MANIPULATION OF AMYLASE REACTION TO IMPROVE THE REDUCING SUGARS PRODUCTION

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MANIPULATION OF AMYLASE REACTION TO IMPROVE THE REDUCING SUGARS PRODUCTION

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To my beloved family

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ABSTRACT

An *Anoxybacillus* strain SK3-4 was previously isolated from Perak Sungai Klah hot spring. The α-amylase gene fragment from *Anoxybacillus* sp. denoted as ASKA was cloned into pET-22b(+) and transformed into *Escherichia coli* BL21 (DE3). However, the reactivity and productivity of this amylase is underexplored. The main objective of this project is to optimize the reducing sugars production using Response Surface Methodology (RSM). The ASKA substrate specificity was determined using soluble starch and nine different commercial starches: corn, tapioca, wheat, potato, rice, sago, rye, green peas and glutinous rice starch. Sago starch was found to be the best substrate with highest reducing sugars production. Variable parameters such as reaction temperature, sago starch and ASKA concentration were screened using one-factor-at-a-time (OFAT) approach before they were optimized through two-level full factorial design and central composite rotatable design (CCRD). Statistical analysis showed that all the three parameters were significant factors in 2^3 full factorial design before further optimized the reducing sugars production with CCRD. The final optimized parameters using CCRD was capable to produce 7.97 g/L reducing sugars with 2.64 % (w/v) sago starch and 0.375 unit ASKA under 66.9 ºC reaction temperature. The hydrolysis products were determined using High Performance Liquid Chromatography (HPLC). Maltose was the major hydrolysis product and no glucose production was detected. As a conclusion, applying experimental designs method was able to improve the efficiency of reducing sugars production for 87.09 % compared with the reference reaction condition with maltose as the major end product.

ABSTRAK

Satu bakteria species *Anoxybacillus* (SK3-4) telah berjaya dipencilkan dari kolam air panas Sungai Klah (SK) di Perak. Species *Anoxybacillus* tersebut mengandungi gen α-amilase yang di namakan sebagai ASKA. Gen α-amilase itu telah diklonkan dalam vektor pET-22b(+) di dalam *E. coli* BL21 (DE3). Namun begitu, tindak balas dan produktiviti α-amilase tersebut masih belum dikaji. Oleh yang demikian, objektif utama kajian ini adalah untuk mengoptimumkan penghasilan oligosakarida dengan menggunakan *Response Surface Methodology* (RSM). Spesifisiti ASKA terhadap substrat telah ditentukan dengan menggunakan kanji terlarut dan sembilan jenis kanji komersil lain iaitu kanji jagung, ubi kayu, gandum, kentang, beras, sagu, rai, kacang hijau dan beras pulut. Kanji sagu dikenal pasti sebagai substrat terbaik dengan penghasilan oligosakarida tertinggi. Tiga jenis faktor iaitu suhu tindak balas, kepekatan kanji sagu dan kepekatan ASKA telah disaring dengan menggunakan kaedah satu-faktor-pada-satu masa (OFAT). Analisis statistikal rekabentuk 2^k faktorial penuh menunjukkan bahawa ketiga-tiga faktor itu adalah signifikan dalam mempengaruhi penghasilan oligosakarida. Ketiga-tiga faktor itu kemudian dimanipulasi menggunakan rekabentuk komposit kebolehputaran pusat (CCRD) untuk mengoptimumkan penghasilan oligosakarida. Keadaan tindak balas yang optimum adalah pada suhu 66.9 ºC, 2.64 % (w/v) kanji sagu dan 0.375 unit ASKA dengan penghasilan oligosakarida sebanyak 7.97 g/L. *High Performance Liquid Chromatography* (HPLC) kemudiannya digunakan bagi menentukan produk hidrolisis itu. Maltosa adalah produk utama hidrolisis dan tiada penghasilan glukosa dicatat. Kesimpulannya, penggunaan rekabentuk eksperimen berjaya meningkatkan penghasilan oligosakarida sebanyak 87.09 % daripada tindak balas rujukan dengan maltosa sebagai produk utama hidrolisis.

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LIST OF SYMBOLS/ ABBREVIATIONS

ANOVA	-	Analysis of variance
ASKA	-	*Anoxybacillus* species SK3-4 alpha-amylase
B.	-	*Bacillus*
Ca^{2+}	-	calcium ion
$CaCl_2$	-	calcium chloride
CCRD	-	central composite rotatable design
C.I.	-	confidence interval
CV	-	coefficient of variation
DNS	-	3,5-dinitrosalicylic acid
E. coli	-	*Escherichia coli*
g	-	gram
G1	-	glucose
G2	-	maltose
G3	-	maltotriose
G4	-	maltotetraose
G5	-	maltopentaose
g/L	-	gram per liter
HCl	-	hydrochloric acid
HPLC	-	High Performance Liquid Chromatography
IPTG	-	isopropyl β-D-thiogalactopyranoside
IU	-	international unit
kDa	-	kilodalton
kPa	-	kilo pascal
L	-	liter
LB	-	Luria-Bertani
mg	-	miligram

min	-	minute(s)
mL	-	mililiter
mm	-	milimeter
mM	-	milimolar
MW	-	molecular weight
MWCO	-	molecular weight cut-off
NaCl	-	sodium chloride
NaOH	-	sodium hydroxide
nm	-	nanometer
OD	-	optical density
OD_{600}	-	optical density at 600 nm
OFAT	-	one-factor-at-a-time
PES	-	polyethersulfone
PRESS	-	predicted residual sum of squares
P-value	-	probability value
R^2	-	coefficient of determination
rpm	-	revolutions per minute
RSM	-	Response Surface Methodology
SK	-	Sungai Klah
sp.	-	species
Tris	-	tris(hydroxymethyl)methylamine
U	-	unit of enzyme activity
v/v	-	Volume per volume
w/v	-	weight per volume
α	-	alpha
µ	-	micro
µg	-	microgram
µL	-	microliter
µm	-	micrometer
µmol	-	micromole
%	-	percentage
ºC	-	degree Celcius
3D	-	three-dimensional

