Pharmacophore, 11(4) 2020, Pages: 22-35

Pharmacophore

ISSN-2229-5402

Journal home page: http://www.pharmacophorejournal.com

Pharmacophore
an International Journal
ISSN 2229-5402

PHENOL BIOREMEDIATION BY A GENETICALLY IMPROVED STRAIN OF *CANDIDA TROPICALIS*

Shaza Y. A. Qattan

Biological Sciences Dept., Faculty of Science, King Abdulaziz University, P.O Box 80203, Jeddah, Kingdom of Saudi Arabia.

ARTICLE INFO

Received:
09 Apr 2020
Received in revised form:
19 July 2020
Accepted:
23 July 2020
Available online:
28 Aug 2020

Keywords: Phenol, Bioremediation, *Candida*, Molecular-identification, Genetic-improvement, ISSR-assay

ABSTRACT

The phenol biosorption is the best alternative to substitute or complete the current treatment forms for the elimination of phenol from the wastewater. A few phenol resistant isolates were obtained from soil specimens gathered from Jeddah, KSA. The yeast ShY-1 isolate removed 1.13 g/l of phenol, and it was considered as the highest phenol-removing isolate. It was identified as *Candida tropicalis* through BLAST analysis of the obtained ribosomal ITS region, then its phenol-uptake capacity was increased in relation to the induced genetic variations. The ShUV10/12 mutant was the best for phenol absorption (94%) compared to wild type strain (45.2%). The next mutant is ShUV10/7 where phenol was uptake by 90.4 % contrasted with the wild strain. Furthermore, protoplast fusion led to producing superior phenol-uptake fusants from yeast. The best phenol-uptake fusant was ShC1/3 which uptake 96.8 % contrasted with the wild strain (45.2%). Moreover, inter-simple sequence repeat (ISSR) techniques using polymerase chain reaction (PCR) on the best mutants and fusant were utilized to correspond the hereditary attributes of the best mutants and fusants with the outcomes acquired through the phenol-uptake experiments in examination with the wild strain. Furthermore, two mutants and the superior fusant comparisons with the wild strain were divided into different clusters. The phylogenetic tree was obtained and the genetic backgrounds of the selected mutants and fusant as well as the wild strain were detected according to the genetic distances.

Copyright © 2013 - All Rights Reserved - Pharmacophore

To Cite This Article: Shaza Y. A. Qattan, (2020), "Phenol bioremediation by a genetically improved strain of *Candida tropicalis*", ***Pharmacophore, 11(4),*** *22-35.*

Introduction

Bioremediation is viewed as a significant removal system in rewarding hydrophobic harmful organic materials in bioreactors [1]. The remediation by microbial uptake is the most recent method to substitute or complete current treatments for wastewater to remove the toxic phenol. [2, 3] When identifying the most dangerous pollutants, it was found that phenolic compounds were the most dangerous. The concentration of phenols in wastewater may differ from 10 to 300 mg/l, but this can reach to 4.5 g/l in the dirty wastewaters. The bioremediation of phenol is an essential process in environmental protection. Phenol is trademark contamination in wastewaters and effluents from raw petroleum, ceramic reactors, steel plants, coal transformation procedures, and phenolic resin industries and has been recognized as of late in river water, and in effluents from wastewater treatment reactors [4]. Moreover, during chlorination of water, the phenol converts to chlorophenols, which have low flavor and odor thresholds (below 1 μg/L) and are believed to be carcinogenic agents. The phenolic derivatives removal from fluid wastewater of factories is of commonsense centrality for environmental health.

In the last years, several manuscripts have focused on some microorganisms including bacteria, fungi and yeast which are able to bioremediate phenols, chloro-phenols, and nitro-phenols [5-8]. Numerous microorganisms capable to degrade phenol have been isolated [9-12]. However, the complete degradation of phenol compounds is not achieved and produces many toxic derivatives [13]. Also, the phenol degradation takes a lot of time. Also, Gonzalez et al. (2001) exhibited the high phenol biodegradation by *Pseudomonas putida*, and 1g L^{-1} phenol was removed after 11 days [14]**.** So, the remediation of phenol by yeast is the best way to eliminate the threat effects of phenol and its derivatives [15, 16].

The development of biosorbents could be accomplished by either screening the isolated microbial strains with elevated capacity, elevated specificity, or by producing improved microbial strains through mutagenesis and protoplast fusion [8, 16-19]. The literature review showed that adsorption investigations of natural operators are exceptionally low and just sorption of selected toxic organic agents onto different types of bacteria, fungi, and yeasts have been studied. The construction of new strains can be provided easily in classical cheap media and have elevated biomass and adsorption ability. So, yeast strains with elevated adsorption ability are important [18, 20]. The mutagenesis, a kind of a simple and attractive developing protocol, has broadly used to choose different sorts of high producer mutants, however, the manuscripts on utilizing them to

Corresponding Author: Shaza Y. A. Qattan, Biological Sciences Dept., Faculty of Science, King Abdulaziz University, P.O Box 80203, Jeddah, Kingdom of Saudi Arabia. Email: S.haza99 @ hotmail.com

improve the uptake of heavy metal were low [16, 21]. The yeast genera, such as *Candida, Rhodosporidium, Trichosporon,* and *Rodotorula* can remove significant amounts of phenol or phenolic agents [22, 23]. The protoplast fusion was also utilized for developing the yeast genomes [24-27].
The present work was directed to isolate different yeast strains under phenol stress from soil samples, Jeddah, KSA, and identify the phenol-uptaking yeast strains by sequencing the ribosomal ITS region. Also, the induction of mutation and protoplast fusion were applied to construct superior strains for the promotion of phenol bioremediation. Finally, the genetic variability of these new yeast strains was assayed by ISSR-analysis.

Materials and methods

Yeasts, media, and growth conditions

Yeast strains were stored on YEP medium (Glucose 20 g/L, Yeast extract 10 g/L, Peptone 10 g/L, and agar 20 g/L) and sub-cultured for 48 hrs at 30º C before using in the uptake experiments. Yeasts isolated from *different* soil samples collected from Jeddah, KSA, *diluted and spread onto the surface of YEP medium supplemented with phenol (1.5 % w/v) for detection of highly phenol-resistant strains.* UV-mutants and fusants obtained after mutagenesis and protoplast fusion of the parental strain(s). The strains were transferred to a fresh medium every month. The protoplast was obtained by protoplast medium [24] and regenerated after protoplast fusion by regeneration medium [25]. The protoplast buffers used for protoplasting and fusion were set up as indicated previously [26, 27].

Molecular identification of the yeast isolates

Firstly, the total DNA was isolated using the DNA extraction Mini BYF Kit (Intron Biotechnology Inc., South Korea). The quantity and purity of the obtained DNA were measured by calculating the UV-absorbance at 260nm and 280nm utilizing spectrophotometer (Shimadzu UV-VIS model UV-240, Shimadzu Scientific Instruments, Maryland, USA) [28]. Secondly, the fungal isolate identification was performed by molecular genetic analysis utilizing the ITS region. Partial sequences of 18S rDNA of the isolates were gotten utilizing a technique dependent on Boekhout et al. (1994) [29]. The amplification was performed utilizing two ITS primers; the forward primer (ITS1) sequence: 5' -TCCGTAGGTGAACCTGCGG-3'; the reverse primer (ITS4) sequence: 5'-TCCTCCGCTTATTGATATGC-3'. All primers were provided by Operon Technologies Company, Netherlands. To every polymerase chain reaction (PCR) bead, 12ng of the applied primer and 40ng of the purified DNA specimen were included. The all-out volume of the PCR mixture was adjusted to 25µl utilizing sterile refined water. The PCR protocol was done as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 1 min; primer annealing at 55°C for 2 min and incubation at 72°C for 2 min. The PCR products were kept at 4ºC till examination. The amplified DNA products were electrophoresed on 1.0% agarose gel and 1X TBE (Tris-borate-EDTA) buffer at a constant 100V for around 2 hrs. The sizes of different bands were resolved by the 100bp ladder. The PCR product was cleaned up using MEGAquick-spin™ Plus Total Fragment DNA Purification Kit (Intron Biotechnology Inc., South Korea). The DNA sequencing of the PCR product was completed by utilizing Applied Biosystems (ABI) 3730xl DNA-sequencer (GATC Biotech, Constance Germany) by using forward and reverse primers. The sequences were aligned with those of reference taxa recovered from open databases. The evolutionary distances were set up dependent on the parameter model and the phylogenetic tree was built by utilizing the neighbor-joining method in MEGA6 [30]. The ITS-DNA sequence was submitted to the NCBI Gene Bank under accession number MT422054.1.

