UTILIZATION OF FOOD WASTE TO PRODUCE α AMYLASE AND SYNTHESIS OF α AMYLASE- NANOPARTICLES COMPOSITE FOR THE DEGRADATION OF TEXTILE DYE

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ABSTRACT

Green synthesis of nanoparticles has bought the trend of using biomaterials to synthesize a variety of metallic nanoparticles. The current study reports the use of α amylase crude extract obtained by *Aspergillus niger* to synthesize copper oxide nanoparticles. Characterization of CuO NPs was carried out using UV-Vis spectroscopy, scanning electron microscopy (SEM), Energy dispersive x-ray (EDX) spectroscopy and Fourier transform infrared spectroscopy, while the effectiveness of CuO NPs as catalytic agents for degradation of textile dye was evaluated. FTIR analysis shows protein molecules were responsible for capping and stabilization of nanoparticles while EDX analysis showed the presence of oxygen with copper. TEM analysis showed that particles size were less than 20nm. 100 µg/ml and 150 µg/ml doses of CuO NPs were found effective to degrade the red dye of different concentrations (1ppm, 2ppm, 5ppm, 10ppm, 15ppm, 20ppm) at different time intervals. 100% dye degradation was achieved at dose of 100 µg/ml dose at 1ppm of dye concentration. This study has demonstrated utilization of food waste into production of a significant and cost-effective product i.e. α amylase-CuO NP composite.

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LIST OF ABBREVIATIONS

α	Alpha
Ag	Silver
Au	Gold
BW	Bread Waste
DLS	Dynamic Light Scattering
EDX	Energy Dispersive X-Ray Spectroscopy
FTIR	Fourier Transform Infrared spectroscopy
HRTEM	High Resolution Transmission Electron Microscopy
NPs	Nanoparticles
PDA	Potato Dextrose Agar
SCB	Sugarcane bagasse
SEM	Scanning Electron Mircroscope
SPR	Surface Plasmon Resonance
SSF	Solid State Fermentation
TEM	Transmission Electron Microscopy
TiO_2	Titanium dioxide
WB	Wheat bran Silver
XRD	X-ray diffraction
UV-VIS	Ultravoilet Visible spectroscopy

CHAPTER 1

INTRODUCTION

Food waste is one of the major sustainability issues causing negative impacts on the environment, food security and economy. It is estimated that about 1.3 billion tons of food waste are generated worldwide and is becoming a serious economic and environmental challenge. In Pakistan, approximately 36 million tons of food go to waste every year [1]. Food waste also involves the waste of resources which are involved in the whole process of food production, packaging and marketing. Food processing and production are resource-intensive, the food waste indirectly leads to a wide range of environmental impacts on water, land and energy resources. Air pollution and greenhouse emissions occur during the processes of food production, storage, transportation and waste management [2-3]. Though food wastes have negative impacts these waste biomaterials can be utilized to produce bioactive products; as food wastes contain valuable compounds like proteins, lipids, dietary fibers and micronutrients which can be converted to produce valuable products through conversion processes [4].

Bread is a staple food that is globally produced at 100 million tons per year but has been one of the highest food waste categories as hundreds of tones are wasted daily [5]. Bread waste (BW) is commonly generated food waste in developed and under-developing countries including European countries, UK and Pakistan. Bread consists of starch through which extraction of fermentable sugars can be easily done therefore, these high-quality sugars and starch composition make bread waste an absolute substrate for the microbial fermentations to produce valuable bio-products such as biofuels, bioplastics, biomolecules (enzymes) and renewable products [6]. BW is considered a rich source of high-quality fermentable sugars from other nutrients as it makes an ideal substrate for biorefineries and metabolic processes [7]. Enzymes are industrial biocatalysts that present notable benefits over traditional chemical processes for process efficiency and productivity [8]. An enzyme is described as a protein that is synthesized as intra and extra-cellular compounds. The

enzyme catalyzes and accelerates a biochemical reaction with high specificity and enhances the rate of reaction. Amylase is an industrial enzyme that breaks down glycogen and starch. The amylase can be yielded from different bio sources like microorganisms, plants and animals. The major benefit of producing amylase from microbial fermentation is economically cheap bulk production and obtaining enzymes of desired characteristics. The microbial amylases significantly meet industrial demands as they have replaced the chemical hydrolysis of starch and are easily commercially available [9]. α-amylase is an enzyme which is currently used in a wide range of industrial processes such as food, textile and other industries for the improvement of product yield and utilization of raw materials i.e. food/ agriculture waste. It is considered a key enzyme as it has a share of around 25% in the enzyme market for its extreme importance in processes and the generation of valuable products [10]. Amylase enzymes are hydrolases that act on α-1, 4-glycosidic bonds. They are subdivided into α, β and γ amylases. α-amylase is an enzyme that acts as a catalyst for hydrolysis of α-linked polysaccharides into anomeric products. The α-amylase is associated with endo-amylases that catalysis the initial hydrolysis of starch into shorter oligosaccharides through the cleavage of α-D-(1-4) glycosidic bonds [11]. The chemical structure of α-amylase is shown in Figure 1.1

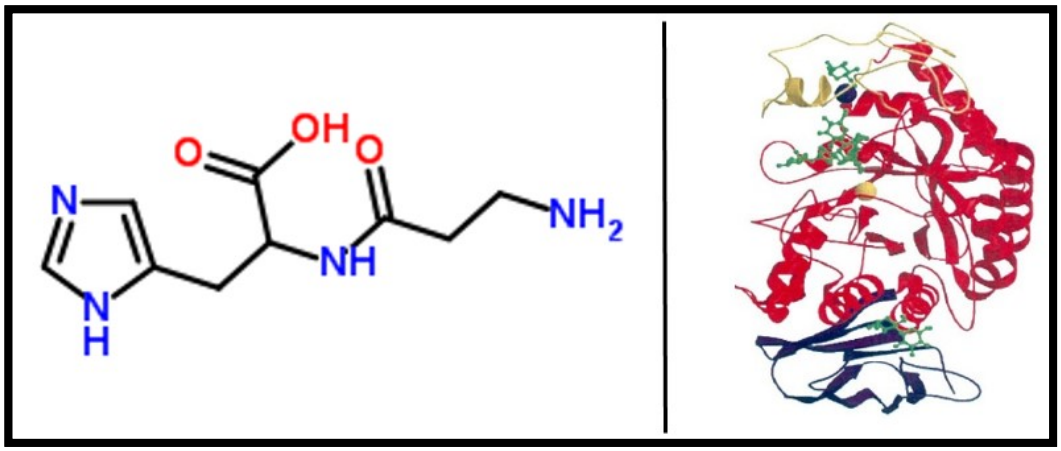


Figure 1.1 Chemical structure of alpha amylase [11]

Due to the starchy composition of BW, it is an ideal substrate for the production of α-amylase by solid-state fermentation (SSF). Bread waste Microbial valorization is a new biotechnological approach to waste processing into renewable raw material for bio based industry. Studies report that bread waste can be valorized by cultivating lactic-acid bacteria and sporulating fungi. Benabda et al. [12] reported the successful utilization of BW for microbial fermentation as media contains different nutrients. Solid-state fermentation is an eco-friendly approach in which low moisture levels can be used with agro-industrial waste as substrate [13]. SSF is the process that takes place in a solid matrix (substrate support), where microorganisms grow in the absence of free water or with low content of free water as the substrate requires moisture for the growth and metabolic activity of microorganisms.

Currently, SSF is gaining more attention because of its wide range of applications in the valorization of inactive organic waste. Different environmental problems are arising around the world due to the high production of biomass, SSF is used for utilizing this biomass and converting it into large-scale production of metabolites like enzymes, biomolecules, antibiotics and biosurfactants etc. [14].

Microorganisms notably used in SSF are fungi (*Aspergillus, Rhizopus, Penicillium, Trichoderma* and *Fusarium*), bacteria (*Bacillus* and *Lactobacillus species*) and yeasts [15]. Many studies have used BW as a substrate for the production of enzymes like α-amylase, glucoamylase and protease by using fungal cultures and bacterial cultures i.e. *Aspergillus, Rhizopus* and *Bacillus spp* [16-18]. Microbial valorization of bread waste is

Furthermore, a lot of cost-effective bio-conversion processes are under investigation which highlights the importance of utilization of food waste, generated worldwide to produce biomolecules i.e. enzymes which have many industrial applications. Figure 1.2 shows bread waste used as the substrate for different microbial cultures for amylase enzyme production.

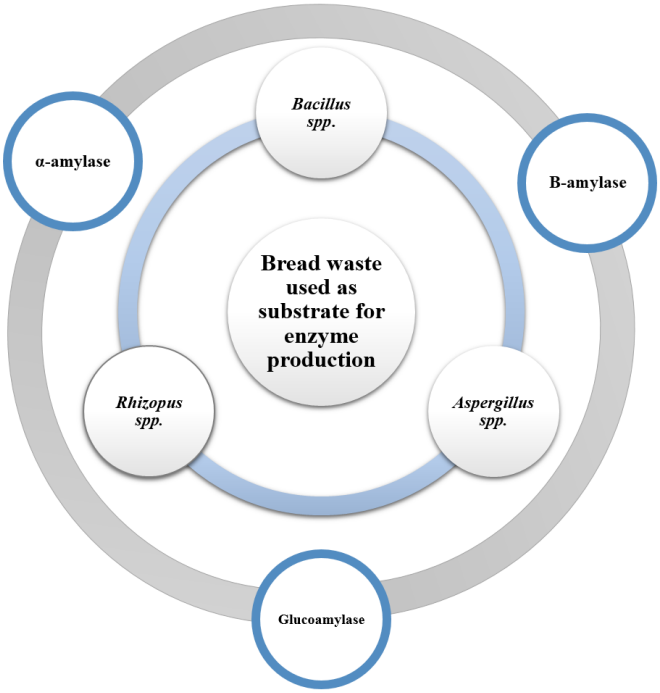


Figure 1.2 Bread waste used for different microbial cultures for enzyme production

Nanotechnology has various applications in multi-disciplinary fields of science such as environment, agriculture and pharmacology. Innovative solutions for different scientific problems have been investigated via nanotechnology and received researchers' attention worldwide. There are physical, chemical and biological methods for the synthesis of NPs, among them biological method is the most easy, fast and eco-friendly method. Physical methods produce high precision and good-quality nanoparticles but these methods are very expensive as the yield obtained is very low [19]. Chemical methods involve toxic and harsh reducing agents like elemental hydrogen, sodium citrate and borohydride though they are easier and faster synthesis is obtained but biological methods are considered reliable and eco-friendly [20]. The increasing demand for nanoparticles must be followed by green synthesis methods as it involves the use of plants, biomolecules, microorganisms and agricultural waste [21]. Nanoparticles produced by biomolecules i.e. enzymes, and proteins lead towards a sustainable approach. Figure no 1.3 shows the non-toxic biological approach in nanotechnology.

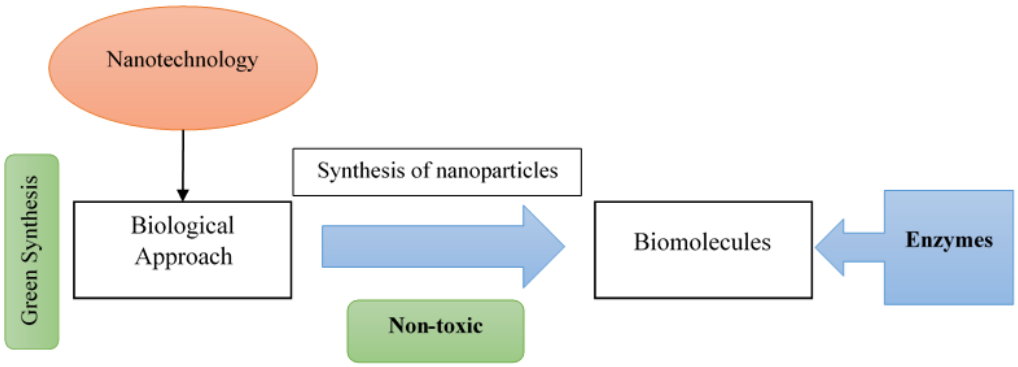


Figure 1.3 Non-toxic biological approach in nanotechnology

Green synthesis of metal nanoparticles is receiving great attention as an emerging field of bionanotechnology as it is a non-toxic approach owning the benefit of cost-effectiveness, environment friendliness, reliability and biocompatibility over physical and chemical methods [22]. According to Anastas and Warner [23], Green chemistry is a set of principles that stimulates the design of products and processes that reduce or eliminates the use and production of hazardous substances. Green synthesis is a bottom-up approach through which excellent nanoparticle synthesis can be obtained; a bottom up approach refers to the synthesis of nanoparticles through chemical reactions among atoms, molecules or ions [24].

