



UNIVERSITI PUTRA MALAYSIA

***INFLUENCE OF CULTIVATION CONDITIONS ON GROWTH,
SPORULATION RATE AND α -ENDOTOXIN SYNTHESIS OF BACILLUS
THURINGIENSIS MPK13***

MOHAMED MAZMIRA BIN MOHD. MASRI

FBSB 2013 26



**INFLUENCE OF CULTIVATION CONDITIONS ON GROWTH,
SPORULATION RATE AND δ -ENDOTOXIN SYNTHESIS OF *BACILLUS
THURINGIENSIS* MPK13**

By

MOHAMED MAZMIRA BIN MOHD. MASRI

**Thesis Submitted to the School of Graduate Studies,
Universiti Putra Malaysia, in Fulfilment of the
Requirements for the Degree of
Doctor of Philosophy**

13 JUNE 2013

Abstract of thesis presented to the Senate of Universiti Putra Malaysia
in fulfilment of the requirement for the degree of Doctor of Philosophy

INFLUENCE OF CULTIVATION CONDITIONS ON GROWTH OF *BACILLUS THURINGIENSIS* MPK13, SPORULATION RATE AND δ -ENDOTOXIN SYNTHESIS

By

MOHAMED MAZMIRA BIN MOHD. MASRI

June 2013

Chair: Professor Arbakariya B. Ariff, PhD

Faculty: Biomolecular Sciences and Biotechnology

The influence of cultivation conditions on growth of Bt MPK13, sporulation rate, δ -endotoxin synthesis and its toxicity was the focus of this study. The Cry+ strain of Bt MPK13 with consistent ability in δ -endotoxin synthesis was selected by repeated isolation with polymerase chain reaction analysis and δ -endotoxin detection. The effect of monosaccharides (glucose, fructose and galactose) and disaccharides (sucrose, lactose and maltose) on growth of Bt MPK13, sporulation and δ -endotoxin synthesis was carried out using shake flask culture. The data generated was also used to find the relationship between intracellular and extracellular total carbon (TC), total nitrogen (TN) and C/N ratio on growth of Bt MPK13, sporulation and δ -endotoxin synthesis. The effect of dissolved oxygen tension (DOT) control strategies and also the influence of different

modes of bioreactor operation (batch, fed-batch and continuous) on the cultivation performance of Bt MPK 13 was investigated using 5 L stirred tank bioreactor. The existence of δ -endotoxin was detected using SDS-PAGE analysis and the toxicity of δ -endotoxin synthesized during different modes of bioreactor operation was carried out against early instars of bagworms, *Metisa plana*.

The selected Cry+ strain of Bt MPK13 was preserved as the stock culture in freeze dried form. The use of Cry+ strain of Bt MPK13 as the stock culture resulted to a consistent δ -endotoxin synthesis in repeated experiments. During cultivation, sucrose, fructose, galactose, lactose and maltose were able to support growth of Bt MPK13, but failed to enhance high percentage of sporulation and the existence of δ -endotoxin was not detected. The synthesis of δ -endotoxin was only detected in cultivation using glucose as the carbon source. Mixture of glucose with other sugars also recorded high cell growth ($> 1.0 \times 10^{12}$ cfu/mL) with high percentage of sporulation ($> 35\%$) however, the synthesis of δ -endotoxin was only detected when glucose concentration used in a mixture was higher than 8 g/L, indicating that high glucose concentration (> 8 g/L) must be present in the culture to trigger δ -endotoxin formation.. The highest initial and final intracellular TC and TN were also recorded in cultivation using glucose as the sole carbon source which corresponded well to high cell growth, high percentage of sporulation and δ -endotoxin synthesis. High DOT level (80% saturation) during active growth phase of Bt MPK13 was compulsory for δ -endotoxin synthesis in batch cultivation using glucose (8 g/L) as a carbon source. Enhanced percentage of sporulation (up to 61%) with early δ -endotoxin synthesis (8 h of cultivation) was obtained in cultivation where the DOT was controlled

