Journal of Advances in Microbiology

4(3): 1-8, 2017; Article no.JAMB.31386
ISSN: 2456-7116

Inhibitory Properties of Lactic Acid Bacteria against Moulds Associated with Spoilage of Bakery Products

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Authors' contributions

This work was carried out in collaboration between all authors. Author IAA designed the study, wrote the protocol and the first draft of the manuscript. Authors AOO and DJA managed the analyses of the study. Author IAA managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMB/2016/31386
Editor(s):
(1) Ana Claudia Correia Coelho, Department of Veterinary Sciences, University of Trás-os-Montes and Alto Douro, Portugal.
Reviewers:
(1) Paul Yahaya Idakwo, University of Maiduguri, Nigeria.
(2) Md. Fakruddin, Bangladesh Council of Scientific and Industrial Research (BCSIR), Bangladesh.
(3) María De Lourdes Reyes Escogido, Universidad de Guanajuato, México.
Complete Peer review History: http://www.sciencedomain.org/review-history/19863

Original Research Article

Received 3rd January 2017
Accepted 18th February 2017
Published 4th July 2017

ABSTRACT

Aim: To evaluate the potentiality of LAB strains isolated from different fermented products to inhibit moulds associated with spoilage of bakery products.
Methodology: Lactic acid bacterial (LAB) strains obtained from fermented products ("burukutu", "pito", yoghurt, and "iru") were screened for antifungal activity against moulds (*Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus repens* and *Penicillium* sp.) isolated from spoilt bakery products. Inhibitory activities of the lactic acid bacterial isolates were determined by the dual agar overlay method and well diffusion method.
Results: Eleven isolates (79%) out of the fourteen lactic acid bacterial strains screened showed antifungal activity against one or more of the moulds when the method used was dual agar overlay method. When agar well diffusion assay was used to check the antifungal activity of the cell-free culture supernatants of these eleven LAB isolates after excluding inhibition due to organic acids and hydrogen peroxide, only four LAB strains (BE1, IO1, PO4 and PO9) continued to show antifungal activity against the moulds. The selected bacteriocin-producing LAB strains (BE1, IO1, PO4, and

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PO9) were identified as *Lactobacillus cellobiosus*, *Pediococcus pentosaceus*, *Lactobacillus rhamnosus*, and *Tetragenococcus halophilus* respectively using API 50 CHL system. The cell-free culture supernatant of *P. pentosaceus* IO1, *T. halophilus* PO9, and *L. cellobiosus* BE1 sprayed on bread surface inhibited the growing of moulds during 14 days of storage in polythene bags.
Conclusion: These LAB isolates from fermented products capable of inhibiting the moulds have potential that could be used as biopreservative agents in bakery products.

Keywords: Antifungal activity; lactic acid bacteria; mould; bakery; spoilage.

1. INTRODUCTION

Bakery products are the important staple foods in most countries and cultures. Bakery products such as breads and cakes are essential food items of the vast majority of the world population [1]. Mould spoilage is the main cause of substantial economic loss in bakery industry and might also cause public health problems due to the production of mycotoxins. The reduction of mould growth in bakery products is thus, of crucial importance and there is great interest to develop safe and efficient strategies for this purpose [2].

Lactic acid bacteria (LAB) have a long history of safe use in food and are generally recognized as safe. LAB are known to produce a wide variety of antagonistic compounds, including lactic acid, hydrogen peroxide, and bacteriocins [3,4]. The use of LAB to control moulds is a good alternative approach to physical and chemical preservation methods normally applied in food products. Careful selection of specific strains of lactic acid bacteria with antifungal properties can allow the reduction of moulds and can, therefore, improve the shelf-life of many fermented products and reduce the presence of mycotoxins [5].

The objective of this study was to evaluate the potentiality of LAB strains isolated from different fermented products to inhibit moulds associated with spoilage of bakery products.

