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Bacillus spp. of Human Origin: A Potential Siderophoregenic Probiotic Bacteria

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Abstract *Bacillus* spp. ST13, isolated from human stool, was evaluated for siderophoregenic and probiotic qualities prior to its possible application for iron nutrition in humans and animals. It was tested for siderophore production in iron-limiting conditions and found to produce catecholate type of siderophore on the basis of high-performance liquid chromatography (HPLC), FT-IR, NMR, and mass spectra analysis. The isolate was screened for probiotic properties as per WHO and FAO guidelines. The strain ST13 can survive stomach acidity, bile salt and partially simulated gastrointestinal tract conditions. It was susceptible to most of the antibiotic tested and showed antimicrobial activity against enteric pathogens like *Salmonella typhimurium*, *Streptococcus pyogenes*, and *Staphylococcus aureus*. Strain ST13 showed close similarity with *Bacillus subtilis* using 16S r-RNA gene sequence analysis and biochemical characterization. The methanolic extract of ST13 siderophore was evaluated for DPPH radical scavenging activity, which showed 94.55±0.9% of radical scavenging effect.

Keywords *Bacillus* spp. · Probiotic · Siderophore · Catecholate · DPPH radical scavenging

Introduction

Probiotics are live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance [1]. While the concept of utilizing probiotics for human health has been around for over 100 years [2]. The food supplements containing potentially beneficial probiotic bacteria such as *Lactobacilli* and *Bacillus* spp. have been promoted for the treatment or prevention of a number of diseases [3] in humans and animals. The mechanism(s) by which they exert their protective effects in the gastrointestinal tract is not well understood, but is likely to involve pathogen control or exclusion as well as protection of host tissues against inflammatory responses [4, 5].

The strains of genus *Bacillus* are aerobic, spore forming, and catalase positive bacteria, commonly associated with soil and faeces of animals [6]. The spores of some species of

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Bacillus genus extensively exploited as probiotics and alternative to antibiotic growth promotants in the live stock industries [7, 8]. On the other hand, some species (*Bacillus anthracis* and *Bacillus cereus*) are pathogenic to humans and/or animals [9]. *Bacillus* sp. posses both positive as well as negative impact on human and animal health.

The potential probiotic attributes exhibited by *Bacillus* included relief of abdominal pain and bloating in patients of irritable bowel syndrome, boosting immune response to viral challenge, reduction in pathogen invasion, and anti-carcinogenic effect [4, 5, 10]. The spores of *Bacillus* spp. can survive extreme physiological conditions such as stomach acidity, bile salt and partially simulated gastro intestinal tract conditions as compare to sensitive strains of *Lactobacillus* and *Bifidobacterium*. In addition, they are stable in different manufacturing methods as well as storage and shipping conditions [11, 12].

Iron is an essential element for most forms of life, but it is too scarce to obtain in sufficient quantity as it is predominantly present in biologically unavailable ferric form [Fe(III)]. It is required for a number of biological reactions, including reduction of the oxygen for synthesis of ATP, reduction of ribotide precursors of DNA, for the formation of heme, detoxification of oxygen radicals, etc. To make an insoluble form of iron soluble, organisms produce organic iron ligands known as siderophores [13]. Siderophores are low molecular weight (<1,000 Da) small peptide molecule, which include catecholate, hydroxamate, a hydroxycarboxylate, or mixed type of ligands that bind to the ferric ions having varied applications in health care and in agriculture. Previously, the genus *Bacillus* has been reported to produce catecholate type of siderophore 2,3-dihydroxybenzoyl glycine under iron-limiting conditions [14, 15]. In this study, it has been observed that *Bacillus* strain of human origin exhibiting good probiotic qualities also do produce 2,3-dihydroxybenzoyl glycine in vitro intestinal conditions.

The aim of the present study was to isolate bacterial strain having siderophoregenic and probiotic qualities prior to its possible application for iron nutrition in humans and animals.

Materials and Methods

Strain Isolation and Identification

Bacterial strain ST13 was isolated from human stool using routine microbiological techniques. In brief, sample was diluted 1:10 (*w*/*v*) in sterile phosphate buffer saline (pH 7.3) and spread on nutrient agar plates. Different colonies grown after a 24-h incubation at 37 °C were isolated on the basis of its morphology, biochemical characteristics such as mannitol utilization, and positive Voges–Proskauer test [16]. The isolates were screened further for siderophore production as mentioned in "Screening for Siderophore Production by Chrome Azurol S Assay". Selected strain was preserved as a frozen stock at −80 °C in nutrient broth containing 30% (*v*/*v*) glycerol and subcultured at the interval of 6 months. Working cultures were prepared by propagating it twice in nutrient broth at 37 °C and maintained on nutrient agar slants.

Media and Growth Conditions

Culture was grown under aerobic (shaking 120 rpm) and microaerophilic (static) conditions at 37 °C in nutrient broth and chemically defined low iron medium (CDLIM) for siderophore production (gm/L) (K_2SO_4, 2.0; K_2HPO_4, 3.0, NaCl−1.0; NH_4Cl, 5.0; $MgSO_4.7H_2O$, 0.08; $ZnSO_4.7H_2O$, 0.002; $CaCl_2.2H_2O$, 0.1; $CuSO_4$, 5.0×10^{-6}; $MnSO_4$.

H_2O, 3.5×10^{-5}; thymine·HCl, 2.0×10^{-4}; and glycerol, 25 ml) pH 7.0±0.1. This medium was further modified with the addition of deferrated 0.3% bile salt.

