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Evaluation of Probiotic Characteristics of Siderophoregenic *Bacillus* spp. Isolated from Dairy Waste

Anil K. Patel · Jayesh J. Ahire · Shrikant P. Pawar · Bhushan L. Chaudhari · Yogesh S. Shouche · Sudhir Bhaskarrao Chincholkar

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Abstract Siderophoregenic *Bacillus* strain DET9 has been selectively isolated from dairy waste. It was evaluated for probiotic characteristics and susceptibility pattern against antibiotics. Its spores showed excellent tolerance to simulated gastrointestinal tract conditions and exhibited antimicrobial activity against organisms such as *Escherichia coli*, *Micrococcus flavus*, and *Staphylococcus aureus.* Its susceptibility to antibiotics reduces the prospect to donate resistance determinants if administered in the form of probiotic preparations. It was observed to produce ~60 mg/l catecholate type of siderophore under iron stressed conditions, identified as a 2,3-dihydroxy benzoic acid by high-performance liquid chromatography, infrared spectroscopy, nuclear magnetic resonance, and mass spectral analysis. Partial 16S-rRNA gene sequencing analysis shows that the isolate exhibited homology with *Bacillus thuringiensis* and *Bacillus weihenstephanensis*, whereas biochemical characterization revealed its novelty. DET9 exhibited no mortality of fishes in a 60-day trial, when fishes (surfi tetra) were challenged up to 100 ppm cell concentration, with their daily diet.

Keywords Probiotic · *Bacillus* · Siderophore · Catecholate · 2,3-Dihydroxybenzoic acid

Introduction

Consumption of assured live microorganisms in some circumstances has proven an advantageous effect on humans and animals. These are the probiotics, or "friendly bacteria," known as inevitable part of functional foods that assert to encourage

A. K. Patel · J. J. Ahire · B. L. Chaudhari · S. B. Chincholkar (✉)
School of Life Sciences, North Maharashtra University, P. O. Box 80, Jalgaon, 425001, Maharashtra, India
e-mail: chincholkar_sb@hotmail.com

S. P. Pawar · Y. S. Shouche
Molecular Biology Unit, National Center for Cell Sciences, Pune 411 007, Maharashtra, India

specific health benefits to the consumers [1]. Probiotics were defined by a group of experts convened by the Food and Agriculture Organization of the United Nations (FAO) as "live microorganisms administered in adequate amounts which confer a beneficial health effect on the host" [2]. Various groups of microbes have been evaluated for a choice of probiotic action, including many species of the genera *Lactobacillus* and *Bifidobacterium*. These genera are the most copious and well established in probiotic-containing food products. But, species of *Enterococcus*, *Saccharomyces*, *Escherichia*, *Sporolactobacillus*, *Brevibacillus*, *Bacillus* have been recommended for probiotic effects. These possess exceptional properties like catalase activity, more immune enhancing ability, easier commercial preparations due to spore formation, etc., which adds on to their importance.

It is often asserted that a novel probiotic organism targeted toward its exploitation in the gastrointestinal tract conditions should be isolated from the gastrointestinal tract of the animal species to which it is intended to be administered. However, impact of probiotic action also may be observed at non-intestinal sites such as mouth, stomach, and vaginal tract. One authentic example of this is the employment of *Saccharomyces boulardii*, a microorganism that is not a general member of human gastrointestinal flora, for the prevention of recurrence of *Clostridium difficile* induced pseudo-membranous colitis. Some of the mechanisms may not require the attributes associated with native flora such as adherence to epithelial cells or colonization of the gastrointestinal tract. One anticipated mechanism for efficacy of this probiotic strain is a protease expressed by *S. boulardii* that cleaves the *C. difficile* toxin A receptor sites from the surface of intestinal epithelial cells [3]. Hence, it appears rational to use the microbes of nonintestinal origin for probiotics.

