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A Comparative Study on Stability and Catalytic Activity of α-Amylase by Iron Oxide, Graphene Oxide and Silver Nanoparticles

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Abstract: The present study analyzed the stability and catalytic activity of α-amylase enzyme by three different nanoparticles Iron oxide, Graphene oxide, and silver nanoparticle. The nanoparticles synthesized were characterized based on their size and magnitude using zeta potential. The activity of α-amylase was done using plate assay to compare the activity of the enzyme with iron oxide nanoparticles, graphene oxide, and silver nanoparticles. The study also confirmed that there was an increase in enzyme catalysis by Iron oxide nanoparticles followed by Graphene and Silver nanoparticles. It was also observed that the silver nanoparticle showed reduced activity when compared to the other two nanoparticles.

Keywords: Enzyme, Iron oxide nanoparticles, Graphene nanoparticles, Silver nanoparticles, Plate assay, Enzyme activity

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Introduction

Enzymes are proteins that catalyze biological reactions; hence they are also called biocatalysts. Enzymes are usually very specific as to which reactions they catalyze and the substances that are involved in the reaction. The internal dynamics of enzymes are connected to their mechanism of catalysis. Internal dynamics are the movement of parts of the enzyme structure such as individual amino acid residues, a group of amino acids or even an entire protein domain. The internal dynamics refer to the movement of individual amino acid residues, groups of amino acids, or even a complete protein domain within the enzyme structure. These movements occur at various time-scales ranging from femtoseconds to second. Network of protein residues throughout an enzyme structure can contribute to catalysis through dynamic motions (Turakhia *et al*., 2019).

α-Amylase is an enzyme that breaks down starch into sugar. Amylase is found in human saliva and initiates the chemical process of

digestion. Foods with a high starch content but low sugar content, such as rice and potatoes, taste slightly sweet when eaten because amylase converts some of their starch into sugar in the mouth. The pancreas also produces amylase (alpha amylase) to break down dietary starch into di- and trisaccharide, which are then turned to glucose by other enzymes to provide energy to the body.

There are three main classes of amylases and are designated by different greek letters (α, β, γ). All amylases are glycoside hydrolases and act on a-1, 4-glycosidic bonds. Amylases are among the most important enzymes and have a significant impact on biotechnology, accounting for approximately 25% of the global enzyme market (Gajbhiye and Sakharwade, 2016).

By acting at random locations along the starch chain, α-amylase breaks down long-chain carbohydrates, ultimately yielding maltotriose and maltose from amylose, or maltose, glucose and "limit dextrin" from amylopectin. In animals, it is a major digestive enzyme and its optimum pH is 6.7-7.0. The sources of α-amylase in humans are saliva and pancreas, also found in plants (barley), fungi (ascomycetes and basidiomycetes), and bacteria (*Bacillus*).

As α-amylase hydrolyzes the α-1,4-glycosidic bonds in starch to produce maltose, glucose, syrup, beer, rice wine, soy sauce, vinegar, and juice and can be regarded as the biomarker for psychological stress like anxiety, and depression. Starch is broken down during seed germination and the early stages of seedling growth by a variety of hydrolases, including α-amylase, β-amylase, debranching enzyme, and α-glucosidase. Also in rice, alpha-amylase isozymes are essential to convert the stored starch into soluble sugar for nourishing the seedling establishment (Ruan *et al*., 2015).

Nanoparticles can be defined as "particulate dispersion or solid particles in the size range of 10-100 mm. Nanoparticles are of great scientific interest as they are effectively a bridge between bulk materials and atomic or molecular structures and atomic or molecular structures. It has become an important field of modern research dealing with the design, synthesis, and manipulation of particle structures ranging from approximately 1-100 nm. The recent advancements in the field of nanotechnology include the preparation of nanoparticles of specific size and shape that exhibit antimicrobial properties. Nanoparticles (NPs) have wide range of applications in areas such as health care, cosmetics, food and feed, environmental health, mechanics, optics, biomedical sciences, chemical industries, electronics, space industries, drug-gene delivery, as have better penetrating and inhibiting effects on tumor tissues due to their smaller sizes, energy science, optoelectronics, catalysis, single electron transistors, light emitters, nonlinear optical devices, and photo-electrochemical applications.

