“Synthesis of Novel Nanoparticles using Silk Worm Fecal Matter: Characterization of Nanoparticles for their Biological Applications”

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in
BIOCHEMISTRY

Submitted by

Mr. Avinash. B M.Sc.

Department of P.G. Studies and Research in Biochemistry
Kuvempu University

Research Guide

Dr. Shivayogeeswar. E. Neelagund

M.Sc. Ph.D., Post Doc

(USA).
Associate Professor
Department of P.G. Studies and Research in Biochemistry
Jnana Sahyadri, Kuvempu University,
Shankaraghatta- 577 451

Declaration

I hereby declare that the Ph.D., thesis entitled **"Synthesis of Novel Nanoparticles using Silk Worm Fecal Matter: Characterization of Nanoparticles for their Biological Applications"** is the results of my original investigations and this has been composed by me under the guidance of **Dr. Shivayogeeswar E. Neelagund,** Associate Professor, Department of PG studies and Research in Biochemistry, Kuvempu University, Jnana Sahyadri, Shankaraghatta-577451, Shivamogga.

I further declare that the results presented in this thesis or any part thereof has not been submitted elsewhere for any other degree, diploma or similar title of any other Universities.

Date: 14-03-2019
Place: **Shankaraghatta**

(Avinash. B)

KUVEMPU UNIVERSITY

Dr. Shivayogeeswar E. Neelagund
M.Sc., Ph.D., Post Doc (USA).
Associate Professor
Department of PG Studies & Research in Biochemistry
Jnana Sahyadri, Shankaraghatta
Karnataka, India
Mobile: 9448234456

RESIDENCE
"Eswara", 12th cross
RMC 60ft Road,
2nd Stage, Vinobhanagar,
Shivamogga-577204

Certificate

This is to certify that the work reported in this thesis entitled "Synthesis of Novel Nanoparticles using Silk Worm Fecal Matter: Characterization of Nanoparticles for their Biological Applications" submitted by Mr. Avinash B. to Kuvempu University, for the award of Doctor of Philosophy in Biochemistry is a record of bonafide and original research work carried out by him under my guidance and direct supervision. The work reported in this thesis has not formed the basis for the award of any degree or diploma or any other similar title in any other institution or university.

Date: 14/03/2019
Place: **Shankaraghatta**

14.3.2019.
(Dr. Shivayogeeswar. E. Neelagund)
Research Guide

Dr. S.E. Neelagund
M.Sc., PhD Postdoc (USA)
Associate Professor
P.G. Dept. Of Biochemistry
Kuvempu University
Shankarghatta, Shivamogga - 577451
Karnataka, India

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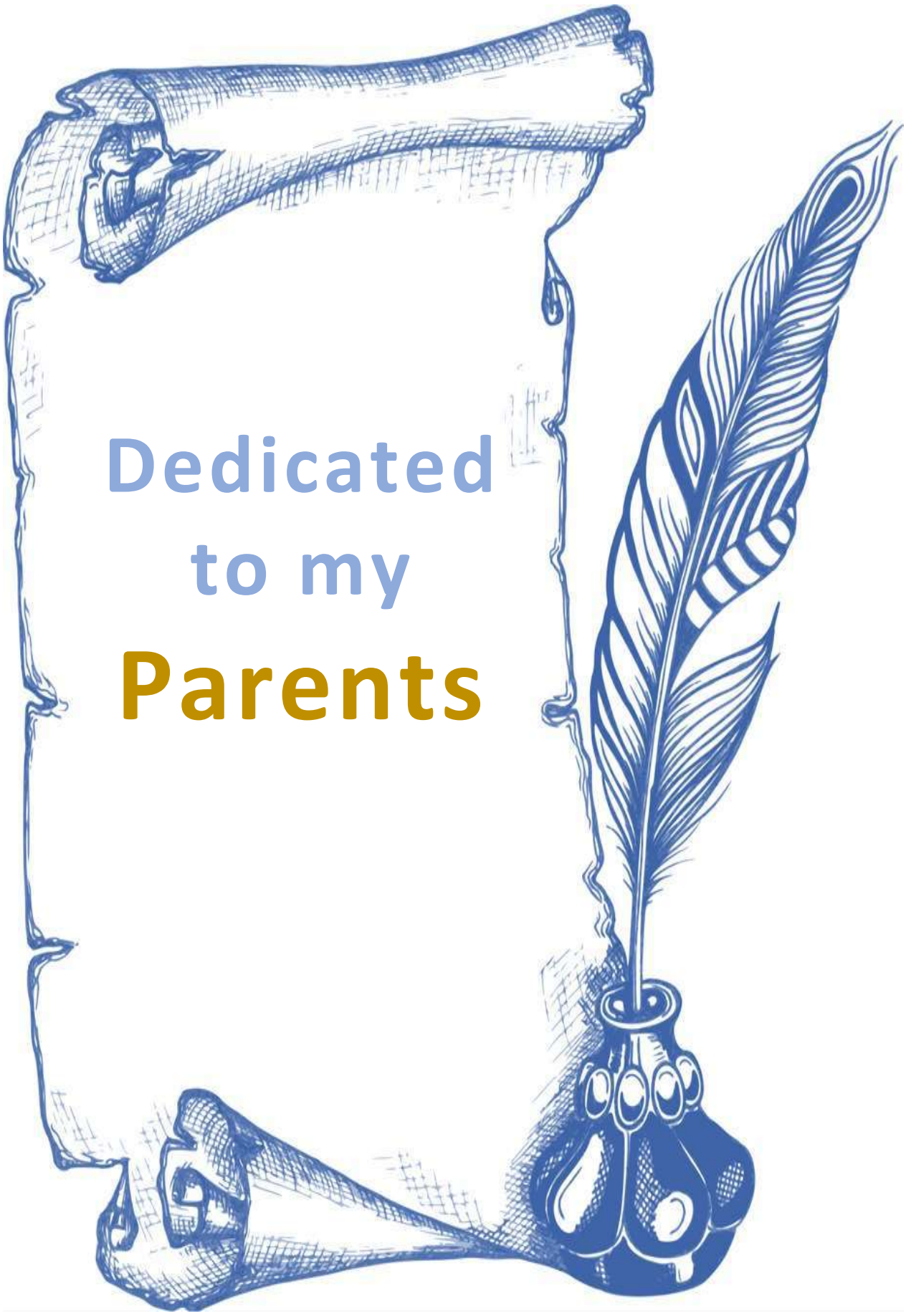

Dedicated
to my
Parents

INDEX

	Contents	Page number
Chapter I: Introduction		**01 – 30**
1.1	Nanoparticles	01
1.2	Nanobiotechnology	03
1.3	Synthesis of nanoparticles	05
1.3.1	Chemical and Physical methods of Synthesis	07
1.3.2	Biological synthesis of nanoparticles	08
1.4	Silk worm fecal matter (*Bombyx mori*)	09
1.5	Silver nanoparticles (AgNPs)	10
1.6	Copper nanoparticles (CuNPs)	11
1.7	Characterization of nanoparticles	12
1.7.1	UV-Vis Spectroscopy	12
1.7.2	Atomic Absorption Spectroscopy	13
1.7.3	X-Ray Diffraction Spectroscopy	13
1.7.4	Fourier Transform Infrared Spectroscopy	14
1.7.5	Scanning Electron Microscopy	14
1.8	Biological applications of nanoparticles	15
1.9	Coconut (*Cococs nucifera*)	16
1.10	Bud Rot disease of *Cocos nucifera*	17
1.11	*Phytophthora palmivora*	19
1.12	Pectinases	20
1.13	Cellulases	22
1.14	Enzyme production	23
1.15	Partial purification of Enzymes	23
1.15.1	Ammonium Sulfate Precipitaion	24
1.15.2	Dialysis	25
1.15.3	Ion Exchange Chromatography	25
1.15.4	SDS-PAGE	26

1.16	Inhibition of Enzymes using nanoparticles	27
1.17	Objectives of present study	30
Chapter II: Materials and Methods		**31-49**
2.1	Synthesis, Characterization and Biological activities of nanoparticles using Silk worm fecal matter	31
2.1.1	Preparation of Silk worm fecal matter aqueous extract	31
2.1.2	Synthesis of nanoparticles	32
	2.1.2.1 Synthesis of Silver nanoparticles (AgNPs)	32
	2.1.2.2 Synthesis of Copper nanoparticles (CuNPs)	32
2.1.3	Characterization of synthesized nanoparticles	33
	2.1.3.1 UV-Vis Spectroscopy	33
	2.1.3.2 Atomic Absorption Spectroscopy	33
	2.1.3.3 X-ray Diffraction measurements	33
	2.1.3.4 Fourier Transform Infrared Spectroscopy	34
	2.1.3.5 Scanning Electron Microscopy	34
2.1.4	Biological Activities of synthesized nanoparticles	35
	2.1.4.1 Antibacterial activity	35
	2.1.4.1.1 AgNPs	35
	2.1.4.1.2 CuNPs	35
	2.1.4.2 Antioxidant assay of AgNPs	36
	2.1.4.2.1 DPPH free radical Scavenging assay	36
	2.1.4.2.2 Hydrogen peroxide (H_2O_2) scavenging assay	37
	2.1.4.3 Cytotoxic effect of biosynthesized nanoparticles	37
2.2	Isolation and Identification of pathogenic fungus causing Bud rot disease in Coconut; Inhibition studies using synthesized nanoparticles	38
2.2.1	Sample Collection	38
2.2.2	Isolation and Identification of pathogenic fungus	39
2.2.3	Molecular identification of isolated fungus	40
2.2.4	Inhibition of *Phytophthora palmivora* using biosynthesized nanoparticles	41

2.3.1	Screening of pathogenic fungus for different cell wall degrading enzymes	42
2.3.2	Optimization and partial purification of Pectinase and Cellulase enzymes	43
	2.3.2.1 Chemicals	43
	2.3.2.2 Optimization of broth conditions for Pectinase production	43
	2.3.2.3 Preparation of Culture supernatant	44
	2.3.2.4 Estimation of Protein concentration	44
	2.3.2.5 Pectinase enzyme assay	44
	2.3.2.6 Partial purification of Pectinase enzyme	45
	2.3.2.7 Optimization of broth conditions for Cellulase production	45
	2.3.2.8 Preparation of Culture supernatant	46
	2.3.2.9 Estimation of Protein concentration	46
	2.3.2.10 Cellulase Enzyme assay	46
	2.3.2.11 Partial purification of Cellulase enzyme	47
	2.3.2.12 SDS-Polyacrylamide Gel Electrophoresis	47
2.4	Inhibition of Pectinase and Cellulase enzymes using synthesized nanoparticles	48
24.1	Inhibition of Pectinase enzyme activity	48
2.4.2	Inhibition of Cellulase enzyme activity	48
Chapter III: Results		**50-95**
3.1	Synthesis, Characterization and Biological activities of nanoparticles using Silk worm fecal matter	50
3.1.1	Characterization of synthesized nanoparticles	51
	3.1.1.1 UV-Vis Spectroscopy	51
	3.1.1.2 Atomic Absorption Spectroscopy	53
	3.1.1.3 X-ray Diffraction measurements	55
	3.1.1.4 Fourier Transform Infrared Spectroscopy	57
	3.1.1.5 Scanning Electron Microscopy	60
3.1.2	Biological Activities of synthesized nanoparticles	61
	3.1.2.1 Antibacterial activity	61

3.1.2.1.1 AgNPs 61

3.1.2.1.2 CuNPs 64

3.1.2.2 Antioxidant assay of AgNPs 66

3.1.2.2.1 DPPH free radical Scavenging assay 66

3.1.2.2.2 Hydrogen peroxide (H_2O_2) scavenging assay 67

3.1.2.3 Cytotoxic effect of biosynthesized nanoparticles 68

3.2 Isolation and Identification of pathogenic fungus causing Bud rot disease in Coconut; Inhibition studies using synthesized nanoparticles 69

3.2.1 Isolation and Identification of pathogenic fungus 69

3.2.2 Molecular Identification of fungus 69

3.2.3 Inhibition of *Phytophthora palmivora* using biosynthesized nanoparticles 73

3.3.1 Screening of pathogenic fungus for different cell wall degrading enzymes 76

3.3.2 Optimization of Pectinase production 78

3.3.2.1 Incubation time v/s Pectinase production 78

3.3.2.2 pH v/s Pectinase production 79

3.3.2.3 Temperature v/s Pectinase production 80

3.3.2.4 Substrate v/s Pectinase production 81

3.3.2.5 Substrate concentration v/s Pectinase production 82

3.3.3 Partial Purification of Pectinase enzyme 83

3.3.4 Optimization of Cellulase production 86

3.3.4.1 Incubation time v/s Pectinase production 86

3.3.4.2 pH v/s Pectinase production 87

3.3.4.3 Temperature v/s Pectinase production 88

3.3.4.4 Substrate v/s Pectinase production 89

3.3.4.5 Substrate concentration v/s Pectinase production 90

3.3.5 Partial purification of Cellulase enzyme 91

3.4 Inhibition of Pectinase and Cellulase enzymes using synthesized nanoparticles 94

3.4.1 Inhibition of Pectinase Enzyme activity 94

3.4.2 Inhibition of Cellulase enzyme activity 95

Chapter IV: Discussion		**96-110**
4.1.1	Synthesis and Characterization of nanoparticles	96
4.1.2	Biological activities of synthesized nanoparticles	100
	4.1.2.1 Antibacterial activity	100
	4.1.2.1.1 AgNPs	100
	4.1.2.1.2 CuNPS	101
	4.1.2.2 Antioxidant assay of AgNPs	102
	4.1.2.2.1 DPPH free radical scavenging assay	102
	4.1.2.2.2 Hydrogen peroxide scavenging assay	103
	4.1.2.3 Cytotoxic effect of synthesized nanoparticles	103
4.2	Isolation, Identification and Inhibition of pathogenic fungus	104
4.2.1	Isolation and Identification	104
4.2.2	Inhibition of *Phytophthora palmivora* using biosynthesized nanoparticles	106
4.3.1	Screening of pathogenic fungus for different cell wall degrading enzymes	107
4.3.2	Optimization and partial purification of Pectinase and Cellulase enzymes	107
4.4	Inhibition of Pectinase and Cellulase enzymes using synthesized nanoparticles	109
Conclusions		**111-114**
References		
List of Publications and Conferences		

List of Tables

4.1	MIC of biosynthesized silver nanoparticles	**63**
4.2	Zone of Inhibition for Copper nanoparticles	**64**
4.3	Sequences showing similarity with the isolated Fungus	**71**
4.4	Percentage of Mycelial Growth Inhibition of *P. palmivora*	**73**
4.5	Steps involved in partial purification of Pectinase	**75**
4.6	Steps involved in partial purification of Cellulase	**93**

List of Figures

3.1 a	UV-Vis spectrum of Silver nanoparticles	**51**
3.1 b	UV-Vis spectrum of Copper nanoparticles	**52**
3.2 a	AAS of Silver nanoparticles	**53**
3.2 b	AAS of Copper nanoparticles	**54**
3.3 a	XRD pattern of biosynthesized Silver nanoparticles	**56**
3.3 b	XRD pattern of biosynthesized Copper nanoparticles	**57**
3.4 a	FTIR spectrum of Silver nanoparticles	**58**
3.4 b	FTIR spectrum of Copper nanoparticles	**59**
3.5 a	SEM image of Silver nanoparticles	**60**
3.5 b	SEM image of Copper nanoparticles	**61**
3.6 a	Antibacterial activity of AgNPs on growth of *S. typhi*	**62**
3.6 b	Antibacterial activity of AgNPs on growth of *S. aureus*	**63**
3.7	Antibacterial activity of CuNPs	**65**
3.8 a	DPPH free radical scavenging assay of AgNPs	**66**
3.8 b	Hydrogen Peroxide Scavenging Activity of AgNPs	**67**
3.9	Bar graph showing efficacy of AgNPs and CuNPs against A549 human alveolar basal epithelial cell lines at different concentration	**68**
3.10	Agarose gel pattern of Phytophthora palmivora genomic DNA	**70**
3.11	PCR amplified product of isolated *Phytophthora* sp. on Agarose gel electrophoresis pattern.	**70**
3.12 a	Picture showing inhibition effect of (1) 1mg/ml AgNPs, (2) 2mg/ml AgNPs, (3) 3mg/ml AgNPs, (F) 3mg/ml Standard and (C) Control	**74**
3.12 b	Picture showing inhibition effect of (1) 1mg/ml CuNPs, (2) 2mg/ml CuNPs, (3) 3mg/ml CuNPs, (F) 3mg/ml Standard and (C) Control	**75**

3.13 a	Screening of fungus for Pectinase production	**76**
3.13 b	Screening of fungus for Cellulase production	**77**
3.14 a	Effect of Incubation time on Pectinase production by *P. palmivora*	**78**
3.14 b	Effect of pH on Pectinase production by *P. palmivora*	**79**
3.14 c	Effect of temperature on Pectinase production by *P. palmivora*	**80**
3.14 d	Effect of different substrates on Pectinase production by *P. palmivora*	**81**
3.14 e	Effect of substrate concentration on Pectinase production by *P. palmivora*	**82**
3.15 a	Pattern of DEAE-Cellulose anion exchange chromatographic purification of Pectinase enzyme using 0.1 to 1.0 M NaCl in citrate buffer	**84**
3.15 b	Polyacrylamide gel electrophoresis analysis of the partially purified Pectinase enzyme	**85**
3.16 a	Effect of Incubation time on Cellulase production by *P. palmivora*	**86**
3.16b	Effect of pH on Cellulase production by *P. palmivora*	**87**
3.16 c	Effect of temperature on Cellulase production by *P. palmivora*	**88**
3.16 d	Effect of different substrates on Cellulase production by *P. palmivora*	**89**
3.16 e	Effect of substrate concentration on Cellulase production by *P. palmivora*	**90**
3.17 a	Pattern of DEAE-Cellulose anion exchange chromatographic purification of Cellulase enzyme using 0.1 to 1.0 M NaCl in acetate buffer	**92**
3.17 b	Polyacrylamide gel electrophoresis analysis of the partially purified Cellulase enzyme	**93**
3.18 a	Decreasing Relative activity of Pectinase enzyme	**94**
3.18 b	Decreasing Relative activity of Cellulase enzyme	**95**

Abbreviations

%	percent
µl	microliter
µmol	micro mole
AAS	Atomic Absortion Spectra
Ag	Silver
AgNPs	Silver nanoparticles
bp	base pair
BSA	Bovine Serum Albumin
cm	centimeter
Cu	Copper
CuNPs	Copper nanoparticles
DEAE	Diethylaminoethyl
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxy ribonucleic acid
DNS	Dinitrosalicylic acid
dNTPs	deoxy Ribonucleotide triphosphate
DPPH	DiphenylPicrylHydrazyl
et al.,	and others
FBS	Fetal bovine serum
fcc	face centered cubic
FTIR	Fourier Transform Infrared
g/l	gram per liter
hr	hour

ITS	Internal Transcribed Spacer
kb	kilo base
KBr	Potassium Bromide
kDa	kilo Dalton
mA	milliampere
mg	milligram
MGI	Mycelial Growth Inhibition
MIC	Minimum Inhibitory Concentration
min	minute
ml	milliliter
mm	milli meter
mM	milli Molar
MO	Miroorganism
MTT	Dimethylthiazole
ncbi	National Centre for Biotechnology Information
ng	nano gram
nm	nanometer
^{o}/min	degree per minute
^{o}C	degree Centigrade
OD	Optical density
PAGE	Poly Acrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
ppm	parts per million
RNA	Ribo Nucleic Acid
rpm	Rotation per minute
SDS	Sodium Dodecyl Sulphate

SEM	Scanning Electron Microscope
sp.	Species
U	Unit
UV	Ultra Violet
v/s	versus
w/v	weight per volume
XRD	X-Ray Diffraction
ZOI	Zone of Inhibition
α	Alpha
μg	micro gram

Chapter 1

Introduction

1.1. Nanoparticles

Nanomaterials are cornerstones of nanoscience and nanotechnology. We specifically considered nanoparticles as clusters of atoms in the size of 1-100 nm. 'Nano' is a Greek word synonymous to dwarf meaning extremely small. Nanostructure science and technology is an expansive and interdisciplinary area of research and development activity that has been growing explosively worldwide in the past few years. The idea of nanotechnology was coined by physicist Professor Richard Feynman (known as "Father of Nanotechnology") in his historic talk "there's plenty of room at the bottom" [Feynman, 1959], though the term nanotechnology was introduced by Tokyo Science University Professor Norio Taniguchi [Taniguchi, 1974].

Richard Feynman

Norio Taniguchi

Nanotechnology aims at combining fundamental principles of physics, chemistry and biology for manufacturing nanosensors, nano medicine, nanomaterials which finds ample applications in the field of energy, medicine, pollution control, computing, etc. Nanotechnology is one of the leading scientific fields today since it combines knowledge from the fields of Physics, Chemistry, Biology, Medicine, Engineering and Informatics. It is an emerging technological field with great potential to lead in great breakthroughs that can be applied in real life [Logothetidis, 2012].

Novel nano biomaterials and nanodevices are fabricated and controlled by nanotechnology tools and techniques, which investigate and tune the properties, responses and functions of living and non-living matter at sizes below 100 nm. It has the potential for revolutionizing the ways in which materials and products are created and the range and nature of functionalities that can be accessed [Alagarasi, 2011]. Nanoscale materials are defined as a set of substances where at least one dimension is less than approximately 100 nanometers which behaves as a whole unit in terms of its transport, properties and unique characteristics like optical, electrical and biological properties. That have attracted significant consideration due to their potential use in many applications such as catalysis, bio-sensing, drug delivery and nanodevice fabrication. Hence nanoparticles have attracted huge attention due to catalytic, electric, optical, photonic, textile, nanofluid and antimicrobial activities which varies depending on their size, shape and neighboring medium [Alagarasi, 2011]. Nanotechnology is considered as an emerging technology because it provides a platform to develop advance well-established products, to create new products with totally new characteristics and functions with enormous potential in a wide range of applications [Logothetidis, 2012]. In addition to various industrial uses, great innovations are prophesied in information and communication technology, in biology and biotechnology, in medicine and medical technology, in metrology, etc. Significant applications of nanosciences and nanoengineering lie in the fields of pharmaceutics, cosmetics, processed food, chemical engineering, high-performance materials, electronics, precision mechanics, optics, energy production and environmental sciences [Mandeep Kaur et *al.*, 2015; Mobaseer *et al.*, 2017].

Current research trend of combating various antimicrobial and antiviral diseases through nanobiotechnology research, which is identified as a prominent area. Nanobiotechnology has emerged as integration among biotechnology and nanotechnology for developing biological synthesis and environmental-benign technology for synthesis of nanomaterials.

1.2. Nanobiotechnology

Nanobiotechnology is one of the most promising areas in modern nanoscience and technology. This emerging area of research intermingles various disciplines of science such as physics, chemistry, biology and material science. Biotechnology and nanotechnology are two of the 21st century's most promising technologies. Nanotechnology is defined as the design, development and application of materials and devices whose least functional make up is on a nanometer scale. Generally, nanotechnology deals with developing materials, structures or other devices possessing at least one dimension sized from 1 to 100 nanometers. Nanobiotechnology is a young and fast evolving field of research in nanoscience. It is an interdisciplinary area which complies advances in Science and Engineering. Nanobiotechnology is a field that concerns the utilization of biological system optimized through evolution, such as cells, cellular components, nucleic acid and proteins to facilitate functional nanostructured and mesoscopic architecture comprised of organic and inorganic materials [Andieveskii, 2003]. Biofunctionlization of nanoparticles is an important contribution of present day nanobiotechnology. Meanwhile, biotechnology deals with physiological and other metabolic processes of biological subjects. Nanobiotechnology is the association of

these two technologies and can play a vital role in developing and implementing many useful tools in the study of life [Thakkar *et al.*, 2010].

The organisms used in nanoparticle formation vary widely from simple prokaryotic bacterial cells to complex eukaryotes. Biosynthesis of gold, silver, gold–silver alloy, selenium, tellurium, platinum, palladium, silica, titania, zirconia, quantum dots, magnetite and uraninite nanoparticles by bacteria, actinomycetes, fungi, yeasts and viruses have been reported [Castro *et al.*, 2014]. The use of organisms in nanotechnology is developing rapidly because of the ease of handling and formation of nanoparticles. Furthermore, biosynthesis of metal nanoparticles was an environmentally friendly method without the use of harsh toxic and expensive chemicals. Although biological methods were regarded as safe, cost-effective, sustainable and clean processes, they also have some drawbacks in culturing of microbes and use biomasses, which was time-consuming and difficult in providing better control over shape, size distribution and crystallinity. Additionaly, biological nanoparticles were not monodispersible and its production rate was slow. These were the main problems that have delayed the biological synthesis approaches [Scheu *et al.*, 2006]. The most relevant aspects to be considered in the biological process to synthesize highly stable and well characterized nanoparticles are suggested the following [Siavash Iravani, 2011]:

(1) Selection of the suitable organisms: Researches have been focused on the important intrinsic properties of the organisms such as enzyme activity and biochemical pathways with the aim of choosing the best candidates for metal nanoparticles production. There is also the possibility of producing genetically

engineered microbes that overexpress specific reducing agents and thereby, can control the size and shape of biological nanoparticles.

(2) Optimal conditions for cell growth and enzyme activity: The nutrients, inoculums size, light, temperature, pH, mixing speed, and buffer strength are very important points that should be controlled. The presence of substrates or related compounds in subtoxic levels from the beginning of the growth would increase the activity of the enzymes.

(3) Optimal reaction conditions: The yield and the production rate are important points to be considered to implement the use of organisms for the synthesis of metal nanoparticle at industrial scale. Bioreduction conditions in the reaction mixture should be carefully optimize. In addition, substrate concentration, biocatalyst concentration, electron donor and its concentration, exposure time, temperature, buffer strength, mixing speed, and light need to be controlled. Moreover, some investigations have shown that the use of some complementary factors such as visible light or microwave irradiation, and boiling could affect the size, morphology, and rate of reaction.

The optimization of these factors are required to implement those approaches in large scale and for commercial applications. It is particularly interesting to develop new low cost processes for the synthesis of metallic nanoparticles.

1.3. Synthesis of Nanoparticles

Novel materials have been developing by material scientists with better properties, more functionality and lower cost than the existing one. To enhance the performance of nanomaterials, several physical and chemical methods have been

developed displaying upgraded properties with the aim to have a better control over the particle size and distribution.

In general, top-down and bottom-up are the two main ways of nanomaterials synthesis.

a. Top-down: Size reduction from bulk materials.

b. Bottom-up: Material synthesis from atomic level.

Top-down routes are included in the typical solid –state processing of the materials. This route is based with the bulk material and making it smaller, thus breaking up larger particles by the use of physical processes like milling, crushing or grinding. Usually this is not suitable route for preparing uniform shaped materials and it is very difficult to realize very small particles even with high energy consumption. The major problem with top-down approach is the imperfection of the surface structure, such imperfection would have a significant impact on surface chemistry and physical properties of nanostructures and nanomaterials. It was well known that the conventional top-down technique can cause significant crystallographic damage to the processed patterns [Arole and Munde, 2014].

Bottom–up approach refers to the build-up of a material from the bottom: atom-by-atom, molecule-by-molecule or cluster-by-cluster. This route is more oftenly used for preparing most of the nano-scale materials with the ability to generate a uniform shape, size and distribution. It successfully covers chemical synthesis and precisely controlled the reaction to inhibit further particle growth. Although the bottom-up approach is nothing new, it plays an important role in the

fabrication and processing of nanostructures and nanomaterials [Yuliang Wang and Younan Xia, 2004].

Including above mentioned approaches, synthesis of metal nanoparticles is mainly divided into three major methods, which includes chemical, physical and biological synthesis.

1.3.1. Chemical and Physical methods of synthesis

A clear understanding of chemical and physical methods of synthesizing metal nanoparticles is needed to study biological synthesis of nanoparticles and to allow contrasts to be made and a root for development to become evident. Plenteous volume of research on the synthesis of metal nanoparticles is available in the literature. Here, we just discussed an overview about them to get insight into non-biological synthesis of nanoparticles. There are many different procedures of chemical and physical methods could be used for synthesis of nanoparticles. Several chemical and physical practices have been exploited in the synthesis of various metal nanoparticles by dry and wet approaches viz., laser ablation, ultraviolet irradiation, lithography, aerosol technologies, ultrasonic fields and photochemical reduction practices [Ankit Chokriwal *et al*., 2014]. Conversely, these practices are burdened with many problems including use of expensive and harmful chemical agents, high energy requirement and creation of hazardous commodities.

However, for nanoparticle production, various chemical methods evidenced many problems with product stability, crystal growth control and particles aggregation on long term exposure. Applications are becoming limited due to use of organic solvents and toxic chemicals during the process of nanoparticles preparation

and their occurrence on the surface of nanoparticles. Therefore, there is an essential requirement to spread out for environmentally benign measures for nanoparticles synthesis. The waste disposal problems in chemical synthesis of nanoparticle, due to high usage of organic solvents, toxic capping and reducing agents and high energy constraint in physical methods of synthesis, both the methods are expensive and generation of toxic by-product are foremost drawbacks of chemical and physical methods of nanoparticle synthesis [Gade *et al.,* 2011: Musarrat *et al.,* 2011; Shedbalkar *et al.,* 2014]. Such disadvantages impose the development of clean, inexpensive, non-hazardous, energy-efficient, biocompatible and eco-friendly approaches for synthesis of nanoparticles. Accordingly, biological systems have been exploited and focused for the nanoparticle synthesis providing a harmless substitute for chemical and physical methods [Gaidhani *et al.,* 2013].

1.3.2. Biological synthesis of nanoparticles

Biological synthesis is a green and environmental friendly approach of synthesizing nanoparticles which are biodegradable and non-toxic to the environment [Kuppuswamy *et al.,* 2016]. Biosynthesis includes using of plants, algae, fungi, actinomycetes, yeast, bacteria, etc. along with precursors to synthesize nanoparticles alternative to convention chemicals for capping and bioreduction purposes. The biologically synthesized nanoparticles finds their own way in biomedical applications due to their unique and enhanced properties [Hasan, 2015]. Nanoparticle biosynthesis is an eye catching area of nanoscience and nanotechnology. It encompass the development of clean, eco-friendly, biocompatible and non-toxic methods for synthesis of nanoparticles in comparison

with the conventional methods like chemical and physical which are often toxic [Ankit Chokriwal *et al*., 2014].

The reduction rate of metal ions using biological agents was found to be pretty faster and also with ambient pressure and temperature conditions. It is widely known that microorganisms such as bacteria, yeast, fungi and algae are capable of adsorbing and accumulating metals [Juibari *et al*., 2011; Longoria *et al*., 2011; Sakaguchi *et al*., 2013]. These biological agents are capable of secreting various number of enzymes, which are potent in hydrolyzing metals and thus brings the enzymatic reduction of metal ions. Even fecal matter contains various useful excretory products and various properties, which may result in the reduction of precursor for nanoparticle production and there was no availability of literature of using fecal matter for the production of nanoparticles. Hence, we have used silk worm fecal matter for biological synthesis of nanoparticles.

1.4. Silk worm fecal matter (*Bombyx mori*)

Bombyx mori is a species under the Phylum: Arthropoda, Class: Insecta, Sub-class: Pterygota, Order: Lepidoptera, Sub-order: Heteroneura, Family: Bombycidae, Genus: *Bombyx* and Species: *mori*, it is an economically significant insect, being a primary producer of silk and these are quite important in the science world.

Silkworm fecal matter was known for large number of biological activities such as anticancer, antibacterial, antiviral, antihypertensive, free radical scavenging and antidiabetic [Wan-Taek Ju *et al*., 2013; L Xia *et al*., 2013; Regina Ines Kunz *et al*., 2016; Hae Yong Kweon *et al*., 2017]. Silkworm fecal matter was also reported

to be one of the richest sources of antiviral and antimicrobial protein (substances). The partially purified protein from silkworm fecal matter was found to be with the association of tetrapyrrole pigment which were the most common group of chlorophyll pigments. The Silk worm fecal matter protein shown an excellent antibacterial activity on gram negative as well as gram positive bacteria and good analgesic activity was also reported [Neelagund *et al*., 2007; Raghavendra *et al*., 2009; Neelagund *et al*., 2011]. As earlier studies said, Chlorophyll a probably is the pigment moiety of the complex which behaves as a single electron transfer oxidation-reduction system [B Kok, 1961]. Silkworm feces act as a raw material for variety of products such as paste chlorophyll and sodium copper chlorophyllin [Raju, 1996]. The use of waste materials not only reduces the cost of synthesis but also minimizes energy requirement in comparison to physical or chemical synthesis methods, the need of using harmful chemicals or byproducts would stimulates 'biological synthesis'. Therefore, we have explored biosynthesis of nanoparticles using silkworm fecal matter as a bioreducing agent.

