UNIVERSITI PUTRA Malaysia

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MASTER OF SCIENCE
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DEVELOPMENT OF FED-BATCH CULTIVATION PROCESS FOR *ESHERICHIA COLI* HARBORING SUPEROXIDE DISMUTASE

By

WAN SITI ATIKAH WAN OMAR

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Master of Science

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DEVELOPMENT OF FED-BATCH CULTIVATION PROCESS FOR *ESCHERICHIA COLI* HARBORING SUPEROXIDE DISMUTASE

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WAN SITI ATIKAH WAN OMAR

October 2010

Chairman : Rosfarizan Mohamad, PhD

Institute : Bioscience

The fed-batch cultivation process for the production of the recombinant protein, superoxide dismutase (SOD) by *E. coli* BL21 (DE3) pLysS was carried out. The cultivation process for recombinant SOD (ESOD) production was optimized through several approaches and strategies. The first approach was to optimize medium and culture conditions of the ESOD culture in shake flask via conventional and the Response Surface Methodology (RSM) methods. Laboratory scale batch cultivation of ESOD was then performed using a 2 L stirred tank bioreactor (STB) in order to further optimize the medium and culture conditions. The effects of glucose concentrations (15 and 20 g/L), agitation speeds (300 – 1000 rpm) and controlled dissolved oxygen tension (DOT) via agitation speeds (20%, 50% and 80%) on the growth performance of the recombinant *E. coli* strain were investigated. In the final stage, fed-batch techniques

were applied to the process for the development of high cell density cultivation. The performance and kinetics of the ESOD fed-batch and batch cultivations were then evaluated and compared.

Plasmid harboring the SOD gene was found to be 100% stable over 200 generations in shake flask culture, which was agitated at 200 rpm and incubated at 37^{o}C for 72 h. A 27kDa protein band representing the intracellular rSOD was detected by SDS-PAGE analysis. Optimized medium and cultural conditions through RSM approach was found at 4.89 g/L of glucose, 21.86 g/L of yeast extract and tryptone (2:1 ratio) and initial pH of 7.84. By using the optimized conditions from the RSM approach, maximum cell concentration of 7.39 g/L was achieved.

Batch cultivations were further performed using 2 L STB in order to investigate the effects of glucose and cultural conditions on the growth performance of ESOD. A concentration of 15 g/L of glucose as a carbon source showed higher maximum cell concentration (14.54 g/L) compared to 20 g/L of glucose. Growth of ESOD was inhibited at high initial glucose concentration supplied to the medium, whereas, controlled DOT via agitation speed at 20% showed the highest cell concentration (7.44 g/L) obtained as compared to other controls. However, there were no significant differences of maximum cell concentration achieved at different DOT controlled cultivations. Meanwhile, a controlled agitation speed at 500 rpm throughout cultivation exhibited a maximum cell concentration of 15.70 g/L, which was two times higher compared to other agitation speeds tested.

Due to the substrate inhibition effect that occurred in the batch cultivation, growth of ESOD and SOD production were further enhanced through constant and pH-stat fed-batch cultivation techniques. In batch and constant fed-batch, significant amount of acetic acid and glucose were found accumulated at the end of cultivation which caused to inhibit ESOD growth and SOD production. Final cell concentration in the culture was significantly improved by the application of pH-stat fed-batch cultivation technique. The maximum cell concentration obtained by such cultivation technique was 5.4 and 2.4 times higher than constant fed-batch and batch cultivation techniques, respectively. Nevertheless, total protein productivity at 0.48 g/L/h was found higher in the batch cultivation compared to fed-batch cultivation techniques owing to the extended culture period.

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

PEMBANGUNAN PROSES PENGKULTURAN SUAPAN SESEKELOMPOK BAGI *ESCHERICHIA COLI* PENGELUAR SUPEROKSIDA DISMUTASE

Oleh

WAN SITI ATIKAH WAN OMAR

Oktober 2009

Pengerusi : Rosfarizan Mohamad, PhD

Institut : Biosains

Pengkulturan suapan sesekelompok protein rekombinan, superoksida dismutase (SOD) oleh *E. coli* BL21 (DE3) pLysS telah dijalankan. Proses pengoptimaan pengkulturan untuk penghasilan rekombinan SOD (ESOD) telah dijalankan dalam beberapa pendekatan dan strategi. Pada peringkat pertama, pengoptimaan media and kondisi ke atas kultur tersebut dijalankan di dalam kelalang kon dengan menggunakan kaedah konvensional dan Kaedah Permukaan Gerakbalas (RSM). Pengkulturan sekelompok skala makmal kemudian dijalankan di dalam 2 L bioreaktor pengaduk (STB) bagi pengoptimaan medium dan kondisi kultur. Kesan pertumbuhan ESOD terhadap kepekatan glukosa (15 g/L dan 20 g/L), kelajuan adukkan (300 – 1000 rpm) dan tekanan oksigen terlarut (DOT) yang dikawal melalui kelajuan adukkan (20%, 50% dan 80%) juga telah dijalankan. Pada peringkat akhir, teknik pengkulturan suapan sesekelompok telah diaplikasi dalam proses bagi pembangunan sel kultur

berketumpatan tinggi. Perkembangan dan kinetik bagi kultur suapan sesekelompok dan sekelompok ESOD telah dinilai dan dibandingkan.

