Determination and Partial Purification of Pectinase Enzyme Produced by Aspergillus Niger using Pineapple Peels as Sole Source of Carbon

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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. The *Aspergillus niger was isolated* from soil containing decomposing pineapple peels and was induced to produce extracellular pectinase in submerged fermentation using pectin extracts from pineapple peels. Then from the extracted enzyme protein concentration was determined and partially purified by ammonium sulphate precipitation and dialysis method.

Keyword- Pineapple peels, Pectinase, *Aspergillus niger,* and Dialysis

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. Pineapple waste can be bio-transformed in to by-products such as pectin, dietary fibers and pectinases. Pectin is one of the major components of the primary cellular walls in the middle lamella of plant tissues. Pectin was first isolated and described in 1825 by Henri Braconnot (Bracconot and Keppler, 1825. Pectinases can be produced by both submerged and solid state fermentation (SSF).

Submerged fermentation is cultivation of microorganisms in liquid broth. It requires high volumes of water, continuous agitation and generates lot of effluents. SSF incorporates microbial growth and product formation on or within particles of a solid substrate (Mudgett, 1986) under aerobic conditions.

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. In fruit juice industry, it is used for clarification; reduction in viscosity is caused which ultimately leads to formation of clear juice.

Abbreviations

UDP-D- Uridinediphosphate
PDA- Potato Dextrose Agar
SmF- Submerged fermentation
SSF- Solid state fermentation
BSA-Bovine serum albumin

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 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).

Plant Cell Wall

Plant cell walls consist of plant middle lamella, primary cell wall and secondary cell wall. The primary walls of enlarging plant cells are composed of approximately 30% cellulose, 30% hemicellulose and 35% pectin with about 1-5% structural protein

(glycoprotein) on a dry weight basis (Cosgrove, 1997).

The Middle Lamella of the Fruit Cell

The middle lamella is the first layer formed during cell division, and can also be seen as the space between the cell walls, and as the connecting region between adjacent cells, binding cells together. The highest concentrations of pectin are found in the middle lamella of cell walls, with a gradual decrease as one passes through the primary wall toward the plasma membrane (Kertesz, 1951).

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 (Table:1). 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.1).

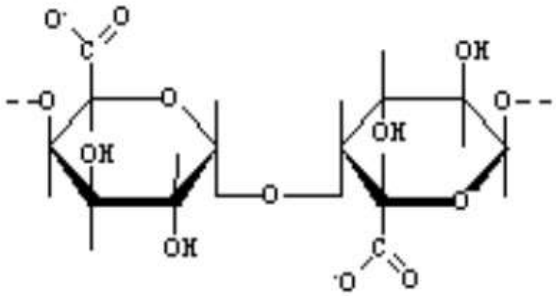

Figure.1: Structure of Galacturonic Acid (Pilnik and Voragen, 1993)

Types of Pectin

The percentage of ester groups is called degree of esterification. High methyl ester pectins are classified in groups according to their gelling temperature as rapid set to slow set pectins. Pectin as extracted normally has more than 50% of the acid units esterified, and is classified as "high methyl ester (HM) pectin" as shown in Fig. 2.

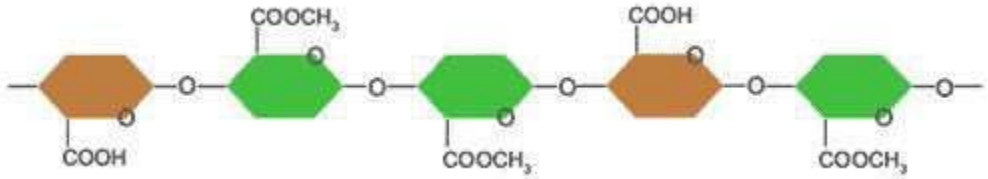

Fig. 2: HM pectin formula (IPPA, 2001)

Modification of the extraction process, or continued acid treatment, will yield "low methyl ester LM pectin" with less than 50% methyl ester groups as observed in Fig. 3.