Nanoparticles have high surface area, which can increase catalytic activity. Nanoparticle catalysts can be easily separated and recycled. They are used under mild conditions to prevent decomposition of the nanoparticles.Thus it proves that nanoparticles can act as highly efficient catalysts. Biocatalysis with nanoparticles promotes green processes due to low chemical consumption and absence of toxic by-products. However, when used in large-scale industrial processes, the enzymes' low stability and reusability result in significant operational costs for the enzyme-catalyzed bioprocesses. An innovative new product called the nanobio-catalyst (NBC) combines advances in nanotechnology and biotechnology. The assembly of enzyme molecules onto nanomaterial carriers known as the NBC formation favors desired chemical kinetics and substrate selectivity (Debnath *et al*., 2021).

Among them, magnetite nanoparticles, graphene and silver nanoparticles have attracted increasing interest due to their unique physical, chemical, catalytic and biological properties. Magnetite nanoparticles are colloidal iron oxide

(Fe_3O_4) materials that exhibit superparamagnetic properties at ambient temperatures. This material is used in nanotoxicology, magnetic nanotechnology research and development. The size, non-toxicity, and super paramagnetic properties of the magnetite nanoparticles, make them of interest for applications in many fields, including catalysis, biosensors, ferrofluids, magnetic separations and MRI contrast agents. Nontoxic conduct and biocompatible applications of magnetic NPs can be enriched further by special surface coating with organic or inorganic molecules, including surfactants, drugs, proteins, starches, enzymes, antibodies, nucleotides, nonionic detergents, and polyelectrolytes. Magnetic NPs can also be directed to an organ, tissue, or tumor using an external magnetic field for hyperthermic treatment of patients. There are numerous *in vivo* and *in vitro* examples of MNPs breaching the BBB to deliver th erapeutic drugs to the central nervous system. Graphene oxide (GO) is of great application due to its low cost, easy access, and widespread ability to convert to graphene. Graphene oxide is graphite that has been oxidized to intersperse the carbon layers with oxygen molecules, and then reduced, to separate the carbon layers completely into individual or few layer graphene. Therefore, the suggested system comprising doxorubicin, graphene oxide, and galactosylated chitosan has the potential to manage the therapy of cancer.

The synthesis of copper nanoparticles on Graphene oxide (GO) was used to create a copper nanoparticle-based heterogeneous catalyst, and the obtained catalyst was discovered to exhibit good activity in the reduction of nitroarenes and organic dyes. GO, the functionalized graphene with oxygen-containing chemical groups, has attracted resurgent interest because of its superior properties such as large surface area, mechanical stability, and catalytic properties (Zhou *et al*., 2010).

With the growing application of nanoparticles in medicine, Ag-NPs exhibit broad-spectrum bactericidal and fungicidal activity that has made them utilized in a diverse range of soaps, pastes, and textiles. AgNPs also play an important role in food, health care, therapeutics, industrial purposes, nanoscience, and nanotechnology, particularly in nano catalysis. By immobilizing amylase on chitosan-coated MNPs, an effective magnetically recoverable nano-biocatalyst for starch hydrolysis under various physicochemical circumstances was produced. FTIR research verified the presence of surface functional groups on chitosan-coated MNPs and the immobilization of α-amylase. When compared to the free enzyme, prepared nanocatalysts showed improved catalytic activity at all temperatures and pH environments. Intrinsic magnetic properties of AMNPs provided an additional advantage of the magnetic separation, which was employed to validate reusability. It was found that AMNPs have better starch digestion capacity at different physicochemical conditions than that of free α-amylase. The immobilized enzyme retained 66 % activity after 20 days compared to 18 % activity of the free enzyme. AMNPs were reused for 20 times, without significant loss of activity, by magnetically separating the nano-biocatalyst from the reaction mixture after each starch hydrolysis cycle (An *et al*., 2020).