Strain Identification

Potential isolate was characterized based on 16S r-RNA gene partial sequencing using universal primer set 16F27N (5′-CCA GAG TTT GAT CMT GGC TCA G-3′) and 16R1525XP (5′-TTC TGC AGT CTA GAA GGA GGT GTW TCC AGC C-3′) [17]. Amplification was performed in thermal cycler following PCR conditions (35 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, with initial denaturation at 95 °C for 5 min and final extension at 72 °C for 10 min) [18]. Amplicons were separated on 1.3% agarose gel electrophoresis (60 V/3 h) with 1 kb DNA ladder.

Screening for Probiotic Properties

Acid Tolerance

Single colony of isolate was inoculated in 20 ml of nutrient broth and incubated overnight at 37 °C. The cell pellet was harvested by centrifugation at 8,000×*g* for 5 min and washed twice with sterile phosphate buffered saline (PBS) pH 7.3. A 1-ml PBS was added to resuspend the cell pellets completely for assay. The strain was diluted 1:100 in PBS at pH 1, 2, 3, and 4. Mixture was incubated at 37 °C, and viable bacterial cells were determined at 0-, 60-, 120-, and 180-min time interval by plating on nutrient agar plates. Growth of bacteria was expressed in colony forming units per milliliter ($\log_{10}$ cfu/ml), and the survival percentage of strain to different pH values was calculated.

Gastric Juice Tolerance

Gastric juice tolerance was determined as per the method of Pedersen et al. [19] with some modifications. Overnight grown culture of isolate was obtained by inoculating a single colony in 20 ml of nutrient broth medium. Cell pellet was washed twice with 10 ml of PBS and resuspended in same followed by centrifugation at 8,000×*g* for 5 min. It was further diluted as 1:10 in synthetic gastric juice (composition (gm⁄L): peptone, −8.3; glucose, −3.5; NaCl, −2.05; KH_2PO_4, −0.6; $CaCl_2$, −0.11; KCl, −0.37; bile, −0.05; lysozyme, −0.1; pepsin, −13.3 mg; distilled water, 1 L, and pH 2.5 adjusted with 1 M HCl) [19] and incubated at 37 °C. Samples were taken at 0-, 30-, 180-min intervals, and survival rate was measured by spreading 100-μl sample on nutrient agar plates, which were incubated at 37 °C for 24 h. Growth of bacteria was expressed in colony forming units per milliliter ($\log_{10}$ cfu/ml) and the survival percentage of strain was calculated.

Bile Salt Tolerance

Bile salt tolerance was determined by inoculating 100-μl overnight grown culture into 900-μl growth medium supplemented with 0.3%, 0.5%, 1.0%, 1.5%, and 2.0% bile salt. It was incubated at 37 °C for 24 h. The viable bacteria were enumerated by plating 100 μl of culture on to the nutrient agar plate incubated at 37 °C for 24 h. The inoculated growth medium without bile salt served as control. Growth of bacteria was expressed in colony forming units per milliliter ($\log_{10}$ cfu/ml), and the survival percentage of strain was then calculated on the basis of control.

Antibiotic Susceptibility Test

Antibiotic drug susceptibility was determined by spreading 0.1 ml (10^6 cells/ml) of overnight grown culture on top of the nutrient agar plate as a lawn. Standard antibiotic discs (Hi-Media, Mumbai) were placed on the surface of the agar medium aseptically. Plates were observed for zones of inhibition after 24 h incubation at 37 °C.

Antimicrobial Activity

An agar spot test was used to detect antimicrobial activities of test organism against potent enteric pathogens. Overnight grown culture was spotted onto the surface of nutrient agar plate and spots were developed by incubation at 37 °C for 24 h. Enteric pathogens *Salmonella typhimurium* NCIM 2501, *Streptococcus pyogenes* NCIM 2608, and *Staphylococcus aureus* NCIM 5021 were inoculated at a concentration of 10^6 cells/ml in 7 ml of soft agar (0.7% agar), overlaying the test organism spots on the top and incubated at 37 °C for 24 h. Growth inhibition of pathogens were observed.

Mucin Binding Assay

The bacteria were grown at 37 °C in nutrient broth medium supplemented with 0.1% mucin type II from porcine stomach (Sigma) for 24 h to induce binding [20]. Wells of micro-titer plate were coated with mucin (100 μg/L in 50 mM Na_2CO_3 buffer, pH 9.7) was added 150 μl per well, incubated overnight at 4 °C with slow rotation [19]. Wells were blocked with PBS with 1% Tween 20 for 1 h and washed with PBS supplemented with 0.05% Tween 20 (PBST; pH 7.3). The bacterial strain was grown as described above and washed once in PBST and diluted to an $A_{595} = 0.5 \pm 0.02$ in the same buffer. The bacterial suspension (100 μl) was added to each well and incubated for 1 h at room temperature. The wells were washed with PBST and binding was examined under an inverted microscope TCM−400 (Labomed, USA). The buffer was poured off and, after wells had dried, the A_{450} was measured using microplate reader (Model 680, Bio-Rad, Japan). All measurements were obtained in triplicate.

Autoaggregation Assay

Autoaggregation assay was performed according to Del Re et al. [21] with certain modifications. Isolate was grown over night at 37 °C in nutrient broth. The cells were pelleted and washed twice with PBS (pH 7.3) and resuspended in PBS to get A_{600} to 0.5 of which 4 ml was mixed by gentle vortexing for 10 s and incubated at 37 °C for 1 h. After incubation absorbance of upper suspension was measured. Autoaggregation percentage was expressed as: $A_0 - (A_t/A_0) \times 100$, where A_t represents the absorbance at time t=1 h and A_0 the absorbance at t=0 h.

