

Characterization of a Newly Isolated Probiotic Strain from *Oecophylla smaragdina*, an Edible Insect Popular Among the Indigenous Communities of Northeast India

Debajit Borah¹ · Pronami Gogoi² · Dipti Agarwal¹ · Ankita Khataniar¹

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Abstract Northeast India is amongst the 34 Biodiversity Hot Spots of the world with large number of unexplored flora and fauna. A total of 74 potential isolates with probiotic properties were isolated from *Oecophylla smaragdina*, an edible insect used by local tribes as a nutritious medicinal food source out of which, the most potential probiotic candidate was identified as *Bacillus* sp. PD6 by 16S rRNA sequencing. Cell surface trait analysis shows hydrophobicity and auto-aggregation percentage of $46.2 \pm 0.32\%$ and $61.41 \pm 0.28\%$ respectively. The cell free extract (CFE) of the isolate shows the presence of essential amino acids viz, lysine, valine, leucine, isoleucine, and threonine indicating its nutritional value. It survives significantly at pH range from 1 to 10 and capable of hydrolyzing bile salt. The CFE shows antagonistic effect against *B. cereus* ATCC 11778, *E. coli* ATCC 25922 and *L. monocytogenes* ATCC BAA751. FTIR spectra of bacterial exo-polysaccharide shows the presence of $\nu_{C=C-H}$ stretch, ν_{N-H} stretch, ν_{C-H} stretch, ν_{C-H} bending and $\nu_{C=O}$ stretch bonds. It was found to be non-cytotoxic against primary mouse liver cell line and sheep erythrocytes and also, sensitive against wide range of broad and narrow spectrum antibiotics making it recommendable for human consumption.

Keywords Edible insect · *Oecophylla smaragdina* · Probiotic · Cytotoxicity assay · Antagonistic effect · *Bacillus* sp. PD6

Introduction

All over the world, especially among ethnic tribes, edible insects are used as a cheap source of protein and wellness food [1]. North Eastern India, the ethereal universe of extraordinary floral and faunal endemism, is a part of Eastern Himalayan Biodiversity hotspot, one of the three biodiversity hotspots in India. In the state of Assam, a part of North Eastern India lays the cradle of numerous economically important flora and fauna, particularly micro flora and fauna, most of which are yet to be explored [2]. The indigenous populations of Assam have long since been consuming various arthropods as a source of nutrition. Of these, *Oecophylla smaragdina* (local name-*Amroli porua*) is considered a local delicacy and consumed either raw or in cooked form and is a good source of edible protein, essential amino acids, antioxidants and minerals [3, 4]. Insects could provide these essential fatty acids to areas with lower access to food sources like fish, as a part of local diet [5]. Many people around the world consume insects as a part of their local food culture, mainly because of the palatability of the insects. Insects are a healthy food source of fatty acids, proteins, vitamins and mineral content. Essential fatty acids are important in development of children and infants [1]. However, the benefits of consuming insects as a food source are largely unknown to general public, as there is almost minimal scientific exploration that determines the utility of the traditional consumption of insects [3, 4, 6].

Debajit Borah, Pronami Gogoi and Dipti Agarwal have been contributed equally to this work.

✉ Debajit Borah
dborah89@gmail.com

¹ Centre for Biotechnology and Bioinformatics, Dibrugarh University, Dibrugarh 786004, India

² Department of Microbiology, Assam Medical College and Hospital, Dibrugarh 786002, India

Table 1 Geographical distribution of the sample collection sites

| Sample ID | Collection site | Geographical position of sample collection site | |
|-----------|-----------------|---|----------|
| | | Longitude | Latitude |
| 1 | Tinsukia | 95°22'E | 27°30'N |
| 2 | Dibrugarh | 94°54'E | 27°29'N |
| 3 | Sivasagar | 94°13'E | 26°59'N |
| 4 | Jorhat | 94°13'E | 26°45'N |
| 5 | Golaghat | 93°58'E | 26°31'N |
| 6 | Nagaon | 92°41'E | 26°21'N |
| 7 | Morigaon | 92°21'E | 26°15'N |
| 8 | Guwahati | 91°44'E | 26°8'N |
| 9 | Nalbari | 91°32'E | 26°36'N |
| 10 | Barpeta | 91°00'E | 26°32'N |
| 11 | Silapathar | 94°76'E | 26°60'N |
| 12 | Dhakuakhana | 94°45'E | 27°31'N |
| 13 | Tezpur | 92°79'E | 25°65'N |
| 14 | Rowta | 92°22'E | 26°71'N |
| 15 | Karbi Anglong | 91°26'E | 26°25'N |
| 16 | Jorabat | 91°86'E | 26°89'N |
| 17 | Dispur | 91°78'E | 26°14'N |
| 18 | Bezera | 91°77'E | 26°34'N |
| 19 | Baihata charali | 91°71'E | 26°34'N |
| 20 | Sipajhar | 91°89'E | 26°40'N |

Probiotic definition, according to World Health Organization (WHO) is “live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host” [5]. The European parliament and the council of the European Union have encouraged the development of alternative products to replace antibiotics as feed supplements for growth promotions [5, 7]. An effective and safe alternative to antibiotic implementation is the use of probiotics, which protects the individual from pathogens by enhancing the microbial balance in the gastro intestinal tract to exclude potentially harmful bacteria [8].

However, the characterization of probiotic microorganism from *O. smaragdina* has not been reported before. Taking into consideration that characterization of probiotic microbes from edible insects has not been adequately studied and also that North East India is a treasure trove of such traditional knowledge, the current study was undertaken for the very first time.