2. MATERIALS AND METHODS

2.1 Lactic Acid Bacterial Strains

Lactic acid bacterial (LAB) strains from fermented products (“burukutu”, “pito”, yoghurt, “wara”, and “iru”) procured from sellers were isolated using de Man Rogosa Sharpe (MRS; Oxoid Ltd) agar and incubated at 30℃ for 48 h under anaerobic conditions [6]. The LAB strains were then stored at 4℃ in MRS broth and MRS agar slants.

2.2 Isolation and Identification of Fungal (mould) Strains

Loaves of bread and cakes were stored at room temperature (28 ± 2℃) for 4 – 7 days to allow mould growth on them. Moulds in pure culture were then isolated from mouldy breads and cakes using streak plate method and inoculated onto the surface of malt extract agar medium, which were incubated afterward for the growth of mould colony at 28 ± 2℃ for 5 days [7]. The mould cultures were preserved at 4℃ and identification was done on the basis of morphological and microscopic characterization [8].

2.2.1 Fungal inocula preparation

Moulds isolated from loaves of bread and cakes were grown on malt extract agar medium at 28 ± 2℃ for 5 days. A sterilized cork borer was used to punch out agar disk containing the spores from agar plate into 5 ml sterile distilled water to disperse the spores. The mould suspension was adjusted to give approximately 10^7 spores/ ml using sterile distilled water [7].

2.3 Antifungal Activity Assay

2.3.1 Initial screening for antifungal activity by overlay method

Inhibitory activity of the lactic acid bacteria isolates was determined by the overlay method as described by Muhialdin and Hassan [9] with a slight modification. Lactic acid bacteria were inoculated in 2 cm lines on MRS agar plates and incubated anaerobically at 30℃ for 24 h. The plates were then overlaid with 10 ml of malt extract soft agar containing 10^7 spores/ml of each mould and incubated aerobically. After 48 h of aerobic incubation at 28 ± 2℃, zone of inhibition was observed. Inhibitory activity was scored as follow: -, no inhibition; +, very weak inhibition; ++, low inhibition with a little clear zone “near the

edge of the colony"; +++, strong inhibition with a large clear zone.

2.3.2 Detection of antifungal activity of cell-free culture supernatant by well-diffusion method

The anti-mould activity of the cell-free supernatant from LAB isolates which showed inhibitory activity was determined by well-diffusion assay as described by Muhialdin and Hassan [9]. The LAB isolates were grown in de Man Rogosa Sharpe (MRS) broth separately for 48 h at 30℃. The broth culture was centrifuged at 4000 x g for 20 min to get the cell-free supernatant. Fungi 10^7 spore/ml was mixed with molten malt extract agar and allowed to solidify. Then, wells of size 6 mm were made using cork borer and 20 µl of malt extract agar was pipette to cover the base of the well to avoid leaking of the supernatant. Eighty (80) microlitres of LAB supernatants (pH-neutralized cell-free culture supernatant with addition of catalase enzyme) were added to different wells and the plates were incubated at room temperature (28 ± 2℃) for 48 h. The zone of inhibition of mycelia growth was measured with a ruler and recorded in millimetres.

2.4 Identification of LAB Isolates

LAB that possessed inhibitory activity after excluding inhibition due to organic acids and H_2O_2 from their supernatants were selected for identification using API 50CH Kit (Bio Merieux, France).

2.5 Screening for Anti-mould Activity of LAB on Bread Surface

Three LAB strains were tested for anti-mould activities on the bread surface using the method described by Cizeikiene et al. [10] with some modifications. Cell-free culture supernatant of the LAB strains grown in MRS broth for 48 h at 30℃ were obtained by removing the cells mass following centrifugation at 4000 g for 15 min and sterilizing the supernatant by filtration through 0.45 µm pore filter (Millipore). The cell-free culture supernatant was divided into two parts. One part of supernatant was used for the determination of antimicrobial activities of LAB supernatant (total metabolites), whereas the other part of supernatant sample was adjusted to pH 6.5 with 1 M NaOH to eliminate the organic acids effect. This part was also treated with 1 mg catalase/ ml (Sigma, St Louis, MO, USA) to eliminate the activity of hydrogen peroxide and thus to evaluate potential activity of bacteriocin.

