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Isolation and characterization of phylloplane yeasts

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Abstract
In this study, yeasts were isolated from different fruit phylloplane during different seasons (summer, winter, and rainy) to study their population density and then brought to the Department of Mycology and Plant Pathology Laboratory, BHU for further experiment. A total of thirteen different yeast isolates were isolated from different phylloplane and then studied for their different characterization like morphological characters *viz;* colony colour, colony appearance, nature of growth, cell shape, type of growth, cell dimension etc. and regarding physiological characters viz., C and N utilization, sensitivity to cycloheximide as well as growth progression at different temperature. The finding to the present investigation revealed that colony colour of yeast isolates vary from cremish (Y1 to Y7, Y10 and *Saccharomyces cerevisiae*) to whitish (Y8, Y11, Y12) and single isolate Y9 gives yellowish colour. Smooth, crusty to granular colony appeared on yeast suitable media (YDPA) and having different growth nature *viz;* isolates (Y2, Y9 and Y14) shows faster growth as compare to isolate (Y1, Y11, Y12 and Y13) which shows slow colony growth. The cell shape and size ranges from spherical, oval to cylindrical and (1.9 x 1.06 µm to 4.51 x 3.14 µm) respectively. The growth of all isolate was found maximum @ 27°C while Cycloheximide @50 µg/ml found more sensitive and gives cent per cent inhibition over control against all the isolates. Among C & N source, Maltose was found more essential for the growth of all the isolate of yeast.

Keywords: Yeasts, phylloplane, isolation, characterization

Introduction
Microorganisms including bacteria, actinomycetes, cyanobacteria, filamentous fungi as well as yeast considered as important natural colonizers of phylloplane. Different aerial plant surface such as flowers, fruits, stems and leaves represented as phylloplane where microorganism population found to be more which vary from unspecific or ephemeral epiphytic to epiphytic residents, endophytes or pathogens. However, microbial populations on phylloplane depend on leaf topography, cuticle composition, availability of nutrients and microclimate. Yeast can be isolated from the fruit skin of different crop *viz;* grapes, apples, peach, and also from plant exudates as plant sap etc. The yeasts are unicellular eukaryotic organisms belonging to kingdom Fungi and are chemo- organotrophs in nature as they use inorganic chemicals fro their energy source. The temperature range of 20 to 28º C and acidic pH of 3.5 to 4 are congenial for their growth [17]. They may be ascomycetous or basidiomycetous due to presence of ascospores and teliospore respectively. They reproduce mainly asexually by the process of fission or budding and form sexual status without fruiting body. In ascomycetes, hologenous budding is found while it is enterogenous budding is common in basidiomycetes.
Economic importance of yeast makes its utilisation in brewing and baking industries [9, 10]. Yeast such as *Saccharomyces cerevisiae* is nutritionally an excellent source of vitamins (Vit. B) and protein. Protein contains in the dried yeast cell contain protein up to 40 to 50 %. In fermentative industries, they are not used only in making bread, wines and beer but also in non-food industries for making bio fuel to produce ethanol. Yeast also helps in the bioremediation process eg. *Yarrowia lipolytica* degrades palm oil and some hydrocarbons such as fatty acids, oils, fats and alkanes, etc. Now a day, only a few yeast-based bio-control products which are available in the market containing mainly *Candida oleophila* was registered by U.S Environmental Protection Agency in 1995 for control of postharvest rots of citrus fruit but is no longer available in the market as it was unable to control previously established as well as latent infection. This was the first launched yeast-based bio-control product. Schemer containing *Metschnikowia fruticola*, Candifruit containing *Candida sake*, ProYeast-ST and ProYeast-ORG containing heat-tolerant strains of *Metschnikowia fruticola* are some yeast-based biocontrol products available in some countries for commercial use but these are yet to become popular among the formers.

Candida saitonana based products, Biocure (derivative of chitosan) and Biocoat (derivative of lysozyme) is still awaiting registration. An experiment was conducted in the Department of Mycology and Plant Pathology, BHU to study the morphological and physiological characters of different isolates of yeast from different phylloplane.

