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# MOLECULAR CHARACTERIZATION OF PROBIOTICS ISOLATED FROM MARINE MUSSELS: *Perna viridis*

Biological Science

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## ABSTRACT

*Perna viridis* (Asian green mussel) is an economically important mussel, a bivalve belonging to the family Mytilidae. It is unique in its nutritional composition and is harvested for consumption. It is rich in protein contents followed by carbohydrates and lipids. It is also a good source of vitamins and minerals. An attempt has been made to isolate and molecular characterize probiotic bacteria from these mussels. Two potential probiotic bacteria *Lactobacillus coryniformis* subsp. *torquens* strain MM1 and *Lactobacillus helveticus* strain MM2 have been identified based on 16S rRNA sequencing. The 16S rRNA sequences determined in this study have been deposited in the NCBI GenBank database and accession numbers have been obtained. The isolates have shown good probiotic properties.

## KEYWORDS

*Lactobacillus coryniformis* subsp. *torquens, Lactobacillus helveticus,* 16SrRNA sequencing, *Perna viridis,* Probiotics

## INTRODUCTION

In a variety of ecological niches, microorganisms compete with each other for survival and through evolution they form unique flora. These microorganisms have a reputation for causing diseases and there is a tendency to perceive all microorganisms as harmful. The number of non pathogenic bacteria far exceeds the number of pathogenic bacteria and many of the non pathogens are in fact useful, even essential for the continued existence of life on earth. These beneficial bacteria inhabit gastrointestinal (GI) tract of human and animals. These bacteria that colonize our GI tract interact dynamically with each other and with our cells. This interaction serves to stimulate the immune system for optimal functioning. Body's many functions are associated with the bacterial communities in GI tract and their interaction with the host system plays a crucial role in the anatomical, physiological and immunological development of the host including roles in host nutrition, intestinal epithelial development and activity, education of the immune system, maintenance of the integrity of the mucosal barrier, and contribution to drug and xenobiotic metabolism.

The traditional methods used for detection of probiotics are identification of colony morphology, fermentation patterns, serotyping or some combination of these. These traditional methods got limitations still they have been used in routine identification process. With the developing technology about the molecular techniques it is getting more reliable to identify and differentiate bacterial strains. Classical microbiological techniques are really important for selection, enumeration and biochemical characterization (fermentation profiles, salt-pH-temperature tolerances) but it is not efficient to classify a culture taxonomically. These method based on phenotypic characteristics employed for identification of bacteria are generally not as accurate as identification based on genotypic methods. Bacterial 16S rRNA gene sequence has emerged as a most potential and preferred genetic technique. 16S rRNA gene sequence analysis technique can better identify poorly described, rarely isolated, or phenotypically aberrant strains and can be routinely used for identification of Lactic Acid Bacteria (LAB). 16S rRNA gene is the most commonly used part of the DNA for taxonomic purposes for bacterial identification[1-6]. Molecular identification techniques provide two primary advantages to phenotypic identification, a more rapid turnaround time and improved accuracy in identification[7].

## MATERIALS AND METHODS

The mussels were randomly picked from rocky sea shores of Dakshina Kannada District of Karnataka and brought to the laboratory alive. They were washed with sterile water to remove the dirt. They were aseptically opened and the whole meat and the fluid were collected and homogenised using mortar and pestle. 1ml of the homogenate was serially diluted till $10^{-8}$. 0.1 ml was spread plated on De Man, Rogosa and Sharpe (MRS) agar plates. All the plates were incubated under anaerobic condition in anaerobic jar at 37°C for 48 h or until the bacterial colonies were of sufficient size.

Colonies were tested for catalase activity with 4% $H_2O_2$ and catalase negative colonies were streaked on MRS agar and incubated at 37°C for 48 h to obtain pure colonies. Pure colonies were again tested for catalase activity and Gram stained. All catalase negative and Gram positive colonies were maintained in MRS broth with 15% of glycerol and kept at-20°C for further study.

The colony characteristics (size, shape, margin and colour) on solid medium, growth pattern in broth and agar slants were recorded. Type and arrangement of cells have been identified. Motility, Gas production, Spore formation, Indole test, MRVP, Starch Hydrolysis, Citrate utilization, Gelatine liquefaction, Arginine hydrolysis have been tested following the standard protocols.

