TWO-STAGE PROCESS FOR AMMONIUM AND NITRATE REMOVAL AND POLYHYDROXYALKANOATE PRODUCTION BY *RHODOBACTER SPHAEROIDES* ADZ101

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Specially dedicated to my entire family members

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ABSTRACT

The application of photosynthetic bacteria in bioremediation is an eco-friendly technique that remains untapped. For the aquaculture industries that are based on high density protein feeding, elevated levels of ammonium and nitrate had been reported. High concentrations of ammonium can cause coma, convulsion and death to aquatic organisms besides eutrophication causing oxygen depletion in water bodies, increasing its harmful effect to aquatic organisms. In view of this, fundamental aspects of nitrogen removal were studied using synthetic medium. The ability of *Rhodobacter* sp. ADZ101, a denitrifying phototrophic bacterium which was successfully isolated and identified using 16S rRNA analysis was investigated for the removal of ammonium and nitrate. Different initial concentrations of ammonium and nitrate were used to determine the nitrogen removal and its reaction kinetics using the Michaelis-Menten rate expression. Results showed that 71% of nitrate was removed at initial concentration of 85 mg/L and 62% of ammonium at initial concentration of 52 mg/L under photoheterotrophic and anoxic dark conditions respectively. The kinetic coefficients of nitrate were determined as: $k = 4.5\times10^{-2}$ g NO_3^- g L^{-1} DCW d^{-1}, K_m = 0.55 g L^{-1}, and that of ammonium as: $k = 4.5\times10^{-3}$g NH_4-N g L^{-1} DCW d^{-1}, K_m = 0.52 g L^{-1}. The yield coefficient of nitrate (Y_N) was 0.15 mg DCW mg $L^{-1}NO_3^-$ and that of ammonium was 0.3 mg DCW mg L^{-1} NH_4-N. Analysis and amplification of the possible genes that are involved in denitrification revealed the presence of both nitrate reductase (*napA*) and nitrite reductase (*nirK*) genes. *Rhodobacter* sp. ADZ101 was also found to produce PHA. Using different carbon and nitrogen sources, acetate and ammonia chloride showed the highest accumulation of PHA of 46% (DCW) with C:N ratio of 32.5 at pH.7. The structural analysis via NMR and GCMS of PHA produced under optimised condition showed that the polymer consisted of PHB/V with methyl esters of butyrate, dodecanoic, hexadecanoic, and heptadecanoic acids as well as oxirane, 2-methyl 2-phenyl, Phenol 2,5 bis (1,1 dimethyl ethyl)-, and benzenepropanoic acid, 3,5-bis (1,1-dimethylethyl)-4-hydroxyl as major monomers. The PHA has molecular weight of 628.55 kDa and maximum decomposition temperature of 395ºC and 454ºC. To incorporate nitrogen removal and production of PHA, a two-stage fermentation process was selected. The two-stage process revealed that the biomass produced during ammonium and nitrate removal enhanced the production of PHA up to 35%. This is the first report of two-stage process of ammonium and nitrate removal with PHA production using *Rhodobacter* sp. ADZ101.

