Original
ISSN 2413-0516

Improved Exopolysaccharide Production from *Lacticaseibacillus paracasei* SH6 Using Mutagen EMS

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(Submitted: 02 November 2024 – Revised version received: 22 November 2024 – Accepted: 10 December 2024 – Published online: 26 February 2025)

Abstract

Objective: This study aims to enhance exopolysaccharide (EPS) production from a local isolate of *Lacticaseibacillus paracasei* through ethyl methanesulfonate (EMS)-induced chemical mutagenesis.

Methods: Two isolates, *L. paracasei* SH5 and SH6, were obtained from fermented beverages (boza and cider) and initially screened for EPS production. The SH6 isolate was subjected to EMS-induced mutation. Antibiotic-resistant mutants were selected from the inhibition zones surrounding antibiotic discs. EPS production was assessed through total soluble carbohydrate content analysis, HPLC calculations, and gene expression studies.

Results: Among the selected mutants, M8 exhibited the highest EPS production, with a yield of 566 mg/100 ml compared to the wild-type isolate, which produced 362 mg/100 ml. Other mutants (M1, M4, and M7) also showed increased EPS levels, reaching 411, 488, and 457 mg/100 ml, respectively. Mutant M8 demonstrated a 1.5-fold increase in EPS production compared to the wild type.

Conclusion: The findings confirm that EMS-induced mutagenesis effectively enhances EPS production in *L. paracasei*. This improvement has significant implications for biotechnological applications, particularly in the pharmaceutical and food industries.

Keywords: *Lacticaseibacillus paracasei,* exopolysaccharide (EPS), ethyl methanesulfonate (EMS), dextran

Introduction

Lacticaseibacillus paracasei, a member of lactic acid bacteria (LAB), exhibits numerous beneficial effects in the food industry as both a starter culture and a preservative. This organism produces a variety of metabolites, including exopolysaccharides, organic acids, fatty acids, and bacteriocins, which positively influence human health; therefore, it is classified as Generally Recognized as Safe (GRAS).[1-3] Exopolysaccharides (EPSs) are high molecular weight biopolymers frequently secreted into the extracellular environment by bacterial cells, notably LAB.[4] EPSs are essential in managing microbial communities by promoting the adhesion of bacteria to the intestinal lining through effective cell recognition processes. This adhesion creates a protective barrier as opposed to harmful elements such as toxins and antibiotics. As a result, this formation improves the tolerance of bacteria to and effectively copes with various stress factors during fermentation, such as pH, temperature, and pressure, and adapts to it effectively.[5,6] Mutations can occur spontaneously and at low frequencies, typically ranging from one mutant cell every 10^6 to 10^7 cells/generation. Mutagenesis uses chemical agents, such as ethyl methanesulfonate (EMS), nitrous acid, etc., or physical agents, such as ultraviolet, called induced mutagenesis. This technique is used frequently to generate novel mutant strains that exhibit enhanced phenotypic characteristics.[7]

Lactobacillus strains are known to produce EPS at a relatively lower level compared to other bacterial species. Therefore, various studies have been conducted to enhance EPS production through physical mutagenesis. The hyper-production of EPS by mutant *Bacillus licheniformis* using ultraviolet irradiation has been reported by Asgher *et al.*[8] Moreover, EPS production was improved after using microwave radiation on *Xanthomonas campestris.*[9] In the current study, the EMS was employed to induce mutations in the LAB strain and to evaluate its effects on EPS production. The function mutagen of ethyl methanesulfonate (EMS) in affecting Lacticaseibacillus strains for generating exopolysaccharide (EPS) has not been studied yet. Consequently, further explorations are required to evaluate the effectiveness of chemical mutagenesis such as EMS in boosting EPS production. In contrast, many beneficial compounds such as bacteriocins have been studied extensively, as in previous studies of Khattab,[10] Khattab,[11] they found the role of EMS in increasing bacteriocin production, as it induces various point mutations in bacterial DNA. These point mutations may generate exceptional mutants directly correlated with the enhancements implemented to augment bacteriocin production. At the laboratory scale, LAB required expensive growth factors, such as casein hydrolysate, yeast extract, beef extract, etc, for fermentation. However, the production of these expensive growth requirements is not feasible. Therefore, it is essential to explore more economical and efficient alternative materials.[12] In this context, sugarcane juice represents an economical component for commercial *Lactobacillus* fermentation, as well as it is considered an alternative source for EPS production because it contains water, sucrose, glucose, fructose, and protein in the following proportions: 80%, 17%, 0.4%, 0.2%, and 0.44–1.06%, respectively, along with various non-sugar organic compounds such as potassium, calcium, and magnesium. The pH of sugarcane juice ranges from 5.0 to

5.5.[13] Previous studies have demonstrated that EPS production by *Lactobacillus confusus* TISTR 1498 can be enhanced using sugarcane juice as an alternative carbon source under optimal conditions (including nitrogen and pH).[14] Finally, the mutagenic chemical ethyl methanesulfonate (EMS) proved a unique capability to increase exopolysaccharide (EPS) synthesis in the isolated M8 mutant compared to the wild-type isolate. The current work aims to assess the scope of EMS-induced mutations to increase EPS production in Lactaseibacillus isolates. because this worthy attention variance has not been studied before; therefore, this emphasizes the importance of utilizing it in many applications, especially in the pharmaceutical and food biotechnology fields.

