PRODUCTION AND CHARACTERIZATION OF AMYLASE FROM D-LACTIC ACID-PRODUCING BACTERIUM *Lactobacillus* sp. SUTWR 73

Ekkasit Kanklang

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Microbiology

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การผลิตและศึกษาคุณลักษณะของอะไมเลสจากแบคทีเรียผลิตกรดดี-แล็กติก *Lactobacillus* sp. SUTWR 73

นายเอกสิทธิ์ แก่นกลาง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

สาขาวิชาจุลชีววิทยา

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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เอกสิทธิ์ แก่นกลาง : การผลิตและศึกษาคุณลักษณะอะไมเลสจากแบคทีเรียผลิตกรดดี-แล็กติก *Lactobacillus* sp. SUTWR 73 (PRODUCTION AND CHARACTERIZATION OF AMYLASE FROM D-LACTIC ACID-PRODUCING BACTERIUM *Lactobacillus* sp. SUTWR 73) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.สุรีลักษณ์ รอดทอง, 191 หน้า.

อะไมเลสเป็นเอนไซม์ที่มีความสำคัญในการผลิตกรดอินทรีย์ในกลุ่มมอนอเมอร์เพื่อการผลิตพลาสติกชีวภาพจากแป้งด้วยจุลินทรีย์ *Lactobacillus* sp. SUTWR 73 เป็นแบคทีเรียสร้างอะไมเลสที่สามารถผลิตกรดดี-แล็กติกได้โดยตรงจากแป้ง การใช้แป้งความเข้มข้นสูงเพื่อให้ได้กรดปริมาณมาก มีปัญหาความหนืดในระยะเริ่มต้นของการผลิตกรดที่ต้องอาศัยอะไมเลสทางการค้าช่วยลดความหนืด การศึกษานี้จึงมุ่งหาสภาวะที่เหมาะสมต่อการผลิตอะไมเลสจาก *Lactobacillus* sp. SUTWR 73 และศึกษาคุณลักษณะของเอนไซม์เพื่อการใช้ประโยชน์ โดยเฉพาะการทดแทนหรือลดการใช้เอนไซม์ทางการค้า ซึ่งพบว่า SUTWR 73 สามารถผลิตอะไมเลสได้ทั้งแบบหลั่งออกนอกเซลล์ (4.23±1.17 หน่วยต่อมิลลิลิตร) และติดอยู่ที่ผิวเซลล์ (0.0854±0.06 หน่วยต่อมิลลิลิตร) พร้อมทั้งได้ปรับปรุงสายพันธุ์ของแบคทีเรียให้แข็งแรงและเสถียรขึ้นโดยกระตุ้นด้วยแสงอัลตร้าไวโอเลต (ยูวี) ซ้ำ 8 ครั้ง คัดเลือกได้สายพันธุ์ SUTWR 73-1 ที่เสถียร ผลิตอะไมเลสได้ 8.79±1.33 หน่วยต่อมิลลิลิตร (4.20±0.061หน่วยต่อมิลลิกรัมโปรตีน) ในอาหารแป้งมันสำปะหลังสูตรเริ่มต้น จากนั้นได้พัฒนาสูตรอาหารเพื่อผลิตอะไมเลสจาก SUTWR 73-1 โดยทดลองแบบแฟกทอเรียลและใช้วิธีการทดสอบแบบพื้นผิวตอบสนอง (อาร์เอสเอ็ม) เพื่อหาปัจจัยสำคัญและระบุสภาวะที่เหมาะสมสำหรับการผลิตอะไมเลส พบว่า ปัจจัยที่สำคัญต่อการผลิตอะไมเลส ได้แก่ ความเข้มข้นของแป้งมันสำปะหลัง ความเข้มข้นของทริปโตน และค่าพีเอชเริ่มต้นของอาหาร สูตรอาหารที่เหมาะสมเป็นสูตรอาหารมาตรฐานเอ็มอาร์เอสที่มีแหล่งคาร์บอนและแหล่งไนโตรเจนคือแป้งมันสำปะหลังและทริปโตนเข้มข้นร้อยละ 10 และ 4.5 ตามลำดับ ที่ค่าพีเอชเริ่มต้นของอาหารเท่ากับ 9.0 นำค่าที่ได้นี้ไปใช้สร้างแบบจำลอง พบว่าแบบจำลองที่ได้สามารถทำนายสภาวะที่เหมาะสมต่อการผลิตอะไมเลสที่ความเข้มข้นของแป้งและทริปโตนร้อยละ 11.70 และ 4.68 ตามลำดับ มีค่าพีเอชเริ่มต้นเท่ากับ 8.87 เมื่อยืนยันโดยทดลองที่ระดับการผลิตเอนไซม์ด้วยอาหารเลี้ยงเชื้อปริมาตร 50 มิลลิลิตร ขวดรูปชมพู่ขนาดบรรจุ 125 มิลลิลิตร ได้อะไมเลสสูงถึง 33.54 หน่วยต่อมิลลิลิตร เมื่อเพิ่มระดับการผลิตเอนไซม์ด้วยการเพาะเลี้ยงในอาหารเลี้ยงเชื้อที่มีแป้งมันสำปะหลังร้อยละ 5 ปริมาตรอาหาร 3 ลิตร ในถังปฏิกรณ์ชีวภาพขนาดบรรจุ 5 ลิตร ได้กิจกรรมของอะไมเลสสูงสุด 17.00±0.44 หน่วยต่อมิลลิลิตร ที่ 18 ชั่วโมงของการเลี้ยงเชื้อ ใกล้เคียง

กับที่ใช้แบบจำลองทำนาย กิจกรรมจำเพาะของอะไมเลสสูงสุดได้จากการทำบริสุทธิ์ด้วยแอมโมเนียมซัลเฟตอิ่มตัวร้อยละ 70 เท่ากับ 17.34 หน่วยต่อมิลลิกรัมโปรตีน อะไมเลสที่ได้มีน้ำหนักโมเลกุล 46.04 กิโลดาลตัน ที่ค่าคงที่ของมิเชลิส-เมนเทนและอัตราความเร็วสูงสุดของอะไมเลสต่อแป้งสับสเตรทเท่ากับ 0.167 มิลลิกรัมต่อมิลลิลิตร และ 1.67×10^{4} ไมโครโมลาร์ต่อนาที ตามลำดับ อะไมเลสที่ได้ทำงานได้ดีที่สุดที่พีเอช 6.8 และยังคงทำงานได้ดีในช่วงพีเอช 6.0 ถึง 9.0 อุณหภูมิที่เหมาะสมเท่ากับ 35 องศาเซลเซียส เมื่อพิจารณาเสถียรภาพของอะไมเลสที่ได้ พบว่าการบ่มเอนไซม์ที่พีเอช 8.0 และ 9.0 เป็นเวลา 30 นาที ให้กิจกรรมสูงกว่าสภาวะเริ่มต้นถึง 1.38 และ 1.33 เท่า ตามลำดับ นอกจากนี้อะไมเลสยังทำงานได้ดีที่สุดภายหลังบ่มก่อนทำปฏิกิริยาที่อุณหภูมิ 25 องศาเซลเซียส เป็นระยะเวลา 50 นาที ซึ่งพบว่าเอนไซม์มีกิจกรรมสูงขึ้นจากสภาวะเริ่มต้นถึง 2.09 เท่า อะไมเลสที่ได้สามารถกระตุ้นด้วยการเติมแบเรียม แมกนีเซียม แมงกานีส และเฟอรัสไอออน และยับยั้งได้โดยเอทิลีนไดเอมีนเตตระอะซิติกแอซิด (อีดีทีเอ) และแคลเซียมไอออน จากผลข้างต้น อะไมเลสที่ผลิตได้จัดเป็นอะไมเลสที่มีเสถียรภาพในสภาวะด่างและทำงานได้ดีที่อุณหภูมิต่ำโดยสามารถกระตุ้นได้โดยไอออนของโลหะ ด้วยสมบัติการเร่งปฏิกิริยาที่อุณหภูมิต่ำและสภาวะด่างทำให้สามารถลดการใช้พลังงานในกระบวนการผลิตที่กำลังการผลิตสูง เช่น การผลิตกรดแล็กติก และยังสามารถต่อยอดไปสู่อุตสาหกรรมอื่นได้

สาขาวิชาปรีคลินิก
ปีการศึกษา 2561

ลายมือชื่อนักศึกษา ศุภลักษณ์ แก่นกลาง
ลายมือชื่ออาจารย์ที่ปรึกษา
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม

EKKASIT KANKLANG : PRODUCTION AND CHARACTERIZATION OF AMYLASE FROM D-LACTIC ACID-PRODUCING BACTERIUM *Lactobacillus* sp. SUTWR 73. THESIS ADVISOR : ASST. PROF. SUREELAK RODTONG, Ph.D. 191 PP.

AMYLASE PRODUCTION/AMYLASE PURIFICATION/*LACTOBACILLUS*/ /AMYLASE/OPTIMIZATION/LACTIC ACID BACTERIUM

Amylase is an important enzyme for the microbial production of organic acids from starch, especially the acids used as monomers for bioplastics production. The bacterium *Lactobacillus* sp. SUTWR 73 can produce amylase, and directly convert starch to D-lactic acid. The utilization of high starch concentration to obtain high acid yield faces the high viscosity problem at initial stage of the acid production. Commercial amylases are then required. This study aimed to determine optimum conditions for amylase production from *Lactobacillus* sp. SUTWR 73, and to characterize the enzyme for further applications, especially the purpose for replacing or reducing the commercial enzymes. It is found that SUTWR 73 could produce both extracellular (4.23±1.17 U/ml) and cell-associated amylase (0.0854±0.06 U/ml). The strain was also improved by repeating UV exposure for 8 times to obtain a strong and stable mutant. The mutant strain SUTWR 73-1 was selected as its capability to produce amylase exhibiting the highest activity of 8.79±1.33 U/ml (0.420±0.061 mg/ml) among other mutants when cultivated in the original cassava starch medium. The amylase production medium for SUTWR 73-1 was then developed to support the maximum enzyme yield using a factorial experiment and response surface method (RSM) for identifying the enzyme production key factors, which were found to be cassava

starch and tryptone concentrations, and initial pH of the medium. The optimized medium contained cassava starch and tryptone as C-and N-sources at 10 and 4.5%(w/v), respectively, with the initial pH of 8.0. These values were used to construct the model, that could predict the optimum condition for amylase production as follows: the suitable medium contained 11.70 and 4.68%(w/v) of cassava starch and tryptone, respectively, at the initial pH of 8.87. When confirmed the conditions by performing two experimental scales, the highest amylase yields of 33.54 U/ml and 17.00±0.44 U/ml were detected at 18 h with 5% starch in 50-ml and 3-l medium in 125-ml flask and 5-l bioreactor, respectively. The results were as predicted by the model. The partially purified enzyme, 46.04-kD molecular weight, was obtained at 70% ammonium sulphate saturation with the highest specific activity of 17.34 U/mg. The kinetic values K_m and V_{max} toward the soluble starch were 0.167 mg/ml and 1.67×10^4 μM/min, respectively. The enzyme effectively performed at a wide pH range (pH 6.0 to 9.0) with the optimum pH of 6.8, and the optimal temperature at 35°C. When incubated the amylase from SUTWR 73-1 at pH 8.0 and 9.0 for 30 min, its activity was higher than the initial activity around 1.38 and 1.33 times, respectively. The highest activity was still obtained with incubating at 25°C for 50 min, which was found to be 2.09 times higher than the initial activity. The amylase was activated by Ba^{2+}, Mg^{2+}, Mn^{2+}, and Fe^{2+}. But it was inhibited by EDTA and Ca^{2+}. The application of this metalloenzyme stable and active in alkali and at low temperature, could decrease energy consumption in large scale operations such as lactic acid production, and could expand to other industry.

School of Preclinic

Academic Year 2018

Student's Signature EKKASIT KANKLANG

Advisor's Signature Sureelak Rodtong

Co-Advisor's Signature

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LIST OF ABBREVIATIONS

α	Alpha
β	Beta
γ	Gamma
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
ATP	Adenosine triphosphate
$BaCl_2$	Barium chloride
°C	Degree Celsius
$CaCl_2$	Calcium chloride
CCD	Central composite design
CFU	Colony forming unit
cm	Centrimeter
Co., Ltd.	Limited company
CRD	Completely randomized design
CuSO4	Copper (II) sulphate
DI	Deionized water
DMRT	Duncan's multiple range test
et al.	et alia (and others)
e.g.	For example
$FeSO_4$	Iron (II) sulphate

LIST OF ABBREVIATIONS (Continued)

(m, μ) g	(milli, micro) Gram
h	Hour
H_2	Hydrogen gas
HCl	Hydrochloric acid
H_2O	Water
HPLC	High performance liquid chromatography
i.e.	That is
Inc.	Incorporation
K_m	Michaelis-Menten constant
KCl	Potassium cloride
kD	Kilo Dalton
K_2HPO_4	Dipotassium hydrogen phosphate
K_2SO_4	Potassium sulphate
LAB	Lactic acid bacteria
l	Liter
μl	Microliter
μm	Micrometer
ml	Milliliter
mm	Millimeter
mM	Millimolar
min	Minute

LIST OF ABBREVIATIONS (Continued)

M	Molar
$MgSO_4$	Magnesium sulphate
$MgCl_2$	Magnesium chloride
$MnCl_2$	Manganese chloride
ng	Nanogram
$NiCl_2$	Nickel chloride
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
Na_2SO_4	Sodium sulphate
OD	Optical density
%	Percentage
P	P-value
RAM	Rogosa agar modified medium
rpm	Round per minute
RSM	Response surface method
SD	Standard deviation
SM	Submerged cultivation
SS	Solid state cultivation
subsp.	Subspecies
*T*m	Melting temperature

LIST OF ABBREVIATIONS (Continued)

U	Unit
U.S.A.	United States of America
UV	Ultraviolet
v/v	Volume by volume
w/v	Weight by volume

CHAPTER I

INTRODUCTION

1.1 Significance of the study

Microorganisms have their ability to produce many enzymes which are widely used in industrial processes. One kind of industrial enzymes is a starch-degrading enzyme which can convert the starch molecule into sugars, syrups, and destrins (Gupta et al., 2003). The starch-degrading enzymes are important in the food, paper, textile pharmaceutical, and sugar industries. The most popular starch-degrading enzyme is an amylase. Many industries use the enzyme to prepare their substrates from starch.

Our previous study used cassava starch as the main carbon source for D-lactic acid production by *Lactobacillus* sp. SUTWR 73. The lactic acid bacteria are important for producing biodegradable plastics because the addition of D-lactic acid is a constitutent of the biodegradable plastics to enhance thermostability of the plastics. Commercial α-amylase is needed to reduce the viscosity of high starch concentrations at >3.0% or 30 g/l (from unpublished data (2015)) for producing optical purity D- or L-lactic acid. Usually, only 10% of cassava starches are applied for D-lactic acid fermentation by our bacterial strain. Commercial α-amylase which is commonly used in our D-lactic acid production is costly. So, if the commercial amylase can be reduced to use in the starch substrate utilization process, the cost will be reduced too.

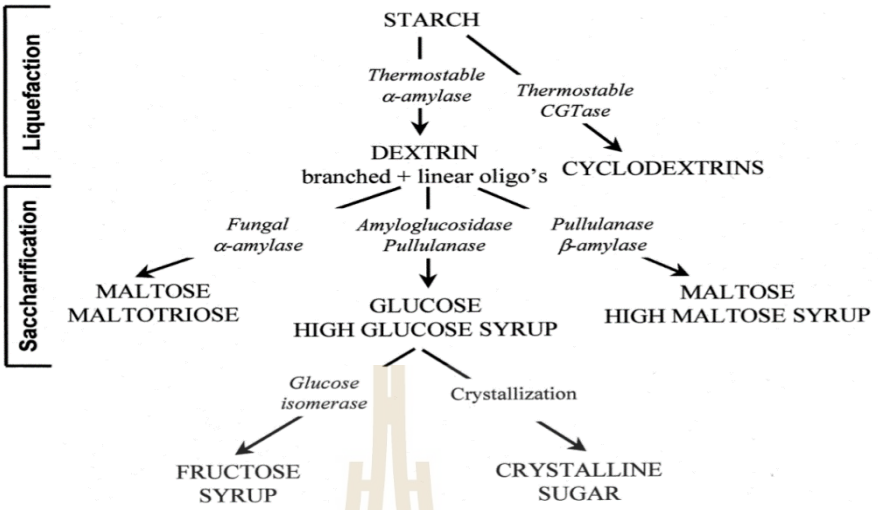


Figure 1.1 Overview of the industrial processing of starch involving with amylase (Van der Maarel et al., 2002).

This research project aimed to investigate amylase enzyme produced by a specific strain of D-lactic acid-producing bacteria. The enzyme could be used to replace the high-cost commercial enzyme currently used in our laboratory, and this enzyme could also be applied in larger laboratory and industrial-scale settings.

1.2 Research objectives

1.2.1 To optimize medium compositions for amylase production by *Lactobacillus* sp. SUTWR 73.

1.2.2 To partially purify amylase produced by *Lactobacillus* sp. SUTWR 73.

1.2.3 To characterize the amylase enzyme produced by *Lactobacillus* sp. SUTWR 73.

1.3 Research hypotheses

Amylase could be potentially produced by D-lactic acid-producing and starch-utilizing bacterium for assisting the starch hydrolysis process, particularly in lactic acid production and bio-plastic industry.

1.4 Scope and limitations of the study

This research project was comprised of steps of detection and location amylase of either extracellular or cell-bound amylase produced by D-lactic acid-producing and starch-utilizing bacterium (*Lactobacillus* sp. SUTWR 73) that obtained from stock cultures of the Microbial Culture Collection and Applications Research Centre, Suranaree University of Technology. The amylase production conditions for the lactic acid bacterium were then optimized by varying cassava starch concentration, nitrogen sources, temperature, and pH. Finally, the production, partial purification, and characterization of the bacterial amylase were performed and preliminarily characterized amylase enzyme.

1.5 Expected results

Amylase from *Lactobacillus* sp. SUTWR 73 could be detected as either extracellular or cell-bound enzyme. The optimum medium for low cost amylase production and the characteristics of the amylase produced by *Lactobacillus* sp. SUTWR 73 would be obtained.

CHAPTER II

LITERATURE REVIEWS

2.1 Amylase

Amylase is a useful enzyme widely used in many industries. The enzyme can hydrolyze starch molecules into diverse small polymers of carbohydrate composed of glucose units and other monosaccharides such as maltose, maltotriose, and maltotetraose as shown in Finore et al. (2016) and Gupta et al. (2003). Nowadays, more industries use it broadly, ranging from pulp and paper, textile, laundry detergents, dairy, baking, juice, and cosmetic industries (Li et al., 2012). Amylase can be derived from many sources due to its role in carbohydrate metabolism of animals, plants, fungi, and microorganisms. It is gradually getting high attention over a year. Lastly, amylase has become a popular enzyme covering 25% of the enzyme world market (Arikan, 2008).

2.1.1 Types of amylase

Amylase can be classified from the cleavage position on the polysaccharide chain and the products of the reaction. There are two types including an exoamylase and an endoamylase. Exoamylase is an enzyme capable of hydrolyzing starch from the ends of the polysaccharide chain. In addition to exoamylase, endoamylase is able to randomly hydrolyze the inner polysaccharide chains. Furthermore, we can also classify amylases into three subtypes α-amylase (EC 3.2.1.1), β-amylase (EC 3.2.1.2), and γ-amylase (EC 3.2.1.3).

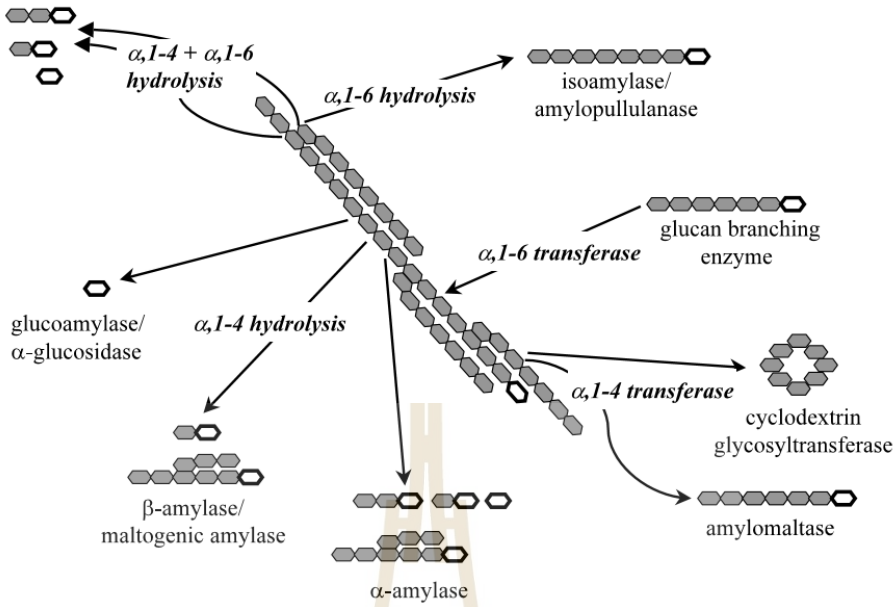


Figure 2.1 α-Amylase involved in the degradation of starch (Van der Maarel et al., 2002).

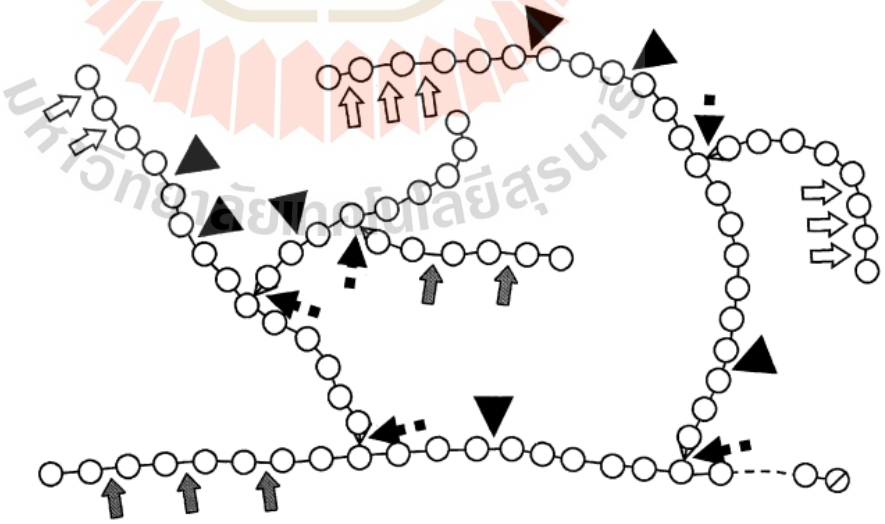


Figure 2.2 Schematic action pattern of amylase (Sakano, 2008).

α-Amylase is known as 1,4-α-D glucan glucanohydrolase or glycogenase. It belongs to the GH family 13 class and is also a calcium metalloenzyme meaning that it is completely unable to function without divalent calcium ion. However, there is some calcium-independent amylase. For example, Wang et al. (2016) found that their α-amylase can act without divalent calcium ion. It acts randomly at locations along the starch chain and breaks down long-chain carbohydrates into smaller molecular sugars. For its potential cleavability, it has become the most popular starch-converting enzyme broadly used on the industrial scale.

β-amylase is alternatively known as 1,4-α-D-glucanmaltohydrolase, glycogenase or saccharogen amylase. It can hydrolyze α-1,4 linkages at the non-reducing end of polysaccharide chain giving maltose as the end product. So, it is classified in GH family 14 similar as α-amylase.

The last, γ-amylase is also known as glucan 1,4-α-glucosidase, amyloglucosidase, exo-1,4-α-glucosidase, glucoamylase, lysosomal α-glucosidase or 1,4-α-D-glucanglucohydrolase. It is a member of GH family 15 not 14 as α-amylase and β-amylase. It cleaves the last α-(1-4)-glycosidic linkages at the non-reducing end of amylose and amylopectin, yielding glucose with a single displacement mechanism and also cleave α-(1-6)-glycosidic linkages (Vaidya et al., 2015).

2.1.2 Sources of amylase

Amylase is found in many organisms with carbohydrate metabolism because of the ability to convert starch into smaller oligosaccharide. Amylase can be derived from several sources, for instance, bacteria, fungi, plant, insect, as well as animal. Some studies report that amylase can be found in bacteria and fungi such as *Bacillus cereus* amy3 (Saha and Mazumdar, 2019), *Bacillus licheniformis* (Hwang et al., 1997),

Bacillus subtilis (Chen et al., 2015), *Geobacillus stearothermophilus* (Fincan and Enez, 2014), *Aspergillus niger* RBP7 (Mukherjee et al., 2019) and *Aspergillus oryzae* (Sahnoun et al., 2012). According to prokaryote, amylase can also be found in eukaryotes such as *Opuntiaficus indica* seeds (Ennouri et al., 2013), barley amylase (Kadziola et al., 1994), *Hyper postica, Eurygaster maura, Pieris brassicae* (Kaur et al., 2014), along with human salivary amylase (Kadziola et al., 1994), and human pancreatic amylase (Brayer et al., 1995). However, the most popular sources widely used in the industrial scale are always from microorganisms.

2.1.3 Uses of amylase

Nowadays, many industries use amylase for processing starch due to the properties to hydrolyze starch into oligosaccharides or smaller carbohydrates such as maltopentaose, maltotetraose, maltotriose, maltose, and glucose. Start from baking and milking industries that use amylase for reducing dough viscosity and accelerating the fermentation process. Even in brewery industries, Amylase has been used of amylase in the mashing process. Moreover, pharmaceutical, clinical industries, and energy companies rely on amylase for processing digestive acid (Naidu, 2013) and accelerate fermentation for producing fuel alcohol. These industries rely on starch processing including the liquefaction, and saccharification processes, where starch is converted into small sugar molecules (Souza, 2010).

2.1.4 Structure of amylase

The structure of amylase is investigated for three decades using amino acid data to determine the enzyme structure. The well-known amylase that used as the reference model of amylase is TAKA amylase. TAKA amylase is established in 1954

(Akabori et al., 1954). The enzyme is from *Aspergillus oryzae*. Since then, the amylase has studied the structure from its protein crystal.

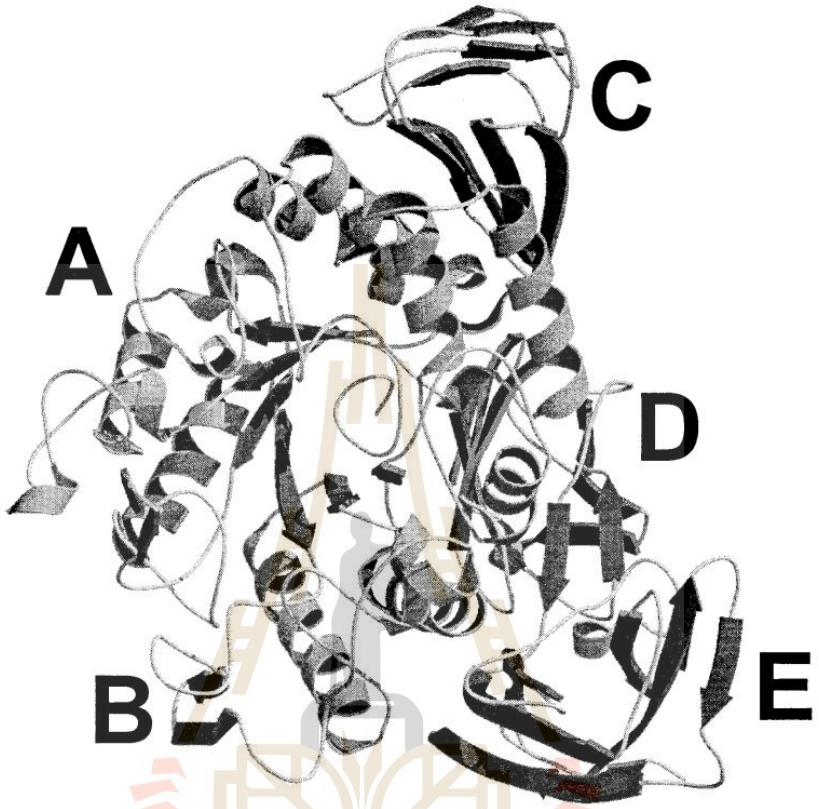


Figure 2.3 The amylase structure, A is the catalytic $(\beta/\alpha)_8$ domain, B is a loop protruding from the domain, C, D, and E are antiparallel β-strand domains which E can bind starch (Lawson et al., 1994).

The three-dimension is generated by X-ray crystallography showing that the amylase contains three domain A, B, and C (Macgregor et al., 2001). The amylase especially α-amylase is multi-domain protein but still shares conserved catalytic region called $(\beta/\alpha)_8$ barrel which is in the A domain. Domain A consists of α-helices surrounded the β-strand as the catalytic region. Firstly, it is found that this structure possessed by only the amylase, for now, some of the member group of the carbohydrate-

active enzyme also consists of this conserved region. Near the active site the loop of β-strand link at the 3rd α-helices. In some case, if it is long enough, the loop would be called domain. Therefore, the loop between β-strand and α-helices is Domain B. This domain is varied from enzyme to enzyme in the irregular form. Furthermore, most amylases are found the domain A at the end of the N-terminal of protein which often found to form the active site. The distance of N-terminal is defined to the substrate preference. Near $(\beta/\alpha)_8$ the domain C constituted of β-strand acts as the stabilizer of the catalytic site, to prevent hydrophobic residues of the domain A. This suggests that the domain A aids the binding of substrate. As shown in Figure 2.3, some amylases have additional β-sheet domain considered as domain D and E. In the Taka amylase one glutamic and two aspartic acid are found to be important part while glutamic acid acts as the proton donor, the first aspartic acid acts as nucleophile, and the last one is believed to be stabilizer in transition state.

2.1.5 Kinetic parameters of amylase.

The Michaelis parameters in this section are included the Michaelis constant, K_m the maximum velocity, v, and the molar (or molecular) activity, k_o (k_{cat}) which defined as follows (Copeland, 2004):

$$v = \frac{V[S]}{K_m+[S]} = k_0[E_0]/(K_m+[s]) \qquad (1)$$

According to the Michaelis-Menten equation, the initial velocity (v) increases hyperbolically with increasing substrate concentration ([S]) to reach the maximum velocity. The substrate concentration that gives ν which is equal to V/2 is defined as the Michaelis constant, K_m. The molar activity, k_o, is obtained from $V/[E]_o$, where $[E]_o$ is the molar concentration of the enzyme, that can be determined from the molecular weight of the enzyme. If the molecular weight is not known, V must be expressed in

the molar concentration of substrate converted to the products per unit time (second or minute) at the fixed concentration of the enzyme in the reaction mixture.

To obtain reliable values of the Michaelis parameters, the following should be remembered. The first, the initial velocity at every substrate concentration must be determined as accurately as possible, and second, the range of [S] must be chosen appropriately (the range from 0.2 to 2 is recommendable). The curve tends to concave upwards with decreasing [S], for which the error involved in obtaining the initial velocity (v) (the slope of the tangent at zero time) becomes larger. It is important to check the proportional between ν and $[E]_o$, to confirm the validity of the procedure for evaluation of vs. Linear plots. The rate equations (Equation 1) are transformed into linear equations as follows.

$$\frac{1}{v} = \left(\frac{K}{V}\right)\left(\frac{1}{[S]}\right) + \frac{1}{v} \qquad (2)$$

These equations can be used to evaluate K_m and V. Equation 2 is most frequently used, known as the Lineweaver-Burk plot (Copeland, 2004). The least-squares method for straight lines (y = ax + b) can be applied to obtain K_m and V values together with their standard deviations. From the statistical viewpoint, Equation 2 is better than Equation 1 in kinetic parameter determination, since the weight is higher for smaller ν values at lower [S] where the error becomes bigger. However, some researcher proposed various novel forms of a plot, but each plot was preferred to describe the standard deviations and V values. To show the reliability of the experimental data, the curve was plotted using least-squares methods to evaluate the Michaelis parameters. A non-linear least-squares method applied directly to the hyperbolic form, Equation 1, is also available. If the [S]-dependence of ν does not fit the hyperbolic form (Equation l)

but shows a sigmoidal curve, suggested that the possible involvement of some kinds of inhibitions.

2.2 Amylase production

Naidu and Saranraj (2013) have described that microorganisms are used to produce various industrial enzymes due to high productivity. The global market for industrial enzyme estimated the value at $2 billion in 2010 and tend to increase in the rate of 3.3% every year. The most usage enzymes are amylolytic enzymes such as amylases which received a great deal of attention because of their perceived technological significance and economic benefits and make it constitutes a class of industrial enzymes having approximately 25% of the enzyme market (Rao et al., 1998; Sidhu et al., 1997)

2.2.1 Amylase production by bacteria

The amylase production normally uses microorganisms as the sources due to the low concentration in plants and low supply in animals. The popular amylase sources can be divided into fungi and bacteria which are described in the next paragraph. Several reports mentioned about how they produced amylases. Some changed nitrogen sources, some changed carbon sources as well as changing ion components. All these factors influenced the amylase production. For examples, Aguilar et al. (2000) studied α-amylase produced by *Lactobacillus manihotivorans* LMG 18010. MRS medium was used and prepared by using 1.5%(w/v) soluble starch as a carbon source and adjusted pH to be 6. Then cultivated 12-h starter in the bioreactor. Lastly, the given amylase has activity around 22 to 25 U/ml when U is defined as the amount of enzyme that permits the hydrolysis of 10 mg of starch in 30 min in the conditions of the assay. Kanpiengjai et al. (2015) reported that the modified

MRS medium (mMRS) was used for the preparation of the seed inoculum and as enzyme production medium. The medium was prepared from the mixtures of peptone, beef extract, yeast extract, tween80, di-potassium hydrogen phosphate (K_2HPO_4), sodium citrate ($CH_3COONa \cdot 3H_2O$), tri-ammonium citrate ($C_6H_5O_7(NH_4)_2H$), magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$), manganese sulphate monohydrate ($MnSO_4 \cdot H_2O$) and soluble starch as the sole carbon source (10.0, 10.0, 5.0, 1.0, 2.0, 5.0, 2.0, 0.2, 0.2, and 10.0 g/l, respectively), then incubated at 37℃ for 48 h. The amylase which was given from this study showed 2.1 U/ml of activity when one unit of amylase activity was defined as the amount of releasing reducing sugars per time, by 1 μmol of reducing sugars (as glucose equivalents) per min under assay condition. Fossi study (Fossi et al., 2014) showed that the commensalism between bacterium and other microorganisms also make its amylase activity different. All the above reports represent that the amylase production can be influenced by many factors not only by one factor.

2.2.2 Amylase production conditions

Amylase production by an organism relies on both chemical and physical factors. Different conditions or factors affect amylases production in various ways, which may increase or decrease amounts of amylases production**.** For instance, Wang et al. (2016) found that *Bacillus* sp. WangLB could potentially produce amylase at 30 to 37℃ meaning that the low optimal temperature of strain WangLB could enable producers to save on the cost of energy consumption and avoid the use of special equipment that tolerates high production temperature. Moreover, Wang et al. (2016) also study the effect of the carbon source concentration and found that at 20 g/l of starch concentration shows the maximum of amylase production. Study of starch concentration provides them the suitable concentration, and it was found that the high

starch concentration does not always give high amylase production. The example shows that chemical and physical studies are important.

2.2.2.1 Chemical factors

Chemicals are always used as nutrients for essentially growing microorganisms. The medium always consists of various components which are important for bacterial amylase production. The components can be divided into three main components, namely nitrogen sources, carbon sources, and ion components. These were identified as key roles in the carbohydrate metabolism of the microorganism (Naidu, 2013).

a. Nitrogen sources

Organic nitrogen sources have been preferred to produce α-amylase. Several sources of organic nitrogen source are investigated for their potential in amylase production, namely yeast extract, tryptone, soybean meal, peptone. Yeast extract has been used as the nitrogen source in the production of α-amylase from *Streptomyces* sp., *Bacillus* sp. and *Halomonas meridian.* In addition to yeast extract, other organic nitrogen sources have also been reported to support the maximum α-amylase production by various bacteria and fungi. However, organic nitrogen sources, for instance, beef extract peptone and com steep liquor were required for maximum α-amylase production by bacterial strains as well as soybean meal and amino acids for *Aspergillus oryzae*. CSL has also been used for the economical and efficient production of α-amylase from a mutant of *Bacillus subtilis*. Furthermore, the organic compound with salt can also be used as nitrogen sources to stimulate amylase production such as ammonium sulfate for *Aspergillus oryzae* and *Aspergillus nidulans*, ammonium nitrate for *Aspergillus oryzae* and Vogel salts for *Aspergillus fumigatus* (Naidu, 2013).

b. Carbon sources

Amylase is an inducible enzyme, activated by the presence of starch or its hydrolytic products. Reportedly, the enzyme could be induced by the oligosaccharides using maltose and starch as substrates. In some cases, these oligosaccharides are recognized as the inducer of the enzyme as found in *Aspergillus oryzae*. In the study of amylase production by *Lactobacillus fermentum*, their finding is about the effect of different carbon sources on amylase production. It gave high enzyme production when using soluble starch as carbon source following by amylose and amylopectin respectively (Fossi and Tavea, 2013). These represented the effect of the high structure.

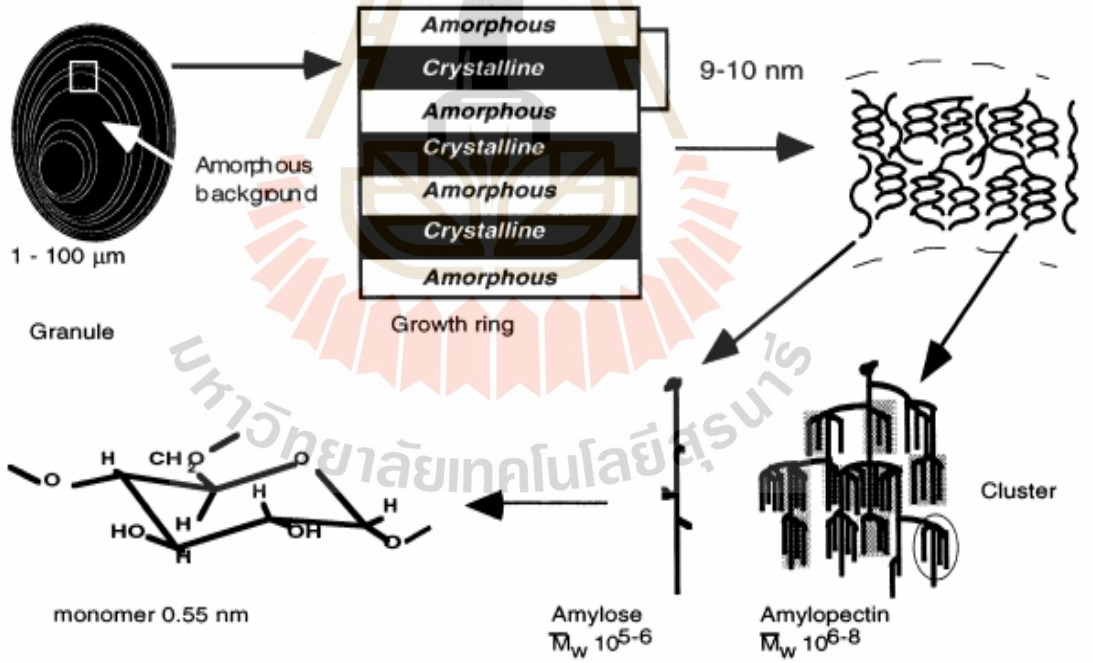


Figure 2.4 Schematic representation of different structural levels of starch which is the most popular carbon source granule and the involvement of amylose and amylopectin (Buleon et al., 1998).

c. Ion components

Seven ions namely, K^+, Na^+, Fe^{2+}, Mn^{2+}, Mo^{2+}, Cl^+, and SO_4^{2+}, are reported to have no effect on amylase production while Ca^{2+} inhibits amylase production from *Aspergillus oryzae*. As well as Mg^{2+}, the amylase production is reduced to 50% when Mg^{2+} is omitted from the medium. However, Na^+ and Mg^{2+} are coordinated for stimulation of amylase produced by *Bacillus* sp.. Naidu (2013) stated the adaptation by adding zeolites to control ammonium ions in *Bacillus amyloliquefaciens* resulted in an increasing yield of α-amylase production.

2.2.2.2 Physical factors

Physical factors affect amylase production in various ways. Either can decrease or increase amylase production in some organisms. However, the physical factors of amylase production depend on a kind of organism. The main physical factors that play an important role in amylase production of microorganisms, are pH and temperature.

a. pH

pH is a physical factor which plays an important role by inducing morphological change in the organisms and related to enzyme secretion. pH also affects the product stability in the medium. Normally, commercial enzymes are from *Bacillus* strains which have optimal pH between 6.0-7.0 for growth and enzyme production. But there is still some exception, in the case of *Aspergills oryzae* and *Bacillus amylolquefaciens* which have the optimal pH 4.2 and 6.8, respectively (Naidu, 2013).

b. Temperature

The effect of temperature on amylase production is related to the growth of the organism. Among the fungi, most amylase productions are derived from

mesophilic fungi at the temperature, ranging from 25 to 35℃. The optimum activity of α-amylase produced by *Aspergillus oryzae* was obtained at around 30 to 37℃. Similarly, α-amylase production from *Thermomonospora fusca* at 55 to 50℃ has been reported as the optimum point. The microorganisms can produce amylase at the wide range of temperature, but the wider range was often from an α-amylase derived from the bacteria. Furthermore, the investigation of amylase from *Bacillus amyloliquefaciens* showed the optimum activity at 36℃. However, it was also found that at the high temperatures as high as 80℃, has been used for amylase production from the hyperthermophile *Thermococcus profundus* (Naidu, 2013).

2.3 Characteristics and properties of amylase

Amylase is an amylolytic enzyme that catalyzes the hydrolysis of glycosidic linkages of starch and related oligo- and polysaccharides by the transfer of a glucosyl residue to water (Whitaker and Wong, 1995). The example of characteristics of amylase in different types, α-amylase is an enzyme that cleaves α-1,4-glycosidic bonds and randomly cleaves from inside the molecule of polysaccharide chain to yield dextrin and oligosaccharide which have C1-OH in the α-configuration. An α-1,4 linkage neighboring an α-1,6 branching point in the substrate is resisted to attack by the enzyme (Hizukuri et al., 1981). Furthermore, the enzyme is metalloenzyme which requires Ca^{2+} for stability and ability. From several reports, the enzyme has a molecular weight around 50 kD. The optimal pH of enzyme varies depending on the enzyme sources from the review article and some book it was found that the optimum pH is 6.0-7.0 for mammalian, 4.8 to 5.8 for *Aspergillus oryzae*, 5.85 to 6.0 for *Bacillus subtilis*, 5.5 to 7.0 for *Bacillus licheniformis*. The optimum temperature varies from 70 to 72℃ for the

a-amylase produced by *Bacillus subtilis*, *Bacillus licheniformis* B4-423 (Wu et al., 2018) and 90℃ for the enzyme from *Bacillus licheniformis* (Wong, 1995) but there are some of them which have higher than 90℃. In β-amylase, the enzyme can cleave α-1,4-glycosidic bond as well as α-amylase but different from the manner of cleaving bond, by start cleaving from the non-reducing end of the polysaccharide chain to yield maltose with the CI-0H in the β-configuration (Thoma et al., 1971). Unlike α-amylase, β-amylase is not able to pass the α-1,6 branching point and stop the action at the location. The enzyme has a molecular weight of 50 kD apart from sweet potato β-amylase, which is a tetramer of 215 kD. The pH optimum for activity ranges from 5.0 for wheat, malt, and sweet potato to 6.0 for soybean and pea. Plant α-amylases often consists of several isoforms. β-amylase has also been found in several species of *Bacillus* sp., including *Bacillus polymyxa*, *Bacillus cereus*, and *Bacillus megaterium*, and in *Clostridium thermosulfurogenes*. These enzymes have a molecular weight of 30 to 160 kD. The last example is a glucoamylase or γ-amylase, the enzyme catalyzes the hydrolysis of both α-D-l,4- and α-D-l,6-g1ucosidic linkages at the branching point, but hydrolysis of the latter occurs at a slower rate. The enzyme has the pH optimum of 4.0 to 4.4 or lower in fungi, with stability over a pH range of 3.5 to 5.5. The enzyme is stable at a temperature range of 40 to 65℃ with optimum range at 58 to 65℃ in the hydrolysis of starch at pH 4.2 (Wong, 1995).

2.4 Lactic acid bacteria

Lactic acid bacteria are capable of producing lactic acid as the main primary fermentation product. Since early times, they have been used in food and feed fermentation and are generally considered beneficial microorganisms, for example, some

strains are health-promoting bacteria. However, some genera also contain species or strains that are human or animal pathogens. Lactic acid-producing bacteria are classified by using morphological, physiological and metabolic characteristics. They are Gram-positive bacteria, non-sporulating, facultative or aerotolerant, cocci or rods, and have lactic acid production ability. Lactic acid-producing bacteria belong to the phylum *Firmicutes*, class *Bacilli*, and order *Lactobacillales*. The different families include *Aerococcaceae, Carnobacteriacea, Enterococcaceae, Lactobacillaceae, Leuconostocaceae, and Streptococcaceae.* They can also be classified by their carbohydrate fermentation capability into two main groups, homofermentative, and heterofermentative lactic acid-producing bacteria. The homofermentatives can produce only lactic acid from fermentation but heterofermentative can produce more than lactic acid, they can produce lactic acid or acetic acid, carbon dioxide and produce ethanol. Furthermore, the type of lactic acid which produced by lactic acid bacteria, can be divided into two enantiomers, D-lactic acid and L-lactic acid (Von Wright and Axelsson, 2012).

