CHAPTER 3

Identification and Characterization of Potential Probiotic Isolate MBTU PBBM1
IDENTIFICATION AND CHARACTERIZATION OF
POTENTIAL PROBIOTIC ISOLATE MBTU PBBM1

3.1 Introduction

Probiotics have been used for many years to aid in restoring and maintaining a healthy intestinal balance in favour of healthful bacteria, which is essential in maintaining good health. A major consideration in the choice of probiotics is to choose a strain that can survive and establish itself under the conditions encountered in the intestinal environment. Therefore, when choosing a probiotic supplement the healthcare professional must consider those qualities and characteristics essential to establishment and activity. The basis for assessing probiotic efficacy in humans requires the understanding of probiotic strains, each of which is unique and different. Novel methods to select and characterize the probiotic strains are therefore needed. Regarding the selection and characterization of probiotic bacteria is of special interest and the criteria used for strain selection are, physiological characteristics, tolerance to conditions of the digestive tract, multiplication and operating capacity in the intestine, the effects on the immune system, antibacterial factors, the ability to colonize, resistance to industrial processing, their efficacy and safety. All bacteria used as probiotics are now selected on the basis of these above criteria’s. Enhancements of colonization resistance and/or direct inhibitory effect against pathogens are important factors where probiotics have reduced the incidence and duration of diseases. Probiotic strains have inhibited pathogenic bacteria both in vivo and in vitro through different mechanisms, offers protection creating a hostile environment for pathogens by the production of inhibitory compounds such as bacteriocins, siderophores,
lysozymes, proteases, or hydrogen peroxide, formation of ammonia and diacetyl, alteration of pH value by the production of organic acids (Verschuere et al., 2000) and competition for essential nutrients and adhesion sites (Vine et al., 2004b). Therefore, screening of candidate probiotics preferably requires various selective criteria such as antagonism, production of beneficial compounds, growth etc. Antagonism toward pathogens is one of the properties of probiotic bacterial strains (Fuller, 1992; Austin et al., 1995; Moriarty, 1999; Atlas and Bartha, 1997). Bacteriocins may play defensive role to hinder the invasion of ecosystem of other strains or species in to an occupied niche (Riley and Wertz, 2002). Members of the genus *Lactobacillus* have traditionally been used in food fermentation and are therefore regarded as GRAS (generally recognized as safe status) by the FDA (Donohue and Salminen, 1996). Antibiotic resistance studies was said to be an important attribute during the probiotic development. Bacterial growth characteristics such as lag period and doubling time also influence in the exclusion of pathogens. Selection of potential probiotic isolate is very important in probiotic development. Based on *in vitro* probiotic characterization studies discussed in chapter one and two, a potential probiotic bacterium was selected as a probiotic candidate. Biochemical and molecular identification of the selected bacteria were done to find species identity. Physiological properties of the isolate were also studied for understanding the probiotic efficiency. For analyzing the general nature and health promoting effects of the MBTU PBBM1, specific tests such as, antibiotic sensitivity, analysis of plasmid profiles, bile salt deconjugation and cholesterol assimilation were performed.
3.2 Literature review

An effective probiotic product requires proper identification and characterization of a bacterial species used. This is very important because there is now a wealth of information from food industry, in regards to probiotic bacteria, which not always corresponds to reality (Temmerman et al., 2003). There are more specific tests such as the ability to hydrolyse bile salts (Lim et al., 2004) or to produce antimicrobial substances (Toure et al., 2003). The viability of a probiotic strain is considered crucial to ensure optimal functionality. This is explained by the fact that after ingestion these bacteria have to survive the inevitable three biological barriers such as salivary lysozyme, the acidic environment of the stomach and to the bile acids in the duodenum (Saarela et al., 2009). Therefore to ensure their survival during passage through the gastrointestinal tract, the probiotic trains are tested in terms of resistance to pH and bile acids. These tests were conducted on several strains and the results were different depending on the species (Tuomola et al., 2001). In general resistance in the digestive environment is low as a result currently investigated novel approaches such as those based on mechanisms to stress adaptation of probiotic bacteria (Collado et al., 2005).

3.2.1 Techniques for the identification of Probiotic Strains

Methods used for detection of probiotics in human gastrointestinal tract are identification of colony morphology, fermentation patterns, serotyping or some combination of these. Although these traditional methods have limitations when they are used for identification. 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. Molecular characterization methods are powerful even between closely related species. There are number of alternative taxonomic classification methods well known including hybridization with species-specific probes and generation of profile PCR applicants by species-specific primers (Klaenhammer and Kullen 1999). Polymerase chain reaction based methods (PCR-RFLP, REP-PCR, PCR ribotyping and RAPD) are mainly used as molecular tools (Bulut 2003).

Characterization of microorganisms according to their 16S rDNA regions sequencing was firstly proposed by Woese in 1987. The 16S rRNA gene is nearly 1540 bases long and includes variable regions while the general structure is highly conserved. Because the probes have the broadest specificity ranging from universal to species specificity, it is possible to use 16S rRNA gene to study phylogenetic relationships between microorganisms and identify them more accurately (Çakır 2003, Holzapfel, et al., 1998, Charteris, et al., 1997). Traditional cultivation and microscopy have been improved by the introduction of genotypic studies. The latest technique involves analysis of ribosomal RNA, specifically to a subunit, called 16S and 32S rRNA for bacteria. This sequence contains hyper variable subunits that are specific to each species. By using universal primers I and with Polymerase Chain Reaction (PCR) we can now determine and identify bacterial species. Currently there are about 16,000 sequences 16S rRNA in the databases associated with new bioinformatics techniques and in parallel with genetic and molecular biology techniques and this are used very easy to identify and characterize strains of probiotic bacteria (Thornton, 2001). There are less time-consuming techniques such as distortion in gradient electrophoresis (DGGE) (Yang et al., 2009).
However the most efficient method is to use species-specific primers that allow direct identification of probiotic organisms. Laboratory procedures according to Bergeys Manual of Determinative bacteriology will help in the identification and determination of physiological and biochemical properties of a selected isolate.

