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Transformation of Lactobacilli Plasmid by Electroporation into Probiotic Strain *Lactobacillus helveticus* MTCC 5463

Mital R Kathiriya*, Kunal M Gawai and J.B. Prajapati

Dairy Microbiology Department, SMC College of Dairy Science, AAU, Anand - 388110, Gujarat, India

*Corresponding author: mital@aau.in

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ABSTRACT

Plasmid DNA was isolated from lactic acid bacteria (LAB) in order to check for plasmid mediated antibiotic resistance. Attempts were made to transfer plasmid with antibiotic resistance to plasmid deficient probiotic strain – *Lactobacillus helveticus* MTCC 5463.

Total 31 LAB isolates were tested for the presence of plasmid DNA using Ultra clean mini plasmid DNA isolation kit (Molbio Laboratories Inc.). After plasmid isolation the samples were run on agarose gel (0.8%) and the gel was observed under UV Transilluminator for the bands of plasmid DNA. The samples showed the presences of plasmid were checked for the plasmid DNA concentration and purity using nanodrop instrument. LAB isolates which showed good plasmid DNA concentration and purity was further taken for identifying plasmid mediated antibiotic resistance. The isolated plasmid was then transferred into plasmid deficient probiotic *Lb. helveticus* MTCC 5463 using electroporator. Successful transfer of plasmid to *Lb. helveticus* MTCC 5463 was checked by isolation of plasmid from transformed cells and then developed antibiotic resistance was determined by disc diffusion assay.

Out of 31, only 7 LAB isolates were containing plasmid. The highest concentration and purity of plasmid DNA was found in *Lb rhamnosus* 52 (155.4/2 µl sample). Then *Lb rhamnosus* 52 plasmid was electroporated successfully in probiotic *Lb. helveticus* MTCC 5463. It was found that antibiotic resistance against many antibiotics was significantly increased in electroporated cells of *Lb. helveticus* MTCC 5463 compared to control cells.

Keywords: *Plasmid, lactic acid bacteria, electroporation, antibiotic, Lb. helveticus* MTCC 5463

Lactic acid bacteria (LAB) are not a collective noun in classification, but are a heterogeneous group of Gram-positive, microaerophilic, non-sporulating and low G + C microorganisms which can ferment various carbohydrates to produce lactic acid. LAB are commonly found in a variety of natural habitats, and are important industrial microbes that are used to produce a variety of industrial fermented food (dairy products, meat, wine, and silage etc.), macromolecules, enzymes, and metabolites. Some of them attract more attention from researchers as probiotics to maintain and regulate the human intestinal microflora (Sun *et al.* 2014; Liu *et al.* 2014). In the LAB group, Lactobacillus is the largest genus having more than 100 species (Sun *et al.* 2014; Dellaglio and Felis, 2005). Lactobacilli are generally associated with habitats rich in nutrients, especially in food products like milk, meat, beverages, fermented foods and vegetables. Some are also members of the normal flora of the mouth, intestine and vagina of mammals (Sun *et al.* 2014; Liu *et al.* 2014). To date, 22 species of Lactobacillus have been identified that contain plasmids. Lactobacillus plasmids vary widely in size (from 1.81–242.96 kb), number (from 1–10 different plasmids in a single strain) and gene content (Wang, and Lee, 1997; Jiménez *et al.* 2010; Pan *et al.* 2011).

Plasmids are defined as double stranded, extra-chromosomal DNA that replicate independently of the host cell chromosome and are stably inherited. The plasmids are not necessary genetic material for the survival of bacteria, but they often carry some special genes. They allow host strains to survive in a harsh environment and give the host strains greater competitiveness than other microorganisms, which are in the same environments (Wegrzyn and Wegrzyn, 2002). Although most plasmids remain cryptic, some plasmids have been found that are associated with many important functions of LAB species, like hydrolysis of proteins, metabolism of amino acid, citrate and carbohydrate (e.g., lactose/ galactose utilization and oligopeptide transport), production of bacteriocin, exopolysaccharide and pigments, resistance to antibiotic, bacteriophage, heavy metal and other stress responses and DNA restriction-modification systems (Gasson, 1990; Zhang and Zhang, 2014; Shareck *et al.* 2004; Schroeter and Klaenhammer, 2009).

