Immobilization of α-amylase onto Magnetic Nanomaterials: Process Optimization and Evaluation of Biocatalytic Activity

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CERTIFICATE

This is to certify that the dissertation entitled **"Immobilization of α-amylase onto Magnetic Nanomaterials: Process Optimization and Evaluation of Biocatalytic Activity"** submitted by **Mr. Yeshaswi Kaushik** (Roll. No. 601704008) in partial fulfillment of the requirement for the award of the degree of **Master of Technology** in Biotechnology, TIET, Patiala (India) is the record of the candidate's own independent and original research work carried out under my supervision and guidance. The matter embodied in this dissertation has not been submitted in part to any other University/Institute for the award of any degree or diploma in India.

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DECLARATION

I hereby declare that the work which is being presented in dissertation entitled **"Immobilisation of α-amylase onto Magnetic Nanomaterials: Process Optimization and Evaluation of Biocatalytic activity"** submitted by me for the award of the degree of **Master of Technology** in Biotechnology, TIET, Patiala (India) is true and original record of my own independent and original research work carried out under the supervision of **Dr. Shekhar Agnihotri**. Further, I declare that no part of this dissertation has been submitted to any other University/Institute for the award of any degree in India or abroad.

Place: Patiala

Dated: 09/08/2019

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List of Abbreviations

BBD	Box-Behnken experimental design
BSA	Bovine serum albumin
D-MHNTs	Dopamine Magnetic Halloysite Nanotubes
DNSA	3,5-dinitrosalicylic acid
FTIR	Fourier Transform Infra-red spectrum
HNTs	Halloysite Nanotubes
HRP	Horseradish Peroxide
mM	millimolar
NBC	Nanobiocatalysts
nm	nanometer
NPs	Nanoparticles
OD	Optical density
pH	Potential of hydrogen
PPW	Potato peel waste
RSM	Response surface methodology
SD	Standard deviation
SEM	Scanning electron microscope
UV	Ultra Violet
VSM	Vibrating Sample Magnetometer

Abstract

Food industry is one of the most important industries in modern society and provides a wide range of products for human need. However, the perishability of crop and food products, results in the generation of significant quantities of food waste. Potato peel waste (PPW) as zero value byproduct generated from food processing industries contains large quantity of starch, non-starch polysaccharides, lignin, protein and lipids. As starch is high yield feedstock, requires enzymatic hydrolysis into reducing sugar before further processing. α-amylase, enzyme cleaves glycosidic linkages in starch and yields dextrins and oligosaccharides. In this work, α-amylase was immobilized onto the dopamine functionalized magnetic halloysite nanotube via Schiff base reaction. The ultimate aim was to develop a nanobiocatalysts (NBC) for efficient starch hydrolysis and determine its industrial capability. The synthesis and functionalization of magnetic carrier for immobilization were examined by UV-visible spectroscopy, FTIR, VSM and FE-SEM. A high enzyme loading of 185.52 mg/g of support matrix was achieved. The immobilized α-amylase displayed optimal activity at pH 6.5 and temperature 50 ℃. Furthermore, the pH and thermal stability of the immobilized enzyme indicates improvement as compared to its free counterpart. The immobilized enzyme had also been demonstrated to be capable of being reused for 10 cycles with 56.7 % retained activity. In addition to that, even after storage time of 30 days the NBC retained almost 64.8 % of initial activity.

Keywords: Potato peel waste, α-amylase, Nanobiocatalysts, Dopamine magnetic halloysite nanotube, Starch hydrolysis.

CHAPTER 1

Introduction

Expanding worldwide population leads to growing demand for food manufacturing which stimuli the growth of food processing industries. According to a study, about one third of total food produced all around the globe finds its way to garbage through supply chain, processing unit or consumption habits. The increasing amount of food waste presents an unavoidable problem because of the limited waste management strategies. Food waste comprise ample amount of complex carbohydrates, proteins, lipids, and nutraceuticals. Disposal of such high nutrition value material is not only waste of resources employed for production, but also adversely affect the balance in ecosystem. The presence of many suspended liquids and solids, bring forth offensive odors and visual discomfort. Furthermore, accumulated food waste, being higher in nutritionary content, provides breeding grounds for disease-causing microorganisms (Rajeev *et al*., 2016). This complex food waste system could be utilized in the form of raw materials for commercially essential products. The thought of converting food waste into industrial products such as chemicals and energy, used in our everyday exercise is a region of research with immense potential and openings.

Potato is placed at fourth spot in list of most consumed agricultural crop after wheat, maize and rice. Potato peeling, is an inevitable step during industrial processing and production, causes loss of biomass anywhere from 15 to 40 % depends on the peeling methods as potato peel waste (PPW) (Wu *et al*., 2016). Every year almost 70-140 thousand tons of PPW as a by-product is generated during industrial potato processing. Traditionally, PPW is used for producing low values animal feed, fertilizers which causes waste of rich nutritive materials having the properties of antioxidants, antibacterial, apoptotic, chemo-preventive and anti-inflammatory (Khawla *et al*., 2014). The compositional analysis of PPW indicates the presence of large amount of starch (52g per 100g dry weight of PPW) along with non-starch polyphenols polysaccharides, lignin, protein and very little amount of lipids. Starch is high yield feedstock, but requires hydrolysis into reducing sugar before further processing. The traditional hydrolysis methods are based on corrosive chemicals, which results in

formation of inhibitory by-products. Downstreaming of the product also results in complications as hazardous chemicals are released into ecosystem. On the other hand, enzymes are highly specific biocatalysts, operate under mild reaction conditions, and possess no environmental threat. In addition to that, their inherent nature, high efficiency, less energy consumption, biodegradable nature, has led to the distributed use of enzymes in industrially. Amylases are hydrolase enzymes that have been industrially used since many years. It randomly cleaves internal glycosidic linkages in starch molecule to hydrolyze them and yields dextrins and oligosaccharides. Among amylases, α-amylase is in the maximum demand due to its wide range of industrial applications. It is observed that acid hydrolysis of PPW liberates less quantity of fermentable sugar and on the other hand enzymatic hydrolysis produces more (Hamid *et al*., 2014). However, their application in industrial sector is hindered by drawbacks associated with them such as long term operational strength, shelf life and reusability. These obstacles can be overcome by employing numerous approaches such as protein engineering, microbiology, chemistry of proteins, immobilization etc. Among all the techniques, immobilization is well known for high binding capacity, simplification of biocatalysts, increasing stability, recycling and smooth downstream processing. The principal elements of an immobilized enzyme system are the mode of attachment, matrix and enzyme (Mohamad *et al*., 2015).

During the last few decades a vast array of materials had been used as support for enzyme immobilization including, organic, inorganic, polymer etc. Ideal support should possess properties such as ease of derivatization, hydrophilicity, biocompatible, inertness toward enzymes and physical resistance to compression (Rueda *et al*., 2016). Different natural polymer materials like pectin, alginate, sepharose etc. have been commonly used for enzyme immobilization. Beside natural polymers, various synthetic polymeric materials have also been used owing to their good mechanical stability, large surface area and ease in modification. Various inorganic supports have also been used for the immobilization of enzymes, e.g., alumina, silica, zeolites, and mesoporous silica (Datta *et al*., 2013). Carriers with high surface area are considered superior to others as they can lead to high enzyme loading.

Over the last years, the use of nanostructures to immobilize enzymes is considered as one of the crucial research development in modern science (Eliane *et al*., 2014). The use of nanomaterials offers numerous advantages such as improved biocatalysts

efficiency, high specific surface area, mass transfer resistance, and effective enzyme loading (Pandey *et al*., 2017). A broad class of nanomaterials had been used as supports for enzyme immobilization like gold nanoparticles, carbon nanotubes, silica nanoparticles, nanofibers, nanosheets etc (Roger *et al*., 2017). They have been used for wide range of applications like starch hydrolysis, proteolysis, biofuel production, bio-magnetic separations etc. In recent time, growing environmental concern about nanomaterial release into natural bodies and feasible/economical barrier associated with their synthesis process have somewhat restricted their use. These problems led to the search of eco-friendly and economical materials which could be utilized for biological means.

The abundance and biocompatible property of naturally occurring clay materials make them a perfect candidate as effective solid supports for the immobilization of enzymes (Tharmavaram *et al*., 2018). Clay is formed through the combination of one or more minerals with traces of metal oxides and organic matters. Naturally occurring clays like bentonite, montmorillonite, kaolinite, halloysite etc. have been exploited for immobilization of biomolecules. Halloysite, have attracted attention of scientific community because of its unique structural and chemical properties.

Halloysite is a form of kaolinite mineral with additional water molecules and a tubular structure formed under specific physiological conditions. They are hollow nanotubes with external diameter of about 100 nm, lumen diameter ranging from 5 to 30 nm and length between 500 nm to 1.2 μm. Their intrinsic features like, biocompatible nature, non-toxic in vivo and in vitro, strong surface adsorption, hollow lumen available for loading of biological molecules, simultaneously plays a crucial role for immobilization of biological molecules. The external surface of halloysite is made up of the siloxane (Si-O-Si) group with low chemical activity, whereas internal surface consist of Aluminol (Al-OH) groups and is comparatively active (Wang *et al*., 2013). Outer surface of halloysite nanotube bears negative charge, while inner core provides positive charge at lower pH. Owing to the above mentioned properties, they have been utilized in different applications like drug delivery, as pharmaceutical excipients, removal of dyes from aqueous environment, biocatalytic applications etc. However, the intrinsic inorganic property and the absence of abundant functional groups on halloysite results in nominal loading and weak bonding between biomolecules and surface (Chao *et al*., 2013). The weak bonding often results in leaching of

biomolecules while washing. Therefore, modification of halloysite surface becomes essential to improve the binding efficiency as well as for high loading of biomolecules.

Different modification strategies introduce either a number of functional group or change the surface morphology of HNTs (Pandey *et al*., 2018). Surface modification like alkali treatment, surfactants, acid etching, polymer coating etc. impart electrical, thermal, and antibacterial properties to the system along with enhancing stability. On the other hand, functionalization of halloysite nanotube could be used as target specific coating to capture special biomolecules. According to previous studies, many modifications like biomimetic molecules, dopamine, organosilanes etc. have been used for functionalization. Surface functionalization with polydopamine is considered as an efficient method to endow biological functionality on inorganic materials. The catechol/quinone moieties in polydopamine facilitate adhesion and deposition on solid surfaces through hydrogen bonding (Wang *et al*., 2016). After the coating of polydopamine on solid surface, enzyme immobilization can be easily achieved through Michael addition or Schiff base reaction.

The aim of the current study is to develop a novel nanobiocatalyst for efficient starch hydrolysis and to determine its capability for industrial process. The nanobiocatalyst was based on magnetically modified halloysite nanotubes. Further to impart functionalization onto MHNTs, dopamine self-polymerization was employed. Finally, alpha amylase was immobilized on magnetic halloysite nanotube via Schiff base reaction. Synthesis and functionalization are characterized through UV-Visible spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), VSM and FE-SEM.

Objectives of the work:

- Synthesis and functionalization of magnetic halloysite nanotubes as carrier for immobilization.
- Determining the favorable parameters for high loading of α-amylase on magnetic support.
- Optimizing the process parameters for efficient conversion of starch.

CHAPTER 2

Literature Review

After the onset of industrial revolution, food industry have become one of the most essential industries in society, as it lead to increase in products for both industrial sector and human need. These industries provide a range of different products for fulfilling the demands and needs of people around the globe like artificial sweeteners, breweries, grain flours, starches, dairy, fruits, vegetables, meats and many more. Owing to perishability of crop and food products, consumer demand and inefficiencies in supply chains, huge amount of waste products are being generated which is not only harmful to the environment but also causes a loss in economic resources (Zhang *et al*., 2016). Few of the harmful effects of these products include greenhouse gas emission like methane because of activities associated with food production and during final disposal in landfills. When exposed to environment, these harmful waste products also lead to depletion of natural resources, decrease in land quality and a disturbance in the biogenic cycles due to intensive agricultural practices to meet the increased demands. Since, food industries are a huge source of revenue, wastage of food can have a huge impact on economy to a lot of people associated with any industry, including owners, farmers and employers (Francesca *et al*., 2015). Some of these foods processing waste (FPW) include carbohydrate polymers like starch and cellulose, proteins, lipids and organic acids.