Enzymes are biomolecules that provide unique benefits over traditional chemical processes for process efficiency, productivity and sustainability. These abundant biomolecules exhibit a unique property that they can function outside of the cell which makes them promising and suitable for bio-nano technological applications [25]. Enzyme-mediated nanoparticles are achieved by catalysis, oxidation and reduction of metals [26]. The biosynthesized nanoparticles can play an important role in commercial-level applications [27]. Nanoparticles produced by microbial synthesis involving the utilization of raw materials and waste is a sustainable approach which has positive benefits. Many studies have documented the synthesis of metallic and metal oxide nanoparticles such as AgNPs, AuNPs, CuO NPs and TiO_2 NPs from bacteria, fungi and algae for various environmental applications like bioremediation, biodegradation and pollutant detection etc. This microbial cultivation and extraction are cost-effective and less time-consuming which can

provide an alternative way for the synthesis of nanoparticles [28]. The biosynthesized nanoparticles play an important role in the removal and degradation of dyes from industrial wastewater [29]. Enzyme nanoparticles reveal functional groups such as –NH2, -COOH, -OH, -SH2 in the side chain of amino acids; serine, aspartic acid, aspargine and cysteine. The enzyme nanoparticles are accumulated forms of enzyme molecules of an applicable grouping in a nano-scale of 10-100nm. Different applications show significant results due to characteristics exhibited by enzyme nanoparticles such as optical, thermal, chemical, electronic, catalytic and mechanical properties [30]. In Figure 1.4, different applications of enzyme-based nanoparticles are shown.

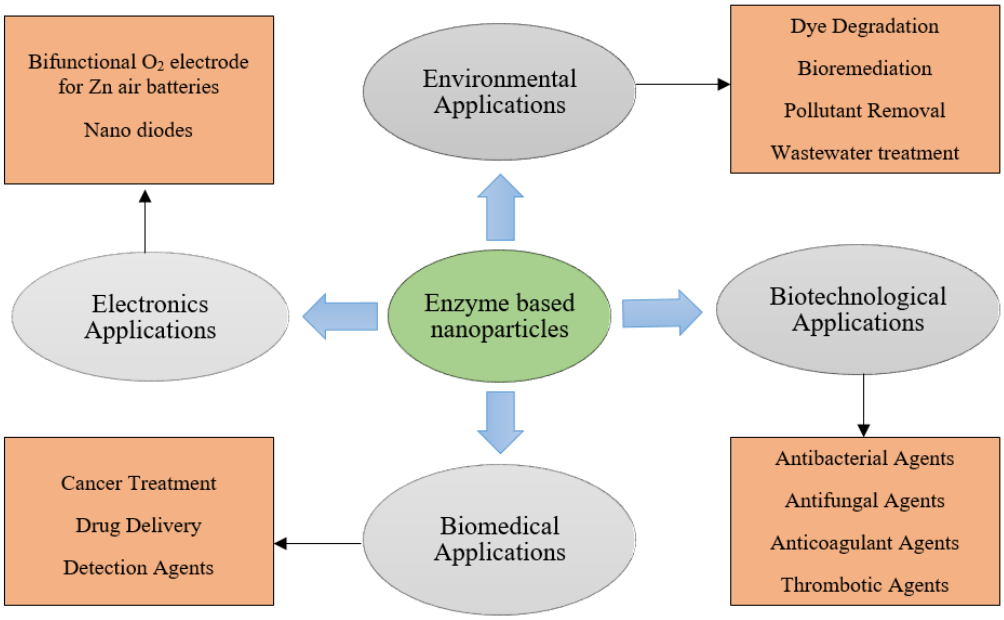


Figure 1.4 Different applications of enzyme based nanoparticles

Nanotechnology has bought new advances in the scientific field for the biodegradation of toxic textiles as these need to be eliminated from the water bodies to reduce pollution; nanomaterials exhibit significant properties like large surface area, chemical reactivity, faster adsorption equilibrium, mechanical strength and higher absorption capacity which make them excellent absorbents significantly for environmental applications. Nanoparticles like AgNPs, CuO NPs, ZnO NPs and

TiO_2 Nps show great antibacterial activity, photocatalytic activity and fast oxidation rate which makes them significant for treating toxic dyes present in industrial water [31].

Copper nanoparticles have gained major interest due to their significance such as cost effectivity, easy availability and similar chemical properties. The biological synthesis of CuO NPs is considerably less toxic, eco-friendly and cost-effective, unlike the physical and chemical synthesis of CuO NPs which is toxic and expensive. Among other nanoparticles, CuO NPs have major potential for applications like bioremediation, biodegradation and biomedical treatments [32]. Many studies have shown effective biodegradation of textile dyes such as methyl red, eosin dye and reactive red 81 dye by biosynthesized copper nanoparticles [33-34]. Figure 1.5 shows different environmental applications of green copper nanoparticles.

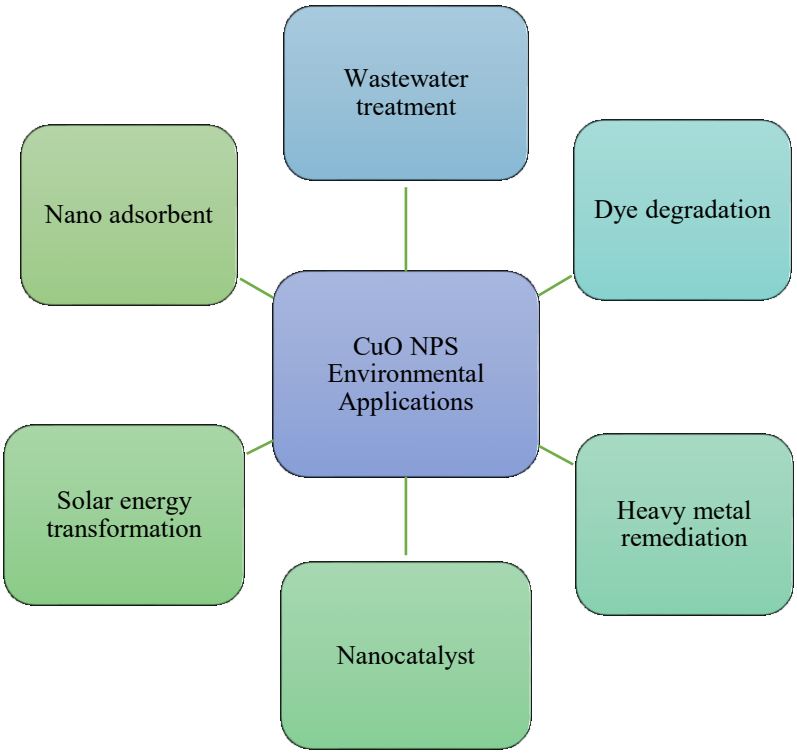


Figure 1.5 Environmental applications of CuO NPs

In this study, α amylase- copper oxide nanoparticles were synthesized and used for the application of biodegradation of red dye. The extraction of α-amylase was done via solid-state fermentation (SSF) by utilizing bread waste (BW) as a substrate medium for *Aspergillus niger*. The amylolytic activity of α-amylase was determined by DNS assay. Copper oxide nanoparticles were synthesized by crude α-amylase extract and applied to different concentrations of red textile dye solution for biodegradation.

RATIONALE

Food waste is a serious challenge worldwide. Annually about one third of the food produced is wasted or lost globally. The amount of food waste has increased due to over population and economic growth. Food waste reutilization strategy received growing interest as it contains high carbohydrate content and the other nutrients. So it could be a valuable resource for the production of value-added molecules or any other biocatalyst like enzymes. Enzymes are biocatalysts that have a significant range of industrial and environmental applications so conversion of food waste into valuable enzymes through fermentation processes is a highly sustainable approach. The aim of this study is the utilization of bread waste as substrate for *Aspergillus niger* for the extraction of α-amylase enzyme and synthesis of metal oxide nanoparticles (CuO) from produced enzyme extract. Further this nanocomposite will be applied for dye degradation. Thus, the main purpose is the utilization and conversion of food waste into production of an effective and cost-effective product.

OBJECTIVES

The objectives of this research study were as follows:

- Production of α-amylase from food waste i.e. bread waste using fungal culture.
- Synthesis of metal oxide nanoparticles using α-amylase extract.
- Application of synthesized nanoparticles for the biodegration of textile dye.

CHAPTER 2

LITERATURE REVIEW

Food waste is a serious problem all over the world. It is estimated that one-third of the food produced around the world is wasted every year. In Pakistan, tons of food go to waste while 43% of its population is food insecure. Food wastage is a major problem that the world must overcome so different studies are being carried out to make use of food waste being utilized as a valuable resource to produce value-added products or biocatalysts like biomolecules, enzymes etc. Enzymes are biocatalysts that have a significant range of industrial and environmental applications which can be helpful in daily life so valorizing and converting the food waste into enzymes through fermentation processes is a highly sustainable approach.

Enzymes are one of the key biomolecules that possess excellent properties for commercial production and environmental applications; α-amylase is considered a substantial enzyme at an industrial level which can be easily extracted through solid-state fermentation by different fungal cultures i.e. *Aspergillus niger, Aspergillus oryzae, Rhizopus oryzae* etc. that use bread waste as substrate which can be further purified to synthesize metal nanoparticles. Benabda et al. [35] extracted α-amylase and protease by administrating solid-state fermentation using humidified bread waste as substrate for *Rhizopus oryzae*. The protease and amylase production in 24 h was 1260 U/g and 16.2 U/g but in 120 h it maximized to 2412 U/g and 100 U/g which showed that BW used as substrate for the production of enzymes through SSF is of great interest in food industries and could be valorized as elevation supplement in bread making process. Similarly, Irshad et al. [36] produced α-amylase by solid-state fermentation using bread waste as substrate for *Ganoderma tsuage*. The enzyme activity achieved for α-amylase was 80 U/ml using BW as substrate medium under optimum temperature of 35^{o}C and maximum activity of 83 U/ml was achieved at pH 6 at 72h times.

In another study, Aliyah et al. [37] produced α amylase and β glucosidase via solid-state fermentation using different types of biomass waste i.e. corncob, sugarcane

bagasse and rice husk as substrate for *Aspergillus niger*. The enzyme activity achieved for α-amylase and β-glucosidase was 81.86 U/ml and 95.02 U/ml using corn cob as substrate; enzyme activity achieved using rice husk as substrate for α-amylase and β-glucosidase production was 65.95 U/ml and 31.91 U/ml and enzyme activity achieved using bagasse as substrate for α-amylase and β-glucosidase production was 75.35 U/ml and 91.67 U/ml. The highest enzyme activity unit was attained using corn cob as a substrate for 6 days of fermentation time.

