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ISOLATION, PURIFICATION AND CHARACTERIZATION OF PECTINASE

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ABSTRACT
Pectinases are the enzymes which breakdown pectin polysaccharide present in plant tissues into simpler molecules like sugar and other useful compounds. Pectinases account for 10% of the total worldwide production of enzymes. In the present study, bacteria were isolated from soil sample and screened for the production and characterization of pectinase. The bacterial isolate was identified as *Bacillus* sp. Maximum yield of poectinase was obtained after 72 hrs incubation., The enzyme activity was performed by titrimetric method using starch as an indicator and polygalactouranase as substrate, The maximum enzyme activity was shown as 40 units/ml, The Optimum temperature and pH for enzyme activity was found to be 35°C, pH-6.0 respectively. The induced stress showed maximum activity at 30mg of salt concentration and the molecular weight of the protein was found to be 40kDa.

KEYWORDS: Bacillus sp., Polygalactouronase, Pectinase, Temperature, pH, Stress.

INTRODUCTION
Pectic substances are the complex polysaccharides present in the middle lamella of plants and are degraded by a group of enzymes, pectinases. (Ramachandran Sandhya *et al*, 2013). Pectinases are the enzymes which breakdown pectin polysaccharide present in plant tissues into simpler molecules like sugar and other useful compounds. Investigation of pectinases is a central issue in enzymology research due to their wide applications in Pharmaceutical, food, Agricultural products and Bioremediation processes. Pectinases account for 10% of the total worldwide production of enzymes. But for both technical and economic aspect microbial source of pectinase has become increasingly important. (Rehman Naziya *et al*. 2015). Pectinases are the group of enzymes that catalyzes the degradation of pectic substances through de-polymerization and de-esterification reactions. Pectinolytic enzymes are classified according to their mode of action on the galacturonan part of the pectin molecule. (Mukesh Kumar *et al*, 2012).

Due to a wide range of applications of pectinases in food industry, the industrial production of pectinases has drawn worldwide attention. The characteristics of enzymes from these sources make them ideally suited to fruit juices and related technologies, where they find many applications. Many studies have been conducted on the production of pectinase from various microorganisms. But a few works have been published about cost-effective production of enzymes. The difficulties to obtain the appropriate substrate might be the biggest problem to develop such studies.

MATERIALS AND METHODS
COLLECTION OF SAMPLE
Soil samples were collected from woody area and brought to the laboratory and were used for isolation.

ISOLATION OF PECTINASE ENZYME PRODUCING MICROBES
The Pectin medium was prepared and sterilized along with required glassware in an autoclave. 1g of soil sample was dissolved in 1% sodium chloride saline solution and mixed thoroughly; 200µl of this solution was suspended on the pectin medium plates and incubated at 37°C for 48 hours.

PREPARATION OF PURE CULTURE
Cultures isolated from soil will contain many bacteria. After suspension culture, a colony can be selected and streaked onto another media to obtain pure colony of the desired bacteria. The requirements are Luria-Bertani [LB] medium with composition of Tryptone - 10.0g, Yeast extract - 5g, NaCl - 10.0g, Agar - 20.0g, Distilled water - 1000ml.

The media was poured into test tubes to prepare slants. After agar solidification, a particular colony from the petriplates containing microbes of desired morphological appearances, isolated from soil was selected and was

streaked onto a media using an inoculation loop. It was then incubated for 24 hours at 37°C.

IDENTIFICATION OF PURE CULTURE

Identification of bacteria was done by biochemical characterization and morphological identification by Gram's staining.

OPTIMIZATIONOF CULTURAL CONDITIONS FOR PECTINASE PRODUCTION

The bacterial isolate was subjected to different culture conditions to derive the optimum conditions for pectinase production. The inoculated broth after incubation was centrifuged at 10,000 rpm for 10 minutes at 4°C and the clear supernatant was used as crude enzyme for enzyme assay.