Bacterial Adhesion to Solvent

Bacterial adhesion to solvent was measured according to Rosenberg et al. [22] with some modifications. Isolate was grown overnight at 37 °C in nutrient broth medium. The cells were pelleted at 8,000×g for 5 min and washed twice with PBS pH 7.3, resuspended in 0.1 M KNO_3 (pH 6.2) and A_{600} was measured as A_0. Absorbance was measured by using spectrophotometer (UV–VIS 1601 Shimadzu, Japan) throughout this study; 1.0 ml solvent was added to 3 ml of cell suspension. After a 10-min preincubation at room temperature,

two phases were mixed by gentle vortexing for 2 min. The aqueous phase was removed after 20 min of incubation at room temperature and A_{600} was measured as A_1. The percentage of bacterial adhesion to solvent was calculated as $(A_0 - A_1/A_0) \times 100$.

Hemolytic Activity

Blood hemolysis was examined on nutrient agar plate supplemented with 5% sheep blood, after incubation at 37 °C for 24 h.

Siderophore Production and Characterization

Screening for Siderophore Production by Chrome Azurol S Assay

Overnight grown culture of isolate was centrifuged at 8,000×g for 5 min. Cell pellet was washed twice with PBS (pH 7.3) and resuspended in the same. One percent of suspension was inoculated in CDLIM to grow at 37 °C for 24 h. The cell free supernatant was collected on centrifugation at 8,000×*g* for 5 min whose pH was adjusted to 7.0 and subjected to universal Chrome Azurol S (CAS) assay for siderophore [23].

Determination of Type of Siderophore

The type of siderophore was determined by Arnow's test for catecholate type [24] and Csaky test for hydroxamate type siderophore [25].

Siderophore Production

Siderophore production was achieved by growing (3% *v*/*v*) culture in CDLIM at 37 °C for 36 h with 120 rpm. The percentage of siderophore unit produced was assessed as per Payne [26]. The culture supernatant was mixed in equal amount with CAS solution. A reference was prepared using uninoculated CDLIM and A_{630} was measured to calculate percentage of siderophore units produced.

Siderophore Production in Artificial Intestinal Stress Conditions

Overnight grown test organism was centrifuged at 8,000×*g* for 5 min and obtained cell pellet was washed twice with PBS (pH 7.3) and resuspended in the same. One percent of organism was inoculated (initial $\log_{10}$ cfu/ml was determined) in siderophore production medium containing 100% glass beads and 0.3% bile salt. The conical flask containing this mix was incubated at 37 °C under static as well as reciprocal shaking (120 rpm) condition for 24 h. The $\log_{10}$ cfu/ml was calculated to determine survival percentage rate and percentage of siderophore production as discussed earlier.

Extraction and Purification of Siderophore

CAS-positive culture supernatant was acidified gently to pH 2.0 with 12 M HCl and extracted with 0.4 volume of monopolar basic solvent ethyl acetate. Ethyl acetate extract was concentrated using rotary vacuum evaporator (Buchi, Switzerland). The concentrated sample was further purified by using XAD-4 column chromatography. The sample was eluted with methanol with flow rate of 1 ml/min. Fractions of 2 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, finally freeze dried using lyophilizer (3.3 L VirTis, USA). The freeze dried sample was stored at −20 °C and subjected to thin layer chromatography using butanol/acetic acid/water (12:3:5 *v*/*v*) as mobile phase.

Characterization of Siderophore

HPLC Analysis High-performance liquid chromatography (HPLC) was carried out on Knauer HPLC (Germany) with isocratic pump using Eurospher C18 reverse phase column (100-5, 250×4.6 mm with pre-column) as stationary phase, equipped with detector (190–600 nm), interrogator (Iris 32, Lite data processor) and acetonitrile/water (95:05 *v*/*v*) as mobile phase with a flow rate of 1 ml⁄min (programmed for 15 min).

IR Analysis Lyophilized siderophore sample was subjected to Perkin Elmer Spectrum, Fourier transform infrared (FT-IR) spectrophotometer one B, calibrated with polystyrene for the determination of functional groups. The sample was thoroughly mixed with nuzol mulling agent on KBr. A spectrum was recorded in 4,000–450⁄cm ranges.

Proton NMR Analysis The sample was dissolved in DMSO and nuclear magnetic resonance (^{1}H) (NMR) was recorded using 5 mm liquid with solid multi-nuclei probe with 400-MHz FT-NMR spectrophotometer (BRUKER, Advance III plus 400, Billerica, MA) frequency in range 1−15 δ.

Mass Spectra The molecular weight of the siderophore was determined using liquid chromatography mass spectrometer (Thermo Scientific, Model LCQ Fleet and TSQ quantum, USA), where, 50–400 *m*⁄*z* mass range was applied.

DPPH Radical Scavenging Effect of Siderophore

The scavenging of α,α-diphenyl-β-picrylhydrazyl (DPPH) radical by methanolic extract of siderophore was analyzed by modifying the method of Shimada et al. [27]. A 0.8 ml of methanolic siderophore extract and 1 ml of freshly prepared DPPH solution (0.2 mM in methanol) were mixed and allowed to react for 30 min in the dark. Blank samples contained pure methanol. The scavenged DPPH was then monitored by measuring the decrease in absorbance at 517 nm. The scavenging ability was defined as follows: $[A_{\text{blank}} - A_{\text{test}}/A_{\text{blank}}] \times 100$.

Results

Strain Isolation and Identification

Isolation of Bacterial Strain

Bacterial cultures were isolated from human stool out of which a culture designated as ST13 was selected for studies based on its ability to produce siderophores and probiotic

characters. The colony of ST13 appeared small with diameter of 2 to 3 mm, white colored with irregular margins, flat, and rough on nutrient agar. Microscopically culture appeared to be spore bearing rod-shaped Gram-positive rods.

