ml]	Activity [U/ml]	Protein [mg/ml]	Specific activity [U/mg]	Total activity [U]	Activity yield [%]	Total protein [mg]	Protein yield [%]
Initial supernatant	**1.000**	**1.22**	**0.95**	**1.28**	**1.220**	**100**	**950**	**100**
Retentate	**50**	**14.69**	**6.76**	**2.17**	**734.5**	**60.2**	**338**	**35.6**

5. Characterization of cellulase and xylanase enzymes

The concentrated cellulase produced by *C. globosum* was biochemically characterized compared with other commercial microbial cellulase and xylanase enzyme. Kinetic constants, activity, optimum temperature, optimum pH and their pH and temperature stabilities were investigated. In addition, the isoelectric point (pI) and the molecular weight of cellulase produced by *C. globosum* were determined.

5.1. Kinetic constants and activities of cellulases and xylanases

The activities and kinetic constants were determined at 30 °C. The Kinetic parameters such as K_m and v_{max} of the enzymes were determined under steady state conditions by Lineweaver-Burk double reciprocal plots. Graphs were drawn using the Enzyme Kinetic Module. The obtained results are summarized in Table 12 and graphically illustrated in Figs. 21- 28.

Table 12. Kinetic constants and activities of cellulases and xylanases

Enzyme	Enzyme source	K_m [mg/ml]	V_{max} [µmol/min/ml]	Activity
1. Cellulase	Produced by *C. globosum*	2.27	0.1194	14.69 U/ml
2. Cellulase	Produced by *T. reesei*	0.39	0.0124	1.500 U/g
3. Xylanase	Produced by *A. niger*	4.92	0.1274	25.238 U/g
4. Xylanase (UF)	Produced by *Disporotrichum sp.*	1.87	0.0361	2175 U/ml
5. Laminex as cellulase	Mixture of cellulase and xylanase	4.59	0.0587	182 U/ml
6. Laminex as xylanase	Mixture of cellulase and xylanase	2.11	0.0368	286 U/ml
7. CWDEC as cellulase	Cell wall degrading enzyme complex of cellulase and xylanase	1.56	0.0304	104 U/ml
8. CWDEC as xylanase	Cell wall degrading enzyme complex of cellulase and xylanase	0.74	0.0947	105 U/ml

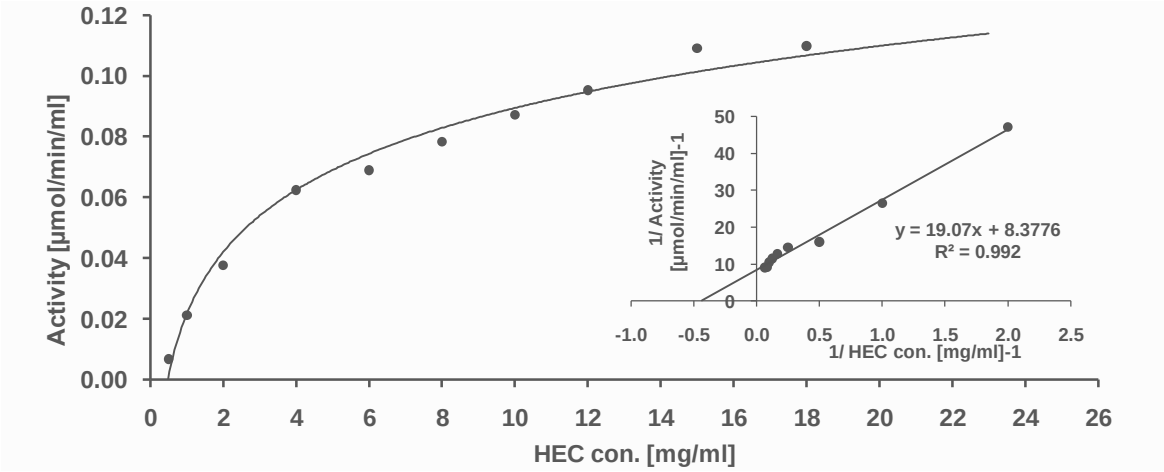
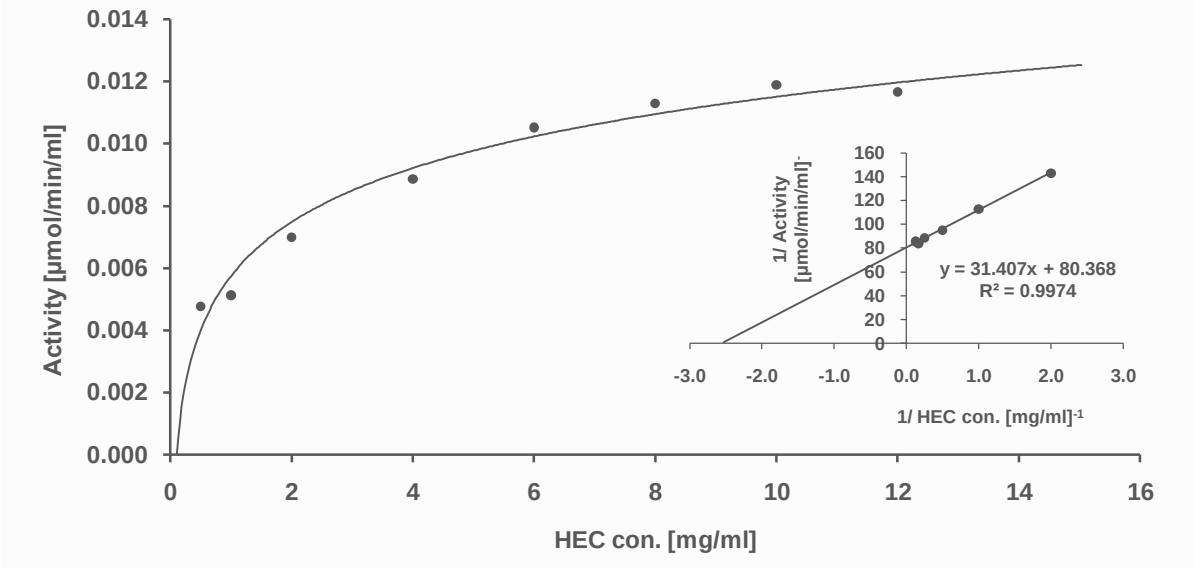
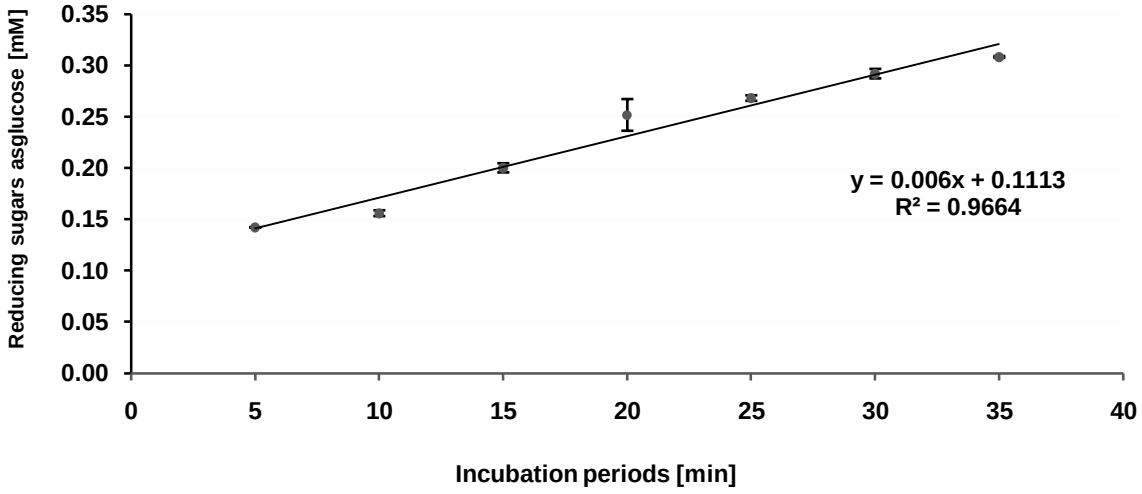


Fig.21.Michaelis-Menten curve and double reciprocal (Lineweaver-Burk) plot with HEC as substrate for cellulase (*C. globosum*)



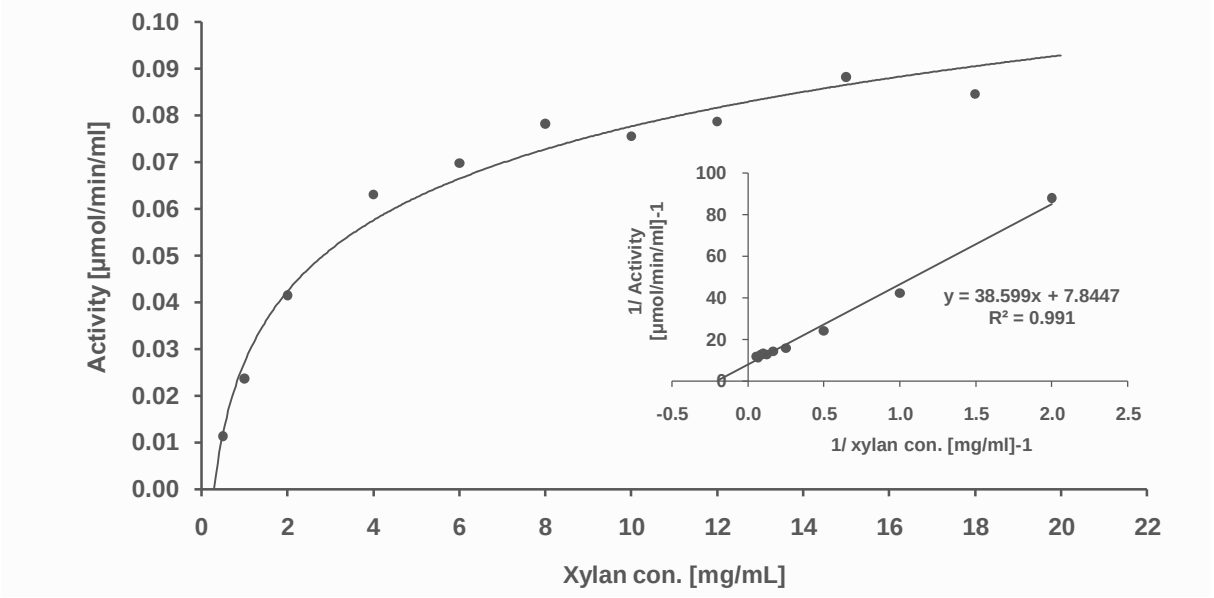
(a)



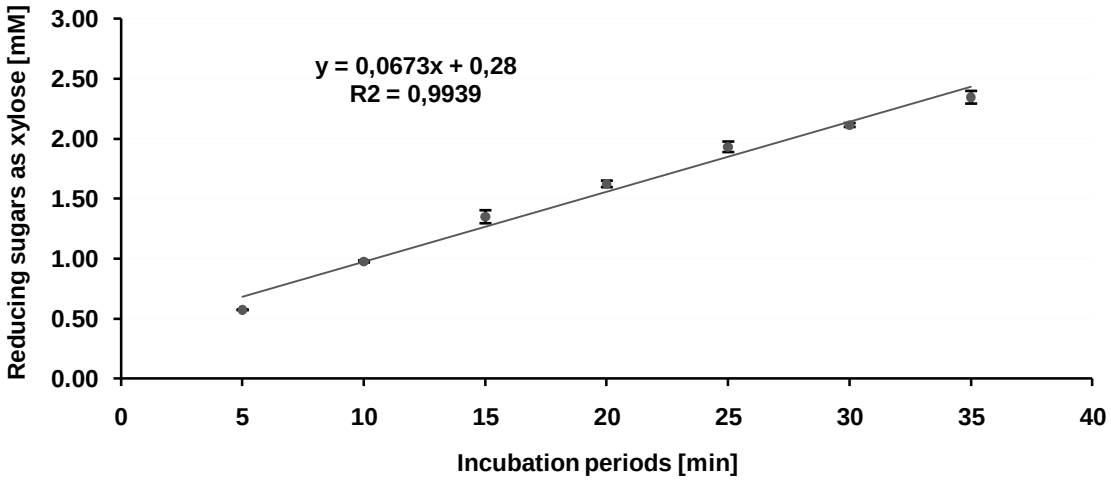
(b)

Fig.22. (a) Michaelis-Menten curve and double reciprocal (Lineweaver-Burk) plot with HEC as substrate for cellulase (*T. reesei*)

