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BIODIVERSITY OF YEAST MYCOFLORA IN SLIME FLUXES OF SOME TREES

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Abstract

A total of 13 species belonging to 9 genera were isolated from slime fluxes of *Araucaria cooki*, *Azadirachta indica*, *Eucalyptus camaldulensis* and *Ficus religiosa*. The isolated yeast species were identified on the basis of morphological and physiological / biochemical characters. *Bullera pseudoalba*, *Candida lyxosophila*, *Cryptococcus gasrticus*, *Pichia anomala*, *P. strasburgensis*, *Sporidiobolus ruineniae* and *Wiliopsis californica* were predominantly isolated from slime fluxes of trees.

Introduction

The flowing of tree sap from a wound (Fluxing) is generally caused by injuries due to boring insects, frost cracks or the breaking of twigs. The flowing sap becomes heavily infested with bacteria, yeasts and protozoa and rarely by filamentous fungi. It usually assumes a thick and slimy consistency, probably due to microbial capsular polysaccharides formation and for this reason it is usually referred to as slime flux. Phaff & Knapp (1956) isolated *Endomycopsis javanensis* (Syn. *Arhtroascus javanensis*) from slime flux of *Quercus kellogii* in Sierra Nevada, California. Subsequently a number of *Pichia* species such as *P. fluxuum*, *P. trehalophila*, *P. salictaria*, *P. angophorae*, *P. scutulata*, *P. anganishii*, *P. veronae* and *P. nakazawae* were reported from slime fluxes of *Abies* sp., *Acer* sp., *Aesculus* sp., *Angophora* sp., *Camellia* sp., *Myoporum* sp., *Populus* sp., *Quercus* spp., and *Salix* sp., from Canada, Japan, and USA (Phaff & Knapp, 1956; Phaff *et al*., 1964; Miller & Baker, 1968; Kodama & Kyono, 1974; Kodama, 1975; Phaff *et al*., 1976). Lodder (1970) reported several species of *Nadsonia* as well as *Saccharomycodes ludwigii* in slime fluxes of birch and oak.

In a previous survey we reported a number of yeast species belonging to several genera from slime fluxes of *Acacia nilotica*, *Albizzia lebbeck* and *Aralia cachemirica* trees (Mushtaq *et al*., 2005). In the present studies efforts have been made to isolate and identify yeast species associated with slime fluxes of *Araucaria cooki*, *Azadirachta indica*, *Eucalyptus camaldulensis* and *Ficus religiosa*.

Materials and Methods

Four samples of slime fluxes from *Araucaria cooki*, 10 from *Azadirachta indica*, 7 from *Eucalyptus camaldulensis* and 4 from *Ficus religiosa* trees were collected from University of Karachi, Karachi. Yeasts associated with these samples were isolated by suspending a known amount of slime flux/gum exudates in sterile distilled water and shaken well to dislodge the adhering cells from residues. Serial dilution was made up to 10,000 and inoculated either on malt-yeast-glucose-peptone (YM), malt extract or yeast

morphology agar medium and incubated for 5-7 days at 25±1°. Three isolates of yeasts per plate were selected, as representatives of the yeast mycoflora, from morphologically similar looking growing colonies, which were further purified and maintained on yeast morphology agar buffered at pH 4.5 (Mushtaq *et al*., 2005). All isolated yeasts were primarily classified into 7 different groups *viz*., pink (group A), methanol assimilating (group B), cap-, hat-, saturn- or walnut- shaped ascospore producing (group C), round-, oval-, conical- or reniform shaped ascospore producing (group D), ballistoconidia forming (group E), basidiomycetous (group F) and glucose fermenting (group G). Identification of yeasts up to species level was carried on the basis of standard morphological and physiological/biochemical tests as proposed for each group (Kurtzman & Fell, 1999; Barnett *et al*., 1990).

