PRODUCTION AND OPTIMIZATION OF INDOLE-3-ACETIC ACID BY *RHODOPSEUDOMONAS PALUSTRIS*

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PRODUCTION AND OPTIMIZATION OF INDOLE-3-ACTIC ACID BY *RHODOPSEUDOMONAS PALUSTRIS*

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A thesis submitted in fulfillment of
the requirements for the award of the degree of
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Specially dedicated to *Mom and Dad*
I really love both of you.

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ABSTRACT

Indole-3-acetic acid (IAA) is known to be an important phytohormone that helps to regulate plant growth and development. In this study, the optimum culture medium for the production of IAA by *Rhodopseusomonaspalustris* in shake flask culture was studied. *Rhodopseudomonaspalustris* is a purple non-sulfurbacteria which has been well recognised as one of the most metabolitically versatile bacteria. The research was divided into three parts. First, a pre-screening process based on Taguchi Design was conducted to identify the significant factors that could affect the production of IAA. The pre-screening indicated that three parameters were found to be significant, which include the concentration of tryptophan, glucose and potassium nitrate. These parameters were selected and used to optimize the production IAA by Response Surface Methodology (RSM). Lastly, a kinetic study for the bacterial growth and IAA production was investigated. The optimal amount of IAA was obtained after incubation of 48 hours at 35 ^{0}C in the presence of 5 g L^{-1} of tryptophan, 4.94 g L^{-1} of glucose and 0.60 g L^{-1} of KNO_3, as recommended by the RSM. Under this condition, the experimental yield of IAA production was 80.77± 2.13 μg mL^{-1}, which was in close agreement with the value predicted by the RSM model (77.64 μg mL^{-1}). This was the highest yield of IAA that was reported compared to the IAA yields obtained from the 20 experiments designed under the RSM. The IAA production depends on growth stage as most of the IAA was produced during the stationary growth phase of *Rhodopseudomonaspalustris*. This study has successfully optimized the production of IAA by *Rhodopseudomonaspalustris* by statistical approach and proved that *Rhodopaeudomonaspalustris* has the potential to be used as plant bioenhancer or biofertiliser for plant growth development.

ABSTRAK

Asid indola-3-asetik (IAA) merupakan fithormon yang dapat menggalakan dan mengawal pertumbuhan dan perkembangan tumbuh-tumbuhan. Dalam kajian ini, medium kultur yang optimum bagi penghasilan IAA oleh *Rhodopseusomonas palustris* dalam kelalang gocang telah dikaji. *Rhodopseudomonas palustris* adalah bakteria ungu bukan sulfur yang telah mempunyai metabolisme yang amat versatil. Kajian ini dibahagikan kepada tiga bahagian. Pertama, proses pra-saringan telah dijalankan untuk mengenalpasti faktor-faktor penting yang menentukan penghasilan IAA. Daripada proses pra-saringan tersebut, tiga parameter telah dikenalpasti sebagai parameter yang signifikan, yakni kepekatan triptofan, glukosa dan kalium nitrat. Pada bahagian yang kedua, ketiga-tiga parameter ini telah dipilih dan digunakan untuk mengoptimumkan penghasilan IAA melalui pendekatan statistik, iaitu Metodologi Permukaan Respon (RSM). Akhirnya, pertumbuhan bakteria dan penghasilan IAA telah dikaji. Penghasilan optimum IAA dicapai selepas pengeraman selama 48 jam pada suhu 35 °C dengan kehadiran 5 g L^{-1} triptofan, 4.94 g L^{-1} glukosa dan 0.60 g L^{-1}kalium nitrat, seperti yang dicadangkan oleh kaedah RSM. Dalam keadaan ini, hasil eksperimen pengeluaran IAA adalah 80.77 ± 2.13 g mL^{-1}, nilai ini agak hampir dengan nilai yang diramalkan oleh model RSM (77.64 g mL^{-1}). Ini adalah nilai hasil IAA yang tertinggi berbanding dengan hasil IAA yang diperoleh daripada 20 ujikaji lain yang disarankan oleh kaedah RSM. Kadar penghasilan IAA juga didapati berkait rapat dengan pertumbuhan sel, dimana kebanyakan IAA telah dihasilkan semasa fasa pertumbuhan statik sel *Rhodopseudomonas palustris*. Kajian ini telah berjaya dioptimumkan pengeluaran IAA dan membuktikan bahawa *Rhodopaeudomonaspalustris* mempunyai potensi untuk digunakan sebagai bio-peringkat tumbuhan atau bio-baja untuk menumbuhkan tumbuh-tumbuhan**.**

