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# Wastewater Effluent Treatment using the Immobilized Glucose Oxidase from *Aspergillus niger*

**K B Sangavi[1*], M. Nirmala[2] and D Somasundaram**
**Department of Electronics and Communication, Sri Shakthi Institute of Engineering and Technology, Coimbatore, India.**

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***Corresponding Author Email:** sangavihere2c@gmail.com

## Abstract

Dwindling water resources is a global problem. Effective effluent treatment is an important step towards conserving our water resources. Some pollutants such as dyestuffs resist degradation by conventional treatment methods and persist in the environment. The paper describes the use of enzymes as an alternative method for treatment of such recalcitrant pollutants. It evaluates different methods in which enzymes can be delivered to the target effluent, including nanoparticles as delivery systems. It also emphasizes the need for current and future research to focus on developing economically feasible and environmentally sustainable wastewater treatment practices. An acid-producing filamentous fungus, Aspergillus Niger NB2, used throughout this study, was maintained on potato-dextrose agar slants and Yeast malt agar. This new glucose method offers an efficient process that preserves these sensitive molecules and is a step-change in analysis of madder dyed textiles as it can provide further information about dye preparation and dyeing processes that Current methods cannot. Dye is an integral part which is used to impart colour to materials. The waste generated during the process and operation of the dyes, contains the inorganic and organic contaminant leading to the hazard to ecosystem and biodiversity causing impact on the environment. The physico-chemical treatment does not remove the color and dye compound concentration. The decolorization of the dye takes place either by adsorption on the microbial biomass or and enzymatic degradation. Bioremediation takes place by anaerobic and/or aerobic process. In the present review the decolorization and degradation of dyes by fungi, algae, yeast and bacteria have been cited.

**Index terms:**
Water treatment, Aspergillus Niger, NB2, effluent treatment, glucose oxidase, Bioremediation.

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## I.INTRODUCTION

The limited availability of fresh water is a global crisis. The growing consumption of fresh water by anthropogenic activities has taken its toll on available water resources. Unfortunately, water bodies are still used as sinks for wastewater from domestic and industrial sources. However, in recent times, the need to replenish our water resources has been receiving increasing attention. This has led to the development of strategies to return water to its source in the least toxic form possible, to enable reutilization of water [1]. These strategies and processes may be collectively termed as 'wastewater treatment'. An anthropogenic activity that produces large volumes of concentrated effluent is the process of dyeing.

Whether applied to fabric, paper, pulp, leather etc., the processes involved in dyeing generate effluents that are rich in colorants. The presence of colorants in wastewater and eventually in receiving waters poses a threat to aquatic life forms [2]. The attempts to explore and evaluate the use of enzymes to degrade or decolorize the dyestuffs in effluents as an alternative to conventional treatment methods. For this water effluent process glucose oxidase enzyme is cultivated and implemented in the project.

The factors affecting decolorization and biodegradation of dye compounds such as pH, temperature, dye concentration, effects of carbondioxide and nitrogen, agitation, effect of dye structure, electron donor and enzymes involved in microbial decolorization of dyes have been also highlightened in the review.

The primary uses of A.niger are for the production of enzymes and organic acids by fermentation. A.niger is also used to produce organic acids such as citric acid and gluconic acid. Macroscopically, this fungus can be identified growing on substrates producing colonies of felt like yellow to white hyphae, turning black with the formation of conidia [5][6].

Microscopically, A.niger can be identified by its hyaline, septate hyphae. Asexual conidiophores can be identified by being long and globose at the tip, with what appears to be a hymenial layer of structures, each "ejecting" its own spore. Which can be identified by lactophenol cotton blue staining method, it will detect the spore formation of A.niger.

## II.METHODOLOGY

The Fungal Strain Aspergillus Niger (MTCC 181) was obtained from KSRCT microbial consortium, Department of Biotechnology, Tiruchengode, India. Basal Salt medium with slight modifications were used for the growth of fungus. Culture conditions like pH (3-5), Temperature (25-50 $^0$C), agitation speed (100-300 rpm) and incubation time (3-7 days) were optimized using one parameter variation at a Time (OVAT) approach for the improvement of growth of the culture and its enzyme production. Glucose oxidase (GOD) is the mainly regular enzyme utilized as the biological entity within glucose biosensors one obstacle: enzymatic reaction depends robustly on the concentration of dissolved oxygen like an electron acceptor.