The objectives of the study were to analyze the stability and catalytic activity of α-amylase enzyme by three different nanoparticles-- Iron oxide, Graphene oxide, and silver nanoparticle. The nanoparticles synthesized were characterized based on their size and magnitude using zeta potential.

Materials and Methods

Synthesis of Magnetite nanoparticles:

Magnetite nanoparticles (MNPs) were prepared by co-precipitation of Fe^{2+} and Fe^{3+} salts in 2:1 molar ratio. Ferric and ferrous chloride ($FeCl_3$.6H2O and $FeCl_2$.4H2O) in 2:1 molar ratio was dissolved in deionized water at a pH 9.5 adjusted by adding 1 N sodium hydroxide (NaOH) solution. After stirring (350 rpm) for 30 min, the black precipitation of iron oxide was formed in

the solution at room temperature. The particles were separated by a high-speed centrifuge at 13,000 x g for 20 min, washed with deionized water three times at pH 8, and dried in hot air oven at 70 °C for 10 h, respectively. The dried contents were ground well to fine powder after drying (Misson *et al*., 2015).

Synthesis of Graphene oxide nanoparticles:

Graphene oxide nanoparticles were prepared by mixing concentrated Sulphuric acid (H_2SO_4) and concentrated Orthophosphoric acid (H_3PO_4) in a 9:1 ratio (90 ml and 10 ml, respectively). Prepared a mixture of graphite powder and Potassium permanganate ($KMnO_4$) in a 1:6 ratio (1 g and 6 g, respectively). The acid mixture was kept on a magnetic stirrer and graphite-$KMnO_4$ mixture was added slowly while stirring. Later, kept on stirrer for 12 h with temperature maintained at 50ºC. 30 ml of 30% H_2O_2 was added slowly into the mixture. The mixture was kept in an ice bath to cool to room temperature and 100 ml of cold distilled water and mixed well. Centrifuged the contents for 5 min at 2500 rpm, removed the supernatant, and kept the pellet. Acid wash with 10 ml of 30% HCl was done twice. Centrifuged after the addition of acid for 10 min at 5000 rpm and discarded the supernatant. Gave ethanol wash with 10 ml of 70-80% ethanol and centrifuged. The pellet was added with 5 ml of 70% ethanol in each tube and mixed well. Then, allowed it to dry at a 50ºC incubator overnight. It was ground well to fine powder after drying (Gupta and Gupta, 2005).

Synthesis of Silver nanoparticle:

About 1.5 g of fresh flower of *Hibiscus rosa-sinensis* was weighed and washed with de-ionized water before use. The crushed flowers were mixed with 100 ml of de-ionized water and allowed to stand for 6 h at room temperature. The mixture was filtered. To 10 ml of this solution, silver nitrate solution was added drop by drop. Keep the beaker still in a magnetic stirrer for 3 h. The reduction of Ag^+ to Ag^0 was confirmed by the color change of the solution from red to brown which indicates the formation of silver nanoparticles (Tartaj *et al*., 2003).

Stability of Nanoparticles:

Dynamic light scattering (*DLS*):

DLS measurements were performed with a Zeta sizer equipped with a maximum 4 mW He–Ne laser, emitting at 633 nm (ZS 90, Malvern Instruments Ltd, Malvern, UK) instrument to determine the nanoparticles' diameters and zeta potentials, respectively. For DLS three different vials of each material were measured in triplicate under repeated conditions for the three nanoparticles (Ali *et al*., 2016).

