Synthesis and characterization of lipase based nanohybrid system

Project report submitted in partial fulfillment of the requirement for the degree of

Master of Science

in

Biotechnology

By

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Under the supervision of

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to

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Candidate's declaration

I hereby declare that the work presented in this report entitled **"Synthesis and characterization of lipase based nanohybrid system"** in partial fulfillment of the requirements for the award of the degree of **Master of Science** in **Biotechnology** submitted to the Department of Biotechnology & Bioinformatics**,** Jaypee University of Information Technology Waknaghat is an authentic record of my own work carried out over a period from July 2022 to May 2023 under the supervision of **Dr. Ashok Kumar Nadda, Department of Biotechnology and Bioinformatics.**

I also authenticate that I have carried out the above-mentioned project work under the proficiency stream.

The matter embodied in the report has not been submitted for the award of any other degree or diploma.

Alka Sharma

217814

Supervisor's certificate

This is to certify that the work which is being presented in the project report entitled **"Synthesis and characterization of lipase based nanohybrid system"** in partial fulfilment of the requirements for the award of the degree of **Masters of Science** in **Biotechnology** submitted to the Department of Biotechnology And Bioinformatics , Jaypee University of Information Technology, Waknaghat is an authentic record of work carried out by during the period from July 2022 to May 2023 under the supervision of **Dr. Ashok Kumar Nadda, Department of Biotechnology And Bioinformatics, Jaypee University of Information Technology, Solan, India.**

This is to certify that the above statement made is correct to the best of my knowledge.

Dr. Ashok Kumar Nadda
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Date:

Acknowledgement

First and foremost, I bow down before **"ALMIGHTY GOD"** for his countless blessings on me.

I express my deep sense of gratitude to **Dr. Sudhir Kumar**, Professor and Head, Department of Biotechnology and Bioinformatics, JUIT, Solan, Himachal Pradesh for his kind support that helped me in carrying out the dissertation work in a good manner.

I thank my project supervisor, **Dr. Ashok Kumar Nadda,** Assistant Professor, Department of Biotechnology and Bioinformatics, JUIT for his guidance, immerse support from starting to the end and always giving suggestions during this project work. I express my deepest sense of gratitude towards him for kind cooperation in every condition.

I would like to express my gratitude to PhD. Scholars **Tanvi Sharma, Kriti Sharma** for their constant encouragement, support and guidance till the completion of my project work.

I would like to appreciate my lab-mates, classmates and my friend **Megha Sharma** for her constant encouragement and support till the completion of my project work.

I am thankful to and fortunate enough to get encouragement, support and guidance from all the technical staff and the laboratory staff of Department of Biotechnology and Bioinformatics unit because it helped me in completing my project work successfully.

Last but not the least I would like to thank my friends and family for their valuable companionship, suggestions, guidance.

Thank you one and all.

Alka Sharma

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List of abbreviation

S. No.	Abbr.	Abbreviation(s)
1.	^{0}C	Degree celcius
2.	BSA	Bovine serum albumin
3.	FTIR	Fourier transform infrared
4.	Gm	Gram
5.	HNFs	Hybrid nanoflowers
6.	Ml	Mili-litre
7.	mM	Mili-molar
8.	PBS	Phosphate buffered saline
9	RPM	Revolution per minute
10.	SEM	Scanning electron microscopy
11.	XRD	X-Ray differaction

Abstract

Lipase (EC 3.1.1.3), enzyme that is extensively used in industries. The lipase can be taken from different sources such as vegetable, animal, bacteria, fungi and has numerous approaches in different industries such as medical industry, leather industry, and food industry and also in waste water management. To improve these industrial applications immobilizations of enzyme is done. Immobilization of enzyme allows the biocatalyst's reusability and also increases their catalytic activity. This immobilization of enzyme can be done by a low-cost, simple and innovative method that is synthesis of nano-flower. This study deals with the synthesis of organic-inorganic nano-flowers using Lipase enzyme and copper metal ions to increase its enzyme activity and stability. In this study, different protein concentrations were used for the synthesis of nano-hybrid system. Among all protein concentrations used for nanoflower synthesis 0.6 mg/ml showed maximum enzyme activity as compared to others. Then effects of different parameters such as temp., pH and substrate conc. were measured. The result showed maximum enzyme activity of 0.98uM/ml/min. The comparison between activity of nanohybrid system and free enzyme showed that protein inorganic nanohybrid was more stable and showed higher enzyme activity. The result demonstrated that the immobilization of Lipase enzyme using nano-hybrid system has potential applications in different industries.

Keywords: *Lipase, Immobilization, Nano-hybrid system, Enzyme activity, Industrial applications*

Chapter 1 Introduction

Enzymes are proteins that catalyze biochemical reactions by increasing their reaction rate. Enzymes work at mild conditions of pH and temp. These were specific to the reaction. Enzymes have various uses in various fields such as the food industry, pharmaceuticals, environmental science, etc. In Free State, enzymes have less stability and low reproducibility and they lose the property of reusability. To overcome this problem, immobilization of enzymes is one of the methods [1]. The conventional method of enzyme immobilization includes insoluble solid support. The solid support needs to be non-toxic, inert, easily accessible, insoluble, eco-friendly, and resistant towards fungal and bacterial attack and has affinity to bind with catalyst which is being utilized. Immobilization of enzymes has numerous utilities which include an increase in stability towards reaction conditions such as increase in pH and temperature, increased catalytic performance, and easy separation and recovery. There are many research works that prove that immobilized enzymes have more stability in comparison with free enzymes but in some cases, immobilization of enzyme leads to low catalytic activity this is cause of change of conformational of the enzyme during immobilization, the active site of an enzyme can be blocked and can lead to contamination of product [2]. The nano-hybrid system provides a solution to overcome the problems caused by the conventional method of immobilization of enzyme.

1.1 Nano-hybrid system

Nano hybrid systems are nano-scalic particles which are composed of organic and inorganic molecules. Organic molecules include protein, DNA, RNA and inorganic molecules include metal ions. This study mainly focused on enzyme based Nano-hybrid system. The system resembles the structure of flower so the nano-hybrid system named as hybrid nano-flower [3]. The synthesis process of hybrid nanoflower is simple. The steps included in the formation of hybrid nano-flower are nucleation, coordination, precipitation and anisotropic growth.

1.1.1 Advantages of hybrid nanoflower

These hybrid Nano- flowers provide large surface area to volume ratio which increases the enzyme activity and lowers mass transfer limitations. These enhance the

reusability of enzyme [4]. Hybrid Nano-flower has numerous applications. These have applications in the area of bio-catalysis, bioremediation, and biosensors [2].

1.2 Lipase enzyme and its application

Lipase belongs to the triacylglycerol ester-hydrolase family. The range of molecular weight of lipases is 19-60 KDa. Lipase enzyme catalyzes various reactions such as inter-esterification, aminolysis, esterification, alcoholysis, and hydrolysis of triglycerides into fatty acids. Lipase enzyme has applications in various industries such as pharmaceuticals, biodiesel, drinks, foods, textile, leather, and detergents [5].