After the purification of produced enzyme by dialysis method. The enzymatic activity was measured by the method described by Bergmeyer et al. (1970) by measuring the increase of absorbance at 436 nm due to the oxidation of guaiacol in the reaction mixture containing peroxidase. Enzyme unit is defined as the amount of the enzyme which liberate one micromole of hydrogen peroxide per minute under the experiment conditions.

The Enzyme which is produced after several process is then treated with Sodium Alginate. The liquid enzyme is transformed into jelly matter on reaction with sodium alginate. Then the jelly matter of enzyme is reacted with Calcium chloride which is responsible for formation of minute beads of enzyme.

The formed beads of enzyme are oven dried under an optimum temperature these beads are used on the dye, based on the methodology described by with modifications, the enzymatic oxidation reactions of the textile dyes were conducted with 1.2 mL Sodium acetatebuffer (pH 5.5) containing 0.4 mL H2O2, different concentration of Reactive Red dye, 1 bead of immobilized enzyme and 0.3mL of para phenylenediamine solution.

The final volume of reaction medium was 3.5 mL. The Enzyme beads of different Ammonium concentration is dropped into dye water (equal number of beads) to find the concentrated bead at which maximum and fast decolourization takes place is used for waste water treatment for more efficiency.

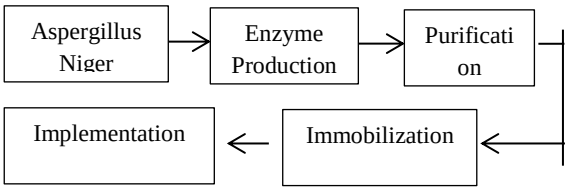


**Figure.1. Block Diagram of Waste Water Treatment Using Immobilized A.niger**

## III.ENZYME PRODUCTION

To produce GOD, we use aspergillus niger as organism and corn steep liquor as substrate. Glucose

oxidase production was optimized using an isolated strain of A.niger and an economical nutrient source, (CSL).

