Biocatalysis and Agricultural Biotechnology 18 (2019) 101008

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Probiotic characterization of indigenous *Bacillus velezensis* strain DU14 isolated from Apong, a traditionally fermented rice beer of Assam

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ARTICLE INFO

Keywords:
Rice beer
Probiotic bacteria
Exopolysaccharide
Antagonistic effect
Bacillus velezensis strain DU14

ABSTRACT

Study was carried out to isolate and characterize novel indigenous probiotic candidates from traditionally prepared rice beer locally known as Apong in Assam, India. The most potential Probiotic candidate was identified as *Bacillus velezensis* strain DU14 (GenBank accession no. MK177191) on the basis of 16S rDNA gene sequencing. It was found to show significant tolerance in a pH range of 2–9 and survived in presence of 1% (*w/v*) of bile salt. It showed sensitivity against both broad and narrow spectrum antibiotics with antagonistic activities against both Gram positive (*Bacillus cereus* ATCC-11778) and negative and (*Escherichia coli* ATCC-25922) pathogens. The isolate was found non haemolytic in nature and the cell-free supernatant concentrate (CFSC) of the isolate showed no cytotoxicity against mouse liver cells. The FTIR analysis of exopolysaccharide synthesized by the isolate showed the presence of $\upsilon_{C=C\text{-}H\ stretch}$, $\upsilon_{C=C\ stretch}$, $\upsilon_{CH2\ symmetrical\ stretch}$, $\upsilon_{C\text{-}H\ bend}$, and $\upsilon_{C\text{-}O\ stretch}$.

1. Introduction

Probiotics have been extensively researched as an adjunct or alternative therapy against drug resistant pathogens (Forestier et al., 2001). Probiotic bacteria have a key role in modifying the intestinal microflora to better maintain digestive health and reducing gut related disorders and heart diseases (Borruel et al., 2002). Some of the most common Probiotics belong to the genera of *Lactobacillus, Leuconostoc, Pediococcus* and *Bifidobacterium* and have also been reported to be exhibit antagonistic effects against various pathogenic species of genera including *Listeria, Clostridium, Salmonella, Shigella, Escherichia, Helicobacter, Campylobacter, Candida etc.* (Collado et al., 2007). Fermented foods and beverages that have been developed throughout the history of human civilization till the present day for sustained nutrition and preserved foods are known as rich source of Probiotic microorganisms (Ray et al., 2016; Nematollahi et al., 2016). There have been extensive studies describing screening characteristics of potential probiotic bacteria from diverse sources such as traditional dairy food, swine origin, cheese, infant gut microbiota *etc.* (Bao et al., 2010; Guo et al., 2010; Zago et al., 2011; Kirtzalidou et al., 2011; Coda et al., 2012).

The state of Assam located in the Northeast region of India is famous not only for its traditionally fermented food and beverages but also blessed with its biodiversity hotspots which are least explored till date. Traditionally fermented rice beers that have been prepared and consumed as a part of their rituals and customs by the ethnic communities since ages, are underexplored for their Probiotic potential (Saikia et al., 2018). One such type of rice beer is locally known as 'Apong'. Rice beer is a fermented beverage which is a source of potential probiotic bacteria. However, limited work has been done in describing the probiotic properties of traditional rice beer. Therefore, this study was undertaken to explore the potential probiotic characteristics of bacteria isolates from rice beer (Saikia et al., 2018). In brewing, the use of starter cultures is aimed to increase the fermentation efficiency, to develop new beers, and to enhance the sensory complexity of the beer that is produced. In response to increased consumer demand toward new products, nowadays, there is a worldwide increase in popularity of craft beers. A new generation of products are now obtained in small breweries that focus on the production of traditional ales, lagers, and beer styles that deviate from mainstream beer types.

Current study exploits the probiotic potential of bacterial isolates from traditionally fermented rice beer of Sivsagar district of Assam

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https://doi.org/10.1016/j.bcab.2019.01.046
Received 8 December 2018; Received in revised form 22 January 2019; Accepted 24 January 2019
Available online 28 January 2019
1878-8181/

Table 1
List of sample collection sites and their geographical locations.

Sl. no.	Names of collection site	Geographical location	
		Latitude	Longitude
1	Dishangmukh	27.04°N	94.33°E
2	Dikhowmukh	27.00°N	94.46°E
3	Bolama Miri Goan	27.06°N	94.58°E
4	Rupahimukhmirigoan	26.95°N	94.42°E
5	Dhai Bari	27.19°N	94.65°E
6	Kawoimarigoan	26.88°N	94.36°E
7	Joysagar	26.95°N	94.62°E
8	Afala	27.04°N	94.56°E
9	Ligiri Bari	26.97°N	94.44°E
10	Sogunparagoan	26.91°N	94.38°E

using *in-vitro* screening tests and at the same time to study the characterization of specific probiotic properties. Investigating the probiotic abilities, stress responses, adhesion and permeability studies of the isolates form a crucial part of this research.