Mutagenesis by UV-light

Three ml of *potent yeast* cells after overnight growth was lighted with Philips TUV-30-W-254 nm Lamp (Philips, Holland) for 5, 10, and 15 min at 20 cm. The irradiated yeast cells were placed (2hrs) in dark to bypass photo-reactivation repair. Suspensions then were diluted and spread onto the surface of YEP medium supplemented with phenol (1.5 % w/v) for selecting exceptionally phenol-resistant colonies. Then, the plates were placed for 3 days at 30°C. The highly well-growing colonies were refined on slants for additional investigations. The selected mutants with a high growth rate were then retested on a similar medium plate for 3 days at 30°C.

Antifungal response of mutants used as parental strains for protoplast fusion

To explore the impact of protoplast combination on phenol-uptake, three mutants that displayed the most elevated phenol-uptake were chosen to be utilized as parental strains for protoplast combination crosses. Besides, an extra-marker, for example, antifungal response to cycloheximide, griseofulvin, nystatin, and miconazole were determined for the selected mutants on YEP medium supplemented with cycloheximide (100 µg/ml), miconazole (5 µg/ml), nystatin 10 µg/ml), and griseofulvin (250 µg/ml). The plates were incubated at 30ºC for 48hrs before recording the antifungal resistance or sensitivity.

Protoplast formation

Yeast cells were cultured in 50 ml of protoplast medium and placed on a rotary shaking incubator (150 rpm) at 30ºC for 18 hrs. Cells were assembled by centrifugation and washed twice with sterile refined water. The washed cells were resuspended in the pre-treatment buffer, and the suspension was placed for 20 min at 35ºC with gentile disturbance. After incubation, cells were centrifuged again and resuspended in protoplast support supplemented with snail enzyme (1%, w/v) and incubated under shaking (120 rpm) in a water bath at 35ºC. The cells were analyzed discontinuously, utilizing a phase-contrast

microscope for the development of protoplasts. The change of cells into protoplasts was done in one hour. Protoplasts were centrifuged at 2500 rpm for 5 min at 4ºC and remixed in protoplast buffer.

Fusion of protoplasts
The combination of different protoplasts after mixing could be done between picked isolates which vary in either their resistance to antifungals and heavy metals or phenol-uptake levels as follow: Protoplasts from the two parents were mixed, centrifuged at 2500 rpm for 5 min and the supernatant was disposed of. Two ml of PEG solution were incorporated and mixed gently with protoplasts. The mixture of protoplasts was incubated up to 40-60 min at 30ºC. By then, the blend was diluted with 0.65 M KCl. 0.1 and 0.5 ml of the dilutions were overlaid on the recovery medium.

Batch phenol-uptake
Phenol (1.5 g/L) was included under aseptic condition after the wild yeast strains were grown on YEP medium for 48 h. at 30°C with shaking (120 rpm). Again, the flasks were incubated under the above same conditions for 24h and the remaining and uptake of phenol were determined. The initial concentration of phenol was 2.5 g/L when the phenol-uptake of mutants and fusants were assayed.

Phenol analysis by HPLC
To isolate the supernatant from the cultures after different *batch phenol-uptake* experiments, specimens were taken and centrifuged for 5 min in a micro-centrifuge at 8000 rpm. The supernatant was isolated utilizing a Millipore 0.22 µm channel. Tests were moved into a glass vial, fixed with a Teflon secured elastic top, and put at 4°C before investigation. *Different* concentrations were determined utilizing a high-performance liquid chromatography (HPLC). Specimens were confined on a Nora Pak Cl8 (3.9 X 150 mm) column. The wavelengths were set at 260 nm for detection. The column was kept up at 40°C and the flow rate of the mobile phase was done at 0.8 ml/min. A solvent gradient utilizing HPLC grade methanol (0-100% in 15 min) and 0.05 M sodium acetate at pH 4.7 was applied.

ISSR-analysis of some superior yeast strains by PCR
Firstly, the DNA from selected novel genetically improved strains was isolated according to the above protocol. Secondly, the molecular analysis by PCR of the mutant was applied using 2x PCR Master Mix Solution (i-StarTaq), Hot-Start (Intron Biotechnology Inc., South Korea Product Catalog No: 25166). The 1x PCR Master Mix containing all reagents, except primer and DNA template, was used for in vitro DNA amplification. In this study, three different primers obtained by Operon Technologies Inc, Venlo, Netherlands were used; first primer (ISSR1) 5'-GAGAGAGAGAGAGAGAT-3', second primer (ISSR2) 5'-CACACACACACACACAG-3' and third primer (ISSR3) 5'-CTCTCTCTCTCTCTC TTT -3'. ISSR-primer (12 ng) and purified DNA specimen (40 ng) were added to each PCR bead. The total 25 µl volume of the PCR-cocktail was adjusted by sterile distilled water. The amplification was performed as per the following protocol: Step 1: Denaturation of double-stranded DNA at 95ºC for 1 minute. Step 2: Primer annealing to template DNA for 1.5 min according to the GC ratio of the applied primer. Step 3: Extension-DNA polymerization by incubation at 72ºC for 1 min. These three steps were repeated 35 times. In the end, the PCR product was kept at 4ºC. The DNA products obtained after ISSR-assay were separated based on size through gel electrophoresis according to the above protocol.

ISSR-data Analysis
Amplification profiles of two mutants and one fusant were differentiated with each other as well as the wild type of *yeast strain*. The DNA fragments were registered as a binary data, where (1) implies nearness and (0) implies nonattendance of the band. The distance coefficients were determined by accompanying statistical equation: F= 2Nxy/ (Nx + Ny); where, F is the separation coefficient wherein Nx and Ny are the quantities of bands in genotypes x and y, respectively, and Nxy is the quantity of bands varied by the two genotypes [31]. The electrophoretic profiles of the reproducible banding *profiles* of every primer created by ISSR were picked for examination. Pairwise comparisons between mutants were made to compute the Jukes-Cantor coefficient utilizing the PAST program (PAleontological Statistics Version 1.94b) balanced by Hammer et al. (2001) [32]. Cluster analysis was performed to make an unweighted pair group method with arithmetical mean (UPGMA) denderogram.

Results

Screening for phenol resistant and uptake
In the beginning, five yeast isolates, which isolated from soil samples, Jeddah, KSA under the stress of 1.5 g/L of phenol, were collected for further studies. These strains exhibited excellent tolerance to the toxicity of the phenol at a concentration of up to1.5 g/L. The phenol-uptake of these strains was evaluated by batch phenol-uptake method under the same concentration of phenol and it was discovered that one yeast isolate (ShY-1) had the highest phenol-uptake as shown in **Table 1**. The yeast isolate (ShY-1) removed 1.13 g/l of phenol, and it was considered as the highest phenol-removing strain. The second highest phenol-removing strain was ShY-5 which removed 1.01 g/l of phenol. **Table 2** presents the phenol remediation potential of the strains in this study and the related previous studies. It is apparent that yeast isolate ShY-1 utilized in this examination has incredible potential for biosorption of phenol.

