Screening and genotypic characterization of lactic cultures isolated from fermented *idli* batter

ABSTRACT

The present study was conducted during 2021-22 at Dairy Science College, Bangalore, India to study the microflora present in fermented in control *idli* batter and batter with added with paneer whey and characterize them to use as solid-state fermentation cultures for idli batter fermentation. After enumeration of microflora in ingredients and fermented batter, eighteen isolates were screened for their phenotypic characteristics. The paneer whey was used @70 % of the water used as value addition and by product utilization for enhancing nutritional quality of idli. Further these lactic cultures were screened for their activity for acid production and DMC in whey based medium. Titratable acidity ranged from 0.42 to 0.70 % lactic acid with highest of 0.70 for Lb1 and lowest of 0.42 for Leu6 and W4 isolates. DMC was highest of 8.01 log10 cells/ml for E1 isolate and lowest of 7.41 log10 cells/ml for W4 with range from 7.41 to 8.01 log10 cells/ml. The isolates with code numbers Lb1, Lb2, Leu2 and E1 showed higher titratable acidity and DMC. DNA extracted from the selected four isolates, was subjected to PCR and PCR products were sequenced. Based on results obtained species of isolates wereidentified as *Leuconostoc* sp. strain Leu2, *Enterococcus* sp. strain E1, *Lactobacillusbrevis* strain Lb1 and *Lactobacilluscasei* strain Lb2. The nucleotide sequences of 16S rRNA were submitted in GenBank of NCBI and obtained accession numbers as MW 386845.1, MW386871.1, MW480882.1 and MW485119.1 respectively. These isolates were further used as solid-state fermentation cultures for *idli* batter fermentation.

Keywords: Screening, lactic cultures, batter, sequencing, fermentation,

1. INTRODUCTION

The microorganisms responsible for characteristic change during fermentation of *idli* batter. There will be sequential change in bacterial flora, and main responsible bacteria for gas production is due leavening action caused by the activity of heterofermentative lactic culture, *Leuconostocmesenteroides*[1].Soni and Sandhu [2]enumerated lactic acid bacteria in the fermented batter ranging from 10^6–10^9/g that included *Leuconostocmesenteroides, Enterococcus faecalis, Lactobacillus fermentum* and*Pediococcus cerevisiae* essential for leavening of batter and acid production in idli.

The predominant yeasts identified belonged to the species of genera namely *Candida*, *Saccharomyces*, *Trichosporon* and *Torulopsis*. *Saccharomyces* sp., were predominantly present and was identified at 0, 8, 16 and 24 h of fermentation. *Candida* sp. was identified at 0 and 8 h, *Trichosporon* sp., at 8 h and *Torulopsis* sp., at 16 h of fermentation [3].Fresh idli batter samples of ten household were analyzed during various fermentation time intervals and isolated 300 pure colonies and characterized by morphological and biochemical methods. Out of the 300 colonies isolated, 40 strains were characterized and identified as *Leuconostoc*spp, *Weissella*spp, *Pediococcus*spp, *Lactococcus*spp, and *Bacillus* spp.[4].

Shukla and Dubey [5] replaced water by whey for soaking the rice and black gram, which had enhanced the mean sensory scores of 9-point hedonic scale to 8-8.5 compared to control of 6.0 -7.9. There was increase of protein by 0.67 %, fat by 0.04 %, carbohydrate by 1.28 % and noticeable increase in calcium content approximately by five folds, i.e. 32.78 mg in control and 161.84 mg in whey based idli batter.In one more study, concentrated paneer whey (15 % TS) was used in complete replacement of water for batter production, which improved nutritional quality of idli and dosa as well as effective utilization of whey by reducing burden on effluent treatment. The idli made with whey concentrate had higher ash content (1.28 %) and acidity (3.81 ml of 0.1 NaOH per g of sample), with higher degree of hardness (21166.07 g). [6].

