PRODUCTION AND CHARACTERIZATION OF AMYLASE FROM L-LACTIC ACID-PRODUCING BACTERIUM *Lactococcus* sp. SUT 513

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การผลิตและศึกษาคุณลักษณะของอะไมเลสจากแบคทีเรียผลิตกรดแอล-แล็กติก *Lactococcus* sp. SUT 513

นายรัฐพร สุมาลุย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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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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เอนไซม์อะไมเลสทางการค้ามีประโยชน์ในอุตสาหกรรมการผลิตกรดแล็กติกที่ต้องย่อยแป้งเพื่อแก้ปัญหาความหนืดสูงจากวัตถุดิบแป้งความเข้มข้นสูง การศึกษาการผลิตอะไมเลสจากแบคทีเรีย *Lactococcus* sp. SUT 513 ที่มีศักยภาพสูงในการผลิตกรดแอล-แล็กติกเพียงไอโซเมอร์เดียวได้โดยตรงจากแป้ง อาจสามารถลดการใช้เอนไซม์อะไมเลสทางการค้า การศึกษาครั้งนี้จึงเริ่มพัฒนาอาหารเลี้ยงเชื้อที่เหมาะสมเพื่อผลิตอะไมเลส ตามส่วนประกอบมาตรฐานที่มีแป้งมันสำปะหลัง ทริปโตน สารสกัดจากยีสต์ ไค-โพแทสเซียมฟอสเฟต ไตร-แอมโมเนียมซิเตรท แมกนีเซียมซัลเฟต แมงกานีสซัลเฟต และเฟอรัสซัลเฟต ปริมาณ 10.0 5.0 3.0 6.0 1.0 0.57 0.12 และ 0.03 กรัมต่อลิตร ตามลำดับ และมีค่าความเป็นกรด-ด่างเริ่มต้นเท่ากับ 7.0 เมื่อพัฒนาสูตรอาหารเลี้ยงเชื้อด้วยวิธีการพื้นผิวตอบสนอง โดยใช้แผนแบบเซ็นทรัลคอมโพสิต และนำมาสร้างแบบจำลองทางสถิติด้วย 3 ตัวแปรหลัก คือ ความเข้มข้นของแป้งมันสำปะหลัง ความเข้มข้นของรำข้าวสกัดน้ำมัน และความเป็นกรด-ด่างเริ่มต้นของอาหารเลี้ยงเชื้อ พบว่าสูตรอาหารที่เหมาะสมประกอบด้วย แป้งมันสำปะหลัง และรำข้าวสกัดน้ำมัน ความเข้มข้น 45.0 และ 6.0 กรัมต่อลิตร ตามลำดับ ที่ค่าความเป็นกรด-ด่างเริ่มต้น 8.5 และจำเป็นต้องเติมไค-โพแทสเซียมฟอสเฟต เข้มข้น 6.0 กรัมต่อลิตร เพื่อให้ได้การเจริญดีที่สุด เมื่อทดลองเลี้ยงแบคทีเรียเป็นเวลา 24 ชั่วโมง ในอาหารเลี้ยงเชื้อที่เหมาะสม ตรวจพบกิจกรรมของอะไมเลสเท่ากับ 9.508±0.022 หน่วยต่อมิลลิลิตร มีโปรตีน 305±53 กรัมต่อลิตร และสามารถลดต้นทุนค่าอาหารเลี้ยงเชื้อลงจากเริ่มต้นร้อยละ 79.0 ผลการศึกษาแสดงถึงศักยภาพสูงในการผลิตอะไมเลสโดย *Lactococcus* sp. SUT 513 เมื่อเลี้ยงเชื้อในถังปฏิกรณ์ชีวภาพขนาด 7.5 ลิตร ด้วยปริมาตรอาหาร 3 ลิตร พบกิจกรรมของอะไมเลสสูงสุด 9.459±0.219 หน่วยต่อมิลลิลิตร ที่ 16 ถึง 18 ชั่วโมงของการเลี้ยงเชื้อ จากการศึกษาคุณลักษณะของเอนไซม์ที่ผ่านการทำบริสุทธิ์ขั้นต้นด้วยวิธีการตกตะกอนแอมโมเนียมซัลเฟตอิ่มตัวร้อยละ 90.0 เมื่อกรองและแยกโปรตีน พบว่าอะไมเลสที่ได้มีน้ำหนักโมเลกุลอยู่ในช่วง 16 ถึง 52 กิโลดัลตัน เอนไซม์สามารถทำงานได้ดีที่สุดที่อุณหภูมิช่วง 50 ถึง 60 องศาเซลเซียส และค่าความเป็นกรด-ด่าง ช่วง 6.0 ถึง 7.0 สามารถทนอุณหภูมิในช่วง 70 ถึง 100 องศาเซลเซียส เป็นเวลา 30 นาที ซึ่งพบค่ากิจกรรมสัมพัทธ์ตั้งแต่ร้อยละ 88 ถึง 95 และพบว่าเอนไซม์นี้สามารถทำงาน

ได้โดยไม่มีแคลเซียม อีกทั้งแคลเซียมยังมีแนวโน้มยับยั้งการทำงานของเอนไซม์ลงประมาณ 2.5 เท่า สำหรับแมกนีเซียมและเฟอรัสไอออนนั้นสามารถกระตุ้นกิจกรรมของเอนไซม์เพิ่มขึ้นประมาณ 2.0 เท่า สำหรับค่าความคงตัวในกรด-ด่าง พบว่าอะไมเลสนั้นตกตะกอนที่ค่าความเป็นกรด-ด่าง 3.0 ถึง 5.0 มีค่ากิจกรรมสัมพัทธ์ร้อยละ 10 ถึง 20 ซึ่งมีความคงตัวสูงสุดที่ค่าความเป็นกรด-ด่าง 6.8 และในตะกอนส่วนนี้มีเอนไซม์ที่สามารถทำงานได้ดีที่สุดที่อุณหภูมิช่วง 15 ถึง 35 องศาเซลเซียส จากการศึกษายังพบว่าจากการแช่แข็งและนำมาละลาย กิจกรรมของเอนไซม์ลดลงร้อยละ 5 ถึง 15 ต่อครั้งของการละลาย และเมื่อติดตามผลเป็นเวลา 26 วัน โดยเก็บเอนไซม์สกัดหยาบที่อุณหภูมิ 2 ถึง 4 องศาเซลเซียส ค่ากิจกรรมลดลงร้อยละ 1.5 ต่อวัน ผลจากการศึกษานี้สามารถใช้ต่อยอดในการผลิตและใช้ประโยชน์เอนไซม์อะไมเลสที่ผลิตจากแบคทีเรียผลิตกรดแอล-แล็กติก อย่างไรก็ตามยังคงต้องศึกษาเพิ่มในขั้นตอนการทำบริสุทธิ์และคุณลักษณะของเอนไซม์ต่อไป

สาขาวิชาปรีคลินิก
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RATTAPORN SUMALU : PRODUCTION AND CHARACTERIZATION OF AMYLASE FROM L-LACTIC ACID-PRODUCING BACTERIUM *Lactococcus* sp. SUT 513. THESIS ADVISOR : ASST. PROF. SUREELAK RODTONG, Ph.D. 133 PP.

AMYLASE PRODUCTION/ENZYME CHARACTERIZATION/L-LACTIC ACID BACTERIA

Commercial amylase enzymes provide benefit to starchy industry for solving viscosity problem that causes by high starch concentration. The potential production study of amylase from *Lactococcus* sp. SUT 513 that directly produces optical pure L-lactic acid at high concentration of cassava starch could reduce the application of commercial enzymes. This study commenced from the development of the optimal amylase production medium using the standard medium composed of cassava starch, tryptone, yeast extract, di-potassium hydrogen phosphate, tri-ammonium citrate, magnesium sulfate, manganese sulfate, and ferrous sulfate at concentrations of 10.0, 5.0, 3.0, 6.0, 1.0, 0.57, 0.12, and 0.03 g/l, respectively, with the initial pH of 7.0. Then, the 3 factors (cassava starch concentration, defatted rice bran concentration, and initial pH) were optimized, and used to build the model using central composite design (CCD) with response surface methodology (RSM). The optimal amylase production medium formula composing of cassava starch, defatted rice bran, and di-potassium hydrogen phosphate at concentrations of 45.0, 6.0, and 6.0 g/l, respectively. When cultivated *Lactococcus* sp. SUT 513 for 24 h in the optimal medium with an initial pH of 8.5, the amylase activity of 9.508±0.022 U/ml with protein concentration 305±0.053 g/l was

obtained. The cost of optimized medium was 79.0% cheaper than the original medium. These results showed the high potential production of amylase by *Lactococcus* sp. SUT 513. The amylase enzyme could produced by *Lactococcus* sp. SUT 513 within shorted cultivation period (16 to 18 h of cultivation) when cultivated in a 3-liter working volume in 7.5-liter bioreactor. The bacterium produced enzyme exhibiting 9.459±0.219 U/ml of amylase activity. For the characterization of partially purified amylase by 90% ammonium sulfate precipitation with ultrafiltration, the molecular weight of amylase was in the range of 16-52 kDa. The amylase had its optimal pH at 6.0 to 7.0, and optimal temperature ranging from 50 to 60°C. The partially purified amylase was stable at 70 to 100°C for 30 min with 88 to 95% relative activity. This amylase was active without adding calcium ion. The calcium ion could slightly inhibit (2.5-fold reduction of activity). The magnesium and ferrous ions acted as activators that could increase its activity to 2.0 fold. The amylase partially purified by pH 3.0 precipitation showed high stability at pH 6.0 to 6.8, low stability at pH 3.0 to 5.0 with 10 to 20% relative activity, this fraction of enzyme showed activity at the optimal temperature ranging from 15 to 35°C. Freezing and thawing of the crude enzyme resulted in amylase reducing activity around 5-15% each time. The activity of crude enzyme reduced 1.5% per day, during storage at 4°C for 26 days. Results from this study provide advantages for future application and production of the enzyme from agricultural waste. Also, further purification and characterization of the enzyme will be necessary to be investigated.

School of Preclinic

Academic Year 2018

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LIST OF ABBREVIATIONS

Conc	Concentration
DNS	3,5-Dinitrosalicylic acid
EDTA	Ethylenediaminetetraacetic acid
%	Percentage
°C	Degree Celsius
µl	Microliter
µM	Micromolar
BSA	Bovine serum albumin
CFU	Colony forming unit
Co., Ltd.	Limited company
CSB	Cassava starch broth
EGTA	Ethylene glycol-bis-(β-aminoethyl ether)-N, N, N', N'-tetraacetic acid
et al.	et alia (and others)
$FeSO_4$	Ferrous sulfate
g	Gram
h	Hour
K_2HPO_4	Dipotassium hydrogen phosphate
kDa	Kilodalton
l	Liter

LIST OF ABBREVIATIONS (Continued)

M	Molar
$MgSO_4$	Magnesium sulfate
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
$MnSO_4$	Manganese sulfate
mol	Mole
nm	Nanometer
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
RAM	Modify Lactobacillus MRS Medium
rpm	Round per minute
SDS	Sodium dodecyl sulfate
SMF	Submerged fermentation
SSF	Solid-state fermentation
TEMED	N,N,N' ,N'-Tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
U	Unit
w/v	Weight/volume

CHAPTER I

INTRODUCTION

1.1 Significance of the study

Amylase is one of the starch-degrading enzymes. This enzyme can randomly hydrolyze starch molecules that cause the diverse product of carbohydrate polymer such as linear, branched oligosaccharides in various lengths composed of dextrin and glucose units (Gupta et al., 2003). Amylase can be obtained from various sources including plants, animals, and microorganisms (Pandey et al., 2000). One of the simplest classifications is based on substrate specificity that can divide amylase into several groups such as α-amylase (EC. 3.2.1.1), β-amylase (EC 3.2.1.2), and γ-amylase (EC 3.2.1.3). Amylase of different origins has been extensively studied. However, for industrial applications, α-amylase is usually produced by bacteria, particularly several species in the genus *Bacillus* such as *B. licheniformis, B. amyloliquefaciens, B. stearothemophilus,* and *B. subtilis* (Nigam and Singh, 1995). α-Amylase has potential applications in a wide variety of industrial processes such as food production, fermentation, textile, paper, detergent, and pharmaceutical industries (Machius et al., 1995; Nigam and Singh, 1995; Pandey et al., 2000; Richardson et al., 2002). From the global alpha-amylase baking enzyme market, microbial maltogenic enzymes accounted for 47,233.8 tons in 2015. The global alpha-amylase baking enzyme market is expected to reach USD 320.1 million by 2024, according to the report by Grand View Research,

Inc. (Sherry James, 2016). The low cost-effective alternative α-amylase for industrial should be investigated.

Currently, our laboratory produces L-lactic acid from cassava starch as a substrate by using *Lactococcus* bacteria. The L-lactic acid is aimed to be used as a monomer for bioplastic polymer that can be processed by extrusions such as 3d printing, injection molding, film, and sheet casting, and spinning, providing access to a wide range of materials. Cassava starch is the primary carbon source in the production medium containing a very high concentration of cassava starch (12%, w/v). The high concentration of cassava starch makes the media difficult to prepare because of its high viscosity resulting in low water activity. The components are also very hard to mix and unsuitable for bacterial cultivation.