at 80% saturation during active growth and then switched to 60% or 40% saturation at mid-exponential growth phase (after 6 h). The application of this optimal DOT control strategy without affecting the cell growth could also be used to enhance the percentage of sporulation (> 45%) and δ -endotoxin synthesis in fed-batch cultivation. The highest viable cell count (1.5×10^{12} cfu/mL) and spore count (7.1×10^{11} spore/mL) with the existence δ -endotoxin was obtained in fed-batch cultivation with constant feeding of 2 L, 8 g/L glucose at 6 h of cultivation, and optimal DOT control strategy was applied. Compared to batch cultivation, fed-batch without DOT control strategy able to increase cell count (24% increment), however spore count was greatly decreased (50% decrement). Feeding of glucose at stationary phase and intermittent feeding resulted to non-existence of δ -endotoxin. In continuous cultivation, the highest cell (5.8×10^{14} cfu/L.h) and spore (1.6×10^{13} spore/L.h) productivities were obtained at the dilution rate (D) of 0.39 h^{-1} and 0.05 h^{-1} , respectively. Although the steady-state viable cell concentration (1.7×10^{12} cfu/mL) was substantially higher than those obtained in batch cultivation, more than 50% decrement in spore count was recorded. It is important to note that δ -endotoxin synthesis was not detected in all D tested in continuous cultivation, indicating that this cultivation technique is not suitable for Bt production.

The culture with the existence of δ -endotoxin recorded 100% mortality towards bagworms (*M. plana*) at 14 DAT which was related to oil palm defoliation, suggesting that Bt MPK13 has potential to be used as industrial biopesticide. The proposed optimal cultivation conditions may be used in the development of large scale cultivation of Bt MPK13 for subsequent use as biopesticides in oil palm plantation

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**PENGARUH KEADAAN PENGKULTURAN TERHADAP PEMBIAKAN,
KADAR PENSPORAAN DAN PENGHASILAN δ -ENDOTOKSIN *BACILLUS
THURINGIENSIS* MPK13**

Oleh

MOHAMED MAZMIRA BIN MOHD. MASRI

Jun 2013

Pengerusi: Professor Arbakariya Ariff, PhD

Fakulti: Sains Biomolekul dan Bioteknologi

Kesan keadaan pengkulturan terhadap pembiakan Bt MPK13, kadar pensporaan, penghasilan δ -endotoxin dan ketoksikannya merupakan fokus kajian ini. Strain Cry+ Bt MPK13 dengan keupayaan untuk hasilkan δ -endotoxin dengan konsisten telah dipilih melalui pemencilan berulang menggunakan analisis tindak balas rantaian polimerase dan pengecaman δ -endotoksin.

Kesan monosakarida (glukosa, fruktosa dan galaktosa) dan disakarida (sukrosa, laktosa dan maltosa) terhadap pembiakan Bt MPK13, kadar pensporaan dan penghasilan δ -endotoksin telah dijalankan menggunakan kelalang goncangan. Data yang diperolehi digunakan untuk mengkaji kaitan di antara jumlah karbon (TC), jumlah nitrogen (TN) dan nisbah C/N intraselular dan ekstraselular terhadap pembiakan Bt MPK13, pensporaan

dan juga penghasilan δ -endotoksin. Kesan strategi kawalan kepekatan oksigen terlarut (DOT) dan juga kesan operasi bioreaktor yang berbeza (kelompok, suap-kelompok dan selanjar) terhadap prestasi pengkulturan Bt MPK 13 juga telah dikaji menggunakan bioreaktor tangki adukan 5 L. Kehadiran δ -endotoksin dalam kultur dikesan menggunakan analisis SDS-PAGE dan ketoksikan δ -endotoksin yang terhasil semasa operasi bioreaktor yang berbeza diuji ke atas larva awal ulat bungkus, *Metisa plana*.