Materials and Methods

Fresh leaf or fruit samples of banana, citrus, mango, apple, grapes were collected during different seasons in separate brown paper packets from well-grown established trees of selected fruit from Banaras Hindu University campus. The standard laboratory techniques were used for the preparation of media, cleaning of glassware's, sterilization, maintenance of yeast culture, with modification whenever necessary.

Isolation of yeast from leaf sample and fruit surfaces by washing

The leaf imprint method as described by [18] was followed with a little modification for isolation of yeasts from leaf sample. Initially, leaf samples were surface sterilized with 0.1% sodium hypochlorite and repeatedly washed in sterile water and then cut into size of 2x2 cm approximately and two pieces of it are placed on Petri plates containing solidified Yeast Dextrose Peptone Agar medium such that upper surface of one piece and lower surface of other piece remain in contact with the medium. After leaving plate undisturbed as such in laminar airflow for 4 hours, sample pieces were removed with the help of sterilized forceps. Then the plate was incubated for 2 days at 28^0C. The same procedure is followed for every sample. The yeasts were also isolated from fruit surfaces by shaking fruits in sterile distilled water for 5 minutes after putting in a flask under aseptic conditions followed by serial dilution by tenfold and streaking the same on Yeast Dextrose Peptone Agar media for each sample collected and after that it is incubated for 2 days [5]. After incubation, isolates were collected by streaking yeast colonies on Yeast Dextrose Peptone Agar medium and then maintained in slants containing same medium (YDPA) and refrigerated for further use. Sub-culturing of the isolates was done as when required.

Morphological characterisation of yeast isolates

The purified yeast isolates from different phylloplane were grown on Yeast Dextrose Peptone Agar medium after sub culturing of culture after few days observations were made for their morphological studies *viz;* colony appearance, shape, nature of growth, type of growth and colony colour. After that isolates were observed under the compound microscope of Olympus Company having model number CH20iBlMF using 10X and 40X lenses for cell dimensions (shape and size) and presence or absence of pseudohyphae was recorded by making slides of yeast isolates. The cell dimension was measured using Zeiss image analysis software.

Physiological characterisation of yeast isolates

Yeast isolates were tested for carbon assimilation capacity, nitrogen assimilation capacity, sensitivity to cycloheximide, fermentative ability and growth capacity at different temperature. For carbon assimilation capacity of different sugars *viz.* maltose, raffinose, galactose, cellobiose, trehalose, mannitol, erythritol, inositol, melibiose and xylose sugars were tested by pour plate auxano graphic method. In this method, the plates were inoculated with the isolated yeast and then incubated for 3 days at 27±1 ºC after which observations were recorded [7]. Further, for nitrogen assimilation capacity test, KNO3 (5 gm /lit) were taken as a source of nitrogen. The highly diluted suspension of yeast isolates were inoculated in a plate containing yeast carbon base agar media and incubated at 27±1 ºC for 5 days after which observations were made. For each isolates three replications were taken [7]. For evaluating the sensitivity of yeast to cycloheximide, the yeast isolates were inoculated on Yeast Dextrose Peptone Agar media containing antifungal antibiotic cycloheximide of different concentration (1 µg, 10 µg, 25µg and 50 µg) after sterilization for cycloheximide sensitivity test. A Control plate was set up for all the isolates. The inoculated plates were then incubated at 28 ± 1 ºC for 3 days. After 3 days plates were observed for positive and negative growth. Fermentative ability was tested through Yeast Dextrose Peptone broth. The quantity of 7 ml of prepared broth was fill in test tubes and then sterilized at 15 psi at 21 ºC for 20 minutes. After broth gets cooled it was inoculated with a highly diluted suspension of yeast isolates containing 1-1.6×108 spores/ml. Spore population was counted using a haemocytometer and Durham tubes were put upside down. Then tubes were incubated at 28 ± 1 ºC for 7 days. The formation of gas was indicated by floating of tubes above the broth. Further, yeast isolates were inoculated in slant having Yeast Dextrose Peptone agar medium and allowed to grow at -25 ºC, 10 ºC, 27 ºC and 35 ºC for testing growth ability at different temperature. The growth was recorded 3 days after inoculation.

