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Optimization and Identification of Lactic Acid Bacteria with Higher Mannitol Production Potential

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ABSTRACT

Background: Lactic acid bacteria (LAB) is considered as food-grade microorganism with generally recognized as safe (GRAS) status. Both hetero-fermentative and homo-fermentative LAB have the ability to produce mannitol as metabolic end product in normal fermentation.

Methods: Ten LAB isolates selected were detected for its mannitol production potential using colorimetric assay in preliminary study. Later selected four isolates of LAB with better mannitol production were further optimized at different culture conditions. A total of 540 mannitol combinations were obtained after optimization. All the observations were statistically analyzed using response surface methodology. Biochemical and molecular assays were carried out to identify the isolates.

Result: The isolate L8 produced mean mannitol content of 1.635 g/L, 0.345967 cell densities, at pH 7.0 and temperature 42°C with agitation of 100 rpm was selected with optimum response surface optimization because of its higher mannitol production. Biochemical and molecular assays identified higher mannitol producers, L4 as *Enterococcus faecium* strain Gr17, L6 as *Lactobacillus rhamnosus* strain 6870, L8 as *Leuconostoc pseudomesenteroides* culture IMAU: 11666 and L9 as *Lactobacillus plantarum* subsp. *plantarum* strain NMB8.

Key words: Biochemical and molecular assay, Colorimetic assay, LAB, Mannitol, Sequencing.

INTRODUCTION

The diversity of natural lactic flora, was dominated by Lactococci, Enterococci, Lactobacilli, Pediococci and Leuconostocs (Dahou *et al.,* 2020). Lactobacilli, Lactococci, Pediococci and Leuconostoc species were also isolated using ripened, aged fruits and vegetables by Padmaja *et al.* (2011). Lactic acid bacteria's functional attributes include probiotic activities, boosting nutritional density by sugar conversion, improving nutrient bioavailability, antibacterial and antioxidant activity, vitamin biosynthesis, antinutritive chemical breakdown and sensory quality enhancement. Several technologically relevant properties of lactic acid bacteria isolated from samples were investigated and exploited in order to maintain the dairy bacteria ecology live and functioning in today's technologically advanced world (Ketrouci *et al.,* 2021). Species from the genera Leuconostoc, Oenococcus and Lactobacillus, in particular, have been found to successfully manufacture mannitol (Von Weymarn *et al.,* 2002).

D-Mannitol is a sugar alcohol with six-carbon, considered to be half sweet as sucrose and have diverse applications in low-calorie foods and pharmaceuticals (Ojamo *et al.,* 2003). Mannitol is partially metabolized by human and reduce hyperglycaemia in diabetic patients (Ruiz Rodriguez *et al.,* 2017). Low-calorie sweeteners are applicable in foods and due to increased demand, wide varieties of low-calorie foods are available in the market. Mannitol production by food grade organism can act as natural sweetener in food products and thereby side effects of other artificial sweeteners can be reduced (Von Weymarn *et al.,* 2002). Some strains of LAB enhanced fructose

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consumption rate and greater mannitol output improved volumetric mannitol productivity as well (Helanto *et al.,* 2005). By recycling intracellular NADH, most heterofermentative lactic acid bacteria convert fructose to mannitol (Otgonbayar *et al.,* 2011). Moreover, it is a food grade sweetener with Food and Drug Administration (FDA) endorsement with ADI of 0-50 mg/kg body weight. Considering these factors, this research work was undertaken with the objectives of identifying LAB with mannitol production potential and there by optimizing its fermentation conditions in a batch fermenter for obtaining maximum mannitol production.

MATERIALS AND METHODS

Preparation of standard curve for mannitol estimation

The experiment was conducted at Department of Dairy Science, College of Veterinary and Animal Sciences, Mannuthy, Kerala, India during 2019-2020. Calibration curve for mannitol was plotted according to Sanchez, (1998).

