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Comparative accounts of probiotic characteristics of *Bacillus* spp. isolated from food wastes

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ABSTRACT

Bacillus strains JHT3, DET6 and DET9 were selectively isolated from food wastes. These isolates exhibited various degrees of essential probiotic qualities and varied level of susceptibility patterns against tested antibiotics. Spores of DET9 elucidated best tolerance against simulated gastro-acidic conditions whereas DET6 showed best steadiness against simulated intestinal conditions. DET6 exhibited better antimicrobial activity than JHT3 and DET9 against unsafe organisms *viz Staphylococcus aureus, Micrococcus flavus, Proteus vulgaris, Salmonella typhi* and *Escherichia coli.* Susceptibility of these isolates to antibiotics decreases the illustration to offer resistance determinants to other organisms if administered in the form of probiotic preparations. JHT3, DET6 and DET9 showed high homology with *Bacillus megaterium,* (98%) *Bacillus subtilis* (99%) and *Bacillus thuringiensis*, respectively, using partial 16S r-RNA gene sequencing. Biochemical characterizations have supported the results of partial 16S r-RNA gene sequencing for JHT3 and DET6 but did not for DET9 and revealed its innovation. These isolates exhibited zero mortality of fishes in a 60 days trial, when fishes (Surfi tetra) were challenged up to 100 ppm cell concentration, with their daily diet.

1. Introduction

The growing health awareness in the consumption of microorganisms as probiotics has encouraged consumers worldwide. To attain health benefits from health friendly organisms, certain instructions were on demand hence a number of organizations to recommend guidelines for their use (FAO/WHO, 2002; Sanders, 2000). Probiotics are dietary supplements and live microorganisms containing potentially beneficial bacteria or yeasts. According to the recent definition by FAO/WHO, probiotics are 'Live microorganisms which when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2001; Sanders, 2003).

Strains of the genera *Lactobacillus* and *Bifidobacterium*, are the most extensively used probiotic bacteria. LAB has been used in the food industry for many years, because they are able to convert sugars (including lactose) and other carbohydrates into lactic acid. Less known genus than that of the *Lactobacillus* and *Bifidobacterium* are certain spore-forming *Bacillus* species which are being used as probiotics, *viz Bacillus clausii, Bacillus subtilis, Bacillus pumilus, Bacillus coagulans* (often mislabelled as '*Lactobacillus sporogenes*') and *Bacillus cereus* (Hong, Duc, & Cutting, 2005; Sanders, Morelli, & Tompkins, 2003).

Commonly probiotic bacterial cultures are aimed to help the body's naturally occurring gut flora. Often it is recommended by doctors and nutritionists to re-establish them when it gets disturbed after a course of antibiotics or as part of the treatment for gut related candidiasis. Claims are made that probiotics strengthen the immune system to combat allergies, excessive alcohol intake, stress, exposure to toxic substances, and other diseases (Nichols, 2007; Sanders, 2003).

The probiotic application of the *Bacillus* species raises safety issues because few species are known to be pathogenic viz *B. cereus, Bacillus thuringiensis, Bacillus pseudomycoides and Bacillus weihenstephanesis.* Majority of the cases *B. cereus* found to be associated in food poising wherein number of enterotoxin production has been verified (From, Pukall, Schumann, Hormazabal, & Granum, 2005; Granum, 2002; Granum & Lund, 1997). Therefore, the use of these bacteria in preparations for humans requires development of strict standards for safety control. Significant progress in legislation concerning this matter has been made in USA, Canada, and Europe (EFSA, 2005; FAO/WHO, 2002).

The aim of the present study was to explore the probiotic qualities of *Bacillus* isolates and their comparative assessment prior to possible probiotic application in animals and humans.

2. Materials and methods

2.1. Strain isolation and identification

2.1.1. Sample collection and isolation

Samples of dairy and mango pulp waste were collected from local dairy and food industries in Jalgaon. One gram of sample was

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dissolved in 1 ml of peptone water (pH 7.0) and spore-formers were isolated by ethanol treatment (Koransky, Allen, & Dowell, 1978) and heat treatment (Nicholson and Setlow, 1990). For heat treatment, diluted suspension (1:10, v/v) in peptone water was incubated at 65 °C for 45 min and for ethanol treatment, peptone water dairy waste suspension was diluted 1:1(v/v) with ethanol and incubated for 1 h at room temperature. Subsequent plating of 0.1 ml aliquots of appropriate dilutions (up to 10^{-5}) was done aseptically on nutrient agar plate and incubated at 37 °C for 24 h. Colonies with different morphologies were selected at random, stored on the same medium and screened further.

2.1.2. Media and growth conditions

All the media ingredients and chemicals used were of pure quality, obtained from Himedia Labs, Mumbai. Isolates were grown at 37 °C aerobically (agitation, 180 rpm/min) and at micro-aerophilic condition (without agitation) in nutrient broth as well as in Chemically Defined Low Iron Medium (CDLIM). Originally, CDLIM medium contained (g/L): K_2SO_4-2.0, K_2HPO_4-3.0, NaCl-1.0, NH_4Cl-5.0, $MgSO_4{\cdot}7H_2O$-0.08, $ZnSO_4{\cdot}7H_2O$-0.002, $CaCl_2{\cdot}2H_2O$-0.1, $CuSO_4$-5.0×10^{-6} $MnSO_4{\cdot}H_2O$-3.5×10^{-5}, thymine·HCl-2.0×10^{-4}, glycerol-25 ml and pH-7.0 ± 0.1 (Muller & Raymond, 1984). It was supplemented with 0.2% deferrated bile salt (to mimic the partial simulated gastrointestinal condition) and 0.5 g casein enzyme hydrolysate prior to attainment of better biomass and then siderophore production was observed after 36 h of growth.

