MEDIUM OPTIMIZATION FOR HIGH CELL MASS AND EXOPOLYSACCHARIDES PRODUCTION USING *PAENIBACILLUS POLYMYXA*

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DEDICATION

La hawla wala quwwata illa billah. There is neither might nor strength except with Allah. This is for my parents En Daud and Puan Zauyah. The brothers Shazwan, Shahidan and Firdaus. My only sister Syahidah. For the deen and for the ummah. Beloved akhawat. May He gather us all in Jannah.

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ABSTRACT

Paenibacillus polymyxa ATCC 842 is one of the beneficial microbes that shows high potential to promote plant growth and enhance soil health by producing a variety of beneficial secondary metabolites specifically exopolysaccharides (EPS). This study aimed to optimize medium composition for enhancing the production of cell mass and EPS by *P. polymyxa* ATCC 842 using statistical methods for agriculture application. At first, the one-factor-at-a-time (OFAT) method was used to screen the cultivation medium of *P. polymyxa* ATCC 842 which was reported previously for high cell mass and EPS production. Then, different ranges concentration of the main components of selected media were investigated such as sucrose, yeast, phosphate and $MgSO_4$. Based on the OFAT results, three factors were selected as the most influence factors: sucrose, yeast extract, and K_2HPO_4 for the medium optimization through statistical approaches by using response surface methodology (RSM). The Box Behnken design (BBD) was used to evaluate the effects of variables on the cell mass and EPS production. The optimal medium composition by RSM composed of sucrose, 30 g L^{-1}; yeast extract, 30 g L^{-1}; K_2HPO_4, 5.72 g L^{-1}; NH_2NO_4, 5 g L^{-1}; KH_2PO_4, 1.9 g L^{-1} and $MgSO_4$, 0.5 g L^{-1} . Under the optimized medium, the cell mass of *P. polymyxa* ATCC 842 about 20.36 g L^{-1}, which was 7.51-fold higher than the unoptimized medium (2.71 gL^{-1}). Meanwhile, the EPS was increased 16.46 g L^{-1}, about 6.71-fold higher than the unoptimized medium (2.45 g L^{-1}) after 36 hours of cultivation at pH 7.0, 30 °C, and agitation speed at 150 rpm. The model was found to be significant and subsequently validated through the growth kinetics studies in the unoptimized and optimized medium by RSM. The result revealed that the optimized medium was able to support better growth of *P. polymyxa* ATCC 842 with a specific growth rate of 1.397 h^{-1} and EPS productivity about 0.68 g $L^{-1}h^{-1}$. Furthermore, to understand the physio-chemical of EPS from *P. polymyxa* ATCC 842, a series of biochemistry characteristics were investigated. High-Performance Liquid Chromatography (HPLC) analysis revealed that EPS of *P. polymyxa* ATCC 842 is composed of monomer fructose and glucose. Meanwhile, Fourier-transforms infrared spectroscopy analysis showed the presence of hydroxyl, carboxyl groups, and glycosidic linkages which showed the composition of the polysaccharide bound by α-(1→6). The scanning electron micrographs analysis showed EPS appears as a homogenous spherical with irregular porosity for high water holding capacity of the soil. The EPS structure could result in a higher water absorption/ retention capacity, which is an attractive feature for a sticky agent for soil aggregation. *P. polymyxa* ATCC 842 with the spent mushroom substrate (SMS) as soil amendment gives the best result in neutralizing the pH and forming soil aggregate including phosphate and potassium improvements. In conclusion, the optimized production medium shows significant effects on enhancing the growth of *P. polymyxa* ATCC 842 and maximum secretion of EPS and indirectly, promotes the application of *P. polymxya* ATCC 842 as the beneficial candidate for sustainable agricultural practices.

