



Microbiology Research Journal International

19(4): 1-9, 2017; Article no.MRJI.33106
Previously known as British Microbiology Research Journal
ISSN: 2231-0886, NLM ID: 101608140



SCIEDOMAIN international
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Characterization of Psychrotrophic Molds from Indigenous Fermented Dairy Products

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Authors' contributions

This work was the collaborative effort of all authors. Author Basavabharati designed the study, performed and wrote the first draft of the manuscript. Authors NS and RP managed the literature search and finalized the draft. All the authors read and approved the final manuscript.

Article Information

DOI: 10.9734/MRJI/2017/33106

Editor(s):

(1) Raúl Rodríguez-Herrera, Autonomous University of Coahuila, México.

Reviewers:

(1) Celenk Molva, Izmir Institute of Technology, Turkey.

(2) Francisco De Assis Baroni, Federal Rural University of Rio de Janeiro, Rio de Janeiro, Brazil.

(3) Pinar Oğuzhan yıldız, Ardahan University, Turkey.

Complete Peer review History: <http://www.sciencedomain.org/review-history/18710>

Original Research Article

Received 30th March 2017
Accepted 17th April 2017
Published 20th April 2017

ABSTRACT

Aim: The aim of the study was to establish the number and type of psychrotrophic molds in Indigenous Fermented Dairy Products (IFDP) of Bengaluru market, Karnataka, India.

Methodology: The study was taken up in the Department of Dairy Microbiology, Dairy Science College, KVAFSU, Hebbal, Bengaluru, 560 024, Karnataka, India between June 2014 to June 2015 as part of post graduate research work. The samples used in the study were unbranded (n=6) and branded (n=15) IFDP such as Mishtidoi, Shrikhand, Lassi and Buttermilk collected from Bengaluru markets. The market samples of IFDP were subjected for isolation on poured sterile malt extract agar of pH 3.5. The isolates were obtained by pour-plate technique on malt extract agar (pH 3.5). The mold isolates were identified by colony morphology, microscopic examination for cell morphology and specific biochemical tests.

Results: Out of total of 21 psychrotrophic mold isolates, 6 and 15 isolates were obtained from

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unbranded and branded samples, respectively. Based on the preliminary identification, the isolates were characterized as *Penicillium* spp. (n=3), *Mucor* spp. (n=2) and *Cladosporium* spp. (n=1) from unbranded samples while branded IFDP samples showed *Penicillium* spp. (n=9 and *Alternaria* spp. (n=6) were isolated from branded samples. According to pheno and genotypic characterization, the isolates were identified as *P. chrysogenum* (57.12%) followed by *A. alternata* (28.56%), *C. cladosporioides* (4.76%) and *M. mehei* (9.52%) at the species level.

Conclusion: Except unbranded mishitdoi, all types of IFDP samples were found to contain psychrotrophic molds. *P. chrysogenum* was the most abundant species especially in branded butter milk indicating longer refrigeration may lead to selection of psychrotrophic molds due to low temperature storage and pH of the IFDP samples.

Keywords: Psychrotrophic mold; indigenous fermented dairy products; characterization and refrigeration.

1. INTRODUCTION

India is the largest milk producing country in the world. Milk is utilized to manufacture a variety of milk products to increase the shelf life such as fermented dairy products, heat desiccated and acid coagulated products. Simple processes are used to preserve milk's nutrition quality to protect and promote health. Indigenous dairy products may be defined as all milk products which are native to India which evolved over ages utilizing locally available fuels and cooking wares [1].

Out of the total production, 50 – 55 per cent is utilized to manufacture indigenous dairy products in which 7 per cent is utilized to manufacture fermented dairy products such as Mishtidoi, Shrikhand, Lassi and Butter milk [2]. Market for indigenous fermented milk products in Bangalore is covered by many brands like AMUL, Danone, Milky Mist, Mother Dairy, Britannia, Nilgiris, K. C. Das and its regional brands such as Nandini. The Indigenous Fermented Dairy Products (IFDPs) need to be stored at low temperature to avoid spoilage by molds [3].

The presence of *Aspergillus*, *Fusarium*, *Mucor* and *Penicillium* spp. in yogurt stored at low temperature was observed [4]. Low temperature storage extends the shelf life of indigenous fermented milk products, significance of psychrotrophic molds and their metabolites need to be considered. Psychrotrophic molds (*Penicillium*, *Rhizopus* and *Aspergillus*) produce proteolytic and lipolytic enzymes even at low temperature that alter the product composition leading to defects [5]. Certain molds like *Penicillium* spp release the mycotoxins such as citrinin, patulin, penicillic acid, ochratoxin A and sterigmocystin in cheese and other fermented

dairy products [6,7] which are difficult to destroy during milk processing to produce fermented milk products.