This research aims to investigate amylase production by *Lactococcus* sp. SUT 513, a specific strain of L-lactic acid-producing bacteria. The strain actively uses cassava starch as the main substrate for optically purified L-lactic acid production. The bacterium could potentially be used for amylase enzyme production to reduce or replace commercial amylases.

1.2 Research objectives

1) To optimize medium composition and cultivation conditions for amylase production by *Lactococcus* sp. SUT 513
2) To partially purify and characterize amylase produced by *Lactococcus* sp. SUT 513

1.3 Research hypotheses

Amylase could be potentially produced by L-lactic acid-producing and starch utilizing bacteria for lactic acid production and food industry. Amylase could be potentially produced by *Lactococcus* sp. SUT 513 using the low-priced modified medium, which could be equal or better than the original medium. The enzyme could have different characteristics when compared with commercial amylase.

1.4 Scope and limitations of the study

1) Optimization of C-source concentrations, N-source types and concentrations, pH, temperature, and inoculum size for amylase production by *Lactococcus* sp. SUT 513.

2) Partial purification of amylase produced by *Lactococcus* sp. SUT 513 using methods such as ammonium sulfate precipitation, pH precipitation, dialysis, and ultrafiltration.

3) Characterization of extracellular amylase from *Lactococcus* sp. SUT 513, and investigation of pH and temperature profiles, and type and properties of amylase.

1.5 Expected results

The optimal medium and cultivation conditions for producing amylase, the partially purified amylase, and characteristics of the partially purified amylase would be obtained.

CHAPTER II

LITERATURE REVIEWS

2.1 Lactic acid bacteria

Lactic acid bacteria are Gram-positive, non-spore forming, catalase-negative, bacillus, or coccus cell shape able to undergo fermentation to produce lactic acid (Fossi and Tavea, 2013). Some lactic acid bacteria are microaerophilic, anaerobic, or facultative anaerobes. They can convert starch or carbohydrate into the lactic acid with 2 conformational isomerism, D-or L-lactic acid. In most cases, a combination of both forms is produced by chemical synthesis. These bacteria can be produced high optical purity of lactic acid. The D-form or L-form lactic acid bacteria can be found in many food products such as sour milk, yogurt, or brown syrup. Reddy et al. (2008) suggested that lactic acid bacteria can use carbohydrates from many sources such as wheat, cassava, and corn. The products are used in a wide variety of industrial sectors such as food, chemical, pharmaceutical, and beverage industries (Ghaffar et al., 2014). One of the most interested in lactic acid application is used in the polymerization of L-lactic acid (monomer) to polylactic acid (PLA, polymer) because it can be biodegradable. By using starch as the substrate (Ilmén et al., 2007) some species of lactic acid bacteria such as *Lactobacillus* sp. (Ghaffar et al., 2014) and *Lactobacillus plantarum* S21 (Kanpiengjai et al., 2015) can produce high amount of lactic acid.

2.2 Amylase enzymes

Amylase is one type of starch-degrading enzymes. Amylase can be classified into two groups as endoamylases and exoamylases. Endoamylases randomly catalyze hydrolysis in the interior of the starch molecule as α-amylase (EC. 3.2.1.1). This action causes the formation of linear and branched oligosaccharides of various chain lengths. Exoamylases hydrolyze from the non-reducing end, successively resulting in short end products as, α-amylase (EC. 3.2.1.1). β-amylase (EC 3.2.1.2) (Gupta et al., 2003; Kelly et al., 1991; Mihajlovski et al., 2016).

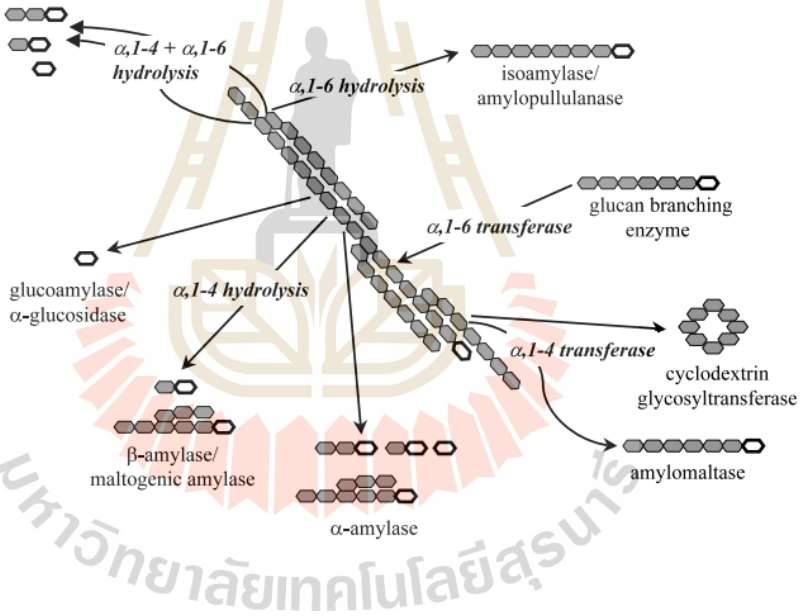


Figure 2.1 Different types of amylase enzymes involved in the degradation of starch. The blank hexagon shape symbolizes the reducing end of a poly-glucose molecule (Van Der Maarel et al., 2002).

α-Amylase (E.C.3.2.1.1) is a hydrolase enzyme that catalyzes the hydrolysis of internal α-1,4-glycosidic linkages in starch to yield products like glucose and maltose. The amylase has been derived from several species of fungi, yeasts, bacteria, and

actinomycetes, however, enzymes from fungal and bacterial sources have dominated applications in industrial sectors (Pandey et al., 2000). Usually, α-amylase is a calcium metalloenzyme. There are 2 types of hydrolases: endo-hydrolase and exo-hydrolase (Gupta et al., 2003). Therefore, at the end of glucose residues and α-1, 6-linkages cannot be cleaved by α-amylase. α-Amylase has become an important enzyme because of starch degradation activity that can be digested. One such action is glucose production and fructose syrup from starch (Gupta et al., 2003).

This is the list of α-amylase molecular mass from lactic-acid bacteria such as 121 kDa from *Lactococcus lactis* IBB500, 135 kDa from *Lactobacillus plantarum* A6, 140 kDa from *Lactobacillus manihotivorans* LMG18010, and 150 kDa from *Lactobacillus amylovorus* NRRL B-4540 (Kanpiengjai et al., 2015).

β-Amylase (EC 3.2.1.2) or glycogenase or 4-alpha-D-glucan maltohydrolase, is an exo-hydrolase that specifically cleaves α-1,4-glycosidic linkages to yield successive maltose units. The monomers inwards from the non-reducing ends of the glucan chain, producing maltose for every successful hydrolysis and then stopping by the presence of branching points (α-1,6-linkages). The main source of β-amylase is the seed of the monocotyledon and sweet potato. During the ripening of fruits, β-amylase breaks down starch into maltose resulting in the sweetness of ripened fruit. The optimal pH of the enzyme ranges from 4.0 to 5.5. In industry, it is used for fermentation in the brewing and distilling industry (Sivaramakrishnan et al., 2006). Also, it is used for starch processing and its main application is for producing maltose syrup which could be used in the food industry (Mihajlovski et al., 2016).

γ-Amylase (EC 3.2.1.3) or glucan 1,4 alpha-glucosidase, hydrolyzes the terminal α-1,6-glycosidic linkages, in addition to cleaving the last α-1,4-glycosidic linkages at

the non-reducing ends of amylose and amylopectin, and release of D-glucose (Kelly et al., 1991). γ-Amylase is most efficient in acidic environments and has an optimum pH of 3.0 (Sivaramakrishnan et al., 2006).

The major advantage of using microorganisms to produce amylase is the economical bulk production capacity and microbes are easy to manipulate to obtain enzymes of desired characteristics (Lonsane and Ramesh, 1990).

2.3 Microbial cultivation process for amylase production

Some microbial species have been used for amylase production for a long time. Not only are used for food production namely, dairy, fish, and meat products but are also involved in textile, alcoholic beverages, energy, and some chemical production. Besides, several additives and supplements are from microbial fermentation, such as antioxidants, flavors, colorants, preservatives, and even in sweeteners. Nowadays, the cultivation of microorganisms is developed to be highly efficient and yielding large amounts of desired products. The large-scale cultivation processes are widely used to produce biomolecules. Some are used in various enzyme productions, such as extracellular amylase, lipase, pectinase, and cellulase. Others are used in organic acid production, for example, lactic acid production and citric acid (Couto et al., 2005).

The process can also be categorized as solid-state fermentation (SSF) or submerged fermentation (SMF), depending on the amount of free water in the medium. In SSF, the process performed on the non-soluble material that acts both as physical support and nutrient source without free-flowing liquid. The microorganisms are grown on a solid substrate which is moistened (Ganesh Karanth et al., 2005; Pandey, 1992). Many kinds of non-soluble materials were used in the process, such as potato peels

(Mukherjee et al., 2017), *Arundo donax* rhizome, banana peel, orange peel, wheat bran, as well as rice bran (Finore et al., 2014; Anto et al., 2006).

Submerged fermentation (SMF) is in which microorganisms grow submerged in a liquid medium where free water is abundant. This is the method of choice for many industrial operations over SSF although SSF is also rapidly gaining interest in the present (Ganesh Karanth et al., 2005). The use of submerged culture is helpful because of the easy sterilization and manipulation system. Depending on the strain and the culture conditions, the enzyme can be constitutive or inducible, showing different production patterns (Kunamneni et al., 2005).

2.4 Optimization of cultivation medium and conditions for amylase production by microorganisms

The low-cost medium is required to produce amylase, especially α-amylase. Both solid-state fermentation (SSF) and submerged fermentation (SMF) used to produce amylases, although traditionally, these have been obtained from submerged cultures because of ease of handling and greater control of environmental factors such as temperature and pH. Mostly synthetic media have been used to produce bacterial amylase through SMF. The compositions of standard media particularly nutrient broth, soluble starch, as well as other components are awfully expensive, and these could be replaced with cheaper agricultural by-products for the reduction of the medium cost. SSF resembles natural microbiological processes such as composting and ensiling, which can be utilized in a controlled way to produce the desired product. SSF has been used for a long time to convert moist agricultural polymeric substrates, e.g.

wheat, rice, soy, cassava, etc. into fermented food products including industrial enzymes (Mielenz, 1983; Sivaramakrishnan et al., 2006).

2.4.1 Carbon source

Common carbon sources used as substrates for supporting microbial growth include maltose, sucrose, and glucose. A different source of carbon such as starch, glucose, galactose, glycogen, and inulin have been reported as suitable substrates to produce α-amylase by *Bacillus licheniformis* and *Bacillus* sp. I-3 (Chandra et al., 1980). Soluble starch has been found as the best substrate for the production of α-amylase by *Bacillus stearothermophilus* (Srivastava and Baruah, 1986). *Aspergillus tamarii* produces α-amylase when cultivated in maltose, starch, and glycogen under static conditions. This yield has increased four times compared to shaking cultures (Kundu and Das, 1970). Wheat bran has been used as a substrate for α-amylase production by *Bacillus licheniformis*, and *Aspergillus niger* (Gangadharan et al., 2006). Wheat bran and banana waste have been used for production by *Bacillus subtilis* (Laderman et al., 1993). *Bacillus* sp. was noted to give a maximum raw starch digesting amylase in a medium containing lactose 1% and yeast extract (Rahardjo et al., 2005). Agricultural wastes were used for reducing the cost of fermentation media. These wastes consist of carbon and nitrogen sources necessary for the growth and metabolism of organisms. For example, orange waste, pearl millet starch, potato, corn, tapioca, wheat, and rice as flours were included in these nutrient sources (Ashraf et al., 2005; Chandra et al., 1980).

2.4.1.1 Cassava and cassava starch

Cassava, tapioca, or mandioca (*Manihot esculenta*) is a tuber native plant from South America that has huge nutritional importance in the tropics. Cassava root

is one of the main sources of carbohydrates. Cassava is produced in almost all mild and tropical countries and grows in degraded soils where almost nothing else can grow (Okudoh et al., 2014). Moreover, cassava tubers can be harvested from 8 to 24 months after planting. Cassava is categorized based on its hydrocyanic acid content into sweet cassava (directly consumed) and bitter cassava for making starch and other industrial purposes (Okudoh et al., 2014).

Not only does cassava root store starch but also contains small amounts of protein, vitamins, and minerals (Tonukari, 2004). The starch content was reported as 32.4% in fresh cassava tuber while the starch contained 80.6% in dry cassava. The protein contents of fresh and dry cassava were also reported as 1% and 1.41%. The fresh cassava tubers had 65% of moisture, 0.9% of ash, and 0.03% of phosphorus (Okudoh et al., 2014).

Cassava crop is very resilient and can be cultivated in a wide variety of agro-ecological zones. It is normally propagated by cut stems and can thrive in marginal environments where crops such as maize, banana, sorghum, sugarcane, and sugar beet, which have a higher yield of carbohydrates per hectare, cannot survive. Besides the fact that only, cassava has the advantage of requiring a low agrochemical input. Cassava is also one of the most drought-tolerant crops and can be successfully grown on marginal soils. Another advantage is that it produces higher yields per hectare of land than another crop in its categories such as maize, yam, rice, and wheat. It is one of the most efficient producers of starch and carbohydrates among all crops (Ezui et al., 2016; Okudoh et al., 2014; Sánchez et al., 2017).