In a study conducted by Escaramboni et al. [38] in which amylase and protease were extracted from FW. A bioprocess was applied involving SSF in which *Rhizopus oligosporus* was cultured using FW as substrate by implementing a cell-substrate recycling system. The highest outcomes were obtained using bioprocess of one cell recycling round using FW as substrate. The highest activity unit for amylase was achieved by the combination of 50% FW, 40% wheat bran and 10% sugar cane bagasse amplified with a salt solution which yielded 260.9 U/g amylase while the same combination was used in protease production with the addition of 20% corn steep liquor which produced 665.5U/g protease. The whole bioprocess showed cost-effectiveness and potential for large-scale commercial purposes.

To support the bioconversion of organic/ agricultural waste to valuable products like enzymes Chilakamarry et al. [39] highlighted the importance of utilization of agricultural waste for the production of valuable products. Utilization of agricultural waste which is organic waste can be used as raw material for biofuels production, bioremediation and biocontrol agents involving microbial action. Solid-state fermentation is a common method used for the bioconversion of agricultural waste by using them as substrates to produce bio-products like enzymes and proteins. SSF is used for the production of commercially important enzymes and secondary metabolites.

In different studies, amylases are partially purified to be administered in different industrial and environmental applications. Fabiane et al. [40] developed a three-stage bioethanol bioprocess by secreting amylases from *Rhizopus microsporus var. oligosporus* where wheat bran was used as substrate in SSF. Partial purification and

characterization of amylases were performed that showed amylase activity in the crude extract was 358 U/g substrate and purified amylase's best activity was in the 4-5.5 pH range. The amylase was thermostable up to 60^{o}C and it was observed that the process in 10 h had 54.9% yield in the conversion of rice residues into reducing sugars. Enzymatic hydrolysis of rice residue using extracted glucoamylases was performed to obtain a high yield of glucose for the production of bioethanol. The glucose solution fermented by Saccharomyces cerevisiae showed high ethanol efficiency with a value of 95.8% which demonstrates a feasible process for the production of bioethanol.

Nowadays, different enzyme-based nanoparticles are studied due to their increasing demand for significant applications. Enzymes catalyze the synthesis of nanoparticles and serve as reducing and stabilizing agents. Mojumdar and Deka [41], α-amylase-gold nanoparticle composites were synthesized by using SSF incorporating agricultural waste-based substrates like potato peels, wheat and rice bran. The specific enzyme activity units were compared while the activity and stability of α-amylase- The results showed that the highest rate of enzyme production was observed in potato peel and wheat bran substrate with the specified enzyme activity of approximately 1.1 U/ug and 1.2 U/ug. The combination of substrates i.e. wheat bran with potato peel showed a high rate of enzyme production of 1.3 U/ug. Wheat bran can be effective substrate as used by itself or in combination with potato peels for pure amylase recovery and the enzyme has the capability for synthesis of gold nanoparticles.

In a study conducted by Mishra and Sardar [42], AgNPs were synthesized by using purified α-amylase produced by *Aspergillus oryzae*. The UV Vis spectroscopy showed absorbance spectra at 525 nm which confirmed the presence of AuNPs in the reaction time of 6 hours. FTIR analysis revealed a distinct and strong peak at 1650 cm-1 which showed α amylase acting as a capping and reducing agent for the synthesis of nanoparticles. Transmission Electron Microscopy showed nanoparticles were spherical and their size ranged from 2-20 nm. The biosynthesized AuNPs were used for the degradation of nitro aromatic pollutants

reducing the high cost drawn in bioremediation processes. Similarly, Mishra and Sardar [43] also synthesized AgNPs by using α-amylase. The α-amylase reduced silver ions for the synthesis of AgNPs at 25ºC after 12h of reaction. The light brownish color indicated the synthesis of AgNPs while UV-Vis spectroscopy confirmed and showed maximum absorbance at 422nm. TEM analysis showed monodispersed particles with triangular and hexagonal shapes, with a size of 22-44nm.

In another study, Ahmad et al. [44], reported biosynthesis of TiO_2 nanoparticles using hydrolytic α-amylase secreted by *Aspergillus oryzae*. The α-amylase consists of 478 amino acid residues; 21 residues were proline and 12 others were exposed out of them. FTIR analysis revealed distinct and strong peaks at 1625 cm^{-1} due to possible stretch in the amide I while 1520 cm^{-1} signifies a characteristic amide II which showed α amylase acting as stabilizing agent for the synthesis of nanoparticles. Antibacterial properties of TiO_2 nanoparticles against grame negative and positive bacteria i.e. *E.coli and S. aureus* were tested.

Similarly, Moshfegh et al. [45], carried out research to use α amylase as a reducing agent for the formation of metallic nanoparticles by examining 5 different metal ions; Ag^{+}, Au^{+3}, Se^{+4}, Cu^{+2}, and Bi^{+4}. The experiment resulted in the successful synthesis of AgNPs, AuNPs, and Ag/AuNPs alloys. The biosynthesized nanoparticles exhibited maximum absorbance at 440nm, 530nm, and 458nm. FTIR analysis showed distinct peaks at 3430 cm^{-1} and 1620 cm^{-1} signifying OH or/and NH and carbonyl groups capping the surface of nanoparticles. The peak at 3286 cm^{-1} revealed the O-H stretch of carboxylic acid or N-H of amines and the peak at 1641 cm^{-1} indicated the C-O stretch of amides or the C-C stretch of alkenes. SEM analysis showed a size of 37nm for AgNPs, 89nm for AuNPs, and 63nm for Ag/AuNPs.

For eco-friendly applications in dye degradation processes, Ghosh and Webster [46], studied nanoparticles with excellent chemical reactivity and surface properties that can act as promising products for biodegradation and removal of dyes at commercial level. Physical and chemical treatments for dye degradation and

removal are extensive and less efficient compared to fungal, bacterial, plant and algae-mediated synthesis of metal oxide nanoparticles. The scope of a pilot and bench-scale studies to employ biogenic nanoparticles in the treatment of contaminated textile industries with toxic dyes is scientifically rationalized.

Different studies show bioremediation and dye degradation applications by green catalysts (enzyme-mediated nanoparticles) like a study conducted by Elegbede et al. [47], which used crude xylanases produced by *Aspergillus niger* (NEA) and *Trichoderma longibrachiatum* (TEA) for the synthesis of AgNPs. The brownish colour indicated the synthesis of AgNPs while UV-Vis spectroscopy confirmed and showed maximum absorbance obtained at 402.5nm for NEA-AuNPs and 410nm for TEA-AuNPs. FTIR analysis showed distinct bands at 3277 cm^{-1}, 3294 cm^{-1} and 1641 cm^{-1} for both NEA-AgNPs and TEA-AgNPs. The peaks at 3277 cm^{-1}, 3294 cm^{-1} and 1641 cm^{-1} signify the N-H bond of amines, the C=C stretch of alkenes or the C=O stretch of amides. Scanning Electron Microscopy showed nanoparticles were spherical and their size ranged from 15.21-77.49 nm. The biosynthesized nanoparticles were applied to malachite green and methylene blue for dye degradation; the AgNPs degraded 64.30-78.97% malachite green while 14.8-25.3% methylene blue.

Similarly, Elegbede et al. [48], also synthesized Ag-AuNPs using crude xylanases produced by *Aspergillus niger L3* and *Trichoderma longibrachiatum L2* through valorization of corn cob as substrate. The biosynthesized silver-gold nanoparticles were ruby red and light purple. The UV Vis spectroscopy showed absorbance at 520 nm for NEAg-AuNPs and 534 nm for TEAg-AuNPs. FTIR analysis showed that protein molecules capped and stabilized the nanoparticles; distinct bands at 3286 cm^{-1} and 1641 cm^{-1} were observed for both NEAg-AuNPs and TEAg-AuNPs. The peak at 3286 cm^{-1} revealed the O-H stretch of carboxylic acid or N-H of amines and the peak at 1641 cm^{-1} indicated the C-O stretch of amides or the C-C stretch of alkenes. Field Emission Scanning Electron Microscopy showed nanoparticles were anisotropic with different sizes ranging from 6.98- 52.51nm. The biosynthesized nanoparticles were applied to malachite green and methylene blue for dye

degradation; After 24 h of reaction, 74.86-91.39% of malachite green while 12.1-47.1% of methylene blue was degraded by Ag-AuNPs.

Enzyme-based copper nanoparticles act as antibacterial and antifungal agents when incorporated into coatings, textiles, and plastics. Furthermore, CuO NPs are used in various environmental applications such as dye degradation, wastewater treatment and bioremediation. Kamal et al. [49], synthesized CuNPs by using carboxymethyl cellulose (CMC) through a microwave-assisted method. The formation of CuNPs was done by mixing $CuSO_4$ in an aqueous solution in the presence of CMC through a microwave heating quick procedure. CuNPs were coated on bacterial cellulose nanofibers that were synthesized by *Gluconacetobacter xylinum*. The UV Vis spectroscopy showed absorbance at 660 nm CuNPs. Field Emission Scanning Electron Microscopy showed nanoparticles were anisotropic with sizes < 20nm. The synthesized CuNPs were used for catalytic reduction of 4-nitrophenol and methylene blue. Similarly, Marimuthu et al. [50] evaluated the potential of Ag nanoparticles to treat dye effluent produced in industries as severe water pollution has occurred due to harmful industrial discharge in water resources causing environmental and health hazards. Ag nanoparticles and Ag nanocomposites provide significant alternatives for the treatment of textile effluents.

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1. Materials and Chemicals

Bread waste, *Aspergillus niger*, Potato dextrose agar (PDA), DNS (3 5-dinitrosalicylic acid) ($C_7H_4N_2O_7$), Acetate buffer, Glacial Acetic acid (CH_3COOH), Sodium acetate ($C_2H_3NaO_2$), Maltose ($C_{12}H_{22}O_{11}$), Starch $(C_6H_{10}O_5)_n$, Copper sulfate pentahydrate ($CuSO_{4.5}H_2Oa$), Red dye ($C_{18}H_{14}N_2Na_2O_8S_2$)

3.2 Production of α- amylase

3.2.1 Microorganism

The microorganism used in this study was *Aspergillus niger* obtained from the Pakistan Council of Scientific and Industrial Research (PCSIR), Lahore, Pakistan. The culture was grown on the slants of Potato Dextrose Agar.

3.2.2 Inoculum Preparation

3.9g of Potato Dextrose Agar was added in 100ml distilled water and mixed thoroughly. The media was autoclaved for 20 min for sterilization. After cooling at room temperature, the slants of media were prepared. For inoculum preparation, *Aspergillus niger* was grown on PDA slants at 37ºC for 4 days for complete sporulation. 5ml autoclaved distilled water was added in slant test tubes and with an inoculating loop the spores were scrapped off under aseptic conditions. The spores were used for the fermentation process.

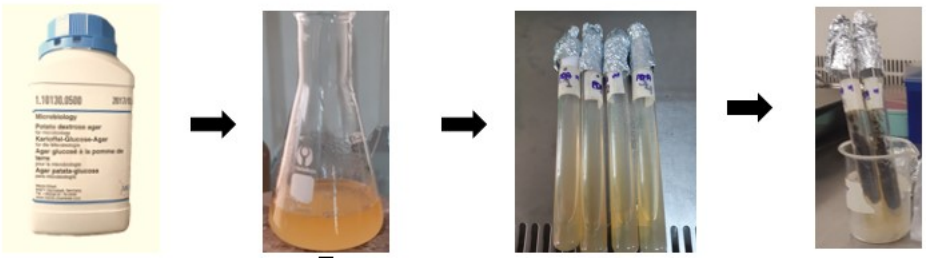


Figure 3.1 Process of inoculum preparation

3.2.3 Collection of food waste and substrate preparation

White bread waste produced at the household level was collected. It was grinded in a grinder and sieved from which a fine powder was obtained. 25g of bread waste powder (substrate) and 15 ml of distilled water was added to a conical flask. The flask was closed with aluminum foil and autoclaved at 121ºC for 20 min.

Figure 3.2 Process of substrate preparation

3.2.4 Fungal Solid State Fermentation (SSF)

The autoclaved bread waste mixture was inoculated with *Aspergillus niger* culture and incubated at 37ºC for 4 days under optimum conditions.

