Original Research Article

Characterization and control of two unknown fungal strains isolated from postharvest mango spoilage

Abstract

Ripened mangoes are perishable sometimes as they contain large amount of water and carbon sources which make it susceptible to spoilage by different fungi. This study was therefore carried out through morphological characteristics, growth characteristics and control measure of two unknown fungal strains isolated from postharvest spoiled 'Gopalvog' and 'Mollica' mango varieties. Both the colony color of fungal strain isolated from 'Gopalvog' and 'Mollica' was initially white. Surprisingly, the colony of fungal strain from 'Gopalvog' became grayish brown after 72 hours. The optimum mycelial growth of fungal strain isolated from 'Gopalvog' was obtained at pH 8, temperature 35°C and 2% glucose concentration. The optimum pH and temperature for growth of the fungal strain isolated from 'Mollica' were 6 and 35 °C respectively. At 6% NaCl concentration, 100% inhibition of growth was obtained for both fungi. Growth of both the fungal strain was inhibited at 2% and 0.5-2% citric acid concentration respectively.

Keywords: Mango, postharvest spoilage, fungi, isolation, inhibition

¶ Both authors are considered as first author

Introduction

Huge amounts of fruits are cultivated across the world. The recommended quantity of fruits to be consumed by a healthy adult is 230 g/day, while the current per capita consumption of fruits is

reported to be less than 160 g/day (1). Mango was originated from India and Southeast Asia and nowadays it is one of the most important fruits cultivated in tropical countries (2).

In accordance with several studies it was reported that di□erent parts of *M. indica* contains phenolic compunds, polyphenols, phenolic acids, hydrocarbons, fatty acid, amino acids and triterpenes etc. and these chemical compounds present in the mango exhibited various biological activities like anticancer, anti-inflammatory, antidiabetic, antioxidant, antibacterial, antifungal, anthelmintic, gastroprotective, hepatoprotective, immunomodulatory, antiplasmodial and antihyperlipemic e□ects. (3)

Extension of mango cultivation has been occurred to several other parts of the world including Africa, the Americas and the Caribbean region (4). Day by day consumption of mango get popularity in the developed countries(5). Among highest mango producing countries, Bangladesh takes 8th position in the world (6). In Bangladesh, total production of mango is 1047849 t annum $^{-1}$ with an average yield of 13.25 t ha^{-1} (6).The potential of mango as a commercial crop is markedly limited because of its high perishability, which results in considerable wastage (7). Disease susceptibility due to microorganism, sensitivity to low storage temperatures and perishability due to ripening and softening are serious causes of postharvest losses in mango which are limiting its handling, storage and transport potential. The postharvest losses of fresh mango fruits are reported to be 25 - 40% in India and 69% in Pakistan; and microbial decay accounts for 17.0 - 26.9% of the total postharvest losses in Asian countries (8). The postharvest spoilage in mangoes has been estimated to be in the range of 25-40% from harvesting till they reach consumers. It is well known that mango is climacteric in nature and ripen quickly after harvest. As a tropical fruit, mango is susceptible to a number of physiological disorders due to low temperature during storage and even suffers from chilling injury (9). At

ambient temperature, harvested mango fruit at the mature stage ripen quickly and have a short postharvest life, which is limited by physiological deterioration related to over ripening and by pathogen development leading to decay (10). Rapid ripening in combination with infection by microorganism is a serious cause of postharvest spoilage in mango (11). Most of cases microorganism responsible for mango spoilage are fungi and bacteria where ripened mangoes are more susceptible to attack by a variety of microorganisms (12). More than 90 fungal strains are responsible for mango spoilage(13).“Gopalvog” and “Mollica” are the two most cultivated mango varieties in Bangladesh. These two varieties are greatly affected by postharvest spoilage. Current study was designed to characterize and control of fungi associated with the spoilage of postharvest mango varieties named Gopalvog and Mollica.

Materials and Method

Collection of fruits

Postharvest spoiled mangoes of Gopalvog and Mollica varieties were collected from Fruit Research Centre, Rajshahi, Bangladesh. The selected mangoes were separated by polyethylene bag for each type of infected fruit.