Strain Identification

Genetic Analysis 16S r-RNA gene analysis of the culture was performed that exhibited close association with known *Bacillus* spp. and sequence is available on NCBI gene bank as GU372084.

Phylogenetic Tree Phylogenetic tree showed its close similarity with *Bacillus subtilis* (Fig. 1).

Biochemical Analysis A specific biochemical test showed culture to be catalase, Voges–Proskauer test positive and was able to produce acid from mannitol [16]. These results are in agreement with its 16S r-RNA gene analysis supported ST13 of being *B. subtilis*.

Studies on Probiotic Properties of Isolate ST13

Acid Tolerance

It was found to be viable at highly acidic conditions like pH 2. The percent survival of ST13 was 60.26% after 180 min of exposure. The values of survival percentage at different pH were depicted in (Table. 1).

Gastric Juice Tolerance

Its survival in simulated gastric juice conditions was studied. It showed 97%, 91% viability at 30 and 180 min with compared with 100% survival was observed at 0-min time interval.

Bile Salt Tolerance

The culture when grown in 0.3% and 0.5% of bile salt concentrations showed 95% and 86% survival, respectively, on a 24-h incubation, but failed to survive in 1.0%, 1.5%, and 2.0% concentrations.

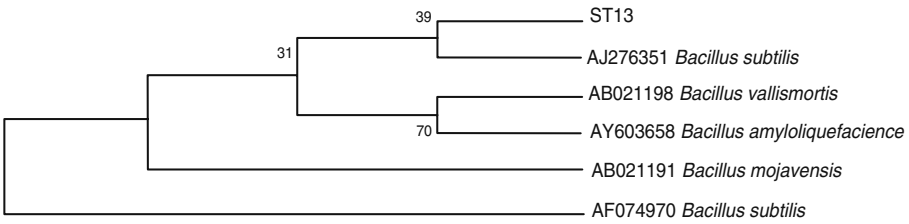

Fig. 1 Phylogenetic tree showing species relatedness of isolate

Table 1 Acid tolerance of ST13

pH	Log_{10} cfu of viable bacteria ml^{-1}				Survival after 180 min (%)
	0 min	60 min	120 min	180 min	
1	8.74±0.13	0±0.00	0±0.00	0±0.00	0.0
2	9.11±0.13	8.68±0.33	5.43±4.71	5.49±4.76	60.26
3	9.25±0.08	8.91±0.08	8.58±0.27	8.67±0.19	93.72
4	9.35±0.11	9.37±0.08	9.12±0.16	9.01±0.12	96.36

Each value is the mean of three experiments ± SD

Antibiotic Susceptibility Test

It was sensitive to variety of antibiotics tested. The degree of sensitivity was quite high to the antibiotics tested viz. amoxicillin (10 μg), amoxyclav (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), tetracyclin (30 μg), gentamycin (10 μg), co-trimoxazole (25 μg), ciprofloxacin (10 μg), clindamycin (2 μg), cloxacillin (1 μg), and cephalexin (30 μg).

Antimicrobial Activity

ST13 showed 5 mm zone of inhibition against *S. typhimurium* NCIM 2501, 4 mm against *S. pyogenes* NCIM 2608, and 4 mm against *S. aureus* NCIM 5021, respectively.

Mucin Binding Assay

It was able to bind mucin coated microtitre plate of which A_{450} was 0.037±0.0 over mucin coated well as blank.

Autoaggregation Assay

Its autoaggregation was determined on the basis of sedimentation rate which was found 96.8±1.05 after 1 h in PBS (pH 7.3).

Bacterial Adhesion to Solvent

It showed remarkable percent adhesion to xylene, chloroform, and ethyl acetate (Table. 2).

Table 2 Percent hydrophobicity of ST13 against various solvents

Solvent	Hydrophobicity (%)
Xylene	27.68±2.27
Chloroform	58.49±1.49
Ethyl acetate	63.34±6.18

Mean ± standard deviation of results from three separate experiments

Hemolytic Activity

It was found to be β-hemolytic on sheep blood agar plate after 24 h of incubation at 37 °C.

Siderophore Production and Characterization

Siderophore Detection and Extraction

The isolate was found positive for siderophore production on microaerophilic as well as on aerobic condition in CDLIM. Qualitatively it was confirmed by change in color of CAS reagent blue to golden yellow. The development of red color in Arnow's test indicated positive reaction while absence of color formation in Csaky test indicated negative. The purity of ethyl acetate extract and XAD-4 column fractions were confirmed on thin layer chromatography, where it showed single spot with R_f value 0.80 in butanol/acetic acid/water (12:3:5 *v*/*v*) as mobile phase under UV light. The freeze dried fractions yielded ~10 mg/L of siderophore.

Siderophore Synthesis Under Artificial Intestinal Stress Conditions

The production of siderophore and survival percentage rate in in vitro intestinal stress conditions was studied. The production of siderophore and survival percentage rate in microaerophilic condition (CB1) in presence of 0.3% bile and glass beads was found to be higher than aerobic conditions (CB2). The comparative results of siderophore and% survival in normal as well as in stress conditions are showed in Fig. 2.