Optimization of culture conditions for maximal mannitol production

Effect of temperature, pH and agitation

Effect of different temperatures, pH and agitation on specific growth rate and mannitol yield of superior LAB strains was studied in MRS growth media at batch bioreactor (Scigenics, India). Temperatures used for the study were 33°C, 37°C and 42°C, while pH used for the study was 6, 6.5 and 7.0. On agitation, 100 rpm were provided. Done research without agitation also.

Identification of LAB isolates

Biochemical identification of LAB isolates

Primary tests

Gram staining, motility test, catalase test, oxidase test, sugar utilization test, effect of different temperature, pH, NaCl concentration on growth of isolates were studied.

Secondary tests

Gelatin liquefaction, production of ammonia from arginine, oligosaccharide production, bile tolerance test, phenylalanine deamination, urease test, indole test, methyl red test, Voges Proskauer test, citrate utilization test, production of gas from glucose and triple sugar iron (TSI) agar test were studied.

Molecular identification of LAB strains

Preparation of bacterial DNA

The method suggested by Salehi *et al.* (2005) was followed.

Primers used

Target	Primer name	Direction	Sequence (5′→3′)
16S rRNA	16S-RS-F	Forward	CAGGCCTAACACATGCAAGTC
	16S-RS-R	Reverse	GGGCGGWGTGTACAAGGC

PCR amplification, detection of PCR products and sequencing

Amplification was carried out in a PCR thermal cycler (T 100 Bio-Rad) with annealing temperature 60°C for 40 seconds in 35 cycles. Later 1.2% agarose gel electrophoresis was performed and visualized in a UV transilluminator (Genei) and Gel documentation system (EZI Imager, Bio-Rad). Finally, were sequenced in Rajiv Gandhi Centre for Biotechnology, Trivandrum.

Statistical analysis

Six replications were carried out and the data was statistically analyzed using SPSS version 24.0. One Way ANOVA and dendrogram were used in the evaluation.

RESULTS AND DISCUSSION

Preliminary screening of ten LAB isolates for mannitol production

Standard mannitol curve (Fig 1), as suggested by Sanchez (1998) was prepared. Mannitol production by isolates was estimated after 18 hours of incubation at 37°C in MRS broth. Quantification of mannitol was done using linear regression equation obtained from standard curve.

$$Y = a + bx,\ a = -706.454,\ b = 11550.159$$

Y = Mannitol concentration.
x = Optical density value.

The mannitol yield is shown in Table 1. The values ranged from 0.36 to 0.65 g/L. Among the screened ones, isolates *viz*., L4, L6, L8 and L9 showed comparatively higher mannitol yield of 0.51, 0.54, 0.65 and 0.57 g/L respectively selected for further study. In a study conducted by *Lactobacillus reuteri* CRL 1101 was shown to be capable of producing mannitol effectively by Ortiz *et al.* (2017). According to Von Weymarn *et al.* (2002) Leuconostoc, Oenococcus and Lactobacillus, can produce mannitol. Cell density shows that mannitol production differs amongst LAB species with identical growth rates (Table 1). Helanto *et al.* (2005) observed maximum cell growth of *Leuconostoc pseudomesenteroides*, along with mannitol production 30°C, pH 5 and 200 rpm.

Optimization of the cultivation conditions for maximum mannitol production

Effect of varying temperature, pH and non-agitation on mannitol yield

Mean mannitol production by selected isolates at 33°C and different pH was in Table 2. All isolates produced significantly higher mannitol at pH 7.0. In fact, increasing the pH from