Determination of α-amylase agar well diffusion assay:

α-amylase enzyme screening plate assay, the agar plates were prepared with 2% of starch and 1.5% of agar. After the solidification of agar, about 10 mm diameter of the well was cut aseptically, using a 6 mm cork borer. Then, the wells were filled with the enzyme concentrations (100 µl,75 µl, 50 µl, respectively). Under sterile conditions, constant µl of nanoparticles were added to the wells and incubated at 40°C for 72 h. Overlayed 1% of iodine solution on the agar and observed for zones of hydrolysis around the wells. The negative control was maintained by adding sterile water in a separate well (D'Agata *et al*., 2017).

Results and Discussion

Zeta potential (Figs. 1, 2) was done to identify the size and potential of Iron nanoparticles. The FeNPs had ZP values of 0.0129mV, average size was 1635 nm, and a PdI of 0.740. The nanoparticles synthesized in our study were confirmed to be stable.

Zeta potential was done to identify the size and potential of Graphene nanoparticles (Figs. 3, 4). The GRNPs was with ZP values of -8.60mV, average size was 6264 nm and PdI of 1.000. All the parameters concluded that the synthesised nanoparticle was stable.

Zeta potential was done to identify the size and potential of silvernanoparticles (Figs. 5, 6). The AgNPs with ZP values of -10.3mV was found to be moderately stable, average size was 165.2

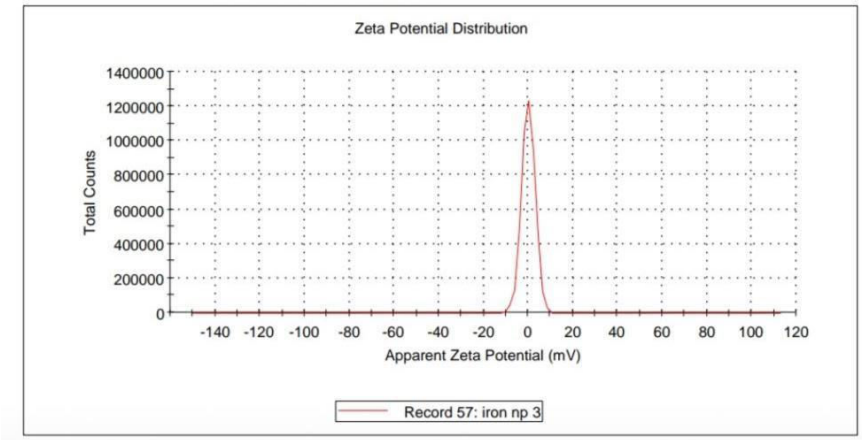

Fig. 1: Analysis of Zeta potential of Iron oxide Nanoparticles.

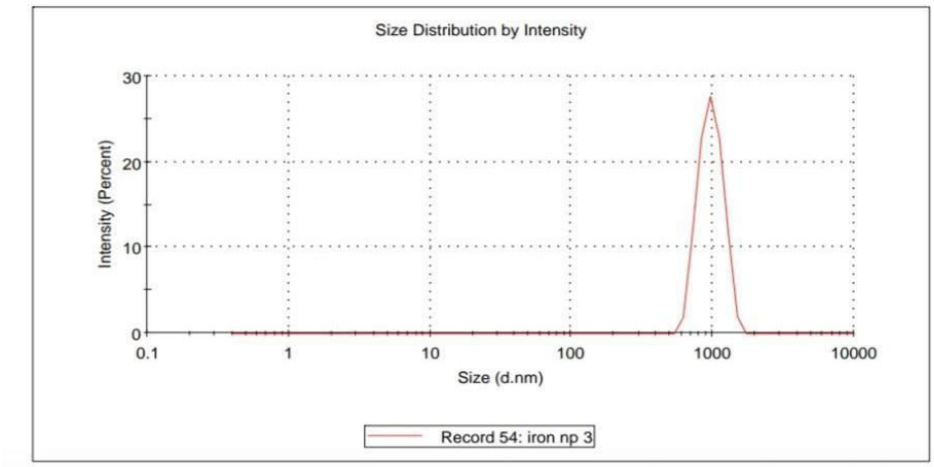

Fig. 2: Analysis of Size distribution of Iron oxide Nanoparticles.

