INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 11, No. 5, p. 260-269, 2017

RESEARCH PAPER OPEN ACCESS

Isolation, characterization and control of a fungus responsible for post-harvest mango spoilage from northern region of Bangladesh

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Key words: Postharvest mango spoilage, 'Totapuri' mango, Fungus, Biological Control measurement, Citric acid.

http://dx.doi.org/10.12692/ijb/11.5.260-269 Article published on November 30, 2017

Abstract

Mango, 'the king of the fruits' is one of the most popular, nutritionally rich fruit in Bangladesh. One of the limiting factors that influence the economic value of mango is its short storage life at ambient temperature due to spoilage, caused by microbial invasion. Fungi are the primary spoiling organism as they are rich in cell wall degrading enzymes. Current study was carried out to isolate, characterize and to find out a cost effective control measure for the fungal strains associated with post-harvest spoilage of mango. One fungal strain was isolated from the spoiled "Totapuri' mango. The primary characterization of the fungal strain shows that the colony diameter, culture characteristics and sporulation of the fungus is greatly influenced by the type of growth medium used. CDA exhibited higher mycelial growth, whereas the PDA culture medium was responsible for heavy sporulation. Molecular confirmation of the isolate as fungus was done by the extraction of genomic DNA and ITS region amplification by PCR. The fungi grew optimally at 25°C, pH 9 and in dextrose carbohydrate source. The fungus exhibited high sensitivity to citric acid and NaCl.. The results of this investigation combined with further studies like ITS region sequencing, can be used in precise identification of organism causing mango spoilage and it will also be possible to decipher a biological control mechanism for this fungus.

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Introduction

Mango (*Mangifera indica L.*) is one of the most important and popular fruits in Bangladesh, which is considered as the "king of fruits". Mango cultivation occupies about an area of 67,842 ha with a production of 889,176 metric tons per season in Bangladesh (BBS, 2012). Alone in Rajshahi region the cultivation area of mango is about 46,602 and the production is 370,513 M. tons (BBS, 2012).There are over 270 varieties of sweet edible mangoes in the Rajshahi region (Agricultural Revolution, 2013).'Totapuri' is one of the widely cultivated varieties of mango found in the northern region of Bangladesh.

Flesh of mango are very much susceptible to postharvest diseases. Fluctuation of temperature, and physical injury also causes enormous economic losses (Al-Najada and Al-Suabeyl 2014). Ripened mango are highly susceptible to microbial invasion (Palejwala *et al.* 1984). The primary spoilage organisms are fungi, moulds and yeasts as well as some bacterial strains in post-harvest stage (Zheng *et al.* 2007). Quantitative and qualitative losses results from rapid and extensive break down of soft tissue or flesh of mango by pathogenic or saprophytic microorganisms (Reddy *et al.* 2008).

The microorganisms which are responsible for spoilage, exploit the host by using extracellular lytic enzymes that degrade principal storage polymers to release water and the fruit's other intracellular constituents for use as nutrients for their growth (Barth *et al.* 2009).

Fungi (both yeasts and molds) are the dominant microorganisms for mango spoilage because fruits contain high levels of sugars and nutrient elements and their low pH values make them particularly vulnerable to fungal decayed (Singh and Sharma 2007). Generally spoiling fungi are considered toxigenic or pathogenic. Earlier, a number of toxigenic fungi have been isolated from spoiling fruits (Stinson *et al.* 1981). Some of them may produce mycotoxins during refrigeration (Tournas and Stack 2001).On the other hand pathogenic fungican cause infections or allergies (Mons 2004). *Aspergillus spp.* are known to produce several toxic metabolites, such as malformins, naphthopyrones (Frisvad and Samson 1991; Pitt and Hocking 1997) and they can produce ochratoxins (OTA), a mycotoxin which is a very important toxin worldwide because of the hazard it possess to human and animal health (Peraica *et al.* 1999; Petzinger and Weidenbach 2002).

There are several fungi which infect the fruits either on the trees or during storage. These include *Aspergillus sp*, *Phomopsis astromella* causing soft rot and scab of fruits respectively. Development of such abnormalities results wastage and low market value of the fruits.

