Int. J. Biol. Res., 6(2): 59-72, 2018.

ROLE OF LACTOCOCCUS LCP-1 IN BIOCONTROL AND *LYCOPERSICON ESCULENTUM* MILLER PLANT GROWTH PROMOTION

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ABSTRACT

Lactic acid bacteria (LAB) are gram positive, catalase negative, non-sporulating, non-motile cocci or bacilli that are abandoned in environment. Lactic acid bacteria are employed in food industries, dairy industries and agriculture because of their health promoting activities. They not only provide health benefits but also enhance plant growth and combat pathogens. This study was conducted to isolate LAB and determine their plant growth promoting attributes. In this study ten LAB strains were isolated from different sources including vegetables, milk, dairy products, chicken, rice-wash and wheat bran by spread plate technique. These isolates were identified to genus level by the help of morphological characteristics and catalase test. The isolated strains were screened for their antibacterial activity against *Bacillus subtilis, Streptococcus faecalis, Klebsiella, Shigella dysenteriae* and *Salmonella typhi.* Out of these 10 isolated strains 40% were found to be effective bacteriocin producers. All the strains showed antibacterial activity against *Salmonella typhi* and almost 80% of the LAB showed antagonistic activity against *Shigella dysenteriae* by their cell free supernatants. Most promising results were given by LBO4, LCP1, LCC5 and LBR10. Lactic acid bacteria are known to solubilize minerals for plants including phosphate and nitrogen. This property was assessed by studying the phosphate solubilization by LAB using NBRIP medium. One of the isolated LAB strain namely LCP1 was used to study the plant growth promoting abilities of Lactic acid bacteria by performing pot trials in which tomato seeds were coated with the LAB strain. A significant increase in shoot and root lengths was noticed in the test pots after 60 days with a high germination percentage (86%) and vigor index (2652.125). Hence, LCP1 could prove to be a promising strain for plant growth promotion after further characterizations.

KEYWORDS: Lactic acid bacteria, Biocontrol, Plant growth promotion**.**

ABBREVIATIONS: LCK1: Lactococcus from chicken, LCP1: Lactococcus from potato, LCT2: Lactococcus from tomato, LCO3: Lactococcus from onion, LBO4: Lactobacillus from onion, LCC5: Lactococcus from cheddar cheese, LCW7: Lactococcus from wheat bran, LCM9: Lactococcus from mozeralla cheese, LBR10: Lactobacillus from rice wash, LCM18: Lactococcus from milk.
NBRIP: National Botanical Research Institute's phosphate.
CSF: Cell free supernatant, NCSF: Neutralized cell free supernatant.

INTRODUCTION

Lactic acid bacteria (LAB) are characterized by several unique properties, one of them is production of lactic acid by fermentation of sugars (Jackson, 2000). Many of the

LAB strains particularly those of genus *Lactobacillus* are thought to have the potential to work as a beneficial dietary component in food industries (Zotta *et al*., 2017). They are gram positive, catalase negative, non-sporing and non-motile microorganisms (Minervini *et al.*, 2015). They are aerotolerant, acid-tolerant, organotrophic and fermentative bacteria. They may be present as rods or cocci having a low G-C content and produce lactic acid as a major end product after fermentation (König *et al.*, 2017). They are generally recognized as safe (GRAS) as they are ubiquitous in food products and human mucosa (Perez *et al.*, 2014). Lactic acid bacteria are classified into two phyla namely Firmicutes and Actinobacteria. Firmicutes contain following genera*; Aerococcus, Carnobacterium, Lactobacillus, Vagococccus, Pediocccus, Streptococcus, Symbiobacterium, Weisella, Tetragenococcus, Oenococcus, Leuconostoc, Enterococcus* and *Alloiococcus* while *Bifidobacterium* from the phyla *Actinobacteria.* (Wenjun *et al*., 2014). LAB are divided into two metabolic groups: homofermentative LAB and heterofermentative LAB (Wee *et al*., 2004). Homofermentative LAB produces lactic acid as a sole end product of glucose fermentation whereas heterofermentative LAB produces lactic acid, ethanol and CO_2 in equimolar quantities. Lactic acid bacteria are usually found in decomposing plants and milk products. They are also found in the normal flora of human intestinal tract, oral cavity and vagina. They are associated with a number of food-related habitat including fruits, vegetables and cereals (Makavora *et al*., 2006). Lactic acid bacteria can also be isolated from sour cabbage, sourdough, sausages, throat and dairy products (Masood *et al.*, 2010). Lactic acid bacteria have the ability to produce bacteriocins and these bacteriocin producing strains can be used as part of adjuncts to starter cultures for fermented foods in order to improve safety and quality. Many organic acids like acetic acid, propionic acid etc. are involved in it which makes the environment acidic and unfavorable for the growth of many pathogens and spoilage causing microorganisms. They not only inhibit gram negative organisms but also act on yeast and molds (Rattanachaikunsopon *et al*., 2010). Lactic acid bacteria along with other Rhizobacteria like *Bacillus* are ubiquitous in soil and provide variety of benefits to the plant (Minervini *et al*., 2015). LAB shows antagonism towards soil pathogens which is directly related to the production of bacteriocins and organic acid form Lactic acid bacteria (Lutz *et al.*, 2012). Lactic acid bacteria and other bacteria promote plant growth by facilitating the uptake of nitrogen, phosphorus and their important minerals or by improving plant hormone status. They also do this indirectly by removing plant pathogens. These bacteria have the ability to colonize the root surfaces, rhizosphere and even intracellular spaces of the plants (Glick *et al.*, 2012). Scientists have studied the antifungal activities of many Lactic acid bacteria including *Lactobacillus lactis* that were originally isolated from the fermented milk products and can be used as effective antifungal and antibacterial agent. Lactic acid bacteria are able to compete with the phytopathogens and kill fruit rot pathogens like pathogen of jackfruit (Ghosh *et al*., 2015). Some of the genera of Lactic acid bacteria are epiphytic or endophytic and provide benefits to the plants; these include *Lactobacillus, Streptococcus, Enterococcus and Lactococcus* (Minervini *et al*., 2015).

