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ISOLATION AND IDENTIFICATION OF TOLERANT BACTERIA FROM ROOTS OF *SCIRPUS GROSSUS* EXPOSED TO MIXED SYNTHETIC DYE

Enas Abdulqader[1,*], Siti Rozaimah Abdullah[1], Hassimi Abu Hasan[1], Reehan Adre ab.Rahim[1], Mushrifah Idris[2], mohd aidil kamaruzzaman[1] and nur izzati ismail[1]

[1]Department of Chemical and Process Engineering, Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia

[2]Tasik Chini Research Center, Faculty of Science and Technology, UniversitiKebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia

*Corresponding author email: enas.phd@gmail.com; rozaimah@ukm.edu.my

Abstract

Aside from plants, rhizobacteria in the rhizosphere play an important symbiotic role in plants to render the toxicity and remove pollutants from contaminated soils and water in phytoremediation. This study screens and identifies effective rhizobacteria that can help plants remove dye in synthetic dye effluent. In this study, during 14 days of a preliminary test, *Scirpus grossus* was exposed to different concentrations (50, 75and 100 mg/L) of mixed synthetic dye of methylene blue and methyl orange in the subsurface flow reed bed. A total of 27 colonies of rhizobacteria were isolated from the roots and the sand surrounding the roots of *S. grossus* with the highest dye concentration of 100 mg/L. The isolates were further analysed for their colony and microscopic morphology to detect any similar bacteria. No similarities exist between them. The bacteria were then exposed to a dye concentration mixture of 100 mg/L to screen for rhizobacteria that are capable of tolerating dye. Six bacteria (A5, B1, B2, B3, C6 and E3) survived in this dye-based concentration according to the CFU results. Subsequently, the capability of the six rhizobacteria to degrade dye was tested with 100 mg/L of dye concentration in 5 days. Only three of the rhizobacteria (B3, C6 and E3) degraded the dye according to UV-Vis spectroscopy results, in which 92%, 58% and 63%of the dye was removed, respectively. The three rhizobacteria were identified to be the same bacteria as *Alcaligenes faecalis* strain II TRM4using polymerase chain reaction.

Keywords: methylene blue, methyl orange, Scirpus grossus , PCR, Alcaligenes faecails strain II TRM4

Introduction

The textile and apparel industry is a diverse sector that involves many processes, from the production of raw materials to processing of yarns, fabrics, dyeing and finishing operations that lead to the final sewing (Parisi *et al.* 2015). The pollutants of textile effluents are toxic and contain supplementary chemicals, dissolved solids, heavy metals and residual chlorine. Textile wastewater treatment has become a global concern and a need exists for new recyclable technologies to decrease massive water consumption. Textile dyes that contain effluents are toxic to the natural flora and fauna and are mutagenic and carcinogenic (Wang *et al.* 2009). Phytoremediation helps prevent landscape demolition and promotes activity and diversity of soil microorganisms to maintain healthy ecosystems. Phytoremediation is an attractive alternative to traditional methods currently used to deal with dye. Interaction of bacteria depends on the conditions, biological availability, root exudate composition and nutrient levels of the soil (Ali *et al.* 2011). In this study, *Scirpus grossus* was used to facilitate phytoremediation. Previous studies used *S. grossus* to remediate wastewater containing Fe-Al (Ismail *et al.* 2015), Pb (Tangahu *et al.* 2013) and hydrocarbons (Al-Baldawi *et al.* 2013). Besides that, Jehawi *et al.* (2015) and Yusoff *et al.* (2019) used *S. grossus* to treat domestic wastewater and recycled pulp and paper effluent respectively. The main objective of this study is to determine, identify and optimise rhizobacteria that can tolerate dye exposure.

Materials and Method

Microbe Study

Figure 1 shows the process of isolating rhizobacteria from the roots of *Scirpus grossus* and the surrounding sand that cover the root. Preliminary tests were performed before this study was conducted to determine rhizobacteria-isolated colonies. Mixed dyes of methylene blue (MB) and methyl orange (MO) were used with *S. grossus* in two systems, namely, SSF and FSF, to identify suitable mixed dye concentrations in which plants can survive. The preliminary test identified three different concentrations of 50, 75 and 100 mg/Lin which plants can live and survive.

