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Preparation of Fermented Ragi Based Beverage Containing Probiotic *Lactobacillus* Culture Producing Folic Acid isolated from Infant Faecal Matter of Preterm Babies

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Abstract: *Lactobacillus* isolate was used for preparation of a ragi based fermented beverage and its fermentative behavior was also studied upto 4 days at room temperature. Different biochemical parameters such as pH, titratable acidity, lactic acid. The isolate grown well in rice and decreased the pH, with an increase of total titratable acidity on account of high yield in lactic acid. Extracellular production of folate by lactic acid bacteria (LAB) has been investigated by isolating LAB from infant faecal matter. Among 70 isolates of LAB, One isolate IFM2 (2) was found to produce high levels of folate i.e. 32 ng/ml, respectively. Extracellular folate produced by isolate IFM2 (2) (*Lactobacillus* spp.) was detected by HPLC and compared with the standards tetrahydrofolate and 5-methyl tetrahydrofolate, which have shown the retention time of 7.615. The chromatogram suggested that IFM2 (2) synthesized 5-methyl tetrahydrofolate form of folate. Further the strain was evaluated for probiotic properties as per WHO and FAO guidelines. The isolate showed 50 % survival at highly acidic condition (pH 2.0) and 95 % survivability at 0.5 % bile salt concentration for 24 h. Isolate was susceptible to many antibiotics (tetracycline, erythromycin, ampicillin, gentamycin, penicillin, etc.), which reduce the prospect to offer resistance determinants to other organisms if administered in form of probiotic preparations. Isolate showed antimicrobial activity against enteric pathogens like *Staphylococcus aureus, Escherichia coli* and *Shigella dysenteriae*. The above profiling revealed that probiotic *Lactobacillus* isolate have significant impact in preparation of ragi beverage improving its functional characteristics digestibility and therapeutic potentialities mainly among tribals of Odisha and Africa.

Introduction

Ragi (*Eleusine coracana*) commonly known as finger millet, widely grown as cereal arid area of Africa and Asia. It remains one of the main staple food in Karnataka and tribal people of Odisha. It is highly nutritious and good substitute for milk powder based beverages. Ragi is mostly fermented with Lactic acid bacteria to ragi fermented beverage / drink. This is very energy rich drink and has many ethino medical importances. It protects from gastrointestinal disorders like indigestion, acid formation, vomiting, etc. Fermentation of ragi with LAB provides health benefits to the consumers producing different organic acids, oligosaccharides and polyphenolic compounds.

Folate is widely distributed in the biological world, intestinal bacteria being one source of this vitamin [14]. Folates represent an essential nutrition component in the human diet, being involved in many metabolic pathways. The daily recommended intake as approved by the European Union is 400 µg/day for adults [7,12]. Efficiency of DNA replication, repair and methylation are affected by folate, therefore high amounts of folate are required by fast proliferating cells such as leucocytes, erythrocytes and enterocytes [9]. Epidemiological studies indicated that folate deficiency is often associated with increased risk of breast cancer and that low folate homeostasis may induce hypomethylation of DNA.

LAB play a significant role in fermented foods and produce antimicrobial metabolites such as

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lactic acid, bacteriocins and hydrogen peroxide [14,17]. LAB predominantly selected from the genera *Lactobacillus* and *Bifidobacterium*, constitute a significant proportion of probiotic cultures in nutritional supplements, pharmaceuticals and functional foods [19]. Probiotics are defined as live microorganisms, which contribute to the health and well being of the hosts by maintaining or improving their intestinal microbial balance [1]. Probiotics confer their health benefits by inhibiting pathogen growth, maintaining health-promoting gut microflora, and stimulating the host's immune response [4,8]. These probiotic cultures include *Lactobacillus rhamnosus* GG, *Saccharomyces cerevisiae Boulardii*, *Lactobacillus casei Shirota* and *Bifidobacterium animalis*, which are by far the most studied probiotics with proven human health efficacy. Considering the above facts in mind, the present study was undertaken to isolate and characterize the probiotic properties of *Lactobacillus* strains producing folic acid.