NaCl tolerance, growth at different pH and temperature, carbohydrate fermentation pattern, acid tolerance, bile tolerance, bile salt hydrolase activity, gastric juice tolerance, intestinal juice tolerance, auto aggregation, co-aggregation, adhesion, hydrophobicity, antibiotic sensitivity, antimicrobial activity, production of bacteriocin, lactic acid and hydrogen peroxides have been studied based on the protocols given elsewhere.

### 16S rRNA sequencing

Only 2 organisms with excellent probiotic properties have been subjected for molecular characterization. Bacterial Genomic DNA was isolated using the InstaGene™ Matrix Genomic DNA isolation kit with the protocol provided. The 16S gene fragment was amplified using 27F/1492R Universal 16S rRNA primers by using MJ Research Peltier Thermal Cycler. The PCR product was sequenced using the 785F/907R primers. Sequencing reactions were performed using a ABI PRISM® BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

The 16S rRNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of our sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences[8]. The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions and divergent regions[9]. Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as substitution model.

## RESULTS AND DISCUSSION

### Morphological and Biochemical Characterization

The isolate MM1 was catalase negative, Gram positive with short rods and positive for MR test. The isolate MM2 was catalase negative, Gram positive rods/chains and positive for MR test. Rest of the biochemical tests were negative for both the organisms. Strain MM1 showed least growth in 6% NaCl whereas strain MM2 showed moderate growth. Strain MM1 could not grow at 50°C, whereas strain

MM2 showed moderate growth. Both the strains could tolerate pH 3. The carbohydrate utilization pattern of the isolates has been given in table no.1.

**Table 1** Carbohydrate utilization pattern of the isolates

| Sugars | Isolate MM1 | Isolate MM2 |
|---|---|---|
| Fructose | + | - |
| Galactose | + | + |
| Cellobiose | - | - |
| Esculin | + | + |
| Inulin | - | - |
| Rhamnose | - | - |
| Melibiose | - | - |
| Mannitol | + | - |
| Maltose | + | + |
| Mannose | + | + |
| Ribose | - | - |
| Trehalose | - | + |
| Arabinose | - | - |
| Lactose | - | + |
| Sucrose | - | - |
| Xylose | - | - |
| Salicin | + | - |
| Sorbitol | + | + |
| Raffinose | - | - |
| Glycerol | - | - |

**Probiotic Properties of the isolates**

To consume them for health benefit, probiotic microorganisms should be capable of surviving passage through the digestive tract and also have the capability to proliferate in the gut. This means they must be resistant to gastric juices and be able to grow in the presence of bile and intestinal juices. The survival of strain MM1 at pH 2 after 3 hours of incubation was 2% whereas MM2 was 7%. The survival of strain MM1 at 1% of bile after 3 hours of incubation was 4% and strain MM2 could show a survival rate of 3%. Both strains showed moderate bile salt hydrolase activity. Strain MM1 showed a survival rate of 4% in gastric juice at pH 2 after 3 hours of incubation and strain MM2 showed 7.50%. Intestinal juice survival by strain MM1 after 6 hours of incubation was 85% and strain MM2 was 75%. This confirms their survival during gastrointestinal transit. Once the probiotic bacteria reach the lower intestine, it is necessary that they colonize there and multiply and form a biofilm to protect the host from colonization by pathogens[10]. Auto aggregation of strain MM1 increased from 12% at first hour of incubation to 47% at fifth hour of incubation and strain MM2 increased from 9% to 68%. Co-aggregation capacity of strain MM1 and MM2 with *S. aureus* MTCC 9760 was 49.72±2.61 and 51.23±1.16 percentage and with *E. coli* MTCC7410 was 12.33±1.33 and 12.45±1.27 percentage. The ability to co-aggregate with other bacteria such as pathogens may form a barrier that prevents colonization by pathogenic microorganisms[11]. Adhesion of probiotics to intestinal mucus and epithelial cells has long been considered one of the most important selection criteria for probiotic microorganisms. Adhesion percentage of strain MM1 was 7.41±1.14 and strain MM2 was 12.65±1.05. Hydrophobicity of organisms could confer a competitive advantage, important for bacterial maintenance in the human gastrointestinal tract[12]. Hydrophobicity of strain MM1 was 19.54% and strain MM2 was 20.14%. Routine antibiotic susceptibility testing of LAB is advisable in a number of instances e.g. for checking the bio-safety of potential probiotic isolates. The strain MM1 was resistant ampicillin, erythromycin, gentamycin, kanamycin, nalidixic acid, neomycin, tertracyclin and vancomycin antibiotics. The strain MM2 was resistant to ampicillin, erythromycin, gentamycin, kanamycin, nalidixic acid, neomycin, lincomycin, penicillin, streptomycin and tetracycline antibiotics. Antimicrobial activity is one of the most important selection criteria for probiotics. Antimicrobial activity targets the enteric undesirables and pathogens[13]. Both the strains MM1 and MM2 inhibited the growth of pathogenic bacteria *S. typhimurium* MTCC 3224, *E. coli* MTCC7410, *P. aeruginosa* MTCC 9499, *B. subtilis* MTCC 8605 and *S. aureus* MTCC 9760 with different degree. None of the isolate inhibit the growth of *K. pneumonia* MTCC 9751. Antimicrobial effects of lactic acid bacteria are formed by producing some substances such as organic acids (lactic, acetic, propionic acids), carbon dioxide, hydrogen peroxide, diacetyl, low molecular weight antimicrobial substances and bacteriocins[14]. The strain MM1 produced 1.75±0.26 Gm/l lactic acid where as strain MM2 produced 3.65±0.25Gm/l. The concentration of $H_2O_2$ produced by strain MM1 was 0.023±0.002 Gm/l and strain MM2 was 0.006±0.001Gm/l.

