DECLARATION

No part of this dissertation has been submitted in support of an application for any degree or qualification of the University of Central Lancashire or any other University or Institute of learning.

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Immobilisation of enzymes on super paramagnetic nanoparticles for biodiesel production

ABSTRACT

Commercial material DAE was modified by the incorporation iron oxide by one pot synthesis, was characterised by several techniques i.e. Energy Dispersive X-ray Analysis (EDAX), Powder X-ray Diffraction (XRD, Scanning Electron Microscope (SEM),) and (Transmission Electron Microscope (TEM). EDAX/SEM analyses confirmed the presence of iron oxide in the nanocomposites, (XRD) measurements showed the crystalline formation of the iron oxide nanocomposite. TEM and SEM with have been used for the determination of sizes, internal structure, and morphology of the composite. Furthermore, TEM images showed the porous structure of the DAE. While, FTIR spectroscopy was used for the determination for the presence to Fe–O bonding due to a specific stretching vibration at 551cm-1.

On one hand, second part of the dissertation was consists of the surface functionalisation of the developed composed material. The composite was functionalised by silanisation (a classical method) with two different amino-silane groups i.e. APTS and APDS and was followed by the conversion of surface amine group into the aldehyde by using glutaraldehyde through chemical conjugation method, The surface functionalization with APTS showed more amine density as compared to APDS due to more number of ethoxy group attached with APTS. The functionalised nanocomposite was prepared on a small scale and was further altered by chemical conjugation with enzymes.

The final part of this project was based on the biolytic applications of the developed and surface functionalised material. In this studies Candida Rugosa lipase (CRL)] were chemically conjugated through glutaraldehyde-modification onto the amino-functionalised nanoparticles for applications such as the formation of p-nitrophenol and palmitic acid, by the hydrolysis of p-nitrophenyl palmitate. It was observed from the bio catalytic reaction (i) that the conversion values given by lipase-immobilised materials were comparable to those given by free lipases with the added advantage of being reusable for further catalytic cycles.

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LIST OF ABBREVIATIONS

DAE	Diatomeous earth
EDAX	Energy Dispersive X-Ray Analysis
FTIR	Fourier transforms infrared spectroscopy
Fe_3O_4	Magnetite
SEM	Scanning Electron Microscopy
SPIONs	Superparamagnetic iron oxide nanoparticles
TEM	Transmission Electron Microscopy
XRD	X-Ray Diffraction
XRF	X-Ray Fluorescence

UNITS

°C	degree Celsius	**mL**	millilitre (1×10^{-3} L)
g	gram	**µL**	microlitre (1×10^{-6} L)
h	hour	**µm**	micrometre (1×10^{-6} m)
K	degree Kelvin	**µmol**	micromoles (1×10^{-6} moles)
kOe	kilooersted (1×10^{3} Oe)	**nm**	nanometre (1×10^{-9} m)
km	kilometre (1×10^{3} m)	**nmol**	nanomoles (1×10^{-9} moles)
kV	kilovolt (1×10^{3} V)	**rpm**	revolutions per minute
m	metre	**v/v**	volume per volume
M	molarity (moles per litre)	**w/v**	weight per volume
mg	milligram (1×10^{-3} g)		

Aims

The purpose of this study is to run transesterification reactions of the palm oil by use of Lipase catalysed where immobilised on iron oxide (Fe_3O_4) nanoparticles. Numerous studies have been conducted for transesterification reaction for different catalyse, alcohol and molar ratios at different temperature. Use of immobilized lipase for transesterification of oils has been a trendy application for producing biodiesel in recent years.

Objectives

1: Lipase analyses immobilization on super paramagnetic nanoparticles by physical adsorption and chemical conjugation.

2: Transesterification palm oil of using immobilized lipase.

3: Optimisation of final product by different techniques.

1 Introduction

1.1 Biodiesel

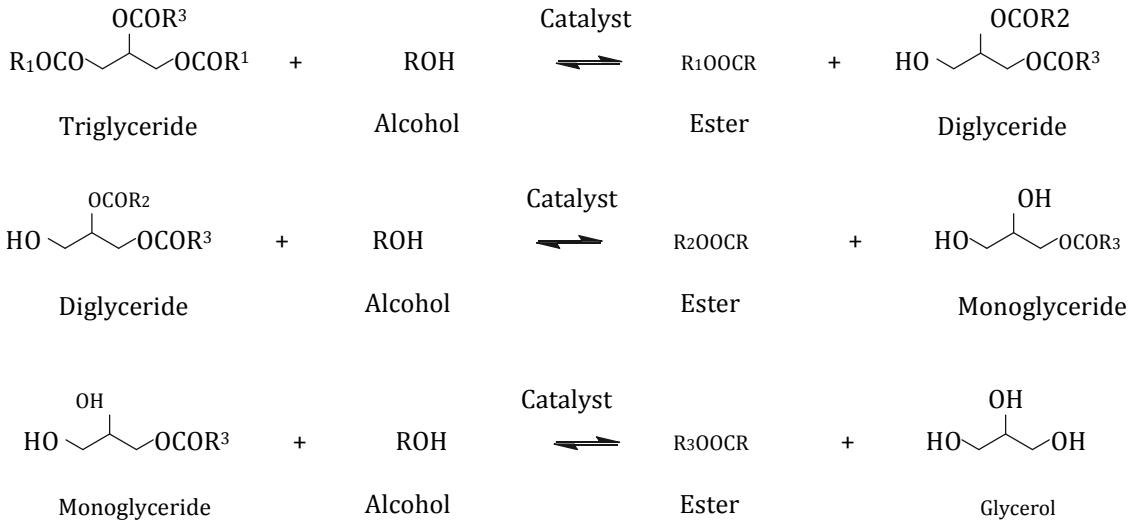
Biodiesel was named and presented by the National Soya Diesel Development Board in the United States in 1992 [1]. The petroleum reserves of the world have been decreasing rapidly in parallel with this the consumption has been increasing rapidly. Biodiesel has become one of significant alternative renewable fuel containing mono-alkyl esters of long chain fatty acids which are produce from vegetable oils (such as rapeseed (canola) oil, palm oil, sunflower oil, cottonseed oil) and animal fats. Biodiesel participates has been reached 10% of the world's diesel demand, and its part is predicted to grow by upcoming years[2] . The marketplace for biodiesel is predicted to reach 37 billion gallons by 2016 with an annual growth of 42%. However the cost of biodiesel is almost 30% higher than petroleum-based diesel[3] .

Biodiesel fuel has same identical feature with petroleum diesel and can be used together as a mixture. Nevertheless biodiesel fuel has some advantages such be as non-toxic, renewable, reproducibility, biodegradable and free of sulphur and aromatics.

1.2 Biodiesel production

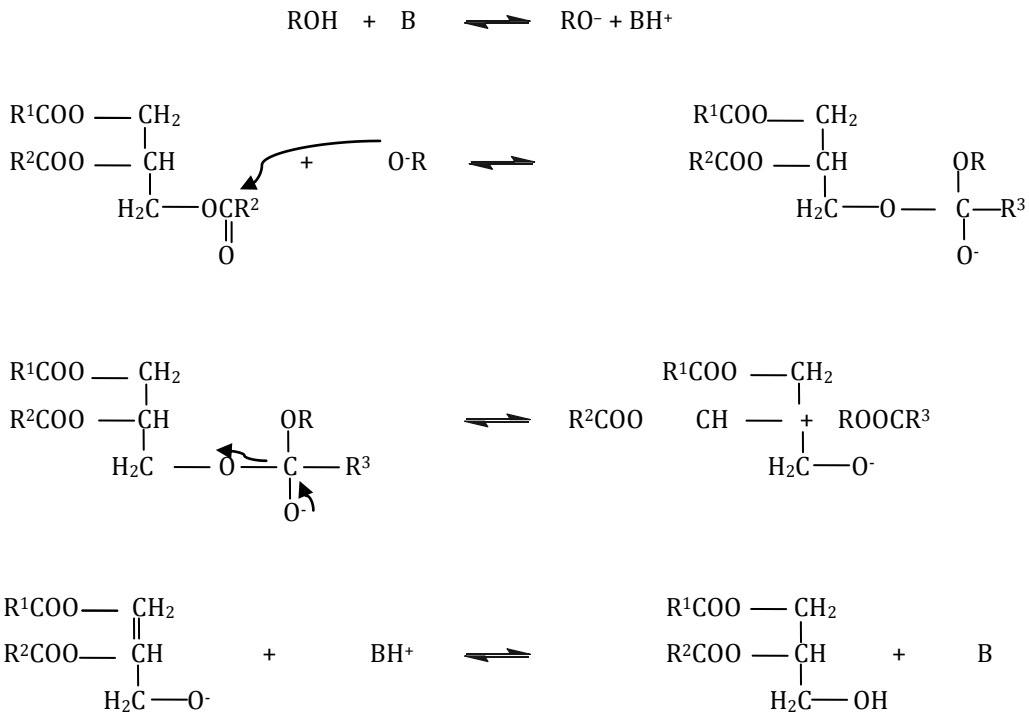
Biodiesel can be produce with three chemical processes: pyrolysis (thermal cracking), micro emulsions, dilution, and transesterification. Transesterification or alcoholises of triglyceride is the most common and inexpensive method in order to manufacture biodiesel.