Ajay *et al.,* (2003) investigate that few number of low cost adsorbents like carbonaceous are prepared from slurry of fertilizer industries and are found to adsorb 198 and 211 mg/g of ethyl orange and metanil yellow dyes respectively. The adsorption data confirmed with good coefficients varying from 0.998 to 0.999 adsorption rate is fast. Overall, it concludes that it can be used for low cost alternative (US ＄100 per ton) for removal from effluents. Thus, finding ways to effectively convert such food wastes into useful industrial products is an region of research with immense potential and can be exploited to not only make products at a low capital cost but will also prove to be safe to the environment (Mattsson *et al*., 2002).

2.1 Potato peel waste (PPW)

Potato is the fourth main crop after rice, maize and wheat as it is a staple food item and plays an important role in human diet all over the world (Di *et al*., 2016). It is used as raw material by a number of industries like food, textile, paper and many more. One disadvantage of these industries is the generation of a high volume of wastes released as by-products which are usually discarded. Most of the wastes from potatoes arise from peeling, trimming, slicing, cleaning, and rinsing operations. One such waste product is potato peels. It mainly comprises of starch, pectin, cellulose, lignin, proteins, lipids and ash (Shaobo *et al*., 2014). Traditionally, potato peel waste is used for the production of animal feed and fertilizers. The waste products released due to the manufacture of these items are advantageous as it can act as an antioxidant, antibacterial agent and had anti-inflammatory properties.

Arka *et al*., (2013) studied that potato and banana peels, after the treatment with hydroxyapatite nanoparticles supplement purified pectate lyase nanoparticles pectate lyase [NP-PL] to produce reducing sugar. At temperature about 50 and 90 °C reducing sugar produced by NP-PL is 2-fold greater than untreated on every 3 peels. The optimum production of reducing sugar for banana and potato were after 24-6 hours and 24-4 hours respectively. This finding concludes that they have potential applications in various industries like textile and paper. In this regard and owing to a huge worldwide demand in alternative forms of energy (renewable), potato peels can serve as a promising alternative carbon resource for the production of various products and chemicals. Shaobo *et al*., (2014) used fermentation technology to convert PPW into crude biofuel via thermo chemical process. PPW contains fermentable carbohydrates that is utilized by fermentation using microbial consortium that yields about 60 % unreacted fermentation residue. After characterization, it concludes that there is phenolic and long fatty acid compounds in PPW and fermentable PPW and have potential for conversion into bio-fuels. Before further processing, starch needs to be converted into a reducing sugar by the process of acid/enzyme hydrolysis (Ben *et al.*, 2013).

Acid hydrolysis requires extreme temperature and pH conditions for which corrosive-resistant equipment are generally employed. On the contrary, biological catalysts called enzymes are universally present in both plants and animals, where they are responsible for enhancing the speed of a large number of reactions in the body. Enzymes can be extracted from the living systems and used in industries like paper, textile, food, pharmaceuticals etc. They have specificity towards a particular substrate and have number of applications in baking, production of dairy products, beverage processing and hydrolysis of starch. The quality of products has greatly been enhanced due to the use of myriad enzymes in various reactions.

2.2 α-Amylase

Starch hydrolyzing enzymes belongs to different subclasses based on the nature of end products formed after enzymatic action. One such hydrolase is amylase which catalyzes the breakdown of starch into low molecular weight dextrins, oligosaccharides and small sugar molecules and plays important role in breakdown of starch, seed growth and maturation (Marc *et al*., 2001). It includes, linear glucose polymer and branched polymer containing α-1,4 linkages i.e amylose and α-1,4 glucose residues linked by α-1,4 linkages i.e amylopectin respectively. The optimum activity of α-amylase varies in very large range from 30 °C up to 110 °C. The thermo-stability of enzyme makes the enzyme feasible to be used at higher temperature. The optimum activity of the enzyme generally occurs in the acidic pH range of 4.8 and 6.5 but it varies depending on the source of enzymes and on the availability of calcium ions. Certainly the presence of calcium ion plays an important role (Swetha *et al*., 2007). In the presence of calcium ions, α-amylase is quite resistant to extremes of temperature, pH and even to treatment with some proteases like pepsin, trypsin and subtilisin. The most distributed application of α-amylase are, starch industries where the enzyme takes part in the hydrolysis of starch in liquefaction process and finally gets converted into fructose and glucose syrups (Paula *et al*., 2010).

For the industrial scale processes, enzymes from *Bacillus* species are desirable because of their extraordinary thermo-stability. Extensively, enzymes in the detergent industries are widely used in respect of quality and quantity (Gupta *et al*., 2003). The use of enzymes in the preparation of detergents improves its ability to remove tough

stains and breaks the residues of starchy food items like potatoes and many more to dextrin and other oligosaccharides and also makes the detergent surroundings safe. Amylases are also extensively working in food-processing industries such as baking, brewing and starch syrups (Couto *et al*., 2006). To degrade the starch into smaller dextrins in the bread, amylase can add to the dough, which would be fermented by yeast. For the improvement of texture of the dough, α-amylase is added results out improvement in rate of fermentation. Besides generating fermentable compounds, α-amylase also has an anti-staling effect in bread baking, and they help the baked goods to retain their softness. Amylases find a wide range of application in textile industries (Feitkenhauer *et al*., 2003). α-amylase in the pulp and paper industry is employed for the modification of coated paper, i.e. for the production of low-viscosity, high molecular weight starch (Maarel *et al*., 2002). Starch also acts as a good sizing agent for providing paper with a good texture and improving its quality. Nevertheless, all the preferred characteristics of the enzyme and their distributed industrial applications are obstructed by inability to recovery, reusability, shelf life and lack of long-term functional stability (Rueda *et al*., 2016). These obstacles can be overcome by immobilization approaches. Immobilization is well known for improving stability of the enzyme enhancing its activity and ability to reuse the enzyme multiple times. Immobilization of an enzyme restricts its mobility in fixed space. An immobilized enzyme can be reused multiple times which helps in avoiding the situation of isolating the enzyme again and again and leads to lower capital cost and a product of better quality (Brady *et al*., 2009). The properties of supports are important to identify the performance of the immobilized enzymes. Various polymer and inorganic matrices like chitosan, pectin, zeolites, silica etc. have been used as support matrices for enzyme immobilization (Datta *et al*., 2013). Various immobilization method were explained in figure 1.

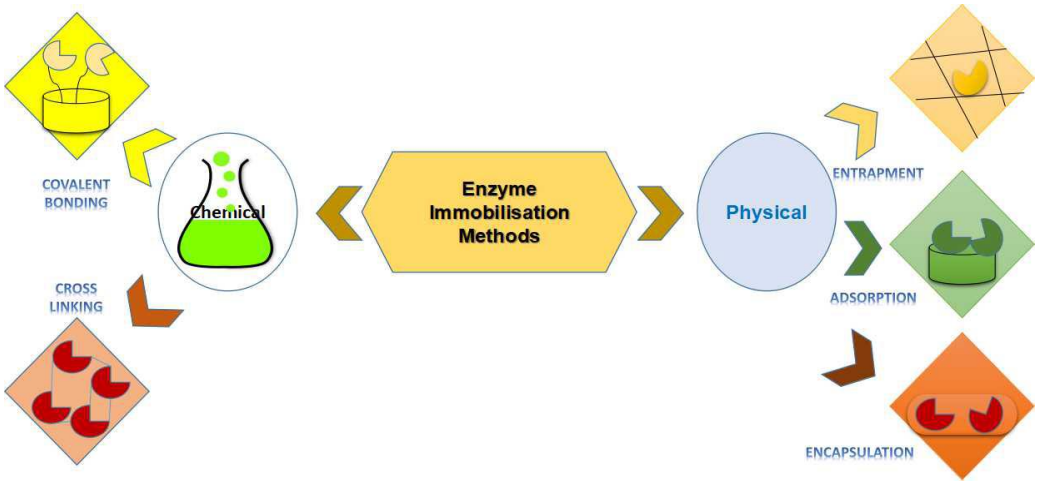

Figure 1: Methods of immobilization

Vezir *et al*., (2007) explored the covalent immobilization of α-amylase on phthaloyl chloride contains amino groups functionalized glass beads. Immobilized α-amylase indicated better thermo stability than free enzyme. Bound amylase was stable up to 25 days with 80 % retained activity while as free enzyme lost activity in 15 days. Immobilized enzyme retained 81.4 % even after 25 runs. On the other hand, Gargi *et al*., (2003) immobilized α-amylase on calcium alginate beads. Immobilized activity was affected by bead size as size of 2 mm is most effective for hydrolysis. Catalytic activity studied in various starchy residues. Optimum pH and temperature was found to be 4.9 and 57 °C on which apparent activity was 25.6 U/g of beads. In a similar study, Jakub *et al*., (2018), immobilized α-amylase on titania/lignin novel hybrid support. The highest activity of immobilized enzyme was achievable at 5 °C, 7.0 pH and 3 hour process duration. Immobilized enzyme retained over 80 % initial activity when stored for 30 days at 4 °C.

2.3 Nanotechnology

Nanotechnology is the branch of engineering science with effective work at biological scale. The idea of nanotechnology was initially examined by “Richard Feynman” honored physicist, in 1959 in his talk “There's Plenty of Room at the Bottom”. He was known as the “Father of Nanotechnology”. National Nanotechnology Initiative said that it is manipulation of matter on molecular and supermolecular scale having at least one dimension sized 1 to 100 nm. Due to the assortment of potential applications, governments have invested billions of dollars in nanotechnology research. For example, it profound influence on medical machineries like imaging probes,

diagnostic biosensors, and systems of drug delivery in the pharmaceutical companies. In cosmetics and food industries it is used for meliorate in shelf life, packaging, production, and bioavailability. It also have impending benefits in the field of medicines like improved drug delivery system, reduced inflammations, antibacterial coating over medical devices, improved surgical healing of tissue, and detection of cancer cells in blood circulation. Be that as it may, because of the absence of complete data about the toxicology of nanomaterials, the potential utilization of nanotechnology is curbed.

Nanomaterials are the cornerstone of nanoscience and nanotechnology and are expected to open new avenues to various rising innovative applications. They developed as an inventive field that has pulled in striking consideration from enzymologists (Kim *et al*., 2008). They have the potential for building up the ways in which materials and products are generated and the scope and nature of functionalities that can accessed (Sutariya *et al*., 2014). Nanomaterials with basic measurements under 100 nm may exhibit superior chemical, biological, mechanical, magnetic and optical properties that are often not quite the same as their corresponding small counter parts. These one of a kind properties relies upon the atomic structure, size confinements, composition, micro structure, defects and interfaces, all of which can be tailored by synthesis and other processes. The examination on nanomaterials is profoundly interdisciplinary in light of the fact that it includes various synthetic methodologies and characterization techniques (Husain *et al*., 2017). Synthesis of nanomaterials play a significant role for cutting edge applications rotates around the issue of collecting atoms or molecules into nanostructures of the desired coordination environment, size and shape.

For the suitable and efficient support of enzyme immobilization, nanomaterials are widely used industrially because of the effective enzyme loading, mass transfer resistance, large surface area and identify biocatalytic efficiency (Cipolatti *et al*., 2014). Immobilization of enzymes to the nanomaterials can increase catalytic efficacy, stability, hyper activity, catalytic efficacy, facile recovery, performance etc. shown in figure 2.

Figure 2: Advantages of nanomaterials as enzyme immobilization platform

Nanoparticles have been studied as potential supports for enzyme immobilization in previous years of both organic and inorganic origin. The synergy between nanomaterials and immobilization techniques improves the efficiency of the biocatalytic system and significantly improves the yield of immobilization (Verma *et al*., 2010). The plus point of nanoparticles over different materials is quality to get low diffusional limitations (Min *et al*., 2014). Nanofibers are also used as support materials for the immobilization of enzyme and can be immobilized inside or outside surface. When immobilization takes place inside the nanofibers, polymer solution is mixed with enzymatic solution after nanofibers are created from the mixture (Cipolatti *et al*., 2014). So, the enzymes are enclosed in between the fiber material and polymer chains. Comparatively, immobilization on outer surface of fiber materials require best quality for enzyme functionality, good stability and higher security.