Strain Cry+ Bt MPK13 yang terpilih disimpan sebagai kultur stok di dalam bentuk sejuk-kering. Penggunaan strain Cry+ Bt MPK13 sebagai kultur stok menjanjikan penghasilan δ -endotoksin yang konsisten dalam eksperimen ulangan. Semasa pengkulturan, sukrosa, fruktosa, galaktosa, laktosa dan maltosa didapati mampu menyokong pembiakan Bt MPK13 yang baik namun tidak mampu untuk meningkatkan pensporaan dan tidak menyokong penghasilan δ -endotoksin. Penghasilan δ -endotoksin hanya dikesan semasa pengkulturan menggunakan glukosa sebagai sumber karbon utama. Campuran glukosa dengan gula yang lain juga merekodkan pembiakan sel yang tinggi ($> 1.0 \times 10^{12}$ cfu/mL) dengan peratus pensporaan yang tinggi ($> 35\%$), walaubagaimanapun penghasilan δ -endotoksin hanya dikesan sekiranya kepekatan glukosa yang digunakan dalam campuran adalah 8 g/L, yang mana ini menunjukkan kepekatan glukosa yang tinggi (> 8 g/L) adalah suatu kemestian dalam kultur untuk mencetuskan penghasilan δ -endotoksin. Nilai permulaan dan nilai akhir TC dan TN tertinggi semasa pengkulturan menggunakan glukosa sebagai sumber karbon utama menyokong pembiakan sel yang tinggi, peratus pensporaan dan tinggi dan juga penghasilan δ -endotoksin. Tahap DOT yang tinggi (80% ketepuan) semasa fasa pembiakan aktif Bt MPK13 adalah suatu kemestian untuk

menyokong penghasilan δ -endotoxin dalam kultur kelompok yang menggunakan glukosa (8 g/L) sebagai sumber karbon utama. Peningkatan pensporaan (sehingga 61%) dengan penghasilan δ -endotoxin yang cepat (seawal 8 j pengkulturan) diperolehi semasa pengkulturan dengan DOT dikawal pada 80% ketepuan semasa fasa pembiakan aktif dan ditukar ke 60% atau 40% ketepuan pada pertengahan fasa pembiakan eksponen (selepas 6 jam pengkulturan). Strategi kawalan DOT yang optimum yang diaplikasi tanpa mengganggu pembiakan sel juga boleh digunakan untuk meningkatkan peratus pensporaan sel (> 45%) dan penghasilan δ -endotoksin semasa pengkulturan suap-kelompok. Nilai sel hidup tertinggi (1.5×10^{12} cfu/mL) dan kiraan spora tertinggi (7.1×10^{11} spore/mL) dengan kehadiran δ -endotoksin diperolehi dalam kultur suap-kelompok suapan tetap dengan suapan 2 L, 8 g/L glukosa pada 6 jam pengkulturan dan aplikasi strategi kawalan DOT yang optima. Berbanding dengan kultur kelompok, kultur suap-kelompok tanpa strategi kawalan DOT mampu meningkatkan kiraan sel hidup (24% kenaikan), walaubagaimanapun kiraan spora didapati menurun dengan mendadak (50% pengurangan). Suapan glukosa pada fasa pembiakan pegun dan suapan berkala semasa pengkulturan didapati tidak menyokong penghasilan δ -endotoksin. Semasa pengkulturan selanjara, produktiviti sel hidup tertinggi (5.8×10^{14} cfu/L.h) dan produktiviti spora tertinggi (1.6×10^{13} spore/L.h) masing-masing diperolehi pada kadar pencairan (D) 0.39 h⁻¹ dan 0.05 h⁻¹. Walaupun kepekatan sel hidup pada keadaan mantap (1.7×10^{12} cfu/mL) adalah lebih tinggi dari yang diperolehi semasa pengkulturan kelompok, lebih daripada 50% penurunan dalam kiraan spora telah direkodkan. Adalah penting diketahui bahawa δ -endotoksin tidak dikesan dalam semua D yang diuji dalam kultur selanjara, justeru menunjukkan teknik pengkulturan ini adalah tidak sesuai untuk penghasilan Bt.

