INTRODUCTION

Enzymes are biocatalysts produced by living cells which govern the chemical reactions for life processes. Enzymes are highly specific in their action on substrates and often many different enzymes are required to bring about, by concerted action, the sequence of metabolic reactions performed by the living cell. All enzymes which have been purified are protein in nature, and they may or may not possess a nonprotein prosthetic group.

Enzyme catalyzed reactions have been utilized by man through the ages. The production of wine, bread and cheese predates biblical times. Means were found to exert a general control over these fermentation processes, although the mechanism of chemical reactions involved was not understood. It was not until nearly the turn of this century that the causative agents or enzymes responsible for bringing about biochemical reactions became known.

Hundreds of scientists have contributed to our knowledge of enzymes, but major credit for having the foresight to apply this knowledge for industrial uses goes to three men. Jokichi Takamine obtained a patent in 1894 for a process of making a distatic enzyme from fungi (1). Otto Röhm found in 1908, that pancreatic enzymes could be

utilized in the bating of hides (2). In 1911, Leo Wallerstein patented a process for the chillproofing of beer by means of the introduction of proteolytic enzymes during processing (3).

The practical application and use of pectin degrading enzymes in the fruit processing industry for the production and clarification of fruit juices dates back about forty years. Advantages of enzymatic treatment of fruit pulps and juices for extraction and clarification respectively, were detailed by Reid (4,8), Kertesz (5), Tressler and Joslyn (6), Charley (7), Sreekantiah et al (9), Joseph et al (10) and Makari et al (11).

Charley (12) as early as 1936 used commercial pectinase enzymes for the treatment of strawberry, raspberry, loganberry, blackberry and blackcurrant pulps. Widmer (13) used a combination of enzyme-gelatin clarification method with apple juice and stated that the removal of pectin is essential for efficient concentration of the clear juice. Coxe (14) destroyed the bothersome quick-setting pectin naturally present in cranberries and then used a slow-setting citrus pectin to make cranberry jelly. According to Serbinova (15) the enzymic clarification with a mold pectinase had no effect on the ascorbic acid content of black-currant juice. In 1950 Cruess et al (16) found that the treatment of the fresh prunes with 0.1% pectinol

permitted the pressing of juice with an 86% yield whereas without the treatment only a negligible quantity of juice could be pressed from the fruit. In addition to their use in fruit juices, pectinases are also extensively used in the clarification of wine by degradation of pectin (17,18) which also helps to enhance the deposition of argols (19), increase the ease of filtration, and improve the bouquet and flavor of the finished product (20); in the retting of textile fibres by partial digestion of the cementing material between the fibres in the bundles (21); in the refinement of vegetable fibres; in the curing of coffee, cocoa and tobacco (22); in the preparation of hydrolyzed products of pectin like galacturonic acid which is used as a substrate for the manufacture of ascorbic acid (24); as an analytical tool in the estimation of certain plant products (24,25) and in the manufacture of starch.

The history of pectic enzymes is characterized by a confusion of nomenclature since the nomenclature of the pectic substances has been very confused. In the plant tissue pectin does not exist free, but in the form of a labile combination with cellulose and possibly other materials such as hemicelluloses of which araban is an example. The pectin material in this state is known as protopectin. The enzyme which liberates pectin from the protopectin molecule is known as protopectinase. The

existence of protopectinase was first suspected by Kolb in 1868 (26) when he was working on the retting of flux. Later on Brown (27), Davison and Willaman (28) established the existence of the macerating enzyme, protopectinase, in fungi. The enzyme is produced by Botrytis cinerea, and species of Rhizopus especially R. tritici which are parasitic on sweet potatoes, Fusarium chronyophthoron and various penicillia. The idea that an enzyme complex consisting of several individual enzyme is involved in the complete hydrolysis of pectin, first emerged from the work of Ehrlich (23). Later on, Kertesz in 1936 (29) postulated six enzymes in the complex, differing according to the groups split. However, in addition to protopectinase, the other two generally accepted enzymes attacking pectin are pectase or pectin-methylesterase and pectinase or pectin-poly-galacturonase. Pectin-methylesterase (30) is the enzyme which catalyzes the hydrolysis of the methoxy groups off from the esterified carboxyl groups of the galacturonic acid residues in the soluble pectin molecule. The enzyme was first described by Frémy (31) in 1940 as responsible for the coagulation of soluble pectin into a gel in the presence of calcium salts. This enzyme occurs commonly in the roots, stems, leaves, and fruits of many higher plants and is also produced by microorganisms (32,33,34). In higher plants, or at least in macerates (32), pectin-methylesterase