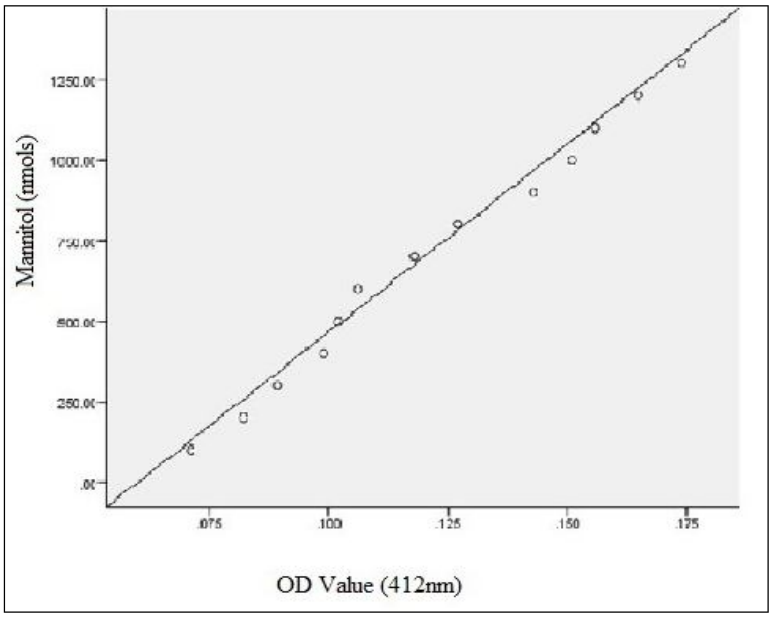

Fig 1: Calibration curve for mannitol estimation.

Table 1: Cell density and corresponding mannitol production of LAB strains.

LAB strains	Mean cell density (OD value)	Mean mannitol (g/L)
L1	0.2341	0.42
L2	0.1269	0.45
L3	0.1038	0.43
L4	0.2684	0.51
L5	0.2257	0.48
L6	0.2109	0.54
L7	0.2781	0.36
L8	0.2971	0.65
L9	0.2506	0.57
L10	0.1942	0.50

6.0 to 7.0 resulted in a small increase in productivity. Likewise, at 37°C all the isolates produced maximum mannitol at pH 7.0. Among that L9 produced higher mannitol of 1.30± 0.27 g/L. Significantly no differences were observed in mannitol yield at different pH. While at 42°C, it is obvious from Table 3 that except L9, all other isolates showed a better productivity at pH 7.0. However, strain L4 showed significant difference in yield at different pH.

Yun and Kim (1998) reported maximum production of mannitol of 73 g and 26 g from *Lactobacillus* species and *Leuconostoc* species respectively, at 35°C and pH 8.0 and 6.0 respectively. In research conducted by Weymarn *et al.* (2002), mannitol production from *Lactobacillus fermentum* were estimated to be 86, 89 and 94 mol per cent at 25°C, 30°C and 35°C respectively. Volumetrically higher mannitol yield was detected at 35°C than at 25°C in *Lactobacillus* and *Leuconostoc* species. In a study conducted by Ojamo *et al.* (2003), higher mannitol production of 20 g/L/h was reported by *Leuconostoc pseudomesenteroides* ATCC 12291 strain at high cell density fermentation.

Effect of different temperature, pH and agitation on mannitol yield

When evaluating effect of agitation, pH 7.0 shown to be the best for all isolates, resulting in significantly higher mannitol production at 33°C (Table 4). The highest mannitol production was shown by isolate L8 (0.99±0.01 g/L) followed by L4, L9, and L6. Production of standard *Leuconostoc mesenteroides* was 0.89 ± 0.02 g/L. At 37°C (Table 5) isolate L8 produced significantly higher mannitol of 1.17±0.04 g/L at pH 6.5. Isolates didn't show any significant variation at pH 6.5 and 7.0. At 42°C with agitation the values ranged from 1.12±0.09 g/L to 1.63±0.16 g/L, where peak mannitol production was by isolate L8 at pH 7.0. Yun and Kim (1998) specified that partial aeration was necessary for higher mannitol yield. Weymarn *et al.* (2002) observed an increase in mannitol production with *Lactobacillus fermentum* from 1.33±0.02 to 1.65±0.06 (g/L/h) at semi anaerobic condition. Sugarcane molasses yielded the highest mannitol concentrations (38 and 41.5 g/L) when cultivated *Lactobacillus reuteri* in agitated cultures at 37°C (Ortiz *et al.*, 2017). This finding was in harmony to the present exploration.