2. Materials and methods

2.1. Chemicals and reagents

All the media used in this research were procured from HiMedia India Pvt. Ltd. And all the chemicals and consumables were procured from Merck India Pvt. Ltd.

2.2. Isolation and screening of probiotic bacteria from Apong (rice beer)

Traditionally fermented beverage locally known as Apong was collected from 10 different villages of Sivasagar district of Assam (Table 1). The samples collected were stored in ice cold condition until future use. Potential microbes were isolated on MRS agar plates maintained at pH 5 by spreading 1 ml of 10^{-5} times diluted sample. The plates were incubated at 37 °C for 24 h. After incubation, individual colonies were selected and transferred into sterile broth media. Pure cultures were obtained by streak plate technique. Colony morphology of all the isolates was observed and recorded. Only Gram positive and catalase negative isolates were processed further. The overall screening methodology of the isolates in the form of graphical representation is shown in Fig. 1.

2.3. Antibiotic susceptibility test as safety assessment

Antibiotic susceptibilities of the isolated strains were examined by Kirby-Bauer's disc diffusion method as per CLSI (Clinical and Laboratory Standards Institute, USA) guidelines (Sweeney, 2018). The strains were tested for their susceptibilities against both broad and narrow spectrum antibiotics as per CLSI guideline. The diameters of zone of inhibition observed after overnight incubation at 37 °C were recorded.

2.4. Evaluation of antagonistic effect of the most potent isolate

Cell free supernatant concentrate (CFSC) of the potential isolates with sensitivity against maximum numbers of antibiotics were screened for their antagonistic potential against both Gram positive (*Bacillus cereus* ATCC no. 11778) and negative (*Escherichia coli* ATCC no. 25922) pathogens by agar well diffusion method. The cell free extract (CFE) was prepared by centrifuging overnight culture of the isolates in MRS broth at 10,000 rpm for 10 min at 4 °C. The CFE was collected and heated to 70 °C (to inactivate proteases) and concentred to 1/10th volume by rotary vacuum evaporator. This cell-free supernatant concentrate (CFSC) was passed through 0.22 μm membrane filters and evaluated for its antimicrobial activities.

2.5. In-vitro cell surface traits analysis

2.5.1. Hydrophobicity assay

Evaluation of bacterial cell surface hydrophobicity was done by determining the microbial adhesion to hydrocarbons following the protocol described by Del Re et al., 2000). Overnight bacterial culture was centrifuged at 10,000 rpm for 5 min. The pellet was washed twice in phosphate buffer saline (PBS) and then suspended in 3 mL of 0.1M KNO_3 solution. The absorbance of the suspension was measured (A_0) 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 kept for 10 min of incubation at room temperature followed by vortex for 2 min. It was again incubated for 30 min at room temperature. After incubation, the water and toluene phases separated, the aqueous phase was then carefully separated and the absorbance was measured at 600 nm (A_1). The percentage of the cell surface hydrophobicity (H) was calculated using the following formula:

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

2.5.2. Auto-aggregation assay

Auto-aggregation was carried out by using the methods described by Del Re et al., 2000) with minor modification. The specific cell–cell interactions were determined using auto-aggregation assay. The bacterial cells were centrifuged at 5000 rpm for 10 min at 4 °C. The pellet was washed with PBS and then re-suspended in PBS to 10^8 cfu/mL. 3 mL of each bacterial suspension was vortex for 10 s 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 formula:

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

Where, A_{2h} = absorbance at 600 nm 2 h of incubation.A_{0h} = absorbance at 600 nm zero hour.

2.6. Safety assessment

2.6.1. Haemolysis assay

The bacterial isolates were evaluated for haemolytic activity using Blood agar base No.2 plates containing 5% (*v/v*) commercially available mammalian blood and incubated at 37 °C for 24 h. Clear zone around the test organism indicated positive test for *β*-haemolysis.

