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Efficiency of Pesticide Degrading Probiotic *Lactobacillus plantarum* Pb3 to Remove Chlorpyriphos on Tomato Fruits

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ABSTRACT: Food contamination is a global major concern in food health and safety. Therefore, the present study was aimed to evaluate the potential ability of probiotics in pesticides detoxification. In this context we investigated the reduction of chlorpyriphos residues on tomato fruits inoculated with Lactic acid bacteria (LAB). *Lactobacillus plantarum* Pb3 isolated from bhendi that received high amount of pesticide residues was used in this study. The strain, *L. plantarum* Pb3 possessed good tolerance to pesticides and the highest chlorpyriphos removal capacity. The *L. plantarum* Pb3 strain can bind chlorpyriphos as indicated by Gas chromatography analysis. The sample were prepared by QuEChERS method for extraction and clean-up process. The strain was employed for further *in vivo* studies on chlorpyriphos binding characteristics on tomato fruits. Water washing removed approximately 34.6% of pesticides added at a final concentration of 1 mg L^{-1} of tomato fruits. However, 67.8% of exogenously added chlorpyriphos was removed within 4h by heat-treated cells of *L. plantarum* Pb3. Heat treatment enhanced chlorpyriphos binding rate. During the biosorption process, tomato fruits did not exhibit any obvious adverse effects. These findings indicated that *L. plantarum* Pb3 may be useful to reduce chlorpyriphos in contaminated agricultural products.

Keywords: Lactic acid bacteria, Pesticide, chlorpyriphos, binding, Tomato.

INTRODUCTION

Pesticides are absolutely necessary in agricultural production. Pesticides are used in the production of approximately one-third of agricultural products (Liu and Liu 2002). Pesticide contamination in foods is an important safety hazard, since residual effect of pesticides still increases the risks to consumer's health (Bonner and Alavanja, 2017). Several studies have reported the presence of organophosphate residues in terrestrial and food chains, such as dimethoate residues in apples (Szpyrka *et al*., 2015), soil (Liu *et al*., 2016), olives (Paiga*et al*., 2016); omethoate residues in various vegetables (Stoleru *et al*., 2015) and phorate residues in green tea (Steiniger *et al*., 2010) and livestock products (Rahman *et al*., 2016).

The broad spectrum organophosphate pesticide chlorpyriphos [O, O-diethyl O-(3, 5, 6-trichloro-2-pyridyl) phosphorothioate] is used against a wide range of insects and pests. Pino and Penuela (2011) examined chlorpyriphos residues on vegetables, and found on raw spinach (1.87 mg kg^{-1}), okra (1.41 mg kg^{-1}) and eggplant (1.25 mg kg^{-1}).

Since several microbes, including LAB, have been implicated in the degradation and removal of pesticides from contaminated food and soil in recent years, there is potential for using this technique to increase chemical safety in the food chain in a way that is efficient, safe, and affordable (Zhang *et al.*, 2016). The fact that several LAB species including Saccharomyces cerevisiae are frequently used in food products and are considered Generally Recognized as Safe (GRAS) organisms makes them an appropriate foundation for techniques designed to reduce oral exposure to chemical pollutants. There are many strains of LAB strains that are safe microorganisms, easy to grow on a large scale, and a by-product of the food processing industry. The ability of many probiotic strains to reduce xenobiotic levels, particularly through binding, is one of their most effective traits (Muhialdin *et al*., 2020). Chemicals may interact with both alive and dead microbial cells through physical adsorption, which is a reversible, metabolically passive physicochemical process. The polysaccharide and teichoic peptide components of *Lactobacillus fermentum* ME3 and *Bifidobacterium longum* 46 are

responsible for binding and removing heavy metals (Teemu *et al*., 2008). It has been demonstrated that the ability of probiotic strains to biosorbxenobiotics is pH dependent. Hence, the high acid producing strain of *L. plantarum* Pb3 was used in this study on chlorpyriphos removal on tomato fruits.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The lactic acid bacterial (LAB) strain *L. plantarum* Pb3 was originally obtained from bhendi. The cell density of approximately 10^6 cfu ml^{-1} was used in binding study. The LAB strain was grown in de Man Rogosa and Sharpe (MRS) medium for 48 h.