Materials and Methods

Chemicals

The materials used in this study were MRS broth, Agar, and Pepton water (Oxoid®, Basingstoke, UK); Methylene blue (Sigma®, Denmark); Ethanol (Applichem®, Indonesia); kits of Anaerogene and cDNA synthesis (Thermofisher®, UK); DNA mini kit, Bacteria Reagent of RNA protect, quantiTect SYBR-green PCR kit, and DNA-Ladders (QIAGEN®, Düsseldorf, Germany); Emerald PCR master-mix (Takara®, Gothenburg, Sweden); Ethylm ethanesulfonate "EMS" (Himedia®, Mumbai, India); Dextran, Tris buffer, Boric acid, EDTA, TCA, Sucrose, Beef extract, Yeast extract, K_2HPO_4, KH_2PO_4, $MgSo_4$, $MnSo_4$, Tween80, and NaCl (Fluka®, London, UK).

Cultivation and Screening of EPS Producers

About two isolates were obtained from the same sources, which were not included, belonging to the genus *Lacticaseibacillus* (formerly *Lactobacillus*). These distinct isolates for *Lacticaseibacillus paracasei* SH5 and SH6 (separated from fermented beverage boza and cider) have been classified in GenBank after identifying the 16S ribosomal-RNA gene. Both isolates have accession numbers, OM489429 for SH5 and OM489430 for SH6 respectively. The two isolates were monthly subcultured in freshly prepared MRS broth media at 37°C for 24–48 hrs and kept at 4°C. The isolated *Lacticaseibacillus* bacteria had been for screening, which was better in growth and EPS production before the mutagenesis process. Therefore, the fermentation was carried out using one-liter sugar cane juice supplemented with 300 g/L sucrose, using a 10% inoculum volume under sterilized conditions. According to the technique described by Manochai et al. and Allaith et al.,[14,15] the extraction and purifying of EPS were performed.

Mutagenesis by Ethyl Methanesulfonate (EMS)

The selected strain for mutagenesis was *L. paracasei* SH6 due to the superior EPS production in the preliminary experiments. A 5 ml aliquot of fresh cultivated SH6 strain (16–18 h) was centrifuged at 5,600 (xg) and 4 (°C) for 3 (min). The resulting pellets were carefully resuspended in 5 ml of sodium phosphate buffer, which had a pH of 7 and a concentration of 50 mM. Ethyl methanesulfonate (EMS) was integrated into this mixture to reach an ultimate concentration of 200 mM. The blend was incubated with kind shaking at 100 rpm and a temperature of 30°C at 20, 40, or 60 (min) intervals. Later, 500 µl of sodium thiosulfate was introduced to prevent and inhibit the reaction of the mutagenic compound. Then, the cells were centrifuged at 5600 × g for 10 min at a cold temperature of 4°C. The cells underwent two wash cycles using the same buffer solution. After that, the cells were resuspended in a phosphate buffer solution and regulated to pH 7 with a concentration of 50 mM.

Decimal mitigation (10^1–10^3) of each sample was precisely dispensed onto MRS agar dishes. The antibiotic discs were selected and calibrated with accurate quantification by micrograms per disc (ten µg Ampicillin, thirty µg Chloramphenicol and Tetracycline, fifteen µg Erythromycin). These discs are meticulously located on the surface of cultured plates, utilizing antiseptic forceps. subsequently, positioned in the aerobic incubator that was calibrated at 37°C for two days. The antibiotic-resistant mutants were prominently manifest from the inhibition zone encircling the discs that were selected and transmitted to MRS dishes for analysis later. The antibiotic sensitivity valuation was performed on the isolated wild-type and the selected mutants to confirm the effectiveness of antibiotics against them before and after EMS-induced mutations. [10, 11]

Fermentation of Sugarcane Juice and Extraction of EPS

A fresh 1 L sugarcane juice obtained from Egyptian Sugar and Integrated Industries Company (ESIIC), Giza, Egypt, was analyzed for pH and Brix before and after the addition of sucrose and the fermentation. Using batch fermentation, 500 ml of sugarcane juice was mixed with a solution containing nutrients estimated by grams per litter for (ten) protease-peptone, (five) beef extract, (five) yeast extract, (two) K_2HPO_4, (two) KH_2PO_4, (two) diammonium hydrogen citrate, (one tenth) $MgSO_4$, (four-tenths) $MnSO_4$, (one) NaCl, and one (ml) Tween80. Additionally, 300 g of sucrose was added to the remaining 500 ml of sugarcane juice, which was sterilized separately to avoid Millard's reaction. After sterilization, the two volumes of sugarcane juice were combined aseptically and distributed into flasks containing 100 ml each.[8,14]

The wild-type L. paracasei SH6 (SH6.WT) fermentation inocula and mutants were first prepared in MRS broth media and kept at 37°C for two days. The visual intensity was modified at 0.8, 650 nm for precise assessment of the premier number of the bacterial population that transferred at a concentration of 10% (v/v) to the fermentation flasks, kept at 30 (°C), shaking at one hundred (rpm) for two-three days.

