Republic of Iraq
Ministry of Higher Education
and Scientific Research
Al- Nahrain University
College of Science
Department of Biotechnology

Production, Characterization and Genetic Study of *Pantoeadispersa* Producing Cellulase and pectinase

A dissertation

Submitted to the Council of theCollege of Science, Al- Nahrain University,in PartialFulfillment of the Requirements for the Degree of Doctorate of PhilosophyinBiotechnology

By

MohmmedLeftaAtala

B.Sc. Biology/Baghdad University/ 1993
M.Sc. Biotechnology/ Baghdad University, 2000

Supervised by

Dr. Hameed M. JasimDr. Kadhim M.Ibrahim ProfessorProfessor

FebruaryRabi'ulThani
2015 1436

بسم الله الرحمن الرحيم

صدق الله العلي العظيم

سورة النمل- الآية (19)

Supervisor (s) certification

We, certify that this dissertation entitled " **Production, Characterization and Genetic Study of *Pantoea dispersa* Producing Cellulase and Pectinase** " was prepared by " **Mohmmed Lefta Atala** "under our supervision at the College of Science, Al- Nahrain University as a partial fulfillment of the requirements for the Degree of Doctorate of Philosophy in Biotechnology.

Signature:

Name: Dr. Hameed M. Jasim

Scientific Degree: Professor

Date:

Signature:

Name: Dr. Kadhim M. Ibrahim

Scientific Degree: Professor

Date:

In view of the available recommendation , I forward this dissertation for debate by the examining committee.

Signature:

Name: Dr. Hameed M. Jasim

Scientific Degree: Professor.

Title : Head of Biotechnology Department

Date:

Committee certification

We, the examining committee, certify that we have read this dissertation entitled " **Production, Characterization and Genetic Study of *Pantoea dispersa* Producing Cellulase and Pectinase** " and examined the student " **Mohmmed Lefta Atala** " in its contents and that in our opinion it is accepted for the degree of Doctorate of Philosophy in Biotechnology.

(**Chairman**)
Signature:
Name: Dr. Majid H. Algelawi
Scientific Degree: (Professor)
Date:

(Member)
Signature:
Name: Dr. Ghazi M.Aziz
Scientific Degree: (Professor)
Date:

(Member)
Signature:
Name: Dr. Sanad B. Arraji
Scientific Degree: (Professor)
Date:

(Member)
Signature:
Name: Dr. Nadhem H. Hyder
Scientific Degree: (Assist. Professor)
Date:

(Member)
Signature:
Name: Dr. Ali Shihab Ahmed
Scientific Degree: (Assist. Professor)
Date:

(Member)
Signature:
Name: Dr. Hameed M. Jasim
Scientific Degree: (Professor)
Date:

(Member)
Signature:
Name: Dr. Kadhim M. Ibrahim
Scientific Degree: (Professor)
Date:

I, hereby certify upon the decision of the examining committee.

Signature:

Name: Dr. Hadi M. A. Abood
Scientific Degree: Assistant professor
Title: Dean of the College of Science
Date:

TO MY PARENTS

Acknowledgements

Praise to God the first cause of all causes the glorious creator of the universe for his mercy and kindness, and blessings upon Mohammed prophet of God and his family.

I wish to express my deep gratitude and appreciation to my supervisors professor Dr. Kadhim M. Ibrahim & professor Dr. Hameed M. Jasim, whom without their continuous advice and guidance, this study wouldn't have been completed. Acknowledgments are due to of Dr. Abdul wahid Shamkhi, Dr. Rehab S. Ramadhan, Dr. Majid Hussein Al-gelawi, and Mr. Zaid Nsaif Abass of the Biotechnology Department, science college, Al-Nahrain University; I would like to express deep gratitude and appreciation to Dr. majeed Arsheed Sabbah of DNA Forensic Center of Al- Nahrain University and to Dr. Rabah Najah Jabbar Al-Gafari of Al- Nahrain Center for Biotechnology and to Dr. Munira CH. Ismail of Biological Researches Unit of Baghdad university.

I am very much thankful to the staff of Center Public Health Lab, especially Mr. Abdl AlKareem Hammed; Mr. Abdul Khaliq Abbas, Mrs. Bayan Hassan Hadi, Dr. kifah Ahmed Jassim, Mr. Mudaffer qader; the staff of Ibn - Albalady Hospital, especially Mr. Haider Zaidan for their kind assistance. My thanks are devoted to the postgraduate students and the staff of Biotechnology Department.

Finally, I wish to thank my family, may God bless them.

MOHAMMED

Summary

Soil samples were collected from fields cultivated with wheat and barley and from spoiled orange, apple fruits and carrot roots to enzyme isolate bacteria producing lytic. Bacterial isolates were screened for enzymes production on selective media containing carboxymethyl cellulose (CMC) or pectin as a sole source for carbon and energy. Results of semi-quantitative screening showed that nine isolates from fifty isolates were able to produce cellulase and pectinase with variable degrees depending on the formation of halo zones around colonies of these isolates. The most efficient isolates in producing cellulase and pectinase were selected and subjected to quantitative screening by determining cellulase and pectinase specific activities in the culture filtrates. Results showed that the isolate symboled A3 was the most efficient among the other isolates in cellulase and pectinase production since it produced the highest yield of these enzymes. The specific activity of cellulase and pectinase reached 3.9 and 1.31U/mg protein respectively.The selected isolate (A3) was identified according to its morphological and biochemical characteristics. Results of identification showed that this isolate was belong to *Pantoea* sp., and identification further confirmed by using Api 20-E and VITEK 2, and identified as *Pantoea dispersa*. Optimum conditions for cellulase production by local isolate *Pantoea dispersa* A3 was studied. Results showed that the optimum conditions include the supplementation of the production medium with CMC as a sole source for carbon and energy at a concentration of 1.5%, ammonium sulfate as a nitrogen source at a concentration of 0.1%; potassium dihydrogen phosphate at a concentration of, 0.1%, in the a medium adjusted to the pH 7, and incubation at 30 ºC for 72 hrs with shaking at 140 rpm. Under these conditions, specific activity of cellulase was increased to 24 U/mg protein. Cellulase produced by *P. dispersa* A3 under the optimum conditions was purified

throughout four purification steps includes ammonium sulfate precipitation step with a saturation ratio of 70% then dialysis step followed by purification with ion exchange chromatography by using DEAE-cellulose, and then gel filtration step throughout sephadex-G200 which allows larger ability of separation with high degree of purification. Results of purification showed that the specific activity of the purified enzyme was 190.9 U/mg protein with a purification fold and yield of 113.6 and 26.2% respectively. Purified cellulase from *P. dispersa* A3 was well characterized by studying some enzyme characteristics. Results showed that the molecular weight of cellulase was 15148 dalton; pH 7.0 was the optimum for enzyme activity and stability, while 30 ºC was also the optimum for enzyme activity and stability. In attempt to increase cellulase productivity from *P. dispersa* A3 by physical mutagenesis, bacterial cells from fresh culture of this isolate was subjected to random mutagenesis by UV-ray to induce random mutations affects positively enzyme production. Results of mutagenesis showed that there were nine of overproducer mutants characterized with their higher cellulase productivity were obtained after irradiation with UV-ray. Specific activity of cellulase produced by these mutants was ranged between 12.48 and 55.65 U/mg protein in comparison with the productivity of wild-type (3.9 U/mg protein). Genomic DNA of *P. dispersa* A3 was extracted by using favorgen extraction kit in order to amplify cellulase gene after designing oligoprimers including genetic elements upstream and downstream sequences of cellulase gene for different species of *Pantoea* spp. according to database found in the website of national center for for biotechnology information (NCBI). After amplification, PCR products were analyzed on agarose gel (0.8%) to identify DNA bands in presence of DNA ladder marker. Results of electrophoresis showed that there were no any PCR products identified for each pair of oligonucleotids primers, which may which may refer that these primers were not specific for amplification of cellulase gene of *P. dispersa* A3.

List of contents

List of contents

List of contents

List of contents

List of contents

List of contents

List of contents

List of contents

List of Tables

List of figures

List of figures

List of abbreviations

Abbreviations	Full name
BSA	Bovine Serum Albumin
CAZy	Carbohydrate-active enzymes
CBH	Cellobiohydrolases
CBMs	Cellulose-binding domains
CMC	Carboxymethyl cellulose
CWDE	Cell wall degrading enzymes
D.D.W	Deionized distilled water
DEAE	Diethylaminoethyl
DNS	Dinitro salycilic acid
EDTA	Ethylenediaminetetracetic acid
EG	Endoglucanase
F	Forward
HMMs	Hidden markov models
IAA	Indole acetic acid
KDa	Kilo dalton
Kb	Kilobase
LD	Lethal dose
MR-VP	Methyl red-Voges Proskauer
MLSA	Multilocus sequence analysis
MLST	Multilocus sequence typing
NCBI	National Center of Biotechnology Information
O. D.	Optical density
PCR	Polymerase chain reaction
PPases	Protopectinases
R	Reverse
rpm	Rotation per minute
SMF	Submerged fermentation
spp	Sub-species

List of abbreviations

SSC	Solid state cultivation
SSF	Solid state fermentation
TBE	Tris Borate EDTA buffer
V_e	Elution volume
GH	Glycoside hydrolase
ppases	Protopectinases
PMG	Polymethylgalacturonases
PG	Polygalacturonases

Chapter One
Introduction

1. Introduction

Pantoea dispersa is a Gram-negative bacteria belong to the family of Enterobacteriaceae. It is rod in shape nonencapsulated, nonsporeforming, motile, peritrichously flagellated, yellow-pigmented bacterium isolated on nutrient agar plates incubated at 4 °C. The identity of the bacterium was confirmed by sequencing of the 16 S rRNA gene. It is capable of growing at temperatures ranging from 4 to 41 °C, but maximum growth was observed at 30 °C. It is endowed with multiple plant growth promotion attributes such as phosphate solubilization, indole acetic acid (IAA) production and siderophore production, which are expressed differentially at sub-optimal temperatures of 15 and 4 °C (Gavini *et al*., 1989; Selvakumar *et al*., 2008).

Cellulases and pectinases are potential plant cell wall degrading enzymes that convert, the most abundant and renewable source of energy on earth to glucose and soluble sugars. Studies have demonstrated that the enzyme-induced bioconversion of lignocelluloses and pectins are rather difficult and uneconomical. Nevertheless, continued research on cellulases, hemicellulases and pectinases revealed their biotechnological potential in various industries, including food, brewery, animal feed, textile and laundry, plant pathogen and disease control, fusion of plant and fungal protoplasts and conversion of cellulosic materials to ethanol (Bajpai, 1999; Taleb *et al*., 2009). Plant cell wall is a natural physical barrier against pathogens and is at the forefront of the contents of plant cells. Many phytopathogenic bacteria produces cell-wall degrading enzymes (CWDE) such as cellulase, pectinase, xylanase and glycosidase. CWDE function is to break down the components of host cell walls and may play a crucial role in virulence and bacterial nutrition (Liu *et al*., 2005; Rajeshwari *et al*.,

2005). The process of photosynthesis is the main route for the acquisition of energy in plant biomass of which cellulose is the major component. Release of energy and return of bio-sequestered carbon to soil environment are primarily the concern of cellulose utilizing special group of microorganisms called cellulolytic organisms (Narasimhan *et al*., 2013; Saha *et al*., 2013). The complete enzymatic hydrolysis of cellulosic material needs three types of enzymes, namely, cellobiohydrolase, endoglucanase or carboxymethylcellulase (CMCase), and beta-glucosidases (Yi *et al*., 1999). The endoglucanase randomly hydrolyzes ∝-1, 4 bonds in the cellulose molecule, and the exocellobiohydrolases in most cases release a cellobiose unit showing a recurrent reaction from chain extremity. Lastly, the cellobiose is converted to glucose by ∝-glucosidase (Bhat, 1997).

Pectinases are the group of enzymes that catalyzes the degradation of pectic substances through de-polymerization and de-esterification reactions. Pectinolytic enzymes are classified according to their mode of action on the galacturonan part of the pectin molecule. Pectinase enzymes are commonly used in degrading of plant materials and in extraction of fruit juice (Pedrolli *et al*., 2009).

Many microorganisms like bacteria, yeast, fungi, could produce cellulases and pectinases. Bacteria from the genus *Pantoea* have become increasingly important plant pathogens around the world. Many plant pathogenic *Pantoea* spp. are seed borne and seed transmitted such as *P. agglomerans* in cotton (Medrano and Bell, 2007). It also causes leaf blight and bulb rot in onion. Strain improvement for cellulase production via UV–light as mutagenic agent has attracted a great attention owing to its

efficiency. UV was also utilized for strain improvement in *Pantoea* spp. The activity of carboxymethylcellulase was improved respectively when compared to parent strain (Sangharak *et al*., 2012).

Plant residues represent major environmental hazard without an appropriate recycling strategy. The huge biomass has to be utilized through carbon and nitrogen cycle. This imposes the need for an efficient microorganism that degrades such residues to useful materials for the industry particularly for biofeul production.

The ability of *P. dispersa* to produce cellulases and pectinases enzymes have not been locally studied. Therefore this study was aimed to:

1- Isolation of bacteria producing cellulase and pectinase.

2- Selection of isolates especially those produce more than one degrading enzymes.

3- Identification of the selected isolate.

4- Improvement of cellulase production by random mutagenesis for the efficient isolate.

5- Molecular detection of gene (s) coding for cellulase by using PCR technique.

Chapter two

Literature review

2. Literature Review

2.1. Genus *Pantoea*

Scientific classification
Kingdom: Bacteria
Phylum : Proteobacteria
Class : Gammaproteobacteria
Order : Enterobacteriales
Family: Enterobacteriaceae
Genus : *Pantoea*
(Selvakumar *et al*., 2008)

The genus *Pantoea* includes several species that are generally associated with plants, either as epiphytes or as pathogens, and some species can cause disease in humans. The *Pantoea* species most commonly isolated from humans, is widely distributed in nature and has been isolated from numerous ecological niches, including plants, water, soil, humans and animals. It is frequently associated with plants as an epiphyte or an endophyte, and some isolates have been reported to be tumorogenic pathogens (Weinthal *et al.*, 2007). As an opportunistic human pathogen, *Pantoea agglomerans* occurs sporadically or in outbreaks. Seven *Pantoea* species are distinguished (Mergaert *et al*., 1993).

- *P. agglomerans*
- *P. ananatis*
- *P. stewartii*

  Subdivided to *P. stewartii* subsp. *stewartii* and *P. stewartii* subsp. *indogenes*
- *P. dispersa*
- *P. punctata*
- *P. terrea*

Pantoea is a genus of Gram-negative bacteria of the family Enterobacteriaceae, recently separated from the *Enterobacter* genus. Much knowledge about the characteristics of the genus *Pantoea* was acquired when all *Pantoea*, *Leclerrcia* and related bacteria were called *Enterobacter agglomerans*. It is difficult to differentiate *Pantoea* spp. from other members of this family, such as *Enterobacter*, *Klebsiella*, and *Serratia* species. However, *Pantoea* does not utilize the amino acids lysine, arginine, and ornithine, a characteristic that sets it apart from the other genera. On nutrient agar the bacteria of *Pantoea* spp. form mucoid colonies, smooth and irregularly round colonies, rough and wrinkled colonies that are difficult to remove with a platinum wire. Morphological and cultural properties for *Pantoea* genus is straight rods, 0.5-1.3×1.0-3.0 μm, nonencapsulated, nonsporeforming, some strains form symplasmata. Most strains are motile and are peritrichously flagellated. Colonies on nutrient agar are smooth, translucent, and more or less convex with entire margins or heterogenous in consistency and adhering to the agar. Colonies are yellow, pale beige to pale reddish yellow, or nonpigmented (Gavini *et al*., 1989).The antigenic structure of *Pantoea* spp. are uncharged capsular polysaccharides. These antigens are not removed from the cells by heating at 100 ºC for 30 min. Most members of the *Pantoea* ssp. are motile, but there is no report on the antigenic structure of the flagella. Most strains produce antigenically similar high molecular weight acidic polysaccharides. Most strains initially identified as *P. agglomerans* by using of API 20-E strips belonged to a compact sequence cluster together with the type strain, but other strains belong to diverse phylogenetic branches corresponding to other species of *Pantoea* or *Enterobacteriaceae*

and probable novel species (Rezzonico *et al*., 2012).

Although members of the genus *Pantoea* are primarily plant pathogens, they occur in many ecological habitats, including in association with soil, water, dairy products, meat, fish, humans, and animals, plant surfaces, seeds, human clinical specimens. Precise knowledge of the phylogenetic relationships and the degree of genetic distinctness among *Pantoea* species is a prerequisite for their correct identification. Phylogenetic relationships among *Pantoea* species were initially based on 16S rRNA analysis, which showed that *P. stewartii*, *P. ananatis* and *P. dispersa* are the most closely related species and meso-tartrate was used by the same group (85% of *P. agglomerans* strains) except for *P. ananatis* (Brady *et al*., 2010; Deletoile *et al*., 2009).

2.2. *Pantoea dispersa*

Pantoea dispersa is a member of Enterobacteriaceae family that inhabits plants, soil and water. *P. agglomerans*, member of this family, has previously been reported presenting as severe neonatal sepsis (Brady *et al*., 2008; Qazi and Stoll, 2009) however, *P. dispersa* has not been reported as a causative organism for neonatal sepsis. There are two neonates reports with early onset sepsis caused by *P. dispersa*. Blood culture report of infected humans showed *P. dispersa* organism is sensitive to amikacin, cefepime, ceftriaxone, ciprofloxacin, meropenem, aztreonam and resistant to cefazolin. *P. dispersa* is a Gram-negative rod-shaped, yellow-pigmented bacterium isolated on nutrient agar plates incubated at 4°C. The identity of the bacterium was confirmed by sequencing of the 16S rRNA gene. It was capable of growing at temperatures ranging from 4 to 41 °C, but maximum growth was observed at 30 °C. It is endowed with multiple plant growth

promotion attributes such as phosphate solubilization, IAA production, siderophore production and HCN production, which are expressed differentially at sub-optimal temperatures (4 and 15 °C). It was able to solubilize phosphate (17.6 µg of P_2O_5 ml^{-1} day^{-1}), and produce IAA (3.7 µg ml^{-1} day^{-1}), at 15 °C.

Qualitative detection of siderophore production and HCN were also observed at 15 °C. At 4 °C, it was found to express all the plant growth promotion attributes. This bacterial isolate was able to positively influence and promote the growth and nutrient uptake parameters of wheat (cv. VL.802) under glass house conditions (Selvakumar *et al*., 2008).

2.3. Cellulases

About 200 gigatons of CO_2 are fixed of earth every year and the equivalent amount of organic material has to be degraded approximately 30% by plants and animals to 70% by microorganisms (Gottschalk, 1988). On average, cellulose accounts as 50% of the dry weight of plant biomass. Such plant biomass is the only foreseeable sustainable source of fuels and materials available to humanity. Agricultural residues are a great source of lignocellulosic biomass which is renewable, chiefly unexploited and inexpensive. (figure 2-1) Generally, cellulases are named (EC 3.2.1.4, endo-1,4-beta-D-glucanase, beta-1,4-glucanase, beta-1,4-endoglucan hydrolase etc….) and refer to a suite of enzymes produced chiefly by fungi, bacteria, and protozoans that catalyze cellulolysis. However, there are also cellulases produced by a few other types of organisms, such as some termites and the microbial intestinal symbionts of other termites (Dashtban *et al*., 2010).While several kinds of cellulases are known, which differ structurally and glycosidic linkages in cellulose, lichenin and cereal beta-D-glucans. Approximately 70%

of plant biomass is locked up in 5- and 6-carbon sugars. These sugars are found in lignocellulosic biomass comprised of mainly cellulose (a homologous polymer of glucose linked by β-1.4 glycosidic bonds) hydrolyzed by a complex enzyme system named as cellulase (exoglucanase, endoglucanase and β-glucosidase etc.).

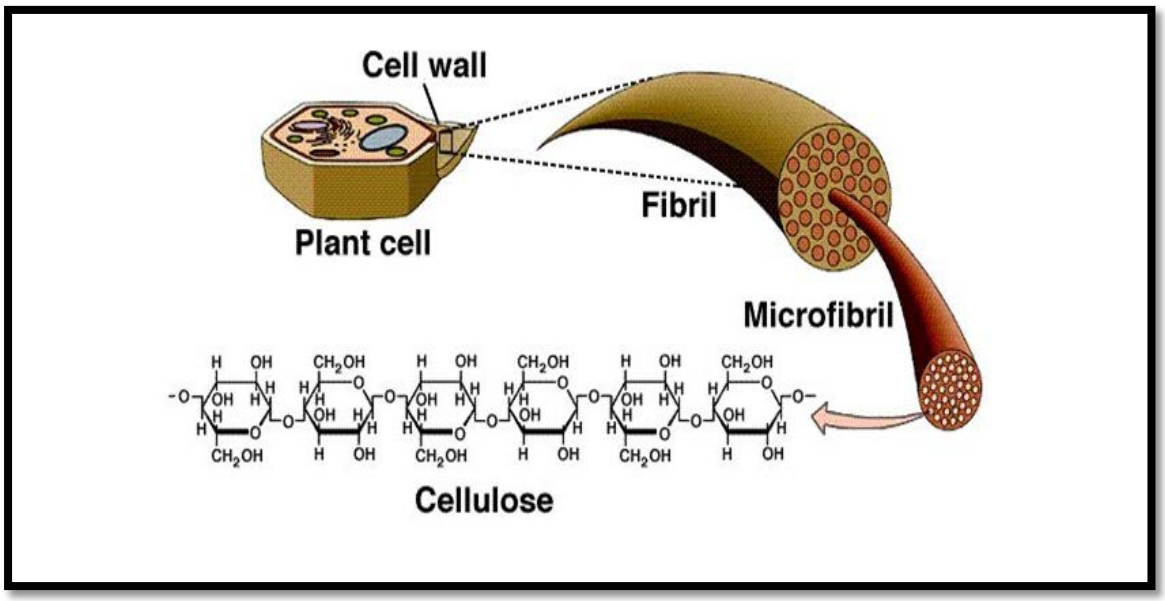

Figure (2-1): Arrangement of fibrils, microfibrils, and cellulose in plant cell wall (Ding *et al*., 2014)

Lesser hemicelluloses (heterologous polymer of 5- and 6-carbon sugars consists of pentoses D-xylose, D-arabinose and hexoses D-mannose, D-glucose, D-galactose with sugar acids); and least of all lignin (a complex aromatic polymer). In hardwoods, hemicellulose contains mainly xylans, while in softwood mainly glucomannans are present. Briefly, xylan degradation requires endo-1,4-β-xylanase, β-xylosidase, α-glucuronidase, α-L-arabinofuranosidase, as well as acetylxylanesterases. In glucomannan degradation β-mannanase and β-mannosidase are required to cleave the polymer backbone. The cellulase market has been estimated in the United

States to be as high as US $ 400 million per year (Zhang *et al*., 2006). In the period 2004-2014, an increase of approximately 100% in the use of cellulase as a specialty enzyme is expected. The biotechnology companies Genencor International and Novozymes Biotech have reported the development of technology that has reduced the cellulase cost for the cellulose-to-ethanol process from US$ 5.40 per gallon of ethanol to approximately 20 cents per gallon of ethanol, in which the two main strategies were an economical improvement in production of cellulase to reduce US$ per gram of enzyme by process and strain enhancement, e.g., cheaper medium from lactose to glucose and alternative inducer system and an improvement in the cellulase enzyme performance to reduce grams of enzyme for achieving equivalent hydrolysis by cocktails and component improvement (Knauf and Moniruzzaman, 2004). Screening for bacterial cellulase activity in microbial isolates is typically performed on plates containing crystalline cellulose or microcrystalline cellulose such as Avicel in the agar at a final concentration of 0.1-0.5% (w/v). After incubation for a suitable period, a zone of clearing surrounding the colonies will be indicated that cellulase producer by using congo red as indicator (Sadhu and Maiti, 2013). In the most familiar case of cellulase activity, the enzyme complex breaks down cellulose to beta-glucose. This type of cellulase is produced mainly by symbiotic bacteria in the ruminating chambers of herbivores. Enzymes that hydrolyze hemicellulose are usually referred to as hemicellulases and are usually classified under cellulase. In general there are two types of cellulases progressive (also known as processive) and non-progressive types. Most fungal cellulases have a two-domain structure, with one catalytic domain and one cellulose binding domain, that are

connected by a flexible linker. These enzymes might have a swelling function. In many bacteria, cellulases in-vivo are complex enzyme structures organized in supramolecular complexes, the cellulosomes. They contain roughly five different enzymatic subunits representing namely endocellulases, exocellulases, cellobiases, oxidative cellulases and cellulose phosphorylases. Recent work on the molecular biology of cellulosomes had led to the discovery of numerous cellulosome-related "signature" sequences known as dockerins and cohesins. Depending on their amino acid sequence and tertiary structures, cellulases are divided into clans and families (Bayer *et al*.,1998; Boraston *et al*., 2004) as shown in figure (2-2).

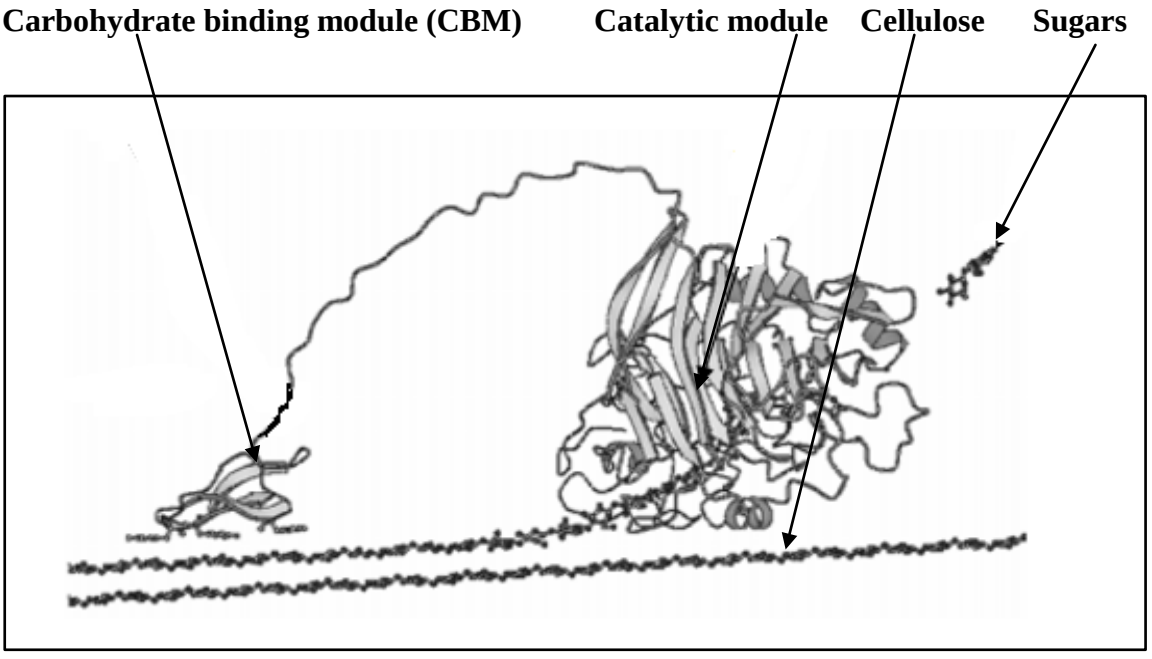

Figure (2-2): Hypothetical structure model for cellulases (Boraston *et al*., 2004)

2.4.Cellulases classification

Microorganisms produced extracellular cellulases that are either free or cell associated to hydrolyze and metabolize insoluble cellulose. Cellulase is

an inducible enzyme complex involving synergistic action. The biochemical analysis of cellulose systems from aerobic and anaerobic bacteria and fungi has been comprehensively reviewed during the past three decades. Catalytic domains of cellulases were first classified into six families with alphabetical letters, i.e. families A to F, based on amino acid sequence homology and hydrophobic cluster analysis. Six families were later added to the classification , on the other hand by using hydrophobic cluster analysis, all the available sequences of glycosyl hydrolases (EC 3.2.1.X) were compared and classified into families with Arabic numerals, currently families 1 to 58 (Henrissat *et al.*, 1989).

Generally cellulases are classified into seven types depend on their catalytic action. Endoglucanases cut at random at internal amorphous sites in the cellulose polysaccharide chain, generating oligosaccharides of various lengths and consequently new chain ends. It is generally active against acid-swollen amorphous cellulose, soluble derivatives of cellulose such as CMC. Exoglucanases act in a possessive manner on the reducing or non-reducing ends of cellulose polysaccharide chains, liberating either glucose by glucanohydrolases or cellobiose by cellobiohydrolaseas major products. These enzymes are active against crystalline substrate such as Avicel, amorphous celluloses and cellooligosaccharides, However, they are inactive against cellobiose or substituted soluble celluloses such as CMC. Cellobiohydrolases, are catalyzes the removal of cellobiose from cellooligosaccharides or from p-nitrophenyl-β –D cellobioside but inactive against amorphous cellulose or CMC. β–Glycosidase hydrolyze soluble cellodextrins and cellobiose to glucose from non-reducing end, but inactive against crystalline or amorphous cellulose. Cellobiase, is catalyzes the

reversible phosphorolytic cleavage of cellobiose. Cellodextrin Phosphorylase, is found in cells of *Clostridium thermocellulam* (Sheth and Alexander, 1969). It does not act on cellobiose but catalyze the reversible phosphorylytic cleavage of cellodextrins ranging from cellotriose to cellohexose and Cellobiose. Epimerase, is found in cells of *Ruminococcus albus* (Tyler and Leatherwood,1967) it catalyzes the Cellobiose to 4-O-β-D-glucosylmannose.

2.5. Source of cellulases

Cellulases are produced naturally by different symbiotic fungi, protozoa, and bacteria that have the ability to breakdown of cellulose, but they are manifest in fungi and microbial sources (Almin *et al*., 1975). Different kinds of bacteria able to produce cellulases like *Pantoea* spp., *Clostridium thermocellum, Pseudomonas fluorescens, Ruminococcus albus* and some kinds of Actinomycetes are able to produces these enzymes like *Streptomyces* spp., *Thermoactinomyces* spp., *Thermomonospora curvata,* also many of fungi produce these enzymes like *Chrysosporium lignorum, Schizophyllum commune, Penicillium notatum, Sporotrichum pulverulentum, Trichoderma reesei, Acremonium cellulolyticus, Aspergillus acculeatus, Aspergillus fumigatus, Aspergillus niger, Fusarium solani, Irpexlacteus, Penicillium funmiculosum, Sclerotium rolfsii, Sporotrichum cellulophilum, Talaromyces emersonii, Thielavia terrestris, Trichoderma koningi, Trichoderma viride* (Agustini *et al*., 2012; Chudasama and Thaker, 2012). However, there are also cellulases produced by a few other types of organisms, such as some termites and the microbial intestinal symbionts of other termites. In reality, there are

different types of cellulases whose activity is pH-dependent; some are more active in an alkaline environment while others are more active in an acidic or neutral one. However, humans and some of animals like cows do not manufacture the cellulase enzyme needed to digest the cellulose fiber and based on the fermentation by the flora in the large intestine of humans and in the ruminary stomach for these animals. This process allows us to break down a small portion of these plant fibers, and using the products as energy source (Akpomie *et al*., 2013).

