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

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

By

FADZLIRAHIMI BIN ISMAIL

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 throughout 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 increase 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

Oleh

FADZLIRAHIMI BIN ISMAIL

Mac 2006

Pengerusi : Professor Madya Raja Noor Zaliha Raja Abd. Rahman, PhD
Fakulti : Bioteknologi dan Sains Biomolekul

Strain rekombinan *Escherichia coli* BL 21(DE 3) plyS (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 aktiviti 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).

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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.

__________________________
FADZLIRAHIMI BIN ISMAIL

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>vi</td>
</tr>
<tr>
<td>APPROVAL</td>
<td>viii</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xv</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xvi</td>
</tr>
</tbody>
</table>

## CHAPTER

1 **INTRODUCTION**

2 **LITERATURE REVIEW**

   2.1 Industrial enzymes
   2.2 Lipases
   2.3 Applications of lipases
   2.4 Lipase producers
      2.4.1 Bacteria
      2.4.2 Fungi
      2.4.3 Recombinant cell
   2.5 Intracellular lipases
   2.6 Extracellular lipase
   2.7 Thermostable lipases
   2.8 Alkalophilic lipases
   2.9 Acidic lipases
   2.10 Large scale lipase production
      2.10.1 Mode of fermenter operation on lipase production
   2.11 Scaling-up procedure
   2.12 Effect of physical factors in fermenter operation on lipases production
      2.12.1 Temperature
      2.12.2 Aeration and agitation
      2.12.3 pH
      2.12.4 Inoculum size
      2.12.5 Time course of enzyme production
2.13 Effect of nutritional factor in fermenter operation on lipase production
   2.13.1 Carbon 43
   2.13.2 Inducers 47

3 GENERAL MATERIALS AND METHODS
   3.1 Experimental programme 50
   3.2 Microorganism 50
   3.3 Fermentation media 52
   3.4 Inoculum 52
   3.5 Fermenter 52
   3.5.1 Effect of preliminary fermenter culturing condition on thermostable lipase production 55
   3.5.2 Identification of important fermenter parameter for thermostable lipase scaling-up 56
   3.6 Analytical procedures
   3.6.1 Cell concentration determination 58
   3.6.2 Preparation of cell and culture supernatants 59
   3.6.3 Lipase activity assay 59
   3.7 Theory and mathematical calculations 60

4 RESULTS AND DISCUSSIONS
   4.1 Effect of preliminary fermenter culturing condition on thermostable lipase production 62
   4.2 Identification of important fermenter parameter for thermostable lipase scaling-up
   4.2.1 Influence of different inoculum age 67
   4.2.2 Influence of different growth temperature 71
   4.2.3 Influence of different airflow rate 75
   4.2.4 Influence of different impeller speed 79
   4.2.5 Influence of different pH control strategy 84
   4.2.6 Influence of different dissolve oxygen tension (DOT) control strategy 87