Citrinin production mainly by *P. citrinicum* and also by *P. chrysogenum* found to be involved in nephrotoxicosis issues by disfunctioning of renal veins of kidney and also caused Balkan nephropathy and Yellow rice fever when the food contaminated with the mold was consumed [8]. Various cytotoxic effects were offered by patulin like damage to the immune system; pancreas; liver and gastrointestinal tract [9]. During storage of shrikhand, an indigenous fermented milk product at refrigeration temperature had shelf life of 10 days with psychrotrophic mold count of $6.25 \times 10^1/g$ [10].

The present study help to know the number and types of psychrotrophic molds occur in IFDP and gives an idea about their metabolites like enzymes that cause spoilage and toxins that pose health hazards when such products are ingested.

2. MATERIALS AND METHODS

2.1 Collection of IFDP

Different varieties of mishtidoi, shrikhand, lassi, buttermilk samples were collected in an ice box from many sweetmeat sellers as well as from retail branded sellers of Banashankari, Hebbal, R.T. Nagar, Majestic areas of Bengaluru city which were considered as unbranded samples and branded samples respectively. The samples were brought to the laboratory for the isolation of psychrotrophic molds using Malt Extract Agar (MEA) adjusted to pH 3.5 [11] followed by incubation at 7°C for 20-25 days.

2.2 Isolation of Psychrotrophic Molds from IFDP

Plates, showing cottony colonies were selected and streaked thrice onto MEA agar plates in order to purify the isolates. The purified isolates were grown on MEA slants, subcultured every month and maintained at 2°C till required.

2.3 Phenotypic Characterization of Psychrotrophic Mold Isolates

2.3.1 Macroscopic characteristics of the isolates

The psychrotrophic mold isolates obtained were characterized macroscopically through the colony morphology, such as colour of colony, front view, reverse view, surface of the colony and any exudate production over the colony.

2.3.2 Microscopic characteristics of the isolates

The isolates were microscopically identified by Wet Mount Staining technique [12].

2.3.3 Specific tests for selective mold isolates

2.3.3.1 Ehrlich test

All the mold isolates were examined for production of cyclopiazonic acid and other alkaloids reacting with Ehrlich using a filter paper method [13]. The appearance of a violet ring for 2- 6 min indicates the presence of cyclopiazonic acid or related alkaloids.

2.3.3.2 Screening of *Penicillium* isolate for penicillin production

Penicillium isolate was tested for penicillin production. After extraction [14] they are tested by β -Lactamase test [15] against *Staphylococcus aureus*. The production of yellow color confirms the presence of penicillin [15].

After the preliminary test, the penicillin extracted was tested for its antibacterial activity against *Staphylococcus aureus* [14]. Zone of inhibition was noted and the activity of the antibiotic was confirmed.

2.3.3.3 Screening of the *Penicillium* isolate for citrinin production

Citrinin production from penicillium isolates were tested against *S. aureus* and *Escherichia coli* [16]. Citrinin was found to inhibit *Staphylococcus*

aureus but not the *Escherichia coli*. The inhibition zones around wells of citrinin inoculated with the *S. aureus* culture confirms the presence of citrinin toxin [17].

2.3.3.4 Genotypic identification of mold isolates

The gene sequencing of mold isolates was outsourced for Serene Biosciences, Molecular Diagnostics Research, Custom services & Training. No. 30, II cross, Marappa Garden, Benson Town Post, Bangalore - 560 046 to determine the genotypic identity [18].

3. RESULTS AND DISCUSSION

3.1 Psychrotrophic Mold Isolated from IFDPs

Out of 21 isolates, 6 and 15 isolates were obtained from unbranded and branded samples, respectively (Table 1). Four different colony morphologies were obtained among the mold isolates. These isolates were further subjected to preliminary, specific tests and characterized by genotyping methods.

3.2 Phenotypic Identification of Psychrotrophic Molds from Selected Indigenous Fermented Dairy Products

The preliminary identification of psychrotrophic molds isolates (n=4) were based on the colony characteristics, cell morphology while specific tests included Ehrlich reaction for screening of toxigenic mold species and antibiotic production by the species (Table 2).

Mold isolate -1 formed colony with greenish on the surface, yellow exudate while citrine yellow colour at the bottom. Microscopic examination of the same revealed septate hyphae bearing conidiospores resembling the characteristics of *Penicillium* sp. with production of penicillin and citrinin with absence of alkaloid (Fig. 1).

