Probiotic and antimicrobial activity of bacteria from fermented toddy of Cocos nucifera

M. Krishnamoorthy and P. Arjun
Microbiology, Plant tissue culture and Food safety lab, National Agro Foundation, Anna University, Taramani Campus, Taramani, Chennai-600113
krish2285@gmail.com; +91 9962200868

Abstract

Two lactic acid bacteria isolated from 20 fermented samples of plant fermented beverages (PFB) were analyzed for probiotic properties. Acid tolerant (pH 3), thirty six Lactobacillus sp. and thirty Streptococcus sp. showed good resistance (2%) in bile salt even after exposure for 48 h. The test organisms showed high specific growth rate and inhibitory action against potent food borne pathogenic bacteria.

Keywords: Lactic acid bacteria, plant fermented beverages, probiotic, Lactobacillus, Streptococcus.

Introduction

Probiotics are growth promoting factors produced by microorganisms (Lilly and Stillwell, 1965). Parker (1974) defined probiotic as “Organisms and substances with beneficial effects on animals by influencing the intestinal microflora”. Maintaining balance of bacteria residing in the intestine is necessary to healthy intestine. Many factors may change the balance away from potentially beneficial, health promoting bacteria like Lactobacilli and bifidobacteria to potentially harmful or pathogenic microorganisms like clostridia, sulphate reducers and bacteroides species. Use of probiotics help to protect the host from various intestinal diseases and disorders while increasing the number of beneficial bacteria and making the balance steady (Fooks et al., 1999). It is believed that most probiotics do not permanently adhere in the intestine, but exert their effects as they metabolize and grow during their passage through the intestine (colonization). Thus, daily consumption of these bacteria is probably the best way to maintain their effectiveness. With the current focus on disease prevention and the quest for optimal health at all ages, the probiotics market potential is enormous. Health professionals are in an ideal position to help guide their clients toward appropriate prophylactic and therapeutic uses of probiotics that deliver the desired beneficial health effects.

Both bacteria and yeasts are generally introduced by the two main ingredients and participate in the fermentation (Kadere et al., 2008). In view of the above facts, this study was aimed to evaluate probiotic activity of isolated bacterial species from coconut toddy and antimicrobial activity.

Materials and methods

Chemicals: Ringer Martin salt, Nutrient agar, MacConkey agar, MRS agar, Nutrient broth, Phosphate Buffer Saline (PBS), ATCC culture of Lactobacilli was obtained from HiMedia lab Pvt. Ltd, India.

Sample collection: Fermented coconut toddy was collected in the early morning near Uthukottai, Tiruvallur. The container was pre-autoclaved with capacity of 300 mL. Samples were maintained between 0-5°C and brought to laboratory with in 2 h.

Saline preparation: Known volume of 8.9% saline was prepared in double distilled water and was autoclaved at 121°C for 15 min at 15 Lbs.

Serial dilution of the sample: Toddy sample (25 mL) was transferred into 225 mL of saline (10^{-1}) from which, 10 mL was transferred to 90 mL of saline (10^{-2}) and from this 1 mL was transferred to 9 mL of saline (10^{-3}) and repeated up to 10^{-7} dilution.

Methodology of inoculation: Sample of 1 mL was taken from 10^{2}, 10^{3}, 10^{4}, 10^{5}, 10^{6}, and 10^{7} dilutions and poured into separate petri plates. Nutrient agar (20 mL) was poured into 6 petri plates. It was then solidified and kept in incubator at 37°C for 48 h in inverted position. Similarly 20 mL of MacConkey agar was solidified and kept for incubation at 37°C for 48 h in inverted position.
Isolation and identification of strain from fermented coconut toddy

Coconut toddy (25 mL) was added to 225 mL saline and blended thoroughly. Appropriate serial dilutions of the blended mixture was plated onto PCA (Plate count agar) and MRS (de Man Rogosa and Sharpe) agar and incubated at 37°C for 48 h. The translucent/opaque colonies with 2-3 mm in diameter having entire margins were taken and suspended in nutrient broth and incubated at 37°C for 48 h. The process was repeated until pure cultures were obtained. These isolated organisms were maintained in nutrient agar slants, by sub-culturing them periodically and stored in at 37°C (Iyer and Ananthanarayan, 2008).