Due to having less information regarding these diseases in mango and due to lack of proper control measure, current study was undertaken to isolate the fungi causing post-harvest spoilage, determine the morphological and biochemical characteristics as well as find out a cost effective control practice for the isolated fugal strain from the 'Totapuri' cultivar of mango.

Materials and method

Collection of infected mango and isolation of fungi

Postharvest spoiled mangoes of 'Totapuri' variety were collected from Fruit Research Centre, Rajshahi, Bangladesh. The fungus responsible for the 'Totapuri' mango spoilage was isolated on PDA (Potato Dextrose Agar) medium by following the standard procedures described by (Agostini and Timmer 1992) with slight modification. The fungus grown from the infected piece was removed and re inoculated on PDA medium for several times to obtain pure culture. Single colony or sweep from the end of a hyphal tip was used as inoculum and inoculated on PDA for pure culture.

Microscopic observation of fungi

Optika digital microscope (Italy) along with photographic unit was used to observed the mycelia from pure cultures and identified by comparing their morphological and cultural characteristics with previously published descriptions (Barnett and Hunter 1998; Hamd *et al.* 2013).

Molecular confirmation of the isolate as fungal strain

After 7 days of incubation of the isolates on potato dextrose broth at 28±2°C, DNA was extracted from mycelium mat by using TIANamp Genomic DNA Kit (TIANGEN Biotech Beijing co. LTD, China). ITS (Internal Transcribed Spacer) region (universal Region) was amplified for the identification of the sample as fungus (Hinrikson *et al.* 2005). Primers used in PCR amplification were ITS 1 (5'-

TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5' – TCCTCCGCTTTATTGATATG-3').Cycling condition was set as initial activation of 5 min at 94°C, followed by 35 cycles of 94°C/30 sec., 52°C /30 sec.,72°C/1min.The amplified PCR products were run into 1% agarose gel along with Tiangen 1KB plus DNA ladder (Beijing, China) as marker. After 50-60 minutes of running, the gel was visualized under UV illuminator using gel documentation system (proteinsimple, Alphaimager mini, USA).

Growth profiling the fungi

Primary colony morphology was studied using Potato Dextrose Agar (PDA) media whereas to compare the morphology with PDA media, Czapeck Dox Agar (CDA) and Sabouraud Dextrose Agar (SDA) media were used. Diverse morphological characteristics of colony such as form, elevation, margin, colour, size, surface, and dry weight were observed on three different media after 7 days of fungal growth on the plates ,and classified according to the cultural characteristics described in (de Hoog and Guarro 1995). Different carbohydrates such as glucose, fructose, sucrose and starch were added as sole carbon source to the medium at 2% concentration instead of dextrose to check the effect of them. The effect of temperature on the growth of fungal strain was identified by incubating the fungus at 5°C, 15°C, 25°C and 35 °C at 28±2°C for 7 days. The effect of pH on the growth of the fungal strain was identified by inoculating into the PDA medium of pH 5.0, 6.0, 7.0, 8.0 and 9.0. Lastly, dry weight of all the fungi was measured.

Study on cellulolytic activity

A cellulose strip of 6cm X 1.5cm was used to study the cellulolytic activity. For this study the strip was placed in the liquid culture of the fungus before incubation. The flasks were observed after 7 days to check the cellulose degrading ability of the fungal stain.

Control measure by aqueous spice and plant extracts

To identify the growth inhibition of the isolated fungus, aqueous extracts of several plant parts such as bulb of *Allium sativum,* root of *Borussus flabellifer* and leaves of *Scaparia dulcis*, *Pandanus odoratissimu* and *Withania somnifera* were used in different concentrations.

Control measure by treating with NaCl

The effect of salinity on the growth of the fungal strains was carried out by incubating the fungus in various NaCl concentrations- 0.5%, 1%, 2%, 4%, 6% (w/v).

Control measure by citric acid

Citric acid is one of the predominant organic acids present in mango. To observe the effect of citric acid, different citric acid concentrations [0.25%, 0.5%, 1% and 2% (w/v)] were added into the potato dextrose liquid medium and pH was adjusted to 6.5. Inhibition percentage of fungal growth were measured by the following formula,

$$\%I = \frac{C-T}{C} \times 100$$

Where, I= Percentage of inhibition, C= radial growth in control, T= radial growth in treatment.

