PHYTOREMEDIATION OF ARSENIC IN CONSTRUCTED WETLAND USING *Pennisetum purpureum* Schumach. AND PLANT GROWTH-PROMOTING RHIZOBACTERIA

By

MD. EKHLASUR RAHMAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

I dedicated my thesis to beloved my parents, my wife and my sons for their prayer, patient and support
During my study to achieve my goal

Grateful to almighty Allah
and
Thanks to my family members as well as my friends who appreciated me about my study

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Nowadays there are many types of technologies to restore contaminated areas with heavy metals. One of the technologies that belong to a green technology is the remediation of soil and groundwater contaminated with heavy metals using plants and plant growth-promoting rhizobacteria (PGPR). This technology is known as PGPR-assisted phytoremediation in the world. Among the advantages of phytoremediation are a cost-effective, environmentally friendly and it can be used as an alternative for bioenergy. The research was conducted to search for potential plants which can be used in phytoremediation mainly for inorganic arsenic in arsenate form [As(V)]. The objectives of this study were to determine the maximum concentration of As that could be taken up by *Pennisetum purpureum* in phytoremediation, to identify the role of rhizobacteria in the root of *P. purpureum* which can enhance phytoremediation process, to determine the mechanisms of As uptake and bioaccumulation by *P. purpureum* and to optimize the As phytoremediation process using pilot reed beds. As phytotoxicity test, rhizobacteria isolation, molecular identification of rhizobacteria, qualitative and quantitative screening of the rhizobacterial isolates for As-tolerance, determination of different plant growth-promoting traits, As phytoremediation in pilot reed beds, determination of different enzyme activity in pilot reed beds and the optimization of phytoremediation process were included in this study. Results of rhizobacteria isolation showed that two gram-positive and seven gram-negative rhizobacteria that resistant to As. Almost all rhizobacteria (nine) through molecular identification showed excellent performance on As tolerance and different plant growth-promoting activity. Among them *Bacillus australimaris* showed the highest performance on As-tolerant and different plant growth-promoting activity. Based on SEM-EDX and TEM-EDX analysis showed that there was a difference between *B. australimaris* in control and exposed with As. *P. purpureum* was able to survive up to 40 mg kg^{-1} As concentration for 77 days of exposure at the time of advanced phytotoxicity test. The amount of bioaccumulation of As in entire *P. purpureum* on 77 days reached 2,323.22 ± 74.34 mg

kg^{-1} dry weight with bioaccumulation factor 0.23 ± 0.006, translocation factor 0.87 ± 0.028, percentages of translocation 42.92 ± 1.2 and calorie value of *P. purpureum* 16,841.32 ± 199.10 J g^{-1}. Results on phytoremediation of As in non-aerated pilot reed beds showed that the highest amount of As bioaccumulation by *P. purpureum* that occurred in the As concentration of 39 mg kg^{-1} at a retention time of 42 days was 5,733.28 ± 68.80 mg kg^{-1} dry weight. The addition of aeration in pilot reed beds could enhance toxic effect of As on *P. purpureum* although the accumulation of As increased. Optimization results on the phytoremediation process with concentration of 39 mg kg^{-1} As showed that addition of nine rhizobacteria consortium and NPKS fertilizer could alleviate the As toxic effect and increase the biomass of *P. purpureum*, however the total As bioaccumulation ability increased and its highest uptake was 6,944.48 ± 69.44 mg kg^{-1} dry weight. Results of SEM-EDX analysis of fresh roots, stems and leaves of *P. purpureum* revealed significant differences in the context on the effect of As on plant tissue and uptake of As between control and different treatments. In conclusion, *P. purpureum* is an As hyperaccumulator plant but it is sensitive to high concentration of As and the addition of PGPR consortium has enhanced both the growth of *P. purpureum* and the As accumulation. These results suggest that PGPR-assisted phytoremediation of As in constructed wetland can be used in As phytoremediation in agricultural polluted areas and anthropogenically polluted environments due to its high capability to uptake and bioaccumulation of As.