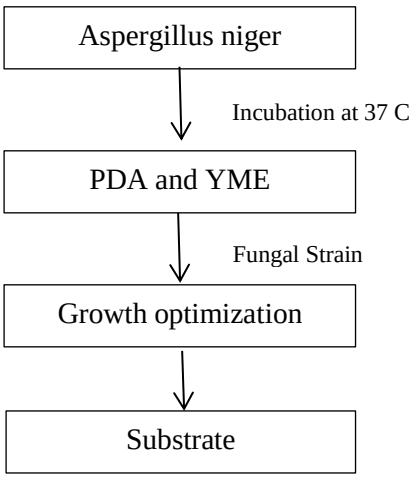


**Figure.2. Flow Diagram of A,niger Production**

Glucose oxidase (GOx), a flavoenzyme, from Aspergillus niger was produced, purified and immobilized for glucose oxidation. Maximum activities of 0.6 and 0.31U mg-1 fungus dry weight were obtained as intracellular and extracellular respectively. Optimal production (4000 IU/L) was achieved when basal salt medium was supplemented with sucrose (7.5%), peptone (1.5%) phosphorus (0.2%) and MgSO4 (0.2%) at pH 5.7 after 48 h at 250 rev. min–1. Enzyme purified by ammonium sulphate precipitation (75%), gel filtration, Q-Sepharose and DEAE Sepharoses has 30.08, 63.3% and 22.3-fold purification, % recovery and specific activity respectively. Enzyme was dimeric consisting of two equal subunits with molecular weight of 80 kDa. and displayed temperature and pH optima 25- 30°C and 5.5-6.0 respectively for the oxidation of-D-glucose. The enzyme was stable at 50 °C for 1 h without any prior stabilization. For the optimization of GOx production basal salt medium (BSM) with slight modification containing (g/l): (NH4)2HPO4 0.4, KH2PO4 0.2, NaNO3 2.0, KCl 0.5, MgSO4 0.2 and pH 5.5, was used for the optimization process parameters for maximum GOx production. All the growth experiments were carried out in 250 ml Erlenmeyer flask containing 50 ml of the medium. The sterilized medium (15 min at 15 psi/cm2) was inoculated with the germinated pre culture spores of 24 h age and incubated in orbital shaker (250 rev. min–1) at 300C. Different physical and chemical process parameters for regulation the enzyme production was optimized by classical methods of medium optimization. The mycelia mass was harvested by centrifugation at 7000x g for 20 min in a centrifuge fitted with fixed angle rotor. The supernatant constituted the extracellular GOx fraction and for the intracellular fraction 50 gm mycelial biomass (wet weight) was suspended in 10 ml sodium citrate buffer pH 5.75(50mM). Liquid nitrogen was added on the fungal biomass and after evaporation of nitrogen, biomass was crushed to powder in buffer. The clear supernatant of the broken cell suspension constituted the intracellular GOx fraction.

**IV.PURIFICATION OF ENZYME**

The various processes used for the actual recovery of useful products from fermentation or any other industrial process together are known as downstream processing. Conventional use of dialysis bags involves the removal of unwanted low molecular weight solute from the sample and replacement with buffer present in the dialysate. High concentrations of salt or organic solvent in the sample cause water to enter the bag before the salt leaves consequently, there is an increase in volume during the early stages if the solute concentration is high. Dialysis bag can be made by following steps,

1) Take 8-10cm of dialysis membrane and boil it in 100ml of distilled water for 10min with slow stirring.
2) Decanted the membrane from boiled water and placed it in 100ml of solution containing 2% sodium carbonate (boiling).
3) After ten minutes with sodium carbonate discard the water
4) Then pour fresh 100ml distilled water and boil it for 10mints. Allow it to cool.
5) Take the membrane, tied with rubber tightly on one side of the membrane then pour the pellet which was dissolved in Tris HCL and prepare the dialysis tube.
6) The dialysis tube was kept overnight with distilled water. For every one hour the buffer is changed.
7) After dialysis, protein solution was centrifuged 6000rpm to remove large molecule.

**Figure.3. Dialysis Bag**

Ammonium sulphate instead takes up the water molecules around the protein exposing hydrophobic sites on the protein. Because hydrophobic groups tend to prefer to be together, the proteins will

aggregate and thus come out solution. Ammonium sulphate is not the only salt that can be used, but it is the cheapest. Desired protein precipitation occurs in the percentage saturation of ammonium sulphate. Culture liquid from two different production media are centrifuged (6000rpm for 10mints) to remove the cells. After the centrifugation of crude extract or filtrate is subjected to ammonium sulphate precipitation. The filtrate is taken and 40% ammonium sulphate was added slowly to the supernatant. While adding ammonium sulphate the culture was kept in ice cubes because it is an exothermic reaction. Then the mixture was incubated for overnight in refrigerator at 4oc. next day the mixture was centrifuged at 12000rpm for 10min. the pellet was collected and dissolved in 10mM Tris HCL.

Ion-Exchange chromatography is a method used to purify individual chemical compounds from mixtures of compounds. The classical preparative chromatography column is a glass tube with a diameter from 5 mm to 50 mm and a height of 5 cm to 1 m with a tap and some kind of a filter (a glass frit or glass wool plug – to prevent the loss of the stationary phase) at the bottom. Two methods are generally used to prepare a column: the dry method and the wet method.

1) For the dry method, the column is first filled with dry stationary phase powder, followed by the addition of mobile phase, which is flushed through the column until it is completely wet, and from this point is never allowed to run dry.

2) For the wet method, a slurry is prepared of the eluent with the stationary phase powder and then carefully poured into the column. Care must be taken to avoid air bubbles.