Among the mold isolates, only *Penicillium spp.* produced typical citrine yellow exudate confirming the species and negative to Ehrlich reaction which are in conformation with the other two studies [13,17]. Isolate was a producer of antibiotic penicillin which showed antibacterial activity against *Staphylococcus aureus* and citrinin showed antibacterial activity against *S. aureus* but not on *E. coli* in addition to positive results of β -lactamase test. Similar characteristics were noticed in one of the study [15] with respect to *Penicillium spp.* Citrinin and

penicillin production by *P. chrysogenum* was determined in the present study while the same things were confirmed in other studies [17,14]. In a study [19], fruity odour from the mold colony showed ter means 3 or quart means 4 branched smooth phialides under microscope. The same was observed in the present isolate hence confirmed as *Penicillium* sp. and the same was in relevance to the features of mold by one more study [20].

The colony of mold isolate – 2 was brownish red at the surface and brownish black colour at the bottom with raise at the center. Microscopic examination of mold showed pear or club shaped spores with both longitudinal septa, cross walls with septate hyphae and mycelium. The mold produced alkaloids determined by positive Ehrlich test. All the features The colony and cell morphology revealed the mold isolate as *Alternaria* sp. (Fig. 2).

Table 1. Psychrotrophic molds isolated from Indigenous fermented dairy products

Sl. no.	Samples	Number of isolates	
		Unbranded	Branded
1	Mishtidoi	-	4
2	Shrikhand	3	4
3	Lassi	2	2
4	Butter milk	1	5
Total isolates obtained		6	15
Types and number of psychrotrophic mold isolates		<i>Penicillium</i> spp.(3) <i>Mucor</i> spp. (2) <i>Cladosporium</i> spp. (1)	<i>Penicillium</i> spp.(9) <i>Alternaria</i> spp.(6)

Table 2. Colony morphology of the isolated psychrotrophic mold isolates from indigenous fermented dairy products

Characteristics	Isolate - 1	Isolate – 2	Isolates - 3	Isolate - 4
Colony morphology				
Diameter on MEA	18 mm X 17 mm	22 mm X 25 mm	9 mm X 10 mm	25 mm X 26 mm
Colour on MEA	Greenish with whitish edges	Brownish red	Blackish with aerial spores	Dark green
Reverse side	Citrine yellow	Brownish black	Dark muddy white	Black
Aspect on MEA	Radially grooved	Flat with raised at the center	Highly raised	Flat spread
Texture of the colony	Grainy, non dissociable spores	Cottony spread	Grainy of aerial spores	Velvety hard , leathery
Cell morphology				
Spore shape	Oval round conidiospores	Beak shaped conidiospores	Round sporangiospores	Round conidiospores
Type of hyphae	Septate	Septate	Non septate	Septate
Type of mycelia	Septate	Septate	Non septate	Septate
Species wise special characteristics				
Exudate production	Typical yellow	-	-	-
Ehrlich reaction	-	+ (reddish pink) indicates alkaloids	-	-
Antibiotic production:				
Penicillin (β-Lactamase test)	+	-	-	-
Citrinin (MH Agar)	+	-	-	-
Identity of the isolate	<i>Penicillium</i> sp.	<i>Alternaria</i> sp.	<i>Mucor</i> sp.	<i>Cladosporium</i> sp.



a) Front view



b) Reverse view



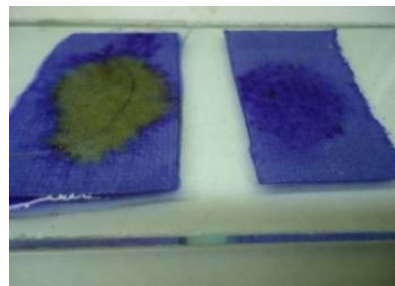
c) Exudate production



d) Cell morphology

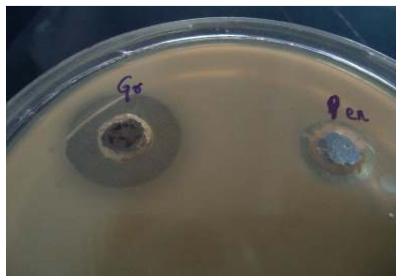


Negative



Positive

e) β - Lactamase test



f) Penicillin production



g) Citrinin production

Fig. 1. Colony and cell morphology of psychrotrophic *Penicillium* sp

The mold was identified based on the asexual spores conidia over conidiophore and confirmed it as *Alternaria* by a study based on cell structure [21] while another study [15] identified the mold based on alkaloid production.

