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Antifungal activity of some food grade lactic acid bacteria

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Five lactic acid bacteria (LAB) with bacteriocin producing capabilities were isolated from different food grade fermented meat products. These isolates were selected and partially identified as different strains of *Pediococcus* based on their morphological, physiological and biochemical properties. When screened for antifungal activity, all the isolates showed prominent activity against different fungi on solid plates. Two of them (LAB 146, LAB 06) also showed anti-candidal activity. Activity was also found in the cell free supernatant of the isolates but with a lesser amount compared to solid plate assay.

Key words : Lactic acid bacteria, *Pediococcus,* antifungal activity

INTRODUCTION

Fungi, a group of living world with heterotrophic absorptive mode of nutrition generally responsible for degradation of biomass, is also known for their pathogenicity to plants and animals, and spoilage of food materials and toxicity leading to health hazards and economic losses (Filtenborg *et al.*, 1996). They rarely cause disease in healthy immunocompetent hosts. Disease results when fungi accidentally penetrate host barriers or when immunologic defects or other debilitating conditions exist that favour fungal entry and growth. *Candida albicans, Aspergillus* sp. (aflatoxin producing), *Fusarium* sp., *Alternaria* sp. are some common pathogenic fungi of human and plants. In food industry fungal contaminations of different foods are very common phenomenon. Common contaminants are moulds and yeasts particularly species of *Mucor, Penicillium, Aspergillus, Rhizopus, Fusarium etc.* Though some chemical compounds such as nitrite, sulphite, calcium propionate, sodium benzoate etc are used widely to combat the pathogenicity and spoilage by these fungal species, scientists are putting increased efforts into the discovery and purification of natural compounds for use as safe alternatives to chemical preservatives.

Lactic acid bacteria (LAB), a large group of beneficial bacteria, are widespread in nature. Most LAB are nonpathogenic and generally regarded as safe (*i.e.*, GRAS) by the U.S.Federal Drug Administration (FDA). They are used in the food industry for several reasons. They protect foods from spoilage and pathogenic microorganisms through the production of bacteriocins (DeVugst, 1994), organic acids, hydrogen per-oxide, di-acetyle (Messens, 2002) and/or antifungal compounds such as fatty acids (Corsetti, 1996) or phenyl lactic acid (Lavermicocca, 2000). Recently there are few reports (Florianowicz, 2001, Magnusson *et al.*, 2003) on production of antifungal compound by some species of lactic acid bacteria. Magnusson and Schnurer (2001) have reported on the production of proteinaceous antifungal compound by *Lactobacillus coryniformis* strain Si3. Sjogren *et al.* (2003) have reported the production of a different kind of antifungal compound, 3- hydroxyl fatty acid from *Lactobacillus plantarum* Mi LAB- 14. Lavermicocca *et al.*(2000, 2003) have reported on the purification and characterization of antifungal phenyl lactic acid and 4-hydroxyl phenyl lactic acid from sourdough *Lactobacillus plantarum* strain 2 IB. In the present investigation attempt has been made to isolate and characterize some food grade microorganisms with a good spectrum of antifungal activity.

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MATERIALS AND METHODS

Media and strains

The stock cultures of lactic acid bacteria including the producer isolates as well as indicator strains were maintained at -20°C on MRS (De Mann *et al.*, 1960) supplemented with 15% glycerol. Working cultures were prepared as slants on MRS agar, and stored at 4°C. *Enterococcus faecalis MBI, Leuconostoc messenteroides Ly* and *Lactococcus lactis* [MTCC 3038] were used as bacteriocin sensitive indicator strains in this study. Fungal strains (Table 2) were grown on malt extract agar slant (2%) and on the medium suggested by MTCC during procurement and stored at 4°C.

Isolation of lactic acid bacteria

From local market different vacuum-packed refrigerated meat products were purchased and stored at 4°C up to one week. A sample of 25 g of each food sample was taken aseptically and homogenized in 25 ml of sterile peptone water. Six 10-fold dilutions of the homogenates were prepared and 100 µl from last two dilutions were spread on MRS agar and TGE agar plates containing cycloheximide 50 µg ml^{-1}. Plates were incubated for 48 hr at 28°C. Colonies with typical characteristics of lactic acid bacteria were randomly selected from plates and streaked on MRS agar to check the purity of the isolates and then stored at -20°C in MRS broth with 15% (v/v) glycerol. They were screened based on their ability to produce clear inhibition zone around their colonies against three indicator lactic acid bacteria.

