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ISOLATION OF FUNGI ASSOCIATED WITH SPOILAGE OF SELECTED FRUIT

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ABSTRACT

The purpose of this investigation was to isolate and identify some fruit spoilage fungi. Fruits due to their low pill, high moisture content and nutrient composition are highly susceptible for the colonization of pathogenic fungi, fruits are colonizedby fungi and other agents different types of disease. The spoiled fruit were like as banana, orange and apple collected from Jwalapur, Haridwar, Uttarakhand state, India. The homogenates were cultured on potato dextrose agar(PDA) plates and incubated in incubator for 4-7 days .Pure culture of the resulting fungal colonies were obtained from primary plates and identified by using morphological and microscopicallycharacterizations. The most common fungi found were *Aspergillus* spp. (27.2%), *Rhizopus* spp.(27.2%), *Mucor* spp.(27.2%), *Helmenthosporium* spp.(1.0%), *Fusrium* spp.(9.09%) and *Penicillium* spp.(1.0%).

INTRODUCTION

Fruits are widely distributed in nature. Fruits play a vital role in human nutrition to keep a good health by supplying the necessary growth factor such as vitamins and essential minerals. A fruit is the edible part of a mature ovary of flowering plants. The primary cell wall of fruits is composed of approximately 10% proteins and 90% poly saccharides which can be divided into three; cellulose, hemicelluloses and pectin (Nathalie.,2006).

Fruit spoilage refers to various changes in which the fruit become the less palatable or taste, smell, appearance or texture (Akinmusire., 2011). It is also estimated that about 20% of all fruits and produced is lost each year due to spoilage (Barth et al., 2009). Fruits are attacked by fungi, bacteria and other agent causing different type of disease over 25 to 30% loss are causing by fungal disease in transit and storage fruit may be affected either by dry or soft rot. Contamination of fruit and other defects of fresh fruit are termed "Market disease". Fruits are highly perishable product; the quality is affected by post –harvest handling, transportation, storage and marketing conditions, or after purchasing by the consumer. Fruits contain high levels of sugars and nutrients element and their low pH values make them particularly desirable to fungal decayed (Singh and Sharma., 2007).

The most common cause of apple rot is from the fungi *Penicillium expansum* and *Monilinia fructigena* (Fiori et al.,2008). Other fungal genera that were isolated from apples include *Colletotrichum xylaria. Botaryospharia* (Camattgri-sartori et al., 2005) and *Rhizopus orygae* (Knon et al., 2011). *Aspergillus spp*. has also been isolation and known to cause infections or allergies (Monso., 2004).In last studies have shown that the *Penicillum* and *Monilinia* species commonly cause spoilage of fruits especially apples.

The national clinical and laboratory standard institute has developed a protocol for testing antifungal susceptibility agented filamentous fungi using a minimum inability concentration (MIC) assay. One of the most important problems occurs in the susceptibility testing of Ketaconazolebecause the partially inhibit fungal growth. The primary objective of virtually all in vitro antimicrobial susceptibility testing is to aid in the prediction of the effect of the antimicrobial agent of interest on the outcome of infection caused by a specific pathogen. This is true whether or not the in vitro susceptibility tests are being for patient care, for drug development or drug discovery, or in epidemiological studies. Regardless of the purpose of antimicrobial susceptibility testing, results obtained in a simple, well-defined, and highly artificial in vitro test system have intrinsic limitation in predicting the outcome of the complex biological processes that clinical infection represent(McCabe et al., 1985). Effort in the United States to develop standardized and clinically useful methods for in vitro susceptibility testing of antifungal agents began in 1982 with the establishment of the national committee for clinical laboratory standards (NCCLS) Subcommittee for antifungal susceptibility testing. Among the reasons for this slow development was the relative

infrequency of fungal infections before the 1980s as well as the limited number of available therapeutic agents (Pfaller et al., 1997).

Material and Methods

Study site:-

The market site selected for collection of fruits samples was Krishi Farm Utpadan Samiti situated along Sarai road, opposite Harilock Colony (Haridwar) Uttarakhand. The site was selected because it serves as the main deport of fruits where the main fruits dealers and suppliers sell to petty traders who in turn circulate the product.

Collection of fruit materials:-

Three types of spoiledfruits banana, orange and apple were purchased from the market of selected site and were collected individually in air tight polyethylene bags.

Preparation of media:-

Potato dextrose agar(PDA) was prepared and sterilized by autoclaving at 121°C for 15 minutes. Then it was transferred in to petriplates (20mleach) and allowed for solidification.

