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RESEARCH ARTICLE

Characterisation of effective antifungal *Lactobacillus* strain isolated from *Chilika curd*

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Abstract: In this study different strains of Lactobacilli isolated from *Chilika curd,* (fermented milk product native to the Chilika region of Odisha, India) were assessed for their antifungal activity. *Limosilactobacillus fermentum* C-14 was shown to have the strongest inhibitory activity against tested yeast and mold cultures. This antifungal activity of cell free supernatant (CFS) Lb-C-14 was found to be stable at high temperature and up to pH 6. Antifungal activity has been observed in the bacterial cell-free supernatant, and the compounds have been identified as Phenyllactic acid (PLA), acetic acid and lactic acid. *L. fermentum* Lb-C-14 has the potential antifungal activity and could be suggested as a protective culture for the biopreservation of fermented foods.

Keywords: Antifungal; *Chilika curd*; Phenyllactic acid; *Limosilactobacillus fermentum*

Introduction

All foods, including cereals, meat, fruits, nuts, milk, and dairy products can support growth of fungi. Food spoilage caused by fungi results in significant financial losses for the food industry and may pose a health risk due to their toxicity and pathogenicity (Cheong et al. 2014). Several chemical and physical preservation techniques are used to increase the shelf-life of milk products. Drying, freeze drying, cold storage, modified atmospheric packaging and heat treatment constitute physical methods; these methods are costly and tend to change the texture of products. Salts of benzoic acid, sorbic acid, propionate, and methyl, ethyl, and propylesters of p-hydroxybenzoic acids are used as antifungal chemical preservatives. Their incorporation at specific concentrations can increase the shelf-life of food products. Nevertheless, there remain some complications associated with the use of these chemicals (Cosentino et al. 2018).

Lactic acid bacteria (LAB) are a prominent group of microbes involved in fermented products as starter cultures to initiate fermentation. In addition to fermentation, their antibacterial and antifungal activities have received substantial research. There are many substances synthesized by LAB with antimicrobial activity such as bacteriocin, H_2O_2, diacetyl, organic acids, carbon dioxide, oleamide, trans-cinnamic acid, and citric acid etc. (Voulgari et al. 2010, Barrios-Roblero et al. 2019, Ramos et al. 202, Peng et al. 2023). Recently there has been research interest into the phenyllactic acid synthesis by LAB due to its effective antimicrobial activity (Bustos et al. 2018). It is produced as a by-product of phenylalanine metabolism in LAB identified in fermented dairy products (Valerio et al. 2016, Jung et al. 2019). The lactate dehydrogenase (LDH) is the main responsible enzyme for the production of PLA in LAB which catalyzes PLA release from the direct precursor Phenylpyruvate. PLA production has been documented in a wide range of LAB such as *L. plantarum, L. fermentum* and *L. casei* (Muhialdin et al. 2011, Cortés-Zavaleta et al. 2014, Jung et al. 2019, Xu et al. 2021). However, its production has been reported as species and strain dependent (Wang et al. 2012, Li et al. 2014, Xu et al. 2021).

One of the oldest and most well-known fermented milk products in the Indian subcontinent is dahi or curd, which is similar to yogurt. The Chilika curd is made by the local ethnic community in and around Chilika Lake region in Odisha, India, typically made from the milk of Chilika breed of buffalo (Singh et al. 2017). The Chilika dahi is extremely distinctive in terms of having an incredibly long shelf life (Nanda et al. 2013, Sahoo et al. 2020). Therefore, it is crucial to understand the wide diversity of LAB in Chilika curd for the nature of such properties that gives its extended shelf life. Previously some of the *Lactobacillus* sp.

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were isolated and screened for antifungal activity but additional studies are required focusing on characterisation of those isolates and acquisition of data regarding antifungal compounds they produce (Nanda et al. 2013). In this study, we have investigated the antifungal activity of Lactobacillus strain that was isolated from Chilika curd and conducted preliminary studies on their ability to produce PLA as a compound of antifungal properties.

