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Evaluation of Lactic acid bacteria for extending the shelf life of fruits and vegetables

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Abstract

The preservation of fruits and vegetables using lactic acid bacteria (LAB) isolated directly from fruits and vegetables is a human-friendly approach. The fruits and vegetables which were available in the nearby local market were used for this experiment. LAB in these fruits and vegetables were enriched using skim milk broth. Then the cultures were isolated and purified. LAB was isolated using MRS media. Then after serious of morphological and biochemical characterization, their antimicrobial property was assessed against *B. subtilis*, *E. coli*, *Pseudomonas* sp., *Xanthomonas* sp., *Serratia* sp. and *Collectotrichum* sp., *Alternaria* sp., *Sclerotia* sp., *Rhizopus* sp. and best isolate which was performed well as selected and polysaccharide was extracted by centrifuge method at 10,000 rpm for 20 minutes. The *Lactobacillus plantarum* LABB3 was found to be the best isolate recorded maximum polysaccharide with antimicrobial activity. Then fruits and vegetables (banana, papaya, carrot, beans) were coated with these polysaccharides (biofilm). It is found that these biofilms reduce the weight loss of fruits and vegetables and also reduce atmospheric microbial attack on fruits and increase the shelf life of fruits and vegetables. Thus these edible biofilms can be used as bio preservatives for increasing the shelf life of fruits and vegetables.

Keywords biofilm, fruits, lactic acid bacteria, polysaccharide, shelf life, vegetables

Introduction

Fruits and vegetables when harvested contain 65 to 95 percent of water. These produce die and decay when water and food reserves are exhausted. Any biotic and abiotic stress that increases the usage of food and water reserves can lead to increases in the likelihood of losses [1]. The FAO (Food and Agricultural Organization) estimated that in the year of 2009, 32% (weight basis) of all food produced around the world was wasted. Fruits and vegetables are also severely affected by pathogens after harvest during storage and marketing, thus causing heavy losses in terms of value [2]. Post-harvest treatments using chemicals such as Sulphur dioxide, Calcium Chloride, Irradiation, Formalin, and Ozone may cause health problems and additional costs. Lactic Acid Bacteria (LAB) is. a suitable candidate to solve these issues since this genus comes under the category of "Generally Recognized as Safe" (GRAS) [3].

LAB belongs to different taxonomic groups of Gram-positive bacteria, with a common characteristic that produces lactic acid as the main (or sole) product during the fermentation of carbohydrates.

Lactobacilli strains are generally referred to as probiotics, which have a considerable role in human diets [4]. LAB cultures and their cell-free supernatants (CFS), which contain bacteriocins, organic acids, biosurfactants, hydrogen peroxide, reuterin, and diacetyl, have been extensively utilized to inhibit and control the growth of pathogenic bacteria and their associated biofilms. LAB can exert both bacteriostatic and bactericidal effects, disrupting the function of the pathogenic cell membrane, leading to increased membrane permeability, cell lysis, loss of cellular contents, and ultimately, cell death [5].

LAB biofilms have been observed to act not only against bacterial pathogens but also against fungi, particularly in clinical settings. The formation of LAB biofilms within the human body plays a significant role in health, as it can influence microbial functionality and help reduce microbial persistence [6].

Probiotics are naturally available live organisms that can confer a benefit to the host when consumed. The application of LAB for the preservation of foodstuffs and prevention of food spoilage is a worldwide known concept but the use of LAB to extend the shelf life of fruits and vegetables is a new approach that we studied here [7]. The objectives of this study are to isolate, characterize and identify the biofilm-forming LAB from fruits and vegetables to screen and separate the biofilm from LAB, and to evaluate their effects in extending the shelf life of fruits and vegetables under in vitro conditions.

Methodology

Sample collection and enrichment

The samples for the isolation of LAB include different fruits like pomegranate, sapota, banana, papaya, guava, apple, and different vegetables like amaranthus, brinjal, chilli, carrot, and beans. These fruits and vegetables were collected from the local market in our surroundings. The fruits and vegetables that are collected are suspended appropriately 10g of each sample in 50ml of 10% of sterile milk powder solution for 48 hours at room temperature for the enrichment and then inoculated in MRS (de Man, Rogosa, and Sharpe) broth at room temperature for 48 hours.