One of the emerging nanomaterials that have been widely used in recent years for immobilization of enzyme is carbon nanotube. They have characteristic like organized nanoporous structure, biocompatibility with larger surface area in both multi walled and single nanotubes shown in figure 3. To enhance the enzyme-matrix interaction carbon nanotubes can also be further functionalized (Besteman *et al*., 2003). They are frequently used for immobilization and in different industrial sectors like, biosensors

for the identification of different compounds like phenol, pharmaceuticals etc.

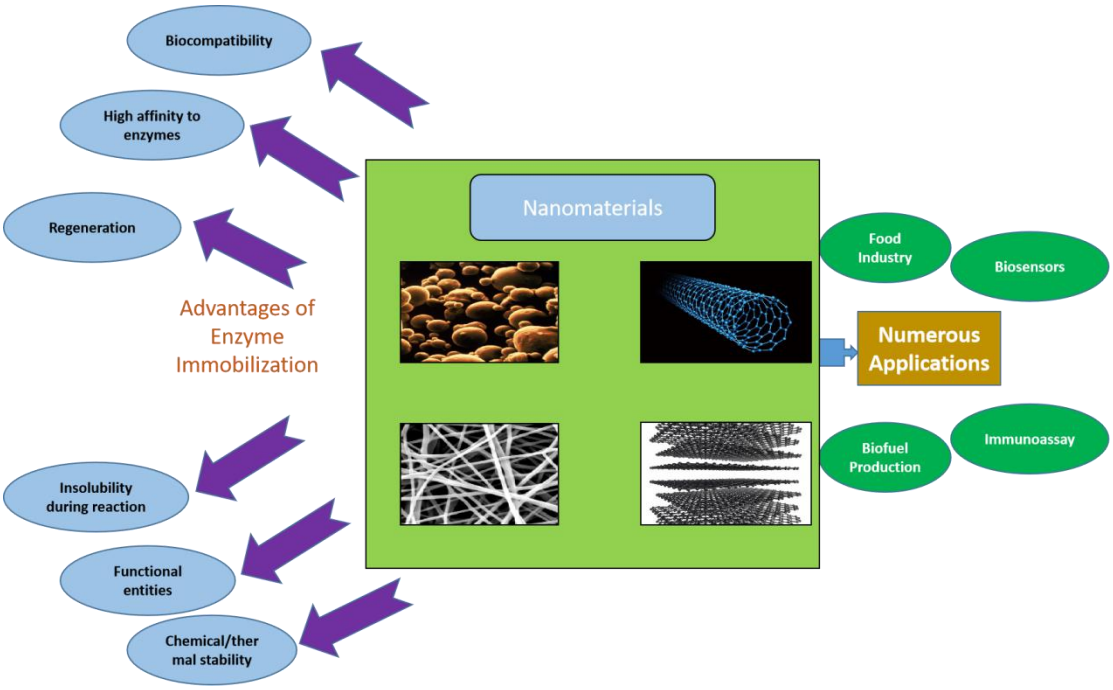

Figure 3: Advantages and applications of different nanomaterials

Nanomaterials have wide range of industrial applications because of their specific physical, chemical and mechanical properties (Neethirajan *et al*., 2011). In the solar cells TiO_2 nanoparticles can be transparent and function as the electrons acceptor. Organic semiconductor layer is being excites by solar radiation which then emitting the electrons and shift to the semiconductor TiO_2 nanomaterials. To increase solar cell efficiency a patterned TiO_2 thin film containing numerous pores a few nm in diameter can be used. Nanomaterials are used in the automotive industry, for construction of lightweight materials, paints, tires, sensors, and covering for windscreen of car body parts.

In pharmaceutical sector nanomaterials are utilized as drug delivery systems, operational agents, medical rapid tests, antimicrobial agents, covering and agents in different therapies. As, nanomaterials have better chemical activity with effective specific properties, they can be used as catalysts to act with toxic gases like nitrogen oxide and carbon monoxide in instruments of the power generation to prevent the environment pollution arising from different burning fuel and coal (Misson *et al*., 2015). Comprehensive utilization of nanomaterials in different sectors and the use of nanomaterials had emerged as a skilled tool for creating superior matrices because of

their specific properties, they serve as novel matrices for enzyme immobilization and target to decrease the biocatalysts cost industrially by aiming to increase enzyme loading, its activity and stability. The high surface to volume ratio results in concentration of immobilized entity being higher. They have been used in diverse range of applications as mentioned in table 1.

Table 1: Enzyme immobilized on nanomaterials & their Industrial application

S.no	Enzyme	Nanoparticle	Bonding	Application	Reference
1	β-glucosidase	Iron oxide nanoparticles	Covalent	Biofuel production	Verma *et al*., 2013
2	Superoxide Dismutase	Nano Fe_3O_4 coated on a gold electrode surface	Covalent	Biosensors	Chen *et al*., 2008
3	Invertase	Chitosan nanoparticles	Covalent	Food Industry	Sun *et al*., 2009
4	α-amylase	Silica nanoparticles	Adsorption	For enhancing removal of starch soils	Ahmad *et al*., 2014
5	Trypsin	Nanodiamond	Covalent	Proteolysis	Wei *et al*., 2010

6	Lysozyme	Chitosan nanofibers	Covalent	Antibacterial	Meyer *et al*., 2014
7	Horseradish peroxidase	Magnetic silica nanoparticles	Entrapment	Immunoassays	Yang *et al*., 2014
8	Cholesterol oxidase	Fe_3O_4 nanoparticles	Adsorption	Total cholesterol in serum	Kouassi *et al*., 2005

2.4 Halloysite Nanotubes (HNTs)

Halloysite nanotubes are naturally occurring clay mineral composed of double layers of aluminium, silicon, hydrogen and oxygen. They are found abundantly around the world with tubular morphology having about 1500 nm lengthwise, 15 nm lumen and 50 nm external diameters. HNT Formation is caused by lattice mismatch between aluminium oxide layers and adjacent silicon dioxide (Zhai *et al*., 2010). Due to their unique properties like high aspect ratio, high porosity, superior loading rates to other carriers, high adsorption capacity, high cation exchange capacity etc. were exploited for various applications explained in figure 4. The external surface of HNTs are composed of silicon oxygen octahedrons i.e. Si-O-Si group and internal surface composed of Al-OH. As HNTs are eco-friendly clay mineral and were confirmed to be non-toxic, hence is widely employed for biological applications. The weak intermolecular forces somewhat limits their application in biological applications (Pandey *et al*., 2017).

To improve the performances, surface modification is very desirable which includes, surfactant modification, coupling agent modification, intercalation modification, dopamine, surface covering modification, free radical modification etc. Surfactant modification means to presence of different groups in the surfactant molecules and can also be modified via electrostatic interaction. Coupling agent modification can be performed via chemical method in which grafting silane coupling agent reacted to the surface (Tharmavaram *et al*., 2018). Modified halloysite nanotubes get large contact area, and great dispersion (Kausar *et al*., 2018). Surface coating modification is done by coating surface with a covering of polymer by way of electrostatic adsorption. Dopamine is a molecule that can be polymerized at an alkaline pH to make

polydopamine (Chao *et al*., 2013). For the covering of polydopamine layer on solid surface, catechol and quinone could cause adhesion and deposition on surface via hydrogen bonding (Wang *et al*., 2017).

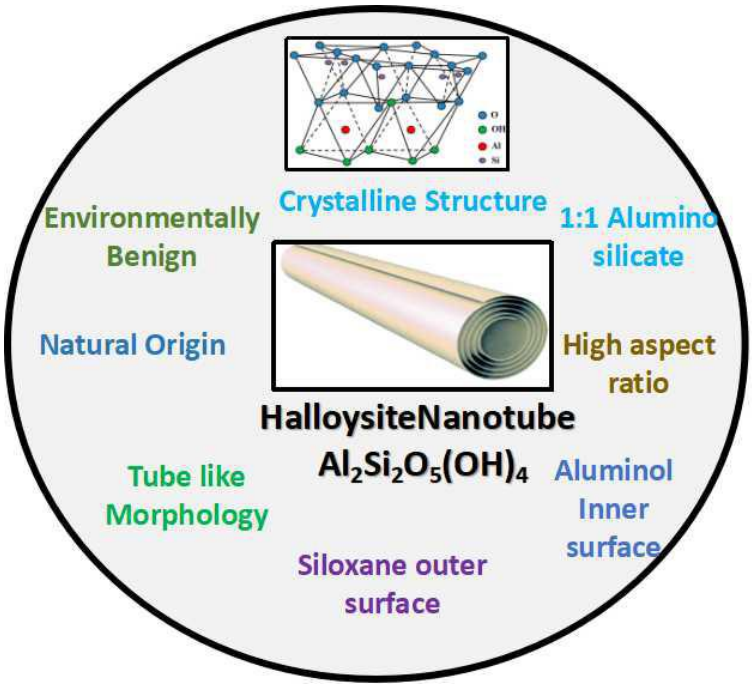

Figure 4: General properties of Halloysite Nanotubes

2.5 HNTs as template for various biological applications

Halloysite nanotube has been widely used in assorted antimicrobial application to immobilize different nanoparticles and having antimicrobial properties. Immobilization improves the antimicrobial characteristics of the materials by accelerating their dispersion and stability (Massaro *et al*., 2017). Jilei *et al*., (2015) used natural resources i.e. halloysite nanotube and chitosan to prepare porous hybrid by cross linking technique. The hybrid could be used as supports for the immobilization of laccase enzyme and shows loading capacity of 123.1 mg/g. Immobilized laccase was employed to remove phenols from waste-water, with removal efficiency as high as 95 %. HNTs due to their specific properties like non-toxicity, drugs can be easily absorbed and existences of different groups on their surface ease the binding of drugs. Different lipophilic and hydrophobic drugs can be easily encapsulated in the lumen of HNTs with which it show long release time and high loading because of which drug dosage can decreased.

Jiajaa *et al*., (2015) studied the clinical applications of HNT based microfibers. They developed electrospining drug loaded halloysite clay nanotube doped into poly microfibers. In the membrane 20wt % nanotube content allows 25wt % metronidazole drug loading. The halloysite doped membrane acts as barriers against cell in grows and have good biocompatibility. The drug loaded halloysite nanotube incorporated in microfibers allows for extent release of drug over 20 days. It indicates capability of HNTs as drug container and can be used in different applications. HNTs have potential for the immobilization of enzymes. In a study Rui *et al*., (2013) employed 3D nanocomposites based on chitosan-halloysite nanotube hybrid with porous structure. It exhibited good capacity for horseradish peroxidase [HRP] immobilization through cross linking by glutaraldehyde. Highest loading of 21.5 mg/g was achieved that is higher than 3.1 mg/g on raw halloysite. Even after 35 days, immobilized HRP retained about 27 % activity. This study concludes that high removal efficiency for phenol from waste water.

CHAPTER 3

Methodology

3.1 Materials

Halloysite nanoclay ($Al_2Si_2O_5(OH)_4.2H_2O$, surface area 64 m^2/g), dopamine hydrochloride, ferric chloride hexahydrate, and ferrous chloride tetrahydrate were procured from Sigma-Aldrich. Starch, DNS and coomassie brilliant blue g-250 were obtained from Hi Media Pvt. Ltd (Mumbai, India). α-amylase from *Bacillus subtilis* (Approximately 165 U mg^{-1}) was purchased from MP Biomedicals. All the chemicals were of analytical grade and used without further purification. Potatoes peels were gathered from local market (Patiala, Punjab). Double deionized water was used thoroughly for all the experiments related to nanomaterials synthesis, functionalization and biological studies.

3.2 Starch extraction from potato peels

Potato peels were washed with deionized water to remove dirt. The peels were chopped down to small size of approximately 4 mm. The chopped peels were then stirred with deionized water for 4 hours. The color of solution changes to white/brown in appearance. The peels were parted with help of sieve. Afterwards, the solution was centrifuged at 800 rpm for 10 minutes to separate the powder. The obtained pellet was washed three times with deionized water to remove other impurities. The resultant powder was dried overnight at 40 °C.

