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Pectinase Production by Fungi and Spoilage of Fruits and Vegetables under Refrigerated Conditions

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Abstract – Refrigeration is believed to be the best practice to keep fruits and vegetables safe from spoilage due to fungi. However, most of the consumers do not know the correct temperature to keep these foodstuffs in refrigerator. The present study showed that more than 10 fungal species can harbour under refrigerated conditions and produce pectinases, responsible for food decay. These pectinases are shown to have optimum pH as 6.5 to 7.5 and optimum temperature as 12-15°C during the study. Further, foods with high sugar and starch are more prone to fungal attack. The study has shown, the even under refrigerated conditions, fungi are able to deteriorate the fruits and vegetables and appropriate measures should be taken while the bulk storage of such fruits and vegetables.

Key Words: Food, Fungi, Food Storage, Pectinase

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INTRODUCTION

Fruits and vegetables are cheaper and natural sources of nutrition and form an important food commodity. Fruits and vegetables have a pivotal role in our daily nutritional needs, as they are the source of many important elements required for growth and metabolism, i.e., vitamins, protein, carbohydrates, fats and nutritionally important minerals. Fruits and vegetables are widely distributed in nature (Al-Hindi et al., 2011).

The spoilage of fruits and vegetables is an important concern, and even though the presence of various technologies for preservation of these foodstuffs, a large proportion is lost to due to spoilage. Microbiological spoilage of food is one of the most talked about, as it spoils the foods from the fields to the cold stage and household refrigeration (Barth et al., 2009). Various reports assessed that in developed countries, 20-25% of the harvested fruits and vegetables are decayed by fungal pathogens, while a much higher percentage is expected in developing countries, including India (Liu et al., 2020).

In this era of time, most of the fruits and vegetables are kept under refrigeration, either at the supermarket or in the household kitchen. Since, enzymes are highly temperature dependent for their activity, the fruits and vegetables stored under refrigeration should not be vulnerable to the degradation by fungi. However, this is not the case. Due to poor refrigeration conditions, power cuts and other circumstances, fungi tend to grow under refrigeration conditions too. Since, the enzyme activities are compromised, the investigations of extracellular enzyme activities will provide a platform to understand, and hence control the fungal degradation of fruits and vegetables stored under refrigeration. The present study is aimed at providing the much needed support in this direction.

MATERIALS AND METHODS

The present work was conducted during the year 2017-2019 in the laboratory of the Swami Vivekanand University, Sagar in collaboration of Excellent Bio Research Solutions Pvt. Ltd., Jabalpur.

Isolation and Identification of fungi from infected fruits and vegetables

The contaminated fruits and vegetables stored under refrigeration were collected from major super markets, departmental stores and house hold kitchens. A variety of fruits and vegetables were collected.

The fungi infected part was cut from such fruits and vegetables and inoculated on potato dextrose agar (HiMedia, India). The plates were incubated for 3 to 5 days at 25°C. The fungal colonies appearing onto

the plate were isolated and plated on a separate PDA plate. The isolation was performed for several generations to achieve the pure culture. The macroscopic and microscopic characters of the isolated fungi were recorded. The fungal identification was done using available scientific literature, books and monographs.

Bulk culture of isolated fungi

Isolated fungi were cultured in bulk in order to get higher amounts of extra cellular enzymes. The bulk growth will be achieved by culturing the fungi in potato dextrose broth with the fruit or vegetable extracts, from which that particular fungus was isolated. Fungi were inoculated under aseptic conditions in 250 ml Erlenmeyer flasks contained 5% fruit/vegetable peels. The inoculated flasks were incubated at 15°C (to mimic the refrigerated conditions) with shaking on a rotary incubator shaker at 150 rpm for 5-10 days.

Extra cellular pectinase activity

Cell wall degrading enzymes i.e., pectinases, cellulases and amylases and proteases from the isolated fungi were produced using their spoilage fruits as culture media in stationary or agitation phases. The fungal filtrate will be used as a source for enzyme activities. The activity of pectinase was screened using standard methods.

Pectinase Enzyme Assay

Pectinase enzyme assay was based on the determination of reducing sugars produced as a result of enzymatic hydrolysis of pectin by dinitrosalicylic acid reagent (DNS) method (Carrasco et al., 2020). For enzyme assay, 1.5 mL of freshly grown culture was taken and centrifuged at 10,000 rpm for 5 min. The supernatant (100 µL) from the culture broth was served as the source of the enzyme. In addition, substrate was prepared by mixing 0.5% (w/v) citrus pectin in 0.1 M of pH 7.5 phosphate buffer.