Global demand for cassava by-products is on the increase as the world leaders in cassava production, Nigeria, Brazil, and Thailand, transform industrial uses of

cassava (Okudoh et al., 2014). Cassava starch has several industrial applications. The raw material is cassava roots. The starch content in the roots ranging from 20% to 32% that varies by region, climate, soil, and cultivation, while the water content in the roots is around 60%. Also, it is a common practice for local farmers to use cassava hay and tubers for animal feed, as it is done worldwide in the Tropics. But it is in times of drought where the importance of this plant arises as it becomes a survival crop for cattle and goats (Dai et al., 2006; Le et al., 2013; Liu et al., 2013; Sánchez et al., 2017).

2.4.2 Nitrogen source

The nitrogen source used to produce α-amylase may be organic or inorganic. The most commonly used organic sources of nitrogen include yeast extract, peptone, and soya bean meal. A few of the inorganic nitrogen sources include ammonium sulfate, ammonium hydrogen phosphate, and ammonium chloride (Sundarram et al., 2014). Soya bean meal was found as the best nitrogen source for α-amylase production by *Bacillus* sp. I-3 (Francis et al., 2003; Goyal et al., 2005; Sodhi et al., 2005). Tanyildizi et al. (2005) suggested that peptone increased enzyme activity, while *Bacillus amylolyticus* secreted the maximum α-amylase in a medium supplemented with 1% peptone, 0.5% yeast extract, and 0.5% maltose under vigorous shaking conditions (Dettori-Campus et al., 1992). Aiyer (2004) compared the influence of organic and inorganic nitrogen sources and reported peptone to be a better nitrogen source for enzyme production by *B. licheniformis* SPT 278 than ammonium hydrogen phosphate, the best among inorganic nitrogen sources. L-Asparagine was reported to be one of the most promising nitrogen sources for α-amylase production by *Thermomyces lanuginosus*. Yeast extract also resulted in significant α-amylase yield (Nguyen et al., 2000). The supplementation of casein hydrolysate to the medium resulted in a 143%

increase in α-amylase productivity by *A. oryzae* A1560 compared to ammonia. Yeast extract along with ammonium sulfate also gave significant enzyme productivity 110% by *A. oryzae* (Pedersen and Nielsen, 2000). Peptone (1%) and ammonium nitrate (1%), when supplemented individually, gave an increase in enzyme yield in SSF using coconut oil cake as a substrate, while sodium nitrate exhibited a negative influence (Ramachandran et al., 2004). The addition of corn steep liquor and ammonium hydrogen phosphate to SSF involving Amaranthus grains as substrate by *Aspergillus flavus* gave the high enzyme yield (Viswanathan and Surlikar, 2001).

2.4.2.1 Rice bran

Rice is grown and consumed widely as a staple food for more than half of the world's population. Rice bran is a source of many antioxidant compounds such as tocopherols, γ-oryzanol, and other phenolic (Aguilar-Garcia et al., 2007), which could help in health effects including lowering blood cholesterol, reducing platelet aggregation, and anti-inflammation (Chotimakorn et al., 2008; Lai et al., 2009)

Rice protein is the second most abundant component of milled rice, following starch. The main source of protein in rice is rice bran. The protein content ranges from 10% to 16% (Cao et al., 2009; Kulp and Ponte, 2000; Faria et al., 2012) depending on its varieties. Rice bran proteins can be divided into four parts according to their solubility, by Osborne fragments, including glutelin, albumin, globulin, and prolamin. The ratio of each protein is different in each rice species (Chanput et al., 2009).

The high amount of rice bran protein was found in pH precipitation by using alginate and carrageenan at pH 3.50 (Fabian et al., 2010). Defatted rice bran is considered as a waste by-product of rice milling. But, defatted rice bran still holds

important nutrients such as protein. Rice bran protein is a high-quality protein with unique nutritional value and nutraceutical properties (Saunders, 1990).

About the protease in rice bran, Sreedhar and Tiku (2016) mentioned that cupincin was found to be a homotrimer, consisting of 3 distinct subunits with molecular masses of 33.45 kDa, 22.35 kDa, and 16.67 kDa. About α-amylase inhibitor protein in rice bran, the 14-to-16-kDa rice salt-soluble protein from *RAG2* gene was found in embryo and seed grain. The 14-to-16-kDa protein can be an α-amylase/trypsin inhibitor of α-amylase from human salivary. The *RAG2* gene was expressed during the accumulation of the starch in rice endosperm (Zhou et al., 2017).

Helm and Burks (1996) suggested that rice bran protein is a mixture of hypoallergenic food that may be useful for infant formula. Recovery of this protein from defatted rice bran (Table 2.1) would lead to the development of new value-added products. For rice bran protein extraction, the yield of rice bran protein was about 40% by using acid precipitation (Chen and Houston, 1970).

Table 2.1 Type and molecular weight of protein in rice bran.

Rice protein	Brief description	Protein content (%) of the total protein	Molecular weight (kDa)	Reference
Glutelin	Major fraction of rice protein	22.7-40.25	10-60	Xia et al. (2012)
			45-150	Hamada (1997)
Albumin	Water-soluble protein	6.24-9.73	18-20	Cao et al. (2009)
		30.9-42.7	30-50	Chanput et al. (2009)
Globulin	Salt-soluble and sulfur-rich	12.5-24.9	15, 30,50	Chanput et al. (2009)
			16-130	Xia et al. (2012)
Prolamin	Alcohol-soluble	3.24-11.6	10, 15, 25	Chanput et al. (2009)
			10, 13, 16	Cao et al. (2009)

2.4.3 Essential elements

Supplementation of salts of certain metal ions could provide good growth of microorganisms and thereby better enzyme production as most amylases. α-Amylase is known to be a metalloenzyme. Ca^{2+} ion is reported to be present in most of these enzymes. The addition of $CaCl_2$ to fermentation media increased enzyme production (Francis et al., 2003; Patel et al., 2005). Positive results of the influence of $CaCl_2$ (0.1%) and NaCl (0.1%) on α-amylase production in SSF using Amaranths grains as substrate were recorded (Viswanathan and Surlikar, 2001). $LiSO_4$ and $MgSO_4$ increased α-amylase production by *Bacillus* sp. I-3 (Goyal et al., 2005; Sodhi et al., 2005), but $FeCl_3$

and $MgSO_4$ exhibited negative influence on α-amylase production (Viswanathan and Surlikar, 2001).

2.4.4 Temperature

Cultivation of certain groups of microorganisms divides the temperature into two temperatures, namely the temperature for the growth of microbial sources and the optimum temperature that the maximum enzyme production occurs. The influence of temperature on amylase production is related to microorganism growth. Hence, the optimum temperature depends on whether the culture is mesophilic or thermophilic. Some of microorganisms producing α-amylase and optimal temperature were listed in the above in Table 2.2. Thermophilic archaeal α-amylases are active and grow at high temperatures.

2.4.5 pH

Optimum pH is an important factor in the stability of enzyme produced. The enzymes are sensitive to pH; therefore, care must be taken to control the pH of the production process (Sundarram et al., 2014). The pH is used for determining the growth and morphology of microorganisms because they are sensitive to the concentration of hydrogen ions present in the medium. Many studies revealed that fungi required slightly acidic pH and bacteria needed neutral pH for optimum growth. pH has been reported to affect the synthesis and secretion of amylase (Sivaramakrishnan et al., 2006). The optimal pH for some microorganisms has been reported (Table 2.3).

2.4.6 Cultivation duration

The duration of fermentation is an important factor in the fermentation process. If this process is carried out in a shorter period than the optimal time, it is not possible to receive the maximum yield. Enzyme activity will increase with increasing

duration until the optimal time. In most cases, enzyme production begins to decrease if the incubation time further increases. That may be due to the reduction of nutrients in the medium or release of toxic substances. (Lévêque et al., 2000; Raul et al., 2014) *Bacillus subtilis*, a well-known producer of α-amylase was studied and revealed a high yield of α-amylase after 48 h of fermentation (Gangadharan et al., 2006). Comparison of different curing times compared with the yield of amylase from *Penicillium fellutanum* isolated from mangrove soil when incubated at 96 h showing maximum activity 136 U/ml. This activity increased by 2 times when compared with the 24 h incubation (68 ± 2.3 U/ml) (Erdal and Taskin, 2010). In the production of α-amylase from *Aspergillus oryzae* using coconut oil cake as a substrate, the maximum activity was found to be 1,752 U/GDS which resulted after 72 h of incubation (Thippeswamy et al., 2014). The optimum α-amylase activity of 62,470 U/g was found after fermentation for 72 h when produced by *Bacillus amyloliquefaciens* (Fabiana et al., 1999).

Table 2.2 Optimal temperature for amylase production by microorganisms.

Microorganism	Temperature (°C)	Reference
Psychrophilic bacteria		
Alteromonas haloplanktis	4	Feller et al. (1998)
Mesophilic bacteria		
Bacillus amyloliquefaciens	37	Syu and Chen (1997)
Bacillus subtilis	37	Mishra et al. (2005)
Bacillus licheniformis	40	Mendu et al. (2005)
Thermophilic bacteria		
Rhodothermus marinus	61	Gomes et al. (2003)
Bacillus stearothermophilus	55	Mielenz (1983)
Hyperthermophilic bacteria		
Thermatoga maritima	80	Vieille and Zeikus (2001)
Thermococcus profundus	80	Vieille and Zeikus (2001)
Thermophilic fungi		
Talaromyces emersonii	80	Bunni et al. (1989)
Thermomyces lanuginosus	50	Jensen and Olsen (1992)
Yeasts		
Saccharomyces cerevisiae	30	Møller et al. (2004)
Saccharomyces cerevisiae Y294	37	Cripwell et al. (2018)
Saccharomyces kluyveri	30	Møller et al. (2004)

Table 2.3 Optimal initial pH of the medium for amylase production by microorganisms.

Microorganism	Initial pH	Reference
Thermophilic anaerobic bacteria		
Clostridium thermosulfurogenes	7.0	Carlsen et al. (1996)
Hyperthermophilic archaea		
Pyrococcus furiosus	5.0	Vieille and Zeikus (2001)
Pyrococcus woesei	5.0	Vieille and Zeikus (2001)
Bacteria		
Bacillus licheniformis	7.0	Syu and Chen (1997)
Bacillus subtilis	7.0	Ashraf et al. (2005)
Bacillus amyloliquefaciens	7.0	Tanyildizi et al. (2005)
Yeasts		
Saccharomyces cerevisiae	5.0	Møller et al. (2004)
Saccharomyces kluyveri	5.0	Knox et al. (2004)
Molds		
Aspergillus oryzae	6.0	Carlsen et al. (1996)
Aspergillus ficuum	6.0	Kammoun et al. (2008)
Aspergillus niger RBP7	3.0	Mukherjee et al. (2017)

2.5 Extraction and purification of microbial amylases

Enzymes used for industrial applications usually crude preparations and require less downstream processing. While the enzymes used in the medical and pharmaceutical industries need to be highly purified. Besides, when used for studying the relationship between structures and biochemical properties, enzymes must be in pure forms (Gupta et al., 2003). After amylase was produced the extracted and purified using the following four major processes will perform centrifugation, precipitation, chromatography, and liquid-liquid extraction, depending on the desired enzyme properties. The combination of the above methods is used in a series of steps to achieve high purity. The number of steps involved in purification will depend on the extent of purity required (Sivaramakrishnan et al., 2007; Gangadharan et al., 2006; Fabiana et al., 1999; Kundu et al., 1970). Centrifugation is the process of using a centrifugal field to separate and select suspended particles in a liquid medium. The selective concentration of the supernatant is best achieved using ultrafiltration which is a process of separating a mixture of particles into respective particle components using membrane filters. Selective precipitation of enzymes involves enzyme precipitation based on solubility (or extent in which it can dissolve in a solvent). Chromatographing of crude enzymes involves the separation of enzymes to solve the problem due to its migration rate along with the stationary phase (Sundarram et al., 2014).

For partial purification, the ammonium sulfate precipitation with a different percentage of saturation is widely used salt because highly soluble at low temperatures. A thermostable acidic α-amylase produced by *Geobacillus bacterium* K1C was precipitated by using ammonium sulfate to 60% saturation at 4°C for 2 h (Sudan et al., 2018). A thermostable and Ca-independent *α*-amylase produced by *Bacillus*

amyloliquefaciens BH072 was precipitated by using ammonium sulfate to 70% saturation for 12 h (Du et al., 2018). A maltose-forming α-amylase produced by amylolytic lactic acid bacterium *Lactobacillus plantarum* S21 was precipitated by using ammonium sulfate to 80% saturation at 4°C for 1 h (Kanpiengjai et al., 2015). For the isoelectric precipitation, it occurs by lowering the overall charge of protein near-zero minimizes and, the stability of the enzyme to be purified must be checked at that pH. The temperature should be low to increase the stability of the enzyme. Isoelectric precipitation is not commonly used for purification of α-amylase. Due to the aggregation of many proteins and may include particulate fragments and protein-nucleic acid complex. If the initial composition of the crude enzyme is changed, the desired enzyme may not show the same solubility behavior. Moreover, isoelectric precipitation can result in the denaturation of proteins or the reduction of bioactivity (Burgess et al., 2009).

