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ORIGINAL ARTICLE

# Improvement of *Lactobacillus brevis* NM101-1 grown on sugarcane molasses for mannitol, lactic and acetic acid production

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**Abstract** The conversion of sugarcane molasses for the production of lactic acid, acetic acid, and mannitol was enhanced by subjecting *Lactobacillus brevis* NM101-1 wild strain to various doses of gamma irradiation. Four mutants (LM-1-LM-4) obtained at gamma ray doses of 30, 60, 90, and 120 Gy produced higher levels of lactic acid, acetic acid, and mannitol than the wild-type. Among all the mutants tested, LM-3 strain showed the highest mannitol and acetic acid production which reached 198.95 and 96.86 g/l, respectively. On the other hand, mutant LM-1strain exhibited the best performance with respect to lactic acid production (143.73 g/l). Random amplified polymorphic DNA polymerase chain reaction technique (RAPD-PCR) using three primers (RP, R5, and M13) was used in order to detect the variation in DNA profile in response to gamma irradiation treatments. RAPD analysis indicated the appearance and disappearance of DNA polymorphic bands at different gamma ray doses. The results showed the potential of these mutants to be potential candidates for economical production of mannitol, lactic and acetic acids from molasses on a commercial scale.

**Keywords** *Lactobacillus brevis* · Mutation · Sugarcane molasses · Mannitol · Lactic acid · Acetic acid

## Introduction

The trend towards environmental sustainability and the development of renewable resources has significantly increased interest in the recovery of fermentation products, such as organic acids, feed or food additives, and industrial chemicals. Consequently, the range of products of fermentation is expanding, and is beginning to compete with traditional synthetic production of commodity chemicals (John and Nampoothiri 2007).

Sugar is one of the most important substrates for human diet. In the past, the sugar industry produced only sugar but nowadays sugar industries are involved in the production of sugar, electricity, and ethanol, and there are several waste products which can be used for other purposes (Chauhan et al. 2011). Molasses is a by-product of the sugar manufacturing process, as a final effluent obtained during the preparation of sugar by repeated crystallization. Molasses consist of: major components (water, sugar, nonsugars) and minor components (trace elements, vitamins, growth substances) which are very useful for the fermentation process (Rodrigues et al. 2006). Molasses is widely used as a fertilizer and animal feed, and in the fermentation industry (Najafpour and Shan 2004; Fisher and Bipp 2005). Its uses in the pharmaceutical industry and for the production of biodegradable plastics or their precursors have also been reported (Wee et al. 2004). A large amount of molasses is produced as a by-product, which provides sufficient raw material for these applications.

Lactic acid (2-hydroxypropanoic acid) is a natural organic acid with a long history of use in the food and non-food industries, including the cosmetics and pharmaceutical industries (John and Nampoothiri 2007; Tashiro et al. 2011; Abdel-Rahman et al. 2011). It has been utilized as a raw material in the production of polylactic acid (PLA), a biodegradable polymer used as an environmental-friendly biodegradable plastic (Calabia and Tokiwa 2007). Lactic acid can be produced either by chemical synthesis or microbial fermentation. Chemical synthesis from petrochemical resources always results in racemic mixture of DL-lactic acid, which is a major disadvantage of this approach (Hofvendahl and Hahn-Hagerdal 2000). Conversely, microbial lactic acid fermentation offers an

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advantage in terms of the utilization of renewable carbohydrate biomass (low cost of substrates), low production temperature, low energy consumption, and the production of optically high pure lactic acid (either D- or L-lactic acid or a mixture of the two isomers) by selecting an appropriate strain (Ilmen et al. 2007; Abdel-Rahman et al. 2011). Only a few lactic acid bacteria (LAB) such as *Lactobacillus brevis*, *L. helveticus*, and *L. delbrueckii* can produce optically pure lactic acid (Benthin and Villadsen 1995).

Mannitol, a naturally occurring polyol, is a sugar alcohol with six carbon atoms. It is widely used in the food, pharmaceutical, medicine, and chemical industries (Saha 2003). The production of mannitol by fermentation has become attractive because of the problems associated with its chemical production. A number of homo- and heterofermentative LAB, yeasts, and filamentous fungi are known to produce mannitol. In particular, several heterofermentative LAB are excellent producers of mannitol from fructose. These bacteria convert fructose to mannitol with 100 % yields from a mixture of glucose and fructose (1:2). Glucose is converted to lactic acid and acetic acid, and fructose is converted to mannitol (Saha and Racine 2011).

In developing countries, simple biotechnological techniques like mutagenesis may be adopted in order to enhance food and goods productivity. Mutation has its harmful and beneficial effects (Allan and Greenwood 2001). There are numerous documented cases where beneficial mutations with survival advantages have arisen in a population. Such beneficial mutations also occur frequently among viruses, bacteria, and higher organisms. For example, mutagenesis has been used in the selection and improvement of LAB starter culture (Harlander 1992). Irradiation by gamma rays may cause some mutations to the genes of cells through the DNA repair mechanisms within cells (Iftikhar et al. 2010). Microbial strains for the overproduction and improvement of industrial products have been the hallmark of all commercial fermentation processes.

Therefore, this study has undertaken a simple biotechnology technique, mutagenesis, to see if it can improve the conversion of sugarcane molasses into mannitol, lactic acid, and acetic acid by *Lactobacillus brevis* NM101-1 isolated from dairy products, with the ultimate objective of making the process more effective and economical.