Bacillus species are considered better probiotics as it bear spores, which offer possible benefit of a live passage through the stomach to the intestine. However, *Bacillus* spp. have been comparatively less studied as a probiotic [4]. Patterson and Burkholder have anticipated that the competitive exclusion of pathogens by *Bacillus* probiotics will result from one or more modes of action, including immune exclusion, competition for adhesion sites, and production of antimicrobial agents, such as bacteriocins [5, 6]. D'Arienzo et al. established a new model of reduction in *C. rodentium* infection, where *Bacillus subtilis* spore predosing was found effective in reducing *C. rodentium* infection in mice and cognate enteropathy, leaving unaltered immune response to the pathogen [7]. Probiotics may even activate macrophages directly [8]. Furthermore, bacilli are known to produce siderophore [9, 10], which may offer additional benefits to the host.

Lankford coined the term siderophore in 1973 from the Greek word meaning "iron bearer" [11]. Siderophores are low molecular weight (<1,000 Da) small peptide molecules produced under iron-limited conditions [12]. Some known siderophores are, e.g., desferrioxamines, pertobactin, rhizobactin, ferribactin, acinetobactin, desferrithiocin, coelibactin, and coelichelin [13]. The threshold value at which the ambient iron concentration represses siderophore production is 5 μM for fungi, and for bacteria, it varies between 1 and 25 μM [13]. Siderophores are useful in health care as a drug, in agriculture for plant disease management and also plant growth improvement [13]. *Bacillus* group is known to produce 2,3 di-hydroxy benzoic acid [9, 10], and in these studies, it has been observed that certain strains exhibit good probiotic qualities.

The aim of the present study was to characterize the probiotic qualities of *Bacillus* isolate and study its siderophores prior to possible siderophoregenic probiotic application for iron nutrition in animals and humans.

Materials and Methods

Strain Isolation and Identification

Sample Collection and Isolation

Samples of dairy waste were collected from a local industry in Jalgaon. Care was taken to avoid gross contamination with environmental material. One gram of sample was dissolved in 1 ml of peptone water (pH 7.0) and spore-formers were isolated by ethanol treatment [14]. 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.

Media and Growth Conditions

All the media ingredients and chemicals used were of pure quality, obtained from Himedia Labs, Mumbai. Isolate was grown at 37°C aerobically (agitation, 180 rpm/min) and at microaerophilic 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 [15]. 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.

Strain Identification

A selected *Bacillus* was identified by complete 16S r-RNA sequence analysis followed by phylogenetic studies. Universal primers 16S27F (5′-CCAGAGTTTGATCMTGGCTCAG-3′) and 16S1488R (5′-ACATTTCACAACAC GAGCTG-3′) [16] were used for the amplification of 16S r-RNA gene of the isolate. Biochemical characterization was also done in order to examine the agreement of genetic analysis and phylogenetic studies.

Screening for Probiotic Properties

Bile Salt and Acid Tolerance

The tolerance of *Bacillus* vegetative cells and spores to bile salts and partially simulated gastrointestinal conditions was assayed as described by Duc et al. [17] with modifications. Fundamentally, 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.

Antimicrobial Properties

The antimicrobial activity of *Bacillus* isolate was assessed by a colony overlay assay, described by Pugsley et al. [18] to screen 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 vapors 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, and *Staphylococcus aureus* (clinical isolate). Zones of inhibition around the isolate at 24 h incubation time at 37°C were scored as positive.

Antibiotic Susceptibility

The susceptibility of the *Bacillus* isolate 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).

Catalase and Hemolytic Tests

The catalase activity of the isolate was detected by resuspending 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 Thapa et al. [19], based on adhesion of cells to organic solvents. The culture was grown in 10 ml of CDLIM broth, centrifuged at 6,000×*g* 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:

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

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 [20]. Microtiter plate wells were coated with mucin (150 µl per well) 100 $\mu g l^{-1}$ in 50 mM Na_2CO_3 buffer, pH 9.7, incubated overnight at 4°C with slow rotation, then blocked with phosphate-buffered saline (PBS) with 1% Tween 20 for 1 h and

washed with PBST (PBS supplemented with 0.05% Tween 20, pH 7.3) [21]. 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).

Autoaggregation Assay

It was performed according to Del Re et al. [22] 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 [23]. 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.