MATERIALS AND METHODS

Isolation of Lactic acid bacteria: Ten different strains of Lactic acid bacteria were isolated from different sources like Lactococcus LCM9 from mozzarella cheese, Lactococcus LCC5 from cheddar cheese, Lactococcus LCM18 from fermented milk,

Lactococcus LCT2 from fermented tomato, Lactococcus LCK1from fermented chicken, Lactococcus LCO3 & Lactobacillus LBO4 from fermented onion, Lactococcus LCP1 from fermented potato, Lactococcus LCW7 from fermented wheat bran and Lactobacillus LBR10 fermented rice wash. After isolating LAB by spread plate technique, Gram staining and catalase test was performed for the isolated colonies to identify the culture.

Screening of LAB for antimicrobial compounds: The antimicrobial activity of LAB isolates against five indicators namely *Streptococcus faecalis, Bacillus subtilis, Shigella dysenteriae, Salmonella typhi, Klebsiella pneumoniae* by agar well diffusion assay was conducted. Indicator organisms were grown in nutrient broth for 24 hours and centrifuged at 3000rpm for 10 minutes. Supernatant of the indicator was discarded and pellet was washed twice to match the turbidity with 0.5 McFarland's index. The cell suspension of indicator organisms was introduces in the semi-solid nutrient agar and after vigorous shaking it was poured on pre-poured nutrient agar plated. LAB isolates were grown in MRS broth for 24 h and centrifuged at 3000 rpm for 15 minutes. The supernatant of LAB isolates was collected in two separate aliquots one of which was subsequently neutralized by using 1N NaOH and its pH was adjusted around 6.5-7. One aliquot was labeled as cell free supernatant (CFS) and other was labeled as neutralized cell free supernatant (NCFS). After solidification of the plates, wells were made by the help of a 10mm borer. The CSF and NCSF (200µL) were added into the respective wells and the plates were kept in refrigerator for the diffusion of supernatant into the media. After 45 minutes the plates were incubated at 37˚C for 24 h. Sterilized MRS broth was used as control and zone of inhibitions were measured in mm (Cizeikiene *et al.,* 2013).

***In vitro* characterization of Lactic acid bacteria as plant growth promoting bacteria**

Phosphate solubilization assay: Twenty four hours old culture was stabbed in National Botanical Research Institute's phosphate growth medium (NBRIP) media plates, incubated at 30˚C for 24 h and clear halos were observed around the colonies of phosphate solubilizing bacteria (Park *et al.,* 2011).

***In vivo* characterization of Lactic acid bacteria as plant growth promoter**

Preparation of inoculum/Optimization of dose: Lactic acid bacteria were grown in 2mL MRS broth for 24 h. On next day 0.1 mL of culture broth was transferred to 2mL of sterilized MRS broth and further incubated for 24 h. The cells were harvested by centrifugation at 3000 rpm for 15 minutes and washed twice with PBS to attain a uniform cell suspension. The optical density of the cell suspension was measured using a spectrophotometer at 650nm. Serial dilutions (100 folds) were made up to dilution 10^{-6}, 0.1 mL of culture from each dilution tube was spread on a pre-poured agar plate to acquire a CFU/mL of 1×10^{8}.

Seed treatment: Fresh tomato seeds were air dried for 24 h and coated with optimized dose of LCP-1 in such a way that 1 seed was provided with 1mL of cell suspension (1×10^{8} CFU/ml).