A solution of the organic material is pipetted on top of the stationary phase. This layer is usually topped with a small layer of sand or with cotton or glass wool to protect the shape of the organic layer from the velocity of newly added eluent. Eluent is slowly passed through the column to advance the organic material. Often a spherical eluent reservoir or an eluent-filled and stoppered separating funnel is put on top of the column.

At the end of the incubation period, the mold biomass was harvested by filtration through Whatmann No. 1 filter paper, washed by potassium phosphate buffer (0.1 M, pH 6) and suspended in the same buffer (75 g biomass + 25 ml buffer), The suspension was freezed at 15 C° for 24 hours and then subjected to sonication at 10 KHz to rupture the cellular walls, Cell-free extract was obtained by centrifugation at 9000×g for 10 minutes using a refrigerated centrifuge. The enzyme was then precipitated by adding cold acetone (1 – 2 volumes) with gentle stirring for 1 hour. The mixture was centrifuged at 10000 rpm for 10 minutes and the precipitate was suspended in a small amount of phosphate buffer and applied on the top of a column (1.6 × 65 cm) of Sephadex G-150 that had been equilibrated with phosphate buffer (0.05 M, pH 6). The column was then eluted with the same buffer at a flow rate of 12 ml / hour. Enzyme contained fractions of 2 ml each were pooled and applied to the top of a column (1.6 × 25 cm) of DEAEcellulose equilibrated with the same buffer. The column was washed with a linear NaCl gradient of 0 – 0.7 M in phosphate buffer at a flow rate of 24 ml / hour. Active fractions of 3 ml each were pooled.

SDS-PAGE, with full name of sodium dodecyl sulfate polyacrylamide gel electrophores, is the most widely used technique to separate proteins from complicated samples of mixture, plays key roles in molecular biology and wide range of subfield of biological research. Being present an electricity, proteins migrate towards the negative anode inside the poly-acrylamide gel under denaturing conditions. In SDS-PAGE, the detergent SDS and a heating step determine that the electrophoretic mobility of a single kind of protein is only affected by its molecular weight in the porous acrylamide gel. This process has two stages, Stacking & Separating. After this process, stop SDS-PAGE running when the down most signs of the protein marker (if no visible sign, inquire the manufacturer) almost reaches the foot line of the glass plate. Generally, about 1 hour for a 120V voltage and a 12% separating gel. For a separating gel possessing higher percentage of acylamide, the time will be longer. The complete sample of purified enzyme will be a product at the end of this process.

**V.IMMOBILIZATION**

An immobilized enzyme is an enzyme that is attached to an inert, insoluble material such as calcium alginate (produced by reacting a mixture of sodium alginate solution and enzyme solution with calcium chloride). This can provide increased resistance to changes in conditions such as pH or temperature. It also allows enzymes to be held in place throughout the reaction, following which they are easily separated from the products and may be used again - a far more efficient process and so is widely used in industry for enzyme catalyzed reactions. An alternative to enzyme immobilization is whole cell immobilization.

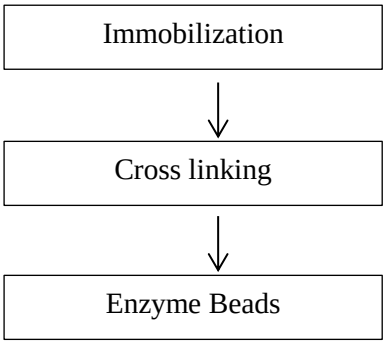


**Figure.4. Immobilization Process**

The functionality of enzymes depends largely on their conformation. Harsh reaction conditions like extreme temperature, very high or low pH, high ionic strength, high concentrations of reactants, and presence of inhibitors can alter the conformation of an enzyme. Enzymes may not function optimally under such drastic conditions which are often encountered in effluent streams. Immobilization methods that increase the reusability of enzymes by preventing the loss of enzyme during the course of the reaction and minimizing the loss of activity of enzymes under harsh treatment conditions have been developed. An enzyme is said to be immobilized when it is physically confined to a certain region of space, retaining its catalytic activity and the capacity to be used repeatedly or continuously. The use of immobilized enzymes in effluent treatment has many important advantages over the use of free enzymes including increased stability, localization, ease of handling, reusability and a consequent decrease in running cost.

