Molecular characterization of the superior lignin peroxidase-producing *Streptomyces lavendulae* R-St-1 mutants and fusants

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Background
The extracellular lignin peroxidase (LiP) secreted by bacterial isolates is the key enzyme in lignin degradation in several species of *Streptomyces* (actinomycetes). Random mutations were induced for bacterial strains using ultraviolet (UV) and ethyl methanesulfonate (EMS). Moreover, protoplast fusion is an important tool in strain improvement to achieve genetic recombination and developing hybrid bacterial strains. The molecular analysis of mutants and fusants by random amplification of polymorphic DNA (RAPD-PCR) was done.
Objective
Streptomyces lavendulae R-St strain, which produces the highest LiP, was discovered and investigated in a previous study by the authors. It has been deposited in NCBI under the accession number 'OL697233.1.' *S. lavendulae* was used in the present study to produce novel, higher LiP-producing mutants using EMS-mutagenesis and UV light. Most mutant strains that produce LiP fuse their protoplasts. To assess the genetic diversity of isolated *S. lavendulae* R-St-1 with its mutants and fusants, RAPD-PCR was used.
Materials and methods
Lignin was extracted and purified from black wood liquor. UV and EMS were used for creating super LiP-producing mutants of *S. lavendulae* R-St. Protoplast fusion between EMS and UV-treated mutants was performed for isolating LiP-productive fusants (s) from *S. lavendulae* R-St-1 as the original isolate. Fermentation medium (FM) (g/l) was used for lignin-degrading bacterial screening after dilution of the soil samples: K_2HPO_4, 4.55, KH_2PO_4, 0.53, $MgSO_4$,0.5, NH_4NO_3, 0.1, yeast extract, 0.1, lignin (0.1% v/v), agar 15, and the pH should be 7.0. The aforementioned FM medium was supplemented with 50 mg/l of azure B and toluidine dyes and 100 mg/l of tannic acid. FM was used without any supplements and agar for the isolation of lignin-degrading bacteria using lignin (0.1% v/v). The molecular analysis of mutants by RAPD-PCR was applied using different primers, and different separate bands were determined.
Results and discussion
S. lavendulae R-St-1 strain was mutagenized with alkylating EMS (200 mm) and UV. Results showed that from the *S. lavendulae* R-St-1 (W.T) isolate, two EMS-treated mutants (Rst/60/7E and Rst/40/8E), which showed activities of 8.5 and 7.3 U/ml, respectively, and two UV-treated mutants (Rst/9/2U and Rst/9/6U), which showed activities of 9.4 and 7.8 U/ml, respectively, were the most efficient ligninolytic mutants. Protoplast fusion between two higher LiP-producing mutants (cross 1 and 2) proved to be the most effective, and the two isolated fusants C1/St/5 and C1/St/6 showed activity of 12.8 and 11.8 U/ml, respectively, after protoplast fusion between Rst/9/6U and Rst/60/7E mutants of *S. lavendulae* R-St-1 (W.T). To determine molecular variability of two EMS mutants, and their recombinant fusants as well as *S. lavendulae* (W.T) (parental), three random primers were used. RAPD primer (P1) was employed. Fusant C1/St/5 shared the parental isolate with the bands 850 and 300 bp, whereas fusant C1/St/6 had five new unique bands (1470, 750, 650, 520, and 250 bp). The DNA loci of the obtained banding profiles using P1, P2, and P3 primers were 12, 17, and three loci after RAPD assay. A total of 14 unique loci were obtained using the primers P1 and P2.

Keywords:
Ethyl methanesulfonate mutagenesis, lignin peroxidase, phylogenetic analysis, protoplast fusion, random amplification of polymorphic DNA-PCR, *Streptomyces*, ultraviolet light

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Introduction

An environmentally friendly approach for promoting environmental sustainability is the conversion of renewable biomass into chemicals and fuels. The

three main components of lignocellulose are cellulose, lignin, and hemicellulose [1]. Although most lignin cannot be used effectively, cellulose and hemicellulose are digested by enzymes into monosaccharides [2,3] and fermented to produce diverse bioproducts [4,5]. Large volumes of lignin ($5–36\times10^8$ tons) are generated every year [6]. The biomass refinery and pulp/paper sectors produce 6.2×10^7 and 5×10^7 tons of lignin per year, respectively, including kraft lignin, lignosulfonate, and soda lignin [7]. Currently, lignin is used to make biofuels out of waste or abandoned materials. Extracellular enzymes from microbes, such as lignin peroxidase (LiP), are primarily responsible for depolymerizing lignin [8]. As Actinobacteria [9], lignin-degradation bacteria have been isolated from a variety of sources, including soil, rotten wood, wastewater treatment plants, and wastewater [10]. According to Kamimura *et al.* [11], the extracellular enzymes released by filamentous *S. viridosporus* T7A break down lignin. Actinomycetes are an appealing category that can be used to produce lignocellulolytic enzymes [12,13], such as the peroxidase-producing *Streptomyces* sp. S19 [14]. According to Batayyib *et al.* [15], *S. lavendulae* R-St-1 was the isolate that produced LiP most frequently.

The commercialization of microbial fermentation processes depends heavily on strain improvement, as wild strains typically generate only small amounts of the required enzyme [6]. The yield can be further improved using mutagens and adopting specialized techniques or procedures for identifying useful mutants. Colonies that demonstrated an increase in the yield can be obtained by straightforward selection methods, such as spreading the culture on particular media [16]. Additionally, the mutant of various *Streptomyces* species produced highly active enzymes [17–19]. Ethyl methanesulfonate (EMS) and ultraviolet (UV) irradiation were used to introduce random mutations in bacterial strains [20–23]. For bringing about genetic recombination and creating hybrid bacterial strains, protoplast fusion is a crucial technique in strain enhancement [21,23–25]. Protoplast fusion approach is thought to be an effective way to manipulate genetic material to encourage recombination. Additionally, the synthesis of improved enzyme-producing fusants from *Streptomyces* was facilitated by protoplast fusion [26,27]. Successful genetic research on the improvement of lignin conversion was made possible by early attempts to improve *Streptomyces* through mutation [28,29].