### 3.2.2 Growth Properties

Growth is an orderly increase in the quantity of cellular constituents. It depends upon the ability of the cell to form new protoplasm from nutrients available in the environment. In most bacteria, growth involves increase in cell mass and number of ribosomes, duplication of the bacterial chromosome, synthesis of new cell wall and plasma membrane, partitioning of two chromosomes, septum formation, and cell division. Theoretically, competition for nutrients can play an important role in the composition of the macrobiota of the intestinal tract. Competition for space (adhesion sites) in the gut or other tissues in the digestive tract would be an antagonistic mechanism to colonization of pathogenic bacteria by probiotics (Verschuere et al., 2000). Successful probiotic bacteria are usually able to colonize the intestine, at least temporarily, by adhering to the intestinal mucosa. Proper growth of the probiotic bacteria in the gastrointestinal tract help in the adhesion and colonization in gastrointestinal tract. The adhesive probiotic bacteria could prevent the attachment of pathogens, such as coliform bacteria and clostridia and stimulate their removal from the infected intestinal tract (Vine et al., 2004b).
3.2.3 Antibiotic resistance and Plasmid curing

The human gastrointestinal tract is a massive reservoir of bacteria with a potential for both receiving and transferring antibiotic resistance genes. The increased use of fermented food products and probiotics, as food supplements and health promoting products containing massive amounts of bacteria acting as either donors and/or recipients of antibiotic resistance genes in the human GI tract, also contributes to the emergence of antibiotic resistant strains (Susanne et al., 2010). Absence of plasmids in probiotic bacteria prevents the bacterial resistant determinants to the host system. The sustainability of antibiotic resistance is partly due to selection of already resistant bacteria that become the new dominant population in the environment. Thus continuous usage and accumulation of antibiotics in the environment has resulted in the increase of antibiotic resistant bacteria.

Curing is the process of removing plasmids from a bacterial cell. This may be observed with high copy number or relaxed plasmids when the bacterial cell is grown for successive generations in the absence of a selective agent (e.g., antibiotic). Treatment at elevated temperature are also applicable for some bacteria (Trevor et al. 1985) Thus resulting bacteria, then, becomes sensitive to the selective agent. Plasmid curing help in detecting plasmid mediated properties. Rasool et al., 2003 studied on indigenous clinical Klebsiella and found that among the isolates, fifty two percent were identified as K. oxytoca, 42% as K. pneumoniae and 6% as K. ozaenae. All the isolates offered different resistance patterns (determined by medium incorporation-replica method) against antibiotics including ampicillin, streptomycin, gentamicin, ofloxacin, tetracycline and chloramphenicol. Some of the representative isolates lost the antibiotic resistance after acridine orange mediated curing.
Molnár et al., 1978 reported curing of an F-prime plasmid by imipramine, was most efficient on bacteria growing semi-anaerobically at 37°C. The plasmid curing effect of imipramine was increased in the presence of methylene blue, whilst fluorescein, chlorpromazine-sulphoxide and tetraoxyanthrachinon antagonized the plasmid curing action of the drug.

3.2.4 Health promoting attributes of probiotic
3.2.4.1 Deconjugation of bile salts and Cholesterol Assimilation

The viability and survival of probiotic bacteria are the most important parameters for providing therapeutic functions as well as nutritional effects. Several factors have been claimed to affect the viability of probiotic bacteria in dairy foods such as yogurt and fermented milks, including low pH and bile salts. Numerous studies have shown promising results in reducing triglyceride, total- and LDL cholesterol levels after consumption of probiotic and/or prebiotics (Jin et al., 1998). Bile, composed mainly of bile salts, is produced by liver cells, and secreted into the duodenum via the bile duct. Bile salts are glycine and taurine conjugates of bile acids, and act as natural ionic detergents. In the intestine, bile salts play an important role in emulsifying lipids, which enable intraluminal lipolysis and absorption of lipolytic products by enterocytes. Cholic acid, one of the most common free bile acids in the intestine, is produced mostly by the deconjugation of bile salts (Kurdi et al., 2003). The major route of cholesterol excretion from humans and other mammals is through faeces. Bile secreted in the small intestine reduces the survival of bacteria by destroying their cell membranes, the major components of which are lipids and fatty acids; these modifications may affect not only the cell permeability and viability, but also the interactions between the
membrane and the environment (Succi et al., 2005). A concentration of 0.15-0.3 \% of bile salt has been recommended as a suitable concentration for selecting probiotic bacteria for human use (Goldin BR and Gorbach 1992).

Cholesterol is the precursor of primary bile salts that are formed in the liver and stored as conjugated bile salts in the gall bladder for secretion in the gastrointestinal tract (Corzo and Gilliland, 1999). The secretion of conjugated bile salts into the small intestine helps absorption of hydrophobic vitamins and other dietary fat soluble compounds. Some probiotics such as \textit{Lactobacillus acidophilus} was found to excrete bile salt hydrolase (BSH) (cholylglycine hydrolase; EC 3.5.1.24), the enzyme that catalyses the hydrolysis of glycine and/or taurine conjugated bile salts into amino acid residues and free bile salts (bile acids) (Corzo and Gilliland, 1999). A study on the formation of deconjugation products reported that cholate, chenodeoxycholate and deoxycholate were found on the agar plugs containing typical colonies on plates containing taurocholic acid, taurochenodeoxycholic acid and taurodeoxycholic acid (Ahn et al., 2003). Most bile salts found in faeces are of deconjugated forms and consist almost entirely of deoxycholate and lithocholate (Korpela et al., 1988). Strains of \textit{L. acidophilus} that could deconjugate bile salt \textit{in-vitro} contributed to higher faecal excretion of deoxycholate in \textit{in-vivo} human trials, which led to the suggestion that the increase of deoxycholate was contributed by deconjugation of glycine or taurine by \textit{L. acidophilus} and the transformation of cholate into deoxycholate through 7\alpha-dehydroxylation of other intestinal bacteria (Ahn et al., 2003).