Materials and methods

Ragi fermentation by Lactobacillus brevis

Ragi powder (10 g) was boiled and then fermented by the isolate, *Lactobacillus brevis* (10^5 cfu) at 37°C for consecutively 4 days. The samples were collected at every 24 h interval and stored in -20°C.

Biochemical analyses of fermented ragi beverage

Data were collected for several parameters such as pH, lactic acid (LA), titratable acidity (TA). The pH was measured by a pH meter [Model 351; Systronics (India) Pvt. Ltd., Ahmadabad, India] using glass electrode. TA and LA contents were measured by titration and spectrophotometric [UV-VIS spectrophotometer, Model 117; Systronics (India) Pvt. Ltd] methods, respectively [2] and the values were expressed in g/100 mL ragi beverage).

Isolation and screening for folic acid production

Samples were collected from infant fecal matter (6 months-2yr old infant). They were diluted by serial dilution method and plated using MRS (Mann Rogassa Sharpe) agar media [21] by spread plate and pour plate method. Plates were then incubated at 37°C for 48 h and after the incubation period the colonies were picked based upon their morphological appearance and characterized by various biochemical tests. The isolates were preserved as frozen glycerol stocks and maintained on MRS agar slants at 4°C and working cultures were prepared by propagating them in MRS broth.

Screening of folate producers

Isolates were screened for folate production, by inoculating a singly colony from MRS agar plate into folic acid assay medium (2 ml) and incubated for 18 h at 37°C.Out of 70 Isolates *Lactobacillus brevis* showing growth in the assay medium and was selected as folate producers and quantitative determination of folate was carried out by microbiological assay.

Microbiological assay for quantification of folate

To quantify the folate, microbiological assay was performed using cryoprotected folate auxotrophic mutant, *Lactobacillus casei* NCIM 2364, as standard. The organism was activated by growing in folic acid assay medium supplemented with 30 ρg of calcium folinic acid and 15 mg of ascorbic acid and incubated for a period of 18 h at 37°C. Various concentrations of folate standard ranging from 50 pM to 300 pM were used for standardization. The sample reaction mixture contained working buffer (16 % sodium ascorbate in 50 mM phosphate buffer pH 6.1), varying concentrations of working standard solution (0.9 nM of calcium salt of folinic acid), double strength folic acid casei medium, sterile water for making up the volume and the culture inoculum. All the above reagents were added in a 96-well microtiter plate and incubated for 18 h at 37°C and the absorbance of the supernatant was read at 655nm using UV-VIS spectrophotometer. Further the isolates producing maximum extracellular folate were identified by 16SrRNA sequencing

Qualitative analysis of folate by HPLC (High pressure liquid chromatography)

To determine different forms of folate produced

by the isolate, the isolate was grown in folic acid assay medium for 7 h. Further the samples were collected, filtered through 0.2 micron filters and analyzed by HPLC (Schimadju HPLC system, Japan). The C18 Nova-Pak (25c m x 4.6 mm, 4 µ spherical packing) was used as the analytical column and 0.2 micron filtered HPLC grade acetonitrile (2.5 %) in 20 mM sodium phosphate buffer pH 6.2 was used as mobile phase. Flow rate through the column was 1 ml/min. A UV-Visible detector was used at 280 nm. Different forms of folate such as tetrahydrofolate (THF) and 5-methyl tetrahydrofolate (5-MeTHF) was procured from Sigma and used as standards.

Strain identification

Identification of isolates producing maximum extracellular folate was carried out based on colony morphology, physiological and biochemical characteristics by referring to Bergey's Manual of Determinative Bacteriology [10]. Further the isolate was confirmed by complete 16S rRNA gene sequence analysis and phylogenetic studies (Macrogen Inc., Seoul, South Korea). Universal primers 518F (52 -CCAgCAgCCgCgg TAATA Cg-32) and 800R (52 -TACCAggg TATC TAATCC- 32) was used for the amplification of 16SrRNA gene of the isolates. Evolutionary analyses were conducted in MEGA 5 software [23]. Evolutionary history was inferred using the Neighbor-Joining method [20] and the evolutionary distances were computed using the Tajima-Nei method [22].