Keywords: Phytoremediation, Arsenic, Constructed wetland, *Pennisetum purpureum*, Plant growth-promoting rhizobacteria

SDG: GOAL 3: Good health and well-being, GOAL 6: Clean water and sanitation

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

FITOREMEDIASI ARSENIK DI TANAH BENCAH YANG DIBINA MENGGUNAKAN *Pennisetum purpureum* Schumach. DAN RHIZOBAKTERIA PENGALAK TUMBESARAN

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Kini terdapat banyak jenis teknologi untuk memulihkan kawasan yang tercemar dengan logam berat. Salah satu teknologi yang tergolong dalam teknologi hijau ialah pembaikan tanah dan air bawah tanah yang tercemar dengan logam berat menggunakan tumbuhan dan rhizobakteria penggalak tumbesaran tumbuhan (PGPR). Teknologi ini dikenali sebagai PGPR dibantu oleh fitoremediasi di dunia. Antara kelebihan fitoremediasi ialah kos efektif, mesra alam dan ia boleh digunakan sebagai alternatif untuk biotenaga. Penyelidikan ini dijalankan untuk mencari tumbuhan berpotensi yang boleh digunakan dalam fitoremediasi terutamanya untuk arsenik tak organik dalam bentuk arsenat [As(V)]. Objektif kajian ini adalah untuk menentukan kepekatan maksimum As yang boleh diambil oleh *Pennisetum purpureum* dalam fitoremediasi, untuk mengenal pasti peranan rhizobakteria dalam akar *P. purpureum* yang boleh meningkatkan proses fitoremediasi, untuk menentukan mekanisme arsenik (As) pengambilan dan bioakumulasi oleh *P. purpureum* dan untuk mengoptimumkan proses fitoremediasi As menggunakan pilot rid bed. Ujian kefitotoksikan, pengasingan rhizobakteria, pengenalpastian molekular rhizobakteria, saringan kualitatif dan kuantitatif bagi pengasingan rhizobakteria untuk toleransi As, penentuan ciri-ciri penggalak pertumbuhan tumbuhan yang berbeza, fitoremediasi dalam pilot rid bed, penentuan aktiviti enzim yang berbeza dalam rid bed dan pengoptimuman proses fitoremediasi turut dijalankan dalam kajian ini. Keputusan pengasingan rhizobakteria menunjukkan dua gram positif dan tujuh gram negatif rhizobakteria yang mempunyai rintangan kepada As. Hampir semua rhizobakteria (sembilan) melalui pengenalpastian molekul menunjukkan prestasi cemerlang pada toleransi As dan aktiviti yang menggalakkan pertumbuhan tumbuhan yang berbeza. Antaranya *Bacillus australimaris* menunjukkan prestasi tertinggi. Berdasarkan analisis SEM-EDX dan TEM-EDX menunjukkan terdapat perbezaan antara *B. australimaris* dalam kawalan dan terdedah dengan As. *P. purpureum* mampu bertahan sehingga 40 mg kg^{-1} As kepekatan selama 77 hari pendedahan pada masa ujian fitotoksisiti lanjutan. Jumlah bioakumulasi As dalam keseluruhan *P. purpureum* pada 77 hari mencapai 2,323.22 ± 74.34 mg kg^{-1} berat kering dengan faktor bioakumulasi 0.23 ± 0.006, faktor translokasi 0.87 ± 0.028, peratusan nilai translokasi 42.92 kalori dan purpureum. 16,841.32 ± 199.10 J g^{-1}. Keputusan fitoremediasi As dalam pilot rid yang tidak berudara menunjukkan jumlah bioakumulasi As tertinggi oleh *P. purpureum* yang berlaku dalam kepekatan As 39 mg kg^{-1} pada masa pengekalan 42 hari ialah 5,733.28 ± 68.80 mg kg^{-1} berat kering. Penambahan pengudaraan dalam pilot rid bed boleh meningkatkan kesan toksik As pada *P. purpureum* walaupun pengumpulan As meningkat. Hasil pengoptimuman terhadap proses fitoremediasi dengan kepekatan 39 mg kg^{-1} Seperti yang menunjukkan bahawa penambahan sembilan konsortium rhizobakteria dan baja NPKS dapat mengurangkan kesan toksik As dan meningkatkan biojisim *P. purpureum*, namun jumlah keupayaan bioakumulasi As meningkat dan pengambilan tertinggi ialah 6,944.48 ± 69.44 mg kg^{-1} berat kering. Keputusan analisis SEM-EDX akar, batang dan daun segar *P. purpureum* mendedahkan perbezaan yang ketara dalam konteks kesan As pada tisu tumbuhan dan pengambilan As antara kawalan dan rawatan yang berbeza. Kesimpulannya, *P. purpureum* adalah tumbuhan hiperakumulator As tetapi ia sensitif kepada As dan penambahan konsortium PGPR telah meningkatkan kedua-dua pertumbuhan *P. purpureum* dan pengumpulan As. Keputusan ini menunjukkan bahawa fitoremediasi As dibantu PGPR dalam tanah lembap yang dibina boleh digunakan dalam fitoremediasi As dalam kepekatan rendah seperti kawasan tercemar pertanian dan kawasan perindustrian kerana keupayaannya yang tinggi untuk menyerap dan bioakumulasi As.

