

Exploring lactic acid bacterial metabolites for antimicrobial activity against spoilage organisms of grapes and potato

Abstract:

Biopreservation of foods is an alternative and novel method of preservation with increased special interest from the consumers. Lactic acid bacteria (LAB) has the potentiality in restricting microbial proliferation of foods, thus preventing spoilage and extending their shelf life. The antimicrobial capabilities of lactic acid bacteria are linked to various actions by the production of antimicrobial compounds such as bacteriocins, hydrogen peroxide, organic acids, etc. The study aims at exploring LAB from traditional foods for biopreservation of grapes and potato. LAB were isolated from twelve different food samples viz., mango pickle, curd, cumbu gruel, neera, cold rice, finger millet, infant feces, milk, lime pickle, fermented cumbu (grain), fermented cumbuflour and whey water and their cell free metabolites were extracted. A total of thirteen bacteria and seven fungal cultures were isolated from spoiled grapes and potato. An *in vitro* experiment was conducted to study the antimicrobial activity of LAB metabolites by performing agar well diffusion assay against the spoilage cultures. The results revealed varied antimicrobial activity of LAB isolates to different spoilage cultures from potato and grapes. Among the LAB isolates, metabolites from curd, cumbu gruel, fermented cumbuflour, finger millet and neera cultures were observed to inhibit more number of spoilage organisms and produced maximum inhibition zone. A consortium was prepared by pooling the metabolites from these promising cultures and assayed for antimicrobial activity which confirmed the above results.

Keywords: Lactic acid bacteria, metabolite, spoilage organisms, consortium

Introduction:

Biopreservation is a new and innovative approach to control food spoilage is growing significance for several industries and consumers. Use of coatings on fruits and vegetables for extending the shelf life is a practice followed for decades. Edible coatings in foods include organic or inorganic compounds, proteins, lipids, waxes, resins either alone or in combination (Hernandez, 1994). Biopreservation could be highly helpful in extending the shelf life of fresh-cut fruits and vegetables by the use of safe, natural or controlled microflora and biologically active compounds of non-toxic nature with food safety.

Lactic acid bacteria constitute a ubiquitous bacterial group that is wide spread in nature in niches of dairy (fermented), meat and vegetable origin, gastrointestinal and urogenital tracts of humans and animals and soil and water. LAB are gram-positive, non-respiring and non-motile microorganisms with optimum pH range between 5.5 and 5.8 that help in extending the shelf life of products. LAB mainly produce lactic acid as by-product during metabolic activities. They play a multifaceted role in agriculture, food and medical fields.

LAB is referred to as probiotics due to their capacity to resist in the gastrointestinal tract or to produce exopolysaccharides, biological preservatives and bacteriocins. These antimicrobial properties can extend the shelf life of fruits and vegetables (Ranadheera *et al.*, 2019). The antimicrobial capabilities of LABs are linked to various actions by the production of antimicrobial compounds such as bacteriocins, hydrogen peroxide, organic acids etc., (Ramos *et al.*, 2020).

Grapes (*Vitis vinifera L.*) are one among the widely consumed fruits in the world. They are highly perishable. Fungal contamination is one of the main causes of economic losses worldwide in the food industry and agriculture (Gerez *et al.*, 2013). Post-harvest decay of grapes is due to grey mold (*Botrytis cinerea*), sour rot (*Aspergillus carbonarius*), *Rhizopus* rot (*Rhizopus stolonifer*) and blue mold (*Penicillium expansum*).

Potato (*Solanum tuberosum L.*) is the world's number one non-grain food commodity predominantly cultivated in Asia and Europe. Difficulties in growing this crop commercially can be partially attributed to its susceptibility to disease, of which late blight caused by the oomycete *Phytophthora infestans* is considered the most important.

There are several drawbacks in using chemical pesticides, particularly for vegetable and fruit production which include fear of general public in using hazardous chemicals, risks of toxic residues in treated products as well as contamination of soil and water. LAB is used as a natural food grade biopreservative against a broad spectrum of undesirable microorganisms. This indicates the potentiality of these bacteria in restricting microbial proliferation of foods, thus preventing spoilage and extending their shelf life.