Chemistry of transesterification process method of triglyceride oil with alcohol reaction in the presence of a catalyst and at the end of reactions mono alkyl esters and glycerol yield. This reactions chain shows triglyceride are changed to diglycerides, monoglycerides and as a final products to glycerol. Another name for this reaction is alcoholysis and using of ethanol or methanol as acyl acceptors.



Chemistry of transesterification process was generated by Christopher, L., Hemanathan Kumar, and Zambare, V. (2014) [[3]].

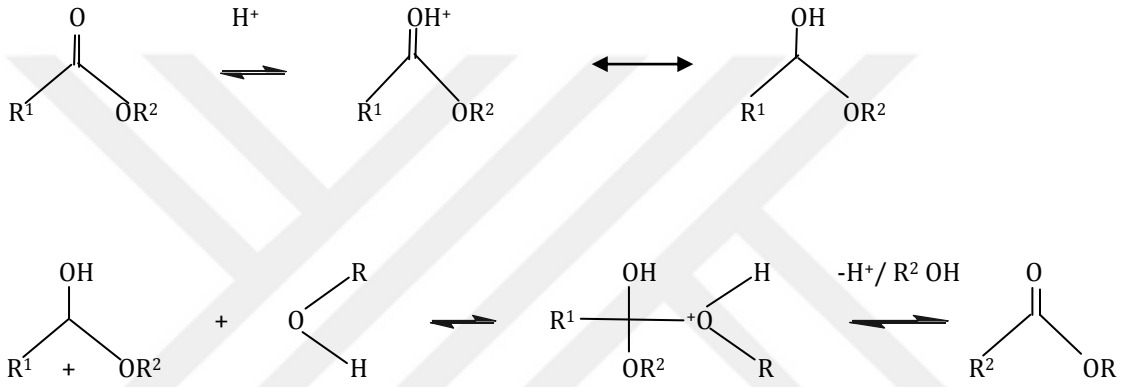
Alkali catalysed transesterification is another transesterification method consist of in three steps as illustrated in equation [1]. In the first step triglyceride is carbon atom attacks by the alcohol ion and intermediate molecule occurs and to form an intermediate reacts with alcohol then again to regenerate alcohol ion. In the last step, intermediate forms to a diglyceride a fatty acid ester.



Reactions of the alkali-catalysed transesterification. [5] [6]

The alkali catalysed provides better transesterification of triglyceride in short reaction times. However the disadvantage of the alkali process is free fatty acids which in oil can be caused to form soap in the transesterification process.

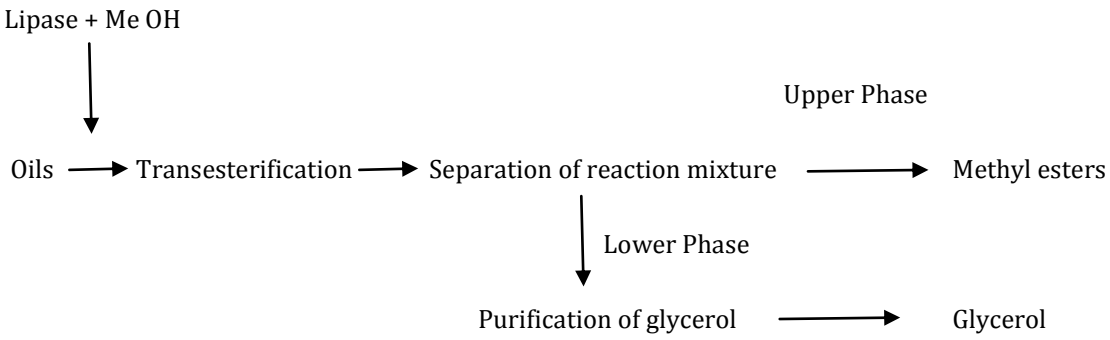
Another method is acid catalysed transesterification of the vegetable oil which is demonstrated in Figure equation [1]]. The particular protonation involving the carbonyl group of this ester brings about this carbocation, which in turn after a nucleophilic strike in the alcohol makes an intermediate. This specific intermediate eliminates glycerol to make an ester and regenerate this catalyst. This method happens almost 4000 times faster than alkali catalyst catalysed with an equal amount of catalyse. [4]



R: alkyl group of the alcohol, R^1 Carbon chain of fatty acid, R^2: glyceride.

Reactions of the Acid catalysed transesterification are generated by Singh, S. and Singh, D. (2010) [1].

Lipase (is an enzyme) catalysed transesterification which is the method applied in this study, is similar to alkali transesterification, the differences are using lipase catalysed, the amount of catalyse used and reaction time. The process is described below [5] .



Biodiesel production process by use of lipase-catalysis are generated by Fukuda H, Kondo A, and Noda 2001 [5].

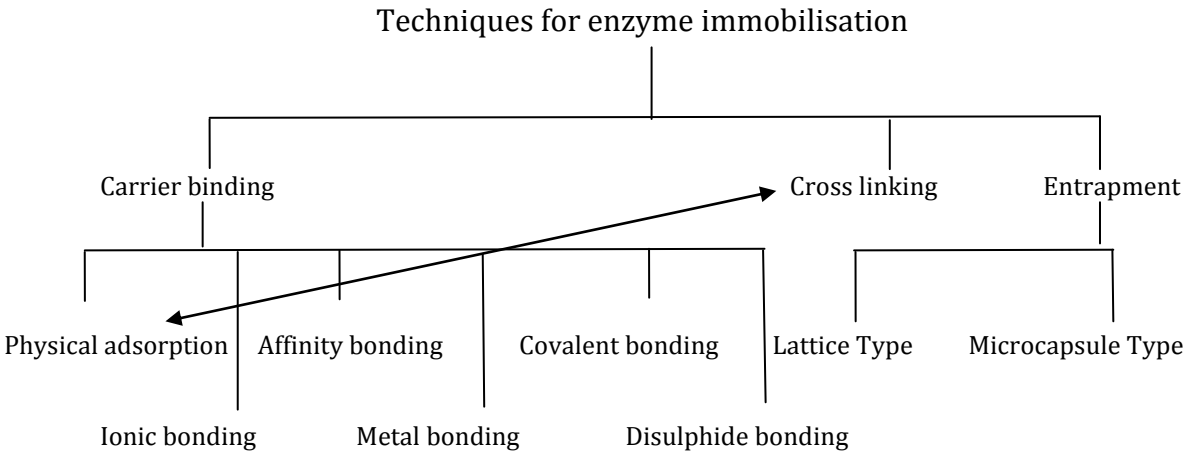
Lipase catalysed transesterification has been most efficient methods for manufacturing of biodiesel. This method has been practised in scientific publications during the last decade. However, just a few plants have applied enzymatic catalysed process (instead of alkalis or acids) for industrial manufacture of biodiesel. The reason for this is because the use of lipase catalysed much more expensive than chemical catalysts.

1.3 Immobilisation of lipase catalyse

As we discussed previously cost of production is very high using enzymatic catalysis for production of biodiesel. By the development of immobilisation of lipase enzyme, running cost of biodiesel production has been reduced. However, the method is still costly and unavailable to use for commercial biodiesel manufacturing.

Immobilised enzyme means the enzyme is physically narrowed to a certain stated area but retaining its maximum catalytic activity. It gives main advantages such as easy to use again, advanced adaptableness for continuous process, to be eco-friendly, thermal and pH stableness and faster and higher catalytic activity. On the other hand the method still has given some disadvantages such as, reduce of enzymatic ability, low stableness in oil–water systems and so on. In recent years immobilisation technologies have been developed by the use of nanoparticles and magnetic in order to reduce cost of biodiesel production.

