Study of yeast flora of fruits and their in vitro screening for antagonistic property against *Penicillium digitatum*

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Abstract: Hundred fifty (150) samples of different types of fruits (thirty samples of each fruit of Orange, Green grape, Fig, Dried date and Apple) were collected from different markets of (N) 24 Parganas of West Bengal in sterile biodegradable polyethylene bags. These were brought to laboratory and the isolation of different yeasts were done from the washing of the fruit samples by using dilution plating method on MA medium. From these samples 480 yeast colonies were isolated. These isolates of yeasts were identified by conventional morphological, microscopical and biochemical testing methods. These colonies comprise of 13 genera and 20 species of yeasts. Maximum number of four species were found in both *Candida* and *Rhodotorula*. The percent of occurrence of each species was calculated. All yeast species isolated were screened by dual culture plate method for their antagonistic property against *Penicillium digitatum*, causal pathogen of *Penicillium* rot of *Citrus*. Out of 20 species of yeasts, 15 species are antagonistic to *Penicillium digitatum*. *Candida guilliermondii* gave maximum percent of radial inhibition of growth (75.50 PIRG) followed by *Candida famata* (70.56 PIRG), *Rhodotorula mucilaginosa* (68.21 PIRG), and *Debaryomyces hansenii (58.00* PIRG*)*. Therefore, these fruits are good habitats of various yeast species and the antagonistic yeasts can be applied as biological control agents against post harvest *Penicillium* rot disease of *Citrus*.
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1. Introduction

Yeasts are unicellular eukaryotic fungi which are generally multiply quickly by budding or fission. Yeasts are generally distributed into two phyla: a) Ascomycota b) Basidiomycota and in mitosporic group-Deuteromycota. Dimorphic yeasts are also present which become filamentous under certain environmental conditions. Ascomycetous are two types: Budding yeasts (*Saccharomyces cerevisiae*) and fission yeasts (*Schizosaccharomyces pombe)*. According to Kurtzman & Fell (1999), there are about 100 genera and 700 species.

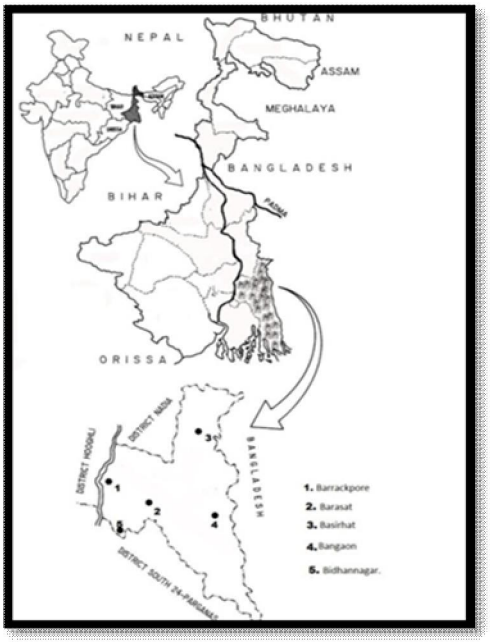
Yeast applications are used in many ways -wine and bakery industry, SCP production, carotenoid production, recombinant vaccine production, in understanding of cell cycle and so on. Modern trend is application of yeasts as biocontrol agents against many post harvest diseases of many fruits and vegetables (Droby et al. 2003; Coelho et al. 2007; Pimenta et al.2008; Kurtzman et al. 2012). Unfortunately yeast flora or diversity is highly neglected in India.

The main objectives of this study are to know the diversity of yeast flora of fruit surfaces in 24-Parganas (N), West Bengal and to find out the antagonistic potential of yeast and their application as biocontrol agent against *Penicillium* fruit rot of orange**.**

2. Materials and methods

Study area

The 24-Parganas (N) district of West Bengal is situated in the tropical zone and extends from latitude$22^0$11′ 6′′ north to $23^0$15′ 2′′north and from longitude $88^0$20′east to 89^o 5′ east. It is bordered to Nadia by north, to Bangladesh (Kulna Division by north and east), to south 24 -Parganas and Kolkata by south and to Kolkata, Howrah and Hooghly by west. The elevation of the district is 2,134m (7001ft). It lies with in the Ganga- Brahmaputra delta. The river Ganga flows along the entire west border of the district. There are many rivers, such as Ichaamoti, Jamuna and Bidyadhuri. Annual rainfall is 1579mm. The weather remains humid and dry during the most time of the year except the rainy seasons. It remains dry during the winter (mid November to mid February) and humid during Summer. Temperature remains 41^0C (maximum) in May and 10^0C (minimum) in January. Relative humidity remains between 50% in March and 90% in July (Fig1)**.** **(**http://famillpedia.wikia.com/wiki/north_24_pargana_district#climate**; State of Forest Report 2003).**



Subdivisions and some study spots:

1. Barrackpore
- a) Naihati
- b) Halishahar

2. Basirhat
- a) Hasnabad
- b) Hingalgunj

3. Barasat
- a) Badu
- b) Nilgunj

4. Bongaon
- a) Duttafulia
- b) Gopalnagar

5. Bidhannagar
- a) Rajarhat
- b) Mohishbathan

Study samples

The collected materials were fruits of Orange, Green grape, Fig, Dried date and Apple from different traders of five sub-divisions of 24-Parganas (N) (Fig1) of West Bengal, India.

