The Pharma Innovation Journal 2019; 8(2): 673-676

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2019; 8(2): 673-676

www.thepharmajournal.com
Received: 12-12-2018
Accepted: 15-01-2019

AS Lejaniya
MVSc Scholar, Department of Dairy Science, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

R Geetha
Department of Dairy Science, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

CT Sathian
Department of Dairy Science, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

K Radha
Department of Dairy Science, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

KK Jayavardhanan
Department of Veterinary Biochemistry, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

KA Mercy
Department of Statistics, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

Correspondence
AS Lejaniya
MVSc Scholar, Department of Dairy Science, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

Mannitol production in lactic acid bacteria

AS Lejaniya, R Geetha, CT Sathian, K Radha, KK Jayavardhanan and KA Mercy

Abstract
As calorie-consciousness and obesity became worldwide crisis day by day, demand for low calorie sweeteners is increasing. Mannitol is a natural sugar alcohol widely used in foods, pharmaceuticals and medical industry and considered to be half as sweet as sucrose. Reduced calorific value of mannitol (1.6 kilocalorie per gram) when compared to other sugars finds its application as a sweetener in low calorie foods. In a study conducted to identify LAB having significant mannitol production potential, *Leuconostoc pseudomesenteroides* IMAU: 11666 was found to be the best among the five unknown LAB isolates screened. Colorimetric assay was adopted for mannitol estimation and a yield of 0.65 g/l was observed from the above strain. The organism was identified by biochemical assays and confirmed by PCR and sequencing. Since production of mannitol by bacterial fermentation is more economical compared to industrial method of production, this specific strain can be utilised for economic production of mannitol.

Keywords: Lactic acid bacteria, mannitol, low calorie sweeteners, colorimetric assay

1. Introduction
D-Mannitol is a six-carbon sugar alcohol, which is considered to be half as sweet as sucrose (4 kilocalorie per gram) and has various applications in foods and pharmaceuticals. Mostly organisms like bacteria, fungi, yeast and some of the plant species have the ability to produce sugar alcohols (polyols) in abundant. Commercially mannitol is produced by catalase (nickel) mediated hydrogenation of glucose and fructose. But economical investment in industrial production is very high especially in purification and separation of end product, sorbitol from mannitol. The yield of crystalline mannitol, in above method is only 17% (w/w) approximately based on substrates. So utilization of useful microorganisms should be considered to produce high quantity of mannitol without much expenditure. In this study microbial production of mannitol by lactic acid bacteria (LAB) were focussed.

2. Materials and methods
Five isolates of LAB maintained in the culture collection of Department of Dairy Science was selected for the study. Selected LAB isolates were screened for mannitol production potential in a batch fermenter, by providing optimal growth condition of 37 °C, pH 6.5, not providing agitation. Mannitol yield was detected by D- Mannitol colorimetric assay (Sanchez, 1998) [7]. The LAB isolate yielded maximum mannitol was identified by biochemical assays, according to Bergey's Manual of Determinative Bacteriology, 8th Ed [1]. Identified LAB isolate with maximum mannitol production was confirmed by PCR and sequencing. The data were subjected to statistical analysis following the procedure described by Snedecor and Cochran (1994) [8] using the SPSS software version 24.0.

3. Results and Discussion
3.1 Mannitol estimation
Five unknown lactic acid bacterial strains isolated from dahi, purified by repeated quadrant streaking were labeled as L1, L2, L3, L4 and L5. Characters of the colonies were observed after streaking on MRS agar and incubating at 37 °C for 24 hours. The colonies appeared as white smooth and round in shape. According to the method suggested by Sanchez (1998) [7] calibration curve (Fig.1) was drawn by plotting different concentration of mannitol (Pure mannitol in a range of 100–1300 nmols) against optical density values obtained at 412 nm in a spectrophotometer.

