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BIOPRESERVATION OF FRESH VEGETABLES USING LACTIC ACID BACTERIA

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ABSTRACT

Biopreservation is a technique of preservation with a natural antibacterial or antifungal agent. Lactic Acid Bacteria (LAB) a known natural biopreservation agent are found in many nutrient-rich environments and occur naturally in the production of fermented foods and are also part of intestinal microflora. The present study was performed at RVS Technical Campus Coimbatore during the year 2021 to check the efficiency of lactic acid bacteria as a biopreservation agent. In this study, Nine LAB isolates were isolated from different natural substrates, plants and leafy vegetables. All the isolates were screened *in vitro* against phytopathogens *viz., Erwinia carotovora* subsp. *Carotovora, Xanthomonas campestris, Rhizoctonia solani, Botrytis cinerea* for its antimicrobial activity. Among the nine LAB isolates screened, isolates LAB2, LAB3 and LAB7 exhibited a significant inhibitory against the pathogens causing vegetable spoilage. The efficiency of the LAB isolates against vegetable rot was also evaluated by spraying the effective LAB strain over tomatoes and capsicum. On comparing the shelf life of samples stored under ambient conditions and LAB sprayed samples the latter showed less deterioration rate. The results of the present study indicated that spraying LAB2 which was identified as Lb. *plantarum* V7B3 strain to tomato and capsicum at a level of 6 log CFU ml^{-1} increased product shelf-life additionally by 7 days. The shelf life study revealed that LAB strain proved to be an efficient and cost-effective method of preserving vegetables.

(Key words: Vegetable spoilage, biopreservation, lactic acid bacteria, shelf life)

INTRODUCTION

India is an agriculture based country. Hundreds of fruits and vegetables are grown in all parts of India and it is the second largest producer of vegetables in the world (ranks next to China) and accounts for about 15% of the world's production of vegetables. The current production level is over 329.86 million tonnes and the total area under vegetable cultivation is around 27.23 million hectares which is about 4% of the total area under cultivation in the country (Anonymous, 2021). Generally, fresh produce losses are higher than those of processed food. This is due to improper harvesting, handling, transportation and distribution of vegetables and fruits result in the significant losses which cause ultimately economic loss. It is estimated that total loss of vegetable and fruits in India due to inadequate post-harvest handling and storage is about 20-25%.

Pathogenic microorganism such as *Botrytis cinerea, Fusarium, Rhizoctonia solani, Erwinia carotovora, Xanthomonas* and *Penicillium* play an undeniable role in the deterioration of the marketable quality of vegetables and constitutes a major economic problem. Physical and chemical methods have been developed to control the occurrence of these microorganisms and their toxins but, no efficient strategy has yet been proposed to reduce the post harvest loss. Moreover, some moulds have acquired the ability to resist chemical treatments and some preservatives. The reduction of such moulds in food production is thus of primary importance and there is great interest in developing efficient and safe strategies for this purpose (Ren *et al.,* 2018).

Recently, LAB has received much attention. Application of LAB in traditional food and feed fermentation and preservation is well documented (Kim, 1993 and Johan and Jesper, 2005). Lactic acid bacteria are harmless and are used to improve human and animal health (probiotics). They have a GRAS (generally recognized as safe) status and it has been estimated that 25 per cent of European diet and 60 per cent of the diet in many developing countries consist of fermented foods.

The effect of antibiotics and other compounds produced by LAB has widely been researched, especially in fermented foods (Johan and Jesper, 2005), silage (Stirling and Whittenbury, 1963 and Woolford, 1994) and as biopreservatives (Johan and Jesper, 2005).

During the past 50 years, many studies were reported about bacterial and fungal diseases as well as the application of different microorganisms as biocontrol agents. However, little information is available on the interactions of LAB with phyto pathogenic bacteria (Stephane *et al.*, 2005 and Ashgar and Mohammad, 2010). A very few *in vitro* studies have been reported about the efficacy of LAB against phyto pathogenic bacteria (Visser *et al.*, 1986 and Rasalia *et al.,* 2008).

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Lactic acid bacteria have been isolated by many workers from various sources such as dairy products, traditional fermented foods, soil, fruits and vegetables. Lactic acid bacteria that have been isolated from fresh vegetables and fruits have been shown to possess bioprotective potentials against food borne human bacterial pathogens through the production of bacteriocins (Pal *et al*., 2005 and Rajaram *et al.*, 2010).

The aim of our investigation is to isolate LAB from natural substrates, plants and leafy vegetables, *in vitro* screening of LAB isolates against bacterial pathogens (*Erwinia carotovora* subsp. *Carotovora, Xanthomonas campestris, Rhizoctonia solani, Botrytis cinerea*) and evaluate the potential of efficient LAB isolates against vegetable rot thereby extending the shelf life of vegetables.