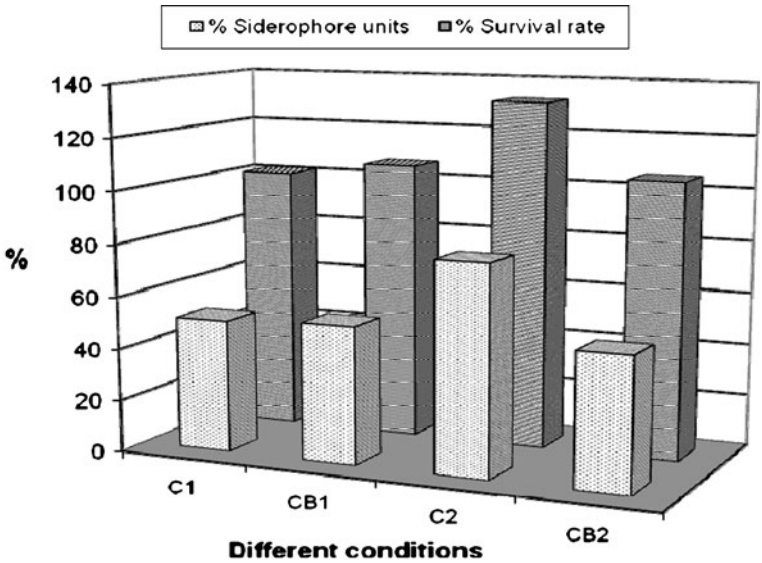

Fig. 2 Siderophore production and percentage of survival rate of isolate ST13 in different conditions: *C1* microaerophilic growth in CDLIM medium, *C2* aerobic growth in CDLIM medium, *CB1* microaerophilic growth in modified CDLIM bile medium with glass beads, *CB2* aerobic growth in modified CDLIM bile medium with glass beads

Characterization of Siderophore

The siderophore of ST13 was catecholate type which on HPLC analysis showed major peak at 3.7 min of retention time (Fig. 3).

Infrared spectroscopy showed the presence of >C–O stretching of COOH group, >C = C− of benzene nuclease, C = N of enamine form of O = C−NH, O = C− stretching of O = C−NH and COOH, and aromatic hydroxyl groups in molecules (Fig. 4).

FT-IR details are shown in the Table 3. The ^{1}H NMR (400 MHz, DMSO, 25 °C) showed δ−4.14 (S, 2H, N–CH_2), 5.21 (bs, 2H, 2–OH), 6.78 (d, 1H, Ar–H), 6.93 (dd, 1H, Ar–H), 7.43 (d, 1H, Ar–H), and 10.82 (S, 1H–COOH). Mass spectral analysis of siderophore sample of ST13 showed molecular weight to be 211.05, which represents the molecular weight of molecule 2,3-dihydroxybenzoyl glycine. The details of the mass spectra are as follows: MS (ESI, 70 ev) *m/z* (100%); 210 (77; M–1); 194 (26; M–OH) (Fig. 5).

The siderophore of ST13 revealed to be 2,3-dihydroxybenzoyl glycine on the basis of data obtained on IR, NMR and Mass spectroscopic studies.

DPPH Radical Scavenging Effect of Siderophore

The methanolic extract of siderophore showed 94.55±0.9% α,α-diphenyl-β-picrylhydrazyl radical scavenging activity.

Discussion

Lactic acid bacteria and Bifidobacteria have been established to be safe and reliable as a probiotics; however, *Bacillus* spp. has been relatively less studied as a probiotic [28, 29]. Recent studies claims that genus *Bacillus* offers beneficial effects over wide range of medical complications in humans and animals, including irritable bowel syndrome, pathogen invasion, small intestinal bacterial over growth and carcinogenicity, etc. [4, 5, 10, 30]. The spore former *Bacillus* preparations have been marked with commercial appearance in global market under various brand names viz. GanedenBC™ in USA [31], Biosporine^{R} in Russia and Ukraine [32], Biosubtyl^{NT}, Biosubtyl^{DL} in UK [33]. In addition strains of *B. subtilis* and *Bacillus indicus* has been approved as a food supplement in Italy. There are some traditional functional foods in Japan like natto comprising safe use of

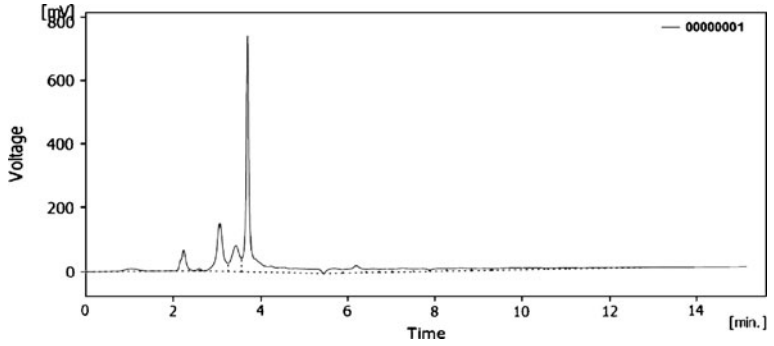

Fig. 3 HPLC analysis of siderophore of ST13

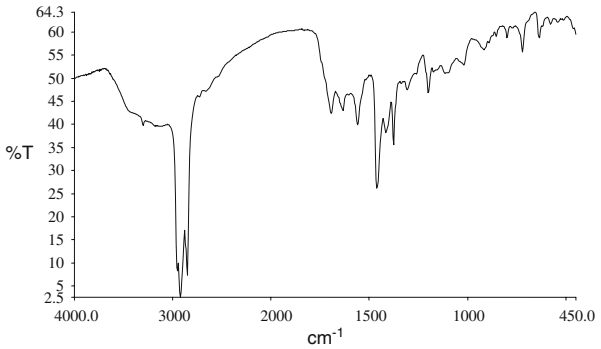

Fig. 4 FT-IR spectra of the siderophore of ST13

Bacillus coagulans along with *Bacillus natto* (*B. subtilis*) in it and few products of spore formers are available commercially [31].