ABSTRAK

Paenibacillus polymyxa ATCC 842 adalah salah satu mikrob bermanfaat yang berpotensi tinggi untuk menggalakkan pertumbuhan tanaman dan meningkatkan kesihatan tanah dengan menghasilkan pelbagai metabolit sekunder yang bermanfaat khususnya eksopolysakarida (EPS) untuk digunakan dalam bidang pertanian. Kajian ini bertujuan untuk mengoptimumkan komposisi medium untuk meningkatkan penghasilan jisim sel dan eksopolisakarida (EPS) oleh *P. polymyxa* ATCC 842 menggunakan kaedah statistik. Pada permulaanya, kaedah satu faktor pada satu masa (OFAT) digunakan untuk menyaring medium pertumbuhan *P. polymyxa* ATCC 842 berdasarkan medium kajian terdahulu untuk penghasilan jisim sel dan EPS. Kemudian, komponen utama medium terpilih tersebut dikaji julat kepekatannya seperti sukrosa, yis, fosfat dan $MgSO_4$. Berdasarkan keputusan OFAT, tiga faktor yang paling mempengaruhi iaitu sucrose, ekstrak yis dan K_2HPO_4 dipilih untuk pengoptimuman medium melalui pendekatan statistik iaitu Kaedah Gerakbalas Permukaan (RSM). Rekabentuk Box Behnken digunakan untuk menilai sama ada pembolehubah mempunyai kesan yang ketara ke atas jisim sel dan EPS. Komposisi medium optimum oleh RSM adalah terdiri daripada sucrosa, 30 g L^{-1}; ektrak yis, 30 g L^{-1}; K_2HPO_4, 5.72 g L^{-1}; NH_2NO_4, 5 g L^{-1}; KH_2PO_4, 1.9 g L^{-1} dan $MgSO_4$, 0.5 g L^{-1}. Medium yang dioptimumkan menghasilkan jisim sel sebanyak 20.36 g L^{-1}, peningkatan 7.51 kali ganda jika dibandingkan dengan medium yang tidak dioptimumkan (2.71 gL^{-1}). Pada masa yang sama penghasilan EPS adalah 16.46 g L^{-1}, peningkatan 6.71 kali ganda lebih tinggi daripada medium yang tidak dioptimumkan (2.45 g L^{-1}) selepas 36 jam pengeraman pada pH 7, 30 °C dan kelajuan kocakkan 150 rpm. Model ini didapati signifikan dan pada masa yang sama disahkan melalui kajian kinetik pertumbuhan melalui medium yang tidak dioptimumkan dan medium yang dioptimumkan melalui RSM. Keputusan menunjukan medium optimum berkebolehan untuk menyokong pertumbuhan yang lebih baik pada kadar pertumbuhan spesifik pada 1.397 h^1 dengan produktiviti penghasilan EPS of 0.68 g $L^{-1}h^{-1}$. Selanjutnya, untuk memahami fisiokimia EPS dari *P. polymyxa* ATCC 842, ciri-ciri biokimia telah diselidiki. Analisis Kromatografi Cecair Berprestasi Tinggi (HPLC) menunjukkan bahawa EPS *P. polymyxa* ATCC 842 terdiri daripada monomer fruktosa dan glukosa. Sementara itu, analisa spektroskopi inframerah transformasi Fourier menunjukkan adanya hidroksil, kumpulan karboksil, dan hubungan glikosidik yang menunjukkan komposisi polisakarida yang diikat oleh α-(1→6). Analisis mikrograf elektron imbasan menunjukkan EPS muncul sebagai sfera homogen dengan rongga-rongga yang tidak teratur untuk menahan air yang tinggi apabila diaplikasi pada tanah. Struktur EPS dapat menghasilkan daya penyerapan/penahan air yang lebih tinggi, yang merupakan ciri menarik bagi agen melekit untuk penggumpalan tanah. *P. polymyxa* ATCC 842 dengan sisa buangan cendawan (SMS) sebagai pengubah tanah memberikan hasil terbaik dalam meneutralkan pH dan membentuk agregat tanah termasuk peningkatan fosfat dan kalium. Medium pengeluaran yang optimum ini menunjukkan kesan yang signifikan terhadap peningkatan pertumbuhan *P. polymyxa* ATCC 842 dan pengeluaran maksimum EPS jika dibandingkan dengan kajian sebelumnya, secara tidak langsung mempromosikan penggunaan *P. polymxya* ATCC 842 sebagai pendekatan yang bermanfaat untuk amalan pertanian lestari.

