



UNIVERSITI PUTRA MALAYSIA

**EFFECT OF PHOSPHOGLUCOISOMERASE (*hasE*) AND HYALURONAN
SYNTHASE (*hasA*) CO-EXPRESSION ON HYALURONIC ACID
PRODUCTION IN *ESCHERICHIA COLI* ROSETTA (DE3)**

MUHAMMAD AZMI BIN SAMSUDIN

FBSB 2013 13



UPM
UNIVERSITI PUTRA MALAYSIA
BERILMU BERBAKTI

**EFFECT OF PHOSPHOGLUCOISOMERASE (*hasE*) AND HYALURONAN
SYNTHASE (*hasA*) CO-EXPRESSION ON HYALURONIC ACID
PRODUCTION IN *ESCHERICHIA COLI* ROSETTA (DE3)**

By

MUHAMMAD AZMI BIN SAMSUDIN

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfillment of the Requirement for the Degree of Master of Science**

October 2013

COPYRIGHT

All material contained within the thesis, including without limitation text, logos, icons, photographs and all other work, is copyright material of Universiti Putra Malaysia unless otherwise stated. Use may be made of any material contained within the thesis for non-commercial purposes from the copyright holder. Commercial use of material may only be made with the express, prior, written permission of Universiti Putra Malaysia.

Copyright © Universiti Putra Malaysia



**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfillment of the Requirement for the Degree of Master of Science**

October 2013

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

**EFFECT OF PHOSPHOGLUCOISOMERASE (*hasE*) AND HYALURONAN
SYNTHASE (*hasA*) CO-EXPRESSION ON HYALURONIC ACID
PRODUCTION IN *ESCHERICHIA COLI* ROSETTA (DE3)**

By

MUHAMMAD AZMI BIN SAMSUDIN

October 2013

Chairman: Raha Abdul Rahim, Ph.D

Faculty: Biotechnology and Biomolecular Sciences

Hyaluronic acid (HA) is one of nature's most versatile and fascinating materials due to its unique behavior and characteristic. This substance has a wide usage in clinical sector and plays a very important role in physiological and cell biological functions. Previously, the main source of this polysaccharide was from animal, but due to ethical issues and viral contamination, bacteria has emerged as an alternative resource for HA. The utilization of bacteria to produce HA is further improved by using heterologous recombinant host such as *Escherichia coli*. This study was aimed to enhance the production and molecular weight of HA in a new recombinant *E. coli* host that posed

useful industrial characteristic. Productions of hyaluronic acid were performed by expression of hyaluronan synthase (*hasA*) and phosphoglucoisomerase (*hasE*) genes in selected expression vectors into *E. coli* Rosetta (DE3) expression host. In this study, genomic DNA was isolated from *Streptococcus zooepidemicus* ATCC 39920. The *hasA* and *hasE* genes were successfully PCR amplified using primers derived from NCBI GeneBank database. The fragments were then cloned into TOP 10 *E. coli* cloning vector. Sequencing results showed that both genes cloned were 100% identical to the published sequences. The inserts from the TOP 10 clones were sub-cloned into two different expression vectors, pRSF-DUET and pCDF. The *hasA* was cloned into pRSF-DUET, while *hasE* gene was cloned into both pRSF-DUET and pCDF producing pRSF-DUET-A, pRSF-DUET-AE and pCDF-E. All clones were transformed into *E. coli* Rosetta (DE3).

Four recombinant clones, *E. coli* Clone A, AE, A-E and AE-E were produced by transforming the recombinant plasmids into the expression host. All clones successfully expressed the recombinant proteins corresponding to the expected sizes of ~42 kDa and ~48 kDa. Both recombinant proteins were confirmed by western blotting using monoclonal anti-His and anti-S antibody. Batch cultivation of all clones in shake-flask showed a rapid decreased in cell growth after induction with Isopropyl β -D-1-thiogalactopyranoside (IPTG). The highest concentration and molecular weight of HA obtained were from clone AE-E at 30°C with the concentration of 0.072 g/L and a molecular weight of 1.1×10^5 Da.

