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Selection of ideal carbon nitrogen source and fruit waste for the induction of enhanced levels of pectinases in case of bacteria

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Abstract

Pectinases are a big group of enzymes that break down pectic polysaccharides of plant tissues into simpler molecules like galacturonic acids. It has long been used to increase yields and clarity of fruit juices. Since pectic substances are a very complex macromolecule group, various pectinolytic enzymes are required to degrade it completely. These enzymes present differences in their cleavage mode and specificity being basically classified into two main groups that act on pectin "smooth" regions or on pectin "hairy" regions. Pectinases are one of the most widely distributed enzymes in bacteria, fungi and plants. This review describes the pectinolytic enzymes and their substrates, the microbial pectinase production and characterization, and the industrial application of these enzymes. Possibility of producing pectinase utilizing fruit wastes of cashew, banana, pineapple, and grape under controlled fermentation with Different bacterial isolates were studied. Among the different media composition tried, medium containing 5 g fruit waste + trace elements supported better growth of the microorganism. Enzyme production was maximum in the medium with grape waste, glucose as carbon sources and peptone as nitrogen sources.

Keywords: Pectinases, glucose, peptone, nitrogen

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Introduction

The potential of the so-called "white biotechnology" as an ecological advantageous and moreover economical beneficial technology is beyond all question. Caused of the ever growing costs for energy and polluted waste water, enzymatic technologies will stay in the focus of science and technique, and their relevance will increase significantly in the future. Enzymes, biological catalysts with high selectivities, have been used in the food industry for hundreds of years, and play an important role in many other industries (washing agents, textile manufacturing, pharmaceuticals, pulp and paper). Currently, enzymes are becoming increasingly important in sustainable technology and green chemistry. In the opinion of many experts and based on different studies, by 2010, 20 % of all chemical products in a dimension of 300 billion US dollar will be produced using biotechnology. This would represent a tenfold increase compared to 2001**.** Micro-organisms are considered to be prospective enzyme producing sources. They have a number of advantages: through the application of selection methods increase of biosynthesis via the conditions of cultivation [1], in-depth interaction on various substrates, wide spectrum of enzyme complex and their application in gene engineering via gene cloning.

Fruit processing industries produce a large amount of waste material in the form of peel, pulp, seeds, etc. Some fresh orange peel is, however, used in shredded form in the preparation of orange-marmalade. This waste material presents considerable disposal problems and ultimately leads to pollution. Dried citrus peel is rich in

carbohydrates, proteins and pectin; the fat content, however, is low. Various microbial transformations have been proposed for the utilization of food processing waste for producing valuable products like biogas, ethanol, citric acid, chemicals, various enzymes, volatile flavoring compounds, fatty acids and microbial biomass . Citrus peel contains an appreciable amount of pectin and thus can be used as a substrate for the production of pectinolytic enzymes by micro-organisms. Pectin acts as the inducer for the production of pectinolytic enzymes by microbial systems .The advantage of using micro-organisms for the production of enzymes is that these are not influenced by climatic and seasonal factors, and can be subjected to genetic and environmental manipulations to increase the yield. Highly productive strains of micro-organisms are required at the industrial level to reduce the production costs.

