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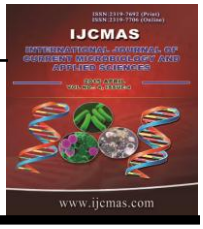

Original Research Article

Evaluation of molecular variations in Probiotic *Bacillus coagulans* and its bacteriophage resistant mutants

Kavita R. Pandey*, Pramod S. Shinde and Babu V. Vakil

Guru Nanak Institute for Research and Development (GNIRD), Guru Nanak Khalsa College, Matunga, Mumbai 400 019, India
**Corresponding author*

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A B S T R A C T

Probiotics are live microorganisms which, when administered in adequate amounts confer health benefit(s) on the host. They are produced by fermentation technology and find applications in production of functional foods and pharmaceutical formulations. They play several roles in promoting & maintaining human health. One of the most notorious contaminants in dairy and probiotic fermentation industries are bacteriophages. A leading Indian pharmaceutical industry was facing the recurring problem of phage contamination of probiotic fermentation batches. Adopting the approach of random mutagenesis, using UV light and MMS, several phage resistant strains were developed. The mutants were evaluated for retention /enhancement of their probiotic attributes, like aggregation potentials, tolerance to acids and bile salts, sporulation efficiencies etc. Genotypic and phenotypic variations and protein characterization was carried out for two mutants labelled as PIII and MIII. The observed phenotypic differences among the strains and variations in MALDI-TOF profiles encouraged us to use the tool of 16SrRNA sequencing to derive the phylogenetic relationship between parental and the mutant strains. One striking observation deduced from the phylogenetic tree analysis is that the parental strain B was an out group in the cluster while mutant PIII was more closely related to other reference taxa and mutant MIII was divergent from the other two sequences B and PIII.

Introduction

Probotics have been defined jointly by FAO and WHO as "live microorganisms which when consumed in adequate amounts, confer various healath benefit(s) on the host" (FAO/WHO Experts' Report, 2001). Probiotics find applications in pharmaceutical, dairy and veterinary productions.

Probiotic consumption claims several health benefits to the hosts, some of which are as follows: having positive influence on the intestinal flora of the host, competitive exclusion of pathogens, stimulating or modulating mucosal immunity, reducing blood cholesterol levels, anti-colon cancer activity, reducing inflammatory or allergic

reactions, alleviating lactose intolerance, reducing the clinical manifestations of atopic dermatitis, Crohn's disease, treatment of diarrhoea, constipation, candidiasis, and urinary tract infections (Harish and Varghese, 2006). This exhaustive list of health benefits exhibited by the probiotics is reflected by the scientific data generated in the field of probiotic research in the last two decades ("Probiotic Lactobacillus" PubMed, 2014).

Combinations of *Lactobacillus* and *Streptococcus* species have traditionally been used as probiotics for dairy fermentations. New genera and strains of probiotics are continuously emerging with more advanced and focused efforts. A leading Indian pharmaceutical industry producing B. *coagulans* as a bulk probiotic for medical purpose, was facing the problem of repeated bacteriophage contamination. *B. coagulans* is an aerobic gram positive, spore forming rod, which produce (+) lactic acid homofermentatively (Jurenka, 2012). Phage once attached to its host enter either lytic or lysogenic cycle and eventually it leads to the cell death by lysis.Therefore a phage contamination generally causes huge financial losses to the dairy and probiotic industries.

One proven way to overcome the risk of phage attack is introduction of mutations in the host bacterium to make it genetically resistant. Industrial strains have been traditionally improved by combination of random mutagenesis followed by rational selection for mutants of interest (Parekh, Vinci and Strobel, 2000). Short wave UV radiation and methyl methane sulphonate (MMS) were used to induce mutations. The bacterial population surviving the mutagenesis was screened to obtain phage resistant mutants (Kavita and Babu, 2010).
Random mutagenesis implies possibilities of several point mutations in the genome of the bacterium and hence phage resistant mutants may also carry other unwanted mutations resulting in loss of several desirable probiotic traits. Hence, the mutants were evaluated for their functional attributes like tolerance to acid and bile salts, sporulation efficiency *etc.* Two of the seven phage resistant mutants- GNKC/PBc/UV m3 [PIII] and GNKC/PBc/MMSm3 [MIII] showed improved probiotic profile and hence were chosen for the further studies.

