COMPARATIVE STUDY OF PROTEIN CONCENTRATION AND ACTIVITY OF THE CRUDE, PRECIPITATED AND PARTIALLY PURIFIED DIALYSED ENZYME PRODUCED FROM *ASPERGILLUS NIGER* USING PINEAPPLE PEELS

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***ABSTRACT*: The agricultural wastes generated from pineapple (*Ananas cosmosus*) represents about 35% of the entire fruit. These wastes can be converted to most useful products such as pectin. Pectin was extracted from pineapple peels with a percentage yield of 8.33% at pH 2.2 and temperature of 70º C. Three pectinolytic fungi: *Aspergillus niger, Aspergillus fumigatus* and *Aspergillus flavus* were isolated from natural sources and tested for their pectinolytic activity. In submerged fermentation system containing pineapple pectin broth, *Aspergillus niger* was inoculated and incubated for 4 days. The crude enzyme pectinase was harvested after fermentation by filteration process. Crude enzyme obtained from *Aspergillus niger* was precipitated with 80% ammonium sulphate saturation. The dialysis is carried out according to Dixon and Webb (1964) for 12 hours to obtain partially purified enzyme. Then the protein concentration and activities of the crude, precipitated and dialyzed pectinase enzymes were compared.**

Keywords: Pineapple peels, Pectin, *Aspergillus niger*, Pectinase and Partial purification.

Introduction:-

Pineapple (*Ananas cosmosus*) belongs to Bromeliaceae family. This is a tropical plant and its edible fruit is a multiple fruit consisting of coalesced berries. However, processing and utilization of pineapple in to various products leads to generation of waste in the form of peels and pomace. Pineapple waste can be conventionally bio-transformed anaerobically in to humus; although valuable by-products such as pectin, dietary fibers and pectinases can be produced from the rich waste.

Pectinases are today one of the upcoming enzymes of the commercial sector. Primarily these enzymes are responsible for the degradation of the long and complex molecules called pectin that occur as structural polysaccharides in the middle lamella and the primary cell walls of young plant cells. Pectinases are a group of enzymes, which cause degradation of pectin that, are chain molecules with a rhamnogalacturonan backbone, associated with other polymers and carbohydrates. These pectinases have wide applications in fruit juice industry and wine industry.

Abbreviations:-

UDP-D- Uridinediphosphate
PDA- Potato Dextrose Agar
SmF- Submerged fermentation
SSF- Solid state fermentation
XGA- Xylogalacturonan
Xyl- Xylose
β-Gal- β-Galactosidase

History and Description of Pineapple:-

Pineapple (*Ananas comosus*) is the common name for a tropical plant and its edible fruit, which is actually a multiple fruit consisting of coalesced berries. It was given the name pine apple due to its resemblance to a pine cone. The pine apple is the most economically important plant in the Bromeliaceae family. (Coppens d'Eeckenbrugge and Leal, 2003). Besides being produced for consumption, it can be grown as an ornamental plant.

The popularity of the pineapple is due to its sweet-sour taste. The core of the pineapple is continuous with the stem supporting the fruit and with the crown, a feature unique among cultivated fruits.

Pectic Substances:-

Pectic substance is the generic name used for the compounds that are acted up on by the pectinolytic enzymes. They are high molecular weight, negatively charged, acidic, complex glycosidic macromolecules (polysaccharides) that are present in the plant kingdom. They are present as the major components of middle lamella between the cells in the form of calcium pectate and magnesium pectate (Rastogi, 1998). The synthesis of pectic substances occurs in the Golgi apparatus from UDP-D-galcturonic acid during early stages of growth in young enlarging cell walls (Sakai *et al*., 1993). Compared with young, actively growing tissues, lignified tissues have a low content of pectic substances. The content of the pectic substances is very low in higher plants usually less than 1%. They are mainly found in fruits and vegetables, constitute a large part of some algal biomass (up to 30%) and occur in low concentration in agricultural residues . Pectic substances account for 0.5–4.0% of the fresh weight of plant material (Kashyap *et al*., 2001; Sakai *et al*., 1993). Contrary to the proteins, lipids and nucleic acids, which are polysaccharides, pectic substances do not have defined molecular masses.