2.6 Characterization of α-amylase

α-Amylase has a variation in many characteristics depending on bacterial producers such as *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus stearothemophilus,* and *Bacillus subtilis* (Nigam and Singh, 1995). α-Amylase shows its highest specificity toward starch compared to other substrates, including amylose, amylopectin, and glycogen (Bendelow, 1963). α-Amylase display activity over a broad pH range from 2.0 to 12.0 (Arnesen et al., 1998). However, the optimal pH of most of the α-amylase falls in the acidic and neutral range, and the optimal temperatures ranging between 45 and 115°C (McMahon et al., 1999). The molecular weights of microbial α-amylases range from 12.5 to 160 kDa. Various cations, substrates, and other stabilizers

influence the thermostability of these enzymes (Arnesen et al., 1998). α-Amylase is metal activated enzymes that have a high affinity for the Ca^{2+} ion (Gupta et al., 2003). On the other hand, many metal cations, especially heavy metal ions, sulphydryl group reagents, *N*-bromosuccinimide, *p*-hydroxyl mercuribenzoic acid, iodoacetate, EDTA, and EGTA inhibit microbial α-amylases. α-Amylase from many microorganisms is also inhibited by Hg^{2+} ion (Hayashida et al., 1988).

2.7 Applications of α-amylase

The first commercial α-amylase was a pharmaceutical aid for the treatment of digestive disorders in 1984. In the present-day scenario, α-amylase is applied in several industrial processes such as in food, detergents, textiles, and the paper industry, for the hydrolysis of starch. In this light, microbial amylases have completely replaced chemical hydrolysis of the starch processing industry. They can also be of potential use in the pharmaceutical and fine chemical industries. Today, amylases have the major world market share of enzymes (Gupta et al., 2003). Microbial amylase, especially thermostable α-amylase, has a wide range of industrial applications due to higher thermal stability than different sugar profiles and long-lasting safety applications (Rao et al., 2007). For industrial applications of α-amylase and the microbial source such as, In the starch conversion process, *Bacillus amyloliquefaciens* and *Bacillus licheniformis* used to hydrolysis of starch into dextrins forming a less viscous starch suspension. In the bakery industry, *Bacillus stearothermophilus* used to convert the starch in the dough to smaller fermentable sugars. In the detergent industry, *Bacillus licheniformis* has also used to digests starch-containing foods to water-soluble dextrin (Gupta et al., 2003). In the textile industry *Bacillus* sp. Used in the removal of starch sizing agents from woven

fabric. For fuel alcohol production, *Escherichia coli*, *Bacillus subtilis* used to convert the starch into smaller fermentable sugars which acted upon by yeast to produce alcohol (Sundarram et al., 2014).

2.8 Determination of α-amylase activity

Enzyme activity is determined by measuring the reducing sugars released due to the action of α-amylase on the starch, the extent of degradation by reading the adsorption of starch-iodine complexes, and reducing the viscosity of the starch. Dinitro salicylic acid method (DNS) and Nelson – Somogyi are the colorimetric assays that need to heat or boiling for color development and detect the level of reducing sugars with absorbance value by spectrophotometer, then calculate amylase activity (Sundarram et al., 2014).

Amylase activity can be determined by the principle that starch and iodine react to form blue complexes. In the process of breakdown, this starch will turn to reddish-brown, then read the absorbance value after the enzyme reaction with the substrate ends, this ability to measure of the amount of starch degradation by α-amylase (Gupta et al., 2003).

The reducing viscosity of the starch suspension, this method is used to check the quality of flour in the baking industry. Two of the methods used to measure enzyme activity in terms of reducing the viscosity of starch suspensions were falling number method and Amylograph/Farinograph tests. In the falling number method, the amount of enzyme-substrate preparation will be estimated at 100°C, generally, malted flour is reduced by about 400. The amylograph test uses the principle of the relationship between the highest viscosity of the starch solution and the activity of the enzyme. The

less viscosity of the starch solution is the activity of the enzyme. The value of the Farinograph 400-600 Brabender is suitable for bread flour (Gupta et al., 2003).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials and instrumentation

The commercial or analytical grade of chemicals, such as 3, 5-Dinitrosalicylic acid (DNS) for DNS method (Bernfeld, 1955) and Bradford reagent (Bradford, 1976), and all culture medium compositions were used and purchased from Himedia (Hi-Media Laboratories Pvt Ltd, Mumbai, India). Instruments for production, purification, and characterization of amylase, and instruments required for all of the research activities were located at the Cassava and Products Research Center building, Suranaree University of Technology, and the Instrument Buildings of the Center for Scientific and Technological Equipment, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

3.1.1 Microorganism and cultivation

A strain of L-lactic acid-producing bacterium (*Lactococcus* sp. SUT 513) was obtained from the previous L-lactic acid project carried out at the Microbial Culture Collection and Applications Research Center, Institute of Science, Suranaree University of Technology. *Lactococcus* sp. SUT 513 was cultivated in cassava starch agar medium (RAM, Appendix A 2.1) under anaerobic condition in an anaerobic chamber (BRACTRON I, Shel Lab, U.S.A.) at 35°C for 24 h (broth) or 48 h (agar medium) for further investigation.

3.1.2 Standard medium for bacterial growth

The suitable medium for L-lactic acid-producing bacterial growth was the modified Rogosa agar (RAM) medium (Appendix A1.2; Rodtong and Ishizaki, 2003). One liter of broth medium composed of tryptone (pancreatic digest of casein), yeast extract, K_2HPO_4, tri-ammonium citrate, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$, $FeSO_4 \cdot 7H_2O$, and Cassava starch at concentration of 5.0, 3.0, 6.0, 1.0, 0.57, 0.12, 0.03, and 10.0 g, respectively. For agar medium preparation, 1.5% of agar was added into the broth. All media containing gelatinized cassava starch were autoclaved at 121°C for 15 min. Tryptone (pancreatic digest of casein) and yeast extract were purchased from Himedia (Hi-Media Laboratories Pvt Ltd, Mumbai, India). The other composition: K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$, $FeSO_4 \cdot 7H_2O$, and tri-ammonium citrate were obtained from Carlo Erba (Carlo Erba Reagents, Milan, Italy). Cassava starch was purchased from Sanguan Wongse Industries Co., Ltd. (Nakhon Ratchasima, Thailand).

3.2 Screening and selection of suitable medium compositions and cultivation conditions for amylase production by *Lactococcus* sp. SUT 513

Effects of medium compositions (individual nutrient particularly carbon and nitrogen sources) for amylase production were investigated. The experiments were conducted in 125 ml Erlenmeyer flasks containing 50 ml of sterilized modified RAM medium with the initial pH at 7.00, then inoculated with the 2% (around 2 x 10^6 cells/ml) inoculum size of *Lactococcus* sp. SUT 513 overnight culture, and incubated for 24 h at 35°C under anaerobic condition in the anaerobic chamber. After

cultivation, the cell-free supernatant (crude amylase) was obtained by centrifugation at 3,500 rpm and used for amylase activity assay. The weight of cell pellet and supernatant, the bacterial growth, and pH of cultered medium were collected and measured from cultivation, at start and end of cultivation. Protein concentration in supernatant was determined by the method of Bradford protein assay.

3.2.1 Concentration of cassava starch

The suitable concentration of cassava starch was studied by varying the starch concentration in basal medium at 0.5 to 2% (w/v), compared to 1% (w/v) of the standard medium. The medium formula that supported the highest amylase activity and good bacterial growth was selected for nitrogen source optimization and medium cost evaluation.

3.2.2 Types of nitrogen source

The suitable cassava starch concentration was selected to prepare basal medium for investigation of nitrogen sources. Rice bran (a local market, Surin province, Thailand), soy protein (Food grade soy protein, Food EQ Co., Ltd, Thailand), spent brewer's yeast (Beer Thai (1991) Public Company Limited, Thailand) (Table 3.1) were tested to replace the commercial tryptone and yeast extract (Hi-Media Laboratories Pvt Ltd, Mumbai, India).

The total nitrogen composition of each nitrogen source in these lists was used for calculating concentration applied in the optimized medium formula. A suitable type of N-source was selected for further investigation.

Table 3.1 Proximate analysis of alternative nitrogen sources (result from our previous research projects).

N-source	Composition (%, w/w)				
	Moisture content	Total C	Total N	Total P	Total K
Soy protein (Food EQ Co., Ltd, Thailand)	8.69	57.86	14.82	0.44	0.06
Spent brewer's yeast (Beer Thai (1991) Public Company Limited, Thailand)	3.66	72.33	5.17	0.89	1.39
Rice bran (a local market, Surin province, Thailand)	9.67	67.77	2.07	0.91	1.19
Sticky rice bran (a local market, Surin province, Thailand)	10.22	65.19	2.14	2.32	1.15

3.2.3 Concentration of the selected nitrogen source

The suitable concentration of the selected nitrogen source was studied by varying concentrations in the investigated medium, then selected one of the best N-source concentration for amylase production.

3.3 Statistical optimization of medium compositions and cultivation conditions for amylase production by response surface methodology (RSM)

In this step, defatted rice bran (Surin Bran Oil Co., Ltd., Buri Ram province, Thailand) was selected to replace rice bran (a local market, Surin province, Thailand) because the defatted rice bran provided longer shelf-life and more homogeneity of medium composition than rice bran. The interaction among three parameters: cassava starch concentration, defatted rice bran (Surin Bran Oil Co., Ltd., Buri Ram province, Thailand) concentration, and initial pH of the production medium were considered for their effects on bacterial amylase production, bacterial growth, and L-lactic acid production of *Lactococcus* sp. SUT 513. The parameters were selected for futher optimization using RSM by designing experiments, building statistical models, and confirmation of the model.

3.3.1 Central composite design (CCD)

To optimize the conditions for amylase production, Design-Expert 11.0 (Stat-Ease, Inc, Minneapolis, USA) was used to design the Central Composite Design (CCD) experiment. The levels of independent factors (cassava starch concentration, rice bran concentration, and initial pH of the production medium) were optimized by studying each factor in design at five different levels ($-\alpha$, -1, 0, +1, $+\alpha$) with α equal to 1.68179 (Table 3.2).

Table 3.2 Range of values for the response surface methodology (RSM).

Independent factor	Range of level				
	-α	-1	0	+1	+α
A: Cassava starch (%, w/v)	0.48	1.50	3.00	4.50	5.52
B: Defatted rice bran (%, w/v)	0.06	0.20	0.40	0.60	0.74
C: Initial pH	4.48	5.50	7.00	8.50	9.52

(The defatted rice bran was selected to replace rice bran)

The optimum value of each factor was taken at a central coded value considered as zero. The minimum (coded as (-α)) and maximum (coded as (+α)) range and the low (coded as (-1)) and high (code as (+1)) of experimental values of each factor used and the full experimental plan for RSM performed with 20 sets per experiment were listed in Table 3.3. This experiment included 8 factorial designs with 14 non-central points and 6 central points. All experiments were carried out in triplicate.

Table 3.3 Central composite design (CCD) for optimization of amylase production medium in coded units and actual value.

Run no.	A		B		C	
	Code	Actual	Code	Actual	Code	Actual
1	0	3.00	+α	0.74	0	7.00
2	-1	1.50	-1	0.20	-1	5.50
3	0	3.00	0	0.40	0	7.00
4	+1	4.50	+1	0.60	-1	5.50
5	-1	1.50	+1	0.60	+1	8.50
6	0	3.00	-α	0.06	0	7.00
7	+1	4.50	-1	0.20	0	8.50
8	0	3.00	0	0.40	-α	4.48
9	+1	4.50	+1	0.60	+1	8.50
10	0	3.00	0	0.40	0	7.00
11	-1	1.50	-1	0.20	+1	8.50
12	0	3.00	0	0.40	0	7.00
13	0	3.00	0	0.40	0	7.00
14	0	3.00	0	0.40	0	7.00
15	+α	5.52	0	0.40	0	7.00
16	0	3.00	0	0.40	+α	9.52
17	0	3.00	0	0.40	0	7.00
18	-α	0.48	0	0.40	0	7.00
19	-1	1.50	+1	0.60	-1	5.50
20	+1	4.50	-1	0.20	-1	5.50

A: Cassava starch concentration, B: Defatted rice bran concentration, C: Initial pH of the amylase production medium

The experiments were conducted in 125 ml Erlenmeyer flasks containing 50 ml of sterilized development medium of different pH 4.48-9.52, inoculated with the freshly prepared 2% ($2x10^6$ cells/ml) inoculum and incubated for 24 h at 35°C under anaerobic condition. After cultivation, the cell-free supernatant was harvested by centrifugation at 3,500 rpm and used for amylase activity detection. Using RSM for result evaluation, the relationship among the factors, as cassava starch concentration, defatted rice bran concentration, and initial pH were showed mathematically in the form of a polynomial model, and process orders with quadratic.

3.3.2 Statistical analysis and modeling

The amylase production data from RSM was imported to Design Expert 11.00 for analysis of variance (ANOVA). The quadratic models for predicting the optimal points were expressed according to the quadratic equation;

$$Y= \beta_0 + \beta_1A + \beta_2B + \beta_3C + \beta_{11}A^2 + \beta_{22} B^2 + \beta_{33}C^2 + \beta_{12}AB + \beta_{13}AC+ \beta_{23}BC$$

Where Y was response variable; β_0 was intercepted; β_1, β_2, and β_3 were linear coefficients; β_{11}, β_{22}, and β_{33} were squared coefficient; β_{12}, β_{13}, and β_{23} were interaction coefficients, and A, B, C, D, A^2, B^2, C^2, AB, AC, and BC were levels of independent factors. The significance of the quadratic model equation was expressed by the coefficient of determination (R-squared) and the statistical significance of the model was confirmed by Fischer's test value (F-value).

