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Screening of *Bacillus* spp. of Human Origin A Potential Siderophorgenic Probiotic Bacteria

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Abstract: Siderophorgenic *Bacillus* strain FM 22 and FM 23 has been isolated from faecal sample of 8-month-old healthy infant. It was evaluated for probiotic characters (WHO guidelines) and siderophore production for iron nutrition in human and animals to make iron more soluble biologically. Partial 16S-rRNA sequencing analysis showed that isolates exhibited homology with *Bacillus subtilis*. Isolates FM 22 and FM 23 were found to produce maximum siderophore ranging from 65-90 % at an optimum pH 7, incubation period of 72 h, agitation speed of 150 rpm and inoculum volume of 15 %. The above two isolates produced hydroxymate type of siderophores under iron stressed conditions. Strains have shown excellent tolerance to acid, bile salt, sodium chloride and phenol, non-haemolytic in nature and exhibited high hydrophobicity and autoaggregation Further, isolates showed antimicrobial activity against enteric pathogen like *Staphylococcus aureus*, *E. coli* and *Klebsiella pneumoniae* and exhibited moderate susceptibility to antibiotics tested.

Key words: Probiotic, lactic acid bacteria, *Bacillus*, siderophore.

Introduction

Probiotics are microorganisms, which confer beneficial effects in the prevention and treatment of certain pathological conditions [24]. Probiotic cultures have been utilized as pharmaceutical preparations or as animal feed additives already for a few decades [27]. Various groups of microbes have been chosen for probiotic action including many species of genera *Lactobacillus* and *Bifidobacterium* which were originally isolated from the human gastrointestinal tract [22, 24]. These genera are the most copious and well established in probiotic containing food products but species of *Enterococcus*, *E.coli*, *Bacillus*, *Brevibacillus*, etc. have also been recommended for probiotic effects [20, 25].

Products containing endospores of members of the genus *Bacillus* (in single doses of up to 10^9 spores g or 10^9 spores/ml) are also used commercially as probiotics [22]. The implementation of *B. subtilis* and *B. indicus* have been approved for application as a food supplement in few European countries like Italy and *B. clausii* is another strain licensed as a prophylactic medicine in the product 'Enterogermina' (manufactured by Sanofi-Aventis, Milan, Italy) [22]. Furthermore, Bacilli are known to produce siderophores, which may offer additional benefits to the host [22, 27].

Microbes require 0.4-1.0 M iron for their optimum growth and to run their number of crucial biochemical reactions including reduction of the oxygen for synthesis of ATP, reduction of ribotide precursors of DNA, etc [15,16]. Siderophores are relatively low-molecular-mass (500-1000 Da) iron-chelating ligands that are synthesized by most microorganisms under iron-limited conditions, which bind ferric ions with high affinity and solubilize the iron in order to make it biologically available[18]. Food even fortified with iron may not be in soluble form and if colon harbors probiotic

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microbes producing siderophores would give bonus to human health correcting the deficiencies of iron for metabolic process such as formation of red blood cells, DNA repair, etc [19].

Materials and methods

Isolation and screening for siderophore production

Fifty samples were collected from infant faecal matter (4 months - 2yr old, Baripada, Odisha, India). Serial dilution method and plating was carried out using MRS agar media (Mann Rogassa Sharpe) (Sharpe and Elizabeth Pyer) [1] by spread plate and pour plate method. Plates were then incubated at 37°C for 48 h and after the incubation period the colonies were picked based upon their morphological appearance and characterized by various biochemical tests. The isolates were preserved as frozen glycerol stocks and maintained on MRS agar slants at 4°C and working cultures were prepared by propagating them in MRS broth.

Inoculum of all the fifty different isolates were prepared in MRS broth and incubated in a rotary shaker maintained at 37°C, 150 rpm for 24 h. Siderophore production was studied using modified succinate medium of Meyer and Abdallah [2].

Qualitative detection of siderophore

Qualitative detection of siderophore was carried out using universal CAS assay as per Schwyn and Neilands [3].

Quantitative detection of siderophore

Quantitative detection of siderophore was carried out as per Payne [4].

Determination of type of siderophore

The type of siderophore was determined by Arrow's test [5] for catecholate type and Csaky test [6] for hydroxymate type.

Arrow's test for catecholate type of siderophores

1 ml of culture supernatant was mixed properly with 1ml of 0.5 mol l^{-1} HCl. Further 1ml of nitrate molybdate reagent (prepared by dissolving 10 g of sodium nitrate and sodium molybdate in 100 ml of water) and 1ml of NaOH was added and then this mixture was allowed to react for 5 min for the reaction to fully occur. Catecholate group can be detected by observation of change in colour. Uninoculated succinate medium used as control, which remains colourless.

Csaky test for hydroxymate type of siderophores

1 ml of culture supernatant was hydrolyzed with 1ml of 6 mol l^{-1} H_2SO_4 in boiling water bath for 6 h or at 130°C for 30 min. The solution was then buffered by adding 3ml of sodium acetate solution. Then 1ml of sulphalinic acid (1g dissolved in 100 ml of 30 % acetic acid) was added followed by 0.5 ml of iodine solution and kept for 3-5 min. Further 1ml of sodium arsenate (2 %) was added for destroying excess iodine. Finally 1ml of naphthalamine solution (3 % in 30 % acetic acid) was added and allowed to react for 20-30 min and then observed for change in colour.

