



UNIVERSITI PUTRA MALAYSIA

***FERMENTATION STRATEGIES FOR PRODUCTION OF HYALURONIC ACID
THROUGH BIOSYNTHESIS BY *Streptococcus zooepidemicus* AND
RECOMBINANT *Esherichia coli* USING STIRRED TANK BIOREACTOR***

LAI ZEE WEI

FBSB 2013 7



**FERMENTATION STRATEGIES FOR PRODUCTION OF HYALURONIC
ACID THROUGH BIOSYNTHESIS BY *Streptococcus zooepidemicus* AND
RECOMBINANT *Esheria coli* USING STIRRED TANK BIOREACTOR**

By

LAI ZEE WEI

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

January 2013

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Doctor of Philosophy

FERMENTATION STRATEGIES FOR PRODUCTION OF HYALURONIC ACID THROUGH BIOSYNTHESIS BY *Streptococcus zooepidemicus* AND RECOMBINANT *Esherichia coli* USING STIRRED TANK BIOREACTOR

By

LAI ZEE WEI

January 2013

Chairman : Associate Professor Rosfarizan Mohamad, PhD

Faculty : Biotechnology and Biomolecular Sciences

Hyaluronic acid (HA) is a high molecular mass, composed of D-glucuronic acid and N-acetyl glucosamine residues linked by β -1-3 and β -1-4 glycosidic bonds. It is a high value biopolymer due to its biological functions and unique physicochemical properties. It has wide variety applications in biomedical, healthcare and cosmetic field. The HA from rooster comb for human therapeutics carries the risk of cross-species viral infection; thus, microbial fermentation is gradually replacing extraction as the preferred source of HA which has the advantages of low production costs and more efficient purification.

In the attempt to achieve effective HA biosynthesis by a microorganism, optimization of medium formulation, development of suitable process strategies and strain improvement are required. In the present study, the experimental data from batch fermentation was analysed in order to form the basis for a kinetic model of the

process. Unstructured kinetic model based on Logistic and Luedeking-piret equations was found suitable to describe the growth, substrate consumption and HA biosynthesis by *S. zooepidemicus* ATCC 39920 in batch fermentation using glucose as a carbon source. From the modelling, it was found that the HA biosynthesis by *S. zooepidemicus* ATCC 39920 was a growth-associated process. The present study was also undertaken to investigate the culture conditions and specific nutritional requirements for the growth and high molecular weight of HA biosynthesis using a stirred-tank bioreactor. Different agitation speeds (200-600 rev/min) were initially investigated using Rushton turbine impeller. The effects of glucose (20, 30, 50 and 60 g/L), nitrogen sources ((NH₄)₂S₂O₈, (NH₄)₂PO₄, yeast extract, and tryptone) and carbon/nitrogen (C/N) ratio on the growth of the strain and on HA biosynthesis were investigated. The HA productivity and molecular weight exhibited by the strain in batch fermentations using Rushton turbine and helical ribbon impellers were compared. The potential use of *n*-dodecane and *n*-hexadecane as oxygen vectors for enhancing HA biosynthesis by *S. zooepidemicus* ATCC 39920 was also investigated using a 2-L stirred-tank bioreactor equipped with helical ribbon or Rushton turbine impellers.

The optimum agitation speed for the HA biosynthesis (0.587 g/L) was obtained at 300 rev/min. Increasing the agitation speed would increase the oxygen transfer rate.

The organic nitrogen sources (yeast extract and tryptone) were proven to be favourable used in the medium for HA biosynthesis compared to inorganic nitrogen sources. About 2.44 g/L of HA with a high molecular weight (4.36×10^6 Da) was synthesised at an optimal C/N of 5:1 (using a mixture of yeast extract and tryptone) in the bioreactor equipped with a Rushton turbine impeller. Helical ribbon impeller

showed efficient mixing in a non-Newtonian HA broth. It was able to improve the HA molecular weight from 4.36×10^6 Da to 5.20×10^6 Da, even though the HA concentrations obtained are almost the same at fixed impeller tip speed (0.785 m/s) using both impellers. Batch HA fermentation with 1% (v/v) *n*-dodecane or 0.5% (v/v) *n*-hexadecane addition was carried out at different impeller tip speeds. The maximum HA concentration (4.25 g/L) and molecular weight (1.54×10^7 Da) were obtained when 0.5% (v/v) *n*-hexadecane and 0.785 m/s impeller tip speed of helical ribbon were used.

