Production and Purification of Glucose Oxidase (GOD) from Pseudomonas sp. in Different Mutagenic Environment

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***Abstract*- The enzyme Glucose oxidase has significant application in therapeutics, agriculture and food industries as well as a growing impact on the bakery industry. Commonly glucose oxidase production is done from fungal source, but we made an attempt to produce this commercially important enzyme from bacterial species, isolated from soil samples which have been collected from different regions from Bangalore, India; It was identified as Pseudomonas sp. by various morphological characteristics and biochemical tests, and ken its optimum culture conditions for production as there is a little work has done on it. In general, the optimization showed the increase in enzyme activity on the basis of the minimally required incubation period of 24 hours at 40°C in alkaline pH level 10. A strain improvement was done through physical and chemical mutations to optimize the production of glucose oxidase as then proceeds to be purified through several processes and thereafter up to expectation molecular weight of the enzyme was set up by SDS-PAGE on comparison with the protein molecular weight marker.**

Index Terms- Biochemical test and staining, salt precipitation, dialysis, de-osmosis ion- exchange chromatography, gel filtration method, SDS-PAGE.

I. INTRODUCTION

The Glucose Oxidase (GOD) enzyme, also known as notatin was first isolated by Detlev Müller from Aspergillus niger. Glucose oxidase is also produced by Penicillium amagasakiensis and present in honey as a natural preservative. It is an oxidoreductase that catalyzes the oxidation of β–D-glucose to D-glucono-δ-lactone and hydrogen peroxide and highly specific for β–D-glucose and does not act on α–D-glucose. Though a prime use of glucose oxidase has been in the determination of free glucose in body fluids, food, and agricultural products, it has been gaining attention in the bakery industry because of its oxidizing effects result in the stronger dough, replacing oxidants such as bromate and L-ascorbic acid. Additional usage of GOD includes the removal of oxygen from food packaging and removal of D-glucose from egg white to prevent browning. It is a dimeric protein that act outside of cells and covered with carbohydrate chains. Growing multitude of different uses in the industries Glucose oxidase has become a commercially important in the last few years that often used in solution phase reactions as well as being immobilized on "dip-sticks" and electrodes. Glucose oxidase is used as an antioxidant in food industries, plays a key role in gluconic acid production and also in clinical analysis. GOD, coupled with a peroxidase, can be used for a color identification test. Such as the disposable glucose sensor strips use by diabetics to monitor serum glucose levels. It is also used as a chemical sensor (oxygen sensor) in oxygen electrodes and hydrogen peroxide electrodes.

Spectrometric assays allow the control of glucose levels in fermentation, bioreactors, and to manage glucose in vegetal raw material and food products. To prevent the alteration in the taste of wine occurs during its long-term storage due to the presence of oxygen, glucose oxidase can be used for the removal of oxygen from wine. For the reason, hydrogen peroxide is a reaction product of glucose oxidase that can act as an oxidizing agent. Enzyme is use in bread making applications to allow the promotion of disulfide bond formation in gluten.

Moreover, it is possible to prepare a natural and potent antibacterial system by combining glucose oxidase with lacto-peroxidase.

II.LITERATURE REVIEW

Arima K. et al. [1], presented that microbial enzymes are in great demand owing to their importance in several industries such as brewing, baking, leather, laundry detergent, dairy, starch processing and textiles besides pharmaceuticals. About 80% of the enzymes produced through fermentation and sold in the industrial scale are hydrolytic enzyme.

Della Pina et al. [2], proposed that extracellular glucose oxidase was examined for various microorganisms and it was found in strains belonging to genus Penicillium except one species of Tallalomyces. The most common sources of GOD were A.niger, Penicillium notatum, Alternaria alternate. Penicillium purogenumand,

Danyk HC et al. [3], described that enzymatic glucose biosensors use an electrode instead of O2 to take up the electrons needed to oxidize glucose and produce an electronic current in proportion to glucose concentration. It is a technology behind the disposable glucose sensor strips used by diabetics to monitor serum glucose levels.