Condition optimization for siderophore production

Optimization for factors like incubation period pH, rpm and inoculum volume was carried out for maximum production of siderophores. The incubation period was optimized by collecting and analyzing the samples at every 24 h using CAS assay. pH was optimized by growing the isolates in the succinate media with a pH range of 5-9 using 1 mol l^{-1} HCl and 1 mol l^{-1} NaOH. Optimization of agitation at different rpm such as 50, 100, 150, 200 and inoculum volume of 5, 10, 15, 20 and 25 % was carried out for maximum siderophore production.

Strain identification

Identification of isolates producing maximum siderophore was carried out by complete 16S rRNA gene sequence analysis and phylogenetic studies (Macrogen Inc., Korea). Universal primers 518F (52 -CCAgCAgCCgCgg TAATA Cg-32) and 800R (52 -TACCAgggTATCTA ATCC-32) were used for the amplification for 16SrRNA gene of the isolates. Evolutionary analyses were conducted in MEGA 5 software [7]. Evolutionary history was inferred using the

Neighbor-Joining method[8] and the evolutionary distances were computed using the Tajima-Nei method [9]

Screening for probiotic properties

Acid tolerance

Isolates were grown overnight in MRS broth at 37°C followed by centrifugation at 8000 g for 5 min. Cell pellet was harvested and washed twice in sterile phosphate buffered saline (PBS) pH 7.3 and resuspended in 1 ml of PBS and the strains were further diluted 1:100 in PBS at pH 1, 2, 3 and 4. Samples were then incubated at 37°C and viable bacterial cells were determined at 0, 60, 120 and 180 min time interval by plating on MRS agar plates. Growth of bacteria was expressed in colony forming units per millilitre ($\log_{10}$ cfu ml^{-1}) and survival % of strains was calculated.

Bile salt tolerance

Bile salt tolerance was determined by inoculating 100 µl overnight grown culture of the isolates into 900 µl MRS broth supplemented with 0.3 %, 0.5 %, 1.0 %, 1.5 %, 2.0 %, 2.5 %, 3 %, 3.5 % and 4 % bile salt (Ox gall, Hi-media) and was incubated at 37°C for 24h. The viable bacteria were enumerated by plating 100 µl of culture onto the MRS agar plates incubated at 37°C for 24 h. Growth of bacteria was expressed in colony forming units per millilitre ($\log_{10}$ cfu ml^{-1}) and survival % of strain was then calculated.

Phenol tolerance

Phenol tolerance was determined by inoculating 100 µl overnight grown culture of the isolates into 900 µl MRS broth supplemented with 0.1-0.5 % of phenol and was incubated at 37°C for 24 h. Tolerance of isolates was analyzed by measuring the absorbance at 600nm and MRS broth without phenol was taken as reference and further the survival % of strain was calculated.

NaCl tolerance

NaCl tolerance was determined by inoculating 100 µl overnight grown culture of the isolates into 900 µl MRS broth supplemented with 2, 4, 6, 8, 10 and 12 % of NaCl and was incubated at 37°C for 24 h. Tolerance of isolates was analyzed by measuring the absorbance at 600nm and MRS broth without phenol was taken as reference and further the survival % of strain was calculated.

Antibiotic susceptibility test

Antibiotic drug susceptibility was determined by spreading overnight grown culture of the isolates on MRS agar plates as a lawn. Standard antibiotic discs (tetracycline, erythromycin, ampicillin, gentamycin, penicillin, chloramphenicol, cefuroxime, cefoperazone, levofloxacin, norfloxacin, Hi-Media, Mumbai) were placed on the surface of the MRS agar medium aseptically. Plates were incubated for 24 h at 37°C and observed for zones of inhibition.

Antimicrobial activity

An agar spot test was used to detect antimicrobial activities of test organism against potent enteric pathogens. Overnight grown culture of test isolates was spotted onto the surface of MRS agar plates and spots were developed by incubation at 37°C for 24 h. Enteric pathogen like and *Staphylococcus aureus* NCIM 5021, *E.coli* NCIM 6145, *Klebsiella* and *Pseudomonas aeruginosa* were inoculated at a concentration of 10^6 cells ml^{-1} in 0.7 % of nutrient agar and was overlayed on the test organism spots and incubated at 37°C for 24 h and observed for growth inhibition of pathogens around the spots.

Autoaggregation assay

Autoaggregation assay was performed according to Del Re *et al*., [10] with certain modifications. Isolates were grown over night at 37°C in MRS broth. The cells were pelleted and washed twice with PBS (pH 7.3) and resuspended in PBS to get an OD of 0.5 at A_{600} and considered as A_0. Four ml of culture was mixed by gentle vortexing for 10s and incubated at 37°C for 1h. After incubation absorbance of upper suspension was measured as A_t.

Autoaggregation % was expressed as: $A_0 - (A_t A_0^{-1}) \times 100$

where A_t represents the absorbance at time t = 1 h and A_0 the absorbance at t = 0 h.