Results and Discussion

Isolation of unicellular fungi during different seasons from fruits and their phylloplane

A total of 13 yeasts (Y1 to Y13) were isolated from fruits and their phylloplane during different season by imprinting washed or unwashed leaf pieces or by fruit washing on Yeast Dextrose Peptone Agar medium and further maintained in slants for further experiment. The source, season of isolation and number of isolates obtained from washed and unwashed samples are presented in Table 1. Among 13 isolates, source of four isolates are banana leaf and two isolates are banana fruit washing, source of one isolate is citrus leaf and one is citrus fruit washing, source of two isolates are grapes fruit washing, source of one isolate is mango leaf and one of mango fruit wash and source of one isolate is apple fruit washing.

Colony and Cell morphology of yeast isolates

All the 13 yeast isolates were observed for their morphological characters like colony colour, colony appearance and nature of growth and were also observed under microscope for cell shape, cell size and presence of pseudo mycelium are presented in Table 1. Most of the isolates colonies were cremish in colour while some were whitish and some were yellowish. One of the isolate (Y13) was red. Yeast isolates were found to be smooth to crusty, chalky and granular. Most of the isolates were found to be fast-growing while few (Y1, Y11, Y12 and Y13) were slow-growing in nature. Isolate (Y5) showed the presence of pseudo mycelium. Cell shape of most the isolates varied from oval to spherical but cylindrical shape was only observed in isolate Y2, Y7 and Y11. The present finding favour the experiment conducted by Gosh (2011) in which yeast isolated from the surface of the Jamun fruit (*Syzygium cumini)* from different zones of West Bengal. They identified yeasts were *Candida famata, Candida ipomoea, Candida succiphila, Rhodotorula mucilaginosa, Debaryomyces hansensii,*

Kodamaea anthophila and *Pichia lachancei.* The isolated yeast species from the leaves of sugarcane and rice collected in Thailand. They determined that two strains represent a novel *Papiliotrema* species based on morphological, biochemical, physiological and chemotaxonomic characteristics, sequence analysis of the D1/D2 region of the large subunit rRNA gene and Internal Transcribed region [19]. The isolated 5 strains representing a single novel anamorphic yeast species from sugarcane, two from tissue (DMKU-SE38, DMKU-SE59(T), two from leaves surface (DMKU-SP385, DMKU-SP403) collected in Thailand and one from rhizoplane (IMUFRJ 52020) collected from organically cultivated field in Brazil. They classified them as a single species of genus *Occultifur* based on sequence analysis of the D1/D2 region of large subunit rRNA gene and the ITS region. They observed that the sequence of the D1/D2 region of the LSU rRNA genes and the ITS region of 5 strains were identical or differed from each other by one nucleotide substitution. And species were more closely related to *Occultifur brasiliensis f.a.* CBS12687 (T) but with 0.7-1.0% nucleotide substitution in D1/D2 region of the LSU rRNA gene and 2.5-2.7% nucleotide substitution in ITS region. So, *Occultifur tropicalis f.a., nov* name was proposed [12].