Probiotic Trial on Pisces

The aim of this experiment was to check mortality of the fishes due to harmful effect from Isolate DET9. Surfi tetra fishes were challenged against DET9 strains for 60 days wherein the fish meal was fortified with culture at 100 ppm cell concentration before feeding, and only fish meal without culture fed fishes were used as control. The trial fishes were dissected after 60 days to remove gut, sliced up sample was mixed by vortexing for 10 s in sterile PBS. Then, it was plated on nutrient agar plate to observe growth after 24 h of incubation at 37°C.

Siderophore Production and Characterization

Screening of Siderophore Production and Partial Characterization

CAS Assay and Siderophore Type Determination Inoculum (2% *v*/*v*) was added to CDLIM and incubated at 37°C on the rotary shaker at 180 rpm for 48 h. The culture biomass was harvested by centrifugation at 8,000×*g* at 4°C for 10 min, and the supernatant was assessed for siderophore production. Qualitatively siderophore was detected using universal CAS assay [24]. The type of siderophore was determined by using Arnow's test for catecholate type [25] and Csaky test for hydroxamate type of siderophores [26].

Growth Pattern and Siderophore Production Growth response of isolate was studied using 500 ml Erlenmeyer flask, carrying 100 ml CDLIM broth, with 2% of 24 h old culture inoculums (initial cell density A_{600} 0.1), incubated for 48 h at 37°C and 180 rpm. Cell density was recorded at an interval of an hour up to 48 h. The relative siderophore production was also checked as a function of time as per Payne [27] wherein 0.5-ml culture supernatant was mixed with 0.5 ml of CAS solution. A reference was prepared using uninoculated CDLIM medium. Both the test and reference were read at 630 nm, and percent siderophore units in the aliquots were calculated.

Extraction and Purification of Siderophore

Solvent Extraction Culture supernatant was acidified to pH 4.0 using 12 M HCl and extracted with 0.4 volume of ethyl acetate. Ethyl acetate fractions were pooled together and

concentrated by using rotary vacuum evaporator (Buchi, Switzerland). Dried fractions were then resuspended in deionized water (1 mg ml^{-1}) and subjected to further purification.

Siderophore Purification by Column Chromatography Solid phase matrix Sephadex LH-20 column was prepared by suspending 50 g material in methanol with gentle shaking for 20 min, and subsequently, material was packed into a 50×1.5 cm column and equilibrated with four bed volumes of methanol. The concentrated sample was then loaded on column and eluted with methanol with flow rate of 1 ml/min. Fractions of 5 ml each were collected and tested for their siderophore content using CAS assay and fractions positive for siderophore were pooled together and evaporated to concentrate using a rotary vacuum evaporator and finally freeze dried to dryness using lyophilizer (VirTis, USA). The dried sample was then stored at –20°C.

Characterization of Siderophore

Thin Layer Chromatography Purified siderophore sample was run on TLC along with 2,3-dihydroxybenzoic acid (DHBA) wherein butanol/acetic acid/water (12:3:5) was a solvent system.

IR Analysis Purified siderophore sample was lyophilized and subjected to Fourier transform-infrared spectroscopy (FT-IR) spectrophotometer one B (Perkin Elmer Spectrum), calibrated with polystyrene for determination of functional groups and sample was thoroughly mixed with nuzol mulling agent. Spectra were recorded in 4,000–450 cm range.

Proton NMR Analysis P-NMR was taken using deuterated methanol (MeOD) solvent as the compound was not soluble in $CDCl_3$, and D_2O. The sample was dissolved in 0.75 ml MeOD and injected through a 5-mm OD NMR tube to record nuclear magnetic resonance (H^1) (NMR) at 300-MHz NMR spectrophotometer (Bruker Model DRX-300). P-NMR spectrum was collected with 65,076 data points and 12 ppm sweep width with P-NMR.

HPLC Analysis It was carried out by using Younglin HPLC, ACME-9000 (South Korea) with isocratic pump. Eurospher C_{18} reverse phase column (250×4 mm, 10 μM integrated pre-column) was employed as stationary phase equipped with detector (190–600 nm, interrogator, (Chemito data processor) and programmed for 20 min with acetonitrile/water (9:1) as mobile phase at a flow rate of 1 ml/min.