Identification of isolates

The isolates were identified according to their morphological, physiological and biochemical properties. Bacterial samples were prepared through pre-fixation (glutaraldehyde), post-fixation (osmium tetroxide), dehydration with alcohol grades (from 30% to 100%) and observed under scanning electron microscope (HITACHI-S30, JAPAN). Gram staining, Catalase test, Carbohydrate utilization (Himedia Hi-carbohydrate™ Kit) tests were performed following standard protocol.

Detection of antifungal activity

Antifungal activity was determined by overlay method and agar well diffusion assay (Magnusson, 2001). In the first method, MRS plates were inoculated centrally with different isolates of LAB as 2 cm long lines and incubated for 48 hr at 28°C. After incubation the plates were overlaid with 10 ml of ME soft agar (2% ME, 0.7% agar) containing 10^4 ml^{-1} fungal spores (CFU for *C. albicans*) and incubated further for 48 hr for appearance of inhibition zones. In the second method, MRS plates were first overlaid with ME soft agar containing fungal spores. Then with a sterile cork borer wells with a diameter of 5 mm were made on the overlaid plates. For sealing of any leakage under wells, one drop of melted agar was added to each well. 50 µl of cell free supernatant of different isolates were inoculated into the well and allowed to diffuse for 5 hr at room temperature. Plates were then incubated at 28°C for 48 hr and observed for any zone of inhibition.

RESULTS AND DISCUSSION

Isolation and identification of LAB

After screening a large number of bacteria from different ready-to-eat quality fermented food sources five efficient bacteriocin producing LAB were isolated. The isolates were characterized morphologically, physiologically and biochemically. Scanning electron microscopic observation showed all the isolates as mixtures of single and paired cocci (Fig. 1, A, B, C). The sizes of cocci of LAB 06, LAB 146 and LAB 186 were slightly greater than the sizes of LAB 147 and LAB 185. The colonies of the isolates were very small (2-3 mm diameter), smooth and pure white. All isolates were Gram positive, catalase negative and non sporing. They could not produce gas from glucose, but produce acid during fermentation. These isolates showed variation in carbohydrate fermentation profile which was performed by using Himedia Hi-carbohydrate™ Kit. (Table 1). The inability to use maltose as carbon source and the profile of other carbon source utilization along with the biochemical parameters of the isolates indicated that the organisms were found to be different strains of *Pediococcus*.

Table 1 : Carbohydrate utilization profile of different isolates of lactic acid bacteria

Carbohydrates	LAB 06	LAB 146	LAB 147	LAB 185	LAB 186
Lactose	-	+	-	-	+
Xylose	+	+	+	-	-
Maltose	-	-	-	-	-
Fructose	+	-	+	-	-
Dextrose	+	+	+	+	+
Galactose	+	+	+	+	+
Raffinose	-	-	-	-	-
Trehalose	+	+	-	-	-
Melibiose	-	-	-	-	-
Sucrose	-	-	-	-	-
L-Arabinose	+	+	+	+	+
Mannose	+/-	+	+	+	+
Inulin	-	-	-	-	-
Sodium gluconate	-	-	-	-	-
Glycerol	+	-	-	-	-
Salicin	+	+	-	-	-
Glucosamine	-	-	+	+	+
Dulcitol	-	-	-	-	-
Inositol	-	-	-	-	-
Sorbitol	+	-	-	-	-
Mannitol	-	-	-	-	-
Adonitol	-	-	-	-	
α-methyl-D-glucoside	+/-	-	+	-	-
Ribose	+	-	+	+	+
Rhamnose	-	-	-	-	-
Cellobiose	+/-	+	+	+	-
Melezitose	+/-	-	+/-	-	+/-
α-methyl-D-mannoside	+	-	+/-	-	-
Xylitol	+	+/-	+/-	-	-
ONPG	-	-	-	-	-
Esculin	+	+	+	+	+
D-Arabinose	+	+/-	+	+/-	+
Citrate	-	+	-	+	+/-
Malonate	-	+	-	+	+/-
Sorbose	-	-	-	-	-

'+' positive; '-' negetive

Assessment of antifungal spectrum

The data in Table 2 showed the antifungal spectrum of the LAB isolates. Among the fungi tested some were isolated in the laboratory from spoiled foods (*Penicillium* sp., *Mucor* sp. *Fusarium* sp. *Rhizopus* sp., *Aspergillus* sp., *Curvularia lunata, Alterania* sp.) and some were procured from Microbial Type Culture Collection [*Aspergillus parasiticus* (MTCC 2796), *Fusarium oxysporum* (MTCC 24W) *Cladosporium herbarum* (MTCC 351), *Altemaria solani* (MTCC 2101), *Candida albicans* (MTCC 308), *Collectotricum accutatum* (MTCC 1037)]. They were all harmful either by being pathogenic to human or plants or causing spoilage to food. Cell free supernatant (CFS) of some isolate showed