1.5. Silver Nanoparticles (AgNPs)

Silver nanoparticles are of focussed because of their unique properties (e.g., electrical, magnetic, size and shape depending optical properties) which could be assimilated into antimicrobial applications, composite fibers, biosensor materials, cosmetic products, cryogenic superconducting materials and electronic components. Among metal nanoparticles, silver nanoparticle (AgNP) has extensive advantages in industry and medicine due to its antibacterial, antifungal, larvicidial and anti-parasitic effects. Because of their widespread applications, which are advantageous to humans, there is a need to develop quick and consistent experimental procedures

for the synthesis of silver nanoparticles (AgNPs). Numerous chemical and physical protocols have been used for synthesizing and stabilizing silver nanoparticles [Klaus *et al*., 1999; Senapati S, 2005]. The most popular chemical approach includes, chemical reduction using varied organic and inorganic reducing agents, physicochemical reduction, electrochemical techniques and radiolysis are extensively used for the synthesis of silver nanoparticles (AgNPs). Recently, nanoparticle synthesis is among the furthermost interesting scientific areas of inquiry and there is a rising attention to synthesize nanoparticles using environmentally friendly approaches (biological synthesis). The chemical methods are the most common and widespread but using toxic chemicals during the synthesis produces toxic by-products [Song JY *et al*., 2009]. The physical methods demand huge quantity of energy to maintain high temperature and pressure required for the reaction [Rajasekharreddy *et al*., 2010]. Thus, the physical and chemical approaches have their own limitations; which are considered as expensive and unsuitable for sustainable ecosystem [Kumar *et al*., 2009]. The Silver nanoparticles (AgNPs) synthesis using biological entities is achieving momentum as; biological synthesis methods are providing non-toxic and environmentally acceptable "green chemistry" procedures.

1.6. Copper Nanoparticles (CuNPs)

Copper nanoparticles (CuNPs) are of great attention just because of their distinctive catalytic, thermal, optical, antimicrobial, magnetic, electronic and electrical conducting properties [Judai *et al*., 2009; Yu *et al*., 2010; Dang *et al*., 2011]. They present a wide range of potential applications in nanotechnology

including catalysts [Judai *et al*., 2009], additives for lubricants, conductive inks, biosensors, heat transfer nanofluids, manufacture of electronic and optical devices, antibiofouling agents, materials for solar energy conversion and cancer cell treatments [Garg et al., 2008; Shi *et al*., 2013]. CuNPs act as antimicrobial agent in various fields. The copper is highly toxic to microorganism such as bacteria and non-toxic to animal cells, due to these phenomena, it was considered to be an effective bactericidal metal and safe for human beings such as food package application and in water treatment. Cu and CuONPs have shown significant antimicrobial property against different bacterial pathogens [H. Raja Naika *et al*., 2015]. Copper nanoparticles have also shown considerable wound healing activity, hence it could be a potent material for wound healing [Tiwari M, 2014] Further, CuNPs could be a promising tool to replace expensive noble metal nanoparticles such as AgNPs and AuNPs [Luo *et al*., 2007; Khanna *et al*., 2011; Suo *et al*., 2013]. The high-performance copper nanostructures reliant on the protocol used for the production, in which a good control over particle's size, shape and spatial distribution is of utmost importance [A Kumar *et al*., 2013]. Hence, the development of new, simple and low-cost aproaches to boost CuNPs properties is essential in order to uphold the production of CuNPs at an industrial level.

1.7. Characterization of Nanoparticles

1.7.1. UV-Vis Spectroscopy

UV-Vis Absorption Spectroscopy gives UV absorption of the amorphous gels and crystalline ceramic samples heated at different temperatures [Thomas *et al*., 1996]. Many molecules absorb visible or ultraviolet light. An absorption spectrum will show some number of absorption bands corresponding to structural groups

within the molecule. When an atom or a molecule absorbs energy, electrons will be excited from their ground state to an excited state. In a molecule, the atoms can vibrate and rotate with respect to each other. These vibrations and rotations also have discrete energy levels, which can be treated as being packed on top of each electronic level. The absorption of Visible or UV radiation occurs to the excitation of outer electrons [Monalisa Pattanayat *et al*., 2013]. UV-Vis spectroscopy can help us to explore particular wavelengths, at which UV-Visible light absorption take place and to detect the optical property of nanoparticles.

1.7.2. Atomic Absorption Spectroscopy

It is a spectroanalytical procedure for the quantitative determination of chemical elements using the absorption of optical radiation (light) by free atoms in the gaseous state. AAS is based on absorption of light by free metallic ions. This technique is used for determining the concentration of a particular element (the analyte) in a sample to be analyzed. The technique basically uses the principle that, free atoms (gas) generated in an atomizer can absorb radiation at specific frequency. The atoms absorb UV or Visible light and make transitions to higher electronic energy levels. The analyte concentration is measured from the amount of absorption. AAS could be useful in nanotechnology, in detecting the concentration of ions present with the precursor solution, which in turn gives knowledge about nanoparticles formation.

1.7.3. X-ray Diffraction spectroscopy

X-ray diffraction (XRD) is the conventional technique for determination of crystallographic structure and morphology. There will be an increase or decrease in intensity of patterns with the amount of constituent. This technique is used to

determine the metallic nature of particles, which gives information on translational symmetry size and shape of the unit cell from peak positions and information on electron density inside the unit cell, namely where the atoms are located from peak intensities. The X-rays are generated by a cathode ray tube, filtered to produce monochromatic radiation, collimated to concentrate and directed towards the sample. It is based on constructive interference of monochromatic X-rays and a crystalline sample [Yelil Arasi *et al*., 2012].

1.7.4. Fourier Transform Infrared Spectroscopy

Measures infrared intensity vs wavelength of light, it is used to determine the nature of accompanied functional groups and structural features of biological extracts with nanoparticles. The resulted spectra clearly reflect the well-known dependence of nanoparticle optical properties. Also, one can use the unique gathering of absorption bands to confirm the identity of a pure compound or to notice the presence of specific impurities. FTIR trusts on the fact, that the most of the molecules absorb light in the infra-red region of the electromagnetic spectrum. This absorption corresponds exactly to the bonds present in the molecule. It also used to obtain an infrared spectrum of absorption or emission of a solid, liquid or gas. An FTIR spectrometer simultaneously collects high-spectral-resolution data over a wide spectral range [Amudha Murugan *et al*., 2014].

1.7.5. Scanning Electron Microscopy

The characterization of Scanning electron microscope analysis is employed to determine the size, shape & morphologies of formed nanoparticle. SEM gives high resolution images of the surface of a sample is desired. The scanning electron

microscope works with the same principle of optical microscope, but it measures the electrons scattered from the sample rather than photon. Because electrons can be accelerated by an electric potential, the wavelength can be made shorter than the one of photons, this makes the SEM capable of magnifying images up to 200.000 times. Measures the particle size and characterization, Conductive or sputter coated sample involved and the sensitivity down to 1nm [Asim Umer *et al*., 2012].

1.8. Biological applications of Nanoparticles

The wide variety of core materials available, coupled with tunable surface properties, make nanoparticles an outstanding platform for a broad range of biomedical and biological applications. Nanoparticles can be engineered with distinctive composition, shape, size and surface chemistry to empower novel techniques in a wide range of biological applications. Nanoparticles has gained immense response in the role of antibacterial, antifungal, antiviral, antihelminthic, larvicidal agents, etc. The main application involved in use of nanoparticles for biomedical applications, such as drug and gene delivery, cancer treatment and diagnostic tools, food etc., [Daisuke Kami *et al*., 2011; Liang Cheng *et al*., 2013] has been extensively studied throughout the past decade and also nanoparticle created a huge interest due to their very small size and large surface-to-volume ratio and they display absolutely novel distinctiveness contrast to the large particles of bulk material [Jitendra Mittal *et al*., 2014]. Very recently, nanoparticles have gained significance in biomedical field [Varahalarao Vadlapudi *et al*., 2013]. Nanoparticles have potential application in medical field including diagnostics and therapeutics [Rajshri *et al*., 2007; Heera *et al*., 2015]. Nanoparticles were also having huge range of applications in cell and molecular biology as sensors, analyte detection, pathogen

detection and separation, cell detection and separation, molecular imaging agents, delivery vehicles and to study biological processes [Edina *et al*., 2014].

1.9. Coconut (*Cocos nucifera*)

The coconut tree (*Cocos nucifera*) is a member of the palm tree family (Arecaceae) and the solitary living species of the genus *Cocos*. The term "coconut" (or the archaic "cocoanut") can refer to the whole coconut palm, the seed, or the fruit, which botanically is a drupe, not a nut. The term is derived from the 16th-century Portuguese and Spanish word *coco* meaning "head" or "skull" after the three indentations on the coconut shell that resemble facial features. Coconut belongs to Kingdom: Plantae, Order: Arecales, Family: Arecaceae, Subfamily: Arecoideae, Genus: *Cocos*, Species: *nucifera*. It is slender, leaning, ringed, the tree rises to a height of up to 80 feet from a swollen base and is surmounted by a graceful mature fruits, ovoid or ellipsoid in shape [Hugh C Harries, 1992].

The coconut palm is the most useful tree in the world. Coconut is deeply rooted in the culture, religion, environment, social status and diet to millions of people of tropics. Coconut is believed to be cultivated in more than 93 countries all over the world in an extent of 12.78 million hectares with an average annual production of 54 billion nuts. India, Indonesia, Philippines and Sri Lanka together account for 78% of total world production, India is the third largest producer of coconut (Asian and Pacific Coconut Community (APCC) Statistical Year Book 2014). The other important coconut producing countries are Thailand, Bangladesh, New Guinea, Vietnam, Papua, Brazil, Myanmar, Tanzania and Mozambique. India and Sri Lanka hold a unique position in having the highest productivity of 7,000

nuts per hectare. India contributes to 15.53% in area and 22.34% in the production of coconut in the world (Horticulture Division, Dept. of Agriculture and Cooperation, Ministry of Agriculture, Government of India). It is a versatile tree in the World. In India it is cultivated in an area about 1.93 million hectare with an estimated production of 12,147 million nuts, has tremendous influence on the socio-economic property of million people. Coconut palm is dominant and essential component of the homesteads and garden lands along the coastal parts of southern India and it plays a vital role in the sociocultural and economic life of huge number of small and marginal farmers [Dagar *et al*., 2014]. Coconut tree has many economic importance as food, drink, health, shelter, medicine, fuel, aesthetics and wealth. Because of its versatile nature it is known as "Kalpa-Vriksha", "Tree of abundance", "Tree of Heaven", "Tree of Life", Nature's super market"', "King of palm", "Tree of virtues " and by many names. It is also known as lazyman's crop [Guptha *et al*., 2013; Loomba *et al*., 2013; Lima *et al.,* 2015].

1.10. Bud Rot disease for *Cocos nucifera*

Coconut production is limited by several factors, among them diseases play a major havoc role. Coconut crop diseases are a major biotic stress, play a great havoc causing considerable reduction in yield level. Due to microbial infections, there are several major diseases reported from our country, causing severe damage to the plant body such as Basal stem rot (Thanjore wilt), Bud rot, Leaf blight, Leaf rot disease, Root wilt and Stem bleeding [Nambiar 1994, Henry Louis 2002]. Among those Bud rot disease caused by *Phytophthora palmivora* was known to be severe of coconut palm. Palms of all ages were susceptible to bud rot disease, but the young

palms were observed to be more susceptible, particularly during monsoon season when the temperature is low and humidity is very high.

Briton- Jones (1940) described the disease symptoms first. The first visible symptom is the withering of the spindle marked by pale colour. The spear leaf or spindle turns brown and bends over. Basal tissues of leaf rots quickly and can be easily detached from the crown. Spindle withers and droops down and one by one, the inner leaves also fall away, leaving only fully matured leaves in the crown. A foul smell is emitted by the rotting tissue [Nambiar, 1994]. The palms succumb to the disease with the death of the spindle [Briton Jones, 1940; Menon and Pandalai, 1958; Lingaraj, 1972]. Later, infection spreads to the older leaves, causing sunken leaf spots covering the entire leaf blade spreading both up and down. Spot margins are irregular and water soaked and when the leaves are unfolded, the characteristic irregular spots are conspicuous on the blade. In severely affected trees, the entire crown may rot and in few months the trees wilt. The heart leaf becomes wilts, chlorotic and collapses. The disease may spread to older, adjacent leaves and spathes, producing a dead centre with a fringe of living leaves. Light brown to yellow, oily, sunken lesions may be seen on leaf bases, stipules or pinnae. Internally, the tissues beneath the bud rot discolored pink to purple with a dark brown border. Infected nuts show brown to black necrotic areas with yellow border rising on the surface. Internally, they have a mottled appearance. Young nuts are highly susceptible to disease and fail to mature and then fall off from the tree. Older, infected nuts ripen normally [Anon, 2000; Srinivasulu, 2007]. Ultimately, the entire crown falls down and the palm dies. In the 1920s “budrot diseases” were identified in Jamaica, Puerto Rico, Africa, Peninsular Malaysia and the Philippines (Menon

and Pandalai, 1958). Later, it was also reported in India, Sri Lanka, Central America, the West Indies, Fiji and Vanuatu. Several practices like spraying chemicals, keeping the garden clean, application of talc powder formulation on rotten areas, etc., were made to avoid the spreading of bud rot disease [B Srinivasulu *et al*., 2008].

1.11. *Phytophthora palmivora*

Phytophthora is a genus of plant-damaging oomycetes (water molds), whose member species are capable of causing huge economic losses on crops worldwide, as well as environmental damage in natural ecosystems. *Phytophthora palmivora* is an oomycete which causes bud-rot of palms, fruit-rot or kole-roga of coconut and areca nut. These are among the most severe diseases caused by fungi and moulds in South India. . The causative organism was first identified as *Phytophthora palmivora* by Butler in 1917. *P. palmivora* belongs to Phylum: Heterokontophyta, Class: Oomycota, Order: Peronosporales, Family: Peronosporaceae, Genus: *Phytophtora*, Species: *palmivora.*

Phytophthora palmivora is an omnipresent pathogen which causes many different diseases on a wide range of plants. The pathogen was assumed to have originated in Southeast Asia but is now pantropical. It causes significant losses to farmers of tropical fruit and vegetable crops. *P. palmivora* infects a thousand or more plant species including horticultural, ornamental and agricultural crops. It is also a common soil inhabitant. Important horticultural hosts include cocoa (black pod, canker, cherelle wilt), papaya (fruit rot), durian (fruit rot, canker) pineapple (heart rot), citrus (canker), black pepper (foot rot) and coconut (bud rot). *P.*

palmivora grow vigorously under humid wet conditions and as a result can cause significant losses in many economically important tropical fruit crops [B Srinivasulu *et al*., 2008]. The main distinguishing feature of the pathogen includes conspicuous papillate sporangia, which can be distinguished from other *Phytophthora* species because they are caducous and have short pedicels. Primary inoculum originates from soil and infected plant material. The pathogen is circulated through rain splash, insects and human activity into the canopy of trees, where symptoms appear. Secondary inoculum spreads rapidly through wind and rain splash, contact and vector activity in humid weather [Drenth and Guest, 2004; R Ramesh *et al.,* 2013]. *Phytophthora palmivora* can be managed using cultural and chemical methods. The use of resistant varieties, sanitation (including complete harvesting and the removal of infected planting material and weeds), improved nursery hygiene, pruning to improve air flow and reduce humidity and boosting soil health by increasing organic matter can be used as part of an integrated management strategy. Chemicals used in the control of *P. palmivora* include metalaxyl, phosphonates or copper hydroxide to paint cankers and phosphonates as a soil drench, trunk injection or foliar spray.

1.12. Pectinases

Pectinases are group of enzymes that degrade the pectic substances, present mostly in plants. Microorganisms and plants mainly synthesizes these group of enzymes. Discovery of pectinases enzymes causes revolution in commercial and economic sector. Depending upon their production, pectic enzymes nature may be divided into alkaline or acidic. Acidic pectinases are produced by eukaryotic organisms, while alkaline are produced by both prokaryotic and eukaryotic microorganisms [Hoondgal *et al*., 2002; Jayarani *et al*., 2005]. Yeasts has distinct

role in the production of pectinases among eukaryotes [Blanco *et al*., 1999; Alimardani-Theuil *et al*., 2011]. Pectinase enzymes can be defined as a heterogeneous enzymes group that hydrolyzes pectic substances, which is the substrate molecule of the same. Pectic substance is a polymer of chain molecules made of rhamnogalacturonan backbone, which is linked with carbohydrates and other polymers [Jayarani *et al*., 2005]. Pectic substance is the common name used to speak of four types of molecules: pectins, pectic acids, pectinic acids and protopectin. Protopectinases are not soluble in water, while others are absolutely or partially soluble [Alkorta *et al*., 1998]. Several pectin rich substrates such as; Citrus peel [Sathvinder *et al*., 2004], Lemon peel [Larios *et al*., 1989], wheat bran [Tara gano *et al*., 1997], coffee pulp [Boccas *et al*., 1994], apple pomace [Hours *et al*., 1988], sugar cane bagasse [Solis-Pereyra *et al*., 1993, 1996] and deseeded sunflower head [Patil, 2004] are using for the microbial pectinases production in both solid-state and submerged conditions.

Pectinolytic enzymes are of noteworthy advantageous in current biotechnological era with their wide-ranging applications in fruit juice extraction and its clarification, degumming of plant fibers, scouring of cotton, vegetable oil extraction, bleaching of paper, Coffee, waste water treatment, cocoa and tea fermentations, in poultry feed additives and in the alcoholic beverages and food industries [Rashida Banu *et al*., 2010; Kubra *et al*., 2018]. Pectinase are now a fundamental part of textile and fruit industries [Kashyap *et al*., 2001] for breaking of tea leaves [Angayarkanni *et al*., 2002], dispensation of cotton fabric along with various biotechnological applications [Alkorta *et al*., 1998; Jacob *et al*., 2006].

Pectinase have shared about 25% in global sales of food enzymes [Rashida Banu *et al*., 2010].

1.13. Cellulases

Cellulases are the enzymes that hydrolyze β -1,4 linkages in cellulose chains. Fungi, bacteria, protozoans, plants and animals produces cellulase enzymes. The catalytic modules of cellulases have been classified into numerous families based on their amino acid sequences and crystal structures. Based on their amino acid sequences, the catalytic modules of cellulases have been classified into numerous families [Henrissat, 1991]. Cellulases contain some noncatalytic carbohydrate-binding modules (CBMs) and/or some functionally known or unknown modules, which might be located at the N- or C- terminal of a catalytic module. In general, complete cellulose (substrate) hydrolysis is facilitated by three main types of cellulases: (1) endoglucanases, (2) exoglucanases, including cellobiohydrolases (CBHs) and (3) β -glucosidase (BG). Cellulose is the most profuse renewable biological source and a low-cost energy basis based on energy contented [Lynd *et al*., 2008; Zhang, 2009]. The production of bioenergy and bio-based products from less expensive renewable lignocellulosic materials would fetch profits to the local economy, environment and national energy security [Zhang, 2008]. To metabolize and hydrolyze insoluble cellulose, the MO's requisite to secrete the cellulases that are either free or cell-surface-bound. Cellulases are progressively being used for a large sections of industrial purposes, in the use for textile and food industry, pulp and paper industry, as well as an additive in detergents and in the improvement in the digestibility of animal feeds. Cellulases are the third most significant industrial

enzyme due to its multipurpose advantages in various industries such as textile, paper and pulp and detergent industry. Now cellulases account for a significant share of the world ' s industrial enzyme market. [Himmel *et al*., 1999; Zaldivar et al., 2001; Bayer *et al*., 2007].

1.14. Enzyme production

The optimizations of fermentation conditions, particularly chemical and physical parameters are significant in the improvement of fermentation processes due to their influence on the practicability and economy of the process [Francis *et al*., 2003]. The role of different factors, including pH of the medium, temperature, carbon and nitrogen source, inoculum age, aeration and agitation have been studied for pectinase and cellulase production. Enzyme production from fungi is more beneficial as the enzyme production rate is much higher in comparison with other microorganisms [Gupta *et al*., 2015]. Due to ease of control of different parameter such as, temperature, pH, aeration and oxygen transfer and moisture, submerged fermentation (SmF) has been traditionally used for the production of industrially important enzymes [Couto and Sanroman, 2006; Gangadharan *et al*., 2008].

1.15. Partial Purification of Enzymes

Enzyme purification is of great importance in acquiring knowledge about structural and functional properties and to prefigure its applications. The main objective behind deciding the strategy for purification is to obtain the utmost possible yield of the desired enzyme with the highest catalytic activity and the greatest possible purity. Most of the purification methods employed in the laboratory

research can be easily scaled to industrial processes [Pratima Bajpai, 2014; Anil Prakash *et al*., 2015]. Industrial enzymes produced in bulk, generally require little downstream processing and hence were relatively crude preparations. Laboratory scale partial purification for enzymes includes various processes such as, Ammonium sulfate precipitation, Dialysis and Ion exchange chromatography. Results were analyzed for each step by performing SDS PAGE.

1.15.1. Ammonium sulfate precipitation

Ammonium sulfate precipitation is one of the most universally used methods for large and laboratory scale purification of protein and fractionation, that can be used to separate proteins by varying their solubility in presence of high salt concentration. Ammonium sulfate is an inorganic salt with a high solubility that disassociates into ammonium (NH_4^+) and sulfate (SO_4^{2-}) in aqueous solutions. Ammonium sulfate is especially beneficial as a precipitant because it is highly soluble, stabilizes protein structure, has a relatively low density, readily available and is relatively inexpensive. As well as other neutral salts, ammonium sulfate stabilizes proteins by preferential solvation. Proteins differ markedly in their solubilities at high ionic strength, therefore, "salting out" is a very useful procedure to assist in the purification of the desired protein. The commonly used salt is ammonium sulfate, because it is very water soluble, forms two ions high in the Hofmeister series and has no adverse effects on enzyme activity. It is generally used as a saturated aqueous solution which is diluted to the required concentration, expressed as a percentage concentration of the saturated solution [Scopes R, 1993]. Ammonium sulfate precipitation is an advantageous technique as an initial step in protein purification because it enables quick and bulk precipitation of cellular proteins.

1.15.2. Dialysis

Dialysis is a separation technique that facilitates the removal of small and unwanted compounds from macromolecules in solution by passive and selective diffusion through a semi-permeable membrane. A dialysis membrane is a semi-permeable film (regularly a sheet of regenerated cellulose) containing various sized pores. Larger molecules than the pores cannot pass through the membrane but small molecules can do so easily. In this manner, dialysis may be used to perform buffer exchange or purification for samples containing macromolecules. Dialysis works by diffusion, a process that results from the random movement of molecules in solution and leads to the net movement from the areas of higher concentration to lower concentration (until an equilibrium is reached). Unwanted molecules present inside a sample-chamber diffuses through a semi-permeable membrane into a second chamber of liquid or dialysate. Due to large size of large molecules, they cannot pass through the pores of the membrane, they will remain in the sample chamber. By contrast, the small molecules will easily diffuse across the membrane and attain equilibrium across the entire solution volume, effectively reducing the concentration of those small molecules within the sample [Walker JM, 2009].

1.15.3. Ion exchange Chromatography

Ion exchange Chromatography is a chromatographic technique that separates ions and polar molecules based on their affinity to the ion exchanger. It works on almost any kind of charged molecule, including small nucleotides, large proteins and amino acids. The two forms of ion exchange chromatography are anion-exchange and cation-exchange. Anion-exchange chromatography is used

when the molecule of interest is negetively charged. Cation-exchange chromatography is vice versa and when the stationary phase is negetively charged and the molecule is positively charged. It works on the principle that, it separates molecules based on particular charged groups, as it preserves analyte molecules on the column based on the coulumbic (ionic) interactions.

Protein purification is possible in ion exchange because most proteins bear non-zero net electrostatic charges at all pH except at pH=pI (isoelectric point). At a pH >pI of an interested protein, that protein becomes negatively charged (an anion), at the pH<pI of that same protein, it becomes positively charged (a cation). Ion exchange chromatography occurs due to electrostatic attraction between oppositely charged binding sites on a solid ion exchange adsorbent and a buffer-dissolved charged proteins. An ion exchange adsorbent (also called media, resin, gel or matrix) usually comprises of spherical porous inert beads with charged groups (functional groups) densely embedded onto the beads surfaces; the charges of functional groups are neutralized by free counter-ions [Ninfa *et al*., 2010].

1.15.4. SDS-PAGE

The process of macro-molecules separation in an electric field is called electrophoresis. A very usual method for separating proteins by electrophoresis uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins. The method is called as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The most frequently used system is also called the Laemmli method after U.K. Laemmli, who

was the first to publish an article employing SDS-PAGE in a scientific study [Laemmli U. K, 1970].

SDS (also called as lauryl sulfate) is an anionic detergent, meaning that when dissolved its molecules have net negative charge within a wide range of pH. A polypeptide chain binds some amounts of SDS in proportion to its relative molecular mass. The negative charges on SDS destroy most of the complex structure of proteins and are strongly attracted towards anode (positively-charged electrode) in an electric field. Polyacrylamide gels lock up larger molecules from migrating as fast as smaller molecules. Because the charge-to-mass ratio is almost the same among SDS-denatured polypeptides, the final separation of proteins is dependent almost wholly on the differences in relative molecular mass of polypeptides. Protein separation by SDS-PAGE can be used to approximate relative molecular mass, to determine the relative abundance of major proteins in a sample and to determine the distribution of proteins among fractions. The purity of protein samples can be judged and the progress of a fractionation or purification procedure can be followed [Smith BJ, 1984].

1.16. Inhibition of Enzymes using nanoparticles

Enzyme activity can be affected by other molecules, an enzyme inhibitor is a molecule, which binds to enzymes and decreases their activity. Since blocking an enzyme's activity can kill a pathogen or correct a metabolic imbalance. Many drugs and poisons are enzyme inhibitors. Activity is also affected by temperature, pressure, chemical environment (eg. pH) and the concentration of substrate. In general, the blocking or stopping of the enzyme action is called as enzyme

inhibition. Indeed, both natural and synthetic inhibitors are available that can be effectively used in therapeutics and as medicine for controlling harmful diseases mediated by specific enzymes. Enzyme inhibition is mainly of two types: reversible and irreversible. In both reversible and irreversible inhibition, the inhibitor need not cover the complete binding (active) site of the enzyme. Binding of the inhibitor to any part of the enzyme or the active site results in bringing a conformational change in the enzyme resulting in distortion of the active site of the enzyme due to which the substrate does not fit in the active site and the enzyme action is inhibited [Ahmed *et al*., 2016]. Nanoparticles (NPs) have some advantages over small organic molecules. First, NPs have large specific surface area for sufficient protein binding and biological interactions. Second, NPs can enter cells easily, in contrast to some small molecules and biological molecules. Third, there has been considerable progress in the synthesis of NPs with well controlled dimensions, geometry and surface properties, to complement the structural complexity of proteins. Recent developments in nanomaterials offer a new pathway for controlling protein behavior through surface interactions [Toogood *et al*., 2002; Gadek *et al*., 2003; Porter *et al*., 2007; Zhaochun *et al*., 2009].

There were so many methods employing for the synthesis of nanoparticles, among those chemical and physical synthesis are prominent, but which were found to be very hazardous to environment and very expensive. To overcome this, currently the novel biological synthesis methods for nanoparticles are dominating. Where in, biological synthesis method is cost effective and environmental friendly, so we have chosen biological synthesis method for the synthesis of nanoparticles using silkworm fecal matter as a reducing agent. The plenty of silkworm fecal

matter is available in the farmer's silkworm rearing houses and they are discarding it, without knowing the importance of fecal matter. From this discarded waste, we had set the biochemical technique to synthesize novel metal nanoparticles and made them easily available, in turn boosted the application of silk worm fecal matter. Nanomaterials were found to be more effective as antibacterial and antifungal agents and also emerged as enzyme inhibitors in recent days. Hence we have used the synthesized nanoparticles to inhibit the fungus causing bud rot disease of coconut and cell wall degrading enzymes produced by the isolated pathogenic fungus. Coconut is one the major economic crop in India, a considerable amount of crop will be lost every year due to bud rot disease. So we have undertaken inhibition studies of synthesized nanoparticles against bud rot disease causing fungi and its enzymes using biologically synthesized novel metal nanoparticles, which would led to develop a new drug and treatment for bud rot disease of coconut. This would in turn may helpful for uplifting of coconut growing farmers economically.

1.17. Objectives of Present Study

- ✓ Synthesis of novel nanoparticles using Silk worm fecal matter extract and their characterization.
- ✓ Bioactivity assays of synthesized nanoparticles.
- ✓ Pathogenic fungus will be screened for different cell-wall degrading enzymes like cellulases, pectinases, xylanases, etc.
- ✓ Validation of above enzymes inhibition using synthesized novel nanoparticles.

Chapter 2

Materials and Methods

2.1. Synthesis, Characterization and biological activities of nanoparticles using silkworm fecal matter

All the materials used in the present study were used as received. Silk worm fecal matter required for the synthesis of nanoparticles was bought from silk worm rearing houses situated in place called Kachinakatte near Shivamogga (Dt), Karnataka, India. Fecal matter was collected, cleaned, shade dried and stored until use. Bacterial strains used for the antibacterial studies were bought from Department of Microbiology, Kuvempu University, Shankarghatta, Shimoga, Karnataka, India. Chemicals used in the study were of analytical grade and highest possible purity. They were purchased from Hi-Media Chemicals (Mumbai, Maharashtra, India), Merck Chemicals (Mumbai, India) and Sigma Chemical Company (St. Louis, Mo. USA).

2.1.1. Preparation of silkworm fecal matter aqueous extract

The Silkworm fecal matter extract used for the synthesis of nanoparticles was prepared from 20gram of thoroughly washed fecal matter. The mixture of fecal matter and 100 ml of double-distilled deionized water in a 250ml Erlenmeyer flask was boiled for 10min in a hot plate at about 80^0C. The mixture was then filtered and centrifuged at 8000rpm for 20min. This solution was stored at 4^0C and used within 1 week.

2.1.2. Synthesis of Nanoparticles

2.1.2.1. Synthesis of Silver nanoparticles (AgNPs)

The Erlenmeyer flask (250ml) was used as the reaction vessel, whereby 10 ml of the aqueous extract of Silkworm fecal matter was made to react with 100 ml of 1mM Silver Nitrate (1mM $AgNO_3$). This setup was subjected to microwave irradiation for bioreduction process at the power rate of 600 watts for 10min and the colour change in the solution indicates the production of nanoparticles. The solution mixture was centrifuged at 10000rpm for 15min and the pellet was collected, further washed to remove impurities present along with the nanoparticles and it was stored for further use.

2.1.2.2. Synthesis of Copper nanoparticles (CuNPs)

1ml of Silkworm fecal matter extract was added drop-wise into 10ml of 1mM aqueous solution of Copper Sulfate (1mM $CuSO_4$) with constant stirring at 80–100^{0}C. Within few hours, the brown solution gradually shows greenish coloration which changed to dark after vigorous stirring for 24hr. The solution mixture was then centrifuged at 10000rpm for 15min and the pellet was collected. The pellet was washed to remove impurities present along with the nanoparticles and it was stored for further use.

2.1.3. Characterization of synthesized nanoparticles

2.1.3.1. UV-Vis Spectroscopy

To detect optical property and to identify the biosynthesized silver and copper nanoparticles. Samples were analyzed for UV–Vis spectroscopic studies (Eppendorf AG 22331, Hamburg) at room temperature operated between 300 to 650 nm range for AgNPs and 500 and 750nm range for CuNPs.

2.1.3.2. Atomic Absorption Spectroscopy (AAS)

Atomic Absorption Spectroscopy was done to study the conversion of $AgNO_3$ and $CuSO_4$ into AgNPs and CuNPs respectively (Thermo AA M5). Samples were withdrawn at various stages of reaction and were centrifuged at 6000 rpm for 10 minutes. The supernatant was subjected to AAS for concentration of Ag and Cu ions in the solution. Ions present in the solution are much smaller and hence would not be separated on centrifugation. Whereas AgNPs and CuNPs were in zero valent metallic form and can be easily separated by centrifugation.

2.1.3.3. X-ray Diffraction Measurements (XRD)

X-ray diffraction measurements of the biologically synthesized AgNps and CuNPs casted onto glass slides were done on a panAnalyticalX'pert pro X-ray diffractometer operating at a voltage of 40 kV and a current of 30 mA with Cu K (α) radiation. The scanning was done in the region of 10^0 to 80^0 at 0.02^0/min and the

time constant was 0.12 seconds in the speed of 10^0/min. Crystallite size was calculated using Debye Scherrer formula.

2.1.3.4. Fourier Transform Infrared Spectroscopy (FTIR)

For Fourier transform infrared spectroscopy measurements, the reaction mixture was centrifuged at 12,000 rpm for 15 min after complete reduction of $AgNO_3$ and $CuSO_4$ by the silk worm fecal matter extract to separate AgNPs and CuNPs. The AgNPs and CuNPs pellet obtained after centrifugation were re-dispersed in water and washed (centrifugation and re-dispersion) with distilled water for three times. Finally, the samples were dried and grounded with KBr pellets and analyzed on a FTIR spectrophotometer (Brucker alpha-T, Germany).

