إقـــــرار

أنا الموقع أدناه مقدم الرسالة التي تحمل العنوان:

Optimization of cellulase enzyme production from *Trichoderma* isolates under submerged fermentation

الظروف المثلي لإنتاج إنزيم السيلوليز من عزلات الترايكوديرما بواسطة التخمر المغمور

أقر بأن ما اشتملت عليه هذه الرسالة إنما هو نتاج جهدي الخاص، باستثناء ما تمت الإشارة إليه حيثما ورد، وإن هذه الرسالة ككل أو أي جزء منها لم يقدم من قبل لنيل درجة أو لقب علمي أو بحثي لدى أي مؤسسة تعليمية أو بحثية أخرى.

DECLARATION

The work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification

Student's name:	اسم الطالب: رهام جواد فايز بدير
Signature:	التوقيع:
Date:	التاريخ: 21 / 11 / 2015

Islamic University-Gaza

Deanship of Graduate Studies

Biological Sciences Master Program

Optimization of cellulase enzyme production from *Trichoderma* isolates under submerged fermentation

الظروف المثلي لإنتاج إنزيم السيلوليز من عزلات الترايكوديرما بواسطة التخمر المغمور

Prepared By:

Rham Jawad Budeir

Student ID: 220110393

Supervisor

Dr. Tarek Elbashiti

Assoc. Prof. of Biotechnology

Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Biological Sciences in Microbiology.

2015 – هـ 1436

بسم الله الرحمن الرحيم

الجامعة الإسلامية – غزة
The Islamic University - Gaza

مكتب نائب الرئيس للبحث العلمي والدراسات العليا هاتف داخلي 1150

الرقم Ref ج س غ/35/
التاريخ Date 2015/11/16م

نتيجة الحكم على أطروحة ماجستير

بناءً على موافقة شئون البحث العلمي والدراسات العليا بالجامعة الإسلامية بغزة على تشكيل لجنة الحكم على أطروحة الباحثة/ **رهام جواد فايز بدير** لنيل درجة الماجستير في **كلية العلوم** قسم **العلوم الحياتية – أحياء دقيقة** وموضوعها:

الظروف المثلى لإنتاج انزيم السيلوليز من عزلات Trichoderma بواسطة التخمر المغمور

Optimization of cellulose enzyme production from Trichoderma isolates under submerged fermentation

وبعد المناقشة العلنية التي تمت اليوم الاثنين 04 صفر 1437هـ، الموافق 2015/11/16م الساعة الواحدة ظهراً بمبنى اللحيدان، اجتمعت لجنة الحكم على الأطروحة والمكونة من:

د. طارق عبد القادر البشيتي	مشرفاً و رئيساً	.....
د. عبود ياسر القيشاوي	مناقشاً داخلياً	.....
د. ناهض علي اللحام	مناقشاً خارجياً	.....

وبعد المداولة أوصت اللجنة بمنح الباحثة درجة الماجستير في **كلية العلوم**/ قسم **العلوم الحياتية – أحياء دقيقة**.

واللجنة إذ تمنحها هذه الدرجة فإنها توصيها بتقوى الله ولزوم طاعته وأن تسخر علمها في خدمة دينها ووطنها.

والله ولي التوفيق،،،

نائب الرئيس لشئون البحث العلمي والدراسات العليا

أ.د. عبدالرؤوف علي المناعمة

P.O. Box 108, Rimal, Gaza, Palestine fax: +970 (8) 286 0800 فاكس Tel: +970 (8) 286 0700 هاتف ص.ب. 108 الرمال، غزة، فلسطين
public@iugaza.edu.ps www.iugaza.edu.ps

بسم الله الرحمن الرحيم

(قَالُواْ سُبْحَانَكَ لاَ عِلْمَ لَنَا إِلاَّ مَا عَلَّمْتَنَا إِنَّكَ أَنتَ الْعَلِيمُ الْحَكِيم)

البقرة(32)

صدق الله العظيم

Optimization of cellulase enzyme production from *Trichoderma* isolates under submerged fermentation

Abstract

Cellulases are enzymes that analyze the biomass of the cellulose and it is used in many fields such as the paper industry, fabric yarn, manufacture of detergents and laundry.

Cellulase enzyme is produced by micro-organisms that grow on cellulosic materials.

The main purpose of this study is to find out the optimal conditions for the production of cellulase enzyme by the local *Trichoderma* isolates. In this study, *Trichoderma* was isolated from air and soil of the Islamic University gaza campus male and female. Three Trichoderma species were isolated and identified as following *Trichoderma harizanium*, *Trichoderma reesi* and *Trichoderma viride*. Congo red and CMC have been used to know the best species of *Trichoderm* have ability to produce cellulase enzyme and *Trichoderma viride* was the best for cellulase production. The produced amount of glucose was examined by cellulase enzyme that produced by *Trichoderma viride* using glucose kit assay. The optimum conditions for cellulase enzyme production were found. The required carbon and nitrogen sources and the environmental conditions such as pH and temperature were optimized.

The results showed that the best activity for cellulase enzyme is at 7 days in basal salt media where the produced amount of glucose reached (30.8 mmol / L), pH of 5.5 and a temperature of 30 °C.

The best source of carbon and nitrogen to produce cellulase enzyme are 1% sucrose and 1% yeast extract respectively. *Trichoderma viride* is considered a unique source for the production of cellulase enzyme in large quantities compared to other *Trichoderma* species.

Keywords: Cellulases, *Trichoderma harizanium*, *Trichoderma reesi*, *Trichoderma viride*, glucose, cellulose, basal salt media.

الظروف المثلي لإنتاج إنزيم السيلوليز من عزلات الترايكوديرما بواسطة التخمر المغمور

الملخص

السليلوزات هي إنزيمات تقوم بتحليل الكتلة الحيوية السليلوزية، ويتم استخدامها في مجالات عديدة مثل صناعة الورق وغزل النسيج وصناعة المنظفات وغسيل الملابس ...الخ، إنزيم السيلوليز يتم انتاجه من قبل الكائنات الحية الدقيقة التي تنمو علي المواد السليلوزية.

الغرض الأساسي لهذه الدراسة هو معرفة الظروف المثلي لإنتاج إنزيم السيلوليز بواسطة الترايكوديرما المعزولة، في هذه الدراسة تم عزل الترايكوديرما من الهواء و التربة من حرم الجامعة الإسلامية غزة للطلاب والطالبات. تم عزل ثلاثة أنواع من الترايكوديرما وهي كالتالي *Trichoderma harizanium*، *Trichoderma reesi*، *Trichoderma viride*.

تم استخدامCMC و Congored لمعرفة أفضل أنواع الترايكوديرما المعزولة لإنتاج إنزيم السيلوليز فكان أفضلها *Trichoderma viride*.

تم عمل فحص لكمية الجلوكوز المنتجة بواسطة إنزيم السيلوليز المنتج بواسطة *Trichoderma viride* باستخدام Glucose kit assay، لإنتاج إنزيم السيلوليز بكميات كبيرة كانت أفضل المعايير المستخدمة مثل مصدر الكربون والنيتروجين ودرجة الحرارة ودرجة الحموضة كل في اطار مفصل.

ولقد دلت النتائج أن أفضل نشاط لإنزيم السيلوليز عند 7 أيام في وسط ملحي أساسي، حيث بلغت كمية الجلوكوز المنتجة (30.8 mmol/L)، درجة حموضة 5.5 و درجة حرارة 30 درجة مئوية.

أفضل مصدر كربون و نيتروجين لإنتاج إنزيم السيلوليز هما 1 % sucrose و1% yeast extract علي التوالي.

تعتبر *Trichoderma viride* مصدر مميز لإنتاج إنزيم السيلوليز بكميات كبيرة مقارنة بالأنواع الأخري من الترايكوديرما.

كلمات مفتاحية: السيلوليز، *Trichoderma harizanium*، *Trichoderma reesi*، *Trichoderma viride*، الجلوكوز، السيلولوز، وسط ملحي أساسي.

DEDICATION

To whom drank the empty cup to irrigate me a drop of love

To whom reaped the thorns from my way to pave me the science road

To whom his fingers were exhausted to bring us a moment of happiness

To the big heart (my dear father)

To whom suckled me love and compassion

To the symbol of love and a healing balm

To the brightening white heart (my beloved mother)

To the spirit that inhabited my soul, dear to my heart (my husband)

To the pure and kind hearts, innocent souls and basils of my life (my brothers)

The sails are now opened, the anchor is raised and the ship is to be launched in a dark large sea. It is the sea of life in which in this darkness there is no shines except the chain of memories. Memories of far brothers to whom I loved and who loved me (my friends).

Acknowledgement

I thank to Allah whose blessings have enabled me to accomplish my thesis work successfully.

I take this opportunity to thank Dr. Tarek El Bashiti, Department of Biology and Biotechnology, for his supervision, supports, encouragements, and guidance. No words can express my sincere and deep sense of reverence for him.

My special thanks to the biotechnology and biology department where I completed the practical part in its laboratories.

Thanks also go to the Interpal Foundation for their support of this research in terms of providing the necessary materials for the practical part, so all thanks and respect for this great institution

Finally, I'm also thankful to the chemistry department who let me use shaker device which was so important to complete my research.

Signature
Rham J.Budeir

Contents

Contents

List of Tables

List of Figures

List of Abbreviations

A…… Absorbance

AP……..... Phenol-aminphenazone

°C ……..…… degree Celsius

C …….…..…..... carbon

Ca Cl_2…… Calcium Chloride

CFU……..……..... Colony forming unit

CMC……..……..... carboxymethyl cellulose

CMCase ……..…… carboxymethyl cellulase

Cm……..……. Centimeter

Con……..... Concentration

Fig ……..….. Figure

FPase ……..…….. Filter Paper Activity Assay

g……..…..…….. gram

g/l……..…..…….. gram per liter

GOD ……..…… Glucose Oxidase

h……...…….. hour

HCl……..…… Hydrochloric acid

H_2O_2……..…….. hydrogen peroxide

H_2SO_4……..…… Sulfuric acid

H_3PO_4……..…… Phosphoric acid

KDa ……..…….kilodalton

KCl ……..……... Potassium chloride

L……..…….. Liter

M…….. molar

Mmole……Millimole

μ ……..…….. Micron

Min…….. Minute

mg/dl …….……... Milligrams per deciliter

ml ……..…….. milliliter

Mmole/L……Millimole per liter

NaCl……..…… Sodium chloride

NaOH……..Sodium Hydroxide

Na_2NO_3……..…… Sodium nitrate

nm…….Nanometer

OD…….…….Optical density

PDA ……..…… Potato Dextrose Agar

% ……. Percent.

POD …….. Peroxidase

pH…….Hydrogen ion concentration

RBA……..…….Rose Bengal Agar

rpm ……..……. rpm

SmF ……..…….. Submerged state fermentation

SSF ……..…….. Solid state fermentation

SBP ……..…….. sugar beet pulp

T……..…….. Temperature

W/V……Weight per volume

Chapter1

Introduction

1.1 Overview

Cellulose is a branched glucose polymer composed of an 1,4 -glucose units linked by 1, 4-ß-D- glycosidic bond **(Han *et al.*, 1995; Gielkens *et al.*, 1999; Acharya *et al.*, 2008)**. Thousands of million tons are being produced by photosynthesis annually and accumulate in large quantity in the form of agricultural forest and municipal residue which deteriorate the environment. Cellulases are the hydrolytic enzymes which are responsible for the decomposition of the natural cellulose polymer (cotton, filter paper or lignocellulosic biomass) by acting at 1,4 β-D-glucosidic linkages thus finally converting into glucose monomer **(Sternberg *et al.*, 2000)**.

Today cellulases are of great attention due to the large industrial applications. These cellulase complex are of three different classes an endo-1,4-ß-glucanase[Carboxymethyl cellulase (EC 3.2.1.4)], a 1,4-ß-cellobiohydrolase [Exoglucanase (EC 3.2.1.91) and a1,4-ß-glucosidase [Cellobiase (EC 3.2.1.21)] which are produced by filamentous fungi **(Zhou *et al.*, 2008)**.

The production and applications of cellulases have central importance in bioprocess industries because it causes 30% more hydrolysis than acids **(Headon and Wash, 1994)**. Mode of action of cellulases in animal feed, fruit processing, textile wet processing, preparation of dehydrated vegetables, food products, essential oils, flavors, starch processing, botanical extracts, pulp and paper production, jams, juice, production of plant protoplast for genetic manipulation, wine production, pharmaceutical and biomass conversion have greatly increased the prospects of enzymatic hydrolysis over chemical processes **(Latif *et al.*, 1998)**.

Although cellulases are distributed throughout the biosphere, they are manifested in fungi, bacteria and a few actinomyctes **(Kim and Wimpenny, 1981; Rojaka and Malik, 1997)**. These microorganisms produce cellulases to release sugar for cell growth and product formation under certain environmental conditions. More than 14,000 species of fungi have been found to be active in cellulose degradation **(Esterbauer, 1991)**. The saprophytic filamentous fungi, especially *Trichoderma* spp., have been extensively studied due to their strong cellulolytic activity against crystalline celluloses which results into saccharification **(Deschamps *et al.*, 1985)**. Among them *Trichoderma viride, T. harzianum, T. reesei* and *T. konigii* have been studied **(Saddler, 1982; Deschamps, 1985; Macris, 1985; Hawary *et al.*, 2001)**. However, *Trichoderma viride* have proven to be an efficient candidate for the biodegradation of pretreated bagasse **(Hawary *et al.*, 2001)**.

During fermentation, cultural parameters and nutritional requirements such as carbon and nitrogen sources, ionic concentration, pH, temperature, cultivation time, aeration, water content of the substrate and inoculum size have fundamental role in the growth of microorganisms and subsequent product formation **(Mangat and Mandahr, 1998; Mekala *et al.*, 2008).**

Enzymes could be produced by submerged state fermentation (SmF) and solid-state fermentation (SSF). SmF includes the production of enzymes by microorganisms in a liquid nutrient media whereas SSF is a fermentation process conducted on a non-soluble material that acts both as physical support and source of nutrients in absence of free flowing liquid **(Pandey, 1992)**.
In our research, SmF method was used to produce cellulase enzyme .

General objective

Optimization of cellulase enzyme production from *Trichoderma* isolates under Submerged fermentation.

Specific objectives

1. Isolation and identification of *Trichderma* spp. from different sources.
2. To evaluate the effect of different carbon and nitrogen sources on the production of cellulase.
3. To optimize the environmental condition (T and pH) for enzyme production.
4. To investigate the effect of different incubation period on enzyme production.

Significance of the Study

This search is considered from the scientific view the first research of this kind in Gaza Strip even in Palestine. To introduce the concept of biotechnology especially from the industrial side in the Palestinian community, we need for such research which the success of its results prove to us that it is possible to start the application of modern techniques and in regards to this area we mean the use of micro-organisms techniques in the industrial field. The political and economic situation that the region going through requires us to start like this applied research to get out of these crises especially that the raw materials are easy to get locally. The industries that depend generally on micro-organisms in their products and fungi enzymes in particular can be done easily and this research is a clear example of this trend.

The monthly average of solid waste amount produced by governmental and civil health facilities in 2014 reached about 381 tons distributed by Palestine (277 tons per month in West Bank, and 104 tons per month in Gaza Strip) **(Palestinian Central Bureau of Statistics, 2014)**. Therefore, cellulolytic microorganism play an important role in the biosphere by reducing cellulose and they also convert cellulose waste into various economically important products like monomeric sugars, single cell proteins or microbial biomass proteins, sugars, alcohol and Bio-ethanol, compost, antibiotics, enzymes, to everyday use for man.