On the other hand, biosynthesis of HA by recombinant *Escherichia coli* ROSETTA (DE3) harbouring *has* genes (*B*, *C* and *D*) from *S. zooepidemicus* ATCC 39920 previously developed in our laboratory was also investigated in batch and fed-batch fermentations. The maximum HA concentration produced by *E. coli* ROSETTA (DE3) was increased by about 16% in the stirred-tank bioreactor (127.00 mg/L) with a controlled dissolved oxygen tension at 30% air saturation *via* cascade control of airflow rate and agitation speed when compared with the shake-flask fermentation. The fed-batch fermentation with constant feeding (2 mL/min) of 10 g/L glucose was not improved neither biosynthesis nor HA molecular weight. Nevertheless, the HA molecular weight was increased by about 42% in the bioreactor experiment compared to shake-flask fermentation. Generally, the HA biosynthesis by *S. zooepidemicus* ATCC 39920 and *E. coli* ROSETTA (DE3) applying an optimal process control strategy of 2-L stirred-tank bioreactor was improved by 487.83% and 46.31%, respectively when compared with the shake-flask experiment of non-optimal condition.

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

**STRATEGI FERMENTASI UNTUK PENGHASILAN MELALUI
BIOSINTESIS ASID HIALURONIK OLEH *Streptococcus zooepidemicus* DAN
REKOMBINAN *Esherichia coli* MENGGUNAKAN BIOREAKTOR TANGKI
BERPENGADUK**

Oleh

LAI ZEE WEI

Januari 2013

Pengerusi : Profesor Madya Rosfarizan Mohamad, PhD

Fakulti : Bioteknologi dan Sains Biomolekul

Asid hialuronik (HA) adalah jisim molekul yang tinggi, terdiri daripada asid D-glukuronik dan sisa N-acetyl glukosamina yang dihubungkan oleh ikatan β -1-3 dan β -1-4 glykosidik. Ia adalah biopolimer bernilai tinggi kerana fungsi biologi dan sifat fizik dan unik. Ia mempunyai pelbagai aplikasi dalam bioperubatan, penjagaan kesihatan dan bidang kosmetik. HA daripada balung ayam untuk terapeutik manusia membawa risiko rentas-spesies jangkitan virus, dengan itu, penapaian mikrob secara beransur-ansur menggantikan pengekstrakan sebagai sumber pilihan HA yang mempunyai kelebihan dari segi kos pengeluaran yang rendah dan penulenan yang lebih cekap.

Dalam usaha untuk mencapai biosintesis HA yang berkesan oleh mikroorganisma, formulasi medium optima dan pembangunan proses strategi yang

sesuai adalah diperlukan. Dalam kajian ini, data eksperimen dari fermentasi sesekelompok telah dianalisis untuk membentuk asas bagi model kinetik proses. Model tidak berstruktur kinetik berdasarkan persamaan Logistik dan Luedeking-piret telah didapati sesuai untuk menggambarkan pertumbuhan, penggunaan substrat dan biosintesis HA oleh *S. zooepidemicus* ATCC 39920 dengan fermentasi sesekelompok menggunakan glukosa sebagai sumber karbon. Daripada permodelan, didapati bahawa biosintesis HA oleh *S. zooepidemicus* ATCC 39920 adalah satu proses yang berkaitan dengan pertumbuhan. Kajian ini juga telah dijalankan untuk mengkaji keadaan kultur dan keperluan pemakanan khusus bagi pertumbuhan dan berat molekul yang tinggi bagi biosintesis HA dengan menggunakan bioreaktor tangki berpengaduk 2-L. Pada mulanya, kelajuan pengadukan yang berbeza (200-600 pusingan/min) telah dikaji menggunakan pengaduk jenis “Rushton”. Kesan glukosa (20, 30, 50 dan 60 g/L), sumber nitrogen ((NH₄)₂S₂O₈, (NH₄)₂PO₄, ekstrak yis, dan tripton) dan nisbah karbon/nitrogen (C/N) terhadap pertumbuhan mikroorganisma dan biosintesis HA telah dikaji. Produktiviti dan berat molekul HA yang dihasilkan oleh strain dalam fermentasi sesekelompok yang menggunakan pengaduk Rushton dan pengaduk reben berlingkar pada bioreaktor tangki berpengaduk 2-L juga telah dibandingkan. Potensi penggunaan *n*-dodecane dan *n*-hexadecane sebagai vektor oksigen untuk meningkatkan biosintesis HA oleh *S. zooepidemicus* ATCC 39920 juga telah dikaji dengan menggunakan bioreaktor 2-L yang dilengkapi dengan pengaduk reben berlingkar atau Rushton.

