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Short Communication

Isolation and Characterizations of Probiotics from Bovine (Cow) Milk

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Abstract

Products extracted from bovine (Cow) origin are the chief vehicle for the delivery of the probiotic. Probiotic is a potential therapeutic for numerous disease now-a-days. Our experiment was designed to find out the beneficial role of probiotic in cow milk. In this study, five isolates were selected from raw cow milk which was initially characterized through staining, different biochemical tests. All the isolates were shown Gram positive, non-endosporer rods in nature. CW1 and CW4 showed maximum bile salt tolerance including antimicrobial activity, whereas only CW1 showed maximum antioxidant activity. Furthermore molecular characterization (16S rRNA sequencing) and phylogenetic tree was analyzed. This unique microflora maybe used as a probiotic to enhance gastrointestinal health, prevention of pathogenic infection including immunomodulation.

Keywords: antioxidant activity, Bovine; Probiotics, 16rRNA

Introduction:

Different traditional dairy products produced in different parts of India as well as other countries enhance benefits, such as improvement of nutrients absorption, inactivation of toxins and anti-pathogenic activities, are used worldwide [1]. Cow milk is a pale liquid produced by the mammary glands of cow. It is the primary source of nutrition for infant mammals before they are able to digest other types of food. It contains many other nutrients including proteins and lactose [2]. Moreover, Milk is also known as one of the natural habitats of Lactic Acid Bacteria (LAB) [3]. Probiotics are defined as live microorganisms, which gives a health benefit to the body by maintaining microbial balance in Gastrointestinal Tract. Lactic Acid Bacteria are studied as one of the main probiotics and as a protective culture, Lactic Acid Bacteria are common probiotic organisms that are considered safe. Few examples of probiotics are *Lactobacillus, Lactococci, Bifidiobacteria and Saccharomyces.* Probiotic bacteria reduce gastrointestinal diseases by increasing benefit microorganisms growth and reducing pathogens population mechanisms. Probiotic releases certain chemical compounds that are inhibitory to both Gram positive bacteria and Gram negative bacteria [4]. Some of the chemical compounds released by probiotics bacteria are – Bacteriocins, sideropheres, Lysozymes, Proteases, Hydrogen peroxide etc. Bacteriocins are proteinaceous compounds

produced by a wide range of bacteria exhibiting antimicrobial activity against selected range of other bacteria [5]. By facilitating the absorption of magnesium and calcium from milk proteins, digesting Lactose and producing foliate and B vitamins; probiotics significantly affect the bioavailability of nutrients in the human body.

Antimicrobial activity, bile salts, acid tolerance are three important features for screening probiotic potential of bacteria to be used. Antimicrobial compounds are prepared from probiotic bacteria to compete and inhibit pathogenic microorganisms which may effects metabolism or toxins of pathogenic bacteria. Probiotics have protective effects in dairy products against harmful bacteria. Probioitics produce organic acids and antifungal compounds such as fatty acids during lactic fermentation. Bacteriocins producing strains are used as bio – preservative.

Today, the probiotic human friendly bacteria are isolated from foods, cheese, yoghurt as well as human himself such as human milk, infant feaces, human vagina etc. Probiotics are widely used for the promotion and improvement of health in humans and animal species. They have been used as a biologically active substance in a large extent of pathologic conditions ranging from antibiotic – associated or travelers diarrhea, Irritable Bowel Syndrome (IBS) and Lactose Intolerance to dental caries, ulcers, intestinal motility disorders [5]. There are also numerous scientific reports about interaction between probiotics and immune systems. Therefore, the aim of the study is to isolate and characterize the probiotics from cow milk.

Materials and Methods

Sample collection

The study was conducted to isolate and identify the naturally occurring probiotic from cow milk of three different places. Three Cow milk samples were collected from lactating cows from Dewandighi, Station Bazar, and GolaCWagh in the nearby area of the city Bardhhaman, West Bengal. Samples were collected using sterilized bottles and brought to laboratory for microbiological investigation. Samples were kept in a refrigerator (4°C) till the further analysis begins.

Isolation of Microbes from cow milk sample

The microbial strains were isolated from local cow milk sample. 1 gram of cow milk sample was weighed, taken aseptically and was diluted from 10^{-1} to 10^{-4}; 0.1 ml of diluted sample was inoculated on Nutrient Agar plates and incubated in incubation at 37°C for 24 hours. The isolated colonies were purified by streaking on Nutrient Agar slant, incubated in incubation at 37°C for 24 hours. The purified colonies isolated on Nutrient Agar slant were inoculated in Nutrient Broth, incubated in incubation at 37°C for 24 hours. The purified bacterial strains were stored at 4°C for further use.

