INTRODUCTION

1- Microbial enzymes in relation to their activities:

Most of the chemical changes that occur in living tissues are regulated by enzymes. Since all living cells produce enzymes, these are obtainable from plant and animal tissues and microorganisms. The quantities of enzymes produced on a commercial scale from plant and animal source are very considerable, but for both technical and economical reasons, microbial enzymes have become more important (Beckhorn, 1960).

There are many criteria that have to be considered: The organisms must grow and produce enzymes on cheap and readily available, low cost material, it must produce enzymes in high amount, the extracellular crude enzyme must be simple to be isolated with a good recovery from the fermentation broth. The organism must be non pathogenic, it should not produce any other secondary biological active material or toxins (Keay, 1972).

2- Pectinases and pectic substances:

Pectinases form the group of enzymes that degrade pectic substances, which are the structural polysaccharides present in vegetable cells, responsible for maintaining the integrity of plant tissues. Pectic substances are characterized by long chains of galacturonic acid residues. On these residues are carboxyl groups, which are sometimes modified by the addition of methyl groups, forming methoxyl groups. Pectic enzymes act by breaking glycosidic bonds of the long carbon chains (polygalacturonase,

pectin lyase and pectate lyase) and by splitting off methoxyl groups (pectin esterase) (Castilho *et al*., 1999).

Pectic and cellulosic substances are the most abundant carbohydrates present in plants. Pectic substances like pectin, protopectin and pectic acids, present in cell wall and middle lamella, contribute firmness and structure to plant tissues. In pectic substances, D-galacturonic acid units are linked together by α-1,4-glycosidic linkages and the carbonyl side groups are 60-90% estrified with methanol. The rhamnose units can be inserted into the main uronide chain and often side chains of arabinan, galactan or arabinogalactan are linked to rhamnose. This indicates that various forms of pectic substances are present in plant cells and for this reason pectinases exist in various forms. Pectinases degrade pectic substances for nutritional purposes and they are responsible for plant pathogenesis. Pectic substances are widely distributed in fruits and vegetables (10-30 % in turnips, peels of orange and in pulps of tomato, pineapple and lemon), hence they form important natural substrates for pectinases (Gummadi and Panda, 2002).

Enzymes which degrade pectic substances are pectinases or pecteolytic enzymes and can be classified into three types. Pectin methyl esterase (PME) hydrolyzes the methyl ester of galacturonide chain liberating methanol. Polygalacturonases (PG) and pectate lyases (PL) split the molecular chains of the respective polymers (Goodman *et al.*, 1986 and Agrios, 1988;).

The array of pecteolytic enzymes is even more complex than the types of molecules they depolymerize. According to Chesson

(1980), the galacturonan chain of the pectic substances is degraded by two groups of enzymes distinguished by the site attached; pectinesterase (PE), responsible for saponification of esterified regions of the chain and the depolymorases responsible for chain cleavage.

The major classes of pectolytic enzymes are listed in table (i):

Noting that:

- Pectin methylesterase: pectin dimethylase that hydrolyzes the methoxyl groups from the 6-carboxyl groups of the galacturonate residues in the pectin chain (Voragen and Pilnik, 1989).
- Protopectinase: Polygalacturonase that hydrolyzes protopectin (the water-insoluble, highly cross-linked parent compound of the pectic substances found in the middle lamella of plants) to a water-soluble form with degrees of polymerization.
- Endopolygalacturonase: Poly(1,4-α-D-glacturonide) glycan-hydrolyase that randomly cleaves polygatacturonates to yield a series of intermediate oligomers.
- Exopolygalacturanase: Poly (1,4-α-D-galacturonide) galacturonohydrlase that cleaves polygalacturonates from the non reducing ends to yield either galacturonate or digalacturonate.
- Pectate lyases: Poly-1,4-D-galacturonide lyases, acting in either an endo or exo manner, that cleave polygalacturonaes via a trans elimination mechanism to yield to double-bonded 4-deoxy-L-threo-5-hexosculose uronic acid.

Table Wide

In general pectinases are classified into de-esterification and depolymerizing enzymes based on the degradation mechanism. Different pectic substances and their mode of reaction are illustrated in fig.(i).

