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Isolation and identification of antimicrobial-producing lactic acid bacteria from fermented cucumber

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Lactic acid bacteria (LAB) responsible for spontaneous fermentation of cucumber were isolated and their antimicrobial producing potentials were screened against 10 indicator strains. 65% of the isolated LAB produced antimicrobial activities against at least two indicator strains. The indicator strains used were: *Escherichia coli*, *Bacillus licheniformis*, *B. cereus*, *Proteus* species, *Staphylococcus aureus*, *Salmonella* species, *Pseudomonas flourescence*, *P. aeruginosa*, *Serratia* species and *Pediococcus acidilactici*. Of the 42 antimicrobial producing isolates characterized, 16, 12, 6 and 8 were identified as *Lactobacillus plantarum, L. fermentum*, *L. acidophilus* and *Leuconostoc mesenteroides*, respectively. Highest lactic acid producers DW7, DT6, DH13 and DF12 were selected for further investigations and were identified as *L. plantarum*. None of the selected *L. plantarum* isolates had antagonistic activity against *S. aureus*, *Salmolnella* species and *P. acidilactici*. The effect of pH and temperature on the antimicrobial activity of selected isolates showed that pH 5.5 and temperature 30°C were the optimum pH and temperature respectively. Thus, the selected *L. plantarum* isolates are good producers of antimicrobial compounds and could be suitable for application in food industry in preservation of vegetables so as to increase their shelf life.

Key words: Cucumber, fermentation, antimicrobial activity, indicator strains, lactic acid bacteria.

INTRODUCTION

Lactic acid bacteria (LAB) are common fermentation microorganisms because of their mechanisms for survival in acidic, high salt environments and the end products they produce through metabolism (Hutkins, 2006). They are intrinsically present in vegetables, plant materials and gastro-intestinal tract of human and animals and dairy products among other foods and have generally recognized as safe (GRAS) status. Consequently, LAB may be added to minimally processed vegetable products intended for consumption (Caplice and Fitzgerald, 1999).

LAB is natural colonizer of fresh vegetables and have been previously described as good antagonist of several bacteria and fungi in different food products (Stiles and Holzapfel, 1997). According to Ogunbanwo et al. (2004), LAB has the potential to inhibit the growth of pathogenic and spoilage bacteria and possibility exist for using them

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to improve the shelf life of different foods.

LAB strains showing functional properties such as degradation of phytic acid in vegetable products were reported to have antimicrobial activities including production of bacteriocin in fermented olive (Rubia-Soria et al., 2006), sauerkraut (Tolonen et al., 2004), fermented carrots, fermented cucumbers and organic leafy vegetables (Ponce et al., 2008). Researchers have shown that LAB can decrease the pathogen numbers in vegetable products and they can develop the immune system of the hosts (Irkin and Songun, 2012).

Lactic acid bacteria (LAB) are generally recognized as safe (GRAS microorganisms) and play an important role in food and feed fermentation and preservation either as the natural microflora or as starter cultures added under controlled conditions. The preservative effect exerted by LAB is mainly due to the production of organic acids (such as lactic acid) which result in lowered pHs (Daeschel, 1989). LAB also produce antimicrobial compounds including hydrogen peroxide, CO_2, diacetyl, acetaldehyde, D-isomers of amino acids, reuterin and bacteriocins (Cintas et al., 2001). Since the isolation and screening of microorganisms from natural sources has always been the most powerful means for obtaining useful and genetically stable strains for industrially important products (Ibourahema et al., 2008), this work therefore aimed at isolation of antimicrobial producing lactic acid bacteria from spontaneously fermenting cucumber and identifying the highest antimicrobial producing isolates.

MATERIALS AND METHODS

Sample collection and processing

Cucumber was obtained from a vegetable Market in Ibadan, Oyo state, Nigeria. It was fermented spontaneously for 96 h and sample was taken at 24 h interval for microbial analysis and pH measurement. Analysis of samples was carried out in triplicate.

Microbial analysis

Isolation was by serial dilution using pour plate method (Harrigan and McCance, 1966). 1 ml of appropriate dilutions was mixed with molten de Mann Rogosa Sharpe agar (Oxoid, Basingstoke, UK) which was aseptically poured into sterile petri dishes. They were incubated at 35±2°C for 48 h under anaerobic condition. Distinct colonies were streaked out in order to obtain pure cultures. Gram positive, catalase negative, non-spore forming rods were selected for further tests. The selected microbes were identified based on their biochemical and physiological characteristics (Kandler and Weiss, 1986).

