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DETECTION OF YEAST MYCOFLORA FROM BUTTER

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Abstract

Thirteen genera and 28 yeast species belonging to teleomorphic and anamorphic, ascomycetous and basidiomycetous yeasts were isolated from samples of butter, and identified on the basis of morphological and physiological/biochemical characteristics. All yeast species appear to be newly reported from butter in Pakistan. The predominant yeast species in the samples of butter were *Debaryomyces vanrijii*, *Pichia lynferdii* and *P. anomala*.

Introduction

Dairy products are favourable environment for growth of yeasts due to their acidic pH (Wickerham, 1966; Rose & Harrison, 1987). Species of *Candida*, *Debaryomyces*, *Galactomyces*, *Fellomyces*, *Pichia* and *Saccharomyces* have been isolated from different dairy products (Wickerham, 1966; Lodder & Kreger-van Rij, 1952; Van Uden & Windisch, 1968; Savova & Nikolova, 2000-2002). There are about 100 genera and 700 species of yeasts (Kurtzman & Fell, 1999) of which only 5 genera and 7 species were enlisted from Pakistan (Mirza & Qureshi, 1978).

In a previous study we isolated and identified 35 yeast species belonging to 14 genera from milk and 16 species belonging to 9 genera from samples of yogurt with predominant occurrence of *Bullera pyricola*¸ *Candida succiphila*, *Debaryomyces castellii*, *D. hansenii* and *Pichia angusta* (Mushtaq *et al*., 2006). We have also isolated and identified a number of yeast species from nectar of flower's (Mushtaq *et al*., 2006), slime fluxes of trees (Mushtaq *et al*., 2005) and soil (Mushtaq *et al*., 2004). The aim of this study was the isolation and taxonomic characterization of yeast species from butter collected from Karachi regions of Pakistan.

Materials and Methods

Twenty-one samples of butter were collected from various localities of Karachi, Pakistan and the 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 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 was used to test the ability of yeast to produce pseudo- or true-hyphae and ballisto- or arthro-conidia. Thin layers of sterile corn meal agar were poured in sterilized Petri plates and dried at room temperature for 2 days before streaking with up to 4 cultures per plate. A sterile cover slip was placed over a part of each streak. After 3-5 days of incubation filamentous growth was observed in the aerobic and anaerobic (covered) portions of the streak (Beech *et al*., 1972). To observe ballistoconida formation, malt extract agar (10 to 15 ml) was poured in Petri plates and dried at room temperature for 2 days. The medium was then inoculated with the yeast to be tested in lines along two diameters at right angles. This inoculated plate was inverted over another Petri plate having a sterile microscope glass slide on the surface of medium. The two plates were taped together all round the circumference and the preparation was incubated up to 3 weeks at 20°C. Discharged ballistoconidia that either formed colonies on medium in the lower Petri plate or collected on the slide were examined microscopically (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, 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 yeast 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 after a positive growth in liquid medium of a sugar or an alditol. One drop of Lugol's iodine solution was shaken with yeast culture in the tube. A blue, purple or green colour indicated that the test result is positive (Cowan & Steel, 1966). Diazonium Blue B (DBB) test was tested on 10-days old culture growing on malt-yeast-glucose-peptone agar. The culture was kept at 55°C for several hrs. and then flooded with ice-cold DBB reagent. If the culture turned dark red within 2 min at room temperature, the result was recorded as positive (Van der Walt & Hopsu-Havu, 1976).

Results and Discussion

Twenty eight yeast species belonging to 13 genera were isolated and identified from samples of butter and presented in terms of mean value of colony forming unit (cfu) with standard error and range (Table 1). Identification of yeasts up to species level was carried out on the basis of their morphological (Table 2) and physiological/biochemical characteristics (Table 3). Teleomorphic ascomycetous species were identified as *Debaryomyces castellii*, *D. hansenii*, *D. nepalensis*, *D. vanrijii*, *Kluyveromyces polysporus*, *Pichia angusta*, *P. anomala*, *P. euphorbiaphila*, *P. guilliermondii*, *P. jadinii*,

P. lynferdii, *P. methanolica*, *P. mexicana*, *P. ohmeri*, *P. strasburgensis*, *Stephanoascus ciferrii* and *Williopsis californica*, whereas, anamorphic ascomycetes included only 3 species of *Candida* viz., *C. friedrichii*, *C. valdiviana* and *C. xestobii*.