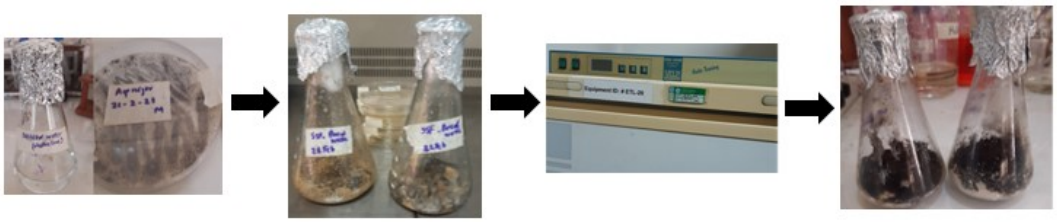


Figure 3.3 Process of solid state fermentation

3.2.5 Extraction of enzyme (α- Amylase)

The enzyme (α- Amylase) was extracted at the end of 4 days using acetate buffer at pH 6.0. The buffer was added to culture flasks and stirred for 30 min. The mixture was filtered using a muslin cloth to obtain filtrate. The filtrate was then centrifuged at 5000rpm for 15 min. The supernatant was collected and used as the crude enzyme source.

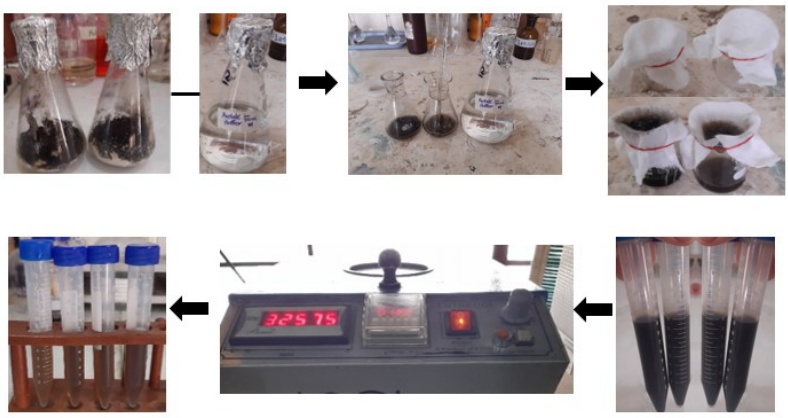


Figure 3.4 Process of enzyme extraction

3.2.6 DNS Assay

α- Amylase activity was assayed using the DNS method after crude extraction of fermented bread waste powder. The enzyme activity was determined by incubating a mixture of 0.1ml enzyme extract and 0.5ml of 1% starch solution at 35ºC for 30min. Then 3ml DNS (3 5-dinitrosalicylic acid) was added to the enzyme mixture followed by boiling for 15 min. After cooling, the amylolytic activity was measured by taking absorbance at 540nm wavelength. The values were obtained and compared with a standard Maltose graph.

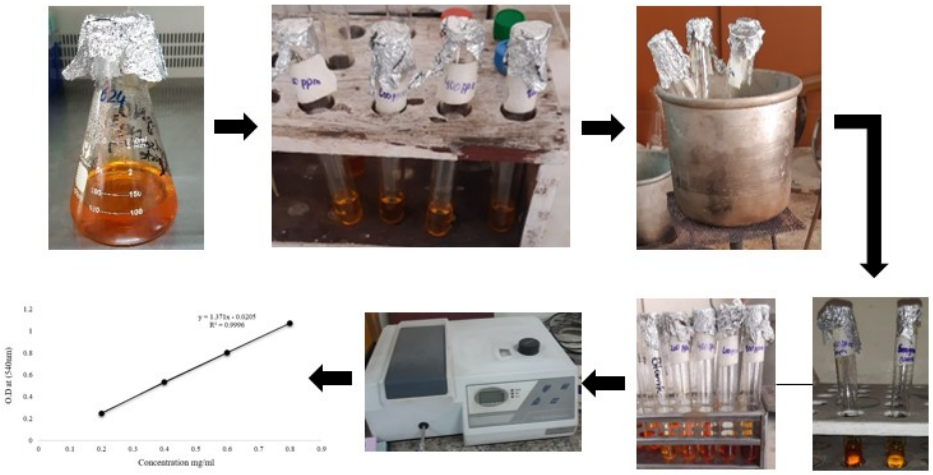


Figure 3.5 Process of DNS assay

3.3 Synthesis of Copper oxide nanoparticles

3.3.1 Biosynthesis of copper oxide nanoparticles using crude α-amylase extract

1M Copper (II) sulfate pentahydrate solution was prepared by adding 12.5 g $CuSO_4.5H_2O$ in 50ml distilled water. 0.05 M aqueous stock solution was prepared by adding 5ml copper (II) sulfate pentahydrate solution in distilled water up to 100ml volumetric flask. 100ml of 0.1 M $CuSO_4.5H_2O$ was added to 50 ml of α-Amylase crude extract and it was incubated at 37ºC for 48 hours. After 48hrs, sample was analyzed in UV-Vis Spectrophotometer in the range of 250-500nm. A defined peak was observed at 310nm indicating the formation of copper nanoparticles.

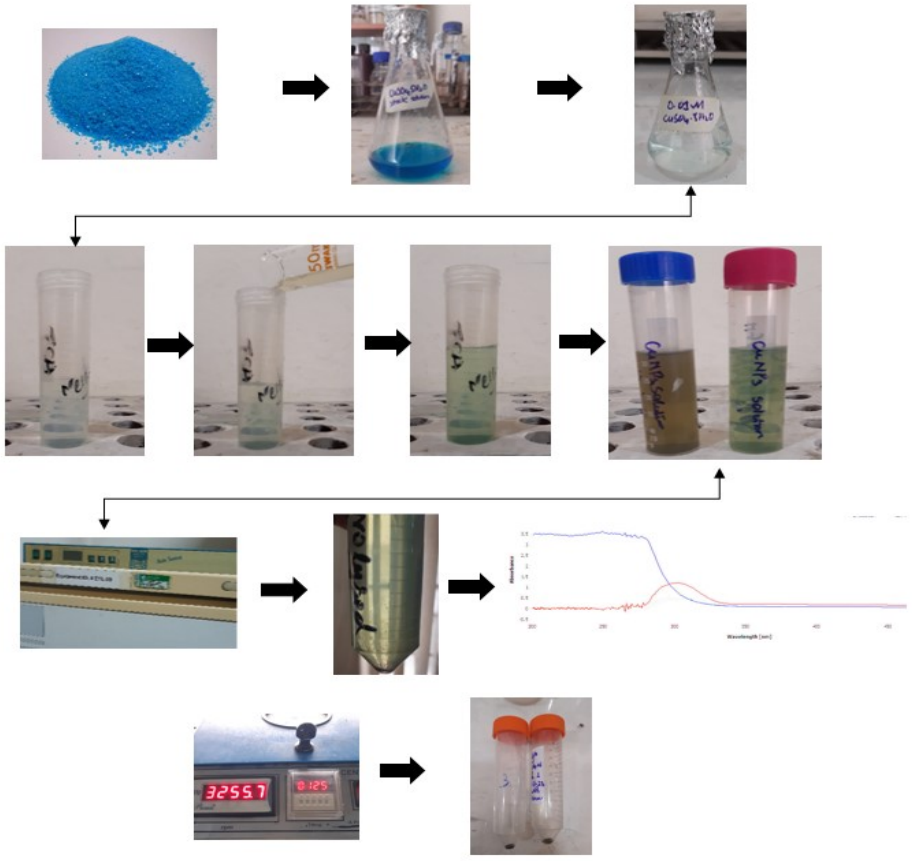


Figure 3.6 Synthesis process of CuO NPs

3.3.2 Washing and Purification of copper oxide nanoparticles

Sample was centrifuged at 5000rpm for 20 min. Pellets were obtained consisting of copper nanoparticles. Supernatant was discarded and pellets were washed with distilled water. The emptied falcon tubes were filled with distilled water and mixed, then centrifuged at 5000rpm for 20 min. This washing procedure was repeated twice. The nanoparticles were dried in hot air oven, after the moisture was evaporated pellets were collected for further characterization.

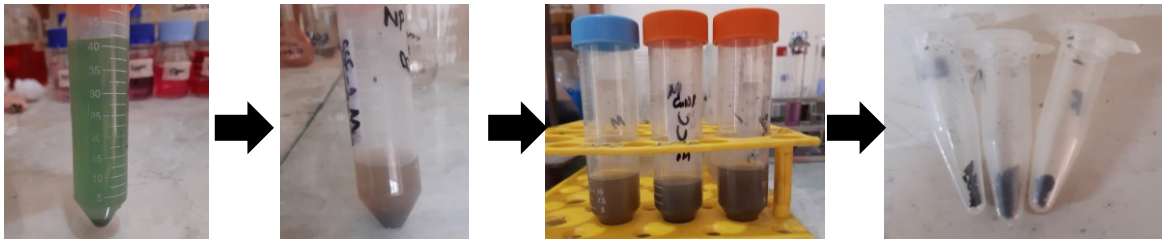


Figure 3.7 Washing process of CuO NPs

3.4 Characterization of α amylase- copper oxide nanoparticles composite

Synthesis of copper nanoparticles was confirmed by measuring absorbance in UV-Vis Spectrophotometer (Specord 200 plus) and morphology of copper nanoparticles was observed by Scanning Electron Microscope (FEI NOVA 450 Nanosem), respectively. To identify capping agents (biomolecules) Fourier Transform Infrared Spectroscopy (Cary 610/620 FTIR Microscope) was performed.

3.5 Application of the α amylase- copper oxide nanoparticles composite on red dye

1mg of red dye was prepared in 1 liter of distilled water. Different concentrations of 1ppm, 2ppm, 5ppm, 10ppm, 15ppm and 20ppm were prepared from the stock solution to make a standard curve. 1ml of α amylase- copper nanoparticles at concentration of 100 µg/ml were mixed with 9ml of 1ppm, 2ppm, 5ppm, 10ppm, 15ppm and 20ppm of the dye solution. The control sample contained 10 ml of dye solution from 1ppm, 2ppm, 5ppm, 10ppm, 15ppm and 20ppm without the addition of nanoparticles. All solution flasks were stirred continuously. At time intervals of

30min, 45min and 24h, absorbance was measured at 540nm wavelength by using a UV-Vis spectrophotometer. The %removal/ dye degradation of red dye was measured by the following formula.

$$\%\text{removal/ dye degradation} = \frac{A_{initial} - A_{final}}{A_{initial}} \times 100$$

Where, A represents the Absorbance value.

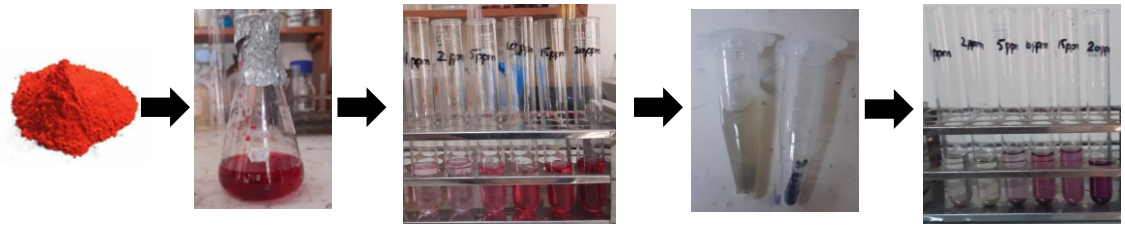


Figure 3.8 Application of CuO NPs on red dye

3.6 Data Analysis and thesis writing

The data (tables and graphs) was analyzed and interpreted on Microsoft Excel. Report writing was done on Microsoft word.