To know the effects of immobilization of lipase enzyme on copper we have carried out the experiments. We studied the effects of temperature, pH and substrate concentration on free enzyme and on nanohybrid system. In this these below mentioned objectives were achieved.

1.3 Objectives

- Screening of lipase producing microbial strain
- Characterization of isolated bacterial strain
- Use of commercial lipase for the synthesis of lipase based nano-hybrid system
- Characterization of nano-hybrid system

Chapter 2 Literature survey

2.1 Enzyme-based nanohybrid systems

Enzyme-based nanohybrid systems have been the subject of extensive research in recent years due to their promising role in biocatalysis and biomedical applications. These systems consist of enzyme molecules that are immobilized onto nanomaterial support, which enhances their stability, reusability, and catalytic activity. The growing demand for eco-friendly and sustainable technologies has also fueled the interest in enzyme-based nanohybrid systems as they are biocompatible, non-toxic, and can function under mild reaction conditions [1]. These nanohybrid systems are composed of numerous layers which looks similar to biological flower. These nanoflowers has large surface area to volume ratio which increases enzyme activity and lowers the mass transfer limitation ultimately leads to increase in reaction rate. Enzymes immobilized on nanomaterials have more stability towards the reaction conditions as compared to free enzymes. Nanoflowers provide the property of the reusability of enzyme.

2.2 Mechanism used for the production of hybrid nanoflower

The process of production of hybrid nanoflower is very simple. The synthesis process includes simple steps like nucleation, coordination and self-assembly [4]. Synthesis process starts with the formation of primary crystals. These formed when metal ions binds with the –vely charged phosphate group electrostatically. After that, the metal phosphate crystals bind with enzyme through coordination bond which is established between metal ions and nitrogen atom. This leads to formation of Nano plates and these nano plates are similar to petals. These petals self-assembled to take the shape of flower. These nanoflowers grow until saturation point. At the end, enzyme hybrid nanoflowers are efficiently synthesized. In this synthesis process, enzyme works as glue which binds phosphate and metal ions together [6].

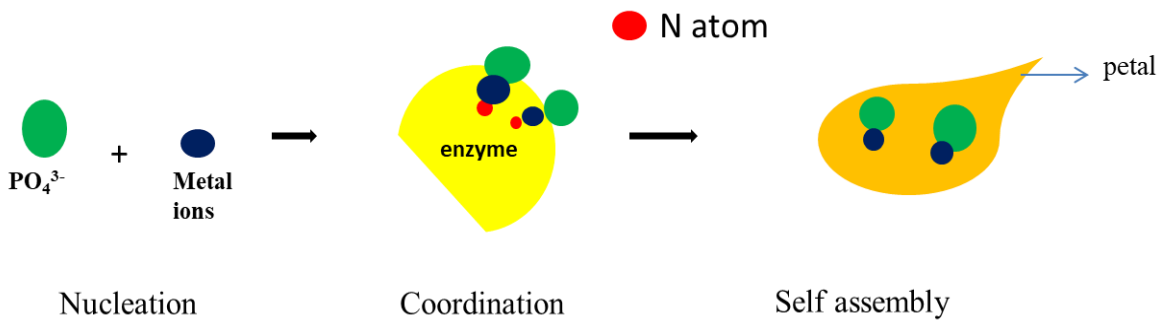

Fig. 2.1: Illustration of synthesis process of hybrid nanoflower

2.3 Methods of synthesis of hybrid nanoflower

Precipitation method

In this method, enzyme was dissolved in PBS which contains metal salt. The reaction mixture was kept at 4^0C or 25^0C for 72 hours. After incubation period, precipitates were observed in solution which were said as hybrid nanoflowers and these were separated from solution by centrifugation then washed with ultrapure water dried and characterization was done [7].

This method does not require any harsh reaction conditions. This method is time consuming, to overcome this limitation sonication method is developed.

Sonication method

In this method, the reaction mixture which contains enzyme, metal salt, and PBS was sonicated for 5 minutes at room temperature. After 5 minutes hybrid nanoflowers observed in solution [6].

2.4 Classification of nanohybrid systems

The classification of nanohybrid systems is based on metal ions and enzymes used in the synthesis process. There are some researches which used multiple metal ions for the immobilization of single enzymes and multiple enzymes were immobilized on single metal ions.

Hybrid nanoflower based on single metal and single enzyme

There are numerous researches which support the synthesis of single metal and single enzyme based nanoflowers. Some of the examples are given below.

Vojdanitalab et al., reported the synthesis of laccase based nanoflower. They used cobalt as inorganic part. They obtained purple coloured nanoflowers. They studied the activity of enzyme, storage stability, stability towards the different ranges of temperature and reusability of immobilized enzyme and found the enhancement in properties. These hybrid nanoflowers utilized for the degradation of moxifloxacin [7].**Sun et al.** claimed the production of hybrid nanoflowers by utilizing polyphosphate kinase enzyme and copper metal. They utilized precipitation method for the production. Hybrid nanoflowers used to regenerate ATP from ADP [8].**Lin et al.** studied the synthesis of nanoflower using HRP enzyme and copper sulphates. They used different concentration of enzyme. They done comparative study of free enzyme and immobilized for colorimetric assay. They found that the immobilized enzyme show fast reaction and detects the substrate in less time period as compared to free enzyme. Hybrid nanoflower used to detect H_2O_2 and phenol [9].**Rai et al.** reported the synthesis of nanoflower by utilizing L- arabinose isomerase enzyme and manganese phosphate metal ions. This assembly was utilized in the synthesis procedure of D- tagatose by utilizing D- galactose as substrate [10].**Duan et al.** reported synthesis of nanoflower using carbonic anhydrase and different metal ions. They studied how metal ion affects the activity of immobilized enzyme. They used three different metal phosphates**.** Metal phosphates added to PBS (pH 7.4) and 10 mg of carbonic anhydrase was added for the synthesis of nanoflower. They compared catalytic activity of nanoflowers of different metal phosphates with free enzyme. They found that Mn-CA hybrid nanoflower has low activity in comparison with free enzyme. Other two immobilized enzyme shows higher activity to the free enzymes. They utilized hybrid nanoflower in hydrolysis of CO_2 to produce carbonate [11].

Hybrid nanoflowers based on two metal ions and single enzyme

Patel et al. reported synthesis of laccase based nanoflower. They utilized the multiple metals for the synthesis .They used copper and zinc for the synthesis. Then they characterized their structure and properties. They observed that the nanoflower with multiple metals show higher activity as compared to the single metal based nanoflower. They synthesize the nanoflower by adding Cu and Zn in 1:1 ratio in PBS with varied conc.

of protein. They used these enzymes for the degradation of bisphenol. A toxic compound which has adverse side effects [12].