Psychrotrophic mold isolate - 3 was black at surface bearing pin heads of the aerial spores, with non septate hyphae bearing round sporangiophore and pheotyped as *Mucor* spp (Fig. 3).

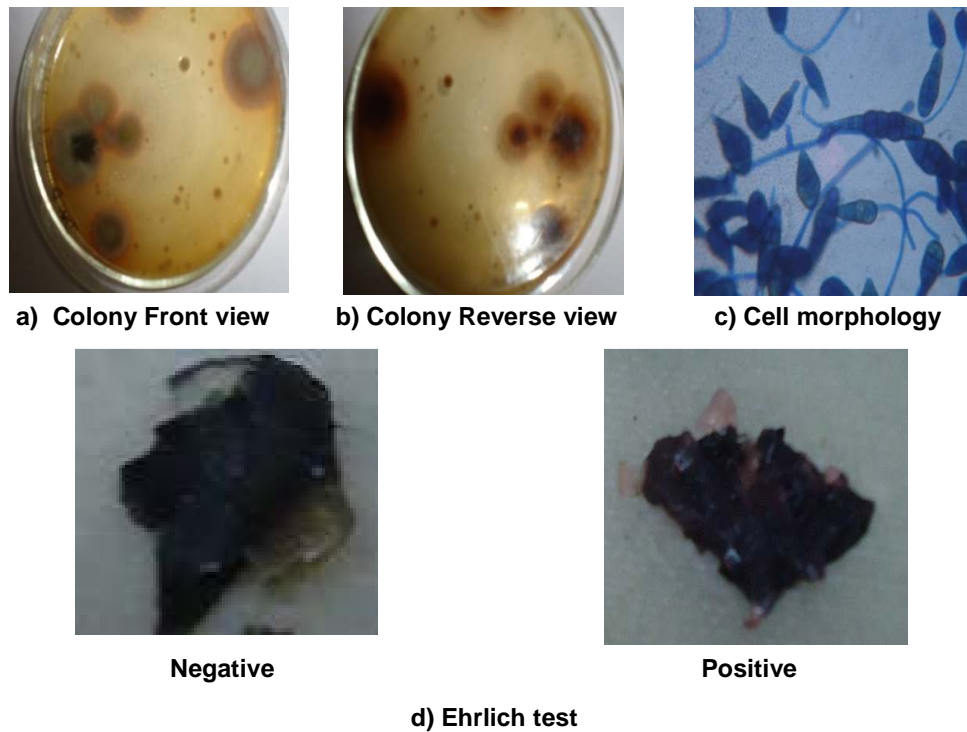


Fig. 2. Colony and cell morphology of *Alternaria* sp

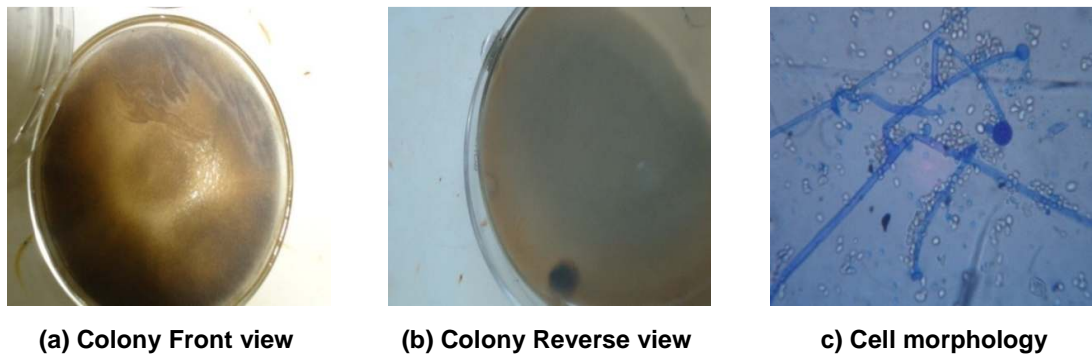


Fig. 3. Colony and cell morphology of *Mucor* sp

Based on a study [22] similar to the present study, the non septate hyaline hyphal elements bearing globose sporangia above the sporangiophore was identified as the *Mucor* spp.

The colony with dark green on surface and back at the bottom with aseptate hyphae i.e., conidiophores bearing 1 to 2 celled conidia in branched chains was observed for mold isolate – 4 under microscope and identified as *Cladosporium* sp (Fig. 4).

Another study [23] which was similar to present study identified the mold isolate - 4 as *Cladosporium* sp. based on co and cell morphology.

