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Isolation and characterization of the new indigenous *Staphylococcus* sp. DBOCP06 as a probiotic bacterium from traditionally fermented fish and meat products of Assam state



Debajit Borah ^{a,*}, Olee Gogoi ^a, Chanakya Adhikari ^a, B.B. Kakoti ^{a,b}

^a Centre for Studies in Biotechnology, Dibrugarh University, 786004, India

^b Department of Pharmaceutical Sciences, Dibrugarh University, 786004, India

ARTICLE INFO

Article history:

Received 29 April 2016

Received in revised form 5 June 2016

Accepted 10 June 2016

Available online 17 June 2016

Keywords:

Probiotic strain

Staphylococcus sp. DBOCP6

Antagonistic effect

Gastrointestinal tract

Commercial potentiality

ABSTRACT

Traditionally fermented fish and meat products are known for their probiotic values. An approach was made for the very first time to isolate and characterize novel indigenous probiotic bacterial strains from traditionally fermented fish and meat products of Assam, India, which is known as the biodiversity hotspot of the country with largely unexplored economically important microorganisms. The most potent probiotic isolate was identified as *Staphylococcus* sp. DBOCP6 (GenBank accession no. KR706310) on the basis of 16S rDNA sequencing technique. The new isolate was found to be non-hemolytic and nonpathogenic on the basis of its susceptibility against broad and narrow spectrum antibiotics. It shows antagonistic effect against *E. coli* MTCC 40. Its viability in presence of lysozyme, bile salt (4%), and a pH range of 1–10 signifies its ability to survive throughout the gastrointestinal tract. It also shows significant value of hydrophobicity (33.1%) along with a higher value of auto-aggregation (71.43%) indicates its ability to adhere to the intestinal wall on the basis of its cell surface traits. The high thermal death point of the isolate (100 °C) indicates its suitability for further commercial exploitation. Based on the above observations, it could be concluded that the novel *Staphylococcus* sp. strain DBOCP6 can serve as a potential probiotic candidate for industrial exploitation.

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1. Introduction

Assam, the state of North-East India is one of the richest site for fresh water fishes and largest dry fish production site in

Asia [1,2]. Moreover, the Northeast region of India is considered as a biodiversity hotspot for flora and fauna, particularly of economically important microbes, that are yet to be explored [3]. The fish and meat are the important source of food across the world. The ethnic people of Assam traditionally

* Corresponding author. Tel.: +91-9706394994.

E-mail address: dborah89@gmail.com (D. Borah).

<http://dx.doi.org/10.1016/j.ejbas.2016.06.001>

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ferment and produce different preparations from the fish and meat such as xukati, hidal, and other dried fish and meat products [4].

It is a well established fact that fermented foods are enriched with health beneficial probiotic microbes. Probiotics have been defined as “a live microbial food supplement that benefits the host by improving the intestinal microbial balance,” and more broadly, as live microorganisms which exerts positive health benefits [5,6]. Metchnikoff in 1907 who first implied that ingested bacteria in the form of fermented foods could beneficially affect the normal gut flora [7]. These properties include the beneficial influences that probiotics apparently exert on the microbial ecology of the host [8,9]. The physicochemical conditions that persuade the composition of the intestinal microbiota include intestinal motility, pH, redox potential, nutrient materials, host secretions (e.g. digestive enzymes, hydrochloric acid, bile and mucus), and the presence of an intact ileocaecal valve [10]. Thus, the gastrointestinal (GI) tract harbors many distinct niches, with diverse microbial ecosystem that increases along the GI tract [11]. Apart from supporting the digestive functions, indigenous microbes also act as a main actor of the immune system by preventing the colonization by pathogenic microorganisms [11–13]. The European Parliament and the Council of the European Union also encourages development of suitable alternative to replace antibiotics [14,15]. The use of probiotics is an effective and safe alternative to antibiotic implementation [14–17]. Since the beneficial effects of probiotic bacteria is mainly centralized in the GI tract, the probiotics should exhibit good surface hydrophobicity and aggregation properties to adhere and colonize in the GI tract [18,19].