## Materials and methods

### Bacterial strain

*Lactobacillus brevis* NM101-1 was isolated from an Egyptian yogurt sample, identified as described by Rushdy and Gomaa (2012), and maintained in 70 % glycerol vials at −80 °C. A loopful of cells was transferred to an agar slant made with De Man, Rogosa, and Sharpe (MRS) medium containing bromocresol green (25 mg/l), based on the method of Dal Bello and Hertel (2006), and cultivated at 35 °C for 24 h. The slants were stored at 4 °C for use in seed culture preparation.

### Molasses

Sugarcane molasses was obtained from the Sugar Refinery Factory at El-Hawamdia, Giza, Egypt. The sugarcane molasses was hydrolyzed by adding 1 ml of 20 % $H_2SO_4$ in 100 ml of sugar solution. The acidified sugar solution was heated in a boiling water bath for 20 min. The pH of the hydrolyzed cane sugar was adjusted to 7.0 (Patil et al. 2006).

### Irradiation and mutant selection

*Lactobacillus brevis* NM101-1 was grown in a 250-ml conical flask containing 100 ml MRS medium at 35 °C for 24 h to cell optical density ($OD_{600}$) of 0.2–0.3. The cells were harvested by centrifugation at 10,000*g* for 20 min and washed twice in 100 ml sterile saline solution. The washed pellets were removed and adjusted with sterile saline to the turbidity of a 0.5 McFarland standard (corresponding to approximately $10^6$ cfu/ml). Five ml of cell suspension was transferred in each vial, sealed with paraffin and exposed to a gamma irradiator. The source of gamma radiation used for irradiation of *Lactobacillus brevis* NM101-1 was Cobalt-60 ($Co^{60}$) Gamma cell GC220, the product of Canada, located at the National Center for Radiation Research and Technology (Nasr City, Cairo, Egypt). This source gave a dose rate of 0.792 Rad/s at the time of the experiment.

A range of doses (20–140 Gy) of gamma radiations at an interval of 10 Gy were tested. The treated cell suspension (0.1 ml) was placed onto plates of agar growth medium. The growth medium consisted of (w/v): hydrolyzed cane sugar molasses 10 % as a carbon source, yeast extract 1 %, and $CaCO_3$ 5 % (Patil et al. 2006). The plates were incubated at 35 °C for 48 h for the appearance of distinct clear zones around colonies.

### Hereditary stability studies of mutants

The mutant strains obtained by the mutation of the aforementioned method that produced much larger zones were studied for their stability for acid production for several generations, then stored in 1 % NaCl for long-term preservation (Kropinski 1975).

### Batch fermentation conditions

The inoculum for fermentation was prepared by transferring a loopful of a fresh culture of *Lactobacillus brevis* NM101-1 (wild and mutant strains) into 50 ml of MRS medium in 250-

ml conical flasks. The flasks were incubated at 35 °C with shaking at 150 rpm for 24 h. Batch fermentation experiments were carried out in 500-ml conical flasks containing 100 ml growth medium. The flasks were inoculated with 5 % inoculum culture of *L. brevis* NM101-1 (wild and mutant strains) and incubated in a shaker incubator (150 rpm) at 35 °C. The culture samples were harvested at various time intervals and centrifuged at 10,000*g* for 20 min to separate the cells. The supernatant was analyzed for sugars, mannitol, and acids (Kadam et al. 2006).

### HPLC analysis

Samples were removed aseptically at various time intervals (12, 24, 48 h) and filtered through a 0.45-μm membrane. Samples were analyzed for sugars (sucrose, lactose, glucose, and fructose), mannitol and organic acids (lactic and acetic) by high-performance liquid chromatography (HPLC), Shimadzu Class-VPV 5.03 (Kyoto, Japan), according to the AOAC Method 982.14 (AOAC 1997; Li et al. 2002). Sugars were extracted into 50 % ethanol, the extract was passed through C18 Sep-Pak cartridge, and then filtered through a 0.45-mm nylon disc. Separation and quantitation were carried out on an amino-bonded column with a mobile phase of $CH_3CN$ and $H_2O$ (80/20 v:v) and detection with a differential refractometer refractive index RID-10A Shimadzu detector. Standard solutions of individual sugars with analytical mannitol, lactic and acetic acids were prepared by diluting each analyzed sugar (2 g) in 100 ml deionized water. Injection volume of each standard was 20 μl.

### Random amplified polymorphic DNA (RAPD) fingerprinting

In the present investigation, the PCR-based RAPD fingerprinting method was used to detect genetic variations among *Lactobacillus brevis* NM101-1 and its mutant strains. The genomic DNA of wild and mutant strains was extracted enzimatically from 800-μl samples of 24-h cultures grown in MRS broth at 35 °C according to the method of Campoccia et al. (2005), and then purified according to the method described by Sieladie et al. (2011). Three primers RP (5′-CAGC ACCCAC-3′), R5 (5′-AACGCGCAAC-3′), and M13 (5′-GAG GGT GGC GGT TCT-3′) (Operon Technology, USA) were used for RAPD-PCR (Huey and Hall 1989; Torriani et al. 1999). Conditions of PCR reactions and amplification were performed as described by Schillinger et al. (2003). Reactions were carried out in 25-μl amplification mixtures with 12.5 μl of 2× Master Mix (Fermentas, USA), 0.5 μl of primer, 1 μl of total DNA, and 11 μl of water. Amplification was performed using the Biomerta gradient thermo cycler at the Biotechnology Lab of the Desert Research Center, Cairo, Egypt.