Structure of Pectic Substances:-

Pectic substances mainly consist of galacturonans and rhamnogalacturonans in which the C-6 carbon of galacturonic acids are oxidized to carboxyl groups, the arabinans and the arabinogalactans as seen in Fig. 1 (Whitaker, 1990). These substances are a group of complex colloidal polymeric materials, composed largely of a backbone of an hydrogalacturonic acid units (Cho*etal*., 2001; Codner, 2001). The carboxyl groups of galacturonic acids are partially esterified by methyl groups and partially or completely neutralized by sodium, potassium or Ammonium ions (Kashyap *et al*., 2001). The primary chain consists of D-galacturonic acid units linked α-(1-4), with 2–4% of L-rhamnose units linked β-(1-2) and β-(1-4) to the galacturonic acid units (Whitaker, 1990). The side chains of arabinan, galactan, arabinogalactan, xylose or fructose are connected to the main chain through their C-1 and C-2 atoms (Blanco *et al*., 1999; Sathyanarayana and Panda, 2003; Vander Vlugt-Bergmans *et al*., 2000). The above description indicates that the pectic substances are present in various forms in plant cells and this is the probable reason for the existence of various forms of pectinolytic enzymes.

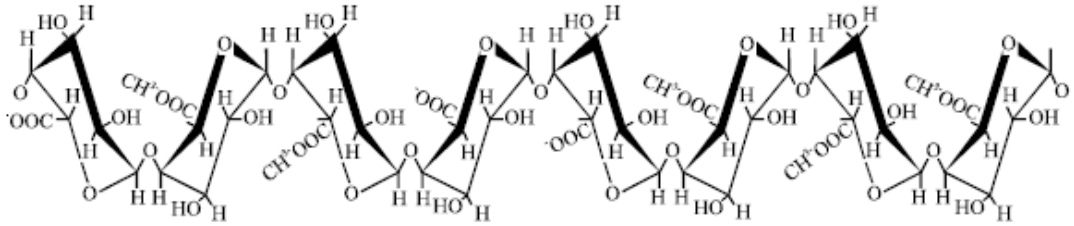

Fig. 1: Primary Structure of Pectic Substances (Pilnik andVoragen, 1993).

Pectin:-

Through various studies, it has been brought in notice that the structure of pectin is difficult to determine because pectin subunit composition can change during isolation from plants, storage and processing of plant material (Novosd' skaya, 2002). Pectin was first isolated and described in 1825 by Henri Braconnot (Braconnot and Keppler., 1825). At present, pectin is thought to consist mainly of D-galacturonic acid (Gal A) units (Sriamornsak, 2002), joined in chains by means of α(1-4) glycosidic linkage (Fig. 2). These uronic acids have carboxyl groups which are naturally present as methyl esters and others which are commercially treated with ammonia to produce carboxiamide group (Sriamornsak., 1998; Yujaroen *et al*., 2008).

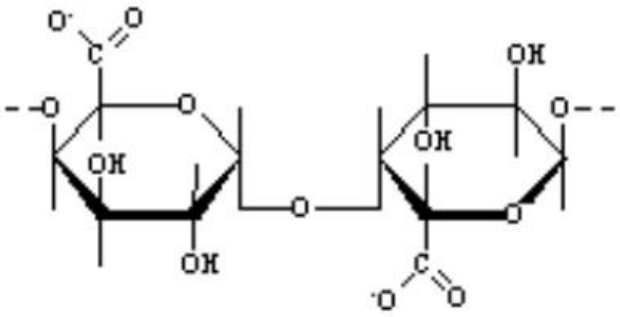

Fig. 2: Structure of Galacturonic Acid (Pilnik and Voragen, 1993)

Pectin is composed of as many as 17 different monosaccharides (Ridley *et al*., 2001; Voragen *et al*., 2003). These monosaccharides are organized in a number of distinct polysaccharides, the structures of which are schematically shown in Fig. 5a→g. Together, these polymers form the pectin network (Visser and Voragen, 1996; Ridley *et al*., 2001; Voragen *et al*., 2003).

Substrates for the Production of Pectinases:-

Substrates that are employed in the production of enzyme should be solid, as solid substrate can encourage the growing cells. Substrates should provide all needed nutrients to the microorganisms for its growth. Other factors like particle size,

moisture levels are also to be taken for consideration. Generally agro-industrial wastes are employed for the pectinase production. Various substrates that are being used are sugarcane bagasse, wheat bran, rice bran, wheat straw, rice straw, sawdust, banana waste, tea waste, sugar beet pulp, apple pomace, orange peel, etc (Pilar *et al*., 1999).