Mass Spectra The system used for siderophore analysis was GCMS 5050A for which mass range 40–750 was applied, and detector voltage was fixed at 1.8 Kv. The sample was injected, and the ion of interest was selected then collided with helium gas to obtain fragments that were analyzed based on *m/z*.

Results

Strain Isolation and Identification

Isolation of Spore Formers

Ethanol treatment was an effective method for the selection of spore formers that also eliminated other non-spore formers from sample based on the level of spore ethanol

resistance between species [14]. Solitary isolate DET9 was employed herein throughout these studies. Morphologically, isolate DET9 appeared off white, circular, rough surfaced, big colony on nutrient agar. Microscopically, it appeared rod-shaped Gram-positive *Bacillus*.

Strain Identification

Genetic Analysis Genetic analysis was performed to identify the selected isolate using 16Sr-RNA gene analysis. Isolate exhibited close association with known *Bacillus* spp. 16S r-RNA gene sequence was submitted to NCBI gene bank which are given below.

16S r-RNA gene sequence of DET9 (Gene Bank accession number EU851974)

GGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTT
ATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACT
CCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGG
CTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACC
AAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACNGCCC
AGACTCCTACGGGAGGCAGCANTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC
GCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAG
TTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGC
CGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGT
TTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTT
GAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAA
CACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGC
AAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTT
CCGCCCTTTANTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTG
AAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAA
CGCGAANAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCTCCTNCGG
GAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC
CGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTTAGTTGGGCACTCTAAGGTGACTGCCG
GTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACA
CACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGA

Phylogenetic Tree Phylogenetic tree showed close similarity of isolate DET9 with *Bacillus thuringiensis and Bacillus weihenstephanensis* (Fig. 1).

Biochemical Analysis Results of the biochemical analysis were not in agreement with 16S r-RNA gene analysis of isolate DET9. It was able to utilize mannitol [28] and lactose [29] which is not a property of either *B. thuringiensis* or *B. weihenstephanensis*. Apart from this, it was found negative to synthesis of protein toxin crystal which is a fundamental property of *B. thuringiensis.* Protein toxin crystal production assay was adopted from Rhodehamel and Harmon [30]. Furthermore, it was Gram-positive, endospore-forming, and catalase-positive; hence, it prompted to be a different *Bacillus* spp than that mentioned above (results not shown).

Probiotic Properties

Acid and Bile Salt Tolerance

Spores of isolate DET9 were found to be tolerant to both partially simulated gastric conditions and bile salts. The calculated $\log_{10}$ CFU of DET9 was 7.74±0.11 and 8.20±0.45 for acid and bile salt tolerance, respectively, and it appeared equal to control after three exposures (mentioned in methods) as depicted in Fig. 2a, b, but not vegetative cells.

