UNIVERSITI PUTRA MALAYSIA

IMPROVEMENT OF CYCLODEXTRIN GLYCOSYLTRANSFERASE BIOSYNTHESIS BY RECOMBINANT Lactococcus lactis NZ:NSP:CGT

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By

AZIN AMIRI

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

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Dedicated to

my beloved parents

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

IMPROVEMENT OF CYCLODEXTRIN GLYCOSYLTRANSFERASE BIOSYNTHESIS BY RECOMBINANT *Lactococcus lactis* NZ:NSP:CGT

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March 2014

Chairman: **Assoc. Prof. Rosfarizan Mohamad, PhD**
Faculty: **Biotechnology and Biomolecular Sciences**

Cyclodextrin glycosyltransferase (CGTase) is a distinctive enzyme that has the capability of producing cyclodextrin (CD) from starch. The CD as the product of CGTase has numerous applications in various industries such as foods, cosmetics and toiletries, textiles and agrochemistry. Therefore, CGTase is considered as an industrially important enzyme and its production improvement is very crucial. So, essential efforts to increase its activity are desirable. CGTase production has never been investigated in Generally Regarded as Safe (GRAS) organism, *Lactococcus lactis* despite its advantages. The CGTase biosynthesis by recombinant *Lactococcus lactis* NZ:NSP:CGT using different carbon sources ((corn starch), potato (dextrin from starch), tapioca starch and several soluble potato starches) and nitrogen sources (yeast extract, meat extract, peptone from meat, peptone from soymeal and peptone from casein) was carried out in batch cultivation using 250 mL shake-flask. Statistical optimization was performed using artificial neural network technique in order to optimize the culture condition (temperature) and medium compositions (carbon and nitrogen sources concentrations) to achieve maximum CGTase production. The experimental data from the aforementioned fermentation experiments were analyzed in order to obtain the kinetic parameter values and establish the basis of a kinetic model. The optimum parameters obtained were used to run batch fermentation in a 2L stirred tank bioreactor. The best carbon source leading to maximum CGTase biosynthesis was determined as Nacalai Tesque GR soluble potato starch. The maximum CGTase activity and productivity obtained by this carbon source were 7.99 U/mL and 1 U/mL.h, respectively. Yeast extract (Merck) was selected as the best nitrogen source due to its highest CGTase activity (9.88 U/mL) and productivity (0.99 U/mL.h) obtained. In screening stage of CGTase fermentation, carbon source concentration, nitrogen source concentration and temperature were recognized as three significant fermentation parameters. The optimum values for these parameters were determined through statistical optimization as 20°C for temperature and 3.82 and 5.67% (w/v) of soluble starch and yeast extract concentrations, respectively. The maximum CGTase activity obtained using the optimum values was 22.09 U/mL, which was closed to the predicted value (24.17 U/mL). The models used in this study were based on unstructured model equations including logistic and Luedeking-Piret, which were suitable to explain the growth, substrate consumption and CGTase production by *L. lactis* NZ:NSP:CGT in batch cultivation. According to the results, CGTase

production is a growth-associated process. Production of CGTase in 2L stirred tank bioreactor (15.36 U/mL) was lower than shake-flask, which shows the essential optimization studies in bioreactor scale.