In the present work, induction of mutation using alkylating chemical EMS and UV and protoplast fusion enabled construction of superior *S. lavendulae* strain for LiP productivity. To improve LiP productivity of *S. lavendulae* R-St-1 previously isolated [15], two mutagens (EMS and UV) were used to induce genetic variability.

Molecular strategies based primarily and totally on genomic DNA have notably been used for the analysis of genetic variation. The random amplified polymorphic DNA (RAPD) approach, which used primers, could be very beneficial because it is fast, cheap, and an easy method in genetic mapping and characterization. One distinguished strength of the RAPD is that it could be carried out effortlessly and employed on harmful gram (–) and gram (+) bacteria [30]. The proof of genetic variety created in *S. lavendulae* DNA following EMS-mutagenesis became corroborated through variations in RAPD patterns. As a result, cluster analysis was used to classify the strains under analysis into clusters that potentially recognize the genetic variety of LiP-producing mutants.

Materials and methods

Lignin preparations

Lignin is separated and purified from black wood liquor according to the method described by Xiong *et al.* [31]. The method of purification was as follows: the pH value of pulping black liquor was adjusted to 3 (20%) using aqueous sulfuric acid. The suspension was stirred for 2 h at 50°C to facilitate lignin precipitation. The precipitates were collected on a Buchner funnel and washed several times with distilled water. Finally, the precipitated lignin was collected and dried overnight in the oven at 70°C.

Source of isolate

The ligninolytic bacterial strain (*S. lavendulae* R-St-1) was previously obtained from Jeddah, KSA [15].

Screening ligninolytic potential of *Streptomyces lavendulae* R-St-1 mutants and fusants

Fermentation medium (FM) (g/l) was used for lignin-degrading bacterial screening after dilution of the soil samples: K_2HPO_4, 4.55; KH_2PO_4, 0.53; $MgSO_4$,0.5; NH_4NO_3, 0.1; yeast extract, 0.1; lignin (0.1% v/v); agar 15; and the pH should be 7.0 [32]. The FM medium was supplemented with 50 mg/l of azure B and toluidine dyes and 100 mg/l of tannic acid.

Assay of lignin peroxidase

The enzyme productivity was determined in the culture supernatant obtained by centrifugation for 5 min at 5000 rpm under cooling in an Eppendorf tube. Half ml

of the clear supernatant was used to assay enzyme activity. LIP activity was measured at 310 nm according to Tien and Kirk [33], with 1 U representing 1 μmol veratryl alcohol oxidized to veratryl aldehyde per minute at 30°C.

Mutation

Two different mutagens (EMS and UV) were used to improve LiP productivity of *S. lavendulae* R-St-1.

Ethyl methanesulfonate mutagenesis

The spores of *S. lavendulae* R-St-1 from old culture (5 days) or 5 ml of 24-h old culture were centrifuged (5600*g*, 4°C for 3 min), suspended in sodium phosphate buffer (0.1 m, pH 7.0), and treated with 200 mm of EMS for 0, 20, 40, and 60 min. Appropriate dilutions were plated on FM medium and incubated at 37°C for 3 days. The growing colonies were counted against the control plates and transferred on a slant for further studies.

Ultraviolet light mutagenicity

A sterile Petri dish with a sterile magnetic rod was used to transfer cells or spores suspended in saline solution. The cells were irradiated using UV lamp with steering at 20-cm distance for 0, 3, 6, and 9 min. The treated cells were kept in dark for 2 h. to avoid photoreactivation. The FM medium plates were inoculated with diluted treated suspensions and incubated at 37°C for 48 h. The grown colonies were counted against *S. lavendulae* R-St-1 (W.T) control of the same dilution and transferred onto slants for further studies.

Protoplast fusion of *Streptomyces*

Test for antibiotic resistance

A preliminary test for antibiotic sensitivity was performed on mutant strains using various antibiotic discs containing (μg/disc) ampicillin (AM), 10; amoxicillin/clavulanic acid (AMC), 30; amoxicillin (AML), 25; chloramphenicol (C), 10; gentamycin (GM), 10; colistin (CT), 25; doxycycline (DO), 30; rifampicin SV (RF), 5; ampicillin/sulbactam (SAM), 20; and streptomycin (S), 10.

Protoplast formation, fusion, and regeneration

Different isolate cultures were cultivated for 40 h at 30°C in 25 ml of trypticase soy broth (40 g/l) and glycine (1.5%). Hopwood *et al.* [34] and Khattab and El-Bondkly [35] described the formation, fusion, and regeneration of protoplasts; nevertheless, P medium included 20% sucrose, and regeneration medium R5 contained 15% sucrose.

Molecular analysis of novel mutants and fusants

Molecular analysis of mutants using PCR was performed by 2×PCR Master Mix Solution (i-StarTaq), Hot-Start (iNtRON Biotechnology Inc., South Korea Product Catalog No: 25166). The 1× PCR Master Mix vials contain all of the necessary reagents, except primer and DNA template, for in vitro DNA amplification. The three different primers used in the study were supplied by Operon Technologies Inc., Venlo, Netherlands. The sequences for the three primers were as follows: first primer (P1) 5′- GTG TTG TGG TCC ACT-3′, second primer (P2) 5′-GTG TTG TGG TCC ACT-3′, and third primer (P3) 5′-AAC CTC CCC CTG ACC-3′. Random primer (12 ng) and purified DNA sample (40 ng) were added to each PCR bead. The total amplification cocktail volume was made to 25 μl using sterile distilled water. The amplification was performed according to the following protocol: step 1: denaturation of double-stranded DNA at 95°C for 1 min; step 2: primer annealing to template DNA for 1.5 min according to GC ratio of each primer; step 3: extension, where DNA polymerization was done by incubation at 72°C for 1 min. These three steps were repeated 35 times. At the end, PCR was kept at hold at 4°C. The amplified DNA products from RAPD analysis were separated based on size through gel electrophoresis. These DNA products were loaded into precast wells on 1.0% agarose gel and 1×Tris/Borate/EDTA buffer, and a current of consistent 100 volts was applied for about 2 h. The different separate bands were determined against 1-kb ladder (iNtRON Biotechnology Inc., South Korea). The separated bands were stained with 0.5 μg/ml ethidium bromide and photographed using Gel Documentation System with a UV transilluminator.