H. Tang et al. [4] told that in food industries, glucose oxidase is used to remove the oxygen from beverages, powdered eggs and as a source of hydrogen peroxide in food preservation. It has been observed that glucose oxidase-glucose system shows an anti-bacterial effect in food poisoning organisms

Johansson et al. [5], introduced One of the first application of thermal monitoring for glucose determination. Glucose oxidase has found applications in the textile industry as a method for producing hydrogen peroxide for bleaching. Covalently immobilized glucose oxidase on alumina and glass supports results in higher recoveries.

Julio Raba et al. [6], proposed review discusses in detail enzyme structure, bio-catalysis, enzymes as analytical reagents, properties of glucose oxidase (including a historical account), and the use of glucose oxidase as a reagent in homogeneous systems as well as it is a very important immobilized reagent.

R.L. Kelley et al. [7], studied that glucose oxidase was purified to electrophoretic homogeneity by a combination of ion-exchange and molecular chromatography. This enzyme is a flavor-protein with a native molecular weight of 1lac and a denatured molecular weight of 8 thousand. This enzyme does not come as a glycoprotein. It gives optimal activity in D-glucose, which is oxidized to D-gluconate. The enzyme's optimum pH range is 4 to 5.

Traeger et al. [8] achieved an enhanced endocellular GOD activity by aerating the reactor with a mixture of pure oxygen and air.

Visser et al. [9] presented that GOD is induced by high levels of oxygen and by a high glucose concentration.

Zetelaki et al. [10], proposed that the role of aeration and agitation on the production of GOD in a submerged culture of A.niger has been reported. The study involved the effect of aeration on respiration, metabolism and GOD synthesis.

III. MATERIALS AND METHOD

There are several methods has used to isolation, collection, production and purification. It has described below-

A. Collection of soil sample-

Five different soil samples were collected from different places in Bangalore, India. Soil samples were collected from Madivala lake-side, Parle G company side area, near market areas, Recipharmaceutical Pvt. Ltd company wastage area. The soil samples were taken in the sterile biodegradable plastics d in a sterile pouch using a spatula from the soil surface (5 cm) at a depth of approximately 20cm and were then taken to a lab in order to carry out the isolation of organisms.

B. Isolation of microorganisms by Pour Plate Method-

For isolation of organism by pour plate method in the process, prepared 10ml of 1% NaCl was autoclaved and cooled. 1g of soil sample was dispersed in the saline solution. Take 10ml saline in each test tubes and autoclaved for 15 min with 15lbs at 121℃ with a cotton plug. After cool, 1g soil samples were added in each test tube and mixed properly used as inoculums. In LAF, Poured 100µl of inoculums from each test tube to Petri-plates by the help of micropipette and spread them. Then poured

autoclaved M9 media and allowed for solidification and incubated for 24-48 hr at 35°C.

C. Selection of colonies-

After incubation bacterial colonies were observed on the media, single colony of an organism selected based on their morphological character sub-cultured on LB agar media. In this process after autoclaving, poured agar in plates and allowed it for solidification. Sub-culturing of bacteria on agar plates was done by taking culture on the sterilized loop from previous day Petri-plates by streaking in a zigzag manner. Incubate for 24 hr at 35°C.

D. Making a pure culture-

After getting the highest value from enzyme assay activity, streaked that selected organism on the plate. Incubate 24hr at 35°C. After incubation, made a slant of an organism and incubated for 24 hr at 35°C.

E. Identification by morphological and biochemical test-

The morphological identification and characterization of isolates take place through gram's staining. The isolates which obtained were biochemically analyzed for the activities of oxidase, catalase, MR-VP, casein hydrolysis test, urease test, carbohydrates (glucose, sucrose, lactose, mannitol), fermentation test, triple sugar iron test, and nitrate reduction test.

F. Strain improvement for production of glucose oxidase-

To optimize the production of glucose oxidase by improving strain were done through physical and chemical mutation. These mutations are mainly two type physical mutation and chemical mutation.

Physical Mutation- To improve the strain for maximum production of GOD, physical mutation was done in two ways. First one was UV mutation and the second one was an x-ray. Chemical Mutation- To improve the strain for the maximum production of the enzyme, the chemical mutation was carried out by the ethidium bromide mutation.