The immobilized enzyme has proved to be an adaptable molecule that can be used in the form of a cell-free crude extract or in an immobilized form entrapped in calcium alginate capsules at a laboratory scale. Fungal laccase immobilized using γ-aluminium oxide pellets, has been reported to decolorize solutions of azo dyes like Ponceau Red (65% decolorization), anthracinoid dyes like lanaset Blue 2R (100% decolorization) and a triphenyl methane dye like crystal violet (98% decolorization) after 48 hours. However, not all enzymes are amenable to immobilization. Some of the methods of immobilization such as adsorption, covalent binding and chemical coupling can adversely affect the catalytic activity of certain enzymes. Adsorption is a widely used immobilization method which is preferred for its simplicity and ease of regeneration. But it has also been found that an immobilized enzyme that adsorbs too strongly to the supporting material may show a loss of functionality. Enzymes immobilized by this method can operate in a relatively narrow range of pH, temperature and ionic strength. Drastic changes in reaction conditions cause desorption of the enzyme from the support material. Covalent binding of enzymes on to a support or matrix may modify the conformation of the enzyme. Considering that the functionality of enzymes depends largely on their conformation, such a change can result in the loss of enzymatic activity. It has been shown that enzymes immobilized by covalent binding can retain their activity more effectively if they are immobilized in the presence of their substrate or a competitive inhibitor since the active site remains protected from conformational changes in the presence of a substrate or its structural analogue. Similarly, the entrapment and encapsulation of enzymes has certain advantages such as large surface area for substrate-enzyme interaction in a relatively small volume. However, the drawbacks of this method are that a high concentration of enzyme is required and that there is occasional inactivation of the enzyme on entrapment. Additionally, the pore size of the (cross linked) polymer has to be very small to retain the enzyme within the capsule. Enzyme entrapped in calcium alginate gel showed lower decolorization (52%) of the azo dye direct yellow than the free enzyme (69%).Immobilization procedures need to be optimized to minimize the loss of enzyme activity and achieve maximum reusability. This method of enzyme delivery holds great potential for the continuous treatment of large volumes of effluent.

**VI.IMPLEMENTATION**

Glucose oxidase (GOD), a flavoenzyme, from Aspergillus Niger was produced, purified and immobilized for glucose oxidation. Maximum activities of 0.6 and 0.31U mg-1 fungus dry weight were obtained as intracellular and extracellular respectively. Optimal production (4000 IU/L) was achieved when basal salt medium was supplemented with sucrose (7.5%), peptone (1.5%) phosphorus (0.2%) and MgSO4 (0.2%) at pH 5.7 after 48 h at 250 rev. min–1. Enzyme was purified by ammonium sulphate precipitation (75%), gel filtration (30.08), Q-Sepharose (63.3%) and DEAE Sepharoses (22.3), specific activity respectively. Enzyme was dimeric consisting of two equal subunits with molecular weight of 80 kDa and displayed temperature and pH optima 25- 30°C and 5.5-6.0 respectively for the oxidation of-D-glucose. The enzyme was stable at 50 °C for 1 h without any prior stabilization. For the optimization of GOD production, basal salt medium

(BSM) with slight modification containing (g/l): (NH4)2HPO4 0.4, KH2PO4 0.2, NaNO3 2.0, KCl 0.5, MgSO4 0.2 and pH 5.5, was used for the optimization process as parameters.

All the growth experiments were carried out in 250 ml Erlenmeyer flask containing 50 ml of the medium. The sterilized medium (15 min at 15 psi/$cm^2$) was inoculated with the germinated pre culture spores of 24 h age and incubated in orbital shaker (250 rev. $min^{-1}$) at 300C.

The mycelia mass was harvested by centrifugation at 7000x g for 20 min in a centrifuge fitted with fixed angle rotor. The supernatant constitutes the extracellular GOD fraction and for the intracellular fraction 50 gm mycelial biomass (wet weight) was suspended in 10 ml sodium citrate buffer pH 5.75(50mM). Liquid nitrogen was added on the fungal biomass and after evaporation of nitrogen, biomass was crushed to powder in buffer. The clear supernatant of the broken cell suspension constitutes the intracellular GOD fraction.

