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Phytase superior *Lactobacillus* isolate (L-Phy5) and its mutants: molecular identification

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Background
Phytase produced by lactic acid bacteria (LAB), which are classified as generally recognized as safe (GRAS), is used in food processing. Phytase produced by *Lactobacillus plantarum* and *acidophilus*, degrades phytic acid, a common anti-nutrient in plant-based foods, offers several key benefits, in food to improve nutrient bioavailability. Phytic acid binds essential minerals like iron, zinc, and calcium, reducing their availability for absorption. Phytase helps release these bound minerals, for absorption in the gut.

Objective
The present study aimed to isolate a probiotic strain that excels in phytase synthesis, identify it at the molecular level, and assess its efficacy as a probiotic. Mutagenesis and protoplast fusion are used in a genetic improvement program to create mutants and fusants with superior enzyme output.

Materials and methods
Different isolates of *Lactobacillus* were isolated from probiotic-rich sources (cheese and yoghurt, from Jeddah, KSA), examined for phytase synthesis and the good candidate was identified using 16SrRNA. To enhance its phytase production, genetic improvement was achieved through physical mutagenesis using ultraviolet (UV) radiation and chemical mutagenesis with ethyl methane sulfonate (EMS). The protoplast fusion between two mutants (PUV12-10 and PMS60-5) was performed to enhance phytase production. The molecular diversity induced by EMS and UV mutagenesis, as well as protoplast fusion in *Lactobacillus reuteri* Pro8 (L-Phy5), was determined using RAPD- PCR (Random Amplification of Polymorphic DNA Polymerase Chain Reaction) analysis with 4random primers.

Results and conclusion
Seven *Lactobacillus* isolates (L-Phy1- L-Phy1-7) were isolated with L-Phy5 identified as *Lactobacillus reuteri* strain Pro8 (PV448931.1), a good candidate for phytase synthesis. Strong probiotic potential, implying its feasibility for incorporation into functional food items. To enhance its phytase production, a genetic improvement (UV) radiation program and EMS were used. Both UV-treated (PUV12-10) and EMS-induced (PMS60-5) mutants exhibited 258.76% and 287.53% phytase increase, respectively compared to the parent strain. The protoplast fusion between PUV12-10 and PMS60-5successfully yielded the fusant (PC1/9) as a promising candidate for further development in phytase-based biotechnological applications. The molecular diversity induced by EMS and UV mutagenesis, as well as protoplast fusion in *Lactobacillus reuteri*Pro8 (L-Phy5) was analyzed using RAPD analysis. Distinct banding patterns were observed among EMS-induced mutants, recombinant fusants, and the parental strain. RAPD-based phylogenetic analysis revealed distinct genetic relationships among *Lactobacillus reuteri*L-Phy5, its mutants, and fusants.

Keywords: *Lactobacillus reuteri*, Genetic Improvement, Phytase Production, Mutagenesis, Protoplast Fusion, Ultraviolet (UV) Radiation, Ethyl methane sulfonate, Phylogenetic analysis, Random Amplification of Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR).

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Introduction

Using lactic acid bacteria (LAB) for phytase production offers several key benefits, especially in food and feed applications aimed at improving nutrient bioavailability and supporting gut health. LAB, such as *Lactobacillus plantarum* and *Lactobacillus acidophilus*, synthesize phytase enzymes capable of degrading phytic acid, a common anti-nutrient in plant-based foods. Phytic

acid binds essential minerals like iron, zinc, and calcium, reducing their availability for absorption. LAB-producing phytase helps release these bound minerals, making them more bioavailable for absorption in the gut [1]. Moreover, LAB are naturally beneficial for gut health, as they produce lactic acid, which helps maintain a healthy gut pH and supports beneficial microbiota. LAB with phytase activity offers a dual benefit by aiding in mineral absorption, naturally reducing phytic acid in grains, legumes, and cereals, enhancing the nutritional profile without needing additional processing steps, and promoting a balanced gut microbiome, making them especially valuable in functional foods [2]. By improving the digestibility of phytate phosphorus in animal feed, LAB-produced phytase reduces the need for inorganic phosphorus supplements. This not only lowers feed costs but also decreases phosphorus excretion in animal waste, reducing environmental pollution and contributing to more sustainable animal agriculture [3].

Extracellular phytase production by probiotics is valuable for food, feed, and pharmaceutical applications due to its ability to break down phytate outside the cell, making essential minerals more accessible. The best probiotics for extracellular phytase production tend to have high enzyme activity in extracellular environments and resilience in various gastrointestinal and food processing conditions. *L. plantarum* has demonstrated good extracellular phytase activity, particularly in fermented foods and plant-based matrices. As a versatile and safe probiotic, it works well in acidic environments, helping to reduce phytate in foods, especially grains and legumes, thus making minerals more bioavailable. This trait is particularly valuable in fermented foods where extracellular phytase can directly reduce anti-nutrients in the product itself. Furthermore, *L. reuteri* is known for both its probiotic benefits and extracellular phytase production. This strain is used in dietary supplements and has applications in improving mineral bioavailability in the gastrointestinal tract. It can degrade phytate effectively outside the cell, which enhances mineral absorption in the gut environment, especially when administered as part of a probiotic blend [4, 5]. Recent advances in mutagenesis of probiotic strains, particularly for phytase production, focus on improving enzyme yield and enhancing the probiotic's overall effectiveness in phosphorus availability. Mutagenesis techniques, including ultraviolet (UV) exposure and ethyl methane sulfonate (EMS), have been widely applied to probiotics like *Lactobacillus* and *Lactococcus lactis*. These approaches increase the breakdown of phytate, which is a primary phosphorus source in grains but is indigestible for many animals [6-8]. UV mutagenesis has been effectively used to enhance phytase production in various microbial strains, including *Lactobacillus* species. The process involves exposing microbial cultures to UV radiation, which induces random mutations that may enhance the strain's ability to produce higher phytase levels. By applying UV mutagenesis, researchers have successfully obtained mutant strains with improved enzyme activity and stability. Ethyl methane sulfonate (EMS) is an effective mutagen used to enhance phytase production in *Lactobacillus* strains. EMS induces random mutations by alkylating guanine bases in DNA, leading to point mutations. These mutations can result in improved enzyme activity or production levels. For example, applying EMS mutagenesis to *Lactobacillus* strains has shown success in enhancing enzyme secretion, including phytase, which breaks down phytic acid, improving nutrient absorption in feed applications. In some studies, EMS mutagenesis led to mutants with up to 60% higher enzyme production compared to wild strains. The procedure involves exposing cultures to different concentrations of EMS to achieve optimal lethality rates (for example, a lethality of 93.6% was recorded with 2% EMS). This method has proven efficient in increasing enzyme activity while maintaining genetic stability over multiple subcultures. By optimizing conditions, such as the concentration of mutagen and exposure time, researchers can significantly improve the production capabilities of *Lactobacillus* strains, making EMS a valuable tool for developing high-phytase-producing probiotics for the food and feed industries [9-11].

The present study aimed to isolate a probiotic strain that excels in phytase synthesis, identify it at the molecular level, and assess its efficacy as a probiotic. Mutagenesis and protoplast fusion were used in a genetic improvement program to create mutants and fusants with superior enzyme output.

Materials and methods

Isolation and screening of probiotic strains exhibiting phytase enzyme activity

To isolate probiotic strains with phytase activity, samples were collected from probiotic-rich sources such as cheese and yoghurt, from Jeddah, KSA. Serial dilutions of the samples were performed, and they were plated on a suitable selective medium, such as MRS agar, to encourage the growth of probiotic bacteria. The plates were incubated under appropriate conditions, such as anaerobic or microaerophilic environments, depending on the target probiotics. The isolated colonies were tested for phytase activity by culturing them on a phytase screening medium (PSM) that included sodium phytate as the substrate [12]. Colonies that showed

clear zones of phytase activity were selected for further characterization and confirmation using quantitative assays, such as measuring released inorganic phosphate.