2.6. Applications of cellulases

The most known applications of cellulases are: (Singh *et al*., 1999; Lynd, *et al*., 2005; Kuhad *et al*., 2011).

2.6.1. Agriculture

The agricultural applications are plant pathogen and disease control; generation of plant and fungal protoplasts; enhanced seed germination and improved root system; enhanced plant growth and flowering; improved soil quality; reduced dependence on mineral fertilizers**.**

2.6.2. Bioconversion

Cellulases are use in conversion of cellulosic materials to ethanol, other solvents, organic acids, single cell protein and lipids; production of energy-rich animal feed; improved nutritional quality of animal feed; improved ruminant performance; improved feed digestion and absorption; preservation of high quality fodder.

2.6.3. Detergents

Cellulases are use in the detergents industries for cellulase - based

detergents; superior cleaning action without damaging fibers; improved color brightness and dirt removal; remove of rough protuberances in cotton fabrics; antiredeposition of ink particles**.**

2.6.4. Fermentation

Cellulases are use in the improvement of fermentation for improved malting and mashing; improved pressing and color extraction of grapes; improved filtration rate.

2.6.5. Food

Cellulases are use in food industries like release of the antioxidants from fruit and vegetable pomace; improvement of yields in starch and protein extraction; improved maceration, pressing, and color extraction of fruits and vegetables; clarification of fruit juices; improved texture and quality of bakery products; improved viscosity fruit purees; improved texture, flavor, aroma, and volatile properties of fruits and vegetables; controlled bitterness of citrus fruits**.**

2.6.6. Pulp and Paper

Cellulases are use in paper industries for most biomechanical pulping; reduced energy requirement; reduced chlorine requirement; improved fiber brightness, strength properties, and cleanliness; improved drainage in paper mills; production of biodegradable cardboard, paper towels, an sanitary paper**.**

2.6.7. Textile

Textile industries are depend on the cellulase as improved agent in

biostoning of jeans; biopolishing of textile fibers; improved fabrics quality; improved absorbance property of fibers; softening of garments; improved stability of cellulosic fabrics; removal of excess dye from fabrics and restoration of color brightness**.**

2.6.8. Others

Cellulases are use in other different applications such as improved carotenoids extraction; improved oxidation and color stability of carotenoids; improved olive oil production of hybrid molecules and production of designer cellulosomes production of hybrid molecules.

2.7. Cellulase Mode of action

Cellulase mode of action based on both endoglucanases and exoglucanases. The exact mode of action of these enzymes is occurs by penetrating the cellulose fibers and attacking at random the β (1, 4) linkages along the cellulose chains by endoglucanases (EG). After this the exocellulase causes depolymerization by cutting the ends of cellulose to produces cellobiose (Shepherd *et al*., 1981).When the exoglucanase acts on the ends of the cellulose chain and releases β-cellobiose as the end product through cleaves two to four units from the ends of the exposed chains produced by endocellulase, resulting in the tetrasaccharides (Deka *et al*., 2011), or disaccharides, such as cellobiose, and there are two main types of exocellulases [or cellobiohydrolases (CBH)] , CBHI works processively from the reducing end, and CBHII works processively from the nonreducing end of cellulose ; EG randomly attacks the internal *O*-glycosidic bonds at amorphous sites that create new chain ends, resulting in glucan chains of different lengths; and the β-glycosidases or

cellobiase act specifically on the *β*-cellobiose disaccharides which is exocellulase product and produce glucose (Bayer *et al*., 1994; Singh, 1999), as shown in figure (2-3). Although the mechanism of cellulose degradation by aerobic bacteria is similar to that of aerobic fungi, it is clear that anaerobic bacteria operate on a different system (Zhang *et al*., 2006). Cellulosomes located on the cell surface mediate adherence of anaerobic cellulolytic bacteria to the substrate, which thereafter undergo a supramolecular reorganization, so that the cellulosomal subunits redistribute to interact with the different target substrates (Bayer *et al*., 2004). The mode of action of highly purified cellulases of exo-type is more effectively than endo-type enzyme and the saccharification activities of both cellulases similarly increased with decreasing crystallinity of cellulose (Hoshino *et al*., 1993).

2.8. Structure of cellulases

The structural and functional features of cellulases are not clear that are critical for decisive computational identification of cellulases and prevent their identification in datasets. Cellulases are defined as enzymes that show biochemical activity on cellulose substrates. All known cellulases have Homologs in their protein folds and even amino acid sequences (Sukharnikov *et al*., 2011). Most fungal cellulases have two-domain structure, with one catalytic domain and one cellulose-binding domain that are connected by a flexible linker. This structure is adapted for working on an insoluble substrate, and it allows the enzyme to diffuse two-dimensionally on a surface in a caterpillar-like fashion. Some of cellulases (mostly endoglucanases) are lack cellulose - binding domains. These

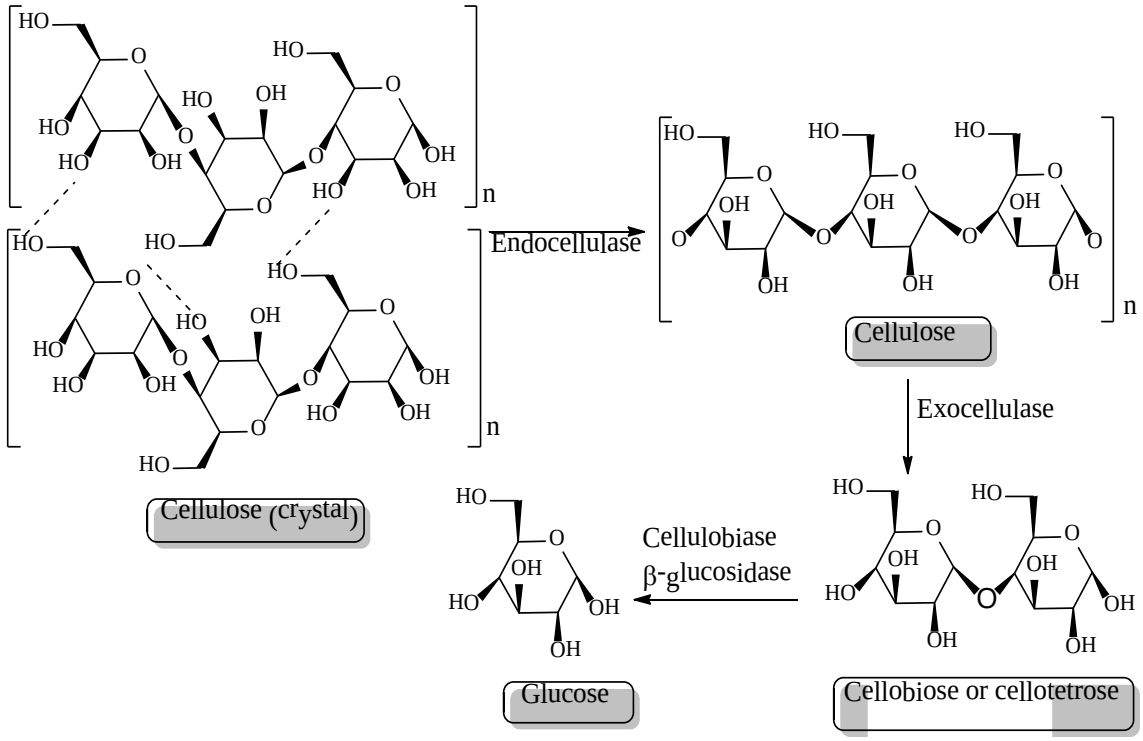

Figure (2-3): Mechanism of cellulolysis (Bayer *et al*., 1998).

enzymes might have a swelling function (Bayer *et al*., 1998).

In many bacteria, cellulases *in-vivo* are complex enzyme structures organized in supramolecular complexes, the cellulosomes. They contain roughly five different enzymatic subunits representing namely endocellulases, exocellulases, cellobiases, oxidative cellulases and cellulose phosphorylases where in only endocellulases and cellobiases participate in the actual hydrolysis of the β (1→ 4) linkage. The work on the molecular biology of cellulosomes had led to the discovery of numerous cellulosome-related "signature" sequences known as dockerins and cohesins. Depending on their amino acid sequence and tertiary

structures, cellulases are divided into clans and families (Bayer *et al*., 1998).

Cellulases and hemicellulases are family members of the broad group of glycoside hydrolases, which catalyze the hydrolysis of oligosaccharides and polysaccharides in general. These enzymes are composed of a series of separate modules. This fact explains the very large size of some of these enzymes and gives us some insight into their complex mode of action. Each module or domain comprises a consecutive portion of the polypeptide chain and forms an independently folding, structurally and functionally distinct unit. Each enzyme contains at least one catalytic module, which catalyzes the actual hydrolysis of the glycosidic bond and provides the basis for classification of the simple enzymes. Other accessory or "helper" domains assist or modify the primary hydrolytic action of the enzyme, thus modulating the overall properties of the enzyme (Bayer *et al*., 2006).

The major difference between free enzymes and cellulosomal enzymes is that the free enzymes contain cellulose-binding domains (CBMs), which are replaced by a dockerin in cellulosomal complex, and a single scaffolding-born CBM. Otherwise, both the free and cellulosomal enzymes contain very similar types of catalytic domains. Today, over 100 glycoside hydrolase families of and over 50 CBM families have been described. Despite the sequence similarity of both enzymes (exo and endo), their respective active-site architecture is different (Kuhad *et al*., 2011).

Structural the endoglucanase exhibits a deep cleft to accommodate the cellulose chain at any point along its length, whereas the active site of the exoglucanase bears an extended loop that forms a tunnel, through which

one of the termini of a cellulose chain can be threaded (figure 2-4).The sequence of amino acids for endoglucanase of *P. dispersa* according to the database of NCBI is:

MRFWRQWMLCFMVIFFSAQAAADGWSSYKSRFMSSDGRISDTAN
NNVSHTEGQGYGMLLAVAYNDRATFDKLTVIDYAGRKVMLPGAQ
GFNKTSYVVLNPSYFLFAAWQEFAQHSHLRVWNTLIDDGLALLGD
MQFGKTGLPLDWVALNADGSVAPAVGYSNRFSYDAIRIPLNIWWY
DPQSLRLVPFQRVWQGYARDTTPAWFDVLANTPAPYNMEGGLTA
VRDLTLNQTGYLSDRLAPEQNYFSASLQLLTWLAYQEKR.

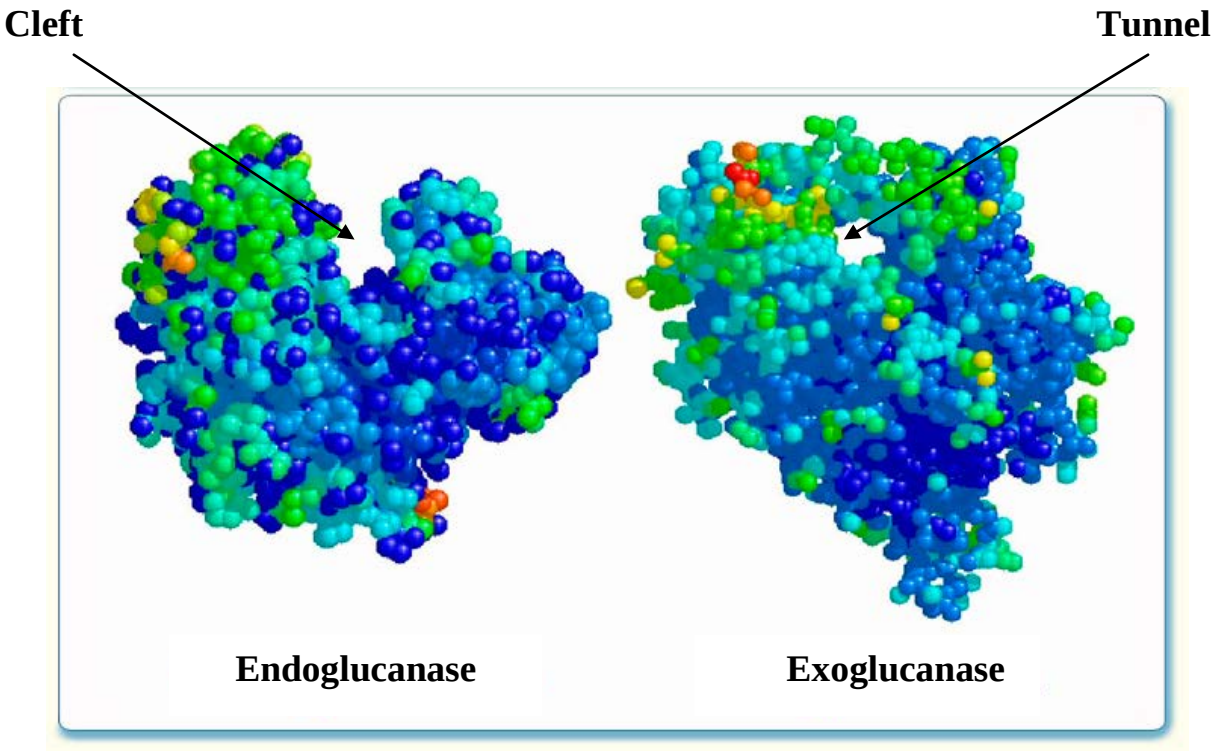

Figure (2-4): Typical cellulase structure (**Bayer *et al*., 2006**).

2.9. Molecular detection of cellulases

The Carbohydrate-Active Enzymes (CAZy) database provides classification of enzymes and substrate-binding modules are involved in

various types of carbohydrate metabolism based on sequence comparison. All known cellulases are found within 12 GH families of the CAZy database, and can be described with two enzyme commission numbers: EC 3.2.1.4 (endoglucanase) and EC 3.2.1.91 (cellobiohydrolase). Families GH5 and GH9 appear to have the largest number of biochemically characterized cellulases. This could be partly because cellulases from these families are abundant in the model cellulolytic bacteria.

Yet, many enzymes that effectively hydrolyze cellulose belong to other, smaller CAZy families, for example, the cellulase from *Rhodothermus marinus* and from *Fusicoccum* spp. (Kanokratana *et al*., 2008). This indicates that the search for potential efficient cellulases should be substantially broadened. Identification of cellulases by domain architecture is problematic because of two characteristics. First, cellulases display an extremely wide diversity of domain architectures even within the same protein family. Second, and more importantly, the domain-specific profile Hidden Markov Models (HMMs) currently available to recognize cellulases are built from multiple alignments that include cellulases and similar-in sequence non-cellulases, and thus are not able to differentiate between members of the same protein family that have different substrate specificities. Therefore, data sets retrieved with the current cellulase domain model might contain primarily non-cellulases and therefore would not be helpful to experimentalists (Zhou *et al.*, 2009). Despite numerous studies of microbial cellulolytic apparatus, only few genomes of known cellulose degraders have been fully sequenced. Recent genomic studies have identified many bacteria that contain arrays of various GHs, and many of which could be cellulases. Therefore, it is likely that only a small fraction of the cellulolytic world has been annotated and studied, and

more experimental and genomic investigation of potential cellulase degraders from diverse taxa and habitats is needed (Sukharnikov, 2011).

2.10. Cellulase production

The production of cellulases are very important, when there are high applications to these enzymes in different fields of industry like extraction of olive oil, improving the quality of bakery products, extraction and clarification of fruit and vegetable juices (Galante *et al*., 1998). It is well known that plants are the most common source of renewable carbon and energy on the earth. Cellulose is the structural component of the primary cell wall of green plants, many forms of algae and the comycetes. Some species of bacteria secrete it to form biofilm. Many microorganisms are able to produce cellulases like *Bacillus licheniformis*, *Bacillus* spp., *B. subtilis*, *Cellulomonas uda*, *Cellvibrio gilvus*, *Microbispora bispora*, *Acetivibrio cellulolyticus, Clostridium josui*, *Pantoea* spp., *Streptomyces* spp., *A. niger* (Chudasama and Thaker, 2012; Devi and Kumar, 2012; Sadhuand Maiti, 2013).

Cellulases are inducible enzymes and there are several types of substrates used as carbon source in the production of these enzymes like molasses, CMC, powder cellulose, wastes of plants as potato waste, where these sources represent inexpensive and economic alternative to synthetic medium for the production of cellulases (Shabeb *et al*., 2010). For many years, cellulose-degrading bacteria have been isolated and characterized for obtaining more effective cellulases from variety of sources such as soil, decayed plant materials, hot spring, organic matters, feces of ruminants and composts. Ray *et al.* (2007) isolated bacteria, which can produce more efficient cellulase enzymes in submerged fermentation.

Fermentation is the technique of biological conversion of complex substrates into simple compounds by various microorganisms. It has been widely used for the production of cellulase for industrial purposes. Over the years, fermentation techniques have gained immense importance due to their economic and environmental advantages. Two broad fermentation techniques have emerged as a result of this rapid development, submerged fermentation (SmF) and solid state fermentation (SSF) or cultivation (SSC) (Sadhu and Maiti, 2013). The bioconversion of cellulosic materials mainly depends on the nature of cellulose sources of cellulolytic enzyme and optimal conditions for catalytic activity and production of enzymes. Cellulose quality, temperature, aeration, carbon source, incubation period, medium additives, pH of the medium and the presence of inducers are important parameters for the optimized production of cellulase enzymes (Immanuel *et al*., 2006).

2.11. Pectinases

Pectinase (EC 3.2.1.4) is an enzyme that breaks down pectin, a polysaccharide found in plant cell walls. Commonly referred to as pectic enzymes, they include pectolyase, pectozyme and polygalacturonase. One of the most studied and widely used commercial pectinases is polygalacturonase. It is useful because pectin is the jelly-like matrix which helps cement plant cells together and in which other cell wall components, such as cellulose fibrils, are embedded. Therefore pectinase enzymes are commonly used in processes involving the degradation of plant materials, such as speeding up the extraction of fruit juice from fruit, including apples and sapota. Pectinases have also been used in wine production since the

1960s.The function of pectinase in brewing is two fold, first it helps breakdown the plant (typically fruit) material and so helps the extraction of flavours from the mash. Secondly the presence of pectin in finished wine causes a haze or slight cloudiness, Pectinase is used to break this down and so clear the wine. It can be extracted from fungi such as *A. niger* and bacteria *Agrobacterium tumefaciens*, *Bacteroides thetaiotamicron*, *Ralstonia solanacearum*, *Bacillus* spp. and *Pantoea* spp. The fungus produces these enzymes to break down the middle lamella in the plant cells so that it can extract nutrients from the plant tissues and insert fungal hyphae. If pectinase is boiled it is denatured (unfolded) making it harder to connect with the pectin at the active site, and produce as much juice. Pectinases are also used for retting. Addition of chelating agents or pretreatment of the plant material with acid enhance the effect of the enzyme (Kumar *et al*., 2012; Qureshi *et al*., 2012).

2.12. Classification of pectinases

According to extensive classification of pectinases, they are classified under three headings according to the following criteria: whether pectin, pectic acid or oligo-D-galacturonate is the preferred substrate, whether pectinases act by trans- elimination or hydrolysis and whether the cleavage is random (endo- liquefying of depolymerizing enzymes) or endwise (exo- or saccharifying enzymes) (Wing *et al*., 1989).

The three major types of pectinases are Protopectinases, Esterases, Depolymerases, the PPases are classified into two types, on the basis of their reaction mechanism. A-type PPases react with the inner site, whereas B-type PPases react on the outer site. A-type PPase are found in the culture filtrates of yeast and yeast-like fungi. They have been isolated from

Kluyveromyces fragilis IFO 0288, *Galactomyces reesei* L. and *Trichosporon penicillatum* SNO 3 and are referred to as PPase-F, -L and -S, respectively. B-type PPases have been reported in *B. subtilis* IFO 12113, *B. subtilis* IFO 3134 and *Trametes* spp., and are referred to as PPase- B, -C and -T, respectively. All three A-type PPases are similar in biological properties and have similar molecular weight of 30 kDa (Rafnar *et al*., 1991; Jayani *et al*., 2005).

2.13. Pectinases sources

Most pectin-degrading organisms are associated with raw agricultural products and with soil. Up to 10% of the organisms in soil have been shown to be pectinolytic (Hankin *et al*., 1999). These include, but are not limited to, bacteria in the genera *Pantoea*, *Achromobacter*, *Aeromonas*, *Arthrobacter*, *Agrobacterium*, *Enterobacter*, *Bacillus*, *Clostridium*, *Erwinia*, *Flavobacterium*, *Pseudomonas*, *Xanthomonas* (Pedrolli *et al*., 2009; Chudasama and Thaker, 2012; Kumar *et al*., 2012). And many yeasts, molds, protozoa and nematodes (Bateman and millar, 1966). Also pectolytic activity was found in a strain of *Leuconostoc mesenteroides* (Juven *et al*., 1985).

Many studies have been conducted on the production of pectinases from various microorganisms. But a few works have been published about cost-effective production of enzymes. The difficulties of obtaining the appropriate substrate might be the biggest problem to develop such studies. There are different low-cost substrates like cassava waste, which is used for cost-effective production of the pectinase enzyme. In addition, the production of pectinases occurs by newly isolated strains of *Bacillus* spp., and *Pantoea* sp. by submerged fermentation using cassava waste

(Sneath *et al*., 1986; Chudasama and Thaker, 2012).

2.14. Pectinase applications

Pectinases are divided to two types, acidic pectinases and alkaline pectinases. Acidic pectic enzymes used in the fruit juice industries and making wine often come from fungal sources, especially from *A. niger*. The juices produced by these industries commercially include: (A) Sparkling clear juices, (apple, pear and grape juices), (B) Juices with clouds (citrus juices, prune juices, tomato juice and nectars), and (C) Unicellular products where the intent is to preserve the integrity of the plant cells by selectively hydrolyzing the polysaccharides of the middle lamella. While alkaline pectinases are mainly used in the degumming and retting of fiber crops and pretreatment of pectic wastewater from fruit juice industries. These enzymes come mostly from bacterial sources. In the industrial sector, alkaline pectinases, mainly from *Bacillus* spp. (De and Pilnik, 1973; Ranadive and Haard, 1973; Kilara, 1982; Askar *et al*., 1990; Schols *et al*., 1990).

Over the years, pectinases have been used in several conventional industrial processes, such as textile processing and bioscouring of cotton fibers, degumming of plant fibers, retting of plant fibers, waste water treatment, coffee and tea fermentation, paper and pulp industry, oil extraction etc. They are yet to be commercialized (Kashyap *et al*., 2001; Jayani *et al*., 2005)**.**

2.15. Mode of action of pectinases

Pectinases are an enzyme group that catalyzes pectic substance degradation through depolymerization (hydrolases and lyases) and

deesterification (esterases) reactions. The well-known pectinolytic enzymes are homogalacturonan degrading enzymes. The action mode of the most studied pectinases is shown in figure (2-5). The pectinolytic enzymes may be divided into three broad groups (Sakai, 1992; Paloma and Saarilahti, 1997), Protopectinases which are degrade the insoluble protopectin and give rise to highly polymerized soluble pectin, Esterases are catalyze the de-esterification of pectin by the removal of methoxy esters and Depolymerases catalyze the hydrolytic cleavage of the ∝-(1-4)-glycosidic bonds in the D-galacturonic acid moieties of the pectic substances (Alkorta *et al*.,1998; Kashyap *et al*., 2001; Pedrolli *et al*., 2009).

2.16. Pectinase production

Pectinolytic enzymes are naturally produced by many organisms like bacteria, fungi, yeast, insects, nematodes, protozoan and plants. Microbial pectinases are important in the phytopathologic process, in plant-microbe symbiosis and in the decomposition of dead plant material, contributing to the natural carbon cycle. Several studies in microbial enzymes have shown the production of multiple pectinase forms which differ on molecular mass and kinetic properties. The production of multiple forms of enzymes improves the microorganism ability to adapt to environmental modifications (Gummadi and Kumar, 2006; Pedrolli *et al*., 2009; Sadhu and Maiti1, 2013). Submerged fermentation (SmF) and solid state fermentation (SSF) have been successfully used in pectinase production by fungi and bacteria. Submerged fermentation is a well developed system used in industrial scale to produce a large variety of microbial metabolites (Silva *et al*., 2002).

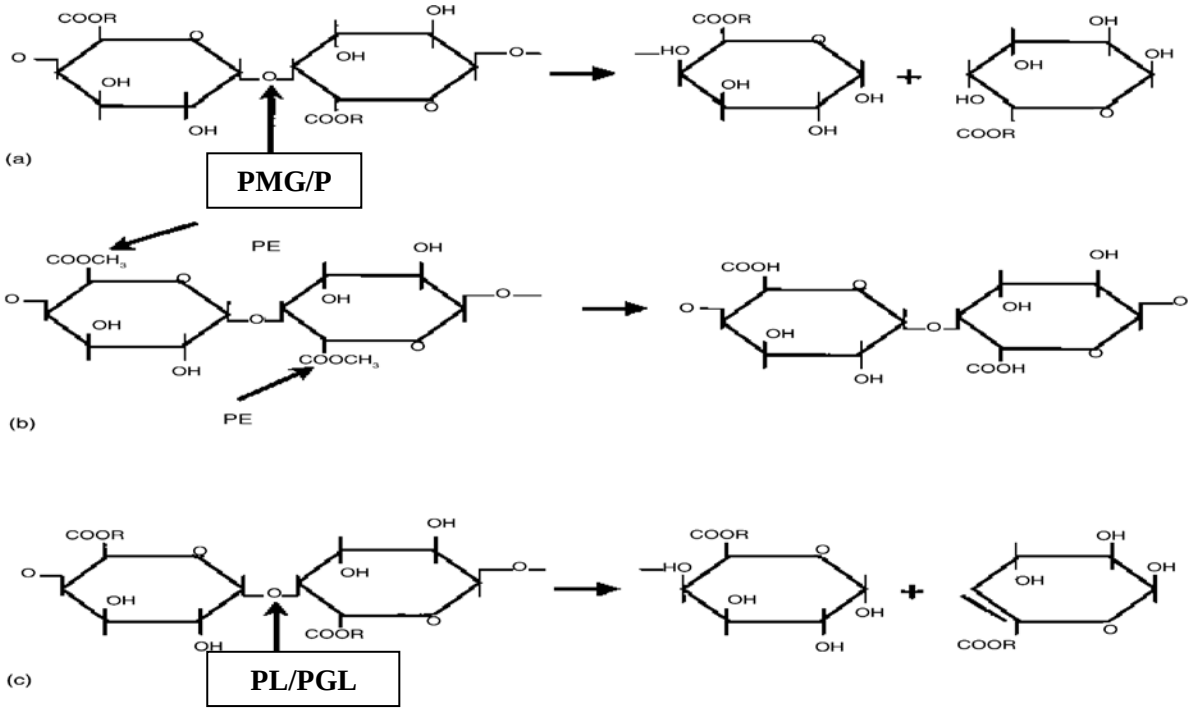

Figure (2-5): Mode of action of pectinases (Reddy and Sreeramulu, 2012)

(a) R = H for PG and CH3 for PMG; (b) PE; and (c) R = H for PGL and CH for PL

Note: Arrows indicate the place where the pectinase reacts with the pectic substances. PMG, polymethylgalacturonases; PG, polygalacturonases (EC 3.2.1.15); PE, pectinesterase (EC 3.1.1.11); PL, pectin lyase (EC-4.2.2.10).

2.17. Mutagenesis

Ultraviolet (UV) radiation has become a global concern due to the fact that it is a major environmental mutagen and carcinogen, leading to conditions such as skin cancer. Coupled with the fact that ozone layer (the atmospheric layer that filters out solar UV radiation from the sun) is continuously depleted by pollution, there is pressure to better understanding the mechanics in how mutations arise so that possible remedies in the future can be devised (Karp *et al*., 2000).

UV radiation may be a hazard to the human population but it is also an environmental stress for other organisms such as bacteria. Such environmental stress caused by UV may in some way induce different evolutionary changes on bacteria that would have otherwise not been selected for. This area thus provides avenues of physiological, ecological, and genetic investigation because mutations play a key role in biological processes such as evolution, carcinogenesis, aging, and generation of somatic genetic diversity (Livneh *et al*., 1993).

For example, the experiments on K-12 strain of *Escherichia coli* were used because of its ease of cultivation, and the most often used organism in UV mutagenesis experiments. The genetic markers chosen for mutational frequency analysis were rpoB (also known as rif) located at about 90 centisomes in the K-12 chromosome, ampA located at about 94 centisomes, and ompF located at about 21centisomes. Mutations at these sites confer resistance to rifampicin (an Antibiotic that strongly inhibits RNA polymerase), ampicillin, and chloramphenicol respectively. The lacZ locus, located at about 8 centisomes (Miller, 1972) was also selected as a genetic marker; $lacZ^{-}$ mutants are not able to use lactose.

When deoxyribonucleic acid (DNA) is exposed to UV light (254nm), the most frequent DNA damage, or lesions, results at dimers of any two adjacent pyrimidine bases (T, thymine; C cytosine) causing T-T, C-T, and C-C dimers, but T-T dimers are the most common cyclobutane pyrimidine dimers. Another type of DNA damage is the 6-4 pyrimidine-pyrimidone photoproducts. These occur at a lesser frequency than cyclobutane pyrimidine dimers but are less mutagenic because they are more efficiently repaired than cyclobutane pyrimidine dimmers. The remaining types of

DNA lesions include pyrimidine hydrates, purine photoproducts, strand brakes, and DNA cross-links. These occur at a much lower frequency. Thus, UV radiation induces mutations such as frame shifts, base substitutions, deletions, recombination and other types of genetic rearrangements (Lin and Wang, 2001).

There are two main categories of repair in bacteria, one is the "error-free" system and is independent of the SOS regulon or response, while the other is the "error-prone" repair system and is part of the SOS regulon. And there are three strategies employed by bacteria that are considered relatively error-free, they are photoreactivation, excision repair and post-replication repair.