is usually strongly adsorbed on the water insoluble cellular components (35), and press juices and extracts often contain only a fraction of total amount of this enzyme present in the tissue (36). The existence of pectin-methylesterase in microorganisms was discovered long after its occurrence in higher plants had been established. Willaman (33) in 1920 first reported the presence of this enzyme in Sclerotinia cinerea and later on various investigators (34,37,38) established the production of pectin-methylesterase along with pectinases by various microorganisms. In fact, the pectin-methylesterase action is a necessary prerequisite for pectinase action, for only deesterified pectin is attacked by the latter enzyme (39). However work in the last decade has shown that several polygalacturonases exist, and commercial preparations frequently contain a mixture of these. These polygalacturonases hydrolyse the α-1,4-glycosidic linkages in the polygalacturonic acid skeleton of pectic acid or pectinic acids with the resulting formation of polygalacturonic acids of smaller molecular sizes and of (mono-) galacturonic acid. The existence of pectinases in barley malt was first reported by Bourquelot and Hérissey in 1898 (40). Later on the enzyme was found to be present in higher plants, especially fruits, in bacteria, yeasts and moulds. According to Demain and Phaff (41) the polygalacturonases may be further differentiated into endoenzymes

which act randomly within the molecule on α-1,4-glycosidic linkages between the galacturonic acids or methylester of galacturonic acids and exoenzymes which catalyzes the stepwise splitting off of the galacturonic acid molecules from the non-reducing end of the chain. A further division arises from the fact that some enzymes act principally on methylated substrates (pectins) while others act on substrates containing free carboxylic acid groups (pectic acids). The enzymes are called accordingly polymethylgalacturonases and polygalacturonases.

In 1963 Neukom (42) reported the presence of another group of enzymes which breaks down the pectin molecule by a nonhydrolytic type of cleavage of the α-1,4-glycosidic linkages resulting in the formation of unsaturated monomeric derivatives of galacturonic acids (4-deoxy-5-ketogalacturonic acid). These enzymes have been called transeliminases. According to Neukom the transeliminases occur in different forms, like that of polygalacturonases, such as pectin transeliminases of both endo- and exo-type which will act specifically on pectin molecule only and pectic acid transeliminases of endo- and exo-type which will act specifically on pectic acid molecule only.

By far the greatest amount of work has been devoted to pectinases, the enzymes responsible for complete rupture of the polymerized pectin molecule into its structural

components. A great variety of strains of bacteria, yeasts and moulds are capable of producing the pectinases. Several bacterial species including Bacillus carotovorus (43), Bacillus subtilis (44), Bacillus mesentericus var. fuscus (45), Clostridium felsineum, Clostridium acetoethylicum (46), Pseudomonas marginalis (48) are capable of producing pectolytic enzymes when cultivated under proper conditions. However the bacterial and plant enzymes have their pH optima in the range of 6.0 and 8.0, and since the pH of fruit juices can not be changed without sacrificing flavour and aroma, enzymes of these origin can not be utilized in fruit processing. Since the optimum pH of mould enzymes lies very near the pH of many fruits which range from 3.0 to 5.0, they are extensively used in the food industry.

Extensive reviews by Demain and Phaff (41) and Endo (49) show that members of the genera Penicillium, Aspergillus and other fungi like Coniothyrium diplodiella, certain members of the genus Rhizopus, Fusarium, Botrytis and Sclerotinia produce pectinases. Among the Aspergillus species those found to produce good amount of pectolytic enzymes are Aspergillus niger (28,46,47a,47b,50,51,64,65) Aspergillus flavus, Aspergillus fumigatus, Aspergillus parasiticus, Aspergillus wentii (50,51), Aspergillus oryzae (52,53,54) and Aspergillus aureus (9). Of the many Penicillium species certain strains of Penicillium glaucum

(51,52), Penicillium ehrlichii (55) and Penicillium chrysogenum (56); among the Rhizopus species, strains of Rhizopus tritici (28,50,51,57) and Rhizopus nigricans (50,51,60); and also certain strains of Monilia fructigena (58), Botrytis cinerea (28,44,46,52,58), Fusarium chroniophthoron (58), Fusarium fructigenum (58) and some Mucor species (52) have been reported to excrete active pectolytic enzymes. In addition many of plant pathogenic fungi and air-borne spores of Aspergillus species are capable of producing pectinases as reported by Puvgi et al (59) and Deuel & Stutz (60).