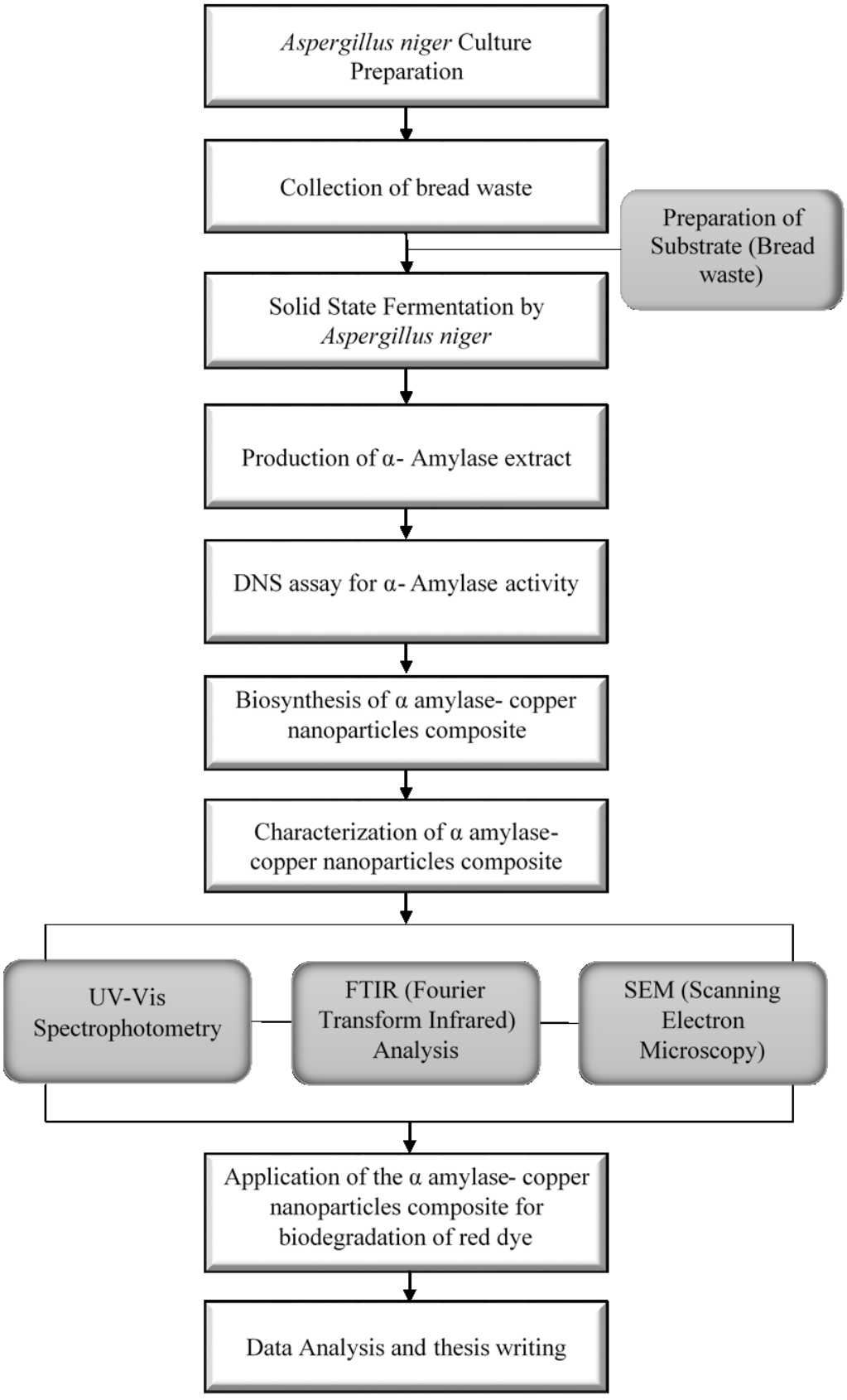


Figure 3.9 Flowchart of summarized research study

CHAPTER 4

RESULTS

4.1 Estimation of amylase enzyme activity from bread waste substrate

Bread waste (BW) is commonly generated food waste around the world that needs to be recycled and managed. Therefore, bread waste was used as substrate for alpha-amylase production using *Aspergillus niger* in SSF. After 4 days of incubation at 37° C, crude enzyme extract was produced and then the enzyme activity was measured by 1% starch solution and DNS solution. The substrate productivity was measured with reference to enzyme activity. One unit of enzyme activity is the amount of enzyme that releases 1µmol of glucose per minute and is expressed in U/ml. The enzyme activity was determined by comparing the observed absorbance with the standard maltose curve as shown in Figure 4.1. [51]

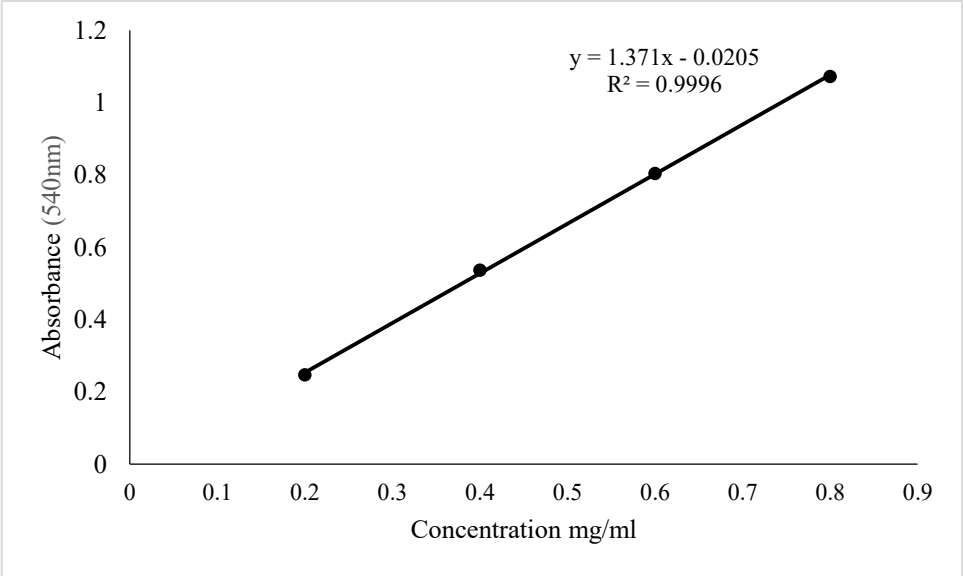


Figure 4.1 Standard curve of Maltose

The α-amylase activity was measured and the enzyme activity obtained from bread waste was 58 mg/ml/min.

The α-amylase activity was recorded after 30 min incubation time at the optimum temperature 35ºC (Figure 4.2 a) and pH 6 (Figure 4.2 b).

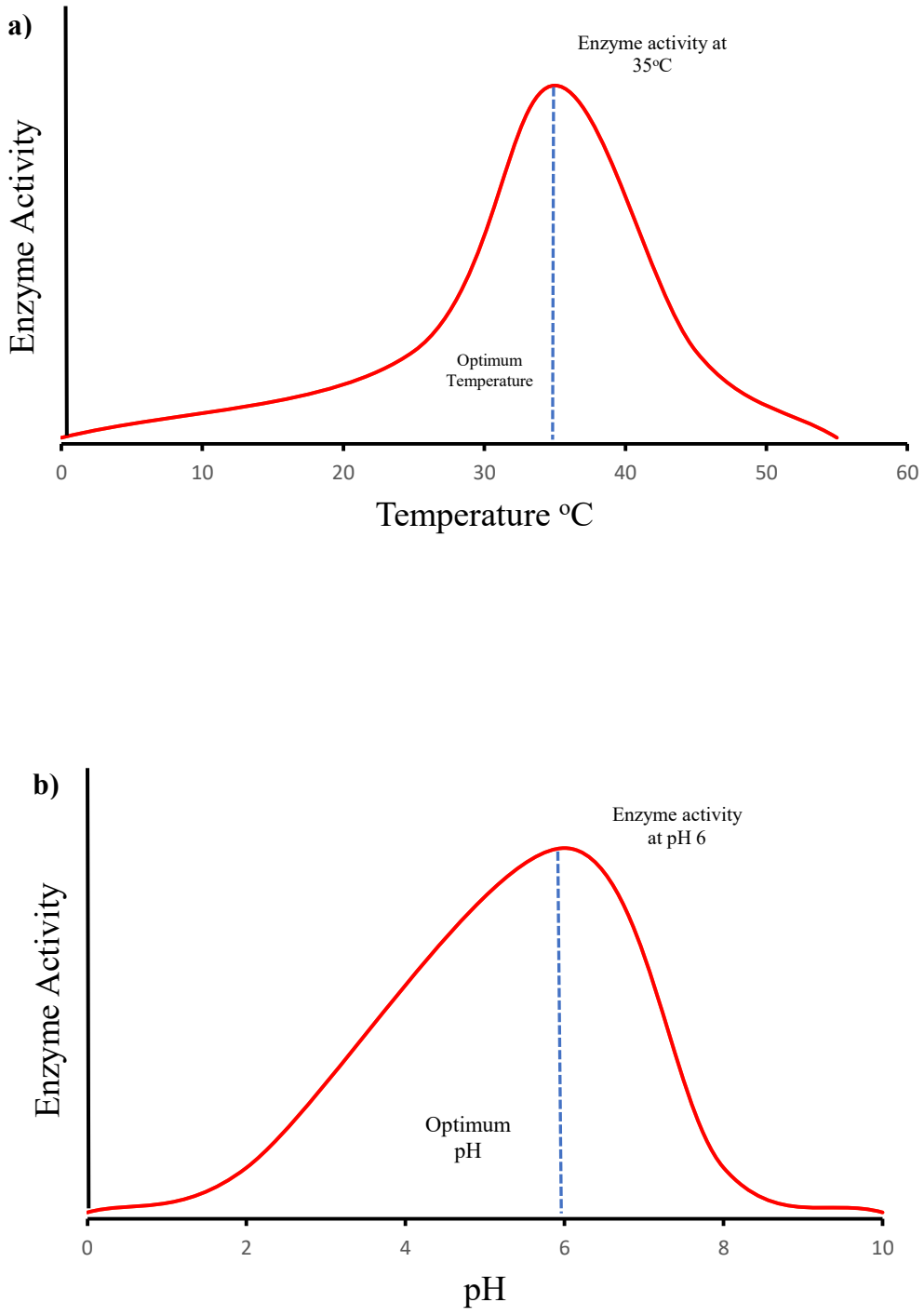


Figure 4.2 Graphs of enzyme activity a) optimum temperature and b) optimum pH

4.2 Synthesis of α amylase-CuO NPs composite

The present study shows the synthesis and characterization of copper nanoparticles from α-amylase extract yielded from bread waste. The study was performed under a controlled environment and optimum conditions for experimentation. The visual observation in the formation of copper nanoparticles was the color change from light blue to blueish green and the settlement of copper nanoparticles in the form of supernatant confirms the full bio-reduction of metal salts present in bread based α-amylase (Figure 4.4). The main advantage of green synthesis of CuNPs is the stabilization [52].