Abstrak tesis yang dikemukakan kepada senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

**KESAN KO-EKSPRESAN GEN PHOSPHOGLUCOISOMERASE (*hasE*) DAN
HYALURONAN SYNTHASE (*hasA*) KE ATAS PRODUKSI ASID
HIALURONIK DI DALAM *ESCHERICHIA COLI* ROSETTA (DE3)**

Oleh

MUHAMMAD AZMI BIN SAMSUDIN

Oktober 2013

Pengerusi: Raha Abdul Rahim, Ph.D

Fakulti: Bioteknologi dan Sains Biomolekul

Asid hialuronik (HA) adalah merupakan bahan semula jadi yang serba guna dan menarik disebabkan oleh sifat dan ciri-cirinya yang istimewa. Bahan ini mempunyai pelbagai kegunaan dalam bidang klinikal dan mempunyai peranan penting dalam fungsi fisiologikal dan biologikal sel. Sebelum ini, sumber utama bagi polisakarida ini adalah dari haiwan. Walau bagaimanapun, isu-isu etika dan pencemaran virus menyebabkan bakteria kini menjadi pilihan sumber alternatif HA. Kegunaan bakteria sebagai sumber kepada HA ditambah baik lagi dengan penggunaan perumah rekombinan heterologos seperti *Escherichia coli*. Kajian ini bertujuan untuk meningkatkan penghasilan dan berat molekul asid hialuronik di dalam *E. coli* rekombinan baru yang berpotensi. Penghasilan HA dilakukan dengan menzahirkan gen hyaluronan synthase (*hasA*) dan gen phosphoglucoisomerase (*hasE*) melalui vektor plasmid zahiran yang sesuai di dalam perumah *E.coli* Rosetta (DE3). Di dalam penyelidikan ini, DNA genomik bakteria

Streptococcus zooepidemicus ATCC 39920 telah dipencilkan. Gen *hasA* dan *hasE* telah berjaya diamplifikasi menggunakan primer yang direka menggunakan jujukan nukleotida di GeneBank, NCBI.

Fragmen-fragmen tersebut kemudian diklonkan di dalam vektor pengklonan. Keputusan penjujukan menunjukkan kedua-dua gen mempunyai 99% kesamaan dengan rekod jujukan di dalam GeneBank NCBI. Gen-gen yang telah diklonkan didalam TOP 10 kemudian diklonkan ke dalam dua vektor ekspresi, pRSF-DUET dan pCDF. Gen *hasA* diklonkan didalam pRSF-DUET manakala *hasE* diklonkan dalam pRSF-DUET dan pCDF menghasilkan pRSF-DUET-A, pRSF-DUET-AE dan pCDF-E. Kesemua klon tersebut ditransformasi ke dalam *E.coli* Rosetta (DE3).

Empat jenis klon, *E. coli* klon A, AE, A-E dan AE-E dihasilkan dengan mentransformasikan klon-klon plasmid ke dalam perumah. Kesemua protin klon telah berjaya dirembeskan dengan saiz protin yang dijangka iaitu ~42 kDa dan ~48 kDa. Kedua-dua protin tersebut dikenalpasti melalui teknik western blotting dengan menggunakan antibodi monoclonal anti-His dan monoclonal anti-S. Keputusan fermentasi menunjukkan pengurangan pertumbuhan sel yang cepat untuk semua klon selepas proses induksi. Kepekatan asid hialuronik dan berat molekul tertinggi diperolehi daripada klon AE-E di suhu 30°C di mana nilai kepekatan adalah 0.072 g/L dengan berat molekul 1.1×10^5 Da.

ACKNOWLEDGEMENTS

In the name of ALLAH The Beneficent, The Merciful

Praise to ALLAH S.W.T for giving me strength to finish this thesis with full heart and determination. I also want to foremost acknowledge my parents, my family and all my friends, Kak Adelene, Kak Shamsiah, Kak Vithya, Kak Noreen, Abang Bakhtiar, Shawal, Menaga, Jeevan, Munir and Kak Yee for the great support and unquantifiable help, opinion, suggestion, advise and for everything during the periods of mental and emotional fluctuations throughout the process of completing this report.