Materials and methods:

1. Isolation of lactic acid bacteria

Lactic acid bacteria were isolated from mango pickle, cumbu, Neera, cold rice, finger millet, infant faeces, milk, lime pickle and whey water. Cumbu grains and cumbu flour were soaked in sterile water. Both these samples were allowed to ferment naturally for a day. From

the fermented water, 1ml was taken and serially diluted in peptone water blanks and plated using spread plate technique in de Man Rogosa and Sharpe (MRS) agar medium and incubated for 48 to 72 hours. Morphologically different colonies were picked and inoculated in MRS agar to obtain pure cultures.

2. Morphological characters of LAB cultures

Lactic acid bacterial cultures were morphologically examined for shape, arrangement, surface, margin and elevation. Gram reaction test was conducted for all the bacterial isolates.

3. Isolation of spoilage organisms

Spoiled samples of grapes and potato were collected from Vegetable and Fruit Market at Matuthavani Madurai, from retail outlets in the locality of Madurai and also from Students Amenity Centre, Agricultural College and Research Institute, Madurai. These spoiled samples of potato and grapes were taken as sources for isolating bacterial and fungal cultures. One gram of each sample was serially diluted upto 10^{-5} dilution in peptone water blanks. From 10^{-5} dilution and 10^{-3} dilution, 0.5ml was taken and plated in petriplates containing nutrient agar medium and Rose Bengal agar (RBA) medium respectively for isolating spoilage bacteria and fungi. Morphologically distinct cultures were selected and purified. Both bacterial and fungal spoilage organisms were morphologically characterized.

4. Extraction of Cell free extracts of LAB

50 ml MRS broth was inoculated with LAB cultures in 1/100 proportion (v/v) and incubated at 37°C for 72 h. After incubation, broth was centrifuged at 4000 rpm for 15 min, and the supernatant with the secreted metabolites of lactic acid bacteria was separated.

5. *In vitro* – Agar well diffusion assay

Agar well diffusion method was conducted to test the antimicrobial activity of LAB metabolites against the isolated bacterial and fungal spoilage organisms. 1 ml of the test bacterial and fungal isolates was seeded in Nutrient Agar (NA) and Potato dextrose agar (PDA) medium, plated and allowed to solidify. Using sterile cork borer, wells were made onto the seeded agar plate and 50µl of cell free supernatant was added into the wells. The plates were incubated at 37°C for 48 h and observed for the formation of inhibition zone (Adeyemo *et al.*, 2018).

6. Statistical analysis

The data collected from the experiments was statistically analyzed using the R software package. At 5% probability level, the critical difference was identified and tabulated (Panse and Sukhatme, 1967).

Result and discussion

1. Isolation and Morphological characterization of lactic acid bacteria

Lactic acid bacteria were isolated in MRS medium from twelve different sources and they were purified in slant cultures. Colonies of lactic acid bacteria appeared colorless, raised and with entire margin. Isolates of mango pickle, curd, cumbu, neera, cold rice, finger millet, infant faeces, milk, lime pickle, fermented cumbuflour, fermented cumbu grains and whey water were slimy. Colonies were circular shaped. They stained purple during Gram reacting, confirming their Gram positive behaviour. Morphological parameters are given in **Table 1**. Chun-lei *et al.* (2014) had isolated eleven lactic acid bacterial cultures from Inner Mongolian traditional yoghurt which were observed to be Gram positive and their colonies were smooth and sticky. Bogdan *et al.*, (2018) isolated five LAB cultures from Kombucha, all the cultures were recorded as Gram positive, with Colorless punctiform colonies and they were cocci and rod shaped. Ismail *et al.* (2018) isolated and characterized two lactic acid bacteria *viz.*, SK-1 and SK-4 from local cow's milk kefir. Morphology of all the cultures were recorded as coccal shaped, with smooth edges and convex elevation. All the isolates were reported to be Gram positive. Bogdan *et al.*, (2018) isolated five LAB cultures from Kombucha, all the cultures were recorded as Gram positive, with Colorless punctiform colonies and they were cocci and rod shaped. Padmavathi *et al.* (2018) isolated 16 LAB cultures from milk, curd and bovine colostrum and their morphological characters were observed. The cells were recorded as coccal to oval or rods, colonies were raised, smooth, margins were entire, with creamy to translucent texture. All the cultures were recorded to be Gram positive.