Cellulases are important agents to solve the problem of accumulated plant wastes, and the cost of cellulases in enzymatic hydrolysis are regarded as a major factor cause many researchers and development attempted to improve cellulase producing microorganism, via mutagenic agents has attracted great attention owing to their efficiency. The use of different mutagenic agents like ultraviolet, X-rays, gamma radiation, and chemicals like ethyl methane sulfonate, N-methyl-N- nitro-N-nitrosoguanidine and mustards, Lead to over-production of the products randomly in some colonies of the mutant (Walter, 1984; Mala *et al*., 2001).

The improvement of cellulase production in different bacteria was occurs by various mutagens like UV and N-methyl-N- nitro-N-nitrosoguanidine, when there are increasing in the production of cellulase in some mutants of *Cellulomonasa* sp p. (Zaldivar *et al*., 2001).

P. dispersa was mutated by physical as well as chemical mutagens. UV and gamma rays were used as mutagens separately for wild type strain of *P. dispersa* and chemical mutagen was used for the further mutation of

mutant obtained from the physical, where chitinase production in *P. dispersa* was improved by UV irradiation. Therefore the mutagenesis in the laboratory is an important technique by which produce mutant proteins with interesting properties, or enhanced or novel functions that may be of commercial use. Mutants strains may also be produced that have practical application or allow the molecular basis of particular cell function to be investigated (Gohel *et al*., 2004).

Chapter Three
Materials
and
Methods

3. Materials and Methods

3.1. Materials

3.1.1.Equipments and apparatus

The following equipments and apparatus were used in this study:

Equipment or apparatus	Company / Origin
Autoclave	Gallenkamp, England
Automatic pipettes	Eppendorf, Germany
Cooling centrifuge	Eppendorf
Compound microscope	Olympus, Japan
Distillator	Gallenkamp
Gel electrophoresis unit	Bio Rad, U.S.A
Gel documentation system	Bio Rad
Hot plate with magnetic stirrer	Gallenkamp
Incubator	Sanyo, Germany
Laminar air flow cabinate	Esco, Singapore
Microwave oven	LG, China
Nanodrop-Spectrophotometer	Thermo Scientific, USA
Oven	Gallenkamp
pH -meter	Jenway, U.K.
Sensitive balance	Sartorius, Germany
Shaker incubator	Gyromax, U.S.A
Thermal cycler	Bio-Rad
UV-Visible- Spectrophotometer	Shimadzu, Japan
VITEK 2 compact	Biomerieux, France
Vortex	INC, U.S.A
Water bath	Memmert, Germany

3.1.2. Chemical and biological materials

The following chemicals and biological materials were used in this study:

Materials	Company/ Origin
K_2HPO_4, KH_2PO_4,Na_2HPO_4, H_3PO_4,NaOH, KCl, $MgSO_4.7H_2O$,$MgSO_4$, KOH, $FeSO_4.7H_2O$,$CaCO_3$,$NaNO_3$, Rochelle salt, Tris-HCl, $MgCl_2$,Sodium metabisulfate, EDTA, Tris-base, Glacial acetic acid	BDH, UK
Agar,Hydrogen peroxide, N,N,N,N,tetramethylenediaminedihydrochloride, Ammonium persulfate, Manitol, Lactose, Sorbitol, Rhamnose, Maltose, Sucrose, Glucose, galacturonic acid, Citrus Pectin, CMC, Yeast extract, BSA, Peptone, Tryptone, Peptone water, Brain heart infusion broth, Gelatin, ρ –Dimethyl-aminobenzaldehyde	Difco, UK
NaCl, Crystal Violet, Safranine, potassium iodide, Tryptone, peptone, Yeast extract, parafilm, Congo-red, Sodium azide, Methyl red	Fluka, Switzerland
DEAE-Cellulose, Sephadex-G200	Pharmacia, Sweeden
Glycerol, Phenol, HCl, Ethanol, isoamyl alcohol	Riedel-Dehaeny, UK
Bovine serum albumin, Trypsin, Hemoglobin, Lysozyme, Ethidium bromide, Pronase, 3,5-DNS,Coomassie brilliant blue G-250,Bromocresolpurpule, Bromophenol blue, Blue dextran	Biolab, USA
DNA ladder, agarose, master mix, Nuclease free D.D.W	Stratagene, USA
Filter paper Whatman No. 1, Millipore filter unit (0.45 μm)	Sigma, USA

3.1.3. Kits

3.1.3.1. Kits used inthe molecular study

Tissue genomic DNA extraction mini kit of Favorgen Company/Australia was used for DNA extraction. This kit was provided with different buffers and lysis solutions, including the followings:

- FATG1 buffer
- FATG2 buffer
- W1 buffer
- Wash buffer
- Elution buffer
- Proteinase K
- RNase

3.1.3.2. Diagnostic kits

3.1.3.2.1.Api 20 E kit (Api Bio merieux, Lyon, France)

Api 20 E kit consists of:

A. Galleries: The gallery is a plastic strip with 20 microtubes containing dehydrated reactive ingredients.

B. Api 20 E Reagent:

1-Kovac's reagent (*p*-dimethylaminobenzaldehyde at 4% in HCl isoamyl alcohol).

2-Oxidase reagent (1% tetra-metheyl-*p*-phenyle-diamine).

3-Voges – proskauer reagent:

- VP1 (40% potassium hydroxide).
- VP2 (5% alpha - naphthol).

4-Ferric chloride 3.4%.

3.1.4. Culture media

3.1.4.1. Ready to made culture media

Medium	Company
Blood agar base	Biolife, Italy
King' B agar	Sigma, U.S.A
MacConkey agar	Biolife
Motility test medium	Himedia, India
Nutrient agar	Biolife
Nutrient broth	Biolife
Simmon citrate agar	Difco,England
Urea agar base	Difco
Brain heart infusion	Difco

These media were prepared according to the manufacturer instructions.

3.1.4.2.Laboratory prepared media

A. CMC agar medium (Bai*et al.*, 2012)

This medium was prepared to be consisted of the following components:

Component	(g/L)
K_2HPO_4	1.00
$MgSO_4$	0.50
KCl	1.00
$FeSO_4.7H_2O$	0.01
Glucose	1.00
Yeast extract	0.50
$NaNO_3$	1.00
Carboxy methyl cellulose	5.00
Agar	16.00

All components were dissolved in 950 ml distilled water, pH was adjusted to 7.0, then the volume was completed to 1L with distilled

water and sterilized by autoclaving.

B. Pectin agar medium (Raju and Divakar, 2013).

This medium was prepared from the following components:

Component	**(g/L)**
Pectin	10.0
$(NH_4)_2SO_4$	3.0
K_2HPO_4	3.0
$MgSO_4$	0.1
KH_2PO_4	2.0
Agar	16.0

All components were dissolved in 950 ml distilled water, pH was adjusted to 6.0, then the volume was completed to 1 L with distilled water andsterilized by autoclaving.

C. Cellulase production medium (Bhale and Chatage, 2013).

This medium was prepared from the following components**:**

Component	**(g/L)**
CMC	10
$NaNO_3$	1
KH_2PO_4	1
$MgSO_4.7H_2O$	5

All components were dissolved in 950 ml distilled water, pH was adjusted to 7.0, then volume was completed to 1 L with distilled water and sterilized by autoclaving.

D. Total cellulases production medium(Barman *et al*., 2011)

This medium was prepared of sterilized distilled water containing stripof filter paper considered as cellulases production medium. The degradation of filter paper represents a positive result for the production of cellulases.

E. Pectinase production medium (Kumar *et al*., 2012)

This medium was preparedfrom the following components.

Component	**(g/L)**
Pectin	0.30
Sucrose	10.00
KNO_3	0.60
$MgSO_4$	0.25
KH_2PO_4	1.00
$CaCl_2$	0.10
$NaNO_3$	2.00
K_2HPO_4	0.50
KCl	0.50
Yeast extract	1.00

All components were dissolved in 950 ml distilled water, pH wasadjusted to 6.0, then the volume was completed to 1 L with distilled water and sterilized by autoclaving.

F. Sugar fermentation medium (Cruickshunk *et al*., 1975)

This medium wasprepared by adding 0.5% of suitable sugar sterilizedby filtration to sterilized peptone water containing 0.005% of bromocresol purple as indicator for pH change.

G. Methyl Red and Voges – Proskauer (MR – VP) medium (Atlas *et al*., 1995)

This medium was prepared from the following components:

Component	(g/L)
K_2HPO_4	5
Peptone	5

All components were dissolved in 900 ml distilled water, pH was adjusted to 7.6, then volume was completed to 950 ml with distilled water and sterilized by autoclaving, then aliquotof 50 ml of 10% glucose solution sterilized by filtration was added.

H. Blood agar medium

This medium was prepared according to Atlas *et al.* (1995) by autoclaving blood base agar medium, cooled to 45°C, then 5% of sterilized blood was added, mixed gently and plated in Petri dishes.

I. Urea agar medium

Urea agar medium was prepared by dissolving 24g of urea agar base in 950 ml of distilled water, pH was adjusted to 6.8-7.0, and sterilized by autoclaving. After cooling to 50 ºC, aliquot of 50 ml of 20% urea solution (sterilized by filtration) was added, mixed gently then the medium was dispensed into sterile test tubes and left to solidify in slant position (Collee *et al*., 1996)**.**

J. Gelatin medium

It was prepared according to Collee*et al.* (1996) by adding 12g of gelatin to 100 ml of brain heart infusion broth, thenthe medium was dispensed into test tube and sterilized by autoclaving and stored at 4°C until use.

3.1.5. Reagents, dyes and solutions

3.1.5.1. Catalase reagent (Atlas *et al*., 1995)

This reagent was prepared to be consisted of 3% hydrogen peroxide.

3.1.5.2. Oxidase reagent (Atlas *et al.,* 1995)

This solution was freshly prepared by dissolving 1g of tetramethyl phenylenediaminedihydrochloride in 100 ml distilled water, then the reagent was kept at 4 ºC until use.

3.1.5.3. Methyl red reagent (Collee *et al***.,** 1996)

This reagent was prepared by dissolving 0.05 g of methyl redin 150 ml of absolute ethanol, then the volume was completed to 250 ml with distilled water.

3.1.5.4. Voges-Proskour reagent (Collee *et al*., 1996)

It was composed of two solutions:

Solution (A): Prepared by dissolving 40 g of KOH in 80 ml of distilled water, then the volume was completed to 100 ml with distilled water.

Solution (B): Prepared by dissolving 5 g of α-naphtholin 100 ml of absolute ethanol.

Equal volumes of solution (A) and (B) were mixed thoroughly and kept at 4 ℃ until use.

3.1.5.5. Ethidium bromide stock solution (10 mg/ml)

It was prepared according to Maniatis *et al*. (1982) by dissolving 1g of ethidium bromide in 100 ml of distilled water in dark container, mixed gently then stirred until complete dissolving and stored at 4°C until use.

3.1.5.6. Loading Buffer (6X)(Maniatis *et al.,* 1982)

This buffer solutionwas prepared by dissolving 0.25%of bromophenol blue and 40 gof sucrose in 80 ml of distilled water, then the volume was completed to 100 ml, and stored at 4°C until use.

3.1.5.7. Tris-EDTA (TE) buffer solution (Maniatis*et al.*, 1982)

This buffer solution was prepared to be consisted of 10 mMTris-HCl, and 1mM EDTA, pH was adjusted to 8.0 and stored at 4°C.

3.1.5.8. Sodium phosphate buffer solution(Maniatis *et al*., 1982)

It was prepared at a concentration 10 mM of sodium phosphate, pH 7.

3.1.5.9. EDTA solution (0.5M) (Maniatis *et al*., 1982)

It was prepared by dissolving 14.61g EDTA in 80 ml distilled water, then pH was adjusted to 8.0,and the volume was completed to 100 ml with distilled water, and sterilized by autoclaving.

3.1.5.10.Tris-Borate-EDTA (TBE) buffersolution

Tris-Borate-EDTA (TBE) Buffer was prepared according to Sambrook and Russell (2001) by dissolving 54 g of Tris-HCl and 27.5 g boric acid in 900ml of D.W, then aliquot of 20 ml of 0.5 M EDTA was added. The volume was completed to 1000 ml with distilled water, then the pH was adjusted to 8 and sterilized by autoclaving.

3.1.5.11.Tris-HCl (0.02M)

It was prepared by dissolving 0.3 g Tris- HCl, in 90 ml of distilled water, then the pH was adjusted to 7 by using 1N of NaOH, then the volume was completed to 100 ml with distilled water.

3.1.5.12. 3, 5-Dintrosalycilic acid solution(DNS) (Ghose, 1987)

It was prepared by mixing 10.6 g of 3, 5-DNS and 19.8g of NaOH in 1416 ml of distilled water, then a quantity of306g of Rochelle salt, 7.6ml phenol and 8.3g Na-meta bisulfite were added, mixed gently until complete dissolving.

3.1.5.13. Congo red solution (0.1%)(Kaur and Arora, 2012)

It was prepared by dissolving 0.1g of Congo red in 100 ml distilled water.

3.1.5.14. Coomassie brilliant blue G-250 (Bradford,1976)

It was prepared by dissolving 0.1 g of the dye in 50 ml of 95% ethanol, Then aliquot of 100ml of 85% phosphoric acid was added with stirring and the volume was completed to 1000 ml with distilled water, then mixture was filtered through Whatman No. 1 filter paper, and stored at 4°C in dark bottle until use.

3.1.5.15. NaCl solution 1M(Nikolskij, 1964)

It was prepared by dissolving 58 g of NaCl in 1L of distilled water.

3.1.5.16. HCl solutions 1N (Nikolskij, 1964)

It was prepared by adding 8.3 ml of concentrated HCl to 100 ml of distilled water.

3.1.5.17. NaOH solution 1N (Nikolskij, 1964)

It was prepared by dissolving 40 g of NaOH in 1L of distilled water.

3.2. Methods

3.2.1. Samples collection

3.2.1.1. Soil samples

For isolation of bacteria producing plant cell wall degrading enzymes, soil samples were collected from fields cultivated with wheat and barley crops located in Al-Jaderia, Baghdad university.

3.2.1.2. Roots and fruits samples

Another samples from spoiled apple and orange fruits and carrot roots were collected from local markets in Baghdad city.

3.2.2. Preparation of samples

A quantity of 1g of soil,fruits and roots samples were suspended in 9 ml of sterilized distilled water and mixed vigorously. After settling, 0.1ml aliquots of supernatant were spreaded on nutrient agar plates, and incubated at 30°C for 24hrs. After incubation, the growing colonies were selected and screened to determine their ability in producing cellulase and pectinase enzymes, then identified according to their cultural, morphological and biochemical characteristics.

3.2.3. Identification of bacterial isolates

3.2.3.1 Cultural and morphological characteristics

To identify bacterial isolates,cultural and morphological characteristics(size, shape, edge, gram staining, and color) were examined according to

Atlas *et al*. (1995).

3.2.3.2. Biochemical tests

3.2.3.2.1. Gram's stain (Harely and Prescott, 1996)

Single colony of each bacterial isolate was transferred and smeared on a clean slide and stained with crystal violet, then slides were treated with iodine solution, decolorized with absolute alcohol, and counterstained with safranine, and examined under light microscope.

3.2.3.2.2. Catalase test (Atlas *et al*., 1995)

This test was performed by adding a few drops of hydrogen peroxide (3%) on a single colony of each isolate usinga clean slide. Production of gaseous bubbles indicates a positive result.

3.2.3.2.3. Oxidase test (Atlas *et al*., 1995)

This test was done by using filter paper moistened with a few drops of freshly prepared solution oftetramethyl-*p*-phenyldiaminedihydrochloride. Aseptically, a clump of bacterial cells was picked up with a sterile wooden stickapplicator and smeared on the filter paper. Development of a violet or purple color within 10 seconds indicates a positive result.

3.2.3.2.4.Indole test reagent

This reagent was prepared according to Atlas *et al*. (1995) by dissolving 10g of *ρ*–Dimethyl-aminobenzaldehyde in 150ml of isoamyl alcohol heated inside a water bath at 50°C and then 50 ml of HCl was added slowly, mixed gently and stored at 4°C until use.

3.2.3.2.5. Voges-Proskauertest (Collee *et al*., 1996)

Aliquot of 5 ml of MR-VP broth was inoculated with a single colony

of each bacterial isolate and incubated at 35°C for 24 hrs., then 1 ml of VP1 and 3ml of VP2 were added to culture broth and shaken for 30 Sec. Formation of pink or red color indicates a positive result.

3.2.3.2.6. Methyl red test (Collee *et al*., 1996)

MR-VP broth were inoculated with a single colony of bacterial isolate and incubated at 35 °C for 48 hrs, then five drops of methyl red reagent was added and mixed gently. Presence of bright red color indicates a positive result.

3.2.3.2.7. Motility test

A semi-solid agar medium was stabbed with each bacterial isolate using a straight wire to make a single stab down the center of the tube to about half depth of the medium. Motile bacteria typically give diffuse, hazy growth that spreads throughout the medium rendering it slightly opaque (Collee *et al*., 1996).

3.2.3.2.8. Ureasetest

Urease test was achieved by streaking Christensen urea agar slants with each bacterial isolate, and incubated at 35°C for 24 hrs. Appearance of a red-violet color indicates a positive result (Atlas *et al*., 1995).

3.2.3.2.9.Indole test

Indole test was achieved by inoculating test tubes containing peptone water with fresh culture of each bacterial isolate and incubated at 35°C for 24 hrs, then 50 µl of Kovac's reagent was added and mixed gently. Appearance of red ring on the surface of the liquid medium indicates a positive result (Collee *et al*., 1996).

3.2.3.2.10. Citrate utilization test

Citrate utilization test was achieved by inoculating fresh culture of each bacterial isolate, and incubated for 24hrsat 35°C. Medium color change from green to blue indicates a positive result (Atlas *et al*., 1995).

3.2.3.2.11.Sugars fermentation test (Cruickshank *et al*., 1975)

Aliquot of 3 ml of sugar medium was inoculated with loop ful of fresh culture of each bacterial isolate grown on a nutrient agar medium, then tubes were incubated at 35°Cfor 24hrs.Changing the color of indicator from purple to yellow is a result of the pH change, indicating a positive result.

3.2.3.2.12.Gelatinase test

This test was achieved by stabbing gelatin medium which prepared in (3.1.4.2. J)with fresh culture of the bacterial isolate and incubated aerobically at 35 ºC for 3d, then tubes were placed inside refrigerator at 4°C for 1h. Medium liquification indicates a positive result (Baron and Finegold, 1998).

3.2.3.2.13. Growth at different temperatures

Pure culture of each bacterial isolate was used to inoculate nutrient agar plates, then plates were incubated separately at 4, 30, 41 and 44 °C for 24 hrs. Presence of heavy growth indicates a positive result (Cruickshank *et al*., 1975).

3.2.3.3. Identification of bacterial isolates by using Api20-E (Overman *et al*.,1985)

1. Preparation of galleries

Aliquot of 5ml D.W. was dispensed inside the incubation tray so as provides a humid atmosphere during incubation.

2. Preparation of bacterial suspension

Loopful of fresh culture from each bacterial isolate was grown for 24hrs at 35°C on MacConkey agar medium suspended in 5 ml of D.W. inside test tubes, mixed thoroughly and used to inoculate the galleries.

3. Reading of the galleries

All the reactions not requiring reagents were recorded first, then the following reagents were added to the corresponding microtubes:

A- One drop of 3.4% ferric chloride to the TDA microtube.

B- One drop of Kovac's reagent to the IND microtube.

C- One drop of Vogas – Proskauer reagent to VP microtube.

3.2.3.4. Identification by using VITEK 2

Each bacterial isolate was re-identified according to the results of 47 type of biochemical tests by using VITEK-2 equipment(Biomericus, USA).

3.2.3.4.1. Preparation of suspension

A sterile swab or wooden applicator stick was used to transfer colonies of a pure culture of each bacterial isolate and suspended in 3.0ml of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a 12 x75 mm clear plastic (polystyrene) tube.

3.2.3.4.2. Inoculation

Identification cards are inoculated with bacterial suspensions using an integrated vacuum apparatus. A test tube containing the bacterial suspension was placed in the neighboring slot while inserting the transfered tubeinto the corresponding suspension tube,then the filled cassette wasplaced into a vacuum chamber station. After the vacuumwas appliedandair was re-introduced into the station, the organism suspension was forced

through the transfer tube into micro-channels that fill all the test wells.

3.2.3.4.3. Card sealing and incubation

Inoculated cards were passed by a mechanism which cuts off the transfertube and seals the card prior to loading into the carousel incubator. The carousel incubator accommodates up to 30 or up to 60 cards. All cards types were incubated on-line at 35.5 +1.0°C, Each card wasremoved from theCarouselincubator once every 15 min., transported to the optical systemfor reaction readings, and then returned to the incubator until the next readtime. Date were collected at 15min. intervals during the entire incubation period.

3.2.3.4.4. Optical system

A transmittance optical system allows interpretation of test reactions usingdifferent wavelengths in the visible spectrum. During incubation, each test reaction was read every 15 min to measure either turbidity or colored products of substrate metabolism. In addition, a special algorithm was used to eliminate false readings due to small babbles that may be present**.**

3.2.3.4.5. Test reactions

Calculations were performed on raw data and compared to thresholds to determine reactions for each test. Once the VITEK 2 Compact, test reaction results appeared as"+", "-", "(-)" or "(+)".

3.2.3.4.6. Database development

The database of the VITEK 2 identification products were constructed with large strain sets of well-characterized microorganisms tested under various culture conditions. These strains were derived from a variety of

clinical andindustrial sources as well as from public (e.g., ATCC) and university culture collections.

3.2.3.4.7. Analytical techniques

Test data from an unknown organism were compared with the respective database to determine a quantitative value for the proximity to each of the databasetaxa. Each of the composite values was compared to the others to determine if the data are sufficiently unique or close to one or more of theother database taxa. If a unique identification pattern is not recognized, a listof possible organisms is given, or the strain is determined to be outside the scope of the database.

3.2.3.4.8. Identification levels(Pincus, 2013)

An unknown biopatternwas compared with the database of reactions foreach taxon; a numerical probability calculation was performed. Various qualitative levels of identification were assigned based on the numerical probability calculation. The following levels and associated information are listed about VITEK 2 kit results (table 3-1).

Table (3-1): Level and association information of VITEK2 kit.

ID Message confidence level	Choice	% Probability
Excellent	1	96-99
Very good	1	93-95
Good	1	89-92
Acceptable	1	85-88
Unidentified	3 or 0	N/a

* N/a = not available.

3.2.4. Preparation of bovine Serum Albumin standard curve (Bradford, 1976)

For preparation of bovine serum albumin (BSA), stock solution of BSA was prepared first by dissolving 1g of BSA in 100 ml of Tris-HCl, then serial concentrations ranged between 0 and1.60 mg/ml were prepared.Absorbance was read at 595 nm after the addition of coomassie brilliant blue for each BSA concentrationas indicated in table (3-2), then standard curve represents the relationship between BSAconcentrations and absorbanceat 595 nmwas plotted (figure 3-1).

Table (3-2): Concentration and absorbance of bovine serum albumin solutions for determination of protein concentration.

Tube No.	BSA stock solution(ml)	buffer (ml)	Final volume (ml)	Final BSA conc. (mg/ml)	Absorbance (595 nm)
1	0.00	10.00	10	0.00	0.000
2	0.16	9.84	10	0.16	0.120
3	0.20	9.80	10	0.20	0.160
4	0.24	9.76	10	0.24	0.190
5	0.32	9.68	10	0.32	0.200
6	0.40	9.60	10	0.40	0.200
7	0.48	9.52	10	0.48	0.300
8	0.64	9.36	10	0.64	0.380
9	0.80	9.20	10	0.80	0.400
10	0.96	9.04	10	0.96	0.440
11	1.28	8.72	10	1.28	0.590
12	1.60	8.40	10	1.60	0.610

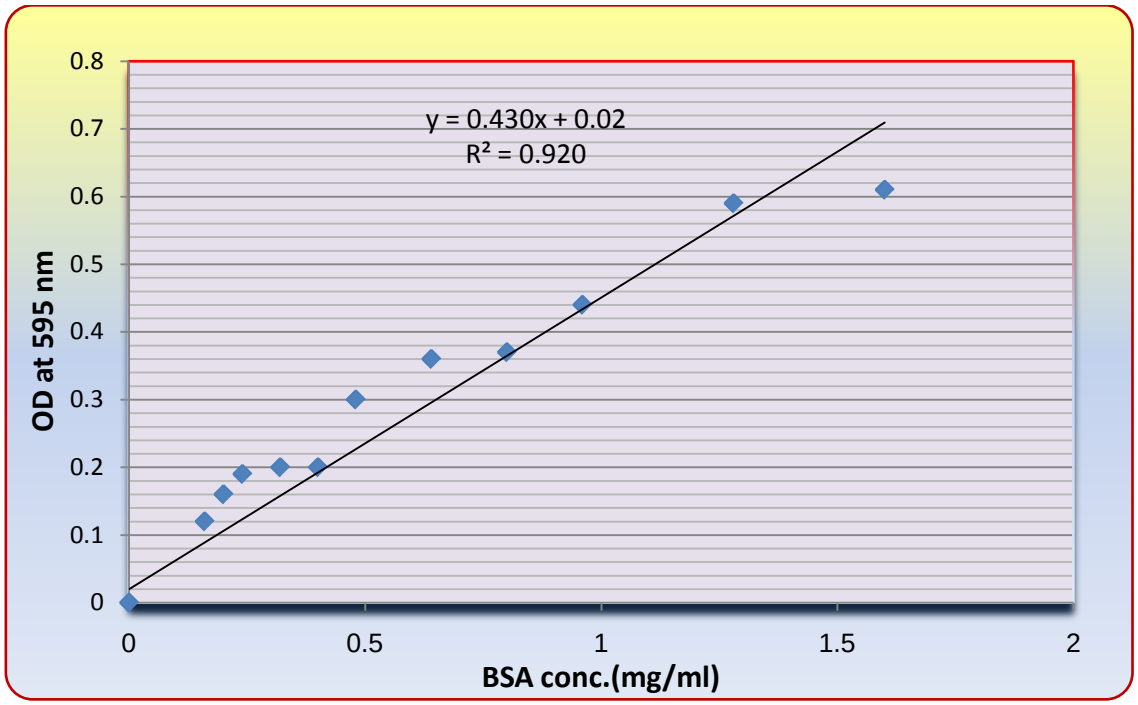

Figure (3-1): Standard curve of bovine serum albumin for determination of protein concentration according to Bradford (1976).

3.2.5. Preparation of glucose standard curve (Otajevwo and Aluyi, 2011)

For preparation of glucose standard curve, glucose stock solution was prepared first by dissolving 1g of glucose in 10 ml of D.W., then serial concentrations were prepared for determination of reducing sugars. Absorbance was measured at 540 nm after the addition of 3,5-DNS reagent and boiling for 5 min for each glucose concentration as indicated in table (3-3). Standard curve representing the relationship between glucose concentrations and absorbancewas plotted (figure 3-2).

Table (3-3): Concentration and absorbence of glucose solutions for determination of reducing sugars.

Tube No.	Glucose stock sol. (ml)	D.W (ml)	Final volume (ml)	Final glucose Conc. (mg/ml)	Absorbance (540 nm)
1	0.00	10.00	10	0	0.000
2	0.01	9.99	10	0.1	0.348
3	0.02	9.98	10	0.2	0.450
4	0.03	9.97	10	0.3	0.590
5	0.04	9.96	10	0.4	0.717
6	0.05	9.95	10	0.5	0.860
7	0.06	9.94	10	0.6	0.949
8	0.07	9.93	10	0.7	1.129
9	0.08	9.92	10	0.8	1.199
10	0.09	9.91	10	0.9	1.332
11	0.10	9.90	10	1.0	1.363

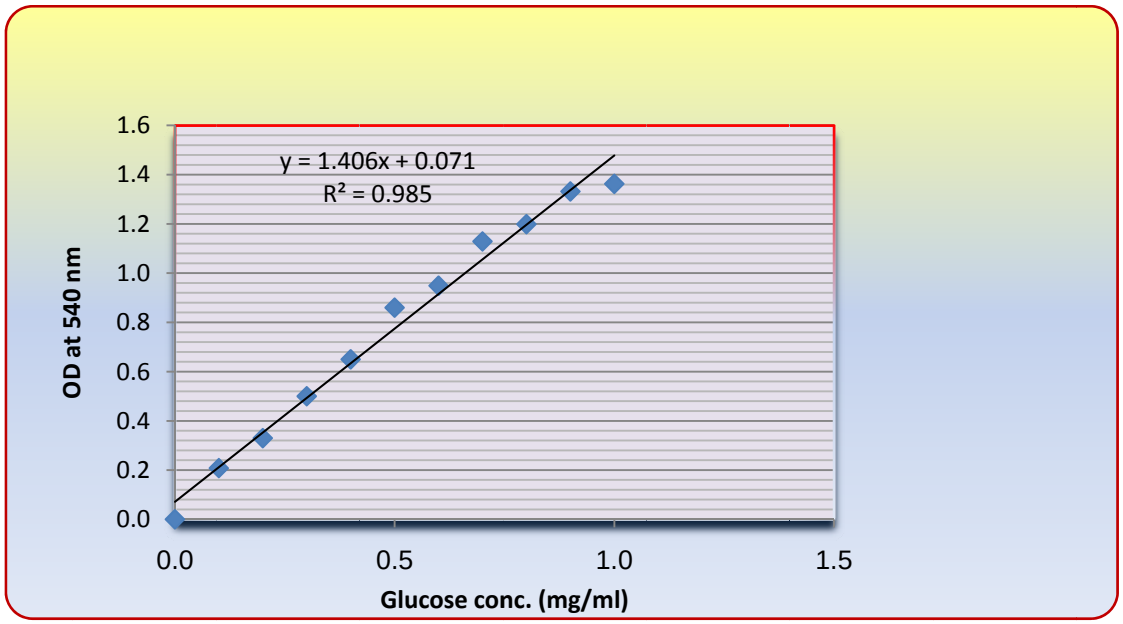

Figure (3-2): Standard curve of glucose for determination of reducing sugar(Otajevwo and Aluyi, 2011).

3.2.6.Galacturonic acid standard curve (Qureshi *et al*., 2012)

For preparation of galacturonic acid standard curve, galacturonic acid stock solution was prepared first by dissolving 1 g of galacturonic acid in 10 ml of D.W., then serial concentrations were prepared for determination of reducing galacturonic acid. Absorbance was measured at 540 nm (table 3-4) after addition of DNS reagent for each galacturonic acid concentration and boiling for 5 min.,then cooled to room temperature. The absorbance was read and compared with a distilled water as a blank. Then standard curve representing the relationship between galacturonic acid concentrations and absorbance was plotted (figure 3-3).