Isolation & Purification of yeasts

Fruit/juice/soil/other samples from different districts of West Bengal, India were collected in separate sterile biodegradable polythene bags and brought in laboratory. Known weight of each sample was washed with known volume of sterilized distilled water by shaking on a rotary shaker. The washings/fruit juices were 10 fold serially diluted and plated on MA medium (MA; malt extract, 2g; Agar, 2g; Distilled water,100ml). The Petri dishes were incubated at 25^{o} C for 3 days. (Beech and Devanport, 1971; Ghosh,2011).

Single cell isolate of each type of yeasts was obtained by streaking loop full of cells on MA medium and transferring well isolated colonies to MA. The isolates were maintained on Malt-Yeast – Glucose- Peptone –Agar medium (Dry malt extract, 3g.; dry yeast, 3g.; Peptone, 5g; D- glucose, 10g; Agar, 20g; Distilled water, 1L) at 28^{o}C with monthly subculturing.

Identification of Yeasts:

Identification of each isolate of yeast up to species level was carried on the basis of standard morphological, and physiological /biochemical tests presented by Barnett et.al (2000); Kurtzman et al. 2012 and Rose & Harisson (1993).

Morphological & Microscopical investigation

The colonies were observed and described on MA and MYGPA medium The isolates were also grown in MA & MYPGA broth for determination of their cultural characteristics (pellicle, sedimentation or ring formation). In certain cases, the isolates were grown on sterile slices of carrot for induction of ascospore formation**.**

Biochemical investigations

For carbon and nitrogen assimilation, the basal medium of Barnett, et al. (2000) was used. the results were determined after the 3th, 7^{th,}, 14th, 21th and 28th day.

The ability of some carbohydrates for anaerobic assimilation (fermentation) was determined by using Durhan glass tubes after 3 weeks. The quantity of the tested carbohydrates was 2%.

For Diazonium blue –B (DBB) test, a ten day old culture on MYPGA was held at 55 ^{0}C for three hours and then flooded with ice –cold DBB reagent. The reagent was prepared by dissolving diazonium blue salt (Sigma) in cold 0.5M-tris –HCL buffer pH 7.0 at 1mg /ml. The reagent was kept ice –cold and used within few minutes of preparation.

Other additional tests such as starch formation, urea hydrolysis, cycloheximide (0.01% or 0.1%), were performed.

Isolation and purification of pathogen from diseased fruit:

The rotten oranges were collected in sterilized polythene bags and carried to laboratory from different markets. Five gram of rotten fruit was aseptically cut out and smashed and mixed in 50 ml sterile distilled water under Laminar air flow. The solution and diluents were streaked on to PDA Petri dishes containing penicillin (1.6ug /ml) and incubated at 28 ^{o}C temperature for 3days. After three days, each colony of the fungus was transferred to PDA slant by inoculating needle and incubated at 28 ^{0}C for one week and stored at 4^{o}C in refrigerator (Hee,2003; Dingra & Sinclair, 1985).

Identification of the pathogen:

The pathogen was identified by its growth pattern and microscopical characteristic consulting with published Keys of *Penicillium* (Nagamani, et al., 2006; Domsch, et al,1980).

Screening of yeasts as antagonists against *Penicillium digitatum*: Dual culture assay:

Five mm diameter of mycelial colony from the margin of actively growing colony of *P. digitatum* was placed on one end of Petri plate and that of yeast was incubated simultaneously at opposite ends of a Petri dish containing 25 ml of sterilized PDA medium. The plates containing the paired culture were incubated at $28^0 \pm 1^0$C for 9 days in a B.O.D. incubator. The five PDA plates inoculating only with 5mm diameter of mycelial colony from the margin of actively growing colony of *P. digitatum were* incubated at $28^0 \pm 1^0$C for 9 days. These were control. The degrees of antagonism were calculated by the formula:

$$PIRG = \frac{R1 - R2}{R1} \times 100$$

Where, R1=Radial growth of *P. digitatum* in control plate.

R2= Radial growth of *P. digitatum* in dual culture plate interacting with yeast.

PIRG=Per cent of Inhibition of Radial growth *of P.digitatum*.

Each treatemt was carried out 5 replications and plates were arranged in randomized Block Design.

