UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF Enterobacter-ENRICHED ALGINATE BEAD BIOFERTILIZER FOR PHOSPHATE AND POTASSIUM ACQUISITION TO IMPROVE OKRA [Abelmoschus esculentus (L.) Moench] GROWTH AND PRODUCTIVITY

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By

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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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July 2022

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Microbial biofertilizer application has been recognized as a sustainable alternative to synthetic fertilizers for the past two decades. The core ingredient in this technology i.e., plant-growth-promoting (PGP) bacteria exerts a broad functional tool for the enrichment of soil fertility. This will help elevate plant-available macronutrient deficiency in soil like phosphate (P) and potassium (K) which is prevalent in many agricultural fields. Deficiency in soil available P and K often hinder optimum growth and productivity in plants. Several issues regarding biofertilizer, such as biocompatibility of formulation and efficient delivery into soil systems, remain a challenge. The general objectives of the present study were to screen for the best candidate of P- and K-solubilizing bacteria, to investigate the effects of seed biopriming on okra seedlings, to formulate a sustainable medium and carrier in maintaining an optimal shelf life of bacteria and eventually evaluate the effects of bacterial inoculation in soil on the growth and yield performance of okra in greenhouse conditions. Out of eighteen isolates, *Enterobacter cloacae* 38, *Enterobacter hormaechei* 15a1 and 40a demonstrated the highest P- and K-solubilizing activities while demonstrating additional PGP potentials such as nitrogen fixation, exopolysaccharide, indole-3-acetic acid, and siderophore production. The okra seed germination assay revealed that all of the *Enterobacter* spp. significantly improved seedling vigor index (19.6%) and exhibited root colonization competence. The bioprimed okra seedlings in the pot experiment showed significant improvement of the plant growth (> 28%), the leaf surface area (> 29%), and the SPAD chlorophyll index (> 9%) which corresponded to the increase of P (> 41%) and K uptakes (> 89%) as compared to the uninoculated control. Strain 40a was selected and further evaluated in a biofertilizer formulation using molasses and defatted soybean meal (DSM). Through the two-level factorial design and central composite design, the optimal formulation and fermentation conditions to achieve maximum cell density of strain 40a were

achieved. The highest cell density of strain 40a in the optimized molasses-DSM (OMD) medium was 12.56 log CFU/mL after 24 h which was 99.7% accuracy towards the predicted value. This formulation was then improvised in the next experiment using the hydrolysate cocktail of DSM and jackfruit peel through the optimized microwave-alkaline hydrolysis to produce an *Enterobacter*-enriched molasses alginate bead. Results show that the P and K solubilization capacity by the encapsulated strain 40a was remarkably maintained and comparable to the free cell counterpart. The performance of both free-cell and encapsulated strain 40a was evaluated on okra plants under greenhouse conditions for 60 days. The treatments given were: Half-dose PK-fertilizer, 3H; half-dose PK-fertilizer and free-cell strain 40a, 3HI; half-dose PK-fertilizer and encapsulated strain 40a, 3HB; full-dose PK-fertilizer, 3F. The results revealed that 3HB had the highest soil available P (SAP) and K (SAK), as well as P and K uptake for all plant organs, followed by 3F, 3HI, and 3H, and improved yield by up to 75.6%. We discovered increased bacterial richness and diversity in both 3HB and 3HI samples compared to uninoculated treatments. Both 3HB and 3F treatments were positively correlated with increasing abundance of *Acidobacteriales, Burkholderia caballeronia paraburkholderia*, *Gemmataceae*, and *Sphingomonas* as well as SAP and SAK. The effect of one-time 3HB treatment on okra growth and yield was comparable to biweekly inoculation in 3HI, suggesting a new cost-effective farming approach in precision agriculture.