Several methods have been developed for lipase immobilisation and usually these can be classified by three type of methods (carrier bonding, entrapment and cross linking).



Techniques for enzyme immobilisation was generated by Singh, S. and Singh, D. (2010) [1].

These methods can be clarify by depending on the sort of connections amongst enzymes and carriers and categorised by irreversible and reversible immobilization methods. In irreversible immobilization, when enzymes are immobilised to support materials, they cannot separate in any conditions. Reversible immobilization method can provide to separate enzymes from the supporting materials under mild circumstances. Cross-linking, entrapment and Covalent bonding are frequently used for irreversible immobilisation of lipases. Reversible immobilisation methods are physical adsorption, affinity bonding and chelation bonding.

With the intention of immobilise lipase enzyme, mostly applies to adsorption method which has more advantages compare to other methods such as setting under easy conditions and low-priced carrier material (polyacrylate, polypropylene, polystyrene etc.) and operation. Lipase attaches the surface of carrier material by van der Walls forces, hydrophobic interactions, electrostatic interaction and hydrogen bonds. Also physical and chemical characteristic specifications of carrier such as surface is, particle size, pore size and etc. effect the activity of lipase immobilisation. In order to increase the efficiency of carrier to lipase immobilisation we will be used super paramagnetic nanoparticles based on (Fe_3O_4) iron oxide. Use of Super paramagnetic nanoparticles has been freshly employed as supporting materials for lipase enzyme, giving significant characteristics, such as increased recovery cycles, large surface area, mobility and high mass transference.

1.4 Superparamagnetic nanocomposites

These developed composite containing nanoparticles of iron oxide inserted and embedded in the oxide of inorganic matrics.an. Due to the dispersing properties of Silica it is used as in organic matrix. Moreover, it has been found that is an efficient and easy for the surface modification. Silica surface is hydrophilic in nature with bio-compatibility hence can be a suitable agent for water treatment processes. The preparation of iron oxide nanoparticles with super paramagnetic properties as well as its internal structure has been focused broadly. On another hand, alike materials with meso [6] and micro porous silica shell have been shown because of their better surface area and size discrimination. Wu *et al* and Wu, Pinggue [7][8] are the pioneer of this sort of material. But the prepared material was a symmetrical. Furthermore, multi steps process has been suggested by Zhao *et al*.[9].The synthesis of nanocrystals could be performed via surfactant templating route has been reported by Sen *et al* [6] and their importance in chemical and biological applications.

First time magnetic separation was introduced by Robinson *et al* [10] in a biotechnological context for the collecting and splitting of cells and bio molecules [6]. These magnetised particles were suggested as magnetic particles due magnetic properties. These kinds of nanoparticles, used in bio-applications, are commonly shown by core-shell structures. Mostly the core shell structure can be formed from magnetite or magnetite, and the shell can be any one of a number of materials, including amorphous silica[11], [12] or porous silica[6],[7] the shape and size of the core are reported to be the fundamental parameters for the morphology of such nanocomposites. Therefore, super paramagnetic core-shell nanoparticles are reported as beneficial in the bio/ life sciences area, both in *vivo* and *in vitro* processes [6]. The general strategy for post-synthesis dispersion of aggregated magnetite suspensions by treatment with commercially available, cheap dispersing agents is also reported [13].

The mesoporous magnetic nanocomposites silica shell around a magnetic core are an exciting type of nanocomposites[14],[6],[12, 15]. It has been used for several purposes due to high magnetic properties and surface area. e.g. magnetically separation of biomolecules,[6] magnetic properties[16],[17] and well-ordered drug delivery[18],[19],[20]and[21]. Deng *et al;* [22] and Zhao *et al;* [9] have reported the immobilization of protein (trypsin) with fabrication of a magnetic core–shell zeolite for the Porous materials from microporous to macroporous (pore diameter > 50 nm) have evolved as a result of extensive research during the last twenty years in order to overcome the diffusional limitation of bulkier biomolecules, i.e. protein and enzymes, through the micro/ mesopores. Sen *et al*[6] pioneered hierarchically ordered porous silica nanocomposites with interconnecting micro, meso and macroporosity using a dual template assisted synthesis protocol. Immobilization of enzymes in mesoporous, macro porous and microcellular silica forms has been studied for bio-catalysis [23] i.e. immobilization of lipase for the hydrolysis of ester (p-nitro phenyl palmitate) [23].

1.5 Applications

Nanoparticles own significant implied importance in all perspectives which include catalytic fields, electronic, biological and chemical, due to their characteristic features, as compared to their bulk analogues[19],[2, 20]. Dyal *et al*[21] for immobilization of lipase (Candida Rugosa) announced nanoparticles for the) and was applied for breaking of ester with water.

The catalysis properties of the superparamagnetic nanocomposite material along with several other properties such as food technology[27],bio separation[23], biomedicine[28],

drug delivery[24], and purification of the environmental contaminants[22], [18][[[have increased the importance of the core-shell nanocomposite material and has made it very important compound in recent years.

1.6 MATERIALS AND METHODS

Table 2: List of commercial host matrices

Sample No	Name	Suppliers	Catalogue No
1	Diatomeous earth	Sigma Aldrich	D3877

Table 3: List of chemicals used

Name of the chemicals	Suppliers Cat. No.	% of Purity	Further modification (concentration, M)
Iron (II) chloride tetra hydrate	Sigma Aldrich. 220299	98%	0.085
Iron (III) chloride hexahydrate	Alfa Aesar A16231	98%	0.160
28%Ammonia NH_3	V.W.R. 21190.326	28%W/V	1.6

1.7 Solutions and Buffers

The solutions were made by using E-pure deionised water, supplied from a Thermo Scientific Barnstead Nanopure Water Deionisation System in UCLan. Moreover, the preparation of the stock solutions and buffers are given in the table below.

The information of the solutions is given in the table below.

Solution	Description	Use	Storage
Coupling solution	1 litre of solution was prepared containing 0.8% w/v acetic acid (glacial) in methanol	UV-Visible colorimetric assays and storage of NH_2 modified nanoparticles	1 litre capped clear glass bottle at 25ºC
Hydrolysis solution	1:1 mixture of methanol and water containing 0.15% acetic acid (glacial)	UV-Visible colorimetric assays	1 litre capped clear glass bottle at 25ºC
4-NBA solution (700 µg/ml)	7 mg of 4-NBA was dissolved in 10 ml coupling solution	UV-Visible colorimetric assays	Centrifuge tubes at 4ºC in the dark (used on the day of production)

Glutaraldehyde solution (5% w/v)	10 ml stock solution was typically prepared containing 1.886 ml glutaraldehyde and 8.114 ml 20×SSC buffer	Conversion of surface amine groups to aldehydes	Centrifuge tubes at -18ºC
20×SSC stock buffer	Stock solution was prepared by dissolving 88.2 g sodium citrate and 175.3 g NaCl in 1 L deionised water. The pH was adjusted to 7.3	Conversion of surface amine groups to aldehydes. Grafting and hybrid capture of oligonucleotides	Capped clear glass bottle at 25ºC
1×SSC and 13×SSC buffer solutions	20×SSC stock buffer solution was diluted respectively to produce 1× and 13×SSC buffers	Conversion of surface amine groups to aldehydes. Grafting and hybrid	Capped clear glass bottle at 25ºC
Reagent A	Gum Arabic (0.0667g), sodium deoxycholate (0.267g), Tris-HCl (12 mL, 250 mM) was added to 48 mL deionised water	Hydrolysis of PNPP reaction solvent in 1:1 mixture with isopropanol	Capped clear glass bottle at 25ºC
PBS Buffer	1×PBS tablet (2 mM KH_2PO_4) , 10 mM Na_2HPO_4, NaCl, 3 mM KCl, 136 mM dissolved in 200mL water	Washing and storage of lipase- materials.	Capped clear glass bottle at 25ºC

1.8 Magnetite nanocomposite

0.5g of solid commercial DAE was introduced in 20ml of magnetite nanoparticle solution and was soaked in it for about 30 minutes. After soaking, the materials were filtered and few drops of (NH4OH 28%) were added onto it.

Its black colour was observed by the pouring of the some drops of reagent (NH4OH 28%) on the filtered magnetite composite, material was dried on the filter paper after adding few drops of the reagent.