3.4 Duration of bacterial growth and amylase production

The optimal complex medium was obtained from RSM experiments (section 3.3), was applied for determination of bacterial growth duration and amylase production. The cultivation was performed in glass fermenter 7.5 liters (New Brunswich, BioFlo®/

CelliGen® 115) with a working volume of 3.0 liters. The fermenter was placed on a magnetic stirrer with agitation speed 150 rpm, and the medium temperature was kept at the optimum point by running water from a water bath act as thermostability of the glass fermenter. The pH in the fermenter was measured by a pH meter. Samples were taken at the bottom by silicon tube. The fermenter was inoculated with 30 ml of 12 h *Lactococcus* sp. SUT 513 inoculum. The starter culture was prepared in the same medium as the amylase production medium.

3.5 Partial purification of amylase produced by *Lactococcus* sp. SUT 513

3.5.1 Ammonium sulfate precipitation

Crude amylase was precipitated by ammonium sulfate (30-100% saturation) at 4°C. Then, the solution was left at 4°C for 30 min before centrifugation at 8,000 rpm for 10 min. The supernatant was discarded; the precipitated protein was dissolved in 50 mM Na-phosphate buffer pH 6.5 and diafiltrated (10 kDa cut-off) against the same buffer at 4°C until equilibrium. An equilibrated solution was clarified by centrifugation to remove insoluble particles, then store at -20°C for further study of amylase characteristics (Kanpiengjai et al., 2015).

3.5.2 Isoelectric precipitation

Rice bran protein can precipitate at pH 3.5 (Fabian et al., 2010). The crude enzyme was precipitated from the culture broth by using 3 M HCl to adjusted pH to acidic condition from pH 3.0-5.0, then incubated for 30 min at 4°C, then centrifugation at 8,000 rpm for 10 min. The supernatant was discarded; the precipitated protein was dissolved in 50 mM tris-maleate buffer pH 6.8 or 100 mM phosphate-buffered saline

(PBS) pH 7.4. An equilibrated solution was clarified by centrifugation to remove insoluble particles, then store at -20℃ for further study of amylase characteristics.

3.6 Characterization of the partially purified amylase

3.6.1 Effects of temperature and pH on amylase activity

To determine the optimal pH, the starch solution (1%, w/v) and enzyme dilutions prepared in 100 mM buffer with pH ranging from 3.0 to 9.0 with citric acid buffer for pH 3.0-5.0, sodium phosphate buffer for pH 6.0-7.0, tris-maleate buffer for pH 6.8 and tris–HCl buffer pH 8-9 were incubated at 35℃ for 30 min according to the assay conditions. The optimal pH for amylase activity was selected for further investigation. To study the effect of temperature on amylase activity, the reaction mixtures prepared at the optimal pH, were incubated at different temperatures ranging from 0℃ to 100℃ and assayed for the amylase activity to find the optimal temperature and compared with their relative activity of the highest activities (taken as 100%).

3.6.2 Effects of temperature and pH on amylase stability

Thermostability of amylase produced by *Lactococcus* sp. SUT 513 was performed by pre-incubating the enzyme sample prepared at the optimal conditions resulted from section 3.6.1, at 0-100℃ for 30 min, and then assayed for α-amylase activity under condition at 35℃ for 30 min. The pH stability of α-amylase was determined by pre-incubating the enzyme with buffer having different pH values of pH 3.0-9.0 (0.1 M of citric acid buffer for pH 3.0-5.0, sodium phosphate buffer for pH 6.0-7.0, tris-maleate buffer for pH 6.8, and tris–HCl buffer for pH 8-9, for 30 min, and then assayed to check for residual α-amylase activity and compared with their relative activity of amylase activity of the control (non-incubating enzyme, taken as 100%).

3.6.3 Effects of metal ions and chelating agent on amylase activity

To determine the effect of metal ions and chelating agent on amylase activity, the alkaline metal ion: Na^{+} (NaCl), alkaline earth metal ions: Mg^{2+} ($MgSO_4$) and Ca^{2+} ($CaCl_2$), transition metal ions: Mn^{2+} ($MnSO_4$) and Fe^{2+} ($FeSO_4$), and chelating agent as EDTA at 5 mM concentration were added to the reaction mixture of substrate and enzyme at the optimal pH. The amylase activity was detected. The enzyme activity under conditions (pH 6.8 and 35℃ for 30 min) was considered as 100% (control) and the activity of the enzyme added with different metal ions, and the chelating agent were shown relative to that of the control.

3.6.4 Stability of amylase produced by *Lactococcus* sp. SUT 513 during storage at 4℃ and effect of freezing and thawing

The stability of the amylase in crude enzyme was determined after stored at 4℃ for 39 days. The relative activity was assessed at desired time intervals. The effect of freezing and thawing was studied by determination of amylase activity from crude amylase, after freezing at -20℃ for 14 days, then thawing with repeating the freezing-thawing for 3 times.

3.6.5 Determination of amylase molecular weight

The molecular weight of amylase in the partially purified enzyme was determined by comparing its electrophoretic mobility with proteins marker that having known molecular weights. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (He, 2011) was performed. For preparing polyacrylamide gel, a polyacrylamide solution (10%) (Appendix A, 3.1) was prepared by gently mix and immediately pour the solution into assembled glass plates on the casting frame until 2 cm below the rim of the small plate, then gently overlay the solution with iso-propanol

and let solidify for 30 min, then remove the iso-propanol and prepare a stacking gel (4%) (Appendix A, 3.1), then gently mix and immediately pour the solution on top of the 10% gel in the assembled glass plates, then insert a comb, and let solidify for 30 min. For electrophoresis was prepared by place the gel in an electrophoresis chamber and fill the chamber with an electrophoresis running buffer (1X) (Appendix A, 3.2), then mix 10 µl of each of the samples with 10 µl of SDS loading buffer (Appendix A, 3.3). The samples were denatured at 95℃ for 5 min and cool down on the ice, then load 20 µl sample per well. For estimating the size of the protein-samples, an SDS-PAGE molecular weight standard solution (PageRuler™ Unstained Protein Ladder, Thermo Fisher Scientific, U.S.A; Bio-Rad Precision Plus, Bio-Rad, U.S.A.) was used. The band of protein in sample was separated by running gel electrophoresis for 15 min at 50 V followed by running constant voltage at 80 V until finished, then stain the gel with staining solution (Appendix A, 3.5), then destain the gel with a destaining solution (Appendix A, 3.6) overnight.

3.7 Detection and analysis

3.7.1 Determination of amylase activity

Amylase activity was determined by measuring reducing sugars produced from soluble starch by the enzyme hydrolysis. The reaction contained 0.5 ml of 1% (w/v) soluble starch in sodium phosphate buffer (pH 6.8) and 0.5 ml of supernatant or cell pellet. The reaction mixture was incubated at 35℃ for 30 min (Takenaka et al., 2015). The reaction stopped by adding 3 ml of 3, 5- dinitrosalicylic acid (DNS) reagents (Appendix A 1.3.1) (Bernfeld, 1955). The reaction mixture was heated for 10 min at 100°C in boiling water bath (1245PC, Shel Lab, U.S.A.), then put in cold water, and

add 10 ml of distilled water. The absorbance was read at 540 nm (Smartspec^{TM}3000, BioRad, U.S.A.) to estimate reducing sugars released. The activity of amylas was determined as U/ml. Enzyme activity was calculated from the amount of reducing sugars produced in 30 min. One unit of amylase was defined as the amount of enzyme release 1 µmol of maltose per min under the assay conditions, using maltose (100–1000 mg) as standard

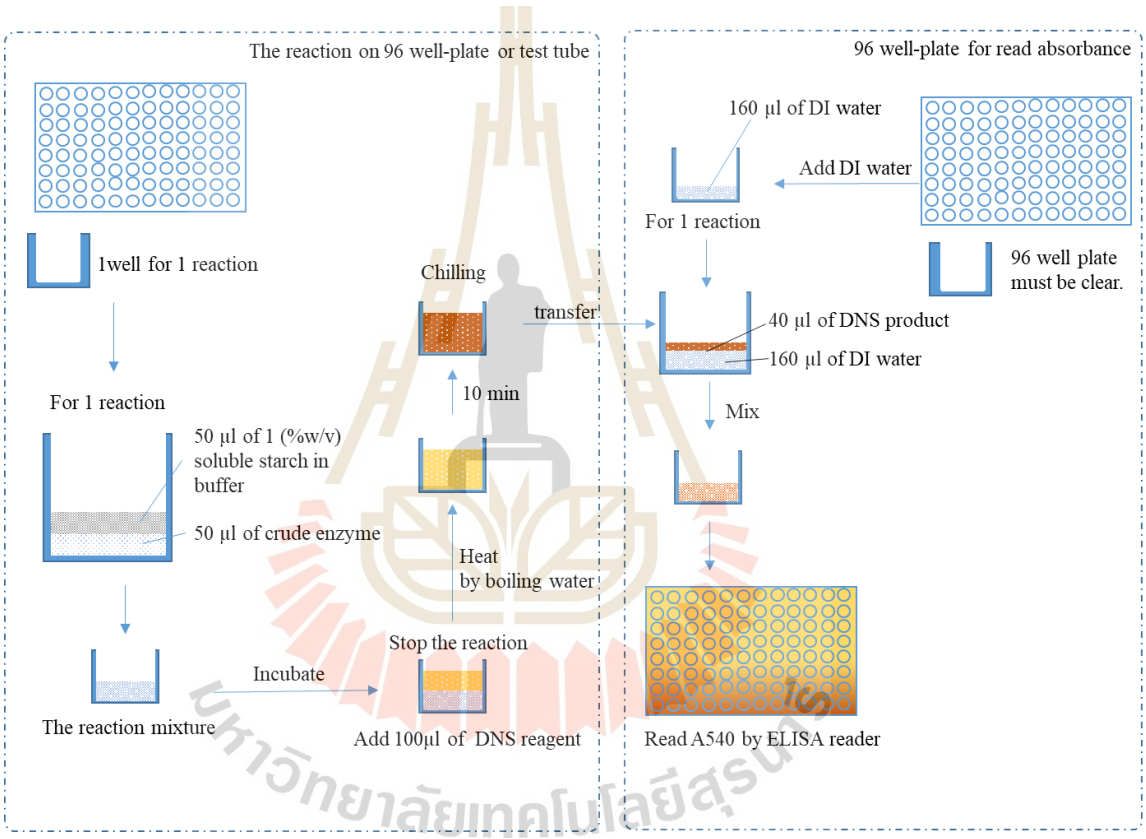


Figure 3.1 Amylase activity procedure in 96-well plate containing 200 µl per well, for amylase reaction and spectrophotometric reading volume.

For 96-well plate, the reaction mixture consisted of 50 µl of crude amylase and 50 µl of soluble starch (1%, w/v) in 50 mM Na-phosphate buffer pH 6.5. The reaction mixture was incubated at 35℃ for 30 min, then stopped by adding 100 µl of DNS reagent, then mixed and incubated at 100℃ in boiling water for 30 min, then put into

cold water. After chilling, 800 µl of DI water was added, mixed, and the absorbance of reducing sugars released from soluble starch was measured at 540 nm.

One unit of amylase activity was defined as the amount of enzyme releasing 1 µmol of reducing sugars (as maltose equivalents) per min under assay condition (sections 3.2-3.6).

$$\text{Enzyme activity (U/ml)} = \frac{\text{Amount of reducing sugars (mM)} \times 1000 \times \text{dilution factor}}{\text{Molecular weight of glucose} \times \text{time} \times \text{enzyme volume}}$$

3.7.2 Determination of protein concentration

The protein concentration in supernatant was measured by Bradford's method (Appendix A 1.2.1) with bovine serum albumin (BSA) as a standard (Bradford, 1976). On 96-well microtiter plate, using 10 µl of protein sample and 250 µl of Bradford's reagent were mixed and incubated at least 5 min but not longer than 1 h. The absorbance was reading at 595 nm.

3.7.3 Enumeration of bacterial growth

Growth of amylase-producing bacterium in media containing different nutrient sources was detected by the standard method (spread plate). Serial dilutions of cultures were performed. One ml of each dilution was transferred and spread onto the cassava starch agar medium (RAM). Drop plate technique was also used for bacterial enumeration of RSM sample, 10 µl of each dilution was transferred and dropped onto the cassava starch agar medium (RAM). The plates were incubated anaerobically at 35°C for 48 h.

3.7.4 Determination of residual substrate concentration

The residual cassava starch in the medium during optimization was performed in the amylase production step. The medium after cultivation was tested for

the starch-iodine complex assay. The iodine reagent was diluted 5 folds by DI water before analysis. The standard curve was prepared by mixing 100 µl of cassava starch at concentration 0.00-0.05 % (w/v) with 100 µl of iodine solution, then read the absorbance at 580 nm before 30 min. The sample was diluted in the range of the standard curve before conducting starch-iodine complex assay (Xiao et al., 2006).

3.8 Statistical analysis

The screening of medium optimization experiment was designed by Completely Randomized Design (CRD), and the response surface methodology (RSM) experiment was planned by the Central Composite Design (CCD). Studies were performed in duplicate or triplicate. Mean and standard deviation values were calculated**.** The student's t-test at a p-value of 0.05 was used to analyze some results of amylase activity in the optimization step at the same number of treatments.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Microorganism and cultivation medium

Amylase and L-lactic acid-producing bacterium, *Lactococcus* sp. SUT 513 was cultivated on starch agar medium (RAM) under an anaerobic condition at 35℃ for 48 h in an anaerobic chamber (Figure 4.1a).