After completion of incubation, in a water bath with shaking fermented flasks were exposed to 100°C for fifteen minutes to denature and inhibit enzymes that break down polysaccharides. Afterward, the flasks were cooled, and a 15% trichloroacetic acid (TCA) solution was added in equal volume and left for 1–2 hrs to precipitate the maximum amount of proteins. To accomplish the separation, the components concerned were exposed to centrifugation for fifteen minutes at four degrees Celsius with a force of six thousand (xg). The supernatant was gathered for further treatment with a double volume of cold ethanol (96%) and stored overnight in a refrigerator. To precipitate EPS from an ethanol solution has been exposed to six thousand (×g) centrifugation forces for ten minutes and four degrees Celsius. The assembled EPS was dissolved in distilled water, freeze-dried to exclude moisture, weighed with milligrams per one hundred milliliters, and kept in a desiccator at room temperature to optimize the preservation guarantee.[14,16,17]

Analysis of Exopolysaccharides (EPS)

Estimation of TSC (Total Soluble Carbohydrate)

The total soluble carbohydrates assay was measured which procedure was summarized by Gerhardt et al.[18] The aggregate volume of 50 µL of 0.25 mg/ml freeze-dried EPS sample was added to the glass tube. Then, 100 µL of 75% (v/v) absolute sulfuric acid solution was added to start the reaction. Subsequently, in the vial, 200 µL of the anthrone reagent (by disbanding five mg of anthrone per ethanol-one hundred µL) was incorporated with 2.4 ml of the 75% (v/v) sulfuric acid solution, heated at 100 (°C) for five minutes, and permitted to chill within room temperature for five minutes. Then, one hundred µL of the blend was relocated to a 96-well plate for six duplicates when the blend coloration changed to green and the absorbance was measured at 578 nm using a microplate reader (FluoStar Omega) employing glucose standardization.

HPLC Analysis

About five samples of the dried EPS that were segregated from the wild-type and the selected mutants (M1, M4, M7, and M8) underwent HPLC analysis to determine the EPS quantitatively and qualitatively, following the method of Allaith *et al.*[15]

The Assessment of Gene Expression

According to the manufacturer's protocols, the total RNA was carefully extracted from two isolates chosen: the wild-type SH6.WT and the elected mutant (M8). Using the device of the MX3005P QPCR System (Agilent Technologies, USA) and Sybr-green PCR set (Qiagen, Germany), real-time experimental procedures PCR were implemented. The *16S ribosomalRNA* gene (housekeeping control) and *the gtf* gene (target gene of glucansucrases responsible for synthesizing EPS, including dextran or glucan). The primers for *16S rRNA* were forward (5-TCCGGATTTATTGGGCGTAAAGCGA-3) and reverse (5TCGAATTAAACCACATGCTCCA-3).[19] The primers for *the gtf* gene were Forward (5-ACACGCAGGGCGTTATTTTG-3) and Revers (5-GCCACCTTCAACGCTTCGTA-3).[20]

The reaction blend was to a volume of twenty-five µL, with three duplicates for each sample, containing 12.5 µL SYBR Green PCR Master Mix, 0.25 µL of reverse transcriptase, 0.5 µL of each primer (F & R), and 3 µL RNA template. The real-time experimental procedure PCR was performed under the following conditions: Reverse transcription was achieved at 50 (°C) for thirty minutes. Followed by a primary denaturation phase at 94°C for fifteen minutes. Followed by forty cycles were conducted, comprising denaturation at 94°C for fifteen seconds, the ranging of annealing temperatures from 58°C to 60°C for forty seconds, and extension at 72°C for forty seconds. A one-cycle subsequent denaturation phase was at 94°C for one minute, at 58°C to 60°C for one minute for the annealing step, and in the last denaturation at 94°C for one minute. The outcomes were analyzed using Stratagene MX3005P software version 1.8 (USA). To evaluate the gene expression of mutant M8 compared to the wild-type SH6.WT, the gene expression variation levels were accurately assessed by applying the ΔΔCt method, which simplified the computation in the fold modification.[21]

$$\Delta\Delta Ct = \Delta Ct \text{ reference} - \Delta Ct \text{ target}$$
$$\Delta Ct \text{ target} = Ct \text{ control} - Ct \text{ treatment and } \Delta Ct \text{ reference} = Ct \text{ control} - Ct \text{ treatment}$$

Statistical Analysis

The results were expressed as means ± standard error at $P < 0.05$ and analysis of variance (ANOVA) with three duplicates. This statistical analysis was done using XLSTAT statistical software version 2014, 5.03 (Addinsoft, USA).