Table 1. Occurrence of yeast mycoflora in terms of mean colony forming units (Mcfu) with standard error (se) and range, isolated from butter.

No.	Yeast species	Occurrence %	*Mcfu ± se **(range)
1.	*Bensingtonia intermedia*	4.8	13.71 ± 13.71^{a} (96.0)
2.	*B. naganoensis*	4.8	0.73 ± 0.73^{b} (5.1)
3.	*Bullera pyricola*	4.8	2.57 ± 1.67^{c} (9.1)
4.	*Candida friedrichii*	4.8	0.70 ± 0.67^{b} (4.9)
5.	*C. valdiviana*	9.5	3.16 ± 2.82^{d} (2.1-20.0)
6.	*C. xestobii*	4.8	1.59 ± 1.59^{e} (11.1)
7.	*Debaryomyces castellii*	14.3	6.11 ± 4.20^{f} (9.3-30.1)
8.	*D. hansenii*	9.5	1.05 ± 0.93^{g} (0.8-6.6)
9.	*D. nepalensis*	4.8	0.62 ± 0.62^{h} (4.4)
10.	*D. vanrijii*	14.3	2.46 ± 1.18^{i} (4.5-6.4)
11.	*Fibulobasidium inconspicuum*	4.8	0.30 ± 0.30^{J} (2.1)
12.	*Filobasidiella neoformans*	4.8	0.40 ± 0.40^{J} (2.8)
13.	*Filobasidium uniguttulatum*	4.8	1.19 ± 1.19^{h} (8.3)
14.	*Kluyveromyces polysporus*	4.8	1.10 ± 1.10^{g} (7.7)
15.	*Phaffia rhodozyma*	4.8	1.01 ± 1.01^{g} (7.1)
16.	*Pichia angusta*	9.5	1.93 ± 1.43^{k} (3.5-10.0)
17.	*P. anomala*	14.3	0.84 ± 0.84^{L} (5.9)
18.	*P. euphorbiaphila*	4.8	11.46 ± 11.46^{m} (80.2)
19.	*P. guilliermondii*	9.5	1.56 ± 1.10^{e} (3.40-7.5)
20.	*P. jadinii*	4.8	1.15 ± 1.15^{g} (8.1)
21.	*P. lynferdii*	23.8	13.48 ± 11.16^{n} (0.6-80.1)
22.	*P. methanolica*	4.8	1.14 ± 1.14^{g} (10.1)
23.	*P. mexicana*	4.8	0.73 ± 0.73^{b} (5.1)
24.	*P. ohmeri*	4.8	0.47 ± 0.47^{J} (3.1)
25.	*P. strasburgensis*	4.8	0.73 ± 0.73^{b} (5.1)
26.	*Sporobolomyces tsugae*	4.8	1.01 ± 1.01^{g} (7.1)
27.	*Stephanoascus ciferrii*	9.5	0.80 ± 0.72^{L} (.5-5.1)
28.	*Williopsis californica*	4.8	1.09 ± 1.09^{g} (7.6)

* 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 characters of yeast species isolated from butter.