Different types of micro-organisms have been exploited for the production of enzymes. Pectinolytic enzymes have been reported to be produced by a large number of bacteria and fungi such as *Bacillus* spp., *Clostridium* spp., *Pseudomonas* spp., *Aspergillus* spp., *Monilla laxa*, *Fusarium spp.*, *Verticillium* spp., *Penicillium* spp., *Sclerotinia libertiana*, *Coniothyrium diplodiella*, *Thermomyces lanuginosus*, *Polyporus squamosus*, *etc*. Pectic enzymes are widely distributed in nature. They mainly occur in plants, bacteria, fungi, yeasts, insects, nematodes and protozoa. Pectic substance is a generic name used for the compounds that are acted upon by the pectinolytic enzymes. These are negatively charged acidic glycosidic macromolecules and have high molecular weight. These are present in the 5 plants as the major component of middle lamella between the cells in the form of calcium pectate and magnesium pectate. Pectinase is an enzyme that breaks down pectins. Pectic substances are glycosidic macromolecules with high molecular weight. They form the major components of the middle lamella and primary plant cell wall. Pectic substance consists of protopectins, pectinic acids, pectins and pectic acids. The main chain of pectin is partially methyl esterified 1, 4-D-galacturonan. Demethylated pectin is known as pectic acid (pectate) or polygalacturonic acid. Pectic substances are naturally degraded by pectinases. The classification of pectic enzymes is based on their attack on the galacturonan backbone of the pectic substance molecule. Basically, there are three types of pectic enzymes; de-esterifying enzymes (pectin esterase), depolymerizing enzymes and protopectinases. Pectin esterases catalyze the hydrolysis of methyl to produce pectic acid and methanol. Depolymerizing enzymes consist of hydrolases and lyases. Lyases are also called transeliminases, which split the glycosidic bonds of either pectate (polygalacturonate) or pectin (polymethylgalacturonate). Pectinases are produced by a large number of organisms, such as bacteria, fungi, actinomycetes and yeast. Pectinases have been used in processes and industries where the elimination of pectin is essential; fruit juice processing, coffee and tea processing, macerating of plants and vegetable tissue, degumming of plant fibers, treatment waste water, extracting vegetable oil, bleaching of paper, adding poultry feed and in the textile, alcoholic beverages and food industries. Pectinolytic enzymes are commonly used during processing of fruits and vegetables for juices and wine. The pectic substances account for about 0.5–4% of the weight of fresh material. The raw pressed juice is rich in insoluble particles mainly made up of pectic substances. When the tissue is ground, the pectin is found in the liquid phase (soluble pectin) causing an increase in viscosity and the pulp particles. It is difficult to extract this juice by pressing or using other mechanical methods. With the addition of pectinases the viscosity of the fruit juice drops, the press ability of the pulp improves, the jelly structure disintegrates and the fruit juice is easily obtained with higher yields, Pectinase are produced during the natural ripening process of same fruits. This can also increase the volume of juice (increase the yield), lowers the viscosity of juice, and reduces the cloudiness of juices, which is caused by suspended pieces of cell wall [2]. Pectinase group of enzymes include polygalacturanases, pectin methyl esterase, pecin lyases. These pectinase enzymes act in different ways and on the pectins. Pectinase are extensively used in fruits juices processing (extraction and clarification) vegetable oil extraction, processing of alcoholic beverages and a variety of applications in food industries. Pectinase have an optimum temperature and pH at which they are most active. The commercial pectinase might typically be activated at 45 to 55C and well work at a pH of 4 to 5. Pectinolytic micro-organism are widely distributed in soil, spoiled fruits, vegetables, decayed leaves and wood and can also be seen in water samples taken from decaying coconut husks, especially in Coastal areas. Intestinal flora of humans also includes pectinolytic micro-organism, mainly bacteria, since pectin the dietary fibre is the substrate for them. Traditionally, commercial source of pectin have been citrus peel and apple pomace. Citrus peel has often

been the preferred material for pectin manufacture due to its high pectin content and good colour properties. Most recently other sources of pectin are sugar beet pectin, sunflower pectin. The amount of pectin from different sources varies considerably.

Apple pomace 10-15%
Citrus peel 25-35%
Sugar beet 10-20%
sunflower 15-25%

Sources of Pectin: The traditional, commercial sources of pectin have been citrus peel and apple pomace. Often this is a waste material from another industry such as apple pomace from must be unlimed and it cannot be enzyme treated. Lime treatment of the peel would hydrolyse all the pectin to pectic acid and peel that has been treated with enzyme to ease the peel removal will have the molecular weight of the pectin reduced [3]. More recently other sources of pectin are beginning to find markets such as sugar beet pectin and sunflower pectin. Sugar beet pectin in particular is finding a niche market due to its unusual emulsification properties. The amounts of pectin from these different sources varies considerably:

- Apple pomace: 10-15%
- Citrus peel: 25-35%
- Sugar beet: 10-20%

Material and methods

The materials and methods used in the present investigation are mentioned under the following heads.