2.6.2. Cytotoxicity assay on primary mouse liver cell line (Mus musculus)

Cytotoxicity assay of CFSC was performed on primary mouse liver cells of *Mus musculus* (albino mouse) by the MTT method (Masters, 2000). Hepatocytes were obtained by extracting the liver from the mouse through dissection (with essential ethical clearance) and subjecting the liver tissue to by physical (mastication) and chemical (0.5% collagenase) breakdown. Hepatic cells population was adjusted to 2×10^6 cells/mL using a haemocytometer in DMEM (Dulbecco's Modified Essential Media). Before proceeding with MTT assay, cell viability was checked by Trypan Blue staining method. The cell containing media was added to a microtitre plate along with different dilutions of the CFSC except in the blank well. All dilutions were taken in triplicates. After incubation with MTT, cell viability was determined by measuring the absorbance at 580 nm.

2.7. pH tolerance test

Growth of the potential isolate in a pH range of 2–10 was monitored by measuring its absorbance at 600 nm followed by inoculation on nutrient agar (NA) plates for the determination of its viability.

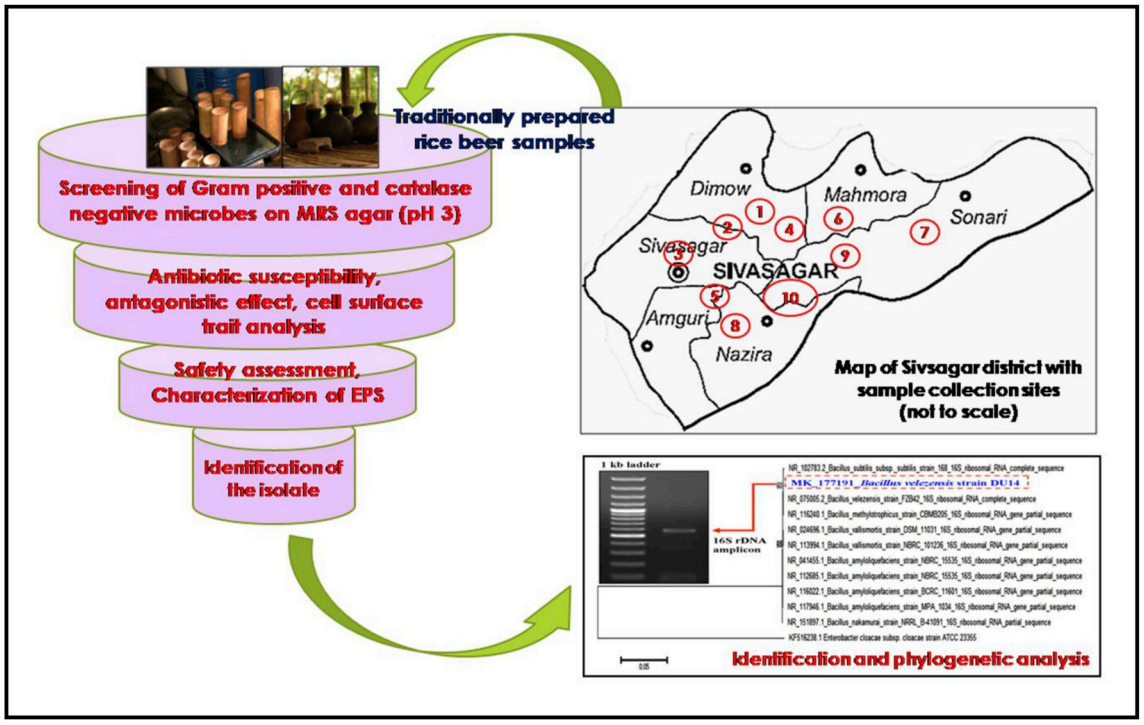


Fig. 1. Overall strategy for the screening of the potential probiotic isolates.

Table 2
Antibiogram of the isolate (as per CLSI guidelines).

Sl. no.	Antibiotic	Abbreviation	Concentration (μg/discs)	Resistance (R)/Sensitive (S)	Zone inhibition (mm)
1	Ampicillin	AMP^{10}	10	S	8
2	Amikacin	AK^{30}	30	S	12
3	Ceftriaxone	CTR^{30}	30	S	17
4	Ceftazidime	CAZ^{30}	30	R	–
5	Ciprofloxacin	CIP^{5}	5	S	38
6	Chloramphenicol	C^{30}	30	S	32
7	Clindamycin	CD^{2}	2	S	36
8	Erythromycin	E^{15}	15	S	32
9	Gentamicin	GEN^{10}	10	S	17
10	Imipenem	IPM^{10}	10	S	40
11	Metronidazole	MT^{5}	5	R	–
12	Ofloxacin	OF^{5}	5	S	32
13	Penicillin	P^{10}	10	S	11
14	Rifampicin	RIF^{5}	5	R	–
15	Vancomycin	VA^{30}	30	S	14

Table 3
Biochemical test of the most potential isolate.