Screening of lactic acid bacteria (LAB) isolates for antimicrobial activity

Antimicrobial activity of LAB isolates was determined by agar well-diffusion method (Tagg and McGiven, 1971). Ten bacterial strains used as indicators to evaluate the antimicrobial activity of LAB included: *E. coli*, *B. licheniformis*, *B. cereus*, *Proteus* sp., *S. aureus*, *Salmonella* sp., *P. flourescence*, *P. aeruginosa*, *Serratia* sp. and *P. acidilactici*. The cell-free supernatants (CFS) of 48 h old LAB culture in MRS broth were tested. All indicator strains were grown in Nutrient broth at 37°C. Mueller Hinton agar (LAB M, Heywood, UK) plates were overlaid with 5 mL of soft agar (0.75%) containing 50 µL of freshly grown culture of indicator organisms. The wells were made in agar and filled with 100 µL of the tested strain CFS. After incubation at 37°C for 24 h, the diameter of the inhibition zones was measured. All antimicrobial tests were performed in triplicate.

Quantitative estimation of lactic acid

Sodium hydroxide (0.1 N) was titrated against 25 ml of broth culture of test organism with 2 drops of phenolphthalein as indicator. Each millilitre of sodium hydroxide (NaOH) is equivalent to 90.08 mg of lactic acid. Titratable acidity of lactic acid was calculated according to AOAC (1980).

Quantitative estimation of hydrogen peroxide

Hydrogen peroxide production was determined by measuring 25 ml of broth cultures of the test organisms into 100 ml flask. To this was added 25 ml of dilute H_2SO_4. This was then titrated with 0.1N potassium permanganate ($KMnO_4$). Each millilitre of 0.1N $KMnO_4$ is equivalent to 1.701 mg of H_2O_2. A decolourization of the sample was regarded as the end point. The volume of H_2O_2 produced was then calculated (AOAC, 1980).

Quantitative estimation of diacetyl

Diacetyl production was determined by transferring 25 ml of broth cultures of test organisms into 100 ml Erlenmeyer flasks. Hydroxylamine solution (7.5 ml) of 1 molar was added to the flask and to a similar flask for residual titration. Both flasks were titrated with 0.1M HCl to a greenish yellow end point using bromophenol blue as indicator. The equivalence factor of HCl is 21.52 mg. The concentration of diacetyl produced was calculated using the following formula (AOAC, 1980).

Effects of different pH on antimicrobial activity of the isolates

The pH of the medium (Mueller Hinton agar) was adjusted to initial pH of 4.5, 5.0, 5.5, 6.0, 6.5 (Hernandez et al., 2005). 24 h culture of indicator organisms ($\times 10^6$ cfu/ml) was seeded into the molten agar, allowed to set and 10 mm well were made in agar with sterile cork borer. About 100 µL of cell free supernatant of 24 h broth of LAB isolates were then poured into the well (Schillinger and Lucke, 1989) and incubated for 24 h. The antimicrobial activity of isolate was determined by the diameter of inhibition zones around indicator strain.

Effects of different temperature on the antimicrobial activity of the isolates

Indicator organisms ($\times 10^6$ cfu/ml) cultured for 24 h were seeded into molten agar of Mueller Hinton (LAB M, Heywood, UK) in which 10 mm agar well were bored after it has set. 24 h culture of cell free supernatant of test isolate's broth (100 µL) was poured into the well. It was then incubated at temperature of 30, 35, 40 and 45°C. The antimicrobial activity of isolate was determined by the diameter of inhibition zones around indicator strain.

Table 1. Total microbial counts and acidity of fermenting cucumber.

Time (h)	LAB isolate count (cfu/ml)	pH	Total titratable acidity
0	9.2×10^5	6.07	3.2
24	2.1×10^7	5.64	3.5
48	1.4×10^8	4.82	3.6
72	1.8×10^8	4.33	4.1

RESULTS

Table 1 shows the total microbial count, pH and total titratable acidity of fermenting cucumber. The microbial counts, total titratable acidity increased with increase in fermentation period whereas, pH decreased with increase in fermentation time. A total of 42 antimicrobial producing LAB was isolated, characterized based on morphological, biochemical and physiological characteristics and identified as *L. plantarum* (38.10%), *L. acidophilus* (14.29%), *L. fermentum* (28.57%) and *L. mesenteroides* (19.05%) with their respective percentage of occurrences.

The quantity of antimicrobial produced by all the LAB isolates increased within 48 h of incubation period and decreased by 72 h (Table 2). The highest lactic acid production of 4.9 g/l was recorded in isolate DF12 followed by 4.5, 4.4 and 3.7g/L by isolates DW7, DT6 and DH13, respectively. Higher lactic acid production was observed in other isolates like DW6, DW11 and DW13 (3.7g/L), DT4 (4.3g/L), DH6 and DH14 (3.8g/L), DF4 (3.8g/L), and DF13 (4.2g/L). Among the 24 h isolates, DW6 and DW12 had the highest diacetyl production while isolates DT5 (48 h), DH6 (72 h) and DF23 (96 h) had highest diacetyl production in their respective groups. The hydrogen peroxide production ranged from 0.02 to 0.13 g/L by all the isolates, with isolate DT5 having the highest (0.13 g/L) at 48 h. Isolates DW6 and DW12 had the highest hydrogen peroxide production among 24 h isolates, DT5 among 48 h isolates, DH6 among 72 h isolates, and DF6 and DF23 among 96 h isolates.