G. Time-Wise Mutation-

The optimization of time-wise mutation for GOD enzyme was done at five different times i.e; 5min,10min,15min,20min,25min with one control. Prepare 6 autoclaved Petri-plates and 2ml of autoclaved saline. Taken 1ml of saline in Eppendorf tube and inoculate a loop of pure culture. Poured the LB agar and solidified them. Poured 100µl of saline and organism mixture on each solidified agar plates .Then swabbed them with the help of cotton swab. After that, ON the UV and blower of LAF and exposed all the plates under UV light except control according to times mentioned above. Incubate for 24 hrs at 40°C. Autoclaved 12 test tubes and 100ml LB agar and make a slant and leave for solidification. Streaking was done on the slants from mutated plates (2 test tubes for 1 plate). Incubated for 24 hrs at 40°C.Make the 25ml M9 broth for each 6 test tubes and autoclaved them. Inoculated the organism from test tube to broth after completely cool down of broth. Incubate them for 24 hrs at 40°C.Analyzed the growth through enzyme assay.

H. Height wise Mutation-

The optimization of height wise mutation for GOD enzyme were done at 30m near to UV at different times i.e;5,10,15,20,25 minutes with one control. Prepare 6 auto-calved Petri-plates and 2ml of autoclaved saline. Took 1ml of saline in Eppendorf tube and inoculate a loop of pure culture. Poured the LB agar and solidified them. After solidification, poured 100µl of saline and organism mixture on each solidified agar plates .Then swabbed them with the help of cotton swab. After that ON the UV and blower of LAF and exposed all the plates under UV light which is 30m near to UV light except control according to times mentioned above. Incubate for 24 hrs at 40°C. Autoclaved 12 test tubes and 100ml LB agar and poured them in all the test tubes and make a slant. Streak on the slants from mutate plates (2 test tubes for 1 plate). Incubated for 24 hrs at 40°C.Made 25ml M9 broth for each 6 conical flask and autoclaved them. Inoculated the inoculums from test tube to broth after completely cool down of broth. Incubate them for 24 hrs at 40°C. The growth was analyzed through enzyme assay.

I. X-Ray Mutation-

The optimization of x-ray mutation for GOD enzyme were done by exposing the organism contain plates under x-ray for different intervals i.e.; 30sec,60sec,and 90sec. Prepared 3 autoclaved Petri-plates and 2ml of autoclaved saline. Took 1ml of saline in eppendorf tube and inoculate a loop of pure culture. Poured the LB agar and solidified them.

After that poured 100µl of saline and organism mixture on each solidified agar plates. Then swab them with the help of cotton. Keep that under x-ray for above mentioned time interval. After that, incubate that for 24 hr at 40°C. Autoclaved 6 test tubes and 100ml LB agar. Poured them in all the test tubes and make a slant and let them for solidification. Do streaking on the slants from mutated plates (2 test tubes for 1 plate). Incubated for 24 hr. Made the each 25ml M9 broth for 6 test tubes And autoclaved them. Inoculated the organism from test tube to broth after completely cool down of broth. Incubate them for 24 hr at 40°C. The growth was analyzed in plates.

J. Ethidium Bromide Mutation-

10ml of LB broth and tubes were autoclaved.1ml of broth was taken in each tubes under LAF. To this, a loop of an organism from slant was inoculated. EtBr was added in each tube as 10, 20,30,40,50 and 60µl except one tube which is considered as control. After this, incubate for 24hrs at 35ºC. The growth was analyzed through enzyme assay.

K. Production, purification and characterization of glucose oxidase-

Production- The production of glucose oxidase was carried out in 200ml M9 broth, inoculated with pure slant culture, then incubated for 24 hr at 40°C. After incubation, took all the broth and centrifuged them for 10 min at 10000rpm. Collected the supernatant into a beaker, kept them in refrigerator and pellets were discarded.

Purification of enzyme- Enzyme purification is a series of process or service that mainly provide purification and quality analysis from a mixture of the sample. All purification steps were carried out at 4°C. The pH of the phosphate buffer is 6.8.

L. Salt Precipitation-

Salt precipitation is mainly used to separate the desired substance from a solution. The effect based on the electrolyte and non-electrolyte interaction. Salt precipitation carried out by preparing 200ml of M9 broth after inoculated the pure culture, incubated for 24hrs at 40°C. Then centrifuged it at 6000 rpm for 10 min, after centrifugation, collected the supernatant from this 2ml supernatant taken out in one tube. The beaker along with ice packs was placed in the bowl to maintain the temperature of the supernatant present in a beaker and kept on a magnetic stirrer with a magnetic bead. Now ammonium sulfate (44%) was added into this pinch by pinch up to 3-4 hrs and allowed it to dissolve completely. Once the process was completed then stored it inside the refrigerator for 24 hrs incubation.

