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Identification and Characterization of *Bacillus* sp. for Probiotic Properties Isolated from Human Faeces

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Among the large number of probiotic products in use today are bacterial spore formers, mostly of the genus *Bacillus*. Used primarily in their spore form, these products have been shown to prevent gastrointestinal disorders and their applications are astonishing. Here we report the isolation and characterization of a probiotic *Bacillus* species isolated from human faeces as per WHO and FAO guidelines. The isolates were identified based on the characteristics on the strains of *Bacillus* sp. according to Bergey's Manual of determinative bacteriology and further confirmed by API 50 CHB test. The *in vitro* studies on the probiotic properties demonstrated that our isolate could prove to be a potential probiotic with spore forming ability coupled with acid and bile tolerance properties and antimicrobial action. It was susceptible to most of the antibiotics tested and showed antimicrobial activity against gut pathogens. In addition, to these characteristics the bacterium also produced enzymes such as amylase, lipase, protease and catalase which can help in improving digestion. This study suggests that bacilli with probiotic properties could be isolated from the human gut.

Key words: *Bacillus*, Probiotics, Spore formers, Screening, Isolation.

According to Food and Agriculture Organization (FAO) and World Health Organization (WHO) probiotics are defined as 'live micro-organisms, which when administered in adequate amounts confers health benefits on the host' (FAO/WHO, 2006). The documented health benefits of probiotics include improvement of the normal microflora, prevention of infectious diseases, prevention of food allergies, reduction of serum cholesterol, anticarcinogenic activity, stabilization of gut mucosal barrier, immune adjuvant properties, alleviation of intestinal bowel disease symptoms and improvement in the digestion of lactose intolerance hosts (Galdeano *et al*. 2007). Probiotics can serve as an alternative to antibiotics in farming and aquaculture and as prophylactics in humans. Moreover, probiotic therapy is very attractive, because it is an effective and non-invasive approach, which attempts to restore the natural flora. The different bacterial genera most commonly used in probiotic preparations are *Lactobacillus, Bifidobacterium, Escherichia, Enterococcus, Bacillus, Streptococcus and Pediococcus (*Adam *et al*., 2012). Moreover, some fungal strains belonging to *Saccharomyces* (*Saccharomyces boulardii*) are now used as a drug to prevent or treat recurrent *Clostridium difficile* infection (CDI), particularly in critically ill patients (Gupta and Garg, 2009; Mc Farland, 2009).

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Recent studies suggest that, members of the spore-forming genus *Bacillus* can inhabit the gastrointestinal tract (GIT) of insects and animals (Hong *et al*. 2008). Furthermore, a number of studies have also recovered *Bacillus* species in mammals, for example members of *Bacillus* were readily recovered in the faeces of broiler chickens (Barbosa *et al*. 2005), deer (Ohba and Lee, 2003) as well as from the mouse GIT (Salzman*et al*. 2002). A recent study has identified *Bacillus* spore-formers in human Faeces (Fakhry *et al*. 2008). It has been shown recently that spores of a laboratory strain of *Bacillus subtilis*, strain PY79 are able to germinate in the jejunum and ileum of mice dosed orally with spores. Surprisingly, germinated spores could outgrow and as they progress into the upper colon re-sporulate. This phenomenon was also observed with other natural isolates of *B. subtilis* that had been recovered from human faeces, suggesting that *B. subtilis* could use the GIT for both growth and sporulation. Only few *Bacillus species* are pathogens of animals (*B. cereus* and *B. anthracis*) or insects (*B. thuringiensis*) while the majority of them are nonpathogenic. Their presence in the GIT has been considered as due to the ingestion of bacteria associated with soil, water, air or foods (Cutting, 2011). There is an increasing interest in the development of the adjunct or alternative therapies based on the bacterial replacement, using probiotics isolated from the natural intestinal flora (Satyabhama *et al*. 2012; Collado *et al*. 2007).

For decades, *Bacillus* and their metabolites have found several biotechnological applications in production of enzymes, amino acids, antibiotics, preparation of fermented foods and as pest control agents (Nithya and Halami, 2013). Selected strains of *Bacillus* are also introduced into various food products as they possess common probiotic features, such as gut viability (Tam *et al*. *2006*), resistance to bile and acid (Hong *et al*. *2005*) and the ability to synthesize different compounds useful to humans. In addition, spore-forming *Bacillus* possess several advantages over other non-spore-formers such as *Lactobacillus* namely (1) Survival in foods requiring harsh processing conditions such as high temperature and pressure (2) withstand harsh gastrointestinal tract (GIT) conditions (3) possess a long shelf-life and remain viable throughout their shelf-life both at room temperature and refrigerated conditions and (4) low dosage requirements providing a cost effective option in formulating probiotic food products (Durkee, 2010).