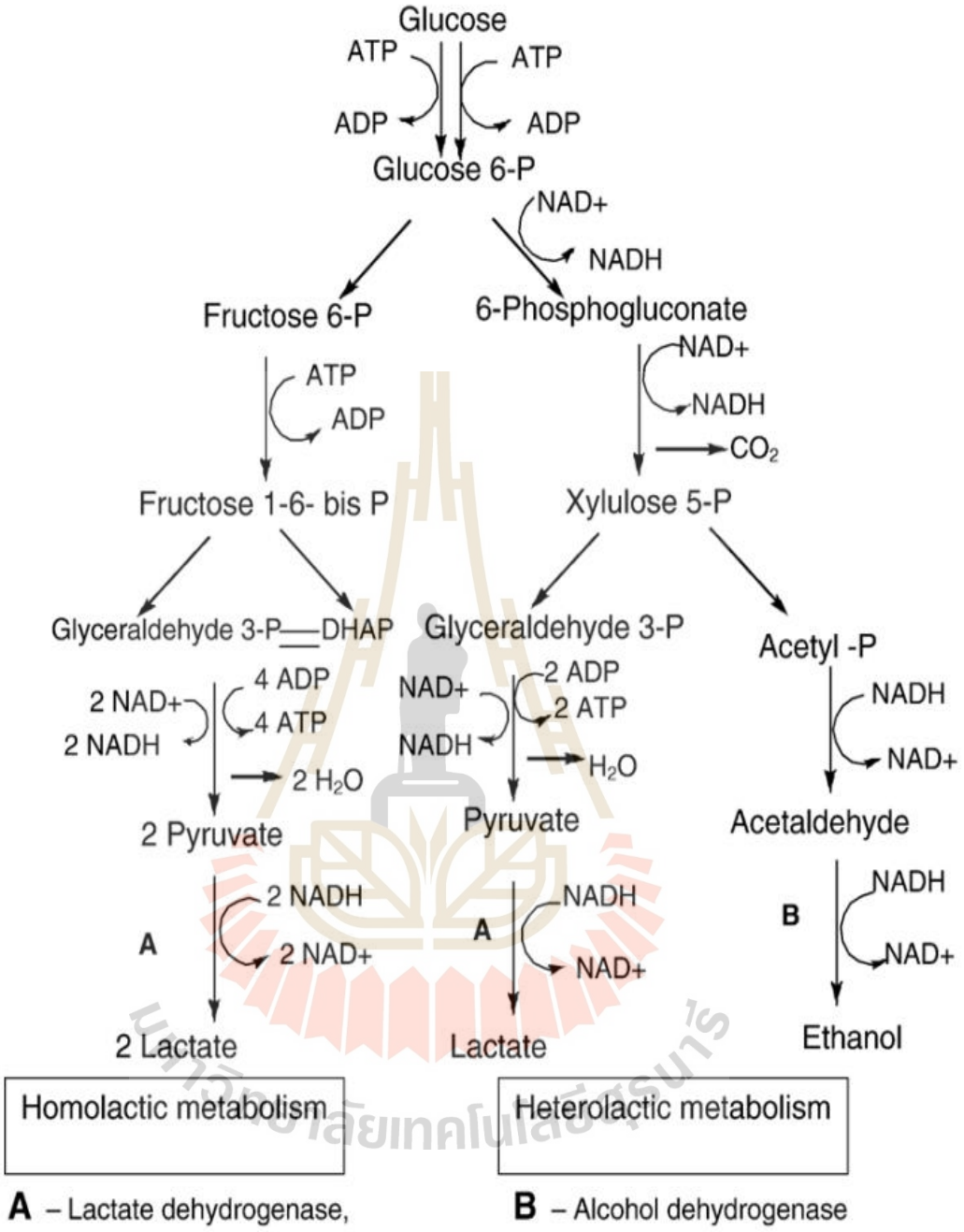


Figure 2.5 Metabolism of lactic acid bacteria (Reddy et al., 2008).

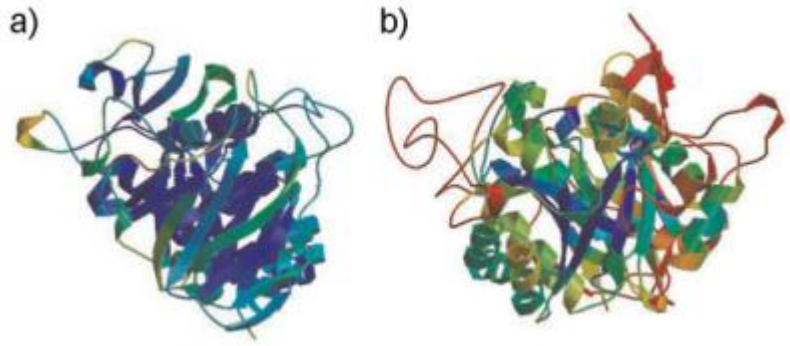


Figure 2.6 Three-dimensional model of amylase from lactic acid bacteria, *Lactobacillus amylovorus* (a) and *Lactobacillus paracasei* (b) (Petrova et al., 2013).

2.5 D-Lactic acid-producing bacteria

D-lactic acid bacteria can produce lactic acid in the D-enantiomer of lactic acid as one of the main products by using D-lactate dehydrogenase (D-LDH). They are valuable because their D-lactic acid can be used as a monomer for biodegradable polylactic acid (PLA) production. D-Lactic acid is generally produced by certain species of the lactic acid bacteria usually in the genus *Lactobacillus* (Carr et al., 2002), such as *Lactobacillus bulgaricus* (Benthin and Villadsen, 1995; Manome et al., 1998) and *Lactobacillus lactis* (Joshi et al., 2010) *Lactobacillus plantarum* S 21 (Unban et al., 2019). Lactic acid production can achieve by fermentation. During the first step, starch is converted to the smaller oligosaccharide called liquefaction, and then gelatinized at high temperatures of 90 to 130℃ for 15 min followed by saccharification using amylolytic enzyme to change the gelatinization products into glucose and subsequently converting glucose to lactic acid (Reddy et al., 2008) by D-lactic acid producing bacteria.

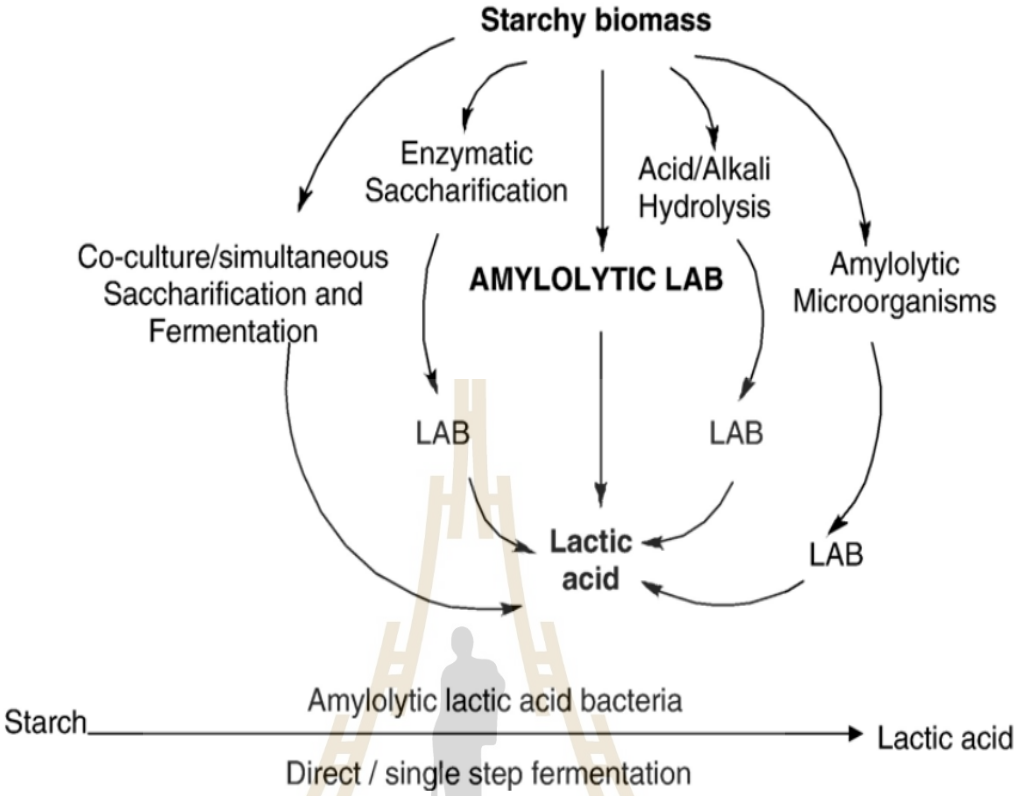


Figure 2.7 Schematic representation of lactic acid production from starch as substrate (Reddy et al., 2008).

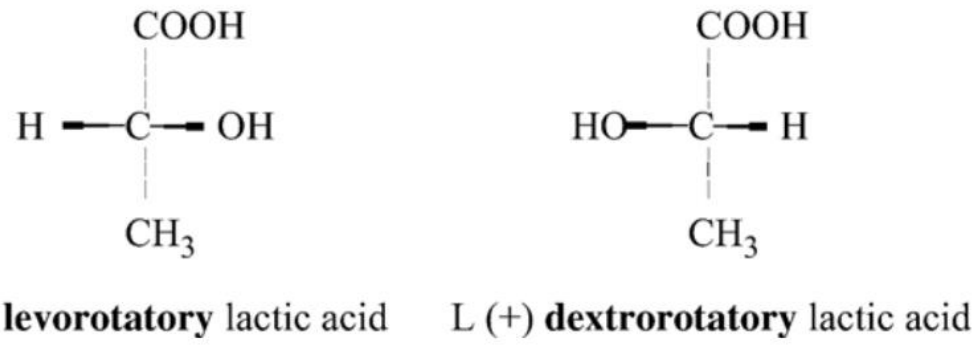


Figure 2.8 Two isomers of lactic acid (Reddy et al., 2008).

2.6 Bacterial cultivation process for amylase production

Microorganisms have been used in the industrial department for a long time. Not only are used for alcoholic beverages production but also involved in other food production namely, dairy, fish, and meat products. Besides, several additives and supplements are from microbial fermentation, such as antioxidants, flavors, colorants, preservatives, and even in sweeteners. Nowadays, cultivation of microorganisms is developed to be highly efficient and yielding plentiful amounts of desired products.

Cultivation processes on a large scale are widely used to produce biomolecules. Some are used in various enzyme-productions, such as extracellular amylase, lipase, pectinase, as well as cellulase. Others are used in organic acid production, for example, lactic acid production and citric acid (Couto et al., 2005).

Now, there are two main types of cultivation processes, solid-state cultivation (SSC) and submerged cultivation (SMC). Solid-stated cultivation is a process performed on the non-soluble material that acts both as physical support and nutrient source without free-flowing liquid (Pandey, 1992). Many kinds of non-soluble materials were used in the process, such as potato peels, *Arundo donax* rhizome, banana peel, orange peel, wheat bran, as well as rice bran (Finore et al., 2014; Anto et al., 2006). In another hand, submerged cultivation is a process performed on the soluble material that acts as nutrients of microorganisms.

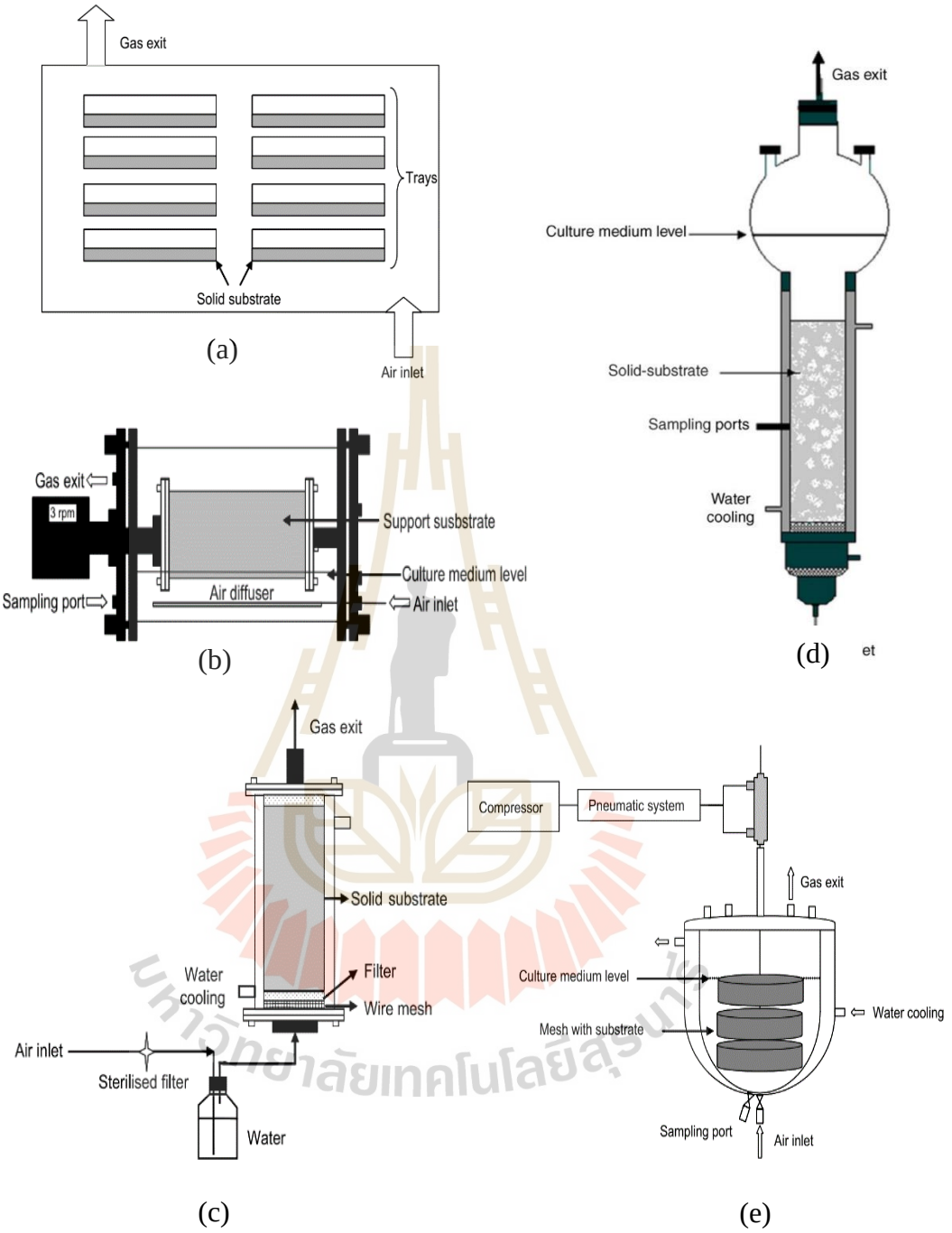


Figure 2.9 Four mainly types of bioreactor for solid-stated cultivation; tray (a), horizontal drum (b), packed-bed (c), fluidized-bed (d), and immersion bioreactor (e), modified from Couto and Sanroman (2006).

2.6.1 Solid-state cultivation

Solid-state cultivation (SSC) was firstly developed in Eastern countries since ancient time. As its name directly represent, this cultivation means cultivation of microorganism on solid, with provided moisture instead of solution containing water as the submerged do. In the cultivation system, the moisture has been carefully controlled by thinking of the final water activities. In some case, SSC could be seen in the presence of the large liquid than normal SSC, this case may use liquid with carries or the solution containing fully substrate (Holker and Lenz, 2005).

The use of SSC for the cultivation of microorganisms is originally used for food production. For instance, the well-known alcoholic beverage, Sake is made from the use of SSC combined with *Aspergillus oryzae* as the starter allowing the fungus to produce the hydrolytic enzyme to saccharify rice into the alcohol. This kind of process of cultivation is common in old time. There is another usage in mold cheese which used SSC for conidiospore production from *Penicillium roqueforti* or *Penicillium camemberti*. For salami, there is used by *Penicillium nalgiovensis* (Grazia et al., 1986). As previously mentioned, the used of SSC for cultivation mostly related to the fungal cultivation owing to the low water available of this system that suits for microorganism grown in low water activity. However, other microorganisms would be cultivated by this method too as shown in Figure 2.9. Among the SSC system, the reactor is also developed in many kinds. Couto and Sanroman (2006) summarized the reactor in 5 main types, including the tray, horizon drum, packed bed, fluidized bed, and immersion bioreactors.

Tray type of SSC consists of flat trays formed into the main part of bioreactor. This tray is overlaid with the substrate with the thin layer for a few centimeters deep.

The moisture is kept in the chamber at constant temperature by circulating air. From details, the construction at high scale for this system is hard to operate. Owing to the larger scale requires the numbers of trays, the configuration of trays is a disadvantage point. The design of the tray may be the point that the users have to consider.

For the packed-bed type, this system normally made from a column of glass or plastic containing the substrate inside by laying on the perforated base. The moisture of the air is continuously circulated. Couto and Sanroman (2006) also suggested that the system could be controlled the temperature by adding a water jacket. However, the weak points of the system are homogeneous of the system, the obstacle when the product has been taken out, the heat transfer problem, and the scale-up problems.

In the case of horizontal drum design, it allows adequate aeration and mixing of the substrate, while limiting the damage to the inoculum or product. Mixing is performed by rotating the entire vessel or by various agitation devices like paddles and baffles. The limit of this design is that the drum has a low capacity, made mixing is inefficient.

The fluidized-bed bioreactor is developed due to previous designs have the drawbacks of adhesion and aggregation of the substrate, to solve these problems. The agitation of this system is controlled through the air inlet. These increase of the mixing of the substrate, the heat and mass transfer. Nevertheless, the higher efficiency also causes the problems especially sheer effect due to the high agitation to the inoculum and the final product.

Immersion bioreactor composed of the cylindrical glass vessels with a round bottle. Inside of the cylindrical glass, mesh baskets contain the support substrate for fungi which could be moved following the control of the pneumatic system. This system

also could be run in a continuous manner which suitable for obtaining a high yield of ligninolytic enzyme activities.

2.6.2 Submerged cultivation

The submerged cultivation or SMC is another well-known cultivation method widely used in western countries. The development of the system is depended on the way to cultivate microorganisms. Three systems that used nowadays, are including batch, continuous, and fed-batch cultivation mentioned below.

Batch cultivation is the simplest method for cultivation in SMC. The starter microorganisms are inoculated into the sterilized medium, then cultivated without the addition of the medium during the cultivation process. The lactic acid bacteria are mostly preferred cultivated by this system than the SSC due to their fastidious characteristics. The weak point of the batch is product inhibition. The inhibition problem is solved by another SMC which is continuous.

Continuous cultivation, the system has been used for a long time. which is mainly focused on the physiological and growth rate studies of the microorganisms. The microorganisms cultivated in this system are kept the growth rate and kinetic parameter especially the concentration of substrate, pH, and waste-product management. Lactic acid bacteria are commonly cultivated in continuous cultivation because the pH can be constantly controlled, decreased the problem of acid inhibition that causes the stress to the lactic acid bacteria. Despite the lactic acid bacteria is strong tolerance to acid but at high concentration, they could be killed by the acid produced by itself. Not only the main advantages in industries, but the continuous is also used in the physiological study of the bacteria.

Fed-batch cultivation is the last three main methods used in SMC. There is the addition of the medium into the reactor while it is running. It seems to be similar as continuous cultivation, but it is performed without removal of the cultivated medium. The biomass and the yield of the metabolite obtained from the fed-batch are higher than continuous whereas the continuous cultivation provides high productivity than the others. Since, fed-batch is developed by the invention of fed-strategies such as pulse, constant feed rate, exponential fed-batches.

Raimbault (1998) said that SMC used much energy, caused more pollution and more effluent than SSC, but was easy to control than SSC, in the view of pH, temperature, and contamination. Goes and Sheppard work (1999) mentioned that the SSC was better than SMC, due to the number of the available substrates and the efficiency converting waste into the desired product, so the cost of SSC was also lower than SMC because many wastes were non-soluble. However, SMC was better on the part of the waste organization because of the low waste-creating process. So, both cultivation types are still being used varying from a kind of substrate and desired product.

Table 2.1 Comparison between submerged and solid-state cultivation which introduced by Raimbault (1998).

Factor	Submerged cultivation	Solid-state cultivation
Substrate	Soluble substrate	Insoluble polymer substrate,
Aseptic condition	Heat sterilization and aseptic technique	Vaporization treatment non-sterile condition
Water	High water requirement	Low water requirement
Metabolic heating	Easy control of temperature	Low heat transfer capacity
Aeration (O_2)	Soluble oxygen requirement	High surface exchange
pH control	Easy control of pH	Control through buffered solid substrate
Mechanical agitation	Good homogenization	Static condition
Scale up	Industrial equipment available	Need the engineering and new equipment design
Inoculation	Easy inoculation, continuous process	Spore inoculation, batch
Cultivated microorganisms	Bacteria	Fungi
Energetic consideration	High energy consumption	Low energy consumption
Volume of equipment	High volume	Low volume
Effluent and pollution	High effluents	Low effluents
Concentration of substrate/product	30-80 g/l	100-300 g/l

Table 2.2 Bioprocesses and product development in solid-state fermentation summarized by Singhania et al. (2009).

Group	Microorganism /biocatalyst	Substrate	Product	Type of bioreactor	Reference
Fungi	*Aspergillus niger*	Buckweed seed	Spores	Packed bed column	Kashyap et al. (2002)
	Aspergillus niger	Cassava bagasse	Citric acid	Column fermenter	Luccio et al. (2004)
	Aspergillus oryzae	Wheat bran + coconut oil cake (3:1)	Neutral metalloprotease	Static flask	Holker and Lenz (2004)
	Monascus purpureus	Jack fruit seed	Pigment	Static flask	Shih et al. (2008)
Bacteria	*Bacillus amyloliquefaciens* ATCC 23842	Groundnut oil cake + wheat (1:1)	Alpha amylase	Static flask	Fujian et al. (2002)
	Bacillus subtilis	Rice bran	Iturin A	Tray	John et al. (2006)
	Bacillus thuringiensis	Wheat bran and bean cake powder	Bt wet powder	Cylindrical steel container	Devi et al. (2005)
	Lactobacillus delbrueckii	Cassava bagasse	Lactic acid	Static flask	Rodriguez et al. (2004)

Table 2.2 (Continued) Bioprocesses and product development in solid-state fermentation summarized by Singhania et al. (2009).

Group	Microorganism /biocatalyst	Substrate	Product	Type of bioreactor	References
Bacteria	*Lactobacillus* sp.	Tamarind seed powder	Tannase	Static flask	Sabu et al. (2006)
	Streptomyces griseoloalbus	Soya-bean flour	Alpha galacto-sidases	Static flask	Ramachandran et al. (2007)
	Streptomyces clavuligerus	Wheat raw with cotton seed cake and sunflower cake	Cephamycin C	Static flask	Hung-Der et al. (2008)
	Erwinia sp.	Sucrose + free and calcium alginate immobilized cells	Palatinose	Packed bed reactor	Kota and Sridah (1999)
	Clostridium tyrobutyricum JM1	Polyurethane foam enriched with glucose medium	Biological hydrogen	Fixed-bed bioreactor	Chang et al. (2007)
Fungi	*Mortierella alpina*	Rice bran	Polyunsaturated fatty acid	Column reactor	Vandenberghe et al. (2000)

Table 2.2 (Continued) Bioprocesses and product development in solid-state fermentation summarized by Singhania et al. (2009).

Group	Microorganism /biocatalyst	Substrate	Product	Type of bioreactor	Reference
Fungi	*Penicillium simplicissimum*	Soybean cake	Lipase	Static flask	Ramachandran et al. (2005)
	Penicillium simplicissimum	Olive oil cake + sugarcane bagasse (1:1)	Lipase	Fixed bed reactor	Anisha et al. (2008)
	Zygosaccharomyces rouxii 2547 NRRL-Y	Sesame oil cake	Glutaminase	Static flask	Sumantha et al. (2005)
	Rhizopus oryzae NRRL 1891	Coconut oil cake + sesame oil cake (1:1)	Phytase	Static flask	Singhania et al. (2007)
	Thermoascus auranticus	Wheat straw	Cellulase	Perforated drum reactor	Xiong et al. (2007)
	Trichoderma reesei	Wheat bran	Cellulase	Static flask	Gangadharan et al. (2005)
	Trichoderma harzianum TUBF 927	Colloidal chitin	Chitobiase	Static flask	Pandey and Soccol (200)
Enzyme	Lipase from *Rhizomucor miehei*	-	Hexyl laurate	Packed-bed reactor	Babitha et al. (2007)
	Lipase producing *Rhizopus oryzae*	Cuboidal polyurethane foam biomass support particles	Bio-diesel fuel	Packed-bed reactor	Sukumaran et al. (2005)

Table 2.2 (Continued) Bioprocesses and product development in solid-state fermentation summarized by Singhania et al. (2009).

Group	Microorganism /biocatalyst	Substrate	Product	Type of bioreactor	Reference
Bio-material	Microbial biofilm developed without prior inoculation	Clay beads + glucose-based	Organic acids and hydrogen	Horizontal packed-bed bioreactor	Hama et al. (2007)
	Hydrogen producing sludge	Carbohydrate substrates enriched polyethylene–octane elastomer immobilized	Hydrogen and ethanol	Fluidized-bed and packed-bed reactor	Singhania et al. (2007)

2.7 Principle and application of response surface method (RSM)

Response surface method (RSM) is a method used in various process optimization. At present, the use of RSM expanded its scope of applications to the biochemical engineering from many years ago. The method can be applied in several processes. For instance, the use of biofuel production, Almengo et al. (2019) has used the method to reduce the hydrogen sulphide released from the biogas production using the chemotroph bacteria, making the product to be friendly to the environment and health. Similarly, there is the use of RSM for removing the chromium out of wastewater from understanding absorption behavior (Khalifa et al., 2019). In the laser-sintered parts investigation, the dimensional accuracy has been optimized using RSM (Singh et al., 2019). To optimize the spray drying of Risperidone (RIS) nanosuspension, Nair et al.

(2019) used RSM for achieving lower particle size, higher yield and good compressibility of spray-dried powder. In the microorganism cultivation, the method is also used for optimized factors in the medium to increase the yield of desirable products such as enzymes, antibiotics, acids, fuels, and sugars as well. Response surface method is used for more than decades, helping to manage cost-yield benefit. Despite the experiment, everything could be possible, but the production at higher scale even in the bioreactor scale, the bulk of the expensive component is not often accepted to be used. If there is another way to improve the yield, it may be the better choice than directly adding substrate. Some case, the growth of the microorganisms largely depended on pH, adjusting pH combined with the system would be higher efficiency or equal to the addition of more substrate. Not only manage cost-benefit effective, but these statistical methods also influence to researchers to conduct their experiment consists of many factors or variables in the brief time instead of doing the classically factorial experiment by changing one independent variable while fixing others at a constant level. For two variables study, the factorial experiment may be accepted, but in case of more than two variables, needing a lot of things including time consumption, numbers of the experimental run, excessive cost from expensive variables, and lack of interaction between variables which could not get from factorial experiments. Hence the statistical method could be effectively used for solving the problems of recent decades. The well-known statistical design is used in optimization including full factorial, fractional factorial, Plackett-Burman, Hadamard matrix, orthogonal array, Central composite, (Latha et al., 2017).

Central composite design (CCD) is a response surface design mostly used to construct the model especially, for the quadratic model. The CCD is developed by Box

and Wilson (1951). It contains three types of the point: factorial point, central point, and axial point. For three to five variables, CCD is appropriate to investigate the effect of factors. The numbers of the run could be calculated from the equation, $N = k^2 + 2k + c_p$ where k is the number of independent variables and c_p is the replicate number of the central point. Nowadays the experimental design could be achieved by many software programs meaning that the design can be easily designed and transformed the data to other available software.

For the statistical calculations, the coded level of each factor was calculated by the following equation.

$$X_i = \frac{x_i - x_0}{\Delta x_i}$$

Where X_i is the independent variable in the coded unit, xi is the real value of the independent variable, x_0 is the real value of the independent variable at the center point, and Δx_i is the step change value. Coded level zero represents the central level of each factor in the intended ranges to assess the background variability in the process; level −1 and +1 are the factorial points, that is the high and low levels of each factor (in the Intended range) to determine the main effects and interactions between them. The empirical data obtained from the CCD design were analyzed by the response surface regression procedure using the second-order polynomial equation followed (Li et al., 2007).

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j i < j$$

where Y is the measured response; β_0, β_i, β_{ii}, β_{ij} are constant and regression coefficients of the model. x_i and x_j are levels (codes values) of independent variables. e

is the random error. The Design Expert was used for regression analysis of the data obtained and to estimate the coefficients of the regression equation. The response surface plots were also obtained by using Design-Expert software to illustrate the relationship between the variables. Accuracy and general ability of the polynomial model were evaluated by coefficient of determination (R^2). The statistical significance of model coefficient was evaluated by analysis of variance (ANOVA) (Soni et al., 2015).

The optimization of RSM could be summarized into seven steps as follows:(1) defining the independent variables, (2) selecting the type of experimental design, (3) performing the regression analysis, calculating the statistical analysis of variance (ANOVA) for the independent input variables and to find which variable shows a critical effect, (5) obtaining the optimal design, and (7) confirming the optimal design.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials and instrumentation

The research was carried out at the Cassava and Products Research Center Building and the Center for Scientific and Technological Equipment Buildings at Suranaree University of Technology, Nakhon Ratchasima, Thailand. Reagents and media used in each step were as follows. To cultivate D-lactic acid-producing bacteria, the bacteria were grown on our starch-based MRS medium called RAM which modified from our previous study. 1 l of our RAM media broth was prepared from pancreatic digest of casein (tryptone) (Hi-Media Laboratories Pvt Ltd, Mumbi, India), di-potassium hydrogen phosphate (Carlo Erba Reagenti, Milan, Italy), yeast extract, tri-ammonium citrate, magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$), manganese sulphate tetrahydrate ($MnSO_4 \cdot 4H_2O$), iron (II) sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) (Ajax, Taren Point, Australia), cassava starch (Sanguan Wongse Industries Co., Ltd., (Nakhon Ratchasima, Thailand)), and agar for 5, 6, 3, 1, 0.57, 0.12, 0.03, 10.00, and 15.00 g, respectively (Appendix A 2.1), to make agar media and 1 l of D-lactic acid production broth medium as shown in appendix a 2.2 was prepared from yeast extract (Himedia laboratories), di-potassium phosphate, magnesium sulphate heptahydrate, manganese sulphate tetrahydrate, iron (II) sulphate heptahydrate, and cassava starch and calcium chloride for 15.00, 6.00, 0.57, 0.12, 0.03, 100.00, and 0.10 g, respectively, with α-amylase pretreatment.

In the part of screening section, lactic acid bacteria were cultured in microbiological media called RAM modified from De Man, Rogosa Sharpe (MRS) medium (De Man, 1960) from our previous study. RAM was prepared from tryptone and yeast extract, di-potassium hydrogen phosphate anhydrous, manganese sulphate monohydrate, iron (II) sulphate, magnesium sulphate monohydrate, cassava starch (Sanguan Wongse Industries Co., Ltd., Nakhon Ratchasima Province, Thailand), and tri-ammonium citrate. The screening included the use of iodine solution.

However, the plate assay was not enough to confirm the potential of amylase production by the lactic acid bacteria. In the broth cultivation, the activity determination was done using 3,5-dinitrosalicylic acid method (DNS)

For the DNS method, the measurement required DNS reagent as the solution in the colorimetric assay, it was prepared by dissolving 1 g of 3,5-dinitrosalicylic acid in 50 ml of deionized water. Then the solution was stirred with magnetic stirrer bar. After that, the solution was added with 30.0 g of sodium potassium tartrate tetrahydrate. Subsequently, the solution was added with 20 ml of 2 N NaOH and adjusted the volume into 100 ml. Finally, 0.2 ml of phenol (Carlo Erba Reagenti, Milan, Italy) was added (Appendix A 1.3.1). The activity can be determined by reacting with 1%(w/v) soluble starch which purchased from (Carlo Erba Reagenti). The standard curved was made from maltose monohydrate purchased from Himedia (Hi-Media Laboratories Pvt Ltd, Mumbi, India).

For protein determination, it was carried out using Bradford reagent (Bradford, 1976) purchased from Bio-Rad (Bio-Rad laboratories, Inc., India). The bovine serum albumin was purchased from Sigma (Sigma Aldrich Chemical Company, Missouri, U.S.A.).

In starch detection, the principle of binding between iodine and starch structure was used for starch determination. The iodine solution was prepared by dissolving 10 g of potassium iodide (Ajax) in 100 ml deionized water. Then it was stirred with the magnetic stirrer bar. Subsequently, 5 g iodine (Carlo Erba) was added into the solution and stirred until the iodine completely dissolved. The final solution was kept in the opaque container to prevent the oxidation by light. However, if there is no availability of opaque container wrapping the container with aluminum foil and storing in a dark close cabinet. The solution was suggested to freshly prepared every single use.

Finally, the total sugar determination was done using the series of sample treatment. There was 5% phenol which could be prepared from solubilizing the 5 g of phenol purchased from Carlo Erba, into the deionized water and made up to 100 ml for the final volume. While the concentrated sulfuric acid was purchased from Carlo Erba (Carlo Erba Reagenti, Milan, Italy).

3.2 Research procedure

3.2.1 Microorganisms and cultivation

Lactic acid-producing bacteria used in this study belonged to D-lactic acid bacteria including *Lactobacillus* sp. SUTWR 73 and *Lactobacillus* sp. SUTWR 81 was carried out at the Microbial Culture Collection and Applications Research Center, Institute of Science, Suranaree University of Technology and cultured on the starch agar and broth media for using in the research. The stock culture was kept at -20℃ in 10% skim milk (Himedia) solution.

D-lactic acid bacteria were cultured in RAM broth medium (Appendix A 2.2) for making inoculum and cultured on RAM agar medium for preparing pure colony before cultured in modified broth media to optimize the amylase production medium.

3.2.2 Detection and selection of bacterial strains for amylase production

The point inoculation technique and the reaction of the starch agar medium with iodine solution (Karnwal and Nigam, 2013) were used for primary screening of starch-hydrolyzing bacteria. If the microorganism could produce extracellular amylase, the starch in the agar medium would be hydrolyzed. The RAM agar (appendix A 2.1) was used for culturing the pure culture of microorganism by point inoculation technique and incubating under anaerobic condition at 35°C for 48 h in anaerobic chamber (Shel LAB, Sheldon Manufacturing Inc, U.S.A.) supplied with a gas mixture of CO_2:H_2:N_2 (5:5:90). After incubation, iodine solution (1%) was overlaid on the surface of agar plate and the clear zone around the bacterial colony was observed and measured.

However, the clear zone size did not clearly imply amylase activity, the broth medium was then used for cultivating the bacterium. The medium for amylase production was prepared as RAM agar but without adding agar. The cultivation was performed in anaerobic condition at 35°C for 24 h. The amylase activity and protein concentration were determined using DNS assay and Bradford method.

3.2.3 Investigation of amylase produced by the selected strain, *Lactobacillus* sp. SUTWR 73

For further investigation, the amylase production was also observed in the lactic acid production medium (Table 4.2), 300-l working volume in 500-l bioreactor inoculated with *Lactobacillus* sp. SUTWR 73. The cultivation was run at constant agitation speed 900 rpm, temperature at 35°C, and pH 7, respectively without aeration

for 24 h. The cultured sample was collected every 4 h, for amylase assay. The cultured media were centrifuged at 8,000 rpm for 10 min to separate cells from supernatant (the clear part). Both bacterial cell pellets and supernatant were collected. Protein in supernatant was determined by Bradford (1976) method for measuring protein concentration in supernatant part. If there was the protein in the sample, there would be the amylase too. In the detection and localization process, amylase activity was determined by using Bernfeld method (1955) or DNS assay. Firstly, 3,5-dinitrosalicylic acid reagent (DNS reagent) was prepared from 3,5-dinitrosalicylic acid, sodium hydroxide, and sodium potassium tartrate. After that, one milliliter reaction mixture was prepared by adding 0.5 ml of the crude enzyme from each part and 0.5 ml of 1%(w/v) soluble starch in sodium phosphate buffer (pH 6.8). Then the reaction mixture was incubated at 30°C for 30 min (Takenaka et al., 2015). The reaction was stopped by adding DNS reagent 3 ml and boiled for 10 min. Finally, the reaction mixture haw been added by deionized water 10 ml, mixed well and read the absorbance at 540 nm to estimate reducing sugars released. One unit of amylase activity was defined as the amount of enzyme that produced 1 μmol of reducing sugars per min. The part that gave the changes of reducing sugars consists of amylase enzyme (external or cell-bounded or intracellular).

3.2.4 Optimization of medium composition for amylase production

3.2.4.1 Factorial experiments

a. Chemical factors: nutrients

The standard medium consisting of pancreatic digest of casein (Hi-Media Laboratories Pvt Ltd, Mumbi, India), di-potassium hydrogen phosphate (Carlo Erba Reagenti, Milan, Italy), yeast extract, tri-ammonium citrate, magnesium sulphate

heptahydrate, manganese sulphate tetrahydrate, iron (II) sulphate heptahydrate (Ajax, Taren Point, Australia), cassava starch (Sanguan Wongse Industries Co., Ltd., (Nakhon Ratchasima, Thailand)), and agar for 5, 6, 3, 1, 0.57, 0.12, 0.03, 10.00, and 15.00 g, respectively (Appendix A 2.1). The medium had been developed from our previous study potentially support D-lactic acid production. The medium was used for commencing the optimization for amylase production.

(1) Carbon source concentration

Cassava starch was the only carbon source because it was cheap and easy to find. The cassava starch concentration was ranging from 0.5 to 15%(w/v) for finding the optimum condition which gave the highest amylase production. Then the amylase activity could be estimated by Bernfeld method (1955).

(2) Nitrogen sources

Different types of nitrogen source were applied into nitrogen source investigation and optimization of nitrogen source concentration. From standard medium compositions, various nitrogen sources were replaced or removed by yeast extract, and pancreatic digestive of casein which were two main nitrogen components in our media and culturing the bacterium in the optimized media. Finally, Bernfeld method (1955) was used to measure the amount of released reducing sugars and determined amylase activity of the enzyme.

Table 3.1 Proximate analysis of different nitrogen sources (data from our previous study) used in the medium optimization for amylase production.

N-source	Composition (%)				
	Moisture content[a]	Total C[b]	Total N[c]	Total P[d]	Total K[e]
Soy protein (food grade, Solca)	8.69	57.86	92.63	0.44	0.06
Spent brewer's yeast (Thailand)	3.66	72.33	32.31	0.89	1.39
Defatted rice bran (Surin, Thailand)	9.67	67.77	12.94	0.91	1.19

[a] Moisture content (AOAC, 1999)

[b] Total organic carbon analyses (Bernard et al., 1995)

[c] Kjeldahl method (AOAC, 2000)

[d] Spectrophotometric method (Bray and Kurtz ,1945)

[e] Flame photometric assay (AOAC, 1990)

Table 3.2 Different medium compositions used in the study.

Component	Amount of component in each medium formula (g/l)				
	RAM	1	2	3	4
Pancreatic digest of casein (Tryptone)	5	-	-	-	-
K_2HPO_4	6	6	6	6	6
Yeast extract	3	-	-	3	3
tri-Ammonium citrate	-	-	-	-	-
$MgSO_4 \cdot 7H_2O$	0.57	0.57	0.57	0.57	0.57
$MnSO_4 \cdot 4H_2O$	0.12	0.12	0.12	0.12	0.12
$FeSO_4 \cdot 4H_2O$	0.03	0.03	0.03	0.03	0.03
Cassava starch	15	15	15	15	15
Soy protein	-	-	8	-	-
Urea	-	-	-	5	8
Defatted rice bran (Surin)	-	8	-	-	-

(3) Essential elements

In the research, the concentration of other components was varied which were not only the carbon source and nitrogen source. From the medium components, the original medium could be changed. The K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ would be varied the concentrations of 7, 6 and 4 g/l, respectively. While the $MgSO_4 \cdot 7H_2O$ was also varied into 0.7, 0.5 and 0.3 g/l, respectively. In another hand, some cost component as tri-ammonium citrate could be cut-off, then the bacterium was also cultured into the modified media. After incubation for 24 h at 30℃, the amylase activity was then determined using Bernfeld method (1955).

b. Physical factors

In addition to medium compositions, physical factors were also important in the amylase production. Physical factors consisting of many factors but in the research, would investigate only pH and temperature for cultivating bacteria.

(1) pH

Normally, pH of the standard medium was adjusted into 6.8 before culturing bacterium. Besides, pH of the media would be varied between 2 to 11 to find the optimal pH condition for amylase production and use the Bernfeld method (Bernfeld, 1955) to measure amylase activity which related to amylase production.

(2) Temperature

In the bacterial cultivation process, the bacterium was cultured in standard media at 35°C. Culturing temperature of the bacterium would be varied by using the temperature from 25 to 90°C to investigate what temperature condition is good for amylase production by *Lactobacillus* sp. SUTWR 73.

3.2.4.2 Statistical experiments using response surface method (RSM)

This section has been conducted as in optimization 3.2.4.1 with the new strain SUTWR 73-1. The critical factor was obtained from this section. Response surface method (RSM) was used to design the experiment based on the data obtained from the factorial experiment. Three factors including cassava starch concentration, tryptone concentration, and pH, were critical factors for cultivating SUTWR 73-1. The central values were used to design the runs using central composite. The 3 variables gave the 20 runs which were conducted. The model construction was shown in the following equation.

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X X_i^2 + \sum_{i<j}^{3} \sum_{j=1}^{4} \beta_{ij} X_i X_j + \sum_{i=1}^{4} \beta_{iii} X X_i^2 + \sum_{i<j}^{3} \sum_{j=1}^{4} \beta_{ij} X_i^2 X_j + \sum_{i<j}^{2} \sum_{j<k}^{3} \sum_{k=1}^{4} \beta_{ijk} X_i X_j X_k$$

Where Y is the predicted response (Amylase activity, U/ml), β_0 is the intercept, β_i is the linear constant coefficient, β_{ii} is the coefficient of the quadratic single term, β_{ij} is the coefficient of the quadratic cross-product term, β_{iii} is the coefficient of the cubic single term, β_{iij} is coefficient of the cubic two cross-product terms, β_{ijk} is the coefficient of the cubic three cross-product terms, and X_i, X_j, and X_k are the coded independent variables. The experiment was performed in three replicates. Response data were analyzed by Design-Expert (Design-Expert version 11, Stat-Ease Corporation, USA). To check the validity of the models, three points in the design space were randomly conducted (Soni et al., 2015).

3.2.5 Production and partial purification of amylase produced by *Lactobacillus* sp. SUTWR 73

After medium optimization had been completely conducted. The modified medium was used in the 3-l scale cultivation in the bioreactor (New Brunswick, BioFlo®/ CelliGen® 115, New Bruswick Scientific, USA) at 35℃ and 150 rpm for agitation speed for 48 h. Each parameter was monitored along with the cultivation.

3.2.5.1 Determination of protein concentration

Bradford reagent was used to determine the protein concentration in the supernatant of cultivated media after centrifugation at 8,000 rpm for 10 min by compared with the standard curve of bovine serum albumin (BSA) concentration. For

the reaction 10 µl of the sample was reacted with the 200 µl of Bradford reagent. Then the absorbance determination was done at 595 nm. The concentration of the protein content was analyzed from the standard curve of bovine serum albumin.

3.2.5.2 Determination of amylase activity

In the processes of detection of amylase and optimization of amylase production step from 3.1.2.3-3.1.2.4, Bernfeld method (1955) was used for measuring the concentration of reducing sugars. The reaction contained 50 µl of 1%(w/v) soluble starch in 50 mM of sodium phosphate buffer (pH 6.8) and 50 µl of crude enzyme. The reaction was left at 35°C for 30 min. Stopping reaction was carried out by adding 100 µl DNS reagent and boiling for 10 min before adding 800 µl of water. Then, the final solution was measured absorbance at 540 nm. Consequently, it was calculated the enzyme activity from the absorbance value at 540 nm. One unit of amylase activity was defined as the amount of enzyme that produced 1 µmol of reducing sugars per min (Takenaka et al., 2015).

3.2.5.3 Detection of residual starch in cultivated broth

The retained starch in cultivated broth was determined using iodine solution. The dilution of iodine solution was reacted with the suitable dilution of the sample broth and suddenly measured absorbance at 570 nm to avoid the loss of color (Swammy et al., 2015).

3.2.5.4 Determination of total sugar in cultivated broth

The total sugar determination given from Dubois et al. (1956) was adapted to micro assay. Twenty-nine microliter of sample reacted with 29 µl of 5% phenol. The reaction mixture was mixed for 1 min. Then, the concentrated sulfuric acid was added into the reaction for 142 µl. The reaction was accelerated the color

development at 80℃ for 30 min. Then the reaction was determined using spectrophotometric technique at 492 nm for wavelength. The concentration of total sugar was calculated from the standard curve of glucose.