Materials and methods

Microbial cultures

The six *Lactobacillus* cultures previously isolated from Chilika dahi were submitted to National Collection of Dairy Cultures (NCDC), ICAR-NDRI, Karnal under the numbers Lb-C-4 (NCDC-847), Lb-C-5 (NCDC-848), Lb-C-6 (NCDC-849), Lb-C-8 (NCDC-850), Lb-C-9 (NCDC-851), and Lb-C-14 (NCDC-852) were used for investigation (Nanda et al. 2013). The freeze-dried cultures of *Lactobacillus* were propagated in de Man, Rogosa and Sharpe broth (MRS, Hi-media, India) and incubated at 37°C for 48 hrs. The stock cultures were stored at -20°C in 15% (v/v) glycerol stocks and cultures were regularly activated in MRS broth.

The fungal cultures used as test microorganisms were *Aspergulls flavus* NCDC 226*, Aspergillus niger* NCDC 315*, Aspergillus niger* NCDC 267*, Aspergillus parasiticus* NCDC 54*, Aspergillus oryzae* NCDC 301*, Penicillium roqueforti* NCDC 170*, Rhizopus oryzae* NCDC 52*, Candida butyri* NCDC 280*,* and *Rhodotorula glutinis* NCDC 51 were procured from National Collection of Dairy Cultures, ICAR-NDRI, Karnal. All the yeast cultures were grown in Yeast extract Peptone Dextrose (YPD) broth and mold cultures were cultivated on Potato Dextrose agar (PDA) pH 4 adjusted and incubated at 30°C for 5 days.

The mold spores were harvested from 5 days old cultures by pouring sterilized peptone water containing 0.05% Tween 80 onto the plates. The number of conidia in the obtained suspension was adjusted to 10^6 conidia/ml using a hemocytometer.

Screening for antifungal activity

Using agar overlay assay described by (Rouse et al. (2008) was followed with minor modifications. Briefly, 20 ml MRS agar was poured into 90 mm petri dish and allowed solidification. Then 4 µL of active inoculum containing 1×10^8 CFU/ml was spotted on the agar surface and plates were incubated at optimum temperature for 48 h. Following the incubation, the surface was covered by 7 ml soft agar seeded with fungal spores or yeast cells at 1×10^4 CFU/ml. After the solidification of the soft agar layer, plates were incubated at 30 °C for 48 h. After incubation, each zone of inhibition was measured. The inhibition level of indicator fungal culture by *Lactobacillus* sp. was graded as follows: (-) no suppression, (+) least inhibition with inhibitory zone diameter < 12 mm, (++) moderate inhibition with inhibitory zone diameter 12-18 mm, (+++) Strong inhibition with inhibitory zone diameter >18 mm.

Molecular identification of *Lactobacillus* sp.

The species of Lb-C-14 strain was identified based on the fermentation profile using API 50CHL test kit (Biomerieux, France) and molecular characterization by partial sequencing of 16S rRNA gene using the primer pairs 27F (5'AGAGTTTGAT(C/T)(A/C)TGGCTCAG3') and S-G-Lab-0677-a-A-17 reverse primer (52 - CACCGCTACACATGGAG-32) was performed as per the method described by Heilig et al. (2002). The 750 bp size amplicon of 16S rRNA gene was sequenced from Automated DNA Sequencing Services provided by Xcleris Lab Ltd. (Ahmadabad, India) and analysed using the BioEdit sequence alignment editor version 7.0.9.0. Basic local alignment search tool (BLAST) was used to check the identity of DNA sequence in the database and for species identification. The obtained nucleotide sequence was submitted to the NCBI Genbank database.

Preparation of cell-free supernatant (CFS)

Overnight active *Lactobacillus* culture suspension was adjusted to 0.5 McFarland standard. The culture was inoculated at 1% into 50 ml sterile MRS broth without sodium acetate and incubated for 48 h at 30 °C. After incubation, cells were removed by centrifugation at 10,000 RPM for 15 min at 4 °C and the supernatant was filter sterilized (0.45 µm pore size) to get CFS. The obtained CFS was freeze dried and reconstituted with Milli-Q water to 10 X concentration was screened for antifungal activity.

Influence of heat and pH on antifungal activity

To evaluate the antifungal compounds in CFS (10X concentrated) for heat and pH, the CFS was subjected to heating (121 °C/15 min/15 psi) and different pH range (4, 5, 6 and 7) and then antifungal activity was determined by agar well diffusion method as described by Roy et al. (1996).