Chapter 2

Literature Review

2.1 Cellulases

The group of hydrolytic enzymes implicated in the bioconversion of celluloses are known as cellulases or cellulose systems. The cellulose system comprises three major highly specific enzymes namely; the endo-glucanases, the exo-glucanases and β-glucosidases. These enzymes are non-constitutive and are produced by many microorganisms such as bacteria, actinomycetes and fungi. The cellulase systems of fungal origin are the most abundant and widely studied. Among the fungi, *Trichoderma* a softwood rotting fungus, is the most potent cellulase-system producer **(Sukumaran *et al.*, 2005).**

The enzyme cellulase, a multi enzyme complex made up of several proteins, catalyses the conversion of cellulose to glucose in an enzymatic hydrolysis **(Aneja, 2005; Zahri *et al.*, 2005).**

Cellulases are extracellular enzymes which released in to the growing media. However, β-glucosidase occurred into three places: cell membrane, intracellular and extracellularly. All cellulytic enzymes share the same chemical specificity for β-1,4-glycosidic bonds, which they cleave by a general acid catalyzed hydrolysis. Most cellulolytic hydrolyses are proteins of 30-40 KDa molecular mass with acidic optima between 2.5 and 4.5 **(Baldrian, 2008)**.

Cellulases are inducible enzymes and their syntheses is strongly repressed by soluble sugars or other easily metabolizable substrates. Therefore, cellulose and Sophorose are natural inducers whereas glucose is catabolite represser for cellulase enzyme production. However, the mechanism of induction by cellulose is still not clear because being large insoluble macromolecules, cellulose cannot go through the plasma membrane directly **(Aneja, 2005; Zahri *et al.*, 2005)**.

2.2 Microbial Sources of Cellulases

Biotechnological conversion of cellulosic biomass is potentially sustainable approach to develop novel bioprocesses and products. Microbial cellulases have become the focal biocatalysts due to their complex nature and wide spread industrial applications. Cellulases enzyme are composed of independently folding, structurally and functionally discrete units called domains or modules, making cellulases enzyme module **(Henrissat *et al.*, 1998)**.

Cellulases are inducible enzymes synthesized by a large diversity of microorganisms including both fungi and bacteria during their growth on cellulosic materials (Table 2.1) **(Kubicek, 1993; Sang and Koo, 2001)**

Table 2.1: Microorganisms having cellulolytic abilities **(Ramesh, 2011)**.

Fungi	Soft rot fungi *Aspergillus niger; A. nidulans; A. oryzae; A. terreus; Fusarium solani; F. oxysporum; Humicola insolens; H. grisea; Melanocarpus albomyces; Penicillium brasilianum; P. occitanis; P. decumbans; Trichoderma reesei; T. longibrachiatum; T. harzianum; Chaetomium cellulyticum; C. thermophilum; Neurospora crassa; P. fumigosum; Thermoascus aurantiacus; Mucor circinelloides; P. janthinellum; Paecilomyces inflatus; P. echinulatum; Trichoderma atroviride* Brown rot fungi *Coniophora puteana; Lanzites trabeum; Poria placenta; Tyromyces palustris; Fomitopsis sp.* White rot fungi *Phanerochaete chrysosporium; Sporotrichum thermophile; Trametes versicolor; Agaricus arvensis; Pleurotus ostreatus; Phlebia gigantea*
Bacteria	Aerobic bacteria *Acinetobacter junii; A. amitratus; Acidothermus cellulolyticus; Anoxybacillus sp.; Bacillus subtilis; B. pumilus; B. amyloliquefaciens; B. licheniformis; B. circulan; B. flexus; Bacteriodes sp.; Cellulomonas biazotea; Cellvibrio gilvus; Eubacterium cellulosolvens; Geobacillus sp.; Microbispora bispora; Paenibacillus curdlanolyticus; Pseudomonas cellulosa; Salinivibrio sp.; Rhodothermus marinus* Anaerobic bacteria *Acetivibrio cellulolyticus; Butyrivibrio fibrisolvens; Clostridium thermocellum; C. cellulolyticum; C. acetobutylium; C. papyrosolvens; Fibrobacter succinogenes; Ruminococcus albus*
Actinomycetes	*Cellulomonas fimi; C. bioazotea; C. uda; Streptomyces drozdowiczii; S. lividans; Thermomonospora fusca; T. curvata*

2.2.1 Fungi

Cellulase are produced in nature by various terrestrial and marine organisms **(Szakacs *et al.*, 2006)**. They are distributed throughout the world, such as plants, animals and microorganisms. Cellulase enzymes produced chiefly by microbial sources, like bacteria, actinomycetes, filamentous fungi and protozoans, which catalyze the cellulose. The most important sources for industrial cellulases production are filamentous fungi, found in the soil, plants and in the marine environments **(Bhat, 2000)**.

Cellulolytic microbes are primary carbohydrate degraders and are generally unable to use proteins and lipids as energy sources for growth. Thus, a large number of bacteria, actinomycetes, and filamentous fungi produce cellulase to degrade cellulose. The ability to secret large amounts of extracellular protein is characteristic of certain fungi and such strains are most suited for production of higher level of extracellular cellulases **(Sukumaran *et al.*, 2005)**.

Although microorganisms capable of complete degradation of native cellulose are widespread in the soil environment, this ability appears to be confined to small number of species, which are predominantly fungi. The potential cellulase producing fungal genera include *Bulgaria, Chaetomium and Trichoderma* (Ascomycetes), *Coriolus, Phaenerochaete, Coriolus, Schizophyllum* (Basidiomycetes), *Aspergillus, Geotrichum and Penicillium* **(Lynd *et al.*, 2002)**.

A large number of fungi produce extracellular cellulases. The ability to fully solubilise the crystalline cellulose is restricted to relatively few of these **(Gow and Gadd, 1996)**.

2.3 *Trichoderma*:

Trichoderma spp. are known as producer of many enzymes. These enzymes include: chitinases, proteases, glucanases, cellulases and xylanase. Among these cellulases are extensively studied and involved in several industrial applications and commonly

found in the cellulolytic materials. *T. harzianum* and *T. reesei* are the potential cellulase producing fungi and the most frequently used species for the production of cellulases for complete hydrolysis of cellulosic substrates into its monomeric glucose, which is a fermentable sugar important for ethanol production **(Zahri *et al.*, 2005; Aneja, 2005)**.

2.4 *Trichoderma*: Diversity and Ecology

Trichoderma are filamentous fungi (Figure 2.1) commonly found in the soil community that are facultative saprophytes. The genus *Trichoderma* currently consists of more than 89 species, which are usually cosmopolitan and typically soil borne or wood decaying anamorph used widely as a bioconversion of lingocellulolytic wastes in to value added products such as enzymes, amino acids, organic acids **(Kubicek, 2004)**.

Colonies growing slowly or rapidly depending on the species, aerial mycelium usually limited, floccose to arachnoid; reverse colourless to dull yellowish. Some isolates with a distinctive aromatic odour resembling coconut. Conidiation variably effuse, loosely tufted, or forming compact pustules; white at first, eventually green (rarely brown). Chlamydospores present in most isolates, frequently abundant. **Conidiophores** usually relatively narrow and flexuous; with primary branches arising at regular intervals, usually paired or in whorls of three, usually short and not extensively rebranched. Phialides mostly in verticils of 2 or 3, in some strains up to 5-verticillate, lageniform to subulate. Conidia green (rarely brownish), smooth walled to distinctly verrucose, subglobose to obovoid or ellipsoid colourless to pale yellowish or greenish, smooth-and sometimes thick-walled (to 4 μm) ***(*Kubicek and Harman, 2002).**

(Rifai, 1969) distinguished nine species differentiated primarily by conidiophore branching patterns and conidium morphology based on microscopic characters; *Trichoderma aureoviride, T. hamatum, T. harzianum, T. koningii, T. longibrachiatum, T. piluliferum, T. polysporum, T. pseudokoningii*, and *T. viride*.

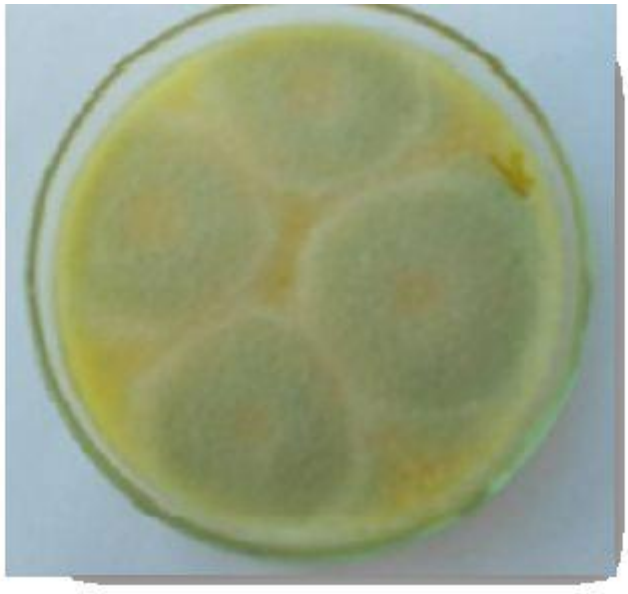

Figure 2.1: *Trichoderma* sp. on potato dextrose agar plate (Neagu, 2012).

The genus *Trichoderma* belongs to the class Deuteromycetes. It was, for the most parts, classified as an imperfect fungus, in that it has no known sexual stage **(Gams & Bisset, 1998).**

A sectional classification was proposed for *Trichoderma* recognizing the following five sections; section *Trichoderma*, *Longibrachiatum*, *Saturnisporum*, *Pachybasium* and *Hypocreanum* (Bissett, 1991a). Twenty species were assigned to *Trichoderma* section *Pachybasium*. They were described and differentiated on the basis of conidiophore and conidium morphology **(Bissett, 1991b)**.

Different media were used for culturing *Trichoderma* isolates for the analysis of their morphology and culture characteristics, e.g. malt extract agar, which is appropriate for conidium production and the observation of conidiophore branching, or potato dextrose agar, which proved useful for observing pigment production **(Hoyos-Carvayal and Bissett, 2011).**

Trichoderma harzianum to establish a mechanism for the aggressiveness towards *Agaricus bisporus* in infested commercial compost (aprotrophic and Mycoparasitic Components of Aggressiveness of *Trichoderma harzianum* Groups toward the Commercial Mushroom *Agaricus bisporus*) **(Williams *et al*., 2003).**

2.4.1 *Trichoderma* spp. applications

2.4.1.1 Bio-control

Trichoderma is a potent bio-control agent and used extensively for post-harvest disease control. It has been used successfully against various plant pathogenic fungi belonging to various genera, viz. *Fusarium*, *Phytophthora*, *Sclerotium*. The mechanisms are antibiotics production, myco-parasitism, nutrient competition and hydrolytic enzyme production **(Papavizas, 1985)**.

2.4.1.2 Transgenic Plants

Introduction of endo-chitinase gene from *Trichoderma* into plants such as tobacco and potato plants has increased their resistance to fungal growth. Selected transgenic lines are highly tolerant to foliar pathogens such as *Alternaria alternata*, *A. solani*, and *Botrytis cinerea* as well as to the soil-borne pathogen, *Rhizectonia* spp. (**Papavizas, 1985)**.

2.4.1.3 Bioremediation

Trichoderma strains play an important role in the bioremediation of soil that are contaminated with pesticides and herbicides. They have the ability to degrade a wide range of insecticides: organochlorines, organophosphates and carbonates **(Papavizas , 1985)**.

2.5 Classification of Cellulases

Based on the site of secretion, cellulase can be classified into two classes: complexed and non-complexed. Complexed cellulases are found in anaerobic bacteria and anaerobic fungi whereas non-complexed cellulase systems are mostly found in filamentous fungi and actinomycete and released in to the surface of the substrate. Organisms that produce noncomplexed cellulase systems are most often used in the industrial production of cellulolytic enzymes, because the secreted enzymes can easily be harvested **(Goldschmidt, 2008)**.

According to their mode of action, cellulolytic enzymes fall into one of two main groups, endogluconase or cellobiohydrolase **(Gow and Gadd, 1996)**. The complete

degradation of cellulose to glucose requires the action of at least three types of enzymes **(Gow and Gadd, 1996)**: endo-β-1,4 -glucanase, exo-β-1,4-glucanase (cellobiohydrolase) and β-glucosidase **(Aneja, 2005; Zahri *et al*., 2005; Miettinen-Oinonen, 2007).**

2.5.1 *Endoglucanase*

Endoglucanase (EG, EC 3.2.1.4):- CMCase, one of the members of cellulase complex, cleaves the internal glycosidic bonds of cellulosic chains yielding celloligosaccharides and acts synergistically with exo-glucanases and β-D-glucosidases during the solubilisation of cellulosic material **(Zhang *et al*., 2006)**. These enzymes are generally inactive towards crystalline cellulose and cellobiose. One important point to note is that endoglucanases do not directly contribute to the generation of soluble saccharides from insoluble cellulose **(Mosier *et al*., 1999; Bhat Hazlewood, 2003)**. Four genes, coding for the two endoglucanases (*EG I* and *EG II*) and the two cellobiohydrolases (*CBH I* and *CBH II*) have been isolated from *T. reesei* **(Aneja, 2005)**.

2.5.2 *Exoglucanase*

Exo-glucanase (CBHs):- like endoglucanases the CBHs are highly active on amorphous and swollen cellulose, but degrade crystalline and cello-oligosaccharides rather poorly **(Bhat and Hazlewood, 2003)**. CBHs release cellobiose, dimer of glucose, from the terminal ends of cellulose. It typically accounts for 80% of the amount of enzyme cellulases produced in *Trichoderma* fermentation. Recent kinetics studies and high resolution structural data confirmed that there are two classes of CBHs, CBH I and CBH II. CBH I attacks the nonreducing ends of cellulose, and CBH II attacks the reducing ends. Most members of *Trichoderma* (*T. reesei*) produce two CBHs which release cellobiose from both ends **(Aneja, 2005)**.

2.5.3 *β-Glucosidase*

β-glucosidase (cellobiase):- β-*glucosidase*, hydrolases cellobiose to glucose, supplying the fungus with an easily-metabolisable carbon source. There are two types of β-glucosidase (BGL I and BGL II). *β*-glucosidase is found in three places,

cell membrane, intracellular and extracellular. Many *Aspergillus* members have been reported to contain multiple β- glucosidases with a large variety in molecular mass. β-glucosidase is an important step because cellobiose inhibits the action of many cellulase components. Some β-glucosidase showed activity towards H_3PO_4-swollen CM-celluloses, but most β-glucosidase are inactive towards these and other polymeric substances such as Avicel, filter paper and cotton **(Bhat and Hazlewood, 2003)**.

2.6 Hydrolytic Mechanism of Cellulases

The cellulolytic enzymes have two different enzymatic mechanisms by which they can hydrolyze the glycosidic bonds in cellulose; the retaining and the inverting mechanisms **(Davies and Henrissat, 1995)**.