Statistical analysis

All data are the average of triplicates. All the graphs and standard error were analyzed using Microsoft Excel 2010.

Results

Isolation and microscopic identification

The unknown fungal strain was from post-harvest spoilage of mangoes of 'Totapuri' variety which is showed Mycelia of the fungus were examined and identified under microscope. The fungal strain could not or rarely sporulation CDA media, while on PDA, it revealed heavy conidial production after seven days of incubation period including few characteristics like thin thread like hyphae, conidia with branched chain of spores and many disperse chain of spores. The diameter of conidia was about 117.20µm and the diameter of hyphae was about 47.01µm in PDA.

In case of SDA culture media, the fungus exhibited poor or moderate sporulation including characteristic thread like mycelium with true hyphae which contain little intercalary spores and single microconidia with conidiophore. The diameter of mycelium was ~ 12.04µm and the conidia were ~44.38µm in SDA. Microscopic view of the fungal strain is given in Fig. 2.

Table 1. Morphological characterization of fungal strain on different growth media.

Characteristics	Potato dextrose agar (PDA)	Sabouraud Dextrose agar (SDA)	CzapekDox Agar (CDA)
Form	Filamentous	Filamentous	Filamentous
Margin	Filiform	Filiform	Filiform
Elevation	Umbonate	Umbonate	Umbonate
Surface	Rough	Rough	Rough with wavy growth
Opacity	Opaque	Opaque	Opaque
Front colour	Light brownish to white	Brownish to white	Pinkish white
Back colour	White with gray center	Brown center with gray center and white border	Yellowish white
Diameter(mm)	59±2.1	72±2.1	78±1.5
Dry weight(gm)	0.256±0.03	0.334±0.03	0.436±0.04

Fig. 1. Isolated fungal strain from the postharvest spoiled mangoes. (A) Postharvest spoiled mango and (B) pure culture of fungal strain isolated from 'Totapuri' variety.

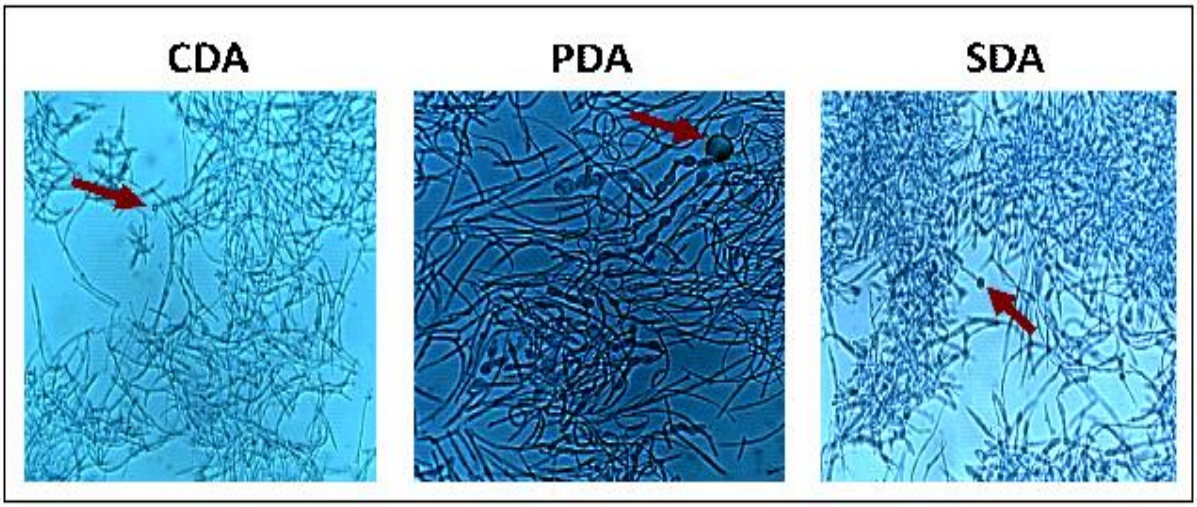

Fig. 2. Microscopic observation of the isolated fungal strains. The red arrows show the conidia of the isolated fungal strain in case of CDA and SDA medium and conidia with branched chain of spores in PDA.