2.1.3.5. Scanning Electron Microscopy (SEM)

Scanning electron microscopy was performed to determine the size and shape of biosynthesized AgNPs and CuNPs. The samples were first sonicated for 15 min and a drop of the solution was loaded on carbon-coated copper grids and solvent was allowed to evaporate under light for 30 min. SEM measurements were performed on VEGA3 model instrument operated at an accelerating voltage at 15.00 kV with 1000X magnification.

2.1.4. Biological activities of synthesized nanoparticles

2.1.4.1. Antibacterial activity

2.1.4.1.1. AgNPs

Biosynthesized AgNPs were investigated for their antibacterial activity against gram-negative *Salmonella typhi* and gram-positive *Staphylococcus aureus*. The AgNPs were dispersed in autoclaved deionized water by ultra-sonication and aqueous dispersions of AgNPs of various concentrations (5, 20, 50, and 100 µg/ml) were made. Stock cultures of *S. typhi* and *S. aureus* were grown separately in liquid nutrient broth medium (containing (g/l): NaCl- 5g, peptone- 10g, and beef extract-2.5g). After incubation of 24 hours, bacterial cultures (50 cells/ml) were added to 25 ml of nutrient broth containing different concentrations of AgNPs in each flask to observe the bacterial cell growth at 37^0C and $AgNO_3$ was taken as a control. Flasks were kept on incubator cum shaker at 150 rpm/min. The growth of bacterial cultures was measured using UV-Vis spectrophotometer (Eppendorf AG 22331, Hamburg) at 600 nm for every 6 hr up to 24 hr. The Minimum Inhibitory Concentration (MIC) of biosynthesized AgNPs was determined as the lowest concentration that inhibited the visible growth of the used bacterium.

2.1.4.1.2. CuNPs

Biosynthesized CuNPs were investigated for their antibacterial activity against gram-negative (*Salmonella typhi* and *Klebsiella pneumonia*) and gram-positive (*Bacillus subtilis*) bacterial cultures. These nanoparticles were dispersed in

autoclaved deionized water by ultra-sonication and aqueous dispersions of CuNPs of various concentrations (10, 25, 50, and 100 µg/ml) were made. To cultivate the bacterial cultures, nutrient agar (containing (g/l): NaCl- 5g, peptone- 10g, and beef extract- 2.5g, Agar- 20g) was prepared and used. After solidification of medium, the disks containing synthesized nanoparticles in different concentration was placed. Autoclaved deionized water soaked discs were used as control. Petri dishes were incubated for 24hours at 37°C. Antibacterial capacity of the copper nanoparticles was measured by standard Zone of Inhibition assay (ZOI).

2.1.4.2. Antioxidant Assays of AgNPs

2.1.4.2.1. DPPH Free Radical Scavenging Assay

DPPH (1,1-Diphenyl-2-picrylhydrazyl) free radical scavenging potential of the AgNPs, were determined using the modified method of Brand-Williams *et al*,. Varied concentrations (10, 20, 40, 60, 80 and 100 µg/ml) of AgNPs and standard Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2- carboxylic acid) were taken in different test tubes. In the above samples, 1 ml of freshly prepared 1mM DPPH was dissolved in methanol, added to the test material and vortexed thoroughly, then the solution was incubated in dark place for 30 min. The absorbance of stable DPPH was recorded at 517 nm. The DPPH (containing no sample) was used as a control. Free radical scavenging activity was expressed as the percentage of inhibition that was calculated using the following equation.

$$\text{DPPH Scavenging Activity (\%)} = \left(\frac{\text{Ac} - \text{As}}{\text{Ac}}\right) \times 100$$

Where, Ac — Absorbance of Control,

As — Absorbance of Sample.

2.1.4.2.2. Hydrogen Peroxide (H_2O_2) Scavenging Assay

The Hydrogen Peroxide scavenging activity was assayed by the modified method of Pick and Mizel. In brief, varied concentrations (10, 20, 40, 60, 80 and 100 µg/mL) of AgNPs and ascorbic acid (Vitamin C) which was taken as standard were mixed with 50 µL of 5mM H_2O_2 solution and incubated at room temperature for 20 min. The absorbance was measured at 610 nm. The percentage of H_2O_2 scavenging was calculated using the following equation.

$$\text{Hydrgen Peroxide Scavenging Activity (\%)} = \left(\frac{\text{Ac} - \text{As}}{\text{Ac}}\right) \times 100$$

Where, Ac—Absorbance of Control,

As—Absorbance of Sample.

2.1.4.3. Cytotoxic effect of biosynthesized nanoparticles

To determine the cytotoxic effect of silver and copper nanoparticles, cell viability study was carried out with the MTT reduction assay. A549 human alveolar basal epithelial cells (human lung adenocarcinoma cell line) were seeded in a 96-well plate at the density of 5×10^3 cells/well. The cells were allowed to attach and grown in 96-well plate for 24h, in 200 µl of DMEM with 10% FBS. After that, the

media were removed and replaced with the suspension of varied concentrations of AgNPs and CuNPs from 10 to 100 µg/ml and the cells were incubated for 48 h. After the addition of MTT (5 mg/ ml), the cells were incubated at 37°C for another 4 hr. The medium was then removed and 200 µl of DMSO added to each well. The optical density (OD) of the formazan product was read at 620 nm using multi-well spectrophotometer. The OD value was subjected to sort-out the percentage of viability by using the following formula,

$$\text{Percentage of Cell viability} = \left(\frac{\text{Ac} - \text{As}}{\text{Ac}}\right) \times 100$$

Where, Ac—Absorbance of Control,

As—Absorbance of Sample.

2.2. Isolation and identification of pathogenic fungus causing bud rot disease in coconut; inhibition studies using synthesized nanoparticles

2.2.1. Sample Collection

During rainy season, bud rot disease infected lesion samples were randomly collected from different regions of Chikkamagaluru districts. If the tissue is fresh and recently infected, isolation of fungus from plant tissue or coconut bud is quite simple. Successful isolation of pathogenic fungus from the disease tissue involves careful selection of freshly infected tissue, it is best to obtain it from actively growing lesions. Collected samples were placed in between the butter paper

or newspaper and placed in separate small sealed envelopes and then they were taken to laboratory and stored at 4^0C for further fungal isolation.

2.2.2. Isolation and identification of pathogenic fungus

The infected plant parts were surface sterilized by dipping in 70% ethanol for 30-60seconds or in 0.5-1% sodium hypochlorite for 3-5minutes, followed by rinsing with distilled water and blotting, then on sterile filter paper.

Primarily two media's were used for the isolation of pathogenic fungus and the composition is listed below:

Composition of Carrot Agar medium

Components	Quantity
Carrot	50gm
Dextrose	2gm
Ampicillin	20mg
Agar	2gm
d. H_2O	100ml

Composition of Potato Dextrose agar medium

Components	Quantity
Potato	20gm
Dextrose	2gm
Ampicillin	20mg
Agar	2gm
d. H_2O	100ml

The infected part of coconut bud was cut into small bits using sterile stainless steel blade, these bits were surface sterilized using sterile distilled water followed by washing in 0.1% mercuric chloride($HgCl_2$) and finally washed with

sterile distilled water (3 times; 1min interval). The surface sterilized bits were inoculated onto the above mentioned sterilized culture plates, the pH of both the medium was adjusted to 7 previously, the plates were covered with radiation resistive laboratory barrier film (Parafilm M) (PPP Company, Menasha, WI) to prevent drying of agar and to avoid contamination of medium and kept for incubation at 24 ±1ºC in biological incubator for 6-7 days. Then the grown colonies were observed under microscope for the identification of pathogenic fungi and subcultured to obtain pure culture of desired fungus. The pure cultures of the fungi were maintained in Potato Dextrose Agar medium (PDA) slants at 4ºC by sub culturing at 2-3 week intervals.

2.2.3. Molecular Identification of isolated fungus

The fungal genomic DNA was extracted from one week old culture maintained in PDA medium. Polymerase Chain Reaction (PCR) was performed with the final volume of 100µl containing approximately 100ng of DNA template, 10mM dNTPs, 400ng of each primers, 10X Chrom Taq DNA Polymerase assay buffer and 3U Taq DNA Polymerase enzyme.

Two universal fungal specific primers: 5'– GRAAGNAHADGTVGKAAYAWSG – 3' (forward) and 5'– TCCTNCGYTKATKGVTADGH – 3' (reverse) were used to amplify in a Biometra T personal thermocycler (Biometra GmbH, Goettingen, Germany). The initial denaturation at 95ºC for 300 sec, followed by 35 cycles of denaturation at 94ºC for 30 sec, primer annealing at 52ºC for 30 sec, elongation at 72ºC for 45 sec with final extension at 72ºC for 420 sec. The PCR products were run

on 1% agarose gel and visualized under UV light. The amplified 1.5 kb product was purified and directly sequenced on ABI 3500XL Genetic Analyzer using a mixture; 4 µl of Big Dye Terminator Ready Reaction Mix, 1 µl of Template, 2 µl of Primers and 3 µl Milli Q water as recommended by the manufacturer.

2.2.4. Inhibition of *Phytophthora palmivora* using biosynthesized nanoparticles

The antifungal activities of the biosynthesized nanoparticles were tested using radial growth technique [V. K. Bajpai *et al.*, 2007]. Petri dishes (9 cm diameter) containing 25 ml of PDA medium were used for anti-fungal activity assay, performed in solid media by disc diffusion method [Duru *et al.*, 2003]. Different concentrations (1mg, 2mg and 3mg/ml) of AgNPs and CuNPs were added to the medium immediately before it was poured into the petri dish. Control was also maintained without any nanoparticles and fungicide (Frucanazole). A diameter of 6 mm disc of *Phytophthora palmivora* was taken from 8 day culture of fungal strain and placed upside down in the centre of the Petri dish. The treatments were kept for incubation at 25ºC for 6-8 days, time by which the growth of control would have reached the edge of petri dish. The inhibition of fungal growth was calculated as percentage of inhibition of radial growth related to control. Percentage of mycelial growth inhibition was calculated using the following formula,

$$\text{Mycelial growth inhibition}(\%) = \left(\text{DC} - \frac{\text{DT}}{\text{DC}}\right) \times 100$$

Where, DC- average diameter of fungal colony of control.

DT- average diameter of fungal colony of treated.

2.3.1. Screening of pathogenic fungus for different cell wall degrading enzymes like Pectinases and Cellulases

Pectinase and Cellulase production of isolated fungus was determined by enrichment culture methods.

For the quantification of Pectinase enzyme, the media components used were 1%(w/v) pectin, 0.1%(w/v) K_2HPO_4, 0.1%(w/v) $CaCl_2$, 0.01%(w/v) $MgSO_4$, 0.01%(w/v) Nacl, 0.5%(w/v) $(NH_4)_2SO_4$, 0.01%(w/v) $FeSO_4$, 3.5%(w/v) Agar and 100ml distilled water.

For Cellulase enzyme quantification, the media component used were 1%(w/v) CarboxyMethylCellulose(CMC), 0.2%(w/v) $NaNO_3$, 0.05%(w/v) $MgSO_4$, 0.02%(w/v) $CaCl_2$, 0.002%(w/v) $MnSO_4$, 0.1%(w/v) K_2HPO_4, 0.1%(w/v) $FeSO_4$, 3.5%(w/v) Agar and 100ml distilled water.

The initial pH of the media was adjusted for three different flasks at 5, 6, 7, 8 and 9. 6 mm disc of the fungal organism were inoculated on to the plates after solidification of autoclaved media and the inoculated plates were incubated for 8 days. On 4^{th}, 6^{th} and 8^{th} days, the plates were removed and enzyme production was observed by flooding the plates with Gram's iodine solution or 1% Congo red solution (later the plates were detained by using 0.1M Nacl) for pectinase enzyme production and in another set Gram's iodine solution was used for the observation of clear zone around the colony for Cellulase enzyme production.

2.3.2. Optimization and Partial purification of Pectinase and Cellulase enzyme

2.3.2.1. Chemicals

All the chemicals used for the experiments were of analytical grade and chemicals were of the highest possible purity. For the preparation of culture media, PDA was purchased from Himedia laboratory (Mumbai, India). Other inorganic salts such as, NaCl, $MgSO_4$, $NaNO_3$, K_2HPO_4, $CaCl_2$, $FeSO_4$, $MgCl_2$ and $MnSO_4$ were purchased from Merck Chemicals (Mumbai, India) and Himedia laboratory (Mumbai, India). $(NH_4)_2SO_4$ was purchased from Himedia laboratory (Mumbai, India) and DEAE-Cellulose anion resin was purchased from Genei (Bangalore, India).

2.3.2.2. Optimization of broth conditions for Pectinase production

Optimization is a process of screening for improvements to a variety of enzyme parameters. The main goal of optimization here is to maximize one or more of the process specifications while keeping all others within their constraints.

For optimization of pectinase enzyme, the used media was composed of 1%(w/v) Pectin, 0.1%(w/v) K_2HPO_4, 0.01%(w/v) $MgSO_4$, 0.01%(w/v) NaCl, 0.5%(w/v) $(NH_4)_2SO_4$, 0.01%(w/v) $FeSO_4$, 0.01%(w/v) $CaCl_2$ and 100ml distilled water. 60ml of above mentioned media was autoclaved at 121ºC for 15 minutes and 5mm disc of *Phytophthora palmivora* culture was inoculated into it. The inoculated media was kept on incubator shaker at 120 rpm and the broth conditions for enzyme

production were optimized for Incubation time, pH, Temperature, Different substrates and Substrate concentration.

2.3.2.3. Preparation of Culture supernatant

Crude culture supernatant of Pectinase from *Phytopthora palmivora* was prepared using previously mentioned medium using 5% orange peel as a substrate. The optimal culture condition was maintained at 30°C, pH of the medium was adjusted to 6.0. After 6 days of incubation, liquid culture was centrifuged at 10,000 X g for 10 min at 4°C to separate the cells from the medium. The supernatant was stored at 4°C and used as enzyme source.

2.3.2.4. Estimation of protein concentration

Protein concentration was measured by the standard procedure of Bradford method (Bradford, 1976). A standard plot was developed using Bovine Serum Albumin (BSA) of different concentration and the protein concentration of test samples was determined for crude preparation and each steps of the purification.

2.3.2.5. Pectinase enzyme assay

The Pectinase activity was determined by a standard colorimetric method of Miller (1959) using 3, 5-dinitrosalicylic acid (DNS) and D-Galacturonic acid as standard. The enzymatic activity of supernatant was expressed as Unit per ml (U/ml), which was defined as the amount of enzyme, which liberates 1 μmole of reducing sugar per mL per minute under assay conditions.

2.3.2.6. Partial purification of Pectinase enzyme

The crude protein was concentrated by the slow addition of solid $(NH_4)_2SO_4$ to the culture supernatant with relaxed stirring to achieve the 75% precipitation level and kept overnight at 4°C. The precipitated supernatant was again subjected to centrifugation at 15,000 X g for 20 min at 4°C and the precipitate was suspended in 0.02M Citrate buffer. The dissolved protein was subjected to Dialysis against large volume of same buffer with continuous stirring overnight. It was possible to remove the left-over salt but also the substances of low molecular weight with dialysis step, buffer solution was renewed for every 4hours. The dialyzed protein was directly applied on DEAE-Cellulose anion resin (Genei, Bangalore, India) in 2 X 15 cm column with the liner increasing gradient of 0.1 to 1 M NaCl containing buffer. The column was previously equilibrated with Citrate buffer of pH 5.5 and the enzyme elution was done using the same buffer with the flow rate of 15 ml/hour. The enzyme activity and protein concentration was determined, the active fraction was dialyzed and the dialysate was concentrated by lyophilization.

2.3.2.7. Optimization of broth conditions for Cellulase production

For optimization of Cellulase enzyme, the used media was composed of 1%(w/v) Carboxymethylcellulose (CMC), 0.2%(w/v) $NaNO_3$, 0.02%(w/v) $MgSO_4$, 0.1%(w/v) $MnSO_4$, 0.1%(w/v) K_2HPO_4, 0.01%(w/v) $FeSO_4$, 0.02%(w/v) $CaCl_2$ and 100ml distilled water. 60ml of above mentioned media was autoclaved at 121ºC for 15 minutes and 5mm disc of *Phytophthora palmivora* culture was inoculated into it.

The inoculated media was kept on incubator shaker at 120 rpm and the broth conditions for enzyme production were optimized for Incubation time, pH, Temperature, Different substrates and Substrate concentration. .

2.3.2.8. Preparation of Culture supernatant

Crude culture supernatant of Cellulase from *Phytopthora palmivora* was prepared using the previously mentioned medium using 1% CarboxyMethylCellulose (CMC) as a substrate. The optimal culture condition was maintained at 32°C, pH of the medium was adjusted to 5.5. After 8 days of incubation, liquid cultures was centrifuged at 10,000 X g for 10 min at 4°C to separate the cells from the medium. The supernatant was stored at 4°C and used as enzyme source.

2.3.2.9. Estimation of protein concentration

Protein concentration was measured by the standard procedure of Bradford method (Bradford, 1976). A standard plot was developed using Bovine Serum Albumin (BSA) of different concentration and the protein concentration of test samples was determined for crude preparation and each steps of the purification.

2.3.2.10. Cellulase enzyme assay

The Cellulase activity was determined by a standard colorimetric method of Miller (1959) using 3, 5-dinitrosalicylic acid (DNS) and Glucose as standard. The enzymatic activity of supernatant was expressed as Unit per ml (U/ml), which was

defined as the amount of enzyme, which liberates 1 μmole of reducing sugar per ml per minute under assay conditions.

2.3.2.11. Partial purification of Cellulase enzyme

The crude protein was precipitated by the slow addition of solid $(NH_4)_2SO_4$ to the culture supernatant with relaxed stirring to achieve the 80% precipitation level and kept overnight at 4°C. The precipitated supernatant was again subjected to centrifugation at 15,000 X g for 20 min at 4°C and the precipitate was suspended in 0.05M Acetate buffer. The dissolved protein was subjected to Dialysis against large volume of same buffer with continuous stirring overnight. It was possible to remove the left-over salt but also the substances of low molecular weight with dialysis step, buffer solution was renewed for every 4 hrs. The dialyzed protein was directly applied on DEAE-Cellulose anion resin (Genei, Bangalore, India) in 2 X 15 cm column with the liner increasing gradient of 0.1 to 1 M NaCl containing buffer. The column was previously equilibrated with the Acetate buffer of pH 6 and the enzyme elution was done using the same buffer with the flow rate of 15 ml/hr. The enzyme activity and protein concentration was determined, the active fraction was dialyzed and the dialysate was concentrated by lyophilization.

2.3.2.12. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were analyzed using polyacrylamide gels containing SDS as denaturing agent essentially as described by Laemmli, (1970). Electrophoresis was carried out at 50 volts through the stacking gel and at 80-100 volts through the

resolving gel, using vertical mini slab gel unit filled with running buffer. After electrophoresis, gels were stained for 1 hr and destained until the clear visualization of protein bands. The apparent molecular weight of the partially purified enzymes was determined by comparing with standard protein marker.

2.4. Inhibition of Pectinase and Cellulase enzymes using synthesized nanoparticles

2.4.1. Inhibition of Pectinase enzyme activity

Enzyme inhibition activity was determined by using a reaction mixture of 1ml Crude Pectinase enzyme, 1ml of 1% Pectin substrate in 0.02 Citrate buffer and different concentrations(0, 20, 40, 60, 80 and 100µg/ml) of Silver and Copper nanoparticles was added separately to the reaction mixtures. These reaction mixtures were incubated at 40^0C for 20min, the reaction was stopped by adding 2ml of DNS and kept in boiling water bath for 10min. The absorbance was measured at 540nm. One unit (U) of enzyme activity was defined as the amount of enzyme causing the release of 1µmole of reducing sugars in 1 min under the assay conditions. The inhibition graph was plotted as Concentration of nanoparticles v/s Relative activity.

2.4.2. Inhibition of Cellulase enzyme activity

Enzyme inhibition activity was determined by using a reaction mixture of 1ml Crude Cellulase enzyme, 1ml of 1% CarboxyMethylCellulose (CMC) substrate in 0.05 Acetate buffer and different concentrations(0, 20, 40, 60, 80 and 100µg/ml) of Silver and Copper nanoparticles was added separately to the reaction mixtures.

These reaction mixtures were incubated at 35^0C for 30min, the reaction was stopped by adding 2ml of DNS and kept in boiling water bath for 10min. The absorbance was measured at 540nm. One unit (U) of enzyme activity was defined as the amount of enzyme causing the release of 1µmole of reducing sugars in 1 min under the assay conditions. The inhibition graph was plotted as Concentration of nanoparticles v/s Relative activity.

Chapter 3

Results

3.1. Synthesis, Characterization and Biological activities of nanoparticles using Silk worm fecal matter

The addition of 10ml aqueous extract of silk worm fecal matter to 100ml of $AgNO_3$ resulted in the colour change from light brown to dark brown after microwave irradiation for bioreduction. The colour change in the solution indicates the production of AgNPs. The yield of nanoparticles was relatively better in comparison of adding 5ml extract and the yield was found same with using 20 and 30 ml of extract. The size of nanoparticles were found to be higher with the increase in the molarity of $AgNO_3$ or increase in the quantity of solution. The size of nanoparticles synthesized using 1mM $AgNO_3$ were considerable. Hence, 10ml silk worm fecal matter and 100ml of 1mM $AgNO_3$ was used for the synthesis.

Similarly, with drop wise addition of 1ml aqueous extract of silk worm fecal matter to 10ml of $CuSO_4$ resulted in the colour change from brown to greenish after about 2 hrs with constant stirring at 80–100^0C, which changed to dark later with continuous stirring. Dark colour indicates the formation of CuNPs, the colour change was due to excitation of Surface Plasmon Resonance (SPR). As said in the previous result, there was no change in the yield of nanoparticles with using more quantity of silk worm fecal matter extract. The size of nanoparticles were found to be higher with the increase in the molarity of $CuSO_4$ or increase in the quantity of solution. Hence, for the synthesis 1ml silk worm fecal matter was used with 1mM $CuSO_4$.

3.1.1. Characterization of synthesized nanoparticles

3.1.1.1. UV-Vis Spectroscopy

AgNPs and CuNPs stretch a characteristic absorbance band due to the excitation mode of their surface plasmons. The SPR of silver and copper nanoparticles produced a peak centered near 412nm and 560nm for AgNPs and CuNPs respectively (Fig 3.1 a & b), which indicates the reduction of $AgNO_3$ and $CuSO_4$.

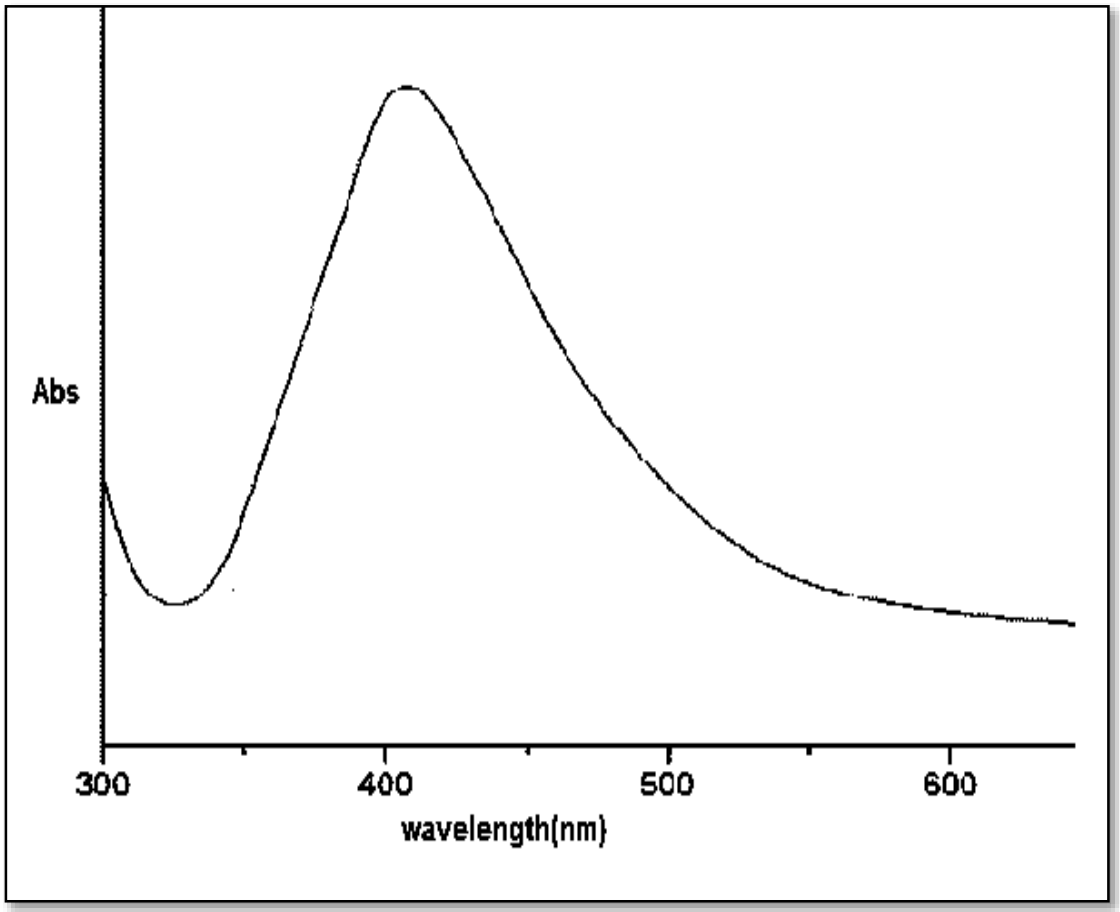


Fig 3.1 a- UV-Vis spectrum of Silver nanoparticles. UV-Vis spectrum taken in aqueous medium containing Silver nanoparticles shown maximum absorption at 412nm.

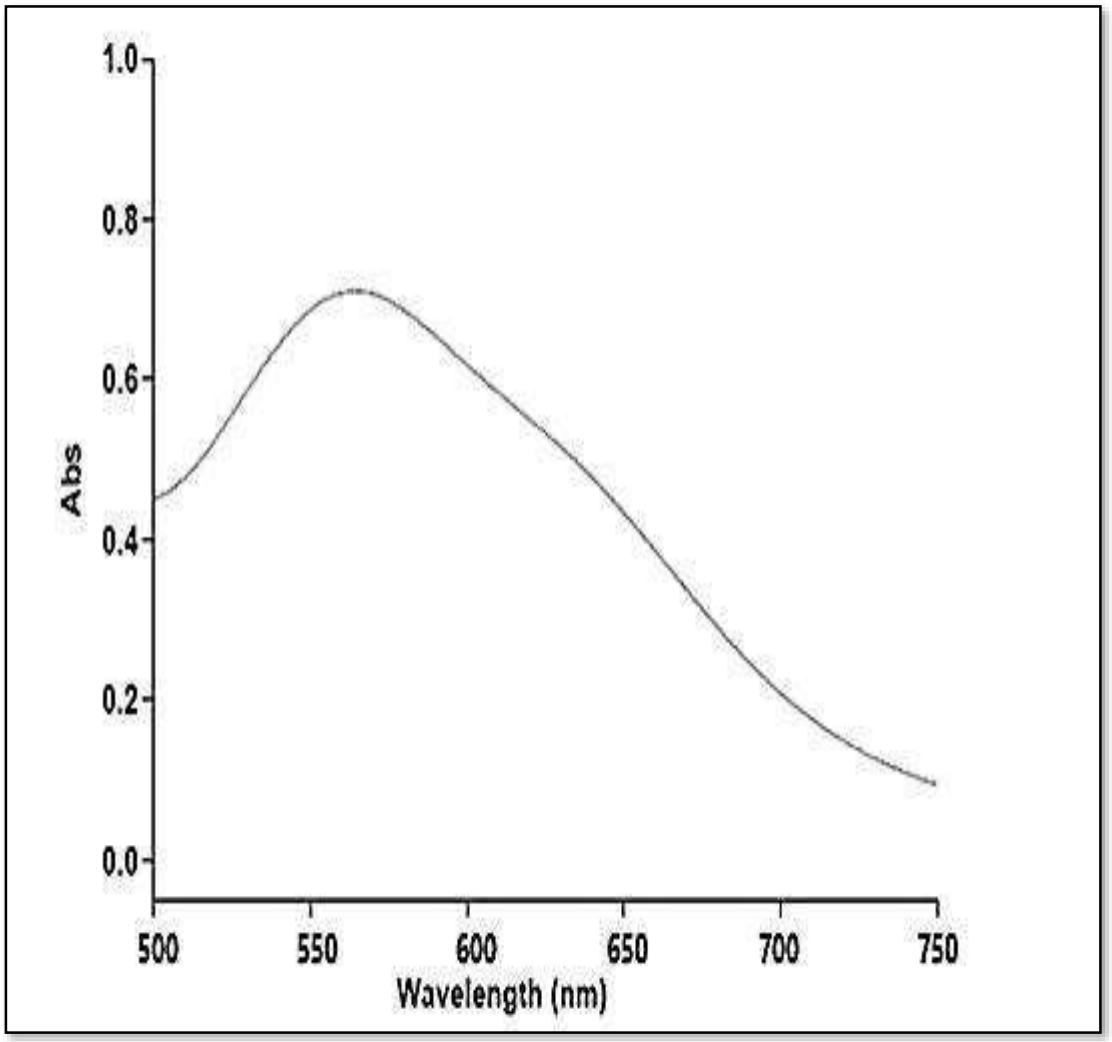


Fig 3.1 b- UV-Vis spectrum of Copper nanoparticles. UV-Vis spectrum taken in aqueous medium containing Copper nanoparticles shown maximum absorption at 560nm.

3.1.1.2. Atomic Absorption Spectroscopy

Silver and copper ion concentration was examined by AAS which showed the conversion of Ag ions into AgNPs and Cu ions into CuNPs. The results showed reduction in the concentration of Ag^+ ions (5.5, 4.41, 3.71, 3.08, 1.68, 0.73, and 0.08 ppm at 0, 2, 4, 5, 6, 7, and 8 min) and Cu^+ ions (5.25, 4.10, 3.73 and 2.75 ppm at 6, 12, 18 and 24hrs) indicating the conversion of Ag^+ ions into AgNPs and Cu^+ into CuNPs (Fig 3.2 a & b), which was due to bio-reduction capability of silkworm fecal matter.

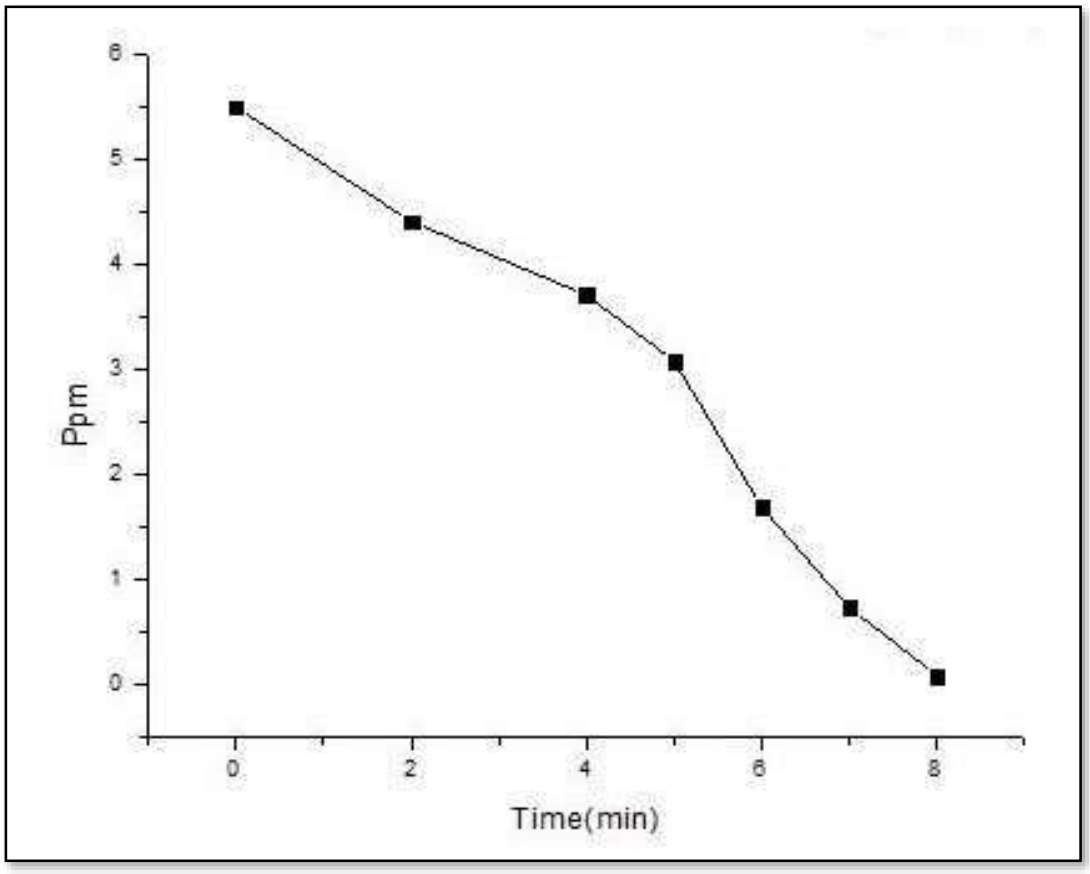


Fig 3.2 a- AAS of Silver nanoparticles. Concentration of Ag^+ ions was measured by withdrawing samples at different stages of reaction and subjecting supernatant to AAS. AAS graph showing decreased concentration of Ag^+ ions during biosynthesis of AgNPs.