2.6.1 Retaining Mechanism

The retaining glycoside hydrolase mechanism leads to a net retention of the configuration at the anomeric carbon (C1) of the substrate after cleavage. This is performed via a double displacement mechanism, i.e., the hydrolysis of a glycosidic bond creates a product with the same configuration at the anomeric carbon as the substrate had before hydrolysis (Fig. 2.1a). The catalytic machinery of these enzymes involves two catalytic carboxylate residues that usually sit at opposite sides of the sugar plane. These are glycosylation and deglycosylation. Glycosylation is a double displacement reaction and a general acid-catalysed leaving group, form a glycosyl-enzyme intermediate. Deglycosylation, the first carboxylate residue now functions as a general base that activates an incoming nucleophile by stealing a proton from it. This activated nucleophile then hydrolyses the glycosyl-enzyme intermediate **(Davies and Henrissat, 1995)**.

2.6.2 Inverting Mechanism

The inverting glycoside hydrolase mechanism (Fig. 2.1b) leads to a net inversion of the configuration at the anomeric carbon (C1) of the substrate after cleavage. This is performed via a single nucleophilic displacement mechanism, that is the hydrolysis of a beta-glycosidic bond creates a product with the alpha-configuration, and vice-

versa. The catalytic machinery of these enzymes involves two catalytic carboxylates. These two carboxylate residues provide a general acid-catalyzed leaving group departure, and a general base-assistance to nucleophilic attack by a water molecule from the opposite side of the sugar ring **(Davies and Henrissat, 1995)**.

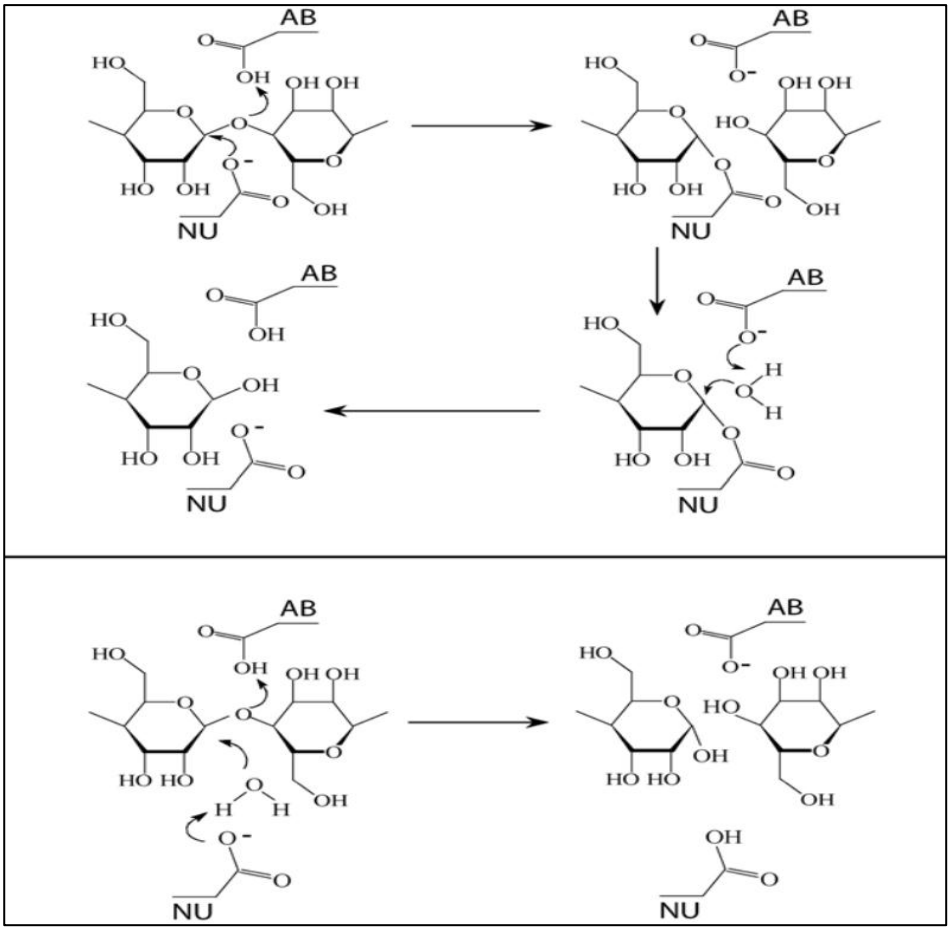

Figure 2.2: Hydrolytic mechanism of cellulases: The two enzyme mechanisms observed for cellulases: (a) the retaining mechanism and (b) the inverting mechanism. In the retaining mechanism the configuration at the anomeric carbon will be in b-configuration after hydrolysis, i.e., the configuration is retained. The distance between the two catalytic carboxylates in the retaining enzymes is ~5.5 Å. In the inverting mechanism the configuration at the anomeric carbon will be changed from b to a configuration upon hydrolysis. The distance between the two catalytic carboxylates in the inverting enzymes usually varies between 6.5 and 9.5 Å **(Davies and Henrissat, 1995).**

2.7 Application of cellulase enzyme

Cellulases have attracted attention of the scientific world due to its varied industrial applications. It has got applications in industries including:

2.7.1 Pulp and Paper Industry

Cellulases have potential in the pulp and paper industry. The mechanical pulping processes (refining and grinding) of woody raw material leads to pulps with high content of fines, bulk and stiffness. Bio-mechanical pulping with cellulases results in substantial energy savings (20-40%) during refining, and improvements in hand-sheet strength properties **(Bhat, 2000)**.

2.7.2 Textile Industry

They have been successfully used for the biostoning of jeans and bio-polishing of cotton and other cellulosic fabrics. During the biostoning process, cellulases break off the small fiber ends on the yarn surface, thereby loosening the dye, which is easily removed by mechanical abrasion in the wash cycle **(Uhlig, 1998 and Sukumaran *et al*., 2005).**

2.7.3 Laundry

The application of cellulases in laundry detergents has recently emerged due to their capacity to modify the cellulosic yarn surface of garments. *Trichoderma reesei* cellulolytic cocktail was employed to modify cellulose fibrils thus improving color brightness, feel, and dirt removal from the cotton blend garments **(Gormsen *et al*., 1998; Kirk *et al*., 2002)**. During the washing step of cotton fabrics, the *T. reesei* cocktails enriched in EGs are able to hydrolyze cellulose within the fibers to effectively remove the visible fuzz (also called "pilling") **(Sukumaran *et al*., 2005)**.

2.7.4 Food Industry

In food industry cellulases are used for productions of fruit nectars and purees and isolation and separation of starch and gluten from wheat flour. Cellulases have been used for extraction, clarification and stabilization of fruit juices and vegetables. Also

cellulases are applied for decreasing viscosity of pulp mush. **(Bhat, 2000; De Carvalho *et al*., 2008).**

2.7.5 Animal Feed Industry

Cellulases have been used for treatment of animal feeds like silage and grain resulting in improvement of their nutritional values **(Bhat, 2000; Dhiman *et al*., 2002).**

2.7.6 Detergent Industry

Cellulases acting under alkaline conditions have been used in detergents for selectively contacting the cellulose within the interior of fibres and removing soil in the inter-fibril spaces. The cellulases are also applied to remove these rough protuberances for a smoother, glossier, and brighter-colored fabric **(Bhat, 2000)**.

2.7.7 Ethanol production

Cellulases help in conversion of cellulosic materials like agricultural wastes to glucose and other fermentable sugars, which can be used as substrates for the production of products like ethanol.

2.8 Previous studies for cellulase production:

In 2003 the investigation made by Andade and Marbach cellulase production by *Trichoderma* spp. using agro-industrial by-products as substrates and observed the influence of the concentration of agricultural wastes on cellulase production. Wheat bran and peptone were the best sources of carbon and nitrogen for the production of cellulase, respectively. Generally, high cellulase enzymes were achieved at high concentration of carbon sources and low amount of nitrogen sources.

The study achieved by Baig in 2005 showed that produced cellulase by growing *T. lingorum* on banana agro-wastes comprising pseudo-stem and leaves as a substrate. Banana leaves and soy peptone was found to be the best carbon and nitrogen sources for cellulase production, respectively. The optimum pH and temperature of the medium was found to be 5.6-5.8 and 45^{o}C, respectively.

(Vyas and Vyas, 2005) studied the production of cellulase by co-culturing *T. viride, Aspergillus terreus* and *A. nidulans*. The combination of *T. viride* and *A. terreus* showed higher cellulase activity as compared to other two combinations. The advantage of mixed culture is more pronounced in SSF condition because the colonization of substrate may be accomplished better in symbiotic association i.e. each species having its own niche for growth and substrate degradation.

(Pothiraj *et al.*, 2006) produced cellulase enzymes from cassava wastes using different fungal cultures such as *Rhizopus stolonifer, Aspergillus niger* and *Aspergillus terreus*. *R. stolonifer* was the most efficient in bio-converting cassava waste into fungal protein (9%) compared to *A. niger* and *A. terreus*. The highest cellulase activity was observed on the 10th day in *R. stolonifer* mediated fermentation.

Cellulase enzyme production with expensive media constituents- celluclast, glucose, yeast extract, peptone, urea, KH_2PO_4, $(NH4)_2SO_4$, $MgSO_4$, $FeSO_4$, $MnSO_4$, $CoCl_2$, $CaCl_2$ etc have been reported by many researchers **(Rashid *et al.*, 2009)**.

In 2007 the investigation made by Moosavi-Nasab and Majdi-Nasab reported that sugar beet pulp as a substrate and used *Trichoderma ressei* as a mushroom to produce cellulase enzyme in the salt solution contains KH_2PO_4, $CaCl_2$, etc., then the researchers have done subculture for fungal cells in orbital shaker (180 rpm) at 30°C for two days and used it as inoculum, and then the researcher has done inoculation in medium center contains sugar beet pulp as a substrate for the production of cellulase. Also, they studied the effect of cellulose concentration in the production of cellulase enzyme by using a mixture of chemical composition of salts and sugar beet pulp as a substrate in fermentation for 4-6 days, a maximum cellulase activity of 0.46 IU/ml of filter paper activity was obtained.

(Gautam *et al.*, 2010) in their study focuses on reducing the cost of cellulases enzyme production using alternative sources for carbon source such as municipal solid waste and optimized fermentation medium for high yielding. They found that

municipal solid waste residue and yeast extract were the best sources of carbon and nitrogen to produce cellulase enzyme by *Trichoderma* spp. The ideal concentration of municipal solid waste and yeast extract was 45% (w/v) and 1.0% (w/v) respectively. It was found that the optimal temperature and pH of the medium for the production of cellulase enzyme by *T. viride* were 45 °C and 6.5 respectively. Cellulase production from *T. viride* can be an advantage as the enzyme production rate is normally higher as compared to other fungi.

(Malik *et al*., 2010) Studied production of cellulase enzyme by *Trichoderma viride* using submerged fermentation. The fermentation process conducted in flasks shaking using pretreated bagasse. The largest production of cellulase enzyme (CMCase 1.57 U/ml/min, FPase 0.921 U/ml/min) was obtained after the fermentation in about 72 hours of incubation at a temperature of 30 °C. Initial pH of the culture medium was also optimized and a pH of 5.5 was found to support maximum growth and enzyme production (CMCase 1.66 U/ml/min and FPase 0.932 U/ml/min) by *T. viride*. Also, they studied the effect of inorganic nitrogen sources on the amount of enzyme production and ammonium sulphate was the best. To promote the production of the enzyme in large quantities, the size of culture medium was 25 ml in flask 250 ml inoculated with 4% conidial inoculum.

(Chinedu *et al*., 2011) used waste cellulosic materials and crystalline cellulose to stimulate the production of cellulase by *Aspergillus niger, Penicillium chrysogenum* and *Trichoderma harzianum*. They isolated them from the wood waste dump in Lagos, Nigeria where cellulose supplemented media gave the maximum cellulase activity of 0.54, 0.67 and 0.39 units/mg Protein for *A. niger*, *P. chrysogenum* and *T. harzianum* respectively. It was found that the best activity for the production of the enzyme was by *Aspergillus niger* at 36 hours. *P. chrysogenum* and *T. harzianum* was given the best activity for the production of the enzyme at 12 and 60 hours respectively. And therefore, it was found that sawdust was the best type of cellulosic waste. Maximum enzyme activity of 0.30, 0.24 and 0.20 units/mg Protein respectively was obtained with *A. niger*, *P. chrysogenum* and *T. harzianum* at 144

hours of cultivation using the substrate. *A. niger* gave the highest enzyme activity with any of the three cellulosic materials followed by *P. chrysogenum*.

(Hussein *et al.*, 2011) isolated 17 mushroom from El-hawia, El-hada, El-kaym and Karwa in Taif governorate in Saudi Arabia where *Alternaria alternata* and *Aspergillus wentii* has the ability to produce cellulase enzyme. They studied some factors such as carbon and wheat bran as a raw material and nitrogen, pH, temperature and incubation time the results indicated that glucose and cellulose were the most effective as a carbon source while, urea was the best nitrogen source for cellulases production. Initial pH 5.0 and incubation temperatures at 25 or 35°C achieved high cellulases production.

(Nathan *et al.*, 2014) isolated 12 mushroom from mangrove plant debris and soil sample collected from Valanthakad Mangroves, Kerala, India, and they found 3 of them has more ability to produce cellulase enzyme and identified the most potent isolate which exhibited maximum cellulolytic activity was identified as *Trichoderma viride* VKF3 [Gene bank accession number- JX683684.1] based on colony morphology, microscopic observation and molecular characterization using D1/D2 region amplification. The isolated *T. viride* VKF3 was found to be non-phytopathogenic against the selected plants. Neighbour joining tree depicted its least divergence rate from the root taxon HM466686.1. *T. viride* VKF3 was grown under dynamic carbon, nitrogen sources, pH and temperature of the medium to draw out the optimum conditions for cellulase production. Protein stability kinetics and biomass production was also studied upto 11th day of incubation. It was evident from the study, that dextrose and beef extract could be used as major carbon and nitrogen sources in submerged fermentation at pH 9.0 and incubation temperature of 25°C to get maximum CMCase yield. Optimum enzyme recovery period was identified between 5th to 9th days of incubation beyond which the enzyme activity was reduced. By comparing two fermentation methods, submerged fermentation was found to be the best for maximum enzyme production. But utilization of substrates like sugarcane bagasse and cassava starch waste in the SSF offers a better scope in biodegradation of solid waste contributing to solid waste management.

Chapter 3

Material and Methods

3.1-Materials

3.1.1 Apparatus

The apparatus that were used are listed in Table 3.1 below.

Table 3.1 List of the apparatus used in this work

Apparatus	**Manufactures**
Vortex mixer	Labret's VX-1OO (USA)
Incubator	N-Biotech (Korea)
Shaking water bath	N-Biotech (Korea)
Autoclave	Bouxum (China)
Microwave oven	JAC (China)
Refrigerator 0℃ up To 15	Boeco (Germany)
Light microscope	Bouxum (China)
Safety cabinet	N-Biotech (Korea)
Hot plate	Scientific company (China)
PH meter	Azzota (U .S .A)
Centrifuge combi 514r	Hanil science industrial (China)
Centrifuge	Scientific company (China)
Spectrophotometer (Ct-220)	ChromTeck (Taiwan)
Ocular micrometer lense	China manufacturer

3.1.2 Equipments

Inoculating loop
Slides
Flasks
Beaker
Funnel
coverslipes
Aluminum paper
Cotton
Filter paper
Tissue paper
Parafilm
Plastic droppers
Plastic Petri plates
pipetted
Plastic Ependroff tubes 1.5 ml
Test tubes
Sterile cotton
Labels

3.1.3 Reagents

Table 3.2: List of the Reagents and Chemicals used in this work

Reagent	**Manufactures**
Sterile distilled water	Islamic University Lab
Oil Immersion	Hi Media Company, India
0.1%Congo red	Hi Media Company, India
Glucose assay kit	Glucose GOD FS 8X50ML (Lab Reagent), Germany
Hydrochloric acid	Hi Media Company, India
Ethyl alcohol	Frutarom "Occupied Palestine"
Hydroxide sodium	Hi Media Company, India
1% Carboxymethyl cellulose	Hi Media Company, India
Cellulose	Hi Media Company, India
1M NaCl	Hi Media Company, India
Sucrose	Hi Media Company, India
Glucose	Hi Media Company, India
Maltose	Hi Media Company, India
Peptone	Sigma (USA)
Beef extract	Hi Media Company, India
Sodium nitrate	Hi Media Company, India
yeast extract	Sigma (USA)

3.1.4 Culture Media

Table 3.3: List of the Culture media used in this work

Media	Manufactures
Rose Bengal Agar	Hi Media Company, India
Potato Dextrose Agar (PDA)	Hi Media Company, India
Basal salt medium	Hi Media Company, India

3.1.5 Microorganisms

Trichoderma harizanium, *Trichoderma reesi* and *Trichoderma viride* used in this study.