The primer RP was used under the following amplification conditions: one cycle 94 °C for 3 min, 45 °C for 45 s, 72 °C for 1 min; 30 cycles 94 °C for 45 s, 45 °C for 45 s, 72 °C for 1 min; one cycle 94 °C for 45 s, 45 °C for 45 s, 72 °C for 5 min. The reaction mixtures with R5 primer, after incubation at 94 °C for 5 min, were cycled through the following temperature profile: 40 cycles 94 °C for 60 s, 29 °C for 90 s, and 72 °C for 2 min. Final extension was carried out at 74 °C for 5 min. The reaction mixtures with M13 primer, after incubation at 94 °C for 2 min, were cycled through the following temperature profile: 40 cycles 94 °C for 60 s, 42 °C for 20 s, and 72 °C for 2 min. Final extension was carried out at 72 °C for 10 min.

The PCR was conducted in a Thermal Cycler (Biometra). Amplification products were separated on a 2 % agarose gel, containing 0.5 μg/ml (w/v) of ethidium bromide (Fermentas). All gels resulting from RAPD electrophoresis were scanned using the Gel Doc-2001 Bio-Rad system. The densitometric scanning of the bands was performed on three directions. Each band was recognized by its length, width, and intensity. Accordingly, relative amounts of each band could be quantified and scored. The gels of DNA were visualized and photographed by a gel documentation system (Gel-Doc Bio-Rad 2000) under UV transilluminator.

For analyses, clustering was performed for RAPD analysis of wild and mutant strains of *Lactobacillus brevis* NM101-1 using three primers (RP, R5, and M13) under various doses of gamma rays by application of the agglomerative clustering analysis technique according to Kruscal (1964) by using the Community Analysis Package (cap) (1999).

## Results and discussion

In this work, we describe the efficient conversion of sugarcane molasses by *Lactobacillus brevis* NM101-1 wild strain and its mutants for the extracellular biosynthesis of lactic acid, mannitol, and acetic acid. The wild culture of *Lactobacillus brevis* NM101-1 was subjected to gamma radiation with a range of doses (20–140 Gy) at an interval of 10 Gy. These irradiated cells were plated onto the fermentation medium to look for the colonies showing larger zones of acid production. Four mutants, designated as LM-1, LM-2, LM-3. and LM-4 obtained at gamma ray doses of 30, 60, 90, and 120 Gy, respectively, produced the highest acid production and were selected for further studies. The stability of these mutants for acid production was determined by successive sub-culturing for several generations. The mutants maintained the same production yield after being sub-cultured several times, indicating that the mutation is stably heritable (data not shown). At 120 Gy, complete death of cells occurred. These results were in accordance with those of Iftikhar et al. (2010) who reported that, with a gradual increase in dose intensity of gamma irradiation, the number of deaths and damage were increased.

*Lactobacillus brevis* NM101-1 wild and mutant strains (LM-1–LM-4) were evaluated for lactic acid, mannitol, and

**Table 1** Comparison of lactic acid, mannitol, and acetic acid production and sucrose, lactose, glucose, and fructose consumption by *Lactobacillus brevis* NM101-1 (wild and mutant strains) grown on sugarcane molasses in batch fermentation

| Parent/mutant strain | Incubation period (h) | Sucrose | Lactose | Glucose | Fructose | Lactic acid | Mannitol | Acetic acid |
|---|---|---|---|---|---|---|---|---|
| Wild | 0 | 82.14±4.90 | 95.20±6.12 | 63.48±4.09 | 206.80±10.01 | 0.00 | 0.00 | 0.00 |
| | 12 | 66.34±2.60 | 76.56±4.80 | 59.80±3.67 | 198.75±8.71 | 33.45±2.06 | 43.00±2.04 | 32.61±2.60 |
| | 24 | 47.83±2.90 | 44.38±3.02 | 40.45±2.41 | 131.06±5.06 | 97.13±5.01 | 51.99±4.06 | 47.30±4.50 |
| | 48 | 21.44±1.02 | 17.17±1.34 | 22.23±1.40 | 120.93±5.02 | 46.45±3.25 | 74.38±5.06 | 51.00±4.20 |
| LM-1 | 12 | 53.17±3.90 | 92.79±4.04 | 53.09±3.25 | 196.14±7.02 | 108.63±5.13 | 55.90±3.07 | 40.15±260 |
| | 24 | 51.23±4.12 | 39.27±2.10 | 38.11±2.64 | 107.25±6.3 | 143.73±6.96 | 114.34±6.00 | 78.59±4.90 |
| | 48 | 20.68±2.05 | 19.23±1.63 | 25.70±1.77 | 172.90±9.93 | 120.87±5.90 | 64.11±3.90 | 32.11±1.10 |
| LM-2 | 12 | 48.48±3.50 | 85.85±5.14 | 51.62±4.40 | 192.66±8.84 | 78.34±4.55 | 98.54±4.03 | 56.52±5.30 |
| | 24 | 39.66±2.74 | 42.30±3.17 | 39.44±3.12 | 171.20±6.14 | 107.09±5.76 | 121.90±6.12 | 87.33±3.70 |
| | 48 | 30.48±2.30 | 30.23±2.03 | 20.93±2.93 | 185.74±10.00 | 66.41±3.30 | 101.74±6.2 | 36.62±2.4 |
| LM-3 | 12 | 53.23±4.86 | 18.71±1.60 | 56.58±5.23 | 165.74±7.6 | 73.87±3.50 | 109.50±7.20 | 75.43±4.73 |
| | 24 | 27.88±1.45 | 16.49±2.71 | 37.12±2.09 | 139.44±6.23 | 103.78±4.57 | 198.95±7.00 | 96.86±5.16 |
| | 48 | 19.70±1.40 | 11.53±1.99 | 15.10±1.84 | 150.49±5.01 | 57.90±3.21 | 117.80±5.20 | 39.34±2.85 |
| LM-4 | 12 | 66.12±5.95 | 28.06±2.80 | 54.50±2.11 | 175.44±7.34 | 70.24±5.95 | 29.40±2.06 | 11.51±1.06 |
| | 24 | 42.17±3.07 | 14.46±1.90 | 43.50±1.40 | 110.34±4.00 | 98.48±6.22 | 177.41±6.95 | 32.22±2.08 |
| | 48 | 34.53±3.09 | 10.01±1.04 | 23.93±1.45 | 133.94±5.24 | 46.66±3.00 | 77.89±4.65 | 7.76±0.89 |