Isolation and purification

Isolation of Lactic Acid Bacteria from the enriched fruit and vegetable samples were carried out using the pour plate technique. Serial dilutions of each sample were prepared up to dilutions of 10-4 and then 1-2 ml of diluted samples were poured into the Petri plates of MRS media and then incubated at room temperature for 48 hours. After 48 hours of incubation, different colonies were selected based on their morphological appearance and they were streaked in a Petri plate and they were further sub-cultured until a single type of colony was found. The pure cultures thus obtained were further subjected to further tests. The cultures were stored in a refrigerated condition of 4°C.

Morphological and cultural identification of lactic acid bacteria

The shape, size, gram staining, and biochemical characteristics of each different isolate were observed and recorded for the identification of species of lactic acid bacteria as per the method [8].

Screening of Lactic acid bacteria

The standard methods used to test the Milk coagulation test, pH tolerance test, and NaCl tolerance test as per the procedure [9].

Isolation of microbial flora from spoiled fruits

Spoiled fruits and vegetables (pomegranate, apple, carrot, beans, papaya) were collected from the nearby local market for the isolation of pathogenic microbes. Fruits and vegetables were swabbed for the collection of pathogenic microbes and then the swabs were added into saline solutions and then

they were inoculated in Nutrient agar and Potato dextrose agar and the plates were incubated at room temperature for 48 hours. After the incubation the colonies were isolated morphological and cultural characters were examined and the organisms were identified.

Assessment of antimicrobial activities of Lactobacillus sp. against pathogenic microbes

The antimicrobial activity of *Lactobacillus* sp. against pathogenic microbes was determined by the well-diffusion method and dual plate method. For this, broths that are inoculated with LAB which are incubated for 48 hours are centrifuged at 10,000 rpm for 20 minutes [10].

Shelf life estimation of fruits and vegetables coated by biofilm of lactic acid bacteria

For the estimation of shelf life, the best-performed cultures were inoculated in MRS broth and then centrifuged at 10,000 rpm for 20 minutes after 48 hours. The supernatant from the centrifuged broth is separated which can act as an approximate coating agent. The fruits and vegetables (papaya, banana, carrot, beans) were dipped in a supernatant containing Bacteriocin-Like Inhibitory Substance (BLIS) and the fruits and vegetables were coated while uncoated fruits and vegetables were dipped in sterile distilled water which can act as the control. After coating, fruits and vegetables were air-dried to remove any moisture on the surface for 1 hour. The coated and uncoated fruits and vegetables were kept at a room temperature of 37°C and were observed until the fruits and vegetables got spoiled [11].

Estimation of weight loss and visual observation

Weight loss of fruits and vegetables were measured with the help of weighing balance at the start of the experiment (at day 0) and thereafter at regular intervals during the storage period. The weight loss is measured as the difference between initial weight and final weight and is expressed in percentage in terms of initial fresh weight [12].

The weight loss can be calculated with,

$$\text{Weight loss} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100$$

Results and Discussion

Isolation and identification of Lactobacillus species

In the present study, for isolating the Lactic acid bacteria several indigenous fruits and vegetables which are locally available in the market are brought and were used for this experiment. The fruits and vegetables were subjected to the enrichment of Lactic acid bacteria through the enrichment technique using skim milk powder. Then colonies were isolated using serial dilution and pour plating techniques and kept in incubation at room temperature. After that those colonies were further purified and sub-cultured for further experiments. The colonies from each sample were subjected to gram staining and were observed under the microscope. Out of the 11 colonies examined 10 colonies were found to be gram-positive and indicate that they were *Lactobacillus* sp., and the strains were named for identification. Vegetables can be used as an alternative source of LAB strains and they also have antimicrobial properties against pathogenic microbes [13].