3.2 Methods

3.2.1 Sample collection

The samples were collected from different places like air and soil of Palestine Gaza. The *Trichoderma* spp. isolated from air from fungus laboratory in biology and biotechnology department Islamic University gaza. The soil samples were obtained from the soil of the Islamic University campus male and female. The soil sample was taken from a depth of 15cm and placed in a sterile polyethylene bag and then transported to the laboratory and stored in the refrigerator at 4 °C until use.

3.2.2 Isolation, identification of *Trichoderma* spp.

Trichoderma was isolated from air by opening petri dish content on PDA in fungus laboratory in biology and biotechnology department Islamic University gaza. Isolation of *Trichoderma* from soil by serial dilution technique and a 10^{-3} dilution of each sample was prepared. One millilitre of each solution was pipetted onto a Rose Bengal Agar (RBA) plate and incubated at 28 °C for 1 week. The culture plates were examined daily and each colony that appeared is considered to be one colony forming unit (cfu). After enumeration of CFU, individual colonies were isolated from the same plates and each uncommon colony was reisolated onto a fresh Potato

Dextrose Agar (PDA) plate. Distinct morphological characteristics were observed for identification according to **(Rahman *et al*., 2009)**, and the plates were stored at 4 °C.

The macroscopic and microscopic characteristic were used for identification of *Trichoderma* spp. The macroscopic characteristic were observed on petri dishes contained PDA after growth for 5 days. In our study used ocular micrometer lense to measure dimensions of phalides and conidia for local *Trichoderma* isolates.

The mode of mycelia growth, colour, odour and changes of medium colour for each isolate were examined every day. For micromorphological studies, a slide culture technique was used **(Leahy and Colwell, 1990)**. Examination of the shape, size, arrangement and development of conidiophores or phialides provided a tentative identification of *Trichoderma* spp. Samples were compared to a taxonomic key for the genus *Trichoderma* **(Rifai, 1969)**.

3.2.3 Screening for Cellulase Enzyme

Trichoderma tested for their ability to produce the hydrolytic enzymes, enrichment procedure was done in minimal medium according to **(Aneja, 2005)** comprising of g/L: Na_2NO_3; 2g, K_2HPO_4; 1g, $MgSO_4$ $7H_2O$; 0.5g, KCl; 0.5g, CMC; 5g and peptone; 2g with 15g agar, pH 5.5.

After incubation for 5 days at 30°C in CMC agar media, 0.1% congo red solution was added and counterstained with 1M NaCl for 15-20min. The zone of cellulose hydrolysis was appeared as a clear area around the colony **(Gautam *et al*., 2011).**

3.2.4 Optimization of culture conditions for enzyme production

3.2.4.1 Effect of incubation period on enzyme production

Fermentation period is important parameter for enzyme production by *Trichoderma* spp. In this study, fermentation experiments were carried out up to 7 days and production rate measured at 24 h intervals **(Gautam *et al*., 2010)**.

3.2.4.2 Effect of pH and temperature on enzyme production

The most suitable pH of the fermentation medium was determined by adjusting the pH of the culture medium at different levels in the range of pH 3.5 to 8.5 using 1% hydrochloric acid and 1% sodium hydroxide. In order to determine the effective temperature for cellulase production by the *Trichoderma* spp., fermentation was carried out at 5 °C intervals in the range of 20 to 50°C**.**

3.2.4.3 Effect of carbon sources on enzyme production

Effect of various carbon compounds on cellulase production, CMC, sucrose and maltose were used for study. The broth was distributed into different flasks and 0.5 to 3.0 % of each carbon sources were then added before inoculation of the strain and after culture inoculation, the flasks were incubated for 7 days at 30° C .

3.2.4.4 Effect of nitrogen sources on enzyme production

In the present study, to detect the appropriate nitrogen source for cellulase production by the *Trichoderma* spp. The influence of peptone, beef extract, sodium nitrate and yeast extract were studied. The fermentation medium was supplemented with the mentioned organic and inorganic compounds at 0.5 to 3.0% level, replacing the prescribed nitrogen source of the fermentation medium according to Gautam *et al*., 2010.

3.2.5 Enzyme production

Cellulase production was carried out by using cellulose as sole carbon source in SmF. The composition of the medium was in g/L: (Na_2NO_3; 2g, K_2HPO_4; 1g, $MgSO_4$ $7H_2HO$; 0.5g, KCl; 0.5g, Cellulose; 5g and peptone; 2g) **(Aneja, 2005).** The pH of the medium was adjusted to 5.5 prior to sterilization. The flask were inoculated with 2 agar discs (2 mm in diameter) of 7 days old culture from PDA plates and were incubated under stationary condition at 30 °C upto 7 days. The crude enzyme were filtered and centrifuged at 11000 x g for 10 min .

3.2.6 Enzyme assay

The amount of reducing sugars was estimated by using glucose assay kit (Glucose GOD FS 8X50ML (Lab Reagent), Germany.

3.2.6. 1 Method

"GOD-POD": Enzymatic photometric test.

3.2.6.2 Principle

Glucose was determined after enzymatic oxidation of glucose by glucose oxidase (GOD) to gluconic acid. The formed hydrogen peroxide (H_2O_2) is detected by a chromogenic oxygen acceptor, phenol-aminphenazone (AP) in the presence of peroxidase (POD):

$$\text{Glucose} + 2H_2O + O_2 \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{Phenol} + (4\text{-AP}) \xrightarrow{\text{POD}} \text{Quinone} + H_2O$$

Using the glucose kit, the produced amount of glucose was measured by the cellulase enzyme which produced by *T. viride* to analyze the existing links in the complex carbohydrates sources like cellulose, sucrose and others. And then turn them into a simple sugar which is glucose and the way was done as following:

Wavelength:……. 640nm.

Cuvette:…….....1 ml.

Temperature: …….37˚C or 15-25 °C.

Measurement:…….Against reagent blank.

Adjust the instrument to zero with Blank of reagent.

	Blank	Sample or standard
Sample or standard	-----	10 ML
Distle water	10 ML	-----
Reagent	1000 ML	1000 ML

Mix and incubate 20 min at 20-25 ºC at 37 °C. Read absorbance against the blank within 60 min. by Spectrophotometer (Ct-220) at length 640 nm.

And when making the calculations to get the amount of glucose produced by gram / ml were as following:

$$\text{Glucose mg/dl} = \frac{\text{Absorbance of sample}}{\text{Absorbance of Standard}} \times \text{concentration of standard /cal [mg/dl]}$$

Conversion factor

Glucose [mg/dl] × 0.05551= Glucose [mmol/L].

3.2.7 Statistical analysis

All experiments and enzyme assays were performed in duplicates, statistically evaluated by Excel (version 16) .

Chapter 4

RESULTS

4.1 Isolation, identification of *Trichoderma* spp.

In Figure 4.1, we observed colonies growing rapidly that were 7-9 cm in diameter, yellowish to dark green that is almost like of *Trichoderma harizanium* .

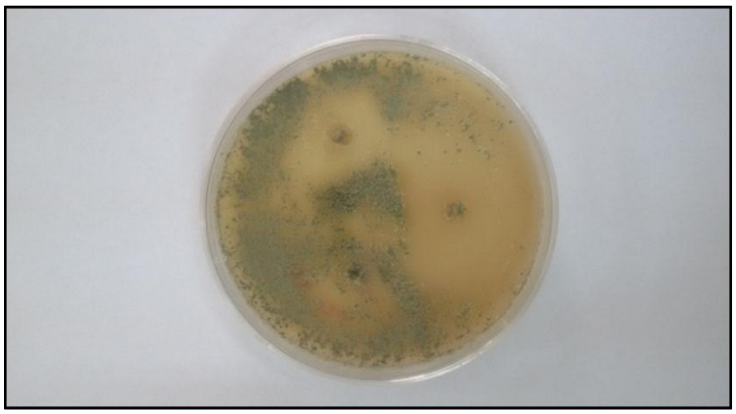

Figure 4.1:The isolated *Trichoderma harizanium* on potato dextrose agar plate.

In Figure 4.2, phalidies is shown growing ampulliform to lageniform that were 7.3 × 2.6 µm.

Microphotograph of *Trichoderma harizanium* under the light microscope show conidia sub globose to obovoid, smooth walled , subhyaline to pale green that were mostly 3× 3.5 µm shown below in Figure 4.2.

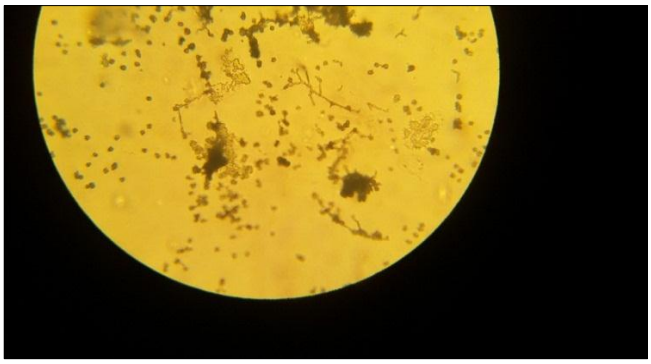

Figure 4.2: Microphotograph of the isolated *Trichoderma harizanium* under the light microscope.

In Figure 4.3, we observed colonies growing rapidly that were mostly 5.5-7 cm in diameter, pale yellow –green that is almost like of *Trichoderma reesi.*

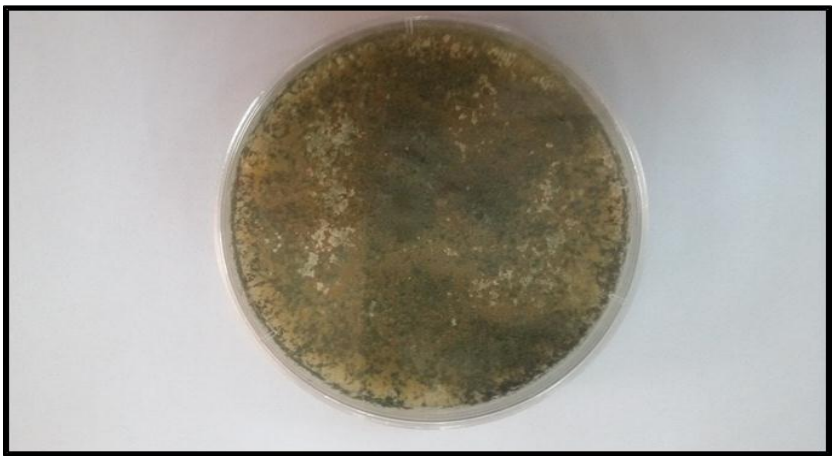

Figure 4.3: The isolated *Trichoderma reesi* on potato dextrose agar plate.

In Figure 4.4, we noticed phalidies growing cylindrical, mostly 7.3×2.2 µ m.

The result showed that the microphotograph of *Trichoderma reesi* under the light microscope represent conidia pale green, ellipsoid that were mostly 3.2× 5.8 µm shown in Figure 4.4.

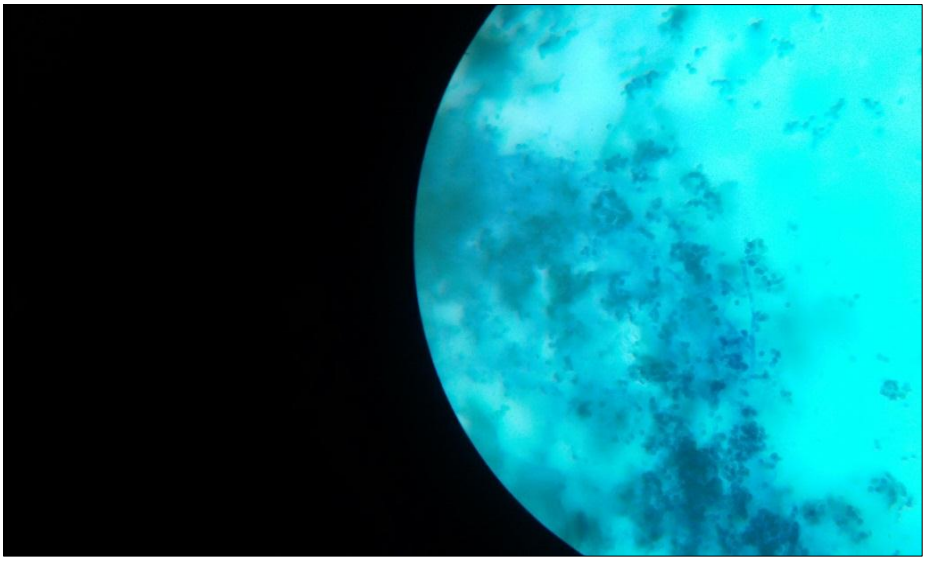

Figure 4.4: Microphotograph of the isolated *Trichoderma reesi* under the light microscope.

In Figure 4.5, we observed colonies glucous to dark green, less often yellowish that is almost like of *Trichoderma viride.*

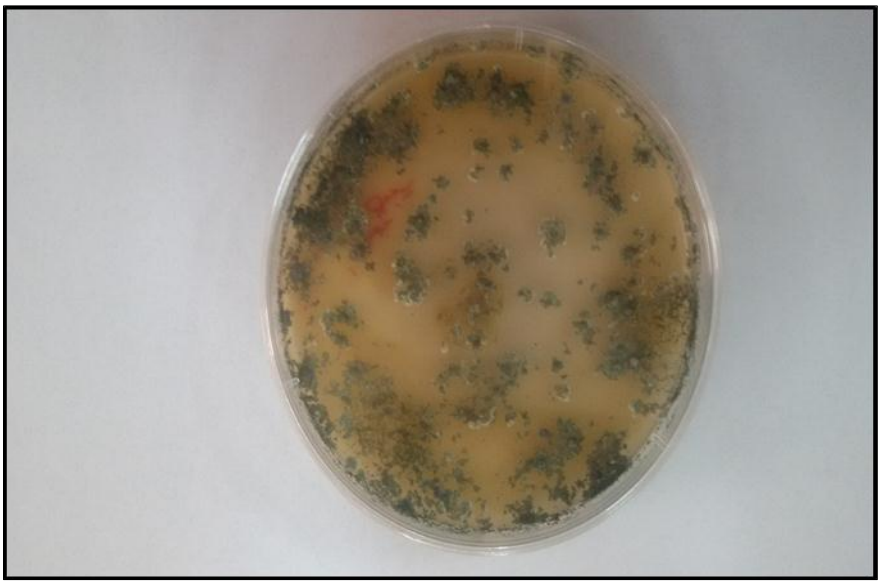

Figure 4.5: The isolated *Trichoderma viride* on potato dextrose agar plate.