No.	Yeast species	Group	Colony color	Shape of cell	Pseudomycelium	Septate hyphae	Ballistoconidia	Symmetric conidia	Ascospores round, oval, conical or reniform	Ascospres cap-, hat-, Saturn- or walnut shaped
1.	*Bensingtonia intermedia*	F	wh.cr.	ov-cy	+	+	+	+	-	-
2.	*B. naganoensis*	E	gr.cr.	ov-ely	-	-	+	+	-	-
3.	*Bullera pyricola*	E	wh.cr.br.yl.	ov-cy	+	-	+	+	-	-
4.	*Candida friedrichii*	G	wh.cr.	ov-cy	+	-	+	+	-	-
5.	*C. valdiviana*	B	wh.cr.	gl-ov	+	-	+	+	-	-
6.	*C. xestobii*	G	wh.cr.	r-ov	-	-	+	+	-	-
7.	*Debaryomyces castellii*	A	or.-pi.	r-ov	-	-	-	-	+	-
8.	*D. hansenii*	D	wh.cr.	r-ov	-	-	-	-	+	-
9.	*D. nepalensis*	D	wh.cr.	r-ov	-	-	-	-	+	-
10.	*D. vanrijii*	D	wh.cr.	r-ov	-	-	-	-	+	-
11.	*Fibulobasidium inconspicuum*	G	wh.cr.	sgl-gl.	-	-	-	-	-	-
12.	*Filobasidiella neoformans*	F	wh.cr.	sgl-gl.	v	-	-	-	-	-
13.	*Filobasidium uniguttulatum*	F	wh.-tan.	sgl-gl.	v	-	-	-	-	-
14.	*Kluyveromyces polysporus*	G	cr.	sph-ov	-	-	-	-	+	-
15.	*Phaffia rhodozyma*	F	Pi.-Red	ov-elo	-	-	-	-	-	-
16.	*Pichia angusta*	D	wh.cr.	sph-ov	-	-	-	-	-	+
17.	*P. anomala*	B	wh.cr.	sph-ov	-	-	-	-	-	+
18.	*P. euphorbiiphila*	C	wh.cr.	r-ov	-	-	-	-	-	+
19.	*P. guilliermondii*	G	wh.cr.	r-ov	-	-	-	-	-	+
20.	*P. jadinii*	C	wh.cr.	sph-ov	-	-	-	-	-	+
21.	*P. lynferdii*	G	wh.cr.	sph-ov	-	-	-	-	-	+
22.	*P. methanolica*	G	wh.cr.	sph-ov	-	-	-	-	-	+
23.	*P. mexicana*	B	wh.cr.	ph-ov	-	-	-	-	-	+
24.	*P. ohmeri*	B	wh.cr.	r-ov	+	-	-	-	-	+
25.	*P. strasburgensis*	C	wh.cr.	r-ov	+	-	-	-	-	+
26.	*Sporobolomyces tsugae*	E	cr.yl.	sgl-gl.	-	-	-	-	-	-
27.	*Stephanoascus ciferrii*	C	yl.br.	r-ov	+	-	-	-	-	+
28.	*Williopsis californica*	G	gr.wh.	r-ov	-	-	-	-	-	+

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

Among basidiomycetous yeast species, *Fibulobasidium inconspicuum*, *Filobasidiella neoformans* and *Filobasidium uniguttulatum* were identified as teleomorphic and anamorphic yeasts identified as *Bensingtonia intermedia*, *B. naganoensis*, *Bullera pyricola*, *Phaffia rhodozyma* and *Sporobolomyces tsugae*. Univariate ANOVA of yeast species isolated from butter revealed that their occurrence was significantly different at $p<0.0001$ (Table 4). Bonferroni test was performed as a post hoc test that also confirmed significant differences of occurrence among yeast species (Table 1). All yeast species appear to be new reports from butter in Pakistan.

Table 4. ANOVA of yeast species isolated from butter.

Source	Sum of squares	df	Mean square	F-value	Probability
Main effects					
Yeasts (A)	2731.427	27	101.164	12991.41	p<0.001
Sample (B)	2008.43	13	154.495	198400.9	p<0.001
A*B	43602.599	351	124.224	159527	p<0.001
Error	305	392	7.79E-04		
Total	**49715.014**	**784**			

Yeast species including *Bullera pyricola*, *Candida valdiviana*, *Debaryomyces castellii*, *D. hansenii*, *D. vanrijii*, *Fibulobasidium inconspicuum*, *Pichia angusta*, *P. anomala*, *P. lynferdii*, *P. mexicana*, *P. ohmeri*, *P. strasburgensis*, *Sporobolomyces tsugae*, *Stephanoascus ciferrii*, *Wiliopsis californica* which were isolated from samples of butter, were also isolated from milk and yogurt in the previous study (Mushtaq *et al*., 2006). However, rest of the yeast species *viz*., *Bensingtonia intermedia*, *B. naganoensis*, *Candida friedrichii*, *C. xestobii*, *D. nepalensis*, *Filobasidiella neoformans*, *Filobasidium uniguttulatum*, *Kluyveromyces polysporus*. *Phaffia rhodozyma*. *P. euphorbiaphila*, *P. guilliermondii*, *P. jadinii* and *P. methanolica* were isolated and identified from samples of butter only and were not found previously in the samples of milk and yogurt (Mushtaq *et al*., 2006).