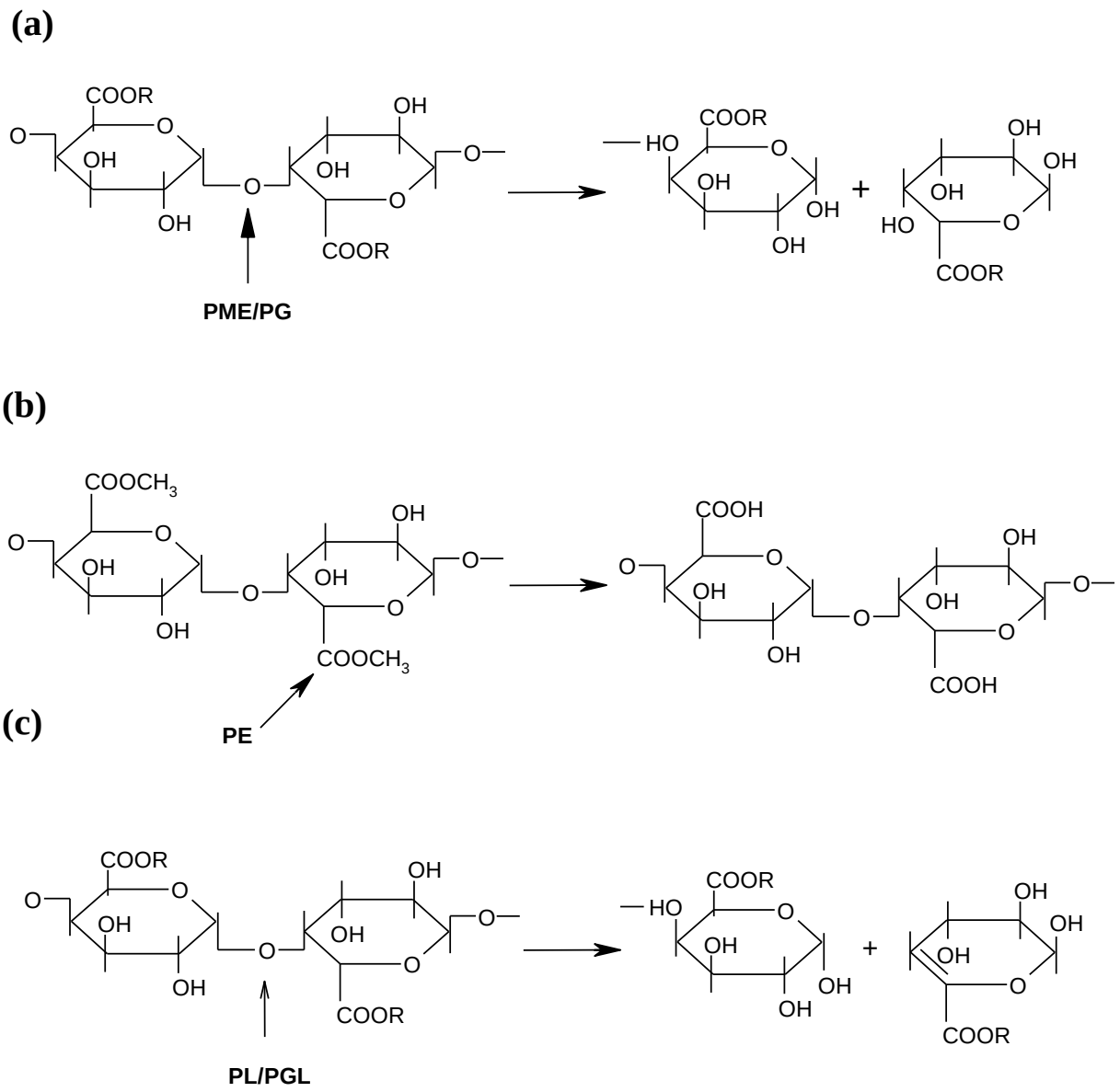

Fig. i: Different types of pectinases and their mode of action on pectic substances (a) R = H for PG and CH_3 for PME (b) PE and (c) R=H for PGL and CH_3 for PL. The arrow indicates the place where the pectinases reacts with pectic substances. PME Polymethylgalacturonase, PG: Polygalacturonase (EC 3.2.1 15). PE : Pectinesterase (EC 3.1.1.11). PL: Pectinlyase (4.2.2.10). Apart from these other pectinases, viz, protopectinase (degrading protopectin), Oilgogalacturonases (degrading D-galactosiduronates) exists. The pectinases are further subclassified based on the nature of reaction. Endopectinases cleaves the substrate in random fashion while exopecxtinases cleaves in end-wise fashion (Gummadi and Panda, 2002).