From the prepared pectin substrate, 900 µl was added to three clean labelled test tubes; one for enzyme, one for enzyme blank, and one for reagent blank. Then, 100 µl of crude enzyme was added to test tube labelled as enzyme and 100 µl of distilled water was added to test tube labelled as regent blank while test tube labelled as enzyme blank remained as it was. Then, the test tubes were incubated at 50°C for 10 minutes in the water bath. After incubation 2000 µl of dinitrosalicylic acid reagent (DNS) was added to the all test tubes to stop the reaction. Meanwhile, into test tube labelled as enzyme blank 100 µl of crude enzyme was added after the DNS. Then, all the test tubes were placed in a boiling water bath (92°C) for 10 minutes. Finally, the tubes were cooled and optical density (OD) was measured using spectrophotometer (EI, India) at 540 nm. Enzyme activity was measured against enzyme blank and reagent blank. The enzyme unit was defined as the amount of enzyme that produces one molecule of glucose per minute (µg min^{-1}) under the assay conditions.

Effect of pH on enzyme activity

Effect of pH on enzyme activity was determined by incubating enzymes (culture supernatant as above) in buffers of different pH for 1 hr during pectinase assay as mentioned earlier. The different pH ranges used for study were 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0. Three buffer systems 100 mM each were used for different pH ranges in this study. They were Citrate buffer (Sodium citrate (Titan Biotech Ltd., India) and Citric acid (Qualigens, India)) for pH ranges 3.0 to 5.0, Phosphate buffer (Sodium dihydrogen phosphate (Thomas Baker, India) and disodium hydrogen phosphate (Merck, India)) for pH ranges 6.0 to 7.0 and Tris/ HCl (Thomas Baker, India) buffer for pH ranges 8.0 to 11.0.

Effect of temperature on enzyme activity

Fungal isolates were inoculated in the above mentioned synthetic medium followed by incubation at 2, 5, 8, 11, 15, 18, 21, 25 and 28°C. Enzyme production was measured after 7 days of incubation.

Effect of substrate (Enzyme Induction Studies)

Selected fungal samples (see above) were inoculated in earlier mentioned synthetic medium containing glucose as carbon source and grown for 3 days at 30°C in shaking water bath (180 rpm). The fungal growths were then separated by centrifugation at 8000 rpm for 10 minutes and washed with sterile distilled water and divided into 5 aliquots each. Each aliquot was inoculated in synthetic medium containing one of the sugar i.e., glucose, fructose, sucrose, maltose, galactose, starch and cellulose, as a sole carbon source respectively. Flasks were incubated at 30°C for 7 days in a shaking water bath followed by determination of enzyme activity in cell free culture supernatant by pectinase activity as above (KC et al., 2020).

RESULTS

The present study was undertaken during the year 2017-2019. The local household kitchens were asked to submit the fungal infested fruits and vegetables, which were stored under refrigeration for a long time. In total, 24 different fruits and vegetable samples were collected from the local households.

The prime fungal strains were isolated from the plates and cultured for several generations to obtain pure cultures. These cultures were identified using the macroscopic characters of the front and back of

fungal colonies on PDA. In total, 10 fungal strains were isolated from fruits and vegetables, which were stored under refrigerated conditions for long.

The pectinolytic activity of cell free extracts of different isolated fungi was assessed using standard methodology. The effects of pH, temperature and carbon source were also studied on pectinolytic activities of different fungi.

The pectinase activity of cell free extract of *Aspergillus flavus* was observed at pH 7.0 (106.80 mg glucose produced min^{-1} mg^{-1} protein), while pH 9.0 had lowest enzyme activity as 20.72 mg glucose produced min^{-1} mg^{-1} protein. *Aspergillus niger* showed maximum Pectinolytic activity at pH 7.0 (118.50 mg glucose produced min^{-1} mg^{-1} protein), while pH 5.0 had lowest enzyme activity as 24.69 mg glucose produced min^{-1} mg^{-1} protein. *Aspergillus fumigatus* had maximum pectinolytic activity at pH 7.5 (117.36 mg glucose produced min^{-1} mg^{-1} protein), while pH 4.0 and 4.5 had lowest enzyme activity as 24.93 and 24.92 mg glucose produced min^{-1} mg^{-1} protein. *Curvularia lunata, Mucor racemosus, Rhizopus oryzae* had optimum pH as 7.0 while *Alternaria alternata* had pH 7.5 (Fig 1).

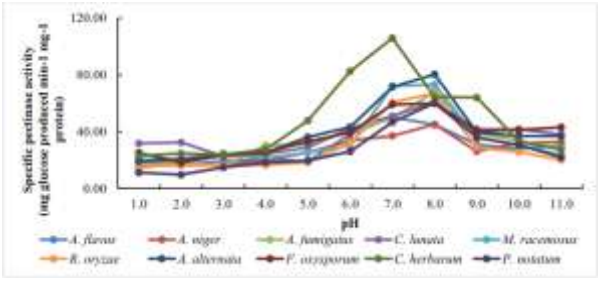

Fig 1: Specific pectinase activity by different fungi at different pH.