Colony morphological characterization

In this study for colony characterization single colony of each isolate was taken and transferred to a petriplate containing MRS media. Out of the 10 colonies 6 colonies were raised (LAB Po1, LAB Pa4, LABA6, LABG7, LABC8, LAB B10) and 4 were flat (LAB S2, LAB B3, LAB G5, LAB Ca9). Most of the

isolated had a creamy to yellowish color except LAB G5 which was whitish in color and most of the colonies were rods and cocci and had shiny surfaces and smooth edges (Table 1).

Table 1. Morphological characteristics of LAB isolates

Isolate identity	Elevation	Gram staining	Colony texture	Edge	Colony colour	Colony shape
LAB Po1	Raised	+VE	Shinny	Rough	Creamy	Short rods
LAB S2	Flat	+VE	Shinny	Rough	Yellowish	Rods
LAB B3	Flat	+VE	Coarse	Rough	Creamy	Rods
LAB Pa 4	Raised	+VE	Shinny	Smooth	Creamy	Cocci
LAB G5	Flat	+VE	Coarse	Smooth	Whitish	Rods
LAB A6	Raised	+VE	Shinny	Smooth	Yellowish surrounded by white	Cocci in chains
LAB G7	Raised	+VE	Shinny	Rough	Creamy	Short rods
LAB C8	Raised	+VE	Shinny	Smooth	Yellowish surrounded by white	Cocci in chains
LAB Ca9	Flat	+VE	Coarse	Rough	Creamy	Cocci in chains
LAB B10	Raised	+VE	Shinny	Smooth	Creamy	Short rods

Biochemical characterization

In the catalase test, there were no bubbles formed over the cultures indicating that these cultures were catalase-negative. It is known that all Lactic acid bacteria are catalase-negative. After that milk coagulation test was performed and was found that all colonies were successfully coagulated with the milk.

All the colonies underwent carbohydrate utilization tests and was found that all the isolates were able to form acid in lactose and fructose and except LAB Ca9 isolate in sucrose. Only LAB S2 and LAB Ca9 were able to form gas in glucose. In the case of fructose LAB B3 only forms gas. LAB Pa4, LAB Pb10 and LAB G5, LAB Ca9 formed gas in sucrose and maltose respectively (Table 2). These biochemical tests revealed that these bacteria belong to the *Lactobacillus* sp.

Table 2. Biochemical characteristics of LAB isolates

Isolate identity	Catalase test	Milk coagulation test	Carbon sources utilization									
			G		F		L		S		M	
			A	G	A	G	A	G	A	G	A	G
LAB Po1	-	+	-	-	+	-	+	-	+	-	+	-
LAB S2	-	+	+	+	+	-	+	+	+	-	-	-
LAB B3	-	+	-	-	+	+	+	-	+	-	-	-
LAB Pa 4	-	+	+	-	+	-	+	+	+	+	-	-
LAB G5	-	+	+	-	+	-	+	-	+	-	-	+
LAB A6	-	+	+	-	+	-	+	-	+	-	+	-
LAB G7	-	+	-	-	+	-	+	-	+	-	+	-
LAB C8	-	+	+	-	+	-	+	+	+	-	+	-
LAB Ca9	-	+	+	+	+	-	+	-	-	-	-	+
LAB B10	-	+	-	-	+	-	+	-	+	+	+	-

G-Glucose, F-Fructose, L-Lactose, S-Sucrose, M-Maltose,
(+)-positive, (-)-negative, A-Acid production, G-Gas production

Screening of Lactic acid bacteria isolates for tolerance to low pH

Before entering the intestinal tract, LAB must survive in the acidic condition of the stomach. In this present study, it is found that most of the isolates were able to grow under more acidic pH. LAB B3 and LAB Ca 9 were not able to grow under pH 4, pH 5 and pH 5, pH 6 respectively and LAB Ca 9 does not survive on pH 5 (Table 3). These results were found similar to [14] in isolation, identification of Lactobacillus species from regional yogurts in Bangladesh. *Lactobacillus* strains isolated from durian fruit have probiotic potential and they found that those strains were able to survive under a low pH of 3.0% in *in vitro* gastrointestinal conditions [15]. The isolates that they tested were able to tolerate low pH and higher bile concentrations such that they can survive in acidic conditions [16]. The experimental results found that *Lactobacillus* sp. can survive under acidic as well as alkaline conditions [17]. The members belonging to the genus Lactobacillus are generally acidophilic and they can grow in pH 4.0 [18]. The growth of lactobacillus sp. was observed maximum in pH 5.5 -6.5 [19].