Pesticide binding studies on tomato fruits

Tomatosample preparation. Lactic acid bacteria were incubated as heat-treated (boiled in 4 mL of phosphate buffer solution for 1 h) bacteria. The bacterial samples were centrifuged at 6,000rpm for 10 min (4°C) and the supernatant was removed prior to binding assays (Wang *et al*. 2016). The tomato samples were collected from organic certified market, Coimbatore, Tamil Nadu, India. Thebacterial cell pellet was resuspended in phosphate buffer solution, the surface sterilized tomato fruits were sprayed with the 10^6 cfu g^{-1} of *L. plantarum* Pb3 incubated at 37°C for four hours. The tomato fruits contain 1 mg L^{-1} of chlorpyriphos, without bacterial inoculation as a control. The pesticide were sterile filtered through 0.22 µm pore size membrane filter before added to the tomato. The extracted from the bacteria (after one time washes with water) using dichloromethane and acetonitrile prior to chlorpyriphos was analysis on GC.

Preparation of reagents and standards solutions. Certified pesticide standards (>90%) of chlorpyriphos, solvents and reagents including Dichloromethane, n-Haxane, sodium chloride and anhydrous magnesium sulphate of analytical grade were purchased from Astron (Ahmedabad, India). Primary Secondary Amine (PSA) (Bondesil 40 µm) and Graphitized Carbon Block (GCB) were purchased from Agilent Technologies, the USA.

a) Primary stock solution. Stock solutions (1000 µg mL^{-1}) of the chlorpyriphos, compounds were prepared individually by dissolving the technical-grade material in acetonitrile (v/v) separately. These were labelled and put in a freezer at a temperature of -20°C. Mixes of the stock solution were used to make the intermediate stock solution and the working standard solution.

b) Intermediate stock solution. One ml of the stock solution was transferred to a 20 ml graduated test tube and diluted to 10 ml with MS grade acetonitrile to create an intermediate stock solution of 100 µg $ml^{-1}$for chlorpyriphos. From there, an intermediate stock solution of 10 µg $ml^{-1}$was created by combining the right amounts of each pesticide stock solution and diluting them appropriately.

c) Working standards. Individual pesticide working standard solutions (0.1 to 1 µg ml^{-1}) were prepared by diluting intermediate stock solution. These working standards were used to quantify residues in samples and determine their retention time. All stock and working standard solutions were kept in a refrigerator at -20°Cuntil further use.

Extraction and clean-up for chlorpyriphos residue from tomato. The QuEChERS method (Anastassiades *et al*., 2003) was employed for extraction and clean-up process. Homogenized tomato (10 g) was weighed out into a 50-mL centrifuge tube. The centrifuge tube was sealed after 20 ml of acetonitrile had been added, and the vortex was agitated for one minute. In addition, 4 g of anhydrous magnesium sulphate and 1 g of sodium chloride were added, and the sample was vortex-shaken for 1 min to mix the sample thoroughly. The samples were centrifuged at 6000 rpm for 10 minutes. In order to eliminate moisture, 9 mL of supernatant was passed through 4 g of anhydrous sodium sulphate, and 6 mL of extract was put into a centrifuge tube containing 100 mg PSA and 600 mg anhydrous magnesium sulphate. The samples were mixed thoroughly by vortexing for 1 min and centrifuged for 10 min at 3000 rpm. Finally, 4 mL of acetonitrile layer was transferred to a clean glass test tube and concentrated using nitrogen gas in a Turbovap (Caliper life sciences, USA) at 40 °C. The residue was redissolved in 5 ml of hexane and injected into GC.