Screening for probiotic properties

Acid Tolerance

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

Bile salt tolerance

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

Phenol tolerance

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

NaCl tolerance

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

Antibiotic susceptibility test

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

Antimicrobial activity

An agar spot test was used to detect antimicrobial activities of test organism against potent enteric pathogens. Overnight grown culture of test

isolates was spotted on to the surface of MRS agar plates and spots were developed by incubation at 37°C for 24 h. Enteric pathogen like and *Staphylococcus aureus* NCIM 5021, *Escherichia coli* NCIM 6145, *Shigella dysenteriae* and *Bacillus subtilis* were inoculated at a concentration of 10^6 cells/ml in 0.7 % of nutrient agar and was overlaid on the test organism spots and incubated at 37°C for 24 h and observed for growth inhibition of pathogens around the spots.

Auto-aggregation assay

Auto-aggregation assay was performed according to Del *et al.*[5] with certain modifications. Isolate was grown over night at 37°C in MRS broth. The cells were pelleted and washed twice with PBS (pH 7.3) and re-suspended in PBS to get an OD of 0.5 at A_{600} and considered as A_0. Four ml of culture was mixed by gentle vortexing for 10 s and incubated at 37°C for 1 h. After incubation absorbance of upper suspension was measured as A_t. Auto-aggregation % was expressed as: A_0 - (A_t/A_0) x100, where A_t represents the absorbance at time t = 1 h and A_0 the absorbance at t = 0 h.

Hydrophobicity of strains

Hydrophobicity of strain was measured according to Rosenberg *et al*. [18] method with some modifications. The isolate was grown overnight at 37°C. The cells were pelleted at 8000 g for 5 min and washed twice with PBS pH 7.3, re-suspended in 0.1 mol/l KNO_3 (pH 6.2). Absorbance at A_{600} was measured as A_0 by using spectrophotometer (UV-VIS 1601 Spectrochem, Mumbai). One ml of solvent (xylene, acetone and heptane) was added to 3 ml of cell suspension. After 10 min pre-incubation at room temperature, two phases were mixed by gentle vortexing for 2 min and incubated at room temperature for 20 min. The aqueous phase was removed after incubation and then A_{600} was measured as A_1. The % of bacterial adhesion to solvent was calculated as (A_0- $A_{1/}A_0$) x 100.

Results and discussion

Changes in p^H, TA, LA and phenol content in fermented ragi beverage

pH is the major factor in lactic acid fermentation in ragi. The increase in bacterial population lead to decrease in pH of ragi beverage fermented for 4 days. The pH decreased to 3.54 in the final fermented product (initial pH was 6.40) by *Lb. brevis*. Similar changes was observed with titratable acidity (TA) content of ragi beverage. Decrease in pH lead to increase in TA content up to 4 days due to production of organic acids by metabolism of LAB (Table 1). Due to lactic acid fermentation by *Lactobacillus spp*. the end product was LA which also increased with increase in fermentation period. Initially the LA content was 0.01gm/100g ragi beverage fermented with *Lb. brevis*.

Strain isolation and identification of folate producing *Lactobacillus*

Selection of isolate was based on the macroscopic differences in the colony morphology and also on the collection of samples. The isolate was qualitatively analyzed for the ability to produce folate by observation of growth of the isolate in the modified folic acid assay media. Among the 70 isolates analyzed, 20 isolates (RM25, H321, C6, RM3, IFM5(1), C10, C4, IFM21, C16, C17, IFM25, RM22, C18, C2, BAH1, C210, C3, BARM, C8, H1I6, IFM2(2)) were found to grow in folic acid assay media indicating their ability to grow without any supplementation of folate (Fig. 1). Among the above isolates the significant

Table 1. Changes in pH, TA, LA content in fermented ragi beverage by *Lactobacillus brevis*

Isolate	Incubation period	pH	TA	LA
IFM2(2) (*Lb. brevis*)	0 hr	6.40	0.76	0.001
	24 hr	5.36	2.34	1.6
	48 hr	5.29	3.47	1.8
	72 hr	4.57	4.12	2.4
	96 hr	3.98	4.55	2.8

folate produced by IFM2 (2) 32 ng/ml.