Table 3. Growth at different pH and tolerance to NaCl

Isolate identity	Growth at different pH			Growth in NaCl			
	pH 4	pH 5	pH 6	2%	4%	6%	8%
LAB Po1	+	+	+	+	+	+	-
LAB S2	+	+	+	+	+	-	-
LAB B3	-	-	+	+	+	-	-
LAB Pa 4	+	-	+	+	+	-	-
LAB G5	+	+	+	+	-	-	-
LAB A6	+	+	+	+	-	+	-
LAB G7	+	+	+	+	+	+	-
LAB C8	+	+	+	+	-	-	-
LAB Ca9	+	-	-	+	+	-	-
LAB B10	+	+	+	+	+	+	-

Tolerance of Lactic acid bacteria to NaCl

In this study in which LAB isolates were subjected to grow in high concentrations of NaCl (2% to 8%), it was found that all colonies were able to grow under 2% NaCl and none of them were grown under 8% NaCl concentration. Only 4 isolates survived under 6% NaCl and 3 colonies failed to survive under 4% NaCl concentration (Table 3). The out of 17 isolates that he isolated 8 isolates were grown under 4% NaCl and Lactobacillus casei was able to grow at 6.5%NaCl [20]. The isolated strains were resistant to NaCl concentrations up to 6% and they were able to survive under acidic conditions with maximum growth in pH 6 [21]. These results have also been found similar to Ma et al., [22].

Assessment of antimicrobial activity of Lactobacillus sp. against pathogenic

The agar well diffusion method and dual plate method were used to assess the antimicrobial activities of the isolates. Their antimicrobial activities were tested against major pathogens which were isolated from the indigenous fruits and vegetables namely *B. subtilis*, *E. coli*, *Pseudomonas* sp., *Xanthomonas* sp., *Serratia* sp. and *Collectotrichum* sp, *Alternaria* sp, *Sclerotia* sp, *Rhizopus* sp. (Table 4 and 5) was used to assess the antimicrobial activity of the selected LAB namely LAB B3, LAB G5, LAB A6 and LAB C8 isolated from varied sources. All the LAB isolates had shown strong inhibition against these microbes and inhibition is mainly due to bacteriocin. There was a difference in the diameter of the zone of inhibition for different colonies. A zone of inhibition of diameter above 1mm is considered a positive result. Only LAB C8 does not show antibacterial activity against bacterial pathogens. LAB Ca9 and LAB A6 did not show antifungal activity). LAB G7 and LAB C8 had shown greater resistance to *E. coli*. These results were found similar [23]. LAB B3 showed maximum inhibitory character on *Collectotrichum* sp. (22 mm) and *Alternaria* sp. (20 mm) and also showed good inhibitory rate in *Sclerotium* sp. (16mm) and *Rhizopus* sp. (11mm).

Table 4. Anti-bacterial activity of isolated bacterial strains by agar well diffusion method

Isolate identity	Zone of inhibition (mm)				
	B. subtilis	*Escherichia coli*	*Pseudomonas sp.*	*Xanthomonas sp.*	*Serratia sp*
LAB Po1	24	21	26	32	7
LAB S2	14	19	21	20	11
LAB B3	21	15	17	20	22
LAB Pa 4	11	14	24	19	2
LAB G5	18	13	16	21	12
LAB A6	10	12	10	18	12
LAB G7	22	29	24	31	11
LAB C8	16	23	17	21	0
LAB Ca9	19	13	16	23	6
LAB B10	18	20	19	27	4

Table 5. Anti-fungal activity of isolated lactic acid bacterial strains by dual culture technique

Isolate identity	Zone of inhibition (mm)			
	Collectotrichum sp	***Alternaria sp***	***Sclertium sp***	***Rhizopus sp***
LAB Po1	8	13	17	3
LAB S2	7	12	14	5
LAB B3	22	20	16	11
LAB Pa 4	11	13	13	10
LAB G5	10	0	11	0
LAB A6	10	8	11	0
LAB G7	5	11	10	10
LAB C8	18	12	12	12
LAB Ca9	11	0	13	7
LAB B10	10	10	12	7

L. collinoides and *L. alimentarius* have strong antimicrobial activity against *Pseudomonas aeruginosa* and *Bacillus subtilis* with an inhibition zone of more than 15mm [24]. The isolates *L. plantarum*, *L. casei*, and *Enterococcus faecium* had probiotic potentials [25].