Hybrid nanoflower based on single metal ions and multiple enzymes

Lou et al. reported the synthesis of nanoflower with multiple enzymes. They utilized polyphosphate kinase and nucleoside kinase for their experiment. They co-immobilized these two enzymes with copper metal. They used these nanoflowers for the production of nucleotides from their corresponding nucleosides [13].**Kong et al.** claimed the synthesis of biosensor by utilizing multienzyme nanoflower. Acetylcholinesterase, choline oxidase, and mimic peroxidase utilized for the nanoflower synthesis. After synthesis different properties of nanoflowers were studied. Hybrid Nano-flowers used for the colorimetic detection and monitoring of acetylcholine which is important for the functioning of nervous system [14].**Rai et al.** reported the synthesis of nanoflower by utilizing dual enzyme system. L- arabinose isomerase enzyme and beta galactosidase enzyme and manganese phosphate as metal ions. They used one pot dual synthesis technique. This assembly was utilized in the production of tagatose by conversion of galactose[15]. **Han et al.** claimed the synthesis of multienzymatic nanoflower to convert starch in gluconic acid. For this purpose they used two enzymes one is glucose oxidase and another one is glucoamylase. These two enzymes are immobilized simultaneously. They used copper ion for this purpose. During synthesis they first immobilized gluco-oxidase on the copper ion via conventional method of 72 hours incubation. And after that the glucoamylase enzyme adsorbed on the synthesized nanoflowers of gluco-oxidase enzyme. By this method they obtain the dual enzymatic nanoflower [16].**Han et al.** reported the synthesis of multiple enzyme immobilization to convert cellulose into glucose. They immobilized three enzymes simultaneously. Cellobiohydrolase, endo-glucanase, and β-glucosidase these three recombinant enzymes were immobilized on the copper metal [17].

2.5 Factors affecting the morphology of hybrid nanoflower

There are so many factors which affect the morphology of hybrid nanoflower. These factors also affect the enzyme activity. These can lead to increase in enzyme activity

but also can decrease the enzyme activity. These factors include protein concentration, metal ions, temperature, and pH.

Effect of enzyme

Enzyme has considerable effect on the structure of hybrid nanoflower. Different kind of enzymes results in the different morphology of nanoflowers. Enzyme helps in the binding of metal ions and phosphates which results in formation of nanocrystals.

Concentration of enzyme affects the morphology of hybrid nanoflower. This influences the density and size of petals. Large amount of enzyme leads to formation of microstructures which are densely packed [6].

Effect of metal ions

Metal ions affects the morphology as well as activity of enzyme. Metal ions have ability to increase the enzyme activity as well as cause decrease in enzyme activity. Duan et al. reported synthesis of nanoflower using carbonic anhydrase and different metal ions. They studied how metal ion affects the activity of immobilized enzyme. They used three different metal phosphates**.** Metal phosphates added to PBS (pH 7.4) and 10 mg of carbonic anhydrase was added for the synthesis of nanoflower. They compared the activity of nanoflowers of different metal phosphates with free enzyme. They found that Mn-CA hybrid nanoflower has low activity as compared to free enzyme. Other two nanoflowers shows increase in activityin comparison with the free enzymes [11]. Thus the selection of metal ion is crucial task.

Effect of synthesis temperature

Synthesis temperature has significant effects on the morphology of hybrid nanoflower. Temperature influences the nucleation rate, growth, and self-assembly. Increase in temperature leads to formation of nanosheets rather than nanoflowers. At low temperature, petals were denser and dispersivity of enzyme is worse. Concentration of enzyme is more around the nucleus [1].

Effect of pH

Reaction pH also affects the morphology of hybrid nanoflower. An enzyme's charge changes with the variation in pH level which leads to different interaction of

enzyme. Various studies reported that there is no nanoflower production takes place at pH 3, 4, and 5. There is nanoflower formation takes place at pH of 6, 7, and 8. As the pH value increases the density of petals decreases [1].

2.6 Advantages of hybrid nanoflower

Catalytic activity

There are so many studies which reported that after the immobilization of enzyme on metal ions catalytic activity of enzymes increases. The immobilized enzyme shows higher catalytic efficiency in comparison with free enzymes. The reasons behind the increase in activity are the large surface area to volume ratio and decrease in mass transfer limitations [7] [8] [10].

Thermal stability

Immobilized enzymes were examined against various parameters thermal stability is one from them. Activity of immobilized and free enzymes was calculated at different temperatures. Results show that immobilized enzymes emits higher activity at high temp. as compared to free enzymes [8]-[17].

Storage stability

Immobilized enzymes were also examined towards the storage stability. Immobilized enzymes and free enzymes were stored at different temperature ranges for some time period. After that time period activity of free and immobilized enzyme were examined. Results show that free enzyme lost more activity when compared with immobilized enzymes [10] [12].

Reusability

Immobilization of enzyme enhances reusability of enzyme. Immobilized enzymes can be reused for the same reaction or different reaction. Free enzymes cannot utilize again. Reusability is advantages of immobilized enzymes over free enzymes [15].

2.7 Applications of nanohybrid systems

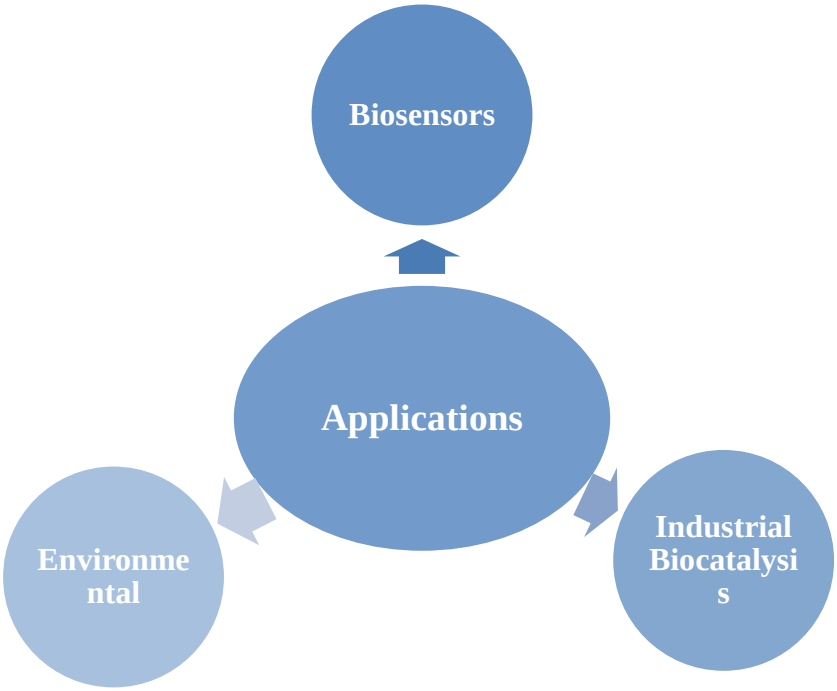

Fig. 2.7: Applications of hybrid nanoflowers

Biosensors

Hybrid nanoflower has various applications. Various studies reported the use of nanoflower as biosensor. Here are some examples Kong et al. claimed the synthesis of biosensor by utilizing multienzyme nanoflower. Acetylcholinesterase, choline oxidase, and mimic peroxidase utilized for the nanoflower synthesis [14]. Lin et al. studied the synthesis of nanoflower using HRP enzyme and copper sulphates. They utilized these nanoflowers for the detection of H_2O_2 and phenol [9].