2. Isolation of Spoilage organisms from grapes and potato

In this study, ten bacterial and ten fungal cultures were isolated from different spoiled potato samples and three bacterial and 7 fungal cultures were isolated from spoiled grapes samples. Colony morphology of the bacterial and fungal spoilage organisms are given in the **Tables 2 and 3**. All the bacterial cultures had translucent colonies with slimy texture. Some cells were cocci shaped whereas some were rods. The cells stained red in gram staining indicating that they are all gram negative in nature. Frances *et al.* (2021) isolated seven spoilage bacterial organisms in grapes which were circular in shape, entire in margin and smooth surfaced. Most of the cultures were found to be gram positive and some were gram negative.

3. Agar well diffusion assay of spoilage organisms with lactic acid bacteria metabolites

3.1. *In vitro* screening of cell free extracts of LAB for antibacterial activity

The present study aimed to investigate the antibacterial activity of lactic acid bacterial metabolites against thirteen spoilage bacteria isolated from grapes and potato by conducting agar well diffusion assay. The results revealed antibacterial activity against four spoilage bacteria viz., POT1B1, POT2B1, POT3B1 and POT8B1 by inhibiting the growth of bacteria with a maximum inhibition zone of 2.8cm diameter (**Fig1, Table 4**). The LAB metabolites of curd, neera, fermented cumbuflour and whey water were found to inhibit bacterial spoilage, of which, neera produced inhibition zone in three bacterial spoilage organisms. This suggested that the lactic acid bacterial metabolites have antibacterial compounds. Research conducted by Jimenez *et al.*, (2017) on safety traits of major LAB species on *Tocosh* (Peruvian traditional fermented potatoes) showed antibacterial activities as well as biogenic amines production capacity. Steglińska *et al.* (2022) analyzed the metabolic profile of LAB strains isolated from pickled vegetables and milk samples and found that it mainly contained lactic acid, acetic acid, propionic acid and ethanol. They had also registered the strain *Lactiplantibacillus plantarum* KB2 LAB 03 to be highly effective in inhibiting potato phytopathogens namely *Alternaria alternata*, *Alternaria tenuissima*, *Fusarium sambucinum*, *Rhizoctonia solani*, *Colletotrichum coccodes*, *Phomaexigua*, *Streptomyces scabies*, *Fusarium oxysporum*, *Alternaria solani* and *Pectobacterium carotovorum*.

3.2. *In vitro* screening of cell free extracts of LAB for antifungal activity

Agar well diffusion method was carried out for testing the antifungal activity of LAB against seventeen isolated fungal spoilage cultures. Results indicated the inhibition of three fungal spoilage organisms namely POT1F3, GRP1F1 and GRP3F2 with a maximum inhibition zone of 2.3 cm diameter (**Fig.2, Table 5**). The LAB metabolites of curd, cumbu, finger millet, milk, lime pickle and fermented cumbuflour were found to inhibit the fungal spoilage organisms. Among them, metabolite from cumbu produced inhibition zone in three fungal spoilage organisms. Saladino *et al.* (2016) had tested sixteen LAB isolates against *Aspergillus parasiticus* and *Penicillium expansum*. At 1:2, 1:5 and 1:10 concentration, they have reported no antifungal activity, whereas at 1:20 concentration, six lactic acid bacterial cultures had exhibited antifungal activity against *Penicillium expansum*. Taurobet *et al.* (2018) reported that *Pediococcus pentosaceus* RG7B strain exhibited antifungal activities against major contaminating fungi *Aspergillus niger* and *Aspergillus carbonarius*. The LAB strain was also identified to have a strong ability to survive in simulated gastric and intestinal fluids and exhibited significant levels of hydrophobicity, making it a promising

candidate for a probiotic and ochratoxin A removal capabilities.

Conclusion

The research paper concludes that the cell free extract containing lactic acid bacterial metabolites exhibited different antimicrobial property against both bacterial and fungal spoilage organisms isolated from spoiled grapes and potato samples. The study could be extended by formulating a product from the metabolites from promising lactic acid bacterial cultures for practical applications for reducing pre and post harvest losses in the crop.