Molecular identification of yeast isolate ShY-1

To identify the isolated yeast strain ShY-1, an ITS-region nucleotides sequences examination was performed. The ITS-forward and reverse primers were used to amplify the ITS-region from the genomic DNA of the strain ShY-1. After the amplification by PCR, a product of ~500 bp was obtained **(Fig. 1).** The BLAST analysis of the amplified ITS-region sequence revealed 98.75% similarity to the ITS-region of *Candida tropicalis* (MH037233.1) strain. Based on this similarity, yeast (ShY-1) strain was identified as *Candida tropicalis* **(Fig. 2).**

Induction of mutation by UV-Light

The cell suspension of *Candida tropicalis* (strain ShY-1) was exposed to UV-mutagen for various periods (5, 10, and 15 min) and spread on the YEP medium after dilution. All colonies which showed up on the YEP medium after UV-exposure were assayed on the YEP and the YEP contained 1.5 g/L of phenol at 30°C. At that point, the colonies which were capable of growth on YEP plates supplemented with 1.5 g/L of phenol were registered as phenol-resistant mutants. It was detected that the percentage of phenol-resistant colonies expanded with the expansion of the exposure time up to 15 min i.e., it was 5.37% for 5 min exposure, 10.46% for 10 min, and 11.84% for 15 min (**Table 3**). On the contrary, the survival percentages were diminished with the expansion of the UV- exposure time i.e., it was 28.19% for 5 min. and diminished radically to arrive at 10.08 and 5.01% after 10 and 15 min of UV-mutagenesis, respectively.

Phenol-uptake after UV-mutagenesis

Phenol absorption of phenol-resistant colonies and wild type taken from every flask was evaluated after 72 hours. For this purpose, the yeast cells were centrifuged and washed multiple times with distilled refined water. The biological uptake of phenol by yeast cells is shown in **Tables 4-6**. The outcomes demonstrated a clear increase in the absorption of phenol by yeast cells after UV treatments. When all colonies were grown on the medium supplemented with phenol (2.5 g/L), the phenol-uptake was elevated compared with the wild isolate and it is ranged from 51.6 to 94.0% in comparison with the wild isolate (45.2%).

The phenol adsorption was evaluated by 23 colonies obtained after exposure to the physical UV-mutagenicity for 5 min (**Table 4).** It was found that only two of these colonies i.e., ShUV5/7 and ShUV5/20 uptake phenol less than the wild isolate. The mutant ShUV5/20 had the lowest absorption of phenol (34.4%) contrasted with the wild isolate (45.2%). Most mutants were superior in phenol absorption compared to the wild isolate, where 21 mutants were superior, and the ShUV5/18 mutant was (78%) compared to wild type isolate (45.2%). The next strain is ShUV5/3 where phenol was uptake by 77.2% contrasted with the wild isolate.

The phenol adsorption was evaluated in 16 colonies obtained after exposure to the physical UV-mutagenicity for 10 min (**Table 5**). It was found that all the obtained mutants are superior in phenol absorption contrasted with the wild strain, and the ShUV10/12 mutant was the best in phenol adsorption (94%) contrasted with the wild strain (45.2%). The next strain is ShUV10/7 where phenol was uptaken by 90.4% contrasted with the wild strain.

Finally, the phenol adsorption was evaluated in 9 colonies obtained after exposure to the physical UV-mutagenicity for 15 min (**Table 6**). It was found that all the obtained mutants were superior in phenol absorption contrasted with the wild strain, and the ShUV15/6 mutant was the best in phenol absorption (90%) contrasted with the wild strain (45.2%). The next strain was ShUV15/5 with phenol absorption of 87.2%.

Antifungal response of excellent mutants

To research the impact of protoplast combination on phenol uptake, three UV-mutants which showed the most noteworthy phenol-uptake were chosen to be applied as parental strains for protoplast fusion. Also, extra-marker, for example, antifungal resistance or sensitivity were measured for the used mutants. Results in **Table 7** demonstrated that the original ShY-1 strain and ShUV10/7 were resistant to cycloheximide, nystatin, and griseofulvin and sensitive to miconazole. Mutant ShUV10/12 was proved to be resistant to miconazole and nystatin and sensitive to cycloheximide and griseofulvin. Finally, the mutant ShUV15/6 was resistant to cycloheximide and griseofulvin and sensitive to miconazole and nystatin.

Phenol uptake after different protoplast fusion crosses

Any experiment with protoplast fusion undergone several stages. In the first stage, genetic markers were determined through resistance and sensitivity to antifungals. After that, the mutants used as parents were converted to protoplasts through the enzymatic treatment by snail enzyme as shown in **Fig. 3**. Through testing the resistance and sensitivity of antifungal agents, two crosses were made using different genetic markers. The first cross was done between mutant ShUV10/12 and mutant ShUV15/6 using the selected antifungal markers (cycloheximide and miconazole) as shown in **Table 8**. The obtained results showed that only one fusant (ShC1/5) exhibited phenol-uptake lower (87.2%) than the lower parent but it still elevated compared with the wild isolate (45.2%). The best fusant was ShC1/3 which uptake 96.8 % contrasted with the wild strain (45.2%). Moreover, the second cross was done between the same above parents but using the selected antifungal markers (nystatin and griseofulvin) as shown in **Table 9**. The obtained results showed that all fusants exhibited phenol uptake higher than the parent. The best phenol-uptaking fusant was ShC2/2 which uptake 96.2 % contrasted with the wild strain (45.2%).

ISSR analysis of the excellent mutants and the best fusant for phenol-uptake

An endeavor was led to assess the genetic impact of mutagenic treatments and protoplast combination on the nucleotide sequence of the selected mutant and fusant DNA against the wild isolate (*C. tropicalis,* ShY-1 strain). Utilizing ISSR-primer No.1 **(Fig. 4)**, it was obviously seen that four bands appeared when DNA of the wild isolate was utilized as a template. The bands' weights were 1250, 850, 700, and 400 bp. Moreover, the band 850bp was absent in all tested mutant and fusant strains. The other three bands found in the wild strain were found in the ShUV15/6 mutant and ShC1/3 fusant (tracks 3 and 4). Then again, in the ShUV10/12 mutant two sharped bands were observed with the weights of 1500 and 1000bp (track 2) which was not present in the wild type and other tested strains. Utilizing ISSR-primer No.2 **(Fig. 5)**, it was seen that four bands were present when DNA of the wild strain and ShUV15/6 mutant were utilized as a template; the bands' weights were 1400, 950, 600, and 500 bp as appeared in tracks 1 and 3. Moreover, the ShUV10/12 mutant and ShC1/3 fusant had two sharped bands with weights of 1400 and 950bp (tracks 2 and 4) which was not present it the wild strain and ShUV15/6 mutant. The two bands (600 and 500bp) were absent in the ShUV10/12 mutant and ShC1/3 fusant (tracks 2 and 4). Finally, using ISSR-primer No.3 **(Fig. 6)**, it was seen that three bands were present when DNA of the wild strain was utilized as a template. The bands' weights were 800, 600, and 300 bp as appeared in track 1. While, ShUV10/12 and ShUV15/6 mutants exhibited the same number of bands (4 bands) with weights 1250, 900, 680, and 300 bp as appeared in tracks 2 to 3. The ShC1/3 fusant (track 4) exhibited three bands with weights 900, 680, and 300 bp.
ISSR-protocol was used for measuring the genetic variation between selected parental mutants and superior fusant in comparison with the wild type strain (**Table 10**). The quantity of the acquired banding profiles with ISSR-1 primer were 12 bands, with ISSR-2 primer were 12 bands, and with ISSR-3 primer were 14 bands. Additionally, the most noteworthy polymorphic bands were acquired with the ISSR-1 primer and the limited polymorphic bands were seen with the ISSR-2 primer. Besides, no unique band was distinguished with the ISSR-2 primer, and the unique bands (3 bands) were identified with the ISSR-1 primer. Two unique bands acquired after utilizing ISSR-3 primer. At last, when utilized the ISSR-2 and ISSR-3 primers, the obtained monomorphic bands were 8 and 4 bands, respectively, but no monomorphic band was seen after the ISSR-1 primer was applied. Finally, the application of the ISSR-1 primer led to the highest polymorphic percentage (75%) followed by 57.14 and 33.33% displayed after using ISSR-3 and ISSR-2 primers, respectively.
As indicated by PCR-ISSR information, the specific connections between various bands can be acquired and through which genetic distances can be concluded as shown in **Table 11**. The genetic distance was high between the wild type strain and ShUV15/6 mutant (96.0%) and afterward (85.7%) between ShUV15/6 and ShC1/3 strains. Then again, a smaller than usual genetic distance matrix (57.1%) was obtained between ShUV10/12 and the wild type *C. tropicalis* ShY-1 strain.
As per the development tree **(Fig. 7)**, the artery design through ISSR distances indicated that the wild type strain and ShUV15/6 mutant are clustered into one cluster and this was related to the ShC1/3 fusant in the second cluster. The connection of the new great ShUV10/12 mutant with the first and second clusters explained the genetic diversity of this new great ShUV10/12 mutant. Moreover, **Fig. 7** revealed that the new genotype (ShUV10/12 mutant) that was thought to be a good phenol-uptaking maker was assembled in one *cluster* with the other strains.