The use of probiotic bacteria of non-human origin are still in controversy, although there are sufficient reports to confer health benefits to humans and animals. In milieu of iron nutrition the spore formers are less investigated [34–36] since *Lactobacillus* [37] and *Bifidobacteria* [38] have been reported as non-siderophoregenic. The present study is an attempt to isolate siderophoregenic probiotic spore forming *Bacillus* spp. for possible iron nutrition in humans and animals. The *Bacillus* spp. ST13 has origin from stool promoted to be *B. subtilis* based on 16S r-RNA gene sequence analysis and biochemical characteristics.

The vegetative cells of strain ST13 showed viability under highly acidic conditions of pH 2 as well as simulated artificial gastric juice containing pepsin, lysozyme. The values of survival percentage were found to be 60% and 91% for 3 h, which were higher than *Bacillus* strains reported earlier by Patel et al. [35] The tolerance to detrimental action of bile salt reflected the potentiality of the strain as a probiotic microorganism, since it survived 0.5% bile salt concentration which was higher than the physiological concentration in the duodenum [39]. It insures the arrival of higher concentration of viable cells in to intestine for its beneficial effects.

Table 3 Group determination by FT-IR of siderophore sample of the isolate

Group	Group cm^{-1} of ST13 siderophore sample
>C−C− stretching of COOH	1,201
>C=C− of benzene nucleus	1,417, 1463
C=N of enamine form of −C(=O)−NH	1,562
−C(=O)− stretching of −C(=O)−NH	1,634
−C(=O)−O stretching of −COOH	1,696
Ar−OH	3,340

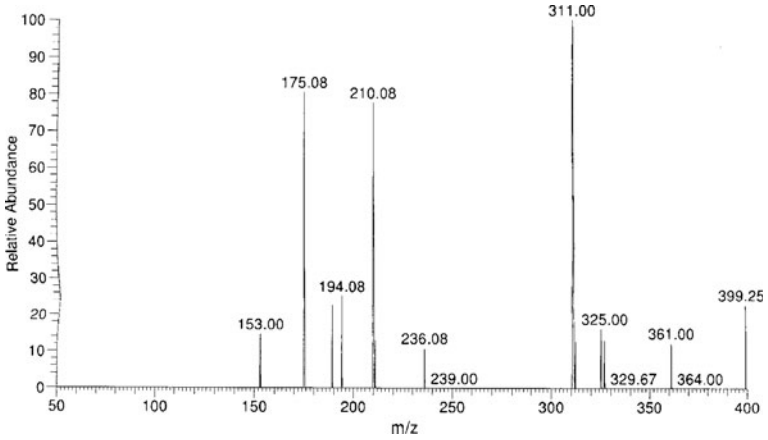

Fig. 5 Mass spectra of ST13 siderophore sample

Isolate ST13 exhibited good antimicrobial activity against potent enteric pathogens *S. typhimurium, S. pyogenes*, and *S. aureus* which can reduce incidence of food born infections and sporadic diseases caused by these strains. The culture was susceptible to most of the antibiotics which belonged to the major class of antibiotics used in human clinical therapy ensuring safety because of its inability to transfer antibiotic resistance determinant to other organisms if administered in the form of probiotic preparations. Isolate ST13 showed β-hemolytic activity on sheep blood, though non-hemolytic strains are desirable.

In order to determine colonization efficiency of probiotic bacteria, it is important to evaluate mucin binding ability, surface properties like autoaggregation and hydrophobicity. The significant values of these properties directly reflect its adhesion ability to enterocytic cellular lines [40]. 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 [41]. The autoaggregation ability of isolate focused on the cell surface hydrophobicity of bacteria. The autoaggregation and cell surface hydrophobicity could be one of the determinants of adhesion to cell monolayers [20]. Furthermore, isolate was able to adhere porcine mucin in vitro. Such abilities of microorganism furnish resistance to peristaltic elimination by providing competitive advantage in ecosystem.

The isolate was found to produce siderophore under artificial gastro intestinal stress conditions. The survival percentage and siderophore activity of this bacterium in in vitro adverse intestinal conditions reflects its ability to survive and produce a significant amount of metabolite. The production of siderophore in stress as well as in normal conditions is affected by number of viable cells. The 2,3-dihydroxybenzoylglycine have been widely reported siderophore from the *Bacillus* spp. [42]. FT-IR and NMR studies conducted in the present study showed it to be the 2,3-dihydroxybenzoylglycine molecule having molecular weight of 211.05 as per mass spectra analysis.

In aerobic conditions Fe(III) form of iron hydroxide is poorly soluble in aqueous solution (as low as 10^{-18} M at pH 7.0), making it basically unavailable for the cells. Under

anaerobic reducing or acidic conditions, the iron equilibrium shifts from the ferric Fe(III) to the ferrous Fe(II) form which is more easily available [43]. But, in intestine, shift in pH from acidic to alkaline leads to increase insoluble form of iron making it unavailable. To overcome these limitations, organisms produce low molecular weight iron chelating compounds known as siderophores. Siderophore-iron complex is soluble although Fe(III) is insoluble. Such chelation may make iron biologically available to the host when supplemented in food. Although, siderophores efficiently provide iron nutrition in plants [44], it is not yet clear whether such siderophore has any potential to serve in iron nutrition. However, it may be inefficient in iron sequestration from mammalian cells [45], but it certainly can sequester the insoluble iron present in food to make it biologically available. In addition methanolic extract of siderophore showed DPPH-free radical scavenging effect which reflected its ability to neutralize or scavenge toxic radicals produced in the body decreasing the risk of free radicals generated by action of xenobiotics on foods.