Table 2 : Antifungal spectrum of different isolates of lactic acid bacteria

Fungus	Characteristics	LAB 06	LAB 146	LAB 147	LAB 185	LAB 186
Penicillium sp. (lab isolate)	Causes food spoilage	++	+++	+++	+++	++
Mucor sp. (lab isolate)	Causes food spoilage	-	+++	+++	+++	++
Aspergillus parasiticus (MTCC 2796)	Produces aflatoxin	+++	++	++	+	+
Aspergillus sp. (lab isolate)	Causes food spoilage	-	+	+	+	-
Fusarium oxysporum (MTCC 2480)	Pathogen of Fusarium wilt of pea	++	+++	++	+	+
Fusarium sp. (lab isolate)	Causes food spoilage	-	+	+	+	+
Candida albicans (MTCC 3018)	Pathogenic, causes candidiasis to human	++	++	-	-	-
Rhizopus sp. (lab isolate)	Causes food spoilage	-	-	-	-	-
Cladosporium herbarum (MTCC 351)	Pathogen of Brown spot of rice	-	+	++	-	++
Collectotricum acutatum (MTCC 1037)	Pathogen of blight of banana	-	-	-	-	-
Curvularia lunata (lab isolate)	Causes food spoilage	+	+	+	-	+
Alternaria sp. *solani* (MTCC 2101)	Pathogen of early blight of potato	++	-	-	++	+
Alterania sp. (lab isolate)	Soil borne fungus	-	+	+	+	-

+++ :: Diameter of inhibition zone >/=15 mm, ++:: Diameter of inhibition zone 8 mm to 15 mm, + :: Diameter of inhibition zone < 8 mm, - :: Absence of inhibition zone

antifungal activity too but the activity was lower than the activity found on solid plate during dual culture assay. It can be concluded that solid condition supported production of more antifungal compound than in broth culture. Previously antifungal activities were obtained mainly from species of *Lactobacillus* (Lavermicocca *et al.*, 2000, 2003), but such activities are very rare from the strains of *Pediococcus*, Only recently antifungal activity of a *Pediococcus* strain with comparatively narrow spectrum had been reported (Mandal *et al.*, 2007). The present isolates showed much broader spectrum with extractable active principle in cell free supernatant. Degree of inhibition zones formed by the isolates was significantly large (Fig.2A-F) even against a human pathogenic yeast *Candida albicans* (Fig.2C). The smaller diameters of inhibition zones produced by the cell free supernatant of the isolates (Fig.3A-C) were probably due to their low concentration. The activity might be increased by concentrating such supernatants. The ability of inhibiting such a vast range of spoilage, plant pathogenic and even human pathogenic fungal organisms by food grade lactic acid bacteria is novel and this could be utilized in future for preservation and control of some unwanted fungal growth if exploited properly.

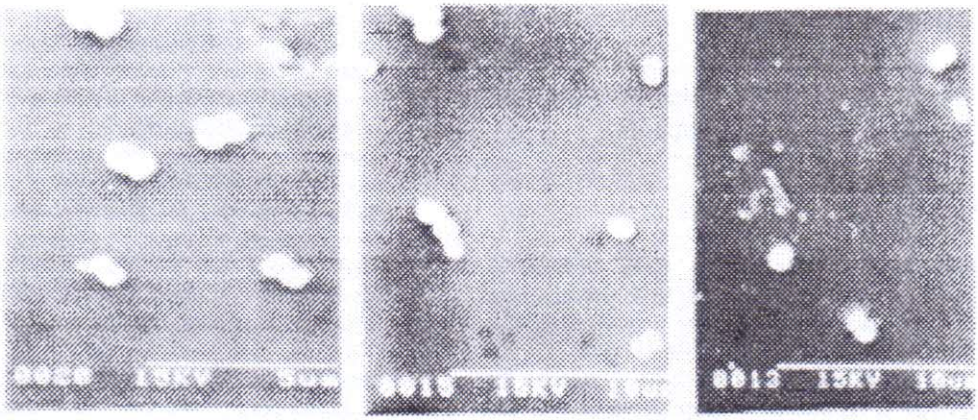

Fig 1. Scanning electron micrograph of isilates of lactic acid bacteria, A, LAB 06, B. LAB 147 and C. LAB 146

