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A Comparative Study of Lytic Enzyme Profiles for Food Borne Fungi

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***Abstract*: Microorganisms, including fungi, are known to exert various functions and are being utilized for various purposes. The fungal invasion of the plants is thought to be completely enzymatic process, which are produced by the invading fungi. The major elements of plant cell walls are polysaccharide, hemicellulose and polymer, with polysaccharide being the foremost abundant part (Hans et al., 2003). Most of the fungi have the ability to penetrate to the cell wall of plants through the actions of different enzymes, that work solely or in combination to allow fungal hypae to invade through the broken cell wall. These enzymes are primarily cellulase, pectinase and amylase enzymes that soften the carbohydrate containing cell wall of plants (Gherbawy, 1998). Further, high water content in most of the fruits and vegetables, coupled with optimum pH conditions provides fungi an added advantage for the invasion and because of this, degradation due to fungi is a more common phenomenon. Refrigeration of fruits and vegetables at low temperatures is thought to be a good technique to avoid fungal invasion, though is not completely foolproof.**

***Keywords*: Amylase, degradation, fungi, lytic enzyme, pectinase.**

1. 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. Food spoilage can occur due to many factors, and at many levels of food transport and storage. These include, but not limited to; damage by insects, rodents, spoilage due to pathogenic bacteria and fungi, spoilage due to use of harsh chemicals, physical spilage due to mishandling during transportation and storage, etc. Microbiological spoilage of food is one of the most talked about, as it spills the foods from the fields to the cold stage and household refrigeration's (Barth et al., 2009).

Out of the many factors responsible for the food spoilage, spoilage due to the fungal attack is more important. These fungi can attack to the fruits and vegetables at various stages, including at the time of agriculture, storage, transport and finally, when these fruits and vegetables are stored under low temperatures for longer shelf life. 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 (Zhu, 2006). In developing countries, higher percentage of spoilage of fruits and vegetables after the harvesting because of inadequate storage and transportation facilities. Further, due to insufficient data, is seems difficult to estimate the proportion of food spoilage due to fungi and to take remedial steps for its prevention. As per the WHO estimation, the developing countries, including India, usually loss more than 50% for fruits and vegetables they produce per year (Broughall and Brown, 1984).

Microorganisms, including fungi, are known to exert various functions and are being utilized for various purposes. The fungal invasion of the plants is thought to be completely enzymatic process, which are produced by the invading fungi. The major elements of plant cell walls are polysaccharide, hemicellulose and polymer, with polysaccharide being the foremost abundant part (Hans et al., 2003). Most of the fungi have the ability to penetrate to the cell wall of plants through the actions of different enzymes, that work solely or in combination to allow fungal hypae to invade through the broken cell wall. These enzymes are primarily cellulase, pectinase and amylase enzymes, that soften the carbohydrate containing cell wall of plants (Gherbawy, 1998). Further, high water content in most of the fruits and vegetables, coupled with optimum pH conditions provides fungi an added advantage for the invasion and because of this, degradation due to fungi is a more common phenomenon. Refrigeration of fruits and vegetables at low temperatures is thought to be a good technique to avoid fungal invasion, though is not completely foolproof.

2. Materials and Methods

A. Experimental Design

The present work was conducted during the year 2023 in the laboratory of Excellent Bio Research Solutions Pvt. Ltd., Jabalpur. The contaminated fruits and vegetables stored under refrigeration were collected from major super markets,

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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. After the serial dilution. The macroscopic and microscopic characters of the isolated fungi were recorded. The fungal identification was done using available scientific literature, books and monographs.

B. Extra Cellular Enzyme Activity

Cell wall degrading enzymes as pectinases, celluloses 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, cellulase and amylase will be screened using standard methods.