Keywords: Phosphate-solubilizing bacteria, potassium-solubilizing bacteria, okra, plant growth-promoting bacteria, bioformulation, encapsulation, biofertilizer

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

PEMBANGUNAN BAJA BIO MANIK ALGINAT YANG DIPERKAYAKAN DENGAN *Enterobacter* UNTUK PEMEROLEHAN FOSFAT DAN KALIUM, BAGI MENAMBAH BAIK PERTUMBUHAN DAN PRODUKTIVITI POKOK BENDI [*Abelmoschus esculentus* (L.) Moench]

Oleh

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Aplikasi baja bio mikrob telah diiktiraf sebagai alternatif yang mampan berbanding baja sintetik sejak dua dekad yang lalu. Bahan teras dalam teknologi ini iaitu bakteria penggalak pertumbuhan pokok (PGP) menjadi alat pelbagai fungsi untuk menambahbaik kesuburan tanah. Hal ini akan membantu meningkatkan defisit makronutrien tanah yang tersedia untuk tumbuhan seperti fosfat (P) dan kalium (K) yang lazimnya berlaku di kebanyakan ladang-ladang pertanian. Kekurangan P dan K yang tersedia di dalam tanah sering menghalang pertumbuhan dan produktiviti yang optimum kepada tumbuhan. Beberapa isu berkenaan baja bio, seperti keserasian-bio formulasi dan pengaplikasian yang berkesan ke dalam sistem tanah kekal sebagai antara permasalahan yang wujud. Objektif umum kajian ini adalah untuk menyaring calon terbaik sebagai bakteria pelarut fosfat (P) dan kalium (K), untuk menyiasat kesan biopriming pada anak benih bendi, untuk memformulasikan medium dan bahan pembawa yang mampan dalam mengekalkan hayat simpanan bakteria yang optimum dan akhirnya menilai kesan inokulasi bakteria ke dalam tanah terhadap pertumbuhan dan prestasi hasil bendi dalam keadaan rumah hijau. Daripada lapan belas pencilan, *Enterobacter cloacae* 38, *Enterobacter hormaechei* 15a1 dan 40a menunjukkan aktiviti pelarutan P dan K yang tertinggi sambil menunjukkan potensi PGP tambahan seperti pengikatan nitrogen, penghasilan eksopolisakarida, asid indol-3-asetik, dan siderofor. Ujian percambahan biji benih bendi mendedahkan bahawa kesemua *Enterobacter* spp. meningkatkan indeks kecergasan anak benih dengan ketara (19.6%) dan mempamerkan keberkesanan kolonisasi akar. Anak benih bendi yang diprim dalam eksperimen berpasu menunjukkan peningkatan yang ketara pada pertumbuhan pokok (> 28%), luas permukaan daun (> 29%), dan indeks klorofil SPAD (> 9%) yang sepadan dengan peningkatan kandungan P (> 41%) dan K (> 89%) berbanding rawatan tanpa inokulasi. Strain 40a telah dipilih dan dinilai selanjutnya dalam formulasi biobaja menggunakan molas dan tepung soya yang dinyahlemak

(DSM). Melalui reka bentuk faktorial dua peringkat dan reka bentuk komposit pusat, formulasi dan keadaan fermentasi yang optimum untuk mencapai ketumpatan sel maksimum bagi strain 40a telah dicapai. Ketumpatan sel tertinggi bagi strain 40a dalam medium molas-DSM (OMD) yang dioptimumkan ialah 12.56 log CFU/mL selepas 24 jam iaitu 99.7% ketepatan terhadap nilai yang diramalkan. Formulasi ini kemudiannya dipertingkat dalam eksperimen seterusnya menggunakan koktel hidrolisis DSM dan kulit nangka melalui hidrolisis beralkali gelombang mikro yang dioptimumkan untuk menghasilkan manik alginat molas yang diperkaya dengan *Enterobacter*. Keputusan ujian menunjukkan bahawa kapasiti pelarutan P dan K oleh kapsul strain 40a adalah setanding dengan sel bebas. Prestasi kedua-dua sel bebas dan kapsul strain 40a telah dinilai ke atas pokok bendi di dalam keadaan rumah hijau selama 60 hari. Rawatan yang diberikan ialah: Baja PK separuh dos, 3H; baja PK separuh dos dan strain sel bebas 40a, 3HI; baja PK separuh dos dan kapsul strain 40a, 3HB; baja PK dos penuh, 3F. Keputusan menunjukkan bahawa tanah 3HB mempunyai P (SAP) dan K tersedia (SAK), serta kandungan P dan K tertinggi untuk semua organ tumbuhan, diikuti oleh 3F, 3HI, dan 3H, di samping meningkatkan hasil tuaian sehingga 75.6%. Kami menemui peningkatan kekayaan dan kepelbagaian bakteria dalam kedua-dua sampel 3HB dan 3HI berbanding dengan rawatan tanpa inokulasi. Kedua-dua rawatan 3HB dan 3F mempunyai kaitan positif dengan peningkatan kelimpahan relatif *Acidobacteriales, Burkholderia caballeronia paraburkholderia, Gemmataceae*, dan *Sphingomonas* serta SAP dan SAK. Kesan rawatan 3HB sekali beri ke atas pertumbuhan dan hasil tuaian bendi adalah setanding dengan inokulasi dua minggu sekali dalam 3HI, dan sekaligus memberi idea pendekatan pertanian kos efektif yang baharu dalam pertanian teliti.