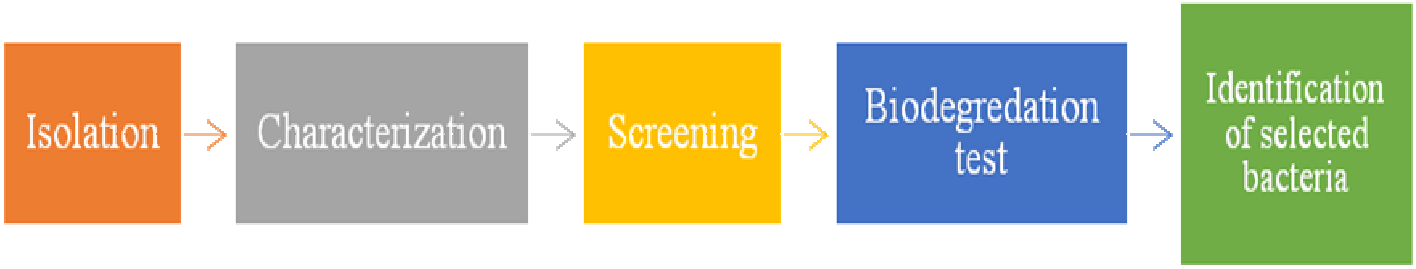


Fig. 1 : Methodology of microbial study

Isolation of Rhizobacteria from *S. grossus*

During the14 days of preliminary testing, *S. grossus* was exposed to varying concentrations of mixed dyes of50, 75, 100 mg/L in SSF.Approximately10 g of *S. grossus* roots and surroundings and were placed in a 250-mL Schott bottle containing 100 mL of sterilised distilled water. The sample was incubated in a rotary shaker (Protech, Model SI-100D, Malaysia) at a temperature of 37 °C at150 rpm for 1 h. Serial dilution method was applied to obtain three suitable dilutions for plating. Then, 1 ml of the sample was pipetted into 9 mL sterile saline water until subsequent dilutions of up to 10^{-4} were achieved. 0.1 mL each of the three dilutions (10^{-2} and 10^{-4} dilution) was pipetted in a sterilised Petri dish with tryptic soya agar (TSA) (Difco, USA). Inside the laminar flow cabinet, the sample was distributed in triplicates with a hooky stick on the TSA and then the Petri dishes were incubated inverted at 37 °C (Al-Baldawi *et al.* 2013; Peng *et al.* 2009). The colonies were isolated and grown in separate TSAs for 24 h.

Characterisation Study of Isolated Rhizobacteria

The different colours and shapes of TSA plates identified the different colonies of rhizobacteria, which were classified based on form, elevation, margin appearance, optical property, pigmentation and texture. The pattern of each cell was observed under a light microscope (Nikon-E100, Tokyo, Japan). The cellular morphology was identified and biochemical tests (Gram stain, catalase, oxidase and motility) were conducted on the pure cultures of bacteria.

Screening of Rhizobacteria Tolerance to Dye

Screening was performed to determine the rhizobacteria that were tolerant to dye. A test was performed in the root colonies using a mineral salt medium (Merck KG, Germany) containing mixed dye(MB+MO) concentration of 100 mg/Lin which *S. grossus* can grow and survive. The dye concentration was selected on the basis of a previous study (preliminary test).

