

**IMMOBILIZATION OF LIPASE FROM *CANDIDA RUGOSA* ONTO
SELECTED MATRICES FOR USE IN ENANTIOSELECTIVE
PREPARATION OF (-)-MENTHYL BUTYRATE**

SITI SALHAH BT. OTHMAN

**DOCTOR OF PHILOSOPHY
UNIVERSITI PUTRA MALAYSIA**

2004

**IMMOBILIZATION OF LIPASE FROM *CANDIDA RUGOSA* ONTO
SELECTED MATRICES FOR USE IN ENANTIOSELECTIVE
PREPARATION OF (-)-MENTHYL BUTYRATE**

By

SITI SALHAH BT. OTHMAN

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

July 2004

ACKNOWLEDGEMENTS

Alhamdulillah, praises to Allah s.w.t., for giving me the strength to endure all challenges and complete this study.

My sincere appreciation goes out to Prof. Dr. Mahiran Basri, Prof. Dr. Abu Bakar Salleh, Prof. Dr. Mohd. Zobir Hussein, Assoc. Prof. Dr. Mohd. Basyaruddin Abd. Rahman and Assoc. Prof. Dr. Raja Noor Zaliha Abd. Rahman of the Enzyme and Microbial Technology Research group for their great concern, advices, patience, persistence encouragement and invaluable assistance from the beginning till the end of this study.

A special thanks to Assoc. Prof. Dr. Fauziah Othman, Ms. Azilah Abd. Jalil and members of the Microscopy and Microanalysis Unit, Institute of Bioscience, whose help and cooperation were invaluable during the course of the study.

Love and thanks to Mama, Abah, Abg. Syukri, Kak CT and An for their love and constant supports. Not forgetting, my beloved husband, Ramli Ahmad and our children, Umairah and Balqis, for simply being there and loving me with all their hearts.

A special thanks also goes out to Kak Nisha, Rozainita, Salina, Yamin, Yati and other members of Lab 401 and Lab 405, without whom life would be a bore and uneventful. Thank you for the bond of friendship and for making my

stay in UPM a bearable one with many sweet memories and experiences.

Thank you for being friends in need.

Thank you also to Kak Hal for her valuable guidance and assistance throughout the period of my study.

Finally, thank you to the Ministry of Science, Technology and Environment, and Universiti Putra Malaysia for their financial support and facilities.

I certify that an Examination Committee met on 5th July 2004 to conduct the final examination of Siti Salhah Bt. Othman on her Doctor of Philosophy thesis entitled “Immobilization of Lipase from *Candida rugosa* onto Selected Matrices for Use in Enantioselective Preparation of (-)-Menthyl butyrate” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

MOHD. ASPOLLAH SUKARI, Ph.D.

Associate Professor
Faculty of Science
Universiti Putra Malaysia
(Chairman)

MAWARDI RAHMANI, Ph.D.

Professor
Faculty of Science
Universiti Putra Malaysia
(Member)

FAUJAN AHMAD, Ph.D.

Associate Professor
Faculty of Science
Universiti Putra Malaysia
(Member)

IBRAHIM CHE OMAR, Ph.D.

Professor
School of Biological Sciences
Universiti Sains Malaysia
(Independent Examiner)

GULAM RUSUL RAHMAT ALI, Ph.D.

Professor/Deputy Dean
School of Graduate Studies,
Universiti Putra Malaysia

Date: 02 NOV 2004

This thesis 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 are as follows:

MAHIRAN BASRI, Ph.D.

Professor
Faculty of Science
Universiti Putra Malaysia
(Chairperson)

MOHD. ZOBIR HUSSEIN, Ph.D.

Professor
Institute of Advanced Technology
Universiti Putra Malaysia
(Member)

ABU BAKAR SALLEH, Ph.D.

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

MOHD. BASYARUDDIN ABD. RAHMAN, Ph.D.

Associate Professor
Faculty of Science
Universiti Putra Malaysia
(Member)

AINI IDERIS, Ph.D.