Physiological characteristics of yeast isolates

All the yeast isolates are tested for utilization of carbon sources by using ten carbon sources *viz.*, Maltose, Raffinose, Galactose, Cellobiose, Trehalose, Mannitol, Erythritol, Inositol, Melibiose, Xylose, Fermentative ability and Nitrogen utilization, the results of which are presented in Table 2. The data revealed that different isolates showed wide variation in carbon utilization. Maltose was utilized by all the 13 isolates while Raffinose was utilized by isolates Y1, Y3, Y5, Y6, Y8, Y10 and Y11. Galactose was utilized by (Y1, Y2, Y3, Y4, Y7, Y8, Y9, Y11 and Y12). Cellobiose was utilized only by isolates Y9 and Y10. Trehalose was utilized by isolates Y1, Y2, Y3, Y4, Y7, Y8, Y9, Y10, Y11, Y12 and Y13 for their growth while, Mannitol was utilized by Y1, Y2, Y3, Y4, Y5, Y6, Y7, Y8, Y9, Y10, Y12 and Y13. The carbon source Erythritol also enhanced the yeast growth as it was utilized by isolates Y4, Y7, Y8, Y9, Y10, Y11, Y12 and Y13. Inositol was utilized by Y1, Y3, Y4, Y5, Y6, Y7, Y8, Y11 and Y12. Melibiose was utilized by Y4, Y6, Y7, Y8, Y11 and Y13 however, the carbon source Xylose utilized by isolates Y1, Y2, Y3, Y6, Y9, Y10 and Y12. The yeast isolates were also tested for fermentative ability. Among 13 isolates, onlyY5 and Y8 showed fermentative capability which is indicated by the formation of gas inside the Durham tube. Yeast isolates were further tested for nitrogen assimilation. Among them, Y1, Y2, Y3, Y4, Y5, Y6, Y10, Y11 and Y12 showed nitrogen utilization from KNO3. The same finding Was found with the work done by many researchers and found that the identification of yeast through microscopic observation, fermentation of sucrose, lactose, glucose and Raffinose and assimilation of nitrogen sources *viz* nitrate, ethylamine, L-lysine, cadaverine and creatine and found 49 isolated yeasts belonged to five genera with seven species. They found that based on nitrogen assimilation test 49 yeasts could be grouped into 18 distinct clusters [8]. The *Saccharomyces cerevisiae* strains isolated from palm wine in the production of burukutu for determining fermentative efficiency. They carried out identification and characterisation by microscopy and conventional biochemical methods and Analytical identification. Biochemical identification and API showed isolate that was Glucose, Galactose, Raffinose, Acetyl D glucosamine positive and Glycerol, Inositol, Sorbital, Arabinose, D-xylose, Adonitol, Xylitol, Celiobiose, 2-Ketoglutanal, Lactose, Maltose, Tretialose, Melezitose negative [6]. The result of fermentative ability of yeast in the present study was favoured by the same work done by Brooks (2008) tested the fermentative ability of eight yeast strains isolated from ripe banana peels. Among 8 isolates, 5 showed enhanced fermentative capacity. The isolates were further assessed for important ethanol fermentation ability *viz* ethanol production ability, ethanol tolerance, flocculence, Thermo- and- osmo tolerance. The isolates that had the potential for ethanol production were *Saccharomyces cerevisiae* R-8, *S. cerevisiae* T-7, *S. cerevisiae* R-2, *Debaryomyces hansenii* and *Saccharomyces kluweri* K-6. The isolated 98 strains of yeast which are belonging to 51 species; in which 32 strains are from fresh fermented orange juice (FSSOJ) while 19 strains are from defective orange juice (DSSOJ). *Candida kruesi* and *Rhodotorula minuta* yeast strain were found to be dominant in FSSOJ while *Candida zeylanoides* and *Candida parapsilosis* were found to be dominant in DSSOJ. *Candida* and *Rhodotorula* were found in both types of juice while *Kodamaea* and *Geotrichum* found only in fresh or healthy orange juice. *Candida Kruesi* had the highest prevalence (57%), *Rhodotorula minuta* (20%), *Candida zeylanoides* (8%), *Candida parapsilosis* (6%), *Geotrichum capitatum* (4%), *Candida norvegensis* and *Kodamea* (1%) respectively. They reported that *Candida lusitaniae*, *Candida parapsilosis* and *Rhodotorula minuta* were able to ferment Xylose [15].

