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UNIVERSITI PUTRA MALAYSIA

CONSTRUCTION OF RECOMBINANT *Escherichia coli* BAD85 FOR THE PRODUCTION OF HIGH PURITY L-LACTIC ACID

TENGKU ELIDA TENGKU ZAINAL MULOK

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By

TENGKU ELIDA TENGKU ZAINAL MULOK

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

May 2008

Especially dedicated to ….

- Almighty God
- My husband and daughters
- My sisters and brother

Thank you for your patience, support, understanding, love, care ….

A BIG THANK YOU

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

CONSTRUCTION OF RECOMBINANT *Escherichia coli* BAD85 FOR THE PRODUCTION OF HIGH PURITY L-LACTIC ACID

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May 2008

Chair : Prof. Mohd. Ali b. Hassan, PhD

Faculty : Biotechnology and Biomolecular Sciences

Lactate dehydrogenase (LDH, EC 1.1.1.27) catalyzes the oxidation of pyruvate to lactate in facultative anaerobes. Two forms of lactate dehydrogenase with different substrate specificities have been identified namely the L-lactate dehydrogenase (EC 1.1.1.27) and D-lactate dehydrogenase (EC 1.1.1.28). The L-lactate dehydrogenase is involved in the reduction of pyruvate into L-lactic acid whilst the D-lactate dehydrogenase is responsible for the formation of D-lactic acid. L-lactic acid is more preferable to D-lactic acid in the production of bioplastics since it is metabolizable in human and animals. The objective of this study is to isolate and clone the *L-lactate dehydrogenase* (*L-ldh*) gene from *Enterococcus faecalis* KK1 and express in *Escherichia coli* SZ85 for the production of L-lactic acid. *E. coli* SZ85 strain has five chromosomal deletions (*pflB, ackA, adhE, ldhA*, and *frdBC*), namely *D-lactate dehydrogenase, pyruvate formate lyase, acetate kinase, alcohol/aldehyde dehydrogenase* and *fumarate reductase* and a chromosomally integrated *L-ldh* gene from *Pediococcus acidilactici.* The 954 bp gene was isolated by using the polymerase chain reaction (PCR). Primers EF.f (forward) and EF.r (reverse) were

designed based on published gene sequence of *L-ldh*, and the PCR amplified *L-ldh* gene was cloned into TOPO TA cloning vector. The DNA sequencing results revealed 99% homology with published sequence in the database. The gene was subcloned into *E. coli* expression vector (pBAD) using the restriction enzymes *Eco* R1 and *Xho* 1. The pBAD-ldh gene was later transformed into *E. coli* SZ85 using electroporation. Sodium dodecyl sulfide-polyacrylamide gel electrophoresis (SDS-PAGE) analyses indicated that L-lactate dehydrogenase recombinant protein was successfully expressed in *E. coli* SZ85 with the expected size of 40 kDa. Western blot analysis revealed an immunoreactive band at 40 kDa size which further confirmed the expression of *L-ldh* gene. In this study, the mouse monoclonal antibody acted as the primary antibody and horse radish peroxidase (HRP), conjugated to the secondary antibody (anti-goat antibody) was used as a probe to confirm the recombinant protein. The recombinant *E. coli* BAD85 underwent fermentation using shake flasks to establish the optimum pH and temperature conditions for lactic acid production from fructose and was conducted at pH between 5.0–7.0 and temperature 30-37ºC. The best condition was later selected to.investigate the effect of temperature and pH on the production of lactic acid using *E. coli* BAD85 in a 2-L bioreactor system. Batch cultivations in 2-L stirred tank fermenter were carried out using the conditions determined during shake flask fermentation to further improve L-lactic acid production by recombinant *E. coli* BAD85. Cultivation of *E. coli* BAD85 at pH 7.0 and incubation temperature of 37ºC was found to be the best condition for producing L-lactic acid. These conditions were able to produce 7.04 gL^{-1} L-lactic acid with a high purity of 98%, 0.70 gg^{-1} yield and productivity of 0.029 gg^{-1} h^{-1}. The recombinant was able to achieve a 98% plasmid stability indicating that the cells were fairly stable for fermentation process.

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

KONSTRUKSI REKOMBINAN *Escherichia coli* BAD85 UNTUK PENGHASILAN ASID L-LAKTIK DENGAN KETULINAN YANG TINGGI

Oleh

TENGKU ELIDA TENGKU ZAINAL MULOK

Mei 2008

Pengerusi : Prof. Mohd. Ali b. Hassan

Fakulti : Bioteknologi dan Sains Biomolekul

Laktat dehidrogenase (LDH, EC 1.1.1.27) memangkinkan pengoksidaan piruvat kepada laktat dalam anaerob fakultatif. Dua jenis laktat dehidrogenase yang sudah dikenalpasti mempunyai pengkhususan terhadap substrat yang berbeza yakni L-laktat dehidrogenase (EC 1.1.1.27) dan D-laktat dehidrogenase (EC 1.1.1.28). L-laktat dehidrogenase terlibat dalam penurunan piruvat kepada asid L-laktik manakala D-laktat dehidrogenase pula terlibat dalam pembentukan asid D-laktik Asid L-laktik adalah lebih baik jika dibandingkan dengan asid D-laktik dari segi penghasilan bioplastik kerana ianya mudah untuk diungkaibina dalam manusia serta haiwan. Objektif kajian ini adalah untuk memencil dan mengklon gen *L-laktat dehidrogenase* (*L-ldh*) daripada strain *Enterococcus faecalis* KK1 dan dizahirkan pada *Escherichia coli* SZ85 untuk penghasilan asid L-laktik. Strain *E. coli* SZ85 mempunyai lima delesi pada kromosom (*pflB, ackA, adhE, ldhA,* and *frdBC*), yakni *D-laktat dehidrogenase, piruvat format liase, acetat kinase, alkohol/aldehid dehidrogenase* dan *fumarat reduktase* dan gen *L-ldh* dari *Pediococcus acidilactici* yang diintegrasikan pada kromosom. Gen *L-ldh*, berukuran 954