Hydrophobicity of strains

Hydrophobicity of strains was measured according to Rosenberg *et al*. method [11] with some modifications. The isolates were grown overnight at 37°C. The cells were pelleted at 8000 g for 5 min and washed twice with PBS pH 7.3, resuspended in 0.1 mol l^{-1} KNO_3 (pH 6.2). Absorbance at A_{600} was measured as A_0 by using spectrophotometer (UV-VIS 1601 Spectrochem, Mumbai). 1.0 ml of solvent (xylene, acetone and heptane) was added to 3 ml of cell suspension. After 10 min pre-incubation at room temperature, two phases were mixed by gentle vortexing for 2 min and incubated at room temperature for 20 min. The aqueous phase was removed after incubation and then A_{600} was measured as A_1. The % of bacterial adhesion to solvent was calculated as $(A_0 - A_1 A_0^{-1}) \times 100$.

Haemolytic activity

Blood haemolysis was examined on MRS agar plates supplemented with 5 % sheep blood, after incubation at 37°C for 24 h.

Statistical analysis

Mean data of at least two independent experiments with three replicates of different characterization studies were used for the evaluation of results. Data were analyzed using ANOVA (*post-hoc*) through General Linear Model procedure to find out the significant difference among the mean values at various pH, incubation period, agitation speed and inoculum volume influencing siderophore production. Correlation analysis was also performed to find out the linear association and to compare the factor level difference among the variables such as tolerance to bile salt, acid, etc by FM 22 and FM 23. All the analysis was carried out by using SPSS software for windows release 19.0 version (SPSS Inc., IBM, NewYork, USA).

Results

Strain isolation and identification

Selection of isolates was based on the macroscopic differences in the colony morphology and also on the collection of samples from different sources. A total number of 50 isolates were screened for siderophore production. Based upon the qualitative detection of siderophore production 4 isolates, RM9 (Raw milk), FM 21, 22 and 23 (Infant faecal matter) were selected for further studies.

Biochemical analysis

The four selected isolates were identified as *Bacillus* spp according to the method described by Bergey's Manual of Determinative Bacteriology [12]. According to the morphological properties the isolated strains RM 9, FM 22, 23 and 24 appeared yellowish roughed surfaced with irregular margins. Microscopically all the four isolates appeared as Gram +ve rods. RM9, FM 21, 22 and 23 were positive for Voges-proskauer test, catalase test and for sugars like mannose, xylose and arabinose, starch and esculin hydrolysis. The bacterial strains can grow at pH 5.5-8.5, temperature of 25-30°C.

Strain identification

Genetic analysis performed using 16S r-RNA gene analysis resulted isolates with expected base pairs 1014 for RM9, 996, 1179, 978 bp for FM 21, 22 and 23, respectively. After performing a BLAST search isolates RM9, FM21, 22 and 23 exhibited close association with known *Bacillus* sp. with GC content of 53 %, 54 %, 55 % and 55 %, respectively. These results were further confirmed by constructing a phylogenetic tree (Fig 1). The optimal tree with the sum of branch length for isolate FM21, 22 and 23 is 0.20663937. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The analysis involved 31 nucleotide sequences, fewer than 5 % alignment gaps, missing data, and ambiguous bases were allowed at any position and there were a total of 44 nucleotide sequences and a total of 878 positions in the final dataset for FM21, 22 and 23.

Qualitative determination of siderophores

Detection of siderophore was carried out using universal CAS assay. This assay is based on the principle of higher affinity of siderophores to acquire iron from its complex with weak chelator