Fermentation Conditions:-

Pectinases are constitutive or inducible enzymes that can be produced either by submerged (Aquilar and Huitron, 1999) or solid state fermentation (Acuna-arguelles *et al*., 1995).Various factors affecting the production of pectinase are concentration of nutrients, pH, temperature, moisture content, influence of extraction parameters on recovery of pectinases and the effects played by the inducers. Both carbon and nitrogen sources show overall effect on the productivity of pectinases (Catarina *et al*., 2003; Almeida and Huber, 2011). Pectin, glucose and sucrose when added to the media in higher concentration have a repression effect on the studied enzyme activity (Maria *et al*., 2000) of the various nitrogenous matters that can be used. Optimum sources are $(NH4)2SO4$, yeast extract, soya bean pulp powder, soya peptone. Temperature and pH are also important parameters, where pH is regulated using a mixture of sources of nitrogen when *Aspergillus niger* is being used, pH turns to be acidic. Moisture content in the substrate also plays a significant role (Martin *et al*., 2004). The previous studies show that it was generally maintained around 50-55% for the production of pectinases by microbial means (Leda *et al*., 2000).

Two types of fermentations can be carried out for pectinase production, they are solid state fermentation and submerged fermentation. The growth of organisms is very high with large quantities of enzyme being produced in solid-state fermentation (Ramanujam and Saritha, 2008). However in the production of extracellular pectinases, submerged fermentation is preferable as the extracellular pectinases are easier and cheaper to use in great quantities. Submerged or solid state mediums are used for producing of the pectinolitic enzymes by fungi (Bali, 2003).

Types of Fermentation:-

i) Solid State Fermentation (SSF)

ii) Submerged Fermentation (SmF)

Solid state fermentation is defined as the cultivation of microorganisms on moist solid supports, either on inert carriers or on insoluble substrates that can be used as carbon and energy source. This process occurs in the absence or near absence of free water in the space between substrate particles. In this system, water is present in the solid substrate whose capacity for liquid retention varies with the type of material (Lonsane *et al*., 1985; Pandey *et al*., 2001).

Submerged liquid fermentation is the cultivation of microorganisms in liquid nutrient broth. Industrial enzymes can be produced using this process. This involves growing carefully selected microorganisms in closed vessels containing a rich broth of nutrient and a high concentration of oxygen (Grigelmo-Migeul and Martin- Belloso, 1998).

There are several disadvantages of SSF which have discouraged the use of this technique for industrial production and therefore have made SmF more applicable in the production of enzymes. These include: the buildup of gradients of temperature, pH, moisture, substrate concentration or CO_2 during cultivation which are difficult to control under limited water availability (Holker *et al*., 2004).

Aim and Objectives of the Study:-

- To precipitate the extracellular pectinase produced by *Aspergillus niger* using ammonium sulphate precipitation method.
- To partially purify the enzyme by dialysis.
- To compare the protein concentration and activity of the crude, precipitated and partially purified pectinase enzyme.

MATERIALS AND METHODS

Chemicals/ Reagents

All the chemicals used in this research work were of analytical grade.

Equipments:-

Autoclave, Centrifuge, Magnetic stirrer, Microscope, Milling machine, Oven, pH meter, Water bath, Weighing balance.

Galacturonic Acid Standard Curve:-

The reaction mixture contained 0.0-1.0ml of galacturonic acid stock solution in test tubes arranged in triplicates. Each test tube was made up to 1ml using freshly prepared 0.05M sodium acetate buffer of pH 5.0. 1ml of DNS reagent was added to each of the test tubes and placed in a boiling water bath for 10min. 1ml of 1.4M Rochelle salt (sodium potassium tartarate) was added to the test tube immediately after heating and the total volume of the solution was adjusted to 4ml with distilled water. The mixture was cooled to room temperature and the absorbance read at 575nm. The concentration of reducing sugar in each of the tubes was calculated using the formula

$$C_1 V_1 = C_2 V_2$$

C_1 = initial concentration of reducing sugar (mM).

C_2 = final concentration of reducing sugar (mM).

V_1 = initial volume of 20mM galacturonic acid preparation measured into the tube.

V_2 = final volume of the preparation.

Using the values obtained from above the calculations, the plot of optical density was constructed and the concentration of galacturonic acid released at a given absorbance was extrapolated (Appendix Two).