3.3 Synthesis of Magnetic Halloysite Nanotubes (MHNTs)

MHNTs were synthesized as follows, ferric chloride hexahydrate (20mM) and ferrous chloride tetrahydrate (10 mM) were dissolved in 100 ml of deionized (DI) water with continuous stirring under nitrogen purging at 60 °C for 1 hour. Then, 500 mg of HNTs were introduced into suspension and was allowed to mix it for two hour. Afterwards, the pH of the solution was adjusted to 9-10, by adding ammonia solution drop-wise. The mixture was aged to 65 °C for 4 hours. As obtained black precipitate was separated with help of magnet and washed 3 times with DI water. The powder was then dried overnight at 60 °C.

3.4 Functionalization of MHNTs (D-MHNTs)

The functionalization of MHNTs was done employing a previously reported method with slight modifications. In brief, 200 mg MHNTs was dispersed in 20 ml Tris-HCl buffer (50 mM, pH 8.5) via ultra-sonication. After 30 minutes of ultra-sonication, 0.2 mg of dopamine hydrochloride was added into the MHNTs suspension. The resultant mixture was shaken in a shaker for 6 hours to obtain MHNTs coated with polydopamine. As obtained black product was separated with the help of a magnet, washed with Tris-HCl buffer and distilled water repeatedly until the solution became colourless and transparent. The resultant powder was denoted as D-MHNTs and was dried at 40 °C for 12 hours.

3.5 Immobilization of Alpha amylase (D-MHNTs-*Amy*)

The loading of enzyme on D-MHNTs was carried out as follows, an appropriate amount of D-MHNTs were mixed with definite amount of enzyme in phosphate buffer (pH 6.5, 50 mM). The solution was left at shaker for 24 hours at suitable

temperature. The enzyme loaded D-MHNTs were recovered with the help of magnet washed by buffer solution and stored at 4 °C for further use.

3.6 Enzyme loading

Enzyme loading on D-MHNTs was determined by Bradford method by measuring initial and final concentration of proteins in the solution. A calibration curve using BSA was plotted beforehand. The enzyme concentration in the initial and final solution was determined with help of UV-spectrophotometry by measuring the absorbance at 595 nm. The amount of enzyme loaded was calculated by the following equation:

$$\text{Enzyme loading (\%)} = \frac{(C_i - C_f)V}{W_{D\text{-}MHNTs}} \times 100$$

……**Eq.1**

Where, $\mathbf{C_i}$ and $\mathbf{C_f}$ are initial and final enzyme concentration respectively

V is solution volume in ml.

$\mathbf{W_{D\text{-}MHNTS}}$ is D-MHNTs amount added in g.

3.7 Determination of free and immobilized alpha-amylases activity

Amylolytic activity was measured by formation of reducing sugars from substrate (1 % w/v starch) in phosphate buffer (pH 6.5, 50 mM). The quantification of reducing sugar was done using millers method. In brief, alpha-amylase and substrate were incubated at 40 °C for 60 minutes and 100 microliter of the solution was taken out. Afterwards 300 microliter of 3,5-dinitrosalicylic acid (DNSA) was introduced into the solution and was mixed with the help of vortex. The resultant solution was kept in boiling water bath for 15 minutes. After cooling, the solution was diluted appropriately and absorbances were recorded at 540 nm using UV-visible spectrophotometer. One unit of enzyme was defined as the amount required to produce 1 micro-mol reducing sugar in one minute at optimum conditions. The initial activity was taken as 100 %, while retained activity were expressed as percentage of initial activity.

3.8 Response Surface Methodology

3.8.1 Single factor study

Single factor study was carried out to determine the effect of individual parameter associated with immobilization such as amount of support matrix, amount of enzyme, pH of the immobilization solution, temperature of the immobilization reaction and immobilization time.

(A) Effect of D-MHNTs amount on immobilization (%) was determined using five different amounts 20, 40, 60, 80, and 100 mg.

(B) Effect of enzyme amount on immobilization (%) was determined using different amounts 10, 20, 30, 40, and 50 mg.

(C) Effect of pH on immobilization (%) was determined using five different pH buffers 5.5, 6, 6.5, 7, and 7.5.

(D) Effect of temperature on immobilization (%) was determined on four different temperature conditions 20, 30 40 and 50 °C.

The factor under examination was varied while all other parameters were kept constant. The outcome of these experiments was employed in Response Surface Methodology (RSM) to determine the optimum condition for high enzyme loading.

3.8.2 RSM optimization

Response Surface Methodology (RSM) was employed using Design Expert software (version 6.0.8, state-Ease Inc, Minnealpolis, USA). Based on the single factor study, three independent factors [D-MHNTs amount (mg), temperature, enzyme amount (mg)] at three levels was used to determine the optimum condition. The factor level were coded as -1 (low), 0 (central point), and +1 (high), for all three parameters as shown table below.

Table 2: The levels and ranges of variables in Box–Behnken statistical experimental design.

Variable	Symbol	Coded level		
		Low	Centre	High
		-1	0	+1
D-MHNTs (mg)	A	50	60	70

Temperature (°C)	B	30	35	40
Enzyme (mg)	C	10	15	20

The response was represented as a second order polynomial equation to express the effect of different variables on the immobilization (%):

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad \text{.....Eq.2}$$

Where, Y represents immobilization (%), X_i and X_j equals to independent variables that affect the response. The β_0 defines regression coefficient for the intercept, β_i for linear, β_{ij} for cross product terms and β_{ii} for quadratic.

To evaluate the model adequacy with experimental data, Analysis of Variance (ANOVA) was employed. Using ANOVA values of various analytical coefficients such as, value of "p", multiple correlation coefficients (R^2), lack of fit and adjusting coefficient of determination (R^2-adj) was determined. In case of three independent factors, 17 set of experiments were designed by Box-Behnken design. All the experiments were conducted in triplicates and average value was filled in the response column. Box-Behnken design then generated a model which was further validated by performing experiments at the optimum condition.

Table 3 : RSM based Box-Behnken experimental design for independent variables

Run	D-MHNTs (mg)	Temperature (°C)	Enzyme (mg)
1	60.00	35.00	15.00
2	70.00	40.00	15.00
3	60.00	40.00	10.00
4	70.00	30.00	15.00
5	60.00	30.00	10.00
6	50.00	40.00	15.00
7	60.00	40.00	20.00
8	60.00	35.00	15.00
9	50.00	30.00	15.00

10	60.00	35.00	15.00
11	60.00	30.00	20.00
12	70.00	35.00	20.00
13	50.00	35.00	10.00
14	60.00	35.00	15.00
15	60.00	35.00	15.00
16	50.00	35.00	20.00
17	70.00	35.00	10.00

3.9 Factors affecting the enzymatic activity of enzyme immobilized

3.9.1 pH

The effect of pH on catalytic activity of free and immobilized alpha-amylase was investigated using different pH buffer solutions (4.5-8.0). 20 mg of nanobiocatalysts was employed for the effect of pH on catalytic activity. 10 ml starch solution (1 % w/v) was prepared in different pH buffers. The enzyme amount equal to loaded on 20 mg NBC was employed for free enzyme activity. The activity was measured by the method described in section 3.7.

3.9.2 Temperature

In order to evaluate the impact of incubation temperature on catalytic activity, free and immobilized enzyme were incubated with 10 ml substrate (1% w/v) solution, in phosphate buffer (pH 6.5, 50 mM) at different temperatures (30-60 ℃) for 60 minutes. The activity was measured by the method described in section 3.7.

3.10 Reusability

For the reusability of immobilized enzyme 50mg of nanobiocatalysts was employed. The NBC was incubated with 100 ml of starch (1% w/v) solution in phosphate buffer (pH 6.5, 50 mM). After every cycle the NBC was separated with help of magnet washed with buffer solution and was introduced to next substrate solution. The

activity of the immobilized enzymes was measured by method described in section 3.7.

3.11 Storage stability

For storage stability, immobilized enzymes and free enzymes solution were incubated with 10 ml of starch (1 % w/v) solution. Aliquots were taken periodically and enzyme activity was determined using the method described in section 3.7.

3.12 Conversion

Evaluate the practical applicability of the NBC, 100 mg of NBC was incubated with potato peel extracted starch solution (1% w/v). Aliquots were taken periodically and productions of reducing sugars were determined using the method described in section 3.7.

CHAPTER 4

Results and Discussion

4.1 UV-Vis Spectroscopy

UV-Visible spectroscopy characterization was performed for halloysite nanotube, iron oxide nanoparticles and magnetic halloysite nanotube to determine the change in intrinsic absorption characteristics. In case of halloysite nanotubes absorption was observed in the UV region along with a weak visible light absorption. The reason can be ascribed to the relation between absorption and the level of impurities. Iron oxide nanoparticles (Fe_3O_4) exhibited no measurable features in the visible range as the

absorbance steadily decreased in visible region. However, a broad peak was witnessed around 370 nm indicating the formation of iron oxide nanostructure. In the case of MHNTs, the mentioned peak red shifted to around 400 nm representing the formation of iron oxide nanoparticles on the surface of HNT. Furthermore, it can be seen from spectroscopic analysis that the characteristic UV-Vis absorbance peak for MHNTs at 260 nm superimposed on the characteristic iron oxide absorption spectrum.

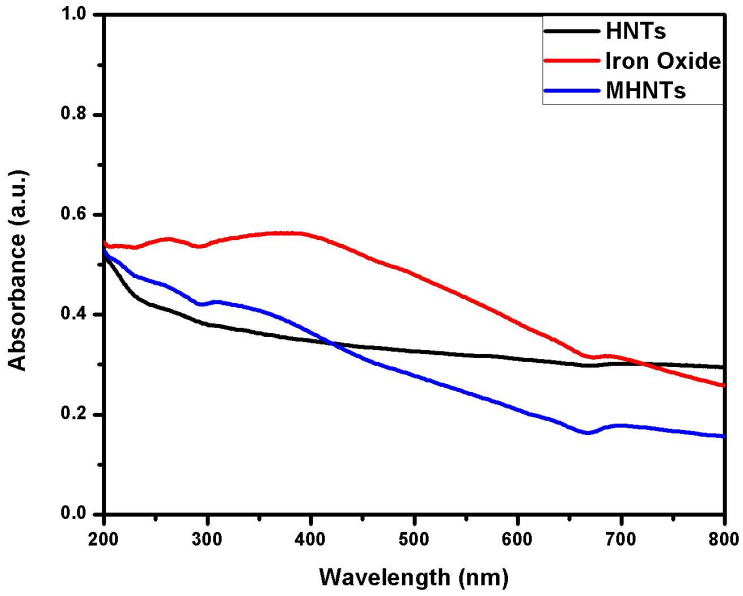

Figure 5: UV-Vis spectra of HNT, iron Oxide NPs and MHNTs

4.2 Vibrating Sample Magnetometer

Iron oxide nanoparticles and MHNTs were investigated by Vibrating sample magnetometer (VSM) for their corresponding magnetic behavior. Figure 6 represents the magnetization curves of the iron oxide nanoparticles and MHNTs, designating sample magnetization (M) in response to the applied magnetic field (G). The obtained results revealed that iron oxide nanoparticles, and MHNTs, have magnetization saturation values of 62.46, and 18.71 emu/g, respectively with zero coercivity and remanence. The zero coercivity and remanence is characteristic feature of super para magnetic materials. The lower magnetic saturation values in case of MHNTs were attributed to the presence of non-magnetic materials in form of HNTs. The separation

capacity of MHNTs however, was not compromised by this behavior and was easily separated as evident form the photographic image below.