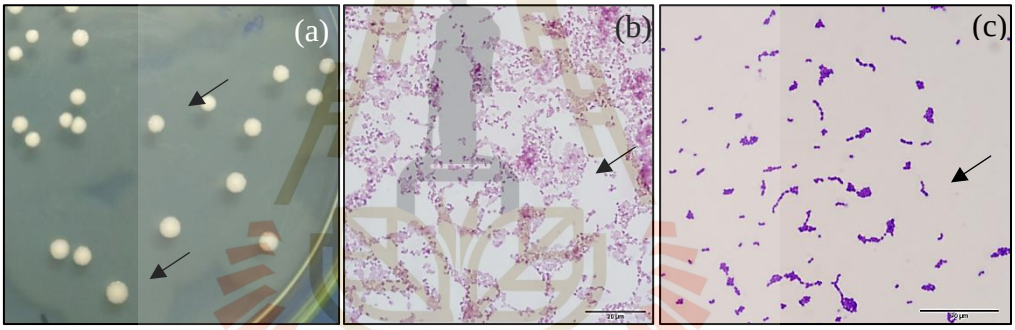


Figure 4.1 Colonies (arrow) and cells (arrow) of *Lactococcus* sp. SUT 513: colonies grown on starch agar medium for 48 h (a). Bacterial cell (arrow) morphology by Gram staining observed under a light microscope at 1,000X (b), and at 2,000X (c).

The colony of *Lactococcus* sp. SUT 513 (Figure 4.1a) was circular form, raised elevation, smooth surface, entire edge or sometimes undulate edge in high water-containing agar surface, and opaque optical. The cultural characteristics of *Lactococcus* sp. SUT 513 were filiform and non-motile which can be observed after streaking and stabbing the bacterium on starch agar, and motility medium, respectively.

However, the colony form depends on the moisture of agar. In broth, this strain was a little turbid and viscous growth, at the bottom, but does not aggregate. Under the light microscope, the bacterium was Gram-positive, non-endospore forming, and non-flagellated, and cell in pair of two or four, and long-chain form.

After point inoculating on cassava starch agar medium and incubating *Lactococcus* sp. SUT 513 exhibited a larger average size of the clear zone (Figure 4.2), which amylases hydrolyze starch molecules than *Lactococcus* sp. SUT 1 approximately 1.98 times.

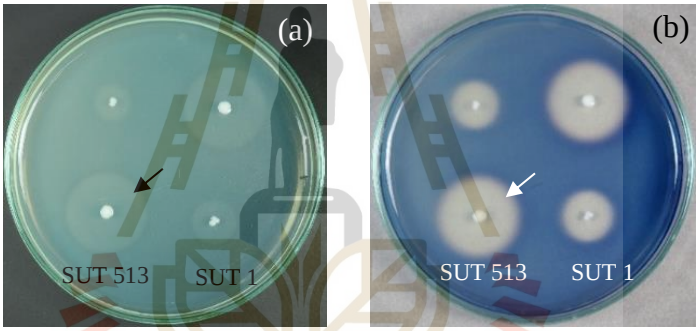


Figure 4.2 Amylase production by *Lactococcus* sp. strain SUT 513 detected on starch agar: the bacterial colony (arrow, a), after point inoculations and incubation for 48 h, then overlaid with 1% iodine solution for the clear zone (arrow, b).

This was a reason for selecting the strain SUT 513 for the amylase production optimization experiment. Bacterial amylase activity was detected in broth with 1.00% (w/v) of cassava starch. The enzyme activity in the supernatant was approximately 4.92 times higher than the cell pellet (Figure 4.3). These reveal that *Lactococcus* sp. SUT 513 had extracellular and cell-bound amylolytic enzymes. The value of the amylase

activity in the supernatant at the beginning was higher than this present because some amylase in the supernatant might be denatured by thawing.

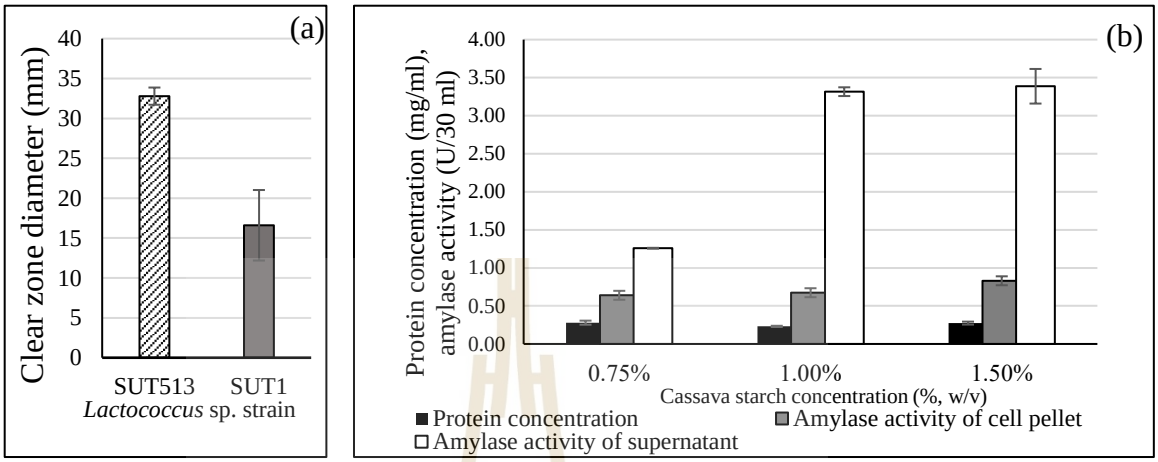


Figure 4.3 Clear zone diameters (mm) from point-inoculation on RAM (a) and protein concentration in supernatant and amylase activity both of supernatant and cell pellet from *Lactococcus* sp. SUT 513 (b).

4.2 Screening and selection of suitable medium compositions and cultivation conditions for amylase production by *Lactococcus* sp. SUT 513

4.2.1 Concentration of cassava starch

Cassava starch was used as a carbon source. The amylase activity was given at different values and different concentrations of cassava starch. The highest amylase activity was exhibited as 0.33 U/ml by 2.00% (w/v) of cassava starch (Figure 4.4). The 2.00% (w/v) of cassava starch was selected to be the best of amylase production. The highest cell growth in 1.00% (w/v) of cassava starch (Figure 4.4) was made amylase activity per cell value lower than 1.00% (w/v) of cassava starch 1.12 times.

When considered bacterial growth and pH value, The bacterial growth decreased when the concentration of cassava starch increased, which might be the effect of L-lactic acid production and starch viscosity were increased. However, amylase activities were directly related to starch concentration range increasing from 1.0 to 2.0% of cassava starch in RAM (Modify Lactobacillus MRS medium) broth.

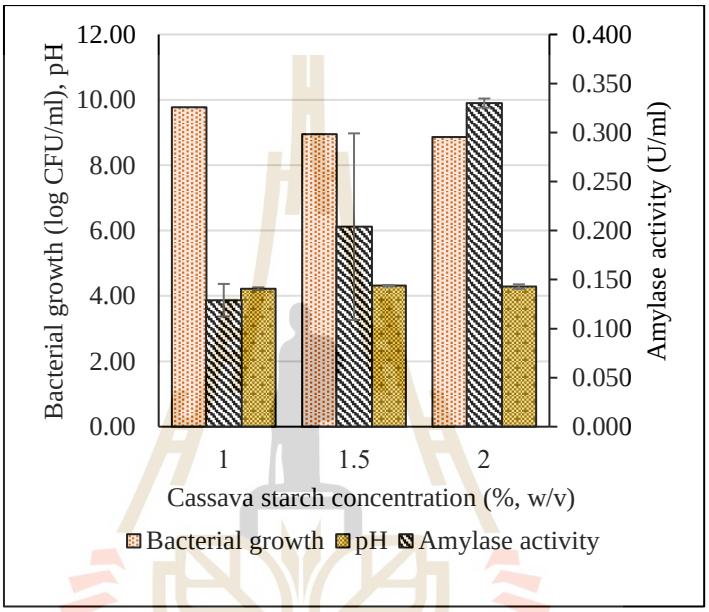


Figure 4.4 Comparison of amylase activity (U/ml) of supernatant and pH after cultivation *Lactococcus* sp. SUT 513 in the RAM medium containing 1.0, 1.5, 2.0% (w/v) of cassava starch under the anaerobic condition at 35°C for 24 h.

From these results, amylase activity increased when the substrate increased that amylase was produced by bacteria in primary metabolite so when the substrate increased, amylase production would be increased too. The enzyme activity will go down at substrate or product inhibition point. The 2.0% (w/v) of cassava starch (Table 4.1) might not be the optimum point for amylase production because the trend line of

amylase activity still going up. The amylase production in higher cassava starch concentration was studied in the Response Surface Methodology part.

Table 4.1 Growth and amylase activity of *Lactococcus* sp. SUT 513 in RAM medium containing cassava starch after cultivation for 24 h**.**

Cassava starch concentration (%, w/v)	pH	Amylase activity (U/ml)	Bacterial growth (log CFU/ml)
1.00	4.22 ± 0.040	0.129 ± 0.016	9.77
1.50	4.31 ± 0.023	0.204 ± 0.096	8.95
2.00	4.29 ± 0.068	0.330 ± 0.004	8.86

4.2.2 Types of nitrogen source

Nitrogen source in the original medium was tryptone and yeast extract. The alternative types of nitrogen source from rice bran, soy protein, splendid yeast, and urea were compared and calculated for the optimal formula (Table 4.2). All formulas contained 2.0% (w/v) of cassava starch.

After cultivation of *Lactococcus* sp. SUT 513 under an anaerobic condition at 35°C for 24 h in a different type of nitrogen source, the RAM medium that contained rice bran 2.0 % (w/v) cassava starch and exhibited the highest amylase activity 0.89 U/ml (Figure 4.5). Therefore, rice bran was selected as a new nitrogen source in the medium.

Table 4.2 The medium composition containing various types and concentrations of nitrogen source.

Component	Medium formula and component concentration (g/l)					
	1	2	3	4	5	RAM
Pancreatic digest of casein	5.00	-	-	-	-	5
K_2HPO_4	6.00	6.00	6.00	6.00	6.00	6.00
Yeast extract	-	-	-	-	3.00	3.00
tri-Ammonium citrate	-	-	-	-	-	1.00
$MgSO_4 \cdot 7H_2O$	0.57	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	0.12
$FeSO_4 \cdot 7H_2O$	0.03	0.03	0.03	0.03	0.03	0.03
Cassava starch	20.00	20.00	20.00	20.00	20.00	20.00
Soy protein	-	8.00	8.00	-	-	-
Spent Brewer's yeast	-	-	-	-	-	-
Rice bran	-	-	-	8.00	-	-
Urea	-	-	-	-	2.00	-

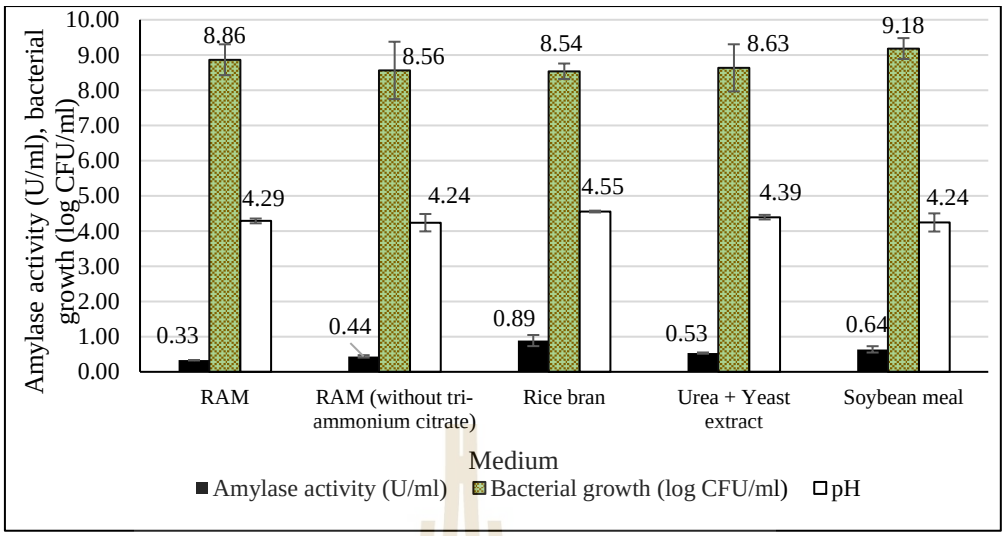


Figure 4.5 Growth, pH, and amylase production by *Lactococcus* sp. SUT 513 in medium containing 2.00% (w/v) of cassava starch with different types of N-source under the anaerobic condition at 35°C for 24 h.