C. Screening for Cellulose Activity

The cellulolytic activity of fungal strains were determined by their ability to grow and form clear zones around colonies on selective medium with carboxymethyl cellulose. The plates containing modified Czapek-mineral salt medium incorporated with 1% carboxymethyl cellulose, were inoculated with isolated and selected fungal cultures and then incubated at 25 ± 2°C in an inverted position for 2-5 days. With little modification the plates were stained with 1% Congo Red solution (Sunchem, India) and incubated for 15 minutes at room temperature. This step was then followed by neutralization with 1M NaCl solution. Plates were incubated for further 10 minutes at room temperature. Formation of yellow coloured halo zone around the colony after flooding with 1% Congo Red solution and washing the dye with 1 M NaCl solution indicates production of cellulase by the fungi i.e., cellulose hydrolysis. The ratio of diameter of the clear zone to the diameter of the colony was then measured.

D. Preparation of Colloidal Chitin

Colloidal chitin was prepared from commercial chitin by the method of Roberts and Selitrennikoff (1988) with a few modifications. In the first step acid hydrolysis of commercial chitin (Titan Biotech Ltd., India) was done by suspending 5.0 g of chitin in 60 ml Conc. HCl by constant stirring using a magnetic stirrer (Sonar, India) at 4°C overnight. Second step was the extraction of colloidal chitin by ethanol neutralization. To the resulting slurry (as obtained in step one), 2000 ml of ice-cold 95% ethanol was added and kept at 26oC for overnight. It was then centrifuged at 3000 rpm for 20 min at 4°C. The pellet was washed with sterile distilled water by centrifugation at 3000 rpm for 5 min at 4°C. The washing of the pellets was done till the smell of alcohol vanished. Colloidal chitin thus obtained was stored at 4°C until further use (Lunge and Patil, 2012).

E. Screening for chitin

Basal chitinase detection medium ((all amounts are per litre) 0.3 g of $MgSO_4.7H_2O$, 3.0 g of $(NH_4)SO_4$, 2.0 g of KH_2PO_4, 1.0 g of citric acid monohydrate, 15 g of agar and 200 ml of Tween-80) was directly supplemented with colloidal chitin (4.5 g/l) and bromocresol purple (Thomas Baker, India) (0.15 g/l). Resulting substrate had a bright yellow colour, and retained enough bromocresol purple even after pH was adjusted to 4.7 and sterilization at 121°C for 15 min. No complicated protocols for dyeing of the chitinous material and mordant to fix colours were required as per previous reports (Gomez et al., 2004; Fen et al., 2006; Wirth & Wolf, 1990). Separated polysaccharide molecules of hydrochloric acid induced colloidal chitin may therefore, form the basis for hydrogen bonds formation between the chitinous matrix and the dye. Chitobiose, as a minimal repeating unit of chitin is formed by two N- acetyl-d-glucosamine (NAGA) molecules linked by a β-1, 4 glycosidic unions and, in every NAGA residue, there are two hydroxyls, one carbonyl and one imines exposed groups that may acts as a reactive binding site for anionic dyes such as bromocresol purple to produce a colour-bound complex (Yellow). Colloidal chitin media containing bromocresol purple (yellow coloured in acidic pH 4.7) were inoculated with previously mentioned isolated fungal cultures and incubated at 25±2°C in an inverted position for 2-5 days. Chitinase producers resulted in breakdown of chitin into N-acetyl-d- glucosamine causing a corresponding shift in pH towards alkalinity and change of colour of pH indicator dye (BCP) from yellow to purple zone (due to increase in pH) surrounding the inoculated fresh culture plugs in the region of chitin utilization. The ratio of diameter of the purple zone to the diameter of the colony was then measured.

3. Conclusion

The results indicate that purified colonies were further subjected to identification using micro-morphology. The fungal colonies were observed under microscope using 40 x magnification. The plates showing only single type of fungus were considered for the further experiments. Overall, three fungi, from such plates were identified and used for further experiments. The fungal characteristics are below:

A. Aspergillus niger

Kingdom-Fungi
Division-Amastigomycotina
Class – Hyphomycetes
Order- Moniliales
Family- Moniliaceae
Genus- *Aspergillus*
Spices- *niger*

1) Cultural characteristics

The Present culture of the fungus *Aspergillus niger* attained maturity in 3- 4 days, color of colony was white, found growing in scattered manner on PDA plates, growth dense, less velvety consistency with irregular margin, powdery in texture.

2) Microscopic features

The hyphen were septet, branched smooth, walled, hyaline, conidiophores cylindrical with broadening distal end bearing a conspicuous conidial head, The phialides arises singly of smaller branches, short.