Strain identification

Genetic analysis performed using 16S r-RNA gene analysis resulted that isolate with expected base pairs 989 bp for IFM2 (2). After performing a BLAST search isolate IFM2 (2) exhibited close association with known *Lactobacillus* sp. The IFM2 (2) strain showed high similarities with *Lb. brevis*. This result was further confirmed by constructing phylogenetic trees for IFM2 (2) (Fig. 2). 16S r-RNA gene sequence of *Lb. brevis* was submitted to NCBI gene bank and the accession number is KP336486.

Probiotic properties

Qualitative determination of forms of folate

Microorganisms synthesize different forms of folate such as tetrahydrofolate, 5-methyl-tetrahydrofolate and 5-formyl tetrahydrofolate.

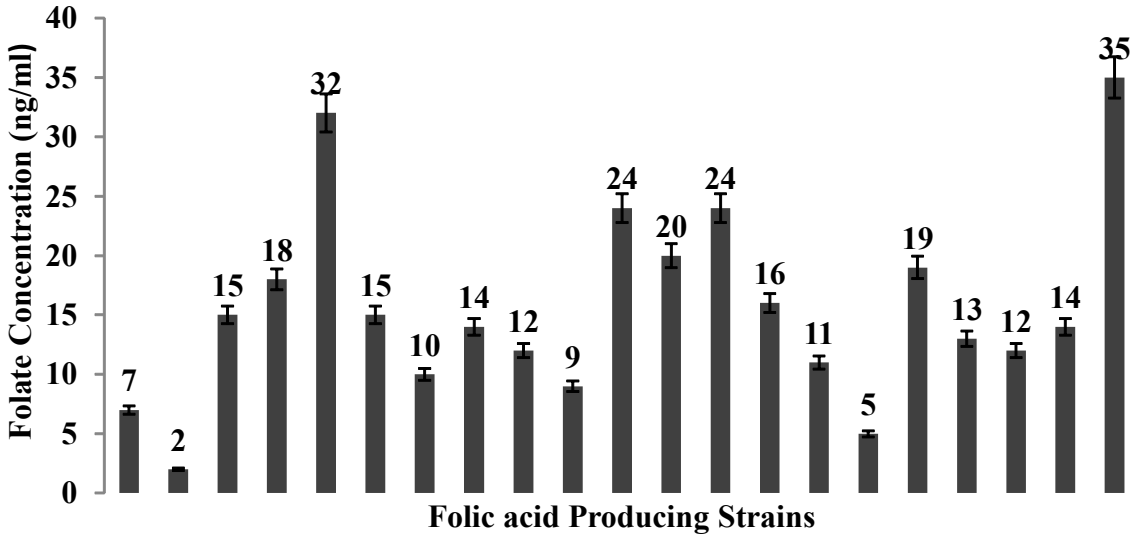

Fig. 1. Folic acid production by different isolates (ng/ml)