ABSTRAK

Penggunaan bakteria fotosintetik dalam bioremediasi adalah satu teknik mesra alam yang masih belum diterokai. Bagi industri akuakultur yang berasaskan pemakanan protein berketumpatan tinggi, peningkatan tahap ammonium dan nitrat telah dilaporkan. Kepekatan tinggi ammonium boleh menyebabkan koma, sawan dan kematian kepada organisma akuatik disamping eutrofikasi yang menyebabkan pengurangan oksigen dalam sumber air, meningkatkan kesan berbahaya kepada organisma akuatik. Memandangkan ini, aspek asas penyingkiran nitrogen telah dikaji menggunakan medium sintetik. Keupayaan *Rhodobacter* sp. ADZ101, bakteria fototrofik penyahnitrat yang telah berjaya dipencilkan dan dikenal pasti menggunakan analisis 16S rRNA telah dikaji untuk penyingkiran ammonium dan nitrat. Kepekatan ammonium dan nitrat yang berbeza telah digunakan untuk menentukan penyingkiran nitrogen dan tindakbalas kinetiknya dengan menggunakan ungkapan kadar Michaelis-Menten. Keputusan menunjukkan bahawa 71% daripada nitrat telah disingkirkan pada kepekatan awal 85 mg/L dan 62% daripada ammonium pada kepekatan awal 52 mg/L masing-masing dalam keadaan fotoheterotrofik dan anoksik gelap. Pekali kinetik nitrat telah ditentukan sebagai: $k = 4.5 \times 10^{-2}$ g NO_3^- g L^{-1} DCW d^{-1}, $K_m = 0.55$ g L^{-1} dan ammonium pula sebagai $k = 4.5\times10^{-3}$ g NH_4-N g L^{-1} DCW d^{-1}, $K_m = 0.52$ g L^{-1}. Pekali hasil nitrat (Y_N) adalah 0.15 mg DCW mg $L^{-1}NO_3^-$ dan ammonium pula adalah 0.3 mg DCW mg L^{-1} NH_4-N. Analisis dan penggandaan gen yang mungkin terlibat dalam penyahnitritan menunjukkan kehadiran kedua-dua gen reduktase nitrat (*napA*) dan reduktase nitrit (*nirK*). *Rhodobacter* sp. ADZ101 juga telah didapati menghasilkan PHA. Menggunakan sumber karbon dan nitrogen sumber yang berbeza, asetat dan ammonium klorida menunjukkan pengumpulan tertinggi PHA iaitu 46% (DCW) dengan nisbah C:N ialah 32.5 pada pH7. Analisis struktur melalui NMR dan GCMS bagi PHA yang dihasilkan pada keadaan optimum menunjukkan bahawa polimer tersebut mengandungi PHB/V dengan metil ester daripada butirat, asid dodekanoik, asid heksadekanoik dan asid heptadekanoik serta oksiran, 2-metil 2-fenil, fenol 2,5 bis (1,1 dimetil etil)-, dan asid benzenepropanoik, 3,5-bis (1,1-dimetiletil)-4-hidroksil sebagai monomer-monomer utama. PHA tersebut mempunyai berat molekul 628.55 kDa dan suhu penguraian maksimum 395ºC dan 454ºC. Bagi menggabungkan penyingkiran nitrogen dengan penghasilan PHA, proses penapaian dua peringkat telah dipilih. Proses dua peringkat tersebut menunjukkan bahawa biojisim yang dihasilkan semasa penyingkiran ammonium dan nitrat meningkatkan pengeluaran PHA sehingga 35%. Ini adalah laporan pertama proses dua peringkat bagi penyingkiran ammonium dan nitrat dengan penghasilan PHA menggunakan *Rhodobacter* sp. ADZ 101.

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LIST OF ABBREVIATION

16S rRNA	-	16 subunit ribosomal ribonucleic acid
2LFD	-	Two level factorial design
ADMI	-	American Dye Manufacturing Index
ANOVA	-	Analysis of variance
BLAST	-	Baslic local alignment search tool
BOD	-	Biochemical oxygen demand
COD	-	Chemical oxygen demand
DO	-	Dissolved oxygen
DNA	-	Deoxyribonucliec acid
DCW	-	Dry cell weight
DTA	-	Differential thermal analysis
EtBr	-	Ethidium bromide
FTIR	-	Fourier transform infrared spectroscopy
GCMS	-	Gas chromatography mass spectrometry
napA	-	Nitrate reductase
NCBI	-	National centre of biotechnology information
nirK	-	Nitrite reductase
PHA	-	Polyhydroxyalkanoate
PCR	-	Polymerase chain reaction
PPB	-	Purple phototrophic bacteria
PB	-	Photosynthetic bacteria
PSB	-	Purple sulphur bacteria
PSNB	-	Purple non-sulphur bacteria
PNSBEM	-	Purple non-sulphur bacteria enrichment medium
SEM	-	Scanning electron microscope
SND	-	Simultaneous nitrification and denitrification

