PRODUCTION OF THERMOSTABLE PULLULANASE FROM *Bacillus flavothermus* KWF-1 IN FED-BATCH CULTURE

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"Specially dedicated to my late grandmother. A small gift for your endless love and care. You always remain in my heart and memory. Thanks to GOD for giving me all the strength I needed."

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ABSTRACT

Optimization of pullulanase production by *Bacillus flavothermus* KWF-1 in fed batch culture was carried out using 2 L bioreactor with working volume of 1.5 L. Fermentation was initiated with batch culture at 50°C and agitation speed of 200 rpm using PYE medium consisted of 2.0% (w/v) sago starch, 1.75% (w/v) peptone, 0.5% (w/v) yeast extract, 0.1% (w/v) KH_2PO_4 and 0.02% (w/v) $MgSO_4.7H_2O$. The initial pH of the medium was adjusted to 7.5 using 0.1M of NaOH. During batch culture fermentation the highest pullulanase activity (0.0803 U/ml) was detected at early stationary phase (t=76h) with specific activity of 0.0213 U/mg. Fed batch culture was initiated after 96 hour when starch was completely depleted (S=0). Optimization of pullulanase production in fed batch culture was performed based on feeding mode, carbon concentration and nitrogen source. Exponential feeding mode with flow rate of 0.01 L/h^{-1} and sago starch at concentration of 2% (w/v) resulted in highest pullulanase activity with 0.171 U/ml and specific activity of 0.066 U/mg which was respectively 2.1 and 3.1 fold higher than in batch culture. Screening of suitable single nitrogen source (organic and inorganic) for enhancement of pullulanase production shown addition of 0.5% (w/v) yeast extract as single nitrogen source gave highest pullulanase activity of 0.133 U/ml which was 1.7 fold higher than in batch culture as compared to other organic and inorganic nitrogen sources. Feeding medium supplemented with 0.5% (w/v) of $(NH_4)_2SO_4$ enhanced pullulanase specific activity by 3.2 fold as compared to batch culture. The optimization of carbon and nitrogen concentration using sago starch and $(NH_4)_2SO_4$ was carried out using Response Surface Methodology (RSM). The optimum conditions obtained were 2.01% (w/v) of sago starch and 0.41% (w/v) of $(NH_4)_2SO_4$. The optimized medium improved pullulanase activity up to 68.8% (0.13557 U/ml) as compared to batch culture.

ABSTRAK

Pengoptimuman pullulanase yang dihasilkan oleh *Bacillus flavathermus* KWF-1 dijalankan dalam kultur suapan sesekelompok menggunakan bioreaktor 2.0L dengan isipadu kerja 1.5L. Fermentasi dimulakan dengan kultur sesekelompok pada suhu 50ºC dan agitasi 200 rpm dengan menggunakan media PYE yang mengandungi 2.0% (b/i) kanji sagu , 1.75% (b/i) pepton, 0.5% (b/i) ekstrak yis, 0.1% (b/i) KH_2PO_4 dan 0.02% (b/i) $MgSO_4.7H_2O$. pH awalan media diselaraskan kepada 7.5 dengan menggunakan larutan 0.1M NaOH. Penghasilan pullulanase yang tertinggi di dalam fermentasi berkelompok 0.0803 U/ml dihasilkan pada awal fasa pegun (t=76 jam) dengan aktiviti specifik 0.0213 U/mg. Kultur suapan balik dimulakan selepas 96 jam apabila kanji di dalam media fermentasi telah kehabisan (S=0). Proses penyaringan dijalankan untuk menentukan jenis suapan, kepekatan sumber karbon dan jenis sumber nitrogen yang terbaik. Suapan secara eksponential dengan kadar aliran 0.01 L/j^{-1} dan kepekatan kanji sagu 2% (b/i) memberikan peningkatan aktiviti pullulanase (0.171 U/ml) dan spesifik aktiviti (0.066 U/mg) masing-masing 2.1 dan 3.1 kali ganda berbanding kultur sesekelompok. Penyaringan untuk sumber nitrogen (organik dan bukan organik) yang dapat meningkatkan penghasilan pullulanase menunjukkan penambahan ekstrak yis meningkatkan aktiviti pullulanase sebanyak 1.7 kali ganda (0.133 U/ml) berbanding kultur sesekelompok. Penambahan 0.5% (b/i) $(NH_4)_2SO_4$ meningkatkan aktiviti spesifik pullulanase sebanyak 3.2 kali ganda berbanding fermentasi berkelompok. Proses pengoptimuman kepekatan sumber karbon (kanji sagu) dan nitrogen $(NH_4)_2SO_4$) dijalankan menggunakan kaedah gerakbalas permukaan (RSM). Keadaan optimum yang diperoleh adalah pengunaan 2.01% (b/i) kanji sagu dan 0.41% (b/i) $(NH_4)_2SO_4$. Penghasilan pullulanase sebanyak 0.13557 U/ml diperolehi dengan peningkatan sebanyak 68.8% berbanding kultur sesekelompok.

