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Extraction, Purification and Enzyme Assay of Pectinase Enzyme from *Aspergillus niger*

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Abstract: ***Enzymes catalyses various reactions involved in the preparation of different food products. It is one of the important tools in modern food industry because while processing many intermediate processes are simplified due to use of enzymes. Pectin is a naturally occurring hetero-polysaccharide which is found in the cell wall (middle lamellae) of plants. It is mainly composed of esterified D-galactournic acid. The pectinases are the enzymes that are capable of hydrolysing the pectin polysaccharide into smaller fragments. Pectin can be used in pharmaceutical industry as well as in the food industry as it can be used as a thickening or solidifying agents. Pectinase is commonly used in degradation of pectin which is generally found in plants. Pectinase has a molecular weight of about 35 KDa. At present, almost all the pectinolytic enzymes used for industrial applications are produced by the fungi, namely Aspergillus sp., Rhizopus stolonifer, Alternaria mali, Fusarium oxysporum, Neurospora crassa, Penicillium italicum, and many others. Aspergillus niger is preferred in industries, since approximately 90% of produced enzymes may be secreted into the culture medium i.e. extracellular production. Orange peels are used as substrate for the growth of Aspergillus niger. Besides, orange peels cause waste disposal problems since they are being thrown around indiscriminately. Agricultural and Industrial wastes pose many disposal problems which can be dealt by treating them with combination of enzymes like cellulase, papain, pectinase etc.Since pectinases are widely used enzyme for different industrial application, it is necessary to use inexpensive and readily available raw material for its production. Hence tomato and orange peels were used as the raw material. In view of the above, the study was focused on analysis of pectinase production by Aspergillus niger when the substrate concentration was varied. The enzyme obtained from different concentrations of the substrate was then used for enzyme assay. Enzyme assay was carried out using DNSA method. The quantitative estimation of the enzyme was carried out using folin-lowry's method. The enzyme activity was checked using orange juice as substrate on appropriate wavelength using colorimetry. It was observed that as the substrate concentration increased , the amount of enzyme produced also increased whereas the specific activity of the enzyme decreased.***

Keywords: ***Extracellular Enzyme, Pectinase, Aspergillus niger, Fruit wastes, purification, Enzyme assay, Immobilization.***

I. INTRODUCTION

The use of enzymes in industrial processes have gained a lot of importance in recent years. The quest for green technology is driving innovation in both the production of specific enzymes and in the use of enzymes already available. Whether it is in the form of using enzymes to make a new use of an old renewable energy source, or simply eliminating the need for extreme temperatures and pressures to synthesize a product, Enzymes are useful in waste degradation such as fruit peels, organic materials, etc. Most commonly used enzymes are lipases, proteolytic and amylases which easily breakdown the complex substances to give desired products in relatively lesser amount of time. Aqueous assisted enzyme extraction are better solution for oil extraction from oilseeds. Enzyme mediated extraction is eco-friendly, can obtain higher yields, cost-effective and aids in obtaining co-products without any damage. Enzyme technology has great potential for oil extraction in oilseed industry. Enzymatic treatment prior to mechanical extraction significantly improves juice recovery compared to any other extraction process. Enzymatic hydrolysis of the cell walls increases the extraction yield, reducing sugars, soluble dry matter content and galacturonic acid content and titrable acidity of the products. Enzymatic degradation of the biomaterial depends upon the type of enzyme, incubation time, incubation temperature, enzyme concentration, agitation, pH and use of different enzyme combinations. We can conclude from the technical literature that use of the enzymes i.e. cellulases, pectinases, amylases and combination of these enzymes can give better juice yield with superior quality of the fruit juice. Pectin is a structural heteropolysaccharide contained in the cell walls of terrestrial plants. Pectinase is an enzyme that breaks down pectin which acts like a cementing agent between two adjacent cell walls. Pectinase enzyme has a molecular weight of 35 Kda. Pectinase enzymes are useful in waste disposal as it degrades the fruit and vegetable peels which contain pectin. The enzyme produced from *Aspergillus* species is normally regarded as safe and as the enzyme production is extracellular, it is easily extracted. Orange peels and spoilt tomatoes are used for obtaining the fungal samples. The import of extracellular pure enzymes increases the cost of production. Majority of pectinolytic enzymes used for industrial purposes are obtained from *Aspergillus sp., Neurospora crassa and penicillium sp.*