The pectinase production was estimated at different pH (4, 5, 6, 7, 8, and 9) and temperature conditions. Optimization was done at various time intervals of 24, 48, 72, 96 hrs. The optimum condition was observed maximum at 72 & 96 hr of incubation. The optimum pH was found to be pH-6.0. The optimum temperature was found to be 35°C. The optimization was done to find out that at which condition the bacteria produces pectinase with various parameters.

EFFECT OF INDUCED STRESS FOR THE PRODUCTION OF PECTINASE ENZYME

INDUCED STRESS BY DIFFERENT SALT CONCENTRATION

Pectin media broth (pH 6.0) was prepared with different concentration of sodium chloride (10, 20, 30, 40, 50 mg). The sample culture was inoculated into different conical flask containing varied concentration of sodium chloride containing pectin broth. The flasks were then incubated at room temperature for 24 hours and 72 hours. After incubation the sample was centrifuged and the enzyme was obtained and pectin activity and assay was carried out and the results were obtained.

INDUCED STRESS BY UV MUTATION

5 plates LB agar was prepared and allowed to solidify. After solidification the saline containing culture was poured and swabbed for uniform spreading. Later the plates containing the culture were exposed to ultra violet radiation under different time intervals (5, 10, 15, 20, and 25) minutes and also at different lengths (cm) of UV exposure.

Pectin media broth (pH 6.0) was prepared and transferred into 5 different conical flasks. The ultra violet exposed samples were inoculated into different conical flasks. The flasks were then incubated for 24 hours at room temperature in the shaker. After incubation pectinase activity was determined by conducting pectin assay.

INDUCED STRESSBY CHEMICAL MUTATION

LB broth was prepared and it was mixed with different concentration of Ethidium bromide ranging from (10, 20, 30, 40, 50µl) and inoculated with bacterial culture. The chemically treated plates were incubated at 37ºC for 24 hours in a shaker. After incubation pectinase activity was determined by conducting pectin assay.

PURIFICATION AND CHARACTERIZATION OF PECTINASE ENZYME

PURIFICATION OF PECTINASE ENZYME

The enzyme solution was stored at 40°C for 2 hours and was centrifuged at 10000 rpm for 10 minutes. Measure the supernatant volume. Keep it in a magnetic stirrer and ammonium sulphate was added slowly pinch by pinch. The protein is further purified by Dialysis, Ion-Exchange chromatography, Gel-filtration chromatography.

PROTEIN ESTIMATION

The protein content was determined by Lowry's method using Bovine serum albumin.

ENZYME ACTIVITY OF PECTINASE ENZYME USING TITRIMETRIC METHOD

The activity of the pectinase enzyme obtained was determined by titrimetric method. Pipette out 4.9 ml of polygalacturonase acid (PGA) into conical flask for sample and added 5 ml of PGA for blank. Equilibrate to 25°C and added 0.1 ml of pectinase only to sample. Mixed them by swirling and incubated at 25°C for exactly 5 minutes. After that added 5 ml of iodine solution and add 1 ml of sodium carbonate in both sample and blank. Mix by swirling and place on a stirrer at room temperature and add 2.0 ml of sulphuric acid. Titrate with sodium thiosulphate until the solution is faint yellow color. Then add 0.1ml of starch and continue to titrate until the solution is colorless. The titration was carried out for various substrates, pH, temperature and the enzymatic activity was observed.

After the production and purification the pectinase activity, specific activity, fold purification and percentage yield was calculated to determine the purity of enzyme.

CHARACTERIZATION OF PECTINASE ENZYME

EFFECT OF TEMPERATURE

The effect of temperature on the enzyme activity at different temperatures starting from 0, 25, 35, 45, 55°C was studied; all the other aspects of the assays were kept identical for those already described for the standard enzyme assay.