Industrial biocatalysis

Enzymes are biological catalyst they catalyses various reactions. Hybrid nanoflowers can be used to catalyse the reaction at industrial level as hybrid nanoflowers have storage stability, reusability and thermal stability. These nanohybrid systems was used in diferent industries. Rai et al. reported the synthesis of nanoflower by utilizing dual enzyme system. L- arabinose isomerase enzyme and beta galactosidase enzyme and manganese phosphate as metal ions. They used one pot dual synthesis technique. This assembly was utilized in the manufacturing of tagatose by conversion of galactose [15].

Environmental applications

Nanoflowers have various applications towards the environment. These can be utilized for the remediation of pollutants, degradation of dyes etc. Patel et al. reported synthesis of laccase nanoflower. These nanoflowers were utilized for degradation of bisphenol [12]. Vojdanitalab et al. claimed the synthesis of laccase based nanoflowers which were utilized for degradation of moxifloxin [7]. Duan et al. synthesized nanoflower by using carbonic anhydrase and different metal ions these were used for the hydrolysis of CO_2 [11].

2.8 Analytical techniques used for characterization of nanoflower-

SEM (Scanning Electron Microscope**)** used to investigate the morphology of synthesized hybrid nanoflower.[10]

Energy dispersive X-Ray spectroscopy used for determination of the composition and spatial distribution of elements on synthesized hybrid nanoflower.[12]

Fourier transform infrared used to analyse different groups of organic and in-organic constituents of hybrid nanoflower.[13]

2.9 Lipase enzyme and its applications

Lipase enzyme belongs to the triacylglycerol ester-hydrolase family. The range of molecular weight of lipases is 19-60 KDa. They works at pH ranges from 4-8. Ni^{2+}, Co^{2+}, Hg^{2+}, and Sn^{2+} inhibited the lipase activities excessively and Zn^{2+}, Mg^{2+}, SDS and EDTA inhibited moderately. Lipase enzyme can be found in animal, microbes and vegetables. Microbial lipase can be obtained from the bacteria as well as fungi. Microbial enzyme has more applications as compared to other lipases. Lipase enzyme catalyzes various reactions such as inter-esterification, aminolysis, esterification, alcoholysis, and hydrolysis of long-chain triglycerides to fatty acids. This enzyme has various industrial applications. These can be consumed in industries such as food and beverages, leather, biodiesel production, textile, and oily waste water treatment [5].

Chapter 3 Materials and methods

Material used

Chemicals and reagents used- Nutrient agar, Nutrient Broth, 4-nitrophenyl acetate (p-NPA), p-NP (para-nitrophenol), Bradford reagent, PBS, NaH_2PO_4, Na_2HPO_4, $CuSO_4$ were purchased from **Himedia**. p-NPP(para – nitrophenylpalmitate), BSA were purchased from **Sigma**. Citric acid, sodium citrate dehydrate, sodium carbonate, sodium bicarbonate were purchased from **Fisher Scientific**. Crystal violet, iodine, safranin were purchased from **Loba chemie**. Lipase enzyme was purchased from **Advance enzyme.** Ethanol.

Instruments used weighing balance, autoclave, laminar air flow (LAF), centrifuge, sonicator, water-bath, incubator, microscope, and spectrophotometer.

3. **Methods**

3.1 **Culturing of microbial strain**

For culturing of microbial strain, first of all nutrient agar media was prepared. 3mM p-NPA was added in the nutrient media. Media was autoclaved and later on poured into petri-plates then wait until it gets solidified. After solidification of media the culture of *Corynebacterium flavescens* was streaked on the plates with the help of inoculation loop[18]. Streaked plates were kept on incubator for 24 hours at 37^0C. After incubation the growth was checked on the plates.

3.2 **Gram-Staining of culture**

To observe the difference between the gram +ve and gram –ve bacteria the Gram Staining method is performed. For this purpose, the smear of culture was prepared on the clean glass slide. After that, smear was fixed by using heat. After heat fixing of smear, crystal violet stain was added to the smear for 60 seconds then washed using water. After that, drop-wise gram's iodine was added for 60 seconds then washed with water. Smear was washed with ethanol for 15 seconds and then rinsed by using water. Later, safranin dye added to smear and kept for 60 seconds. After that, smear was washed. Glass slide was air dried and observed under microscope.

3.3 **Extraction of enzyme**

First of all, 50 ml of nutrient broth was prepared by mixing 0.65gm nutrient broth in 50 ml of distilled water. After mixing the broth was autoclaved and then loop-full culture of strain was inoculated in the broth with the help of inoculation loop. This is said as seed culture. Culture was placed in shaker incubator for 24 hours at 30^0C. After 24 hours turbidity was observed in seed culture. Production media was prepared by inoculating 2%V/V of seed culture. Media was kept in shaker incubator at 30^0C for 35 hours. After incubation production media was centrifuged at 8000 rpm at 4^0C for 10 min. After centrifugation the pellet was suspended in phosphate buffer (pH 7). Later suspension was sonicated using 35% amplitude for 10 minutes at 4^0C. After sonication, suspension was centrifuged at 8000 rpm for 20 min. at 4^0C. Supernatant collected said as crude enzyme extract [19]. After extraction, enzyme activity and protein concentration of crude enzyme were estimated. The methodology of extraction of crude enzyme from bacterial strain is strain in Fig. 3.3 A-E.