The World Health Organization has developed guidelines for the evaluation of probiotics in food (FAO/WHO, 2006), consequently probiotic organisms used in food must be capable of surviving passage through the gut; i.e., they must have the ability to resist gastric juices and exposure to bile. Furthermore, they must be able to proliferate and colonize the digestive tract. In addition, they must be safe and effective, and maintain their effectiveness and potency for the duration of the shelf-life of the product. Although *in vitro* studies mimic *in situ* conditions of gut ecosystem (Dunne *et al*. 2001), these studies can be important for the screening of numerous samples and the selection of potential microbes for conducting further *in vivo* safety and clinical studies and eventually to develop product for human use.

From the above understanding of probiotic applications of *Bacillus*, we aimed to isolate and evaluate probiotic characteristics of *Bacillus* sp. from human faeces since it has been suggested that probiotic strains originate from the target animal flora (Barbosa *et al*. 2005).

MATERIALS AND METHODS

Sample collection, Isolation and Screening of spore formers

Samples of freshly voided faecal material were collected from 20 healthy volunteers at a Public Hospital in Durban, South Africa and stored frozen until processing. Ethical clearance for the study was obtained and all donors gave free consent for their samples to be used for the isolation and characterization of bacteria. No volunteer had taken any form of probiotics or antibiotics within the preceding 12 months. A total of 1 g samples were homogenized in PBS, heated for 1 h at 65°C, serially diluted and plated for single colonies. After aerobic incubation, selected colonies from each sample were re-streaked on Difco Sporulation Medium (DSM)agarplates to isolate single colonies and checked for formation of phase-bright spores and their catalase reaction; sixty isolates in total were short-listed for further analysis.

Microbiological and Biochemical Characterization

The isolates were characterized upto genus level on the basis of Gram stain, spore stain, motility, oxidase, catalase activities using Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). Morphology of the different isolates was studied by growing them in different temperatures, salinities, pH, aerobic and anaerobic condition. The ability of the isolates to elaborate various hydrolytic enzymes such as amylase, protease was determined by plate assay. The isolates characterized preliminary as *Bacillus* sp. were selected and further confirmed by API 50 CHB (bioMerieux) test results.

In vitro studies of the probiotic properties of the isolate

Acid tolerance test

The acid tolerance of *Bacillus subtilis* JK-04 was studied by growing the organism in different pH solutions by the method described by Hyronimus *et al*. (2000). Briefly, the solutions with varying pH values of 1.0, 2.0, 3.0 and 4.0 were prepared by adding 0.1 N HCl to double distilled water. Sterile double distilled water (pH 6.5) was used as the control. A cell suspension containing about 10^{10} cells ml^{-1} was mixed and incubated with each of the solutions of pH and the control (as mentioned above). One milliliter of sample from each solution was drawn at 0 h, 1,2, 3 and 4 h interval time and subsequently serial dilutions were prepared using sterile normal saline. Appropriate dilutions were pour-plated on nutrient agar and incubated at 37°C for 48 h.

Bile tolerance test

The bile tolerance test was performed based on a method published earlier by Sen *et al*. (2010). The bile salt solutions were prepared by dissolving 10 g of Oxgall (Sigma, USA) dry powder in 90 ml of distilled water. From this solution, final concentrations of 1 and 2% were prepared. Sterile double distilled water without Oxgall (pH 7.0) served as control. All solutions were autoclaved and stored at room temperature until further use. Ten milliliters of each solution was transferred into sterile test tubes. Cell suspensions containing about 10^{10} cells ml^{-1} were added to each solution, i.e., 1, 2, 3, 4, 5% and control, and incubated at 37°C aerobically. One milliliter of culture sample was withdrawn from each tube at 0, 3, 6, 9 and 12 h interval and dilutions were prepared in 9 ml of sterile normal saline. Dilutions were plated on nutrient agar and were incubated at 37°C for 48 h.

Antimicrobial activity by agar diffusion method

The antimicrobial activity of *Bacillus* isolate was assessed by agar diffusion method described by Sen *et al*. (2010). The Muller-Hinton (MH) agar plates were overlaid with 7 ml of soft nutrient agar inoculated with 20 µl of overnight active culture of indicator strains. Different wells were made in agar. Wells were filled with 50 µl cell-free broth of 24-h-old cultures obtained by centrifuging the culture broth at 5,000 rpm for 15 min. The diameter of the zones of inhibition extending laterally around the well was measured, and a clear zone of 1 mm ormore was considered positive inhibition

Antibiotic Susceptibility

The susceptibility of the *Bacillus* isolate to ampicillin,, tetracycline, chloramphenicol, gentamycin, kanamycin, ciprofloxacin, cloxacilin, erythromycin, vancomycin were determined in Oxoid Muller–Hinton (MH) agar plates as described by Patel *et al*. 2010.