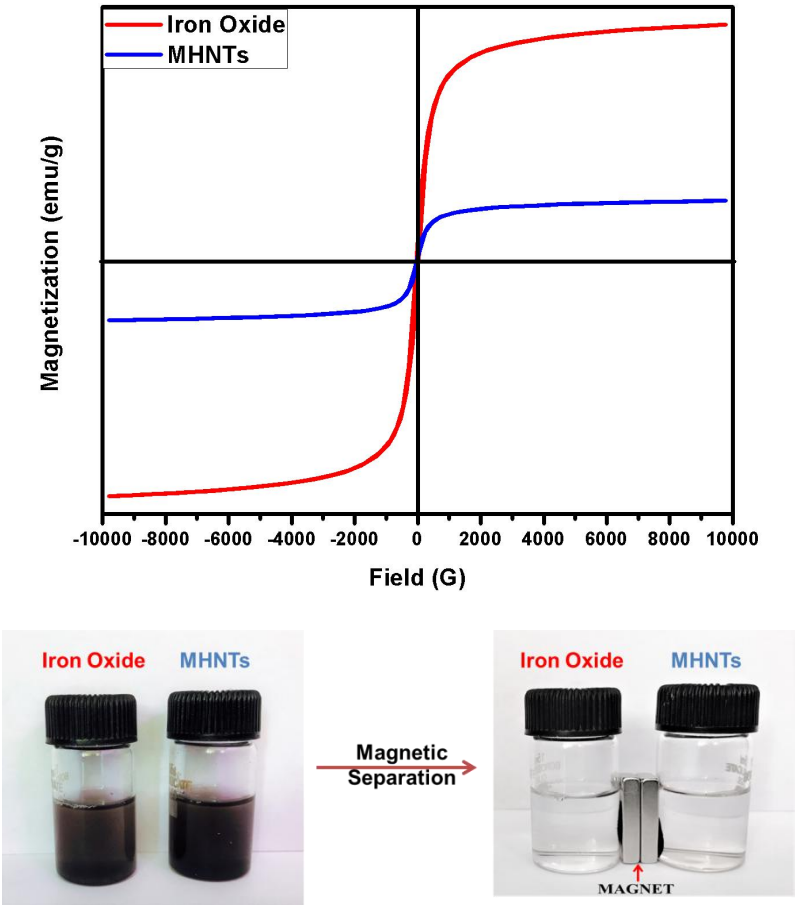

Figure 6: Magnetization curve of iron oxide, MHNTs and magnetic separation efficiency

4.3 SEM Analysis

The size distribution and the morphology of the Iron oxide nanoparticles were examined by SEM analysis. As can be seen, the structure of the halloysite nanotube was stable consisting of rough and irregular shape on the surface i.e. iron oxide nanoparticles.

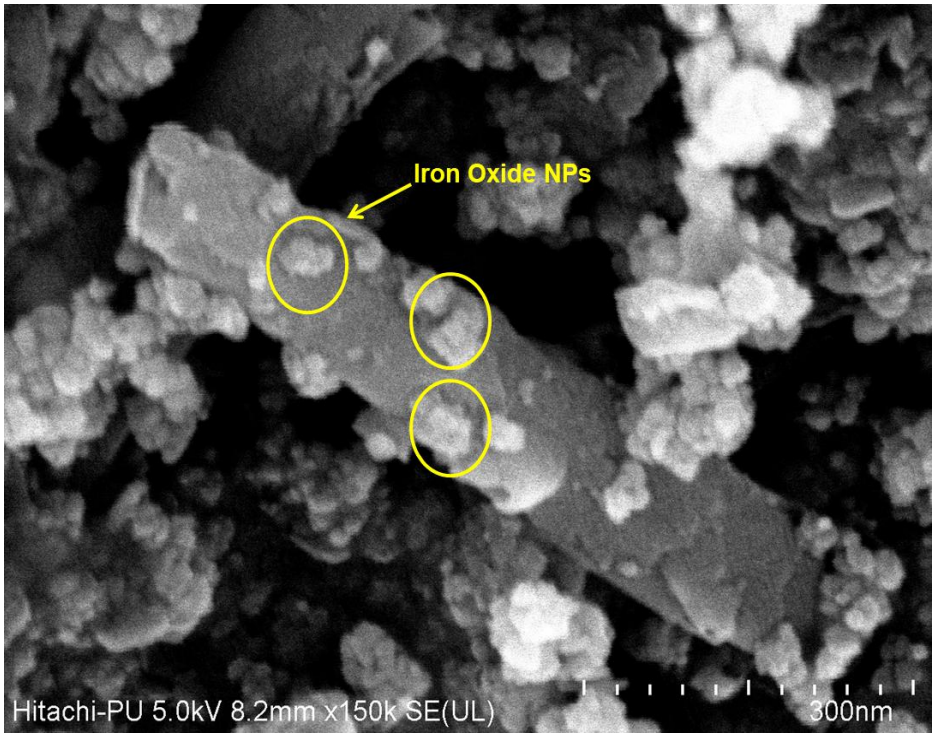

Figure 7: SEM micrograph of MHNTs

4.4 FTIR Analysis

FTIR analysis was performed to determine functional changes in HNTs, Iron oxide, MHNTs, α-amylase and D-MHNTs-amylase. As shown in the figure 8, spectra of HNTs have characteristic peaks at 1035 cm^{-1} attributed to Si-O stretching of bonds. The spectra of iron oxide show an absorption band at around 590 cm^{-1} which is assigned as unique peak of Fe-O band stretching vibrations. The presence of the above band stretching in spectra of MHNTs indicates the successful incorporation of iron oxide nanoparticles on HNTs surface. The appearance of peaks associated with HNTs and iron oxide along with characteristic peaks of α-amylase at 1650cm^{-1} in D-MHNTs-Amylase indicates the immobilization of α-amylase on D-MHNTs. Based on the above observations, it is concluded that functionalization with dopamine and α-amylase immobilization is successfully achieved.

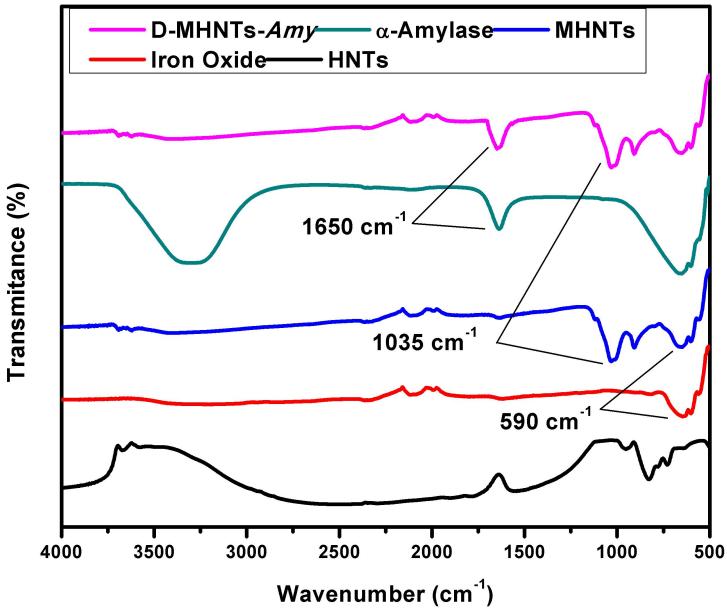

Figure 8: FTIR spectra of HNTs, iron oxide NPs, MHNTs, α-Amylase, and D-MHNTs-Amylase

4.5 Response Surface Methodology

A few preliminary studies were performed using single factor studies at one time as explained following;

The investigation of individual factor's effect on enzyme loading (%) was carried out via determining effect of, matrix amount, enzyme amount, pH of the solution, temperature of the immobilization solution. As can be interpreted from figure 9**,** with increase in immobilization matrix amount the enzyme loading increases, because of increasing surface area for immobilization. With use of 60 mg D-MHNTs 66.67 % enzyme loading was achieved, however further increase in D-MHNTs have no significant effect if the enzyme amount and support matrix amount ratio is taken into consideration. Hence, the range used for optimization was chosen 50-70 mg of D-MHNTs. In case of enzyme amount, the loading shows an inversely proportional relationship with enzyme concentration. As the enzyme concentration is increased the loading starts declining, which is attributed to the limited sites available for immobilisation of fixed amount of D-MHNTs. However based on the close inspection range 10-20 mg of enzyme was chosen for further RSM designing. The single factor study on pH indicated no significant effect on the enzyme loading, nevertheless

highest loading (65.58 %) was achieved at pH 6. For the sake of preventing enzyme from pH shock furhter experiments for immobilisation were carried out at pH 6.5 as the enzyme activity was also measured at the same pH. The results of single factor study on temperature of immobilisation solution reveled an interesting pattern, as the temperature of immobilisation is increased the loading increase until 40 °C after that it strats to decrease. Based on the observed results, the range of temperatre was taken as 30-40 °C.

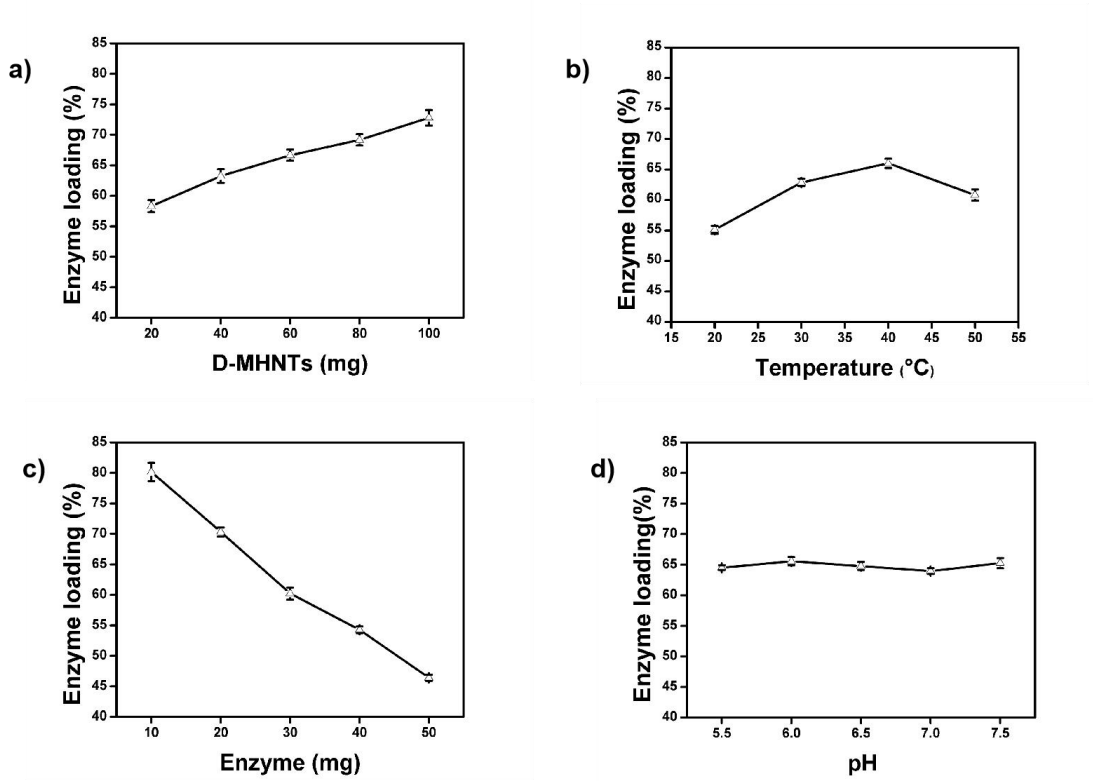

Figure 9: Effect of (a) D-MHNTs amount (mg), (b) Temperature (°C), (c) α-amylase amount (mg), and (d) pH, on enzyme loading (%)

Model fitting and statistical analysis

Experiments were conducted on the 17 runs provided via BBD design of design expert software. The results as actual experimental values and predicted values by the design of enzyme loading (%) are listed in table 4**.** Analysis of multiple regressions was employed to determine the polynomial coefficients for every term using the design expert 6.0.8. The final model for determining enzyme loading (%) is represented in term of equation with actual factors.

$$\text{Enzyme Loading (\%)} = -143.21775+4.76702A+2.80235B+1.11440C-0.033545A^2-0.023380B^2 -0.078880C^2+0.014000AB-8.50000E-004AC+0.011500BC \quad \text{……..Eq.3}$$

Where, A is D-MHNTs in mg; B is temperature in °C; and C is α-amylase in mg.