Data analysis:

The percent of occurrence of each species was determined by total number of yeast colony divided by number of colony of each species and the result is multiplied by hundred.

The Berger –Parker Dominance index (D) was caculated or measured (Harrison, et al.2004) by the formula: D = n/N, where n indicated the No. of individuals in a species and N represented as total No. of individual. The Berger – Parker dominance was calculated in species level. The species with D>0.1 were considered as Dominant, the speices with lying between 0.1 & 0.05 were called General and the species with D <0.05 were considered as Rare.

3. Results & Discusion

The data presented in the Table 1 showed that from 150 samples of different natural sources, 480 yeast colonies were isolated. These yeasts were identified by conventional morphological, microscopical and biochemical testing methods. These colonies comprise of 13 genera and 20species of yeasts. Maximum number of species were found in *Candida* (4) and *Rhodotorula* (4) and *Cryptococcus* (2). Out of 20 isolated & identified species, 12 are ascomycetous while 08 are basidiomycetous yeasts (Table1). The data of table 1 indicated the distribution or habitats of isolated & identified yeasts. Some yeasts were isolated from restricted fruits' surface and some were isolated from maximum fruits' surface. Twelve species of yeast and eight species of yeast were isolated from green grape and apple respectively. Green grape and apple fruit surfaces were good habitats The, percent of occurrence of *Saccharomyces cerevisiae*, *Candida famata* and *Rhodotorula mucilaginosa* were 10.62, 10.00 and 9.37 respectively. The relative occurrence of these three yeasts were 'Dominant' where as other seven species were 'General' and other ten species were 'Rare' in occurrence by calculating the Berger – Parker dominance index. The least percent of occurrence was recorded in *Filobasidium uniguttulatum* (Table2). Ghosh and Sammader (1987) isolated different types of yeasts from different sources like palm juice, toddy, molasses, grape and cane juice. *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Saccharomyces kluveri, Rhodotorula sp, Candida* sp were isolated and identified by them. Identification of yeasts up to species level was carried on the basis of standard morphological and physiological/ biochemical tests proposed for each group by Barnett et al (2000) and Kartzman and Fell (1999). Isolation and taxonomic study of yeast strains from Bulgarian dairy products were reported by Savova (2002). They identified *Debaryomyces hansenii*, *Candida famata, Rhodotorula muiilaginosa, Kluveromyces lactis, Kluveromyces maximus etc.* Isolation and identification of yeast flora from the soil was reported by Mushtaq et al (2004). Ghosh (2011) isolated and identified five genera and seven species of yeast from fruit surface of *Syzygium cumini*.

All yeast species isolated were screened by dual culture plate method for their antagonistic property against *Penicillium digitatum*, causal pathogen of *Penicillium* rot of *Citrus*. The data presented in the table 3 showed that out of 38 species of yeasts, 15 species are antagonistic to *Penicillium digitatum.*

Candida guillarmondii gave maximum percent of radial inhibition (75.50 PIRG) followed by *Candida famata* (70.56 PIRG, *Rhodotorula mucilaginosa* (68.21 PIRG) *Pichia membranifaciens* (67.21 PIRG), *Debaryomyces hansensii* (58.00 PIRG), *Saccharomyces cerevisae* (47.23 PIRG) and *R. minua* (37.44 PIRG) (Table 2). *Kluveromyces lactis* and *Zygosaccharomyces lentus* gave minimum inhibition (03.41and 6.00 PIRG respectively). Potential use of yeasts as biocontrol agents of soil born fungal plant pathogens and as plant growth promoters were recent investigated by EL-

Table 1 Isolated, identified species of yeasts and their distribution in different natural sources

S.No.	Yeast sps	Phylum/ Group (Stage)	Sources/Samples
1	*Aureobasidium pollulens*	Ascomycota (Anamorph)	Apple, green grapes.
2	*Bensingtonia phyllada*	Basidiomycota (Anamorph)	Green grapes,
3	*Candida famata*	Ascomycota (Anamorph)	Apple, green grapes date fruits,
4	*C. guillarmondii*	Ascomycota (Anamorph)	Apple, fig fruit, Orange
5	*C. spherical*	Ascomycota (Anamorph)	Apple, green grapes
6	*C krusei*	Ascomycota (Anamorph)	Apple, fig
7	*Cryptococcus albidus*	Basidiomycota (Anamorph)	Apple
8	*Cryptococcus victoriae*	Basidiomycota (Anamorph)	Fig
9	*Debaryomyces hansensii*	Ascomycota (Telomorph)	Fig
10	*Filobasidium uniguttulatum*	Basidiomycota (Telomorph)	Green grape
11	*Kloeckera apiculata*	Ascomycota (Anamorph)	Green grape
12	*Kluveromyces lactis*	Ascomycota (Telomorph)	Apple, orange
13	*P. membranifaciencs*	Ascomycota (Telomorph)	Apple, orange
14	*Rhodotorula. minua*	Basidiomycota (Anamorph)	Date fruit & fig
15	*R. mucilaginosa*	Basidiomycota (Anamorph)	Green grapes, orange
16	*R.rubra*	Basidiomycota (Anamorph	Apple, green grapes
17	*R.toruloides*	Basidiomycota (Anamorph)	Apple, green grapes,
18	*Saccharomyces cerevisae*	Ascomycota (Telomorph)	Apple, green grapes date fruits
19	*Saccharomycodes ludwigii*	Ascomycota (Telomorph)	Green grapes
20	*Zygosaccharomyces lentus*	Ascomycota (Telomorph)	Green grapes.