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Pectinase enzyme can give maximum juice yield i.e. 92.4% at 360 minutes incubation time, 37°C incubation temperature and 5 mg/100 g of enzyme concentration. Whereas the combination of two enzymes i.e. pectin methyl esterase (PME) and polygalacturonase (PG) at 120 minutes of incubation time, 50°C of incubation temperature and 0.05 mg/100 gm of enzymatic concentration can give the maximum yield of 96.8% for plum fruits.This study is therefore aimed at the production and extraction of pectinase from *Aspergillus niger* by increasing the substrate (pectin) concentration to check the effect in the enzyme activity and the quantity of the enzyme produced. The extracted enzyme is also used to check it's effect on clearance of orange juice.

II. MATERIALS & METHODS

A. *Isolation of the Fungi from the Fruit Peels*

The samples used for obtaining the fungal growth were orange peels and spoilt tomatoes collected from the local fruit market. The peels and the tomatoes were kept in damp zip lock bags till fungal growth was seen on them. This fungal growth was then cultured on PDA and Sabouraud Agar plates and kept for incubation at room temperature for 3-4 days. PDA and Sabouraud Agar plates were used as isolation media as they exclusively support the growth of fungi and bacterial contamination is avoided. After the incubation, it was seen that PDA plates had luxurious growth of fungi as compared to Sabouraud Agar plates which did not show growth after 3-4 days. Hence PDA was used as isolation media for further studies.

B. *Screening for the Pectinase Enzyme Production*

One of the media used for screening of the pectinase producing fungi was Pectinase screening Agar media (PSAM). This media contains pectin which helps to isolate the pectinase producing fungi. Another media was used for the screening process. This media is Mineral Salt Agar media (MSAM). This media also contains pectin present in it. After the preparation and sterilization of the media, the fungal isolates from the master plate were sub cultured on the petri plates containing PSAM and MSAM by simple streak plate technique. The plates were then kept for incubation at room temperature in inverted position for 3- 4 days. After incubation, zones of hydrolysis were observed around the fungal colonies which indicates pectinase production by the fungi.

C. *Colony Morphology of the Culture and Microscopy*

The screening plate contain different zones of microbial growth. Colonies from various areas of the plate were picked up with the help of sterile forceps under aseptic conditions and a slide was prepared with the fungal isolates. This slide was stained using Lacto-phenol Cotton Blue. This slide was then observed under microscope at 40 X magnification. The green coloured sporangiophores with the sporangiospores at the top of sporangium along with septate hyphae was observed. Black coloured spores were also observed around the sporangiospores. These characteristics were distinct of *Aspergillus niger* and hence it was confirmed that the fungal isolates obtained were of *Aspergillus niger*.

D. *Pectinase Enzyme Production by Liquid State Fermentation*

The media used for liquid state fermentation was PSM as significantly dense growth was observed on PSAM plates as compared to MSAM plates. First an aliquot was prepared of the *Aspergillus niger* in 5 ml of media. After growth was observed, this aliquot was added in 45 ml of sterile PSM medium. After 2-3 days spherical fungal growth was observed in the media. These fungal growth were inoculated in 250 ml conical flasks containing 100 ml of the sterile media. 3 conical flasks were prepared with increasing concentration of pectin from 1 to 5 % and the flasks were kept for incubation. After 2-3 days, dense growth was observed in the flasks.

E. *Crude Enzyme Extraction by Filtration*

Separation of the liquid mixture from the mycelia and the spores was achieved by using filtration technique. Whattman filter paper no. 1 was used for filtration. After filtration, the filtrate was subjected to centrifugation at 8000 rpm for 10 min to separate any mycelia which may have escaped into the filtrate. This procedure was repeated for all the 3 flasks. This crude enzyme is used for measuring pectinase activity.

F. *Ammonium Sulfate Precipitation*

Ammonium sulphate salt was used for partial purification of the crude enzyme. Pinch by pinch addition of the salt to the crude enzyme was carried out to obtain complete saturation while maintaining ice cold conditions. This technique is very useful for obtaining partially pure proteins as it separates the unwanted contaminants by precipitation. After this technique, centrifugation at 8000 rpm for 10 min is carried out and the obtained pellet is dissolved in acetate buffer (pH= 4.8).

