RESEARCH ARTICLE

Role of Bacterial-Fungal Consortium for Enhancement in the Degradation of Industrial Dyes

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Abstract: ***Background*:** The presence of anthraquinone (Disperse blue 64) and azodyes (Acid yellow 17) in a waterbody are considered among the most dangerous pollutants.

***Methods*:** In this study, two different isolated microbes, bacterium and fungus, were individually and as a co-culture applied for the degradation of Disperse Blue 64 (DB 64) and Acid Yellow 17 (AY 17) dyes. The isolates were genetically identified based upon 16S (for bacteria) and ITS/5.8S (for fungus) rRNA genes sequences as *Pseudomoans aeruginosa* and *Aspergillus flavus,* respectively.

***Results*:** The fungal/bacterial consortium exhibited a higher percentage of dyes degradation than the individual strains, even at a high concentration of 300 mg/L. Azoreductase could be identified as the main catabolic enzyme and the consortium could induce azoreductase enzyme in the presence of both dyes. However, the specific substrate which achieved the highest azoreductase specific activity was Methyl red (MR) (3.5 U/mg protein). The tentatively proposed metabolites that were detected by HPLC/MS suggested that the reduction process catalyzed the degradation of dyes. The metabolites produced by the action consortium on two dyes were safe on *Vicia faba* and *Triticum vulgaris* germination and health of seedlings. Toxicity of the dyes and their degradation products on the plant was different according to the type and chemistry of these compounds as well as the type of irrigated seeds.

***Conclusion*:** We submit that the effective microbial degradation of DB64 and AY17 dyes will lead to safer metabolic products.

Keywords: *Aspergillus*, disperse blue 64, acid yellow 17, *Pseudomonas*, HPLC/MS, 16S and ITS/5.8S rRNA genes sequences.

1. INTRODUCTION

Due to the ubiquitous application of synthetic dyes in many commercial products, water contaminated with dyes became one of the most dangerous problems [1]. About 10,000 types of dyes and pigments are used in different industries, and 0.7 million tons of dyes produced per year [2, 3]. Azodyes and anthraquinone dyes have a wide range of concerns in wastewater treatment due to their toxicity to animals and humans as they have mutagenic effects, in addition to intense coloration and biorecalcitrance issues [4, 5]. Many strategies have been developed for the removal of these dyes from a water body, but they face several technical and economic limitations and lead to the generation of toxic by-products, in some cases with carcinogenic properties [6]. Microbiological method is the most promising tool suggested for the efficient removal of dyes because it is characterized by lower cost of treatment, eco-friendly and amenability to easy scale up [7]. Various studies have been carried out with isolated, mutated and genetically modified microorganisms for high performance bioremediation to achieve the highest degradation percentage of xenobiotic compounds [8]. Moreover, pure strain is difficult to be scaled up or maintained in large-scale operations typical of effluent treatment systems [9]. Instead, microbial consortia could be attractive tools to improve the rate of decolorization processes with better suitability in large-scale applications.

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Recent studies reported that co-microbial culture may be more suitable for the degradation of different types of dyes [5]. Within the consortium, one strain may degrade the dye compound first or may use the degradation products produced by another strain for further decomposition [9, 10]. Previously, a bacterial consortium (*Enterobacter dissolvens* and *Pseudomonas aeruginosa*) was used for the degradation of diazo dye Acid Maroon V, which showed a 2.15-fold increase in decolorization efficiency when compared to results using an individual microorganism. Yeast species, including *Galactomyces geotrichum, Candida tropicalis,* and *Saccharomyces cerevisiae* were effectively used for dye degradation [5, 8].

The principle aim of this study is to compare the ability of bacterial and fungal strains, individually and as a consortium of both, to degrade anthraquinone dye Disperse blue 64 and diazodye Acid yellow 17.

2. MATERIALS AND METHODS

2.1. Industrial Dyes

Disperse Blue 64 (DB 64) was purchased from a local company for dyes, the chemical formula is $C_{28}H_{22}N_2NaO_4$, the molecular mass (g/mol) is 450.49 and CAS number 12222-77-4 with 98.5% purity. Acid yellow 17 was purchase from a local company, the chemical formula is $C_{16}H_{10}Cl_2N_4Na_2O_7S_2$, and the molecular mass (g/mol) is 551.29 and CAS number 6359-98-4 98.2% purity.

2.2. Isolation of Dyes Degrading Microorganisms

The 10% of industrial dye effluent was inoculated on minimal basal salt (MBS) medium containing (g/L) NaCl – 28.4, K_2HPO_4 – 4.74, KH_2PO_4 – 0.56, $MgSO_4$ – 0.5, $CaCO_3$ – 0.1, NH_4NO_3 – 2.5 and supplemented with 0.1% glucose as an energy source and 100 mg/L of either DB 64 or AY 17 at 30°C, pH 7.0 and 120 rpm shaker. After 48 h, a batch of 1mL was transferred to the solid MBS medium for isolation of bacteria. However, for isolation of fungi the dye effluent consisting media were incubated for 7-15 days. The growing microbial isolates were purified by streak-plate method and each pure isolate was preserved on slant agar tube containing the same previously mentioned medium contents for further use.

2.3. PCR-based Methods for Dyes Microbial Identification

2.3.1. DNA Extraction

Genomic DNA was extracted from fungus and bacterium isolates. The method described in detail by Hesham [11] was used for bacterial genomic extraction, while a DNeasy plant mini kit (Qiagen, USA) was used for fungal genomic DNA according to the company's instructions. The purity and quantity of DNA were examined by recording UV absorption spectra and performing 1% agarose gel electrophoresis.

2.3.2. PCR Amplification

Bacterium isolate was identified by the amplification and sequencing of the 16S rRNA gene using F8 primer (5′-AGA GTT TGA TCC TGG CTC-3′) and R1525 primer (5′-AAG GAG GTG ATC CAG CC-3′) [12] and PCR conditions as

described by Hesham (11). Fungus isolate was identified by sequencing the ITS1, ITS2 and 5.8S rRNA regions using the primers ITS1-F (CTTGGT CAT TTA GAG GAA GTA A) and ITS4 R (TCC TCCGCT TAT TGA TAT GC) with PCR conditions as described by White *et al.* [13].

2.3.3. Nucleotide Sequencing, Alignment and Phylogenetic Analysis

PCR products were analyzed using gel electrophoresis, and the correctly sized product was purified and sequenced (ABI automated sequencer; Macrogen Company, Seoul, Korea). The obtained sequences from the isolates were aligned and compared with the known 16S rRNA and ITS1-5.8S rRNA-ITS2 sequences for bacteria and fungi, respectively in GenBank using the BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) to specify the closest available database sequences. To detect the exact taxonomic position of the isolates, phylogenetic trees were constructed with the MEGA program version 4.0 using a neighbor-joining algorithm, plus the Jukes-Cantor distance estimation method with 1,000 bootstrap replicates was performed [8].