Results and discussion

The importance of isolation of new strains of *Streptomyces* from nature becomes a very important program to produce new high-productivity LiP strains. In the present study, *Streptomyces* strain was selected as a high LiP producer and exposed to random mutagenesis with both EMS and UV rays, and mutants were screened to improve strain ability for LiP production before the advent of protoplast fusion technology. Different authors reported that *Streptomyces* are capable of degrading lignin [9]. Pasti *et al.* [36] isolated 11 Actinomycete strains that degrade lignocellulose, lignin, and carbohydrates. *Streptomyces spp*. have also been isolated from the soil and screened for lignin degradation [37–42]. The highest LiP producer *S.*

lavendulae R-St previously obtained [15] was used to construct new higher LiP-producing mutant strains via EMS and UV rays. Protoplast fusion of most LiP production mutant strains was performed followed by application of RAPD-PCR to study the genetic diversity of the wild *S. lavendulan* R-St-1 compared with its mutants and fusants.

Streptomyces lavendulae R-St-1 (W.T) mutation using ethyl methanesulfonate

The original culture of *S. lavendulae* R-St-1 (W.T) was treated with 200 mm of EMS mutagen for 20, 40, and 60 min (Table 1) to show the effects of EMS on survival percentages. Spore suspension of *S. lavendulae* R-St-1 (W.T) exposed to EMS resulted in 63.2, 84.55, and 94.01% lethality rates at 20, 40, and 60 min, respectively. The results showed that the survival percentages decreased sharply by increasing exposure time to EMS. EL-Shaer *et al.* [43] reported also that the survival percentages significantly decreased with increasing doses of treatment when *Streptomyces* was exposed to EMS mutagen. Results are similar to other authors [35,44], who reported that both EMS and UV were the most used mutagens for improvement of bacterial strain by producing a variety of point mutations in the DNA of *Streptomyces*.

Ultraviolet light

Spore suspension of *S. lavendulae* R-St-1 (W.T) exposed to UV light showed clearly that the survival percentages decreased gradually by increasing the exposure period to UV light (Table 2). The survival percentages were 38.82 and 4.44 after 3 and 9 min, respectively. Strain-improvement strategies, especially mutagenesis and screening of hyper-producing mutants, are very important in secondary metabolite production [45]. To induce random mutations, UV radiation is the most common physical way [46]. In the present study, spore suspension of *S. lavendulae* R-St-1 (W.T) exposed to UV light resulted in 61.18, 86.63, and 95.56% lethality rates at 3, 6, and 9 min, respectively. The increase in the time duration of UV radiation led to a decrease in the number of mutant colonies of *S. lavendulae* R-St-1, which is in agreement with previous work [47], who reported that UV radiation of *Streptomyces griseorubens* E44G for the time duration of 5, 10, and 15 min resulted in 57, 62, and 87% lethality rates, respectively.

Lignin peroxidase productivity after Ethyl methanesulfonate treatment

The LiP activity of the wild-type and mutant strains of *S. lavendulae* R-St-1 (W.T) after treatments with 200 mm of EMS (20 min) is shown in Table 3. The results revealed that eight of 10 mutants showed high activity of LiP. The highest yield (6.4, 6.2 and 5.8 U/ml) was obtained by Rb/20/9E, Rb/20/6E, and Rst/20/10E (mutants), respectively, compared with 4.7 U/ml obtained by *S. lavendulae* R-St-1 (W.T), which enhanced the LiP productivity up to 36.17, 31.91, and 23.40%, respectively, over the original isolate. The results obtained by the eight mutants have shown an improved enzyme production over the wild type, and the two mutants showed lower LiP activity than the wild type. The results after 40 min of treatments with 200 mm of EMS (Table 4) showed that nine of 10 mutants produced LiP higher than their parental isolate. The highest yield (7.3, 7, 6. 7, and 6.6 U/ml) was obtained by Rb/40/8E, Rb/40/5E, Rst/40/6E, and Rst/40/7E (mutants), respectively, compared with 4.3 U/ml obtained by *S. lavendulae* R-St-1 (W.T). These mutants gave a higher activity of LiP, which exceeded their original isolates, with 155.32 and 148.94, 142.55, 140.43%, respectively. The results after 60 min of treatment (Table 5) showed that 10 of 10 mutants produced LiP higher than their parental

Table 1 Survival of *Streptomyces lavendulae* R-St-1 (W.T) spores after ethyl methanesulfonate exposure

Exposure time (min)	Counted colonies	Survival (%)
0	2272	100
20	836	36.80
40	351	15.45
60	136	5.99

Table 2 Survival of *Streptomyces lavendulae* R-St-1 (W.T) spores after ultraviolet light exposure

Exposure time (min)	Counted colonies	Survival (%)
0	1937	100
3	752	38.82
6	259	13.37
9	86	4.44

Table 3 Lignin peroxidase of the selected *S. lavendulae* R-St-1 ethyl methane sulfunate mutants at 20 min ethyl methane sulfunate exposure