Bile salt hydrolases (BSH) are active on both glycine and taurine conjugated bile salts. However, in experiments resembling human intestinal
pH of 6.5 and glycocholate to taurocholate ratio of 2:3, glycine conjugated bile salt was found to be more efficiently deconjugated by strains of *L. acidophilus* from both human and porcine origins than taurine conjugated bile salt (Corzo and Gilliland, 1999). It was postulated that this contributed to the solubility of both conjugated bile salts under acidic conditions. At the normal pH of the upper intestinal tract (5.50-6.50), about 50 percent of free bile salts and a small amount of glycine-conjugated bile salts were found to be protonated (nonionized), while no protonation occurred in taurine-conjugated bile salt (Carey and Cahalane, 1988). This can be explained by the pKa values of taurine and glycine conjugated bile salts, and of deconjugated bile salts, which are at pH 1.9, 3.9 and 5.0, respectively. Thus, at acidic pH, deconjugated bile salts are protonated and precipitated, while taurine-conjugated bile salts remain ionized in solution, and glycine-conjugated bile salts are partially precipitated without hydrolysis (Dashkevics and Feighner, 1989). It was previously reported that the removal of cholesterol from a medium was contributed by the disruption of destabilized cholesterol micelles as a result of bile salt deconjugation, followed by the precipitation of cholesterol with the free bile salts as pH decreased (Klaver and Van der Meer, 1993). However, cholesterol was also reported to be removed *in-vitro* by *L. acidophilus* strains when the pH was maintained at 6.0 (Noh *et al.*, 1997). It was hypothesized that cholesterol removal was not solely contributed by bile salt deconjugation, co-precipitation of cholesterol with deconjugated bile salt or destabilization of cholesterol micelles (Brashears *et al.*, 1998). Nevertheless, low pH and strong deconjugation activities were reported to be important factors for the precipitation of soluble cholesterol *in-vitro* (Ahn *et al.*, 2003). The regulation of BSH activity by pH is still unclear although BSH activities
were shown to be higher in lower pH values. Various strains of the same bacterial species also exhibited different BSH activity under similar pH levels (Corzo and Gilliland, 1999; Lunden and Salvage, 1990).

Considering that sodium glycocholate predominates in the human intestine, it was postulated that strains that prefer to deconjugate sodium glycocholate may have better potential to lower serum cholesterol concentrations if the deconjugation mechanism is important in decreasing serum cholesterol (Brashears et al., 1998). In addition, it was proposed that higher substrate specificity was obtained toward glycine conjugates than taurine conjugates. It has been found that the enzyme hydrolysed cholyglycine with the Michaelis-Menten kinetics and there was competitive inhibition by cholytaurine, as if both conjugated bile salts were hydrolysed at a single site. The products of hydrolysis, taurine and cholic acid, were found to inhibit the hydrolysis of cholyglycine (Dean et al., 2002). In contrast, using crude bovine bile, another study reported that the enzyme complete hydrolysed all the conjugated bile salts without interference by other bile components (cholesterol, phospholipids) or reaction products (taurine, glycine, cholic acid) (De Smet et al., 1995). Until now, the molecular fate of the amino acid released by the deconjugation by the bacterial cell and the benefit the organism may derive from such activity is unclear. A mechanistic approach using isolated and purified BSH from B. longum SBT 2928 found that BSH was an intracellular enzyme and hydrolysis of bile salts makes the amino acid nitrogen atoms of the released amino acids available for cells (Tanaka et al., 2000). Most of the probiotic organisms that originated from human/mammalian intestines and faeces exhibited BSH activity. However, it must also be noted that not all strains isolated from the intestine or faeces have BSH activity, suggesting that
bacteria without this enzyme could survive through bile acids environment (Tanaka et al., 1999). Deconjugation of bile salts also exhibit host specificity.

In mammalian host, the exact location of deconjugation is dependent on the host species. In mice, *Lactobacillus* flora is present in the small intestine where bile salt deconjugation starts (Tannock et al., 1994), while in humans, a significant flora starts only at the end of the ileum and fully developed in the large intestines, indicating that bile salt deconjugation activities begin at the end of the ileum and active in the large bowel (Marteau et al., 1995). Thus, if the deconjugation of bile is an essential mechanism for lowering cholesterol levels, it would be an important consideration that cultures used for human *in-vivo* trials are selected from suitable origins. Dambekodi and Gilliland (1998) have shown no relationship between the amount of cholesterol removal *in-vitro* and the degree of bile salt deconjugation, which led to another hypothesis that cholesterol removal may be related to assimilation of cholesterol. *In-vitro* experiments showed that strains of *lactobacilli* that were able to assimilate cholesterol were also able to reduce cholesterol *in-vivo*. Cholesterol assimilation was associated with the presence of bile salts and cholesterol removal from the medium increased with increasing concentration of bile salt (Rasic et al., 1992; Tahri et al., 1996). However, bile tolerant strains of *L. acidophilus* did not exhibit greatest ability to assimilate cholesterol, while strains that possessed minimal bile tolerant abilities actually assimilated more cholesterol (Dambekodi and Gilliland, 1998). Cholesterol assimilation by strains of *L. acidophilus* during refrigerated storage of non-fermented milk suggested that cholesterol uptake was associated with bacterial growth and their viability (Piston and Gilliland, 1994). Similarly,
Pereira and Gibson (2002) suggested that the cholesterol assimilation ability of the bacteria was growth dependent.

3.3 **Materials and methods**

3.3.1 **Selection of potential probiotic isolate**

   Based on *In vitro* probiotic characterization studies in chapter I, and II a potential probiotic isolate MBTU PBBM1 from milk was selected.

