Chapter – 6

Comparative account of *in vitro* probiotic properties of isolated *Bacillus* species
6.1. Abstract

Since the *Bacillus* cultures obtained in this study was from food source, it was planned to investigate the functional properties of these cultures as probiotics *in vitro*, as an additional application study. *Bacillus* probiotics have established their efficacy as dietary adjuncts providing health benefits to the consumers. However, selection of probiotics before incorporation into diet requires close scrutiny in the form of *in vitro* as well as *in vivo* tests. In this chapter, evaluation of the probiotic properties of seven *Bacillus* spp. obtained from different food sources (as reported in Chapter 2) were documented. *B. coagulans*, a known probiotic strain was used as a positive control. Among the tested strains, the culture *B. licheniformis* Me1 was found to be the most acid-tolerant strain exhibiting 89% of survivability after 3 h of incubation at pH 3. The cultures Me1, Ec1 and Ik11 were found to be bile tolerant. Among the strains screened for BSH activity, the cultures, Me1, Ik11, Ec1, Bk1 and *B. coagulans* showed positive, whereas culture *B. flexus* Hk1 exhibited a negative result. Among the tested *Bacillus* spp., the cultures, Me1, Bk1 and Pk12 were sensitive to all the antibiotics tested, while the culture HK1 and the control probiotic *B. coagulans* exhibited resistances to some of the antibiotics. A marked difference in adhesion to hydrocarbons and auto-aggregation property from 10-80% and 60-99%, respectively, were observed for the tested cultures. Highest antioxidant activity was measured for the culture Hk1 (66.6%), whereas least activity was noted for Ik11 and Bk1. In co-cultivation studies, *B. licheniformis* Me1 completely inhibited the growth of indicator pathogen, *L. monocytogenes* Scott A. Based on the comparison of *in vitro* probiotic properties of the *Bacillus* spp., the culture *B. licheniformis* Me1 exhibited remarkable *in vitro* probiotic properties and thus can be considered a positive trait for supplementation in food products as a probiotic.
6.2. Introduction

Probiotics are live microbial feed supplements which when administered confers health benefit to the host (FAO/WHO 2002; Fuller 1989). Documented benefits include modulation of the immune system, lowering of the serum cholesterol level and alleviation or prevention of intestinal disorders, such as diarrhea or lactose intolerance or antibody-associated diarrhea (Guo et al. 2010). Probiotics are being developed commercially for human use as novel foods or dietary supplements, and as well as incorporated animal feeds for the prevention of gastrointestinal infections (Rolfe 2000; Rowland 1999). Probiotics can also serve as an alternative to antibiotics in farming and aquaculture, and as well as prophylactics in humans. Moreover, the probiotic therapy is very attractive because it is an effective and non-invasive approach as it imparts health benefit by improving the intestinal microbial ‘balance’. The majority of the probiotics studied and commercialised are lactic acid producing bacteria (De Vecchi and Drago 2006; Kabir et al. 1997; Lee and Salminen 1995). Less well known than Lactobacillus spp. and Bifidobacterium spp., certain species of the spore forming Bacillus genus which includes B. subtilis, B. licheniformis, B. clausii, B. coagulans, B. cereus, B. pumilus and B. laterosporus are also being used as probiotics (Hong et al. 2008; Sanders et al. 2003; Sorokulova et al. 2008; Urdaci and Pinchuk 2004).

The Bacillus genus is known to possess features quite common to most commonly used probiotic cultures. These common characters includes i) gut viability (Tam et al. 2006), ii) resistance to bile and acid (Hong et al. 2005) and iii) production of metabolites, such as antimicrobial compounds (Urdaci and Pinchuk 2004), enzymes (Inatsu et al. 2006), and essential amino acids and vitamins (Salvetti et al. 2003). The other health benefits of probiotic Bacillus include decrease in the level of cholesterol in blood (Kim et al. 2002a), antimutagenic effects (Caldini et al. 2002) and stimulation of nonspecific immunoreactivity (Caruso et al. 1993). These findings show the possibility that Bacillus strains with a wide spectrum of biological activity can be selected as probiotics. Moreover, such Bacillus spp. can also act as biopreservatives in food products (Beaumont 2002). It is also well-known that different species belonging to the same genus may have different biological characteristics. Thus, various strains of Bacillus exhibiting inhibitory activity against wide array of pathogens should be tested for in vitro and in vivo probiotic properties before application as food supplements (Endres et al. 2009; Sorokulova et al. 2008).
Products containing *Bacillus* endospores used commercially as probiotics contain a single doses of $10^9$ spores/g or $10^9$ spores/ml (Mazza 1994). The composition of currently-used probiotics varies from those containing a mixture of many strains to those containing single strain. *Bacillus coagulans* GBI-30, 6086, marketed as GanedenBC30® (Ganeden Biotech, Mayfield Heights, Ohio) is a novel *Bacillus* strain which is supplemented in several food products as probiotic and has been documented to have beneficial impact on both digestive system and immunity (Hun 2009). *B. subtilis* and *B. licheniformis* are compounds of two probiotic products, namely BioPlus 2B® and Biosporin (Gracheva et al. 1996; Sorokulova 1998). The former probiotic product is used in animal feed, while the later is licensed as a medicine for the efficient prevention and treatment of GI disorders in Ukraine and Russia.

