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Study of Lactobacillus species MTCC 10093 as Probiotic Bacteria

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Abstract

Due to inhibitory effects of arsenic the selected *lactobacilli* bacteria species, MTCC 10093 was studied as biological preservative probiotic. For the purpose of innovative information regarding referential lactic acid producing bacteria, MTCC 10093 species were isolated from different agricultural soil fields were identified by their biochemical tests through comparing their sugar differential fermentation patterns. Antibacterial activities were confirmed by agar spots, diffusion illustrations and through the blank disk method. Enzymatic sensitivities of supernatant fluids and concentrated cells free culture after treatment with α-amylase, lysozyme and trypsin were determined. It indicated that the isolated bacteria had strong activity against indicator strains and their antibacterial activity was stable at 100ºC for 10 mints whereas at 56ºC for 30 mints their activities were lost after autoclaving. On this basis, maximum production of plantaricin was obtained at 25 - 30ºC at pH 6.5 and thus the *lactobacilli* MTCC 10097 species were produced antimicrobial activity with heat stability bacteriocin, hence, these specified species of *lactobacilli* may be considered as healthy and effective probiotic diet.

Keywords: Lactobacillus, *Probiotic, Antibacterial activity*

Introduction

Probiotic cultures have been associated historically with cultured of milk and dairy products which is substantial evidence for their positive effects on human health and generalwell-being (1, 2). For most of in vitro and in vivo experiments on antagonism of different *Lactobacillus* strains against *Helicobacter pylori*, *Clostridium difficile*, *Campylobacter jejuni*, and *E.coli* performed positive effects on all tested human being by *Lactobacillus* strains and were able to inhibit their growth of all related strains of anaerobic in human gastro-intestinal pathogens (3, 4). In addition, bacteriocins have properties such as antitumour and anticholesterolemic activities. Hence, the chemical reactions associated with reduction of nitrate, improved immunological status and adsorption of vitamin-B group member metabolites (5) and transit lactic acid bacteria strains in gastrointestinal tract to deliver enzymes and other substances for possibly help in controlling intestinal flora (6). Hence in these antioxidative activities, the lactic acid bacteria were reported (7) to induce inhibitory effects as selected probiotic lactobacilli identified species which may be used as biological preservatives as the aims and objectives of this article as their antimicrobial activity, effects of

pH, heat, and sensitivity of proteolytic enzymes as probiotic-by-products of isolated species of *lactobacilli* MTCC 10097 in growth culture media.