Instrumentation. To confirm the presence of insecticide residues detected through the water droplet method, representative sample from each test category was subjected to GC.

GC analysis. The residue of a test insecticide was estimated using GC- ECD operated under the following conditions. Model - GC-VARIAN 3800, Column - Capillary: DB-5, 30 m × 0.25 mm × 0.25 µm, Detector Electron - Capture Detector (ECD), Flow rate - 1.0 ml min^{-1}, Carrier gas -Nitrogen, Sample injection volume - 1 µL and Total run time - 30 min.

Calculating the pesticide residues recovery. The amount of insecticide residues recovered was quantified by comparison of peak area of standard with that of unknown sample under identical condition of operation. The amount of residues recovered in ppm was calculated as follows,

$$\text{Residue (ppm)} = \frac{As}{A_{std}} \times \frac{W_{std}}{W_s} \times V_s \times \frac{Astdj}{A_{sj}}$$

Where, A_s - Peak area of the sample, A_{std}- Peak area of the standard, W_{std}- Weight of the standard in ng, W_s- Weight of the sample in g, V_s-Volume of the sample (final extract in ml), A_{sj}- Aliquot of the sample injected in µl, A_{stdj} - Aliquot of the standard injected in µl.

The chlorpyriphos binding capacity of the strains was expressed as the binding rate, which was calculated as follows:

Binding rate (%) = (1-*C*1/*C*0) × 100%

Where, *C*1 is the residual chlorpyriphos concentration after removal, and *C*0 is the chlorpyriphos concentration in the positive control (Wang *et al.* 2016).

RESULTS AND DISCUSSION

Chlorpyriphos dissipation by binding with *L. plantarum* Pb3 on tomato fruits. In the present study, a modified QuEChERS sample preparation procedure for GC analysis ofchlorpyriphos residue on tomato fruits was followed. According to studies, organic contents co-extracted with residues in plant samples of cereals such as rice, wheat, and oats act as interferences in chromatograms (Koesukwiwat *et al*., 2010).

Binding capacity of chlorpyriphos by the probiotic strain *L. plantarum* Pb3 was studied, *in vivo* using tomato fruits. Tomato fruits were sprayed with the 10^6 cfu g^{-1} of *L. plantarum* Pb3. The Experimental findings showed that chlorpyriphos levels had decreased in the treatment with addition of 1 mg L^{-1} of chlorpyriphos. The residual of chlorpyriphos was estimated after 4 h of standing time using Gas-Chromatography binding capacity of the exogenously added pesticide by the LAB cells was demonstrated. The chromatograms of the samples analyzed and shown in Fig. 1. The results indicated the possibility of decontamination of chlorpyriphos on fresh tomato fruits. Water washing removed approximately 34.6% of pesticides added at a final concentration of 1 mg L^{-1} of tomato fruits. However, 67.8% of exogenously added chlorpyriphos was removed within 4 h by heat-treated cells of *L. plantarum* Pb3. In this study, we found LAB had bound and decontaminated the chlorpyriphos (Fig. 2). The present results are in agreement with earlier reports in which 10-33% reduction of chlorpyriphos residues in vegetables were achieved by washing and 12-48% reductions were achieved by washing followed by cooking (Randhawa *et al*., 2007).