As there are many probiotic (the bacteria provides several health benefits beyond nutrition when added in to the food) strains of lactobacilli which do not harbor their own plasmid. In such strains by transforming potential plasmid will further improve the cells properties. Hence the aim of this study was to investigate the transformation (by electroporation) ability in indigenous probiotic strain with lactobacilli plasmid.

MATERIALS AND METHODS

Microorganisms and growth conditions

The cultures used in the present study were obtained from the Culture Collection of Dairy Microbiology, SMC College of Dairy Science, Anand Agricultural University, Anand, India. All the cultures were maintained by propagating them in sterile MRS (deMan, Rogosa and Sharpe) or M17 broth and they were preserved at 5±1ºC.

Screening of LAB for plasmid

All 31 strains were screened for their plasmid using alkaline lysis method described by Sambrook and Russell (1989) with slight modification. The cultures were grown in 10ml MRS/M17 broth for 15 hours at their optimum temperature. Two ml active culture was transferred to collection tube and subjected to centrifugation at 12000 rpm for 2 min at 4ºC. Supernatant was discarded and cell pellet was re-suspended in 50 µl suspension buffer (50mM Tris pH 8.0 with HCl, 10mM EDTA, 100ug/ml RNase A). Then 100 µl alkaline lysis solution (200 mM NaOH, 1% SDS) and 325 µl neutralization solution (3.0 M Potassium Acetate pH 5.5 with glacial acetic acid) were added and mixed by gently inverting the tube. Bacterial lysate was centrifuged at 12000 rpm for 2 min at 4ºC. Supernatant was transferred to spin filter (contains silica membrane). Spin filter was centrifuged at 10000 rpm for 1 min at 4ºC. Flow through was discarded and 300 µl wash solution (50 % ethanol) was added to spin filter. It was centrifuged again at 10000 rpm for 1 min at 4ºC. Spin filter basket was the transferred to new collection tube and 50µl of TE buffer (pH 8.0) was added. It was centrifuged at 10000 rpm for 30 sec at 4ºC. The spin filter basket was removed and DNA sample was store at -30 ºC.

Agarose gel electrophoresis

Agarose Gel was made in 1X TAE buffer. 100 ml of 1X TBE buffer was taken and 0.9 g agarose was added to it. Buffer with agarose was heated to dissolve agarose. When it cools up to 50ºC, 5µl of Ethidium bromide was added into it. The gel was poured into gel caster with comb. When gel got solidified, the comb was removed gently and gel was transferred into gel tank. 10µl of sample was mixed with 2µl of 6X gel loading dye and was loaded into different wells. Samples were run at 80V till the dye reaches 1/3 of the gel. Then power was turned off, gel was removed and observed under UV transilluminator.

Determination of plasmid DNA concentration

Lactobacilli cultures those found with the presence of plasmid were checked for the concentration using thermo fishers' Nano drop 1000 spectrophotometer instrument.

Plasmid transformation into L. helveticus MTCC 5463 by electroporator

Electro-transformation of plasmid in *Lb. helveticus* 5463 isolate was attempted by a modified protocol adopted from Landete *et al*. (2014). For the preparation of competent cell, an overnight culture of *Lb. helveticus* 5463 was inoculated 1:50 in MRS broth containing 1% glycine and 0.5 M sucrose and incubated at 37 °C until an OD600 of 0.6 was reached. Bacterial cells were collected by centrifugation at 10,000 rpm for 10 min at 4°C and the pellet was washed three times in a washing solution (5 mM KH_2PO_4, 2 mM $MgCl_2$, 10% glycerol (v/v)) containing 0.3 M sucrose. Bacteria were re-suspended 1:100 in the same solution and a volume of 80 µl was taken for the electroporation. 2 µl of plasmid DNA isolated from *Lb. rhamnosus* 52 was added to the 80 µl aliquot of the re-suspended *Lb. helveticus* 5463 strain and was electroporated in 2 mm cuvette using a BTX Gene Pulser. Electroporation was performed at following parameters

Voltage (V)	900	1700	2400
Resistance (Ω)	200	200	200
µF	25	25	25
Time (msec)	1.7	4.9	0.9

Isolation of transformed cells of Lb. helveticus MTCC 5463

After electroporation, the cell suspension was transferred to MRS broth containing 0.3 M sucrose, 20 mM $MgCl_2$ and 2 mM $CaCl_2$ and incubated anaerobically at 37 °C for 2.5 h. Following the incubation, transformed strain was pour plated on MRS agar containing 0.3 M sucrose supplemented with streptomycin (1 µg/ml). The plates were incubated at 37 °C for 3 days under anaerobic condition. A colony was picked from each petri plate and transferred to MRS broth containing antibiotic and the broth tubes were incubated for 24 h at 37°C. After incubation, a colony was picked from the plate and transferred to MRS broth containing streptomycin. Incubated at 37°C for 24 h then plasmid was isolated by alkaline lysis method and analysed using agarose gel electrophoresis.