Kata kunci: Fitoremediasi, Arsenik, Tanah bencah yang dibina, *Pennisetum purpureum,* Rhizobakteria pengalak tumbesaran

SDG: GOAL 3: Kesihatan dan kesejahteraan yang baik, GOAL 6: Air bersih dan sanitasi

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LIST OF ABBREVIATIONS

kg	Kilogram
g	Gram
µg	Microgram
mL	Milliliter
µL	Microliter
ppm	Parts per million
CFU	Colony-forming unit
spp.	Species
COD	Chemical oxygen demand
BOD	Biochemical oxygen demand
TSS	Total suspended solids
TKN	Total Kjeldahl nitrogen
TP	Total phosphate
CO_2	Carbon dioxide
DMDSe	Dimethyldiselenide
DMSe	Dimethylselenide
HMs	Heavy metals
As (III)	Arsenic trivalent (arsenite)

As (V)	Arsenic pentavalent (arsenate)
N	Nitrogen
P	Phosphorus
K	Potassium
S	Sulfur
Na	Sodium
Ca	Calcium
Cl	Chlorine
Mg	Magnesium
Ni	Nickel
Cr (VI)	Hexavalent chromium
Cr (III)	Trivalent chromium
Pb	Lead
Fe	Iron
Co	Cobalt
Al	Aluminium
Se	Selenium
Sr	Strontium
US	United States

UK	United Kingdom
USA	United States of America
pH	Potential of hydrogen
mV	Millivolt
<	Less than
>	Greater than
rpm	Revolution per minute
MWH	Megawatt hour
kWh	Kilowatt hour
ATP	Adenosine triphosphate
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
nm	Nanometer
MW	Molecular weight
min	Minutes
EDTA	Ethylenediamine tetra acetic acid
M	Molar
°C	Degree Celsius
PCR	Polymerase Chain Reaction
mM	Millimolar

bp	Base pair
kb	Kilobases
w/v	Weight per volume
v/v	Volume per volume
HNO_3	Nitric acid
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
NaOH	Sodium hydroxide
KH_2PO_4	Potassium dihydrogen phosphate
NaCl	Sodium chloride
KCl	Potassium chloride
$Na_2HPO_4.7H_2O$	Sodium phosphate dibasic heptahydrate
$MgSO_4$	Magnesium Sulfate
$FeCl_3$	Ferric chloride
$CaCO_3$	Calcium carbonate
$NaNO_3$	Sodium nitrate
$MgSO_4$	Magnesium sulfate
KOH	Potassium hydroxide
OD	Optical density
NBRIP	National Botanical Research Institute's phosphate growth medium
SAS	Statistical analysis software

CHAPTER 1

INTRODUCTION

1.1 Research background

Arsenic (As) is a hazardous, cancer-causing, as well as widespread metalloid in the ecosystem. Owing to industrial usage, the total global arsenic production was anticipated to reach 8.61 million tons (Statista, 2023). As polluted soil, sludge, as well as sediment are the primary factors to the arsenic pollution of drinkable water, aquifers, as well as the food cycle. Arsenite (As (III)) as well as arsenate (As (V)) are the main abundant types of arsenic in environment (Pillewan et al., 2014). It is reported to reduce crop production in plants and prolonged contact to arsenic at levels more than 50 mg L^{-1} can cause a wide array of diseases in humans, including different types of cancers, premature delivery, stillbirth, and spontaneous abortion (Beniwal, Yadav and Ramakrishna 2023). More than 140 million inhabitants in seventy countries are reportedly impacted through arsenic contamination, according to a United Nations Children's Fund (UNICEF) report in 2007. Due to this issue, there is growing awareness in using various treatment methods to get rid of arsenic from polluted water. Although it is well recognized that traditional designed treatment methods are expensive and have issues with sludge formation and disposal. It is crucial to discover onsite, decentralized, as well as ecologically safe treatment processes that are reliable, have little need for maintenance, and are inexpensive to operate.