Sl. no.	Name of the biochemical test	Result	Carbohydrate fermentation test	
			Name of the carbohydrate used	Result
1	Arginine test	-ve	Adonitol	+ve
2	Citrate utilization test	-ve	Arabinose	+ve
3	Gelatine hydrolysis test	-ve	Glucose	+ve
4	Hydrogen sulphide production test	-ve	Inocitol	+ve
5	Indol test	-ve	Lactose	+ve
6	Lysine decarboxylase test	-ve	mannitol	+ve
7	malonate test	-ve	Raffinose	+ve
8	Nitrate reduction test	-ve	Rhamnose	+ve
9	ONPG test	+ve	Rhamnose	+ve
10	Ornithie test	-ve	Rhamnose	+ve
11	Starch hydrolysis test	-ve	Rhamnose	+ve
12	Urease test	-ve	Rhamnose	+ve
13	Voges Poskauer's test	-ve		

2.8. Bile salt tolerance test

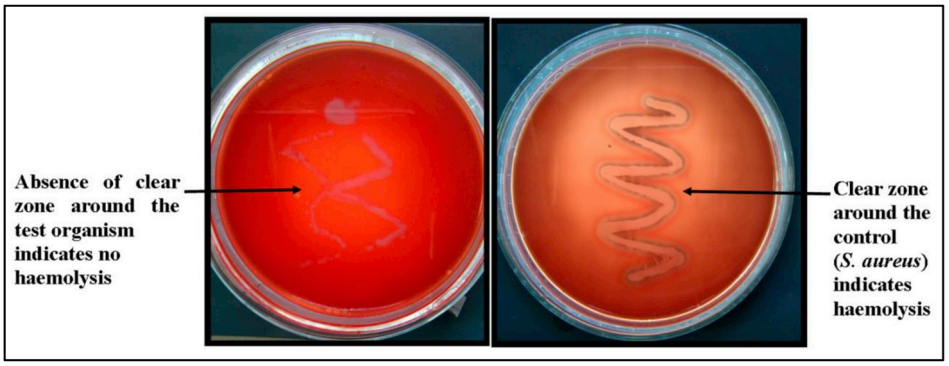
The ability of the isolates to grow in the presence of bile salt was determined by measuring its growth in terms of *cfu* count by inoculating the isolate in bile salt supplemented (0.2, 0.4 … 1.0% *w/v*) MRS broth.

2.9. Lysozyme susceptibility test

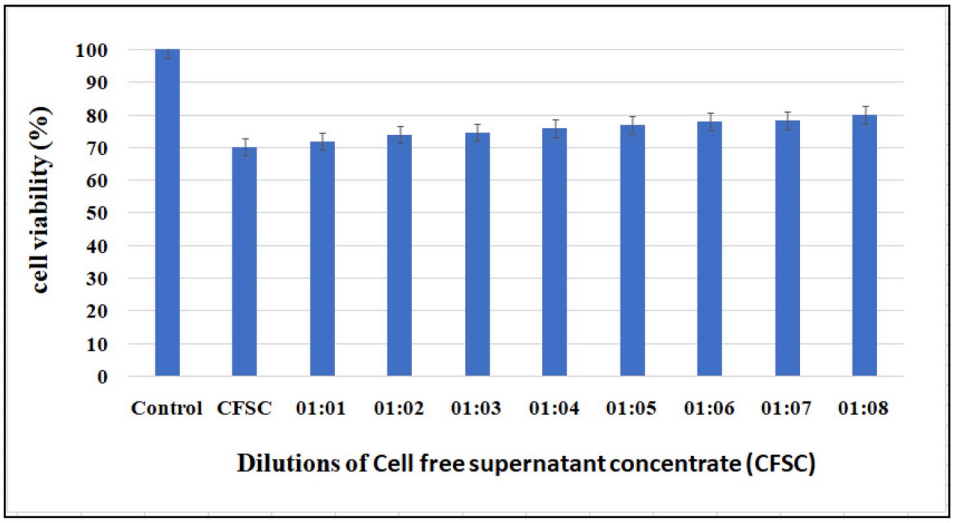
Sensitivity of the most potent isolate to lysozyme was determined by inoculating the isolate in MRS broth supplemented with 400μg/ml lysozyme. Control samples did not contain lysozyme. Viability of the isolate was checked by plating on NA plates with appropriate dilution.

2.10. Growth at different NaCl concentrations

Fresh overnight culture of bacteria was inoculated into MRS broth containing NaCl concentrations of 0.3%, 0.5%, 0.8% and 1.0%. The broths were then incubated at 37 °C. Viability of the isolate was checked



(a)



(b)

Fig. 2. (a) The isolate shows no haemolysis on blood agar (on left), whereas the control (*S. aureus*) on right shows clear zones around the colony indicates haemolysis; (b) CFSC extracted from the isolate shows no cytotoxicity against mouse liver cells.

by plating on NA plates with appropriate dilution.