Conclusion

The genus *Bacillus* claims its beneficial effects over wide range of medical complications in humans and animals. Although, there is no evidence on the role of siderophoregenic bacteria in iron nutrition in animals or humans, these studies clearly showed the synthesis of siderophore under the partially simulated gut conditions. This provokes possibility of making iron bioavailable under in vivo conditions. The isolate showed production of catecholate siderophores in microaerophilic and artificial intestinal tract stress conditions, in addition this siderophore showed extraordinary free radical scavenging effect. The in vitro parameters studied suggested that the culture ST13 may be possible probiotic candidate, in the context of iron nutrition only after conducting a proper animal and human trial.

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References

1. Fuller, R. (1991). Probiotics in human medicine. *Gut, 32*, 439–442.
2. De Vecchi, E., & Drago, L. (2006). *Lactobacillus sporogenes* or *Bacillus coagulans*: misidentification or mislabelling? *Int J Probiot Prebioti, 1*, 3–10.
3. Hong, H. A., Duc, L. H., & Cutting, S. M. (2005). The use of bacterial spore formers as probiotics. *FEMS Microbiology Reviews, 29*, 813–835.
4. Vilà, B., Fontgibell, A., Badiola, I., Esteve-Garcia, E., Jiménez, G., Castillo, M., et al. (2009). Reduction of *Salmonella enterica* var. Enteritidis colonization and invasion by *Bacillus cereus* var. *toyoi* inclusion in poultry feeds. *Poultry Science, 88*(5), 975–979.
5. Hun, L. (2009). *Bacillus coagulans* significantly improved abdominal pain and bloating in patients with IBS. *Journal of Postgraduate Medicine, 121*(2), 119–124.
6. Wu, X.-Y., Walker, M., Vanselow, B., Chao, R.-L., & Chin, J. (2007). Characterization of mesophilic bacilli in faeces of feedlot cattle. *Journal of Applied Microbiology, 102*, 872–879.
7. Mazza, P. (1994). The use of *Bacillus subtilis* as an antidiarrhoeal microorganism. *Bollettino Chimico Farmaceutico, 133*, 3–18.
8. Alexopoulos, C., Karagiannidis, A., Kritas, S. K., Boscos, C., Georgoulakis, I. E., & Kyriakis, S. C. (2001). Field evaluation of a bioregulator containing live *Bacillus cereus* spores on health status and performance of sows and their litters. *J Veter Med Physiol Pathol Clin Med, 48*, 137–145.

9. Shangkuan, Y. H., Yang, J. F., Lin, H. C., & Shaio, M. F. (2000). Comparison of PCR RFLP, ribotyping and ERIC-PCR for typing *Bacillus anthracis* and *Bacillus cereus* strains. *Journal of Applied Microbiology, 89*, 452–462.
10. Lee, N.-K., Park, J.-S., Park, E., & Paik, H.-D. (2007). Adherence and anticarcinogenic effects of *Bacillus polyfermenticus* SCD in the large intestine. *Letters in Applied Microbiology, 44*, 274–278.
11. Ljungh, A., & Wadstrom, T. (2006). Lactic acid bacteria as probiotics. *Current Issues in Intestinal Microbiology, 7*, 73–89.
12. Graff, S. (2008). Formulations for protecting the probiotic *Saccharomyces boulardii* from degradation in acidic condition. *Biological & Pharmaceutical Bulletin, 31*, 266–272.
13. Sandy, M., & Butler, A. (2009). Microbial iron acquisition: marine and terrestrial siderophores. *Chemical Reviews, 109*(10), 4580–4595.
14. May, J. J., Wendrich, T. M., & Marahiel, M. A. (2001). The dhb operon of Bacillus subtilis encodes the biosynthetic template for the catecholic siderophore 2, 3- dihydroxybenzoate-glycine-threonine trimeric ester bacillibactin. *The Journal of Biological Chemistry, 276*, 7209–7217.
15. Zawadzka, A. M., Abergel, R. J., Nichiporuk, R., Andersen, U. N., & Raymond, K. N. (2009). Siderophore-mediated iron acquisition systems in *Bacillus cereus*: identification of receptors for anthrax virulence-associated petrobactin. *Biochemistry, 48*, 3645–3657.
16. Claus, D., & Berkeley, R. C. W. (1986). Genus *Bacillus* Cohn 1872. In P. H. A. Sneath (Ed.), *Bergey's manual of systematic bacteriology, Section 13, vol. 2* (pp. 1105–1139). Baltimore: Williams & Wilkins.
17. Brosius, J. M., Palmer, L., Kennedy, P., & Noller, H. F. (1978). Complete nucleotide sequence of the 16S ribosomal DNA gene from *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America, 75*, 4801–4805.
18. Pidiyar, V. J., Jangid, K., Dayananda, K. M., Kaznowski, A., Gonzalez, J. M., Patole, M. S., et al. (2003). Phylogenetic affiliation of *Aeromonas culicicola* MTCC 3249(T) based on gyrB gene sequence and PCR-amplicon sequence analysis of cytolytic enterotoxin gene. *Systematic and Applied Microbiology, 26*(2), 197–202.
19. Pedersen, C., Lindberg, E., & Roos, S. (2004). Microbiological characterization of wet wheat distillers' grain with focus on isolation of Lactobacilli with potential as probiotics. *Applied and Environmental Microbiology, 70*(3), 1522–1527.
20. Jonsson, H., Strom, F., & Roos, S. (2001). Addition of mucin to the growth medium triggers mucus-binding activity in different strains of *Lactobacillus reuteri* in vitro. *FEMS Microbiology Letters, 204*, 19–22.
21. Del Re, B., Sgorbati, B., Miglioli, M., & Palenzona, D. (2000). Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. *Letters in Applied Microbiology, 31*, 438–442.
22. Rosenberg, M., Gutnick, D., & Rosenberg, E. (1980). Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiology Letters, 9*, 29–33.
23. Schwyn, B., & Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry, 160*, 47–56.
24. Arnow, L. E. (1937). Colorimetric determination of the components of 3, 4 Dihydroxyphenyl alanine–tyrosine mixtures. *The Journal of Biological Chemistry, 118*, 531–537.
25. Csaky, T. Z. (1948). On the estimation of bound hydroxylamine in biological materials. *Acta Chemica Scandinavica, 2*, 450–454.
26. Payne, S. M. (1994). Detection, isolation and characterization of Siderophores. *Method Enzymol, 235*, 329–344.
27. Shimada, K., Fujikawa, K., Yahara, K., & Nakamura, T. (1992). Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *Journal of Agricultural and Food Chemistry, 40*, 945–948.
28. Patterson, J. A., & Burkholder, K. M. (2003). Application of prebiotics and probiotics in poultry production. *Poultry Science, 82*, 627–631.
29. Sanders, M. E., Morelli, L., & Tompkins, T. A. (2003). Sporeformers as human probiotics: Bacillus, Sporolactobacillus, and Brevibacillus. *Compre Rev Food Sci Food Safety, 2*, 101–110.
30. Gabrielli, M., Lauritano, E. C., Scarpellini, E., Lupascu, A., Ojetti, V., Gasbarrini, G., et al. (2009). *Bacillus clausii* as a treatment of small intestinal bacterial overgrowth. *The American Journal of Gastroenterology, 104*, 1327–1328.
31. Endres, J. R., Clewell, A., Jade, K. A., Farber, T., Hauswirth, J., & Schauss, A. G. (2009). Safety assessment of a proprietary preparation of a novel Probiotic, *Bacillus coagulans*, as a food ingredient. *Food and Chemical Toxicology, 47*(6), 1231–1238.
32. Gracheva, N. M., Gavrilov, A. F., Solov'eva, A. I., Smirnov, V. V., Sorokulova, I. B., Reznik, S. R., et al. (1996). The efficacy of the new bacterial preparation biosporin in treating acute intestinal infections. *Zhurnal Mikrobiol Epidemiol Immunobiol, 1*, 75–77.