Isolation of fruit spoilage fungi:-

Firstly, the fruits were washed with running water and the surface contaminants were removed. Serial dilution and washing off methods were carried out individually for fungi isolation, by incubation of the intact fruits after injuring their surface.

Serial dilution method: - Stock solution was prepared by dissolving of 1gm spoiled part of fruit pulp and fruit peel in 9ml of distilled water.Agitated vigorously with the help of stirrer tomixed properly.7 test tube containing 9ml of sterile distilled water placed on test tube stand.1 ml from stock solution was transferred aseptically into the first test tube and vortex mix.1ml from first test tube was then transferred to the second test tube and mixed and this procedure was repeated till the last test tube. 1ml solution from last tube was discarded.0.1ml solution from 10^{-3}, 10^{-4} and 10^{-5} dilution was spreaded on solidified PDA medium (Spreading plate method).The plates were incubated at 25°C ± 28°C for 3-7days.

Identification of fungi:-The isolated fungi were identified microscopically using lactophenol cotton blue staining method.

Measurement of fungal growth by mycelium weight:-

The direct measurement of an increase in fungal weight can be determined by inoculated culture flasks with a known volume of broth followed by filtering off mycelium.

Microorganism used:-Fungal isolates *Aspergillus*spp. *Mucor* spp. and *Rhizopus* spp. were used for dry mycelium weight determination.

Preparation of medium:-Sabouraud's dextrose broth was prepared and autoclaved.

Fresh sterile conical flask containing 20 ml Sabouraud's dextrose broth was taken in duplicate for each organism. The flasks were plugged with cotton plugs and autoclaved at (121°C) for 15 minutes. Each flask was inoculated with 8mm diameter disc, cut from the periphery of the actively growing colony of each fungus. The inoculated flasks were incubated at 25°C for 20 days.After 5 days of incubation remove six inoculated flask (2 flask each fungi) were taken out from incubator.The contents of flasks were filtered through a pre-weighed what man filter paper.The dried filter paper was in an oven at 105°C for 48 hours.Reweigh the dried filter paper was weighed along with the mycelium.The step (5) to (8) was repeated through at each 5-days interval for a period of 20 days.

Antifungal activity by agar well diffusion method:-

Media used:-Sabouraud's dextrose liquid medium was prepared and transferred in culture tube (10ml each) sterilized by autoclaving at 121°C for 15 minutes.

Microorganism used:-Fungal isolates *Aspergillus*spp. *Mucor* spp. *and Rhizopus* spp. were used for antifungal activity test.Theseweresselectedbecause these were dominant fungal spices ineachfruitandtheirpeel.

Antifungal used: Two concentration(5mg/ml and 10mg/ml) of three type antifungal (fluconazole, ketoconazole and terbinafine) used to susceptibility test.

Pure culture of fungi in broth: - 10 ml Sabouraud's broth containing culture tube (1 tube for each fungus) inoculated fungi with the help of sterile cork Bauer and agitator vigorously with the help of stirrer so it mixed properly.In this method, Sabouraud's ager (SDA) media were poured in sterile petriplates and allow to solidification. 0.1ml pure culture of fungi was spreaded on solidified media.After the SDA surface had been dried for about 5-10 minutes. A size of 8mm well was made with the help of cork borer and antifungal solution was put in with help of 1ml micropipette.Then, the petriplates were immediately placed in an incubator at 25°C ± 28°C for 3-7 days.After the incubation time, the diameters zones of inhibition around the well were measured.Inhibition zone diameters were then interpreted into the degree of microbial resistance that relates zone diameter to the degree of microbial resistance.The values can be derived by finding the diameter of the zone of microbial pathogens (R.P Singh, 2009).Depending on the strain and the drug used, a qualitative of susceptible, intermediate or resistant can be obtained.

Determination of Minimum inhibitory concentration (MIC) by Broth dilution method:-

Test organisms:-Fungal isolates *Aspergillus* spp., *Mucor* spp. and *Rhizopus* spp. for used MIC and MFC test.

Mediaused- Sabouraud's dextrose liquid medium was prepared and autoclaved.

Preparation of stock solution-A stock solution of Terbinafine was prepared with concentration 100 mg/ml.7Fresh sterile test tube containing 9 ml Sabouraud's dextrose liquid medium was taken.Initial

dilution was prepared by adding 1ml of Stock solution into 9 ml of Sabouraud's dextrose liquid medium in first test tube thus diluting the original solution 10 times (10^{-1}) and mixed properly.The first test tube was transferred 1ml of solution in next test tube making concentration (10^{-2}).This procedure was repeated till the test tube to achieve a final concentration of 10^{-7}.After dilution 0.1 ml of test fungal specimens were transferred into each test tube.After that inoculated test tubes were incubated at 25°C ± 28°C for 2-4 days.