Before the strains of probiotics are able to exert proposed benefits in the intestine, they need to fulfill several criteria as well as to survive the harsh environments of the gastrointestinal tract, which include the acidic conditions of the stomach and bile salts. Furthermore, gut colonization helps to increase the time of influence of gastrointestinal immune system and microbiota of the host by the probiotic microorganism (Forestier et al. 2000; Kirjavanainen et al. 1998). The World Health Organization developed guidelines for the evaluation of probiotics in food, which indicates parameters for preclinical testing of probiotics, clinical trials, and labeling (FAO/WHO 2006). The Indian Council of Medical Research - Department of Biotechnology (ICMR-DBT), India (2011) has also proposed guidelines for evaluating probiotic in foods. The number of requirements which have been identified for classifying strains to be an effective probiotic microorganism includes survival through the gastrointestinal tract (Maruo et al. 2006), resistance to gastric acid and physiological concentrations of bile, production of antimicrobial substances, adherence to intestinal epithelial cells (Schillinger et al. 2005), sensitiveness to antibiotics (FAO/WHO 2006) and co-aggregation to form a barrier which prevents colonization by the pathogens. Although, *in vitro* studies partially mimic *in situ* conditions in the gut ecosystem (Dunne et al. 2001), these studies can be useful tools for the screening of numerous samples and the selection of microbes for further *in vivo* safety and clinical trials and eventually for use in humans.
The inhibitory spectrum of the seven selected native *Bacillus* cultures isolates against food-borne pathogens and the technological properties of their ABP, as were studied and are included in Chapter 2 and Chapter 3. Keeping in view these findings and considering the increasing demand and potential health benefits of *Bacillus* spp. as probiotics, in this Chapter, the probiotic characteristics of selected *Bacillus* spp. obtained from different food sources were evaluated. Various criteria for selection of a probiotic strain were investigated and compared.

### 6.3. Materials and methods

#### 6.3.1. Chemicals, reagents and bacteriological media

Taurodeoxycholic acid sodium salt (TDCA), 2- diphenyl-1 picrylhydrazyl (DPPH) and ruthedium red were purchased from Sigma, India. The antibiotics used for antibiotic susceptibility test were also purchased from the same source. Chemicals, such as sodium phytate ammonium molybdate, ammonium meta-vanadate cobalt chloride used for phytase assay were also obtained from Himedia, India. Muller-Hinton (MH) agar was also purchased from the same source. Solvents, like xylene and methanol were obtained from Rankem, India.

#### 6.3.2. Bacterial strains and culture conditions

Seven *Bacillus* spp. selected for determination of their *in vitro* probiotic properties included *B. cereus* Ik11, *B. flexus* Hk1, *B. licheniformis* Me1, *B. subtilis* Ec1, *B. thuringiensis* Ik15, *B. amyloliquefaciens* Pk12, and *B. megaterium* Bk1. A known probiotic strain, *B. coagulans* was used as a positive control to compare the probiotic properties of the selected cultures. The test cultures and the food-borne pathogen *L. monocytogenes* Scott A used in this study were maintained and propagated before use as described in section 2.3.2.

