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ENRICHMENT, ISOLATION, AND SCREENING OF DYE-DECOLORIZING HALOTOLERANT MICROBIAL STRAINS

Aya G. Faramawy [1*], Mona E.M. Mabrouk [1], Soraya A. Sabry [2], Mervat A. Arayes [1]

[1]Department of Botany and Microbiology, Faculty of Science, Damanhour University, Damanhour, Egypt. [2]Department of Botany and Microbiology, Faculty of Science, Alexandria University, Alexandria, Egypt

***Corresponding author: Email:** mervat.amen@sci.dmu.edu.eg

***ORCID**: https://orcid.org/0000-0001-7860-817X

ABSTRACT

Recently, environmental pollution has increased and become one of the serious aspects facing the world due to industrialization and rapid population, this led to the emergence of many problems that have affected the life of living organisms. Among various pollutants are dyes which have multiple industrial uses. Azo dyes are carcinogenic and mutagenic and pose hazards to humans, animals, and aquatic life. It is more difficult to degrade these dyes because they are recalcitrant. Microbial degradation has been extensively explored. Fungi are an efficient tool in dye decolorization due to their various mechanical features. The purpose of this article is a trial to enrich and isolate different microbial strains inhabiting the Egyptian samples and to highlight their potential for remediation by decolorizing dyeing waters. Here, thirteen halotolerant strains were isolated from water and sediment samples of Kom Belal Lake of Idko, Al-Beheira, Egypt, under

saline conditions. Out of 13 isolates, eight strains were able to decolorize most of the dyes with different degrees of removal. Strain A11 was selected due to its high potential to decolorize (congo red, malachite green, and methylene blue). The maximum diameter of the clear zone created by strain A11 was recorded in the presence of congo red azo dye (35 mm). The potent strain A11 which has the highest potential to remove the dye was morphologically identified as *Aspergillus* sp. during the screening process. Based on the obtained results presented here, *Aspergillus* sp. will be a candidate for further biotechnological research on the decontamination of dyeing industrial effluents. It is worth mentioning the safety of utilizing decolorized wastewater in vegetable cultivation which considers not only the nutrient needs of the crops but may also act as an alternate source of irrigation water.

Keywords: Decolorization, Azo dyes, *Aspergillus* sp., Congo red, Halotolerant, Lake of Idko

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INTRODUCTION

Microbial communities are widely distributed in our natural ecosystem like soil and water. These microorganisms are a precious natural gift. They may easily adapt to harsh environmental parameters and have a wide range of nutritional requirements. Additionally, microbes have a large number of internal and extracellular enzymes that mineralize complex contaminants as a source of carbon and energy (**Thakur *et al.*, 2019**).

The diversity of microbes that currently inhabit the Earth is known to be high and is believed to be massive. Identification of microbes and

investigation of their potential applications rely on their isolation and growth in pure culture. It is noteworthy that low biodiversity is observed in all saline habitats (**Edbeib *et al.*, 2016**). Moderately halophiles are a category of microorganisms represented by archaea, bacteria, and eukaryotes that have been recovered from adverse extreme environments, and they can grow in a medium containing between 0.5 M and 2.5 M NaCl (**Ventosa *et al.*, 1998**). They provide unique physiological functions under conditions of salinity making them interesting models for fundamental research, exploration of biotechnological potential, and their utilization in remediation. Halophiles participate in the degradation of a wide variety of aromatic hydrocarbons (**Dutta and Bandopadhyay, 2022**).

Synthetic dyes are chemical groups, that binds to an aromatic ring, with lots of commercial interest. They are widely used in the textile, paint, cosmetics, pharmaceutics, plastic, leather, and printing industries. Unsurprisingly, azo dyes accounted for more than 60% of synthetic colors (**Pattanaik *et al*., 2020**). Large amounts of these dyes are directly discharged through waste streams from the industry into natural water systems without treatment (**Qiu *et al*., 2022**), resulting in more serious environmental pollution (**Tang *et al*., 2022)**.

The chemical toxicity of different synthetic aromatic dyes poses multiple hazards to humans due to their possibility to dissolve in water which can absorb easily by the skin leading to the risk of cancer and allergic reactions. Besides, it may be lethal to some aquatic animals and plants. They could be inherently toxic, mutagenic, and carcinogenic, and their persistent nature could prolong their ecotoxicity (**Sandhya, 2010; Kurade *et al*., 2015; Salem *et al.,* 2019**). The complicated and stable chemical structure of dyes makes them difficult to degrade such as azo days, the

largest class of synthetic aromatic dyes composed of one or more (N=N) groups and sulfonic ($-SO^{3-}$) which act as a main problem in the environment (**Sudha *et al.*, 2014**).