The study was conducted to characterize the bacteriocinogenic lactobacilli from fermented idli batter which could find application in bio-preservation and biomedicine. Isolates of 8 numbers out of 22 were characterized based on the various classical phenotypic, physiological and biochemical tests including various carbohydrate utilization profiles. All isolates were homofermentative, catalase and gelatin negative. Molecular characterization was performed by Random Amplification of Polymorphic DNA (RAPD), 16S rRNA analysis, Amplified rDNA restriction analysis (16S ARDRA) and Multiplex PCR for species identification. RAPD was carried out using the primer R2 5'-GGCGACCACTAG 3' and M13 5' GAGGGTGGCGGTTCT-3'. 16S rRNA analysis showed 99 to 100 % homology towards *Lactobacillusplantarum*. Among the five clusters obtained in RAPD, three clusters were clearly identified as *Lactobacillusplantarum* ssp. *plantarum*, *Lactobacilluspentosus*, and *Lactobacillusplantarum* ssp. *argentoratensis*[7]

Saravanan[8] carried out enumeration of the bacterial diversity of idli batter during fermentation and to characterize the potential functional properties of selected isolates. Total of 47 isolates were selected randomly, 16S rRNA was amplified and the sequences were analyzed by BLAST to identify up to strain level and the sequences submitted to NCBI Gene Bank. The 47 isolates represented 10 genera and 15 species. Majorly, Bacillus spp., Weissella spp. *Leuconostocs* spp., *Pediococcus* spp*., Lactococus spp.,Micrococcus* spp., *Enterobacter* spp., *Chryseobacterium*spp and *Acinetobacter*spp were identified. The evolutionary distances were computed using the Maximum Composite Likelihood method and were in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There was a total of 624 positions in the final dataset and phylogenetic analyses were conducted in MEGA 4.

In the present study, lactic cultures were isolated from fermented idli batter using plating method with selective growth media. Selected colonies were further purified by streaking, subjected to screening for various phenotypic characteristics [9]. These isolates were screened for titratable acidity and cell count in whey medium. Further shortlisted four isolates with good biomass and acid development were taken for DNA extraction. Extracted DNA was subjected PCR reaction, and obtained PCR products were sequenced in external lab. The sequence data was analyzed by using BLAST from NCBI website.

2. MATERIAL AND METHODS

Typical colonies from viable count plates were studied for their colony characteristics as well as cell morphology. Total 18 isolates were selected based on colony morphology, isolates were obtained both from control and as well as whey-based idli batter. They were transferred to yeast glucose broth and incubated at 30 ºC for 24 h. Further they were streaked thrice on poured plates of Yeast Glucose Agar (YGA) and purified isolates were maintained in YGA stabs as stock and yeast glucose broth as working cultures. The phenotypic characterization of isolates was carried out by using preliminary tests, viz. Gram's staining, catalase test,

$CO_2$from glucose, growth in litmus milk and specific tests like dextran production on sucrose agar for sugar fermentation[9].

2.1 SCREENING OF ISOLATES FOR CELL COUNT AND ACID PRODUCTION

After phenotypic characterization, lactic isolates were subjected to the growth study in sterile whey medium. Young isolates (24 h) were inoculated individually in a 20 ml sterile whey medium at 1 % rate and incubated at 30 ℃ for 24 h. After 24 h, the whey medium was analyzed for Direct Microscopic count (DMC)[10] and counts expressed as $\log_{10}$ cells/ml and titratable acidity[10] was analyzed and expressed as % lactic acid. The highest values were compared with rest values, screened and selected top four isolates.

2.2 GENOTYPIC CHARACTERISATION OF THE SELECTED ISOLATES

The selected four isolates Lb1, Lb2, Leu2 and E1 among 18 isolates that showed good biomass and acidity were further subcultured, in yeast glucose broth and taken for DNA extraction. The cultures were subjected to DNA extraction using ready to use DNA extraction kit from Genei Laboratories, Bangalore. Further this genomic DNA of isolates was subjected to PCR. The reaction mixture comprising 10X PCR buffer (containing $MgCl_2$), dNTPs, primers and taq polymerase was prepared and distributed to reaction tubes according to the requirements. The final volume of PCR mix was adjusted to 25 µl and the PCR tubes were transferred to thermocycler (S-96 Satellite Gradient Thermal Cycler). The PCR Cycling steps comprised of one cycle of initial denaturation (9 min at 94 ºC), followed by 45 cycles each of denaturation (30 s at 94 ºC), primer annealing (30 s at 50 ºC) and extension (30 s at 72 ºC) followed by a single cycle of final extension of 7 min at 72 ºC. The reaction was terminated by cooling the contents to 4 ºC. After the run was over, the amplified PCR products were kept at -20 °C after dissolving in 20 µl of TE buffer until further use [11].