TAE	-	Tris-acetate-EDTA
TBP	-	Tributyle phosphate
TEM	-	Transmission electron microscope
TDS	-	Total dissolved solids
TGA	-	Thermal gravimetric analysis
TOC	-	Total organic compounds
TSS	-	Total suspended solids
VOA	-	Volatile organic acids
VOC	-	Volatile organic compounds
RNA.	-	Ribonucleic acid

LIST OF SYMBOLS

d	-	Day
$(DCW)_F$	-	Final dry cell weight
$(DCW)_I$	-	Initial dry cell weight
g/L	-	Gram per Litre
h	-	Hour
k	-	Rate constant
K_m	-	Saturation constant
mg/L	-	Milligram per Litre
min	-	Minute
mL	-	Millilitre
N_0	-	Initial nitrogen concentration
nm	-	Nanometre
R_{max}	-	Maximum substrate removal
R_{mo}	-	Initial nitrogen removal rate
R_X	-	Initial nitrogen removal rate
RX_i	-	Specific rate of nitrogen removal
S	-	Effluent concentration
T_{max}	-	Maximum temperature
X_0	-	Initial biomass concentration
Y_N	-	Yield coefficient
^0C	-	Degree Celsius
%	-	Percent

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CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Nitrogen pollution is generally linked to agricultural activities such as fertilizer application, discharge from aquaculture and animal wastes. However non-agricultural sources such as sewage, abandoned landfills, industrial wastes and atmospheric disposition (Wakida and Lerner, 2005) also released large quantities of nitrogenous compounds into the environment. Analysis on the effect of nitrogen to health revealed that acute exposure to excess ammonium resulted in nervous dysfunction, kidney damage and lung oedema. While short time exposure led to increased blood pressure and acidosis. Other health related problems associated with ammonium include the enlargement of uterus and ovaries as well as mutagenicity (WHO 1986), (EPA 1989). Nitrate contributes to the formation of nitrosamines leading to the development of digestive tract cancer (Nash, 1993). In infants, nitrate can cause methemoglobinemia via its conversion to nitrite which results in the blockage of oxygen-carrying capacity of haemoglobin (Wolfe and Patz, 2002). Its major impact on aquatic animals involved the conversion of haemoglobin and haemocyanin into a form that is incapable of carrying oxygen, (methemoglobin) (Scott and Crunkilton, 2000). Hence for sustainable growth and development of any society, an effective and cheap method of managing nitrogen pollution is highly required.

The biological method of managing pollution in general offers a better solution to the conventional methods in terms of cost, *in-situ* application and complete degradation of pollutant to harmless substances. During bioremediation process, microorganisms consume the organic material present in the contaminants. The contaminants can then serve as a source of carbon for growth and energy by breaking the chemical bonds and transfer electrons into the microbial metabolic pathways. The energy gained from the electron transfer is used to produce more cells. The process may involve the manipulation of environmental parameters because it is only effective when environmental conditions permit microbial growth and activity. This in turn results in microbial growth and degradation to proceed at a faster rate. The microorganisms then multiply in the presence of contaminant and favourable environmental conditions. The end products of the process include harmless substances such as cell biomass, water and carbon dioxide.

The advantage of bioremediation over the chemical process include low capital and operating costs, reduction of aquatic toxicity, reduction of sludge production, and reduction of filamentous growth (Muchie, 2010). However, the successful application of bioremediation process is much dependent on the microorganisms exploited in the system. Furthermore, the choice of adaptable, robust and effective natural occurring microorganisms that can breakdown or utilise the pollutants is a major challenge. One group of microorganism that is viable with vast metabolic activities and has the potential of removing toxic compounds from the environment concomitantly generating value added products is photosynthetic bacteria. They have the potential of degrading pollutants yielding products of high value. Most of these products can be used as food supplement for animals. Photosynthetic bacteria produce biomass rich in carotenoids, vitamins, proteins that can be used as animal feed (Ponsano *et al.*, 2003). But due to the diversity and the complexity of pollutants, each microorganism and each type of pollutant require a separate study. This study therefore investigated the potential application of photosynthetic bacteria for ammonium and nitrate removal with polyhydroxyalakanoate (PHA) production via two-stage process.