Amylase obtained from the amylase production step was low purity by containing undesired proteins, so purification step was needed in this step to reduce the proteins and make the higher amylase purity. The ammonium sulphate precipitation (Burgess, 2009) was used to precipitate the protein by adding gently ammonium sulphate (Sigma Aldrich Chemical Company, Missouri, U.S.A.) in the supernatant and then incubated for 30 min. The method relied on the fundamental of the salt effect in solution. At different salt concentration, the obtained precipitate proteins were different. In the research, the crude enzyme (supernatant part) was added ammonium sulphate in the crude enzyme to be saturated with 50, 60, 70, 80 and 90% ammonium sulphate, and then incubated at 4℃ for 30 min. Subsequently, the solution was centrifuged to separate the precipitated proteins from the solution. Then, the precipitate was resuspended in 1 ml of 50 mM Tris-maleate pH 6.8. Solution containing precipitate protein was dialyzed using 30 kD molecular weight cut-off dialysis membrane to eliminate the undesired ions that inhibited amylase activity. Finally, precipitated protein from each saturation of ammonium sulphate were detected amylase activity and determined amount of protein by using Bradford method for callulating specific activity. The percentage of ammonium sulphate which provided the highest specific amylase activity was chosen for characterization of amylase produced by SUTWR 73.

3.2.6 Characterization of amylase produced by *Lactobacillus* sp. SUTWR 73

Amylase was preliminarily characterized in a few primary aspects such as the molecular weight, stability at various pH conditions, and various temperature conditions.

3.2.6.1 Molecular weight determination

In the part of amylase molecular weight determination, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) from Laemmli (1979) was used to determine the molecular weight of protein by comparing with the protein marker which had a various specific size of the proteins. The crude enzyme was mixed with a loading buffer (4% SDS (Sigma), 10% 2-mercaptoethanol (β-ME purchased from Sigma), 20% glycerol (Carlo Erba), 125 mM Tris-HCl (pH 6.8), 0.1% bromophenol blue (Sigma)) and heat at 95℃ for 5 min. Owing to the zymogram technique was not required the denaturation of the enzyme allowing it to perform activity. Hence, the zymogram was not required to treat the protein with the β-ME and heat. After that the treated enzyme was run parallelly in two SDS gel with the constant voltage at 70V. After running the protein through the 10% SDS gel, the gel was then stained with 0.1% Coomassie Blue R-250 (Sigma) dye in the methanol-acetic acid-water mixture (4:1:5, v/v/v) for 30 to 40 min, these chemicals were purchased from Carlo Erba (Carlo Erba Reagenti). The developed color was destained using in the methanol-acetic acid-water mixture (4:1:5, v/v/v) without Coomassie blue R-250 for 3 h. Changing destanined buffer was done every time that the buffer changes to be strongly blue. For another gel it would be brought to eliminate the SDS by immersing the gel in 1% triton-X (Sigma) for 1 h. The gel contained the amylase band was transfered to the box containing the 1% soluble starch and left it for 1 h allowing the

gel saturated with starch at 4℃. The in-gel reaction was conducted by incubated gel in 50 mM tris-maleate buffer (pH 6.8) on the shaker at 35℃ for 1 h before checking the result using iodine solution (1.3% I_2 and 3% KI) for detection of activity (Manchenko, 2002). The band consisted of amylase that would show the clear band. The molecular weight of the protein was compared with the marker in the SDS gel. The protein marker PageRuler™ Unstained Protein Ladder was purchased from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA) contained different 14 proteins varying in size, including 200, 150, 120, 100, 85, 70, 60, 50, 40, 30, 25, 20, 15, and 10 kD. For the easier way, the molecular weight analysis can be determined using gel documentation system (Gel Doc EZ, Bio-Rad, USA).

Table 3.3 The summarized volume of each component for preparing stacking gel and resolving gel in SDS-PAGE.

Gel	Composition	Volume
Stacking gel (10%)	Deionized water (ml)	6.00
	Tris buffer (1.5M pH 8.8) (ml)	2.50
	Acrylamide : Bis-acrylamide 30% (ml)	1.33
	10% SDS (µl)	100
	10% Ammonium persulphate (µl)	50
	N, N, N', N'-tetramethyl ethylene diamine (TEMED) (µl)	15
Resolving gel (4%)	Deionized water (ml)	4.00
	Tris buffer (1.5M pH 8.8) (ml)	2.50
	Acrylamide : Bis-acrylamide 30% (ml)	3.33
	10% SDS (µl)	100
	10% Ammonium persulphate (µl)	50
	N, N, N,' N'-tetramethyl ethylene diamine (TEMED) (µl)	15

3.2.6.2 Kinetic parameter determination

The kinetic parameters of the amylase produced by SUTWR 73-1 which were determined in this section were K_m and V_{max}, when K_m was a Michaelis constant defined as the substrate concentration at 1/2 the maximum velocity and V_{max} was the maximum velocity. Two kinetic parameters were calculated from the second-order of Michaelis-Menten (MM) equation or the inversion of the MM equation given from the Lineweaver-Burk plot. For the investigation, the amount of the enzyme was kept constant and the substrate was varied the concentration from 0 to 0.5 mg/ml. The activity of each soluble starch concentration was checked using DNS method. Then, the graph was plotted between $1/K_m$ and 1/[S]. The linear part of graph was used to determine the K_m and V_{max}.

3.2.6.3 Study of amylase profile

In this section, fifty microliters of partially purified amylase was reacted with 50 µl of 1%(w/v) soluble starch at the different temperatures ranging from 25 to 100℃. The amylase activity was determined using DNS method. The residual activity was compared to the initial activity. For the pH profile construction, the reaction has been carried out at different pH by mixing the amylase with the various pH buffers ranging from pH 3 to pH 12 and made up the final buffer concentration to 50 mM. Then, amylase activity at different points was measured.

3.2.6.4 Detection of amylase stability

Physical factors were considered in the term of pH and temperature. To find the amylase stability, the enzyme was incubated at different conditions and times as mentioned in 3.2.6.3, and allowed the enzyme reacted to the 1%(w/v) soluble starch. The DNS method was used to determine amylase activity.

3.2.6.5 Effect of ions on amylase activity

The effect of ions was investigated by focusing on ions formerly constituted in RAM medium. The reaction was conducted by mixing 50 µl of amylase with 50 µl of 1% soluble starch in the presence of different ions with 2, 5, and 10 mM. The DNS method was used to determine amylase activity.

3.2.7 Statistical analysis

The optimization of some amylase production conditions was performed in triplicates and used statistical analysis for providing the standard deviation of data. Analysis of Variance (ANOVA) and means comparison by Duncan's Multiple Range Test (DMRT) were used to determine differences between mean at $P<0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Detection and investigation of bacterial strains for amylase production

4.1.1 Starch agar medium

The selected bacteria *Lactobacillus* sp. SUTWR 73 and SUTWR 81 were tested for their cassava starch utilization capabilities on RAM agar containing 1%(w/v) cassava starch as a carbon source. It was found that *Lactobacillus* sp. SUTWR 73 could utilize cassava starch as a carbon source better than SUTWR 81. It showed a clear zone around colonies when overlaid with 1% iodine solution on RAM agar (Figure 4.2). So, our bacterium can utilize cassava starch. That means the bacterium can also produce an amylolytic enzyme.

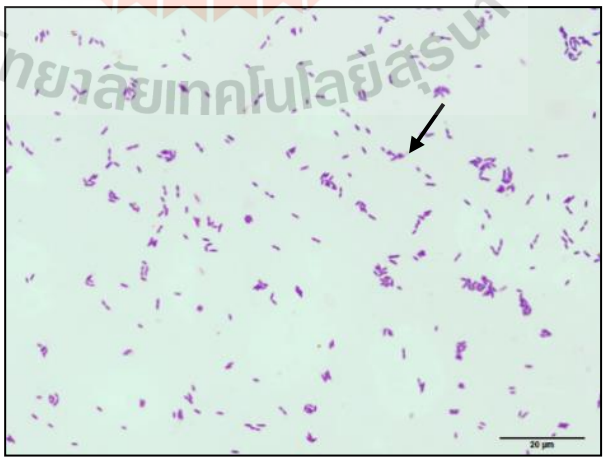


Figure 4.1 Cell morphology of *Lactobacillus* sp. SUTWR 73 from Gram's staining (arrow) under light microscope (1,000X).

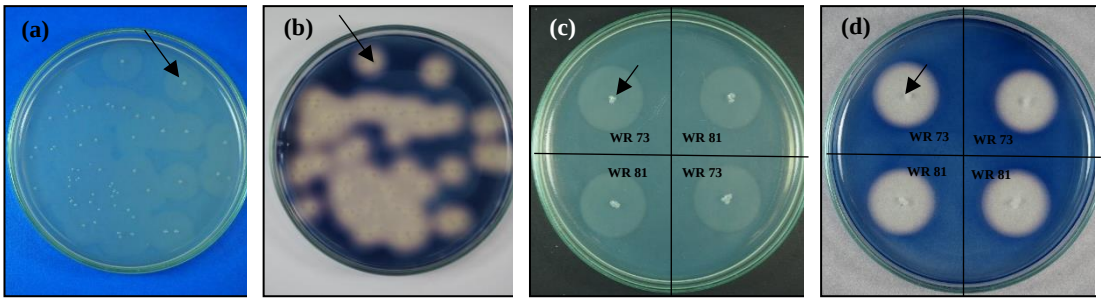


Figure 4.2 Growth and cassava starch hydrolysis of the selected bacterial isolates SUTWR 73 and SUTWR 81 (a) and (c). Growth on RAM medium containing 1% cassava starch (b) and (d). Positive cassava starch hydrolysis colony after adding iodine solution onto the RAM agar surface of (a) and (d).

Table 4.1 Diameters of colony and clear zone from starch hydrolysis after overlaid with the iodine solution and incubation at 30°C for 24 h.

Bacterial strain	Colony	Colony diameter (mm)			Clear zone diameter (mm)		
		X	Y	Average	X	Y	Average
SUTWR 81	1	2.0	3.0	2.5	19.0	20.0	19.5
	2	2.5	3.0	2.8	17.0	19.0	18.0
SUTWR 73	1	3.0	3.0	3.0	21.0	21.0	21.0
	2	4.0	2.0	3.0	19.0	20.0	19.5

The clear zone of starch hydrolysis after overlaid with iodine solution of bacterial strain SUTWR 73 was larger than SUTWR 81 and the clear zone is larger than at 5.27 times of colony diameter.

4.1.2 Starch broth medium

Lactobacillus sp. SUTWR 73 was cultivated in RAM broth containing 1%(w/v) cassava starch, then the amylase activity in the cultivated medium was assayed to determine a concentration of reducing sugars by using DNS method. The enzyme activity of 5.44 U/ml was detected.

From the broth cultivation, it was found that there was amylase activity in the starch cultured broth medium by giving 8.02±2.13 U/ml for the activity at 24 h. The results confirmed that *Lactobacillus* sp. SUTWR 73 can produce extracellular amylase. Due to its amylase activity of conversion of starch to dextrin brought to the conclusion that the *Lactobacillus* sp. SUTWR 73 was an amylolytic lactic acid bacterium. The amylolytic lactic acid bacteria were the group of lactic acid bacteria possesses amylolytic enzyme supporting the direct conversion of the starch to lactic acid (Ray and Montet, 2016). In the group of lactic acid bacteria, the amylolytic activity was mostly belonged to genera *Lactobacillus, Lactococcus, Streptoccocus, Pediococcus, Carnobacterium*, and *Weissella* (Bhanwar and Ganguli, 2014).

From this study, *Lactobacillus* sp. SUTWR 73 can produce the amylase both in broth and on agar medium.

While running the cultivation in 5-ml RAM broth containing 1%(w/v) cassava starch, it revealed that SUTWR 73 took a few hours to be in lag phase and reaching the initial log phase when the time passed for 4 h. The maximum growth was obtained at 16 h with 1×10^{10} CFU/ml and less decreased after that (shown in Figure 4.3). However, SUTWR 73 produced the highest amylase at 22 h 9.00±1.47 U/ml with growth of 1.79×10^{9} CFU/ml, in the original medium (RAM) containing 1% of cassava starch (Figure 4.3). For pH which represented the acid production, it was found that the pH

dropped from the beginning of the experiment representing that SUTWR 73 could produce acid as metabolite and could live in the acidic condition or could be said that it was acid-tolerant bacterium (Hyronimus et al., 2000). The lactic acid bacteria tolerated to the acid environment through two mechanisms. Firstly, the acid tolerance was induced during the logarithm phase with the non-lethal induction, then increased with the general response mechanism. Another response was pH-independent response such as the response from starvation which providing the cross-talk mechanism. For some reason, the third may be purposed as the biofilm formation.

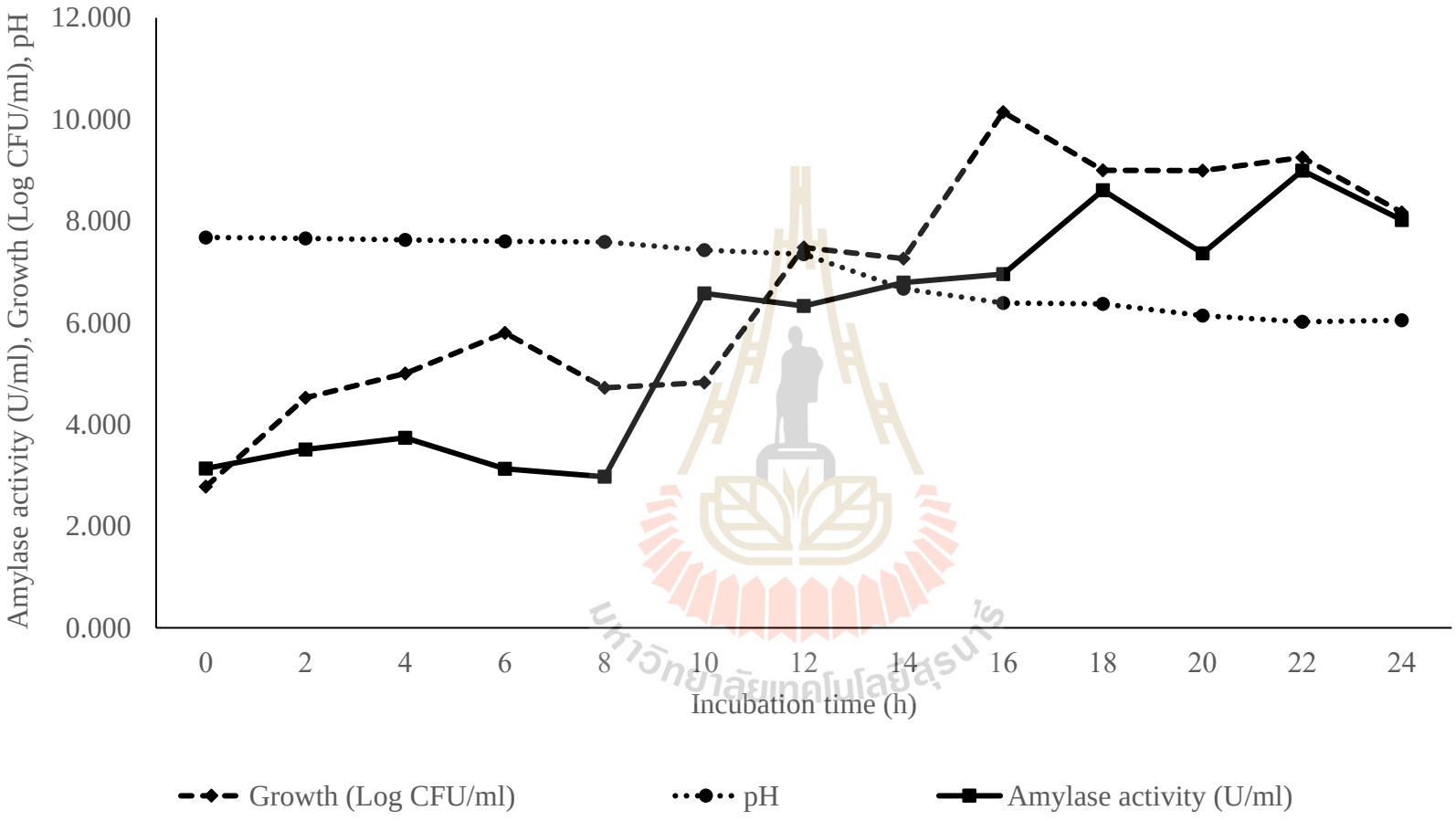


Figure 4.3 Amylase activity, pH, and growth (Log CFU/ml) of SUTWR 73 cultivated in 10-ml RAM broth with agitation speed 150 rpm at 35℃ and pH 8 for 24 h.

The relationship between growth (Log CFU/ml) and OD_{600} was investigated as shown in Figure 4.4. The linear equation given from the relationship has been used to prepare the desired cell concentration. Furthermore, the numbers of the bacterial cell can be estimated from these equations.

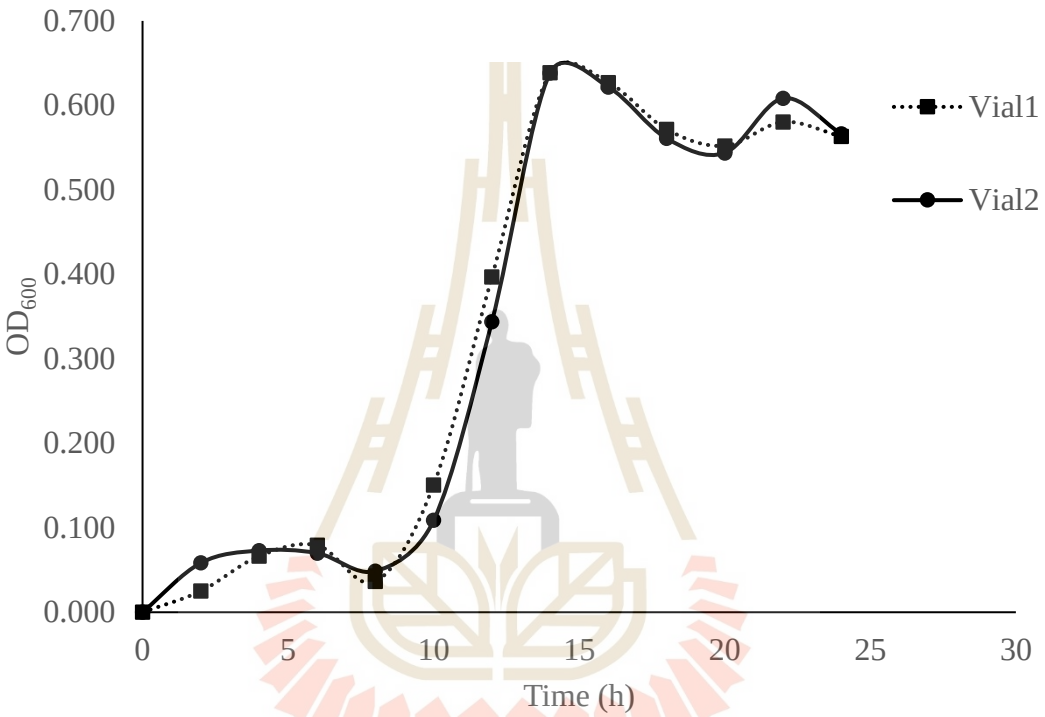


Figure 4.4 Relationship between incubation time and growth (OD_{600}) of SUTWR 73 in RAM medium.

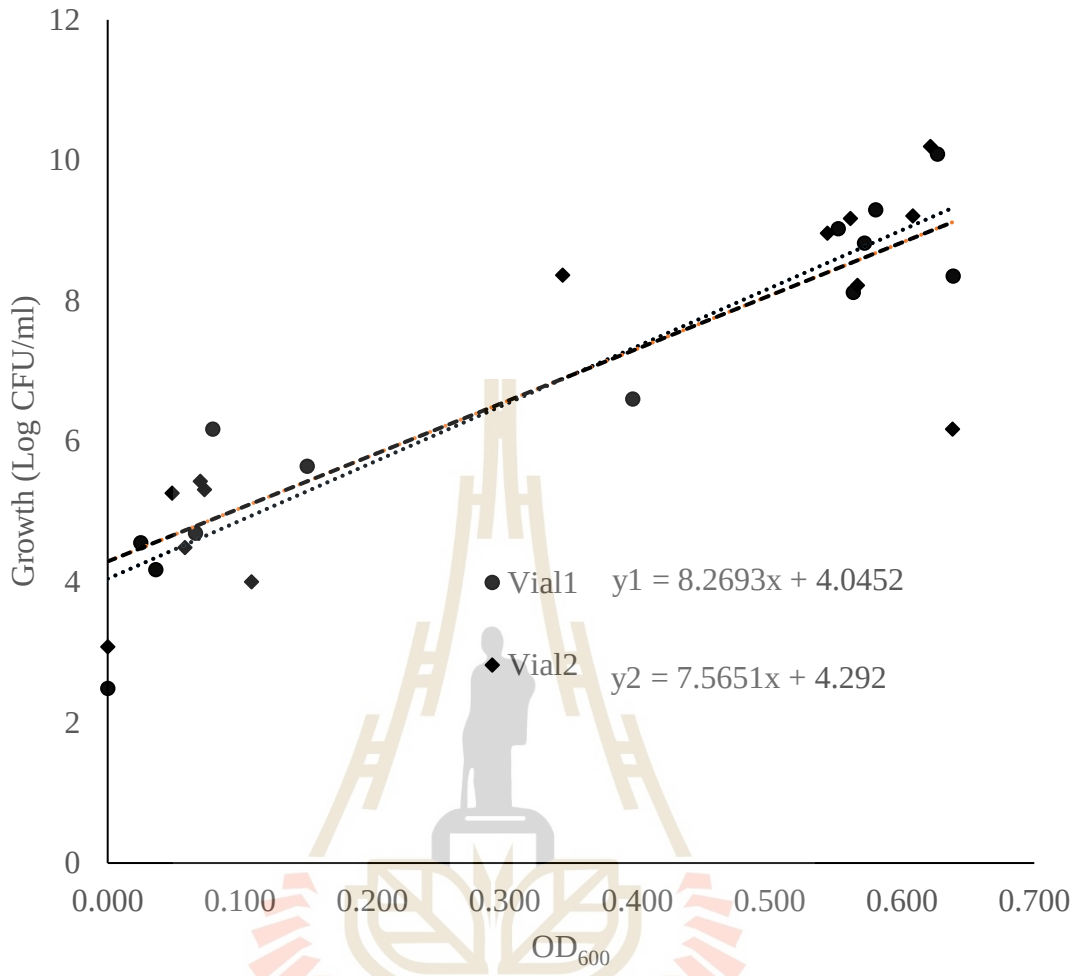


Figure 4.5 Relationship between OD_{600} and Growth (Log CFU/ml) of SUTWR 73 in RAM medium.

To monitor amylase production during cultivation of SUTWR 73, the protein concentration was detected. It was found that the concentration of protein reduced continually to 8 h and stayed constantly until completing 24 h. It could be interpreted that there was usage of protein at earlier, to supplemented growth of the bacterium, 4.4 while the secreted protein was lower than the consumed protein.

When considering amylase activity along with protein concentration, there was something interesting. The change of amount protein coincidence with the change of amylase activity at 6 to 8 h of cultivation time, revealed that after 10 h, the high proportion of protein in the cultivated medium was amylase compared to the earlier (Figure 4.6). Beside this, it also related to the growth of the bacterium which produced amylase at the mid-log phase.

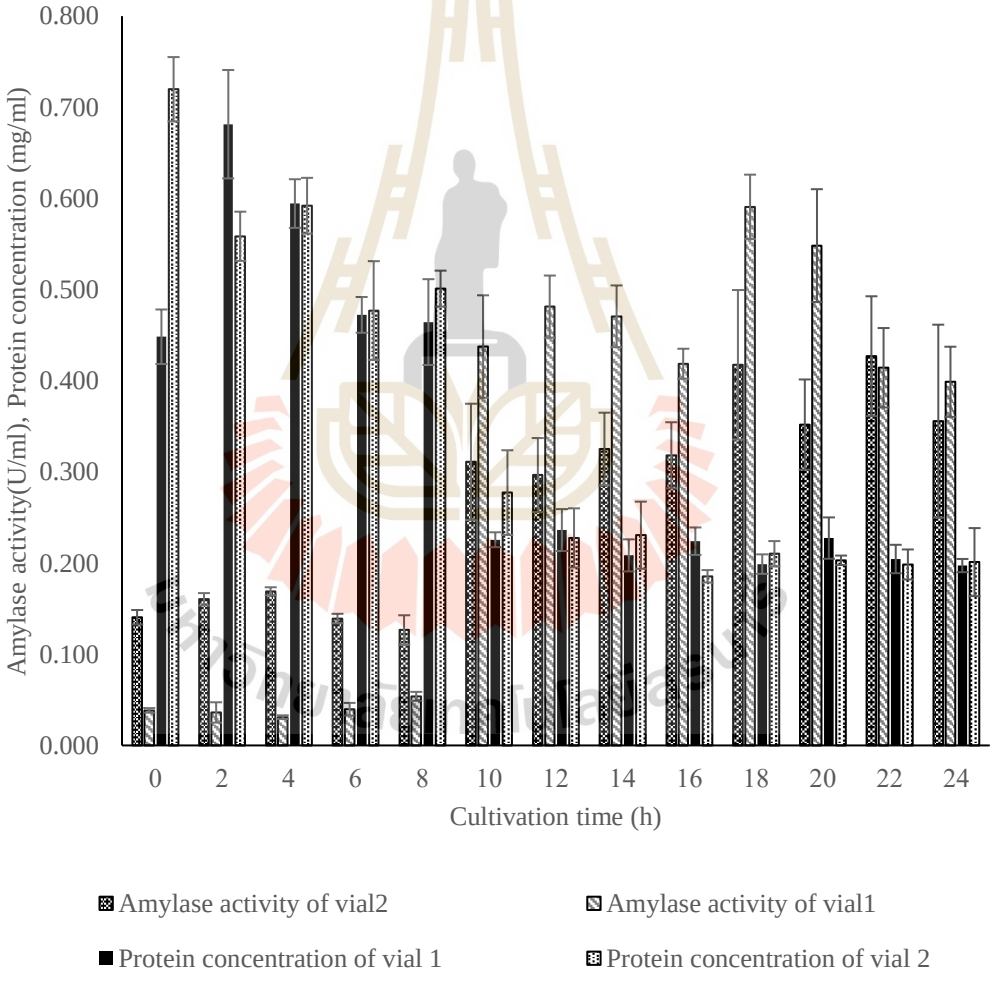


Figure 4.6 Amylase activity and protein concentration of cultured medium with SUTWR 73 at various cultivation times.

The production of amylase was also observed at larger scale (300-l working volume) production of lactic acid. The lactic acid production medium contained yeast extract (Himedia laboratories), K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$, $FeSO_4 \cdot 7H_2O$, cassava starch, and $CaCl_2$ of 15, 6, 0.57, 0.12, 0.03, 100, and 0.10, respectively (Table 4.2). The medium was treated with heat and 0.10 ml of α-amylase before sterilization. pH of the medium was maintained in 6 to 7 (Table 4.2).

Table 4.2 Lactic acid production medium components (Rodtong, 2017).

Component	Concentration (g/l)
Tryptone	-
Yeast extract	15
Soy protein isolate	-
Ammonium sulphate	-
di-Potassium phosphate	6
$MgSO_4 \cdot 7H_2O$	0.57
$MnSO_4 \cdot 4H_2O$	0.12
$FeSO_4 \cdot 7H_2O$	0.03
Tapioca starch	100
$CaCl_2$	0.1

*α-Amylase was used for liquefaction of the gelatinized starch.

Supernatant at different times was separated from crude cultured media and determined the total activity by calculating from the amount of released reducing

sugars. The results reveal that at 8 h of cultivation provided the highest amylase activity from all three fermentation experiments which were 3.03±0.04, 5.35±0.14, and 4.31±0.12 U/ml, respectively (Figure 4.7). However, after 8 h of cultivation, the total amylase activity slightly decreased over time.

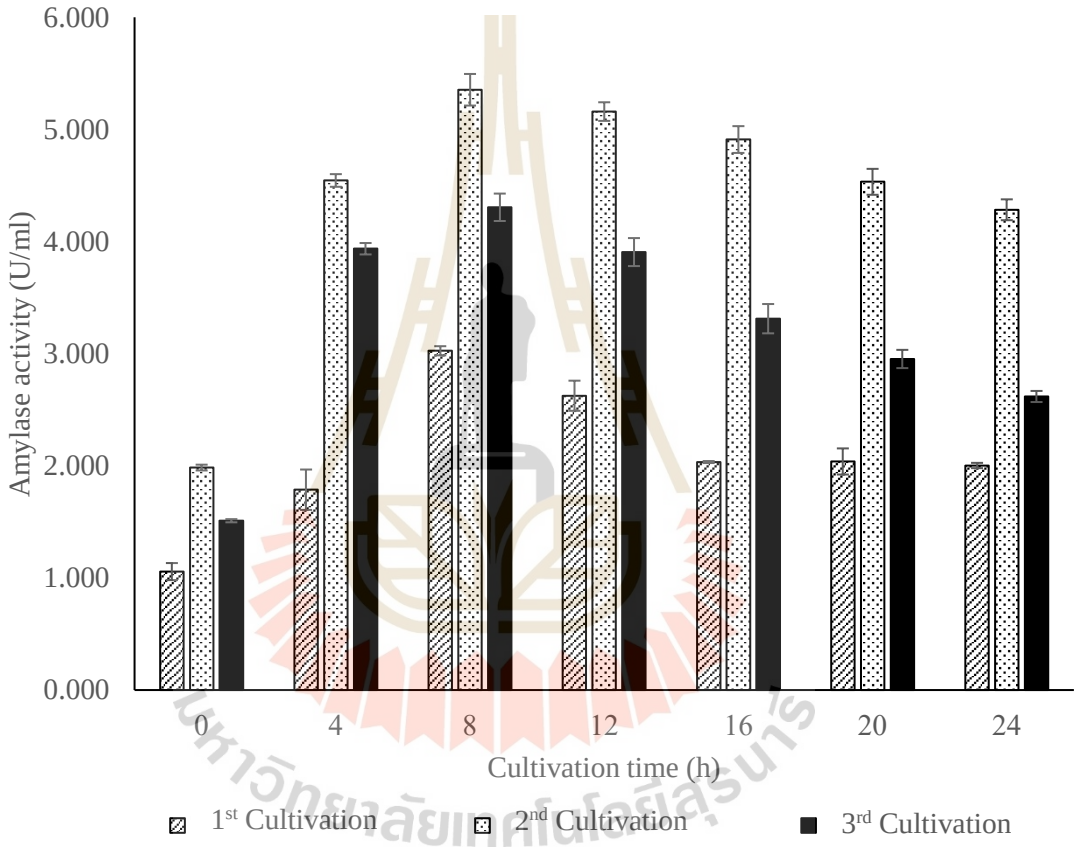


Figure 4.7 Total amylase activity of supernatant collected at various cultivation times.

The remaining cell pellets from the supernatant separation step and supernatant at different cultivation times were also determined for the total activity. The results showed that the highest amylase activity was detected at 8 to 16 h for all three

cultivations but at the 12-h incubation from the second cultivation (Figure 4.8), the low total amylase activity was obtained due to reducing sugar quantification (DNS method) assay having heat and time as the difficult control factors resulting in the different result.

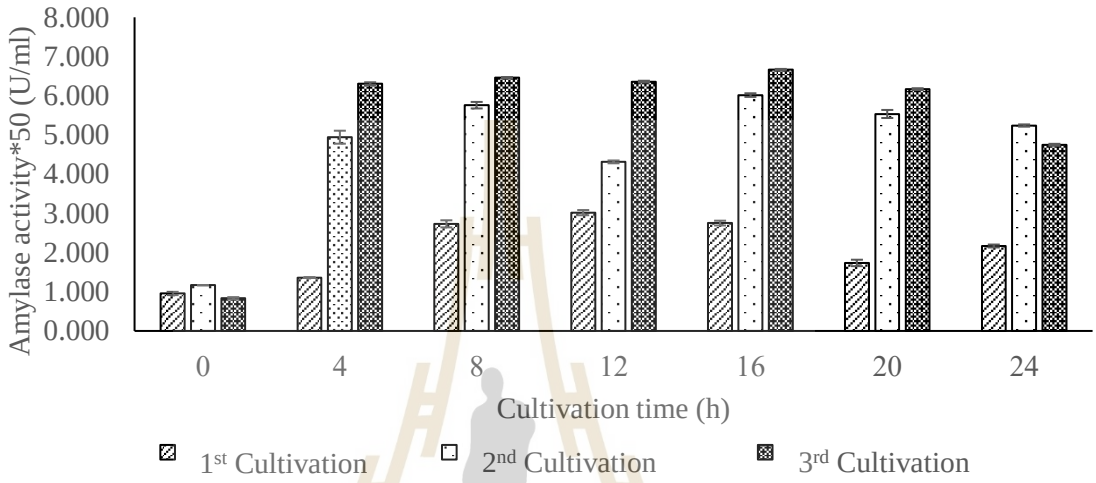


Figure 4.8 Amylase activity of cell pellets in various cultivation times

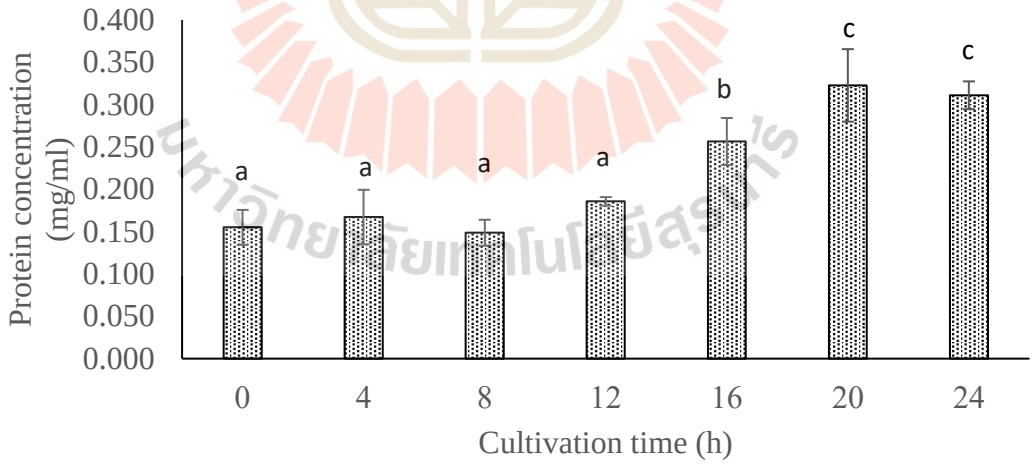


Figure 4.9 Protein concentrations at various cultivation periods. Means with different letters (a, b, and c) showed significantly different means (Duncan's Multiple Range Test (DMRT), $p<0.05$).

The protein concentrations at different cultivation times were determined. It was found that at the initial cultivation time of 0 to 4 h, the protein concentration decreased, while it increased after 8 h tend. These mean that at the initial stage, the bacteria consume protein in the cultivation medium for growth, and secreted protein enzyme for substrate hydrolysis. The secreted protein could be the enzyme or any protein due to the analysis method (Bradford, 1976) can determined the total protein.

From three sets of fermentation mentioned above, the activity data were just appearance activity which contains reducing sugars in the samples. Nevertheless, the trend of appearance activity can be interpreted from the given values. Lastly, the fourth fermentation was done and interpreted in the real activity which was shown below.

As shown in Figure 4.10, the amylase activity profile from this bioreactor was different from the others because the high amylase activity was detected from the beginning of cultivation, and the late incubation in which the trend was not the same as the first, the second and the third bioreactors.

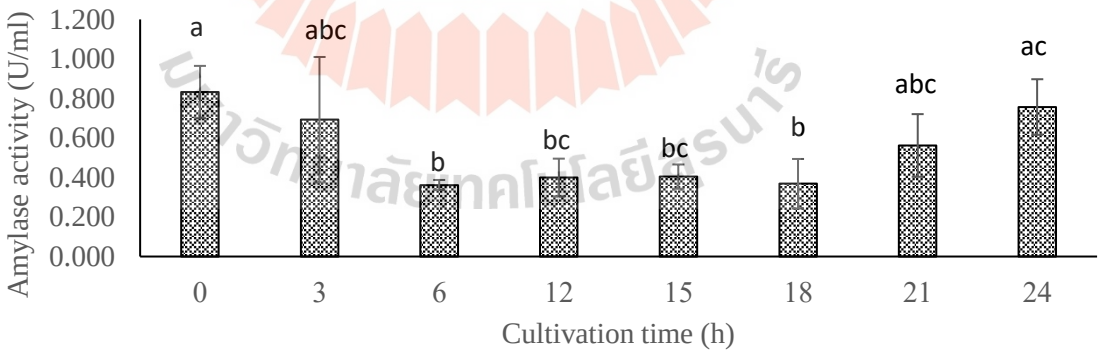


Figure 4.10 Amylase activity at different cultivation times of *Lactobacillus* sp. SUTWR 73 in lactic acid production medium (the fourth cultivation). Means with different letters (a, b, and c) showed significantly different means (Duncan's Multiple Range Test (DMRT), $p<0.05$).

From Figure 4.11, the harvested samples at different cultivation times were separated into supernatant and cell pellets. In supernatant, the highest amylase activity was obtained at an 8-h incubation time of 4.23±1.17 U/ml, with 4.44×10^{8} CFU/ml and 0.15 g/l for growth and protein concentration. After an 8-h incubation time, the amylase activity slightly decreased. However, the highest growth was observed at 20-h incubation. It was an evidence to support that the enzyme was secreted at the early log phase due to the induction of starch similar to the report by Ensari et al. (1995). For substrate consumption after added 10% cassava starch to the cultivation medium, the total sugar decreased continually until reaching 24 h. In contrast, the reducing sugars remained in the broth increase overtime. Coincidentally, the amylase activity detected from the supernatant was slightly getting low meaning that there was product inhibition (Figure 4.11).

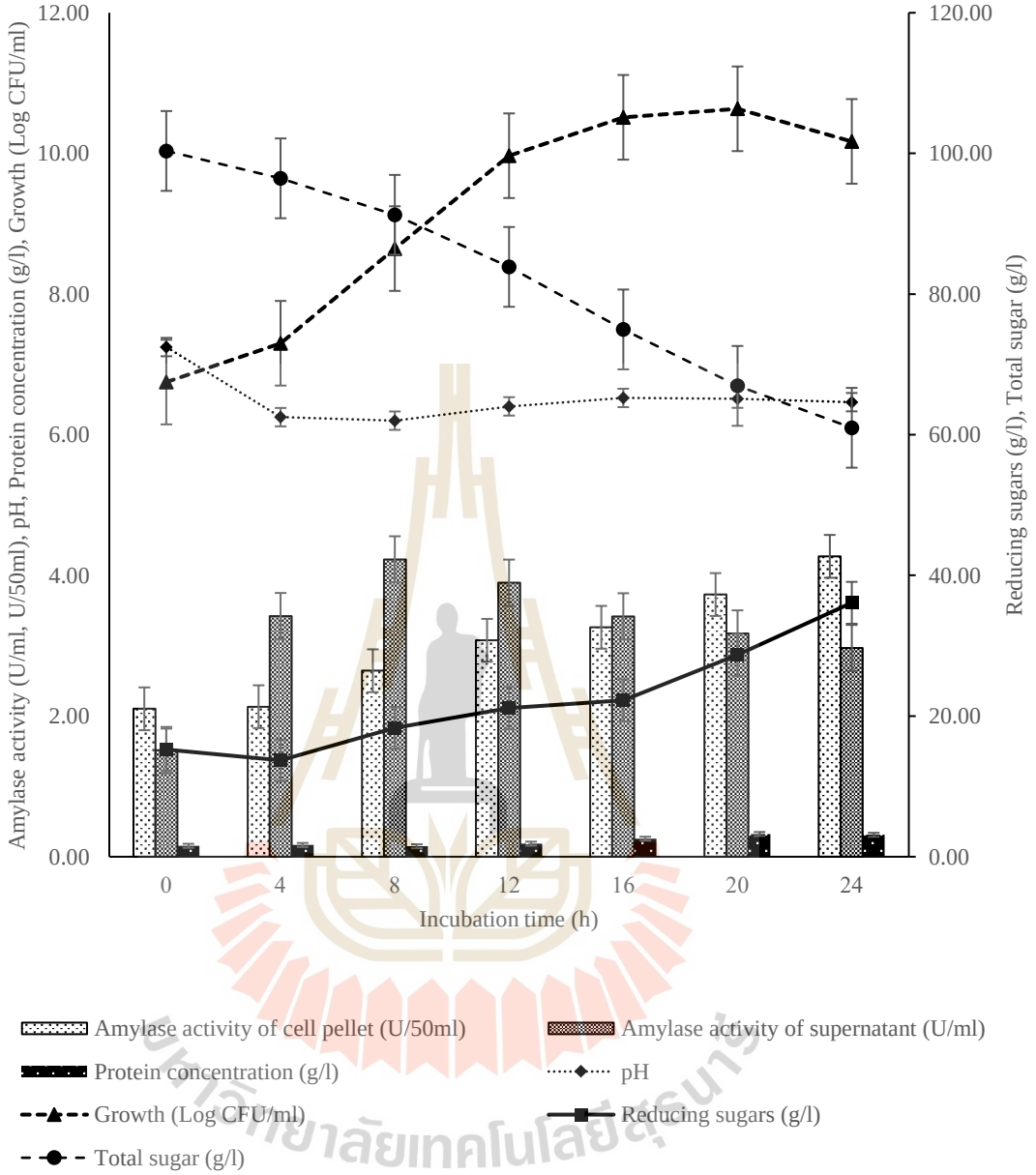


Figure 4.11 Amylase activity of supernatant and cell pellet, protein concentration, pH, growth of SUTWR 73, reducing sugars, and total sugar in 500 l-fermenter with 300 l working volume cultivated at 35℃ for 24 h, 900 rpm for agitation speed with constant pH, around pH 6-7 and without aeration.

Detection of amylase on bacterial cell, the amylase activity was detected on the cell pellet after separating from the cultivated medium. The highest amylase activity of 4.27±0.06 U/50 ml with 1.48×10^{10} CFU/ml, was detected from the cell pellet the enzyme activity increased parallelly to the growth of the bacteria, the higher growth with the higher cell mass contained higher amylase activity. The results suggested that the amylase produced by SUTWR 73 would be cell-associated amylase which rarely found in this group of bacteria. The cell-associated enzyme was also found in the S-layer of *Bacillus stearothermophillus* ATCC 12980 (Egelseer et al., 1996). As the cell-associated amylase had also been found in Gram-positive bacteria, Gram-negative bacteria also had as found in *Bacteroides thetaiotaomicron* (Koropatkin and Smith, 2010). In this study, in comparison to the growth in RAM medium, the lactic acid production medium exhibited no lag phase and the shorten log phase since the cassava starch was gelatinized by the commercial amylase and the large inoculum size (5%) used. The protein concentration in the supernatant at different cultivation times has been considered. It was found that at the initial cultivation time (0 to 4 h) the protein concentration decreased while after 8 h tended to increase, and level off. These mean that at initial stage the bacteria consume the protein for growing, they will secrete protein out from the cell. The secreted protein could be the enzyme or any protein due to the Bradford method can determine the total protein.

Table 4.3 Specific growth rate and D-lactic acid production of *Lactobacillus* sp. SUTWR 73 in standard medium containing 100 g/l cassava starch (Rodtong, 2017) using 300 l in 500-l bioreactor for 48 h.

Experiment	Specific growth rate (μ_{max}) (h^{-1})	D-Lactic acid concentration (LA_{max}) (g/l)	Productivity (P_{LA}) (g/l*h)	
			Maximum	Average
This study	0.3664	50.35	1.995	1.1679
Our previous study (Rodtong, 2011) (48 h)	0.4243	51.92	1.4915	1.2673

Note: μ_{max}, Highest specific growth rate; LA_{max}, Lactic acid at 24-h cultivation time; and P_{LA}, Lactic acid production rate

4.2 Preliminary investigation of medium composition for amylase production by *Lactobacillus* sp. SUTWR 73

To obtain the substrate concentration for the maximum amylase production by *Lactobacillus* sp. SUTWR 73, some components (carbon and nitrogen sources) as nutrients and initial pH of the bacterial cultivation medium for amylase production were investigated. The cost of the amylase production medium was then evaluated as follow:

4.2.1 Nutrients

4.2.1.1 Carbon source concentration

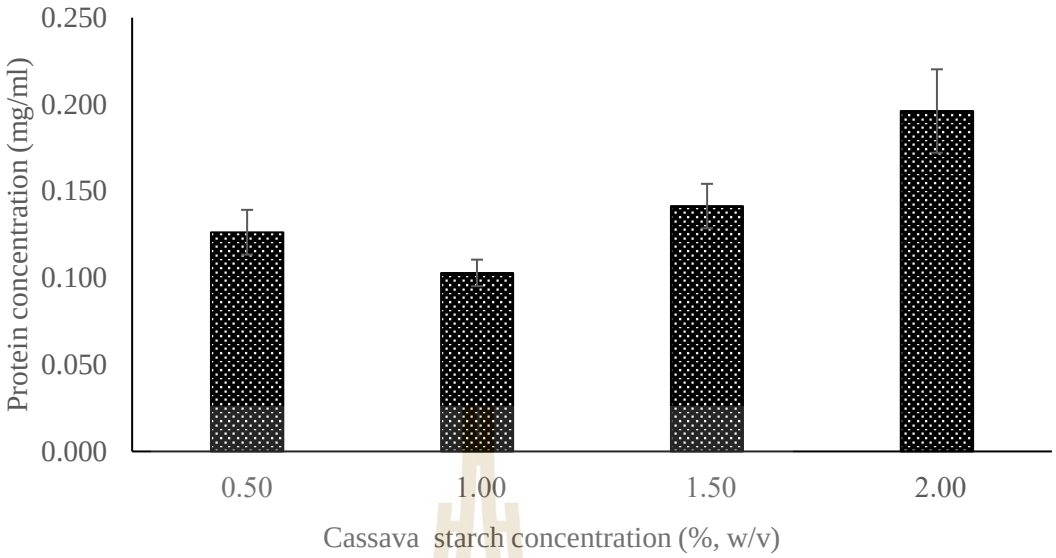
The suitable concentration of cassava starch for amylase production by the selected isolate was determined using RAM medium containing 0.5, 1.0, 1.5,

and 2.0% of cassava starch. The maximum protein of 0.196±0.024 mg/ml was obtained from RAM medium containing 2.0% of cassava starch (Figure 4.12). The protein concentration per cell of 23.00±3.00 µg/cell. This might be due to the substrate concentration which induced the production of amylase (Ensari et al., 1995) which was enough for hydrolyzing the cassava starch substrate. Protein concentration is high in the range of cassava starch in the range of 0.5-2.0% provided the highest concentration of the protein. The extracellular amylase activity per cell was higher than the cell-bound enzyme at all cassava starch concentrations. At 0.5, 1.0, 1.5, and 2.0% cassava starch in the amylase production medium, the extracellular amylase activity was 37.64, 88.08, 31.61 and 32.67 times higher than the amylase activity of the cell, respectively. The broth medium containing 2%(w/v) starch showed the highest amylase activity and bacterial growth of 7.26±0.83 U/ml and 2.38×10^{8} CFU/ml, respectively. The trend was similar to the studies of *Bacillus* sp. I-3 (Gül et al., 2005) and *Lactobacillus fermentum* 04BBA19 (Fossi and Tavea, 2013) which reported that the starch act as the initial inducer for stimulating the bacteria to secrete the amylases outside their cells.