Proteomic studies help in relating the changes in protein expression to the mutations in genome. So we analyzed the whole cell lysates of the two mutants using MALDI-TOF/MS and compared with the parental profile (Lay, 2001).

The current investigation was carried out with the primary aim of comparing the probiotic *B. coagulans* and its phage resistant mutants for their phenotypic and genotypic variations and16S rRNA gene sequencing was used to study the bacterial phylogeny (Jandaand Abbott, 2007).

Diversity within a given community is usually characterized using the phylogenetic distance scale. To study the evolution rate UPGMA (Unweighted Pair Group Method with Arithmetic Mean) tool was adopted which is a simple bottom-up hierarchical clustering method. The data was further verified by bootstrapping to prevent the random sampling errors. Molecular Evolutionary Genetics Analysis (MEGA) 6.0.5 was the statistical tool used to trace the molecular evolution and construction of a phylogenetic tree (Kumar, 2013). Tajima test was applied for studying the neutrality in DNA evolutionary pattern (Aquadro, 1995).

Materials and Methods

Bacterial strain and Culture conditions:

Bacillus coagulans the parental probiotic organism under study, was procured from a private probiotic industry. It was maintained on Glucose yeast extract agar (GYEA) slants [medium composition: glucose 5g, yeast extract 5g, peptone 5g, K_2HPO_4 0.5g, KH_2PO_4 0.5g, $MgSO_4$ 0.3g and mineral salt solution- 1ml, in 1000 ml of distilled water. Mineral Salt solution composition: $FeSO_4$ 1.8g, NaCl 1g, $MnSO_4$ 1g, $ZnSO_4$ 160mg, $CuSO_4$ 160mg and $CoSO_4$ 160mg in 100ml distilled water]. The bacterial strain and mutants were was preserved in 15% glycerol, at -20°C.

Random Mutagenesis:

i. Ultraviolet light**:** short wave radiation-260nm, from a 4 W germicidal lamp fixed at a height of 18cm from working surface area of the Laminar Air Flow work station was employed for inducing random mutations. The host culture was exposed to UV-radiations for time interval range from 30sec to 8min

ii. methyl methane sulphonate (MMS) **:** –was used at concentrations range of 5-50 µl/ml of cell suspension for 2h under static conditions to introduce mutations in the host bacterial cells

The mutants obtained by several rounds of MMS and UV mutagenesis were then screened for bacteriophage resistance by plaque assay.The genetic stability of the mutants was assessed by reversion test (Kavita and Babu, 2010). Out of about 1000 survivors obtained, 10 were phage resistant of which 3 were genetically unstable (revertants) hence were discarded. Out of the 7, mutants MIII and PIII were phage resistant and showed similar profile like parental type, hence were selected for the further characterization (Kavita and Babu, 2010).

Functional Characterization of the phage resistant mutants of B. coagulans:

Acid and bile salt tolerance

Forty eight hours old bacillus culture having 10^8 to 10^9 spores/ml in seed medium (composition g/l: Glucose: 5, peptone: 5, yeast extract: 5, calcium chloride: 0.38g and magnesium sulphate: 0.28g) were used for the assays (Kavita and Babu, 2010).

Acid tolerance: Spores were inoculate in seed medium of pH 2, containing 1 mg/ml pepsinand incubated at 37°C, at 150 rpm for 3h. Appropriate 10-fold dilutions were made for the samples withdrawn at regular intervals and, suitable dilutions were plated on GYEA medium and CFU were determined after incubation at 37°C for 24-48h.

Bile salt tolerance: Spores were inoculated in seed medium containing 0.2% bile salts (50% sodium-cholate and 50% sodium deoxycholate) and incubated at 37°C, at 150 rpm for 3h. CFU were determined as mentioned for acid tolerance assay.