2.11. Extraction of bacterial exo-polysaccharide (EPS)

Exopolysaccharide (EPS) was extracted from the most potential isolate by using the method described by cold acetone precipitation method (Nanda and Raghavan, 2014). Briefly, the most potential probiotic bacteria were inoculated in 100 ml of Yeast mannitol glucose broth and incubated at 37 °C and 135 rpm overnight. After incubation, 100 µl was aseptically transferred to 500 ml of a fresh media in a conical flask and was incubated for 5 days at 37 °C. Samples from flasks were separated and concentrated to small volumes. For the precipitation of EPS, equal volume of alcohol was added to the concentrated samples. The mixture was agitated during addition of alcohol to prevent local high concentration of the precipitate and left-over night at 4 °C. The mixture was then centrifuged at 7000 rpm for 20 min. After centrifugation, the precipitate was collected in Petri plates and dried at 60 °C.

2.12. FTIR analysis of the EPS produced

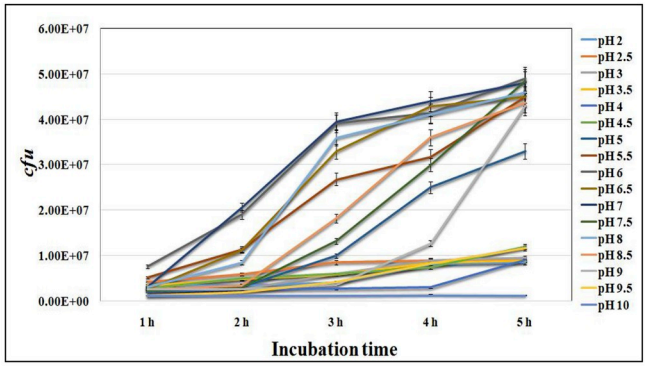
IR spectroscopes of bacterial EPS along with a standard, dextran sulphate (DS) were performed in a frequency range of 400 and 4000 cm^{-1}.

2.13. Identification of the potential isolate by biochemical characterization and 16S rDNA gene sequencing

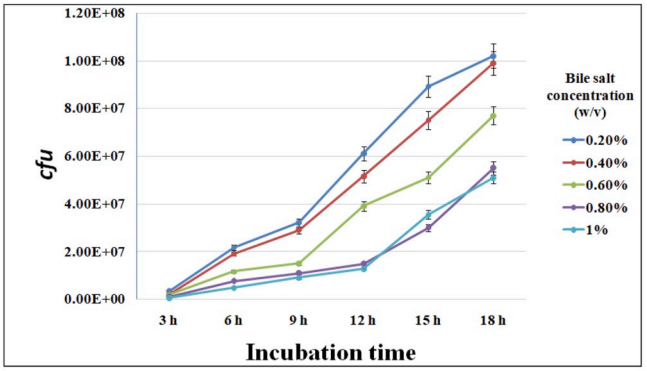
The potential Probiotic candidate was identified on the basis of biochemical characterization as prescribed by Bergey's Manual of Systematic Bacteriology (Table 3) followed by 16S rDNA gene sequencing. The 16S rDNA gene was amplified by using P3 forward (5′-AGAGTTTGATCATGGCTCAG-3′) and P13 reverse primers (5′-GGTTACCTTGTTACGACTT-3′) and the sequencing was done by outsourcing the amplicon to Pentavalent Bio Sciences Ltd., Bangalore. The phylogenetic analysis of the consensus sequence hence generated from forward and reverse sequence was carried out in MEGA6 software by Neigbour-Joining method with the top 10 most closely related strains based on BLAST hits. The bootstrap value was set at 1000 and *Enterobacter cloacae* ATCC 23355 was forced as out group.

2.14. Statistical analysis

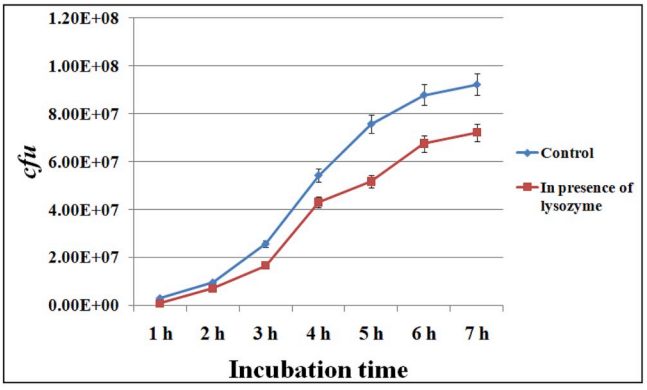
The results are the mean of experiments performed in triplicate with standard deviation. Student's *t*-test was performed to see the significance (p-value < 0.05) differences in findings. All the statistical analysis were performed by Graph padTM online tool (http://www.graphpad.com).