#### 6.3.3. Acid tolerance test

Tolerance to low pH was determined as described by Teh et al. (2009). From the freshly grown *Bacillus* cultures (16 ± 2 h), cells were harvested by centrifugation at 10,000 g and 4°C for 15 min. After washing in phosphate buffered saline (PBS) (0.1 M PBS at pH 7.2), the cell pellets were resuspended in 0.1 M PBS (pH 3) and kept for incubation at 37°C. The survival of the test cultures at 0, 1, 2 and 3 h of
incubation was analyzed by plating the samples in LB agar and by determining the colony forming unit (CFU/ml). The percentage of survival was calculated using the formula (Fuller, 1989),

\[
\text{Survival (\%)} = \frac{\text{Log number of viable cells survived (CFU/ml)} \times 100}{\text{Log number of initial viable cells inoculated (CFU/ml)}}
\]

6.3.4. Bile tolerance test

6.3.4.1. Growth at different bile concentrations

LB broths prepared with different concentration of Ox-bile [0.1, 0.2, 0.3, 0.6, 0.8, 1.0, 2.0, 4.0, 6.0 and 8.0% (w/v)] were inoculated with freshly grown (incubated for 16 ± 2 h at 37°C) *Bacillus* cultures. After 24 h of incubation at 37°C, the growth of the test cultures at different bile concentrations was monitored by measuring the optical density (OD) at 650 nm using spectrophotometer (Shimadzu, Japan). LB broth without Ox-bile served as negative control.

6.3.4.2. Bile tolerance

Growth in the presence of 0.3% (w/v) Ox-bile was analyzed as described by Gilliland et al. (1984). Freshly grown (incubated for 16 ± 2 h at 37°C) cultures were centrifuged for 15 min at 10,000 g and 4°C, and the harvested cell pellet was resuspended in the same volume of saline (0.85% NaCl). Fresh LB broth (5 ml) without Ox-bile (for negative control) and LB broth (5 ml) containing 0.3% (w/v) Ox-bile were inoculated with 250 µl (5% v/v) of cell suspension. The growth was monitored by measuring the OD at 600 nm using spectrophotometer. Time lag to reach log phase at various bile concentrations was determined to ascertain bile tolerance or sensitivity of the cultures (Chateau et al. 1994).

6.3.5. Bile salt hydrolase (BSH) assay

BSH activity of the culture was evaluated using the procedure described by Pereira et al. (2003). The 16 ± 2 h grown cultures were streaked on LB agar plates supplemented with 0.5% (w/v) TDCA and incubated at 37°C for 72 h. After incubation, the plates were observed for the presence or absence of the white precipitate around the colonies and were scored as positive. The LB agar plates without the supplementation of TDCA were used as the negative control.
6.3.6. **Antibiotic susceptibility test**

Susceptibility of the cultures to antibiotics was determined by using the disc diffusion method according to the recommendations of European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2011). The antibiotics are listed in Table 6.2. Cells from the 16 ± 2 h old cultures were suspended to density of a McFarland 0.5 turbidity standard. This suspension was diluted 1:100 and was seeded on MH agar plates using sterile cotton wool swab. Antibiotic-impregnated discs were placed on seeded plates within 15 min of swabbing and the zone of growth inhibition was measured after 24 h of incubation at 37°C. The results were recorded as sensitive (S) and resistance (R) based on the diameter of zone of inhibition.

6.3.7. **Phytase activity**

The phytate degrading ability of the cultures was performed according to the method as described by Raghavendra and Halami (2009), using a modified LB medium containing 0.625 g of sodium phytate. Active cultures (incubated for 16 ± 2 h at 37°C) were centrifuged for 15 min at 10,000 g and 4°C. The harvested cell pellet was then resuspended in the same volume of saline (0.85% NaCl). Harvested cell suspension (3 μl of $10^7$-$10^8$ CFU/ml of the culture) was spotted onto the surface of modified LB agar and incubated for 24 h at 37°C. After incubation, the colonies were washed from the agar surface using sterile double distilled water and flooded with 2% (w/v) aqueous cobalt chloride solution (Bae et al. 1999). After 5 min of incubation at 27 ± 2°C, the cobalt chloride solution was replaced with a freshly prepared solution containing equal volumes of 6.25% (w/v) aqueous ammonium molybdate solution and 0.42% (w/v) ammonium meta vanadate solution. After 5 min of incubation, the immersed solution was removed and the plates were examined for zones of phytate hydrolysis.