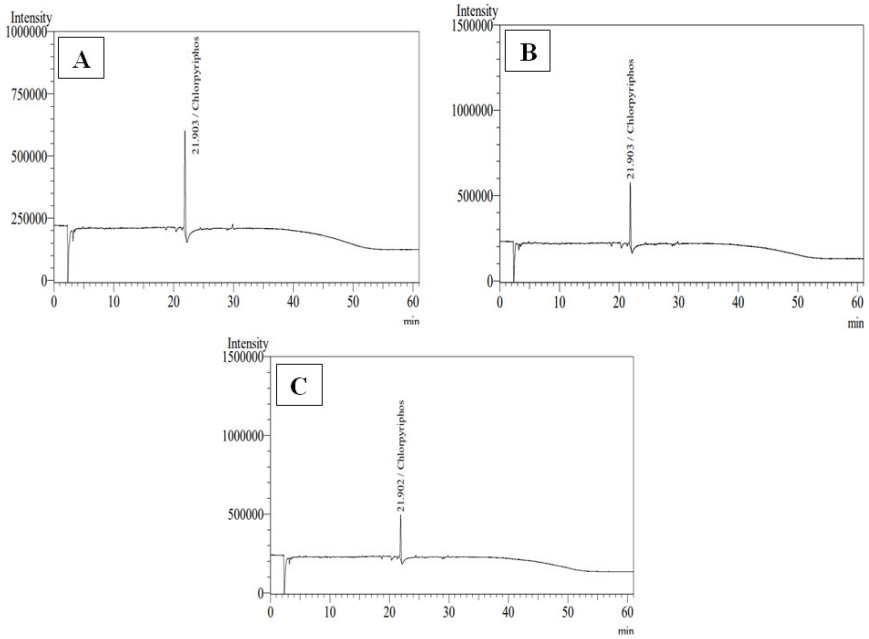
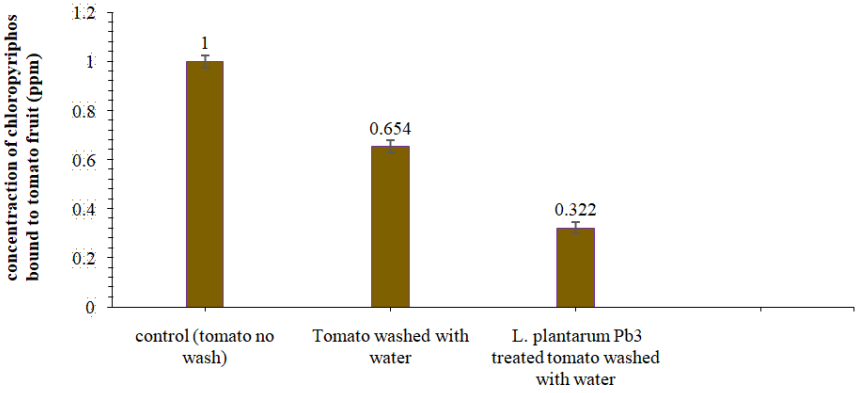
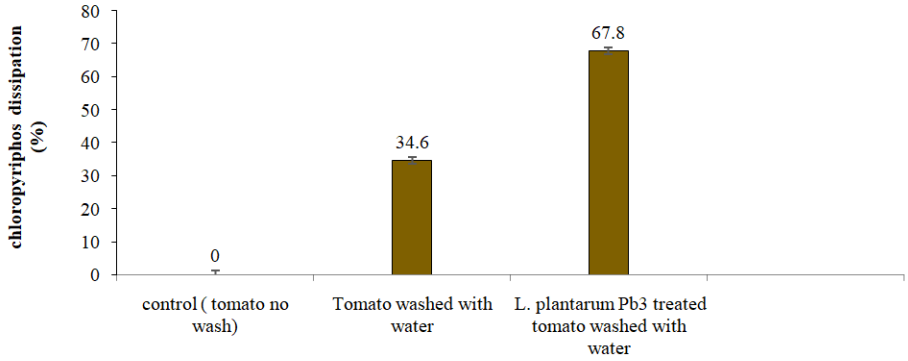


Fig. 1. GC analysis of chlorpyriphos binding to *L. plantarum* Pb3 on tomato. (A) Tomato no water wash, (B) Tomato washed with water and (C) *L. plantarum* Pb3 + Tomato washed with water.