Antibiotic resistance of control and transformed cells of Lb. helveticus MTCC 5463

Transformed cells were also checked for the improved antibiotic resistance pattern by disc diffusion method recommended by Clinical and Laboratory Standards Institute (CLSI, 2007). A total of 6 antibiotic discs (HiMedia Ltd, Mumbai, India) of ampicillin, tetracycline, kanamycin, streptomycin, Neomucin and vancomycin were used. MRS agar was tempered at 45°C and seeded with 200 µl of active cultures. Agar was poured in petriplates and allowed to solidify. Hi-Media antibiotic discs were dispensed onto agar using disc dispenser under aseptic conditions. The agar plates were incubated at 37°C for 24 h. Diameter (mm) of zone of inhibition around the antibiotic discs was measured using antibiotic zone scale.

Statistical Analysis

All the data were subjected to statistical analysis using Completely Randomized Design (CRD) as per the methods described in Steel and Torrie (1980). The significance was tested at 5 % level of significance using mean value; co-efficient of variance (C.V.) and critical difference (C.D.) were determined. The values for microbial counts were log transformed before analysis.

RESULT AND DISCUSSION

Screening of LAB for plasmid and agarose gel electrophoresis

With primary objective to screen LAB isolates for the presence of plasmid, a total of 31 LAB were collected for the study. Detailed information of LAB taken for the initial screening is shown in table 1.

Table 1: List of the LAB isolates taken for the screening

Sl. No.	Code	Name of Isolate	Source of Isolation	Plasmid Present Yes/No
1	B1	*Lactobacilli*	Butter milk	No
2	B3	*Lactobacilli*	Butter milk	No
3	B4	*Lactobacilli*	Butter milk	No

4	C2	*Lb. rhamnosus*	Butter milk	No
5	C4	*Lactobacilli*	Butter milk	No
6	D2	*Lactobacilli*	Dahi	No
7	D6	*Lactobacilli*	Dahi	No
8	52	*Lb. rhamnosus*	Traditional dahi	Yes
9	CM3	*Lactococcus lactis*	Cow milk	No
10	CM4	*Lactococcus lactis*	Cow milk	No
11	CM7	*Lactococcus lactis*	Cow milk	No
12	CM8	*Lactococcus lactis*	Cow milk	Yes
13	NS6	*Lb. rhamnosus*	Shrikhand	Yes
14	V3	*Lb. helveticus*	Human vagina	No
15	I4	*Lb. rhamnosus*	Human intestinal tract	No
16	MD2	*S. thermophilus*	Market dahi	No
17	MD8	*S. thermophilus*	Market dahi	No
18	AI2	*Lb. fermentum*	Dhokla batter	Yes
19	AI3	*Lb. fermentum*	Dhokla batter	Yes
20	138	*Lb. fermentum*	Fresh turmeric	Yes
21	142	*Weissella cibaria*	Cucumber	No
22	145	*Weissella cibaria*	Fermented vegetable	Yes
23	LCM7	*Lactococci*	Cow milk	No
24	51	*Lb helveticus*	Traditional dahi	No
25	NS2	*Lactobacilli*	Shrikhand	No
26	NS4	*Lb. rhamnosus*	Shrikhand	No
27	NK2	*Lb. rhamnosus*	Dahi	No
28	Nk9	*Lb. casei*	Dahi	No
29	NK10	*Lb. rhamnosus*	Dahi	No
30	M20	*Lb. plantarum*	Dahi	No
31	M22	*Lb. pentosus*	Dahi	No

All the LAB isolates were subjected to Plasmid DNA isolation by alkaline lysis method. The extracted sample were then run on agarose gel and observed under UV Transilluminator. The DNA obtained from all the isolates on agarose gel are shown in Fig. 1. The bright band shows the presence of plasmid.