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G. Dialysis

This technique involves the activation of the membrane by keeping it in 70 % ethanol. Once the membrane is activated, the extract is poured in the membrane and then kept in distilled water. The water is changed daily. Biuret and nessler's reagent test are carried out daily to check the presence of proteins and ammonium salts in the water respectively. Once the nessler's reagent test comes negative, it is considered that purification of the enzyme has been completed and the extract is removed out of the membrane and transferred into a test tube. This purified enzyme is stored at 4 °C.

H. Pectin Enzyme Assay

Pectin enzyme assay is carried out using DNSA method. The principle behind this technique is that the enzyme degrades the pectin to give reducing sugars which are estimated colorimetrically at 530 nm. 0.25 ml enzyme is added to 0.75 ml of the substrate, which is prepared in acetate buffer to maintain the pH conditions of the enzyme. This test sample is then kept at 37° C for 30 min. Meanwhile, standards are prepared including a blank tube by using standard glucose stock solution (1000 µg/ ml). The standards were in the range of 200 to 1000 with an interval of 200 in between. The test tube with 1 ml of distilled water serves as blank. After incubation, 1ml of DNSA reagent was added to all the tubes and the tubes were kept in boiling water bath till colour change was observed. The tubes were cooled and were diluted using distilled water. Then absorbance was checked at 530 nm and a standard graph was plotted. The concentration of the product in the test tubes containing the sample was used to determine the enzyme activity.

I. Estimation of the Protein Concentration by Lowry's Method

Lowry's method is a very sensitive and easy method for estimation of protein concentration. In this method, $CuSO_4$ under alkaline conditions along with folin's reagent is used. Standards are prepared using Bovine Serum Albumin (BSA) as stock solution (200 µg/ml). The test tube with 1 ml of distilled water serves as blank. 5ml alk. $CuSO_4$ is added to all the tubes and kept for incubation at R.T for 30 min. Then, 1 ml of folin's reagent is added and further incubation of 10 min is carried out. Absorbance is taken at 660 nm in a colorimeter. A standard graph is plotted and the concentration of the purified enzymes is determined.

III. RESULTS

A. Isolation of Aspergillus niger from Contaminated Peel

The PDA plates showed black coloured fungal growth after 3 – 4 days of incubation at R.T. The fungal isolates were stained with Lacto-phenol cotton blue and observed under 40 X microscope. The presence of septate hyphae, black spores and sporangiospores on sporangium confirmed that the isolated species is *Aspergillus niger*.

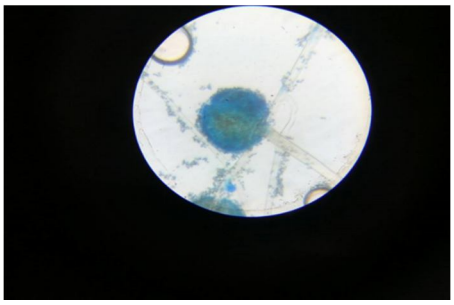


Fig .1.*Aspergillus niger* as observed under microscope

B. Screening for the Pectin Hydrolysis by the Fungi

The PSA and MSA plates showed zones of hydrolysis around the fungal colonies which confirms that the species produced pectinase enzyme. Microscopic examination of the colonies showed presence of *Aspergillus niger.*



Fig .2.Fungal colonies as observed on PSA and MSA plates respectively.

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C. Pectinase Enzyme Production by Submerged Liquid State Fermentation

The aliquot was added into the conical flasks containing pectin in increasing concentration from 1 to 5%. The broth was kept for incubation at R. T for 4 days for complete production of the enzyme in the extracellular media.



Fig .3. Crude enzyme 1 – 5 % serially arranged.