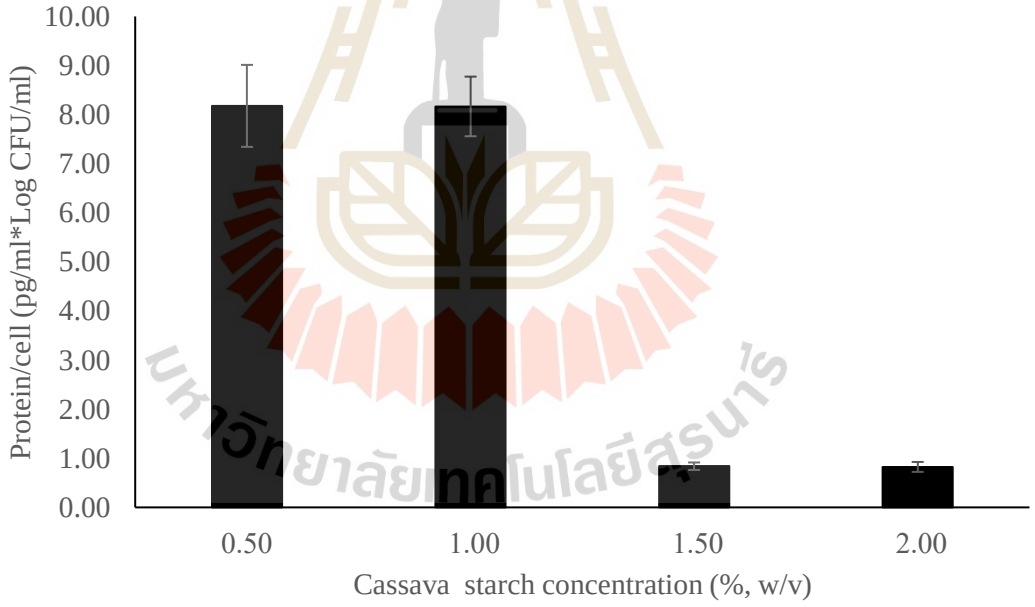
Table 4.4 The average concentrations of protein after culturing *Lactobacillus* sp. SUTWR 73 at 35°C for 24 h.

Medium formula	Bacterial growth (CFU/ml)	Protein concentration (mg/ml) in supernatant	Protein concentration per Log CFU/ml (pg)
1, containing cassava starch concentration (%, w/v)			
0.50	1.54×10^{7}	0126±0.013	8.18±0.83
1.00	1.26×10^{7}	0.103±0.008	8.17±0.61
1.50	2.68×10^{8}	0.141±0.013	0.84±0.08
2.00	3.38×10^{8}	0.196±0.024	0.82±0.10
2, containing cassava starch concentration (%, w/v)			
0.50	nd	0.011±0.005	nd
1.00	nd	0.008±0.007	nd
1.50	nd	0.018±0.008	nd
2.00	nd	0.011±0.002	nd
2.50	nd	0.020±0.004	nd
3.00	nd	0.013±0.009	nd

*nd means not determined.



(a)



(b)

Figure 4.12 Concentrations of protein (a) and protein per cell (b) in supernatants of each media after are cultured with *Lactobacillus* sp. SUTWR 73 in broth that contained different cassava starch concentrations (%, w/v) for 24 h.

From the previous results, the data showed only the amount of protein after culturing the strain SUTWR 73 for 24 h. Since protein concentration in the negative control did not perform. The next experiment also aimed to determine protein concentration both for negative control and the treatments after culturing the bacterium for 24 h.

For all supernatants from cultivated medium (at starch concentration 0.5, 1.0 1.5, and 2.0%), they were measured amylase activity. The average amylase activity values were 1.80, 3.89, 6.75, and 6.82 U/ml, respectively (Table 4.5).

Table 4.5 Amylase activities using Bernfeld method of crude amylase and average activities per cell of crude enzyme in supernatant at different concentrations of cassava starch after cultivating *Lactobacillus* sp. SUTWR 73 for 24 h.

Cassava starch concentration (%, w/v) in the cultivation medium	Amylase activity (U/ml)	Amylase activity per cell (U/ Log CFU/ml)
0.50	1.80±0.30	0.25±0.04
1.00	3.89±0.34	0.55±0.05
1.50	6.75±0.68	0.8 ±0.01
2.00	6.82±1.09	0.82±0.13

*One Unit of amylase activity was defined as the amount of enzyme that produced 1 μmol of reducing sugars per min at pH 6.8, 35℃.

Table 4.6 Maltose concentrations and activities of crude amylase and average amylase activities per cell from bacterial cell pellets after cultivation in the medium containing at different concentrations of cassava starch using DNS assay.

Cassava starch concentration (%, w/v)	Amylase activity*50 (U/ml)	Amylase activity per cell*50 (μU/ml*Log CFU/ml)
0.50	1.89±0.19	0.26±0.03
1.00	2.25±0.25	0.32±0.04
1.50	4.32±0.21	0.53±0.03
2.00	6.31±0.67	0.75±0.08

*One unit of amylase activity was defined as the amount of enzyme that produced 1 μmol of reducing sugars per min at pH 6.8, 35°C.

Table 4.7 Amylase activity of the cell pellets after cultivating *Lactobacillus* sp. SUTWR 73 in the optimized medium containing different cassava starch concentrations at a 100-ml flask.

Cassava starch concentration (%, w/v)	No.	A_{540}	Maltose concentration (mg/ml)	Amylase activity*50 (U/ml)	Average*50 (U/ml)	Cell growth (Log CFU/ml)	Amylase activity per cell*50 (U/Log CFU/ml)	Average*50 (U/Log CFU/ml)
0.50	1	0.252	1.79	1.64	1.89±0.19	7.1889	0.23	0.26±0.03
	2	0.123	2.20	2.02		7.1889	0.28	
	3	0.317	2.19	2.01		7.1889	0.28	
1.00	1	0.586	2.56	2.38	2.25±0.25	7.1004	0.33	0.32±0.04
	2	0.602	2.64	2.44		7.1004	0.34	
	3	0.469	2.01	1.92		7.1004	0.27	
1.50	1	0.289	4.67	4.32	4.32±0.21	8.2266	0.52	0.53±0.03
	2	0.369	4.40	4.08		8.2266	0.50	
	3	0.317	4.95	4.57		8.2266	0.56	
2.00	1	0.456	7.50	6.95	6.31±0.67	8.3766	0.83	0.75±0.08
	2	0.337	5.89	5.45		8.3766	0.65	
	3	0.434	7.06	6.53		8.3766	0.78	

*One Unit of amylase activity was defined as the amount of enzyme that produced 1 μmol of reducing sugars per min at pH 6.8, 35℃ for 30 min.

From *Lactobacillus* sp. SUTWR 73 cultivation medium, amylolytic activities were detected both in the supernatant and on cell pellet. The results revealed that the SUTWR 73 could produce extracellular amylase and the enzyme attached to its cell. However, the types of extracellular and cell-bounded amylases are still needed to investigate. For this investigation, the SDS-PAGE technique combined with high-performance liquid chromatography (HPLC) or thin-layer chromatography (TLC) for analyzing, and identifying the product from starch hydrolysis of amylase from both extracellular and the cell-bounded amylases as well as the molecular weight of protein, respectively.

From the optimization of amylase condition production, it was found that amylase activity per cell in the supernatant was higher than the cell pellet in all cassava starch concentrations applied. The supernatants obtained from cultivation medium containing 0.5, 1.0, 1.5, and 2.0% of cassava starch, performed amylase activity when were higher than the cell pellets around 37.64, 88.08, 31.61, and 32.67 folds, respectively. The medium contained 2% starch gave the highest amylase activity per cell (6.82±1.09 U/ml*Log CFU/ml) similar to the studies of *Bacillus* sp. I-3 (Gül et al., 2005) and *Lactobacillus fermentum* 04BBA19 (Fossi and Tavea, 2013) which reported that the starch act as the initial inducer for stimulating bacteria to secrete the amylase outside the cell.

However, if the activity was considered without a cell number by studying at various cassava starch concentrations, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0%(w/v). The results showed that the total amylase activity increased while starch concentration was increased in the medium (Figure 4.13). The highest amylase activity obtained from the cultivated medium containing 3.00%(w/v) of cassava starch, was around 12.80 U/ml

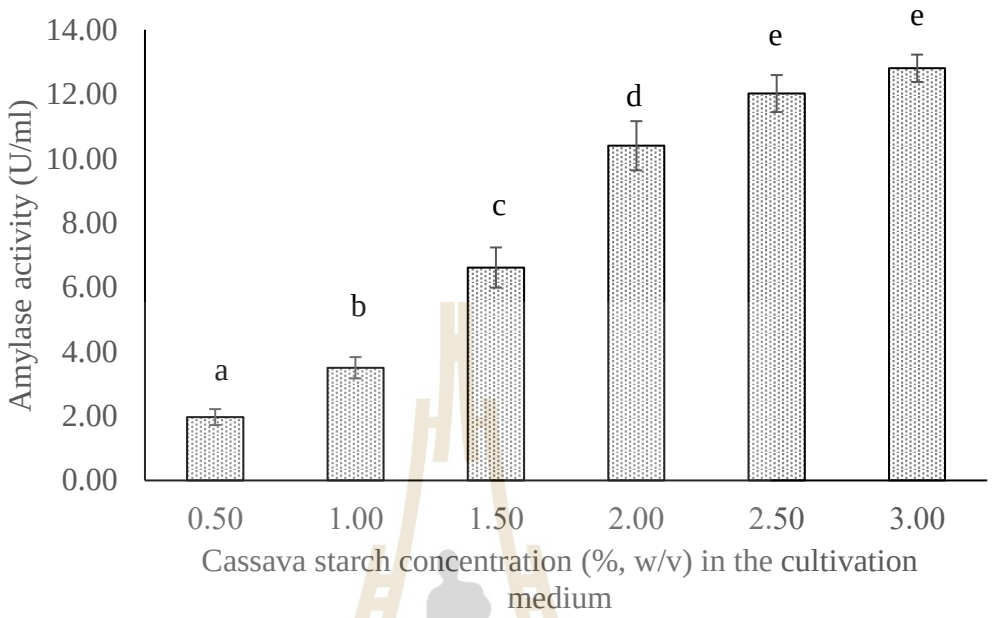


Figure 4.13 Total amylase activity in the medium containing 0.5-3.0% cassava starch after culturing *Lactobacillus* sp. SUTWR 73 for 24 h. Means with different letters (a, b, c, d, and e) showed significantly different means (Duncan's Multiple Range Test (DMRT), $p<0.05$).

4.2.1.2 Nitrogen sources

Three types of nitrogen sources; soy protein, urea, and rice bran (Table 4.8) were tested to optimize conditions for amylase production. The main compositions of the nitrogen sources (Table 4.9). These modified media were used for investigating the nitrogen source effect in this study by removing partially or completely nitrogen sources in the original medium, RAM.

Table 4.8 Media designed for cultivation of *Lactobacillus* sp. SUTWR 73 for optimization of amylase production condition.

Composition	Amount of component in each medium (g/l)				
	RAM	1	2	3	4
Pancreatic digest of casein	5	-	-	-	-
K_2HPO_4	6	6	6	6	6
Yeast extract	3	-	-	3	3
Tri-ammonium citrate	-	-	-	-	-
$MgSO_4 \cdot 7H_2O$	0.57	0.57	0.57	0.57	0.57
$MnSO_4 \cdot 4H_2O$	0.12	0.12	0.12	0.12	0.12
$FeSO_4 \cdot 4H_2O$	0.03	0.03	0.03	0.03	0.03
Cassava starch	15	15	15	15	15
Soy protein	-	-	8	-	-
Urea	-	-	-	5	8
Rice bran	-	8	-	-	-

Table 4.9 Nitrogen source compositions.

N-source	Composition (%)				
	Moisture dry weight[a]	Total C[b]	Total N[c]	Total P[d]	Total K[e]
Soy bean meal (food grade Solca, Spain)	8.69	57.86	14.82	0.44	0.06
Spent brewer's Yeast (Thaibev, Thailand)	3.66	72.33	5.17	0.89	1.39
Rice bran (Surin, Thailand)	9.67	67.77	2.07	0.91	1.19

[a] Moisture content (AOAC, 1999)

[b] Total organiccarbon analyses (Bernard et al., 1995)

[c] Kjeldahl method (AOAC, 2000)

[d] Spectrophotometric method (Bray and Kurtz ,1945)

[e] Flame photometric assay (AOAC, 1990)

Table 4.10 Average concentrations of protein in the cultivation medium containing different cassava starch concentration after culturing *Lactobacillus* sp.SUTWR 73 at 35°C for 24 h.

Cultivation medium	Absorbance at 595 nm	Protein concentration (mg/ml)	SD
Original medium (RAM)	0.020	0.025	0.005
Modified medium with the nitrogen sources			
Rice bran	0.050	0.045	0.006
Soy protein	0.398	0.406	0.018
Urea plus yeast extract	0.018	0.023	0.007

From Figure 4.15**,** the highest amount of total protein (0.41±0.02 mg/ml) was obtained from soy protein followed by rice bran (0.05±0.01 mg/ml), RAM (0.03±0.01 mg/ml), and urea plus yeast medium (0.02±0.01 mg/ml). The total protein result of soy protein medium was not related with the total amylase activity in the supernatant because of the low total protein in RAM. The results indicated that nitrogen sources have other components supporting amylase production rather than the total protein content.

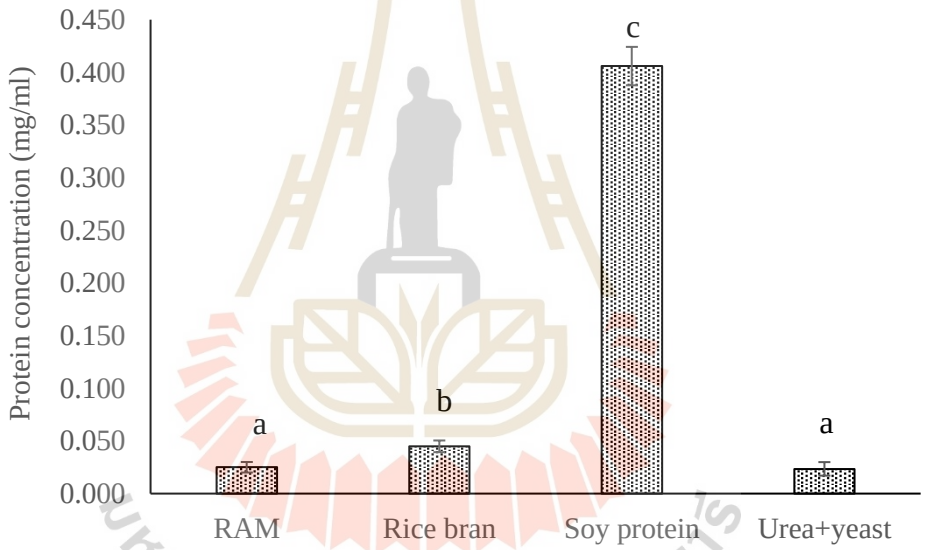


Figure 4.14 Concentrations of protein in supernatants of each medium containing each type of nitrogen source, after cultivation of *Lactobacillus* sp. SUTWR 73 at different nitrogen sources for 24 h. Means with different letters (a, b, and c) showed significantly different means (Duncan's Multiple Range Test (DMRT), $p<0.05$).

The three modified media (Rice bran, soy protein, and urea plus yeast extract) were inoculated with SUTWR 73, then incubated for 24 h, the results showed that RAM

medium exhibited the highest amylase activity (8.06±0.04 U/ml), followed by rice bran (7.54±0.02 U/ml), soy protein (6.73±0.08 U/ml), urea (7.11±0.01 U/ml), and urea plus yeast extract (4.46±0.07 U/ml). The medium with rice bran medium seemed to be the suitable for low-cost amylase production compared to the report that contained protein content of 10 to 16%(w/w), and 40 to 50%(w/w) of carbohydrate as the carbon source which was favorable as an alternative nitrogen source (Abdul-Hamid and Luan, 2000; Moongngarm et al., 2012). The rice bran used in the study also nearly the same as the previous report (Table 10). Some vitamins were also supplied as the growth factors of bacteria such as vitamin E (Hu et al., 1996; Pourali et al., 2009).

According to the results from the first experiment, the second experiment was done to obtain the cheapest medium by either substitution or reduction of yeast extract and tryptone by urea. RAM was still the best cultivation medium by providing the total activity of about 8.06±0.42 U/ml. The second-choice medium was urea plus yeast extract, followed by the medium containing soy protein and the urea medium. From the first and the second time of cultivation of the strain SUTWR 73, the rice bran medium seemed to be suitable for low cost amylase production, but urea plus yeast and soy protein media could also be used for amylase production. Tryptone and yeast extract were expensive which cost about 4.17 Baht/g and 3.96 Baht/g, respectively.

Since the growth of SUTWR 73 did not detect in cassava starch media during optimization study which was important for making the decision to be used in the nitrogen source experiment made us choose the old results which had growth data. From the results in section 4.2.1.1, the media containing 2% and 1.5%(w/v) of cassava starch were not significantly different. Thus, 1.5% of cassava starch was selected for the

nitrogen source study because of the lower cost. The results of the modified medium cultivation for nitrogen source study represented in Figure 4.15.

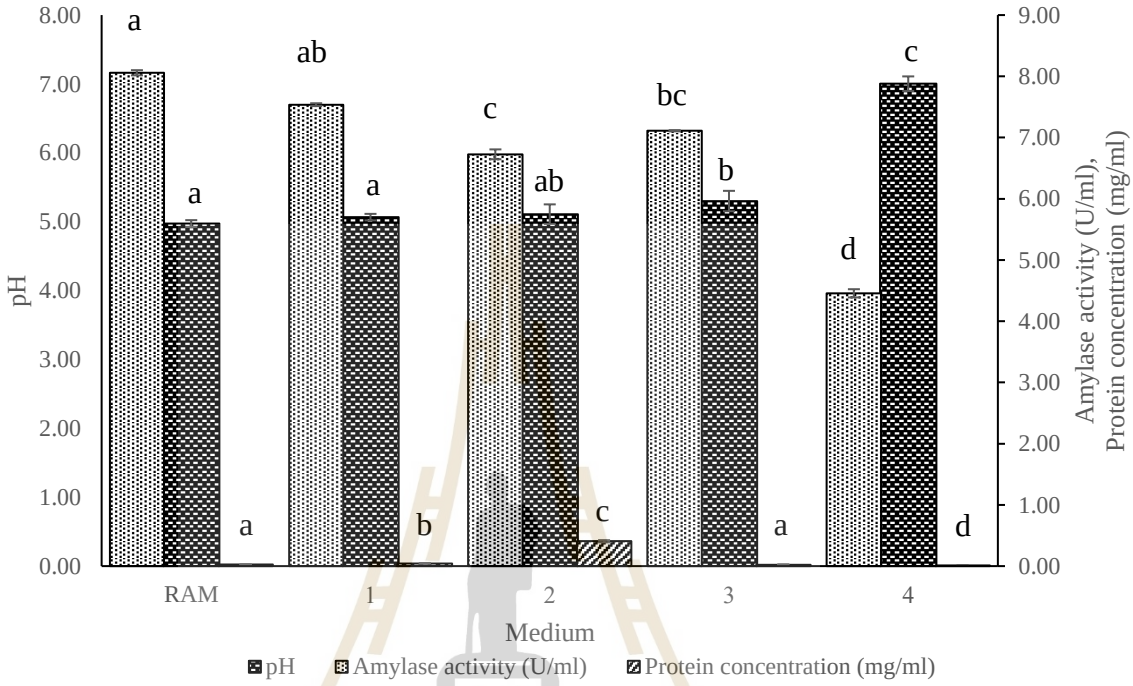


Figure 4.15 Protein concentration, amylase activity and pH of the modified media containing various types of nitrogen source: rice bran (1), soy protein (2), urea and yeast (3), urea (4) after cultivating SUTWR 73. Means with different letters (a, b, c, and d) showed significantly different means (Duncan's Multiple Range Test (DMRT), $p<0.05$).

The medium containing rice bran supported the production of amylase similar to the original medium (RAM) amylase activity of 7.54±0.02 U/ml. Hence, the rice bran medium was selected for determining the substrate concentration.

In comparison to RAM, the medium containing rice bran was more suitable for amylase production because of the lower cost. However, the other modified medium

(soy protein and urea plus yeast) could be used for amylase production. Even though the others did not support amylase yield as high as the rice bran.

However, the soy protein (the commercial nitrogen source) could also be used as a rice bran but it was not suitable for amylase purification because of the problems of high protein background. Urea was considered a poor effect on amylase production. Das et al. (2004) and Swain et al. (2006) reported that urea inhibited the α-amylase activity of *Bacillus subtilis* which is similar to our findings. This study was not only a study of nitrogen source but also study the effect of tri-ammonium citrate by eliminating from the original medium. It was found that the amylase activity was almost the same as the original medium with tri-ammonium citrate. After choosing the rice bran as a nitrogen source, concentration optimization was needed for investigating the optimum point which could be either reduce the cost of the medium or increase of amylase yield.

The amount of rice bran affected amylase production of SUTWR 73. The rice bran concentration at 0.4%(w/v) in the range of 0.4 to 1.6%(w/v) exhibited the highest amylase activity of 6.99±0.50 U/ml (Figure 4.17). These suggested that the rice bran used in the study would consist of ingredients such as zinc, copper, iron, nickel ions, and phenolic compounds which were reported as amylase inhibitors (Muralikrishna and Nirmala, 2005; Sivaramakrishnan et al., 2006; Boue et al., 2016; Quan et al., 2019).

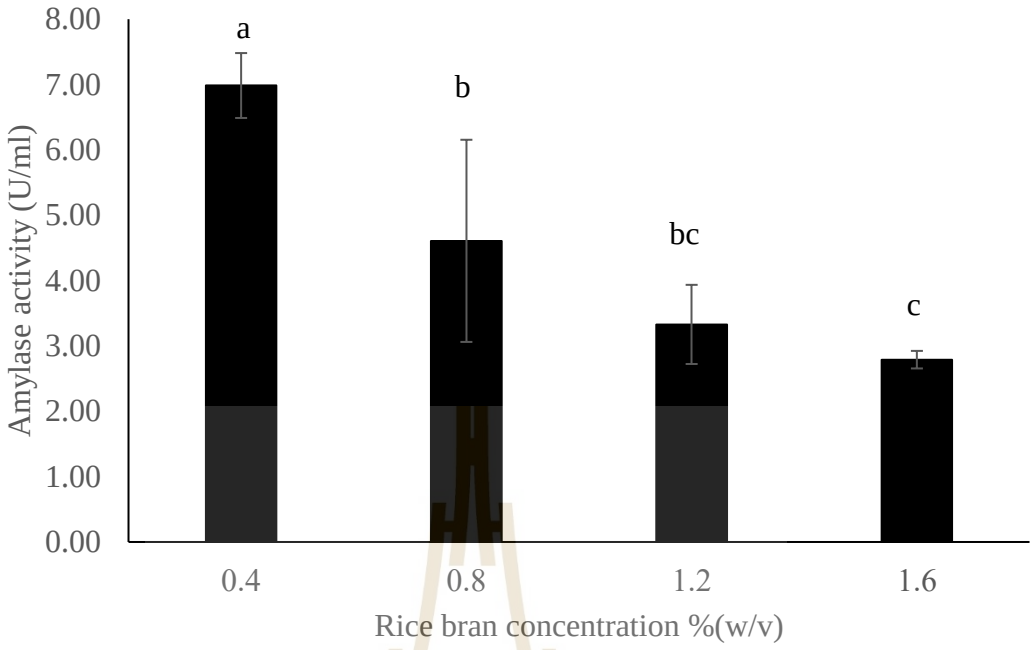


Figure 4.16 Amylase activity harvested from the modified rice bran medium with different rice bran concentrations. Means with different letters (a, b, and c) showed significantly different means (Duncan's Multiple Range Test (DMRT), $p<0.05$).

According to the type of nitrogen source, the amylase activity given from rice bran medium was higher than the other media in this study, because the different lots of rice bran recognized had the variation of components making problems of feedstock for producing amylase. The rice bran properties varied through its harvest season and the shelf life (storage time). Moreover, the variety of rice bran might also display the variation from species to species as investigated by Zarei et al. (2018), their finding showed the core composition of rice bran and the alteration composition through gene expression. These factors were hard to control of each lot. The approximate analysis was needed to detect for each lot for the rice bran collection.

Rice bran obtained from a plant has variation from season to season, even in the same season, the quality of rice bran was different. The approximate analysis was required for analysis of some main components. To improve the utilization of rice bran of the bacterium without causing the higher cost, the mild treatment of substrate especially with the alkali and acid treatment would give the positive effect to the bacterial growth of the bacteria owning to the high accessible of bacterial amylase to the substrate (Begum and Alimon, 2017; Novea et al., 2019).

Table 4.11 Composition of defatted rice bran used in this study.

Composition	Rice bran	
	Lot1	Lot2
Protein (%)	13.15	12.82
Fat (%)	14.24	12.60
Fiber (%)	6.55	7.80
Phosphorous (g/kg)	17.75	13.63
Potassium (g/kg)	19.62	15.67

*Rice bran was obtained from Surin province.

After obtaining the new modified medium which could support amylase production of *Lactobacillus* sp. SUTWR 73, the next part was the comparison of medium-cost between the original medium (RAM) and the modified medium.

4.2.2 Cost evaluation of amylase production medium

The modified medium-cost was lower than the original medium (RAM) 84%. While, the rice bran was 380 times cheaper than yeast extract and tryptone (Table 4.12).

The modified medium could be the alternative medium even it did not support amylase production as the original medium did, but the enzyme production cost could provide the change of large-scale production which could solve the problem of low yield of amylase production.

Table 4.12 Medium cost comparison between the original and the modified media

Composition	Original medium or RAM (Baht)	Modified medium (Baht)
Tryptone	20.87	-
di-Potassium phosphate	5.39	5.39
Yeast extract	11.88	-
tri-Ammonium citrate	23.54	-
Magnesium sulphate heptahydrate	0.40	0.40
Manganese sulphate tetrahydrate	0.15	0.15
Iron (II) sulphate heptahydrate	0.02	0.02
Cassava starch	0.13	0.34
Rice bran	-	0.09
Total (Baht)	62.38	6.39

4.2.3 Amylase production in 7.5-l bioreactor with 3-l working volume

The modified medium was prepared for amylase production at bioreactor using a 7.5-l bioreactor (New Brunswick, BioFlo®/ CelliGen® 115, New Brunswick Scientific, USA) with 3 l of working volume, incubated at 35℃, agitation speed at 150

rpm without aeration for 28 h. When considered reducing sugar concentration of each incubation time, it showed that the amount of initial reducing sugars increased from 0 to 8 h. During 8 to 28 h of cultivation, it decreased and increased repeatedly, meaning that SUTWR 73 produced amylase for digesting starch and giving reducing sugars as the product before consuming. The rate of taking reducing sugars was not equal to the rate of starch digesting starch which caused the accumulation of residual reducing sugars to be different at each point of incubation time. Due to the high demand for energy sources of SUTWR 73 for growing at the initial phase (during 0 to 8 h of incubation time), the rate of taking reducing sugars was a little bit slower than the rate of reducing sugars forming making the remain reducing sugars were low concentration. In contrast, the rate of taking reducing sugars was low after 8 h causing the higher remain reducing sugar concentration. Nevertheless, these things mentioned above was just a possible assumption, and the reasons were still not reasonable enough. To obtain the support evidence supported, the amount of substrate should be monitored over cultivation time to observed SUTWR 73 behavior.

The amylase activity investigated from cultivation in a 7.5-l bioreactor with 3-l working volume increased by 2 h and was constant during 2-8 h of cultivation 8 h. At 10 h the amylase activity increased to be the maximum activity (6.04 U/ml) at 14 h cultivation (Figure 4.18). Then, it was lightly lowering. The amount of protein from bioreactor seem to be not significantly different as three bioreactors mentioned before. At 0 h to 12 h of cultivation, the amount of protein slightly increased over time until reaching the 14 h with the highest amount of protein (0.18 mg/ml). After that, it decreased slightly until the end of cultivation. If considering both amylase activity and protein concentration, it was found that at 0 to 12 h, the major proteins were not the

amylase enzyme, while after 14 h, almost proteins were amylase and had low protein background which was suitable for purifying protein than at the initial time.

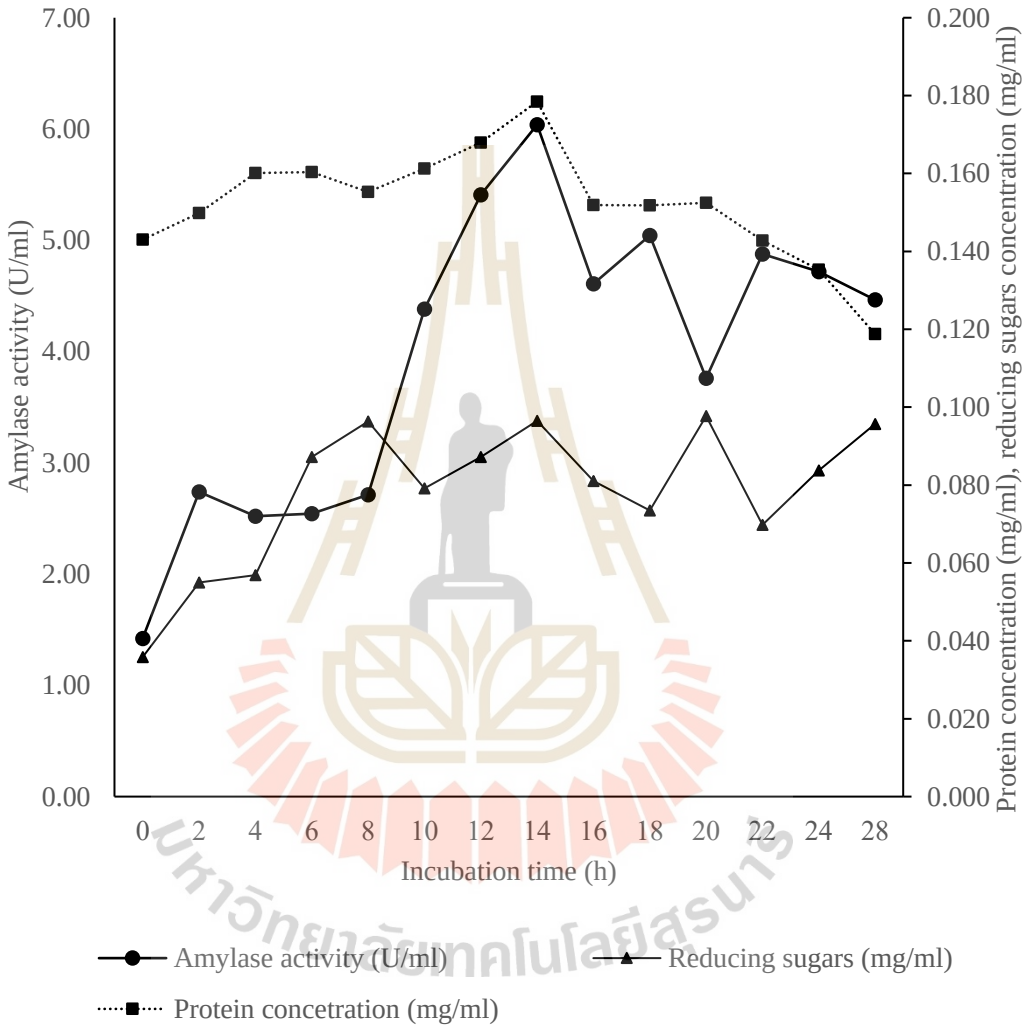


Figure 4.17 Amylase activity at different periods of cultivation time of *Lactobacillus* sp. SUTWR 73 in a 7.5-l fermenter with 3 l for working volume at 35℃ with agitation speed 150 rpm, pH controlling at pH 7.0 without aeration.

4.2.4 Partial purification of amylase produced by *Lactobacillus* sp. SUTWR 73

The obtained crude enzyme was firstly precipitated using ammonium sulphate at 70, 80 and 90% of saturation. Then the precipitated protein was resuspended and dialyzed against the same buffer (50 mM sodium phosphate buffer) to eliminate the ammonium and sulphate ions inhibited protein activity. Both crude and the partially purified enzymes were detected for its activity by reacting with the 1%(w/v) soluble starch in 50 mM sodium phosphate buffer at 30°C for 30 min. The crude enzyme provided the highest activity (0.77 U/ml) compared to partially purified enzyme 12.87, 13 U/ml at 70, 80, and 90% of saturation, respectively (Figure 4.18). This needed to be realized that it was the first purification step, the desired amylase did not 100% precipitated, but partially precipitated making the activity of partially purified amylase at lower activity than it was. Thus, the obtained and the activity did not mean that the higher activity was obtained, the higher purity of the enzyme exhibited. The purity of the enzyme could be calculated from the total activity divided by the total of protein, this value called specific activity which gave the activity per amount of protein.

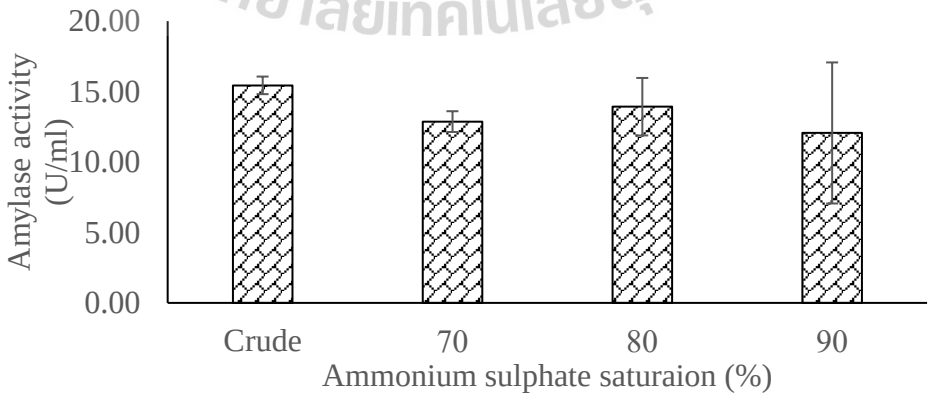


Figure 4.18 Amylase activity of partially-purified amylase from different ammonium sulphate saturation.

Table 4.13 Total activity, protein concentration, and specific amylase activity of cultured medium before and after partial purified by ammonium sulphate precipitation.

Step	Total activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)	Fold	Recovery (%)
Crude enzyme	15.44	0.080	193.10	1.00	100
Ammonium sulphate precipitation (%)					
70	12.87	0.026	494.91	2.56	83.34
80	13.93	0.072	193.89	1.00	90.19
90	12.06	0.118	101.81	0.53	78.12

The specific amylase activity demonstrated the ratio of the activity of the enzyme to the amount of protein. The high specific activity meant the high protein enzyme was high proportion to the protein background (undesired protein). As shown in the table 4.13, ammonium sulphate (70%) was suitable for partial purification because of the highest specific activity (494.91 U/mg) with 83.34% for recovery compared to 80 and 90%, even there was a loss of some amylase than at 90% saturation which had 90% recovery of amylase but incorporated with many protein wastes.

4.3 Preliminary characterization of amylase produced by *Lactobacillus* sp. SUTWR 73

In the amylase enzyme characterization part, it was aimed to obtain data for further investigation and application of the enzyme. Gül et al. (2005) mentioned the reason for the pH reduction of the medium due to the organic acid such as lactic acid from microbial metabolism. When compare between pH of the medium and amylase activity as shown in Figure 4.19, it was found that the pH did not correlate with amylase activity. The average pH of the medium was about 5.21 and constant at all starch concentrations but it tended to decrease while starch concentration was increased.

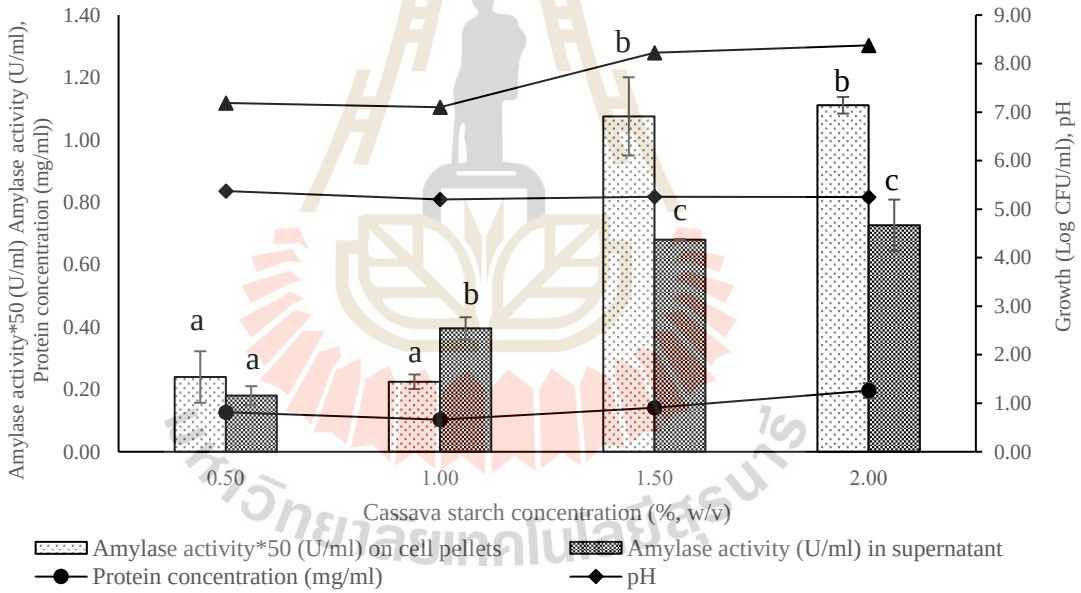


Figure 4.19 Amylase activity and pH of supernatant and cell pellets from 24 h-cultivated medium containing different cassava starch concentrations (%, w/v). Means with different letters (a, b, and c) showed significantly different means (Duncan's Multiple Range Test (DMRT), $p<0.05$).

4.3.1 Calcium ion requirement

It was found that, in the presence of 10 mM Ca^{2+}, the amylase activity from cultivation of SUTWR 73 was 3.35±0.28 U/ml which was not different from the absence of Ca^{2+} (3.40±0.30 U/ml). There were two possible reasons; the amylase was in the crude cultured medium with saturated of calcium ion and did not affect to the additional calcium ion making the amylase activity of crude equal to the crude with additional of calcium chloride as shown in the Figure 4.20 (Bisswanger, 2014).

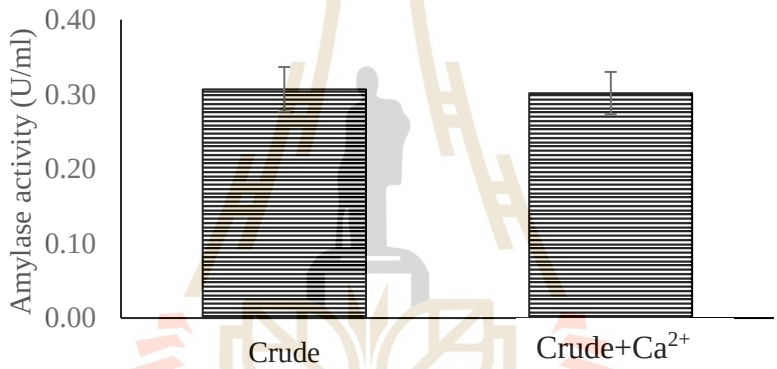


Figure 4.20 Comparison of amylase activity from cultured medium in the reactions with Ca^{2+} and without Ca^{2+}.

So, the amylase was calcium-independent which differed from the report which was calcium-dependent. In the Lactobacillus group, the calcium-independent amylase was rarely found. In contrast, the calcium-independent was usually found in the Bacillus group such as *Bacillus amyloliquefaciens* TSW K1-1 (Kikani and Singh, 2011), *Bacillus* sp. KR-8104 (Sajedi et al., 2004), *Anoxybacillus beppuensis* TSSC-1 (Kikani and Singh, 2012). Since the crude was partially purified, some ions might retain in the amylase solution. The modified medium should to be proved that the modified medium

did not consist of calcium ion. If modified medium could not be proved, there would be another way which having EDTA involvement. EDTA could be used as a chelating agent for chelating with and allow the enzyme to be Ca^{2+} free enzyme before do the reaction with soluble starch-containing $CaCl_2$. Another thing to recognize that EDTA can trap divalent metal ions, hence not only Ca^{2+} was trapped but others were also. If the amylase was activated by other divalent metal ions, its activity may reduce compared to the control without EDTA. However, in the medium possesses monophosphate ion, even it has a weak capacity, it can also sequester the essential ion as Ca^{2+}. This could be considered as another problem because monophosphate ion could chelate Ca^{2+} which prevented amylase from binding to the ion. That was why the increase in calcium chloride did not affect the activity of the enzyme (Bisswanger, 2014).

4.3.2 Temperature profile of crude amylase activity

The crude enzyme collected from cultured medium (rice bran), was subsequently reacted with the 1%(w/v) soluble starch at different temperatures for 30 min for determining its temperature profile. From Figure 4.21, the amylase activity increased from the treatments of 30 to 60℃. The highest enzyme activity was obtained at 60℃ incubation (9.54±1.21 U/ml). This results was similar to the finding in *Lactobacillus manihotivorans* (Aguilar et al., 2000), *Lactobacillus plantarum* S21 (Kanpiengjai et al., 2015), *Lactobacillus plantarum* A6 (Giraud et al., 1993), and also found in the high potential bacterium, *Bacillus amyloliquefaciens* BH072 (Du et al., 2018), which had the optimum temperatures at 55, 45, 60, and 60℃, respectively. Furthermore, the optimal temperature of amylase obtained from SUTWR 73 was higher than some lactobacilli such as *Lactobacillus plantarum* subsp. *plantarum* ST-III, which

has the optimum amylase activity at 35℃ (Jeon et al., 2016). The enzyme activity increased from 30 to 60℃ and dropped deeply down until reaching the lowest activity (5.096±0.171 U/ml) at 80℃. The velocity of the reaction increased making the enzyme was in the highly active stage to quickly convert substrates into products (Bisswanger, 2011). In some points, the temperature raised high enough for making the hydrogen bond in the protein structure was not stable anymore and eventually broken, losing catalytic activity (Takasaki, 1982). Loss of the catalytic activity activated form and low efficiency to convert substrate products.

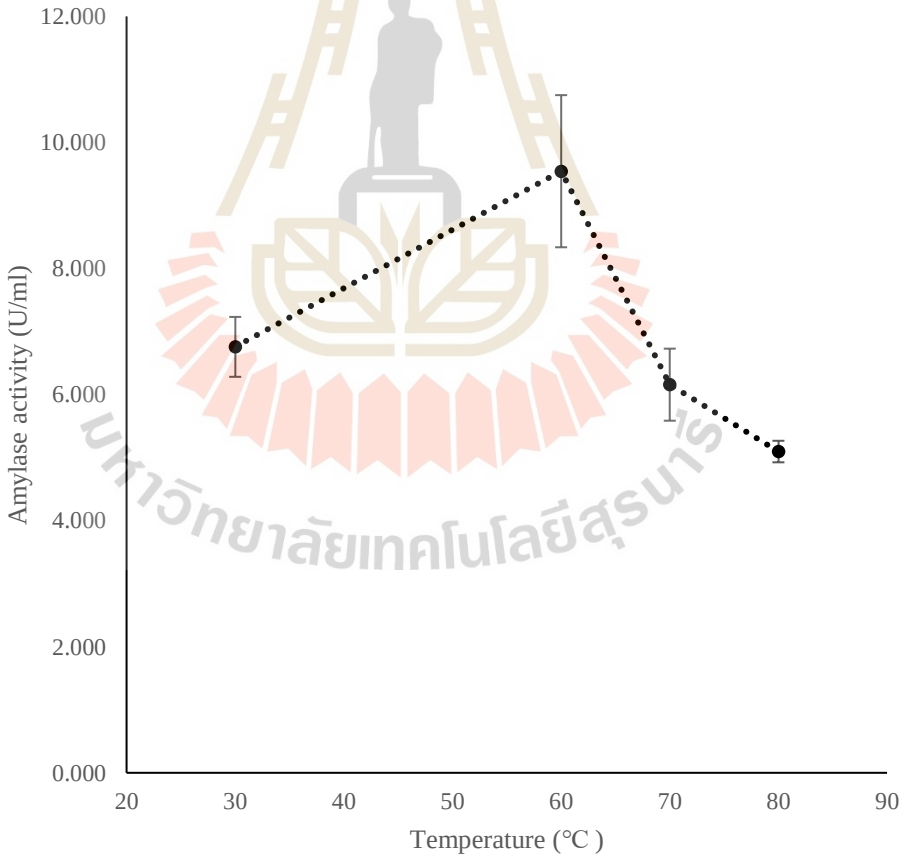


Figure 4.21 Amylase activity of crude enzyme from SUTWR 73 reacted with soluble starch at different incubation temperature.

