OPTIMIZATION OF BIOMASS PRODUCTION OF *Pseudomonas fluorescens* IN A STIRRED TANK BIOREACTOR

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To my beloved mother and father

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ABSTRACT

Pseudomonas fluorescens (*P. fluorescens*) is a denitrification bacterium that able to convert nitrate compounds into nitrogen gas. This process is always slow due to limitation of organic sources that are present in wastewater treatment plant. The efficiency of *P. fluorescens* as a nitrate removal strongly depends on the physiological status of cells and the amount of microorganism added to the treatment plant. Therefore, the objectives of this study are to optimize the culture medium in shake flask and to develop cultivation strategy for the biomass production of *P. fluorescens* in a pilot scale 16-L bioreactor. The medium composition was first optimized using one factor at time (OFAT) and response surface methodology (RSM) methods, which the Box-Behnken experimental design was employed. Analysis of variance (ANOVA) showed significance of findings for each factors with high coefficient of determination (R^2) of 95.58 %. The optimum medium composition of biomass production was composed of: sucrose, 8.0 g L^{-1}; yeast extract, 3.0 g L^{-1}; di-potassium phosphate, 2.0 g L^{-1}; and magnesium sulfate heptahydrate, 1.5 g L^{-1}. This medium gave biomass of 3.28 g L^{-1} (about 57.6 % higher compared to un-optimized medium). After this step, the optimized medium was used to cultivate the cells in batch mode with and without pH control in a 16-L stirred tank bioreactor. It was found that controlling the culture pH at 7.2 during cultivation increased biomass by 39.56 %. In addition, a series of constant feeding strategy in combination with a control pH 7.2 was carried out to increase the biomass production. Both sucrose and full medium feeding were applied, and both yielded biomass of 8.46 g L^{-1} and 14.98 g L^{-1}, respectively. Under constant feeding strategy of full medium, nutrients were consumed after 10 hours of feeding. Therefore, a gradual increase of medium feeding rate was applied to increase the biomass. The highest biomass obtained using increased rate feeding strategy was 33.5 g L^{-1}. In conclusion, the medium optimization accompanied by bioprocess optimizations in terms of pH control and applying fed batch cultivation strategy in the 16-L bioreactor enhanced the growth rate and biomass production of *P. fluorescens.*

ABSTRAK

Pseudomonas fluorescens (*P. fluorescens*) adalah bakteria yang dapat menukar sebatian nitrat kepada gas nitrogen melalui proses pendenitratan. Proses ini adalah perlahan kerana kehadiran sumber organik yang terhad di dalam loji rawatan kumbahan. Kecekapan *P. fluorescens* untuk menyingkirkan nitrat sangat bergantung kepada fisiologi sel dan jumlah mikroorganisma yang berada di dalam loji rawatan kumbahan. Oleh itu, objektif kajian ini adalah untuk mengoptimumkan media kultur dalam kelalang kon dan strategi pembangunan dan pengeluaran biojisim *P. fluorescens* dalam bioreaktor yang berkapasiti 16-L. Komposisi media dioptimumkan dengan menggunakan teknik ubahan faktor satu per satu (OFAT) dan kaedah statistik tindak balas permukaan (RSM) di mana eksperimen Box-Behnken digunakan. Analisis varian (ANOVA) menunjukkan kepentingan penemuan bagi setiap faktor dengan pekali tinggi penentuan (R^2) sebanyak 95.58 %. Nilai optimum untuk menghasilkan biojisim terdiri daripada: sukrosa, 8.0 g L^{-1}; ekstrak yis, 3.0 g L^{-1}; dipotasium fosfat, 2.0 g L^{-1}; dan magnesium sulfat heptahidrat, 1.5 g L^{-1}. Media ini memberikan biojisim 3.28 g L^{-1} (peningkatan kira-kira 57.6 % berbanding media yang belum dioptimumkan). Selepas langkah ini, media yang telah dioptimum digunakan untuk pengkulturan dalam dua keadaan iaitu; dengan kawalan pH dan tanpa kawalan pH di dalam tangki bioreaktor 16-L. Hasil pemerhatian mendapati dengan mengawal pH media pada 7.2 semasa proses fermentasi ini telah meningkatkan biojisim sebanyak 39.56 %. Seterusnya, kaedah suap kelompok berskala tetap pada pH 7.2 telah digunakan untuk meningkatkan penghasilan biojisim. Kaedah pengkulturan suap kelompok telah dilakukan dengan menggunakan sukrosa dan media lengkap telah menghasilkan biojisim masing-masing 8.46 g L^{-1} dan 14.98 g L^{-1}. Semasa kaedah ini dijalankan, didapati bahawa nutrisi bagi media lengkap adalah terhad selepas 10 jam fermentasi. Oleh itu, pemberian media lengkap ditambah secara beransur-ansur telah dilaksanakan sebagai strategi suap kelompok. Biojisim bagi kaedah ini mencapai hasil sebanyak 33.5 g L^{-1}. Sebagai kesimpulannya, penghasilan biojisim bagi *P. fluorescens* dapat dicapai melalui kaedah pengoptimuman media dengan kawalan pH dan strategi suap kelompok di dalam bioreaktor 16 L.