Experimental site: Experiments pertaining to the present research work were carried out at Watson Life Sciences, Tirupati, Andhra Pradesh.

Climate: The experimental sites come under Semi-Arid Tropical Southern Zone of Andhra Pradesh based on Agro-climatic conditions. Southern Zone is characterized by fairly hot summer (>40°C) and rainfall receiving in two spells viz., South-West (June–September) and North–East (October–January) monsoon period. Normally the rainfall is more in North–East monsoon than South–West monsoon period.

Glassware: Borosil/Corning makes flasks (250, 500 and 100 ml) Petri plates (90 mm diameter) test tubes (150x15 mm) microscope slides (2.5x7x2 mm) and pipettes (1, 5 and 10 ml) were used throughout the research work.

Chemicals and equipments: Chemicals used were of 'Analytical Grade' (E. Merck). The pH of the medium was adjusted to the required level with N/10 NaOH and N/10 HCl. Compound microscope (100X, 400X magnification) was used for observing the causal agents. Trinocular research microscope attached with camera and Adobe Photoshop software was used for micro-photography. Weighments were done on single pan electronic balance with a sensitivity of 0.0001 g. The spore concentration of the test pathogen was determined by using a haemocytometer. Portable soil temperature thermometers with stainless steel probes were used to record soil temperature.

Laboratory techniques: General laboratory techniques followed were those described by Dingra and Sinclair (1995) with slight modification wherever necessary.

Isolation of pectinase producing micro-organism: -

Samples from waste, soil etc. will be collected from different places. For isolation of bacteria, suspension of samples will be prepared in sterile distilled water, which will then be placed on modified pectin agar medium .Isolates A loop of the homogenate was then streaked onto nutrient medium and incubated at 30ºC for 24 to 72h. All morphological contrasting colonies were purified by repeated streaking. Pure cultures were sub-cultured onto slants media.

Screening for Pectinase Activity

The isolates were inoculated in medium. After 3-4 days incubation at 500C, 1% (w/v) cetyltrimethylammoniumbromide (CTAB) solution was poured onto the colonies. After 10 min incubation at room temparature, colonies with clear zones were taken as pectinase producers (Kobayashi *et al.*,1999).

Production of pectic enzyme on solid-state (SSF) and submerged (SmF) fermentation: Strains presenting large clearing zones were used for enzyme production assays on liquid and solid medium. The liquid medium containing 1% citrus pectin, 0.14% (NH4)2SO4, 0.6% K2HPO4, 0.20% KH2PO4 and 0.01% MgSO4 7H2O, pH 6.0 was inoculated with a suspension containing 106.cells/ ml. Cultures were grown in 125ml Erlenmeyers flasks with 25 ml of medium in a rotary shaker (150rpm) at 30ºC. After 48h the biomass was separated by centrifugation at 1000xg for 20 min and the supernatant was used to evaluate polygalacturonase (PG) activity. The SSF was done using a 250 ml Erlenmeyer flask containing 5g of Banana peel, Orange peel, Grape peel, pine apple peel and 10 ml of 1% (NH4)2SO4 and 0.02% MgSO4 (67% of moisture). 106 cells per gram were added to each flask and maintained at 30ºC. After

72h, 8 fermented materials was mixed with 40 ml distilled water and filtered at vacuum and centrifuged. The supernatant was used to evaluate PG activity.

Different Nitrogen Sources:

Production medium was supplemented with different nitrogen sources at an equimolecular amount of nitrogen that present in sodium nitrate (0.2%, w/v) in basal medium. Peptone, yeast extract, Urea and casein were introduced as organic nitrogen source at the level of 2 % and the control was devoid from any nitrogen source.