Discussions

The present work was directed to isolate different yeast strains under phenol stress from soil samples, Jeddah, KSA. The isolated yeast colonies resistant to high phenol levels are candidates for phenol bioremediation [33]. In the related previous studies, Varma and Gaikwad (2008) isolated *C. tropicalis* from sludge which degraded 2 g/L of phenol after 48 hrs of growth [34]. Yan et al. (2005) isolated *C. tropicalis* strain from sludge which degraded 2 g/L of phenol after 66 hrs of growth [35]. Rigo and Alegre (2004) isolated *C. tropicalis* strain which removed 2 g/L of phenol after 48 hrs of growth [36]. Tsai et al. (2005) isolated *C. albicans* strain which removed 2.4 g/L of phenol after 250 hrs of growth [37]. Stoilova et al. (2006) reported that the *Aspergillus awamori* NRRL 3112 had degraded 300 mg/L phenol in 60 h, 600 mg/L in 72 h, and 1000 mg/L in 7–8 days [38]. There are various reports on phenol bioremediation by *pseudomonas putida* strain [39]; also, around 0.45 g/L of phenol concentration has been accomplished according to Juang and Tsai (2006) [40]. Yeast isolate ShY-1 used in this assessment has inconceivable potential for phenol biosorption.
The molecular methods were used to identify the isolate ShY-1. Based on this similarity, yeast strain ShY-1 was identified as of *Candida tropicalis.* Yeast identification can take the usefulness of molecular protocols, especially the analysis of the rDNA-loci polymorphism sequence at the region ITS1-5.8S-ITS2 [41-45].
When yeast isolate ShY-1 was identified at the molecular level, a genetic improvement program was introduced to increase the ability of the parental strain (*Candida tropicalis* ShY-1) to get rid of larger quantities of phenol. Therefore, the treatment was applied with physical mutagens such as UV rays. It was detected that the percentage of phenol-resistant mutants was elevated with the increase of the exposure time from 5 up to 15 min of UV rays and the percentage of survival was decreased with the increase of the exposure time from 5 up to 15 min of UV rays. This reduction in survival rate may be ascribed to the harm of nucleic acid or potentially surrenders in other cell parts caused after UV-mutagenesis. The above results were in harmony with those exhibited by Khattab et al. (2012) and Gangopadhyay et al. (2007) [8, 46]. Also, the effect of UV rays on the phenol biosorption of the parental strain (*Candida tropicalis* ShY-1) led to the increase of the biosorption potential of the parental strain and new mutant strains were found for phenol adsorption by 94% compared to wild type strain. The induction of mutations in genetic material can be induced by exposure to physical or chemical agents. UV-light is one of the physical methodologies for starting change in DNA by forming pyrimidine dimerization and cross-connects. Mutation induction by physical UV mutagenicity has been suggested as a tool to increase the biosorption and biodegradation [8, 22,

47-49]. Also, UV-mutants were more stable [50]. Furthermore, Alexievaa et al., (2004) rewarded the parent strain cells with NTG to acquire the mutants of *T. cutaneum* R57. This mutant had higher specific growth and degradation rates than those of the wild strain as the phenol hydroxylase activity was expanded. Khattab et al. (2012) recommended that the application of UV-mutagenesis protocol is the best way for increasing phenol-uptake up to 100.00% in comparison with the wild type (34.18%) by *R. toruloides.* Finally, mutational methodologies have been utilized for the increment of novel isolates, for example, upgraded bioremediation of a sulfonated azo color, Green-HE4B, in *Pseudomonas* sp. LBC1 [51], improved heterologous amylase creation in *Saccharomyces cerevisiae* [52], and upgrading polygalacturonase creation by *Aspergillus sojae* [53]. These transformations realized variations in making microbial products and enzymes, which can be utilized in industry.

The utilization of protoplast fusion as a tool in microbial hereditary qualities has applied for the breeding of commercial strains and gives a method for accomplishing the aims stated above. In the beginning, an extra-marker of antifungal response was measured for the used mutants after that the mutants used as parents were converted to protoplasts through the enzymatic treatment by snail enzyme and finally two crosses were made using different genetic markers. The best phenol-uptake fusant was ShC1/3 which uptake 96.8 % contrasted with the wild strain (45.2%). The outcomes concurred with those acquired by Chang *et al*. (1995). They increased phenol removal by *C. tropicalis* and obtained different mutants from a phenol-utilizing wild strain which was treated by UV-light and N nitrosoguanidine (NTG). Many of protoplast-fusants with high phenol-removing ability were recognized after protoplast combination happened between certain mutants. Two of the fusants had about 1.9 and 2.2 times elevated phenol hydroxylase compared with the wild isolate. Besides, the excellent phenol removing recognized with the fusant and lower sensitivity to phenol inhibition when contrasted with the original strain of *C. tropicalis* [54].

In the ISSR analysis to detect the changes in the nucleotide sequence of the selected mutant and fusant DNA against the wild isolate (*C. tropicalis,* ShY-1 strain), many different variations were detected and recorded. Also, two mutants and the best fusant comparison with the wild strain divided them into different clusters. The phylogenetic tree was prepared and the genetic backgrounds of the selected mutants and fusant as well as the wild strain were detected. *The above outcomes are in a similar trend as those displayed by* Schlick et al. (1994), Dassanayake and Samaranayake (2003), Barcelos et al. (2011), Khattab and Abd El-Salam (2012), Khattab et al. (2012), and Gallardo et al. (2014) [8, 55-59].

Conclusion

This investigation performed the screening on phenol-resistant colonies, their molecular identification, and the improvement of strong *C. tropicalis* strains for high phenol-uptake after UV-mutagenesis and protoplast fusion. Additionally, the ISSR-profiles changes of superior mutants and the excellent fusant in comparison with the wild strain were recognized and these progressions simultaneously demonstrated as biomarkers of genetic variations of superior mutants and excellent fusant of *C. tropicalis*. The assorted diversity of the genetically improved phenol-uptake strains into clusters through the phylogenetic program was applied and reflected the high genetic changes of some improved phenol-uptake strains.