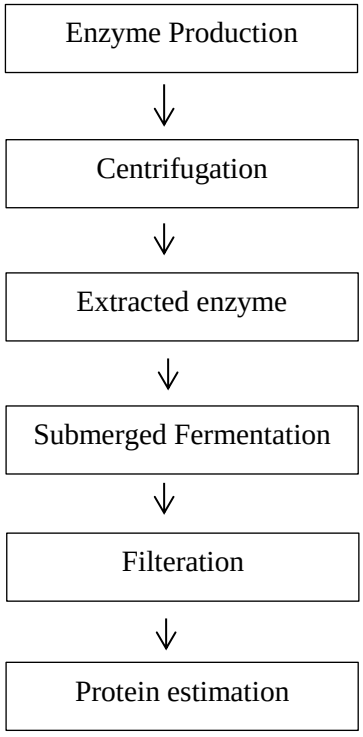


**Figure.5 Flow chart for Enzyme Production**

Centrifugation is a process which involves the application of the centrifugal force for the sedimentation of heterogeneous mixtures with a centrifuge, and is used in industrial and laboratory settings. This process is used to separate two miscible substances, but also to analyze the hydrodynamic properties of macromolecules. More-dense components of the mixture migrate away from the axis of the centrifuge, while less-dense components of the mixture migrate towards the axis. Chemists and biologists may increase the effective gravitational force on a test tube which causes the precipitate (pellet) to gather on the bottom of the tube more rapidly and completely.

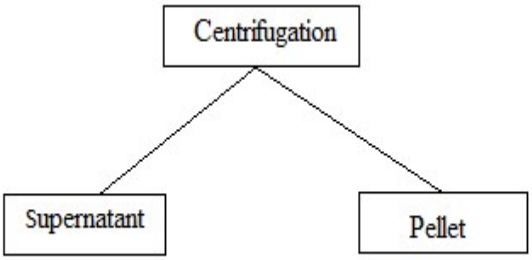


**Figure.6. Centrifugation byproducts**

Entrapment is caging of enzymes by covalent or non-covalent bonds within gels or fibers. Efficient encapsulation has been achieved with alginate–gelatin–calcium hybrid carriers that prevent enzyme leakage and provide increased mechanical stability. Entrapment by nanostructured supports like electrospun nanofibers and pristine materials have revolutionalized the world of enzyme immobilization with their wide-ranging applications in the field of fine chemistry, biomedicine biosensors and biofuels. Prevention of friability, leaching and augmentation of entrapment the enzyme activity by Candida rugosa*lipase* entrapped in chitosan have been reported. This support has also been reported to be non-toxic, biocompatible and amenable to chemical modification and highly affinitive to protein due to its hydrophilic nature. Here Entrapment method is used to immobilize the GOD enzyme for better sensitivity and accuracy in the results. Immobilization plays an efficient role in whole process, making the enzyme to be held in place throughout the reaction, following which they are easily separated from the products and may be used again. Immobilization procedures need to be optimized to minimize the loss of enzyme activity and achieve maximum reusability. This method of enzyme delivery holds great potential for the continuous treatment of large volumes of effluent.

**Figure.7. Immobilized Enzyme**

**VII.RESULT**

An Optimum pH, Temperature, Agitation Speed and Incubation time is set to have a good enzyme growth.

1. pH is a determination factor in the expression of enzymatic activity as it alters the ionization state of amino side chains or the ionization of the substrate. pH of about 5.5 is maintained for good enzyme growth.