In order to eradicate environmental pollution, multiple strategies were used to remove dyes from wastewater, typically consisting of physical, chemical, and biological treatments (**Tang *et al*., 2018**). The physical approaches include adsorption, filtration, photocatalysis, coagulation, and ozone oxidation (**Piaskowski *et al*., 2018; Ledakowicz *et al*., 2021)**. These traditional technologies of dye processing have many disadvantages that make them unsuitable and unfavorable due to high electricity and chemical requirements (**Imran *et al*., 2015**), time-consuming, low efficiency, the toxicity of by-products, high cost and labor-intensive (**Sivarajasekar and Rajoo, 2015; Bhatia *et al*., 2017; Martorell *et al*., 2017; Piaskowski *et al.*, 2018**).

Recently, effective biological treatments such as bioremediation have received increasing attention. It is a feasible alternative to physicochemical methods of dyeing wastewater owing to its simplicity, low disposal costs, environmental friendliness, and generation of less secondary waste **(Kaur and Sarao, 2022; Tang and Kristanti, 2022)**. The removal of synthetic dyes by bacteria, fungi, and algae has more efficiency and advantages.

Different reports had been reviewed a wide variety of azo dye decolorizing microbial strains including bacteria, fungi, actinomycetes, algae, and plants (**Chen, 2006; Ali *et al*., 2019; El-Badan *et al*., 2020; Abd El-Rahim *et al*., 2021; Pinheiro *et al*., 2022; Shackira *et al*., 2022; Tang *et al*., 2022)** for example; *Pseudomonas extremorientalis*;

Geobacillus thermocatenulatus; yeasts like *Geotrichum candidum*; *Cunninghamella elegans*; *Trametes versicolor,* and *Bierkandera adusta.*

Consequently, fungi are commonly utilized to manage different environmental effluents **(Derhab *et al.,* 2022)**. Filamentous fungi play a vital role in the degradation and decolorization of toxic azo dyes, which convert them into less toxic compounds and gentle metabolites released to the environment using their enzymatic system (**Khan *et al*., 2020)** or by adsorbing dyes in their cellular biomass (**Singh *et al*., 2020**). The abundance, cost-effectiveness, desirable mechanical properties, and chemical stability make them good candidates for the biosorption of dyes **(Tang *et al.,* 2022).**

The present research studied the enrichment and isolation of different microbial halotolerant strains, including bacteria and fungi, isolated from Egyptian Kom Belal Lake, Idko, El-Beheira. This article extended to screen their potential to decolorize different synthetic dyes under saline conditions.

MATERIALS and METHODS

Samples Collection

Samples used in this study were collected from Kom Belal Lake of Idko, Al-Beheira, Egypt which is located at 31°17'13.5" North, 30°16'31.2"East). Water and sediment samples were, collected at (10-15cm) depth and kept on sterile labeled bottles, immediately stored at 4 ℃. The samples were transported aseptically to the laboratory to measure pH by using a pH meter (Milwaukee).

Enrichment of halotolerant microbial strains

In order to isolate the inhabitant microbial cells, one ml of wastewater or one gram of sediment was suspended in 9 ml of distilled sterilized water and then mixed vigorously. Serial dilution method of samples was performed up to 10^{-4}. The enrichment culture was initially conducted by adding 1ml of previously diluted samples into 250 ml Erlenmyer flasks having 50 ml of autoclaved culture medium (M) containing the following (g/L): 10 g glucose, 5 g peptone, 5 g yeast extract, 1 g K_2HPO_4, 0.2 g $MgSO_4$. $7H_2O$ and supplemented with 50 g/L of NaCl. pH value was adjusted to 7 by using sterile 1N HCL and 1N NaOH. All Flasks were incubated at 30°C in an orbital shaker at 150 rpm for 5 days till the occurrence of turbidity as an indication of microbial growth.

Isolation of microbial strains

Aliquots of 200 µL of the previously enriched cultures, which served as the microbial source, were inoculated into the M- agar plates by using a pour plate technique. The plates were incubated at 30 °C for 3–5 days and examined for growth.

Purification and preservation

Colonies of different distinct morphological characteristics were selected and picked to re-streak several times by streak plate method to confirm the purification. The purity of colonies was examined visually and microscopically. Subsequently, the distinct purified isolates were preserved on agar slants and stored at 4ºC until use, while the stocks were maintained in 20% glycerol at -80ºC.