2.3SEQUENCING AND ANALYSIS USING BLAST

The PCR products obtained were sent to external laboratory, Theracues Innovations Pvt. Ltd, Bangalore for sequencing. After getting sequence data from the external lab, analysis was performed using Basic Local Alignment Search Tool (BLAST) from NCBI (https:/www.ncbi.nlm.nih.gov.>BLAST) website. Based on results obtained, species of isolates were identified. The nucleotide sequences of 16s rRNA were deposited in GenBank of NCBI and obtained accession number.

3. RESULTS AND DISCUSSION

3.1 SCREENING OF ISOLATES FOR CELL COUNT AND ACID PRODUCTION

After phenotypic characterization, the selected 18 isolates were screened for their activity and growth in whey medium. They were inoculated at 1 % rate in the sterile whey medium. They were incubated at 30 ℃/ 24 h in candle jar. Their activity was determined by titratable acidity and Direct Microscopic Count (DMC). The titratable acidity (% LA) was 0.58, 0.63, 0.56, 0.46, 0.54 and 0.42; DMC ($\log_{10}$ cells/ml) was 7.43, 7.90, 7.61, 7.82, 7.72, 7.87 and 7.60 for isolates Leu1, Leu2, Leu3, Leu4, Leu5, Leu6, and Leu7, respectively. Isolates P1, P2, P3, and P4 showed titratable acidity of 0.59, 0.59, 0.59, and 0.55 % lactic acid, whereas DMC ($\log_{10}$ cells/ml) was 7.67, 7.79, 7.87 and 7.83, respectively. The isolate E1, exhibited titratable acidity of 0.59 % LA and DMC ($\log_{10}$ cells/ml) of 8.01. The titratable acidity was 0.46, 0.44, 0.46 and 0.42 % lactic acid, whereas DMC ($\log_{10}$ cells/ml) was 7.74, 7.41, 7.56, and 7.65 for isolates W1, W2, W3 and W4, respectively. Lactobacillus isolates Lb1, Lb2

showed titratable acidity of 0.70, 0.60 and DMC of 7.91 and 7.98 log_{10} cells/ml, respectively (Table 1 and Fig 1).

Table 1: Screening of batter isolates for titratable acidity and cell count in whey medium

Sl. No	Name of the isolate	Isolate code	Titratable Acidity (% LA)	DMC (log_{10} cells/ml)
1	*Leuconostocsp.*	Leu1	0.58^{cd}	7.43^{f}
2	*Leuconostocsp.*	Leu2	0.63^{b}	7.90^{ab}
3	*Leuconostocsp.*	Leu3	0.56^{de}	7.61^{def}
4	*Leuconostocsp.*	Leu4	0.46^{g}	7.82^{abcd}
5	*Leuconostocsp.*	Leu5	0.54^{e}	7.72^{bcde}
6	*Leuconostocsp.*	Leu6	0.42^{h}	7.87^{abc}
7	*Leuconostocsp.*	Leu7	0.49^{f}	7.60^{def}
8	*Pediococcuspentosaceus*	P1	0.59^{c}	7.67^{bcde}
9	*Pediococcuspentosaceus*	P2	0.59^{c}	7.79^{abcde}
10	*Pediococcuspentosaceus*	P3	0.59^{c}	7.87^{abc}
11	*Pediococcuspentosaceus*	P4	0.55^{de}	7.83^{abcd}
12	*Enterococcus* sp.	E1	0.59^{c}	8.01^{a}
13	*Weissellaconfusa*	W1	0.46^{g}	7.74^{bcde}
14	*Weissellaconfusa*	W2	0.44^{gh}	7.41^{f}
15	*Weissellaconfusa*	W3	0.46^{g}	7.56^{ef}
16	*Weissellaconfusa*	W4	0.42^{h}	7.65^{cdef}
17	*Lactobacillus brevis*	Lb1	0.70^{a}	7.91^{ab}
18	*Lactobacillus casei*	Lb2	0.60^{c}	7.98^{a}
CD (*P*=.05)			0.02	0.28

Note:

- The values were average of three trials
- CD – Critical difference
- Inoculum level was 1 % with incubation anaerobically in candle jar at 30 ºC/24 h in whey medium
- Highest value was compared with other values
- Same superscripts in the column indicate non-significance while different superscripts indicate significant difference