Gargouri *et al.* (2011) and Purwanti *et al.* (2012)conducted studies in which the MSM containing 1.2 g KH_2PO_4, 1.8g K_2HPO_4, 4.0 g NH_4Cl, 0.2 g $MgSO_4$, 0.7g H_2O, 0.01g $FeSO_4$, 0.7g H_2O and 0.1 g NaCl and 0.1% of trace elements was added to 0.1 $\frac{g}{L}MnSO_4.H_2O$, 0.025 $\frac{g}{L}CuCl_2$, 0.025 $\frac{g}{L}(NH_4)_6MO_7.O_{24}$, 0.025 $\frac{g}{L}[CO(NO)_9]_2$, 0.$\frac{6g}{L}H_2O$, 0.025g $\frac{g}{L}ZnCl$, 0.01$\frac{g}{L}NH_4NO_3$ in 1 L of distilled water. The medium was then autoclaved at 121 °C for 15 min. The isolated bacteria were streaked in sterilised tryptic soya broth (Difco, USA) (50 mL) and incubated in a rotary shaker (Protech, Model SI-100D, Malaysia) at 37 °C at150 rpm for approximately18 h to 24 h. Then, the sample was centrifuged at4000 rpm at 4 °C for 10 min using the Eppendorf Centrifuge T-type 5810 R (USA).The supernatants were separated to prepare for standard inoculation from the pellet. In 250-mL conical flask, the bacteria was filled with 50 mL of MSM containing 10% standard inoculation rhizobacteria with 100 mg/L of the dye concentration for screening and incubated in a shaker at 150 rpm at37°C for 5 d. Figure 2 shows an example of the capability of rhizobacteria to grow and survive in dye. The samples were examined through the growth of bacteria (colony-forming unit CFU/mL count) count on the TSA on whether the ratings were very good growth (+++), good growth (++), poor growth (+) and no growth (-) (Purwanti *et al.* 2012).