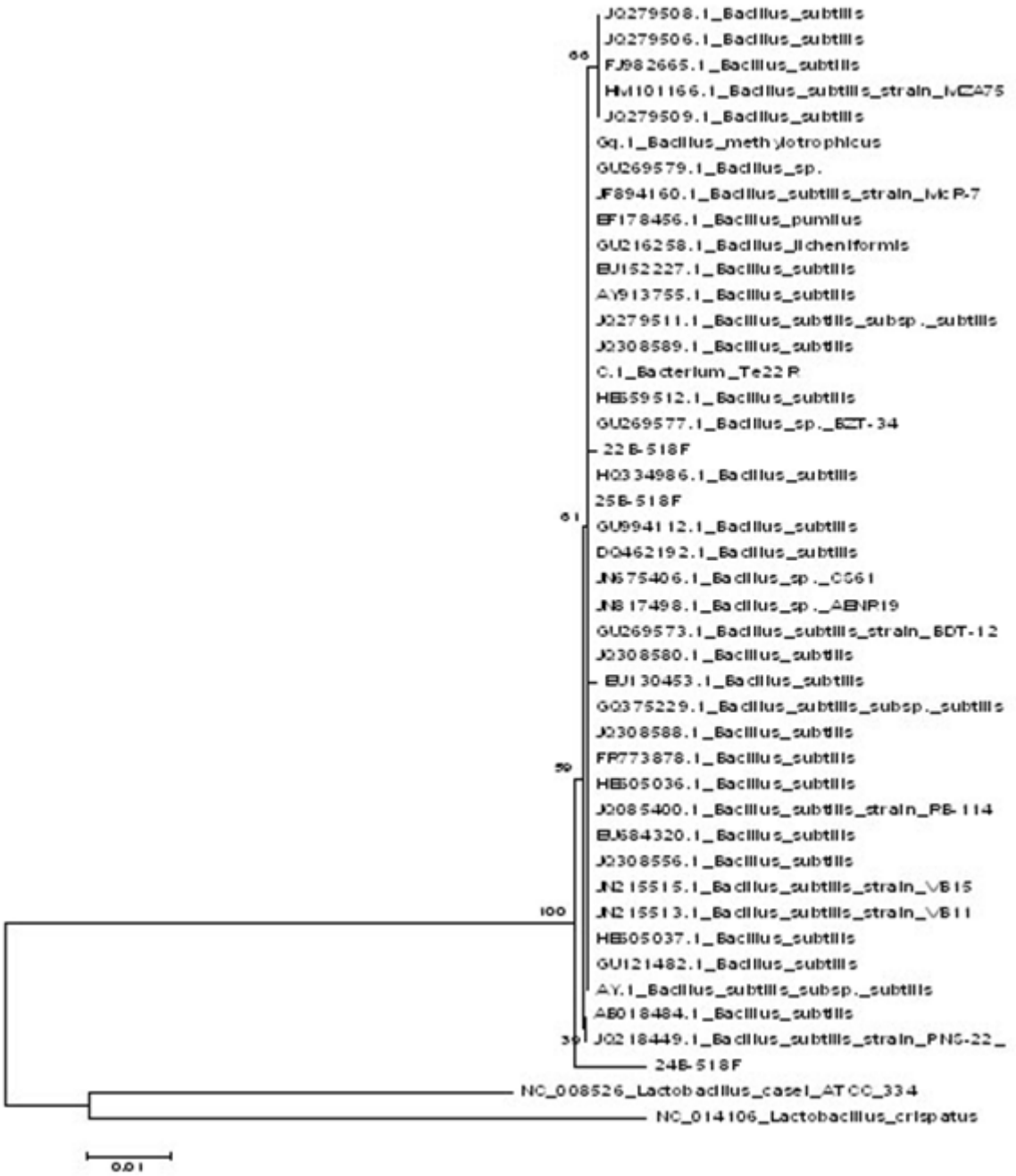

Figure 1. Phylogeny tree of RM 9, FM 22 and FM23

in the reagent due to which it undergoes decolourization. A positive siderophore production is confirmed by change of colour from blue to golden yellow. Out of 50 isolates, change in color was observed only in four isolates (RM9, FM 21, 22 and 23).

Characterization of siderophores

Isolate RM9 was found to possess catecholate type of siderophore whereas FM 21, 22 and 23 possess hydroxymate type of siderophore.

Quantitative determination of siderophores

Production of siderophore was quantified using CAS reagent and % of siderophore unit was determined. Four isolates (RM9, FM21, 22, 23) were producing siderophores in the range of 65-90 % siderophore units. Out of four isolates, FM

22 and FM23 were found to produce high amount of siderophores, hence used for further studies.

Optimization studies

Factors such as pH, inoculum volume, agitation speed and incubation period were optimized to increase the production of siderophores.

The two isolates showed maximum siderophore production, ranged from 89-90 % units at pH 7. Siderophore production was found to increase with incubation period up to 72 h for *Bacillus subtilis* and declined thereafter. The amount of siderophore produced by the two isolates varied and was found to be positively related to their growth (Fig. 2).

When inoculum volume in the range of 5- 25 % was used for siderophore production, 15 % was found to be optimum for maximum siderophore units i.e 90- 91 %. Siderophore production was too negligible when carried out at static conditions whereas when agitated at different rpm (50-200), 150 rpm was found to be optimum for high production of siderophores units. i.e. (Fig. 3).

Probiotic properties

Acid tolerance

Among the isolates evaluated for acid tolerance more than 80 % viability was observed at pH 3 and nearly 70 % viability at pH 2 after 120 min of exposure (Fig. 4).

Bile salt tolerance

Viability of the two isolates when tested for bile salts (0-4 %) it was found that a constant survival rate of 50-80 % was maintained in the bile salt range of 0.3-2% and the viability declined as the concentration of bile salt increased (Fig.5).

Phenol tolerance

Results of phenol resistance exhibited relatively high survival rate at 0.2 % while the two isolates showed 60 % survival at 0.4 % phenol and gradually decreased to less than 10 % at 0.5 % phenol (Fig 6).