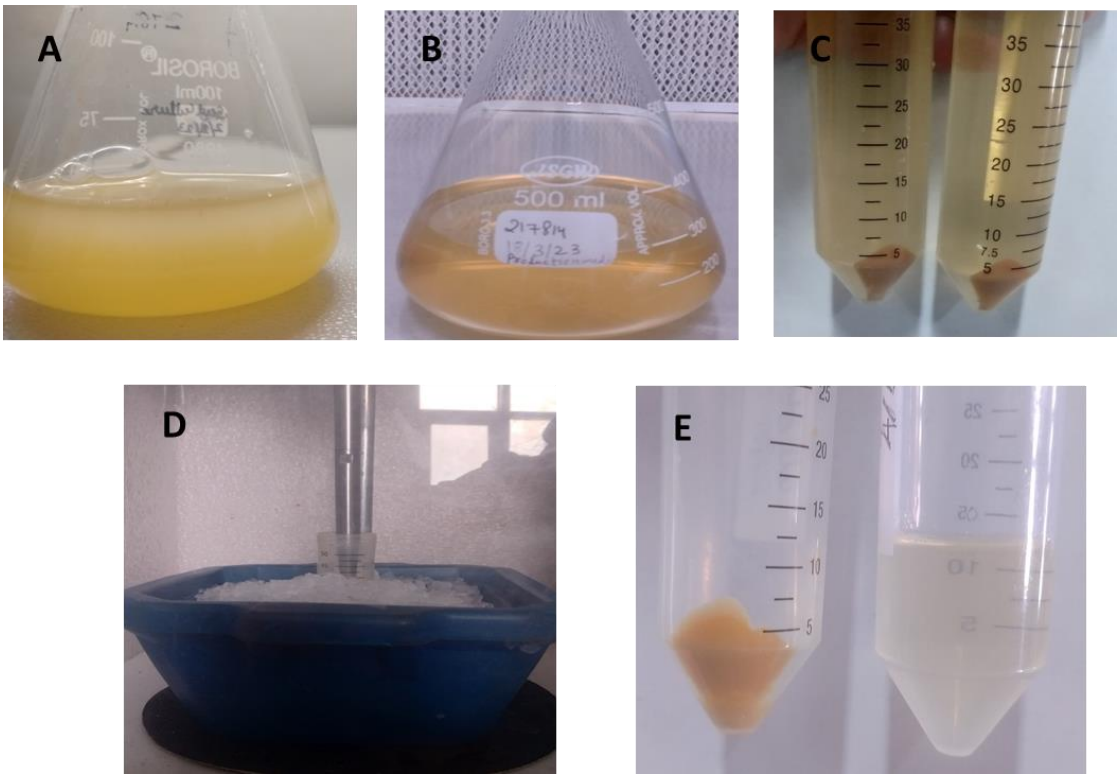

Fig. 3.3.A- seed culture, B- production media before inoculation of seed culture, C- After centrifugation, pellet observed in production media, D- Cell lysis via sonication, E- after centrifugation, separated pellet and supernatant

3.4 Determination of enzyme activity

Enzyme activity was calculated by release of 4-nitrophenol from p-nitrophenyl acetate. To determine the enzyme activity the standard curve of p-NP was prepared. For the preparation of standard curve 10 mM stock solution of p-NP was prepared by mixing p-NP in isopropanol. Different dilutions of p-NP were prepared and mixed in different volume of 50 mM phosphate buffer. The prepared reaction mixture incubated for 10 min. at 37^0C. After incubation O.D. of reaction mixture was measured at 410nm. Enzyme activity was determined by use of 825ul buffer, 175ul substrate and 25ul crude enzyme. Reaction mixture incubated for 5 minutes and absorbance recorded at 410 nm [19].

$$Enzyme\ activity\ = \frac{OD_T - OD_C \times value\ from\ graph \times reaction\ volume}{molecular\ weight\ of\ product \times incubation\ time \times volume\ of\ enzyme}$$

3.5 Determination of protein amount

Protein amount was measured by using Bradford method. In this method, 1mg/ml stock solution of BSA was prepared. After that different dilution of BSA was prepared. Bradford reagent was mixed into the dilutions. Absorbance of each sample was taken at 595nm by using spectrophotometer. Later on the graph was plotted between absorbance and concentrations of BSA. For protein estimation in extracted supernatant, 100ul of supernatant was added to 900ul of Bradford reagent. The sample was loaded in 96- well plate and absorbance recorded at 595 nm via spectrophotometer[20].

3.6 Synthesis of nano-hybrid systems

For the synthesis of Nano-hybrid system precipitation method was used. First of all, stock solutions of different protein concentrations (1mg/ml-10mg/ml) were prepared. 2 mM solution of $CuSO_4$ was prepared.100ul of Enzyme and copper sulphate added to 1x PBS solution. The reaction mixture was kept at 4^0C for 72 hours. Then mixture was centrifuged at 8000rpm for 10 minutes at 4^0C. Blue coloured pellet was observed. Pellet was washed with nuclease free water and dissolved in 500ul PBS solution. Characterisation of nanohybrid system was done[21].

3.7 Characterization of nanohybrid system

3.7.1 Determination of enzyme activity and protein concentration

For the determination of enzyme activity p-NPP was used as substrate. 10 mM stock solution of p-NPP was prepared by dissolving p-NPP in isopropanol. 175ul of p-NPP dissolved in 800ul 50mM phosphate buffer and then 25ul of enzyme was added in the reaction mixture. The mixture incubated at 37^0C for 10 minutes. After 10 minutes reaction mixture was loaded in 96-well plate and absorbance was recorded at 410nm [23].

For protein estimation the Bradford method was used. 100ul of sample was dissolved in 900ul of Bradford's reagent and then absorbance was recorded at 595nm.

3.7.2 Determination of immobilization yield and loading efficiency

Immobilization yield and loading efficiency were calculated by given formulas

$$Immobilization\ yield = \frac{A_0}{A_1} \times 100$$

Where A_0 denotes activity of immobilized and A_1 denotes activity of free enzyme.

$$Loading\ efficiency\ = \frac{B_{0\ -B_1}}{B_0} \times 100$$

Where B_0 denotes the total protein loaded and B_1 denotes the protein concentration in supernatant.

3.7.3 Biochemical characterization of lipase based nanohybrid system

3.7.3.1 Effect of temp. on lipase activity

The activity of immobilized enzyme was calculated on different temp. Para-nitrophenyl palmitate utilized as substrate for determination of enzyme activity. 10mM stock solution was prepared by dissolving p-NPP in isopropanol. 175ul substrate was mixed in the 800ul of 50 mM of phosphate buffer (pH 7). 25ul of immobilized enzyme was added to reaction mixture. Reaction mixture incubated for 10 minutes at different temperatures (25, 35, 45, 55, and 65). After incubation, reaction mixture was loaded in plate and absorbance was measured at 410 nm.

3.7.3.2 Effect of pH

The activity of nanohybrid system was determined by using different pH systems at 37^0C. Buffers of different pH were used. The pH ranges from 4-9. 175ul substrate was

mixed in the 800ul of 50 mM buffer of different pH. 25ul of immobilized enzyme was added to mixture. Reaction mixture incubated for 10 minutes at 37^0C. After incubation, reaction mixture was loaded in plate and absorbance was measured at 410 nm.

3.7.3.3 Effect of substrate concentration

The activity of nanohybrid system also determined by using varied concentration of substrate. The concentration of substrate varies from 10mM to 50mM. The reaction performed at 37^0C and 7 pH.

Chapter 4 Results and discussion

4.1 Culturing of microbial strain

The microbial strain of *Corynebacterium flavescens* was picked from master plate by using the inoculation loop and streaked on solidified nutrient agar media containing 3mM p-NPA. The plates were kept in incubator for 24 hours at 37^0C. After incubation growth was observed.

