UNIVERSITI PUTRA MALAYSIA

PRODUCTION AND ELECTROPHORESIS-BASED PURIFICATION OF RECOMBINANT GREEN FLUORESCENT PROTEIN FROM Escherichia coli

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PRODUCTION AND ELECTROPHORESIS-BASED PURIFICATION OF RECOMBINANT GREEN FLUORESCENT PROTEIN FROM *Escherichia coli*

By

CHEW FEW NE

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

August 2011

This thesis is dedicated to my beloved family,

for years of love and support,

and to Joseph,

for being there for me through everything.

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

PRODUCTION AND ELECTROPHORESIS-BASED PURIFICATION OF RECOMBINANT GREEN FLUORESCENT PROTEIN FROM *Escherichia coli*

By

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August 2011

Chairman: Associate Professor Tey Beng Ti, PhD

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Green fluorescent protein (GFP) can be used as markers in numerous bioscience applications due to its visible fluorescence. Availability of a reliable and affordable GFP quantitation device is important for these applications. GFP can be quantitated with a spectrofluorometer, but the accuracy of spectrofluorometric method is affected by the present of biomass and it cannot be used to differentiate the nativity of a GFP. Therefore, one of the objectives of this study was to develop a GFP quantitation method using gel-based imaging analysis. The precision, detection limit, linearity, reproducibility and accuracy of this quantitation method were investigated to demonstrate its reliability and suitability for practical application. Besides, the sensitivity and GFP nativity differentiation test were performed and compared with those obtained with a spectrofluorometer.

The cultivation conditions for the GFP production in *Escherichia coli* BL21(DE3) were optimized using statistical experimental designs. The process variables include

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agitation rate, temperature, pH of medium, inducer concentration, induction time and inoculum density were screened using the fractional factorial design. The screening step identified that agitation rate, temperature and cell density for protein expression have significant effects on GFP production. Hence, these process variables were further optimized using the Box-Behnken design. The optimal cultivation conditions were 206 rpm agitation rate at 31°C and cell density ($OD_{600\ nm}$) of 1.04 for protein expression. The predicted GFP yield (0.234 g/L) is corresponded well with that obtained experimentally (0.241 g/L). The GFP yield achieved under the optimized conditions is 9-folds higher than that of the unoptimized conditions (0.025 g/L). A similar protein yield was achieved when these optimized conditions were applied in a 2-liter stirred tank bioreactor fermentation.

Cell disruption is an important step in intracellular protein purification. However, this additional step resulted in a substantial protein loss and increased the overall processing time. Hence, a direct purification method of recombinant GFP from intact *E. coli* cells was developed using a preparative native polyacrylamide gel electrophoresis. This direct purification process has eliminated the cell disruption step. The cellular content of *E. coli* was drifted out from cells by the electrophoretic force. The GFP was further separated from other intracellular proteins through a gel and recovered by electrophoretic elution. The effects of the operating parameters including the feedstock volume, feedstock concentration, concentration of resolving gel and height of resolving gel on the purity and yield of GFP were further investigated. At 100 µL feedstock volume, 15% (w/v) feedstock concentration, 12% (w/v) resolving gel concentration and 2 cm resolving gel height, the purity and yield

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of GFP achieved were 98 and 88%, respectively. The scale-up study has demonstrated that the ratio of feedstock volume to column cross-sectional area at optimal ratio of 44 μL/cm^2 was an important factor for scaling-up the purification process.

The present study focused on gel electrophoresis technique, which provides the efficient ways to measure the amount of GFP and to purify the intracellular GFP. Besides, an optimal GFP cultivation condition was identified by using statistical experimental designs for maximum GFP production.

