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BIODIVERSITY OF YEAST MYCOFLORA IN NECTAR OF *HIBISCUS ROSA-SINENSIS* AND *IXORA COCCINEA* FLOWERS

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Abstract

A total of 17 species belonging to 10 genera were isolated from 11 samples of nectar of *Hibiscus rosa-sinensis* and 10 species belonging to 7 genera from 5 samples of nectar of *Ixora coccinea* flowers. The isolated yeast species were identified on the basis of morphological and physiological/biochemical characters. *Bullera pyricola* was predominantly isolated from nectar of *Hibiscus rosa-sinensis*.

Introduction

Flowers' nectar has long been thought as an ideal habitat for yeasts because of rich sugar contents and for many years yeasts were not believed to occur elsewhere. However, the single cell habit allows yeasts to attain a much wider ecological distribution than mycelial forms. Being devoid of photosynthetic power, yeasts depend strictly on the presence of organic carbon as an energy and carbon source. Species of *Saccharomyces* and *Pichia* that assimilate few carbon compounds are abundant in fruit juices and sugary plant exudates such as flowers' nectar (Rose & Harrison, 1987). *Candida ipomoeae* and several species of *Metschnikowia* such as *M. hawaiiensis, M. continentalis, M. drosophilae* and *M. lochheadii* are known to occur on morning glories and insects found on these flowers (Lachance *et al*., 1998b; Lachance *et al*., 1990, Lachance *et al*., 2001a,b). There are about 100 genera and 700 species of yeasts (Kurtzman & Fell, 1999) of which Mirza & Qureshi (1978) enlisted only 5 genera and 7 species from Pakistan. In a recent study we have reported 28 yeast species belonging to 13 genera from sample of butter (Mushtaq *et al*., 2007), 16 yeast species belonging to 10 genera from nectar of *Bombax cieba* and 25 species belonging to 15 genera from nectar of *Canna indica* flowers (Mushtaq *et al*., 2006b). In other studies we recorded 35 yeast species belonging to 14 genera from milk, 16 species and 9 genera from yogurt (Mushtaq *et al*., 2006a), 15 species and 9 genera from slime fluxes of trees (Mushtaq *et al*., 2005), 4 genera and 5 species from cultivated soil and 16 species and 12 genera from garden soil (Mushtaq *et al*., 2004). In the present study, an effort has been made to isolate and identify yeast mycoflora associated with nectar of *Hibiscus rosa-sinensis* and *Ixora coccinea* flowers.

Materials and Methods

Eleven samples of nectar from *Hibiscus rosa-sinensis* and 5 from *Ixora coccinea* flower were collected from plants growing at the University of Karachi, Pakistan. Yeasts

associated with these samples were isolated by modified serial dilution method (Harrigan & McCance, 1976). A known amount of sample was diluted up to 10,000 using double distilled sterilized water 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°C. 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. 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 from 2-3 days old cultures growing on YM (malt-yeast-glucose-peptone) agar, 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). Ballistoconidia 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. In all tests, media and reagents were prepared in double distilled sterilized water, and filter-sterilized through 0.45µ filter paper using Millipore glass filtration apparatus. 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 made 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 12 genera and 22 yeast species were isolated from nectar of *Hibiscus rosa-sinensis* and *Ixora coccinea* flowers (Table 1) where 17 species belonging to 10 genera were isolated from 11 samples of *Hibiscus rosa-sinensis* and 10 species belonging to 7 genera from 5 samples of *Ixora coccinea* and identified on the basis of their morphological (Table 2) and physiological/biochemical characters (Table 3). *Bullera pyricola* was predominantly isolated from nectar of *Hibiscus rosa-sinensis*.