2.0 CHARACTERISATION

Characterisation has been carried out for pure commercial material and modified with iron oxide batches. TEM and SEM were used to study the size, internal structure, morphology and elemental detection while FTIR was used for the functional group analysis. XRD was engaged to confirm the crystalline structure of the nanocomposite;

2.1 TRANSMISSION ELECTRON MICROSCOPY (TEM)

It is widely applied for the determination of the internal structure, particle size incorporated into the matrix and shape of the nanoparticles [30][31], [32][30][33]. In this project, it has been practiced to analyse the particle size, shape and incorporated magnetite nanoparticles into commercial material which has been used as matrix or filler.

Images of pure DAE were taken by an electron microscope at 200 kV, (TEM a JEOL JEM-2000 EX). Gatan Digital Micrograph Software was used to manage the TEM images. Prior to analysis, deionised water was used for the dilution of the nanocomposite suspensions 50 times. For TEM sample, carbon coated copper grid (400 mesh, Agar Scientific, UK) was utilised and around 2 μL of diluted suspension of the composite was introduced on it which was then dried in air for about 20- 30 minutes.

2.2 SCANNING ELECTRON MICROSCOPY (SEM)

SEM is employed for the determination of the surface morphology of the fine fabrication and used for EDAX analysis of the nanocomposite and other materials. SEM forms images of microscopic surfaces at magnifications generally range of x 20 to x 100,000 with the best useful magnification being x 20,000 to x 50,000 subject to the type of sample and the assembly of the instrument [24]. Analysis was performed by using (SEM, FEI QUANTA 200).

For the sample preparation of the SEM imaging, the composite materials were ground up and the smallest possible quantity was placed on a carbon grid mounted on aluminium holder.

The sample was introduced in to the vacuum chamber of the SEM machine and was clicked to run. For the morphology analysis images were taken while for the composition of the composite materials, EDX was performed.

2.3 X-RAY DIFFRACTION (XRD)

The crystalline structure of materials is generally determined by the X-Ray Diffraction (XRD) [34], [35] [33],[36]. This characterization technique was operated on pure commercial and modified the commercial material.

The x-ray diffraction (XRD) technique was utilized for the confirmation of the crystalline structure of DAE and modified with magnetite. the sample was dried at room

temperature and then processed to a fine powder which were introduced and pressed into a clear silicon single crystal low background holder (size: 24.5 mm diameter, 1 mm depth). The holder was then kept in the spinning auto sampler of a Bruker D2 Phaser desktop x-ray diffractometer and scanned constantly between 5-80º at a scan axis 2θ measuring 3715 steps at 0.9 seconds per step (each step size was 0.02º) with a 5o detector window.

2.4 FOURIER TRANSFORMS INFRARED SPECTROSCOPY (FTIR)

This is controlling instrument to analyse the bond between the atoms by producing the absorption spectrum, it is similar to the fingerprint. FTIR shows the quick surface analysis of the materials.

The nanocomposite material and modified commercial Diatomaceous earth was characterized by FT-IR spectroscopy (Nicolet IR200 FT-IR), for functional group analysis, pinch of all the developed and commercial samples were used, after 16 scans the FTIR spectra were collected at RT.

3.0 RESULTS AND DISCUSSION

Scanning electron microscopy (SEM)

The morphology of the synthesized nanocomposite material was examined by Scanning Electron Microscopy. Smple was introduced in to the vacuum chamber of the SEM machine and was clicked to run. For the morphology analysis images were taken while for the composition of the composite materials, EDX was performed.

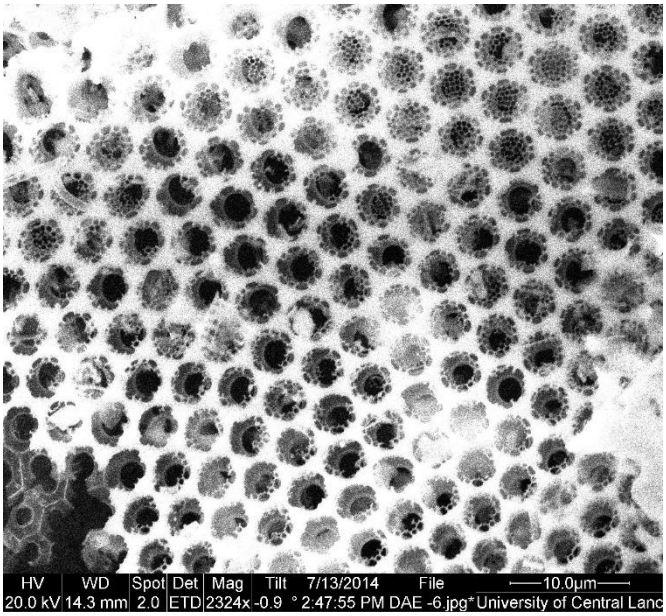


Fig. SEM DAE commercial material

For the morphology analysis images were taken according to the technique described in Methodology section[25], [26], for the composition of the composite materials, EDX was performed to determine the elemental composition of the nanocomposite as it has been used by many researchers [27].Fig.1 is showing the surface morphology of the DAE pure commercial material, the porous surface can be observed.

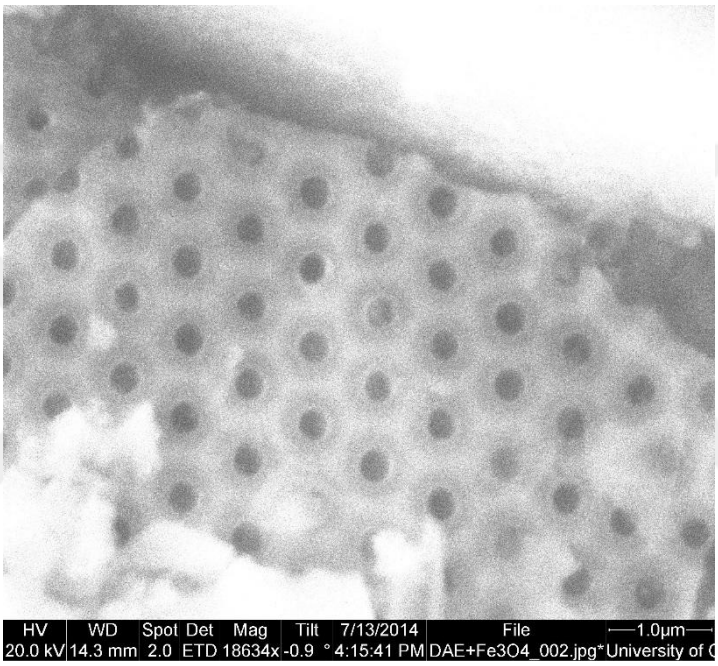


Fig.2 SEM of DAE containing iron oxide composite material

Fig.2 shows the surface morphology of the DAE containing iron oxide composite material. The experiment was performed at 20 KeV with 50000 at 1um and 1500 with 20 um magnifications. From Fig.3 small holes can be seen clearly with dots and spots, these dots are the magnetite particles as suggested [28].

3.1 Energy Dispersive X-Ray Analysis (EDAX)

This technique is applied for a determination of the elemental composition of the sample; it is generally fixed with SEM or TEM. This analytical technique offers broad information for the elemental analysis, composition of the compound and mapping. EDAX was carried out for the elemental detection in the material, the spectrum clearly showed the signals**.**

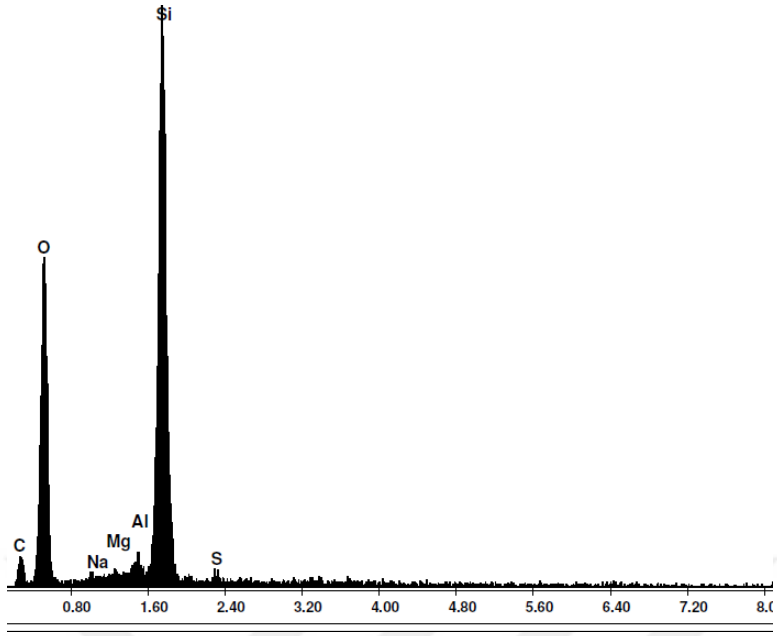


Fig: 3 Spectrum of commercial DAE.