2.4. Preparation of Consortium

The consortium was prepared by the modified protocol reported by Kurade *et al.* [5]. A volume of 1 cm pieces of mycelium of fungal isolate AUMC-10515 was cut from agar slants and cultivated in 100 mL MBS medium supplemented with 0.1% glucose in 250 mL Erlenmeyer flasks at 30°C at 150 rpm rotatory shaker for 72 h. The bacterial isolate AUMC B-171 was grown on Luria-Bertani broth (LB) for 24h at 30°C at 150 rpm rotatory shaker. The cells were harvested and centrifuged for 10 min at 10000 rpm.

For the preparation of bacterial-fungal consortium, the mycelium of AUMC-10515 culture was aseptically transferred MBS medium containing isolate AUMC B-171 cells (OD_{600} 0.8).

2.5. Dyes Biodegradation Studies

All isolates were evaluated spectrophotometrically for their dye decolorizing abilities by determination of the decreasing dye absorbance against time (*i.e.* the disappearance of dye color). The highest decolorizing isolates were selected for detailed degradation study.

A volume of 100 mg/L of either BD 64 or AY 17 was separately added to the conical flask containing the developing consortium. They were separately incubated for 48 h at 30°C under static condition. An aliquot was withdrawn at 06 h intervals and then centrifuged at 5000 rpm for 10 min to obtain a clear supernatant to enable determination of the degradation percentage. The degradation percentage was measured at the maximum absorbance λ_{max} of the dye (DB 64, 603 nm; AY 17, 398 nm). All degradation experiments were performed in triplicate. The degradation percentage is expressed as follows:

Dye degradation percentage (%) = [(initial absorbance) – (final absorbance)]*100/(initial absorbance).

To determine the effect of initial dye concentration on the degradation percentage, different concentration of dyes was prepared from 50 to 300 mg/L.

2.6. Enzymes Responsible for Dyes Biodegradation

Each strain was grown in MBS liquid medium amended with 0.1% glucose and 100 mg/L of methyl red to enhance the induction of azoreductase enzyme. The cells were harvested by centrifugation and suspended in potassium phosphate buffer (50 mM, pH 7.4). The cells were gently crushed by ultrasonication for 2 min (30 second interval) at 35 amplitude keeping the temperature at 4°C. The homogenate was then centrifuged at 5000 rpm for 30 min under cooling.

The total protein concentration was determined by the method of Bradford [9], with bovine serum albumin as a standard.

2.7. Azoreductase Specific Activity and Substrate Specificity

The azoreductase specific activity was assessed by the reduction of methyl red (MR) as the main substrate and oxidation of NADH using a modified procedure described by [10] at 430 nm. The enzyme assays mixture (1 mL) contain 600 µL phosphate buffer (50 mM and pH 7.5), 100 µL of MR (50 µM) and 100 µL of cell free extract. The reaction was started by the addition of 200 µL NADH (200 µM).

DB 64 and AY 17 were also used as a specific substrate to determine the specific azoreductase activity and detect substrate specificity. One unit of enzyme activity was defined as one microgram of the substrate to be reduced per minute per milligram protein. All enzyme assays were run in triplicate.

2.8. Factors Affecting Enzyme Stability

For the determination of the effect of pH on the enzyme activities, the buffer pH was prepared from pH 5.5-9. The NADH and MR were also prepared in these buffers. The acetate buffer was used for pH (5.5), phosphate buffer for pH (6.5-7.5) and tris-HCL buffer for pH (8-9).

To determine the effect of temperature on the enzymes activity, temperature values were set in the range from (20-60°C) during the measurement by UV- Vis Spectrophotometry. All the enzyme's reaction mixture was put in the water path at the desired temperature except NADH.

2.9. Enzymes Kinetics

The series concentration of MR (17-3500 mM) was prepared to determine the azoreductase kinetics, and all other constituents of the reaction mixture were constant. Kinetic values such as Michaelis-Menten constant (K max) and maximal velocity (Vmax) were calculated from Line weaver-Burk doubles reciprocal plots [11].

2.10. Extraction of Dyes Metabolic Products

A volume of 10 mL of the metabolites supernatant was extracted with three equal volumes of ethyl acetate. The combined residual extracts were dried over anhydrous sodium sulfate, filtered and evaporated by rotatory evaporation at 40°C to a volume of 10 mL. The samples were dried further in a vacuum and stored at –20ºC until use.

2.11. Detection of Metabolic Products by HPLC/MS

The extracted metabolites produced by single strains or the consortium were analyzed by high performance liquid chromatography/mass spectrometry. They were analyzed by Waters Alliance Acquity UPLC - Xevo G2S-QTof instrument. Extracts were dissolved in 1 mL of 1:1 MeCN:H_2O. A ten-microliter aliquot was taken and diluted with 90 µL 1:1 MeCN: H_2O. Two microliters were injected for LC-MS analysis. The LC conditions: A = H_2O + 0.1% HCOOH; B = MeCN + 0.1% HCOOH. 5% B – 95% B: 0-12 min 95% B: 12-17 min 5% B : 17-20 min. The flow rate was 0.2 mL/min, column temperature was 40°C using the Acquity UPLC BEH C18 column, 1.7 µm, 2.1 x 50 mm.

The MS conditions: range: 100-1200 Da, capillary: 3.5V, Sampling cone: 40V, source temperature: 120°C, desolvation temperature: 200°C, Cone gas: 25 L/h, Desolvation: 600 L/h and MSe: ramp collision energy from 15-45V. The MS was performed by electrospray ionization in positive mode [ESI$^+$] for DB 64 and negative mode [ESI$^-$] for AY 17.

2.12. Phytotoxicity Studies

The toxicity of dyes and metabolic products was determined by acute toxicity test with one of the most popular crop plants in Egypt; monocot wheat (*Triticum vulgaris*) and dicot, field bean (*Vicia faba*). Briefly, the dyes degraded broth was centrifuged at 10000 rpm for 20 min under cooling to separate the bacterial cells. The supernatant was collected and sterilized by passing through a 0.45 µm pore size filter. The sterilized filtrate is then ready for use. About 10 plant seeds were germinated in a sterilized petri dish and irrigated with dye filtrate (10 mL) at 30°C for 7 days. Then the germination percentage, the root, and shoot length were determined. A positive control was dyes before treatment with bacteria, and negative control was sterilized dist. H_2O.

2.13. Statistical Analyses

Data analysis was done using Graphpad Prism 5.0 software (GraphPad, San Diego, California, USA). Treated samples were statistically analyzed by one-tailed student's t-test ($P < 0.05$). ($P < 0.05$) was considered significant.

3. RESULTS

3.1. Isolation and Identification of Dye Degrading Microorganisms

The degradation of dyes is directly dependent upon the catalytic potential of a microorganism. Among 60 bacterial and fungal isolates, bacterial AUMC B-171 and fungal AUMC-10515 isolates exhibited the highest dyes degradation capability. They were selected for dyes biodegradation studies. The purified isolates were deposited in Assiut University Mycological Center.

Molecular Identification of AUMC B-171 and AUMC-10515 isolates was done based on rRNA gene sequences. Our Alignment results of the 16S rRNA gene sequences of the bacterium isolate AUMC B-171 with the sequences obtained by completing a BLAST search showed that the strain AUMC B-171 is highly homologous to *Pseudomonas aeruginosa* with 99% similarity. To confirm the accurate position of this strain in the phylogenic tree, several sequences repre-

sentative of *Pseudomonas* species were selected from the Genbank database for the construction of the phylogenetic tree. As shown in Fig. (**1a**), the strain AUMC B-171 shares *P. aeruginosa* in the same clusters. Consequently, the strain AUMC B-171 was identified as *P. aeruginosa*.