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LIST OF ABBREVIATIONS

3D	-	Three-Dimensional
ACC	-	1-Aminocyclopropane-1-Carboxylate
ALA	-	5-Aminolevulonic Acid
ANOVA	-	Analysis Of Variance
AO1	-	Aldehyde Protein
AR	-	Analytical Reagent
C18	-	Column 18
CCD	-	Central Composite Design
DOE	-	Design Of Experiment
EM	-	Effective Microorganism
Eq	-	Equation
GC-MS	-	Gas Chromatography-Mass Spectrometry
H_2	-	Hydrogen Gas
H_2O	-	Water
HPLC	-	High-Performance Liquid Chromatography
IAA	-	Indole-3-Acetic Acid
IAAld	-	Indole -3-Acetaldehyde
IAM	-	Indole-3-Acetamide
IAN	-	Indole -3-Acetonitrile
IAOx	-	Indole-3-Acetaldoxime

IPA	-	Indole-3-Pyruvic Acid
KNO_3	-	Potassium Nitrate
LC-MS	-	Liquid Chromatography-Mass Spectrometry
m/z	-	Mass-To-Charged
MRM	-	Multiple Reaction Monitoring
$NaNO_3$	-	Nitrate
OD	-	Optical Density
OFAT	-	One-Factor-At-A-Time
PGPB	-	Plant Growth-Promoting Bacteria
p-values	-	Probability Value
R. palustris	-	*Rhodopseudomonas palustris*
RNA	-	Ribonucleic Acid
RSM	-	Response Surface Methodology
S/N	-	Signal-To-Noise
sp	-	Species
sur1	-	Supperroot
TAM	-	Tryptamine
Tryp	-	Tryptophan
UV	-	Ultra Violet
YUCCA	-	Favin Monooxygenase-Like Protein

LIST OF SYMBOLS

a.u.	-	Arbitrary units
%	-	Percentage
v/v	-	Volume per volume
min	-	Minute
v	-	Voltage
SS	-	Sum of square
R^2	-	Regression coefficient
df	-	Degree of freedom
h	-	Hour
^{0}C	-	Degree Celsius
rpm	-	Rotation per minute
g L^{-1}	-	Gram per liter

CHAPTER 1

INTRODUCTION

a. 1.1 Research Background

One of the major challenges for the twenty first century will be to create an environmental and sustaianble crop production. In the current agricultural practices, improper using of the chemical fertiliser and pesticide have lead to a long list of environmental and health problems (Gunnell *et al.*, 2007; Leach and Mumford, 2008). Moreover, new emerging and treatening plant disease continue to challenge the plant biosecurity and health worldwide (Miller *et al.*, 2009). Altogether was caused increasing the demand of using ecologically compatible strategies in the agricultural sectors, for example using the beneficial bacterial to increase the crop productivity. Plant growth-promoting bacteria (PGPB), is a group of beneficial bacteria which can offer a diverse functions for plant growth and at the same time fullfill a promising solution for an environentally friendly and sustainable agriculture. The PGPB able to enhance the plant growth by increasing the nutrient availability, release of phytohormone for phytostimulation, plant strengthening and biocontroling.

Phytohormones are low molecular weight signal molecules that are naturally occurring and capable to influence plant growth and development in low concentration. Among the five major groups of phytohormones, indole-3-acetic acid (IAA) has been well recognized for pivotal functions in nearly every aspect related to plant growth and architecture. For examples, cell division, cell elongation, apical dominance, adventitious and lateral roots initiation, and cell and vascular differentiation (Chen *et al.*, 2009).

Biosynthesis of IAA can be found in the plants and microorganisms. The biosynthesis process occurs through several pathways, such as indole-3-pyruvic acid (IPA) pathway, tryptamine (TAM) pathway, indole-3-acetamide pathway (IAM) and indole-3-acetaldoxime (IAOx) pathways. The biosynthesis of IAA in the microorganism could be very comprehensive because the level of the IAA biosynthesis can be altered by various environmental and genetic factors. The environmental factors include the presence of tryptophan, carbon source, nitrogen source, pH, temperature. The production of IAA by *Az. Braisilense* and the expression of key gene *ipdC* have been found to be decreased during the reduction of growth rate, carbon limitation and under acidic condition (Ositadinma Ona *et al.*, 2005; Vande Broek *et al.*, 2005). In term of genetic factor, it was found that the biosynthesis of IAA can be affected by the gene location, mode of gene expression and presence of transcriptional regulators across the microorganism (Spaepen *et al.*, 2007).

Basically, auxin-type plant regulators are the oldest compounds that used in the agricultural sectors. After IAA was identified, it was chemically synthesized into the industry (Hofrichter, 2010). Currently a number of synthetic auxin-like substances such as indole-butyric acid (IBA), 2,4dichorophenoxyacetic acid (2,4-D), and naphthalene acid (NAA) were found to be have similar effects to IAA on plant growth development (Hofrichter, 2010). The applications of these auxin-like substances in the agricultural sector were shown in Table 1.1.

