



Isolation, characterization and antimicrobial activity of *Lactobacillus species* (K 3) from fermented toddy of *Cocos nucifera*

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ABSTRACT

The present study focused on probiotic, isolated from *Cocos nucifera*'s toddy. Six Organisms were isolated from toddy and it was characterized and optimized with various parameters to show the organism is probiotic. Among the six isolate one strain was confirmed as *Lactobacillus species* by various Bio Chemical and Gram staining and named as **K 3**. Characterization and optimization study such as pH, bile salt and salt tolerant was performed to confirm it as probiotic. In optimization studies the pH range of about 4 and salt tolerance 7 % concentration and bile salt concentration range about 4% showed maximum bacterial population thus the organism K3 (*Lactobacillus sps*) was taken for further study to determine its antimicrobial efficacy against human pathogens.

Key words: Probiotics, *Lactobacillus sps*, *Cocos nucifera*, bile salt, NaCl, pH

INTRODUCTION

An alcoholic beverage is a potable drink, which contains alcohol as an ingredient. The alcoholic beverages are broadly classified into three categories such as fermented beverages, distilled spirits and fortified wines. The fermented beverages are those which are produced from fruit juice or plant sap by natural fermentation. In India, *Phoenix sylvestris* (wild dates), *Cocos nucifera* (coconut palm), *B. flabellifer* (palmyra) palms etc. are frequently used for this purpose (Shamala and Sreekantiah, 1988). The fermented beverages contain only very low (2.7-8%) content of alcohol (Child, 1972).

Borassus flabellifer (Palmyra) grown in arid regions in Chennai. India holds a prominent importance in Indian Economy for jaggery (Ghosh, and Ghose, 1995) and for local consumption of fermented products (Toddy, Nira, alcohol and vinegar) in Southern parts of India. This type of wine is cheap and represents an important part of income in rural areas and it's the most popular alcoholic beverages consumed by socio-economic strata society in Tamilnadu. Unfortunately this wine produced is not stable, the fermentation process continues until the quality becomes unacceptable. Everyday huge quantities are poured away. Palm wine is consumed for its nutritional effect because of its probiotic content. Palm wine is a beverage produced by fermentation of sugars present in sap of palm trees to ethanol by the yeast and bacteria have by all means been exposed to varying concentration of ethanol and acids. The strains that survive to any extent in that wine must have some degree of ethanol tolerance, which is a monumental importance in choosing a yeast strain for industrial ethanol fermentation (Cassey and Ingledew, 1986) Probiotics are lactic acid bacteria (LAB), which confer health benefit on the host in adequate amounts. They can impose the effect by different ways, including preventing proliferation of pathogens, suppressing production of

virulence factors by pathogens, or modulating immune response. LABs consist a variety of Gram positive, acid-tolerant, generally non sporulating, non-respiring rod or cocci bacteria, including *Bacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* that have the property of producing lactic acid from sugars (pH 5.5-5.8) by a process called 'fermentation'. The bacteria are divided into three groups based on fermentation patterns; Homo fermentative produce more than 85% lactic acid from glucose, while Hetero fermentative produce only 50% lactic acid and considerable amounts of ethanol, acetic acid and carbon dioxide. There is a less well-known hetero fermentative species which produce dL-lactic acid, acetic acid and carbon dioxide.

The study aimed to isolate suitable bacteria from toddy to be used as a probiotic supplement. For the purpose bacteria need to have some special properties like tolerating to pH, Bile salt, body temperature, salt content, inhabitation and antimicrobial activity.

EXPERIMENTAL SECTION

Preparation of samples

Fermented coconut toddy was collected in the early morning near Uthukottai, Tiruvallur (DT), Tamil Nadu, India. Samples were maintained between 0-5°C and brought to laboratory within 2-3 h.