Physiological and Biochemical Characteristics Test of Probiotic Isolate

Gram's Staining Test

Gram staining test was performed for all isolated strains according to the standard procedure. The slide was observed under microscope for the result [6].

Endospore Staining Test

Endospore staining is a differential staining technique done for the purposes of distinguishing between vegetative cells and endospores. For endospores staining, a smear was prepared on a clear grease free

slide with malachite gree (in boiling condition) and saffranine [7].

Determination of optimum pH and temperature of Microbial Strains

The nutrient broth of pH3, pH7, and pH9 were prepared and adjusted with Hydrochloride (HCl) and NaOH for determining the optimum pH. Fresh probiotic isolates (CW1-CW5) were inoculated into Nutrient Broth in test tubes and incubated in incubation at 37°C for 24 hours.

For determination of optimum temperature of probiotic isolates, fresh probiotic isolates (CW1-CW5) were inoculated into Nutrient Broth of 3 test tubes, incubated at three different temperatures – 4°C, 37°C and 50°C for 24 hours.

NaCl Tolerance Test

For determination of NaCl tolerance of five probiotic isolates, 3 conical flasks containing Nutrient Broth for each probiotic were adjusted at three different concentration – 3%, 6%, and 9% respectively [7]

IMViC Test (Indol Methyl Red Vosges Proskauer Citrate Utilising Test)

The IMViC test comprises of four different tests, such as; Indole test, Methyl Red Test, Vosges Proskauer Test and Citrate Utilizing Test [3]. The IMViC tests were performed with all the five probiotics.

Acid-Gas Production Test

For this test, sterile lactose broth was prepared into test tubes with durhum's tubes and was inoculated with all five isolates [8].

Catalase Test

For catalase test all the five isolates were inoculated in the medium and after 4 hours incubation the plates were flooded with 3ml of hydrogen peroxide [9]

Amylase Test

For this test, starch agar medium was prepared, autoclaved and inoculates with five isolates (CW1- CW5). After 24 hours incubation, 3 ml of Gram's Iodine was added to all the culture tubes.

Cellulose Test

Czapek-Mineral salt Agar medium was prepared, autoclaved and inoculated with five isolates (CW1-CW5). After 48 hours of incubation, all the petriplates were pipette with hexadecyltrimethyl ammonium bromide solution and the result was observed.

Arginine test

Ammonia production by arginine hydrolysis was performed in MRS broth containing 0.3% arginine and 0.2 % sodium citrate replacing ammonium citrate. Production of ammonia was detected by using Nessler's reagent of all the five isolates.

Bile tolerance test

The bile salt solutions were prepared using Ox-gall powder (HI-Media). All the 5 isolates was subjected for growth in the MRS media supplemented with varying percentages of bile salt in it, such as; 0.2, 0.4, 0.6and 0.8 % (w/v). The varied concentrations of the bile salt will prove the isolates' resistance power over it which took them towards a potent probiotics.

Antimicrobial activity of Probiotics over *Pseudomonas sp.* and *Vibrio sp.*

The probiotic isolated strains were analyzed for antimicrobial activity against *Pseudomonas sp.* and *Vibrio sp.* using Agar cup well diffusion method. To check the antimicrobial activity, the Nutrient Agar plates were prepared and inoculated with *Pseudomonas sp.* and *Vibrio sp.* separately in

different plates and wells were loaded with the samples (CW1-CW5). One plate is kept for control. After incubation the diameter of zone of inhibition was measured.

Antioxidant Assay:

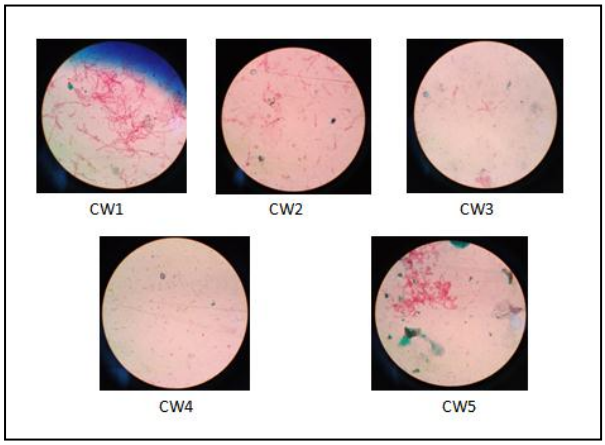
The scavenging effect of all the five isolates on the free radical DPPH was measured. CW samples in MRS broth (1 mL) were mixed with freshly prepared 1 ml DPPH (Sigma—Aldrich) solution (0.2 mM). After shaking the mixture it was left for 30 mins incubation in the dark at room temperature. The control sample only contained deionized water. The scavenged DPPH was then monitored by determining the absorbance at 517 nm using Spectrophotometer (Systronics, India). The radical scavenging activity was quantified as units/mL (U/mL).

