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Determination of Various Parameters of Crude and Partially Purified Dialysed Pectinase 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 70° 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 and dialyzed pectinase enzymes were compared.

Keywords : Pineapple Peels, Pectin, Aspergillus niger, Pectinase and Partial Purification.

I. 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

II. HISTORY AND DESCRIPTION OF PINEAPPLE

Pineapple (Ananas cosmosus) 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. The word "pineapple" in English was first recorded in 1398, when it was originally used to describe the reproductive organs of conifer trees. The term pine cone for the productive organ of conifer trees was first recorded in 1694. When European explorers discovered this tropical fruit, they called them pineapples (Wikipedia, 2011). 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. The stems and leaves of the pineapple plant are sources of fiber, which can be processed in to paper and cloth. The cloth made from pineapple fiber is known as 'pina cloth'and was in use as early as 1571. Parts of the pineapple plant (Fig.1) are used as silage and hay for cattle feed such as the processed wastes in the form of pomace or centrifuged solids from juice production (Wikipedia, 2011).

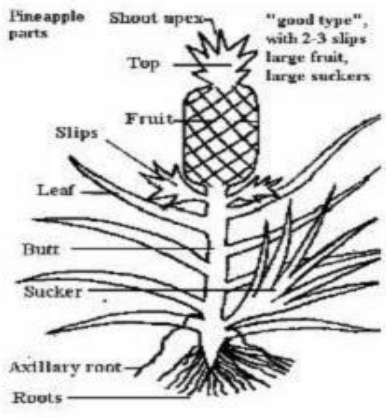

Figure 1. Parts of a pineapple fruit (Elfick, 2007)

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.

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).

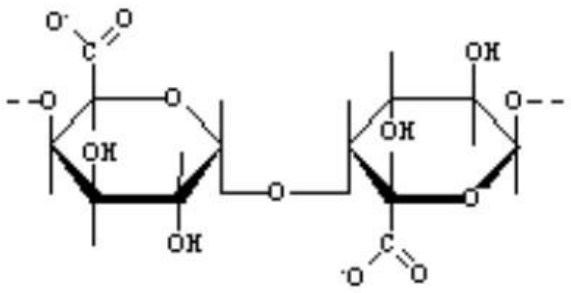

Figure 2. Structure of Galacturonic Acid (Pilnik and Voragen, 1993)

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

1. Solid State Fermentation (SSF)
2. 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 CO2 during cultivation which are difficult to control under limited water availability (Holker et al., 2004).

MICROORGANISMS COMMONLY USED IN SUBMERGED AND SOLID STATE FERMENTATION FOR PECTINASES PRODUCTION

Microorganisms are currently the primary source of industrial enzymes: 50% originate from fungi and yeast; 35% from bacteria, while the remaining 15% are either of plant or animal origin. Filamentous microorganisms are most widely used in submerged and solid-state fermentation for pectinase production. Ability of such microbes to colonize the substrate by apical growth and penetration gives them a considerable ecological advantage over non-motile bacteria and yeast, which are less able to multiply and colonize on low moisture substrate (Smith and Aidoo, 1988). Among filamentous fungi three classes have gained the most practical importance in SSF; the phycomycetes such as genera Mucor, the ascomycetes genera Aspergillus and Basidiomycetes especially the white and rot fungi (Young et al., 1983). Bacteria and yeasts usually grow on solid substrates at the 40% to70% moisture levels (Young et al., 1983). Common bacteria in use are (Bacillus licheniformis, Aeromonas cavi and Lactobacillus) and common yeasts in use are Saccharomyces and Candida. Pectinase production by Aspergillus strains has been observed to be higher in solid-state fermentation than in submerged process (Solis et al., 1996).

AIM AND OBJECTIVES OF THE STUDY

- ✓ To partially purify the enzyme by dialysis.
- ✓ To compare the protein concentration, total activity, specific activity and purification fold of the crude and partially purified pectinase enzyme.