Phytase production

Two different fermentation media were utilized for phytase production. The first was a modified MRS broth, known as MRS-MOPS, where inorganic phosphate (KH_2PO_4) was replaced with 0.65 g/L of sodium phytate. To ensure pH stability and promote optimal enzyme function, the medium was buffered with 0.1 M MOPS [3-(N-morpholino) propane sulfonic acid]. To decrease residual phosphate levels and improve phytase production, the amounts of yeast extract, beef extract, and glucose were adjusted to 2g/L, 4 g/L, and 10 g/L, respectively. The MRS-MOPS medium was then inoculated with 10% (v/v) of a 48-hour-old bacterial culture (approximately 10^8 CFU/mL) and incubated anaerobically at 37 °C. The second medium, referred to as Phytase Screening Medium (PSM), contained 0.1% sodium phytate, 1.5% glucose 0.2 % ammonium nitrate (NH_4NO_3), 0.05% potassium chloride (KCl), 0.05% magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 0.03% manganese sulfate ($MnSO_4$), and 0.03% ferrous sulfate ($FeSO_4 \cdot 7H_2O$), with the pH adjusted to 7.5 [12].

Phytase assay

Phytase activity was assessed by quantifying the inorganic phosphate liberated from sodium phytate. The assay mixture consisted of 800 µL of a 1% (w/v) sodium phytate solution prepared in 0.2 M sodium acetate buffer at pH 5.5, combined with 200 µL of the crude extracellular enzyme extract. The enzymatic reaction was carried out at 37 °C for 1 hour and then halted by subjecting the mixture to 100 °C for 10 minutes. Phosphate released during the reaction was measured spectrophotometrically by mixing 100 µl of the reaction solution with 900 µl of a colorimetric reagent containing 1.0 M sulfuric acid, 10% ascorbic acid, and 2.5% ammonium molybdate in a volumetric ratio of 3:1:0.1. The resulting mixture was incubated at 50 °C for 20 minutes, and absorbance was measured at 820 nm. A calibration curve was generated using potassium dihydrogen phosphate (KH_2PO_4) as the standard. One unit of phytase activity was defined as the quantity of enzyme that liberates 1 µmol of inorganic phosphate per minute under the assay conditions [13].

Evaluation of *Lactobacillus* isolate (L-Phy5) for its probiotic efficacy

Acid tolerance

The probiotic strain was initially cultured in MRS broth at 37°C for 24 h. After incubation, 0.1 mL from each culture was inoculated into 10 mL of MRS broth and adjusted to a pH of 2. These samples were then incubated at 37°C for 5 h. Bacterial growth was assessed by recording optical density at 600 nm with a UV-visible spectrophotometer.

Bile salt tolerance

Bile salt tolerance was assessed by directly plating the samples onto MRS agar containing 1.0% Oxgall, followed by anaerobic incubation at 37°C for 24 h. Control plates, which did not contain bile salts, were also prepared, and incubated under identical conditions. The presence of colony formation on the bile-containing plates demonstrated the strain's resistance to bile salts.

Hydrogen peroxide tolerance

An overnight culture of the isolated probiotic strain was grown in MRS broth and then divided into two groups. The control group consisted of 100 mL of the probiotic culture without any additives. In the test group, 230 µL of a 3% (882 mM) hydrogen peroxide solution was added to 100 mL of the culture, resulting in a final concentration of 2 mM hydrogen peroxide. The samples were maintained at 37°C for 3 h. Following incubation, the extent of bacterial growth was determined by recording the absorbance at 600 nm with a UV-visible spectrophotometer.

Lysozyme resistance

To assess lysozyme resistance, bacterial cells were suspended in 10 mL of sterile saline. For the control group, the suspension was centrifuged at 4,500 ×g for 10 minutes. A separate sample was prepared with the addition of lysozyme at a concentration of 200 µg/mL, followed by (4,500 ×g for 10 min. centrifugation. Both treated and untreated samples were then incubated at 37°C for 16 h. The growth of bacteria was monitored by recording the absorbance at 600 nm.

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The antioxidant activity of the probiotic strain was assessed based on its ability to scavenge DPPH (2,2-diphenyl-1-picrylhydrazyl) free radicals. Cultures (0.1 mL) grown for 24 h in MRS broth at 37°C were mixed with 3.9 mL of a 20 mM DPPH solution. The solution was incubated in the dark at 25°C for 30 minutes. Absorbance was then recorded at 517 nm using a UV-visible spectrophotometer. A control containing only ethanol and DPPH solution was used to calculate scavenging activity as follows: Scavenging activity (%) = $[(A_{blank} - A_{sample}) / A_{blank}] \times 100$

Molecular identification of phytase superior isolate (L-Phy5)

To molecularly identify a superior phytase-producing probiotic strain, genomic DNA was isolated from the selected strain using a reliable DNA extraction protocol. The 16S rRNA gene, a highly conserved region, was amplified through PCR using universal primers: forward primer-5'-CGGGATCCAGAGTTTGATCCTGGTCAGAAC GAACGCT-3' and reverse primer-5'-CGGGATCCTACGGCTACCTTGTTACGACTTC ACCCC-3'. The amplified product was sequenced and compared against reference sequences in NCBI BLAST database to confirm the strain's identity.

Ultraviolet radiation mutagenesis of *Lactobacillus* (L-Phy5)

To perform UV mutagenesis in probiotics, the probiotic strain was first grown to the exponential phase, where cells were metabolically active. The culture was then exposed to a UV light source, typically at a wavelength of 254 nm, for a controlled duration (0 to 12 min), with the exposure time and distance (20 cm) adjusted to regulate the intensity and mutation rate. After UV treatment, the cells were promptly plated or allowed to recover in a growth medium to repair some of the UV-induced damage. Finally, the treated cells were screened for desired mutants exhibiting target traits, such as enhanced enzyme production or improved acid tolerance. For survival analysis, the treated cells were serially diluted, plated on agar plates, incubated, and the resulting colonies were counted to calculate the survival percentage using the formula: Survival Percentage=
(CFU after UV treatment / CFU of untreated control) X 100.

Ethyl methane sulfonate mutagenesis of *Lactobacillus* (L-Phy5)

To perform EMS mutagenesis of a probiotic strain, the bacterial culture was first grown to the mid-logarithmic phase to ensure active metabolism, followed by centrifugation and resuspension in a suitable buffer, such as phosphate-buffered saline (PBS). EMS was then added to the suspension to achieve a final concentration, typically between 1–5% (v/v) and incubated at 30–37°C for 30 minutes to 2 h, depending on the strain's tolerance. The reaction was quenched by adding a neutralizing agent like 5% sodium thiosulfate, which inactivated EMS and prevented further mutagenesis. The cells were washed multiple times to remove residual EMS, resuspended in fresh medium, and allowed to recover under optimal growth conditions. For survival analysis, the treated cells were serially diluted, plated on agar plates, incubated, and the resulting colonies counted to calculate the survival percentage using the same formula as for UV-mutagenesis. Observations typically showed that high EMS concentrations or prolonged exposure reduced survival rates to below 1% due to lethal mutations.

Protoplast fusion

Protoplast formation buffer

The buffer used for protoplast formation (PB), as described by [14], was composed of 1 mM $MgCl_2$, 300 mM raffinose, 0.5% gelatin, and 20 mM HEPES, adjusted to pH 7.0.