Identification of microbial isolates

Bacterial morphology including colony (form, elevation, margin, and pigmentation) was performed according to Berge's Manual of Determinative Bacteriology (**Bilal et al., 2015**). On the other hand, the selected fungal strains were subcultured and sporulated on PDA agar plates to study their morphological features. For fungal microscopic examination, lacto phenol cotton blue stain was used as the mounting fluid. The prepared fungal cell slides were identified with the help of standard fungal identification manuals (**Singh *et al*., 2017).**

Preparation of Dye Solution

The standard stock solution of (Congo red, Malachite green, and Methylene blue) dyes were prepared by dissolving 100 mg of dry powdered dye in 10 ml of distilled water. Dye solutions were sterilized using a 0.2 µm minisart filter unit.

Screening of isolated strains for dye decolorization

To test the ability of isolated pure strains as a dye decolorizer, the isolates were spotted on M-agar plates, using sterile toothpicks, amended with 50 mg/L of individually different dyes including congo red, malachite green, and methylene blue (**Table 1**). All plates were incubated at 30 °C and were examined daily. Negative controls that contained each dye in respective concentrations without inoculum were also included. Subsequently, the capacity of dye decolorization of the obtained microbial isolates on agar plates was assessed by measuring the diameter of the clear zone (mm). The strain which has the highest ability to decolorize dye was selected for identification.

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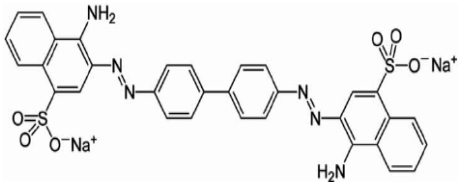
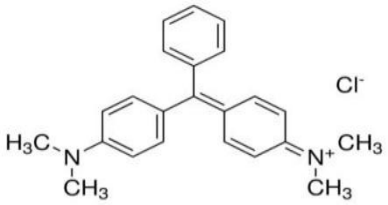
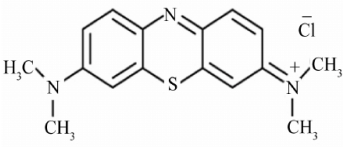
Tables 1. Chemical structures, molecular formula, and maximum wavelength of tested dyes (Khan et al., 2009; Swan et al., 2019; Kaur et al., 2022).

Dye	Chemical structure	Molecular formula	Maximum wavelength (nm)
Congo red		$C_{32}H_{22}N_6Na_2O_6S_2$	490
Malachite green		$C_{23}H_{25}ClN_2$	625
Methylene blue		$C_{16}H_{18}ClN_3S$	663

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RESULTS

Enrichment and isolation of microbial strains

The aim of this part of the work was to isolate and encourage the growth of the desired organisms that can grow aerobically in the presence of salt, the samples, which act as a source of microbes, was enriched on neutral broth media at 30°C. Growth was monitored in the form of turbidity in comparison with control. As a result, thirteen pure halotolerant strains were isolated from water and sediment samples of Kom Belal Lake of Idko, Al-Beheira, Egypt. Data depicted in **Figure. 2** illustrated the higher percentage of bacterial isolates than fungi (77%, 23%, respectively).

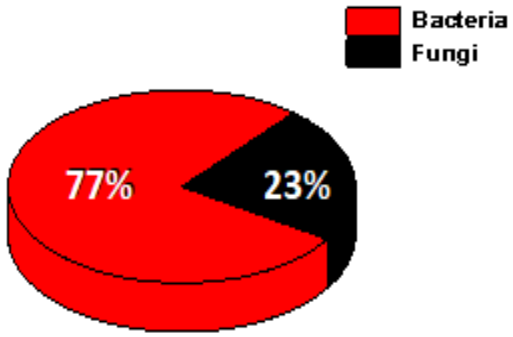

Figure 2. Pie chart representing the percentage of isolates enriched from different local samples.

The results of some morphological characters of selected isolates are summarized in **Table 2.** It was cleared that all colonies of the purified isolates were mostly pigmented.