The morphological, biochemical and physiological characterization of the isolates revealed that all the isolates that produced highest lactic acid among each group are *L. plantarum* (DW7, DT6, DH13 and DF12), while the highest producers of diacetyl (DW6, DW12, DT5, DH6 and DF23) and hydrogen peroxide are identified as *L. mesenteroides*.

Figures 1A to E showed the antagonistic activity of four isolates with the highest lactic acid contents among the LAB isolates against indicator organisms at different pH. The diameter of zones of inhibition ranged between 0 and 21 mm. Antagonistic activity of selected LAB isolates at pH 4.5 is shown in Figure 1A. Isolate DF12 had highest antimicrobial ability against most of the indicator organisms however; DT6 had antagonistic activity against *Proteus* species which no other isolate did. Isolate DW7 and DF12 had the highest antagonistic activity (16 mm) against *B. licheniformis*. Isolate DH13 recorded highest antimicrobial ability (7 mm) against *P. fluorescence* compared to other LAB isolates. None of the LAB isolates recorded antagonistic activity against *S. aureus*, *Salmonella* sp. and *P. acidilactici* at pH of 4.5.

At pH 5.0, all the LAB isolates showed non-antagonistic activity against *Proteus* sp., *S. aureus*, *Salmonella* sp. and *P. acidilactici* except isolate DT6 which had antagonistic activity against *Proteus* sp. and no activity against *Serratia* sp., *P. fluorescence* and *P. aeruginosa*. The highest antagonistic activity against *B. licheniformis* (17 mm) was recorded by isolates DW7 and DF12.

Figure 1C showed the antagonistic activity of LAB isolates at pH 5.5, from the Figure, all the isolates had antagonistic activity against most of the indicator organism. Isolate DT6 was the only isolate which had activity against *Proteus* sp. but had no activity against *S. aureus*, *Salmonella* sp., *P. fluorescence* and *P. acidilactici*. Highest antagonistic activity against all the indicator organisms was observed with isolate DF12. None of the LAB isolates had activity against *S. aureus*, *Salmonella* sp. and *P. acidilactici*.

The effect of adjusting the medium pH to 6 on the antagonistic activity of the cell free supernatant of the selected LAB isolates is as shown in Figure 1D. Higher antimicrobial activity was observed in all LAB isolates against the indicator organisms except *S. aureus*, *Salmonella* sp. and *P. acidilactici* which were resistant. Only isolate DT6 has antagonistic effect on *Proteus* sp. at this pH and no activity on *P. fluorescence* and *P. aeruginosa*.

At pH 6.5, none of the LAB isolate had inhibitory effect on *S. aureus, Salmonella* sp., *P. aeroginosa* and *P. acidilactici* (Figure 1E). Isolate DT6 had no antimicrobial activity against *P. flourescence*. Generally, at all tested pH, highest antimicrobial activity was recorded by isolate DF12 while only isolate DT6 had inhibitory effect on *Proteus* species.

The effect of varying incubation temperature on the antimicrobial abilities of isolate DW7 on selected indicator organisms is as shown in Figure 2A. Increase in temperature from 30 to 45°C resulted in a decreased antimicrobial activity with the least activity observed at 40ºC and the best at 30°C

The result of the antimicrobial potentials of isolate DT6 at different incubation temperature showed that the

Table 2. Quantitative Determination (g/l) of Antimicrobial production.