Lactic acid bacteria protoplasting medium

The composition of the protoplasting medium used for *Lactobacillus* strains was based on a modified protocol reported by Efthymiou and Hansen [15]. The medium contained (per liter): 20 g glucose, 10 g trypticase peptone, 5 g yeast extract, 3 g tryptose, 3 g dipotassium phosphate (K_2HPO_4), 3 g monopotassium phosphate (KH_2PO_4), 2 g ammonium citrate, 1 g sodium acetate, 1 g Tween-80, and 0.2 g L-cysteine hydrochloride monohydrate. The pH was adjusted to 6.8. Furthermore, 5 mL of a mineral supplement per liter was added, comprising (per 100 mL): 11.5 g magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 1.68 g ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), and 2.4 g manganese sulfate monohydrate ($MnSO_4 \cdot H_2O$).

Protoplast regeneration medium and fusion protocol

The protoplast regeneration medium (PRM) was prepared using LCM as the base, excluding Tween 80 from the formulation. Additives included 1% glucose, 0.5% bovine serum albumin (BSA), 2.5% gelatin, 2.5% magnesium chloride ($Mg\ Cl_2$), 25 mM calcium chloride ($Ca\ Cl_2$), 300 mM raffinose, and 2% agar. To begin the preparation, raffinose and gelatin were dissolved in a portion of the Tween 80-free LCM under constant stirring and mild heating at 50 °C using a water bath. Once dissolved, agar was added, and the mixture was autoclaved at 121 °C for 15 minutes. In parallel, glucose, BSA, and the salts were dissolved in the remaining LCM and sterilized through a 0.45 µm membrane filter. These components were then combined with the molten agar solution at 50 °C before being dispensed into plates or containers.

Selection of parental strains for fusion

To determine suitable parental strains for protoplast fusion, an antibiotic susceptibility assay was performed using a range of antibiotic discs: tobramycin (10 µg), chloramphenicol (30 µg), gentamycin (10 µg), colistin (25 µg), doxycycline

(30 µg), rifampicin (30 µg), and streptomycin (10 µg). The results guided the selection of appropriate strain combinations for crossing.

Protoplast fusion procedure

The fusion protocol was based on the method outlined by Chassy [16], where overnight cultures of each parental strain were cultivated in MRS broth at 37°C. Subsequently, 1.5 mL of each culture was transferred into 50 mL of LCM medium supplemented with 0.1% glucose, 1% glycine, and 20 mM threonine, and incubated at 37 °C for 4 h. Cells were harvested, washed with phosphate buffer (PB), and treated with a lytic enzyme mixture containing 400,000 U of lysozyme and 50 U of mutanolysin in PB to facilitate protoplast formation, which was monitored microscopically. Within one hour, protoplasts formed and were collected by centrifugation (4,000 × g, 10 min, 4 °C), and resuspended in PB. Equal volumes of protoplast suspensions from two parental strains were combined, centrifuged, and the pellet was re-suspended in 1 mL PB containing 100 µL of 40% polyethylene glycol (PEG 6000) for 6 minutes to induce fusion. The mixture was centrifuged again, and the pellet was re-suspended in 3 mL PB. A volume of 0.5 mL of this protoplast mixture was combined with 5 mL of soft PRM (0.4% agar) at 45 °C, plated on solid PRM plates, and incubated at 37 °C. Antibiotic discs were placed on the plates, which were incubated for three days to allow for the selection of resistant fusants. Colonies that developed in the presence of antibiotics were separated, re-cultured, assessed for antimicrobial properties, and preserved at 4 °C.

Fingerprinting of mutants and fusants

To perform RAPD fingerprinting of mutants and fusants, a DNA extraction was first carried out from the mutant and fusant strains. After extracting the DNA, a PCR reaction was set up using random primers (Table 1), typically 15-mer sequences, to amplify the genomic DNA. The sequences for the three primers are as follows: first primer (P1) 5′-GGG GTT TGC CAC TGG-3', second primer (P2) 5'-CAT ACC CCC GCC GTT-3', and third primer (P3) 5'-GTG TAC GTG TCC ACT-3', and fourth primer (P4) 5′-TGA GTG GTC TAC GTG-3'. The PCR conditions, including the annealing temperature and cycle number, were optimized for each specific sample. The resulting amplification products, which varied in size due to genetic differences, were then separated by agarose gel electrophoresis, allowing the generation of distinct band patterns. These banding patterns, or "fingerprints," were analyzed to identify polymorphisms between the mutants, fusants, and the parental strains. The comparison of RAPD profiles revealed genetic diversity and potential variations in the genomic structure, which are valuable for distinguishing and characterizing the mutant and fusant strains in relation to the original strain (L-Phy5).

Statistical analysis

The data were analyzed using one-way ANOVA with Costat software [17]. Results were presented as mean values ± standard deviation, with all experiments conducted in triplicate.

Results and discussions

Evaluation of phytase activity secreted by lactic acid bacteria

Evaluating lactic acid bacteria (LAB) for their ability to produce extracellular phytase is a crucial step in selecting strains that can degrade phytate, a plant-based form of phosphorus storage, and release bioavailable phosphate. This ability holds significant potential for developing phytase-producing probiotics. Such probiotics can play a vital role in enhancing mineral bioavailability, including calcium, zinc, and iron, thereby benefiting both animal nutrition and human health. Data in Table 1 provided a comparative overview of phytase production among seven *Lactobacillus* isolates (L-Phy1 to L-Phy7) collected from Jeddah, KSA, grown in two fermentation media MRS-MOPS and PMS (Phytase Modified Medium). Across all isolates, phytase production was consistently higher in PMS, indicating its superior suitability for enzyme biosynthesis. The most productive strain, L-Phy5, showed a notable increase from 564.5 U/mL in MRS-MOPS to 673.8 U/mL in PMS, while other strains such as L-Phy4, L-Phy6, and L-Phy3 also exhibited significant gains, ranging from approximately 10 to 30%, likely due to PMS's optimized nutrients composition, including phytate as an inducer and effective buffering systems. Among the isolates, L-Phy5 emerged as the top phytase producer, followed by L-Phy6 and L-Phy4, making them promising candidates for future enhancement. In contrast, L-Phy1 and L-Phy2 consistently showed the lowest activity, suggesting limited potential or adaptability. Interestingly, L-Phy7 demonstrated a marked improvement in PMS, reaching 212.7 U/despite moderate performance in MRS-MOPS, underscoring the critical influence of culture conditions. These findings emphasize that both genetic potential and fermentation environment significantly impact phytase yield, and they support the selection of a high-yielding isolate like L-Phy5 for further development through mutagenesis or protoplast fusion strategies. The seven LAB strains used in this investigation were obtained from cheese and yoghurt. A previous study showed that

phytase activity differs among lactic acid bacteria (LAB) strains depending on their origin. For instance, Karaman et al. [18] identified 49 LAB and 53 yeast strains from Turkish sourdough, reporting phytase activities ranging from 703 to 1154 U/mL for LAB and 352 to 943 U/mL for yeast. LAB phytases were traditionally characterized by intracellular or cell-bound activity [18, 19]. Interestingly, the majority of strains exhibited increased extracellular activity, consistent with the findings of Nuobariene et al. [20], who also identified both types of phytase activity in several LAB strains, *Lactobacillus panis*, *L. reuteri*, *L. fermentum*, and *Pediococcus spentosaceus*, derived from Lithuanian sourdough.

Table 1 Phytase production of the original isolates obtained from Jeddah cultured in two different fermentation media.