I also would like to remark my full gratitude to my co-supervisors, Professor Dr. Arbakariya Ariff and Associate Professor Dr. Rosfarizan Mohamad. To my supervisor Professor Dr. Raha Bt Abd Rahim, I would like to express my outmost thank you for all the advise, opinion, supervision and kindness from her that really guided me to finish this study. Not to forget to all people in UPM who have stretched the meaning of generosity by allowing me to have the opportunity to carry out my research and finally finish it.

Lastly, I would like to remind myself and everyone that we all could do worse, as writers, as readers and as human beings in general because every human being is not perfect and full perfection is ALLAH S.W.T alone.

"He who leaves home in search of knowledge, walk in the path of God"
Muhammad Bin Abdullah (570 – 632)

I certify that an Examination committee has met on 18 October 2013 to conduct the final examination of Muhammad Azmi B. Samsudin on his thesis entitled “Effect of phosphoglucosomerase (*hasE*) and hyaluronan synthase (*hasA*) co-expression on hyaluronic acid production in *Escherichia coli* Rosetta (DE3)” in accordance with the 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 degree of Master of Science.

Members of Examination Committee were as follows:

Norazizah Bt. Shafee, PhD

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

Dato' Abu Bakar B. Salleh, PhD

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

Foo Hooi Ling, PhD

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

Abdul Munir B. Abdul Murad, PhD

Associate Professor
Faculty of Science and Technology
Universiti Kebangsaan Malaysia
(External Examiner)

NORITAH OMAR, PhD

Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 17 February 2014

This thesis was submitted to the Senate of University Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows.

Raha Bt. Abdul Rahim, PhD

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

Arbakariya B. Ariff, PhD

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

Rosfarizan Bt. Mohamad, PhD

Associate Professor
Faculty 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 hereby confirm that:

- this thesis is my original work;
- quotations, illustrations and citations have been fully referenced;
- this thesis has not been submitted previously or concurrently for any other degree at other institutions;
- intellectual property from the thesis and copyright of thesis are fully owned by Universiti Putra Malaysia, as according to the Universiti Putra Malaysia (Research) Rules 2012;
- written permission must be obtained from supervisor and the office of Deputy Vice-Chancellor (Research and Innovation) before thesis is published in book form;
- there is no plagiarism or data falsification/fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software.

Signature: _____

Date: 18 October 2013

Name and Matric No.: Muhammad Azmi bin Samsudin GS 22964

This to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

Signature: _____
Name of
Chairman of
Supervisory
Committee: Prof Raha binti Abdul Rahim

Signature: _____
Name of
Member of
Supervisory
Committee: Prof Arbakariya bin Ariff

Signature: _____
Name of
Member of
Supervisory
Committee: Assoc Prof Rosfarizan binti Mohamad

TABLE OF CONTENTS

	Page
ABSTRACT	ii
ABSTRAK	iv
ACKNOWLEDGEMENTS	vi
APPROVAL	vii
DECLARATION	ix
LIST OF TABLES	xiii
LIST OF FIGURES	xvi
LIST OF ABBREVIATIONS AND UNITS	xvii
CHAPTER	
1 INTRODUCTION	1
2 LITERATURE REVIEW	4
2.1 Hyaluronic acid	4
2.1.1 Biological synthesis of hyaluronic acid	6
2.1.2 Chemical and physical structure of hyaluronic acid	9
2.2 Function of hyaluronic acid	10
2.2.1 Biological function of hyaluronic acid in living organism	10
2.2.2 Clinical use of hyaluronic acid	12
2.3 Source of hyaluronic acid	15
2.4 Hyaluronic acid production by bacterial fermentation	16
2.5 Production of hyaluronic acid by Streptococci	17
2.6 Heterogously expression of HAS genes and production of hyaluronic acid	18
2.7 Hyaluronic acid synthesis pathway in recombinant <i>E. coli</i>	19
2.8 Expression strategy to enhance the hyaluronic acid production in recombinant bacteria	24
2.9 Co-expression of protein complexes in <i>E. coli</i>	25
2.9.1 Multiple vectors	25
2.9.2 Single vector, single RNA transcript	25
2.9.3 Single vector, multiple RNA transcripts	26