Materials and Methods

Chemicals and Reagents

Chemicals and consumables were procured from Merck India Pvt. Ltd. and all microbiological media used in this study were purchased from Hi Media India Pvt. Ltd.

Collection of Samples

Oecophylla smaragdina samples were collected during summer from 20 different areas of Assam and samples were kept in $-20\text{ }^{\circ}\text{C}$ for future use. The taxonomic identification of the collected insect samples was done in consultation with the Entomologists of the Department of Life Sciences, Dibrugarh University. Detailed geographical distributions of the sample collection sites are shown in Table 1.

Isolation and Screening of Probiotic Bacteria

Augmentation of samples were done by grounding 5 g of each sample into paste followed by inoculation in 100 ml of MRS (Man Rogosa Sharpe) broth maintained at pH-3 followed by incubation at $37\text{ }^{\circ}\text{C}$ and 135 rpm for 48 h. Two fold dilutions of the augmented samples were placed on MRS agar followed by incubation. Only the Gram positive and catalase negative isolates were allowed to be screened on Bile salt agar [9, 10].

Safety Assessment in Terms of Antibiotic Sensitivity Assay

Antibiotic sensitivity of the bacterial isolates was tested against commercially available antibiotic discs with

defined concentration as per CLSI (Clinical and Laboratory Standards Institute, USA) standards [11]. Isolates with sensitivity against maximum number of antibiotics were screened for further studies.

Evaluation of Antagonistic Effect of the Most Potential Isolates

The antimicrobial assay of the most potential isolates were carried out by agar well diffusion method by loading the cell free extract of the isolates against six pathogens viz., *B. cereus* (ATCC 11778), *B. subtilis* (ATCC 6051), *E. coli* (ATCC 25922), *L. monocytogenes* (ATCCBAA-751), *S. aureus* (MTCC 9542), and *Salmonella enterica* subsp. *enterica* Serovar Typhimurium (ATCC 49416). The most potential isolates were screened on the basis of the diameter of zone of inhibitions obtained after incubation.

In-Vitro Determination of Cell Surface Traits of the Isolate

Hydrophobicity Assay

Hydrophobicity of the bacterial isolate was assessed by the test microbial adhesion to hydrocarbon (MATH) as described by Del Re et al., 2000 [12]. Overnight bacterial broth was centrifuged at 10,000 rpm for 10 min and the pellet was re-suspended in 3 ml of 0.1 M KNO₃ solution and the absorbance (A₀) was measured at 600 nm. 1 ml of toluene was added to the cell pellet in order to form a two phase system and incubated for 10 min at room temperature followed by vortex for 2 min and again incubated for 30 min at room temperature until the water and toluene gets separated. Absorbance (A₁) of the aqueous phase was taken at 600 nm. Cell surface hydrophobicity was determined by the following formula [12]:

$$H = (1 - A_1/A_0) \times 100$$

where A₀ = OD of the aqueous phase at 600 nm before incubation, A₁ = OD of the aqueous phase at 600 nm after incubation.

Auto-Aggregation Assay

Auto-aggregation capabilities of the most potent isolates were carried out by the method of Del Re et al. [12]. The bacterial culture was harvested by centrifugation at 10,000 rpm for 10 min at 4 °C the pellet was washed twice with PBS and again re-suspended in PBS and adjusted the bacterial population to 10⁸ cfu/ml. 3 ml of the bacterial suspension was vortex for 10 s and incubated at 37 °C for 2 h. Absorbance of the supernatant before incubation (A_{0h}) and after 2 h of incubation (A_{2h}) at room temperature was

measured at 600 nm and calculated the auto-aggregation using following formula using following formula:

$$\text{Auto-aggregation (\%)} = (1 - A_{2h}/A_{0h}) \times 100$$

Lysozyme Susceptibility Test

Tolerance to lysozyme was assessed by spread plating the isolate on nutrient agar plates supplemented with lysozyme (400 µg/ml of molten nutrient agar just before casting), taking lysozyme free plates as control [13]. Luxuriant growth of the isolate just after 24 h of incubation at 37 °C indicates lysozyme tolerance of the isolate.

Safety Assessment in Terms of Haemolysis Assay

Hemolytic activity of the most potential isolate was performed using 5% (w/v) of defibrinated sheep blood (E & O Laboratories Ltd., New Delhi) in Blood Agar Base No 2 taking *Staphylococcus aureus* (MTCC 9542) as control [14].