Table 4: Box-Behnken experimental design for (Enzyme loading (%)) of α-amylase immobilization on D-MHNTs

Run	D-MHNTs (mg)	Temperature (°C)	α-amylase (mg)	Enzyme loading (%)	
				Actual	Predicted
1	60.00	35.00	15.00	**66.27**	**66.32**
2	70.00	40.00	15.00	**67.19**	**66.56**
3	60.00	40.00	10.00	**70.72**	**70.47**
4	70.00	30.00	15.00	**62.88**	**62.98**
5	60.00	30.00	10.00	**67.04**	**66.07**
6	50.00	40.00	15.00	**63.29**	**63.19**
7	60.00	40.00	20.00	**61.07**	**62.04**
8	60.00	35.00	15.00	**65.92**	**66.32**
9	50.00	30.00	15.00	**56.18**	**56.80**
10	60.00	35.00	15.00	**67.11**	**66.32**
11	60.00	30.00	20.00	**56.24**	**56.49**
12	70.00	35.00	20.00	**59.19**	**58.84**
13	50.00	35.00	10.00	**62.72**	**63.07**
14	60.00	35.00	15.00	**66.83**	**66.32**
15	60.00	35.00	15.00	**65.49**	**66.32**
16	50.00	35.00	20.00	**55.02**	**54.15**
17	70.00	35.00	10.00	**67.06**	**67.93**

Model Validation

Competency of the model generated by BBD was evaluated using variance analysis. The as obtained ANOVA results are shown in Table 5**.** The obtained F-value 40.62 with a p-value less than 0.0001 for the generated model suggests the significance of model with 95 % level of significance. F-Test was used to determine lack of fit (variation of data around the fitted model). Lack of Fit F-value was found to be 3.52, which signifies irrelevance at a 95 % confidence level. The harmony of the generated

model with experimental data can be interpreted by the low p-value and insignificant lack of fit F-value. It also endorses the fact that variation in observations are because of real cause, i.e. change in the independent variables, not by any kind of error or noise.

Table 5: ANOVA results for coefficients of response surface quadratic model

Source	Sum of Square	DF	Mean Square	F Value	Prob>F	Remarks
Model	329.93	9	36.66	40.62	< 0.0001	significant
A	45.65	1	45.65	50.59	0.0002	
B	49.65	1	49.65	55.02	0.0001	
C	162.18	1	162.18	179.72	< 0.0001	
A2	47.38	1	47.38	52.50	0.0002	
B2	1.44	1	1.44	1.59	0.2472	
C2	16.37	1	16.37	18.14	0.0037	
AB	1.96	1	1.96	2.17	0.1840	
AC	7.225E-00 3	1	7.225E-00 3	8.006E-00 3	0.9312	
BC	0.33	1	0.33	0.37	0.5641	
Residual	6.32	7	0.90			
Lack of Fit	4.58	3	1.53	3.52	0.1278	not significant
Pure Error	1.74	4	0.43			
Cor Total	336.25	16				

The F-values of 50.59, 55.02 and 179.72 obtained in the case of D–MHNTs amount, temperature and α-amylase amount, respectively indicates that α-amylase amount has the highest impact on the enzyme loading (%), which is in good corroboration with single factor studies. The quadratic term (A^2 and C^2) also has a significant effect owing to their F-values of 52.50 and 18.14 respectively. The obtained R^2 value of 0.9812 as shown in table 6, indicates a great correlation between predicted and experimental values. It shows that the model can predict 98.12 % of the response and only 1.88 % data is not explained by the model. The values of Adj-R^2 and Pre-R^2 0.9571 and 0.7739 respectively were also in reasonable agreement, indicating the

suitability of generated model. The signal to noise ratio was fund to be 22.407, which further confirms the good efficiency of created model.

Table 6: ANOVA results of response surface quadratic model

Std. Dev.	0.95	**R-Squared**	0.9812
Mean	63.54	**Adj R-Squared**	0.9571
C.V.	1.49	**Pred R-Squared**	0.7739
PRESS	76.01	**Adeq Precision**	22.407

Effect of variables

The model adequacy was investigated by the residuals (difference between the predicted and observed response). The predicted values by model for enzyme loading (%) are plotted versus experimentally obtained values as shown in Figure 10**.** The position of residuals lying around the line indicates great competence of the BBD generated model.

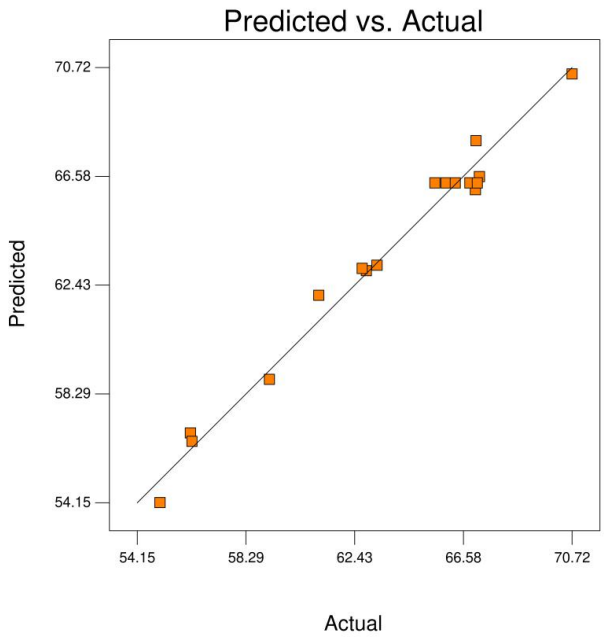

Figure 10: Comparison of the experimental results of enzyme loading (%) with the predicted values.

The effect of individual variable on enzyme loading was also examined by perturbation plots. Perturbation plots are employed to determine the most influential factors for the response. A steep curvature or slope is the indication that the response is subtle to that particular variable. While, a straight line specifies that response is insensitive to the variation in that variable. The observations confirm that α-amylase amount has the highest impact on the final enzyme loading (%)

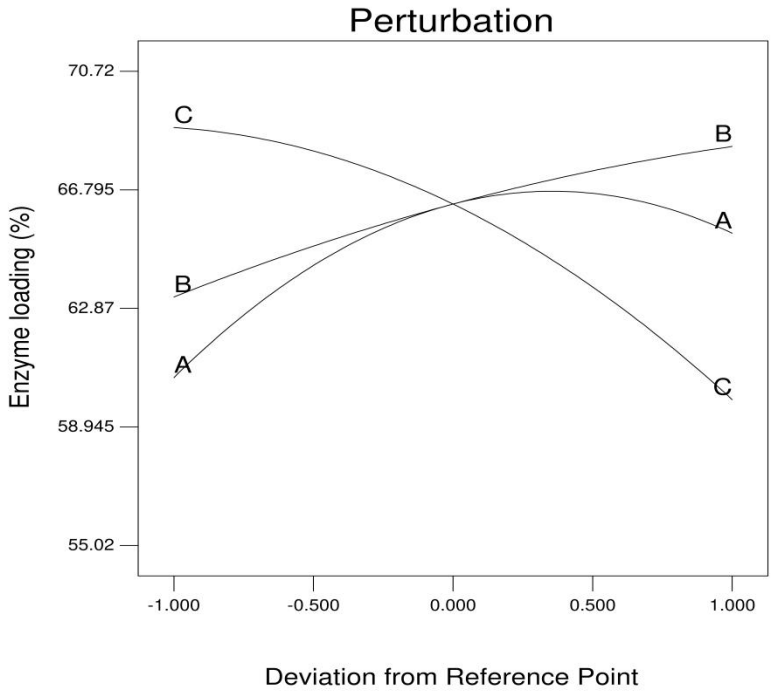

Figure 11: Perturbation plots for the α-amylase loading (%). A, D-MHNTs amount; B, temperature; and C is α-amylase amount.

The response for the independent variables was plotted as contour plot by keeping the one variable constant and the other 2 variables varying within the experimental ranges. The combined effect of D-MHNTs amount and immobilization temperature on enzyme loading (%) is plotted in Figure 12. The enzyme factor is kept constant at center point (15 mg). The enzyme loading (%) increases with increasing support matrix (D-MHNTs) amount, which is attributed to the availability of Schiff base bonding sites greater than before. Enzyme loading (%) follow the same trend with increase in temperature.

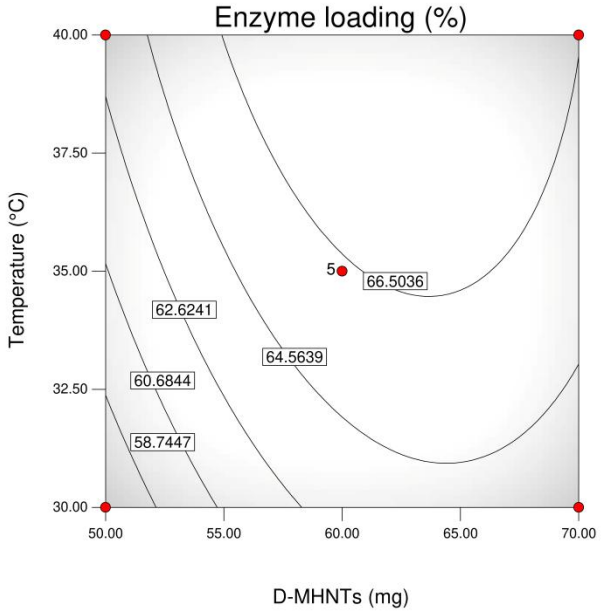

Figure 12: The contour plot of the enzyme loading (%) as the function of D-MHNTs amount (mg) and reaction temperature (°C) (at α-Amylase = 15 mg).

The combinational effect of support matrix amount and enzyme amount as shown in Figure 13. However the availability of enzyme for immobilization has different impact on enzyme loading (%). The enzyme loading (%) increase with increase in enzyme amount to a certain point which is dependent on the availability of support matrix. Any increase beyond that point does not increase the enzyme loading (%).

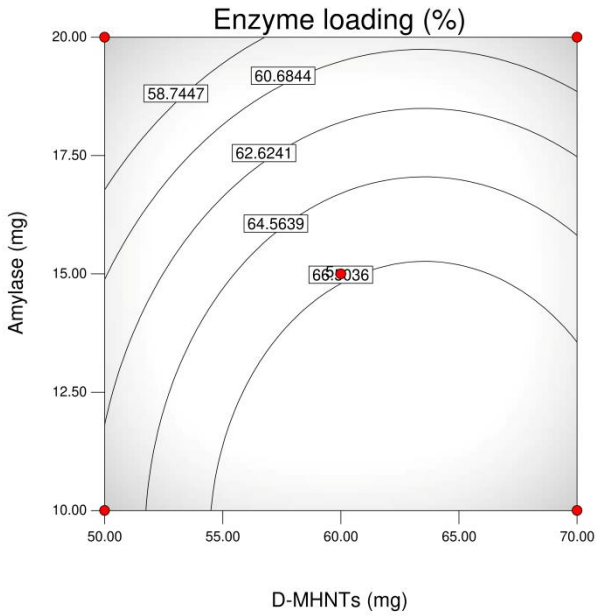

Figure 13: The contour plot of the enzyme loading (%) as the function of D-MHNTs amount (mg) and enzyme availability (mg) (at temperature = 35 °C).

It was found from plotting counter plot between reaction temperature (°C) and enzyme availability (mg) at a constant support matrix (D-MHNTs=60 mg) that the enzyme loading (%) was significantly altered by change in both factors. Enzyme loading (%) increases with increase in reaction temperature irrespective of the enzyme availability. Increase in enzyme amount on the other hand has contrary effect on enzyme loading (%) at any particular temperature point as shown in figure 14.