Out of 22 yeast species, teleomorphic ascomycetous yeasts were identified as *Debaryomyces castellii*, *D. hansenii*, *Pichia angusta*, *P. jadinii*, *P. lynferdii*, *P. methanolica*, *P. ofunaensis* and *Saccharomyces kluyveri*, whereas among anamorphic ascomycetous yeasts only two species of *Candida* viz., *Candida magnoliae* and *C. succiphila*, were isolated and identified. On the other hand among teleomorphic basidiomycetous yeasts, *Mrakia frigida*, *Rhodosporidium toruloides* and *Sporidiobolus*

ruineniae were identified and among anamorphic basidiomycetous yeasts *Bensingtonia miscanthi*, *Bullera megalospora*, *B. pyricola*, *B. pseudoalba*, *Cryptococcus albidus*, *C. curvatus*, *C. laurentii*, *Phaffia rhodozyma Rhodotorula fragaria* and *R. hinnulea* were identified. All yeast species appear to be newly reported from nectar of *Hibiscus rosa-sinensis* and *Ixora coccinea* flowers in Pakistan. Univariate ANOVA of yeast species revealed that their occurrence was significantly different at $p<0.0001$ in nectars' samples of both *Hibiscus rosa-sinensis* and *Ixora coccinea* flowers (Table 4). Bonferroni test also confirmed significant differences among yeast species (Table 1).

Mrakia frigida, *Pichia angusta*, *P. lynferdii*, *P. methanolica* and *Sporidiobolus ruineniae* were commonly isolated from nectar of *Hibiscus rosa-sinensis* and *Ixora coccinea* flowers. *Bullera pyricola* was predominantly isolated from nectar of *Hibiscus rosa-sinensis*. In a survey Lachance *et al*., (1999) identified two new ascomycetous yeast species *viz*., *Kodamaea kakaduensis* and *Candida tolerans*, from Australian Hibiscus flowers.

Table 1. Occurrence of yeast mycoflora in terms of mean colony forming units (mcfu) with standard error (se) and range, isolated from nectar of *Hibiscus rosa-sinensis* and *Ixora coccinea* flowers.

No.	Yeast species	*Hibiscus rosa-sinensis*		*Ixora coccinea*	
		Occurrence %	*mcfu±se **(range)	Occurrence %	*mcfu±se **(range)
1.	*Bensingtonia miscanthi*	----	---- ----	20.0	0.76 ± 0.76^{a} (3.80)
2.	*Bullera megalospora*	9.1	0.55 ± 0.55^{a} (6.1)	----	---- ----
3.	*B. pyricola*	54.5	3.25 ± 1.09^{b} (2.6-8.3)	----	---- ----
4.	*Candida magnoliae*	9.1	0.46 ± 0.46^{c} (5.1)	----	---- ----
5.	*C. succiphila*	----	---- ----	20.0	1.84 ± 1.15^{b} (4.60)
6.	*Cryptococcus albidus*	9.1	0.33 ± 0.33^{d} (3.6)	----	---- ----
7.	*C. curvatus*	9.1	0.54 ± 0.54^{a} (6.0)	----	---- ----
8.	*C. laurentii*	9.1	0.87 ± 0.87^{e} (9.6)	----	---- ----
9.	*Debaryomyces castellii*	18.2	0.89 ± 0.67^{e} (2.6-7.2)	----	---- ----
10.	*D. hansenii*	18.2	1.44 ± 0.98^{f} (6.7-9.1)	----	---- ----
11.	*Mrakia frigida*	9.1	0.46 ± 0.46^{c} (5.1)	20.0	1.56 ± 1.56^{c} (7.80)
12.	*Phaffia rhodozyma*	----	---- ----	20.0	1.12 ± 1.12^{d} (5.60)
13.	*Pichia angusta*	27.3	2.69 ± 1.72^{g} (7.8-8.3)	20.0	0.76 ± 0.76^{a} (3.82)
14.	*P. jadinii*	18.2	1.16 ± 0.76^{h} (3.1-7.3)	----	---- ----
15.	*P. lynferdii*	18.2	1.16 ± 0.79^{h} (5.1-8.3)	20.0	0.36 ± 0.36^{e} (1.80)
16.	*P. methanolica*	18.2	1.54 ± 1.03^{i} (7.8-9.1)	20.0	1.12 ± 1.12^{d} (5.60)
17.	*P. ofunaensis*	----	---- ----	20.0	0.92 ± 0.92^{b} (4.60)
18.	*Rhodosporidium toruloides*	9.1	0.33 ± 0.33^{d} (3.6)	----	---- ----
19.	*Rhodotorula fragaria*	----	---- ----	20.0	1.46 ± 1.46^{f} (7.30)
20.	*R. hinnulea*	27.3	2.03 ± 1.14^{J} (3.6-9.6)	----	---- ----
21.	*Saccharomyces kluyveri*	9.1	0.34 ± 0.34^{d} (3.8)	----	---- ----
22.	*Sporidiobolus ruineniae*	18.2	0.85 ± 0.58^{e} (3.6-5.7)	20.0	0.58 ± 0.58^{g} (2.69)