Growth at different temperature

Yeast isolated are tested for their growth at -25 ºC, 10 ºC, 27 ºC and 35 ºC, the results of which are presented in Table 2. No one isolates showed growth at -25 ºC but at10 ºC isolates Y3, Y8 and Y11 growth was found progressive as compared to other isolates. At 27 ºC temperature, growth was seen in almost all yeast isolates. The yeast isolates *viz;* Y5 and Y8 having fermentative ability also gives growth at 35 ºC temperature. For temperature -25 ºC and 35 ºC, the growth was re-observed one week after incubation. The present experiment was the same as the work done by another researcher in the same field as classified yeast based on temperature limits of growth found by [2]. The 156 strain of yeast was isolated from phylloplane of 85 rice leaf samples. They determine the growth of strains at a high temperature of 35, 37 and 40 ºC. They reported that all strains could grow at 35 ºC, however all strains of *Rhodotorula taiwanensis,* one strain of *Cryptococcus aff. Laurentii* and one strain of *Cryptococcus flavescens* showed weak growth. At 37 ºC most strains grew except all strains of *R. taiwanensis, Cryptococcus aff.* laurentii and *Cryp Flavescens*. At 40 ºC more than 50% of strains could grow [13]. The isolated yeasts from *Arabidopsis thaliana* were tested 11 selected strains for their growth at different temperature *viz*., 8 ºC, 21 ºC, 30 ºC and 37 ºC. They reported that 7 strains were cold-adapted and were able to grow at 8 ºC. They observed that OTU4 and OTU26 showed better growth at low temperature than room temperature and only one strain was able to grow at 30 ºC and 37 ºC [20].

Sensitivity of yeast isolates to cycloheximide

Yeast isolates were also tested for sensitivity to antifungal antibiotic cycloheximide. All 13 isolates were tested for sensitivity at 1µg/ml, 10µg/ml, 25µg/ml and 50µg/ml concentration of cycloheximide on Yeast Dextrose Peptone Agar Medium. The data in table no. 2 reveals that most of the isolates were sensitive to cycloheximide at 10 µg/ml

concentration except isolates Y4, Y7 and Y11. However, isolate Y11 @25µg/ml shows tolerance up to a certain level. The first report that yeasts varied in their sensitivity to the cycloheximide given by [21]. The isolated yeasts from the soil, leaves, fruits, flowers and processed product and identified 4 strains of *Rhodotorula mucilaginosa* and one strain of *R. graminis*. Among all strains, only strain L 125 of *Rhodotorula graminis* showed growth in the presence of cycloheximide while others showed no growth [14]. The isolated yeast strains from a decaying mangosteen fruit. They found that strains were sensitive to 0.1% cycloheximide and resistant to 0.01% cycloheximide [3]. The fourteen cultures were isolated from soil samples, fruits and fermented products. Among 14 cultures, 7 were yeast. Seven isolates of yeast were namely, S1, S2, S3, TA, C2, K1, K2 and MTCC170. Among these isolates, S1, S2 and K1 isolates were not sensitive to cycloheximide [1].

Conclusion

In the present investigation, it was concluded that all the 13 yeast isolates were isolated from four fruits and their phylloplane (Banana, Mango, Grapes, Citrus and Apple) during different seasons. Yeast isolates were characterized for their morphological and physiological aspects with an aim of classification. Yeast colony colour varies from cremish to whitish colour. One of the isolates had an orange colony colour. Yeast cell shape varies from spherical to cylindrical and cell size varies from 2.51×1.17µm, being the smallest to 4.05×2.19µm, being the longest. Yeast isolates were studied for utilization of carbon, nitrogen and fermentative ability. Carbon assimilation pattern was investigated by using the following sources: maltose, Raffinose, galactose, Cellobiose, trehalose, Mannitol, Erythritol, Inositol, Melibiose and Xylose. Yeast isolates were studied for the utilization of nitrogen by using KNO_3. They varied concerning the utilization of carbon sources, nitrogen sources and fermentative ability. All yeast isolates mainly utilized maltose for their growth. All yeast isolates were able to grow at 27 ºC. The yeast at 10µg/ml of concentration found more sensitive toward antifungal antibiotic cycloheximide.