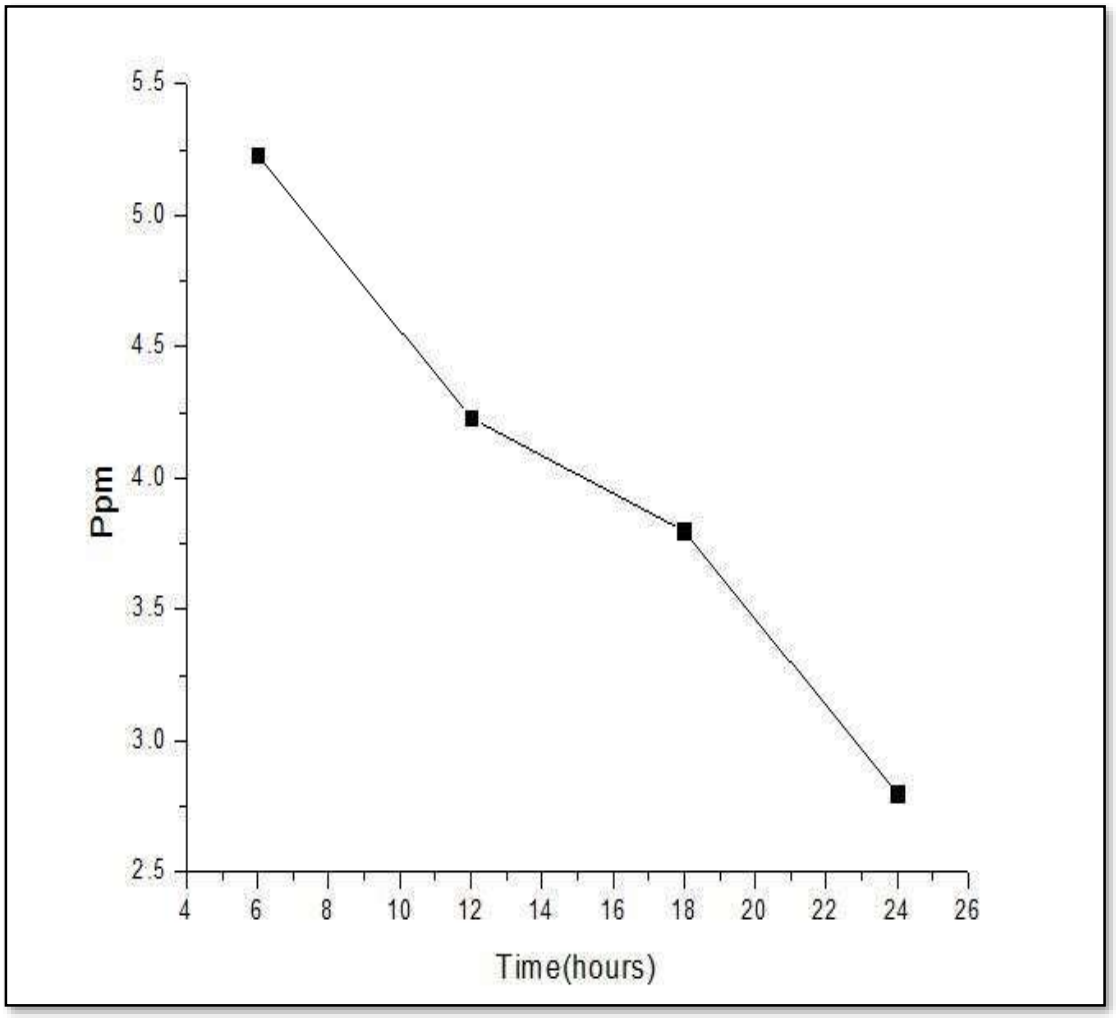


Fig 3.2 b- AAS of Copper nanoparticles. Concentration of Cu^{+} ions was measured by withdrawing samples at different stages of reaction and subjecting supernatant to AAS. AAS graph showing decreased concentration of Cu^{+} ions during biosynthesis of CuNPs.

3.1.1.3. X-ray Diffraction Measurements

The synthesis of silver and copper nanostructures by employing silk worm fecal matter extract was further demonstrated and confirmed by the characteristic peaks observed in the XRD pattern. The XRD analysis showed diffraction peaks corresponding to fcc structure of silver and copper, Intense peaks were observed in the whole spectrum of 2θ value ranging from 10 to 80 at 38.1800º, 44.3600º, 64.5600º and 77.5200º for AgNPs and 14.2380º, 20.0100º, 26.5620º and 32.1520º for CuNPs (Fig), corresponding to 111, 200, 220 and 311 Bragg's reflection, respectively. The typical XRD patterns (Fig 3.3 a & b) showed the sample contains a mixed phase (cubic and hexagonal) structure of AgNPs and CuNPs, the broadening of the Bragg peaks indicates the formation of nanoparticles. Full width at half maximum (FWHM) data were used with Debye-Scherrer's formula to determine the average particle size. The average particle size calculated was approximately 32nm and 34nm for AgNPs and CuNPs respectively.

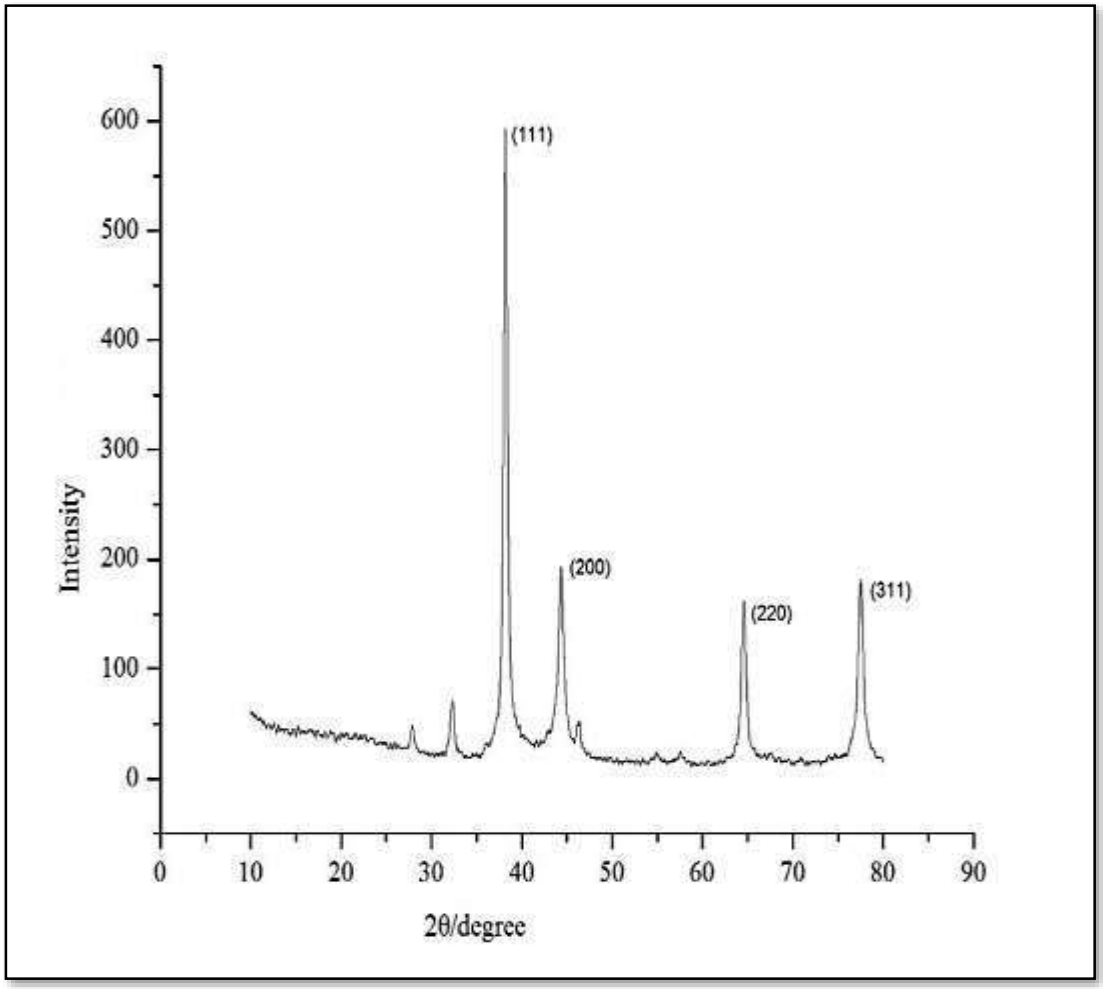


Fig 3.3 a- XRD pattern of biosynthesized Silver nanoparticles. XRD analysis of Silver nanoparticles casted on glass slide was operating at a voltage of 40 kV and a current of 30 mA and scanning was done in the region of 10^0 to 80^0.

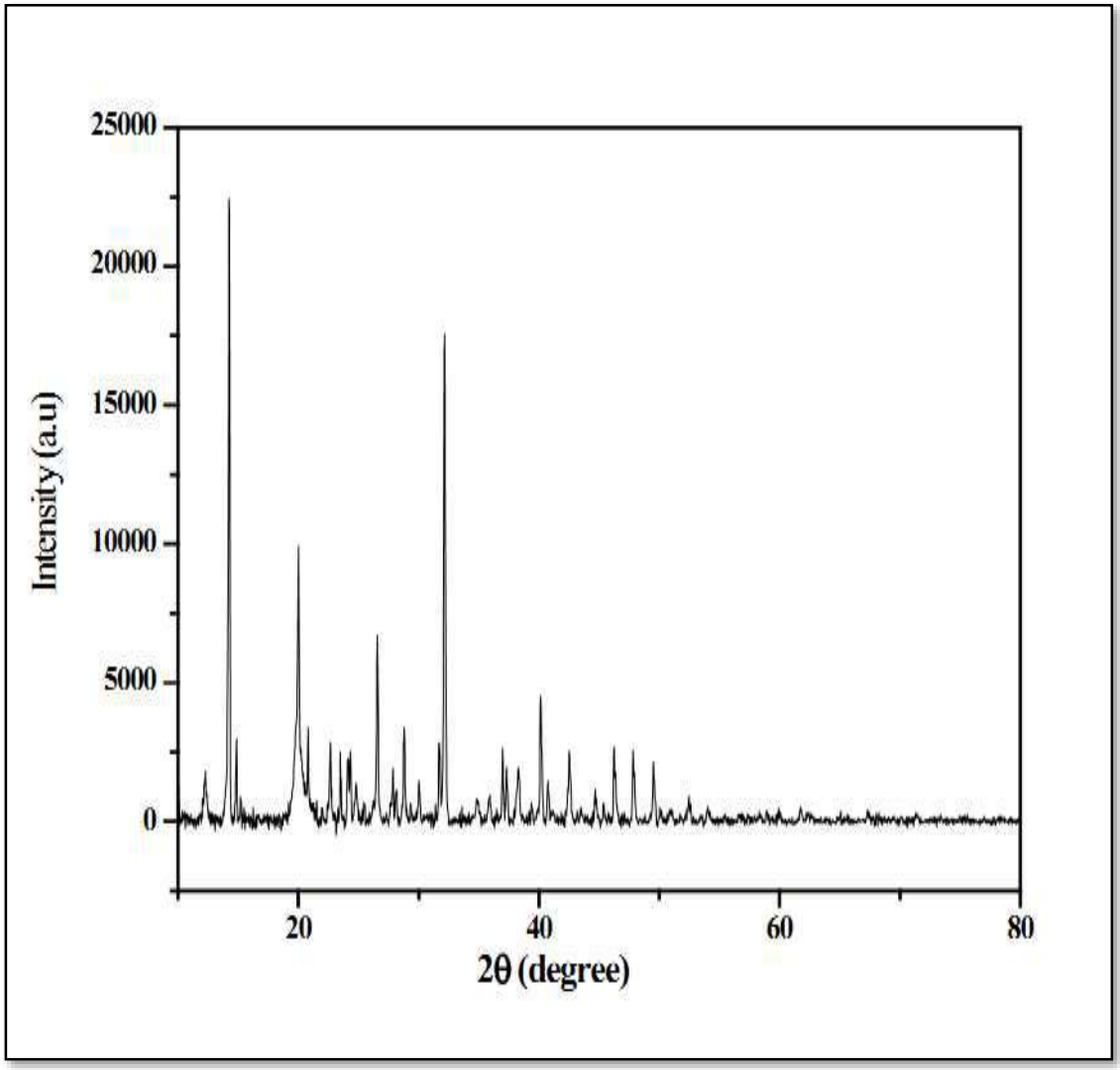


Fig 3.3 b- XRD pattern of biosynthesized Copper nanoparticles. XRD analysis of Copper nanoparticles casted on glass slide was operating at a voltage of 40 kV and a current of 30 mA and scanning was done in the region of 10^0 to 80^0.

3.1.1.4. Fourier transform infrared spectroscopy

Results of FTIR (Fig 3.4 a & b) study showed sharp absorption peaks located at about 3368.11, 2929.68, 1599.99, 1393.20 and 1078.49 cm^{-1} for AgNPs and 3383.40, 2926.56, 1639.89, 1405.69 and 1079.81 cm^{-1} for CuNPs. These absorbance bands were known to be allied with the stretching vibrations for range of functional groups like –C–H (alkane H), C-O stretch in esters, –C–C– [(in-ring) aromatic], –C–C– [(in-ring) aromatic] and C–O (polyols), respectively.

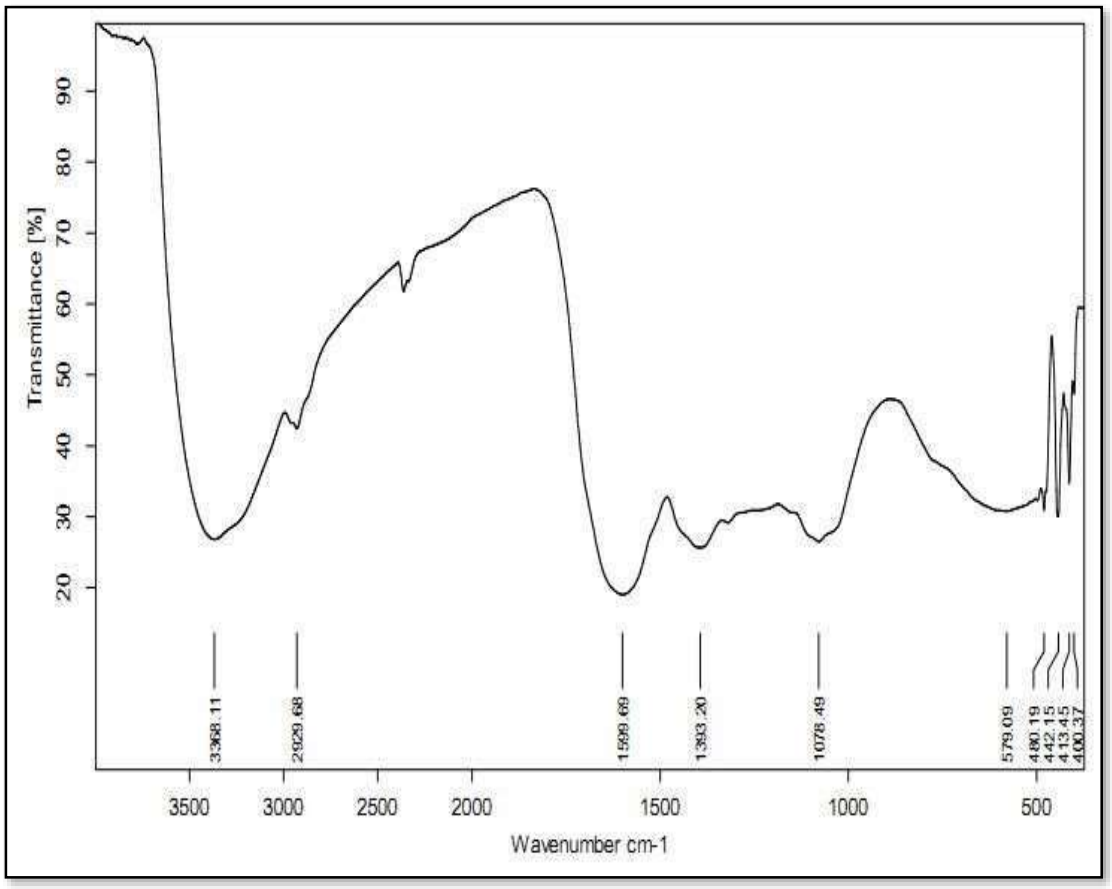


Fig 3.4 a- FTIR spectrum of Silver nanoparticles. FTIR spectrum was measured by grinding dried sample of synthesized silver nanoparticles with KBr pellet and analyzed on FTIR spectrophotometer.

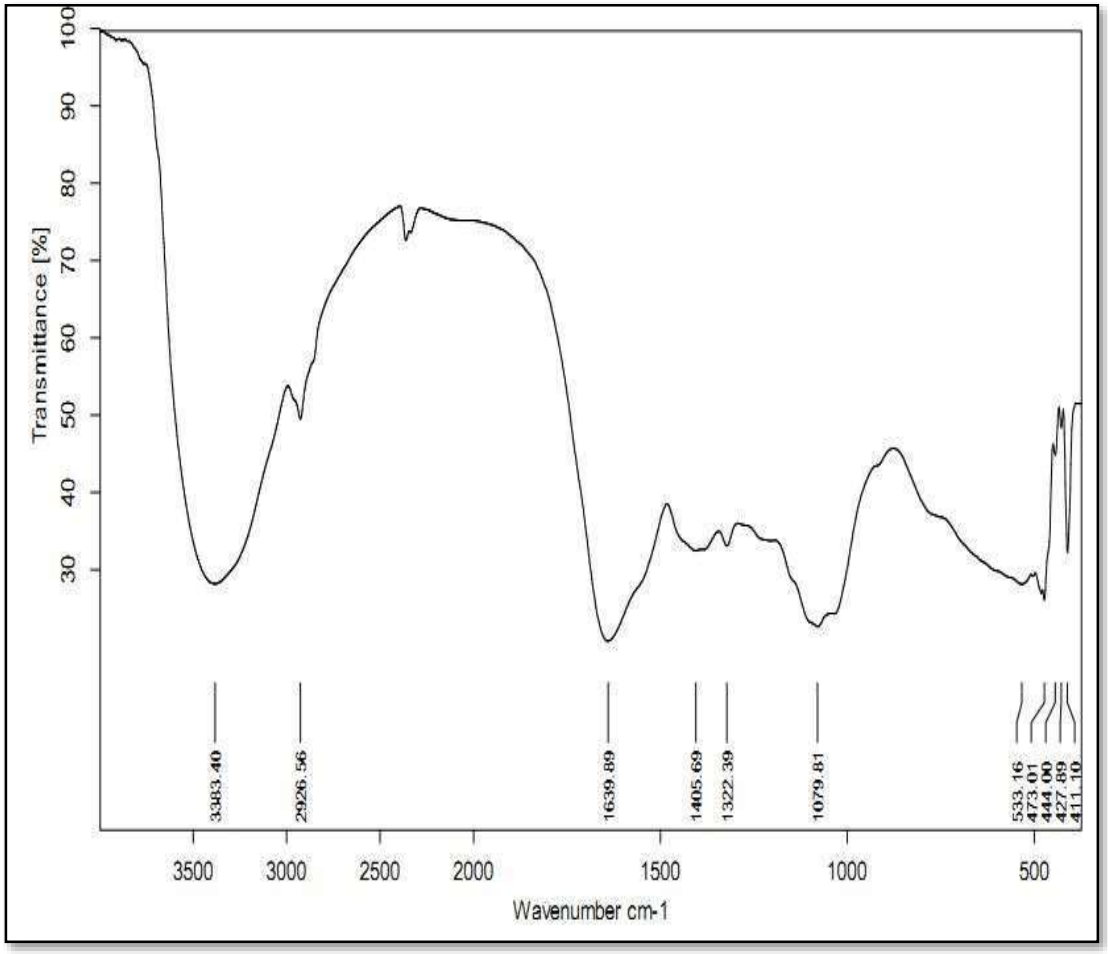


Fig 3.4 b- FTIR spectrum of Copper nanoparticles. FTIR spectrum was measured by grinding dried sample of synthesized Copper nanoparticles with KBr pellet and analyzed on FTIR spectrophotometer.

3.1.1.5. Scanning Electron Microscopy

Scanning electron microscopy provided further vision into the size and morphology details of the AgNPs and CuNPs. However, further observation with high magnification reveals that these Ag and Cu nanoclusters were assembled by smaller nanoparticles whose size distribution ranges from 21-80nm and 20-60nm for AgNPs and CuNPs respectively, which exhibits good uniformity and the shape was in mixed phase as shown in (Fig 3.5 a & b).

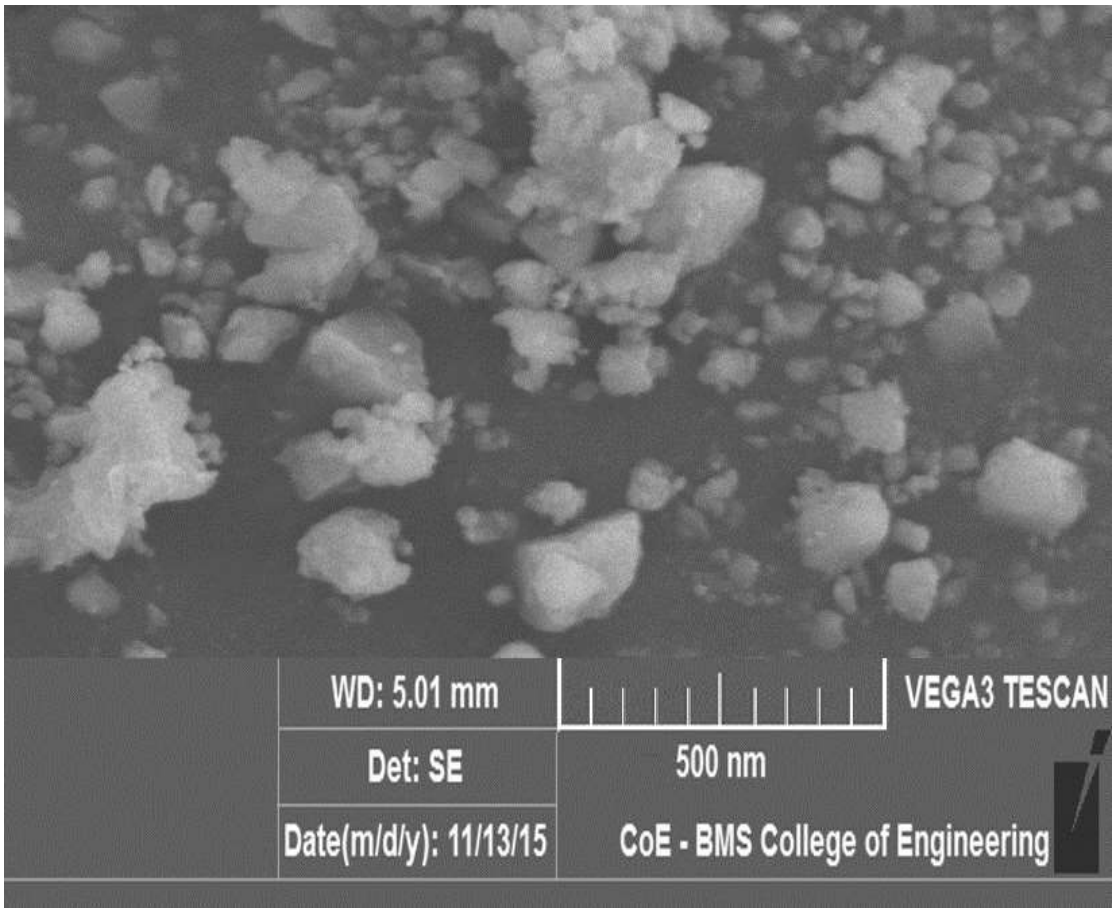


Fig 3.5 a- SEM image of Silver nanoparticles. SEM was performed by loading a drop of sonicated sample on carbon-coated copper grids and allowed to evaporate for 30min. It was carried out on VEGA3 model instrument operated at an accelerating voltage at 15.00 kV with 1000X magnification.

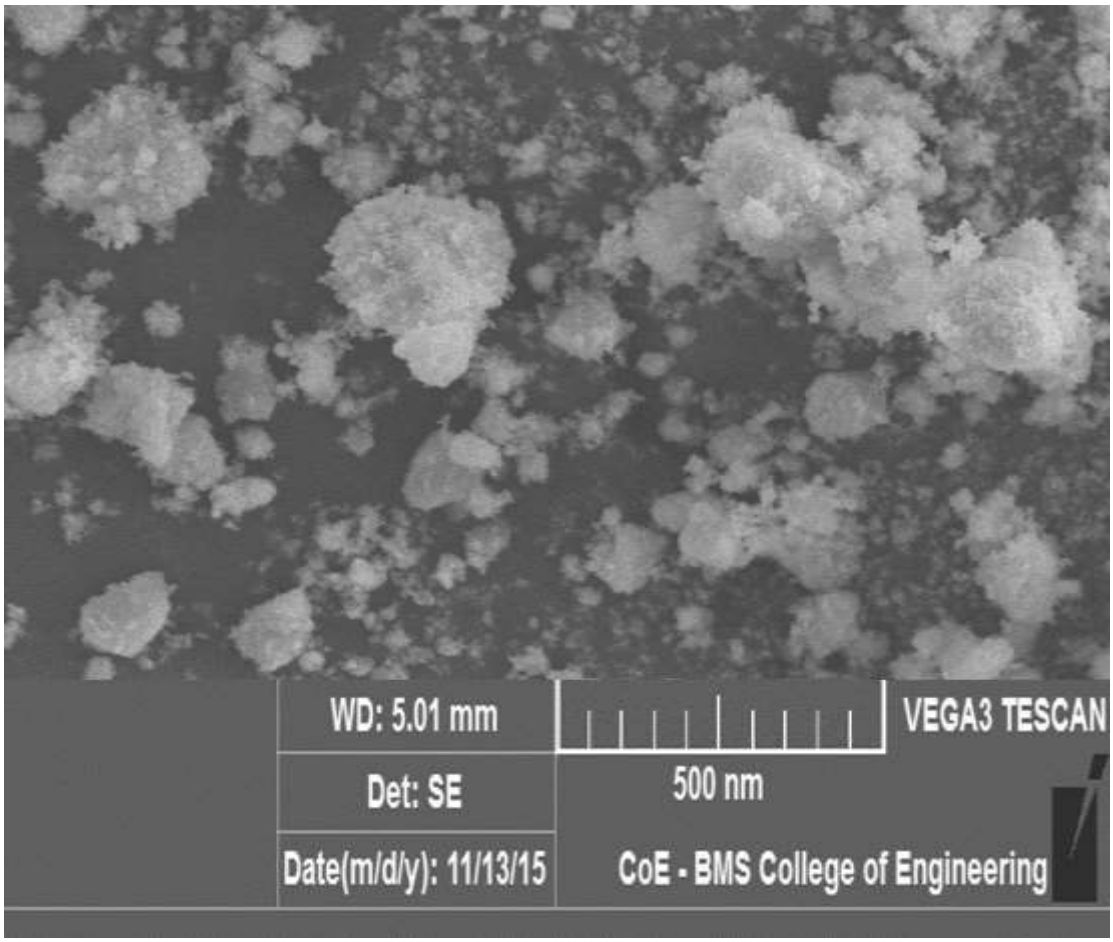


Fig 3.5 b- SEM image of Copper nanoparticles. SEM was performed by loading a drop of sonicated sample on carbon-coated copper grids and allowed to evaporate for 30min. It was carried out on VEGA3 model instrument operated at an accelerating voltage at 15.00 kV with 1000X magnification.

3.1.2. Biological activities of synthesized nanoparticles

3.1.2.1. Antibacterial activity

3.1.2.1.1 AgNPs

Antibacterial assay of biosynthesized AgNPs against both gram-negative (*S. typhi*) and gram-positive (*S. aureus*) microorganisms at different concentrations were showed strong dose-dependent antimicrobial activity (Fig 3.6 a & b). It was observed

that, as the concentration of biosynthesized nanoparticles increased, microbial growth decreases in both cases. Biosynthesized AgNPs were exhibited more antimicrobial activity on gram-negative bacteria than gram-positive one. The MIC of AgNPs against *S. typhi* and *S. aureus* is shown in (Table 4.1). From the antibacterial analysis important point was observed that antibacterial activity of AgNPs synthesized using Silkworm fecal matter was superior to silver nitrate as well as standard antibiotic Ampicillin.

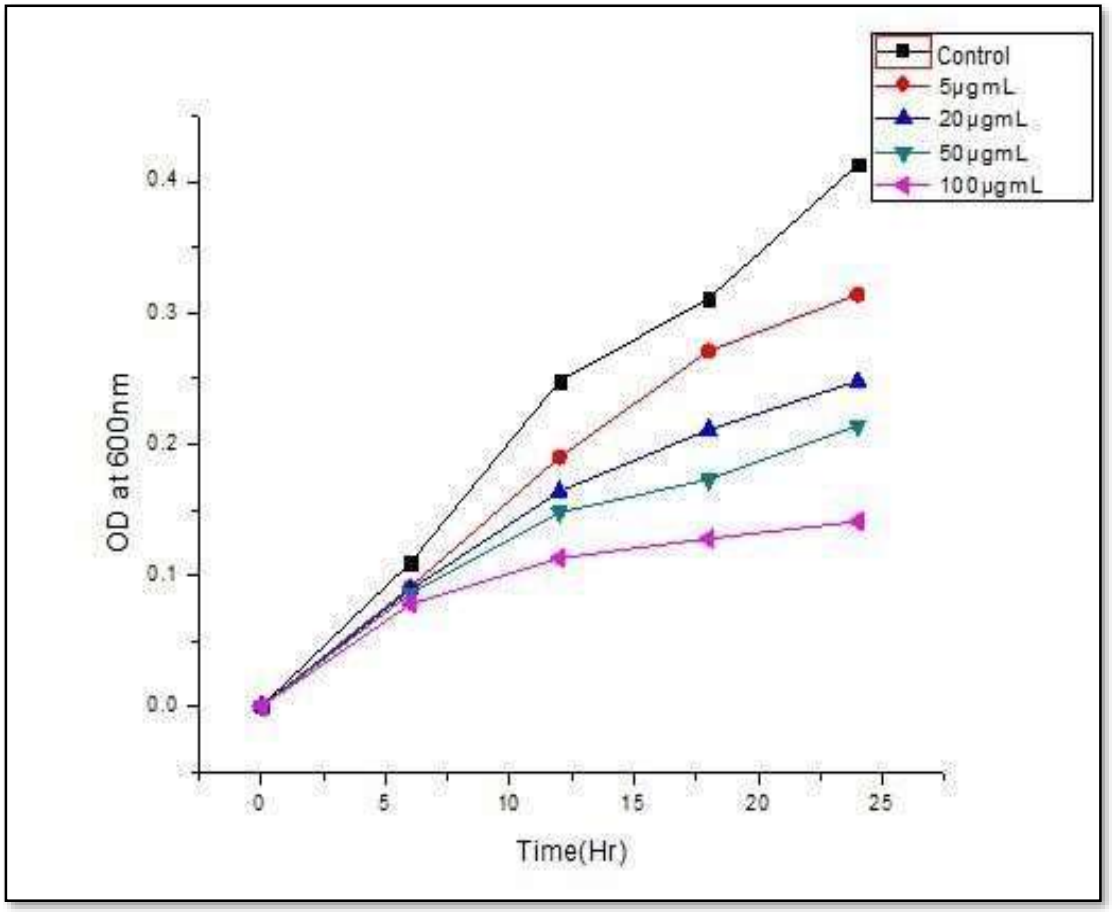


Fig 3.6 a- Antibacterial activity of AgNPs on growth of *S. typhi*.

The effect of Silver nanoparticles on growth of *Salmonella typhi* was conducted using liquid nutrient broth medium containing various concentrations of AgNPs. The growth of bacterial cultures was measured using UV-Vis spectrophotometer at 600 nm for every 6 hr up to 24 hr.