Professor/Dean
School of Graduate Studies,
Universiti Putra Malaysia

Date:

DECLARATION

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

SITI SALHAH BT. OTHMAN

Date:

TABLE OF CONTENTS

	Page
ABSTRACT	ii
ABSTRAK	v
ACKNOWLEDGEMENTS	ix
APPROVAL	xi
DECLARATION	xiii
LIST OF TABLES	xvii
LIST OF FIGURES	xix
LIST OF SCHEMES	xxii
LIST OF ABBREVIATIONS	xxiii
CHAPTER	
1 INTRODUCTION	1
1.1 Research Scope	5
1.2 Research Objectives	6
2 LITERATURE REVIEW	7
2.1 Enzymes as Catalyst	7
2.2 Lipases	9
2.3 Sources of Lipase	11
2.3.1 Animal Lipases	11
2.3.2 Plant Lipases	12
2.3.3 Microbial Lipases	13
2.3.4 Lipase from <i>Candida rugosa</i>	14
2.4 Unique Properties of Lipases for Biotransformation	17
2.4.1 Enantioselectivity	17
2.4.2 Positional Selectivity	19
2.4.3 Substrate Specificity	21
2.5 Mechanism of Action of Lipases	22
2.5.1 The Active Site of Lipases	22
2.5.2 The Tunnel in <i>Candida rugosa</i> Lipase	23
2.5.3 The Ping Pong Bi Bi Reaction Mechanism	27
2.6 Enantiomerically Pure Products as Important Compounds	30
2.6.1 Application of Lipase Enantioselectivity	32
2.6.2 Enantioselective Enzymatic Synthesis of Menthyl Esters	35
2.6.3 Measure of Enantioselectivity	39
2.7 Factors Influencing Lipase-Catalyzed Reactions in Organic Solvent	41
2.7.1 Selection of Organic Solvent	41
2.7.2 Control of Water Activity (A_w)	44
2.7.3 Influence of Temperature	46
2.7.4 The Lipase Structure	47

2.8	Immobilization Techniques	49
2.8.1	Nature of Immobilization Supports	51
2.8.2	Amberlite XAD7	57
2.8.3	Eupergit C and C 250 L	57
2.8.4	Hydrotalcite	59
3	MATERIALS AND METHODS	61
3.1	Materials	61
3.1.1	Sources of Enzymes and Proteins	61
3.1.2	Supports for Immobilization	62
3.1.3	Chemicals for Synthesis of Mg/Al-Hydrotalcite	62
3.1.4	Chemicals for Protein Assay	62
3.1.5	Substrates and Internal Standard	63
3.1.6	Solvents	63
3.1.7	Salts for Initial Water Activity (A_w) Study	63
3.1.8	Instrumentations	64
3.2	Methods	65
3.2.1	Preparation of Lipase Solution	65
3.2.2	Synthesis and Characterization of Mg/Al-Hydrotalcite	65
3.2.3	Immobilization of Lipase	67
3.2.4	Protein Assay	68
3.2.5	Enzymatic Esterification	68
3.2.6	Enantioselective Analysis of Menthyl Esters by Chiral Capillary Gas Chromatography (GC)	69
3.2.7	Preparation of Standard Menthyl Esters	71
3.3	Optimization of the Enantioselective Esterification Reaction	72
3.3.1	Time Course Study	72
3.3.2	Effect of Incubation Temperature	72
3.3.3	Effect of Various Organic Solvents	73
3.3.4	Effect of Initial Water Activity (a_w)	73
3.4	Stability of Immobilized <i>Candida rugosa</i> Lipase	76
3.4.1	Thermostability	76
3.4.2	Storage Stability	76
3.4.3	Stability in Organic Solvent	77
3.4.4	Operational Stability	77
4	RESULTS AND DISCUSSION	79
4.1	Screening of Enzymes	79
4.2	Characterization of Mg/Al-Hydrotalcite	86
4.3	Immobilization of Lipase	94
4.4	Morphology Analysis of Lipase and Supports	106
4.5	Analysis of Products	113
4.6	Optimization of the Enantioselective Esterification Reaction	119
4.6.1	Effect of Reaction Time	119
4.6.2	Effect of Incubation Temperature	124

4.6.3	Effect of Various Organic Solvents	131
4.6.4	Effect of Initial Water Activity (a_w)	138
4.7	Stability of Immobilized <i>Candida rugosa</i> Lipase	147
4.7.1	Thermostability	147
4.7.2	Storage Stability	154
4.7.3	Stability in Organic Solvent	159
4.7.4	Operational Stability	166
5	CONCLUSION	170
5.1	Recommendation for Further Studies	174
	BIBLIOGRAPHY	176
	APPENDICES	190
	BIODATA OF THE AUTHOR	201