Kelajuan pengadukan optimum untuk biosintesis HA (0.587 g/L) telah diperoleh pada 300 pusingan/min. Peningkatan kelajuan pengadukan akan meningkatkan kadar pemindahan oksigen. Sumber-sumber nitrogen organik (ekstrak yis dan tryptone) telah terbukti baik digunakan dalam medium untuk biosintesis HA berbanding

dengan sumber nitrogen bukan organik. Kira-kira 2.44 g/L HA dengan berat molekul yang tinggi (4.36×10^6 Da) telah disintesis pada C/N optimal 5:1 (menggunakan campuran ekstrak yis dan tryptone) dalam bioreactor tangki berpengaduk 2-L yang dilengkapi dengan pengaduk Rushton. Pengaduk reben berlingkar menunjukkan percampuran berkesan dalam air jenis bukan-Newtonian. Ia dapat meningkatkan berat molekul HA daripada 4.36×10^6 Da kepada 5.20×10^6 Da, walaupun kepekatan HA yang diperoleh adalah hampir sama pada kelajuan hujung pengaduk yang sama (0.785 m/s) dengan menggunakan kedua-dua pengaduk. Pecahan isipadu vektor oksigen adalah positif mempengaruhi koefisi volumetric pemindahan oksigen gas-cecair. Fermentasi sesekelompok HA dengan 1% (v/v) *n*-dodecane atau 0.5% (v/v) *n*-hexadecane telah dijalankan pada kelajuan hujung pengaduk yang berlainan. Kepekatan HA (4.25 g/L) dan berat molekul tertinggi (1.54×10^7 Da) telah berjaya diperoleh apabila 0.5% (v/v) *n*-hexadecane dan 0.785 m/s kelajuan hujung pengaduk dengan menggunakan pengaduk reben berlingkar.

Selain itu, biosintesis HA oleh *Escherichia coli* ROSETTA (DE3) rekombinan yang mempunyai gen *has* (*B*, *C* dan *D*) dari *S. zooepidemicus* ATCC 39920 yang sebelumnya dibina di dalam makmal kami juga telah dikaji dalam fermentasi sesekelompok dan fermentasi suapan sesekelompok. Kepekatan HA maksimum yang dihasilkan oleh *E. coli* ROSETTA (DE3) telah meningkat sebanyak 16% dalam bioreactor tangki pengaduk (127.00 mg/L) dengan kawalan tekanan oksigen terlarut pada ketepuan udara 30% melalui kawalan “cascade” kadar pengaliran udara dan kelajuan pengaduk berbanding dengan fermentasi kelalang bergoncang. Fermentasi suapan sesekelompok dengan suapan tetap (2 mL/min) 10 g/L glukosa tidak meningkatkan biosintesis HA mahupun berat molekulnya. Walaubagaimanapun, berat molekul HA telah meningkat sebanyak 42% dalam eksperimen bioreactor

berbanding fermentasi di dalam kelalang bergoncang. Secara umumnya, biosintesis HA oleh *S. zooepidemicus* ATCC 39920 dan *E. coli* ROSETTA (DE3) dengan aplikasi kawalan strategi proses yang optima menggunakan bioreaktor tangki berpengaduk 2-L telah meningkat sebanyak 487.83% dan 46.31%, masing-masing berbanding eksperimen di dalam kelalang bergoncang menggunakan keadaan yang tidak optima.