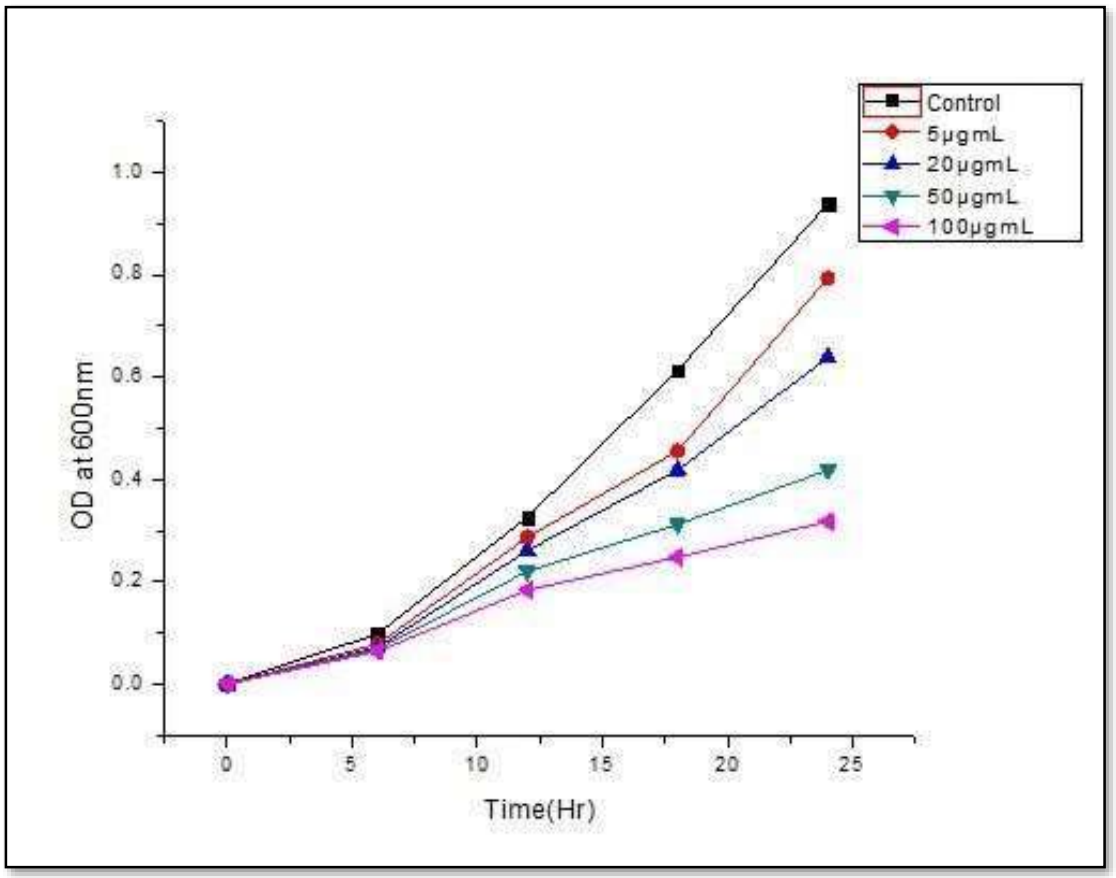


Fig 3.6 b- Antibacterial activity of AgNPs on growth of *S. aureus*. The effect of Silver nanoparticles on growth of *Staphylococcus aureus* was conducted using liquid nutrient broth medium containing various concentrations of AgNPs. The growth of bacterial cultures was measured using UV-Vis spectrophotometer at 600 nm for every 6 hr up to 24 hr.

Table 4.1- Minimum Inhibitory Concentration of biosynthesized Silver nanoparticles. MIC of AgNPs was determined as the lowest concentration that inhibited the visible growth of the used bacterium.

Minimum inhibitory concentration (µg/ml)			
Microorganisms	**Ampicillin**	**Silver Nitrate**	**Silver nanoparticles**
Salmonella typhi	6.25	1.25	3.14
Staphylococcus aureus	12.5	25	6.25

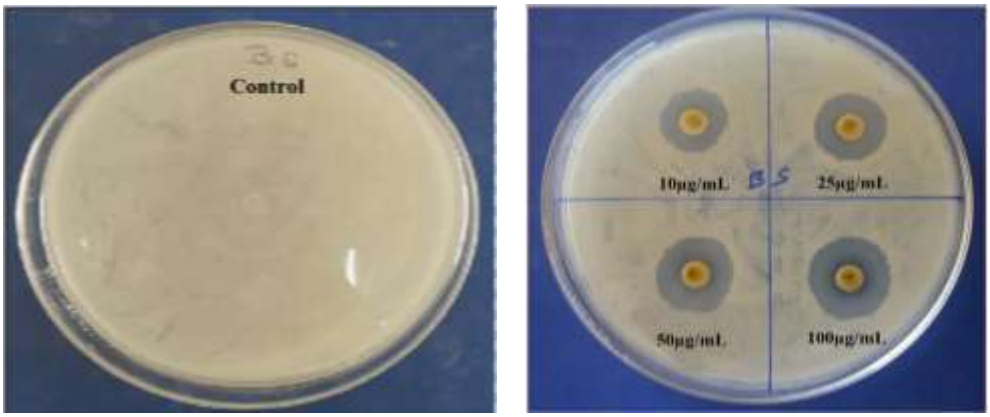
3.1.2.1.2. CuNPs

The antibacterial study of biosynthesized CuNPs were established against both gram negative and gram positive pathogenic bacteria such as *Bacillus subtilis* (gram positive), *Salmonella typhi* and *Klebsiella pneumoniae* (gram negative) using disk diffusion method as shown in Table 4.2 and Fig 3.7, shows the zone of inhibition (ZOI) of copper nanoparticles for different pathogens, while there was no zone observed for control. This result was effective, when the concentration of copper nanoparticles was increased, there was observed to be increase in the zone of inhibition. Conversely, the zone of inhibition was witnessed to be more in gram negative bacteria when compared to gram positive bacteria. This is mainly due to the differences in bacterial pathogen's membrane structures. The maximum ZOI values were observed as 11mm in *Klebsiella pneumoniae* bacteria for100µg/ml concentration of CuNPs shown in Table 1. The ZOI values observed by *Salmonella typhi* and *Bacillus subtilis* was found to be 10 and 8mm respectively as shown in (Table 4.2).

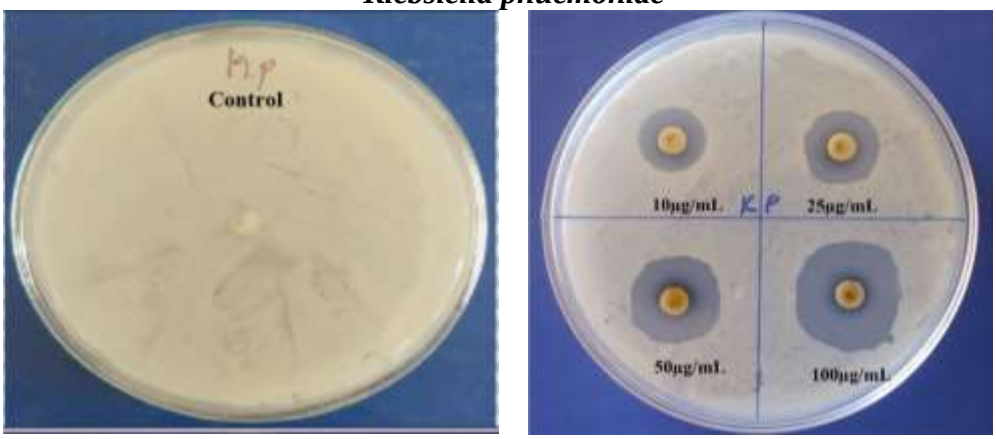
Table 4.2- Zone of Inhibition of Copper nanoparticles on different pathogens (in mm). The maximum ZOI value was observed as 11mm in *Klebsiella pneumoniae* bacteria for100µg/ml concentration of CuNPs.

Species	**10µg/ml**	**25 µg/ml**	**50 µg/ml**	**100 µg/ml**
Bacillus subtilis	3±1	4±1	6±1	8±1
Klebsiella pneumoniae	3±1	5±1	8±1	11±1
Salmonella typhi	4±1	5±1	7±1	10±1
Control	-	-	-	-

Bacillus subtilis



Klebsiella pnuemoniae



Salmonella typhi

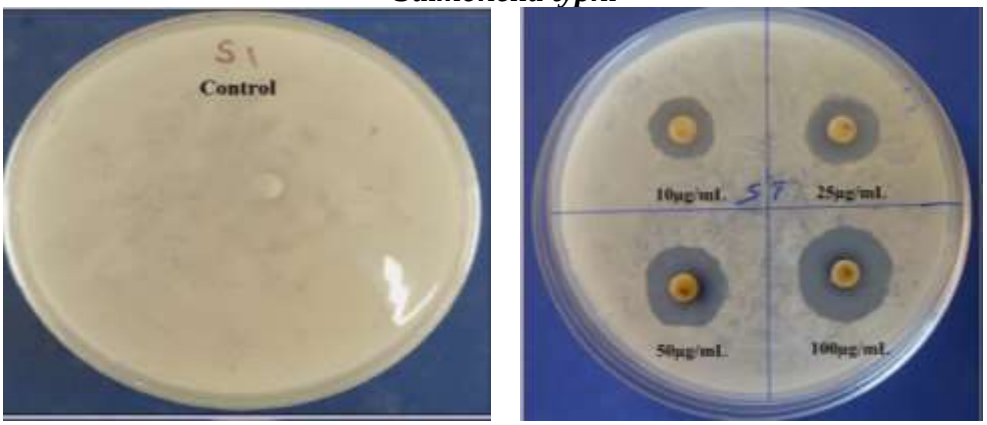


Fig 3.7- Antibacterial activity of CuNPs. The effect of CuNPs on growth of against gram-negative (*Salmonella typhi* and *Klebsiella pneumonia*) and gram-positive (*Bacillus subtilis*) bacterial cultures was measured using disc diffusion method with various concentrations of synthesized CuNPs. The antibacterial capacity of CuNPs was witnessed to be more in gram-negative bacteria when compared to gram-positive bacteria. There was no zone of inhibition around the discs were observed for Control.

3.1.2.2. Antioxidant Assays of AgNPs

3.1.2.2.1. DPPH Free Radical Scavenging Assay

DPPH (1, 1-Diphenyl-2-picrylhydrazyl) is a more stable and well-known free radical based on the reduction of accepting hydrogen or electron from donors. The DPPH reducing ability of the AgNPs was evaluated by observing colour change. The DPPH scavenging assay exhibited effective inhibitory activity of AgNPs when compared with the standard Trolox (Fig 3.8 a). When AgNPs were added to DPPH solution, color change observed which was due to the scavenging of DPPH through donation of hydrogen atom to stable DPPH molecule which was responsible for the absorbance of 517 nm. The DPPH activity of the AgNPs was establish to increase in a dose-dependent manner. The antioxidant potential of AgNPs could be attributed to functional groups adhered to them which originated from the fecal matter extract.

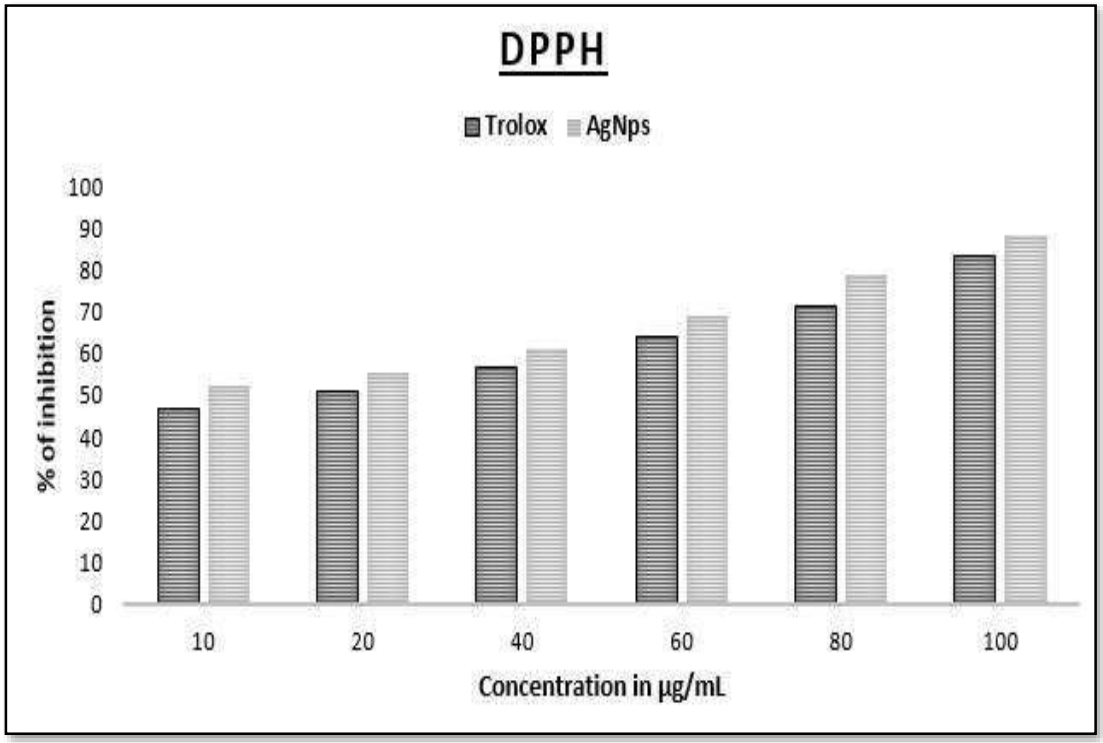


Fig 3.8 a- DPPH free radical scavenging assay of AgNPs. The DPPH scavenging assay exhibited effective inhibitory activity of AgNPs when compared with the standard Trolox. The DPPH activity of the AgNPs was establish to increase in a dose-dependent manner.

3.1.2.2.2. Hydrogen Peroxide Scavenging Activity

The hydrogen peroxide scavenging activity of AgNPs was evaluated spectrophotometrically using ascorbic acid as a standard. The concentrations 100 µg/ml produced inhibitions of 86.98 and 84.68% for AgNPs and ascorbic acid, respectively (fig 3.8 b). Astonishingly the AgNPs showed moderately better reducing power than ascorbic acid due to the structure and characterization of the AgNPs.

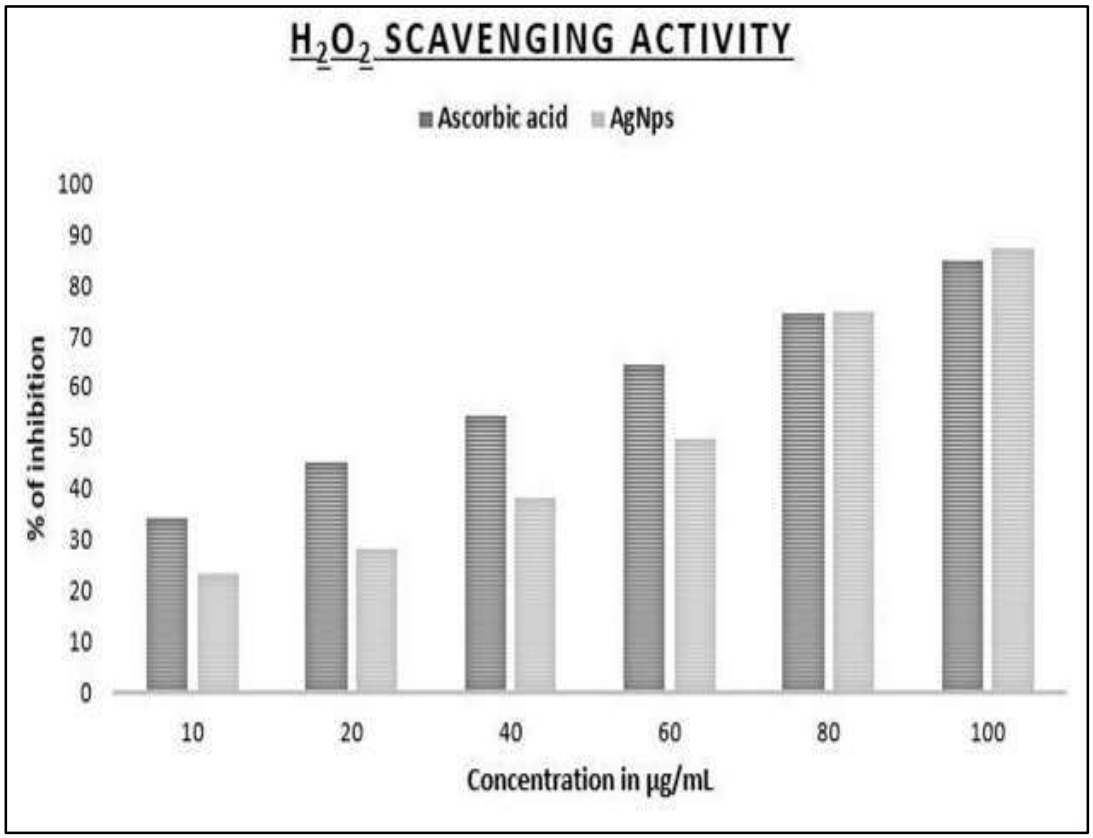


Fig 3.8 b- Hydrogen Peroxide Scavenging Activity of AgNPs. At initial stages, Standard Ascorbic acid was showing moderately higher scavenging activity than synthesized AgNPs and at the concentration of 100 µg/ml, AgNPs attained more activity than standard. The concentrations 100 µg/ml produced inhibitions of 86.98 and 84.68% for AgNPs and ascorbic acid, respectively.

3.1.2.3. Cytotoxic effect of synthesized nanoparticles

The cytotoxicity of the silver and copper nanoparticles was studied against the A549 human alveolar basal epithelial cell lines by MTT assay (Fig 3.9). The cytotoxicity effect was studied at different concentration (10 µg, 20 µg, 30 µg, 40 µg, 50 µg, 60 µg, 70 µg, 80 µg, 90 µg and 100 µg). The bar diagram shows the efficacy of biosynthesized AgNPs and CuNPs against A549 human alveolar basal epithelial cells at different concentration as shown in the Fig 3.9. The results showed that, the synthesized nanoparticles were significantly inhibited the cell growth in a dose-dependent manner, as the concentration of nanoparticles increases there will be decrease in viability of cells.

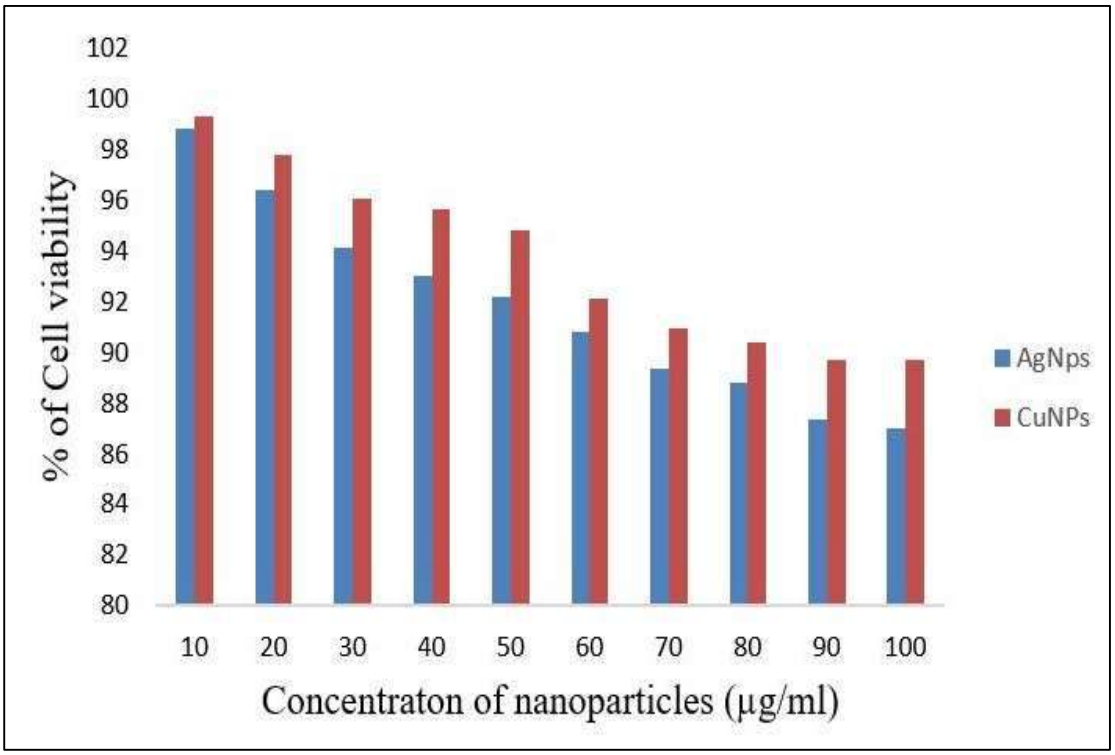


Fig 3.9- Efficacy of AgNPs and CuNPs against A549 human alveolar basal epithelial cell lines. The cytotoxicity effect using MTT assay was studied at different concentrations of synthesized AgNPs and CuNPs. The results showed the dose-dependent inhibition of cell growth which we can observe in the figure, as the concentrations of nanoparticles increases there will be decrease in cell growth.

3.2. Isolation and identification of pathogenic fungus causing bud rot disease in coconut; inhibition studies using synthesized nanoparticles

3.2.1. Isolation and identification of pathogenic fungus

Mixed colonies were obtained when fungi were first isolated on Carrot agar and potato dextrose agar medium. Pure cultures of bud rot causing fungi were identified based its routine cultural and morphological characteristics and the pathogenic fungi was sub-cultured on freshly prepared medium. The pure cultures of the fungi were maintained in Potato Dextrose Agar medium (PDA) slants at 4ºC by sub culturing at 2-3 week intervals. The pure culture of bud rot causing fungi was sent for molecular identification.

3.2.2. Molecular Identification of Fungus

Fungal isolate was identified and confirmed on the basis of its molecular characterization. Genomic DNA was successfully isolated from fungal species and the purity of the same was determined on 1% Agarose gel. After optimization of the PCR condition, the ITS1/ITS4 region DNA was successfully amplified from the genomic DNA using specific primers as described earlier. The PCR product of species had a length of ~700bp. The partial sequence of obtained 18S rRNA was aligned with the available 18S rRNA sequences on ncbi website and compared for homology. The isolated strain exhibited high level of 18s rRNA similarity of 98% with *Phytophthora palmivora* isolate (Genebank accession no. MH401199.1). hence, the molecular characterization confirmed the isolated fungus was *Phytophthora palmivora*.

Fig 3.10- Agarose gel pattern of *Phytophthora palmivora* genomic DNA. DNA loaded on 1% Agarose gel

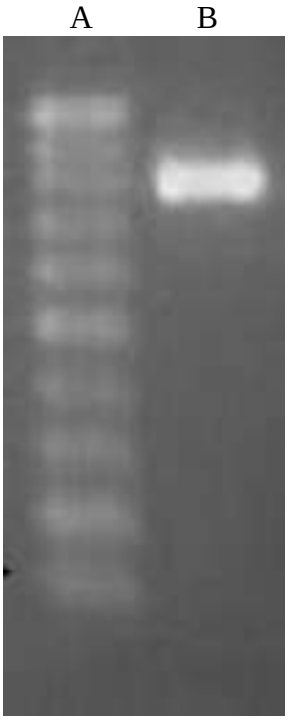


Fig 3.11- PCR amplified product of isolated *Phytophthora* sp. on Agarose gel electrophoresis pattern. Lane A: Standard molecular weight marker (1kb to 100bp), Lane B: Amplified DNA fragments from fungal isolate.

Table 4.3- Sequences showing similarity with the isolated Fungus. The partial sequence of obtained 18S rRNA was aligned with the available 18S rRNA sequences on ncbi website and compared for homology.

Sl No	Description	Similarity	GeneBank Accession number
1	*Phytophthora palmivora* strain PPC3614L 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	98%	MH401199.1
2	*Phytophthora palmivora* isolate FG-12 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	98%	MF370567.1
3	*Phytophthora palmivora* isolate FG-11 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	98%	MF370566.1
4	*Phytophthora palmivora* strain PPG13 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	98%	KY475632.1

5	*Phytophthora palmivora* isolate TW183 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	98%	KU682577.1
6	*Phytophthora palmivora* genomic DNA containing ITS1, 5.8S rRNA gene and ITS2, strain C011	98%	LM650992.1
7	*Phytophthora palmivora* isolate NRCPh-138 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	98%	KF010307.1
8	*Phytophthora palmivora* genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, strain CPPHN02	98%	HE580280.1
9	*Phytophthora palmivora* isolate Dal1b 18S small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S large subunit ribosomal RNA gene, partial sequence	97%	MH219904.1
10	*Phytophthora palmivora* voucher LSVM1405 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	97%	MG956799.1

3.2.3. Inhibition of *Phytophthora palmivora* using biosynthesized nanoparticles

Inhibition study of *Phytophthora palmivora* using synthesized AgNPs and CuNPs at different concentrations were showed strong dose-dependent antifungal activity (Fig 3.12 a & b). It was observed that, as the concentration of biosynthesized nanoparticles increases, there will be decrease in the mycelial growth of pathogenic fungi for both the nanoparticles. Comparatively, AgNPs were exhibited more inhibition effect on *Phytophthora palmivora* than CuNPs. AgNPs has shown some remarkable better activity than the standard fungicide, which we can observe in the figure and table. The percentage of mycelial growth inhibition, which was calculated using the formula is depicted in (Table 4.4).

Table 4.4- Percentage of Mycelial Growth Inhibition (MGI). The percentage of MGI in *Phytophthora palmivora* was observed to be more for AgNPs 86% at 3mg/ml followed by standard Fungicide Fluconazole 80% and CuNPs 70%.

Components	Concentration	MGI (%)
AgNPs	1mg/ml	**30**
	2mg/ml	**58**
	3mg/ml	**86**
CuNPs	1mg/ml	**18**
	2mg/ml	**48**
	3mg/ml	**70**
Fungicide	3mg/ml	**80**

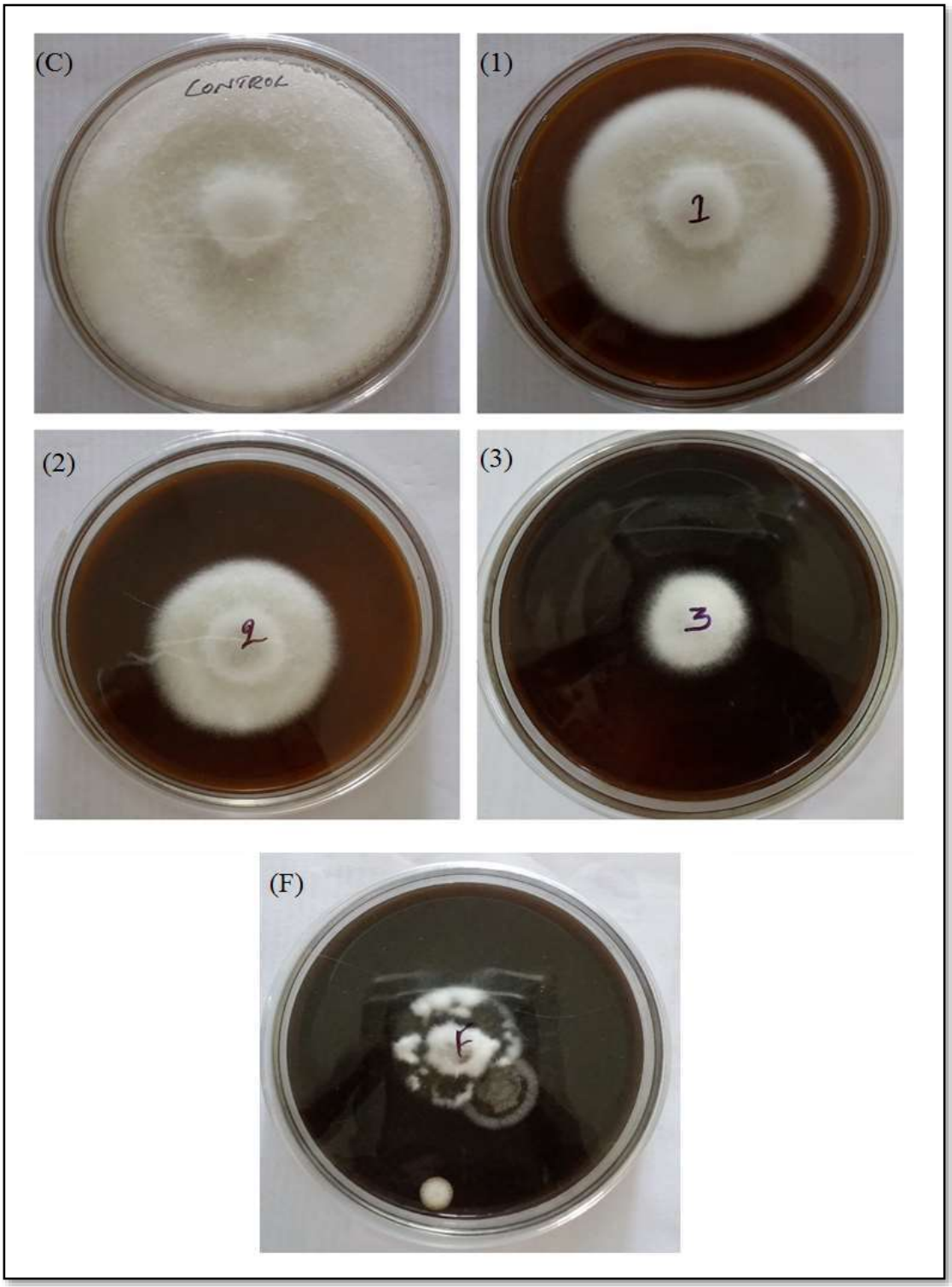


Fig 3.12 a- Picture showing inhibition effect of (1) 1mg/ml AgNPs, (2) 2mg/ml AgNPs, (3) 3mg/ml AgNPs, (F) 3mg/ml Standard and (C) Control. Antifungal effect of AgNPs was measured using Radial growth technique, *Phytophthora Palmivora* showing strong dose-dependent response, with the increase in the concentration of AgNPs there is an increase in the percentage of inhibition.

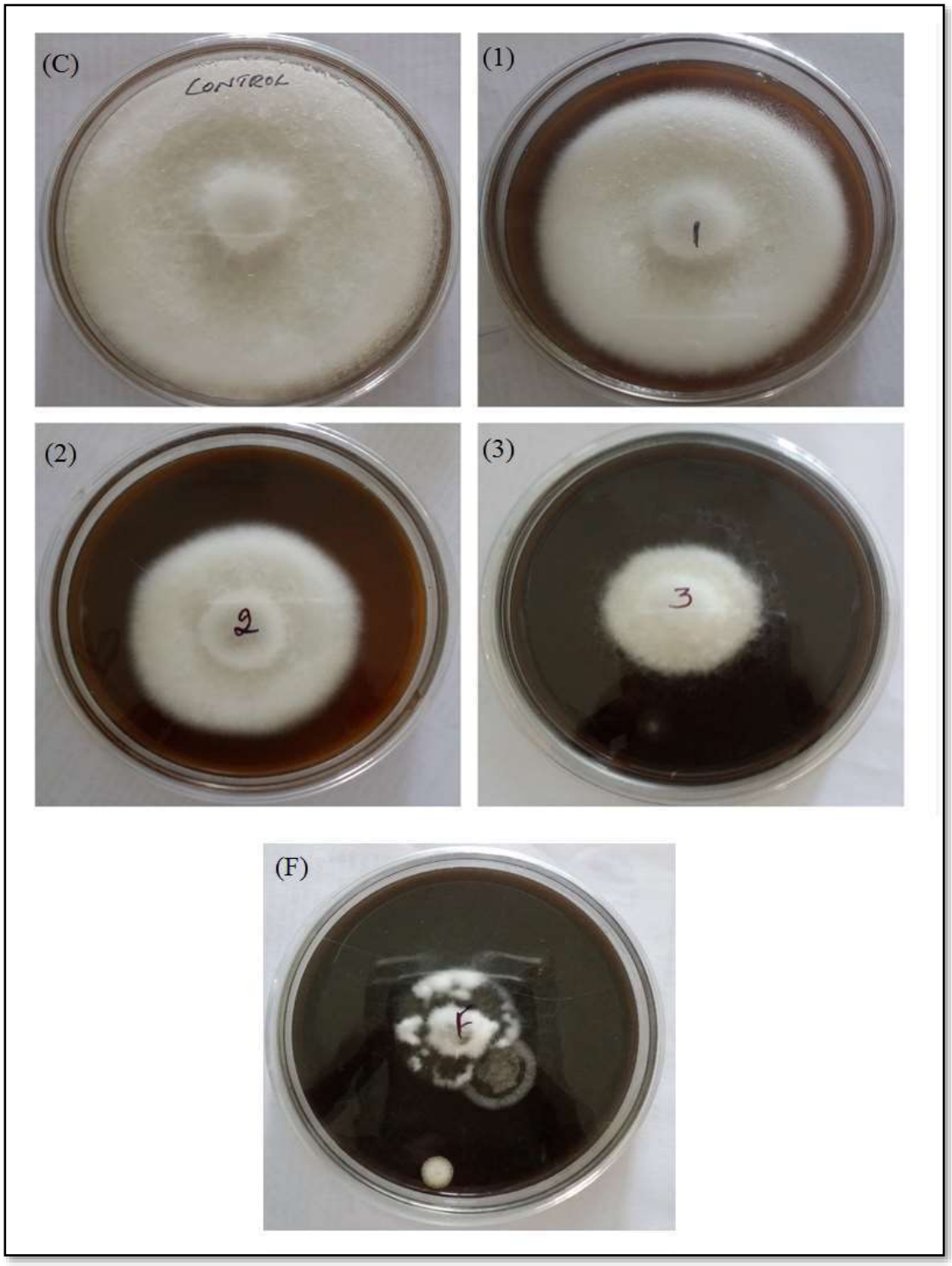


Fig 3.12 b- Picture showing inhibition effect of (1) 1mg/ml CuNPs, (2) 2mg/ml CuNPs, (3) 3mg/ml CuNPs, (F) 3mg/ml Standard and (C) Control. Antifungal effect of CuNPs was measured using Radial growth technique, *Phytophthora Palmivora* showing strong dose-dependent response, with the increase in the concentration of CuNPs there is an increase in the percentage of inhibition.