Minimum fungal concentration (MFC) test:-

Sterile Sabouraud's dextrose agar plates were inoculated separately with fungal suspension from $10^{-3,}$ and 10^{-4} test tubes and incubated at25°C ± 28°C for 3-7 days.

Pathogenic estimation of isolated fungus:-

Test organisms- Fungal isolates *Aspergillus* spp., *Mucor* spp., *Rhizopus* spp., *Fusarium* spp., *Helmenthosporium* spp. and *Penicillum* spp. for used pathogenic estimation.

Test sample: Fresh apple, banana and orange fruits were used for the test of pathogenicity.

Apple, banana and orange fruits were washed with tap water and rinsed with distilled water after which they were surface sterilized with 75% ethanol.A sterile 4mm Cork borer was used to make holes in each of the fruits.The colony of fungi isolate (from each pure culture) was used to inoculate the fruits and the core of the fruits were replaced.The point of inoculation was sealed with petroleum jelly to prevent contamination.Controls consisted of three fruits each of apple, banana and orange wounded with the sterilized cork borer but not inoculated.The inoculated fruits and the controls were placed in clean polythene beg (one fruit per beg).The inoculated fruit were incubated at 30±1°C for 5 days.

OBSERVATIONS

The purpose of using spoiled fruits was to isolate different fungal species. The weights of spoiled fruit parts from total weight of fruit samples are mentioned in **Table 1.** Different colonies were observed at the end of the procedure. A total of 428 fungal colonies were isolated obtained from the three type of fruit (Apple, orange and Banana) from both part of the fruit (pulp and peel), the highest colonies were isolated from orange fruit as shown in **Table 2.** After performing fungal staining (Lactophenol cotton blue stain) these isolated fungi were identified on the bases of morphological characteristics as *Aspergillus* spp, *Mucor* spp, *Rhizopus* spp, *Fusarium* spp, *Helmenthosporium* spp. and *Penicillium* spp. as shown in **Table 3and4.** The total percentage of each fungus evolved in fruit spoilage in shown **Table 5.**

Table 1:-Showing the weight of fruits samples.

S. No	Sample name	Sample code	Total weight of fruit sample(gm)	Weight of spoiled part of fruits sample(gm)
(1)	Apple	A	130	25
(2)	Orange	B	150	50
(3)	Banana	C	125	40

Table 2:-Showing the total number of fungal isolates.

Fruit sample	Total no of isolates	Mean ±S.E
(A)Apple		
Fruit pulp (a)	28	23±5.0
(b)	18	
Fruit peel (a)	13	14.5±1.5
(b)	19	
(B)Orange		
Fruit pulp (a)	86	90.5±4.5
(b)	95	
Fruit peel (a)	35	32.5±2.5
(b)	30	
(C)Banana		
Fruit pulp (a)	30	17.5±5.0
(b)	40	
Fruit peel (a)	17	18.5±1.5
(b)	2	

Table 3:-Showing the fungal spp. in different fruit sample.

Fruit sample	Isolates					
	Mucor spp.	*Aspergillus* spp.	*Fusarium* spp.	*Rhizopus* spp.	*Helmenthosporium* spp.	*Penicillium* spp.
(A)Apple Fruit pulp	+	+	_	+	_	_
Fruit peel	+	+	+	+	_	_
(B)Orange						
Fruit pulp	+	+	_	+	+	_
Fruit peel	+	+	_	+	_	_
(C)Banana						
Fruit pulp	+	+	_	+	_	+
Fruit peel	+	+	+	+	_	_

Table 4:-Colonial and Morphological characteristics of fungi associated with the spoilage.

Identify fungi	Colony shape	Colony colour	Texture	Microscopic Description
Mucor spp.	Circular	Greenish-Black	Rough	Nonseptate mycelium with root like rhizoids; black columellate, sporangiophores, in clusters.
Rhizopus spp.	Circular	White mycelia with Black spores	Smooth	A bundle of sporangiophore was formed. Sporangiophore is smooth walled, aseptate, and light brown, simple. Sporangia globose or sub-globose with some flattened base, contained many spores.

Aspergillus spp.	Circular	Light-Green to Bluish green	Rough	Conidia were round, globose, rough with radiated conidial heads; septate hyphae smooth long and hyaline conidiophores.
Fusarium spp.	Circular	Pink	Smooth	Oval microconidia. Microconidia produced on richly branched conidiophores. Cylindrical to facilitate.
Helmenthosporium spp.	Circular	Black	Rough	Transversely septate conidia.
Penicillium spp.	Circular	Greenish Blue	Rough	Conidiophores smooth, relatively short. Penicillia mycelia arranged very irregular and asymmetrical with branches of various lengths. Sparse and irregular metulae with phialides on them, conidia smooth and ellipsoidal.