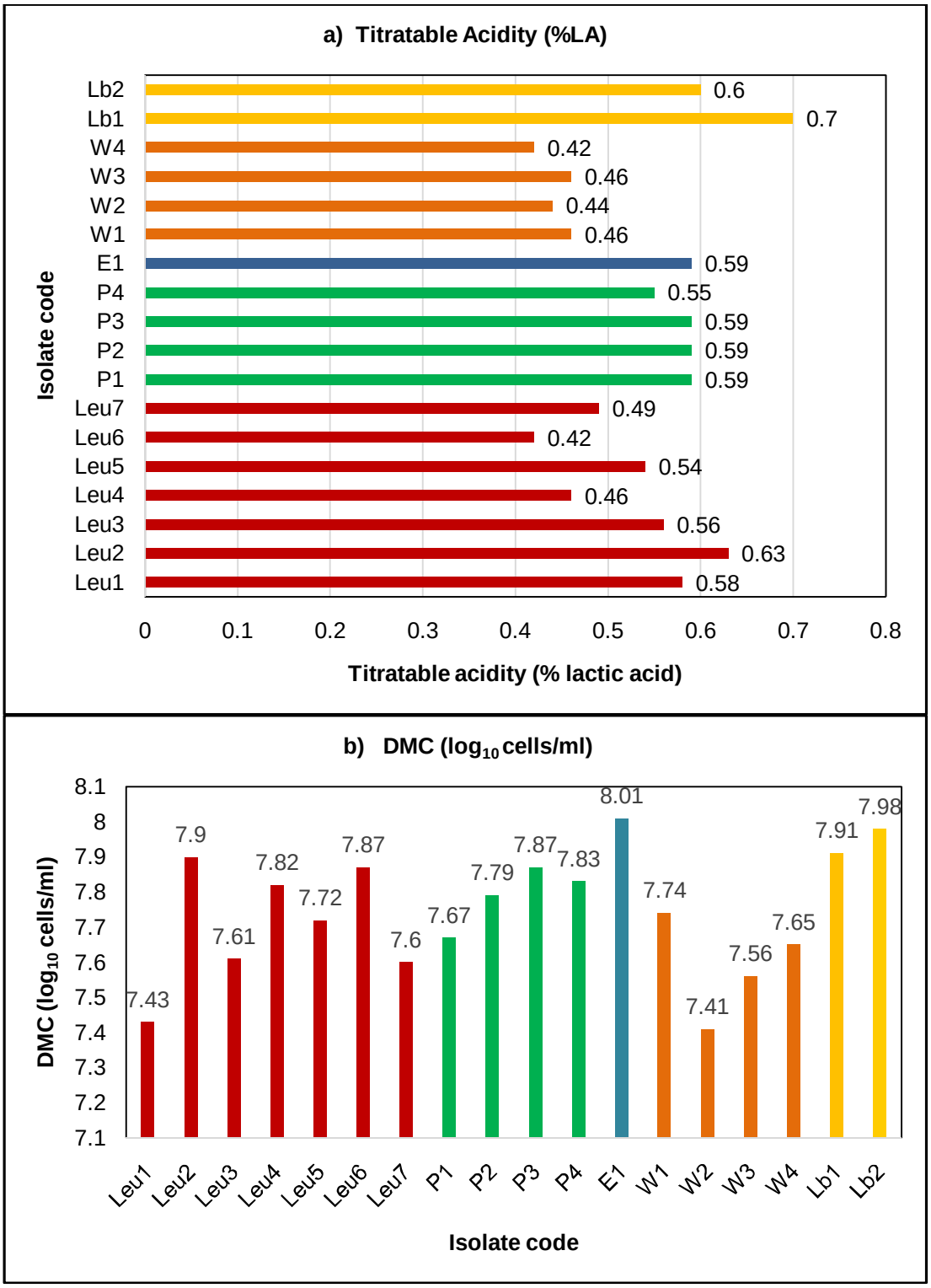

Fig 1: Titratable acidity and DMC of lactic isolates in sterile whey medium

Titratable acidity ranged from 0.42 to 0.70 % lactic acid and DMC from 7.41 to 8.01 log_{10} cells/ml. The highest titratable acidity was found with Lb1 of 0.70 % LA, lowest was 0.42 by Leu6 and W4. The DMC was highest for E1, 8.01 log_{10} cells/ml and lowest for Leu1 of 7.43 log_{10} cells/ml. There was a significant difference in the values from one isolate to other with few exceptions (P=.05). From the above results, it was found that the isolates with code numbers Lb1, Lb2, Leu2 and E1 showed higher titratable acidity and DMC and were selected for further study as solid-state fermentation cultures.