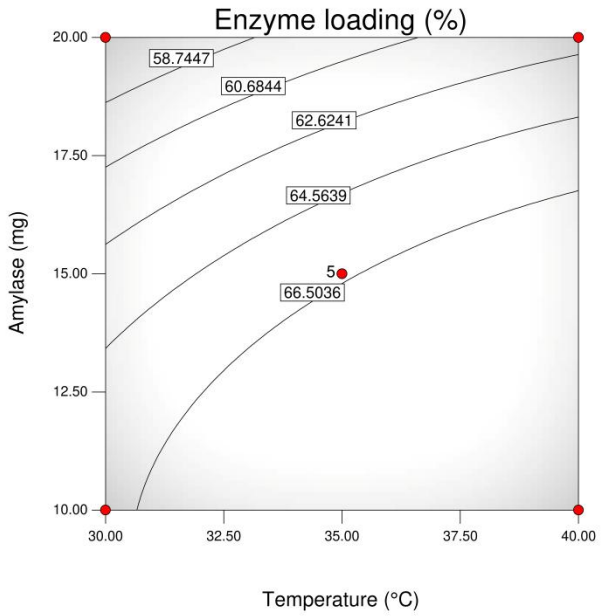

Figure 14: The contour plot of the enzyme loading (%) as the function of reaction temperature (°C) and enzyme availability (mg) (at temperature = 35 °C).

Optimization of enzyme loading (%)

The main objective of employing RSM was to determine the optimum values of operational parameters that will result in highest enzyme loading (%). With Design expert Version 6.0.8, numerical optimization was carried out by taking three independent variables. To confirm the model suitability the optimization condition obtained from the model was verified by conducting experiments under the obtained optimal conditions as shown in Table 7. The harmony between the observed and the predicted values confirmed the legitimacy of the generated model for stimulating the enzyme loading (%). Experiments were accompanied in triplicates and the average values are listed.

Table 7: Obtained optimum values of the process variables and response

Variable	Value	Enzyme loading (%)	
		Predicted	Experimental
D-MHNTs (mg)	62.48		
Temperature (°C)	40	65.32	64.73±0.49
α-amylase (mg)	17.91		

4.6 Enzyme loading and activity

The immobilization reaction was carried out on the reaction parameter gained from the RSM modeling. The enzyme loading on D-MHNTs was measured with respect to time as shown in figure 15 (a). Loaded enzyme amount was calculated via the procedure explained in section 3.6. The enzyme loading increases with reaction time, achieving maximum loading value of 70.76 % in 24 hours. However, a close inspection reveals that the reaction time doesn't have significant effect on enzyme loading after 8 hour (64.73±0.49 %) of immobilization reaction. The enzyme loading on D-MHNTs is attributed to Schiff base binding between functional moieties of enzyme and polydopamine layer. After 8 hours there are very little functionalities left for the binding hence there is no substantial change in enzyme loading.

The total enzyme loaded on the employed D-MHNTs (62.48 mg) was calculated to be 11.59 mg. Further calculations; provide the enzyme loading value of 185.52 mg per gram of D-MHNTs.

Activity retained by immobilized enzyme is the most important factor for industrial applications. The retained activity was calculated via the method explained in section 3.7. The NBC retained 89.34±1.79 % of activity as compared to the same amount of free counterpart.

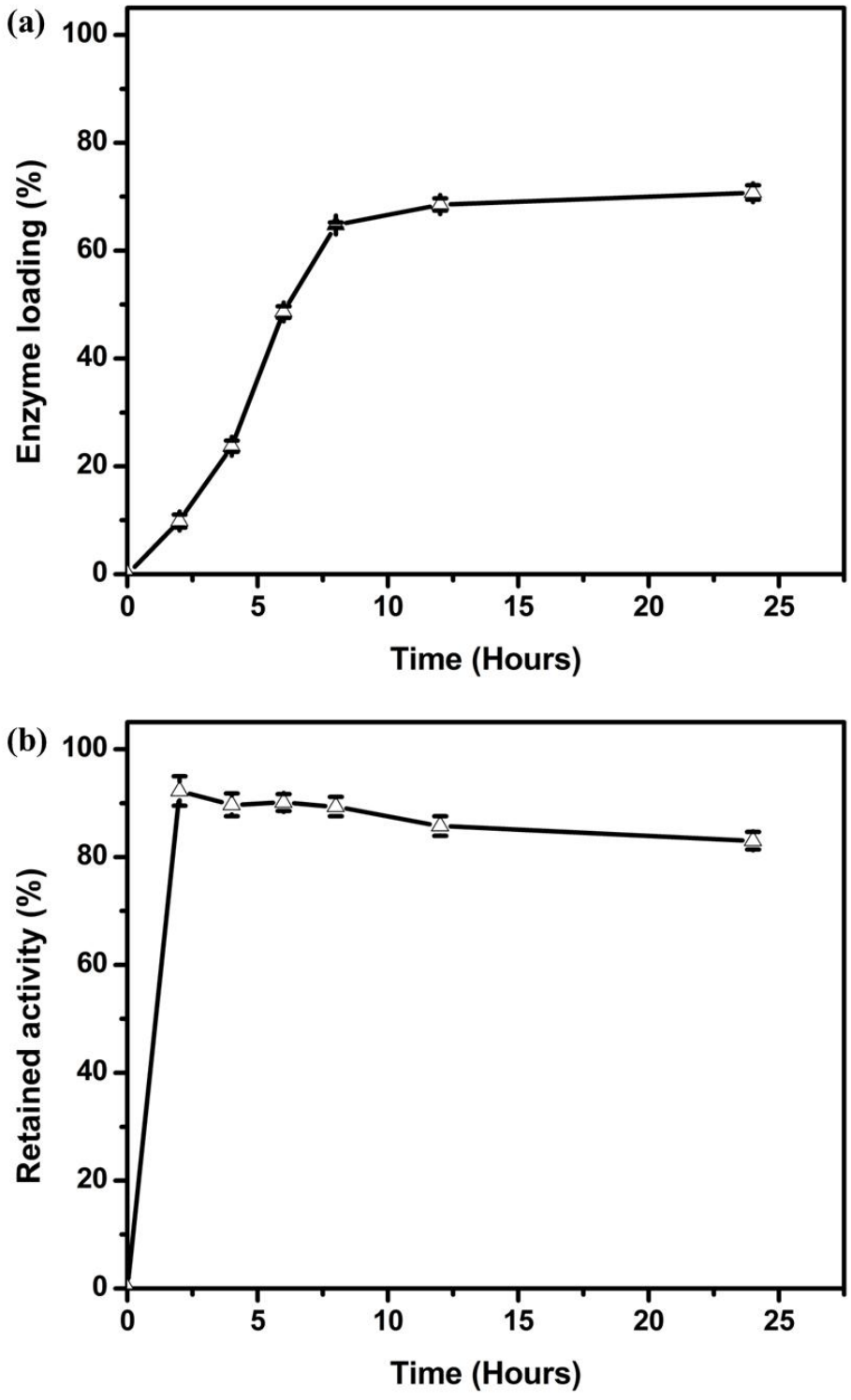

Figure 15: (a) Enzyme loading (%) on D-MHNTs and (b) retained activity of immobilized enzyme

4.7 Effect of various factors on enzymatic activity

4.7.1 pH

The effect of pH on the enzymatic activity of free and immobilized α-amylase is shown in figure 16. The shift in optimum pH after immobilization, where the enzyme activity was highest, was observed. In case of free enzyme highest activity of enzyme was obtained at pH 6.0, which shifted to pH 6.5 in case of enzyme immobilized on D-MHNTs. The overall activity of enzyme has also improved, as can be seen in the graph. At pH 5 free enzyme retained about 46.63 % activity whereas after immobilization the enzyme exhibit 62.64 % activity. In a similar way, at pH 7.5 the immobilized enzyme exhibit 72.11% activity in comparison with its free counterpart which has only 56.36 % activity. It can be concluded from overall observations that immobilized enzyme has improved resistance towards pH change in the reaction mixture. The improved pH activity can play a vital role for the application of α-amylase in industrial sector.

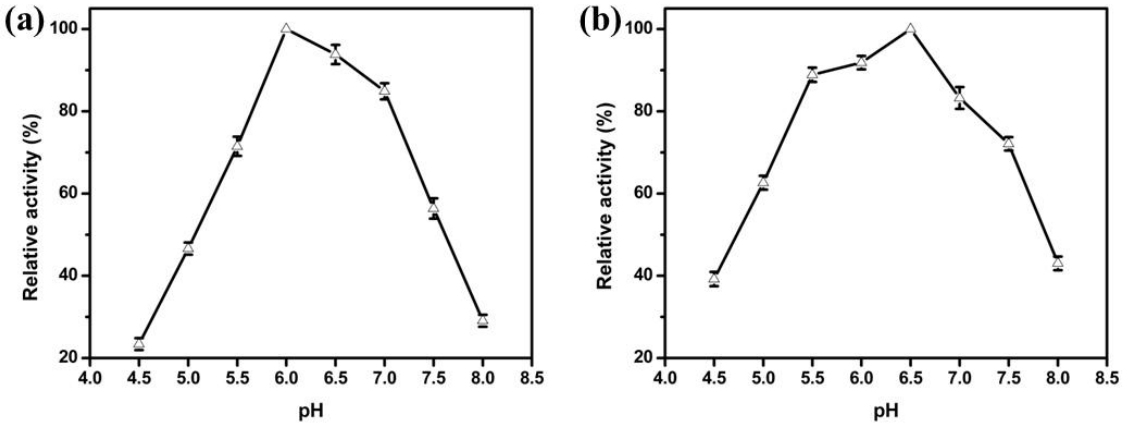

Figure 16: Effect of pH on catalytic activity of (a) free enzyme and (b) immobilized enzyme

4.7.2 Temperature

The impact of temperature on free and immobilized enzyme was determined as in some industrial sectors the temperature of the reaction may vary. In case of free enzyme the enzyme activity increases with increase in temperature till 40 °C, which is the optimum temperature for highest catalytic activity. With increase in temperature beyond 40 °C the activity starts to decline, retaining only 57.49 % activity at 60 °C.

The decrease in the activity of enzyme could be attributed to the breakage of weaker hydrogen and ionic bonds within the enzyme, leading to change in shape of the active sites and ultimately triggering the denaturation of the enzyme. The immobilized enzyme also follows the same trend with slight change in the optimum temperature, which is shifted to 50 °C. However, the activity of enzyme at every observed temperature point indicated improved catalytic activity than free enzyme. The immobilized enzyme retained more than 77.43 % of catalytic activity even at 60 °C. The enhanced thermal stability can improve the applicability of α-amylase in industrial applications.

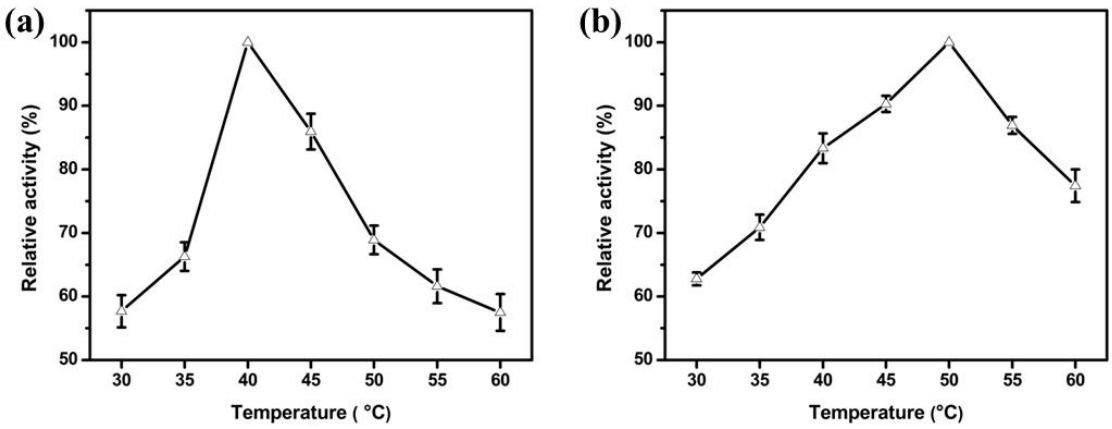

Figure 17: Effect of temperature on catalytic activity of (a) free enzyme and (b) immobilized enzyme

4.8 Reusability

Other important objective of immobilization is to achieve reusability for enzyme based systems. The reusability potential of NBC was determined by plotting the residual activity as a function for the number of cycles. As can be seen in Figure 18 immobilized enzyme retained 56.79 % of initial activity even after recycled for 10 cycles. The high substrate concentrations and the damage to the support material have a role in the declined enzymatic activity observed after recycling cycles.