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Tables

Table 1: morphological characterization of lactic acid bacteria

S.No	LAB cultures	Colour	Margin	Elevation	Texture	Shape	Gram staining
1.	Mango pickle	Colorless	Entire	Raised	Slimy	Round	+ve , Rod shape
2.	Curd	Colorless	Entire	Raised	Slimy, moist	Round	+ve , Rod shape
3.	Cumbu	Colorless	Entire	Raised	Slimy	Round	+ve , Cocci shape

4.	Neera	Colorless	Entire	Raised	Slimy	Round	+ve , Cocci shape
5.	Cold rice	Colorless	Entire	Raised	Slimy	Round	+ve , Rod shape
6.	Finger millet	Colorless	Entire	Raised	Slimy, moist	Round	+ve , Cocci shape
7.	Infant faeces	Colorless	Entire	Raised	Slimy, moist	Round	+ve , Rod shape
8.	Milk	Colorless	Entire	Raised	Slimy, moist	Round	+ve , Rod shape
9.	Lime pickle	Colorless	Entire	Raised	Slimy	Irregular	+ve , Rod shape
10	Fermented cumbu powder	Colorless	Entire	Raised	Slimy	Irregular	+ve , Rod shape
11	Fermented cumbu Grain	Colorless	Entire	Raised	Slimy	Irregular	+ve , Rod shape
12	Whey water	Colorless	Entire	Raised	Slimy	Round	+ve , Rod shape

Table 2: Morphological characterization of bacterial spoilage organism

S. No	Spoilage bacterial cultures	Source	Colour	Margin	Elevation	Texture	Shape	Gram staining & shape
1.	POT1B1	Potato	Translucent	Entire	Raised	Slimy	Round	-ve , Cocci
2.	POT1B2	Potato	Translucent	Entire	Raised	Slimy	Round	-ve , Cocci
3.	POT2B1	Potato	Translucent	Entire	Raised	Slimy	Round	-ve , Cocci
4.	POT3B1	Potato	Translucent	Entire	Raised	Slimy	Round	-ve , Cocci
5.	POT4B1	Potato	Colorless	Entire	Raised	Slimy	Round	-ve , Cocci
6.	POT5B1	Potato	Translucent	Entire	Raised	Slimy	Round	+ve , Rod
7.	POT6B1	Potato	Translucent	Entire	Raised	Slimy	Round	-ve , Cocci
8.	POT7B1	Potato	Translucent	Entire	Raised	Slimy	Round	+ve , Rod
9.	POT8B1	Potato	Translucent	Entire	Raised	Slimy	Round	-ve , Cocci
10.	POT8B2	Potato	Translucent	Entire	Raised	Slimy	Round	-ve , Cocci
11.	GRP1B1	Grapes	Translucent	Entire	Raised	Slimy	Round	+ve , Rod
12.	GRP2B1	Grapes	Translucent	Entire	Raised	Slimy	Round	+ve , Rod

13.	GRP2B2	Grapes	Translucent	Entire	Raised	Slimy	Round	+ve , Rod
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Table 3: Characterization of fungal spoilage organism

S.No	Spoilage fungal cultures	Source	Colony colour
1.	POTF1	Potato	Colorless colour
2.	POTF2	Potato	Colorless colour with yellow centre
3.	POT1F3	Potato	Colorless and brown in colour
4.	POT3F1	Potato	Black colour
5.	POT4F1	Potato	Colorless colony with slightly pink colour
6.	POT5F1	Potato	Colorless colony
7.	POT6F1	Potato	Purplish Colorless colonies
8.	POT6F2	Potato	Blackish colony
9.	POT8F1	Potato	Colorless colony
10	POT8F2	Potato	Colorless colony
11	GRP1F1	Grapes	Colorless colony with black spores
12	GRP1F2	Grapes	Colorless colony with greyish centre
13	GRP1F3	Grapes	Black coloured colony
14	GRP2F1	Grapes	Colorless colony with black colour in middle
15	GRP3F1	Grapes	Colorless colour colony
16	GRP3F2	Grapes	Green powdery colony
17	GRP4F1	Grapes	Blackish colony

Table 4: *In vitro* screening of cell free extracts of LAB for antibacterial activity

Lactic acid bacterial metabolites	Area of Inhibition zone (cm ²)			
	Spoilage bacterial cultures			
	POT1B1*	POT2B1*	POT3B1*	POT8B1*
MP	0.00	0.00	0.00	0.00
Curd	0.00	2.04±0.008 ^a	0.00	0.00
Cumbu	0.00	0.00	0.00	0.00
Neera	1.04±0.024 ^a	1.51±0.028 ^c	0.00	5.80±0.057 ^a
CR	0.00	0.00	0.00	0.00
FM	0.00	0.00	0.00	0.00
IF	0.00	0.00	0.00	0.00
Milk	0.00	0.00	0.00	0.00
LP	0.00	0.00	0.00	0.00
FCG	0.00	0.00	0.00	0.00
FCP	0.00	1.77±0.008 ^b	5.65±0.003 ^a	0.00
WW	0.00	0.00	0.82±0.005 ^b	0.00
S. Ed	0.300	0.810	1.627	1.666
CD(P=0.05)	0.021	0.026	0.005	0.048

