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Functional Characterization of Bacteriophage Resistant Mutants of Probiotic *B. coagulans*

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Abstract

Bacillus coagulans, a probiotic phage sensitive organism was subjected to random mutagenesis and several phage resistant mutants were isolated. Evaluation of mutants, in line with the WHO/FAO guidelines for probiotics, showed that Lactic acid production was not affected. Maximum sporulation efficiency was seen in mutant MIII (81% ± 2). Mutant MII showed least acid tolerance and tolerance to bile salts was lower in all the mutants compared to the parent (76% ± 0.5). All the cultures exhibited similar antibacterial activity (zones: 13-15 mm) against the pathogens used. For all the mutants bile salt hydrolase activity was positive and antibiotic susceptibility was similar to parental culture and all these mutants showed no hemolysis.

Auto-aggregation potential of the mutants was somewhat higher than the parental strain except for mutant PIII (29.5% ± 1.2) whereas mutant PIII displayed highest co-aggregation potential (52% ± 0.7). MATS assay showed that mutant PIII had highest affinity for xylene (65% ± 2.1) and mutant MV for chloroform (66.1 ± 0.8%). Adhesion to Caco2 cell was highest for mutant MIII (20.4%). Comparison of the lipid profiles showed that Alpha-linoleic acid was produced only by the parent whereas PUFA like EPA was produced by only mutants PIII and MII. Mutants PIII also produced DHA. Parental strains as well as all the 7 mutants were found to be negative for siderophore production whereas Mutants MV showed highest anti-oxidative potential (32.3% ± 1.6). Thus, thorough functional characterization of the phage resistant mutants as probiotics coupled with evaluation for additional desirable attributes has helped in better understanding of these mutants in terms of their true potential.

Keywords: Functional characterization; Probiotics; *Bacillus coagulans*; Bacteriophage resistance; Random mutagenesis; PUFA; Antioxidant potential; Siderophores

Introduction

Probiotics have been defined jointly by FAO and WHO as "Live microorganisms which when administered in adequate amounts confer a health benefit to the host" [1]. Numerous probiotic microorganisms (e.g., *L. rhamnosus*, *L. reuteri*, *bifidobacteria* and certain strains of *L. casei*, *B.coagulans, E.coli* strain Nissle 1917, certain *enterococci*, especially *E. faecium* SF68 and the probiotic yeast *S. boulardii*) are used in probiotic food, particularly fermented milk products [2].

Several mechanisms have been ascribed to beneficial probiotic effect such as, competitive exclusion, production of antimicrobial compounds, modulation of immune response, alternation of intestinal bacterial metabolic activity, its microecology and inhibition of bacterial translocation [3-6]. The beneficial effects depend on effective colonization of probiotics in the gut and their antagonistic effect against pathogens. To be able to impart the health benefits, probiotic strains need to possess certain functional properties [7]. However, there are lacunae in the present evaluation systems to establish the safety and efficacy of probiotic strains [8].

The FAO and WHO experts recognized the need for guidelines to set out a systematic approach for the evaluation of probiotics in food, leading to the substantiation of health claims. Hence, the recommended criteria and evaluation methodology for probiotic evaluation were put forward jointly by FAO/WHO [1]. *In vitro* evaluation tests are pivotal not only in gaining knowledge about the strains but also in understanding the underlying mechanisms of probiotic effects [8]. Possession of these properties assist the probiotics in imparting variety of health benefits to the host, like enhanced availability of Fe, prevention of autoimmune disorders, cardiovascular benefits because of production of PUFA, *etc* [9].

Probiotics being live microorganism are mass produced by the fermentation technology. Like any fermentation process, dairy and probiotic industry also has threat of contamination [10]. In addition to the risk of suffering from contamination resulting from the ingress of microbes in the production system, the cultures used as probiotics or dairy cultures also have the additional risk of attack by bacteriophages [11,12].

B. coagulans is produced commercially as a probiotic. The commercial production process is known to suffer from recurring problem of bacteriophage contamination. Phage infections, if not controlled can lead to lysis of host cells resulting in drainage of entire batch causing huge financial losses. One established and proven economic way to overcome the risk of phage attack is introduction of mutations to make the bacterial host genetically resistant to such attacks [13]. Strain improvement using random mutagenesis to introduce permanent genetic changes is the central part of development of fermentation processes for commercial purpose [14].