(b) Kinetic of cellulase (*T. reesei*) at different incubation periods



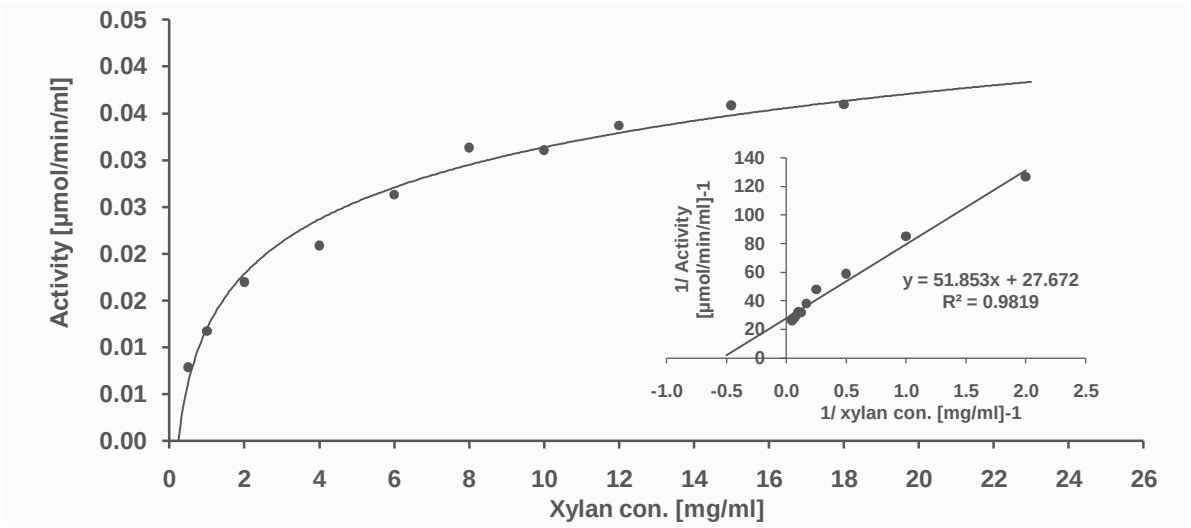
(a)



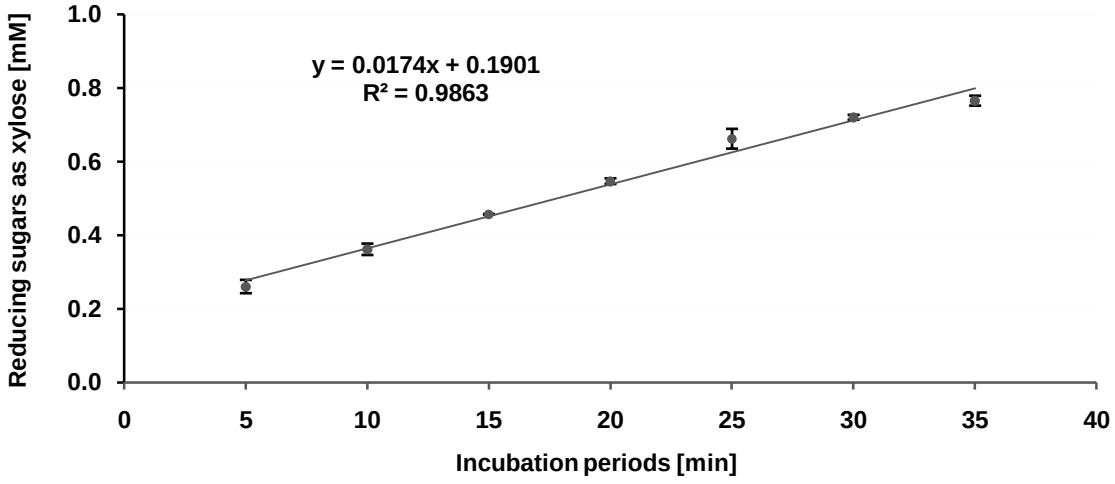
(b)

Fig.23. (a) Michaelis-Menten curve and double reciprocal (Lineweaver-Burk) plot with xylan as substrate for xylanase (*A. niger*)

(b) Kinetic of xylanase (*A. niger*) at different incubation periods



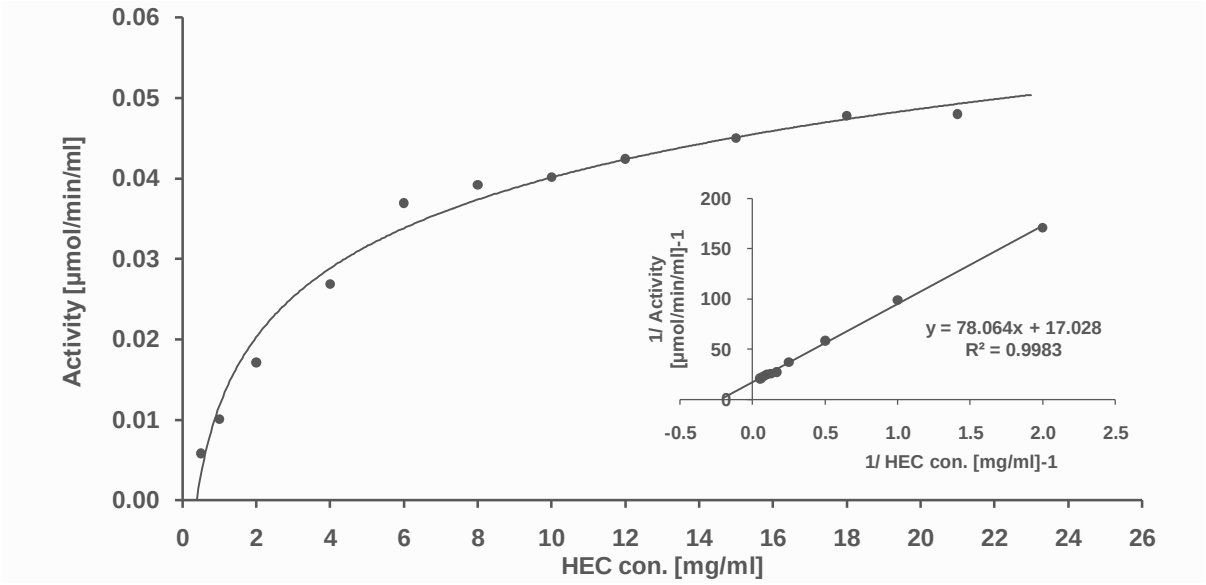
(a)



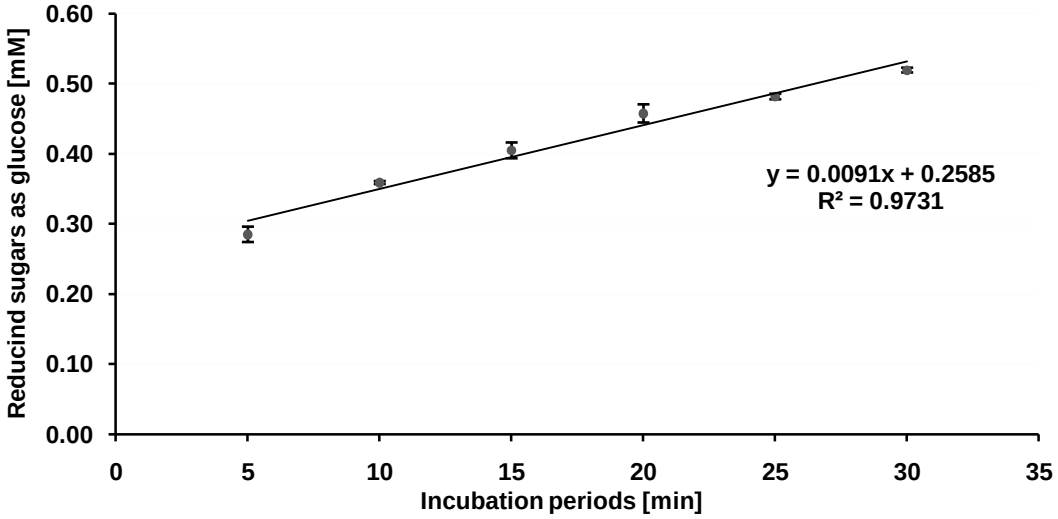
(b)

Fig.24. (a) Michaelis-Menten curve and double reciprocal (Lineweaver-Burk) plot with xylan as substrate for xylanase (UF)

(b) Kinetic of xylanase (UF) at different incubation periods



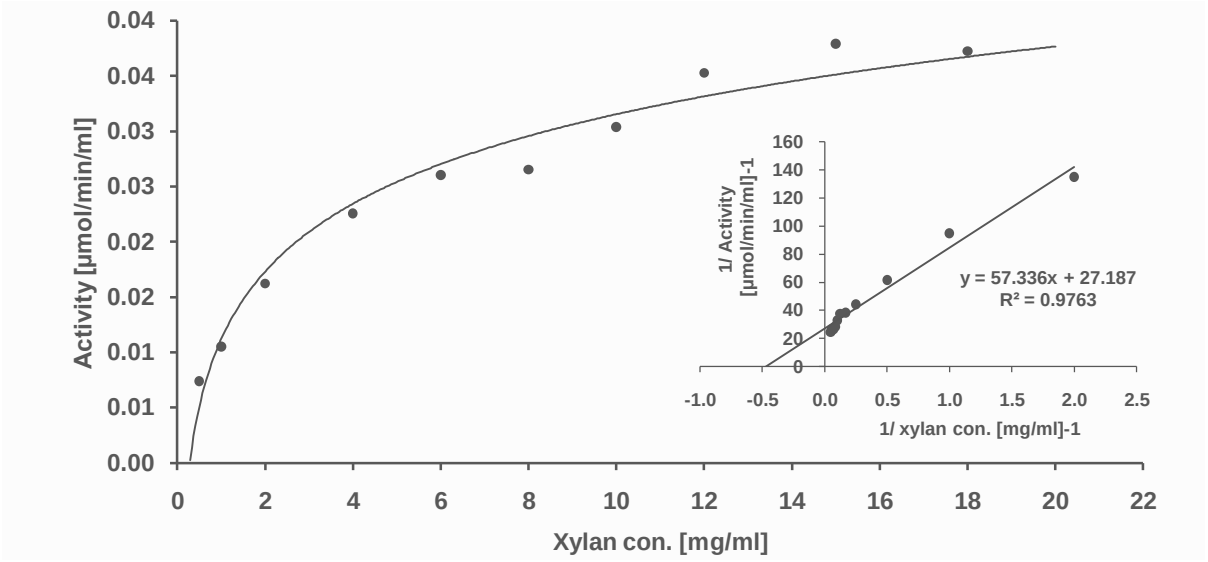
(a)



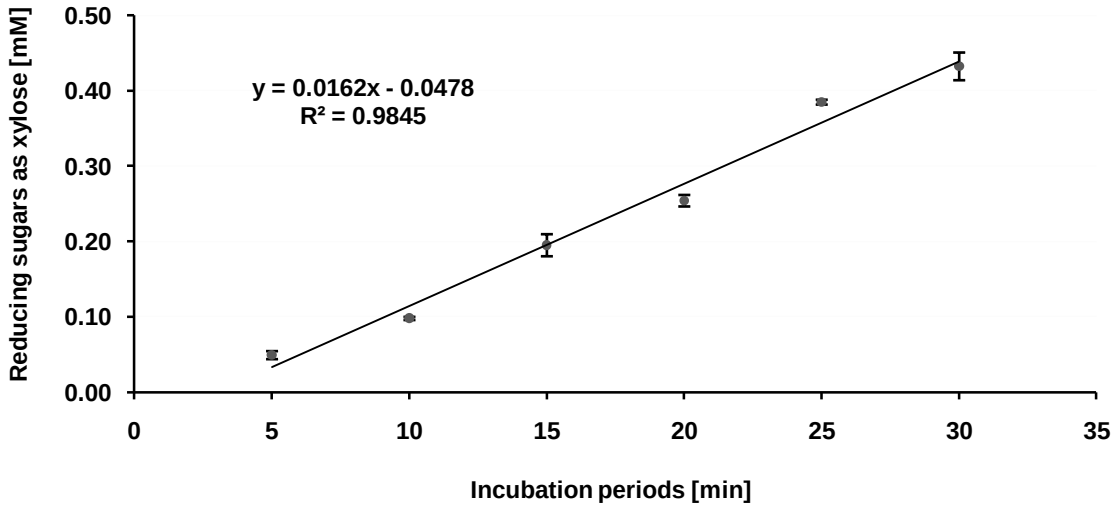
(b)

Fig.25. (a) Michaelis-Menten curve and double reciprocal (Lineweaver-Burk) plot with HEC as substrate for laminex as cellulase

(b) Kinetic of laminex as cellulase at different incubation periods



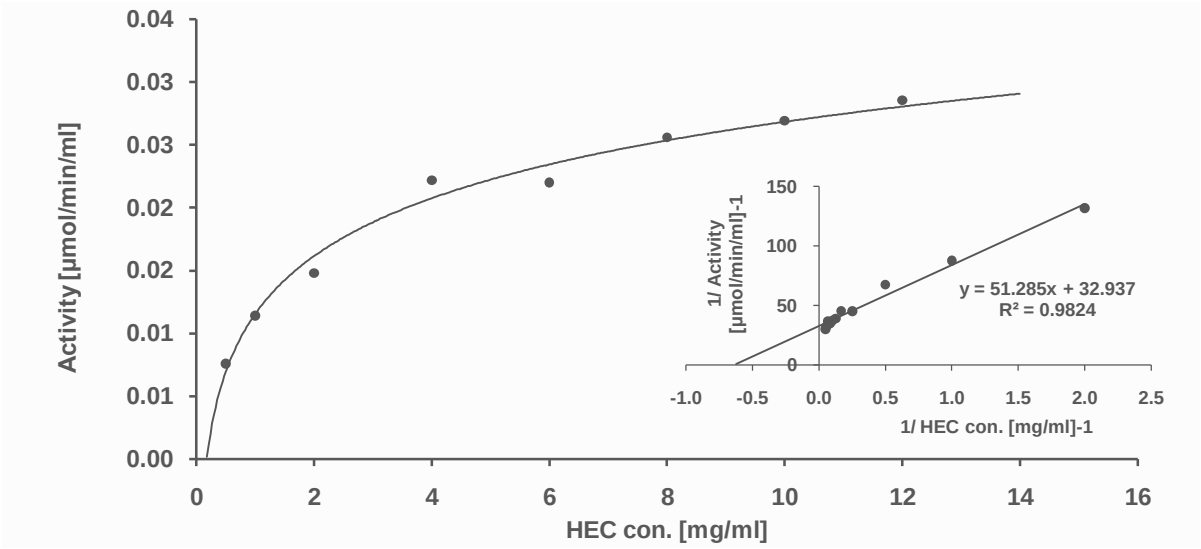
(a)