The sequences of rDNA-ITS for fungal strain AUMC-10515 were compared with the sequences of ITS regions in GenBank database. Results show that the ITS sequence of the isolated strain was highly homologous to *Aspergillus flavus*, with 99% sequence similarity. The constructed phylogenetic tree of AUMC-10515 sequence with a number of some *Aspergillus spp.* sequences were confirmed the right taxonomic position of the fungus isolate as *Aspergillus flavus,* as illustrated in Fig. (**1b**).

The partial sequences of 16S rRNA gene and ITS regions reported in this paper for strains AUMC B-171 and AUMC-10515 have been deposited in the DDBJ (www.ddbj.nig.ac.jp/), EMBL (www.embl.de/), and GenBank nucleotide sequence databases (http://www.ncbi.nlm.nih.gov) under accession numbers KU900217 and KT001520, respectively.

3.2. Biodegradation Studies

The initial dosage of dyes concentration is very important to detect the ability of microbial strains to degrade higher dye concentration. The results in Fig. (**2a** and **2b**) illustrated

that the degradation performance decreased by increasing the dye concentration (Fig. **2**). Stains AUMC B-171 and AUMC-10515 were separately tested for dye degradation studies as shown in Fig. (**2a** and **2b**). The results showed that they could individually remove 96.7 and 95.8% of DB 64 and 90.1 and 89.7% of AY 17 when the initial concentration for each dye was 50 mg/L (Fig. **2a** and **2b**). However, each of the single strain could only remove 30.3 and 22.4% of DB 64 and 4.6 and 7.5% of AY 17 when the initial concentration for each dye was 300 mg/L (Fig. **2a** and **2b**).

On the other hand, the consortium of AUMC B-171 and AUMC-10515 exhibited complete >99% ±0.8 removal of DB 64 and AY 17 when the initial concentration was 50 and 100 mg/L. Although the degradation percentage of both dyes reduced to 55.2 and 57.6% when the initial dyes concentration was 300 mg/L, respectively.

The results, therefore, showed that the consortium enhanced the biodegradation of the two dyes, particularly at a high concentration of dyes, when compared to the biodegradation achieved with the individual strains, as shown in Fig. (**2a** and **2b**).

3.3. Enzymes Activity and Substrate Specificity

The microbial degradation process is mainly catalyzed by several enzymes. Azoreductase is a key enzyme which cata-
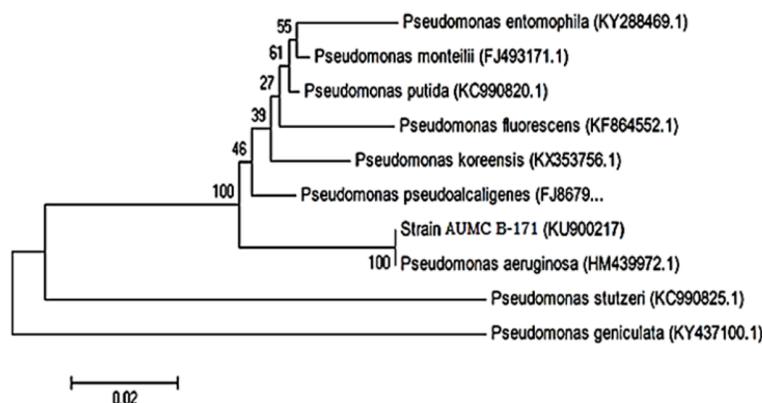
Fig. (1a). Phylogenetic relationship between the strain AUMC B-171 and other 16S rRNA gene sequences of published *Pseudomonas* spp.
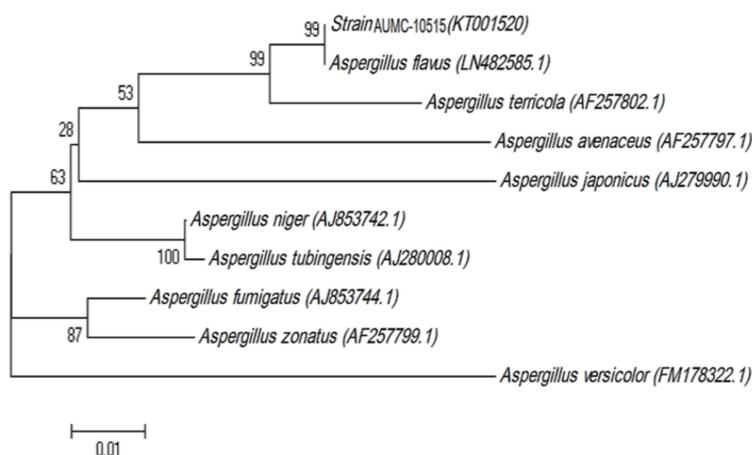
Fig. (1b). Phylogenetic relationship between the strain AUMC-10515 and other ITS rDNA sequences of published *Aspergillus* spp.
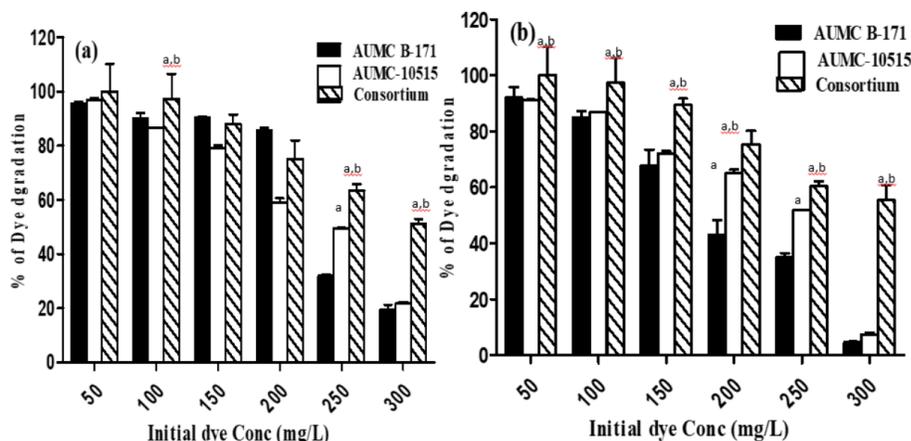
Fig. (2). Effect of initial dye concentration on the degradation percentage of DB 64 (**a**) and AY 17 (**b**) at the static condition for bacterial isolate AUMC B-171, fungal isolate AUMC-10515 and consortium. Error bars represent the standard deviation (SD±) of three replications.a; significant increase (P < 0.05) in percentage of degradation of AUMC-10515 compared to AUMC-B-171. a,b; a significant increase (P < 0.05) in percentage of degradation of consortium compared to the AUMC-B-171 and AUMC-10515.