33. Duc, L. H., Hong, H. A., Barbosa, T. M., Henriques, A. O., & Cutting, S. M. (2004). Characterization of *Bacillus* Probiotics Available for Human Use. *J Appl Environ Microbiol, 70*(4), 2161–2171.
34. Patel, A. K., Deshattiwar, M. K., Chaudhari, B. L., & Chincholkar, S. B. (2009). Production, purification and chemical characterization of the catecholate siderophore from potent probiotic strains of *Bacillus* spp. *Bioresource Technology, 100*, 368–373.
35. Patel, A. K., Ahire, J. J., Pawar, S., Chaudhari, B. L., & Chincholkar, S. B. (2009). Comparative accounts of probiotic characteristics of *Bacillus* spp. isolated from food wastes. *Food Research International, 42*(4), 505–510.
36. Patel, A. K., Ahire, J. J., Pawar, S., Chaudhari, B. L., Shouche, Y. S., & Chincholkar, S. B. (2010). Evaluation of the probiotic characteristics of siderophoregenic *Bacillus* spp isolated from dairy waste. *Applied Biochemistry and Biotechnology, 160*, 140–155.
37. Pandey, A., Bringel, F., & Meyer, J. M. (1994). Iron requirement and search for siderophores in lactic acid bacteria. *Applied Microbiology and Biotechnology, 40*(5), 735–739.
38. Bezkorovainy, A. (1989). Iron transport and utilization by Bifidobacteria. In: A. Bezkorovainy, R. Miller-Catchpole (Eds.), *Biochemistry and physiology of bifidobacteria* (pp. 147–176). CRC Press.
39. Brashears, M. M., Jaron, D., & Trimble, J. (2003). Isolation, selection and characterization of lactic acid bacteria for a competitive exclusion product to reduce shedding of *Escherichia coli* O157:H7in cattle. *Journal of Food Protection, 66*, 355–363.
40. Pérez, P. F., Minnaard, A. Y., Disalvo, A., & De Antoni, G. L. (1998). Surface properties of bifidobacterial strains of human origin. *Applied and Environmental Microbiology, 64*, 21–26.
41. Bellon-Fontaine, M. N., Rault, J., & van Oss, C. J. (1996). Microbial adhesion to solvents: a novel method to determine the electrondonor/electron-acceptor or Lewis acid-base properties of microbial cells. *Colloids and Surfaces, 7*, 47–53.
42. Peters, W. J., & Warren, R. A. J. (1968). Itoic acid synthesis in *Bacillus subtilis*. *Journal of Bacteriology, 95*(2), 360–366.
43. Grass, G. (2006). Iron transport in *Escherichia coli*: All has not been said and done. *Biometals, 19*, 159–172.
44. Poole, K., & McKay, G. A. (2003). Iron acquisition and its control in *Pseudomonas aeruginosa*: Many roads lead to Rome. *Frontiers in Bioscience, 8*, 661–686.
45. Fischbach, M. A., Lin, H., Liu, D. R., & Walsh, C. T. (2006). How pathogenic bacteria evade mammalian sabotage in the battle for iron. *Nature Chemical Biology, 2*, 132–138.