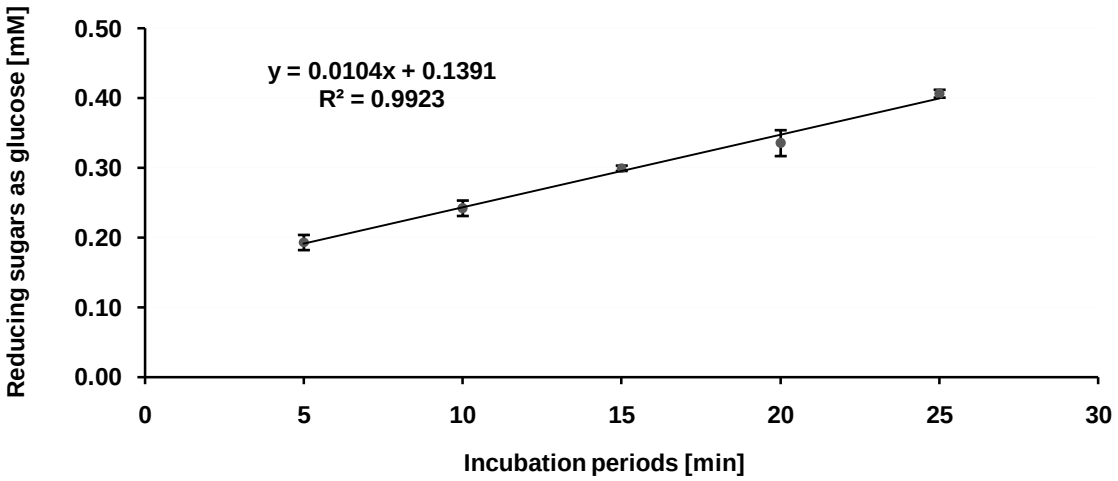
(b)

Fig.26. (a) Michaelis-Menten curve and double reciprocal (Lineweaver-Burk) plot with xylan as substrate for laminex as xylanase

(b) Kinetic of laminex as cellulase at different incubation periods



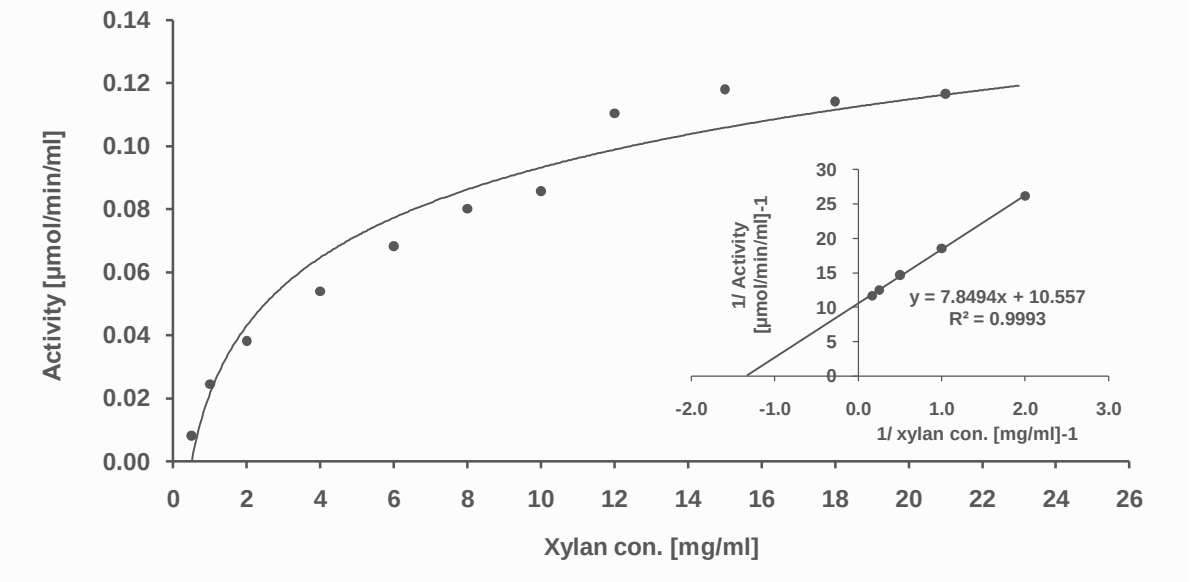
(a)



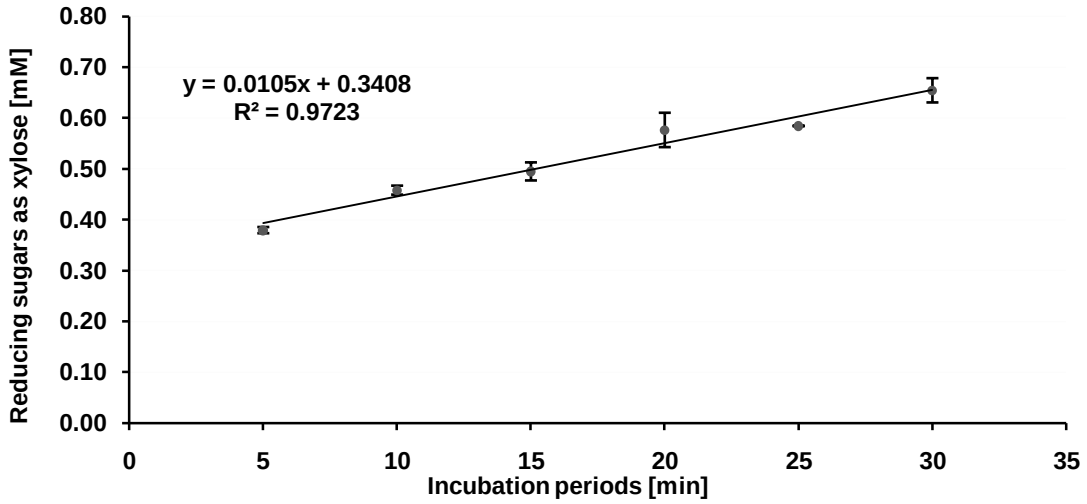
(b)

Fig.27. (a) Michaelis-Menten curve and double reciprocal (Lineweaver-Burk) plot with HEC as substrate for CWDEC as cellulase

(b) Kinetic of CWDEC as cellulase at different incubation periods



(a)



(b)

Fig.28. (a) Michaelis-Menten curve and double reciprocal (Lineweaver-Burk) plot with xylan as substrate for CWDEC as xylanase

(b) Kinetic of CWDEC as xylanase at different incubation periods

5.2. Effect of temperature on cellulases and xylanases activity and thermal stability

Optimum temperature and thermal stability were studied for cellulase produced by *C. globosum* and all lignocellulase degrading enzymes. In all the determinations, cellulase and xylanase activity was measured by using hydroxyl ethyl cellulose (HEC) and xylan as substrates, respectively. For estimation of the temperature optima, the activity was determined by carrying out standard assays at several temperatures degrees (30-75°C).

The loss of enzyme activity depends both on time and temperature, it is therefore important to investigate the stability of the enzyme with respect to these factors. Temperature optima and the thermal stability of the enzymes were studied over a certain period. To estimate the temperature stability, the residual activity after incubation for 12 h. at the enzymes temperature optima was determined under standard conditions. The obtained data was illustrated and recorded in Figs.29a, 29b & Tables 13a, 13b.

Under the assay conditions used, crude preparations of endogluconase and other enzymes showed high activity over a wide range of temperature between 30 and 75°C. The optimum temperature for the cellulases from *T. reesei* and *C. globosum* was 65 °C, while 60 °C was optimum for the xylanase activities of laminex and CWDEC. Xylanase (*A. niger*), laminex, and CWDEC showed their maximum activities at 50 °C. Also, 55 °C was found to be the optimum temperature for xylanase (UF) activity. The optimum temperature of endogluconase produced from *C. globosum* was found to be 65 °C. This value is higher than that of commercial cellulose activity (laminex and CWDEC) which was 50 °C. These results are in good agreement with those reported by **Rao *et al.*, (2003)** and **Wang *et al.*, (2008).** Moreover, **Liang *et al.*, (2010)** reported that the cellulase enzymes produced by *Anoxybacillus sp.* 527 demonstrated good activities between 50 and 70 °C with the maximum activity at 70 °C.

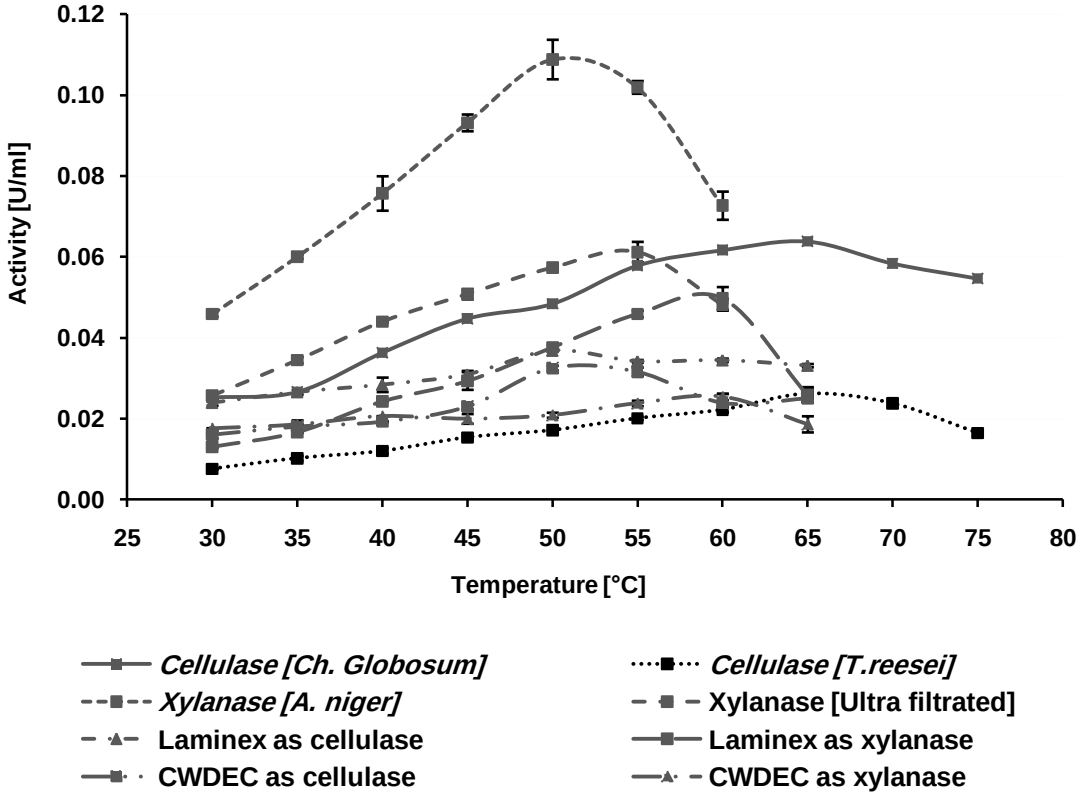


Fig.29a. Effect of the temperature on cellulase and xylanase activities

Table 13a. Temperature optima of cellulases and xylanases

Enzyme	Optimum temperature
1. Cellulase (*C. globosum*)	65 ºC
2. Cellulase (*T. reesei*)	65 ºC
3. Xylanase (*A. niger*)	50 ºC
4. Xylanase (U.F.)	55 ºC
5. Laminex (as cellulase)	50 ºC
6. Laminex (as xylanase)	60 ºC
7. CWDEC (as cellulase)	50 ºC
8. CWDEC (as xylanase)	60 ºC

The use of thermostable enzymes to carry out hydrolysis at high temperature is advantageous because they speed up the reaction rate and prevent microbial contamination **(Raza and Ur-Rehman, 2009)**.

Data recorded in Table 13b and illustrated at Fig 29b show that laminex as cellulase retained 89 and 80% of the initial activity after 2 and 12 h incubation at its optimal temperature, respectively. This indicates high thermostability of laminex as cellulose. On the other hand, laminex as xylanase was rapidly inactivated and retained about 13and 8% of the initial activity after 2 and 12 h, respectively.

Cellulase from *T. reesei* retained about 85and 19% of the initial activity after 2 and 12 hours, respectively. About 42% residual activity was obtained for cellulase from *C. globosum* and CWDEC as cellulase after 12 h. High losses of enzyme activity (about 93%) were detected when xylanase (*A. niger*) was incubated at 50 °C for 12 h. Finally, CWDEC as xylanase retained around 28and 22% of the initial activity after 2 and 12 hours, respectively.

