UNIVERSITI PUTRA MALAYSIA

EFFECTS OF FERMENTATION CONDITIONS ON THE PRODUCTION OF THERMOSTABLE LIPASE BY RECOMBINANT *E. COLI*

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By

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

March 2006

Specially dedicated to my beloved father, mother, brothers, sisters

my dearly wife and our little darling

Anis Sulwani…

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

EFFECTS OF FERMENTATION CONDITIONS ON THE PRODUCTION OF THERMOSTABLE LIPASE BY RECOMBINANT *E. COLI*

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March 2006

Chairman : Associate Professor Raja Noor Zaliha Raja Abd. Rahman, PhD

Faculty : Biotechnology and Biomolecular Sciences

A recombinant *Escherichia coli* BL21 (DE3) plysS (pGEX / T1S) strain harbouring thermostable lipase (E.C.3.1.1.3) gene from *Geobacillus sp*. was used through out this study. Lipase production was investigated using 2 L fermenter with 1.5 L working volume. Initial fermenter operation resulted in 11.07 U/mL activity with 24 h inoculum, temperature of 30°C, 250 rpm impeller speed and without pH and dissolve oxygen tension (DOT) control strategy.

Optimisation of fermentation operation conditions such as inoculum age, temperature, impeller speed, airflow rate, pH and dissolve oxygen tension (DOT) control strategy throughout the fermentation were carried out and compared. A substantial high activity of lipase was detected during fermentation with 10 h inoculum. Lipase production was 41.18 U/mL activity which was comparable to previous optimised shake bottle and was

3.7 times higher than with 24 h inoculum. Increasing the cultivation temperature to 37°C, resulted in an increased of lipase production to 89.82 U/mL activity with highest cell mass attained of 6.63 g/L. However, no significant difference (41.18 and 46.71 U/mL activity) in lipase production was observed at temperature 30°C and 40°C, respectively. Lipase production increased with an increasing airflow rate, with highest production of 89.82 U/mL activity observed at 1.5 L/min (1 vvm). In contrast, lipase production decreased to 47.30 U/mL activity with higher airflow rate, which was 2 times lower than those obtained at low airflow rates.

Further experiment on impeller speed showed only slight increased in lipase production. When impeller speed increased from 250 to 350 rpm, only slight increase of lipase production observed, from 89.82 U/mL to 93.03 U/mL activity. Higher and lower impeller speed showed no improvement in lipase production. Production time was reduced from 8 h to 5 h. Maximum cell mass decreased with increasing controlled pH and no improvement in overall productivity observed. At all dissolve oxygen control (DOT) strategy studied, no improvement in lipase production was observed. Highest production was observed in fermentation when DOT was controlled at 80% saturation which gave productivity of 4.85 U/mL.h.

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

KESAN PROSES FERMENTASI KE ATAS PENGHASILAN LIPASE STABIL HABA DARIPADA *E. COLI* REKOMBINAN

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Strain rekombinan *Escherichia coli* BL 21(DE 3) plysS (pGEX / T1S) yang mengandungi gene lipase (E.C.3.1.1.3) stabil haba daripada *Geobacillus sp*. adalah strain yang digunakan dalam kajian ini. Penghasilan enzim lipase hanyalah pada nilai activiti 11.07 U/mL apabila percubaan pertama menggunakan fermenter dijalankan, dengan inokulum 24 jam, suhu 30°C, halaju pengaduk 250 ppm, kadar kemasukan udara 1.5 L/min dan tanpa sebarang kawalan tetap terhadap pH media dan oksigen terlarut.

Pengoptimuman usia inokulum, suhu, kadar kemasukan udara ke dalam sistem fermenter, kelajuan pengaduk, pH medium yang terkawal tetap, dan kesan tekanan oksigen terlarut terkawal tetap terhadap penghasilan lipase intrasellular di kaji menggunakan fermenter 2 L dengan jumlah media 1.5 L. Aktiviti lipase yang tinggi dikesan dengan menggunakan usia inokulum 10 jam. Penghasilan adalah pada nilai aktiviti 41.18 U/mL yang mana tidak jauh berbeza dengan keputusan optimum skala botol bergoncang. Ia adalah 3.7 kali

lebih tinggi daripada menggunakan usia inokulum 24 jam. Kenaikan ketara aktiviti lipase diperolehi setelah suhu pengkulturan dinaikkan ke 37°C (89.82 U/mL aktiviti). Pada kajian kadar kemasukan udara, penghasilan lipase meningkat dengan peningkatan nilai kadar kemasukan udara, dengan nilai maksima masih kekal pada kadar kemasukan udara 1.5 L/min (1 vvm). Namun begitu, nilai kemasukan udara yang lebih tinggi telah menurunkan penghasilan lipase sebanyak hampir 2 kali ganda (47.30 U/mL aktiviti).