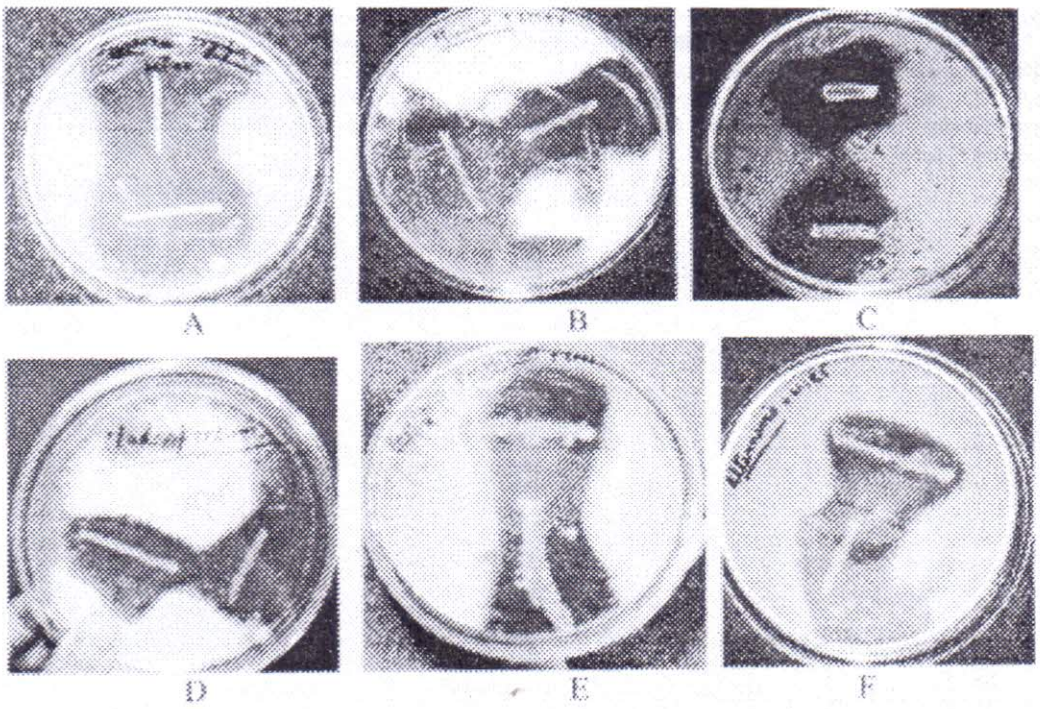

Fig 2. Inhibition zones produced by different LAB isolates. A. Activity of LAB 146 against *Fusarium oxysporum;* B. Activity of LAB 147 against *Mucor* sp.; C. Activity of LAB 06 against *Candida albicans;* D. Activity of LAB 186 against *Cladosporium herbarum;* E. Activity of LAB 06 against *Aspergillus parasiticus;* F. Activity of LAB 185 against *Alternaria solani.*

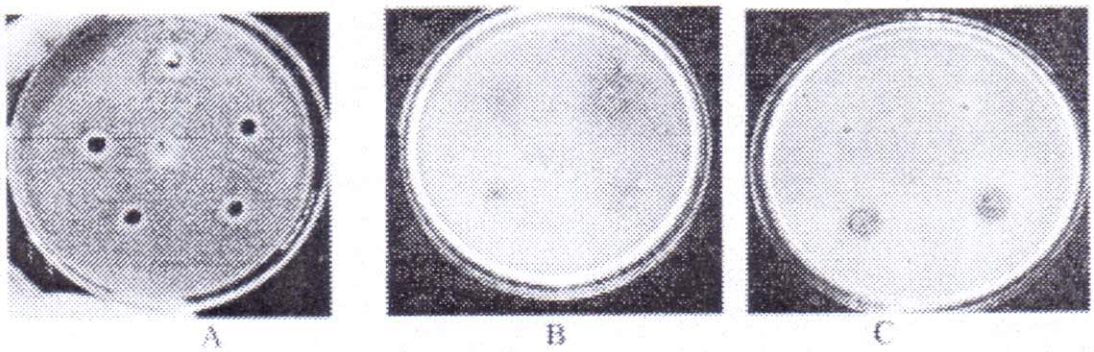

Fig 3. Inhibition zones produced by different LAB isolates. A. Activity of LAB 146 against *Fusarium oxysporum;* B. Activity of LAB 147 against *Mucor* sp.; C. Activity of LAB 06 against *Candida albicans;* D. Activity of LAB 186 against *Cladosporium herbarum;* E. Activity of LAB 06 against *Aspergillus parasiticus;* F. Activity of LAB 185 against *Alternaria solani.*

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