D. Assay of Pectinase Activity

In order to estimate enzyme activity, DNSA method was used. DNSA reagent was used and with the help of standards, concentration of the reduced sugars was found out. The enzyme activity was found out using the following formula:

Specific activity = enzyme activity/protein concentration

$$\text{Enzyme activity} = \frac{Ab \times Vf}{T \times \sum \times Vs \times d}$$

where,

Ab = absorbance at 530 nm

Vf = final volume including DNSA

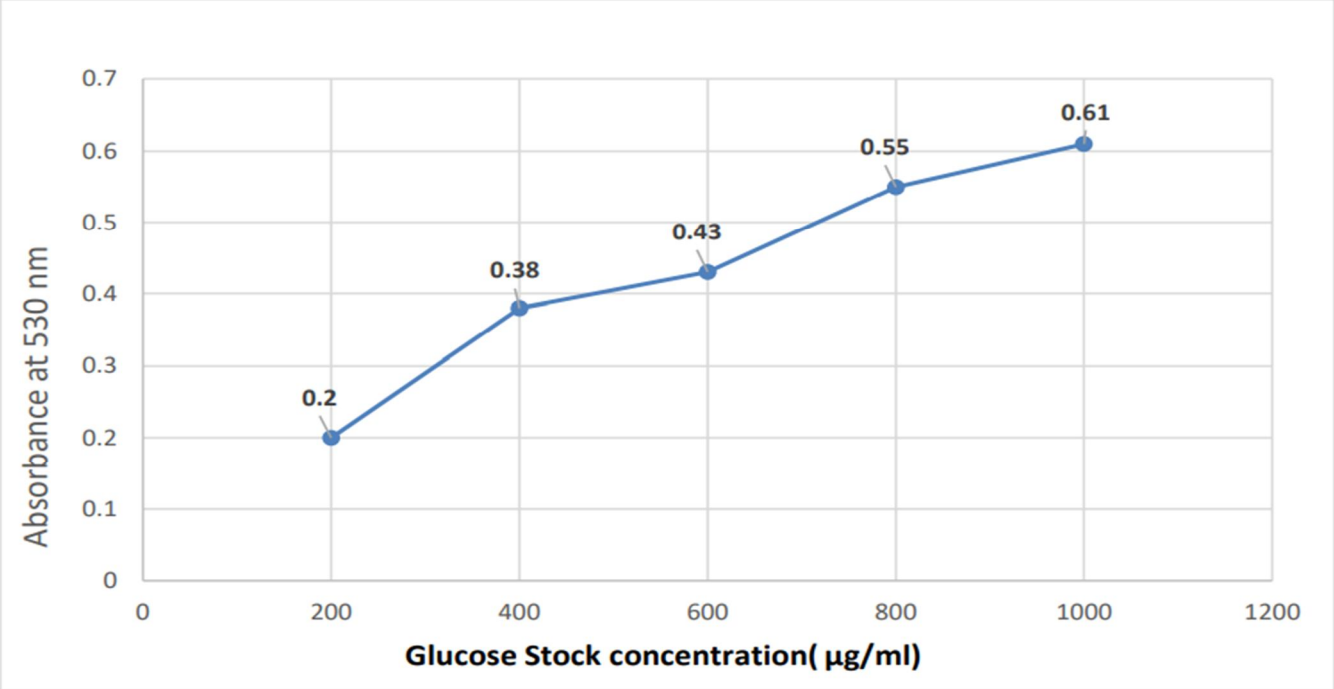
d = diameter of cuvette (1cm)