Results and Discussions

Induction of EMS Mutations for Hyper-EPS Production

Based on the initial screening, strain SH6 was selected for its superior EPS production, yielding 260 mg/100 ml, compared to SH5, which produced 130 mg/100 ml. In this regard, previous research indicates the significance of screening LAB isolates on semi-defined media to enhance β-glucan production as EPS.[15] Ethyl methanesulfonate (EMS) is a widely recognized chemical mutagen that induces random transitional mutations from G/C to A/T.[22] The wild-type isolate L. paracasei SH6 was exposed to EMS to induce random mutagenesis and potentially invigorate hyper-EPS production. Various decimal dilutions were cultivated at multiple time intervals, and the mutants were selected based on their response toward tested antibiotics post-mutation, which served as a preliminary indication for the occurrence of a genetic change. Nine antibiotic-resistant mutants (M1 to M9) were located within the zone of inhibition of tested antibiotic discs: M1 and M5 were within the zone of the Ampicillin disc; M2, M6, and M8 within the Erythromycin disc; M3 and M7 within the Tetracycline disc; and M4 and M9 within Chloramphenicol disc. Notably, the wild-type (SH6.WT) was resistant only to chloramphenicol and sensitive to other antibiotics. However, after EMS exposure, the chloramphenicol-resistant trait was lost, and the sensitivity to different antibiotics was increased by 1 to 3 folds ($P < 0.05$) in comparison to the wild-type (Table 1). Similarly,

Table 1. **Antibiotics response of *L. paracasei* SH6 before and after induction of mutations**

Isolates ID	Antibiotic disc (µg/disc)			
	A	C	E	T
SH6.WT	*16.2 ± 0.5^{f}	R^{h}	8 ± 0.5^{f}	10 ± 0.5^{f}
M1	26.5 ± 1.0^{a}	24 ± 0.5^{a}	23 ± 0.5^{a}	24 ± 0.5^{a}
M2	23 ± 0.7^{c}	15 ± 0.5^{f}	20 ± 0.5^{c}	18.5 ± 0.8^{d}
M3	19.5 ± 0.5^{e}	13 ± 0.5^{g}	18 ± 0.5^{d}	ND
M4	23 ± 0.7^{c}	23 ± 0.5^{b}	22 ± 0.5^{b}	20 ± 0.5^{c}
M5	20 ± 0.7^{d}	17 ± 0.5^{e}	16 ± 0.5^{e}	18 ± 0.5^{d}
M6	ND	15.5 ± 0.5^{f}	ND	14 ± 0.5^{e}
M7	25 ± 0.5^{b}	22 ± 0.5^{c}	23 ± 0.5^{a}	22 ± 0.5^{b}
M8	20.5 ± 0.5^{d}	20 ± 0.5^{d}	22 ± 0.5^{b}	20 ± 0.5^{c}
M9	ND	ND	ND	ND

A, Ampicillin; C, Chloramphenicol; E, Erythromycin; T, Tetracycline; Data represent means ± SE of three duplicates at $P < 0.05$ that are significantly illustrated with small letters (a, b, c, ……. etc); R, resisted (no clear zone); ND: Not detected.

Asgher *et al.*[8] reported improved EPS production in *Bacillus licheniformis* MS3 as a physical mutagen after UV radiation.

Moreover, the viable bacterial cell counts were decreased gradually to non-existent as the exposure time to EMS increased. Notably, no inhibition zone was observed in dilution 10^{-3} of the exposure time 40 and 60 min due to the absence of bacterial growth. Radwan *et al.*[23] explained that the ratio of viable cells of *Streptomyces exfoliates* 15/G710 decreased as the duration of EMS exposure increased, and they proved to have a complete loss of viability after 60 min of exposure. In general, measuring the inhibition zone diameters of different antibiotics in this study indicates mutagenesis occurrence rather than evidence of increased EPS production. Novel LAB mutants with enhanced productivity are often generated using induced mutagenic agents such as EMS, followed by intergeneric protoplast fusion to improve bacteriocin production. Mutant and fusant cells were characterized by their response to various antibiotic discs.[24]

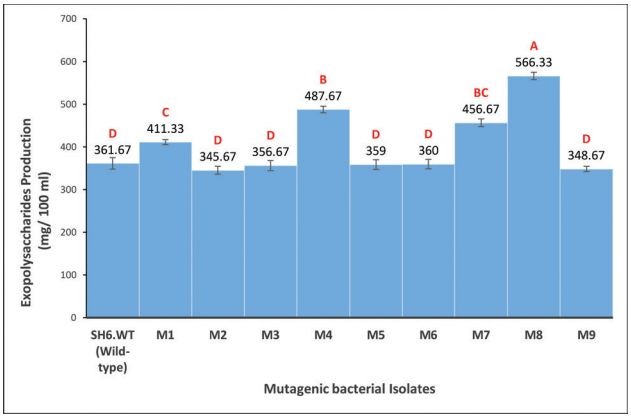

Fig. 1 **Quantity of the produced EPS by *L. paracasei* SH6 (wild-type, SH6.WT) and obtained mutants (nine mutants, M1-M9) after EMS mutagenesis. Data represent means ± SE of three duplicates at $P < 0.05$.**

Evaluation of EMS Mutants

The resulting mutants (nine mutants, M1-M9) from EMS mutagenesis were evaluated based on their EPS yield (mg/100 ml) after cultivation on sugarcane juice medium. Figure 1 shows significant differences among four mutants, i.e., M1, M4, M7, and M8. Mutant M8 exhibited the highest EPS production, reaching 566 mg/100 ml, followed by M4, M7, and M1, which were 488, 457, and 411 mg/100 ml, respectively. Conversely, the wild-type and remaining mutants produced EPS levels ranging from 362 to 346 mg/100 ml.