Kultur yang mengandungi δ -endotoksin merekodkan kematian 100% terhadap ulat bungkus (*Metisa plana*) pada 14 hari selepas rawatan (HSR) yang mana ulat bungkus ini adalah penyebab utama kerosakan daun sawit dan ini menunjukkan bahawa Bt MPK13 mempunyai potensi yang tinggi untuk digunakan sebagai biopestisid industri. Keadaan pengkulturan yang optimal yang dicadangkan boleh digunakan dalam pembangunan pengkulturan Bt MPK13 skala besar untuk digunakan sebagai biopestisid di ladang-ladang sawit.



ACKNOWLEDGEMENTS

First of all, I thank Allah for giving me strength and ability to complete this study. I would like to express my special appreciation and deep gratitude to Professor Dr. Arbakariya Ariff, who has supported me throughout my thesis with his patience and knowledge whilst allowing me the room to work in my own way. I attribute the level of my PhD degree to his encouragement, guidance and effort and without him this thesis, too, would not have been completed or written. One simply could not wish for a better supervisor and teacher.

I also would like to thank the members of my PhD committee, Associate Prof. Dr. Rosfarizan Mohamad, Professor Dr. Ling Tau Chuan and Dr. Siti Ramlah Ahmad Ali for their comments and help since the beginning of this study. I would like to thank MPOB and the management for giving me the opportunity and support to complete this study. I would also like to thank MPOB MICROTEC staff (Zamri, Aminshah and Adhni) for lending a hand during the research works.

I am greatly indebted an appreciation to my beloved wife, Sitti Rahma Abdul Hafid for her encouragement, support and sacrifices through out the study. To my lovely daughter, Yasmeen Zulaikha that inspired me to complete this thesis. Lastly, I am very grateful to my parents, Mohd. Masri bin Shamsuddin and Rusmenah binti Idris, for their love, prayers and sacrifices for preparing and educating me for my future. I would like to take this opportunity again to acknowledge and thank those who made this work possible.

I certify that a Thesis Examination Committee has met on (inset the data of vive voce) to conduct the final examination of (Mohamed Mazmira bin Mohd. Masri) on his thesis entitled “Production of Bacillus thuringiensis using different mode of fermentation” in accordance with the University and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

Suhaimi bin Mustapha, PhD

Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Umi Kalsom Md. Shah, PhD

Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Siti Mazlina binti Mustapha Kamal, PhD

Associate Professor
Faculty of Engineering
Universiti Putra Malaysia
(Internal Examiner)

Murat Elibol, PhD

Professor
Department of Bioengineering
Ege University
Turkey
(External Examiner)

SEOW HENG FONG, PhD

Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Arbakariya bin Ariff, PhD

Professor
Faculty of Biotechnology and Biomolecular Science
Universiti Putra Malaysia
(Chairman)

Rosfarizan Mohamad, PhD

Associate Professor
Faculty of Biotechnology and Biomolecular Science
Universiti Putra Malaysia
(Member)

Ling Tau Chuan, PhD

Professor
Institute of Biological Science
Faculty of Science
University of Malaya
(Member)

Siti Ramlah Ahmad Ali, PhD

Malaysian Palm Oil Board
(Member)