Cytotoxicity Assay

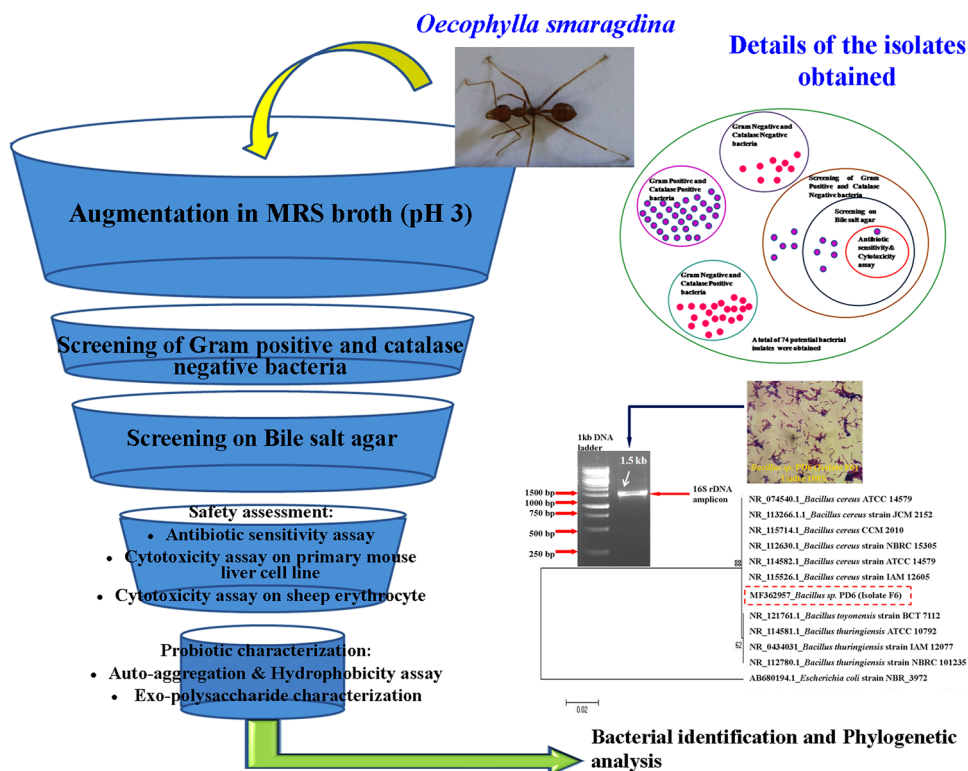
On Primary Liver Cell Line of Mouse (*Mus musculus*)

Cytotoxicity assay of the cell free extract of the potential isolate was done by MTT method against primary culture of liver cells (hepatocytes) of *Mus musculus* (albino mouse) maintained and locally bred in the institutional animal house facility. Only one such animal was sacrificed with essential ethical clearance for the experiment. Hepatic cells population was adjusted to 2x10⁶ cells/mL in Dulbecco's Modified Essential Media (DMEM) using a haemocytometer. 100µL of hepatic cells containing media were loaded into wells of a microtitre plate followed by addition of 100 µL cell free extract (CFE) of different dilutions (crude CFE, 1:1, 1:2,...1:7 in PBS) in each well except in the control well. Viability of the cells was observed after overnight incubation (36.5 °C, with 95% humidified air: 5% CO₂ incubator) in terms of absorbance measured at 600 nm using PBS as blank. Viability of the cells was also determined by transferring 50 µL of hepatic cell suspension mixed in equal volume of trypan blue stain to a haemocytometer. The number of cells per mL and viable cell percentage was calculated using the following formula [15]:

$$\text{Total cells/mL} = \frac{\text{Total cells counted} \times \text{Dilution Factor}}{\text{No. of squares in haemocytometer} \times 10000 \text{ cells}}$$

$$\% \text{ viability of cells} = \frac{\text{Live Cell Count}}{\text{Total Cell Count}} \times 100$$

Fig. 1 Overall flowchart of the study and details of the isolates fulfilling all the basic criteria to be considered as a potential probiotic candidate are represented in the form of Venn diagram



On Sheep Erythrocytes

The cytotoxicity assay at different concentration of CEF was further examined by detecting the haemolysis of sheep blood collected from the jugular vein [15]. Briefly, 3 mL of freshly obtained sheep blood was placed in heparinized tubes to avoid coagulation, gently mixed and poured into a sterile 15 mL Falcon tube and centrifuged for 5 min at 1000 rpm. The supernatant was poured off and RBCs were washed three times with 5 mL chilled sterile PBS solution adjusted to pH 7.4. The washed RBCs were suspended in 20 mL chilled PBS. The RBC count was maintained to 7×10^8 cells/mL PBS for each assay. Different dilutions (1:1, 1:2...1:6) of cell free extract in PBS (total volume 20 μ L) were taken in 2 mL Eppendorf tubes and then 180 μ L of blood cell suspension was added. The samples were incubated for 35 min at 37 °C. After incubation and agitation for 10 min, the tubes were placed on ice for 5 min and centrifuged at 1000 rpm for 5 min. After centrifugation, 100 μ L of supernatant was taken and diluted with 900 μ L chilled PBS. 200 μ L from each tube was transferred to 96 well plates. For each assay, 0.1% Triton X-100 was taken as a positive control and phosphate buffer saline (PBS) as a negative control. The absorbance of each tube was recorded at 600 nm.

Biochemical Characterization and Molecular Identification of the Isolate

Gelatin hydrolysis, Indole, methyl red, Voges Prokauer test, citrate utilization, nitrate utilization, oxidase, starch hydrolysis, Urease, and sugar fermentation (Arbinose, Fructose, Galactose, Glucose, Lactose, Maltose, Mannitol, Sucrose, Xylose) test were performed by standard method [16]. Grams staining and endospore staining were also performed for phenotypic identification of the isolate.

The most potential probiotic isolate was finally identified by 16S rRNA gene sequencing by outsourcing the isolate to Pentavalent Bio Sciences Ltd., Bangalore and the consensus sequence hence generated was aligned by using multiple alignment software program ClustalWTM [17]. Phylogenetic tree was constructed using MEGA6 software by Neighbour-Joining method with 1000 bootstrap value forcing *E. coli* NBR 3972 (GenBank accession no. AB680194.1) as out group.