Table 1.   **The aerobic azoreductase specific activity (U/ mg protein) of two strains on different substrates at 30°C, pH 7.0, 50 μM substrate concentration and 200 μM NADH.**

	Azoreductase Specific Activity (U/mg Protein)		
Substrate Strain	AUMC-10515	AUMC B-171	Consortium
MR	NA	0.92 ± 0.11	3.14[a] ± 0.97
DB 64	NA	0.54 ± 0.102	1.7[a] ±0.42
AY 17	NA	0.312±0.058	0.97[a]±0.01

NA; no activity determined and ±; represent the standard deviation of three replications. [a]:Significant increase (P < 0.05) in the specific activity of azoreductase produced by consortium compared to AUMC-B-171.

lyzes the reduction of not only azodyes but also other type of dyes (Kumar *et al.*, 2017) in versatile microorganisms. The individual strain of AUMC-10515 has no activity of azoreductase on MR, DB 64 or AY 17. Nevertheless, when AUMC-10515 was used in the consortium with AUMC B-171, the highest values of azoreductase specific activity 3.14, 1.7 and 0.97 U/mg protein were recorded on MR, DB 64 and AY 17, respectively as illustrated in Table **1**. In addition, using MR as a substrate stimulated the highest induction of azoreducase by the individual strain AUMC B-171 (0.92 U/mg protein) and consortium (3.14 U/mg protein). The lowest induction of azoreductase was achieved when AY 17 was used as a substrate (0.125 and 0.312 U/mg protein).

The results indicated that the preferred specific substrate selected to determine the enzyme kinetics should be MR also, that the bacterial-fungal consortium enhanced the induction of azoreductase enzyme rather than the individual strain alone (Table **1**).

3.4. Factors Affecting Enzymes Stability

The most important conditions affecting the enzymes activity are pH and temperature. Different pH values were tested to determine the optimum pH for azoreductase activi-

ty. The results showed that the maximum induction of azoreductase (0.15 and 0.25 U/min) produced by individual AUMC B-171 or consortium, respectively, was detected at pH 7.5 when MR was used as a specific substrate (Fig. **3a**).

Regarding the temperature study, the results showed that the maximum induction of azoreductase activity (0.125 and 0.35 U/min) was detected at 40°C for individual AUMC B - 171 and consortium respectively (Fig. **3b**).

3.5. Enzyme Kinetics

Using different concentrations of substrate (MR) is the main factor required for determining the azoreductase enzyme kinetics. The kinetics parameters, V_{max} (maximum velocity) and K_m (Michaelis constant) were estimated from Linveweaver-Burk plot (Fig. **4**).

The maximum velocity of azoreductase in AUMC B -171 was 12.6 μM/min/mg while it was 0.5 μM/min/mg for the consortium. On the other hand, the value K_m values were 120 and 350 μM, for AUMC B -171 and the consortium, respectively (Fig. **4a** and **4b**). The small value of K_m indicated that the affinity of the substrate to azoreductase in the consortium was higher than AUMC B -171.
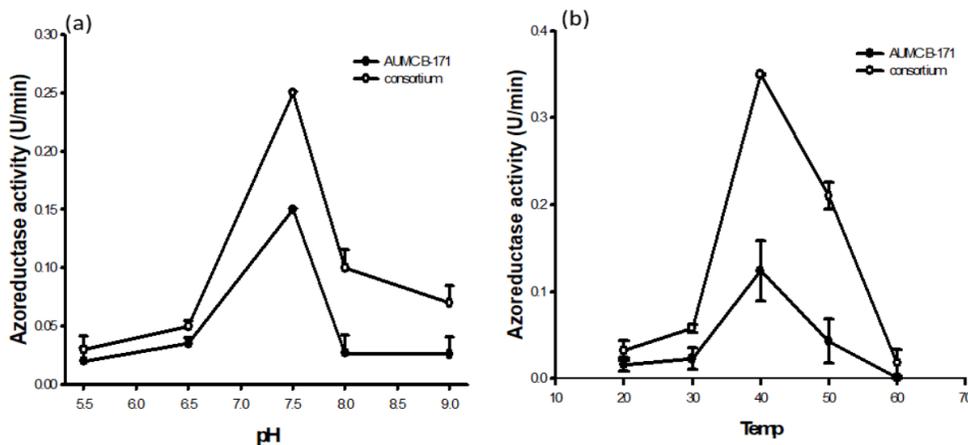
Fig. (3). Effect of pH (**a**) and temperature (**b**) on the azoreductase activity (U/min) in strains AUMC B-171 and consortium when the MR was a specific substrate 50 µM at 430 nm. Error bars represent the standard deviation (SD±) of three replications.
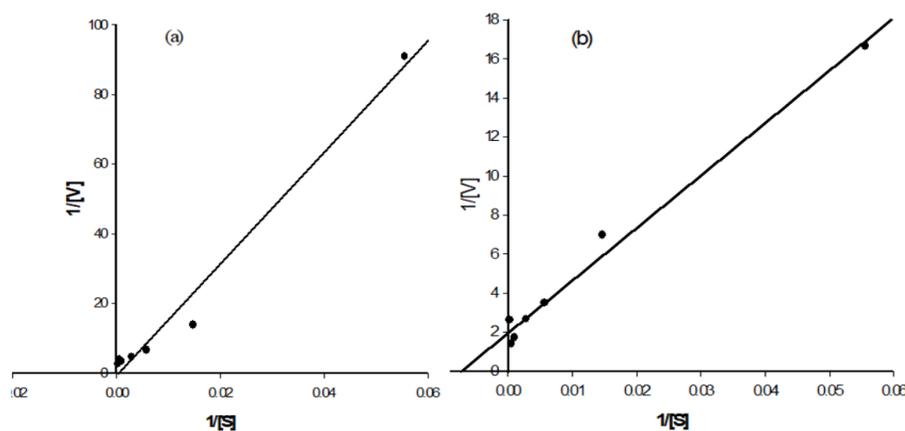
Fig. (4). A Lineweaver-Burk plot for determining the kinetic parameters of azoreductase in AUMC B-171 (**a**) and consortium (**b**) at pH 7.5 and Temperature 40°C.

3.6. Metabolic Products by HPLC–MS

HPLC-MS was used to analyze and tentatively identify the metabolic products which formed during the degradation of DB 64 and AY by either individual AUMC B -171, AUMC 10515 or consortium. The tentative assignment of seven compounds that have a good match with the structure of DB 64 is listed in Table **2**. Five compounds were detected from the degradation on DB 64 by consortium. These compounds indicated that the main mechanism for degradation of DB 64 was the reduction of anthraquinone ring. The proposed products suggest that there were two expected metabolic pathways for degradation of DB 64, 1) reduction through azoreductase *via* two electrons transfer that leads to the formation of a hydroquinone (Table **2**; No. 4 and 5), together with reduction of the C-N bond to release anilines (Table **2**; No. 3, 4, 5, 6 and 7), then further reduction resulting in ring cleavage (Table **2**; No. 6 and 7), and 2) elimination of the methoxy (Table **2**; No. 2, 4 and 7) and methyl groups (Table **2**; No. 3).

In addition, two tentative metabolites were detected from the degradation of AY17. The chemical structures of these produced metabolites indicated the reduction of diazo groups and the formation of aromatic amines (Table **3**; No. 2 and 3).

The results support our claim that the presence of co-culture in a medium consisting of DB 64 or AY 17 catalyzed higher degradation performances of these dyes than when using the individual strains.