$\sum$ = extinction co-efficient

T = incubation time in min

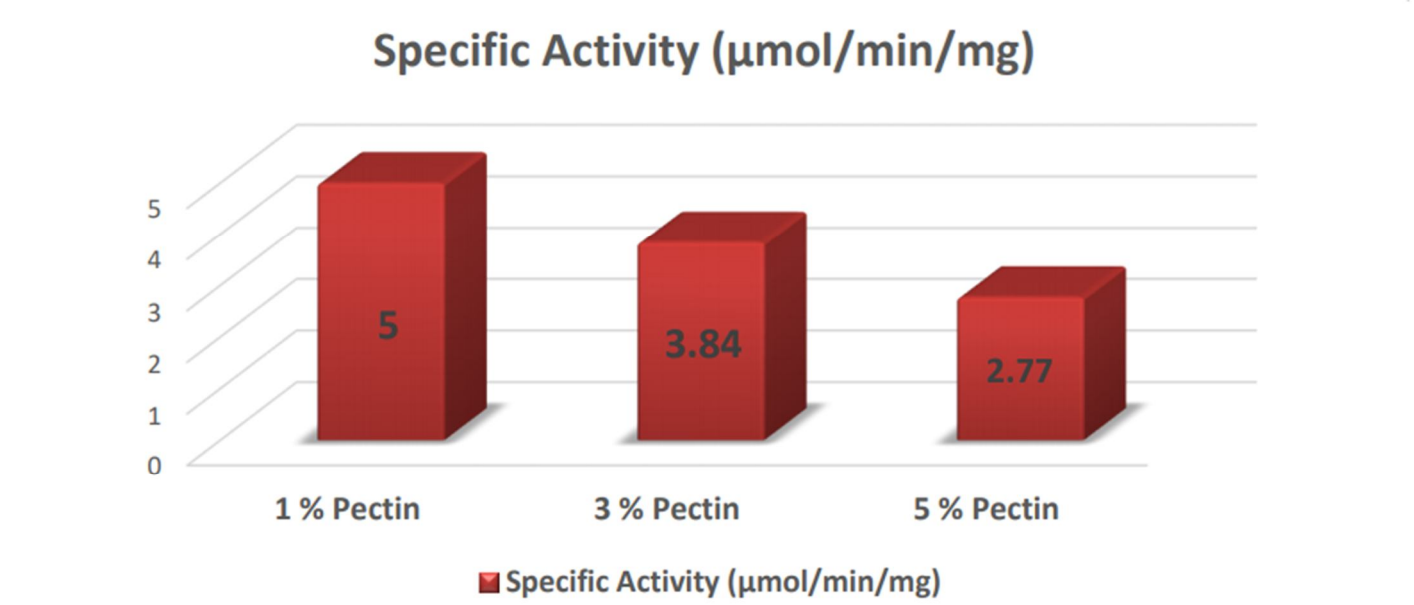
By beer's law

$$\text{Extinction co-efficient } \sum = \frac{\text{absorbance}}{\text{Concentration of sample} \times \text{path length}}$$



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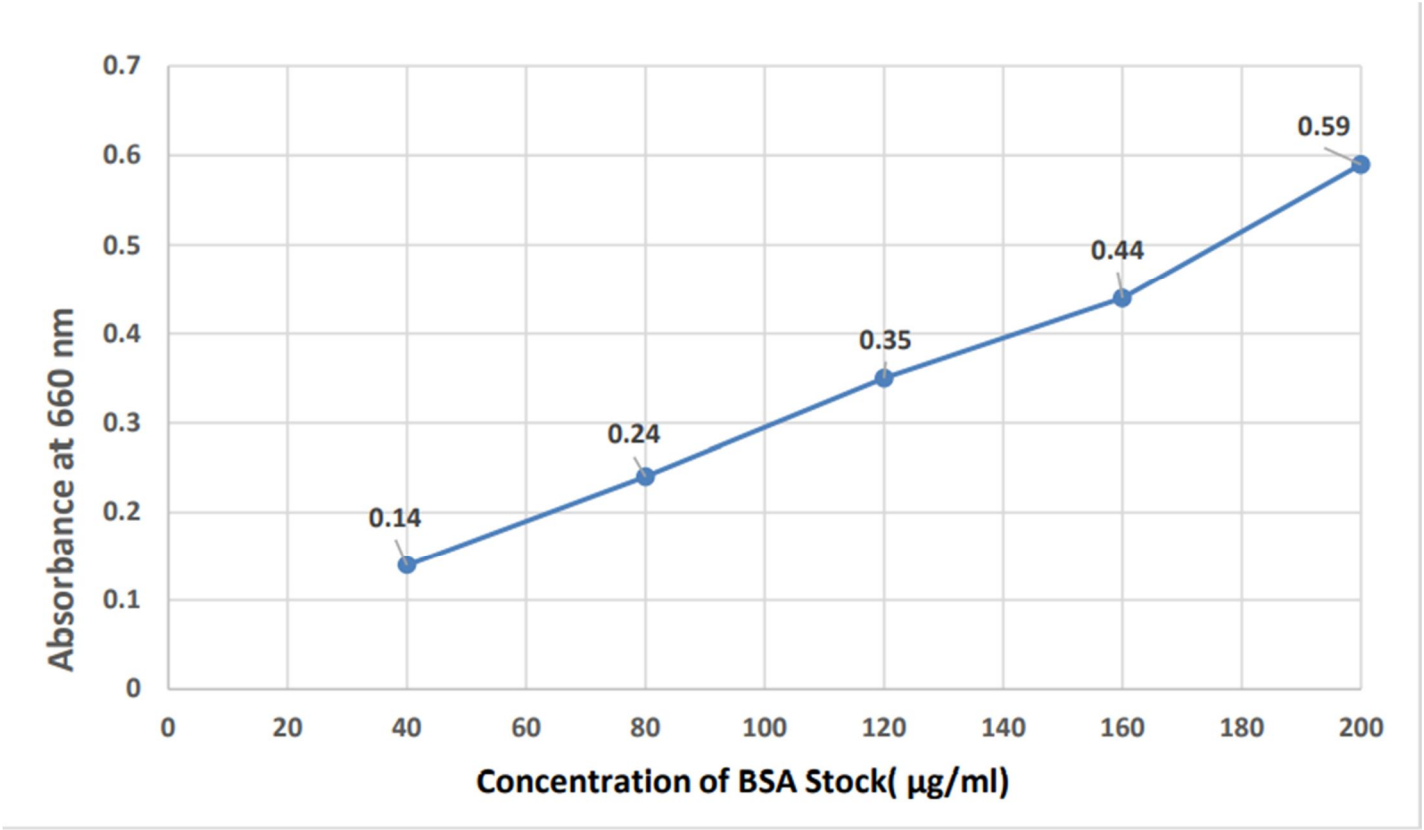