Polyhydroxyalkanoates are degradable polymer accumulated by bacteria. Unlike synthetic plastic which are produced from fossil fuel, PHA are produced by living organisms, as such they renewable and sustainable. They are easily degraded in soil by bacteria that possess the enzyme, PHA polymerase. This enzyme catalyses the ester bonds of PHA to its oligomers and water-soluble monomers. Microorganisms further metabolize these products into H_2O and CO_2 (Chanprateep, 2010). Their biodegradable and thermoplastic properties make them an ideal substitute for petroleum-based synthetic plastic. Other properties such as biocompatibility and elastomeric, broadened their application to industries and medicine. In medicine they are used to develop cardiovascular patches, articular cartilage repair device and bone-marrow scaffolds (Valappil *et al.*, 2006).

1.2 Problem Statement

The presence of ammonium in wastewater affects both aquatic organisms and the surrounding environment. High concentration of ammonium causes coma, convulsion and death to aquatic organisms. It also causes eutrophication, which promotes algal growth and cause oxygen depletion in water bodies thereby increasing its harmful effect to aquatic organisms (Randall and Tsui 2002). The conventional (traditional) method of removing ammonium from wastewater involve nitrification and denitrification by nitrifying and denitrifying bacteria respectively. Since the processes are operated under two distinct conditions by two different groups of bacteria, two separate systems are required. The drawback of this system includes the complexity of separating the two systems (Ahn, 2006). In a natural environment, the nitrification process is also inhibited by high concentration of ammonium leading to water hypoxia, fish poisoning, reduced water purification capability and pollution of the entire water body (Juan *et al.*, 1998). In addition, the process produces nitrate and nitrite which are also harmful to the environment. Thus microorganisms that are capable of assimilating ammonium without the accumulation of nitrate and nitrite can overcome these problems and therefore needed for effective biological ammonium removal.

The major challenges of biological nitrate removal (denitrification) are slow denitrification and nitrite accumulation. Nitrite inhibits cell growth via its conversion from nitrate (Almeida *et al.*, 1995). It can also be mutagenic in the form of HNO_2 (Krishnamachari and Clarkson, 1993). The presence and accumulation of nitrite prolong the complete biological denitrification process (Foglar *et al.*, 2005). In addition, industrial wastewater contains high amount of nitrate, hence difficult for biological denitrification (Smith *et al.*, 1994). Thus microorganisms that have the ability to remove nitrate by both dissimilatory and assimilatory means are highly required in wastewater treatment plants. During dissimilatory and assimilatory processes, nitrate inside the bacterial cell is converted to ammonium via nitrite before it is incorporated into organic nitrogen which is needed for growth (Moreno-Vivián and Flores, 2006). This process is also beneficial where there is need to use the captured nitrogen from the biomass (Lies and Oduor, 2014). Thus this process overcomes the problem of nitrite accumulation as such it is highly required for effective biological nitrate removal.

Furthermore, Hassan *et al.,* (1996) had proposed as two-stage process for PHA production. In the first stage, organic acids were recovered from POME and later used as carbon source in the second stage. A three-stage process for PHA production from paper mill wastewater was also proposed by Bengtsson *et al.,* (2008). Organic matter was converted to volatile fatty acids in the first stage. The second stage involved the enrichment of PHA producing organisms followed by the accumulation of PHA. These studies focused on obtaining organic and fatty acids in the first stage from wastewaters and subsequently used for PHA production in the second stage. The recovery of cell biomass and the removal of toxic ammonium and nitrate were not incorporated in the process. And since PHA is accumulated intracellularly, the amount of cell biomass used as inoculum will definitely enhance PHA yield. It has been reported that a single stage fermentation process of PHA production under limited nitrogen yield low amount of polymer due to low biomass accumulation (Katırcıoğlu *et al.,* 2003). Low PHA productivity was also reported for single batch fermentation process because the accumulated PHA may be degraded by the bacteria resulting in low yield (Zinn *et al.,* 2001). In two-stage process however, the growth phase is separated from PHA production phase, hence degradation of accumulated PHA is limited. Large amount of

