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Application of Antifungal Products from Lactic Acid Bacteria for Post-harvest Disease Management in Vegetables and Fruits

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ABSTRACT

Lactic Acid Bacteria (LAB) is one of the most diverse groups of bacteria. They have been studied extensively for their cultural, biochemical characters and for a wide variety of compounds which they produce. Studies have reported the antagonistic activity of their products against a wide variety of pathogens. The LAB produce antifungal compounds which can be used for preservation of fresh agricultural produce.

A wide variety of antifungal compounds are known to be produced by Lactic acid bacteria. This property confers biopreservation potential to lactic acid bacteria. The biocontrol potential of lactic acid bacteria can be established for the prevention of fungal infections of agricultural produce specially fruits and vegetables. Thus, living cells or product formulations of antifungal lactic acid bacteria may be prepared and used as an alternative biocontrol technology. The use of these antifungal compounds produced by LAB will reduce the use of chemical fungicides and hence aid in control of chemical pollution.
The LAB belong to GRAS (Generally Regarded As Safe) category and hence will not be harmful at the consumers end.

In the present study LAB were inoculated in vegetables and fruits in three different forms, as cells, CFS and a mixture of cells and broth. Good fungal inhibition was observed when compared with chemical fungicide Carbendezim Mencozab mixture.When used as spray formulation inhibition was seen to decrease as compared to chemical fungicide. This could be attributed to poor adhesion on the surface of fruits and vegetables.

INTRODUCTION

Fungicides are a primary means of controlling postharvest diseases [1] and are commonly used as spray formulations. However, as harvested fruits and vegetables are commonly treated with fungicides to retard postharvest diseases, there is a greater likelihood of direct, human exposure to them. Hence biological control becomes the most favourable approach to combat post-harvest fungal diseases.

Microbial antagonists have been reported to control several rot pathogens on diverse post-harvest commodities. Yeasts and yeast like organisms play an important role in controlling post-harvest spoilage. *Pichia guilliermondii* reported by Wickerham, isolated and developed by [2] and subsequent coworkers [3],[4],[5], for control of postharvest rots of citrus and other fruits; *Acremonium breve* isolated by [6]; and several species of Cryptococcus, isolated by [7], for control of postharvest rots of apple.

Protective LAB cultures will find more applications in biocontrol technologies as the use of chemical agents will continuously decrease and demands for safer alternatives goes on increasing with cheaper processing at industrial scale. The “generally recognized as safe” (GRAS) status of LAB, offers the potential to use these bacteria as biological control agents in post harvest produce to prevent fungal growth. [8] have used LAB for the control of fungal rot caused by *Penicillium expansum* on apples and iceberg lettuce. [9] have shown *Lactobacillus plantarum* to be effective for the post harvest control of *A.flavus* and *F. graminearum*, *Rhizopus stolonifer* and, *Bt. cinerea* when used for cucumber. Fresh mango was protected using *Lb. acidilophidus NCDC 291* from spoilage by *A. alternate* by [10],[11],[12], used *Pediococcus pentosaceous* against *P. expansum* as biocontrol agent for pear, plum and grapes. [10] have shown *Lb. plantarum IMAU10014* to control *Bt. cinerea* on tomato leaves.

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MATERIALS AND METHODS

Comparison with standard antifungal compound
In order to compare the potency of LAB in biocontrol, commercially available fungicide preparation SAAF which is a Carbendezim Mencozeb mixture was used. For this the MIC value of this fungicide was determined against *F. Oxysporum* by using dual culture technic as described.