In Figure 4.6, Through working in the laboratory we found phalidies mostly 16.7× 2.3 µm.

Microscopic examination had also revealed that the isolate *Trichoderma viride* under the light microscope show conidia dark green that were mostly 2.6× 2.2 µm shown in Figure 4.6.

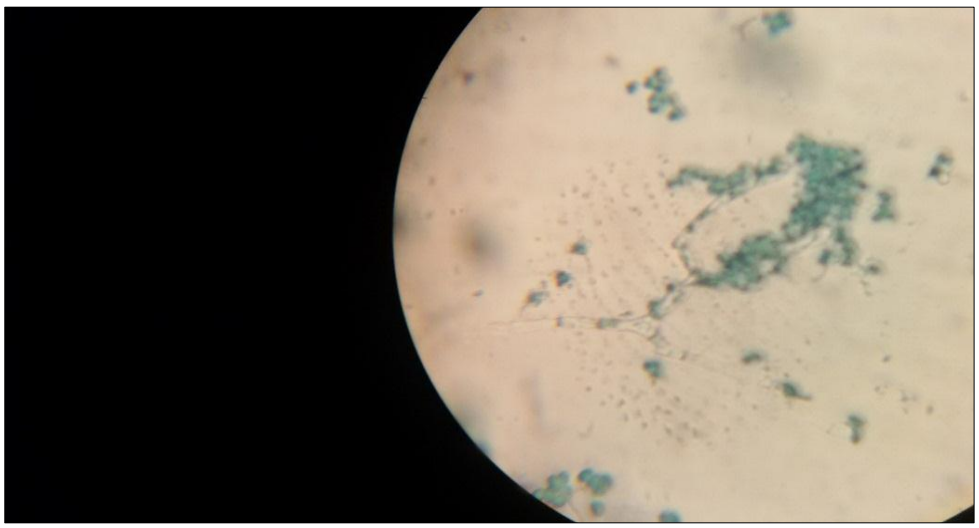

Figure 4.6: Microphotograph of the isolated *Trichoderma viride* under the light microscope

.

4.2 Screening for Cellulase Enzyme

All *Trichoderma* isolates were screened for cellulase activity using CMC agar (selective agar). All isolates of *Trichoderma* were positive for cellulase enzyme production. However, isolates were differing in their ability to produce cellulose degrading enzymes (Figure 4.7 and table 4.1). The isolate from Islamic university/ campus Males was showed the highest hallow zone on the CM-cellulose agar media (6.7cm) whereas the isolate from air showed the least clear zone diameter (5.9cm).

Table 4.1: Screening for Cellulase Enzyme

Site of Trichoderma isolation	1	2	3	Average(cm)
air	3.95	5.75	8.1	5.9
Islamic university/campus Females	7.25	4.15	7.5	6.3
Islamic university/ campus Males	4.85	7.15	8	6.7

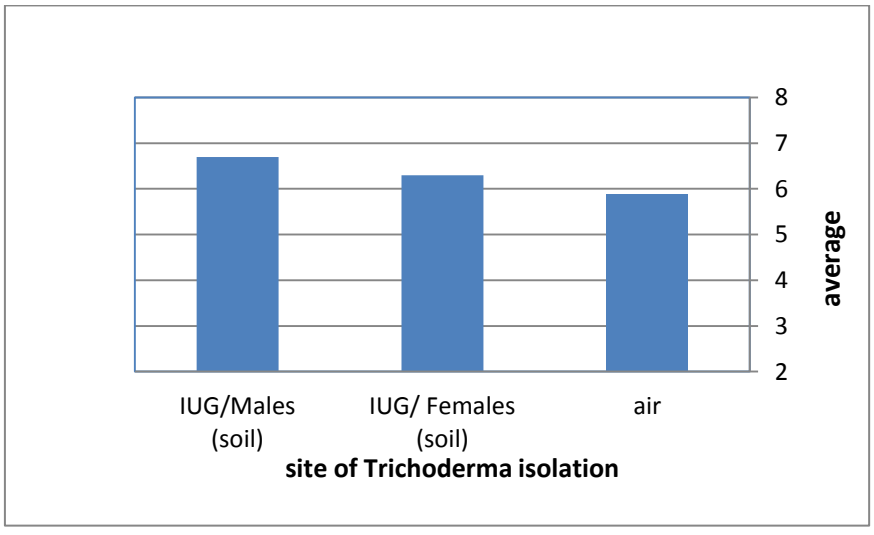

Figure 4.7: Site of *Trichoderma* spp. Isolation.

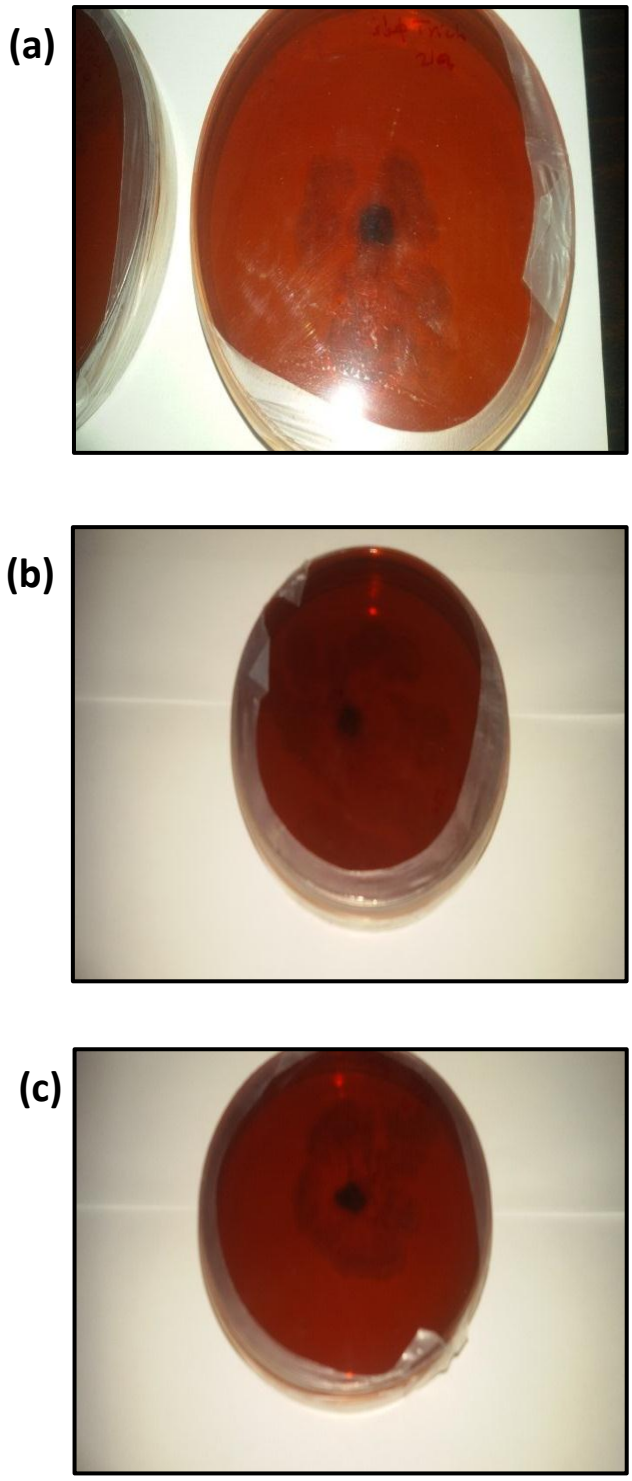

Figure 4.8: Screening of cellulase producing *Trichoderma* isolates by Congo Red reagents (a) *Trichoderma harizanium* (b) *Trichoderma reesi* (c) *Trichoderma viride*

4.3 Optimization of culture conditions for enzyme production

Several factors affect the production of cellulase enzymes by *T. viride*. Among the factors determined in this study were temperature, carbon sources, nitrogen sources, pH and incubation period under SmF.

4.3.1 Effect of incubation period on enzyme production

The effect of incubation period is very clear on the production of cellulase enzyme where the best incubation period was at 7 days as shown in figure 4.9 and table 4.2.

Table 4.2: Effect of incubation period on enzyme production

Days	Reading the device	Glucose [mg/dl]	Glucose [mmol/L]
1	0.019	41.3	2.3
2	0.042	91.3	5
3	0.056	121.7	6.7
4	0.073	158.7	8.8
5	0.128	278.2	15.5
6	0.207	450	25
7	**0.255**	**554.3**	**30.8**
8	0.204	443.5	24.6
9	0.147	319.6	17.7
10	0.070	152.2	8.4

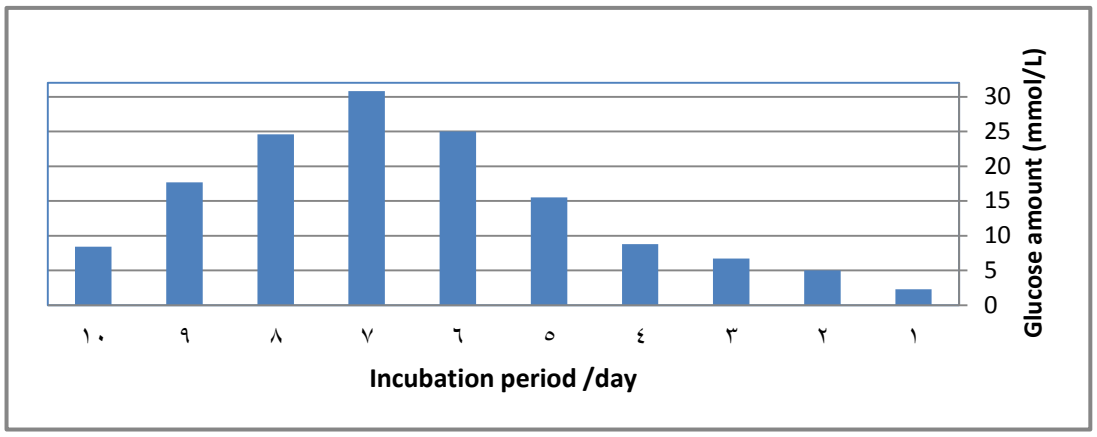

Figure 4.9: The effect of incubation period on the production of cellulase enzymes from *Trichoderma viride*.

4.3.2 Effect of pH on enzyme production

Figure 4.10 and table 4.3 show the effect of different PH on the cellulase enzyme production and the optimal pH for cellulase enzyme production form *T. viride* was 5.5.

Table 4.3: Effect of different pH on enzyme production

PH	Reading the device	Glucose [mg/dl]	Glucose [mmol/L]
3.5	0.044	95.65	5.3
4.5	0.048	104.34	5.8
5.5	**0.257**	**558.7**	**31**
6.5	0.184	400	22.2
7.5	0.114	247.8	13.8
8.5	0.105	228.2	12.7

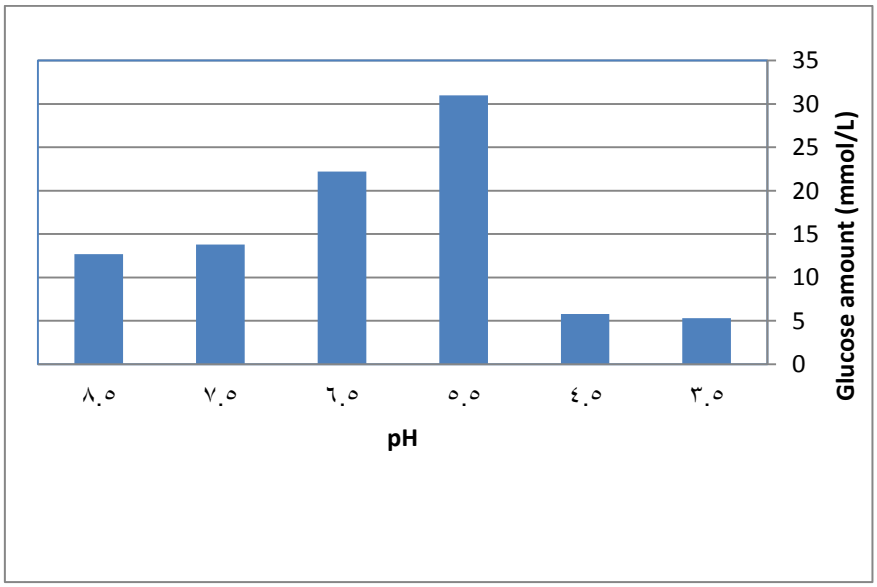

Figure 4.10: The effect of pH on the production of cellulase enzymes from *Trichoderma viride*.

4.3.3 Effect of temperature on enzyme production

The results indicate that the important influence of temperature on cellulase production and the enzyme activity increased as the temperature increase up to 30°C then after it began to decrease when the temperature raising above 30°C. The best temperature for producing cellulase was at 30 °C (table 4.4 and figure 4.11).

Table 4.4: Effect of different temperature on enzyme production

Temperature (°C)	Reading the device	Glucose [mg/dl]	Glucose [mmol/L]
20	0.040	86.95	4.82
25	0.185	402.2	22.3
30	**0.235**	**510.86**	**28.4**
35	0.155	336.95	18.7
40	0.102	221.7	12.3
45	0.084	182.6	10.13

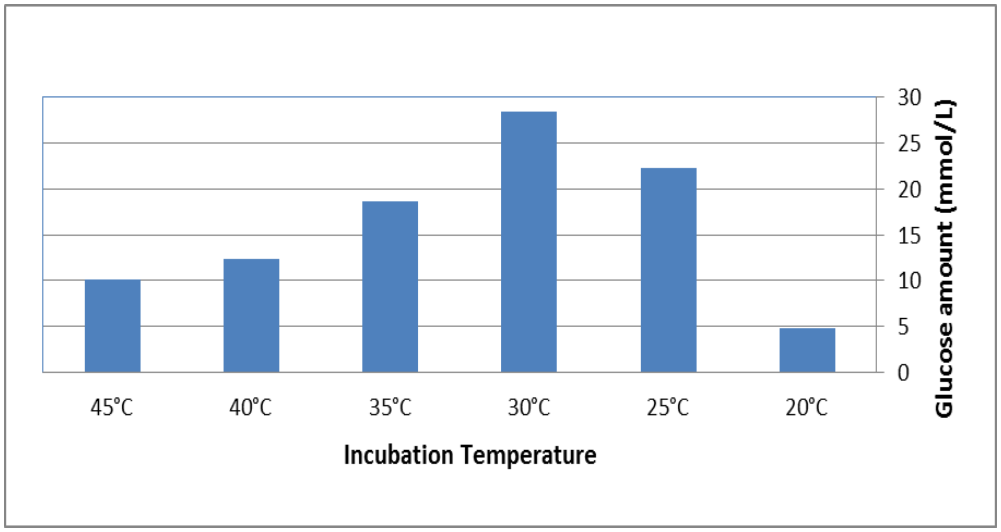

Figure 4.11: The effect of incubation temperature on the production of cellulase enzymes from *Trichoderma viride.*

4.3.4 Effect of carbon sources on enzyme production

4.3.4.1 cellulose

Results of the effect of different cellulose concentration on the production of cellulase enzymes from *T. viride*. The best concentration of cellulose on cellulase enzyme production was 1% as shown in table 4.5 and figure 4.12. Further increased in cellulose concentration beyond the level of 1% the production of cellulase enzyme begun to decrease.