Microbial counts, laboratory isolation and identification

Ten milliliter of sample was homogenized in 90 mL sterile salt peptone solution containing 0.1% bacteriological peptone and 0.9% NaCl as the 1:10 dilution. After serial dilution, Aerobic mesophilic bacteria were enumerated by pour plate on Plate Count Agar (PCA) incubated aerobically at 37°C for 3 d. Then it was enumerated on MRS agar supplemented with cycloheximide (0.005%). Plates were incubated at 30°C for 2 d under aerobic conditions. The organisms were phenotypically characterized by Gram staining. Determination of morphology was done by phase-contrast microscopy. Only gram-positive, catalase negative, non motile rod and cocci isolates strains were selected. The presence of catalase activity was assessed by the formation of gas bubbles after the suspension of bacterial cells in a droplet of 3% hydrogen peroxide on MRS. Stock cultures of the isolates were stored in MRS broth containing 15% glycerol at 80°C. Carbohydrate fermentation pattern of lactic acid bacteria used for sap fermentation were determined according to the manufacturer's instructions (Amoa-Awua et al., 2007).

Determination of antimicrobial activity

Antimicrobial activity was assayed by an adaptation of the critical dilution assay method according to Mayr-Harting et al. (1972). The 48 h culture grown in medium was spread on 2% Nutrient agar (10 mL) was overlaid with nutrient agar 1% (5 mL) inoculated with overnight grown culture suspensions of the indicator organisms. The plates were allowed to solidify and wells of 6 mm diameter were punched into them with a sterile cork borer. Cell free extract (100 μL) was poured in each of the wells and the plates were placed in the refrigerator at 4°C for 20 min to enhance diffusion of sample. The plates were then incubated at 37°C. The plates were then incubated at 37°C for 24 h and examined for zone of inhibition.

Sugar fermentation test

Peptone water (5 mL) was taken in test tube with 1% sugar solution (Glucose and Lactose) and placed on Durham’s tube in inverted position.

The test tube was autoclaved at 121°C for 15 min. The test culture was inoculated into Durham’s tube and incubated at 37°C. The results were confirmed based on the presence of turbidity and gas production.

Aerobic and anaerobic test

MRS agar (0.56 g) was dissolved in 10 mL of distilled water, and autoclaved at 121°C for 15 min in 15 Lbs, then the agar was poured in two petri plates, one for aerobic growth and the other for the anaerobic growth of the organism. The test organisms were taken from the agar's land and was streaked on the MRS agars plates. The Aerobic plate was incubated at 48°C and the anaerobic plate was incubated at 72°C for 48 h in an anaerobic chamber (This slows down the growth). The colony morphology was then identified.

Sodium chloride tolerance test

One gram of 5% Sodium Chloride salt was mixed with 5 mL of MRS broth in a test tube, subsequently tube with 5 mL of MRS broth without salt was taken. With a circular loop, the test cultures were inoculated into the broth (with and without salt) and incubated at 37°C for 48 h.

Bile tolerance isolates

The isolates were grown in MRS broth containing 2% (w/v) of bile salts mixture at 37°C for 24 and 48 h. The growth was checked using the pour plate technique (Seeley and VanDemark, 1981) wherein 1 mL of culture of appropriate dilutions was overlaid with MRS agar. The plates were incubated at 37°C for 48 h and the cell count was compared with that of the control MRS agar plates (containing cultures grown in MRS medium without bile salts mixture). Bacterial growth was expressed as colony forming units per mL (CFU/mL) and the survival percentage (% ± SD) of strains to bile salts was calculated as given below.