Isolation of fungi from infected fruits

The fungi responsible for the spoiled Gopalvog and Mollica mangoes were isolated on PDA (Potato Dextrose Agar, (Hi-Media, India) medium by following the standard procedures described by (14) with a slight modification.

Purification of culture

The fungus growing from the infected piece was removed and re inoculated on PDA medium for several times for pure culture. Single colony or sweep from the end of a hyphal tip was used as inoculum and inoculated on PDA for pure culture of respective fungus.

Microscopic observation of fungi

Mycelia from pure cultures were examined under Optika digital microscope (Italy) and was identified by comparing their morphological and cultural characteristics with previously published descriptions (15, 16)

Molecular Identification of selected fungal isolates

After 7 days of incubation of two fungal isolates on potato dextrose broth at 28±2^{O}C, DNA was isolated from mycelium mat by using TIANamp Genomic DNA Kit (TIANGEN Biotech Beijing co. LTD) using manufacturer's guidelines. The quality of the isolated DNA was determined using 1% agarose (Sigma-Aldrich, Switzerland) gel electrophoresis. The primer pair ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5' –TCCTCCGCTTTATTGATATG-3') were used to amplify the ITS (Internal Transcribed Spacer) region which is the universal fungal primers using for identification of fungi, purchased from IDT, Malaysia. (17). The PCR amplification was carried out by following the Cycling condition where initial activation was at 94°C for 5 min., followed by 35 cycles at 94°C for 30 sec., annealing was at 52°C for 30 sec, and final extension was set at 72°C for 1min.

Growth profiling of both fungi

Potato Dextrose Agar (PDA) media was used to study the colony morphology whereas Czapeck Dox Agar (CDA) (Hi-Media, India) and Sabouraud Dextrose Agar (SDA) (Hi-Media, India) media were prepared to compare the morphology with PDA media. After 7 days of growth of

fungi on the plates, different morphological characteristics of colony such as form, elevation, margin, colour, size, surface, and dry weight were observed on three different media and classified according to the cultural characteristics described in (18).Different characteristics were identified for the growth profiling of the two fungal strains. 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 fungi was identified by a incubating both the fungi at 5^0C, 15^0C, 25^0C and 35 ^{0}C at 28±2^0C for 7 days. The effect of pH on the growth of the two fungal strains was identified by inoculating both the fungi into the PDA medium of pH of 6.0, 7.0, 8.0 and 9.0. Lastly, dry weight of all the fungi was measured.

Study on cellulolytic activity

Cellulolytic activity of the fungi was tested using Potato Dextrose liquid medium in which sterilized 3mm filter paper was inserted as a source of cellulose. Then, 5 mm diameter plug of a 7 days old colony of both fungal isolates were inoculated in the PDA liquid and incubated at 28±2^0C for 7 days and lastly flasks were observed to check the cellulose degrading ability of both fungi.

Control Measure by aqueous of spice and plants extract

Aqueous extracts of bulb of *Allium sativum,* root of *Borussus flabellifer* and leaves of *Scaparia dulcis*, *Pandanus odoratissimus* and *Withania somnifera* were used to investigate their effectiveness on the growth of the fungal strains.

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 (Carl Roth, Germany) 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 of 0.25%, 0.5%, 1% and 2% (w/v) were added into the potato dextrose liquid medium and pH was adjusted to 6.5. All the inhibition percentage 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 2016.

Results

Isolation of fungi

The two unknown fungal strains i.e. fungal strain-1 and fungal strain-2 were from post-harvest spoilage of mangoes of Gopalvog and Mollica varieties which is showed in figure 1.