Isolate code no	Phytase production in MRS-MOPS medium*	Phytase production in PMS medium*
Lactobacillus isolate L-Phy1	138.6^{*k}±0.03	199.4^{i}±0.03
Lactobacillus isolates L-Phy2	86.2^{m}±0.03	106.5^{l}±0.03
Lactobacillus isolates L-Phy3	177.5^{j}±0.03	208.6^{h}±0.06
Lactobacillus isolates L-Phy4	294.3^{f}±0.03	324.9^{e}±0.03
Lactobacillus isolates L-Phy5	564.5^{b}±0.03	673.8^{a}±0.03
Lactobacillus isolates L-Phy6	395.8^{d}±0.03	407.3^{c}±0.04
Lactobacillus isolates L-Phy7	176.1^{j}±0.03	212.7^{g}±0.05

* Units.mL^{-1}

Evaluation of a potential probiotic L-Phy5 strain

Exploring natural environments for novel probiotic strains remains a crucial focus in microbiological, enzymes production and health-related research. Probiotics are beneficial microorganisms known for supporting human health, and current studies are dedicated to isolate promising candidates from diverse natural sources. This study aimed to identify bacterial strains capable of withstanding challenging environmental conditions. Among the isolates, a strain designated as L-Phy5 was selected for phytase production. Its ability to tolerate stress was assessed through various tests as shown in Figure 1, including exposure to acidic conditions (pH 2 for 5 h), bile salts (1% for 24 h), hydrogen peroxide (2 mM for 3 h), lysozyme (200 µg/mL for 16 h), and its antioxidant potential measured by DPPH scavenging activity. The probiotic strain L-Phy5 demonstrates strong stress tolerance and functional attributes, making it a promising candidate for probiotic applications. It exhibited high acid tolerance with a 78.26% survival rate at pH 2, indicating its potential to withstand the harsh gastric environment and reach the intestines intact. Similarly, it showed a 75.63% survival rate in the presence of 1% bile salts over 24 h, suggesting good resilience in the intestinal tract. Notably, L-Phy5 displayed the highest survival rate (82.50%) under oxidative stress induced by hydrogen peroxide, highlighting a strong defense mechanism against reactive oxygen species. Its moderate resistance to lysozyme (68.76%) implies that it can survive initial exposure to host enzymes found in secretions like saliva and mucus. Additionally, the strain showed a DPPH radical scavenging activity of 50.50%, reflecting moderate antioxidant potential and functional bioactivity beyond mere survival, which may contribute to host health by combating oxidative stress.

Overall, L-Phy5 demonstrated a well-rounded probiotic profile, with strong resistance to acidic and oxidative stress, good bile salt tolerance, moderate lysozyme resistance, and fair antioxidant capacity. These characteristics support its potential as a viable and functional probiotic candidate, capable of surviving in the gastrointestinal tract and possibly offering health-promoting effects. L-Phy5 exhibits traits comparable to well-established probiotics like *Lactobacillus reuteri*, which tolerates up to 5% bile due to genetic adaptations such as multidrug resistance transporters. Its high oxidative stress tolerance suggests a strong defense against reactive oxygen species, consistent with other LAB strains. The strain's moderate lysozyme resistance (68.76%) indicates potential survival during early host interactions, while its DPPH scavenging activity (50.50%) reflects moderate antioxidant capacity, aligning with the health-promoting properties observed in many *Lactobacillus* strains [21-23]. On the other hand, the probiotic strain L-Phy5 demonstrates strong stress tolerance and functional properties, aligning with previous findings on the other species of lactic acid bacteria (LAB). Its high acid tolerance (78.26% at pH 2) is comparable to *Lactobacillus fermentum* strains, while its bile salt resistance

(75.63% in 1% bile) matches the resilience of LAB strains known to withstand up to 2.0% bile [24]. L-Phy5 demonstrated superior oxidative stress tolerance, with 82.50% survival under 2 mM H_2O_2, compared to *Lactobacillus plantarum* strains, as well as moderate lysozyme resistance (68.76%), indicating its potential for survival during early host interactions [25]. Its antioxidant activity (50.50% DPPH scavenging) reflects functional bioactivity similar to other LAB strains [26]. These attributes highlight L-Phy5 as a promising probiotic candidate with comparable or superior performance to the established the LAB strains.

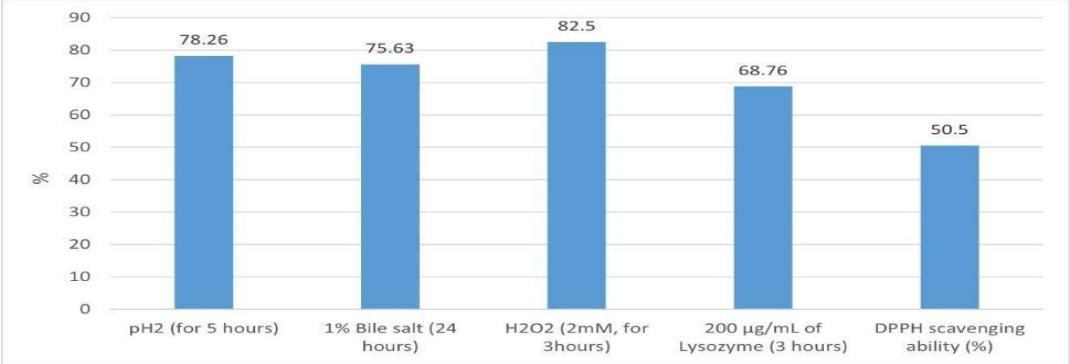

Fig. 1 Quality Assessment of the Probiotic Strain L-Phy5

Molecular identification of the phytase superior isolate (L-Phy5)

To accurately identify the isolated strain (L-Phy5), the 16S rRNA gene was analyzed. The 16S-specific primers were employed to amplify the gene fragment from the total genomic DNA of *Lactobacillus* (L-Phy5). The PCR process yielded an amplicon of approximately 1500 base pairs (Figure 2). *Lactobacillus reuteri* strain Pro8 clustered closely within the *Limosilactobacillu sreuteri* group, showing 94% sequence similarity with strain F275 (NR_025911.1), which confirmed its classification within the same species or a closely related lineage. This phylogenetic positioning (Figure 3) supported the reclassification of *L. reuteri* under the *Limosilactobacillus* genus, as proposed by Zheng et al. [27]. The main *L. reuteri* clade included reference strains such as NBRC 15892, DSM 20016, BG-AF3-A, and WF-MT5-A, all of which shared 94–97% sequence identity, reflecting conserved genetic traits across the lineage. A distinct sister clade comprising *L. urinaemulieris*, *L. rudi*, *L. caviae*, and *L. albertensis* showed 93–94% similarity, indicating greater genetic divergence and reinforcing the species-level diversity within *Limosilactobacillus* [28]. The close evolutionary relationship of Pro8 to strains like F275 and DSM 20016 suggested that it might exhibit similar probiotic functions, including gastrointestinal resilience, antimicrobial properties, and immune modulation, highlighting its potential for application in functional foods or therapeutic contexts.

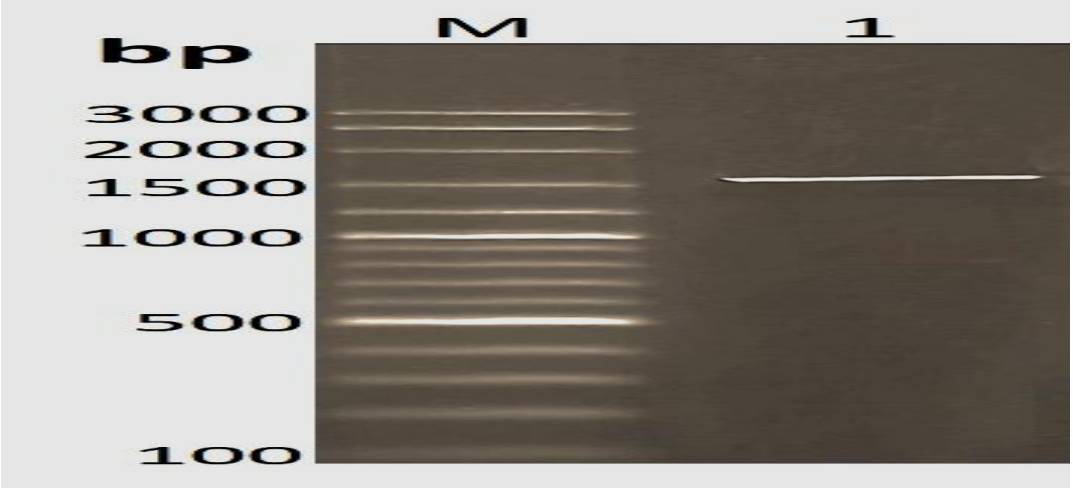

Fig. 2 Photograph of the amplified 16S rDNA band of the *Lactobacillus* strain Pro8 (lane 1), compared with a 100 bp DNA ladder.