According to Mourad and Nour-Eddine (2006), the percentage survival \[
\frac{1}{2} \log CN_{0} = \log CN_{1} \times 10^{10}
\]
Where, \(N_{0}\) is viable count after exposure to bile salts \(N_{1}\) is viable count without exposure to bile salts

Tolerance to acidic pH values

Isolates were grown in MRS broth at 37°C for 48 h. The cultures were centrifuged at 8,000 rpm for 10 min at 4°C. The pellets were washed twice in sterile phosphate-buffered saline (PBS, pH 7) and re-suspended (1:100) in PBS to achieve a cell density of 1 × 10^{12} cells/mL. This was employed for setting up the experimental control and studying survival of isolates at low pH (pH 1, 2 and 3 prepared in PBS). The suspensions were incubated at 37°C and samples were removed after every 1 h to 4 h. Counts of surviving cells were determined by plating on MRS agar using the procedure followed in bile tolerance assay.
Results and discussion

The existence of two beneficial microorganisms were confirmed by different tests namely gram’s staining, motility, catalase, oxidase tests, biochemical tests, indole methyl red, triple sugar iron, lactic acid confirmatory test, sugar fermentation, aerobic, anaerobic, NaCl tolerant, probiotic confirmatory tests, bile salt tolerant and bile tolerant. Gram’s staining results showed the isolated culture was purple coloured, non-sporulating and rod shaped. Gram’s staining test showed positive for organisms 1 (Lactobacillus) and 2 (Streptococcus) (Fig. 1). A total of 10 organisms were isolated from different samples of fermented coconut toddy. Two bacterial strains which were clear, round, opaque, white to yellow colour colonies, 2-3 mm in diameter having entire margins from PCA and yellow colonies and pink colonies from MAC were taken for the study. The isolates were tested for the gram nature and catalase negative (Table 1; Fig. 1).

![Biological characterization of strains isolated from toddy.](image)

The isolates which are gram positive in nature and catalase negative were studied further. Some of the cultures were bacilli (short rods), the others were cocci and one was a coccobacilli. Based on the Gram nature, morphology and catalase test, the cultures were observed. Biochemical tests; citrate, indole showed negative, colour, ring was not observed and in methyl red test showed ring test was positive (Table 1). In trible sugar iron tests, H$_2$S showed negative for both 1 and 2. Gas test showed 1 positive and 2 negative (Table 2). Organisms 1 and 2 showed fermenting property with the production of gas, sugar fermentation test namely glucose and lactose showed positive (Table 3). No colour changes in citrate utilization test. In agar plug test, there is no gas formation and it was homo-fermentative. The isolates grown on MRS broth were treated with 5% of NaCl at 37°C for 48 h. Both the isolates showed good resistance to 5% NaCl even after exposure for 48 h (Table 1). The growth was checked using the pour plate technique (Seeley and VanDemark, 1971). One of the important criteria to be fulfilled and can be used as a probiotic is its ability to resist the effect of bile salts in the gastrointestinal tract (Lee and Salminen, 1995). However, there are no reports on the exact concentration to which a selected strain should be tolerant.