Numerous studies from subsequent research programs have reported a considerable number of species belonging to *Lactobacillus*, *Bifidobacterium*, and *Enterococcus* that are being typically

used as probiotic bacteria in procurement of yogurt and cheese [20]. The microbiota of fermented fish and meat, the ethnic food of Assam, India is almost next to untouched. Thus, an attempt was made to identify potent indigenous probiotic candidate in traditionally prepared fermented fish and meat products through a comprehensive approach which would originally consider the safety features of the strain and potential application of the isolated strain and finally its ability to survive through the entire GIT in order to deliver the health benefits.

2. Experiment and method

2.1. Materials

Chemicals and consumables used in the study were purchased from Merck India Pvt. Ltd., and all the microbiological media were purchased from HiMedia India Pvt. Ltd. Bacterial growth was monitored at 600 nm wave length using UV-Vis spectrophotometer (Shimadzu UV-1800, Japan). Centrifugations were carried out in Sigma 3-30K centrifuge, Germany.

2.2. Collection of fermented fish and meat samples

20 traditionally prepared fermented fish and meat samples were collected from different tribes, communities, and local markets of the state Assam, India. Details of the collected samples and their geographical distribution are shown in Table 1 and Fig. 1.

2.3. Isolation and screening of probiotic bacteria from the collected samples

10% (w/v) of finely powdered each of the fish and meat samples were inoculated individually in 100 mL MRS (de Man Rogosa

Table 1 – List of samples with the details of the collection sites.

Local name of the preparation	Key mode of preparation	Name of the collection sites	Geographical position of the sample collection sites	
			Longitude	Latitude
Hukati	Smoke dried, crushed & fermented in bamboo tubes	Dibrugarh	94° 54'E	27° 29'N
Hukati	Sun dried	Sonapur	91° 45'E	26° 10'N
Hukati	Sun dried	Sonapur	91° 45'E	26° 10'N
Hukati	Sun dried	Sonapur	91° 45'E	26° 10'N
Hukati	Sun dried	Sonapur	91° 45'E	26° 10'N
Hukati	Sun dried	Sonapur	91° 45'E	26° 10'N
Hukati	Sun dried	Nagaon	92° 41'E	26° 21'N
Hukati	Sun dried	Nagaon	92° 41'E	26° 21'N
Hukati	Smoke dried, crushed & fermented in bamboo tubes	Jorhat	94° 13'E	26° 45'N
Hukati	Smoke dried, crushed & fermented in bamboo tubes	Lakhimpur	94° 15'E	27° 14'N
Hidal	Sun dried	Guwahati	91° 44'E	26° 11'N
Hidal	Sun dried	Guwahati	91° 44'E	26° 11'N
Hidal	Sun dried	Guwahati	91° 44'E	26° 11'N
Hidal	Pressed and fermented in earthen pots	Goalpara	90° 37'E	26° 10'N
Halis	Sun dried	Guwahati	91° 44'E	26° 11'N
Halis	Pressed and fermented in earthen pots	Goalpara	90° 37'E	26° 10'N
Wild chicken	Sun dried	North Lakhimpur	94° 07'E	27° 14'N
Halis	Sun dried	Dergaon	93° 58'E	26° 31'N
Mutton	Sun dried	Dhemaji	94° 20'E	27° 53'N
Pork	Sun dried	Tinsukia	95° 22'E	27° 30'N