6.3.8. **Exopolysaccharide production**

The ability to produce exopolysaccharide (EPS) by the tested cultures was examined as described by Mora et al. (2002). Briefly, overnight grown (16 ± 2 h) *Bacillus* cultures were streaked on the surface of plates containing ruthedium red milk (10% w/v, skim milk powder, 1% w/v, sucrose and 0.08 g/L ruthedium red, 1.5% w/v agar). After incubation at 37°C for 24 h, ropy white colonies were scored as EPS producing strains.
6.3.9. Bacterial adhesion to hydrocarbons (BATH)

BATH test was performed using xylene as a hydrocarbon to assess the adherence ability of the isolates according to the procedure described by Canzi et al. (2005). For this purpose, the cells were harvested from 16 ± 2 h grown cultures as described above and the cell pellet were washed with 0.1 M PBS (Appendix I) and resuspended in the same buffer to an absorbance (A$_{600}$ nm) of 0.5. To this suspension, equal volume of xylene was added and the two phase system was thoroughly mixed by vortexing for 3 min. The aqueous phase was removed after 1 hour of incubation at 27 ± 2°C and its absorbance was measured at A$_{600}$. Adhesion percentage was calculated using the formula,

$$\text{Adhesion percentage} = \left(\frac{A_0 - A}{A_0}\right) \times 100$$

Where, A$_0$ and A were absorbance (A$_{600}$) before and after extraction with organic solvents respectively.

6.3.10. Auto aggregation tests

Auto aggregation ability of the cultures was assessed according to the method as described by Canzi et al. (2005). Briefly, 10 ml of bacterial culture was kept in static condition for 3 h at 15°C. After incubation, 1 ml of the broth from the upper suspension was transferred to another test tube and OD was measured at A$_{600}$. Auto aggregation was calculated by using the equation,

$$\text{Auto aggregation \%} = 1 - \frac{\text{OD of upper suspension}}{\text{OD of total culture}} \times 100$$

6.3.11. Antioxidant activity of the cultures

The antioxidant activity was evaluated by determining the DPPH free radical scavenging activity of the cultures as described by Ganeshan et al. (2008). The 16 ± 2 h grown Bacillus cultures were centrifuged for 15 min at 10000 g and the cell free supernatant (CFS) was collected. Test sample was prepared by adding 2 ml of DPPH solution (6 mg/100 ml of methanol) to the tube containing 500 μl of CFS which was previously mixed with 1.5 ml of sterile double distilled water. Similarly, blank solution was prepared by adding 2 ml of methanol instead of DPPH solution. Negative control was prepared by adding 2 ml of DPPH and 2 ml of double distilled water. All the tubes were mixed thoroughly for 3 min and incubated at 27°C for 30 min in dark. After incubation absorbance was measured at 517 nm. Scavenging effect of the culture was calculated in percentage using the formula,

$$\text{Scavenging effect (\%)} = \left[1 - \frac{\text{sample-blank/control}}{\text{control}}\right] \times 100$$
6.3.12. Co-cultivation of Bacillus isolates with indicator *L. monocytogenes*

For co-cultivation studies, to the 16 ± 2 h grown bacterial cultures, approximately $10^6$ CFU/ml of indicator microorganism *L. monocytogenes* Scott A was added. Samples were withdrawn every 2 h over an incubation period of 6 h and the survivability of *L. monocytogenes* Scott A was checked by plating on *Listeria* selective media. The plates were incubated at 37°C for 24 h and the number of colonies (CFU/ml) were counted and recorded.

6.4. Results

6.4.1. Resistance to Low pH

The survival of bacteria in gastric juice depends on their ability to tolerate low pH which is an important probiotic characteristic. Among the tested strains, the culture *B. licheniformis* Me1 was found to be the most acid-tolerant strain exhibiting 89% of survivability after 3 h of incubation at pH 3. The other cultures which exhibited above 80% survivability includes *B. flexus* Hk1, *B. subtilis* Ec1, *B. cereus* Ik11 and *B. coagulans* (Fig. 6.1). The cultures *B. thuringiensis* Ik15, *B. megaterium* Pk12 and *B. amyloliqufaciens* Bk1 exhibited less than 80% survivability. This result indicates that most of the cultures were able to withstand and survive in high acidic conditions.

![Figure 6.1](image)

Figure 6.1. Percentage of surviving *Bacillus* cultures at acidic pH (pH 3) for 3 h of incubation. Each value is the mean ± SEM of three independent experiments.
6.4.2. Growth in presence of different bile concentrations

The cultures exhibited difference in growth at tested concentrations of Ox-bile (0.1-8% w/v) (Fig. 6.2). The cultures Me1, Ik15 and Ik11 showed growth up to 6% w/v of Ox-bile and exhibited a reduction in growth at 8%. While, the cultures Ec1, Hk1 and *B. coagulans* showed stability in growth only up to 1% and reduced growth was observed with further increase in Ox-bile concentration. The cultures Bk1 and Pk12 were found to be less bile tolerant and showed growth only up to 0.8%.