Extraction and Characterization of Bacterial Exo-Polysaccharide (EPS)

Bacterial EPS was extracted by cold acetone precipitation method followed by the evaporation of the solvent by freeze drying [18]. The bacterial EPS characterized by FTIR (Fourier transform infrared spectroscopy) along with a standard dextran sulfate (DS) using Perkin-Elmer FTIR

Table 2 Antibiotic sensitivity assay result of the isolate F6

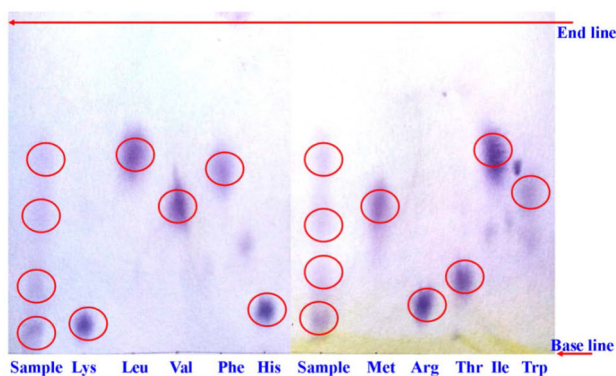
| Sl. no. | Antibiotic | Abbreviation | Concentration | Resistant (R)/sensitive (S) | Zone of inhibition (mm) |
|---------|-----------------|--------------------|---------------|-----------------------------|-------------------------|
| 1 | Ampicillin | AMP ¹⁰ | 10 mcg/discs | S | 8 ± 2 |
| 2 | Amikacin | AK ³⁰ | 30 mcg/disc | S | 17 ± 3 |
| 3 | Ceftriaxone | CTR ³⁰ | 30 mcg/discs | R | – |
| 4 | Ceftazidime | CAZ ³⁰ | 30 mcg/discs | R | – |
| 5 | Ciprofloxacin | CF ³⁰ | 30 mcg/discs | S | 25 ± 3 |
| 6 | Chloramphenicol | C ³⁰ | 30 mcg/discs | S | 24 ± 2 |
| 7 | Clindamycin | CD ² | 2 mcg/disc | R | – |
| 8 | Erythromycin | E ¹⁵ | 15 mcg/disc | S | 10 ± 2 |
| 9 | Gentamicin | GEN ¹⁰ | 10 mcg/disc | S | 9 ± 2 |
| 10 | Kanamycin | K ³⁰ | 30 mcg/disc | S | 15 ± 3 |
| 11 | Penicillin | P ² | 2 unit/disc | S | 15 ± 2 |
| 12 | Streptomycine | HLS ³⁰⁰ | 300 mcg/disc | S | 18 ± 3 |
| 13 | Vancomycin | VA ³⁰ | 30 mcg/disc | S | 17 ± 3 |

* Results are in mean ± SD

Table 3 Antagonistic activity of the isolate F6 against different bacterial strain in terms of zone of inhibition

| Sl. No. | Bacterial strains | Zone of inhibition (mm) including diameter of well |
|---------|-------------------------------------|--|
| 1 | <i>B. cereus</i> ATCC 11778 | 15 ± 2 |
| 2 | <i>E. coli</i> ATCC 25922 | 13 ± 3 |
| 3 | <i>L. monocytogenes</i> ATCC BAA751 | 17 ± 2 |

* Results are in mean ± SD

**Fig. 2** Paper chromatogram showing four distinct spots representing the presence of four essential amino acids present in the CFE of the isolate F6 (sample)

instrument by mixing the EPS with 99 parts of dried potassium bromide (KBr) and IR spectra was measured in the frequency range of 400 and 4000 cm^{-1} (Nanda et al., 2014). Estimation of carbohydrate in the EPS was estimated by Anthrone method [19].

Estimation of Protein and Essential Amino Acids in the Bacterial Cell Free Extract

Protein present in the cell free extract of the sample was estimated by Lowry's method [18]. Bacterial isolate was allowed to grown in Minimal broth (composition (g/l): Dextrose-1.0, K_2HPO_4 -7, KH_2PO_4 -2.0, $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ -0.5, MgSO_4 -0.1, $(\text{NH}_4)_2\text{SO}_4$ -1.0, pH-7.0 ± 0.2). Presence of essential amino acids in the CFE was estimated by paper chromatography [19].

Growth Profiling at Different pH

The most potential probiotic strain was inoculated at Luria–Bertani broth, maintained in a pH range of 1–10 in different test tubes. The growth was monitored at 1 h intervals at 600 nm against suitable blank and the viability of the cells was confirmed by streaking on MRS agar plates followed by incubation.

Overall flowchart of the study is shown in Fig. 1.

Statistical Analysis

All experiments were performed in triplicates and the results were expressed in terms of mean ± SD. One-way ANOVA was applied to the results. Differences in means were considered to be significant for p values < 0.05. A Student's t test was used for statistical comparisons of two means and was done by Graph padTM online tool (<http://www.graphpad.com>).

Fig. 3 Cytotoxicity assay results shows viability rate (%) of healthy erythrocytes in different dilutions of CFE of the isolate F6. Inset figure shows haemolysis test results of the isolate F6 on blood agar