Preparation of seedlings and pot trial: Three sets of seeds were coated with the test strain (LCP-1), uncoated and sterilized seeds. The experiment was performed in triplicate

and one pot was sown with 10 seeds i.e. 10 coated seeds in a 30 cm diameter pot. Moisture level was appropriately maintained throughout the duration of experiment (Abdel-Aziz *et al*., 2014). Germination % and vigor index was calculated by the following formula:

$$\text{Germination\%} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \times 100$$

$$\text{Vigor index} = \frac{\text{Mean root length (cm)+ mean shoot length (cm)}}{\text{Germination\%}}$$

RESULTS

Isolation of LAB strains: A total of 10 LAB strains were isolated from different sources. Out of these 10 LAB isolates, 40% were isolated from fermented vegetables, 30% were isolated from cheese and chicken, 10% from milk, 10% from rice wash and 10% from wheat bran (Fig. 1). Those giving catalase negative test were expected to show Lactic acid bacteria (LAB) characteristics morphologically and were identified on the basis of their size, shape and arrangement in microscopic field. Among the isolated LAB strains 70% were identified as cocci, 10% as coccobacilli and 20% as bacilli (Fig. 2)

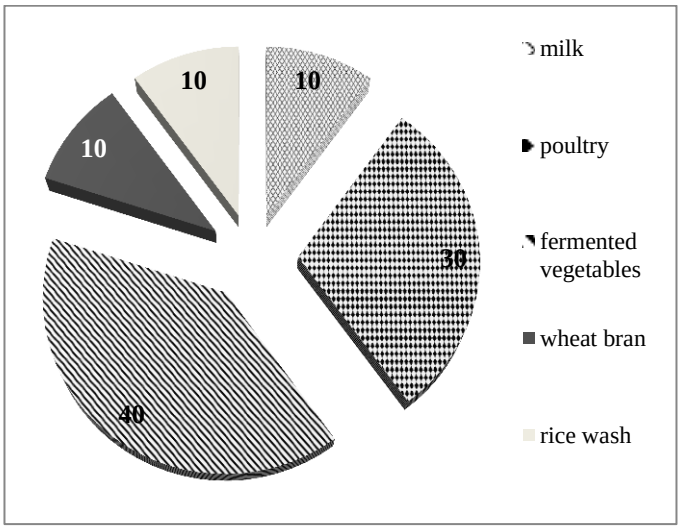

Fig. 1. Sources of LAB.

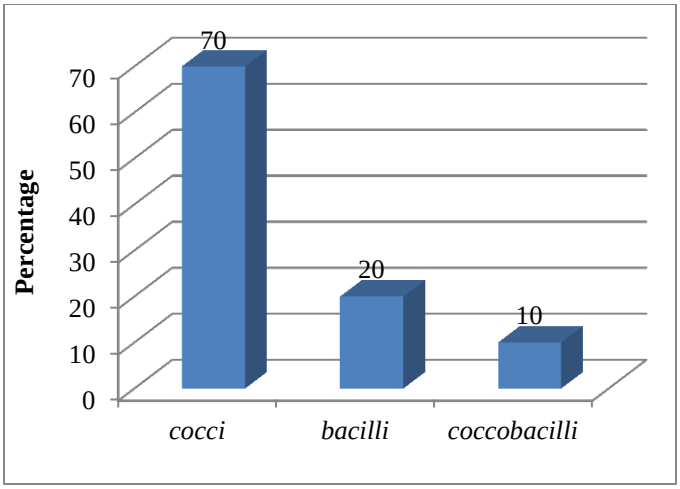

Fig. 2. Percentage of isolated LAB in different morphological forms.

Screening for antibacterial compounds: Antibacterial activity of isolated LAB strains were determined against Gram positive including *Bacillus subtilis* and *Streptococcus faecalis* and Gram negative organisms including *Shigella dysenteriae, Salmonella typhi* and *Klebsiella pneumonia* (Fig. 4a, 4b, 4c and 4d). Among these 10 strains 40% were found to be bacteriocin producers. All the strains showed antibacterial activity against *Salmonella typhi* and almost 80% showed antibacterial activity against *Shigella dysenteriae* and *Klebsiella pneumonia*, pronounced results were displayed by LBO4, LCP1, LCC5 and LBR10 (Fig. 3a, 3d and 3e). Promising results were displayed by LBR10 and LCC5 against *Bacillus subtilis* and *Streptococcus faecalis*, respectively (Fig. 3b and 3c).

Phosphate solubilization by isolated LAB strains: Isolated LAB strains were tested for their ability to solubilize phosphate i.e. to convert it into a soluble form. Maximum phosphate solubilization ability was demonstrated by LBR10 and then LCC5, LBO4, LCK1 LCP1 and LCT2 (Fig. 5a and 5b).

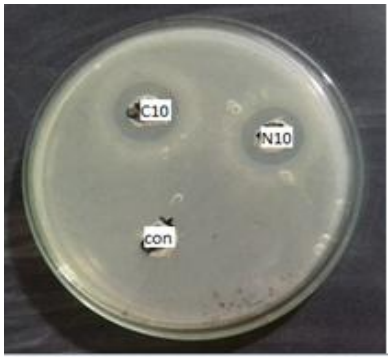

Fig. 3a. Antagonistic activity of LAB towards *Shigella dysenteriae*

Fig. 3b. Antagonistic activiy of LAB towards *Bacillus subtilis*.