Data (in g/l) represent the mean of 3 different readings ± standard deviation

acetic acid production in sugarcane molasses-based fermentation medium. Sugarcane molasses is cheap and abundant and contains ahigh concentration of sucrose, thus batch fermentation of molasses was carried out using this substrate as a carbon source. The buffering capacity of the molasses could be an advantage in maintaining the pH of the medium above pH 5.3 during fermentation (Dumbrepatil et al. 2008). LAB are generally fastidious microorganisms requiring complex nutrients such as amino acids and vitamins for their growth. Molasses was proven to be an economically feasible raw material for industrial production of lactic acid, since it is fortified with enough nutrients necessary for the growth of LAB. This raw material is usually supplied by a complex nitrogen source like yeast extract. Yeast extract is the most commonly used nitrogen source which provides a vitamin B complex content in addition to organic nitrogen to LAB (Yoo et al. 1997). For the fermentation of glucose and soybean hydrolysate by *Lactobacillus rhamnosus*, Kwon et al. (2000) found that the supplementation of the medium with vitamins or yeast extract was essential for lactic acid production when soybean was used as the organic nitrogen source.

A commonly used method for the determination of acid content is alkaline titration using an appropriate visual indicator. However, the titration methods are generally not selective

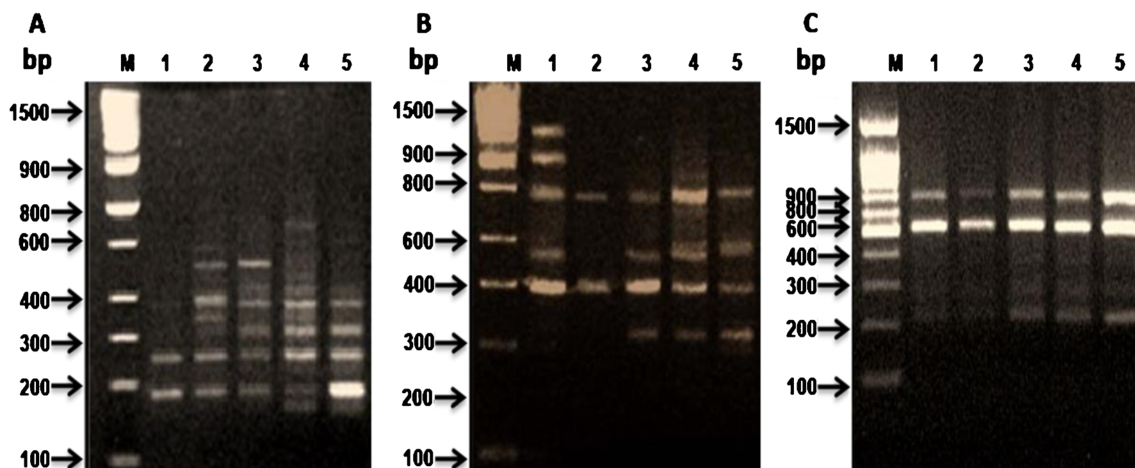


**Fig. 1** Agarose gel showing RAPD fingerprints for detection of DNA profiles changes between wild and mutant strains of *Lactobacillus brevis* NM101-1 using three primers (RP (**a**), R5 (**b**) and M13 (**c**)). *M* Marker, *Lane 1* wild, *Lane 2* LM-1 (30 Gy), *Lane 3* LM-2 (60 Gy), *Lane 4* LM-3 (90 Gy), *Lane 5* LM-4 (120 Gy)