MATERIALS AND METHODS

The materials and methods for isolation of LAB from natural substrates namely curd, leafy vegetable such as cabbage, coriander, curry leaves, plantain leaves and pickle followed by characterization, screening and study of the effect of the most effective LAB substrates on the shelf life extension of vegetable is given below.

Sample collection

Nine LAB substrates were collected from various natural substrates such as curd, cabbage, coriander, curry leaves, plantain leaves and pickle. The isolated LAB were inoculated to MRS broth for multiplication.

Sample preparation

A known surface area of each vegetable sample was individually swabbed aseptically in laminar flow.

Characterization of LAB isolates

All the isolates were identified based on morphology tests which comprised of examining cell shape and gram staining method, biochemical tests was also performed which included catalase test, motility test, endospore test, sugar fermentation test suggested by Wood and Holzapfel (1995);Holt *et al*. (1994), acid production, gas production test as suggested by (Becking,1974 ; Harringan, 1998). Isolates were classified into homo and heterofermentative groups (Zuniga *et al*., 1993).

***In vitro* screening of LAB isolates for antimicrobial activity**

The antimicrobial efficiency of LAB isolates against various vegetable spoiling pathogenic fungi *viz*., *Botrytis cinerea, Rhizoctonia solani* and pathogenic bacteria *viz*., *Erwinia carotovora* subsp. *Carotovora, Xanthomonas compestris* was carried out by Agar Well Diffusion assay (Bali *et al.,* 2011) and Dual agar overlay method (Ganesan and Gnanamanikkam, 1987).

Study of efficacy of LAB isolates in the extension of shelf life of vegetables

The vegetables were sprayed with the LAB strains which were identified to have good inhibitive effect against the selected bacterial and fungal strain based on the *in-vitro* screening test following the method of Frances *et al.* (2006).

RESULTS AND DISCUSSION

The study was conducted to isolate and identify LAB spp. present in different natural sources like vegetables, fermented products and leaf surfaces. The inhibitory effect of LAB substrates on *Erwinia carotovora* subsp. *Xanthomonas campestris, Rhizoctonia solani* and *Botrytis cinerea* was studied and the potential of efficient LAB isolates against vegetable rot was evaluated.

Isolation of lactic acid bacteria from different natural sources

LAB spp. isolated from different natural sources were cultured by standard dilution plate count method and sub cultured on MRS medium under aseptic condition. The number of isolates obtained was found to be highest in curd followed by fermented pickle while least count was observed in vegetable and plant source.

Identification of the isolates upto generic level

The following morphological, physiological and biochemical studies were performed for confirmation of the extracted isolates as lactic acid bacteria. (Table 1).

Acid and gas production test

Though all the extracted 9 isolates produced acid only 3 LAB substrates were observed for significant generation of gas. The isolates were classified as homofermentative and heterofermentative based on acid and gas generation pattern (Table 2).

Sugar fermentation test

Sugar fermentation test was done to test the ability of carbon utilization of LAB isolates. The results showed that LAB isolates have the ability to survive on various carbon sources (Table 3).

***In vitro* screening of LAB isolates pathogenic micro-organisms**

Screening of LAB isolates against *Erwinia carotovora* subsp.*, Xanthomonas campestris, Rhizoctonia solani* and *Botrytis cinerea* were performed to find the effectiveness of the isolates in inhibiting the growth of pathogenic microorganisms (Table 4). *In vitro* screening against *Erwinia carotovora* subsp. had the highest zone of inhibition against LAB 3 with 23.6 mm followed by LAB 7 and LAB 2. While *Xanthomonas campestris* exhibited maximum inhibitory zone of 20.4 mm against LAB 7 followed by LAB 3 and LAB 2. The effect against *Rhizoctonia solani* was found to be maximum in LAB 3 with 13.4 mm followed by LAB 2 and LAB 7. The inhibitory zone was the highest in LAB 3 followed by LAB 2 and LAB 7 against *Botrytis cinerea*.

Study of efficacy of LAB isolates in the extension of shelf life of vegetables

In vitro screening against pathogenic indicated that LAB2, LAB3 and LAB 7 were most effective in inhibiting the growth of pathogens. Hence, these isolates were sprayed at a level of 6 log CFU ml^{-1} on tomatoes and capsicum. The sprayed vegetable samples and control samples were studied for shelf life based on the physiological parameters such as physiological weight loss and color.