Non treated spores were used as control. % efficiency was carried out as shown below:
The flasks containing the mixture of spores with acid or bile salts were incubated

$$\text{Tolerance to acids/ bile salts (\%efficiency)} = \frac{\text{CFU after treatment}}{\text{CFU before treatment}} \times 100$$

Biomass analysis and lactic acid estimation

Biomass was estimated on the basis of dry

cell weight (Ref). Lactic acid was estimated as per the method presented in table (Taylor, K. A. C. C., 1996).

A) ***Dry cell weight***

In a pre-weighed 1.5ml tube, one ml of culture broth was added and centrifuged in a microfuge at 10,000 rpm for 10min at 4˚C. The supernatant was used for the pH and lactic acid estimation. Excess supernatant was blotted out using tissue paper and the tube was incubated at 60°C for dying till three consecutive readings showed constant weight.

B) Lactic acid estimation:

The Lactic acid estimation was given in table 1.

Sporulation Efficiency:

Bacillus spores from 48h old culture were incubated in a water bath set at 75°C for 30 min to kill the vegetative cells followed by pour plate method to enumerate the spore count. The difference between CFU/ml of the untreated and the heat treated cells gave value of sporulating efficiency of the culture. The sporulation efficiency was calculated as follows:

$$\text{Sporulation efficiency (\%)} = \frac{\text{CFU of heat treated cells}}{\text{CFU of non-treated cells}} \times 100$$

Aggregation assays:

For the aggregation assay a loopful of organism was inoculated in seed medium and incubated at 37°C, 150 rpm for 18hr. The cells (A_{600nm} = 0.5) were pelleted down in a microfuge at 10,000rpm for 10min at 4°C. The pellet was washed twice with phosphate buffered saline (PBS) pH 7.3 and re-centrifuged under the same conditions. The pellet was suspended in PBS pH 7.3 (≈OD 0.5) and vortexed for 10sec (for the Auto-aggregation assay: 4ml of the re-suspended pellet was used and for Co-aggregation assay a mixture of 2ml each of pathogenic strain and the probiotic strain was used) and this was incubated undisturbed at 37°C for 3 hours. After every hour, upper volume of the tube was carefully pipetted and absorbance was recorded using UV-Visible spectrophotometer (Shimadzu) (Patel *et.al*, 2010).

The auto-aggregation and co-aggregation potentials were calculated using the formulae given below:

$$\text{Auto-aggregation (\%)} = A_0 - [A_t / A_0] \times 100$$

(Where A_0 and $A_t$are absorbance at 0hr and time (1hr, 2hr and 3hr)

$$\text{Co-aggregation (\%)} = \frac{[A_x + A_y] - A_{x+y}}{[A_x + A_y]} \times 100$$

(Where: $A_x$and A_y are absorbance of probiotic control and pathogenic control, $A_x + A_y$is the absorbance of the mixture)

Proteomic fingerprinting of the phage resistant strains:

The protein fingerprint of the bacterial whole cell lysate was developed using Matrix- Associated Laser-Desorption /Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF/ MS) instrument (BrukerDaltonics- Microflex, Germany) (Li and Gross, 2004).

Proteins were extracted using combination of lysozyme (10mg/ml) treatment (2h at 37°C) followed by ultra-sonication (40% Amplitude, 10sec ON, 5sec OFF cycle for

20minutes). The content was centrifuged at 10,000rpm for 5min at 4°C; supernatant served as the protein sample. The sample aliquots were stored at -20°C till further use. The extracted proteins were dissolved in 10mM ammonium hydroxide / 10mM acetic acid mixture. A mixture of 1ul each of the dissolved protein and matrix i.e. α-Cyano-4-Hydroxy-Cinnamic Acid (CHCA): Sinapinic acid (SA) was mixed thoroughly and sample was spotted onto MALDI ground steel target plate, dried and analyzed in MALDI-TOF/MS instrument (Li and Gross, 2004).

The mass / charge region 1000 to 10000 was selected to obtain protein fingerprint pattern. Fingerprint patterns for the mutant strains were compared with the parental type.