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Table 1: Isolation and morphological character of a different isolate of yeast

Isolate	Season of isolation*	Source	*Morphological characters of yeast						
			Colony colour	Colony appearance	Nature of growth	Cell shape	Type of growth	Cell dimension (µm)	Pseudo mycelium**
Y1	Summer	Banana leaf	Cremish	Crusty	Slow	Spherical	Flat	2.34×1.05	-
Y2	Summer	Banana leaf	Cremish	Smooth	Fast	Cylindrical	Raised	4.01×2.07	-
Y3	Summer	Banana leaf	Cremish	Crusty	Fast	Oval	Raised with margin	2.93×1.13	-
Y4	Summer	Banana leaf	Cremish	Granular	Fast	Oval	Flat	2.02×1.09	-
Y5	Winter	Grape Fruit washing	Cremish	Smooth	Fast	Oval	Flat	2.06×1.27	+
Y6	Summer	Mango fruit washing	Cremish	Smooth	Fast	Spherical	Raised	2.25×1.15	-
Y7	Rains	Banana fruit washing	Cremish	Smooth	Fast	Cylindrical	Flat with spreading	3.87×2.01	-
Y8	Winter	Grape fruit washing	Whitish	Chalky	Fast	Oval	Raised	2.01×1.12	-
Y9	Winter	Citrus fruit washing	Yellowish	Smooth	Fast	Spherical	Raised	1.92×1.06	-
Y10	Winter	Citrus leaf	Cremish	Granular	Fast	Spherical	Raised	2.43×1.13	-
Y11	Winter	Banana fruit washing	Whitish	Smooth	Slow	Cylindrical	Raised with margin	3.89×2.14	-
Y12	Winter	Mango leaf	Whitish	Chalky	Slow	Oval	Flat	2.97×1.03	-
Y13	Winter	Apple fruit washing	Orange	Smooth	Slow	Spherical	Raised	2.99×1.10	-
Y14	Local Standard Culture		Cremish	Smooth	Fast	Oval to Spherical	Raised	4.51×3.14	-

*Mean of three replication; *summer – march to may; winter – october to december and Rains – july to september.
** (+) denotes presence of pseudomycelium, (-) denotes absence of pseudomycelium.

Table 2: Physiological characteristics of yeast isolates

Isolate	*Growth at different Temperature (°C)				**Sensitivity to Cycloheximides (µg/ml)					Carbon source***										Fermentative ability***	Nitrogen utilization***
	-25	10	27	35	1	10	25	50	Control	Maltose	Raffinose	Galactose	Cellobiose	Trehalose	Mannitol	Erythritol	Inositol	Melibiose	Xylose		
Y1	-	-	+	-	+++	-	-	-	++++	+	+	+	-	+	+	-	+	-	+	-	+
Y2	-		+	-	+++	-	-	-	++++	+	-	+	-	+	+	-	-	-	+	-	+
Y3	-	+	+	-	+++	-	-	-	++++	+	+	+	-	+	+	-	+	-	+	-	+
Y4	-	-	+	-	+++	++	-	-	++++	+	-	+	-	+	+	+	+	+	-	-	+
Y5	-	-	+	+	+++	-	-	-	++++	+	+	-	-	-	+	-	+	-	-	+	+
Y6	-	-	+	-	+++	-	-	-	++++	+	+	-	-	-	+	-	+	+	+	-	+
Y7	-	-	+	-	+++	++	-	-	++++	+	-	+	-	+	+	+	+	+	-	-	-
Y8	-	+	+	+	+++	-	-	-	++++	+	+	+	-	+	+	+	+	+	-	+	-
Y9	-	-	+	-	+++	-	-	-	++++	+	-	+	+	+	+	+	-	-	+	-	-
Y10	-	-	+	-	+++	-	-	-	++++	+	+	-	+	+	+	+	-	-	+	-	+
Y11	-	+	+	-	+++	++	+	-	++++	+	+	+	-	+	+	+	+	+	-	-	+
Y12	-	-	+	-	+++	-	-	-	++++	+	-	+	-	+	+	+	+	-	+	-	+
Y13	-	-	+	-	+++	-	-	-	++++	+	-	-	-	+	-	+	-	+	-	-	-
Y14	-	-	+	-	+++	-	-	-	++++	+	+	+	±	+	+	-	-	-	+	+	±

*Mean of three replications.
* Represents "+"= growth; "-"= no growth
** Represents "+, ++, +++, ++++"= extends of growth
*** Represents "+"= utilized; "-"= not utilized and "±"= doubtful

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