The determination of % inhibition was done by performing the Dual culture Technique in liquid media. This was done by inoculating LAB (10^4cfu/ml) in 10 ml aliquots. The tubes were incubated at 30^0C for 48 hours. The test fungus 10µl of 10^8 cfu/ml was inoculated in each tube with LAB growth. The tubes were again incubated at 30^0C for 48 hours. After incubation the growth from each tube was filtered through Whatman Filter paper, dried and weighed. Controls were maintained for LAB and for test fungus. This was done for all the above media. The antifungal activity was calculated using the formula:

Control – (Control)

F. Oxysporum was grown separately on C-Dox agar plates for eight days at 28^oC.The spores were harvested and suspended in sterile C-Dox broth with Tween 80 to a final density of 10^6spores /ml.This was dispensed as 180 µl aliquots to each tube to which 20 µl of fungicide solution from stock solution to get a desired concentrations in the range of 10 to 1000 µg /ml. Positive control consisted of 180 µl of C-Dox broth and 20 µl of fungicide preparation. The negative control consisted of 180 µl of C-Dox broth containing spores and 20 µl of sterile distilled water. The tubes were incubated for three days at 28^oC and checked for fungal growth on dry weight basis. The experiment was carried out in triplicates and MIC was determined as the lowest concentration of fungicide that inhibits spore germination after three days of incubation. This concentration of fungicide was used in fungicide treatment during biocontrol experiment on fruits and vegetables.

Application on vegetables (Beans and Cucumber)
In this study all the LAB isolates were tested for their ability to prevent the rot caused by *Fusarium oxysporum*. The trials were conducted as described by [9] with a few modifications.

For studies on beans, fresh beans were disinfected with ethanol swabs followed by exposure to uv light for 10 min. Three wounds were made at different locations with a sterile cork borer of diameter 5mm about 3mm deep. Every experiment consisted of 20 treatments and each treatment consisted of four bean pods. The wounds of pods of treatment one (*F. oxysporum*) were inoculated with 10 µl of fungal spore suspension only and in treatment two (control)the wounds were inoculated with 10 µl of LAB suspension only. In treatment three (CFS control) the wounds were inoculated with 10 µl of CFS and in treatment four (broth suspension of LAB control) the wounds were inoculated with 10 µl of broth suspension of LAB. In wounds of fifth FL3 (3), sixth PL2 (3), seventh PFL4, eighth F3415 and ninth FL2 (5) 10 µl of bacterial suspension (10^o6 cfu/ml) of each of the LAB was inoculated. In wounds of tenth FL3 (3), 11th PL2 (3) ,12th PFL4 ,13th F3415 and 14th FL2 (5) 10 µl of CFS of each of the LAB was inoculated. In wounds of 15th FL3 (3), 16th PL2 (3) ,17th PFL4 ,18th F3415 and 19th FL2 (5) 10 µl of broth suspension of each of the LAB was inoculated.

In the 20th treatment pods were inoculated with 10 µl of Carbendezim Mencozeb mixture (SAAF) equivalent to MIC value. After 2h the wounds were challenged with 10 µl of spore suspension (10^4 spores /ml) of fungal pathogen separately. All the inoculated pods were placed in sterile polypropylene bags separately and incubated at 28^0C for 10 days. After every 48 h infected wounds from each treatment were counted till end of 10 days. All the experiments were done in triplicates.

For studies on cucumber fresh cucumbers were disinfected with ethanol swabs followed by exposure to uv light for 10 min. Four wounds were made at different locations with a sterile cork borer of diameter 5mm about 5mm deep. Every experiment consisted of 20 treatments and each treatment consisted of three cucumbers. The wounds of cucumber of treatment one (*F. oxysporum*) were inoculated with 10 µl of fungal spore suspension only and in treatment two (control) the wounds were inoculated with 10 µl of LAB suspension only. In treatment three (CFS control) the wounds were inoculated with 10 µl of CFS and in treatment four (broth suspension of LAB control) the wounds were inoculated with 10 µl of broth suspension of LAB. In wounds of fifth FL3 (3), sixth PL2 (3), seventh PFL4, eighth F3415 and ninth FL2 (5) 10 µl of bacterial suspension (10^o6 cfu/ml) of each of the LAB was inoculated. In wounds of tenth FL3 (3), 11th PL2 (3) ,12th PFL4 ,13th F3415 and 14th FL2 (5) 10 µl of CFS of each of the LAB was inoculated. In wounds of 15th FL3 (3), 16th PL2 (3) ,17th PFL4 ,18th F3415 and 19th FL2 (5) 10 µl of broth suspension of each of the LAB was inoculated.