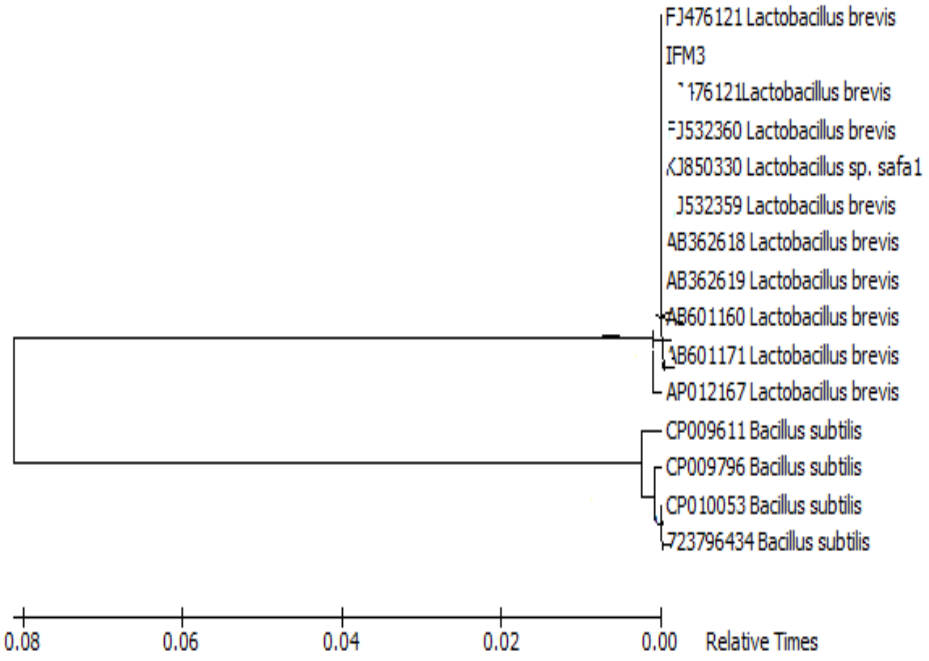

Fig. 2. Phylogenetic tree of *Lactobacillus brevis*

Extracellular folate produced by isolate IFM2 (2) (*Lactobacillus* spp.) was detected by HPLC and compared with the standards tetrahydrofolate and 5-methyl tetrahydrofolate, which have shown the retention time of 7.615. The chromatogram suggested that IFM2 (2) synthesized 5-methyl tetrahydrofolate form of folate (Fig. 3 a, b, c, d).

Acid tolerance

The effect of pH (1.0 - 4.0) on *Lactobacillus* strains was tested and the number of viable cells and survival percentage at each pH value was determined. Among the isolates, Lactobacillus brevis was evaluated for acid tolerance more than 70 % viability and it was observed at pH 3 and nearly 50 % viability at pH 2 and 40 % viability at pH 1 after 1h of incubation (Table 2). Acidity of human gastrointestinal tract varies from 1.5 to 4.5 therefore the *in vitro* studies were mostly performed at a pH range of 1.0 - 5.0.

Bile salt tolerance

The survival percentage at different concentrations of bile salts (0.3 - 4 %) were selected. The results are shown in (Fig. 4). The results indicate that IFM 2(2) showed high survival rate (72 %) at 2 % bile salt concentration. Viability of the isolate remained constant up to 3 % bile salt concentration and it got decreased as bile salt concentration was increased. Bile salt plays a fundamental role in the specific and non-specific defense mechanisms of the gut, the magnitude of its inhibitory effects is determined primarily by the concentration of bile salts [6,24].

Phenol tolerance

Results of phenol resistance exhibited relatively high survival rate at 0.1 % for the isolate i.e. IFM2 (2) and gradually decreased to less than 40 % at 0.5 % phenol (Fig. 5). Resistance to phenol is also an important factor for probiotic bacteria as some aromatic amino acids derived from dietary or endogenously produced proteins can be deaminated in the gut by bacteria leading to the formation of phenols [15].

NaCl tolerance

The results of salt tolerance studies revealed that

Table 2. Isolates incubated at different pH ranging from 6.8 to 1.0 and the number of viable cells (log CFU/ml) and survival percentage at each pH value

Isolates	Control pH 6.8	Log CFU/ml pH 4	*Percent Inhibition (%)	LogCFU/ml pH3	Percent Inhibition (%)	LogCFU/ml pH 2	Percent Inhibition (%)	LogCFU/ml pH 1	Percent Inhibition(%)
IFM2(2)	10.6	6.9± 0.1	67	5.8±0.5	54	4.5 ±0.2	36	4 ±0.0	31