5 CONCLUSIONS AND SUGGESTION FOR FUTURE WORK 92

REFERENCES 95
APPENDICES 109
BIODATA OF STUDENT 112
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fields of applications of enzymes</td>
</tr>
<tr>
<td>2</td>
<td>Industrial applications of microbial lipases</td>
</tr>
<tr>
<td>3</td>
<td>Some commercially available microbial lipases</td>
</tr>
<tr>
<td>4</td>
<td>Recombinant lipases</td>
</tr>
<tr>
<td>5</td>
<td>Intracellular lipases</td>
</tr>
<tr>
<td>6</td>
<td>Extracellular lipases</td>
</tr>
<tr>
<td>7</td>
<td>Thermostable lipases</td>
</tr>
<tr>
<td>8</td>
<td>Alkalophilic lipases</td>
</tr>
<tr>
<td>9</td>
<td>Lipases production with batch fermentation of various types of microorganism</td>
</tr>
<tr>
<td>10</td>
<td>Cultivation temperature for lipases production of various types of microorganism</td>
</tr>
<tr>
<td>11</td>
<td>Aeration and agitation speed for lipases production of various types of microorganism</td>
</tr>
<tr>
<td>12</td>
<td>Time course for lipases production of various types of microorganism</td>
</tr>
<tr>
<td>13</td>
<td>Carbon source for lipases production of various types of microorganism</td>
</tr>
<tr>
<td>14</td>
<td>Inducers for lipase production by various type of microorganism</td>
</tr>
<tr>
<td>15</td>
<td>Kinetic parameter values on time course production of thermostable lipase production by recombinant <em>E. coli</em> in batch submerged fermentation using 2 L fermenter</td>
</tr>
<tr>
<td>16</td>
<td>Kinetic parameter values on the effect of different inoculum age used on lipase production</td>
</tr>
</tbody>
</table>
17 Kinetic parameter values on the effect of temperature on lipase production  73
18 Kinetic parameter values on the effect of various air flow rate on lipase production  77
19 Kinetic parameter values on the effect of various impeller speed on lipase production  82
20 Kinetic parameter values on the effect of pH control strategy on lipase production  86
21 Kinetic parameter values on the effect of dissolve oxygen control strategy on lipase production  89
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Basic types of lipase enzymatic reactions</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>Flow diagram of experimental plan</td>
<td>51</td>
</tr>
<tr>
<td>3</td>
<td>2 L stirred tank fermenter</td>
<td>53</td>
</tr>
<tr>
<td>4</td>
<td>Schematic diagram and dimensions of 2 L stirred tank fermenter used in this study</td>
<td>54</td>
</tr>
<tr>
<td>5</td>
<td>Time course production of thermostable lipase production by recombinant <em>E. coli</em> in batch submerged fermentation using 2 L fermenter</td>
<td>63</td>
</tr>
<tr>
<td>6</td>
<td>Effect of different inoculum ages on lipase fermentation by recombinant <em>E. coli</em> in batch submerged fermentation using 2 L fermenter</td>
<td>68</td>
</tr>
<tr>
<td>7</td>
<td>Effect of different cultivation temperature on lipase fermentation by recombinant <em>E. coli</em> in batch submerged fermentation using 2 L fermenter</td>
<td>72</td>
</tr>
<tr>
<td>8</td>
<td>Effect of different airflow rates on lipase fermentation by recombinant <em>E. coli</em> in batch submerged fermentation using 2 L fermenter</td>
<td>76</td>
</tr>
<tr>
<td>9</td>
<td>Effect of different impeller speeds on lipase fermentation by recombinant <em>E. coli</em> in batch submerged fermentation using 2 L fermenter</td>
<td>81</td>
</tr>
<tr>
<td>10</td>
<td>Effect of different pHs control strategy on lipase fermentation by recombinant <em>E. coli</em> in batch submerged fermentation using 2 L fermenter</td>
<td>85</td>
</tr>
<tr>
<td>11</td>
<td>Effect of different dissolve oxygen tension (DOT) controlled strategy on lipase fermentation by recombinant <em>E. coli</em> in batch submerged fermentation using 2 L fermenter</td>
<td>88</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

DCU  Digital control unit
Di   impeller diameter
Dt   tank/vessel diameter
DOT  dissolve oxygen tension
Hi   impeller high
HL   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)
Pr   productivity
Q    volumetric airflow rate/liquid volume (vvm)
rpm  revolution per minute
t    incubation time (h)
V    impeller tip speed (ms\(^{-1}\))
Wi   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*. 
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)

<table>
<thead>
<tr>
<th>Scientific research</th>
<th>Cosmetic applications</th>
<th>Medical diagnostics and chemical analyses</th>
<th>Therapeutic applications</th>
<th>Industrial catalysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymes are used as research tools for hydrolysis, synthesis, analysis, biotransformations, and affinity separation.</td>
<td>Preparations for skin; denture cleansers.</td>
<td>Blood glucose, urea, cholesterol; ELISA systems; enzyme electrodes and assay kits.</td>
<td>Antithrombosis agents, anti tumor treatments, anti-inflammatory agents, digestive aids, etc.</td>
<td>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.</td>
</tr>
</tbody>
</table>

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](image)

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