M. Dialysis-

Activation of membrane- Taken 100ml of boiling water in a beaker, dialysis bag was added into this and left for 10minutes. To this 2% of sodium carbonate was added and left for 10 minutes boiling. The dialysis bag was transferred into another beaker containing boiled water and left for 10 minutes. After that, the dialysis bag was allowed to cool in the same water. Once cooling was done, the bag is tied at one end with thread and checked for any leakage with water. Then nest is de-osmosis procedure.

N. De-osmosis-

After overnight incubation, water was changed with fresh water and kept on a magnetic stirrer with a magnetic bead. For each and every half an hour till 2 hours, water was changed and de-osmosis was retained in an tube for further uses and the remaining solution was taken for ion exchange chromatography.

O. Ion -Exchange Chromatography-

Firstly, cleaned the ion exchange column with methanol then the column was filled with water and sonicated for 15 min. This process should be carried on until the flow of water from the column became smooth. Then eluted the water from column completely and filled with DEAE gel. It was allowed to settle down. Now seven different dilution using Tris-HCl, NaCl, and water was prepared. After the gel settles down elution 1ml was added and completely eluted out to equilibrate the column. Dialysis sample was added and the upper layer of the sample was marked on the column with the help of the marker. Now each of the remaining elution's was added and collected in their respective tubes until the elution reached the top layer of the sample.

P. Protein estimation by Lowry's method-

Protease enzyme assay was performed using samples (crude, salt precipitation, dialysis) and all seven elution samples to determine the highest enzyme activity value. The amount of protein was evaluated

by Lowry's method. 200µg/ml of working solution was prepared from 1mg/ml of stock BSA and different volumes of this solution were taken in test tubes i.e., 0.2, 0.4, 0.6, 0.8 and 1.0. The volume was made up to 1 ml with distilled water and 5ml of alkaline copper reagent was added and incubated for 10 minutes at room temperature. Then 0.5ml of FC reagent added and incubated for 30 minutes at room temperature and absorbance was read at 660nm. Protein reacts with FC reagent to give a blue colored complex.

Q. Gel filtration-

The gel filtration column was cleaned with methanol and filled with water and sonicated for 5 minutes. It was filled with Sephadex gel and allowed to settle down. Elution sample was added and the upper layer was marked on the column with a marker. 25ml of phosphate buffer was added above the sample with pH 7. Allowed the sample to passes through the Sephadex gel, after that1ml of the elution was collected in Eppendorf tubes 25 times then protein estimation of all 25 samples was observed at 280nm.

R. Characterization of enzyme-

Characterization was performed using purified enzymes and carrying out protease assay. Protease assay of pH and substrate concentration for characterization of enzyme were done.

S. Effect of pH-

The assay was performed using acetate buffer (pH 4, pH 5 & pH 6), Phosphate buffer (pH 7 & pH 8), and Glycine buffer (pH 9 & pH 10)for blanks and a mix of a 100µl sample and phosphate buffer for the test sample. The pH with the highest reading is considered for the next process.

T. Effect of substrate concentration-

Substrate concentration was performed by varying the concentration of glucose (0.25%, 0.5%, 0.75%, 1%, 1.25%, 1.5%, 2.0% and 2.5%) observed the optimum conditions at 280nm.

U. Molecule determination by SDS-PAGE-

SDS-PAGE, with a full name of sodium dodecyl sulfate-polyacrylamide gel electrophoresis, is the most widely used technique to separate proteins from complicated samples of a mixture, plays key roles in molecular biology and a wide range of subfield of biological research. Being present a electricity, proteins migrate towards the negative anode inside the polyacrylamide gel under denaturing conditions. SDS PAGE electrophoresis system should include- a tank, lid with power cables, electrode assembly, cell buffer dam, casting stands, casting frames, combs(usually 10-well), and glass plates (thickness 0.75mm or 1.0mm).