Effect of temperature, pH and agitation on bacterial cell density

Growth rate of all isolates was significantly different (P≥0.05) in different conditions. While considering the non-agitated condition, isolate L8 showed significant difference in cell density on different pH at 33°C and 42°C. L6 showed significant difference in cell density at 33°C and 37°C, whereas L4 showed significant difference in cell density only

at 42°C. With respect to condition of agitation at 100 rpm, significant difference was noted in cell density for isolate L9 and L6 at all temperatures studied. Isolate L4 showed significant difference at 33°C and 37°C, whereas L8 at 33°C and 42°C. Significantly higher growth rate was observed in agitated cultures than non-agitated. Hence by proper agitation, temperature and pH the yield of mannitol by different lactic acid bacteria can be improved.

Grobben *et al.* (2001) opined higher production of mannitol of 75 mM by *Leuconostoc pseudomesenteroides* with high cell density at 30°C and pH 4.5. Weymarn *et al.* (2002) observed that growth rate of all strains increased in semi anaerobic condition, in accordance to present findings. Ojamo *et al.* (2003) reported higher mannitol production by *Leuconostoc pseudomesenteroides* with high cell density. Helanto *et al.* (2005) observed maximum cell growth of *Leuconostoc pseudomesenteroides*, at 30°C temperature, pH 5 and 200 rpm agitation.

Selection of superior lab isolate by response surface methodology

A total of 540 combinations were obtained and statistically analyzed using response surface methodology (Kumari *et al.,* 2016). Dendrogram is shown in Fig 2. The 16th cluster, produced higher mannitol concentration of 1.6209 g/L with 0.2799 optical density of cell growth. Sixteenth cluster included 2 combination studies with agitation (100 rpm), of isolate L8 with 1.6067 g/L mannitol and 0.213767 OD value at pH 6.5, 42°C, later with1.635 g/L mannitol and 0.345967 OD at pH 7.0, 42°C. From these second one was selected as the best. Using cluster and dendrogram analysis, mannitol producer (9.46 0.27 g/l) strain *F. tropaeoli* was discovered from fruits by Ruiz Rodriguez *et al.* (2017). With a maximum volumetric productivity of 2.36 g/l h and the highest yield, of mannitol was obtained for *L. fructosum* NRRL B-2041 according to Carvalheiro *et al.* (2011). Also, different *Leuconostoc pseudomesenteroides* strains were studied for its higher mannitol production potential at different growth conditions (Bhatt *et al.,* 2013).

Identification of lab isolates

Biochemical identification

Most of the phenotypical and biochemical characteristics of strains, L4, L6, L8 and L9 were explained in Fig 3. Sugar fermentation tests, growth condition studies and other biochemical studies proved that the isolate L4 was

Table 2: Mean mannitol production by LAB strains at 33°C and different pH.

pH	S (g/L)	L4 (g/L)	L6 (g/L)	L8 (g/L)	L9 (g/L)
6.0	1.16±0.07^{ns}	0.93±0 .1^{b}	0.96±0.03^{c}	0.95±0.05^{b}	0.92±0.02^{b}
6.5	1.25±0.04^{ns}	1.11±0.05^{ab}	1.17±0.03^{b}	1.29±0.02^{a}	1.18±0.05^{a}
7.0	1.31±0.04^{ns}	1.22±0.04^{a}	1.28±0.02^{a}	1.26±0.04^{a}	1.23±0.04^{a}

Each value is a mean of six observations with SE, means with different superscript in same column differ significantly (p≤0.05), ns-non significant (p>0.05).