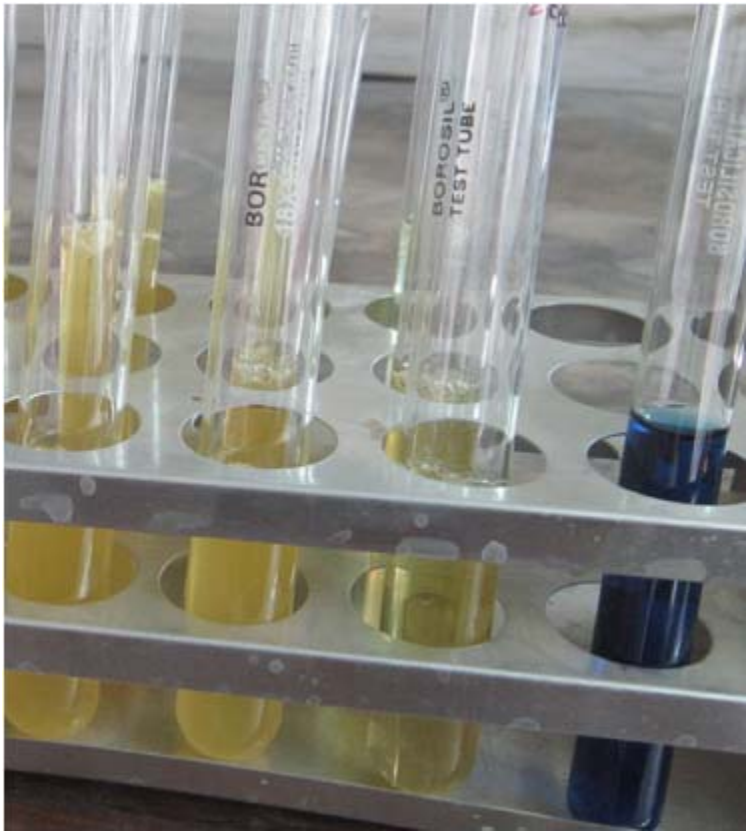

Figure 2. Siderophore production by isolates

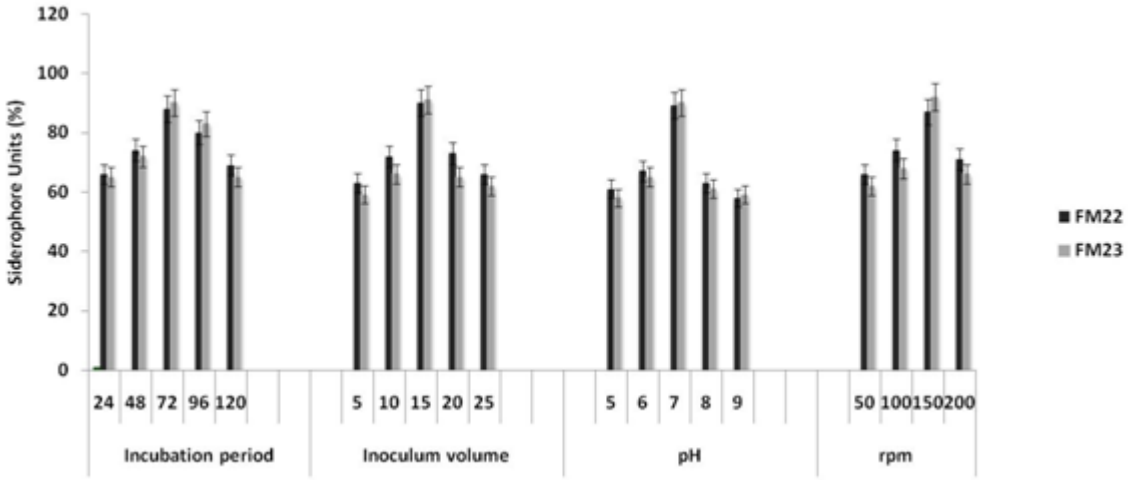

Figure 3. Production of siderophore by isolates at different incubation period, inoculum volume, pH and agitation speed (rmp)

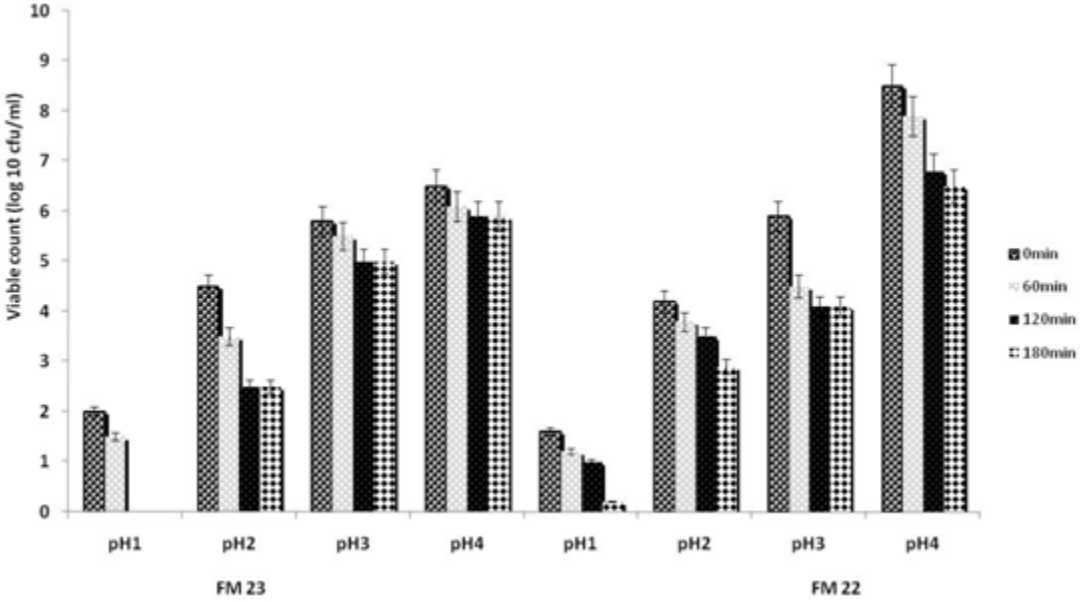

Figure 4. Survival % of the two isolates at different pH and different time of exposure