IV. RESULT AND DISCUSSION

A. Collection and Isolation of the microorganisms-

Five different soil samples were collected from different places in Bangalore, India. The soil sample is rich with nutrients susceptible to the growth of distinguishing micro-organism.M9 media was used for the isolation of bacterial colonies from the soil sample. After incubation, the plates were examined for the presence of colonies of Pseudomonas species. The culture of the organism was obtained in Petri-plates by streaking. No contamination was there and only the culture of the strain grew. Growth was seen after 24 hours of incubation.

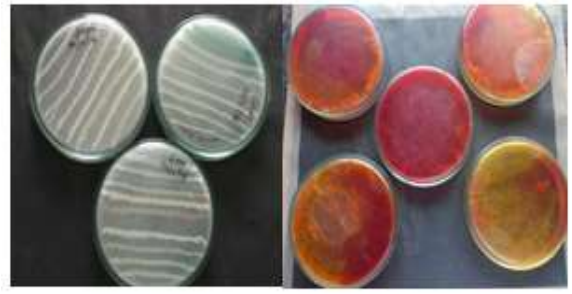

Fig 1 - Isolation of microorganism (Pseudomonas sp.)

B. Identification by morphological, staining and bio-chemical test-

Identification of the organism was carried out on the basis of morphological characteristics, microscopic observation (gram staining) and biochemical tests for further confirmation from the previous culture. Gram staining was done with the culture and viewed under a microscope. Initially, a mixture of gram-positive tubular along with some filamentous bacteria was observed from which filamentous colonies were selected and purely cultured. Finally, gram-positive bacteria were observed in the microscopic study of gram staining slide with purple color and filamentous

structure from the pure culture maintained without any contamination.

Table 1: Biochemical test and Stain test results

Test	Result
Gram stain	+
Catalase	+
Urease	-
Citrate	+
Vogas Proskour	-
Methyi-Red	-
Indole, Lactose, Sucrose	-

C. Effect of physicochemical parameters on microbes for the production of glucose oxidase-

The optimum pH and temperature required for enhanced production of glucose oxidase were determined by preparing the culture medium for 24 hours in different pH and incubating the culture media for 24 hours in different temperatures respectively. Enzyme assay was carried out and the optimum pH was found to be 10 and temperature was found to be 40°C.

D. Effect of incubation-

The effect of incubation time was investigated by determining the number of colonies that were visible on the plate on daily intervals. The colony counts for different days are different according to the type of media. Nutrient agar and Plate count agar (PCA) resulted in increasing counts with increasing incubation time. While working with M9 media, the effect of incubation time was analyzed by considering three incubation periods (24, 48 and 72 hours). Pseudomonas species showed enhancement in 24 hours only. Hence, the optimum time was considered to be 24 hours and further studies were carried out.

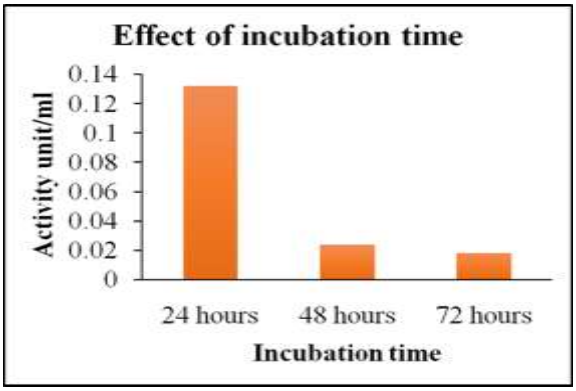

Fig2- Effect of incubation time against activity

E. Effect of pH-

Bacteria are generally neutrophils. The neutral pH is close to 7.0. The optimum growth pH is most favorable for the growth of an organism. The lowest pH value that an organism can tolerate is called the minimum growth and the highest pH value is the maximum growth pH. The range varies with species, for example, the optimum growth ph of Pseudomonas spp. Is 7.5-8.5, but the minimum growth pH is closer to 5.8. In this study, the effect of pH was investigated by considering a wide range of pH (6–10). Pseudomonas species showed enhanced enzyme activity when the pH was 10 and the enzyme activity was suppressed when the pH further decreased.