Fig. 3 Phylogenetic Analysis of *L. reuteri* Strain Pro8 (PV448931.1) Using Maximum Likelihood.

Genetic improvement of phytase production by *Lactobacillus* (LAB-Phy5)

Genetic improvement of phytase production in *Lactobacillus* species can enhance their ability to degrade phytate, making them more effective for applications in food, feed, and probiotic development. Several molecular and genetic strategies can be employed to achieve this, including traditional genetic manipulation, such as mutagenesis and protoplast fusion.

Ultraviolet radiation mutagenesis

Ultraviolet radiation (UV) mutagenesis was a widely used method for inducing random genetic mutations in microorganisms, including *Lactobacillus*, to improve traits such as phytase production. This method employed ultraviolet (UV) radiation to create DNA damage, leading to genetic diversity and the potential for enhanced enzymatic activity, stability, and production. UV mutagenesis required minimal equipment and reagents (UV lamp -254 nm), Petri dishes, and growth medium), making it an accessible and cost-effective method for strain improvement. Since UV mutagenesis did not involve inserting foreign DNA, the resulting strains were considered non-GMO (genetically modified organisms), making them more acceptable for regulatory and consumer applications.

The data in Table 2 illustrates a marked decline in the viability of *Lactobacillus* strainL-Phy5 with prolonged exposure to ultraviolet (UV) radiation, highlighting its vulnerability to oxidative DNA damage induced by UV light. A complete survival rate (100%) was recorded at 0 minutes (1563 CFU/mL), which rapidly decreased to 45.55% (712 CFU/mL) after 4 minutes of exposure. This sharp decline continued, with viability dropping to 18.11% (283 CFU/mL) at 8 minutes and reaching as low as5.31% (83 CFU/mL) after 12 minutes. These results are consistent with findings from previous studies, which have shown that prolonged UV exposure significantly impairs the viability of lactic acid bacteria due to damage to nucleic acids and essential cellular components. Similar trends were demonstrated that *Lactobacillus rhamnosus* experienced a dramatic decrease in survival when subjected to UV-C radiation, with longer exposure times leading to extensive DNA fragmentation [29,30]. Prolonged UV exposure significantly enhanced phytase production in mutants of the *Lactobacillus* strain L-Phy5, with a clear trend of increased productivity correlating with longer exposure times.

Initially, a large number of mutants were evaluated on Petri plates and mutants with a large lytic zone on phytase screening medium (PSM) were selected as an indicator of high phytase production. This was used to evaluate a small number of distinct mutants (13 mutants) after each treatment. The provided data in Tables (3-5) demonstrated the phytase productivity of *Lactobacillus* (L-Phy5) mutants generated via UV-light mutagenesis at varying exposure times (4, 8, and 12 minutes). After 4 minutes of UV treatment, mutant PUV4-8 demonstrated a 211.01% increase in phytase activity compared to the wild-type (L-Phy5). This improvement was surpassed by mutant PUV8-4 (233.59%) after 8 minutes and peaked at 258.76% in mutant PUV12-10 following 12 minutes of exposure, suggesting that UV mutagenesis effectively boosts enzyme yields up to a certain

threshold. However, variability among mutants within the same exposure group, such as PUV12-10 significantly outperforming PUV12-11 (158.60%), highlights the random nature of UV-induced mutations, where only a subset confers beneficial traits [31-33]. Mechanistically, these enhancements may result from gene upregulation, structural mutations improving enzyme stability, or activation of stress responses [33]. From an industrial perspective, high-performing mutants like PUV12-10 offer promising applications in animal feed, potentially lowering production costs and improving nutrient bioavailability, as supported by similar gains in phytase activity observed in UV-mutated *Aspergillus* strains [34, 35].

Table 2 Effect of ultraviolet (UV) radiation on *Lactobacillus* (LAB-Phy5) survival

Exposure time (min.)	**Number of viable colonies (CFU/mL) and%**	
	No.	%
0.0	1563	100.00
4.0	712	45.55
8.0	283	18.11
12.0	83	5.31

Table 3 Phytase productivity of potent mutants after mutagenesis of *Lactobacillus* (LAB-Phy5) with ultraviolet (UV) radiation light for 4 min

Isolate No.	Phytase production*	% toL-Phy5 strain
L-Phy5	673.8^{n}±0.02	100.00
PUV4-1	798.3^{m}±0.04	118.48
PUV4-2	1291.5^{d}±0.1	191.68
PUV4-3	963.5^{l}±0.02	142.99
PUV4-4	878.3^{j}±0.03	130.35
PUV4-5	1094.2^{f}±0.01	162.39
PUV4-6	806.8^{l}±0.03	119.74
PUV4-7	1326.5^{c}±0.08	196.87
PUV4-8	1421.8^{a}±0.03	211.01
PUV4-9	983.9^{h}±0.04	146.02
PUV4-10	1175.6^{e}±0.01	174.47
PUV4-11	1359.5^{b}±0.02	201.77
PUV4-12	867.4^{k}±0.03	128.73
PUV4-13	985.5^{g}±0.05	146.26

*Units.mL^{-1}

Table 4 Phytase productivity of potent mutants after mutagenesis of *Lactobacillus* (LAB-Phy5) with ultraviolet (UV) radiation light for 8 min

Isolate No.	**Phytase production ***	**% To L-Phy5 strain**
L-Phy5	673.8^{n}±0.01	100.00
PUV8-1	1392.6^{d}±0.04	206.68
PUV8-2	1242.5^{l}±0.04	184.40
PUV8-3	871.8^{m}±0.07	129.39
PUV8-4	1573.9^{a}±0.02	233.59
PUV8-5	1281.5^{g}±0.01	190.19
PUV8-6	968.9^{l}±0.04	143.80
PUV8-7	1416.5^{c}±0.02	210.23
PUV8-8	1448.6^{b}±0.06	214.99
PUV8-9	969.6^{k}±0.03	143.80
PUV8-10	1385.5^{e}±0.02	205.62
PUV8-11	1269.5^{h}±0.08	188.41
PUV8-12	1337.8^{f}±0.05	198.55
PUV8-13	1035.7^{j}±0.03	153.71

* Units.mL^{-1}

Table 5 Phytase productivity of potent mutants after mutagenesis of *Lactobacillus* (LAB-Phy5) with ultraviolet (UV) radiation light for 12 min

Isolate No.	Phytase production *	% to L-Phy5 strain
L-Phy5	673.8^{n}±0.02	100.00
PUV12-1	1569.5^{e}±0.02	232.93
PUV12-2	1271.3^{h}±0.05	188.68
PUV12-3	938.6^{m}±0.07	139.30
PUV12-4	1433.5^{f}±0.03	212.75
PUV12-5	1328.5^{g}±0.02	197.17
PUV12-6	1264.7^{i}±0.04	187.70
PUV12-7	1701.5^{b}±0.08	252.52
PUV12-8	1639.8^{d}±0.02	243.37
PUV12-9	1164.4^{j}±0.09	172.81
PUV12-10	1743.5^{a}±0.02	258.76
PUV12-11	1068.7^{l}±0.01	158.60
PUV12-12	1648.5^{c}±0.02	244.67
PUV12-13	1084.6^{k}±0.06	160.97

*Units.mL^{-1}

Ethyl methane sulfonate mutagenesis

Ethyl methane sulfonate (EMS) mutagenesis was a chemical method frequently employed to induce random point mutations in the DNA of microorganisms, including *Lactobacillus*. EMS mutagenesis offered several advantages for improving phytase production, making it a valuable tool in strain development. EMS specifically induced point mutations (e.g., G-C to A-T transitions), which could lead to alterations in gene function or regulatory regions. These mutations might have improved phytase activity, stability, or production efficiency. By inducing random point mutations, EMS generated genetic diversity that could be exploited to enhance enzyme activity, stability, and production. With effective screening and characterization, EMS mutagenesis led to the development of *Lactobacillus* strains with superior phytase properties for different applications.

