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BIODIVERSITY OF YEAST MYCOFLORA IN NECTAR OF *MALVAVISCUS ARBOREUS* AND *PANCRATIUM BIFLORUM* FLOWERS

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Abstract

A total of 11 species belonging to 8 genera of yeasts were isolated from nectar of *Malvaviscus arboreus* and 26 species belonging to 12 genera from nectar of *Pancratium biflorum* flowers. The isolated yeast species were identified on the basis of morphological and physiological / biochemical characters. *Cryptococcus albidus*, *C. laurentii*, *C. macerans*, *Debaryomyces castellii*, *Phaffia rhodozyma* and *Pseudozyma fusiformata* were predominantly isolated from nectar of *Malvaviscus arboreus* and *Pancratium biflorum*.

Introduction

Flowers' nectar has long been thought as an ideal habitat for yeasts because of rich sugar contents; however, the single cell habit allows yeasts to attain a much wider ecological distribution than mycelial forms. We have reported a number of yeast species belonging to several genera from nectar of *Bombax cieba*, *Canna indica*, *Hibiscus rosa-sinensis* and *Ixora coccinea* flowers (Mushtaq *et al*., 2006, 2007). Being devoid of photosynthetic power, yeasts depend strictly on the presence of organic carbon as an energy and carbon source. Apart from nectar, we have also recorded several yeast species belonging various genera from butter (Mushtaq *et al*., 2007), milk and yogurt (Mushtaq *et al*., 2006), slime fluxes of trees (Mushtaq *et al*., 2005) and from cultivated and garden soil (Mushtaq *et al*., 2004). In the present study, efforts has been made to isolate and identify yeast mycoflora associated with nectar of *Malvaviscus arboreus* and *Pancratium biflorum* flowers.

Materials and Methods

Nine samples of nectar from *Malvaviscus arboreus* and 16 from *Pancratium biflorum* flowers were collected from University of Karachi, Pakistan. Yeasts associated with these samples were isolated by modified serial dilution method as described earlier (Mushtaq *et al*., 2007; Harrigan & McCance, 1976) and further purified and maintained on yeast-morphology agar buffered at pH 4.5. 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 out 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 & 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 positive growth in liquid medium of a sugar or an alditol (Cowan & Steel, 1966). Diazonium Blue B (DBB) test was performed 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 15 genera and 34 yeast species were isolated from nectar of *Malvaviscus arboreus* and *Pancratium biflorum* flowers (Table 1). 11 species belonging to 8 enera isolated from 9 samples of *Malvaviscus arboreus* and 26 species belonging to 12 genera from 16 samples of *Pancratium biflorum* and identified on the basis of their morphological (Table 2) and physiological / biochemical characters (Table 3).

Out of 34 yeast species, teleomorphic ascomycetous yeasts were identified as *Debaryomyces castellii*, *D. hansenii*, *D. vanrijii*, *Issatchenkia occidentalis*, *Lipomyces starkeyi*, *Pichia angusta*, *P. fabianii*, *P. jadinii*, *P. lynferdii*, *Williopsis californica* and *W. pratensis*, whereas among anamorphic ascomycetous yeasts only species of *Candida* viz., *Candida gropengiesseri*, *C. rhagii*, *C. succiphila*, *C. valdiviana* and *C. xestobii* were isolated and identified. On the other hand among teleomorphic basidiomycetous yeasts, *Cystofilobasidium bisporidii*, *Fibulobasidium inconspicuum*, *Mrakia frigida*, *Rhodosporidium toruloides* and *Sporidiobolus ruineniae* were identified and among anamorphic basidiomycetous yeasts *Bullera pseudoalba*, *B. pyricola*, *Cryptococcus albidus*, *C. curvatus*, *C. flavus*, *C. heveanensis*, *C. humicolus*, *C. hungaricus*, *C. laurentii*, *C. macerans*¸ *Phaffia rhodozyma*, *Pseudozyma Antarctica* and *P. fusiformata* were identified. All yeast species appeared newly reported from nectar of *Malvaviscus arboreus* and *Pancratium biflorum* flowers in Pakistan. Univariate ANOVA of yeast species revealed that their occurrences were significantly different at $p<0.001$ in nectars' samples of both *Malvaviscus arboreus* and *Pancratium biflorum* flowers (Table 4). Bonferroni test also confirmed significant differences among yeast species (Table 1).