Eksperimen selanjutnya menunjukkan hanya sedikit kenaikan pada nilai aktiviti lipase, iaitu daripada 89.82 U/mL ke 93.03 U/mL apabila halaju pengaduk dinaikkan daripada 250 ppm ke 350 ppm. Kecekapan sel dalam penghasilan enzim dan nilai pengeluaran meningkat kesan daripada peningkatan penghasilan lipase iaitu 14.33 U.L/g dan 11.2 U/mL.h, masing-masing. Semasa proses fermentasi menggunakan strategi pH terkawal tetap sepanjang fermentasi, nilai kepekatan sel maksima menurun dengan nilai pH kawalan meningkat. Penghasilan lipase didapati menurun dengan kenaikan nilai kawalan pH. Daya pengeluaran enzim lipase menggunakan pendekatan ini menunjukkan tiada pembaikan. Keputusan yang hampir sama diperolehi apabila strategi tekanan oksigen terlarut terkawal tetap dijalankan sepanjang tempoh fermentasi. Pada semua kajian strategi kawalan ini, tiada pembaikan dalam penghasilan lipase dikesan. Penghasilan tertinggi di kesan pada kawalan 80% oksigen terlarut dengan daya pengeluaran sebanyak 4.85 U/mL.h.

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I certify that a Thesis Examination Committee has met on 13th March 2006 to conduct the final examination of Fadzlirahimi bin Ismail on his Master of Science thesis entitled "Effect of Fermentation Conditions on the Production of Thermostable Lipase by Recombinant *E. 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 Master of Science.

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DECALARATION

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 UPM or other institutions.

____

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LIST OF ABBREVIATIONS

DCU	Digital control unit
D_i	impeller diameter
D_t	tank/vessel diameter
DOT	dissolve oxygen tension
H_i	impeller high
H_L	liquid high
h	hour/s
L	baffle width
L	litre
mL	millilitre
μ	specific growth rate (h^{-1})
µL	microlitre
P	lipase production (U/mL)
P_{max}	maximum lipase production
Pr	productivity
Q	volumetric airflow rate/ liquid volume (vvm)
rpm	revolution per minute
t	incubation time (h)
V	impeller tip speed (ms^{-1})
W_i	impeller high
X	cell mass (g/L)
X_{max}	maximum cell mass

CHAPTER 1

INTRODUCTION

Lipases (acylglycerol – acylhydrolase, E. C. 3.1.1.3) have been traditionally obtained from animal pancreas as a digestive aid for human consumption. Initial interest in microbial lipase was generated as a result of shortage of pancreas and difficulties in collecting available materials (Falk *et al*., 1991). Lipases are enzymes of considerable physiological significance and industrial potential (Sharma *et al*., 2001). Lipases are serine hydrolase, catalyse the hydrolysis of triglycerides to glycerol and free fatty acid at oil/water interface (Sugihara *et al*., 1995; Sharma *et al*., 2001).

Compared to mammalian and plant, microbial lipases are much more diverse in their enzymatic properties and substrate specificities, which make them attractive for industrial applications (Sugihara *et al*., 1995). Lipases of microbial origin are the most versatile enzymes and are known to bring about a range of bioconversion reactions, which includes hydrolysis, interesterification, esterification, alcoholysis, acidolysis and aminolysis (Haki and Rakshit, 2003). Due to their extracellular nature, most of microbial lipases can be produce in large quantities and are quite stable under non-natural conditions such as high temperatures and nonaqueous organic solvents employed in many applications.

Their stability, inexpensive manufacturing, as well as their broad synthetic potential make microbial lipases as an ideal biocatalyst for oleochemistry and organic synthesis. Application in organic chemical processing, detergent formulations, synthesis of biosurfactants, oleochemical industry, dairy industry, agrochemical industry, paper manufacture, nutrition, cosmetics, and pharmaceutical processing, have recently been reviewed (Schmidt-Dannert, 1999; Sharma *et al*., 2001).