Table1. Characterization of LAB isolates based on morphological and biochemical properties

Sr. No.	Isolate No.	Gram reaction	Shape	Mobility	Catalase	Endospore
1.	LAB1	+	Rod	-	-	-
2.	LAB2	+	Rod	-	-	-
3.	LAB3	+	Rod	-	-	-
4.	LAB4	+	Rod	-	-	-
5.	LAB5	+	Rod	-	-	-
6.	LAB6	+	Rod	-	-	-
7.	LAB7	+	Rod	-	-	-
8.	LAB8	+	Rod	-	-	-
9.	LAB9	+	Rod	-	-	-

Table 2. Acid and gas production test for LAB isolates

Sr.No.	Isolate No.	Acid production	Gas production	Homo fermentative	Hetero fermentative
1.	LAB1	+	-	-	-
2.	LAB2	+	+	-	-
3.	LAB3	+	+	-	-
4.	LAB4	+	-	-	-
5.	LAB5	+	-	-	-
6.	LAB6	+	-	-	-
7.	LAB7	+	+	-	-
8.	LAB8	+	-	-	-
9.	LAB9	+	-	-	-

Table 3. Sugar fermentation test

Sr. No.	Isolate No.	Glucose	Galactose	Fructose	Maltose	Sucrose
1.	LAB1	+	-	-	-	-
2.	LAB2	+	+	-	+	+
3.	LAB3	+	+	+	+	+
4.	LAB4	+	-	-	-	+
5.	LAB5	+	-	-	+	-
6.	LAB6	+	+	-	-	
7.	LAB7	+	+	+	-	+
8.	LAB8	+	-	+	-	-
9.	LAB9	+	-	-	-	-

Table 4. *In vitro* screening of LAB isolates

S.No.	Isolate No.	Area of zone of inhibition (sq. cm)			
		***Erwinia carotovora* subsp.**	***Xanthomonas campestris***	***Rhizoctonia solani***	***Botrytis cinerea***
1.	LAB1	15.2	14.2	7.4	4.9
2.	LAB2	19.3	18.3	11.6	11.4
3.	LAB3	23.6	19.8	13.4	12.5
4.	LAB4	11.6	10.4	4.5	3.4
5.	LAB5	7.5	6.5	6.5	7.6
6.	LAB6	8.4	7.4	4.5	4.5
7.	LAB7	18.4	20.4	9.9	10.6
8.	LAB8	10.7	10.7	3.4	2.6
9.	LAB9	8.5	8.5	4.5	3.8

Physiological weight loss

Physiological weight loss of tomatoes kept under control had a weight loss of 15% while LAB isolates sprayed tomatoes exhibited maximum weight loss of 7%. Among the LAB isolates LAB2 was found to be more efficient with a weight loss of 4.44%. Capsicums had a weight loss of 25% and 18% respectively for control and sprayed samples. LAB2 isolate sprayed samples showed a minimum weight loss of 14% (Fig. 1 and 2).

Effect of LAB substrate on the color

Color changes were observed visually. Tomatoes under control turned from yellowish red to dark red on the fifth day while the LAB sprayed tomatoes showed a significant change in color only from fifth day and changed to red color on the twelfth day. Capsicums exhibited faster color change when placed under control. The green colour started turning yellow on the third day and completely turned yellow with shrinkage on the fifth day, while the samples with LAB isolates sprayed condition remained stable and started turning yellow only on the fifth day and completely became yellow on the twelfth day. The samples sprayed with LAB2 exhibited slower color changes compared to LAB3 and LAB7 for both tomatoes and capsicum samples.

The keeping quality of the vegetables sprayed with LAB strain were found to be increased because of the inhibiting properties of LAB. Ramos *et al*. (2020) isolated LAB strains from rye sour dough. *L. plantarum*, *L. casei, L curvatus*, *L. paracasei* and *L. coryniforms* exhibited antifungal properties against *Aspergillus nidulans, Penicillium funiculosum, Fusarium poae* .