3.3.2 **Identification of probiotic isolate MBTU PBBM1**

3.3.2.1 Biochemical and physiological properties of MBTU PBBM1

3.3.2.1.1 **Indole production Test.**

   The overnight grown culture was inoculated in test tube containing sterilized tryptone broth and incubated for 24-48 hr at 37°C. After incubation, tube was added with ten drops of Kovac’s reagent and observed for any colour production.

3.3.2.1.2 **Methyl Red Test**

   The overnight grown culture was inoculated in test tube containing sterilized MR-VP medium and incubated for 24-48 hr at 37°C. After incubation, 2.5 ml of inoculated broth was transferred to another tube and added five drops of methyl red. Rolled between the palms of hands to disperse methyl red and observed for any colour production.

3.3.2.1.3 **Voges Proskauer Test**

   The overnight grown culture was inoculated in test tube containing sterilized MR-VP medium and incubated for 24-48 hr at 37°C. After incubation, 2.5 ml of inoculated broth was transferred to another tube and added six drops of Barritt’s Reagent A and two drops of Barritt’s Reagent B
Gently mixed and let it sit for 10-15 minutes to allow time for any colour development.

3.3.2.1.4 Citrate Utilization

Citrate utilization test was performed to find out the ability of the bacterial isolate to utilize or ferment citrate as the sole source of carbon. It was done on the Simmon’s Citrate agars slants and slants were observed for any change in the colour of the medium.

3.3.2.1.5 Urea Utilization

The overnight grown culture was inoculated to the test tube containing sterilized urea broth and incubated for 24-48 hr at 28°C and observed for any colour development.

3.3.2.1.6 Nitrate Reduction test

The overnight grown culture was inoculated in test tubes containing sterilized nitrate broth and incubated for 24-48 hr at 37°C. One ml of sulfanilic acid was added followed by the addition of one ml of dimethyl 1-naphthylamine solution; Mixed well and medium was noted for any colour change

3.3.2.1.7 Mannitol Fermentation

Mannitol salt agar slants was streaked with the test isolate and incubated at 37°C for 48 hrs. Medium was noted for colour change.

3.3.2.1.8 Starch Hydrolysis

The isolate was made a single streak on starch agar plate for 72-96 hr at 25⁰C in an inverted position. Grams iodine solution was flooded on the surface of the plates for 30 seconds. The plates were examined for the starch
hydrolysis around the line of growth of each isolates i.e., for the color change of the medium. Clear zone surrounding the microbial colonies is a typical positive starch hydrolysis

3.3.2.1.9 Lipase activity

Lipolytic organism was screened by qualitative plate assay. Isolates were grown on Tributyrin agar base plates and incubated at 37°C for 2 days. Medium was observed for the zone of clearance around the colony.

3.3.2.1.10 Gelatin Hydrolysis

The isolate was inoculated on gelatin agar deep tubes and gelatin agar medium plates at 37°C, for 4-7 days. After incubation, the tubes were placed in a refrigerator for 15 minutes and observed for liquefaction of gelatin.

3.3.2.1.11 Casein Hydrolysis

Overnight grown cultures of the test isolate was spotted on skimmed milk agar plates and incubated at 280°C for 48 hours. The production of halo zone around the colony was taken as positive for the test.

3.3.2.1.12 Motility Test

The isolate was streaked deep with a needle into a tube containing nutrient agar. Tube was incubated at 37°C for 24-48 hours. Medium was observed for motility.

3.3.2.1.13 Oxidase test

A strip of filter paper soaked in oxidase reagent p-phenyldiamine and placed in a sterile petri dish and the colony was smeared on the paper. The smeared area was noted for the colour change.
3.3.2.2 Molecular identification

3.3.2.2.1 Bacterial genomic DNA isolation (Sambrook et al., 1989)

Bacterial genomic DNA isolation of the MBTU PBBM1 was performed according to the procedure given below.

1. Overnight culture (25 ml in LB broth suspension) was taken and centrifuged at 10,000 rpm for 10 min (4 °C).
2. The pellet was resuspended in 10 mM Tris hydrochloride and 100 mM NaCl (2.5 to 3 mL each) and centrifuged at 10,000 rpm for 10 min (4 °C).
3. The pellet was resuspended in 2.5 ml TE (see Appendix A) and 50 µL lysozyme from stock (50 mg/mL) and incubated at 37 °C for 20 min.
4. RNAase 25 µL from stock (100 mg/mL) was added and incubated at room temperature for 10 min.
5. 2.5 mL of SDS, i.e. 2% in TE, was added and incubated at 50 °C for 45 min.
6. Proteinase K (50 µL) from stock (20 mg/mL) was added and incubated at 50-55 °C for 10 min.
7. Equal volume of phenol was added, mixed gently and centrifuged at 10,000 rpm for 10 min (4 °C). Aqueous phase was transferred to a fresh tube.
8. Equal volume of phenol: chloroform (1:1) was added, mixed gently and centrifuged.
9. Equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed gently and centrifuged.

10. 1/10 volume of 3 M sodium acetate was added and kept in ice for 20 min.

11. Equal volume of isopropanol was added and left for 2-3 min, centrifuged and supernatant was decanted.

12. Washed the pellet in 70% alcohol, centrifuged for 3 min and supernatant was decanted.

13. Dried the DNA and dissolved in 25-30 µL TE, stored at 4 ºC until electrophoresis.

3.3.2.2.2 Preparation and examination of agarose gel. (Sambrook et al., 1989)

Preparation and examinations of agarose gel of the sample was performed according to the procedure given below.

1. The edges of a clean, dry glass plate were sealed with tape so as to form a mold. The mold was set on a horizontal section of the bench.

2. Sufficient electrophoresis buffer was prepared (0.5x TBE- see Appendix A) to fill the electrophoresis tank and to cast the gel. The correct amount of powdered agarose was added to a measured quantity of electrophoresis buffer in an Erlenmeyer flask. The buffer did not occupy more than 50% of the volume of the flask.