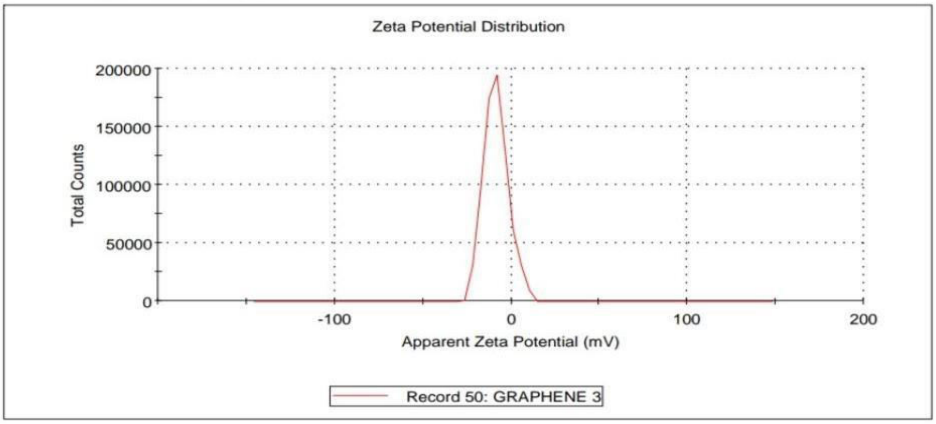

Fig. 3: Analysis of Zeta potential of Graphene Nanoparticles.

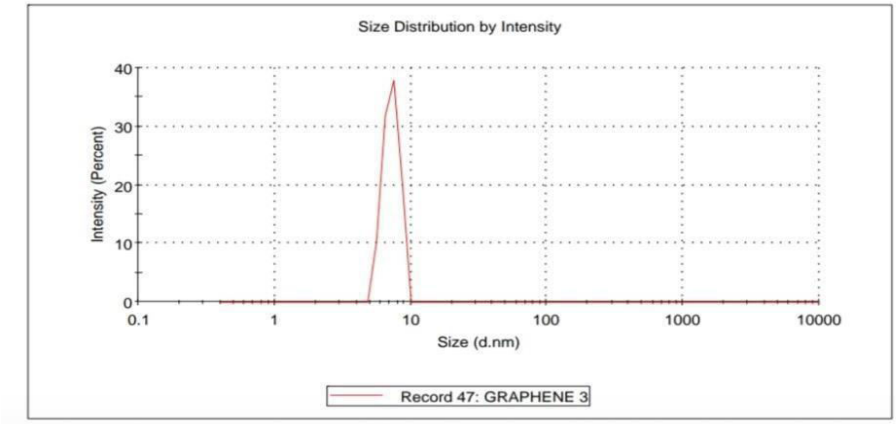

Fig. 4: Analysis of Size distribution report of Graphene Nanoparticles.

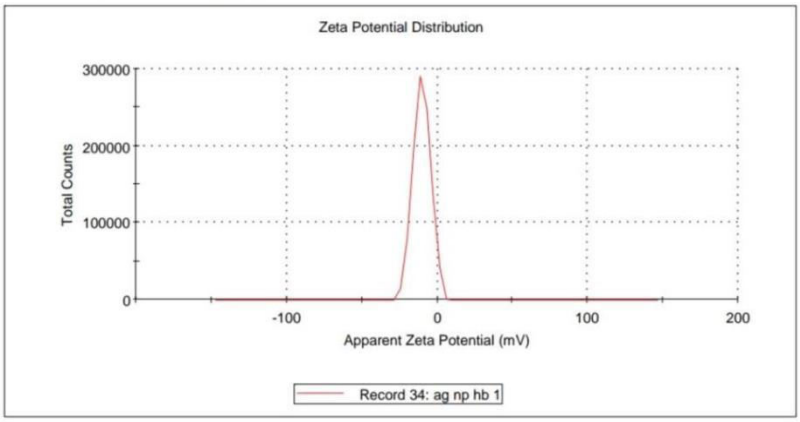

Fig. 5: Analysis Zeta potential report of Silver Nanoparticles.