![Figure 6.2](image_url)  
*Figure 6.2.* Percentage of survival of *Bacillus* cultures in LB broths supplemented with different concentrations of Ox-bile. Each point is the mean ± SEM of three independent experiments.

6.4.3. Bile tolerance test

The concentration of bile to be used for determining the bile tolerance in the selection of probiotic species for human beings is considered to be 0.3% (w/v) (Brashears et al. 2003). Hence, these native cultures were grown in the presence of 0.3% Ox-bile. Table 6.1 shows the bile tolerance of the *Bacillus* isolates under the human proximal intestinal conditions. It was observed that the cultures Me1, Ik11 and Ec1 when grown in LB broth with 0.3% Ox-bile showed a 30 min delay in growth as compared to growth in normal LB broth and thus categorized as bile resistant. In contrast, *B. flexus* Hk1 and *B. amyloliquefaciens* Bk1 exhibited 70 min and 120 min
delay in growth, respectively, and hence, categorized as sensitive. The standard probiotic strain \( B. \ coagulans \), as well as \( B. \ thuringiensis \) Ik15 showed a \( \sim 60 \) min delay in growth, indicating the strains were weakly tolerant. \( Bacillus \) species tested in the present study were found to be falling in both the ‘tolerant’ and ‘sensitive’ groups.

**Table 6.1.** Tolerance of \( Bacillus \) cultures in the presence of 0.3% Ox-bile

<table>
<thead>
<tr>
<th>Test cultures</th>
<th>Delay in growth (min)</th>
<th>Result*</th>
</tr>
</thead>
<tbody>
<tr>
<td>( B. \ coagulans )</td>
<td>50</td>
<td>Weakly Tolerant</td>
</tr>
<tr>
<td>( B. \ licheniformis ) Me1</td>
<td>30</td>
<td>Tolerant</td>
</tr>
<tr>
<td>( B. \ thuringiensis ) Ik15</td>
<td>60</td>
<td>Weakly Tolerant</td>
</tr>
<tr>
<td>( B. \ cereus ) Ik11</td>
<td>30</td>
<td>Tolerant</td>
</tr>
<tr>
<td>( B. \ flexus ) Hk1</td>
<td>70</td>
<td>Sensitive</td>
</tr>
<tr>
<td>( B. \ megaterium ) Pk12</td>
<td>60</td>
<td>Weakly Tolerant</td>
</tr>
<tr>
<td>( B. \ amyloloquefaciens ) Bk1</td>
<td>120</td>
<td>Sensitive</td>
</tr>
<tr>
<td>( B. \ subtilis ) Ec1</td>
<td>30</td>
<td>Tolerant</td>
</tr>
</tbody>
</table>

*The cultures were categorized into four groups according to the observed delay of growth (d) in presence of Ox-bile: resistant strains (\( d \leq 15 \) min), tolerant strains (\( 15 < d \leq 40 \) min), weakly tolerant strains (\( 40 < d < 60 \) min) and sensitive strains (\( d \geq 60 \) min) (Chateau et al. 1994).*

6.4.4. **Antibiogram of the cultures**

Among the \( Bacillus \) spp., the cultures Me1, Bk1 and Pk12 were sensitive to all the antibiotics tested, while the cultures HK1, Ik15 and \( B. \ coagulans \) exhibited resistances to some of the antibiotics (Table 6.2). The culture Hk1 was resistant to penicillin, methicillin and ampicillin, indicating its resistance to penicillin group of \( \beta \)-lactum antibiotics. \( B. \ coagulans \) also showed resistance to penicillin, methicillin and was moderately sensitive to ampicillin (10 mcg). Both of these cultures also exhibited resistance to the antibiotic co-trimoxazole. The cultures \( B. \ subtilis \) Ec1 exhibited resistance only against ampicillin.

6.4.5. **Comparison of the culture for BSH activity**

Of the \( Bacillus \) cultures screened for BSH activity using sodium deoxytaurocholate containing plates, except Hk1, Pk12 and Ik15, all the other cultures showed positive results with formation of white precipitate around the colonies (Fig. 6.3).
In vitro probiotic properties of *Bacillus* species