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LIST OF ABBREVIATIONS

3D	-	Three dimensions
ABA	-	Abscisic acid
ACC	-	1-aminocyclopropane-1-carboxylate
ANOVA	-	Analysis of variance
ARA	-	Acetylene reduction assay
BNF	-	Biological Nitrogen Fixation
C: N	-	Carbon to nitrogen ratio
CCD	-	Central Composite Design
CDW	-	Cell Dry Weight
DPPH	-	1,1-diphenyl-2-picrylhydrazyl
EC	-	Electrical conductivity
EFB	-	Empty fruit bunch
EM	-	Effective Microorganisms
EPS	-	Exopolysaccharide
FOS	-	Fructooligosaccharide
FRS	-	Free radical scavenging
FTIR	-	Fourier Transform Infrared Spectroscopy
GA	-	Gibberellic acid
GC-MS	-	Gas chromatography–mass spectrometry
GLM	-	General linear model
GOS	-	Galactooligosaccharide
GRAS	-	Generally Regarded as Safe
HA	-	Humid acid
HGT	-	Horizontal gene transfer
HPLC	-	High Performance Liquid Chromatography
IAA	-	Indole -3- acetic acid
ICA	-	Innovation Centre in Agritechnology for Advanced Bioprocessing
IMO	-	Isomaltooligosaccharide
ISR	-	Induces systemic resistance

NMR	-	Nuclear Magnetic Resonance
NPK	-	Nitrogen Phosphate Potassium
OFAT	-	One Factor at One Time
PGPB	-	Plant growth-promoting bacteria
PL	-	Pineapple Leaves
rRNA	-	Ribosomal ribonucleic acid
RSM	-	Research surface methodology
RT	-	Room temperature
SEM	-	Scanning Electron Micrograph
SMS	-	Spend mushroom substrate
SPSS	-	Statistical Package for the Social Sciences
UTM	-	Universiti Teknologi Malaysia
UV-VIS	-	Ultraviolet–visible spectroscopy

LIST OF SYMBOLS

%	-	Percentage
P	-	Phosphate
μg ml^{-1}	-	Microgram Per Millilitre
g L^{-1}	-	Gram Per Litre
Fe^{3+}	-	Ferric Ion
Fe^{2+}	-	Ferrous
kDa	-	Kilo Dalton
K	-	Potassium
Na	-	Sodium
P	-	Phosphate
Cl	-	Chloride
S	-	Sulphur
Mg	-	Magnesium
cm^{-1}	-	Per Centimetre
R-COOH	-	Carboxyl
R-OH	-	Hydroxyl
R-C=O-R’	-	Ketone
R-NH2	-	Amines
mg kg^{-1}	-	Milligram Per Kilogram
g kg^{-1}	-	Gram Per Kilogram
g L^{-1}	-	Gram Per Litre
K_2HPO_4	-	Dipotassium Hydrogen Phosphate
rpm	-	Revolutions Per Minute
^oC	-	Degree Celsius
H_2SO_4	-	Sulphuric Acid.
NaOH	-	Sodium Hydroxide
KBr	-	Potassium Bromide
h	-	Hour
CFU mL^{-1}	-	Colony-Forming Units Per Millilitre
w/v	-	Weight Per Volume

NH_4NO_2	-	Ammonium Nitrite
CH^2	-	Aliphatic
ppm	-	Parts Per Million

CHAPTER 1

INTRODUCTION

1.1 Introduction

Hunger prevention is one of the sustainable development goals (SDG) established by the United Nations in 2015. To feed 10 billion people by 2050, we must strike the correct balance between sustainability, food security, and food safety, as well as make better use of food that has already been produced. Limiting food losses and waste, consuming more plant-based meals, and recycling foodstuffs are just a few of the ways that may be taken to achieve sustainability and food security. Sustainable food security necessitates adequate food production in terms of nutrition. Unfortunately, the widespread use of artificial fertiliser reduces the nutritional value of vegetables and fruits in agriculture. Food insecurity disproportionately impacts low-income people, putting them at risk of hunger and malnutrition. As a result, there is a need to devise a strategy for replacing chemical fertiliser that practised by farmers with application of beneficial microbes formulize biofertilizer.