Isolates code	Lactic acid (g/l)			Diacetyl (g/l)			Hydrogen peroxide (g/l)		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
DW1	3.1	3.6	3.4	2.4	2.9	2.6	0.02	0.05	0.03
DW2	2.5	2.9	2.6	2.5	3.3	2.7	0.03	0.07	0.04
DW3	3.1	3.5	3.3	2.2	2.8	2.4	0.04	0.09	0.05
DW4	2.4	2.9	2.6	2.1	2.7	2.4	0.03	0.08	0.05
DW5	2.9	2.5	3.1	2.2	2.7	2.5	0.03	0.09	0.07
DW6	3.1	3.7	3.4	2.8	3.4	3.1	0.07	0.12	0.09
DW7	3.8	4.5	4.2	2.4	2.9	2.7	0.02	0.05	0.04
DW8	2.0	2.6	2.5	2.2	2.7	2.6	0.03	0.08	0.04
DW9	2.1	2.8	2.4	2.5	2.9	2.6	0.03	0.09	0.05
DW10	2.2	2.7	2.5	2.4	3.0	2.8	0.03	0.08	0.05
DW11	3.1	3.7	3.5	2.2	2.8	2.4	0.02	0.06	0.04
DW12	3.0	3.6	3.5	2.7	3.5	2.9	0.07	0.12	0.09
DW13	3.0	3.7	3.4	2.6	3.4	2.8	0.07	0.11	0.09
DW14	2.9	3.5	3.4	2.2	2.7	2.6	0.02	0.05	0.04
DT3	1.9	2.5	2.2	2.3	2.9	2.8	0.03	0.08	0.05
DT4	3.7	4.3	3.9	2.4	3.2	2.9	0.02	0.05	0.03
DT5	2.5	3.2	2.7	2.7	3.6	3.2	0.07	0.13	0.09
DT6	3.8	4.4	3.0	2.2	2.9	2.4	0.02	0.05	0.03
DT7	2.9	3.5	3.1	2.2	2.8	2.4	0.05	0.09	0.07
DT8	2.4	2.9	2.6	2.3	3.0	2.5	0.03	0.08	0.05
DH4	2.6	3.2	2.8	2.5	3.2	2.7	0.07	0.12	0.08
DH5	1.9	2.5	2.2	2.2	2.8	2.4	0.03	0.07	0.04
DH6	3.0	3.8	3.4	2.8	3.6	3.2	0.07	0.12	0.09
DH9	2.4	2.9	2.7	2.1	2.7	2.5	0.04	0.09	0.05
DH10	1.8	2.2	2.0	2.2	2.8	2.5	0.03	0.09	0.05
DH13	3.3	3.7	3.5	2.4	3.2	2.9	0.02	0.06	0.04
DH14	3.0	3.8	3.5	2.2	2.8	2.4	0.02	0.07	0.04
DF2	3.0	3.6	3.5	2.4	3.2	2.9	0.02	0.06	0.05
DF3	1.8	2.5	2.1	2.2	2.8	2.4	0.03	0.09	0.06
DF4	3.0	3.8	3.5	2.0	2.6	2.3	0.02	0.07	0.04
DF5	2.9	3.4	3.1	2.1	2.7	2.4	0.04	0.09	0.06
DF6	2.5	3.0	2.7	2.4	2.9	2.6	0.07	0.12	0.09
DF9	2.1	2.8	2.4	2.1	2.7	2.5	0.04	0.08	0.07
DF10	2.4	3.0	2.8	2.2	2.8	2.7	0.03	0.08	0.06
DF11	2.4	2.9	2.6	2.2	2.9	2.6	0.03	0.08	0.05
DF12	4.2	4.9	4.4	2.4	3.1	2.7	0.03	0.08	0.06
DF13	3.5	4.2	3.8	2.2	2.9	2.5	0.04	0.09	0.07
DF15	2.9	3.5	3.1	2.2	2.9	2.6	0.04	0.08	0.06
DF17	2.7	3.4	2.9	2.1	2.6	2.5	0.04	0.09	0.07
DF19	3.0	3.7	3.4	2.4	3.0	2.8	0.02	0.06	0.05
DF22	2.0	2.7	2.5	2.2	2.8	2.7	0.03	0.07	0.05
DF23	3.1	3.6	3.5	2.6	3.2	2.9	0.07	0.12	0.09

L. plantarum, DW1, DW3, DW7, DW11, DW14, DT4, DT6, DH13, DH14, DF2, DF4, DF12, DF15, DF13, DF19, DF22; *L. fermentum,* DW2, DW4, DW8, DW9, DW10, DT3, DT8, DH5, DH10, DF3, DF10, DF11; *L. acidophilus,* DW5, DT7, DH9, DF5, DF9, DF17; *Leuconostoc mesenteroide,* DW6, DW12, DW13, DT5, DH4, DH6, DF6, DF23.

optimum antimicrobial activity is at 30°C (Figure 2B). As the incubation temperature increased, antimicrobial activity decreased with no activity on most of the indicator organisms at 45°C. Increasing temperature has drastic