The LAB supernatant and pH-neutralized supernatant treated with catalase (crude bacteriocins) were sprayed on the surface of baked bread (1 ml per sliced bread). For the control sample, the LAB supernatant was not used. The bread samples were stored for 14 days at room temperature (27 ± 2℃) in transparent nylons. The loaves (sliced bread) were examined physically/ externally for presence of mould according to the following scale: (-) no mould growth; (+) small visible mould growth; (++) large mould growth.

3. RESULTS

The colonial and morphological features of the fungal isolates from spoilt bakery products are shown in Table 1. The fungal isolates B1, B2, B4, and C1 were identified as *Aspergillus fumigatus*, *Aspergillus repens*, *Penicillium* sp. and *Aspergillus flavus*. Table 2 shows the inhibitory activities of lactic acid bacterial (LAB) isolates against spoilage moulds as determined by the overlay method. Eleven (11) isolates out of the fourteen (14) LAB strains screened showed antifungal activity against one or more of the moulds. Although the inhibition zones varied with the lactic acid bacteria as well as with the mould strain tested. *Aspergillus flavus*, which was the most sensitive indicator strain, was inhibited by most of the LAB strains. Fig. 1 shows the clear zones of inhibition of growth of *A. flavus* formed around colonies of LAB strains (IO1, BE1, and PO9) on MRS agar.

When agar well diffusion assay was used to check the antifungal activity of the cell-free culture supernatants of the eleven LAB isolates after excluding inhibition due to organic acids and hydrogen peroxide, only four LAB strains (BE1, IO1, PO4, and PO9) continued to show antifungal activity against the moulds (Fig. 2).

Identification using apiweb^{TM} software version 5.1 showed that LAB strains BE1, IO1, PO4, and PO9 were identified as *Lactobacillus cellobiosus*, *Pediococcus pentosaceus*, *Lactobacillus rhamnosus*, and *Tetragenococcus halophilus* respectively (Table 3).

Table 1. Colonial and morphological features of the fungal isolates from spoilt bakery products

Fungal isolate	Morphological features	Microscopic characteristics	Suspected organism
B1	Fluffy surface with dark brown-coloured spore heads	Conidiophores upright, terminating in clavate swelling bearing philides at the apex.	*Aspergillus fumigatus*
B2	Green coloured spore heads	Hyphae are septate, simple and thick-walled. Conidiophores bearing conidial heads containing conidia are seen.	*Aspergillus repens*
B4	Gray-green-coloured spore heads	Hyphae are septate, with smooth-walled conidiophores bearing long chains of conidia.	*Penicillium* sp.
C1	Fluffy white surface with yellowish green-coloured spore heads	Conidia are one-celled, globose in dry biaseptal chain. Conidiophores are upright, radiating from the entire surface.	*Aspergillus flavus*