(a)



(b)



(c)

Fig. 3. Growth profile of the isolate in (a) different pH; (b) different concentrations of bile salt; and (c) in presence and absence (control) of lysozyme.

3. Results and discussion

More than 400 bacterial species are known to be existing in human intestinal tract (Naidu et al., 1999). The composition of the gut micro flora is constant but can be affected by some factors such as; age, diet, environment, stress and medication (Conlon and Bird, 2015). It is difficult to maintain a healthy balance of the bacteria present in intestine with the ever changing lifestyles. Lot of factors may shift the balance away from potentially beneficial or health promoting bacteria to potentially harmful or pathogenic microorganisms like sulphate reducers and Bacteroides species. Use of probiotics help to protect the host from various intestinal diseases and disorders while increasing the number of beneficial bacteria and make the balance steady again and hence they are suggested as food to provide for the balance of intestinal flora (Conlon and Bird, 2015).

In the current study, a total of 889 bacterial isolates with 40 different types of colony morphology were obtained from 10 rice beer samples on MRS agar plates maintained at pH 5. Out of these only 15 types of isolates were confirmed as Gram positive and catalase negative and were processed further. However no fungal strains were obtained. The isolate with sensitivity against maximum number of antibiotics *i.e.*, 12 out of 15 antibiotics tests was considered for further processing.

A key property of probiotic bacteria is that they should not carry any transmissible antibiotic resistance genes (Gueimonde et al., 2013). Result from this study shows the most potential bacterial candidate is sensitive to 10 different antibiotics. It showed susceptibility against Ampicillin (10 μg/disc), Amikacin (30 μg/disc), Ceftriaxone (30 μg/disc), Ciprofloxacin (5 μg/disc), Chloramphenicol (30 μg/disc), Clindamycin (30 μg/disc), Erythromycin (15 μg/disc), Gentamycin (10 μg/disc), Imipenem (10 μg/disc), Ofloxacin (5 μg/disc), Penicillin (10 μg/disc), and Vancomycin (30 μg/disc) and the respective zone of inhibition obtained are shown in Table 2.

Antimicrobial activity may also be considered as one of the most important selection criteria for probiotics. Antimicrobial activity targets the enteric undesirables and pathogens (Conlon and Bird, 2015). Antimicrobial effects of "Probiotics" may be due to release of some substances such as organic acids, hydrogen peroxide, low molecular weight antimicrobial substances, bacteriocins *etc* (Ouwehand et al., 2008; Çakir, 2003). The antagonistic effect of the isolate was tested against both Gram positive and negative pathogens by using cell free extract. It showed effective diameter of zone of inhibition of 16 ± 2 and 11 ± 1.5 mm respectively against *B. cereus* ATCC 11778 and *E. coli* ATCC 25922. Whereas, showing no haemolysis on Blood Agar plates advocates its non cytotoxic nature (Fig. 2a). Cytotoxicity assay is one of the most important parameters for the demonstration of safety assessment for a potential Probiotic candidate (Botta et al., 2014). The CFSC extracted from the isolate shows no cytotoxicity against mouse liver cells which advocates its potential for further research and development (Fig. 2b).

A probiotic microorganism should survive and grow under stressful conditions. They must resist gastric acidity and the presence of lysozyme, bile salts and pancreatic enzymes (Salminen et al., 1996). Stomach acidities and the high concentration of bile in the intestine are the first factors to consider in probiotic selection. Resistance to pH 2–3 is often used as *in-vitro* assays for the determination of resistance against stomach pH (Shaikh and Shah, 2013). At the same time it should also be capable of tolerating high pH and emulsification due to the presence of bile salt. The most potential isolate was also found to be survive significantly at up to pH 9 ($p < 0.05$) at least for 5 h which is sufficient for a probiotic microbe to reach colon (Fig. 3a). It could also withstand 0.2–1.0% (*w/v*) bile salt for 18 h (Fig. 3b).