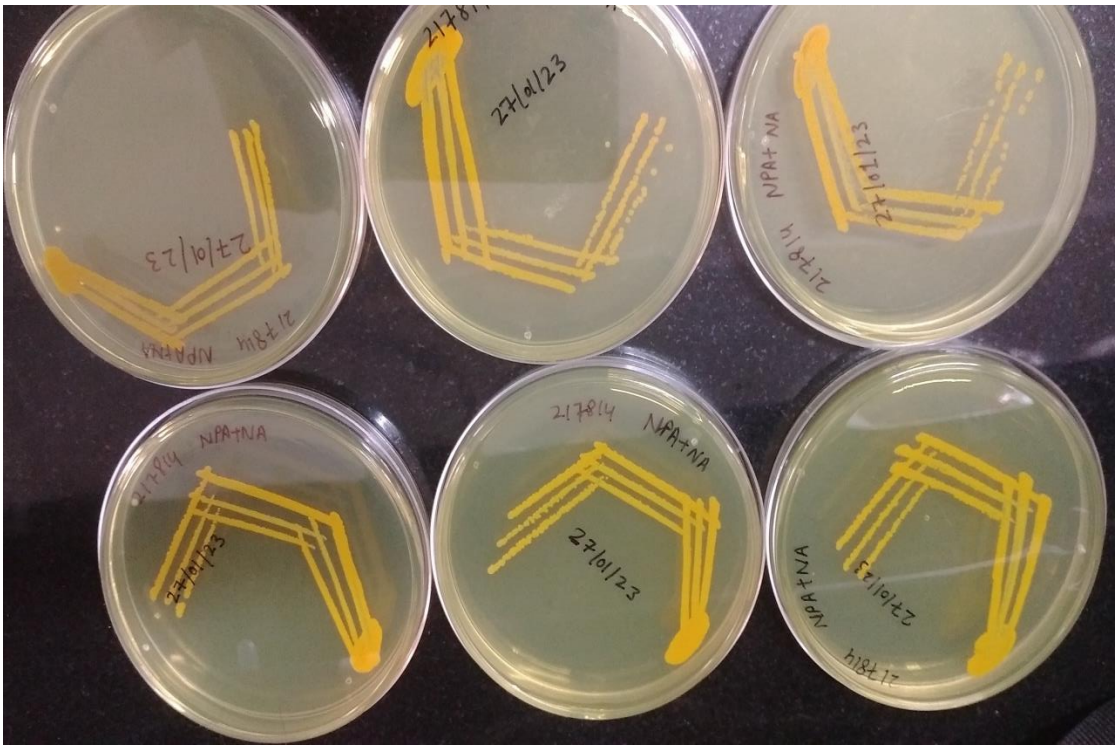

Fig. 4.1. Growth of *Corynebacterium flavescens* on nutrient agar plates

4.2 Gram staining of culture

Morphological features and the classification of bacteria were examined by gram staining of strain. The culture was mounted on the glass slide and treated with different stains. The slide was air dried and observed under microscope at 100X. Purple coloured and rod shaped organisms were observed. Purple colour indicates that the cultured strain is gram positive.

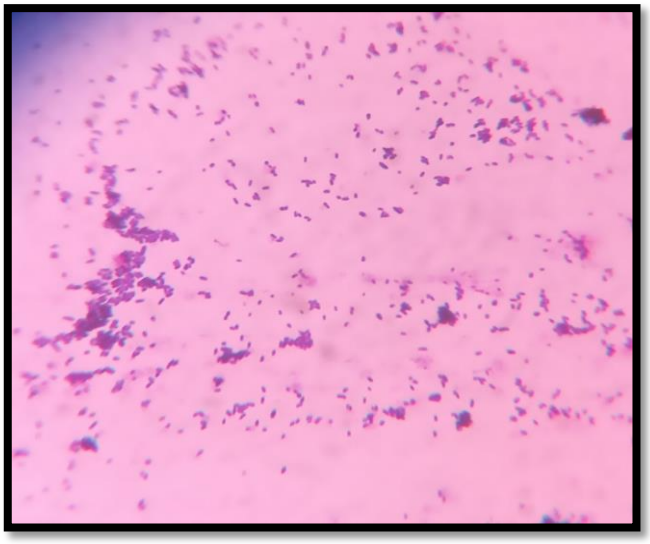

Fig. 4.2. Purple coloured and rod shaped organisms were observe at 100X under microscope

4.3 Extraction of crude enzyme

Crude enzyme was extracted and examined for the enzyme activity and concentration of protein in sample.

Table 4.3- enzyme activity and protein conc. of extracted enzyme :-

	Enzyme activity (uM/min./ml)	**Protein concentration (mg/ml)**
Sample 1	0.89	1.62
Sample 2	0.11	0.39

In sample 2 enzyme activity and protein concentration was less. The reason for this is cell lysis is not proper.

4.4 Synthesis of nanohybrid system

Nanohybrid system was synthesized by dissolving protein and copper salt in PBS. The mixture was kept for 72 hours. After incubation the reaction mixture centrifuged at 8000rpm for 10 minutes at 4^{o}C. After centrifugation blue coloured pellet was observed which is assumed as nanohybrid system. The pellet was washed with ultrapure water and dissolved in PBS. Estimation of different parameters were done.

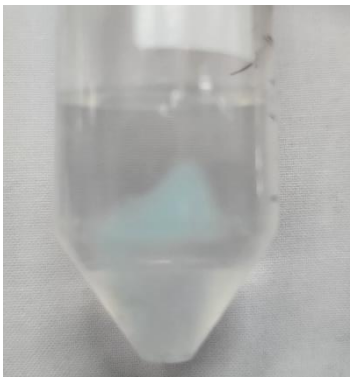

Fig.4.4: Blue coloured pellet observed after centrifugation.

4.5 Comparison of enzyme activity of free and immobilized enzyme

The activity of immobilized and free enzyme examined by using p-NPP as substrate. The reaction was done on optimum conditions.