Table 5:-Percentage occurrence of fungal isolates associated with the spoilage of fruits.

Fungal isolates	Total no in all fruits sample	Percentage occurrence in all fruits sample (%)
Mucor spp.	6	27.2
Aspergillus spp.	6	27.2
Fusarium spp.	2	9.09
Rhizopus spp.	6	27.2
Helmenthosporium spp.	1	1.0
Penicillium spp.	1	1.0
Total	**22**	**100.0**

Measurement of fungi dry mycelium weight of fungi:-

The growth increment was measured at intervals of 5, 10, 15 and 20 days. The weight of mycelium was calculated by subtracting the weight of fitter paper (from the weight of fitter paper +weight of mycelium) and the mean for each Reading was fined. According to reading graph was prepared for *Aspergillus* spp., *Mucor* spp., and*Rhizopus*spp. as shown in **Table 6.**

Table 6:-Showing the dry mycelium weight of fungal spp. at different time intervals.

S.No	Fungal spices	Dry mycelium weight(gm.)				
			5Days	10Days	15Days	20Days
(1)	*Aspergillus* spp.	(a)	0.584	1.006	0.603	0.505
		(b)	0.551	0.754	0.578	0.401
		Mean	0.5675	0.88	0.5905	0.453
(2)	*Mucor* spp.	(a)	0.155	0.325	0.589	0.372
		(b)	0.078	0.28	0.543	0.239
		Mean	0.1165	0.3025	0.566	0.3055
(3)	*Rhizopus* spp.	(a)	0.21	0.323	0.506	0.408
		(b)	0.23	0.309	0.456	0.336
		Mean	0.22	0.316	0.481	0.372

Antifungal sensitivity test:-

The antifungal activity of three types commercial antifungal (Fluconazole, ketaconazole and Terbinafine) were assayed by agar well diffusion Method. Approximately 80% of the fungal isolates have shown the sensitive against fluconazoleand ketoconazole.The diameter of inhibition zones observed in different type of antifungal used at different concentration. But *Rhizopus* spp. shown antifungal activity against terbinafine.

The diameter of inhibition zones were observed in fluconazole, ketoconazole and terbinafine against the growth of *Aspergillus spp., Mucor spp. and Rhizopus spp.* as shown in **Table 7.**

Table 7:-showing the antifungal sensitivity test against fungal species.

S. No	Antifungal drugs	Concentration (mg/ml)	Zone of inhibition(mm)					
			Aspergillus spp.	**Mean± S.E**	***Mucor spp.***	**Mean± S.E**	***Rhizopus Spp.***	**Mean± S.E**
(1)	Fluconazole	5(a)	32	30.75±4.25	19	19±0.0	-	-
		(b)	29.5		19			
		10(a)	42	42±0.0	12	23.75±11.75		
		(b)	42		35.5			
(2)	Ketoconazole	5(a)	14	14±0.0	6	7±1.0	-	-
		(b)	14		8			
		10(a)	14	15±1.0	2	58±2.6	-	-
		(b)	16		54			
(3)	Terbinafine	5(a)	59.5	59.75±0.25	39.5	48.25±9.25	-	-
		(b)	60	61.75±3.75	57			
		10(a)	65.5		59	53±6.0	14	13.5±0.5
		(b)	58		47		13	

Measurement of MIC and MFC test:-

The minimum inhibitory concentration (MIC) of Terbinafine against *Aspergillus* spp., *Mucor* spp. and*Rhizopus* spp. was observed at 72 hours using micro dilution method and minimum fungal concentration was observed in agar plates. The MIC and MFC tests indicated that the antifungal agent Terbinafine exhibited the minimal values of MIC in concentration (0.01mg/ml) and MFC in concentration (0.1mg/ml) against *Aspergillus* spp. and *Mucor* spp. But in case of *Rhizopus spp.*was exhibited of MIC was observed at concentration (0.1mg/ml) and MFC at concentration (1mg/ml).(**Table 8 and 9)**

Table 8:- Showing the MIC and MFC value of Terbinafine against *Aspergillus* spp., *Mucor* spp. and *Rhizopus* spp.

Antifungal agent	MIC(mg/ml)			MFC(mg/ml)		
	Aspergillus spp.	***Mucor spp.***	***Rhizopus spp.***	***Aspergillus spp.***	***Mucor spp.***	***Rhizopus spp.***
Terbinafine	0.01	0.01	0.1	0.1	0.1	1

Table 9:- Showing the value minimum inhibitory concentration (MIC).