3.7. Phytotoxicity Test

The grains of monocot grain wheat *(Triticum vulgaris)* and dicot field bean *Vicia faba* seeds were selected to study the effect of dyes and the metabolic products on the germination of crop plants seeds. The results listed in Table **4** demonstrated that the treatments of *Triticum vulgaris* and *Vicia faba* with dye solution significantly decreased the germination rate and percentage compared to 1) negative control samples and 2) the metabolic products produced after biodegradation of the dyes. The highest germination percentage was achieved when the DB64 consortium metabolic products were applied on *Triticum vulgaris* and *Vicia faba*, 98 and 99%, respectively (Table **4**). The length of shoot and root was also positively affected.

The results showed that the significant variation in the germination percentages of *T. vulgaris* 87, 83 and 99%, when the grains were treated with metabolites of AUMC

Table 2.   The tentative intermediate compounds detected by HPLC/MS from the degradation of DB 64 by AUMC B-171, AUMC-10515 and consortium.

Product No.	Chemical Structure	ES $^+$	Chemical Formula	Appearance
1 **DB 64**		451 [MW+H]$^+$	$C_{28}H_{22}N_2O_4$	-
2		391 [MW+H]$^+$	$C_{26}H_{18}N_2O_2$	AUMC 10515 and Consortium
3		316 [MW+H]$^+$	$C_{20}H_{13}NO_3$	AUMC B-171 and Consortium
4		302 [MW+H]$^+$ Con	$C_{20}H_{15}NO_2$	AUMC B-171
5		332 [MW+H]$^+$	$C_{21}H_{17}NO_3$	Consortium
6		256 [MW+H]$^+$ ASU-6	$C_{15}H_{13}NO_3$	Consortium
7		226 [MW+H]$^+$ Cons	$C_{14}H_{11}NO_2$	All
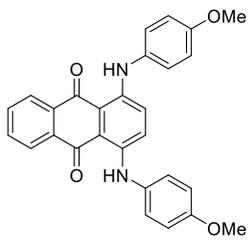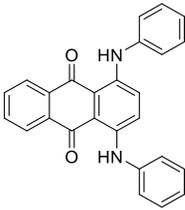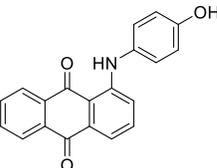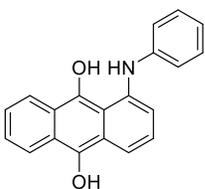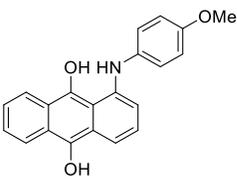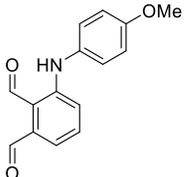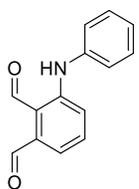
Table 3. The tentative intermediate compounds detected by HPLC/MS from the degradation of AY17 by AUMC B-171, AUMC-10515 and consortium.

Product No.	Chemical Structure	ES $^+$	Chemical Formula	Appearance
1 **AY 17**		551 [MW+H]$^+$	C$_{16}$H$_{10}$Cl$_2$N$_4$Na$_2$O$_7$S$_2$	**1** **AY 17**
2		230 [MW+H]$^+$	C$_6$H$_5$ClNNaO$_3$S	Consortium
3		337 [MW+H]$^+$	C$_9$H$_{11}$Cl$_2$N$_2$NaO$_4$S	All
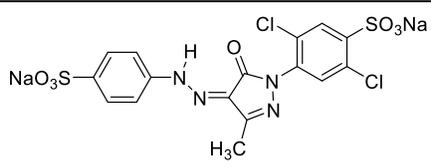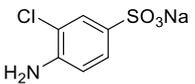
B-171, AUMC-10515 and consortium, respectively compared to a positive control (DB 64 alone) 21%. However, a significant difference trend was observed for the germination percentages of *V. faba* (*i.e.*, 80, 37 and 97%, when treated by metabolites of AUMC B-171, AUMC-10515 and consortium, respectively) compared to positive control DB 64 alone 27 (Table **2** and Fig. **5**).

A similar trend was observed when the seeds of *T. vulgaris* and *V. faba* were treated with AY 17 alone, metabolites of AUMC B-171, AUMC-10515 and consortium. The germination percentages were 24, 75, 90 and 98% (*T. vulgaris)* and 40, 70, 87 and 96% (*V. faba),* (Table **2** and Fig. **5**).

Compared to the negative control (where seeds were treated by distilled water), there was a significant enhancement in the shoot and root lengths when they were treated with metabolites of dye degradation by microorganisms., the treatment by the consortium metabolites increased shoot and root lengths by about 10 cm and 7 cm, respectively as shown in Table **4** and Fig. (**5**).

Besides that, the individual AUMC B-171's metabolites were safer on the seeds germination and the health of the growing seedling than AUMC-10515's metabolites. Overall, the results demonstrated that the crude dye generally inhibited the growth of most germinated seeds. In the case of the germinated seeds, the seedling was characterized by several weaknesses, such as thin black shoot and short root without any root hairs.

4. DISCUSSION

Azodyes and anthraquinone dyes have different concerns in wastewater treatment due to their toxicity to animals and humans [14-17]. The degradation of dyes is directly dependent upon the catalytic potential of a microorganism. In this study, two different isolated microbes, bacterium and fungus,

were individually and as a consortium applied for degradation of DB 64 and AY 17 dyes. The isolates were genetically identified based upon 16S (for bacteria) and ITS/5.8S (for fungus) rRNA genes sequences as *Pseudomoans aeruginosa* AUMC B-171 and *Aspergillus flavus* AUMC-10515, respectively. The use of 16SrRNA gene sequences for bacterial identifications is a more accurate method than traditional methods included in Bergey's Manual of Systematic Bacteriology [18-21]. The nuclear ribosomal Internal Transcribed Spacer ITS region is widely used as a DNA meta-barcoding marker to identify fungal isolates [13, 22-24].

Our results depicted the consortium culture could endure a high concentration of dyes with higher degradation capability than the individual strains studied. This may be due to the synergetic effect of the two microorganisms or due to increasing the induction of dye degrading enzymes [5]. The gradual decrease in the degradation performance by increasing the dye concentration was attributed to the toxic effect of dye concentration on the individual microbial cells [5, 25]. The results of degradation of DB 64 and AY17 by our consortium was in good agreement with the results of Kurade *et al.* [5]. They reported that the consortium of *Brevibacillus laterosporus* and *Galactomyces geotrichum* exhibited 92% decolorization of Remazole Red 198 within 18 h of initial dye concentration of 50 mg/L under optimized conditions (pH 7 and 40°C, under static condition). In contrast, the degradation percentage of the same dye by the individual *B. laterosporus* and *G. geotrichum* microorganisms exhibited 58 and 42% respectively, under analogous experimental conditions. In addition, a consortium of *Aspergillus terreus* and *Rhizopus oryzae* enhanced the removal of metals and dyes and achieved higher degradation percentage than reported in the previous literature [26].