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LIST OF SYMBOLS / ABBREVIATIONS

CCD	-	Central composite design
D	-	Dilution rate
DO	-	Dissolved Oxygen
F	-	Flowrate
g	-	Gram
H	-	Height
H_0	-	Null Hypothesis
H_1	-	Alternative Hypothesis
hr^{-1}	-	Per hour
hr	-	Hour
L	-	Liter
M	-	Molar
mg	-	Milligram
min	-	Minutes
ml	-	Milliliter
mM	-	MiliMolar
nm	-	Nanometer
S	-	Substrate concentration
S_0	-	Initial Substrate Concentration
R^2	-	Regression Coefficient
RSM	-	Response Surface Methodology
rpm	-	Round per minute
t	-	Time
T	-	Temperature

U	-	Unit (enzyme activity)
V	-	Volume
V_0	-	Initial Volume
v/v	-	Volume per volume
w/v	-	Weight per volume
X_{max}	-	Maximum biomass concentration
μm	-	Micrometer
μg	-	Microgram
μ	-	Specific growth
ºC	-	Degree Celcius
%	-	Percentage

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CHAPTER 1

INTRODUCTION

1.1 Introduction and Problem Statement

Starch is the most abundant storage carbohydrate in plants. Starch is found in various plants as a food storage polysaccharide, and it contributes as carbon as well as an energy source for a variety of microorganisms. Polysaccharide is composed of two high-molecular-weight compounds, amylose (15 to 25%) and amylopectin (75–85%). Starch is primarily composed of amylopectin, a branched polymer of glucose in which α-1,4-linked glucan chains are connected by α-1,6-bonds (branch points). Pullulanase can be used with glucoamylase or ß-amylase for the production of high glucose and high maltose syrups during starch saccharification process (Jensen, B.F. and Norman, 1984).

Thermostable and thermoactive amylolytic enzymes are required in the bioprocess of starch since elevated processing temperatures enhance the solubility of starch, reduce its viscosity, limit microbial contamination, reduce reaction times and processing cost is more economical. In 1961, an enzyme capable of hydrolysing α-l,6-glycosidic linkages in pullulan was first explained by Bender and Wallenfels (1961). Pullulanases are produced by several types of microorganisms when they are fed with starch as the main source of carbohydrate.

Mesophilic bacteria such as *Klebsiella pneumoniae* (Bender and Wallenfels, 1961), formerly classified as *Aerobacter aerogenes* (Vihinen and Montsolo, 1989), *Streptococcus mitis*, (Walker, 1968) and *Bacillus sp*. (Nakamura *et al.,* 1975) produce pullulanase that hydrolyze α-l,6 linkages. Several thermophiles such as *Bacillus flavocaldurius* (Suzuki *et al.,* 1991), *Thermoactinomyces thalpophilus* (Odibo and Obi, 1988), *Fervidobacterium pennavoruns* (Koch *et al.,* 1997) and *Bacillus acidopullulyticus* (Kusano *et al.,* 1988) can also produce pullulanase. Research on the production of thermostable pullulanase by microorganisms such as *Thermoanaerobium*, *Clostridium*, *Bacillus, Pyrococcus* and *Fervidobacterium* (Plant *et al.,* 1987; Saha *et al.*, 1988; Rudiger *et al.,* 1995; Koch *et al.,* 1997) has given new hope to starch processing industries.

In order to enhance pullulanase production, parameters such as medium composition need to be optimized. The appropriate fermentation medium should be created as it can extensively affect biomass and product concentration. From the viewpoint of industries, cost of medium can substantially affect the economics of production.

The production of pullulanase is normally carried out in batch culture. No research had been performed regarding the production of pullulanase using fed batch system. Many authors have reported that pullulanase production was highly dependent on strain, medium composition such as substrate concentration and nitrogen sources and also culture conditions such as temperature, agitation and aeration. Fed batch system is theoretically better than batch system as it can extend product formation stage besides helping to reduce medium viscosity and eliminate repressive effects of rapidly utilized carbon sources. The fed batch method has also been practiced to improve the production of phycocyanin (Zhang *et al.,* 1998), recombinant β-1,3glucanase (Shene *et al.,* 1999), protease from *Bacillus sphaericus* (Singh *et al.,* 2004), polysaccharide and ganoderic acid from *Ganoderma lucidum* (Tang and Zhong., 2002) and xylitol from *Bacillus licheniformis* (Yoon *et al.,* 2000).

Pullulanase is mainly used in food industries to produce simple sugars such as glucose, maltose and fructose from starch. Properties of starch and the process for the production require harsh conditions such as high temperature. The main purpose of this study is to reveal the capability of the thermophilic microorganism *Bacillus flavothermus* KWF-1 to produce thermostable pullulanase. Previous study by Rozaimi (2006) on this strain shows that the pullulanase produced is thermostable as the optimum temperature for the crude enzyme was at 80°C. His study also shows that at 90°C the pullulanase activity retained at 90%. This can contribute to the enhancement of product production by accelerating reaction rate and reducing production cost and time. Besides that, the usage of sago starch as the limiting substrate in fed batch system can contribute to our nation's economy as Malaysia is one of the leading producers of sago.

1.2 Objectives of Study

The objective of this research is to enhance the production of thermostable pullulanase under optimized conditions in fed batch culture using *Bacillus flavothermus* KWF-1 and sago starch as the limiting substrate. Response Surface Methodology will be used to optimize the fermentation parameters. Thermostable pullulanase would aid the starch processing industries that require elevated temperature.

1.3 Scope of Study

The scope of the research consists of five parts:

i. To carry out a comparative study of pullulanase production in batch and fed-batch fermentation

ii. To investigate the best carbon concentration and nitrogen sources that give the

highest increment for pullulanase production

iii. To assess different feeding strategies in fed batch culture for highest production of pullulanase

iv. To optimize the carbon and nitrogen concentration in the feed medium to yield maximum pullulanase production using Central Composite Design

v. Detection of different polyoses produced using HPLC

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