Genomic characterization of the phage resistant strains

16SrRNA sequencing

A single isolated colony of each of the 3 strains-B (parental strain), and mutants MIII and PIII were picked up from the plate. Quiagen mini-prep kit was employed for the DNA extraction. RNA was extracted by Phenol-Chloroform method for PCR Template preparation. Amplification was achieved by using 16SrRNA primer regions: 16F27 and 16R907.

After confirming the amplification on agarose gel, the amplified PCR product was purified using the PEG-NaCl method following the Turners protocol (Jandaand Abbott, 2007).Cycle sequencing was performed using the primers 8F and 907R, the amplified samples were then loaded on ABI 3730XL Sequencing machine.

Nucleotide sequence deposition

The 16SrRNA sequences of the mutants GNKC/PBc/UV m3 and GNKC/PBc/MMSm3 (abbreviated as PIII and MIII respectively) were submitted to GenBank using the BankIt tool.

Comparative sequence similarity analysis:

The three partial 16SrRNA sequences of B, PIII and MIII were the test sequences chosen for phylogenetic analysis. The Reference dataset was generated using the testing data against BLAST utility available at DDBJ (http://blast.ddbj.nig.ac.jp/) aligned with annotated 16SrRNAdatabase. A sequence identity threshold of 97% was applied for the sequences and homologous sequences were not selected for the further analysis. These selected sequences considered as the reference data in the sequence driven analysis.

Sequence driven phylogenetic analysis

Sequences from testing dataset were assembled and aligned with the reference sequences. Multiple sequence alignment was performed using MUSCLE algorithm with default parameters from MEGA 6.0.5. Phylogenetic tree was constructed by UPGMA clustering analysis with Maximum Composite likelihood method. This was further verified by performing Bootstrap analysis with 500 cycles and uniform rates were given amongst sites Tajima relativity test was then applied to check the neutrality of the test sequences.

Result and Discussion

Functional Characterization of the phage resistant mutants of *B. coagulans*:
Acid and Bile Salt Tolerance:

For the successful colonization of the probiotic cells in the gastrointestinal tract, it

is essential for probiotic bacteria to be resistant to the gastric juices in order to survive the passage through the harsh milieu of the stomach and also to be resistant to the strongly antibacterial properties of the bile. *Bacillus coagulans* is resistant to gastric conditions and the bile salts (Kavita and Babu, 2010). As evident in figure I, no significant changes were observed in the acid tolerance and bile salt tolerance abilities of mutant strains MIII and PIII when compared to the parental culture. These results are encouraging for mutants as their tolerance towards acid and bile salt is not altered due to mutagenic treatment.

Biomass analysis and lactic acid estimation

B. coagulans produces only L-Lactic acid hence easily used by the body unlike the racemic mixture or Lactic acid that cause acidosis. The lactic acid that is produced lowers the pH in the GIT and vagina, thereby inhibiting the growth of the pathogens, hence an important probiotic characteristic to be present in the phage resistant strain (Teuber, 1993).

Figure II demonstrates that all the 3 strains have almost similar lactic acid producing capacity (1.2±0.2g/l). These results demonstrate that the lactic acid producing capacity of the mutants have not been altered by random mutagenesis. Biomass or yield of biomass/batch is the most important criteria for any manufacturer. Interestingly, the yield of biomass of the mutants exhibited similar productivity of 11±1g/l as that of the parental strain.

Sporulation efficiency

Sporulation efficiency is an important attribute for the spore forming probiotics. On oral administration, these spores help in surviving the acidic gastric environment. The spore coat imbibes water, swells and the increased water content causes a rise in the metabolic rate of the sporulated bacilli. Outgrowths begin to protrude from the spore coats. The spores pass on to the duodenum where the outgrown cells germinate and transform into viable vegetative cells. They begin to proliferate in the small intestine, multiplying rapidly, producing lactic acid and bacteriocins *etc.* which render the intestinal environment non-conducive for the growth of harmful pathogenic bacteria (Teuber, 1993).

A significant reduction is seen in the sporulating efficiency of the mutants (Fig: I) which can be correlated to the adverse impact of random mutations.