III. 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.

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 0.2 mg of protein stock solution (2 mg/mL BSA) and brought up to 1 mL with distilled water. For the test mixture, 0.5 mL of sodium acetate buffer pH 5.5 was added to 0.5 mL 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 min. 0.5 mL of Solution C (dilute Folin-ciocalteau reagent) was added and then the solution was mixed thoroughly and allowed to stand for 30 min under room temperature. The absorbance was read at 750nm and the protein concentration was determined.

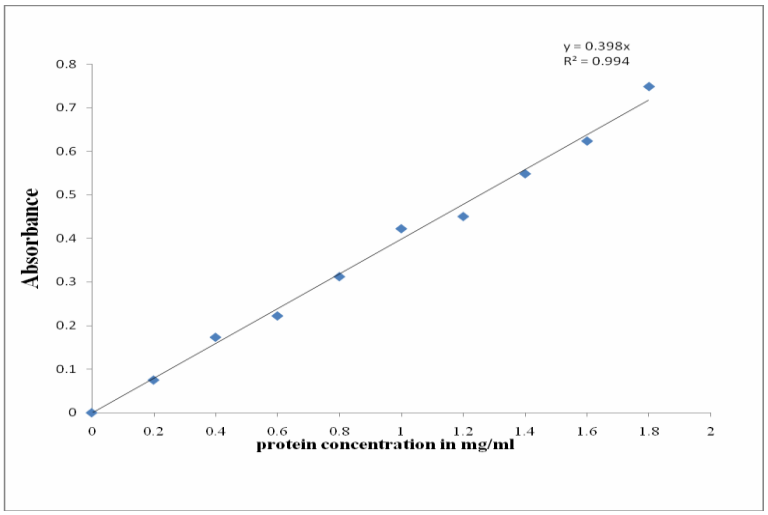

Figure 3. Protein Standard Curve, Using 2 mg/mL Bovine Serum Albumin (BSA)

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 10 mL 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 30h at 4°C. The test tubes were centrifuged at 3500 rpm for 30 min 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 h at 4°C. It is then centrifuged at 3500 rpm for 30 min. 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 0.05M 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 -24°C.

IV. RESULT AND ANALYSIS

Pineapple Pectin Extraction:-

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

Selection of Pectinolytic Fungi:-

Three species of fungi namely: Aspergillus niger, Aspergillus fumigatus and Aspergillus flavus were isolated from natural source of soil containing

decaying pineapple peels. These organisms were qualitatively screened for pectinolytic activity on selective media and their isolation was based on the similarities of their morphological features in both test cultures containing pineapple pectin and the standard culture containing apple pectin as carbon respectively.

Macroscopic and Microscopic Features of Fungal Isolates:-

Genus identification was by examining both macroscopic and microscopic features of a three day old pure culture. Color, texture, nature of mycelia and/or spores produced, growth pattern in addition to microscopic features such as separation and spore shapes were examined. Based on these characteristics, Aspergillus niger, Aspergillus fumigatus and Aspergillus flavus were confirmed as the three pectinolytic fungal isolates, respectively. However, Aspergillus niger showed relatively higher pectinase activity and was selected for further studies.

Pectinase Production under Submerged Fermentation System:-

A volume of 2 litres of crude enzyme was harvested after 4 days of submerged fermentation using Aspergillus niger.

Ammonium Sulphate Precipitation:-

Crude enzyme obtained from Aspergillus niger was precipitated within the range of 20-100% at an interval of 10%.

Assays Carried Out on Pectinase Obtained:-

Protein Concentration of the Crude and Dialyzed Pectinases

In Figure 4: initial protein concentration of pectinase in the crude state was 10.42 mg/mL but after dialysis, the concentration increased to 12.84 mg/mlL.