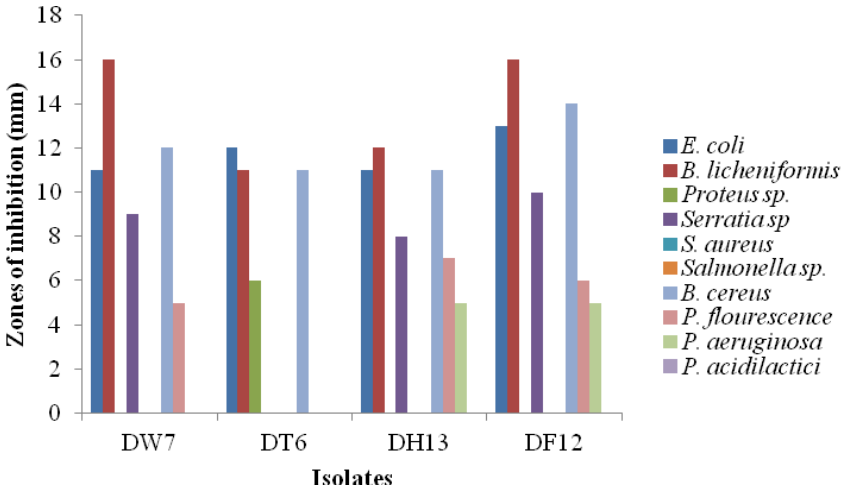

Figure 1A. Antagonistic activity of LAB isolate at pH 4.5.

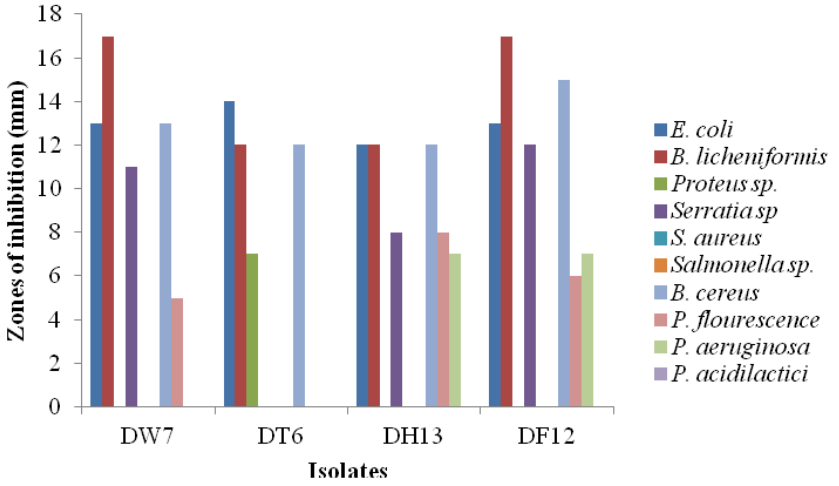

Figure 1B. Antagonistic activity of LAB isolates at pH 5.0.

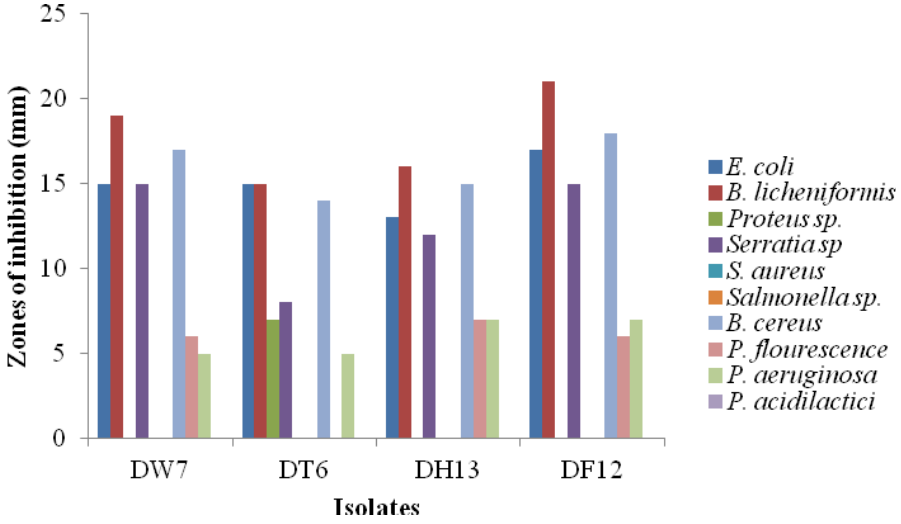

Figure 1C. Antagonistic activity of LAB isolates at pH 5.5.