Determination of antifungal components

The PLA and organic acids (lactic and acetic acid) in the CFS of *Lactobacillus* strain was determined using HPLC method as described in Mu et al. (2012) and Kishore et al. (2013), respectively, with slight modifications. All culture was grown in MRS broth and incubated at 30 °C for 48 h. The CFS obtained was treated with acetone to remove protein content, one volume of CFS was treated with three volumes of cooled acetone (acetone stored at -20 ºC overnight) and then vigorously shaken for 2 min, after that, it was kept at -20°C for overnight). The precipitated protein was removed by centrifugation at 12000 RPM for 15 min at 4°C and the rest of the supernatant was dried. The dried samples were reconstituted in HPLC grade 50 mM phosphate buffer (6.5 pH) and filter sterilized (0.22 µm) before injection in the UFLC

system (Schimadzu) equipped with equipped with Phenomenex C18 (250 X 4.6 mm) column.

SEM analysis of fungal hyphae

The hyphae samples harvested after treated with CFS for 24 h in YPD broth were fixed on a glass coverslip with 2.5% glutaraldehyde for 4 h at 4°C. The samples were subsequently washed with 0.2 mol/L sodium phosphate buffer for 20 min and incubated in 1% osmic acid solution at 4°C overnight. The samples were first dehydrated for 20 minutes in 50% ethanol with two repeats, then for 20 minutes in 50, 60, 70, 80, 90, and 100% ethanol, respectively. The fixed samples were soaked in 100% isoamyl acetate twice for 20 min each and finally dried. Then the samples were sputter coated with palladium gold in Emitech K550 and the results were observed using SEM (Zeiss DSM 940 A) (Ahmad Rather et al. 2013).

Statistical Analysis

The experiments were performed in triplicates and the data are expressed as mean ± standard deviation. The obtained data was analysed for analysis of variance and significance was determined at confidence level of 95% using SAS Software.

Results and discussion

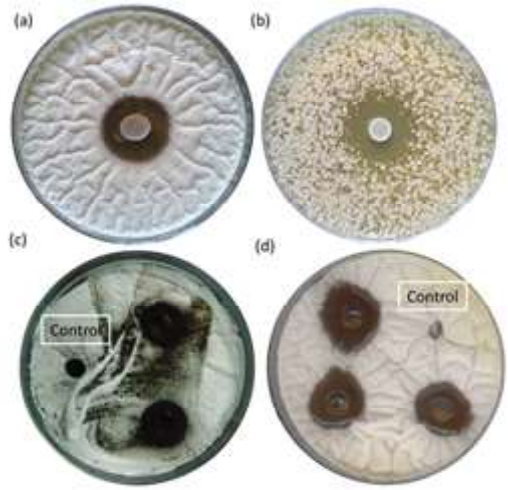

Fig. 1 Antifungal activity of CFS of *Lactobacillus* C-14 against a) *A. niger* NCDC 315 b) *C. butyri* by overlay assay and against c) *A. flavus d) A. niger* NCDC 267 by well assay

The preliminary screening of the antifungal activity of among the strains revealed growth of all fungi was inhibited in the presence of lactobacilli strains (table 1). The strains C9 and C14 demonstrated the strongest activity against *A. niger* mold. *G. candidum* was less sensitive toward all the studied strains. The