Yeast counts (colony forming units, cfu) of most of the yeast species isolated from butter were between 10^3 and 10^5 cells g^{-1}. Highest yeast counts were observed in *Bensingtonia intermedia*, *Candida valdiviana*, *Debaryomyces castellii* and *Pichia lynferdii* (Table 1). However their overall occurrences in samples of butter was found lesser as compared to samples of milk and yogurt isolated in the previous study (Mushtaq *et al*., 2006). Occurrence of yeasts in such counts from dairy products has been recorded in Australia (Fleet & Mian. 1987; Suriyarachchi & Fleet, 1981), Nigeria (Green & Ibe, 1987) and Egypt (Haridy, 1993). However examples of yeast occurrences with more than 10^6 cells g^{-1} (Rohm *et al*., 1990; Van-Uden & Carmo-Sousa, 1957) and 10^3 cells g^{-1} or lesser have also been recorded from various countries such as UK, Canada, USA and the Netherlands (Arnott *et al*., 1974; Davis, 1975; Saad *et al*., 1987). Warmer weather, inadequate refrigeration and improper storage are the principal causes of higher levels of contamination, increased diversity and change in yeast mycoflora (Moreira *et al*., 2001). Ideally, the population of yeast species in dairy products should not exceed 10 yeast cells g^{-1} and values higher than this will probably mean that the product may spoil before refrigeration (Moreira *et al*., 2001).

Several species of *Candida* and *Debaryomyces* isolated from butter have already been reported as contaminant in milk and yogurt (Mushtaq *et al*., 2006; Rose, 1982; Wood, 1985). They are mainly involve in deterioration (Fleet, 1990, 1992) and also responsible for off-flavors and loss of texture quality due to gas production during lactose assimilation (Foschino *et al*., 1993)**.** Yeast species mainly representatives of the genera *Candida (C. sphaeric*a), *Debaryomyce*s, *Mycoderm*a, *Saccharomyces (S. dairensis. S. unisporu*s) and *Rhodotorula* decrease quality of dairy products by lactose assimilation (Kurtzman, 1990; Mossel, 1980; Wood, 1998). It is established that yeast species such as *C. sphaerica* ferment lactose, owing to gas formation in dairy products. Their detrimental effect leads to preparing of non-quality products in the milk processing (Fleet, 1990).

Some representatives of the genus *Rhodotorula* cause staining and give a bitter taste to the products. For example, from the fermented cream it is hard to obtain a churned butter. From the curd, which is a secondary product of the white and yellow cheese processing, the presence of yeasts leads to the so-called "yeast taste". This reflects on the taste quality of the curd if these microorganisms exceed ten thousands per gram product (Savova & Nikolova, 2000-2002).

During this study, 4 methanol assimilating (methylotrophic) yeasts *viz*., *Candida valdiviana*, *Pichia anomala*, *P. mexicana* and *P. ohmeri* were isolated from samples of butter (Table 2). Methylotrophic yeasts are the ideal host for the production of heterologous proteins such as harmones – somatostatin, tumor necrosis factor, and many others, because when carbon source is switched from glucose to methanol (or alkanes) during assimilation, the cells of these yeast species become packed with microbodies (peroxysomes) that contain enzyme, alcohol oxidase. Moreover, as the yeast cells are eukaryotes, they can form secondary and tertiary protein structure that bacterial cells cannot. It is interesting that *P. mexicana* is also reported as lipolytic yeast (Spencer & Spencer, 1997). This yeast species has very strong activity against monoesters of short-chain fatty acids (C8, C10, C12) with triterpenes and sterols.

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