Bullera pyricola, *Cryptococcus laurentii* and *Pichia angusta* were commonly isolated from nectar of both *Malvaviscus arboreus* and *Pancratium biflorum* flowers. *Cryptococcus macerans*, *Phaffia rhodozyma* and *Pseudozyma fusiformata* were predominantly isolated from nectar of *Malvaviscus arboreus*, whereas *Cryptococcus albidus*, *C. laurentii* and *Debaryomyces castellii* were predominant in nectar of *Pancratium biflorum* (Table 1).

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

No.	Yeast species	*Malvaviscus arboreus*		*Pancratium biflorum*	
		Occ. %	*mcfu±se **(range)	Occ. %	*mcfu±se **(range)
1.	*Bullera pseudoalba*	----	---- ----	12.5	0.45 ±0.31[b] (3.4-3.8)
2.	*B. pyricola*	33.3	2.53 ± 2.53[a] (7.6)	18.8	1.18 ± 0.63[a] (5.7-7.0)
3.	*Candida gropengiesseri*	----	---- ----	6.3	0.24 ± 0.20[c] (3.8)
4.	*C. rhagii*	----	---- ----	6.25	0.23 ± 0.23[c] (3.7)
5.	*C. succiphila*	----	---- ----	18.8	1.25 ± 0.68[d] (5.8-8.3)
6.	*C. valdiviana*	----	---- ----	6.3	0.37 ± 0.37[e] (5.9)
7.	*C. xestobii*	----	---- ----	6.3	0.39 ± 0.39[e] (6.3)
8.	*Cryptococcus albidus*	----	---- ----	68.8	3.09 ± 0.68[f] (2.3-8.1)
9.	*C. curvatus*	----	---- ----	12.5	1.22 ± 0.84[g] (5.2-8.2)
10.	*C. flavus*	33.3	1.90 ± 1.90[b] (5.7)	----	---- ----
11.	*C. heveanensis*	33.3	2.20 ± 2.20[c] (6.6)	----	---- ----
12.	*C. humicolus*	----	---- ----	6.3	0.25 ± 0.25[h] (2.7)
13.	*C. hungaricus*	----	---- ----	12.5	0.71 ± 0.55[i] (2.8-8.6)
14.	*C. laurentii*	33.3	2.03 ± 2.03[d] (6.1)	100	5.76 ± 0.57[J] (2.7-8.6)
15.	*C. macerans*	100	6.47 ± 2.13[e] (2.3-9.3)	----	---- ----
16.	*Cystofilobasidium bisporidii*	33.3	2.32 ± 2.32[f] (7.0)	----	---- ----
17.	*Debaryomyces castellii*	----	---- ----	56.3	2.92 ± 0.74[k] (3.1-8.6)
18.	*D. hansenii*	33.3	1.90 ± 1.90[b] (5.7)	----	---- ----
19.	*D. vanrijii*	----	---- ----	6.3	0.48 ± 0.48[L] (7.8)
20.	*Fibulobasidium inconspicuum*	----	---- ----	25.0	1.69 ± 0.79[m] (3.8-7.8)
21.	*Issatchenkia occidentalis*	----	---- ----	6.3	0.54 ± 0.54[n] (8.7)
22.	*Lipomyces starkeyi*	----	---- ----	6.3	0.38 ± 0.38[e] (6.1)
23.	*Mrakia frigida*	33.3	1.87 ± 2.87[b] (5.)	----	---- ----
24.	*Phaffia rhodozyma*	66.7	4.94 ± 2.48[g] (7.0-7.8)	----	---- ----
25.	*Pichia angusta*	33.3	1.73 ± 1.73[h] (5.2)	25.0	1.73 ± 0.82[o] (3.9-9.7)
26.	*P. fabianii*	----	---- ----	6.3	0.18 ± 0.18[h] (2.8)
27.	*P. jadinii*	----	---- ----	6.3	0.49 ± 0.49[L] (7.8)
28.	*P. lynferdii*	----	---- ----	6.3	4.07 ± 0.88[p] (4.0-9.1)
29.	*Pseudozyma antarctica*	----	---- ----	6.3	0.24 ± 0.24[c] (3.8)
30.	*P. fusiformata*	66.7	3.36 ± 2.31[a] (2.3-7.8)	----	---- ----
31.	*Rhodosporidium toruloides*	----	---- ----	25.0	0.36 ± 0.36[e] (5.7)
32.	*Sporidiobolus ruineniae*	----	---- ----	31.3	2.14 ± 0.88[q] (2.7-8.6)
33.	*Williopsis californica*	----	---- ----	6.3	0.49 ± 0.49[L] (7.8)
34.	*W. pratensis*	----	---- ----	6.3	0.29 ± 0.29[r] (4.7)