The loss of enzyme activity can be attributed to protein denaturation. The aforementioned results are in accordance with those obtained by**, Reis *et al.*, (2003); Whiteley *et al.*, (2003)** and **Wang *et al.*, (2008)** who stated that the loss of enzyme activity at elevated temperatures is a consequence of thermal denaturation of protein.

The obtained data revealed that cellulase produced from *C. globosum* has higher residual activity after 12 h and thermostable at 65 °C. This comparison shows that heat stability of our enzyme from *C. globosum* is higher than that of the enzymes mentioned above except cellulase of laminex enzyme. These results are in harmony with those recorded by **Raza and Ur-Rehman, (2009)** they recorded that endocellulase of *Chaetomium thermophile* retained its 100 and 71% activity at 60 and 80°C, respectively, for 15 min. Thus extracellular enzyme from *C. thermophile* was found stable at high temperature.

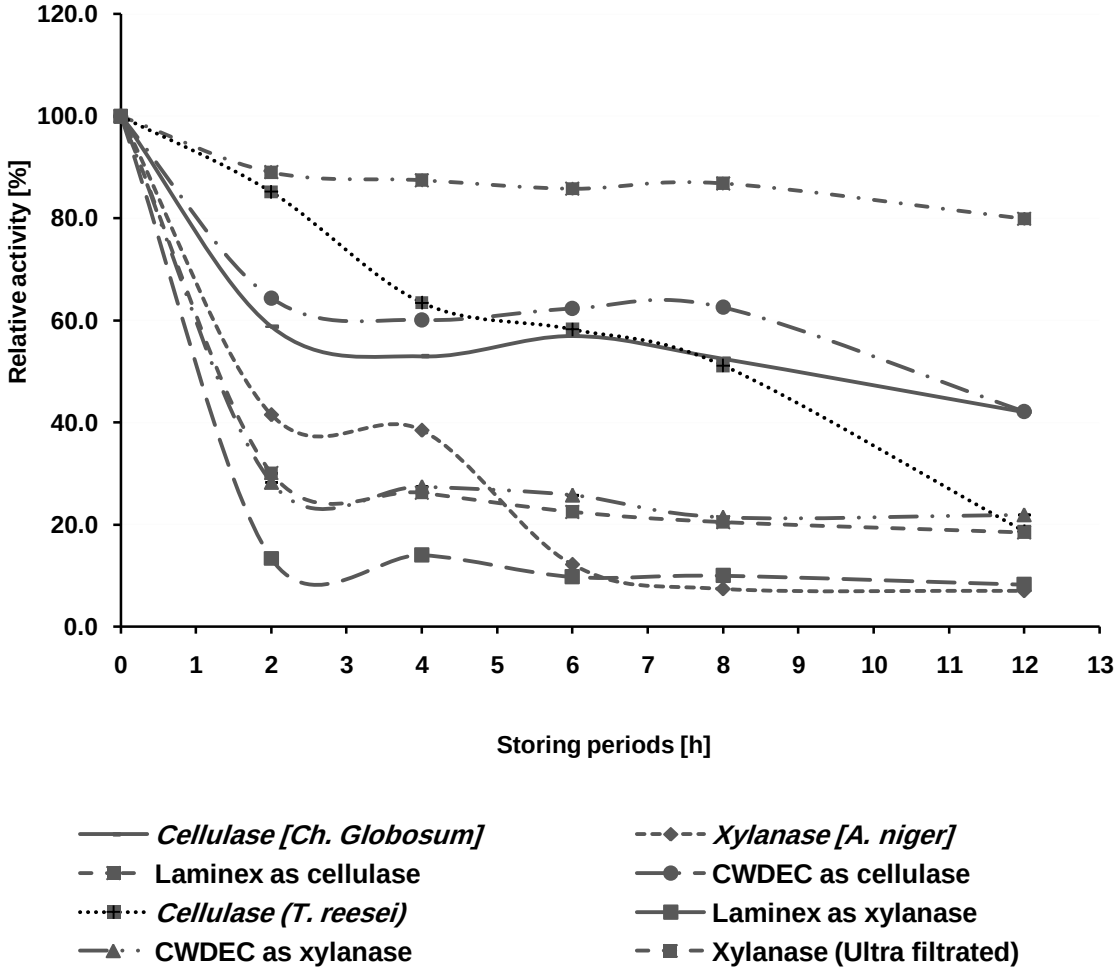


Fig.29b. Thermal stability of cellulase and xylanase enzymes

Table 13b. Thermal stability of cellulases and xylanases

Enzyme	Residual activity [%] after				
	2 [h]	4 [h]	6 [h]	8 [h]	12 [h]
1. Cellulase (*C. globosum*)	59	53	52	51	42
2. Cellulase (*T. reesei*)	85	63	58	51	19
3. Xylanase (*A. niger*)	42	38	12	8	7
4. Xylanase (U.F.)	30	26	22	20	18
5. Laminex (as cellulase)	89	87	86	86	80
6. Laminex (as xylanase)	13	13	10	10	8
7. CWDEC (as cellulase)	64	60	60	59	42
8. CWDEC (as xylanase)	28	26	25	21	22

5.3. Effect of pH on cellulase and xylanase activities and pH stability

The ionization state of amino-acid residues of an enzyme depends on the pH value. Since catalytic activity is dependent on the state of ionization of these residues, enzyme activity is consequently pH dependent. Enzymes are often active over a narrow pH range with a specific pH optimum at which their catalytic activity is maximal **(Wilson, 2000).** Therefore, it was of interest to determine the pH optima and the pH stability of the cellulases and xylanases under investigation (Figs.30a, 30b and Table 14).

The cellulases and xylanases enzymes were active in a broad pH range of 3.0 to 6.0. pH 4.0 was favorable for the activity of the cellulase from *T. reesei*, the xylanase from *A. niger*, and for laminex as cellulase. CWDEC as cellulase and xylanase exhibited the maximum activity at pH 3.0.

The cellulase from *C. globosum* exhibited high activities in the pH range from 3.0 - 6.0 with an optimum at pH 5.0, while pH 4.5 and 5.5 were found to be the optimum for the activity of xylanase (UF) and laminex (as xylanase), respectively. These results are in good agreement with those obtained by **Fadel, (2001)** who produced xylanase by *Trichoderma harzianum* and studied the effect of pH on the enzyme's activity. The enzyme exhibited high activity in a pH range of 3.0-10.0 with an optimum at pH 5.5. Also, **Coral *et al.*, (2002)** studied the pH dependence of an endo cellulase from a wild type strain of *Aspergillus niger*. They reported a broad activity range of 3.0 - 9.0 with a maximum activity at pH 4.5. Moreover, **Liang *et al.*, (2010)** reported that the cellulase enzymes produced by *Anoxybacillus sp.* were active at a broad range of pH (5–7) with optimal pH as 5.0.

All of the enzymes tested were stable for 12 h. when incubated at their pH optima (Fig. 30b). The obtained results are in good agreement with those recorded by **Raza and Ur-Rehman, (2009)** they recorded that endocellulase of *Chaetomium thermophile* retained its 100% activity at pH values from 6.0 - 7.5.

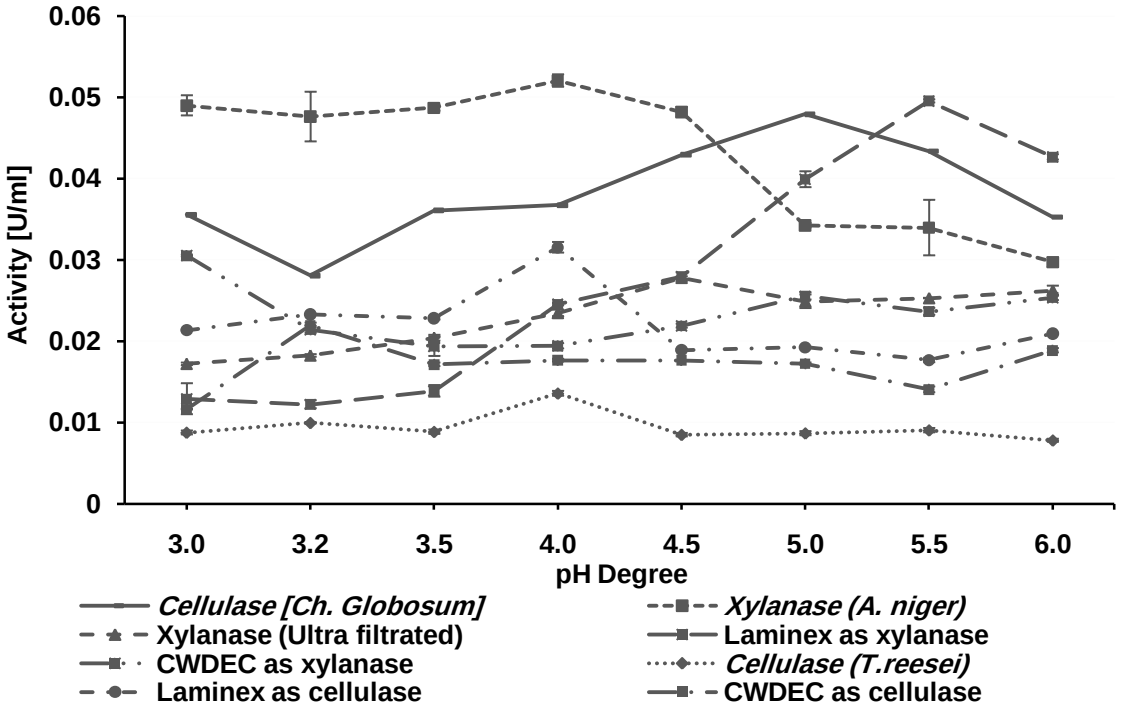


Fig.30a. Effect of pH on cellulase and xylanase activities

Table 14. pH optima of cellulases and xylanases

Enzyme	Optimum pH
1. Cellulase (*C. globosum*)	5.0
2. Cellulase (*T. reesei*)	4.0
3. Xylanase (*A. niger*)	4.0
4. Xylanase (U.F.)	4.5
5. Laminex (as cellulase)	4.0
6. Laminex (as xylanase)	5.5
7. CWDEC (as cellulase)	3.2
8. CWDEC (as xylanase)	3.0

