



Original Research Article

Molecular Characterization and Health Promoting Attributes of MBTUPBBM1 Isolated from Cow Milk

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ABSTRACT

Keywords

Bacillus subtilis, Sporeformer, Probiotic, Bilesalt deconjugation, Cholesterol assimilation

MBTU PBBM1 (previously named as BM-3) is a potential probiotic strain with strong probiotic properties. *In vitro* studies on health promoting properties revealed that MBTU PBBM1 can assimilate cholesterol from media in presence of bile salts. The strain exhibited a better growth in the presence of cholesterol. Strain also possessed capability to deconjugate bile salts. 16S rRNA genes of MBTU PBBM1 was sequenced to analyze species similarity. Phylogenetic analysis showed that MBTU PBBM1 has a good phylogenetic relationship with the *B. subtilis*.

Introduction

“Bacteria keeps us from heaven and puts us there,” is a well known saying by Martin H. Fischer. This word emphasizes the importance of microorganisms in our human body. The criteria for the microbes to be treated as probiotic include: capability to withstand in gastric acid and secretions of bile and pancreas. Withstanding capability in these fluids are necessary to reach the small and large intestines. Probiotics should be non-pathogenic and non-toxic, remain viable during transport and storage, exert beneficial effects on the host; stabilize the intestinal microflora, adhere to the intestinal epithelial cell lining and produce antimicrobial substances towards pathogen (Fuller, 1987,

Atlas 1997, Austin 1995)). *Bacillus* spores are being used as human and animal probiotics. The members of genus *Bacillus* are Gram-positive, aerobic or facultative anaerobic, catalase positive, rod-shaped endospore forming bacteria. Health promoting effects such as cholesterol removing capability from growth media enhances the microorganisms to be used as probiotic. Proposed mechanisms of microorganism in lowering cholesterol includes the assimilation of cholesterol by the cell wall during growth (Buck *et al* 1994) and deconjugation of bile salts by bacteria producing bile salt hydrolase. (Al-Saleh *et al* 2006). MBTU PBBM1

(previously named as BM-3) is a potential probiotic strain with strong probiotic properties (Anu *et al* 2012,2014). Strain had strong probiotic properties such as tolerance to acid and bile, nonhemolytic, lecithinase negative, resistance to artificial gastric and intestinal fluid and antagonism to the enteric pathogens such as *Salmonella typhi*, *Salmonella paratyphi A* and *Vibrio cholera*. (Anu *et al* 2012) The strain possessed superior adhesion and cell surface properties than selected enteric pathogens. (Anu *et al* 2012). Immunomodulatory studies also revealed that this strain possessed a very good immunomodulation in Balb/c mice. (Anu *et al* 2014) Hence the further studies were aimed on detecting the *In vitro* health promoting properties of this potential probiotic candidate bacterium

Materials and Methods

MBTU PBBM1 (previously named as BM-3) is a potential probiotic strain with strong probiotic properties (Anu *et al* ;a,b 2012,c;2014). This Strain was grown in MRS broth and was studied for health promoting properties

Cholesterol removal Assay

Cholesterol removal assay of MBTU PBBM1 were measured according to Rudel *et al* 1973. Cholesterol solution (10 mg/ml in 96% ethyl alcohol) was prepared and filter sterilized. 70 µl of cholesterol solution was added to MRS broth (final cholesterol concentration 70 µg/ml) separately containing 0.2% , 0.4% bile, (oxgall) and without bile salts . To the MRS broth, 1% of freshly grown culture was added and incubated anaerobically at 37°C for 20 h. Uninoculated broth was used as control. After incubation the cells were removed by centrifugation at 10,000 g for 10 min at 4°C and filter sterilized. Cholesterol was determined in the supernatant using modified Rudel and Morris method in which

3 ml of supernatant, 2 ml of 33% (wt/vol) KOH and 3 ml 96% ethanol were placed in a capped test tube, vortexed for 20 second and incubated for 15 min at 60°C in a water bath. After incubation, the mixture was removed and cooled under tap water, then 5 ml of hexane and 3 ml of water were added and vortexed for one min. One milliliter of the hexane layer was transferred into a dry clean test tube and evaporated under nitrogen gas. One milliliters of cholesterol liquicolor enzymatic kit (Life span diagnostics) was added. The solution was mixed and left for 5-10 min at 37°C and absorbance was measured at 500 nm with a spectrophotometer. The ability of bacterial strain to remove cholesterol from media was calculated as percentage from the following equation:

$$A=100-(B/C)*100$$

Where A=% of cholesterol removed,
B=absorbance of the sample containing the cells and

C=absorbance of the sample without cells.