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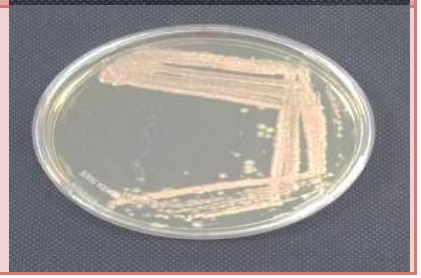
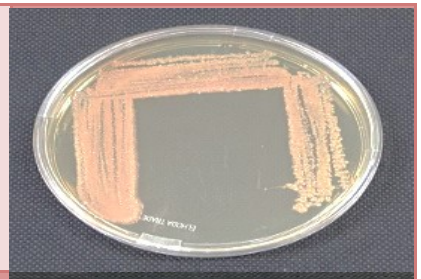
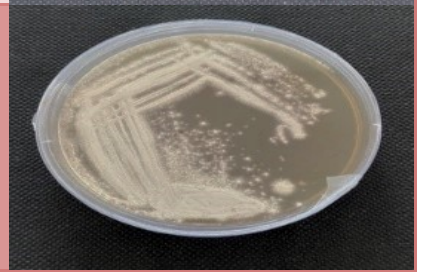
Table.2. Morphological characters of the total number of microbial isolates isolated from Kom Belal Lake of Idko, Al-Beheira, Egypt.

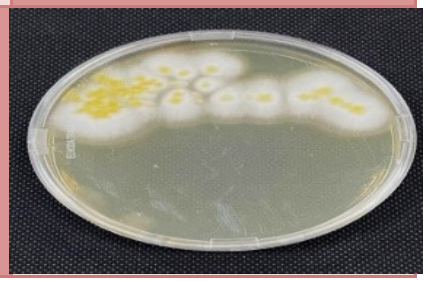
NO.	Isolates	Colony color	Texture	Margin	Image
1	A1	Beige	Flat rough	Entire	
2	A2	Pink	Raised smooth	Entire	

3	A3	Whitish creamy	Flat rough	Round irregular	
4	A4	Beige	Raised smooth	Entire	

5	A5	Slight brown colony	Raised smooth	Entire	
6	A6	Beige colony with white center	Raised	Entire	
7	A7	Beige	Flat smooth	Entire	

8	**A8**	**Beige colony**	**Raised rough**	**Entire**	
9	**A9**	**Beige**	**Convex smooth**	**Entire**	

10	**A10**	**Pink**	**Raised**	**Entire**	
11	**A11**	**White**	**Powdery**	**Flat**	

12	A12	Green with white margin	Cottony growth	Round, even	
13	A13	White colony with yellowish center	Cottony growth	Round, even	

Assessment of dye decolorization

The screening experiment of all isolates was completed in the presence of 50 mg/L of each tested dyes. The results indicated that out of thirteen isolates, only eight were able to decolorize the dyes successfully with different degree according to the type of tested dye (**Table 3)**. All isolates were achieved varied positively results with congo red, and malachite green but only five isolates were able to decolorize methylene blue.

Table 3. Screening of some isolated strains to decolorize different dyes.

Clear zone diameter (mm)			
Strain Code	**Congo Red**	**Malachite Green**	**Methylene Blue**
A2	14	9	-
A3	5	8.5	-
A4	10	9.5	-
A5	9	8	11
A6	25	7	10
A7	8.5	13	7
A8	7.5	15	10
A11	35	24	20

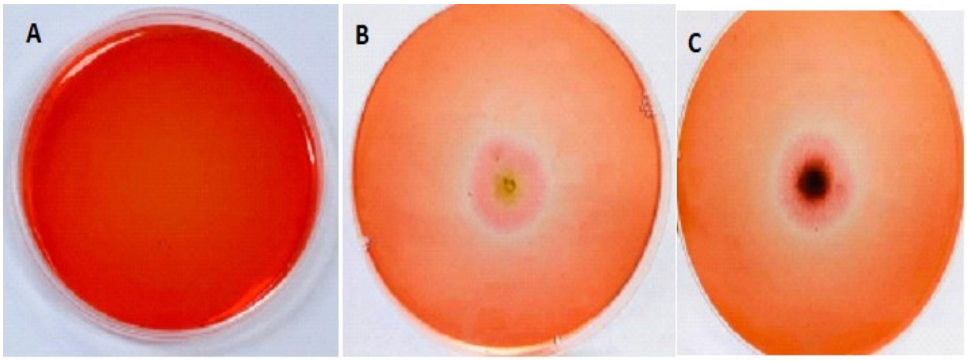


Figure 3. Decolorization potential of the most potent selected fungal strain A11 grown on agar plates amended with 50 mg/L of congo red; A) control plate; B) top view of colony with clear zone formation; c) reverse view of colony.