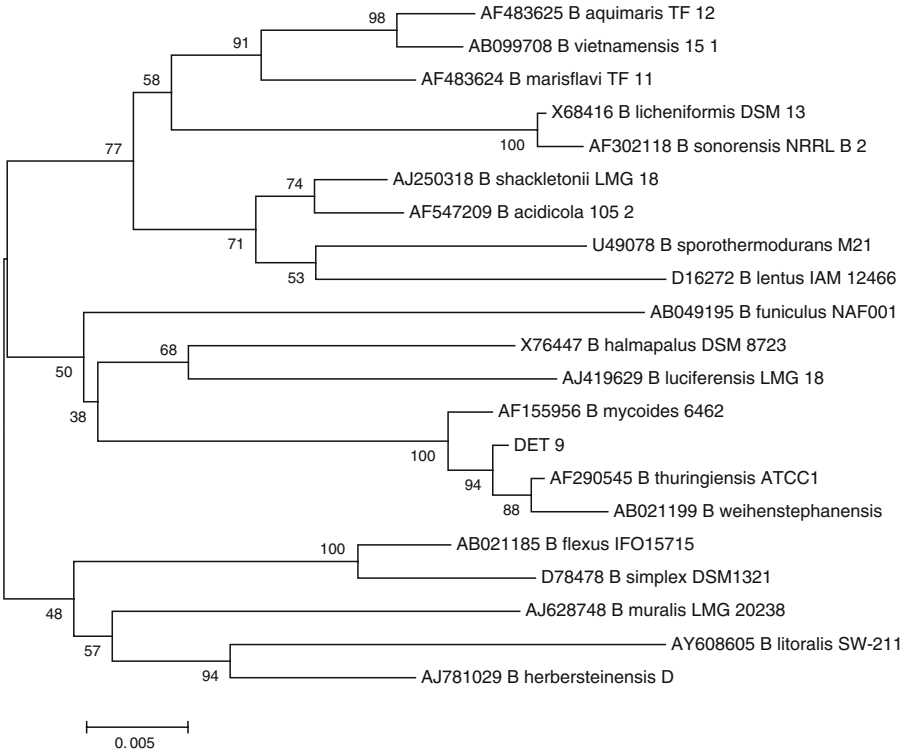

Fig. 1 Phylogenetic tree showing species relatedness of isolate

Vegetative cell viability in both cases was only 5 min on exposure to the experimental conditions.

Antimicrobial Activity

DET9 showed 0.5 mm inhibition zone against *E. coli* NCIM 6145 and 1 mm inhibition zone against *M. flavus* NCIM 2976 and *S. aureus* (clinical isolate), respectively.

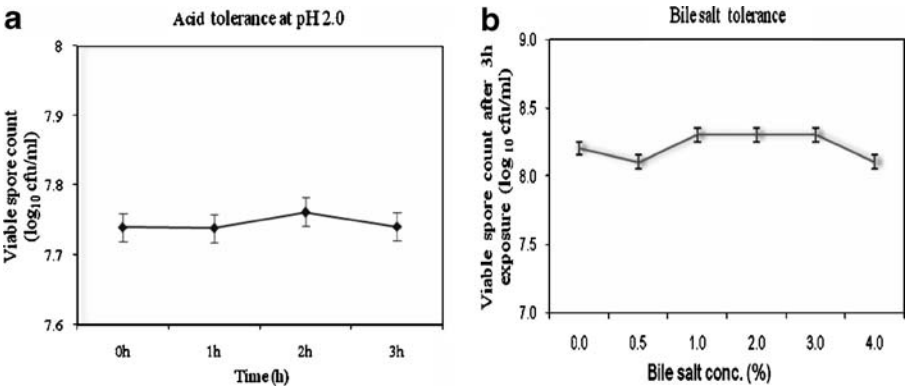

Fig. 2 **a** Acid and **b** bile salt tolerance of DET9

Antibiotic Susceptibility

DET9 culture was susceptible to all tested antibiotics in various degrees. These isolates were highly susceptible (more than 10 mm of zone of inhibition) to ciprofloxacin, tetracycline, erythromycin, gentamicin chloramphenicol, and lincomycin. Cephalexin, cephalothin, and novobiocin also inhibited it effectively (more than 6–9 mm of zone of inhibition), whereas penicillin G, ampicillin, amoxicillin, co-trimoxacin, and cloxacillin also showed moderate inhibition (less than 6 mm inhibition zone) of this isolate.

Catalase and Hemolytic Test

Isolate found to be catalase-positive and β-hemolytic on human blood agar (brain heart infusion) plate after 24 h of incubation at 37°C.

Hydrophobicity Test

DET9 showed remarkable percent hydrophobicity in the xylene, chloroform, and ethyl acetate (Table 1).

Adhesion Assay

The adhesion of isolate DET9 was determined 0.031±0.01 in micro titre plate at 450 nm.

Aggregation Assay

The autoaggregation was investigated on the basis of sedimentation characteristics of isolate and was found to be 32.6±2.77%, where suspension showed both a precipitate and constant turbidity.

DET9 Trial on Pisces

There was no mortality observed when Surfi tetra fishes were challenged with 100 ppm DET9 cell concentration during a thriving trial for 60 days. DET9 colonies were observed largely on nutrient agar plate streaked with fish gut sample. Test fishes also observed for improved weight by 5% (±0.52) than that of the control during 60 days trial.