Molecular confirmation of the isolate as fungal strain

DNA isolated from the fungal strain showed high molecular weight and bright band on 1% agarose gel electrophoresis where 1 kb plus DNA ladder was used as a marker showed in Fig. 3.The consensus primers ITS1 and ITS4 were used to amplify a region of the rDNA gene repeat unit. The isolate yielded a single band of ~550 bp.

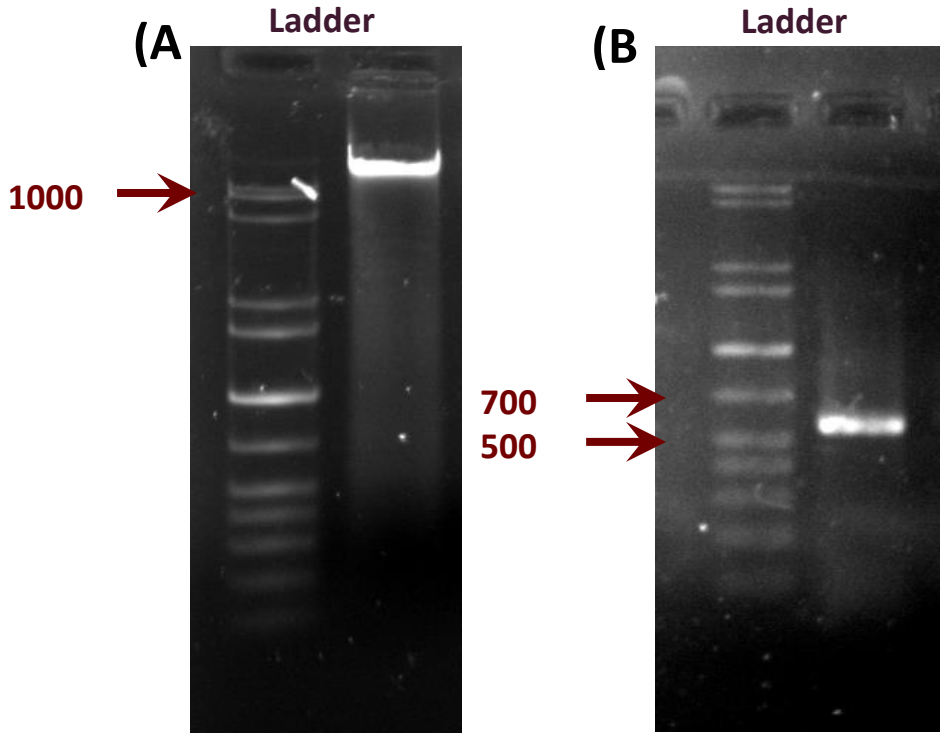

Fig. 3. Molecular confirmation of the isolate as fungal strain. Ladder and sample indicate the molecular marker and fungal strain respectively. (A) Isolated DNA; High molecular weight DNA band with ladder,(B) PCR amplification of ITS region showed expected band of ~ 550 bp which confirms the isolate as fungus.

Colony characterization on different media

According to (de Hoog and Guarro 1995)the colony of the fungal strain was characterizedby culturing them on three different types of media i.e. Potato Dextrose Agar, Czapek Dox Agar, Sabouraud Dextrose Agar. Among three types of media, CDA media increased the growth of the fungal strain. The results are shown in table 1 and in Fig. 4 (A).

Effect of carbohydrate on the growth of the fungal strain

Different carbohydrates such as glucose, fructose, sucrose and starch were added as sole carbon source to the medium at 2% concentration instead of dextrose. Result showed that carbohydrates stimulated the growth of the isolate where sucrose was more stimulatory after dextrose than the other carbohydrates for the growth of fungal isolate. The results are showed in Fig. 4 (B).

Effect of temperature on the growth of the fungal strain

After 7 days ofincubation at 5°C, 25°C, 30°C, and 35°C temperature the fungal growth was observed. Interestingly, the fungal strains showed maximum mycelial growth at 25°C temperature. The results are showed in Fig. 4 (C).

Effect of pH on the growth of the fungal strains

The growth of any fungi was affected by the action of pH. The mycelia growth of the fungal strains was observed in pH values of 5.0, 6.0, 7.0, 8.0 and 9.0. It was found that the fungal strain showed maximum growth at pH 9.0 showed in Fig. 4(D).