The use of random mutagenesis process for development of phage resistance in probiotic strains is a result of mutations at multiple points

in the DNA [15]. There is always possibility that random nature of mutagenesis may lead to other non-specific alterations in the host DNA causing undesirable changes in the properties of the culture under development. Hence the phage resistant mutants obtained by the use of random mutagenesis need to be functionally characterized to make certain that their functional abilities are not impaired and they remain suitable for use on commercial production scale [16].

Hence, the phage resistant mutants need to be characterized as per FAO/WHO guidelines for probiotic attributes like tolerance to acids and bile salt, lactic acid production, sporulation efficiency, adhesion and aggregation potentials, susceptibility to antibiotics, production of antimicrobial compounds, presence of bile salt hydrolase and hemolytic activity. Literature also cites some additional evaluation tests for further characterization of probiotics like desirable lipid profile in term of production of PUFA, ability to produce siderophores and to have antioxidant potential [1].

Materials and Methods

All chemicals and reagents used were procured from Merck India Ltd., Genetix Pvt. Ltd. India or from SRL Chemicals, India from where microbial media like Brain heart infusion, etc. were procured.

Bacterial strains and growth conditions

B. coagulans the phage sensitive probiotic organism under study was obtained from a privately held probiotic company from Hyderabad, India. Phage resistant strains were developed using random mutagenesis technique as described earlier [17]. The original phase sensitive parent culture as well as phage resistant mutants were maintained on glucose yeast extract agar (GYEA) slants and were preserved in 15% glycerol, at -20°C.

The pathogenic strains: *E. coli* NCIM 1025 and *S. typhi* NCIM 1250 were obtained from the School of Life Sciences, North Maharashtra University, Jalgaon, Maharashtra, India and maintained on nutrient agar and brain heart infusion agar, respectively. A total of 7 phage resistant mutants were evaluated for their suitability as probiotics by carrying out several tests.

Growth of cultures and sample preparation: probiotic cultures

Density of cell suspensions of 18-24 h age and in actively growing state of *B. coagulans* and its phage resistant mutants was adjusted to OD at $A_{540\ nm}$=0.5 (about 1.1×10^9 cells/ml) and used as samples.

Pathogenic cultures: Cell suspensions of *E. coli* NCIM 1025 and *S. typhi* NCIM 1250 were grown in Nutrient medium and brain heart infusion medium respectively and diluted to A_{540nm}=0.5 (approximately 0.8×10^9 cells/ml) with PBS (pH 7.3) and used for the assays.

Functional characterization

Preliminary studies: The functional characterization was carried out as described in guidelines by FAO/WHO (2001). Assessment of lactic acid productivity [18], sporulation efficiency, tolerance to acids and bile salts and aggregation potentials have been carried out for mutants PII, MI, MII, MIV and MV as per the methods and procedures described [19,20]. The studies on some of the properties of the phage sensitive parents and its 2 phage resistant mutants PIII and MIII was described earlier.

Aggregation studies: Auto-aggregation and co-aggregation studies were carried out as described-by Pandey et al. [20] briefly. Autoaggregation studies were carried out with cultures grown as described under sample preparation and 4 ml of resuspended cells was incubated undisturbed at 37°C for 3h whereas co-aggregation assay was performed by mixing 2 ml each of cells of probiotics and pathogenic cells grown as described under sample preparation. The mixed cultures were incubated undisturbed at 37°C for 3 h.

Adhesion studies

MATS assay: Probiotic cells were suspended in 0.1M KNO_3 (pH 6.2). O.D. of the suspension was adjusted to 0.5 at A_{600nm} [21]. To 3 ml cell suspension, 1ml of solvent (xylene, chloroform or ethyl acetate) was added. The content was homogeneously mixed by vortexing for 2 min and then allowed to stand for 20 min in an incubator set at 37°C. The lower aqueous layer was carefully removed and the absorbance was spectrophotometrically read at 600 nm. Percentage adhesion to solvents was calculated using the formula:

$$\% \text{ Adhesion} = (A_0\text{-}A_1 / A_0) \times 100$$

(Where A_0 and A_1 are absorbance at 600 nm at 0 and 20 min, respectively)

Adhesion to Caco-2 cell line: Human epithelial cell line Caco-2 (adherent) was procured in actively growing state, from National center for cell sciences (NCCS), Pune, India. The adherent monolayer was dislodged from the substratum of T-75 flask by trypsinization. Caco-2 cells were routinely cultured in Dulbecco Modified Eagle's Minimal Essential Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum. The T-flasks were incubated at 37°C in CO_2 incubator having 5% CO_2 and 95% air atmosphere [22,23].