In the 20th treatment cucumber wounds were inoculated with 10 µl of Carbendezim Mencozeb mixture (SAAF) equivalent to MIC value. After 2h the wounds were challenged with 10 µl of spore suspension (10^4 spores /ml) of fungal pathogen separately. All the inoculated cucumbers were placed in sterile polypropylene bags separately and incubated at 28^0C for 10 days. After every 48 h infected wounds from each treatment were counted till end of 10 days. All the experiments were done in triplicates.

Application on fruits (Pomegranate and apple)

For studies on pomegranates, fresh pomegranates were disinfected with ethanol swabs followed by exposure to uv light for 10 min. Five wounds were made at different locations with a sterile cork borer of diameter 5mm about 5mm deep. Every experiment consisted of 20 treatments and each treatment consisted of two fruits. The wounds of fruits of treatment one (*F. oxysporum*) were inoculated with 10 µl of fungal spore suspension only and in treatment two (control) the wounds were inoculated with 10 µl of LAB suspension only. In treatment three (CFS control) the wounds were inoculated with 10 µl of CFS and in treatment four (broth suspension of LAB control) the wounds were inoculated with 10 µl of broth suspension of LAB. In wounds of fifth FL3 (3), sixth PL2 (3), seventh PFL4, eighth F3415 and ninth FL2 (5) 10 µl of bacterial suspension (10^o6 cfu/ml) of each of the LAB was inoculated. In wounds of tenth FL3 (3), 11th PL2 (3), 12th PFL4, 13th F3415 and 14th FL2 (5) 10 µl of CFS of each of the LAB was inoculated. In wounds of 15th FL3 (3), 16th PL2 (3), 17th PFL4, 18th F3415 and 19th FL2 (5) 10 µl of broth suspension of each of the LAB was inoculated.

In the 20th treatment fruits were inoculated with 10 µl of Carbendezim Mencozeb mixture (SAAF) equivalent to MIC value. After 2h the wounds were challenged with 10 µl of spore suspension (10^4 spores /ml) of fungal pathogen separately. All the inoculated fruits were placed in sterile polypropylene bags separately and incubated at 28^0C for 10 days. After every 48 h infected wounds from each treatment were counted till end of 10 days. All the experiments were done in triplicates.

For studies on apples, fresh apples were disinfected with ethanol swabs followed by exposure to uv light for 10 min.Five wounds were made at different locations with a sterile cork borer of diameter 5mm about 5mm deep. Every experiment consisted of 20 treatments and each treatment consisted of two fruits. The wounds of fruits of treatment one (*F. oxysporum*) were inoculated with 10 µl of fungal spore suspension only and in treatment two (control) the wounds were inoculated with 10 µl of LAB suspension only. In treatment three (CFS control) the wounds were inoculated with 10 µl of CFS and in treatment four (broth suspension of LAB control) the wounds were inoculated with 10 µl of broth suspension of LAB. In wounds of fifth FL3 (3) , sixth PL2 (3) ,seventh PFL4 ,eighth F3415 and ninth FL2 (5) 10 µl of bacterial suspension (10^o6 cfu/ml) of each of the LAB was inoculated. In wounds of tenth FL3 (3), 11th PL2 (3), 12th PFL4, 13th F3415 and 14th FL2 (5) 10 µl of CFS of each of the LAB was inoculated. In wounds of 15th FL3 (3), 16th PL2 (3), 17th PFL4, 18th F3415 and 19th FL2 (5) 10 µl of broth suspension of each of the LAB was inoculated.

In the 20th treatment fruits were inoculated with 10 µl of Carbendezim Mencozeb mixture (SAAF) equivalent to MIC value. After 2h the wounds were challenged with 10 µl of spore suspension (10^4 spores /ml) of fungal pathogen separately. All the inoculated fruits were placed in sterile polypropylene bags separately and incubated at 28^0C for 10 days. After every 48 h infected wounds from each treatment were counted till end of 10 days. All the experiments were done in triplicates.

Spray formulations for biocontrol

Five LAB isolates, their cell free supernatants and broth suspensions were used in the study.