Studies conducted with LAB on chlorpyriphos decontamination in fermenting silage indicatedthe morphological changes in LAB cells that might be caused by chlorpyriphos and that chlorpyriphos most likely adheres to superficial cellular structures. Because LAB cell walls are predominantly composed of peptidoglycan chains, as well as other components such as teichoic, lipoteichoic acids, S-layer proteins, and neutral polysaccharides, they can effectively adsorb specific chemicals (Wang *et al*., 2016). The authors discovered that heat and acid treatments had a significant effect on the amount of bound chlorpyriphos in cells. The viable cells retained 33.3-42.0% of the initially bound chlorpyriphos, with only a minor amount (15.2%) eluted. The chlorpyriphos binding rate was 32.0% for acid-treated cells and 77.2% for heat-treated cells, respectively. Hence, in the case of LAB cells of viable as well as acid and heat treated cells were able to bind chlorpyriphos. However, no significant differences between viable and acid-treated cells of *Lactobacillus casei*WYS3 in decreasing of chlorpyriphos contents. According to the authors, heat-treated cells significantly displayed a higher chlorpyrifos binding rate, likely because more binding sites were exposed (Wang *et al*., 2016).



A. Concentration of Chlorpyriphos bound to tomato fruits



B. Percentage of chlorpyriphos dissipation.

Fig. 2. Binding capacity of *L. plantarum* Pb3 on tomato fruits added with 1 mg kg^{-1} chlorpyriphos.

Moreover, Zhao *et al.* (2013) proposed that physisorption may also occur, with peptidoglycans serving as the primary binding site for benzo (a) pyrene. In comparison to other treatments, the heat-treated cells had a greater binding rate ($P < 0.05$). Franco *et al.* (2011) also demonstrated that heat-inactivated LAB had a significant effect on mycotoxin removal. This effect could be due to the fact that more LAB binding sites are revealed after heat treatment. As heat treated cells of *L. plantarum* Pb3 was used in the present study, LAB bound tightly to chlorpyriphos as most of the pesticide might have been adsorbed on the cell wall even after washing and vigorous mixing. Furthermore, it has been found in several experiments that LAB adheres to aflatoxins with great stability. After washing, the amounts of Aflatoxin B1 (AFB1) that were originally bound to live cells and cells that had undergone heat or acid treatment were 38-50%, 66-71%, and 71%, respectively. Additional autoclaving and sonication did not result in the release of any additional AFB1 from viable or nonviable cells (Haskard *et al.*, 2001).

In the present study the cells of *L. plantarum* Pb3 were inoculated for 4 h time with chlorpyriphos. Another important factor influencing the effectiveness of probiotic strains in xenobiotic biosorption is incubation time (Ameen *et al.*, 2020). The strain of *Lactococcus lactis* removed 90% of zearalenone (ZEN) during the first 20 minutes of incubation, while *Bifidobacterium* spp. biosorption increased linearly and reached 88% ZEN removal after 12 hours of incubation. Patulin shows reduced binding under less acidic conditions by *Bifidobacterium bifidum* 6071, *B. animalis* 6165, *Lactobacillus rhamnosus*6149, *L. delbrueckii* sp. *lactis* 22170 and *L. delbrueckii* sp. *lactis* 22165 (Krol *et al.*, 2018). Therefore, bacterial decontamination of food before ingestion may be a more effective decontamination technique.

CONCLUSIONS

In summary, the chlorpyripos binding capacity of the probiotic strain of *L. plantarum* Pb3 on tomato was determined in this study. The results showed that *L. plantarum* Pb3 manifested relatively excellent binding capacity to chlorpyriphos. The binding capacity would have been influenced by some factors, for instance, heat treatment enhanced the binding capacity. The binding process was reversible, and the bound chlorpyriphos could be washed. This study indicated that this strain is promising for reducing chlorpyriphos contamination on other food products also and decreasing the bioavailability of chlorpyriphos in food. In this approach cost-effective, ecological, and non-destructive when compared to physical and chemical remediation methods.

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Conflict of Interest. None.

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