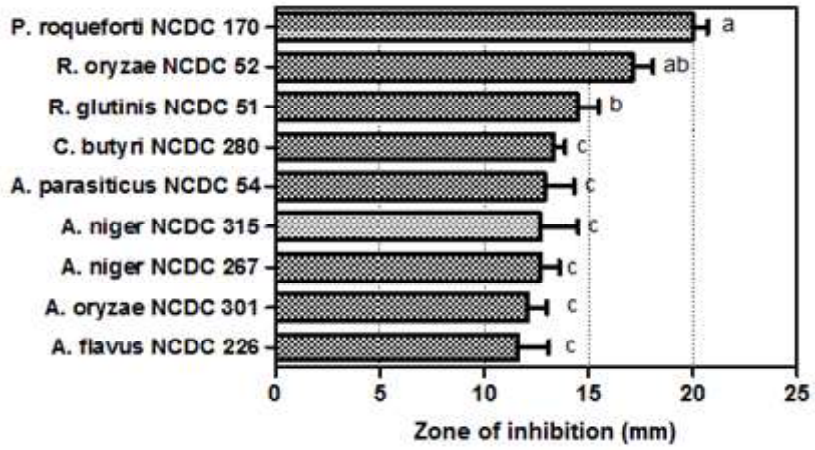

Fig. 2 The antifungal activity of CFS of *L. fermentum* C-14 against the selected fungal species. Data are mean ± standard deviation (n=3). The bars with the same lowercase letter are significantly different (P<0.05).

Table 1 Inhibition spectrum of *Lactobacillus* species against different fungi

Lactobacillus cultures	Fungal species		
	C. butyri NCDC 280	*G. candidum* NCDC 228	*A. niger* NCDC 315
Lb- C4	++	+	++
Lb- C5	++	+	++
Lb- C6	++	+	++
Lb- C9	++	+	+++
Lb- C10	++	+	++
Lb- C14	+++	+	+++

strain Lb C-14 had the highest antifungal activity against *A. niger, C. butyri,* and *G. candidum.* The study of antifungal activity of Lb C-14 was deepened on different fungi by well diffusion assay and results showed a consistent inhibition of growth (Fig 1).

Table 2 Fermentation profile of *Lactobacillus sp.* C-14 strains as per API test

Sugar	*Lactobacillus sp.* C-14
Control	-
Glycerol	-
Erythritol	-
D-Arabinose	-
L-Arabinose	-
D-Ribose	+
D-Xylose	-
L-Xylose	-
D-Adonitol	-
Methyl-âD-Xylopyranoside	-
D-Galactose	+
D-Glucose	+
D-Fructose	+
D-Mannose	-
L-Sorbose	-
L-Rhamnose	-
Dulcitol	-
Inositol	-
D-Mannitol	-
D-Sorbitol	-
Methyl-áD-Mannopyranoside	-
Methyl-áD Glucopyranoside	-
N-AcetylGlucosamine	-
Amygdalin	-
Arbutin	-
Esculin ferric citrate	-
Salicin	-
D-Cellobiose	-
D-Maltose	-
D-Lactose(bovine origin)	+
D-Mellibiose	+
D-Saccharose	+
D-Trehalose	-
Inulin	-
D-Melezitose	-
D-Raffinose	+
Amidon (starch)	-
Glycogen	-
Xylitol	-
Gentibiose	-
D-Turanose	-
D-Lyxose	-
D-Tagatose	-
D-Fucose	-
L-Fucose	-
D-Arabitol	-
L-Arabitol	-
Potassium Gluconate	-
Potassium 2-KetoGluconate	-
Potassium 5-KetoGluconate	-

Among the fungal cultures tested better inhibition was recorded against *P. roqueforti* with the zone of inhibition 20±0.7 mm and thus can be considered as the most sensitive mold towards *L. fermentum* C 14 (Fig 2). Therefore *L. fermentum* C 14 isolated from Chilika dahi shown a high inhibitory activity against different fungi growth and was used for further study of antifungal properties.

Muhialdin et al. (2011) studied the antifungal activity of 137 LAB isolated from Malaysian fruits and fermented foods by using agar overlay method against *A. oryzae*, reported that *L. fermentum* Te007, *P. pentosaceus* Te010, *L. pentosus* G004, and *L. paracasei* D5 were the most effective. Bazukyan et al. (2018) screened eight strains of *Lactobacillus* sp. isolated from Armenian dairy products to assess their antifungal activity in MRS media. The authors reported that *L. rhamnosus* MDC 961 was effective against *G. candidum, T. viride* and *A. flavus* and recognized the activity of proteinaceous compounds present in CFS. Recently, out of 351 LAB strains isolated from naturally fermented Chinese vegetable samples only *L. crustorum* NWAFU 1060 strain was reported to produce the highest producer of PLA as an antifungal compound (Xu et al. 2021).