According to López-Legarda *et al.,* (2020), in fermentation industry one of the major aims is to produce a large amount of biomass and products. Companies, on the other hand, are constantly pushed to create huge cell mass in a cost-effective manner. High cell mass production as a desirable response necessitates considerable research into establishing a suitable industrial medium, optimising the industrial media, and employing submerges fermentation methods such as batch and fed batch fermentation (Vassilev *et al.*, 2017). When designing a process to create a high cell mass, bioprocess optimization includes addressing numerous difficulties and studying the connections between variables and parameters (Zeng *et al.*, 2020). In order to create high cell mass for efficient productivity, a thorough screening and optimization of an industrial medium is required in the fermentation process (Elsayed *et al.*, 2014). Several investigations done by researchers shown that the creation of optimal industrial media

is critical as a medium to increase the production of high cell mass (Zhang *et al.*, 2018; Mohd Din *et al.*, 2020). According to the evidence, the ideal medium composition and concentration for carbon, nitrogen, and minerals may influence cell mass development. Excessive medium composition may result in a longer lag phase since it takes longer phase for cells to adjust to a new environment, whereas restricting medium components (limited substrate) may result in a lower specific growth rate of the cells (Dailin *et al.*, 2014). The technique of the statistical method employing response surface methodology (RSM) might improve cell mass manufacturing. According to certain publications, cell culture in un-optimized media may result in lower biomass than cell cultivation in optimal medium.

Paenibacillus polymyxa, is the bacterial strain that were able to fix nitrogen in atmosphere. Furthermore, *P. polymyxa* is the most species of *Paenibacillus* that shows the ability to promote plant growth and soil fertility by different mechanisms (Wu *et al.*, 2019). According to Kiran *et al.*, (2017), the ability of *P. polymyxa* to colonize, fix nitrogen, and promote growth of most important agronomic crops such as corn and canola is an effective option for sustainable agriculture practices. *P. polymyxa* originated from soil and rhizosphere, and because of that it have high adaptability to soil that is proven due to their multiple beneficial effects towards agriculture. Currently, research using microbial polysaccharides such as exopolysaccharides (EPS) from *P. polymyxa* is rapidly growing as a significant source of natural biopolymer materials (Saha *et al.*, 2020). EPS is an extracellular polymer made up of a long chain of repeating monosaccharide units (Deka *et al.*, 2019). EPS is konown as Generally Regarded As Safe (GRAS) biomaterials and this polysaccharides polymer is now used in the food, nutraceutical, and cosmeceutical sectors (Boukhelata *et al.*, 2019). Furthermore, it has been demonstrated to have several functional qualities in agricultural applications, including the ability to assist soil aggregation and regulate pH fluctuations (Medrano *et al.*, 2011).

However, current data in the literature clearly show that no standard media has been created for this procedure so far. The kind of strain employed, the physical conditions maintained during fermentation, and the medium components used for production all have a significant impact on EPS production (Shen *et al.*, 2013).This

study focuses on the production of a suitable medium for cell mass production co-currently production of EPS in a submerged cultivation method utilising *P. polymyxa* ATCC 842. In the shake flask cultivatio, two optimization approaches, OFAT and statistical experimental design, were employed. The OFAT optimization was performed in a shake flask by optimising one component at a time. Minitab V17 software was used to design the experiment for the statistical approach in order to maximise multiple factors at the same time and analyse the elements' interactions (Then *et al.*, 2016). The OFAT and statistical technique findings were compared to determine the best medium for maximum cell mass and EPS production.

The functional characteristics of the EPS produce by *P. polymyxa* ATCC 842 was discussed in this study. The analysis was involved the determination of the monosaccharide composition, identify the functional bond by FTIR and EPS morphology by SEM. From the functional characteristics study of EPS, it can be concluded that EPS is a very strong polymer chain. It is potentially bind with element in the soil because of its variety of functional groups and it also has high water holding capacity because of its high porosity structure.

1.2 Problem Background

Recent studies of *P. polymyxa* were motivated for its role as plant growth promoting bacteria with direct and indirect action and mechanisms. Many researchers worldwide have published data on the effectiveness of *P. polymyxa* and its production of exopolysaccharide (EPS) but their research is focusing on the use of EPS in cosmetic, food and pharmacy industries, despite of its uses in agriculture. Present research also shows lack of efficient cultivation medium for production of high cell mass and EPS production by *P. polymyxa* ATCC 842 that can give benefit in agriculture.

However, there are so little information on producing higher cell mass and concurrently high EPS cultivation strategy. Through optimizing medium composition

in this work, the optimized condition for high cell mass and high EPS production were studied to formulate production medium towards industrial agriculture applications. This followed by analysis of functional characteristic of EPS and the analysis of produced *P. polymyxa* on field that gave high impact to the agriculture yet food security.

