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

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By

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

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Chairman : Associate Professor Rosfarizan Mohamad, PhD

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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 ($(NH_4)_2S_2O_8$, $(NH_4)_2PO_4$, 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 x 10^6 Da to 5.20 x 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 x 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 *Escherichia coli* MENGGUNAKAN BIOREAKTOR TANGKI BERPENGADUK

Oleh

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Januari 2013

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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 sunber 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 ($(NH_4)_2S_2O_8$, $(NH_4)_2PO_4$, 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 penggaduk 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 x 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 x 10^6 Da kepada 5.20 x 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 koeffisi 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 x 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 eksperiment 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.

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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 *Escherichia 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.

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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:

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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

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