Plasmid pengeluar SOD gen telah menunjukkan 100% stabil sehingga 200 generasi di dalam kultur kelalang kon dengan kelajuan adukkan pada 200 rpm dan dieram pada 37°C selama 72 jam. Jalur protein pada lebih kurang 27kDa mewakili intracellular rSOD didapati melalui analisis SDS-PAGE. Media and kondisi yang dioptimakan menggunakan RSM telah dicapai dengan menggunakan 4.89 g/L glukosa, 21.86 g/L yis ekstrak dan triptone (nisbah 2:1) dan pH awal, 7.84. Dengan menggunakan optimum media daripada pendekatan RSM ini, kepekatan maksima sel 7.39 g/L telah diperoleh.

Kultur sekelompok seterusnya dijalankan dengan menggunakan 2 L STB bagi menyelidik kesan glukosa dan kondisi kultur terhadap pertumbuhan ESOD. Glukosa 15 g/L merupakan sumber karbon telah menghasilkan kepekatan sel maksima yang lebih tinggi (14.54 g/L) berbanding glukosa 20 g/L. Pertumbuhan ESOD telah direncatkan oleh kepekatan glukosa yang tinggi pada awal medium, sementara itu, DOT 20% yang dikawal oleh kelajuan adukkan menunjukkan kepekatan sel yang paling tinggi (7.44 g/L) terhasil berbanding dengan kawalan yang lain. Bagaimanapun, tiada perbezaan ketara bagi kepekatan sel maksima yang terhasil pada kultivasi yang dikawal pada DOT yang berbeza. Di samping itu, kawalan kelajuan adukkan pada 500 rpm telah menghasilkan kepekatan sel maksima 15.7 g/L , yang mana dua kali lebih tinggi berbanding dengan kawalan adukkan lain yang diuji.

Oleh kerana kesan perencatan substrat berlaku dalam kultur sekelompok; pertumbuhan ESOD dan penghasilan SOD seterusnya ditingkatkan dengan menggunakan teknik pengkulturan suapan sesekelompok konstan dan pH-stat. Di dalam pengulkuturan sekelompok dan suapan sesekelompok konstan, asid asetik dan glukosa telah dijumpai terkumpul sehingga akhir kultivasi yang mana membuatkan pertumbuhan ESOD dan pengeluaran SOD terencat. Kepekatan sel telah bertambah dengan ketara melalui aplikasi teknik suapan sesekelompok pH-stat. Sel maksima yang diperoleh daripada teknik ini adalah masing-masing 5.4 dan 2.4 lebih tinggi daripada teknik kultur suapan sesekelompok konstan dan kultur sekelompok. Walau bagaimanapun, produktiviti protein pada 0.48 g/L/h adalah lebih tinggi dalam kultur sekelompok kerana masa kultur yang lebih panjang dalam teknik suapan sesekelompok.

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I certify that an Examination Committee met on **28th October 2009** to conduct the final examination of **Wan Siti Atikah Wan Omar** on her degree thesis entitled "Development of Fed-batch Cultivation Process for *Escherichia coli* Harboring Superoxide Dismutase" 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:

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Date: 12th February 2010

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

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Date: 17th March 2010

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 concurrently submitted for any other degree at UPM or other institutions.

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WAN SITI ATIKAH WAN OMAR

Date: 28 October 2009

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LIST OF ABBREVIATIONS

C/N	Carbon to nitrogen ratio of medium in mm basis
D_i	Impeller diameter
DOT	Dissolved Oxygen Tension
μ	Specific growth rate (h^{-1})
μ_{max}	Maximum specific growth rate
m	Growth associated rate constant for production (g product/g cell)
S_i	Initial substrate concentration (g/L)
T	Fermentation time (h)
UiTM	Universiti Teknologi MARA
UPM	Universiti Putra Malaysia
X	cell concentration (g/L)
X_i	Initial cell concentration (g/L)
X_{max}	Maximum cell concentration (g/L)
$Y_{x/s}$	Yield of cell on basis of consumed substrate (g cell/ g substrate)

CHAPTER ONE

INTRODUCTION

Antioxidants are reducing agents that can slow down or prevent the oxidation of other molecules. Although oxidation reactions are crucial for life, they can also be damaging since the world wide pollutions level becoming a serious problem. Most people nowadays consume vitamin C as their supplementary diet for prevention and treatment deficiency such as scurvy. It also acts as an antioxidant that helps to prevent oxidative damage to cellular components such as DNA, proteins and lipids. Lately, the injection of vitamin C into the face skin is introduced by women to make their skin look youthful and firm.