To a sterile 24 well tissue culture plate containing about 2 × 105 Caco-2 cells, approximately 2 × 109 actively growing bacterial probiotic cells were seeded (10,000:1). The assay plate was incubated at 37°C for 60 min with intermittent shaking. The non-adherent bacterial cells were removed by two washings with sterile 0.1% peptone water. The Caco-2 cells with adhered probiotic cells, were trypsinized and serially diluted in peptone water and spread plated with 100µl of the cell suspension, on GYEA medium plates and was incubated at 37°C for 48 h. Percentage adhesion was estimated using the formula:

$$\% \text{ Adhesion} = (B_1/B_0) \times 100$$

Where B_0 and B_1 are the probiotic CFU of the inoculum and counts after assay respectively.

Assessment of antimicrobial activity

Ten µl of the actively growing test cultures were spot inoculated on GYEA plates and incubated at 37°C overnight. The next day growth was observed at the inoculated spot. One ml each of *E. coli* NCIM and *S. typhi* NCIM suspensions (OD_{540nm}=0.1) were inoculated in 5 ml of 0.7% molten nutrient agar. The content was mixed and poured over the GYEA plates. The agar was allowed to solidify and plates were incubated at 37°C. The next day plates were observed for zones of inhibition (mm) around the spots inoculated with probiotic cells [24].

BSH activity

B. coagulans and its phage resistant mutants were inoculated in the MRS-taurodeoxycholic acid broth and incubated overnight at 37°C, 150 rpm. The next day, probiotic cells were sub-cultured using a loopful of growth to inoculate fresh MRS-taurodeoxycholic acid broth and incubated for another 24 h. On the third day, a loopful of growth was streaked on MRS- Taurodeoxycholic acid agar plates and incubated at 37°C overnight. The plates were observed for precipitation around the streaked area indicating BSH activity [25].

Antibiotic susceptibility

Hundred µl of the culture (A_{540nm}=0.1) was spread plated on GYEA plates. After about 15 min, sterile discs of select antibiotics were placed on agar surface of plates and the plates were incubated for 24-48 h and observed for zones of inhibitions (mm), as indication of antibiotic sensitivity. The zones obtained were compared to the M_2A_4 performance standard chart to classify the organisms as sensitive or resistant towards the antibiotic under study [26].

Hemolytic activity

The blood agar plates were prepared and a loopful of actively growing test strains was streaked and the plates were incubated at 37°C for 48-72 h and checked for haemolysis [27].

Antioxidant potential

To 100 ml flask containing modified MRS medium (MRS medium devoid of salts-$MgSO_4$, $MnSO_4$ and K_2HPO_4), loopful of probiotic cultures were inoculated and incubated at 37°C, 150 rpm for 18-20 h. The culture was centrifuged at 13000 g for 5 min at 4°C. The pellet was resuspended in PBS buffer (pH 7.3) after two washes and served as the sample. The antioxidative potential of probiotic strains was assessed by DPPH (2,2-diphenyl-1-picrylhydrazyl) assay [28].

Ascorbic acid was used as the reference standard for constructing a standard graph. The percentage scavenging activity of the sample was calculated as per the formula given below:

$$\% \text{ scavenging activity} = [A_{(blank)} - A_{(test)} / A_{(blank)}] \times 100$$

Where $A_{(blank)}$ and $A_{(test)}$ are absorbance of blank and test respectively.

Lipid profile

Lipid extraction and quantification: Twenty five ml of actively growing culture was centrifuged at about 13,000 g and resuspended in 20 times its volume in methanol: chloroform (2:1 v/v) contained in a sealed conical flask. Lipids were extracted into the organic solvent mixture by shaking at 60 rpm overnight at 25°C. The suspension was filtered through Whatmann No.1 filter paper. The organic layer containing lipids was separated and evaporated on a water bath set at 80°C. The recovered lipids were derivatized as shown below [29].

Derivatization of lipids to fatty acid methyl esters (FAME): The lipid extract was suspended in methanolic HCl, placed in screw capped 20 ml glass vial and incubated in a water bath at 80°C for 2 h for derivatization [30]. FAMEs were extracted in hexane and dried under the stream of nitrogen. The dried product- FAME, was reconstituted in 30µl of chloroform for GC analysis. GC analysis conditions have been enlisted in Table 1.