The sugar fermentation pattern of *Lactobacillus* C-14 strain is shown in Table 2. The 16s rRNA gene sequence obtained in this study were analysed Insilco using BLAST of NCBI, the cultures *Lactobacillus* C-14 showed 98% similarity to *L. fermentum* (Fig 3). The analysed sequence of *Limosilactobacillus fermentum* C-14 has been submitted to the GenBank database under accession number KC713956.

To assess the characteristics of antifungal compounds, the CFS from *L. fermentum* C-14 was subjected to high temperature (121°C for 15 min) and pH modifications (pH from 4 to 7). Overall the CFS has retained its activity against *P. roqueforti* NCDC 170 after heat treatment ($P>0.05$). But the pH modifications revealed that pH above 6 has significantly caused the reduction in antimold activity. These results suggest that antifungal compounds in CFS can tolerate heat denaturation and are likely to be acidic compounds. In previous studies (Zaiton et al. 2011, Bazukyan et al. 2018, Ramos et al. 2021) similar approach has been reported the use of heat and pH stability to assess the antifungal characteristics of CFS of *L. fermentum* te007*, L. pentosus* g004*, L. rhamnosus* MDC 9661 and *P. pentosaceus* te010 against *A. niger, A.oryzae, P. aurantioviolaceum* and *M. plumbeusin* and attributed their antifungal activity to the production of organic acids. In this study, the MRS broth medium fermented by *L. fermentum* C-14 was acidified to a pH of 4 to 4.5. When the pH was increased to neutral the antifungal property of CFS was lost. Similarly, Cortés-Zavaleta et al. (2014) observed that the antifungal activity of culture filtrate of *L. acidophilus* ATCC 4495 was stable at low pH and was drastically reduced at pH 6.5. The possible reason behind the antifungal activity at low pH could be the involvement of organic acids in the antifungal activity.

Fig. 3 Phylogenetic analysis of *L. fermentum C 14*

Table 3: Organic acids present in CFS of *L. fermentum* C-14

Compound	Concentration
Lactic acid	16.3 ± 0.32 mM
Phenyl lactic acid	0.324 ± 0.01 mM
Acetic acid	5.7± 0.81mM

Fig 4 is the UFLC chromatogram of CFS from *L. fermentum* C14 grown in MRS broth at 30 °C for 48 h. The chromatogram revealed that retention time of PLA (15.1 min) was similar for the retention time of compound in CFS of *L. fermentum* C14. Previous studies (Valerio et al. 2004, Gerez et al. 2013, Zhao et al. 2023) have reported PLA production as a fermentation product by *Lactobacillus* sp and major compound responsible for inhibitory effects against a wide range of fungi. Cortés-Zavaleta et al. (2014) reported PLA production 20 and 21.5 mg/L in *L. fermentum* NRRL B-1932 and *L. fermentum* ATCC 11976, respectively. Likewise, in the present study, *L. fermentum* C-14 was able to produce PLA at a relatively higher level up to 0.32±0.01 mM (54.7 mg/L) (Table 3). In addition, UFLC chromatogram of CFS of *L. fermentum* C-14 showed a sharp peak at a retention time 6.31 min, the same retention as it recorded for pure acetic acid with a retention time of 6.31 min. About 5.7± 0.81mM of Acetate was quantified in the processed CFS of *L. fermentum* C-14. The yield of L-Lactate was 16.3 ± 0.32 mM. Thus PLA need not be the only metabolite in CFS responsible for antifungal property as reported by previous researchers. Bian et al. (2016) identified the inhibitory activity of cheese isolate *L. helveticus* KLDS 1.8701 against *Penicillium* species, the responsible antifungal compound was identified as Lactic and acetic acid. Other researchers (Cortés-Zavaleta et al. 2014, Jung et al. 2019, Riolo et al. 2023) identified organic acids and ethanol etc along with lactic acid as fermentation products of LAB having antimicrobial activity and highlighted the possibility of a synergistic effect between the compounds. Thus, knowing the chemical structure and properties of metabolites of LAB is necessary for a complete understanding of possible interactions.