The difference of the enzymes activity on different substrates indicated that the azoreductase enzyme was an inducible

Table 4.   Phytotoxicity test on *Triricum vulgaris* and *Vicia faba* seeds growing on the tested dyes and its metabolic products of AUMC B-171, AUMC-10515 and consortium. ±; represent the standard deviation of five replications. The p-value for a, b, and c indicate the significance of treatment compared to negative control (-ve), positive controls (+ve) and AUMC B-171 treatment, respectively.

-	Parameters		% Germination	Shoot Length	Root Length
Triticum vulgaris	Consortium	AY 17	98[a] ± 11	15.6 [a,b,c] ± 1.8	12.3 [b] ± 1.5
		DB 64	99[a,b,c] ± 6	16.5 [a,b] ±1.4	12.8[a,b] ±2.8
	AUMC-10515	AY 17	90[b] ± 0	10.5 [a],[b] ± 1.4	9.3 [b] ± 0.8
		DB 64	83 [b] ± 6	10.6 [b] ± 0.81	9.8[b]±2.7
	AUMC B-171	AY 17	75 [a,b] ± 6	15.2 [a,b] ± 1.3	12.5 [b] ± 1.7
		DB 64	87[a,b] ± 5.8	16 [a],[b]±1	7.3 [b] ± 3.8
	AY 17 Cont.		24[a] ± 7	4.1 ± 1.3	4 ± 0.57
	DB 64 Cont.		21[a] ± 6	4.2 ± 0.78	3.1 ± 0.44
	-ve Contr.		60 ± 3	5.1 ± 1.4	7.5 ±1.2
Parameters			**% Germination**	**Shoot length**	**Root length**
Vicia faba	Consortium	AY 17	96 [a,b,c] ± 1.85	14.5[a,b] ± 0.74	10.5 [a]± 1.8
		DB 64	97[a,b,c] ± 5.8	10.2 [a,b]± 1.8	9.8 ± 2.6
	AUMC-10515	AY 17	87[b] ± 6	9.7[b] ± 1	7.7± 1.8
		DB 64	37 ±10	12.1[b]± 1.3	4.8 ± 0.8
	AUMC B-171	AY 17	70[b] ± 0	7.9 [b] ± 1.9	5.4 [b] ± 1.42
		DB 64	80[b]± 10	11.4[b] ± 1.7	8.3 [b] ± 2.5
	AY 17 Cont.		40 [a] ± 10	3.9 ± 0.9	3.8 [a] ± 0.4
	DB 64 Cont.		27[a] ± 6	2.8 [a] ±1.2	3.4 [a] ± 0.7
	-ve Contr.		80 ± 5	4.5 ± 0.5	8 ± 1.2

enzyme; the presence of dyes, as substrates, stimulated the induction of this enzyme. The aerobic azo reductases were able to use both NAD(P)H and NADH as cofactors and reductively cleaved not only the carboxylated growth substrates of the bacteria but also the sulfonated structural analogues. Many researchers have cloned and characterized the genetic code of the aerobic azo reductase from *Pagmenti phagakullae* K24 [27, 28]. However, only a few bacteria with specialized azo dye reducing enzymes were found to degrade azo dyes under fully aerobic conditions [29, 30]. Among the main factors affecting the degradation of dyes is the type of dye. The molecular structure also influences the stability, reactivity with electrons and the binding affinity to degrading enzymes [31]. This knowledge can also be used to design dying processes with dyes that microorganisms can degrade more easily [32]. Many studies reported that azoreductase enzyme was capable of reduction of azo dyes, and also reducing several naphtho-, benzo-, and anthraqui-
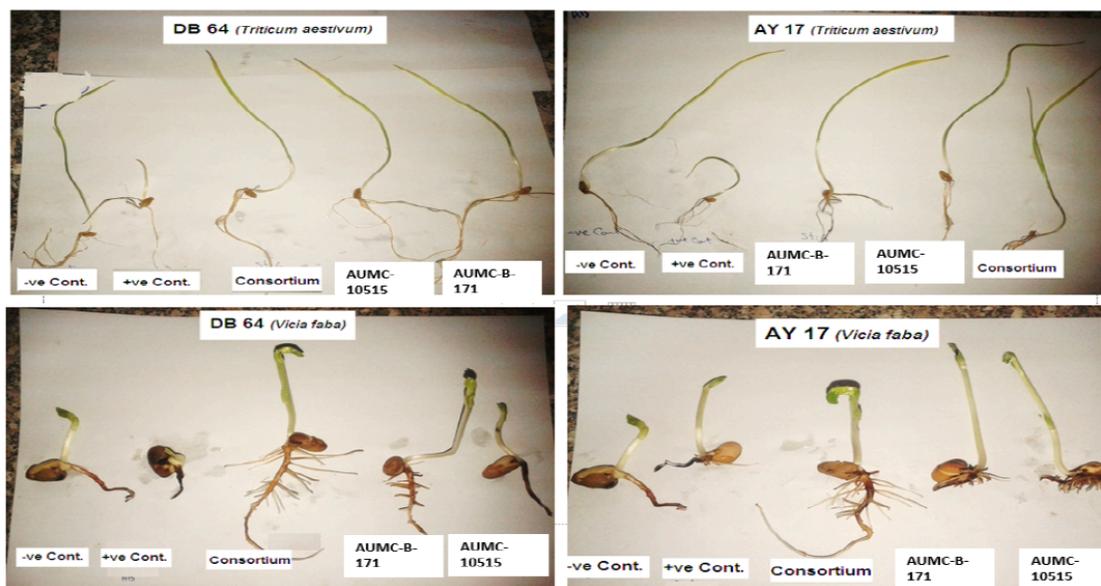
Fig. (5). The effect of dist. H$_2$O (-ve control), DB 64 and AY 17 (+ve control) and dyes metabolites produced from AUMC B-171, AUMC-10515 and Consortium on the *Triticum vulgaris* and *Vicia faba* seedling shoot and root lengths. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

none compounds [33]. This may explain the induction of azoreductase in the presence of DB 64 (anthraquinone dye).

Our results demonstrated that the presence of consortium enhanced the induction of azoreductase. This may be due to degradation products produced by AUMC 10515, which stimulated more induction of azoreductase by AUMC B-171 than observed using an individual strain. We propose that these degradation products act as a good substrate for enzyme, even better than the dye [10, 34]. It is crucial to determine the optimal conditions for azoreductase operation. Saratale *et al.* [35] stated that the isolated azoreductase displays an optimum performance in the pH range 7-9.5, which is confirmed in our study. The degradation rate was low in the pH range 5.5-6.5, however on increasing the pH to 7.5, the degradation rate improved to reach the maximum value. Furthermore, to sustain activity, most bacteria utilize electrons from carbon sources other than the dye and their metabolites [36]. All the results of temperature dependence are in agreement with reports in literature, which study the optimum condition for azoreductase [7, 37]. The presence of redox mediators, such as flavins and quinones, are known to enhance decolorization [35], due to competition for the consumption of NADH by aerobic respiration, which make the electron transfer from NADH to oxygen in order to synthesize ATP [12]. Many bacteria possess a specific cytoplasmic azoreductases system that catalyzes the electron transfer from NADH (electron donor) to azo bonds. So, the presence of NADH is very significant for the reduction of azodyes by aerobic bacteria [7]. Thus, it could be concluded that the aerobic azoreductase enzyme is an inducible enzyme required redox mediator for the best performance.