2.1.3. Strain identification

Selected isolates were identified by complete 16S r-RNA sequence analysis followed by phylogenetic studies. Universal primers 16F27 N (5′-CCAGAGTTTGATCMTGGCTCAG-3′) and 16R1525XP (5′-TTCTGCAGTCTAGAAGGAGGTGTWTCCAGCC-3′) were used for the amplification of 16S r-RNA gene sequencing of JHT3 and DET6 (Brosies, Palmer, Kennedy, & Noller, 1978) whereas for DET9 16S27F (5′-CCAGAGTTTGATCMTGGCTCAG-3′) and 16S1488R (5′-ACATTTCACAACAC GAGCTG-3′) (Pidiyar, Kaznowski, Narayan, Patole, & Shouche, 2002) were used. Biochemical characterization was also done in order to examine the agreement of genetic analysis and phylogenetic studies.

2.2. Screening for probiotic properties

2.2.1. Bile salt and acid tolerance

The tolerance of vegetative cells and spores of these isolates to bile salts and partially simulated gastrointestinal conditions was assayed as per Duc, Hong, Barbosa, Henriques, & Cutting, 2004, with certain modifications. Initially, 1 ml of 48-h old culture, bearing spores was resuspended in an isotonic buffer (Bott and Wilson salts (%): K_2HPO_4-1.24, KH_2PO_4-0.76, Trisodium citrate-0.1, $[NH_4]_2SO_4$-0.6, pH 6.7) containing bile salts 1–4% (sodium cholate 50%, sodium deoxycholate 50%) or in 0.85% NaCl, pH 2, containing 1 mg/ml pepsin and incubated at 37 °C with agitation. Aliquots were taken immediately then at an interval of 1 and 3 h for bile tolerance and after 30 min and 1 h for acid tolerance. Appropriate dilutions were plated directly onto nutrient agar plates. These plates were incubated at 37 °C for 24 h to determine CFU while controls were set up in parallel where spores were resuspended in Bott and Wilson salts or 0.85% NaCl only.

2.2.2. Antimicrobial properties

The antimicrobial activities of isolates were assessed by a colony overlay assay, as per Pugsley, 1985, against various microorganisms. A 24 h grown culture in nutrient broth was spot inoculated on nutrient agar plates and incubated at 37 °C for 24 h prior to killing of the cells by exposure to chloroform vapours for 30 min. Plate covers were replaced, and the plates were aerated for 20 min by keeping it in sterile air before overlaying with 0.7% nutrient agar inoculated with an overnight grown culture of *Escherichia coli* NCIM 6145, *Micrococcus flavus* NCIM 2976, *Staphylococcus aureus* (clinical isolate), *Proteus vulgaris* and *Salmonella typhi*. Zones of inhibition around the isolates at 24 h incubation time at 37 °C were scored as positive.

2.2.3. Antibiotic susceptibility

The susceptibility of the isolates to amoxicillin, ampicillin, cephalothin, co-trimoxacin, cephalexin, lincomycin, cloxacillin, novobiocin, penicillin G, tetracycline, chloramphenicol, gentamicin, ciprofloxacin, and erythromycin were determined in Oxoid Muller-Hinton (MH) agar plates with octadisc (Himedia).

2.2.4. Catalase and haemolytic tests

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

2.2.5. Hydrophobicity test

The degree of hydrophobicity of the isolates was determined by employing the method described by Thapa, Pal, Pal, & Tamang, 2004, based on adhesion of cells to organic solvents. The cultures were grown in 10 ml of CDLIM broth, centrifuged at 6000g 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 A_o was measured. Cell suspension was then mixed with equal volume of solvent and mixed thoroughly by vortexing for 2 min. wherein xylene (an apolar 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 A_1. The hydrophobicity of strain adhering to solvent was calculated as:

$$\% \text{ Hydrophobicity} = (1 - A_1/A_0) \times 100$$

2.2.6. Mucin binding assay

The isolates were grown at 37 °C in nutrient broth supplemented with 0.1% mucin (Sigma) for 24 h to induce binding (Jonsson, Strom, & Roos, 2001). Microtiter plate wells were coated with mucin (150 μl per well) 100 μg l^{-1} in 50 mM Na_2CO_3 buffer, pH 9.7, incubated overnight at 4 °C with slow rotation then blocked with PBS with 1% Tween 20 for 1 h and washed with PBST (PBS supplemented with 0.05% Tween 20, pH 7.3) (Roos, Karner, Axelsson, & Jonsson, 2000). The bacterial strains were grown as described above, washed once in PBST and diluted to an A_{595} = 0.5 ± 0.02 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 (Labomed, USA). The buffer was poured off to dry wells followed by A_{450} determination by using Microplate Reader (Model 680, BioRad, Japan).

2.2.7. Autoaggregation assay

The assay was performed according to Del Re, Sgorbati, Miglioli, & Palenzona, 2000, with certain 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. Then A_{595} of upper layer was measured (Kos et al., 2003). Autoaggregation percentage was expressed as: $1 - (A_t/A_o) \times 100$, where A_t represents the absorbance at time t = 1 h and A_o the absorbance at t = 0.