3- Application of pectinases:-

The synergetic action of pectic enzymes is industrially used for the extraction, clarification and concertration of fruit juices, for the clarification of wines, for the extraction of oils, flavours and pigments from plant materials and for the preparation of cellulose fibres for linen, jute and hemp manufacture (Coelho *et al.*, 1995).

Since the 1940s, pectinases have been exploited for many industrial applications. Pectinases are mainly used for increasing filtration efficiency and clarification of fruit juices (Joslyn *et al*., 1952), in wood preservation (Fogarty, 1973) and used in maceration, liquefaction and extraction of vegetable tissues (Charley, 1969 and Bohziewiez and Bodzek, 1994). Various literature reports and reviews are available on the production and applications of pectinases (Rambouts and Pilnik, 1980; Ward and Young, 1989; Sakai *et al*., 1993a; Kashyap *et al.*, 2001). Also, pectinases have been used in the paper and pulp industry in addition to cellulases (Reid and Ricard, 2000), plant pathology (Hershonhorn *et al.*, 1990 and Lang and Dornenburg, 2000) and in protoplast fusion technology (Takabe *et al*., 1968). Few recent reviews have highlighted the biological and technological importance of pectinases. (Hershonhorn *et al.*, 1990; Reid and Ricard, 2000; Kashyap *et al*., 2001).

On the bases of their applications, pectinases are mainly of two types: acidic pectinases and alkaline pectinases. The important producers of these pectinases as reported in the literature are given in table (ii) (Kashyap *et al*., 2000).

Table (ii): Characterization of microbial pectinases

Producer	Type of pectinase	Opti. pH for activity	Opti. temp. for activity (°C)	References
Acidic pectinases				
Aspergillus niger CH_4	Endo-pectinase Exo-pectinase	4.5-6.0 3.5-5.0	Below 50	Auna-Arguelles *et al.*, (1995).
Penicillium frequentans	Endopolygalacturonase (Endo-PG)	4.5-4.7	50	Borin *et al.*, (1996).
Sclerotium rolfsii	Endo-PG	3.5	55	Channe and Shewal, (1995).
Rhizoctonia solani	Endo-PG	4.8	50	Marcus *et al.*, (1986).
Mucor pusilus	PG	5.0	40	Al-Obaidi *et al.*, (1987).
Clostridium thermosaccharolyticum	Polygalacturonate hydrolase	5.5-7.0	30-40	Rijssel *et al.*, (1993).
Alkaline pectinases				
Bacillus sp. RK9	PGL	10.0		Fogarty and Kelly, (1983).
Bacillus sp. NT-33	PG	10.5	75	Cao *et al.*, (1992).
Bacillus polymysa	PG	8.4-9.4	45	Nagel and Vaughn, (1961).
Bacillus pumilis	PATE	8.0-8.5	60	Dave and Vaughn, (1971).
Amucola sp.	Pectate lyase (PAL)	10.25	70	Bruhlmann *et al.*, (1994).
Xanthomonas compestris	PATE	9.5	25-30	Nasumo and Starr, (1967).
Bacillus No.P-4-N	PG	10-10.5	65	Horikoshi, (1990).
Bacillus stearothermophillus	PATE	9.0	70	Karbassi and Vaughn, (1980).
Penicillium italicum CECT 22941	Pectin lyase	8.0	50	Alana *et al.*, (1990).
Bacillus sp. DT7	Pectin lyase	8.0	60	Kashyap *et al.* (2000).
Bacillus subtilis	PAL	8.5	60-65	Chesson and Codner, (1978).
Pseudomonas syringae pv Glycinea	PAL	8.0	30-40	Magro *et al.*, (1994).