The enzyme, which has a low value of Km, achieves maximum catalytic efficiency only at low substrate concentrations. Thus azoreductase induced by consortium is more effective as a catalyst than that induced by the individual strain AUMC B-171.

The use of untreated or treated wastewater contaminated with dyes in the land may have an adverse effect on the fertility and productivity of the soil. The presence of bacteria AUMC B-171, fungi AUMC 10515 or consortium of both with dyes, enhanced the dye degradation to decrease the toxicity of dye and produce safer metabolic products. Comparing to the negative control, in which the seeds were treated by distilled water, there was a significant enhancement in the shoot and root lengths when they were treated with metabolites of dye degradation by microorganisms. The toxicity of dyes and their degradation products on the plant was different according to the type and chemistry of these compounds as well as the type of irrigated seeds. Of note, our results are in agreement with the phytotoxicity study on different plant species *Triticum aestivum* and *Phaseolus mungo*, which revealed the germination (%) of both species was less in the presence of methyl orange treatment as compared to its degradation product and distilled water [38, 39].

CONCLUSION

Our investigations confirm that the fungal-bacterial consortium exhibited a higher percentage of dyes degradation than the individual strains. Azoreductase plays a significant role as the catabolic enzyme responsible for the degradation of dyes. Additionally, the toxicity of dyes and its degradation products on the plant was different according to the type and chemistry of these compounds as well as the type of irrigated seeds. Hence, we submit that further studies on effective microbial degradation of dyes through novel fungal-bacterial consortium will lead to safer metabolic products and reduce damage to the environment.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data supporting the findings of the article is available in the DDBJ (www.ddbj.nig.ac.jp/), EMBL (www.embl.de/), and GenBank nucleotide sequence databases (http://www.ncbi.nlm.nih.gov) under accession numbers KU900217 and KT001520, respectively.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

REFERENCES

[1]   dos Santos, A.B.; Cervantes, F.J.; van Lier, J.B. Review paper on current technologies for decolourisation of textile wastewaters: perspectives for anaerobic biotechnology. *Bioresour. Technol.,* **2007**, *98*(12), 2369-2385.
http://dx.doi.org/10.1016/j.biortech.2006.11.013 PMID: 17204423

[2]   Robinson, T.; McMullan, G.; Marchant, R.; Nigam, P. Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative. *Bioresour. Technol.,* **2001**, *77*(3), 247-255.
http://dx.doi.org/10.1016/S0960-8524(00)00080-8 PMID: 11272011

[3]   Ogugbue, C.J.; Sawidis, T. Bioremediation and detoxification of synthetic wastewater containing triarylmethane dyes by *Aeromonas hydrophila* isolated from industrial effluent. *Biotechnol. Res. Int.,* **2011**, *2011*, 967925.
http://dx.doi.org/10.4061/2011/967925 PMID: 21808740

[4]   Sendelbach, L.E. A review of the toxicity and carcinogenicity of anthraquinone derivatives. *Toxicology,* **1989**, *57*(3), 227-240.
http://dx.doi.org/10.1016/0300-483X(89)90113-3 PMID: 2667196

[5]   Kurade, M.B.; Waghmode, T.R.; Jadhav, M.U.; Jeon, B.H.; Govindwar, S.P. Bacterial-yeast consortium as an effective biocatalyst for biodegradation of sulphonated azo dye Reactive Red 198. *RSC Adv.,* **2015**, *5*, 23046-23056.
http://dx.doi.org/10.1039/C4RA15834B

[6]   Yoo, E.; Libra, J.; Wiesmann, U. Reduction of azo dyes by *Desulfovibrio desulfuricans*. *Water Sci. Technol.,* **2000**, *41*, 15-22.
http://dx.doi.org/10.2166/wst.2000.0231

[7]   Shah, M.P.; Patel, K.A.; Nair, S.S.; Darji, A.; Maharaul, S. Microbial degradation of azo dye by *Pseudomonas* spp. MPS-2 by an application of sequential microaerophilic & aerobic process. *Afr. J. Microbiol. Res.,* **2013**, *1*, 105-112.

[8]   Govindwar, S.P.; Kurade, M.B.; Tamboli, D.P.; Kabra, A.N.; Kim, P.J.; Waghmode, T.R. Decolorization and degradation of xenobiotic azo dye Reactive Yellow-84A and textile effluent by *Galactomyces geotrichum*. *Chemosphere,* **2014**, *109*, 234-238.

http://dx.doi.org/10.1016/j.chemosphere.2014.02.009   PMID: 24630455

[9]   Dafale, N.; Rao, N.N.; Meshram, S.U.; Wate, S.R. Decolorization of azo dyes and simulated dye bath wastewater using acclimatized microbial consortium--biostimulation and halo tolerance. *Bioresour. Technol.,* **2008**, *99*(7), 2552-2558.
http://dx.doi.org/10.1016/j.biortech.2007.04.044 PMID: 17566726

[10] Forgacs, E.; Cserháti, T.; Oros, G. Removal of synthetic dyes from wastewaters: a review. *Environ. Int.,* **2004**, *30*(7), 953-971.
http://dx.doi.org/10.1016/j.envint.2004.02.001 PMID: 15196844

[11] Hesham, A. New safety and rapid method for extraction ofgenomic DNA from bacteria and yeast strains suitable for PCR amplifications. *J. Pure Appl. Microbiol.,* **2014**, *8*, 383-388.

[12] Beumer, A.; Robinson, J.B. A broad-host-range, generalized transducing phage (SN-T) acquires 16S rRNA genes from different genera of bacteria. *Appl. Environ. Microbiol.,* **2005**, *71*(12), 8301-8304.
http://dx.doi.org/10.1128/AEM.71.12.8301-8304.2005   PMID: 16332816

[13] White, T.J.; Bruns, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications,* **1990**, *18*, 315-322.
http://dx.doi.org/10.1016/B978-0-12-372180-8.50042-1

[14] Hesham, A.-L.; Khan, S.; Tao, Y.; Li, D.; Zhang, Y.; Yang, M. Biodegradation of high molecular weight PAHs using isolated yeast mixtures: application of meta-genomic methods for community structure analyses. *Environ. Sci. Pollut. Res. Int.,* **2012**, *19*(8), 3568-3578.
http://dx.doi.org/10.1007/s11356-012-0919-8 PMID: 22535224

[15] Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **1976**, *72*, 248-254.
http://dx.doi.org/10.1016/0003-2697(76)90527-3 PMID: 942051

[16] Zhao, M.; Sun, P.F.; Du, L.N.; Wang, G.; Jia, X.M.; Zhao, Y.H. Biodegradation of methyl red by *Bacillus* sp. strain UN2: decolorization capacity, metabolites characterization, and enzyme analysis. *Environ. Sci. Pollut. Res. Int.,* **2014**, *21*(9), 6136-6145.
http://dx.doi.org/10.1007/s11356-014-2579-3 PMID: 24474566

[17] Wilkinson, G.N. Statistical estimations in enzyme kinetics. *Biochem. J.,* **1961**, *80*, 324-332.
http://dx.doi.org/10.1042/bj0800324 PMID: 13785321

[18] Woo, P.C.; Lau, S.K.; Teng, J.L.; Tse, H.; Yuen, K.Y. Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. *Clin. Microbiol. Infect.,* **2008**, *14*(10), 908-934.
http://dx.doi.org/10.1111/j.1469-0691.2008.02070.x   PMID: 18828852

[19] Leelakriangsak, M. Molecular approaches for bacterial azoreductases. *Songklanakarin J. Sci. Technol.,* **2013**, *35*, 647-657.