Serial dilution

Toddy sample (25 mL) was transferred into 225 mL of saline 8.9% (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

One mL of sample was taken from 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , and 10^{-7} dilutions and poured into separate petriplates. Plate Count Agar (20 mL) and de Man Rogosa Sharpe Agar (20 mL) and was poured into 12 petriplates (for both medium). It was then solidified and kept in incubator at 37°C for 48 h in inverted position.

Isolation of potential bacterial strains

Palm toddy (25 mL) was added to 225 mL of saline and blended thoroughly. Appropriate serial dilutions of the blended mixture was plated onto Plate Count Agar and de Man Rogosa Sharpe agar and incubated at 37°C for 48 h. The colonies was flat, circular, irregular to rough, translucent and non pigmented colony with 2-3 mm in diameter 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 at 37°C (Iyer and Ananthanarayan, 2008).

The organisms were phenotypically characterized by Gram staining. Determination of morphology and motility was done by phase-contrast microscopy by hanging drop mechanism. Only gram positive, catalase negative, non-motile rod shape bacteria were selected. The presence of catalase activity was assessed by the formation of gas bubbles after the suspension of bacterial cells is passed into the droplet of 3% hydrogen peroxide taken on slide. Carbohydrate fermentation pattern of lactic acid bacteria used for sap fermentation were determined according to the manufacturer's instructions (Amoa-Awwa *et al.*, 2007). Stock cultures of the isolates were stored in de Man Rogosa Sharpe broth containing 15% glycerol at -80°C.

In vitro assessment of potential probiotic bacterial strains

As stated earlier, several research groups have recommended that the assessment of potential probiotics involve assessment of resistance to gastric acidity and bile toxicity, production of antimicrobial substance and ability to grow in anaerobic condition.

Resistance to gastric acidity

The primary character of probiotic bacterial strains is to resist the intestinal acidity because before entering into the intestine the organism must pass through the physical condition of stomach here the secretion of gastric juice play a vital role in defense mechanism during ingestion of microbes, thus primarily the organism must resist the acid condition.

de Man Rogosa Sharpe broth is taken and there pH is adjusted to 2-4% and the test organism was incubated. The growth was observed every 1-4 hour interval of incubation by direct plating method and Spectrophotometer method for its OD value at 600nm.

Bile acid resistance

Bile acids are synthesized in liver from cholesterol and are secreted from gall bladder into the duodenum in conjugated form. These acids have more inhibitory action against gram positive than gram negative bacteria (Floch *et al.*, 1972). Thus the isolates were grown in Muller Hinton broth containing from 2 to 4% (w/v) of bile salt concentration, the test organisms are inoculated into this mixture and incubated at 37°C, at every hour from the tube 1ml of appropriate dilution is taken from the tube and pour plate technique is commenced using Muller Hinton agar (Seeley and Van Demark 1981) numbers are counted using Colony counter. Bacterial growth was expressed as colony forming units per mL (CFU/mL) and the survival percentage. Meanwhile the same percentage is taken in test tube containing de Man Rogosa Sharpe Broth and they are checked from 1 to 4 h interval for its percentage at 600 nm.

Anaerobic tolerant

Intestinal ambient condition is aerobic and for fodder animals have anaerobic condition thus if the organism is used as feed for animals means it should threshold the condition of anaerobic condition for this it anaerobic character is checked. Muller Hinton Agar prepared and autoclaved at 121°C for 15 min in 15 Lbs, then the agar was poured in petriplates for its anaerobic growth character of the organism. The test organism was taken from the agar slant and was streaked on the Muller Hinton Agar plates. The plate was incubated for 48 h in an anaerobic chamber (This slows down the growth). The colony morphology was then identified and compared with a control plate grown in anaerobic condition.

Sodium chloride tolerance test

3%, 5% & 7% sodium chloride salt was taken and mixed in 5 ml of Muller Hinton broth in a test tube, subsequently without Muller Hinton broth without salt was taken along with and the test organisms is also incubated into this tube (with and without salt) and incubated at 37°C for 48 h.