Fig.3 demonstrates the EDX analysis of commercially purchased DAE, peaks of silicon and oxygen are cearly seen in this figure. The energy value of produced peaks were matched with the X-ray emitted wave length for this analysis.

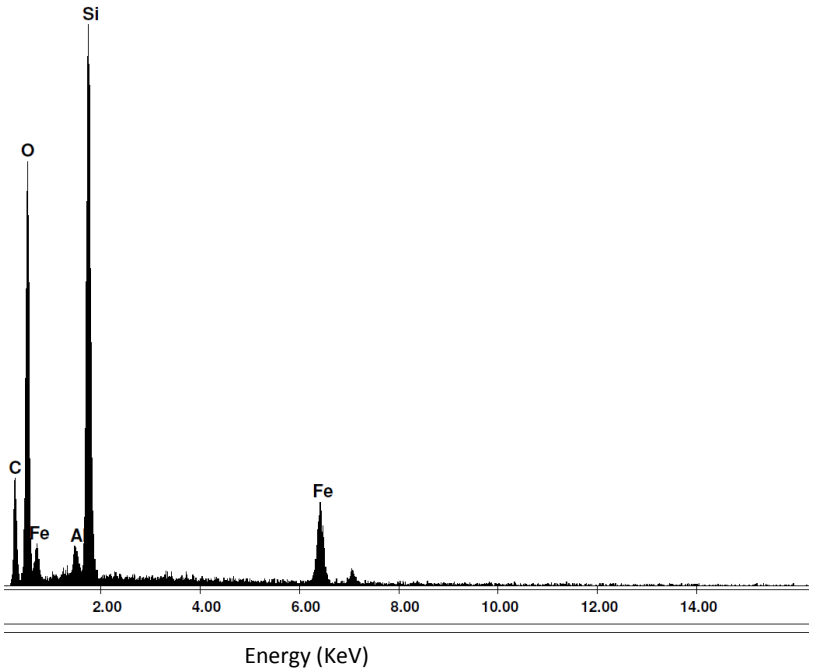


Fig.4 Spectrum of DAE containing iron oxide

Fig.4 represents the spectrum of DAE coated magnetite, it can be clearly seen that iron is located at 6.3 KeV.

3.3 FTIR Analysis

The Fourier transform infrared (FT-IR) spectra were recorded in the range 400–2000cm-^{1} for the bonding between the atoms and presence of functional groups.

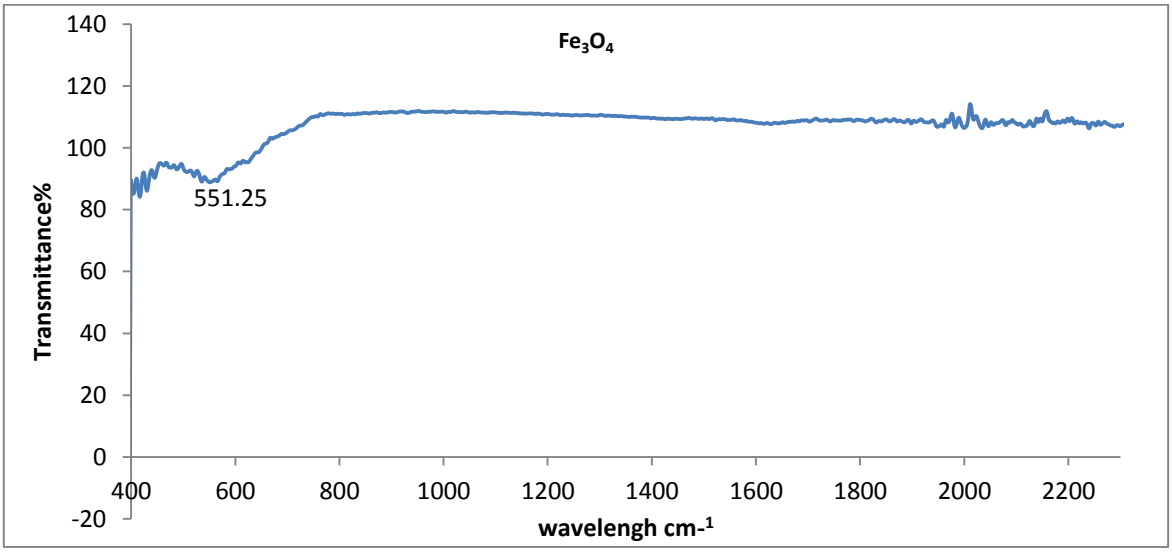


Fig.5 FTIR of magnetite

Fig.5 is showing the stretching vibration of the Fe – O bond at 551cm.

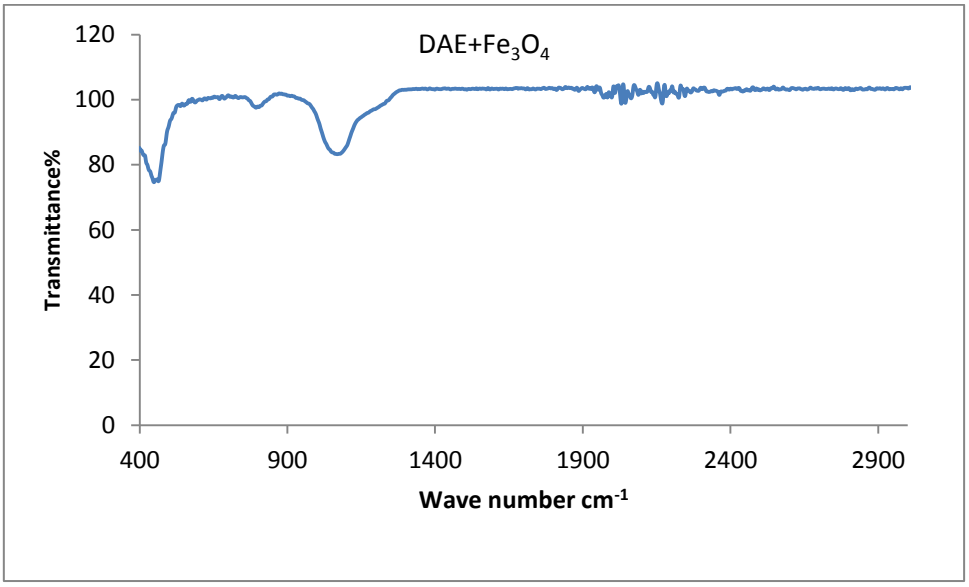


Fig.5 FTIR DAE magnetite composite

Fig.6 shows the stretching vibration of the Fe – O bond at 551 cm-^{1}.

The evidence for the presence of Fe – O bond was obtained by the FTIR analysis of the nanocomposite materials. The peaks for the iron oxide ranges from 460 to 580 cm-^{1}due to stretching vibration of Fe - O, [38] and 1633 cm-1 are described as Hydroxyl (H-O) adsorbed bending vibration of water [41]. For the iron oxide peaks in DAE composite was at 551cm^{1}.By looking at the figures 6, the peak of Fe – O can be seen which is attributed to the iron oxide peak, this result coincides with EDAX analysis of the composite in this project.

3.4 XRD Analysis

The crystalline phase of the composite and pure commercial material were performed with the XRD, the method is given above in characterisation section. Results have shown that the incorporation of the magnetite particles in to the matrix caused the material crystalline While, incorporated magnetite nanoparticles has shown the peaks and caused the material crystalline.

Results are shown below.