<table>
<thead>
<tr>
<th>Types of tests</th>
<th>Observation</th>
<th>Sample 1 and 2</th>
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<tbody>
<tr>
<td>Motility test</td>
<td>Non motile</td>
<td>Non motile</td>
</tr>
<tr>
<td>Catalase test</td>
<td>Bubbles release was not observed</td>
<td>Negative</td>
</tr>
<tr>
<td>Oxidase test</td>
<td>Purple colour was not observed</td>
<td>Negative</td>
</tr>
<tr>
<td>Citrate test</td>
<td>Green colour was not observed</td>
<td>Negative</td>
</tr>
<tr>
<td>Indole test</td>
<td>Red ring was not observed</td>
<td>Negative</td>
</tr>
<tr>
<td>Methyl red test</td>
<td>Red ring was observed</td>
<td>Positive</td>
</tr>
<tr>
<td>Fermentation type</td>
<td>Glucose phosphate broth</td>
<td>MR positive</td>
</tr>
<tr>
<td></td>
<td>Homo–hetero</td>
<td>(Mixed acid fermentation)</td>
</tr>
<tr>
<td>Agar plug test</td>
<td>Homo–hetero</td>
<td>Fermentative medium. No gas formation homo fermentative)</td>
</tr>
<tr>
<td></td>
<td>fermentation medium</td>
<td></td>
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<tr>
<td>Triple sugar utilization</td>
<td>TSI agar</td>
<td>Yellow coloured slant and butt (Utilizes all sugar without H$_2$S and gas. No utilization of N source after exhaustion of C source</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>Simmon’s citrate utilization.</td>
<td>No change in colour</td>
</tr>
<tr>
<td>Agar test citrate as carbon source</td>
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<table>
<thead>
<tr>
<th>Trible sugar iron tests</th>
<th>Results</th>
<th>Sample 1</th>
<th>Sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$S</td>
<td>Negative</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Gas</td>
<td>Negative</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>Butt</td>
<td>Acid</td>
<td></td>
<td>Acid</td>
</tr>
<tr>
<td>Slant</td>
<td>Alkaline</td>
<td></td>
<td>Alkaline</td>
</tr>
</tbody>
</table>

The physiological concentration of bile salts in the small intestine is anywhere between 0.2 and 2.0% (Gunn, 2000). Therefore, the isolates were treated with 2% bile as it is the highest concentration obtained in animal and human intestine during digestion process (Gotcheva et al., 2002). Both the isolates showed good resistance to 2% bile salt even after exposure for 48 h. Resistance to bile is related to bile salt hydrolase (BSH), an enzyme which helps in hydrolyzing conjugated bile, thus reducing its toxic effect (Du Toit et al., 1998). This differs significantly among the lab species and their strains. Similar results were also reported by Mourad and Nour-Eddine (2006) who found one of their isolated strains L. plantarum showed 65% survival rate on exposure to 2% bile salt.
A probiotic strain should survive transit through the stomach where the pH is low around 1.5 to 3 (Table 4). Hence, tolerance to extremely acidic conditions is another important feature of probiotic strain (Dunne et al., 2001; Guo et al., 2009). It was observed that at pH 3.0, lactobacillus showed better survival, even after 4 h of incubation. However, it was noted that the percentage of survival decreased with decrease in pH.

### Antimicrobial activity

An important feature of probiotic culture is its ability to kill pathogens which infect the gastrointestinal tract. The isolates were checked for their antimicrobial activity against B. cereus, L. monocytogenes and E. coli which are common food borne pathogens that infect the gastrointestinal tract (Table 5). The results showed that two of the ten isolates could inhibit the indicator organisms, however, at different inhibition levels. Several researchers have observed that strains which can produce antimicrobial substances are active against pathogenic bacteria (Topisirovic et al., 2006). The differences in inhibition potential among the selected isolates could be due to different intrinsic factors induced by food origins (Klayraung et al., 2008).

### Conclusion

Bacterial species isolated from fermented coconut toddy were confirmed by ATCC (American type colony control) gram staining, catalase, motility, sugar fermentation, aerobic/anaerobic test and salt tolerance. The isolated lactobacillus strain had the ability to tolerate high bile salt concentration and low pH. Based on these in vitro tests, there is a high possibility that the isolates would be able to reach the intestinal tract in good numbers.

Both the isolates were good lactic acid producers and also showed antibacterial activity against pathogenic microorganisms. The ability of the isolates to produce vitamin B12 and β-galactosidase should be investigated in future since this is essential in improving digestion and metabolism. This should be considered as a positive trait for microorganisms which are used as starter cultures and in manufacturing of probiotic and novel functional foods. However, the isolated strains need to be further investigated using in vivo experiments to establish their potential health benefits.

### References


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