BUJANG BIN KIM HUAT, PhD

Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

MOHAMED MAZMIRA BIN MOHD MASRI

Date: 13 June 2013

TABLE OF CONTENTS

	Page
ABSTRACT	ii
ABSTRAK	v
ACKNOWLEDGEMENTS	ix
APPROVAL	x
DECLARATION	xii
LIST OF TABLES	xviii
LIST OF FIGURES	xx
LIST OF ABBREVIATIONS	xxiv
CHAPTER	
1 INTRODUCTION	1
1.1 Background	
1.2 Objectives	5
2 LITERATURE REVIEW	
2.1 The origin of <i>Bacillus thuringiensis</i> (Bt)	6
2.2 Development of Bt as commercial biopesticides	7
2.3 The search for novel Bt isolate	14
2.4 Classification of Bt subspecies	17
2.5 Bt Insecticidal Crystal Proteins (ICP)	18
2.6 Classification of <i>cry</i> genes	22
2.7 The mechanism of action against target insects	25
2.8 Industrial production of Bt as commercial biopesticides	27
2.8.1 Production processes and formulations	27
2.8.2 Nutritional requirement	28
2.8.3 Spores and crystals production via cultivation	30
2.8.4 Effect of physical parameters	33
2.9 Effects of using Bt as biopesticides on the environment	35
2.10 Effects of using Bt as biopesticides on human and animals	37
2.11 Bt as bioinsecticides in Malaysia: The best control for bagworm outbreak in oil palm plantation	43
2.12 Application of Bt biopesticides on other crops	47
2.13 Concluding remarks	48

3	GENERAL MATERIALS AND METHODS	
3.1	General methods and analysis	49
3.2	Experimental overview	49
3.2.1	Microorganisms and inoculum preparation	51
3.2.2	Analytical Procedures	51
3.2.2.1	Total Viable Cell Count	51
3.2.2.2	Spore Count	52
3.2.2.3	Microscopic observation	52
3.2.3	Medium preparation	52
3.2.4	SDS PAGE analyses	53
4	PREPARATION OF COMPETENT CRY+ <i>BACILLUS THURINGIENSIS</i> STOCK CULTURE FOR SUBSEQUENT USED IN CULTIVATION WITH CONSISTENT PRODUCTION OF δ-ENDOTOXIN	
4.1	Introduction	55
4.2	Materials and Methods	57
4.2.1	Microorganism and inoculum preparation	57
4.2.2	Isolation of Bt Cry+ strain	57
4.2.3	Preparation of culture in freeze dried form	60
4.2.4	Production of δ -endotoxin in 5 L stirred tank bioreactor	61
4.2.5	PCR analysis	62
4.2.6	Analytical procedures	63
4.3	Result	63
4.3.1	Isolation of Bt MPK13 Cry+ strain	63
4.3.2	Effect of different protective agents on the survival of Bt MPK13 cells before and after freeze drying	67
4.3.3	Effect of glucose in medium and DOT control strategies on the synthesis of δ -endotoxin by Bt MPK13 Cry+ strain	70
4.3.4	Effect of different stock culture preparations on δ -endotoxin production by Bt MPK13	75
4.4	Discussion	78
4.5	Conclusion	81
5	EFFECT OF DIFFERENT MONOSACCHARIDES AND DISACCHARIDES ON <i>BACILLUS THURINGIENSIS</i> CELL GROWTH, SPORULATION RATE AND δ-ENDOTOXIN SYNTHESIS	
5.1	Introduction	83
5.2	Materials and Methods	85
5.2.1	Microorganism and inoculum preparation	85
5.2.2	Medium and cultivation	85