Mutant no.	Lignin peroxidase (U/ml)	Percentage from W.T
R-St-1 (W.T)	4.7	100
Rst/20/1 E	5.4	114.89
Rst/20/2 E	4.8	102.13
Rst/20/3 E	4.5	95.74
Rst/20/4 E	5.6	119.15
Rst/20/5 E	5.0	106.38
Rst/20/6 E	6.2	131.91
Rst/20/7 E	5.6	119.15
Rst/20/8 E	3.5	74.47
Rst/20/9 E	6.4	136.17
Rst/20/10 E	5.8	123.40

Table 4 Lignin peroxidase of the selected *Streptomyces lavendulae* R-St-1 ethyl methane sulfunatemutants for 40 min ethyl methane sulfunate exposure

Mutant number	Lignin peroxidase (U/ml)	Percentage from W.T
R-St-1 (W.T)	4.7	100
Rst/40/1 E	6.2	131.91
Rst/40/2 E	3.8	80.85
Rst/40/3 E	5.8	123.40
Rst/40/4 E	4.8	102.13
Rst/40/5 E	7.0	148.94
Rst/40/6 E	6.7	142.55
Rst/40/7 E	6.6	140.43
Rst/40/8 E	7.3	155.32
Rst/40/9 E	6.4	136.17
Rst/40/10 E	5.5	117.02

Table 5 Lignin peroxidase of the selected *Streptomyces lavendulae* R-St-1 ethyl methane sulfunate mutants for 60 min ethyl methane sulfunate exposure

Mutant number	Lignin peroxidase (U/ml)	Percentage from W.T
R-St-1 (W.T)	4.7	100
Rst/60/1 E	5.8	123.40
Rst/60/2 E	5.2	110.64
Rst/60/3 E	7.3	155.32
Rst/60/4 E	6.8	144.68
Rst/60/5 E	8.2	174.47
Rst/60/6 E	7.6	161.70
Rst/60/7 E	8.5	180.85
Rst/60/8 E	6.2	131.91
Rst/60/9 E	5.8	123.40
Rst/60/10 E	6.3	134.04

Table 6 Lignin peroxidase of selected selected *Streptomyces lavendulae* R-St-1 ultraviolet mutants at 3 min ultraviolet –light exposure

Mutant number	Lignin peroxidase (U/ml)	Percentage from W.T
R-St-1 (W.T)	4.7	100
Rst/3/1 U	3.8	80.85
Rst/3/2 U	5.3	112.77
Rst/3/3 U	4.8	102.13
Rst/3/4 U	6.2	131.91
Rst/3/5 U	6.0	127.66
Rst/3/6 U	5.8	123.40
Rst/3/7 U	5.2	110.64
Rst/3/8 U	4.6	97.87
Rst/3/9 U	5.7	121.28
Rst/3/10 U	5.4	114.89

Table 7 Lignin peroxidase of the selected *Streptomyces lavendulae* R-St-1 ultraviolet mutants at 6 min ultraviolet –light exposure

Mutant number	Lignin peroxidase (U/ml)	Percentage from W.T
R-St-1 (W.T)	4.7	100
Rst/6/1 U	6.3	134.04
Rst/6/2 U	5.6	119.15
Rst/6/3 U	5.3	112.77
Rst/6/4 U	5.7	121.28
Rst/6/5 U	5.2	110.64
Rst/6/6 U	5.8	123.40
Rst/6/7 U	7.0	148.94
Rst/6/8 U	5.2	110.64
Rst/6/9 U	4.6	97.87
Rst/6/10 U	6.8	144.68

isolate. The highest yields of 8.5 and 8.3 U/ml were shown by Rb/60/7E and Rb/60/5E (mutants), respectively, compared with 4.7 U/ml by *S. lavendulae* R-St-1 (W.T). These two mutants (Rb/40/7E and Rb/40/5E) gave an activity of LiP that exceeded their original isolate, with 80.85 and 74.47%, respectively. It could be concluded that the highest promising mutants for LiP production were three mutants as a result of 20 min of exposure time, that is, Rst /20/9E, Rst/20/6E, and Rst/20/10 E, which produced 6.4, 6.2, and 5.8, respectively. Three mutants, that is, Rst/40/8E, Rst/40/5E and Rst/40/6E, as a result of 40 min of exposure produced 55.32, 48.94, and 42.55%, respectively, being higher than the original isolate. Two mutants were obtained from 60 min of exposure time, that is, Rst/60/7E and Rst/60/5E, which produced 80.85 and 74.47%, respectively, being higher than the wild-type isolate. The highest production of activity of LiP could be a result of the improvement in the efficiency of lignin-degrading ability of *Streptomyces* sp. strain with mutation. It is one of the best strategies for Actinomycetes' efficiency enhancement, where EMS causes GC to AT transversions [Lanoot et al. 2005] [48] induced genetic variability in different *Streptomyces spp*. through chemical mutagenic agents [27–29], and gene cloning [49] paved the way for successful genetic studies for lignin conversion improvement.

Lignin peroxidase productivity after ultraviolet treatments

Table 6 represents the LiP production of selected mutants out of the survived isolates after exposure of *S. lavendulae* R-St-1 (W.T) to UV light for 3.0 min compared with the untreated original isolate. The results indicated that most mutants produced LiP higher than their original parental isolate, where Rst/3/4 U, Rst/3/5U, and Rst/3/6 U showed 131.91, 127.66, and 123.40% enhancement in LiP productivity, respectively.