Shapes and structures of vegetative yeast cells were examined microscopically, whereas Dalmau Plate Culture method on corn meal agar was used to test the ability of yeast to produce pseudo- or true-hyphae and arthro-conidia (Beech *et al*., 1972). Ballistoconida formation was observed on malt extract medium (Barnett *et al*., 1990). Assimilation of carbon and nitrogenous compounds were simultaneously tested in liquid yeast nitrogen base and yeast carbon base supplemented with 50mM carbon/nitrogen source to be tested. Growth at different temperatures (25°C, 30°C, 35°C, 37°C and 40°C), in the presence of Cycloheximide (0.1% & 0.01%) and D-glucose (50% & 60%) were also tested in liquid yeast nitrogen base (used for carbon assimilation). Ability of yeasts to grow without added vitamin(s) was tested in liquid vitamin free yeast base. Production of extra-cellular starch-like compounds was observed using Lugol's iodine solution after a positive growth in liquid medium of a sugar or an alditol (Cowan & Steel, 1966). Diazonium Blue B (DBB) test was tested on 10-days old culture growing on malt-yeast-glucose-peptone agar after drying at 55°C for several hours using ice-cold DBB reagent (Van der Walt & Hopsu-Havu, 1976).

Result and Discussion

A total of 9 genera and 13 yeast species were isolated from 25 samples of slime fluxes of 4 different trees (Table 1). Two species belonging to 2 genera were isolated from 4 slime flux samples of *Araucaria cooki*, 8 species belonging to 7 genera from 10 samples of *Azadirachta indica*, 3 genera and 3 species from 7 samples of *Eucalyptus camaldulensis* and 4 species belonging to 4 genera from 4 slime flux samples of *Ficus religiosa*. The isolated yeasts species were identified on the basis of their morphological (Table 2) and physiological / biochemical characters (Table 3).

Out of 13 yeast species, teleomorphic ascomycetous yeasts were identified as *Pichia anomala*, *P. lynferdii*, *P. strasburgensis, Williopsis californica* whereas among anamorphic ascomycetous yeasts *Candida lyxosophila*, *C. succiphila* and *Saitoella complicata* were isolated and identified. On the other hand among teleomorphic basidiomycetous yeasts, *Fibulobasidium inconspicuum*, *Rhodosporidium toruloides* and *Sporidiobolus ruineniae* were identified and among anamorphic basidiomycetous yeasts *Bullera pseudoalba*, *Cryptococcus albidus* and *C. gastricus* were identified. All yeast species appeared newly reported from slime fluxes of *Araucaria cooki*, *Azadirachta indica*, *Eucalyptus camaldulensis* and *Ficus religiosa* in Pakistan. Univariate ANOVA of yeast species revealed that their occurrence was significantly different at $p<0.001$ in slime flux samples of all trees (Table 4). Bonferroni test also confirmed significant differences among yeast species (Table 1).

Table 1. Occurrence of yeast mycoflora in terms of mean colony forming units (mcfu) with standard error (se) and range, isolated from slime fluxes of different trees.