Polygalacturonase Assay:-

Polygalacturonase activity was determined by measuring the release of reducing sugars from the pineapple pectin using the 3,5-dinitrosalicyclic acid reagent assay as described by Miller, (1959).

Procedure for Polygalacturonase Assay:-

This was carried out by the method described by Miller, (1959) as contained in Wang *et al.*, (1997) with slight modifications. The reaction mixture contained 0.5ml of 0.5% pineapple pectin in 0.05M sodium acetate buffer pH 5.0 and 0.5ml of the crude enzyme solution. After one hour incubation time, 1ml of DNS reagent was added and the reaction was stopped by boiling for 10mins at 70ºC.

The total volume was brought up to 4ml by adding 1ml of rochelles salt and 1ml of distilled water. The reaction mixture was allowed to cool and then absorbance was read at 575nm. One unit of enzyme was defined as the amount of enzyme that catalyzes the formation of 1 micromole of galacturonic acid per minute.

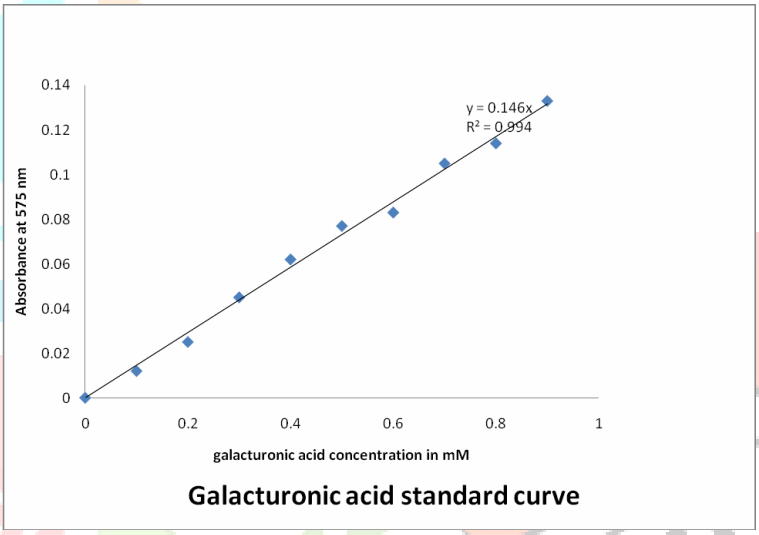

Fig.3: Galaturonic Acid Standard Curve, Using 20mM D-(+)-Galacturonic Acid Monohydrate

Protein Determination:-

Protein content of the enzyme was determined by the method of Lowry *et al.* (1951), using Bovine Serum Albumin as standard.

Procedure for Protein Determination:-

For the reaction mixture, test tubes were arranged in duplicates containing 0.0- 1.0 ml of 0.2mg of protein stock solution (2mg/ml BSA) and brought up to 1ml with distilled water. For the test mixture, 0.5ml of sodium acetate buffer pH 5.5 was added to 0.5ml of the crude enzyme. To both the reaction and test mixture, 5 ml of solution D was added soon after and the mixture was allowed to stand for 10 mins. 0.5ml of Solution C (dilute Folin-ciocalteau reagent) was added and then the solution was mixed thoroughly and allowed to stand for 30mins under room temperature. The absorbance was read at 750nm and the protein concentration was determined.

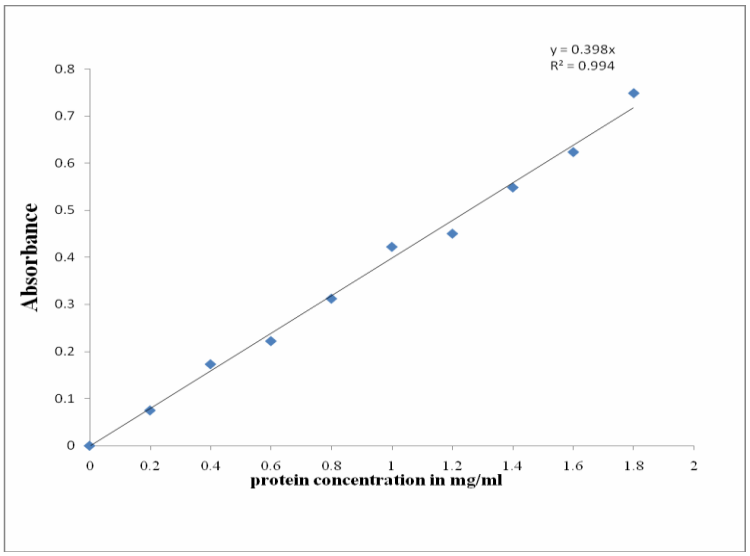

Fig.4: Protein Standard Curve, Using 2mg/ml Bovine Serum Albumin (BSA)