Conflict of interests

The author has no competing interests to declare.

References

1. Zaidi H, Khiari A. Reduction Remediation of Hexavalent Chromium by Indigenous Bacteria. World Journal of Environmental Biosciences. 2018;7(3):43-5.
2. Tolon MA, Mirzaei H, Matin AA, Javadi A, Amani-gadim A. Environmental Monitoring of Bisphenol A in Ardabil's drinking water source as san endocrine disrupting chemical. Arch. Pharm. Pract. 2019;10(2):56-60.
3. Mohammadi H, Salehzadeh H, Khezri SM, Zanganeh M, Soori MM, Keykhosravi SS, Kamarehie B. Improving Nitrification and Denitrification Processes in Urban Wastewater Treatment Using Optimized MBBR Reactor Design Considerations. J. Biochem. Technol. 2020;11(1):85-100.
4. Jiang Y., Jianping, W., Jing, B., Daoquan W., Zongding H. Phenol biodegradation by the yeast Candida tropicalis in the presence of m-cresol. Biochem. Eng. 2006; J. 29: 227–234.
5. Aksu, Z., Yener, J. Investigation of the biosorption of phenol and monochlorinated phenols on the dried activated sludge. Process Biochem. 1998; 33: 649-655.
6. Bell, J.P., Tsezos, M. Removal of hazardous organic pollutants by adsorption on microbial biomass. Water Sci. Tech., 1987; 19: 409-416.
7. Wang, J.L., Qian, Y., Horan, N., Stentiford, E. Bioadsorption of pentachlorophenol (PCP) from aqueous solution by activated sludge biomass. Bioresour. Technol. 2000; 75: 157-161.
8. Khattab A.A., Abdel Ghafar H.H. Ibrahim, M.I.M. Enhancement of phenol biosorption by genetically improved of Rhodosporidium toruloides strains. J. Appl. Sci. Res. 2012; 8: 3600-3607.
9. Bayly, R.C., Wigmore, G.J. Metabolism of phenol and cresols by mutants of Pseudomonas putida. J. Bacteriol. 1973; 113:1112-1120.
10. Antai, S.P., Crawford, D.L. Degradation of phenol by Streptomyces setonii. Can. J. Microbiol. 1983; 29:142-143.

11. Berquist, P.L., Love, D.R., Croft, J.E., Streiff, M. B. Genetics and potential biotechnological application of thermophilic and extremely thermophilic microorganisms. Biotechnol. Genet. Eng. Rev. 1987; 5:199-204.
12. Pernyeszi, T., Farkas, V., Felinger, A., Boros, B., Imre, D. Use of nonliving lyophilized Phanerochaete chrysosporium cultivated in various media for phenol removal. Environ. Sci. Poll. Res. 2018; 25:8550–8562.
13. Rehfuss, M., Urban J. Alcaligenes faecalis subsp. phenolicus subsp. nov. a phenol-degrading, denitrifying bacterium isolated from a graywater bioprocessor. Syst. Appl. Microbiol. 2005; 28: 421–429.
14. Gonzalez G., Herrera, G., Garcıa, M.T., Pena, M. Biodegradation of phenol industrial wastewater in a fluidized bed bioreactor with immobilized cells of Pseudomonas putida, Bioresource Technol. 2001; 80: 137–142.
15. Varma, R., Gaikwad B. Biodegradation and phenol tolerance by recycled cells of Candida tropicalis NCIM 3556. Int. Biodeter. Biodegr. 2009; 63: 539–542.
16. Khattab A. A., Ihab A. M., Mohamed K. A. Molecular analysis of genetically improved therapeutic Saccharomyces cerevisiae strains with high selenium uptake. J. Amer. Sci. 2010; 6: 326-337.
17. Bae, W., Mulchandani, A., Chen, W. Cell surface display of synthetic phytochelatins using ice nucleation protein for enhanced heavy metal bioaccumulation. J. Inorg. Biochem. 2002; 88: 223-227.
18. Huang, C.C., Su, C.C., Hsieh, J.L., Tseng, C.P., Lin, P.L., Chang, J.S. Polypeptides for heavy-metal uptake: capacity and specificity of two heterogeneous MerP proteins. Enz. Microb. Technol. 2003; 33: 379-385.
19. Jiang Y., Wen, J., Jia, X., Caiyin, Q., Hu, Z. Mutation of Candida tropicalis by irradiation with a He-Ne laser to increase its ability to degrade phenol. Appl. Environ. Microbiol., 2007; 73: 226–23.
20. Yavuz, H., Denizli, A., Gungunes, H., Safarikova, M., Safarik, I. Biosorption of mercury on magnetically modified yeast cells. Sep. Purif. Technol. 2006; 52: 253-260.
21. Hedman, J. M., Eggleston, M.D., Attryde, A. L., Marshall, P.A. Prevacuolar compartment morphology in vps mutants of Saccharomyces cerevisiae. Cell Biol. Int. 2007; 31: 1237-1244.
22. Chang, S.Y., Li, C. T., Hiang, S.Y., Chang, M.C. Intraspecific protoplast fusion of Candida tropicalis for enhancing phenol degradation. Appl. Microbiol. Biotechnol. 1995; 43:534–538.
23. Alexievaa Z., Gerginova, M., Zlateva, P., Peneva, N. Comparison of growth kinetics and phenol metabolizing enzymes of Trichosporon cutaneum R57 and mutants with modified degradation abilities. Enzyme Microb. Technol. 2004; 34:242–247.
24. Khattab, A.A. Improvement of ethanol production by Sacharomyces cerevisiae using microbial biotechnology techniques. M.Sc., Genetics Dept., Fac. of Agric., Tanta Univ., Egypt, 1997.
25. Takagi, A., Harashima, S., Oshima, Y. Construction and characterization of isogenic series of S. cerevisiae polyploidy strain. Appl. Environ. Microbiol. 1983; 45: 1034-1040.
26. Spencer, T.F.T., Laud, P., Spencer, D.M. The use of mitochondria mutants in the isolation of hybrid involving industrial yeast strain. II: Use in isolation of hybrid obtained by protoplast fusion. Mol. Gen. Genet. 1980; 179:651-654.
27. Farahanak F., Seki, T., Ryu, D.D.Y., Ogrydziak, D. Construction of lactose-assimilating and high ethanol-producing yeasts by protoplast fusion. Appl. Environ. Microbiol. 1986; 51: 362- 367.
28. Sambrook, J., Fritsch, E.F., Maniatis, T. Molecular Cloning; A Laboratory Manual, Second Edition Cold Spring Harbor, 1989.
29. Boekhout, T., Kurtzman, C.P., O'Donnell, K., Smith, M.T. Phylogeny of the yeast genera Hanseniaspora (anamorph Kloeckera), Dekkera (anamorph Brettanomyces), and Eeniella as inferred from partial 26S ribosomal DNA nucleotide sequences. Int. J. Syst. Microbiol. 1994; 44: 781–786.
30. Saitou, N., Nei, M. The neighbor joining method: a new method for constructing phylogenetic trees. Mol. Biol. Evol. 1987; 4:406-425.
31. Lynch, M. The similarity index and DNA fingerprinting. Mol. Biol. 1990; 7: 478-484.
32. Hammer, Ø., Harper, D.A.T., Ryan P.D. PAST: Paleontological Statistics Software Package for Education and Data Analysis. Palaeontol. Electron. 2001; 4: 1-9.
33. Liu H., Yu, Q. J., Wang, G., Ye, F., Cong, Y. Biodegradation of phenol at high concentration by a novel yeast Trichosporon montevideense PHE1. Process Biochem. 2011; 46: 1678–1681.
34. Varma, R., Gaikwad B. Rapid and high biodegradation of phenols catalyzed by Candida tropicalis NCIM 3556 cells. Enz. Microb. Technol. 2008; 43: 431–435.
35. Yan J., Jianping, W., Hongmei, L., Suliang, Y., Zongding, H. The biodegradation of phenol at high concentration by the yeast Candida tropicalis. Biochem. Eng. J. 2005; 24:243–247.
36. Rigo, M., Alegre R.M. Isolation and selection of phenol-degrading microorganisms from industrial waste waters and kinetics of the biodegradation. Folia Microbiol. 2004; 49:41–45.
37. Tsai S.C., Tsai, L.D., Li, Y.K. An isolated Candida albicans TL3 capable of degrading phenol at large concentration. Biosci. Biotechnol. Biochem. 2005; 69:2358–2367.
38. Stoilova I., Krastanov, A., Stanchev, V., Daniel, D., Gerginova, M., Alexieva, Z. Biodegradation of high amounts of phenol, catechol, 2,4-dichlorophenol and 2,6 dimmethoxyphenol by Aspergillus awamori cells. Enz. Microb. Technol. 2006; 39:1036–41.
39. Shahreza H, Sepahy AA, Hosseini F, Nejad RK. Molecular Identification of Pseudomonas Strains with Polyethylene Degradation Ability from Soil and Cloning of alkB Gene. Arch. Pharm. Pract. 2019;10(4):43-8.