Result and Discussion

The present study described isolation and characterizations of probiotics from bovine (cow) milk. The cultural characteristics of the five isolates after Gram staining and Endospore staining are tabulated in the following table (Table1). From the result it can be concluded that all the five isolates are gram positive rods and are non-endospore former which is observed under light microscope (Fig. 1a,b). From the optical density noted (Table 2), it can be concluded that the optimum pH of the five isolates is pH 7 though they also showed a slight rowth in pH 3, whereas; the optimum growth temperature determined is 37°C and the NaCl which is used as a prominent constituent of growth is considered as their optimum. (Fig. 2). All the five isolates showed positive result for Methyl Test, Citrate Utilizing Test, Acid-Gas Production Test and catalase test, whereas the rest of the tests showed negative result.

Table No. 1:The Gram character and the endospore formation is tabulated below of all isolates.

Samples	Gram Staining	Endospore Staining	Morphology
CW1	Gram (+) ve	Negative	Rods in chain
CW2	Gram (+) ve	Negative	Rods in chain
CW3	Gram (+) ve	Negative	Rods in single
CW4	Gram (+) ve	Negative	Rods in chain
CW5	Gram (+) ve	Negative	Rods in single



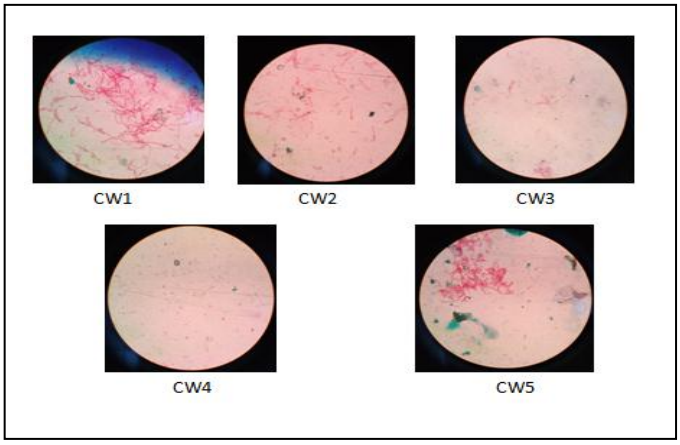


Fig 1a: Observations of Gram Staining. **Fig 1b**: Observations of Endospore Staining

Table No. 2: The optical density of the five isolates is tabulated here to determine the optimum pH, temperature and NaCl concentrations of CW1 to CW5.

Samples	**Optimum pH**			**Optimum Temperature**			**Optimum NaCl Concentration**		
	pH 3	pH 7	pH 9	4°C	37°C	50°C	3%	6%	9%
CW 1	0.12	0.25	0.02	0.03	0.22	0.09	0.21	0.12	0.03
CW 2	0.15	0.27	0.03	0.02	0.25	0.08	0.22	0.14	0.02
CW 3	0.13	0.29	0.02	0.02	0.24	0.10	0.26	0.16	0.03
CW 4	0.14	0.28	0.03	0.01	0.21	0.09	0.19	0.15	0.01
CW 5	0.15	0.27	0.01	0.03	0.23	0.08	0.20	0.11	0.02