biomass obtained during the growth phase can serve as inoculum for the second stage, resulting to an increase in productivity since PHA is accumulated intracellularly. Furthermore, incorporating ammonium and nitrate removal will provide an opportunity to wastewater treatment plants to obtain PHA as a value added product. Polyhydroxyalkanoate has also been reported as a good carbon source for heterotrophic denitrification (Boley *et al.*, 2000). Hence the possibility of using the accumulated PHA as a carbon source for ammonium and nitrate removal is also feasible.

Therefore, the isolation of new bacterial strains that have the capability for ammonium and nitrate removal without the accumulation of nitrite and their potential application in the two-stage process can improve ammonium and nitrate removal as well as PHA production. Hence this study proposed a two-stage process for ammonium and nitrate removal with PHA production by locally isolated strain of photosynthetic bacterium, *Rhodobacter* sp. ADZ101. The first stage involved ammonium and nitrate removal and the accumulated biomass was used as inoculum to produce PHA in the second stage. The advantage of this process is having more biomass during the nitrogen removal that can subsequently be used for PHA production. It provides a high volumetric productivity which enhanced the PHA production. Hence, it has both environmental and economic benefits.

1.3 Objectives of the Study

This study was aimed at investigating the application of photosynthetic bacteria for ammonium and nitrate removal with PHA production via two-stage process. The study was specifically designed to address the following objectives:

i. To isolate and characterise denitrifying photosynthetic bacteria for the removal of ammonium and nitrate

ii. To determine the kinetic coefficients for ammonium and nitrate removal by the isolated bacteria

iii. To assess the production of PHA and to characterise the PHA produced by the isolated bacteria

1.4 Scope of the Study

The study covered the isolation of denitrifying photosynthetic bacteria, in which a purple non-sulphur phototrophic bacterium was isolated and identified by molecular technique using the 16S rRNA analysis as *Rhodobacter* sp. ADZ101. The photosynthetic and denitrifying ability of the bacterium were determined by the detection of photosynthetic pigments and amplification of denitrifying genes respectively. The bioremoval and kinetic coefficients of nitrate and ammonium removal were determined by Michaelis-Menten rate expression using synthetic medium containing different concentrations of nitrate and ammonium. The use of synthetic medium revealed the exact composition and component of the wastewater and determined the types of biochemical reactions occurring in the process. It also provides an effective means of adjusting the concentration of ammonium and nitrate as required in the kinetic study. The ability of the bacterium to produce PHA and the optimum condition of PHA production were also covered. The ability of the bacterium

to accumulate PHA and the optimum condition of PHA accumulation were also covered using two-level factorial design. The accumulated PHA was then characterised by means of FTIR, SEM, GCMS, TEM, NMR and TGA analyses. The study also covered the two-stage process of ammonium and nitrate removal with PHA accumulation.

1.5 Significance of the Study

The significance of this study was to isolate and characterise denitrifying photosynthetic bacteria and to investigate their potential application in ammonium and nitrate removal with PHA production via two-stage process. The two-stage process enhanced PHA production by providing huge amount biomass that can be used in PHA accumulation. The possibility of using the accumulated PHA as a carbon source for ammonium and nitrate removal is also feasible. The study provided important basic information on the potential application of photosynthetic bacteria for ammonium and nitrate removal with PHA production. The reaction kinetic data for ammonium and nitrate removal by the *Rhodobacter* sp. ADZ101 was also provided. Information on the application of *Rhodobacter* sp. ADZ101 to produce PHA and optimum condition of PHA production as well as the characteristics of the PHA was also provided.

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