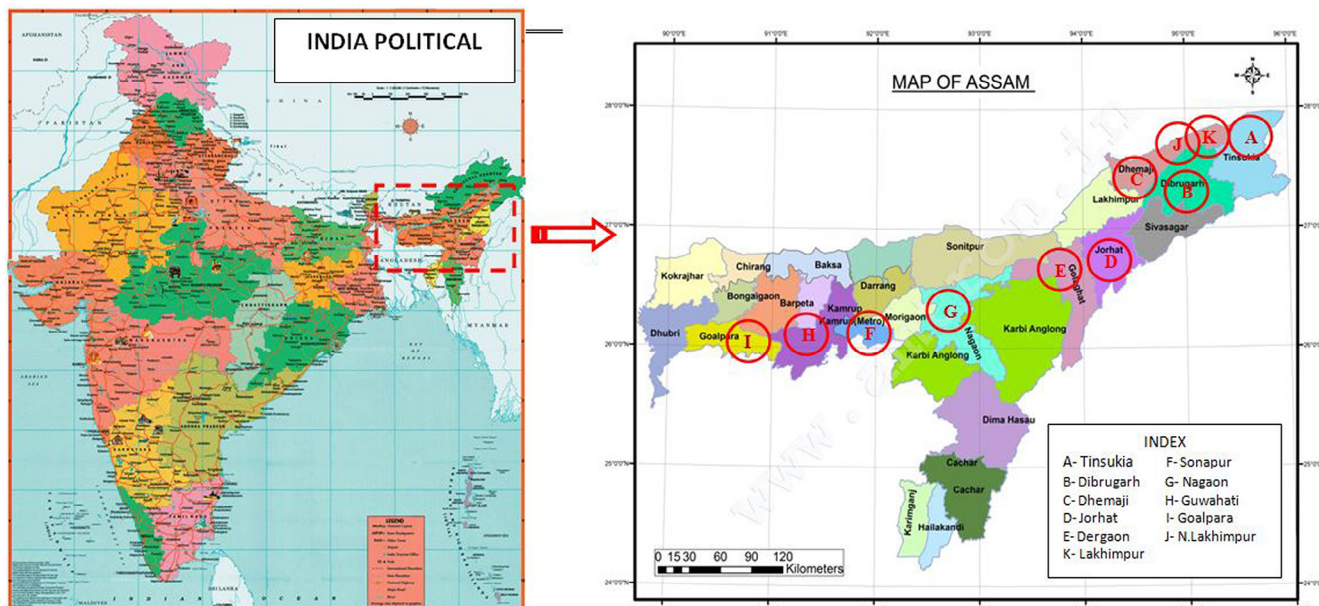


Fig. 1 – Geographical location of the sample collection sites shown in the map.

Sharpe) broth maintained at pH –3 and incubated at 37 ± 2 °C at 135 rpm for 24 hr (CERTOMAT@BS-1 shaker incubator, Sartorius Stedim Germany Ltd.). These were then inoculated on MRS agar (pH-3) plates to obtain acid tolerant bacterial strains. These were further screened by inoculated on bile esculin agar media. Nonpathogenic probiotic strains were screened by treating isolates with broad and narrow spectrum commercially available antibiotic discs belonging to the clinically most relevant antibiotic classes, viz., ciprofloxacin (5 mcg), kanamycin (30 mcg), ampicillin (10 mcg), clindamycin (2 mcg), vancomycin (30 mcg), erythromycin (15 mcg), gentamicin (10 mcg), and tetracycline (30 mcg) by Kirby Bauer's disc diffusion method.

2.4. Viability in acidic and alkaline conditions

The viability of the probiotic candidates were further evaluated in a pH range of 1–10 individually. The growth was measured at absorbance 600 nm at an interval of 1h, and the viability of the cells was confirmed by inoculating onto MRS agar plates by spread plate method.

2.5. Hemolysis assay

Hemolytic activity of the isolates were evaluated using Blood agar base No.2 plates containing 5% (v/v) defibrinated sheep blood, and incubated at 37 °C for 24 h. The assay was performed in triplicate [21].

2.6. Test for the production of bacteriocin

The most potent isolate was grown in MRS broth at 37 °C for 48 h and were harvested at 10,000 rpm for 5 min at 4 °C. The supernatant was heated to 70 °C (to inactivate proteases) and concentrated to 1/10th volume by rotary vacuum evaporator. This cell-free supernatant concentrate (CFSC), passed through

0.22 μ m membrane filters (Millipore, Germany) and evaluated for its antimicrobial activities [22]. The antimicrobial activity was evaluated by agar-well diffusion assay. Briefly, the MH agar medium was poured onto the petri dishes and upon solidification the test organism *E. coli* MTCC 40 (0.5 McFarland standard dilution) was inoculated onto each plate. Wells with 8 mm diameter were made in the plates and filled with 50 μ L of cell free content of the culture of the strain and left to diffuse for 18 hours at 37 ± 2 °C overnight and were examined for zone of inhibition around the wells.

2.7. Evaluation of antagonistic property in terms of minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) the probiotic isolate against *E. coli* (MTCC40)

Aliquots of the overnight broth of the potential isolate (O.D. = 1.0) was inoculated (40, 80, 120, 160, and 200 μ L) to a total volume of 10 mL overnight MRS broth of *E. coli* MTCC40 (as per 0.5 McFarland standard dilution) individually at different test tubes maintained at 135 rpm at 37 °C for the determination of MIC and MBC values. The growth of the pathogen was monitored by measuring the OD at 600 nm at each interval of 2 h. The viability of the test organism was further examined by inoculating on nutrient agar plates by spread plating method.