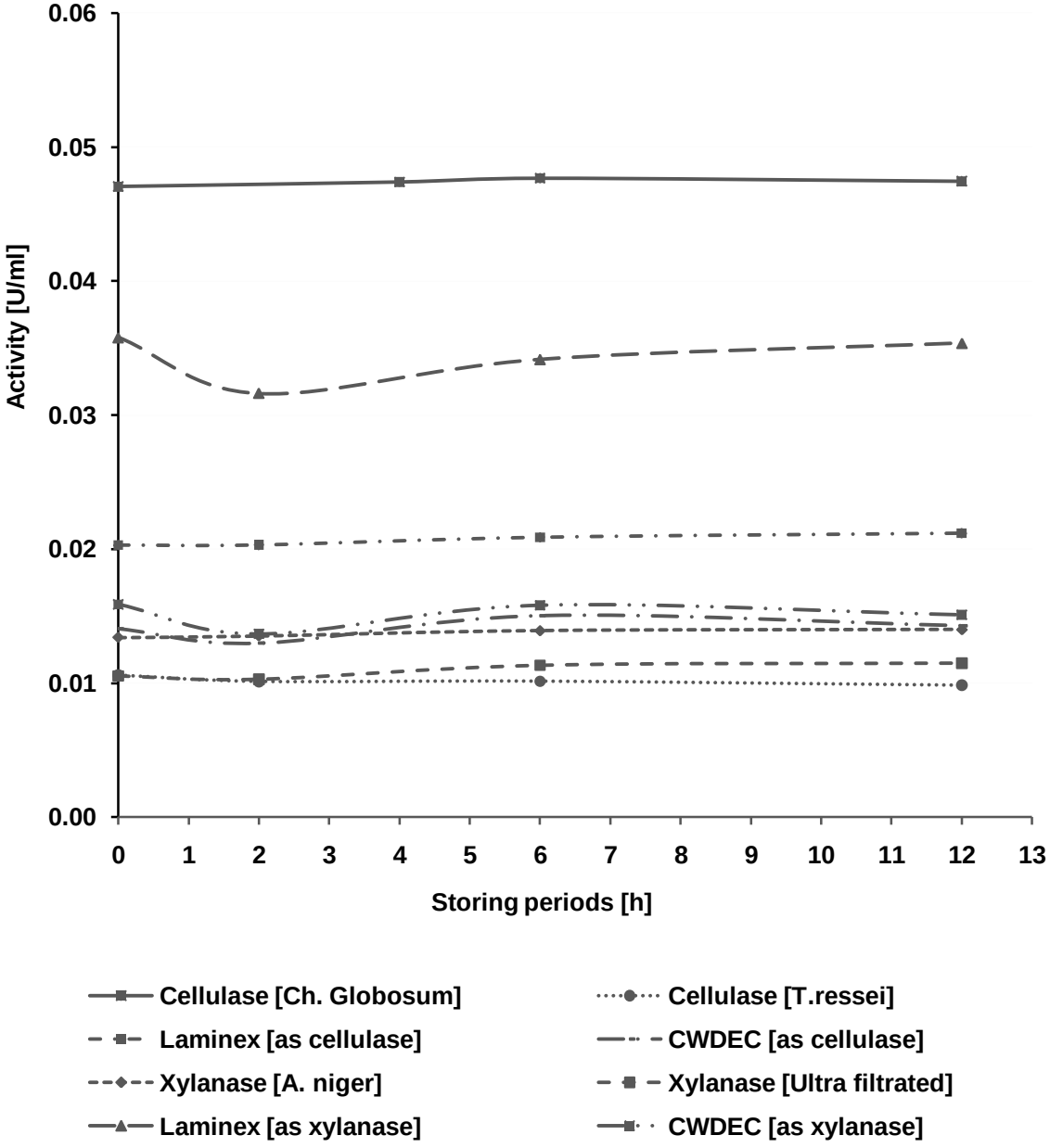
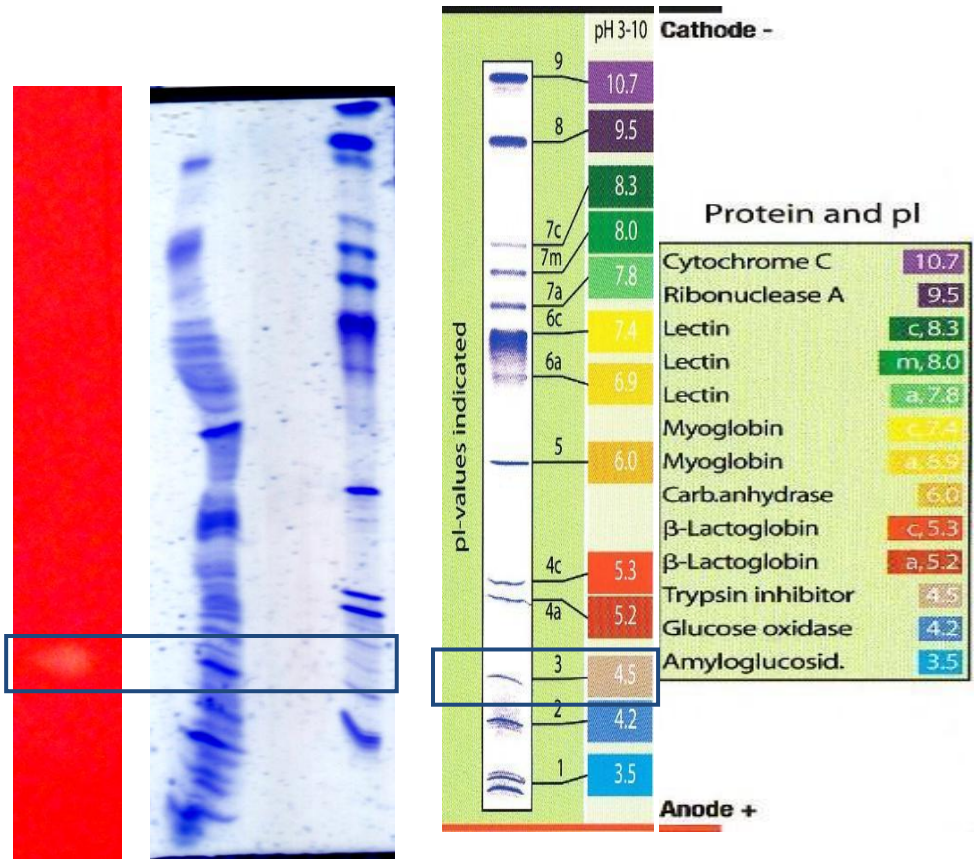


Fig.30b. pH stability of cellulases and xylanases

5.4. Isoelectric focusing (IEF) of cellulase produced by *C. globosum*

Cellulase produced by *C. globosum* was analyzed by isoelectric focusing electrophoresis (IEF) with Coomassie and activity staining (Fig. 31). A clear (unstained) zone on the agarose plate indicated the presence of cellulase activity. An isoelectric point (pI) around 4.5 was determined for the cellulase produced by *C. globosum*.



(a) **(b1)** **(b2)** **(c)**

Fig. 31. Analysis of cellulase (*C. globosum*) by IEF electrophoresis, (a): activity staining with congo red, (b1, b2): Coomassie staining of sample (6µl) and protein marker (5µl), respectively. (c): protein marker

5.5. SDS –PAGE analysis of the cellulase produced by *C. globosum*

Molecular weight was determined by SDS-PAGE. Analyses of the enzyme by SDS-PAGE revealed one band which shows cellulolytic activity on the gel as shown in Fig 32. The molecular weight of this band was detected to be 39 kDa.

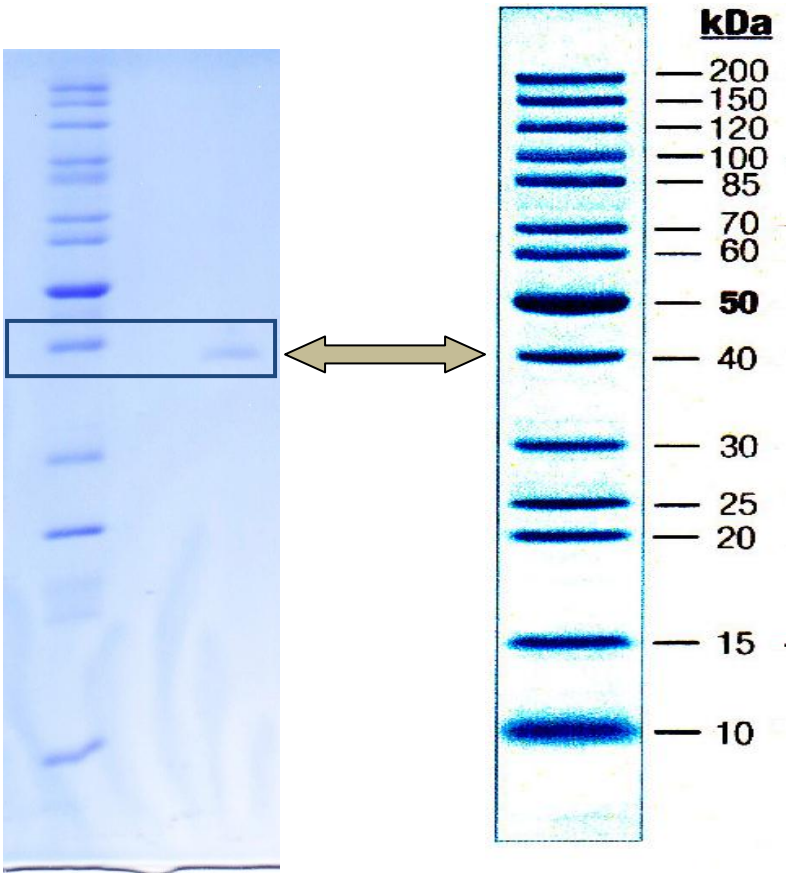


Fig. 32. SDS-PAGE analysis of a cellulase secreted by *C. globosum*

6. Application of lignocellulose degrading enzymes in corn stover degradation

Corn stover is an abundant agricultural residue that is left behind after corn grain harvest. It is a heterogeneous substrate containing stalks and leaves. The use of microorganisms or their enzymes for the conversion of cellulose into fermentable carbohydrates is receiving increased attention **(Sheehan *et al.*, 2004** and **Wayman *et al.*, 2005)**.

The plant cell wall is a composite material in which cellulose, hemicellulose (mainly xylan) and lignin are closely associated. The three major constituents of lignocellulose are cellulose (35-50%), hemicellulose (20-30%, mainly xylan), and lignin (20-30%) **(Subramaniyan** and **Prema, 2002).**

Vast quantities of waste residues are generated by the forestry and agricultural industries, making cellulose a cheap raw material that may be converted to attractive biotechnological products. There is currently great interest in the degradation of lignocellulosic materials to provide suitable raw materials for the production of food, fuels, and enzymes. Much of this interest is centered on the enzymatic degradation of lignocellulose by cellulases, xylanases and ligninases.

6.1. Application of cellulases and xylanases in corn stover degradation

The formation of fermentable sugars by cellulase and hemicellulase catalysed hydrolysis of pretreated corn stover was investigated using cellulase produced from *C. globosum* and the aforementioned five lingocellulolytic enzymes. Data were illustrated in Fig. (33).

The obtained data show that in all treatments, the release of reducing sugars increased with increasing incubation period until 30 h.

Laminex exhibited the highest capacity of corn stover degradation to reducing sugars.

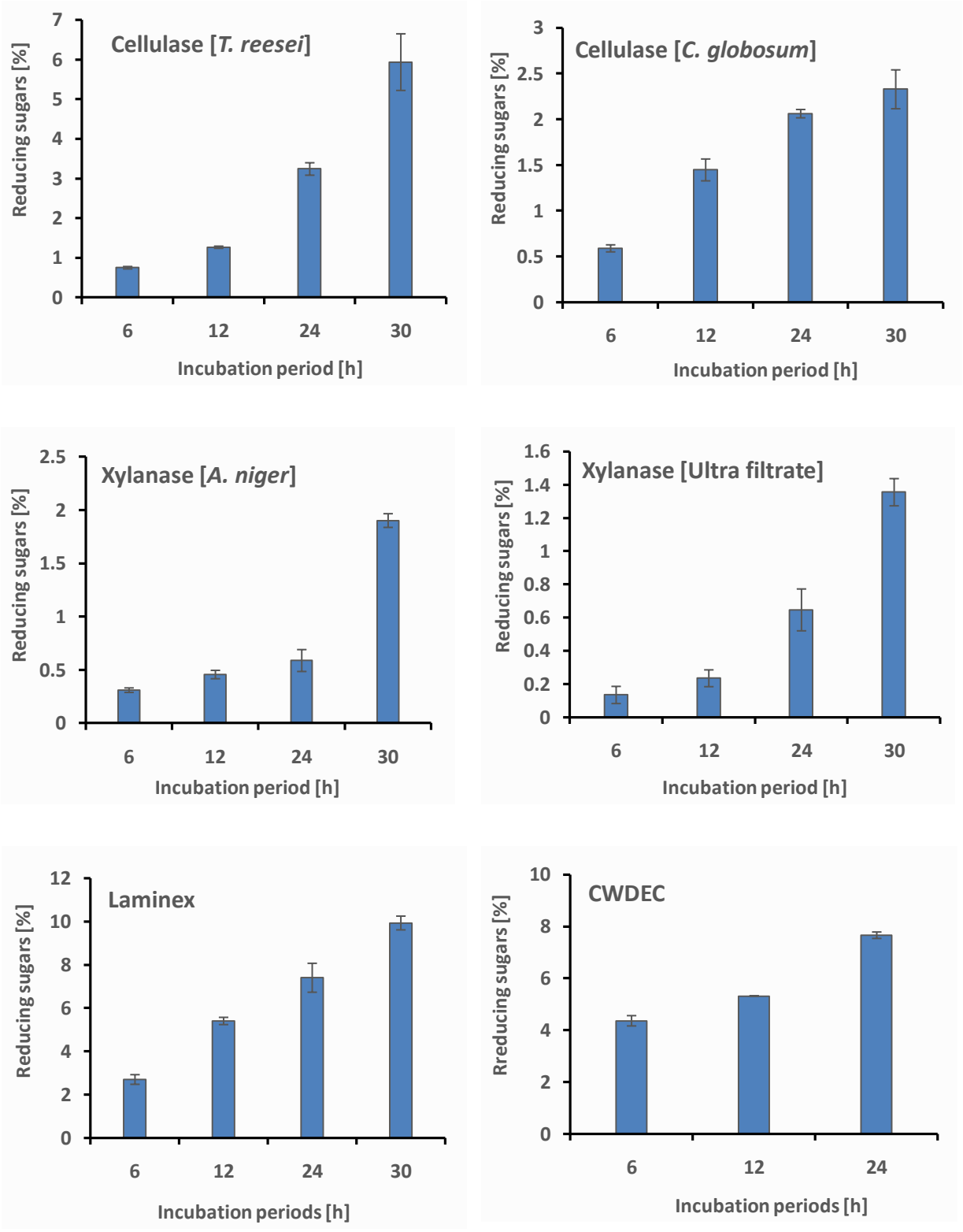


Fig.33. Application of cellulases and xylanases in corn stover degradation

A maximum of 10% reducing sugars were detected after 30 hours of incubation. Using the cellulases from *T. reesei*, and *C. globosum* and CWDEC, about 6.0, 2.5, and 8.0% reducing sugars were formed, respectively.

On the other hand, the xylanases (*A. niger* and UF) produced the lowest amounts of reducing sugars from corn stover (2.0 and 1.4%, respectively). These results are similar to those reported by **Berlin *et al.*, (2007).** They found that ability of a commercial *T. reesei* cellulase preparation to hydrolyze cellulose of pretreated corn stover was significantly improved by supplementation with three types of crude commercial enzyme preparations enriched in xylanase, pectinase, and β-glucosidase. It was suggested that so-called 'accessory' enzymes such as xylanase and pectinase stimulate cellulose hydrolysis by removing non-cellulosic polysaccharides that coat cellulose fibers. Moreover, according to the type of pretreatment applied, biomass of the same type may require different enzyme blends to be efficiently hydrolyzed **(Barros *et al.*, 2010).**

6.2. Application of peroxidases in corn stover degradation

The biodegradation of plant biomass is a slow process as lignin restricts the access of hydrolytic enzymes to the polysaccharide components. Therefore, pretreatment of lignocellulotic materials with lignin degrading enzymes, such as peroxidases were employed to improve the accessibility of lignocelluloses yielding lignocellulosic materials that are much more susceptible to cellulases and hemicellulases attack.