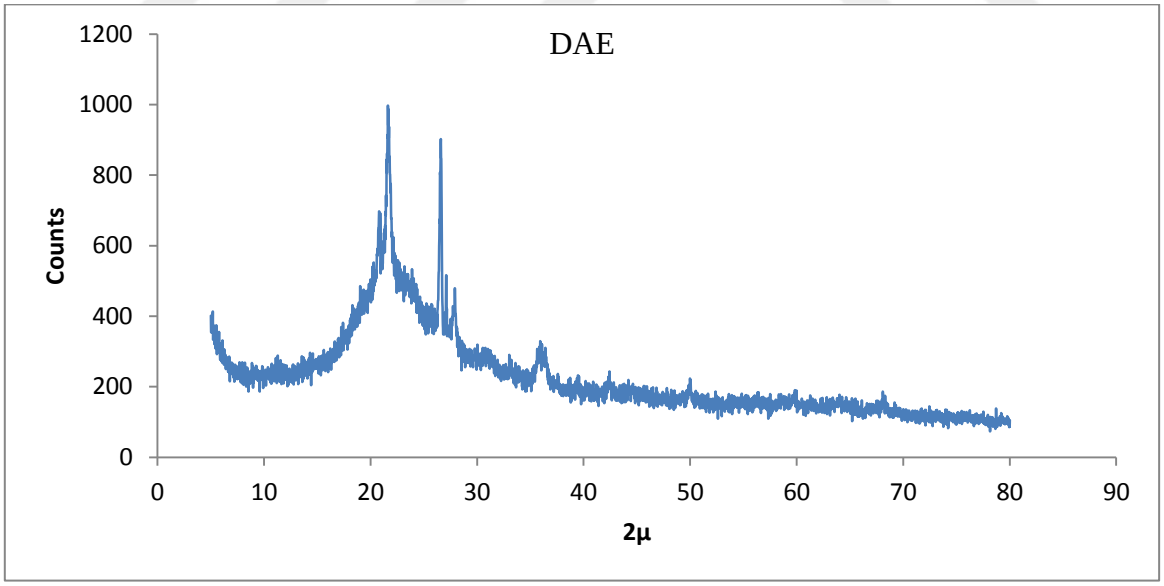


Fig.7 FTIR DAE magnetite composite

Fig.7 is showing the crystalline structure of the diatomeous earth and crystalline structure of the modified diatomeous earth with magnetite nanoparticles. The embedded nanoparticles caused the material crystalline.

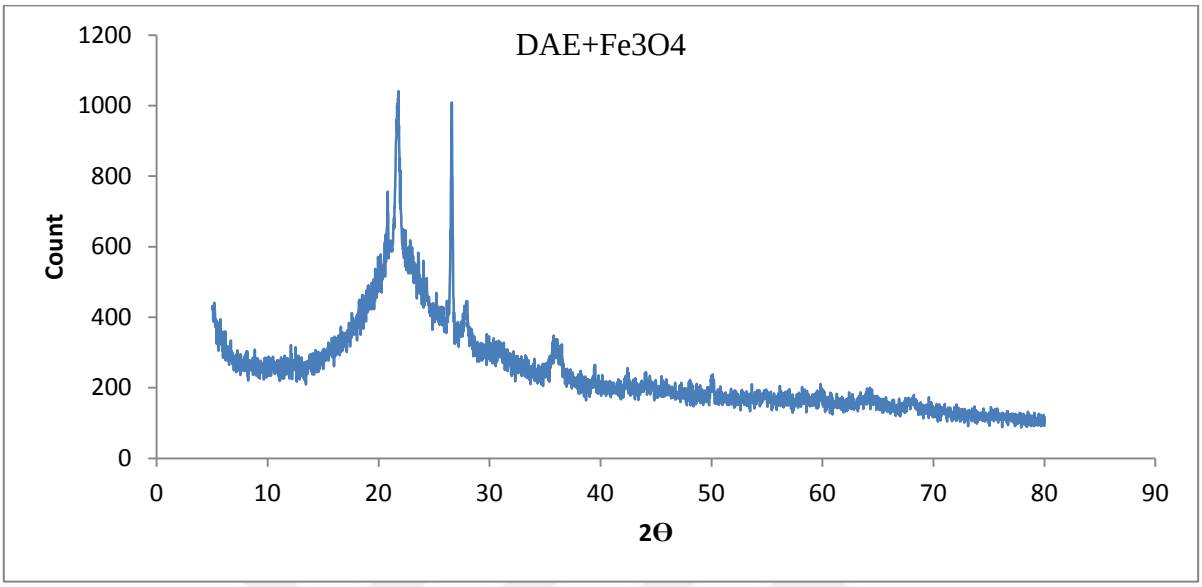


Fig.8 XRD pattern DAE containing magnetite nanoparticles.

The crystalline structure of the composite material and pure commercial material was examined by XRD .Fig.8 shows the typical XRD pattern of the pure commercial DAE, its intensity has been recorded 80 counts.

Fig.7 XRD pattern of the DAE with magnetite shows the reduction in the intensity and little increase in the peak size as compare to commercial DAE, this is due to the embedded magnetite nanoparticles inside the matrix which caused the material crystalline.

4.0 Introduction to Surface Functionalisation

Managing the surface chemistry of any materials can guide to reforms in the way they associate with different materials. In the connection of this work, amino silanes (APTS and APDS,) very important compounds for the functionalisation of the composite material containing magnetic nanoparticles. They are valuable as they can conjugate a broad range of biomolecules (such as enzymes, proteins, and oligonucleotides) to surfaces which have amine or carboxyl groups present [42]. They are also easily accessible and comfortable to use.

Generally silanes are bi-functional reagents, represented by the general formula X-(CH2)n-SiRn(OR′)3-n. (CH2)n is any alkyl chain spacer group, Si(OR′)n groups are support

groups which connect to the silanol hydroxyl groups on the silica surface following hydrolysis of the alkoxy (OR′) group and X represents the head group, Aminosilanes are simply organosilanes holding an amino group as the X functionality. They are commercially prepared and are generally applied in the silanisation of surfaces by organic phase, aqueous phase or chemical vapour ousting processes.[43] Three commonly used amino silanes for surface functionalisation [(3-aminopropyl)-triethoxysilane (APTS), (3-aminopropyl)-diethoxymethylsilane (APDS) and (3-aminopropyl)-monoethoxydimethylsilane (APMS)].

4.1 Strategies and Optimisation

There are several methods for the immobilisation of the enzymes with the composite; the foremost methods include adsorption (non-covalent), binding (covalent), self-immobilisation, entrapment and encapsulation.[29] An essential point to think is selecting the accurate assistance to optimise catalytic activity and stability of the enzyme when it is being immobilised. Captures frequently use either an organic polymer network or sol-gel matrix and are often performed in situ [30]. The captured enzymes are then preserved from the effects of gas bubbles, mechanical erosion and hydrophobic solvents, but entrapped enzymes can experience from bulk transfer conditions and lower enzyme loadings[31] than other immobilisation techniques.[32] Encapsulation is alike to entrapment as the enzyme is preserved from many outside factors but has restricted use in bio-catalysis of large substrates due to mass transfer limitations again.[31],[32] Self-immobilisation is generally conducted out in the case of carrier-free immobilisation, made feasible using a bi-functional cross-linking molecule, i.e. glutaraldehyde, to attach enzymes to each other without any assistance.[32]. For example, cross-linked enzyme crystals

An illustration of several immobilisation techniques being used in the identical reaction is inscribed with PFL. The kinetic determination of numerous 3-aryl-3-hydroxypropanoates has been reported for the immobilisation via adsorption, cross-linked enzyme adsorption and sol-gel encapsulation [33] Immobilisation has been revealed to increase stability, activity and re-usability. The bimolecular form [34] showed increase thermal resistance and activity in catalysing the trans-esterification of olive oil with benzyl alcohol.

4.2 Surface Functionalisation of diatomeous earth containing magnetite nanoparticles

4.2.1 Silanisation of composite by using 99% methanol

Surface modification (silanisation) of diatomaceous earth magnetite nanoparticles by using two amino silanes i.e. APTS and APDS were made as follows, practicing a changed method from that developed by Bruce et al.80 diatomaceous magnetite nanoparticles (150 mg) were introduced to 35 mL of amino silanes in methanol (APTS, APDS , 2% w/v) in a 50 mL screw-capped centrifuge tube. The composite was allowed to react for 24 hours in an incubator at 40 rpm with gentle end-over-end rotation at 50ºC. Silanised diatomaceous magnetite solution of nanoparticles were washed with 3×10 mL with (0.8% v/v glacial acetic acid in methanol) coupling solution and re-suspended in 10 mL of the same solution at 5ºC. Surface amine densities were determined by a colorimetric assay using 4-NBA.88, [50]. It was observed in a detailed study by De Water beemd [51] that surface amine density experienced ample reductions after lengthy storage and as a result, fresh batches of surface-functionalised nanocomposite were produced every month. The calibration of the solution is given below.