2.8. Molecular identification of the most potent isolate

The potential probiotic isolate was identified on the basis of various techniques prescribed by Bergey's Manual of Systematic Bacteriology and finally by 16S rDNA sequencing. 16S rDNA fragment of the isolate was amplified by using forward 704F (5'-GTA GCG GTG AAA TGC GTA GA-3') and reverse 907R (5'-CCG TCA ATT CMT TTG AGT TT-3') primer, and a 909 bp long consensus sequence was generated using aligner software. The 16S rDNA sequence was used to carry out BLAST with the

non-redundant 16S rRNA sequence (bacteria and archaea) database of NCBI GenBank. First ten sequences were selected on the basis of maximum identity scores and aligned using multiple alignment software program ClustalW™. The phylogenetic tree was constructed using MEGA6 software using the maximum likelihood method. The bootstrap value was set at 1000, and percentage values are given at the nodes [23–25]. The 16S rDNA sequence obtained was submitted to GenBank database. The evolutionary distances were computed using the Kimura 2-parameter method [26].

2.9. In-vitro determination of cell surface traits of the most potent isolate

2.9.1. Hydrophobicity assay

Bacterial cell surface hydrophobicity was measured by determining the microbial adhesion to hydrocarbons using the protocol described by Crow et al. with minor modification [27]. Briefly, overnight bacterial culture was centrifuged at 10,000X g for 5 min. The pellet was washed twice in phosphate buffer saline (PBS) and suspended in 3 mL of 0.1M KNO₃ solution. The absorbance was measured (A₀) at 600 nm. 1 mL of toluene was added to the cell suspension in order to form a two-phase system. The two-phase system was vortex for 2 min after 10 min of pre-incubation at room temperature. After 30 min of incubation at room temperature, the water and toluene phases got separated. The aqueous phase was then carefully separated and the absorbance was measured at 600 nm (A₁). The percentage of the cell surface hydrophobicity (H) was calculated using the following formula [28]:

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

2.9.2. Auto-aggregation assay of the potential probiotic isolate with other pathogens

The specific cell–cell interactions were determined using auto-aggregation assay [18]. The bacterial cells were harvested at 5000X g for 10 min at 4 °C and washed with PBS and re-suspended in PBS to 10⁸ cfu/mL. For the auto-aggregation assay, 3 mL of each bacterial suspension was vortex for 10 seconds and incubated at 37 °C for 2 h. The absorbance of the supernatant was measured at 600 nm using spectrophotometer. The auto-aggregation was calculated with the following [29]:

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

Where, A_{2h} = absorbance at 600 nm two hours of incubation

and A_{0h} = absorbance at 600 nm zero hour

2.9.3. Lysozyme susceptibility test

Fresh 30 mL un-inoculated LB broth with 9 mL of egg white lysozyme was inoculated in 3 mL of overnight culture of the most potent isolate and incubated at 37 °C for 1 h. The survival of the isolate was tested by plating on nutrient agar plates [30].

2.9.4. Determination of thermal death point of the potential isolate

5 mL of overnight most potent bacterial isolate was treated at different temperatures for 10 min then streaked onto nutrient

agar plate and incubated overnight at 37 ± 2 °C overnight to see the temperature at which growth ceases [31].

2.10. Statistical analysis

All the experiments were performed in triplicates and results were expressed in terms of mean ± SD. Student's t-test was performed to see the significance of the results obtained.

3. Results

A total of 120 probiotic bacterial candidates were isolated on MRS agar plates maintained at pH 3. The strains were further screened on the basis of their ability to grow on bile esculin agar (Fig. 2a). Only 12 strains were considered for further studies on the basis of their satisfactory growth on bile esculin agar. The tested strains displayed no hemolysis (γ-hemolysis) when challenged with human blood (Fig. 2b) indicate its nonpathogenic character [32].