Catalase and Hemolytic Tests

The catalase activity of the isolate was detected by re-suspending the culture in a 3% solution of hydrogen peroxide. Hemolysis was determined on brain heart infusion agar supplemented with 5% human blood after incubation at 37°C for 24 h.

Hydrophobicity Test

The degree of hydrophobicity of the isolate was determined by employing the method described by Patel *et al*., 2010, based on adhesion of cells to organic solvents. The culture was grown in 10 ml of Brain heart infusion broth, centrifuged at 6000 rpm for 5 min for cell separation. The pellet was washed, resuspended in 10 ml of Ringer's solution, and absorbance of this aqueous phase at 600 nm as Ao was measured. Cell suspension was then mixed with equal volume of solvent and mixed thoroughly by vortexing for 2 min wherein xylene (a polar solvent), chloroform (a monopolar and acidic solvent), and ethyl acetate (a monopolar and basic solvent) were employed. The two phases were allowed to separate for 30 min, and absorbance at 600 nm of non-aqueous was recorded as A1. The hydrophobicity of strain adhering to solvent was calculated as

% Hydrophobicity = $(1-A_1/Ao) \times 100$

Mucin Binding Assay

The isolate was grown at 37°C in nutrient broth supplemented with 0.1% mucin (Sigma) for 24 h to induce binding (Jonnson *et al*. 2001). Microtitre plate wells were coated with mucin (150 µl per well) 100 μgl^{-1} in 50 mM Na_2CO_3 buffer, pH 9.7 and incubated overnight at 4°C with slow rotation, then blocked with phosphate-buffered saline (PBS) containing 1% Tween 20 for 1 h and washed with PBST (PBS supplemented with 0.05% Tween 20, pH 7.3). The bacterial strains grown as described above, were washed once in PBST, and diluted to an absorbance 0.5± 0.02 at 595 nm in the same buffer. Bacterial suspension (100 µl) was added to each well and incubated for 1 h at 30°C. The wells were washed with PBST, and binding was examined with an inverted microscope TCM-400 (Nikon). The buffer was poured off to dry wells followed by absorbance at 450 nm using a Microplate Reader (Mindray MR-96A, Japan)

Autoaggregation Assay

It was performed according to Del Re *et al*. (2000) with modifications. Overnight grown *Bacillus* culture at 37°C in nutrient broth was pelleted and washed twice with PBS (pH 7.3) and resuspended in PBS to get absorbance 0.5 at 595 nm. Cell suspension (4 ml) was mixed by vortexing for 10 s followed by incubation at 37°C for 1 h. Thereafter, absorbance of upper layer was measured at OD 595 nm. Autoaggregation percentage was expressed as: 1-(At/Ao) ×100, where At represents the absorbance at time t=1 h and Ao the absorbance at t=0.

RESULTS AND DISCUSSION

Microbiological and Biochemical Characteristics

The culture was Gram-positive, rod shaped, motile with peritrichous flagella as observed under themicroscope. The microbiological and biochemical characteristics of the isolate are summarized in Table 1 which showed that our strain was catalase, oxidase and Voges-Proskauer reactions positive but methyl red and indole test negative. The strain was capable of using different sugars as sole carbon source. It was able to grow at temperatures as high as 50°C and in salt containing nutrient media. It could grow at pH values ranging from 5.7 to 7. The bacterium grew using cellulose, tributyrin and peptone, respectively indicating it produces cellulose, lipase and protease. Together with the spore-formers, other bacteria were also isolated (data not shown), but only partially characterized and not used in the present study. Those organisms were either members of thermophilic species or mesophilic but probably part of abundant population not totally killed by the heat-treatment. The identification of the isolate to species level was done according to Bergey's manual and confirmed by API tests and the profiles were interpreted by means of interpretation software. The results showed that