Siderophore Production and Characterization

Screening of Siderophore Production and Partial Characterization

CAS Assay and Siderophore Type Determination The isolates were found positive for siderophore production on aerobic as well as on static condition (microaerophilic condition)

Table 1 Percent hydrophobicity of DET9 against various solvents.

Solvent	Percent hydrophobicity
Xylene	62±1.23
Chloroform	86±2.10
Ethyl acetate	12.5±0.45

in CDLIM supplemented with 0.2% bile salts. Qualitatively, it was detected by Universal Chemical assay for siderophores [24]. It turned red orange when the supernatant and CAS reagent were added equally which confirmed the biosynthesis of siderophore. Further positive Arnow's assay [25] precisely confirmed the presence of catecholate group in the siderophore having as its λ_{max} at 276 nm.

Growth Pattern and Siderophore Production The growth pattern of isolate showed long lag phase up to 5 h, and log phase was up to 13 h, followed by stationary phase when studied on shake flask method where siderophore production was also studied. The comparative account as percent siderophore units were calculated as per Payne [27] wherein maximum siderophore secretion was recorded at about 36 h (Fig. 3).

Extraction and Purification

Out of 30 fractions of 5 ml each obtained on column chromatography of siderophores, early fractions [12–16] had a yellow tinge, while later [24–27] had an orange tinge. The yellow-colored fractions were siderophore negative, while fractions [24–27] positive for siderophore were pooled together for further analysis.

Characterization of Siderophore

Preliminary characterization of siderophore confirmed the presence of catecholate group in the molecule. Hence, the expected siderophore in the fractions was either 2,3-DHBA, or its derivative. Thin layer chromatography of siderophore matched with 2,3-DHBA sample, had R_f 0.90 in butanol/acetic acid/water (12:3:5) as solvent system.

Infrared spectroscopy showed the presence of aromatic hydroxyl group, >C=O, >C=C–, >C–O stretching of COOH group, aromatic–O (Ar–O) stretching of phenolic OH group and *m*-disubstituted position of the groups in the molecule as per FT-IR details in Table 2. The NMR showed the presence of benzene ring, followed by the positive peaks of aromatic OH, >C=C–, and COOH noticeably. The NMR chromatogram details are explained in Table 3 (spectra not shown). This also explained it to be 2,3-dihydroxy benzoic acid molecules. The mass spectrum analysis of the test siderophore sample gave 154.05 molecular weight, which represented the molecular weight of molecule 2,3-DHBA (spectra not shown). HPLC analysis of the sample showed peak at 2.15 min of retention time which was the exact

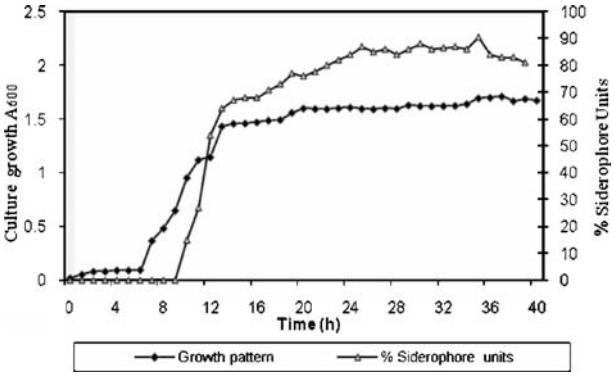

Fig. 3 Growth pattern and siderophore production by DET9

Table 2 Functional group determined in siderophore sample based on FT-IR.

Group	Group cm^{-1} of DET9 siderophore sample
Ar–OH	3,370.65
>C=O	1,643.02
>C=C– of benzene nucleus	1,405.25, 1,458.43
>C–O– stretching of COOH atom	1,261.45
Ar–O– stretching of phenolic OH atom	1,042.83
m substitute (partial)	852.42, 923.63

retention time of the standard 2,3-dihydroxy benzoic acid (spectra not shown). These collectively explained the presence of 2,3-dihydroxy-benzoic acid in siderophore sample.