40. Juang RS, Tsai S.Y. Growth kinetics of Pseudomonas putida in biodegradation of single and mixed phenol and sodium salicylate. Biochem. Bioeng. J. 2006; 31:133–40.
41. Kurtzman, C.P. rRNA sequence comparisons for assessing phylogenetic relationships among yeasts. Int. J. Syst. Bacteriol. 1992; 42: 1-6.
42. Esteve-Zarzoso, B., Belloch, C., Uruburu, F., Querol, A. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and two ribosomal internal transcribed spacers. Int. J. Syst. Bacteriol., 1999; 49: 329-337.
43. Schuller, D., Valero, E., Dequin, S., Casal, M. Survey of molecular methods for the typing of wine yeast strains. FEMS Microbiol. Lett. 2004; 231: 19-26.
44. Souza-Liberal, A.T., Silva Filho, E.A., Morais, J.O.F., Simo˜es, D.A., Morais, J.M.A. Contaminant yeast detection in industrial ethanol fermentation must by rDNA-PCR. Lett. Appl. Microbiol. 2005; 40: 19-23.
45. Khattab, A. A., Khatab, I.A. Molecular identification of new high ethanol production isolate of Saccharomyces cerevisiae. Aust. J. Basic Appl. Sci. 2012; 6: 16- 24.
46. Gangopadhyay S., Nandi, S., Ghosh, S. Biooxidation of fatty acid distillates to dibasic acids by a mutant of Candida tropicalis. J. Oleo. Sci. 2007; 56: 13-17.
47. Ramsay, L. M., Gadd, G.M. Mutants of Saccharomyces cerevisiae defective in vacuolar function confirm a role for the vacuole in toxic metal ion detoxification. FEMS Microbiol. Lett. 1997; 152: 293-298.
48. Gharieb, M.M., Gadd, G.M. Evidence for the involvement of vacuolar activity in metal (Loid) tolerance: Vacuolar-lacking and defective mutants of Saccharomyces cerevisiae display higher sensitivity to chromate, tellurite, and selenite. BioMetals. 1998; 11: 101-106.
49. Hua Y., He, B., Lu, X., Peng, H., Ye, J., Yang, F. Improvement of chromium biosorption by UV–HNO2 cooperative mutagenesis in Candida utilis. Water Res. 2008; 42: 3981-3989.
50. Thoma, R.W. Use of mutagens in the improvement of production strains of microorganisms. Folia Microbiol. 1971; 16: 197-204.
51. Joshi, S.M., Inamdar, S.A., Jadhav, J.P., Govindwar, S.P. Random UV mutagenesis approach for enhanced biodegradation of sulfonated azo dye, green HE4B. Appl. Biochem. Biotechnol. 2013; 169: 1467–1481.
52. Liu, Z.H., Liu, L.F., Sterlund, T.O., Hou, J., Huang, M.T., Fagerberg, L., Petranovic, D., Uhlen, M., Nielsen, J. Improved production of a heterologous amylase in Saccharomyces cerevisiae by inverse metabolic engineering. Appl. Environ. Microbiol. 2014; 80: 5542–5550.
53. Heerd, D., Tari, C., Fernandez-Lahore M. Microbial strain improvement for enhanced polygalacturonase production by Aspergillus sojae. Appl. Microbiol. Biotechnol. 2014; 98: 7471–7481.
54. Chang, Y.H., Li, C.T., Chang, M.C., Shoieh, W.K. Batch phenol degradation by Candida tropicalis and its fusant. Biotechnol. Bioeng. 1998; 60: 391-395.
55. Schlick, A., Kuhls, K., Meyer, W., Lieckfeldt, E., Borner, T., Messner, K. Fingerprinting reveals gamma ray induced mutations in fungal DNA: implications for identification of patent strains of Trichoderma harzianum. Curr. Genet. 1994; 26: 74-78.
56. Dassanayake, R.S., Samaranayake, L P. Amplification-based nucleic acid scanning techniques to assess genetic polymorphism in Candida. Cri. Rev. Microbiol. 2003; 29:1–24.
57. Barcelos, Q.L., Souza, E.A., Damasceno-Silva, K.J. Vegetative compatibility and genetic analysis of Colletotrichum lindemuthianum isolates from Brazil. Genet. Molec. Res. 2011; 10: 230-242.
58. Khattab, A.A., Abd El-Salam, I.S. Construction of new mutants of Mucor racemosus to improve progesterone biotransformation. Aust. J. Basic Appl. Sci. 2012; 6: 356-363.
59. Gallardo G., Ruiz-Moyano, S., Hernández, A., Benito, M.J., Córdoba, M.G., Pérez-Nevado, F., Martín, A. Application of ISSR-PCR for rapid strain typing of Debaryomyces hansenii isolated from dry-cured Iberian ham. Food Microbiol. 2014; 42: 205-211.

Table 1: Batch phenol-uptake by different isolated yeast strains.

Yeast strain code	Phenol conc. (g/L)			% Uptake
	Initial	Residual	Uptake	
ShY-1	1.5	0.37	1.13	75.33
ShY-2	1.5	0.83	0.67	44.66
ShY-3	1.5	0.50	1.00	66.66
ShY-4	1.5	0.74	0.76	50.66
ShY-5	1.5	0.49	1.01	67.33

Table 2: Bioremediation of phenol by different microorganisms against yeast ShY-1 strain.