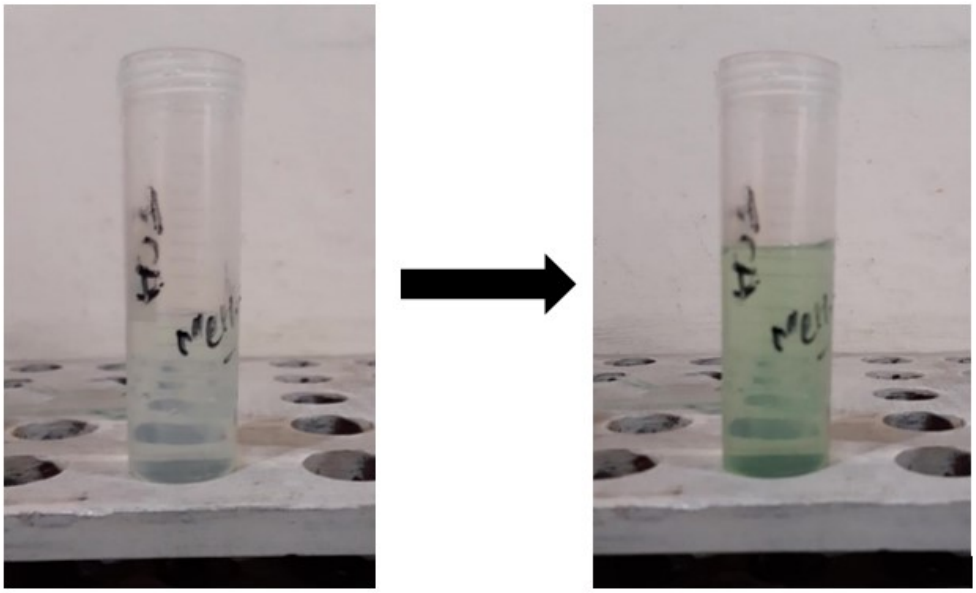


Figure 4.3 Picture showing color change after synthesis of α amylase- CuNPs composite

4.3 Characterization of α amylase-CuO NPs

4.3.1 UV-Vis Spectroscopy analysis of CuO NPs

UV–Vis spectroscopy is the most significant characterization technique for the determination of copper nanoparticles. This technique is basically used for the depiction of the synthesis nanoparticles and analyzing their stabilization in aqueous

solution. According to literature, CuO NPs display a distinctive surface plasmon resonance (SPR) peak in the range of 220-540 nm wavelength [53].

The results showed characteristic SPR peak at 300 nm with the absorbance of 0.767, specifying the existence of CuO NPs synthesized in the solution. The absorbance is due to the formation of CuO NPs by bio-reduction of metal salts. The broad peak shown in Figure 4.5 depicts the synthesis of CuO NPs.

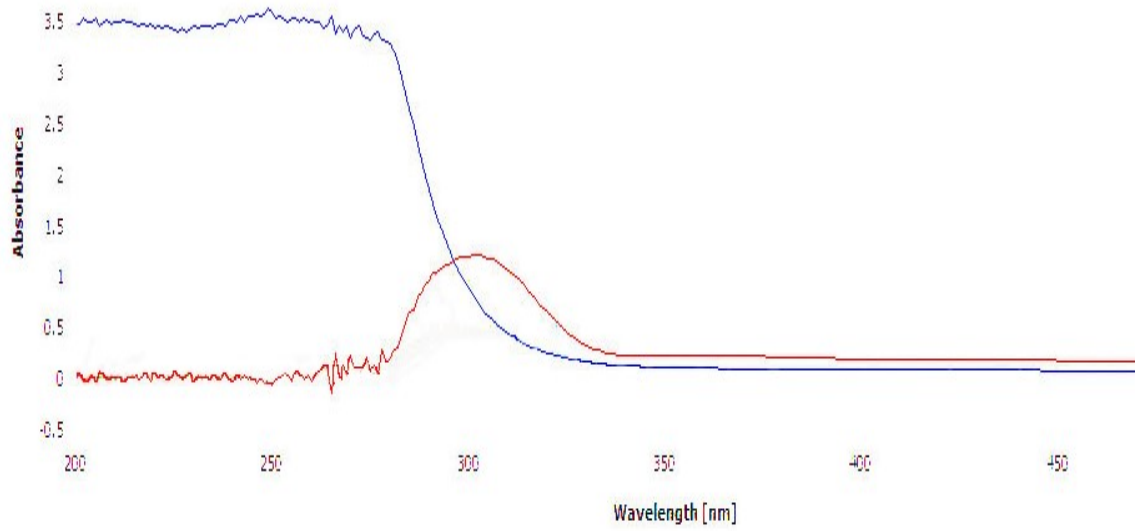


Figure 4.4 UV-Vis spectra indicating synthesis of α amylase- CuO NPs composite

4.3.2 SEM, TEM and EDX analysis of CuO NPs

SEM and TEM characterization was carried out to determine the morphology and size of synthesized nanoparticles. The results of SEM analysis were not clear and material was found to be aggregated. TEM analysis showed that nanoparticles were spherical and clustered with size range less than 20nm.

EDX confirms the formation of copper nanoparticles (CuO NPs) by showing peaks at 8 keV as shown in Figure 4.7.

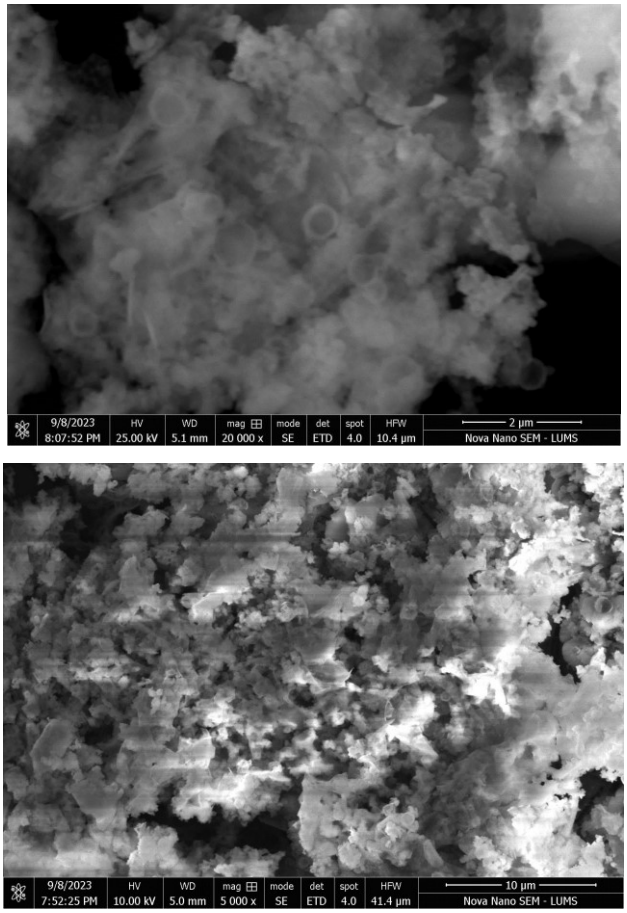


Figure 4.5 SEM images of α amylase- CuO NPs composite

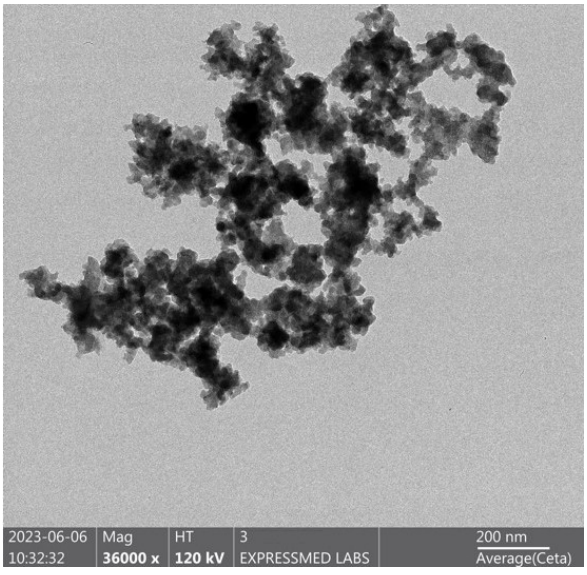
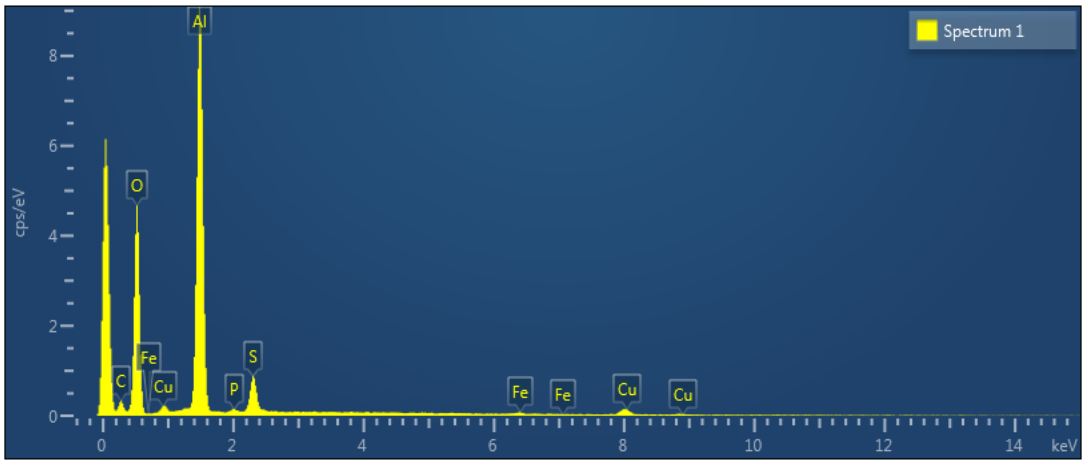


Figure 4.6 TEM image of α amylase- CuO NPs composite

a)



b)

Element	Apparent Concentration	k Ratio	Wt%	Standard Label
C	2.45	0.0245	8.9	C Vit
O	115.14	0.38745	57.88	SiO_2
Al	45.99	0.33035	28.3	Al_2O_3
P	0.66	0.00369	0.33	GaP
S	4.44	0.03827	2.81	FeS_2
Fe	0.38	0.00382	0.21	Fe
Cu	2.68	0.02679	1.57	Cu
Total:			100	

Figure 4.7 (a) EDX spectrum showing different elements (b) Elemental composition of α amylase- CuO NPs composite

4.3.3 Fourier Transform Infrared Spectroscopy (FTIR) of CuO NPs

FTIR spectra of biosynthesized α amylase-CuO NPs composite showed distinct peaks at 1144.3 cm^{-1}, 1075.3 cm^{-1} and 997.1 cm^{-1} as shown in Figure 4.8. Also, FTIR analysis revealed strong peaks at 2922.2 cm^{-1}, 2849.5 cm^{-1} and 1636.3 cm^{-1} as shown in Figure. The prominent bands at 1144.3 $cm^{-1,}$ 1075.3 cm^{-1} relates to the

C-F stretch of aliphatic organohalogen compounds, and 997.1 cm^{-1} indicates cyclohexane ring vibrations. The broad bands at 2922.2 indicates C-H stretch of methylene, 2849.5 relates to C-H stretch of methyl and 1636.3 cm^{-1} indicates C=C stretch of alkenes. It showed that C-H stretch of methylene, C-H stretch of methyl and C=C stretch of alkenes from α amylase were the biomolecules that reduced Cu^{+2} and acted as capping agents for CuNPs.