The LAB were grown in sterile MRS broth for 48h at 28^oC.Cells were harvested by centrifugation at 10,000 rpm for 25 min. Bacterial cell suspension was prepared in distilled water to which Cobawet (Commonly used surfactant in agrochemical sprays to enhance adherence and spread of formulations) was added (0.015%). The final cell concentration was adjusted to 10^6cfu/ml. Cobawet was added in similar concentrations to CFS and broth suspension of LAB.

The fruits and vegetables were prepared in the same way as described before.

For the actual application the experiment consisted of three sets, each with eight treatments (in multiples of 10). Treatment one was the control which consisted of the fruit or vegetable sprayed with saline. Treatment two was surfactant control where the fruit or vegetable was sprayed with Cobawet.Treatment three was fungal control where the fruit or vegetable was sprayed with fungal spores (prepared as 4.2.1). Treatment four to eight was appropriately sprayed with LAB cells, CFS and broth suspension of LAB. Approximately 2ml was used for cucumber and the fruits and 1ml for bean pod which was sprayed with a hand-held spray bottle. Treatment four to eight were challenged with fungal spore suspension to which also the surfactant was added. The samples were dried for 20 mins and then packed in sterile polypropylene bags. All the trials were done in triplicate.

Development of fungal growth was observed after 5 and then after 10 days.

RESULTS AND DISCUSSION

The LAB were actually applied on vegetables, beans and cucumbers and fruits, pomegranates and apples. This was done to see their effect in actual preservation of vegetables and fruits. The application was done in three ways, using cells, using cell free supernatant and a mixture of cells and MRS broth. Spores of test fungus *F. oxysporum* were used to challenge the antifungal preparations. Carbendezim Mencozeb mixture was used as chemical control.

Also, the growth of *F. oxysporum* was checked on the said fruits and vegetables without action of antifungal from LAB as well as chemicals. Similarly, it was checked if the antifungals in all three combinations had an effect on fruits and vegetables alone.

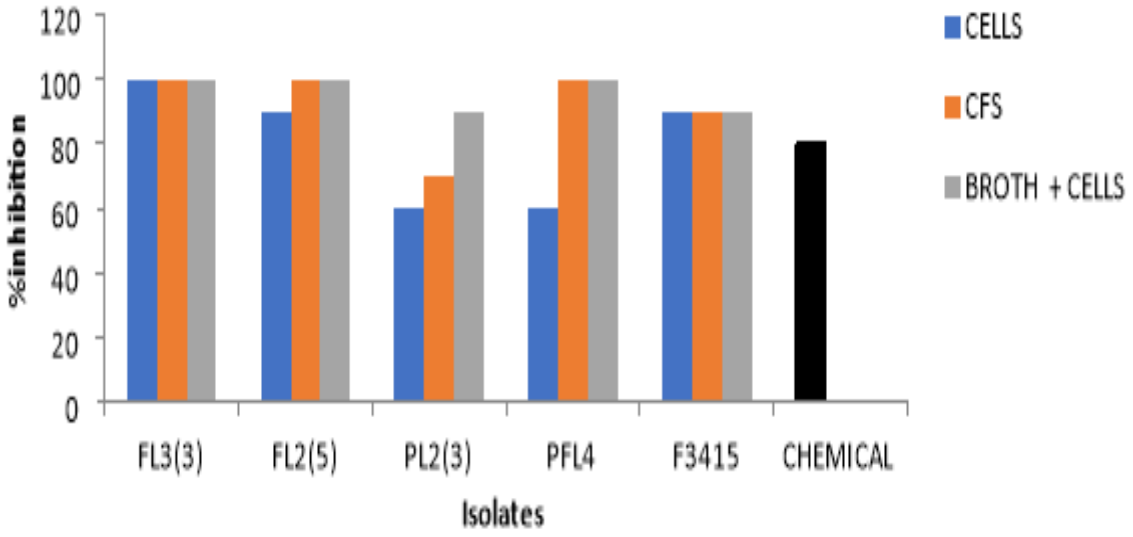

Fig1: % Inhibition of *Fusarium oxysporum* on beans

The results obtained on application of beans suggested successful role of LAB in their preservation. For *Leuconostoc mesenteroides* FL3(3) the application of cells against the test fungus showed 100% inhibition of the fungus and same results were obtained for CFS and broth and cells mixture. As compared to this the chemical used showed a 80% inhibition which is less than that showed by FL3(3). No changes were observed on the beans when the cells, CFS or broth and cells mixture was applied which proves harmless nature of LAB application.