* Percent inhibition = final (CFU/ml)/control (CFU/ml) x 100

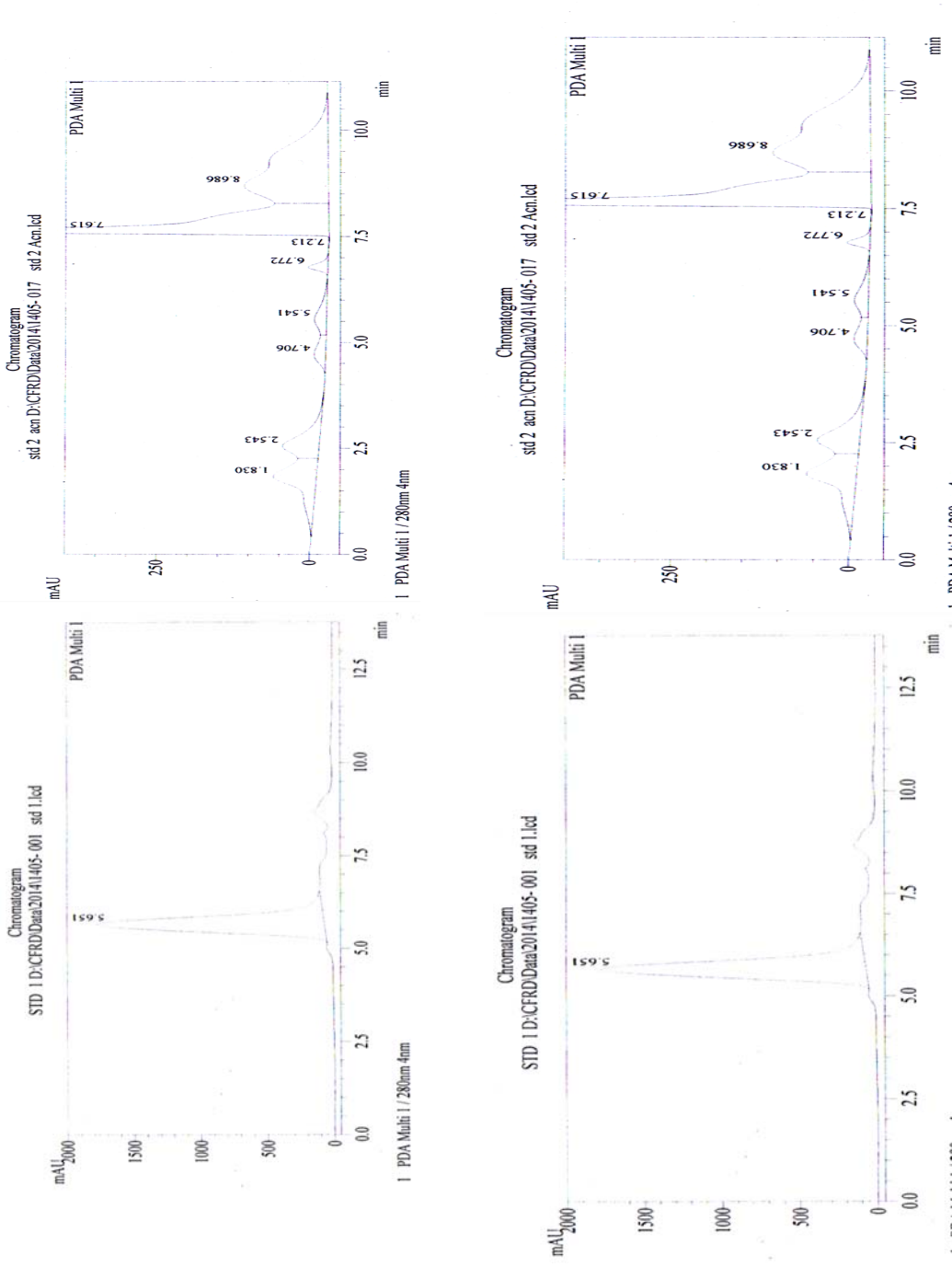

Fig. 3. Chromatogram of standard 5-methyl tetrahydrofolate; **b** Chromatogram of standard tetrahydrofolate; **c, d** Chromatogram of 5-methyl tetrahydrofolate produced by the isolate IFM2(2)