B. Mucor sp.

Kingdom - Fungi
Phylum – Zygomycota
Class – Zygomycetes
Order – Mucorales
Family – Mucoraceae
Genus – *Mucor*

1) Cultural characteristics

The present culture of the fungus Mucor sp. was found growing very fast at 28±1ºC on PDA plate, from the front, the color was white initially and becomes grayish brown later on while from reverse it dull white, fluffy, cottony growth, growing radially, with irregular margin.

2) Microscopic features

Hyphae non-septate, broad, hyaline, sporangiophores, hyaline, long, erect, taper towards their apices, branched; columella, hyaline; sporangia, round, in diameter, gray to black in color, filled with sporangiospores.

C. Penicillium sp.

Kingdom: Fungi
Division: Ascomycota
Class: Eurotiomycetes
Order: Eurotiales
Family: Trichocomaceae
Genus*: Penicillium*
Species: sp.

1) Cultural characters

The fungal colonies were fast spreading, floccose, with bright blue green surface, later becoming darker, back of the colonies were yellowish.

2) Microscopic characters

Microscopic characters were shown by conidiophores arising from submerged mycelium, sometimes branched up to 750µ long × 2.8-4.6µ broad. Conidia globose to oval, 2.6-3.2µ in diameter. The cellulolytic activity of different isolated fungi was assessed using standard methodology. When the cellulose activity was screened, highest cellulase activity was shown by *Mucor* sp. When chitinase activity was screened, all three isolated fungi showed good chitinase activity (Clear zone around the colonies), highest with *Aspergillus niger*.

Fig. 1. Collection of food samples for isolation of fungi are activity

Basal chitinase detection medium (all amounts are per litre) 0.3 g of $MgSO_4.7H_2O$, 3.0 g of $(NH_4)SO_4$, 2.0 g of KH_2PO_4, 1.0 g of citric acid monohydrate, 15 g of agar and 200 ml of Tween-80) was directly supplemented with colloidal chitin (4.5 g/l) and bromocresol purple (Thomas Baker, India) (0.15 g/l). Resulting substrate had a bright yellow colour, and retained enough bromocresol purple even after pH was adjusted to 4.7 and sterilization at 121°C for 15 min. No complicated protocols for dyeing of the chitinous material and mordant to fix colours were required as per previous reports (Gomez et al., 2004; Fen et al., 2006; Wirth & Wolf, 1990).