3. The neck of the Erlenmeyer flask was loosely plugged with Kim wipes. The slurry was heated in a boiling water bath until the agarose dissolved.

4. The flask was transferred into a water bath at 55 ºC. When the molten gel had cooled, ethidium bromide was added to a final concentration of
0.5µg/mL and the gel solution was mixed thoroughly by gentle swirling.

5. The comb was positioned 0.5 - 1.0 mm above the plate so that a complete well is formed when the agarose is added to the mold.

6. The warm agarose solution was poured into the mold.

7. After the gel was completely set (30-45 min at room temperature), a small amount of electrophoresis buffer was poured on the top of the gel and the comb was removed. The electrophoresis buffer was poured off and the tape was removed. The gel was mounted in the electrophoresis tank.

8. Just enough electrophoresis buffer was added to cover the gel to a depth of about 1 mm.

9. The DNA samples were mixed with the desired volume of the gel-loading buffer (see Appendix A). The sample mixture was loaded into the slots of the submerged gel using a micropipette.

10. The lid of the gel tank was closed and the electrical leads were attached so that the DNA will migrate towards the anode (red lead). A voltage of 1-5 V/cm (measured as the distance between the electrodes) was applied. The gel was run until the bromophenol blue and xylene cyanol FF have migrated the appropriate distance through the gel.

11. The electric current was turned off and the leads and lid were removed from the gel tank. The gel was examined by UV light and photographed.
3.3.2.2.3 16S r DNA analysis of the isolates

(i) 16S rDNA primers

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

16S R 5´-GGT TAC CTT GTT ACG ACT T-3´

(ii) PCR reaction mixture

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA</td>
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</tr>
<tr>
<td>Taq buffer</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>dNTPs</td>
<td>1 µL</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>1.5 µL</td>
</tr>
<tr>
<td>Forward primer</td>
<td>1 µL</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>1 µL</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>12 µL</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.3 µL</td>
</tr>
</tbody>
</table>

PCR reaction was carried out in Eppendorf AG 22331 Thermal Cycler.

(iii) PCR reaction cycle

The PCR reaction cycle is given in Table 3.1. The PCR products were size fractionated on 1.5% agarose gel stained with ethidium bromide to check the amplification.
Table 3.1 PCR reaction cycle

<table>
<thead>
<tr>
<th>Step No.</th>
<th>Temperature (°C)</th>
<th>Duration</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>2 min</td>
</tr>
<tr>
<td>2</td>
<td>94</td>
<td>45s</td>
</tr>
<tr>
<td>3</td>
<td>55</td>
<td>45s</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>1 min 30 s</td>
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<td>5</td>
<td>Go to step 2</td>
<td>Repeat 35 times</td>
</tr>
<tr>
<td>6</td>
<td>72</td>
<td>10 min</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>10 min</td>
</tr>
</tbody>
</table>

3.3.2.2.4 Sequence similarities and phylogenetic analysis

The sequence of the insert was determined using the automated DNA sequencing service provided at SciGenomics Lab Pvt Ltd., Cochin, India. 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 phylogeny were evaluated using ClustalW2 at the European Bioinformatics Institute (http://www.ebi.ac.uk). The sequences (see Appendix B) were deposited in the National Centre for Biotechnology Information (NCBI) gene bank database. The phylogenetic tree was displayed using the Molecular Evolutionary Genetics Analysis (MEGA) version 5.05 program (http://www.megasoftware.net).

3.3.3 Growth curve assay

MBTU PBBM1 was individually grown in nutrient broth medium. To obtain the growth profiles of this strain the nutrient broth was inoculated with 50µL of 24 h culture and incubated for 72 h at 37 ºC. Each culture was inoculated in triplicate and the readings of the profiles were averaged during
which the optical density (OD) was read and recorded at every 1 hour. Absorbance was measured at 640 nm against the corresponding non-inoculated blanks.

3.3.4 Spore growth and stability at different temperatures

50 µl of MBTU PBBM1 spores were inoculated to tubes containing 5 ml of sterile nutrient broth and incubated for 3 hrs at 10°C, 15°C, 20°C 25°C, 30°C, 35°C, 40°C 0, 45°C, 50°C 55°C 60°C,65°C,70 °C, 75°C, 80°C, 90°C. During these incubation time cell growths were measured by absorbance at 640 nm against the corresponding non-inoculated blanks.

3.3.5 Antibiotic sensitivity test

Antibiograms of the selected isolates and enteric pathogens were prepared using selected antibiotic discs (Gentamicin (10 mcg/disc), Chloramphenicol (10 mcg/disc), Streptomycin (25 mcg/disc), Tetracycline (10 mcg/disc), Vancomycin (10 mcg/disc), Carbenicillin (100 mcg/disc), Kanamycin (10 mcg/disc), Methicillin (5 mcg/disc), Ciprofloxacin (30 mcg/disc), Erythromycin (10 mcg/disc), Amikacin (30 mcg/disc), Ampicillin (10 mcg/disc), Penicillin G (10 mcg/disc)) which were placed on Muller-Hinton agar (HiMedia, India) plates previously swabbed with the isolated bacterial strains. Plates were incubated at 37 °C for 24 hr. Resistant bacterial strain grows around the antibiotic discs. Clear zones around the discs indicate the sensitivity of the bacterial strains against the antibiotics.
3.3.6 Analysis of plasmid DNA

3.3.6.1 Isolation of plasmid DNA (Maniatis et al. 1982)

Small-scale preparation of plasmid DNA was performed using rapid alkaline lysis procedure.

1) A single bacterial colony was inoculated into 2 mL of LB medium and the cultures were incubated overnight with vigorous shaking.

2) 1.5 mL of the culture was poured into a micro centrifuge tube and centrifuged at 12,000 rpm for 30 sec at 4 ºC.

3) The supernatant was removed leaving the bacterial pellet as dry as possible.

4) The bacterial pellet was resuspended in 100 µl of ice cold solution I (see Appendix A) by vigorous vortexing.