Sr.No	Aspects of GC analysis	
1	Carrier gas	Nitrogen
2	Injection volume	0.2µl
3	Initial column temperature	140°C
4	Temperature ramping to 230°C for analysis @ 4°C/min and final hold of 5 min.	
5	Injector temperature	240°C
6	Detector temperature	

Table 1: GC conditions for FAMEs analysis

FAME samples were analyzed by Shimadzu 2014 Gas Chromatograph equipped with capillary column (30 m × 0.32 mm ID × 0.2 µm df) and flame ionization detector and Rtx®-2330 (Restek Corporation, USA). Standard (obtained from IISER, Pune) used in the study was mixture of 37 fatty acids [22].

Siderophore activity

Hundred µl of actively growing cells (A_{540nm}=0.5) was inoculated to 50 ml of sterile SAM (composition g/l: KH_2PO_4:6, K_2HPO_4:3, $(NH_4)_2SO_4$:1, $MgSO_4.7H_2O$:0.2, Sodium Succinate: 4, pH: 7.0 ± 0.2). The flasks were incubated on an orbital shaker cum incubator at 37°C, 150 rpm for 24-48 h (until growth was visible). The growth was centrifuged at approx. 13000 g for 10 min at 4°C. The cell-free supernatant acted as the source of siderophore. One ml of test sample was mixed with 1 ml of the CAS reagent. Siderophore production was indicated by the change in colour of the CAS reagent from blue to orange [31].

Results and Discussion

Preliminary studies

Lactic acid productivity, tolerance to acids and bile salts, sporulation efficiencies and aggregation potentials of the parental probiotic *B. coagulans* and its 7 phage resistant mutants showed variation as discussed below.

Lactic acid productivity

Table 2 displays the lactic acid productivity of *B. coagulans* parental and mutant strains. As reported earlier, the parental type produced 3.7 ± 1.2 g/l of lactic acid and mutants PIII and M III produced 4.7 ± 0.9 and 4.3 ± 0.7 g/l of lactic acid (Pandey et al.). The results with remaining mutants show that their ability to produce lactic acid was not seriously affected and they produced similar amount of lactic acid like the parental phage sensitive culture. The property of lactic acid production is the key to effectiveness of the probiotics in the local microflora environment of gut as it suppresses growth of many pathogenic or undesirable bacteria. At low pH, a large amount of lactic acid is in the undissociated form, and it is toxic to many bacteria, fungi and yeasts [32]. In addition, the stereoisomers of lactic acid also differ in antimicrobial activity, L-lactic acid being comparatively more inhibitory than the D-isomer [33]. Considering the role of lactic acid in probiotic action it is desirable that cultures produce more lactic acid.

Sporulation efficiency

Sporulation efficiency of the parental type was 66 ± 2% (Pl refer to Table 2). Maximum sporulation efficiency (81 ± 2%) was seen in mutant MIII, followed by mutant PII (75 ± 2.1%) and the lowest efficiency was noted for the mutant MIV (39 ± 1.6%). Higher sporulation efficiency increases the chances of cultures surviving the processes like spray drying, storage subsequent to formulation and on oral administration the passage through the intestine. The rather wide variation seen in the sporulation efficiency of mutants can be attributed to the non-specific nature of random mutagenesis. This decrease in sporulation efficiency could be a result of mutations in the genes involved in spore coat formation or the genes regulating dipicolinic content of the spore coat [34].

Tolerance to acids and bile salts

There was not much variation seen in the acid tolerance capacities of mutants except mutant MII which showed least acid tolerance [25].

Tolerance to bile salts was lower in all the mutants as compared to the parental type (76 ± 0.5%). The worst adversely affected mutants were mutant PII (35.5 ± 0.6%) and mutant MIV (12.5 ± 1.2%). Their quite increased sensitivity towards bile salts could be assigned to the structural changes in the membrane proteins and lipids due to random mutagenesis [10].