Partial Purification of Protein:-

Ammonium Sulphate Precipitation Profile:-

This procedure is carried out in order to know the percentage of ammonium sulphate concentration is suitable to precipitate the most protein from the crude enzyme. Nine test tubes were used containing 10ml of the crude enzyme and the enzyme was precipitated from 20% - 100% saturation of solid ammonium sulphate at 10% interval in each test tube.

The contents of the tubes were mixed thoroughly to ensure that the salts were dissolved and then allowed to stand for 30hrs at 4ºC. The test tubes were centrifuged at 3500 rpm for 30 mins and the filtrates were decanted while the pellets were re-dissolved in equal volume of 0.05M sodium acetate buffer pH 5.0. Pectinase activity was determined on the contents of each tube.

Ammonium Sulphate Precipitation:-

After determining the percentage saturation of ammonium sulphate salts that gave the highest activity, the equivalent amount of salt for 1 litre of crude enzyme is added. The salt is allowed to dissolve completely and the mixture is allowed to stand for 30 hrs at 4ºC. It is then centrifuged at 3500 rpm for 30mins. The pellets are collected and stored in a cool place for further studies.

Dialysis:-

Dialysis tubes stored in 90% ethanol were used. However the tubes were rinsed thoroughly with distilled water and finally with 0.05M sodium acetate buffer in order to remove traces of ethanol. An amount of the precipitated enzyme is poured into the dialysis tubes and placed in a beaker containing 0.05 M sodium acetate buffer. The beaker is placed on a magnetic stirrer which allows for a homogenous environment. The dialysis is carried out according to Dixon and Webb (1964) for 12 hours and the buffer is changed after 6 hours which allows for the exchange of low molecular weight substances and left over ammonium sulphate salts that may interfere with the activity. After dialysis, the partially purified enzyme is stored frozen at -24ºC.

Result and Analysis:-

Pineapple Pectin Extraction:-

Pectin extraction yield was found to be 8.33% at pH 2.2, temperature of 70^{O} C and extraction time of 1 hour.

Ammonium Sulphate Precipitation:-

Crude enzyme obtained from *Aspergillus niger* was precipitated within the range of 20-100% at an interval of 10%. At 80% ammonium sulphate saturation, the highest activity was obtained at 81.62U/ml as shown in Fig.5: thus, the percentage was used for the precipitation of pectinases.

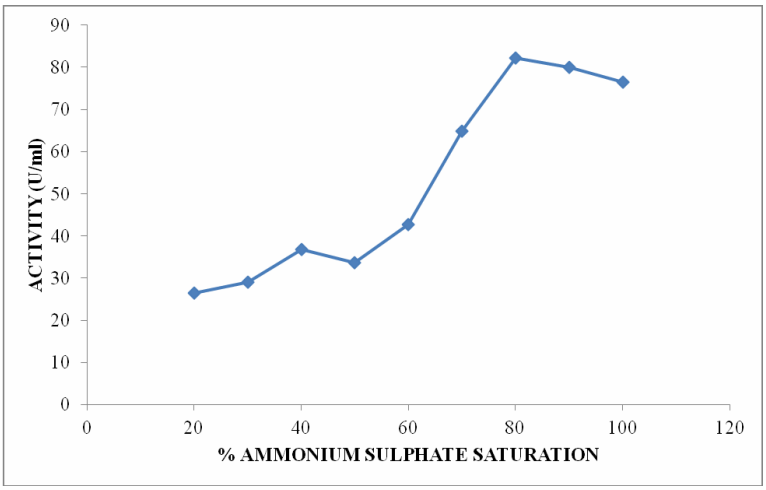

Fig.5: Ammonium sulphate precipitation profiling of pectinases obtained from *Aspergillus niger*.

Assays Carried Out on Pectinase Obtained:-

Protein Concentration of the Crude, Precipitated and Dialyzed Pectinases:-

In Fig.6: initial protein concentration of pectinase in the crude state was 10.42mg/ml but after ammonium sulphate precipitation and dialysis, the concentration increased to 15.82mg/ml and later decreased to 12.84mg/ml.