Preliminary and specific tests revealed the identity of psychrotrophic molds as *Penicillium* spp. (12 nos.), *Alternaria* spp. (6 nos.), *Mucor* spp. (2 nos.) and *Cladosporium* sp (1 no.).

3.3 Genotypic Identity of the Isolates

Predominating phenotyped psychrotrophic molds 1, 2, 3 and 4 isolated from Indigenous Fermented Milk Products were identified based on 18S rRNA sequencing [24] as *Penicillium chrysogenum*, *Alternaria alternata*, *Cladosporium cladosporioides* and *Mucor mehei* respectively (Table 3).

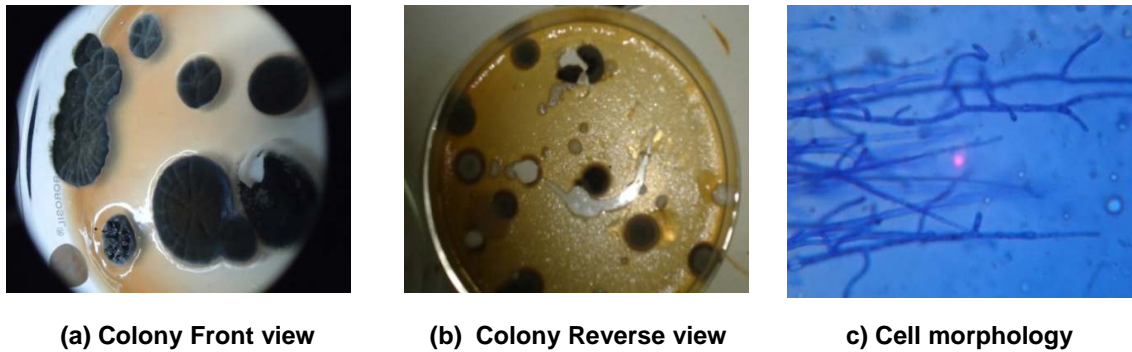


Fig. 4. Colony and cell morphology of *Cladosporium* sp

Table 3. Genotypic identity of the predominating psychrotrophic mold isolates with the accession number

Isolate	Accession number	Identity
Isolate – 1	JQ015265.1	<i>Penicillium chrysogenum</i>
Isolate – 2	JN210895.1	<i>Alternaria alternata</i>
Isolate – 3	KF983515.1	<i>Cladosporium cladosporioides</i>
Isolate – 4	KG983517.1	<i>Mucor mehei</i>

The same identification tool of 18s rRNA sequencing was used in the recent mold classification by one more study [24].

Out of the psychrotrophic mold isolated (21 nos.) from refrigerated Indigenous Fermented Dairy Products, *Penicillium* spp. (12 nos.) predominated followed by *Alternaria* spp. (6 nos.), *Mucor* spp. (2 nos.) and *Cladosporium* spp. (1 no.) in the present study. These molds may enter from the air, utensils used, sugar added for preparation and unhygienic handler as mentioned in one of the study [25]. Post processing contamination especially by local producers may introduce the spores of molds into product, low pH and low temperature help in favouring their growth.

On par with the present study, another study [26] also revealed predominance of *Penicillium* spp. in Yoghurt and cheese samples stored at refrigeration condition followed by *Aspergillus* spp. whereas one more study [27] though observed predominance of *Penicillium* but followed by *Monilia* in cheese. On the contrary to the present study, another study [4] showed predominance of *Aspergillus* as the psychrotrophic mold species followed by *Fusarium*, *Mucor* and *Penicillium* in case of low temperature stored Yoghurt samples while in milk samples occurrence of the *Alternaria* was observed.

4. CONCLUSION

A total of 21 psychrotrophic mold isolates were obtained from the Indigenous Fermented Dairy Products such as Mishti Doi, Shrikhand, Lassi and Buttermilk collected from Bengaluru market, Karnataka, India, out of which 6 isolates were from unbranded samples while 15 from branded samples. Pheno and genotypic characterization of the isolated psychrotrophic molds from indigenous fermented milk products revealed predominance of *Penicillium chrysogenum* (12 nos.) followed by *Alternaria alternata* (6 nos.), *Mucor mehei* (2 nos.) and *Cladosporium cladosporioides* (1 no.) among isolated psychrotrophic molds.

ACKNOWLEDGEMENT

Authors are grateful to sweetmeat sellers as well as retail branded sellers of Banashankari, Hebbal, R.T. Nagar, Majestic areas of Bengaluru city market from whom samples of Indigenous Fermented Dairy Products such as Mishtidoi, Shrikhand, Lassi and Buttermilk for the present study were purchased.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:

The peer review history for this paper can be accessed here:
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