3.2 GENOTYPIC CHARACTERIZATION OF SELECTED ISOLATES

Based on results, isolates were correctly identified as *Leuconostoc* sp. strain Leu2, *Enterococcus* sp. strain E1, *Lactobacillusbrevis* strain Lb1 and *Lactobacilluscasei* strain Lb2. The nucleotide sequences of 16s rRNA were submitted in GenBank of NCBI and obtained accession numbers for Leu2, E1, Lb1 and Lb2 as MW386845.1, MW386871.1, MW480882.1 and MW485119.1 respectively (MW in accession number indicates direct submission to GenBank) (Fig 2-4,Table2).
These isolates were used for further studies for employing them solid state fermentation cultures for *idli* batter fermentation.

Table 2: Genotypic identity of selected lactic isolates obtained from idli batter

Sl. No	Isolate code	Source	Name of isolate	Accession number
1	Leu2	Control batter (100 % water)	*Leuconostoc sp.*	MW386845.1
2	E1		*Enterococcus sp*	MW386871.1
3	Lb1	Whey based batter (70 % paneer whey)	*Lactobacillus brevis*	MW480882.1
4	Lb2		*Lactobacillus casei*	MW485119.1

4. CONCLUSION

After phenotypic characterization, the selected 18 isolates were screened for DMC and titratable acidity in whey medium. Titratable acidity ranged from 0.42 to 0.70 % lactic acid with highest of 0.70 for Lb1 and lowest of 0.42 for Leu6 and W4 isolates. DMC was highest of 8.01 log10 cells/ml for E1 isolate and lowest of 7.41 log10 cells/ml for W4 with range from 7.41 to 8.01 log10 cells/ml. The isolates with code numbers Lb1, Lb2, Leu2 and E1 showed higher titratable acidity and DMC and hence they were selected for further study for solid state fermentation on black gram dhal.Genomic DNA extracted from the selected four isolates, was amplified through PCR and PCR products outsourced for gene sequencing. After receiving the sequence data, analysis was done using Basic Local Alignment Search Tool (BLAST) from NCBI website. Based on results obtained species of isolates were correctly identified as *Leuconostoc* sp. strain Leu2, *Enterococcus* sp. strain E1, *Lactobacillus brevis* strain Lb1 and *Lactobacilluscasei* strain Lb2. The nucleotide sequences of 16S rRNA were submitted in GenBank of NCBI and obtained accession numbers as MW 386845.1, MW386871.1, MW480882.1 and MW485119.1 respectively.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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APPENDIX