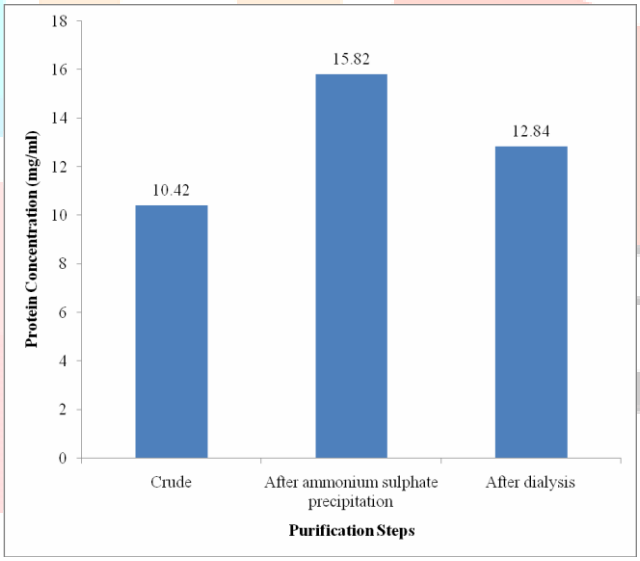

Fig.6: Comparison in the protein concentration of the crude, precipitated and dialyzed pectinases

Activity of Pectinase in the Crude, Precipitated and Dialyzed Enzyme:-

The activities of the enzymes increased progressively from one purification step to the other with the corresponding values of 116.12U/ml, 272.98U/ml and 634.56U/ml respectively as shown in

Fig. 7.

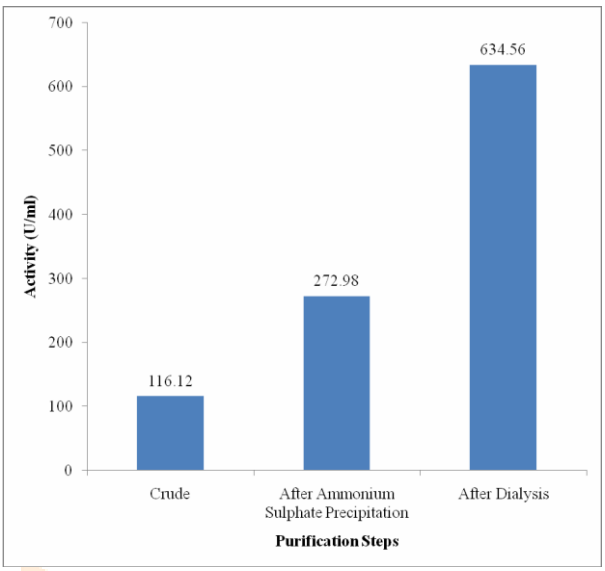

Fig.7: Comparison in the activities obtained from the crude, precipitated and dialyzed pectinases

Total Protein Content of the Crude, Precipitated and Dialyzed Enzymes:-

The total protein content of the crude enzyme reduced from the crude to the precipitated and the dialyzed enzyme with the respective values of 10420mg, 1012.48mg and 48226.56mg as seen in Fig.8.

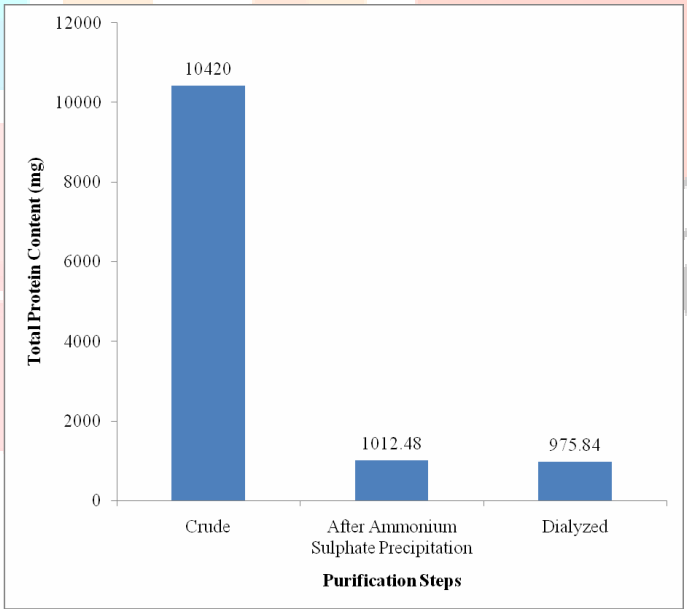

Fig.8: Comparison in the total protein content of the crude, precipitated and dialyzed pectinases