It was noticeable that the most promising isolate designated as A11, which can decolorize all three synthetic dyes, was selected to identify. According to the morphological and microscopic features, strain A11 was identified as *Aspergillus* sp. The maximum diameter of the clear zone created by *Aspergillus* sp. was observed clearly in the presence of congo red azo dye (35 mm) (**Figure 3)**. The obtained data proved the efficiency and immense potential of the candidate *Aspergillus* sp. to remove toxic dyes from contaminated wastewater.

DISCUSSION

Microorganisms are interesting for basic science inquiry as well as for investigating their possible biotechnological applications or remediation. The diversity and abundance of microorganisms that depend on culture techniques is a crucial tool for discovering new genera and species (**Arayes *et al.*, 2021**). Since the industrial effluents contained

significant quantities of salts, selecting a salt-tolerant microbe is typically regarded as an aspect of bioremediation research improvement **(Taskin and Erdal, 2010; Pourbabaee *et al*., 2011; Kaushik and Malik, 2010)**.

Dyes are extensively used for multiple commercial purposes **(Sundarajoo *et al*., 2022)**. However, the complexity, toxicity, and stability of dyes have increased in parallel with the industrial demand for dye color and fastness, which simultaneously results in more serious environmental pollution.

Thousands of varied synthetic coloring agents are used by dye-consuming industries for diverse operations (**Laraib *et al*., 2020**). According to statistics, 30–50% of these dyes are untreated and discharged into natural water systems (**Giovanella *et al.,* 2020**), leading to serious water contamination in the surroundings of the printing and dyeing industries. In addition to the carcinogenicity of dyes, they are harmful to aquatic life, as well as human life **(Lellis *et al*., 2019)**.

Nowadays, the effective biological method based on the microbial removal of dyes has attracted much attention due to their low cost, environmentally friendly, low sludge, and high efficiency to remove dye **(Adenan *et al*., 2021)**. The bioremediation of extreme environments requires microbial populations that are adapted to the environment. Many bacteria and filamentous fungi produce a powerful biodegradation ability to degrade toxic azo dyes **(Kang *et al.,* 2018; Ngo and Tischler, 2022)**.

Elimination of a wide variety of dyes using fungi and other microbes was performed through different bio-processes such as biosorption, detoxification, bio-degradation, bioaccumulation, and enzymatic mineralization **(Monsalve *et al.,* 2017)**. They have the capability

to release several extracellular enzymes, such as manganese peroxidase, lignin peroxidase, and laccase which play a vital role in dye degradation **(Kang *et al.,* 2018; Souza *et al.,* 2022)**.

Several fungal strains have been reported to degrade the color of dyeing wastewater including *Aspergillus niger* **(Asses *et al*., 2018),** *Phanerochaete chrysosporium* **(Spadaro *et al*., 1992; Pazarlioglu *et al.,* 2005),** *Emmia latemarginata*, *Mucor circinelloides* **(Hernández *et al.,* 2021),** *Aspergillus fumigatus* **(Jin *et al.,* 2007; Karim *et al.,* 2017**), *Cryptococcus spp*. **(Olufunke *et al.,* 2016**), *Candida albican* **(Sudiana *et al.,* 2022),** *Penicillium geastrivorus* **(Yang *et al.,* 2003; Das and Das, 2017),** and *P. ochrochloron* **(Shedbalkar and Jadhav, 2011; Aytar *et al.,* 2016).**

The current study showed that several halotolerant microbial strains including the bacteria and fungi, were successfully isolated and enriched from the local samples by relying on the growth in culture media amended with salt which indicates the halotolerant nature of our local isolates. The high percentage of bacterial isolates was an indicator of the wide distribution of bacteria in the collected samples. The obtained results pointed out that *Aspergillus* sp. was the most promising isolate that has the potential to decolorize different types of dyes. This difference might be due to that filamentous fungi normally display biodegradation and biosorption activities because they contain a large specific surface area and lignin-modifying enzymes **(Kang *et al.,* 2018)**. The capability of *Aspergillus* sp. to remove congo red under saline conditions in the dark at 30 °C, was detected without abiotic loss of congo red in the control test.

*Aspergillus s*pp. such as *A. terreus, A. niger* have been reported before in previous research to indicate their natural potential to remove azo

dyes **(Jin *et al*., 2007; Ilyas and Rehman, 2013; Asses *et al.,* 2018; Almeida and Corso, 2019; Salem *et al*., 2019; Sundarajoo *et al.,* 2022)**. The variation in decolorization efficiency and the time required for the decolorization of various dyes may be due to the molecular complexity of the dyes, culture conditions, and the enzyme system produced by fungi **(Karim *et al.,* 2020)**.