From the result, the type of nitrogen source was one of the important medium components and expensive. The rice bran was very cheap and efficient for using in amylase production medium. The cost of tryptone, yeast extract, and tri-ammonium citrate per gram were 4.00, 3.50, and 22.00 Baht/g that means the new rice bran formula could reduce cost from these components by completely replacing in the medium. The rice bran composed of the pericarp, seed coat, nucellus, aleurone layer, and a little bit of endosperm that may be a growth factor for microbial growth. But rice bran still has the consistency of raw materials problem, unlike urea and soybean meal with more consistency. This problem was solved by using rice varieties from the same source and the same milling plant to reduce variability as much as possible. Another problem was the rice bran component not homogeneous as the other alternative nitrogen sources. This problem was solved by sieving with sake sieve pore size 150 µm (Table

4.3). After screening, the rice bran component not only going to the same size and became homogeneous, but also remove Rice weevil, *Sitophilus oryzae,* an egg that help longer storage time of rice bran.

Table 4.3 Growth and amylase activity of *Lactococcus* sp. SUT 513 in the medium containing cassava starch.

Medium	Amylase activity In supernatant (U/ml)	Bacterial growth (log CFU/ml)	pH
RAM	0.330 ±0.004	8.865 ± 0.438	4.29 ± 0.068
RAM (without tri ammonium citrate)	0.438 ±0.034	8.561 ± 0.815	4.24 ± 0.247
Rice bran (150 µm size screening)	0.888 ±0.158	8.538 ± 0.219	4.55 ± 0.025
0.2% Urea + Yeast extract	0.533 ±0.017	8.635 ± 0.669	4.39 ± 0.065
Soy protein	0.638 ±0.090	9.182 ± 0.296	4.24 ± 0.258

One unit of amylase (U) was defined as the amount of enzyme release 1 µM of maltose per min under the reaction at pH 6.8, 35°C for 30 min.

4.2.3 Concentration of the selected nitrogen source

Rice bran was selected as a nitrogen source since it provides the maximum amylase activity. For finding suitable amylase production and investment, after cultivation *Lactococcus* sp. SUT 513 for 24 h in different concentrations of rice bran medium. 0.4% (w/v) of rice bran exhibited the highest amylase activity at 1.054 U/ml (Figure 4.6)

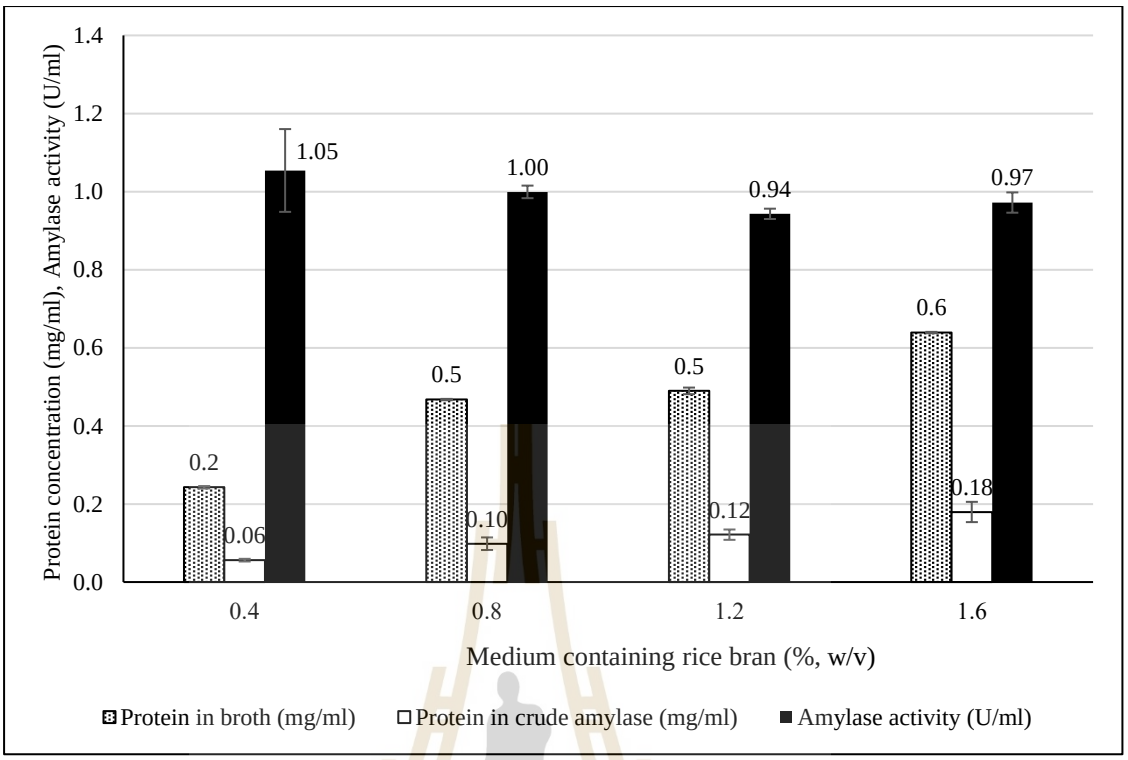


Figure 4.6 Apparent amylase activity and pH after cultivation in 2% (w/v) of cassava starch, and rice bran at various concentrations: 0.4, 0.80, 1.20, 1.6% (w/v) by *Lactococcus* sp. SUT 513 under the anaerobic condition at 35°C for 24 h.

The amylase activity in the crude enzyme was not significantly different among 4 concentrations (Table 4.4) that look like the amylase production was the same, but if we considered on specific activity 0.4% (w/v) which gave the maximum value of 18.75 U/mg. Sivaramakrishnan et al. (2006) suggested that bacteria needed neutral pH for optimum growth. This bacterium had a range of growth around pH 4.3-7.0. This bacterium could be acidophilic.

Table 4.4 Protein concentration and amylase activity of *Lactococcus* sp. SUT 513 in medium containing rice bran.

Rice bran (%, w/v)	Protein in medium (mg/ml)	Protein in crude amylase (mg/ml)	Amylase activity (U/ml)	pH
0.4	0.243	0.056 ± 0.0034	1.05 ± 0.106	4.57 ± 0.036
0.8	0.467	0.098 ± 0.0161	1.00 ± 0.016	4.57 ± 0.010
1.2	0.490	0.121 ± 0.0132	0.94 ± 0.013	4.54 ± 0.000
1.6	0.639	0.179 ± 0.0260	0.97 ± 0.026	4.58 ± 0.006

One unit of amylase (U) was defined as the amount of enzyme release 1 µM of maltose per min under the reaction at pH 6.8, 35°C for 30 min.

4.2.4 Effect of selected nitrogen source on medium characteristics, bacterial growth, and amylase production

At 24 h cultivation, the bacterial growth and amylase activity showed non-significantly different among 4 concentrations of rice bran (Figure 4.7). Rice bran could, therefore, have a small effect on amylase production. The medium contained a high solid particle of rice bran at 1.6 % (w/v), but this particle was able to easily separate by centrifugation. The developed medium showed the dropping of pH after autoclaving when increased rice bran concentration that showed in Table 4.5. pH of the medium was adjusted to pH 7 after preparing. The pH of medium slightly dropped to pH 6.97-6.66 after autoclaving along with rice bran concentrations from 0.4-1.6% (w/v). The property of rice bran and pH was used to the information for the Response Surface Methodology (RSM) step.

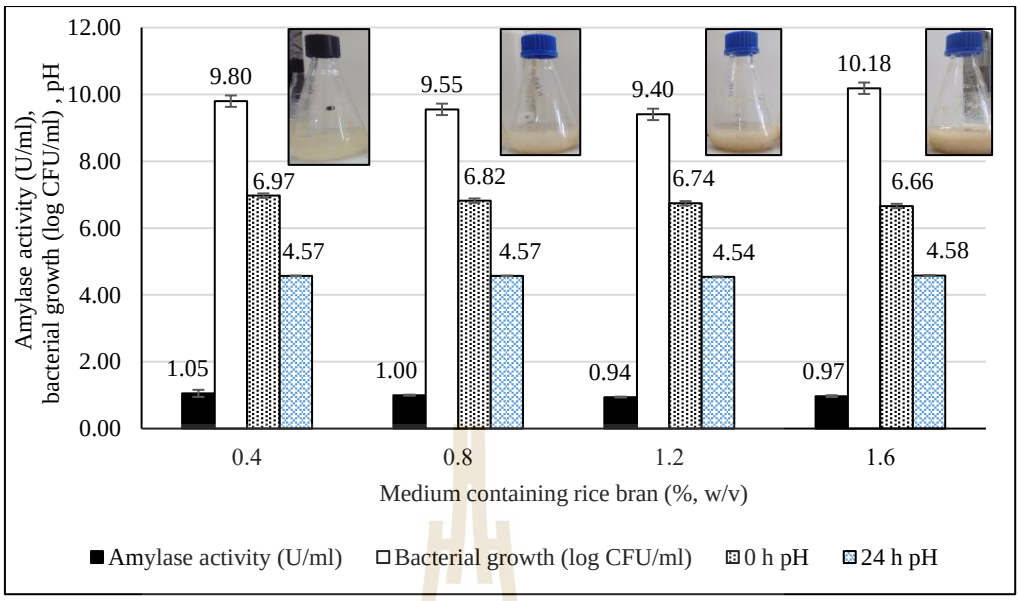


Figure 4.7 Comparison of amylase activity, growth, pH, and appearance of the medium after cultivating *Lactococcus* sp. SUT 513 in 2% (w/v) of cassava starch with 0.4-1.6% (w/v) of rice bran under anaerobic condition at 35℃ for 24 h.

Table 4.5 Growth and pH of *Lactococcus* sp. SUT 513 in the RAM medium containing rice bran at various concentrations.

Rice bran (%, w/v)	pH (pre-autoclave)	pH (post-autoclave and inoculate)		Bacterial growth (log CFU/ml)
		0 h	24 h	
0.4	7.00	6.97	4.57 ± 0.036	9.80 ± 0.086
0.8	7.00	6.82	4.57 ± 0.010	9.55 ± 0.325
1.2	7.00	6.74	4.54 ± 0.000	9.40 ± 0.600
1.6	7.00	6.66	4.58 ± 0.005	10.18 ± 0.351

One unit of amylase (U) was defined as the amount of enzyme release 1 µM of maltose per min under the reaction at pH 6.8, 35°C for 30 min

4.2.5 Cost evaluation of amylase production medium

The composition detail original and developed medium was shown in Table 4.6. The developed medium was more simple than the original medium because eliminating yeast extract, tryptone, and tri-ammonium citrate. The rice bran was applied as a nitrogen source. The cost of the medium was considered. The original medium was around 58.00 baht/l and the developed medium was around 12.50 baht/l which was around 79% cheaper than the original medium. The developed medium was cheap and provided amylase production by *Lactococcus* sp. SUT 513. The amylase activity, bacterial growth, and cost of developed medium composition were supported RSM study to the obtained wide range and more diverse of optimal condition.

Table 4.6 Comparison between the composition of original and developed medium for.

Component	Original medium (RAM)		Developed medium	
	Composition (g/l)	Cost (bath)	Composition (g/l)	Cost (bath)
Yeast extract	3.00	21.00	-	-
Tryptone	5.00	2.50	-	-
K_2HPO_4	6.00	11.00	6.00	11.00
tri-Ammonium citrate	1.00	23.00	-	-
$MgSO_4 \cdot 7H_2O$	0.57	0.75	0.57	0.75
$MnSO_4 \cdot 4H_2O$	0.12	0.07	0.12	0.07
$FeSO_4 \cdot 7H_2O$	0.03	0.05	0.03	0.05
Cassava starch	10.00	0.12	20.00	0.12
Rice bran	-	-	4.00	0.24

4.3 Amylase production by *Lactococcus* sp. SUT 513 in 3 liters of the suitable L-lactic acid production medium

The amylase production of *Lactococcus* sp. SUT 1 in 300 liters of L-lactic acid production medium was used to estimate the duration of amylase production by *Lactococcus* sp. SUT 513. The apparent amylase activity of *Lactococcus* sp. SUT 1 from the L-lactic production process was measured during 48 h cultivation in a pilot plant (Figure 4.8). The concentration of cassava starch in this medium was 12% (w/v). When incubating at 14 - 18 h, amylase activity exhibited at 1.11-1.14 U, after 18 h cultivation the amylase activity was decreased.

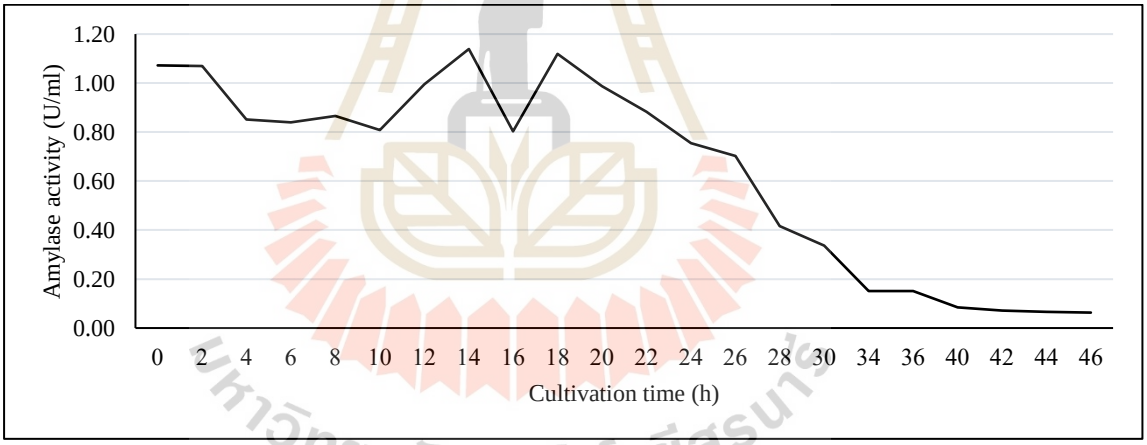


Figure 4.8 Amylase activity (U/ml) detected in the supernatant of *Lactococcus* sp. SUT 1 cultivated in 300 l of the medium containing 12% (w/v) cassava starch in a 500-l fermenter at 35°C for 48 h.