Constructed wetlands are a reliable and economically viable natural process that have been helpful in eliminating a variety of contaminants, including arsenic (Ayangbenro and Babalola, 2017). Constructed wetlands (CWs) are artificial processes that have been created to utilize the natural processes incorporating the plants, soils, as well as related microbial populations of wetlands to help purify wastewater (Hammer, 2020). Arsenic and other metals and metalloids might be effectively removed by this technique (Buddhawong et al., 2005). Different types of plants have been found useful for phytoremediation of heavy metals in the CWs such as *Pennisetum purpureum*, *Scirpus grossus, Ludwigia octovalvis, Melastoma malabathricum* etc. (Rahman et al., 2020). Napier grass (*Pennisetum purpureum*) is a perennial grass, has lately gained international interest as a process for bioremediation of heavy metals (Zhang et al., 2010). Juel, Dey & Akash 2018 studied Napier grass (*P. purpureum*) and Indian mustard (*Brassica juncea*) plants grew well on tannery waste and gathered large levels of heavy metals in various areas of the plant. Yun and Ali, 2019 directed a research on *Pennisetum purpureum* (elephant grass) for phytoremediation of cadmium. They discovered that the maximum cadmium ion elimination percentages for both untreated as well as treated *P. purpureum* were 92% and 98%, respectively. Although, Napier grass is efficient in removing toxic heavy metal and nutrients. However, the capability and efficiencies of Napier grass in removing arsenic in constructed wetland is not much reported in the literature.

Recently, the probable synergies between phytoremediation including bioenergy generation have been progressively studied (Kumar et al., 2017). By using the biomass that is collected during the phytoremediation operation, it is possible to create renewable bioenergy, like biogas, while simultaneously removing hazardous toxins as well as improving the condition of the soil (Hunce et al., 2019). As 100% of the biomass is being used, this is also one of the zero-waste management concepts (Osmana et al., 2020). Due to *P. purpureum* has been extensively investigated and may be used as a feedstock for the manufacture of biofuels, its effectiveness for bioenergy generation cannot be disputed in this aspect (Takara and Khanal, 2015; Mohammed et al., 2019). Many investigations have been done on the process of making ethanol from *P. purpureum*. According to Bensah et al. (2015), when compared to other biomass kinds investigated, *P. purpureum* had the greatest ethanol generation of 65.1% (bamboo wood, rubber wood, Siam weed, including coconut husk). Theoretically, the highest ethanol output is 35%. Research proved that this plant is a good resource for biofuel generation (Osmana et al., 2020). Napier grass obtained as a by-product after removing of arsenic in constructed wetland and then its substantial cellulose level allowed for the production of biofuels (Takara and Khanal, 2015). Its abundant cellulose may be used as a reservoir of carbon in the synthesis of biofuels like ethanol as well as butanol (He et al., 2017).

Previously, there are various methods used to get rid of that pollution from the environment. Amongst the approaches, phytoremediation is a green and an awful lot convincing device for clean-up of arsenic. Nevertheless, the implementation of phytoremediation in polluted places is constrained by dual major issues as for example i) Slow and steady expansion rate at stronger heavy metals polluted places and ii) Systemic absorption of heavy metals. This situation will be minimized and hasten the phytoremediation effectiveness by integrating the latent rhizobacteria as a complementary method. For this reason, to remove arsenic from environment rhizobacteria assisted phytoremediation of arsenic in Constructed wetland (CW) are very important at this moment. Plant growth promoting rhizobacteria (PGPR) assisted phytoremediation in CW is a system utilized to eliminate ecological pollutants from the environment and utilized the biological mechanisms inherent in microbes and plants eradicated harmful contaminants and restored the ecosystem to its original state (Ayangbenro and Babalola, 2017). Additionally, constructed wetlands are designed to mimic natural chemical, microbiological, and physical methods. The process is dependent on 3 factors: contact with soil microorganisms or rhizobacteria, chemical as well as physical properties of the reed bed, along with the identity of the plant itself.