For *Lactobacillus brevis* FL2(5) the application of cells against the test fungus, showed 90% inhibition of the fungus and whereas for CFS and broth and cells mixture it was 100%. As compared to this the chemical used showed a 80% inhibition which is less than that showed by FL2(5).No changes were observed on the beans when the cells ,CFS or broth and cells mixture was applied which proves harmless nature of LAB application.

The application of *Leuconostoc mesenteroides* PL2(3) was less effective than FL3(3) and FL2(5). The application of cells against the test fungus, showed 60% inhibition of the fungus and whereas for CFS it was 70% and for broth and cells mixture it was 90%. It is less than that showed by the chemical (80%) but still comparable to it. No changes were observed on the beans when the cells, CFS or broth and cells mixture was applied which again proves harmless nature of LAB application.

The application of *Leuconostoc mesenteroides* PFL4 was as effective as FL3(3) and FL2(5) and more than PL2(3). The application of cells against the test fungus, showed 60% inhibition of the fungus and whereas for CFS it was 100% and for broth and cells mixture it was again 100%. With the cells It is less than that showed by the chemical (80%) but with the CFS and broth and cells mixture the inhibition is higher. No changes were observed on the beans when the cells, CFS or broth and cells mixture was applied which again proves harmless nature of LAB application.

The application of *Leuconostoc mesenteroides* F3415 was found to be more effective than the chemical in all three types of applications. The application of cells against the test fungus, showed 90% inhibition of the fungus and whereas

for CFS it was 90% and for broth and cells mixture it was again 90%. No changes were observed on the beans when the cells, CFS or broth and cells mixture was applied which again proves harmless nature of LAB application.