Surface functionalization of the nanocomposite with aminosilanes

4-Nitrobenzaldehyde Calibration: Coupling solution

No	**4-Nitrobenzaldehyde (µl)**	**Coupling solution (µl)**	**Absorbance 282nm**	**Concentration nmol /mL**
1	10	990	0.376	46.00
2	20	980	0.619	92.66
3	30	970	1.001	139.12
4	40	960	1.297	185.30
5	50	950	1.553	231.64
6	60	940	1.753	278.07

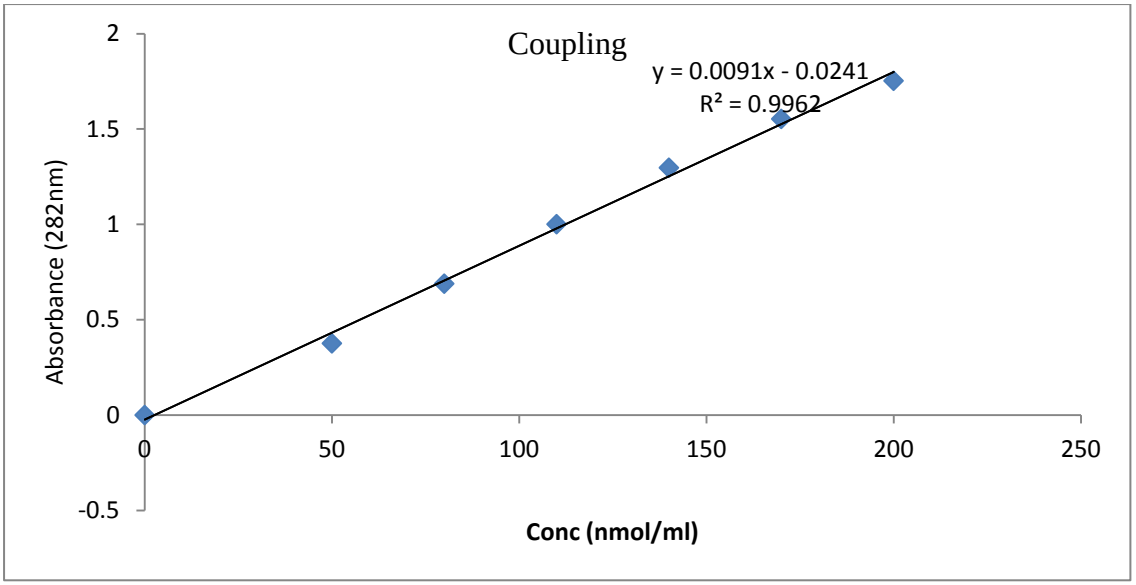


Figure 9: 4-Nitrobenzaldehyde Calibration: Coupling solution

4-Nitrobenzaldehyde Calibration: Hydrolysis solution

No	4-Nitrobenzaldehyde (µl)	Hydrolysis solution (µl)	Absorbance	Concentration nmol/mL
1	10	990	0.382	46.00
2	20	980	0.595	92.66
3	30	970	0.987	139.12
4	40	960	1.200	185.30
5	50	950	1.485	231.64
6	60	940	1.808	278.07

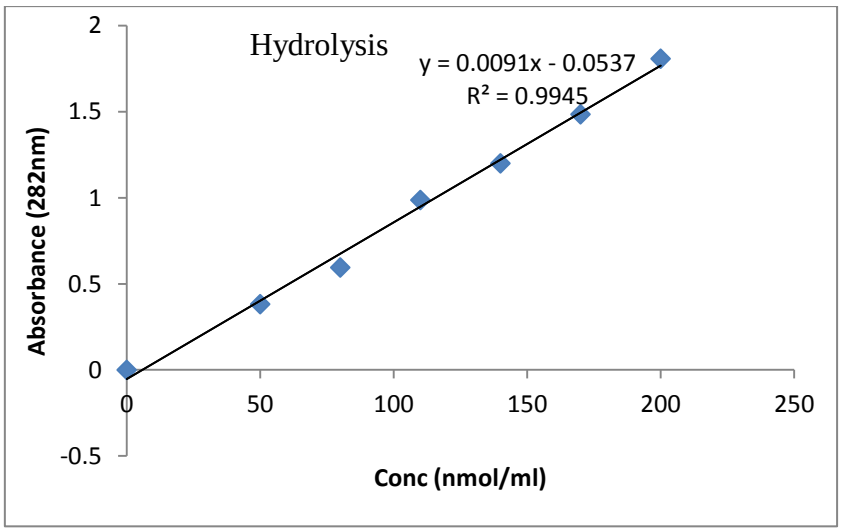


Figure 10: 4-Nitrobenzaldehyde Calibration: Hydrolysis solution

DAE+Fe_3O_4 with APTS

Sample	Supernatant (µl)	Coupling solution(µl	Absorbance 282nm	Concentration nmol/mL
1	50	950	1.326	148.36
2	50	950	1.278	143
3	50	950	1.232	143.08
Avg			1.279	143.19

DAE+Fe_3O_4 with APDS

Sample	Supernatant (µl)	Coupling solution(µl	Absorbance	Concentration nmol /mL
1	**50**	**950**	**1.328**	148.58
2	**50**	**950**	**1.335**	149.35
3	**50**	**950**	**1.337**	149.57
Avg			**1.333**	149.13

Hydrolysis

DAE+Fe_3O_4 with APTS

Sample	Supernatant (µl)	Absorbance	Concentration nmol/mL
1	800	0.582	69.85
2	800	0.572	68.75
3	800	0.498	60.62
Avg	800	0.550	66.34

DAE+Fe_3O_4 with APDS

Sample	Supernatant (µl)	Absorbance	Concentration nmol/mL
1	800	0.362	45.68
2	800	0.423	52.38
3	800	0.415	51.50
Avg	800	0.400	49.85

4.3 Conversion of Surface Amine Groups to Aldehyde Groups Using Glutaraldehyde80

(20×) SSC buffer was prepared as stock solution by dissolving sodium citrate (88.2 g) and sodium chloride (175.3 g) in 1 litre distilled; deionised water (final pH 7.3) was adjusted by adding dilute HCl. Again that concentrated buffer was diluted 20 times to prepare (1×) SSC buffer by adding 50 mL 20×SSC buffer into 1 litre distilled, deionised water. 50mg of amino surface functionalised diatomaceous composite material was washed three times with 1×SSC buffer to remove the excess of the silanes in the composite and the supernatants separated, while, Glutaraldehyde solution were freshly prepared promptly before its use. The suspension was incubated at (40 rpm) for 3 hours at 18ºC with end-over-end rotation after the addition of 4 mL of a 5% w/v Glutaraldehyde solution (in 20×SSC buffer) with the amino-modified composite. The supernatant was separated magnetically. The composite was followed by washing three times with 4 times with 1×SSC buffer to discard surplus Glutaraldehyde and then washed 3 times with 5 mL of PBS buffer (pH 7.2) prior to immobilisation of lipase.

4.4 Immobilisation of the Enzyme

50 mg Glutaraldehyde-modified composite material was magnetically collected and the supernatant removed. To these 50mg of the solid Glutaraldehyde-modified composite, 4 mL of CRL in PBS buffer (1mg/mL) lipase solution was introduced with the mixture and at 18ºC was incubated for overnight with end-over-end rotation (40 rpm). The quantity of lipase in solution before and after immobilisation was determined by measuring absorption at 595 nm using UV-Visible spectrophotometer using a modified version of the Bradford Assay.

The complete mechanism, from surface functionalisation to Glutaraldehyde surface modification and enzyme immobilisation is shown in Scheme.

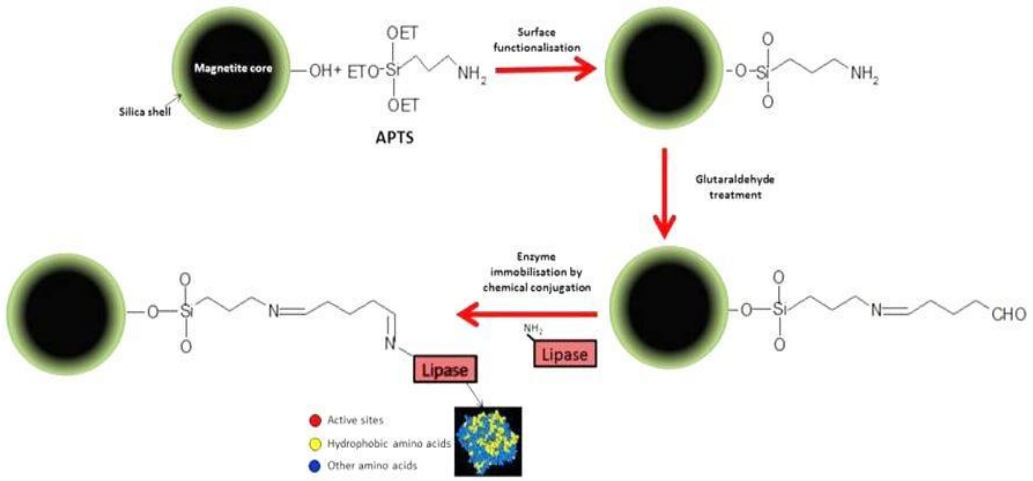


Figure11: A schematic diagram of surface functionalisation, glutaraldehyde surface modification and enzyme immobilisation on core-shell silica-magnetite nanoparticles[35].