In a related study, the creation of EPS by Bacillus licheniformis MS3 was increased to 4.6 g/L after UV radiation for 60 min, compared to 3.4 g/L produced by the wild-type.[8] Additionally, EMS was used as a chemical mutagen to modify the genetic material of two LAB strains, followed by intergeneric protoplast fusion to boost the production of bacteriocins.[24] Furthermore, mutant EMS7 showed a significant 1.96-fold increase in proteolytic activity compared to the wild-type after EMS exposure.[23] Generally, the observed hyper-production EPS by mutants M1, M4, M7, and M8 can be attributed to transitional mutations. These mutations, a type of substitution mutation, involve alteration in a single codon (where a purine base is substituted for another purine or a pyrimidine base for another pyrimidine). This substitution alters the native amino acid with a mutant one, which subsequently impacts the organism's phenotype and productivity.[22]

The sugarcane juice here was assessed immediately for pH and Brix° values and after sucrose addition and fermentation. The initial values were pH 5.2 and Brix° 22°. The sucrose addition increased the Brix° to 40°, while fermentation decreased the pH to 3.0 for all treatments compared to pH 4.0 of the control. Previous studies reported a similar starting pH of 5.5.[14] The sugar concentration in sugarcane molasses, a fermentation medium used for EPS production from various LAB species, was estimated at 40° Brix°.[15] In this study, the EPS yield of the wild-type SH6. WT was relatively low, reaching 260 mg/100 ml when sugarcane juice was supplemented only with sucrose crystals. However, when the medium was fortified with additional nutrients and mineral elements along with 300 g/L sucrose, as mentioned above, the EPS yield of the same strain doubled to 360 mg\100 ml. The EPS production by *L. confusus* TISTR 1498 was improved under optimal conditions to the predicted level of EPS of 107.5 g/L using sugarcane juice supplemented with 300 g/L of crystallized sucrose.[14] Similarly, mutant *B. licheniformis* MS3 exhibited a third-fold increase, from 3.4 to 4.6 g/L, when cultured on a fermentation medium composed of mango peels supplemented with various ratios of yeast extract, beef extract, KH_2PO_4, K_2HPO_4, $MgSO_4$, NaCl, $FeSO_4$.[8] The EPS production in the present study was carried out in a shaking incubator at 30°C and one hundred rpm for three days. Previous research revealed that 30°C incubation with shaking enhanced the EPS yield by *Lacticaseibacillus* sp.[25]

TSC Estimation of Best Mutants

The total soluble carbohydrates (TSC) content in EPS by the elected mutants (M1, M4, M7, and M8) was estimated and compared with the wild-type (SH6.WT) as indicated in Figure 2. The results revealed significant differences among the means of the selected mutants, which were 4.6, 6.2, 4.4, and 10.7 g/L, respectively, compared to the wild-type, which exhibited a TSC of 3.10 g/L. Notably, mutant M8 demonstrated the highest TSC content. This increase in carbohydrate production can be attributed to EMS mutagenesis, which likely enhanced the overall carbohydrate content, particularly in mutant M8. Similarly, the highest amount of EPS observed in a mutant of *Bacillus licheniformis* MS3 reached 4.6 g/L with 4.69% reducing ends.[8] Another study estimated the percentage of total carbohydrates in EPS produced from *L. paracasei* after being grown in chemical-define media containing 5% glucose and other carbohydrates.[26] In addition, Allaith *et al.*[15] measured the total carbohydrate percentage in EPS extracted and partially purified from four LAB strains.

Determination of EPS as a Dextran by HPLC

The dextran content in EPS of mutants (M1, M4, M7, M8) and the wild-type (SH6.WT) was determined quantitatively and qualitatively by HPLC, see Figure 3 and Table 2. As shown, M8 exhibited the highest dextran content in EPS, reaching 7.8 ± 0.2%, followed by M4, M1, and M7 with 4.4 ± 0.1%, 4.2 ± 0.12%, and 3.6 ± 0.11%, respectively. In contrast, the dextran content in EPS of wild-type strain SH6.WT was 3.3 ± 0.11 %. The retention time for mutants and wild-type EPS ranged from