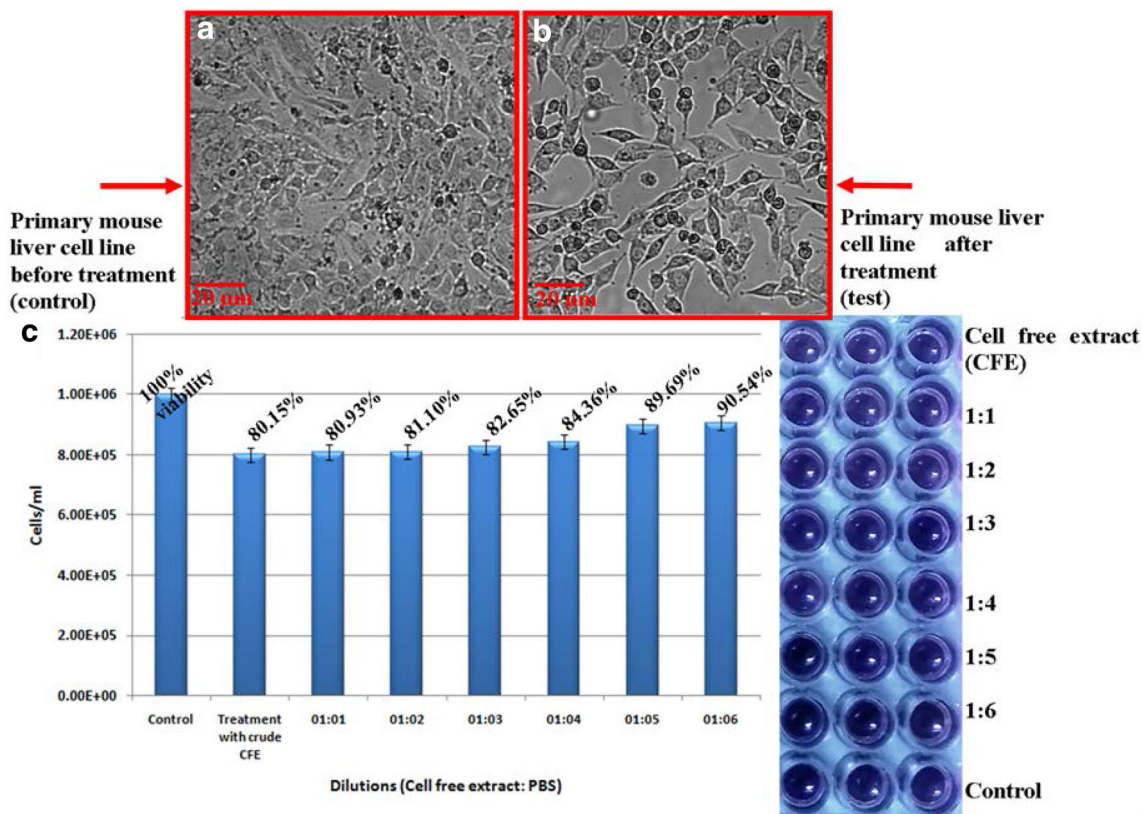
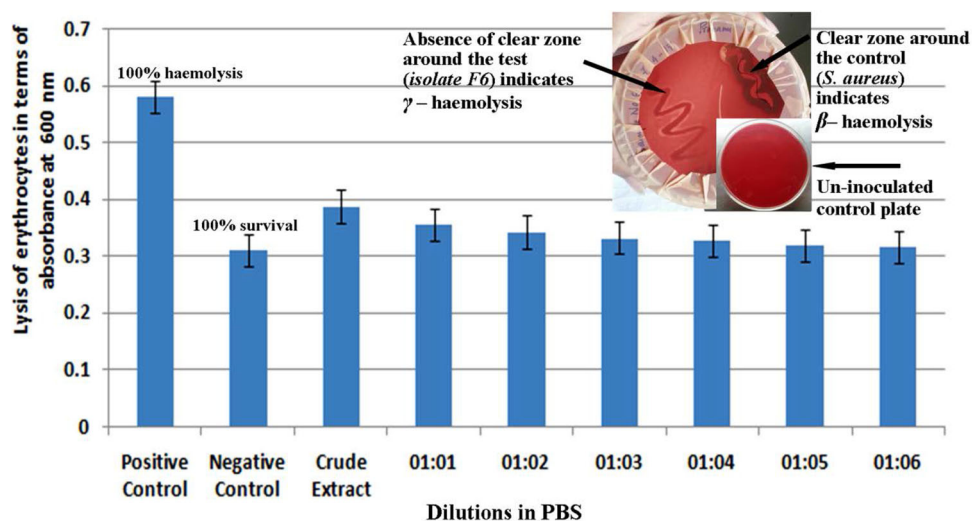


Fig. 4 a-Primary mouse liver cell line before treatment and b-after treatment with crude CFE of isolate F6. c-shows cytotoxicity assay results of primary mouse liver cell line in terms of viability rate (%) at

different dilutions of CFE in PBS along with the colour intensity in respective micro-titre plate (inset)

Results and Discussion

Isolation and Screening of Potential Probiotic Microorganisms

A total of 74 different types of bacterial colonies on the basis of their colony morphology were obtained after initial

screening of all the isolates on MRS agar, maintained at pH 3, whereas fungal colonies and yeast-like colonies were not obtained. Out of these, 11 colonies were found to be Gram positive and catalase negative in nature and only six of these isolates could hydrolyze the bile salt agar. Antibiotic susceptibility test result shows that the isolate F6 has susceptibility against maximum number of antibiotics viz.,

Table 4 Biochemical test results

| Sl. No. | Name of the biochemical test | Results |
|--------------------------------|------------------------------|---------|
| 1 | Catalase production | - |
| 2 | Voges Proskauer(VP) | + |
| 3 | Methyl Red | + |
| 4 | Indole production | - |
| 5 | Citrate utilization | + |
| 6 | Starch hydrolysis | + |
| 7 | Urease production | + |
| 8 | Gelatin hydrolysis | + |
| <i>Sugar fermentation test</i> | | |
| 9 | Glucose | + |
| 10 | Fructose | + |
| 11 | Galactose | + |
| 12 | Maltose | + |
| 13 | Arbinose | - |
| 14 | Lactose | + |
| 15 | Mannitol | - |
| 16 | Sucrose | + |
| 17 | Xylose | - |
| 18 | Glucose | + |