Table (3-4): Concentration and absorbence of galacturonic acid solutions for determination of reducing galacturonic acid.

Tube No.	Glalacturonic acid stock sol. (ml)	D.W (ml)	Final volume (ml)	Final glalacturonic acid Conc. (mg/ml)	Absorbance (540 nm)
1	0.00	10.00	10	0.0	0.000
2	0.01	9.99	10	0.1	0.324
3	0.02	9.98	10	0.2	0.476
4	0.03	9.97	10	0.3	0.646
5	0.04	9.96	10	0.4	0.822
6	0.05	9.95	10	0.5	0.883
7	0.06	9.94	10	0.6	1.026
8	0.07	9.93	10	0.7	1.01
9	0.08	9.92	10	0.8	1.214
10	0.09	9.91	10	0.9	1.330
11	0.10	9.90	10	1.0	1.427
12	0.11	9.89	10	1.1	1.565
13	0.12	9.88	10	1.2	1.800
14	0.13	9.87	10	1.3	1.702
15	0.14	9.86	10	1.4	1.841

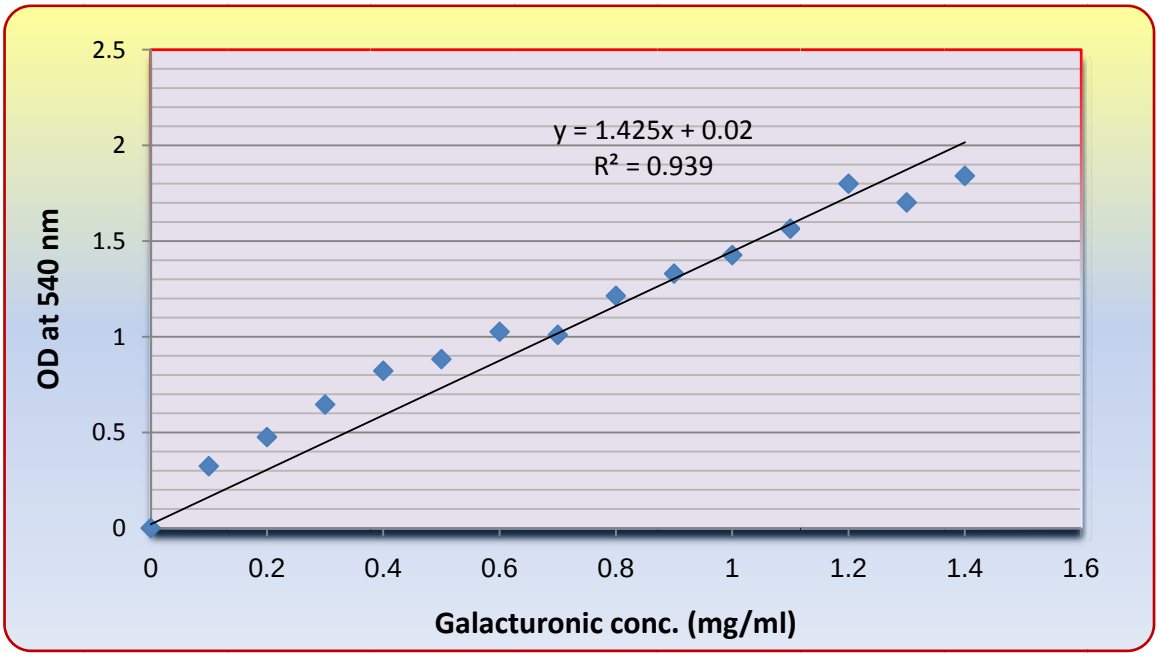

Figure (3-3): Standard curve of galacturonic acid for determination of reducing galacturonic acid (Qureshi *et al*., 2012).

3.2.7. Ability of local isolatesin production of cell wall degrading enzymes

3.2.7.1. Cellulase production

The ability of the selected isolates in cellulase production was detected according to Irfan *et al*. (2012) by using carboxymethylcellulase (CMC) as a suitable substrate for assaying enzyme activity as follows:

3.2.7.1.1. Qualitative screening (Bai *et al*., 2012)

The ability of the selected isolates for cellulase production on a solid medium was examined by culturing single colony of each bacterial isolate on CMC agar plates, incubated at 30 ºC for 24 hrs., then plates were flooded with 0.1% congo red solution and left to stand for 15 min., then plates were destained with1M NaCl solution. Appearance of hydrolysis halo around

colonies due to cellulase production indicates positive result.

3.2.7.1.2.Semi-quantitative screening(Jakucs and Varallyay, 1995)

Cellulase activity was measured by placing 20 ml of CMC agar assay medium in each pre-sterilizedpetriplates. After solidifying,a cavity or hole was made in the center by using cork borer (8mm).The central cavity was filled with 100 µl crude filtrate of fresh culture of each bacterial isolate, incubated at 30°C for 24hrs. Then the plates were flooded with 0.1% congo red for 15 min at room temperature and allowed to counterstaining with 1M NaCl. Appearance of a clear zone of hydrolysis around the well is due to cellulase production indicating a positive result.

3.2.7.1.3.Quantitative screening(Samira *et al*., 2011)

Quantitative screening for cellulase production by the bacterial isolates were achieved by determining cellulase activity in culture filtrate for each bacterial isolate after propagation in CMC broth medium (3.1.4.2- C) at 35 ºC for 24 hrs. Cellulase activity was measured by mixing 0.1 ml of enzyme (crude filtrate) with 0.1 ml of 1% (w/v) CMC prepared in 10mM sodium phosphate buffer, the pH was adjusted to7.0 at 37°C for 60min. The reaction was stopped by adding 1.0 ml 3, 5- dinitro salicylic acid (DNS) reagent. Then mixture was placed in a boiling water bath for 10min.and left to cool at room temperature, and the absorbance at 540 nm was read. Cellulase activity was calculated in accordance with glucose standard curve.

Cellulase activity (U/ml) = absorbance at 540 nm / slope of glucose standard curve ×1000/MW of glucose × 0.1×60 min.

Enzyme activity unit was defined as the amount of enzyme that releases 1µmol of glucose per min.

3.2.7.1.4. Total cellulases production (Barman *et al*., 2011)

Test tubes containing total cellulases production medium (as mentioned in 3.1.4.2. D) were inoculated with a single colony of each bacterial isolate, incubated at 30 ºC for 15d. Degradation of the filter paper is due to the activity of cellulase produced by bacterial isolates, indicating a positive result.

3.2.7.2.Detection of pectinase production

3.2.7.2.1. Pectinase production on a solid medium(Soares*et al*., 1999; Raju and Divakar, 2013)

Pectin agar medium was used to assay the abilityofbacterial isolates for pectinase production medium. This was achieved by transferringa single colony of each bacterial isolate onto the medium containing 1% citrus pectin as a suitable substrate, incubated at 30°C for 48hrs, then the plates were flooded with iodine solution.Appearance of hydrolysis zone around the colonies is due to pectinase production indicating a positive result.

3.2.7.2.2. Pectinase assay by cup-plate method(Jakucs and Varallyay, 1995; Soares*et al* ., 1999)

In this method, pectinase activity was assayed by transferring 100 µl crude filtrate of a fresh culture from each bacterial isolate into the wells center done in the pectin agar plates, and incubated at 30 ºC for 48 hrs, plates wereflooded with iodine solution for zoneclearance of hydrolysis then counterstained with D.W. Appearance of hydrolysis zone around

each well is due to pectinase activity indicating a positive result

3.2.7.2.3. Pectinase assay (Qureshi *et al*., 2012)

Pectinase activity was assayed by adding 1ml of enzyme solution (bacterial crude filtrate)to 1ml of the substrate solution (0.01 mg/ml pectin) and incubated at 37°C for 15 min, then the reaction was stopped by adding 2 ml of dinitro salysilic acid solution. The mixture was boiled for 5 min. After cooling,absorbance at 540 nm was recorded against a blank D.W.Pectinase activity was calculated in accordance of galacturonic acid standard curveand the following formula:

Pectinase activity (U/ml) = OD at 540 nm / Slope of galacturonic acid standard curve ×1000/MW of galacturonic acid × 1×15 min.

Enzyme activity unit was defined as the amount of enzyme required for liberating 1µg of galacturonic acid per min. under the assay conditions.

3.2.8. Estimation of protein concentration (Bradford, 1976)

Protein concentration was determined by adding 0.1 ml of crude enzyme (crude filtrate) to0.4 ml of Tris-HClthen 2.5 ml of Coomassie brilliant blue was added. After shaking for 2min., absorbance at 595 nm was read, and the protein concentration was calculated from the standard curve for BSA.

3.2.9. Enzyme specific activity

Cellulase and pectinase specific activity was calculated according to the following formula (Whitaker, 1972):

$$\text{Specific activity (U/mg)} = \frac{\text{Enzyme activity (U/ml)}}{\text{Protein conc. (mg/ml)}} = \text{U/mg}$$

3.2.10. Maintenance of bacterial isolates

Maintenance of bacterial isolates was preformed according to Maniatis *et al.* (1982) as follows:

3.2.10.1. Short term storage

Bacterial isolates were maintained for a few weeks on nutrient agar plates. The plates were tightly warped with parafilm, and stored at 4°C.

3.2.10.2. Medium term storage

Bacterial isolates were maintained by stabbing on nutrient agar medium using a small screw- capped bottles then stored at 4 ºC.

3.2.10.3. Long term storage

Bacteria can be maintained for many years in a medium containing 15% glycerol at low temperature without significant loss of viability. This was done by adding 1.5 ml of sterilized glycerol to the exponential growth of bacterial isolates in a screw- capped bottle with a final volume 10ml and stored at -20°C.

3.2.11. Sterilization methods

3.2.11.1. Autoclaving

Media and solutions were sterilized by autoclaving at 121°C (15 Ib/In^2) for15 min.

3.2.11.2. Dry heat sterilization

Glassware were sterilized at 180 °C using an oven for 3 hrs.

3.2.11.3. Membrane sterilization

All heat sensitive solutions were sterilized by filtration using Millipore filters (0.22 μM).

3.2.12. Optimum conditions for cellulase production

Optimum conditions for cellulase production by the selected isolate were determined by inoculating 100 ml of the production medium (as in 3.1.4.2. C) with 1 ml of fresh culture (OD =0.6) of the bacterial isolate and incubated at 35 ºC for 24 hrs, in shaker incubator at 140 rpm, then cellulase activity and protein concentration in crude filtrate were estimated.

3.2.12.1. Optimum CMC concentration (%)

Optimum concentration of CMC for cellulase production was determined for the selected isolate. CMC was added to the production medium at the concentrations of 0.5, 1, 1.5, 2 and 2.5% to determine the optimum concentration of CMC for cellulase production.

3.2.12.2. Optimum nitrogen source

Optimum nitrogen source was determined by adding 0.1% of different nitrogen sources (Sodium nitrate,Ammonium sulfate, Yeast extract, Peptone, and Tryptone) to the production medium in order to determine the best nitrogen sourcefor cellulase production.

3.2.12.3. Optimum nitrogen source concentration

To determine optimum nitrogen source concentration for cellulase

production by theselected isolate,different concentrations of 0.05, 0.1, 0.15, 0.2 and 0.25% of ammonium sulfate were added to the production medium.

3.2.12.4. Optimum phosphate source

Optimum phosphate source for cellulase production by the selected isolate was determined by adding the phosphate sources(Potassium dihydrogen phosphate, Dipotassium hydrogen phosphate,and Disodium hydrogen phosphate) to the production medium at a concentration of 0.1% to determine the bestphosphate source for cellulase production.

3.2.12.5. Optimum phosphate source concentration

To determine optimum phosphate source concentration for cellulase production, different concentrations of 0.05, 0.1, 0.15, 0.2 and 0.25% potassium dihydrogen phosphate were added to the production medium.

3.2.12.6. Optimum temperature

Optimum temperature for cellulose production by the selected isolate was determined by incubating the isolateat different temperatures (25, 28, 30, 32 and 35°C) in the production medium to determine the best temperature for cellulase production.

3.2.12.7. Optimum pH

Optimum pH for cellulase production by the selected isolate was determined by incubating the isolateat different pH values (5, 6, 7 and7.5) in the production medium to determine the best pH for cellulase production.

3.2.12.8. Optimum incubation period

Optimum incubation period for cellulase production by the selected isolate was determined by incubating the isolateat different incubation periods (24, 36, 48, 60, 72 and 96 hrs) in production medium to determine the best incubation time for cellulase production.

3.2.12.9. Optimum agitation speed

Optimumshaking speedfor cellulase production by the selected isolate was examined by incubating the production medium at different shaking speeds(100, 140, 150, 200and 250 rpm) to determine the optimum speed for cellulase production.

3.2.13. Cellulase purification(Yin *et al*., 2010)

More than one-step (using different purification techniques)were used to purify cellulose which produced by the locally isolated *P. dispersa* A3.

3.2.13.1. Ammonium sulfate precipitation

The first step of cellulase purification was achieved by precipitation with ammonium sulfate produced by *P.dispersa* under the optimum conditions.Culture medium was centrifuged at 7500rpm for 15min.at 4ºC. Ammonium sulfate was addedto the supernatant (crude enzyme) with gradual saturation ranging between 60% and 80% after the addition of ammonium sulfate to crude enzyme at each saturation ratio. The mixture was mixed gently on a magnatic stirrer at 4ºC for 20min. The precipitated proteins were dissolved in a suitable volume of 0.02 M Tris-HCl buffer at pH 8. Enzyme activity and protein concentration were estimated before and after ammonium sulfate precipitation.

3.2.13.2. Dialysis of crude enzyme

Cellulase crude enzyme precipitated with a70% saturation percentage of ammonium sulfate wasredissolved in 0.02 M Tris-HClbuffer solution,pH 8 and dialyzed at 4ºC for overnight against the same buffer solution with three increments of substitutions. Also the dialysis tubes were used in the concentration of the products of the enzymes.The dialyzed enzyme was then kept at 4ºC for the next purification steps.

3.2.13.3. Purification by ion exchange chromatography

- **DEAE-Cellulose preparation**

A DEAE-Cellulose column was prepared according to Whitaker and Bernard, (1972) by dissolving 20 g of resin in 1L of D.W. beads were then left to settle down, washed several times with D.W until getting clear appearance. The suspension was filtered throughout Whattman No. 1 using Buchner funnel under discharging. The resin was resuspended in 0.25 M NaCl and NaOH solutions. The suspension was filtered again as mentioned above and washed several times with 0.25 M HCl solution and next by D.W. before it was equilibrated with 0.02 M Tris-HCl buffer solution pH 8. After preparation, dialyzed cellulase was concentrated by sucrose to 19 ml of partial purified enzyme. An enzyme concentrate was then applied to ion exchange chromatographycolumn (1.75×20 cm) packed with DEAE-Cellulose equilibrated previously with 0.02 MTris-HCl buffer pH8. Then column was washed with an equal volume of the same buffer, while attached proteins were stepwise eluted with gradual concentrations of NaCl (0.1, 0.2, 0.3, and 0.4 M respectively). Flow rate throughout the column was 3ml/fraction and the absorbance of each fraction was measured at 280 nm using UV-VIS spectrophotometer. Cellulase activity was determined in

each fraction according to assay method described in 3.2.6.1.3. Fractions representing cellulase activity were pooled together and concentrated for the last step of purification by gel filtration chromatography.

3.2.13.4. Purification by gel filtration chromatography

- **Preparation of Sephadex G-200**

SephadexG-200 was prepared as recommended by Pharmacia FineChemicals Company. A quantity of Sephadex G-200 was suspended in 0.0.2 M Tris-HCl buffer solution pH 8 for 24 hr, degassed, and packed in a glass column (1.25 × 30 cm), and equilibrated with 0.02 M Tris-HCl buffer solution pH 8. Aliquot of concentrated cellulase obtained after the ion exchange purification step was applied ontoSephadexG-200 column equilibrated previously with 0.02 M Tris-HClbuffer solutionpH8. Elution was achieved at a flow rate of 3 ml/fraction using the same buffer for equilibration. Absorbance of each fraction was measured at 280 nm. Cellulase activity was also determined in each fraction.

3.2.14. Determination of Molecular weight

Molecular weight of pure cellulase was determined by gel filtration chromatographyby using the same column that used for purification (Sephadex G-200). Pure cellulase was left to flow throughthe glass column ,and eluted in a flow rate of 3ml / fraction. Standard proteins (Lysozyme,Trypsin, Pronase, Hemoglobin and Bovin Serum Albumine) at a concentration of 3 mg/ml were also applied separately through the same column and were eluted in the same manner of cellulase elution. Absorbance at 280 nm was measured for each fraction, while blue dextran

was applied and eluted to determine the void volume, then the linear relationship between v_e /$v_o$against log molecular weight for each standard protein was plotted.

3.2.15. Determination of optimal pH for cellulase activity

Determination of optimal pHfor cellulase activity was achieved by using phosphate buffer solutions prepared in 3.1.5.6 which had been distributed evenly into aliquots. pH was adjusted in each one according to the required value, so the substrate (CMC) was added to buffer solutions at different pH values (6-8) to produce 0.01 mg/ml of substrate. Test tubes were incubated in a water bath at 37ºC for 10 min.,and the enzyme solution was added to the reaction mixture and incubated for1hr. The reaction was stopped and the activity of cellulase was assayed and plotted against optimalpH for cellulase activity.

3.2.16. Determination of optimal pH for cellulase stability

Purified enzyme was incubated at different pH values ranging between 6 and 8 at room temperature for 1 hr. The remaining activity was then measured after assaying enzyme activity.

3.2.17. Determination of optimal temperature for cellulase activity

Cellulase activity was determined after incubation of the purified enzyme with CMC at different temperature (20, 25, 30, 35, 40, 45, 50, 55 and 60ºC) for 10 min. Then cellulase activity was assayed after each incubation temperature.

3.2.18. Determination of optimal temperature for cellulase stability

A purified enzyme was incubatedin a water bath for different

temperatures (20, 25, 30, 35, 40, 45, 50, 55 and 60 ºC) for 1 hr, then immediately transferred into an ice bath. Activity was assayed for each treatment. The remaining activity (%) for cellulase was plotted against the temperature.

3.2.19. Mutagenesis of the selected isolate

Mutagenesis of the selected bacterial isolate was achieved according to Xu *et al*. (2011) by subjecting fresh cultures of bacterial growth to UV light in a dark place.

A portion of 1 ml of overnight bacterial isolate culture was inoculated into 100 ml of nutrient broth for 6 hrs at 37 ºC. Then 10 ml of the cell suspension was transferred into a sterilized petridish and placed in the tray of the irradiation unit. The distance between tray and source of UV ray was 11 cm.

Cell suspension was subjected to UV irradiation for different periods for 15 sec. with time intervals of 1 sec. For each time interval, aliquot of 100 µl of cell suspension was taken, serially diluted and spread on nutrient agar plate and incubated in dark at 37 ºC for 24 hrs to estimate survivals and detection of mutants at the killing percentage of more than 90%. Suspected mutants were screened for their ability in cellulase production.

3.2.20. Isolation of genomic DNA

Genomic DNA of the selected isolate was extracted according to Blood, Cultured Cell Handbook protocol using Favorgen-Kit as follows:

1-Aliquot of 1 ml of an overnight culture of the selected isolate was centrifuged at 7500 rpm, and the bacterial cells were collected.

2-Pelleted cells were resuspended in eppendorf tube containing 200µl FATG1 solution, mixed gently, then 20µl of Proteinase K (10mg/ml) was added, mixedthoroughly by vortexing, and incubated at 60°C with vortexing every 10 min until complete lysis.

3-Microtubes were briefly spined to remove drops from the inside of the lid, then 4 µl of RNase solution (100 mg/ml) was added and incubated at a room temperature for 2 min.

4- The mixture wasresuspendedin 200 µl of FATG2 solution and mixedthoroughly by pulse-vortexing and incubated at 70°C for 10 min.

5-Aliqout of 200 µl of absolute ethanol was added, mixed thoroughly by pulse- vortexing, and spined briefly to remove drops from the inside of the lid.

6- Eppendorf content was pipetted into FATG mini-column in a 2 ml collection tube, and centrifuged at 8000 rpm for 1min.The supernatant was then discarded.

7- FATG mini column was placed in a new 2 ml collection tube, then 500 µl of buffer W1 was added and centrifuged at 6000 rpm for 1min. Then supernatant was discarded.

8- FATG mini-column was inserted in a new 2 ml collection tube, and aliquot of 750 µl of washing buffer was added, centrifuged at 14000 rpm for 1min. Then supernatant was discard, left to stand for 3 min. to dry off the column.

9- FAGT mini-column was placed in 1.5 ml elution tube, then 50 µl of elution buffer was added directly onto themembrane and incubatedatroom temperature for 3 min., and centrifuged at 6000 rpm for 2min. to elute DNA, and stored at 4°C.

3.2.21. Determination of DNA purity and concentration

Purity and concentration of DNA solutions were measured by using nanodrop spectrophotometer, by placing 2µl of DNA solution into the photocell of the apparatus, then optical density was measured at 260 and 280 nm.

DNA purity was calculated according to the following formula (Maniatis *et al*., 1982)

$$\text{DNA purity} = \frac{\text{Abs. at 260 nm}}{\text{Abs. at 280nm}}$$

DNA solution was pure when this ratio is ranging between 1.8 and 2.0
DNA concentration was calculated according to the following formula:

DNA concentration(ng/µl) = Abs (260 nm) x 50 µg/ml

Note: Absorbency (260 nm) of 1 equal to 50 µg/ml of pure DNA.

3.2.22. Amplification (Maniatis *et al*., 1982)

In order to amplify cellulase gene of the selected isolate, this isolate was first identified as *Pantoeadispersa* and the DNA primers were designed according to the nucleotide sequence of the cellulase gene for this bacterial species that available in the web site of National Center of Biotechnology Information (NCBI). Results of surveying the cellulase gene nucleotide sequence showed there was no any nucleotide sequence matched for cellulase gene of this bacterium. Accordingly, nucleotide sequence of *Pantoea* spp. cellulase gene available in the NCBI was accreditate to design the following primers (table 3-5):

Table (3-5): Primers were used in the cellulase gene amplification.

Oligonucleotide primer	Sequence(5' ⟶3')	Size (bp)
Forward	AGTTAAGCCAGTGTGGTCCG	20
Reverse	CCCATGCAATCAGCACATCG	20
Forward	GCTTACGCTGGAAAAGCTCG	20
Reverse	TGAATACGATCCGACGCCTG	20
Forward	GGGCTGGATCTGCTCTGTTT	20
Reverse	GCTCTCCACCTGCCATTTCT	20
Forward	CGTTTGACAGGTTGTGGCAG	20
Reverse	ATAGCCGCCAAAGTTCGTGA	20
Forward	GGCAGGTGGAGAGTTACCAG	20
Reverse	GGCCGGTTTTACCAAAGCTC	20
Forward	GGGCTGGATCTGGTCTGTTT	20
Reverse	CTGCCACAACCTGTCAAACG	20

The reaction mixture (25µl) for amplification by PCR was consisted of thefollowing components:

Component	Final Conc.	Volume (µl)
Go Taq-Green master mix, 2×	1×	12.5µl
Upstream primer, 10µM	0.1-1.0µM	1µL
Downstream primer, 10µM	0.1-1.0µM	1µL
DNA template	<250 ng	3µl
Nuclease-Free water	N.A.	7.5µl

Amplification procedure was achieved according to the following program:

Step	Temperature(°C)	Time (min.)	No. of cycles
Initial denaturation	95	2	1
Denaturation	95	1	35
Annealing	*	0.5	35
Extension	72	1	35
Final extension	72	5	1

depends on optimum temperature for each primer.

3.2.23. Gel electrophoresis for PCR

PCR products were analyzed on agarose gel (0.8%) using horizontal electrophoresis unit, gel was left to solidify in the tray, then the tray was transferred to the tank of the device and immersed with 1X TBE buffer, DNA samples were then loaded into the wells. Electrophoresis was carried out for 2 hrs at 5v/cm. After electrophoresis, gel was stained with ethidium bromide stock solution for 10min., and then DNA bands were visualized by using UV transilluminator at 365 nm. (Maniatis *et al*., 1982).

Chapter Four
Results
and
Discussion

4. Results and Discussion

4.1.Isolation of cellulase and pectinase enzymes producing bacteria

In order to obtain bacterialisolates producing plant cell wall degrading enzymes (cellulase and pectinase),soil samples were collected fromfields of wheatand barley crops located in Al-Jaderia, Baghdad university. Othersamples were collected fromspoiledorange and apple fruits and carrot roots. Results in table(4-1) showed that 19 bacterial isolates were obtained from soil samples, and 31 bacterial isolates were obtained from spoiled roots and fruits samples. This isolates were maintain on nutrient agar medium for further using.

Table (4-1): Bacterial isolates obtained from soil samples and spoiled fruits and roots.

Sample source	Type of sample	No. of isolate
Soil	Wheat crop	9
	Barley crop	10
Spoiled fruits and roots	Apple	13
	Orange	8
	Carrot	10
Total		50

These isolates were screened to examine their ability in producing cellulase and pectinase enzymes.

4.2. Ability of local isolates in producing cellulases and pectinases enzymes

4.2.1. Screening of isolates for cellulase production

To select the most efficient isolates forcellulase production, the local isolates were screened using solid and liquid medium supplemented with CMC as sole source of carbon.

4.2.1.1. Qualitative screening

Qualitative screening for cellulose production by local isolates was achieved by detecting their ability to grow on CMC- agar medium. Results mentioned in table (4-2) showed the ability of a these isolates to grow on cellulose agar medium with variable degrees according to the formation of clear halo around the colonies of each bacterial isolate. Results displayed in table (4-2)also showedthat the diameters of hydrolysis zone ranged between 10 and 14 mm for different isolates. The isolate A3was the most efficient in cellulase production sinceit gave thehigherof hydrolysis zone on cellulose agar medium (14 mm). This result indicated that local isolates were able to produce cellulose by converting the cellulosic materials to soluble reducing sugars. Congo redis an indicatorfor attachment to the cellulosic material formingclear halo zones. Similar results have been reported by Krootdilaganandh (2000), and Nurachman *et al*.(2010).

4.2.1.2. Semi-quantitative screening

The isolates which were exhibited higher hydrolysis zones more than 10 mm were screened semi-quantitatively in CMC agar medium for cellulase production. Results showed in table (4-3) the presence of clear zones of hydrolysis around each well on CMC–agar medium due to the activity of cellulase produced by individual isolate. Different diameter of clear zone ranged between 8 and 20 mm were observed, due to the differences in the ability of local isolates for cellulase production. Among these isolates, the isolate A3 was the most efficient in cellulase production since the crude filtrate of this isolate caused the highest hydrolysis zone reached 20 mm on CMC agar medium due to the hydrolysis of cellulose to different

reducing sugars as reported by Devi and Kumar (2012).

Table(4-2):Ability of local isolates to cellulase production expressed by zone of hydrolysis on CMC agar medium after incubation for 24 hours at 30 ºC.

Isolate symbol	Source	Diameter of clear zone (mm)	Isolate symbol	Source	Diameter of clear zone (mm)
S1	Soil	11	O1	Orange	13
S2	Soil	10	O2	Orange	0
S3	Soil	12	O3	Orange	0
S4	Soil	0	O4	Orange	0
S5	Soil	0	O5	Orange	0
S6	Soil	10	O6	Orange	0
S7	Soil	0	O7	Orange	0
S8	Soil	0	O8	Orange	0
S9	Soil	0	A1	Apple	0
S10	Soil	0	A2	Apple	12
S11	Soil	0	A3	Apple	**14**
S12	Soil	0	A4	Apple	10
S13	Soil	0	A5	Apple	0
S14	Soil	0	A6	Apple	0
S15	Soil	0	A7	Apple	0
S16	Soil	0	A8	Apple	0
S17	Soil	0	A9	Apple	0
S18	Soil	0	A10	Apple	0
S19	Soil	0	A11	Apple	0
C1	Carrot	0	A12	Apple	0
C2	Carrot	10	A13	Apple	0
C3	Carrot	0	-	-	-
C4	Carrot	0	-	-	-
C5	Carrot	0	-	-	-
C6	Carrot	0	-	-	-
C7	Carrot	0	-	-	-
C8	Carrot	0	-	-	-
C9	Carrot	0	-	-	-
C10	Carrot	0	-	-	-

Table (4-3): Semi-quantitative activity of cellulase enzyme produced by bacterial isolates.

Isolate symbol	Inhibition zone (mm)
A3	**20**
A2	11
S1	8
S3	9
O1	14

4.2.1.3. Quantitative screening for cellulase production

Quantitative screening of local isolates forcellulase production was achieved by determining enzyme specific activity in culture filtrates in cellulase production medium at 35 °C for 24 hrs. Results displayed in table (4-4) showed that all bacterial isolates are cellulase producers with variable degrees in the specific activity of cellulase ranged between 0.12-3.90 U/mg. Among these isolates, also the isolateA3 isolated from spoiled apple fruits was the most efficient in cellulase production sincethe specific activity of the isolate reached 3.90 U/mg. According to these results, the isolate A3 was selected for further investigations.

Table (4-4): Specific activity of cellulase produced by different bacterial isolates.

Isolate symbol	Protein conc. (mg/ml)	Activity (U/ml)	Specific activity (U/mg)
A3	0.122	0.474	**3.90**
A2	0.162	0.04	0.24
S1	0.150	0.041	0.27
S3	0.134	0.025	0.19
O1	0.138	0.017	0.12

4.2.1.4. Ability of the selected isolate for total cellulase production

Total cellulase production of the selected isolate was achieved according to Barman *et al*. (2011), by examining its ability to grow in broth medium containing Whatman No.1 filter paper as a sole source for carbon and energy. Results showed the occurrence of a heavy growth due to converting and utilizing cellulosic materials of the filter paper to simple saccharides (triose, pentose, hexose, …… etc.) offering a suitable medium for its growth and multiplication as has been mentioned by Chudasama and Thaker (2012).

4.2.2. Pectinase production

The ability of local isolates for pectinase production was examined to select the most efficient isolate using qualitative, semi-quantitative and quantitative screening methods.

4.2.2.1. Qualitative screening

Qualitative screening for pectinase production by local isolates,was achieved by detecting their ability to grow on pectin agar medium. Results exhibited in table (4-5) showed that these isolates were able to grow in pectin agar medium after the formation of hydrolysis zones around the colony for each bacterial isolate. The diameter of hydrolysis zone was ranged between 5 and 12 mm depending on the isolate ability to hydrolyze the pectin.Accordingly, the isolate A3was found to be the most efficient one in pectinase production sinceit produced the highest halo zone diameter on pectin agar medium (12 mm) due to the activity of pectinase in convertingpectin to galacturonic acid at pH 6.0. Similar results have been reported by Soares *et al*. (1999).