PGPR is a set of bacteria that can be obtained in the rhizosphere (Ahmad et al., 2008). Soil rhizobacteria may also have a direct influence on metal dissolvability through affecting the speciation of heavy metals in the root zone, as well as metal bioavailability through modifying their chemical characteristics (Jing et al., 2007). Due to the absence of beneficial microorganisms, polluted soils are frequently lacking in nutrients. Nevertheless, such soils may be rendered nutrient-rich through introducing metal-resistant microorganisms, particularly PGPR, which not only offer critical nutrients to plants, but also enable plants to extract heavy metals, which can then be used in agricultural production or phytoremediation of polluted soil.

The interactions between plant and rhizobacteria have widely been applied in farming activities by providing plant with nitrogen sources and thus stimulate plant growth. This relationship has been applied in heavy metal contaminated soils to enhance soil fertility and to increase bioavailability of the metals through nitrogen fixation along with generation of the plant growth promoting factors like generation of carboxylic acid, solubilize insoluble phosphate, siderophores, indole acetic acid including 1-aminocyclopropane-1-carboxylate deaminase. However, the mechanisms of PGPR from Napier grass in assisting arsenic (As) uptake has never been studied. Therefore, in this study, PGPR will be isolated and characterized from Napier grass rhizosphere environment in constructed wetland. The functions and mechanisms of isolated rhizobacteria to enhance arsenic uptake will be investigated.

1.2 Problem statement

Amongst the Potentially toxic elements (PTEs), Arsenic (As) was identified as one of the most extremely hazardous as well as cancer causing chemicals (Niazi et al., 2017; Mehmood et al., 2017). Arsenic along with its components were classified as a category 1 human carcinogens by the US Environmental Protection Agency including the International Agency for Research on Cancer (Niazi et al., 2018). As species are harmful to the humans, animals and plant varieties (Quaghebeur and Rengel, 2005). As poisoning in both groundwater including soil has considered a major health along with ecological issue around the globe, particularly in South as well as Southeast Asia (Podgorski et al., 2017; Beniwal, Yadav and Ramakrishna 2023). As levels in drinkable water in certain emerging countries, such as Bangladesh, India, as well as China, surpass requirements for human health safety, resulting in significant toxicity including probably death (Srivastava et al., 2012).

Previously, there are various methods used to remediate arsenic from the environment. These include physical, chemical and biological methods. These are overpriced, environmentally not so safe and inadequate in performance but we need to find out such method which is being aesthetically pleasing, sustainable, environmentally friendly, easy to operate and economically viable. So, an importance has been averted towards the development of another technologies, like PGPR assisted phytoremediation, which uses various living organisms and plants for the elimination of toxic metals and covers all the aforesaid side. Besides, by removing arsenic from the soil, it will be possible to protect the health of the soil; crops will be conserved from the phytotoxic effect of arsenic; production of crops will be enhanced as well as prevent the entry of arsenic in our food chain. Continuation of this study will make it possible to grow arsenic free crops in healthy soil. As a result, overall ecosystem will be protected and food security will be ensured.

There have been very few studies on the elimination of heavy metals including various pollutants utilizing exclusively *P. purpureum*, but no research has been done yet on arsenic removal in constructed wetland utilizing *P. purpureum* and plant growth promoting rhizobacteria that were the novelty of my research.

1.3 Objectives

1. To determine the maximum concentration of As that *P. purpureum* can survive and assess the capability of As uptake through preliminary and advanced phytotoxicity test for phytoremediation application.
2. To isolate, screen, identify and characterize the As resistant PGPR from *P. purpureum* and determine their capability for biosorption of As.
3. To design, operate and assess the capability of arsenic uptake with different rate of aeration in CW using *P. purpureum* and PGPR.
4. To optimize some factors (As loading, retention time including rate of aeration) and determine the effects of applying PGPR consortium, NPKS fertilizers as well as PGPR consortium including NPKS fertilizers together on the growth of *P. purpureum* and phytoextraction of As in CW.

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