5.2.3	Analytical procedures	86
5.2.4	Measurement of cell length	86
5.2.5	Kinetic data analysis	86
5.2.6	Determination of saccharides concentration	87
5.2.7	SDS-PAGE analysis	87
5.3	Results	
5.3.1	Time course of Bt MPK13 cultivation in different monosaccharides and disaccharides	88
5.3.2	Effect of different individual saccharides on growth and sporulation of Bt MPK13	90
5.3.3	Glucose residual after the cultivation	92
5.3.4	Mixing different saccharides with glucose and its effect on cell growth and sporulation of Bt MPK13	95
5.3.5	Effect of different saccharides on cell size and growth morphology of Bt	96
5.3.6	δ -endotoxin synthesis during the cultivation	99
5.4	Discussion	101
5.5	Conclusion	104
6	RELATIONSHIP BETWEEN TOTAL CARBON, TOTAL NITROGEN AND CARBON TO NITROGEN RATIO ON GROWTH, SPORULATION RATE AND δ-ENDOTOXIN SYNTHESIS OF <i>BACILLUS THURINGIENSIS</i>	
6.1	Introduction	105
6.2	Materials and Methods	108
6.2.1	Microorganism and inoculum preparation	108
6.2.2	Medium and cultivation	108
6.2.3	Analytical procedures	108
6.2.4	Preparation of Intracellular and Extracellular TC and TN samples	109
6.2.5	Determination of TC and TN	109
6.2.6	δ -endotoxin determination	109
6.3	Results	
6.3.1	Profile of Intracellular TC, TN and C/N ratio	110
6.3.2	Profile of Extracellular TC, TN and C/N ratio	112
6.3.3	Relationship between TC, TN and C/N ratio with cell growth, sporulation and δ -endotoxin production	115
6.4	Discussion	119
6.5	Conclusion	121

7	AERATION REQUIREMENT FOR ENHANCEMENT OF GROWTH OF <i>BACILLUS THURINGIENSIS</i> MPK13, SPORULATION RATE AND δ-ENDOTOXIN SYNTHESIS	
7.1	Introduction	122
7.2	Materials and Methods	124
7.2.1	Microorganism	124
7.2.2	Cultivation media	124
7.2.3	Cultivation using 5L stirred tank bioreactor	124
7.2.4	Analysis of gaseous exchange during cultivation	126
7.2.5	Analytical procedures	126
7.2.6	Kinetic data analysis	127
7.3	Results	
7.3.1	Time course of Bt MPK13 cultivation without DOT control	127
7.3.2	Effect of single-phase DOT control on growth of Bt MPK13, sporulation and δ -endotoxin synthesis	131
7.3.3	Effect of two-phase DOT control strategy on growth of Bt MPK13, sporulation and δ -endotoxin synthesis	134
7.3.4	Relationship between exhaust gas analysis and Bt cultivation performance	135
7.4	Discussion	139
7.5	Conclusion	142
8	INFLUENCE OF DIFFERENT MODES OF BIOREACTOR OPERATION ON <i>BACILLUS THURINGIENSIS</i> MPK13 GROWTH, SPORULATION RATE, δ-ENDOTOXIN SYNTHESIS AND TOXICITY	
8.1	Introduction	143
8.2	Materials and Methods	146
8.2.1	Microorganism	146
8.2.2	Medium formulation	146
8.2.3	Stirred Tank Bioreactor	147
8.2.4	Batch cultivation	147
8.2.5	Fed-batch cultivation	148
8.2.6	Continuous cultivation	150
8.2.7	Continuous cultivation model	152
8.2.8	Analytical procedures	153
8.2.9	Laboratory bioassay towards bagworm, <i>Metisa plana</i>	153
8.3	Results	154
8.3.1	Batch cultivation of Bt MPK 13	154
8.3.2	Fed-batch cultivation of <i>B. thuringiensis</i> MPK13	157
8.3.2.1	Feeding during lag growth phase	157
8.3.2.2	Feeding during exponential growth phase	159
8.3.2.3	Feeding during stationary growth phase	160
8.3.2.4	Intermittent feeding during log and stationary growth phase	160

8.3.3	Fed-batch with optimal DOT control strategy	162
8.3.4	Continuous cultivation of Bt MPK13	164
8.3.5	Comparison of cultivation performance in different modes of bioreactor operation	167
8.3.6	Synthesis of δ -endotoxin	169
8.3.7	Toxicity against <i>Metisa plana</i>	173
8.4	Discussion	174
8.5	Conclusion	178
9	CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH	179
	REFERENCES/BIBLIOGRAPHY	184
	APPENDICES	219
	BIODATA OF STUDENT	222
	LIST OF PUBLICATIONS	223