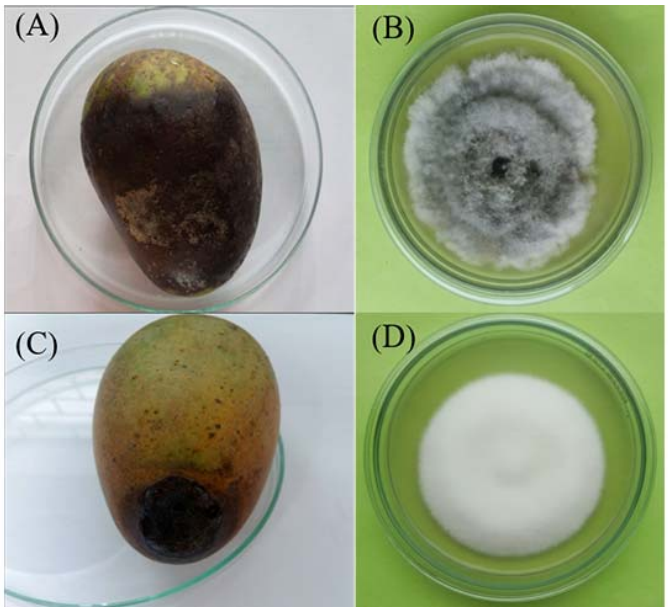

Figure 1. Isolated strains from the postharvest spoiled mangoes. (A) And (C) are the selected postharvest spoiled mangoes. (B) and (D) are the pure culture of fungal strains named stain-1 and strain-2 isolated from Gopalvog and Mollica respectively.

Microscopic Identification

Mycelia of the two fungi were examined and identified under microscope. In fungal strain 1, colonies grew faster, mycelium was fine threaded shape and the color was white from the front initially and became grayish brown in time. In addition, hyphae of the fungal strain-1 were branched, broad and the diameter of hyphae was about 63.35µm and no spore was appeared. On the other hand, in fungal strain-2, colonies were very fast growing and appeared cottony to fluffy, colony color was white from the front and the shape of hyphae was very fine thin thread like. Hyphae were about 14µm in diameter and spore was not found**.** Microscopic view of both the fungal strain are given in figure 2.

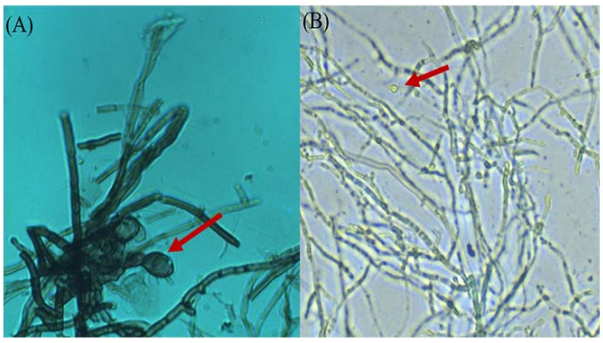

Figure 2. Microscopic observation of the isolated fungal strains. (A) Shows thicker hyphae with conidia indicated by arrow. (B) Shows thinner hyphae with spore indicated by the arrow.

Molecular Identification

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

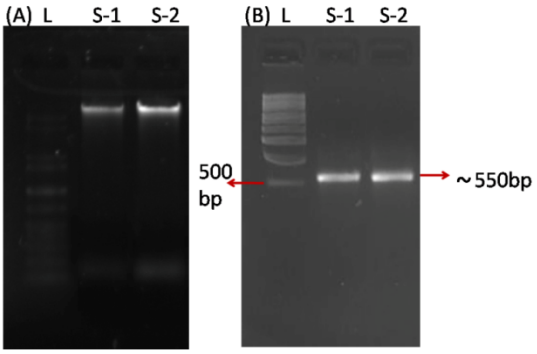

Figure 3. Molecular identification of the isolated fungal strains. L, S-1 and S-2 indicate the Ladder, Strain-1 and Strain-2 respectively. (A) High molecular weight DNA band with ladder (B) PCR amplification of ITS region showed around 550 bp band in both strain.

Colony Characterization on different media

Characterization of the colony of the two fungi were done according to (18) by culturing them on three different types of media i.e. Potato Dextrose Agar, Czapek Dox Agar, Sabouraud Dextrose Agar. Among three types of media, SDA media increased growth of the fungal strain-1 where the growth of the fungal strain-2 was promoted by PDA media. The results are shown in table 1 & 2 and in figure 4 (A).