Polygalacturonase :PG, Pectate lyase: PAL,: polygalacturonase: PGL (Kashyap *et al.*, 2000)

4. Solid state fermentation (SSF) processes:

4.1. Definition of the (SSF) methods:

Solid-state fermentation (SSF) is a microbial process in which a solid material is used as the substrate or the inert support of microorganisms growing on it. In SSF, microorganisms can sometimes grow well and produce larger amounts of extracellular enzymes and other metabolites than they do in submerged (liquid) fermentation. Although SSF was developed for the manufacturing of traditional foods and alcoholic beverages, its application has been

extended to the pharmaceutical and biochemical industries (Sato and Sudo, 1999).

4.2. Characteristics:

SSF processes present a series of advantages over submerged fermentations (Hesseltine, 1972).

Product concentrations after extraction are usually larger than those of products obtained by submerged fermentation and the quantity of liquid waste generated is lower. Additionally, these processes are of special economic interest for countries with abundance of biomass and agro-industrial residues, as these can be used as cheap raw materials (Castillho *et al*., 1999b).

The industrial utilization of SSF has expanded to include composting, mushroom cultivation and the production of other foods such as bread and mold-ripened cheese (Sato and Sudo, 1999).

The basic differences between SSF and submerged fermentation are summarized as follows:

a) In SSF, the microbial distribution occurs on the solid surface and the microbial growth and product formation also occur mainly on the surface. The substrate is not uniform and not easily agitated. The culture environment is therefore heterogeneous.
b) The moisture content of a solid substrate is normally low, depending on the physical or chemical characteristics of the substrate. For the media with a high moisture content, steady aeration throughout the substrate bed is difficult, and channeling of airflow often occurs.

c) Heat derived from the metabolism and growth of the microorganisms raises the temperature of the solid substrate bed and causes the loss of moisture. This phenomenon creates challenges for the control of the SSF process.

d) SSF substrates are usually natural materials, e.g. cereals, soybeans, agricultural biomass and solid waste. Sometimes the product is the entire fermented substrate, as in the case of traditional foods, e.g., miso, natto and tempeh. (Sato and Sudo, 1999).

4.3. Advantages of SSF:

In the industrial production of extracellular enzymes such as amylase, cellulose and pectinase, both SSF and submerged fermentation are used. The decision is likely based on the cost and efficiency of the process. It is therefore important to know the advantages of SSF as compared with submerged fermentation (Sato and Sudo, 1999), as follow:

a) SSF is relatively resistant to bacterial contamination since bacterial growth is restricted by low water activity, serious contamination on a solid medium rarely occurs.

b) Ferment or the fermentative facilities are compact. The volumetric loading of the substrate is much higher in SSF than in submerged fermentation because the moisture content of the solid substrate is lower.

c) If extraction of the product from SSF is necessary, it requires much less solvent and lower recover cost than from submerged fermentation.

d) Treatment of the fermented residue is very simple. Since the moisture content of the fermented residue is very low, it can be dried and used as animal feed or fertilizer.

e) Microbial utilization of gaseous oxygen reduces the energy cost of aeration. The air supply and temperature of the solid substrate bed can be controlled by forced aeration, in which the large surface area of the solid substrate promotes heat transfer and gas exchange of oxygen and carbon dioxide (Sato and Sudo, 1999).

The main objective of the present work was an investigation of screening, production, purification and characterization of pectinases enzymes produced by some bacterial strains and its application in food industries technology.

Acidic pectinases:

Acidic pectic enzymes used in the fruit juice industries and wine making often come from fungal sources, especially from *Aspergillus niger*. The juice produced by these industries commercially include: (A) Sparkling clear juices, (apple, pear and grape juices, (B) Juices with clouds (citrus juices, prune juices, tomato juice and nectarts), and (C) Unicellular products where the intent is to preserve the integrity of the plant cells by selectively hydrolyzing the polysaccharides of the middle lamella.

The objectives of adding enzymes differ in these three types of fruit and vegetable juices (Kashyap *et al*., 2001).