Aggregation assays

Colonization in the intestinal wall is one of the most desirable property of probiotic bacteria in order to exert its beneficial effects. Auto-aggregation is a property where the probiotic cells aggregate together to form a clump or mass. This when passes through the harsh environment, only a small number of cells die, hence the number of cells surviving and the colonizing the gut will be higher.
As depicted in figure III, mutant PIII shows a drop in the auto-aggregation potentials while MIII has aggregation potential similar to B.

Co-aggregation efficiency of the probiotic culture reflects the property of these cells to hinder the adherence of harmful bacteria like pathogenic strain of *E coli, S. aureus etc.*, to the epithelia. As seen in figure III, the aggregation potential of the mutants show significant differences compared to the parental strain. The event of random mutation seems to have enhanced the co-aggregation capability of both the probiotic

mutnts-52 and 51.5% as against the parental type (38%).

Proteomic fingerprinting of the phage resistant strains

There are several variations in the MALDI-TOF/MS profiles of the parental and the 2 mutants. Table II enlists the significant peaks showing alterations in each profile. MALDI-TO F/MS assisted in easy visualization of difference in protein make-up of the mutants. When compared to the protein pattern of parent strain, the mutant cultures had few missing peaks indicating possible deletion mutation.

Genomic Characterization of the phage resistant strains:

16SrRNA sequencing

With the rapid accumulation of 16S rRNA gene sequences in public databases, the technique of 16S rRNA sequencing has been widely used when designating the phylogenetic position of prokaryotic organisms and constitutes the basis of the modern bacterial taxonomy (Janda and Abbott, 2007). The sequencing details have been tabulated in table III.

Nucleotide sequence deposition

The 16S rRNA sequences of the mutant strains which had been submitted to GenBank were accepted and provided with unique accession numbers, as enlisted below in table IV.

Comparative Sequence Similarity Analysis

BLAST was performed for all the three test sequences (B, PIII and MIII). The sequences were aligned with annotated 16S rRNA database to minimize the error. To describe species in molecular surveys that sequence the 16S rRNA gene a threshold value of 97% homology was chosen for the phylogenetic analysis. Since, 16S rRNA sequences are termed to be highly conserved across species, constituting high similarity would prescribe detailed information of minute differences which are observed in evolutionary spread which can be understood using phylogenetic tree.

Sequence driven phylogenetic analysis:

Multiple alignment was performed using the MUSCLE algorithm, which is a statistically sound, easy to use algorithm for the non-coding 16S rRNA sequences. The evolutionary history was inferred using the UPGMA method, which is a clustering method of phylogenetic analysis performed to study the divergence between species (Huson and Bryant, 2006).

The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum composite likelihood method (Firth et.al., 2011), and are in the units of the number of base substitutions per site. The analysis involved 22 nucleotide sequences including 19 reference and 3 test sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. Thus, the noise generated during multiple sequence alignment was eluded. There were a total of 805 positions in the final dataset (Tamuraet.al., 2013). Bootstrap analysis with 500 cycles ensured the statistical connotation of the phylogenetic data obtained.

The optimal tree with the sum of branch length = 0.88037341 showed a bidirectional

separation in evolutionary distinction one with 0.4261 for MIII and other with 0.4111 for remaining taxa, this reflected the efficiency of mutagens to generate variations. We observed that the parental strain B is an out-group(figure IV), with minimum phylogenetic distances from other reference taxa and the two mutants.

A noteworthy observation made in the phylogenetic tree is that mutant PIII is more closely related to other taxa than the parental strain B, while MIII is divergent from the other two sequences- B and PIII. The parental culture of *Bacillus coagulans* (B) is an industrial strain used by a leading probiotic industry. Under the improvement program several mutations have been incorporated in its genome and hence with time it has evolved and showing divergence from the other taxa in the cluster.

The event of mutation is affected by two factors: the relative impact of mutagen; and the efficiency of damage repair. Both of these factors can vary between species (Bromham, 2009). Mutant PIII, which was obtained on UV exposure was found to be closer to the reference taxa in the cluster and was distant from the parental strain B. On UV irradiation, many of the alterations in strain B might have reverted to the wild type or nullified and hence PIII has entered the cluster and in fact closer to other taxa compared to parental strain B. On the contrary, mutant MIII obtained by MMS exposure had new alterations in the DNA leading to drifting away of the MIII sequence in the phylogenetic tree compared to PIII and B.