Moreover, the results indicated that two *S. lavendulae* R-St-1 UV mutants at 3 min of UV-light exposure produced lower LiP than their parental isolate. Table 7

represents the LiP productivity of 10 selected *S. lavendulae* R-St-1 UV mutants at 6 min of UV-light exposure compared with its parental isolate productivity. The obtained results showed that the majority (nine out of 10) of mutants produced LiP higher than their original parental strain. The highest record of the LiP production was 7.0 U/ml (with 48.94% more than the original untreated isolate), which was obtained from the mutant Rst/6/7U. Table 8 shows the LiP activity of the selected mutants after treatment of *S. lavendulae* R-St-1 with UV-light for 9 min. The results revealed that all of the other mutants produced more LiP than the untreated original isolate. In the meantime, the percentages that increased ranged from 14.89 (mutant Rst/9/4U) to 65.96 (mutant Rst/9/2 U). Regarding the results of *S. lavendulae* R-St-1 (W.T) exposed to different UV exposure times, it could be clearly concluded that the highest promising mutants for LiP production were one mutant, that is, Rst/3/4U, after 3 min exposure time, which produced 131.91%; two mutants, that is, Rst/6/7 U and Rst/6/10 U, after 6 min of exposure time, which produced 148.94 and 144.68, respectively; and one excellent mutant, that is, Rst/9/6U, after 9 min of exposure time, which produced 200.00%. The results are similar to those of Khattab and El-Bondkly [35] and EL-Sherbini and Khattab [44], who reported that UV and EMS are two of the most used mutagens for bacterial strain improvement, producing a variety of point mutations in *Streptomyces* bacterial DNA. Increasing the enzyme productivity of *Streptomyces* using UV light and EMS as a mutation-inducer has been recommended [17,18]. The mutants induced by UV were more stable through long-term generation and subculturing [50]. In addition, UV light also induced tolerance to different environmental stresses and changes in the synthesis of protein [51]. Furthermore, UV light is safe in use and causes no pollution. Genetic improvement of the chitinolytic activity of *S. griseorubens* E44G was done using chemical, physical, and site-directed mutagenesis [48]. Although UV radiation, as a physical mutagen, was irradiated on the tested bacteria for different durations (5, 10, and 15 min), no change in the chitinolytic activity was observed when compared with the wild type. Physical and chemical mutagens have been used to obtain new microorganisms with improved biocontrol potentiality and/or antibiotics production [45].

Protoplast fusion of *Streptomyces lavendulae* R-St-1 and lignin peroxidase productivity

As previously stated, protoplast fusion was the primary method for transferring genetic material between two cell types and obtaining new gene recombinants for the purpose of isolating greater LiP-producing fusants (s).

Response of some *Streptomyces lavendulae* R-St-1 and selected mutants to different antibiotics

S. lavendulae R-St-1 as the original isolate; two EMS-treated mutants as the best ligninolytic efficiency mutants designated as Rst/60/7E and Rst/40/8E, which showed activity of 8.5 and 7.3 U/ml, respectively; and two UV-treated mutants Rst/9/2U and Rst/9/6U, which showed an activity of 9.4 and 7.8 U/ml, respectively, were chosen to assess antibiotic resistance or sensitivity as an additional selection marker to investigate the influence of intraspecific protoplast fusion on LiP production (Table 9).

Table 8 Lignin peroxidase of the selected *Streptomyces lavendulae* R-St-1 ultraviolet mutants at 9 min ultraviolet light exposure

Mutant number	Lignin peroxidase (U/ml)	Percentage from W.T
R-St-1 (W.T)	4.7	100
Rst/9/1 U	6.2	131.91
Rst/9/2 U	7.8	165.96
Rst/9/3 U	6.5	138.30
Rst/9/4 U	5.4	114.89
Rst/9/5 U	5.6	119.15
Rst/9/6 U	9.4	200.00
Rst/9/7 U	7.7	163.83
Rst/9/8 U	6.4	136.17
Rst/9/9U	6.3	134.04
Rst/9/10 U	7.6	161.70

Table 9 Antibiotic susceptibility of the four selected *Streptomyces lavendulae* R-St-1 hyper-producing ethyl methane sulfunate mutants

Isolate	Antibiotic disc								
	*AM +10	AMC 30	AML 25	CN 10	CT 25	DO 30	RF 30	SAM 20	S 10
R-St-1 (W.T)	+	+	–	–	+	+	–	–	+
Rst/40/8E	+	+	–	+	+	–	+	+	+
Rst/60/7E	+	+	–	+	+	–	–	+	+
Rst/9/2U	+	–	+	+	+	+	+	+	+
Rst/9/6U	+	+	–	–	+	–	+	+	+

AMC, amoxicillin; AML, amoxicillin/clavulanic acid; CN, gentamycin; CT, colistin; DO, doxycycline; RF, rifampicin SV; SAM, ampicillin/sulbactam; S, streptomycin. *R*, resisted (no clear zone). +, concentration (μg/disc). *AM, ampicillin. **Means of diameter of inhibition zones (mm).

Streptomyces lavendulae R-St-1 mutants' protoplasts, fusion, and regeneration

Enzymatic treatments and subsequent analysis of the treated bacterial cells using a phase-contrast microscope revealed that the progressive disintegration of the bacterial cell wall began after lysozyme addition, incubated at 37°C for 60 min, as shown in Fig. 1, which described the protoplasting of *S. lavendulae*. For protoplast fusion and regeneration experiments, aliquots (500 µl) of both parental protoplast suspensions were used.

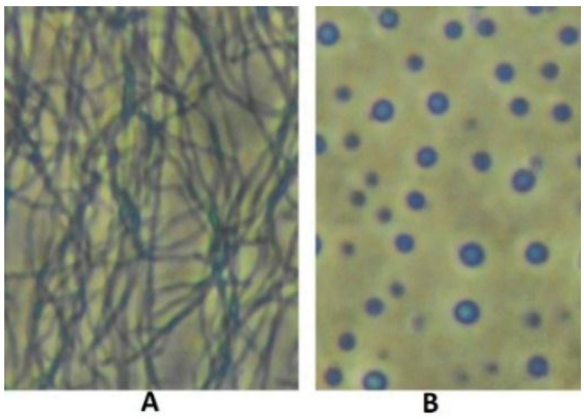
Lignin peroxidase productivity of fusants obtained after intraspecific protoplast fusion between *Streptomyces lavendulae* R-St-1mutants