The survival and productivity data from EMS-treated *Lactobacillus* strain LAB-Phy5 reveals key insights into the mutagenic efficiency and biotechnological potential of chemical mutagenesis using (EMS). Data in Table 6 about survival rate under EMS exposure showed that as EMS exposure increased, a sharp decline in colony viability was observed. At 40 minutes of treatment, viability dropped to 39.58%, and further decreased to 5.73% at 60 minutes. This is consistent with the known dose-dependent cytotoxicity of EMS, which induces random point mutations, predominantly G→A transitions, often leading to gene inactivation or altered gene regulation. Similar survival reductions have been reported in *Lactobacillus plantarum* and *Bacillus subtilis* under EMS treatment [36-38].

Table 6 Effect of ethyl methane sulfonate (EMS) on *Lactobacillus* (LAB-Phy5) survival.

Exposure time (min.)	**Number of viable colonies (CFU/mL) and %**	
	No.	%
0.0	1675	100.00
40.0	663	39.58
60.0	96	5.73

Table 7 Phytase productivity of mutants after mutagenesis of *Lactobacillus* (LAB-Phy5) with ethyl methane sulfonate (EMS) for 40 min

Isolate No.	**Phytase production ***	**% to L-Phy5 strain**
L-Phy5	673.8^{n}±0.02	100.00
PE40-1	1348.4^{g}±0.05	200.12
PE40-2	1158.6^{k}±0.04	171.95
PE40-3	1137.2^{l}±0.02	168.77
PE40-4	1594.7^{b}±0.01	236.67
PE40-5	1641.5^{a}±0.05	243.62

PE40-6	1027.5^{m}±0.06	152.49
PE40-7	1366.5^{e}±0.05	202.80
PE40-8	1528.4^{d}±0.04	226.83
PE40-9	1175.6^{j}±0.01	174.47
PE40-10	1295.8^{h}±0.03	192.31
PE40-11	1361.4^{f}±0.05	202.05
PE40-12	1542.5^{c}±0.04	228.93
PE40-13	1233.6^{i}±0.07	183.08

*Units.mL^{-1}

Results from the EMS mutagenesis experiments on *Lactobacillus* strains for enhanced phytase production in Tables (7 & 8) revealed compelling trends that underline the mutagenic potential of EMS. The 40-minute EMS treatment produced mutants with phytase activity ranging from 152.49 to 243.62% relative to the wild-type (L-Phy5), with PE40-5 being the most productive (1641.5 U/mL). This result indicates that even moderate EMS exposure can yield a significant pool of beneficial mutations. The high variability within this group supports the random nature of EMS-induced mutations, where most are either neutral or deleterious, but some confer advantageous phenotypes such as enzyme overproduction [35, 36]. The 60-minute EMS exposure group demonstrated even greater enhancement in phytase productivity. PMS60-5 stood out, producing 1937.4 U/mL, 287.53% of the wild-type activity. Other mutants, such as PMS60-11 (272.84%) and PMS60-9 (272.40%), reinforced the trend that longer EMS exposure, though more cytotoxic, can result in the isolation of highly productive strains. This observation resonates with recent reports suggesting that extended mutagenic exposure may improve the frequency of beneficial mutations, as long as viability is not severely compromised [39-41].

Table 8 Phytase productivity of resistant mutants after mutagenesis of *Lactobacillus (*LAB-Phy5) with ethyl methane sulfonate (EMS) for 60 min

Isolate No.	Phytase production *	% To L-Phy5 strain
L-Phy5	673.8^{n}±0.02	100.00
PMS60-1	987.6^{m}±0.03	146.57
PMS60-2	1558.5^{g}±0.04	231.30
PMS60-3	1171.5^{k}±0.08	173.86
PMS60-4	1045.4^{l}±0.06	155.15
PMS60-5	1937.4^{a}±0.02	287.53
PMS60-6	1635.7^{f}±0.04	242.76
PMS60-7	1447.9^{h}±0.05	214.89
PMS60-8	1701.5^{e}±0.09	252.52
PMS60-9	1835.4^{c}±0.02	272.40
PMS60-10	1364.6^{i}±0.02	202.52
PMS60-11	1838.4^{b}±0.08	272.84
PMS60-12	1285.8^{j}±0.05	190.83
PMS60-13	1738.6^{d}±0.04	258.03

*Units.mL^{-1}

Protoplast fusion

Protoplast fusion was considered a powerful technique for enhancing phytase production in *Lactobacillus*. It allowed the combination of genetic material from two or more strains by fusing their protoplasts, bypassing traditional reproductive barriers. This method was particularly useful for strain improvement, as it could combine desirable traits such as higher phytase activity, stress tolerance, and faster growth.

Antibiotic marker for selection of fusants

Based on Table 9 following mutagenesis, notable changes in antibiotic susceptibility were observed among the phytase hyper-producing mutants. The UV-induced mutant PUV12-10 developed resistance to tobramycin but exhibited increased sensitivity to most antibiotics compared to the parental strain L-Phy5, particularly chloramphenicol, gentamicin, and cefotaxime, while retaining high-though slightly reduced-susceptibility to doxycycline, rifampicin, and

streptomycin. The EMS-derived mutant PMS60-5 retained or gained resistance, notably to gentamicin and cefotaxime, while showing lower sensitivity to most antibiotics, with the exception of tobramycin, to which it was more sensitive than the original strain. PMS60-11, another EMS mutant, also developed resistance to tobramycin, with marginal improvements in sensitivity to gentamicin and cefotaxime but a moderate decline in response to rifampicin and streptomycin. These variations were attributed to random mutations induced by UV or EMS treatments, which likely altered genes associated with membrane permeability, efflux mechanisms, ribosomal targets, or regulatory networks governing antibiotic resistance. The increased susceptibility in PUV12-10 suggested that UV mutagenesis had disrupted specific resistance pathways, while the mixed susceptibility patterns in EMS mutants indicated broader, less targeted changes, potentially resulting from nonspecific alkylation of genetic material.

Table 9 Comparison of antibiotic susceptibility among the original strain (L-Phy5) and three selected phytase hyper-producing mutants generated via ultraviolet radiation and ethyl methane sulfonate.

Strain code	Antibiotic disc						
	*TOB +10	C 30	CN 10	CT 25	DO 30	RF 30	S 10
L-Phy5	**18	22	21	R	40	42	44
PUV12-10	R	32	29	15	36	38	37
PMS60-5	28	18	R	10	20	20	25
PMS60-11	R	19	30	17	27	32	22

*TOB, tobramycin; C, chloramphenicol; CN, gentamycin; CT, colistin; DO, doxycycline; RF, rifampicin SV; S, streptomycin. + Concentration (µg/disc). R= resisted (no clear zone). **Means of diameter of inhibition zones (mm).

The previous studies collectively indicated that mutagenesis methods like UV and EMS induced genetic changes in *Lactobacillus reuteri*, leading to variations in antibiotic susceptibility. Such alterations might have resulted from mutations affecting membrane permeability, efflux mechanisms, ribosomal targets, or regulatory networks governing antibiotic resistance [42,43]. Immediately before protoplast fusion, live protoplasts were prepared from the parental strains involved in the fusion, as shown in Figure 4.