3	MATERIALS AND METHOD	27
3.1	Bacteria strains, plasmid and media	27
3.2	Vectors and recombinant plasmids	28
3.3	PCR primers and characteristics	28
3.4	Extraction of <i>S. zooepidemicus</i> ATCC 39920 genomic DNA	31
3.5	Preparation of competent <i>E. coli</i> cells	32
3.6	Transformation of <i>E. coli</i>	33
3.7	Plasmid DNA extraction by Modified Alkaline Lysis method for <i>E. coli</i>	33
3.8	Polymerase Chain Reaction (PCR)	35
3.8.1	PCR amplification of hyaluronan synthase gene (<i>hasA</i>) phosphoglucoisomerase genes (<i>hasE</i>)	35
3.9	Agarose gel electrophoresis	36
3.10	Quantification of DNA concentration	37
3.11	Purification of PCR amplified <i>hasA</i> , <i>hasE</i> and <i>hasE</i> -DUET genes for cloning and sequencing	37
3.12	Restriction enzyme digestion	38
3.13	Sub-Cloning of the PCR-amplified <i>hasA</i> , <i>hasE</i> and <i>hasE</i> -DUET genes into cloning vector, pTZ	39
3.14	Analysis and sequencing of positive pTZ-A, pTZ-E and pTZ-E-DUET clones	39
3.15	Sub-cloning of <i>hasA</i> , <i>hasE</i> and <i>hasE</i> -DUET genes into expression vector	40
3.16	Protein induction and extraction	41
3.16.1	Extracellular fraction	41
3.16.2	Intracellular fraction	41
3.17	SDS-PAGE	42
3.18	Western blot	44
3.18.1	His-Tag	44
3.18.2	S-Tag	45
3.19	Batch Cultivation of recombinant <i>E. coli</i>	46
3.20	Hyaluronic acid quantification and molecular weight analysis	46
4	RESULTS AND DISCUSSION	48
4.1	<i>S. zooepidemicus</i> ATCC 39920 DNA genome extraction	48
4.2	Amplification of hyaluronan synthase (<i>hasA</i>), and two sets of phosphoglucoisomerase (<i>hasE/hasE</i> -DUET) genes by PCR	50
4.3	Cloning of hyaluronan synthase (<i>hasA</i>) and two set of phoglucoisomerase (<i>hasE/hasE</i> -DUET) genes into <i>E. coli</i> by using pTZ57R/T cloning vector	52

4.3.1	Plasmid transformation verification	53
4.3.2	Restriction enzyme verification	54
4.4	Sub-cloning of genes of interest into <i>E. coli</i> expression vectors, pRSF-DUET and pCDF	57
4.4.1	Sub-cloning of gene coding hyaluronan synthase (<i>hasA</i>) and phosphoglucoisomerase (<i>hasE</i> -DUET) into pRSF-DUET	57
4.4.2	Sub-cloning of phosphoglucoisomerase (<i>hasE</i>) into pCDF	58
4.4.3	Transformation of digested inserts into <i>E. coli</i> Rosetta (DE3) expression host	59
4.5	DNA sequence determination and analysis	70
4.6	SDS-PAGE and Western blot analysis of recombinant clones from <i>E. coli</i> Rosetta (DE3)	70
4.7	Batch cultivation of recombinant clones	76
4.8	Biosynthesis of HA	81
4.9	Hyaluronic acid molecular weight	86
4.10	Correlation between cell growth and HA concentration and molecular weight	89
5	CONCLUSIONS AND RECOMMENDATION FOR FUTURE WORK	96
5.1	Strategies for improving HA production	97
5.1.1	Gene manipulation	97
5.1.2	Induction Strategies	97
5.1.3	Growth Control	98
	REFERENCES	99
	APPENDICES	114
	BIODATA OF STUDENT	125
	LIST OF PUBLICATIONS	126