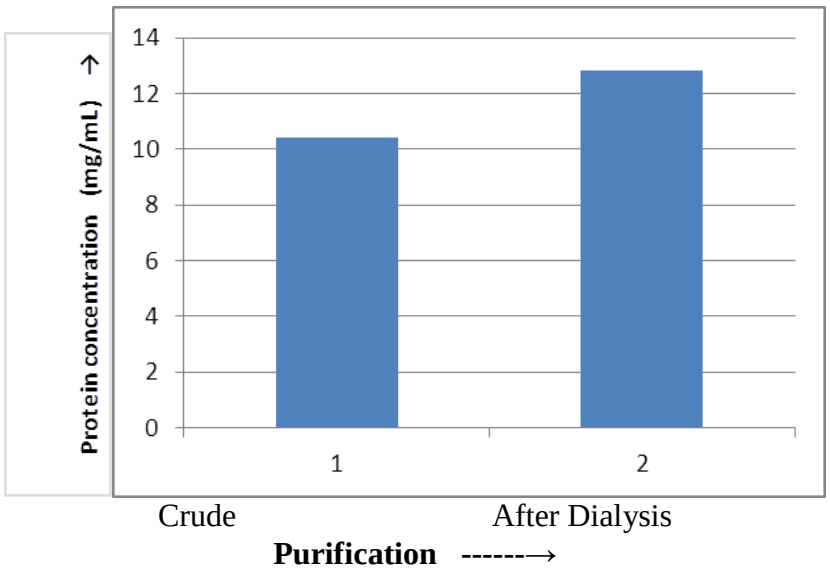

Figure 4. Comparison in the protein concentration of the crude and dialyzed pectinases

Activity of Pectinase in the Crude and Dialyzed Enzyme

The activities of the enzymes increased from one purification step to the other with the corresponding values of 116.12 U/mL and 634.56 U/mL respectively as shown in Figure 5.

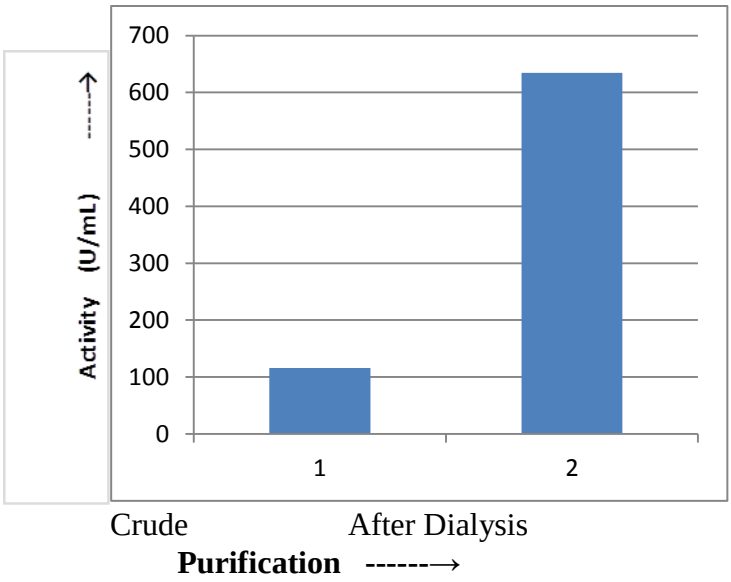

Figure 5. Comparison in the activities obtained from the crude and dialyzed pectinases

Total Protein Content of the Crude and Dialyzed Enzymes

The total protein content of the crude enzyme reduced from the crude to the dialyzed enzyme with the respective values of 10420 mg and 48226.56 mg respectively as seen in Figure 6.

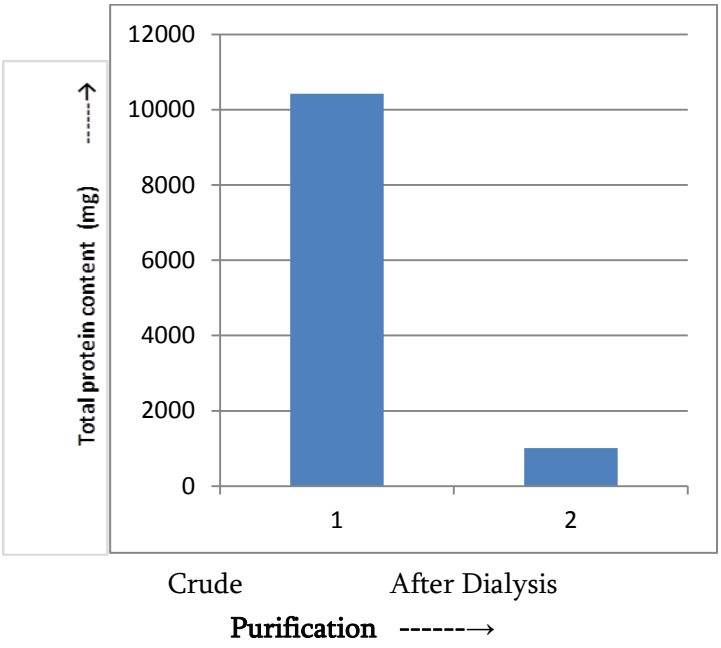

Figure 6. Comparison in the total protein content of the crude and dialyzed pectinases