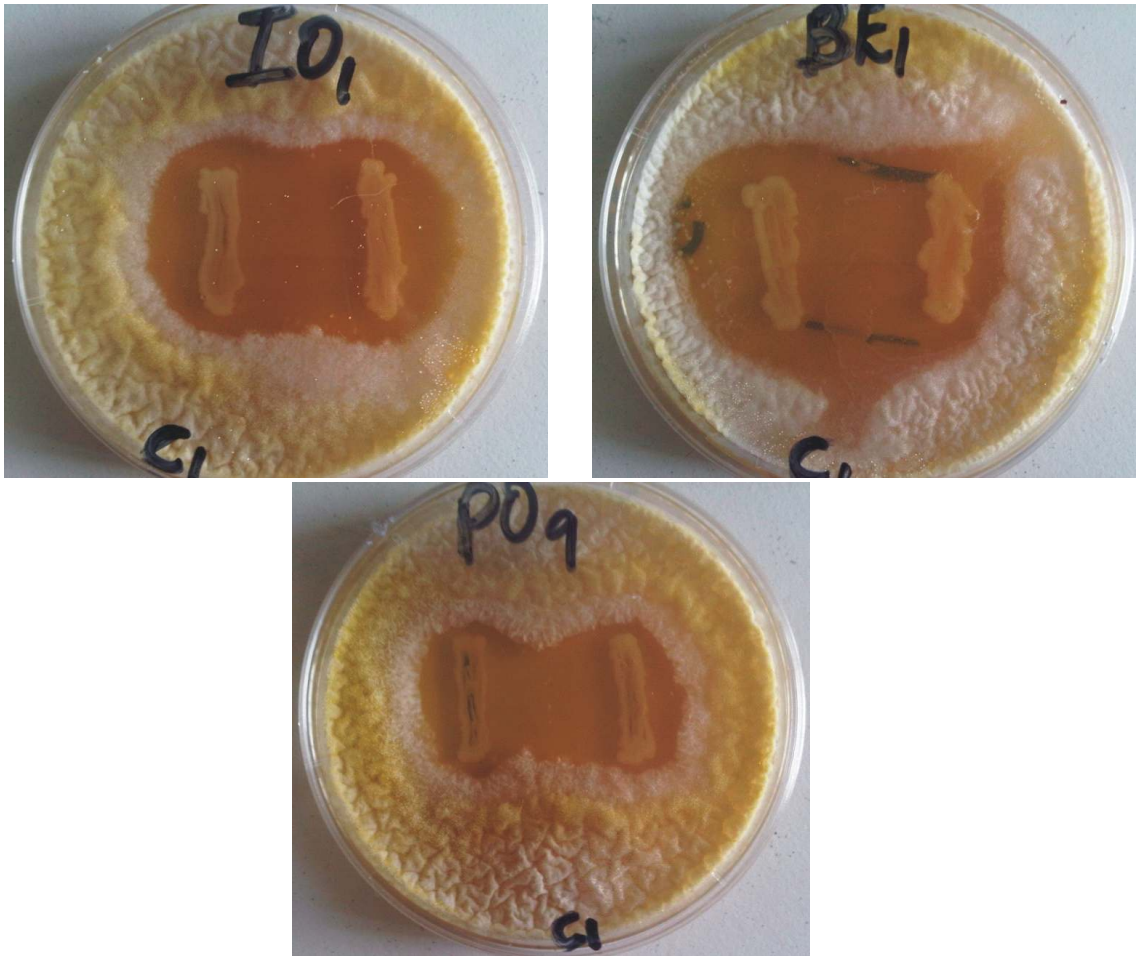

Fig. 1. Clear zones of inhibition of growth of *A. flavus* formed around colonies of LAB strains (IO1, BE1, and PO9) on MRS agar

Table 2. Inhibitory activity of LAB isolates against spoilage moulds as determined by the overlay method

LAB strains	Moulds			
	Aspergillus fumigatus	***A. repens***	***Penicillium* sp.**	***A. flavus***
IO1	++	+++	++	+++
BO2	+++	+	+	+++
BE1	+	+++	+	+++
YO5	++	++	++	+++
YO7	++	+++	+	++
YO9	-	-	-	++
YO2	-	-	-	+
YO4	-	-	-	-
PO4	++	++	++	-
PO9	+	++	-	++
BR1	-	-	-	-
BR2	-	-	-	-
BR3	-	-	-	+
BR4	-	+	-	+

Key: -, No inhibition; +, very weak inhibition; ++, low inhibition with a little clear zone; +++, strong inhibition with a large clear zone