Study of cellulolytic activity

In this study, after 7 days of inoculation of fungus, it was observed that the filter papers in the cultural flasks were not degraded which indicates that the strain do not have any ability to degrade cellulose showed in Fig. 5.

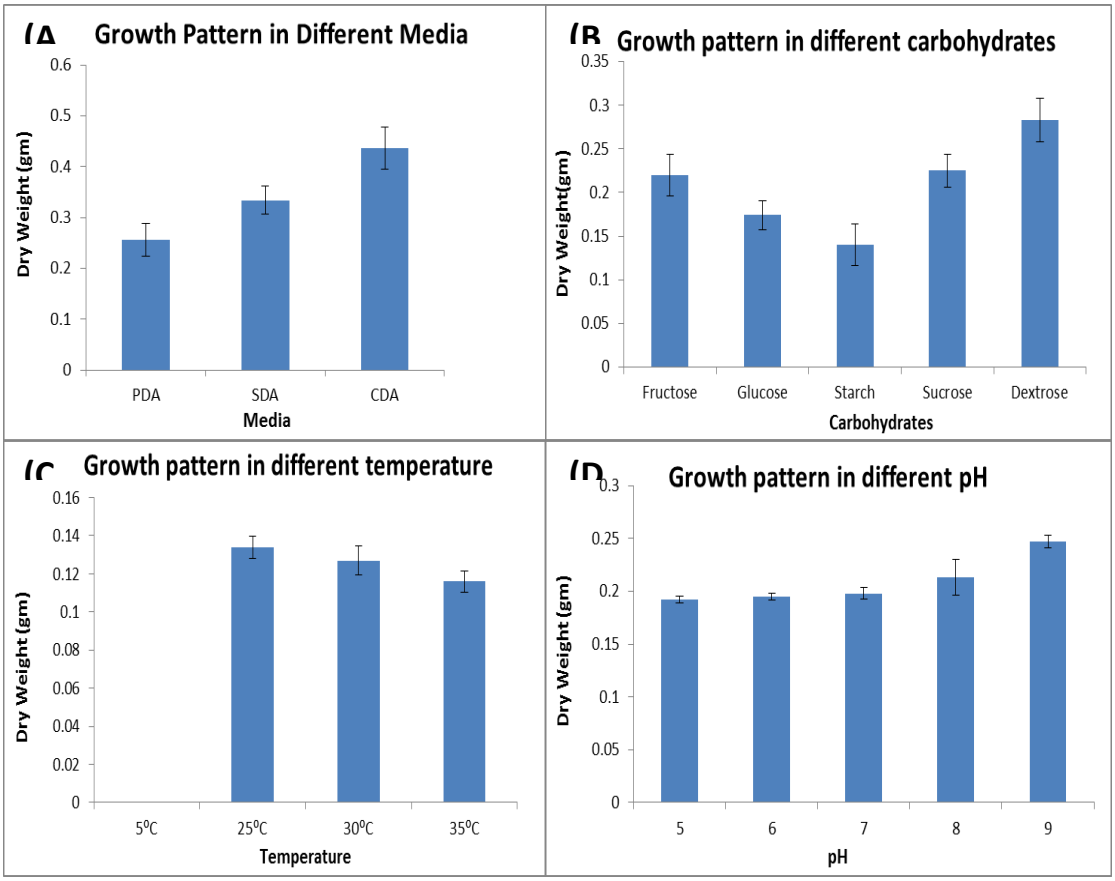

Fig. 4. Growth profiling of the isolated fungal strains. (A) Growth of fungi in three different media; Highest growth was observed on CDA for the isolated fungal strain, (B) Effect of carbohydrate on growth pattern of the isolated fungal strain; the strain showed highest growth in Dextrose media (C) Growth parameter of the fungal strain in different temperature, 25°C was found to be the optimal temperature for its growth. (D) Effect of pH on growth parameter; optimum pH for the strain was 9.