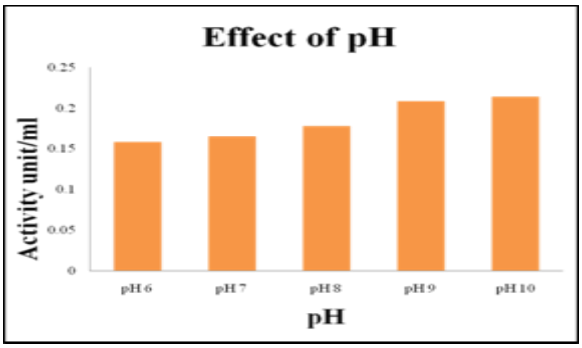

Fig 3- Effect of pH against activity

F. Effect of temperature-

At lower temperature molecules move slower, enzymes cannot mediate in chemical reactions, and eventually, the viscosity of the cell interior brings all activity to a halt. Bacteria grow most rapidly in the range of temperature between 4.4C – 60C doubling in number in as little as 20 minutes. Therefore, growth rate, biomass yield and reaction mechanism need optimum temperature for their maximum efficiency. The highest optimum efficiency of the species was found at 40ºC which favored the growth of bacteria.

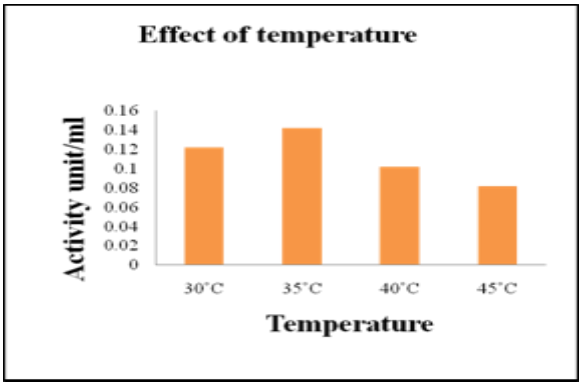

Fig 4- Effect of temperature against activity

G. Strain improvement for maximum production of GOD by UV mutation-

To enhance the enzymatic activity UV mutation was done. Absorbance was taken after 24hr. Since enzymatic activity was higher of the organism which kept for 20min in under UV radiations it showed the highest percentage enzymatic activity.

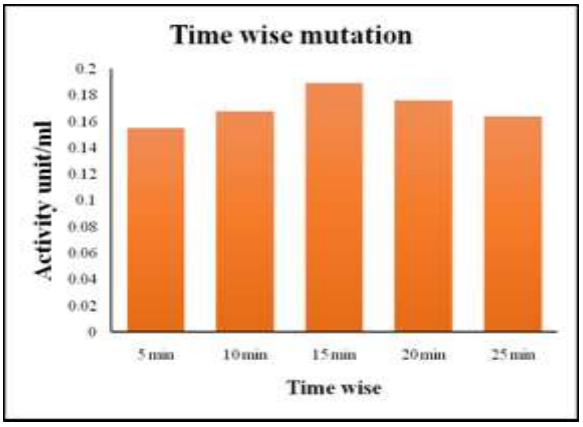

Fig 5- Time wise mutation against activity

H. Height wise UV mutation-

The organism which showed the highest percentage of an enzyme activity in UV mutation was kept at some height under UV radiation to enhance the enzyme activity. Since the enzymatic activity of organism which kept for 5 min at the height of 30 meters under UV radiations showed the highest percentage of enzyme activity after 24hrs.

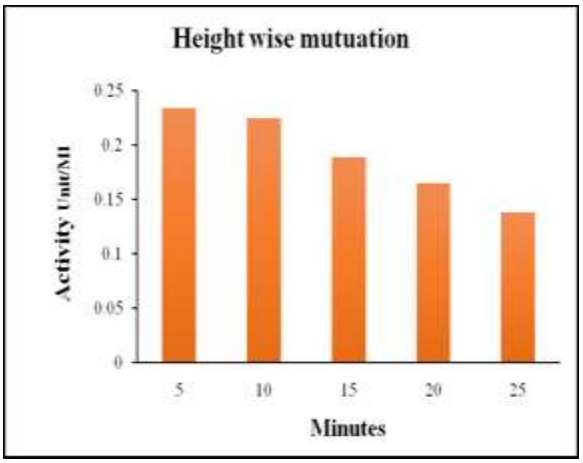

Fig 6- Height wise mutation against activity

I. X-Ray mutation-

Percentage of enzyme activity by UV mutation on height-wise was higher than the time-wise mutation.. The highest enzyme activity was shown for 90 seconds after incubation of 24 hours at 40ºC.