**Table 2** RAPD analysis of wild and mutant strains of *Lactobacillus brevis* NM101-1 using three primers (RP, R5, and M13) under various doses of gamma rays

| Marker | Wild | | LM-1 | | LM-2 | | LM-3 | | LM-4 | |
|---|---|---|---|---|---|---|---|---|---|---|
| MW[a] (bp) | MW[a] (bp) | A[b] | MW[a] (bp) | A[b] | MW[a] (bp) | A[b] | MW[a] (bp) | A[b] | MW[a] (bp) | A[b] |
| RP | | | | | | | | | | |
| 1500 | – | – | – | – | – | – | – | – | – | – |
| 900 | – | – | – | – | – | – | – | – | – | – |
| 800 | – | – | – | – | – | – | – | – | – | – |
| – | – | – | – | – | – | – | 706 | 5.19 | – | – |
| 600 | – | – | 592 | 3.68 | – | – | 600 | 3.36 | – | – |
| – | – | – | 525 | 6.07 | 525 | 9.08 | 533 | 5.26 | – | – |
| – | – | – | 467 | 3.2 | – | – | 467 | 4.4 | – | – |
| – | – | – | – | – | 442 | 8.79 | 433 | 5.48 | 425 | 4.41 |
| 400 | – | – | 394 | 12 | 389 | 7.76 | 383 | 8.85 | 389 | 6.71 |
| – | – | – | 350 | 5.64 | – | – | 350 | 4.11 | – | – |
| 300 | – | – | 317 | 4.78 | 322 | 10.7 | 322 | 9.97 | 322 | 11.1 |
| – | 255 | 10.9 | 260 | 8.94 | 260 | 8.01 | 260 | 11.4 | 260 | 12.4 |
| 200 | 185 | 17 | 188 | 10.2 | 191 | 7.35 | 191 | 4.17 | 191 | 20.2 |
| – | – | | – | – | – | – | 174 | 4.21 | – | – |
| 100 | – | – | – | – | – | – | – | – | – | – |
| Total | 2 | | 8 | | 6 | | 11 | | 5 | |
| R5 | | | | | | | | | | |
| 1500 | – | – | – | – | – | – | – | – | – | – |
| – | 1149 | 12.4 | – | – | – | – | – | – | – | – |
| 900 | 895 | 13.6 | – | – | – | – | – | – | – | – |
| – | – | – | – | – | – | – | 843 | 6.06 | – | – |
| 800 | 777 | 14 | 766 | 11.7 | 766 | 12.1 | 771 | 20.7 | 783 | 15.9 |
| – | – | – | – | – | – | – | 709 | 5.85 | – | – |
| – | – | – | – | – | – | – | 657 | 5.67 | – | – |
| 600 | – | – | – | – | – | – | – | – | 567 | 17.7 |
| – | 533 | 9.57 | – | – | 527 | 9.85 | 533 | 14 | – | – |
| 400 | 400 | 21.2 | 395 | 20.5 | 395 | 25.4 | 393 | 12.7 | 393 | 11.2 |
| 300 | – | – | – | – | 317 | 6.44 | 315 | 4.39 | 317 | 8.94 |
| 200 | – | – | – | – | – | – | – | – | – | – |
| 100 | – | – | – | – | – | – | – | – | – | – |
| Total | 5 | | 2 | | 4 | | 7 | | 4 | |
| M13 | | | | | | | | | | |
| 1500 | – | – | – | – | – | – | – | – | – | – |
| 900 | 900 | 11 | 919 | 7.03 | 919 | 16.9 | 900 | 16.2 | 900 | 23.4 |
| 800 | – | – | – | – | – | – | – | – | – | – |
| 600 | 600 | 22.7 | 667 | 20.7 | 667 | 22.3 | 600 | 21.6 | 633 | 23.5 |
| 400 | – | – | – | – | 392 | 4.96 | 385 | 3.18 | 400 | 3.7 |
| 300 | – | – | – | – | 300 | 4.95 | 289 | 5.37 | 294 | 3.64 |
| – | 261 | 3.56 | 267 | 2.05 | – | – | – | – | – | – |
| 200 | 228 | 3.37 | 228 | 2.32 | 228 | 5.72 | 222 | 5.33 | 217 | 5.9 |
| 100 | – | – | – | – | – | – | – | – | – | – |
| Total | 4 | | 4 | | 5 | | 5 | | 5 | |

[a] *MW* molecular weight

[b] *A* amount

nor sufficiently sensitive or precise to detect a small acid content (Casella and Gatta 2002). To avoid these problems, gas chromatography and high-performance liquid chromatography (HPLC) methods have gained importance in organic acid analysis due to their speed, selectivity, sensitivity, reliability, and simple sample preparation methods. In the present study, HPLC has been used with a refractive index (HPLC-RID) with the aim of monitoring the sugars (sucrose, lactose, glucose, and fructose), mannitol, and acids (lactic and acetic) during the batch fermentation process.