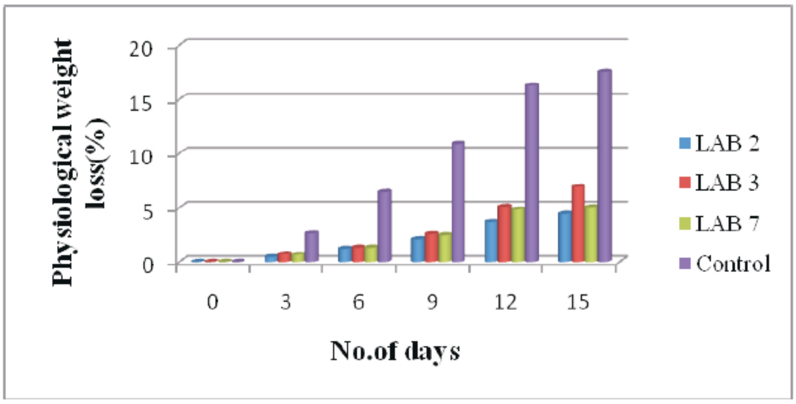

Fig. 1. Physiological weight loss of tomatoes sprayed with LAB isolates

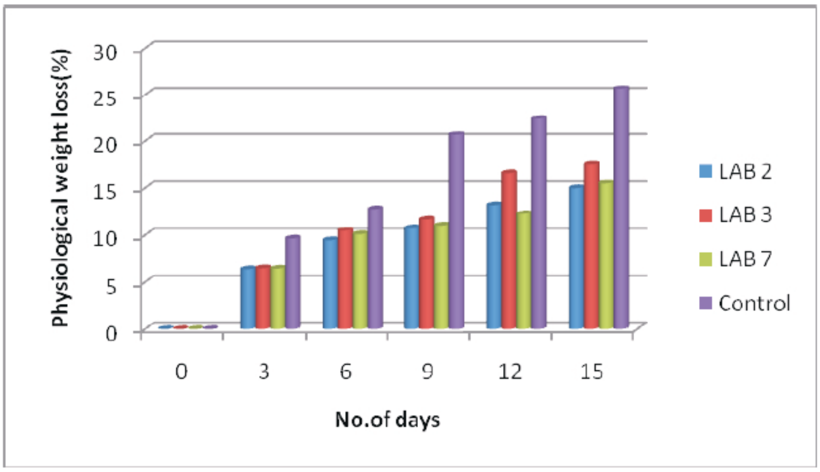

Fig. 2. Physiological weight loss of capsicum sprayed with LAB isolates

Among natural biological antagonists, Lactic Acid Bacteria (LAB) has several potential applications. These microorganisms are found in many nutrient rich environments and occur naturally in production of fermented foods and are also part of intestinal micro flora. Research reports indicate that LAB has beneficial health effects in humans and also have a long history of use in foods. They produce some antagonistic compounds which are able to control pathogenic bacteria and other undesirable spoilage micro flora. Using LAB to control mould growth could be an interesting alternative to physical and chemical methods because these bacteria have been reported to have strong antimicrobial properties. A limited number of reports have shown that a good selection of LAB could allow the control of mould growth and improve the shelf life of many fermented products and, therefore, reduce health risks due to exposure to mycotoxins (Perczak *et al.,* 2018).

Antimicrobial production from LAB isolates creates competition for bacterial growth. *Lactobascillus sp.* produce bacteriocins, a protein which are generally produced at the end of exponential growth of bacteria. This interferes the metabolic activities of both Gram positive and Gram negative bacteria (Martin *et al*., 2022). The synthesis of cell wall is disrupted by inducing pore formation on bacterial cell membrane thereby inactivating and affecting the metabolism of bacteria (Costa *et al*., 2019). LAB isolates used as active packaging against *L. monocytogenes* proves to be an effective antimicrobial approach, LAB also acts as probiotics thus improving host immune system (Webb *et al*., 2022).

Lactic acid bacteria (LAB) are known to preserve foods from spoilage, prolong the shelf life and provide better quality and safe foods. The present study focused on the use of lactic acid bacteria as bioprotective agents to inhibit vegetable spoilage microorganisms. A total of 9 isolates were isolated from different natural sources and subsequently they were characterized for various morphological and biochemical characters. They were tested under *in-vitro* conditions against four phytopathogens *viz*., *Erwinia carotovora*. *Xanthomonas campestris, Rhizoctonia solani* and *Botrytis cinerea*. The bacteria *Erwinia carotovora* and *Xanthomonas campestris* were the most sensitive to LAB whereas *Rhizoctonia solani* and *Botrytis cinerea* were less resistant compared to bacterial pathogens. Out of all the isolates, 3 efficient isolates LAB2, LAB3 and LAB7, which showed the highest zone of inhibition against spoilage organisms were selected to test their efficacy against vegetable spoilage microorganisms. Significant inhibitory activity was observed by LAB3 followed by LAB7 and LAB2. These efficient isolates which were promising against spoilage microorganisms under *in-vitro* conditions were chosen for shelf life enhancement study of tomato and capsicum under incentive condition. Out of 3 selected isolates, LAB2 was more effective in controlling the spoilage of tomato and capsicum, which increased the product shelf-life additionally by 7 days when sprayed at a level of 6 log CFU ml^{-1}. The isolate LAB2 has been identified as *Lb. plantarum* V7B3 strain through genetic studies. The strain did not produce any undesirable effects on vegetables, such as browning and off odours.

Lactic acid bacteria strains could survive at postharvest conditions and were effective in controlling the spoilage microorganisms. The isolated lactic acid bacteria did not produce undesirable visible effects on vegetables, such as browning and off odours. The bacteria *Erwinia carotovora* and *Xanthomonas campestris* were most sensitive to LAB. *Rhizoctonia solani* and *Botrytis cinerea* were a little resistant compared to bacterial pathogens. The selected LAB isolates reduced the growth of the test bacteria, and had bactericidal effect. Thus, it can be concluded that the efficacy of LAB isolates against vegetable spoilage microorganisms is high and can be used as biopreservative agent in extending the shelf life of vegetables.

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