5) 200 µl of freshly prepared solution II (see Appendix A) was added. The tube was closed tightly and the contents were mixed by inverting the tube rapidly 5 times. The tube was stored on ice.

6) 150 µl of ice-cold solution III (see Appendix A) was added. The tube was closed and vortexed gently in an inverted position for 10 s to disperse solution III through the viscous bacterial lysate. The tube was stored on ice for 3-5 min.

7) Centrifugation was done at 12,000 rpm for 5 min at 4 ºC in a microfuge. The supernatant was transferred to a fresh tube.

8) The DNA was precipitated with 2 volumes of ethanol at room temperature and mixed by vortexing. The mixture was allowed to stand for 2 min at room temperature.
9) The samples were centrifuged at 12,000 rpm for 5 min at 4 ºC in a microfuge.

10) The supernatant was removed by gentle aspiration. The tubes were kept in an inverted position on a paper towel to allow all of the fluid to drain away.

11) The DNA pellet was rinsed with 1 mL of 70% ethanol at 4 ºC. The supernatant was removed as described in step 10 and the pellet was dried in air for 10 min.

12) DNA was re dissolved in 50µl of TE (pH 8.0) and vortexed briefly. The DNA was stored at -20 ºC.

The isolated plasmid profile were separated on an agarose gel(0.8% w/v) prepared using TBE buffer. The electrophoresis was carried out at 75 V for 1 hr (see section 3.3.2.1.2).

Molecular weight of the isolated plasmid was determined using Lambda DNA/ EcoR I/Hind III Double Digest markers and by using gel scanners.

3.3.7 Plasmid curing (Trevors 1986)

Plasmid curing was performed according to the procedure given below.

1. Logarithmically growing overnight cultures were used to inoculate a series of culture flasks containing LB broth amended with a range of curing agent concentrations.

2. The inoculated flasks were incubated overnight at the appropriate temperature for the bacteria under investigation.
3. Flasks displaying observable turbidity and containing the highest concentration of curing agent was used to plate out dilutions on Muller Hinton agar.

4. Individual colonies were patch-plated to a selective agar medium to screen for lose of plasmid-encoded trait (e.g., antibiotic sensitivity or metal sensitivity) and/or individual colonies were tested for plasmid loss using plasmid isolation procedure (section 3.3.6.1). The curing of plasmid was carried out by applying intercalating dye acridine orange, at elevated growth temperature. The concentration ranges of mutagen was 100 µg/ mL to 800 µg/ mL with increments of 100 µg/ ml for acridine orange. For curing by elevated temperature, cultures was incubated at the desired temperature 40ºC overnight, re inoculated in fresh LB medium and re incubated at the corresponding temperature until late log phase. Samples from culture flasks which displayed observable turbidity were plated on nutrient agar and incubated overnight at 37 ºC. Colonies developed in the nutrient agar mother plate were patch plated on Methicillin supplemented nutrient agar and incubated. (MBTU PBBM1 showed Methicillin resistance) The percentage curing efficiency was expressed as 100 × (number of cured colonies/total number of colonies used). To check the role of plasmids, cured Methicillin sensitive colonies were selected and inoculated in nutrient broth medium and tested for inhibitory activities in well diffusion assay (See section1.3.4.1.2) against the indicator pathogens *Salmonella typhi, Salmonella para typhi A, Vibrio cholerae.*
3.3.8 Health promoting attributes of probiotic

3.3.8.1 Cholesterol removal (Rudel and Morris 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:

\[ A = 100 - \left( \frac{B}{C} \right) \times 100 \]

Where \( A \) =% of cholesterol removed, \( B \) =absorbance of the sample containing the cells and 
\( C \) =absorbance of the sample without cells.
3.3.8.2 Deconjugation of bile salts (Ahn et al., 2003)

Deconjugation of bile salts by MBTU PBBM1 was tested qualitatively through the plate assay. 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.

3.4 Results

3.4.1 Biochemical and Physiological properties

With reference to biochemical and physiological properties, as outlined in the Bergey’s manual of determinative bacteriology, the bacteria was showing more similarity to the *Bacillus subtilis*. The results of biochemical and physiological studies are summarized in Table 3.2

<table>
<thead>
<tr>
<th>Biochemical and physiological properties of MBTU PBBM1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Indole production Test</td>
<td>Negative</td>
</tr>
<tr>
<td>Methyl red test</td>
<td>Positive</td>
</tr>
<tr>
<td>Voges Proskauer Test</td>
<td>Positive</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>Positive</td>
</tr>
<tr>
<td>Urea utilization</td>
<td>Negative</td>
</tr>
<tr>
<td>Nitrate reduction test</td>
<td>Positive</td>
</tr>
<tr>
<td>Mannitol fermentation</td>
<td>Positive</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>Positive</td>
</tr>
<tr>
<td>Lipase activity</td>
<td>Positive</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>Negative</td>
</tr>
<tr>
<td>Casein hydrolysis</td>
<td>Negative</td>
</tr>
<tr>
<td>Oxidase activity</td>
<td>Negative</td>
</tr>
<tr>
<td>Motility test</td>
<td>Motile</td>
</tr>
</tbody>
</table>
3.4.1.1 Molecular identification

3.4.1.1.1 Genomic DNA Isolation

Isolated genomic DNA of MBTU PBBM1 was visualized by agarose gel electrophoresis under UV light. Then they were amplified by PCR method.

3.4.1.1.2 16S rDNA sequence analysis of the isolate

The genomic DNA of MBTU PBBM1 was amplified with 16S rDNA primers and result is shown in Figure 3.1.

Figure 3.1. Genomic DNA amplified with 16S F and R primers
M- DNA Ladder; 1-MBTU PBBM1
Result of molecular identification of the isolate by 16S rDNA sequencing are summarized in Table 3.3.