In an attempt to evaluate the effect of incubation period on the production of lactic acid, mannitol, and acetic acid by wild and mutant strains of *Lactobacillus brevis* NM101-1, grown on sugarcane molasses in batch fermentation, the samples were withdrawn at specified time intervals (12, 24, and 48 h) and analyzed by HPLC. As evident from results cited in Table 1, an increase in sugars utilization and subsequent lactic acid, mannitol, and acetic acid production was found up to 24 and 48 h. This could be attributed to the growth of the culture reaching the stationary phase and as a consequence of metabolism. Generally, all the mutant strains grew more efficiently and produced more lactic acid, mannitol, and acetic acid than the parent strain. Similar results of high lactic acid production were reported by Kadam et al. (2006), when using four mutant strains of *Lactobacillus delbrueckii* NCIM 2365 grown on a medium containing (100 g/1) hydrolyzed cane sugar (the mutation was made by ultraviolet rays). The LM-3 mutant strain was proved to be an efficient strain for mannitol and acetic acid production, reaching 198.95 and 96.86 g/l, respectively. On the other hand, the LM-1 mutant strain showed the best performance with respect to lactic acid at 143.73 g/l. Moreover, maximum production of lactic acid, mannitol, and acetic acid by the mutant strains was observed after 24 h of incubation. The reduction in fermentation period is additionally advantageous to improve the economics of the process. Thus, the reduction in the fermentation period along with high sugar conversion to these fermentation products areis the advantages of the developed process.

It is noteworthy that *Lactobacillus brevis* NM101-1 mutant strains were able to preferentially utilize glucose rapidly in media containing hydrolyzed cane sugar. It utilized fructose at a much lower rate than that of glucose, leading to an accumulation of fructose and lactic acid in the fermented broth. There are many reports on the co-production of fructose and ethanol from sucrose by fructose nonutilizing mutants. These mutants were capable of utilizing glucose selectively from media with glucose/fructose mixtures or sucrose, and producing fructose and ethanol. The majority of the microorganisms used in these processes produced unwanted byproducts, such as sorbitol and glycerol, along with fructose and ethanol. Saha and Nakamura (2003) have reported the production of mannitol from fructose by *Lactobacillus intermedius* NRRLB-3693. The bacterium produced mannitol, lactic acid, and acetic acid from fructose. However, there are no reports in the literature on the co-production of lactic acid and fructose from sucrose. The results obtained in the present study demonstrated that *L. brevis* NM101-1 mutant strains were able to produce lactic acid and fructose from media containing hydrolyzed cane sugar.

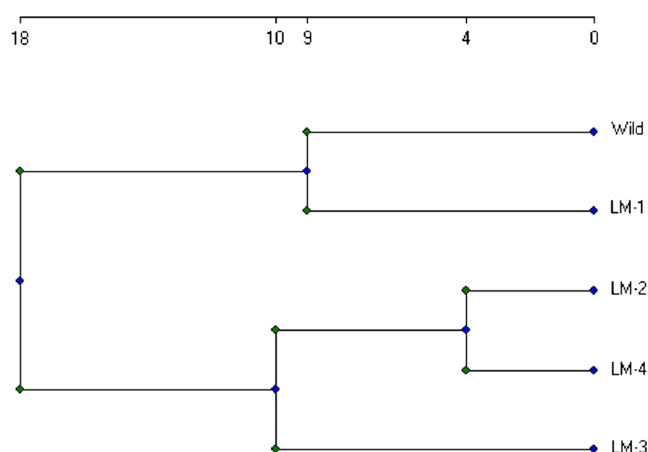


**Fig. 2** Dendrogram obtained by using RAPD patterns generated with three primers (RP, R5, and M13) from wild and mutant strains of *Lactobacillus brevis* NM101-1

Due to increasing consumer demands for more natural, tasty, and healthy food, the traditional process of bread production has enjoyed renewed success in recent years (Lopez et al. 2003). In some products of dough, such as Sourdough (a microbial ecosystem of LAB and yeasts in a matrix of mainly cereal flour), the production of organic acids is the main attribute resulting in a sour taste of the dough (Vogel et al. 1999). So, *Lactobacillus brevis* NM101-1 may be added to baker's yeast as a probiotic addition in order to produce organic acids (lactic and acetic acids) and improve the quality, taste, and flavor of wheat breads.

**Table 3** Pattern of monomorphic and polymorphic bands generated by three primers in parent and mutant strains of *Lactobacillus brevis* NM101-1 under various doses of gamma rays

| Primer code | Total no. of bands | Monomorphic bands | | Polymorphic bands | | | |
|---|---|---|---|---|---|---|---|
| | | No. | % | Shared bands | Unique bands | No. | % |
| M13 | 6 | 3 | 50 | 3 | – | 3 | 50 |
| R5 | 10 | 2 | 20 | 2 | 6 | 8 | 80 |
| RP | 11 | 2 | 18.2 | 7 | 2 | 9 | 81.81 |

Molecular analysis (RAPD-PCR)

In the present study, because the abilities of parent and mutant strains of *Lactobacillus brevis* NM101-1 for the production of lactic acid, mannitol, and acetic acid were different, the need for molecular differentiation was necessary. Random amplified polymorphic DNA (RAPD) fingerprinting is a modification of the polymerase chain reaction (PCR), which utilizes a single, arbitrarily-chosen primer to amplify a number of fragments from a given template DNA to generate a discrete "fingerprint" when resolved by gel electrophoresis. Alterations by as little as a single base in the primer sequence lead to marked alterations in the fingerprints generated with a given template under optimized conditions (Jones and Kortenkamp 2000).

New PCR amplification products may reveal a change in some oligonucleotide priming sites due to mutations and/or homologous recombination. On the other hand, the disappeared bands could be attributed to DNA damage (e.g., single- and double-strand breaks, modified bases, abasic sites, oxidized bases, bulky adducts, DNA protein cross-links), point mutations, and/or complex chromosomal rearrangements induced by genotoxins (Atienzar and Jha 2006).