Table 4.5: Effect of different concentrations of cellulose on enzyme production

Cellulose concentration (g/L)	Reading the device	Glucose [mg/dl]	Glucose [mmol/L]
0.5	0.108	215.2	12
1	**0.193**	**419.56**	**23.3**
1.5	0.176	382.6	21.23
2	0.160	347.82	19.3
2.5	0.147	319.56	17.7
3	0.088	191.3	10.6

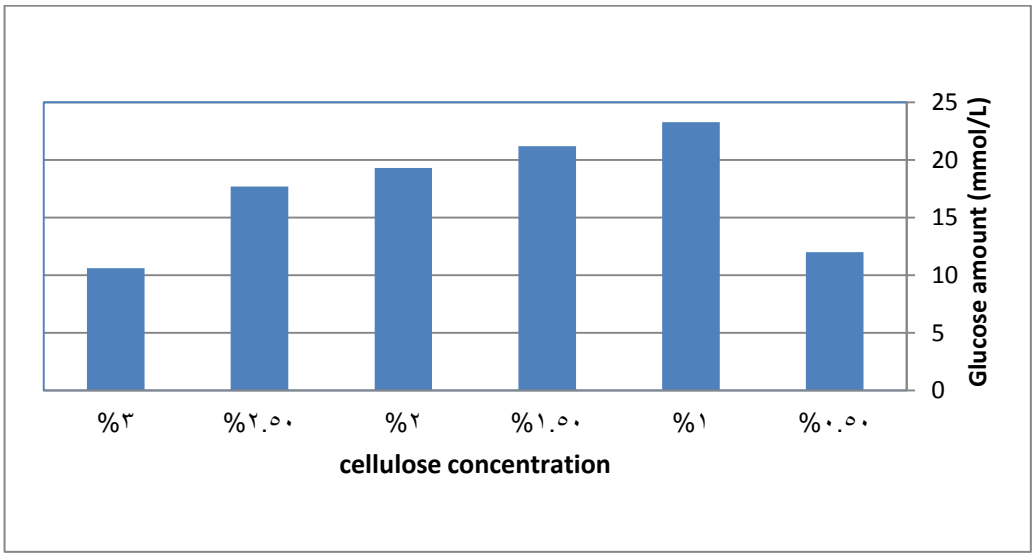

Figure 4.12: The effect of cellulose concentration on the production of cellulase enzymes from *Trichoderma viride*.

4.3.4.2 Carboxymethylecellulose (CMC)

As shown in Table (4.6 and Figure 4.13), 1% CMC is the best concentration of the used Carboxymethylecellulose concentrations for the production of the highest concentration of cellulase enzyme.

Table 4.6: Effect of different concentrations of Carboxymethylecellulose on enzyme production

CMC concentration (g/L)	Reading the device	Glucose [mg/dl]	Glucose [mmol/L]
0.5	0.091	197.82	11
1	**0.139**	**439.13**	**24.4**
1.5	0.163	354.34	19.7
2	0.154	334.78	18.6
2.5	0.143	310.86	17.25
3	0.102	221.73	12.3

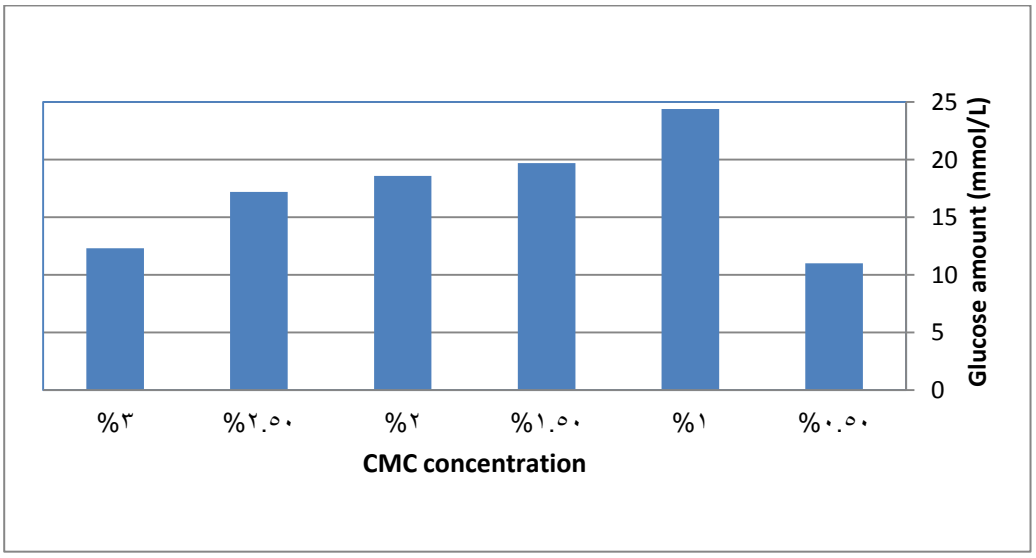

Figure 4.13: The effect of CMC concentration on the production of cellulase enzymes from *Trichoderma viride*.

4.3.4.3 Maltose

The concentration of 1% maltose was the best concentration to produce a greater amount of cellulase enzyme as shown in table 4.7 and figure 4.14.

Table 4.7: Effect of different concentrations of Maltose on enzyme production

Maltose concentration (g/L)	Reading the device	Glucose [mg/dl]	Glucose [mmol/L]
0.5	0.061	132.6	7.3
1	**0.200**	**434.6**	**24.13**
1.5	0.192	417.4	23.2
2	0.124	269.6	15
2.5	0.068	147.8	8.2
3	0.053	115.2	6.4

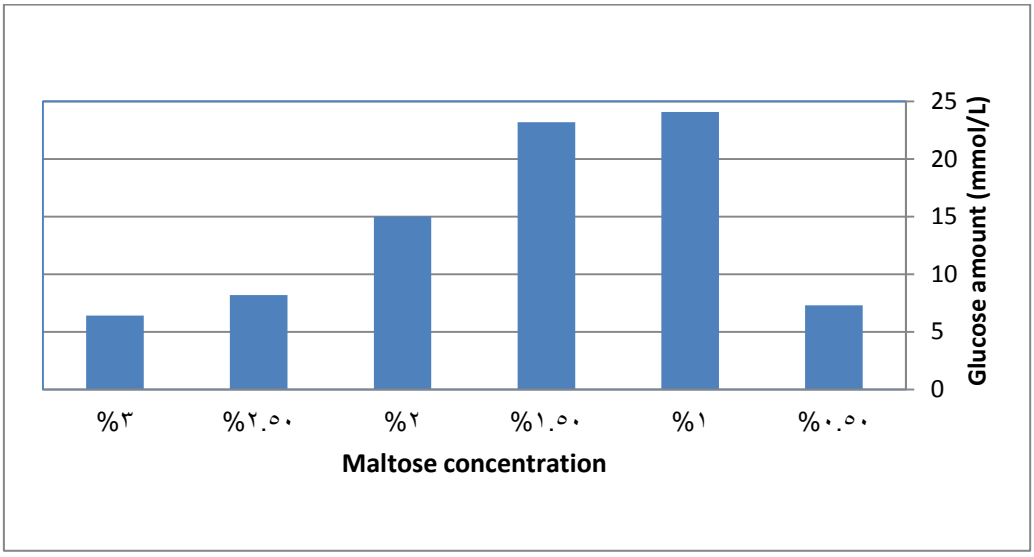

Figure 4.14: The effect of Maltose concentration on the production of cellulase enzymes from *Trichoderma viride.*

4.3.4.4 Sucrose

The results showed that there was a significant increase concentration of glucose amount when used sucrose as a carbon source. Sucrose was the best source of carbon sources that used in this study at a concentration of 1% as shown in the figure 4.15 and table 4.8.

Table 4.8: Effect of different concentrations of Sucrose on enzyme production

Sucrose concentration (g/L)	Reading the device	Glucose [mg/dl]	Glucose [mmol/L]
0.5	0.143	310.9	17.3
1	**0.321**	**697.8**	**38.7**
1.5	0.300	652	36.2
2	0.163	354.34	19.7
2.5	0.096	208.7	11.6
3	0.084	182.6	10.1

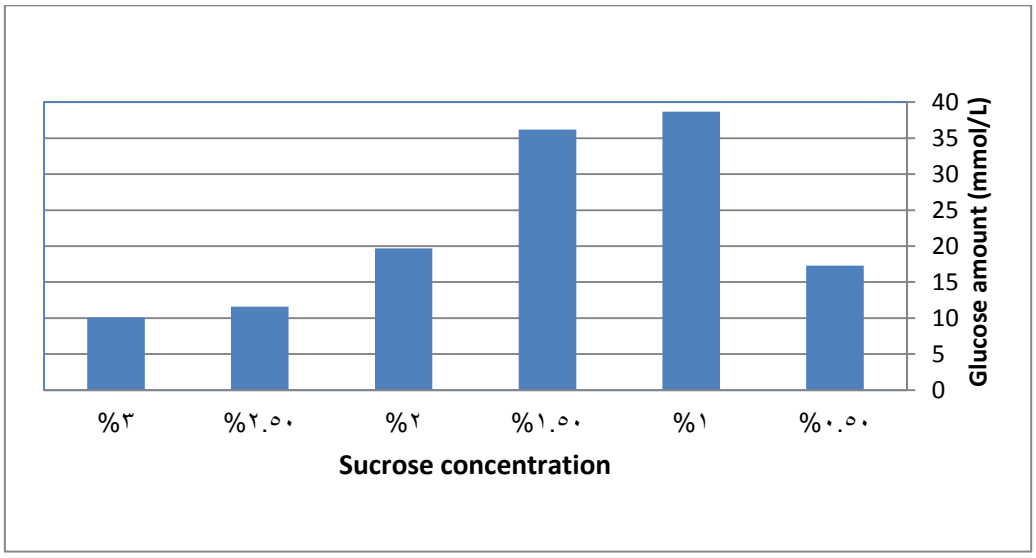

Figure 4.15: The effect of sucrose concentration on the production of cellulase enzymes from *Trichoderma viride*.

4.3.5 Effect of nitrogen sources on enzyme production

4.3.5.1 Peptone

Figure (4.16 and table 4.9) show the effect of different peptone concentration on the cellulase enzyme production and the best concentration was 1% peptone.

Table 4.9: Effect of different concentrations of Peptone on enzyme production

Peptone concentration (g/L)	Reading the device	Glucose [mg/dl]	Glucose [mmol/L]
0.5	0.054	117.4	6.5
1	**0.111**	**241.3**	**13.4**
1.5	0.106	230.43	12.8
2	0.098	213	11.8
2.5	0.086	187	10.4
3	0.063	137	7.6

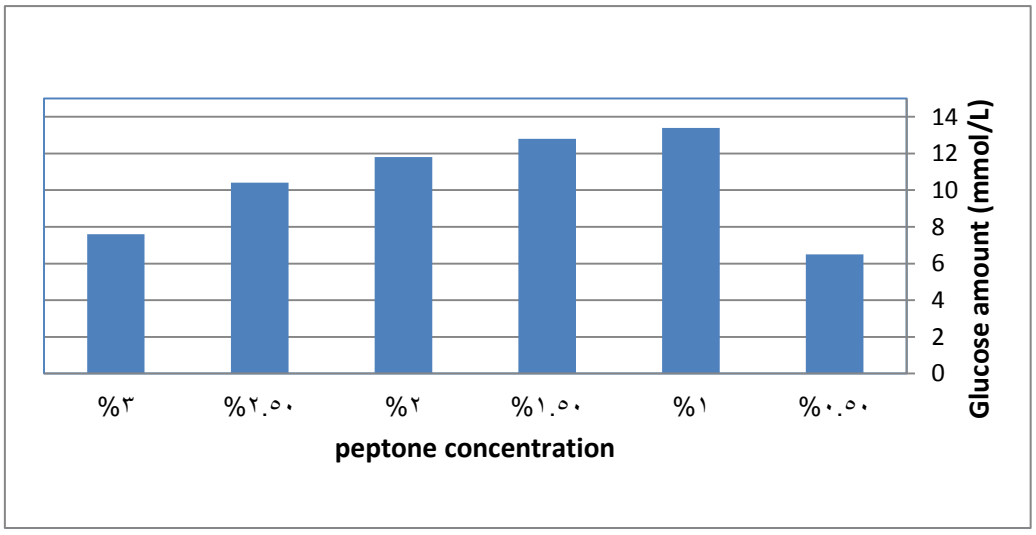

Figure 4.16: The effect of peptone concentration on the production of cellulase enzymes from *Trichoderma viride.*

4.3.5.2 Yeast Extract

Results of the effect of different yeast extract concentration on the production of cellulase enzymes from *T. viride*. The best concentration of yeast extract on cellulase enzyme production was 1% give greater amount of glucose **(23.8 mmol/L)** as show in the table 4.10 and figure 4.17.

Table 4.10: Effect of different concentrations of Yeast Extract on enzyme production

Yeast Extract concentration (g/L)	**Reading the device**	**Glucose [mg/dl]**	**Glucose [mmol/L]**
0.5	0.054	117.4	6.5
1	**0.197**	**428.3**	**23.8**
1.5	0.179	389.13	21.6
2	0.102	221.7	12.3
2.5	0.092	200	11.1
3	0.080	174	9.7

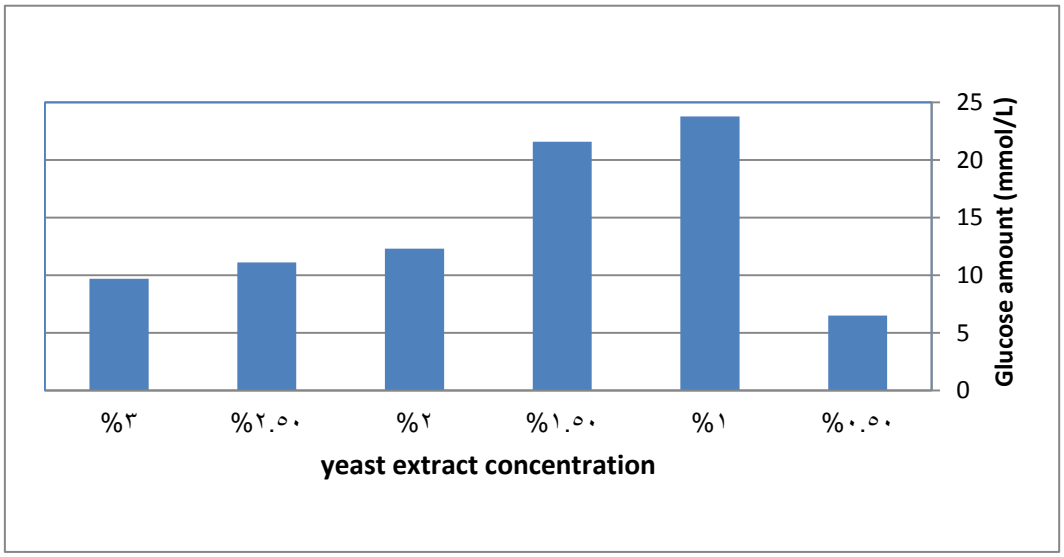

Figure 4.17: The effect of Yeast Extract concentration on the production of cellulase enzymes from *Trichoderma viride*.

4.3.5.3 Beef Extract

Table 4.11 and Figure 4.18 show the effect of different concentrations of beef Extract on cellulase enzyme production and the best concentration was of 1% beef Extract.