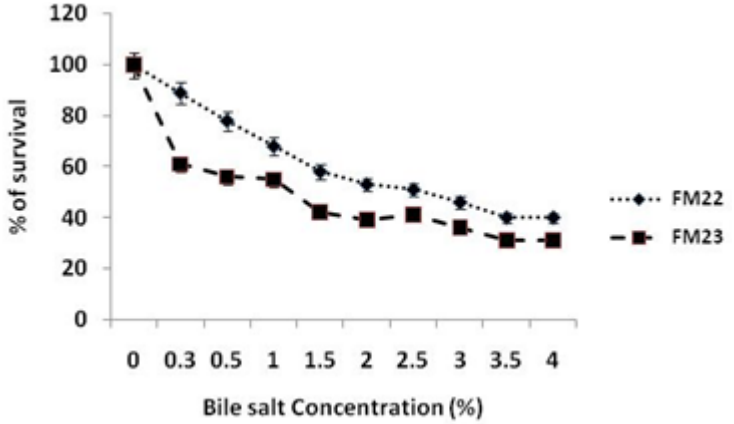

Figure 5. Survival % of the two isolates at bile salt range of 0-4 %

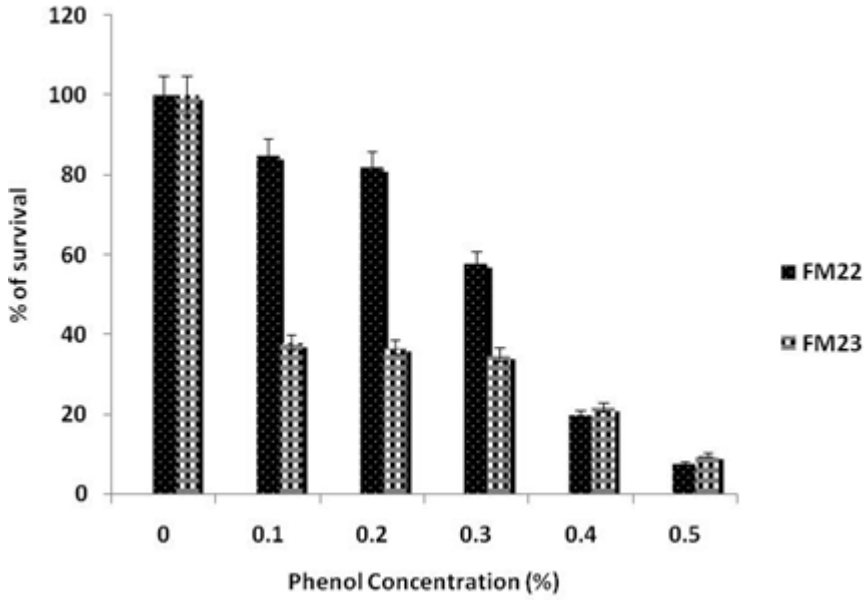

Figure 6. Survival % of the two isolates at a phenol range of 0-0.5 %

NaCl tolerance

The results of salt tolerance studies in the Fig 7 showed 59-99 % viability at 2 % and 4 % salt concentration by *Bacillus subtilis*. The viability reduced to 50 % and 30 % at 6 % and 8 %, respectively and the growth was totally inhibited at 12 % NaCl.

Antibiotic resistance study

The two strains of *Bacillus subtilis* were highly susceptible to Levofloxacin, chloramphenicol and tetracycline and moderately susceptible to all other antibiotic tested (Table 1).

Antimicrobial activity

The two isolates of *Bacillus subtilis* showed moderate antimicrobial activity against *S. aureus*, *Klebsiella* and *E. coli*, respectively. Gomes *et al*.[13] reported antimicrobial activity of *Enterococcus* spp against Gram +ve bacteria such as *Bacillus cereus*, *Clostridium botulinum*, *Staphylococcus aureus* and also against some Gram –ve bacteria spp.

Autoaggregation assay

Autoaggregation is an important property of probiotic bacteria because they reflects its