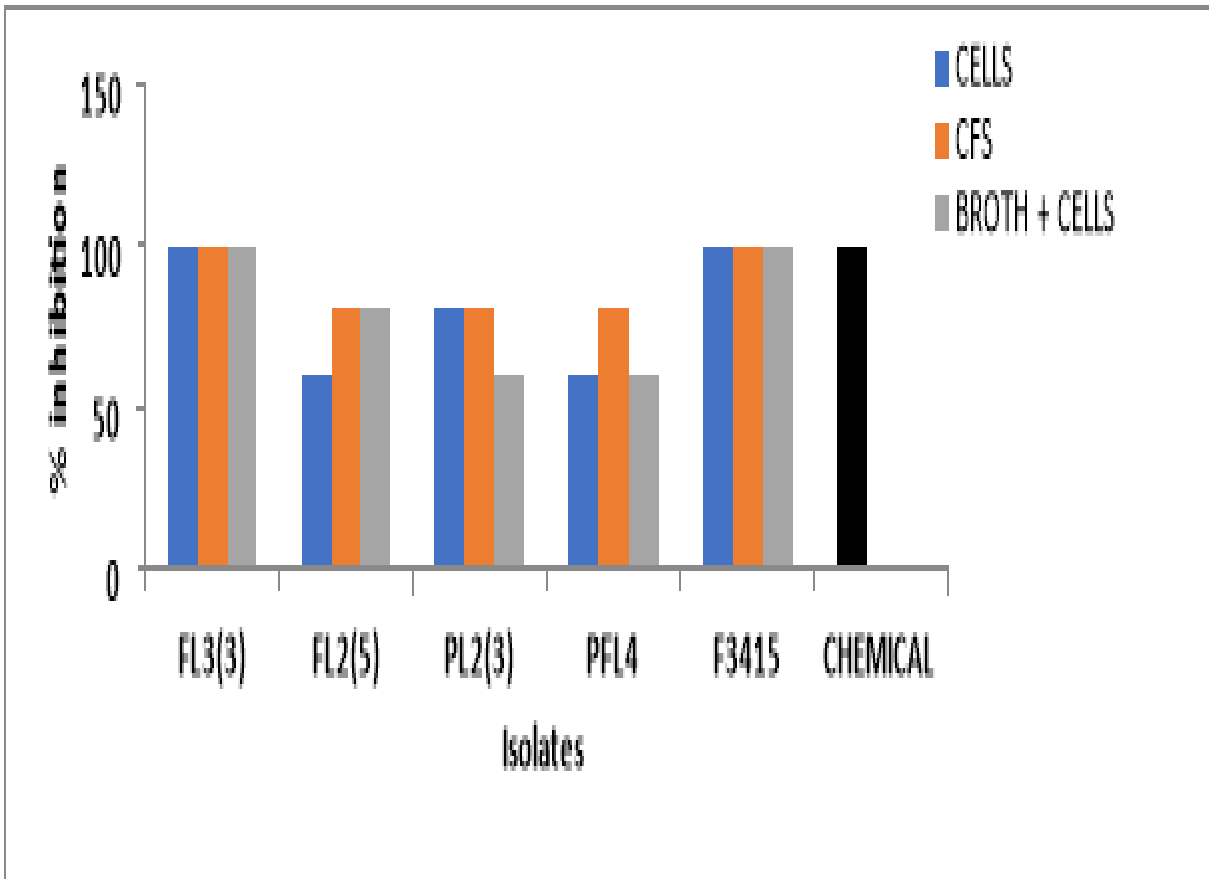

Fig 2: % Inhibition of *Fusarium oxysporum* on Cucumber

The results obtained on application of cucumber suggested successful application of LAB in their preservation. For *Leuconostoc mesenteroides* FL3(3) the application of cells against the test fungus showed 100% inhibition of the fungus and same results were obtained for CFS and broth and cells mixture. The chemical used also showed a 100% inhibition which is similar to that showed by FL3(3). No changes were observed on the cucumber when the cells, CFS or broth and cells mixture was applied which proves harmless nature of LAB application.

For *Lactobacillus brevis* FL2(5) the application of cells against the test fungus, showed 60% inhibition of the fungus and whereas for CFS and broth and cells mixture it was 80%. As compared to this the chemical used showed a 100% inhibition which is more than that showed by FL2(5) and hence here it proves better than LAB.No changes were observed on cucumber when the cells ,CFS or broth and cells mixture was applied which proves harmless nature of LAB application.

The application of *Leuconostoc mesenteroides* PL2(3) was less effective the chemical application. The application of cells against the test fungus, showed 80% inhibition of the fungus and whereas for CFS it was 80% and for broth and cells mixture it was 60%. It is less than that showed by the chemical (100%). No changes were observed on the cucumber when the cells, CFS or broth and cells mixture was applied which again proves harmless nature of LAB application

The application of *Leuconostoc mesenteroides* PFL4 was less effective .The application of cells against the test fungus, showed 60% inhibition of the fungus and whereas for CFS it was 80% and for broth and cells mixture it was again 60%. it is less than that showed by the chemical (100%). No changes were observed on the cucumber when the cells, CFS or broth and cells mixture was applied which again proves harmless nature of LAB application.

The application of *Leuconostoc mesenteroides* F3415 was found to be as effective as the chemical in all three types of applications. .The inhibition showed by cells, CFS and broth and cells mixture against the test fungus, was 100%. No

changes were observed on cucumber when the cells, CFS or broth and cells mixture was applied which again proves harmless nature of LAB application.