Table 6.2. Antibiotic susceptibility of the *Bacillus* cultures

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>B. flexus</em> Ik1</td>
</tr>
<tr>
<td>Amikacin (30 mcg)</td>
<td>S+++</td>
</tr>
<tr>
<td>Ampicillin (10 mcg)</td>
<td>R</td>
</tr>
<tr>
<td>Cefalaxine (10 mcg)</td>
<td>S+++</td>
</tr>
<tr>
<td>Cefixime (10 mcg)</td>
<td>S+</td>
</tr>
<tr>
<td>Cephalexine (10 mcg)</td>
<td>S+</td>
</tr>
<tr>
<td>Chloramphenicol (25 mcg)</td>
<td>S+++</td>
</tr>
<tr>
<td>Ciprofloxacin (10 mcg)</td>
<td>S+++</td>
</tr>
<tr>
<td>Co-trimoxazole (25 mcg)</td>
<td>R</td>
</tr>
<tr>
<td>Erythromycin (5 mcg)</td>
<td>S+++</td>
</tr>
<tr>
<td>Fusidic acid (10 mcg)</td>
<td>S+</td>
</tr>
<tr>
<td>Gentamycin (10 mcg)</td>
<td>S+++</td>
</tr>
<tr>
<td>Kanamycin (30 mcg)</td>
<td>S+++</td>
</tr>
<tr>
<td>Levofloxacin (10 mcg)</td>
<td>S+++</td>
</tr>
<tr>
<td>Methicillin (10 mcg)</td>
<td>R</td>
</tr>
<tr>
<td>Norfloxacin (10 mcg)</td>
<td>S+++</td>
</tr>
<tr>
<td>Novobiocin (5 mcg)</td>
<td>S+++</td>
</tr>
<tr>
<td>Penicillin (1 unit)</td>
<td>R</td>
</tr>
<tr>
<td>Streptomycin (25 mcg)</td>
<td>S+++</td>
</tr>
<tr>
<td>Tetracycline (25 mcg)</td>
<td>S+++</td>
</tr>
</tbody>
</table>

0 – 5 mm: Resistance (R); 6 – 15 mm: Sensitive (S+); 16 – 25 mm: Sensitive (S++); 26 – 35 mm: Sensitive (S+++).
In vitro probiotic properties of *Bacillus* species

Figure 6.3. BSH activity of the *Bacillus* cultures. a) *B. megaterium* Pk12, b) *B. coagulans*, c) *B. licheniformis* Me1, d) *B. amyloliquefaciens* Bk1, e) *B. flexus* Hk1, and f) *B. subtilis* Ec1.

6.4.6. Adhesion to hydrocarbons and Auto-aggregation

BATH and auto-aggregation test were studied as an index for adhesion property. The cultures affinity for water was evaluated by thoroughly mixing a culture with hydrocarbon suspension and then evaluating the difference in OD of the culture phase. The decrease in OD correlates with the hydrophobicity of the isolates and thus adhesion to hydrocarbons. In the present study, a marked difference in adhesion from 30-80% was observed between the tested cultures (Fig. 6.4). The cultures Hk1, Me1 and Pk12 showed better adhesion to hydrocarbons (> 70%) as compared to the reference strain, *B. coagulans* suggesting that these native cultures have increased level of adherence and colonization ability. The cultures Ik15 and Bk1 showed very low level of adhesion (≤ 30%). Similar to cell surface hydrophobicity, auto-aggregation property of the tested cultures also varied from 60-99% (Fig. 6.4). The culture Hk1 showed the highest auto aggregation (98.2%), while the culture Pk12 showed the lowest auto aggregation property (< 20%).
In vitro probiotic properties of *Bacillus* species

**Figure 6.4.** Adhesion and auto-aggregation property of the *Bacillus* cultures. Each value is the mean ± SEM of three independent experiments.

### 6.4.7. DPPH radical scavenging activity

The DPPH scavenging activity of the *Bacillus* isolates is shown in Figure 6.5. All the strains tested showed varying degrees of scavenging activity (50-70%). The highest scavenging activity was measured for the culture Hk1 (66.6%), while the least activity was observed for the cultures Ik11 and Bk1 (53%). The culture Me1 and the reference strain *B. coagulans* exhibited almost similar DPPH radical scavenging activity (63%).

**Figure 6.5.** DPPH radical scavenging activity of the culture filtrates of *Bacillus* species. Each value is the mean ± SEM of three independent experiments.
6.4.8. Co-cultivation of the cultures with pathogens

The ability of the probiotic to inhibit the ingested pathogenic microorganism in the gastrointestinal tract (GIT) is an important aspect. Survivability of the pathogen *L. monocytogenes* Scott A by growing the cultures together with pathogenic bacteria was determined by checking the CFU/ml, in the selective differential media. It was found that count of the indicator organism *L. monocytogenes* ScottA decreased with increase in time, showing that cultures are antagonistic to the tested pathogen. When co-cultured with *B. licheniformis* Me1, *B. subtilis* Ec1, *B. cereus* Ik11, *B. thuringiensis* Ik15 and *B. coagulans*, complete inhibition of the indicator organism was observed. However, in case of *B. megaterium* Pk12 and *B. flexus* Hk1, 75% and 65% of the indicator organism survived after 6 h of incubation, respectively. Only 51% of inhibition of growth of *L. monocytogenes* occurred due to the culture *B. amyloliquefaciens* Bk1.