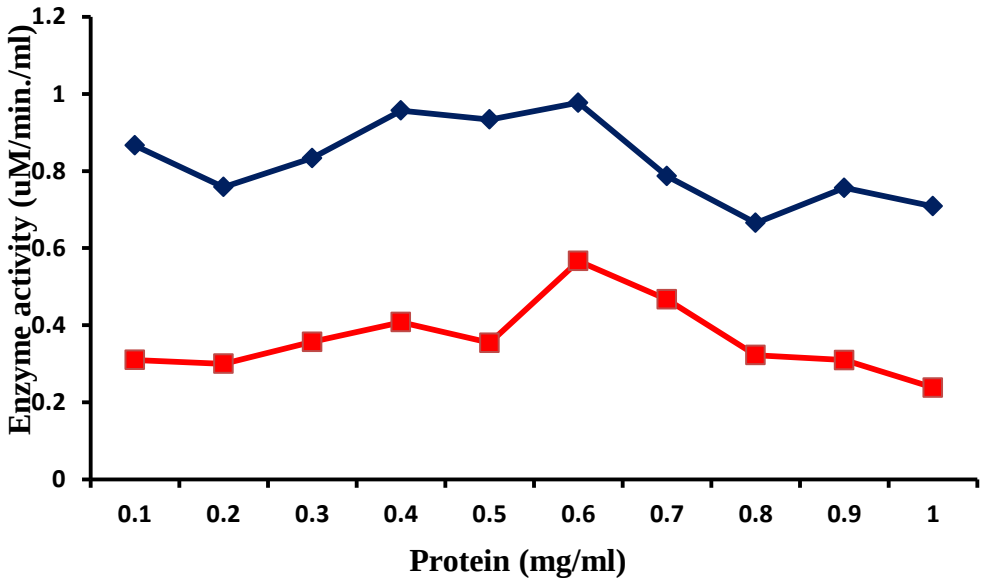

Fig.4.5. Activity of free and immobilized enzyme

The results depicted that immobilized enzyme has more enzyme activity when compared with free. The nanohybrid system which was synthesized by using 0.6 mg protein showed maximum activity as compared to other nanohybrid systems.

4.6 Effect of temperature

The activity of immobilized and free enzyme measured at different temperature ranges (25-65).

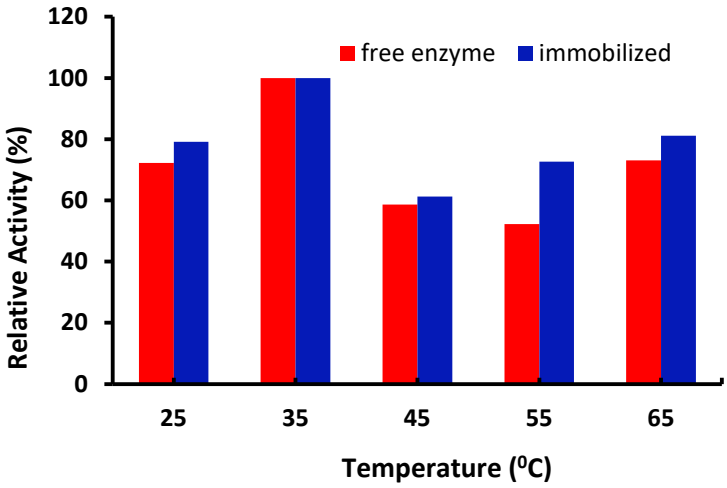

Fig.4.6. Graph showing relative activity of enzymes on different temperatures

The optimum temp. was 35^0C for both free as well as immobilized enzyme. The relative activity of immobilized at 25^0C was 79.09% and at 65^0C was 81.07%. The relative activity of free enzyme at 25^0C was 72.09% and at 65^0C was 73.03%. These results depicts that immobilized enzyme has higher activity as compared to free enzymes on different temperatures.

4.7 Effects of different pH

Enzyme activity was determined at different pH ranges which are 4-9.

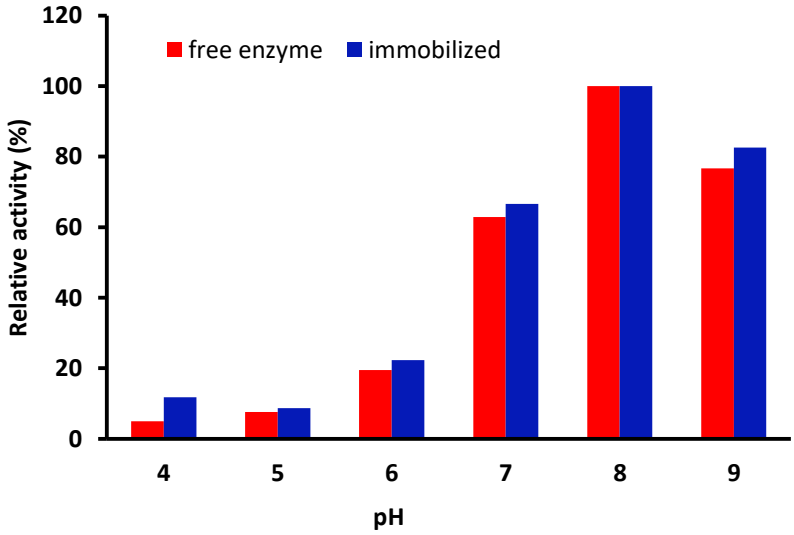

Fig.4.7. Graph showing the relative activity of enzymes at different pH

The optimum pH was 8 for both the immobilized and free enzyme. The relative activity of free and immobilized enzyme was 4.99% and 11.72% at pH 4 respectively. The relative activity at pH 9 was 76.61% of free enzyme and 82.567 of immobilized enzyme.

4.8 Effect of substrate concentration

For determination of the effect of substrate concentration on the activity of enzyme was measured by using different concentrations of substrate which vary from 10mM - 50mM.

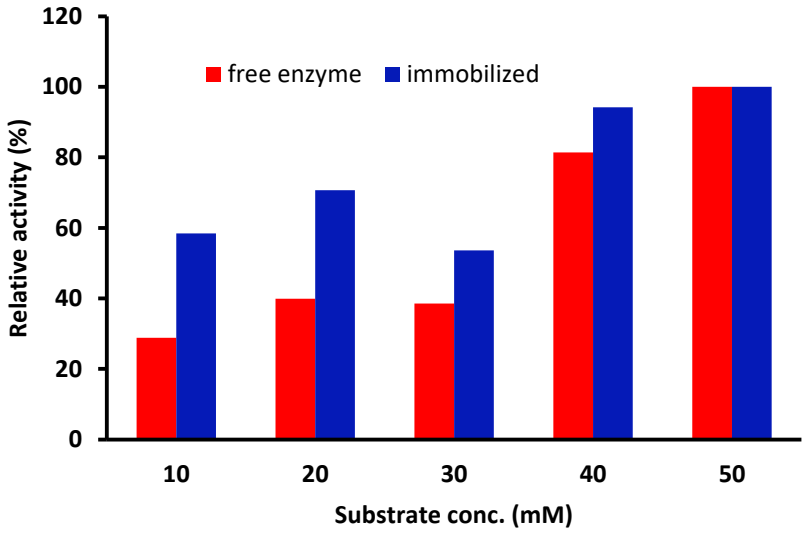

Fig.4.8. Graph showing the relative activity of enzymes on different substrate concentrations

Immobilized and free enzyme shows maximum activity at 50mM conc. The results showed that Immobilized enzymes hydrolysed substrate more readily as compared to free enzymes.