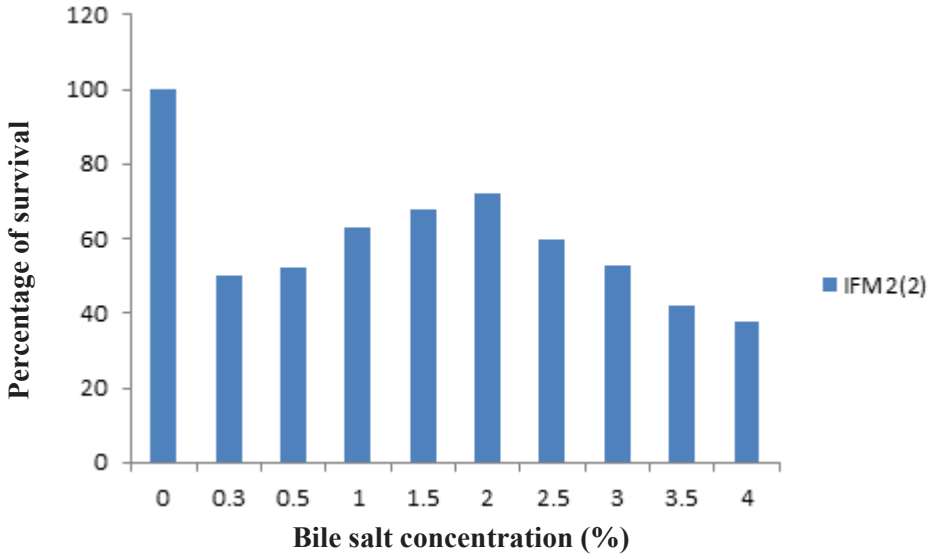

Fig. 4. Survival percentage of IFM2(2) at bile salt range of 0-0.4 %

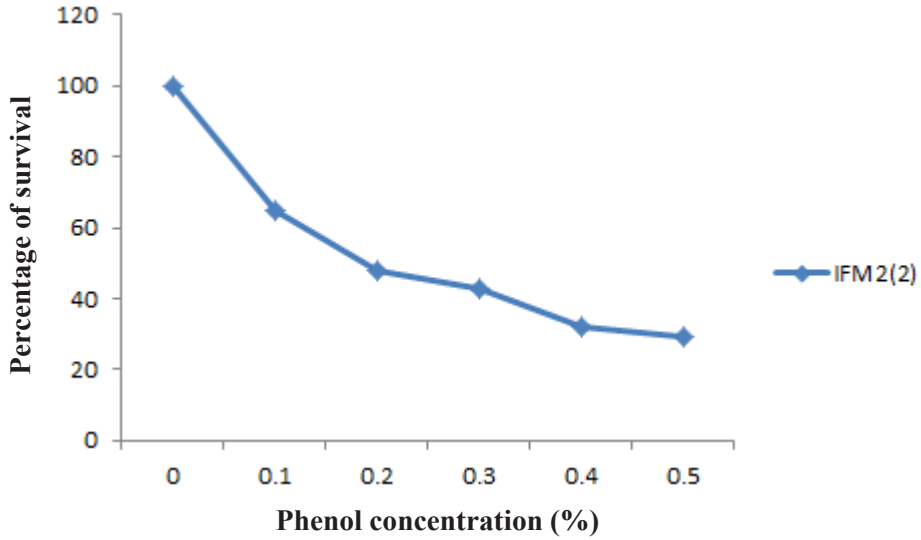

Fig. 5. Survival percentage of IFM2 (2) at phenol range of 0-0.5 %

the one isolate i.e. IFM2 (2) showed 80- 90 % viability at 2 %, 4 % and 6 % salt concentration and further reduced to 30 % at 12 % NaCl concentration. NaCl is an inhibitory substance which may inhibit growth of certain types of bacteria [11]. Tolerance to high salt concentrations is useful to help in the initiation of metabolism.

Antibiotic susceptibility study

The isolate IFM2 (2) was found to be moderately susceptible to most of the antibiotic tested i.e Norfloxacin (10 µg), Levofloxacin (5 µg), Ampicillin (10/10 µg), etc. However, the above isolate was resistant to Penicillin (10 µg) and Erythromycin (15 µg) (Table 3). In our study, the isolate was showed high sensitivity to most of the antibiotics like norfloxacin, gentamycin, tetracycline, etc and resistant to penicillin (inhibitor of cell wall synthesis) and erythromycin.