The isolates were screened for nonpathogenic strains on the basis of their susceptibility against standard clinically significant antibiotics. The most potent isolate was found to be sensitive against all the antibiotics used in the study. The candidate probiotic strain was not found to be producing bacteriocin. As far as direct pathogen inhibition is apprehensive, many probiotic LAB have been observed to produce antimicrobial substances, and mainly organic acids, especially lactic and acetic acids, etc. [29]. The antagonistic property of the potent probiotic candidate was checked against the most common GI tract bacterial species *E. coli* (MTCC40). The MIC and MBC values of the potent probiotic candidate were found to be 120 and 200 μL/mL, respectively, against *E. coli* (MTCC40) (Fig. 3).

It shows maximum viability at a pH of 2–8, however also shows its viability at pH 1 and 10 (Fig. 4). Here, the isolates were grown in bile esculine agar (4% Ox bile), where they hydrolyse bile esculin producing dark brown to black coloration of the media (Fig. 2a). Isolates split the esculin molecules and use the liberated glucose to supply energy needs to release esculin into the medium where it reacts with ferric citrate to form a phenolic iron complex, which turns the agar medium from

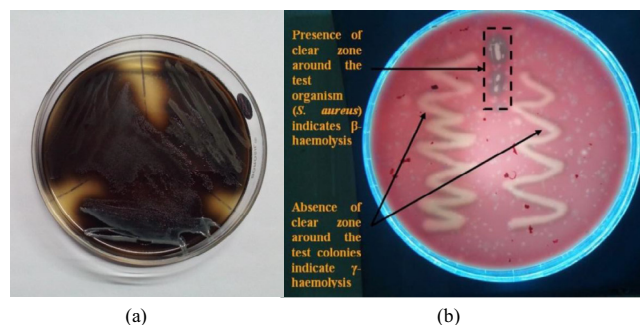


Fig. 2 – Appearance of black in bile esculin agar in (a) indicates hydrolysis of esculin and (b) shows γ-hemolysis by the potent isolate against a suitable reference strain.

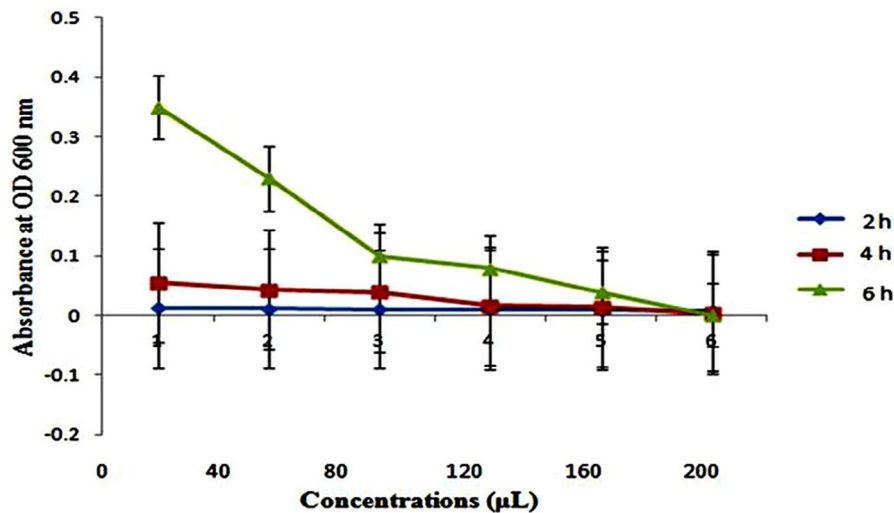


Fig. 3 – Antagonistic effect of the potent isolate against *E. coli* (MTCC40) at different time interval.

dark brown to black. The strain shows $33.1 \pm 0.5\%$ hydrophobicity along with higher values of auto-aggregation ($71.43 \pm 0.2\%$) as compared to the test pathogen *E. coli* MTCC40 ($55.5 \pm 0.4\%$). The auto-aggregation value of the most potent strain obtained in our study is compared with some of the established probiotic isolates worldwide, viz, *Bifidobacterium longum* B6, *Lactobacillus acidophilus* ADH, *Lactobacillus paracasei* ATCC 25598, *Lact. rhamnosus* GG, *Lactobacillus brevis* (KACC 10553), *Lactobacillus casei* (KACC 12413), *Leuconostoc mesenteroides* (KACC 12312), *Pediococcus acidilactici* (KACC 12307), *L. monocytogenes* (KACC 12671); and also with some of the pathogens viz, *S. aureus* (KACC 10768), *Shigella boydii* (KACC 10792) and *Salmonella typhimurium* (KCCM 40253), *E. coli* MTCC40 (Fig. 5). The values used for comparison were taken from documented literature [29].