Ampicillin, Amikacin, Ceftriaxone, Ceftazidime, Ciproflaxacin, Chloramphenicol, Erythromycin, Gentamicin, Kanamycin, Penicillin, Streptomycin and Vancomycin as shown in Table 2. The isolates fulfilling all the basic criteria to be considered as a potential probiotic candidate is

Fig. 5 Phylogenetic analysis of the isolate F6 (*Bacillus* sp. PD6) with 10 most closely related species shows 88% similarity index. The tree was constructed by Neighbour-Joining method and bootstrap value was set at 1000 by forcing *E. coli* strain NBR_3972 as out group and the scale bar indicates the number of nucleotide substitution per site

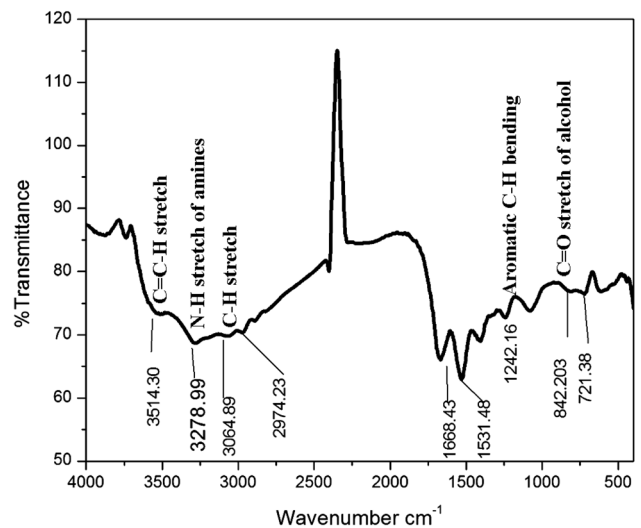
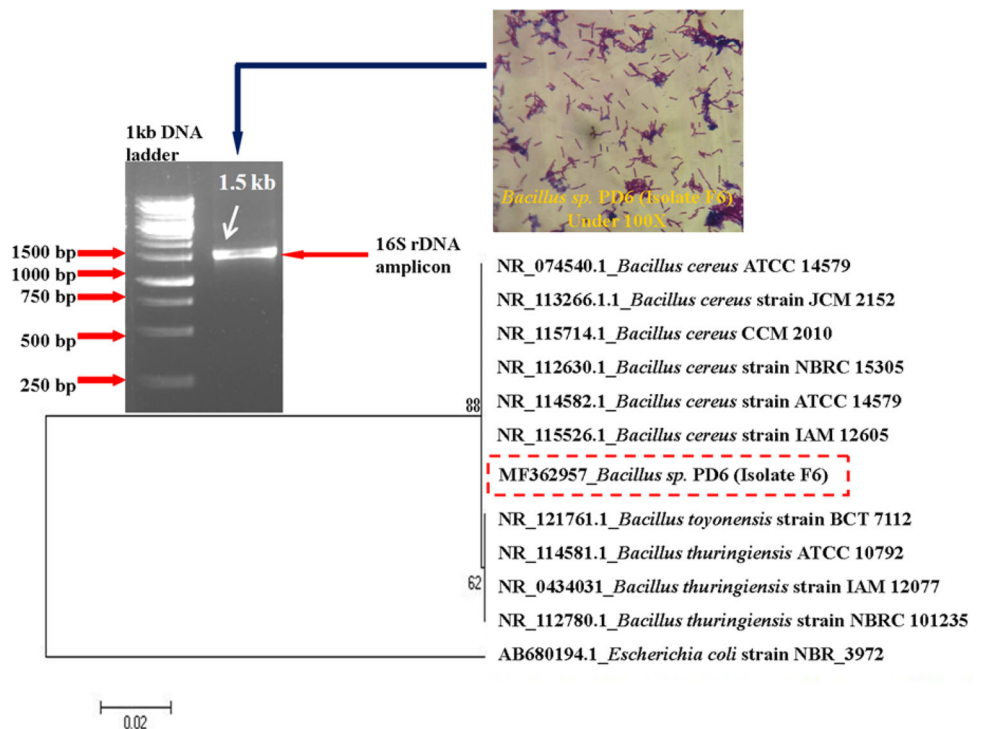


Fig. 6 FTIR absorption analysis of exo-polysaccharide produced by the most potential probiotic isolate F6 (*Bacillus* sp. PD6)

represented by a Venn diagram within the overall flowchart of the study (Fig. 1).

Further, the cell free extract of the isolate F6 shows antimicrobial activity against *B. cereus*, *E. coli* and *L. monocytogenes*. The zone of inhibitions created by the CFE of the isolate is shown in Table 3.