Table 1: Summary of the Parameters Determined From the Crude, Precipitated and Dialyzed Pectinases:-

Purification Step	Volume (ml)	Protein Conc. (mg/ml)	Activity (U/ml)	Total Protein (mg)
Crude Enzyme	**1000**	**10.42**	**116.12**	**10420**
80% ammonium Sulphate	**64**	**15.82**	**272.98**	**1012.48**
Dialyzed Enzyme	**76**	**12.84**	**634.56**	**975.84**

µmole/min= Unit (U)

According to Table 1, the crude state of pectinase had the least value for activity of 116.12U/ml unlike the precipitated and dialyzed, which were 272.98U/ml and 634.56U/ml respectively. Also, the protein concentration increased after ammonium sulphate precipitation due to the precipitated proteins. However, the protein concentration reduced after dialysis to 12.84mg/ml. Thus it can be said that protein concentration is inversely relative to activity.

Discussion:-

Pineapple (*Ananas cosmosus*) peels as agricultural wastes represent about 35% of the fruit mass. During extraction of pectin from pineapple peels, the yield of pectin extracted was 8.33% at pH2.2, temperature of 70º C and extraction time of 1 hour using the method as described by Mc.Cready (1970). The yield could be affected by the pH of the extraction medium and extraction time.

Three fungal species *Aspergillus niger* was isolated from natural waste source selected including 2 other sps ie.,*Aspergillus fumigatus* and *Aspergillus flavus* which showed low pectinase activity in the fermentation process when compared to *A.niger*. In a fermentation process substrate should provide all nutrients needed to the microorganisms for its growth. The accumulation of maximum extracellular pectinase was observed after 96 hours of fermentation. The period of fermentation depends on the nature of medium, fermenting organisms, concentration of nutrients and the process physiological conditions (Patil and Dayanand, 2006).

Submerged fermentation is the cultivation of microorganisms in liquid nutrient broth. In a submerged fermentation microorganisms are grown in closed vessels containing a rich broth of nutrients and a high concentration of oxygen (Grigelmo - Migeul and Martin-Belloso, 1998).

Ammonium sulphate precipitation profile was carried out on the crude enzyme and the activities of the pellets obtained from the different percentages of saturation from 20%-100% was determined. Fig.5 shows that 80% had the highest activity and was therefore used for the actual precipitation process of the enzyme. Buga *et al*. (2010) reported 70% ammonium sulphate saturation for pectinase from *Aspergillus. niger* (SA6) while Adejuwon and Olutiola, 2007 reported 90% ammonium sulphate saturation for pectinase from *Lasidioplodia theobromae*.

After the protein was precipitated, one way to remove this excess salt is to dialyse the protein. Also it changes the buffer composition of solutions of biomolecules too large to pass through the membrane (Rosenberg, 2004). It was observed that there was an increase in volume of the enzyme after dialysis which was done for 12 hours; this may be due to the buffer that entered from the dialyzing medium during the process.

The protein concentration increased from 10.42 to 15.82 mg/ml after ammonium sulphate precipitation and then there was a decrease in the concentration to 12.84mg/ml after dialysis as seen in Fig.6. This may be due to the removal of other proteins of lower molecular weight during dialysis that was not the protein of choice.

Fig.7 shows the pectinase activity which increased from 116.12 to 272.98 U/ml after precipitation and further increased to 634.56 U/ml after dialysis. This may be due to the removal of impurities during dialysis such as other proteins which may have affected the enzyme activity negatively (Lukong *et al*., 2007). Fig. 8 shows the total protein content of the crude enzyme reduced from the crude to the precipitated and the dialyzed enzyme.

Conclusion:-

From these investigations it is evidenced that the pineapple peels with 8.33% pectin content were successfully used to induce the production of pectinase under submerged fermentation process. By Ammonium sulphate precipitation method and by dialysis the partially purified pectinase enzyme pellets has shown the increase in concentration and activity comparatively. The enzymes obtained can be industrially used in the production of fruit juice, paper making, retting of plant fibers, etc. Ultimately, the rationale behind this research was the conversion of waste to wealth which could increase the revenue base of any establishment or country obtained and also geared towards a cleaner and safer environment.

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