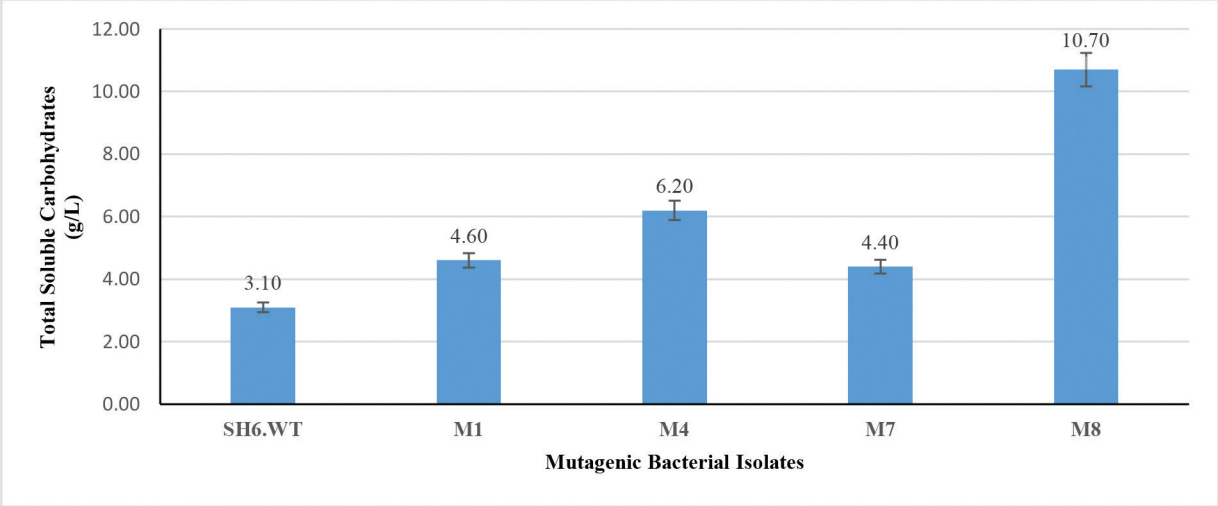

Fig. 2 **Total soluble carbohydrates (g/L) content of EPS of *L. paracasei* SH6 (wild-type, SH6.WT) and the best mutants of EMS mutagenesis (M1, M4, M7, and M8). Data represent means ± SE of three independent experiments.**

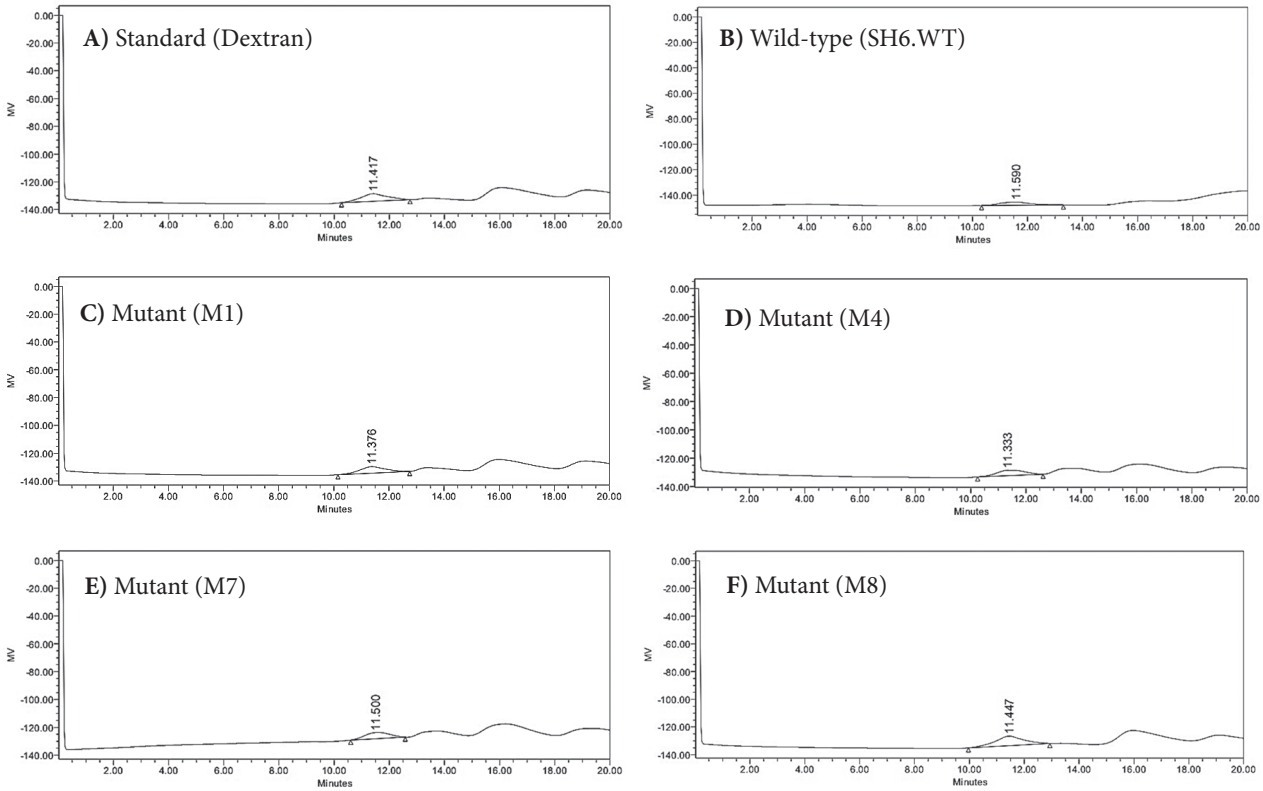

Fig. 3 **HPLC analysis for extracted EPS from selected mutants and wild-type of *L. paracasei* SH6, using dextran as a standard.**