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LIST OF SYMBOLS

%	-	Percentage
>	-	Greater than
μ	-	Specific growth rate (h^{-1})
μ_{max}	-	Maximum specific growth rate (h^{-1})
F	-	Feed rate ($g\ L^{-1}h^{-1}$)
K_s	-	Substrate utilization constant ($g\ L^{-1}h^{-1}$)
m	-	Maintenance coefficient (mol substrate g^{-1} cell L^{-1})
q_p	-	Specific rate of product formation (mg product g^{-1} biomass h^{-1})
S	-	Substrate concentration ($g\ L^{-1}$)
S_o	-	Initial feed substrate concentration (g substrate L^{-1})
t	-	Time interval (h)
t_0	-	Initial time (h)
V	-	Volume of reactor (L)
v/v	-	Volume per volume
vvm	-	Volume per volume per minute
X	-	Biomass concentration ($g\ L^{-1}$)
$Y_{X/S}$	-	Substrate yield coefficient (g biomass per g substrate)
β_o	-	Regression constant
β_i	-	Linear regression coefficient
β_{ii}	-	Quadratic regression coefficient
°C	-	Degree Celsius
g	-	Gram
h	-	Hour
kg	-	Kilogram
L	-	Litre

M	-	Molarity
min	-	Minute
ml	-	Millilitre
rpm	-	Revolutions per minutes

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LIST OF ABBREVIATIONS

CDW	-	Cell dry weight
DO	-	Dissolve oxygen
OD	-	Optical density
OD_{600}	-	Optical density at 600nm
Sp	-	Species
BOD	-	Biochemical oxygen demand
OFAT	-	One factor at time
$(NH_4)_2SO_4$	-	Ammonium sulfate
C	-	Carbon
$CaCl_2$	-	Calcium chloride anhydrous
$CaCl_2.2H_2O$	-	Calcium chloride dihydrate
$CaSO_4$	-	Calcium sulfate
CoA	-	Coenzyme A
$CoCl_2$	-	Cobalt (II) chloride
Cyt	-	Cytochrome
DHAP	-	Dihydroxyacetone phosphate
DNS	-	3,5-dinitro-salicylic acid
$FeCl_3$	-	Iron (III) chloride anhydrous
$FeSO_4.7H_2O$	-	Iron (II) sulfate heptahydrate
$FeSO_4$	-	Iron (II) sulphate anhydrous
GDP	-	Guanosine diphosphate
H^+	-	Proton
H^+/e	-	Proton to electron ratio
H_2	-	Hydrogen
H_3BO_3	-	Boric acid

HCl	-	Hydrocloric acid
KNO_3	-	Potassium nitrate
K_2HPO_4	-	Dipotassium hydrogen phosphate
KH_2PO_4	-	Potassium dihydrogen phosphate
$MgSO_4.7H_2O$	-	Magnesium sulphate heptahydrate
$MnSO_4.4H_2O$	-	Manganese (II) sulfate heptahydrate
MSG	-	Monosodium glutamate
N	-	Nitrogen
N_2	-	Dinitrogen
Na	-	Sodium
Na_2HPO_4	-	Disodium hydrogen phosphate
$Na_2MoO_4.2H_2O$	-	Sodium molybate dihydrate
NaCl	-	Sodium chloride
NADH	-	Reduce nicotinamide adenine dinucleotide
NADHP	-	Reduce nicotinamide adenine diphosphate
NDH I	-	NADH dehydrogenase I
NDH II	-	NADH dehydrogenase II
NH_3	-	Ammonia
NO	-	Nitrogen oxide
N_2O	-	Nitrous oxide
O_2	-	Oxygen
P	-	Phosphate
PHA	-	Poly-beta-hydroxybutyrate
P_i	-	Inorganic phosphate group
TCA	-	Tricarboxylic acid
$ZnSO_4.7H_2O$	-	Zinc sulfate heptahydrate