S.No	Antifungal agent (Terbinafine) concentration (mg/ml)	Growth of Fungus		
		Aspergillus spp.	***Mucor spp.***	***Rhizopus spp.***
(1)	10	-	-	-
(2)	1	-	-	-
(3)	0.1	-	-	-
(4)	0.01	-	-	+
(5)	0.001	+	+	+
(6)	0.0001	+	+	+
(7)	0.00001	+	+	+

Pathogenic estimation of isolated fungal spp.

After observing the fruits inoculated with isolated fungus spoiled fruits the same symptoms of pathogens obtained in the fresh fruit samples. As shown in **Table 10.**

Table 10:- Pathogenic symptoms of fresh fruit samples.

S. No	Fresh fruit samples	Symptoms
1.	Apple	Brown Spot
2.	Banana	Black Spot
3.	Orange	White Spot

Abbreviation: MIC-minimum inhibitory concentration, MFC-minimum fungal concentration, SE-standard error,(+)-Positive /(-)-Negative

RESULTS AND DISCUSSION

The purpose of this study was to isolate fungi from selected spoiled fruits on PDA medium by serially diluted method. In this study total of 428 fungal colonies were isolated from the study site. The isolates were identified Aspergillus spp., *Mucor* spp., *Fusarium* spp., *Penicillium* spp., *Rhizopus* spp. and *Helmenthosporium* spp. But in previous study Sani. I., 2014 reported total of 486 fungal isolates were obtained from the 10 different spoilt fruit from the three markets. The isolates were classified by grouping into 8 taxonomic genera namely; *Aspergillus*,*Rhizopus*, *Mucor*,*Alterneria*, *Neurospora*, *Penicillum*, *Cladosporium* and *Fusarium*. According to Beiyewa *et al*., 2007 reported *Aspergillus* spp., *Mucor* spp. and *Rhizopus* spp. successfully take part in the fruit spoilage confirmed as the caused organisms of fruit decay.

In our study the percentage occurrence of fungi isolated associated with the spoilage of fruits were *Aspergillus* spp. (27.2%), *Rhizopus* spp.(27.2%), *Penicillum* spp.(1.0%) and *Fusarium* spp.(9.09%) but in previous study, Arena. A., 2011 reported the percentage occurrence of fungi isolated with spoiled fruit were *Aspergillus* spp. (33.3%), *Rhizopus* spp. (50.0%), *Penicillum* spp.(100.0%) and *Fusarium* spp.(50.0%)

During antibiotic sensitivity, different antibiotics were used to check the activity of these antibiotics against fungal species. *Aspergillus* spp. and *Mucor* spp. were sensitive against all antibiotics used in test and *Rhizopus* spp. was sensitive only against terbinafine at 10mg/ml concentration. The highest zone of inhibition was appeared in *Aspergillus* spp. against terbinafine at 10mg/ml concentration. The minimum inhibitory concentration was performed by micro dilution method and minimum fungal concentration was SDA plates. *Aspergillus* and *Mucor* were inhibited at 0.01mg/ml concentration but Rhizopus was inhibited at 0.1mg/ml concentration. The minimal fungal concentration was observed at 0.1mg/ml concentration by *Aspergillus* and *Mucor* but *Rhizopus* at 1mg/ml concentration. We performed pathogenicity test to check the rot symptoms obtained were similar to those observed previously on the fruits when subjected to identification procedures by examining their morphological characteristic

CONCLUSION

The results of this study suggest that decaying fruits nourishes various types of fungi which may cause harm to the consumers. *Aspergillus*, *Fusarium* and *Penicillium* spp. are commonly occurring filamentous fungi and their growth may result in producing of toxins known as mycotoxins. This can cause a variety of ill effect in human from allergic responses to immune suppression and cancer. It showed their fungi were involved in the spoilage of many fruits. Mechanical injuries such as bruises or cuts that occur during harvesting or post-harvesting, grading and packing could provide infection sites for spoilage pathogens. Fruit spoilage however can be controlled by the following practices: washing of harvested fruit with clean or pure water; proper cleaning and sanitation of warehouses and disinfection of packaging and transit containers; proper handling of the fruit during harvest to prevent bruises and scars or other mechanical injuries; Inhibition of fungal growth by lowering storage temperatures through storage under refrigeration and the use of fungicides. It is therefore important that both the farmer who harvests the fruits into bags for transportation, the markets and consumers take necessary precaution in preventing contamination and eating of contaminated fruits.

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