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

PENGHASILAN DAN PENULENAN BERASASKAN ELEKTROFORESIS PROTEIN PENDARFLUOR HIJAU REKOMBINAN DARIPADA *Escherichia coli*

Oleh

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Protein pendarfluor hijau (GFP) boleh digunakan sebagai petanda dalam pelbagai penggunaan biosains disebabkan oleh pendarfluornya yang dapat dilihat. Keperolehan alat kuantifikasi GFP yang boleh dipercayai dan mampu dimiliki adalah penting untuk kegunaan tersebut. GFP boleh dikuantifikasi dengan menggunakan spektrofluorometer, tetapi ketepatannya dipengaruhi oleh kehadiran biojisim dan ia tidak dapat digunakan untuk membezakan keaslian GFP. Oleh yang demikian, salah satu objektif kajian ini adalah untuk membangunkan satu kaedah kuantifikasi GFP dengan menggunakan analisis pengimejan berasaskan gel. Kejituan, had pengesahan, kelinearan, kebolehulangan dan ketepatan bagi kaedah kuantifikasi ini telah dikaji bagi menunjukkan kebolehpercayaan dan kesesuaiannya untuk penggunaan praktikal. Selain itu, ujian kepekaan dan pembezaan keaslian GFP telah dijalankan dan dibandingkan dengan spektrofluorometer.

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Keadaan pengkulturan untuk penghasilan GFP dalam *Escherichia coli* telah dioptimumkan dengan menggunakan ujikaji reka bentuk statistik. Pembolehubah proses termasuk kadar pengadukan, suhu, pH medium, kepekatan penggalak, masa aruhan dan kepekatan inokulum telah disaring dengan menggunakan reka bentuk pecahan faktorial. Langkah penyaringan mengenalpasti kadar pengadukan, suhu dan kepekatan sel untuk pengekspresan protein mempunyai kesan ketara ke atas penghasilan GFP. Oleh yang demikian, pembolehubah proses ini dioptimumkan selanjutnya dengan menggunakan reka bentuk Box-Behnken. Keadaan pengkulturan optimum adalah kadar pengadukan 206 rpm pada 31°C dan kepekatan sel (OD_{600nm}) pada 1.04 bagi pengekspresan protein. Hasil GFP yang diramalkan (0.234 g/L) adalah setara dengan yang diperolehi secara ujikaji (0.241 g/L). Hasil GFP yang dicapai dalam keadaan optimum adalah 9 kali ganda lebih tinggi berbanding tanpa pengoptimuman (0.025 g/L). Hasil protein yang sama telah dicapai apabila keadaan optimum ini digunakan dalam bioreaktor tangki berpengaduk 2-liter.

Pemecahan sel adalah langkah yang penting dalam penulenan protein intrasel. Walau bagaimanapun, langkah tambahan ini mengakibatkan kehilangan protein yang banyak dan memanjangkan masa pemprosesan keseluruhan. Oleh yang demikian, kaedah penulenan secara langsung GFP rekombinan daripada sel *E. coli* yang utuh telah dibangunkan dengan menggunakan elektroforesis sediaan dengan gel poliakrilamida asli. Proses penulenan secara langsung ini menyingkirkan langkah pemecahan sel. Kandungan sel *E. coli* ditarik keluar daripada sel oleh daya elektroforesis. GFP dipisah selanjutnya daripada protein intrasel yang lain melalui gel dan diperolehi semula dengan elusi elektroforesis. Kesan parameter pengendalian

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termasuk isipadu suapan, kepekatan suapan, kepekatan gel leraian dan ketinggian gel leraian terhadap ketulenan and hasil GFP telah dikaji selanjutnya. Pada isipadu suapan sebanyak 100 µL, kepekatan suapan sebanyak 15% (w/v), kepekatan gel leraian sebanyak 12% (w/v) dan ketinggian gel leraian setinggi 2 cm, ketulenan and hasil GFP yang dicapai masing-masing adalah 98 dan 88%. Kajian skala naik menunjukkan nisbah isipadu suapan terhadap luas keratan rentas turus pada nisbah optimum 44 µL/cm^2 adalah faktor yang penting untuk skala naik proses penulenan ini.

Kajian ini tertumpu pada teknik elektroforesis gel di mana memberi kaedah yang cekap untuk menyukat jumlah GFP dan menulen protein intrasel. Selain itu, keadaan pengkulturan GFP optimum telah ditentukan dengan menggunakan reka bentuk ujikaji statistik untuk penghasilan GFP maksimum.

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I certify that a Thesis Examination Committee has met on 18 August 2011 to conduct the final examination of Chew Few Ne on her thesis entitled "Production and Electrophoresis-Based Purification of Recombinant Green Fluorescent Protein from *Escherichia coli*" 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 Doctor of Philosophy.

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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 at any other institution.

_____

CHEW FEW NE

Date: 18 August 2011

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