Table 3.3. 16S rDNA identification of bacterial isolate

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Gene Bank accession number</th>
<th>Bacterial genus</th>
<th>Phylogenetic Relationship</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBTUPBBM1</td>
<td>JN873913</td>
<td>Bacillus</td>
<td>Bacillus subtilis</td>
</tr>
</tbody>
</table>

3.4.1.1.1.3 Phylogenetic tree and sequence similarities of the isolated strain

Figure 3.2 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.

Figure 3.2   Phylogenetic tree of the isolated strain MBTU PBBM1
3.4.2 Growth Curve

Growth curve of the MBTU PBBM1 is shown in Fig.3.3. In the given growth conditions, the lag phase was from 0-3 h. The exponential phase started from the 4th hr and extended up to 48 hr. After that, the bacterium entered into stationary phase (48 -72 hrs) of growth. By 72 hrs of growth, the spores were found to have fully formed. After that the stationary phase growth were found to be stable.

![Growth curve of MBTU PBBM1](image)

**Figure 3.3: Growth Curve of MBTU PBBM1**
3.4.3 Spore growth and stability at different temperatures

Growth of spores at different temperature reveals the ideal temperature for the growth of MBTU PBBM1. From growth studies at different temperatures, (See figure 3.4) it was inferred that the ideal temperature of MBTU PBBM1 was in and around 35°C. Absorbance was measured at 600 nm against the corresponding un inoculated blank. Spore growth was limited in the low and high temperatures. Optical density measurement at different temperature reveals that extreme low and high temperatures will not affect the viability but it will not favours progressive cell growth rate.

![Growth of spores at different temperatures](image)

**Figure 3.4: Growth of spores at different temperatures**

3.4.4 Antibiotic sensitivity test

The results obtained (see Table 3.4) after the antibiotic sensitivity test on Muller-Hinton agar plates showed that isolate MBTU PBBM1 was sensitive to all the investigated antibiotics except Methicillin. However, the
indicator strain *V. cholerae* was sensitive to all the antibiotics, except Penicillin. Further, *Salmonella typhi* was resistant against Chloramphenicol and Penicillin. While *Salmonella para typhi A* was resistant against, Chloramphenicol Carbencillin, Ciprofloxacin and Penicillin.

**Table 3:4 Antibiotic sensitivity test of bacterial strain MBTU PBBM1**

<table>
<thead>
<tr>
<th>Antibiotic (conc.)</th>
<th>MBTU PBBM1</th>
<th>V.cholerae</th>
<th>Sal.typhi</th>
<th>Sal.paratyphi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamicin (10 mcg/disc)</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Chloramphenicol (10 mcg/disc)</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Streptomycin (25 mcg/disc)</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Tetracycline (10 mcg/disc)</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Vancomycin (10 mcg/disc)</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Carbenicillin (100 mcg/disc)</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Kanamycin (10 mcg/disc)</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Methicillin (5 mcg/disc)</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Ciprofloxacin (30 mcg/disc)</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>Erythromycin (10 mcg/disc)</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Amikacin (30 mcg/disc)</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Ampicillin (10 mcg/disc)</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Penicillin G (10 mcg/disc)</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
</tbody>
</table>

S –Sensitive  R - Resistant
3.4.5 Analysis of plasmid DNA

3.4.5.1 Isolation of plasmid

Small-scale preparation of plasmid DNA was performed using rapid alkaline lysis procedure of Maniatis et al. 1982. Molecular weight of the plasmid was determined using λDNA digested with Hind III size markers and by using gel scanners. Presence of plasmid in the selected isolate was checked and the result is given in Figure 3.5. Result indicated the presence of plasmid and has molecular weight of ~12 kb.

![Figure 3.5 Plasmid DNA profile of MBTU PBBM1.](image)

i) M - λDNA / EcoR I/Hind III Double Digest; ii) MBTU PBBM1

iii) MBTU PBBM1(C) - Plasmid cured strain of MBTU PBBM1
3.4.6 Plasmid curing

Isolated strain was tested for inhibitory activities in well diffusion assay against the indicator strains after the successful curing of plasmid DNA. The cured derivative of the strain MBTU PBBM1 was obtained by incubation with 600 µg/ mL acridine orange at an elevated temperature of 40°C. Individual colonies after curing were patch plated on methicillin supplemented nutrient agar plate and those failed to develop colonies on methicillin plate was selected from the mother plate as cured cells. (See figure 3:6). The percentages of plasmid curing (number of cured colonies per 100 colonies tested) was determined and it was about 51% for MBTU PBBM1 (See Table 3.5). Plasmid curing was confirmed by agarose gel electrophoresis. (See Figure 3.5). Cured derivative of MBTU PBBM1 was found to be sensitive to methicillin. This proved that methicillin resistance was plasmid mediated. The antibacterial activity of the cured strain was found to be unaltered when tested for the inhibitory activity against indicator pathogens. This reveals that antibacterial activity of MBTU PBBM1 against indicator pathogens are not plasmid mediated.

Figure 3.6 Plasmid cured colonies of MBTU PBBM1
Table 3.5: Effect of curing agent on curing ability of selected strain

<table>
<thead>
<tr>
<th>Bacterial isolate</th>
<th>Curing agents</th>
<th>Concentration/Temperature</th>
<th>Percentage of curing</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBTU PBBM1</td>
<td>Acridine orange and elevated temperature</td>
<td>600 µg/mL, 40°C</td>
<td>51</td>
</tr>
</tbody>
</table>

3.4.7 Health promoting attributes of probiotic

3.4.7.1 Cholesterol assimilation

Cholesterol assimilation was associated with the presence of bile salts. Probiotic strains assimilate cholesterol for their own metabolism. The percentage of cholesterol assimilated by MBTU PBBM1 during 20 hr growth at 37°C in MRS broth is given in Figure 3.7. 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%.

Figure 3.7 Cholesterol assimilation of MBTU PBBM1
3.4.7.2 Deconjugation of bile salts

Bile salt deconjugation capability of probiotic strain help in the reduction of serum cholesterol. 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 3.8.