3.3.1. Screening of pathogenic fungus for different cell wall degrading enzymes like Pectinases and Cellulases

Phytophthora palmivora was screened for Pectinase and Cellulase enzyme production. Initial pH of both the media were maintained at 5, 6, 7, 8 and 9 and the substrate concentration was 1%. Clear zone was observed around the fungal colony in solid media after staining with Gram's iodine (Fig 3.13 a & b). Clear zone around the colony indicates the degradation of substrate added to the media along with other composition. Hence, it confirms the production of Pectinase and Cellulase by pathogenic fungi.

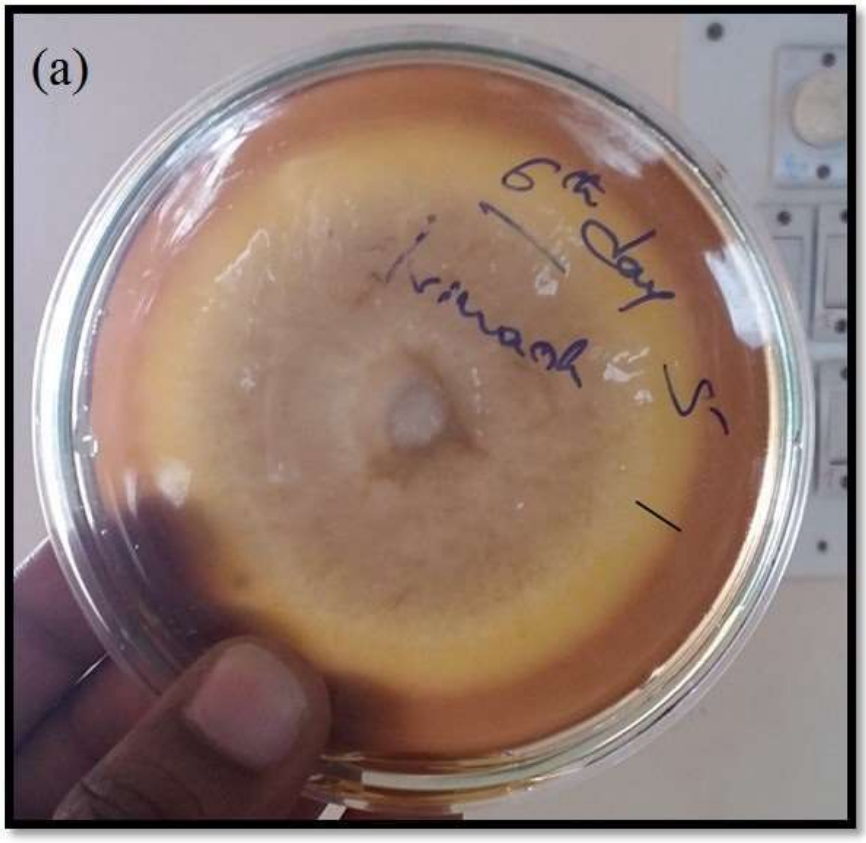


Fig 3.13 a- Screening of fungus for Pectinase production. Screening for the production was done using cup plate assay and components with pectin as substrate, clear zone around the colony indicates the production of Pectinase from *Phytophthora palmivora* by degrading the substrate in the media.

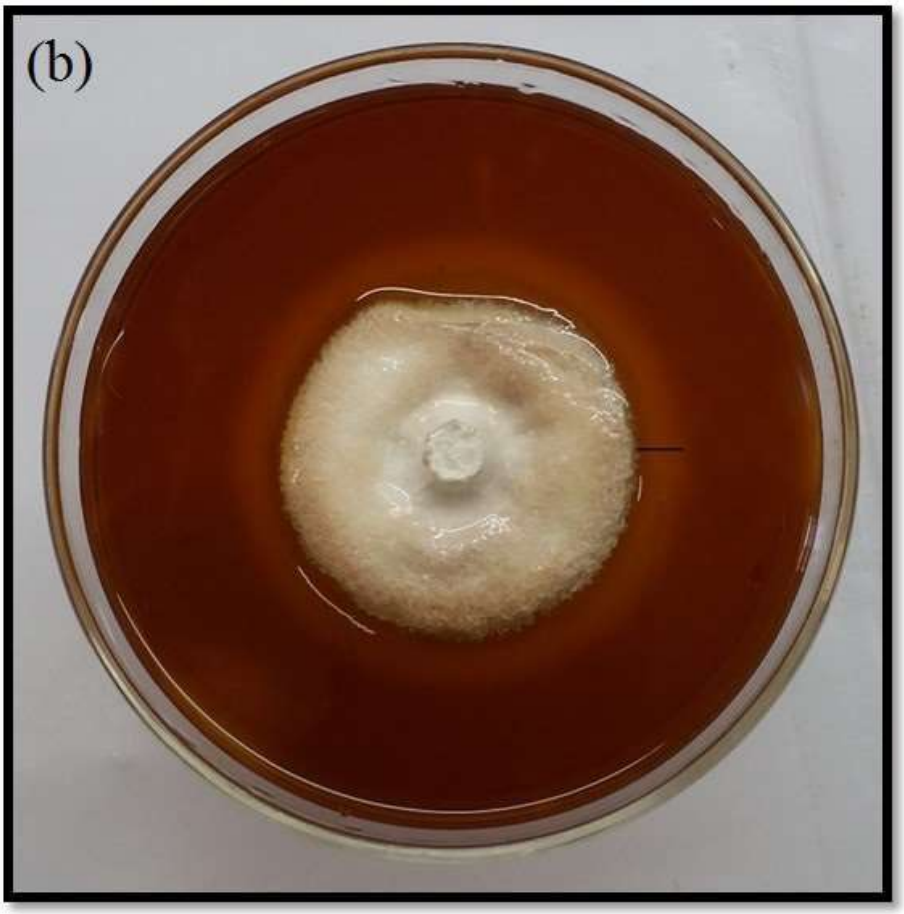


Fig 3.13 b- Screening of fungus for Cellulase production. Screening for the production was done using cup plate assay and components with CMC as substrate, clear zone around the colony indicates the production of Cellulase from *Phytophthora palmivora* by degrading the substrate in the media.

3.3.2. Optimization of Pectinase production

3.3.2.1. Incubation time v/s Pectinase production

The pectinase production was optimized by supplementation with different incubation time of production, range from 4 to 14 days. The maximum pectinase production was noticed at 6th day of production (fig 3.14 a).

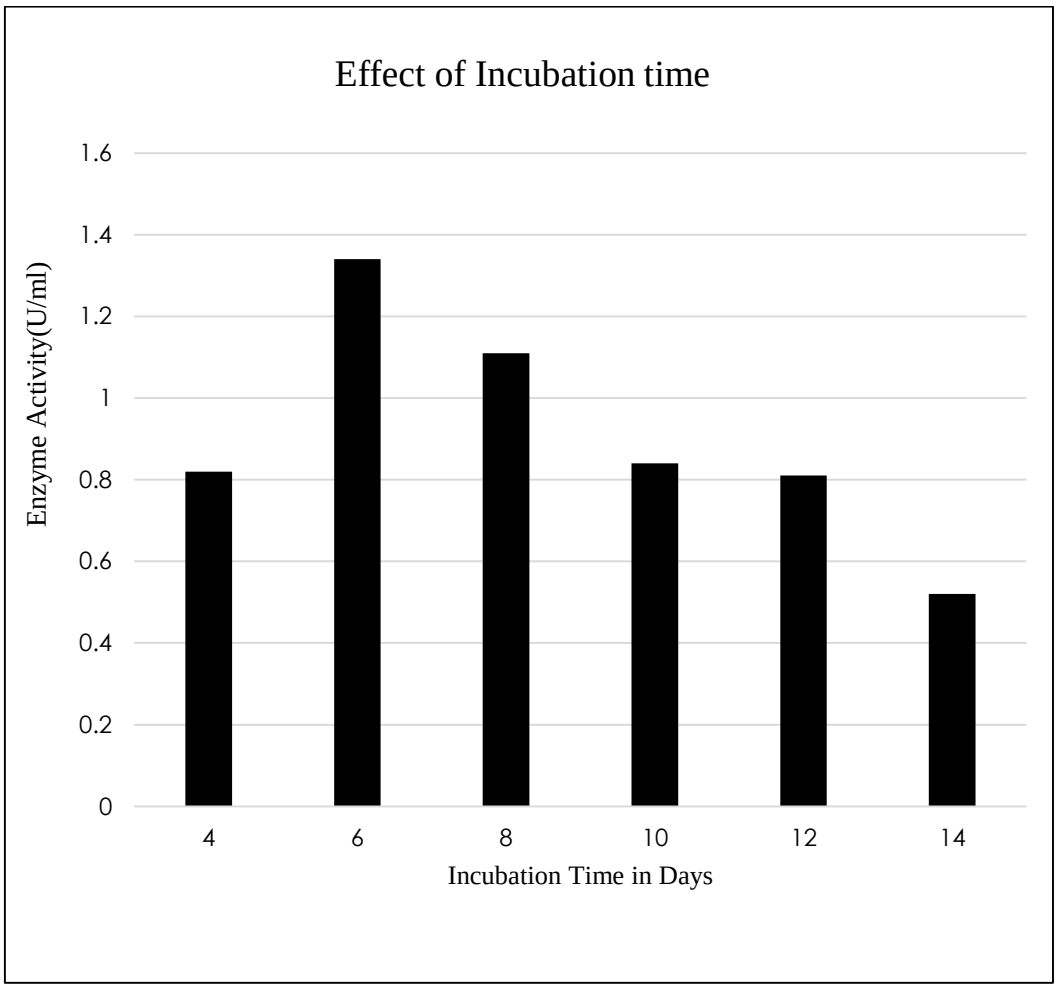


Fig 3.14 a- Effect of Incubation time on Pectinase production by *P. palmivora*. Incubation time for production of pectinase was optimized by checking enzyme activity at different days by keeping other parameters constant. Enzyme activity was moderately high at 6th day.

3.3.2.2. pH v/s Pectinase production

The pectinase production was optimized by supplementation using different pH range of medium from 4 to 9. The maximum pectinase production was noticed at pH 6 (fig 3.14 b).

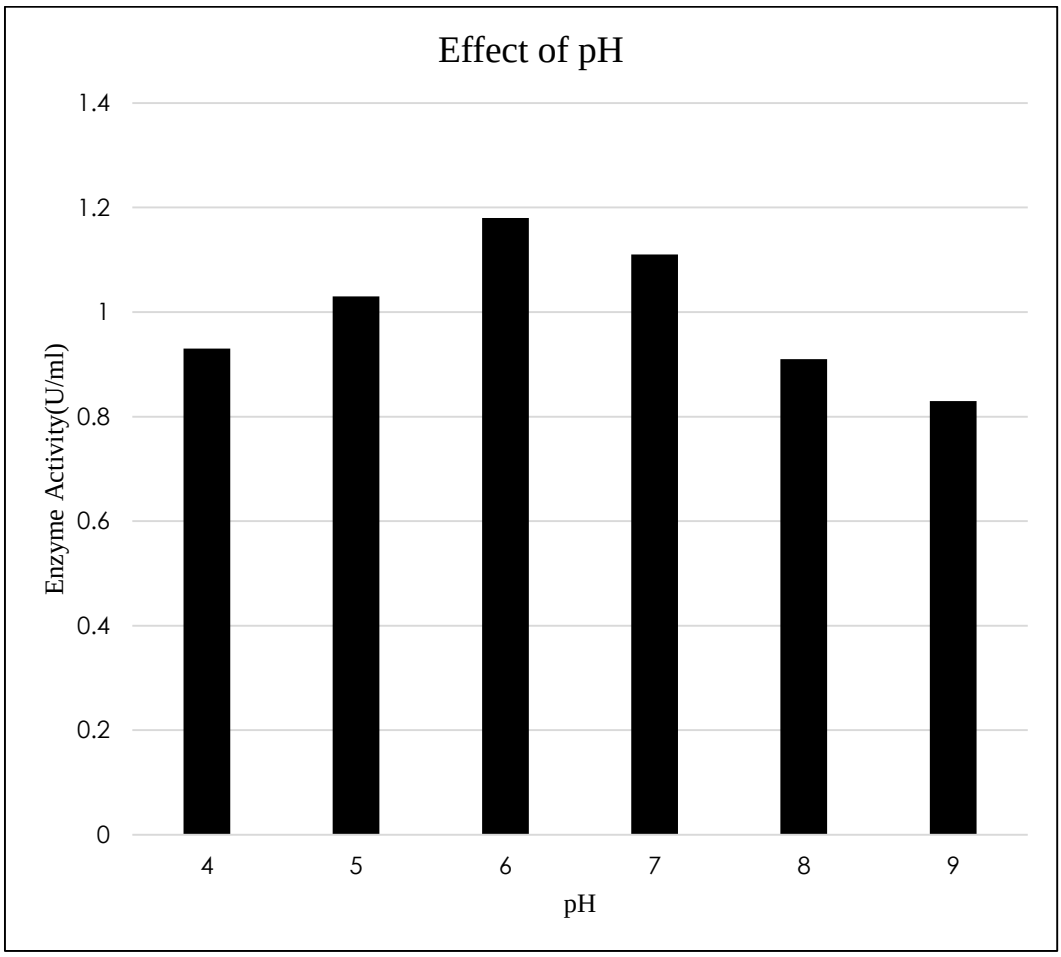


Fig 3.14 b- Effect of pH on Pectinase production by *P. palmivora*. pH for the production of pectinase was optimized by varying the pH of production medium by keeping other parameters constant. Optimum pH of the production media was found to be 6.

3.3.2.3. Temperature v/s Pectinase production

The pectinase production was optimized using different temperature range of medium from 28ºC to 36ºC. The maximum activity for pectinase production was observed at 30ºC (Fig 3.14 c).

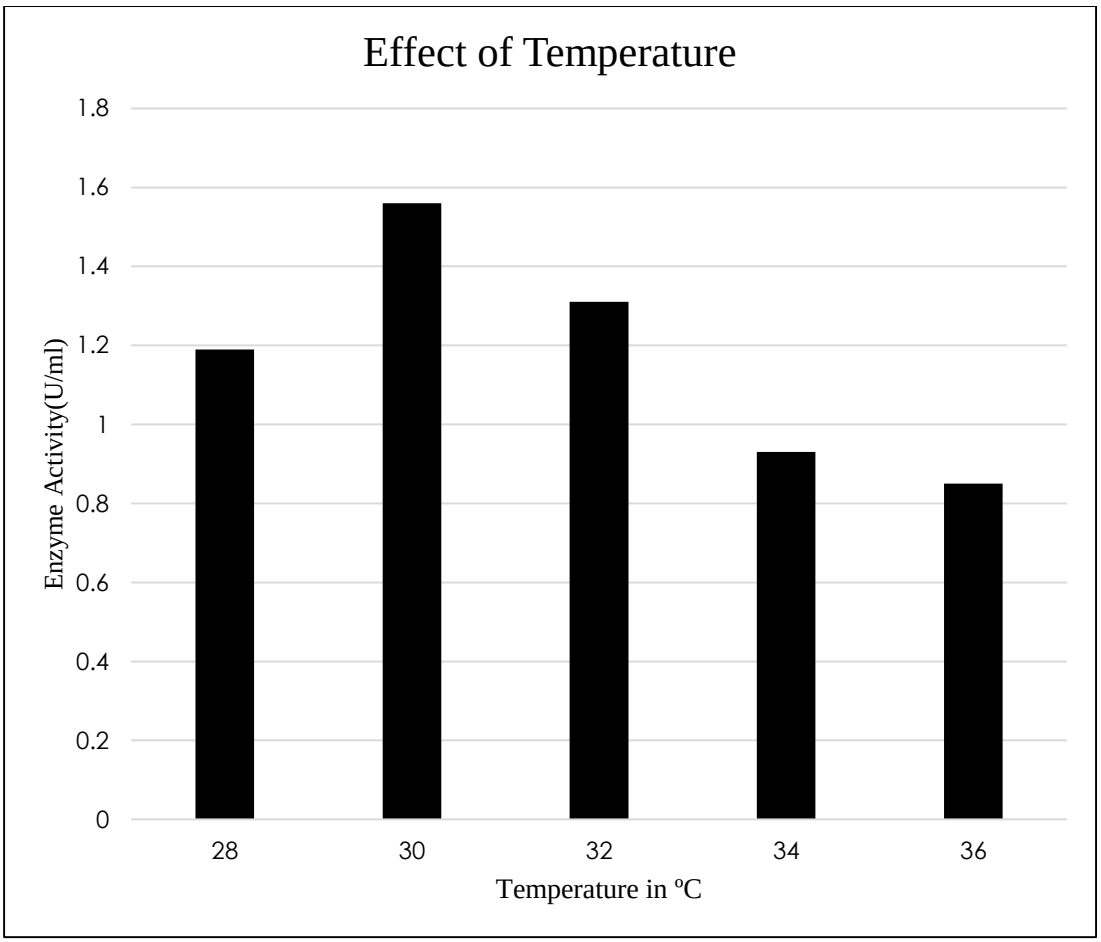


Fig 3.14 c- Effect of temperature on Pectinase production by *P. palmivora*. The optimum temperature for the production of pectinase was determined by varying the temperature of medium during production of enzyme by keeping other parameters constant. Optimum temperature for the production was found to be 30ºC.

3.3.2.4. Substrate v/s Pectinase production

The pectinase production was optimized by supplementation using different pectic substrates such as Areca husk, Wheat straw, Pectin and Orange peel. The maximum pectinase production was noticed for Orange peel (fig 3.14 d).

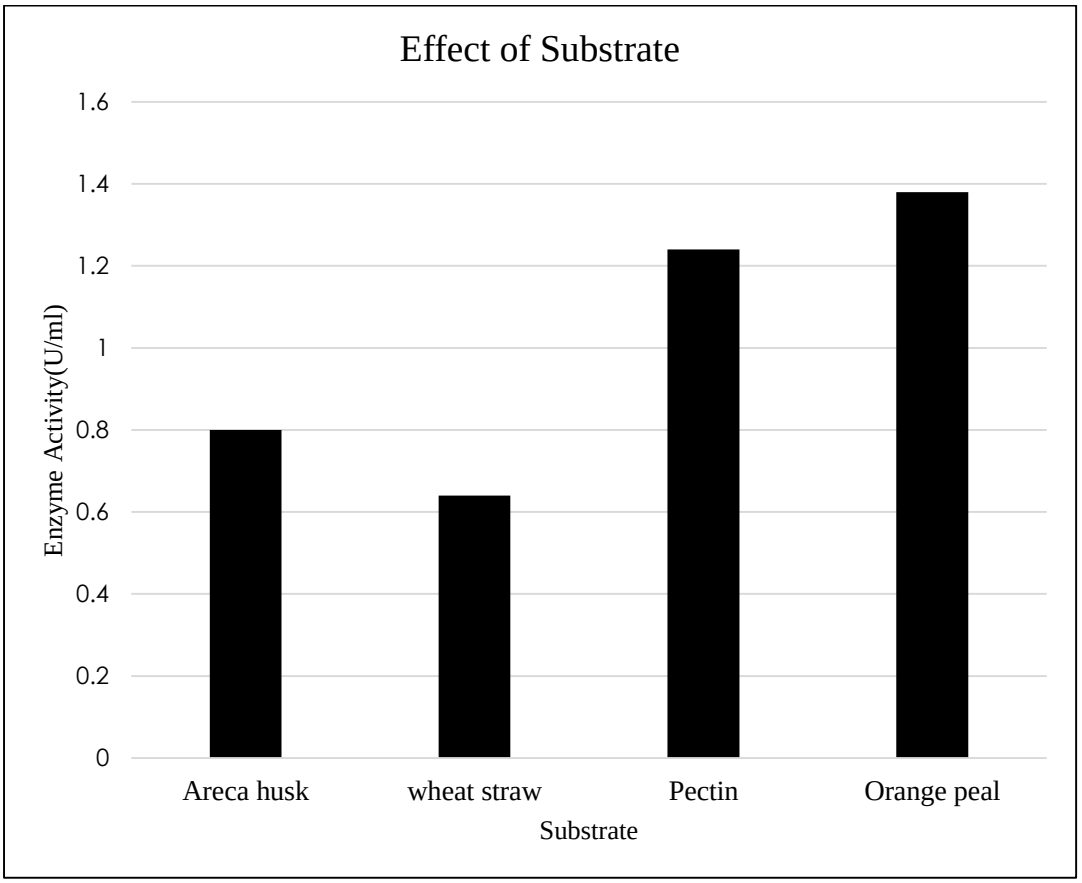


Fig 3.14 d- Effect of different substrates on Pectinase production by *P. palmivora.* By keeping other parameters constant, substrates for the medium were varied. Enzyme activity was moderately high for Orange peel.

3.3.2.5. Substrate concentration v/s Pectinase production

The pectinase production was optimized by supplementation with different substrate concentration in the range of 1 to 6%. The maximum pectinase production was noticed at the concentration of 5% (fig 3.14 e).

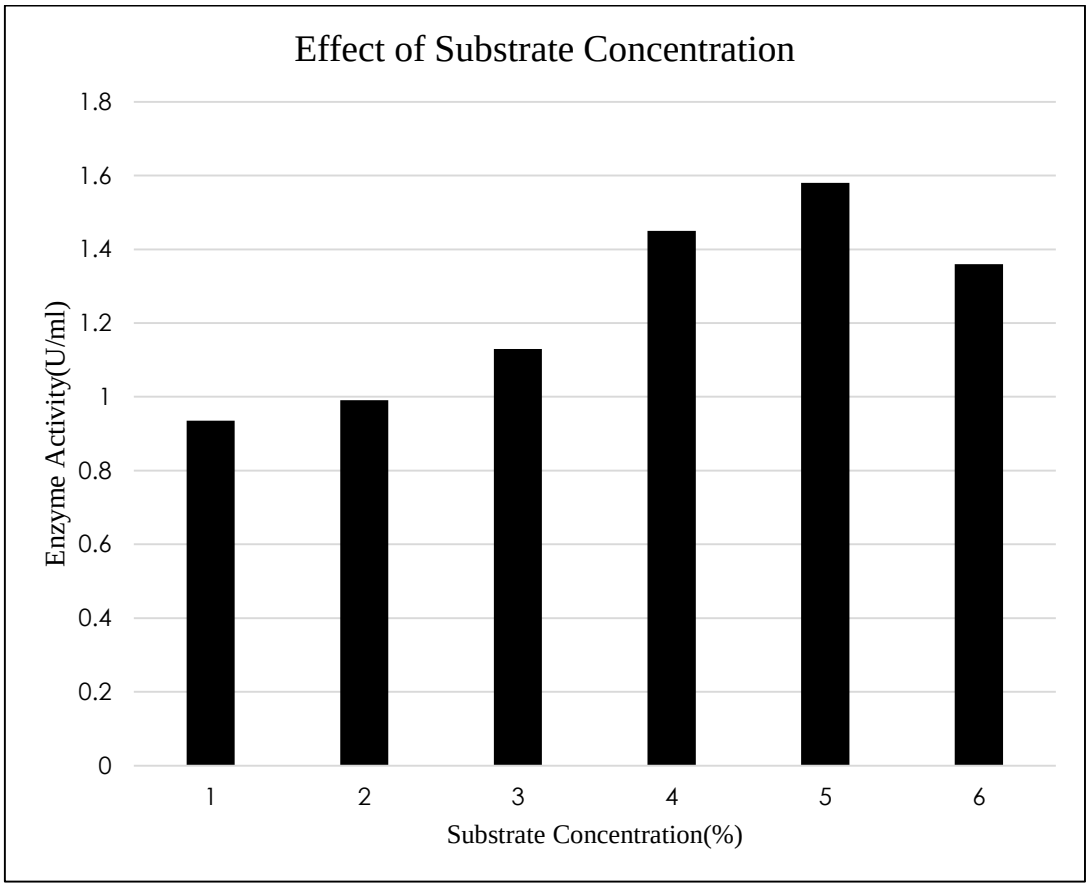


Fig 3.14 e- Effect of substrate concentration on Pectinase production by *P. palmivora*. Substrate concentration was optimized by varying the concentration of substrate and keeping the other parameters constant. 5% orange peel was found to be effective on production.

3.3.3. Partial purification of Pectinase enzyme

Pectinase enzyme produced from *Phytophthora palmivora* was partially purified by employing DEAE-Cellulose anion resin (Genei, Bangalore, India) in 2 X 15 cm column with the liner increasing gradient of 0.1 to 1 M NaCl containing buffer (Fig 3.15 a).

During the initial extraction steps, the protein concentration was observed to be high (approximately 2.3 mg/ml) and the activity of pectinase enzyme was at 32 U/ml. At this stage, specific activity was determined to be 14 U/mg and fold purity was considered as 1.0 for 100% yield. After 75% ammonium sulfate precipitation of the culture supernatant, the specific activity of pectinase during desalting steps was observed to be 148 U/mg and the purification fold was found to be 10.57 with the yield of 65.62%. Further purification was achieved using DEAE-Cellulose ion exchange chromatography with linear increasing gradient of 0.1 to 1.0 M NaCl. After eluting the sample from DEAE-Cellulose anion resin, the specific activity of pectinase was determined to be 212.5 U/mg and the purification fold was enhanced upto 15. The detailed purification factors were summarized in the (Table 4.5). The molecular weight of partially purified pectinase was determined by comparison with standard molecular marker in 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Approximate molecular weight of partially purified pectinase was 25 kDa (fig 3.15 b).

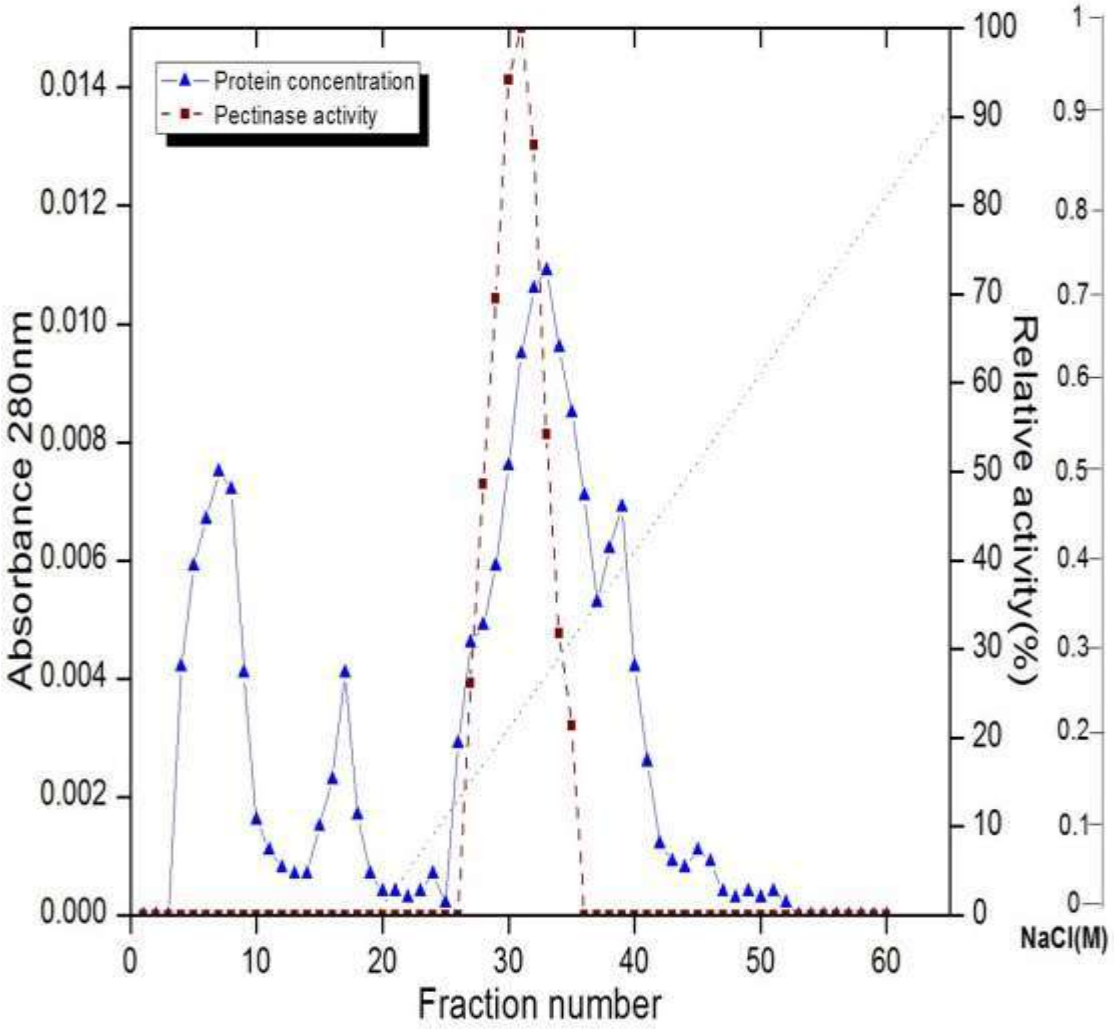


Fig 3.15 a- Pattern of DEAE-Cellulose anion exchange chromatographic purification of Pectinase enzyme using 0.1 to 1.0 M NaCl in citrate buffer. The column was previously equilibrated with 0.02M Citrate buffer of pH 5.5 and the enzyme elution was done using the same buffer with the flow rate of 15 ml/hour.

Table 4.5 - Steps involved in partial purification of Pectinase from *Phytophthora palmivora.*

Purification steps	***Activity (U/ml)***	***Total protein (mg/ml)***	***Specific Activity (U/mg)***	***Purification fold***	***Yield (%)***
Crude	32	2.3	14	1	100
Dialyzed	21	0.15	148	10.57	65.62
DEAE Cellulose	17	0.08	212.5	15.13	53.12

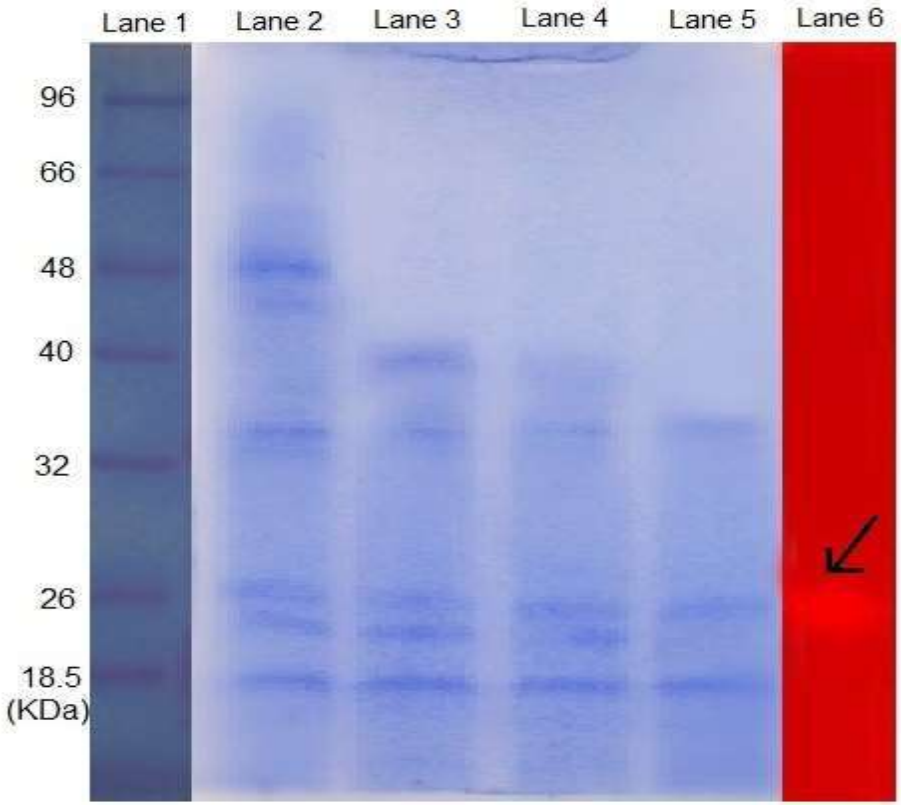


Fig 3.15 b- Polyacrylamide gel electrophoresis analysis of the partially purified Pectinase enzyme. Lane 1-Standard molecular weight, Lane 2-Crude sample, Lane 3-Ammonium sulphate precipitated, Lane 4-Dialysed sample, Lane 5-Partially purified and Lane 6-Zymogram of Pectinase.