The potent probiotic candidate was challenged with 400 µg lysozyme/mL for 60 minutes where it shows moderate

resistance to the enzyme (Fig. 6). This concentration was used in characterizing *Bifidobacterium* strains in simulated GI tract passage by Rada et al. [30]. On the basis of various staining techniques and their biochemical tests, the isolate 9F was found to be gram positive, catalase positive cocci. The most potent isolate 9F was identified to be novel species of *Staphylococcus* sp. DBOCP6 on the basis of 16S rDNA sequencing. The evolutionary history was inferred using the maximum likelihood method [24]. The optimized phylogenetic tree shows maximum similarity score of 70% with *S. pasteurii* ATCC 51129 (GenBank Accession Number: NR 024669.1) by forcing *Lactobacillus paracasei* strain R094 (GenBank Accession Number: NR 025880.1) as outgroup with 1000 bootstrap value (Fig. 7). The GenBank accession number KR706310 was received successfully for the novel probiotic isolate *Staphylococcus* sp. DBOCP6.

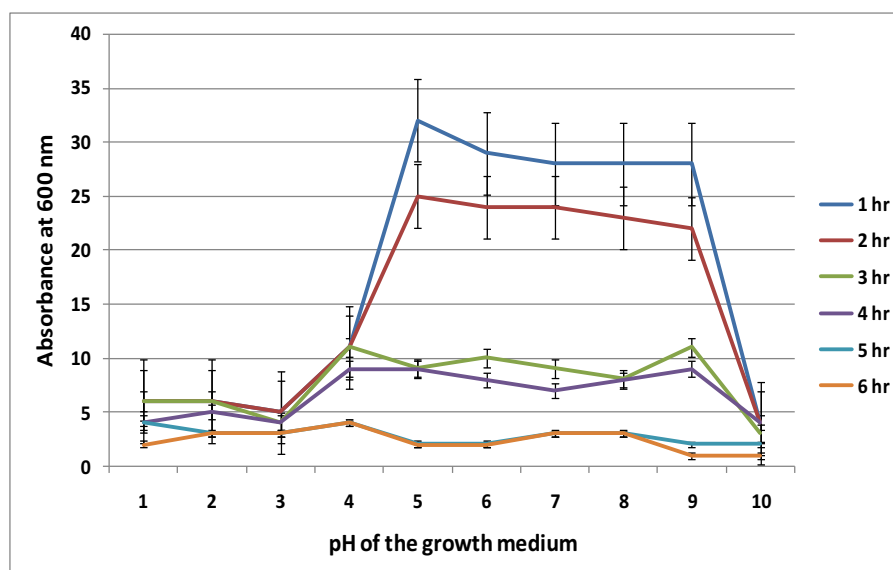


Fig. 4 – Growth profile of the most potent isolate at different pH and time interval.

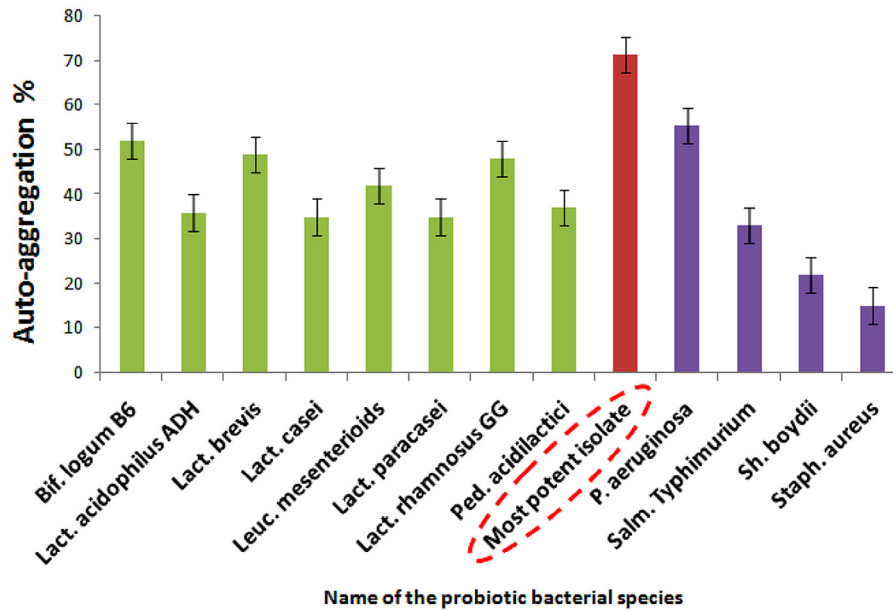


Fig. 5 – Graph showing the comparison of auto-aggregation values by the most potent isolate in the current study with other known probiotic bacteria.