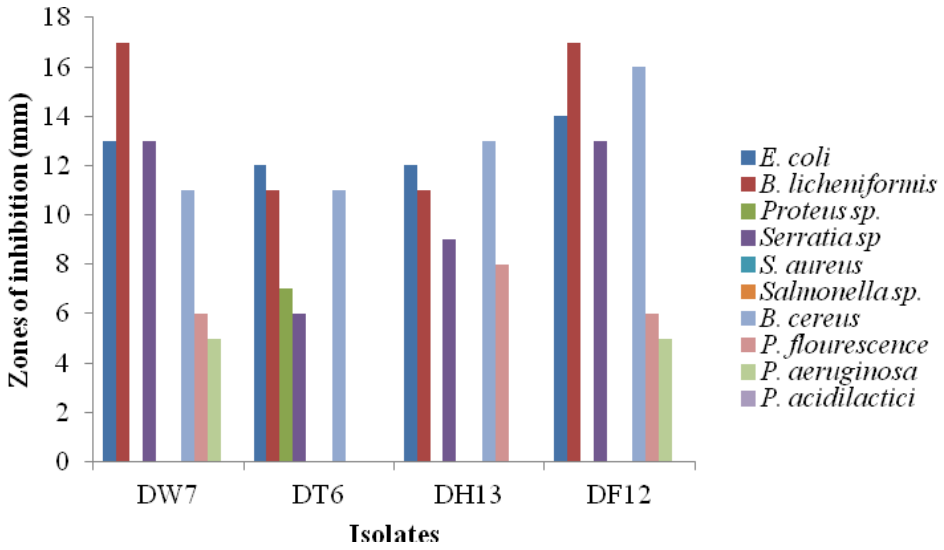

Figure 1D. Antagonistic activity of LAB isolates at pH 6.0.

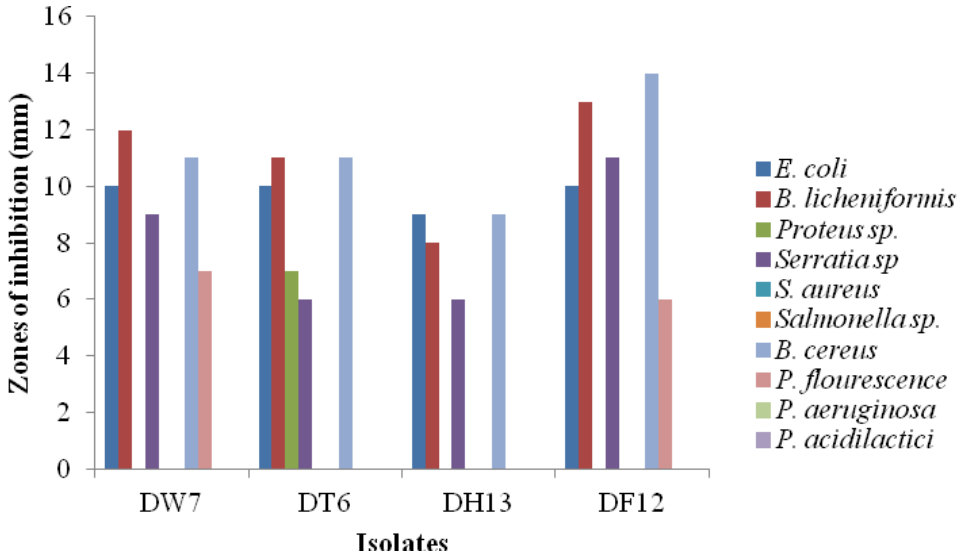

Figure 1E. Antagonistic activity of LAB isolates at pH 6.5.

drastic effect on the antimicrobial abilities of isolate DT6.

Figure 2C shows the antagonistic spectrum of LAB isolate DH13 at different incubation temperature. The antagonistic activity of the organism decreased with increase in incubation temperature. The optimum and least antimicrobial activity were recorded in 30 and 45°C. It had wide range of antimicrobial activities against indicator organisms at all incubation period.

The antagonistic spectrum of LAB isolate DF12 at different incubation temperature is shown in Figure 2D. Antagonistic properties also decreased with increase in incubation temperature. The best antimicrobial ability was recorded at 30°C. The highest antimicrobial activity (21 mm) was recorded against *B. licheniformis* at 30°C.

DISCUSSION

The lactic acid bacteria were identified according to Kandler and Weiss (1986). *L. plantarum* was found to have a dominant role in cucumber fermentations due to its tolerance for high acidity (Fleming and McFeeters, 1981) which is in support of this work. *L. plantarum* isolated in this work is in line with the work of Lu et al.

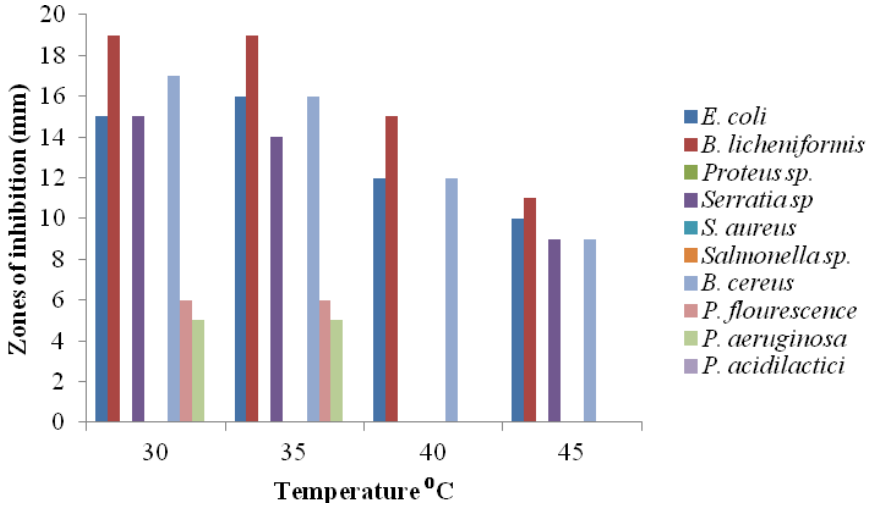

Figure 2A. Antagonistic effect of LAB isolate DW7 at different incubation temperature