3.3.4. Optimization of Cellulase production

3.3.4.1. Incubation time v/s Cellulase production

The cellulase production was optimized by supplementation with different incubation time of production, range from 4 to 14 days. The maximum cellulase production was noticed at 8th day of production (fig 3.16 a).

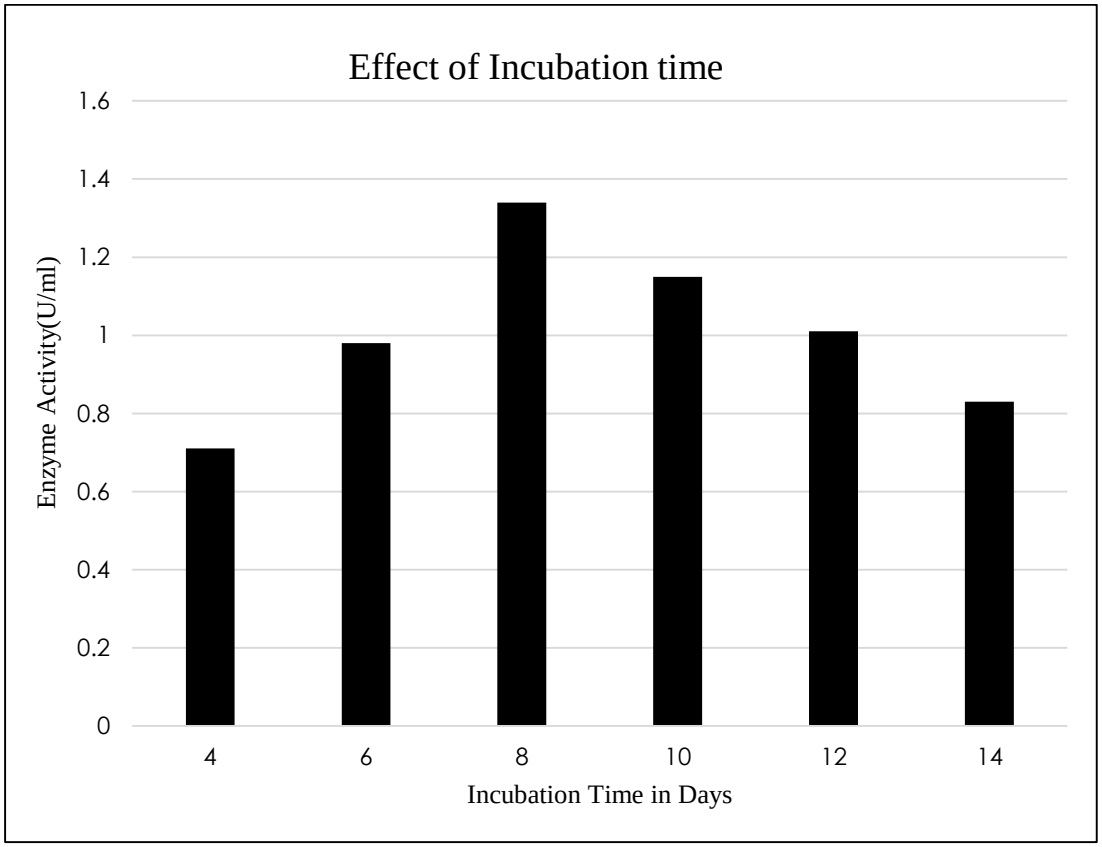


Fig 3.16 a- Effect of Incubation time on Cellulase production by *P. palmivora.* Incubation time for production of cellulase was optimized by checking enzyme activity at different days by keeping other parameters constant. Enzyme activity was found to be high at 8th day.

3.3.4.2. pH v/s Cellulase production

The Cellulase production was optimized by supplementation using different pH range of medium from 4 to 9. The maximum Cellulase production was noticed at pH 6 (fig 3.16 b).

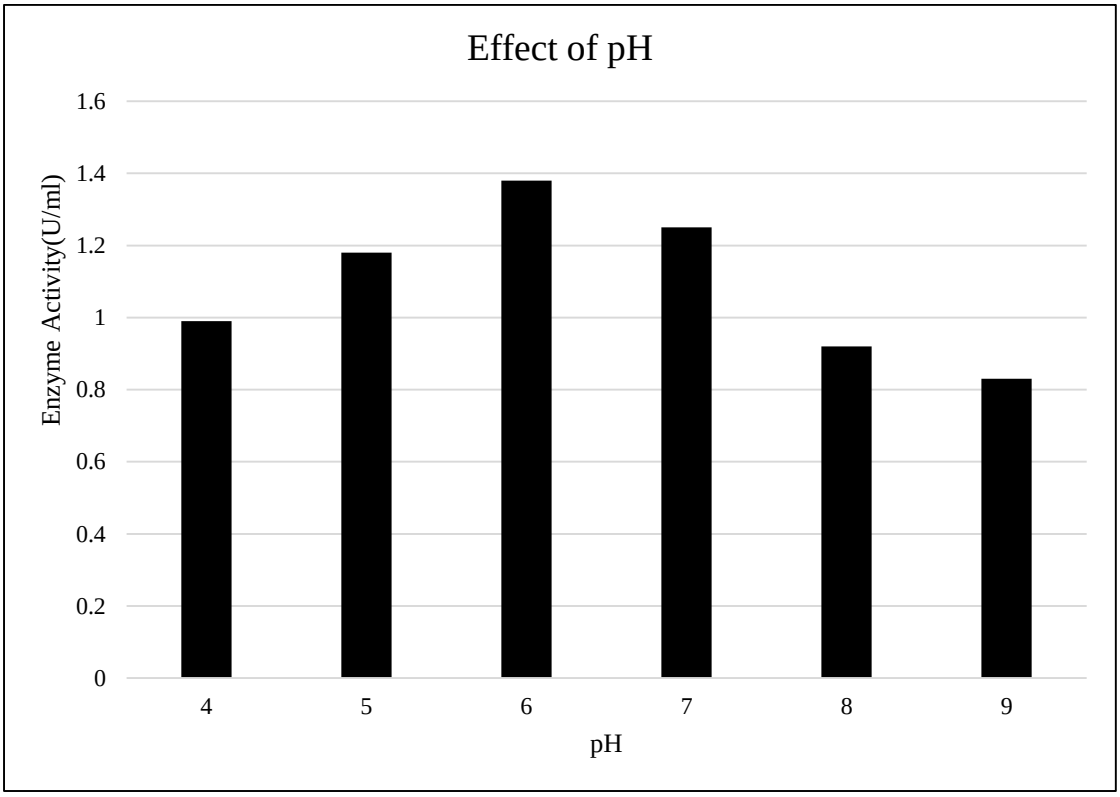


Fig 3.16 b- Effect of pH on Cellulase production by *P. palmivora*.pH for the production of cellulase was optimized by varying the pH of production medium by keeping other parameters constant. Optimum pH was found to be 6.

3.3.4.3. Temperature v/s Cellulase production

The Cellulase production was optimized using different temperature range of medium from 28ºC to 36ºC. The maximum activity for cellulase production was observed at 32ºC (Fig 3.16 c).

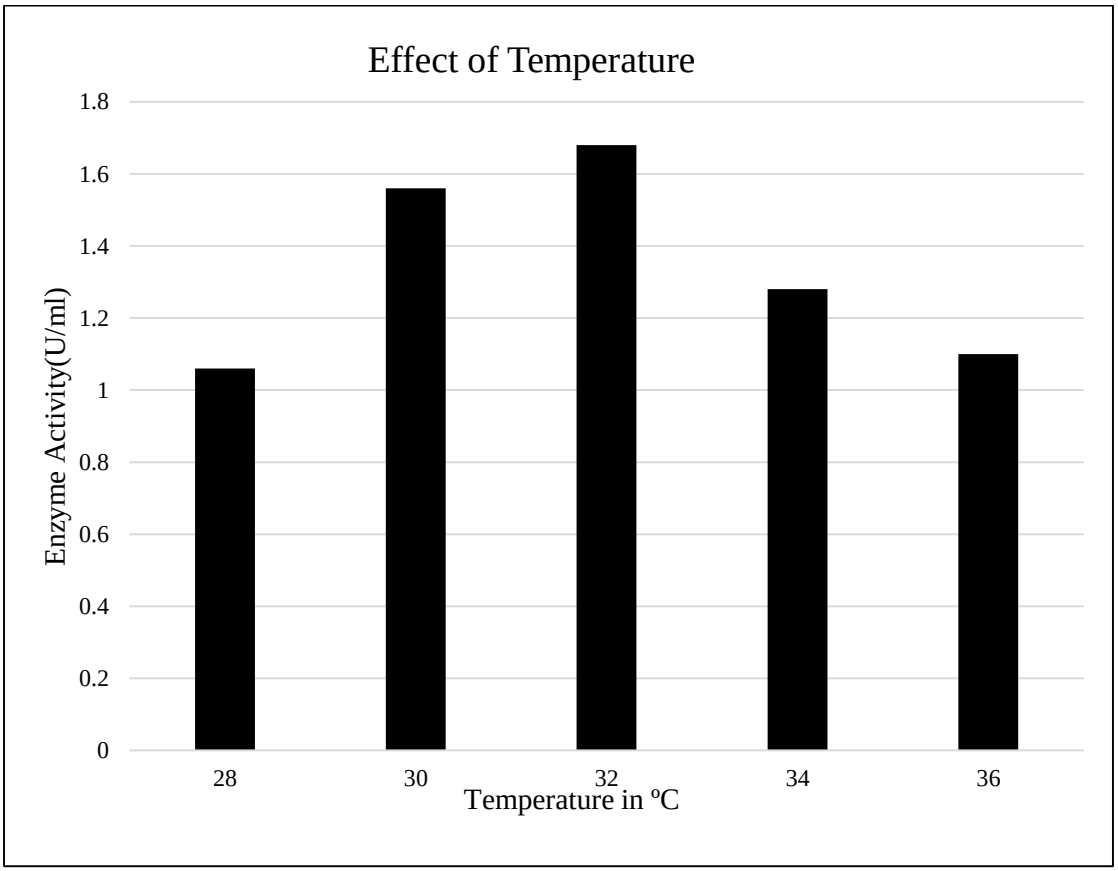


Fig 3.16 c- Effect of temperature on Cellulase production by *P. palmivora*. The optimum temperature for the production of cellulase was determined by varying the temperature of medium during production of enzyme by keeping other parameters constant. Optimum temperature was found to be 32ºC.

3.3.4.4. Substrate v/s Cellulase production

The Cellulase production was optimized by supplementation using different substrates such as Corncob, Carboxymethylcellulose (CMC) and Sodium Carboxymethylcellulose (SMC). The maximum Cellulase production was noticed for Carboxymethylcellulose (CMC) (fig 3.16 d).

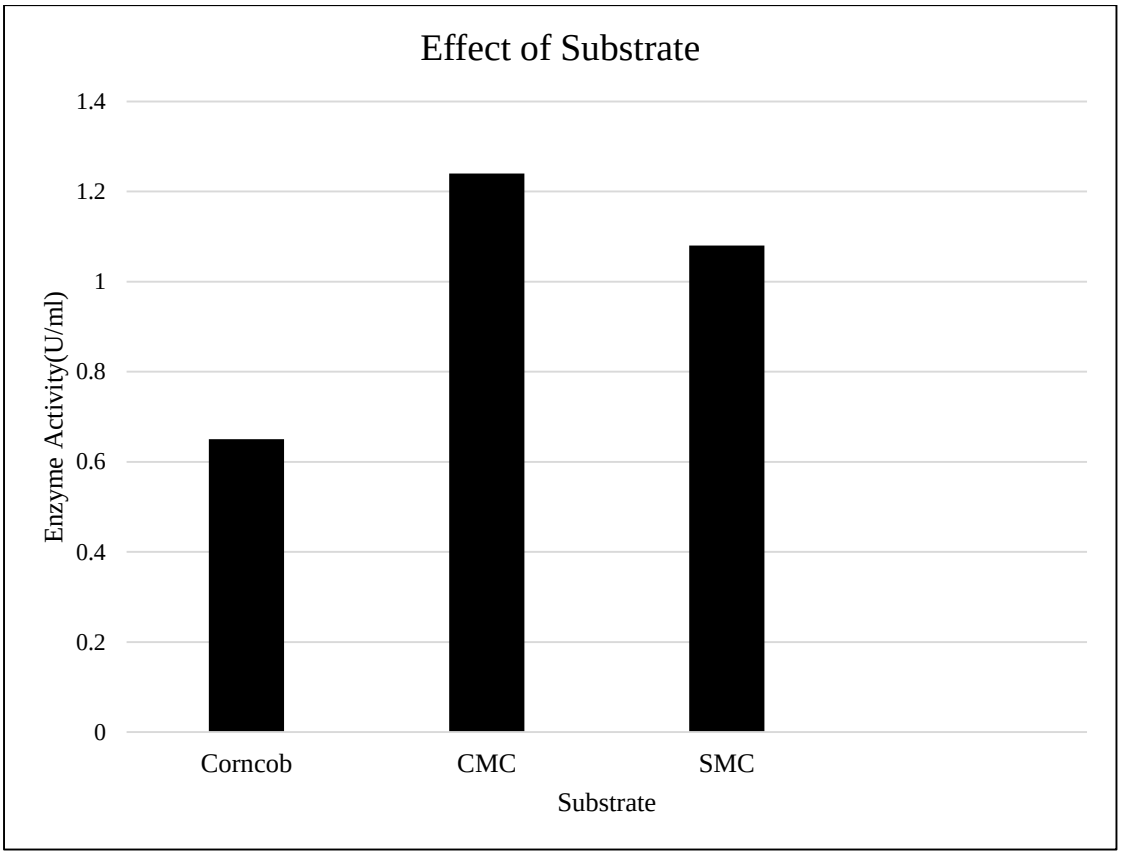


Fig 3.16 d- Effect of different substrates on Cellulase production by *P. palmivora.*

By keeping other parameters constant, substrates for the medium were varied. Enzyme activity was moderately high for CMC.

3.3.4.5. Substrate concentration v/s Cellulase production

The cellulase production was optimized by supplementation with different substrate concentration in the range of 0.5 to 3%. A slight maximum cellulase production was noticed at the concentration of 1% (fig 3.16 e).

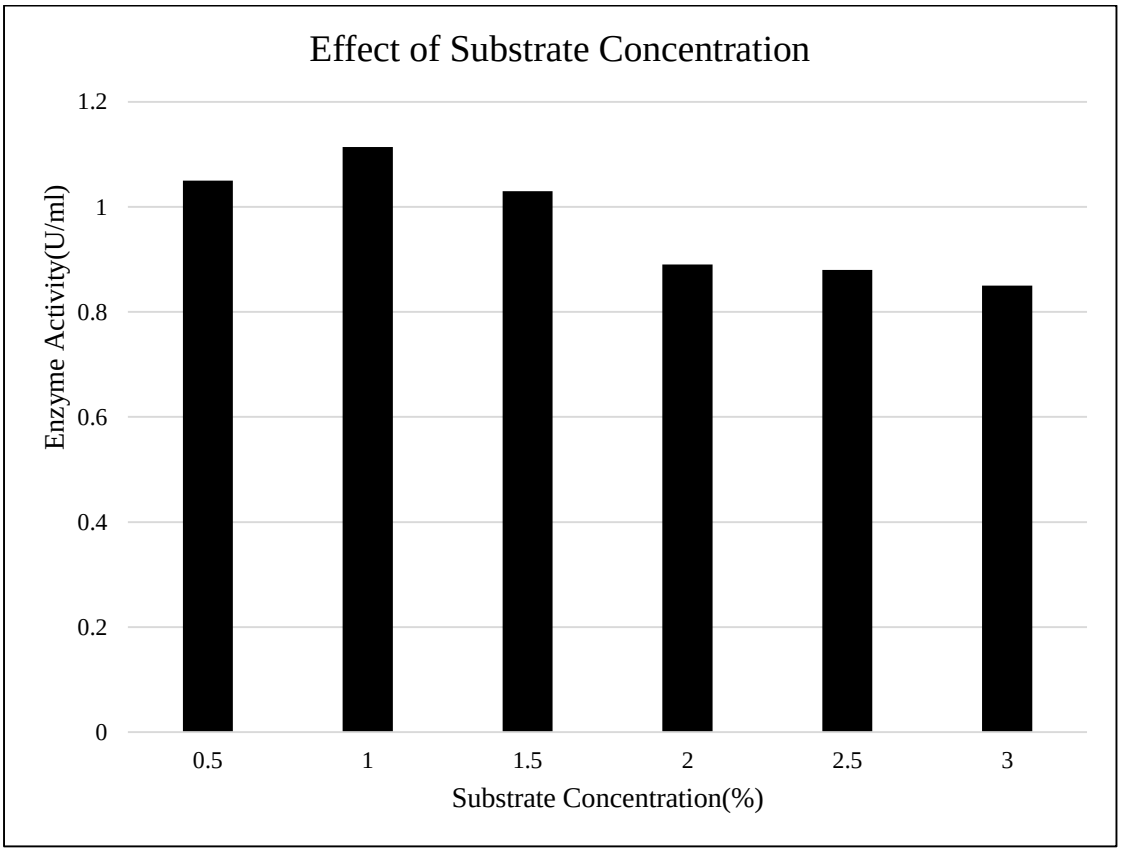


Fig 3.16 e- Effect of substrate concentration on Cellulase production by *P. palmivora*. Substrate concentration was optimized by varying the concentration of substrate and keeping the other parameters constant. 1% CMC was found to be moderately effective on production.

3.3.5. Partial purification of Cellulase enzyme

Cellulase enzyme produced from *Phytophthora palmivora* was partially purified by employing DEAE-Cellulose anion resin (Genei, Bangalore, India) in 2 X 15 cm column with the liner increasing gradient of 0.1 to 1 M NaCl containing buffer (Fig 3.17 a).

During the initial extraction steps, the protein concentration was observed to be high (approximately 3.8 mg/ml) and the activity of cellulase enzyme was at 47 U/ml. At this stage, specific activity was determined to be 12 U/mg and fold purity was considered as 1.0 for 100% yield. After 80% ammonium sulfate precipitation of the culture supernatant, the specific activity of cellulase enzyme during desalting steps was observed to be 28 U/mg and the purification fold was found to be 2.3 with the yield of 68.08%. Further purification was achieved using DEAE-Cellulose ion exchange chromatography with linear increasing gradient of 0.1 to 1.0 M NaCl. After eluting the sample from DEAE-Cellulose anion resin, the specific activity of cellulase was determined to be 328 U/mg and the purification fold was enhanced upto 27 with the yield of 48.93%. The detailed purification factors were summarized in the (Table 4.6). The molecular weight of partially purified cellulase was determined in comparison with standard molecular weight marker in 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The approximate molecular weight of partially purified cellulase was 40 kDa (fig 3.17 b).

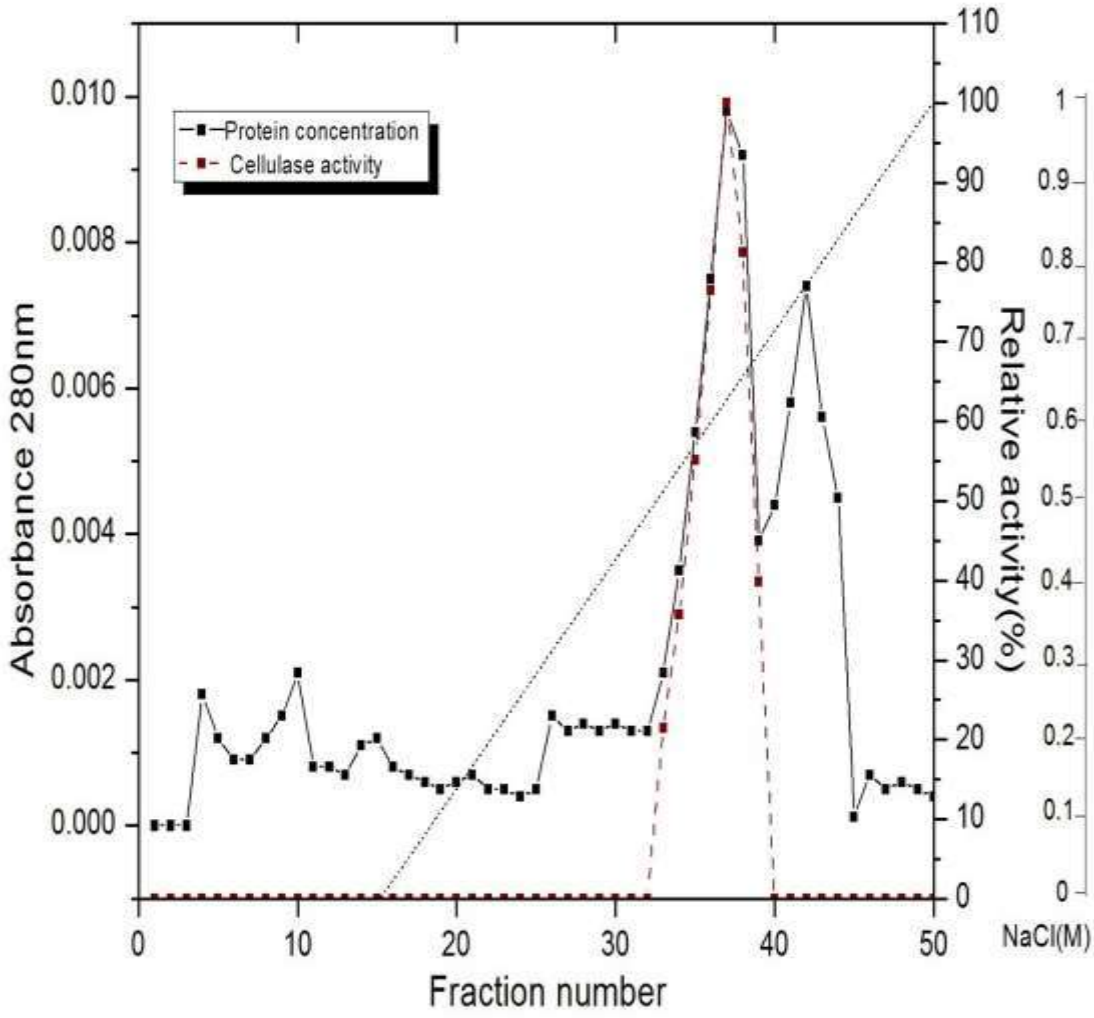


Fig 3.17 a- Pattern of DEAE-Cellulose anion exchange chromatographic purification of Cellulase enzyme using 0.1 to 1.0 M NaCl in acetate buffer. The column was previously equilibrated with 0.02M 0.05M Acetate buffer of pH 6 and the enzyme elution was done using the same buffer with the flow rate of 15 ml/hour.

Table 4.6- Steps involved in partial purification of Cellulase from *Phytophthora palmivora*.

Purification steps	***Activity (U/ml)***	***Total protein (mg/ml)***	***Specific Activity (U/mg)***	***Purification fold***	***Yield (%)***
Crude	47	3.8	12	1	100
Dialyzed	32	1.14	28	2.3	68.08
DEAE Cellulose	23	0.07	328	27	48.93

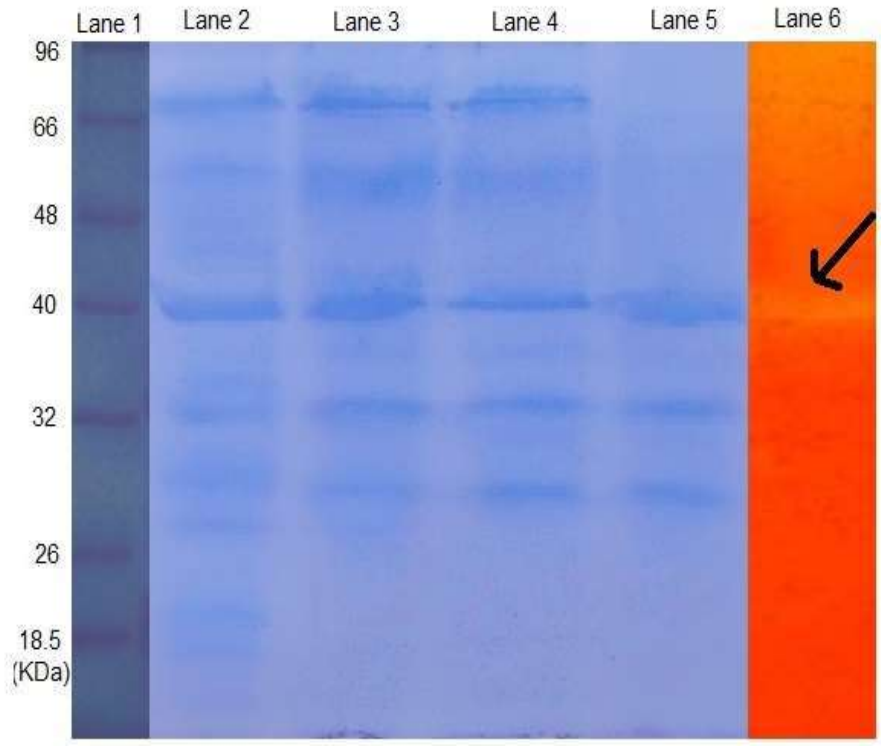


Fig 3.17 b- Polyacrylamide gel electrophoresis analysis of the partially purified Cellulase enzyme. Lane 1-Standard molecular weight, Lane 2-Crude sample, Lane 3-Ammonium sulphate precipitated, Lane 4-Dialysed sample, Lane 5-Partially purified and Lane 6-Zymogram of Cellulase.

3.4. Inhibition of Pectinase and Cellulase enzymes using synthesized nanoparticles

3.4.1. Inhibition of Pectinase enzyme activity

Inhibition assay of AgNPs and CuNPs against pectinase enzyme activity was studied at different concentrations of nanoparticles (20 µg, 40 µg, 60 µg, 80 µg and 100 µg). In turn, it was observed that NPs have shown strong dose-dependent inhibition activity on pectinase activity. When the concentration of biosynthesized nanoparticles increased, there will be decrease in relative activity of pectinase in both the cases of AgNPs and CuNPs. In comparison, the inhibition activity of CuNPs was much greater than that of AgNPs (fig 3.18 a).

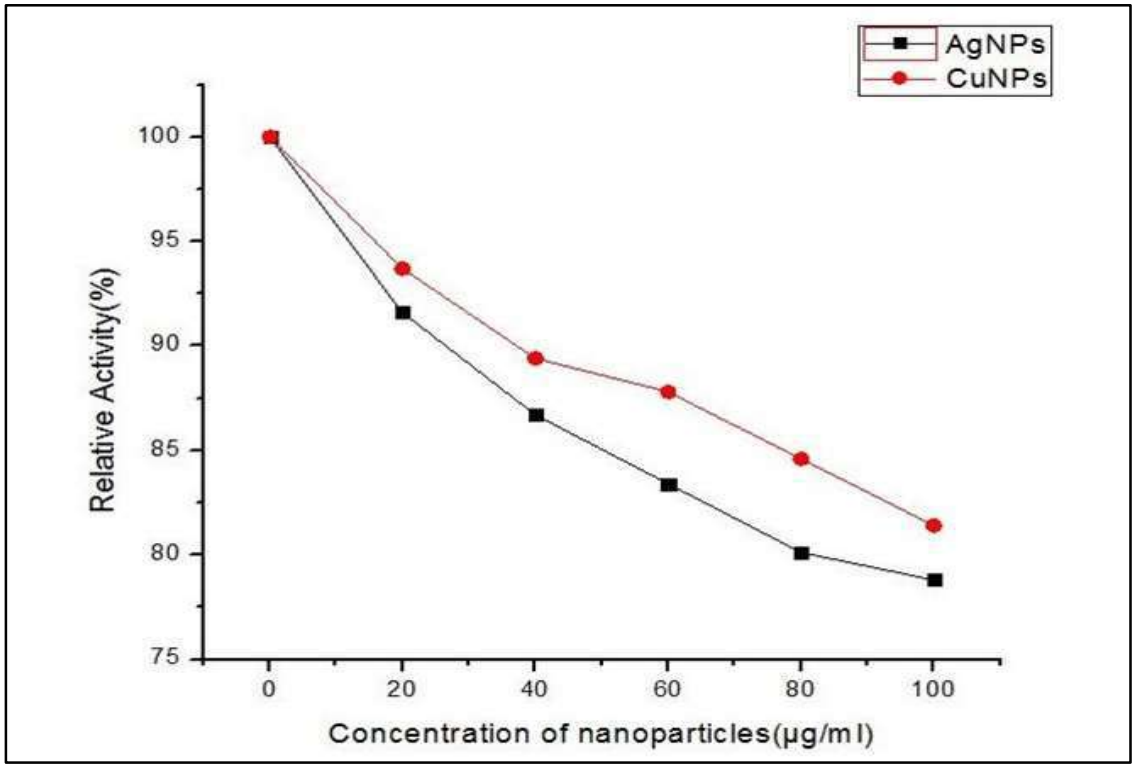


Fig 3.18 a- Decreasing Relative activity of Pectinase enzyme. The inhibition effect of synthesized nanoparticles on Pectinase enzyme activity was determined using DNS method of estimating reducing sugar released during the reaction. Pectinase Enzyme activity has shown dose-dependent response on addition of nanoparticles. The inhibition activity of CuNPs was much greater than that of AgNPs on Pectinase enzyme activity.

3.4.2. Inhibition of Cellulase enzyme activity

Inhibition assay of AgNPs and CuNPs against cellulase enzyme activity was studied at different concentrations of nanoparticles (20 µg, 40 µg, 60 µg, 80 µg and 100 µg). In turn, it was observed that NPs have shown strong dose-dependent inhibition activity on cellulase activity. There was observed to be gradual decrease in the relative activity of enzyme, with the increase in concentration of both nanoparticles. In comparison, the inhibition activity of AgNPs was much greater than that of CuNPs (fig 3.18 b).

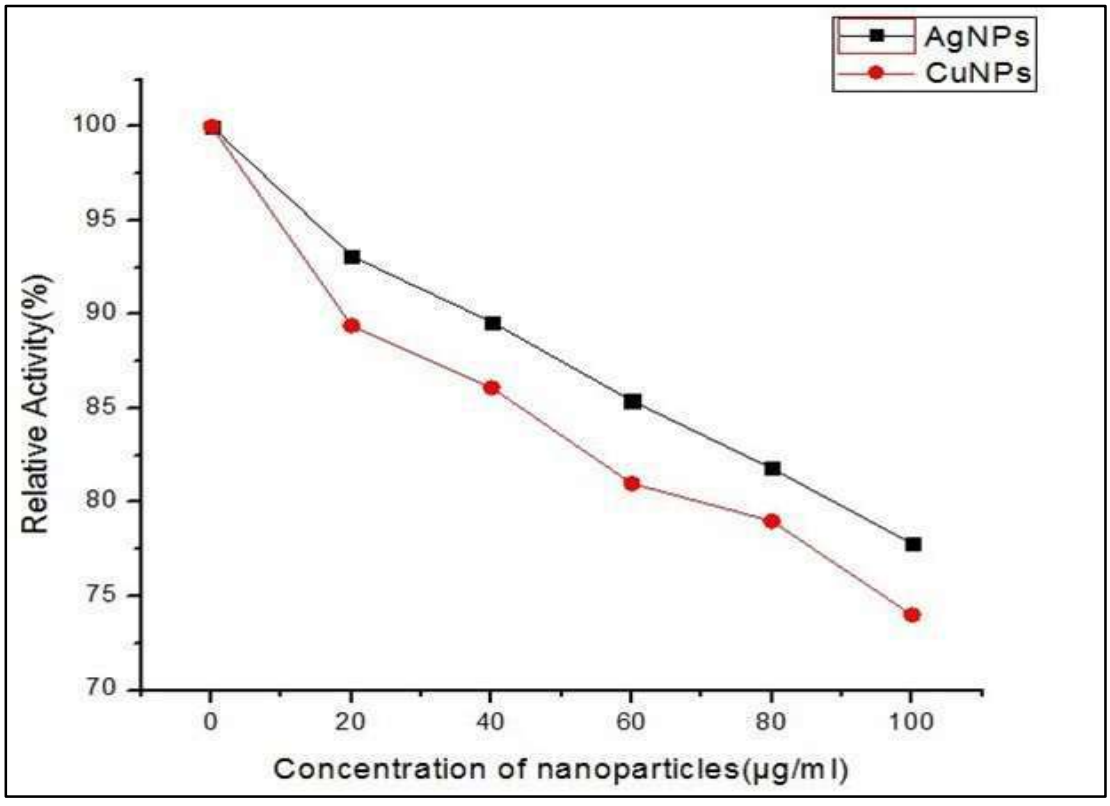


Fig 3.18 b- Decreasing Relative activity of Cellulase enzyme. The inhibition effect of synthesized nanoparticles on Cellulase enzyme activity was determined using DNS method of estimating reducing sugar released during the reaction. Cellulase Enzyme activity has shown dose-dependent response on addition of nanoparticles. The inhibition activity of AgNPs was much greater than that of CuNPs on Cellulase enzyme activity.