In theory, the fermentative production of microbial enzymes is a simple matter, requiring an appropriate organism grown on a medium of optimum composition under optimum conditions. Among the more important of the factors influencing enzyme yield are mash design, oxygen supply, temperature, pH and method of cultivation. A suitable mash must first of all support good growth of the microorganism. Unfortunately, this alone is not enough to ensure good enzyme yield. Commonly, the best enzyme yield is obtained on a mash which supports marginal growth of the organism. Mashes are composed of mixtures of carbohydrates, nitrogenous compounds, minerals and growth stimulants. For the production of adaptive enzymes, the enzyme substrate must be included.

Microorganisms vary widely in their oxygen requirements. Most of the species used in industrial enzyme production are highly aerobic and thus require large amounts of oxygen. The amount of oxygen required may be a key factor in determining the method of cultivation to be employed.

Temperature has a direct influence on the rate of microbial growth, the rate of enzyme synthesis and the rate of enzyme inactivation. The establishment of the temperature giving highest enzyme yield is often a compromise between these three effects of temperature. As is the case with temperature, there is one optimum pH for microbial growth, another for enzyme production and a third for enzyme stability. The pH in production may be a compromise among these three or the pH may be changed during the course of the fermentation to coincide with a change in the phase of enzyme production.

Either surface or submerged culture method may be employed for the production of most microbial enzymes. Usually different cultures must be used for maximum enzyme yields by the two methods, although there are exceptions to this rule. In the laboratory, submerged cultures are grown in shake flasks or in aerated bottles or flasks. The

choice between the two methods of cultivation is governed by the environmental requirements of the micro-organisms, plant equipment, convenience, relative yields and application.

The production of pectinases by microorganisms is dependent to a large extent on the metabolic characteristics of the culture. According to Kertesz (61) a variation in the composition of the medium will cause a shift in the metabolic products produced by the organism, which have got a profound effect on the rate of production of the enzymes. Cultural conditions also determine the proportion of enzyme which remains in the microorganism and that which is excreted into the nutrient solution. The variations in this respect are considerable. Phaff (56) in 1947 reported that for cultures of a strain of Penicillium chrysogenum most of the pectin polygalacturonases could be found in the nutrient solution. The effect of different carbon and nitrogen compounds on the production of pectinases by fungi was studied by Fernando (44), Willaman and Kertesz (51), Phaff (56), Puvgi et al (59), Gupta (62), Chatterjee and Bose (63), Tuttobello and Mill (64), Sreekantiah et al (65), Makari et al (66) and Moldabaeva (67).. Saito (47b) studied the factors affecting the production of enzymes in shake flask cultures of Aspergillus niger and has reported that endopolygalacturonase is adaptive to the presence of pectic substances.

Kantio (68) has reported that the pectolytic enzymes of Aspergillus niger are adaptive to the presence of pectin. Phaff (56) has demonstrated that both pectin esterase and polygalacturonase of Penicillium chrysogenum are induced by pectic materials, D-galacturonic acid, mucic acid and L-galacturonic acid. Harter and Weimer (69), Proskuriakov and Ossipov (52), Gäumann and Böhni (70) also reported that the pectolytic enzymes are adaptive enzymes since they are found only when the organism grows in the presence of specific substrates or greatly increased by the addition of pectin. This situation is not entirely clear, however, and the situation may be different in different fungi. Gäumann and Böhni (70) found that pectinases of Botrytis cinerea are constitutive enzymes and pectase of the same organism is strongly adaptive. In each case they studied the extracellular enzymes. In synthetic medium with glucose as the sole carbon source pectinases were strong but there was practically no pectase formed. When pectin was present either in the presence or absence of glucose, abundant pectinases were formed. However, Sreekantiah et al (65) reported that pectinases as produced by Aspergillus aureus Nakazawa and Penicillium expansum in shake flasks are constitutive in nature.