Table 1.1: Application of auxin-like substances in agriculture (Hofrichter, 2010)

Auxin-like compounds	Application
Indole-3-acetic acid	Cell enlarger, disease controller, anti-transpirant fruit ripening inhibitor
indole-butyric acid (IBA)	Stimulation of root development in the propagation of stem cuttings
2,4 dichorophenoxyacetic acid (2,4-D)	As herbicide: stimulates uncontrolled growth in broadleaf weeds in grasses
naphthalene acid (NAA)	Reduction of excessive fruit set to avoid development of many small fruits
4-Chlorophenoxyacetic acid (4-CPA)	Increase of fruit set in tomato and other solanaceous plants
Indole-butyric acid (IBA) 2,4-D together with gibberellic acid (GA_3)	Prolongation of the pre-harvest and post-harvest life of navel oranges
2,4-Dichorophenozyacetic acid (2,4-D) naphthalene-acetic acid (NAA)	Delay of fruit abscission and senescence of the fruit button in grapefruit, prevention of fruit frop of apple, pear and lemon

In order to meet the demand from the market, a statistical approach was used to optimize the production of IAA. The Taguchi Design and Response Surface Methodology (RSM) are powerful statistical tools used for identifying the significant factors and optimization of the production. Both techniques have significant advantages compared to the conventional methods. For examples, they require less labour and time compared to other approaches. These methods have successfully been applied for the optimization of media and culture conditions in many cultivation processes for the production of primary and secondary metabolites, for instance enzymes, amino acids, ethanol and flavouring compounds.

b. **1.2 Problem Statement**

Auxin is an important plant hormone that could influence the physiological processes of plant growth by modulating their development events, such as embryogenesis, root initiation, apical dominance, gravitropism and phototropism. Currently, chemical auxins are the most common phytohormone used in the market such as indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxy acetic acid, α-naphthalene acetic acid, 2-methoxy-3,6-dichlorobenzoic acid and 4-amino-3,5,6-trichloropicolinic acid for promoting plant growth. However excessive use of these chemical or synthetic auxins are not sustainable for soil and environment as the chemical auxin could increase heavy metal in the soil, nutrient imbalance and soil acidification by changing the aggregation degree of the potassium nitrate (KNO_3) and nitrate ($NaNO_3$) in the soil (Savci, 2012). In addition, public are increasing concern on the quality of foods and health, as well as on their nutritional properties which stimulate the agriculture trend to a more sustainable and environmental friendly approach. In this context, application of soil microorganisms with beneficial activities on plant growth represents an attractive alternative approach as compared to the conventional agriculture that uses chemical or synthetic fertilizer (Choudhary *et al.*, 2011; Miransari, 2011; Verma *et al.*, 2011).

The release of tryptophan in the root exudates may results in its conversion into IAA by PGPB. In the previous study found that PGPB from different genera (Alcali gene faecalis, Enterobacter, Azospirillum, Klebsiella) and fungi have shown to enhance plant growth by synthesis of IAA (Reinekei*et al*. 2008; Torres-Rubio *et al*., 2008). The *Stretomyceteslydicus* WYEC108 and *Streptomycetesgriseoviridis* K61 are used commercially for IAA production under the trade of Mycostop(Khamna*et al*. 2010).

*Rhodopseudomonaspalustris,*a purple nonsulfur photosynthetic bacteria with extraordinary diversity of enzymes, can be considered as one of the promising PGPB for natural soil enhancer compared to other microorganisms. It is not only able to fix carbon dioxide from atmosphere into biomass, but also fixing the nitrogen gas to form ammonia source for plants (Larimer *et al*., 2004). Nevertheless, there are evidences from the genetic traits showing the capability of *R. palustris* to synthesis IAA. These features enable *R. palustris* to exhibit potential to enhance plant productivity by increasing the nitrogen availability, release of functional phytohormones and even detoxifying contaminated soil from overuse of chemical fertilizers and pesticides (Chen *et al.*, 2007; Elder and Kelly, 1994; Kim *et al.*, 2004; Liu *et al.*, 2011). To date, the use of *R. palustris* for agriculture application is seldom been reported, particularly for the production of phytohormone such as the IAA. Therefore, in this study, the *R. palustris* strain NRRL-B4276 was chosen for validation as a candidate strain to produce IAA via submerged fermentation

The level of IAA biosynthesis by bacteria can be influenced by a number of factors, such as bacteria strain, concentration of tryptophan, nutrient availability, pH, temperature and time of incubation (Apine and Jadhav, 2011; Chaiharn and Lumyong, 2011). Thus, it is necessary to consider which type of factors would affect the production of IAA in the *R. palustris*. In the present study, four key factors: the amount of tryptophan, types of carbon and nitrogen sources and pH was chosen by the Taguchi Design under the pre-screening process. Subsequently, only the significant factors were chosen and proceeded for the optimisation study by RSM analysis.

c. 1.3 Research Objective

The objective of this study is to optimize the key parameters for the production of IAA by *Rhodopseudomonaspalustris* under submerged fermentation using statistical analysis approach.

d. 1.4 Scopes of Study

The scopes of the study are to:

a) determine the significant factors that affect the production of IAA by *R. palustri*using Taguchi Design as pre-screening step;

b) optimize the parameters of IAA production by *R. palustris*using RSM analysis

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