6.2.1. Application of MsP1 in corn stover degradation

MsP1 represents a peroxidase originally derived from the basidiomycete *Marasmius scorodonius* and heterologously expressed in *A. niger*. About 1.5 units of MsP1 were used for the degradation of corn stover lignin, followed by treatment with cellulases and hemicellulases (Fig.34); the total consumption of hydrogen peroxide is summarized in Table 15.

Results show that higher concentrations of reducing sugars were released after pretreatment with peroxidase (MsP1) than untreated ones. This may be attributed to the direct attack of MsP1 on the lignin fraction of corn stover. Also lignin is the most recalcitrant biomaterial for degradation because of its highly ordered crystalline structure. Enzymes are preferred for lignin degradation since they operate under mild reaction conditions, do not produce undesirable side-products and are environmentally compatible **(Howard *et al.*, 2003).**

Two families of lignolytic enzymes are widely considered to play a key role in the enzymatic degradation of lignin: phenol oxidase (laccase) and peroxidases **(Krause *et al.*, 2003; Malherbe and Cloete, 2003)**. Moreover, **Martinez *et al.*, (2005)** stated that peroxidase was described as true ligninases because of its high redox potential that degrades non-phenolic lignin units (up to 90% of the polymer).

Data also show that the amounts of released reducing sugars increased with increasing the incubation period up to 24 h. Laminex and CWDEC enzymes (commercial cellulases and xylanases) showed the highest catalytic activity to hydrolyze polymeric carbohydrates of corn stover compared with cellulase of either *C. globosum* or *T. reesei*. These results may be explained by the fact that Laminex and CWDEC both exhibit cellulase and hemicellulase activity.

These results are in accordance with those obtained by **Ohgrena *et al.*, (2007)** who recorded that high glucose yields from corn stover are obtained if xylanases are used to supplement cellulases during hydrolysis. Xylanases hydrolyse residual hemicellulose, thereby improving the access of enzymes to cellulose**.**

Enzymatic hydrolysis of pretreated lignocellulose is more challenging than enzymatic hydrolysis of pure cellulose because any remaining lignin and residual hemicellulose could adsorb cellulase components and thereby block or impede cellulose hydrolysis **(Berlin *et al.*, 2007)**

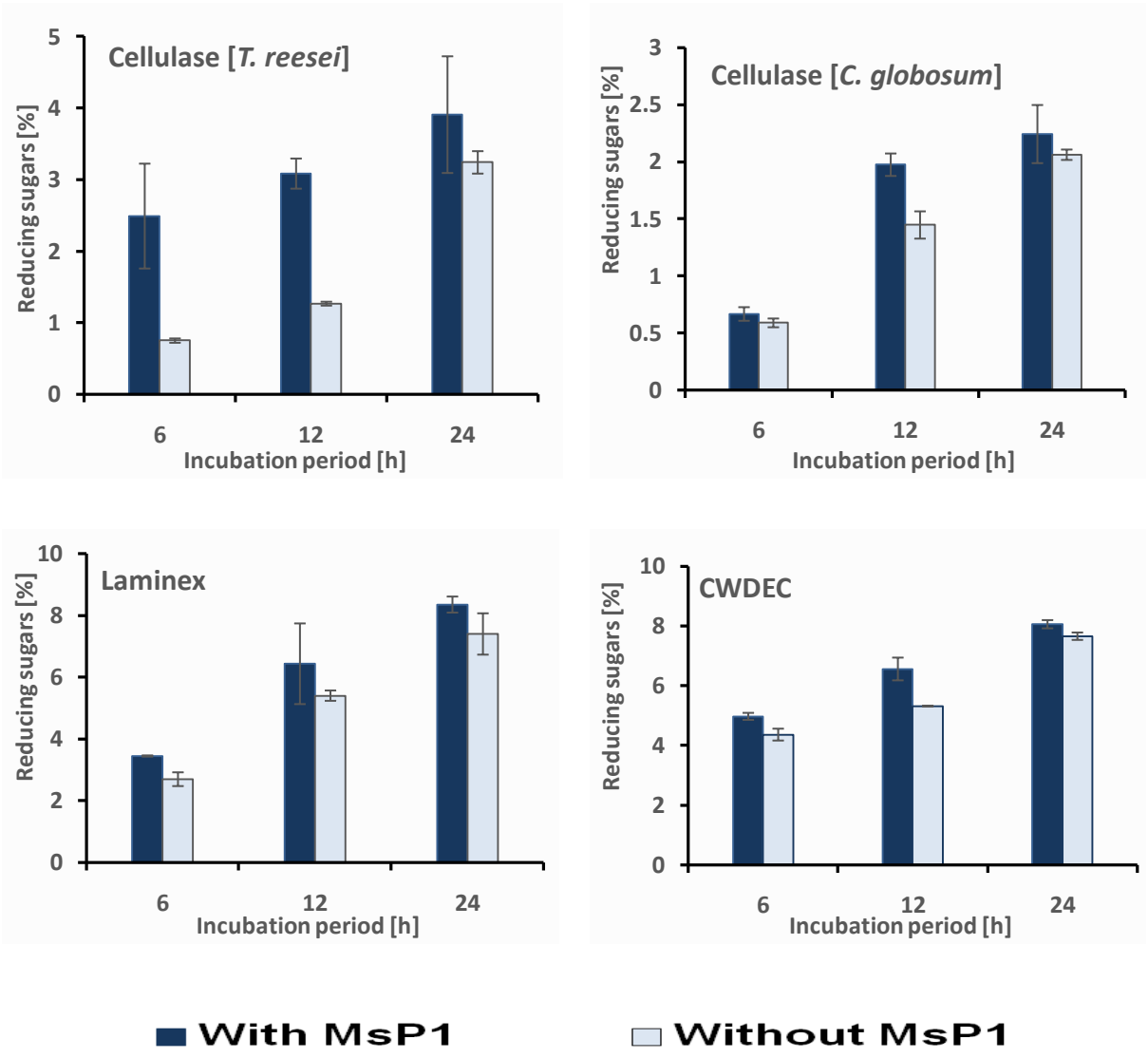


Fig.34. Application of MsP1 enzymes in corn stover degradation

Table 15. Total consumption of hydrogen peroxide

Treatment	Total consumption of 20 mM H_2O_2
MsP1 + Cellulase (*T. reesei*)	900 µl
MsP1 + Cellulase (*C. globosum*)	1000 µl
MsP1 + laminex	1000 µl
MsP1 + CWDEC	900 µl

6.2.1.1. Determination of the optimum pH of MsP1 enzyme

Since MsP1 was capable to degrade lignin, it was of interest to determine the optimum conditions for maximum MsP1 activity. The optimum pH of MsP1 was investigated at pH values from 3–6. The obtained results are graphically illustrated in Fig. (35).

pH 6 was not suitable for MsP1 as the activity decreased with increasing the pH value. Maximum activity of MsP1was found at pH 3.

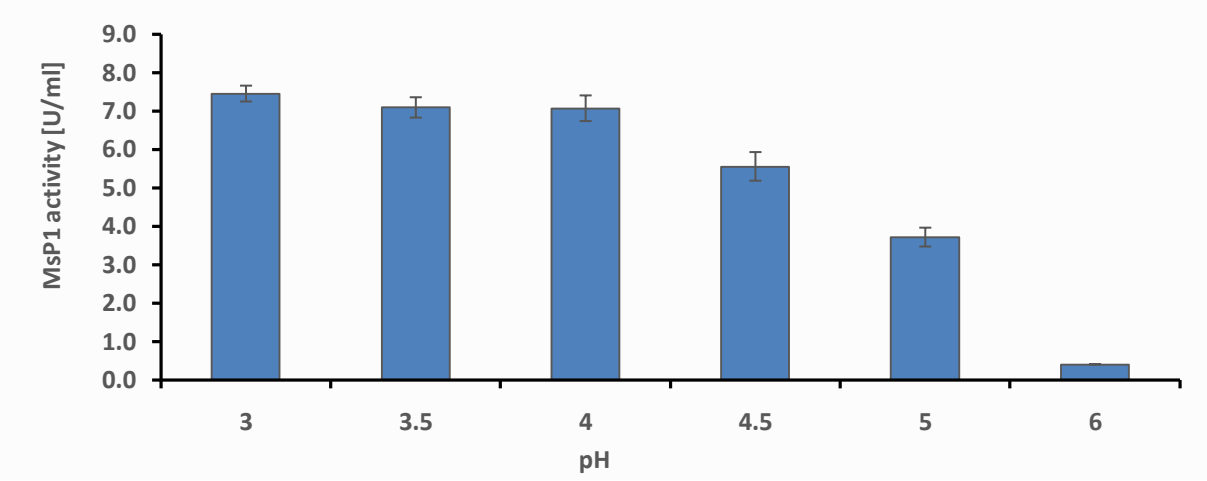


Fig. 35. Determination of the optimum pH of MsP1

6.2.1.2. Application of MsP1 at different pH values and laminex in corn stover degradation

MsP1 was employed for the degradation of corn stover lignin at pH 3.0, 4.0, and 5.0 in 50 mM Na-malonate buffer. Then, laminex (the highest degrading enzyme) was applied to hydrolyze the cellulose and hemicellulose fraction in corn stover. For the activity of MsP1 enzyme, hydrogen peroxide was added periodically after its complete consumption (Fig. 36); the total consumption of hydrogen peroxide is summarized in Table 16.

Obtained results show that though only minor differences in the amounts of produced reducing sugars were observed, the consumption of hydrogen peroxide increased with increasing the pH.

Generally, the released amounts of reducing sugars from corn stover increased with increasing the incubation period up to 24 hours for all pH values either with untreated and treated corn stover with MsP1.

Effect of laminex enzyme on corn stover, which treated with MsP1, gave high values of reducing sugars more than that untreated with MsP1. The lowest reducing sugars percentage was obtained when MsP1 was used at pH 5. While, at pH 4 and after 6 h. MsP1 showed the highest catalytic activity to degrade corn stover yielding lignocellulosic materials that are much more susceptible to laminex enzyme attack. Moreover, after 12 and 24 h incubation, MsP1 showed the highest catalytic activity at pH 3 to degrade corn stover.

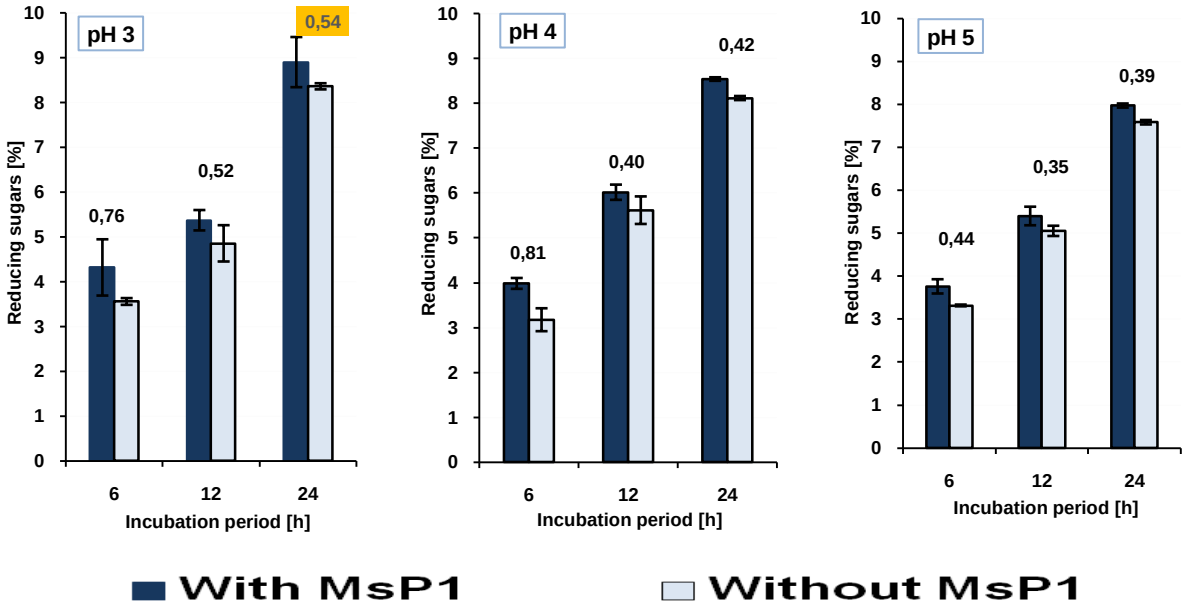


Fig. 36. Application of MsP1 and laminex in corn stover degradation at different pH values

Table 16. Total consumption of hydrogen peroxide

Treatment	Total consumption of 20 mM H_2O_2
MsP1, pH 3	700 µl
MsP1, pH 4	800 µl
MsP1, pH 5	1100 µl

6.2.1.3. Application of different amounts of MsP1 in corn stover degradation

Different amounts of MsP1 were examined for the degradation of corn stover lignin. 1.5, 3.0 and 6.0 units of MsP1 enzyme were applied using 50 mM Na-acetate buffer at pH 3.0. For the activity of MsP1, hydrogen peroxide was periodically added after its complete consumption. Then, laminex enzyme was applied to hydrolyze cellulose and hemicellulose of corn stover (Fig. 37). The total consumption of hydrogen peroxide was summarized in Table 17.