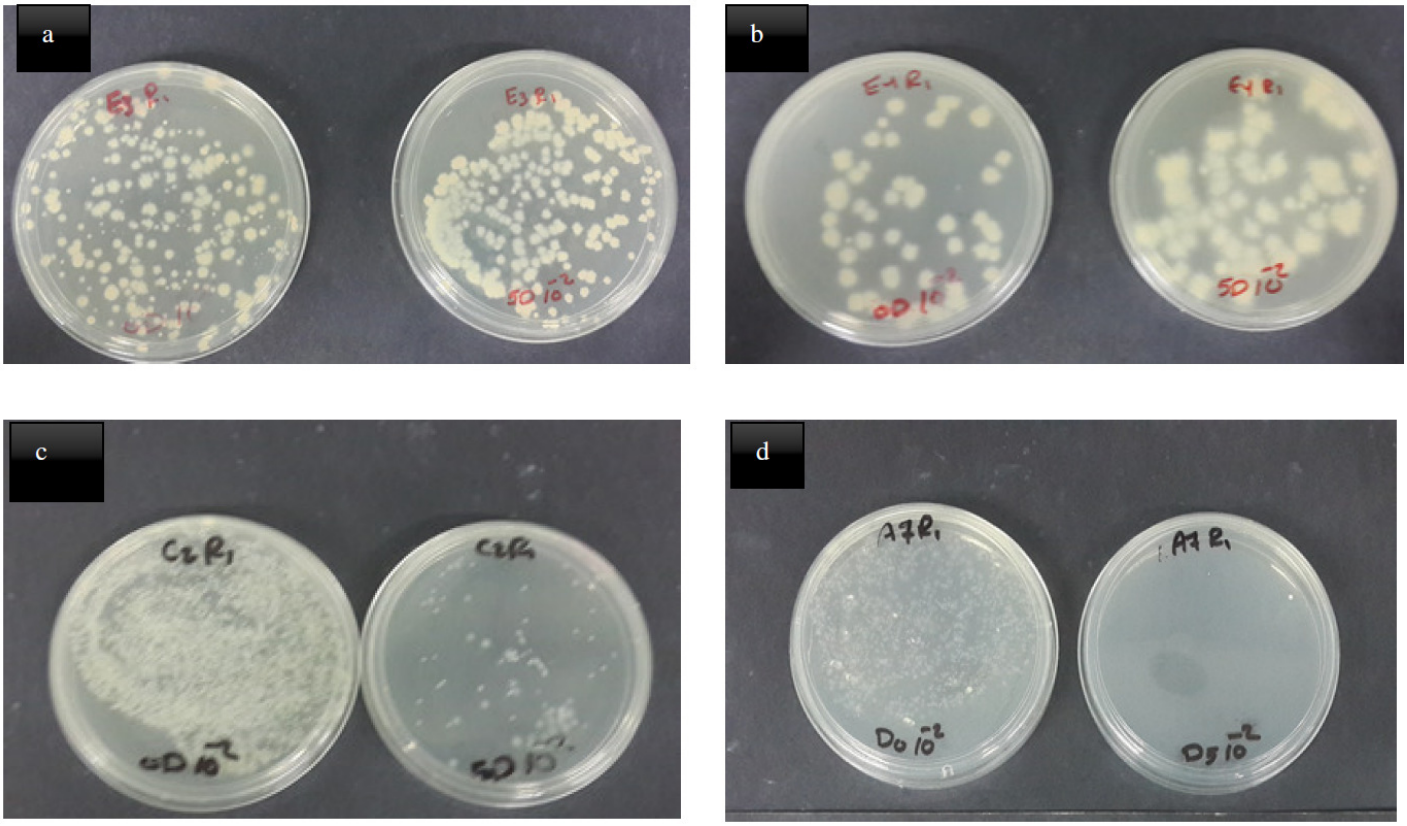


Fig. 2 : Rhizobacteria spread after five days of incubation in MSM + dye (a) very good growth (+++), (b) good growth (++), (c) medium growth (+), (d) no growth (-)

Biodegradation Test of Selected Rhizobacteria

Biodegradation test measures the capability of selected rhizobacteria from the screening test to degrade the dye mixture using UV–Vis spectroscopic analysis (DR 3900 HACH). MSM broth containing a dye concentration of 100 mg/L (50 mL of MSM containing 4.5 mg MB+ 4.5 mg MO,10% (v/v) rhizobacteria of 5 mL inoculum of rhizobacteria with an optical density of 550 (OD550) and 45 mL of MSM)was used in this test. The mixture was placed in a 250mL conical flask and incubated in a shaker at 150 rpm at37 °C for 5 d. At zero and 5days, two conical flasks of MSM broth were harvested for every bacterium through centrifugation (Eppendorf/Centrifuge 5418) at 4000 rpm at 4 °C for 10 min.

The samples were then obtained from the conical flask at day 0.After 5 days of incubation, the samples were measured using UV–Vis to compare the removal of dye at days 0 and 5.The percentage removal of the dye mixture for each sampling at days 0 and 5 was determined by using the following Equation (1):

$$\text{Dye bio deg radation}(\%) = \frac{\text{Initial colour} - \text{Final colour}}{\text{Initial colour}} \times 100 \quad (1)$$

with, Initial colour= initial colour value at day 0

Final colour= value obtained after day 5.

Identification of Selected Rhizobacteria

The selected rhizobacteria from screening step were subjected to 16S rRNA. This method consists of a series analyses, DNA extraction, amplification of 16S rRNAgens by PCR, PCR product purification and sequencing and DNA analyses. DNA was extracted from rhizobacteria suspensions in a TSB medium of incubation at 37°C for 24 h.Genomic DNA was extracted from rhizobacteria culture using RTP® Bacteria DNA Mini Kit.

DNA extracts are stored in the Eppendorf tube at −20 °C. After genomic extraction, the 16S rRNA was amplified. The protocol used for this reaction was One Taq® 2X Master Mix (New England BioLabs, USA) with a primer pair of 27F universal primer (primer forward, 5'-AGA GTTTGATCCTGGCTCAG-3') and 1492R (primary reverse, 5'-GGTTAC CTTGTTACGACTT-3'). PCR was conducted using a Mastercycler (Epgradient S, Eppendorf, and Version 3608) for30 cycles. The amplification was initiated with the start of the curing at 94 °C from 30 s to 1min, followed by disassembly for 30 cycles at 94 °C for 30 s, annealing at 50 °C for 20 s and elongation at 68 °C for 1 min. Then, extended elongation at 68 °C for 5 min. The PCR product obtained was purified using a purifying kit PureLink TM Quick PCR (Invitrogen, Germany) on the basis of the protocol proposed by the manufacturer. The success of PCR product production and its size is determined by electrophoresis in 0.8% agarose gel in 1x TBE with 80V power.

In DNA sequencing and analysis, the PCR product obtained was sent to the First BASE Laboratories Sdn. Bhd (Kuala Lumpur, Malaysia) for 16S rRNA sequencing. The sequence obtained was manually edited based on chromatography before aligning with the sequence found in the GenBank database using the Blast search tool from the National Centre for Biotechnology Information (NCBI, United States, http://www.ncbi.nlm.nih.gov).

A phylogenic tree for each 16S rRNA sequence was constructed using neighbour-joining model using software package MEGA 6.0(Tamura *et al.* 2013).Bootstrapping was also performed in 1000 replicates.