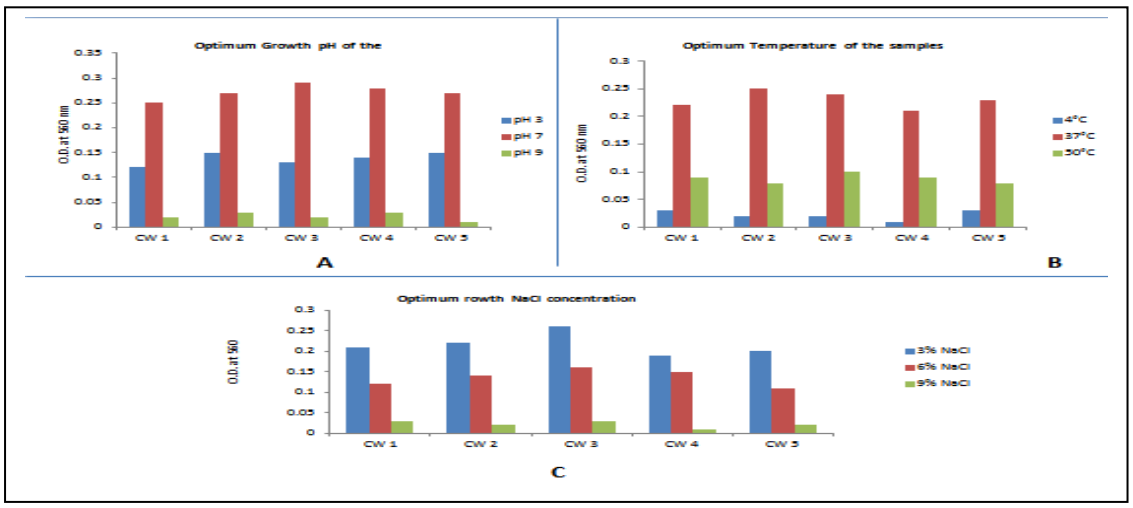


Fig 2: The Graphical Representation of O.D. values at different growth pH, temperature and NaCl concentrations for the determination of its optimum values.

Table No. 3: Different biochemical test of the five isolates, CW1 to CW5.

Samples	Indole Test	Methyl Red Test	Voges Praskauer Test	Citrate Utilizing Test	Acid-Gas Production Test	Arginine Hydrolase Test	Catalase Test	Amylase Test	Cellulase Test
CW 1	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve
CW 2	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve
CW 3	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve
CW 4	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve
CW 5	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve

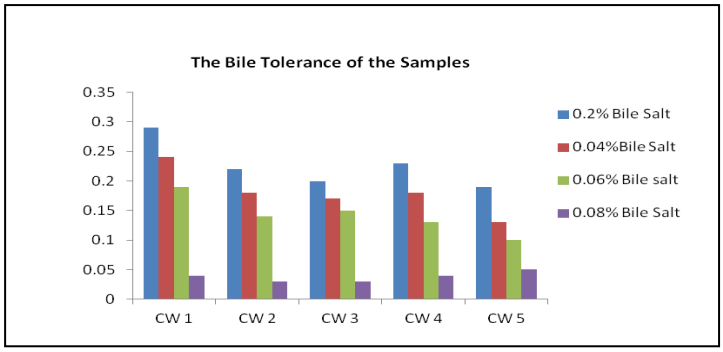


Fig 3: The Graphical Representations of Bile Salt tolerance.

The result depicted that all the five isolated organism showed decreasing resistance against bile salt of 0.2%, 0.4% and 0.6% (w/v) respectively. And the isolates showed very less growth in presence of bile salt in and above 0.8% (w/v). But among these all the best resistance is observed in the sample CW 1 which showed maximum resistance over every applied bile salt concentration in the medium.

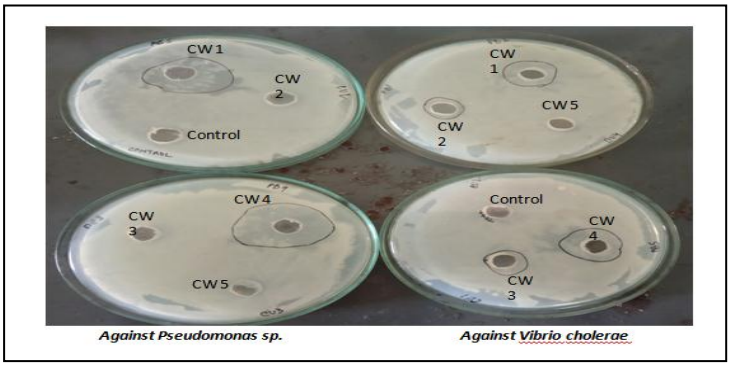


Fig 4: The anti-microbial activity of the five isolates against *Pseudomonas alcaligenes* (ATCC 14909) and *Vibrio Cholera* (ATCC 14035).

Table 4: The diameter of the zone of inhibition of five isolates against *Pseudomonas alcaligenes* and *Vibrio cholera*.

Samples	Zone of Inhibition against *Pseudomonas sp.*(cm)	INDEX	Zone of Inhibition against *Vibrio cholera* (cm)	INDEX
Control	0.00	Weakest	0.00	Weakest
CW 1	2.3	Strong	1.4	Strong
CW 2	0.00	Weakest	0.3	Weak
CW 3	0.00	Weakest	0.9	Weak
CW 4	1.7	Strong	1.1	Strong
CW 5	0.00	Weakest	0.00	Weakest

[**Note:** Weakest – 0 to 0.2 cm; Weak – 0.3 to 0.9 cm; Strong – 1.0 to1.9 cm; Strongest – 2.0 cm and above.]