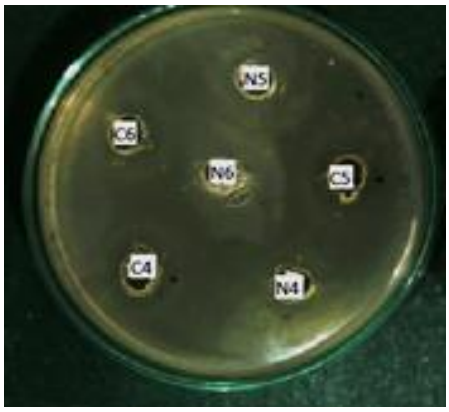

Fig. 3c. Antagonistic activity of LAB towards *Streptococcus faecali*.

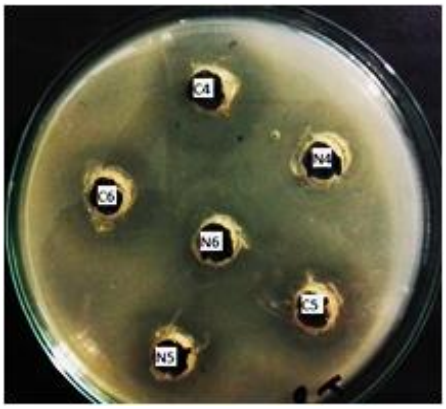

Fig. 3d. Antagonistic activity of LAB towards *Salmonella typhi*.

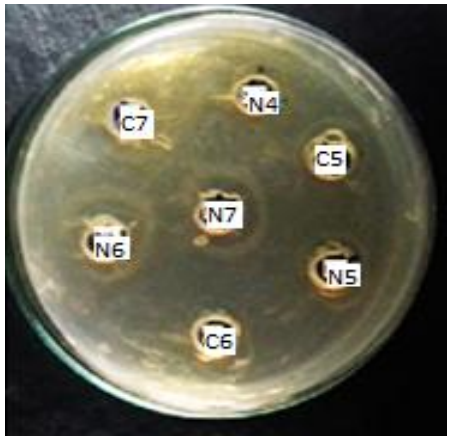

Fig. 3e. Antagonistic activity of LAB towards *Klebsiella pneumonia.*

KEY

1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
LCK1	LCT2	LCO3	LBO4	LCC5	LCP1	LCW7	LCMI8	LCMC9	LBR10

C= Cell free supenatant
N= Neutralized cell free supernatant

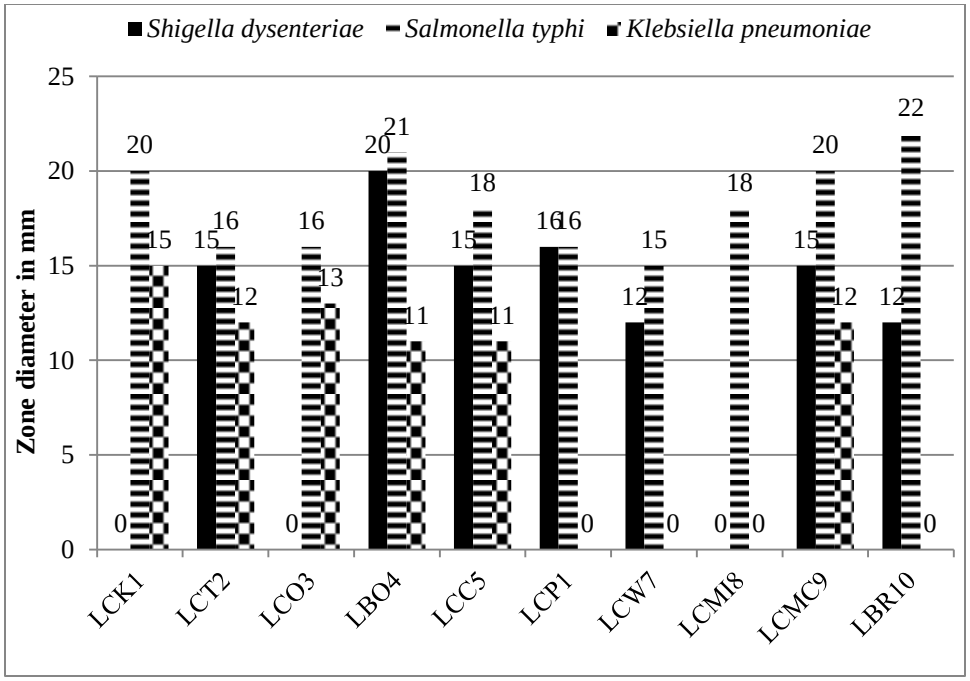

Fig. 4a. Antimicrobial potential of CFS from LAB against gram negative indicators