Cultures	Lactic acid production (g/l)	Sporulation efficiency (%)	Acid tolerance (%)	Bile salt tolerance (%)
B. coagulans	3.7 ± 1.2	81 ± 2.0	66.8 ± 0.6	74 ± 0.5
Mutant P II	3.45 ± 0.6	75.0 ± 2.1	64.0 ± 0.2	35.5 ± 0.6
Mutant P III	4.50 ± 0.9	73.0 ± 1.3	65.0 ± 0.5	76.0 ± 1.1
Mutant M I	3.85 ± 1.0	69.5 ± 0.5	60.0 ± 1.2	34.5 ± 0.8
Mutant MII	3.65 ± 0.3	58.0 ± 2.3	47.0 ± 0.3	63.0 ± 0.7
Mutant MIII	4.40 ± 0.7	66.0 ± 2.0	68.6 ± 2.1	63.8 ± 0.4
Mutant MIV	3.50 ± 1.1	39.0 ± 1.6	58.5 ± 2.0	12.5 ± 1.2
Mutant MV	3.80 ± 0.2	47.0 ± 1.1	58.5 ± 1.5	54.5 ± 0.8

Table 2: Assessment of *B. coagulans* and phage resistant mutants for Lactic acid productivity, sporulation efficiency and tolerance to acids and bile salts (n*=3).

Note: Control samples were set up in parallel where spores were suspended in seed medium devoid of acids /bile salts/heat treatment and for the sake of comparison, the data shown for parental culture *B. coagulans* and mutants PIII and MIII are reproduced from Pandey et al. [20].

Aggregation studies

Aggregation properties are useful characteristics of probiotic cultures [35]. Aggregation happens because of clumping of the probiotic cells resulting in larger cell aggregates. Strains with higher auto-aggregation potential tend to adhere better to the gut lining and therefore are able to exert better, the probiotic benefits [36].

Auto-aggregation: Cultures with higher auto-aggregation ability are expected to survive better during the transit through the intestine and then colonize the gut more efficiently [28,37]. Auto-aggregation potentials of all the mutants were higher than the parental strain (44 ± 0.7%), except mutant PIII (29.5 ± 1.2). Remaining mutants displayed similar capacity to parental culture (Figure 1). Thus, all the strains may survive the transit through the intestinal canal and colonize the intestine as effectively as the parental culture.

Co-aggregation assay: Substantial differences in co-aggregation potentials of probiotic strains were not observed. Mutant PIII displayed highest co-aggregation potential (52 ± 0.7%) followed by mutant MIII (51.5 ± 0.4%) and MII (47 ± 0.5%) while rest of the strains showed almost similar profiles (about 40%), mutant MV showing the least activity (32 ± 1.0%). Through the phenomenon of co-aggregation, probiotic cells form a physical-chemical barrier due to stearic hindrances or blockage of cell receptors, around the pathogen preventing them from adhesion or colonization [29]. The surface structures of probiotic cells (example: lectin like adhesins and certain receptor moieties) have the potential to neutralize the surface molecules of pathogens and thus prevent pathogens from adhering to the gut lining [27]. Figure 1 summarizes the comparative aggregation (auto and co-aggregation) potentials of the probiotic mutants.

Mutants MII and MIII exhibited high auto as well as co-aggregation potentials. Hence, they might be superior to the other strains. In general, higher aggregation potentials (auto and co-aggregation) of the strains indicate that they can survive the harsh conditions prevalent in GIT.

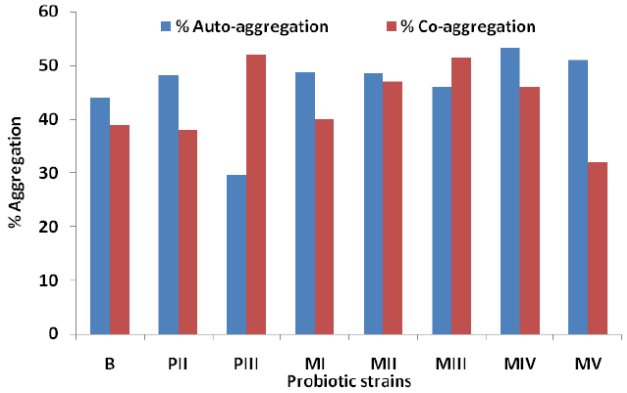

Figure 1: Comparative profiles of auto and co-aggregation potentials of the parental strain *B. coagulans* and its phage resistant mutants.

Key: B- B. coagulans parent strain; PII, PIII and MI-MV are phage resistant mutants. Note: For the comparative purpose, the data shown for parental culture *B. coagulans* and mutants PIII and MIII is quoted from Pandey et al. [20].