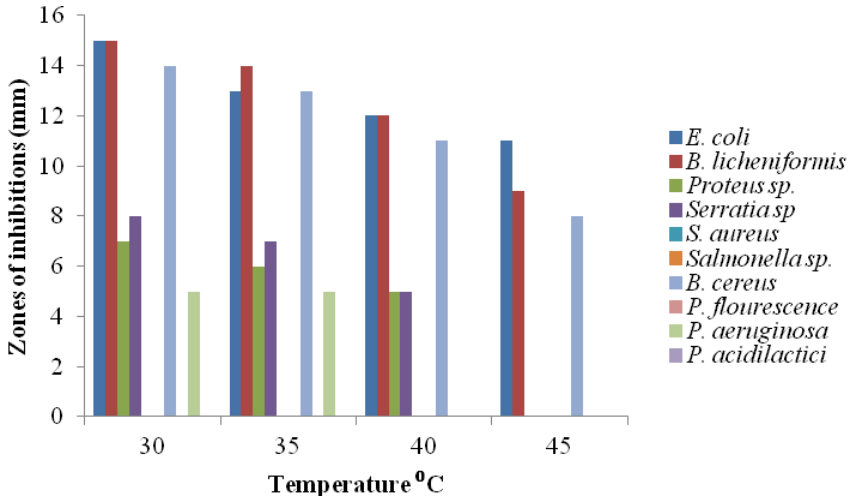

Figure 2B. Antagonistic effect of LAB isolate DT6 at different incubation temperature

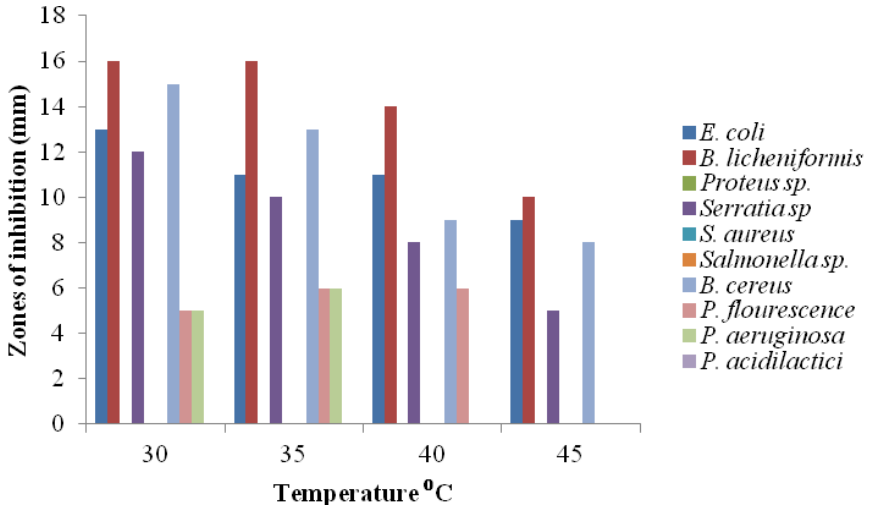

Figure 2C. Antagonistic effect of LAB isolate DH13 at different incubation temperature.