The scanning electron microscope (SEM) was used to reveal the morphological changes on the hyphae of *P. roqueforti* NCDC 170 (Fig 5). In control group, the hyphae was smooth and had tubular morphology. Whereas the hyphae treated with CFS had a rough and disrupted surface and there was cytoplasmic leakage around the hyphae. Previous literature on the investigation of antifungal mechanism has also shown damaged and distorted hyphae with shrivelled and crinkled cell walls, flattened hyphae and reduced hyphae in mold hyphae treated with CFS of *L. plantarum* strains (Sangmanee and Hongpattarakere, 2014). However, the mechanism behind the disruption of hyphae is not fully understood. Only a few mechanisms have been proposed by researchers, *L. plantarum* 29 first attaches to *Penicillium* species hyphae and then colonizes on it, their colonization results in the formation of depressions on hyphae as results damage of hyphae that leads to the inhibition of mould (Sorrentino et al. 2013). Our results also indicate the antifungal compounds in CFS of *L. fermentum* C-14 damage the structural integrity of *P. roqueforti* NCDC 170 and significantly affect the development of mold mycelia.

The fact that LAB produces a significant quantity of acidic metabolites from sugar in the medium that varying antimicrobial activity is well recognized. *Lactobacillus* sp. can produce organic acids that are antifungal such as lactate, acetate, PLA, succinic acids etc. The low pH of 3 to 5 pH favours the undissociated state of organic acids to enter the fungal cells and dissociate within higher pH cytosol causing acidification of the cytoplasm. It can cause the fungal cell to be suppressed or die (Batish et al. 1997, Li et al. 2014, Jung et al. 2019). However many of them are active against bacteria and some organic acid compounds are active against yeast and molds (Wang et al. 2012, Valerio et al. 2016). For many decades the extended shelf life of fermented milk products was attributed to lactic acid and acetic acid produced by LAB. Studies have shown that lactic acid is less effective