Antimicrobial activity

Lactic acid bacteria tend to produce many metabolic compounds (including organic acid, fatty acid, hydrogen peroxide and diacetyl) they all have antimicrobial effect (Ouweland, 1998). Thus the organism isolated from toddy sample was assayed for its antimicrobial activity against a range of common food pathogens. 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% Muller Hinton Agar (20 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 diameters 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 for 24 h and examined for zone of inhibition.

RESULTS AND DISCUSSION

Among the six isolated organisms, K3 identified as *Lactobacillus species* by their biochemical and physiological characteristics. This organism are important beneficial bacteria and are found invading the micro population of our intestine and the probiotic conformation analysis by gram staining, motility, catalase, oxidase tests, biochemical tests, citrate, indole, methyl red, triple sugar iron, lactic acid confirmatory test, sugar fermentation, aerobic & anaerobic, NaCl tolerant, probiotic confirmatory tests and bile tolerant.

Biochemical tests

Gram's staining, motility, catalase and Oxidase test were done for the isolated 6 organism, out of this K3 have been selected based upon the Probiotic criteria among all the isolates and K3 have been identified as *Lactobacillus species*. (Table1)

Table 1: Biochemical Test of organism isolated from toddy

Parameter	K 3
Gram Staining	+
Motility	-
Catalase	-
Oxidase	-
Indole	-
Methyl Red	-
Voges Proskauer	-
Citrate	+
Urease	+
Triple Sugar Iron	A/K
	H ₂ S -
	Gas +

The various biochemical tests were done according to the standard procedure referred from Bergeys Manual of determinative bacteriology.

Acidic tolerant test

Acidic tolerant was tested against the selected K 3 organism, pH was adjusted between 1- 3 and the growth of the organism was tested from 1 – 4 hours interval. Among which organism K3 showed a good set of tolerance against the acidic condition. (Fig. 2)

Figure1. Acid tolerant for K3

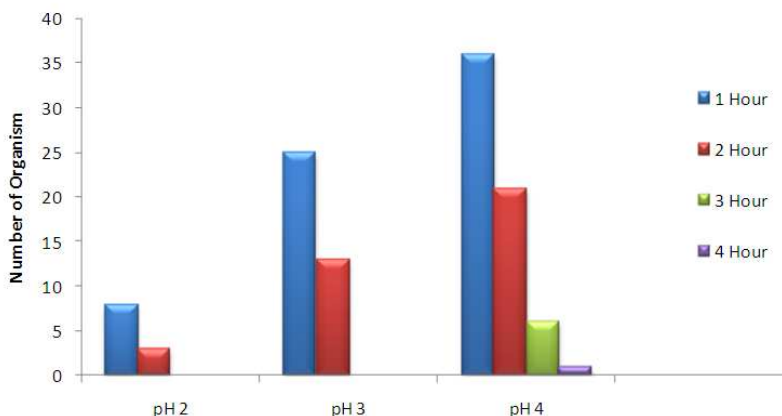


Table 2: Acidic Tolerant Test – UV Spectrophotometer (600nm) for K 3

pH	1 Hour	2 Hour	3 Hour	4 Hour
pH 2	0.168	0.137	0.076	0.060
pH 3	0.266	0.247	0.159	0.132
pH4	0.347	0.302	0.178	0.148

Acid Tolerant was done against K 3 and they showed a good tolerance at pH 4 in 3 hour period.

Survival of probiotics during storage is considerably affected by pH and titratable acidity of the products (Mortazavian *et al.*, 2010). The pH of gastric juice is often kept at 3, and pH 2 for 3 h is often used as an extreme condition to simulate the conditions in the stomach (Tsai *et al.*, 2005).