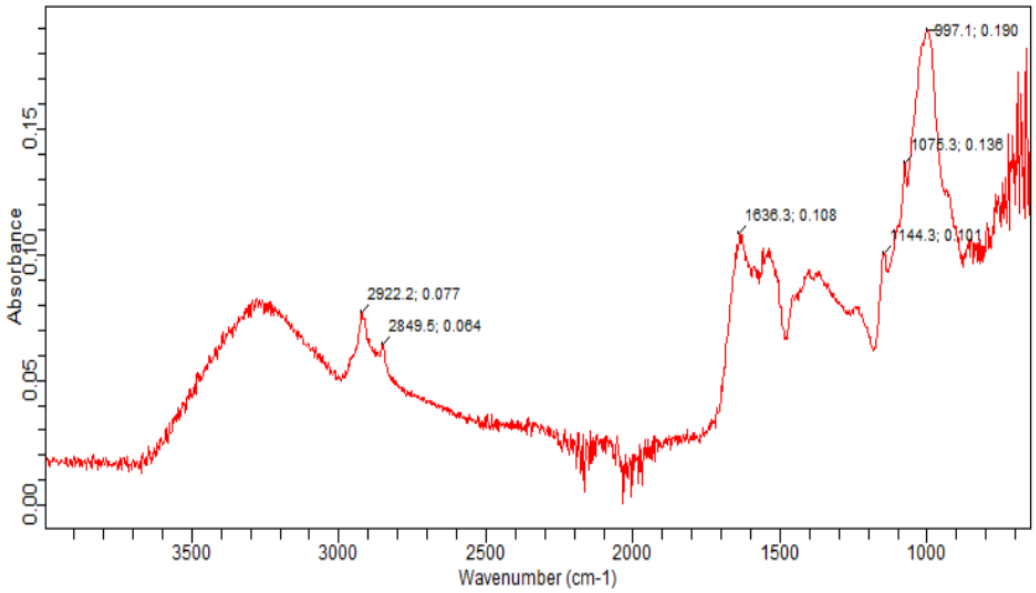


Figure 4.8 FTIR spectra of CuO NPs

4.4 Degradation of Red dye

4.4.1 Mechanism of Catalytic Degradation

As per the earlier studies, catalytic dye degradation was explained using the following mechanism. A catalytic reaction occurred on the surface of the metals present in the dye solution. Enhancing the surface area of the nanoparticles increases the efficiency of the catalyst used for the dye degradation. On the other hand, declining the size of the catalyst also enhances the catalytic reaction**.**

4.4.2 Degradation of Red dye

The catalytic activity of biosynthesized copper nanoparticles was determined by the degradation of red dye.

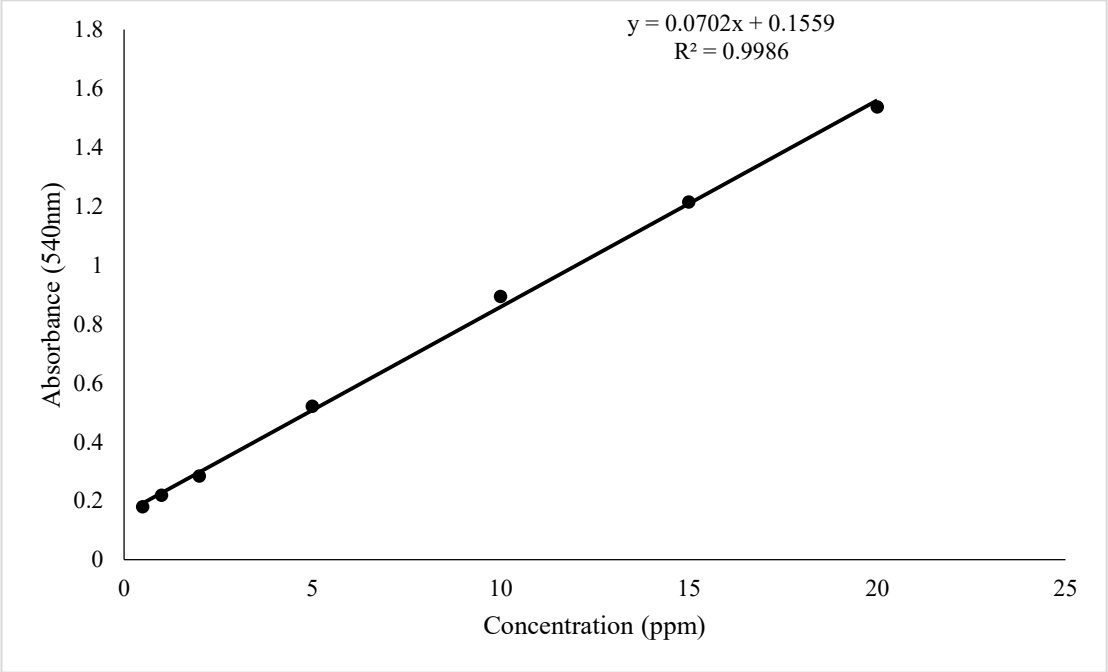


Figure 4.9 Standard curve of Red dye

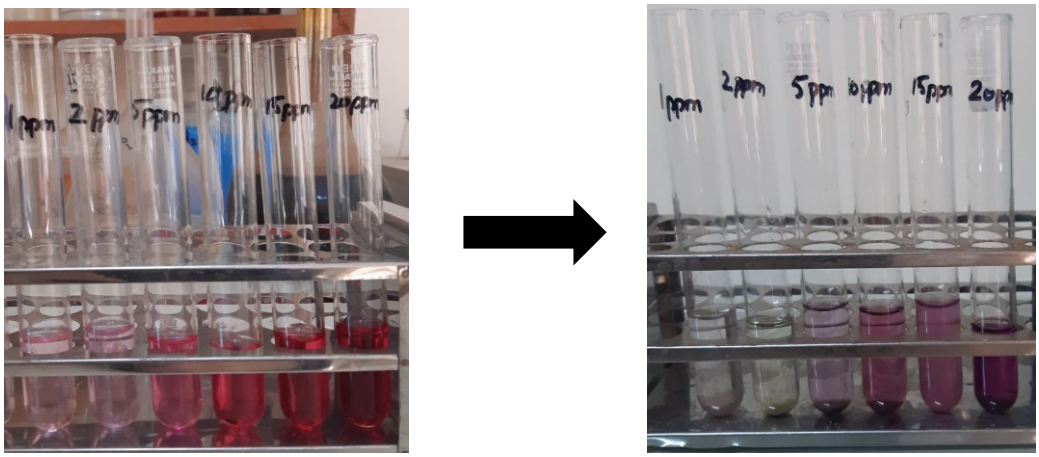


Figure 4.10 Color change of red dye after degradation

The degradation of dye concentration in solution by using copper oxide nanoparticles was observed by color change. The color of dye solution at different concentrations of 1ppm, 2ppm, 5ppm, 10ppm, 15ppm and 20ppm changed as shown in Figure 4.10 indicates the % removal of dye. The progress of dye degradation was recorded using an Ultraviolet-Visible spectrophotometer in the range of 400–600 nm. The maximum absorbance was recorded at 540nm. The CuO NPs degraded red textile dye of different initial concentrations (1ppm, 2ppm, 5ppm, 10ppm, 15ppm, 20ppm) at different time intervals as shown in Figure 4.11 and 4.12. The % dye degradation by CuO NPs at dose of 100 µg/ml concentration resulted in 100% at initial concentration of 1ppm while it decreased to 61.50% at 2ppm. As the initial concentration increased % dye degradation decreased to 30.01% at 20ppm of dye concentration at 30 min, % dye degradation resulted in 100% at initial concentration of 1ppm while it decreased to 62% at 2ppm. As the initial concentration increased % dye degradation decreased to 30.5 % at 20ppm of dye concentration at 45 min and after 24 hrs, 100% dye degradation at initial concentration of 1ppm was achieved while it decreased to 62% at 2ppm which further decreased to 31% at 20ppm of dye concentration.

Furthermore, 100 % dye degradation was achieved by 150 µg/ml dose of CuO NPs at dye concentrations of 1ppm and 2ppm after 30 min, 45 min and 24hrs. Then initially, the % dye degradation resulted in 78.4% at 30min for 5ppm concentration which increased to 81% after 24 hrs, for 10ppm % dye degradation resulted in 68% at 30min which increased to 73% after 24 hrs, for 15ppm % dye degradation resulted in 54% at 30min which increased to 59% at 45min and 65% after 24 hrs and 45% dye degradation resulted at 30min for 20ppm concentration which increased to 51% after 24 hrs. From this, it has been revealed that green-synthesized α amylase- copper oxide nanoparticles have the ability to degrade red textile dye at lower concentrations.

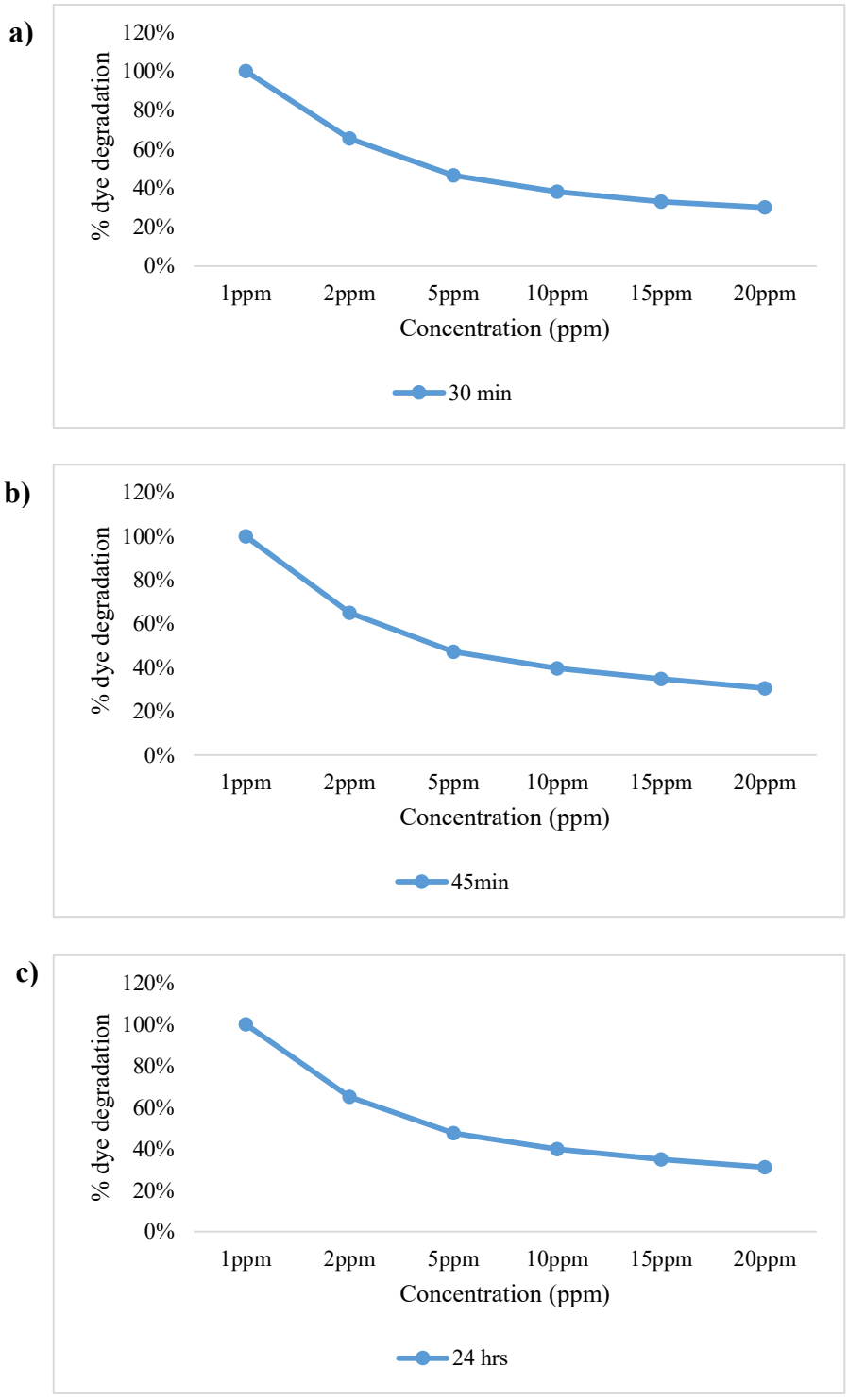


Figure 4.11 % dye degradation at different time intervals at 100 µg/ml NPs dose a) At 30 min, b) At 45 min, c) At 24 hrs