Hydrophobicity and auto-aggregation percentage of the isolate were found to be 39.2 and 51.66% respectively. Cell adhesion is a multi-step process involving contact of the bacterial cell membrane and interacting surfaces. The ability of probiotic bacteria to form cellular aggregates is considered a desirable characteristic, as they can potentially inhibit adherence of pathogenic bacteria to intestinal mucosa either by forming a barrier via self-aggregation or co-aggregation with commensal organisms on the intestinal mucosa or by direct coaggregation with the pathogens to facilitate clearance (Bujnakova and Kmet, 2002; Voltan et al., 2007).

Swallowed probiotic bacteria encounter the first biological barrier of lysozyme of saliva in the mouth which may hydrolys β-(1,4) linkage between N-acetylglucosamine and N-acetylmuramic acid in bacterial (Rada et al., 2010; Bera et al., 2007). The isolate shows significant level of growth ($p < 0.05$) even in presence of lysozyme as compared to control which further may help the isolate against digestion if administered orally (Fig. 3c).

It is universally recognised that probiotics must be able to colonize the digestive tract (Pascual et al., 2008; Rivera-Espinoza and Gallardo-Navarro, 2010). In our study toluene was used for assess hydrophobicity/hydrophilic characteristics of most potent probiotic bacterial cell surface. Exopolysaccharide (EPS) are polymeric substance of

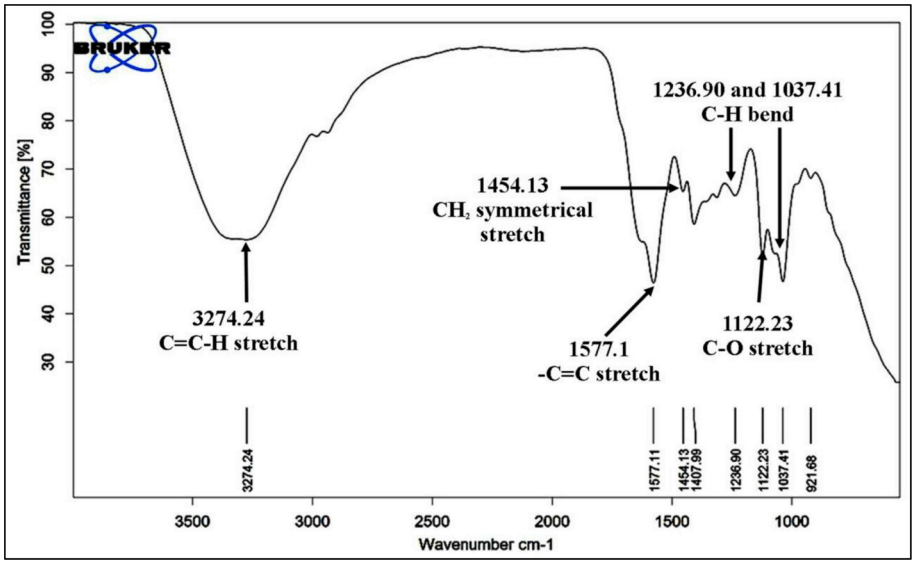


Fig. 4. FTIR spectrum of the bacterial exopolysaccharide (EPS).

Table 4
Some of the recent reports on probiotic characterization of various microbial isolates.

Sl no.	Name of the isolate	Country origin	*In-vitro* cell surface property		Antimicrobial assay		References
			Hydrophobicity (%)	Autoaggregation (%)	Test microbes	Zone of inhibition (mm)	
1	*Lactobacillus sp.* KKL1	India	31.03	18.03	–	–	Ghosh et al., (2015)
2	*Saccharomyces cerevisiae* ARDMC1 *Saccharomyces boulardii*	India	61.40 58.59	43.19 40.40	– –	– –	Saikia et al., (2018)
3	*L. delbrueckii* LA4	India	66.02	–	*Staphylococcus aureus* ATCC 9144, *Aeromonas hydrophila* ATCC 35654, *Yersinia enterolitica* ATCC 9610, *Enterobacter* (Chronobacter) *sakazakii* ATCC 51329, *Shigella flexneri* 2a, *Salmonella typhimurium* ATCC 19585, *Listeria monocytogenes* ATCC 19111, *Escherichia coli* 057:H7 ATCC 43895	> 11 > 20 > 20 > 20 > 20 > 11 > 11	Kumar et al., (2012)
4	*Lactobacillus plantarum* L7	India	61.40	39.40	–	–	Giri et al., (2018)
5	*Bacillus velezensis* DU14	India	39.20	51.66	*Bacillus cereus* ATCC-11778, *Escherichia coli* ATCC-25922	16 11	Present study

microorganisms of high molecular weight and long chain composed of sugar residue secreted by them into the surrounding environment. Bacterial EPS are complex mixture of macro molecular poly electrolytes including polysaccharides, protein and nucleic acids, each composition of variable molecular mass and structural properties. The FTIR result of the EPS of isolate shows the presence of distinct peaks at 3274.24, 1577.1, 1454.13, 1236.9 and 1122.23 which represents $\upsilon_{C=C\text{-}H\ stretch}$, $\upsilon_{C=C\ stretch}$, $\upsilon_{CH2\ symmetrical\ stretch}$, $\upsilon_{C\text{-}H\ bend}$, and $\upsilon_{C\text{-}O\ stretch}$ respectively (Fig. 4).