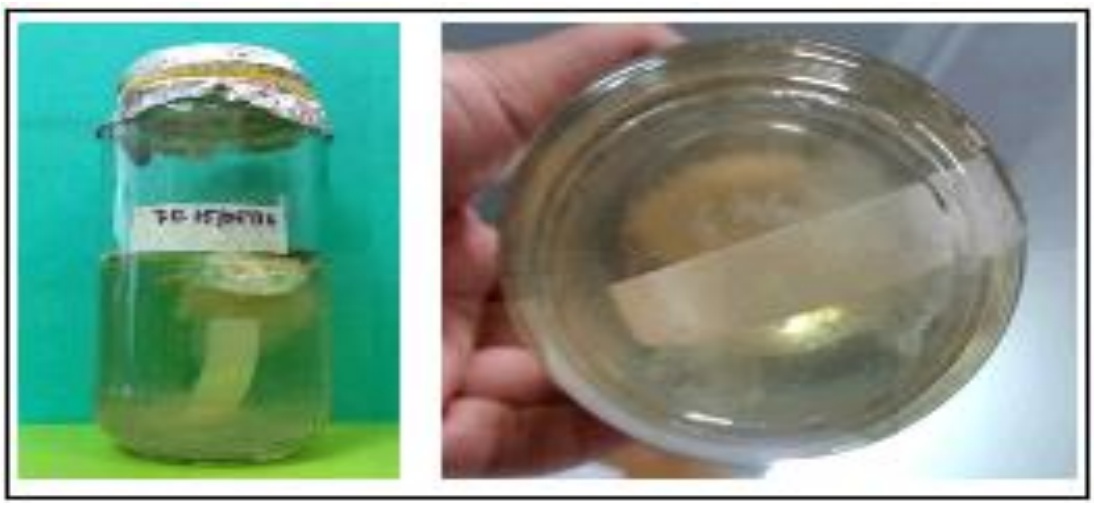

Fig. 5. Cellulolytic activity of the fungal strain. The fungal strain showed no cellulolytic activity to degrade cellulose.

Control measure by treating with plant extracts

Different concentrations of aqueous extracts of plant parts such as *Allium sativum, Scaparia dulcis, Borussus flabellifer, Pandanus odoratissimus*and *Withania somnifera* were used to investigate the inhibition rate on fungus. In the present study, growth of the fungus could not be controlled by 10%, 15%, 20% concentrations of aqueous extracts of the above plants except *Pandanus*

odoratissimus. The highest rate of was observed in 20% aqueous extract of *Pandanus odoratissimus* showed 27.5% for the fungal growth inhibition as shown in Fig. 6 (A).

Control measure by treating with NaCl

It was found that the increasing concentration of NaCl had a greater inhibitory effect on the growth of the fungus. Percentage of growth inhibition was increased with the increment of NaCl concentration. At 6% NaCl concentration, 55% growth inhibition was observed which is showed in Fig. 6(B).

Control measure by treating with organic acid

Since citric acid is one of the predominant organic acids present in mangoes, their effect on the growth of the fungal strain was studied using different concentrations. It was observed that the growth of the fungus decreased as the concentrations of citric acid was increased. Citric acid at 2% concentration inhibited the growth of the fungus completely which is showed in Fig. 6 (C).

Discussion

Several studies revealed that the microorganisms like fungi, bacteria cause mango spoilage because the flesh of the ripened mango is very much susceptible to theses microorganisms. (Barth *et al.* 2009; Jamalizadeh *et al.* 2011).

This microbial attack to the ripened mango makes economical loses and makes the fruit less nutritive to the consumers. 'Totapuri' a widely cultivated mango variety in Northern region of Bangladesh is also affected by several post-harvest spoilage.