CHAPTER 1

INTRODUCTION

1.1 Research Background

In natural environment *Pseudomonas fluorescens* is present as a soil micro-organism that living in symbiosis with plants where it promotes the growth of fungi and providing nitrogen source for plant roots (Roca and Olsson, 2001). *P. fluorescens* is able to utilize NO_3 as an electron acceptor in place of O_2 and some others is an obligate aerobe. Physiological and genetic features of *Pseudomonas* make it a promising agent for utilization in biotechnology, agriculture and environmental bioremediation applications. Thus *P. fluorescens* show a significant role in the bioremediation of nitrogen cycle especially in denitrification steps (Hayat *et al*., 2010). Oxidation of ammonia to nitrate is known as nitrification and reduction of nitrate to nitrogen gas via nitrite is known as denitrification (Kim *et al*., 2008). Because of the variability and unique characteristic of bacteria in wastewater treatment, the most effective treatment solution is to be specific. As the present of denitrifying bacteria is ubiquitous in wastewater treatment, thus it is the most environmental friendly method and could provide assuring chance of nitrate waste treatment (Yang *et al*., 2011).

Currently, the process for nitrogen removal in wastewater treatment plants is substantive based on the natural process of nitrifying and denitrifying microorganism (Takaya *et al*., 2003). However, from the schematic cycle of the microorganisms it is still not sufficient due to the complexity in the environment such as uneven distribution

of dissolve oxygen (DO) and inadequate amount of substrate (Patureau *et al*., 2000). Most of the researcher have found groups of heterotrophic nitrification and aerobic denitrification microorganisms, such as *Paracoccus denitrificans* (formerly known as *Thiosphaera pantotropha*), *Alcaligenes faecalis*, *Pseudomonas stutzeri*, *Microvirgula aerodenitrificans* and *Bacillus* isolated from soils and wastewater treatment (Joo *et al*., 2006).

Globally, treatment by microorganisms has received wide attention due to their efficiency (Jechalke *et al*., 2010; Perelo, 2010). Unfortunately, the natural process is slow before the clean water is discharge to the environment. These may cause from the low amount of biomass from denitrifying bacteria and the condition inside treatment plant may inhibit their growth. It is believed that *P. fluorescens* required a substantial study on improving the growth kinetic and further understanding and high cell mass production. The high yield of *P. fluorescens* biomass is generally depends on well-defined condition type of carbon source, nitrogen source and minerals necessity. Further investigation is needed to figure out the effect of each chemical ingredient on *P. fluorescens* growth kinetic. According to the analysis by Roca and Olsson (2001) reaction of *P. fluorescens* could be quantified. Glucose is mainly converted through glycolysis pathway, succinate and citrate through the Tricarboxylic Acid cycle whereas acetate is used as a gluconeogenic substrate. Moreover according to Chawla *et al.*, (2009) nitrogen source favors the biomass production for bacteria. This is because the nature of these substrates has the ability and characteristic in metabolism of microorganism. There are various type of nitrogen source such as yeast extract, soy flour, corn steep powder, peptone and a few inorganic nitrogen sources. Even though complex media would give significant support to enhance the growth and production of biomass but the necessities of economic value, knowing the entire chemical composition and to have purification of product make chemically defined medium is important for industrial use.

1.2 Problem Statement

Pseudomonas fluorescens is an effective denitrification bacterium in wastewater. It can utilize nitrate and convert to nitrogen gas. The higher cell mass of denitrification bacteria, the more of nitrate will be utilize. However, the studies of biomass production from *P. fluorescens* are very limited. There is little information available about medium composition for cell mass production. Most researchers are either focused on genetic enrichment of denitrifying activities or production of secondary metabolites. Therefore, it is important to determine the optimum cultivation medium for high cell mass production of *P. fluorescens* particularly the requirement for the most effective carbon and nitrogen source. It is also important to look for new chemically defined medium or semi-defined medium to produce high cell density of *P. fluorescens*. In addition, dissolve oxygen and pH condition could be the limiting growth factors for *P. fluorescens*. Thus, further studies on cultivation strategy are needed to increase biomass production.

1.3 Objective

The main objective of the present work is to develop an optimum culture medium and cultivation strategy for biomass production of *P. fluorescens* as nitrate removal microorganisms in wastewater treatment.

1.4 Scopes of Research

The scopes of the research are:

a) Media screening for high cell mass production of *P. fluorescens*.
b) Media optimization for high cell mass production of *P. fluorescens* using one factor at time (OFAT) and statistical approaches (RSM).
c) Batch cultivation of *P. fluorescens* in a 16-L pilot scale stirred tank bioreactor for high cell mass production under controlled and uncontrolled pH condition.
d) Fed-batch cultivation of *P. fluorescens* in a 16-L pilot scale stirred tank bioreactor for high cell biomass production.

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