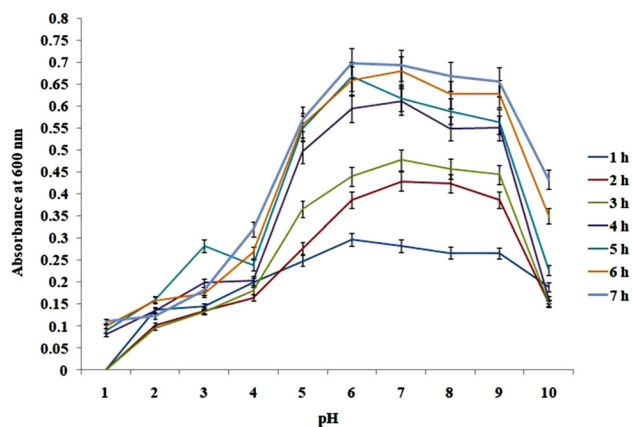


Fig. 7 Growth profile of the isolate F6 (*Bacillus* sp. PD6) in a pH range of 1–10

Nutritional and Cell Surface Trait Analysis of the Most Potential Probiotic Candidate

The CFE shows the presence of 1.9 ± 0.22 mg/mL of protein content and also shows the presence of essential amino acids viz, lysine, valine, leucine, isoleucine, and threonine (Fig. 2). In-vitro cell surface trait analysis of the isolate shows $46.2 \pm 0.32\%$ hydrophobicity and $61.41 \pm 0.28\%$ auto-aggregation property. Moreover, it shows resistance against lysozymal degradation.

Safety Assessment

As a part of safety assessment, the isolate shows no hemolysis on blood agar as well as no cytotoxicity by the cell free extract (CFE) of the isolate on sheep erythrocytes (Fig. 3). Also, the crude CFE of isolate F6 shows no cytotoxicity on primary mouse liver cell lines (Fig. 4).

Identification of the Most Potential Isolate

The isolate F6 was initially identified on the basis of staining and biochemical tests which show it as Gram positive rod shaped bacteria, capable of forming endospores. It gives positive results for MR-VP, citrate utilization, starch hydrolysis, urease production and gelatin hydrolysis test whereas negative for catalase and Indole test (Table 4). It also ferments glucose, fructose, galactose, maltose, lactose, and sucrose (Table 4). 16S rRNA sequencing of the most potential isolate F6 confirms it as a novel strain of *Bacillus* sp. PD6 with 88% sequence similarity with 10 most closely related *Bacillus* sp. (GenBank accession no. MF362957) (Fig. 5).

Characterization of Bacterial EPS

Appearance of intense green colour during Anthrone test confirms the presence of carbohydrates in the bacterial exopolysaccharide and FTIR spectra in Fig. 6 shows the presence of $\nu_{C=C-H}$ stretch (3514.30 cm^{-1}), ν_{N-H} stretch (3278.99 cm^{-1}), ν_{C-H} stretch (3064.89 and 2974.23 cm^{-1}), ν_{C-H} bending (1242.16 cm^{-1}), $\nu_{C=O}$ stretch (842.203 and 721.38 cm^{-1}).

Growth Characterization of the Most Potential Isolate in Different pH

Evaluation of growth of the potential probiotic isolate in different pH shows the maximum growth at pH 6 after as 7 h of incubation but significant growth even in a wide range of pH 1–10 (Fig. 7).

Isolation and characterization of probiotic properties of *Bacillus* sp. PD6 from an edible insect *Oecophylla smaragdina* of North East, India, was carried out for the very first time [20]. Microorganisms to be applied as probiotic must overcome the uncongenial conditions of human gastrointestinal tract (GIT) and subsequently colonize the intestinal tract. Therefore, the pH of gastric juice is the main factor that determines the survival of bacteria that pass from the stomach to the intestine. In this study, the potential bacterial isolate *Bacillus* sp. PD6 (GenBank accession no. MF362957) shows significant growth in a pH range of 1–10 indicating its potentiality to survive in GI tract of host [21]. Bile plays a fundamental role in specific and non-specific defense mechanisms in gut [21, 22]. The isolates F6 which was later identified as *Bacillus* sp. PD6 was capable of hydrolyzing bile esculine agar (4% bile) whereas in human body the relevant physiological concentrations of human bile ranges from 0.3 to 0.5% only [23].

A key property of probiotic strain is that they should not carry any transmissible antibiotic-resistant genes [23]. Results from this study shows that potential probiotic candidate is susceptible to eight antibiotics to the entire broad and narrow spectrum antibiotics tested (Table 2). On the other hand the novel isolate *Bacillus* sp. PD6 exhibits antagonistic activity against both gram positive (*B. cereus* ATCC 11778 and *L. monocytogenes* ATCC BAA751) and negative pathogen (*E. coli* ATCC 25922).

It is universally recognized that probiotics must be able to colonize the digestive tract to adhere and colonize the intestine apart from being resistant to GI condition which is considered as a crucial factor for the selection of new probiotics [24–26]. The studied bacterial isolate shows

Table 5 Some of the recent reports on probiotic characterization of various microbial isolates