TABLE 1.Morphological characterization of fungal strain-1 on different growth media

Characteristics	**Potato dextrose agar (PDA)**	**Czapek Dox Agar (CDA)**	**Sabouraud Dextrose agar (SDA)**
1.Form	Irregular and Filamentous	Irregular and Filamentous	Irregular
2.Elevation	Raised	Cateriform	Convex
3.Margin	Filiform	Filiform	Undulated
4.Surface	Smooth	Smooth	Smooth
5.Color	Greyish White	Greyish White	Greyish White
6.Size (cm)	6.65cm	5.9cm	8.6cm
7. Dry weight (gm)	0.1464gm	0.1020gm	0.2845gm

TABLE 2.Morphological characterization of fungal strain-2 on different growth media

Characteristics	**Potato dextrose agar (PDA)**	**Czapek Dox Agar (CDA)**	**Sabouraud Dextrose agar (SDA)**
1.Form	Irregular and Filamentous	Irregular and Filamentous	Irregular and Filamentous
2.Elevation	Nmbonate	Nmbonate	Convex
3.Margin	Undulated	Undulated	Undulated
4.Surface	Smooth	Smooth	Smooth
5.Color	white	White	White
6.Size (cm)	4.45	2.9	4.35
7.Dry weight (gm)	0.1020gm	0.0262gm	0.0870gm

Effect of Carbohydrate on the growth of two selected fungal strains

To identify the effect of different carbohydrates on the growth of the two fungal strains, different carbohydrates such as glucose, fructose, sucrose and starch were added as sole carbon source to the medium at 2% concentration instead of dextrose. It was found that all the carbohydrates stimulated the growth of both isolates but glucose was more stimulatory than the other carbohydrates for the growth of fungal isolate-1 where sucrose enhanced the growth of fungal isolate-2 comparative to other carbohydrates. So, the obtained result is much closed to the referred one. The results are showed in in figure 4 (B).

Effect of Temperature on the growth of two selected fungal strains

The effect of different temperatures on the growth of both fungal strains were observed after incubation both of them at 5°C, 15°C, 30°C, and 35°C temperature for 7 days. Interestingly, both the fungal strains showed maximum mycelial growth at 35°C temperature. The results are showed in figure 4 (C).

Effect of pH on the growth of two selected fungal strains

pH is one of the major criteria for the optimal growth of any fungi. The mycelial growth of the two fungal strains was observed in pH values of 6.0, 7.0, 8.0 and 9.0. It was found that the fungal strain-1 showed maximum growth at pH 8.0. On the other hand, the fungal strain-2 showed maximum growth at pH 6.0. The results are showed in figure 4 (D).

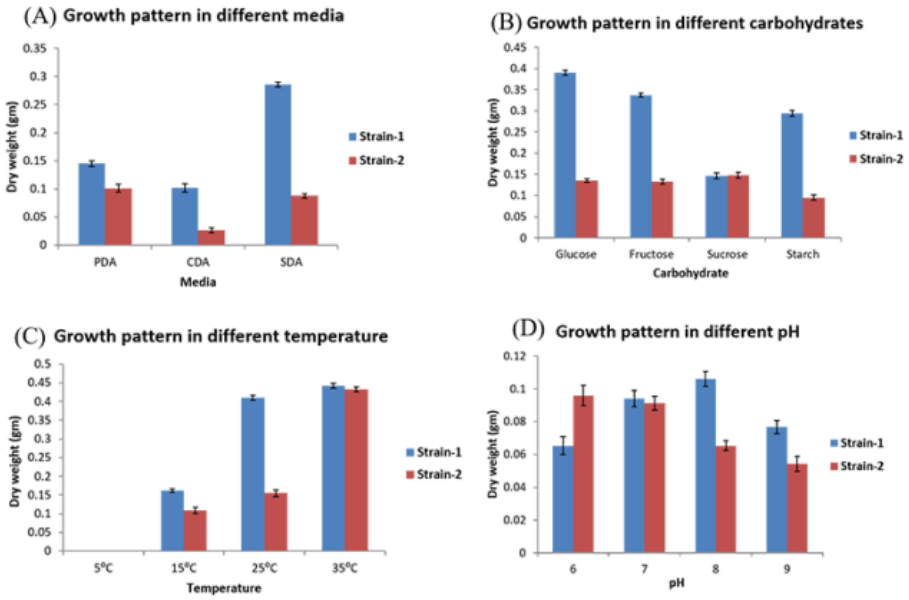

Figure 4. Growth profiling of the isolated fungal strains. (A) Highest growth was found on SDA in case of strain 1 whereas strain-1 exhibited highest growth on PDA. (B) Different growth pattern showed on different carbohydrate level. (C) Showed similar growth at 35°C temperature& (D) Optimum pH for strain-1 and strain-2 was 8 and 6 respectively.