The optimum temperature for pectinase activity of cell free extract of most of the fungi were at 12-15 ºC, except *Fusarium oxysporum* and *Cladosporiuum herbarum* which had optimum temperature at 18ºC. *Penicillium notatum* had maximum pectinolytic activity at 5ºC (37.64 mg glucose produced min^{-1} mg^{-1} protein) (Fig 2). Among various carbon sources, highest pectinolytic activity was observed of starch and glucose.

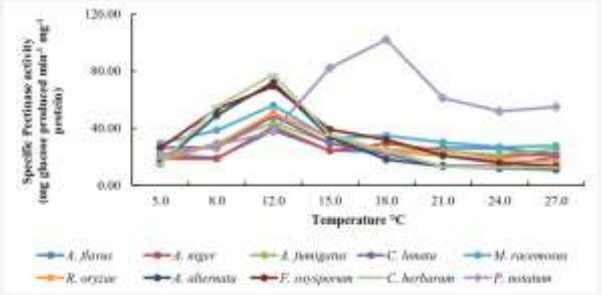

Fig 2: Specific pectinase activity at different temperatures by isolated fungi

DISCUSSION

The evolution of human beings has relied heavily on their food and the ability to cultivate the food material. Still, almost large if not all human populations depends on food that is cultivated in various parts of the world by employing different and expensive cultivation techniques (Dijksterhui*.,* 2017).

Spoilage of food, especially fruits and vegetables, is a major problem in all over the world, studying the cause of food spoilage is of high importance. Food is a very important source of energy human being, plants, animals and all living organisms. Food is a substance that contains and provides all the essential nutrients such as carbohydrates, fats, protein, vitamin, or minerals. The food is consumed by the organisms and assimilated to produce energy to maintain and stimulate the growth (Benedict *et al.,* 2016).

Fungi grow on food with fuzzy or cottony appearance and sometimes leave coloured spots that are also visible in the food product. Fungi destroy the enzymatic activity of food. They also produce various types of allergic products along with toxins. Fungi from a diverse group are known to cause food spoilage all over the world. Most of these fungal species have omnipresence and include the genera like *Aspergillus*, *Alternaria*, *Fusarium*, *Mucor*, *Phytophtora*, *Penicillium*, *Cladosporium*, *Curvularia*, *Rhizopus* and *Trichoderma* etc (Edward & Alcamo, 2001).

The fungal deterioration of raw vegetables and fruits may result from the action of the fungal enzymes. The spoilage of fruits and vegetables due to fungal attack may vary with type of fruit or vegetable as well as with the specific varieties, as some of the varieties are more resistant towards fungal attack. However, when it comes to the fungal deterioration of fruits and vegetables under refrigerated conditions, it is important to note that the fungi responsible to contaminate a particular fruit or vegetable under normal or ambient temperature, may not act similarly under refrigeration conditions. The extra cellular enzymes, thus should have capabilities of acting under cold conditions. The present study is thus important to analyze the role of different extracellular enzymes under refrigerated conditions.

Pectinases are a heterogeneous group of enzymes that are responsible to degrade the pectin by the hydrolytic actions. Pectin is one of the major substances of the plant cell walls. Since, plant cell wall present the first defence against fungal invasion, hence the activity of pectinases are an important tool to under the mode of fungal invasion for spoilage of fruits and vegetables (Sandri *et al*. 2011).

Further, as per our study, it was shown that the optimum temperature range for most of the isolated fungi was between 8-15°C. This means, one should know and regulate the temperature of the refrigerator and the fruits and vegetables should be stored under 8°C, in order to reduce the incidents of fungal storage. However, most of the households are not aware of the temperature of the refrigerator, and also do not keep fruits and vegetables at the proper temperature, which results in faster deterioration of them due to fungi. The fruits and vegetable trays supplied in the refrigerator, usually has a temperature of 10-15°C, allowing the fungal growth. Further, fruits and vegetables kept in the door of the refrigerator, allow more suitable temperature for the fungal growth. An awareness program is required for the refrigerator owners to keep the fruits and vegetables at lower temperature, to keep them safe and stay longer. Such an approach is highly useful for the industrial storage and transportation in order to increase the shelf life of these crucial agricultural products. Since fungal invasion can be at any stage of handling of fruits and vegetables such as harvesting, cleaning, sorting, packaging, transport and storage, it is possible that the fruits and vegetables kept under refrigeration in the household are already infected, and even the refrigeration process cannot guarantee the safety of these edible items.

The present study was able to demonstrate the ability of different isolated fungi to degrade the various biomolecules on offer under varied pH and temperature conditions. The study has shown, the even under refrigerated conditions, fungi are able to deteriorate the fruits and vegetables and appropriate measures should be taken while the bulk storage of such fruits and vegetables.

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