Kata kunci: bakteria pelarut fosfat, bakteria pelarut kalium, bendi, bakteria penggalak pertumbuhan pokok, bioformulasi, pengkapsulan, bio baja

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Declaration by Graduate Student

I hereby confirm that:

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treatments; B, soil P and K status; C, soil culturable bacteria.

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
CAS	Chrome Azurol S
CCD	Central composite design
CFU	Colony-forming unit
CMC	Carboxymethyl cellulose
CRD	Completely randomized design
DNA	Deoxyribonucleic acid
DSM	Defatted soybean meal
EPS	Exopolysaccharide
GA3	Gibberellic acid
Gly	Glycerol
h	Hour
IAA	Indole-3-acetic acid
ICP-EOS	Inductively coupled plasma optical emission spectrophotometer
JP	Jackfruit peel
KSB	Potassium-solubilizing bacteria
KSI	Potassium solubilization index
min	Minute
NADPH	Nicotinamide adenine dinucleotide phosphate
NBRIP	National Botanical Research Institute's Phosphate
NFb	N-free malate broth
NGS	Next-Generation Sequencing
OMD	Optimized molasses-DSM

OPEFB	Oil palm empty fruit bunch
PCR	Polymerase chain reaction
PGP	Plant-growth promoting/ promotion
PGPB	Plant-growth promoting bacteria
Pi	Orthophosphate ions
PSB	Phosphate-solubilizing bacteria
PSI	Phosphate solubilization index
RDA	Redundancy analysis
RNA	Ribonucleic acid
RSM	Response surface methodology
s	Second
SA	Sodium alginate
SEM	Scanning electron microscopy
SJMo-Alg	Soybean-jackfruit-molasses-alginate bead
TLFD	Two-level factorial design
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

There may be a preamble at the beginning of a chapter. The purpose may be to introduce the themes of the main headings. Conventional agriculture employs large amounts of synthetic chemical fertilizers to support growth requirement of crops. However, excessive use of chemical fertilizers, particularly by large commercial farms, has polluted the environment and hindered economic progress. Nutrient leaching e.g., nitrogen (N), phosphate (P), and potassium (K) has caused groundwater and waterway contamination, especially in places with high rainfall and near streams (Islam *et al.*, 2015; Summers & Kingdon, 2019). Finding new ways to boost crop yields while protecting the environment is thus crucial. The use of plant growth-promoting bacteria (PGPB) is a suitable alternative for more sustainable agricultural output while reducing chemical fertilizer application (Kuan *et al*., 2016; Shen *et al*., 2016; Suleman *et al*., 2018). PGPB, also called biofertilizers promote plant growth by enhancing soil nutrient acquisition through N_2 fixation, P and K solubilization; phytohormone regulation and phytopathogen inhibition (Shen *et al*., 2016).