Table 2. **The quantity of dextran (%) in extracted EPS from selected mutants and wild-type of *L. paracasei* SH6.**

	Code	Estimated EPS as a dextran (%)
Wild-type	SH6.WT	3.3 ± 0.10^{e}
Mutants	M1	4.2 ± 0.12^{c}
	M4	4.4 ± 0.12^{b}
	M7	3.6 ± 0.11^{d}
	M8	7.8 ± 0.2^{a}

Data represent means ± SE of three duplicates at $P < 0.05$ that are significantly illustrated with small letters (a, b, c, d, and e).

11.333 to 11.590 min, similar to the standard dextran with a retention time of 11.417 min. Previous studies have identified dextran as a major component of EPS from *L. kunkeei* AK1 and *Leuconostoc citreum* HE29, at a concentration of 4.78% and 5.3%, respectively, as determined by HPLC and NMR.[27,28] Additionally, the EPS from *L. paraplantarum* KM1 was determined by HPLC to consist of monosaccharides such as glucose, galactose, and mannose.[29] Furthermore, quantitative and qualitative analyses via HPLC have revealed that the EPS from certain LAB strains is β-glucan.[30,15]

Expression Analysis of Mutant M8 by qRT-PCR

Based on the results obtained, mutant M8 was selected as the superior strain for EPS production, yielding 566 mg/100 ml. This mutant was subsequently subjected to gene expression analysis for the glucosyltransferase (*gtf*) gene, which is involved in EPS production of certain LAB strains, to evaluate the impact of EMS mutagenesis on expression levels of this gene and its correlation with the observed increase in EPS production. The *gtf* genes encode enzymes known as glucansucrases, which are involved in synthesizing high molecular weight α-glucan from sucrose in various LAB strains. The α-glucan is a typical example of EPS, categorized mainly into four types based on their significant linkage, such as dextran, which has α-(1→6) linkage between glucopyranosyl units.[31,32]

Figure 4 and Table 3 present the qRT-PCR results for expression analysis of the *gtf* gene in mutant M8 and the wild-type SH6. WT, as well as the housekeeping control, 16S rRNA gene expression. The results confirmed a significant enhancement in EPS production in mutant M8, as verified by a 7.0128-fold rise in expression of the *gtf* gene compared to the wild type and 16S rRNA gene.

The expression analysis of the *gtf* gene has been utilized in numerous studies regarding EPS production.