EFFECT OF pH

The effect of pH on the enzyme activity at different pH starting from 4 to 10 was studied; all the other aspects of the assays were kept identical to those already described from the standard enzyme assay.

EFFECT OF CONCENTRATION

The effect of concentration on the enzyme activity at different concentrations starting from 2-6ml was studied;

all the other aspects of the assay were kept identical to those already described for the standard enzyme assay.

EFFECT OF INCUBATION TIME

The effect of incubation time on the enzyme activity at different time intervals starting from 5-25 minutes was studied; all other aspects of the assay were kept identical to those already described for the standard enzyme assay.

SDS-PAGE was performed for separation of proteins.

RESULT

ISOLATION AND IDENTIFICATION OF PECTINASE ENZYME PRODUCING MICROBES

Isolated colonies were obtained and pure cultures were grown as shown in **figure-1and2.**

Figure 1: Isolated bacterial colonies.

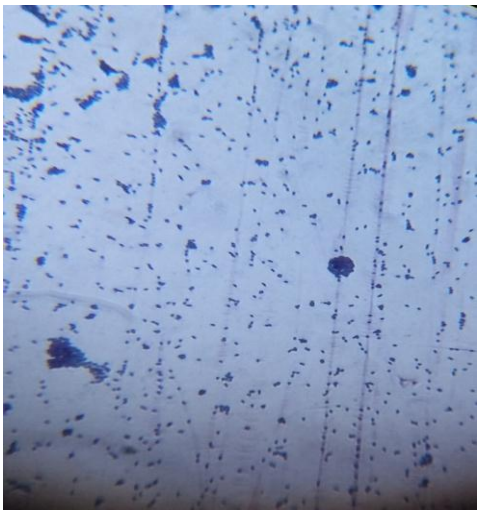

Figure 2: Microscopic view of Gram positive *Bacilli*

PRODUCTION OF PECTINASE ENZYME

After isolation and optimization the enzyme obtained was used for production and purification. The crude form of pectinase was obtained by collecting the supernatant when the broth was centrifuged at 10,000 rpm at 10 minutes.

ENZYME ACTIVITY

Production media was inoculated with Pectinase microbe. After incubation period enzyme activity was performed. The enzyme activity was performed by titrimetric method using starch as an indicator and polygalactouranase as substrate. The maximum activity of the enzyme was found to be.

FORMULA FOR CALCULATING PECTINASE ASSAY

$$\text{Units/ml of enzyme} = \frac{(\text{Ml of titrant for blank- ml of titrant for test}) \; 1 * df * 100}{0.100 * 5.0 * 2}$$