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

PENAMBAHBAIKAN BIOSINTESIS SIKLODEKSTRIN GLIKOSILTRANSFERASE OLEH *Lactococcus lactis* NZ:NSP:CGT REKOMBINAN

Oleh

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Mac 2014

Pengerusi: **Assoc. Prof. Rosfarizan Mohamad, PhD**
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Siklodekstrin glikosiltranferase (CGTase) ialah enzim yang jelas berbeza dan berkeupayaan menghasilkan siklodekstrin (CD) daripada kanji. CD sebagai produk CGTase mempunyai banyak kegunaan dalam pelbagai industri seperti industri makanan, kecantikan dan kelengkapan kebersihan diri, pakaian dan agrokimia. Oleh itu, CGTase adalah dianggap sebagai enzim yang penting dalam industri dan penghasilannya adalah amat penting. Justeru itu, penghasilan CGTase daripada organisma yang boleh dianggap selamat (GRAS) seperti *Lactococcus lactis* NZ:NSP:CGT menggunakan sumber karbon berbeza; (kanji jagung, ubi kentang (dekstrin daripada kanji), kanji ubi kayu dan beberapa jenis kanji boleh larut daripada ubi kayu) dan sumber nitrogen (ekstrak yis, ekstrak daging, pepton daging, pepton minyak kacang soya dan pepton kasein) telah dilakukan dalam pengkulturan sesekelompok menggunakan 250 mL kelalang kon. Pengoptimuman secara statistik telah dibuat menggunakan teknik rangkaian neural tiruan untuk mengoptimumkan keadaan kultur dan komposisi media untuk memperoleh penghasilan CGTase yang paling maksima. Data eksperimen daripada eksperimen fermentasi yang disebutkan telah dianalisis untuk memperoleh nilai parameter kinetik dan membuat satu model kinetik asas. Parameter optimum yang diperoleh telah digunakan untuk melakukan fermentasi kelompok di dalam bioreaktor tangki berpengaduk 2L. Sumber karbon terbaik yang dapat menghasilkan CGTase secara maksima adalah kanji boleh larut daripada ubi kayu Nacalai Tesque GR. Aktiviti dan penghasilan maksima CGTase yang diperoleh menerusi sumber karbon ini adalah 7.99 U/mL bagi aktiviti dan 1.00 U/mL.h untuk penghasilannya. Ekstrak yis (Merck) dipilih sebagai sumber nitrogen terbaik disebabkan aktiviti (9.88 U/mL) dan produktiviti (0.99 U/mL.h) CGTase yang tinggi diperoleh daripadanya. Dalam peringkat saringan fermentasi CGTase, kepekatan sumber karbon, kepekatan sumber nitrogen dan suhu telah dikenalpasti sebagai parameter fermentasi yang penting. Nilai optimum untuk parameter ini telah ditentukan menerusi pengoptimuman secara statistik di mana suhunya adalah 20°C, 3.82% (w/v) kanji boleh larut dan 5.67% (w/v) kepekatan ekstrak yis. Aktiviti CGTase paling maksima yang diperoleh dengan menggunakan nilai optimum adalah 22.09 U/mL, di mana berdekatan dengan nilai jangkaan (24.17 U/mL). Model yang digunakan dalam kajian ini adalah berdasarkan persamaan model tidak berstruktur termasuk Logistik dan Luedeking-Piret yang sesuai untuk menerangkan proses pertumbuhan, penggunaan sumber dan penghasilan CGTase oleh *L. lactis*

NZ:NSP:CGT dalam pengkulturan sesekelompok. Berdasarkan kepada keputusan, penghasilan CGTase adalah proses berkaitan dengan pertumbuhan. Penghasilan CGTase di dalam bioreaktor tangki berpengaduk 2L (15.36 U/mL) adalah lebih rendah berbanding di dalam kelalang kon dimana menunjukkan pengoptimuman adalah penting untuk dikaji dalam skala bioreaktor.

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I certify that a Thesis Examination Committee has met on (**6 of March**) to conduct the final examination of Azin Amiri on her thesis entitled "**Improvement of Cyclodextrin Glycosyltransferase Biosynthesis by Recombinant *Lactococcus lactis* NZ:NSP:CGT**" in accordance with the University 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 M.Sc degree.

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LIST OF ABBREVIATIONS

AAD	Absolute Average Deviation
CCD	Central Composite Design
CD	Cyclodextrin
CGTase	Cyclodextrin Glycosyltransferase
DCW	Dry Cell Weight
DOT	Dissolved Oxygen Tension
E. coli	*Escherichia coli*
EC	Enzyme Classification
Eq	Equation
F. S.	Friendemann Schmidt
GRAS	Generally Recognized as Safe
IBP	Incremental Back Propagation
IUBMB	International Union of Biochemistry and Molecular Biology
L. lactis	*Lactococcus lactis*
LAB	Lactic Acid Bacteria
max	Maximum
OD	Optical Density
PBD	Placket-Burman Design
Pr	Productivity
RMSE	Minimum Root Square Error
RSM	Response Surface Methodology
μ	Specific Growth Rate

CHAPTER 1

INTRODUCTION

Cyclodextrin glycosyltransferase (EC 2.4.1.19; CGTase), is a "carbohydrate-converting", "bacterial glycosyltransferase" (Subramaniam *et al.*, 2012). CGTase is an enzyme that catalyzes the conversion of starch and related substances to cyclodextrins through cyclization reaction (Ibrahim *et al*., 2005). It catalyzes other transferase reactions including disproportion and coupling beside cyclization. CGTase displays minor hydrolysis activity as well (Rahman *et al.* 2004).