Deconjugation of bile salts

Deconjugation of bile salts by MBTU PBBM1 was tested qualitatively through the plate assay according to Ahn *et al.*, 2003). To MRS agar containing 0.5 g/l cysteine, 1mM of sodium taurocholate. (Sigma Chemical Co., USA) was added. After autoclaving and solidifying, the plates were incubated anaerobically for 48 h before use. The plates were inoculated with active culture (20 µl) and incubated for 72 h at 37°C. Colonies were observed for precipitation.

Molecular identification

Bacterial genomic DNA isolation of the MBTU PBBM1 was performed according to (Sambrook *et al.*, 1989)

16S r DNA analysis of the isolate

For 16S r DNA analysis, rDNA primers used were as following,

16S F 5'-AGA GTT TGA TCC TGG CTC AG-3'

16S R 5'-GGT TAC CTT GTT ACG ACT T-3'

PCR reaction was carried out in Eppendorf AG 22331 Thermal Cycler. The PCR reaction cycle is given in Table 1. The PCR products were size fractionated on 1.5% agarose gel stained with ethidium bromide to check the amplification.

Sequence similarities and phylogenetic analysis

The BLAST program (www.ncbi.nlm.nih.gov/blst) was employed in order to assess the degree of DNA similarity. Multiple sequence alignments and molecular phylogenies were evaluated using ClustalW2 at the European Bioinformatics Institute (<http://www.ebi.ac.uk>). The sequences were deposited in the National Centre for Biotechnology Information (NCBI) gene bank data base. The phylogenetic tree was displayed using the Molecular Evolutionary Genetics Analysis (MEGA) version 5.05 program (<http://www.megasoftware.net>).

Results and Discussion

Cholesterol assimilation

The percentage of cholesterol assimilated by MBTU PBBM1 during 20 hr growth at 37°C in MRS broth is given in Figure 1. Tested strain MBTU PBBM1 was able to assimilate cholesterol, and uptake of cholesterol was higher in the medium containing 0.4% oxgall bile than 0.2%.

Deconjugation of bile salts

MBTU PBBM1 grown on sodium taurocholate-MRS agar plates formed fine white precipitated granules around and within the colonies. These white precipitate have been reported to be related to the solubility of bile salt. Deconjugation of bile salts is shown in Figure 2

Molecular identification

Isolated genomic DNA of MBTU PBBM1 was visualized by agarose gel electrophoresis under UV light. Then they were amplified by PCR method. The genomic DNA of MBTU PBBM1 was amplified with 16S rDNA primers. Result of molecular identification of the isolate by 16S rDNA sequencing are summarized in Table 2. Figure 3 displays the phylogenetic tree of MBTU PBBM1. The phylogenetic analysis revealed that the strain MBTUPBBM1 was closest to *Bacillus subtilis* (Gene Bank accession no. HQ 718411) and showed 98% identity.

Probiotic strains are unique and different in their properties and characteristics. Hypercholesterolemia is considered as a major risk factor for the development of coronary heart disease (Pereira *et al.*, 2003). Therapeutic drugs are available to relieve this problem, but they are often expensive and can have side effects.

The use of probiotic bacteria in reducing serum cholesterol levels has attracted much attention. MBTU PBBM1 exhibited a better growth in the presence of cholesterol, indicating that cholesterol stimulated its growth. Results revealed that addition of bile salts greatly improved the uptake of cholesterol from the media. This was in good support with studies of Rasic *et al.*, 1992; Tahri *et al.*, 1996.