Adhesion potential

Bacterial adhesion is initially based on non-specific physical interactions between two bacterial surfaces, which then enable specific interactions between adhesins (usually proteins) and complementary receptors [38,39]. The initial and reversible stage is mediated by complex physicochemical interactions, including hydrophobic and

charge based interactions which are thought to be nonspecific but important initiation properties for successful adhesion to occur [40].

Cell surface hydrophobicity: Basic or acidic cell surface characteristics of probiotic strains were studied by measuring the partitioning of cells between the aqueous phase and organic phase (ethyl acetate, xylene or chloroform (Table 3)). Significant differences were observed in the affinities of probiotic mutants towards the 3 solvents. Mutant PII showed highest adhesion to ethyl acetate but the least towards xylene and chloroform whereas mutant MV showed highest affinity towards chloroform. This variation in the extent of adhesion can be attributed to the nonspecific reaction by changed charges and hydrophobicity of the membrane surfaces of mutants under study [28].

The MATS test has been extensively used for measuring cell surface hydrophobicity in Lactobacilli and Bifidobacteria [41,42]. Adhesion of probiotic cells to xylene (a non-polar solvent) at high ionic strength of 0.1 M KNO_3 (pH 6.2) reflects the cell surface hydrophobicity [43].

Culture	% Microbial adhesion to solvent		
	Ethyl acetate	Xylene	Chloroform
Parent *B. coagulans*	44.3 ± 2.1	38.8 ± 0.2	32.7 ± 2.8
Mutant PII	72.5 ± 0.8	16.2 ± 2.1	19.0 ± 2.0
Mutant PIII	32.0 ± 1.3	65.0 ± 2.1	47.0 ± 1.9
Mutant MI	39.6 ± 1.0	29.8 ± 0.5	44.2 ± 0.4
Mutant MII	44.1 ± 1.4	34.5 ± 2.0	58.3 ± 3.0
Mutant MIII	54.0 ± 1.6	40.2 ± 1.7	26.0 ± 2.4
Mutant MIV	52.1 ± 1.0	21.3 ± 1.8	46.3 ± 1.3
Mutant MV	37.4 ± 0.9	48.7 ± 1.7	66.1 ± 0.8

Table 3: Hydrophobicity of *B. coagulans* and mutants, based on adhesion (%) to solvents (n*=3)

For the comparative purpose, the data shown for parental culture *B. coagulans* and mutants PIII and MIII is quoted from Pandey et al. [20].

Mutant PIII had highest affinity for xylene (65 ± 2.1%) and mutant MV for chloroform (66.1 ± 0.8%). Overall comparison reveals that mutants PIII and MV were the best strains exhibiting higher hydrophobicity than the rest of the mutants. Cells having high affinity towards non-polar solvent, display higher hydrophobicity and hence better adhesion capability. The high or low affinity for one solvent did not exclude different affinity for the other, suggesting a high complexity of the cell surface. The increased hydrophobicity can be attributed to higher concentration of glycol-proteinaceous material and high levels of fatty acids particularly lipotechoic acid at the cell surface [44]. Predominance of hydrophilic polysaccharide structures might have led to reduced affinity of mutants PII (16.2 ± 2.1%) towards xylene [15].

Adhesion to Caco-2 cell line: The Caco-2 cells were grown for 12 days before performing the adhesion assay, this is because this cell line exhibits characteristics of small-intestinal epithelium such as brush-border microvilli, tight junctions, dome formation, and vectorial transport of both cations and anions when cultured continuously at confluence for about 2 weeks [45,46]. A comparison was made between adhesion potentials of probiotic strains to xylene and number of cells adhered to Caco-2 cell line.

Figure 2 displays the % adhesion of probiotic cells to xylene and Caco-2 (bars) and their counts adhering to Caco-2 cell line (line graph with marker). Adherence of B. coagulans to Caco-2 cells is not very efficient (range: 12-20%). Strains exhibiting high affinity to xylene (most hydrophobic solvent) were the ones with higher adherence to Caco-2 cell line- mutants MIII (40.2 v/s 20.4%), MV (48.7 v/s 18.4%) and PIII (65.0% v/s 12.2%). The most promising strain with best adherence potential was mutant MIII followed by mutants MV and PIII.