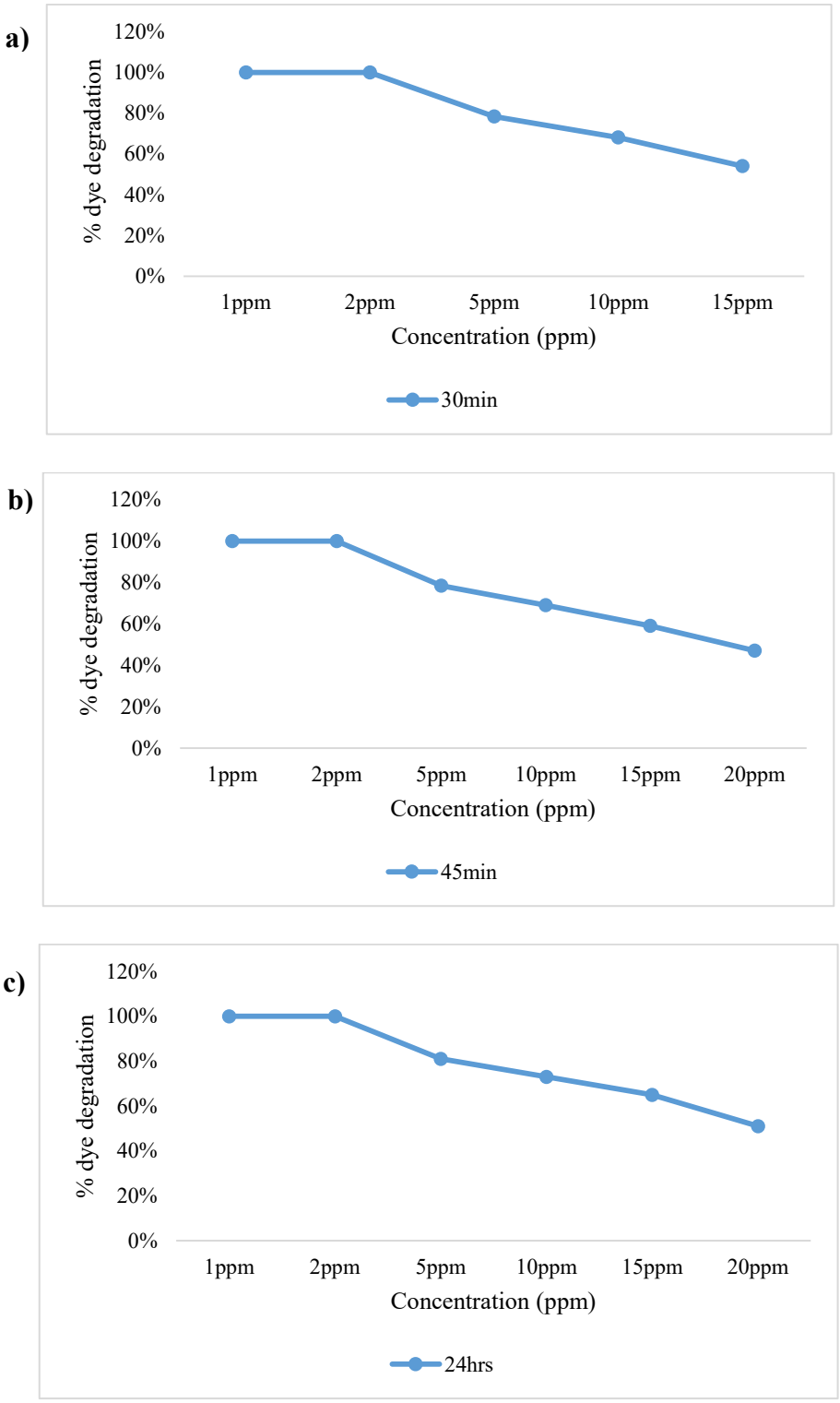


Figure 4.12 % dye degradation at different time intervals at 150 µg/ml NPs dose a) At 30 min, b) At 45 min, c) At 24 hrs

CHAPTER 5

DISCUSSION

Enzymes are biocatalysts that have a significant range of industrial and environmental applications so conversion of food waste into valuable enzymes through fermentation processes is a highly sustainable approach. This research study reports utilization of bread waste as substrate for *Aspergillus niger* for the extraction of α-amylase enzyme and biosynthesis of copper nanoparticles from crude enzyme extract. This nanocomposite is applied for the dye degradation of red dye.

The α-amylase obtained from bread waste using *Aspergillus niger* in SSF was 58 mg/ml. The α-amylase activity was recorded after 30 min incubation time at the optimum temperature 35^{o}C and pH 6 as it is extensively reported in previous studies [54-57]. The enzyme activity of this crude extract was measured by using 1% starch solution and DNS solution.

Nanotechnology is gaining significant attention around the world as many of its applications are involved in different industrial sectors such as healthcare, electronics, wastewater treatment, food safety, agriculture, energy storage, cosmetics, textile, cancer treatment and drug delivery. Nowadays, more attention is drawn towards bio-nanotechnology (synthesis based on microorganisms, enzymes and plants) as chemical synthesis of nanomaterials is toxic, time consuming and expensive. Copper nanoparticles are well known for their antimicrobial, anti-inflammatory and pollutant degradation applications. The copper nanoparticles can be easily synthesized through green synthesis and it is cost effective than silver or gold nanoparticles [58]**.** Nowadays, green synthesis of copper nanoparticles has gained much attention around the world [59]. Green synthesis of nanoparticles is trending than chemical methods because biomolecules like enzymes and plants are ecofriendly and act as reducing and capping agents [60].

The environmentally friendly synthesis of copper nanoparticles using crude α-amylase extract is a simple, economical and cost-effective process as α-amylase

extract is produced through valorization of bread waste. In our study the color change from light blue to blueish green indicates the synthesis of CuO NPs which is in accordance with previous studies [61, 62]. Maximum absorbance is measured at 300nm by UV-Vis spectra depicting the synthesis of copper nanoparticles as reported in previous studies [63,64]. FTIR spectra of biosynthesized α amylase-CuO NPs composite showed distinct peaks at 1144.3 cm^{-1}, 1075.3 cm^{-1} and 997.1 cm^{-1}. Also, FTIR analysis revealed strong peaks at 2922.2 cm^{-1}, 2849.5 cm^{-1} and 1636.3 cm^{-1}. The prominent bands at 1144.3 $cm^{-1,}$ 1075.3 cm^{-1} relates to C-F stretch of aliphatic organohalogen compounds, and 997.1 cm^{-1} indicates cyclohexane ring vibrations. The broad bands at 2922.2 indicates C-H stretch of methylene, 2849.5 relates to C-H stretch of methyl and 1636.3 cm^{-1} indicates C=C stretch of alkenes similar to previous studies in which the ftir analysis of CuO NPs synthesized by *Citrus Aurantifolia* enzyme extract showed similar peaks at 2922 and 2851 cm^{-1} indicating the presence of methylene groups and 1744 cm^{-1} band due the C=O stretching which concludes the alkyne bonds and carboxylate groups are responsible for capping and stabilizing agents [61, 65] Similarly, ftir analysis of xylanase mediated silver nanoparticles resulted in peaks at 1641 cm^{-1}, 2154-2187 cm^{-1} indicated O-H vibrations of alcohols, aromatic N compounds, C ≡ C stretch of alkynes and N-H bend of 1^{o} amine [66, 67].

Copper nanoparticles have gained researchers' interest worldwide as they show excellent catalytic properties; they are easily available, have similar properties to other metal nanoparticles and are cost effective. These nanoparticles are widely used in antimicrobial agents, sensors, heating transfer fluids and catalysis [68].

The reduction of dye concentration in the solution by using copper nanoparticles was observed by color change. The % dye degradation by CuO NPs at dose of 100 µg/ml concentration resulted in 100% at initial concentration of 1ppm while it decreased to 30.01% at 20ppm of dye concentration at 30 min, % dye degradation resulted in 100% at initial concentration of 1ppm while it decreased to 30.5 % at 20ppm of dye concentration at 45 min and after 24 hrs, 100% dye degradation at

initial concentration of 1ppm was achieved while it decreased to 31% at 20ppm of dye concentration.

By applying 150 µg/ml dose of CuO NPs at dye concentrations of 1ppm and 2ppm, 100 % dye degradation was achieved after 30 min, 45 min and 24hrs. 78.4% dye degradation was achieved for 5ppm concentration which decreased to 51% for 20ppm concentration after 24 hrs. Similarly, reactive red 81 dye degradation was reported using CuNPs of 0.005mg/L, the optimum decolonization was measured 72.7% and it obtained at a dose of 0.02%. The degradation of dye was reduced as the concentration of reactive red 81 dye increased which is like this research study as the higher concentration of dye the lower the discoloration and degradation of dye. The catalyst efficiency was reduced when the amount red 81 dye was greatest [69].

In another similar study, xylanase-based silver nanoparticles degraded methylene blue and malachite green dyes. The AgNPs of different concentrations decolorized methylene blue within the range of 14.8-25.3% and malachite green within the range of 64.30-78.97%. Catalysis reaction of dye degradation is attained through redox reaction as nanoparticles act as electron transfer mediators between the biomolecules on dye surface and nanoparticles [47]. Other green synthesis using plant extract for copper nanoparticles has been reported and nanoparticles applied for different dye degradation [70, 71] but no previous study reported which showed synthesis of CuO nanoparticles from alpha amylase. It is recommended that yield for production of alpha amylase from bread waste should be increased so that good quantity of enzyme can be produced to synthesis reactive metal nanoparticles and they will be used as environmental friendly material to remediate pollutants.

CONCLUSION

In this research, bread waste was utilized as substrate by *Aspergillus niger* to produce α-amylase which was further used for green synthesis of copper oxide nanoparticles. The spherical nanoparticles in agglomerated form of size less than 20nm were biosynthesized and showed significant ability for degradation of red textile dye. The CuO NPs degraded red textile dye of different concentrations (1ppm, 2ppm, 5ppm, 10ppm, 15ppm, 20ppm) at different time intervals. The dye degradation resulted in 100% removal of 1ppm concentration by 100 µg/ml dose of CuO NPs and 100% dye degradation of 1ppm and 2ppm concentration was achieved by 150 µg/ml dose of CuO NPs. Therefore, this study concludes promising environmental and nano-biotechnological applications of fungal α-amylase synthesized copper oxide nanoparticles.

RECOMMENDATIONS

1. The experiments should be carried out to increase the yield of enzymes as well as nanoparticles.
2. α amylase- CuO NPs composite efficiency should be checked for degradation of other textile dyes.
3. Further characterization studies should be performed to understand the characteristics of α amylase- CuO NPs composite.

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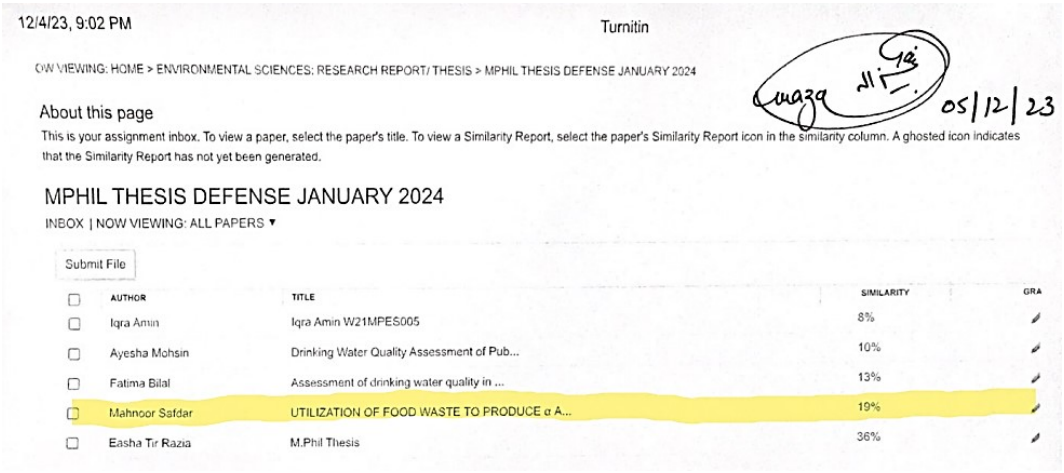

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