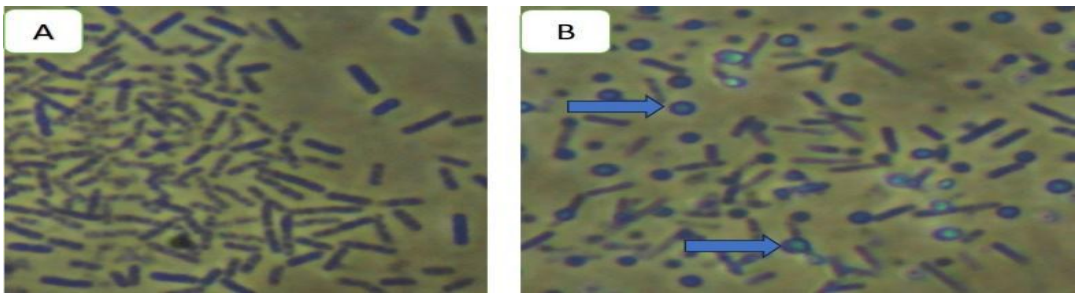

Fig. 4 The obtained protoplast from parental strain (B) in compassion of the original strain (A).

Table 10 Phytase productivity of fusants after protoplast fusion among the superior phytase mutants.

Isolate No.	Phytase production *	% To L-Phy5 strain	% To PMS60-5 strain
L-Phy5	673.8^{m}±0.03	100.00	34.78
PUV12-10	1743.5^{l}±0.02	258.76	89.99
PMS60-5	1937.4^{g}±0.02	287.53	100.00
PC1/1	2065.4^{c}±0.05	306.53	106.61
PC1/2	1968.3^{e}±0.07	292.12	101.59
PC1/3	1897.3^{i}±0.01	281.58	97.93
PC1/4	2005.9^{d}±0.06	297.70	103.54
PC1/5	1853.8^{j}±0.05	275.13	95.68
PC1/6	2186.5^{b}±0.06	324.50	112.86
PC1/7	1843.8^{k}±0.07	273.64	95.17
PC1/8	1931.6^{h}±0.08	286.67	99.70
PC1/9	2347.9^{a}±0.02	348.46	121.19
PC1/10	1964.7^{f}±0.03	291.59	101.41

*Units.mL^{-1}

Table 10 presents the phytase activity of fusants derived from protoplast fusion between two high-performing *Lactobacillus reuteri* mutants, PUV12-10 (UV-induced) and PMS60-5 (EMS-induced), in comparison with the original wild-type strain L-Phy5. The parental strain L-Phy5 produced 673.8 U/mL of phytase, serving as the baseline for productivity comparisons. PUV12-10 and PMS60-5 showed significantly higher activities, producing 1743.5 U/mL (258.76%) and 1937.4 U/mL (287.53%), respectively. All fusants (PC1/1 to PC1/10) outperformed the original strain, and most exceeded the parent mutants, indicating successful trait enhancement through fusion. Among these, PC1/9 was the most productive, yielding 2347.9 U/mL (348.46% of L-Phy5 and 121.19% of PMS60-5), followed by PC1/6 (2186.5 U/mL) and PC1/1 (2065.4 U/mL). These improvements suggest effective genetic recombination and synergistic interaction between the fused genomes, likely due to genetic complementation, increased gene dosage of phytase-encoding regions, elimination of suppressor genes, or enhanced promoter activity. Variations among fusants may reflect partial incompatibility or differential expression of the fused genetic elements. Protoplast fusion has thus proven to be a powerful strategy for enhancing phytase biosynthesis in *L. reuteri*, as supported by previous findings showing the creation of stable, high-yielding hybrids with improved metabolic traits [44-47].

Random amplified polymorphic DNA fingerprinting of mutants and fusants

The Random Amplified Polymorphic DNA (RAPD) technique is a widely used molecular tool for genotypic fingerprinting of probiotics, including mutants and fusants. This technique employs short, arbitrary primers to amplify random regions of the genome, providing unique DNA profiles that can be used to differentiate between strains. The RAPD technique is a powerful and cost-effective tool for genotypic fingerprinting of probiotic mutants and fusants. It allowed to differentiate, characterize, and monitor genetic variations resulting from mutagenesis or protoplast fusion. By providing unique DNA profiles, RAPD helps in verifying the success of strain improvement programs and ensuring the stability of beneficial traits in probiotic strains.

To assess molecular variations induced by different mutagenesis treatments and protoplast fusion cross, four random primers were employed to analyze two UV and EMS-induced mutants, two recombinant fusants, and the original *L. reuteri* strain Pro8 (L-Phy5). Using RAPD primer P1, distinct banding patterns were generated for the mutants, fusants, and the original strain, as illustrated in Figure 5 A. There is clear band pattern variability among the strains, indicating that both EMS mutagenesis and protoplast fusion introduced genetic changes. Furthermore, the discriminatory bands at 5000 bp and 1500 bp were particularly informative for differentiating fusants and a specific mutant (PMS60-5), respectively. Also, a number of shared bands (2500, 1400, 700, 600, 300, and 100 bp) were also found common to all strains, indicating conserved genomic regions.

P2 primer was also used with the tested mutants and fusants in comparison to the parental-strain of *L. reuteri*Pro8 to discover molecular differences between mutants, fusants, and the parental-strain (Figure 5 B). The analysis revealed that bands at 4000 bp and 250 bp were consistently present across all strains, indicating highly conserved regions. Unique bands observed at 1450, 1000, 800, and 450 bp were specific to the EMS mutant PMS60-5, while L-Phy5 and PUV12-10 shared a common band at 700 bp but lacked several bands found in PMS60-5 and the fusants. Fusant-specific bands in 1900, 1400, 900, and 550 bp were detected exclusively in PC1/9 and PC1/6, suggesting successful recombination and the presence of distinct fusion markers. Overall, the notable variations in band presence and absence, particularly within the 500–2000 bp range, reflected substantial genetic divergence between the EMS-induced mutants and the recombinant fusants.

Moreover, primer 3 (Figure 5 C) effectively differentiated between EMS-induced mutants and protoplast fusion-derived fusants. Conserved bands at 4000 bp and 250 bp were present in all strains and served as reliable reference points for assessing genomic integrity. PMS60-5 exhibited several unique bands (1450, 1000, 800, 450 bp), indicating a high level of divergence likely caused by EMS-induced point mutations. In contrast, the fusants PC1/9 and PC1/6 displayed distinct bands at 1900, 1400, 900, and 550 bp, which were absent in the mutants, confirming successful protoplast fusion and the formation of novel genetic profiles. These fusion-specific bands served as excellent markers for distinguishing fusants from EMS mutants, while bands at 700 and 500 bp represented EMS-mutant signatures that were lost following fusion. Overall, P3 proved to be a powerful tool for identifying genetic variation introduced by both mutagenesis and fusion techniques.

In the analysis with the P4 (Figure 5 D), no conserved bands were observed, as no single band was present across all strains. Each strain, with the exception of PC1/6, exhibited at least one unique band. L-Phy5 showed specific bands at 2250 bp, 900 bp, 650 bp, and 250 bp, while PMS60-5 had a distinct band at 800 bp. PC1/9 displayed a unique band at 200 bp, and PUV12-10 exhibited a band at 150 bp. PC1/6 did not present any unique bands; instead, it shared a 2000 bp band exclusively with PC1/9.