However, the higher activity was found while the temperature increased from 90 to 100℃. This could be explained from the reduction of volume caused the sample more concentrate, and finally displayed the strong positive results than the actual.

4.3.3 pH profile of crude amylase activity

The pH profile should be clarified for further application of the enzyme. However, this study worked with a crude enzyme which comprised lots of components from the cultured broth including the hydrogen ion, the residual component which would act as buffers such as protein and di-potassium hydrogen phosphate. These factors made the problems in pH profile construction, because its properties made the use of high concentration of buffer due to the high requirement of the buffer capacity, to keep the pH constant along with the cultivation. The increase of buffer concentration directly affected to the ionic strength which may cause damages to the protein structure. In contrast, low ionic strength did not mean to be always good because it can destabilize the protein structure leading to low activity (Bisswanger, 2014). From the preliminary investigation (data not shown) the experiment failed due to the high interference from the buffer system. The high concentration (more than 100 mM) of the buffer made the results to be unreliable because when buffer concentration increases, each condition displayed different colors both in the negative control and the reaction, even they came from the same sample. It was concluded that the high concentration of buffer interfered with the development of reaction color.

4.4 Strain improvement of lactic acid-producing bacterium

The D-lactic acid-bacterium, *Lactobacillus* sp. SUTWR 73, was fastidious. It was microaerophilic which did not require a high amount of oxygen, causing the obstacle

during performed its cultivation. Hence, the improvement of the strain would be necessary for potential use. The ultraviolet mutation (Yu et al., 2008) was chosen for improving the *Lactobacillus* sp. SUTWR 73.

Firstly, the inoculum of SUTWR 73 was prepared by adding 1 loopful of the 48 h culture on RAM medium, in 5 ml RAM broth having cassava starch, then incubating in anaerobic chamber at 35℃ for 18 h. The 18-h suspension of SUTWR 73 cell suspension was subsequently transfer to the sterile petri dishes for 1 ml per dish, and put the dish beneath under ultraviolet-light source. SUTWR 73 was treated with ultraviolet light at 15 cm for the distance between UV source and surface of the SUTWR 73 dish. The experiment was performed under concerning the safety of people by using the closed laminar flow. The UV exposure time was 15 min. Then 0.1 ml was transfer to RAM agar, and gently spread until the surface was not wet. At 1, 5, 10, and 15 min of incubation time, the UV-treated cell suspension was spread on RAM agar and incubated under anaerobic condition at 35℃ for 48 h. At 48-h incubation time, the survival colonies grown on agar were counted. The survival curve was constructed based on growth results and the curve have been used for further strain improvement. As shown in Figure 4.23, the exposure time that colonies could survive less than 1 Log CFU/ml was at 3 min. Hence, the 3-min time was chosen for the bacterial strain improvement.

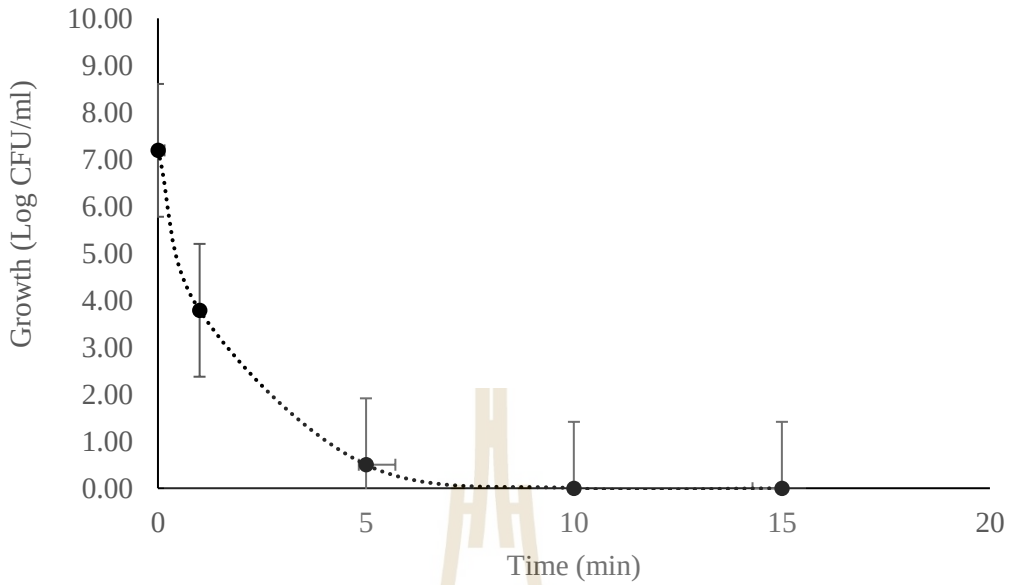


Figure 4.22 Survival curve of UV-treated SUTWR 73 for strain improvement.

In the strain improvement step, the inoculum was prepared as previously mentioned. The 18-h SUTWR 73 cell suspension was exposed to UV for 3 min then the treated cells were spread onto RAM agar and incubated under the anaerobic condition at 35℃ for 48 h. The survived colonies were collected and compared for their amylase production capability. The potential amylase producer was repeatedly treated with UV 8 times to obtain a stable mutant. After the last UV-treatment, the survive colonies were tested for their growth ability and its amylase. Three mutants; WR 73-1, WR 73-2, and WR 73-3 were selected due to they performed the highest amylase activity and good growth. The strain, SUTWR 73-1 exhibited high amylase activity among four selected strains with 8.79±1.33 U/ml. For the protein detection, SUTWR 73-1 cultivated medium was also found to have the lower protein concentration, 0.420±0.061 mg/ml (Figure 4.23) meaning that SUTWR 73-1 may secrete the extracellular amylase with the low amount based on the protein concentration compared with the original strain,

SUTWR 73. However, due to its high potential in amylase production and specific activity among three strains, it showed that amylase produced by this strain may have highly active amylase. For the future, if the amylase was needed to be purified, SUTWR 73-1 would be the choice. For the bacterial growth, it was found that the SUTWR 73-1 also showed higher growth than the original strain.

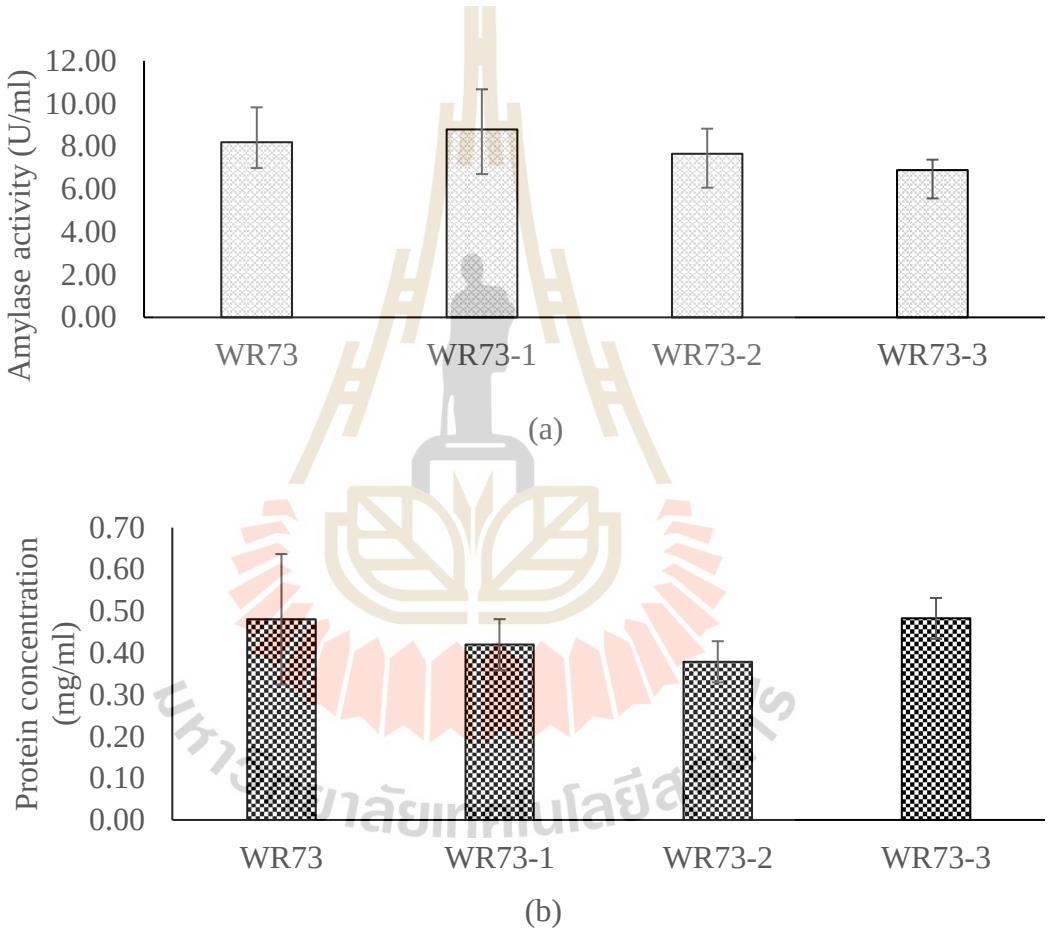


Figure 4.23 Yield of amylase and protein concentration from D-lactic acid producing bacterium, SUTWR 73, and the improved strains after cultivation in RAM medium under anaerobic condition at 35℃ for 48 h.

4.5 Optimization of medium composition for amylase production

4.5.1 Factorial experiments

4.5.1.1 Concentration of carbon source

RAM medium (Appendix A 2.1) was used for composition optimization. Cassava starch was the main carbon source, due to its low cost, and dominant around the campus by covering with many cassava fields and cassava starch industries. The cassava starch concentration in RAM recipe was varied from 1 to 15%(w/v) by increasing the concentration until decrease in amylase activity was exhibited.

Figure 4.24 showed the highest amylase obtained from the RAM medium containing 10%(w/v) of cassava starch by giving amylase activity around 30.43±3.47 U/ml with 4.32×10^{7} CFU/ml. At this starch concentration the highest amylase was detected outside the cell to access the high substrate viscosity due to after gelatinization of the medium preparation, the available water was trapped inside the starch structure. If the bacterial could not access the starch, their growth would be disrupted and gradually decrease over time. Surprisingly, the SUTWR 73-1 had high capability to digest high viscosity of cassava starch, their amylase production was dramatically induced by the substrate. This finding was supported by several reports of amylase mentioned that the amylase could be induced by the high complex carbohydrate like starchy biomass (Sreekanth et al., 2013), and some reports additionally revealed that the enzyme could be induced by oligosaccharide namely, maltose, lactose, and galactose as well. The induction of the amylase production was varied from substrate to substrate the higher complex of substrate tended to induce amylase production better than the smaller like maltose, lactose, galactose, fructose and glucose (Ashwini et al.,

2011). In other hand, the smallest unit as glucose was also reported as the amylase production repressor.

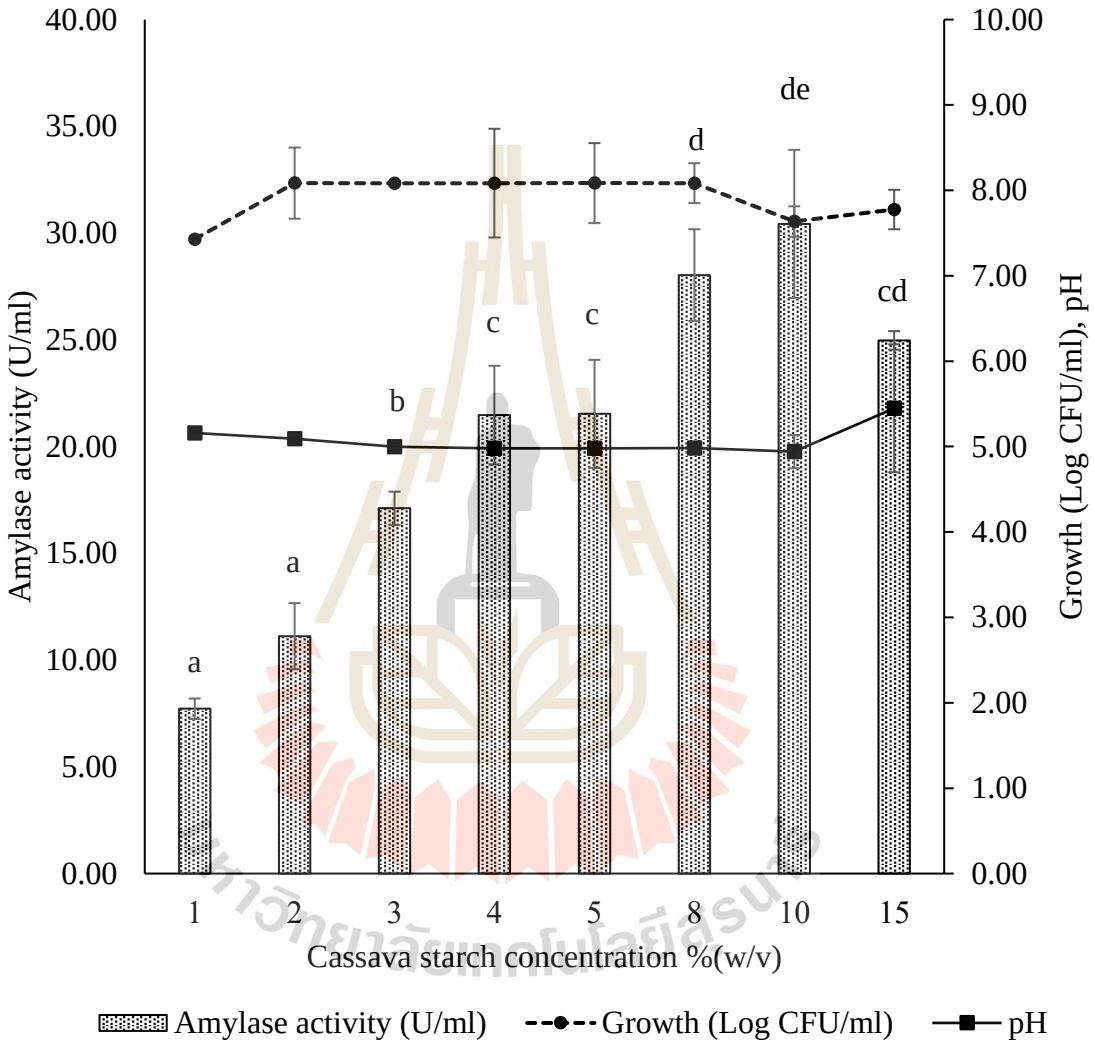


Figure 4.24 Effect of cassava starch concentration on amylase produced by SUTWR 73-1 cultivated at 35℃ pH 7 for 24 h without aeration. Means with different letters (a, b, c, d, and e) showed significantly different means (Duncan's Multiple Range Test (DMRT), $p<0.05$).

4.5.1.2 Type and concentration of nitrogen source

The RAM medium containing the optimal concentration of cassava starch was used as the initial recipe. The critical nitrogen sources were studied by adding, replacing, and removing the tryptone and yeast extract. The combination of the medium used in this section was shown in Table 4.14.

Table 4.14 Medium recipe for studying type and concentration of nitrogen source and amylase activity of the strain SUTWR 73-1.

Component	Medium formula no.						
	1	2	3	4	5	6	7
Yeast extract (g/l)	3	-	3	-	-	3	-
Tryptone (g/l)	5	5	-	-	5	-	-
Defatted rice bran (g/l)	-	11.85	23.31	35.16	-	-	-
Soy protein (g/l)	-	-	-	-	2.52	4.95	7.47
K_2HPO_4 (g/l)	6	6	6	6	6	6	6
Triammonium citrate (g/l)	1	1	1	1	1	1	1
$MgSO_4 \cdot 7H_2O$ (g/l)	0.57	0.57	0.57	0.57	0.57	0.57	0.57
$MnSO_4 \cdot 4H_2O$ (g/l)	0.12	0.12	0.12	0.12	0.12	0.12	0.12
$FeSO_4 \cdot 7H_2O$ (g/l)	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Cassava starch (g/l)	40	40	40	40	40	40	40
SUTWR 73-1 Amylase activity (U/ml)	33.09	43.64	16.50	16.201	17.12	12.95	8.90
Standard deviation	1.76	8.50	3.35	2.05	4.57	4.57	2.91

After SUTWR 73-1cultivation in the 7 medium formulas, the highest amylase activity was observed from formula no.2, 43.64±8.50 U/ml with 0.20±0.03 mg/ml for

protein concentration, which had been eliminated yeast extract, and replaced by defatted rice bran with the same amount of total nitrogen content. In the part of the control (Formula no.1), it was found to have amylase activity (33.09±1.76 U/ml) higher than the other except the formula no.2, meaning that the defatted rice bran might act as the activator for amylase production. Nevertheless, this experiment was still lack of the formula without yeast extract and defatted rice bran while adding tryptone as the only main nitrogen source because the highest activity of the formula no.2 would be from the cutting out of yeast extract or adding of defatted rice bran. So, the next experiment had been conducted, testing the doubt effect of nitrogen sources (Figure 4.25).

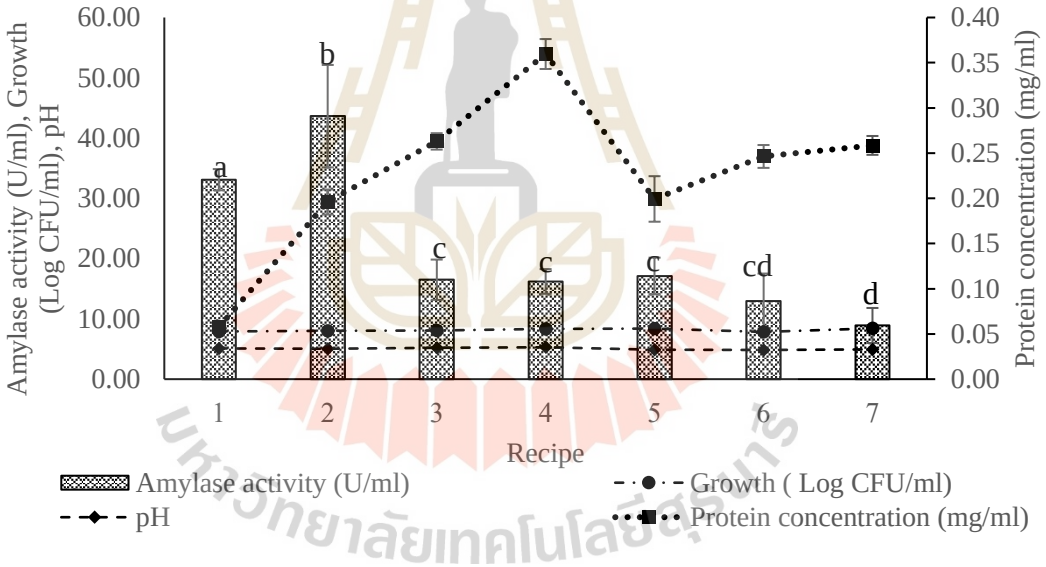


Figure 4.25 Amylase activity, growth, pH and protein concentration of the 7 formulas after cultivating SUTWR 73-1 under anaerobic condition at 35℃ for 24 h. Means with different letters (a, b, c, d, and e) showed significantly different means (Duncan's Multiple Range Test (DMRT), $p<0.05$).

It was also found that the medium without rice bran (formula no.2) showed the lowest amylase activity (15.26±1.78 U/ml) compared to the other formulas, and also gave the lowest protein concentration with 0.018±0.01 mg/ml (Figure 4.26). This represented the low impact of yeast extract on the bacterial amylase production. As found in the yeast extract, defatted rice bran did not affect amylase production. The amylase production depended on tryptone as a critical factor. Furthermore, the amount of protein after the addition of defatted rice bran showed the possibility that most of the defatted rice bran protein or nitrogen-containing components that were not effectively used by SUTWR 73-1. Hence, the formula no.2 was chosen for studying the effect of concentration on amylase production. The amylase production by using tryptone as a nitrogen source has been reported for a decade about its stimulatory effect. Sharma et al. (2012) provided the use of organic nitrogen sources like tryptone having the effect on enzyme production, and also suggested that the concentration of the nitrogen source was important. The lower levels of nitrogen and also excess nitrogen were equally detrimental causing enzyme inhibition. In Okolo et al. (1996) finding, tryptone highly affected amylase production from bacteria. However, some reports also claimed that the yeast extract was a good choice similar to our study, by supporting the enzyme production in *Bacillus amyloliquifaciens* (Sharma et al., 2012). Also, an inorganic nitrogen source showed the stimulatory effect on amylase production. According to Deb et al. (2013), ammonium salts were found to be stimulators of amylase synthesis from *Bacillus* sp., which was in the same way with Coleman and Eliot (1962) finding. Nevertheless, Sharma et al. (2012) reported that concentration of nitrogen source should be in the point that did not too excessive or lower, hence the concentration optimization was assessed in the next experiment.

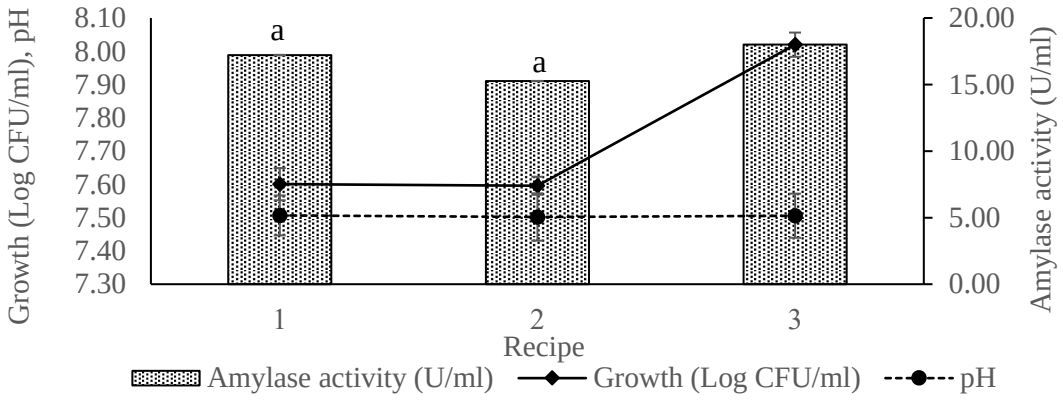


Figure 4.26 Comparison of amylase activity after cultivating SUTWR 73-1 at 35℃ pH 7 for 24 h under anaerobic condition. Means with different letters (a) showed significantly different means (Duncan's Multiple Range Test (DMRT), $p<0.05$).

Tryptone was considered as a critical nitrogen source. Its concentration was varied from 0.05 to 10%(w/v). After the cultivation of SUTWR 73-1 in the medium containing different tryptone concentrations, tryptone 4.5%(w/v) provided the highest amylase activity, 35.47±1.79 U/ml (Figure 4.27). For the bacterial growth comparison, the higher tryptone concentration was added, the lower growth of SUTWR 73-1 was observed. It was the evidence supporting the situation of metabolic drove by inducing the usage of the substrate. The carbon source was not the only factor affecting the metabolic process, but nitrogen source was another one to be considered, due to both sources were required for high energy compound and coenzyme productions. The flux of substrate into SUTWR 73-1 cell drove the growth mechanism, and eventually lead to cell age resulting in growth at 24-h incubation time, which was not good as the lower tryptone concentration. Acid production was also affected. The effect of tryptone to acid production was reported by Huang et al. (2005), they mentioned that lactic acid

bacteria required substrates with high nitrogen content, and it has a particular demand for vitamins B21. The nitrogen sources were required for the fermentation medium by supplying in forms of yeast extract, cotton oil, soy flour, tryptone, and peptone. Acid directly affected the cell weakness because of the concentration of proton excessing in the cultured medium penetrated through the cell. Each metabolic pathway inside the cell has its pH for working. The high proton caused proton motive force between inside and outside the bacterial cell change. If the balance of proton was broken, the homeostasis of the cell membrane would fail and lead to cell death (Huang et al., 2005).

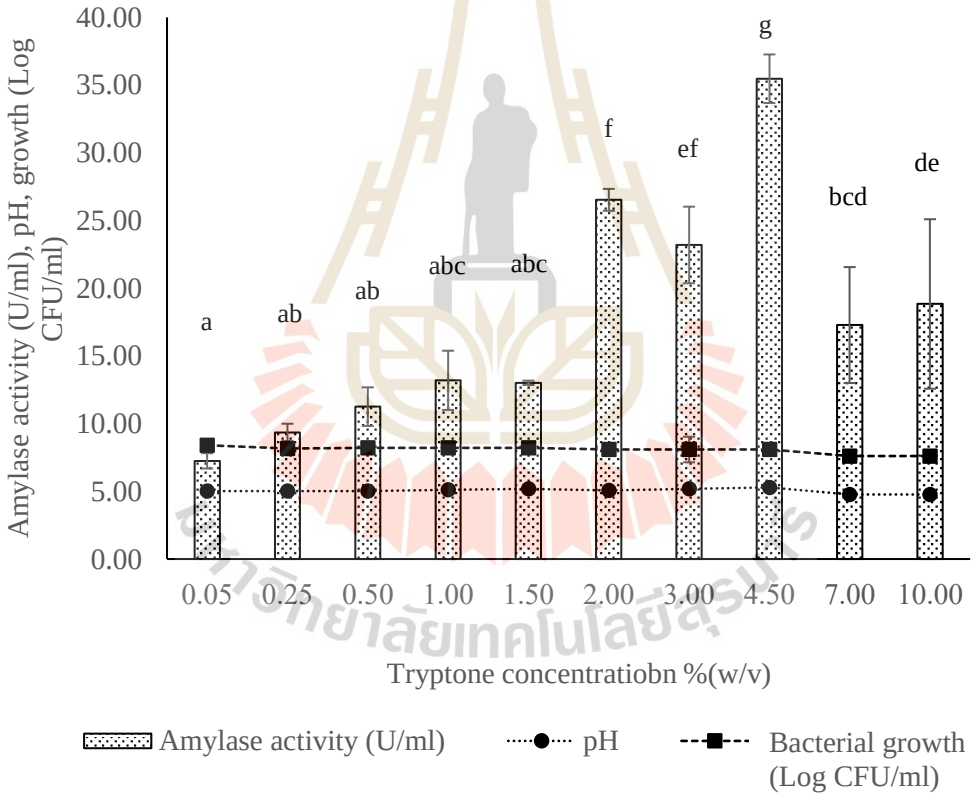


Figure 4.27 Comparison of amylase activity after cultivating SUTWR 73-1 in different tryptone concentration at 35℃ pH 7 for 24 h under anaerobic condition. Means with different letters (a, b, c, d, and e) showed significantly different means (Duncan's Multiple Range Test (DMRT), $p<0.05$).

4.5.1.3 pH of the amylase production medium

From previous results, pH also related to the bacterial growth. At low pH, the concentration of acid made the low survival of SUTWR 73-1 after cultivation at 35℃ under anaerobic condition for 24 h. Then pH was also investigated for its effect on amylase production. Selection of pH was based on data collected from our previous experiments. 4 points of pH were chosen including pH 7, 8, 9, and 10. These selections were investigated because the high pH could neutralize the acid produced during cultivation of SUTWR 73-1 and there would be possibilities that the bacterium could produce amylase, effectively. The pH of the medium was adjusted only the initial pH without controlling while the cultivation was performed.

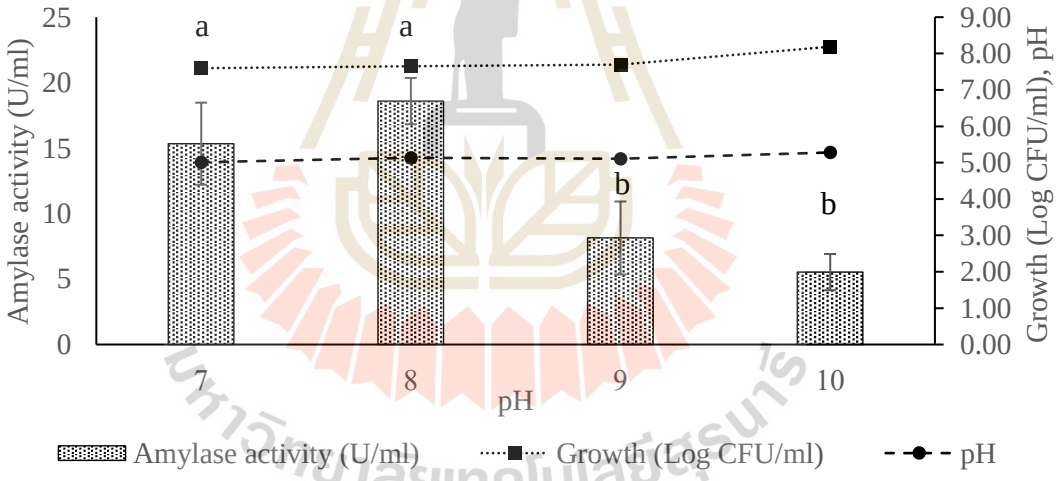


Figure 4.28 Comparison of amylase activity after cultivating SUTWR 73-1 at different initial pH, and at 35℃ for 24 h without aeration. Means with different letters (a, b, c, d, and e) showed significantly different means (Duncan's Multiple Range Test (DMRT), $p<0.05$).

The results showed that the highest amylase activity was obtained from pH 8 with 18.59±1.77 U/ml for activity. While the higher pH (pH 9 and 10) had been

decreased followed by the increase of pH, meaning that the lactic acid bacteria preferred the little alkali condition. This finding was the same trend that was found in a general lactic acid bacterium which prefers acidic to the neutral condition. The Final pH tends to increase as the initial pH. At higher initial pH the alkali or OH- would neutralize by abstracting the proton from acid produced by SUTWR 73-1. However, the high potential acid production of lactic acid bacterium could decrease the pH of the medium after cultivation. The highest pH after cultivation was found at the initial pH of the medium of 10 and decreased to pH 5.28±0.02. That means the high acid concentration was produced resulting in the largest change of pH, and the amylase activity may have high at earlier because the high acid production was driven from the high substrate flux into the cellular metabolic pathway. Therefore, the complex substrate as cassava starch had to be cleaved into the small dextrin, maltose, and glucose by high activity or high amount of amylase. Another remark was the trend and relationship between growth and final pH at 24-h cultivation time, if initial pH was low, the final pH tended to be low as pH coincident with the growth that would be inhibited. This finding had been reported (Hutkins and Nannen, 1993), the growth of lactobacilli was found to be inhibited when the pH nearly dropped to pH 5. At pH 5, the leakage of solute and derangement of cell membrane occurred, but the leakage of ions like potassium ion and magnesium ion was occurred varied from species to species. As mentioned in this paragraph, it could be concluded that the initial pH was suitable for amylase production by SUTWR 73-1.

4.5.2 Statistical experiments using response surface method (RSM)

Statistical optimization using response surface method (RSM) was performed for the study of three main factors including cassava starch concentration,

tryptone concentration, and pH. The statistical was applied in the experimental design, which would give the model that could be used at every point on the surface area for amylase production. The model was better in cost-benefit comparison, which differs from the previous factorial design which showed only the point that has been examined. The central value of each critical factor was based on the factorial experiment, which was 10%(w/v), 4.5%(w/v), and 8, for cassava starch concentration, tryptone concentration, and pH, respectively. Then, the experiment was designed and through Design-Expert 11. The set of conditions was generated to twenty runs (shown in Table 4.15 and 4.16).

Table 4.15 Building information in Design-Expert 11 for model construction.

Detail	Set up
Study type	Response surface
Design type	Central composite
Design model	Quadratic
Build time (ms)	3.00
Sub type	Randomized
Run	20
Blocks	No blocks

Table 4.16 Coding factors from Design-Expert 11.

Factor	Coded symbols	Decoding values				
		-α	-1	0	+1	+α
Starch concentration (%)	X_1	1.59	5.00	10.0	15.00	18.41
Tryptone concentration (%)	X_2	0.30	2.00	4.50	7.00	8.70
pH	X_3	4.64	6.00	8.00	10.00	11.36

Table 4.17 Experimental design from Design-Expert 11 and the results.

Run	Cassava starch concentration (%)	Tryptone concentration (%)	pH	Amylase activity (U/ml)
1	0	+α	0	19.20
2	0	0	0	31.82
3	+1	-1	-1	13.60
4	+α	0	0	25.53
5	+1	+1	-1	16.35
6	0	0	0	30.80
7	0	0	+α	26.34
8	0	0	0	33.36
9	+1	-1	+1	25.41
10	+1	+1	+1	24.69
11	-1	-1	+1	18.37
12	-1	-1	-1	10.43
13	-1	+1	+1	20.08
14	0	-α	0	12.51
15	0	0	0	30.43
16	-1	+1	-1	13.44
17	0	0	-α	11.70
18	-α	0	0	13.62
19	0	0	0	33.56
20	0	0	0	31.87

Table 4.18 Summary of model type analysis from Design-Expert 11.

Response 1: Amylase activity					
Source	**Sequential p-value**	**Lack of Fit p-value**	**Adjusted R^2**	**Predicted R^2**	**Analysis result**
Linear	0.1091	0.0002	0.1776	0.0659	
2FI	0.9895	0.0001	-0.0033	-0.6781	
Quadratic	**< 0.0001**	**0.3634**	**0.9702**	**0.9204**	**Suggested**
Cubic	0.2501	0.5309	0.9773	0.8593	Aliased

Sequential model sum of square [Type-1]						
Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	**Analysis result**
Mean vs Total	9816.60	1	9816.60			
Linear vs Mean	385.96	3	128.65	2.37	0.1091	
2FI vs Linear	7.63	3	2.54	0.0384	0.9895	
Quadratic vs 2FI	**841.97**	**3**	**280.66**	**142.32**	**< 0.0001**	**Suggested**
Cubic vs Quadratic	10.72	4	2.68	1.79	0.2501	Aliased
Residual	9.00	6	1.50			
Total	11071.88	20	553.59			

Lack of fit test						
Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	**Analysis result**
Mean vs Total	9816.60	1	9816.60			
Linear vs Mean	385.96	3	128.65	2.37	0.1091	
2FI vs Linear	7.63	3	2.54	0.0384	0.9895	
Quadratic vs 2FI	**841.97**	**3**	**280.66**	**142.32**	**< 0.0001**	**Suggested**
Cubic vs Quadratic	10.72	4	2.68	1.79	0.2501	Aliased
Residual	9.00	6	1.50			
Total	11071.88	20	553.59			

After the experimental data were used to construct the model, the data were used to look for the most fitted models among linear, 2FI, quadratic, and cubic model. The results showed that the most fitted model was quadratic which gave the lowest p-value (below than 0.0001). The lack of fit p-value was 0.3634 meaning that there was 36.34% for a chance of model noise occurrence making lack of fit. For the R^2, the model which provided the highest both predicted and adjusted R^2 was selected for constructing the model equation. Therefore, the quadratic type was selected due to the adjusted and predicted R^2 was highest than the others, which were 0.9702 and 0.9204, respectively.

Table 4.19 Statistical analysis (ANOVA) for quadratic model from Design-Expert 11.

Source	Sum of squares	df	Mean square	F-value	P-value	Difference at α=0.05
Model	1235.558	9	137.2842	69.61543	7.81E-08	Significant
A: Starch concentration	104.4464	1	104.4464	52.9637	2.67E-05	
B: Tryptone concentration	23.74666	1	23.74666	12.04169	0.00602	
C: pH	257.7642	1	257.7642	130.7096	4.6E-07	
AB	0.902454	1	0.902454	0.457625	0.514078	
AC	3.887347	1	3.887347	1.971234	0.190603	
BC	2.83799	1	2.83799	1.439116	0.257933	
A^2	264.2309	1	264.2309	133.9888	4.1E-07	
B^2	451.3169	1	451.3169	228.8582	3.22E-08	
C^2	288.8366	1	288.8366	146.4661	2.7E-07	
Residual	19.72037	10	1.972037			
Lack of fit	11.4678	5	2.293561	1.389604	0.363438	Not significant
Pure error	8.252568	5	1.650514			
Cor total	1255.278	19				

The constructed quadratic model was also tested for statistical ANOVA. The selected model showed F-value equal to 69.62 which implied that the model was significant. There is only a 0.01% chance that F-value could occur due to noise. The P-value of this model was less than 0.0500 indicated the model terms were significant

including A, B, C, A^2, B^2, and C^2. Values were greater than 0.1000 indicated that the model terms were not significant. If there were significant model terms, the reduction of the term may improve the model more reliable. The F-value of lack of fit was found of 1.39 suggested that lack of fit was not significant relative to the pure error. There was a 36.34% chance that the lack of fit F-value could occur due to noise. Furthermore, the mathematical model also showed that adjusted R^2 equaled to0.9702 differed from Predicted R^2 equaled to 0.9204 less than 0.2 meaning that the constructed model has no problem from the block effect or the data, making the model could precisely forecast the actual value. The signal to noise ratio greater than 4, indicated an adequate signal supporting the model to be used to navigate the design space.

As shown in Table 4.20, the coefficient was estimated. The mean coefficient also represented the impact of these factors, how much is influenced by these factors. The coefficient itself represented the expected change in response per unit change in factor value when all factors were kept constant. The intercept in an orthogonal design was the all average response of all runs. The coefficients were adjusted around that average based on the factor setting. Thus, the coefficient of pH had the big critical to amylase production because it was the 4.34 which was the largest among three factors followed by cassava starch, and tryptone concentration which had 2.77 and 1.32, respectively. However, the VIF consideration revealed that when the VIFs was 1, the factor was orthogonal. In contrast, the VIF was greater than 1 indicated multi-collinearity. The higher the VIF, the more severe the correlation of factors, but the rough rule, VIF less than 10 were still tolerated and acceptable.

Table 4.20 Coefficients in termed of coded factors.

Factor	Coefficient estimate	df	Standard error	95% CI low	95% CI high	VIF
Intercept	31.96	1	0.5727	30.68	33.23	
A: Starch concentration	2.77	1	0.3800	1.92	3.61	1.0000
B: Tryptone concentration	1.32	1	0.3800	0.4719	2.17	1.0000
C: pH	4.34	1	0.3800	3.50	5.19	1.0000
AB	-0.3359	1	0.4965	-1.44	0.77	1.0000
AC	0.6971	1	0.4965	-0.41	1.80	1.0000
BC	-0.5956	1	0.4965	-1.70	0.51	1.0000
A^2	-4.28	1	0.3699	-5.11	-3.46	1.02
B^2	-5.60	1	0.3699	-6.42	-4.77	1.02
C^2	-4.48	1	0.3699	-5.30	-3.65	1.02

Note: df, degree of freedom
CI, Coefficient interval
VIF, Variance inflation factor

Table 4.21 Comparison the value of amylase activity between the observed and predicted by the model.

Run no.	Observed (U/ml)	Predicted (U/ml)
1	19.20	18.35
2	31.82	31.96
3	13.60	13.75
4	25.53	24.50
5	16.35	16.90
6	30.80	31.96
7	26.34	26.60
8	33.36	31.96
9	25.41	25.02
10	24.69	25.80
11	18.37	17.42
12	10.43	8.94
13	20.08	19.54
14	12.51	13.91
15	30.43	31.96
16	13.44	13.44
17	11.70	11.99
18	13.62	15.19
19	33.56	31.96
20	31.87	31.96

The quadratic graph was constructed from the model and shown in the 3D surfaces and contour plots (Figure 4.29). It indicated that the center values given from factorial design were also nearly the same point of the optimum condition at the central of the slope. The central slope was placed on the central map meaning that this model showed the optimum condition as expected from factorial results. All mentioned data were tested using a statistical way to construct the model and lead to the final equation which covered enough for actual usage. The model equation was amylase activity equaled to $31.96+2.77(A)+1.32(B)+4.34(C)-0.3359(AB)+0.6971(AC)-0.5956(BC)-4.28(A^2)-5.60(B^2)-4.48(C^2)$.

Finally, the equation of the model must be validated for its optimum point in real use by keeping two-sided confidence of 95%. The generated confirmation conditions were conducted in 3 conditions as shown in Table 4.22.

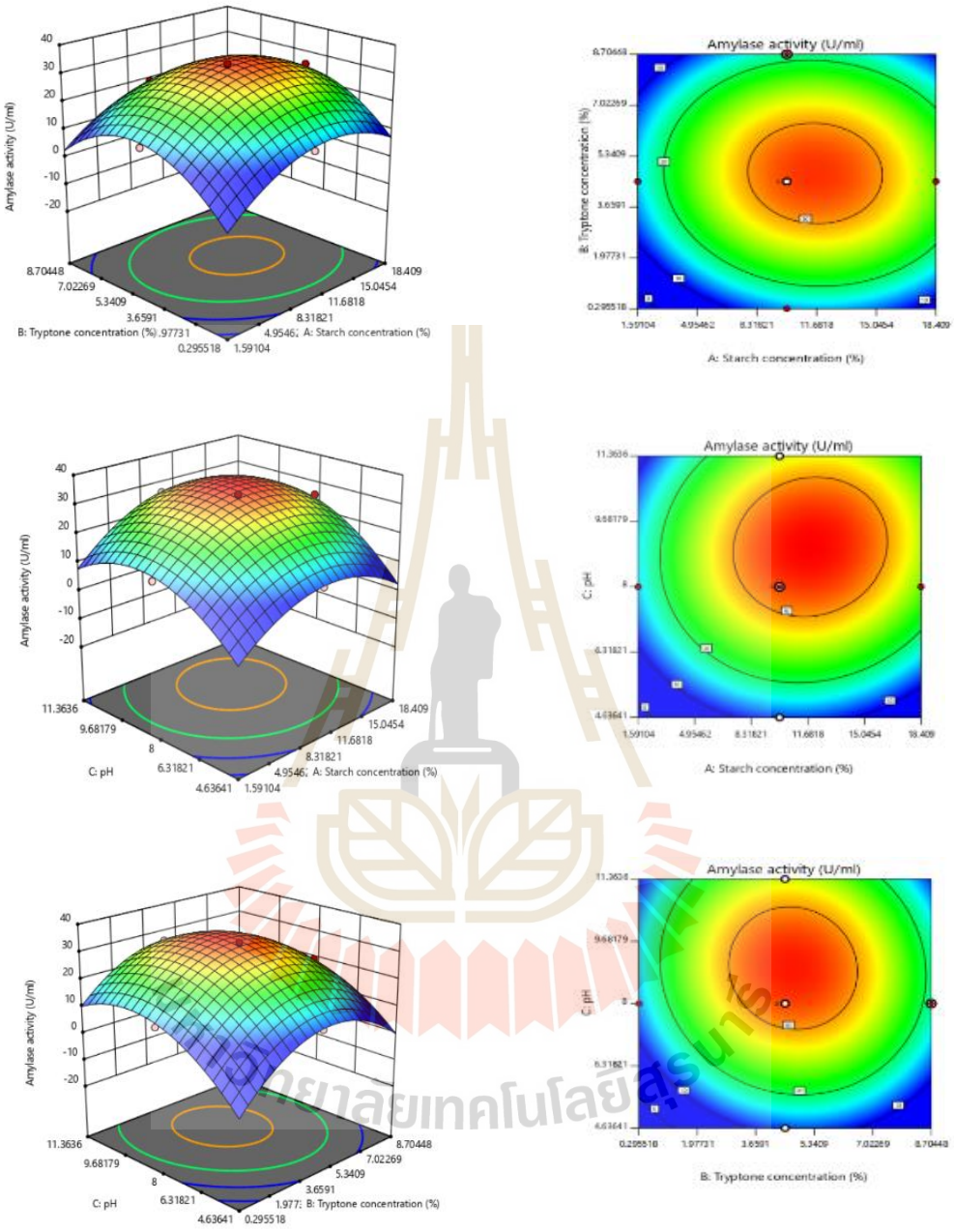


Figure 4.29 3D surfaces and contour map of the quadratic model constructed from Design-Expert 11 using the response surface method of amylase production optimization by SUTWR 73-1.

Table 4.22 Confirmation results from three random conditions at the optimum point by using Design-Expert 11.

Condition	Cassava starch concentration (%)	Tryptone concentration (%)	pH	Predicted amylase activity (U/ml)	Amylase activity (U/ml)	Protein concentration (mg/ml)
1	11.69	4.57	8.95	33.53	34.52±6.51	0.503±0.190
2	11.69	4.79	8.96	33.60	31.10±4.32	0.367±0.025
3	11.77	4.69	8.80	33.56	31.40±5.83	0.398±0.100

The concentration of tryptone and cassava starch as well as pH were randomly generated from each condition using the model, and also predicted the possibilities of amylase activity. Then, the amylase activities of three conditions were obtained from the experiment by having 34.52±6.51, 31.10±4.32, and 31.40±5.83 U/ml for the 1st, 2nd, and 3rd conditions.

Table 4.23 revealed the result of confirmation, showing the predicted value of each condition and the data from cultivation. It was found that the amylase activity from cultivations was nearly the predicted values. To clarify the difference, the statistical method was used to identify by one-way t-test at 95% for a confident level. The test showed no significant difference between the predicted and the actual values. This could be concluded that the model was reliable enough for using.