Chapter 5 Conclusion

Nanohybrid system was synthesized using different concentration of protein which vary from 0.1mg/ml to 1mg/ml. The different concentration of lipase enzyme was dissolved in PBS containing 2mM $CuSO_4$. The reaction mixture was kept at 4^0C for 72 hours after incubation the mixture was centrifuged and nanohybrid system was collected. The activity of nanohybrid system and free enzymes was calculated where the activity of nanohybrid system containg 0.6mg protein was highest. Later, the effect of different pH, temperature and substrate concentration on the enzyme activity of immobilized enzyme and free enzyme was examined. The optimum temperature and pH for nanohybrid system and free enzyme was 35^0C and 8 respectively. The activity was highest at 50 mM substrate concentration. The overall result indicated that the nanohybrid system has shown higher activity in comparison with free enzyme. So, the nanohybrid system can be utilized for the industrial applications.

Summary

Enzymes are biological catalysts which were utilized to increase the reaction rate. Enzymes are unstable at free state. They can't works well at harsh conditions. They lose their activity at harsh environmental conditions. To solve these problems immobilization of enzyme was used. In conventional method of immobilization the enzymes were immobilized on the solid support. The solid support has to be inert and has binding affinity towards enzyme. The conventional method of enzyme immobilization includes insoluble solid support. The solid support needs to be non-toxic, inert, easily accessible, insoluble, eco-friendly, and resistant to fungal and bacterial attack and has affinity to bind with the catalyst which is being utilized. Immobilization of enzymes has numerous utilities which include an increase in stability towards reaction conditions such as increase in pH and temperature, increased catalytic performance, and easy separation and recovery. The immobilization of enzyme increases its stability, activity. But in some cases the immobilization of enzyme leads to lose of activity of enzyme. The possible reason for this is change in confirmation of active site of enzyme. They also exhibit poor mass transfer which decreases the reaction rate. The solid support used for immobilization can cause product contamination which is also a thing of concern. To solve the problem caused by conventional methods the nanohybrid system comes to the picture. Nanohybrid systems were made up of the organic part and inorganic part. Organic part includes enzymes and inorganic part includes metal ions. These were synthesized by simple method like precipitation. Their synthesis process includes steps like nucleation, coordination and self assembly they took shape similar to flower hence named as nanoflowers. These nanohybrid systems shows higher enzyme activity as compared to free enzymes and there is no risk of product contamination. These can be reused and works well at extreme conditions of environment.

Lipase enzyme (EC 3.1.1.3) belongs to the triacylglycerol ester-hydrolase family. The range of molecular weight of lipases is 19-60 KDa. Lipase enzyme catalyzes various reactions such as inter-esterification, aminolysis, esterification, alcoholysis, and hydrolysis of triglycerides into fatty acids. Lipase enzyme has applications in various industries such as pharmaceuticals, biodiesel, drinks, foods, textile, leather, and detergents.

In this research we synthesized the nanohybrid system of lipase and copper metal ion. This system was synthesized by precipitation method this includes the incubation of reaction mixture for 72 hours. After synthesis the characterization of nanohybrid system was done.

We studied the effects of different temperatures and pH on the nanohybrid system and free enzymes.

The optimum pH and temperature for free enzyme and nanohybrid system was 8 and 35^0C respectively. The enzyme activity was highest at 50 mM substrate concentration. The overall result depicts that the immobilized enzyme has shown more activity in comparison with free enzyme. So, the nanohybrid system can be utilized for the industrial applications.

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Annexure

Standard curve of p-NP

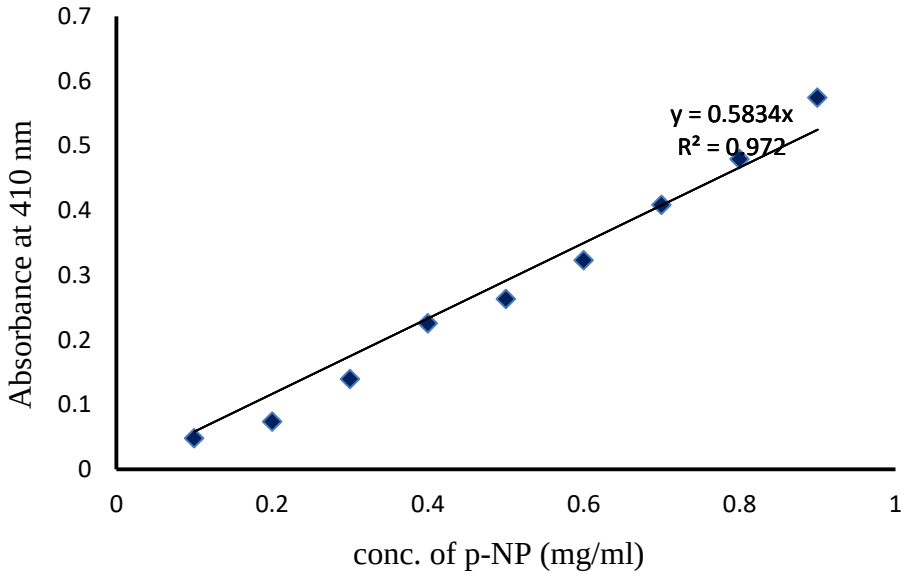

Fig.1 standard plot of p-NP (0.1-0.9 mg/ml)

Standard plot of BSA

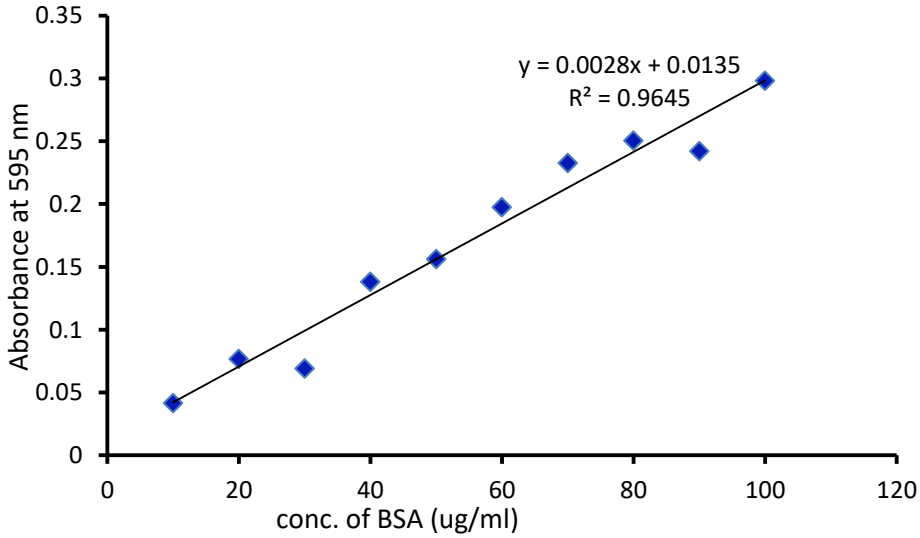

Fig.2 Standard plot of BSA (10-100ug/ml)