In this study, improvement of *S. lavendulae* R-St-1 mutants for ligninolytic activity by protoplast fusion was attempted. Two distinct crosses were carried out to examine the influence of intraspecific protoplast fusion on LiP productivity. The parents were dissimilar in terms of productivity and antibiotic sensitivities. At the level of intraspecific protoplast fusion, Table 10 shows the LiP productivity of the parents and fusants chosen based on the screening assay after cross 1. Cross 1 was carried out between the two highest LiP generating mutants (Rst/9/6U and Rst/60/7E). The results revealed that seven out of the seven fusants produced more LiP than both parents. The fusant C1/St/5 produced 172.34% more LiP activity than the original wild-type isolate and 36.17% more than its higher producer parent (Rst/9/6U). Table 11 shows the LiP productivity of seven fusants chosen based on the screening assay after intraspecific protoplast fusion between two high LiP-producing mutants, Rst/9/6U, and Rst/9/2U. Seven fusants were detected, all of which produced more LiP than the initial isolate.

Six of the seven isolates had better LiP productivity than their superior parent (Rst/9/6U). C2/St/3 was the best fusant in this cross, producing 146.81% more LiP than the original isolate and 23.40%more than the higher parent (Rst/9/6U). In general, it could be concluded that intraspecific protoplast fusion proved to be an effective tool to improve LiP production. In addition, protoplast fusion between two higher LiP-producing mutants (cross 1 and 2) proved to be the most effective one since the enhancement of LiP productivity reached up to 172.34 and 151.06% higher than the original isolate for the two fusants (C1/St/5 and C1/St/6), which showed activity of 12.8 and 11.8 U/ml, respectively, of the selected fusants after a protoplast fusion between Rst/9/6U and Rst/60/7 E mutants of *S. lavendulae* R-St-1 (W.T).

Protoplast fusion recombinants were developed and assayed for their ligninolytic activity and production of LiP. In comparison with the mutants and wild strain, fusion recombinant C1/St/5 and C1/St/6 showed higher peroxidase activity. Highly enzyme activity was produced by the mutant of different species of

Figure 1



Photomicrographs of *S. lavendulae* R-St-1 protoplasts (B) in comparison with normal mycelia (A). *S. lavendulae, Streptomyces lavendulae.*

Table 10 Lignin peroxidase of the selected *Streptomyces lavendulae* R-St-1 fusants of Rst/9/6U and Rst/60/7E mutants

Fusant number	Lignin peroxidase (U/ml)	Percentage from higher P1	Percentage from W.T
R-St-1 (W.T)	4.7	50.00	100.00
P1-Rst/9/6U	9.4	100.00	200.00
P2-Rst/60/7E	8.5	90.43	180.85
C1/St/1	8.8	93.62	187.23
C1/St/2	9.7	103.19	206.38
C1/St/3	11.3	120.21	240.43
C1/St/4	8.9	94.68	189.36
C1/St/5	12.8	136.17	272.34
C1/St/6	11.8	125.53	251.06
C1/St/7	10.4	110.64	221.28

Table 11 Lignin peroxidase of the selected *Streptomyces lavendulae* R-St-1 fusants between Rst/9/6 U and Rst/9/2U mutants

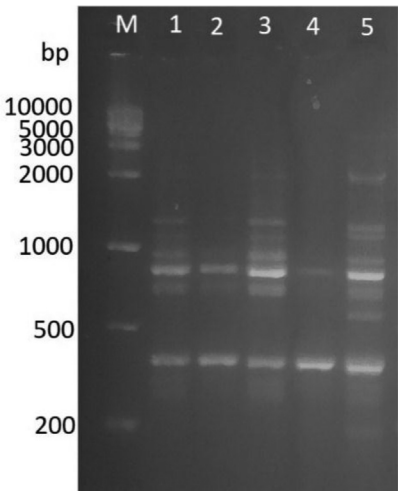
Fusant number	Lignin peroxidase (U/ml)	Percentage from higher P1	Percentage from W.T
R-St-1 (W.T)	4.7	50.00	100.00
P1- Rst/9/6U	9.4	100.00	200.00
P2- Rst/9/2U	7.8	82.98	165.60
C2/St/1	8.4	89.36	178.72
C2/St/2	9.6	102.13	204.26
C2/St/3	11.6	123.40	246.81
C2/St/4	10.6	112.77	225.53
C2/St/5	9.7	103.19	206.38
C2/St/6	10.6	112.77	225.53
C2/St/7	9.8	104.26	208.51

Streptomyces [17–19]. Moreover, one of the methods to obtain genetic manipulation is protoplast fusion, which promotes recombination. Furthermore, protoplast fusion led to producing superior enzyme-producing *Streptomyces* fusants [26,27]. In the present work, we demonstrated that by induction of mutation using EMS and ultraviolet rays and then protoplast fusion, we can continuously construct superior strains for LiP productivity. Moreover, to enhance *S. noursei* NRRL 1714 antifungal productivity (nystatin), it was treated with UV and then followed by intraspecific protoplast fusion; three of 114 mutants produced at least 49% more nystatin than the wild strain [35]. For the identification of protoplast fusion recombinants, some kind of marker is necessary [52]. Sivakumar *et al.* [27] reported that recombinants from fusion were screened and then the positive strains were later assayed for ligninolytic enzyme production, and 3 of 13 recombinants showed high ligninolytic activity. In comparison with the mutants and wild strain, fusion recombinant F4 showed higher laccase activity and lower peroxidase activity.