Sparkling clear juices:

In the case of sparkling clear juices enzymes are added in order to increase the juice yield during pressing and straining of the juice and to remove suspended matter to give sparkling clear juices (free of haze). Some examples of such juices and their further uses are described below.

Apple:

Apple juice is manufactured as natural, unfiltered and unclarified, juice containing a high percentage of pulp; as a hazy juice that has been centrifuged to remove coarse particles but not filtered; and finally as filtered clear and amber-colored juice prepared by enzymatic treatment (Kilara, 1982). Although pectinases that can depolymerize highly esterified pectin are the major types of enzymes used in apple juice processing they are by no means the only enzymes used for this purpose. A combination of pectinases and cellulases has been reported to give a juice yield up to 100% (Alkorta *et al*., 1998). Another potential contributor to the haziness is starch. Unripe apples may contain up to 15% starch. This can be broken down using an amylase (strictly speaking, an amyloglucosidase) active at the pH of apple juice, added at the same time as the pectinases.

Juice extraction. The initial steps in the extraction of juice from apples include washing sorting and crushing of apples in a mill. Although pectinases are often added at this stage, better results are achieved if the apple pulp is first stirred in a holding tank for 15-20 min so that enzyme inhibitors (polyphenols) are oxidized by naturally occurring poly-phenol oxidase present in the fruit. As these

polyphenols in the pulp inhibit pectic enzymes, addition of microbial polyphenol oxidases to the fruit juices has also been suggested. An alternative way to remove polyphenols in the fruit juices is the addition of polyvinyl pyrolidone (PVP) which oxidizes the phenolic substances hence creating an environment for the action of pectic enzymes (De Vos and Pilnik, 1973).

Pectic enzymes are used in apple juice prepration to facilitate pressing or juice extraction and to aid in the separation of a flocculent precipitate by sedimentation, filtration or centrifugation. Schols *et al*., (1990) described a new pectinase called rhamnogalacturonase and its role in the maceration of apple tissue. This enzyme was found in *Aspergillus aculeatus* initially, but it seems also to be produced by other *Aspergillus* sp. Treatment with pectinase takes anything from 15 min to 2h depending upon the extract nature of the enzyme and how much is used, the reaction temperature and the variety of apple chosen (Kilara, 1982).

Clarification: Clarification is affected by pH, temperature, contact time and enzyme concentration. A juice with low pH will clarify more readily than one with a higher pH, and as the temperature increases the rate of clarification also increase as long as the temperature is below denaturation temperature for the enzyme (40-60 °C) (Kilara, 1982). In general, the time required to obtain clarification is inversely proportional to the concentration of enzyme used at constant temperature over the range of 5-50 °C and treatment time of 2-16 h (Kashyap *et al*., 2001).

If a cloudy product is required, the juice is pasteurized immediately after pressing, to denature any residual enzymes.

Centrifugation then removes large pieces of debris, leaving most of the small particles in suspension. For a clear juice these suspended particles have to be removed. A dose of commercial enzyme e.g. "Rapidase pomaliq, which contains pectinases, hemicellulases, and cellulases is the accepted way of removing suspended particles (Grassin and Fauqembergue, 1996a). This enzyme is supplied by Gist-Brocades and is used at 200-600g per ton of apples while stirring the pomace at 40-60 rpm for approximately 3h at 50 °C. The liquidifed pomace is centrifuged. Fig. (ii) shows the important steps involved in production of apple juice.