The isolate was identified on the basis of biochemical tests and phylogenetic analysis. The isolate shows positive result for ONPG (o-Nitrophenyl-β-D-Galactopyranoside) test but negative results for all other tests (Table 4). Phylogenetic tree constructed by using 16S rDNA sequence of the most potential isolate with 10 most closely related strains based on BLAST hits confirms the identity of the isolate as *Bacillus velezensis* strain DU14 and the accession no. MK177191 was received from NCBI GenBank (Fig. 5). Probiotic and antimicrobial potential of various strains of *Bacillus velezensis* were also discussed and established by other researchers (Gao et al., 2017; Yi et al., 2018). However, it was not reported to be present as a probiotic candidate in fermented rice beer. Moreover, the isolate shows probiotic efficacy in terms of hydrophobicity, autoaggregation, antimicrobial potential and safety assessment in accordance with other reported probiotic candidates isolated from traditionally fermented rice beer advocates its future scopes (Table 4).

4. Conclusion

Isolation and characterization of indigenous probiotic candidates from fermented rice beer was carried out. The most potential isolate was identified as *Bacillus velezensis* strain DU14 on the basis of 16S rDNA gene sequencing which was found to be fulfilling all the basic criteria to be considered as a potential Probiotic candidate. The non-haemolytic and non-cytotoxic nature of the isolate further advocates its potential application for commercial exploitation after further research.

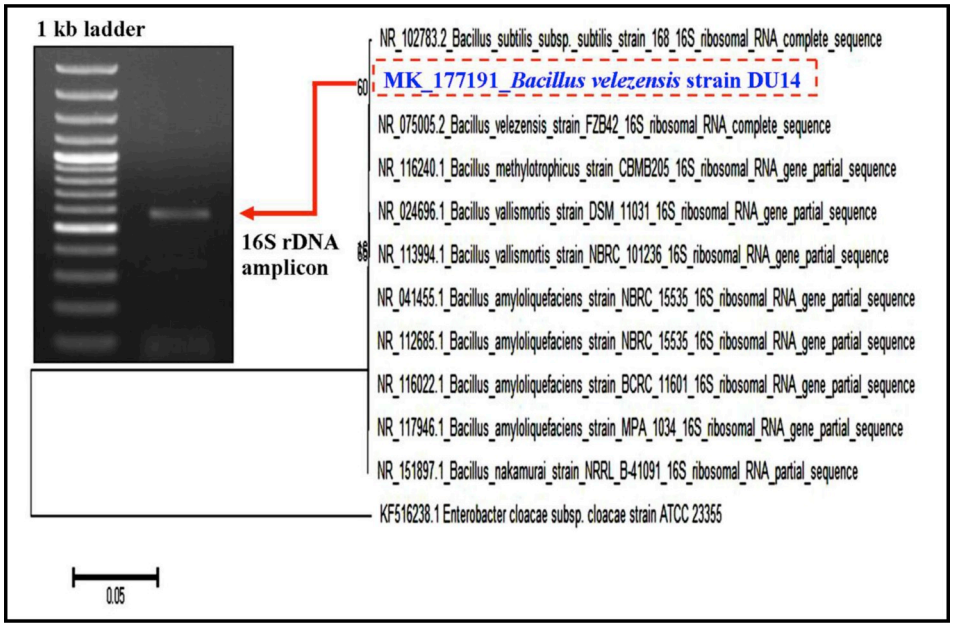


Fig. 5. Phylogenetic analysis of the isolate *Bacillus velezensis* strain DU14 (GenBank accession MK177191) with 10 most closely related species.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

The author acknowledges Department of Biotechnology (DBT-HRD), Govt. of India (Grant No. BT/04/NE/2009) for providing infrastructure facilities at Centre for Biotechnology and Bioinformatics, Dibrugarh University, DBT-BIF facility (Grant no. BT/BI/13/035/2017), DBT-Delcon facilities for providing access to online journals and departmental Animal Cell Culture facility to carry out cytotoxicity assay. Author also acknowledges Pentavalent Bio Sciences Pvt. Ltd., Bangalore for providing 16S rDNA gene sequencing services.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.01.046.

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