Total Activity of the Crude and Dialyzed Enzymes

Figure 7 shows activity-dependent decrease from the crude enzyme across dialysed sample. The crude enzyme had the highest total enzyme activity of 116120 U with after dialysed sample having the least enzyme activity of 4822.56 U.

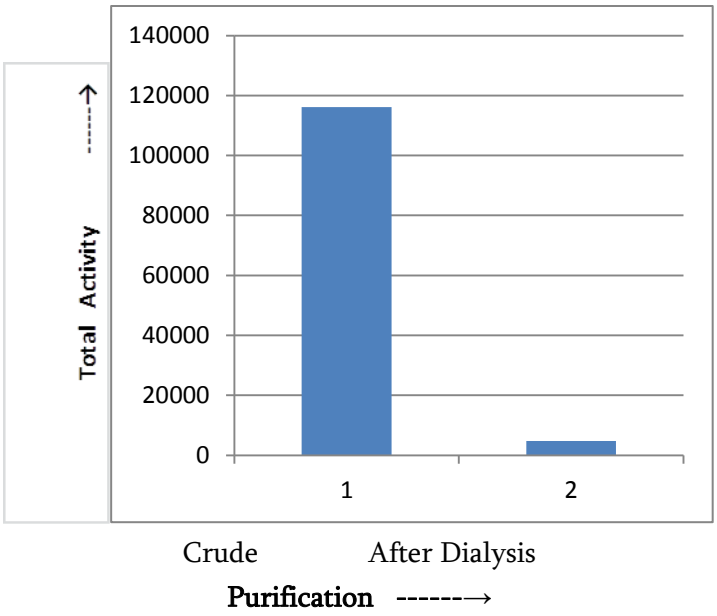

Figure 7. Comparison in the total activity of the crude and dialyzed pectinases

Specific Activity Determined on the Crude and Dialyzed Enzymes

Specific activity-dependent increase was observed in Figure 8 with crude enzymes having the lowest activity and after, dialyzed sample had the highest enzyme activity with values of 11.14 U/mg and 49.42 U/mg respectively. This further explains that the enzyme was in a purer state after dialysis.

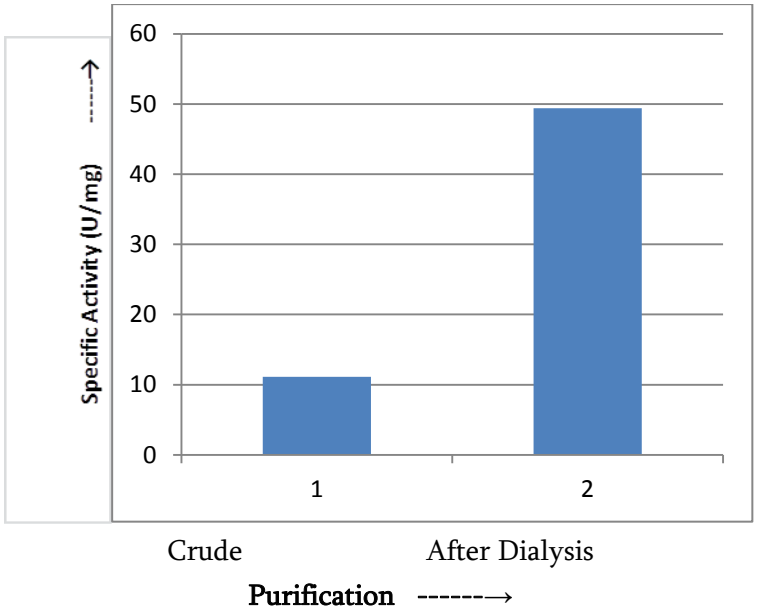

Figure 8. Comparison in the Specific Activities of the crude, and dialyzed pectinases

Purification Folds of the Partially Purified Enzymes

As shown in Figure 9, after dialysis the purification fold increased from 1.55 after ammonium sulphate precipitation and further increased to 4.43 after dialysis which infers that the enzyme had undergone a 4.43 purification fold.