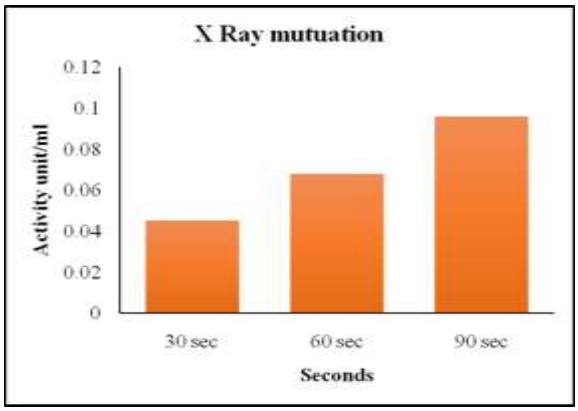

Fig 7- Height wise mutation against activity

J. Chemical mutation by Ethidium bromide methods-

Mutant Pseudomonas sp was obtained by random mutagenesis of wild Pseudomonas sp. using UV and EtBr. It is thought that due to some alterations in the gene Bacillus species, the enzyme activity is improved. With the concentration of 20µl, get 0.144 U/ml.

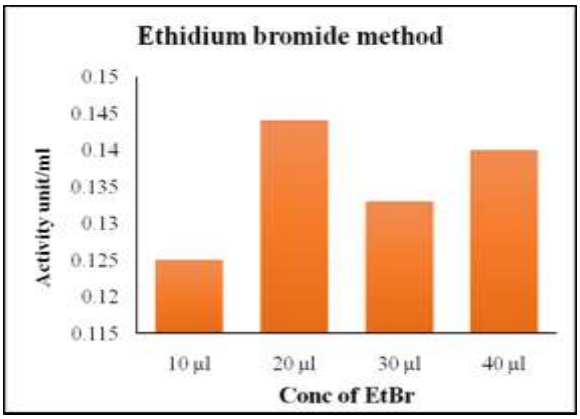

Fig 8- Mutation by EtBr against activity

K. Purification of glucose oxidase by salt precipitation-

At higher salt concentration, protein solubility usually decreases leading to precipitation. Most of the proteins precipitate at 70% saturation of ammonium sulfate salt.

L. Dialysis-

Dialysis was performed to remove excess ammonium sulfate from the sample through diffusion. The dialysis sample was processed for enzyme activity and protein estimation.

M. Ion Exchange Chromatography-

Ion exchange chromatography is a chromatography that separates ions and polar molecules based on their affinity to the ion exchange. The stationary phase is positively charged and negatively charged molecules are loaded to attract it.

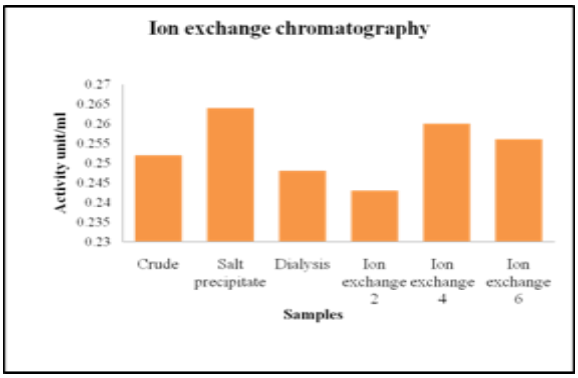

Fig 9- Ion exchange chromatography

N. Protein estimation-

The estimation of protein has done by Lowry's method at 660nm.Graph plotted here by concentration against OD.