Molecular characterization of the superior lignin peroxidase-producing mutants and fusants of *Streptomyces lavendulae* R-St-1 (W.T)

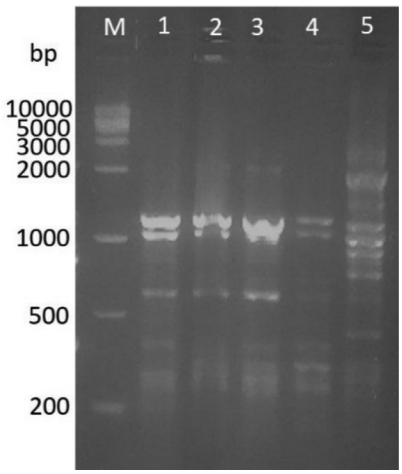
To study the change of mutagenesis treatments and protoplast fusion crosses on the nucleotide sequence of the two (Rst/9/6U, Rst/60/7E) DNA mutants and two (C1/St/5 and C1/St/6) fusants with the greatest LiP production relative to the original strain *S. lavendulae*, RAPD analysis utilizing three 15-mer random primers was used. Using primer-1, the results revealed obvious changes in band number and size between the original strain, its mutants, and fusants (Fig. 2). Six amplified bands with sizes of 1600, 1330, 110, 850, 730, and 300 bp were found (lane 1) when the template used was the wild type strain DNA. The same amplified three band sizes (850, 730, and 300 bp) of wild-type strain were found with Rst/9/6U mutant (lane 2). Six bands were detected for the mutant Rst/60/7E (lane 3), as the same as the parental isolate, and an additional band was also detected with size of 2700 bp. The same amplified three band sizes of 850 and 300 bp of wild-type strain were found with fusant C1/St/5 (lanes 4), whereas fusant C1/St/6 had five new unique bands, with molecular weights of 1470, 750, 650, 520, and 250 bp. Using primer 2, the results revealed changes in band number and size between *S. lavendulae* R-St-original strain, its mutants, and fusants (Fig. 3). Five amplified bands with sizes of 1350, 1100, 650, 350, and 190 bp were observed (lane 1) when the template used was the wild-type strain DNA. The same amplified three band sizes of 1350, 1100, and 650 bp of wild-type strain were found with Rst/9/6U mutant (lane 2), as the same as the parental isolate, and an additional band was also detected with size of 2100. Seven bands (2100, 1350, 1100, 350, 320, and 190 bp) were detected in lane 3 for the mutant Rst/60/7. Sex bands (2100, 1350, 1100, 350, 320, and 190 bp) were detected for the mutant Rst/60/7E (lanes 3 and 4), as the same as the parental isolate, and two additional bands was also detected with size of 320–2100 bp. The same amplified three band sizes of 1350, 350, 320, and 190 bp of wild-type strain were found with fusant

Figure 2



RAPD-amplified banding profiles using a primer (P1) for two different mutants and fusants (lanes 2–5) *S. lavendulae* R-St-1(W.T) (lane 1) opposite to DNA Marker,iNtRON Biotechnology Inc., South Korea, (lane M). The mutants and fusants sequence is as follows: Rst/9/6U, Rst/60/7E, C1/St/5, and C1/St/6. RAPD, random amplified polymorphic DNA.

Figure 3

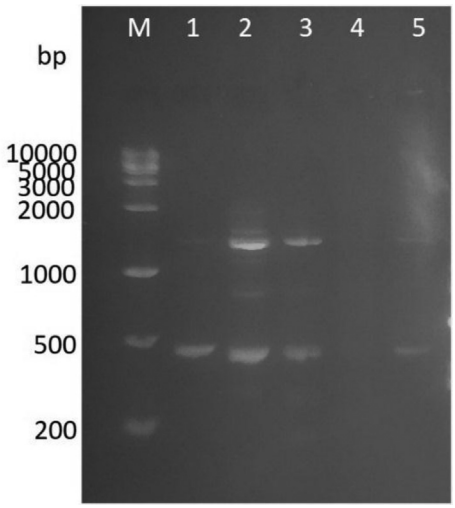


RAPD-amplified banding profiles using primer (P2) for two different mutants and fusants (lanes 2–5) *Streptomyces lavendulae* R-St-1 (W.T) (lane 1) opposite to DNA Marker (lane M).The mutants and fusants sequence are as follows Rst/9/6U, Rst/60/7E, C1/St/5, and C1/St/6. RAPD, random amplified polymorphic DNA.

C1/St/5 (lanes 4), whereas fusant C1/St/6 had 7 new five unique bands, with molecular weights of 2650, 1800, 1500, 1200, 900, 750, and 400 bp. Using primer-3, the results revealed changes in band number and size between *S. lavendulae* R-St-original strain, its mutants, and fusants (Fig. 4). In the parental isolate, one band (430 bp) was discovered (lane 1). The mutants Rst/9/6U (lanes 2 and 3) produced the same molecular weight bands (2050, 950, 430 bp) as the Rst/60/7E. Unlike the Fusant C1/St/5, which had no bands, the fusant C1/St/5 had two molecular weight bands (2050 and 430 bp).

The DNA loci of obtained banding profiles when using primer P1 were 12 loci, primer P2 were 17 loci, and primer P3 were three loci after RAPD assay, as shown in Table 12. Furthermore, primer P2 produced the most polymorphic loci, whereas primer P3 produced the least polymorphic bands. After using the primers P1 and P2, 14 unique loci were obtained, whereas the P3 primer had no loci. After all, primers had been applied, and there were no monomorphic loci visible.

Figure 4



RAPD-DNA amplified banding profiles using a primer (P3) for two different mutants and fusants (lanes 2–5) *Streptomyces lavendulae* R-St-1(W.T) (lane 1) opposite to DNA Marker (lane M). The mutants and fusants are as follows: Rst/9/6U, Rst/60/7E, C1/St/5, and C1/St/6.