Table 4.11: Effect of different concentrations of Beef Extract on enzyme production

Beef Extract Concentration (g/L)	Reading the device	Glucose [mg/dl]	Glucose [mmol/L]
0.5	0.030	65.2	3.6
1	**0.134**	**291.3**	**16.2**
1.5	0.100	217.4	12
2	0.091	197.8	11
2.5	0.084	182.6	10.1
3	0.073	158.7	8.8

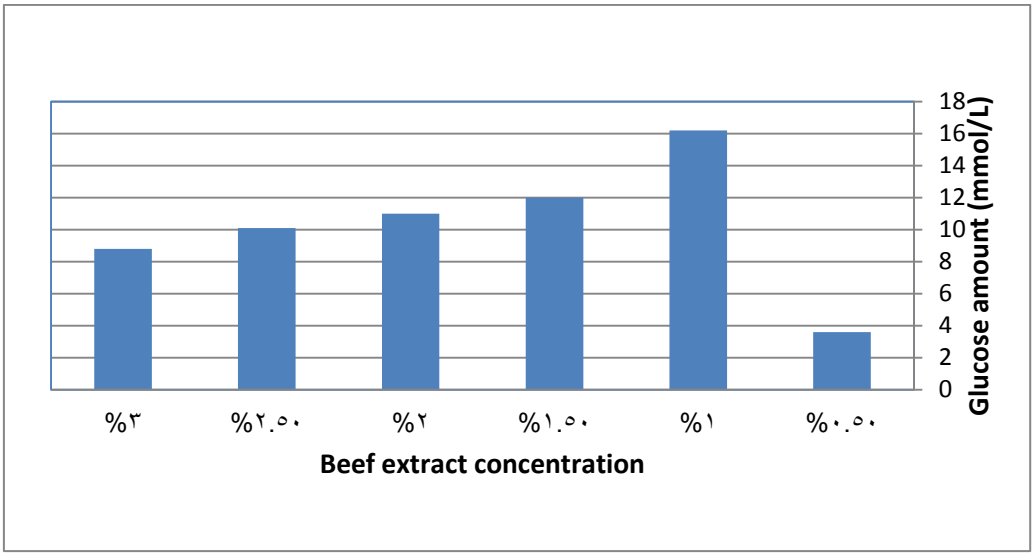

Figure 4.18: The effect of Beef extract concentration on the production of cellulase enzymes from *Trichoderma viride*.

4.3.5.4 Sodium Nitrate

The results indicate that the less amount of glucose was produced when using sodium nitrate as a source of nitrogen . The table 4.12 and figure 4.19 illustrate the effect of sodium nitrate on cellulase enzyme and the 1% concentration was the best of the used concentrations of sodium nitrate on the production of cellulase enzyme.

Table 4.12: Effect of different concentrations of Sodium Nitrate on enzyme production

Sodium nitrate concentration (g/L)	**Reading the device**	**Glucose [mg/dl]**	**Glucose [mmol/L]**
0.5	0.042	91.3	5
1	**0.092**	**200**	**11.1**
1.5	0.085	184	10.2
2	0.073	158.7	8.8
2.5	0.069	150	8.3
3	0.066	134.5	7.5

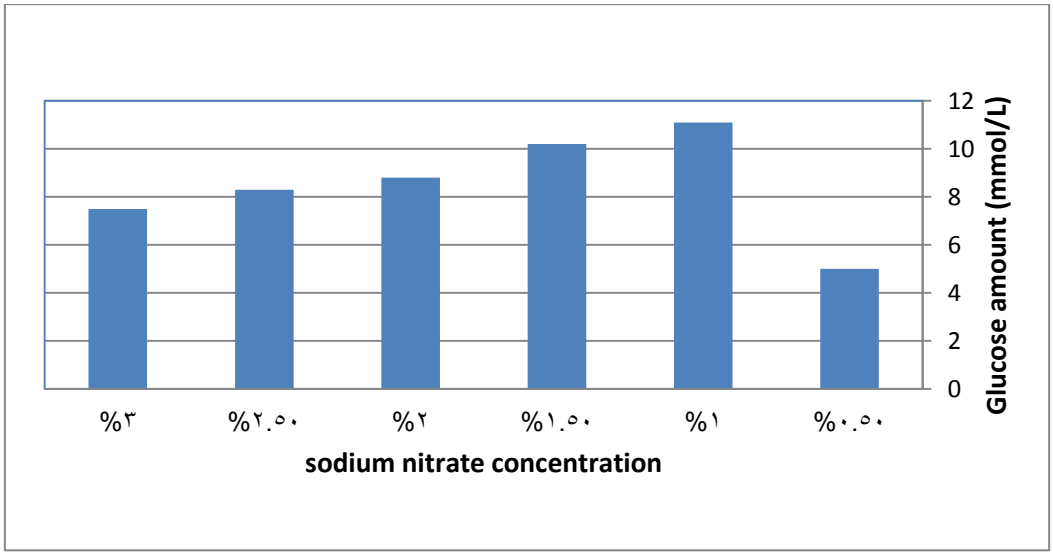

Figure 4.19: The effect of Sodium nitrate concentration on the production of cellulase enzymes from *Trichoderma viride*.

Chapter 5

Discussion

5.1 Isolation, identification of *Trichoderma* spp.

In this study, the largest amount of cellulase enzyme was produced by *Trichoderma viride* isolated from air and soil of the Islamic University gaza.

According to **(Rahman *et al.*, 2009)** *Trichoderma* spp. isolated from soil was more prominent than that of the strains isolated from other sources.

The result regarding the identification of *Trichoderma* have been described in the result section (4.1). The *Trichoderma* spp. isolated and identified using various techniques including macroscopic and microscopic characteristics, which facilitate the identification process.

T. harizanium as colonies growing rapidly (most isolates 7-9 cm). Conidation predominantly effuse appearing granular or powdery due to dense conidation, rapidly turning yellowish green to dark green. Phalides ampulliform to lageniform mostly 7.3×2.6 µm. Conidia subglobose to obovoid, mostly 3×3.5 µm, smooth walled subhylaine to pale green, As well described *T. ressei* like this colonies growing rapidly (most isolates 5.5-7cm), phalides cylindrical or slightly inflated mostly 7.3×2.2 µm. Conidia pale green, ellipsoid 3.2×5.8 µm. Likewise described *T. viride* based on the microscopic and macroscopic morphology which observed on figures (4.5 and 4.6). as colonies growing rapidly, conidiation appearing granular or crusty in age initially glaucous rapidly turning to dark green. Conidia mostly 2.6×2.2 µm and phalides mostly 16.7×2.3 µm, our results strongly agreed with these finding which described by **(kubicek and Harman, 2002).**

5.2 Effect of incubation period on enzyme production

The incubation period is directly related with the production of enzyme and other metabolic activities up to a certain extent. *Trichoderma* sp. showed the different activities of cellulolytic species along with different incubation period.

The effect of incubation period on the production of cellulase enzyme by *T. viride* were shown in figure 4.9. We observed the best production at 7 days of incubation which is almost like with previous study **(Khare and Upadhyay, 2011)** which have reported that the maximum production of cellulases by *T. viride* was observed after 6 days of incubation.

In this study increase in the incubation period however, resulted in the gradual decrease in the production of cellulase. Therefore incubation period of 7 days was found to be optimal for cellulase production by *T. viride*.

The incubation period to achieve peak cellulase activity by the isolated *Trichoderma* spp. was six days which suitable from commercial point of view **(Kang *et al*., 2004).** It might be due to the depletion of nutrients in the medium which stressed the fungal physiology resulting in the inactivation of secretary machinery of the enzymes **(Nochure *et al*., 1993)**.

This is due to the fact that during this phase the microbes were in stationary phase. The results are in accordance with those of earlier work conducted on celluloytic fungi by **(Aurangzeb *et al*., 1997)**.

Further increase in the incubation period did not show any enhancement for cellulase production **(Haq *et al*., 2005).**

5.3 Effect of pH on enzyme production

pH is important for the growth of organism and the production of the enzyme. In this study *T. viride* has the ability to grow between 3.5-8.5 pH and we found in this study that the best pH suitable for the *T. viride* growth is at pH 5.5 and this is clear in Figure 4.10.

This result also agree with the previous reported results by Gatuam *et al*. in 2010 where the production of the enzyme increases at pH 5-6 and the largest amount enzyme production was at pH 5.5.

Effect of pH on cellulase production by these fungi is supported by the findings of previous study **(Lee *et al*., 2002)** who reported that CMCase, Avicelase and FPase activities exhibit an optimum pH of approximately 4, while the pH optimum of β-glucosidase was between pH 5 to 6.

Our study agree **(Haltrich *et al*., 1996)** optimum pH for fungal cellulase varies from species to species. Both high acidic and high basic pH shows negative effects, but a medium with low acidic pH 5.5 was ideal for enzyme production. This might be due to the fact that fungal cultures require slightly acidic pH for their growth and enzyme biosynthesis.

5.4 Effect of temperature on enzyme production

Temperature is also an important environmental factor that influences the cellulase yield.

In this study *T. viride* has the ability to grow at a broader range of temperature from 20-45°C (Figure 4.4). It was found in this study that the best temperature for the production of cellulase enzyme is at 30°C and for the growth of *T. viride* as well.

This result is almost like previous study made by (Shafique *et al*., 2009) where it was found that the optimum temperature to produce a greater amount of cellulase enzyme by *T.viride* is between 40-50 °C.

The optimum temperature for cellulase production from *T. reesei* was 15-28°C and optimal temperature for its growth was 30 °C **(Esterbaur *et al*., 1991)**.

The study achieved by Smith and Wood in 1991 showed the optimum temperature of 30°C and 35°C for the production of extracellular xylanase and xylosidase by *Aspergillus awamori* .

Many workers have reported different temperatures for maximum cellulase production either in flask or in fermentor studies using *Trichoderma* sp. suggesting that the optimal temperature for cellulase production also depends on the strain variation of the microorganism **(Murao *et al*., 1988; Lu *et al*., 2003)**.

5.5 Effect of carbon sources on enzyme production

Carbon sources play a vital role in the cell metabolism and synthesis of cellulase enzyme. The effect of carbon sources on the production of cellulase enzyme by *T. viride* was investigated.

Carbon sources tested for production of cellulase enzyme by *T. viride* were sucrose, cellulose, carboxymethylcellulose and maltose ranging from 0.5 to 3 % (w/v).

We found that sucrose (1.0%) is the best carbon source for the production of cellulase enzyme. Cellulase production increased with increasing the initial sugar concentration from 1.0-1.5% while further increase in sugar concentration slightly reduce the yeild and this result is agree with the previous study **(Gatum *et al*., 2010)**. It was found that sucrose is more efficient as a sole carbon source in the center for the production of cellulase enzyme.

(Mendals and Reese, 1957) also reported that maximum yields of cellulases were obtained on 1% different carbon sources using cellulase production commended on reaching nitrogen limiting conditions and the yield of cellulase decreased when excess peptone was presented **(Solomon *et al*., 1997; Lee *et al*., 2010; Sherief *et al*., 2010).**

In the present study, the media containing sucrose and maltose induced the production of cellulase. Our results in accordance with previous studies which reported that lactose, avicel and Carboxymethylcellulose induced the production of cellulase by *Trichoderma* spp. **(Baig, 2005; Szakacs *et al*., 2006)**.

5.6 Effect of nitrogen sources on enzyme production

To study the effect of different nitrogen sources on the production of cellulase enzyme, different sources of nitrogen were used in this study where it has been replaced by nitrogen sources in the basal salt media by sources of nitrogen such as peptone, sodium nitrate, beef extract and yeast extract at different concentrations from 0.5 to 3.0% (w/v).

we found that the highest cellulase enzyme production on yeast extract (1.0%) and this is clear in Figure 4.17. It is evident from Figure 4.19 the least cellulase enzyme production was recorded on sodium nitrate medium Similarly, Ahamed and Vermette (2008) revealed that yeast extract yielded the highest CMCase activity by *T. reesei* RUT-C30.

The maximum enzyme activities were obtained with yeast extract (1.0%) which brought about an improvement in all the three cellulase components, including exoglucanase (2.40 U/ml), endoglucanase (2.28 U/ml) and β-glucosidase (1.99 U/ml) where peptone also produce second most cellulase producing nitrogen source by *Trichoderma* sp. It was reported that good cellulase yield can be obtained with ammonium compound as the nitrogen source **(Gatum *et al*., 2010)**.

Though the addition of organic nitrogen sources such as beef extract and peptone resulted in increased growth and enzyme production, as reported before, they were not an effective replacement for inorganic nitrogen sources because of their higher cost **(Sun *et al*., 1999)**.

In 1998 Mangat and Mandahr showed that nitrogen sources greatly influence cellulase biosynthesis hence they should be used with proper concentration. High concentration of nitrogen sources may lead to virtification (the medium appears to be yellow and glassy) that is usually unstable for he microorganisms.

Chapter 6

Conclusion and recommendation

6.1 Conclusion

Cellulase is an enzymes catalyse reaction that degrade insoluble cellulose to soluble carbohydrates. In recent years attention in cellulase has increased because of the many potential applications for these types of enzymes for example cellulases are engaged in research and development and in the production of bioenergy and in textile, laundary, pulp, paper etc,...…

Solid waste is the largest collection of waste that causes environmental pollution. Cellulose which occupies a large part of the waste is fiber, insoluble and has a high molecular weight. It also an excellent source of carbon for the production of cellulase enzyme by fungi including *Trichoderma* spp.

It is known that *Trichoderma* is an excellent source for the production of cellulase enzyme. Our study focuses on isolating Trichoderma from various sources such as air and soil. We have succesieded to isolate three species of Trichoderma that have been identified in two ways, macroscopic and microscopic as *T.harizanium*, *T. reesi*, *T. viride*. During the screening stage to find out which species of *Trichoderma* is more productive to get cellulase enzyme. The result was *T. viride* and therefore cellulase enzyme has been produced by *T. viride* in basal salt media.

In the present study, the effect of environmental factors such as pH and temperature on *Trichoderma* spp. were studied. For estimation of glucose amount produced by cellulase enzyme, a glucose kit assay method was used.

6.2 Recommendations

The present work is part of extensive research devoted to meeting challenges and requirements that human society currently encounters in sustainable development. The production of cellulase enzyme become an important issue, so further studies should be performed and the following recommendations should be taken in account:

1. It is possible to depend on this results in the process of industrial application where several factors were identified for the best production of cellulase enzyme.

2. The use of solid state fermentation method for the production of cellulase enzyme.
3. Evaluation of the Industrial and agricultural waste for the production of cellulase.
4. Purification and characterization of Cellulase enzyme produced by *Trichoderma* spp.
5. Studies should be required to determine the inhibitory concentration of simple sugars for the production of cellulase by *Trichoderma* isolates.
6. The Cellulase enzyme should be evaluated for industrial purposes such as textile industry, animal feed and ethanol.

References

Acharya, P., Acharya, D. K. and Modi, H. A. (2008). **Optimization for cellulase production by *Aspergillus niger* using saw dust as substrate**. *African Journal of Biotechnology*, 7(22): 4147- 4152.

Aneja, K. R. (2005). **Experiments in Microbiology, Plant Pathology and Biotechnology**. *New Age International*, 4th Ed, New Delhi.

Andade, J. P. and Marbach, P. A. S. (2003). **Production and partial characterization of cellulases from *Trichoderma* spp. IS-05 isolated from sandy coastal plains of Northeast Brazil**. *Universidade Federal do Recôncavo da Bahia (UFRB), Centro de Ciências Agrárias*, pp.1-24.