6.4.9. Phytase activity and Exopolysaccharide production

Phytase activity of the strains was screened for their ability to degrade myo-inositol hexakisphosphate (IP6) by cobalt chloride qualitative staining method (plate assay). Except the cultures, Hk1, Ik15 and Pk12, all other *Bacillus* spp. were found to produce phytase, observed by the zone of clearance in the sodium phytate added plate. Only the cultures Me1 and *B. coagulans* were found to produce EPS, which can be used to impart functional effects to foods and further confer health benefits.

6.5. Discussion

In order to select an efficient probiotic candidate, a comparative account of *in vitro* probiotic properties of seven different *Bacillus* spp. isolated from different food sources (Chapter 2) was done. The cultures were tested for acid and bile tolerance, hydrophobicity, and as well as other health benefits imparting factors like bile salt hydrolase activity, antioxidant properties, and antimicrobial compounds and enzyme productions.

It is very important to characterize the organism for its gastrointestinal persistence before application as probiotics. During passage through the upper alimentary tract, the microorganisms are subjected to several stress factors, such as gastric and bile acids. In *in vitro* studies, hydrogen chloride (HCl) and bile salts are the two chemicals which are usually used to imitate the unfavourable conditions that exist in the GIT. Hence, to be selected as a potential probiotic, the bacterial culture must possess the properties of acid and bile tolerance, which allows them to survive...
and grow in the adverse conditions found in the gut and further confer health benefits (Gibson et al. 2000). Three hours at pH 3 was chosen to determine acid resistance, as this simulates residence time in the stomach (Olejnik et al. 2005). All the tested Bacillus strains showed tolerance to pH 3 for 3 h despite variations in the degree of viability. The existence of a similar heterogeneity in response to acid environments is also suggested elsewhere within the Bacillus species. Hyronimus et al. (2000) carried out acid tolerance test of B. coagulans, B. laevolacticus and B. racemilacticus and demonstrated that only B. laevolacticus showed significant survival rate at pH 2.5. Likewise acid tolerance, resistance to bile salts is also generally considered as an essential property for the probiotic strains to survive the conditions in the small intestine and is an important characteristic feature to be evaluated for a probiotic (Havenaar et al. 1992). Moreover, the presence of bile salts in the environment of bacteria cultures is much more detrimental than the effect of low pH. Ox-bile concentration of 0.3% is considered to be a crucial concentration to evaluate a bile tolerant probiotic (Gilliland et al. 1984) and also 0.3% of ox bile has similarity to human bile juice (Brashears et al. 2003; Chou and Weimer 1999). In this study, among seven isolates three cultures (viz. Me1, Ik11 and Ec1) were found tolerant to 0.3% Ox-bile, while rest of the cultures were grouped as sensitive or weakly tolerant, suggesting that the Bacillus spp. exhibited a wide variation in bile tolerance. These results are in agreement with those observed with other cultures, such as B. subtilis and B. toyoi (Cosson and Deschamps 1994) and B. coagulans strains (Hyronimus et al. 2000), and as well as among lactobacillus acidophilus and Enterococcus faecium strains (Gilliland et al. 1984; Taranto et al. 1997).