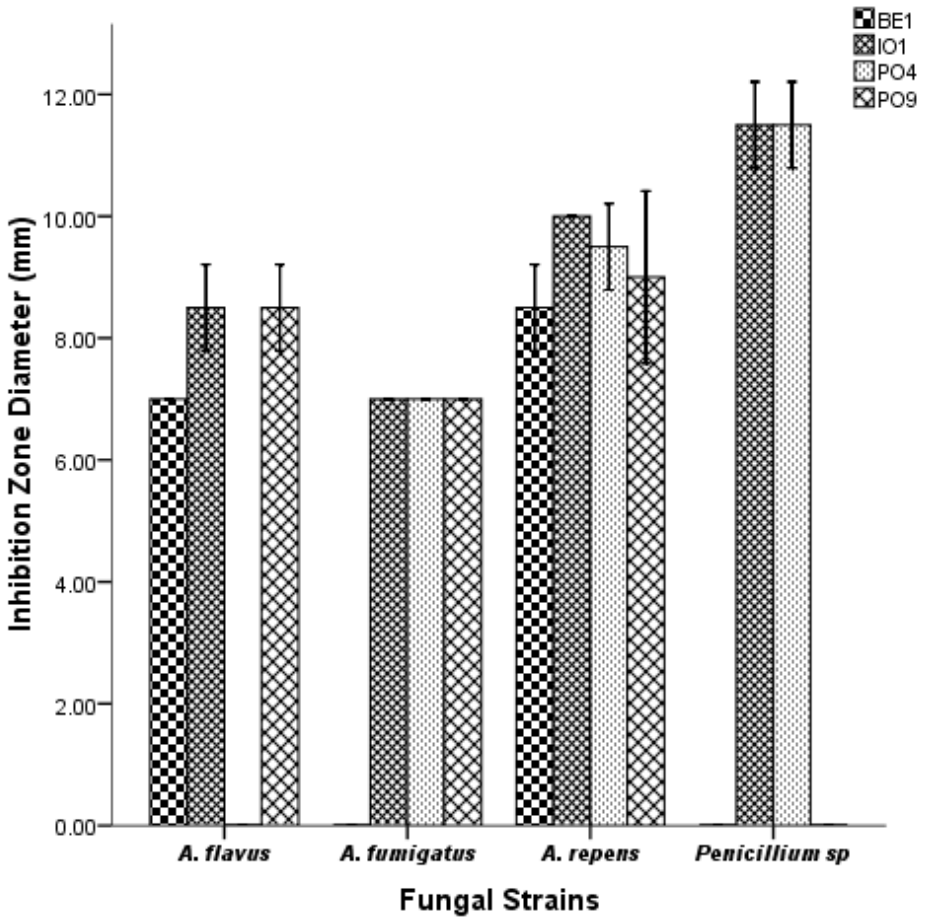

Fig. 2. Inhibition of moulds by bacteriocin-containing cell-free culture supernatants of four selected lactic acid bacteria

The LAB supernatants and crude bacteriocins of *P. pentosaceus* IO1, *T. halophilus* PO9, and *L. cellobiosus* BE1, sprayed on bread surface, inhibited the growing of moulds during 14 days of storage (Table 4); whereas on the control bread sample, large mould growths were detected after 14 days of storage.

Table 3. Identification of isolates using apiweb software v. 5.1

LAB isolate	Source	Identified isolate
BE1	Burukutu	*Lactobacillus cellobiosus*
IO1	Iru	*Pediococcus pentosaceus*
PO9	Pito	*Tetragenococcus halophilus*
PO4	Pito	*Lactobacillus rhamnosus*

Table 4. Effect of cell-free culture supernatant of LAB isolates on mould growth of bread surface after 14 days storage

LAB strains	Treatment	
	Supernatant (total metabolites)	Crude bacteriocin
P. pentosaceus IO1	-	-
T. halophilus PO9	-	+
L. cellobiosus BE1	-	+
Control	++ (MRS broth)	++ (Not sprayed)