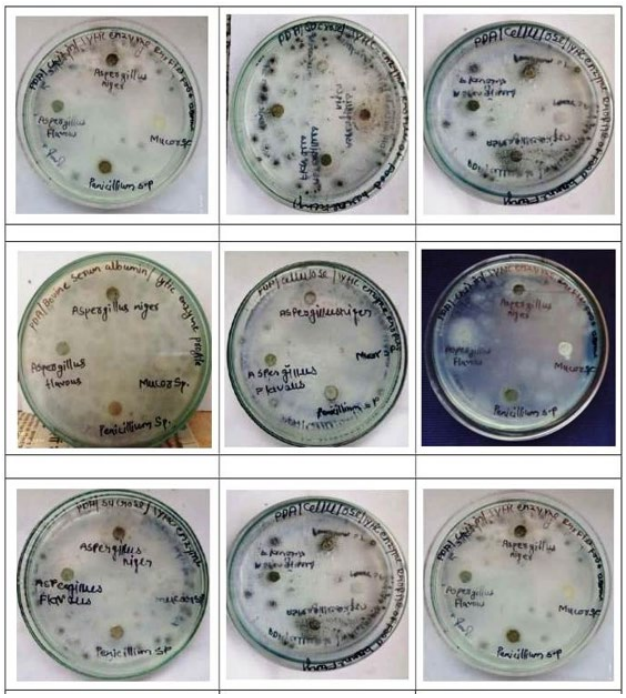

Fig. 2. Appearance of fungal colonies on PDA

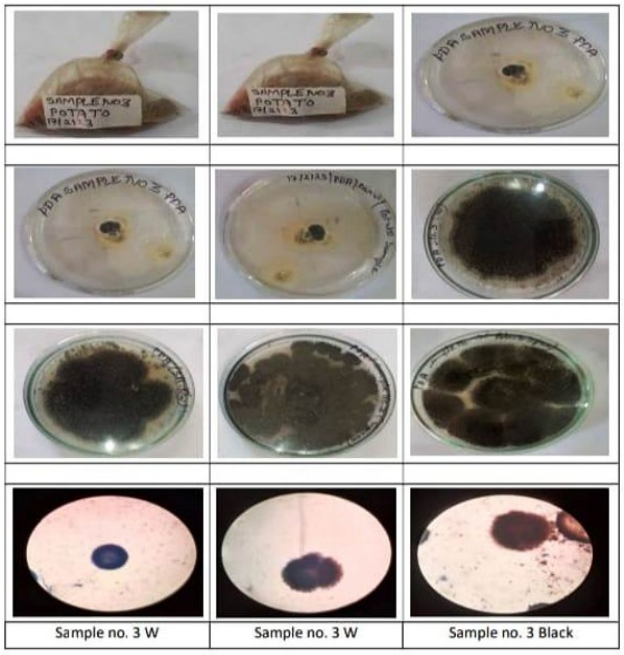

Fig. 3. Microscopic characters of fungi cultured from isolated pure colonies

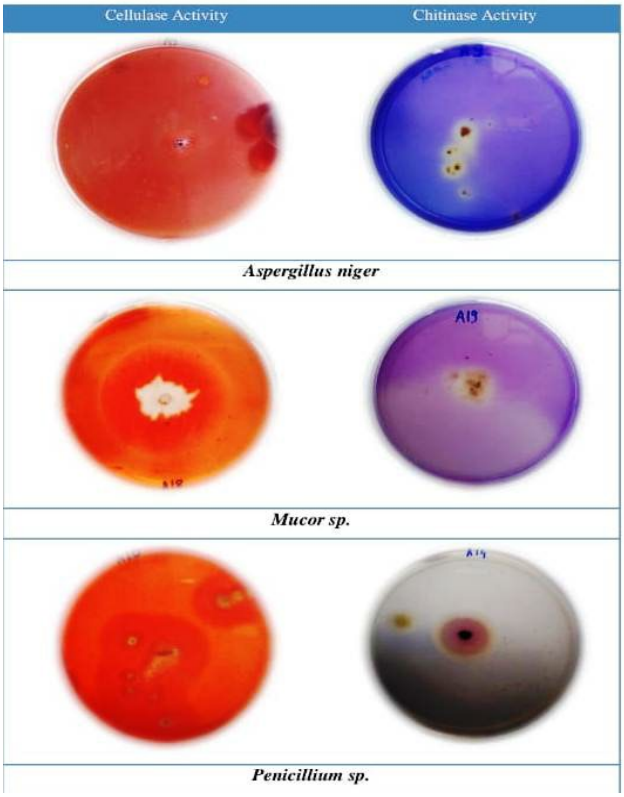

Fig. 4. In vitro lytic enzyme screening for fungi isolated from food samples

Separated polysaccharide molecules of hydrochloric acid induced colloidal chitin may therefore, form the basis for hydrogen bonds formation between the chitinous matrix and the dye. Chitobiose, as a minimal repeating unit of chitin is formed by two N- acetyl-d- glucosamine (NAGA) molecules linked by a β-1, 4 glycosidic unions and, in every NAGA residue, there are two hydroxyls, one carbonyl and one imines exposed groups that may acts as a reactive binding site for anionic dyes such as bromocresol purple to produce a colour-bound complex (Yellow). Colloidal chitin media containing bromocresol purple (yellow coloured in acidic pH 4.7) were inoculated with previously mentioned isolated fungal cultures and incubated at 25±2°C in an inverted position for 2-5 days. Chitinase producers resulted in breakdown of chitin into N-acetyl-d-glucosamine causing a corresponding shift in pH towards alkalinity and change of colour of pH indicator dye (BCP) from yellow to purple zone (due to increase in pH) surrounding the inoculated fresh culture plugs in the region of chitin utilization. The ratio of diameter of the purple zone to the diameter of the colony was then measured.

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