As reported by Hegazi and Hamideldin (2010), the RAPD technique has been successfully utilized to detect various types of DNA damage and mutation induced by gamma rays. We utilized this technique to compare the appearance and disappearance of DNA polymorphic bands and their amount between the *L. brevis* NM101-1 parent strain and its mutant strains created by exposure to different doses of gamma radiation.

The results cited in Fig. 1 and Tables 2 and 3 show that the three primers used (RP, R5, and M13) resulted in the appearance of PCR products with a variable number of bands. Also, the sizes of amplified fragments differed from wild and mutant strains for the same primer and ranged from 174 to 1,149 bp.

From this RAPD dataset, the primer RP indicated the presence of two monomorphic bands (18.2 %) and nine polymorphic bands (81.81 %). Two unique bands with molecular weight 706 and 174 bp were genotype-specific bands for the LM-3 mutant strain. Primer R5 resulted in the appearance of two monomorphic bands (20 %) and eight polymorphic bands (80 %). Six unique bands, two for the wild strain (1,149 and 895 bp), three for the LM-3 strain (843, 709 and 657 bp) ,and one for the LM-4 strain (567 bp) were scored. On the other hand, the results of primer M13 indicated the production of three monomorphic bands (50 %) and three polymorphic (shared only) bands (50 %).

As reported above, the LM-3 mutant strain showed the best performance with respect to mannitol and acetic acid production. This may be related to the highest number of bands (11, 7, and 5) detected with RP, R5, and 13 primers, respectively, and the appearance of three unique bands with the R5 primer and two with the RP primer, whilst the LM-1 mutant strain showing the highest lactic acid production had eight bands with the RP primer and two bands with the R5 primer.

In order to classify the analyzed bacteria, a hierarchical cluster analysis was performed. With the help of this cluster algorithm, the most similar groups are not clustered but the most feasible homogeneous clusters are formed. The dendrogram of the resulting classification of the *Lactobacillus brevis* NM101-1 wild strain and its mutants are shown in Fig. 2. There is a distinction into the two main clusters. One cluster consists of two strains that are *Lactobacillus brevis* NM101-1 wild strain and LM-1 mutant strain. The other group is formed by LM-2, LM-3, and LM-4 mutant strains. Cluster B contained two sub-clusters: LM-2 and LM-4 mutant strains lie in one subcluster, shild the other included LM-3.

## References


Abdel-Rahman MA, Tashiro Y, Sonomoto K (2011) Lactic acid production from lignocellulose-derived sugars using lactic acid bacteria: overview and limits. J Biotechnol 156:286–301

Allan R, Greenwood T (2001) Advanced Biology 1: Student Resource and Activity Manual. Biozone, New Zealand, pp 126–158

AOAC (1997) Official Methods of Analysis, 16th edn. AOAC international, Gaithersburg

Atienzar FA, Jha AN (2006) The random amplified polymorphic DNA (RAPD) assay and related techniques applied to genotoxicity and carcinogenesis studies: a critical review. Mutat Res 613:76–102

Benthin S, Villadsen J (1995) Production of optically pure D-lactate by Lactobacillus bulgaricus and purification by crystallization and liquid/liquid extraction. Appl Microbiol Biotechnol 42:426–9

Calabia BP, Tokiwa Y (2007) Production of D-lactic acid from sugarcane molasses, sugarcane juice and sugar beet juice by *Lactobacillus delbrueckii*. Biotechnol Lett 29:1329–1332

Campoccia D, Montanaro L, Baldassarri L, An YH, Arciola CR (2005) Antibiotic resistance in *Staphylococcus aureus* and *Staphylococcus epidermidis* clinical isolates from implant orthopedic infections. Int J Artif Organs 28:1186–1191

Casella IG, Gatta M (2002) Determination of aliphatic organic acids by high-performance liquid chromatography with pulsed electrochemical detection. J Agric Food Chem 50:23–28

Chauhan MK, Chaudhary VS, Samar SK (2011) Life cycle assessment of sugar industry: a review. Renew Sust Energ Rev 15(7):3445–3453

Dal Bello F, Hertel C (2006) Oral cavity as natural reservoir for intestinal lactobacilli. Syst Appl Microbiol 29:69–76

Dumbrepatil A, Adsul M, Chaudhari S, Khire J, Gokhale D (2008) Utilization of molasses sugar for lactic acid production by *Lactobacillus delbrueckii* subsp. delbrueckii mutant Uc-3 in batch fermentation. Appl Environ Microbiol 74:333–335

Fisher K, Bipp HP (2005) Generation of organic acids and monosaccharides by hydrolytic and oxidative transformations of food processing residues. Bioresour Technol 96:831–842

Harlander SK (1992) Genetic Improvement of Microbial Starter Culture. In: Application of Biotechnology to Traditional Fermented Foods. Report of an Ad-Hoc Panel of the Board on Science and Technology for International Development. National Academic Press, Washington D.C, pp 20–26

Hegazi AZ, Hamideldin N (2010) The effect of gamma irradiation on enhancement of growth and seed yield of okra [*Abelmoschus esculentus* (L.) Monech] and associated molecular changes. J Hortic For 2(3):38–51