Table 3: Mannitol production by selected lactic acid bacterial strains at 42°C and different pH.

pH	S (g/L)	L4 (g/L)	L6 (g/L)	L8 (g/L)	L9 (g/L)
6.0	0.82±0.01^{b}	0.56±0 .06^{b}	0.73±0.09^{ns}	0.83±0.08^{ns}	0.81±0.11^{ns}
6.5	0.99±0.01^{a}	0.76±0.10^{ab}	0.86±0.11^{ns}	1.00±0.11^{ns}	1.04±0.15^{ns}
7.0	1.02±0.07^{a}	0.91±0.13^{a}	0.88±0.12^{ns}	1.13±0.13^{ns}	0.87±0.11^{ns}

Each value is a mean of six observations with SE, means with different superscript in same column differ significantly (p≤0.05), ns-non significant (p>0.05).

Table 4: Mean mannitol production by selected lactic acid bacterial strains at 33°C and different pH on agitation.

pH	S (g/L)	L4 (g/L)	L6 (g/L)	L8 (g/L)	L9 (g/L)
6.0	0.72±0.02^{b}	0.73±0 .0.2^{c}	0.65±0.01^{c}	0.72±0.02^{c}	0.66±0.01^{c}
6.5	0.75±0.03^{b}	0.83±0.02^{b}	0.72±0.01^{b}	0.91±0.02^{b}	0.81±0.01^{b}
7.0	0.89±0.02^{a}	0.93±0.03^{a}	0.81±0.01^{a}	0.99±0.01^{a}	0.91±0.01^{a}

Each value is a mean of six observations with SE, means with different superscript in same column differ significantly (p≤0.05).

Table 5: Mean mannitol production by selected lactic acid bacterial strains at 37°C and different pH on agitation.

pH	S (g/L)	L4 (g/L)	L6 (g/L)	L8 (g/L)	L9 (g/L)
6.0	0.78±0.01^{b}	0.80±0 .02^{b}	0.87±0.03^{b}	0.90±0.03^{b}	0.86±0.03^{b}
6.5	1.03±0.03^{a}	1.05±0.05^{a}	1.00±0.05^{a}	1.17±0.04^{a}	0.99±0.03^{a}
7.0	1.12±0.04^{a}	1.03±0.04^{a}	1.04±0.05^{a}	1.16±0.04^{a}	1.08±0.03^{a}

Each value is a mean of six observations with SE, means with different superscript in same column differ significantly (p≤0.05).