![Figure 3.8 Bile salt deconjugation of MBTU PBBM1](image)

3.5 Discussion

Probiotic strains are unique and different in their properties and characteristics. Based on *In vitro* probiotic characterization studies best performing isolate MBTU PBBM1 from milk was selected and identified. With reference to colony morphology, biochemical tests as outlined in the Bergey’s manual of bacteriology, the bacteria ‘MBTU PBBM1’ was more similar to *Bacillus subtilis*. Accurate species labeling is important in the field of probiotics because it was important in quality control efforts, confidence
Identification and Characterization of Potential Probiotic Isolate MBTU PBBM1

in product labeling, and for safety considerations. To establish the relatedness of the strains at the genetic level, 16S rRNA genes of MBTU PBBM1 was sequenced to analyze species similarity. PCR product was sequenced by using an automated sequencer and phylogenetic analysis showed that MBTU PBBM1 has phylogenetic relationship with the *B. subtilis*. A short lag period and short doubling time has a better chance to out compete other bacteria based on their growth characteristics (Vine *et al*., 2004a). *In vitro* growth is one important factor for the selection of probiotics. Strain MBTU PBBM1 has got a short lag phase and had a short doubling time. Short lag phase and short doubling time are said to be the probiotic features to outcompete pathogens (Aparna *et al*., 2011) There was no decline phase in the growth curve because after, exhaustion of the nutrients, the cells started sporulating and the number of colony forming units remained constant. Spores of bacteria are safe for probiotic applications because they can tolerate harsh conditions of gastrointestinal tract. (Anu *et al*., 2012). Hence spores of MBTU PBBM1 were studied for the stability and growth at different temperatures. Three hour spore growth at different temperatures, revealed that growth rate at lower and higher temperatures was found to be less than the intermediate temperatures. The maximal growth was found to be at 35 °C. Growth rate was found to be descending with increase in temperature. Optimal growth of this strain is in and around to body temperature of human reveals the strains capability to grow efficiently in human body. Antibiotic resistance of microorganisms used as probiotic agents is an area of growing concern. Antibiotic resistance of probiotic should be studied because novel probiotic microorganism should not be resistant for the antibiotics (Anil *et al*., 2010). It is believed that antibiotic used for food-producing animals can promote the emergence of antibiotic resistance in bacteria present in the intestinal
Chapter 3

microflora. Then, the antibiotic-resistant bacteria can transfer the resistance factor to other pathogenic bacteria through the exchange of genetic material (Mathur and Singh, 2005). Hence isolate MBTU PBBM1 was studied for antibiotic susceptibility and found that it was sensitive to all the tested antibiotics except Methicillin. This reveals the wild nature of the isolate. Resistance of MBTU PBBM1 strain to Methicillin may be due to its natural and intrinsic resistance, probably due to the cell wall structure and membrane impermeability. The antibiotic resistance profile of microorganisms depends largely on the previous exposure histories of the microorganisms, e.g. to the type of antibiotics, period of exposure, and contact with other resistant microorganisms (Schwarz and Chaslus-Dancla, 2001). Plasmid isolation study revealed that MBTU PBBM1 possessed plasmid. Plasmid curing experiment was performed in order to check the role of plasmid in mediating antibiotic resistance and antagonistic activity. Several chemical curing agents, including novobiocin, acriflavin, acridine orange, ethidium bromide, and SDS, have been successfully used in the curing of gram-positive bacterial plasmids (Caro et al., 1984). The effectiveness of curing methods depends on the nature of the bacterial host and/or plasmids. (Ghosh et al., 2000, Bringel et al., 1989) Present study found that application of intercalating agent acridine orange at an elevated temperature (40°C) was found to be effective in curing the plasmid. The percentage of plasmid curing was determined and it was about 51% for MBTU PBBM1. Plasmid curing experiment inferred that the Methicillin resistance of MBTU PBBM1 was plasmid mediated, because cured colonies had no Methicillin resistance. Antagonistic activity against indicator pathogen of cured strain was found to be unaltered. This proves that antagonistic activity of MBTU PBBM1 was not mediated by plasmid. Cured strain of MBTU PBBM1 was Methicillin
sensitive and antagonistic toward enteric pathogens *Salmonella typhi*, *Salmonella paratyphi* A, and *Vibrio cholera*. These features add probiotic value of MBTU PBBM1 to be used as probiotic and this may limit the transfer of many antibiotic resistance genes between pathogenic and nonpathogenic bacteria. Hypercholesterolemia is considered as a major risk factor for the development of coronary heart disease (Pereira *et al.*, 2003). Although therapeutic drugs are available to relieve this problem, they are often expensive and can have side effects. The use of probiotic bacteria in reducing serum cholesterol levels has attracted much attention. 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. Gram positive bacteria have been reported to be capable of hydrolyzing amide bond of conjugated bile salts liberating free bile salts with lower detergent properties. Cholesterol is the precursor of primary bile salts that are formed in the liver and are stored as conjugated bile salts in the gall bladder for secretion in the gastrointestinal tract (Corzo & Gilliland, 1999). MBTU PBBM1 showed capability bile salt deconjugation and the strain 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 agreement with studies of Rasic *et al.*, 1992; Tahri *et al.*, 1996. Fine precipitated white granules observed around and within the colonies during deconjugation test indicates bile salt hydrolase activity of the strain. 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. 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). Thus health promoting effects such as bile salt deconjugation and cholesterol assimilation capability of MBTU PBBM1 may help in the reduction of serum cholesterol. Chapter 3 summarizes that the strain MBTU PBBM1 shows more similarity to *Bacillus subtilis* and possessed a number of interesting properties that constitute the basis for their use as health-promoting bacteria and warrant further clinical investigations. Hence future *in vivo* studies are aimed at persistence of the strain in the gastrointestinal tract of Balb/c mice and nature of immunostimulation induced by this candidate bacterium.