*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 nectar of *Hibiscus rosa-sinensis* and *Ixora coccinea* flowers.

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. *Bensingtonia miscanthi*	E	gr.cr.	ov-cy	-	-	+	+	-	-
2. *Bullera megalospora*	E	gr.cr.	eli	-	+	+	+	-	-
3. *B. pyricola*	E	wh.cr.br.yl.	ov-cy	+	-	+	+	-	-
4. *Candida magnoliae*	G	wh.cr.	gl-ov	-	-	-	-	-	-
5. *C. succiphila*	B	wh.cr.	sgl-gl.	-	-	+	+	-	-
6. *Cryptococcus albidus*	F	cr.	gl-ov	-	-	-	-	-	-
7. *C. curvatus*	F	br.yl.	ov	+	-	-	-	-	-
8. *C. laurentii*	F	pi.	sph-ov	-	-	-	-	-	-
9. *Debaryomyces castellii*	D	or.-pi.	r-ov	-	-	-	-	+	-
10. *D. hansenii*	D	wh.cr.	r-ov	-	-	-	-	+	-
11. *Mrakia frigida*	F	wh.cr.	ov-elo	+	-	+	+	-	-
12. *Phaffia rhodozyma*	F	pi.-red	ov-elo	-	-	-	-	-	-
13. *Pichia angusta*	D	wh.cr.	sph-ov	-	-	-	-	-	+
14. *P. jadinii*	C	wh.cr.	sph-ov	-	-	-	-	-	+
15. *P. lynferdii*	G	wh.cr.	sph-ov	-	-	-	-	-	+
16. *P. methanolica*	G	wh.cr.	sph-ov	-	-	-	-	-	+
17. *P. ofunaensis*	C	wh.-tan.	sph-ov	-	-	-	-	-	-
18. *Rhodosporidium toruloides*	A	pi.-red	sph-elo	+	-	-	-	-	-
19. *Rhodotorula fragaria*	F	wh.cr.	ov-cy	+	-	-	-	-	-
20. *R. hinnulea*	F	wh.cr.	ov	-	-	-	-	-	-
21. *Saccharomyces kluyveri*	F	wh.cr.	sph.ov	+	-	-	-	+	-
22. *Sporidiobolus ruineniae*	A	pi.	ov-cy	-	+	-	-	-	-

colony color: wh=white; cr=cream, yl=yellow; br=brown; gr=gray; br=bright; cr-tan= cream to tan; or=orange; pi=pink; t.wh=tanish white.
shape of cell: r=round; ov=oval; gl=globose; sgl= sub globose; sph=spherical; elo=elongated; eli=eliptical; cyl=cylindrical; le=lemon

The total colony forming units (cfu g^{-1}) of yeasts ranged from $0.33x10^4$ g^{-1} (*Cryptococcus albidus* and *Rhodosporidium toruloides*) to $3.25x10^4$ g^{-1} (*Bullera pyricola*) in nectar of *Hibiscus rosa-sinensis* flowers and $0.36x10^4$ g^{-1} (*Pichia lynferdii*) to $1.84x10^4$ g^{-1} (*Candida succiphila*) in nectar of *Ixora coccinea* flowers (Table 1). It may be noted that distribution of yeast species in nectar of *Ixora coccinea* was found only in one sample (Table 1) but showed extensive physiological activities (Table 3).