Different carbon sources:

Different carbon sources were introduced into the production medium at an equimolecular amount located at 1% (w/v) glucose. Parallel experiment was made with no sugar as a control. The carbon sources were represented by fructose, sucrose and cellulose was introduced at the level of 1% (w/v). In all cases, other previously mentioned optimal conditions were taken into consideration.

Results

The serially diluted sample was spreader on pectin screening media. Among 4 isolates (B1-B4), B2 isolate was identified to be the best bacterial isolated secreting pectinase as maximum halozone of hydrolysis was found around B2 colony (Figure1).

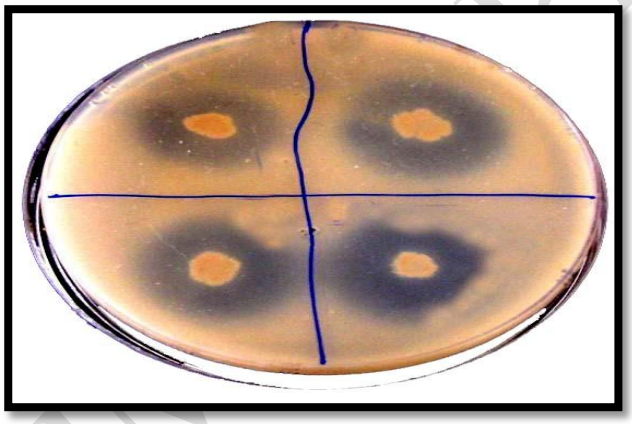

Figure 1: Bacterial isolated secreting pectinase

Influence of different fruit wastes as substance on pectinase production have been investigated among the four fruit wastes it have been found that grape waste induce maximum pectinase activity (0.23U/mg¹)followed by orange waste (0.20U/mg).least pectinase activity was shown by bacteria when pineapple waste used as substrate . the growth of microorganism is an indication of utilization of pectin(Figure 2).

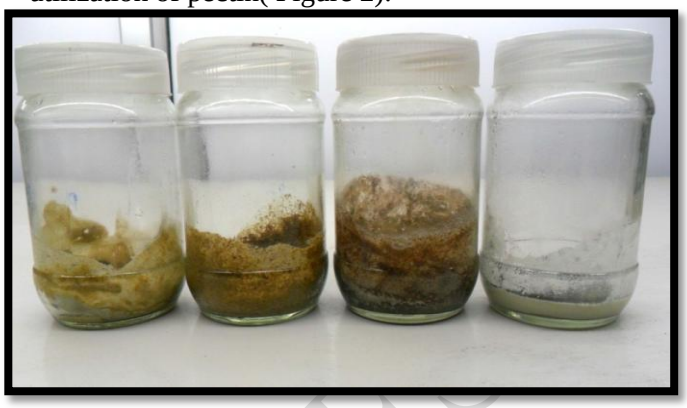

Figure 1: Effect of different carbon sources on levels of pectinase secretion

Effect of carbon source

Glucose, fructose ,sucrose cellobiose were the different carbon sources supplemented .it was observed the growth of bacteria is much faster when glucose is used as carbon source also highest pectinase activity of (0.92u/ml). Cellobiose is the carbon source that was least utilized by the bacteria. Very low enzyme activities were identified in case of carbon sources sucrose (0.83u/ml)and cellobiose (0.64U/ml) among the nitrogen sources supplemented peptone is selected as the best source as it produced highest levels of pectinase (1.32u/ml) urea and yeast extract gave similar pectinase activity results both secreting (1.28u/ml) of enzyme casein is noticed to be not a good nitrogen source for the bacteria is isolated as only (1.0u/m) of pectinase is produced when casein was used as nitrogen source (Figure 3).

Figure 3: Effect of different carbon sources on levels of pectinase secretion

Effect of Nitrogen

Finally it can be concluded that grape wastes when used as substrate supplemented with glucose as

carbon source and peptone as nitrogen source is best suited for the present isolated bacteria to produce significant levels of pectinase (Figure 4).

