UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION AND GROWTH KINETICS OF LOCAL N_2-FIXING BACTERIUM, *BACILLUS SP*. UPMB10

OOI TZE CHEAN

CHARACTERIZATION AND GROWTH KINETICS OF LOCAL N_2-FIXING BACTERIUM, *BACILLUS SP.* UPMB10

By

OOI,TZE CHEAN

Thesis Submitted in Fulfillment of the Requirements for the Degree of Master of Science in the Faculty of Food Science and Biotechnology Universiti Putra Malaysia

April 2002

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

CHARACTERIZATION AND GROWTH KINETICS OF LOCAL N_2-FIXING BACTERIUM, *BACILLUS SP.* UPMB10

By

OOI TZE CHEAN

April 2002

Chairman : Associate Professor Dr. Arbakariya Ariff

Faculty : Food Science and Biotechnology

The production of locally isolated N_2-fixing bacteria was undertaken in Fermentation Technology Unit, Enzyme and Microbial Technology Laboratory, Institute of Bioscience, Universiti Putra Malaysia. Cellular studies and biochemical tests conducted on *Bacillus sp* UPMB10 suggests that the bacterium is *Bacillus sphaericus.* Optimization of medium for cultivation of the N_2-fixing *Bacillus* was achieved using 1.4 g/L of glycerol and 2.0 g/L of yeast extract. Addition of biotin and thiamine did not improve growth of the bacteria. Optimum culture condition for growth of UPMB10 in the 2L: stirred tank fermenter was obtained at initial pH range between pH 6.0-8.0, 30°C, at agitation speed of 600 rpm and airflow rate of 0.5 vvm. Viable cell counts obtained under these conditions were approximately 3.5 X 10^9 cfu/mL.

A model employing the logistic equation was proposed to describe growth of this newly isolated *Bacillus*. The values of the general kinetic parameters were calculated from the analysis of experimental data obtained from a number of culture using batch fermentation. The specific growth rates of 0.40 h^{-1} and 0.45 h^{-1} were employed for modeling of bacteria growth in a shake flask and in 2 L fermenter, respectively. The proposed model consisting of general kinetic parameters and the specific growth rate was adequate to describe the fermentation data with sufficient accuracy for prediction of biomass production and substrate consumption.

Due to substrate inhibition, production of the bacteria was further enhanced using an exponential fed-batch fermentation technique. With the specific growth rate maintained at 0.4 h^{-1}, viable cells obtained using fed-batch fermentation was four times higher than batch cultivation of the bacteria. Cell density and productivity was improved by three fold compared to batch cultivation. In all experiments acetylene reduction assay (ARA) levels remained unchanged and was maintained at 20 nmol C_2H_2/hr/mL

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENCIRIAN DAN KINETIK PERTUMBUHAN BACTERIA PENGIKAT NITROGEN TEMPATAN, *BACILLUS SP.* UPMB10

oleh

OOI TZE CHEAN

April 2002

Pengerusi : Professor Madya Dr. Arbakariya Ariff

Faculti : Sains Makanan dan Bioteknologi

Pengeluaran bacteria pengikat nitrogen tempatan telah dijalankan di Unit Teknologi Fermentasi, Makmal Teknologi Enzim dan Microb, Institut Biosains, Universiti Putra Malaysia. Kajian sel dan ujikaji biokimia ke atas bacteria *Bacillus sp* UPMB10 mendapati bahawa bacterium tersebut adalah *Bacillus sphaericus*. Media yang optimum untuk pengkulturan bacilli pengikat nitrogen telah dicapai dengan menggunakan 1.4 g/L glycerol dan 2.0 g/L yeast extract. Penambahan biotina dan thiamina tidak memperbaiki pertumbuhan bacteria. Keadaan fermentasi yang optima telah dicapai dengan pH permulaan di antara pH 6.0-8.0, 30°C dan kadar pemutaran 600 rpm dengan 0.5 vvm pengaliran udara. Anggaran kiraan sel hidup yang diperolehi dalam keadaan demikian adalah 3.5 X 10^9 cfu/mL.