4.5 Bio-catalytic applications of enzyme grafted nanocomposite material

There are several applications of the Magnetic nanocomposite grafted enzymes [53]. Past work in the field has revealed that magnetic nanocomposite grafted with enzyme such as, amorphous silica [54] have been practiced in the separation of bio molecules, for instance, drug delivery[58] DNA, extraction of nucleic acids from soil,[57] genomic[55], removal of phenolic compounds from environmental water[59]. and plasmid[56] There are numerous reports that MNP's coated with mesoporous silica,[60] have been observed to be helpful in controlled drug delivery,[61][62] hyperthermia[63], removal of mercury from- and desulfurization of -industrial effluent[64], magnetic resonance imaging2[65]70,fluorescence, and separation of plasmid and genomic DNA related to MNP's incorporated with amorphous silica.

They are favourable to use in drug delivery due to the immense surface area of the nanocomposite material. The strength to cross cellular and tissue walls and endurance to bio-degradation have made them very important [51]. Sen et al have reported the hierarchically ordered synthesis for template assisted porous magnetic nanocomposites and as supports for biocatalysis100. They examined the catalytic activity of CRL immobilised on hierarchically ordered porous magnetic nanocomposites to convert p-nitrophenyl palmitate (PNPP) to palmitic acid and p-nitrophenol via ester hydrolysis.

4.6 Calculation of p-nitrophenol (PNP) concentration by UV-Visible Assay.

A standard catalysis reaction was performed for the determination o of lipase immobilised nanocomposite as an experiment for their performance. Hydrolysis of PNPP was carried out in order to produce palmitic acid and PNP. It is UV-Visible effective molecule and its absorbance can be determined at 410 nm. Therefore, standard curves were created using a range of dilutions of PNP dissolved in a 1:1 mixture of reagent A (as given on the solution chart) and isopropanol. Absorbance was calculated by UV-Visible spectrophotometer at 410 nm.

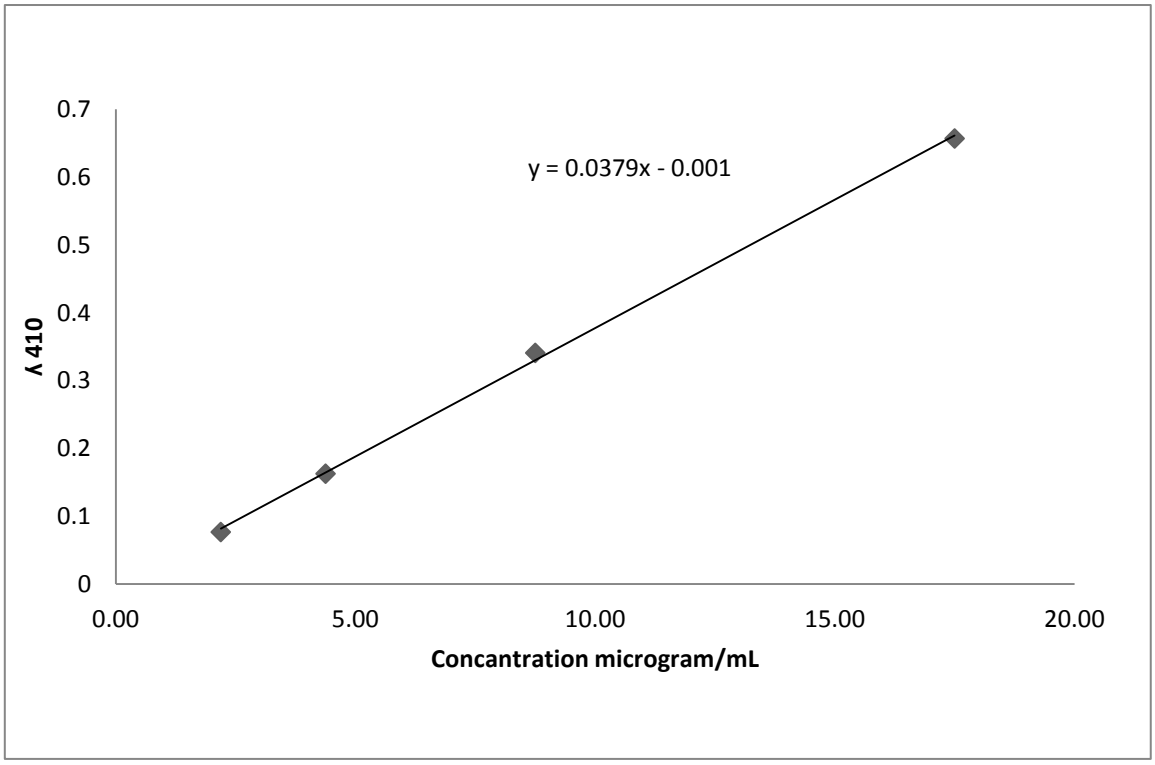


Figure 12 4- nitrophenol calibration curve

90 minutes

Non funclised	ʎ1.835	48.44 microgram/ml
Funclised	ʎ0.053	1.42 microgram/ml

120 minutes

Non funclized	ʎ2.492	65.77 microgram/ml
Funclised	ʎ0.484	12.80 microgram/ml

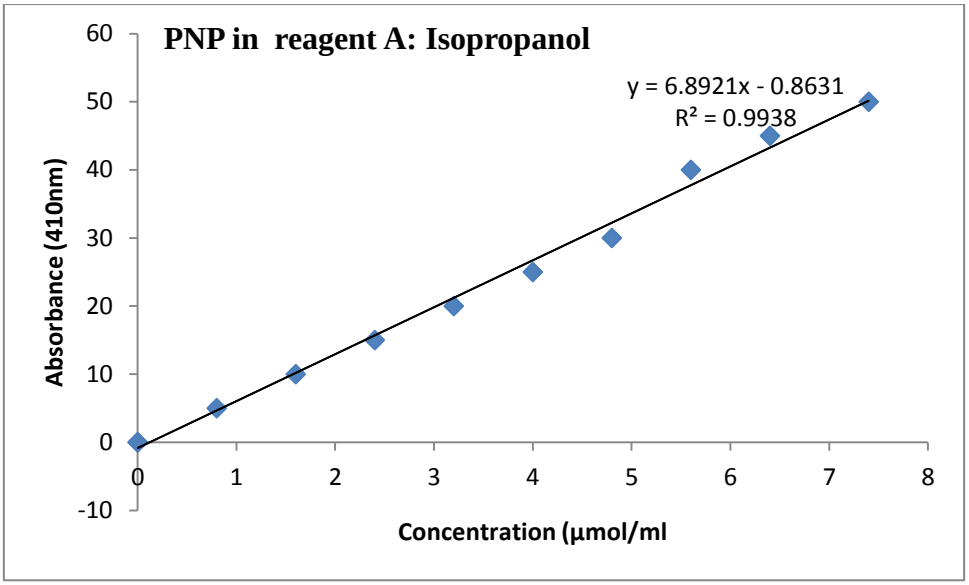


Figure 13: : Calibration curves for PNP in a 1:1 mixture of reagent A: Isopropanol.

It explains that linearity has been seen over the whole range of 0-8 µmol/mL for the whole series of solutions. As the PNP is UV-active, amounts as low as 1 µmol/mL needed dilutions up to 20 times. The calibration curve was applied to measure the quantity of PNP hydrolysed by lipase-immobilised nanoparticles.

5.0 CONCLUSIONS

For this project, novel multifunctional nanocomposites have been development by one-step reduction method. By modifying the Diatomaceous earth cheap commercial material with magnetite nanoparticles. Moreover, material has been surface functionalised with Enzyme for biocatalysts. A result showed that Enzyme grafted magnetite nanocomposite material is worth to use as biocatalyst reagent.

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