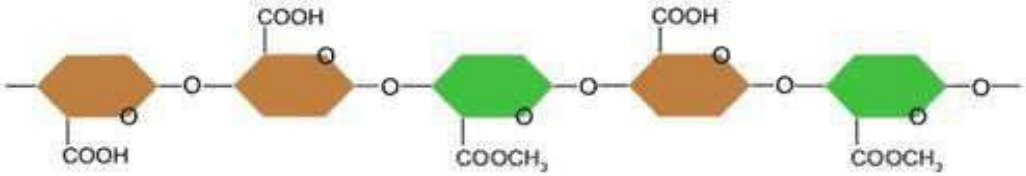

Fig. 3: LM pectin formula (IPPA, 2001)

Some pectins are treated during manufacture with ammonia to produce amidated pectins, which have particular advantages in some applications are observed in Fig.4.

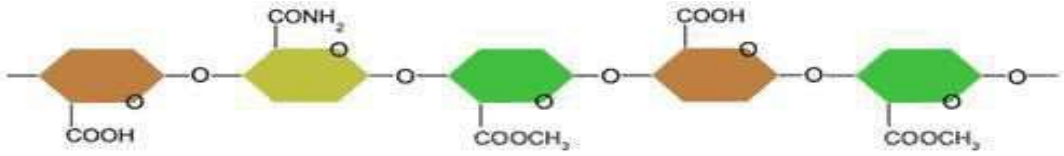

Fig. 4: Amidated pectin formula (IPPA, 2001)

General Properties of Pectins

Pectin is soluble in pure water as monovalent cation (alkali metal) salts of pectinic and pectic acids; are usually soluble in water unlike di- and trivalent cation salts that are weakly soluble or insoluble (Sriamornsak, 1998). Dilute pectin solutions are Newtonian but at a moderate concentration, they exhibit the non-Newtonian, pseudo plastic behaviour and characteristics.

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 $CO2$ during cultivation which are difficult to control under limited water availability (Holker *et al*., 2004).

Aim and Objectives of the Study

- To produce extracellular pectinase by inducing *Aspergillus niger* in submerged fermentation with pectin extracts from pineapple peels.
- To precipitate the extracellular pectinase by carrying out ammonium sulphate precipitation.
- To partially purify the enzyme by dialysis.

MATERIALS AND METHODS

Chemicals/ Reagents

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

Apparatus/ Equipment

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

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 30 hrs at 4ºC. 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 hr at 4ºC. It is then centrifuged at 3500 rpm for 30min. 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.

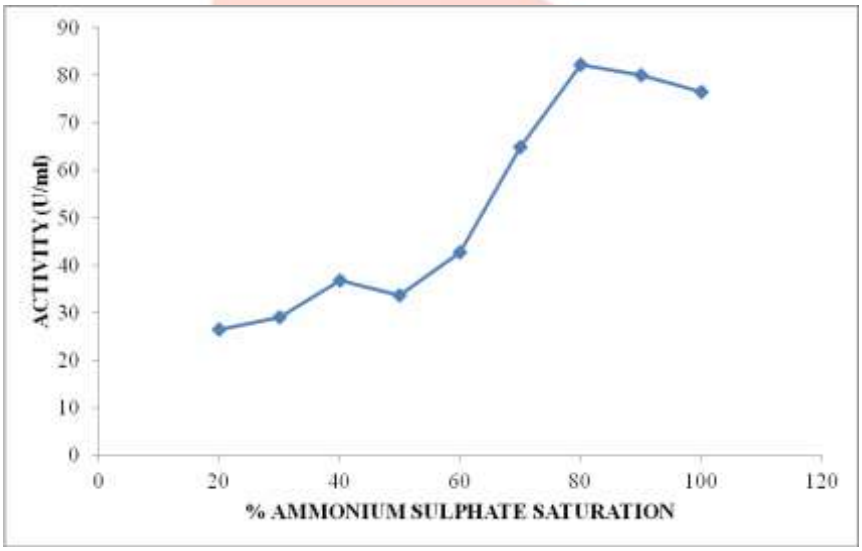

Fig.5: Ammonium sulphate precipitation profiling of pectinases obtained from *Aspergillus niger*.

Dialysis:-

By using dialysis method the precipitayed enzyme is partially purified and stored at -24ºC for further research.

Discussion:-

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. 12 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 dialyze 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.

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. Thus the crude Pectinase enzyme obtained was

partially purified by ammonium precipitation and dialysis methods and can be further characterized for its purity and activity at various physiological conditions. The enzymes obtained can be industrially used in the production of fruit juice, paper making, retting of plant fibres, 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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