The superoxide dismutase (SOD) is an enzyme that catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. As such, it is an important antioxidant defense in nearly all cells exposed to oxygen. Naturally, SOD would be the antioxidants in aqueous compartments for example in the cytosol and the extracellular fluids in human cell that defends against reactive oxygen species (Valentine e*t al.,* 1998). This enzyme is used in pharmaceutical products and also in cosmetic products as antioxidant that can help to protect against cell destruction.

SOD was first discovered almost five decades ago by McCord and Fridovich, 1969 (Fridovich, 1974). SOD is divided by five metalloforms; two containing copper and zinc, one manganese, one iron and one nickel. Yi Sun (1990) stated that different SOD type would be found in different type of species. In prokaryotic cells, there are three forms of SOD, which are FeSOD, MnSOD and NiSOD (Yi Sun, 1990 and Youn *et al.,* 1996). The latter SOD is the latest found in several types of *Streptomyces sp.* .While in eukaryotic cells, three forms of SOD are known to exist; Cu ZnSOD, ECSOD, and FeSOD. On the other hand, Kinnula and Crapo (2003) stated three types of SOD found in mammalian cell, Cu ZnSOD, MnSOD and ECSOD. These SODs would act as anticarcinogens, the inhibitors at the initiation stage of mutation, protectors against oxidative damage and also indicator of abnormal level of SOD secreted by the cell. As the SOD would feasibly become one of the important proteins in human care products, it would be needed in large amount and carefully cultivated in control and optimize production cost.

The advent of molecular biology makes manipulation of microorganism and the production of recombinant proteins possible. Tan (2009) has successfully cloned and expressed a gene coding for SOD from *Lactoccocus lactis* M4 in *Escherichia coli.* The purification and characterization of SOD are explained in detail by Tan (2009). The SOD was confirmed visualized by Western Blot analysis at 27 kDa. *E. coli* has been well characterized found to be a good host for expression of heterologous protein and numerous proteins have been studied using *E. coli* on the expression vehicle. This recombinant was the first that carrying SOD gene from *Lactoccocus sp*., compared

others that taken from human gene (recombinant human SOD, rhSOD) (Monica *et al.,* 2002; Steinhorn *et al.,* 2001). The expression of the protein in laboratory scale cultivation was therefore come into highlight as the potential of SOD in the therapeutic and pharmaceutical as well as cosmetic products.

High cell density cultivation may be achieved using fed-batch culture if the growth of the cultured microorganism is subjected to substrate inhibition. The main advantage of the high density cell cultivation technique is the possibility of reducing the size of the production bioreactor for production of proteins. By employing laboratory scale bioreactor, the cost of production can be reduced greatly to enable economic production of the recombinant SOD. In addition, the fed-batch techniques also enhanced downstream processing and reduced wastewater (Yee and Blanch, 1992). Most proteins are accumulated intracellularly in recombinant *E. coli*, hence the productivity is proportional to the final cell density and the specific productivity. Cell concentrations of greater than 50 grams dry cell weight per liter (g/L) can be routinely obtained by fed-batch culture of both non-recombinant and recombinant *E. coli* (Riesenberg, 1999, Kim *et al.,* 2004). However, this technique has several drawbacks, including: substrate inhibition, limited oxygen transfer capacity, the formation of growth-inhibitory by-products and limited heat dissipation (Kilikian *et al.,* 2000).

Regardless of the disadvantages, genetic alteration, medium optimization and cultivation techniques would be crucial in maximizing the yield of the proteins on interest. Thus, scope of this study focuses on the development process aimed at

establishing high cultivation of cell density of recombinant *E. coli* harboring SOD (ESOD) using fed-batch culture technique. Therefore, the objectives of this research are;

1. To optimize the medium and cultural conditions of ESOD cultivation using Response Surface Methodology.

2. To investigate the effect of glucose and cultural conditions (DOT and agitation speed) in batch cultivation of ESOD using 2 L Stirred Tank Bioreactor.

3. To develop fed-batch cultivation process of ESOD with an optimal controlled strategy to achieve high cell density cultivation.

In this thesis, literature review is covered in chapter two with detailed discussion on various aspects of research on the development of ESOD involving small to large scale production, including details of the current state of knowledge of superoxide dismutase application. In chapter three, general materials and methods used in this study have been briefly described where all analytical procedures were described. The results and discussion of the research have been prepared and divided into several chapters (chapter four to six). Chapter four covers the study of culture media and condition of ESOD in shake flasks by using conventional method and response surface methodology (RSM).

Cultural condition optimization of batch cultivation in 2 L stirred tank bioreactor is discussed in chapter five, which also includes the comparison performance of ESOD cultivation by different concentration of glucose, different agitation speed and control of dissolved oxygen tension. Meanwhile, chapter six focuses on the development of fed-batch cultivation that aims to achieve HCDC by different feeding mode (pH-stat and constant) approached to ESOD. In chapter seven, general discussion is explaining more detail of the results in each chapter. Finally, chapter eight concludes the whole study and included suggestions for further work.