ACKNOWLEDGEMENTS

The successful completion of this thesis would not had seen the light if it wasn't supported by the continuous motivation, constructive guidance and a little bit of coaxing by the ever patient Assoc. Prof. Dr. Rosfarizan Mohamad as my main supervisor throughout the course of this study. I sincerely express my deepest gratitude and appreciation to Assoc. Prof. Dr. Rosfarizan Mohamad for her invaluable guidance, constant encouragement and constructive suggestions throughout my study. My appreciations and gratitude also goes to the members of my supervisory committee, Professor Dr Arbakariya Ariff and Professor Dr Raha Abdul Rahim for their support, encouragement and willingness to share their views throughout the project.

Heartfelt appreciation is also due to all faculty members of FBSB, laboratory staffs and fellow graduate students of Fermentation Technology Unit for their kind co-operation and assistance during the period of this study. Special thanks to my friends: Yee Lian Ngit; thank you for your moral support and practical handyman tips and approaches during the study; and Wong Teck Sec for her willingness and sacrifices during the period of this study.

Special appreciation and deepest gratitude is extended to my family for their love, patient, caring, understanding and support. Acknowledgement is also due to those who are involved directly or indirectly in the completion of this study. Last but not least, to Kelvin How, thank you for sharing my bitterness and happiness throughout my study and loving me.

I certify that a Thesis Examination Committee has met on 10 January 2013 to conduct the final examination of Lai Zee Wei on her Ph.D thesis entitled “Fermentation strategies for production of hyaluronic acid biosynthesis by *Streptococcus zooepidemicus* and recombinant *Esherichia coli* using stirred-tank bioreactor” in accordance with Universities 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:

Mohd Yunus Abd Shukor, Ph.D

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

Luqman Chuah Abdullah, Ph.D

Professor
Faculty of Engineering
Universiti Putra Malaysia
(Internal Examiner)

Umi Kalsom binti Md Shah, Ph.D

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

Ian Maddox, Ph.D

Professor
School of Engineering & Advanced Technology
Massey University
(External Examiner)

SEOW HENG FONG, Ph.D

Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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

Rosfarizan Mohamad, PhD

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

Arbakariya Ariff, PhD

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

Raha Abdul Rahim, PhD

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

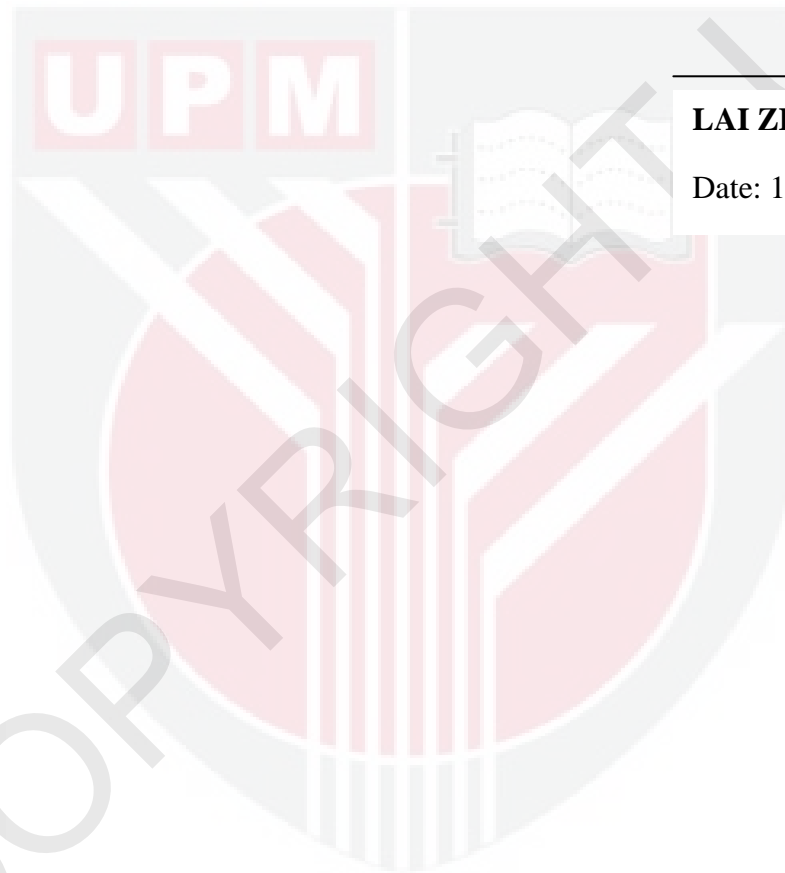
BUJANG BIN KIM HUAT, PhD

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 is not concurrently, submitted for any other degree at Universiti Putra Malaysia or other institution.