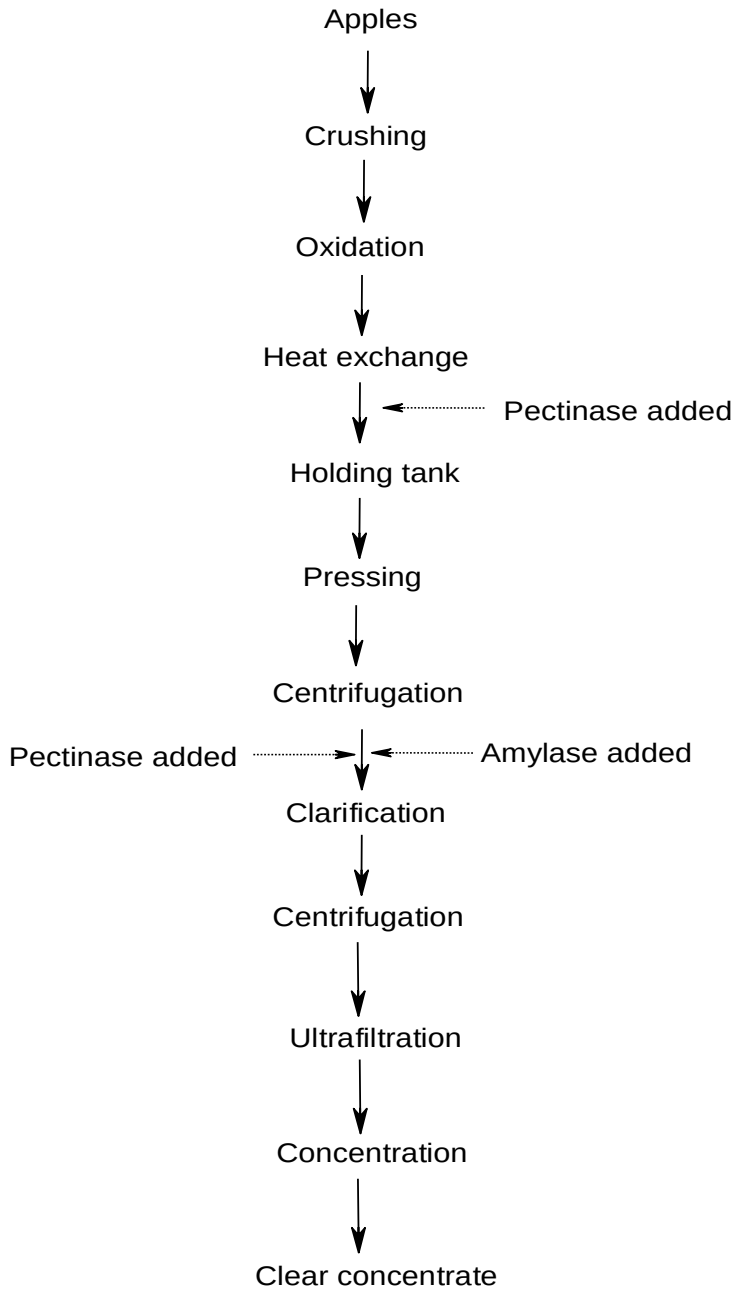

Fig. (ii): Production of apple juice (Grassin and Fauquembergue, 1996a).

Yamaski *et al.* (1964) showed that clarification of apple juice could be obtained by a mixture of PG and PME alone without the presence of contaminating enzymes from the apples. Recent work has shown that purified pectin lyase (PL) used at pH 3-4 can clarify apple juice (Ishii and Vokotsuka, 1971, 1976). This research has also

shown that apple juice containing 91-92 % esterified pectin can be easily clarified by pure PL.

Clear apple juice can sometimes develop a haze during storage, especially refrigerated storage. This defect is termed "After Haze" and results from juice processed at temperature higher than storage temperature. The haze may result from polymerization of polyphenols and oxidation of proanthocyanidins during milling and pressing. Another appearance defect called "Starch Haze" may sometimes develop in apple juice prepared from early-season apples (contain up to 15% starch). Starch haze can be removed by fungal amylases (diastase) added to juices heated to 77 °C to gelatinize the starch and then cooled to 52 °C before diastase addition. Pectic enzyme is added at the same time as the diastase (Kashyap *et al.*, 2001).

In conclusion, advantages of the use of pectinases in apple juice are the following: their availability allows the producer to diversify the type of products, i.e., cloudy, clear juices, clear concentrates,… etc., and to increase the value of this raw material. In practice, the total time for the juice extraction is shorter than the classical process. Enzymes also help in production of juices and concentrates that are very stable and have a good state with a reduced pomace waste, and reduced production cost (higher yield, less equipment, less labor in a concentration process). Finally, they also permit the processing of different fruits by the same process (Kashyap *et al.*, 2001).