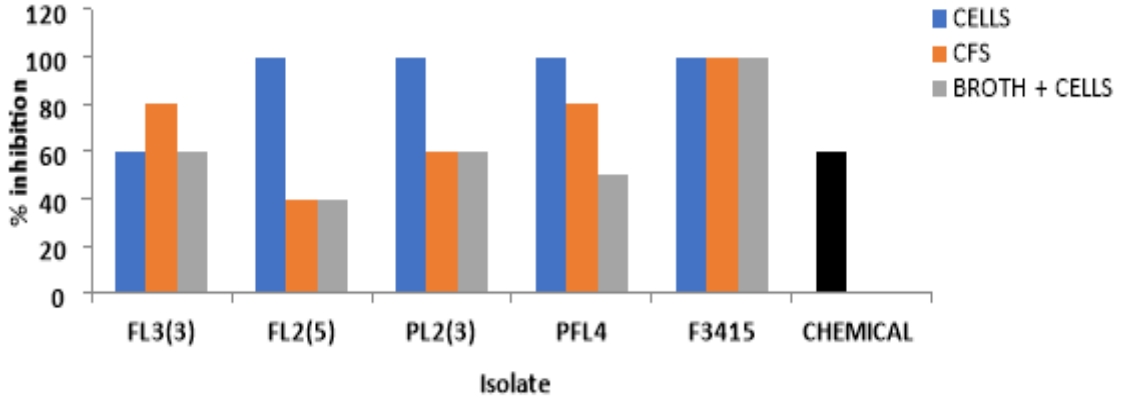

Fig 3: % Inhibition of *Fusarium oxysporum* on Apples

The results obtained on application of apples suggested successful application of LAB in their preservation. For *Leuconostoc mesenteroides* FL3(3) the application of cells against the test fungus showed 60% inhibition of the fungus ,80% inhibition was obtained for CFS and 60% inhibition in case of broth and cells mixture. The chemical used showed a 60% inhibition which is similar to that showed by FL3(3). No changes were observed on the apples when the cells ,CFS or broth and cells mixture was applied which proves harmless nature of LAB application.

For *Lactobacillus brevis* FL2(5) the application of cells against the test fungus, showed 100% inhibition of the fungus and whereas for CFS and broth and cells mixture it was 40%.As compared to this the chemical used showed a 60% inhibition which is similar to that showed by FL2(5) and hence here it proves better than LAB. No changes were observed on apples when the cells, CFS or broth and cells mixture was applied which proves harmless nature of LAB application.

The application of *Leuconostoc mesenteroides* PL2(3) was less effective the chemical application. The application of cells against the test fungus, showed 100% inhibition of the fungus and whereas for CFS it was and broth and cells mixture it was 60%. It is similar to that showed by the chemical (60%). No changes were observed on the apples when the cells, CFS or broth and cells mixture was applied which again proves harmless nature of LAB application

The application of *Leuconostoc mesenteroides* PFL4 was also effective .The application of cells against the test fungus, showed 100% inhibition of the fungus and whereas for CFS it was 80% and for broth and cells mixture it was again 50%. It is same as that showed by the chemical (60%). No changes were observed on the apples when the cells, CFS or broth and cells mixture was applied which again proves harmless nature of LAB application.

The application of *Leuconostoc mesenteroides* F3415 was found to be more effective than the chemical in all three types of applications. . The inhibition showed by cells, CFS and broth and cells mixture against the test fungus, was 100%. No changes were observed on apples when the cells, CFS or broth and cells mixture was applied which again proves harmless nature of LAB application.

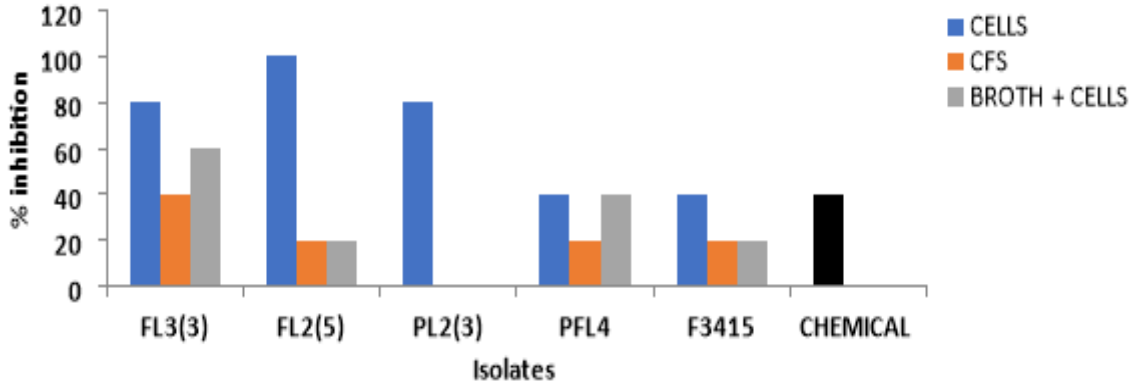

Fig 4: % Inhibition of *Fusarium oxysporum* on Pomegranates

The results obtained on application of pomegranate suggested successful application of LAB in their preservation. For *Leuconostoc mesenteroides* FL3(3) the application of cells against the test fungus showed 80% inhibition of the fungus ,40% inhibition obtained for CFS and 60% shown for broth and cells mixture. The chemical used also showed a 40% inhibition which is less than that is showed by FL3 (3).No changes were observed on the pomegranates when the cells, CFS or broth and cells mixture was applied which proves harmless nature of LAB application.