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CHAPTER 1

INTRODUCTION

1.1 Background of research

Starch is one of the most abundant natural storage polysaccharides synthesized by plants. The hydrolysis of the complex starch structure required amylolytic enzymes to depolymerise it and form oligosaccharides and small sugars. The world today shows an increasing interest in investigating the usage of amylolytic enzymes for biorefinery in varieties of industries; include the food product and non-food product industries. Amylolytic enzymes act on starch and can be categorized into four different groups, i.e. the exo acting amylases, endo acting amylases, debranching amylases and cyclodextrinases (Nigam and Pandey, 2009). α-Amylase (EC 3.2.1.1) is one of the endo acting amylases (endo-1,4-α-D-glucan glucohydrolase) which is capable to hydrolyze internal α-D-1,4-glycosidic linkages in amylopectin and glycogen (Richardson *et al.*, 2002).

Alpha-amylase can be found in plants, animals and microorganisms as it plays a dominant role in their carbohydrate metabolism. Since 1980, mesophile *Bacillus licheniformis* (Richardson *et al.*, 2002) is highly used for industrial application due to its extreme thermostability. Others α-amylase producers include *B. subtilis* (Konsula and Liakopoulou-Kyriakides, 2003), *B. amyloliquefaciens* (Demirkan, 2005), *B. stearothermophilus* (Kim *et al.*, 1989), *Aspergillus* species and *Penicillium* sp. (Gouda and Elbahloul, 2008). Thermophilic *Anoxybacillus* which was first described by Pikuta *et al.* (2000) also contains the ability to undergo extra-cellular amylase activity (Poli *et al.*, 2006).

Amylases have been applied in varieties of industries; include food, textile, paper, pharmaceutical and detergent industries (Shigechi *et al.*, 2004). High demand of amylases has encouraged the discovery of new amylases from different microorganisms sources with an aim to find alternative that could lower the cost and power requirement. Amylase reaction condition is also playing an important role for enzyme stabilizing, which will subsequently increase the enzyme reactivity and influence the products formation (Sivaramakrishnan *et al.*, 2006).

An in-house *Anoxybacillus* strain SK3-4 was previously isolated from Sungai Klah (Perak) hot spring. The α-amylase gene fragment from *Anoxybacillus* sp. was cloned into pET-22b(+) and transformed into *E. coli* BL21 (DE3) (Chai, 2012). The recombinant α-amylase (denoted as ASKA) has an optimum activity of pH 8 and 60 °C.

Physical and chemical parameters are two categories that influence the enzymatic hydrolysis reaction (Agrawal *et al.*, 2005). The physical parameters include starch source, starch condition, pH of the reaction mixture, reaction temperature and the incubation period for enzymatic reaction. While chemical parameters are starch concentration, enzyme concentration, presence and the concentration of divalent ions and other stabilizing agents (Richardson *et al.*, 2002; Sivaramakrishnan *et al.*, 2006; Tester *et al.*, 2006; Tamilarasan *et al.*, 2010).

Conventional one-factor-at-a-time approach for optimization process is time consuming and tedious. Therefore, response surface methodology (RSM) which designs and analyzes the experimental result through mathematical and statistical techniques can be useful to solve the complexity of one-factor-at-a-time approach and optimize the response. In this study, two-level-full-factorial and central composite design (CCD) will be applied to optimize the reducing sugars production which involves various factors such as reaction temperature, starch and α-amylase concentration.

1.2 Problem statement

The study of amylase from *Anoxybacillus* is an interesting field since the function and reactivity of this amylase is underexplored. The application of ASKA is an economic alternative for high temperature liquefaction process. Thus, optimize the reducing sugars production by novel recombinant amylase is important. This ultimately provides an alternative to produce high amount of reducing sugars with less expenditures.

1.3 Objectives

i. To identify the best substrate for *Anoxybacillus* sp. amylase (ASKA).
ii. To screen the variable parameters that will influence the reducing sugars production.
iii. To optimize the relevant factors that involve in reducing sugars production by ASKA reaction through two-level full factorial and central composite rotatable design (CCD).
iv. To determine the end product of ASKA hydrolysis reaction using HPLC.

1.4 Scopes of research

i. Determination of the best substrate for ASKA using nine food-grade starches.
ii. Possible reducing sugars production ranges determination using conventional one-factor-at-a-time (OFAT).
iii. Optimization and validation of reducing sugars production by ASKA enzymatic reaction through 2^3 full factorial design and central composite design (CCD).
iv. Analysis of ASKA reaction products by HPLC.

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