Discussion

Lactobacillus and *Bifidobacterium* are safer and well-recognized genera of probiotic, available in commercially probiotic products. Use of spore formers as probiotics has not been established well because of their non-indigenous origin, and it is scarcely studied in the various vital aspects where other important genus has failed to perform, like siderophore synthesis. Lactic acid bacteria [31] and Bifidobacteria [32] have been reported as non-siderophoregenic; therefore, *Bacillus* claims its importance and poses demand. Although reports related to positive health effects from spore-forming "probiotics" are few, and FDA has not granted generally regarded as safe (GRAS) status for any spore formers to be used as probiotic, it might be because a few bacilli are associated with food poisoning, but its importance in the context of much required fundamental aspects like iron nutrition firmly claims to have their benign role. Bacilli proved improved health and no visible side effects after conducting a trial on fishes. The inherent resistance of spores to environmental stress is an attractive attribute for commercial probiotic preparations. Moreover, there are some of the functional food like natto of Japan which comprises the use of bacilli in it [33], and varieties of probiotic products that contain spore formers are available commercially [17]. Some of the commercial preparations do contain *Lactobacillus planterum* 299 v that claims increased iron absorption in the host gut although the mechanism remains unexplained, as the earlier reports quoted that *Lactobacillus* do not produce siderophores when grown under iron-deficient or iron-rich media [31].

One aspect must be considered for *Bacillus* probiotic that non-intestinal sites like mouth, stomach, vaginal tract, etc., where probiotic attribute do not employ, are connected to native flora and adherence to intestinal epithelial cell. *Bacillus* probiotics must be relevant, since these shows higher protease activity [34] and antimicrobial property against various Gram-positive and Gram-negative pathogens [6].

Table 3 ^{1}H NMR chemical shift assignments of siderophore sample of DET9 dissolved in MeOD.

Atom	^{1}H, ppm of DET9 siderophore sample
Ar–OH	8.0
Ar–H of benzene nucleus	6.8, 6.9, 7.5
COOH	11.3

Spores of isolate under study showed excellent resistance to bile and partially simulated gastric conditions, but the vegetative cells have not shown much challenging marks. Acid and bile salt tolerance studies suggested that, for probiotic applications, spores would render the best result. Casula and Cutting [35] 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. Moreover *B. subtilis* and *Bacillus cereus* have also been reported to grow anaerobically by Nakano and Zuber [36], which favors *Bacillus* strains to be used as probiotics.

Although the phylogenetic tree of DET9 showed its close similarity with *B. thuringiensis and B. weihenstephanensis* on the basis of partial 16Sr-RNA analysis, whereas biochemical studies did not support it as being close to *B. thuringiensis and B. weihenstephanensis*, as it was observed not to produce protein toxin crystals like *B. thuringiensis*. This entire scenario explains this culture to be a new strain. Susceptibility to all tested antibiotics and no mortality of fishes during trial by this *Bacillus* supported novelty and safe use of this isolate for aquaculture and perhaps animal application.

There are a number of *B. cereus*-containing probiotic products being sold for humans in developing countries [37]. A commercial product contains a strain of *B. cereus* var *toyoi* that has been deemed safe for animal use because of its failure to produce enterotoxins and its failure to transfer antibiotic resistance [38, 39], which is recommended for calves, poultries, swine, rabbits and for aquaculture.

Hydrophobicity, autoaggregation, and mucin adhesion are important attributes which help in the attachment of various substrata that explain the probiotic nature of the microorganism and bacterial adhesion to xylene reflects cell surface hydrophobicity. The values of microbial adhesion to solvent (MATS) obtained with the two other solvents, chloroform and ethyl acetate, were regarded as a measure of electron donor (basic) and electron acceptor (acidic) characteristics of bacteria, respectively [40]. Bacterial adhesion can also determine the colonization capability of a microorganism in the gastrointestinal tract preventing their immediate elimination by peristalsis and providing a competitive advantage in this ecosystem [41, 42, 43]. Through adhesion ability and colonization on tissues, probiotic microorganisms can prevent pathogen access by steric interactions or specific blockage on cell receptors [44]. Rijnaarts et al. observed that as the hydrophobicity of the cell increases, the level of adhesion also increases [45].