bp, telah dipencilkan dengan menggunakan kaedah reaksi berantai polimeres (PCR). Primer EF.f (ke hadapan) dan EF.r (terbalik) telah direka berdasarkan kepada jujukan yang telah diterbitkan dan gen *L-ldh* yang telah digandakan melalui PCR telah diklonkan ke dalam vector pengklonan TOPO TA. Keputusan daripada penjujukan DNA menunjukkan 99% persamaan dengan jujukan yang telah diterbitkan dalam pengkalan data. Gen ini disubklonkan ke dalam vector penzahir *E. coli* (pBAD) dengan menggunakan enzim penghazaman *Eco* R1 dan *Xho* 1. Gen pBAD-ldh kemudiannya ditransformasikan pada *E. coli* SZ85 melalui elektroporasi. Penganalisaan melaui elektroforesis jel natrium dodesil sulfid-poliakrilamid (SDS-PAGE) menunjukkan bahawa protein rekombinan L-laktat dehidrogenase telah berjaya dizahirkan pada *E. coli* SZ85 dengan jangkaan saiz 40 kDa. Analisis daripada blot Western menunjukkan kehadiran protein aktif imun berukuran 40 kDa dan selanjutnya membuktikan sekali lagi penzahiran gen *L-ldh*. Dalam kajian ini, antibodi monoklon tikus bertindak sebagai antibodi primer dan peroksidase lobak putih (HRP), dikonjugasikan pada antibodi sekunder (antibodi anti-kambing), telah digunakan sebagai prob untuk pengesahan terhadap protein rekombinan. Rekombinan *E. coli* BAD85 menjalankan fermentasi dengan menggunakan kelalang kon untuk menyelidiki keadaan pH dan suhu optima untuk penghasilan asid laktik dari fruktos dan telah dikendalikan pada pH di antara 5.0–7.0 dan suhu 30-37ºC. Keadaan yang terbaik kemudiannya dipilih dalam kajian kesan suhu dan pH terhadap penghasilan asid laktik menggunakan *E. coli* BAD85 dalam fermenter berpengaduk 2-L. Fermentasi sesekelompok dalam fermenter berpengaduk 2-L telah dijalankan di dalam keadaan yang telah ditentukan semasa fermentasi kelalang kon untuk meningkatkan penghasilan L-asid laktik oleh rekombinan *E. coli* BAD85. Pertumbuhan *E. coli* BAD85 dalam fermenter berpengaduk 2-L pada pH 7.0 dan suhu

eraman 37ºC didapati keadaan terbaik untuk penghasilan asid L-laktik. Keadaan ini telah menghasilkan 7.04 gL^{-1} asid L-laktik dengan ketulinan 98%, penghasilan nisbah asid laktik dengan berat kering sel sebanyak 0.70 gg^{-1} dan 0.029 gg^{-1} h^{-1} produktiviti. Rekombinan berjaya mencapai 98% kestabilan plasmid yang menunjukkan sel boleh dikatakan stabil untuk proses fermentasi.

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Pursuing a doctorate is always a challenging experience, in this case, it was also a pleasure.

I certify that an Examination Committee has met on _____ to conduct the final examination of Tengku Elida Tengku Zainal Mulok on her Doctor of Philosophy thesis entitled "Construction of recombinant *Escherichia coli* BAD85 for the production of high purity L-lactic acid" 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:

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

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

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

_____
(External Examiner)

_____
HASANAH MOHD GHAZALI, PhD
Professor 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 are as follows:

Mohd. Ali Hassan, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Raha Abdul Rahim, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Suraini Abdul Aziz, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

_____
AINI IDERIS, PhD
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 currently submitted for any other degree at Universiti Putra Malaysia or other institutions.

_____

TENGKU ELIDA TENGKU ZAINAL MULOK

Date :

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ATP	Adenosine triphosphate
Bp	Base pair
CFU/L	Colony forming unit per liter
°C	Degree celcius
DNA	Deoxyribonucleotide acid
g	Gram
g/L	Gram per liter
GRAS	Generally regarded as safe
h	Hour
H_20	Water
kDa	Kilo Dalton
L	Liter
LB	Luria Bertani
MCS	Multiple cloning site
mg	Milligram
mL	Milliliter
mM	Millimolar
µg	Microgram
µL	Microliter
PCR	Polymerase chain reaction
Taq	*Thermus aquaticus*
v/v	volume over volume
OD	Optical density
rpm	Rotation per minute

BSA	Bovine serum albumin
s	Second
h	Hour
M	Molar
mol	mole
min	Minute
U/L	Unit per liter
w/v	Weight/volume
g	Acceleration of gravity (~9.8 m/s/s)
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
dH_2O	Distilled water