Results and Discussion

Isolation and Characterisation of Rhizobacteria

Isolation of rhizobacteria was conducted to obtain the rhizobacteria located in the roots of the *S. grossus* plant and surrounding sand. The rhizobacteria was then exposed to various concentrations of MB and MO mixtures in varying concentrations of 50, 75 and 100 (mg/L) and plant control (PC) during the preliminary study. In this experiment, 27 different types of bacterial colonies were isolated. The colonies were examined under light microscopy (Nikon-E100, Tokyo, Japan) to describe the colonies on the basis of their form/shape, elevation, margin, appearance, pigmentation (colour) and texture and to determine the similarities among the colonies. Figure 3 summarises the results obtained from the biochemical test of the colonies (Gram stain, catalase, oxidase and motility).

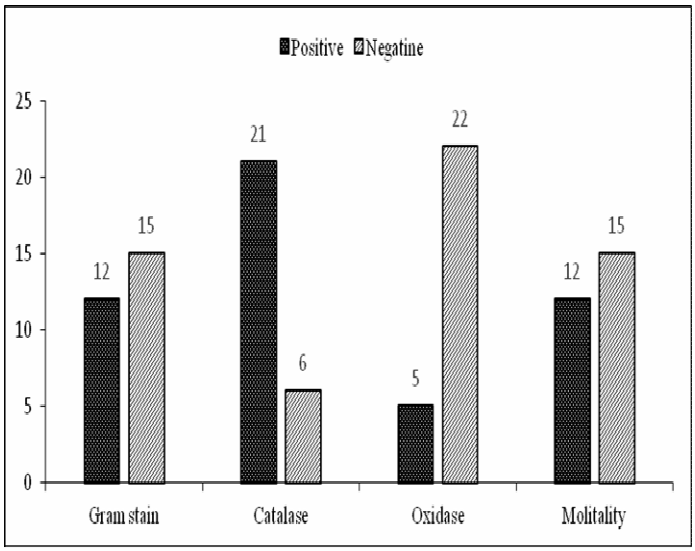


Fig. 3 : Biochemical test results on the rhizobacteria of *S. grossus*

Screening of Rhizobacteria through Dye Exposure

In this step, the isolated rhizobacteria were tested for tolerance to dye concentration on the basis of growth intensity (CFU/mL) on TSA after 5 days of exposure to the dye that showed no, good, very good or medium growth. The results of the bacteria growth showed that six out of 23 isolated rhizobacteria had good growth (+++). Table 1 lists the CFU of the rhizobacteria (A5, B1, B2, B3, C6 and E3) after 5 days of exposure. Degradation test was then performed on the rhizobacteria.

Table 1 : CFU of rhizobacteria after 5 days of exposure to 100 mg/L of dye mixture concentration .

Rhizobacteria code	Dye concentration (100)mg/L
A5	+++
B1	+++
B2	+++
B3	+++
C6	+++
E3	+++

Biodegradation of Dye Based on the Selected Rhizobacteria

Based on the screening test, biodegradation test was conducted on the six selected bacteria. The colour removal in the samples at days 0and 5 were measured by UV–Vis. The six bacteria are capable of degrading dye during exposure. Figure 5 shows that bacteria B3, E3 and C6 exhibited the best colour removal percentages of 92%, 63% and 58%, respectively. Bacteria B2, B1 and A5 only had removal percentages of 30%, 40% and 20%, respectively. Table 2 lists the qualities and characteristics of the six rhizobacteria.