Yeast species	*Araucaria cooki*		*Azadirachta indica*		*Eucalyptus camaldulensis*		*Ficus religiosa*	
	Occ. %	*mcfu±se **(range)	Occ. %	*mcfu±se **(range)	Occ. %	*mcfu±se **(range)	Occ. %	*mcfu±se **(range)
Bullera pseudoalba	--	-- --	--	-- --	--	-- --	100.0	63.5 ± 1.50[a] (62.0-65.0)
Candida lyxosophila	--	-- --	--	-- --	--	-- --	100.0	5.75 ± 0.05[b] (5.7-5.8)
C. succiphila	--	-- --	10.0	0.20 ± 0.20[a] (2.0)	--	-- --	--	-- --
Cryptococcus albidus	--	-- --	10.0	0.75 ± 0.75[b] (7.5)	14.3	1.06 ± 1.06[a] (7.4)	--	-- --
C. gastricus	--	-- --	--	-- --	--	-- --	100.0	5.2 ± 0.10[b] (5.1-5.3)
Fibulobasidium inconspicuum	--	-- --	10.0	0.94 ± 0.94[c] (9.4)	14.3	0.53 ± 0.53[b] (3.7)	--	-- --
Pichia anomala	--	-- --	40.0	2.49 ± 1.02[d] (5.7-7.1)	--	-- --	--	-- --
P. lynferdii	--	-- --	--	-- --	14.3	9.43 ± 9.43[c] (6.6)	--	-- --
P. strasburgensis	50.0	5.35 ± 0.05 (5.3)	10.0	0.75 ± 0.75[b] (7.5)	--	-- --	--	-- --
Rhodosporidium toruloides	--	-- --	10.0	0.83 ± 0.83[e] (8.3)	--	-- --	--	-- --
Saitoella complicata	--	-- --	10.0	0.92 ± 0.92[c] (9.2)	--	-- --	--	-- --
Sporidiobolus ruineniae	50.0	6.30 ± 0.10 (6.4)	--	-- --	--	-- --	--	-- --
Wiliopsis californica	--	-- --	10.0	0.94 ± 0.94[c] (9.4)	--	-- --	100.0	0.70 ± 0.10[c] (0.6-0.8)

* Values are in 10,000; ** single values in parentheses indicates that yeast species was isolated only from 1 sample. Mean values in each column having different letters are significantly different at $p<0.001$ (Bonferoni test).

Table 2. Morphological Characteristics of yeast species isolated from slime fluxes of trees.

Morphological characters	Colony color	Shape of cell	Splitting cells	Pseudomycelium	Septate hyphae	Arthroconidia	Ballistoconidia	Symmetric conidia	Ascospores round, oval, conical or reniform	Ascospres cap-, hat-, Saturnm- or walnut shaped
Bullera pseudoalba	wh.cr.	eli	-	+	+	-	+	+	-	-
Candida lyxosophila	wh.cr.	sgl-gl.	-	+	-	-	-	-	-	-
C. succiphila	wh.cr.	sgl-gl.	-	-	-	-	+	+	-	-
Cryptococcus albidus	cr.	gl-ov	-	-	-	-	-	-	-	-
C. gastricus	cr.-tan.	gl.	-	-	-	-	-	-	-	-
Fibulobasidium inconspicuum	wh.cr.	sgl-gl.	-	-	-	-	-	-	-	-
P. anomala	wh.cr.	sph-ov	-	-	-	-	-	-	-	+
P. lynferdii	wh.cr.	sph-ov	-	-	-	-	-	-	-	+
P. strasburgensis	wh.cr.	r-ov	-	+	-	-	-	-	-	+
Rhodosporidium toruloides	pi.-red	sph-elo	-	+	-	-	-	-	-	-
Saitoella complicata	pi.	ov-eli	-	-	-	-	-	-	-	-
Sporidiobolus ruineniae	pi.	ov-cy	-	-	+	-	-	-	-	-
Williopsis californica	gr.wh.	r-ov	-	-	-	-	-	-	-	+

Colony color: wh=white; cr=cream; gr=gray; cr-tan= cream to tan; pi=pink;
Shape of cell: r=round; ov=oval; gl=globose; sgl= sub globose; sph=spherical; elo=elongated; eli=eliptical; cy=cylindrical

Bullera pseudoalba, *Candida lyxosophila*, *Cryptococcus gasrticus* and *Wiliopsis californica* were predominantly isolated from slime fluxes of *Ficus religiosa*. The total colony forming units (cfu) per grams of sample ranged from $63.5x10^4$ g^{-1} (*Bullera pseudoalba*) to $0.7x10^4$ g^{-1} (*Wiliopsis californica*). Similarly in slime flux of *Azadirachta indica*, *Pichia anomala* was predominant and the range of cfu g^{-1} was recorded between $2.49x10^4$ g^{-1} (*Pichia anomala*) and $0.2x10^4$ g^{-1} (*Candida succiphila*). Only two yeast species viz., *P. strasburgensis* ($5.35x10^4$ g^{-1}) and *Sporidiobolus ruinenae* ($6.3x10^4$ g^{-1}) were recorded from slime flux samples of *Araucaria cooki*. The total colony forming units of yeasts in slime flux samples of *Eucalyptus camaldulensis* ranged from $0.53x10^4$ g^{-1} (*Fibulobasidium inconspicuum*) to $9.43x10^4$ g^{-1} (*Pichia lynferdii*) (Table 1). It is clear that the number of yeast species isolated from slime flux of *Ficus religiosa* were exceptionally higher than yeast species isolated from other trees.