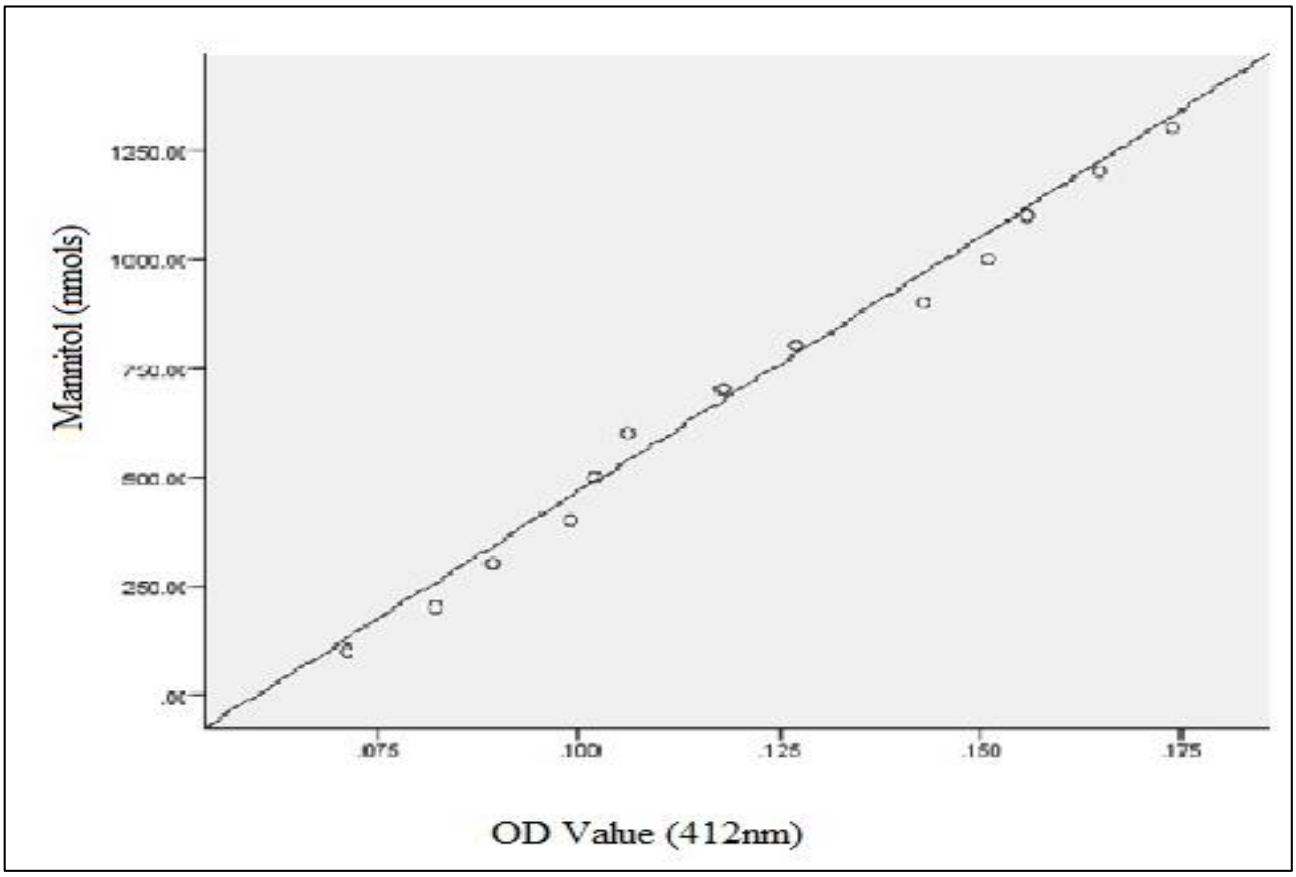

Fig 1: Caliberation curve for mannitol estimation

The cultures were inoculated in MRS broth, incubated at 37 °C for 18 hrs. in a batch bioreactor and collected the cell free supernatant. Mannitol yield from screened lactic acid bacterial strains was estimated using linear regression equation obtained from standard mannitol curve. Cell density was measured in spectrophotometer at 600nm. It is evident from table 1 that all the LAB strains screened for the study were able to produce mannitol in minor quantity on incubating for 18 hrs. Among them, the isolate L3 showed higher mannitol yield of 0.65 gram per litre. The cell density of screened LAB isolates is also mentioned in table 1. A direct co-relation observed between growth rate of LAB and mannitol yield. In a study conducted by Martinez *et al.*, 1963 [3] it was observed that *Lactobacillus breves* strain ATCC 367, a hetero fermentative species had mannitol dehydrogenase enzyme, promoting the production of mannitol. Yum and Kim (1998) [10] also observed that *Lactobacillus* sp. Y-107 and *Leuconostoc* sp. Y-002 isolated from kimchi possess the ability to yield mannitol. However mannitol yield can vary based on different culture environments such as media selected, substrate addition (glucose or fructose), culture methods (batch vs. fed-batch), and phase of growth of cells (growing vs stationary phase).