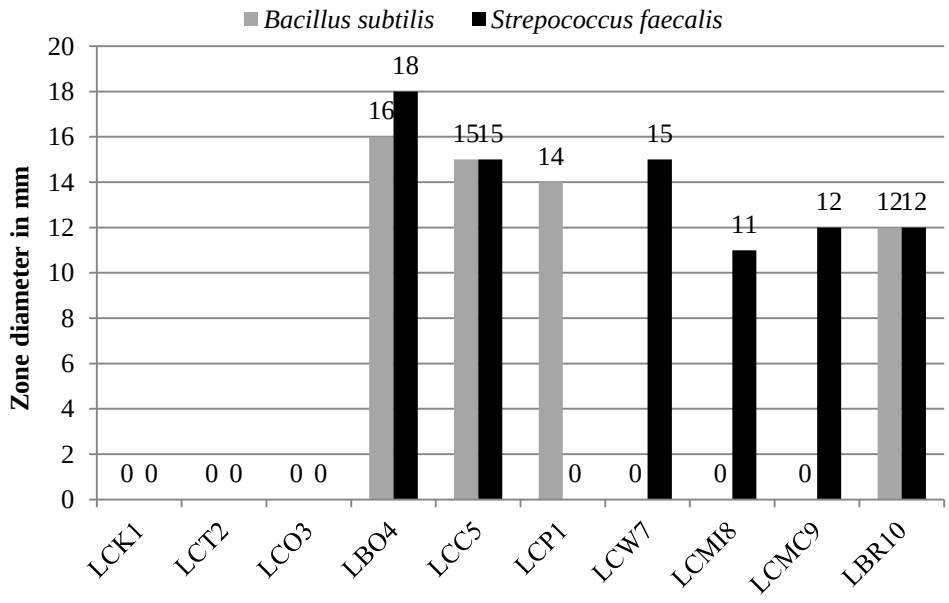

Fig. 4b. Antimicrobial potential of CFS of LAB against gram positive indicators.

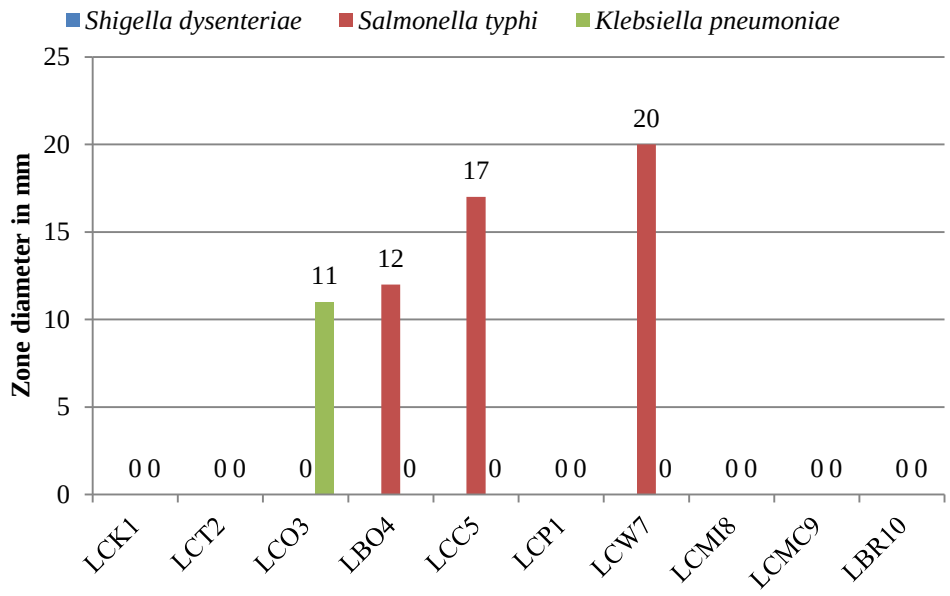

Fig. 4c. Antimicrobial potential of NCFS of LAB against gram negative indicators.

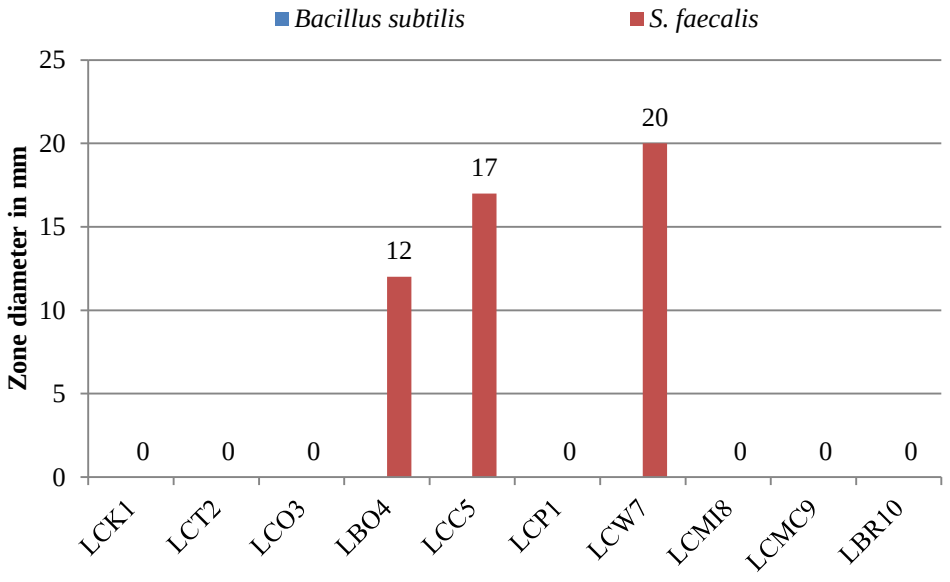

Fig. 4d. Antimicrobial potential of NCFS from LAB isolates against gram positive indicators.