Tajima analysis involved only the 3 nucleotide sequences of B, PIII and MIII and supported the above observations with 380 Identical, 7 Divergent sites detected in all three sequences. A stretch was observed in the global alignment of the three sequences, which was higher in mutant MIII with 278 indels and relatively very low indels 04 in PIII and B sequences. Thus, MIII showed much divergent evolutionary pattern than PIII and B, which were relatively closely related.

The equality of evolutionary rates between sequences MIII and PIII, with sequence B used as an out-group in Tajima's relative rate test gave a P-value<0.05. Hence, the null hypothesis of equal rates between lineages was rejected. The composite likelihood method was used to correct the probability that multiple nucleotide changes occurred at the same site and that divergence is thus not linear with evolutionary time.

In conclusion, permanent phage resistant strains were developed by introducing suitable genetic changes in the host organism. The mutants have been characterized and possess the required attributes to remain a probiotic culture. This gives long term relief to the probiotic industry at a nominal onetime cost as the mutant is phage resistant unlike the parental strain that was vulnerable to phage attack.

The probiotic attributes of the mutants like yield, acid tolerance and lactic acid productivity were not altered significantly. The bile salt tolerance showed slight alterations. Significant changes were observed in the aggregation potentials of the mutants. Aggregation capability of a strain is attributed to the surface structures of the cell. As observed in this study, random mutagenesis might have led to alterations in the membrane structures, which in turn led to development of phage resistance. The mutants showed phenotypic and genotypic heterogeneity when compared to the parental type. The mutants exhibited better aggregation potentials and thus possibly better colonization efficiencies in the gut compared to the parental type.

The 16S rRNA gene sequences allowed the study of relatedness of the parental strain to the mutant varieties. One of the observations was that, for *Bacillus coagulans*, MMS mutagen not only helped in development of phage resistance, but also induced several rearrangements in the genome. The phylogenetic analysis of *B. coagulans* and mutants revealed that the parental strain was distant from the other taxa-an out group in the phylogenetic tree cluster, while mutant PIII was closer to them. Probably MMS exposure, which led to development of phage resistance in mutant PIII also contributed to mutational rearrangements in the DNA, because of which PIII is a part of the cluster, closer to the other taxa. However, this fact needs to be established with more evidences and studies with other strains as well.

The mutants MIII and PIII were very close to the parental strain in their functional attributes, but the phylogenetic analysis reflected their distant relatedness in the evolutionary tree. This can be indicative of the fact that the mutations have not altered the genes controlling selected probiotic attributes of the strains. Hence, the mutants need further characterization to understand the sites of alterations and the effects of those alterations on the organism.

Table.1 Protocol for colorimetric estimation of lactic acid

Conc. Of Lactic acid (µg)	**Vol. of lactic acid (µl)**	**Vol. of d/w (µl)**	**Vol. of (96%) H_2SO_4**	**Mix well &incubate at 95°C to 100°C for 10min in a boiling water bath.**	**Vol. of (4%) $CuSO_4$ (µl)**	**Vol. of (1.5%) p-Phenyl Phenol (µl)**	**Mix well & incubate at Room Temp. For 30 min. Measure OD 600nm**	**Read at Delete this column**
0	0	500	**3ml**		**50µl**	**100µl**		
5	300	200						
10	250	250						
15	200	300						
20	150	350						
25	100	400						
30	50	450						

Table.2: Comparative table showing the peculiar peaks of each strain, which is missing in the other two profiles

B	**PIII**	**MIII**
2159	-	-
-	-	**3024**
4645	-	-
-	**6071.01**	-
-	-	**7155.719**
7476.13	-	-
-	-	**8173.2**
8475.425	-	-
	9073	-

Table.3 Sequencing conditions for the partial 16SrRNA sequencing of B, PIII and MIII strains.