Table (4-5): Ability of local isolates in pectinase production on pectin-agar ((pH 6) medium after incubation at 30 ºC for 48 hrs.

Isolate symbol	Source	Diameter of clear zone (mm)	Isolate symbol	Source	Diameter of clear zone (mm)
S1	Soil	7	O1	Orange	11
S2	Soil	0	O2	Orange	0
S3	Soil	10	O3	Orange	0
S4	Soil	6	O4	Orange	0
S5	Soil	0	O5	Orange	0
S6	Soil	0	O6	Orange	0
S7	Soil	0	O7	Orange	0
S8	Soil	0	O8	Orange	0
S9	Soil	0	A1	Apple	0
S10	Soil	0	A2	Apple	6
S11	Soil	0	A3	Apple	**12**
S12	Soil	0	A4	Apple	5
S13	Soil	0	A5	Apple	9
S14	Soil	0	A6	Apple	0
S15	Soil	0	A7	Apple	0
S16	Soil	0	A8	Apple	0
S17	Soil	0	A9	Apple	0
S18	Soil	0	A10	Apple	0
S19	Soil	0	A11	Apple	0
C1	Carrot	0	A12	Apple	0
C2	Carrot	0	A13	Apple	0
C3	Carrot	0	-	-	-
C4	Carrot	9	-	-	-
C5	Carrot	0	-	-	-
C6	Carrot	0	-	-	-
C7	Carrot	0	-	-	-
C8	Carrot	0	-	-	-
C9	Carrot	0	-	-	-
C10	Carrot	0	-	-	-

4.2.2.2. Semi-quantitative screening

Semi-quantitative screening for pectinase production by five selected local isolates, was achieved by testing their ability to grow on a pectin-agar medium. Table (4-6) shows that crude filtrates of these isolates caused hydrolysis around the wells with different diameters ranging between 7 and 25 mm. Among these isolates, the isolate A3was the most efficient in pectinase production producing the highest clear zone diameter (25 mm) on pectin-agar medium after flooding the plates with iodine solution. These results confirm that pectinase produced by this isolate catalyzes the hydrolysis of pectin in to polygalacturonic acid at pH 6.0 as reported by Raju and Divakar(2013).

4.2.2.3. Quantitative screening for pectinase production

Quantitative screening for pectinase production by five selected local isolates was carried out by determining enzyme specific activity in culture filtrate of these isolates after culturing in pectin broth medium at 30°C for 48 hours. Results showed in table (4-7) that these isolates were able to produce pectinase in a pectin broth medium. Specific activity of pectinase in culture filtrates was ranged between 0.74-1.31 U/mg protein. Among these isolates, also the isolate A3 was the most efficient in pectinase production with specific activity of pectinase reached 1.31 U/mg protein. Accordingly,the isolate A3 was selected for further studies.

According to the results of different screening methods for detecting the ability of local isolates in producing cellulase and pectinase, the isolate A3

Table (4-6): Diameters of hydrolysis zones around wells on pectin-agar medium caused by crude filtrates of bacterial isolates.

Isolate symbol	Diameter of clear zone (mm)
A3	25
A2	20
S1	10
S3	7
O1	16

Table (4-7): Specific activity for pectinase produced by local bacterial isolates.

Isolate symbol	Specific activity (unit/mg)
A3	**1.31**
A2	0.86
S1	0.75
S3	0.74
O1	0.89

It was subjected to full identification including genus and species to characterize this bacterial isolate.

4.3. Identification of bacterial isolates

The selected isolate was subjected to identification according to its morphological, cultural and biochemical tests. Additional confirmation was carried out using Api E 20 and VITEK 2.

4.3.1.Morphological and cultural characteristics

Different morphological characteristics were studied to identify the selected isolate. Results showed that this isolate was appeared asstraight rods in shape, motile, non-haemolytic, non-capsulated, non-sporeforming. Colonies of this isolate were smooth, mucoid, yellow pale pigmented, irregularly round, rough and wrinkled thatare difficult to remove with a platinum wire. It was also able to produce yellow pigment, and grows at 30 ºC and 41 ºC and unable to grow at 4 ºC and 44 ºC.

4.3.2 Biochemical identification

Biochemical tests were achieved to identify the selected isolate. The results in table (4-8) showed that this isolate (A3) was positive for catalase, voges proskauer, urease and gelatinase tests. Utilizes citrate and motile, while it was negative for Gram staining, oxidase, methyl red and indole production tests. Results of carbohydrate fermentation tests showed that this isolate was able to ferment manitol, lactose, rhamnose, maltose, sucrose and salicin, while it was unable to ferment sorbitol as indicated in table (4-8). These results confirm that this isolate belongs to *Pantoea* sp. as it was mentioned by Deletoile *et al*. (2009), and Alexandra *et al*. (2010).

Identification of the bacterial isolate as*Pantoea*sp. was confirmed by using Api 20E and VITEK2 identification system. Results indicated in table (4-9) and appendix (1) showed that this isolate was positive for β-galactosidase, ferment glucose, inositol, rhamnose, sucrose, melibiose, arabinose and manitol, while it was negative for arginine dehydrolase, lysine decarboxylase, ornithine decarboxylase, hydrogen sulfide production, tryptophan deaminase, ferment sorbitol and amygdalin.

According to these results it could be concluded that this isolate

Table (4-8): Biochemical tests for identification of the selected bacterial isolate (A3).

Biochemical test	Result
Gram stain	-
Yellow pigment	+
Catalase	+
Oxidase	-
Motility	+
VP (Acetoin production)	+d
Methyl red	-
Indole	-
Urease	-
Gelatinase	+
Citrate utilization	+
Fermentation / Oxidation of	
Mannitol	+
Lactose	+
Rhamnose	+
Maltose	+
Sucrose	+
Salicin	+
Sorbitol	-
Growth at	
30 °C	+
41°C	+
4 °C	-
44 °C	-

(-) : Negative result; (+) : positive result; (+d) : positive during 1-4days; (+): strains positive during 1-2 days; (d): Positive during 4-5days.

Table (4-9): Identification of locally isolated *Pantoea* spp. using Api 20E identification system kit.

Test	Result
β-Galactosidase	+
Arginine dihydrolase	-
Lysine decarboxylase	-
Ornithine decarboxylase	-
Citrate utilization	-
Hydrogen sulphide	-
Urease	-
Tryptophan deaminase	-
Indole	-
Voges-Proskauer	+
Gelatinase	-
Glucose fermentation	+
Inositol fermentation	+
Sorbitol fermentation	-
Rhamnose fermentation	+
Sucrose fermentation	+
Melibiose fermentation	+
Amygdalin fermentation	-
Arabinose fermentation	+
Mannitol fermentation	+

(-): Negative result. (+): Positive result.

is characterized as *Pantoea* sp., then it was differentiated from other *Pantoea* spp. as *Pantoea dispersa* by its ability to metabolize malonate, melibiose, 5-ketogluconate and growth at 41 ºC but not 44 ºC. These results are in accordance with those of Selvakumar *et al*. (2008) who mentioned that these biochemical characteristics are applied to *P. dispersa*.

The isolation of *P. dispersa* from the plant products is so important, since *P. dispersa* produces chitinase and protease. It was found that *Pantoea* spp. Cause crop disordersas a result of damaging components, of plant yield like cotton by losing 10-15% of cotton yield, due to plant cell wall degrading enzymes. In addition to their medical disorders for humans like urinary tract infection and arthritis (Gavini *et al*., 1989; Champs *et al*., 2000; Gohel*et al*., 2004; Medrano and Bell, 2007).

4.4. Optimum conditions for cellulase production

Nutritional and cultural conditions were studied to determine the optimum conditions for cellulase production by *P.dispersa* A3. Each Experiment was achieved by inoculating the production medium with fresh culture (O. D = 0.6) of bacterial isolate and incubation under the studied conditions as follows:

4.4.1. Effect of inducer concentration(CMC)

In order to determine the optimum of inducer concentration (CMC) for cellulase production by *P. dispersa* A3, five concentrations (0.5, 1.0, 1.5, 2.0 and 2.5%) of CMC were used. Results illustrated in figure (4-1) showed that specific activity of cellulase production by *P.dispersa* A3 was varied according to the concentration of the inducer, and the maximum production of cellulase was achieved when the production medium was supplemented with 1.5% CMC. At this concentration, cellulase specific activity reached 5.2 U/mg. This result agrees with the results reported by Verma *et al*. (2012) who mentioned that the optimal concentration of CMC for cellulase production from *Bacillus* spp. is 1.5%. A significant decrease in cellulase specific activity was observed when the

concentration of the substrate was either increased or decreased (figure 4-1). This observation suggests that scaling up of cellulase production by this organism could be done with 1.5% CMC concentration as reported by Sakthivel*et al*.(2010).

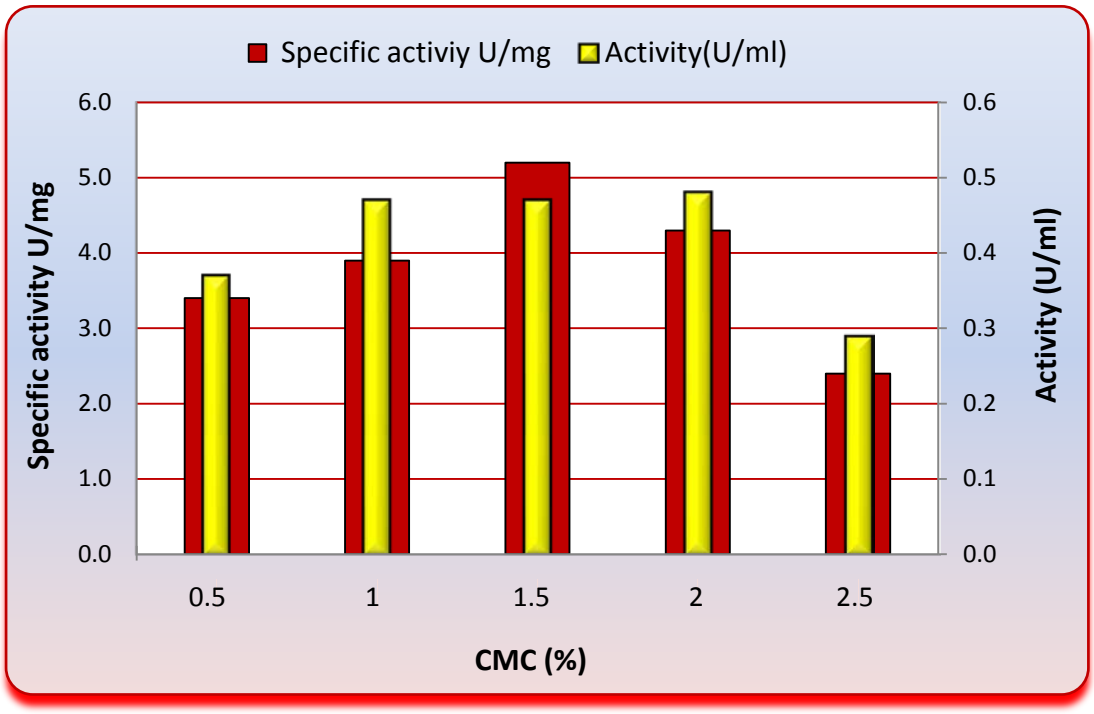

Figure (4-1):Effect of CMC concentration on cellulase production by local isolate *P. dispersa* A3 after incubation for 24 hours at 35 °C, inshaker incubator at 140 rpm.

The bacterium under study is heterotrophs requires organic carbon for growth, and the organic carbon must be in a form that can be assimilated. Glucose, supports the fermentative or respiratory enhancement of many organisms. It is crucial that a substrate should be supplied at levels appropriate for the microbial strain that is being grown. Levels that support the growth of one organism may inhibit the growth of another organism, on the other hand cellulose production increased with the

increase in initial CMC concentration from 1.0 to 1.5%, while further increases in CMC concentration slightly reduced the yield. A decrease in cellulase production above 1.5% concentration of CMC was noticed by Kock (1997).

4.4.2. Effect of nitrogen source

In order to determine the optimum nitrogen source for cellulase production by local isolate *P. dispersa* A3, five nitrogen sources including sodium nitrate, ammonium sulfate, yeast extract, peptone and tryptone were used. These nitrogen sources were added to the production medium at a concentration of 0.1%. Results illustrated in figure (4-2) show that the production of cellulaseby *P. dispersa* A3 varied according to the type of nitrogen source. Inorganic nitrogen sources are better than organic ones in encouraging cellulase production.

Maximum production of cellulase was achieved when the production medium was supplemented with ammonium sulfate. Enzyme specific activity reached 9.1U/mg when this nitrogen source was used. This result is similar to those of Sethi *et al*. (2013) who reported that the cellulase production is sensitive to repression by different nitrogen sources. Mandels (1975) found that replacing peptone by different nitrogen sources, among them ammonium sulfate, was suitable nitrogen source for cellulase production. Nitrogen is one of the major cell protein components and stimulates cellulase activity.Ammonium sulfate salt might have a direct involvement in protein synthesis.

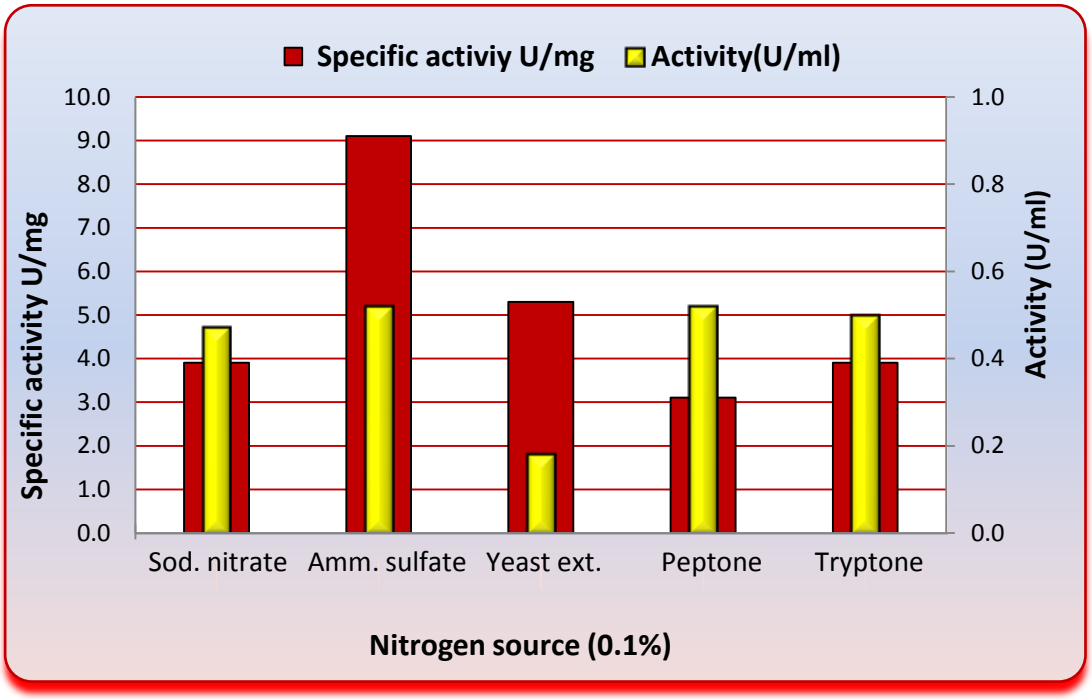

Figure (4-2): Effect of nitrogen source on cellulase production by the local isolate *P.dispersa* A3 after incubation for 24 hours at 35°C, inshaker incubator at 140 rpm.

4.4.3. Effect of ammonium sulfate concentration

In order to determine the optimum concentration of ammonium sulfate for cellulase production by isolate *P. dispersa* A3, different concentrations (0.05, 0.1, 0.15, 0.2 and 0.25%) of ammonium sulfate as a nitrogen source were used. Results in figure (4-3) showed that maximum production of cellulase was obtained when ammonium sulfate was added to the production medium at a concentration of0.1%. At this concentration, cellulase specific activity in culture filtrate recorded 9.1 U/mg. This finding is in accordance with Mandels (1975); Bhale and Chatage (2013), who found that the best concentration of ammonium sulfate to cellulase production is 0.1%.

This results also agrees with the results reported by Sherief *et al*. (2010) who found that cellulose production commended by nitrogen-limiting

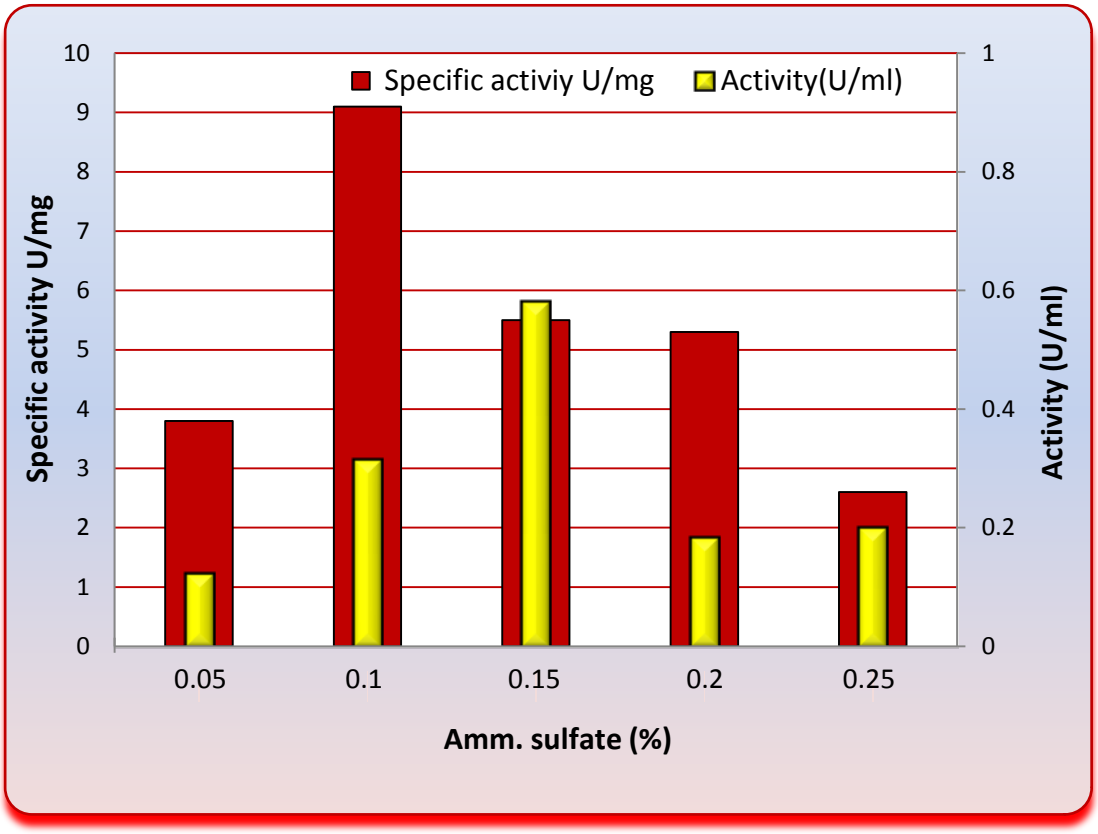

Figure (4-3): Effect of ammonium sulfate concentration on cellulase production by the local isolate *P.dispersa* A3 after incubation for 24 hours at 35 °C, in shaker incubator at 140 rpm.

conditions and the yield of cellulase decreased when excess peptone was supplemented tothe growth medium.

4.4.4. Effect of phosphate source

In order to determine the optimum phosphate source for cellulase production by isolate *P. dispersa* A3, three phosphate sources were used. These phosphate sources included potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), and disodium hydrogen phosphate (Na_2HPO_4). They were added to the production medium at 0.1%.

Results illustrated in figure (4-4) exhibited that the production of cellulase by *P. dispersa* A3 varied according to the type of phosphate source. Maximum production of cellulase was achieved when the production medium was supplemented with KH_2PO_4. Enzyme specific activity reached 9.1 U/mg, when the above phosphate source was used. The composition of nutritional substances and growth factors in the medium, represents the suitable environmental conditions forthe microorganism.It controlled the rate of growth and the final harvest.

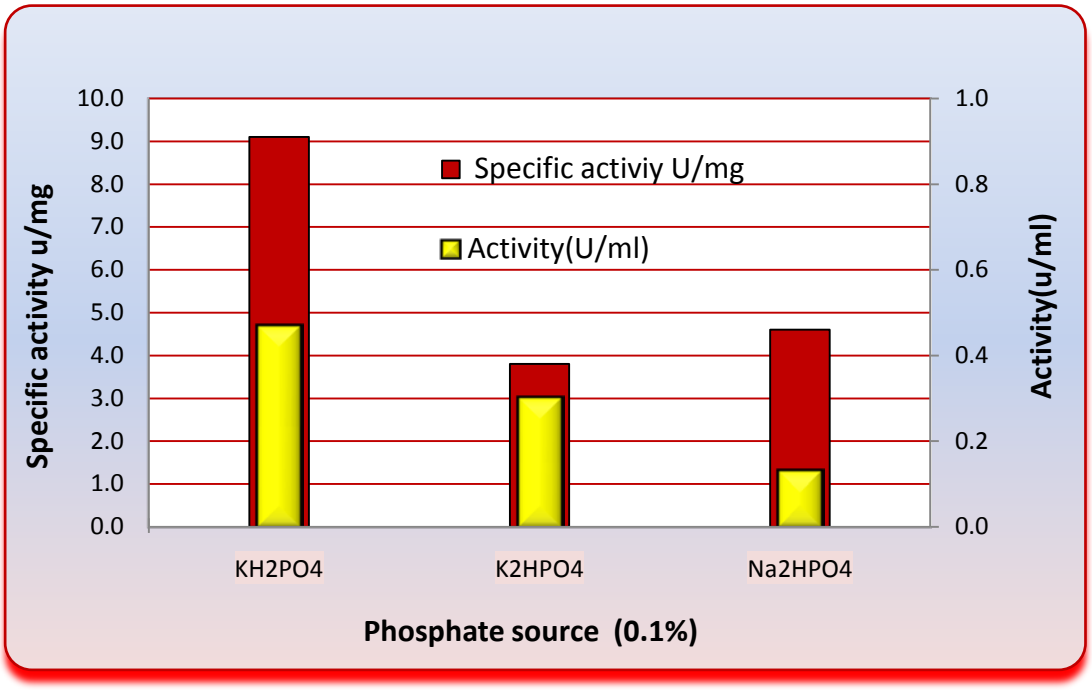

Figure (4-4): Effect of phosphate sources on cellulase production by the local isolate *P. dispersa* A3 after incubation for 24 hours at 35 °C, in shaker incubator at 140 rpm.

4.4.5. Effect of KH_2PO_4 concentration

Optimum concentration of $KH_2PO_4$for cellulase production by isolate *P. dispersa* A3 was examined using various concentrations (0.05, 0.1, 0.15, 0.2, and 0.25%) of KH_2PO_4 for cellulase production. Results indicated in figure (4-5) show that maximum production of cellulase

was obtained when KH_2PO_4 was used to supplement the production medium at a concentration of 0.1%. Cellulase specific activity in culture filtrate reached 9.1U/mg at the above concentration. This suggests that scaling up of cellulase production by this organism could be done with 0.1% of KH_2PO_4. This result was similar to those of Sakthivel *et al*. (2010) who reported that the best concentration of KH_2PO_4 to cellulase production was 0.1%.

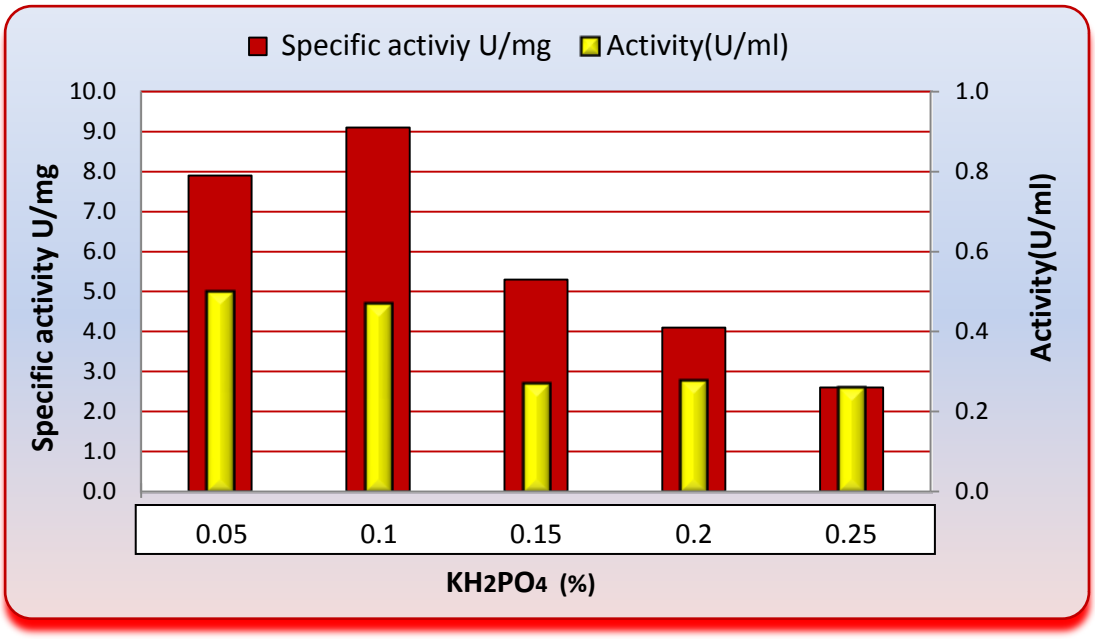

Figure (4-5): Effect of KH_2PO_4 concentration on cellulase production by the local isolate *P. dispersa* A3 after incubation for 24 hours at 35 °C, in shaker incubator at 140 rpm.

4.4.6. Effect of the pH

The effect of the pH on the production of cellulase by *P. dispersa* A3, was also studied.The pHof the medium was adjusted to different values ranging between 5 and 7.5. Results illustrated in figure (4-6) showed that maximum cellulase production was obtained when the pH value was adjusted to 7. At this pH value the enzyme specific activity in culture medium recorded 9.1 U/mg. This obtained result was in accordance

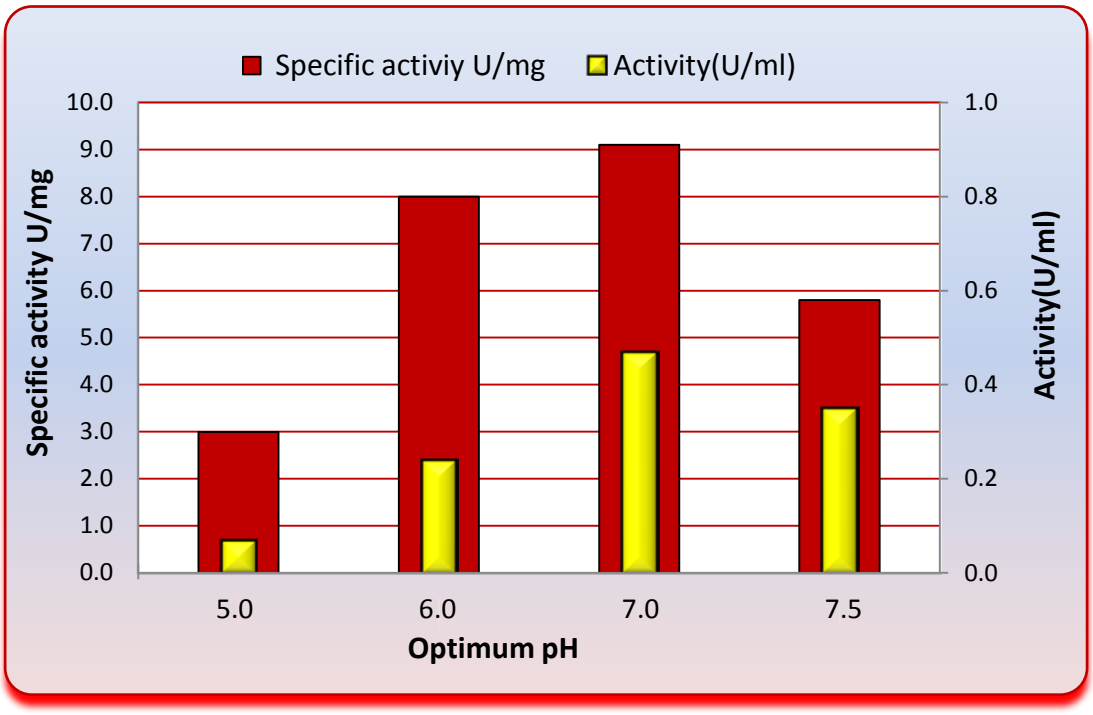

Figure (4-6): Effect of pH values on cellulase production by the local isolate *P. Dispersa* A3 after incubation for 24 hours at 35°C, in shaker incubator at 140 rpm.

with Bai *et al*. (2012), they reported that the maximum production of cellulase by *B. subtilis* isolated from cow dung was obtained when the pH medium was adjusted to pH 7. The decrease in cellulase production in culture medium below or over pH 7 may be attributed to its effect on the metabolism of microorganism, ionization, stability and solubility of the bio-molecules.Also a decrease or increase in hydrogen ions concentration causing pH changes, which may lead to drastic changes in the three-dimentional structure of proteins because hydrogen and/or hydroxyl ions compete the hydrogen bonds and ionic bonds in an enzyme structure (Tortora *et al*., 1970; Hammami *et al*., 2007). Therefore, the effect of pH on enzyme production results from its role in the solubility of the nutritional substances and its effect on the ionization of the substrate

and its availability to the microorganism, in addition to its effect on the stability of the produced enzyme as has been documented previously by Bull and Bushnel (1976).

4.4.7. Effect of incubation temperature

In this study different incubation temperatures (25, 28, 30, 32, and 35 °C) were used to determine the optimal temperature for cellulase production by *P. dispersa* A3. Results displayed in figure(4-7) denote that maximum production of cellulase was obtained when the isolate was grown in the production medium at 30 °C. Under these conditions, cellulase specific activity reached12.0U/mg. Similar finding was proposed by Jaradat *et al.* (2008) and Bai*et al.* (2012),they mentioned that the optimum temperature for cellulase production by *Bacillus subtilis* was 30 °C.

Verma *et al*. (2012), found that the optimum temperature for cellulase production by *Bacillus* spp. isolated from agricultural fields, was 45 °C, therefore the optimum temperature required for enzyme production depends on the species of microorganism and its cultural and physiological requirements.