Table 1: Corelation between cell density and mannitol production

LAB strains	Cell density (OD value)	Mannitol (g/l)
L1	0.1269±0.01^{d}	0.45±0.02^{d}
L2	0.1038±0.02^{d}	0.43±0.03^{d}
L3	0.2971±0.02^{a}	0.65±0.01^{a}
L4	0.2506±0.03^{b}	0.57±0.01^{b}
L5	0.1942±0.01^{c}	0.50±0.02^{c}

Each value is a mean of six observations with SE
Means with different superscript in same column differ significantly ($p \leq 0.05$)

3.2 Biochemical identification

The isolate L3 which showed comparatively high mannitol yield was biochemically characterised to identify the genus. Characters of the colony were observed by plating on MRS agar and studying the peculiarities (Table 2). Morphological examination was carried out by performing Gram staining and microscopical assessment. The colony was smooth, round small and greyish white in colour. The isolate appeared as Gram positive cocci (Fig. 2), was negative for motility, catalase and oxidase test. Under secondary identification tests, different sugars were used to determine the fermentation profile of isolate. Moreover effect of different temperature, NaCl concentrations and pH on growth rate was also observed. All these studies proved that the isolate L3 as *Leuconostoc* sp. according to Bergey's Manual of Determinative Bacteriology.

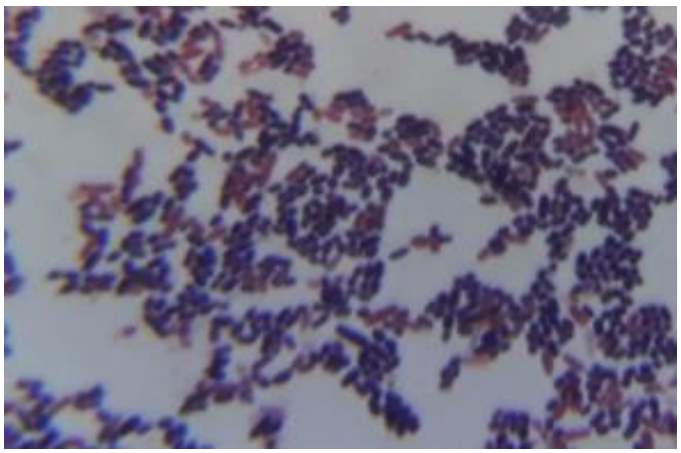

Fig 2: Gram's reaction of L3 isolate

Table 2: Biochemical test results

Tests	L3	TESTS	L3	Parameters	L3
Esculin Hydrolysis	+	Inuline	-	Growth at temperature 15 °C	+
Xylose	+	Sodium gluconate	-	33 °C	+
Cellobiose	+	Glycerol	-	37 °C	+
Arabinose	+	Salicin	-	42 °C	+
Maltose	+	Dulcitol	-	45 °C	+
Galactose	+	Inositol	-	Growth at different NaCl Concentrations (2 percent)	+
Mannose	+	Sorbitol	-	3 percent	_
Mellibiose	+	Mannitol	-	4 percent	_
Raffinose	+	Adonitol	-	6.5 percent	_
Sucrose	+	Arabitol	-	Growth at pH 4.5	_
Trehalose	+	Erythritol	-	pH 6.5	+
Oligosaccharide production	+	Tomato juice agar test	+	pH 9.6	+

Patil *et al.* (2010) [6] identified *Lactobacillus, Pedococcus* and *Weissella* from curd and cucumber by morphological and biochemical analysis. 50 colonies were isolated and all the LAB strains were observed as gram positive and catalase negative. Narwade *et al.* (2015) [5] done morphological and biochemical studies of organism which appeared as yellowish round colonies in MRS agar. The organism was gram positive in reaction, rod shaped and identified as *Lactobacillus*. Mithun *et al.* (2015) [4] in their studies also characterized the microbes isolated from raw milk sold from Aarey Milk Colony, the major supplier in the city of Mumbai as *Lactobacillus* species by routine microbiological and biochemical assays. In a study conducted by Makanjuola and Springham (1984) [2] *Leuconostoc* sp. was found to be the predominant one among the LAB strains isolated from distillery fermentations. They were able to produce gas from glucose, ammonia from arginine, showed growth at 15 and 45 °C.

3.3 Molecular characterization of LAB strain

DNA of isolate L3 was subjected to quality check using agarose gel electrophoresis. Clear band of DNA were obtained for the isolate. PCR amplification reactions were carried out by designing 16S rRNA primer and standardized the annealing temperature 60 °C for 40 seconds. Product obtained after 35 cycles were electrophoresed. The gels obtained were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (EZI Imager, Bio-Rad) and the picture obtained was represented in Fig. 3. A clear band on 1.5 kbp region was observed.