The yeast species including *Fibulobasidium inconspicuum*, *Pichia anomala*, *P. lynferdii* and *Sporidiobolus reineniae* isolated during this study were also isolated from slime fluxes of *Acacia nilotica*, *Albizzia lebbeck* and *Aralia cachemirica* in the previous study (Mushtaq *et al*., 2005). However rest of the yeast species have not been reported earlier from slime fluxes of trees and appeared to be new report from slime fluxes of *Araucaria cooki*, *Azadirachta indica*, *Eucalyptus camaldulensis* and *Ficus religiosa* in Pakistan. It is very likely that insects which are known to breed in slime fluxes to

Table 3. Morphological and physiological/biochemical characteristics* of yeast mycoflora isolated from slime fluxes of different trees.

	Yeast species												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Type of test performed	***Bullera pseudoalba***	***Candida lyxosophila***	***C. succiphila***	***Cryptococcus albidus***	***C. gastricus***	***Fibulobasidium inconspicuum***	***Pichia anomala***	***P. lynferdii***	***P. strasburgensis***	***Rhodosporidium toruloides***	***Saitoella complicate***	***Sporidiobolus ruineniae***	***Wiliopsis californica***
Group →	**E**	**G**	**B**	**D**	**A**	**F**	**G**	**B**	**C**	**A**	**D**	**A**	**G**
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	v	+	+	+	n	+	-(+)
L-Sorbose	-	-	+	+	v	+(-)	+(-)	+(-)	-	+	n	+	+
D-Glucosamine	+	-	-	v	v	n		n	n	+	n	n	n
D-Xylose	+(-)	v	+	v	+	-(+)	V	v	v	-	-	+	v
L-Arabinose	n	+	n	+	+	+(-)	-(+)	-(+)	+	+	+	n	v
D-Arabinose	n	-	n	+(-)	v	+(-)	-(+)	-(+)	v	+	+	n	-
L-Rhamnose	+	-	+(-)	v	+(-)	+	-	-	+	+(-)	n	n	-
Sucrose	n	+	n	+	+(-)	+	+	+(-)	+	+	n	+	+
Maltose	+	+	v	+	+	+	+	+	+	+	n	+	+
α,α-Trehalose	n	+	+	n	n	n	+	+	+	n	n	n	+
Methyl α-D-glucoside	+	+	n	n	n	n	+(-)	+(-)	+	n	n	n	+
Cellobiose		+	+	n	n	n	+(-)	+	+	n	n	n	+
Arbutin	n	+	n	n	n	n	+(-)	+	v	n	+	+	+
Melibiose	+	-	n	v	v	-(+)	-	-	-	+	+	+(-)	-
Lactose	+	-	n	v	-(+)	-	-	-(+)	-	v	+	+(-)	-
Raffinose	+	-	-	+	+	+	+	+	+	v	+	+	+
Melezitose	+	v	+	+	+	+	+(-)	+(-)	+	+	+	-(+)	-
Inulin	-	-	n	n	n	n	-	+	+	n	+	n	+
Starch	n	+	n	v	+	+	+(-)	-(+)	v	+	+	n	-
Glycerol	n	n	n	n	n	n	n	n	n	n	n	+	n
Erythritol	-	-	+	v	-	-	+	+	-	-	-	-	-
Xylitol	n	-	n	n	n	n	-(+)	v	+	n	n	n	v
L-Arabinitol	+	n	n	n	n	n	n	n	n	n	n	+	n
D-Glucitol	n	n	n	+	v	n	n	n	n	n	n	n	n
D-Mannitol	n	+	+	+	v	+	+(-)	v	+	+	+	n	v
Galactitol	+	n	+	v	-	+(-)	n	n	n	-(+)	-	+	n
myo-inositol	+	-	-	+	+	n	n	-	n	n	n	-	n
2-Keto-D-gluconate	n	+	+	n	n	n	-	n	-	-	n	-	n
5-Keto-D-Gluconate	n	n	n	n	n	n	n	n	n	-	n	n	n
D-Gluconate	n	-	+	+	+	n	v	-(+)	-	n	n	n	-