Model menggunakan persamaan logistik telah dicadangkan untuk mewakili pertumbuhan bacilli yang baru ditemui. Nilai parameter kinetik am telah dikira daripada analisis banyak data eksperimen fermentasi sekelompok. Nilai kadar pertumbukan spesifik 0.40 j^{-1} dan 0.45 j^{-1} telah digunakan untuk penghasilan model pertumbuhan bakteria dalam 'shake flask' dan dalam fermenter 2 liter. Model dicadangkan yang mengandungi parameter kinetik am dan kadar pertumbuhan spesifik adalah memadai untuk mewakili data fermentasi dengan tepat untuk ramalan penghasilan biojisim dan penggunaan substrat

Oleh kerana perencatan substrat, pengeluaran bakteria boleh ditingkatkan dengan menggunakan teknik fermentasi suapan sekelompok eksponen. Dengan kadar pertumbuhan spesifik ditetapkan pada 0.4 j^{-1}, sel hidup yang diperolehi daripada fermentasi suapan sekelompok eksponen adalah empat kali lebih tinggi berbanding pertumbuhan bakteria sekelompok. Kepekatan sel dan produktiviti menggunakan fermentasi suapan sekelompok telah ditingkatkan sebanyak tiga kali berbanding pertumbuhan bakteria sekelompok. Dalam semua eksperimen, kadar penurunan acetylene (ARA) tidak berubah dan tetap pada 20 nmol C_2H_2/hr/mL.

ACKNOWLEDGEMENTS

I wish to thank my all postgraduate students and laboratory staff of Fermentation Technology Unit, Enzyme and Microbial Technology Laboratory for their assistance throughout the length of my research. I like to express my deepest gratitude towards my father, mother and also See Pao Theen for always giving me the love and support I need.

I would especially like to thank my supervisor Prof. Madya Dr. Arbakariya b. Ariff and the supervisory committee for their guidance, support and encouragement. Without them I would not have been able to complete my study.

Above all, I praise GOD for His guidance and continuous blessings upon my life

This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfillment of the requirement of the degree of Master of Science

AINI IDERIS, Ph.D.
Professor/Dean
School of Graduate Studies
Universiti Putra Malysia

Date:

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been dully acknowledge. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

OOI TZE CHEAN

Date:

TABLE OF CONTENTS

LISTS OF TABLES

LISTS OF FIGURES

LIST OF ABBREVIATIONS

N_2	Molecular dinitrogen
C/N	Carbon to nitrogen ratio of medium in mM basis
D_i	Impeller diameter
DOT	Dissolved oxygen tension
μ_{max}	Maximum or initial specific growth rate (h^{-1})
S_i	Initial substrate concentration (g/L)
t	Fermentation time (hr^{-1})
X	Cell concentration (g/L)
X_i	Initial cell concentration (g/L)
X_m	Maximum cell concentration (g/L)
μ	Growth rate (h^{-1})

CHAPTER 1

INTRODUCTION

Although nitrogen gas (N_2) makes up 78% of the atmosphere, plants and other microorganisms cannot use it as a source of nitrogen. Nitrogen fixation plays an important role in the conversion of 'inert' N_2 from the atmosphere into usable ammonia. In the twentieth century, increase in crop yield requires that biological N_2 fixation be supplemented increasingly with the use of fixed nitrogen from chemical fertilizers. The development of Haber-Bosch process for catalytically combined atmospheric nitrogen with hydrogen from fossil fuels to produce ammonia has enabled increases in crop yields. This process now accounts for almost all nitrogenous fertilizer production and absorbs approximately 1.5% of the world's energy consumption (Cocking, 2000). In 1990, world consumption of fertilizer nitrogen was about 80 million tons (Brown, 1994).

Nitrogen fertilizer application is essential in Malaysian agriculture as it plays a major role in improving plant growth and yield on highly leached, infertile acid tropical soils. The common sources of nitrogen being used in this country are ammonium sulphate (21% N), ammonium nitrate (26% N), ammonium chloride (25%) and urea (46% N). Vast areas are cultivated with perennial tree crops like oil palm where large quantities of fertilizer are required annually to sustain high crop yields and profitability. At present, Malaysia has a total land area of about 329,733 km^2 while those cultivated with industrial crops, namely, oil palm, rubber, cocoa and paddy, amounted to 5.53 million hectares (5530 km^2). Of this, oil palm accounts for approximately 50% of the cultivated area

(Raof *et al.*, 1999). In oil palm production alone, the estimated total N fertilizer cost is RM 470 million per year (Amir, 2001).

Recently, environmental and energy concerns has risen from the overuse of nitrogenous fertilizers. Fertilizer nitrogen is normally applied in the zone of diminishing returns in order to maximize yield. There may be as much as 50% inefficiency in terms of plant uptake with resultant nitrate contaminating ground water. Conversion of excess nitrate to nitrous oxide (N_20) by denitrification also produces a greenhouse gas 180 times more potent than carbon dioxide per molecule. It is well known that the Haber-Bosch process is energy expensive. Bockman (1997) pointed out that it takes 1.3 tons of oil to deliver 1 ton of fertilizer nitrogen. At present, the annual cost of fertilizer nitrogen is US$20-60 billion worldwide (Hardy, 1997). This has highlighted the need for plants to obtain more of their nitrogen from biological N_2 fixation.