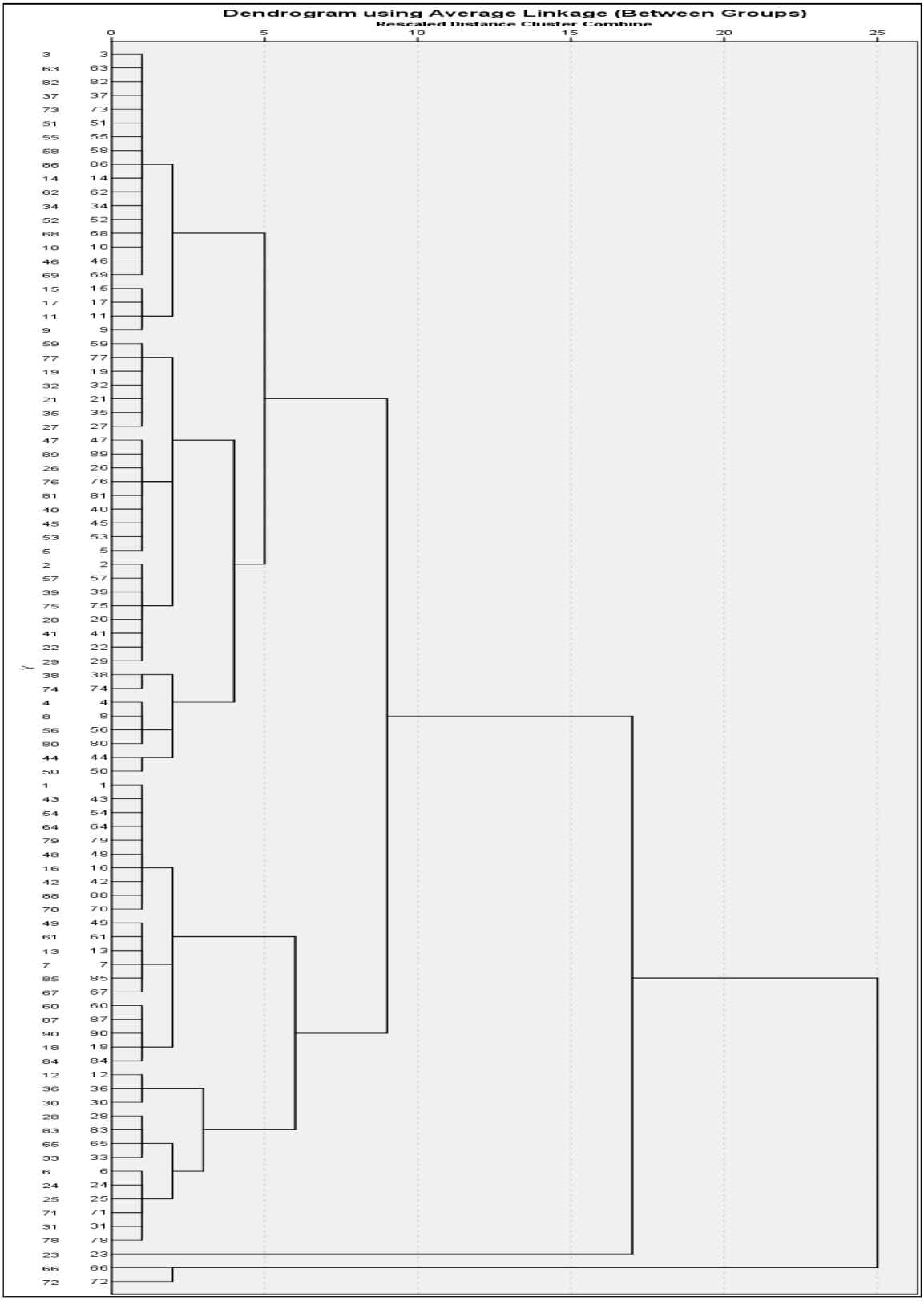

Fig 2: Representation of dendrogram.

Enterococcus sp. L8 was *Leuconostoc* sp. and L6 and L9 belong to *Lactobacillus* sp. according to Bergey's Manual of Determinative Bacteriology.

In a study conducted by Makanjuola and Springham (1984), *Leuconostoc* sp. was able to produce gas from glucose, ammonia from arginine and also showed growth at 15°C and 45°C. Fermentation results of rhamnose and arabinose support the present study to characterize L8 isolate as *Leuconostoc* sp. Gancel *et al.* (1997) identified genus *Lactobacillus* from vacuum packed fish meat by biochemical characterization with tolerance of high levels of NaCl, bile salts and CO_2 production. But in present study all the isolates of LAB L4, L6, L8 and L9 were intolerant to higher concentration NaCl. In different studies (Azadnia and Nazer, 2009; Nair and Surendran, 2005) fermentation of specific sugars were observed for Lactobacillus species especially *L. plantarum*. These observations are related to the results obtained for L9 isolate in the present study. Also, Bhatt *et al.* (2012) used bile tolerance test in LAB identification. According to Hassanzadazar *et al.* (2012) all LAB strains isolated could not tolerate the pH ≤ 2 and the presence of bile salts, which agree with the existing results.