RAPD banding patterns differ owing to variations in DNA nucleotides (fingerprints). Changes in the primer's annealing nucleotide sequences may prevent annealing, as seen by the removal of the identical amplified band. Mutations can induce new annealing sites to appear in the template (genome), resulting in new bands. RAPD assays have been used in a few studies to detect bacterial isolates, mutations, and fusants.Phylogenetic tree of *Streptomyces lavendulae* mutants and fusants based on PCR-random amplified polymorphic DNA assay

The exact relationships between different band types may be acquired using PCR-RAPD data, and genetic links can be derived as in Table 13. The genetic relationship between the mutants Rst/9/6U and Rst/60/7E was significant (72.00%), and then between mutant Rst/60/7E and the parental isolate was (62.10%). A small genetic relation matrix (8.3%) was established between fusantsC1/St/5 and C1/St/6, on the contrary. The artery pattern using RAPD distances revealed that the mutants Rst/9/6U and Rst/60/7E are clustered into one first cluster, which is related to the parental isolate, which is located in the separate second group, according to the evolution tree (Fig. 5). Moreover, both fusants C1/St/5 and C1/St/6 fall into separate groups but are related to the parent isolate and the two excellent mutants (Rst/9/6U and Rst/60/7E). The aforementioned results showed complete conformity with the previous studies, which have demonstrated the genetic effects of mutagenic treatments by EMS or UV on mutants of *S. lavendulae* R-St-1 in comparison with their original strains. These investigations' findings, which included differences in band counts and sizes between the original strain and its mutations as determined by RAPD analysis, followed the same general pattern as our study [53–55]. In addition, a number of differences between mutants and the original strain have been found in another investigation [56], which also reported that with the use of UV radiation to induce mutation, these changes in RAPD patterns demonstrated the confirmation of genetic changes in the DNA in mutants. Additionally, it has been shown that applying the RAPD method on some superior

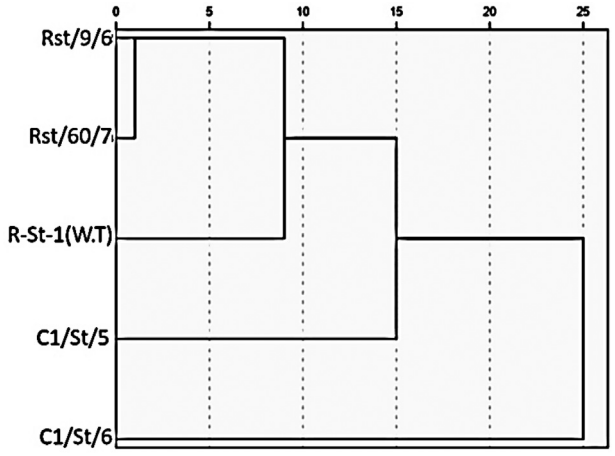
Table 12 Total amplified DNA loci and the percentages of polymorphic loci of *Streptomyces lavendulae* mutants and fusants observed using random amplification of polymorphic DNA assay with three random primers

Primer code	Unique loci	Polymorphic loci	Monomorphic loci	Total loci	Polymorphic percentage
P1	5	7	0	12	58.33
P2	9	8	0	17	47.06
P3	0	3	0	3	100.00
Total	14	18	0	32	–

Table 13 The proximity matrix between the tested mutants, fusants, and the parental *Streptomyces lavendulae* R-St-1 mutants and fusants based on random amplification of polymorphic DNA assay

Isolate code	R-St-1(W.T)	Rst/9/6 U	Rst/60/7E	C1/St/5	C1/St/6
R-St-1(W.T)	1.000	0.545	0.621	0.400	0.400
Rst/9/6U	0.545	1.000	0.720	0.500	0.308
Rst/60/7E	0.621	0.720	1.000	0.522	0.424
C1/St/5	0.400	0.500	0.522	1.000	0.083
C1/St/6	0.400	0.308	0.424	0.083	1.000

Figure 5



Phylogenetic tree between the tested mutants, fusants, and the parental *Streptomyces lavendulae* R-St-1 through RAPD method. RAPD, random amplified polymorphic DNA.

mutants leads to connecting the DNA changes of the mutants with a significant capacity of biosorption potential. Additionally, Gomaa and El-Mahdy [57] showed that the biocontrol efficiency was statistically much superior in mutant strains compared with wild-type strains. In addition to gamma radiation treatments, they also used RAPD-PCR technique to identify variations in DNA profiles between mutant and wild-type organisms.

As a result of findings and analysis, we may conclude that our research discovered a novel mutant and LiP-active fusants strain of *S. lavendulae*. The characterization of these superior mutants by RAPD fingerprint confirmed the genetic changes in the mutant with high LiP activity. The aforementioned findings show that mutagenesis is a viable method to enhance LiP activity for agricultural, industrial, and other objectives.

Conclusion

Streptomyces lavendulae R-St-1 strain was mutagenized with alkylating chemical EMS (200 mm) and UV. Results indicated that from the *S. lavendulae* R-St-1 (W.T) isolate, two EMS-treated mutants (Rst/60/7E and Rst/40/8E), which showed activity of 8.5 and 7.3 U/ml, respectively, and two UV-treated mutants (Rst/9/2U and Rst/9/6U), which showed an activity of 9.4 and 7.8 U/ml, respectively, were the most efficient ligninolytic mutants. Protoplast fusion between two higher LiP-producing mutants (cross 1 and 2) proved to be the most effective, and two isolated fusants C1/St/5 and C1/St/6 showed activities of 12.8 and 11.8 U/ml, respectively. after protoplast fusion between Rst/9/6U and Rst/60/7E mutants of *S. lavendulae* R-St-1 (W.T). To determine the molecular variability of two EMS mutants, and their recombinant fusants as well as *S. lavendulae* (W.T) (parental), three random primers were used. RAPD primer (P1) was employed. Fusant C1/St/5 shared the parental isolate with the bands of 850 and 300 bp, whereas fusant C1/St/6 had five new unique bands (1470, 750, 650, 520, and 250 bp). The DNA loci of obtained banding profiles using P1, P2, and P3 primers were 12, 17, and three loci after RAPD assay. Fourteen unique loci were obtained using the primers P1 and P2.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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