P- and K-solubilizing bacteria are among common PGPB used in agriculture. They cleave and dissolve insoluble organic and inorganic compounds bound to P and K in soil via several metabolic processes (Alori *et al*., 2017; Etesami *et al*., 2017). They comprise those from genera *Pseudomonas* (Fenta & Assefa, 2017), *Burkholderia* (Kour *et al*., 2020), *Bacillus* (Pande *et al*., 2017), *Serratia* (Parastesh *et al*., 2019), *Mycobacterium* (Seshachala & Tallapragada, 2012), *Enterobacter* and *Paenibacillus* (Wang *et al.,* 2017). Application of P- and K-solubilizing bacteria could be a viable alternative to reducing chemical fertilizer use and improving P and K levels in soil. The seed biopriming method can be used to determine the compatibility of specific P- and K-solubilizing bacteria on plant seeds. This method involves coatings seeds with selected PGPBs to allow rapid bacteria colonization (Liu *et al*., 2019). Seed biopriming boosts stress tolerance in seeds prior to germination by assuring early protein and DNA synthesis and promoting mitochondrial biogenesis (Chakraborti *et al*., 2022). Earlier researches have shown that this strategy improves germination rate and seedling vigour, as well as reducing the occurrence of plant diseases (Amruta *et al*., 2019; Mnasri *et al*., 2017).

One of the challenges in biofertilizer application is delivering sufficient viable cells into the soil to assure an efficient colonization rate. To address this issue, biofertilizer formulation must be tailored accordingly to ensure high quality and viability of bioinoculant cells, particularly for non-sporulating bacteria (Stephens & Rask, 2000). In this case, priority should be given to carbon (C) and N composition in the liquid medium since they heavily affect cell proliferation and preservation. These sources can be derived from low-cost, natural biomass, such as agricultural wastes (Singh *et al*., 2019; Wang *et al*., 2015), animal manure (Unsoed *et al*., 2017), and food waste (Cano *et al*., 2020). While the use

of diverse raw materials offers an environmentally sustainable solution to natural waste management, it also serves a platform to recycle nutrients that can replenish soils, particularly those in tropical locations with low fertility conditions (Cajamarca *et al*., 2019).

Okra is a popular annual vegetable crop in tropical and subtropical regions around the world (Kumari *et al*., 2017). The fibrous fruits or pods containing round, white seeds are in high demand since they are utilized in soups, salads, and as a seasoning when dried and powdered. In particular, the demand for okra pods in Malaysia is increasing annually, owing to the growing population of this country. As a result, since 2010, okra production has steadily increased, reaching over 60,000 mt per year in 2021 (DOA, 2021). Previous research centred on expanding okra yield by optimizing inorganic fertilizer requirements, among other things (Ferdous *et al*., 2017; Khandaker *et al*., 2017; Nagegowda *et al*., 2020; Rahman & Akter, 2012). Alternatively, introducing P- and K-solubilizing bacteria to okra soil may be a viable approach to improve okra production through amelioration of P and K availability.

In light of the above context, the present study was designed to achieve the following objectives:

1. To screen pre-isolated bacteria for PGP properties particularly P- and K-solubilizing activities and identify them through 16S rRNA gene molecular sequencing.
2. To determine the effect of seed biopriming using the selected P- and K-solubilizing *Enterobacter* sp. on seedling germination, early vegetative growth, and P and K uptake of okra.
3. To screen and characterize the suitable molasses-based media supplemented with different carbon and nitrogen sources in addition to cell protectant to sustain high cell density of bioinoculant.
4. To formulate biofertilizer alginate capsule containing high viable cell immobilized bioinoculant with hydrolysate of defatted soybean meal and jackfruit peel biomass as additives treated using microwave-alkaline hydrolysis.
5. To unravel the impacts of free cell and encapsulated bioinoculant application with different rate of inorganic fertilizers input on okra growth, productivity and soil rhizosphere bacterial community in greenhouse conditions.

As such, the hypotheses of this project were outlined as follows:

1. The screened pre-isolated bacteria are able to demonstrate PGP properties particularly P- and K-solubilizing activities.
2. The selected bacteria are able to improve germination parameters, growth and P and K acquisition of okra seedlings.
3. The selected carbon and nitrogen sources supplemented in the molasses-based media in addition to the selected cell protectant are able to sustain high cell density of bioinoculant.

4. The formulated nutrient-rich hydrolysate extracted from the defatted soybean meal and jackfruit peel biomass is biocompatible with selected bioinoculant as an additive in alginate capsule.
5. The formulated bioinoculants in both free cell and encapsulated forms are capable to improve growth and yield of okra under greenhouse experiments.

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