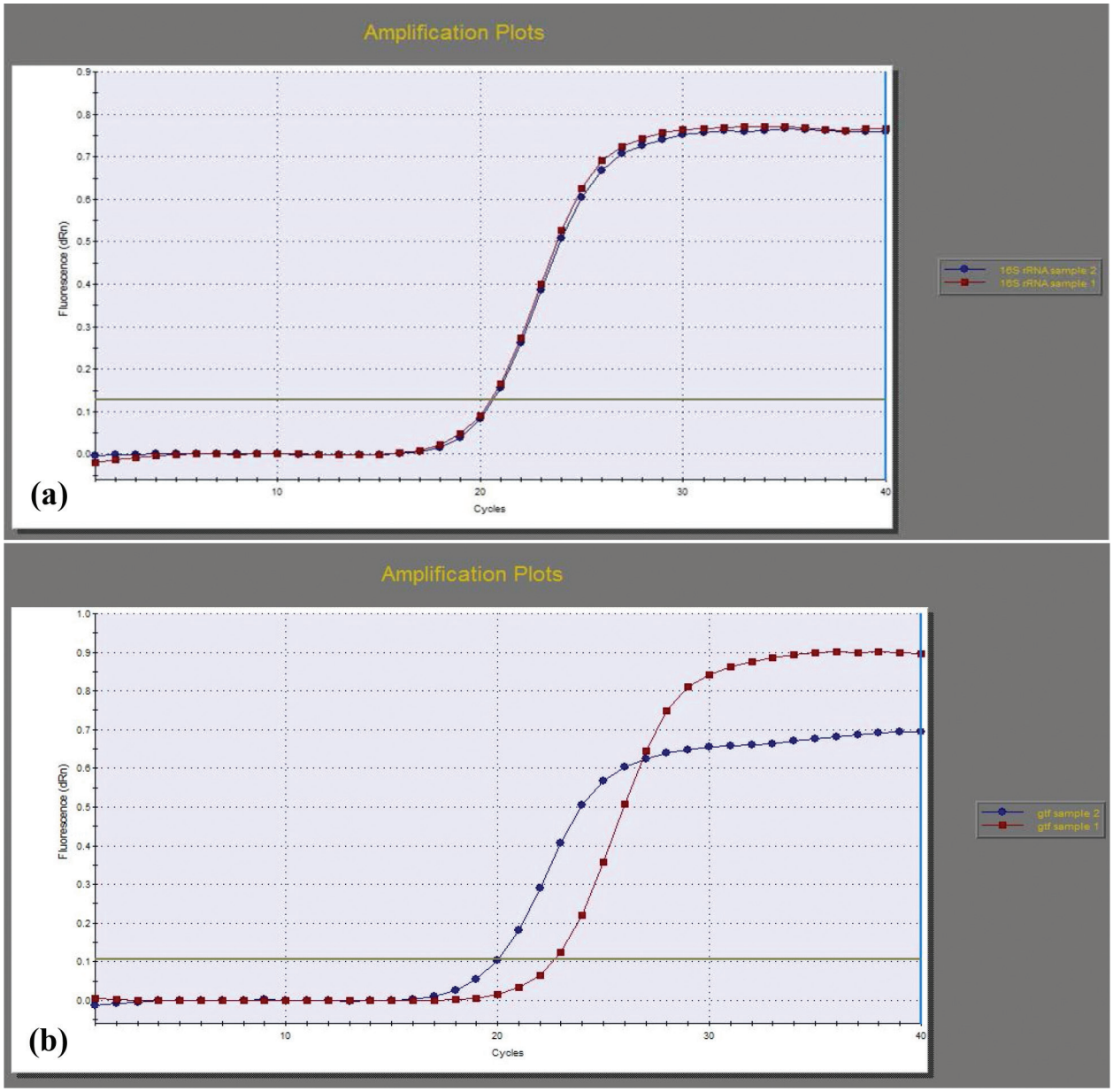

Fig. 4 **Amplification plots of qRT-PCR reactions for housekeeping control (a) 16S ribosomal RNA gene that is targeted glucantransferase; (b) gtf gene; red line for the wild-type *L. paracasei* SH6; blue line for the mutant M8.**

Table 3. **Expression analysis of the *gtf* gene in mutant M8 and wild-type strain of *L. paracasei* SH6**

	Code	Housekeeping control *16S rRNA gene* *Mean Ct	Target gene *gtf gene* Mean Ct	Fold change in level of expression ($P < 0.05$)
Wild-type	**SH6.WT**	22.19	20.05	$1 \pm 0.000^{b**}$
Mutants	**M8**	20.14	20.81	7.0128 ± 0.0001^{a}

Data represent means ± SE of three duplicates at $P < 0.05$ that are significantly illustrated with small letters (a, and b).

For instance, beta-glucan formation in four native LAB isolates and *L. rhamnosus* NRRL 1937 (LGG) was evaluated using the *gtf* as a target gene and the16S rRNA as a control. The results of this analysis for the four strains (B6, C9, A3, and D6) were 14.7230-, 10.1730-, 8.6139-, and 0.8566-fold, respectively.[15] In a similar context, expression analysis of *gtf* genes in *Streptococcus mutans* demonstrated the crucial role of both soluble and insoluble polysaccharides of *L. paracasei* ET-22 in inhibiting biofilm formation.[33] Wasfi *et al.*[34] reported a significant drop in the expression of gtfB, gtfC, and gtfD genes participatory in EPS formation by S. mutans in the manifestation of four supernatants from different Lactobacillus Sp.

The results obtained here from expression analysis of the *gtf* gene, which showed a seven-time increase in mutant M8 and the observed increase in EPS production, provide insight into the effects of EMS on bacterial cells. This enhancement, in comparison to the wild-type SH6.WT is likely a direct consequence of the genetic alterations induced by the chemical mutagen EMS. This follows those reported by Radwan *et al.*,[23] Bazaraa *et al.*,[24] Singh and Sharma,[35] Khattab *et al.*,[36] Jung *et al.*,[37] and Mukherjee *et al.*[38] However, the current study is the first to apply EMS mutagenesis on *the L. paracasei* strain for EPS hyper-production and to examine the improved mutant by gene expression analysis.

Conclusion

This study highlights the increased EPS productivity of the *Lacticasibacillus paracasei* SH6 (SH6.WT) strain through exposure it to the EMS mutation. This progress underscores the exciting potential of genetic modification to improve microbial production, seek other factors, and audition various carbon sources to elevate EPS production. For the first time, the EMS-induced mutation in *Lacticasibacillus paracasei* SH6 strain has been demonstrated to improve EPS production, reaching 566 mg/100 ml for the M8 mutant compared to the wild-type (SH6.WT), which produced 362 mg/100 ml using fermented sugarcane juice medium. The results of the total soluble carbohydrates content, HPLC estimations, and gene expression outcomes confirmed the M8 mutant that was elected as the most efficient, reaching 10.70 g/L and 7.8% and 7-fold, respectively.

Also, the study emphasizes that sugarcane juice can be an economical medium that congresses the biological demands for improving EPS production and can be commercially used in various foods, pharmaceuticals, and probiotic industries. Subsequently, bioreactors can be used for EPS production in large quantities of the M8 mutant of L. paracasei SH6. The biotechnology application is promising, can quickly bridge the gap in industry sectors, and can be transmitted from laboratory-scale to industrial-scale for EPS synthesis. The authors recommend further guidance, involving useful other sources of EPS production and utilizing mutation-consolidated and optimized strategies with Lactobacilli species to maximize the productivity of microbial EPS.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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