For *Lactobacillus brevis* FL2(5) the application of cells against the test fungus, showed 100% inhibition of the fungus and whereas for CFS it was 20% and for broth and cells mixture it was 20%. As compared to this the chemical used showed a 40% inhibition which is more than that showed by FL2(5) and hence here it proves better than LAB. No changes were observed on pomegranate when the cells, CFS or broth and cells mixture was applied which proves harmless nature of LAB application.

The application of *Leuconostoc mesenteroides* PL2(3) was much less effective the chemical application. The application of cells against the test fungus, showed 80% inhibition of the fungus and whereas for CFS and for broth and cells mixture no inhibition was observed. It is less than that showed by the chemical (40%). No changes were observed on the pomegranate when the cells, CFS or broth and cells mixture was applied which again proves harmless nature of LAB application

The application of *Leuconostoc mesenteroides* PFL4 was less effective. The application of cells against the test fungus, showed 40% inhibition of the fungus and whereas for CFS it was 20% and for broth and cells mixture it was again 40%. It is similar to that showed by the chemical (40%). No changes were observed on the pomegranate when the cells, CFS or broth and cells mixture was applied which again proves harmless nature of LAB application.

The application of *Leuconostoc mesenteroides* F3415 was found to be less effective than the chemical in all three types of applications. The inhibition showed by cells was 40%, for CFS 20% and for broth and cells mixture against the test fungus, it was 20%. No changes were observed on pomegranate when the cells, CFS or broth and cells mixture was applied which again proves harmless nature of LAB application.

From the above results it was generally observed that the application of whole cells was more effective than CFS or cells and broth mixture. Also protection offered to the vegetables was more than that seen for fruits.

[14] have successfully used *Lactobacillus sp.*for preserving tomatoes. They have suggested the use of these LAB in form of aerosols or used in modified atmosphere for packaging which will help to improve the shelf life of tomatoes. Similarly [15] have shown role of *Lactobacillus sp* and *Pediococcus pentocaseus* in preservation of tomato puree. *L. plantarum* CUK-501 was used against *Aspergillus flavus* , *F.graminearum* , *Rhizopus stolonifer* , and *Botrytis cinerea* by [10]where cucumber was used as a vegetable model for preservation. [16],[17],used *Lactobacillus sp* to preserve grass silage against spoilage by *Aspergillus flavus* .[18],[19], tested *Lactobacillus delbrueckii* FERM BP-10663 in animal feed for successful preservation.

The above studies have shown that antifungal properties of LAB can be used as alternatives for the commonly used chemicals. The number of patents in bio preservatives has significantly increased in last few years.

[20] have reported *Lactobacillus plantarum* CUK 501to delay spoilage of apples, pears and grapes. [23] have used five psychrotrophic strains of lactic acid bacteria to preserve salads and vegetable juices.[9] have shown application of LAB against fruit rot. [23] have suggested use of LAB against *Penicillium expansum* to preserve fruits and vegetables against its mycotoxigenic effects. [23] have demonstrated preservation of litchi using LAB. [24] have used *Lactobacillus* sp against *Aspergillus* to protect tomato puree and tomato sauce. Use of LAB against *Colletotrichum gloiesporoides* to preserve chilli seeds has been done by [25].

CONCLUSION

Application of LAB on fruits and vegetables is highly effective. Spray formulations need to be improvised with respect to adhesion properties to make them equally effective.

Hence LAB can be effectively used for preservation of vegetables and fruits as they are safe, natural, nonpathogenic, nontoxic and easy to handle and use.

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