Aurangzeb, M., Jalal, N. and Kauser, T. (1997). **Isolation of cellulolytic fungi from indigenous sources and shake flask studies on their nutritional requirements for enzyme production**. *Biologia*, 43(1): 113-119.

Baig, M. M. V. (2005). **Cellulolytic enzymes of *Trichoderm lignorum* produced on banana agrowaste: optimization of culture medium and conditions**. *Journal of Scientific and Industrial Research*, 64:57-60.

Bhat, M. K. (2000). **Cellulases and related enzymes in biotechnology**. *Biotechnology advances*, 18:355-383.

Bhat, M. K. and Hazlewood, G. P. (2003). **Enzymology and other characteristics of cellulases and xylanases**. **(**Bedford M. R. and Partridge

G.G. (Eds.), **In: Enzymes in Farm Animal Nutrition,** pp: 11-60, *CAB international*, Cambradge.

Baldrian, P. (2008). **Enzymes of sprotrophic basidiomycetes. In: Ecology of Saprotriphic Basidiomycetes**. *Elsevier Ltd*.,1 st Edition, London.

Bissett, J. (1991b). **A revision of the genud *Trichoderma* III. Section Pachybasium**. *Canadian Journal of Botany* 69, 2373-2417.

Chinedu, S. N., Okochi, V. I. and Omidiji, O. (2011). **Cellulase Production By Wild Strains of *Aspergillus niger*, *Pencillium chrysogenum* and *Trichoderma harzianum* Grown On Waste Cellulosic Materials**. *Journal of Science*, 13(1):57-65.

Davies, G. and Henrissat, B. (1995). **Structures and mechanisms of glycosyl hydrolases**. *Advances in Applied Microbiology*, 56: 853-859 .

DeCarvalho, L. M. J., deCastro, I. M. and DaSilva, C.A.B. (2008). **A study of retention of sugars in the process of clarification of pineapple juice (Ananas comosus, L. Merril) by micro- and ultra-filtration**. *Journal of Food Engineering*, 87 (4): 447–454.

Demain, A. L., and Newcomb, M. (2005). **Cellulase, clostridia, and ethanol**. *Microbiology and Molecular Biology Reviews*, 69(1): 124-154

Deschamps, F., Giuliano, C., Asther, Ma., Huet, M. (1985). **Cellulases production by *Trichoderma harzianum* in static and mixed solid state fermentation reactor under nonasceptic conditions**. *Biotechnology and Bioengineering*, 27(13):28-31.

Esterbauer, H., Steiner, W., Labudova, I., Heermann, A. and Hayn, M. (1991). **Production of *Trichoderma* cellulases in laboratory and pilot scale**. *bioresource technology*, 36: 51-56.

Gautam, S. P., Bundela, P. S., Pandey, A. K., Jamaluddin Awasthi, M. K., and Sarsaiya, S. (2010). **Optimization of the medium for the production of cellulase by the *Trichoderma viride* using submerged fermentation**. *International Journal of Environmental Sciences*, 1(4): 656- 665.

Gams, W. and Bissett, J. (1998). **Morphology and identification of *Trichoderma***. In G. E. Harman, & C. P. Kubicek (Eds.), ***Trichoderma* & *Gliocladium***, Vol. 1 (pp. 3-43). London: *G. E. Taylor and Francis*.

Gielkens, M. C., Dekkers, E., Visser, J. and Graaff, H. (1999). **Two cellobiohydrolase encoding gene from *Aspergillus niger* require Dxylose and the xylanolytic transcriptional activator Xln R for their expression**. *Applied and Environmental Microbiology*, 65(10): 4340-4345.

Goldschmidt, F. (2008). **From Cellulose to Ethanol: Engineering Microorganisms to Produce Biofuel**. *Institute of Biogeochemistry and Pollutant Dynamics*, pp:1-17.

Gormsen, E., Marcussen, E., Damhus, T. (1998). **Enzymes in: Powdered Detergents**. *Surfactant Science Series*, Dekker, New York, pp: 137–163.

Haltrich, D., Nidetzky, B., Kulbe, K., Steiner, W. and Zupancic, S. (1996). **Production of fundal xylanases**. *Bioresource Technology*, 58(2):137-161.

Han, S. J., Yao, Y. J. and Kang, H. S. (1995). **Characterization of a bifunctional cellulose and its structural gene**. *Journal of Biological Chemistry*, 270 (43): 26012- 26019.

Haq, I., Hameed, U., Shahzadi, K., Javed, M.M., Ali, S., Qadeer, M.A. (2005). **Cotton saccharifing activity of cellulases by *Trichoderma harzianum* UM–11 in shake flask**. *International Journal of Botany*, 1(1): 19-22.

Hawary, F. I. and Mostafa, Y. S. (2001). **Factors affecting cellulases production by *Trichoderma koningii***. *Acta Alimentaria*, 30(1):3-13.

Headon, D. R. and Walsh, G. (1994). **The industrial production of enzymes.** *Biotechnology*, 12: 635-646.

Henrissat, B., Teeri, T. T., and Warren, R. A. J.(**1998**). **Ascheme for designating enzymes that hydrolyse the polysaccharides in the cell walls of plants**. *FEBS Letters*, 425(2): 352 - 354.

Hoyos-Carvajal, L., Orduz, S., Bissett, J. (2009). **Genetic and metabolic biodiversity of Trichoderma from Colombia and adjacent neotropic regions**. *Fungal Genetics and Biology*, 46: 615‑631.

Hussein, N. A., Sohair, N. A., Abozaid, A. A. and Alsalemi, F. A. (2011). **Cellulase production by two fungal strains isolated from Taif in Saudi Arabia**. *Journal of Agricultural Science*, 19(1): 171-178.

Kang, S. W., Park, Y. S., Lee, J. S., Hong, S. I., and Kim, S. W. (2004). ***Production of cellulases and hemicellulases by Aspergillus niger* KK2 *from lignocellulosic biomass***. *Bioresource Technology*, 91: 153-156.

Khare, A. and Upadhyay, R. S. (2011). **Influence of some cultural factors on production of cellulase and β-1,3-glucanase by the mutant strains of *Trichoderma viride* 1433**. *Journal of Agricultural Technology*, 7(2): 403-412.

Kim, B. H. and Wimpenny, J. W. T. (1981). **Growth and cellulytic activity of *Cellulomonas flavisin***. *Canadian Journal of Microbiology*, 27: 1260-1266.

Kirk, O., Borchert, T. V., Fuglsang, C. C. (2002). **Industrial enzyme applications**. *Current Opinion in Biotechnology*, 13(4):345-51.

Kubicek P. C. (2002). ***Trichoderma* and *Gliocladium* Volume 1**. *Taylor & Francise-Library*, London, P: 8-9.

Kubicek C. P. (1993). **From cellulose to cellulase inducers: facts and fiction in Proceedings of the 2nd Symposium *Trichoderma Reesei* Cellulases and Other Hydrolases**, In P. Suominen and T. Reinikainen, (Eds.), **Foundation for Biotechnical and Industrial Fermentation Research**, pp. 181–188. *Espoo*, Finland,**.**

Latif, F., Plus, J. and Malik, K. A. (1998). **Effect of alkali pretreatment on enzymatic hydrolysis of plant growing on saline lands**. *Biomass*., 17: 105-114.

Leahy, J. G., Colwell, R. R. (1990). **Microbial degradation of hydrocarbons in the environment**. *Microbiology review journals*, 54(3): 305-315.

Lee, R. L., Paul, J. W., vanZyl, W. H., and Pretorius, I.S. (2002). **Microbial cellulose utilization: Fundamentals and biotechnology**. *Microbiology and Molecular Biology Reviews*, 66: 506-577.

Lee, B. H., Kim, B. K., Lee, Y. J., Chung, C. H. and Lee, J. W. (2010). **Industrial scale of optimization for the production of carboxymethylcellulase from rice bran by marine bacterium, *Bacillus subtilis* subsp. *subtilis* A53**. *Enzyme Microbiol*. *Technology*, 48: 38-42.

Lu, W., Li, D., and Wu, Y. (2003). **Influence of water activity and temperature on xylanase biosynthesis in pilotscale solidstate fermentation by *Aspergillus sulphurous***. *Enzyme and Microbial Technology*, 32: 305-311.

Macris, H. J., Paspaliari, M. and Kekos, D. (1985). **Production and cross synergistics action of cellulytic enzymes from certain fungi mutants grown on cotton and straw**. *Biotechnology Letters*, 7:183-186.

Malik, S. K., Mukhtar, H. and Haq, I. U. (2010). **Optimization of process parameters for the biosynthesis of cellulase by *Trichoderma viride***. *Pakistan Journal of Botany*, 42(6): 4243-4251**.**

Mandels, M., and Reese, E. T. (1957). **Induction of cellulase in *Trichoderma viride* as influenced by carbon sources and metals**. *Journal of Bacteriology* , 73: 269-278.

Mangat, M. K. and Mandhar, C. L. (1998). **Effect of cultural conditions on production of cellulases by *Helminthosporium teres*: *Res. Bull***. *Panjab University Research*, 46(1): 139-145.

Mekala, N. K., Singhania, R. R., Sukumaran, R. K. and Pandey. (2008). **Cellulose production under solid-state fermentation by *Trichoderma ressei* RUT C30: Statistical optimization of process parameters**. *Applied Biochemistry and Biotechnology*, 151(2-3): 122-31.

Moosavi-Nasab, M. and Majdi-Nasab, M. (2007). **Cellulase Production by *Trichoderma reesei* using Sugar Beet Pulp**. *Iran Agricultural Research*, 25(2): 107-116.

Murao, S., Sakamoto, R. and Arai, M. (1988). **Cellulase of *Aspergillus aculeatus***. In Wood, W.A. and Kellog, S.T., (Eds.), **Methods in Enzymology,**. *Academic Press Inc*., London, 160: 275-284.

Nathan, V. K., Rani, M. E., Rathinasamy, G., Dhiraviam K. N. and Sridhar, J. (2004). **Process optimization and production kinetics for cellulase production by *Trichoderma viride* VKF3**. *SpringerPlus*. 3:92

.

Neagu A. D., Destain J., Thonart P., Socaciu C.(2012). ***Trichoderma Reesei* Cellulase Produced by Submerged Versus Solid State Fermentations.** *Bulletin UASVM Agriculture,* 69(2)/2012

Nochure, S.V., Roberts, M. F. and Demain, A. I. (1993). **True cellulases production by *Clostridium thermocellum* grown on different carbon sources**, *Biotechnology Letters*, 15: 641-646.

Palestinian Central Bureau of Statistics, the Palestinian Ministry of Health. (2014). **Environment survey for the governmental and civil health facilities**, *basic results. Ramallah* - Palestine.

Pandey, A. (1992). **Recent process developments in solid state fermentation**. *Process Biochemistry*, 26: 355 -361.

Papavizas, G. C. (1985). ***Trichoderma* and *Gliocladium*: biology, ecology, and potential for biocontrol**. *Annual Review of Phytopathology*, 23: 23-54.

Pothiraj, C., Balaji, P. and Eyini, M. (2006). **Enhanced production of cellulases by various fungal cultures in solid state fermentation of cassava waste**. *African Journal of Biotechnology*, 5:1882-1885.

Rahman, A., Begum, F., Rahman, M., Bari, M., Ilias, G. and Alam, M. (2009). **Isolation and identification of *Trichoderma* species from different habitats and their use for bioconversion of solid waste**. *Tubitak*, 35 (2011): 183-194.

Rajoka, M. I. and Malik, K. A. (1997). **cellulase production by *Cellulomonas biazotea* cultured in media containing different cellulosic substrates**. *Bioresource technology*, 59: 21-27.

Ramesh, C.K., Gupta, R. and Singh, A. (2011). **Microbial cellulases and their industrial applications**. *Enzyme research* , 2011:1-10.

Rifai, M. A. (1969). **A revision of the genus *Trichoderma***. *Commonwealth Mycological Institute*, Kew Surrey, England, Mycological papers no. 116-156.

Saddler, J. N., Hogan, C. M., Chan, M. K. H. and Louis, S. G. (1982). **Ethanol fermentation by enzymatically hydrolyzed and pretreated wood fractions using *Trichoderma* cellulases, *Zymomonas mobilis* and *Saccharomyces cerevisiae***. *Canadian Journal of Microbiology*, 28: 1311.

Sang-Mok, L. and Koo, Y. M. (**2001**). **Pilot-scale production of cellulase using *Trichoderma reesei* Rut C-30 in fed-batch mode**. *Journal of Microbiology and Biotechnology*, 11 (2): 229–233.

Sherief, A. A., ElTanash, A. B., and Atin, N. (2010). **Cellulase production by *Aspergillus fumigatus* grown on mixed substrate of rice straw and wheat bran**. *Research Journal Microbiology*, 5(3):199-211.

Smith, D. C. and Wood, T. M. (1991). **Xylanase production of *Aspergillus awamori* development of medium and optimization of the fermentation parameters for the production of extra cellular xylanase of ß–xylosidase while maintaining low protease production**. *Journal of Bioscience and Bioengineering*, 38: 838–90

Solomon, B. O., Amigun, B., Betiku, E., Ojumu, T. V. and Layokun, S. K., (1997). **Optimization of cellulase production by *Aspergillus flavus* Linn**

isolates NSPR 101 grown on bagasse. *Journal of Nigerian Society of Chemical Engineering*, 16: 61–68.

Sternberg, K., Bollok, M., Reczey, K., Galbe, M. and Zacchi, G. (2000). **Effect of substrate and cellulase concentration on simultaneous sacchrarification and fermentation of steam pretreated softwood for ethanol production**. *Biotechnology and Bioengineering*, 62(2): 204-210.

Sukumaran, R. K., Singhania, R. R., Pandey, A. (2005). **Microbial cellulases: production, applications and challenges**. *Journal of Scientific and Industrial Research*, 64: 832–844.

Sun, T., Liu, B. H., Li, Z. H. and Liu, D. M. (1999). **Effect of air pressure amplitude on cellulase production by *Trichoderma viride* SL1 in periodic pressure solid state fermentation**. *Process Biochemistry*, 34: 25-29.

Szakacs, G., Tengerdy, R. T. and Nagy, V. (2006). **Cellulases**. (Pandey, A., Colin, W., Soccol, C. R. and Lorroche, C., (Eds) **In: Enzyme Technology,** *Spring Science +Besiness Media, Inc. and Asiatech*.

Vyas, A. and Vyas, D. (2005). **Production of cellulases by solid state bio-of**

ground nut shell wastes. *Journal of Scientific and Industrial Research*, 64:767770.

Williams, J., Clarkson, J. M., Mills, P. R., and Cooper, R. M., (2003). **A Selective Medium for Quantitative Reisolation of *Trichoderma harzianum* from *Agaricus bisporus* Compost**. *Applied and Environmental Microbiology*, 69(7): 4190–4191.

Zahri, S. S., Reza, M. Z., Motallebi, M. and Sadeghi, M. (2005). **Cloning and characterization of cbhII gene from *Trichoderma parceramosum* and**

its expression in Pichia pastoris. *Iranian Journal of Biotechnology*, 3:204-215.

Zhou, J., Wang, Y. H., Chu, J., Zhuang, Y. P., Zhang, S. L. and Yin, P. (2008). **Identification and purification of the main components of cellulases from a mutant strain of *Trichoderma viride* T 100-14**. *Bioresource Technology*, 99: 6826-6833.