Table 1. Physiological and biochemical characteristics of isolate JK-04

Characteristics	Isolate JK-04
Gram Staining	+
Spore staining	+
Motility	+
Growth at different pH	
5.7	+
6.8	+
7	—
Growth on Nacl %	
2.5	+
5	+
7	+
10	+
Growth at 50°C	+
Casein hydrolysis	+
Starch hydrolysis	+
Reduction of Nitrate to nitrite	+
Denitrification	-
Hydrogen sulphide gas	+
Methyl Red	-
Voges-proskauer	+
Indole	-
Gas from glucose	-
Urease	-
Citrate Utilisation	+
Aerobic growth	+
Anaerobic growth	+
Gelatin liquefactions	+
Catalase	+
Oxidase	+
Lactose	-
Commercially important enzyme production	
Cellulase	+
Lipase	+
Protease	+
Amylase	+

isolate JK-04 was far more closely related to *Bacillus subtilis*, with an identification percentage of 97.6 and a T-value of 0.84, than to any other species. The comment made by the interpretation software was 'Good identification'. On the basis of the experimental results, the isolate has been designated as *B. subtilis* JK-04. In the present study, we found that our strain can produce amylase, cellulase, lipase and protease which are very important from industrial point of view during bulk biomass production of bacteria by fermenter (Das *et al*. 2010). Furthermore lipase can hydrolyze oil and hence can reduce oil absorption from oil-rich spicy food chimed in gastro intestinal tract (GIT), resulting in lowering lipid and cholesterol level in blood, as some other probiotics have been reported to show the similar benefit (Sanders 2000).

Table 2. Acid tolerance of *Bacillus subtilis* JK-04

pH	Time (h)				
	1	3	6	9	12
1	4.6	2.5	-	-	-
2	7.2	6.8	4	3	2.2
3	8.8	7.6	6.2	4.4	3.6
4	9.2	9.0	6.8	6.1	5.8

- No growth or less than 10^4cfu/ml

Table 3. Bile tolerance of *Bacillus subtilis* JK-04

Bile	Time (h)			
Concentration	3	6	9	12
1	8.9	7.8	5.6	4.6
2	7.2	6.9	4.8	3.2
3	1.4	-	-	-
4	1.2	-	-	
5	1.1	-	-	-

- No growth or less than 10^4cfu/ml

Table 4. Antimicrobial activity of cell free upernatant of *Bacillus subtilis* isolate JK-04 against test organisms

Test Organism	Zone diameter in mm
B. cereus	+++
B. pumilis	++
E. coli	+++
L. brevis	+
Micrococcus flavus	+
S. typhimurium	+++
S. aureus	+
Serratia marcescens	++
L. mesenteroides	+++

+: 1.0-2.0 mm; ++: 2.1-4.0 mm; +++: more than 4.0 mm

In Vitro Screening of Probiotic Properties of Bacillus subtilis JK-04

Acid tolerance and Bile tolerance

The survival *of Bacillus subtilis* JK-04 at pH 1, 2, 3 and 4 and its resistance to oxgall bile were observed from 0 to 12 h of growth. The results showed that the isolate could survive at very low pH levels (Table 2). There were 2.5x 10^4cfu / ml at pH 1.0 at 3 h, which clearly shows that the organism is tolerant to highly acidic conditions. *B. subtilis* JK-04 also exhibited resistance up to 4% bile concentration till 12 h. There were no colonies found at 5% bile concentration after 6 h, but considerable number of colonies was observed at 3 h at the same bile concentration (Table 3). This indicates that it can survive in human stomach at a very low pH and is tolerant to bile secretion. Acid and bile salt tolerance studies suggested that, for probiotic applications, spores would render the best result. Casula and Cutting (2002) have reported thriving germination of *Bacillus* spore in the mice gastrointestinal tract, which made it the aspirant to become probiotics. It was tested when a chimeric gene was successfully expressed in mice gut, which is possible when the fed *B. subtilis* spores do germinate and transform itself to vegetative cells in the gastrointestinal tract. The inherent resistance of spores to environmental stress is an attractive attribute for commercial probiotic preparations. Moreover, a functional food like Natto of Japan comprises the use of bacilli in it (Naidu *et al*. 2012).

Antimicrobial property

The microbial growth inhibitory activity of cell-free supernatant (pH adjusted to 7) of *B. subtilis* JK-04 isolate was assayed against standard gut pathogens (Table 4). The supernatant showed maximum inhibitory activity against *Bacillus cereus, E.coli, Salmonella typhimurium, Leuconostoc mesenteroides* and significant inhibitory activity against *B.pumilis*, *Serratia marcescens* and least inhibitory activity against *L. brevis, Micrococcus flavus* and *S. aureus.* The results indicated that the supernatant was active against gut pathogens. Antagonistic activities of

probiotic strains are essential to prevent the infection or invasion of pathogenic bacteria. These are the most important and desirable properties of an organism to qualify as a potential probiotic. Members of the genus *Bacillus* are known to produce a wide range of antimicrobial substances, including peptide and lipopeptide antibiotics (Abriouel *et al*. 2011; Ricca *et al*. 2004). These antimicrobial agents are active mostly against gram-positive bacteria, but some are active against gram-negative bacteria which are capable of colonizing the gut. This specific and limited spectrum of inhibition may be due to the synthesis of bacteriocin-like inhibitory substances (BLIS).