Table 3. (Cont'd.).

Type of test performed	Yeast species												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
D-Glucuronate	v	-	n	+	+	+(-)	n	n	n	-(+)	n	-	n
D-Galacturonate	+	-	+	n	-	+	-(+)	-(+)	v	-	n	n	-
DL-Lactate	+	+	+	n	-	n	+	+	+	n	n	n	+
Succinate	n	N	n	n	n	n	n	n	n	n	n	n	n
Citrate	+	+	+	-(+)	-	-	+	+	+	-	+	+	+
Methanol	-	-	+	-	-	-	-	-(+)	-	-	-	-	-
Ethanol	+(-)	N	+	+(-)	+	+	n	+	n	+(-)	+	+	+
Propane 1,2-diol	n	N	n	n	n	n	n	n	n	n	n	n	n
Butane 2,3 diol	n	N	n	n	n	n	n	+	+	n	n	n	n
Fermentation													
D-Glucose	n	N	+	n	n	n	+	+	n	-	n	n	+
D-Galactose	n	+	+(-)	n	n	n	n	n	n	n	n	n	n
Methyl ᾱ D-glucoside	n	+	n	n	n	n	+(-)	v	v	n	n	n	v
Sucrose	n	+	n	n	n	n	+(-)	v	+	n	n	n	-(+)
Inulin	n	-	n	n	n	n	w/-	v	w/-	n	n	n	-(+)
Assiml. of N comp.													
Nitrate	-	-	w/-	v	-	-	+	+	-	+(-)	+	+	+
Ethylamine	n	n	+	-(+)	-(+)	v	+	+	n	+(-)	-(+)	n	n
L-Lysine	n	+	-	+	n	n	+	+	+	n	n	n	+
Cadaverine	+	+	n	-(+)	+	+	+(-)	+(-)	+	+	+	+	+
Glucosamine	+	-	n	-(+)	+	v	v	-(+)	+	v	n	n	-
Growth without vitamin(s)													
Without vitamins	n	-	v	-	n	n	+	n	n	+	-(+)	+	-
Without *myo*-Inositol	n	+	n	n	n	n	+	+	+	n	n	n	+
Without Biotin	n	+	n	n	n	n	+	+	+	n	n	+	+
Without Thiamin	n	+	+	-(+)	+	+	+	+	+	+	n	+	+
Without Biotin & Thiamin	+	n	n	n	n	n	n	n	n	n	n	+	n
Without Pyridoxine	n	+	n	n	n	n	+	+	-(+)	+	n	n	+
Without Thiamin & Pyridoxine	n	n	n	n	n	n	n	n	n	n	n	n	n
Without Niacin	+	+	n	n	n	n	+	+	+	n	n	n	+
Without *P*-aminobenzoate	+	n	n	+	+	+	n	n	n	+	+	n	n
Additional tests													
With 0.01% (w/v) cycloheximide	n	v	v	n	n	n	n	n	n	n	n	n	n
With 0.10% (w/v) cycloheximide	n	n	n	n	n	n	n	n	n	.-	-	n	n
With 1% Acetic Acid	n	-	-	n	n	n	-	-	-	n	n	n	-
With 50% (w/v) D-glucose	n	+	+	n	n	n	+(-)	+	+	n	n	n	+
With 60% (w/v) D-glucose	n	-	-	n	n	n	-	+(-)	-	n	n	n	-
Starch formation	-	n	n	+(-)	+(-)	-	n	n	n	-	-	n	n
Diazonium Blue B Reaction	n	-	n	-	-	v	n	n	n	+	-	n	n

Responses: + = positive; +(-) = mostly positive with some negative; -(+) = mostly negative with some positive; w/- =weak or negtive, w/+ =weak or positive, - = negtive, n=not determined

Table 4. ANOVA of yeast species isolated from slime fluxes of trees.