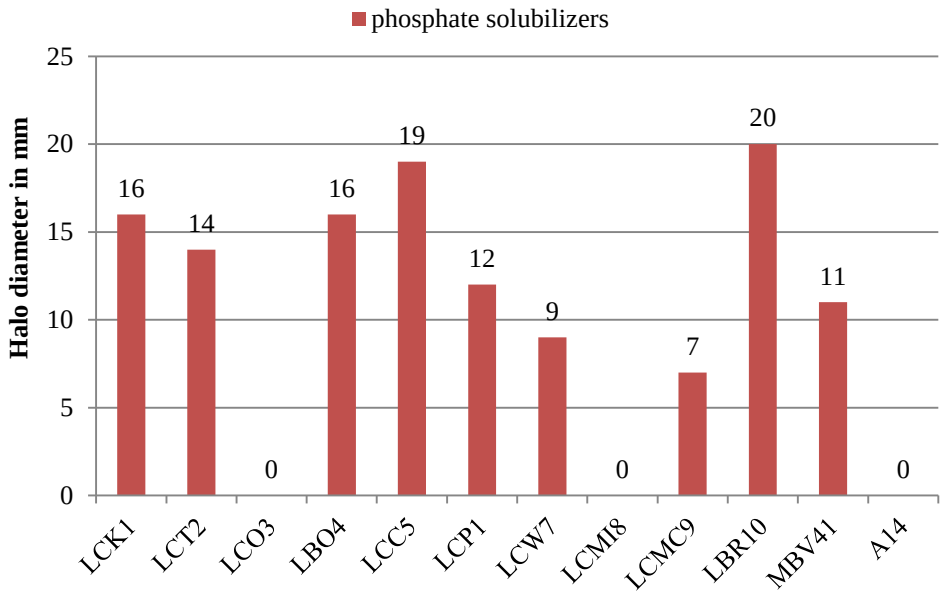

Fig. 5a. Phosphate solubilization by LAB strains.

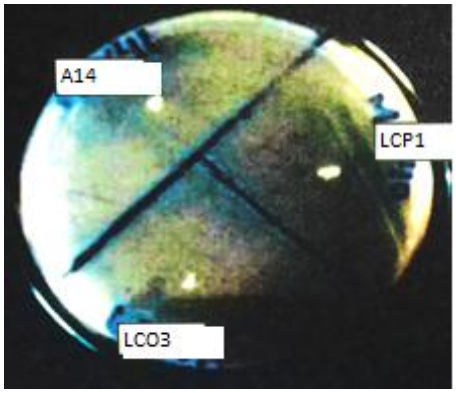

Fig. 5b. Phosphate solubilization by isolated LAB strains.

***In vivo* assessment for plant growth promotion:** Based on results obtained after agar well diffusion and phosphate solubilization, LAB isolate LCP1 was coated on tomato seeds to check its ability to promote plant growth. A significance increase in the growth rate of plants of coated seeds was seen as compared to that of uncoated and sterilized seeds. Furthermore, great difference was seen in the germination percentage of coated seeds as compared to that of other two sets. Most promising results were seen in pot 2 with a germination percentage of 100% and vigor index of about 2652.125. On the other hand uncoated fresh tomato seeds showed reduced growth as compared to pot 1, 2 and 3 (test). Although it showed reduced growth rate than the test but its growth rate was

comparatively higher than sterilized seeds which hardly got germinated. Only 13% of the sterilized seeds were germinated after two months. In case of fresh uncoated seeds trial the most promising results were observed in pot 7and 8 with a germination percentage of 60% and vigor index of 963 (Fig. 6a, 6b and 6c).

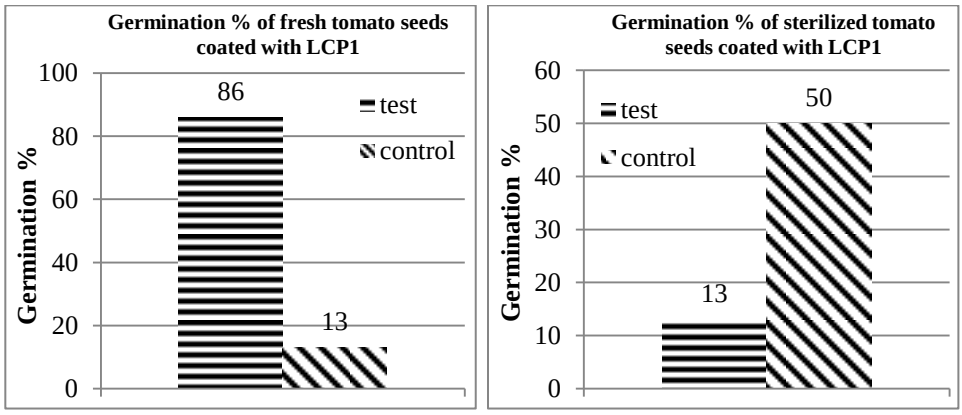

Fig. 6a. Germination % of fresh and sterilized tomato seeds coated with LCP1.