Study of cellulolytic activity

Cellulolytic activity is the ability of the cellulose enzyme to degrade cellulose. In this study, after 7 days of inoculation of fungi, it was observed that the filter papers in the cultural flasks were not degraded which indicates that both of the strains do not have any ability to degrade cellulose.

Control Measurement by treating with plant extracts

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

Growths of both fungi cultured with aqueous extract were close to control where the aqueous extract was absent.

Control Measurement by treating with NaCl

It was found that the increasing concentration of NaCl had a greater inhibitory effect on the growth of both fungi. It was observed that the percentage of inhibition of growth rose with the increase of the concentration of NaCl. At 6% concentration, 100% inhibition of growth of both fungal strains was observed respectively. The results are showed in figure 5.

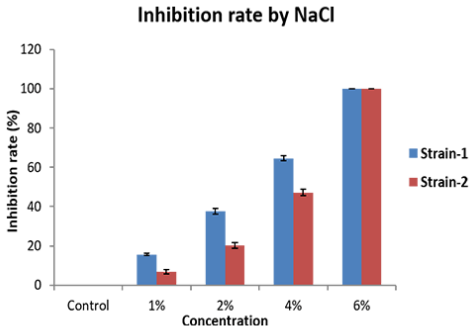

Figure 5. Inhibition of the fungal isolates by NaCl. 6% NaCl showed highest inhibition for the growth of both fungal strains.

Control Measurement by treating with organic acid

To identify the effect of organic acids on the growth of both the fungi, different concentrations of citric acid e.g., 0.25%, 0.5%, 1%, 2% w/v were added to the potato dextrose liquid media. No growth of the fungal strain-1 was observed at 2% concentration of citric acid. On the other hand, fungal strain-2 did not show significant growth at 0.25% concentration and no growth was noticed at 0.5-2% concentration. The results are showed in figure 6.

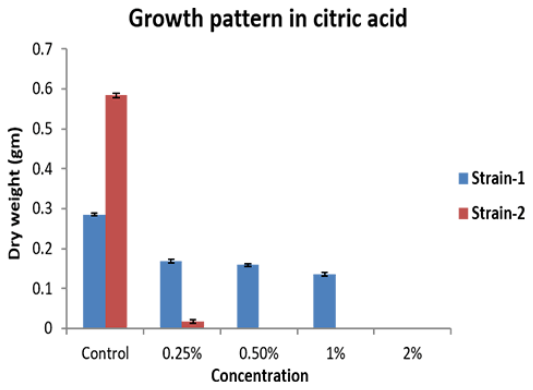

Figure 6. Growth pattern of the fungal isolates in different citric acid concentration. 2% and 0.5-2% citric acid inhibited 100% growth of fungal strain-1 and strain-2 respectively

Discussion

Mango is one of the most popular fruits in the tropical region and its consuming rate is increasing in the developed countries day by day (5). But one of the reasons for not being economically much important fruit in the world is its susceptibility to postharvest diseases (19). Ripened mangoes are more susceptible to attack by a variety of microorganisms and several studies found that the main microorganisms that cause mango spoilage are fungi and bacteria (12, 13). “Gopalvog” and “Mollica” are two most cultivated varieties of mango in northern region of Bangladesh. Postharvest spoiled mango of those two varieties was collected and two unknown fungal strains i.e. fungal strain-1 and fungal strain-2 were isolated from them in PDA media. Mycelia of the two fungi were identified by comparing with the previously published descriptions in several studies (15, 16) and it was found that both the colonies of fungal strain 1 and 2 show different characteristics. Isolated DNA from both fungi were amplified and run on gel electrophoresis which confirms the presence of the region which are specific for fungi. Colony characterization of the two fungi were done according to (18) by culturing them on three