Key: (-) No mould growth; (+) Small visible mould growth; (++) Large mould growth.
The dimension of sliced bread was 1.3x11x10.5 cm

4. DISCUSSION

The four most common fungal isolates that were obtained from spoilt bread and cake samples in this study were identified as *Aspergillus fumigatus*, *A. repens*, *A. flavus* and *Penicillium* sp. Similarly, Mishra et al. [11] isolated *Aspergillus flavus* and *Penicillium oxalicum* from spoiled bread and cake samples. Abellana et al. [12] had reported *Aspergillus* and *Penicillium* isolates which spoiled sponge cake. Filamentous fungi (moulds) involved in spoilage of bakery products include *Aspergillus* sp., *Penicillium* sp., *Rhizopus* sp., *Monilia* sp., *Mucor* sp., and *Eurotium* sp. [1]. In addition to the economic losses associated with the spoilage of bakery products by moulds, another concern is the possibility of mycotoxins production [1]. The occurrence of mycotoxinogenic moulds such as *Aspergillus* and *Penicillium* in foods is potentially dangerous for public health and also constitutes a major economic problem [13]. The screening of some selected lactic acid bacteria strains for antifungal activity against the spoilage moulds using agar-well diffusion method demonstrated that only four LAB strains; *Lactobacillus cellobiosus* BE1, *Pediococcus pentosaceus* IO1, *L. rhamnosus* PO4, *Tetragenococcus halophilus* PO9 were able to produce bacteriocins with antifungal activities. *Pediococcus pentosaceus* IO1 was most effective in the inhibition of *Aspergillus repens* when compared with the other LAB isolates.

Studies concerning antibacterial proteinaceous compounds, such as bacteriocins, are extensive in comparison to proteins with antifungal properties, although during the last decade various LAB-derived proteinaceous compounds with anti-yeast and anti-mould abilities have been identified [14-16]. Adebayo and Aderiye [15] reported that only 17 of the 25 bacteriocin-producing LAB isolates from some fermented foods (“eko”, “fufu”, “iru”, and “ogi”) possessed antifungal activity against *Penicillium citrinum*, *Aspergillus niger* and *A. flavus*. *Lactobacillus rhamnosus* VT1 was reported to inhibit the growth of spoilage and toxigenic fungi species in the genera *Aspergillus*, *Penicillium*, and *Fusarium* [17]. Muhialdin and Hassan [9] found that *Lactobacillus brevis* G004, *Lactobacillus fermentum* Te007 and *Pediococcus pentosaceus* Te010 isolated from fermented guava juice and tempeh possessed strong antifungal activity against *Aspergillus oryzae*. Kim [18] also reported that the five *Lactobacillus* strains isolated from Kimchi exhibited antifungal activity against several fungi and concluded that some different compounds produced by the bacteria caused the inhibitory activity.

Lactic acid bacteria are well known for their antifungal activity, which is related to the production of a variety of compounds including organic acids, diacetyl, reuterin, hydrogen peroxide, phenyllactic acid, bacteriocins and cycle peptides [19-23]. The organic acids are active at low pH and the activity relies on the un-dissociated form of the acids. Recently, interest has dramatically increased in the use of bioactive peptides produced by LAB as an antifungal agent. The use of protein-like compounds such as bacteriocins are preferred over the use of acids because their activity is present over a wide range of pH and they are heat stable compounds which are ideal for use in heat processed foods [24].

The findings in this study using sprayed LAB supernatant on bread surface is in agreement with the report of Cizeikiene et al. [10] which indicated that effective inhibition of undersirable microorganisms could be achieved by LAB for preventing bread moulding.

5. CONCLUSION

Based on the findings of this study, it can be concluded that the selected LAB strains from different fermented products possess inhibitory activity against the spoilage moulds and could be used as biopreservatives in bakery products.

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:
http://sciencedomain.org/review-history/19863