From the observation (**Fig 4 and Table 4**), it can be concluded that CW 1 and CW 4 showed a prominent clear zone of inhibition against both of the pathogens, such as, *Pseudomonas alcaligenes* (ATCC 14909) and *Vibrio Cholera* (ATCC 14035). Though the diameter of the clear zone of inhibition is much more in case of *Pseudomonas alcaligenes* in compare to the *Vibrio cholera* but resistivity is also observed in other two isolates namely (CW 2 and CW 3) against *Vibrio cholera*. Thus, CW1 and CW 4 are considered as a potent anti-microbial agent against the pathogens previously mentioned in the context. All these observation were in consonance with previous reported studies [10-12]

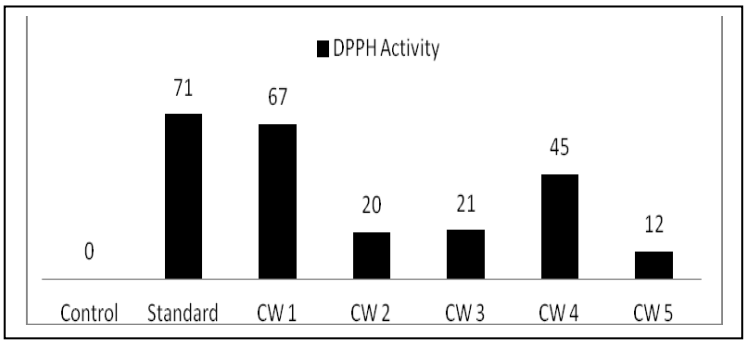
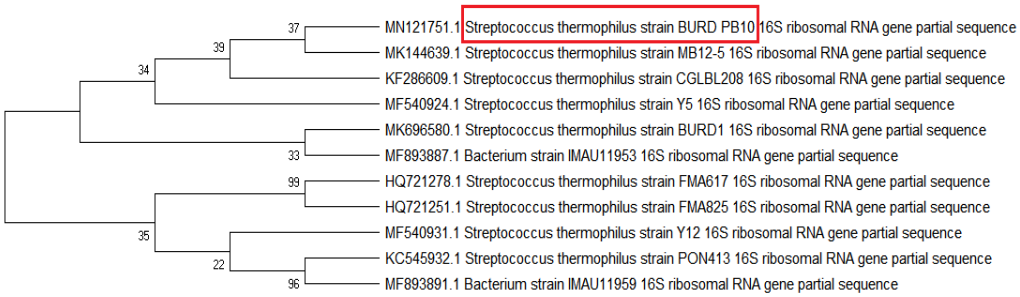


Fig. 5: The scavenging activity of the five isolates (CW1 to CW5) along with ascorbic acid act as a standard anti-oxidant.

From the scavenging activity of the five isolates along with the ascorbic acid used as a standard it is clearly observed that the anti-oxidant activity of the CW 1 is much more high among the five isolates and is nearly similar to the standard used, Ascorbic Acid which is proved to be one of the best source of anti-oxidant. **(Fig. 5).** Among the five isolates, only CW 1 showed the maximum bile salt tolerance, antimicrobial activity against *Pseudomonas alcaligenes* & *Vibrio cholera* and scavenging activity. Thus,

the DNA of the isolate CW 1 was isolated and purified for further analysis. It was the sent to KPC Medical College, Kolkata for 16S rRNA sequencing which produce a partial sequence that were used as a query sequence to find out the homologous sequence present in database. The similar sequences were retrieved through BLAST and the phylogenetic tree was revealed that the PCR sequence is *Streptococcus thermophilus* BURD PB 10. The sequence of the PCR product was submitted in NCBI and the final accession number is **MN121751**.



Conclusion

From the above experiments, it can be concluded that all the five isolates were Gram positive rods and were non-endospore former. The optimum pH, temperature and NaCl concentrations of every isolates were pH 7, 37°C and 3% respectively. And thus, this result supports the growth of the isolates in the GI tract of human body. Along with this, they are also acid producer and gives positive result for cellulase test and catalase test. But among all the five isolates (CW1 to CW 5), only CW1 gives highest bile salt tolerance, antimicrobial activity and anti-oxidant activity. These indicates the CW1 to be a potent probiotic, which after 16S rRNA sequencing was assigned as a new strain of *Streptococcus thermophilus* BURD PB 10 and the accession no. is **MN121751.**

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Conflict of interest

Authors declares no conflict of interest

Compliance with Ethical Standards

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors

Author contributions

AR: did experiment work

AP: manuscript editing

MM: manuscript editing

BB: designed the study and prepared manuscript

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