* 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).

The total colony forming units (cfu g^{-1}) of yeasts ranged from $1.73x10^4$ g^{-1} (*Pichia angusta*) to $6.47x10^4$ g^{-1} (*Cryptococcus macerans*) in nectar of *Malvaviscus arboreus* flowers and $0.18x10^4$ g^{-1} (*Pichia fabianii*) to $5.76x10^4$ g^{-1} (*Cryptococcus laurentii*) in nectar of *Pancratium biflorum* flowers (Table 1). It is clear that the number of yeast species isolated from nectar of *Pancratium biflorum* were exceptionally higher than yeast species isolated from *Malvaviscus arboreus*. Furthermore it may be noted that all species of *Candida*, *Cystofiloabasidium bisporidii*, *Filobasidium inconspicuum*, *Issatchenkia occidentalis*, *Lipomyces starkeyi*, all species of *Pichia* except *P. angusta*, *Rhodosporidium toruloides*, *Sporidiobolus ruineniae*, *Williopsis californica* and *W. pratensis* were isolated only from nectar of *Pancratium biflorum* flowers (Table 1).

Table 2. Morphological characteristics of yeast species.

No.	Yeast species	Group	Colony color	Shape of cell	Pseudomycelium	Septate hyphae	Ballistoconidia	Symmetric conidia	Ascospores round, oval, conical or reniform	Ascospres cap-, hat-, Saturnm- or walnut shaped
1.	*Bullera pseudoalba*	E	wh.cr.	eli	+	+	+	+	-	-
2.	*B. pyricola*	E	wh.cr.br.yl.	ov-cy	+	-	+	+	-	-
3.	*Candida gropengiesseri*	G	wh.cr.	ov	-	-	-	-	-	-
4.	*C. rhagii*	G	wh.cr.	gl-ov	+	-	+	+	-	-
5.	*C. succiphila*	B,G	wh.cr.	sgl-gl.	-	-	+	+	-	-
6.	*C. valdiviana*	G	wh.cr.	gl-ov	+	-	+	+	-	-
7.	*C. xestobii*	G	wh.cr.	r-ov	-	-	+	+	-	-
8.	*Cryptococcus albidus*	F	cr.	gl-ov	-	-	-	-	-	-
9.	*C. curvatus*	F	br.yl.	ov	+	-	-	-	-	-
10.	*C. flavus*	F	br.yl.	ov	-	-	-	-	-	-
11.	*C. heveanensis*	F	cr.	ov-elo	-	-	-	-	-	-
12.	*C. humicolus*	F	ye.-tan.	ov-le	+	+	-	-	-	-
13.	*C. hungaricus*	F	red-or.	gl-ov	-	-	-	-	-	-
14.	*C. laurentii*	F	pi.	ph-ov	-	-	-	-	-	-
15.	*C. macerans*	A,F	cr.	gl-ov		-	-	-	-	-
16.	*Cystofilobasidium bisporidii*	A	cr.	sgl-elo	+	+	-	-	-	-
17.	*Debaryomyces castellii*	D	or.-pi.	r-ov	-	-	-	-	+	-
18.	*D. hansenii*	D	wh.cr.	r-ov	-	-	-	-	+	-
19.	*D. vanrijii*	D	wh.cr.	r-ov	-	-	-	-	+	-
20.	*Fibulobasidium inconspicuum*	F,G	wh.cr.	sgl-gl.	-	-	-	-	-	-
21.	*Issatchenkia occidentalis*	F	wh.	sph-ov	-	-	-	-	+	-
22.	*Lipomyces starkeyi*	D	wh.cr.	sph-ov	-	-	-	-	+	-
23.	*Mrakia frigida*	F	wh.cr.	ov-elo	+	-	+	+	-	-
24.	*Phaffia rhodozyma*	F,G	pi.-red	ov-elo	-	-	-	-	-	-
25.	*Pichia angusta*	B,F	wh.cr.	sph-ov	-	-	-	-	-	+
26.	*P. fabianii*	G	wh.cr.	r-ov	-	-	-	-	-	+
27.	*P. jadinii*	C	wh.cr.	sph-ov	-	-	-	-	-	+
28.	*P. lynferdii*	G	wh.cr.	sph-ov	-	-	-	-	-	+
29.	*Pseudozyma antarctica*	G	wh.cr.	cly.ely	-	+	-	-	-	-
30.	*P. fusiformata*	F	wh.cr.	cly.ely	-	+	-	-	-	-
31.	*Rhodosporidium toruloides*	A	pi.-red	sph-elo	+	-	-	-	-	-
32.	*Sporidiobolus ruineniae*	A	pi.	ov-cy	-	+	-	-	-	-
33.	*Williopsis californica*	G	gr.wh.	r-ov	-	-	-	-	-	+
34.	*W. pratensis*	G	t.wh.	r-ov	-	-	-	-	+	-