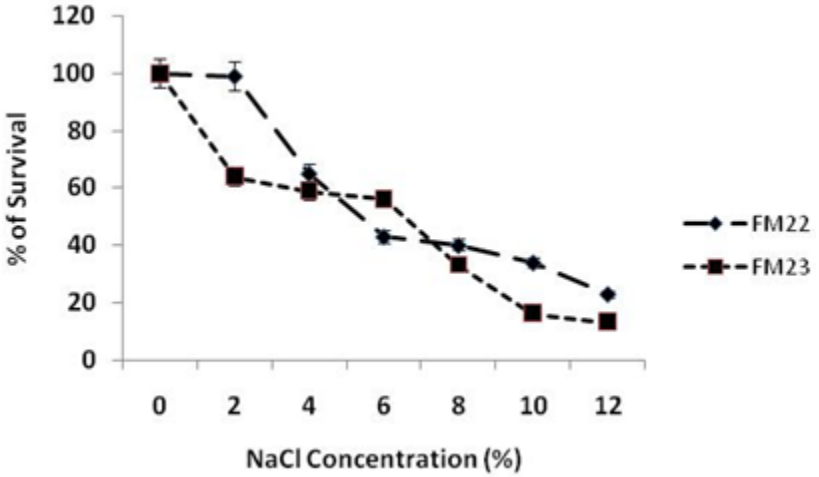

Figure 7. Survival % of the two isolates at a NaCl range of 0-12 %

Table 1. Antibiotic sensitivity tests of the two isolates

Isolates	Name of the Antibiotic (mcg)	Diameter of inhibition zone (mm)	Sensitivity
FM22	Levofloxacin (5)	33	+++
	Cefuroxime (30)	23	++
	Norfloxacin (10)	14	++
	Erythromycin (15)	15	++
	Ampicillin (10/10)	20	++
	Gentamycin (10)	16	++
	Chloramphenicol (30)	29	+++
FM23	Norfloxacin (10)	24	++
	Ampicillin (10/10)	31	+++
	Tetracyclin (10)	32	+++
	Gentamycin (10)	17	++
	Chloramphenicol (30)	24	++
	Erythromycin (15)	17	++
	Levofloxacin (5)	30	+++
	Cefuroxime (30)	24	++

Highly sensitive: +++
Moderately sensitive: ++
Less sensitive :+

adhesion ability to enterocytic cellular lines and also provides resistance to peristaltic elimination [14]. Autoaggregation was investigated on the basis of sedimentation characteristics of isolates. The two strains of *Bacillus subtilis* showed more than 30 % of autoaggregation ability.

Hydrophobicity of strains

The hydrophobicity of the isolate FM 22 was higher with acetone. However, for isolate FM 23 hydrophobicity was higher with xylene (Table 2).

Haemolytic activity

The two isolates of *Bacillus subtilis* were found to be non-haemolytic (γ- haemolysis) on 5 % sheep blood agar. The absence of haemolytic nature is considered to be a positive trait for bacteria to be used as a probiotic strain.

Statistical analysis

The ANOVA results revealed that siderophore production by *Bacillus subtilis* was highly significant at pH7 [F (4, 14) = 433.400 and 542.067, respectively; P<0.001], inoculum volume at 15 % [F (4, 14) = 426.375 and 593.250, respectively; P<0.001] and agitation speed at 150rpm [F (3, 11) =338.800 and 220.00, respectively; P<0.001]. Further, for *Bacillus subtilis* the siderophore

Table 2. Percent hydrophobicity of the two isolates against different solvents

Isolates	Solvents	Initial OD at 600 nm	Final OD at 600 nm	% of Hydrophobicity
FM22	Heptane	0.168	0.155	7.7
	Xylene	0.168	0.148	11.9
	Acetone	0.168	0.099	44.0
FM23	Heptane	0.193	0.163	15.5
	Xylene	0.193	0.183	51.8
	Acetone	0.193	0.112	41.9

production was highly significant at incubation period of 72 h [F (4, 14) = 439.0, 543.0, respectively; P<0.001]. The post hoc analyses revealed that all the factors such as pH at 7, incubation period at 72 h, inoculum volume at 15 % and agitation speed at 150 rpm had significant effect on siderophore production (P< 0.05; Tukey HSD). Pearson's correlation coefficient (r2) analysis was used for the measurement of linear association between the survibility of all the three isolates and variables like bile salt, phenol and NaCl. The analysis showed that all the above variables had significant negative correlation with the two isolates i.e when the concentration of bile salt, NaCl and phenol increased the survibility rate of the isolates decreased. For example- *Bacillus subtilis* -bile salt tolerance (-0.947, P< 0.01), *Bacillus subtilis* -phenol tolerance (-0.977, P< 0.01) and *Bacillus subtilis* - NaCl tolerance (-0.960, P< 0.01).

Discussion

pH plays a vital role in the solubility of iron in production media and thereby siderophore production. Iron is insoluble at neutral to alkaline pH, hence shows increased siderophore production [15]. Solubility increases at acidic pH, so at acidic condition iron is available to micro-organism, which in turn reduces the siderophore production. At highly alkaline pH siderophore yield should increase as available iron is less but as the growth of the organism decreased, siderophore production was found to be decreased. Bendale *et al.*[16] reported highest siderophore production of 93 % units at pH 8 using *Streptomyces fulvissimus*. Lisiecki *et al.*,[17] reported siderophore units of 45.7 % at pH 7.2 by *Enterococci* spp which was in close agreement with our results. In our study all the six isolates shown maximum siderophore production with 15 % inoculum volume. However, Sayyed and Chincholkar [18] reported optimum siderophore production with only 5 % inoculum level in *P. fluorescens* NCIM 5096. Lisiecki *et al.*[17] also reported siderophore units of 45.7 % at 120 rpm with 5 % inoculum volume using *Enterococci* spp. Difference in the quantity of siderophore production is a logical observation and several reports have indicated variations in siderophore production with time, space and environment[19]. Patel *et al.*[20,21] reported highest production of siderophore units of 80 % at an incubation period of 36 h using *Bacillus* sp in contrast to our study, siderophore production was found to increase with incubation period up to 72 h for *Bacillus subtilis* and declined thereafter.