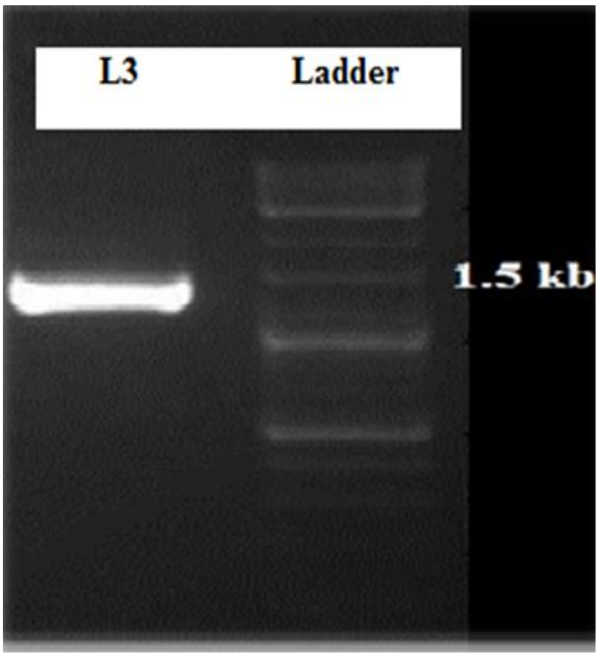

Fig 3: Gel picture of PCR

PCR products of isolate L3 was further sealed and send for sequencing in Rajiv Gandhi Centre for biotechnology, Trivandrum. The obtained sequences were blasted in NCBI nucleotide blast. Blast result obtained confirmed L3 as *Leuconostoc pseudomesenteroides* IMAU: 11666.The isolate identified showed resemblance with the biochemical results. The sequence attached was represented in figure 4. Recently, bacterial species identification by means of 16S rDNA-based method is almost accepted, as large public-domain sequence databases are accessible in Gene Bank for comparison. Tannock (1999) [9] confirmed the Lactobacillus species genotypically by means of 16S and 23S rRNA gene sequence as primer, in polymerase chain reaction (PCR). Primer amplified at 1.35 kbp region. Patil *et al.* (2010) [6] used 16S rRNA gene sequences as universal primer for the identification of *Lactobacillus*, *Pedococcus* and *Weissella* from curd and cucumber.

AACAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACCCGC
GGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCTACGATGCATAGCCGACCTGAGAGGG
TGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTC
GGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACT
CTGTTGTTAGAGAAGAACAAGGATGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGC
CACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGG
GCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGT
CATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGC
GTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCG
AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGT
GTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGAC
CGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
GAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTCCCC
TTCGGGGGCAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAG
TCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCCATCATTCAGTTGGGCACTCTAGCAAGACTGC
CGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACA
CACGTGCTACAATGGGAAGTACAACGAGTCGCGAAGTCGCGAGGCTAAGCTAATCTCTTAAAGCTT
CTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGT

Fig 4: Sequence of *Leuconostoc pseudomesenteroides* culture IMAU:11666

4. Conclusion

In the present study five lactic acid bacterial isolates from curd cultures available in the department were screened to find out excellent mannitol producers. An attempt was made to identify the isolate by molecular assay. Among the five isolates screened, L3 showed significantly higher mannitol yield of 0.65 g/l. The isolate was identified by biochemical tests and confirmed by PCR assay and sequencing as *Leuconostoc pseudomesenteroides* IMAU:11666. As mannitol is a food grade sweetener with Food and Drug Administration (FDA) endorsement, the newly identified LAB strain can be used to develop low calorie dairy products with beneficial effects. Side effects of other artificial sweeteners can be reduced also.

5. References

1. Buchanan RE, Gibbons NE. *Bergey's* Manual of Determinative Bacteriology. (8th Ed.). Williams and Wilkins, Philadelphia, 1974.
2. Makanjuola DB, Springham DG. Identification of lactic acid bacteria isolated from different stages of malt whisky distillery fermentations. J Inst. Brewing. 1984. 90:13-19.
3. Martinez G, Barker HA, Horecker BL. A specific mannitol dehydrogenase from *Lactobacillus brevis*. J. Biological Chem. 1963; 238:1598-1603.
4. Mithun S, Dipak V, Sheela S. Isolation and Identification of lactobacilli from raw milk samples obtained from Aarey Milk Colony. Int. J. Scient. Res. Publs. 2015; 5:1-5.
5. Narwade RB, Kasare JD, Choudhary RS. Isolation, Screening and Characterization of Lactobacilli from Cow Milk. Int. J Agric. Innovations Res. 2015. 3:1635-1637.
6. Patil MM, Pal A, Anand T, Ramana KV. Isolation and characterization of lactic acid bacteria from curd and cucumber. Indian J Biotech. 2010. 9:166-172.
7. Sanchez J. Colorimetric assay of alditols in complex biological samples. J Agric. Food Chem. 1998. 46:157-160.
8. Snedecor GW, Cochran WG. Statistical methods. (8th Ed.). Oxford and IBH Publishing Company, Culcutta, 1994, 564.
9. Tannock GW. Identification of lactobacilli and bifidobacteria. Curr. Issues Mol. Biol. 1999; 1:53-64.
10. Yun JW, Kim DH. A comparative study of mannitol production by two lactic acid bacteria. J Fermentation Bioengineering. 1998; 85:203-208.