Graph no 1. Graph showing pectinase activity.

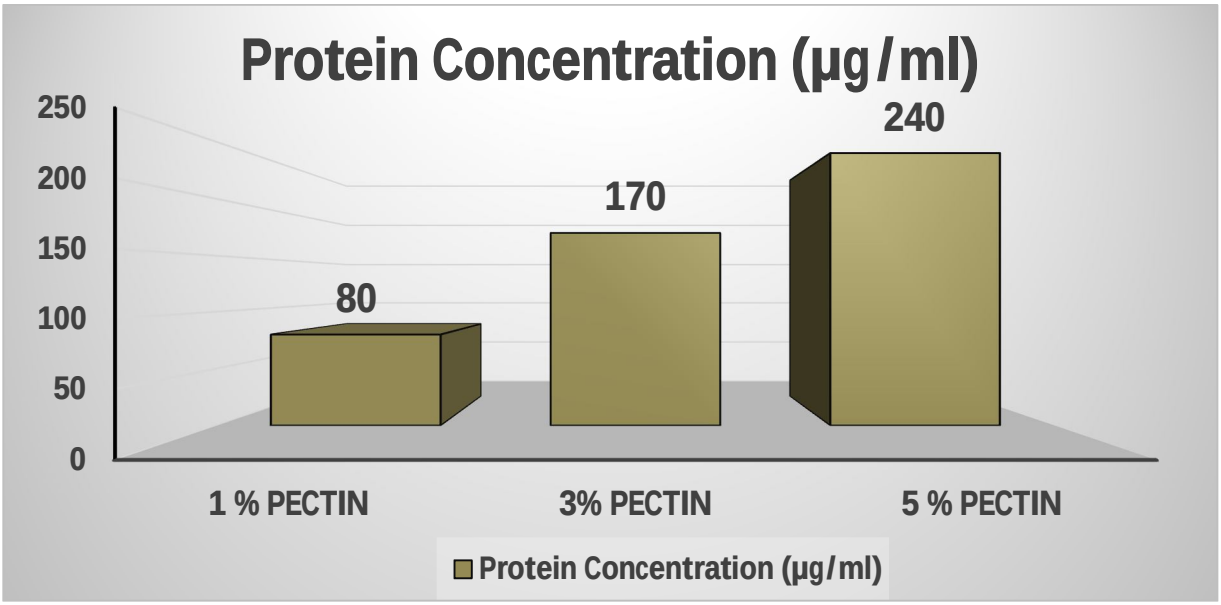
E. Estimation of Protein Concentration of the Extracted Enzyme.