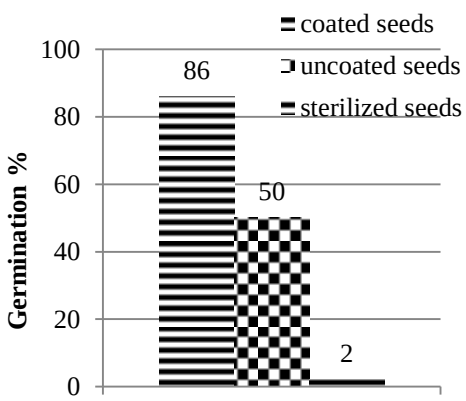

Fig. 6b. Comparison between the germination % of coated, uncoated and sterilized seeds.

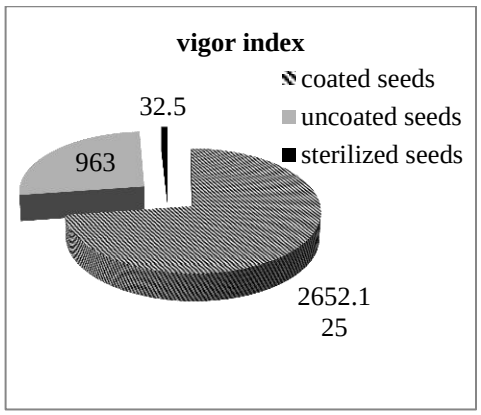

Fig. 6c.Seedling vigor index of the seeds coated with LCP1.

DISCUSSION

Lactic acid bacteria perform a variety of functions when it comes to food supplementation and preservation. It has the ability to preserve food and provide essentials nutrients and vitamins (Axelsson *et al.,* 2000). Lactic acid bacteria have ancient history as it is used in industries for fermentation and production of beverages. They have the ability to carry out acidification of medium by producing organic acids, some aroma producing compounds and most importantly lactic acid (Leroy *et al*., 2004). Since Lactic acid bacteria have a wide range of habitat, in this study LAB was isolated from various

sources including fermented vegetables, wheat bran, milk, rice wash and poultry. Almost 40% of the LAB were isolated from fermented vegetables including onions, potatoes and tomatoes. A group of scientists worked on the isolation of LAB from various vegetables (Tamang *et al*., 2005). Lactic acid bacteria are producers of acids and other antimicrobial compounds by which they prevent the colonization of phytopathogens thus overcoming the spoilage of vegetables. In a similar study scientist found that LAB is present on the surface of variety of vegetables including cabbage, cucumber, capsicum, carrot, cauliflower, cowpea, cluster beans, French beans, lady finger and spinach leaves (Sathe *et al*., 2007). In a similar study 40% LAB were isolated from vegetables of which $1/4^{th}$ were isolated from potatoes. Since, LAB are efficient in metabolism of carbohydrate their presence is obvious on the surface of starchy vegetables like potatoes. Recent studies showed that incorporation of fibers from potatoes and other vegetables along with fermentation efficient LAB has reduced the complication of gastrointestinal tract to a greater extent (Kim *et al.,* 2000). Poultry and dairy products including mozzarella cheese, chicken and cheddar cheese carried 30% LAB. Lactic acid bacteria are considered as the most abundant microbe present in dairy products including cheese, yoghurt, fermented milk and butter etc. Scientists studied that dairy products are rich in LAB and its antimicrobial compounds (Smitt *et al*, 2005). A group of researchers isolated LAB from cheese and other dairy products to study their antimicrobial activities (Sintubin *et al*., 2009). In our study almost 40% of the LAB isolated from vegetables have antimicrobial activity against gram positive as well as gram negative organisms. Scientists isolated Lactic acid bacteria including *Lactobacillus casei* and *Lactobacillus dulbrueckii* from kitchen vegetable waste and performed agar well diffusion assay to assess the antimicrobial activity of the isolated LAB strains. The study was found to be extremely promising in terms of probiotic potential of Lactic acid bacteria (Rauta *et al*., 2013). Our present study employed Lactic acid bacteria isolated from dairy products to check their bacteriocin producing ability. Similarly scientist isolated Lactic acid bacteria from dairy and non-dairy products to screen for the presence of antimicrobial compounds. They isolated *Lactobacillus fermentum, Lactobacillus brevis, Lactobacillus casei, Lactobacillus plantarum, Leuconostoc mesenteroides.* Out of these strains most pronounced results were obtained by *Lactobacillus plantarum* (Adebayo-tayo *et al*., 2008). Some gram positive and gram negative indicators were used to study the antagonistic activity of Lactic acid bacteria including *Bacillus subtilis, Streptococcus faecalis, Salmonella typhi, Shigella dysenteriae* and *Klebsiella pneumoniae.* Screening of Lactic acid bacteria against *Salmonella typhi and Shigella dysenteriae* showed the most promising results by cell free supernatant as compared to neutralized cell free supernatant. A similar research was done by a group of scientists who isolated Lactic acid bacteria from different food sources and tested their antagonistic ability towards *Salmonella typhimurium, Salmonella enteritidis, Bacillus cereus* and they found the cell free supernatants of LAB more active against these pathogens (Tripathy *et al.,* 2012). Lactic acid bacteria play a key role in inhibiting hazardous microorganisms coupled to the preservation of food by the production of certain metabolites (Ham *et al.,* 2003). This ability of Lactic acid bacteria was employed in our study to determine it antagonistic activity against *Bacillus subtilis.* Some of the Lactic acid bacterial isolates were found to be effective in eliminating the growth of *Bacillus subtilis.* Similar work reported the antagonistic activity of LAB strains namely *Pediococcus pentosaceus, Lactobacillus*