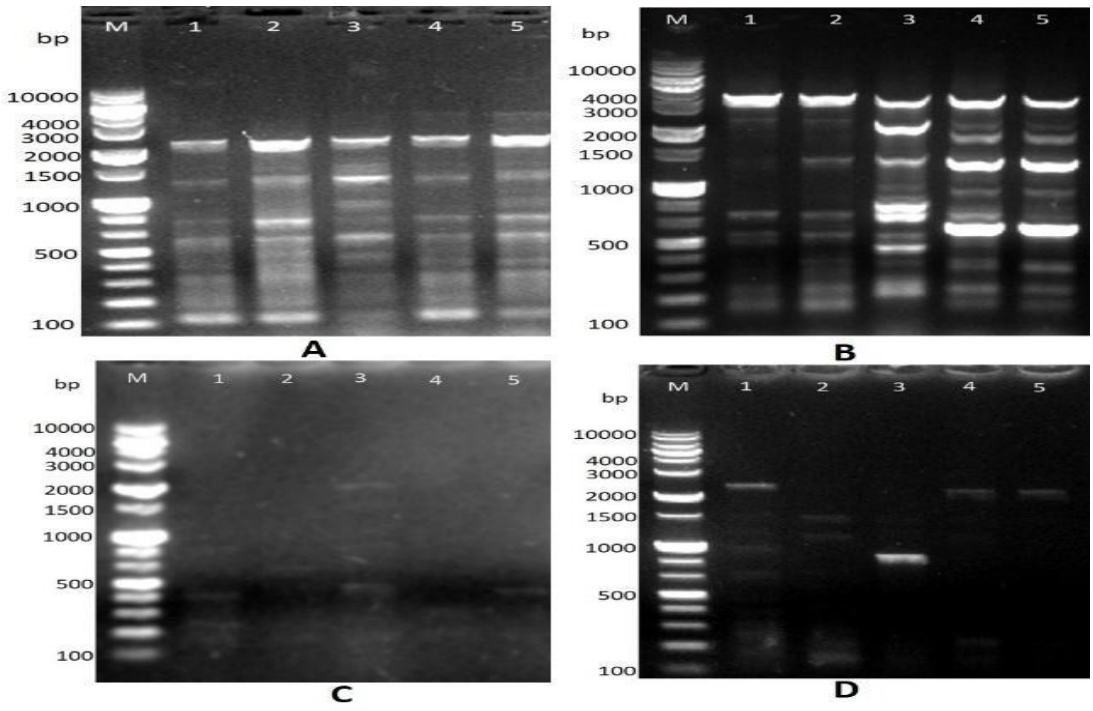

Fig. 5 Random amplified polymorphic DNA (RAPD)amplification using four primers—P1 (A), P2 (B), P3 (C), and P4 (D) for two different mutants and two fusants (Lanes 2 to 5), *L. reuteri* strain Pro8 [L-Phy5] (lane 1) opposite to DNA Marker (lane M). The strains were arranged in the following order: L-Phy5, PUV12-10, PMS60-5, PC1/6, and PC1/9.

Phylogenetic tree in relation to random amplified polymorphic DNA assay

The data in Table 11 exhibited a proximity matrix from a RAPD assay, used to compare genetic similarities among different *Lactobacillus reuteri* strains, including mutants, fusants, and the parental strain L-Phy5. The parental strain L-Phy5 exhibited the highest genetic similarity with PUV12-10 (0.789), indicating that this mutant retains much of the parental genome, while it was least similar to PMS60-5 (0.522), suggesting significant genetic divergence. PUV12-10 displayed moderate similarity to all other strains (0.571–0.789), with the closest relationship to L-Phy5, reinforcing its close genetic affiliation. In contrast, PMS60-5 was the most genetically distinct strain, showing the lowest similarity values (around 0.52–0.57) with both the parent and other fusants. Meanwhile, the fusants PC1/6 and PC1/9 showed a high degree of similarity to each other (0.818) and moderate similarity to both L-Phy5 and PUV12-10, suggesting they may be sister fusants or recombinants derived from successful protoplast fusion.

The phylogenetic tree (Figure 6) constructed using the RAPD method showed that L-Phy5 and PUV12-10 clustered closely together, supporting the earlier proximity matrix data, and indicating that PUV12-10 was genetically closest to the parental strain L-Phy5, and likely representing a mild or low-level mutant. PMS60-5 formed a distinct branch, reflecting a greater evolutionary distance from both the parental strain and the other derivatives, which suggested substantial genomic alterations potentially caused by strong mutagenic pressure. PC1/6 and PC1/9 formed a tight cluster, appearing more closely related to each other than to any other strain, reinforcing the possibility that they were sister fusants or products of a shared recombination event. Their moderate divergence from the parental strain indicated partial genetic separation. Overall, the phylogenetic analysis corroborated the RAPD proximity matrix, showing that PUV12-10 was a near-parental variant, PMS60-5 was a distinct and highly divergent outlier, and PC1/6 and PC1/9 were genetically similar fusants derived through a fusion cross. These findings are consistent with the patterns reported in previous studies [49-51].

Table 11 A proximity matrix random amplified polymorphic DNA (RAPD) assay to compare the tested mutants, fusants, and the parental *L. reuteri*Pro8 [L-Phy5] strain.

Strain code	L-Phy5	PUV12-10	PMS60-5	PC1/6	PC1/9
L-Phy5	1.000	0.789	0.522	0.533	0.537
PUV12-10	0.789	1.000	0.571	0.634	0.595
PMS60-5	0.522	0.571	1.000	0.531	0.533
PC1/6	0.533	0.634	0.531	1.000	0.818
PC1/9	0.537	0.595	0.533	0.818	1.000

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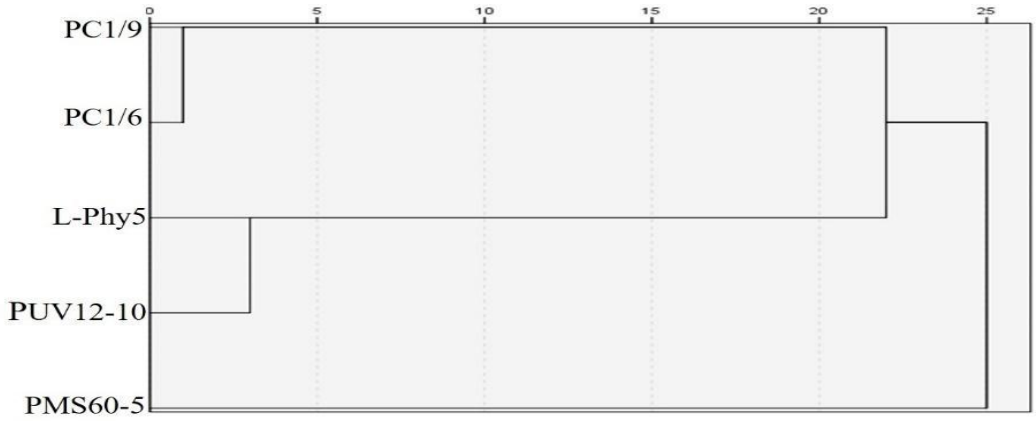

Fig. 6 A phylogenetic tree using the RAPD method to analyze the genetic relationships among the tested mutants, fusants, and the parental *Lactobacillus reuteri* Pro8 [L-Phy5] strain.

Conclusion

Phytase-producing lactic acid bacteria represent a sustainable, health-promoting, and multifunctional resource for food, feed, and environmental applications. This study involved the screening and molecular identification of phytase-producing colonies, followed by the enhancement of a high-yielding *Lactobacillus reuteri*Pro8 [L-Phy5] strain through ultraviolet radiation and ethyl methane sulfonate mutagenesis as well as protoplast fusion. The random amplification of polymorphic DNA profiles of the resulting superior mutants and fusants displayed notable variations compared to the wild-type strain, serving as indicators of underlying genetic modifications. Phylogenetic analysis further grouped these improved phytase-producing strains into distinct clusters, highlighting the extent of genetic divergence achieved through the applied enhancement techniques.

Conflict of interest

The authors declare that they have no conflict of interest.

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