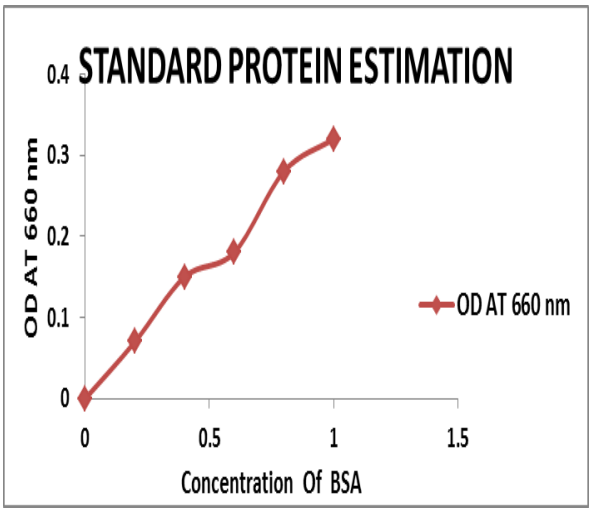

Figure 3: Standard protein graph

INDUCED STRESS FOR THE PRODUCTION OF PECTINASE ENZYME

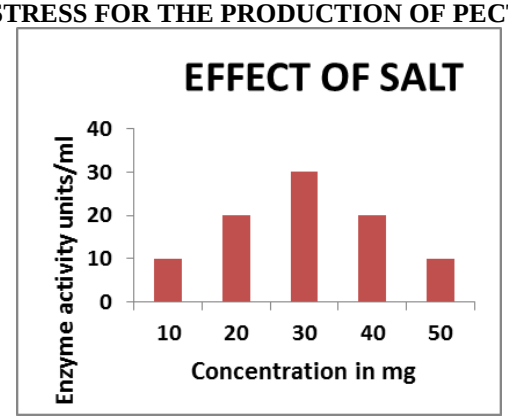

Figure 4: Effect of salt

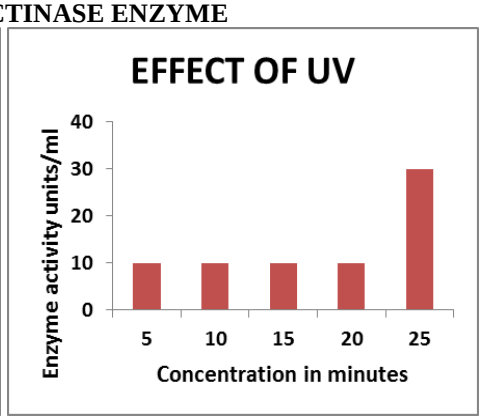

Figure 5: Effect of UV

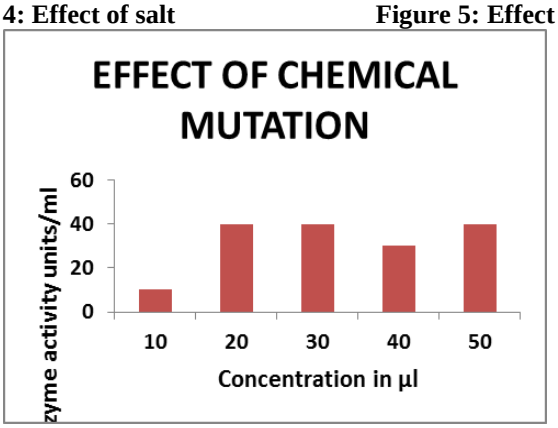

Figure 6: EFFECT OF CHEMICAL MUTATION

CHARACTERIZATION OF PECTINASE ENZYME

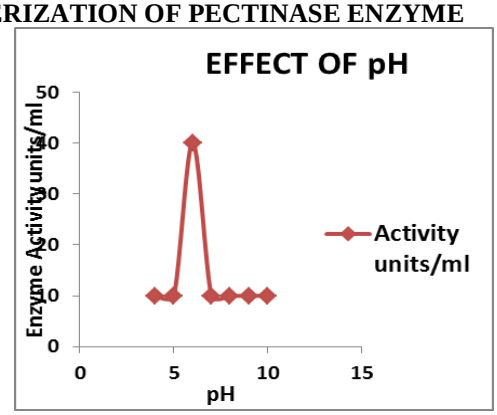

Figure 7: EFFECT OF pH

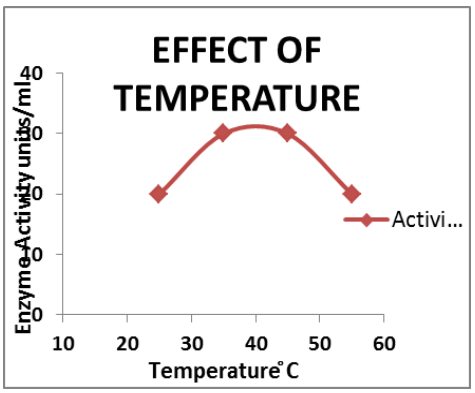

FIGURE 8: EFFECT OF TEMPERATURE

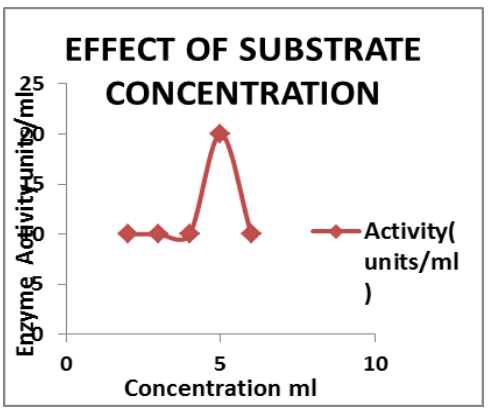

FIGURE 9: EFFECT OF CONCENTRATION

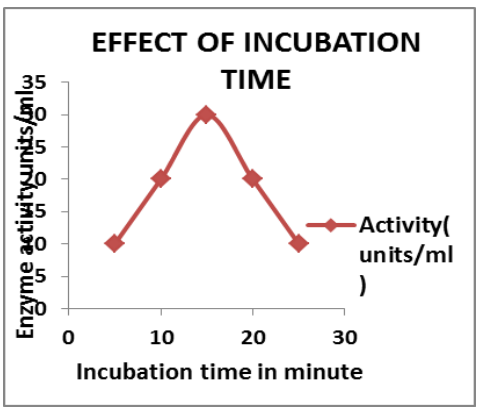

FIGURE 10: EFFECT OF INCUBATION TIME

DISCUSSION

The microscopic identification of isolated bacterial samples were carried out by Gram staining technique and confirmed that the organism was Gram Positive bacteria, short *Bacilli*.

The induced stress by different salt concentration showed varied enzyme activity in pectinase producing bacteria. The activity increased with increase in concentration of salt and further increase in concentration showed decrease in activity. The maximum activity was observed with 30mg of salt concentration.

The induced stress by UV mutation in pectinase producing bacteria showed increase in enzyme activity under different time interval of UV exposure. The maximum activity of pectinase producing bacteria was seen at 25 minutes of UV exposure. The induced stress by chemical mutation using ethidium bromide in pectinase producing bacteria showed varied enzyme activity. The maximum enzyme activity was shown as 40units/ml. Pectinase produced from *Bacillus* species had an optimum pH of 6. The activity exhibited a steady increase as the pH increased from 1-6. Further increase in pH led to decrease in the activity and it remained constant. This shows that the pectinase enzyme can be produced by maintaining the pH of 6. Pectinase enzyme produced by *Bacillus* species had an optimum temperature