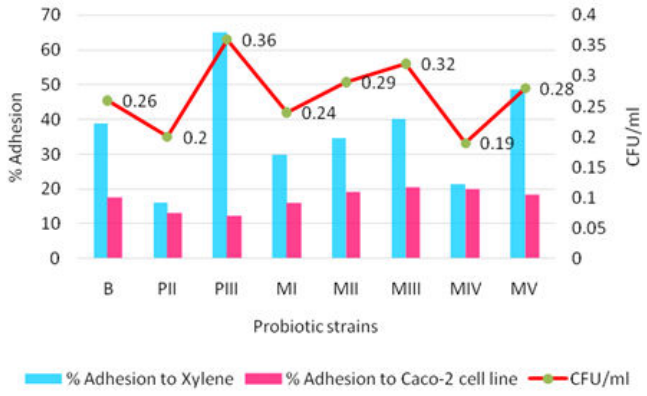

Figure 2: Comparative adhesion profiles of probiotic *B. coagulans* (parental strain) and phage resistant mutants.

Several reports show that higher the hydrophobic property of the strains the stronger is its adhesive capability [47-49]. Results of current study do not lead to any clear-cut correlation between adhesions to xylene and Caco-2 cell line. Possible causes for poor correlation could be alterations in cell surface characteristics, binding properties and /or adhesion abilities of the probiotic strains reacting differently towards solvents in in vitro MATS assay and Caco-2 cell line. The hydrophobic and hydrophilic properties of cells are known to be related to the proteins and polysaccharides present on the bacterial cell [40]. Changes in these structures due to induced mutations, might have led to the alterations in adhesion potential of the mutants under investigation.

Antimicrobial activity

All the cultures exhibited similar antibacterial activity-(zones of 13-15 mm) against the pathogens *E. coli* NCIM 1025 and *S. typhi* NCIM 1 and the response to antibiotics was considered as sensitive as per the M_2A_4–performance standards for antimicrobial disc susceptibility tests, 4th edition, approved standards [50].

Bile salt hydrolase activity

All the strains were found to be positive for BSH activity. As the test is qualitative the data is not shown.

Antibiotic susceptibility

Circulation of genes coding for antibiotic resistance from beneficial LAB in the food chain *via* animals to humans is a complex problem [51]. Hence, there is a need to evaluate the safety of probiotic strains by establishing their inability to acquire and disseminate antibiotic resistance.

The sensitivity pattern for all the *B. coagulans* mutants was identical to that of parent (Data not shown). It can be assumed that mutations had no adverse effect on antibiotic resistance of the cell. The probiotic strains were sensitive to all the antibiotics tested (except Ampicillin). Therefore, they may not be able to transmit drug resistance genes to other intestinal and/or food borne pathogens, in the food matrix or, more importantly, in the GIT, when introduced as probiotics

Hemolytic activity

All the strains tested were found to be negative for hemolytic ability (data not shown) hence can be considered safe for probiotic applications. Hemolysis assay is a test recommended by European Food Safety Authority (EFSA) to detect toxigenic potential [52]. EFSA guidelines state that Bacillus strains proven to be hemolytic are not recommended for use as feed additives. Therefore, it would be preferable to select only the non-hemolytic or perhaps weakly/very weakly hemolytic isolates for probiotic use [19]. Therefore, the mutants can be considered as safe probiotics.

Lipid profile of parent *B. coagulans* and mutants

Identification of fatty acid methyl esters (FAMEs) derived from the hexane extracts of bacteria was based on the identity of GC retention time's vis-à-vis those of standards. In general the FAMEs elute in order of increasing carbon number. However, the polyunsaturated esters exhibited higher retention times since these compounds have greater polarity compared to same carbon number FAMEs that are saturated or have lesser number of double bonds [36].

GC profile of FAMEs from reference material demonstrated resolved peaks with their relative retention times. It included about 35 peaks of saturated (even and odd carbons) and unsaturated fatty acids of varying degrees, monounsaturated fatty acids like palmitoleic and eicosanoic acid and PUFAs like docosahaexenoic and α-linolenic acids.