Even though lipases have been used traditionally for decades, the range of application and the volume of lipases manufactured have been limited. Accordingly, the economic importance of lipases to the enzyme industry has been quite restricted when compared with major industrial enzymes such as proteases and carbohydrases. Recently, however, many new potential applications of lipases have been proposed and, since the methods for lipase production have been improved, some of these new uses will be economically viable. In 1995, lipases are considered to be the third largest group based on total sales volume (Jaeger *et al*., 1997). It is likely, therefore, that lipases will become of increasing importance, not only within areas traditionally employing large quantities of enzymes such as, for example, the detergent and food industries (Björkling *et al*., 1991).

Thermostable enzymes are particularly attractive for industrial applications because of their high activities at the elevated temperatures and stabilities in organic solvents (Lee *et al*., 2001). Most of the industrial processes in which lipases are employed function at temperature exceeding 45°C. The enzymes, thus, are required to exhibit an optimum temperature around 50°C. Some enzymatic processes for the physical refining of seed

oils have four distinct requirements. These include pH of 5.0 and optimal temperature of around 65°C, adding an enzyme solution and enzyme reaction followed by the separation of the lysophosphatide from the oil at about 75°C. Another best known examples probably being those used in starch hydrolysis for the production of high fructose syrup and the use of proteases in detergents. These reactions, therefore, are enhanced through the utilization of thermo-tolerant lipases (Kristjansson, 1989; Haki and Rakshit, 2003). Thermostable are one of the main desirable characteristics that commercial lipases should exhibit.

Thermostable enzymes are mainly derived from thermophilic microorganism. As isolation and cultivation of the extreme thermophiles often requiring anaerobic conditions at 100°C or above is much more difficult to operate, therefore, genetic engineering and cloning enzymes from thermophiles into appropriate mesophiles host is the best approach to express the selected protein. Recently, *Escherichia coli* is still the most important and favorable host organism for recombinant protein production (Korz *et al*., 1995). As most *E. coli* strain are aerobic mesophile, culturing in a fully equipped fermenter is the most suitable approach to enhance growth and protein production. In *E. coli*, the amount of carbon source and level of oxygen play an important role in the metabolic fluxes associated to its growth. Oxygen is a growth limiting factor and below a critical value affects the growth rate but at the same time can have inhibitory effects when present in excess. Similarly, low levels of dissolved carbon dioxide are reported to stimulate growth, meanwhile increasing levels have progressive inhibitory effects. The levels of dissolved oxygen and carbon dioxide are affected by the consumption or production

respectively, and the transfer rate between phases. In aerobic systems, fermenter can work in optimal conditions for gas-liquid transfer by means of agitation and aeration (Alba and Calvo, 2000).

The scope of this study was focused on development of process aimed at establishing high performance thermostable lipase fermentation using local recombinant strain, *E. coli* BL21 (DE3) plysS (PGEX / T1 S). Therefore, the objectives of this research are;

1. to compare and analyze experimental data of batch lipase fermentation of 1 L screw cap media – lab bottle and 2 L fermenter
2. to improve thermostable lipase production using 2 L stirred tank fermenter
3. to identify important fermenter parameters on maximum thermostable lipase production by recombinant *E. coli.*

CHAPTER 2

LITERATURE REVIEW

2.1 Industrial enzymes

Today, nearly 4000 enzymes are known, and of these, about 200 are in commercial use. Until the 1960s, the total sales of enzymes were only a few million dollars annually, but the market has since grown spectacularly (Godfrey and West, 1996).

Twelve major producers and 400 minor suppliers satisfy the world enzyme demand. Around 60% of the total world supply of industrial enzymes is produced in Europe. At least 75% of all industrial enzymes are hydrolytic in action. Proteases dominate the market, accounting for approximately 40% of all enzyme sales. As depicted in Table 1, most enzymes are used as industrial catalysis. As an example, lipase have an important applications in food industry as additives to modify the food flavour as well as in laundry detergents due to it tendency toward lower washing temperatures to save energy (Sokolovska *et al*., 1998). Indeed, the single biggest market of their use is in the detergent formulations. While in therapeutic applications, penicillin G acylase (PGA) hydrolyses penicillin G to 6-aminopenicillanic acid (6APA), which is used in the semi-synthetic antibiotic industry (Liu *et al*., 2000). Other applications of enzymes are summarized in Table 1.

Table 1: Fields of applications of enzymes (Sharma *et al*., 2001)

Scientific research: Enzymes are used as research tools for hydrolysis, synthesis, analysis, biotransformations, and affinity separation.