different types of media where SDA and PDA media increased growth of the fungal strain-1 and fungal strain-2 respectively. The results showed that all the carbohydrates stimulated the growth of both isolates but glucose was more stimulatory than the other carbohydrates for the growth of fungal isolate-1 where sucrose enhanced the growth of fungal isolate-2 comparative to other carbohydrates which are close previous studies(20, 21). Both the fungal strains showed maximum mycelial growth at 35°C temperature which were also showed in several that fungi may grow well from temperature of 25 ^{0}C to 37^{0}C (22, 23). The mycelial growth of the two fungal strains showed maximum growth at pH 8.0 and 6.0 respectively. (22, 23). The isolated fungi strains are not cellulolytic as they cannot produce cellulase enzyme like *Trichoderma*, *Humicola*, *Penicillium* and *Aspergillus* (24). Growth of both the fungi could not be controlled by different concentrations of aqueous extracts of the part extracts of *Allium sativum, Scapariadulcis, Borussus flabellifer, Pandanus odoratissimus* and *Withania somnifera* plants which all have the antifungal properties described in several studies (25-28). NaCl has the ability to apply stress in the growth of fungi and it was found that the increasing concentration of NaCl had a greater inhibitory effect on the growth of both fungi and at 6% concentration, 100% inhibition of growth of both fungal strains was observed. Several studies have been done on the effect of those organic acids on the growth of the fungi (29). No growth of both the fungal strains was observed at 2% concentration of citric acid.

Conclusion

In the present study, colony morphology of both fungi grown on different media showed different characteristics. Similar characteristics were also noticed especially in color. The maximum growth of the fungal strains was achieved at Potato dextrose agar and Sabouraud dextrose agar media respectively. The optimum temperature (35°C) and pH (8 and 6) for growth

of the fungal strains were successfully identified. The most efficient carbohydrates (glucose and sucrose) for growth of the fungal strains were investigated. No cellulose degrading activity was shown by both fungi. It was identified that growth of both fungi could not be controlled by aqueous extracts of five types of plant. The control measurement of growth of the fungal strains was carried out with the treatment of NaCl. With the increase in the concentration of NaCl, the percentage of growth inhibition was increased. It was noticed that the growth of both fungal strain decreased with the increase in the concentration of organic acid. These findings will assist to prevent postharvest mango spoilage attacked by the both fungal strains. If we can interfere the conditions that increase the growth of the fungi, it is possible to prevent mango spoilage.

References

1. Abdulla M, Andersson I, Asp N-G, Berthelsen K, Birkhed D, Dencker I, et al. Nutrient intake and health status of vegans. Chemical analyses of diets using the duplicate portion sampling technique. The American journal of clinical nutrition. 1981;34(11):2464-77.
2. Mukherjee S. Origin of mango (Mangifera indica). Economic Botany. 1972;26(3):260-4.
3. Ediriweera MK, Tennekoon KH, Samarakoon SR. A Review on Ethnopharmacological Applications, Pharmacological Activities, and Bioactive Compounds of Mangifera indica (Mango). Evidence-Based Complementary and Alternative Medicine. 2017;2017.
4. Vietmeyer ND. Lesser-known plants of potential use in agriculture and forestry. Science. 1986;232:1379-85.
5. Diedhiou P, Mbaye N, Drame A, Samb P. Alteration of post harvest diseases of mango Mangifera indica through production practices and climatic factors. African journal of biotechnology. 2007;6(9).
6. Amin M. Studies on physico-chemical and microbiological qualities of some selected brand of mango fruit juice of Bangladesh: BRAC University; 2015.
7. Mootoo A. Efforts to improve the post-harvest technology of mangoes and heliconias. Post-harvest Management of Tropical Fruits and Ornamentals in the Caribbean Region; Trinidad; 18-22 November 1991. 1991.