For any enzymatic reaction, elevation of temperature below or above the optimal drastically reduces the rate of reaction. This may be due to the enzyme denaturation, or losing the three-dimentional structure characteristics. Denaturation of a protein involves the breakage of hydrogen bonds and other non-covalent bonds (Tortora *et al*., 1970).

Temperature affects microorganism growth offering oxygen solubility in the medium, kinetic energy of molecules, or the reaction velocity of molecules. In other study, it was found that cellulose produced by

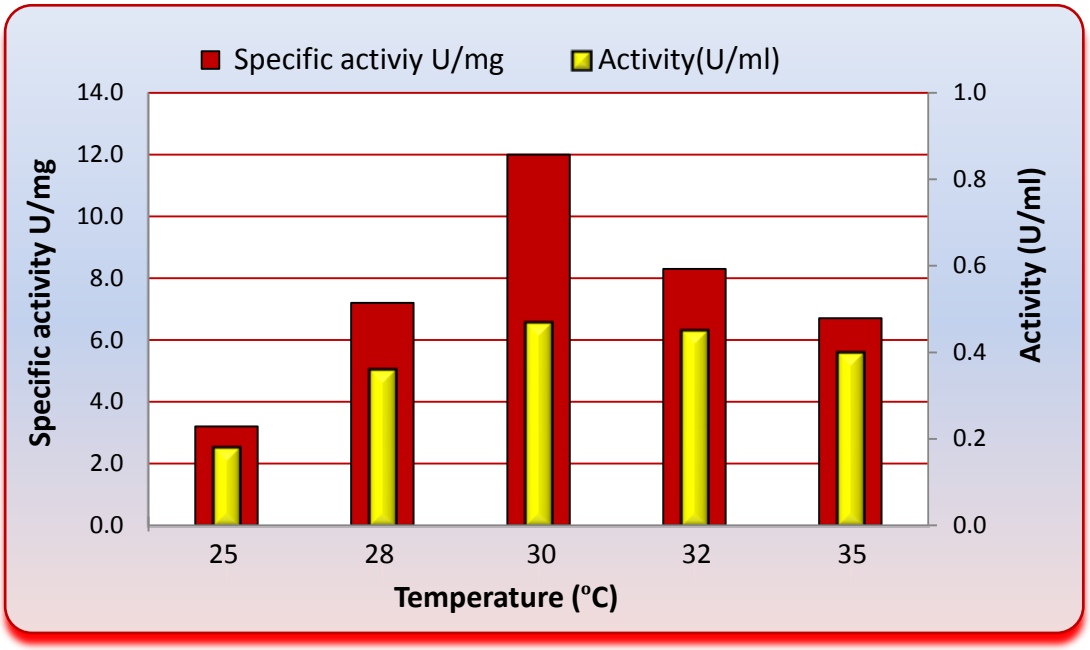

Figure (4-7): Effect of temperature on cellulase production by the local isolate *P. dispersa* A3 after incubation for 24 hours, in shaker incubator at 140 rpm.

Pseudomonas spp. at 40 °C possessed a higher cellulose production (Sethi *et al*.,2013). Irfan *et al*. (2012) reported that optimum production temperature for cellulose production by *Cellulomonas* spp. isolated from soil was at 60 °C.

Temperature has a significant effect on enzyme structure and enzyme production. High temperature affects metabolism of the living cells and may destroy many sensitive enzymes used in cellulase production. Furthermore, it may change enzyme structure and reduces its activity or by influencing extracellular enzyme secretion, possibly by changing the physical properties of the cell membrane (Prescott *et al*., 2005).

4.4.8. Effect of incubation period

Optimum cellulase production by local isolate *P.dispersa*A3 was determined after different incubation periods (24, 36, 48, 60, 72, and

96 hours). Results presented in figure (4-8) showed that enzyme production was initiated after the first 24 hrs of incubation with gradual increase in productivity with the increase in the incubation period. Enzyme specific activity reached 24U/mg after 72 hours of incubation, then it began to decline reaching 14 U/mg after 96 hours of incubation. The decline after 96 hours of incubation might be referred to the adverse effect of the metabolism products in culture medium which were produced continuously throughout growth of bacteria (Heinemann and Howrd, 1969).

Accordingly, Jaradat *et al*. (2008) found that the production of cellulasefrom *B.subtilis* increased gradually after 12 hours of incubation, and the maximum production was occurred after 72 hours of incubation.

4.4.9. Effect of agitation speed

Effect of agitation speed on cellulase production by local isolate *P. dispersa* A3 was examined throughout incubation at different speeds of agitation (100, 140, 150, 200, and 250 rpm) in ashaker incubator. Results illustrated in figure (4-9) showan increase in the cellulase production and enzyme specific activity reached 24 U/mg when the culture medium was incubated in a shaker incubation at 140 rpm, then it began to decrease with the increase in the agitation speed. This may due to the moderate agitation speed that supplies growth medium with sufficient amount of oxygen for growth and production of different metabolites.

This result is approaching other study which reported an increase in bacterial growth under shaking conditions compared to static. Maximum enzyme production observed when agitation speed at ranged between 100 to 200 rpm. Khan *et al*. (2006) achieved maximum enzyme product at

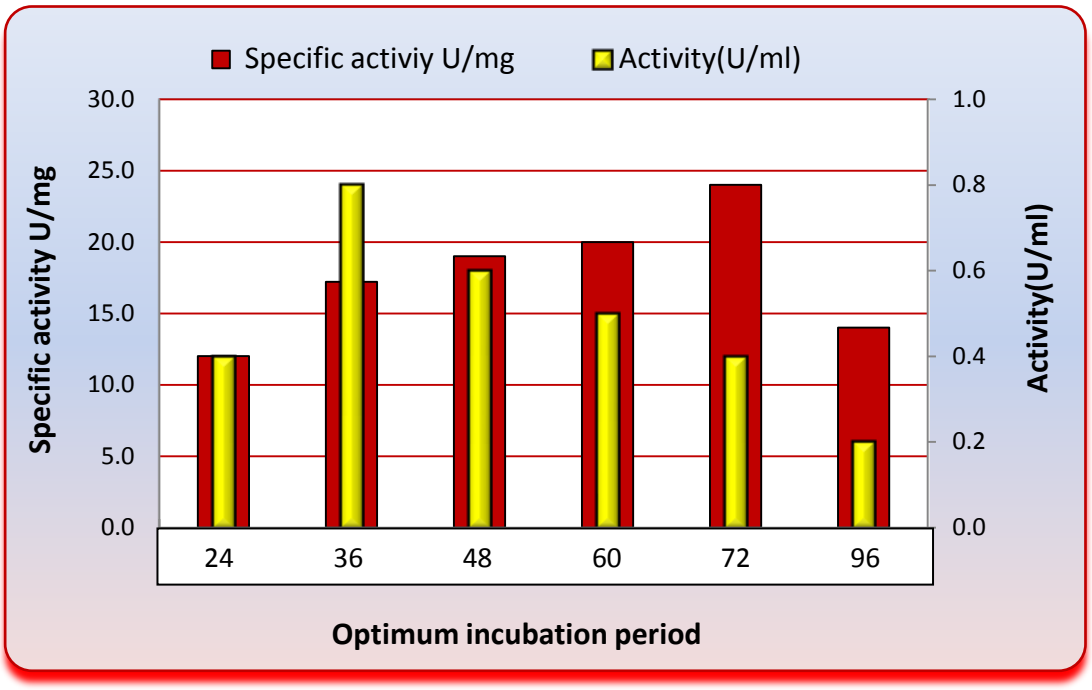

Figure (4-8): Effect of incubation period on cellulase production by the local isolate *P. dispersa* A3 after incubation at 30 °C, in a shaker incubator at 140 rpm.

100-200 rpm.Purwanto (2009) found that vigorous agitation suppressed enzymes production causing mechanical inactivation of the enzymes. Vigorous agitation seems to affect the catalytic activity of the enzymes.

4.5. Purification of cellulase

To purify cellulase produced by locally isolated *P. dispersa* A3 under the optimum conditions, four purification steps were used, they included the following:

4.5.1. Ammonium sulfate precipitation

In this experiment, ammonium sulfate precipitation method was used as the first step in cellulose purification. The ammonium sulfate

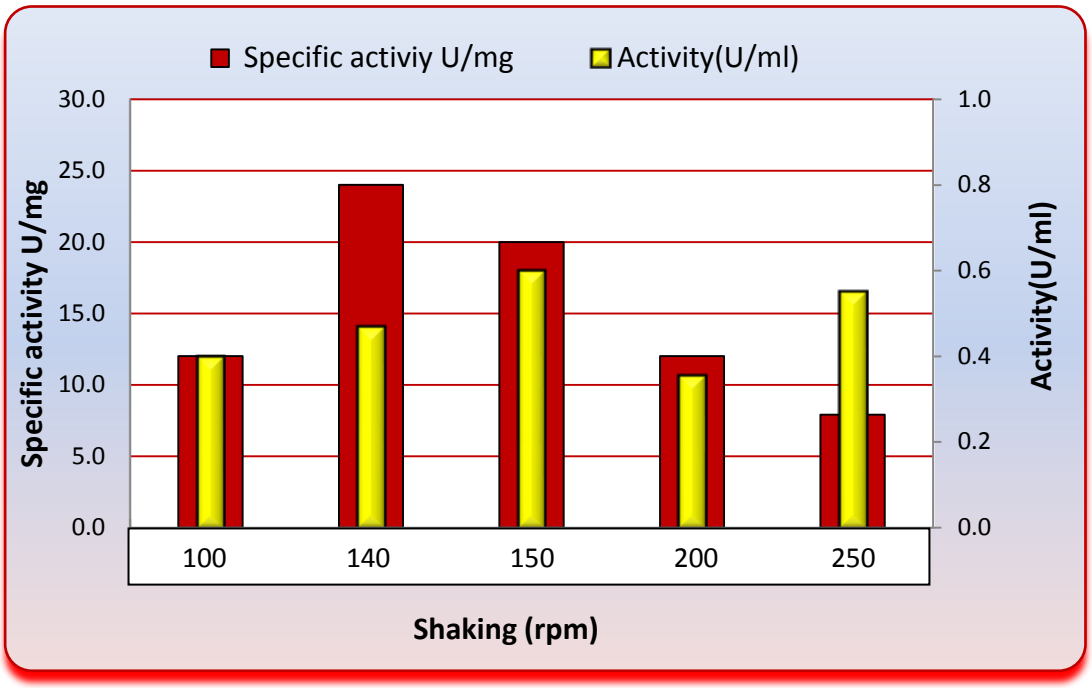

Figure (4-9): Effect of shaking on cellulase production by the local isolate *P. dispersa* A3 after incubation for 72hours at 30 °C, in a shaker incubator at different speeds .

saturation for enzyme solution was done at 65-70% for precipitation of the crude enzyme (cell free culture filtrate). Results showthat 70% of saturation with ammonium sulfate precipitated the enzyme, under the optimum conditions, and the specific activity of cellulase was 4.0 U/mg with a purification fold of 2.32. Ammonium sulfate is a common salt used in protein precipitation due to its high solubility, stabilized protein structure and cheap. Therefore, it was used in precipitating cellulase and different enzymes (Mawadza, 2000).Saha (2004) found that 50.4 fold of purification and 42.7% of cellulase yield were obtained from *B. subtilis* YJ1 after ammonium sulfate precipitation with a saturation percent reached 60-80%.

After ammonium sulfate precipitation, the precipitate was redissolved

in small amount of 0.02 M Tris-HCl buffer pH 8 and dialyzed against the same buffer. Results showed that after dialysis, cellulase specific activity and the unit activity increased to5.0 U/mg, 1.5 U/ml respectively.

4.5.2. Ion exchange chromatography

Ion exchange chromatography technique was used to purify cellulase produced by local isolate *P. dispersa* A3 after ammonium sulfate precipitation and dialysis step. In this technique, the dialyzed cellulase was applied onto DEAE-cellulose column, then the column was equilibrated and washed with an equal volume of 0.02 M Tris-HCl buffer solution at pH 8 to wash uncharged and positively charged proteins in protein mixture of crude cellulase. The negatively charged bound proteins were then eluted using gradient concentration of sodium chloride ranged between 0.1 and 0.4 M. Cellulase activity and absorbance at 280 nm were measured at each fraction. Results in figure (4-10) showed the appearance of one protein peak in the washing fractions, while two protein peaks appeared after elution by gradient concentrations of sodium chloride. Cellulase activity was measured in the fractions of these three protein peaks. Data indicated that cellulase activity occurred in the fraction of the wash step and confined between fraction No.23 and fraction No.34.

The highest cellulase activity was in the fraction No.32. Accordingly, fractions represent cellulase activity were pooled and concentrated to 5ml, then protein concentration, cellulase activity and specific activity were measured. It was concluded from the separation step by using DEAE-cellulose, that cellulase has a positive net charge, since it was not bound with the same charge of the ionic matrix (anionic ion exchanger) of DEAE-cellulose.

The proposed data in table (4-10) also show that protein concentration, cellulase activity and specific activity in this step were 0.024 mg/ml, 3.1U/ml, 129.1 U/mg protein respectively, with purification fold of 76.8 and enzyme yield of 27.6%.These results were in agreement with Mirzaakhmedov *et al*. (2007) who reported an increase in cellulase activity after purification by using anionic ion exchanger of DEAE-cellulose. Yin *et al*. (2010) also purified cellulase using the same method, and the enzyme was eluted fromthe columnin the elution fractions ion exchanger enzyme was washed when they purified the cellulase from *B.subtilis*.

After purification by ion exchange chromatography step, cellulase was further purified using the gel filtration chromatography technique to purify the enzyme for further enzyme characterization studies.

4.5.3. Gel filtration chromatography

The gel filtration chromatography technique was the final step in the purification of cellulase produced by the locally isolated *P. dispersa* A3. After purification by the ion exchange purification step, fractions representing cellulase activity were collected, pooled and concentrated to be applied in sephadex-G200 previously equilibrated with 0.02 M Tris-HCl bufferpH 8. Sephadex-G200 has separation limits ranging between (5000-600,000 dalton) allowing larger capacity of separation with a high purification degree.

A aliquot of 3 ml of cellulase concentrate (3 mg/ml) was injected into the column and eluted with the same buffer. Results displayed infigure (4-11) show two protein peaks appeared after elution with

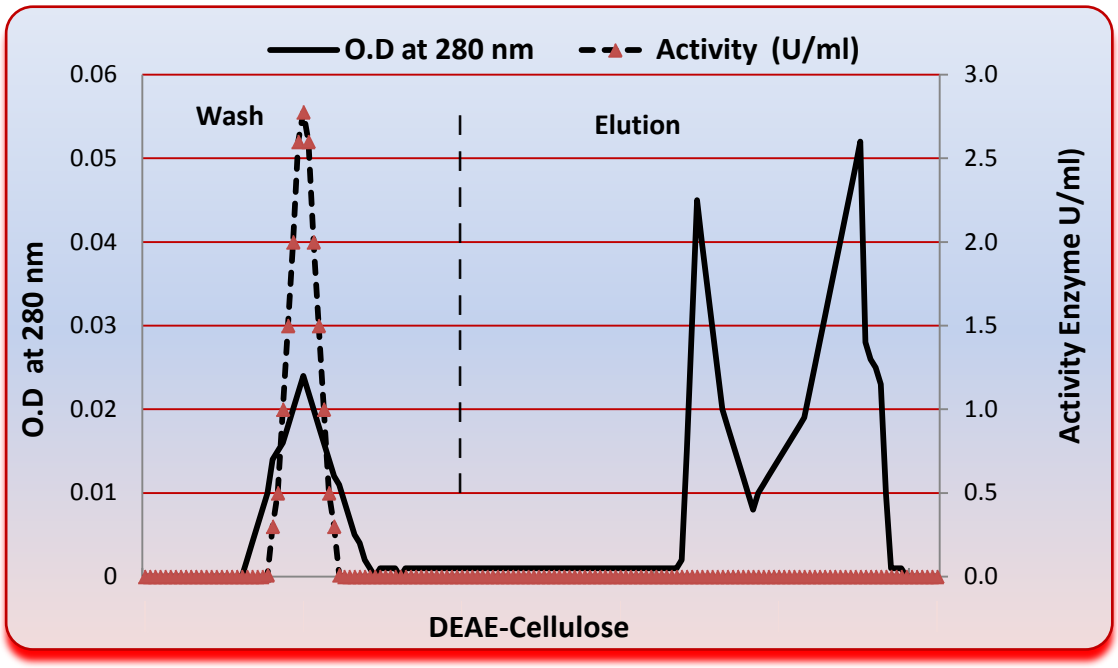

Figure (4-10): Ion exchanger chromatography of cellulase produced by the locally isolated *P. dispersa* A3 using DEAE-Cellulose column (1.75x20cm) with a flow rate of 30 ml/hour.

buffer solution, one of these representscellulase activity manifested after elution with Tris-HCl buffer at fraction number 36. Fractions represented cellulase activity were pooled and concentrated by sucrose. Protein concentration, activity and specific activity were measured in 7 ml aliquot of concentrated enzyme. Table (4-10) confirms an increase in the specific activity of the purified enzyme (190.9 U/mg) recording purification fold reached 113.6 producing 26.2%cellulase.

In another study, cellulase produced by *Aspergillusterreus* was purified by gel filtration using Bio-Gel P-100 in five steps resulting in an enzyme with specific activity of 7.001 U/mg and 9.7% yield (Yin *et al*., 2010). Mirzaakhmedov *et al*. (2007) purified the enzyme by gel filtration achieving specific activity of 1.70 U/mg and a purification up to 2.47 folds.

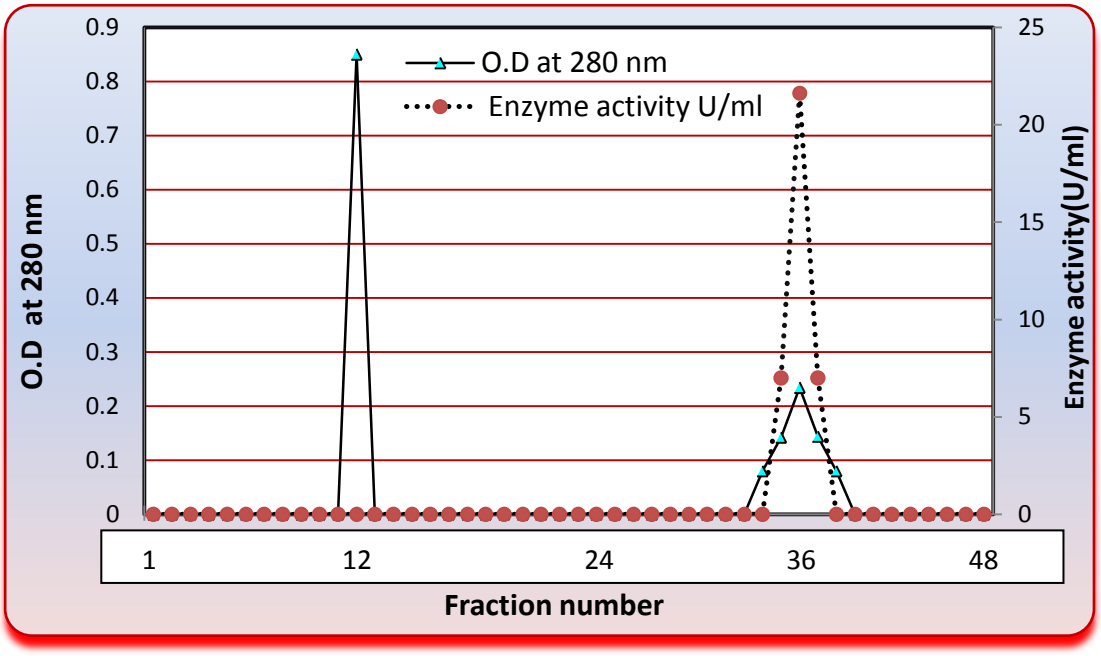

Figure (4-11): Gel filtration chromatography of cellulase produced by the locally isolated *P. dispersa* A3 using sephadex-G200 column (1.25 cm x 30 cm) equilibrated with Tris-HCl buffer solution pH, fraction volume was 3 ml at flow rate of 30 ml/hours.

Table (4-10): Purification steps for cellulase produced by locally isolated *P. dispersa*A3.

Purification steps	Vol. (ml)	Enzyme Activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification (fold)	Yield (%)
Crude Enzyme	156	0.36	0.214	1.68	56.1	1.00	100
$(NH_4)SO_4$ Precipitation (65-70%)	30	1.0	0.25	4.0	30	2.38	53.4
Dialysis	19	1.5	0.300	5.0	28.5	2.97	50.8
Ion exchanger after concentration	5	3.1	0.024	129.1	15.5	76.8	27.6
Gel filtration	7	2.1	0.011	190.9	14.7	113.6	26.2

4.6. Characterization of purified cellulase

4.6.1. Molecular weight of cellulase

Molecular weight of the purified cellulase produced by *P. dispersa* A3 was determined by gel filtration using sephadex-G200 in the presence of five standard proteins (Lysozyme, Trypsin, Pronase, Hemoglobin and BSA).

Cellulase and standard proteins were applied and eluted individually under the same conditions.

Figure (4-12) denotes that cellulose has a molecular weight of 15148

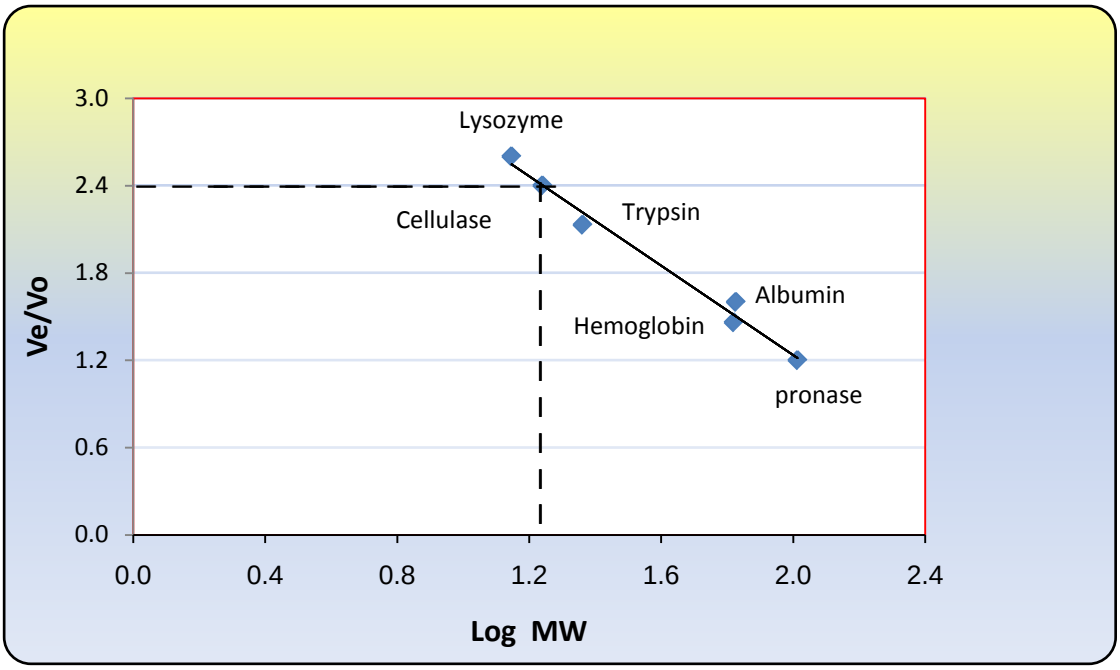

Figure (4-12): The relation ship between the v_e/v_o and log MW for molecular weight determination of purified cellulase produced by the locally isolated *P.dispersa* A3 by gel fitration using sephadex-G200 (30 x 1.25 cm).

dalton. Molecular weight of cellulase differs from microorganism to other. Haung and Monk, (2004) found that molecular weight of the cellulase produced by bacteria *Bacillus* spp.Was 32500 dalton, while Beldman *et al*. (1985) found that the molecular weight of cellulase

produced by *Trichoderma reesei* was 60500-62000 dalton. According to our manually mathematical calculation depending on the information of NCBI database, there are differences in the molecular weights of cellulases produced by *P. dispersa* strains and other strain of *Pantoea* spp. Lee and Fan (1980) found cellulase molecular weight ranged from 5300 - 55000 dalton.

4.6.2. Optimum pH for enzyme activity

Optimum pH for cellulase activity was determined by incubation of the purified cellulase with its substrate (CMC) at different pH values ranging from pH 6 to pH8. Results in (figure 4-13) showed that cellulase was active over a wide range of pH (6-8) with an optimum activity of 4.79 U/ml at pH 7. But it decreased to 4.484 U/ml at pH 8 as illustrated in figure (4-13). Shanmugapriya *et al*. (2012) reported that optimum enzyme activity for cellulase in *Bacillus* spp. Increased at pH ranged 5 to 8, with a maximum enzyme activity at pH 6 or pH 7. Robson and Chambliss (1989) concluded that a variety of factors influence cellulase activity, the pH is one of these crucial factors.

4.6.3. Optimum pH for enzyme stability

To determine the optimum pH for cellulase stability, purified cellulase was incubated at different pH values. The remaining activity was then determined after assaying enzyme activity. Results in figure(4-14) showed that cellulase was more stable at pH 7 since at this pH,enzyme gains maximum remaining activity (100%). While The remaining activity decreased when the enzyme was incubated at pH values less or more than 7.

Ogura *et al*. (2006) found that optimum pH for cellulase stability in *Lysobacter*spp. was at pH 5-7. While Akiba*et al*. (1995) found that optimum pH for stability of cellulase from *Aspergillusniger* ranged between 6 and 7. Therefore the optimum pH for enzyme stability may differ among enzyme types and the microorganism. Enzymes are proteins stable over only a limited rangeofpH.Outside this range, changes in the chargesonionisable residues result in modifications ofthe tertiary structure of the protein and eventually cause denaturation (Zubay, 1993).

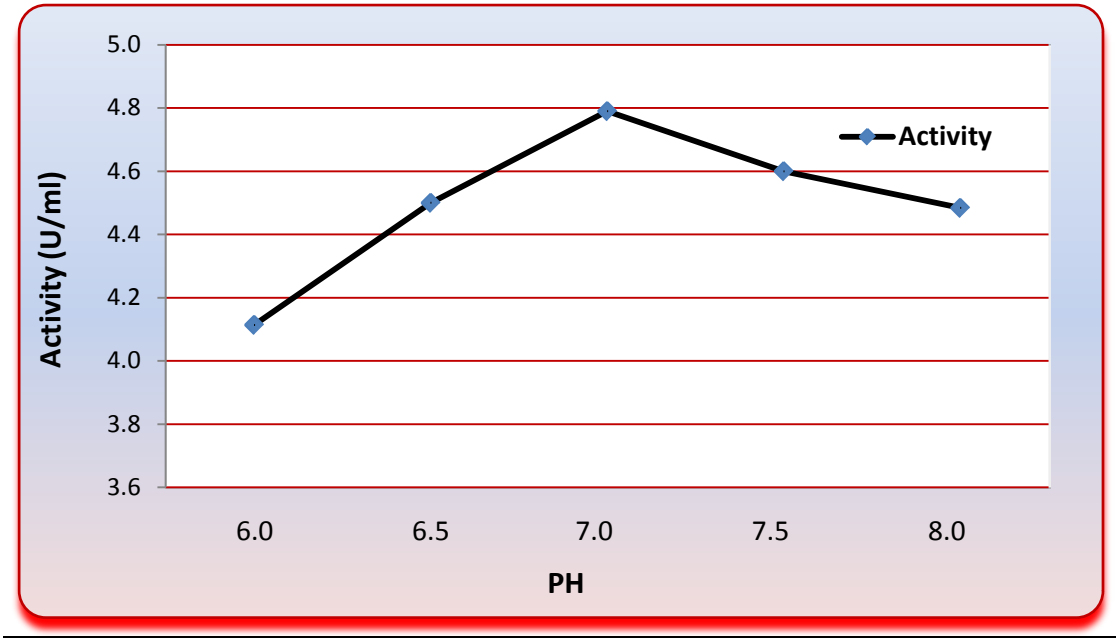

Figure (4-13): Effect of different pH values on activity of purified cellulase produced by locally isolated *P. dispersa* A3.

4.6.4. Optimum temperature for enzyme activity

Optimum temperature for cellulase activity was examined at different temperatures ranging between 20 - 60 °C. Results showed that 30 °C was

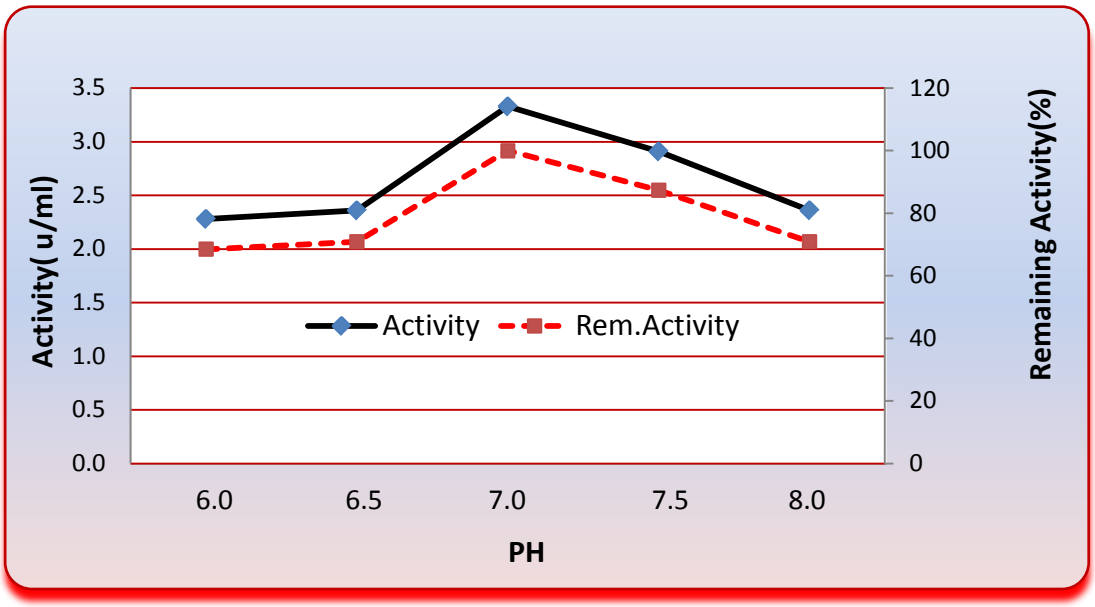

Figure (4-14): Effect of pH on stability of purified cellulase produced by the local isolate *P.dispersa* A3 after 1 hr of incubation at 30 ºC.

the optimum temperature for cellulase activity (figure 4-15). Activity decreased after enzyme incubation at values less or more than 30°C. Most enzyme reactions are found to be accelerated with the increase in temperature up to a limit. Other studies indicated that optimum activity of purified cellulase was obtained at 60 °C for thermophilic*Bacillus*spp. indicating that results depend on the kind of microorganism.Whilethe optimum temperature forpurified cellulasein some*Bacillus* strains was65 °C for CH43 and 70 °C for RH68, which is higher than 55 ºC in *Mucorcircinelloides* (Saha, 2004).