A number of tests are recommended for screening putative probiotic strains. The isolates should pass all the recommended tests to select as a potential probiotics. The present study has generated a whole data set on the ability of isolated strains to survive *in vitro* conditions that mimic individual physiological environments of the intestinal tract. The choice of *in vitro* systems allowed direct comparison between bacterial strains. The isolates have been subjected to *in vitro* tests as the guidelines of FAO-WHO and ICMR-DBT.

**Molecular Characterization**

The isolates in present study were identified at species/sub species level by aligning of 16S rRNA sequences with those from NCBI gene bank. The molecular confirmations of present work were in agreement with many of the previous reports[15-17]. The comparative evaluation of phenotypic and genotypic results confirmed that the phenotypic test, in spite of giving information on the biochemical and metabolic traits of LAB, are not reliable enough for the identification of these microorganisms, although it is a useful tool for presumptive classification. The advantages of genotyping include the stability of genomic DNA, its composition being independent of cultural conditions or preparation methods, and amenability to automation and statistical data analysis[18].

The isolates were identified as *Lactobacillus coryniformis* subsp. *torquens* strain MM1 and *Lactobacillus helveticus* strain MM2. Strain MM1 showed 99% similarity with the types of strains present in the GenBank and strain MM2 showed 100% homology with the types of strains in NCBI GenBank.

**Nucleotide sequence accession number**

The 16S rRNA sequences determined in this study have been deposited in the NCBI GenBank database. The accession numbers assigned to the identified sequence of *Lactobacillus coryniformis* subsp. *torquens* strain MM1 is KX430830.1 and *Lactobacillus helveticus* strain MM2 is KX430831.1

Sequence analysis of conserved "housekeeping" genes such as the bacterial 16S rRNA gene are increasingly being used to identify bacterial species in the scientific investigations[19-20]. In the case of 16S rRNA analysis, species identification is easiest when most or the entire gene can be sequenced.

## CONCLUSION

Nowadays, probiotics gained much more consideration in the health and well being of humans. Morphological and biochemical characteristics have been used largely for their identification. These identification strategies may not be enough to assign a organism definitely to a particular species. Since the isolated LAB from marine mussels were characterized with phenotypic and genotypic methods in this study, it can finally be informed that the phenotypic methods should be supported with the genotypic ones[21-22]. Misidentifications of bacterial species using biochemical methods are common as phenotypic characterization is unreliable and is affected by changes of the environmental conditions[23]. In the case of multi-strain probiotic, which has been reported to be more effective than mono-strain probiotic[24], enumeration of a particular species by conventional method cannot be achieved since the phenotypic traits of the different species are not clearly distinguishable. This leads to difficulty in monitoring the quality of the final products. These factors have encouraged the development of molecular-based identification and detection methods as an alternative to the phenotypic identification.

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