Protein concentration of the enzyme was estimated using lowry's method.

BSA was used as stock and concentration was found out using a standard graph.



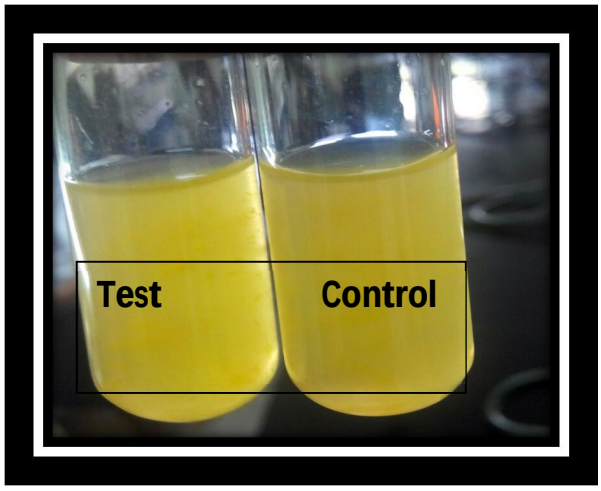
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Graph no.2 Graph showing concentration of pectinase.

IV. APPLICATION

A. *Clarification of Orange Juice*



The λ max of the orange juice was found out to be 420 nm and absorbance at this wavelength was taken for test sample. The absorbance values of the crude and pure enzymes were compared with control and it was found that the pure enzyme showed increased activity.

B. *Determination of λ max*

Wavelength	Absorbance
400	0.69
420	1.01 (λ max)
470	0.99
500	0.96
530	0.86
620	0.69

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660	0.63
700	**0.53**

Orange juice	Crude	Pure
Control	0.5	0.5
Test	0.43	0.36

Absorbance of orange juice at λ max (420 nm).

V. CONCLUSION

Pectinase is an extracellular enzyme, which is produced from various organisms including bacteria, fungi, and some actinomycetes. In the current work Pectinase enzyme was produced by *Aspergillus niger* which in turn was obtained from the decaying Fruit peels and rotten tomatoes. The Culture was allowed to grow on PDA media and on PSAM and MSAM media containing Pectinase to selectively allow only the Pectinase producers to grow. The mycelia thus obtained after 3 days was stained and observed microscopically. The mycelium was identified as *Aspergillus niger*. The culture was further used for the production of the enzyme using PSM medium. The culture was incubated for 4 days for the complete production of the extracellular enzyme. The enzyme thus produced was purified by several techniques like Filtration, Centrifugation, Salt precipitation and Dialysis. The purified product was used for the Enzyme assay which showed that the activity of the enzyme produced, decreased as the substrate concentration increased. Quantitative estimation of the enzyme was performed by Lowry's Method which showed that the concentration of the enzyme increased with increase in pectin concentration. The extracted enzyme was used in clarification of orange juice. The λ max value of the orange juice was determined which was found to be 420 nm. The crude and purified enzyme showed decreased absorbance as compared to the control. The purified enzyme showed increased enzyme activity as compared to the crude enzyme.

Pectin concentration	Crude	Pure
1 %	0.662	5
3 %	0.602	3.84
5 %	0.0913	2.77

Table no.1. Enzyme activity (in µmol/min/mg) with increase in concentration of pectin.

Pectin concentration	Crude	Purified
1 %	330	80
3 %	930	170
5 %	1460	240

Table. no. 2 protein concentration of the crude and purified enzyme.

VI. FUTURE PROSPECTS

A. Molecular Weight Determination Using Gel Electrophoresis Method

Polyacrylamide Gel Electrophoresis can be used as enzymes are proteins in nature and PAGE is exclusively used for proteins. The sample needs to be run in the gel alongside a protein marker. By comparing with the protein marker, the molecular weight of the extracted enzyme can be determined.

B. Intensification And Stabilization Of Colour In Wine Production

The enzyme can be used in wine production prior to the fermentation process. The enzyme interacts with the fruit and breaks down pectin present in the fruit skin. This allows the yeast to act on the fruits with better efficiency and this results in improved colour, tannins and juice extraction.