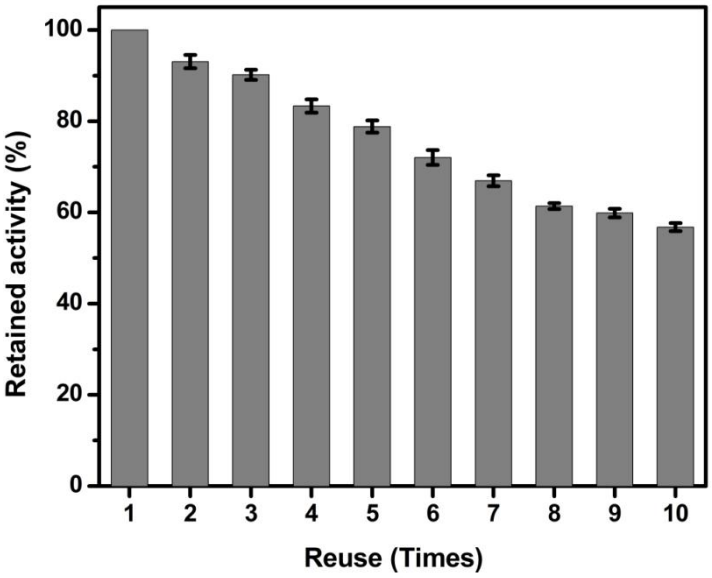

Figure 18: Reusability potential of immobilized enzyme

4.9 Storage stability

One of the goals of immobilization is to improve the storage stability of enzyme as the catalytic activity of free enzyme is prone to decrease over storage time. The storage stability of free and immobilized enzyme was determined by calculating the retained activity at optimum temperature and pH. Immobilized enzyme had about 64.87 % of its original activity after 30 days of storage. However, the free enzyme only retained about 18.54 % of its original activity. The study concludes that NBC displayed better stability than free enzyme.

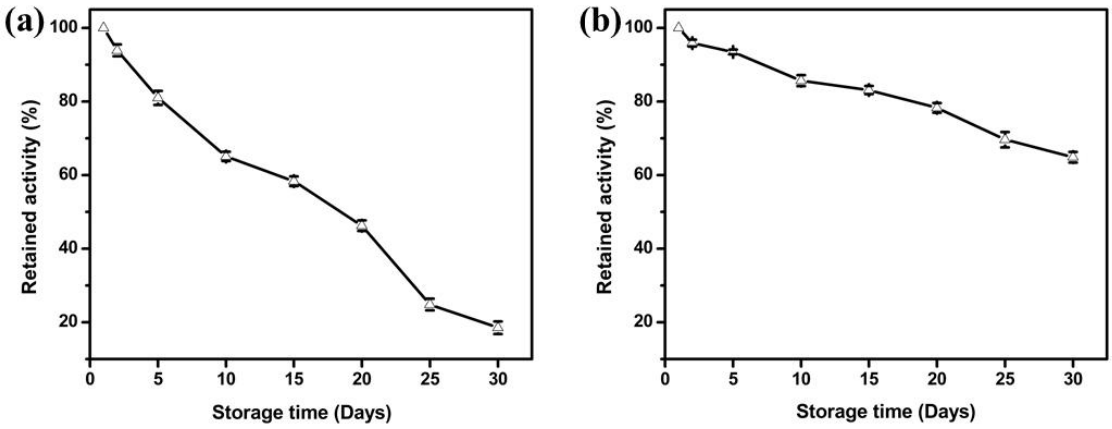

Figure 19: Effect of storage time on catalytic activity of (a) free enzyme and (b) immobilized enzyme

4.10 Conversion

The immobilized α-amylase was employed for conversion of PPW extracted starch into reducing sugars in 24 hours of reaction time. The figure 20 represents the production reducing sugar (mg) per gram of substrate with time of incubation. Production of 105.39±2.50 mg/per gram of starch was achieved in 24 hours reaction time. The production of reducing sugars was lower than previously reported studies, which can be attributed to purity of extracted starch. The additional impurities might have captured the catalytic sites of NBC, hence lowering the hydrolysis of starch into reducing sugars.

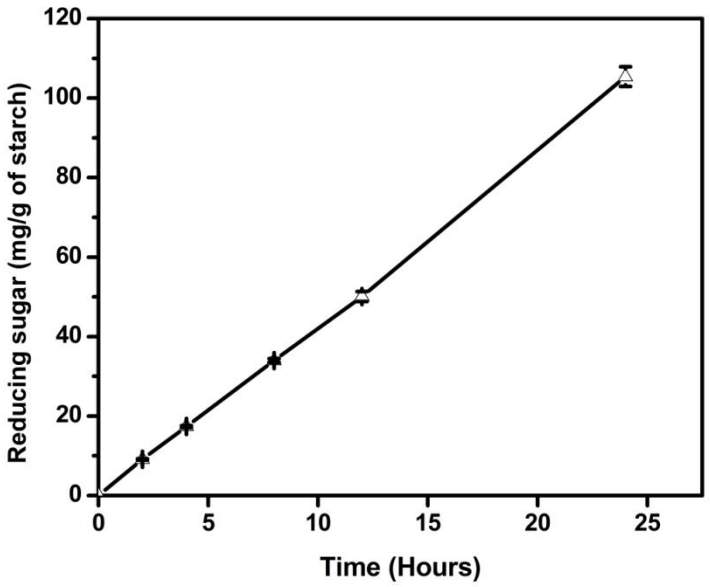

Figure 20: Production of reducing sugar via hydrolysis of potato peel extracted starch using immobilized α-amylase

CHAPTER 5

Conclusions and Future scope

Enzyme immobilization techniques are employed worldwide to improve the operational performances and stability of enzymes in sustainable industrial biocatalytic processes. It leads to easy recycling, recovery from reaction mixture and multi-time usability of biocatalyst via restricting the enzyme mobility in fixed space. It entails the interaction of two species, the enzyme and the carrier. Various supports like organic, inorganic, natural have been used for enzyme immobilization. Over the last few decades, immobilization on nanomaterials have been widely studied due to their superior activity. Their characteristics like high specific surface area, mass transfer resistance, and high loading capacity, present them as ideal support materials for immobilization of biological molecules. Broad class of nanomaterials had been used as supports i.e. carbon nanotubes, silica nanoparticles, nanofibers, nanosheets etc. On the other hand, growing environmental concern and economical barriers associated with nanomaterials synthesis process restricts their usage at large scale. Hence, quest for alternative naturally occuring materials with similar properties has been a major goal of scientific community. Clay materials are considered appropriate for biological applications owing to their wide availability and biocompatible property.

Naturally occurring clays like bentonite, montmorillonite, kaolinite, halloysite etc. have been exploited for immobilization. Among them, Halloysite nanotubes have been studied widely for various biological and non-biological applications because of their unique structural and chemical properties. Their intrinsic features like, biocompatible nature, non toxicity, surface adsorption, hollow lumen available for loading of biological molecules, simultaneously plays a crucial role for enzyme immobilization.

The aim of the current study is to develop a nanobiocatalysts based on magnetically modified HNTs for efficient starch hydrolysis and to determine its capability on industrial scale. In this work, α-amylase was successfully immobilized on dopamine functionalised magnetic HNTs via schiff base reaction. Synthesis and functionalisation were characterized through different characterization techniques like

UV- visible spectroscopy, VSM, SEM analysis, FTIR analysis. UV-visible shows the formation of iron oxide nanoparticles on the surface of HNTs. VSM showed lower magnetic saturation value in case of MHNTs, however the separation of MHNTs from solution mixture was not compromised. Presence of iron oxide nanoparticles on the surface of halloysite nanotube were also confirmed by SEM analysis. The loading of α-amylase on dopamine functionalized magnetic halloysite nanotubes was furhter assured by the presence of various functional groups associated with enzyme. The high enzyme loading was achieved successfully with retained activity of 89.34 % after 8 hours immobilization reaction time. Compared to the free enzyme, the immobilized enzyme exhibits better resistance to pH and also exhibit significantly higher thermal stability. Furthermore, it showed higher storage stability than free enzyme and preservers its activity via retaining comparatively higher activity after 10 cycles. In conclusion, the obtained NBC showed improved catalytic activity which could be good candidate for industrial processes.

Immobilized α-amylase was found more successful in hydrolyzing higher amount of starch as compared to their free counterpart. This work concluded that enzyme immobilization provides stabilisation and improved catalytic activity for industrial. In future there is scope for various other immobilization approaches for potential applications in bio-sensing and bioremediation, baking and brewing, pharmaceuticals, biofuel production, immunoassays, detergent formulation and many other fields.

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APPENDIX-I

Standard graphs used for enzyme loading and activity calculations

BSA standard for enzyme loading

Standard curve of BSA was prepared with different concentration i.e. 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/ml. Absorbance was taken at 595 nm.

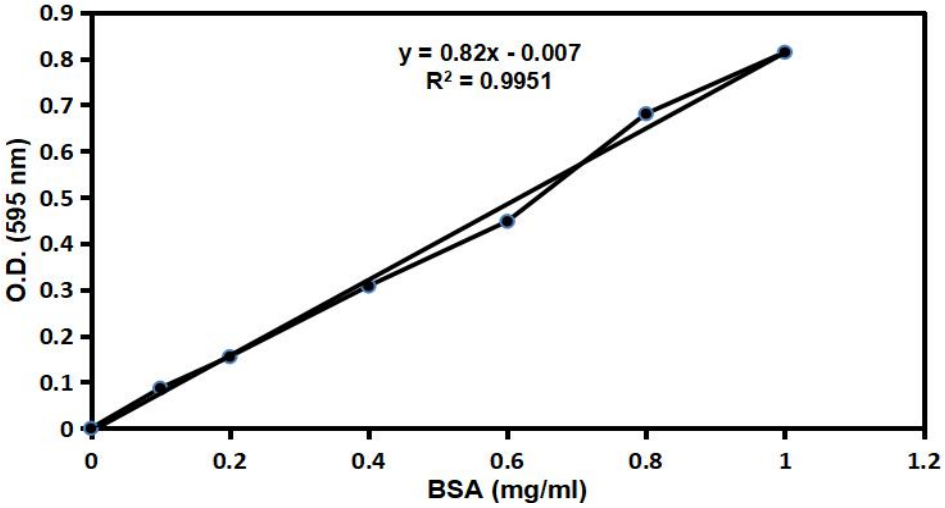

Figure 21: Standard curve of BSA

Maltose standard for enzymatic activity

Standard curve of maltose was prepared with different concentration i.e. 0.1, 0.2, 0.3, 0.4 and 0.5 mM. Absorbance was taken at 540 nm.

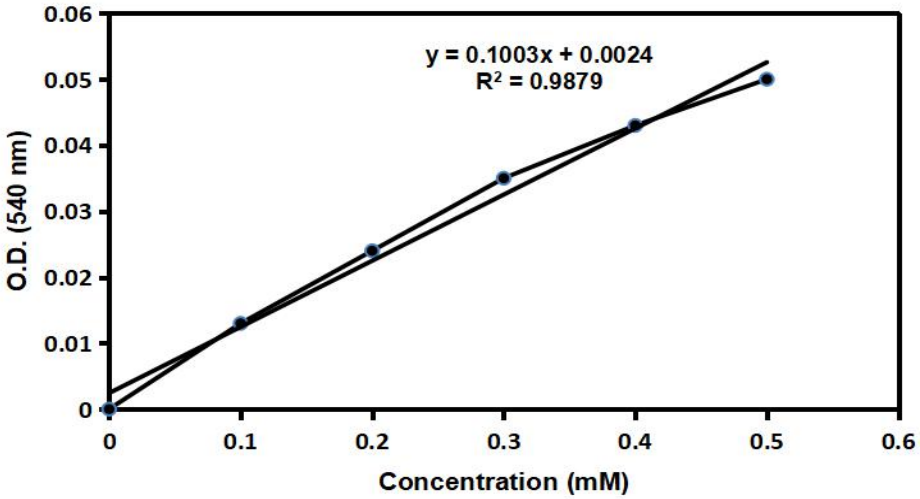

Figure 22: Standard curve of Maltose

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