Out of total LAB, 3 strains of *Lb. fermentum* (AI2, AI3 AND 138), 2 strains of *Lb. rhamnosus* (52, NS6), 1 strain of *Lactococcus lactis* (CM8) and 1 strain of *Weissella cibaria* (145) showed the presence of plasmid DNA. While no plasmid found in species of *streptococcs*. The result agrees with those of Soomaro and Masud (2007). They found that plasmids are present in wide range of lactobacilli isolated from the dahi while no plasmid were present in lactococci and *Streptococcus thermophilus* strains isolated from the same source. They took 8 strains of *S. thermophilus* for the study. None were harboring plasmid. In addition to this, Turgeon and Moineau (2001) found that the species of streptococcus was naturally poor in the plasmid composition and most of the industrial strains of *S. thermophilus* were plasmid-free. On the contrary few researchers have reported small and single plasmid in *S. thermophilus.* In one of the study it was reported that *L. lactis* strains isolated from dairy products carry number of plasmids in their host cells (Mills *et al.* 2006; Ainsworth *et al.* 2014). Similar result was observed in our study i.e. *Lactococcus lactis* CM8 isolated from cow milk showed the presence of plasmid.

Determination of plasmid DNA concentration

Seven lactobacilli which have shown the presence of plasmid DNA were checked for the concentration and purity of plasmid using Nano drop 1000 spectrophotometer. The observation is shown in table 2.

Table 2: Concentration and purity of plasmid in extracted DNA sample

Name of Isolate	Concentration of plasmid (ng/2µl sample)	Purity of plasmid (260/280 ratio)
Lb. fermentum AI2	71.40 ± 2.90	1.43 ± 0.12
Lb. fermentum AI3	125.50 ± 1.50	1.70 ± 0.20
Lb. fermentum 138	82.41 ± 2.91	1.30 ± 0.20
*Weissella cibaria*145	101.69 ± 3.29	1.67 ± 0.49
Lb. rhamnosus 52	158.97 ± 3.57	1.95 ± 0.13
Lb. rhamnosus NS6	146.35 ± 1.85	1.68 ± 0.13
Lactococcus lactis CM8	33.64 ± 3.14	1.24 ± 0.23
SEm	1.63	0.14
CD (0.05)	4.95	0.43
CV %	2.75	15.66

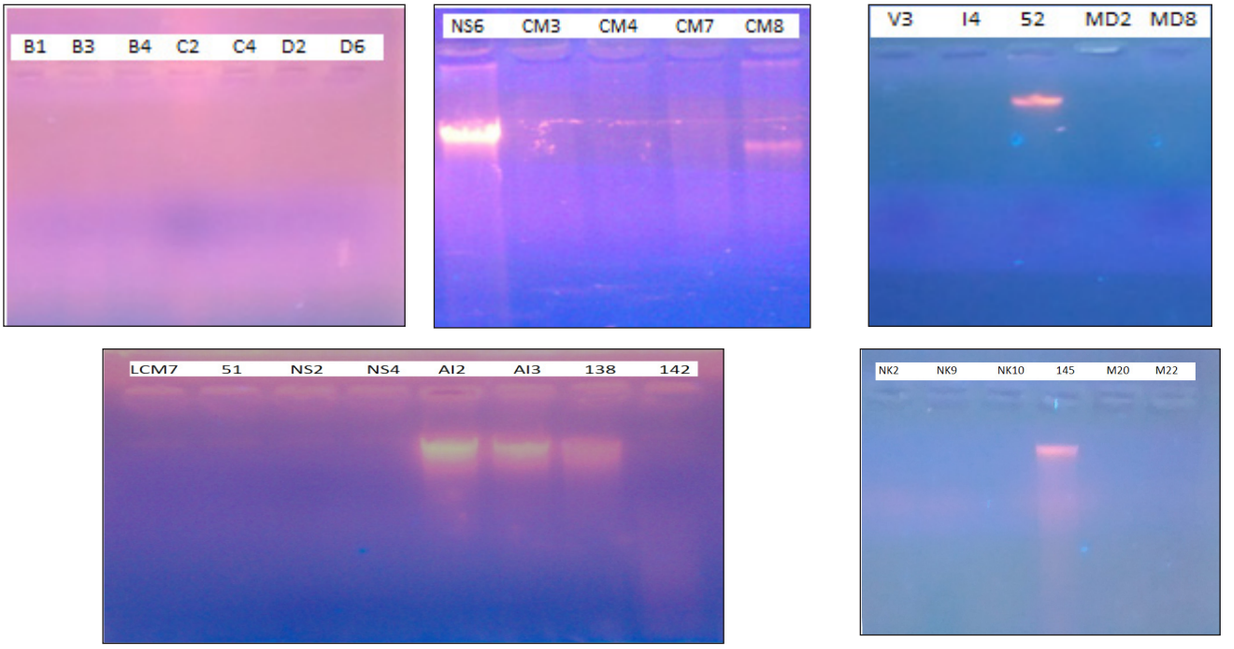

Fig. 1: Images of different LAB plasmid DNA on Agarose gel under UV transilluminator