Antibiotic susceptibility

The culture JK-04 was susceptible to all tested antibiotics which supported the ideal probiotic characteristics (Table 5). The isolate was more susceptible (more than 10 mm zone of inhibition) totetracycline, chloramphenicol, gentamycin, kanamycin, and ciprofloxacin, whereas ampicillin, cloxacilin, erythromycin and vancomycin showed moderate inhibition (less than 6 mm inhibition zone). Pathogenicity and enterotoxin production are closely associated with occurrence of plasmid (Pannucci *et al*. 2002) and as the culture under study is susceptible to each tested antibiotic which ensures its inability to transfer antibiotic resistance.

Table 5. Antibiotic susceptibility of JK-04 culture against different antibiotics

Antibiotic (µg)	JK-04
Vancomycin (30)	S^{++}
Trimethoprim (5)	S^{++++}
Penicillin (10)	S^{+++}
Chloramphenicol (30)	S^{++++}
Gentamicin (10)	S^{+++}
Streptomycin (10)	S^{++}
Tetracycline (30)	S^{+++}
Novobiocin (5)	S^{++}

0-5 mm: Sensitive (S+); 6-15 mm: Sensitive (S++); 16-25 mm: Sensitive (S+++); 26-35 mm: Sensitive (S++++)

Table 6. Percent hydrophobicity of JK-04 against various solvents

Solvent	Percent Hydrophobicity
Xylene	58 ± 1.2
Ethyl acetate	82 ± 2.1
Chloroform	12.4 ±0.4

Catalase and Hemolytic Test

Isolate was found to be catalase-positive and β-hemolytic on human blood agar (brain heart infusion) plate after 24 h of incubation at 37°C. Catalase production may further add to the antioxidant and radical scavenging abilities of the bacterium during its transit through GI tract. The culture exhibited β-hemolytic activity on human blood, though γ-hemolytic strains are desirable (Patel *et al*. 2010). This property is not very significant as blood cells never come in contact with gastrointestinal tract.

Hydrophobicity, Binding and Autoaggregation assay

Hydrophobicity, autoaggregation and mucin binding were studied to understand bacterial surface characteristics and adhesion ability of the isolate. The isolate JK-04 showed notable percent hydrobicity in the Xylene, chloroform and ethyl acetate solvents (Table 6). Bacterial surface properties have been associated with attachment to a variety of substrates, which in turn is associated with hydrophobicity (Gilbert *et al*. 1991). The values obtained from microbial adhesion to solvents showed significant difference in accordance with measures of electron donor (basic) and acceptor (acidic) characteristics of bacteria. This reflects physiochemical properties of cell surfaces which showed presence of glyco-proteinaceous material contributing higher hydrophobicity, whereas hydrophilic surfaces are associated with the presence of polysaccharides (Bellon-Fontaine *et al*. 1996; Ahire *et al*. 2010).

The Autoaggregation was investigated on the basis of sedimentation characteristics of isolate and was found to be 40.2 ± 2.77%, where suspension showed both a precipitate and constant turbidity. Furthermore, JK-04 was able to bind mucin coated microtitre plate which was 0.034±0.0 at optical density 495 nm over mucincoated well which served as blank. The autoaggregation and cell surface hydrophobicity could be one of the determinants of adhesion of probiotics to cell monolayers. Such abilities of microorganism furnish resistance to peristaltic elimination by providing competitive advantage in ecosystem (Otero *et al*. 2004).

CONCLUSION

This study was the first phase of multi-phase study aimed to develop a novel, safe and efficient prophylactic agent against gastrointestinal disorders using probiotic *Bacillus* sp. In this current study, faecal samples of healthy volunteers were screened and a promising probiotic strain was selected for further studies. Our results evidently demonstrate the potential of bioprospection in the obtainment of new *Bacillus* strains for biotechnological applications. Furthermore, our study shows that *B. subtilis* is present in human GIT as diverse population of strains. These seem to be armed with attributes that could enable survival within the GIT and support the hypothesis that this species, and probably other *Bacillus* species, are gut commensals. This strain is being further assessed for molecular characterization and *in vivo* safety and use as a culture for the development of new products for human use.

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