[20] Hesham, A.; Asmaa, M.M.; Yasser, M.M.; Shoriet, A. Biodegradation ability and catabolic genes of petroleum-degrading *Sphingomonas koreensis* strain ASU-06 isolated from Egyptian oily soil. *BioMed Res. Int.,* **2014**, *2014*, 127674.
http://dx.doi.org/10.1155/2014/127674

[21] Hesham, A.; Asmaa, M.M.; Yasser, M.; Shoriet, A. study of enhancement and inhibition phenomena and genes relating to degradation of petroleum polycyclic aromatic hydrocarbons in isolated bacteria. *Microbiol.,* **2014b**, *83*, 599-607.
http://dx.doi.org/10.1134/S0026261714050129

[22] Hoorfar, J.; Cook, N.; Malorny, B.; Wagner, M.; De Medici, D.; Abdulmawjood, A.; Fach, P. Diagnostic PCR: making internal amplification control mandatory. *Lett. Appl. Microbiol.,* **2004**, *38*(2), 79-80.
http://dx.doi.org/10.1046/j.1472-765X.2003.01456.x   PMID: 14746535

[23] Borman, A.M.; Linton, C.J.; Miles, S.-J.; Johnson, E.M. Molecular identification of pathogenic fungi. *J. Antimicrob. Chemother.,* **2008**, *61*(Suppl. 1), i7-i12.
http://dx.doi.org/10.1093/jac/dkm425 PMID: 18063605

[24] Hesham, A.; Mohamed, E.; Asmaa, M.M.; Elfarash, A.; Abd El-Fattah, B.; El-Rawy, M. Molecular characterization of *Fusarium Solani* degrades a mixture of low and high molecular weight polycyclic aromatic hydrocarbons. *Open Biotechnol. J.,* **2017**, *11*, 27-35.
http://dx.doi.org/10.2174/1874070701711010027

[25]   Chen, K.C.; Wu, J.Y.; Liou, D.J.; Hwang, S.C.J. Decolorization of the textile dyes by newly isolated bacterial strains. *J. Biotechnol.,* **2003**, *101*(1), 57-68.
http://dx.doi.org/10.1016/S0168-1656(02)00303-6   PMID: 12523970

[26]   Mishra, A.; Malik, A. Novel fungal consortium for bioremediation of metals and dyes from mixed waste stream. *Bioresour. Technol.,* **2014**, *171*, 217-226.
http://dx.doi.org/10.1016/j.biortech.2014.08.047 PMID: 25203229

[27]   Stolz, A. Basic and applied aspects in the microbial degradation of azo dyes. *Appl. Microbiol. Biotechnol.,* **2001**, *56*(1-2), 69-80.
http://dx.doi.org/10.1007/s002530100686 PMID: 11499949

[28]   Blümel, S.; Knackmuss, H.-J.; Stolz, A. Molecular cloning and characterization of the gene coding for the aerobic azoreductase from *Xenophilus azovorans* KF46F. *Appl. Environ. Microbiol.,* **2002**, *68*(8), 3948-3955.
http://dx.doi.org/10.1128/AEM.68.8.3948-3955.2002   PMID: 12147495

[29]   Zimmermann, T.; Kulla, H.G.; Leisinger, T. Properties of purified Orange II azoreductase, the enzyme initiating azo dye degradation by *Pseudomonas* KF46. *Eur. J. Biochem.,* **1982**, *129*(1), 197-203.
http://dx.doi.org/10.1111/j.1432-1033.1982.tb07040.x   PMID: 7160382

[30]   Valli, N. Biodegradation of acid blue 113 containing textile effluent by constructed aerobic bacterial consortia. Optimization And Mechanism. *J. Bioremed. Biodegrad.,* **2012**, *3*, 162.

[31]   Pasti-Grigsby, M.B.; Paszczynski, A.; Goszczynski, S.; Crawford, D.L.; Crawford, R.L. Influence of aromatic substitution patterns on azo dye degradability by *Streptomyces* spp. and *Phanerochaete chrysosporium. Appl. Environ. Microbiol.,* **1992**, *58*(11), 3605-3613.
http://dx.doi.org/10.1128/AEM.58.11.3605-3613.1992   PMID: 1482183

[32]   Chengalroyen, M.D.; Dabbs, E.R. The microbial degradation of azo dyes: minireview. *World J. Microbiol. Biotechnol.,* **2013**, *29*(3), 389-399.
http://dx.doi.org/10.1007/s11274-012-1198-8 PMID: 23108664

[33]   Leelakriangsak, M.; Borisut, S. Characterization of the decolorizing activity of azo dyes by *Bacillus subtilis* azoreductase AzoR1. *Songklanakarin J. Sci. Technol.,* **2012**, *34*, 509-516.

[34]   Pearce, C.; Lloyd, J.; Guthrie, J. The removal of colour from textile wastewater using whole bacterial cells: a review. *Dyes Pigments,* **2003**, *58*, 179-196.
http://dx.doi.org/10.1016/S0143-7208(03)00064-0

[35]   Saratale, R.; Saratale, G.; Chang, J.; Govindwar, S. Bacterial decolorization and degradation of azo dyes: a review. *J. Taiwan Institute Chem. Eng.,* **2011**, *42*, 138-157.
http://dx.doi.org/10.1016/j.jtice.2010.06.006

[36]   Moosvi, S.; Kher, X.; Madamwar, D. Isolation, characterization and decolorization of textile dyes by a mixed bacterial consortium JW-2. *Dyes Pigments,* **2007**, *74*, 723-729.
http://dx.doi.org/10.1016/j.dyepig.2006.05.005

[37]   Supaka, N.; Juntongjin, K.; Damronglerd, S.; Delia, M.L.; Strehaiano, P. Microbial decolorization of reactive azo dyes in a sequential anaerobic-aerobic system. *Chem. Eng. J.,* **2004**, *99*, 169-17.
http://dx.doi.org/10.1016/j.cej.2003.09.010

[38]   Yemashova, N.; Kalyuzhnyi, S. Microbial conversion of selected azo dyes and their breakdown products. *Water Sci. Technol.,* **2006**, *53*(11), 163-171.
http://dx.doi.org/10.2166/wst.2006.349 PMID: 16862786

[39]   Parshetti, G.K.; Telke, A.A.; Kalyani, D.C.; Govindwar, S.P. Decolorization and detoxification of sulfonated azo dye methyl orange by *Kocuria rosea* MTCC 1532. *J. Hazard. Mater.,* **2010**, *176*(1-3), 503-509.
http://dx.doi.org/10.1016/j.jhazmat.2009.11.058 PMID: 19969416