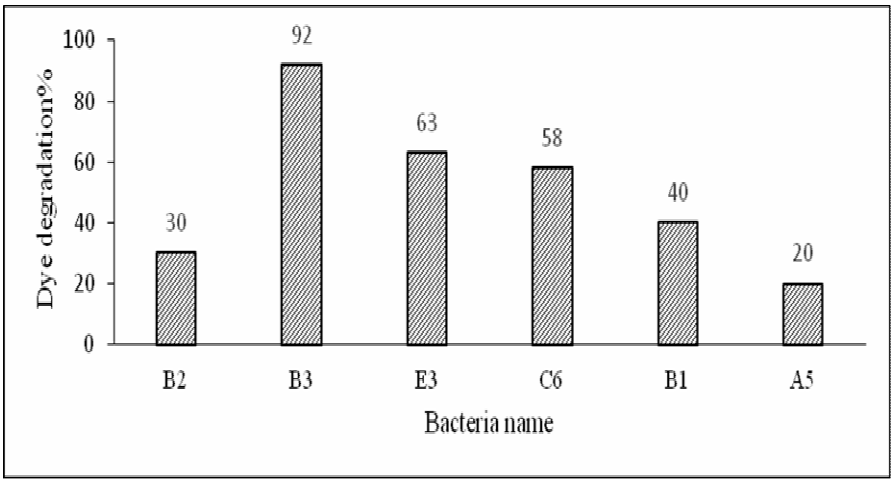
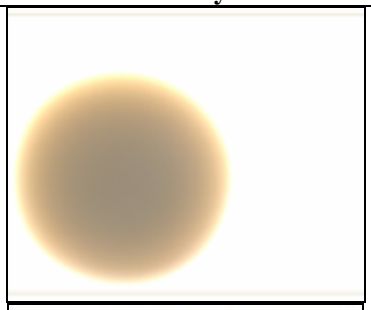
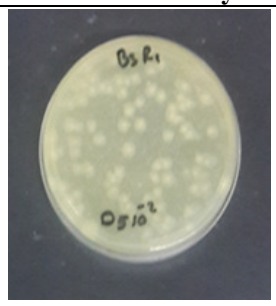
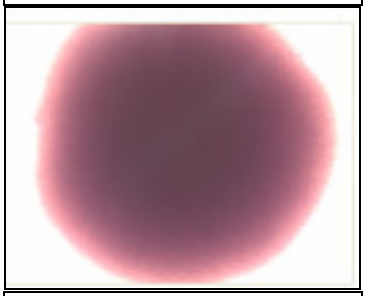
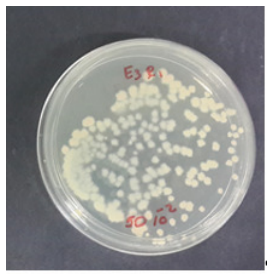
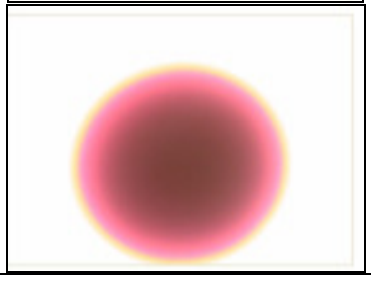
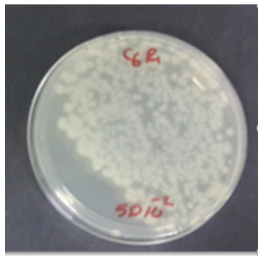


Fig. 5 : Removal percentage of dye by each bacterium after five days of exposure

Table 2 : Colony of selected bacteria (B3, E3 and C6)

Rhizobacteria Code	Colony	Gram Stain	CFU after 5 day
B3		Negative	
E3		Negative	
C6		Positive	

Identification of Selected Rhizobacteria

A phylogenetic tree was constructed to identify the selected rhizobacteria B3, C6 and E3 up to their species level. The identification of impurities was performed using molecular biological methods. Figure 6 shows that isolate bacteria B3 and C3anE3 showed a strong relationship with a bootstrap value 91% similar to *Alcaligenes faecalis* strain II TRM4. Therefore, this isolate was assigned to the *Alcaligenes faecalis* strain EAQ.

Saratale *et al.* (2011) conducted a study on *Alcaligenes faecalis* using decolourised Direct blue-15 and Remazol black. Five other isolates (*Bacillus vallismortis, B. pumilus, B. cereus, B. subtilis and B. megaterium*)were degraded using Direct red 28 (Tony *et al.* 2009).