Materials and Methods

For the purpose of research, the lactic acid bacteria were isolated from soil by using MRS (pepton, meat extract, yeast extract, glucose, tween 80) medium. Briefly, 1g of sausage was mixed and vortexed into MRS broth medium, incubated at 37ºC for 24 h. Growth from MRS broth cultures was used to streak on MRS agar plate. *Lactobacillus* species of these isolates were identified by comparing their sugar fermentation patterns with the scheme described in Bergey's Manual of Systematic Bacteriology (8). *Lactobacilli* were grown in MRS broth or MRS agar. One ml of an overnight culture of *lactobacillus* was used to inoculate 100 ml of MRS broth and incubation was continued at 20, 25, 30, 35, 37, 40, 45ºC for 24 h. Samples were removed at regular intervals (30 min) for the determination of turbidity (measured at 570 nm), culture pH and antibacterial activity. The experiment was repeated with broth in which the initial pH was adjusted to 2 to 12 with HCl or NaCl. Initial and final pH of all samples was also measured. Culture supernatant (200µl) was heated in a boiling water bath for 10 min and cooled rapidly on ice. Serial twofold dilutions of the heated supernatants were made in 0.2 N HCl, and 10µl of each dilution was spotted on to fresh, duplicate indicator lawns. Cultures were incubated for 24 h. For the effects of different sugar and NaCl concentration on production of bacteriocin, the isolated *lactobacillus* was grown in MRS broth without beef extract, supplemented with different concentration of glucose, xylose, sucrose, furoctose, galactose, maltose and NaCl. Then, remaining activity against indicator strains was assayed. For the preparation of culture supernatant, the bacteriocins producing strain was grown in MRS broth for 24 h at 25ºC. A cell free solution was obtained by centrifuge the culture, followed by filtration of the supernatant through a 0.2 µl pore size filter. The supernatant was adjusted to pH 6.5 or dialyzed for 24 h against MRS broth at 4ºC where the Mode of action as explained in one ml of cell free culture supernatant of isolated lactobacilli was added to10 ml of a fresh culture logarithmic phase of indicator bacteria. Culture optical density were determined (at 570 nm) at appropriate intervals where the antimicrobial activities where detection of antagonistic activities, an agar spot procedure, well diffusion assay and blank disk method were used. For the agar spot test, supernatant of overnight cultures of *lactobacillus* strains were spotted (1mm) onto the surface of BHI agar plates of indicator strains and incubated for 24 h at 37ºC to allow colony develop. For the agar well diffusion assay, an overnight culture of the indicator strain was used to inoculate agar growth media at 37ºC. Wells of 5mm diameter w ere cut into agar plates and 50 µl of culture supernatant fluid containing antibacterial activity were added to each well. Supernatant fluid was obtained by growing the inhibitory producer strain overnight in MRS broth at 30ºC. Cells were then removed by centrifugation and the supernatant fluid placed in the wells and allowed to diffuse into the agar for 24 h at 4ºC. The plates were then incubated at optimum growth temperature of the indicator strains and examined after 24 h for inhibition zone. Five sterile paper blank disks were placed on the agar plate which was inoculated by indicator strains and 20 µl of the filtered supernatant of lactobacilli was applied. Plates were incubated and observed for zones inhibition. Indicator strains used as indicator organisms for bacteriocin screening were *Staphylococcusaureus, Salmonella typhi, Yersinia enterocolitica,Bacillus subtilis, Listeria monocytogenes* and other lactobacilli isolated from sausage without antibacterial activity. The plates were incubated at 30ºC for 24-48 h or until growth of the test organism could be easily observed with naked eye.

For the sensitivity to pH and heat, the supernatant was adjusted to pHs between 2 to 12 with HCl or NaOH and incubated. To test heat stability, the supernatant fluid was heated in boiling water for 10 min, at 56ºC for 15 min, or autoclaved at 121ºC for 15min. In all cases, the activity remaining after treatment was measured by spotting procedure. This experiment repeated and the solutions were kept at 4 and -20ºC for 4 weeks, then antibacterial activity was measured. Sensitivity of proteolytic enzymes, the cell free culture supernatant fluid was treated for 1h at 30ºC with trypsin, α-amylase, lysozyme at final concentration

of0.5mg/ml, 220 IU/mg/ml and 22IU/mg/ml, and incubated at 37ºC for 1 h. Concentrated cell free culture supernatants were heated at 100ºC for20 min and the remaining activity was determined by spotted For Bacteriocin concentration procedure**,** one litre lactobacilli culture was grown in MRS broth at30ºC until the late logarithmic phase. The cellremoved by centrifugation for 12 min. at 4ºC,and ammonium sulphate was gradually addedto achieve 40% saturation. The sample was keptat 4ºC with stirring for 30 min. After centrifugation for 30 min, the resulting pellet wasmixed and solubilized in 120 ml of 10 mM sodium phosphate buffer, pH 5.8. Then antimicrobial activity was measured against indicator bacteria (9).

Results

A Lactobacillus species MTCC 10093 lactic acid bacteria isolated from Soil was tested for antimicrobial activity. It shown to produce abacteriocin-like substance. Their sensitivity varied greatly and produced more heat stable bacteriocin than the other isolated strains, which exhibited a broad spectrumof inhibitory activity. The antibacterial activity of plantaricin was more potent than theother isolated strains when sensitive strainswere in the logarithmic growth phase, including cell lysis, as observed by decreased in optical density. No bacteriocin activity was found in cultures grown at 4 or 8ºC. However, bacteriocin production was observed at 20, 25, 30, 37, 40 and 45ºC. At all of these temperatures, the maximum antimicrobial activity in the growth medium was obtained in the late logarithmic growth and early of stationary phases where the amounts of bacteriocin produced at 25 and 30ºC were similar. The bacteriocin activity in the supernatant was stable and no decrease in activity was detected after 5 days at 25ºC. The antibacterial activity was stable at 100ºC for 10 min and at 56ºC for 30 min, but all activity was lost after autoclaving. The antibacterial activity was not lost by freezing and thawing, and long-term storage at 4 and -20ºC. When the supernatants of the cultures containing *Lacto. Plantarum* were checked, a small zone of inhibition was first observed on plates after 6 h at 25ºC and larger zones of inhibition were detected after 24 h (Figure 1)