Bile tolerant test

Bile tolerance was tested against the isolated organisms among which K3 showed a good set of tolerance against 4 percent concentration exposed up to 4 hour. For the bile tolerance study, 0.3% is considered to be a critical concentration for the selection of resistant strains (Gilliland, *et al.*, 1984). The result of isolates high tolerance to bile in the study was different from other reports (Mishra and Prasad, 2005).

Figure 2: Bile Tolerance for K 3

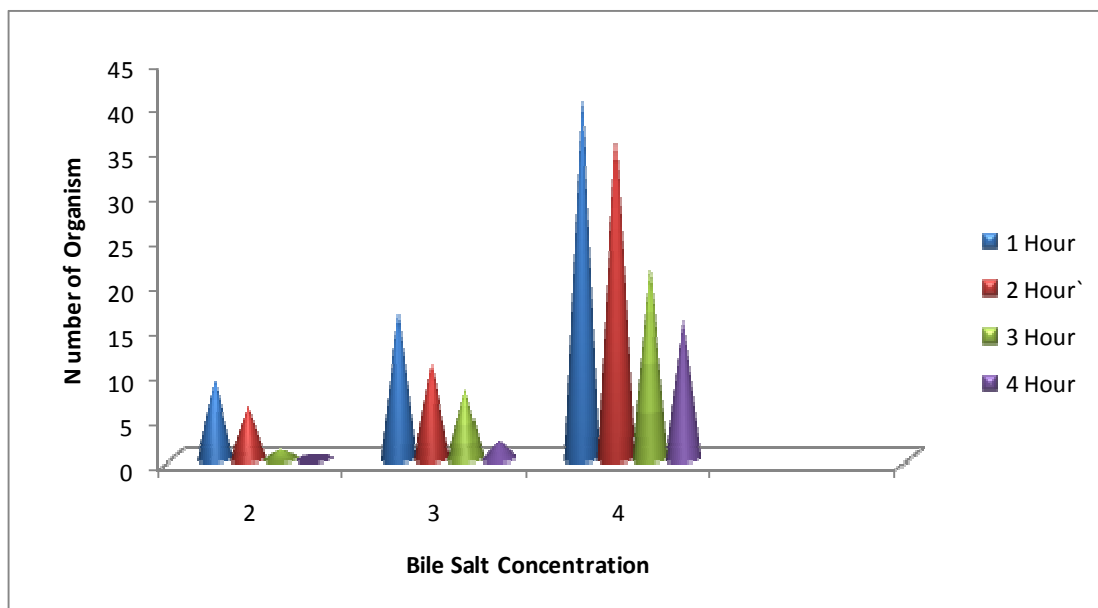
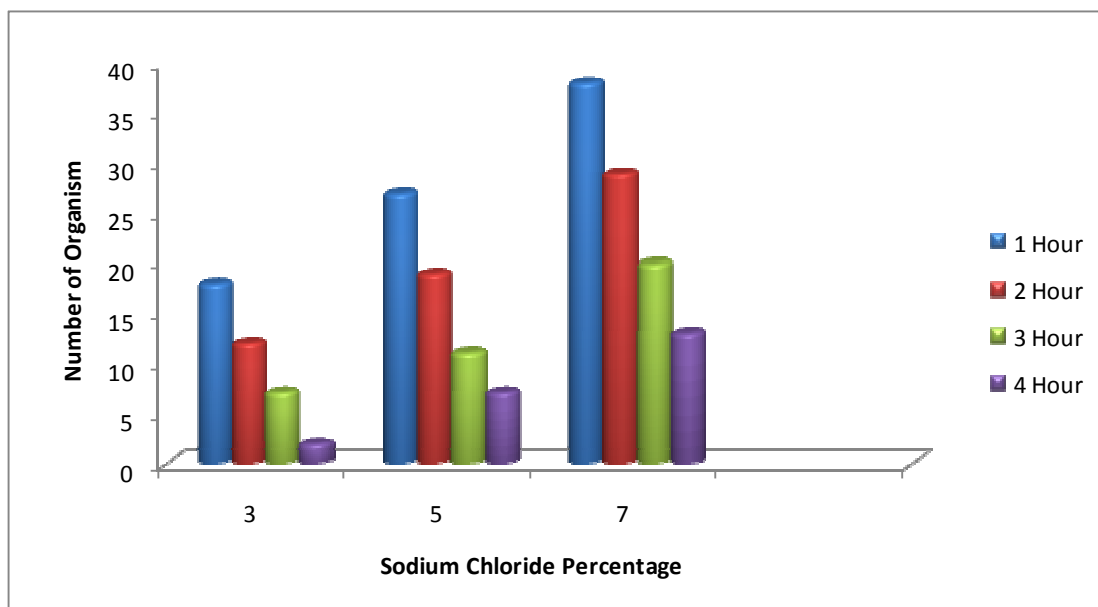