C. Extrction of Vegetable Oils

The enzyme can be used to degrade the pectin which is present in the coating of many plant seeds used for oil extraction. It can be used to increase the yield of the oil as compared to the conventional methods. The yield of oil is seen to be higher when enzymatic processes are used.

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D. Clarification of Fruit Juices

Pectinase enzyme can be used for the process of clarification in fruit juices. The enzyme degrades the pectin and increases the clarity of the juice thus enhancing the appearance.

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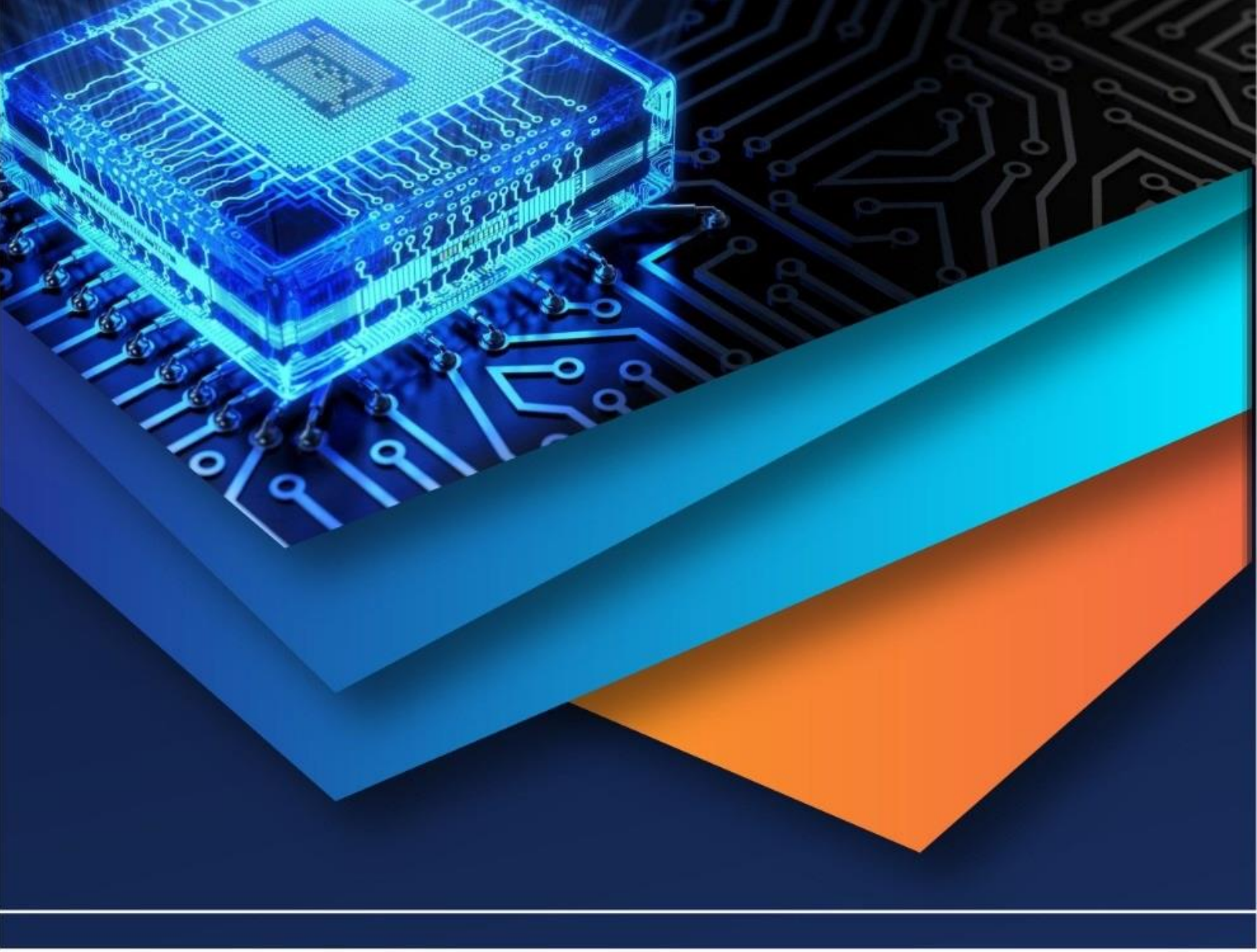
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