Figure 1. Zone inhibition of *Staph aureus* against supernatant of *Lacto. species MTCC 10093* by agar spot method, a) *Lacto. Plantarum*, b) *Lacto. delbruekii,* c) *Lacto. acidophilus,* d) *Lacto. brevis,* e) *Lacto. Casei.*

The antibacterial activity of bacteriocin was destroyed by trypsin treatment, but was unaffected by α-amylase and lysozyme. The inhibitory activity remained stable over the pH ranges 2 to10, but was lost after incubation at pH 12, indicating its sensitivity to alkali treatment.All activity was lost after autoclaving.The antimicrobial properties of the *lactobacillus* strains tested were very variable.Many of the strains showed weak or no inhibition of the pathogenic strains. Only 4 strains (14.3%) inhibited the growth of pathogenic bacteria broadly. The maximum production of the bacteriocin was obtained at 25ºCat pH 6.5. Maximum production of bacteriocin was obtained in MRS broth containing at least 1-2% glucose or xylose, also MRS medium with 1% NaCl found that the antibacterial activity increased where the inhibitory activity was maximal at the beginning of the stationary phase and remained stable long after growth had ceased, even in

the presence of the producer cells where the zone inhibition of *Staph aureus* against supernatant of lactobacilli by agar spot method, blank disk, and agar well diffusion assay (Figure-2).

Figure 2. Zone inhibition *Staph. aureus* against supernatant of *Lacto. species MTCC 10093* by blank disk method, a) *Lacto. plantarum*, b) *Lacto. delbruekii,* c) *Lacto. acidophilus,* d) *Lacto. brevis,* e) *Lacto. Casei.*

Discussions

Our results showed that bactericidal actionof the bacteriocin against indicator strains wereon logarithmic phase and early stationary phaseand cell lyses in actively growing cells, therebycausing a decrease in culture optical density.This was also confirmed by Gao et al (13).In this study, production of plantaricin was bestin MRS broth, or in a medium containing peptone, yeast extract, beef extract, glucose, sodium acetate and Tween 80. Glucose couldbe replaced by xylose without a decrease in the amount of plantaricin, but other carbohydrates resulted in less bacteriocin being produced where the maximum production was coincided with onset of logarithmic phase and early of stationary phases and these conditions of low pH and highcell number have also been found to be necessaryfor the production of high levels of bacteriocins. Hence, the maximum production of plantaricin KW30 (9) in bacteriocin of *Lacto and Delbrueckii* (14) were in MRS broth. Their results were similar to our results. Our results showed that bacteriocin activity were very stable under a series of different conditions such as storage at room temperature for 5 days at 4ºC and -20ºC, and heating (100ºC for 10min or 56ºC for 30 min). This is confirmed by Rekhif et al (15).

In general, bacteriocin are from lactobacilli specially *Lacto. plantarum* relatively heat stable with promising inhibitory spectra of antimicrobial activities. Their general heat stability is an advantage, temperature stability being a very importantparameter if a bacteriocin is to be used as a food preservative because many processing procedures involve a heating step.However, the bacteriocins from *Lacto. Plantarum* described in this paper appear quite promising as potential bio-preservatives. Our results are confirmed by some researchers (9, 11, 15). Lactic acid bacteria originally isolated frommeat, meat products and dairy products are probably the best candidates as probiotic bacteria to improve the microbiological safety ofthese foods. Since, they are well adapted to theconditions in meats and dairy products andshould therefore be more competitive than from other sources. Interest in lactic acid bacteria is growing. Also, bacteriocins produced by lactic acid bacteria aregreat interest to the food fermentation industry because they may inhibit the growth of many food spoilage and pathogenic bacteria, therefore an investigation of bacteriocin in lactic acid bacteria may offer potential applicability in food preservation.

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