Table 4. ANOVA of yeast species isolated from nectar of *Hibiscus rosa-sinensis* and *Ixora coccinea* flowers.

Source	Sum of Squares	df	Mean Square	F	Probability
Main effects					
	Hibiscus rosa-sinensis				
Yeasts (A)	259.949	16	16.228	13793.84	p<0.0001
Sample (B)	864.38	10	86.438	73472.313	p<0.0001
A*B	1300.006	160	8.125	6906.282	p<0.0001
Error	0.22	187	1.18E-03		
Total	2858.331	374			
	Ixora coccinea				
Yeasts (A)	12.803	9	1.423	3556.366	p<0.0001
Sample (B)	210.217	4	52.554	131385.8	p<0.0001
A*B	203.668	36	5.657	14143.638	p<0.0001
Error	2.00E-02	50	4.00E-04		
Total	517.377	100			

Yeast species *viz*., *Bullera megalospora*, *B. pyricola*, *Cryptococcus albidus*, *C. laurentii*, *Debaryomyces castellii*, *D. hansenii*, *Mrakia frigida*, *Pichia lynferdii*, *Rhodosporidium toruloides*, *Rhodotorula fragaria* isolated from nectar of *Hisbiscus rosa-sinensis* and *Ixora coccinea* were common to that previously isolated from nectar of *Bombax cieba* and *Canna indica* flowers (Mushtaq *et al*., 2006b). Whereas, a number of yeast species such as *Bensingtonia miscanthi*¸ *Candida magnoliae*, *C. succiphila*, *Cryptococcus curvatus*, *Phaffia rhodozyma*, *Pichia angusta*, *P. jadinii*, *P. methanolica*, *P. ofunaensis*, *Rhodotorula hinnulea*, *Saccharomyces kluyveri* and *Sporidiobolus ruineniae* appear to be newly reported from nectar of *Hisbiscus rosa-sinensis* and *Ixora coccinea* and were not found in nectar of *Bombax cieba* and *Canna indica* flowers (Mushtaq *et al*., 2006b). On the other hand *Bullera pyricola, Candida succiphila, Cryptococcus albidus, Debaryomyces castellii, D. hansenii, Mrakia frigida*, *Phaffia rhodozyma*, *Pichia angusta*, *P. jadinii, P. lynferdii*, *P. methanolica*, *Rhodosporidium toruloides* and *Sporidiobolus ruinenae* have been already reported from slime fluxes of *Accacia nilotica*, *Albizzia lebbeck* and *Aralia cachemirica* (Mushtaq *et al*., 2005) and from soil (Mushtaq *et al*., 2004). It is established that transmission of yeast species from nectar of a flower to another or to other substrates such as slime fluxes of trees and soil is carried out by insects including drosophilids, beetles and bees, found on these flowers (Lachance *et al*., 2001b). In a survey Hong *et al*., (2003) isolated a novel asexual ascomycetous yeast, *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.

All yeast species assimilated D-glucose and positively grow at temperatures 25°C, 30°C, 35°C, 37°C and 40°C. During assimilation tests, *Candida succiphila*, *Pichia angusta*, *P. lynferdii*, *P. methanolica* and *P. ofunaensis* appeared to utilized methanol aerobically. Methylotrophic yeasts can assimilate methanol (or alkanes) as sole carbon and energy source in absence of a carbon source such as glucose and glycerol (FitzGerald *et al*., 2004; Cereghino & Cregg, 2000). 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 on their potential clinical and biotechnological applications such as the production of interferons active against viruses (eg. dengue, HIV and influenza) and bacteria, production of tumor necrosis factor, harmones-somatostatin, bacterial toxins (causative agents of human diseases such as tetanus, botulism and cholera) and their derivatives (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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