Table 3: Bile Tolerance Test – UV Spectrophotometer (600nm) for K 3

Concentration in %	1 Hour	2 Hour	3 Hour	4 Hour
Bile Salt 2 %	0.079	0.067	0.052	0.039
Bile Salt 3 %	0.163	0.148	0.126	0.110
Bile Salt 4 %	0.103	0.091	0.078	0.058

Figure 3: NaCl Tolerant Test for K 3



Sodium chloride tolerant test

Sodium chloride tolerance was tested at 3, 5 and 7 percent concentration of NaCl. The organism was inoculated, and at every hour 1ml of medium is taken out and pour plate technique is performed using Muller Hinton agar. From the

same tube 1ml is taken and they are subjected to UV Spectrophotometer and observed at 600nm. K3 showed good set of tolerance at 5 percent concentration of NaCl.

Table 4: Sodium Chloride Test – UV Spectrophotometer (600nm) for K 3

Concentration	1 Hour	2 Hour	3 Hour	4 Hour
NaCl 3 %	0.327	0.271	0.226	0.130
NaCl 5 %	0.199	0.183	0.169	0.117
NaCl 7 %	0.172	0.154	0.141	0.086

Anaerobic growth characteristic

The test organism were streaked on Muller Hinton agar and incubated in anaerobic condition and they are compared with a standard aerobic plate which is incubated in an aerobic condition. K3 showed growth in both extremities.

Antibacterial activity

Production of antimicrobial substances is one of the principle probiotic properties for strain selection. In this study the test organisms K3 was tested against ten human pathogens; *Bacillus*, *Escheria*, *Staphylococcus*, *Salmonella*, *Proteus*, *Micrococcus*, *Klebshiella*, *Shigella*, *Enterobacter* and *Listeria* spp. It showed highest bacterial activity against *Staphylococcus* (51 mm), followed by *Micrococcus* and *Enterobacter* (44 mm), *Escherichia* (32 mm). Lowest activity was observed against *Salmonella* (19 mm)

Table 5: Antimicrobial activity of isolated strains

Pathogens	Result in (mm)
	K3
<i>Staphylococcus</i>	19mm
<i>Micrococcus</i>	16mm
<i>Salmonella</i>	13mm
<i>Proteus</i>	-
<i>Listeria</i>	11mm
<i>Klebshiella</i>	-
<i>Bacillus</i>	13mm
<i>Escheria</i>	9mm
<i>Shigella</i>	9mm
<i>Enterobacter</i>	7mm

CONCLUSION

Probiotics are being included in different food systems apart from the traditional fermented dairy products, and numbers of such probiotic foods are available in the market nowadays.

From the present study, K3 was isolated from *Cocus nucifera* toddy, confirmed its potential probiotics culture of *Lactobacillus species* properties including different biochemical tests, tolerance to acidic and intestinal bile concentration at different ranges, and antimicrobial activity analyzed. Thus the isolated **K3** organism *Lactobacillus species* showed good Probiotic characteristics and Antimicrobial activity.

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