Source	Sum of squares	df	Mean square	F	Probability
Araucaria cooki					
Main effects					
Yeasts (A)	0.605	1	0.605	30.25	p<0.005
Sample (B)	0.605	1	0.605	30.25	p<0.005
A*B	68.445	1	68.445	3422.25	p<0.001
Error	8.00E-02	4	2.00E-02		
Total	138.180	8			
Azadirachta indica					
Main effects					
Yeasts (A)	60.1105	7	8.586	5648.989	p<0.001
Sample (B)	115.302	9	12.811	8428.494	p<0.001
A*B	876.862	63	13.918	9156.876	p<0.001
Error	0.122	80	1.52E-03		
Total	1205.272	160			
Eucalyptus camaldulensis					
Main effects					
Yeasts (A)	7.823	2	3.911	4107.000	p<0.001
Sample (B)	33.899	6	5.650	5932.333	p<0.001
A*B	83.444	12	6.954	7301.333	p<0.001
Error	2.00E-02	21	9.52E-04		
Total	136.92	42			
Ficus religiosa					
Main effects					
Yeasts (A)	1421.991	25	56.880	59154.820	p<0.001
Sample (B)	1048.527	15	69.902	72697.901	p<0.001
A*B	2620.334	375	6.988	7267.058	p<0.001
Error	0.400	416	9.62E-04		
Total	6201.900	832			

complete their life cycle are responsible for introducing the yeasts to these niches (Phaff & Starmer, 1987). It is interesting that in a survey Hong *et al*., (2003) isolated *Candida kunwiensis*, which is phylogenetically related to the genus *Metschnikowia* from sweet potato (*Ipomoea batatas*) flowers in Korea and from the body surface of pollinating bumblebees in Germany indicating role of insects in the transmission of yeasts.

Physiologically all yeast species assimilated D-glucose and positively grow at temperatures 25°C, 30°C, 35°C, 37°C and 40°C. A great variation in biochemical and physiological tests was observed during identification of yeast species including *Candida succiphila* and *Pichia lynferdii* which were observed to assimilate methanol as sole carbon and energy source in absence of a carbon source. During assimilation, methanol is oxidized to formaldehyde and also generates hydrogen peroxide (H_2O_2) using molecular oxygen by the enzyme alcohol oxidase (AOX), within peroxisomes (to avoid H_2O_2 toxicity from rest of the cell). Since AOX has a poor affinity for oxygen, the methylotrophic yeasts compensate by generating large amounts of the enzyme, which can accumulate to comprise up to 30% of total cell protein (TCP) during induction with methanol (Macauley-Patrick *et al*., 2005). Nowadays, more than 500 proteins of viruses, bacteria, fungi, protists, plants, animals and even of humans have been cloned and expressed using this system in methylotrophic yeasts. Special emphasis have been laid down to produce therapeutic proteins for their potential clinical and biotechnological

applications such as the production of human insulin, interferons, tumor necrosis factor, hormones and bacterial toxins which are causative agents of human diseases such as tetanus, botulism and cholera (Harakuni *et al*., 2005; Macauley-Patrick *et al*., 2005; FitzGerald *et al*., 2004; Smith *et al*., 2004; Byrne & Smith, 2000; Himani *et al*., 2002; Cereghino & Cregg, 2000; Hwang *et al*., 2000; Liu *et al*., 1998; Cregg *et al*., 1993; Scorer *et al*., 1993).

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