The consumption of hydrogen peroxide increased with increasing enzyme concentrations and reached 4,300µl of hydrogen peroxide when 6.0 units of MsP1 were used. This high value of hydrogen peroxide consumption could be attributed to the high MsP1 catalytic activity against lignin of corn stover.

The lowest lignin degradation was obtained when 1.5 units of MsP1 were used. While, the highest degradation rates were found after 12 and 24 hours incubation when 6.0 and 3.0 units of MsP1 were used, respectively. These results indicate that the maximum level of lignin degradation can be reached after 24 hours with using the half unit's level of MsP1.

Table 17. Total consumption of hydrogen peroxide

MsP1 (Units)	Total consumption of 20 mM H_2O_2
1.5	1200 µl
3.0	3600 µl
6.0	4300µl

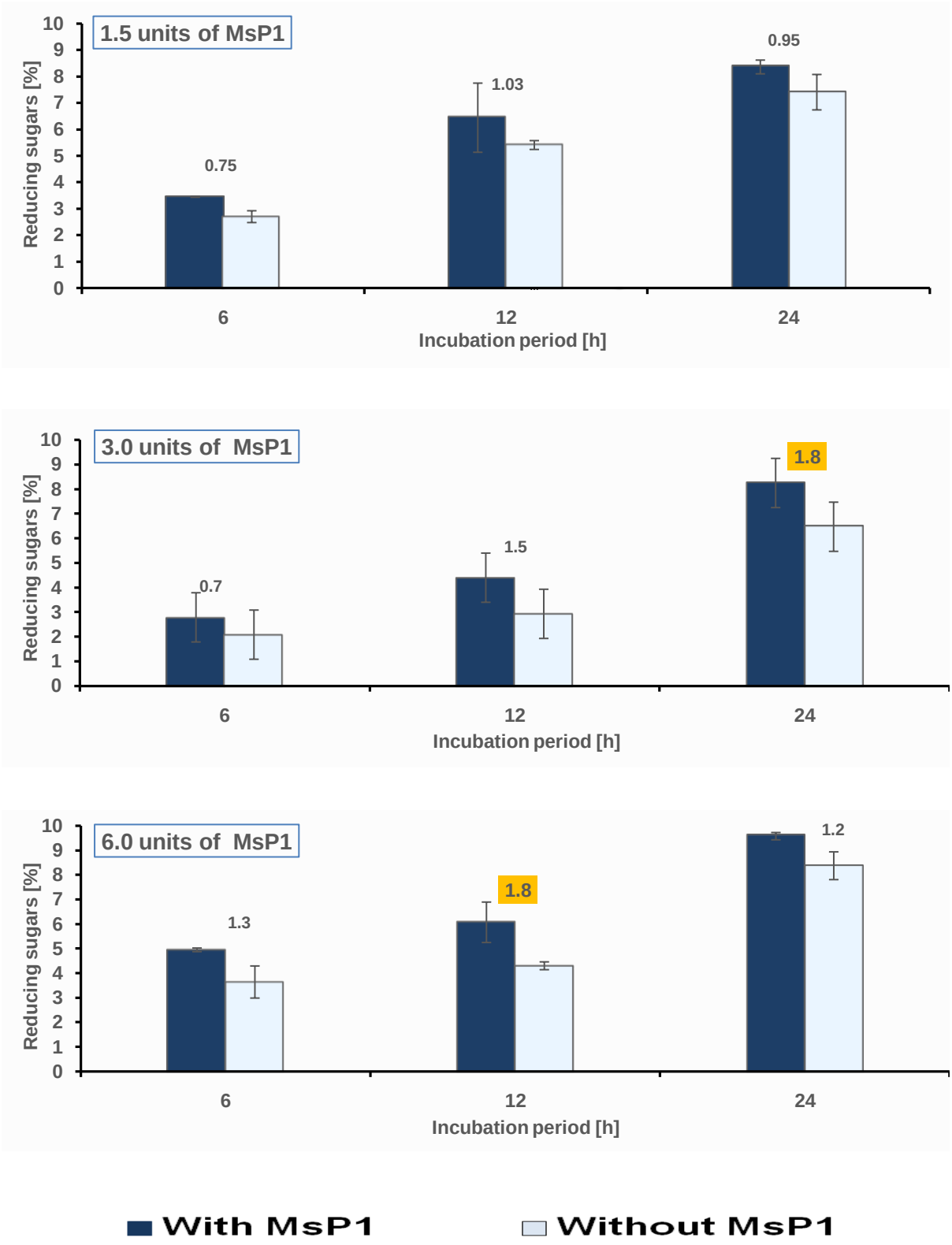


Fig. 37. Application of MsP1 at different levels in corn stover degradation

6.2.2. Application of MsP1 and Manganese-peroxidase in corn stover degradation

MsP1 was capable of lignin degradation, yielding lignocellulosic materials more susceptible to cellulase and hemicellulase enzymes attack. Therefore, it was of interest to investigate the effect of other enzymes of the peroxidase family as manganese-peroxidase (Mn-P) on lignin degradation.

6.2.2.1. Determination of the optimum pH of Mn-Peroxidase

The activity increased with increasing pH value up to 5.0 (Fig. 38). Higher pH values were not considered as the enzyme should be used to synergistically increase the activity of MsP1.

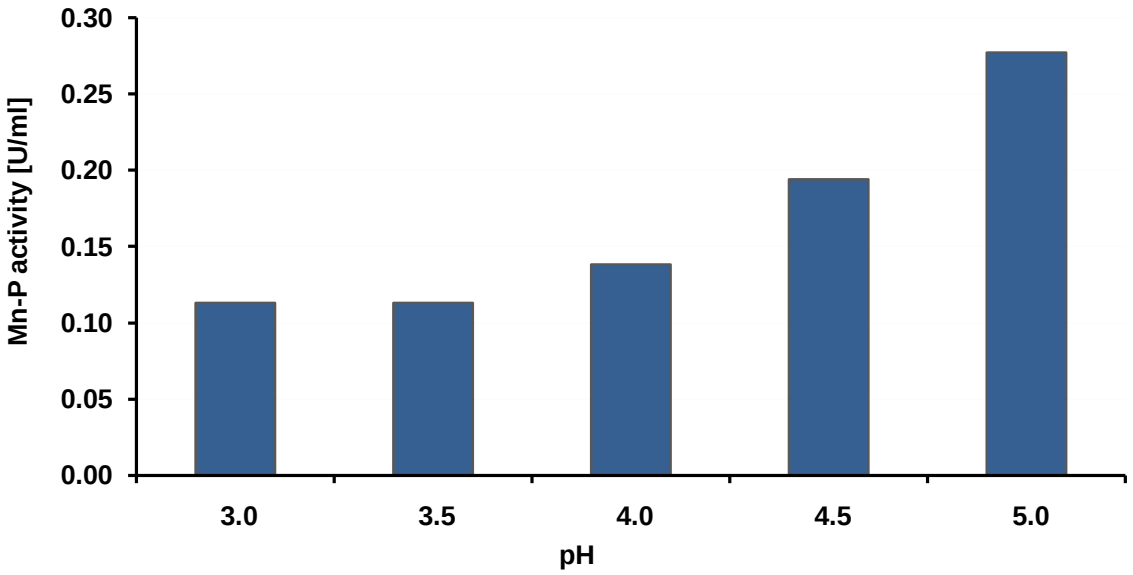


Fig. 38. Determination of the optimum pH of Mn-peroxidase

6.2.2.2. Application of MsP1 and manganese-peroxidase (MnP) in corn stover degradation

The effect of MsP1 and Mn-P on lignin degradation and the enzymatic hydrolysis of cellulose and hemicellulose by laminex enzyme were investigated. MsP1 and Mn-P were applied to corn stover individually or combined. Concerning the combination of MsP1 and Mn-P, two pH values (3.5 and 5.0) were used.

All treatments were incubated at 30 ºC for 24 hours under shaking. For the activity of MsP1 and MnP, 20 mM H_2O_2 was periodically added. Moreover, manganese chloride was added to the reaction mixture to reach 2mM as final concentration which is needed for Mn-P activity. After treatment with peroxidases, laminex enzyme was used to hydrolyse the cellulose and hemicellulose fractions (Figs. 39a, 39b, Table 18).

Compared to the application of MsP1 individually, the consumption of H_2O_2 was lower with Mn-P individually or combined with MsP1.

In all experiments, the amounts of released reducing sugars increased with increasing the incubation period until 24 hours. The highest amounts of reducing sugars were formed by laminex after pretreatment of corn stover with MsP1. On the other hand, the lowest yields of reducing sugars were obtained with Mn-P treated corn stover.

Compared to the controls, the combination of MsP1 and Mn-P resulted in lower yields of reducing sugars than at both pH values.

Generally, the beneficial effect of the pretreatment with peroxidase was recorded by **Martinez *et al.* (2005)** who stated that peroxidase was described as true ligninases because of its high redox potential. Mn-P generates Mn^{3+}, which acts as a diffusible oxidizer on phenolic or non-phenolic lignin units via lipid peroxidation reactions.

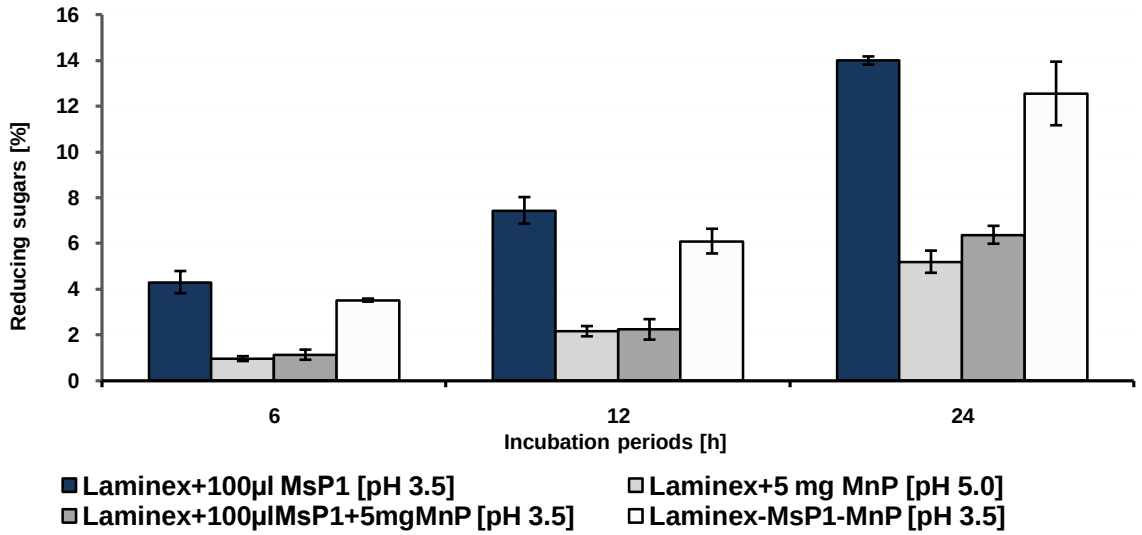


Fig. 39a. Effect of MsP1 and Mn-P on the degradation of lignocellulose at pH 3.5

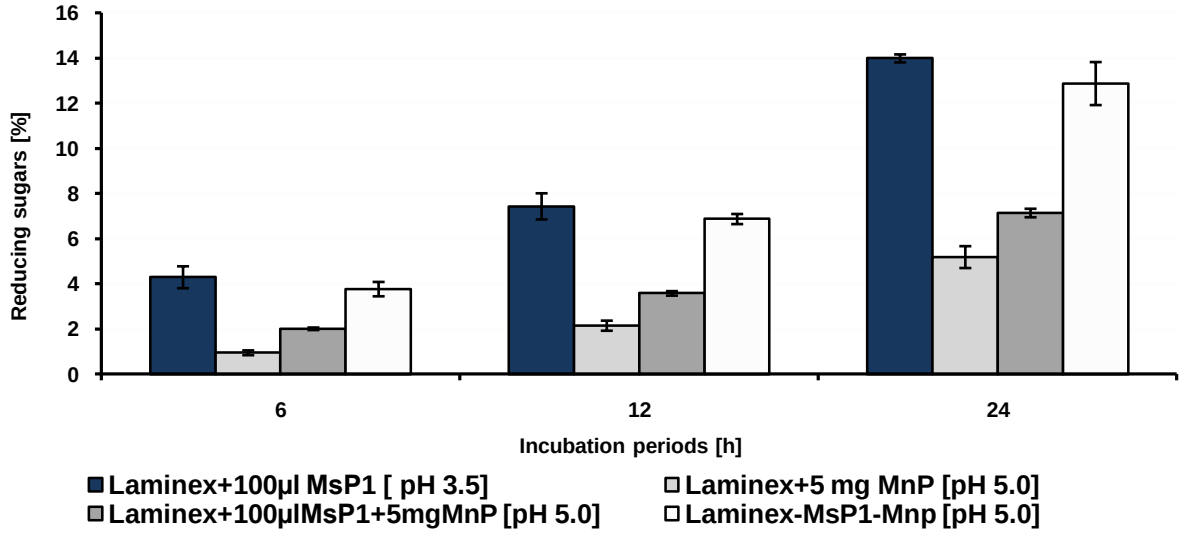


Fig. 39b. Effect of MsP1 and Mn-P on the degradation of lignocelluloses at pH 5.0

Table 18. Total consumption of hydrogen peroxide

Treatment	Total consumption of 20 mM H_2O_2
MsP1	1200 µl
MnP	300 µl
MsP1+ MnP pH 3.5	300 µl
MsP1+ MnP pH 5.0	300 µl

SUMMARY

5. SUMMARY

This work deals with isolation and screening of cellulase producing microorganisms. Moreover, the optimization of cellulase production conditions was studied to reach the maximum enzyme production. The produced cellulase enzyme as well as some other lignocellulose degrading enzymes were applied in some lignocellulosic materials (corn stover) degradation.