Biological N_2 fixation through legume-*Rhizobium* symbiosis sustains soil N balances in temperate regions. In the tropics, however, legumes contribute little to the soil nitrogen economy (Dart, 1986). Non-legumes in association with free-living or associative bacteria types are capable of N_2 fixation. This N_2-fixing system plays an important role in maintaining N levels in tropical soils. Free-living and associative N_2-fixing bacteria can be found in the genera *Azospirillum*, *Azotobacter*, *Beijerinckia*, *Derxia*, *Archromobacter*, *Mycobacterium*, *Arthrobacter*, *Bacillus*, *Klebsiella*, *Pseudomonas*, *Clostridium*, *Chromatium*, *Rhodopseudomonas*, *Desulfovibrio*, *Rhodomicrobium*, *Chlorobium* and *Rhodospirillum* (Subba Rao, 1996). Recently, a

newly isolated strain from the genera *Bacillus* (catalogued as UPMB10) has the ability for high rates of N_2-fixation (Shamsuddin *et al.*, 1999a). This strain has been tested on local oil palms (Amir, 2001) and banana crops (Mia *et al*, 1999) and was found to significantly enhance their growths. The preliminary results indicate that *Bacillus sp.* (UPMB10) can be used as a potential biological fertilizer to reduce the usage of chemical N fertilizers.

Biofertilizer refers to microorganisms which increase crop growth through biological N_2 fixation, growth-promoting or hormonal substances, increase availability of soil nutrients, and/or control diseases (Ladha, 1997). The application of biofertilizer for improvement of crop yield and productivity has been studied extensively (Dobereiner and Pedrosa, 1987; Okon and Hadar *et al.*, 1987; Arsac, *et al.*, 1990). For the preparation of biofertilizer, large-scale production of starter cultures or microbial inoculants containing either single pure strain or mixed culture is essential before inoculation into suitable solid substrate for composting. In most cases, fermentation deals with a metabolite or a product of the cell. In the case of microbial inoculants, the cell is the product and, accordingly, fermentation should be altered to optimize cell mass production (Bowers, 1982). One of the important approaches for the development of efficient cultivation process for mass production of cell culture is the optimization of medium and cultural conditions. Since starter cultures for biofertilizer are classified as low end products, cheap industrial medium using locally available carbon and nitrogen sources should be used. For aerobic N_2-fixing bacteria, such as *Azospirillum, Azotobacter* and *Bacillus sp.*, it has been found that concentrations of dissolve oxygen (DO) play an important part in determining

biomass yield and N_2-fixing efficiency of the strain. Optimization may be carried out using systematic experimental design or mathematical models.

Starter culture, containing high-density cells is normally produced by mass cultivation of the microorganism in submerged cultivation using a fermenter. Several modes of fermenter operation, such as batch, continuous and fed-batch cultures are possible for improvement of yield, productivity and reduction of production cost. Although, reports on the application of various modes of fermenter operation for large-scale cultivation of microbial cell are available elsewhere in the literature (e.g. Harwood and Pirt, 1972) this nature of work is scarce for the production of N_2-fixing microorganisms. Few studies have been devoted to the physiology of N_2-fixing bacteria in fermenters for biomass production. This is true even for *Azospirillum*, a well-studied N_2 fixer (Fages, 1994). Most of these physiological studies were related with N_2-fixation activity. For biomass production growth must not be limited by nitrogen, thus a nitrogen source must be added into the medium. Carbon and energy sources, oligoelements and vitamins are also important components of a growth medium.

This study is aimed at the development of an efficient cultivation technique for the production of locally isolated N_2-fixing *Bacillus sp* (UPMB10) in the fermenter, as microbial inoculants for subsequence use in the preparation of biofertilizers. Thus, the objectives of the study are;

(i) To conduct basic identification of *Bacillus sp.*

(ii) To optimize the cultural conditions for enhancement of growth of *Bacillus sp* (UPMB10).

(iii) To propose models that can be used to describe growth of *Bacillus sp* (UPMB10) and substrate utilization for better understanding of the process.

(iv) To investigate the feasibility of using a fed-batch fermentation technique for improvement in yield and productivity of the cultivation of *Bacillus sp* (UPMB10) cells.