Biofilm coating and estimation of shelf life

Among the isolates, the most promising *L. plantarum* (LAB B3) which survived well in antimicrobial activity and other tests was taken and mass multiplied using the centrifuge method. Supernatant which contains polysaccharide or bacteriocin was taken and was coated in papaya, banana, carrot, and beans. Weight loss estimation and visual observation were taken into account for estimating the shelf life and it was found that there is a significant decrease in weight in control when compared to biofilm-coated fruits and vegetables.

In the non-coated carrot, there was severe microbial growth was observed and in the coated carrot, it was not noticed due to the antagonistic activity of LAB. Similarly, microbial growth was observed in the non-coated papaya. In bananas, black spots were more predominant in non-coated fruits. In the banana, there was a drastic decrease in weight in control (19.3%) when compared to the coated banana (8.60%). In carrot weight loss is 15% in control and 8.66 % in treatment and weight loss is 8.9% in control and 6.20% in treated papaya. In beans, weight loss is 22.9% in control and 15.5% in coated beans (Table 6 and Figure 1.). In the case of the carrot, when compared to the non-biofilm control carrot, the coated carrot in seven days did not deteriorate and the non-coated carrot had severely deteriorated. Papaya and beans also showed promising results. Bananas also performed well when coated. Thus when compared to the non-coated fruits, the coated fruits show

Table 6. Effect of lactic acid bacteria on the storage of fruits and vegetables

Isolate identity	Uncoated (wt. in g)		Coated(wt. in g)	
	Day of treatment	Day 7	Day of treatment	Day 7
Banana	43	36	37	34
	50	39	56	51
Carrot	74	64	68	62
	65	53	59	54
Papaya	184	167	172	163
	165	151	147	136
Beans	53	41	58	49

Figure 1. LAB coated and uncoated vegetables (Carrot and Beans) and fruits (Papaya and Banana) after 0 and 7 DAI (Days After Inoculation)

increased shelf life and reduced physical and microbial spoilage. These results were found similar to Pham et al., [26]. *L. equigeneosi* and *B. smithii* are suitable for pomegranate preservation [7]. The edible coating can also act as a barrier to microbial attack [27]. The biofilm coat of *B. smithii* was found suitable for extending the shelf life of strawberries up to seven days [7]. LAB coating forms a film on the surface thus it inhibits the fruit-spoiling bacteria and extends the shelf life of fruits and vegetables.

Conclusion

Fruits and vegetables coated with Bacteriocin Crude extract from lactic acid bacteria lead to inhibition of microorganism growth that is responsible for spoilage of fruits, vegetables and it is very active in reducing weight loss in fruits during marketing which leads to extending the shelf life of fruits and vegetables. The fruits and vegetables i.e., papaya, banana, carrot, and beans which were treated (coated) had shown a reduced reduction in weight when compared to the fruits and vegetables that were untreated (control). It is also seen that the fruits and vegetables that are kept under control show severe microbial growth due to the absence of edible polysaccharide coating from LAB whereas fruits and vegetables that are coated show less microbial growth in their surface this indicates that the atmospheric microbes which come in contact with the fruits and vegetables which were coated were subjected to death due to antagonistic effects (bacteriocin). There is also severe shrinkage noted in control but it is less in treated.

Thus the isolated *L. plantarum* LAB B3was found antimicrobial activity against fruit flora as well as pathogenic microorganisms and was revealed as the best agent for fruit and vegetable preservatives and extends the shelf life of fruit. They were applicable as the biological preservative towards fruits and vegetables as well as this secondary role of probiotics are very advantageous. This edible coating also does not affect the taste and flavor of fruits and vegetables.

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