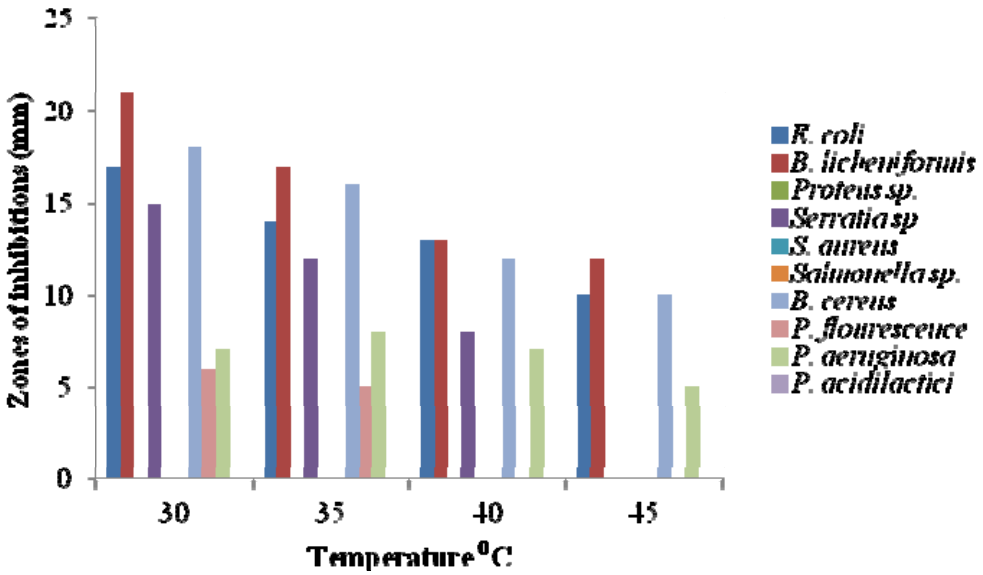

Figure 2D. Antagonistic effect of LAB isolate DF12 at different incubation temperature.

(2003) who isolated *L. plantarum* from commercial cucumber fermentation.

The ability of some species of LAB particularly *L. plantarum* in acidification of the substrates is significant in food preservation (Ammor and Mayo, 2007). It was found that some LAB strains isolated from fermented bamboo products were able to lower the pH to 3.8 (Tamang and Sarkar, 1996). LAB cultures were used effectively against Gram positive pathogens, coliforms, *Aeromonas hydrophila*, *S. aureus*, *Salmonella typhi* and *Listeria monocytogenes* in ready to use vegetables (Vescovo et al., 1995).

It is well documented that lactic acid bacteria (LAB) were involved in vegetable fermentation, so this genera had been extensively studied with the aim of using them as starter culture (Leal-Sanchez et al., 2002). Of the 42 LAB isolates screened, all exhibited a broad activity against indicator culture. Similar results have been reported by Geis et al. (1983) and Klaenhammer (1988) who detected activity only in agar medium. Other authors have also found that cell-free culture supernatant were inhibitory (Schillinger and Lucke 1989; Stiles and Holzapfel, 1997).

The isolated and identified *Lactobacillus* strains exhibited different levels of antimicrobial activity against the selected indicator strains. Among the isolates, *L. plantarum* (DW7, DT6, DH13 and DF12) recorded higher inhibitory activity compared to the others, suggesting that those strain could be used as starter culture especially because of the high inhibitory effect of these strains on the pathogen bacteria such as *Bacillus* species and *E. coli* and this can increase their importance for the industrial applications. Gagiu et al. (2013) showed that *L. plantarum* can be used to develop a wheat sourdough with inhibitory activity against bread spoilage fungi. Bamidele et al. (2013) reported that LAB isolated from vegetable exhibited varied spectra of inhibition against the test MRSA which was resistant to cloxacillin, augmentin, streptomycin, tetracycline and cotrimozaxole.

L. plantarum produced high lactic acid content in this study because under anaerobic conditions, *L. plantarum* had homofermentative pathway, thus produce only lactic acid (Holzer et al., 2003) which may be the reason for their being suggested as antagonistic starter culture for their high lactic acid production and various inhibitory metabolites (Caplice and Fitzgerald, 1999). The ability of a rapid and high acid production has been demanded for lactic cultures to be used as starter in the vegetable fermentation technology (Buckenhuskes, 1993).

From the work carried out by Trias et al. (2008) LAB isolates like *L. plantarum* isolated from ready to eat vegetables, inhibit the growth of food borne pathogens like *Listeria monocytogenes*, *Salmonella typhii* and *E. coli* but in the present study the isolate did not inhibit *Salmonella* sp. Okereke et al. (2012) also reported that the LAB isolated in their study inhibited the growth of *S. aureus*, *E. coli* and *B. cereus* however; the LAB isolated in this research work was not able to inhibit *S. aureus* but was able to inhibit both *E. coli* and *B. cereus*.

Effect of varying pH on antimicrobial ability was observed in this study which showed that pH had significant effect on antimicrobial activities. The optimum pH recorded was 5.5. This is in line with the work of Oskay (2011) who reported that varying pH had significant effect on antimicrobial activities of microorganisms; however, his optimum pH (7.5) was contrary to this work.

It was observed in this work that changed in incubation

temperature had drastic effect on antimicrobial activities. The antimicrobial ability decreased with increase in incubation temperature. The optimum antimicrobial activity was recorded at 30°C. This is in relation to the work of Oskay (2011) who reported that temperature had a significant effect on antimicrobial ability. He also reported 30°C as the best temperature for antimicrobial activity, below and above which there will be reduction in microbial ability of the microbe.

In conclusion, antimicrobial producing LAB can be isolated from fermenting cucumber and best activity can be achieved with *L. plantarum* at pH 5.5 and temperature 30°C. This shows the possible use as potential starter culture in vegetable product preservation.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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