LAI ZEE WEI

Date: 10th January 2013

TABLE OF CONTENTS

	Page
ABSTRACT	ii
ABSTRAK	v
ACKNOWLEDGEMENTS	ix
APPROVAL	x
DECLARATION	xii
LIST OF TABLES	xvii
LIST OF FIGURES	xix
LIST OF ABBREVIATIONS	xxi
CHAPTER	
1. INTRODUCTION	1
2. LITERATURE REVIEW	7
2.1 Microbial Polysaccharides	7
2.1.1 Extracellular Polysaccharides	8
2.1.2 Functions of Extracellular Polysaccharides	10
2.1.3 Hyaluronic Acid	11
2.1.3.1 History	11
2.1.3.2 Structure	12
2.1.3.3 Sources	13
2.1.3.5 Isolation and Purification	15
2.1.3.6 HA in Human Body	18
2.1.3.7 Biosynthesis Pathway	19
2.1.3.8 Applications	22
2.1.4 Hyaluronan Synthase (HAS)	27
2.2 Hyaluronic Acid Producers	28
2.2.1 <i>Streptococcus spp.</i>	28
2.2.1.1 <i>Streptococcus zooepidemicus</i>	29
2.2.2 Recombinant Microorganism	30
2.2.2.1 <i>Escherichia coli</i>	30
2.2.2.2 <i>Bacillus subtilis</i>	31
2.2.2.3 <i>Lactococcus lactis</i>	32
2.3 Production of HA via Fermentation Process	32
2.3.1 Medium and Culture Conditions Optimization	32
2.3.1.1 Carbon Source	33
2.3.1.2 Nitrogen Source	34
2.3.1.3 Agitation and Aeration	36
2.3.1.4 Oxygen Role in HA Production	37
2.3.2 Oxygen Vector	38
2.3.2.1 Perfluorocarbon	40
2.3.2.2 Hydrocarbon	41
2.3.3 Mode of Bioreactor Operation	44
2.3.3.1 Batch Culture	46
2.3.2.2 Fed-Batch Culture	46

2.3.4	Impeller Design and Classification	49
2.3.4.1	Remote Clearance Impeller	49
2.3.4.2	Close Clearance Impeller`	50
2.3.5	Helical Ribbon Impeller	50
2.3.6	Flow Pattern	52
2.3.6.1	Fluid Flow and Mixing	52
2.4	Concluding Remarks	54
3.	GENERAL MATERIALS AND METHODS	56
3.1	Microorganism	56
3.2	Inoculum Preparation	56
3.3	Medium Composition	57
3.4	Stirred Tank Bioreactor	57
3.5	General Experimental Work	60
3.6	Analytical Assay	62
3.6.1	Cell Concentration	62
3.6.2	Reducing Sugar Concentration	63
3.6.3	Hyaluronic Acid Determination	63
3.6.4	Molecular weight of Hyaluronic Acid	64
4.	KINETICS AND MODELLING OF HA BIOSYNTHESIS BY <i>STREPTOCOCCUS ZOOEPIDEMICUS</i> ATCC 39920	65
4.1	Introduction	65
4.2	Materials and Methods	67
4.2.1	Microorganism and Medium	67
4.2.2	Fermentation	67
4.2.3	Analytical Methods	68
4.2.4	General Balance Equation	68
4.2.5	Kinetic Models	69
4.2.6	Mathematical Method	73
4.3	Results and Discussion	74
4.3.1	Kinetic Analysis of Microbial Growth	74
4.3.2	Kinetic Analysis of HA Biosynthesis	75
4.3.3	Kinetic Analysis of Substrate Consumption	76
4.4	Conclusion	81
5.	INFLUENCE OF AGITATION SPEED ON HA BIOSYNTHESIS BY <i>STREPTOCOCCUS ZOOEPIDEMICUS</i> ATCC 39920 IN 2-L STIRRED TANK BIOREACTOR	82
5.1	Introduction	82
5.2	Materials and Methods	83
5.2.1	Microorganism and Medium	83
5.2.2	Fermentations	83
5.2.3	Analytical methods	84
5.3	Results and Discussion	84
5.3.1	Effect of Agitation Speed on Cell Growth and HA Biosynthesis	84
5.3.2	Effect of Two-Stages Agitation Speed Control on HA Biosynthesis	89