B. coagulans parent and mutants produced, as expected, a mixture of different saturated and unsaturated fatty acids. Majority of saturated fatty acids (with even carbon) like hexanoic, octanoic, myristic acid, etc. were produced by the parental strain IIIb , The three mutants whose lipid profiles were different from the parental strain were mutants MI, MIII and MIV. Figure 3c represents a comparison of lipid profiles of mutants MIII, PIII with the parental strain *B. coagulans*. Most of the mutants showed presence of C_6-C_{24} compounds similar to the parental type. Alpha-linoleic acid, an essential ω-3 fatty acid, was produced only by the parental type and no other mutant strain whereas mutants PIII and MII produced Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA) which have roles in brain development and preventing neurological disorders like Alzheimer's disease [53]. The loss of fatty acids like dodecanoic acid, pentadecanoic acid etc. in mutants MII and MV might have been a result of deletion mutations due to mutagenic treatment of the host. Similarly, EPA (20:5) an omega-3 fatty acid, was produced by mutants PIII and MII, but not by the parental strain.

As is evident from the discussion that mutants MIII and PIII have improved lipid profiles compared to parent and hence are more promising cultures for commercial applications.

All the FAMEs were chromatographically resolved. The GC chromatograms of probiotic FAMEs were compared to the standard as shown in Figure 3.

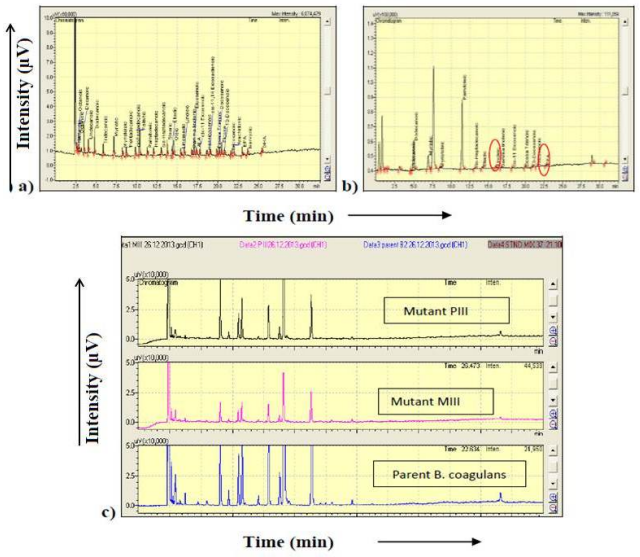

Figure 3: Gas Chromatogram of a) FAMEs standard used in the analysis, b) *B. coagulans* (parental type) profile, and c) Comparisons of mutants PIII and MIII with the parental profile.

Siderophores activity

In this study parental strain as well as all the 7 mutants were found to be negative for siderophore production. We are of the opinion that it is desirable to have siderphoric activity for the probiotic strains as such probiotics may show benefit in terms of better adsorption of iron from the intestine thus helping subjects suffering from anemia

Antioxidative potential

The antioxidative activity of parent and mutants is summarized in Table 4**.** Scavenging activity of probiotic cultures was in the range of 13.7-32.3%. Scavenging potential of parental type (20 ± 2.3%) was lower than potentials of mutant MV (32.3 ± 1.6%), MI (30.3 ± 2.9%), MIII (28.5 ± 2%) and PIII (27.5 ± 1.7%). Thus, these mutants have better scavenging activity compared to the phage sensitive parental culture. This scavenging action could be a contribution by lipid and protein molecules at the cell surface [54].

Oxidative damage is related to many diseases like atherosclerosis, rheumatoid arthritis, etc. Scavenging of different types of reactive oxygen species is thought to be one of the main antioxidant mechanisms exhibited by lactic acid bacteria [55]. Such radical scavenging active fractions and cultures can possibly be used in animal feed formulations as a supplement to relieve oxidative stress [16].

Cultures	Parent *B. coagulans*	Mutant PII	Mutant PIII	Mutant MI	Mutant MII	Mutant MIII	Mutant MIV	Mutant MV
% scavenging Activity	20 ± 2.3	13.7 ± 3.1	27.5 ± 1.7	30.3 ± 2.9	20 ± 3.5	28.5 ± 2	14.7 ± 1.3	32.3 ± 1.6

Table 4: Spectrophotometric estimation of scavenging potential of the probiotics

The data presented for the phage sensitive parental strain *B. coagulans* and its phage resistant mutants shows that there is improvement in some of the characteristics of a good probiotic. In addition, some mutants like mutant PIII and mutant MII have shown to be capable of producing some of the useful polyunsaturated fatty acids (PUFA). Scavenging potentials of some mutants was higher than parental activity. The work clearly demonstrates that it is quite feasible to mutate current probiotic cultures by simple and proven straightforward approach of random mutagenesis and obtain mutants with more enhanced probiotic attributes.

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