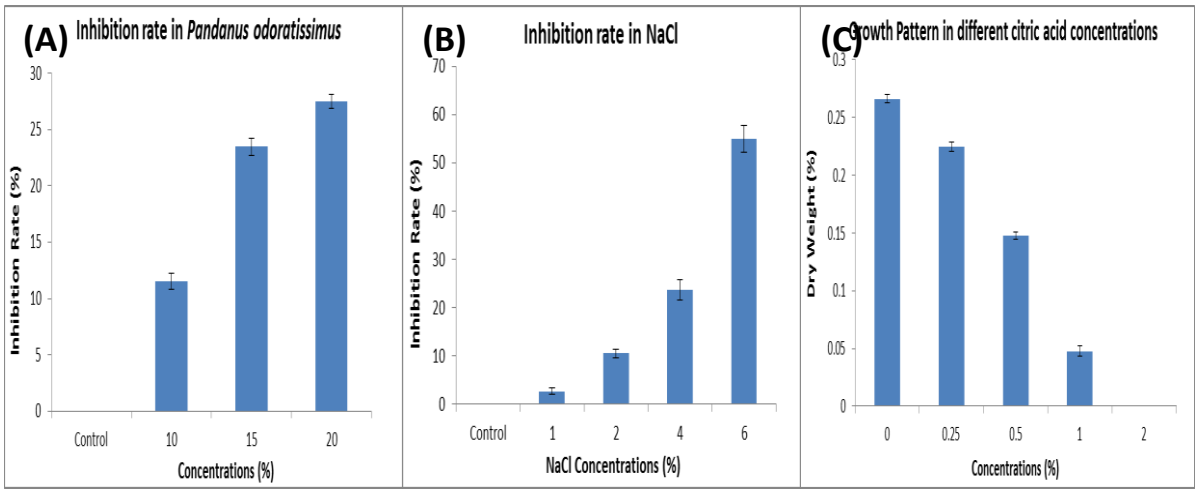

Fig. 6. Control measure of the fungal strain. (A) Growth inhibition of the fungal strain against the plant extracts where 20% aqueous solution of *Pandanus odoratissimus* showed highest inhibition for the growth, (B) Control measure of the fungal strain using NaCl. 6% NaCl showed highest inhibition for the growth of the fungal strain and (C) Growth pattern in different citric acids concentration where 2% citric acid inhibited 100% growth of fungal strain.

In the present study, the gross appearance of colonies developed on agar media recommended for this particular fungal genus (PDA, SDA and CDA) is considerable important in identification showed in Table 1.

Our findings revealed marked differences in the sporulation patterns of the fungus on three culture media used where least sporulation with maximum mycelial growth on CDA, heavy sporulation with minimum growth on SDA and poor or moderate sporulation was found on PDA. Several workers have recognized the importance of reproductive structures for inoculums production and studies have been conducted on the effects of various media components along with important physiological parameters that lead to maximum sporulation (Kim *et al.* 2005; Saha *et al.* 2008; Saxena *et al.* 2001).

Molecular identification of most fungal species relies on the amplification and sequencing of the internal transcribed spacer (ITS) region of the fungal genome, which is highly variable among species or even populations of the same species (Hibbett 1992; Horton

and Bruns 2001). PCR amplification of the isolated DNA confirms the isolated organism as fungal stain by molecular means.

Present study evaluates the effect of the different temperature ranges (5°C, 25°C, 30°C, 35°C) on the fungal strain. The fungal strain had optimal growth at 25°C and poor growth at 35°C which indicates the isolated fungus belonging Mesophiles group (Burge 2006).It was reported that each enzyme system of organisms has a particular pH range in which it can function(Bhatia *et al.* 2014). The fungal strain showed the maximum growth at alkaline pH i.e. 9. The current investigation showed the effect of different carbohydrates on fungal growth where it was observed that the growth was highest on dextrose source and lowest on starch source. The isolated fungal strains are not cellulolytic as they cannot produce cellulase enzyme like *Trichoderma*, *Humicola*, *Penicillium* and *Aspergillus* (Lynd *et al.* 2002).

Different studies reported that the growth of the several fungi was controlled using the extract of natural plant parts(Andriani *et al.* 2015; Javadian *et al.* 2016; Shams-Ghahfarokhi *et al.* 2006). The present study revealed the mycelium growth of the fungal strain was limitedly inhibited only by *Pandanus odoratissimus* among five plant extracts used in the experiment.It was also found that 6% NaCl was able to inhibit ~ 55% fungal growth, which can be a great promising factor for successful future control practice. Harmless organic acids like citric acid can also be a fair and easy control of the fungal strain which showed a significant growth inhibition at 2% concentration.

Conclusion

In conclusion, it was confirmed by microscopic observation and molecular means that the 'Totapuri' variety of mango is invaded/infected by a fungal strain which causes post-harvest spoilage and causes severe economic loss. The growth characteristics of the fungal strain were determined. *Pandanus odoratissimus* extract, 2 % citric acid and 6% NaCl solution can be used as a cost effective, cheaper and user friendly control measure which can save us from the economic losses and maintain the nutritive value of the postharvest 'Totapuri' mango.

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