Cosmetic applications: Preparations for skin; denture cleansers.

Medical diagnostics and chemical analyses: Blood glucose, urea, cholesterol; ELISA systems; enzyme electrodes and assay kits.

Therapeutic applications: Antithrombosis agents, anti tumor treatments, anti-inflammatory agents, digestive aids, etc.

Industrial catalysis: In speciality synthesis; brewing and wine making; dairy processing; fruit, meat and vegetable processing; starch modifications; leather processing; pulp and paper manufacture; sugar and confectionery processing; production of fructose; detergents and cleaning agents; synthesis of amino acids and bulk chemicals; wastewater treatment; desizing of cotton.

2.2 Lipases

Lipases are ubiquitous enzymes of considerable physiological significance and industrial potential (Sharma *et al*., 2001). Lipases are classified as serine hydrolases because the active site is generally characterized by the triad composed of serine, histidine and aspartate (Sharma *et al*., 2001; Reetz, 2002; Tyndall *et al*., 2002).

Due to the flexibility of protein chains, lipases can catalyse three basic types of enzymatic reactions on the esteric bond between fatty acid and alcohol – hydrolysis, esterification and interesterification (Figure 1) (Sokolovská *et al*., 1998). They usually

retain their structure and activity in organic solvents. They also have several advantages over chemical catalyst: substrate specificity, regio- and enantio-selectivity, lower temperature and pressure requirements (Vanot *et al.,* 2001).

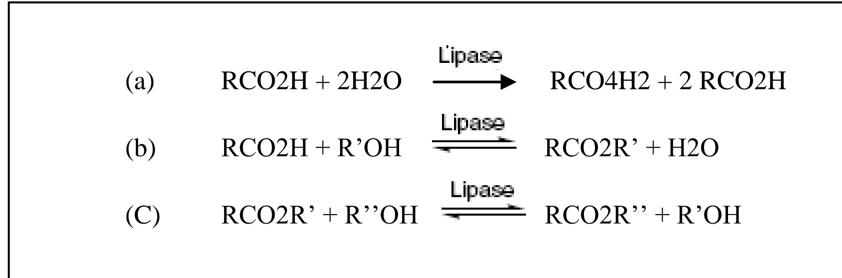

Figure 1: Basic types of lipase enzymatic reactions: (a) hydrolysis (b) esterification and (c) transesterification

Lipases catalyse the hydrolysis of triacylglycerols to glycerol and free fatty acids. In contrast to esterases, lipases are activated only when adsorbed to an oil-water interface (Martinelle *et al*., 1995) and do not hydrolysed dissolve substrates in the bulk fluid. Interfacial activation of lipases that occurs at this lipid-water interface is a phenomenon that can be traced to the unique structural characteristics of this class of enzymes. Lipases contain a helical oligopeptide unit that shields the active site. This so-called lid, upon interaction with hydrophobic interface such as lipid droplet, undergoes movement in such a way that exposes the active site providing free access for the substrates (interfacial activation) (Reetz, 2002; Tyndall *et al*., 2002).

Although lipases have been found in many species of animals, plants, yeast and fungi, the enzymes from microbial sources are currently receiving particular attention because their actual and potential application in the industry and different operating strategies have been evaluated to improve the yield of this enzyme (Sarkar *et al*., 1998; Sokolovská *et al*., 1998). The yeast *Candida lipolytica* and *Candida cylindracea* have been identified as the most important microorganism to produce lipases (Falk *et al*., 1991). However, there was also an interest in using microbial strain as they generally offer higher activities compared to yeast (Frost and Moss, 1987).

Over the past decade, a number of bacterial lipases have been purified and characterized. However, as reported earlier, crude lipase preparations are among the commercial lipases most often employed in the hydrolysis and synthesis of a wide range of esters of commercial interest (Tripathi *et al*., 2004). Lesser amounts of lipases are used in oleochemical transformations (Sharma *et al*., 2001).

2.3 Applications of lipases

Lipases find promising applications in organic chemical processing, detergent formulations, synthesis of biosurfactants, the oleochemical industry, the dairy industry, the agrochemical industry, paper manufacture, nutrition, cosmetics, and pharmaceutical processing (Sharma *et al*., 2001, Björkling *et al*., 1991). The major commercial application for hydrolytic lipases is their use in laundry detergents. Detergent enzymes