4.6.5. Optimum temperature for enzyme stability

Different temperature regimes were used to determine the optimum temperature for cellulase stability (figure 4-16).Enzyme was incubated at a range of temperatures and the remaining activity was determined.

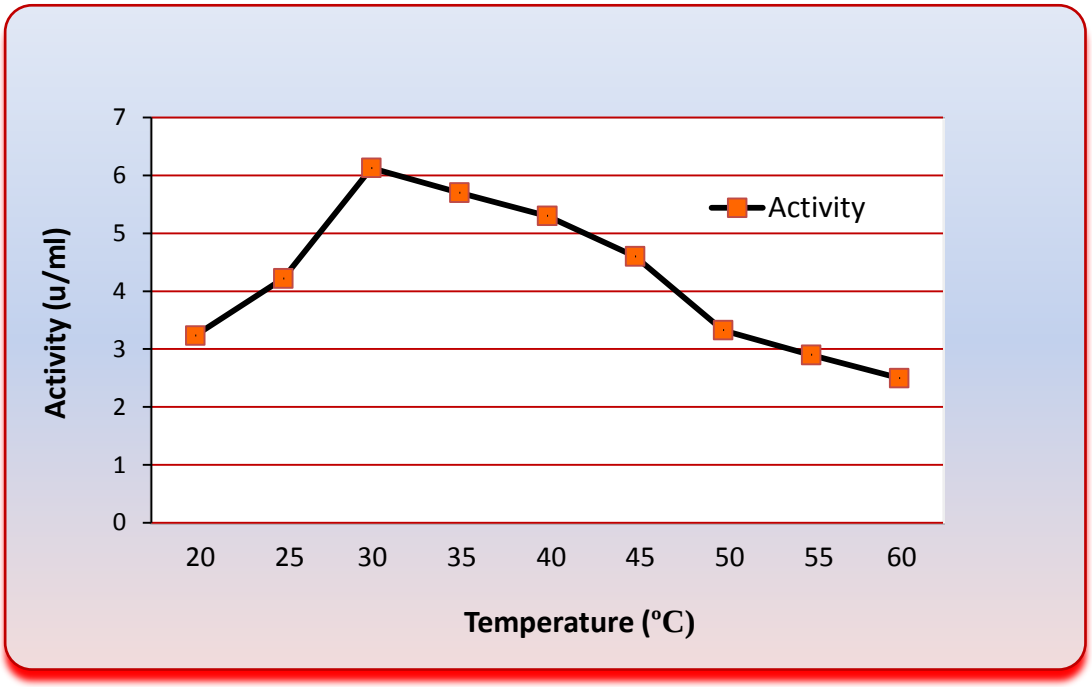

Figure (4-15): Effect of temperature on activity of purified cellulase produced by the locally isolated *P. dispersa* A3.

The enzyme was stable at a temperature ranged between (30 – 50) ºC, while maximum remaining activity (100%) was gained at 30 ºC. The remaining activity decreased slowly when the enzyme was incubated at temperature values exceeds or less than the optimum temperature for stability (30 °C). Raising the temperature may be lead to denaturation of enzyme by destructing the threedimensional structures of the protein and formation of random polypeptide chains. This may cause a change in the active site which leads to inactivation of the enzyme at high temperatures. Literature documented heat stable enzymes of thermophiles organisms which may not be affected by high temperature due to its hydrophobic and disulfide bonds which strengthen their structure (Price and Steven, 1982; Prescott *et al*., 2005). Segel, (1976) stated that crude and partially purified enzymes are more stable than purified ones due to the existence of carbohydrates and other proteins offering protection.

Saha, (2004) found that thermal stability of purified cellulase (0-50 °C) for *Bacillus* spp. AdditionallyMawadza*et al*. (2000) reported that thermal stability of purified cellulaseobtained from*Mucorcircinelloides*ranged between 0-70 °C.

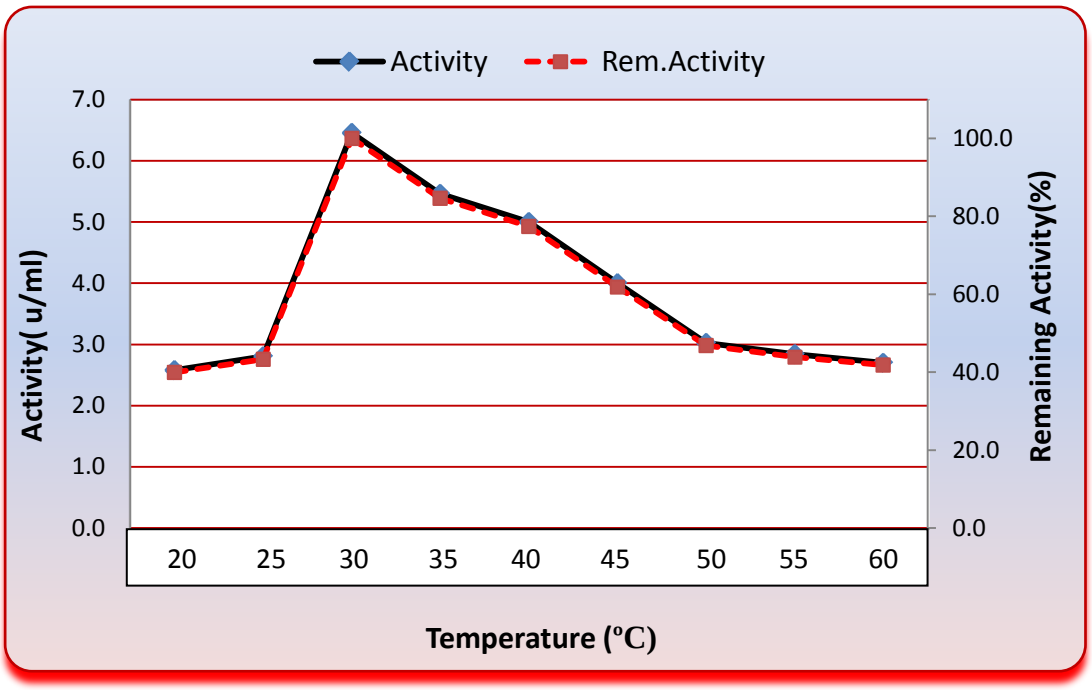

Figure (4-16): Effect of temperature on stability of purified cellulase produced by the locally isolated *P. dispersa*A3.

4.7. Mutagenesis of *P. dispersa*A3

To enhance the ability of the locally isolated *P. dispersa*A3 forcellulaseproduction, this isolate was subjected to physical mutagens (UV) in order to induce random mutations may affectpositively on cellulase production then selecting the over producer mutant according to the increase in cellulase specific activity in culture filtrates of these mutants.

Physical mutagenesis was achieved after fresh culture of *P. dispersa* A3 suspension was subjected to UV-light type (c) at a 254 nm wave length

for length for different durations (1, 2, 3, 4, 5, 10 and 15 seconds), then 100 µl aliquots from each treatment were taken and spread separately on a nutrient agar medium. Results indicated in figure (4-17) indicate that LD90 was approached after 3 seconds of irradiation under UV-light, and most of A3 cultivability was lost after 5 seconds of irradiation.

Survivals of irradiated bacterial cells obtained after subjection to LD90 of UV-light were selected and screened to detect their ability for cellulase production. As indicated in table (4-11) and appendix (2), only nine out of one hundred mutants exhibited an increase in cellulase production compared with the wild type. On the other hand, all other mutants showed lower cellulase production. This result is similar to those of Siddalingshwara *et al*. (2010) who stated that UV rays with wave lengths shorter than the visible light are mild mutagens, hence they are considered to be ideal for induction of mutations.

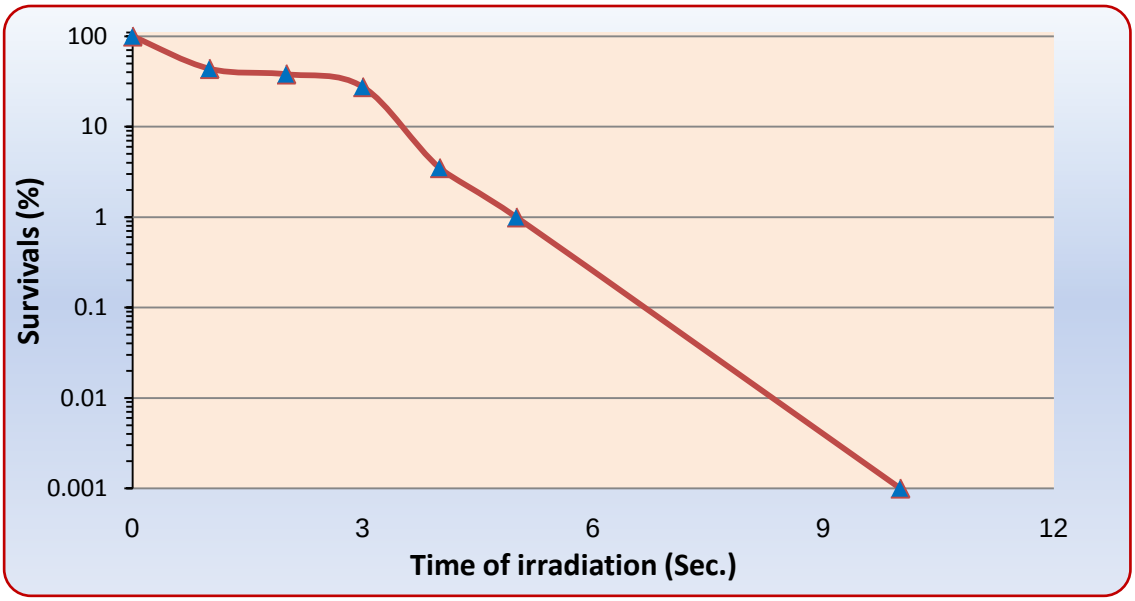

Figure (4-17): Survivals of *P. dispersa* A3 after UV irradiation for different times of period.

Table (4-11): Cellulase specific activity for mutants and wild type.

Mutant No.	Specific activity (U/mg)	Fold ofincrease
Wild type	3.90	1
*Pantoeadispersa*A3 M40	12.48	3.2
*Pantoeadispersa*A3 M41	24.10	6.1
*Pantoeadispersa*A3 M53	28.50	7.3
*Pantoeadispersa*A3 M69	26.70	6.8
*Pantoeadispersa*A3 M71	55.65	**14.2**
*Pantoeadispersa*A3 M72	34.10	8.7
*Pantoeadispersa*A3 M74	20.50	5.2
*Pantoeadispersa*A3 M86	49.83	12.7
*Pantoeadispersa*A3 M87	22.19	5.6

Also this results agrees with the results reported by Sangkharak *et al*. (2012) who mentioned that mutants areeither increase or decrease the enzyme specific activity after subjection of the wild type to UV light as a mutagen.

4.8. Isolation of genomic DNA

Genomic DNA of the *P.dispersa* A3 was extracted in order to amplify cellulase gene by using polymerase chain reaction. Results of extraction using favorgen kit showed that DNA was extracted in a pure form according to the absorbance ratio (260/280) which was 1.8-2.0 with a concentration of 50 µg/ml. Results of electrophoresis on agarose gel (0.8%) are illustrated in figure (4-18) which show that only one DNA band with high resolution represents genomic DNA extracted from the wild typebacterial isolate. Aliqoutes of thisDNA were taken and used for amplification of cellulase gene (s).

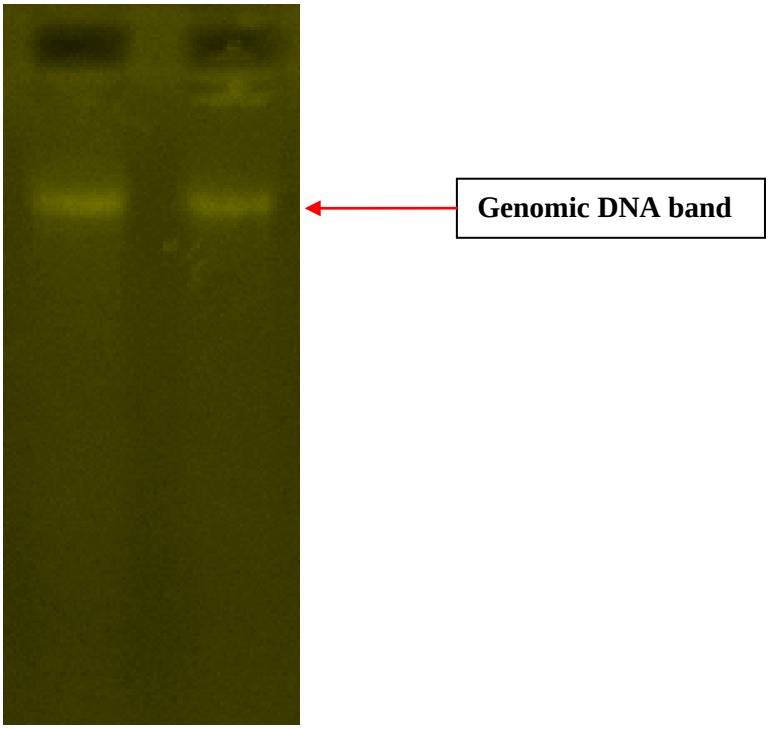

Figure (4-18): Genomic DNA of *P. dispersa* after electrophoresis on agarose gel (0.8%) at 70 volt for 2 hours.

4.9. Amplification of cellulase gene(s)

Genomic DNA of *P.dispersa* A3 was used to amplify cellulase gene. Primers used for amplification were designed to include genetic elements upstream and downstream of cellulase gene for different species of *Pantoea*sppwith the aim of amplifyingcellulase gene of *P. dispersa* A3 according to the informationrecorded in NCBI concerning DNA sequences of cellulase enzyme (s) in these species.No data were found in NCBIabout DNA sequencefor cellulase gene (s)in*P. dispersa*. According to this fact, nucleotide sequence of therelated species was used to design the primers. DNA samples from genomic DNA stock solution were amplified by using different primers mentioned in table (3-4), then PCR products were analyzed on agarose gel (0.8%) to

identify the product of amplified fragments in the presence of 1Kb DNA landmark ladder.

Results of gel electrophoresis are documented in figure (4-19) and figure (4-20) showing none PCR product of the amplification reactions for each set of primers. This may be attributed that these primers which were specific for amplification of cellulase gene in *Pantoea* spp. were not specific for amplification of the same gene in *P. dispersa*. The complete DNA sequences of this bacterium have not stated in the NCBI on the web site yet. It may need more investigations.

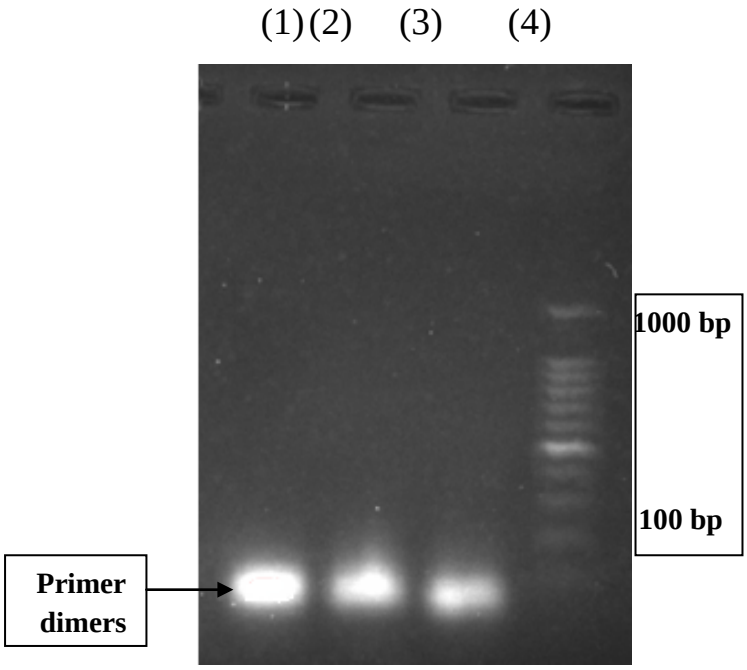

Figure (4-19): Agarose gel electrophoresis for amplification of cellulase gene of *P. dispersa* A3 on (0.8%) agrose gel, 70 voltage for 90 minutes.
Lane (1, 2, 3): DNA primer dimmers after amplification using primers mentioned in table (3-4)respectively.
Lane (4): Land marker ladder (1Kb).

(1) (2) (3) (4) (5)

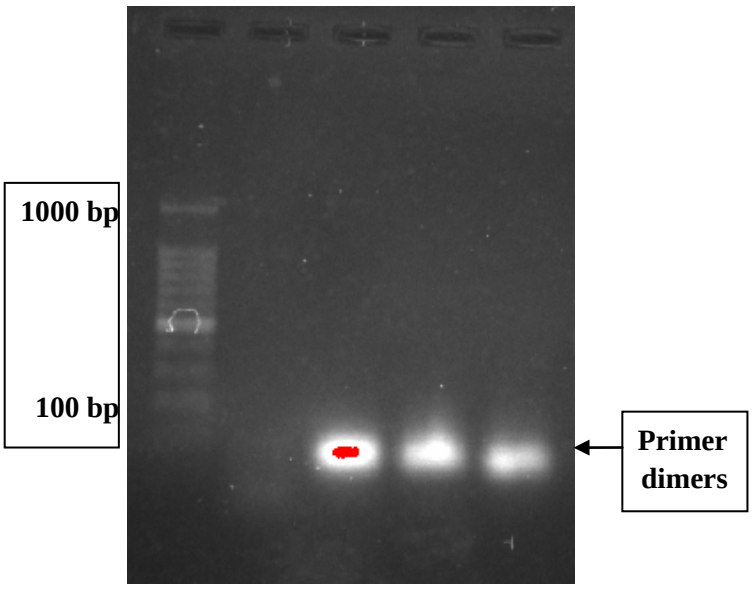

Figure (4-20): Agarose gel electrophoresis for amplification of cellulase gene of *P. dispersa* A3 on (0.8%) agrose gel, 70 voltage for 90 minutes.
Lane (1): Landmarker ladder (1Kb).
Lane (2): --
Lane (3, 4, 5): DNA primer dimmers after amplification using primers mentioned in table (3-4)respectively.

Conclusions and Recommendations

Conclusions

- *Pantoea dispersa* is one of the most efficient bacteria in producing cellulase and pectinase.

- Higher production of cellulase from *P. dispersa* was achieved under optimum growth conditions adjusted to increase productivity of cellulase.

- Cellulase produced by *Pantoea dispersa* under optimum conditions can be fully-purified throughout four steps of purification including the ammonium sulphate precipitation step, dialysis step, ion exchange chromatography step by using DEAE-cellulose, and gel filtration step throughout sephadex-G200.

- Productivity of cellulase by *P. dispersa* can be increased thirty four fold by random mutagenesis using irradiation with UV-ray.

Recommendations

1- Studying the kinetics of cellulase and pectinase produced by *P. dispersa* A3.

2- Investigating the affinity of cellulase and pectinase toward its substrates by site-direct mutagenesis.

3- Immobilization of cellulase and pectinase produced by *P. dispersa* A3 for large scale treatment of cellulotic materials, with the potential of using fermenters for large scale cellulase production.

4- Studying the genetic map of *P. dispersa* A3 and determine different genes sequences and locations.

5- Sequencing cellulase and pectinase genes of *P. dispersa* A3.

6- Studying the optimum conditions for pectinase production by *Pantoea dispersa* A3.

7- Purification of pectinase produced by *P. dispersa* A3.

8- Exploitation of the current results for increase in the yield more than 14 folds compared with the wild type for commercialization of cellulase.

References

Agustini, L.; Efiyanti, L.; Faulina, S. A., and Erdy, S. (2012). Isolation and characterization of cellulase- and xylanase- producing microbes isolated from tropical forests in java and Sumatra. Int. J. of Env. and Bioen., 3(3): 154-167.

Akiba, S.; Kimura, Y.; Yamamoto, K., and Kumagai, H. (1995). Purification and characterization of a protease-resistant cellulase from *Aspergillus niger*. J. of Ferm. and Bioeng., 79(2):125-130.

Akpomie, O. OF.; Ubogun, E. and Ubogun, M. (2013). Determination of the cellulolytic activities of microorganisms isolated from poultry litter for sawdust degradation. J. of Environ. Sci. and Water Res., 2 (2): 62-66.

Alkorta, I.; Garbisu, C.; Llama, M. J. and Serra, J. L. (1998). "Industrial Applications of Pectic Enzymes: A Review, Process Biochem., 33: 21-28.

Alexandra, P.; Cleenwerck, L.; Iversen, C.; Vos, P. D. and Stephen, R. (2010). *Pantoea gaviniae* sp. nov. and *Pantoea calida* sp. nov., isolated from infant formula and an infant formula production environment. Inter. J. of Syst. and Evol. Microbiol., 60: 2786-2792.

Almin, K.E.; Eriksson, K.E. and Petersson, B. (1975). Extracellular enzyme system utilized by the Fungus *Sporotirichum pulverulentum*(*Chrysosprium lignorum*) for the breakdown of cellulose- 2 Activites of the five endo-1,4-beta-glucanases towards carboxymethylcellulose. Eur. J. Biochem., 51: 207-211.

Askar, A.; Gierschener, K.; Siliha, H. and El-Zoghbi, M. (1990). Polysaccharides and cloud stability of tropical nectars. In: Proceedings of the XX international federation of fruit juice producers symposium. Paris. 207-223.

Atlas, M.; Parks, C. and Brown, A. (1995). Laboratory Manual of Experimental Microbiology. Mosby-Year-Book, Inc., USA.

Bai, S.; Ravi kumar, M.; Mukeshkumar, D. J.; Balashanmugam, P.; Balakumaran, M. D. and Kalaichelvan, P. T. (2012). Cellulase production by *Bacillus subtilis* isolated from cow dung. Arch. of Appl. Sci. Res., 4(1):269-279.

Bajpai, P. (1999). Application of enzymes in the pulp and paper industry. Biotechnol. Prog., 15: 57-147.

Barman, D.; Saud, Z. A.; Habib, M. R.; Islam, M. F.; Hossain, K. and Yeasmin, T. (2011). Isolation of cellulytic bacterial strains from soil for effective and efficient bioconversion of solid waste. Life Sci. and Medi. Res., 2011: 1-7.

Baron, P., Finegold. (1998). Diagnostic Microbiology. In Bailey & Scott, Mosby-Year Book, Inc., St. Louis, Mo. 2. Wickerham. 1951.

Bayer, E. A.; Chanzy, H.; Lamed, R. and Shoham Y. (1998). Cellulose, Cellulases and Cellulosomes, Curr. Opin. Struc. Biol., 8: 548–557.

Bayer, E. A.; Morag, F. and Lamed, R. (1994). The cellulosome-a treasure-trove for biotechnology. Trends in Biotechnol., 12(9): 379–386.

Bayer, E. A.; Belaich, J. P.; Shoham, Y. and Lamed, R. (2004). The cellulosomes: multienzyme machines for degradation of plant cell wall polysaccharides. Ann. Rev. of Microbiol., 58: 521–554.

Bayer, E. A.; Shoham, Y. and Lamed, R. (2006).Cellulose-decomposing Bacteria and Their Enzyme Systems. The Prokaryotes. 2: 578-617.

Beldman, G.; Rombouts, F. M.; Voragen, A. G. J. and Pilnik, W. (1985). Application of cellulase and pectinase from funjl origin for the liquification and sachharification of biomass. Enz. Microbiol. Technol., 6:503-507.

Bhat, M. K**,** and Bhat, S. (1997). Cellulose degrading enzymes and their potential industrial applications. Biotech. Adv.,15: 583-620.

Bhale, U. N. and Chatage, V. S. (2013). Phytotoxic activity of fungal pathogens inciting IVY gourd fruit. Asian J. of Biochem. and Pharmac. Res., 3: 86-93.

Boraston, A. B.; Bolam, D. N.; Gilbert, H. J. and Davies, G. J. (2004) Carbohydrate-binding modules: fine-tuning polysaccharide recognition. Biochem. J., 382: 769–781.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of Protein-Dye Binding. Analy. Biochem., 72: 248-254.

Brady, C. L.; Cleenwerck, L.; Stephanus, N.V.; Engelbeen, K.; Vos, P. D. and Coutinho, T. A. (2010). Emended description of the genus *Pantoea*, description of four species from human clinical samples, *Pantoea septic* sp. nov., *Pantoea eucrina* sp. nov., *Pantoea brenneri* sp. nov. and *Pantoea conspicua* sp. nov., and transfer of *Pectobacterium cypripedii* (Hori 1911) Brenner *et al*., 1973 emend. Hauben *et al*., 1998 to genus as *Pantoea cypripedii* comb. Nov. Inter. J. of Syst. and Evol. Microbiol., 60: 2430-2440.

Brady, C.; Cleenwerck, I. and Venter, S.(2008). Phylogeny and identification of *Pantoea* species associated with plants, humans and the natural environment based on multilocus sequence analysis (MLSA). Syst. Appl. Microbiol., 31:60–447.

Bull, A. and Bushnel, M. (1976). Environmental control of fungal growth. In:The filamentous fungal (eds. Smith, J. E. and Berry, D. E.), 2P: 1-26. Edward Arnold, London.

Champs, C. DE.; Seaux, S. LE.; Dubost, J. J.; Boisgard, S.; Sauvezie, B. and Sirot, J. (2000).Isolation of *Pantoea agglomerans* in two cases of septic monoarthritis after plant thorn and wood sliver injuries. J. of Clin. Microbiol., 38(1): 400-461.

Chudasama, K.S. and Thaker, V.S. (2012). Secretion of type II extracellular cell wall degrading enzymes from *Pantoea agglomerans* phytopathogen., Asian J. of Plant and Res., 2(5):559-565.

Collee, J.; Miles, R. and Watt, B. (1996). Tests for the identification of bacteria. In: Practical Medical Microbiology. Collee, J.; Fraser, A.; Marmion, B. and Simmons, A. (eds). 4th edition. Churchill Livingstone, U. K.

Cruickshank, R.; Duguid, J.; Marimon, B. and Swain, R. (1975). Medical Microbiology. 12th. Ed. Vol. 11. Churchill Living stone. New York.

Dashtban, M.; Maki, M.; Leung, K. T.; Mao, C. and Qin, W. (2010). Cellulase activities in biomass conversion: measurement methods and comparison. Critical Reviews in Biotechnology. Review Article. 1-8.

De, V. L. and Pilnik, W. (1973). Pectolytic enzymes in apple juice extraction. Proc. Biochem., 8: 18-19.

Deka, D.; Bhargavi, P.; Sharma, A.; Goyal, D.; Jawed, M. and Goyal. (2011). Enhancement of cellulase activity from a new strain of *Bacillus subtilis* by medium optimization and analysis with various cellosic substrates. Article. 1-8.

Deletoile, A.; Decre, D.; Courant, S.; Passet, V.; Audo, J.; Grimont, P.; Arlet, G. and Brisse, S. (2009). Phylogeny and identification of *Pantoea* species and typing of *Pantoea agglomerans* strains by multilocus gene sequencing. J. of clin. Microbial., 47(2): 300-310.

Devi, M. C. and Kumar, M. S. (2012). Production, optimization and partial purification of cellulase by *Aspergillus niger* fermented with paper and timber saw mill industrial wastes. J. Microbiol. Biotech. Res., 2(1): 120-128.

Ding,S. Y.;Zhao, S. and Zeng,Y. (2014). Size, shape, and arrangement of native cellulose fibrils in maize cell walls. J. of Cellulose., 21:87-863.

Galante, Y. M.; De, C. A. and Monteverdi, R. (1998). Application of Trichoderma enzymes in food and feed industries. Biolog. control and Commer. appl., 2: 42-327.

Gavini, F.; Mergaert, J.; Beji, A.; Mielcarek, C.; Izard, D.; Kersters, K. and De Ley, J. (1989). Transfer of *Enterobacter agglomerans* (Beijerinck1988) Ewing and Fife 1972 to Pantoea gen. nov. as *Pantoea agglomerans* comb. nov. and description of *Pantoea dispersa* sp. nov. Int. J. Syst. Bacteriol., 39 : 337–345.

Ghose, T. K. (1987). Measurement of cellulose activities. Pure and Appl. Chem., 59(2): 257-268.

Gohel, V.; Megha, C.; Vyas, P. and Chhatpar, H. S. (2004). Strain improvement of chitinolytic enzyme producing isolate *P. dispersa* for

enhancing it biocontrol potential against fungal plant pathogens. Ann. of Microbiol., 54(4): 503-515.

Gottschalk, G. (1988). FEMS Symp. Biochemistry and Genetics of Cellulose Degradation. Aubert JP, Beguin P, Millet J (Eds), Academic Press London p.3.

Gummadi, S. N. and Kumar, D. S. (2006). Optimization of chemical and physical parameters affecting the activity of pectin lyase and pectatelyase from *Debaryomyces nepalensis*: a statistical approach. Biochem. Eng. J., 30: 130-7.

Hammami, R.;Zouhir, A.; Ban Hamida, J. and Fliss, I. (2007). Bacteriocin characterization. BMC Microbiol., 17: 89-99.

Hankin, L.; Zucker, M. and Sands, D.C.(1999). Improved solid medium for the detection and enumeration of pectolytic bacteria. Appl. Microbiol., 38: 205-209.

Harely, P. J. and Prescott, M. L. (1996). Laboratory exercises in microbiology. McGraw-Hill. USA.

Haung, XP. and Monk, C. (2004). Purification and characterization of a cellulase from a newly isolated thermophilic aerobic bacterium *Caldibacillus cellulovorans* gen. nov. sp. World J. of Microbiol. and Biotech., 20: 85-92.

Heinman, B. and Haward, A. J. (1969). Production of neoplastic tumor inhibitory enzyme 1-asparaginase by submerged growth of *Serratia marcescens*. Applied Microbiology. 18: 550-554.

Henrissat, B.; Claeyssens, M.;Tomme, P.; Lemesle, L. and Mornon, J.P. (1989). Cellulase families revealed by hydrophobic cluster analysis. Gene 81: 83-95.

Hoshino, E.; Sasaki, Y.; Okazaki, M.; Nisizawa, K. and Kanda, T. (1993). Mode of action of exo- and endo – type cellulases from irpex lacteus in the hydrolysis of cellulose with different crystallinities. J. of Biochem., 114(2): 5-230.