Figure 4: Effect of different nitrogensources on levels of pectinase secretion

Discussion

Enzyme production is a growing field of biotechnology and the world market for enzyme is1.5 billion and it is anticipated to double by the year 2008.The majority of the industrial enzymes are of microbial origin. In the present study, four isolates were isolated from different places. These isolates were grown on different carbon and nitrogen sources to be able to produce a polygalacturonase which favourable to be used as additive for clarification of juice. A screening of pectinolytic productivities of the isolates showed that one of them gave good pectinolytic productivities. The nature of solid substrate is the most important factor in solid state fermentation. This, not only supplies the nutrient to the culture but also serve as anchorage for the growth of microbial cell [4]. The selection of substrate SSFdepends upon several factors mainly with the cost of availability and this may involve the screening of several fruit juice residues.

An optimum substrate provides all necessary nutrients to the micro organism for optimum function. However, some of the nutrients may be available in suboptimal concentrations or even not present in the substrate in such cases, it would be necessary to supplement them externally. Indeed 30-40% of the production cost for industrial enzymes is accounted for the cost of the culture medium [5]. In order to reduce medium costs we screened different low cost substrates like fruit juice residues and in the course of this we identified grape waste to be cost effective production of the pectinase enzyme under study. SSF is receiving a renewed surge of interest, primarly because increased productivity and prospect of using a wide fruit juice residues as substrate [6, 7]. From industrial point of view, in order to achieve production of low cost of enzymes, these isolates under study were allowed to grow. The selection of the substrate for the process of enzyme biosynthesis was based on the following factors i.e.

1) They represent the cheapest substrates.
2) They are available at any time of the year.
3) Their storage represents no problem in comparison with other substrate.
4) They resist any drastic effect due to exposure to other environmental conditions. E.g: Temperature variation in the weather from season to season and from day to night.

SSF are usually simple and can use waste of agro-industrial substrates for enzyme production. The minimal amount of water allows the production of metabolites, less time consuming and less expensive Higher production of pectinase in SSF process may be due to the reason that solid substrate not only supplies the nutrient to the microbial culture growing in it, but also serves as anchorage for the cell allowing them to utilize the substrate effectively [8]. The environmental conditions in SSF conditions can stimulate the microbe to produce the extra cellular enzymes with different properties other than those of enzymes produced by same organism under the conditions performed in submerged fermentation. In this field many workers dealt with the main different factors that effects the enzyme productions such as temperature, pH, aeration, addition of different carbon and nitrogen sources [9, 10]]. Although such factors were previously studied, still we need for more investigation seems to be continuously required to give a chance to isolate more. The present work is to determine the ideal substrate for the enzyme. On the other hand, the economic feasibility of the microbial enzymes production application generally depends on the cost of its production processes. In order to obtain high and commercially viable yields of pectinases enzyme, it is essential to optimise the fermentation medium used for growth and enzyme production. Optimal parameters of the pectinases enzyme biosynthesis from microbial origin varied greatly, with the variation of the producing strain, environmental and nutritional conditions.

Conclusion

Pectinases are a big group of enzymes that break down pectic polysaccharides of plant tissues

into simpler molecules like galacturonic acids. It has long been used to increase yields and clarity of fruit juices. Since pectic substances are a very complex macromolecule group, various pectinolytic enzymes are required to degrade it completely. These enzymes present differences in their cleavage mode and specificity being basically classified into two main groups that act on pectin "smooth" regions or on pectin "hairy" regions. Pectinases are one of the most widely distributed enzymes in bacteria, fungi and plants. This review describes the pectinolytic enzymes and their substrates, the microbial pectinase production and characterization, and the industrial application of these enzymes. Possibility of producing pectinase utilizing fruit wastes of cashew, banana, pineapple, and grape under controlled fermentation with Different bacterial isolates were studied. Among the different media composition tried, medium containing 5 g fruit waste + trace elements supported better growth of the microorganism. Enzyme production was maximum in the medium with grape waste, glucose as carbon sources and peptone as nitrogen sources.

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