Universal Primers for 16SrRNA sequencing		**PCR program**	**Reference**
8F	**5'AGAGTTTGATC-CTGGCTCAG 3'**	94°C for 3 min 94°C for 1 min 55°C for 1 min 72°C for 1 min **repeated for 35cycles** 72°C for 10 min 4°C ∞.	**(Vogel, E., and Martin Sicken, P. R. 2007)**
907R	**5'CCGTCAATTCM-TTTRAGTTT 3'**		**(Goodfellow, M. and Stackebrandt, E., 1991)**

Table.4 Accession numbers obtained on GenBank submission of the partial 16SrRNA sequences of B, PIII and MIII

Sr No	**Strain Identity**	**Name code**	**Abbreviation**	**Accession number**
1	*B. coagulans* mutant	GNKC/PBc/UVm3	PIII	BankIt1761411 Bacillus KM652655
2	*B. coagulans* mutant	GNKC/PBc/MMSm3	MIII	BankIt1761402 Bacillus KM652654

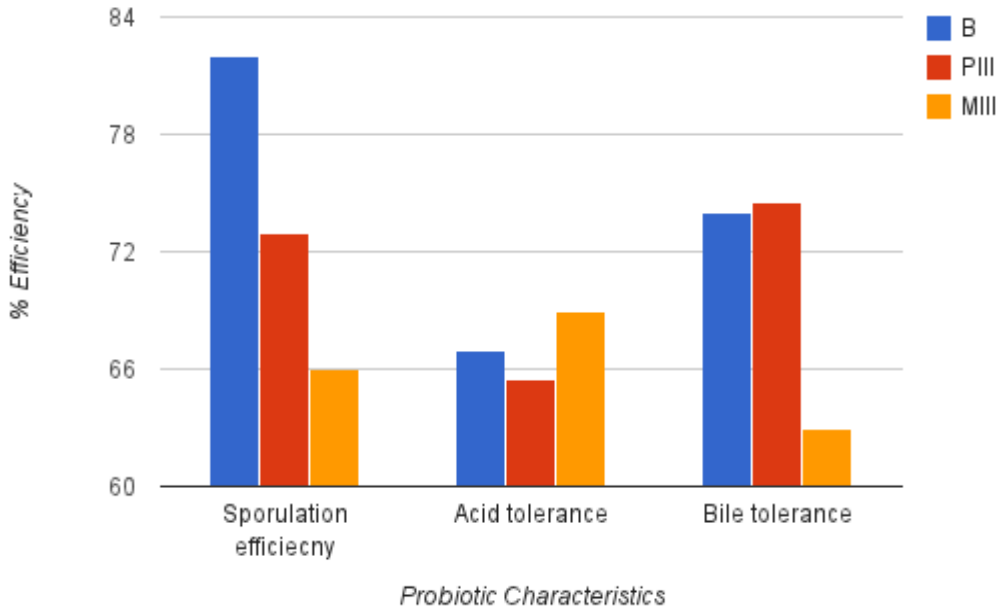

Fig.I Histograms showing profile of probiotic attributes of *Bacillus coagulans* and its phage resistant mutants.

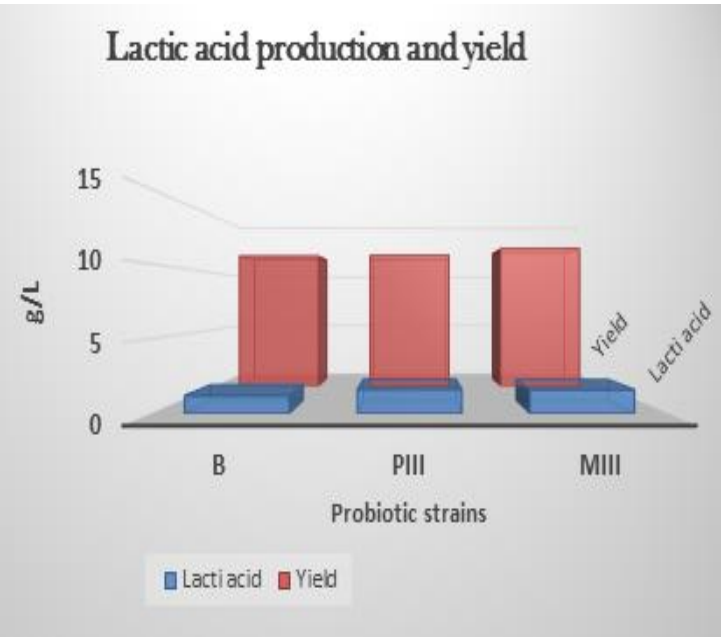

Fig.2 Histograms showing profile of probiotic attributes of *Bacillus coagulans* and its phage resistant mutants