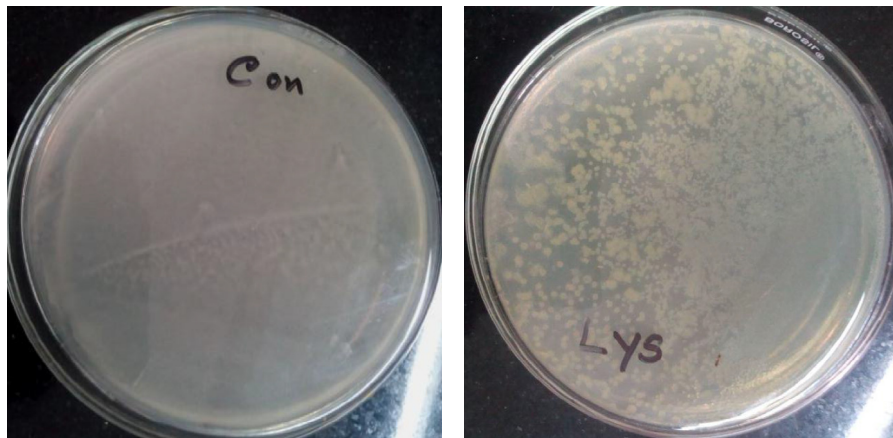


Fig. 6 – Considerable growth (right image) of the isolate in presence of lysozyme as compared to the control plate (left image).

4. Discussion

A large number of authors reported the occurrence of food borne pathogens causing health hazards [33–35]. With the present status of escalating drug resistant microorganism and the side effects caused by these drugs, there is a fervent need for the development of unconventional natural food products with health promoting properties [36,37]. Only one of the isolates was found to be susceptible to the entire broad and narrow spectrum antibiotics tested, which indicates that the isolate is nonpathogenic.

Bacteriocins actively produced *in vitro* may not necessarily be sufficiently high in quantities, or at all, within the GIT [28,38]. Therefore, the production of bacteriocins might not be considered as a fundamental tract for a new probiotic candidates [20].

Gastric juice in stomach is a biological barrier where the pH is between 1.5 and 3.0 and the candidate isolate was able to survive and grow at pH range of 1–10 till 6 hr of incubation, proving its capacity to transit through the acidic environment of stomach and also to survive in the alkaline conditions of GI tract [39]. Bile is another biological barrier encountered by the candidate microbe in the upper part of the small intestine. In the human body, the relevant physiological concentration of human bile ranges from 0.3 to 0.5% [38,40]. The inhibition of common intestinal bacteria had been related to the presence of deconjugated bile acids rather than conjugated ones [41]. The ability to hydrolyze bile salts can help the microorganism to sustain the balance of the gut microflora [42]. Bile salt hydrolase (BSH) activity is also correlated to a decrease in cholesterol level in humans [43,44].