Table 4.23 Statistical results from one sample t-test of three conditions using SPSS 23 at 95% for confident level.

Condition	Test value	t	df	Sig. (2-tailed)	Mean difference	95% Confidence interval of the difference	
						Lower	Upper
1	33.52	.245	2	.829	.92233	-15.2489	17.0936
2	33.60	-1.402	2	.296	-2.49667	-10.1604	5.1671
3	33.56	-1.833	2	.208	-2.15823	-7.2247	2.9083

Note: t, t-test value

Df, degree of freedom

Sig., significantly different

4.6 Production and partial purification of amylase produced by SUTWR 73-1

After achieving the optimum condition and the reliable equation, the model was used to perform the experiment by conducting in the 7.5-l bioreactor with a working volume of 3 l. At this scale, the medium used in this step was reduced the cassava starch concentration to 5%(w/v) to avoid the problem of high viscosity which was found at the flask scale. In the bioreactor scale, there was the addition of factor which was agitation speed, it was set at 150 rpm. The cultivation was conducted without adjustment of acidity, and the addition of medium. For 48 h of cultivation time, change of amylase activity, growth, pH, protein concentration, and remaining starch, were monitored (shown in Figure 4.30).

Figure 4.30 showed the kinetic value and the change of each parameter. The highest activity and the protein concentration obtained at 18 h with the activity of 17.00 U/ml and 217.79 µg/l, respectively. Interestingly, the amylase was a growth-dependent enzyme, but the shape of the curve did not go parallelly with growth. The amylase activity dropped after firstly surging at 18 h, and then went up to 15.99 U/ml at 48 h. This finding looked like the pulsed production similar to the operating in a continuous system, but it was found in the batch cultivation. The phenomena could be interpreted in two ways. Firstly, the amylase was produced in a high rate to digest the high concentration of the starch due to the small requirement of bacteria, a high amount of starch causing the imbalance between the rate of consumption and reducing sugar accumulation. The high accumulation of reducing sugars then inhibited the amylase production as reported by Negi and Banerjee (2010) and Teodoro and Martins (2000). After the reducing sugars were consumed by the bacteria reaching at one level, the effect of inhibition decreased allowing the bacterium to produce amylase again making higher amylase at 48 h which was not different as at 18 h. Another explanation for the phenomena, relied on the finding of enolase in the sample at 48-h incubation time which has been mentioned in the molecular weight determination section. Normally, the enolase was synthesized and anchored inside the bacterial cell. If the enzyme was found outside the cell meaning that there was the leakage of content from the cell, there was a chance that the other kind of amylase could represent the activity at the second surged while the bacteria have been in the death phase.

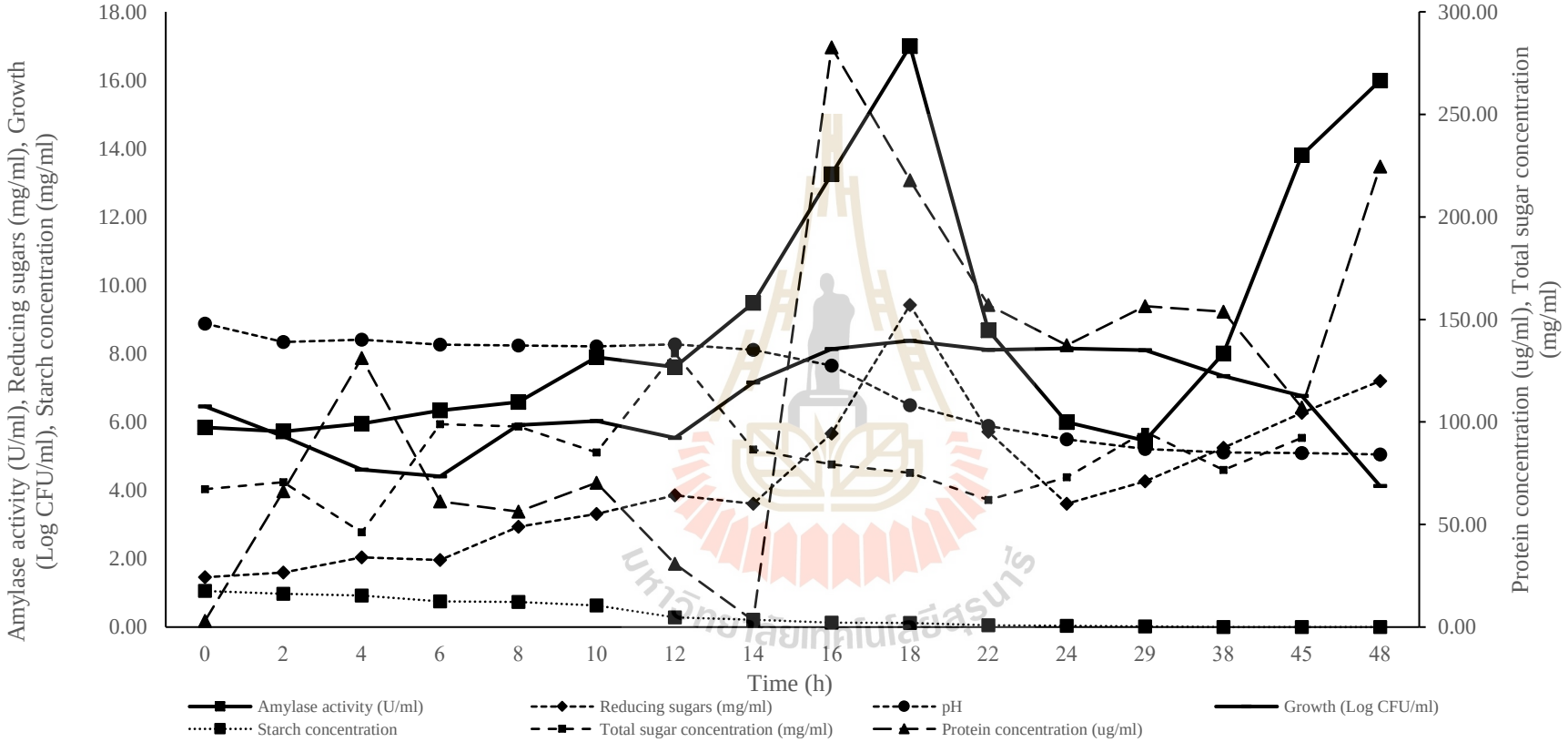


Figure 4.30 Amylase activity, pH, reducing sugars, total sugars, starch, protein concentration and growth of SUTWR 73-1 obtained from 2-l cultivation at 150 rpm, 35°C without aeration for 48 h.

Table 4.24 Amylase activity, pH, reducing sugars, total sugar, starch, protein concentration and growth of SUTWR 73-1 obtained from 2-l cultivation at 150 rpm, 35℃ without aeration for 48 h.

No.	Time (h)	Amylase activity (U/ml)	pH	Growth		Total sugar concentration (mg/ml)	Protein concentration (ug/ml)	Starch concentration (mg/ml)
				(CFU/ml)	(Log CFU/ml)			
1	0	5.84	8.88	2.83×10^{6}	6.45	67.19	3.02	1.056
2	2	5.73	8.34	3.80×10^{5}	5.58	70.67	66.23	0.974
3	4	5.96	8.41	4.00×10^{4}	4.60	46.16	131.12	0.922
4	6	6.34	8.26	2.55×10^{4}	4.41	98.85	61.24	0.748
5	8	6.58	8.24	8.11×10^{5}	5.91	97.85	56.25	0.736
6	10	7.89	8.21	1.06×10^{6}	6.03	85.21	70.32	0.636
7	12	7.60	8.27	3.45×10^{5}	5.54	133.52	30.84	0.287
8	14	9.48	8.11	1.42×10^{7}	7.15	86.53	3.16	0.217
9	16	13.25	7.65	1.37×10^{8}	8.14	79.37	282.68	0.131
10	18	16.99	6.49	2.38×10^{8}	8.38	75.16	217.79	0.118
11	21	8.68	5.88	1.29×10^{8}	8.11	61.99	156.99	0.049
12	24	6.00	5.49	1.41×10^{8}	8.15	73.11	137.47	0.039
13	28	5.45	5.21	1.25×10^{8}	8.10	95.23	156.53	0.016
14	38	8.00	5.11	2.18×10^{7}	7.34	76.63	153.81	0.000
15	45	13.81	5.09	5.80×10^{6}	6.76	92.28	107.07	0.000
16	48	15.99	5.05	1.35×10^{4}	4.13	75.89	224.60	0.000

Since the cultivations both in small or large-volume scale have been conducted at the beginning of the experiment, it was noticed that optimum point of amylase production was not the same point of the lactic acid production, and the highest growth was also different from the condition used in amylase and lactic acid production. Sreekanth et al. (2013) stated that the optimal growth and amylase production was not at the same point, but both were influenced by substrates, carbon, and nitrogen source. The medium prepared from glucose resulted in higher amylase yield while the maximum growth was observed with corn starch. In contrast, glucose was found to repress the amylase production in *Bacillus* sp. (Thippeswamy et al., 2006). Not only the glucose, but the lactose was also reported as an inducer of amylase production (Sudharhsan et al., 2007), and the fructose was identified as a repressor. Negi and Banerjee (2010) also supported in the same way as Sreekanth et al. (2013) did, by suggesting that not every carbon source acted as an enhancer of amylase production. The structure of the carbon source of the substrate was important to amylase production. It tended to be that the complex carbohydrates as soluble starch (Sreekanth et al., 2013), exhibited the higher amylase activity than small oligosaccharide and monosaccharide, respectively (Ashwini et al., 2011). In the presence of complex carbohydrate, the bacteria had to produce the high amylase for digesting, after that the happened oligosaccharide or monosaccharide was then absorbed for growth. If there was no complex carbohydrates, the bacteria did not need to produce amylase for digesting, the bacteria preferred to have oligosaccharide and monosaccharide for growing than amylase production. Similarly, Teodoro and Martins (2000) described that synthesis of carbohydrate degrading enzyme in most species of genus *Bacillus* leads to catabolic repression by readily metabolizable substrates such as glucose and fructose. From

Sreekanth et al. (2013), the nitrogen sources were tested for their effects on growth and the amylase production, it showed that nitrogen sources like tryptone, peptone, yeast extract, soybean meal, and beef extract, resulted in the production of the higher amount of amylase than simple inorganic sources of nitrogen such as various salts of ammonia and potassium. Various combinations of carbon and nitrogen sources significantly affected the difference in growth and amylase production. These could be concluded that the substrate preference acted on growth and metabolite differently leading the optimal condition for amylase production to differ from the growth of the organisms.

Lactic acid production from microorganisms was explained as amylase production, but it was related to the capabilities of the microorganisms resistant to the pH in the environment. The high amount of substrate made high acid production. If the environment constituted of the metabolizable substrate like glucose, fructose, lactose, and galactose, the microorganisms could suddenly use these substrates to produce lactic acid. However, the high acid production was not good for microbial metabolism, the proton fluxed into the cell causing the change of membrane permeability and high concentration of intracellular proton. The high proton actively damaged various enzymes in metabolic pathways, meaning that the microorganisms could not retain effective metabolism for growing as reported by Huang et al. (2005)'s report.

In the study, the bacterium could produce amylase and lactic acid, so the competition of carbon source between lactic acid production, growth, and amylase production would occur. If the higher amylase was obtained, the others were reduced. Nevertheless, there report about the effect of substrate competition on growth, it suggested that the growth would be affected less than metabolite production. Hence the optimal lactic acid production condition did not seem to be at the same point of amylase

production but possible for the growth of the microorganism as shown in Huang et al. (2005)'s results. However, to the study of amylase, the partially purified amylase tended to give a clear result of the amylase characteristics than was done in the crude enzyme.

Protein purification was needed for effectively investigating the characteristics of the enzyme. Several reports recommended the amylase precipitation by adding the ammonium sulphate until the crude protein solution saturated with 80%-saturation. However, this study has planned to study the efficiency of precipitation at different saturation including 60, 70, 80, and 90%. The precipitate of the amylase was resuspended and dialyzed through ultrafiltration (30 kD molecular weight cut-off).

From Figure 4.32, the amylase was precipitated by 70% ammonium sulphate giving the highest amylase activity. The saturation at 60, 80, and 90% ammonium sulphate showed the relative activities of 38.76, 68.94, and 6.96%, respectively. For enzyme purification, the elimination of undesirable protein was needed. So, the specific activity showed the activity to protein ratio, which meant that the higher specific activity the lower of undesirable protein. At 70% ammonium sulphate saturation the specific activity was 17.34 U/ml followed by 60, 80, and 90% which were 14.27, 13.85, and 2.80 U/ml, respectively. In comparison to crude, the 70% saturation could reduce the undesirable protein from 6.136 mg/ml to 0.593 mg/ml and kept the amylase 56.71% of the initial crude which considered as 100%. The purification fold of ammonium sulphate precipitation at 70% showed 5.9 folds greater than crude amylase.

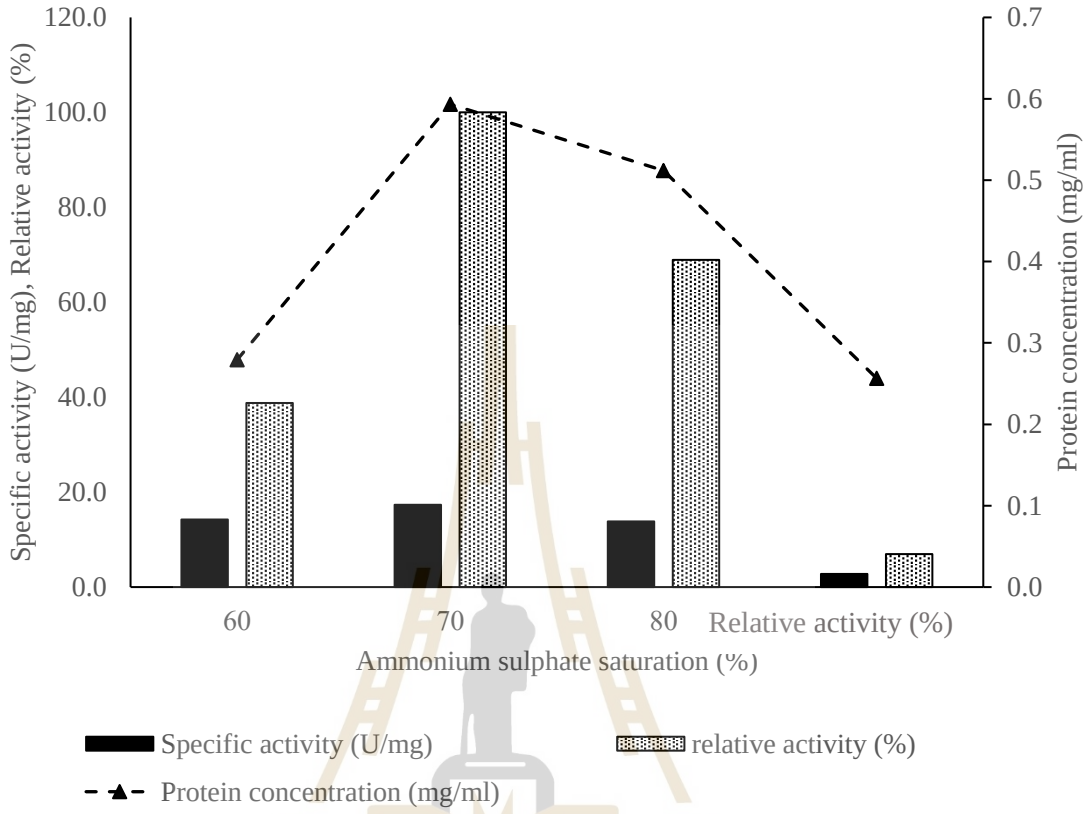


Figure 4.31 Protein concentration, specific, and relative activities of the partially purified amylase of SUTWR 73-1 with different ammonium sulphate saturation.

The below data came from partially purified for more than 7 days. This let the activity of the amylase was being lost during stored in the 4℃-refrigerator without the addition of enzyme protectant. Afterward, each time of precipitation still showed low recovery. This may be from incompletely desalting out from the precipitated protein or disappear of the cofactor required by the enzyme to stabilize its structure for working properly.

Table 4.25 Purification table of crude and partially purified amylase produced by SUTWR 73-1.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Fold
Crude	6.136	18.139	2.956	100.0	1.0
Ammonium sulphate precipitation					
60%	0.279	3.986	14.266	22.0	4.8
70%	0.593	10.286	17.337	56.7	5.9
80%	0.512	7.091	13.851	39.1	4.7
90%	0.256	0.719	2.802	4.0	0.9

The precipitation was done again to measure the most reliable specific activity. This time, the specific activity was high with 17.337 U/mg, but the yield was only 20% of the initial crude. This could be interpreted that if the problem of the dialysis occurred, the real amylase activity that should have after dialysis, may have higher activity than this observed. Likewise, the specific activity might have higher than the one presented in Table 4.26.

Table 4.26 Purification table of crude and 70% ammonium sulphate-precipitated amylase produced by SUTWR 73-1.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold
Crude	1.539	1,539.59	963.64	100.0	1.0
70%-Ammonium sulphate precipitation	0.127	313.34	2,462.29	20.4	2.6

The problem of impurities in this section would be solved using fractionation of ammonium sulphate precipitation to decrease most of the undesirable protein followed by performing dialysis to remove excess ammonium sulphate which was found to inhibit the amylase in this study. In the bulk scale of the enzyme purification, membrane filtration could be another choice that decreases chemical use, time, and cost.

4.7 Characterization of amylase produced by SUTWR 73-1

The partially purified amylase from ammonium sulphate precipitation was consequently characterized for its molecular weight, kinetic parameters, pH and temperature profile and stability, and ion effect. The results were shown in the next section.

4.7.1 Molecular weight

Due to the amylase was partially purified, the additional separation technique was necessary. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

(SDS-PAGE) was used for protein band separation. But SDS-PAGE could not clarify the molecular weight directly in the case of the crude amylase consisted of some other proteins. The amylase in SDS-PAGE was partially in the inactive form because SDS and beta-mercaptoethanol caused the protein denaturation. For this reason, the native gel would be another parallel gel electrophoresis technique, because it did not treat protein with beta-mercaptoethanol which was highly active in protein denaturation, but still used SDS as the detergent to keep the protein charge constantly to length ratio, let it flowed regularly as was conducted in SDS-PAGE. This technique was also called non-reducing native gel electrophoresis. The native gel electrophoresis was combined with the additional step of staining, the activity staining using soluble starch as the amylase substrate. The activity staining would show the clear result that which band was and the band of the amylase from SUTWR 73-1.

As clearly shown in Figure 4.32, the amylase band would be the upper band from two major bands. The molecular weight of the amylase was estimated from moving distance measurement, and then plotting the distance vs. the logarithm value of the molecular weight of the known standard protein marker.

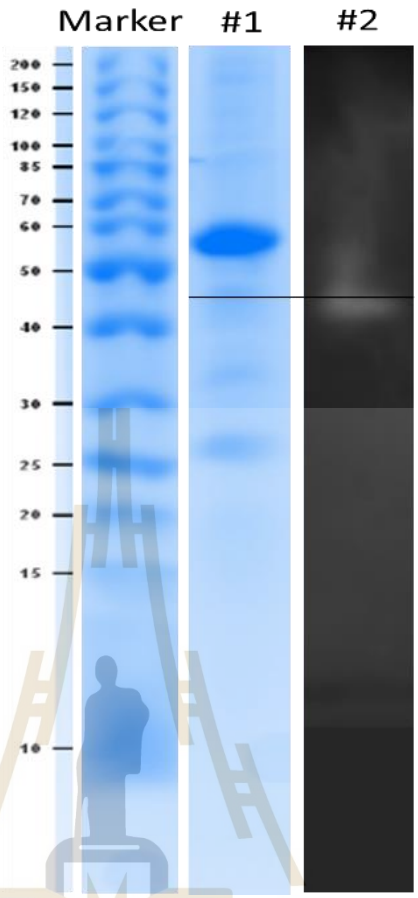


Figure 4.32 The SUTWR 73-1 Amylase protein band in the SDS-gel and the non-reducing gel after activity staining.

The equation was used to estimate the molecular weight of amylase, which was calculated to 46.04 kD. However, there would be used another way for measuring this through the software of gel documentation system. The software required filling the data of protein marker and marking the target band before the molecular weight estimation. The result of the software estimation was as shown in Figure 4.34 which represented the molecular weight around 45.1 kD. Both estimations gave the molecular weight nearly the same. Due to the crude have been through only two steps including ammonium sulphate precipitation and dialysis, the impurities were still presented. The

impurities could observe primarily from the numbers of bands after gel electrophoresis. Figure 4.34 showed the major band at 56.3 kD, but also provided the minor proteins at least 8 bands that could be detected by the Coomassie blue staining method.

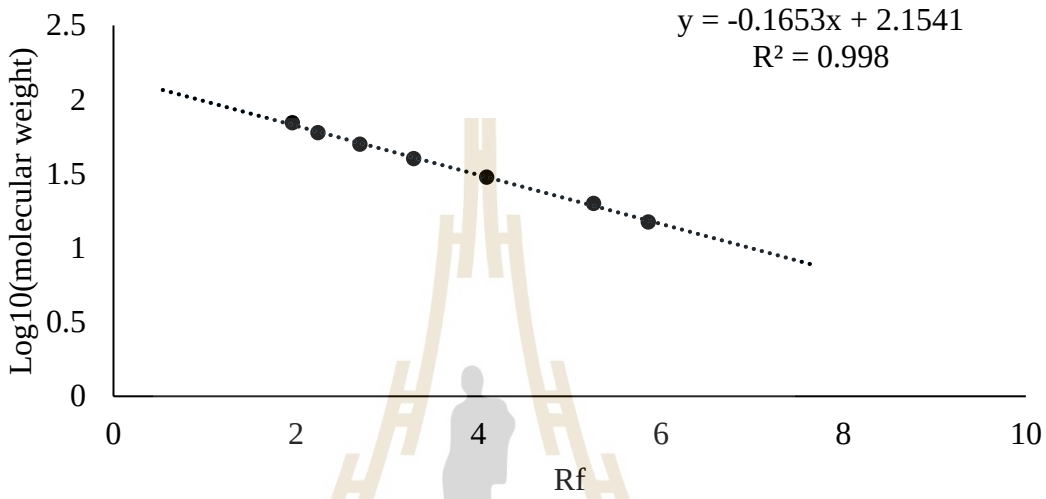


Figure 4.33 Standard curve of the molecular weights vs. the distances for molecular weight estimation.

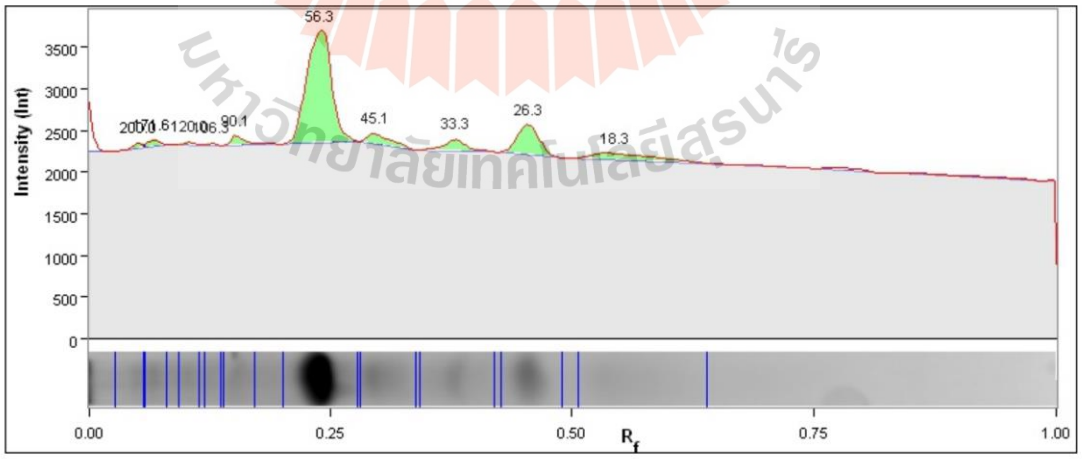


Figure 4.34 The result of molecular weight determination by software of gel documentation system (Gel-doc^{TM} EZ).

In this section, the major band of protein was focused. From the experiment, the main protein at this condition should be the extracellular amylase produced from SUTWR 73 but this condition simultaneously supported the expression of other proteins outside the cell in the supernatant. The proteomics study of 56.3-kD protein was performed. The excision of the major band was done and washed with DI before kept in the 1%(v/v) of acetic acid. Then it was sent to Research Instrument Center, Khon Kaen University, Thailand, for proteomics analysis using in-gel digestion technique combined with LC/ESI-MS/MS (liquid chromatography-electrospray ionization tandem mass spectrophotometry) analysis. Then the LC-MS/MS spectra were used to identified and analyzed using MASCOT program with NCBI and SwissProt database. The results of peptide fingerprint matching were shown in Figures 4.36, 438, and 4.39. Surprisingly, the closest protein of 56.3-kD protein was identified as enolase or phosphopyruvate hydratase from *Tolumonas auensis* strain DSM 9187. The enolase was well known in the role of glycolysis which could converse 2-phosphoglycerate to phosphoenolpyruvate. From Mascot score histogram, it revealed that the observation was a random event by having individual ion score larger than 41 implied the identity and extensive homology at a confident level of 95%. The searching protein results were similar with 8 peptides of *Tolumonas auensis* DSM 9187 which were shown in Figure 4.36, consisting of DAGYTAVISHR (1,188.587 Da) for amino acid sequence. The interesting was that enolase was found in the extracellular which normally expected to be in cytoplasmic. However, there was a report introducing the surface-associated enolase (Antikainen et al., 2007). The paper stated that enolase was produced a lot in the fermentation and glycolysis during growth. The evidence of the high amount of enolase may be affected from the optimal medium with high C-source

and N-source concentration accelerating the enolase production in the late milestone step of glycolysis and the bacterium SUTWR 73-1 simultaneously produced acid that harmful to itself, causing the bacteria stress and leak of protein component from the cell which has been mentioned in the pH optimization section.

Search title : 1
MS data file : 1_B6_01_116.mgf
Database : SwissProt 2019_05 (560292 sequences; 201357942 residues)
Taxonomy : Bacteria (Eubacteria) (334059 sequences)
Timestamp : 19 Jun 2019 at 09:52:55 GMT
Protein hits :

ENO_TOLAT	Enolase OS=Tolumonas auensis (strain DSM 9187 / TA4) OX=595494 GN=eno PE=3 SV=1
EFTU_BUCAI	Elongation factor Tu OS=Buchnera aphidicola subsp. Acyrthosiphon pisum (strain APS) OX=107806 GN=tuf PE=3 SV=2
ENO_VIBPA	Enolase OS=Vibrio parahaemolyticus serotype O3:K6 (strain RIMD 2210633) OX=223926 GN=eno PE=3 SV=1
ENO_SALAR	Enolase OS=Salmonella arizonae (strain ATCC BAA-731 / CDC346-86 / RSK2980) OX=41514 GN=eno PE=3 SV=1
ENO_SACD2	Enolase OS=Saccharophagus degradans (strain 2-40 / ATCC 43961 / DSM 17024) OX=203122 GN=eno PE=3 SV=1
ENO_HAEIE	Enolase OS=Haemophilus influenzae (strain PittEE) OX=374930 GN=eno PE=3 SV=1
EFTU_HAMD5	Elongation factor Tu OS=Hamiltonella defensa subsp. Acyrthosiphon pisum (strain 5AT) OX=572265 GN=tuf PE=3 SV=1
ENO_ACTP7	Enolase OS=Actinobacillus pleuropneumoniae serotype 3 (strain JL03) OX=434271 GN=eno PE=3 SV=1
ENO_ALIFM	Enolase OS=Aliivibrio fischeri (strain MJ11) OX=388396 GN=eno PE=3 SV=1
ENO_PHOPR	Enolase OS=Photobacterium profundum (strain SS9) OX=298386 GN=eno PE=3 SV=1
EFTU1_PHOLL	Elongation factor Tu 1 OS=Photorhabdus luminescens subsp. laumondii (strain DSM 15139 / CIP 105565 / TT01) OX=243265 GN=tuf1
EFTU_RICAH	Elongation factor Tu OS=Rickettsia akari (strain Hartford) OX=293614 GN=tuf PE=3 SV=1
ENO_HAEPS	Enolase OS=Haemophilus parasuis serovar 5 (strain SH0165) OX=557723 GN=eno PE=3 SV=1
CH60_TOLAT	60 kDa chaperonin OS=Tolumonas auensis (strain DSM 9187 / TA4) OX=595494 GN=groL PE=3 SV=1
CH60_AERHH	60 kDa chaperonin OS=Aeromonas hydrophila subsp. hydrophila (strain ATCC 7966 / DSM 30187 / JCM 1027 / KCTC 2358 / NCIMB 924
EFTU1_YERE8	Elongation factor Tu 1 OS=Yersinia enterocolitica serotype O:8 / biotype 1B (strain NCTC 13174 / 8081) OX=393305 GN=tuf1 PE=
CH60_PHOLL	60 kDa chaperonin OS=Photorhabdus luminescens subsp. laumondii (strain DSM 15139 / CIP 105565 / TT01) OX=243265 GN=groL PE=3
CH60_BUCPP	60 kDa chaperonin OS=Buchnera aphidicola subsp. Pterocomma populeum OX=98792 GN=groL PE=3 SV=1
CH60_ERWT9	60 kDa chaperonin OS=Erwinia tasmaniensis (strain DSM 17950 / CIP 109463 / Et1/99) OX=465817 GN=groL PE=3 SV=1
FLAD_VIBCH	Flagellin D OS=Vibrio cholerae serotype O1 (strain ATCC 39315 / El Tor Inaba N16961) OX=243277 GN=flaD PE=1 SV=1
FLAA_VIBPA	Polar flagellin A OS=Vibrio parahaemolyticus serotype O3:K6 (strain RIMD 2210633) OX=223926 GN=flaA PE=3 SV=1
EFTU_BACAA	Elongation factor Tu OS=Bacillus anthracis (strain A0248) OX=592021 GN=tuf PE=3 SV=1
ENO_SHEON	Enolase OS=Shewanella oneidensis (strain MR-1) OX=211586 GN=eno PE=3 SV=2
EFTU_COXBN	Elongation factor Tu OS=Coxiella burnetii (strain Dugway 5J108-111) OX=434922 GN=tuf1 PE=3 SV=1
EFTU_RICRH	Elongation factor Tu OS=Rickettsia rhipicephali OX=33992 GN=tuf PE=3 SV=1
EFTU_LACF3	Elongation factor Tu OS=Lactobacillus fermentum (strain NBRC 3956 / LMG 18251) OX=334390 GN=tuf PE=3 SV=1
RS5_BACAH	30S ribosomal protein S5 OS=Bacillus thuringiensis (strain Al Hakam) OX=412694 GN=rpsE PE=3 SV=1
SAS2_BACCE	Small, acid-soluble spore protein 2 OS=Bacillus cereus OX=1396 GN=sasP-2 PE=3 SV=1
ENO_IDILO	Enolase OS=Idiomarina loihiensis (strain ATCC BAA-735 / DSM 15497 / L2-TR) OX=283942 GN=eno PE=3 SV=1
EFTU_BAUCH	Elongation factor Tu OS=Baumannia cicadellinicola subsp. Homalodisca coagulata OX=374463 GN=tuf PE=3 SV=1
EFTU_PARDP	Elongation factor Tu OS=Paracoccus denitrificans (strain Pd 1222) OX=318586 GN=tuf1 PE=3 SV=1
CYSI_METNO	Sulfite reductase [NADPH] hemoprotein beta-component OS=Methylobacterium nodulans (strain LMG 21967 / CNCM I-2342 / ORS 2060
MNMA_LACP3	tRNA-specific 2-thiouridylase MnmA OS=Lactobacillus paracasei (strain ATCC 334 / BCRC 17002 / CIP 107868 / KCTC 3260 / NRRL
ISPDF_DESMR	Bifunctional enzyme IspD/IspF OS=Desulfovibrio magneticus (strain ATCC 700980 / DSM 13731 / RS-1) OX=573370 GN=ispDF PE=3 SV
G3P1_ECO57	Glyceraldehyde-3-phosphate dehydrogenase A OS=Escherichia coli O157:H7 OX=83334 GN=gapA PE=3 SV=2
G3P1_STRAW	Glyceraldehyde-3-phosphate dehydrogenase 1 OS=Streptomyces avermitilis (strain ATCC 31267 / DSM 46492 / JCM 5070 / NBRC 1489
G3P_GEOSE	Glyceraldehyde-3-phosphate dehydrogenase OS=Geobacillus stearothermophilus OX=1422 GN=gap PE=1 SV=5
SYT_PROM0	Threonine--tRNA ligase OS=Prochlorococcus marinus (strain MIT 9301) OX=167546 GN=thrS PE=3 SV=1
TRUD_CAMJD	tRNA pseudouridine synthase D OS=Campylobacter jejuni subsp. doylei (strain ATCC BAA-1458 / RM4099 / 269.97) OX=360109 GN=tr
Y4WC_SINFN	Uncharacterized HTH-type transcriptional regulator y4wC OS=Sinorhizobium fredii (strain NBRC 101917 / NGR234) OX=394 GN=NGR_
RPOB_PEDPA	DNA-directed RNA polymerase subunit beta OS=Pediococcus pentosaceus (strain ATCC 25745 / CCUG 21536 / LMG 10740 / 183-1w) OX
MALE_ECOL1	Maltose/maltodextrin-binding periplasmic protein OS=Escherichia coli (strain K12) OX=83333 GN=malE PE=1 SV=1
PFLB_ECOLI	Formate acetyltransferase 1 OS=Escherichia coli (strain K12) OX=83333 GN=pflB PE=1 SV=2
TOLB_AERHH	Tol-Pal system protein TolB OS=Aeromonas hydrophila subsp. hydrophila (strain ATCC 7966 / DSM 30187 / JCM 1027 / KCTC 2358 /
LAMB2_YERP3	Maltoporin 2 OS=Yersinia pseudotuberculosis serotype O:1b (strain IP 31758) OX=349747 GN=lamB2 PE=3 SV=1
RUVA_ALIFM	Holliday junction ATP-dependent DNA helicase RuvA OS=Aliivibrio fischeri (strain MJ11) OX=388396 GN=ruvA PE=3 SV=1
RUVA_PSEHT	Holliday junction ATP-dependent DNA helicase RuvA OS=Pseudoalteromonas haloplanktis (strain TAC 125) OX=326442 GN=ruvA PE=3

Figure 4.35 The result of protein fingerprint matching of the SUTWR 73-1 protein and the proteins from SwissProt database.

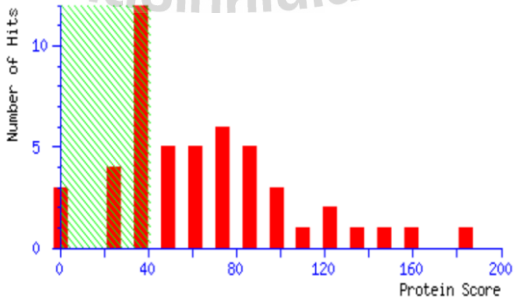


Figure 4.36 Mascot score histogram of searching identity protein of SUTWR 73-1 protein and the proteins from the database.

1. ENO_TOLAT **Mass:** 45582 **Score:** 184 **Matches:** 8(5) **Sequences:** 6(4) **emPAI:** 0.36
Enolase OS=Tolumonas auensis (strain DSM 9187 / TA4) OX=595494 GN=eno PE=3 SV=1
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
467	595.3050	1188.5954	1188.5887	0.0067	0	74	2.9e-05	1		K.DAGYTAVISHR.S
708	781.9370	1561.8594	1561.8352	0.0243	0	37	0.13	1	U	K.IQIVGDDLFVTNTK.I
739	830.9630	1659.9114	1659.8944	0.0171	0	62	0.00036	1		K.SNFGANAILAVSLANAK.A
740	554.3270	1659.9592	1659.8944	0.0648	0	(25)	1.9	1		K.SNFGANAILAVSLANAK.A
762	855.3970	1708.7794	1708.8057	-0.0262	0	59	0.00071	1		K.GYNTAVGDEGGFAPNLK.S
763	570.6370	1708.8892	1708.8057	0.0835	0	(56)	0.0015	1		K.GYNTAVGDEGGFAPNLK.S
871	679.3040	2034.8902	2034.8550	0.0351	0	15	16	1	U	K.DVTLAMDCAASEFYDAEK.K
896	706.7250	2117.1532	2117.0488	0.1044	0	44	0.021	1		R.SGETEDATIADLAVGTAAGQIK.T

Figure 4.37 Comparison of protein fingerprint homology of 56-kD protein of SUTWR 73-1 to the enolase of *Tolumonas auensis* in SwissProt database using monoisotopic mass of neutral peptide.

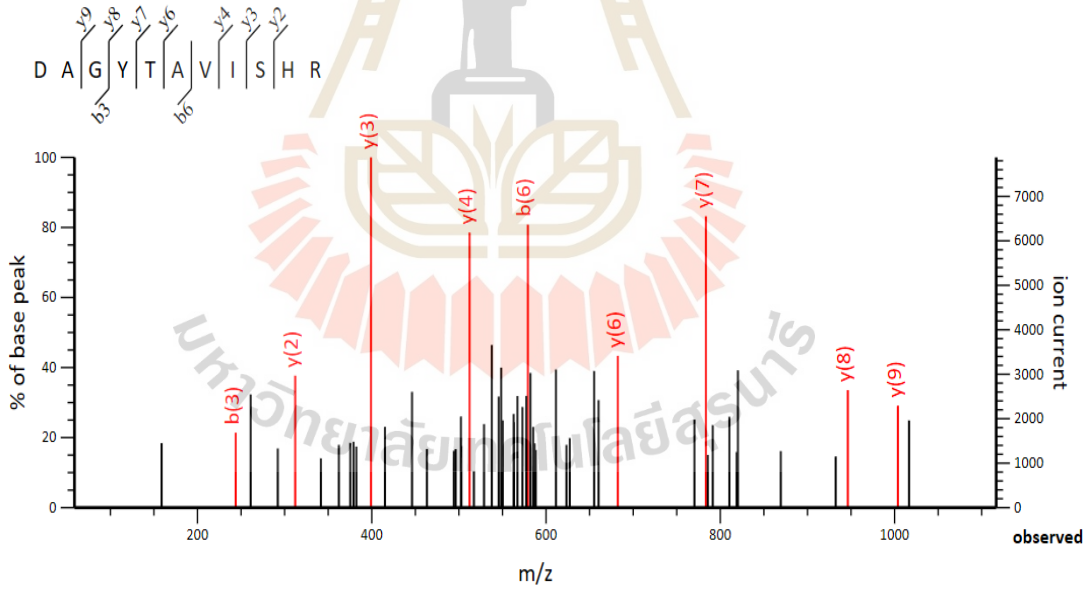


Figure 4.38 Comparison of protein fingerprint homology of 56-kD protein of SUTWR 73-1 to the enolase of *Tolumonas auensis* in SwissProt database using monoisotopic mass of neutral peptide.

4.7.2 Kinetic parameters

The Kinetic parameters of the amylase produced by SUTWR 73-1 which were determined in this section were K_m and V_{max}, when K_m was a Michaelis constant defined as the substrate concentration at 1/2 the maximum velocity, and V_{max} was the maximum velocity. Two kinetic parameters were calculated from the second-order of Michaelis-Menten equation or the inversion of the MM equation given from the Lineweaver-Burk plot. For the investigation, the amount of the enzyme was kept constant and the substrate concentration was varied from 0 to 0.5 mg/ml, then chose the part that was the representative of the enzyme kinetic before reaching the steady-state for calculating the kinetic parameters.

The K_m and V_{max} were calculated using the linear equation as follows:

$$\frac{1}{V} = \frac{K_m}{V_{max}} \cdot \frac{1}{S} + \frac{1}{V_{max}}$$

The slope of the graph is the term of $\frac{K_m}{V_{max}}$ while Y intercept was $\frac{1}{V_{max}}$. For the x-intercept was considered as $\frac{-1}{K_m}$. From the Y-intercept equaled to $\frac{1}{V_{max}}$, then the V_{max} was equal to $\frac{1}{Y-intercept}$ which was 1.67×10^4 µM/min. The K_m could be calculated in two ways, both from graph by continually drawn line intercept with x-axis and brought to calculate in equation x-intercept equaled to $\frac{-1}{K_m}$, or calculate directly by insert the value of the known V_{max} in the slope equaled to $\frac{K_m}{V_{max}}$. Here, the K_m was calculated in the 2nd way, which gave theK_m equaled to 0.167 mg/ml. Therefore, the K_m and V_{max} of the amylase produced by SUTWR 73-1 were 0.167 mg/ml and 1.67×10^4 µM/min, respectively.

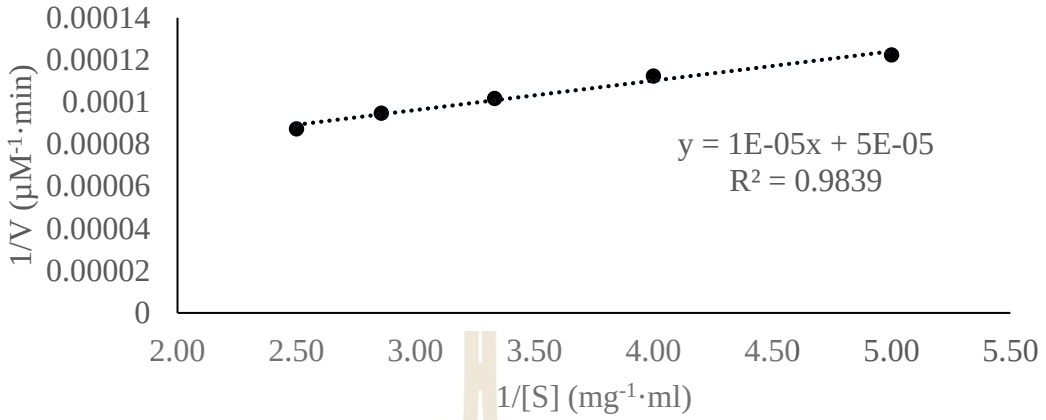


Figure 4.39 Lineweaver-Burk plot for the Michaelis-Menten constant (K_m) and the maximum activity (V_{max}) with soluble starch as substrate.

Table 4.27 Kinetic parameter at different concentration of substrate (soluble starch).

Soluble starch concentration (mg/ml)	Amylase activity (U/ml)	V (μM/min)	1/V ($\mu M^{-1}\cdot min$)	1/[S] ($mg^{-1}\cdot ml$)
0.20	8.131±0.160	8169.093	1.22×10^{-4}	5.000
0.25	8.897±0.458	8897.296	1.12×10^{-4}	4.000
0.30	9.823±0.290	9822.720	1.02×10^{-4}	3.333
0.35	10.559±0.093	10558.51	9.47×10^{-5}	2.857
0.40	11.446±0.322	11446.00	8.74×10^{-5}	2.500

Note: K_m defined as the concentration of substrate at half of maximum velocity called Michaelis-Menten constant.

V defined as the velocity of the enzyme reaction toward substrate

[S] defined as substrate concentration

4.7.3 Amylase profile

Temperature and pH were mostly considered as the main effects which directly interact with the action of the enzyme. These might cause structural change and the improper fold of the enzyme leading the action on the substrate dropped from normal activity. However, in some cases, the extreme condition would give the surprising results that enhance the performance of the enzyme in the substrate to product conversion.

4.7.3.1 pH

The amylase obtained from *Lactobacillus* sp. SUTWR 73-1 was partially purified by ammonium sulphate precipitation previously. The enzyme displayed a good activity at alkaline pH levels, with an optimum activity at pH 6.8 (100%) (Figure 4.40). The enzyme could effectively perform at a wide range, ranging from pH 6 to pH 9 and fall at pH 11 and 12 with 35% remaining activity. Similarly, the optimal pH of amylase produced by lactobacilli was around neutral. For instance, the amylase derived from *Lactobacillus fermentum* 04BBA19 (Fossi and Tavea, 2013) was active at a wide range of pH ranging from pH 4 to 6 and the optimal was obtained at pH 6. Despite the optimal pH was closed to strain 04BBA19, but the range of activities covered both the alkali and acidic range which commonly found in bacilli such as *Bacillus lehensis* G1 expressed in *Escherichia coli* and *Bacillus subtilis* N8 which were highly active at wide pH range (Manas et al., 2014; Arabaci and Arikan, 2018). The widely active enzyme could be described in 3 aspects. The enzyme consisted of both acidic and basic side chain amino acid meaning that these side chain could be deprotonated and protonated. When the amino acid side chains were changed, the high order of protein structure was affected making them either greatly binding or

dramatically losing its activity. For the specific situation, the changes may occur at the critical site like the binding site or active site, changing properties of the amino acid at these areas resulted in a huge change of activity without 3D-structural change. In the other hand, the enzyme would not be affected by pH but the substrate did the making of the changes of substrate binding.