sakei and *Pediococcus acidilactici* against many pathogens including *Bacillus* sp., *Pseudomonas* sp., *Listeria* sp. and *Escherichia* sp. They found out that introducing *Pediococcus pentosaceus* in bread can inhibit its spoilage by *Bacillus subtilis* spores after 6 days at room temperature. They sprayed the single cell suspension of *P. acidilactici KTU05-7, P. pentosaceus KTU05-8* and *KTU05-10* on the surface of bread which inhibited the growth of fungus after even keeping it in polythene bags for 8 days (Cizeikiene *et al.,* 2013). Our study found Lactic acid bacteria to be more active against gram negative organisms which is in contrast to a report demonstrating the effectiveness of Lactic acid bacteria towards gram positive (Mezaini *et al.,* 2009). Phosphorus is one of the key nutrients required by plants, it is abandoned in soil in its insoluble form and needs to be solubilized to be converted into a usable form that will be easily assimilated by plants. Appropriate supply of phosphorous throughout the life span of the plant leads to the increased growth and enhanced resistance towards plant diseases (Sharma *et al.,* 2013). Most of the microorganisms have the ability to convert the insoluble form of phosphate into soluble form by the production of enzymes and organic acids and make it available for plants (Qureshi *et al.,* 2012). We isolated Lactic acid bacteria from potato, tomato, chicken and rice-wash that showed pronounced results of phosphate solubilization, while some of the LAB strains were unable to solubilize phosphate. One such study isolated Lactic acid bacteria from fermented food items and only one was able to solubilize phosphate (Giassi *et al.,* 2016). Similarly, another study was conducted in which scientist determined the effect of phosphate solubilizing bacteria namely *Lactobacillus* to check its effect on growth of spinach plant. After 45 days they found that the germination percentage of the LAB treated seeds were 100% with greater number of leaves (23/plant) and high chlorophyll content (Uma *et al.,* 2018). A study determined the phosphate solubilization by microbes in the presence of organic acids (oxalic and malic acid), where organic acids increased the phosphate solubilization by bacteria without affecting the pH of the soil and also enhanced the shoot length and growth rate of plant (Panhwar *et al.,* 2013). In our study tomato seeds coated with LCP-1 increased the root length and shoot length of the plant with a germination percentage of 86% and vigor index of 2625.1. Similar research was conducted by a group of researchers where they isolated 8 LAB strains from sugarcane liquor, 11 *Bacillus* strains from strawberry leaves and 11 *Actinobacteria* from citrus growing soil then observed the effect on plant growth in terms of leaves, shoot and height. They used other plant growth promoting bacteria as well but species of LAB was the only one to promote the plant growth to such an extent with greater number of leaves as compared to others (Giassi *et al.,* 2016). Similarly a study was conducted to observe the plant growth promotion by LAB, where the seeds treated with LAB showed improved germination percentage with a maximum of 79.76% and vigor index 1130.20 (Murthy *et al.,* 2012). Pot trials on LAB coated seeds demonstrated an increased germination percentage, root and shoot length and vigor index with a marked increase in plant dry weight (Abdel-Aziz *et al.,* 2014).

CONCLUSION

It is important to consider the role of particular biofertilizer which must have the ability to solubilize minerals for plants and aids in growth promotion. The isolated LAB strains possessed the ability to solubilize phosphate for plants. Current study reveals that

LCP-1 could be used as a potential biofertilizer and a plant growth promoting candidate. However, further research should be carried out to investigate the pathogen inhibiting properties of these biofertilizer.

Future perspective

The isolated LAB strains have proved to be good biofertilizers after their *In vivo* and *In vitro* characterization. Moreover, these strains must be tested for their other bactericidal and fungicidal properties.

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(Received 28 June 2018; Accepted 30 August 2018)