As a nitrogen source in the fermentative production of pectinases, ammonium sulphate (56), yeast extract (44), potato decoction, asparagine, peptone, ammonium tartarate

(44); and gelatin or casein (71) have been recommended. According to Fernando (44) the best nitrogen sources (for Botrytis strains) for the production of pectolytic enzymes are those which cause a pH shift to the alkaline side. As a result of the action of excreted enzymes on pectin, the pH drops considerably due to the liberation of free carboxyl groups (47a).

Details of commercial production of pectinases are rather scanty but there are two examples in the B.I.O.S. report (72). Limited information is available on the culture methods employed for the commercial production of pectic enzymes. Some of these are apparantly produced on solid bran cultures (73,74) and the mycelium is often mechanically disintegrated and then extracted to obtain intracellular enzymes. It is not clear, for example, what organisms are used at the present time for the production of this enzyme. The patents name a long series of organisms, among them Aspergillus and Penicillium species including Aspergillus wentii, Aspergillus niger, Aspergillus flavus, Aspergillus oryzae, Aspergillus fumigatus, as well as aspergilli of the parasiticus and tamari types; Penicillium glaucum and Rhizopus trifici, Rhizopus nigricans (75). In Germany a mold culture designated as Aspergillus aureus Nakazawa and Aspergillus wentii were used in the manufacture of pectinases (73). It is quite clear that any given culture which

proves to be suitable for the manufacture of pectolytic enzymes under any given set of manufacturing conditions might not necessarily be as useful and successful under a different set of conditions. Most laboratory studies have been made with either surface cultures or shake flasks (68,76,77). According to Brooks and Reid (76) Aspergillus foetidus produces both endopolygalacturonase and exopolygalacturonase in surface cultures but only endo-polygalacturonase in submerged cultures. However, the food technologists are concerned with the action of commercial enzyme preparations which are complex mixtures of enzymes, on fruit juices and pulps etc., that are mixtures of heterogenous substances (78,79).

Now considering the importance of studying fermentative production of the pectinases by fungi, the present work was undertaken. As the first step in the manufacture of any commercial enzyme is the selection of the proper organism, attempts were first made to screen out fungi from decaying fruits, soils of West Bengal (India) for the selection of the potent organism giving high yield of pectinases. Among the isolates, a strain of Aspergillus niger JU, being the most potent organism, was selected and further studied in details to determine optimum environ-mental conditions for the production of pectin degrading enzymes.

SCHEME OF WORK

The work embodied in this thesis is a study on the fermentative production of pectinases, for which soil samples collected from different parts of West Bengal (India) and decaying fruits have been screened. This investigation has resulted in the isolation of a strain of Aspergillus niger JU which gives a moderate yield of pectinases in synthetic media. The fermentation conditions for optimum production of pectinases were studied in details. The following scheme of work was followed in course of these studies.

Chapter 1 - Screening of natural sources for pectinase producing organisms.

(i) Isolation of fungi from natural sources.

(ii) Pectinase activity of the fungal isolates.

(iii) Selection of most potent strain amongst the isolates.

(iv) Characterization of the most active isolate.

Chapter 2 - Determination of optimum cultural conditions for the production of pectinases by Aspergillus niger JU with particular reference to (1) initial pH, (2) period of fermentation and method

of cultivation (3) temperature of incubation (4) aeration and (5) volume of inoculum.

Chapter 3 - Effect of carbon and nitrogen sources on the production of pectinases by Aspergillus niger JU.

(i) Effect of different carbon sources on the production of pectinases by A. niger JU.

(ii) Effect of different nitrogen sources on the production of pectinases by A. niger JU.

(iii) Effect of C/N ratio on the production of pectinases by A. niger JU.

Chapter 4 - Studies on the mineral requirements of A. niger JU for the production of pectinases.

Chapter 5 - Effect of some growth factors like para-aminobenzoic acid, yeast extract and sodium phytate and metabolic inhibitors like sodium fluoride, sodium azide, methylene blue and 2:6-dichlorophenol indophenol on the production of pectinases by A. niger JU.

Chapter 6 - Metabolic changes during the production of pectinases by A. niger JU.

(i) Rate of elaboration of pectinases in relation to pH, galacturonic acid (free and combined) concentration and cellular growth.

(ii) Nitrogen balance during the production of pectinases.