of 35°C. The activity exhibited a steady increase as the temperature increased from 0-45, further increase in temperature led to a decrease in activity of the enzyme. This shows that pectinase production from bacteria produces at 35^{o}C. Pectinase produced by *Bacillus* species showed an increase in activity with increase in concentration. Activity was found to increase with increase in concentration from 0-6 ml. activity was found to be maximum with 5 ml of sample concentration. Pectinase produced by Bacillus species showed an increase in activity with increase in time intervals. The activity was found to increase with increase in incubation time from 0-25 minutes. The activity reached maximum with 15 minute incubation. Further increase of incubation time lead to decrease in the enzyme activity. The molecular weight of the protein was found to be 40kDa.

CONCLUSION

The present study made a successful primary attempt to enrich and isolate the potential bacterial strain from the natural reservoir producing industrially important pectinase enzyme. The isolates which showed higher pectinase activity were selected for biochemical characterization and identification. A screening of pectinolytic productivities of the isolates showed that many of them gave good pectinolytic productivities. The isolated bacterial strains were identified as *Bacillus* species. On the basis of data obtained in the present work it shows that *Bacillus* species isolates can be employed in the production of pectinase. The production and optimization studies revealed that isolates requires 35°C, pH 6.0, 72 Hrs. of incubation time.

Thus, this study could provide a good platform for further investigations into microbial identification, biochemical characterization of enzyme and optimization of culture conditions. Further research needs to be followed in these lines, to scale up the commercial implications of these microbes.

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