1.3 Research Objectives

The objectives of the research were:

1. To optimize production medium for production of high cell mass and EPS by *P. polymyxa* ATCC 842
2. To characterise the EPS produced by the *P. polymyxa* ATCC 842 under optimized medium formulation.
3. To analyse the effect of *P. polymyxa* ATCC 842 application on sandy soil and plant growth performance.

1.4 Scope of Research

To accomplish the objectives, there are four research scopes were applied:

4. Screening and optimization of medium formulation using classical and statistical methods for high yield of cell mass and EPS production by *P. polymyxa* ATCC 842 in the shake flask scale.
5. To characterise the produced EPS by *P. polymyxa* ATCC 842 including monosaccharide composition, physiochemical properties in term of physical morphology and chemical functional group using High Performance Liquid Chromatography (HPLC), Fourier-Transform Infrared Spectroscopy (FTIR), and Scanning Electron Microscope (SEM).

6. To analyse the sandy soil characteristic including soil pH, EC, moisture content, soil aggregation, water holding content of soil, and CNPK availability under application of resulted *P. polymyxa* with different types of soil amendments.

7. To analyse the Pak Choy performance including plant height, root length and number of leaves under application of resulted *P. polymyxa* ATCC 842.

1.5 Significance of Study

Plant growth-promoting bacteria is a potential microorganism that can allows a promotion for plants without no side effects for plant, soil, and human, compared to the usage of chemical fertilizer which have a negative side effect. More importantly the formulated media should be maximizing the *P. polymyxa* ATCC 842 viability during processing and storage. It should survive throughout the treatment and storage process. By confirmed it in the large numbers and still have beneficial effects to the plants.

The use of *P. polymyxa* ATCC 842 as inoculants provides an environmentally sustainable approach to increase crop production. *P. polymyxa* ATCC 842 directly promote plant growth by helping in nutrient acquisition, enhancing root permeability and soil porosity. Whereas, *P. polymyxa* ATCC 842 indirectly enhancing plant growth by suppression of diseases caused by plant pathogens. The exopolysaccharide generated by *P. polymyxa* ATCC 842 has the potential to be utilised as an active biofertilizer and form a biofilm that help in soil aggregation, nutrient retention (Yegorenkova *et al.*, 2008) and bioremediation agent for decontamination of lead in the contaminated soil (Han *et al.*, 2020).

This study would help to understand the effect of development culture medium for high cell mass and high EPS production. EPS is known non-toxic biomaterials, biodegradable, environmentally friendly and offers numerous applications in fermentation, pharmacy and bioremediation and soil health (Daud *et al.*, 2019a).

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LIST OF PUBLICATIONS

1. **Nur Sazwani Daud,** Abd Rahman Jabir Mohd Din, Mohamad Azzuan Rosli, Zaheda Mohamad Azam, Nor Zalina Othman, Mohamad Roji Sarmidi. (2019) *Paenibacillus polymyxa* bioactive compund for agricultural and biotechnological application. 10.1016/j.bcab.2019.101092. *Biocatalysis and Agricultural* Biotechnology

2. Nur Hidayah Shadan, Zaheda Mohamad Azam, **Nur Sazwani Daud**, Leong Hong Yeng, Nor Zalina Othman **(2020).** Growth Development of Pak Choy (*Brassica rapa l.)* under drought stress when treated with plant growth consortium microorganisms (Pro-BacY) isolated from agrowaste. *E- proceeding ICARS 2020*

3. **Nur Sazwani Daud**, *Z*aheda Muhamad Azam, Nur Hidayah Sahadan, Nor Zalina Othman (2021). Optimization of Exopolysaccharide Production and its Partial Characterization from *Paenibacillus polymyxa* for Agricultural Application. *E Proceeding ICrest UiTM*

4. **Nur Sazwani Daud**, *Z*aheda Muhamad Azam, Khairilanuar Mohd Hanim, Mohamad Azzuan Rosli, Nur Hidayah Sahadan, Leong Hong Yeng, Nor Zalina Othman (2020). Enhancement of the Cell Growth and Exopolysaccharide Production by *Paenibacillus polymyxa* through the Optimization of Medium Composition for Sustainable Agricultural Application