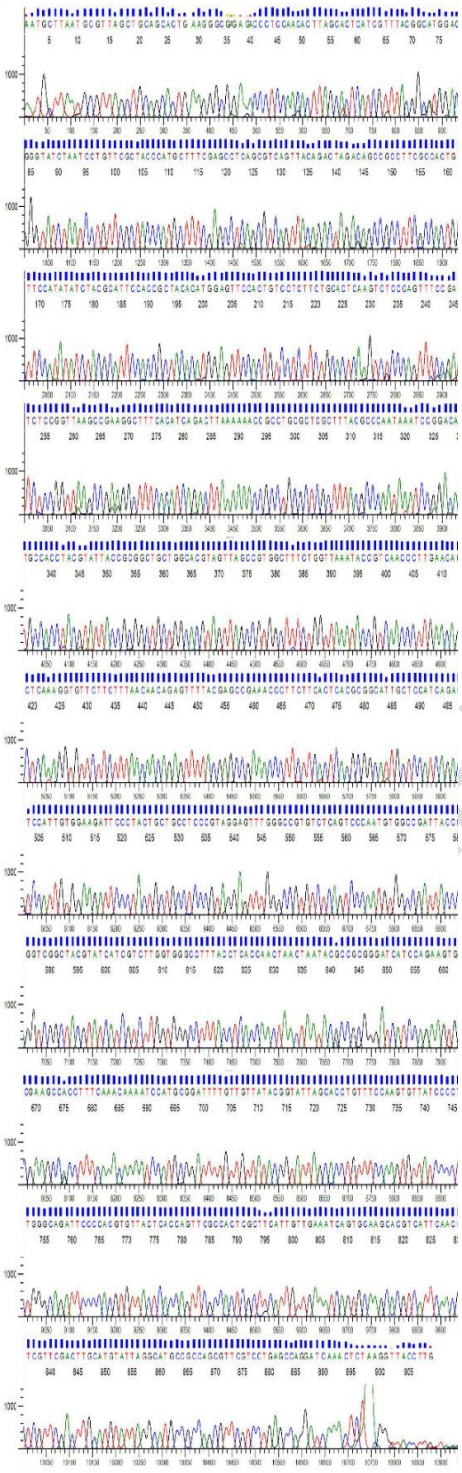

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Fig. 2: 16s rRNA sequence *Leuconostoc*sp. Leu2 with Accession number – MW 386845.1

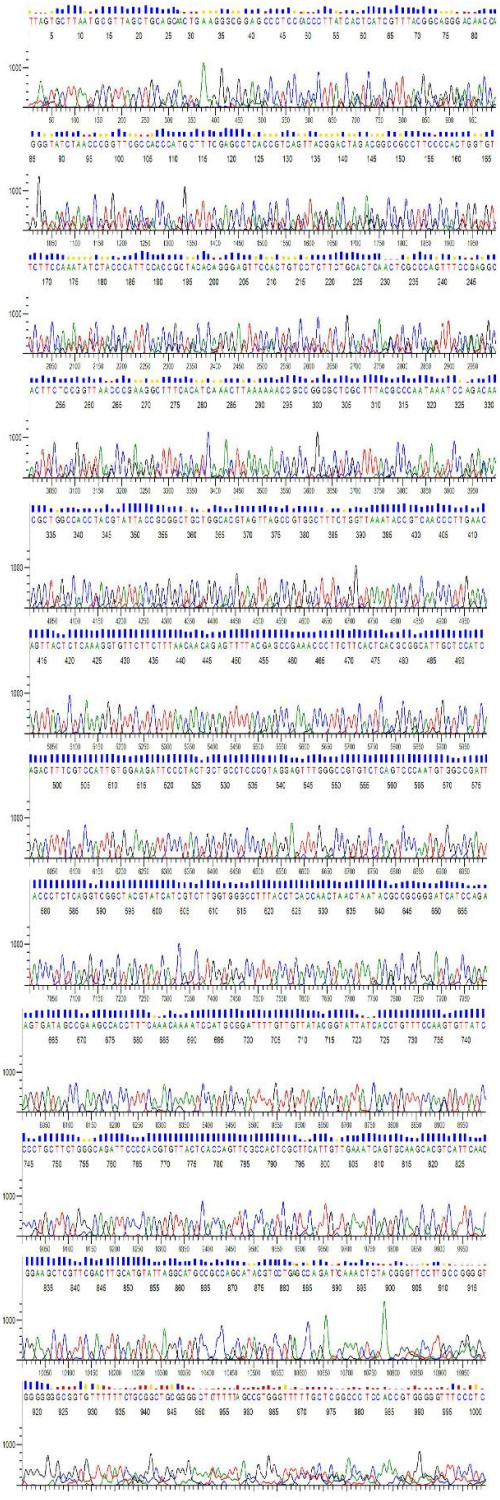

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Fig. 3: 16s rRNA sequence of *Enterococcus* sp. E1 with Accession number – MW386871.1