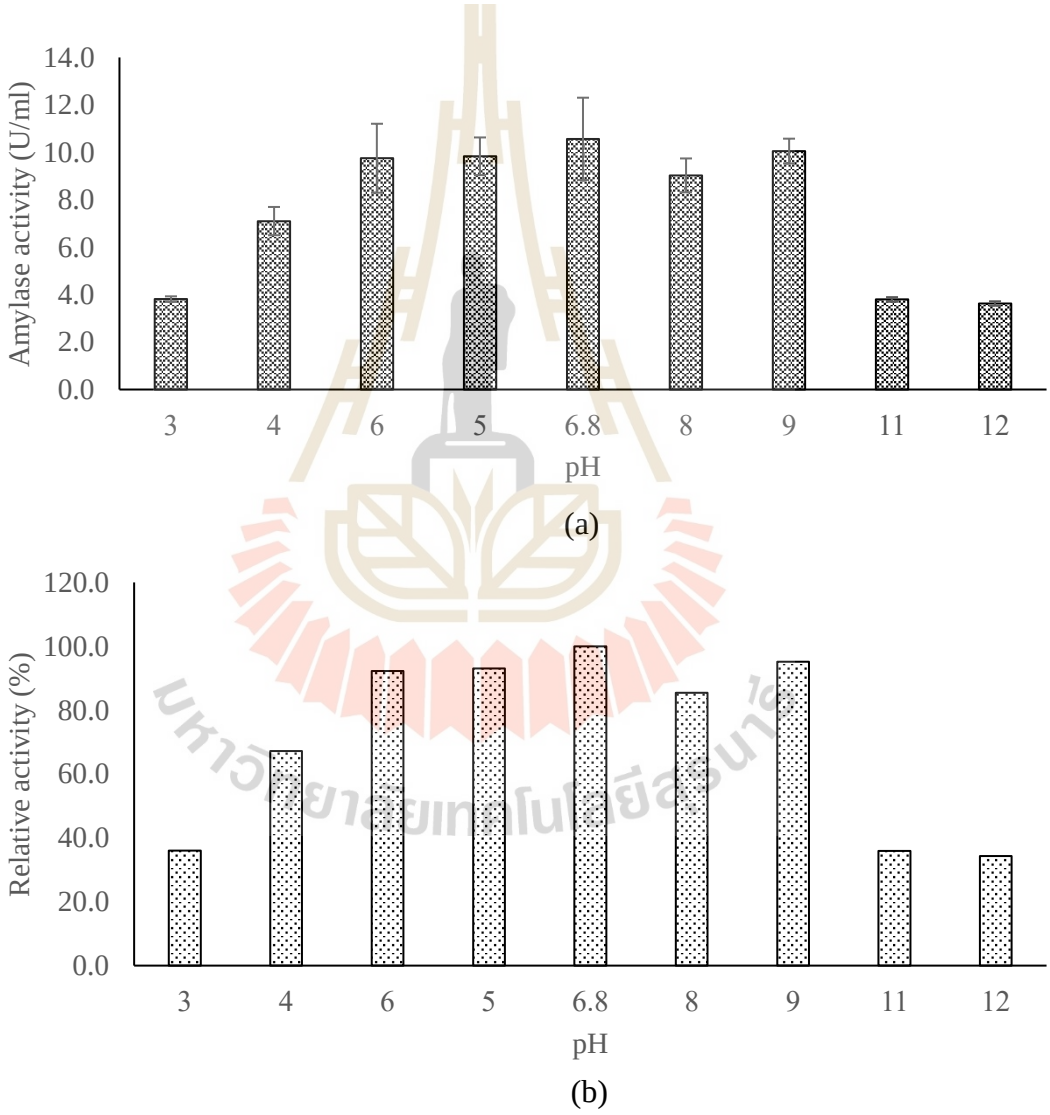


Figure 4.40 pH profile showing the activity and relative activity of amylase produced by SUTWR 73-1 (a and b, respectively).

4.7.3.2 Temperature

The amylase from SUTWR 73 had an average activity of 74% at the temperature range 25-50℃ and the optimal temperature at 35℃ (Figure 4.42). The activity decreased dramatically upper than 50℃ with the retained activity of 42% and only 31.79% at 90℃. This situation could be explained simply as several studies mentioned that the temperature increases the kinetic energy leading the enzyme was activated, at one point the higher temperature may not support anymore, hence the enzyme gradually loosed of its activity. Despite this reason, the effect of temperature could be explained in the details in the ratio of the active and inactive enzyme in the environment, meaning that the temperature did not degrade all enzymes, some of them were still active. Thus, the change in the ratio of two forms at each temperature changed also caused different on the amylase activity measurement. At high temperature, the enzyme partially loses its activity as shown in Figure 4.41. The enzyme could perform at high temperature by retaining the 30% of relative activity (Daniel and Danson, 2013).

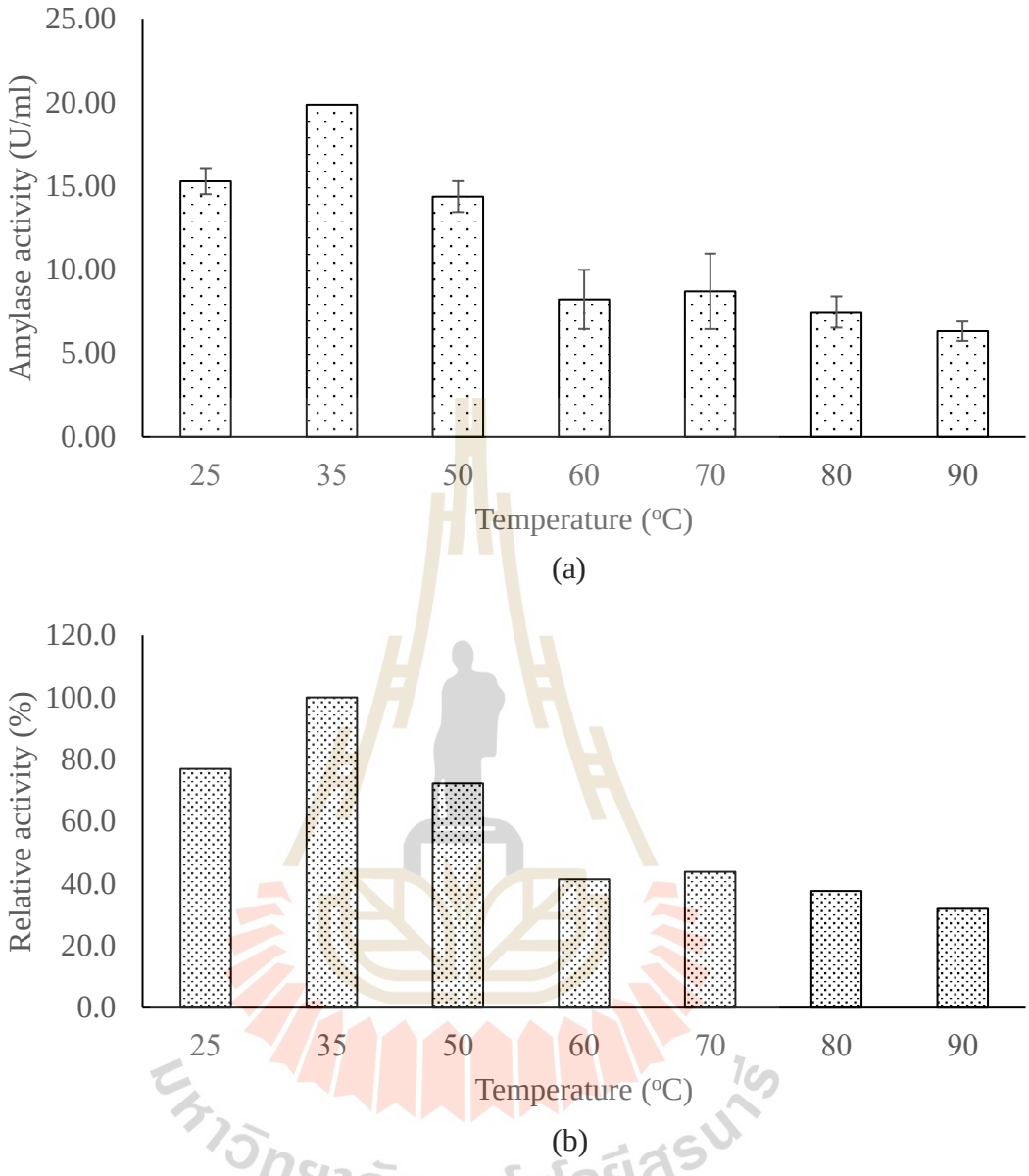


Figure 4.41 Temperature profile showing the activity and relative activity of amylase produced by SUTWR 73-1 (a and b, respectively).

4.7.4 Amylase stability

Despite the profile of SUTWR 73-1 amylase was studied but the amylase was still needed to show its potential in terms of stability. To find the enzyme stability, the enzyme was incubated at different conditions and detected for its activity.

4.7.4.1 pH

It was found that the amylase from SUTWR 73-1 tended to decrease over incubation time for 18 h at different pH especially dramatically decreased at pH 5 that the incubation passed only 30 min by retaining activity around 83% (Figure 4.42). Moreover, the activity gradually decreased while the pH was decreased, the stability at pH 8 and 9 possessed activity around 138 and 133%. Hence the enzyme preferred alkali environment meaning that it could be defined as alkali stable amylase similar to the amylase produced by *Bacillus subtilis* N8 (Abaci and Arikan, 2018), *Bacillus lehensis* G1 (Manas et al., 2014), and *Bacillus circulans* (Joshi, 2011).

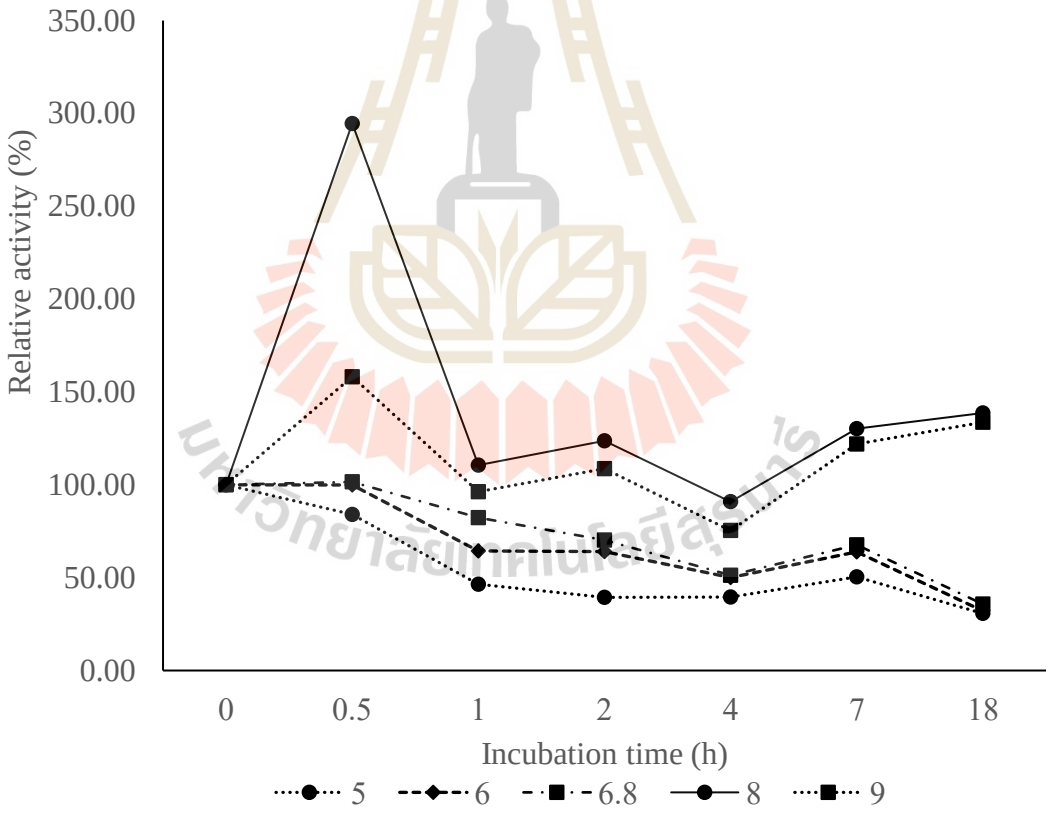


Figure 4.42 pH stability showing the activity of the amylase produced by SUTWR 73-1 at different incubation periods.

4.7.4.2 Temperature

From the temperature profile for amylase, the activity was increased after incubating enzyme for 50 min at 25℃ meaning that the amylase could be used at low temperature which would adapt for future uses. The enzyme showed good stability at 35℃. In the case of high temperatures, between 50 to 90℃ the activity remained at 3 to 16%. The highest retained activity after pre-incubating for 90 min was found at 25℃. Similar results were also reported by these outcomes to confirm that the enzyme has catalytic activity at low temperatures and is a cold-active enzyme. This cold-active amylase has been reported by the effect of the constituents of amylase with a few salt bridges, aromatic interactions, small hydrophobic cluster (including alanine, valine, isoleucine, leucine, phenylalanine, proline, and methionine), few arginine residues and weak stabilization of helix dipoles from the study of *Alteromonas haloplanctis* A23 amylase (Feller et al., 1992; Aghajari et al., 1998). After the pre-incubation period, the enzyme showed 7.25 ± 0.39 and 7.72 ± 0.38 U/ml for activity at temperatures between 25 and 35°C for 60 min, and the 56% of retained activity was measured at 40℃ (Figure 4.43).

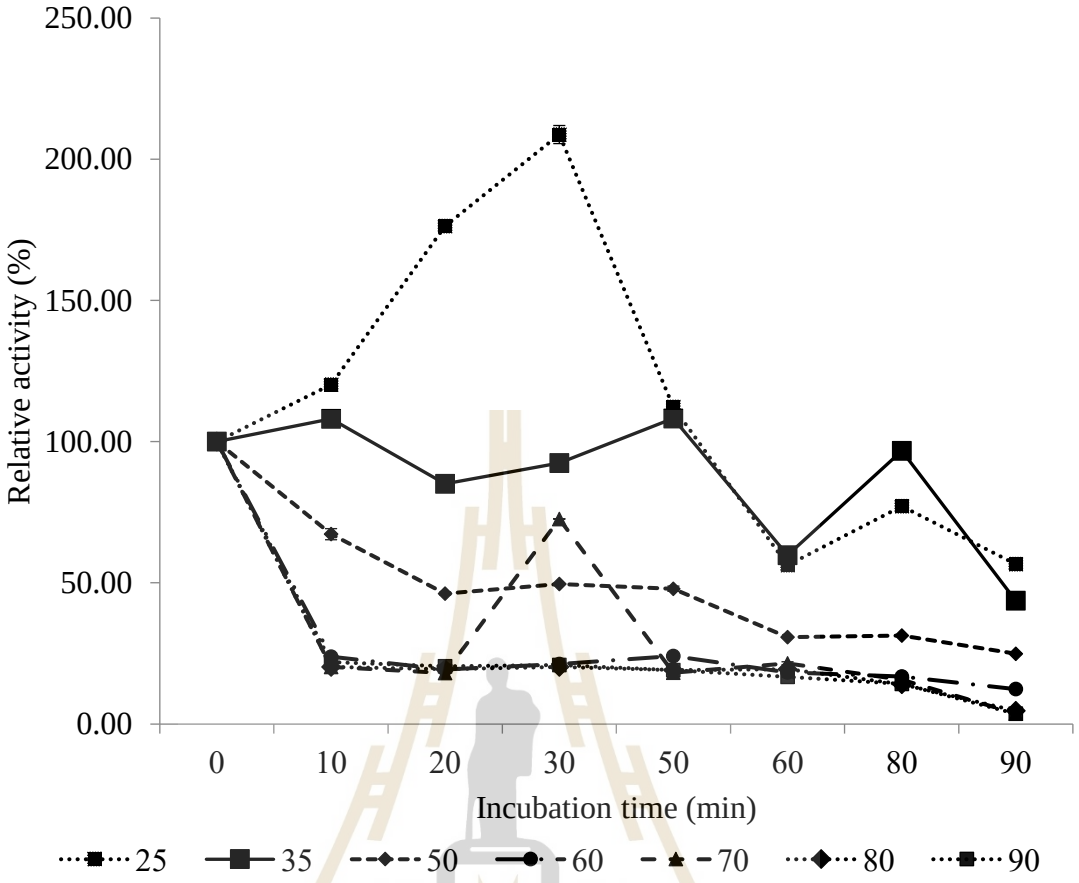


Figure 4.43 Temperature stability showing the activity of amylase produced by SUTWR 73-1 at different incubation periods.

4.7.5 Effect of ions on amylase activity

It was needed to know the effect of ions on amylase activities to manage the enzyme application. The amylase produced by SUTWR 73-1 was highly enhanced by Ba^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, and Cl^{-} while was strongly inhibited by EDTA which was a chelating agent that entrapped the ion from the amylase structure. The amylase was also mildly inhibited by SO_4^{2-}, and K^{+}. Surprisingly it was found that Ca^{2+} inhibited the amylase activity which contrasts to several reports claimed that the amylase was the calcium-dependent enzyme requiring it in substrate enzyme interaction. From Figure

4.45 the activity was mainly based on the metal ion while the negative ion was considered as almost no-effect, however, the negative ion still supported the amylase activity like Cl^-. The chloride ion was reported to act as an activator for amylase enzyme due to its allosterically binding near the center of the active site. The binding of Cl^- helped to polarize hydrolytic water molecule and enhance the rate of 2^{nd} step catalytic reaction as stated by Aghajari et al. (2002). The results clearly showed that the amylase produced by SUTWR 73-1 was a metalloenzyme which requires the metal ions to perform its activity. From the ion effect section, various ions enhanced the amylase activity, implying that the medium could be further modified at high cassava concentration by improving the use of carbon source, metal ion acted as activator would be added into the modified medium. Results from this study revealed that 10 mM of $BaCl_2$, $MnCl_2$, $CaCl_2$, $MnSO_4$, $FeSO_4$, and $MgSO_4$ provided the highest activity which was around 2-3 times better than the absence of ions, so the addition of these salts in the medium would increase the rate of starch utilization at an early stage of cultivation. The high rate of starch hydrolysis allowed the microorganisms accessed into the substrate making the growth at lag phase shorten and might support high amylase production quicker than was found in the former medium. Ions in the modified medium consisted of $MnSO_4$, $FeSO_4$, and $MgSO_4$ at the concentrations of 0.53, 0.134, and 2.31 mM, respectively. The new medium would contain $MnSO_4$, $FeSO_4$, and $MgSO_4$ around 10 mM by weighing the chemicals 2.23, 2.24, and 2.46 g/l. The addition of $BaCl_2$ would be necessary. All of the additional ions were not only for growing but for supporting the amylase activity. A higher rate of starch hydrolysis occurred causing the higher rate of sugars consumption allowing the high amount of amylase produced. These strategies could be applied for lactic acid production to yield high amounts of acid too.

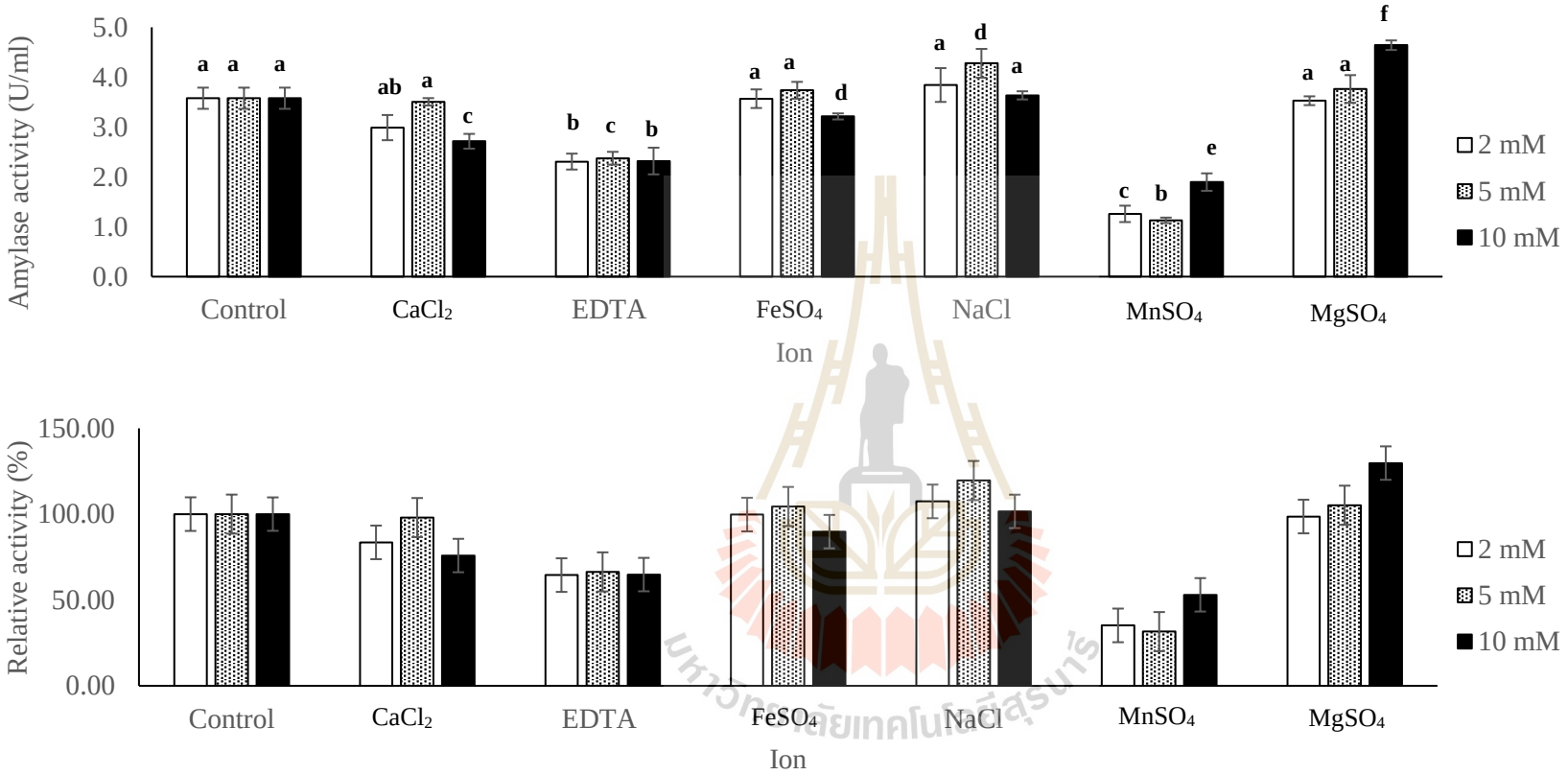


Figure 4.44 Amylase activity produced by SUTWR 73-1 in the presence of different ions, compared to the absence of additional ions (control). Means with different letters (a, b, c, d, e, and f) showed significantly different means (Duncan's Multiple Range Test (DMRT), $p<0.05$).

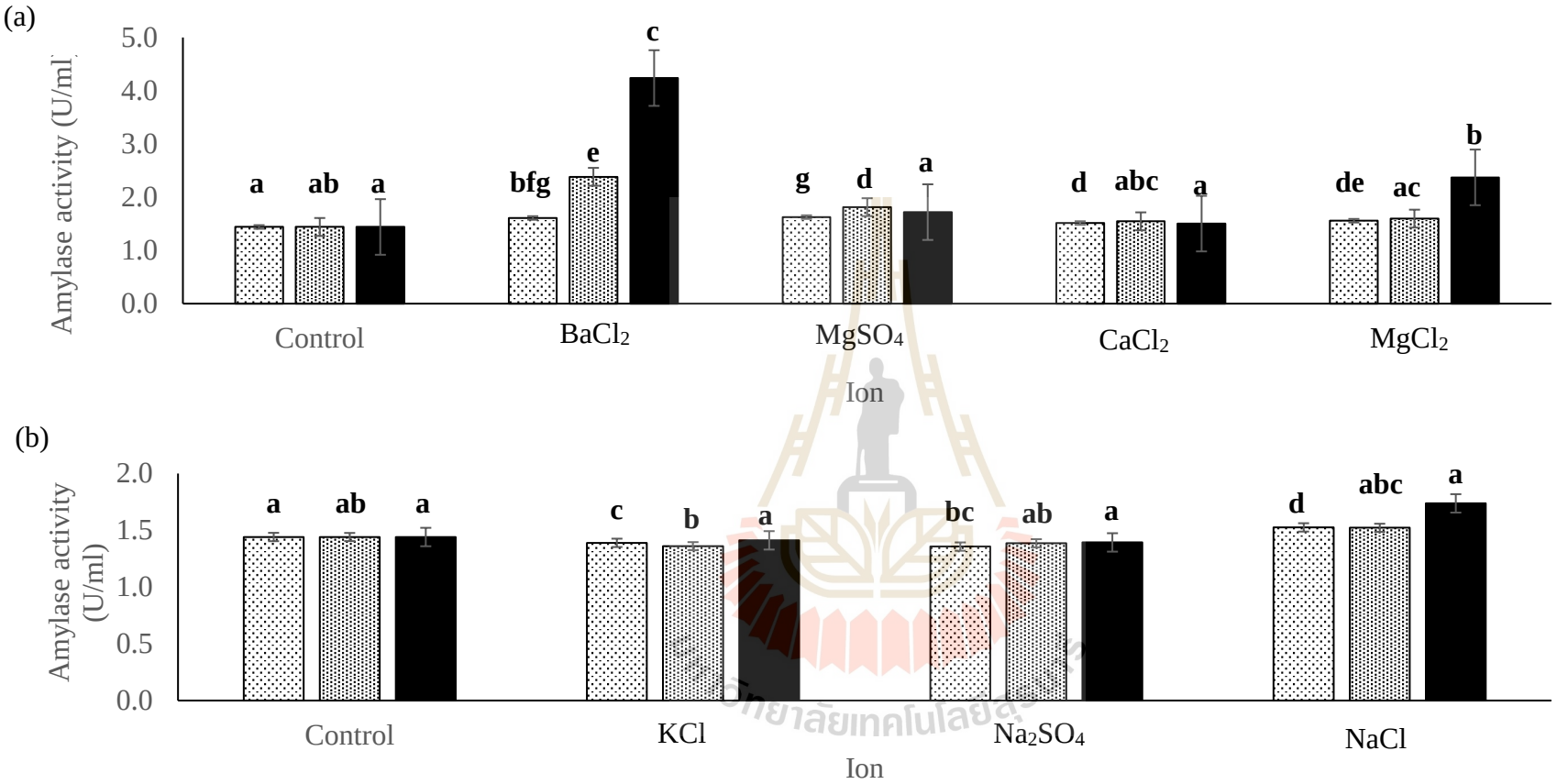


Figure 4.45 Amylase activity produced by SUTWR 73-1 in the presence of different ions, shown in amylase activity (a and b, respectively) compared to the absence of ions (control). Means with different letters (a, b, c, d, e, f, and g) showed significantly different means (Duncan's Multiple Range Test (DMRT), $p<0.05$).

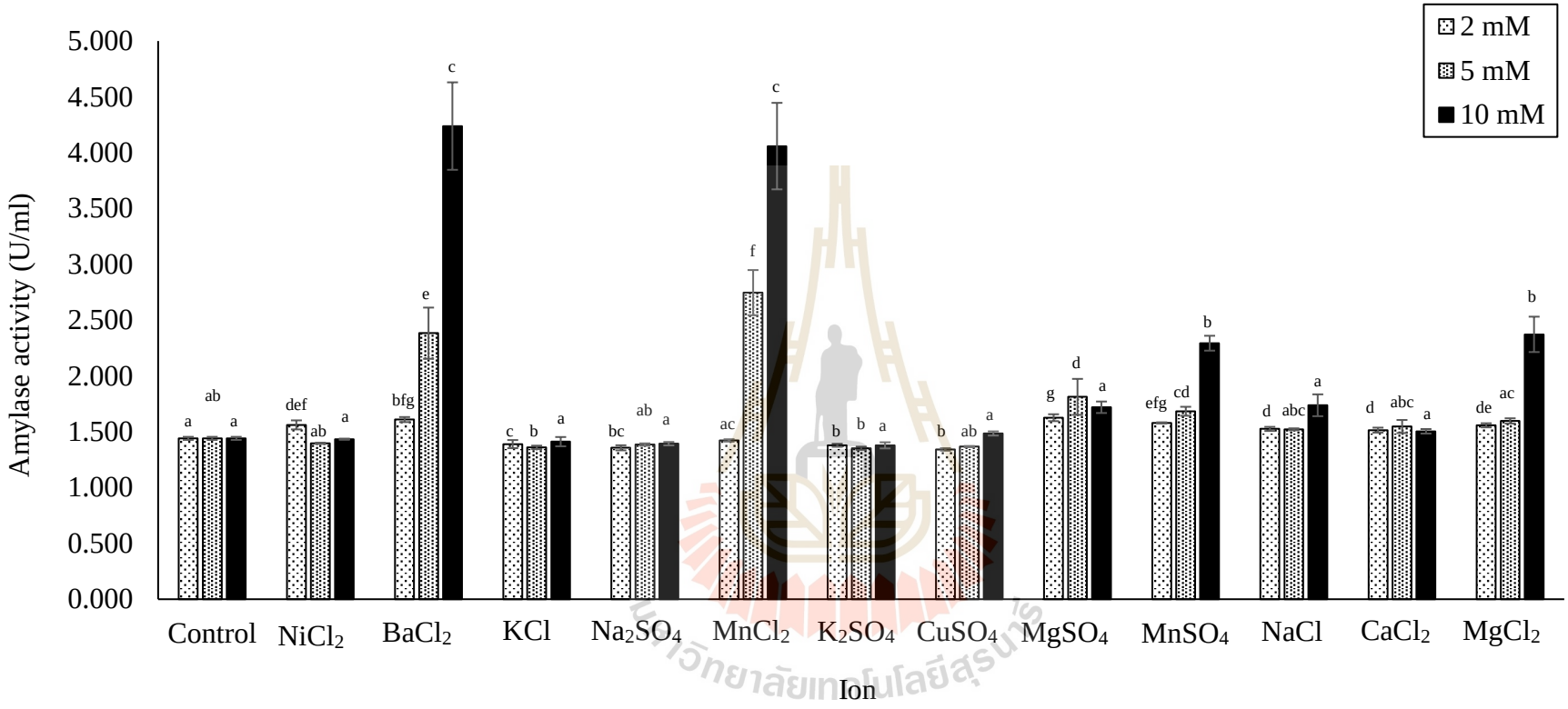


Figure 4.46 Amylase activity produced by SUTWR 73-1 in the presence of different ions compared to the absence of additional ions (control). Means with different letters (a, b, c, d, e, f, and g) showed significantly different means (Duncan's Multiple Range Test (DMRT), $p<0.05$).

CHAPTER V

CONCLUSION

This research project aimed to investigate the amylase enzyme produced by a specific strain of D-lactic acid-producing bacteria, *Lactobacillus* sp. SUTWR 73, to replace the high-cost commercial enzyme currently used in our laboratory, applied in larger laboratory and industrial-scale settings. Hence, the optimization of medium compositions for amylase production by SUTWR 73, to obtain the maximum enzyme production was needed. The enzyme had been then partially purified and characterized in this study. The selected bacteria *Lactobacillus* sp. SUTWR 73 were tested for their amylase production on RAM agar and broth containing 1%(w/v) cassava starch as a carbon source. It was found that *Lactobacillus* sp. SUTWR 73 could utilize cassava starch as a carbon source by showing the clear zone on cassava starch agar and the amylase activity in the starch cultured RAM at 8.02±2.13 U/ml for the activity after cultivating for 24 h. The maximum growth was obtained at 16 h with 1×10^{10} CFU/ml. However, SUTWR 73 produced the highest amylase at 22 h 9.00±1.47 U/ml with a growth of 1.79×10^{9} CFU/ml. In the supernatant, the highest amylase activity was obtained at an 8-h incubation time of 4.23±1.17 U/ml, with 4.44×10^{8} CFU/ml and 0.15 g/l for growth and protein concentration. The highest amylase activity of 4.27±0.06 U/50 ml with 1.48×10^{10} CFU/ml, was detected from the cell pellet. The results suggested that the amylase produced by SUTWR 73 was detected both extracellular

and cell-associated enzymes which rarely found in lacttobacilli. Preliminary optimization, the maximum protein of 0.196±0.024 mg/ml was obtained from RAM medium containing 2.0% which was 32.67 times higher than the amylase activity of the cell by giving 6.82 U/ml. Three agricultural wastes were tested for their potential in amylase production from SUTWR 73. The three modified media (Rice bran, soy protein, and urea plus yeast extract) were inoculated with SUTWR 73, the results showed that RAM medium exhibited the highest amylase activity (8.06±0.04 U/ml), followed by rice bran (7.54±0.02 U/ml), soy protein (6.73±0.08 U/ml), urea (7.11±0.01 U/ml), and urea plus yeast extract (4.46±0.07 U/ml). The highest amount of total protein (0.41±0.02 mg/ml) was obtained from soy protein followed by rice bran (0.05±0.01 mg/ml), RAM (0.03±0.01 mg/ml), and urea plus yeast medium (0.02±0.01 mg/ml). RAM was still the best cultivation medium by providing the total activity of about 8.06±0.42 U/ml. However, tryptone and yeast extract in RAM, was expensive (4.17 Baht/g and 3.96 Baht/g, respectively). The second-choice medium was urea plus yeast extract (7.54±0.02 U/ml). Hence, the rice bran medium was selected for determining the substrate concentration. The rice bran concentration at 0.4%(w/v) in the range of 0.4 to 1.6%(w/v) exhibited the highest amylase activity of 6.99±0.50 U/ml. The modified medium-cost was lower than the original medium (RAM) 84%. While, the rice bran was 380 times cheaper than yeast extract and tryptone. At the bioreactor cultivation, the modified medium was used for amylase production using a 7.5-l bioreactor with 3 l of working volume, incubated at 35°C, agitation speed at 150 rpm without aeration for 28 h. The amylase activity increased to be the maximum activity (6.04 U/ml) at 14 h cultivation with the highest amount of protein (0.18 mg/ml). Ammonium sulphate was used to precipitate protein. Ammonium sulphate (70%) was

suitable for partial purification because of the highest specific activity (494.91 U/mg) with 83.34% for recovery compared to 80 and 90%, even there was a loss of some amylase than at 90% saturation which had 90% recovery of amylase but incorporated with many protein wastes. The precipitated amylase was tested for its characteristics. Interestingly, in the presence of 10 mM Ca^{2+}, the amylase activity was 3.35±0.28 U/ml which was not different from the absence of Ca^{2+} (3.40±0.30 U/ml). The enzyme showed the highest enzyme activity at 60℃ (9.54±1.21 U/ml).

Due to its fastidious characteristics, the strain improvement was done through UV exposure for repeating 8 times to obtain the stable mutant. Three mutants; WR 73-1, WR 73-2, and WR 73-3 were selected due to they performed the highest amylase activity and good growth. The strain, SUTWR 73-1 exhibited the high amylase activity among four selected strains with 8.79±1.33 U/ml while producing the lower protein concentration, 0.420±0.061 mg/ml meaning that SUTWR 73-1 may secrete the extracellular amylase with the low amount based on the protein concentration compared with the original strain, SUTWR 73. The amylase production medium was then developed using factorial experiment to roughly screen the medium composition which suitable for amylase production, and response surface method (RSM) to identify the key composition affected to amylase production from SUTWR 73-1. The highest amylase obtained from the RAM medium containing 10%(w/v) of cassava starch by giving amylase activity around 30.43±3.47 U/ml with 4.32×10^{7} CFU/ml. The bacterium was cultivated in 7 medium formulas, the highest amylase activity was observed from formula no.2, 43.64±8.50 U/ml with 0.20±0.03 mg/ml for protein concentration, which had been eliminated yeast extract, and replaced by defatted rice bran with the same amount of total nitrogen content. Further investigation showed that

the medium without rice bran showed the lowest amylase activity (15.26±1.78 U/ml) compared to the other, and also gave the lowest protein concentration with 0.018±0.01 mg/ml. This represented the low impact of yeast extract to the bacterial amylase production. As found in the yeast extract, defatted rice bran did not affect amylase production. The amylase production depended on tryptone as a critical factor. Tryptone concentration was varied from 0.05 to 10%(w/v). Tryptone at the concentration of 4.5%(w/v) provided the highest amylase activity, 35.47±1.79 U/ml. For the bacterial growth comparison, the higher tryptone concentration was added, the lower growth of SUTWR 73-1 was observed. It was the evidence supporting the situation of metabolic drove by inducing the usage of the substrate to produce the metabolite, especially acid production. Four points of pH were chosen including pH 7, 8, 9, and 10. The results showed that the highest amylase activity was 18.59±1.77 U/ml at pH 8.These three values obtained from a factorial experiment which was 10%(w/v) of cassava starch, 4.5%(w/v) of tryptone, and pH 8, for cassava starch concentration, tryptone concentration, and pH, respectively, were used to optimize the medium composition by using response surface method (RSM). The results showed that the most fitted model was quadratic which gave the lowest p-value (below than 0.0001). The lack of fit p-value was 0.3634. Therefore, the quadratic type was selected due to the adjusted and predicted R^2 was highest than the others, which were 0.9702 and 0.9204, respectively. The coefficients showed that the pH 4.34 had the largest impact on amylase production among three factors followed by cassava starch, and tryptone concentration which had 2.77 and 1.32, respectively. The model equation was amylase activity equaled to $31.96+2.77(A)+1.32(B)+4.34(C)-0.3359(AB)+0.6971(AC)-0.5956(BC)-4.28(A^2)-5.60(B^2)-4.48(C^2)$. Finally, the model was validated at its optimum point in

real use by keeping two-sided confidence of 95%. And conducting in 3 conditions. The amylase activities of three conditions were obtained from the experiment by having 34.52±6.51, 31.10±4.32, and 31.40±5.83 U/ml for the 1st, 2nd, and 3rd conditions. It was found that the amylase activity from cultivations was nearly the predicted values. After achieving the optimal condition and the reliable equation, the model was used to perform the experiment by conducting in the 7.5-l bioreactor with a working volume of 3 l at 5%(w/v) of cassava starch concentration with agitation speed of 150 rpm for 48 h. The highest activity and the protein concentration obtained at 18 h with the activity of 17.00 U/ml and 217.79 µg/l, respectively. The amylase was a growth-dependent enzyme. The amylase from SUTWR 73-1 cultivation was partially purified using ammonium sulphate. At 70% ammonium sulphate saturation the specific activity was 17.34 U/ml. In comparison to crude, the 70% saturation could reduce the undesirable protein from 6.136 mg/ml to 0.593 mg/ml and kept the amylase 56.71% of the initial crude. The purification fold of ammonium sulphate precipitation at 70% showed 5.9 folds greater than crude amylase. Partially purified amylase of SUTWR 73-1 was characterized using native gel electrophoresis combined with the additional step of staining using soluble starch as the amylase substrate. The molecular weight of the SUTWR 73-1 amylase was 46.04 kD. The kinetic values toward the soluble starch as substrate including K_m and V_{max}, were 0.167 mg/ml and 1.67×10^4 µM/min, respectively. The optimum activity was at pH 6.8 (100%). The enzyme could effectively perform at a wide range, ranging from pH 6 to pH 9 and fall at pH 11 and 12 with 35% remaining activity. The amylase from SUTWR 73 had the average activity of 74% at a temperature range 25-50℃ and the optimal temperature at 35℃. The activity decreased dramatically upper than 50°C with the retained activity of 42% and

only 31.79% at 90℃. Stability of the amylase from SUTWR 73-1 tended to decrease over incubation time for 18 h at different pH especially dramatically decreased at pH 5 that the incubation passed only 30 min by retaining activity around 83%. The stability at pH 8 and 9 possessed activity around 138 and 133%. alkali stable amylase. The temperature stability of the activity was increased after incubating enzyme for 50 min at 25℃, and could be used at a low temperature which would be useful for further application. The enzyme showed good stability at 35℃. The highest retained activity after pre-incubating for 90 min was found at 25℃. The enzyme had catalytic activity at a low temperature and is a cold-active enzyme. After the pre-incubation period, the enzyme showed 7.25±0.39 and 7.72±0.38 U/ml for activity at a temperature between 25 and 35℃ for 60 min, and the 56% of retained activity was measured at 40℃. It was needed to know the effect of ions on amylase activities to manage the enzyme application. The amylase was highly enhanced by Ba^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, and Cl^{-} while was strongly inhibited by EDTA. The amylase was also mildly inhibited by SO_4^{2-}, and K^{+}. Surprisingly it was found that Ca^{2+} inhibited the amylase activity which was the calcium-dependent enzyme. From the ion effect section, various ions enhanced the amylase activity, implying that the medium could be further modified at high cassava concentration by improving the use of carbon source, metal ion acted as activator would be added into the modified medium. Results from this study revealed that 10 mM of $BaCl_2$, $MnCl_2$, $CaCl_2$, $MnSO_4$, $FeSO_4$, and $MgSO_4$ provided the highest activity which was around 2-3 times better than the absence of ions.

The modified medium consisted of cassava starch, tryptone, tri-ammonium citrate, di-potassium phosphate, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$, and $FeSO_4 \cdot 7H_2O$ for 11.70, 4.68, 0.1, 0.6, 0.057, 0.012, and 0.003%(w/v), respectively. The optimized pH

of the medium was 8.87. This medium supported the highest amylase production of SUTWR 73-1, 33.54 U/ml. However, the practical cassava concentration was 5% due to the viscosity of the starch. The optimized medium was then adjusted the cassava starch concentration, and the highest yield of amylase was 17.00±0.44 U/ml obtained at 18 h of cultivation time. The amylase produced by SUTWR 73-1 was characterized into the alkali-stable and cold-active enzyme, because its hydrolytic capability was still active (100% relative activity) in the range of pH 6 to pH 9 for 18 h, while the a low-temperature preincubation at 25ºC for 50 min, enhancing its stability higher than 2.08 folds than the initial activity. The optimum temperature was found at 35℃. The molecular weight of the enzyme was 46.01 kD and its kinetic parameters towards soluble starch were 0.167 mg/ml and 1.67×10^4 μM/min for the kinetic constant and maximum velocity, respectively. Furthermore, the amylase of SUTWR 73-1 was the metalloenzyme, because its activity was activated by a divalent metal ion, especially barium ion. The higher rate of starch hydrolysis occurred causing s higher rate of sugars consumption allowing the high amount of amylase produced. These strategies could be applied for enzyme production as well as lactic acid production using a high concentration of cassava starch to obtain the high lactic acid yield. The hydrolytic activity at a low temperature could decrease the energy consumption at the large-scale operation such as ethanol production that requires the energy to activate the amylase for directly converting the starch into sugars. In bioremediation, the wastewater could be treated using the enzyme. Its alkali-stable may lead to the use of the enzyme in detergent for cold washing. The cold-active and alkali stable amylase would play an important role in a wider range for the future to reduce energy consumption in the processes.

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APPENDICES

APPENDIX A

CULTURE MEDIA AND REAGENTS

1. REAGENTS USED FOR EXPERIMENTS

1.1 Gram's staining for D-Lactic acid bacteria

1.1.1 Crystal violet (Gram stain)

Crystal violet	2.00	g
Ethanol	20.00	g
Mixed thoroughly		
Ammonium oxalate (1% Aqueous solution)	80.00	ml

1.1.2 Safranin

Safranin O (2.5% solution in 95% Ethanol)	10.00	ml
Distilled water	90.00	ml

1.1.3 Iodine (Gram's iodine)

Iodine	1.00	g
Potassium iodide	2.00	g
Added distilled water and brought volume up to	300.00	ml

1.2 Protein determination

1.2.1 Bradford reagent

Coomassie blue	100.00 mg
Ethanol (95%, prepared from dissolve absolute ethanol in DI water)	50.00 ml
Phosphoric acid	100.00 ml

The compositions were suspended in 1,000 ml of distilled water.

1.3 Reducing sugars for amylase activity determination

1.3.1 Dinitrosalicylic reagent

3,5-Dinitrosalicylic acid	10.00 g
NaOH	19.00 g
Potassium sodium tartrate	30.60 g
Phenol	7.60 g
Sodium metabisulfite	8.30 g

The compositions were suspended in 1,416 ml of distilled water.

2. CULTURE MEDIA FOR LACTIC ACID BACTERIUM CULTURING AND AMYLASE PRODUCTION

2.1 Rogosa agar medium with modification (RAM) (Rodtong and Ishizaki, 2003)

Pancreatic digest of casein or tryptone	5.00	g
Potassium hydrogen phosphate	6.00	g
Yeast extract	3.00	g
tri-Ammonium citrate	1.00	g
$MgSO_4.7H_2O$	0.57	g
$MnSO_4.4H_2O$	0.12	g
$FeSO_4.7H_2O$	0.03	g
Cassava starch	10.00	g

Final pH 7.0±0.2 at 25℃

The compositions were suspended in 1,000 ml of distilled water. Bring to the boil until dissolved completely. For solid medium was obtained by adding 15 g/l agar. Sterilization was done by autoclaving for 15 min at 121℃.

2.2 D-Lactic acid production broth medium

Potassium hydrogen phosphate	6.00	g
Yeast extract	15.00	g
tri-Ammonium citrate	1.00	g
$MgSO_4.7H_2O$	0.57	g
$MnSO_4.4H_2O$	0.12	g
$FeSO_4.7H_2O$	0.03	g
Cassava starch	100.00	g
α-Amylase	0.10	g

Final pH 7.0±0.2 at 25℃

The compositions were suspended in 1,000 ml of distilled water. Bring to the boil until dissolved completely. Sterilization was done by autoclaving for 15 min at 121℃.