5.4	Conclusion	93
6.	INFLUENCE OF MEDIUM FORMULATION ON HA BIOSYNTHESIS USING <i>STREPTOCOCCUS ZOOEPIDEMICUS</i> ATCC 39920 USING 2-L STIRRED TANK BIOREACTOR	
6.1	Introduction	94
6.2	Materials and Methods	95
6.2.1	Microorganism and Medium	95
6.2.2	Fermentations	96
6.2.3	Analytical Methods	97
6.3	Results and Discussion	97
6.3.1	Effect of Glucose Concentration	97
6.3.2	Effect of Nitrogen Source	100
6.3.3	Effect of C/N Ratio	105
6.3.4	Influence of Medium Formulation on HA Molecular Weight	109
6.4	Conclusion	111
7.	INFLUENCE OF IMPELLER DESIGN ON HA BIOSYNTHESIS BY <i>STREPTOCOCCUS ZOOEPIDEMICUS</i> ATCC 39920 USING 2L STIRRED TANK BIOREACTOR	
7.1	Introduction	112
7.2	Materials and Methods	114
7.2.1	Microorganism and Medium	114
7.2.2	Fermentations	114
7.2.3	Analytical Methods	115
7.2.4	K_{La} Measurement	116
7.3	Results and Discussion	117
7.3.1	Effect of Agitation Speed and Impeller Configuration on K_{La}	117
7.3.2	Influence of Impeller Design on HA Biosynthesis	119
7.3.3	Influence of Impeller Design on HA Molecular Weight	125
7.4	Conclusion	126
8.	INFLUENCE OF OXYGEN VECTOR AND IMPELLER TIP SPEED OF HELICAL RIBBON IMPELLER ON HA BIOSYNTHESIS BY <i>Streptococcus zooepidemicus</i> ATCC 39920 USING 2-L STIRRED TANK BIOREACTOR	
8.1	Introduction	127
8.2	Materials and Methods	129
8.2.1	Microorganism and Medium	129
8.2.2	Fermentations	130
8.2.3	K_{La} Measurement	130
8.2.4	Analytical Methods	130
8.2.5	Broth Rheology	131
8.3	Results and Discussion	131
8.3.1	Effects of Oxygen Vectors on K_{La} using Different Type Impellers	131
8.3.2	Influence of Oxygen Vectors and Impeller	135

	Design on Broth Rheology	
8.3.3	Influence of <i>n</i> -dodecane and Helical Ribbon Impeller on HA Biosynthesis	137
8.3.4	Influence of <i>n</i> -hexadecane and Helical Ribbon Impeller on HA Biosynthesis	142
8.3.5	Influence of Oxygen Vectors and Helical Ribbon Impeller on HA Molecular Weight	146
8.4	Conclusion	147
9.	COMPARISON OF HA BIOSYNTHESIS BY <i>Escherichia coli</i> ROSETTA STRAINS IN DIFFERENT MODE OF BIOREACTOR OPERATION	
9.1	Introduction	148
9.2	Materials and Methods	150
9.2.1	Microorganism and Medium	150
9.2.2	Fermentation in Shake-flask	151
9.2.3	Fermentation in 2-L Stirred tank Bioreactor	152
9.2.4	Analytical Methods	153
9.3	Results and Discussion	154
9.3.1	Fermentation in Shake-flask	154
9.3.2	Fermentation in 2-L Stirred-tank Bioreactor	163
9.4	Conclusion	172
10.	DISCUSSION, CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK	173
	REFERENCES	179
	APPENDICS	195
	BIODATA OF STUDENT	206
	LIST OF PUBLICATIONS	207