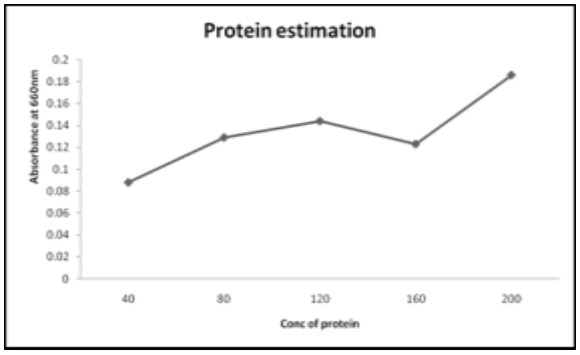

Fig 10: Protein estimation

O. Gel filtration-

The highest elution of ion exchange chromatography with higher activity was subjected to gel filtration. After the enzyme assay was done for 25 Eppendorf tubes sample first 3 samples was highest and sample 9, 10, 11, 12 was mixed and used for enzyme assay.

P. Characterization of glucose oxidase by the effect of pH and substrate concentration-

Effect of pH: The highest activity was 0.078 U/ml noted at pH 6. The lowest activity was 0.036U/ml at pH 8. Thus in the present study pH, 6 was found to be ideal for an enzyme activity.

Substrate concentration: The activity of the enzyme at various substrate concentrations was calculated and the results are presented in the table. The highest activity was 0.072 U/ml noted at a concentration of 1.5µM.

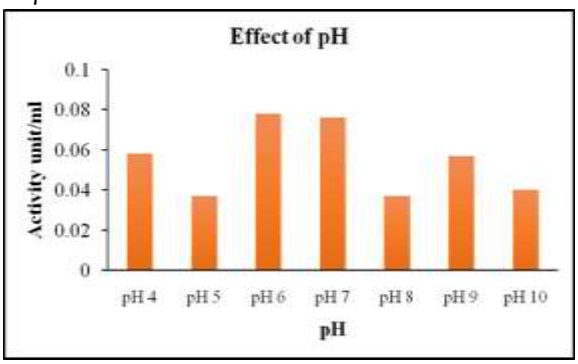

Fig 11- pH against activity

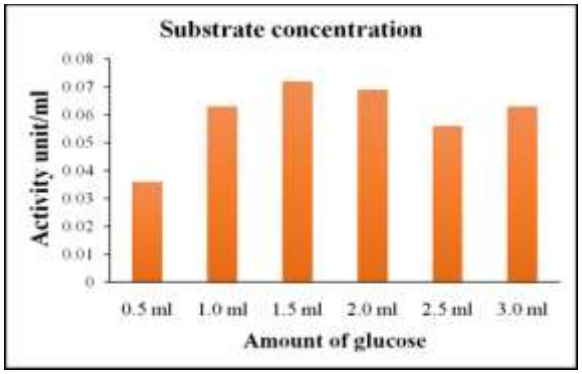

Fig 12- Amount of glucose (substrate) against activity

Q. SDS-PAGE electrophoresis-

The glucose oxides enzyme was viewed as a major separating protein on the electrophoresis polyacrylamide gel which was stained with coo massive brilliant blue along with other minor protein impurities as the enzyme was not fully purified. In comparison with the protein molecular weight marker, the glucose oxidase was found to have a molecular weight of approximately 31KDa. Molecular weight of marker is 66kDa. Molecular weight of dialysis is 62kDa. Molecular weight of glucose oxidase (sample) is 31kDa.

V. CONCLUSION

Glucose oxidase producing organism was isolated from soil samples which have been collected from different regions of Bangalore and was tentatively identified as Pseudomonas species by various morphological characteristics and biochemical tests. Enzyme activity was tested against various test organisms. On confirmation of an enzyme activity of

the organism further optimization of the media was carried which showed the increase in growth of the organism as tested by well diffusion method. The optimization of various factors of the medium more prominent and clear zone can be observed. The optimization showed that there was an increase in an enzyme activity indicating that the organism requires minimum of one days (24hrs) incubation, 40°C incubation temperature, alkaline pH (pH 10), 15 minutes of time-wise mutation, 5 minutes for height-wise mutation, 90 seconds for x-rays, 20µl EtBr for were found to be most optimum parameters for the organism to show maximum enzyme activity. It was found that glucose oxidase showed maximum activity at 1.5% of glucose for substrate concentration. Significant change observed at 500nm. For purification, ammonium salt precipitation, dialysis, ion exchange chromatography, and gel filtration chromatography were done. In comparison with the protein molecular weight marker, the glucose oxidase was found to have a molecular weight of approximately 31KDa.

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