APPENDIX B

STANDARD CURVES

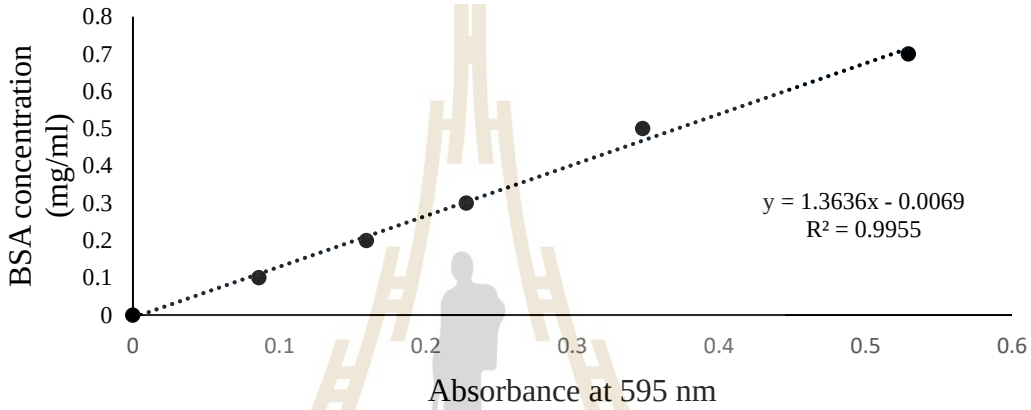

Figure 1B Standard curve of bovine serum albumin concentration using Bradford method (Bradford, 1976).

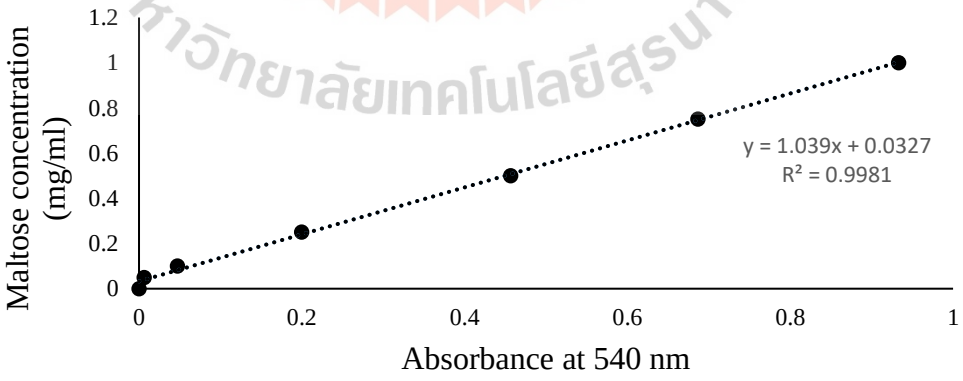

Figure 2B Standard curve of maltose concentration using Bernfeld method (Bernfeld, 1955).

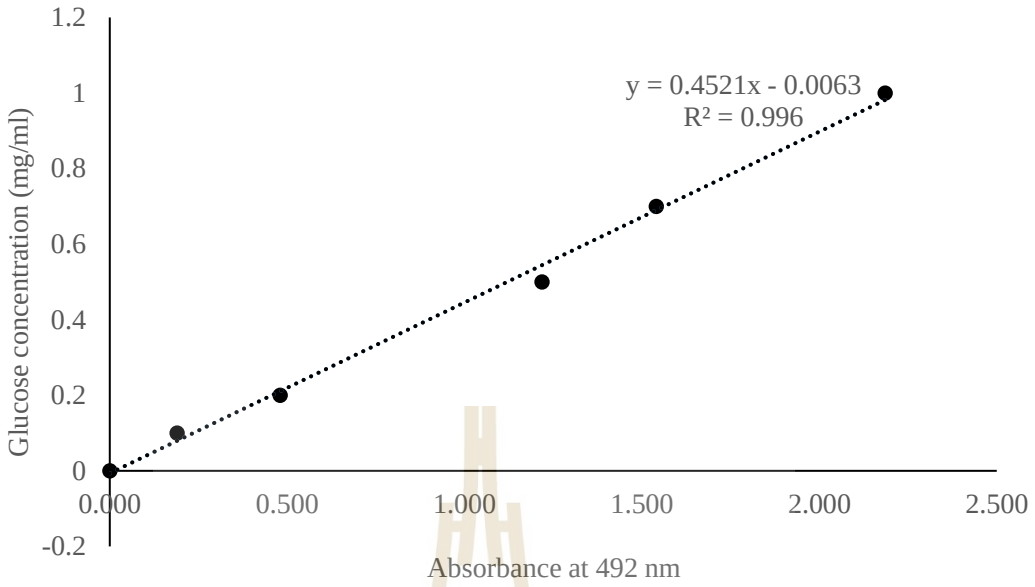

Figure 3B Standard curve of glucose concentration using total sugar method (Dubois et al.,1956).

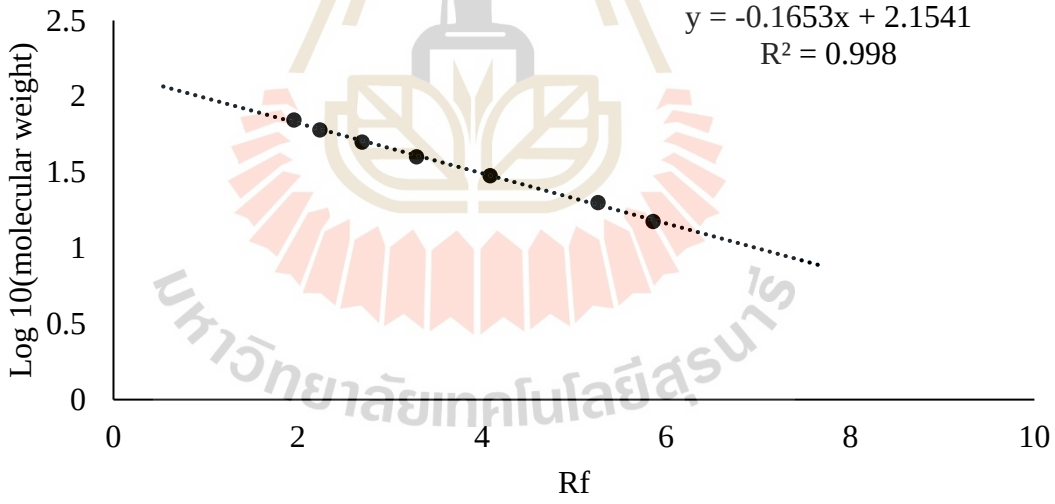

Figure 4B Standard curve of the Log 10(molecular weights) of protein marker vs. the distances for molecular weight estimation.

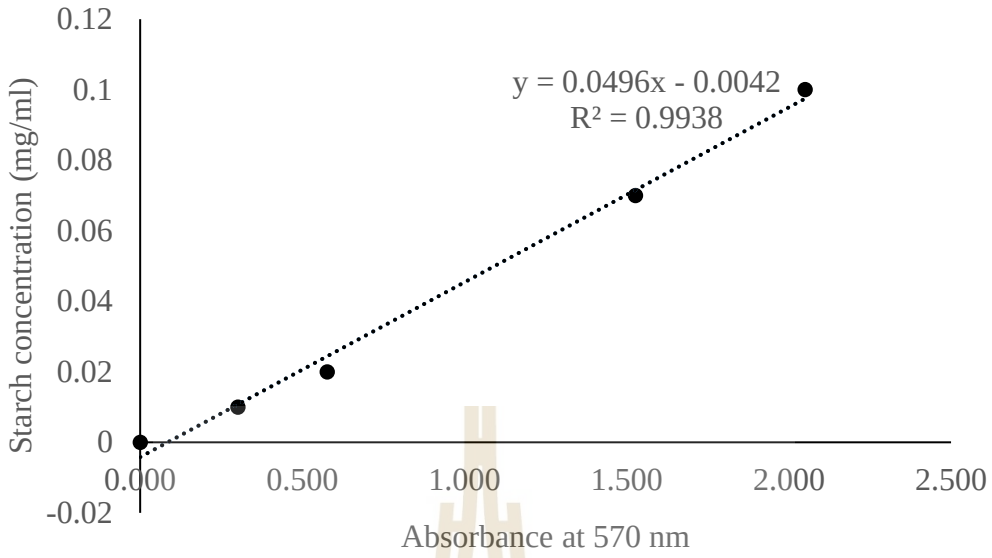

Figure 5B Standard curve of starch concentration using iodine method (Swamy et al., 2015).

APPENDIX C

AMYLASE ACTIVITY FROM 300-L CULTIVATED MEDIUM OF *Lactobacillus* sp. SUTWR 73

Table 1C Total amylase activity at various times of cultivation period from supernatant of each batch cultivation of *Lactobacillus* sp. SUTWR 73.

Bioreactor	Time (h)	Amylase activity (U/ml)	Bioreactor	Time (h)	Amylase activity (U/ml)	Bioreactor	Time (h)	Amylase activity (U/ml)
1	0	1.06±0.08	2	0	1.98±0.03	3	0	1.51±0.01
	4	1.79±0.18		4	4.54±0.06		4	3.94±0.05
	8	3.03±0.04		8	5.35±0.14		8	4.31±0.12
	12	2.62±0.13		12	5.16±0.08		12	3.91±0.12
	16	2.03±0.01		16	4.91±0.12		16	3.31±0.13
	20	2.04±0.12		20	4.53±0.12		20	2.95±0.08
	24	12.00±0.24		24	4.28±0.09		24	2.62±0.05

Table 2C Total amylase activity at various times of cultivation period from cell pellet of *Lactobacillus* sp. SUTWR 73.

Bioreactor	Time (h)	Amylase activity*50 (U/ml)	Bioreactor	Time (h)	Amylase activity (U/50 ml)	Bioreactor	Time (h)	Amylase activity*50 (U/ml)
1	0	0.96±0.04	2	0	1.17±0.00	3	0	0.83±0.03
	4	1.36±0.01		4	4.94±0.17		4	6.31±0.03
	8	2.73±0.09		8	5.76±0.08		8	6.46±0.02
	12	3.02±0.07		12	4.31±0.04		12	6.36±0.02
	16	2.75±0.06		16	6.02±0.05		16	6.66±0.02
	20	1.73±0.08		20	5.53±0.10		20	6.17±0.02
	24	2.17±0.04		24	5.24±0.03		24	4.75±0.02

APPENDIX D

AMYLASE ACTIVITY FROM 50-ML CULTIVATED MEDIUM OF SUTWR 73

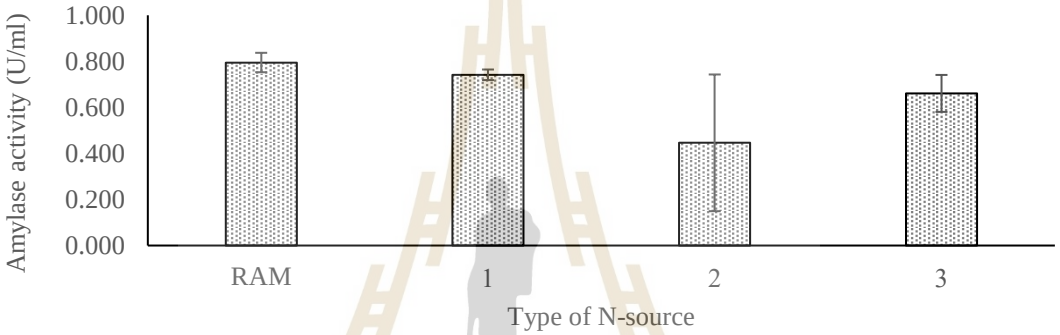

Figure 1D Total amylase activity of supernatant from various modified media including tryptone (1), defatted rice bran (2), and tryptone plus defatted rice bran (3) recipes after cultivating Lactobacillus sp. SUTWR 73 for 24h.

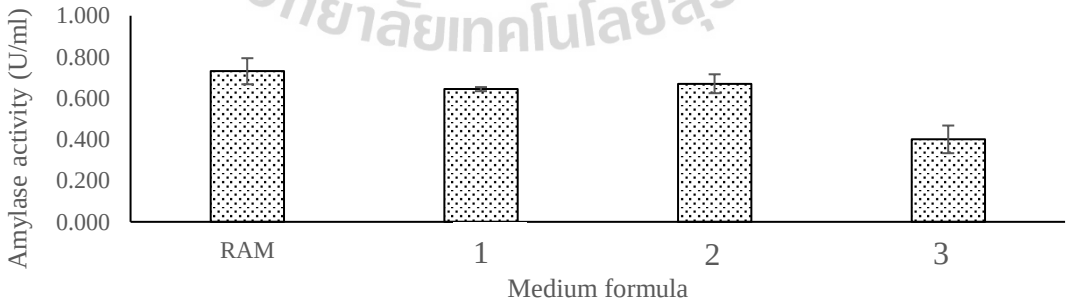

Figure 2D Amylase activity of supernatant from various modified media: in the second cultivation of Lactobacillus sp. SUTWR 73 including tryptone (1), tryptone plus defatted rice bran(2), and rice bran (3) recipes.

APPENDIX E

AMYLASE ACTIVITY FROM 3-L CULTIVATED MEDIUM OF *Lactobacillus* sp. SUTWR 73

Table 1E Growth, pH, amylase activity, protein concentration and growth (OD_{600}) relationship of cultured medium in vials 1 and 2.

Time (h)	Growth (OD_{600})				Protein concentration (mg/ml)		Amylase activity (U/ml)		pH	
	OD_{600}		Log CFU/ml							
	1	2	1	2	1	2	1	2	1	2
0	0.000±0.000	0.000±0.000	2.4843	3.0755	0.448±0.030	0.720±0.035	0.039±0.003	0.141±0.008	7.68	7.68
2	0.025±0.000	0.058±0.022	4.5563	4.4900	0.681±0.060	0.559±0.027	0.036±0.011	0.160±0.007	7.65	7.66
4	0.066±0.004	0.073±0.002	4.6946	5.3139	0.594±0.027	0.592±0.031	0.031±0.002	0.169±0.004	7.57	7.63
6	0.079±0.009	0.070±0.001	6.1761	5.4314	0.472±0.020	0.477±0.054	0.040±0.007	0.139±0.005	7.59	7.60
8	0.036±0.006	0.052±0.006	4.1761	5.2625	0.464±0.047	0.501±0.020	0.054±0.005	0.127±0.016	7.58	7.59
10	0.151±0.006	0.151±0.006	5.6449	4.0000	0.226±0.008	0.277±0.046	0.438±0.056	0.311±0.064	7.48	7.43
12	0.397±0.002	0.344±0.010	6.6021	8.3646	0.236±0.023	0.228±0.032	0.481±0.034	0.297±0.041	7.25	7.35
14	0.638±0.005	0.638±0.006	8.3541	6.1761	0.209±0.018	0.231±0.037	0.471±0.034	0.325±0.040	6.59	6.67
16	0.627±0.001	0.622±0.003	10.0871	10.1992	0.224±0.015	0.186±0.007	0.419±0.016	0.318±0.036	6.42	6.39
18	0.572±0.003	0.561±0.004	8.8235	9.1738	0.199±0.011	0.210±0.014	0.591±0.035	0.418±0.082	6.33	6.37
20	0.552±0.003	0.544±0.006	9.0269	8.9657	0.227±0.023	0.203±0.005	0.548±0.062	0.352±0.050	6.13	6.14
22	0.580±0.015	0.608±0.002	9.2960	9.2096	0.205±0.016	0.199±0.017	0.414±0.044	0.427±0.066	6.01	6.02
24	0.563±0.001	0.566±0.011	8.1186	8.2222	0.197±0.007	0.201±0.037	0.399±0.038	0.356±0.106	6.05	6.05

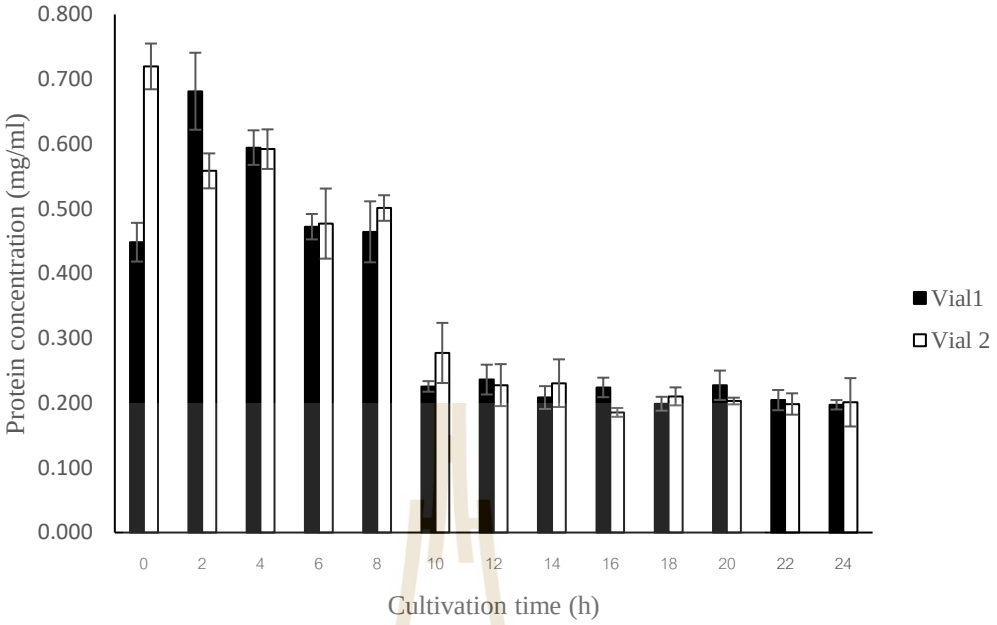

Figure 1E Protein concentration in RAM medium at various cultivation times of *Lactobacillus* sp. SUTWR 73.

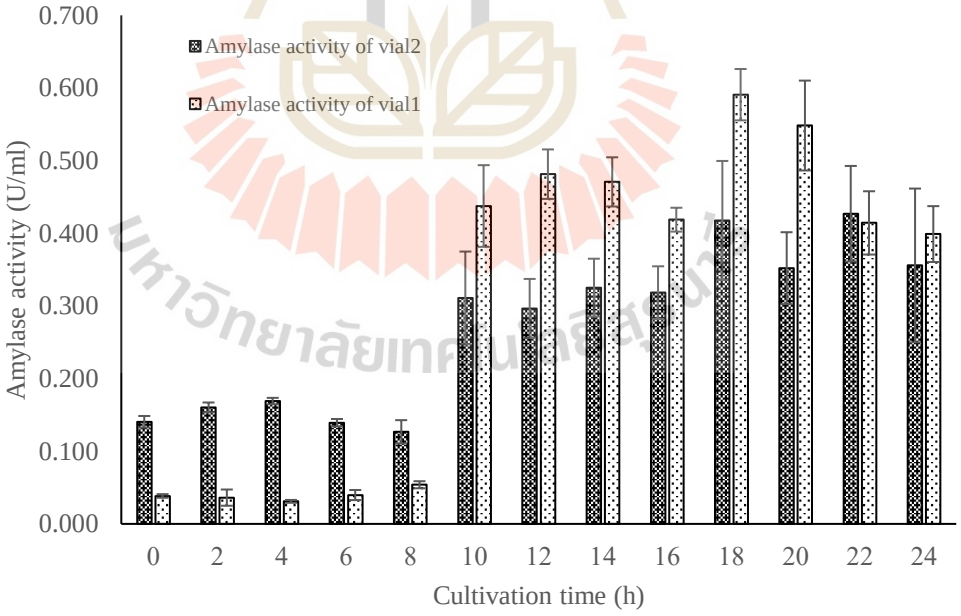

Figure 2E Amylase activity of cultured medium at various cultivation times of SUTWR 73 cultivation.

Table 2E Protein concentration in RAM medium at various period of SUTWR 73 cultivation.

Cultivation time (h)	A_{595}	Protein concentration (mg/ml)
0	0.140	0.155±0.021
4	0.148	0.167±0.032
8	0.136	0.149±0.015
12	0.161	0.186±0.005
16	0.209	0.256±0.028
20	0.255	0.323±0.043
24	0.247	0.311±0.016

Table 3E Reducing sugar concentration and amylase activity at different cultivation times during lactic acid fermentation by SUTWR 73 in lactic acid production medium (the fourth cultivation).

Cultivation time (h)	Reducing sugar concentration (mg/ml)	Amylase activity (U/ml)
0	8.998±1.752	0.83±0.13
3	7.485±4.203	0.69±0.32
6	3.911±0.340	0.36±0.03
12	4.323±1.264	0.40±0.10
15	4.371±0.813	0.40±0.06
18	3.985±1.657	0.37±0.13
21	6.075±2.100	0.56±0.16
24	8.177±1.864	0.76±0.14

APPENDIX F

LACTIC ACID PRODUCTION BY SUTWR 73 USING 300-L FERMENTATION MEDIUM IN 500-L BIOREACTOR

Table 1F Growth and D-lactic acid production of *Lactobacillus* sp. SUTWR 73 (the first-run cultivation) in the fermentation medium containing 10% cassava starch and using α-amylase for viscosity reduction in 500-l bioreactor with 300-l working volume. The inoculum size of 5% (approximately 10^6 cells/ml) was used. The cultivation conditions were at 35°C, 900 rpm of agitation speed, and 6.0-7.0 of pH controlled by 5 N NaOH without aeration for 48 h (Rodtong, 2017).

Cultivation time (h)	Growth (Log CFU/ml)	pH	Total acidity[a] (%)	D-Lactic acid[b] (g/l)	Total lactic acid[c] (g/l)	Reducing sugars[d] (g/l)	Total sugar[e] (g/l)
0	6.7634	7.24	0.00	0.00	0.00	15.85	99.26
2	6.7731	7.10	0.01	0.00	0.05	15.37	98.52
4	7.0792	5.88	0.09	0.89	0.95	14.45	95.97
6	7.9294	5.96	0.14	1.36	1.38	12.76	93.85
8	8.4456	6.22	0.56	5.58	5.62	20.50	90.50
10	9.5843	5.96	1.22	12.21	12.57	18.23	86.81
12	10.2648	6.57	1.61	16.13	16.61	21.49	82.29
14	10.1761	6.50	2.22	22.31	22.98	19.72	75.61
16	10.7782	6.57	2.79	28.04	28.88	24.60	70.54
18	10.3617	6.53	3.24	32.56	33.54	26.31	66.39
20	10.3979	6.45	3.65	36.63	37.73	29.15	61.98
22	10.5809	6.50	3.83	38.44	39.59	30.25	58.40
24	9.8938	6.42	4.02	40.40	41.01	36.45	56.53
26	9.6180	6.30	4.25	42.66	43.30	35.10	53.90
28	9.6532	6.50	4.32	43.42	44.07	34.58	50.31
30	8.7243	6.55	4.39	44.19	44.85	34.02	49.05
32	8.7782	6.50	4.41	44.32	44.98	35.38	47.87

Table 1F (Continued) Growth and D-lactic acid production of *Lactobacillus* sp. SUTWR 73 (the first-run cultivation) in the fermentation medium containing 10% cassava starch and using α-amylase for viscosity reduction in 500-l bioreactor with 300-l working volume. The inoculum size of 5% (approximately 10^6 cells/ml) was used. The cultivation conditions were at 35°C, 900 rpm of agitation speed, and 6.0-7.0 of pH controlled by 5 N NaOH without aeration for 48 h (Rodtong, 2017).

Cultivation time (h)	Growth (Log CFU/ml)	pH	Total acidity [a] (%)	D-Lactic acid [b] (g/l)	Total lactic acid [c] (g/l)	Reducing sugars [d] (g/l)	Total sugar [e] (g/l)
34	8.7709	6.48	4.50	45.23	45.91	35.90	46.50
36	7.5623	6.50	4.58	46.03	46.72	36.10	46.12
38	7.7993	6.50	4.62	46.38	47.08	37.59	45.57
40	7.5441	6.50	4.65	46.73	47.43	35.15	44.51
42	7.1761	6.50	4.79	48.10	48.82	38.45	43.38
44	6.2577	6.50	4.90	49.30	49.45	38.60	42.15
46	5.9731	6.50	4.95	49.75	50.49	36.15	40.95
48	5.9395	6.50	5.01	50.35	51.11	37.49	40.79

[a] Titration method (AOAC International, 2000)

[b] HPLC analysis (Yang and Chung, 2007)

[c] Colorimetric assay (Kimberley and Taylor, 1996)

[d] Dinitrosalicylic acid reagent (Miller, 1959)

[e] Colorimetric (phenol-sulphuric acid) method (Dubois *et al*., 1956)

Table 2F Growth and D-lactic acid production of *Lactobacillus* sp. SUTWR 73 (the second-run cultivation) in the fermentation medium containing 10% cassava starch and using α-amylase for viscosity reduction in 500-l bioreactor with 300-l working volume. The inoculum size of 5% (approximately 10^6 cells/ml) was used. The cultivation conditions were at 35°C, 900 rpm of agitation speed, and 6.0-7.0 of pH controlled by 5 N NaOH without aeration for 48 h (Rodtong, 2017).

Cultivation time (h)	Growth (Log CFU/ml)	pH	Total acidity[a] (%)	D-Lactic acid[b] (g/l)	Total lactic acid[c] (g/l)	Reducing sugars[d] (g/l)	Total sugar[e] (g/l)
0	6.7559	7.20	0.00	0.00	0.00	14.78	100.82
2	6.7959	7.06	0.00	0.00	0.01	15.35	99.50
4	7.3181	6.32	0.05	0.40	0.45	14.11	97.75
6	8.4232	6.40	0.20	2.01	2.11	10.84	95.92
8	8.5563	6.15	0.48	4.94	5.19	16.26	93.10
10	9.2405	6.37	0.99	10.20	10.71	19.53	88.25
12	9.7076	6.43	1.28	13.21	13.87	20.51	86.20
14	10.4031	6.47	1.65	17.00	17.85	18.27	80.65
16	10.5670	6.48	1.96	20.19	21.20	21.58	78.52
18	10.7634	6.40	2.36	24.26	25.47	25.79	73.11
20	10.8633	6.40	2.56	26.37	27.69	28.30	70.09
22	10.5809	6.43	2.76	28.40	29.82	30.95	67.53
24	10.8388	6.40	2.93	32.16	31.67	35.46	65.15
26	10.4683	6.48	3.09	34.83	33.42	32.68	62.80
28	9.5119	6.50	3.29	37.85	37.36	30.50	61.76
30	9.7160	6.50	3.44	40.38	39.56	33.02	58.71

Table 2F (Continued) Growth and D-lactic acid production of *Lactobacillus* sp. SUTWR 73 (the second-run cultivation) in the fermentation medium containing 10% cassava starch and using α-amylase for viscosity reduction in 500-l bioreactor with 300-l working volume. The inoculum size of 5% (approximately 10^6 cells/ml) was used. The cultivation conditions were at 35°C, 900 rpm of agitation speed, and 6.0-7.0 of pH controlled by 5 N NaOH without aeration for 48 h (Rodtong, 2017).

Cultivation time (h)	Growth (Log CFU/ml)	pH	Total acidity[a] (%)	D-Lactic acid[b] (g/l)	Total lactic acid[c] (g/l)	Reducing sugars[d] (g/l)	Total sugar[e] (g/l)
32	8.9031	6.50	3.61	44.18	34.59	36.38	52.30
34	8.5682	6.55	6.68	48.29	49.01	35.55	48.65
36	8.2405	6.48	4.73	48.72	49.45	34.10	46.25
38	7.6721	6.50	4.78	49.25	49.98	35.75	45.12
40	6.9731	6.50	4.81	49.50	50.24	35.12	43.89
42	6.7126	6.50	4.90	49.74	50.49	34.46	42.50
44	6.6665	6.50	4.93	50.72	51.48	36.06	40.42
46	5.9294	6.55	5.07	51.46	52.23	35.85	39.15
48	5.9196	6.50	5.12	51.92	52.70	35.20	38.60

[a] Titration method (AOAC International, 2000)

[b] HPLC analysis (Yang and Chung, 2007)

[c] Colorimetric assay (Kimberley and Taylor, 1996)

[d] Dinitrosalicylic acid reagent (Miller, 1959)

[e] Colorimetric (phenol-sulphuric acid) method (Dubois *et al*., 1956)

Table 3F Growth and D-lactic acid production of *Lactobacillus* sp. SUTWR 73 (the third-run cultivation) in the fermentation medium containing 10% cassava starch and using α-amylase for viscosity reduction in 500-l bioreactor with 300-l working volume. The inoculum size of 5% (approximately 10^6 cells/ml) was used. The cultivation conditions were at 35°C, 900 rpm of agitation speed, and 6.0-7.0 of pH controlled by 5 N NaOH without aeration for 48 h (Rodtong, 2017).

Cultivation time (h)	Growth (Log CFU/ml)	pH	Total acidity[a] (%)	D-Lactic acid[b] (g/l)	Total lactic acid[c] (g/l)	Reducing sugars[d] (g/l)	Total sugar[e] (g/l)
0	6.7284	7.30	0.00	0.00	0.00	15.10	100.97
2	6.7404	7.20	0.02	0.21	0.23	14.52	97.36
4	7.5051	6.55	0.08	0.77	0.81	12.56	95.65
6	7.8162	5.80	0.27	2.78	2.92	12.90	94.13
8	8.9395	6.23	0.69	7.12	7.48	18.20	90.21
10	9.7782	6.51	1.01	10.35	10.87	16.91	88.59
12	9.9294	6.21	1.38	14.21	15.05	21.45	83.11
14	10.1461	6.36	1.77	18.23	19.14	19.62	79.75
16	10.1959	6.52	2.04	21.04	21.52	20.54	75.86
18	10.5105	6.58	2.27	23.33	24.50	26.33	71.57
20	10.6424	6.69	2.45	25.20	26.46	28.63	68.77
22	10.1038	6.53	2.60	26.73	27.65	29.90	65.21
24	9.7810	6.57	2.72	27.96	28.10	36.40	61.29
26	9.4728	6.80	3.05	31.42	31.58	34.69	58.47
28	9.5575	6.50	3.27	33.65	35.33	36.24	55.62
30	8.8325	6.45	3.51	36.17	36.35	37.05	52.90
32	8.3579	6.50	3.87	39.86	40.46	37.76	50.45

Table 3F (Continued) Growth and D-lactic acid production of *Lactobacillus* sp. SUTWR 73 (the third-run cultivation) in the fermentation medium containing 10% cassava starch and using α-amylase for viscosity reduction in 500-l bioreactor with 300-l working volume. The inoculum size of 5% (approximately 10^6 cells/ml) was used. The cultivation conditions were at 35°C, 900 rpm of agitation speed, and 6.0-7.0 of pH controlled by 5 N NaOH without aeration for 48 h (Rodtong, 2017).

Cultivation time (h)	Growth (Log CFU/ml)	pH	Total acidity[a] (%)	D-Lactic acid[b] (g/l)	Total lactic acid[c] (g/l)	Reducing sugars[d] (g/l)	Total sugar[e] (g/l)
34	8.7993	6.50	4.13	42.49	43.13	35.05	48.16
36	7.7076	6.55	4.29	44.18	44.48	36.10	45.18
38	7.6866	6.50	4.42	45.48	46.16	35.80	43.57
40	7.3284	6.50	4.60	46.64	47.34	34.37	42.85
42	6.8692	6.50	4.71	47.83	38.55	35.20	42.15
44	6.0607	6.52	4.87	50.11	50.86	35.82	40.93
46	5.3579	6.50	4.91	50.57	51.33	35.17	40.35
48	5.3201	6.50	5.10	51.19	52.28	35.05	39.74

[a] Titration method (AOAC International, 2000)

[b] HPLC analysis (Yang and Chung, 2007)

[c] Colorimetric assay (Kimberley and Taylor, 1996)

[d] Dinitrosalicylic acid reagent (Miller, 1959)

[e] Colorimetric (phenol-sulphuric acid) method (Dubois *et al*., 1956)

APPENDIX G

AMYLASE PRODUCTION MEDIUM OPTIMIZATION

Table 1G Amylase activity, protein concentration, and pH of modified media (50 ml in 125-ml flasks) after cultivation of SUTWR 73 (2% inoculum size, approximately 10^6 cells/ml) at 35°C under anaerobic condition for 24 h.

Medium no.	Amylase activity (U/ml)	Protein concentration (mg/ml)	pH of the medium
RAM	8.06±0.04	0.025±0.005	4.97±0.05
1 (rice bran)	7.54±0.02	0.045±0.006	5.07±0.05
2 (soy protein)	6.73±0.08	0.406±0.018	5.11±0.14
3 (urea plus yeast)	7.11±0.01	0.024±0.006	5.30±0.15
4 (urea)	4.46±0.07	0.012±0.001	7.00±0.11

Table 2G Reducing sugar concentration and amylase activity of rice bran medium (50 ml in 125-ml flasks) after cultivation *Lactobacillus* sp. SUTWR 73 (2% inoculum size, approximately 10^6 cells/ml) at 35°C under anaerobic condition for 24 h.

Rice bran concentration (%, w/v)	No.	Reducing sugar concentration (mg/ml)	Amylase activity (U/ml)
0.4	1	3.031±0.083	0.28±0.01
	2	3.361±0.065	0.31±0.01
	3	2.843±0.180	0.26±0.02
0.8	1	0.897±0.011	0.08±0.00
	2	2.219±0.029	0.21±0.00
	3	2.378±0.100	0.22±0.01
1.2	1	1.541±0.029	0.14±0.00
	2	1.032±0.083	0.10±0.01
	3	0.860±0.033	0.08±0.00
1.6	1	0.785±0.027	0.07±0.00
	2	0.844±0.008	0.08±0.01
	3	0.926±0.043	0.09±0.00

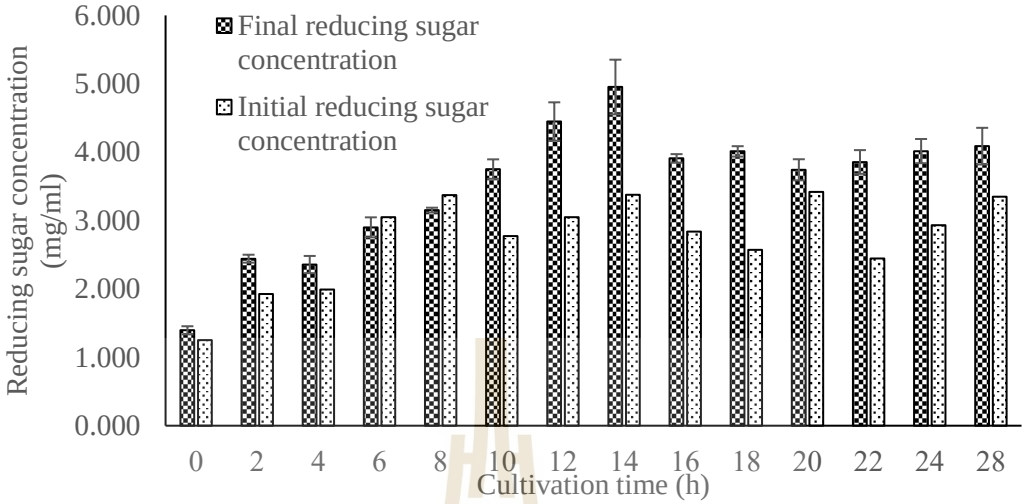

Figure 1G The changes in reducing sugar concentration in 30 min of hydrolysis reaction compared to the initial reducing sugar concentration from 7.5-l bioreactor with 3-l working volume at 35°C with agitation speed 150 rpm, pH controlling at pH 7.0 without aeration.

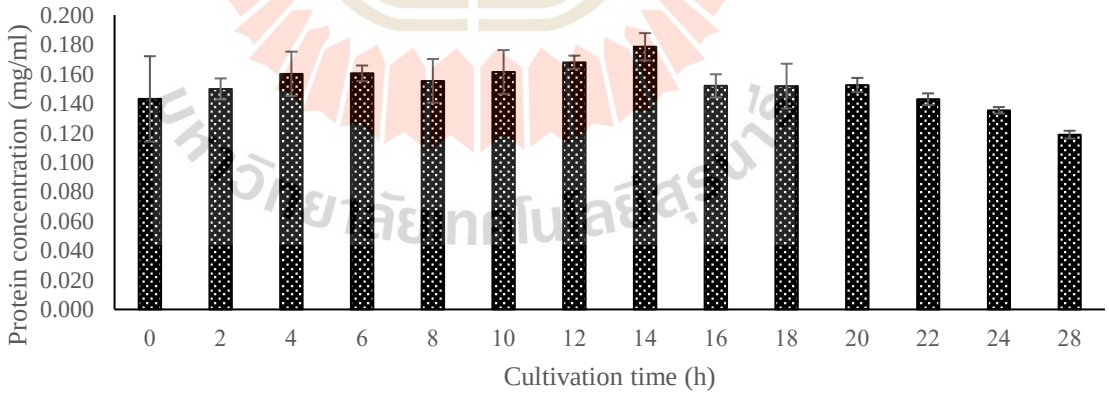

Figure 2G Protein concentrations at different cultivation times of the modified medium cultured with *Lactobacillus* sp. SUTWR 73 in 7.5-l bioreactor with 3-l working volume at 35°C with agitation speed 150 rpm, pH controlling at pH 7.0 without aeration.

APPENDIX H

LIST OF PRESENTATIONS

Oral presentation

Kanklang, E., Songsiriritthigul, C., and Rodtong, S. (2017). **Production of Amylase from the D-Lactic Acid-Producing Bacterium,** *Lactobacillus* sp. SUTWR73. The 12th Conference on Science and Technology for Youth, 2-4 June 2017, BITEC, Bangkok, Thailand.

Poster Presentation

Kanklang, E., Songsiriritthigul, C., and Rodtong, S. (2017). **Production of Amylase from the D-Lactic Acid-Producing Bacterium,** *Lactobacillus* sp. SUTWR73. 3rd Prize Winner of poster presentation. Biology Section. The 12th Conference on Science and Technology for Youth, 2-4 June 2017, BITEC, Bangkok, Thailand.

Kanklang, E., Songsiriritthigul, C., Yongswatdigul, J., and Rodtong, S. (2019). **Low Cost Medium for Amylase Production by the Lactic Acid Bacterium,** *Lactobacillus* sp. SUTWR73. Honorable mention award of poster presentation. Food Innovation Asia Conference 2019, 13-15 June 2019, BITEC, Bangkok, Thailand.

การผลิตอะไมเลสจากแบคทีเรียที่ผลิตกรดดี-แล็กติกสายพันธุ์ *Lactobacillus* sp. SUTWR 73

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บทคัดย่อ: อะไมเลสเป็นเอนไซม์ย่อยแป้งที่ใช้กันอย่างแพร่หลายในอุตสาหกรรมหลายประเภท *Lactobacillus* sp. SUTWR 73 เป็นแบคทีเรียสร้างอะไมเลส ที่สามารถผลิตกรดดี-แล็กติกได้โดยตรงจากแป้ง การผลิตอะไมเลสจากแบคทีเรียจึงเป็นประโยชน์ในการผลิตดี-แล็กติกจากแป้งความเข้มข้นสูงที่มีปัญหาความหนืด จากการศึกษาเพื่อให้ได้อาหารเลี้ยงเชื้อที่เหมาะสมในการผลิตอะไมเลส พบว่าแป้งมันสำปะหลังเข้มข้นร้อยละ 2.0 จากที่ศึกษาช่วงร้อยละ 0.5-3.0 น้ำหนักต่อปริมาตร เหมาะสมในอาหารที่มีทริปโตนและสารสกัดจากยีสต์เป็นแหล่งไนโตรเจนและสารส่งเสริมการเจริญ ได้กิจกรรมของเอนไซม์สูงสุดเท่ากับ 0.726±0.083 หน่วยต่อมิลลิลิตร ที่มีโปรตีน 0.1962±0.024 กรัมต่อลิตร เมื่อทดแทนแหล่งไนโตรเจนทั้งหมดด้วยรำข้าวซึ่งมีราคาถูกลงไม่น้อยกว่า 380 เท่า ด้วยความเข้มข้นเท่ากัน พร้อมทั้งไม่เติมไดแอมโมเนียมซิเตรตจากที่เติมในอาหารเริ่มต้น พบว่ายังคงมีกิจกรรมของเอนไซม์สูง 0.74±0.02 หน่วยต่อมิลลิลิตร มีโปรตีน 0.0449±0.006 กรัมต่อลิตร และลดต้นทุนของอาหารเลี้ยงเชื้อลงร้อยละ 84 เอนไซม์ที่ผลิตได้ มีแนวโน้มใช้ทดแทนอะไมเลสทางการค้า

คำสำคัญ: อะไมเลสจากแบคทีเรีย, *Lactobacillus*, แบคทีเรียผลิตกรดดี-แล็กติก

Production of Amylase from the D-Lactic Acid-Producing Bacterium, *Lactobacillus* sp. SUTWR 73

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Abstract: Amylase, a starch-degrading enzyme, is widely used in several industries. The amylase-producing *Lactobacillus* sp. SUTWR 73 can efficiently directly produce D-lactic acid from starch. The study of bacterial amylase production would be useful for producing D-lactic acid from high starch concentrations with the high viscosity problem. From the investigation to obtain the suitable medium for the maximum bacterial amylase production, the medium composed of cassava starch at 2.0% (w/v) obtained from the range of 0.5-3.0% w/v, with tryptone and yeast extract as nitrogen sources and growth factors, was found to support the highest amylase yield at activity of 0.726±0.083 U/ml with 0.1962±0.024 g/l of protein. When replaced all nitrogen sources with rice bran at the same total concentration having at least 380 times cheaper, and without tri-ammonium citrate, the amylase activity was still high at 0.74±0.02 U/ml with 0.0449±0.006 g/l of protein. The optimized medium is 84% cheaper than the original medium, and the amylase produced has a tendency to replace the commercial amylase.

Keywords: Bacterial amylase, *Lactobacillus*, D-Lactic acid-producing bacteria

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increased after 1-h treatment. The results suggest that the addition of EGCg or theaflavins caused immediate stress to *S. aureus*, leading to temporally increased in the transcription of *sea*. However, EGCg addition reduced the production of SEA, which would be consistent with the reduction of *sea* transcription after 4–h EGCg treatment. These findings suggest that EGCg and theaflavins are promising natural antibacterial agent to control *S. aureus* and its enterotoxin production.

Keywords: *EGCg, Theaflavins, Staphylococcus aureus,*

DPB112

Screening of Bile Salt Hydrolase Activity and Cholesterol Assimilation of Lactic Acid Bacteria isolated from Plant Samples

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Generally, lactic acid bacteria (LAB) are found and isolated from fermented foods, dairy products, gastrointestinal tract of humans and animals, and plants. They play beneficial role as health promoter on their host. Nowadays, the interested ability of probiotic is bile salt hydrolase (BSH) has become the focus of attention on account of its influence on cholesterol metabolism. The bile salt hydrolase activity of probiotic bacteria residing in gastrointestinal tract have often being associated with their cholesterol lowering effects. Therefore, this study aims 1) to isolate LAB from plant samples, 2) to screen BSH activity, and 3) to investigate cholesterol assimilation ability. Twenty-five were screened from flowers, rice seed, and tree bark for the BSH activity on MRS agar supplemented with 0.5% (w/v) of taurodeoxycholic acid (TDCA) as a conjugated bile salt. Only seven isolates including FM1-1, FM1-2, FM2-3, FM3-1, FM11-2, FM12-1, and FM12-2 exhibited bile salt hydrolase activity. After that, all LAB isolates were determined the cholesterol assimilation. The results showed that their assimilated ability varied from 9.57 to 51.69%. The isolate FM11-2 could efficiently assimilate the most cholesterol, with 51.69%; on the contrary, the isolate FM11-3 assimilated the least cholesterol, with 9.57%. The isolate FM11-2 was identified as *Enterococcus lactis* (99.77% similarity). The isolate FM1-1, FM1-2, FM12-1 and FM12-2 were identified as *E. durans* (100% similarity). The isolate FM2-3 was identified as *E. gallinarum* (99.92% similarity); while the isolate FM3-1 was identified as *Lactobacillus plantarum* subsp. *plantarum* (100% similarity) based on 16S rRNA gene sequencing analysis. For the further study, they will be also tested for other probiotic properties. Hopefully, all of these isolates could be possibly used as probiotics in order to reduce cholesterol and the risk of heart disease in the future.

Keywords: *Lactic acid bacteria (LAB), Bile salt hydrolase (BSH), Cholesterol assimilation.*

DPB131

Low Cost Medium for Amylase Production by the Lactic Acid Bacterium, *Lactobacillus* sp. SUTWR73

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Amylase, an enzyme catalyzing the degradation of the large carbohydrate molecule, starch, to oligosaccharides, glucose, and maltose, is widely used in the food industry. Glucose and maltose syrup production, for example, is important to the production of gum, adhesive, ice-cream, candy, and a muesli bar. The enzyme target bond varies from type to type of amylase. This study aims to develop a low-cost medium suitable for amylase production by the amylolytic lactic acid bacterium, *Lactobacillus* sp. SUTWR73. The De Man, Rogosa and Sharpe medium (MRS) was used as the standard medium for lactic acid bacterium cultivation. The medium was then modified through substitution of the carbon and nitrogen sources with low-cost agricultural products, and elimination of unnecessary components using factorial experiments. Results showed that the medium composed of 2% cassava starch to replace glucose and 0.4% defatted rice bran to replace tryptone and yeast extract, without adding tri-ammonium citrate as MRS, provided the high potential production of amylase. The lactic acid bacteria, *Lactobacillus* sp. SUTWR73, could produce the enzyme of 0.74±0.02 U/ml (with 0.0449±0.006 mg/ml). The cost of the developed medium was 90% lower than the MRS standard medium. The crude amylase produced by using the developed medium, could perform its activity without Ca^{2+} ion at 60°C for 30 min. The results reveal that the amylase produced by SUTWR73 in the low-cost medium showed activity at in the range of starch gelatinization temperature. These results support the application of the modified medium for further large scale production of amylase.

Keywords: *Amylase, Lactic acid bacteria, Amylase production medium*

DPB149

Amylase Production by the Potential Strain of L-Lactic Acid-Producing Bacterium in Cassava Starch Medium Containing Rice Bran

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