Cyclodextrin (CD) is a cyclic malto-oligosaccharide molecule, which is formed of 6 to 60 glucose monomers (Vassileva *at al.*, 2003). CDs most commonly synthesized are α-, β- and γ-CDs, which consist of 6, 7 and 8 glucose units (Mahat *et al.*, 2004). CD holds a hydrophobic central cavity and a hydrophilic outer surface (Vassileva *et al.*, 2003).

According to Dodziuk (2006a, b) and Uekama *et al.* (2006), various molecules can enter the CDs' cavity and almost all the applications of CDs include their inclusion complex formation capability leading to their wide uses in different industries. For instance, CDs are applied in foods, cosmetics and toiletries, textiles and agrochemistry. In food industry, CDs are employed for stabilization by powdering (flavor or spices, fish oil, coffee, green tea), taste modification, anti-oxidation and improvement of bioavailability. CDs applications are also expanded to pharmaceuticals. They are employed to study different properties of drugs such as release control, site-specific drug delivery, absorption enhancement and so on. They can also be utilized in gene therapies. CDs also assist in improving the solubility and stability, reducing volatility and masking odors and tastes which result in increased popularity with their extensive use in various industries (Sian *et al.*, 2005). The vast applications of CD, increases the attentions focused on CGTase.

Microorganisms synthesize CGTase in order to catalyze the conversion of starch present in their environment to cyclodextrin for the purpose of growth and survival (Wang *et al.*, 2005). Although, enhancement of CGTase biosynthesis is of great interest (due to improvement of CD production), there is no report on CGTase production by *L. lactis* species. Therefore, no studies on optimization of culture conditions and medium compositions for the purpose of maximum CGTase production by *L. lactis* have been conducted. Recently, Subramaniam *et al.* (2012) has constructed a recombinant *L. lactis* strain capable of producing CGTase. The CGTase gene originally from *Bacillus* sp. G1 (Illias *et al.*, 2003) was cloned in *Escherichia coli* (Ong *et al.*, 2008). According to Subramaniam *et al.* (2012), the CGTase quality might be reduced in *Bacillus* and *E. coli* due to presence of some impurities such as proteases. Therefore, CGTase production studies in *L. lactis* are desirable.

The CGTase activity obtained by recombinant *L. lactis* NZ:NSP:CGT strain was very low and needed improvement through fermentation techniques. There are various methods available for fermentation optimization. One of the useful techniques is mathematical optimization with different tools available. Artificial

neural network (ANN) is one of the softwares employed for optimization of various product formation processes through fermentation process. There is no literature available in regard to CGTase biosynthesis using ANN. In this study, ANN was employed for the purpose of optimization of CGTase biosynthesis by recombinant *L. lactis* NZ:NP:CGT.

In most fermentation processes, mathematical models are required to control, optimize, simulate and scale up of the process in lots of unit operations (Rosfarizan, 2000). Information on the kinetics and modeling of CGTase production is very scarce. Therefore, a set of experiments is necessary in order to develop better understanding of CGTase fermentation process. The results could be used for estimation of kinetic parameters, which are prerequisite for mathematical model development. The model helps in better understanding of the whole process as well as control of CGTase biosynthesis by *L. lactis* NZ:NSP:CGT. This study is one of the first attempts in terms of studying the CGTase fermentation kinetics and modeling.

Generally, the focus of this study was on development of a process aimed at establishing high performance CGTase fermentation using a newly constructed recombinant *L. lactis* NZ:NSP:CGT strain. The objectives of this research were;

1. To evaluate the influences of different types of carbon and nitrogen sources on CGTase biosynthesis by *L. lactis* NZ:NSP:CGT in shake-flask culture.

2. To improve the CGTase biosynthesis by *L. lactis* NZ:NSP:CGT through medium compositions and environmental conditions optimization by statistical experimental design techniques.

3. To perform the kinetics and modeling of CGTase fermentation by *L. lactis* NZ:NSP:CGT in shake-flask scale followed by evaluation of CGTase biosynthesis in a 2L stirred tank bioreactor system.

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