Chapter 4

Discussion

4.1.1. Synthesis and characterization of nanoparticles

Biological synthesis of metal nanoparticles is an eye-catching area of nanoscience and technology. It comprehend the expansion of clean, eco-friendly, biocompatible and non-toxic methods for synthesis of nanoparticles in comparison with current conventional methods like chemical and physical which are often toxic [Ankit Chokriwal *et al*., 2014]. Biological synthesis uses plants, algae, fungi, actinomycetes, yeast, bacteria, etc. along with precursors to synthesize nanoparticles. We have studied biological synthesis of nanoparticles using Silk worm fecal matter, May be the water soluble organics present in the silk work fecal matter were mainly responsible for the reduction Ag^{+} and Cu^{+} ions to AgNPs and CuNPs respectively. During synthesis, the formation of nanoparticles was due to reduction of ions to nanoparticles [Garima Singhal *et al*., 2011]. Colour change in the solution mixture occurs which indicates the formation of nanoparticles, this colour change was due to excitation of surface plasmon vibrations with the respective nanoparticles which was also reported by Afreen *et al*.,(2011), Dattu *et al*.,(2013) and Ranganath *et al*.,(2016).

In the present study, The UV-Vis spectrum of AgNPs and CuNPs were obtained by exposing the sample to UV-light. A specific SPR is responsible for their unique remarkable optical phenomenon. A single peak observed maximum at 412 and 560nm corresponds to SPR of AgNPs and CuNPs, can be observed in the UV-Vis spectrum. Absorbance spectroscopy is used to determine the optical properties of sample. The optical measurement of UV-visible spectrophotometer for AgNPs has different absorbance peaks like 413nm with the addition of *Ocimum sanctum* (Tulsi) extract [Garima Singhal *et al*., 2011], 410nm when treated with the Nerium Obander

plant extract [Subbaiya *et al*., 2014] and 370nm when treated with *Mentha arvensis* extract [Shivakumar *et al*., 2015]. Our results for optical measurement of UV-visible spectrophotometer for CuNPs exactly correlates with Hyo-Jeoung Lee *et al*., (2013), who reported that CuNPs shows maximum absorption peak at 560 when synthesized using *Magnolia kobus* leaf extract, while K. Saranyaadevi *et al*., (2014) reported that CuNPs shows maximum absorption peak at 531nm when synthesized using *Capparis zeylanica* leaf Extract and absorption maxima of copper nanoparticles was at 570 nm when synthesized using *Syzygium aromaticum* (Cloves) Aqueous Extract [Ipsa Subhankari and P.L. Nayak, 2018]. Ag and Cu ions concentration was also examined by AAS by withdrawing solution mixture at various stages of reaction followed by centrifugation. Ions are much smaller, they cannot be separated by centrifugation but nanoparticles are in zero valent metallic form, those can be easily separated. It was showed gradual decrease in concentration of ions which in turn indicates the conversion of ions to nanoparticles which was due to bioreduction capacity of silkworm fecal matter. Our AAS results are in good accordance with the reports of Garima Singhal *et al*., (2011), Javier Suarez-Cerda *et al*., (2017), Madiha Batoool and Bilal Masood (2017).

XRD (X-Ray Diffractometer) analysis reveals the crystalline nature of biosynthesized nanoparticles. The diffracted intensities were recorded from 0 to 80º (2θ), the intense XRD peaks for AgNPs were observed corresponding to (111) and (200) planes at 38º and 44º and intense XRD peaks for CuNPs were observed corresponding to (111) and (311) planes at 14º and 32º of 2θ. Results showed that the synthesized particles have mixed phase (cubic and hexagonal). Guangquan *et al*., (2011) and Peter Logeswari *et al*., (2015) reported the XRD pattern of AgNPs which

showed the presence of sharp absorption peaks (111), (200), (220) and (311). Our results correlates with them all the facets. Our results also corroborates with Asmathunisha Nabikhan *et al*., (2010) who reported pure silver facets with main single peak at 38º, which is in good contract with the published XRD standard for AgNPs. Vasudev D *et al*., (2013) reported crystalline nature of CuNPs in the fcc structural confirmation by peaks at 2θ values of 22.3°, 25.9°, 28.3° and 44.8° in the XRD pattern corresponding to (111), (200), (220) and (311) planes using *Ocimum sanctum* leaf extract. Pramod Kulkarni and Vasudev Kulkarni (2014) reports the sample synthesized using *aegle marmelos* which established a high crystallinity level with diffraction angles of 21.09°, 28.8°, 29.20° and 34.10° which agree the characteristic fcc of CuNPs indexed at (111), (211), (211) and (220). Here our results resembles the previous available reports. Full width at half maximum (FWHM) data were used with Scherrer's formula to determine the average particle size and which was calculated as approximately 32 nm and 34nm for AgNPs and CuNPs respectively.

Fourier-Transform Infrared Spectroscopy (FTIR) is a technique widely used to obtain an infrared spectrum of emission or absorption of a solid, liquid or gas. It is rapid technique which can detect a range of functional groups present along with interested sample. In present study, AgNPs and CuNPs synthesized using silk worm fecal matter extract were centrifuged at 12,000 rpm for 15min and dried samples analysis were recorded on Brucker alpha-T FTIR spectrophotometer in the range of 400 to 4000 cm^{-1}. Results of FTIR study revealed the presence of different functional groups located at about 3368.11, 2929.68, 1599.99, 1393.20 and 1078.49 cm^{-1} for AgNPs and at 3383.40, 2926.56, 1639.89, 1405.69 and 1079.81 cm^{-1} for CuNPs.

These absorbance bands were known to be allied with the stretching vibrations for –C–H (alkane H), C-O stretch in esters, –C–C– [(in-ring) aromatic], –C–C– [(in-ring) aromatic] and C–O (polyols), respectively. In particular, the 1393.20 cm^{-1} band arises most probably from the C–O group of polyols such as hydroxyl flavones and catechins and the absorption peak at 3760 cm^{-1} indicates the presence of phenols (O–H group) and the presence of phenolic compound in the fecal extract. In adding to that it was assumed that the phenolic contents present in the extract might be involved in the bioreduction and production of AgNPs and CuNPs. Our results of FTIR spectroscopy coincides with the results of Suresh *et al.*, (2014). Vandana *et al*., (2011) reported the proteins present in the extract can bind to nanoparticles through either carboxyl groups or free amino acids in the proteins and also reports dissimilar functional groups absorbing representative frequencies of FTIR radiation. Gopal Suresh *et al.,* (2014) reported the possible biomolecules responsible for the reduction AgNPs by *Delphinium denudatum* root extract, using FTIR spectra by comparing the absence of functional groups in the extract and synthesized nanoparticles. Our findings also supports the results of Garima Singhal *et al*., (2011), Gopinath *et al*., (2014), Vasudeo Kulkarni *et al*., (2014) and Olajire *et al*., (2018). Thus, FTIR is an important tool for compound identification and structural elucidation. Scanning Electron Microscopy (SEM) was used to record the photomicrograph of biosynthesized AgNPs and CuNPs. A small volume of nanoparticles suspension were taken for SEM analysis on electromicroscope stub. The stubs were dried and pictures were taken by random scanning of stubs. The resulted SEM images shows the distribution of AgNPs and CuNPs synthesized using silk worm fecal matter extract. SEM micrograph of synthesized nanoparticles reveals that these nanoclusters were aggregated with smaller nanoparticles, which exhibits good uniformity and the shapes

were found to be in mixed phase. The average diameter of synthesized nanoparticles were observed about 21-80nm and 20-60nm for AgNPs and CuNPs respectively. Monali *et al.,* (2009) and Krishnaraj *et al*., (2010) have reported fungus mediated and *Acalypha indica* leaf extract mediated synthesis of AgNPs having polydisperse with spherical nature. Khan *et al*., (2018) reported that the most of the AgNPs were predominantly in spherical shape and have smooth surface with close compact arrangement and well dispersed. M Jayandran *et al*., (2015) reported that the biosynthesized CuNPs were clearly showed mixed phase containing cubic and rod shaped morphology. P Heera *et al.,* (2015) and G. Valli and S. Geetha, (2016) reported that plant extract mediated synthesis of CuNPs having uniformed spherical shape and were assembled by the aggregation of smaller nanoparticles. Our results are in accordance with the previous results that the synthesized AgNPs and CuNPs contain mixed phase of spherical and other natures.

4.1.2. Biological activities of synthesized nanoparticles

4.1.2.1. Antibacterial activity

4.1.2.1.1 AgNPs

In present study, Antibacterial effect of synthesized AgNPs against both gram-negative (*S. typhi*) and gram-positive (*S. aureus*) microorganisms were carried out at varied concentrations of AgNPs in liquid broth medium. Effect of synthesized AgNPs was witnessed in dose-dependent manner, it was observed that, as the concentration of biosynthesized AgNPs increased, bacterial growth decreases in both cases of *Salmonella typhi* and *Staphylococcus aureus*. Effect of AgNPs was observed more on gram-negative microorganism in comparision with the gram-positive one. From the antibacterial analysis important point was observed that antibacterial activity of

AgNPs synthesized using Silkworm fecal matter was superior to silver nitrate as well as standard antibiotic Ampicillin, these results were showing stronger activity than Garima Singhal *et al*., (2011) report on antibacterial activity of AgNPs synthesized using *Ocimum sanctum* leaf extract, the method which we followed to conduct antibacterial assay. I Maliszewska and Z Sadowski, (2009) reported fungal mediated synthesis of AgNPs which were more active against gram-positive *Staphylococcus aureus* and *Bacillus cereus*. Erick Pazos-Ortiz *et al*., (2017) reported dose-dependent antimicrobial effect of chemically synthesized AgNPs against four bacterial pathogens using disk diffusion method. Ravindra B *et al*., (2012) had reported plant mediated AgNPs showing more antibacterial activity on Gram-negative bacteria the Gram-positive ones. Our findings are quite superior to previous ones which we have reported here.

4.1.2.1.2. CuNPs

In present study, the antibacterial study of biosynthesized CuNPs was conducted against both gram negative and gram positive pathogenic bacteria such as *Bacillus subtilis* (gram positive), *Salmonella typhi* and *Klebsiella pneumoniae* (gram negative) using disk diffusion method. These results were effective and has shown dose-dependent growth inhibition of bacterial pathogens, when the concentration of CuNPs was increased, there was observed to be increase in the zone of inhibition. The effect of biosynthesized CuNPs was observed to be more on gram negative bacteria when compared to gram positive bacteria. This was mainly due to the differences in bacterial pathogen's membrane structures. The maximum ZOI values observed was 11mm in *Klebsiella pneumoniae* bacteria for 100µg/mL concentration of CuNPs. The ZOI values observed by *Salmonella typhi* and *Bacillus subtilis* was found to be 10 and

8mm respectively. In the previous reports of Sapna Thakur *et al*., (2014) and M Goinath *et al*., (2014), the Zone of Inhibition was observed more for *Staphylococcus aureus* in comparision with other tested pathogens for medicinal plants leaf mediated synthesis of CuNPs and the nanoparticles were more active on Gram-positive bacteria. Li Q *et al*., (2019) and Appu M *et al*., (2014) reported that the biosynthesized CuNPs were more active on inhibiting the growth of Gram-negative bacteria when compared with the Gram-positive ones.

4.1.2.2. Antioxidant assay of AgNPs

4.1.2.2.1. DPPH Free Radical Scavenging Assay

The significant antioxidant potential of AgNPs was investigated by DPPH radical scavenging assay. Trolox was used as a standard. The purple solution containing DPPH turns yellow on addition of AgNPs, which indicates the scavenging of free radicals and reducing ability of nanoparticles [K Singh *et al*., 2014]. Our findings for DPPH scavenging assay exhibited effective inhibitory activity of AgNPs when compared with the standard Trolox in dose-dependant manner. Kharat and Mendhulkar, (2016) reported the antioxidant activity of synthesized AgNPs using DPPH assay and observed the antioxidant capabilities of photosynthesized nanoparticles. They suggested that photosynthesized NPs could be used as a potential free radical scavenger. Priya *et al*., (2016) reported in vitro antioxidant assay of biosynthesized nanoparticles from *P. pinnata* extract and found significant free radical scavenging potential. Patra and Baek, (2016) validated the presence of strong antioxidant activity in terms of DPPH radical scavenging. The previous and our results strongly recommend the application of AgNPs as convenient natural

antioxidants for health safeguarding against many oxidative stress allied with degenerative diseases.

4.1.2.2.2. Hydrogen Peroxide Scavenging Assay

In living systems, uninhibited accumulation of H_2O_2 leads to the expansion of oxygen free radicals like hydroxyl and peroxide radicals which could cause huge damage to cell membranes. In present study, at the initial stages Ascorbic acid was showing moderately higher scavenging activity than synthesized AgNPs and at the concentration of 100 µg/ml, AgNPs attained more activity than the standard. Hence, the results confirms that the AgNPs has 86.98% hydrogen peroxide scavenging activity while the standard Ascorbic acid has 84.68% hydrogen peroxide scavenging activity. These properties of silver nanoparticles occur due to the presence of functional groups on the surface of silver nanoparticles [Anand kumar Keshari *et al*., 2018]. In an earlier report, *S. torvum* fruits were used for the synthesis of AgNPs, further the hydroxyl scavenging activity was observed in dose dependent manner [B Sundararajan *et al*., 2016]. Vanmathi *et al*., (2014) further demonstrated the hygraogen peroxide scavenging activity of fungus mediated biosynthesized AgNPs, which in turn strengthen the fact of AgNPs scavenging activity. In fact, antioxidant evaluation is necessary for AgNPs before its use in in-vivo models and as well as human applications.

4.1.2.3. Cytotoxic effect of synthesized nanoparticles

Cytotoxic effect of AgNPs and CuNPs have shown very significant result on human lung adenocarcinoma cell line (A549 human alveolar basal epithelial cells), these are one of the causes for human lung cancer. Lung cancer, typically non-small

cell lung cancer (NSCLC), is the foremost cause of cancer deaths; however, proficient treatments for NSCLC remain insufficient. In present study, both synthesized nanoparticles depicted very good dose-dependent cytotoxic effect against cell line, percentage of viable cells was decreased with the increased concentration of nanoparticles. However, AgNPs were found comparatively more toxic to human lung adenocarcinoma cell line than CuNPs. Several earlier studies have been carried out by the researchers to evaluate the cytotoxic effect of nanoparticles on different cell lines, most of them are succeeded with their results [Sankar R *et al*., 2013 and Ill-min Chung *et al*., 2017]. Sima *et al*., (2017) reported cytotoxic effect of green synthesized CuNPs on normal human peripheral blood cells using MTT assay, they found 83% of viable cells for 100µg/ml. Teresa Ostaszewska *et al*., (2018) depicted upright cytotoxic effect of AgNPs and CuNPs on rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Seoyoung Park *et al*., reported about cellular toxicity of various metal nanoparticles on human alveolar epithelial cells and they were succeeded in inhibiting the growth of cells [Seoyoung Park *et al*., 2017]. In recent days, exposure to biosynthesized nanoparticles indicated a range of cytotoxic responses to different number of cancer cell lines. Results of this study demonstrated that the MTT assay could be implemented as sensitive and effective tool to assess cytotoxicity of AgNPs and CuNPs on human alveolar basal epithelial cell lines.

4.2. Isolation, identification and inhibition of pathogenic fungus

4.2.1. Isolation and identification

In the present investigation, the fungal species was isolated from bud rot affected coconut plant using PDA medium. The fungal species was identified as *Phytophthora palmivora* by its molecular characterization. The sequence of the

isolated has shown upto 98% identity with other *Phytophthora* species. It is well-known that molecular classification is a fast procedure which requires least management of pathogens and also supports in distinguishing morphologically, similar fungal species. Similar applications of PCR technology were used for detection and identification of fungi, by using an internal transcribed spacer (ITS). *Phytophthora palmivora* is the main cause for Bud rot disease in coconut, which causes huge loss to economy of farmers. Rashmi *et al*., (2010) and K M Sharadraj *et al*., (2016) and many other researchers reported the isolation of *Phytophthora palmivora* which caused bud rot and root rot in coconut. In 2008, B Srinivasulu and coworkers made a complete report on *Phytophthora palmivora* isolation from bud rot caused tissue of coconut and well written about the disease management [B Srinivasulu *et al*., (2008)]. Including Bud rot of coconut, *P. palmivora* is one of the reason for many deadly diseases of other plants. In the same way, Hung *et al*., (2015) identified *P. palmivora* from root rot symptoms of Citrus maxima in Thailand. Serious root rot disease of citrus and papaya caused by *P. palmivora* has been recorded in India [Graham and Timmer, 1992] and America [Zitko *et al*., 1991 and Naqvi, 2004]. Mounde LG *et al*., (2012) also isolated and characterized *Phytophthora* species which caused Citrus Gummosis in Kenya. Suskiri *et al*., (2018) reported *Phytophthora* form Durian Orchard in Chumphon Province, Thailand. Root rot and stem rot are the major disease of durian orchard, which is caused by *Phytophthora* species. Root rot of Pomelo caused by this pathogen was observed in Thailand [Phung Manh Hung *et al*., 2015]. There are many other major diseases are associated with *Phytophthora* species which causing major loss to agricultural and ornamental crops. Hence, the fungus should be inhibited to avoid loss for economy.

4.2.2. Inhibition of *Phytophthora palmivora* using biosynthesized nanoparticles

Biosynthesized nanoparticles showed an effective range of antifungal activity against *Phytopthora palmivora.* Antifungal activity of synthesized nanoparticles was determined using Radial Growth Technique [V. K. Bajpai *et al.*, 2007]. As *P.Palmivora* shows strong dose dependent response over the addition of AgNPs and CuNPs. There was an increase in the percentage of inhibition with the increase in the concentration of biosynthesized nanoparticles. In present study, both AgNPs and CuNPs were proven to be good antifungal agents, where AgNPs established remarkable inhibition activity against the pathogenic fungi *P. palmivora* than CuNPs and standard Fungicide (Fluconazole). As previous reports suggests, biosynthesized nanoparticles were already proved that they are remarkable antifungal agents against many pathogenic fungus like *Candida albicans* and *Aspergillus niger* [Kumarasamyraja *et al*., 2013] and *Rhizoctonia solani*, *Fusarium oxysporum*, *Sclerotinia sclerotiorum* and *Sclerotium rolfsii* [Kaman *et al*., 2018]. Nida *et al*., reported antifungal activity against *Candida glabrata*, *Candida albicans* and *Candida tropicalis* using fungal mediated nanoparticles in disc diffusion method [Nida et al., 2016]. However, there is a report available on antifungal activity of nanoparticles against pathogenic fungus causing red root rot disease in tea plants [Ponnuswamy *et al*., 2016]. This study is the first trial of biosynthesized nanoparticles as antifungal agents against this bud rot causing pathogenic organism *Phytophthora palmivora*. Our inhibition study stands in front with the previous reports on inhibition of *P. palmivora* reported by M. Senthil *et al.,* (2013) and Gills-Alex Pakora *et al.,* (2017).

4.3.1. Screening of pathogenic fungus for different cell wall degrading enzymes like Pectinases and Cellulases

There are many different methods were employing for screening of microbes for the production of enzymes. In present study, the isolated pathogenic fungus *Phytophthora palmivora* was screened for the production of Pectinases and Cellulases enzyme using cup plate assay and components with suitable substrate. Clear zone was observed around colony of an organism after staining with gram's iodine for pectinase and cellulase enzyme. Clear zone indicates the capability of pathogenic fungus to produce pectinase and cellulase enzyme, this clear zone around the colony was due to utilization of substrate by the enzyme secreted by the fungus. On the basis of screening program, *P. Palmivora* was proceeded for further production. Cup plate assay is a common technique which is normally used for the screening of microbes for their capability to produce enzymes [John H carder, 1986]. Whether the microbe is poor or rich producer can be decided by the diameter of zone around the colony [Devu *et al*., 2018]. The plate assay approach which is used in this study has been employed for screening and isolating many enzyme producers [Hankin *et al*., 1971].

4.3.2. Optimization and partial purification of Pectinase and Cellulase enzymes

For optimization of pectinase and cellulase enzymes, the flasks were observed for 14 days and they were withdrawn for every alternative days to check the enzyme activity using DNS method (Miller, 1959). By changing different parameters one at a time by keeping others constant, the production media was optimized [Suhaimi *et al*., 2016].

For pectinase enzyme production, the optimized parameters were as follows: the optimized days of incubation for highest enzyme activity was 6, similarly optimum pH of the medium was 6, optimum temperature for enzyme production was found to be 30ºC, similarly maximum enzyme activity was observed for 5% Orange peel substrate. Partially purified Pectinase enzyme produced from *Phytophthora palmivora* has shown the molecular weight of around 25 kDa. 34kDa pectinase form *Aspergillus niger* using banana peel as substrate was partially purified and shown relatively enhanced activity at optimum temperature of 32ºC [Sumi Barman *et al*., 2015]. *Neurospora crassa* produces a 37 kDa low molecular weight Pectinase was isolated and characterized [Maria de Lourdes *et al*., 1991]. Fungal mediated production of Pectinases is almost of the commercial preparations and *A. niger* is the most common fungal species for the industrial production of pectinases [Gummadi and Panda, 2003]. Microbial Pectinase can be listed as the most significant enzyme for the juice industry. Although, production of pectinase is an inherent property of most of the organisms, only those microbes that produce a considerable amount of extracellular pectinases are of industrial prominence and have been used commercially [Yogesh Khairnar *et al*., 2017].

For cellulase enzyme production, the optimized parameters were as follows: the optimized days of incubation for highest enzyme activity was 8, similarly optimum pH of the medium was 6, optimum temperature for enzyme production was found to be 32ºC, similarly maximum enzyme activity was observed for 1% CMC (Carboxymethylcellulose). Here, CMC indicated as a good producer of cellulolytic activity. Partially purified Cellulase enzyme produced from *Phytophthora palmivora* has shown the molecular weight of around 40 kDa. Chethan Gupta and coworkers

reported the optimum temperature for fungal mediated 38 kDa cellulase production was 32ºC [Chethan Gupta *et al*., 2015]. Considerable number of cellulase has been produced from fungal species such as *Penicillium* sp. [Picart *et al*., 2007, Prasanna *et al*., 2016], *Aspergillus terreus* M1 [Gao *et al*., 2008], *Aspergillus niger* [Baig and Saleem, 2012], *Aspergillus ornatus* [Toor and Ilyas, 2014], *Aspergillus terreus* MS105 [Sohail *et al*., 2016], *Aspergillus niger* and *Rhizopus* sp. [Santos *et al*.,2016], *Trichoderma longibrachiatum* [Pachauri *et al*., 2017] and *Beauveria Bassiana* [Petlamul *et al*., 2017]. For the past few decades, cellulases has been widely studied for their importance in textile industry, paper industry, animal feed, bio-fuel as renewable energy source and detergents [Muhammad *et al*., 2016]. Due to overwhelming demand for natural products, significance of industrial enzymes has been elevated, among them cellulases occupy a pivotal position [Sajith S *et al*., 2016].

4.4. Inhibition of Pectinase and Cellulase enzymes using synthesized nanoparticles

Enzyme activity inhibition of biosynthesised silver and copper nanoparticles against fungal cell wall degrading enzymes has been studied. The inhibition effect of synthesized nanoparticles were determined using DNS method of estimating reducing sugar released during the reaction (Miller, 1959). Enzyme activity has shown dose dependent response on addition of nanoparticles, as the concentration of NPs increases, inhibition of enzyme activity increases. Nowadays inhibitory effect of heavy metals and other chemical inhibitors on enzyme activity of industrial important enzymes produced from various microorganism, nanoparticles mediated enzyme inhibition studies are very few. In this point of view, synthesized silver and copper nanoparticles were evaluated against fungal mediated cell wall degrading enzymes

under laboratory condition. AgNPs have shown more inhibitory effect on Cellulase enzyme than CuNPs comparatively, where CuNPs shown more effect on pectinase enzyme than AgNPs. Nicholas S Wigginton *et al*., (2010) discussed about the binding of silver nanoparticles to bacterial proteins and their enzyme inhibition depends on surface modifications. Sang-Ho Cha *et al*., (2015) also reported about the Shape-Dependent Biomimetic Inhibition of Enzyme by Nanoparticles with chemically synthesized nanoparticles. There is a report on evaluation of Enzyme activity inhibition of biosynthesized nanoparticles against some microbial extracellular enzymes using DNS method [S. Karthick Raja Namasivayam *et al*., 2016]. From the available literature, this is very rare approach of inhibiting cell wall degrading enzymes from biosynthesized nanoparticles and we have got significant results for nanoparticles on inhibiting cell wall degrading enzymes produced from pathogenic fungi *Phytophthora palmivora* which causes bud rot disease of coconut.

Conclusions

The development of eco-friendly methods in nanomaterial synthesis is of considerable significance to expand their biological applications. This investigation on biosynthesis of nanoparticles has greatly increased our understanding of their applications like antibacterial, antioxidant, cytotoxicity, antifungal and most importantly inhibition of cell wall degrading enzymes produced from pathogenic fungus. Nanotechnology offer diverse potential applications for many biotechnological as well as industrial processes. Thus, the search and discovery of novel nanoparticles and their applications has become challenge for many industries. The advancement in the discovery of biosynthesized nanoparticles using plant extracts, microbes, etc., has resulted by their increased use in numerous novel applications and it delivers clear contribution in expansion of new technologies.

Biosynthesized Silver and Copper nanoparticles have also made a noteworthy impact in industrial and biotechnological processes. In relate to this, we undertook the investigation on synthesis of silver and copper nanoparticles using silk worm fecal matter and characterization of nanoparticles for their biological applications. The AgNPs and CuNPs were synthesized using aqueous extract of silk worm fecal matter as a reducing agent with suitable procedure. The synthesized nanoparticles were characterized, UV-Vis absorption peak at 412nm confirmed the production of AgNPs and absorption maximum at 560nm confirmed the production of CuNPs. The reduction in the concentration of silver ions and copper ions during synthesis, which in turn indicates the production of nanoparticles was monitored using AAS, which was due to bio-reduction capability of silk worm fecal matter. FTIR characterization of synthesized AgNPs and CuNPs detected the functional groups present along with the nanoparticles. XRD further confirmed the

nanoparticles with the sharp characteristic peaks of AgNPs and CuNPs. XRD and SEM resulted in the size of crystallite and particles respectively. Synthesized AgNPs were tested for their antibacterial activity against both gram-negative and gram-positive bacteria in liquid broth culture. The effect was observed in dose dependent manner and AgNPs were shown excellent activity against gram-negative bacteria and gram-positive and the activity of AgNPs was superior to $AgNO_3$ and standard Ampicillin. Similarly, antibacterial activity of CuNPs was evaluated using disc diffusion method. Like AgNPs, the effect of CuNPs was also in dose dependent manner and was observed to be more on gram-negative bacteria. The maximum Zone of Inhibition values observed was 11mm in *Klebsiella pneumoniae* for 100µg/ml concentration of CuNPs. The synthesized AgNPs were evaluated for their antioxidant property. The assay was conducted using two methods namely, DPPH Free Radical Scavenging Assay and Hydrogen Peroxide Scavenging Assay. AgNPs were found to be potential antioxidants than standard Trolox in a dose dependent manner in DPPH Free Radical Scavenging Assay. Where as in Hydrogen Peroxide Scavenging Assay, the activity of Ascorbic acid was slightly higher than AgNPs up to the concentration of 90µg/ml and at the concentration of 100µg/ml, the activity of AgNPs was higher than standard Ascorbic acid. Cytotoxic effect of biosynthesized AgNPs and CuNPs were evaluated on human lung adenocarcinoma cell line (A549 human alveolar basal epithelial cells) and they have shown very significant result, as the concentration of nanoparticles was increased, there was observed to be decrease in viability of cells. AgNPs exhibited more toxic effect on human lung adenocarcinoma cell line than CuNPs.

Bud rot causing fungus was isolated from infected tissue of coconut using suitable method. The molecular characterization identified the isolated fungus species as *Phytophthora palmivora*, in which the sequence has 98% homology with the similar *Phytophthora* species. The isolated species was found to be the main reason for bud rot disease of coconut and there was a demand for the inhibition of *Phytophthora palmivora*. In present study, the inhibition effect of AgNPs and CuNPs synthesized using silk worm fecal matter was studied against *Phytophthora palmivora* using radial growth technique. The pathogenic fungus showed dose dependent response on addition of nanoparticles. In our study, both synthesized AgNPs and CuNPs were observed as very good antifungal agents. The Percentage of Mycelial Growth Inhibition of fungus was 86%, 70% and 80% for AgNPs, CuNPs and Standard Fungicide respectively for the concentration of 3mg/ml and AgNPs were found as potent antifungal agent against *Phytophthora palmivora* than CuNPs and standard Fungicide. Cell wall degrading enzymes produced by pathogenic fungus were found to play an important role in penetration of organism inside the plant tissue, hence it is critical to inhibit those enzymes using synthesized nanoparticles. Hence, the isolated *Phytophthora palmivora* was screened for different cell wall degrading enzymes, clear zone was observed around the fungal colony for pectin and CMC substrate, which indicated the production of Pectinase and Cellulase enzyme. The production parameters of pectinase and cellulase enzyme were optimized by changing different parameters one at a time by keeping others constant and the enzyme activity was checked using DNS method. The pectinase enzyme was produced from *Phytophthora palmivora* using 5% Orange peel as substrate and it was partially purified using anion exchange chromatography. Similarly, the cellulase enzyme was produced using 1% CMC and it was partially

purified using anion exchange chromatography. The molecular weights of Pectinase and Cellulase were determined to be 25 kDa and 40 kDa. The inhibition of partially purified cell wall degrading enzymes was evaluated using synthesized AgNPs and CuNPs as enzyme inhibitors. It was observed that, as the concentration of nanoparticles increased there was observed to be increase in inhibition of enzyme activity of both pectinase and cellulase. Comparatively, AgNPs were found more effective in inhibiting enzyme activity of Cellulase enzyme than CuNPs and CuNPs were found to be effective on inhibiting Pectinase enzyme activity than AgNPs. In future, these nanoparticles could be mold for best antibacterial, antioxidant, cytotoxic, antifungal and enzyme inhibition agents. So these novel nanoparticles could be used as useful drug for bud rot disease, which in turn help the farmers to get good yield in coconut crop and it could boost the economy. Finally, this piece of investigation is useful in many industrial and biotechnological applications. This work will elongate some of the natural resources, which could exploit green synthesis of novel nanoparticles for useful applications in the field of agriculture and medicine.

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List of Papers

Papers Published

1. **Avinash Basavarajappa** & Shivayogeeswar E Neelagund. "An Investigation on Antibacterial Efficacy of Biosynthesized Novel Copper nanoparticles using Silkworm fecal matter". *Imperial Journal of Interdisciplinary Research* (IJIR), Vol-2, Issue-12, 2016. ISSN: 2454-1362.
2. **Avinash B** & S E Neelagund. "An Investigation on Antibacterial and Free Radical Scavenging Efficacy of Biosynthesized Silver Nanoparticles Using Silkworm Fecal Matter (Bombyx mori-L)". *Journal of Bionanoscience*, Vol. 11, Issue-01, 2017. ISSN: 1557-7910.
3. Kotresh K Rajashekarappa, Shivayogeeswar Neelagund, M.C. Mahesh, **Avinash Basavarajappa**. "Immobilization of Hyperthermostable α -Amylase Using Magnetite [Fe_3O_4] Nano Particle to Promote the Properties for Industrial Applications". *Journal of Bionanoscience*, Vol. 12, Issue-05, 2018. ISSN: 1557-7910

Papers Communicated

1. Isolation, identification and molecular characterization of pathogenic fungus causing bud rot disease in coconut and its inhibition using nanoparticles synthesized using Silkworm fecal matter.
2. Screening of *Phytophtora palmivora* for different cell wall degrading enzymes like Pectinases and Cellulases and their optimization of different parameters of production.
3. Partial purification of Pectinases and Cellulases from bud rot disease causing fungi *Phytopthora palmivora* and their inhibition by nanoparticles synthesized using Silkworm fecal matter.

Poster and Oral presentations in National and International Conferences

1. **Oral presentation** at National conference on **"Green Chemistry-Need of the Universe"**, held on 28th February 2015, at Sri Shivalingeshwara Swamy Govt. First Grade College & PG Centre, Chennagiri, Davangere, Karnataka.
2. **Poster presentation** at International conference on at **"Nanoscience, Nanotechnology and Advanced Materials"** held on14th, 15th, 16th and 17th December 2015, at GITAM University, Vishakhapatnam, Andhra Pradesh.
3. **Oral presentation** at International conference on **"Nanotechnology: the fruition of science ICON-2017"**, held on 15th and 16th February 2017, at Nesamony Memorial Christian college, Marthandam, Tamilnadu
4. **Poster presentation** at International conference on **"Green Chemical and Nanotechnology Opportunity and Challenges-2017"**, held on February 27th and 28th 2017, at St. Allosius College, Mangalore, Karnataka
5. **Poster presentation** at National Conference on **"Recent trends in Applied Science & Technology (RTAST-2017)"**, held on Oct 26th and 27th 2017, at Alliance College of Engineering & Design, Bangalore.