Several studies on the cholesterol lowering effect of probiotic cultures have been reported (De Smet 1994; 1998). The deconjugated bile salts produced due to BSH activity are less efficiently reabsorbed and hence these bile salts are excreted through faeces. Moreover, the free bile salts are less efficient in solubilisation and absorption of lipids in the gut. Hence, the BSH activity increases the utilization of cholesterol for Denovo synthesis of bile salts to maintain the loss through faeces and reduces the absorption of cholesterol by decreasing the solubility of cholesterol in the gut (Begley et al. 2006). The formation of white precipitate around the colonies shows that these strains were able to enzymatically deconjugate bile salts to primary bile salts. Thus, when ingested as probiotics, these strains may exert beneficial health effect by lowering the serum cholesterol level.
The sensitivity patterns for *B. flexus* Hk1, *B. thuriengensis* Ik15 and *B. coagulans* was of almost similar, indicating that they may harbour similar antibiotic resistance mechanism. The resistance of these cultures to β-lactum antibiotics might be primarily due the expression of β-lactamase and hydrolysis of antibiotics or else due to cellular permeability and mutational events resulting in the modification of penicillin binding proteins of the cultures (Alanis 2005). Further studies are required to elucidate this perception. However, the intrinsic resistance and susceptibility of these strains to a range of antibiotics is important, since *Bacillus* strains that show resistance to a specific antibiotic can be given at the time of antibiotic treatment (Hoa et al. 2000). Sorokulova et al. (2008) also reported antibiotic resistant probiotic *B. subtilis* 3 and *B. licheniformis* 31 strains which are compounds of probiotic Biosporine®. However, among the strains tested, three of the cultures were found to be sensitive to all of the antibiotics tested, thereby suggesting that they would not be responsible for transmissible drug resistance genes to other intestinal and/or food-borne pathogens, in the food matrix or, more importantly, in the gastrointestinal tract, if introduced as probiotics.

One of the important criteria to consider a culture as probiotic is its ability to adhere to the epithelial cells and intestinal mucosa. Bacterial surface properties have been associated with attachment to a variety of substrate, which in turn is associated with hydrophobicity (Gilbert et al. 1991). Bacterial adhesion can also determine the colonization ability of a microorganism which can prevent pathogenic access by steric interactions or specific blockage on cell receptors (Otero et al. 2004). The BATH test has been extensively used for measuring cell surface hydrophobicity in *Lactobacillus* (Dharmawan et al. 2006; Vinderola 2004) and *Bifidobacteria* (Holzapfel 1998; Zavaglia et al. 2002). Thus, BATH test was used for determining the hydrophobic surface characteristics of bacterial cells. A marked difference in adhesion was observed between our tested strains. This difference in the level of adhesion among the tested bacterial cultures can be attributed to several factors, such as the non-specific reaction by charge and hydrophobicity. Collado et al. (2007) stated that aggregation is useful for preliminary screening in order to identify the potent probiotic strains suitable for food, human or animal use. Thus, higher aggregation property of the cultures Hk1, Me1 and Pk12 represents its characteristic feature for interaction with pathogens, which is highly important from the application point of view in both food preservation and therapeutic impact of food on intestinal microbiota.
The application of probiotic bacteria with antioxidant activity in foods increases their biological activity and quality, as well as their shelf-life (Nedelcheva et al. 2010). DPPH has been used extensively as a free radical to evaluate reducing substances (Cotelle et al. 1996) and is a useful reagent for investigating the free radical scavenging activities of compounds (Duan et al. 2006). Scavenging of different types of reactive oxygen species is thought to be one of the main antioxidant mechanisms exhibited by lactic acid bacteria (Stecchini et al. 2001). The CFS of the tested strains exhibited antioxidant activity possibly due to the presence of water-soluble protein and peptides produced by the cultures. Such radical scavenging active fractions and cultures can possibly be used in animal feed formulations as a supplement to relieve oxidative stress.

The co-cultivation studies resulted in the decrease in the CFU of the indicator organisms (*L. monocytogenes* Scott A) with increase in time, indicating that isolates are antagonistic to the pathogen. Thus, these isolates can serve as strong and potent cultures against different pathogens indicating its potentiality as use in biopreservation.

### 6.6. Conclusion

In conclusion, most of *Bacillus* spp. showed desirable and promising *in vitro* probiotic properties. The survivability of the *Bacillus* isolates in the conditions of high bile salt concentration and low pH values indicates that the strains will reach the small intestine and colon, and thereby contributing to the balance of the intestinal microflora. All the tested cultures showed good bile salt hydrolase activity, hydrophobicity towards hydrocarbons, antioxidant properties, as well as exhibited antibacterial activity against food-borne pathogens. Particularly, the cultures *B. licheniformis* Me1 and *B. flexus* Hk1 exhibited remarkable *in vitro* probiotic properties and thus can be used as a probiotic feed supplement. However, *B. flexus* Hk1 exhibited less potential antibacterial activity as compared to *B. licheniformis* Me1. Moreover, the isolate *B. licheniformis* Me1 was found to be safe for food industry application. Hence, the culture, *B. licheniformis* Me1 is a more suitable candidate for probiotic application. However, before commercialization of this culture as probiotic, several other criteria need to be evaluated including *in vivo* efficacy studies in animal models and human.