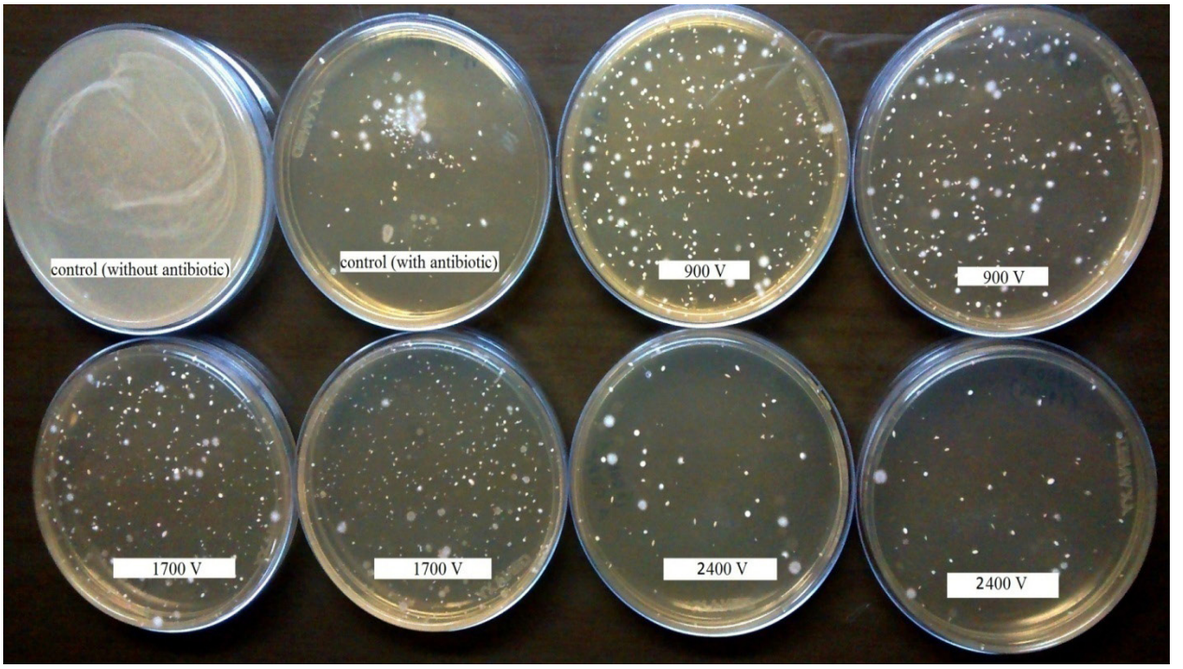

Fig. 2: Petri plates containing transformed (at 900, 1700 and 2400 voltage) as well as control *Lb. helveticus* MTCC 5463 colonies on MRS agar in duplicate

Quantity and purity of plasmid yielded from *Lb. rhamnosus* 52 was significantly higher while *Lactococcus lactis* CM8 yielded lowest plasmid DNA concentration. As the purity of *Lb. rhamnosus* 52 plasmid was found higher, it was further taken to check the transformation ability of the plasmid in to probiotic strain *Lb. helveticus* MTCC 5463.

Plasmid transformation into *L. helveticus* MTCC 5463 by electroporator

It is clear from the Fig. 2 that with increasing the voltage, viability of cells decreased. From the each plate typical colony was picked to check the presence of transformed plasmid. It is shown in Fig. 3. After isolation of plasmid DNA from control and transformed *Lb. helveticus* MTCC 5463 cells, the samples were run on agarose gel. It was found that the cells transformed at 1700 V showed the presence of plasmid.