| Sl No. | Name of the bacteria | Isolation source | In-vitro cell surface property | | Antimicrobial assay | Zone of inhibition (mm) | Lists of essential amino acids released by the isolate(s) | References |
|--------|---|--|---------------------------------------|--------------------------------------|--|--------------------------------------|---|------------|
| | | | Hydrophobicity (%) | Autoaggregation (%) | | | | |
| 1 | <i>B. subtilis</i> | Gut of fish (<i>Labeo rohita</i>) | – | – | <i>A. hydrophila</i> <i>A. submonitidia</i> <i>V. harveyi</i> <i>V. alginolyticus</i> | >5 mm >2 mm >2 mm – | – | [33] |
| 2 | <i>S. succinus</i> | Faeces of various human group | 69.11 | – | <i>E. coli</i> <i>Salmonella</i> sp. <i>Enterococcus</i> sp. <i>Klebsiella</i> sp. <i>S. aureus</i> | – 4 ± 0.7 – – 5 ± 0.6 | – | [34] |
| 3 | <i>E. fecium</i> | | 30.12 | – | <i>E. coli</i> <i>Salmonella</i> sp. <i>Enterococcus</i> sp. <i>Klebsiella</i> sp. | 5 ± 0.6 10 ± 1.2 12 ± 0.9 – | – | [34] |
| 4 | <i>L. plantarum</i> | Fermented foods & beverages | 35.74 | 15.45/3 h | <i>S. aureus</i> <i>S. aureus</i> <i>B. cereus</i> <i>S. dysenteriae</i> | 4 ± 0.2 >5 mm >5 mm >5 mm | – | [10] |
| 5 | <i>L. mesenteroides</i> <i>L. plantarum</i> | Kimchi, traditional fermented food | – | – | – | – | – | [35] |
| 6 | <i>Bacillus</i> sp. strain MKSK-E1 | Korean traditional soy sauce | 25.7 ± 10.0 | 89.6 ± 2.0 | <i>S. aureus</i> , <i>B. cereus</i> , <i>E. coli</i> , <i>S. sonnei</i> , <i>S. flexneri</i> , <i>Pseudo. fluorescens</i> , and <i>K. pneumoniae</i> | – | – | [35] |
| 7 | <i>L. plantarum</i> <i>L. plantarum</i> <i>Fructobacillus fructosus</i> | <i>Opuntia ficus-indica</i> fruits | 10.2 ± 0.5 3.5 ± 0.3 64.3 ± 3.7 | 1.5 ± 0.1 9.9 ± 0.3 25.1 ± 2.1 | <i>Bacillus</i> sp. | 4.0 2.0 3.0 | – | [36] |
| 8 | <i>Bacillus subtilis</i> subsp. spizizenii ATCC 6633 | Purchased from the Iranian Organizations for Science and Technology (Tehran, Iran) | – | – | <i>C. perfringens</i> | – | – | [37] |
| 9 | <i>Bacillus subtilis</i> | Cheonggukjang (Sobean fermented food) | – | 49.18 ± 0.99 | – | – | – | [38] |

Table 5 continued

| Sl No. | Name of the bacteria | Isolation source | In-vitro cell surface property | | Antimicrobial assay | | Zone of inhibition (mm) | Lists of essential amino acids released by the isolate(s) | References |
|--------|--------------------------------------|------------------------------|--------------------------------|---------------------|--|---|--|---|------------|
| | | | Hydrophobicity (%) | Autoaggregation (%) | Test microbes | | | | |
| 10 | <i>Bacillus velezensis</i> strain JW | Fish | – | – | <i>A. hydrophila</i> , <i>Aeromonas salmonicida</i> , <i>Lactococcus garvieae</i> , <i>Streptococcus agalactiae</i> , and <i>Vibrio parahaemolyticus</i> | – | – | [39] | |
| 11 | <i>Bacillus</i> sp. | <i>Oecophylla smaragdina</i> | 61.41 ± 0.28 | 46.2 ± 0.32 | <i>E. coli</i> <i>B. cereus</i> <i>L. monocytogenes</i> <i>B. subtilis</i> <i>S. aureus</i> <i>S. typhimurium</i> | 13 ± 3 15 ± 2 17 ± 2 – – – | lysine, valine, leucine, isoleucine, and threonine | In this study | |

concurrent or even higher percentages of hydrophobicity ($46.2 \pm 0.32\%$) and auto-aggregation ($61.41 \pm 0.28\%$) compared to other reported probiotic isolates (Table 5) [10, 27, 28]. The comparison table shows *S. succinus* with highest value of hydrophobicity (69.11%) but shows poor antimicrobial potential as compared to the current isolate *Bacillus* sp. PD6. The hydrophobic and hydrophilic properties are a result from proteins and exo-polysaccharides on the bacterial cell surface and the CFE of the isolate shows the presence of 1.9 ± 0.22 mg/mL of protein along with five essential amino acids advocate its nutraceutical importance also [25]. Presence of sugar moiety in the bacterial EPS was evaluated by Anthrone method and FTIR spectra also confirms the presence of $\nu_{C=C-H}$ stretch, ν_{N-H} stretch, ν_{C-H} stretch, ν_{C-H} bending and $\nu_{C=O}$ stretch functional groups which correlates with the findings of other researchers [18]. It may also be noted that EPS is one of the key factors that helps in cell adhesion with host intestinal lining and FTIR analysis attributes important data for chemical characterization of EPS produced by the bacterial cells [29].

Swallowed probiotic microbes encounter the first biological barrier of lysozyme of saliva in the mouth but the isolate can successfully tolerate lysozymal activity making it suitable for oral administration [13, 30]. On the other hand, non cytotoxicity is one of the most important criteria to be considered as a potential probiotic candidate [31, 32]. The potential isolate *Bacillus* sp. PD6 shows no haemolysis on blood agar and further different dilutions of the CFE of the isolate shows significant level of survival as compared to positive control which represents 100% haemolysis and negative control with 100% survival of sheep erythrocytes. Further, the crude CFE itself shows $80.15 \pm 1.02\%$ viability in primary mouse liver cell line makes it suitable to be considered as potential probiotic candidate.

In conclusion, the probiotic characterization of edible insect *Oecophylla smaragdina* was carried out for the very first time. The novel isolate *Bacillus* sp. PD6 found to be fulfilled all the basic characteristics to be considered as a potential probiotic with the ability to produce exopolysaccharides. Moreover, the cell free extract of the isolate was found to be non-cytotoxic against primary mouse liver cell line and sheep erythrocytes makes it safe and suitable for further commercial exploitation.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

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