Table 2. Isolated and identified yeasts, their number of colony and their percent of occurrence, Berger – Parker dominance index and Relative dominance

S.No.	Yeast sps	Number of colony	Occurrence (%)	Berger- Parker dominance index (D)	Relative dominance
1	*Aureobasidium pollulens*	17	03.54	0.0354	R
2	*Bensingtonia phyllada*	10	02,09	0.0209	R
3	*Candida famata*	48	10.00	0.1000	D
4	*C guillarmondii*	28	05.71	0.0571	G
5	*C. spherical*	13	02.70	0.0270	R
6	*C krusei*	15	03.10	0.0310	R
7	*Crytococcus albidus*	15	03.10	0.0310	R
8	*Crytococcus victoriae*	15	03.10	0.0310	R
9	*Debaryomyces hansensii*	31	06.04	0.0604	G
10	*Filobasidium uniguttulatum*	08	01.60	0.0160	R
11	*Kloeckera apiculata*	25	05.20	0.5200	G
12	*Kluveromyces lactis*	14	02.90	0.0290	R
13	*P. membranifaciencs*	30	06.02	0.0602	G
14	*Rhodotorula mucilaginosa*	45	09.37	0.0937	D
15	*R.toruloides*	26	05.4	0.0540	G
16	*R. minua*	25	05.20	0.0520	G
17	*R.rubra*	32	06.74	0.0674	G
18	*Saccharomyces cerevisae*	51	10.62	0.1062	D
19	*Saccharomycodes ludwigii*	13	02.70	0.0270	R
20	*Zygosaccharomyces lentus*	19	03.70	0.0370	R
	Total	480			

Tarabily and Sivasiyhamparam (2006). Wide variety of yeasts have been used extensively for biological control of post harvest diseases of fruits & vegetables (Punja, 1997). The yeast *Torulapsis candida (Candida famata*) effectively controls *Penicillium digitatum* infection on *Citrus* fruits (Arras et al, 1999). *Candida saitoana* & *C. oleophila* control post harvest diseases of apple & *Citrus* fruits (El-Ghaouth et al. 2000). Ghosh *et al* (2013) reported that *Candida famata* gave maximum percent of radial

inhibition (70.24 PIRG) followed by *Pichia membranifaciens* (68.21 PIRG), *Rhodotorula mucilaginosa* (60.56 PIRG), *Debaryomyces hansensii* (55.40 PIRG), against *P. digitatum*. The yeast elicited production of phytoalexins, scopoletin and scoparine and did not produce toxic substances against the pathogens *Penicillium digitatum* and *Botrytis cinerea*. It suggested that the yeast may reduce fungal infection by activating host's defense mechanisms (Arras & Arru, 1999). Fifteen antagonistic yeasts showed inhibitory activity against *P. expensum* based on nutrition and space competition (Coelho, 2007). The expression of an antifungal peptide (cecropin- A based peptide) in *Saccharomyces cerevisae*, inhibited the germination of *Colletotrichum coccodes* (causal organism of tomato fruit decay) was reported (Jones & Prusky,2005). Capdeville et al (2007) worked on the interaction between *C. magnus* and *C. gloeosporioides* on papaya fruits under electron microscope and reported that the yeast colonized wound surface of fruits much faster than the pathogen, overcompeting the latter for space and nutrition. Moreover, *C. magnus* produced a flocculent matrix, which affected hypal integrity of the pathogen. *Rhodotorula* sp produces rhodotorulic acid which inhibits the growth of the pathogen. In this experiment the mechanism of inhibition of growth of *P. digitatum* may be due to antifungal substances secreted by yeasts.

This work including the diversity of yeast flora in fruit surfaces in 24-Parganas of West Bengal and screening of antagonistic yeasts against *P. digitatum* is new in its kind in India.

4. Conclusion

Therefore, these fruits (Orange, apple, green grapes, date fruits, and fig) of 24 –Parganas.

(N), West Bengal are good habitats of various yeast species including antagonistic yeasts and the antagonistic species specially *C guillarmondii* can be applied as biological control agents against post harvest *Penicillium* rot disease of *Citrus*.

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