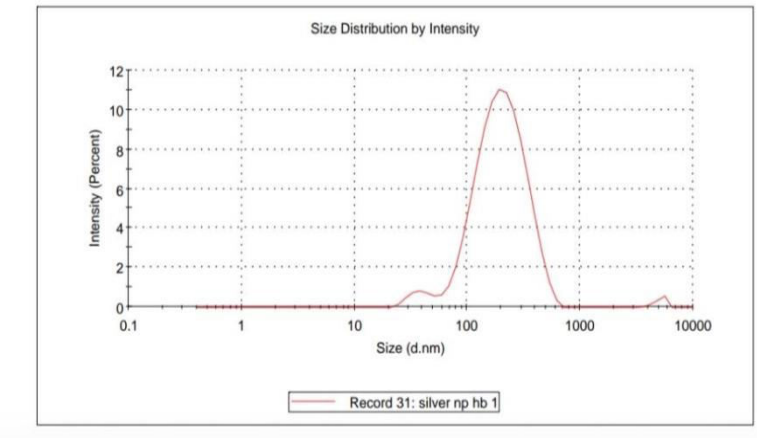

Fig. 6: Analysis of Size distribution report of silver Nanoparticles.

Table 1: Catalytic activity of α- amylase by iron oxide, graphene oxide and silver nanoparticles

Nanoparticle	Zone of Hydrolysis in mm		
	100 µl	75 µl	50 µl
Standard Enzyme Sample	25	22	19
FeNPs	38	35	33
GR NPs	32	24	22
AgNPs	27	25	23

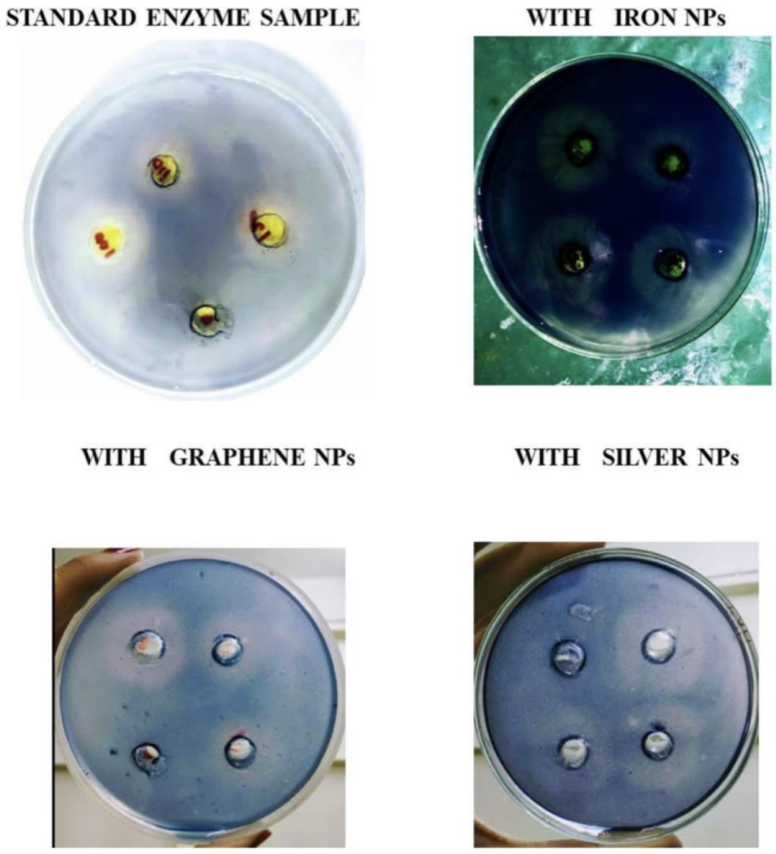

Fig. 7: Agar well diffusion assay with Zone of hydrolysis.