TESTS	L4	L6	L8	L9
Esculin Hydrolysis	+	+	+	+
Xylose	+	–	+	–
Cellobiose	+	–	+	+
Arabinose	+	–	+	+
Maltose	+	+	+	+
Galactose	+	–	+	+
Mannose	+	–	+	+
Mellibiose	+	–	+	+
Raffinose	+	–	+	+
Sucrose	+	+	+	+
Trehalose	+	–	+	+

PART A

TESTS	L4	L6	L8	L9
Inuline	+	+	–	+
Sodium gluconate	+	+	–	+
Glycerol	+	+	–	+
Salicin	+	+	–	+
Dulcitol	+	+	–	+
Inositol	+	+	–	+
Sorbitol	+	+	–	+
Mannitol	+	+	–	+
Adonitol	+	+	–	+
Arabitol	+	+	–	+
Erythritol	+	+	–	+
α – methyl D-glucoside	+	+	–	+

PART B

TESTS	L4	L6	L8	L9
Rhamnose	+	+	–	–
Melezitose	–	+	–	+
α- methyl D-mannoside	+	+	–	+
Xylitol	+	+	–	+
ONPG	–	–	–	–
Malonate	–	–	–	–
Sorbose	+	+	–	+

PART C

SUGAR FERMENTATION TEST- PART A, PART B & PART C

PARAMETERS	L4	L6	L8	L9
Temperature 15 °C	+	+	+	+
33 °C	+	+	+	+
37 °C	+	+	+	+
42 °C	+	+	+	+
45 °C	+	+	+	–
Sodium chloride concentrations (2 per cent)	+	+	+	+
3 per cent	+	+	–	–
4 per cent	+	+	–	–
6.5 per cent	+	–	–	–
pH pH4.5	–	+	–	+
pH6.5	+	+	+	+
pH9.6	+	+	+	+

Growth at different temperature NaCl concentrations and pH

TESTS	L4	L6	L8	L9
Gelatin Liquefaction	–	–	–	–
Production of ammonia from arginine	+	–	–	–
Oligosaccharide production	Slime	slime	slime	slime
Bile tolerance test (0.05, 0.1, 0.15, 0.3 and 0.5 per cent)	–	–	–	–
Phenyl alanine deamination	–	–	–	–
Urease test	–	–	–	–
Citrate Utilization Test	–	–	–	–
Gas from Glucose	–	–	–	–
Indole test	–	–	–	–
Methyl Red test	+	+	+	+
Voges proskaure test	–	–	–	–
Triple Sugar Iron (TSI) Agar Test	Acid	Acid	–	Acid
Tomato juice agar test	+	+	+	+

Secondary identification test results

Fig 3: Results of biochemical tests.

Sugar fermentation assays and growth of LAB at different conditions in the above investigation were supportive to the present study.

Molecular characterization of LAB strains

Sequencing result confirmed L4 as *Enterococcus faecium* strain Gr17, L6 as *Lactobacillus rhamnosus* strain 6870, L8 as *Leuconostoc pseudomesenteroides* culture IMAU: 11666 and L9 as *Lactobacillus plantarum* subsp. *plantarum* strain NMB8. Bacterial species identification by 16S rRNA-based technique is the most accepted, as large public-domain sequence databases are accessible in Gene Bank for comparison (Morgan *et al.,* 2009; Sharif *et al.*, 2018).

CONCLUSION

In the present study an attempt was made to isolate and characterize LAB, which show higher mannitol production. Ten isolates were screened for mannitol production potential. The effects of temperature, pH and agitation on mannitol production were studied. Where *Leuconostoc mesenteroides* was used as control. Isolate L8 was identified as the most superior mannitol producer among the screened strains. Higher mannitol producing four isolates (L4, L6, L8, L9) were characterized biochemically as *Enterococcus* sp., *Lactobacillus* sp. and *Leuconostoc* sp. Later on, L4, L6, L8, L9 were identified as *Enterococcus faecium* strain Gr17, *Lactobacillus rhamnosus* strain 6870, *Leuconostoc pseudomesenteroides* culture IMAU: 11666 and *Lactobacillus plantarum* subsp. *plantarum* strain NMB8 respectively *via* molecular method.

Conflict of interest

The authors declare that they have no conflict of interest.

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