Major characteristic used for *in-vitro* screening of probiotic bacteria is its resistance to acidity of the stomach to exert their beneficial effects in the gut [22,23]. Acidity of human gastrointestinal tract varies from 1.5 to 4.5 but the *in-vitro* studies were mostly performed at pH 3 as the viability below pH 3 is very low [24]. Gangadharan *et al.* [25] reported 80 % viability of *Lactococcus* spp. at pH 3 and 60 % viability at pH 2. Kumar *et al.* [26] reported that at pH 2.0 *E. coli* strains 10, 20 and 16 showed higher acid tolerance and strains 3, 44, 45, 14 and 17 showed poor acid tolerance whereas, at pH 3.0, all the isolates showed good acid tolerance. Probiotic strains need to survive bile salts in the duodenum to exert their beneficial effects in the gut. Hence bile salt tolerance is considered one of the most important properties of probiotic microorganism as it allows them to survive and colonize the gastrointestinal tract by enterocytes adhesion [21,27]. Bile salt tolerance studies were mostly carried out using Oxgall bile salt because of their similarity to human bile juice. Bhakta *et al.* [23] reported lactic acid bacteria (LAB) have the highest bile salt (4 gl^{-1}) tolerance. The *Lactobacillus* strains can grow in MRS agar supplemented with 3gl^{-1} bile salt [28]. The strains *Pediococcus acidilactici* (P2), *Lactobacillus curvatus* (RM 10) and *Lactobacillus sake* (L2), were resistant to 3g l^{-1} bile salt at pH 6 [29].

Resistance to phenol is also an important factor for probiotic bacteria as some aromatic amino acids are derived from dietary or endogenously produced proteins can be deaminated in the gut by bacteria leading to the formation of phenols [30]. Phenolic compounds can exert bacteriostatic effects, thus testing for resistance to phenol generates information on potential for survival in the gastrointestinal conditions, thereby proving to be the best probiotic strain. Gangadharan *et al.* [25] has reported relatively high survibility at 0.2 % phenol that has been decreased to 50 % at 0.4 % phenol and to 10% survival at 0.6 % phenol by

Lactococcus spp. Tolerance to high salt concentrations is useful to help in the initiation of metabolism [31]. Gomes *et al*. [13] reported that *E. faecium* and related species were resistant to ampicillin, tetracycline, chloramphenicol, trimethoprim/ sulfamethozaxole, quinolones, and streptogramins. Antimicrobial activity may be as a result of organic acids i.e. lactic acid, acetic acid and formic acid produced or because of diecetyl, hydrogen peroxide and CO_2 alone or in combination[32,25].

Gomes *et al*.,[13] reported antimicrobial activity of *Enterococcus* spp against Gram +ve bacteria such as *Bacillus cereus*, *Clostridium botulinum*, *Staphylococcus aureus* and also against some Gram –ve bacteria spp. Qing *et al*. [33] has reported wider range (5-30 μg ml^{-1}) of antibiotic resistance by *Enterobacter cloacae* against ampicillin, erythromycin, kanamycin and rifampicin. The antibiotic resistant properties also indicated that the isolated LAB strains would be able to survive in the environment and intestinal milieu by withstanding the undesirable situation occurred because of occasional high antibiotic concentrations.

Autoaggregation is an important property of probiotic bacteria because they reflects its adhesion ability to enterocytic cellular lines and also provides resistance to peristaltic elimination[14]. Autoaggregation was investigated on the basis of sedimentation characteristics of isolates. Ahire *et al*., [24] reported 18.23 % and Patel *et al*., [21] reported 32.6 % of autoaggregation for *E. coli* and *Bacillus*, respectively. Colonization of the mucosal surfaces and adhesion of bacteria to gastrointestinal host epithelial cells and extracellular matrix proteins is dependent on the cell surface hydrophobicity [25]. The colonization of the tissues by probiotic microbes can prevent pathogen access by steric interactions or specific blockage on cell receptors [34]. High hydrophobicity indicates the presence of hydrophobic molecules on the bacterial surface like array proteins, wall intercalated proteins, cytoplasmic membrane protein and lipids. Kos *et al*. [35] reported the hydrophobicity of *Lactobacillus* towards xylene and chloroform as 70.96 % and 36.06 %, respectively and *Enterococcus* towards chloroform as 61.21 %. Hamadi and Latrache [36] reported adhesion of *Bacillus subtilis* towards solvents like chloroform and hexane with 19 and 11.46 %, respectively.

Conclusion

Probiotics have been studied in various aspects including feed supplements and therapeutic application but not in the context of iron nutrition. Since *Lactobacillus* and *Bifidobacterium* have been reported as non- siderophorogenic, the present study mainly focused on siderophore production by isolate *Bacillus*. 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 two strains of *Bacillus subtilis* have been proved to be potential probiotic strains due to their survival under highly acidic conditions, higher tolerance to bile salt, NaCl and phenol. In addition, its colonization efficiency was proved by exhibiting high autoaggregation and hydrophobicity. Further absence of haemolytic nature and antibiotic resistance with maximum siderophore production made them to be considered as potential probiotic strains, but their applications have to be determined by conducting proper animal and human trials. Hence probiotic bacteria with all the special abilities will not only give advantage of all the probiotic features but also correct the deficiencies of iron.

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