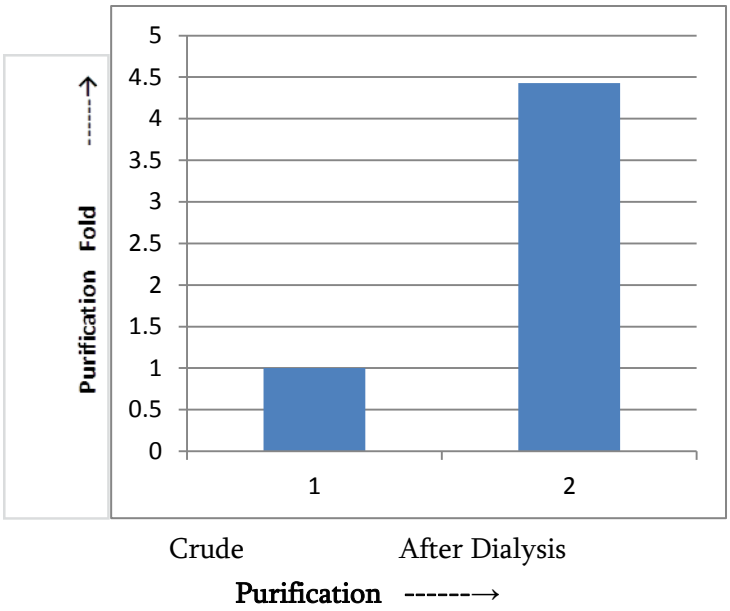

Figure 9. Purification folds of the pectinase after dialysis

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

Purification Step	Crude Enzyme	Dialyzed Enzyme
Volume (mL)	1000	76
Protein Conc. (mg/mL)	10.42	12.84
Activity (U/mL)	116.12	634.56
Total Protein(mg)	10420	975.84
Total Activity (U)	116120	48226.56
Specific Activity (U/mg)	11.14	49.42
Purification fold	1	4.43

μ mole/min= Unit (U)

According to Table 1, the crude state of pectinase had the least value for activity of 116.12 U/mL unlike the dialyzed, which was 634.56 U/mL. However, the protein concentration increased due to the precipitated proteins. However, the protein concentration increased after dialysis to 12.84 mg/mL.

V. 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 70° 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).

The protein concentration increased from 10.42 to 12.84 mg/mL after dialysis as seen in Figure 4. This may be due to the removal of other proteins of lower molecular weight during dialysis that was not the protein of choice.

Figure 5 shows the pectinase activity which increased from 116.12 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). Figure 8 shows the total protein content of the crude enzyme reduced from the crude to the dialyzed enzyme.

The total protein content of the crude enzyme reduced from the crude to the dialyzed enzyme with the respective values of 10420 mg and 48226.56 mg respectively as seen in Figure 6.

Figure 7 shows activity-dependent decrease from the crude enzyme across precipitated enzyme to after dialysed sample. The crude enzyme had the highest total enzyme activity of 116120 U with after dialysed sample having the least enzyme activity of 4822.56 U. Specific activity-dependent increase was observed in Figure 8 with crude enzymes having the lowest activity and after, dialyzed sample had the highest enzyme activity with values of 11.14 U/mg and 49.42 U/mg respectively. This further explains that the enzyme was in a purer state after dialysis.

As shown in Figure 9, after dialysis the purification fold increased from 1.55 to 4.43 after dialysis which infers that the enzyme had undergone a 4.43 purification fold.

VI. 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 dialysis method the partially purified pectinase enzyme has shown the increase in protein concentration, total activity and specific activity and in purification fold when compared with the crude enzyme. 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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