Hofvendahl K, Hahn-Hagerdal B (2000) Factors affecting the fermentative lactic acid production from renewable resources. Enzyme Microb Technol 26:87–107

Huey B, Hall J (1989) Hypervariable DNA fingerprinting in *Escherichia coli* minisatellite probe from bacteriophage M13. J Bacteriol 171: 2528–2532

Iftikhar T, Niaz M, Hussain Y, Abbas SQ, Ashraf I, Zia MA (2010) Improvement of selected strains through gamma irradiation for enhanced lipolytic potential. Pak J Bot 42(4):2257–2267

Ilmen M, Koivuranta K, Ruohonen L, Suominen P, Penttila M (2007) Efficient production of l-lactic acid from xylose by *Pichia stipitis*. Appl Environ Microbiol 73:117–123

John RP, Nampoothiri KM (2007) Fermentative production of lactic acid from biomass: an overview on process developments and future perspectives. Appl Microbiol Biotechnol 74:524–534

Jones C, Kortenkamp A (2000) RAPD library fingerprinting of bacterial and human DNA: applications in mutation detection. Teratog Carcinog Mutagen 20(2):49–63

Kadam SR, Patil SS, Bastawade KB, Khire JM, Gokhale DV (2006) Strain improvement of *Lactobacillus delbrueckii* NCIM 2365 for lactic acid production. Process Biochem 41:120–126

Kropinski AM (1975) Stability of bacterial mutants in saline. Appl Microbiol 29(4):448–450

Kruscal JB (1964) Nonmetric multidimensional scaling: a numerical method. Psychometrika 29:115–129

Kwon S, Lee PC, Lee EG, Chang YK, Chang N (2000) Production of lactic acid by *Lactobacillus rhamnosus* with vitamin-supplemented soybean hydrolysate. Enzyme Microb Technol 26:209–215

Li BW, Andrewsw KW, Pehrssonw PR (2002) Individual sugars, soluble, and insoluble dietary fiber contents of 70 high consumption foods. J Food Compos Anal 15:715–723

Lopez HW, Duclos V, Coudray C, Krespine V, Feillet-Coudray C, Messager A, Demigné C, Rémésy C (2003) Making bread with sourdough improves mineral bioavailability from reconstituted whole wheat flour in rats. Nutrition 19:524–530

Najafpour GD, Shan CP (2004) Enzymatic hydrolysis of molasses. Bioresour Technol 86:91–94

Patil SS, Kadam SR, Patil SS, Bastawde KB, Khire JM, Gokhale DV (2006) Production of lactic acid and fructose from media with cane sugar using mutant of *Lactobacillus delbrueckii* NCIM 2365. Lett Appl Microbiol 43:53–57

Rodrigues LR, Teixeira JA, Oliveira R (2006) Low-cost fermentative medium for biosurfactant production by probiotic bacteria. Biochem Eng J 32:135–142

Rushdy AA, Gomaa EZ (2012) Antimicrobial compounds produced by probiotic *Lactobacillus brevis* isolated from dairy products. Ann Microbiol. doi:10.1007/s13213-012-0447-2

Saha BC (2003) Production of mannitol by fermentation. In: Saha BC (ed) Fermentation biotechnology. American Chemical Society, Washington, DC, pp 67–85

Saha BC, Nakamura LK (2003) Production of mannitol and lactic acid by fermentation with *Lactobacillus intermedius* NRRL B- 3693. Biotechnol Bioeng 82:864–871

Saha BC, Racine FM (2011) Biotechnological production of mannitol and its applications. Appl Microbiol Biotechnol 89:879–891

Schillinger U, Yousif NMK, Sesar L, Franz CMAP (2003) Use of group specific and RAPDPCR analyses for rapid differentiation of *Lactobacillus* strains from probiotic yogurts. Curr Microbiol 47: 453–456

Sieladie DV, Zambou NF, Kaktcham PM, Cresci A, Fonteh F (2011) Probiotic properties of Lactobacilli strains isolated from raw cow milk in the western highlands of Cameroon. Innov Rom Food Biotechnol 9:12–28

Tashiro Y, Kaneko W, Sun Y, Shibata K, Inokuma K, Zendo T, Sonomoto K (2011) Continuous d-lactic acid production by a novel thermotolerant *Lactobacillus delbrueckii* subsp lactis QU 41. Appl Microbiol Biotechnol 89:1741–1750

Torriani S, Zapparoli G, Dellaglio F (1999) Use of PCR-based methods for rapid differentiation of *Lactobacillus delbrueckii* subsp. *Bulgaricus* and *L. delbrueckii* subsp. *Lactis*. Appl Environ Microbiol 65:4351–4356

Vogel RF, Knorr R, Müller MRA, Steudel U, Gänzle MG, Ehrmann MA (1999) Non-dairy lactic fermentations: the cereal world. Antonie Van Leeuwenhoek 76:403–411

Wee YJ, Kim JN, Yun JS, Ryu HW (2004) Utilization of sugar molasses for economical L(+)-lactic acid production by batch fermentation of *Enterococcus faecalis*. Enzyme Microb Technol 35: 568–573

Yoo IK, Cheng NN, Lee EG, Cheng YK, Moon SH (1997) Effect of B vitamin supplementation on lactic acid production by *Lactobacillus casei*. J Ferment Bioeng 84:172–175