8. Prabakar K, Raguchander T, Parthiban V, Muthulakshmi P, Prakasam V. Post harvest fungal spoilage in mango at different levels marketing. Madras Agric J. 2005;92(1-3):42-8.
9. Ding ZS, Tian SP, Zheng XL, Zhou ZW, Xu Y. Responses of reactive oxygen metabolism and quality in mango fruit to exogenous oxalic acid or salicylic acid under chilling temperature stress. Physiologia Plantarum. 2007;130(1):112-21.
10. Johnson G, Coates L. Postharvest diseases of mango. Postharvest news and information. 1993;4(1).
11. Zheng X, Tian S, Gidley MJ, Yue H, Li B. Effects of exogenous oxalic acid on ripening and decay incidence in mango fruit during storage at room temperature. Postharvest biology and technology. 2007;45(2):281-4.
12. Barth M, Hankinson TR, Zhuang H, Breidt F. Microbiological spoilage of fruits and vegetables. Compendium of the microbiological spoilage of foods and beverages: Springer; 2009. p. 135-83.
13. Jamalizadeh M, Etebarian H, Aminian H, Alizadeh A. A review of mechanisms of action of biological control organisms against post-harvest fruit spoilage. EPPO Bulletin. 2011;41(1):65-71.
14. Agostini JP, Timmer LW. Selective isolation procedures for differentiation of two strains of Colletotrichum gloeosporioides from citrus. Plant disease. 1992;76(11):1176-8.
15. Hamd M, Shazia I, Iftikhar A, Fateh F, Kazmi M. Identification and characterization of post harvest fungal pathogens of mango from domestic markets of Punjab. International Journal of Agronomy and Plant Production. 2013;4(4):650-8.
16. Barnett HL, Hunter BB. Illustrated genera of imperfect fungi: American Phytopathological Society (APS Press); 1998.
17. Hinrikson H, Hurst S, De Aguirre L, Hinrikson H, Hurst S, de Aguirre L, et al. Molecular methods for the identification of Aspergillus species. Medical mycology. 2005;43(sup1):129-37.
18. de Hoog GS, Guarro J. Atlas of clinical fungi: Centraalbureau voor Schimmelcultures; 1995.
19. Al-Najada AR, Al-Suabeyl MS. Isolation and classification of fungi associated with spoilage of post-harvest mango (Mangifera indica L.) in Saudi Arabia. African Journal of Microbiology Research. 2014;8(7):685-8.

20. Devi SS, Sreenivasulu Y, Rao KB. Talaromyces verruculosus, a novel marine fungi as a potent polyhydroxybutyrate degrader. Research Journal of Pharmacy and Technology. 2014;7(4):433-8.

21. Li H, Fu Z, Zhang X, Li H, Shi J, Xu Z. The efficient production of 3β, 7α, 15α-trihydroxy-5-androsten-17-one from dehydroepiandrosterone by Gibberella intermedia. Applied biochemistry and biotechnology. 2014;174(8):2960-71.

22. Goyari S, Devi SS, Kalita MC, Talukdar NC. Population, diversity and characteristics of cellulolytic microorganisms from the Indo-Burma Biodiversity hotspot. SpringerPlus. 2014;3(1):700.

23. Pitt JI, Hocking AD. Fungi and food spoilage: Springer; 2009.

24. Lynd LR, Weimer PJ, Van Zyl WH, Pretorius IS. Microbial cellulose utilization: fundamentals and biotechnology. Microbiology and molecular biology reviews. 2002;66(3):506-77.

25. Andriani Y, Ramli NM, Syamsumir DF, Kassim MNI, Jaafar J, Aziz NA, et al. Phytochemical analysis, antioxidant, antibacterial and cytotoxicity properties of keys and cores part of Pandanus tectorius fruits. Arabian Journal of Chemistry. 2015.

26. Javadian F, Sepehri Z, Saeidi S, Hassanshahian M. Antifungal effects of the extract of the Withania somnifera on Candida albicans. Advanced Herbal Medicine. 2016;2(1):31-7.

27. Shams-Ghahfarokhi M, Shokoohamiri M-R, Amirrajab N, Moghadasi B, Ghajari A, Zeini F, et al. In vitro antifungal activities of Allium cepa, Allium sativum and ketoconazole against some pathogenic yeasts and dermatophytes. Fitoterapia. 2006;77(4):321-3.

28. Singh T, Verma AK, Haq SIU, Mounika N. Evaluation and Determination of Antifungal Potentials of Sap of Borassus Flabellifer.

29. Mattoo A, Murata T, Pantastico EB, Chachin K, Ogata K, Phan C. Chemical changes during ripening and senescence. Postharvest Physiol, Handling and Util of Tropical and Subtropical Fruits and Vegetables EB Pantastico, ed. 1975.