Microorganism	Phenol bioremediation		Reference
	Initial conc. (g/L)	Removing time (h)	
Yeast ShY-1 strain	1.5	24	This study
Candida tropicalis	2.0	48	Yan et al., (2005)
Candida tropicalis	2.0	66	Varma and Gaikwad (2008)
Candida parapsilopsis	0.39	18	Rigo and Alegre (2004)
Candida albicans	2.4	250	Tsai et al. (2005)
Aspergillus awamori	0.6	72	Stoilova et al. (2006)
Pseudomonas putida	0.45	36	Juang and Tsai (2006)

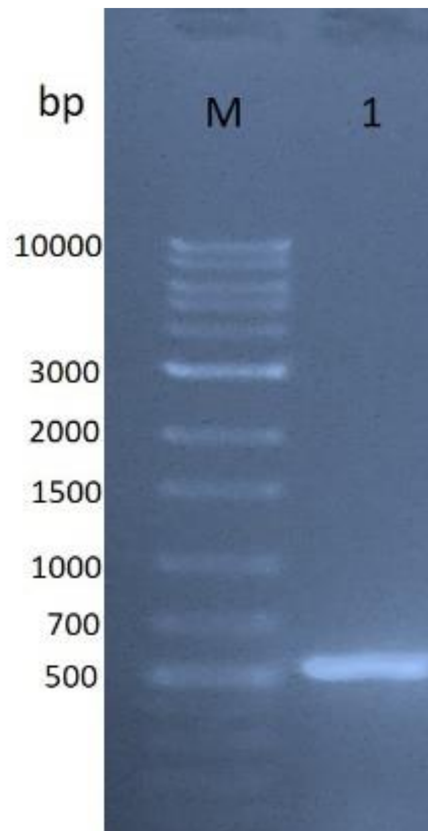

Fig. 1. ITS-DNA amplified band for yeast strain (ShY-1) isolated from soil (lane 1) using ITS1 and ITS4 primers against 100 bp ladder DNA marker (lane M).

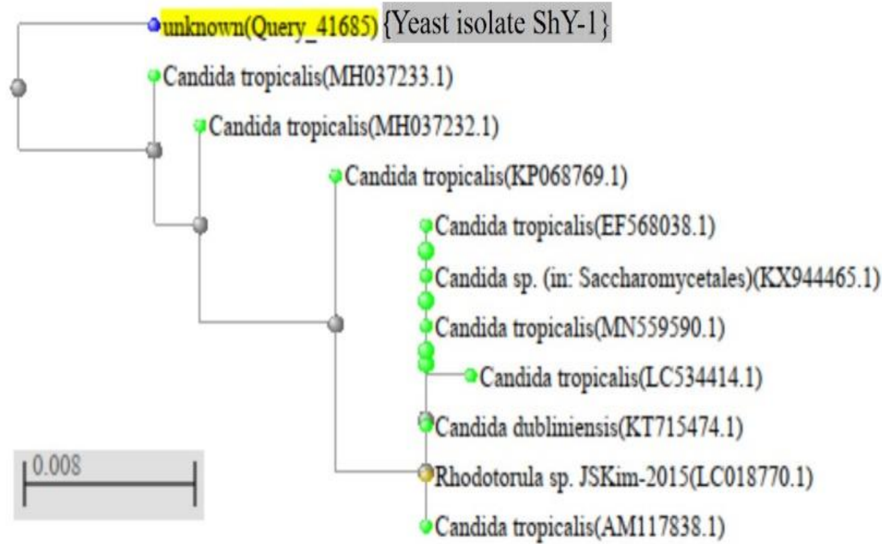

Fig. 2. Phylogenetic dendrogram showing the taxonomic position of *Candida tropicalis* ShY-1 strain (accession number MT422054.1), based on the ITS-sequences and most related *candida* species (*Candida tropicalis*) in NCBI-database GenBank database.

Table 3: Statement of the phenol resistant mutants and survival of yeast ShY-1 strain after utilizing different time intervals of UV-rays.

Time interval (min)	Survival		Phenol resistant colonies	
	No.	%	No.	%
0	1518	100.00	0	0
5	428	28.19	23	5.37
10	153	10.08	16	10.46
15	76	5.01	9	11.84

Table 4: Batch phenol-uptake by different mutants obtained after 5 min UV-treatment under initial phenol concentration of 2.5 g/L.

Mutant No.	Phenol conc. (g/L)		Uptake % to ICP*	Mutant No.	Phenol conc. (g/L)		Uptake % to ICP*
	Residual	Uptake			Residual	Uptake	
ShY-1 (W.T)	1.37	1.13	45.2	ShUV5/12	0.77	1.73	69.2
ShUV5/1	1.02	1.48	59.2	ShUV5/13	0.99	1.51	60.4
ShUV5/2	0.93	1.57	62.8	ShUV5/14	0.90	1.60	64.0
ShUV5/3	0.57	1.93	77.2	ShUV5/15	0.92	1.58	63.2
ShUV5/4	0.88	1.62	64.8	ShUV5/16	1.04	1.46	58.4
ShUV5/5	1.01	1.49	59.6	ShUV5/17	1.18	1.32	52.8
ShUV5/6	0.67	1.83	73.2	ShUV5/18	0.55	1.95	78.0
ShUV5/7	1.53	0.97	38.8	ShUV5/19	0.90	1.60	64.0
ShUV5/8	1.05	1.45	58.0	ShUV5/20	1.64	0.86	34.4
ShUV5/9	0.64	1.86	74.4	ShUV5/21	1.10	1.40	56.0
ShUV5/10	0.92	1.58	63.2	ShUV5/22	0.62	1.88	75.2
ShUV5/11	1.03	1.47	58.8	ShUV5/23	1.21	1.29	51.6

ICP* means the initial concentration of phenol (2.5 g/L)

Table 5: Batch phenol-uptake by different mutants obtained after 10 min UV-treatment under initial phenol concentration of 2.5 g/L.

Mutant No.	Phenol conc. (g/L)		Uptake % to ICP*
	Residual	Uptake	
ShY-1 (W.T)	1.37	1.13	45.2
ShUV10/1	0.74	1.76	70.4

ShUV10/2	1.21	1.29	51.6
ShUV10/3	0.40	2.10	84.0
ShUV10/4	0.55	1.95	78.0
ShUV10/5	0.92	1.58	63.2
ShUV10/6	0.35	2.15	86.0
ShUV10/7	0.24	2.26	90.4
ShUV10/8	1.12	1.38	55.2
ShUV10/9	1.03	1.47	58.8
ShUV10/10	0.31	2.19	87.6
ShUV10/11	0.96	1.54	61.6
ShUV10/12	0.15	2.35	94.0
ShUV10/13	0.80	1.70	68.0
ShUV10/14	0.35	2.15	86.0
ShUV10/15	1.04	1.46	58.4
ShUV10/16	0.30	2.20	88.0

ICP* means the initial concentration of phenol (2.5 g/L)

Table 6: Batch phenol-uptake by different mutants obtained after 15 min UV-treatment under initial phenol concentration of 2.5 g/L.

Mutant No.	**Phenol conc. (g/L)**		**Uptake % to ICP***
	Residual	**Uptake**	
ShY-1 (W.T)	1.37	1.13	45.2
ShUV15/1	0.70	1.80	72.0
ShUV15/2	0.93	1.57	62.8
ShUV15/3	1.04	1.46	58.4
ShUV15/4	0.77	1.73	69.2
ShUV15/5	0.32	2.18	87.2
ShUV15/6	0.25	2.25	90.0
ShUV15/7	0.65	1.85	74.0
ShUV15/8	0.73	1.77	70.8
ShUV15/9	0.37	2.13	85.4

ICP* means the initial concentration of phenol (2.5 g/L)

Table 7: The antifungal reaction of the wild strain and the predominant mutants chosen for protoplast combination.

Mutant code	**Antifungal agents' responses**			
	C	M	N	G
ShY-1 (W.T)	+	-	+	+
ShUV10/7	+	-	+	+
ShUV10/12	-	+	+	-
ShUV15/6	+	-	-	+

C: Cycloheximide (100 µg/ml), M: Miconazole (5 µg/ml), N: Nystatin 10 µg/ml), G: Griseofulvin (250 µg/ml) (+): Resistant, (-): Sensitive

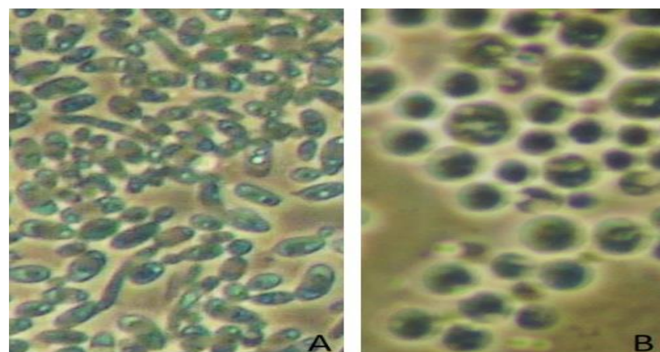

Fig. 3. The formation of the protoplasts (B) against the original wild type (A).