Knowledge of biodegradation, which uses microscopic biological reactors, is not only helpful in pollution abatement but also in the production of bio-friendly products. To confirm the potentiality of water reuse, it is critical to demonstrate a reduction in dye-decolorizing wastewater toxicity towards seed germination and plant growth through phytotoxicity analysis performance. The current study was a trial to explore the halotolerant microbes with the potential to bioremediate the recalcitrant toxic compounds such as azo dyes from the surrounding natural environment (**Ali, 2010; Amin *et al*., 2020).**

CONCLUSION

The generated wastes, as a result of growing industries, are the main contributors to water pollution. Therefore, bioremediation is as eco-friendly, easily applicable, cost-effective process that has been recommended and received increasing attention in the treatment of pollutants. There were great efforts that have to be endeavored microbiologically to manage the industrial effluents. The current study employed a candidate local isolate *Aspergillus* sp. as a green biocatalyst to degrade and decolorize Congo red under saline conditions. This article was a potential technology way to detoxify the contaminated wastewater containing dyes.

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الملخص العربي

إثراء وعزل واختبار السلالات الميكروبية المتحملة للملوحة المزيلة للأصباغ

آيه جمال فرماوي [1*]- مني السيد مبروك [1] - ثريا عبدالفتاح صبري[2] - ميرفت أمين عرايس[1]

[1] قسم النبات والأحياء الدقيقة ، كلية العلوم ، جامعة دمنهور، دمنهور، مصر [2] قسم النبات والأحياء الدقيقة ، كلية العلوم ، جامعه الاسكندية ، الاسكندرية ، مصر

***Corresponding author: Email:** mervat.amen@sci.dmu.edu.eg

ORCID: https://orcid.org/0000-0001-7860-817X

يشكل التلوث البيئي مخاطر عديدة تواجه العالم بسبب تزايد عمليات التصنيع والنمو السكاني ، مما أدي الي انتشار العديد من المشكلات البيئية والتي أثرت بطبيعة الحال علي حياة الكائنات الحية. وتعتبر الأصباغ إحدي الملوثات البيئية والتي تتعدد في إستخدامها صناعيا.وتشكل مجموعة أصباغ الآزو المسرطنة مخاطر علي الانسان والحيوان والكائنات المائية. حيث تمثل عملية إزالة هذه الأصباغ صعوبة شديدة .

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ومن الممكن استخدام الميكروبات في إزالة أصباغ الآزو والتخلص منها. وتحتل الفطريات كإحدي الكائنات الدقيقة الهامة والمستخدمة بيولوجيا، دورا مهما للتخلص من الأصباغ وذلك لتعدد وتنوع خصائصها الميكانيكية. والهدف من هذا العمل هو محاولة لإثراء وعزل السلالات الميكروبية المختلفة والكامنة في العينات المجمعة محليا وتسليط الضوء علي مدي إمكانية استخدام هذه الميكروبات للتخلص من الأصباغ الملوثة للمياه. وفي هذه الدراسة, تم عزل 13 سلالة نقية متحملة للملوحة من مياه ورواسب بحيرة كوم بلال ، إدكو ، محافظة البحيرة ، مصر. نجحت ثمان من تلك العزلات في التخلص من معظم الأصباغ المختبرة وبنسب مختلفة. وأوضحت النتائج أن السلالة (A11) أثبتت قدره عالية في إزالة صبغ الكونجو الأحمر، وأخضرالمالاكيت ، وأزرق الميثيلين.وكانت أعلي قدره لهذه السلاله في إزالة صبغ الكونجو الأحمر وسجلت أعلي قيمة لها بقياس قطر المنطقه الرائقه من الصبغه والتى بلغت (35 مم). وتم تعريف هذه السلاله باستخدام الطرق المورفولوجية والتي أثبتت انها تنتمي إلي .*Aspergillus* sp و أثبتت النتائج التي تم الحصول عليها أن هذا الفطر يعد أحد الكائنات الدقيقة الواعدة والمرشحة للمزيد من التطبيقات التكنولوجية والحيوية للتخلص من الأصباغ الملوثة للمياه والمنتشرة في المخلفات الصناعية واستخدام المياه المعالجة من الأصباغ في زراعة النباتات.