Antimicrobial activity

Isolate IFM2 (2) showed good antimicrobial activity against, *Bacillus subtilis Shigella dysenteriae*, *Staphylococcus aureus* and *E. coli* (Table 4). Isolates IFM2 (2) exhibited good antimicrobial activity against *Shigella dysenteriae*, *S.*

Table 3. Antibiotic sensitivity test of IFM2 (2)

Isolates	Name of the antibiotic (µg)	Diameter of inhibition zone (mm)	Sensitivity
IFM2(2)	Norfloxacin (10)	34 ± 3.0	++
	Levofloxacin (75)	40 ± 1.0	+++
	Cefoperazone (75 (5)	12 ± 1.0	+
	Erythromycin (15)	No zone	-
	Ampicillin (10/10)	22 ± 2.0	++
	Chloramphenicol (30)	12 ± 1.0	+
	Gentamycin (10)	18 ± 1.5	++
	Penicillin (10)	No zone	-

Susceptibility is expressed as resistant (-), low susceptible (+), moderately susceptible (++) and highly susceptible (+++)

aureus, etc. These results indicate the ability of isolate to reduce food- borne out breaks or sporadic cases caused by these enteric pathogens.

Auto-aggregation assay

In auto-aggregation study, cell sedimentation rate was found to be 44.44 % for IFM2 (2), after 1h incubation in PBS (pH 7.3) (Table 5). Auto-aggregation of probiotic strains appears necessary for their adhesion to intestinal epithelial cells with presenting a barrier that prevents colonization by pathogenic microorganisms [4,19].

Cell surface hydrophobicity

In this study, the *in vitro* determination of microbial adhesion to propanol, acetone, xylene and ether droplets was carried out. The results revealed that, in comparison to other solvents, the highest value of 66 % hydrophobicity was found in IFM2 (2) with propanol (Table 6). Cell hydrophobicity is one of the factors that may contribute to adhesion of bacterial cells to host tissues [13,16]. High hydrophobicity indicates the presence of hydrophobic molecules on the bacterial surface like array proteins, wall intercalated proteins, cytoplasmic membrane protein and lipids.

Conclusions

Ragi is well known traditional food among most of the tribal people in Odisha, Africa and is syn-

Table 4. Antimicrobial activity against tests of IFM2 (2)

Isolates	Bacterial strains	Zone size (mm)	Activity
IFM2(2)	*Bacillus substilis*	7±0.1	+
	Shigella dysenteriae	21±1.0	+++
	S. aureus	7±0.1	+
	E.coli	20±1.5	++++

Activity is expressed as low activity, (+), moderate activity, (++) and high activity (++)

Table 5. Percentage of autoaggregation by IFM2 (2)

Isolate	Initial OD at 600 nm	Final OD at 600 nm	Percentage of Autoaggregation (%)
IFM2(2)	0.5	0.28	44.44

Table 6. Precentage of hydrophobicity of IFM2(2) against different solvents

Isolates	Solvents	Initial OD at 600 nm	Final OD at 600 nm	Percentage of Hydrophobicity (%)
IFM2(2)	Xylene	0.06	0.04	33
	Propanol	0.06	0.02	66
	Acetone	0.06	0.04	33
	Ether	0.06	0.04	33

onymous with food for every Indian. Further, the wet processing of ragi with a culture of fermentable organism (*L. brevis*) improves its functional compositions (nutrients and minerals), digestibility and therapeutic potentialities. These evidences indicate that the isolated organism has significant fortifying roles in ragi fermentation. Besides, the organism itself exerts significant probiotic characteristics, which further added advantage of physiological functions.

The probiotic effects have also been reported that they produce antimicrobial substances such as H_2O_2, lactic acid, alcohols and other metabolites. These metabolites inhibit undesirable pathogens causing diarrhea or other diseases in the human intestine. *Lactobacillus* and *Bifidobacterium* are safer and well-recognized genera of probiotic, available in commercially probiotic products. *In vitro* tests showed that the isolate IFM2 (2) have high tolerance to the technological challenges assessed. The strain proved to be potential probiotic strain due to survival under highly acidic conditions, higher tolerance to bile salt, NaCl and phenol. In addition, its colonization efficiency was proved by exhibiting high auto-aggregation and hydrophobicity.

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