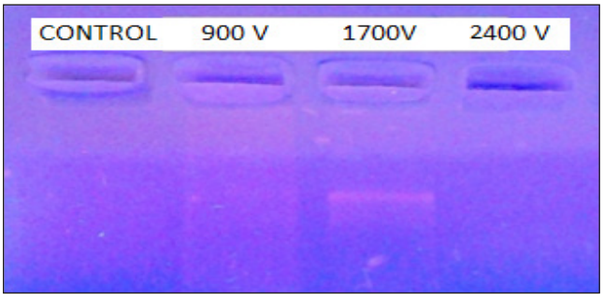

Fig. 3: Plasmid isolated from transformed *Lb. helveticus* MTCC 5463 on agarose gel

Agarose gel electrophoresis of transformed cells of *Lb. helveticus* MTCC 5463

Landete *et al.* (2014) Successfully transformed plasmid DNA by electroporation into Lactobacillus, Pediococcus, Enterococcus Leuconostoc and Streptococcus strains at 1700 V, when cells grown in MRS containing 1% glycine and 0.3 M sucrose, cell pellet washed using 0.3 M sucrose, 10% glycerol, 5 mM KH_2PO_4, 2 mM $MgCl_2$, pH 7.4 and re-suspended in MRS, 0.3 M sucrose, 20 mM $MgCl_2$, 2 mM $CaCl_2$. Same protocol was followed in the present study for the transformation of plasmid in to *Lb. helveticus* MTCC 5463. Many researchers have reported the transfer of plasmid by electroporation method in lactic acid bacteria at varying rage of voltage and resistance (Chassy and Flickinger, 1987; Holo and Nes, 1989; Ahrné *et al.* 1992; Kim *et al.* 1992; Varmanen *et al.* 1998; Serror *et al.* 2002; Leathers *et al.* 2004). It was also reported that electroporation is found to be efficient method for the transfer of plasmid DNA into lactic acid bacteria (Berthier *et al.* 1996; Bringel and Hubert, 1990; Thompson and Collins, 1996).

Improvement in antibiotic resistivity in transformed cell

The resistance in *Lb. helveticus* MTCC 5463 after transformation (at 1700 V) of plasmid against six antibiotic discs was checked by the disc diffusion assay which is shown in the table 4.

Table 3: Antibiotic resistance profiles of control and transformed *Lb. helveticus* MTCC 5463

Antibiotic	Zone of Inhibition (mm)	
	Control *Lb. helveticus* MTCC 5463	Transformed *Lb. helveticus* MTCC 5463
Neomycin(10 µg)	3.80 ± 5.22	0.00 ± 0.00
Tetracyclin (30 µg)	36.80 ± 2.39	21.00 ± 2.24
Streptomycin (10 µg)	21.80 ± 2.59	7.40 ± 4.16
Vancomycin (30 µg)	26.20 ± 2.28	12.40 ±1.14
Kanamycin (30 µg)	9.60 ± 0.89	0.00 ± 0.00
Ampicillin (10 µg)	34.80 ± 1.92	21.00 ± 1.58

The resistance against antibiotic discs was found to increase in transformed cells of *Lb. helveticus* MTCC 5463. That shows that the plasmid which is transformed in to *Lb. helveticus* MTCC 5463 is consist of multiple antibiotic resistance genes. Supporting our results Aukrust and Nes (1988) transformed erythromycin resistant plasmid DNA into *L. plantarum* ATCC 8014 GenePulser ™ supplied by Bio-Rad Laboratories U.S.A.

One of the researcher reported that most lactobacilli strains consist of one or more plasmid naturally (Pouwels and Leer, 1993) and one of the common trait found in these plasmids is resistance to antibiotics (Posno *et al.* 1991). Charteris *et al.* (1998) observed that Lactobacillus strains isolated from both the human gastrointestinal tract and dairy products showed resistance to many antibiotics like cefoxitin, aztreonam, amikacin, gentamicin, kanamycin, streptomycin, sulphamethoxazole, trimethoprim, co-trimoxazole, metronidazole, polymyxin B and colistin sulphate.

CONCLUSION

It can be concluded that the indigenous probiotic strain *Lb. helveticus* MTCC 5463 can be transformed with the lactobacilli plasmid containing multiple antibiotic resistance or any other beneficial properties, through electroporation method. Trials can also be conducted to check the transformation ability of plasmid other than *Lb. rhamnosus* 52, having comparatively good concentration and purity. This method can be used for other LAB species or genera. Lactobacilli plasmid can also be studied for the plasmid mediated characteristics other than antibiotic resistance like bacteriocin production, digestion of sugar and protein, resistance to bacteriophage, heavy metals, drugs etc. using various bioinformatics tools.

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