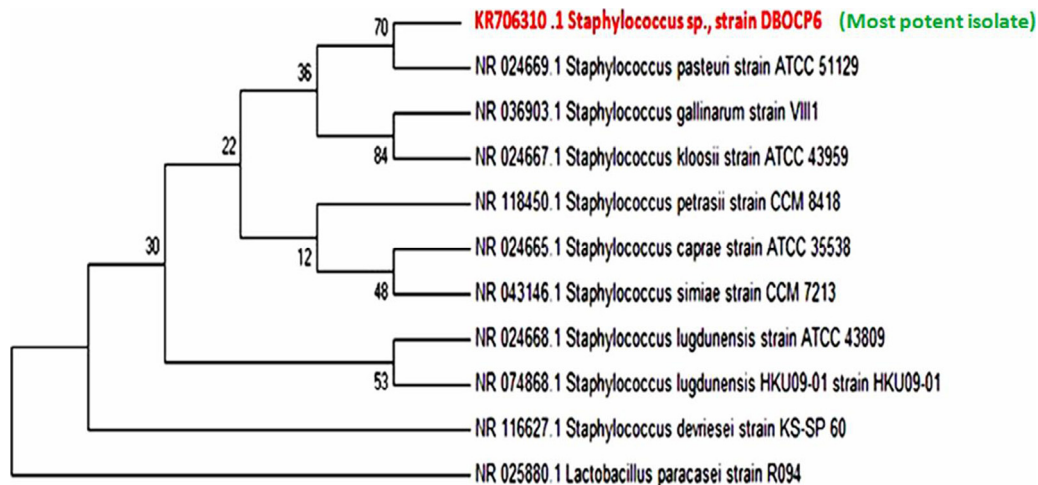


Fig. 7 – Phylogenetic relationships of the new potent isolate *Staphylococcus sp.* DBOCP6 (GenBank accession no. KR706310) with ten other most closely related *Staphylococcus* species based on 16S rDNA sequencing. The data set resembled 1000 times using the bootstrap option, and percentage values are given at the nodes.

The strain with promising probiotic characters was tested for its cell surface adhesion properties. It is globally recognized that probiotics must be able to colonize themselves inside digestive tract of the host [45,46]. Consequently, adhesion properties have been anticipated as a crucial factor for the selection of new probiotics [28,47]. For probiotics to exert maximum effect on the host, it should have the aptitude to adhere and colonize the intestine apart from being resistant to GI conditions [36]. Toluene is used to assess the hydrophobic or hydrophilic characteristics of bacterial cell surface, which are attributed to carboxylic groups on the cell surface [29]. Functional effects of probiotic bacteria comprise adherence to the intestinal wall for colonization in the gastrointestinal tract with capacity to prevent pathogenic adherence or pathogen activation [48]. The hydrophobic and hydrophilic properties may result from proteins and polysaccharides on the bacterial cell surface [49]. Hydrophobicity is one of the physico-chemical properties that assist the first contact between the microorganisms and the host cells [36]. The high cell surface hydrophobicity of the strain could indicate its capability to attach to the epithelial cell lining of the intestine and resist the movement of digested food materials [49]. The probiotic candidate showed reasonably higher auto-aggregation than the pathogens, suggesting the specific binding capabilities of probiotics in the GIT.

Lysozyme is the first biological barrier in the human digestive system encountered by the swallowed probiotic microbes [50]. Viability of the isolate indicates its ability to survive if it is administered orally.

5. Conclusion

This study extensively documented the isolation and screening of probiotic bacteria from traditionally prepared fermented fish and meat of Assam, India. The most potent isolate was found to be a novel gram positive, cocci shaped, catalase positive, non-hemolytic bacteria fulfilling all basic criteria to be considered as a potential probiotic candidate. The isolate was

identified to be novel species of *Staphylococcus* species on the basis of 16S rDNA sequencing technique. It shows satisfactory growth in presence of lysozyme, capable of surviving in a wide range pH range and also can hydrolyze bile esculin, indicating its potential to survive throughout the mammalian food tract. The isolated strain was also found to be nonpathogenic on the basis of susceptibility to both the broad and narrow spectrum antibiotics and non-hemolytic property. It shows antagonistic activity against *E. coli*. It displayed good hydrophobicity values with a higher value of auto-aggregation. Even though the isolate was not found to be producing any antimicrobial bacteriocin, it is capable to withstand high temperature with thermal death point of 100 °C, indicating it encompasses the vulnerability of probiotic bacteria during the food processing techniques, making it suitable for industrial exploitation. Based on the above observations, it could be concluded that *Staphylococcus sp.* strain DBOCP6 can serve as a potential probiotic candidate for industrial exploitation.

Acknowledgements

The author acknowledge DBT-MHRD, Govt. of India (Grant No. BT/04/NE/2009) and DBT-IBTHub projects (BT/04/NE.2009) for providing the funds and infrastructure to carry out the study at Centre for Studies in Biotechnology, Dibrugarh University. Authors also acknowledge Delcon facilities for providing access to more than 900 e-journals to Dibrugarh University, and Xcelris Labs. Pvt. Ltd., Ahmedabad for successfully performing 16S rDNA sequencing presented in the manuscript.

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