Isolate DET9 did not show any negative side effects and mortality of fishes when it was challenged up to 100 ppm cell concentration during 60 days trial which was colonized in fish gut successfully and exhibited improved health than that of the control. No mortality and weight gain in this trial certainly supported the notion that enterotoxin might not have been produced in the gut, and trial also ensured this strain to be used as a probiotic for aquaculture and animal farming for better health and iron nutrition.

Bacilli are efficient producers of catalase which certainly can reduce the harmful effects of active oxygen molecules or free radicals viz. H_2O_2, O_2^{2+}, O_2^{2-}, OH^-, etc. which are generated during metabolic process which are injurious to the host health; this potent probiotic strain if inhabited in the gut could act as a good antioxidant. DET9 showed β-hemolytic activity on human blood, though γ-hemolytic strains are desirable. This property is not very significant as blood cells never come in contact with gastrointestinal tract.

The culture DET9 was susceptible to all tested antibiotics which supported the ideal probiotic characteristics. Pathogenicity and enterotoxin production are closely associated with occurrence of plasmid [46] and as DET9 is susceptible to each tested antibiotic which

ensures its inability to transfer antibiotic resistance as well. In various reports, several bacilli have established its antimicrobial properties against various Gram-positive and Gram-negative pathogenic bacteria [47]. The production of antimicrobials is considered to be inhibiting mechanism exhibited by probiotic organisms, and such compounds have been shown to be produced by some spore formers. Dozens of different peptide antibiotics have been observed of exhibiting antagonism against a broad spectrum of microbes. Bacilli are involved in the production of bacteriocins, polymyxin, amicoumacin A [6], polyfermenticin SCD from *Bacillus polyfermenticus* [48], and coagulin from *Bacillus coagulans* [49] etc.

The isolate was found to produce siderophore at static conditions under partially simulated gastrointestinal tract conditions. FT-IR, HPLC, and NMR herein noticeably explained it of being 2,3-DHBA in the siderophore of this *Bacillus* isolate having 154.04 molecular weight as per Mass spectra. 2,3-Dihydroxy benzoic acid (2,3-DHBA) and its derivatives have been scarcely reported to be produced by bacilli [9, 10].

The concentration of available form of iron is less than 10^{-18} M; moreover, it is highly needed for metabolic processes. Thus, iron nutrition is a challenge for microorganisms, and siderophore production is the most suitable aid for assimilation of iron under aerobic conditions. [50] Almost all aerobic organisms are known to produce siderophores, and a few have the ability to employ siderophores of other organisms [51]. Literature indicates that plants combat iron deficiency through siderophoregenic plant growth promoters [52]; it is speculated that siderophores can act as biocontrol molecules only under soluble iron scarce environment and when phytopathogen(s) fail to utilize those ferreted siderophores. Since bacilli are good producers of siderophore in simulated gastrointestinal condition, it can play the same role as it exists in rhizospheric region of the plant to combat iron and antagonize to the pathogens in the colon by making them iron unavailable for their survival and produce some other antagonistic metabolites consequently leading to the pathogen's death and offer betterment of the host. Furthermore, it may serve to iron nutrition for the host.

Conclusion

The context of iron nutrition among probiotic organisms has been explored scarcely, wherein there were a few *Lactobacillus* spp. have been reported with phytic acid degradation ability where chelated iron becomes available biologically. *Bacillus* is an important genus among probiotics which are siderophoregenic of which no toxicity data were published with respect to its use as human probiotic. In such connection, these studies focus widely on siderophore and probiotic qualities of *Bacillus* isolate DET9. This isolate was able to produce ~60 mg/l of 2,3 dihydroxybenzioc acid in microaerophilic conditions which certainly can help in iron nutrition if administered to animals or human. Siderophoregenic probiotic DET9 may scavenge total iron and make unavailable for survival of other pathogenic strains in the colon and help antagonize. The in vitro studies supported siderophore production at simulated gastrointestinal condition, a short 60-day trial on fisheries showed no mortality of fishes, it could be claimed herein on the basis of present investigations that isolate *Bacillus* spp DET9 could be a good possible probiotic candidate for iron nutrition in animals after more studies.

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