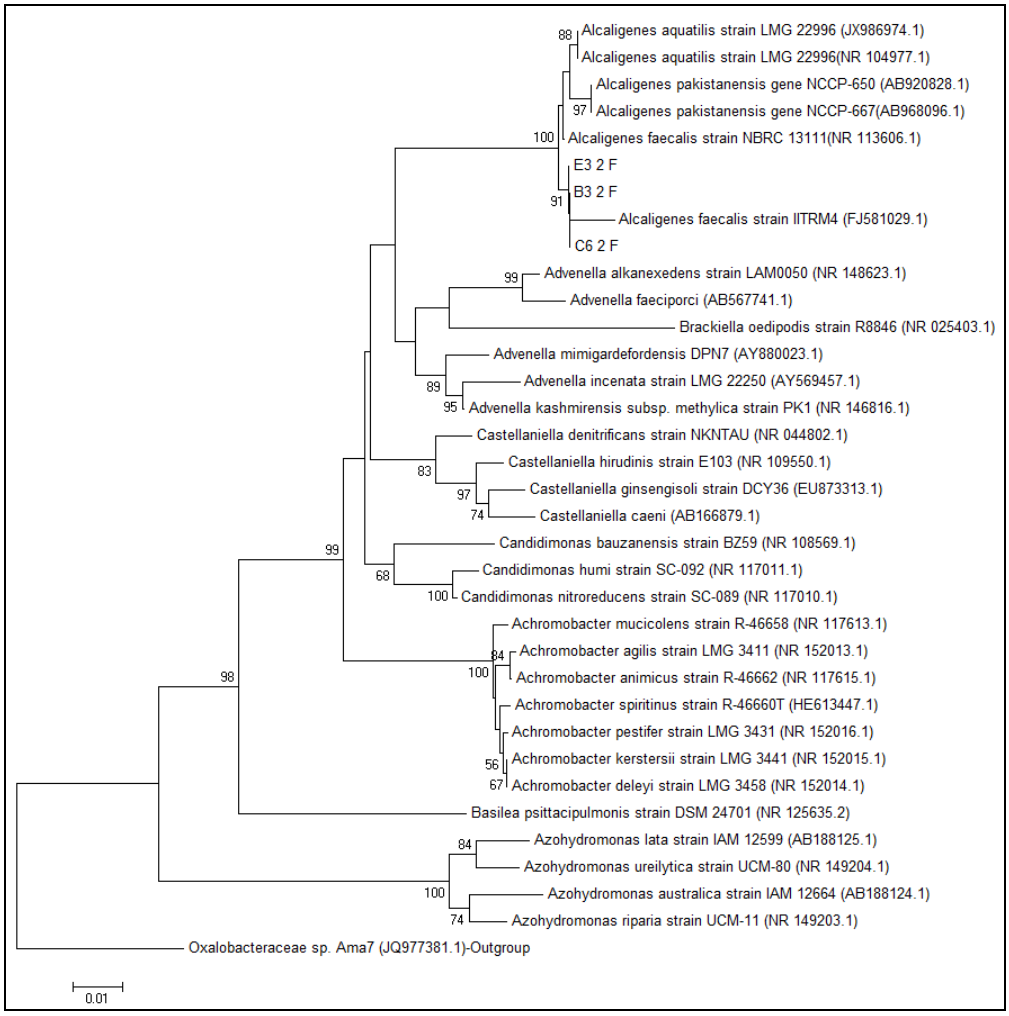


Fig. 6 : Phylogenetic tree (neighbour-joining method) on the three rhizobacteria (B3, C6 and E3) and other bacteria based on the 16S rRNA gene sequence analysis. (The internal labels on the branch refer to the bootstrap value.)

Conclusions

A total of 27 rhizobacteria colonies were isolated from the roots and the sand surrounding the roots of *S. grossus* with a dye concentration of 100 mg/L. The isolates were tested for colony morphology, microscopic morphology, and then examined to detect any similar bacteria. No similarities existed among them. The bacteria were then exposed to 100 mg/L of dye mixture concentration to determining which bacteria are capable of tolerating dye. Six bacteria (A5, B1, B2, B3, C6 and E3) grew and survived in this dye concentration based on the CFU results. Subsequently, the capability of the six rhizobacteria to remove dye was tested with 100 mg/L of dye concentration for5 days. Only three of the rhizobacteria (B3, C6 and E3) could successfully remove the dye with removal percentages of 92%, 58% and 63%, respectively. PCR identified that the three rhizobacteria belonged to the *Alcaligene sfaecalis* strain II TRM4.

Acknowledgements

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