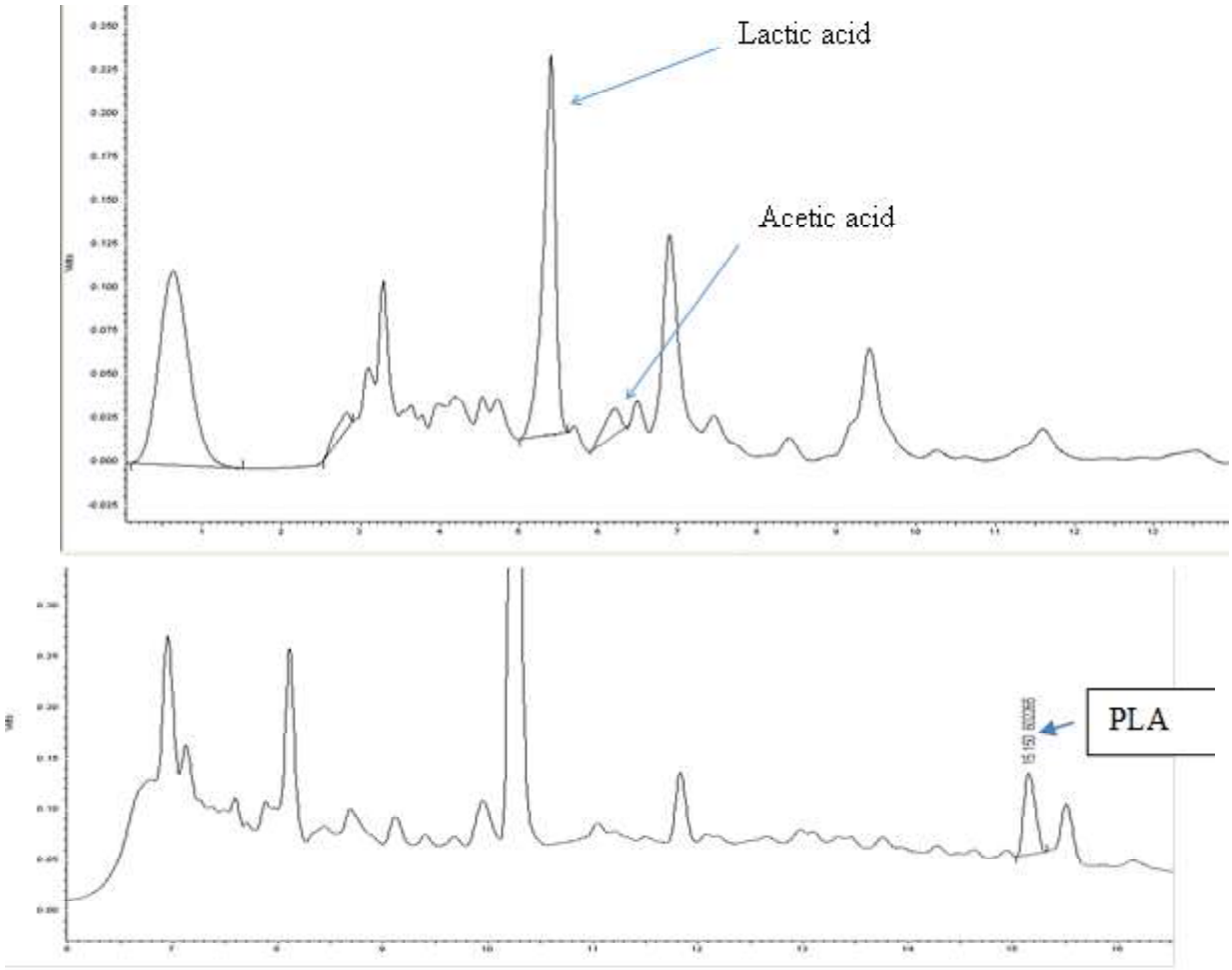

Fig. 4 UFLC chromatograms of lactic acid and acetic acid (A) and PLA (B) in CFS of *Lactobacillus* sp. C-14

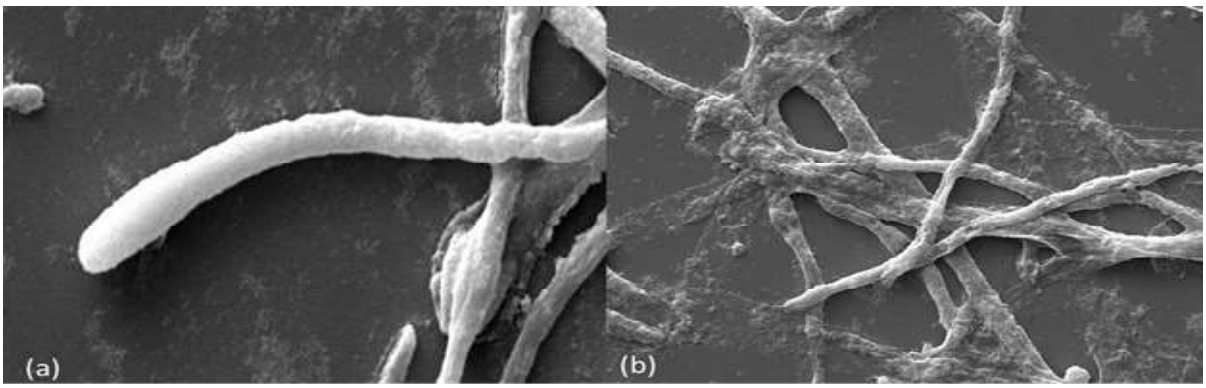

Fig. 5 Scanning electron microscope images of effect of *L. fermentum* C-14 on hyphae of *P. roqueforti* 170. a) Control group b) Experimental group

against fungi while acetic acid and phenyl lactic acid are strongly inhibitory to fungi (Jung et al. 2019, Xu et al. 2021). This study also highlights the importance of studies to discover new LAB strains that have better antifungal activity and the nature of metabolism products.

Conclusions

The *Lactobacilli* sp. isolated from Chilika dahi have shown antifungal activity especially *Limosilactobacillus fermentum* C-14 shown the highest activity. The antifungal activity of this strain remained stable to high temperature and at low pH levels

confirmed the acidic nature of antifungal compounds. The results confirmed that mixture of different organic acids produced are responsible for inhibition of molds. The SEM images showed distorting of the morphology of mold hyphae as a result of treatment by CFS. A further investigation into the structure and interaction between the compounds in CFS on antifungal activity is required. *L. fermentum* C-14 with high antifungal activity has the potential to be exploited as a promising bio-preservative for acidic food products.

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