Table 8: Batch phenol-uptake by different fusants obtained after protoplast fusion using cycloheximide and miconazole as selectable markers (Cross 1).

Fusant No.	Phenol conc. (g/L)		Uptake % to ICP*
	Residual	Uptake	
ShUV10/12 (P1)	0.15	2.35	94.0
ShUV15/6 (P2)	0.25	2.25	90.0
ShC1/1	0.22	2.28	91.2
ShC1/2	0.13	2.37	94.8
ShC1/3	0.08	2.42	96.8
ShC1/4	0.25	2.25	90.0
ShC1/5	0.32	2.18	87.2
ShC1/6	0.10	2.40	96.0
ShC1/7	0.18	2.32	92.8

ICP* means initial concentration of phenol (2.5 g/L)

Table 9: Batch phenol-uptake by different fusants obtained after protoplast fusion using nystatin and griseofulvin as selectable markers (Cross 2).

Fusant No.	Phenol conc. (g/L)		Uptake % to ICP*
	Residual	Uptake	
ShUV10/12 (P1)	0.15	2.35	94.0
ShUV15/6 (P2)	0.25	2.25	90.0
ShC2/1	0.20	2.30	92.0
ShC2/2	0.09	2.41	96.2
ShC2/3	0.24	2.26	90.4
ShC2/4	0.15	2.35	94.0
ShC2/5	0.22	2.28	91.2

ICP* means initial concentration of phenol (2.5 g/L)

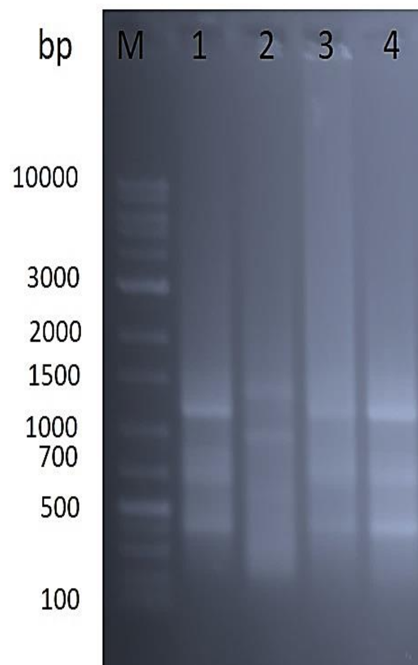

Fig. 4. ISSR-amplified banding patterns using primer (ISSR1) for two different mutants, ShUV10/12; ShUV15/6 (tracks 2 to 3) *C. tropicalis* (ShY-1) (track 1) and fusant, ShC1/3 (track 4) opposite to DNA Marker, Intron Biotechnology Inc., South Korea, (track M).

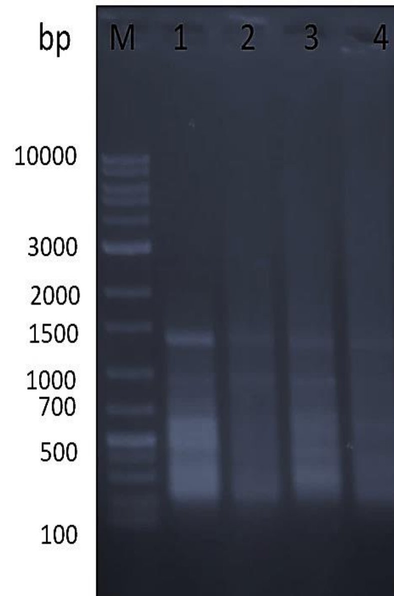

Fig. 5. ISSR-amplified banding patterns using primer (ISSR2) for two different mutants, ShUV10/12; ShUV15/6 (tracks 2 to 3) *C. tropicalis* (ShY-1) (track 1) and fusant, ShC1/3 (track 4) opposite to DNA Marker, Intron Biotechnology Inc., South Korea, (track M).

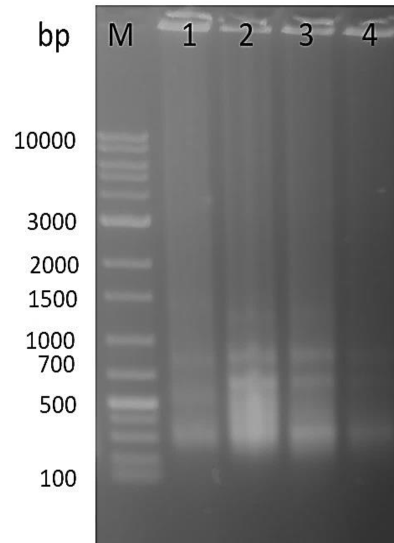

Fig. 6. ISSR-amplified banding patterns using primer (ISSR3) for two different mutants, ShUV10/12; ShUV15/6 (tracks 2 to 3) *C. tropicalis* (ShY-1) (track 1) and fusant, ShC1/3 (track 4) opposite to DNA Marker, Intron Biotechnology Inc., South Korea, (track M).

Table 10: Different-amplified DNA-bands and the observed polymorphic bands' percentages using three ISSR-primers.

Primer code	Total band No.	Unique band No.	Monomorphic band No.	Polymorphic band No.	Polymorphic %
ISSR-1	*12*	*3*	*0*	*9*	*75.00*
ISSR-2	*12*	*0*	*8*	*4*	*33.33*
ISSR-3	*14*	*2*	*4*	*8*	*57.14*
Total	*34*	*5*	*12*	*21*	-

Table 11: Proximity matrix between the tested mutants, fusant and *C. tropicalis* (ShY-1 strain) according to ISSR-assay.

Strain code	ShY-1 (W.T)	ShUV10/12	ShUV15/6	ShC1/3
ShY-1 (W.T)	1.000	0. 571	0.960	0. 818
ShUV10/12	0. 571	1.000	0. 600	0. 706
ShUV15/6	0. 960	0. 600	1.000	0. 857
ShC1/3	0. 818	0. 706	0. 857	1.000

Pharmacophore, 11(4) 2020, Pages 22-35

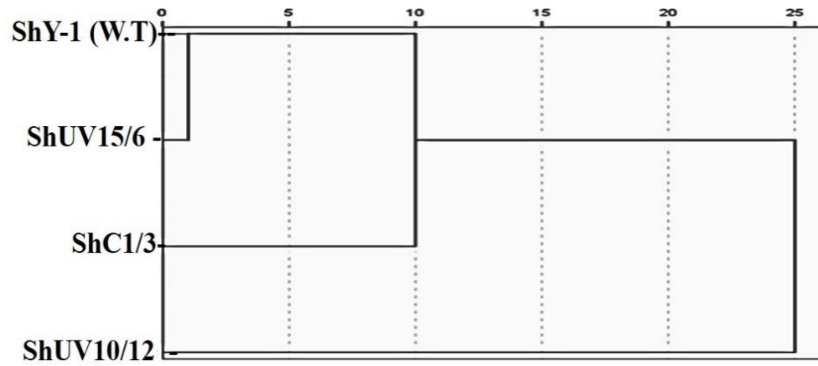

Fig. 7. Phylogenetic and clusters via Average Linkage dendrogram of two novel mutants and fusant against *C. tropicalis* (ShY-1 strain) through ISSR method.