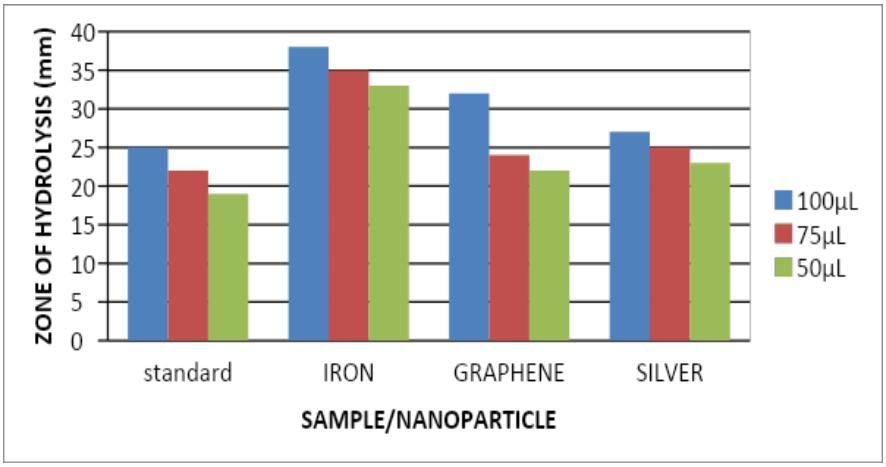

Fig. 8: Catalytic efficiency of nanoparticles of α-amylase by iron oxide, graphene oxide and silver nanoparticles.

nm and PdI of 0.267 (Sintov *et al*., 2016). Silver nanoparticles having PdI value of more than 0 (Arsalan and Younus, 2018). 20 shows it is highly polydispersed and in accordance with previous studies.

Table 1 illustrates the zone of hydrolysis in mm of enzyme concentrations (100 µl, 75 µl, and 50 µl, respectively) and Figure 7 indicates the clear zone around the samples showing catalytic activity (Paulchamy *et al*., 2015). Figure 8 shows the catalytic efficiency of Iron oxide, Graphene oxide, and silver nanoparticles on the hydrolytic activity of α- amylase (Naghdi *et al*., 2018). It is observed that all three nanoparticles showed increased activity in comparison with the standard. As the catalysis of an enzyme is influenced by its surface area and hence in the current study, the synthesized nanoparticles would have enhanced the catalytic activity of α-amylase (Wang *et al*., 2018). In this study, iron oxide nanoparticles showed the highest efficiency among the others (Nayak *et al*., 2015).

Conclusion

Amylase is an important enzyme used in the industry and accounts for approximately 25% of the enzyme market. As the nanoparticles could enhance the activity of enzymes, this study was designed to synthesize iron, graphene, and silver nanoparticles of α- amylase which is an important enzyme in the industry. The synthesized nanoparticles were characterized by determining their zeta potential and size, which determined the stability of the nanoparticles. The stable nanoparticles of iron, graphene, and silver nanoparticles were analyzed for their catalytic activity by the well diffusion method. The zone of hydrolysis diameters was measured and it was found that Iron had more catalytic activity than silver and graphene oxide nanoparticles. Among the three nanoparticles, the iron oxide particles showed increased activity followed by graphene nanoparticles. The silver nanoparticles do not show much activity in comparison with standard α-amylase activity. Hence, the study suggests that the iron and graphene nanoparticles of α-amylase could be used in industries for better performance.

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