From fifty isolates, two isolates showed maximum zones of clearance around their colonies and were thus found to have the highest cellulase activity. These two isolates which designated as A7 and B25 were selected and identified as *Streptomyces griseus* and *Paenibacillus polymyxa*, respectively. In addition, ten different microbial strains were tested for their cellulase and protein production capability, *T. viride* and *C. globosum* were selected for further cellulase production studies.

Reese and Mandels basal medium was the best medium for growth, protein synthesis and cellulase production for all of the investigated strains. glucose was found to induce cellulase formation and protein synthesis of *C. globosum* and *T. viride* when used at concentrations of 0.10 and 0.15%, respectively. 2.0% cellulose was the optimum concentration for enzyme activity and protein synthesis of *T. viride* and *S. griseus*. While, the maximum cellulase activity of *P. polymyxa* (0.26 U/ml) was found at 1.0% cellulose. Among the tested organic and inorganic nitrogen sources, urea was found to be optimal for growth, protein synthesis, and cellulase production by *C. globosum* and *T. viride*. Yeast extract was shown to be the most suitable nitrogen source for *Paenibacillus polymyxa* and *Streptomyces griseus*.

Initial pH 6 was the optimum for *C. globosum* and *S. griseus*, while pH 5 was found to be optimal for growth, protein synthesis and cellulase production by *T. viride*. Extracellular cellulase activity of *P. polymyxa* reached a maximum of 0.51 U/ml at pH 7. The maximum cellulase activities of *C. globosum* and *T. viride* was obtained after 8 days of fermentation. The highest cellulase activities and protein concentrations of *P. polymyxa* were obtained after 4 and 10 days, respectively. Six days of fermentation were best suitable for the formation of cellulase by *S. griseus*.

Cellulase activities of *C. globosum* and *S. griseus* reached maximum activities of 1.45 and 1.06 U/ml, respectively, at 8% (v/v) inoculum volume. An inoculum volume of 5% was more favorable for formation of cellulase by *T. viride* and *P. polymyxa* (1.16 and 0.55 U/ml) respectively.

The cellulase activity increased with increasing concentrations of Tween 80 up to 1.5 ml/L for *T. viride* and *P. polymyxa* with maximum activities of 1.29 and 0.64 U/ml, respectively. In addition, a concentration of 1.0 ml/L was found to be the most suitable for cellulase formation by *C. globosum* and *S. griseus*.

The culture supernatant of *C. globosum* was concentrated by ultrafiltration. Cellulase and protein yields after concentration were 60.2 and 35.6%, respectively. The obtained specific activity was 2.17 U/mg.

Kinetic studies of cellulase produced by *C. globosum* revealed 2.27 mg/ml as K_m and 0.1194 µmol/ml/min as V_{max}. Moreover, pH 5.0 and 65 °C were found to be optimum for the activity. Cellulase of *C. globosum* retained about 60 % of its activity when incubated at 65 °C for 2 h. Moreover, the enzyme showed high stability for pH 5 when incubated for 12h. An isoelectric point (pI) around 4.5 was detected for cellulase of *C. globosum*. The molecular weight was calculated to be about 40 KDa.

The optimum temperature for the cellulase from *T. reesei* was 65 °C, while 60 °C was optimum for the xylanase activities of laminex and CWDEC.

Cellulase from *T. reesei* retained about 85and 19% of the initial activity after 2 and 12 hours, respectively. While, laminex as cellulase retained 89 and 80% of the initial activity after 2 and 12 h incubation at its optimal temperature, respectively.

pH 4.0 was favorable for the activity of the cellulase from *T. reesei*, and for laminex as cellulase. CWDEC as cellulase and xylanase exhibited the maximum activity at pH 3.0. All of the enzymes tested were stable for 12 h. when incubated at their pH optima.

Application of cellulase, produced from *C. globosum*, on corn stover was assessed compared with cellulase from (*T. reesei*) , xylanase (*A. niger* and the UF), laminex and CWDEC enzymes. All enzymes gave higher concentrations of reducing sugars when corn stover was pretreated with peroxidase (MsP1) than untreated one. Laminex enzyme showed the highest catalytic activity to hydrolyze polymeric carbohydrates of corn stover. Maximum level of lignin degradation can be reached after 24 h. with using 3 units of MsP1.

The highest amounts of reducing sugars were formed by laminex after pretreatment of corn stover with MsP1. On the other hand, the lowest yields of reducing sugars were obtained with Mn-P treated corn stover.

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ARABIC

SUMMARY

الملخص العربي

استخدام الإنزيمات الميكروبية لتحليل اللجنوسليولوز

تهدف هذه الدراسة إلي الحصول علي كائنات حية دقيقة ذات قدرة عالية علي إنتاج إنزيم السليولين ودراسة الظروف المثلي للوصول إلي اعلي إنتاجية من الإنزيم وكذلك استخدام بعض الإنزيمات المحللة للجنوسليولوز لتحليل مطحون مخلفات نبات الذرة.

وخلال هذه الدراسة تم عزل خمسين عزلة ميكروبية لها القدرة علي إنتاج إنزيم السليوليز بكميات متفاوته. وكانت العزلتين A7 و B25 الاعلي إنتاجا لإنزيم السليوليز لذلك تم اختيارهما وتعريفهما فكانت السلالة الاولي هي *Streptomyces griseus* والاخري كانت *Paenibacillus polymyxa* علي التوالي.

بالإضافة إلي ذلك تم الحصول علي سلالات ميكروبية من أماكن مختلفة وباختبار قدرتهم علي إنتاج إنزيم السليوليز أظهرت النتائج أن كل من السلالة الفطرية *Trichoderma viride* و *Chaetomium globosum* كانتا الاعلي في إنتاج الإنزيم. لذلك تم اختيارهما لاستخدامها في الدراسات اللاحقة.

وبدراسة انسب البيئات الغذائية لإنتاج إنزيم السليوليز بواسطة السلالات المنتخبة أظهرت النتائج المتحصل عليها أن بيئة Reese and Mandels basal medium كانت أفضل البيئات الغذائية لنمو الميكروب و إنتاج إنزيم السليوليز و ذلك لجميع السلالات تحت الدراسة . كما أوضحت النتائج ان الجلوكوز شجع علي إنتاج إنزيم السليوليز من السلالة الفطرية *T. viride* و *C. globosum* عند استخدامه بتركيزات 0,15 و 0,10% علي التوالي. في حين كان التركيز الأمثل من السليولوز للحصول علي اعلي نشاط إنزيمي من السلالتين السابقتين هو تركيز 2% . و من الجدير بالذكر انه عند دراسة تأثير مصادر النيتروجين العضوية و المعدنية علي نمو الميكروبات المستخدمة و إنتاجها لإنزيم السليوليز أظهرت النتائج أن اليوريا هى المصدر المناسب للنيتروجين بالنسبة للسلالتين الفطريتين *T. viride* و *C. globosum* . في حين كان مستخلص الخميرة هو أفضل مصادر النيتروجين لإنتاج إنزيم السليوليز من السلالتين *P. polymyxa* و *S. griseus* .

و عند دراسة تأثير pH البيئة الغذائية علي إنتاج الإنزيم من السلالات الميكروبية المختارة أظهرت النتائج إن امثل درجة pH للسلالتين *C. globosum* و *S. griseus* هو pH 6. في حين كان pH 5 هو الأمثل لإنتاج إنزيم السليوليز من السلالة *T. viride* و كانت درجة ال pH 7 هي المثلي للسلالة *P polymyxa*.

و من الجدير بالذكر إن لفترات التحضين تأثير علي إنتاج و نشاط إنزيم السليوليز فقد أوضحت النتائج إن فترة التحضين لمدة 8 أيام أدت إلي الحصول علي اعلي نشاط من إنزيم السليوليز المنتج من السلالتين الفطريتين *T. viride* و *C. globosum* . وأظهرت النتائج أن اعلي نشاط لإنزيم السليوليز و تركيز البروتين في البيئة الغذا ية للسلالة *P. polymyxa* كان بعد فترة تحضين 4 و 10 أيام علي التوالى. في حين كان التحضين لمدة 6 أيام هي أفضل فترات التحضين لإنتاج إنزيم السليوليز من السلالة *S. griseus* .

و عند دراسة تأثير كمية اللقاح علي نشاط إنزيم السليوليز فقد توصلت النتائج ان اضافة اللقاح بتركيز 8 % أدت إلي الحصول الي اعلي نشاط من الإنزيم المنتج من *C. globosum* و *S. griseus* في حين كان إضافة اللقاح بنسبة 5% لكلا من *T. viride* و *P. polymyxa* هي الأفضل للحصول علي اعلي نشاط إنزيمي.

و من الجدير بالذكر انه يزداد نشاط إنزيم السليوليز بزيادة تركيز Tween 80 عن 5 , 1 ملي/لتر لكلا من *T. viride* و *P. polymyxa* حيث كان اعلي نشاط للإنزيم هو 29, 1 و 64, 0 وحدة نشاط انزيمي لكل ملي . بالاضافة لذلك فان تركيز ال Tween 80 بمقدار 1 ملي/ لتر كان الافضل لانتاج انزيم السليوليز من *C. globosum* و *S. griseus*.

عند تركيز راشح مزرعة الفطر *C. globosum* بواسطة الترشيح الفائق زاد محصول الانزيم و البروتين بنسبة 2, 60 % و 35,6 % علي التوالي و النشاط المتخصص للإنزيم كان 2,17 وحدة / ملليجرام .

و خلال ه ذه الدراسة تم دراسة النشاط الحركي لإنزيم السليوليز المنتج بواسطة *C. globosum* حيث كانت السرعة القصوي للنشاط الانزيمي (V_{max}) هي 0,1194 ميكرومول/ ملي/ق و ثابت ميكالس (K_m) هو 2,27 مليجرام/ملي. و كان أفضل pH هو 5 و درجة حرارة هي 65° م. كما وجد أن إنزيم السليوليز المنتج بواسطة *C. globosum* يحتفظ بـ 60 % من نشاطه عند تحضينه على 65° م لمدة ساعتين. و علاوة علي ذلك اظهر ثبات عالي في نشاطه عند تحضينه علي درجة pH 5 لمدة 12 ساعة. و كانت نقطة التعادل الكهربي لهذا الإنزيم حول 4,5 أما الوزن الجزيئي فكان 40 كيلودالتون.

كانت درجة الحرارة المثلي لن شراط انزيم السليوليز المنتج من *T. reesei* هي 65° م و استرجع هذا الإنزيم حوالي 85% و 19% من النشاط الأصلي عند التحضين علي 65° م لمدة 2 و 12 ساعة علي التوالي . يعتبر pH 4 هو الأمثل لنشاط إنزيم السليوليز المنتج من *T. reesei* و اللامينيكس كمصدر للسليوليز. بينما كان pH 3 هو الأمثل لنشاط الإنزيم المحلل للجدر الخلوية . كل الإنزيمات تحت الدراسة أظهرت ثبات لدرجة ال pH المثلي حتي 12 ساعة تحضين علي درجة الحرارة المثلي.

تم تطبيق إنزيم السليوليز المنتج من *C. globosum* علاوة علي مجموعة إنزيمات أخري محللة للجنوسليولوز مثل السليوليز (منتج من *T. reesei*) و زيلانيز (منتج من *A. niger*) زيلانيز المركز بالترشح و اللامينيكس و إنزيم (CWDEC) المحلل للجدر الخلوية علي مخلف نبات الذرة. أظهرت النتائج أن كل المعاملات الإنزيمية أعطت تركيزات عالية من السكرات المختزلة في حالة معاملة مخلف نبات الذرة معاملة مبدئية بواسطة إنزيم البيروكسيديز (MsP1). كما اظهر إنزيم اللامينيكس اعلي معدل تحليل للمخلف . أما أقصي معدل تحليل للجنين يمكن الحصول علية بعد 24 ساعة باستخدام 3 وحدات من انزيم البيروكسيديز (Msp1) . أظهرت النتائج أفضلية إنزيم البيروكسيديز(Msp1) عن إنزيم البيروكسيديز(Mn-P) و ذلك من خلال كمية السكريات المختزلة المنتجة عند معاملة مخلف نبات الذرة بواسطة انزيم اللامينيكس.