Immanuel, G.; Dhanusha, R.; Prema, P. and Palavesam, A. (2006). Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. Inter. J. Environ. Sci. Tech., 3: 2.

Irfan, M.; Safdar, A.; Syed, Q. and Nadeem, M. (2012). Isolation and screening of cellulytic bacteria from soil and optimization of cellulase production and activity. Turkish J. of Biochem., 37(3): 287-293.

Jakucs, E. and Várallyay, E. (1995).A simple and rapid semi-quantitative method for measuring cellulaseactivityin agar media. Acta.Microbiol.Immunol. Hung., 42(1): 77–80.

Jaradat, Z.;Dawagreh, A.; Ababneh, Q. and Saadoum, I. (2008). Influence of culture conditions on cellulase production by *Streptomyces* sp. (Strain J2). Jordan J. of Biol. Sci., 1(4):141-146.

Jayani, R. S.; Saxena, S. and Ranveer Singh Tayani, R. G. (2005).Microbial Pectinolytic Enzymes: A review. J. of Proc.Biochem., 40:2931-2940.

Juven, B. J.; Lindner, P. and Weisslowicz, H. (1985). Pectin degradation in plant by *Leuconostoc mesenteroides*. J. Appl. Bacteriol., 58: 533.

Kanokratana, P. (2008). Identification and expression of cellobiohydrolase (CBHI) gene from an endophytic fungus. *Fusicoccum* sp. (BCC4124) in *Pichia pastoris*. Protein Expr. Purif., 58: 148-153.

Karp, P.D.; Riley, M.; Saier, M.; Paulsen, I. T.; Parley, S. M. and Pellegrini-Toole, A. (2000). The EcoCyc and MetaCyc databases. Nucleic Acid Res., 28(1):56-59.

Kaur, M. and Arora, S. (2012). Isolation and screening of cellulose degrading bacteria in kitchen waste and detecting their degrading potential. J. of Mech. and Civil Engin., 1: 33-35.

Kashyap, D. R.; Vohra, P. K.; Chopra, S. and Tewari, R. (2001). Applications of pectinases in the commercial sector: arev. Bioresou. Technol.,77: 215- 227.

Kilara, A. (1982). Enzymes and their uses in the processed apple industry

A Review. Proc. Biochem., 23: 35-41.

Khan, F. A. B. A. and Husaini, A. A. S. A. (2006). Enhancing alpha-amylase and cellulase *in vivo* enzyme expressions on sago pith residue using *Bacillus amyloliquefaciens* UMAS 12002. Biotechnol., 5(3): 391-403.

Kock, AL. (1997). Microbial physiology and ecology of slow growth. Microbiol. Molec. Biol. Rev., 61: 305.

Knauf, M. and Moniruzzaman, M.(2004). Lignocellulosic biomass processing: a perspective. Int. Sugar J.,106:147–50.

Krootdilaganandh, J. (2000). Isolation and selection of thermotolerant bacteria capable of producing cellulase. Chiang Mai: Chiang Mai Univ. Prees., 20-21.

Kumar, M. D. J.; Saranya, G. M.; Suresh, K.; Priyadharshini, A. D.; Rajakumar, R. and Kalaichelvan, P. T. (2012). Production and optimization of pectinase from *Bacillus* sp. MFW7 using cassava waste. Asian J. of plant sci. and Res., 2(3):369-375.

Kuhad, R.C.; Gupta, R. and Singh, A. (2011). Microbial cellulases and their industrial applications. Enzyme Research. Rev. Article., 1-10.

Lee, Y. H. and Fan, L. T. (1980). Properties and mode of action of cellulase. Adv. in Biochem. Engin.,17: 101-129.

Lin, K. and Wang, A. (2001). UV mutagenesis in Escherichia coli K-12: Cell survival and mutation frequency of the chromosomal genes lacZ, rpoB, ompF, and ampA. J. of Exper. Microbiol. and Immunol (JEMI)., 1:32-46.

Liu, H.;Zhang. S.; Schell, M.A. and Denny, T.P. (2005).Pyramiding unmarked deletions in *Ralstonia solanacearum* shows that secreted proteins in addition to plant cell-wall-degrading enzymes contribute to virulence.Mol. Plant-Microbe Interact., 18: 1296-1305.

Livneh, Z.; Cohen-Fix, O.; Skaliter, R. and Elizur. T. (1993). Replication of damaged DNA and the molecular mechanism of ultraviolet light mutagenesis. Crit. Rev. in Biochem. and Molec. Biol., 28(6):465-513.

Lynd, L.R.; Van, Zyl. WH. and McBride, J.E. (2005). Laser M. Consolidated bioprocessing of cellulosic biomass: an update. Curr. Opin. Biotechnol., 16(5):577-583.

Mala, JGS.; Kamini, NR. And Puvanakrishnan, R. (2001). Strain improvement of *Aspergillus niger* for enhanced lipase production. J. Gen. Appl. Microbiol., 47: 181-186.

Mandels, M. (1975). Microbial source of cellulose. Biotech. and Bioengin., 5: 81-105.

Maniatis, T.; Fritch, E. and Sambrock, J. (1982). Molecular cloning: A Laboratory Manual. Gold Spring Harbor Laboratory. N.Y., USA.

Mawadza, C.; Hatti-Kaul, R.; Zvauya, R. and Mattiasson, B. (2000). Purification and characterization of cellulases produced by two *Bacillus* strain. J. of Biotechnol., 83(3): 177-187.

Medrano, E. G. and Bell, A. A. (2007). Role of *Pantoea agglomerans* in opportunistic bacterial seed and boll rot of cotton (*Gossypium hirsutum*) grown in the field. J. of Appl. Microbiol., 102: 134-143.

Mergaert, J.; Verdonck, L. and K. Kersters. (1993).Transfer of *Erwinia ananas* (synomym, *Erwinia uredovora*) and *Erwinia stewartii* to the genus *Pantoea emend*. as *Pantoea ananas* (Serrano 1928) comb. nov., and *Pantoea stewartii* (Smith 1898) comb. nov., respectively, and description of *Pantoea stewartii* subsp. *Indologenes* subsp. nov. Int. J. Syst. Bacteriol., 43:162–173.

Miller, J. H. (1972). Experiment 32: Isolation of valine-resistant and antibiotic-resistant mutants. Pages 221-229 in J.H. Miller, Experiments in Molecular Genetics. Cold Spring Harbour Laboratory Press, New York.

Mirzaakhmedov, S. Y.; Ziyavitdinov, Z. F.; Akhmedova, Z. R.; Saliev, A. B.; Ruzmetova, D. T.; Ashurov, K. B.; Fessas, D. and Iametti, S. (2007). Isolation, *Aspergillus terreus*. Chem. of Compounds., 43(5): 594-597.

Narasimhan, A.; Bist, D.; Suresh, S. and Shivakumar, S. (2013). Optimization of mycolytic enzymes (Chitinase, Ɓ 1,3- Glucanase and Cellulase) production by *Bacillus subtilis*, a potential biocontrol agent using one-factor approach. J.of Sci. and Indust. Res., 72: 172-178.

Nikolskij, B. P. (1964). Reference Book of Chemist. Volume III, "Chemistry" , Moscow-Leningrad, 2nd edition. Ed. B. P. Nikolskij.

Nochur, S. V.; Roberts, M. F. and Demain, A. L. (1993). True cellulase production by *clostridium thermocellum* grown on different carbon sources," Biotech. Letters., 15(6): 641–646.

Nurachman, Z.; Kurniasih, S. D.;Puspitawati, F.;Hadi, S.; Radjasa, O. K. and Natalia, D. (2010). Cloning of the endoglucanase gene from a *Bacillus amyloliquefaciens* PSM 3.1 in *E. coli* revealed catalytic triad residues Thr-His-Glu. Americ. J. of Biochem. and Biotech., 6(4): 268-274.

Ogura, J.; Toyoda, A.; Kurosawa, T.; Chong, A. L.; Chohnan, S. and Masaki, T. (2006). Purification, characterization, and gene analsis of cellulase (Cel8A) from *Lysobacter* sp. IB-9374. Biosci. Biotech. and Biochem., 70(10): 2420-2428.

Otajevwo, F. D. and Aluyi, H. S. A. (2011). Cultural conditions necessary for optimal cellulase yield by cellulolytic bacterial organisms as they relate to residual sugars released in broth medium. Modern Appl. Sci., 5(3): 141-151.

Overman, T. L.; Kessler, J. and Seabolt, J. P. (1985). Comparison of APIE20, API Rapid E, and API Rapid NFT for identification of members ofthe family Vibrionaceae. J. of Clin. Microbiol., 22:778–781.

Paloma, ki. T. and Saarilahti, H. T. (1997). Isolation and Characterization of New C Terminal Substitution Mutation Affecting Secretion of Polygalactur-Onases in *Erwinia Carotovora* ssp. *Carotovora*. FEBS Lett., 400:122-126.

Pedrolli, D. B.; Monteiro, A. C.; Gomes, E. and Carmona, C. C. (2009). Pectin and Pectinases: Production, Characterization and Industrial Application of Microbial Pectinolytic Enzymes. Open Biotech. J., 3: 9-18.

Pincus, D.H. 2013. Microbial identification using the biomerieuxVITEK® 2 system. In: Miller, M.J. Encyclopedia of rapid microbiological methods volume 4. DHI Publishing, LL River Grove, IL, USA

Prescott, L. M.; Harley, J. P. and Klein, D. A. (2005). Microbiology. (6th Ed.). Pubished by McGraw Hill. New York.

Price, N. C. and Stevens, L. (1982). Fundamental of enzymology. Oxford, University Press.

Purwanto, L. A. D.; Ibrahim, D. and Sudrajat, H. (2009). Effect of Agitation Speed on Morphological Changes in *Aspergillus niger* Hyphae During Production of Tannase. World J. of Chem., 4 (1): 34-38.

Qureshi, A. S.; Bhutto, M. A.; Chisti, Y.;Khushk, I.; Dahot, M. U. and Bano, S.(2012).Production of pectinase by *Bacillus subtilis* EFRL 01 in a date syrup medium. Afric. J. of Biotech., 11(62):12563-12570.

Qazi, S. A. and Stoll, B.J. (2009). Neonatal sepsis: a major global public health challenge. Pediatr. Infect. Dis. J., 28:S1–2.

Rajeshwari, R. Jha. G. and Sonti, R.V. (2005). Role of an in *planta* expressed xylanase of *Xanthomonas oryzae*pv. *oryzae*in promoting virulence on Rice. Mol. Plant-Microbe Interact**.,**18(8): 830-837.

Rafnar, T.; Griffith, L. J.; Kuo, M. C.; Bond, J. F.; Rogers, B. L. and Klapper, D. G. (1991). Cloning of *Amb al* (antigen E), the major family of short ragweed pollen. J. Biol. Chem., 266 : 1229–1236.

Ranadive, A. andHaard, N. (1973). Chemical nature of stone cells from pear juice. J. Food Sci., 38: 331-333.

Raju, E. V. N. and Divakar, G. (2013). Screening and isolation of pectinase producing bacteria from various regions in Bangalore. Inter. J. of Res. in Pharmac. and Biomed. Sci., 4(1):151-154.

Ray, A.K.; Bairagi, K. S.; Ghosh, A. and Sen, S. K.(2007). Optimization of fermentation conditions for cellulase production by *Bacillus subtilis* CY5 and *Bacillus circulans* TP3 isolated from fish gut. Acat. Icht. Et. Pist., 37: 47-53.

Reddy, P. L. and Sreeramulu, A. (2012). Isolation, identification and screening of pectinolytic funji from different oil samples of chittoordistrict. Int. J. LifeSci. Bt. And Pharmac. Res., 1(3): 186-193.

Rezzonico, F.V.O.; Stockwell, M.; Tonolla, B. and Duffy, T.H.M. (2012). *Pantoea* clinical isolates cannot be accurately assigned to species based on metabolic profiling. Transplant Infec. Disease., 14: 220-221.

Robson, LM. and Chambliss, GH. (1989). Characterization of the cellulolytic activity of a *Bacillus* isolate. Appl. Environ. Micrbiol., 47: 1039-1046.

Sadhu, S. and Maiti, T. K. (2013). Cellulase Production by Bacteria: A Review. British Microbiol. Res. J., 3(3): 235-258.

Saha, B. C. (2004). Production, purification and properties of endoglucanase from a newly isolated strain of *Mucor circinelloides*. Process Biochem., 39(12): 1871-1876.

Saha, N.; Wirth, S. and Ulrich, A. (2013). Cellulolytic bacterial biodiversity in long-term manure experimental sites. African. J. of Agricul. Res., 8(3): 299-307.

Sakai, T. (1992). "Degradation of Pectins", in Winkelmann G (Ed.), "Microbial Degradation of Natural Products", Weinheim, VCH. pp. 57-81.

Sakthivel, M.; karthikeyan, N.; Jayaveny, R. and Palani, P. (2010). Optimum of culture conditions for the production of extracellular cellulase from *Corynebacterium lipopbiloflavum.* J. of Ecobiotechnol., 2/9:6-13.

Sambrook, T. and Russell, D. W. (2001). Molecular cloning: a laboratory manual. Cold spring Harbor laboratory press, cold spring Habor, N. Y.

Samira, M.; Mohammad, R. and Gholamreza, G. (2011). Carboxymethyl-cellulase and filter-paperase activity of new strains isolated from Persian gulf. Microbiol. J., 1(1): 8-16.

Sangkharak, K.;Vangsirikul, P. and Janthachat, S. (2012). Strain improvement and optimization for enhanced production of cellulase in *Cellulomonas* sp. TSU-03. Afric. J. of microbial. Res., 6(5): 1079-1084.

Schols, H.;Geraeds, C.; Searle- Van-Leeuwen, M. and Komelink, F. (1990). Rhamnogalacturonase: A novel enzyme that degrades the hairy region of pectins. Carbohydr. Res., 206: 105-115.

Sege
l, L. H. (1976). Biochemical calculation. John Whiley and Sons. Inc. New York.

Selvakumar, G.; Kundu, S.; Joshi, P.; Nazim, S. A. D.; Mishra, P. K. and

Gupta, H.S. (2008). Characterization of a cold-tolerant plant growth-promoting bacteria *Pantoea dispersa* 1A isolated from a sub-apine soil in the north western Indian Hiimalagas. J. Microbiol. Biotechnol., 24: 955-960.

Sethi, S.; Datta, A.; Gupta, B. L. and Gupta, S. (2013). Optimization of cellulase production from bacteria isolated from soil. Biotech. Res., 1-7.

Shabeb, M. S. A.; Magdi, A. M.; Younis, F. F. H. and Nour-Eldein, M.A. (2010). Production of cellulase in low- cost medium by *Bacillus subtilis* KO Strain. World Appl. Sci. J., 8(1): 35-42.

Shanmugapriya, K.; Saravana, P.; Krishnapriya, M.; Mythili, A. and Joseph, S. (2012). Isolation, screening and partial purification of cellulase from cellulase producing bacteria. Int. J. of Adv. Biotech. And Res., 3(1):509-514.

Shepherd, G. M.; Tong, C. C. and Cole, A. L. (1981). Substrate specificity and mode of action of the cellulases from the thermophilic fungus *Thermoascus aurantiacus*. Biochem. J., 193: 67-74.

Sheth, K. and Alexander, J.K. (1969). Purification and properties of ß-1, 4-oligoglucan: orthophosphate glucosyl transferase from *Clostridium thermocellum*. J. Biol. Chem., 244:457-464.

Siddalieshwara, K. G.; Devi, D. N.; Sudipta, K. M.; Lodh, S.; Sumanata, K. P.; Sunil, D. P. L. (2010). Mutational study on glutaminase a tumor inhibitor from microbial origin. Article. 4(2): 1`76-179.

Silva, D.; Martins, E. S.; Silva, R. and Gomes, E. (2002). Pectinase production by *Penicillium viridicatum* RFC3 by solid state fermentation using agricultural wastes and agro-industrial by-products. Braz. J Microbiol., 33: 318-24.

Singh, A. (1999). Engineering enzyme properties. Indian J. of Microbiol., 39(2): 65–77.

Sneath, H. A.; Peter, M. S.; Nicolas, H.; Hold, G. L. (1986). Bergy's manual of systemic bacteriology, (vols. 1 and 2) (William and Wilkins Company, Baltmore, MS, USA).

Soares, M. M. C. N.; Silva, R. D. and Gomes, E. (1999). Screening of bacterial strains for pectinolytic activity: characterization of the polgalacturonase produced *Bacillus* SP. Revista de Microbiol., 30: 299-303.

Sukharnikov, L. O.; Cantwell, B. J.; Podar, M. and Zhulin, I. B. (2011). Cellulases: ambiguous nonhomologous enzymes in a genomic perspective. Trends Biotech., 29: 473-479.

Taleb, A.; Khadigs, A. A.; Mashhoor, W. A.; Sohair, N.A.; Sharaf, M. S. and Hoda, A. H. H. M. (2009). Nutritional and environmental factors affecting cellulase production by two strains of cellulolytic bacilli. Austr. J. of Basic and Appl. Sci., 3(3): 2429-2436.

Tortora, G.J.; Funke, B. R. and Case, C. L. (1970). Microbiology. (8^{th} ed.) Pearson Education, Inc. San Francisco. New York.

Tyler, T.R. and Leatherwood, J.M. (1967). Epimerization of disaccharides by enzyme preparations from *Ruminococcu salbus*. Arch. Biochem. Biophys., 119:363-367.

Verma, V.; Verma, A. and Kushwaha, A. (2012). Isolation and production of cellulase enzyme from bacteria isolated from agricultural field in district hardoi, uttar Pradesh. Advances in Appl. Sci. Res., 3(1): 171-174.

Walter, G.C. (1984). Mutagenesisand inducible responses to deoxyribonucleic acid damage in Escherichia coli. Microbiol. Rev., 48(1):60-93.

Weinthal, D. M.; Barash, I.; Panijel, M.; Valinsky, L.; Gaba, V.and S. Manulis- Sasson. (2007). Distribution and replication of the pathogenicity plasmid pPATH in diverse populations of the gall-forming bacterium *Pantoea agglomerans*. Appl. Environ. Microbiol., 73:7552–7561.

Werkman, G. H and Wilson, P. W. (1953). Bacterial physiology. Academic press Inc. New York.

Whitaker, J. R. (1972). Priniciple of enzymology for the food science. Marcel Dekker, Inc. New York.

Wing, R. A.; Yamaguchi, J.; Larabell, S. K.; Ursin, V. M. and McCormick, S. (1989). Molecular and genetic characterization of two pollen-expressed genes that have sequence similarity to pectatelyases of the plant pathogen. *Erwinia*. Plant Mol. Biol., 14:17–28.

Xu, H.; Jia, S. and Liu, J. (2011). Development of a mutant strain of *Bacillus subtilis* showing enhanced production of acetoin. African J. of Biotech., 10(5): 779-788.

Yi, J. C.; Sandra, A. B. J. and Shu, T. C. (1999). Production and distribution of endoglucanase, cellobiohydrolase, and Ɓ-glucosidase components of the cellulolytic system of *volvariella volvacea*, the edible straw mushroom. Appl. Environ. Microbiol. 65: 553-559.

Yin, L.; Lin, H. and Xiao, Z. (2010). Purification and characterization of a cellulase from *Bacillus subtilis* YJ1. J. of Marine Sci. and Technol., 18(3): 466-471.

Zaldivar, M.; Velasquez, JC.; Contreras, I.; Perez, LM. (2001). *Trichoderma aureoviride* 7-121 a mutant with enhanced production of lytic enzymes its potential use in waste cellulose degradation and/or biocontrol. Electron J. Biotechnol., 4:1-9.

Zhang, Y. H. P.; Himmel, M.E. and Mielenz, J. R. (2006). Outlook of cellulase improvement: screening and selection strategies. Biotech. adv., 24:452-481.

Zhou, Z. G.; Li, F. Y.; Yi, T.; Huang, G. H. (2009). Large-scale analyses of glycosylation in cellulases .Genomics Proteomics Bioinfor., 7:194–199.

Zubay, G.(1993). Biochemistry (3rd ed.)., I. W. M. C. Bown Communications. Inc. USA.

Appendices

Appendices

Appendix (1): VITEK 2 result for *Pantoea dispersa*.

Number	Test	Result	Number	Test	Result
1	APPA	-	26	dTAG	-
2	ADO	-	27	dTRE	+
3	PyrA	+	28	CIT	+
4	IARL	-	29	MNT	-
5	dCEL	+	30	5KG	-
6	BGAL	+	31	ILATK	+
7	H2S	-	32	AGLU	-
8	BNAG	-	33	SUCT	+
9	AGLTp	-	34	NAGA	-
10	dGLU	+	35	AGAL	-
11	GGT	+	36	PHOS	+
12	OFF	+	37	GLyA	-
13	BGLU	-	38	ODC	-
14	dMAL	+	39	LDC	-
15	dMAN	+	40	IHISa	-
16	dMNE	+	41	CMT	-
17	BXYL	-	42	BGUR	-
18	BAIap	-	43	O129R	+
19	ProA	-	44	GGAA	-
20	LIP	-	45	IMLTa	-
21	PLE	-	46	ELLM	-
22	TyrA	+	47	ILATa	-
23	URE	-			
24	dSOR	-			
25	SAC	+			

Appendices

Appendix (2): Specific activity of cellulase in the mutants of cellulase gene.

Mutant number	Protein concentration (mg/ml)	Specific activity (u/mg)
wild	0.122	3.900
1	0.549	0.783
2	0.533	1.408
3	0.517	1.062
4	o.476	1.230
5	0.527	1.250
6	0.546	1.148
7	0.723	0.645
8	0.635	1.590
9	0.513	1.370
10	0.568	1.369
11	0.577	1.440
12	0.625	1.450
13	0.625	1.340
14	0.609	1.277
15	0.638	1.550
16	0.651	1.600
17	1.512	0.611
18	0.118	0.660
19	0.474	1.477
20	0.636	1.064
21	0.578	1.480
22	0.66	1.138
23	0.649	1.396
24	0.626	1.125
25	0.627	1.432
26	0.638	1.312
27	0.631	1.356
28	0.635	1.400
29	1.555	0.544
30	0.643	0.761
31	0.62	1.169
32	0.578	0.790
33	0.646	1.303
34	0.619	0.953

Appendices

Mutant number	Protein concentration (mg/ml)	Specific activity (u/mg)
35	0.196	1.550
36	0.194	1.580
37	0.113	1.154
38	0.099	1.176
39	0.135	0.542
40	0.077	**12.48**
41	0.0023	**24.10**
42	0.106	0.949
43	0.104	1.408
44	0.129	1.100
45	0.187	1.220
46	0.400	1.540
47	0.234	0.840
48	0.336	0.558
49	0.105	1.065
50	0.114	1.000
51	0.143	0.800
52	0.195	1.149
53	0.057	28.50
54	0.358	0.255
55	0.381	0.720
56	0.392	0.303
57	0.391	0.201
58	0.405	0.790
59	0.356	0.269
60	0.380	0.505
61	0.216	1.230
62	0.210	1.221
63	0.231	1.330
64	0.196	1.100
65	0.208	1.550
66	0.204	1.232
67	0.201	1.340
68	0.199	1.440
69	0.019	**26.70**
70	0.200	1.120
71	0.0023	**55.65**
72	0.015	**34.10**

Appendices

Mutant number	Protein concentration (mg/ml)	Specific activity (u/mg)
73	0.201	1.441
74	0.025	51.25
75	0.196	1.404
76	0.197	1.04
77	0.119	1.430
78	0.196	1.100
79	0.120	1.221
80	0.192	0.900
81	0.192	1.080
82	0.189	0.990
83	0.192	0.553
84	0.151	0.654
85	0.189	0.220
86	0.009	**49.83**
87	0.005	**22.19**
88	0.194	0.980
89	0.193	1.105
90	0.157	1.460
91	0.131	0.660
92	0.193	0.760
93	0.254	1.711
94	0.348	1.315
95	0.312	1.236
96	0.361	1.521
97	0.202	1.342
98	0.409	1.118
99	0.497	1.022
100	0.349	1.570

الخلاصة العربية

جمعت عينات مختلفة من ترب المناطق المزروعة بنباتي الحنطة والشعير، كما جمعت عينات اخرى ومن نماذج الفاكهة التالفة كالبرتقال والتفاح ومن جذور الجزرلعزل البكتريا المنتجة للانزيمات المحللة. وقد غربلة تنمية العزلات البكتيرية على الاوساط الانتقائية الحاوية على السيليلوز او البكتين كمصادر وحيدة للكاربون والطاقة لعزل البكتريا المنتجة لانزيمي السيليليز والبكتنيز. أظهرت نتائج الغربلة شبة الكمية قدرة تسعة عزلات بكتيرية من بين خمسين عزلة على انتاج انزيمي السيليليز والبكتنيز وبدرجات متفاوتة وفقا لأقطار الهالات الشفافة المتكونة حول مستعمرات. أخضعت العزلات الاكثر انتاجية لانزيمي السيليليز والبكتنيز الى الغربلة الكمية وذلك بتقدير الفعالية النوعية لكلا الانزيمين في الرائق الخام لمزارع تلك العزلات. أشار ت النتائج إلى إن العزلة البكتيرية المرمزة A3 كانت هي الأكفأ من بين العزلات الاخرى في انتاج انزيمي السيليليز والبكتنيز وذلك للانتاجية العالية لكلا الانزيمين. إذ بلغت الفعالية النوعية للسيليليز والبكتنيز في الرائق الخام للمزرعة البكتيرية لتلك العزلة 3.9 و 1.31 وحدة/ملغم بروتين على التوالي. تم تشخيص العزلة البكتيرية المنتجة (A3) وفقا لخصائصها المظهرية وصفاتها الكيموحيوية. وقد أشارت نتائج التشخيص إلى أن هذه العزلة تعود إلى جنس *Pantoea* spp.، وقد تم تاكيد نتائج التشخيص باستخدام نظام العدة التشخيصية Api 20-E والـ VITEK 2 ووشخصت العزلة على انها *Pantoea dispersa* . درست الظروف المثلى لإنتاج أنزيم السيليليز بوساطة العزلة البكتيرية *P. dispersa* A3 . وقد أشارة النتائج إلى أن الظروف المثلى كانت تتضمن تدعيم وسط الإنتاج بمادة كاربوكسي مثيل سليلوز (CMC) مصدرا وحيدا للكاربون والطاقة بتركيز 1.5%، وكبريتات الامونيوم مصدرا نيتروجي نيبتركيز `0.1%، وفوسفات البوتاسيوم ثنائية الهيدروجين بتركيز 0.1% و تنظيم الرقم الهيدروجين للوسط الى pH 7 والحضن بدرجة 30 °م في الحاضن الهزاز بسرعة 140 دورة/دقيقة لمدة 72 ساعة. وقد بلغت الفعالية النوعية لأنزيم السيليليز المنتج تحت هذه الظروف 24 وحدة/ملغم بروتين. تم تنقية السيليليز المنتج من قبل العزلة البكتيرية *P. dispersa* A3 تحت الظروف المثلى باربعة خطوات تضمنت خطوة الترسيب بكبريتات الامونيوم بنسبة أشباع 70%، ثم خطوة الديلزة، وخطوة التبادل الأيوني باستخدام المبادل DEAE-cellulose ثم خطوة الترشيح الهلامي خلال هلام السيفادكس G200. وقد أشارة نتائج التنقية الى أن الفعالية النوعية للأنزيم المنقى كانت 190.9 وحدة/ملغم بروتين بعدد مرات تنقية وحصيلة أنزيمية مقدارها 113.6 و 26.2% على التوالي. تم توصيف انزيم السيليليز المنقى من العزلة *Pantoea dispersa* A3 وذلك بدراسة بعض خصائص الأنزيم. وقد أشارت النتائج الى أن الوزن الجزيئي للأنزيم هو 15148 دالتون، وأن الرقم الهيدروجيني الأمثل

لفعالية وثبات الأنزيم هو pH 7 وان الدرجة الحرارية المثلى لفعالية وثبات الأنزيم أيضا كانت 30 °م. وفي محاولة لزيادة أنتاج السيليليز بواسطة العزلة البكتيرية *P. dispersa* A3 بالتطفير الفيزيائي، فقد تم تعريض الخلايا البكتيرية لمزرعة فتية لهذة العزلة الى التطفير العشوائي بالاشعة فوق البنفسجية لأحداث الطفرات الوراثية المؤثرة ايجابا في انتاج الأنزيم. وقد أشارت نتائج التطفير الى الحصول على تسعة طافرات بكتيرية متميزة بإنتاجيتها العالية للسيليليز بعد التشعيع با لأشعة فوق البنفسجية. اذ تراوحت الفعالية النوعية للأنزيم المنتج بوساطة هذة الطافرات بين 12.48 و 55.65 وحدة/ملغم بروتين مقارنة بأنتاجية النوع البري (3.9 وحدة/ملغم بروتين). تم أستخلاص الدنا المجيني للعزلة A3 *P. dispersa* بأستخدام عدة الأستخلاص (Favorgen kit) وذلك لتضخيم جين السيليليز بتصميم بادئات نكليوتيدية تتضمن التعاقبات النكليوتيدية للعناصر الوراثية أسفل وأعلى مجرى جين السيليليز لأنواع بكتريا .*Pantoea* sp ووفقا لقواعد البيانات الموجودة في المركز العالمي لمعلومات التقانة الأحيائية (NCBI) على موقعة في شبكة الأنترنيت. وبعد ترحيل نواتج التضخيم على هلام الأكاروز (0.8%) لتمييز الحزم بوجود دليل دنا حجمي. أشارت نتائج الترحيل الى عدم ظهور أي حزمة دنا يمكن تمييزها على أنها ناتج لتفاعل البلمرة لكل زوج من البادئات النكليوتيدية، مما يشير الى أن هذة البادئات لم تكن متخصصة لتضخيم جين السيليليز للعزلة البكتيرية A3 *P. dispersa*.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الاحيائية

أنتاج وتوصيف ودراسة جينية لبكتريا *Pantoeadispersa* المنتجة لأنزيمات السيليليزوالبكتنيز

اطروحة

مقدمة إلى مجلس كلية العلوم، جامعة النهرين وهي جزء من متطلبات نيل درجة

دكتوراه علوم فلسفة في التقانة الأحيائية

من قبل

محمد لفته عطا الله

بكالوريوس أحياء مجهرية، جامعة بغداد (1994)

ماجستير تقنيات إحيائية،جامعة بغداد (2000)

بأشراف

د. حميد مجيد جاسم د.كاظم محمد إبراهيم

أستاذأستاذ

ربيع الثاني
1436

شباط
2015