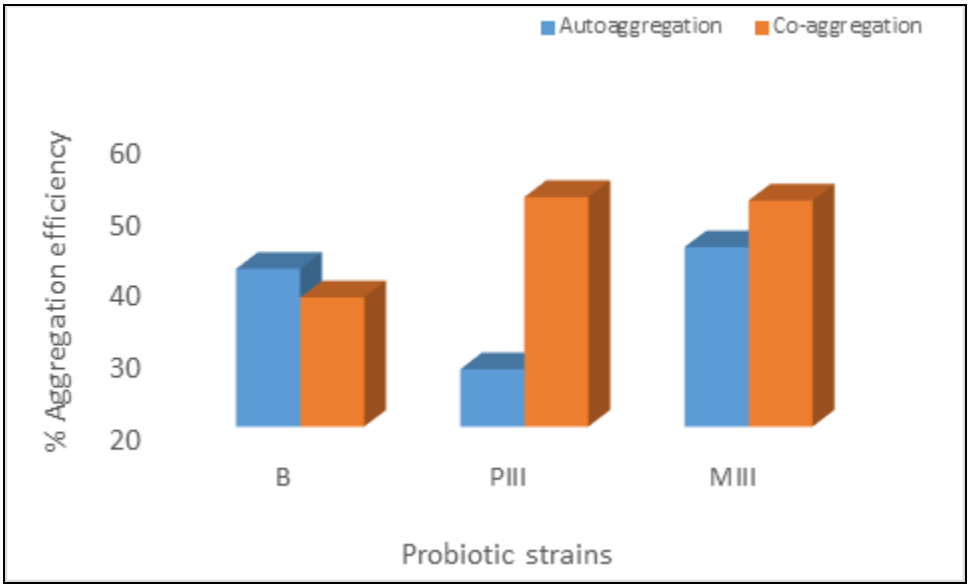

Fig.3 The aggregation potentials of the probiotic strain and its phage resistant mutants

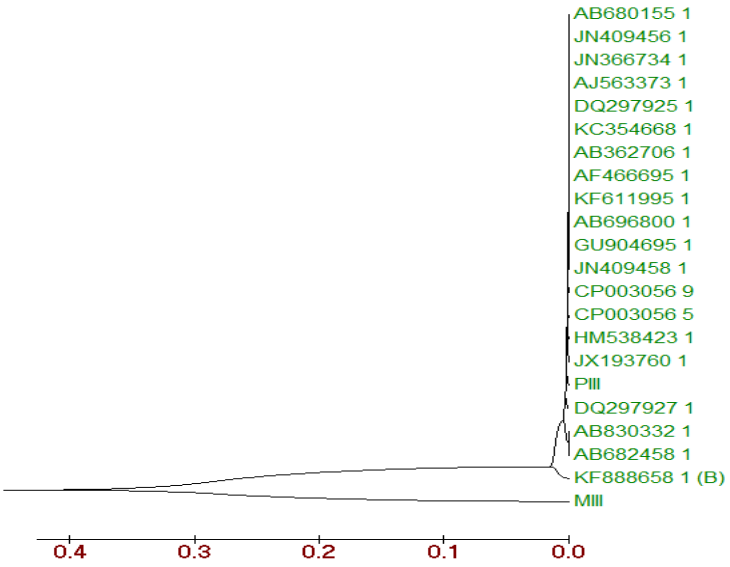

Fig.4 Evolutionary relationships of taxa where MIII, B, PIII are test and others are reference sequences (Below scale is showing the phylogenetic distance amongst all taxa)

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