Table.1 PCR reaction cycle

Step No.	Temperature (°C)	Duration
1	95	2 min
2	94	45s
3	55	45s
4	72	1min 30 s
5	Go to step 2	Repeat 35 times
6	72	10 min
7	4	10 min

Table.2 16S rDNA identification of bacterial isolate

Isolate	Gene Bank accession number	Bacterial genus	Phylogenetic Relationship
MBTUPBBM1	JN873913	<i>Bacillus</i>	<i>Bacillus subtilis</i>

Figure.1 Cholesterol assimilation of MBTU PBBM1

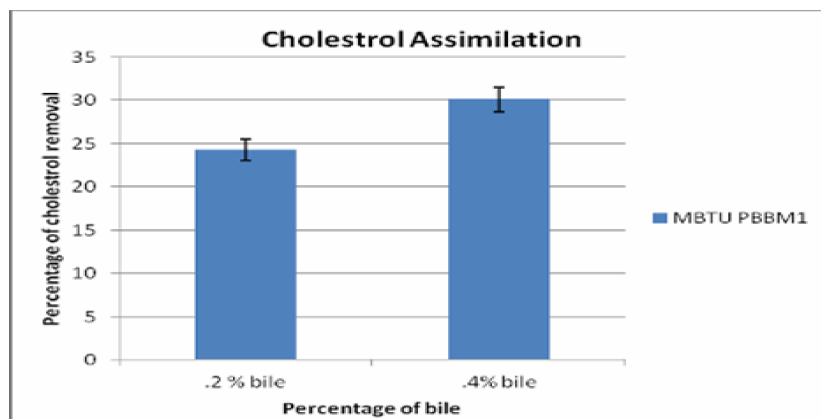
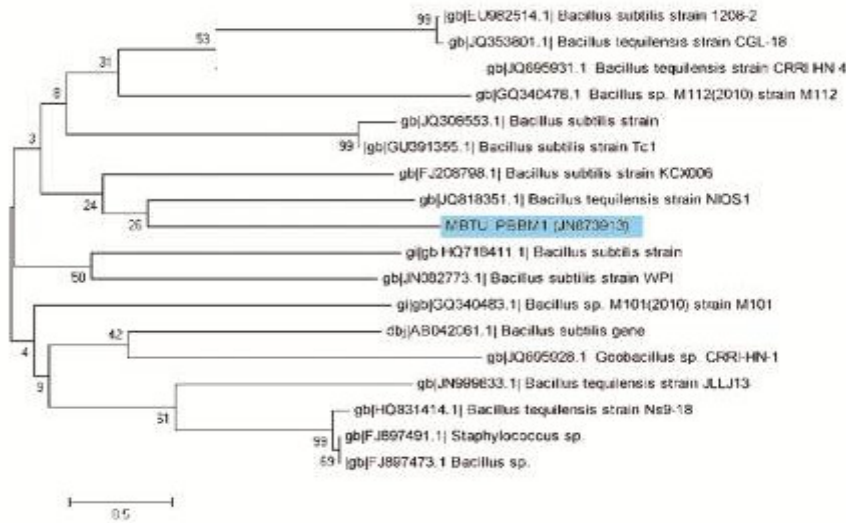


Figure.2 Deconjugation of bile salts



Figure.3 Phylogenetic tree of the probiotic strain MBTU PBBM1



Probiotic strains assimilate cholesterol for their own metabolism. The organism binds to the cholesterol molecule, degrading it to its catabolic products. Cholesterol level gets reduced indirectly by deconjugating the cholesterol to bile acids. Increased uptake of cholesterol in presence of more bile salts may due to the co precipitation of cholesterol with deconjugated bile salts. Fine precipitated white granules observed around and within the colonies during deconjugation test indicates bile salt hydrolase activity of the strain. Deconjugation of bile salts in a mammalian host takes place in the small and large intestines. In a steady state situation, deconjugation of bile can reduce serum cholesterol levels by increasing the formation of new bile acids that are needed to replace those that have escaped the enterohepatic circulation (Reynier *et al.*, 1981) Free bile acids formed by the deconjugation of conjugated bile salts are less soluble and are less likely to be reabsorbed by the intestinal lumen compared to their conjugated counterpart, and are lost from the human body through feces (Center *et al.*, 1993). This could lead to a higher metabolism of cholesterol and,

subsequently, the reduction of serum cholesterol (Reynier *et al.*, 1981). Bile salt deconjugation and cholesterol assimilation capability of MBTU PBBM1 may help in the reduction of serum cholesterol. To establish the relatedness of the strains at the genetic level, 16S rRNA genes of MBTU PBBM1 was sequenced to analyze species similarity. Phylogenetic analysis showed that MBTU PBBM1 has phylogenetic relationship with the *B. subtilis*. Probiotic bacteria are mostly delivered in a food system and must be acid and bile tolerant in order to survive in the human gastrointestinal tract.

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