*Mean for three replications

Table 5: *In vitro* screening of cell free extracts of LAB for antifungal activity

Lactic acid bacterial metabolites	Area of Inhibition zone (cm ²)		
	Spoilage fungal cultures		
	POT1F3*	GRP1F1*	GRP3F2*
MP	0.00	0.00	0.00
Curd	4.40±0.005 ^a	0.00	2.96±0.054 ^a
Cumbu	1.04±0.027 ^c	1.04±0.008 ^c	1.77±0.018 ^b
Neera	0.00	0.00	0.00
CR	2.64±0.011 ^c	0.00	0.00
FM	2.96±0.029 ^b	0.00	0.82±0.006 ^c
IF	2.04±0.037 ^d	0.00	0.00
Milk	0.00	1.26±0.029 ^b	0.00
LP	0.00	0.00	1.26±0.003 ^d
FCG	0.63±0.013 ^e	0.00	0.00
FCP	2.96±0.052 ^b	0.00	1.51±0.015 ^c
WW	0.00	0.00	0.00
S. Ed	1.546	0.450	1.002
CD(P=0.05)	0.065	0.025	0.050

*Mean for three replications

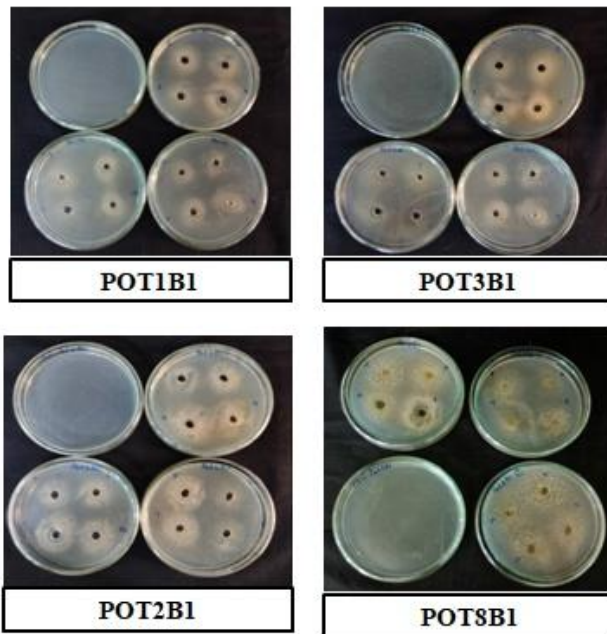


Fig.1. *In vitro* screening Cell free extracts of LAB for antibacterial activity

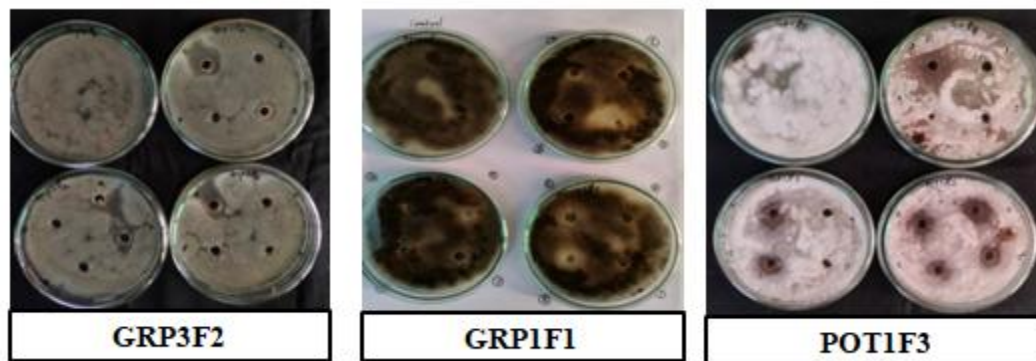


Fig.2. *In vitro* screening Cell free extracts of LAB for antifungal activity