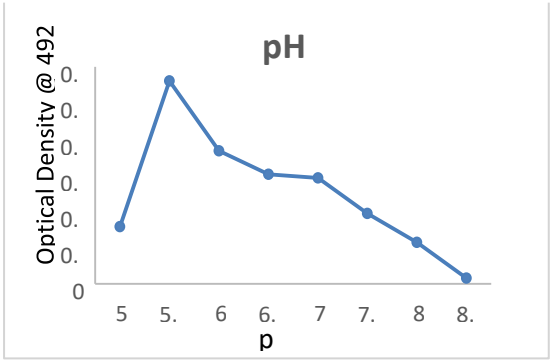


**Figure.8. Effect of pH on growth**

2. The enzyme activity against temperature depends on the source of the enzyme as well as on the assay conditions, particularly pH and substrate. An optimum Temperature was found to be 30° C.

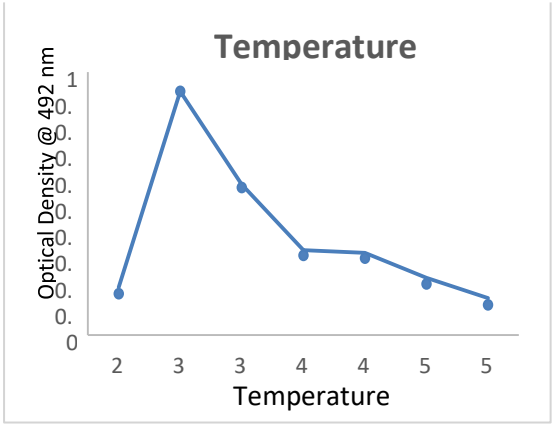


**Figure.9. Effect of Temperature on growth**

3. Agitation Speed of 150 rpm is maintained

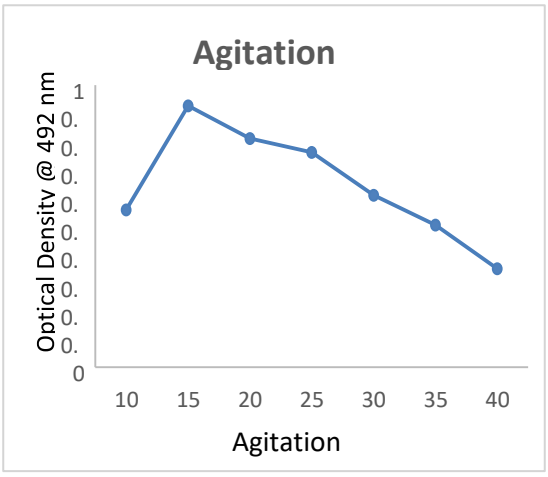


**Figure.10. Effect of Agitation speed on growth**

Based on the methodology described with modifications, the enzymatic oxidation reactions of the textile dyes were conducted with 1.2 ml Sodium acetate buffer (pH 5.5) containing 0.4 ml $H_2O_2$, different concentration of Reactive Red dye, 1 bead of immobilized enzyme and 0.3ml of paraphenylene diamine solution. The final volume of reaction medium was 3.5 ml.

The plant enzymatic extract is considered most appropriate for use in the oxidation of the dyes, presenting higher enzymatic activity. The reaction mixture was incubated in a spectrophotometer and the absorbance of the dye was measured at different times during the experiments. Monitoring of the oxidation was done at 518 nm, the maximum wavelength for Remazol Reactive Red dye.

**Figure.11. Decolorized dye water after five days**

## VIII.CONCLUSION

The Glucose oxidase enzyme was isolated from Aspergillus Niger was dialyzed and purified using Ion exchange chromatography The color removal pattern with free and immobilized GOD was found to be significantly different. The waste waters from textile industries are mostly contains the higher amount of dyes, aliphatic and aromatic polymeric substances and polyphenols. These dye molecules are often toxic and hard to degrade in the conventional waste water treatment. This immobilized GO has ability to decolourize Reactive red dye. This result has shown the GO enzyme possess the ability to decolourize the dyes. This immobilized GO finds application in many industries. All tested dyes were more efficiently decolorized by immobilized GO as compared to its free counterpart. Immobilization prevents enzyme washouts and allows a high enzyme concentration to be maintained in a continuous reactor. Since the catalytic stability is often improved by immobilization, enzymes may degrade a higher concentration of toxic compounds then their free counterpart. By degrading these toxic compounds from dye water it can be reused for Plantations, Agriculture etc.

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