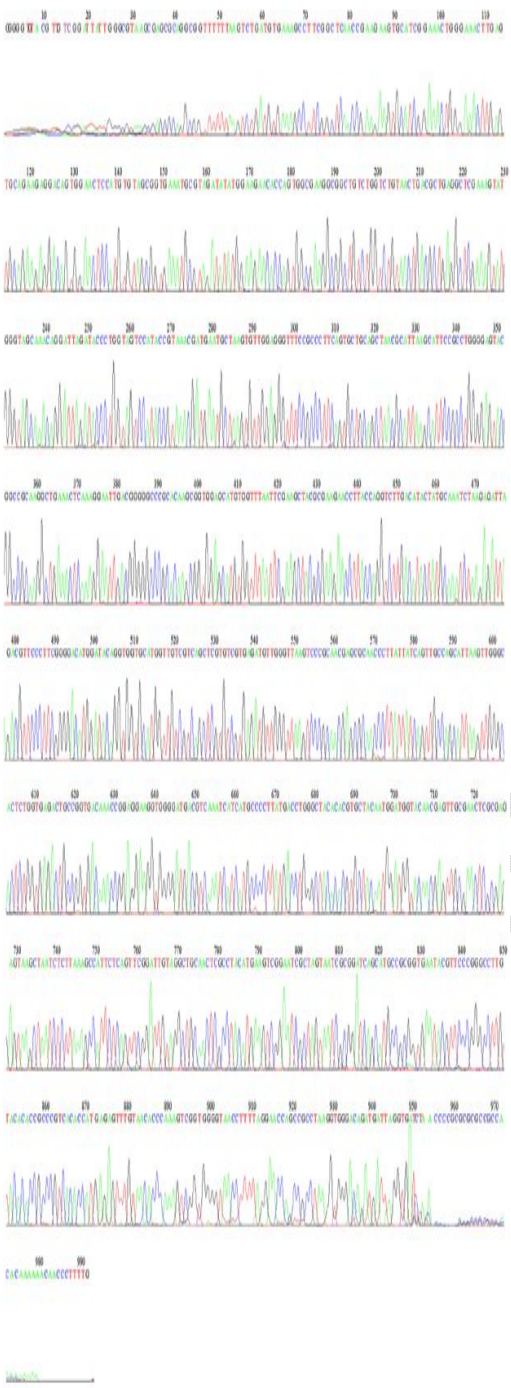

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Fig.4: 16s rRNA sequence of *Lactobacillus brevis* Lb1 with Accession number – MW480882.1

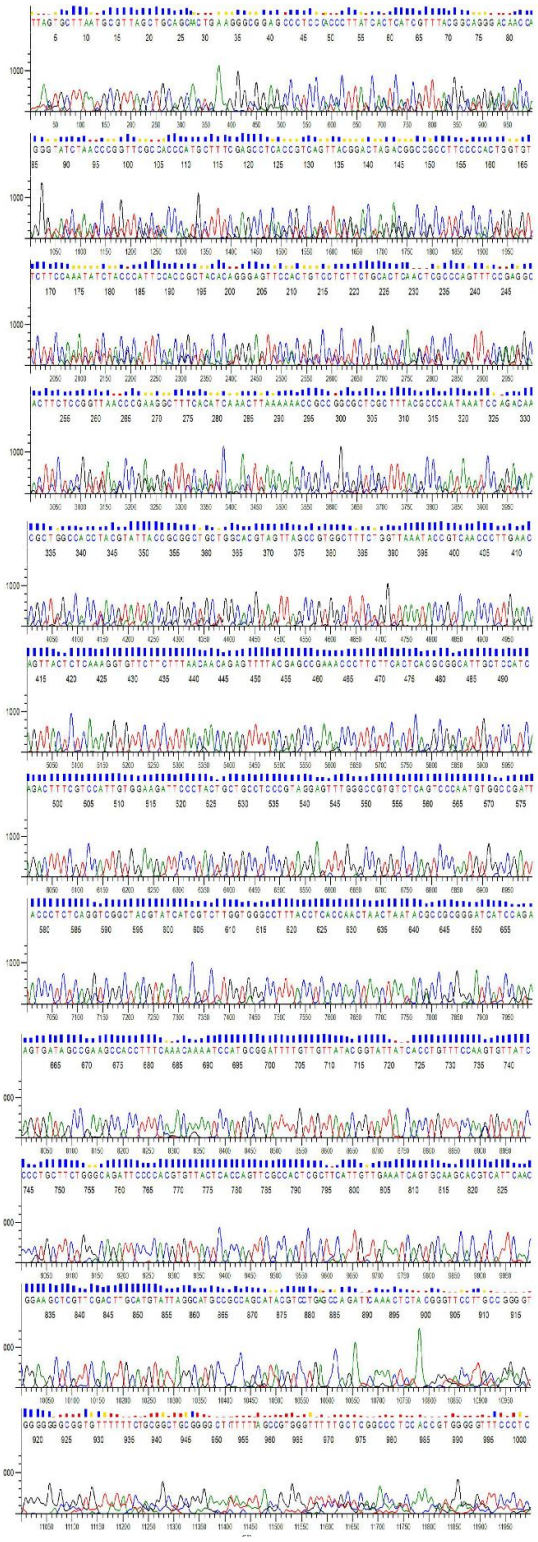

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Fig .5: 16s rRNA sequence of *Lactobacillus casei* Lb2 with Accession number – MW485119.1