shape of cell: r=round; ov=oval; gl=globose; sgl= sub globose; sph=spherical; elo=elongated; eli=eliptical; cyl=cylindrical; le=lemon

colony color: wh=white; cr=cream, yl=yellow; br=brown; gr=gray; br=bright; cr-tan= tan; or=orange; pi=pink; t.wh=tanish white.

A number of yeast species isolated during this study have already been recorded in the previous studies from nectar of *Bombax cieba*, *Canna indica*, *Hibiscus rosa-sinensis* and *Ixora coccinea* flowers (Mushtaq *et al*., 2007, Mushtaq *et al*., 2006). However the yeast species including *Candida gropengiesseri*, *C. xestobii*, *Cryptococcus heveanensis*, *C. humicolus*, *C. hungaricus*, *C. macerans*, *Cystofilobasidium bisporidii*, *Issatchenkia occidentalis*, *Lipomyces starkeyi*, *Pichia fabianii* and *Williopsis pratensis* have not been reported earlier from nectar of flowers, and appeared to be newly reported from nectar of *M. arboreus* and *P. biflorum* flowers in Pakistan.

Table 4. ANOVA of yeast species isolated from flowers' nectar.

Malvaviscus arboreus					
Source	**Sum of squares**	**df**	**Mean square**	**F**	**Probability**
Main effects					
Yeasts (A)	1421.991	10	13.771	-1.3E+30	p<0.001
Sample (B)	1048.527	2	200912	2.85E-288	p<0.001
A*B	2620.334	20	11.057	-1.15E+09	p<0.001
Error	0.400	33	1.212.367		
Total	**6201.900**	**66**			
Pencratium biflorum					
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**			

The similarity of occurrence of large number of yeast species is due to their transmission carried out by insects including drosophilids, beetles and bees that visit these flowers especially for sucking the flower's nectar (Lachance *et al*., 2001b). 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 resulted in the identification of a large number of yeast species, where some of the species viz., *Candida succiphila*, *Pichia angusta* and *P. lynferdii* were also found 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, harmones, bacterial toxins (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 and Smith, 2000; Himani *et al*., 2002; Cereghino and Cregg, 2000; Hwang *et al*., 2000; Liu *et al*., 1998; Cregg *et al*., 1993; Scorer *et al*., 1993).

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