The amylase activity of *Lactococcus* sp. SUT 1 was similar to *Lactococcus* sp. SUT 513 strain that commonly used in L-lactic acid production with high concentration

of cassava starch. These data were used to estimate amylase production condition of strain SUT 513

The developed complex medium was performed using the RAM medium containing a mixture of cassava starch (Nakhon Ratchasima, Thailand), rice bran (Surin, Thailand), K_2HPO_4, $MgSO_4$, $MnSO_4$, $FeSO_4$ with 20, 4, 6, 0.57, 0.12, 0.03 g/l, respectively).

The time-courses of growth and amylase production by *Lactococcus* sp. SUT 513 was shown in Figure 4.9. Amylase production was initiated during the exponential growth phase of the strain, and began to increase rapidly in the late exponential growth phase. Amylase production reached its maximum activity of 4.848 U/ml at 18 h of incubation time, which corresponds to the end of the exponential growth phase.

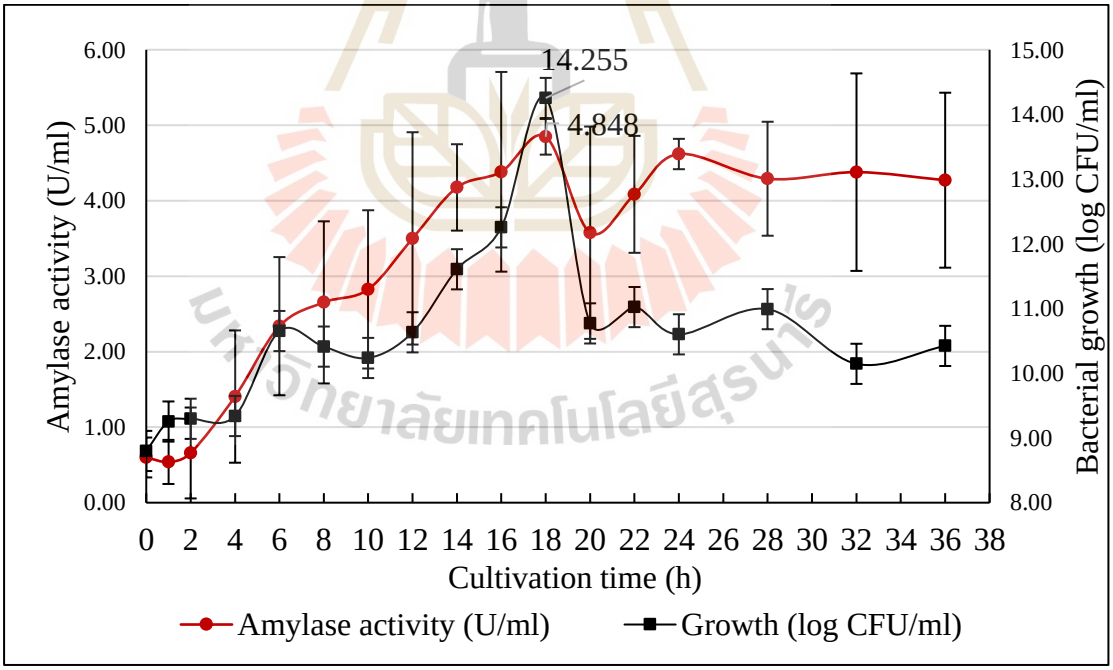


Figure 4.9 Time courses of growth of *Lactococcus* sp. SUT 513 (filled squares) and the production of amylase (filled circles) during cultivation in a 3-liter starch medium in the 5-liter fermenter with 150 rpm agitation at 35°C for 24 h.

For the result of 3-liter cultivation, the growth of bacterium was followed by viable cell counts, using the spread plate technique. At the start, bacterial growth was estimated to be 8.80 log CFU/ml. At the early of incubation time, growth slightly increased to 9.30 at 2 h and increase to 10.24 log CFU/ml at 10 h cultivation. From 10-18 h cultivation, bacterial growth rapidly increased to the maximum of 14.26 log CFU/ml at 18 h cultivation. At cultivation time of 20-36 h, growth quickly declined to 10.42-10.77 log CFU/ml.

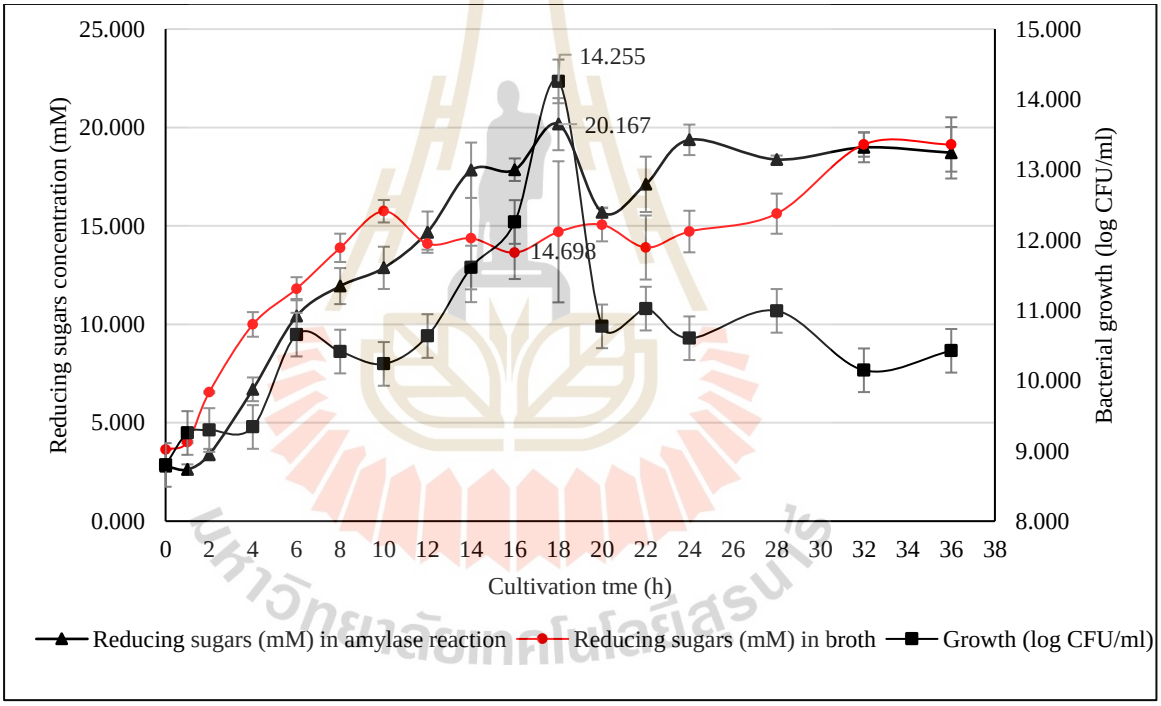


Figure 4.10 Time courses of growth of *Lactococcus* sp. SUT 513 (filled squares), the concentration of reducing sugars in the fermenter (filled triangles) during cultivation and reducing sugars in amylase activity assay (filled circles) during cultivation in a 3-liter starch medium in the 5-liter fermenter with 150 rpm agitation at 35℃ for 24 h.

The result of amylase production showed a positive relation between bacterial growth and amylase production so that the highest amylase activity exhibited by 4.848 U/ml at 18 h of incubation time.

When considering reducing sugars production and consumption in 3-liter working volume fermenter (Figure 4.10), reducing sugars concentration was high at the beginning of cultivation from 0 to 10 h that might be because of the bacterium, with slightly increasing of growth, consume reducing sugars lower than the production of reducing sugars. When bacterial growth quickly from 10-18 h cultivation, the level of reducing sugars in the fermenter was lower than the level of reducing sugars in a reaction that might be because of the bacterium consumed reducing sugars at a higher rate than production rate. However, after 20 h cultivation, the level of reducing sugars in the fermenter was increased while the growth of bacterium was decreased that might be because amylase was still active to produce reducing sugars but the rate of consumption was slow down or might be because of contamination of yeast or bacteria, which produce starch hydrolysis enzyme, in fermenter problem. The experiment of amylase activity was performed in extra-triplicate.

When compared amylase production by *Lactococcus* sp. SUT 513 with other bacteria such as *Lactobacillus plantarum* S21 with amylase activity of 2.1 U/ml from 36 h of incubation time (Kanpiengjai et al., 2015), *Lactobacillus plantarum* ST-II with amylase activity of 2.88 U/mg (Jeon et al., 2016), *Lactobacillus acidophilus* GV6 with amylase activity of 0.28 U/ml at 24 h of cultivation time (Vishnu et al., 2000), *Bacillus methylotrophicus* strain P11-2 exhibited 117.0 U/ml from 72 h of cultivation (Xie et al., 2014). We found amylase activity produced by *Lactococcus* sp. SUT 513 showed to shorten the time of cultivation, the potential to grow and produce amylase in low-cost

complex medium, and one important point also utilized starch to the high optical purity of L-lactic acid. Then, the batch of L-lactic acid fermentation could be developed.

4.4 Optimization of amylase production by response surface methodology (RSM)

4.4.1 Central composite design (CCD)

Batch fermentation of 50 ml in 125 ml Erlenmeyer flask was used to produce extracellular amylase from *Lactococcus* sp. SUT 513. Preliminary experiments on amylase production from the bacterial strain indicated that the important factors were cassava starch concentration, rice bran concentration, and pH. Therefore, these three factors were considered as the independent variables, and their effect on amylase production was studied using a CCD of RSM. The rice bran was replaced by defatted rice bran that gives the advantage of shelf-life, low rancidity, and more homogenous of material.

4.4.2 Analysis of variance (ANOVA) and modeling

The results of CCD experiments for studying the effects of three independent variables, cassava starch concentration, defatted rice bran concentration, and pH on amylase production were shown in Table 4.7 along with the actual and predicted value. The standard deviations on the observed responses were also presented in Table 4.7. The maximum predicted activity of 9.84 U/ml was observed for central points while actual experimental results showed the maximum activity of 9.80 U/ml for run number 4.

Table 4.7 Actual and predicted of amylase activity values.

Run order	Factor			Amylase activity (U/ml)		Residual standard deviation
	A	B	C	Actual value	Predicted value	
1	3.00	0.40	7.00	7.87	7.48	0.3875
2	1.50	0.60	8.50	4.46	4.45	0.0084
3	3.00	0.40	7.00	7.68	7.48	0.1973
4	4.50	0.60	8.50	9.80	9.84	-0.0386
5	3.00	0.40	9.52	5.70	6.27	-0.5661
6	3.00	0.40	7.00	6.23	7.48	-1.2500
7	0.48	0.40	7.00	1.94	1.67	0.2704
8	1.50	0.20	8.50	3.47	3.04	0.4356
9	3.00	0.74	7.00	7.62	6.80	0.8167
10	4.50	0.60	5.50	0.75	2.21	-1.4600
11	3.00	0.40	7.00	7.68	7.48	0.1976
12	4.50	0.20	8.50	7.51	7.79	-0.2858
13	1.50	0.20	5.50	0.72	1.71	-0.9885
14	4.50	0.2	5.50	1.01	2.04	-1.0400
15	1.50	0.6	5.50	0.52	1.26	-0.7412
16	3.00	0.4	7.00	9.10	7.48	1.6200
17	3.00	0.06	7.00	6.06	5.45	0.6049
18	3.00	0.4	7.00	6.10	7.48	-1.3800
19	5.52	0.4	7.00	7.65	6.47	1.1900
20	3.00	0.40	4.48	0.77	-1.25	2.0200

A: Cassava starch concentration, B: Defatted rice bran concentration, C: Initial pH of the amylase production medium

Table 4.8 Fit summary of response 1: amylase activity**.**

Source	p-value		R^2		Result
	Sequential	Lack of fit	Adjust	Predict	
Linear	0.0071	0.0297	0.4304	0.2623	
2FI	0.5990	0.0221	0.3900	-0.3689	
Quadratic	0.0016	0.2514	0.8163	0.4726	Suggested
Cubic	0.5341	0.0973	0.8060	-5.1478	Aliased

From the fit summary (Table 4.8), the data of amylase activity with the quadratic model was suggested with the lowest of Sequential p-value (0.0016) and highest adjust (0.8163) and predict R^2 (0.4726). The results of the second-order quadratic model have performed an analysis of variance (ANOVA) (Table 4.9). When considered at the model F-value (10.38), it implies the significant model. There was only a 0.05% chance that an F-value this large could occur due to noise. p-values less than 0.05 indicate significant model terms.

In this case, A, C, AC, A^2, C^2 were significant model terms. Values greater than 0.1 indicate the model terms that were not significant. If there were many insignificant model terms, model reduction may improve this model. The lack of fit F-value of 1.89 implies the lack of fit that was not significant relative to the pure error. Non-significant lack of fit was good. The fitting of the model was checked by the determination coefficient (R^2 in Table 4.10).

Table 4.9 ANOVA for response 1: amylase activity quadratic model.

Source	df	Sum of squares	Mean square	F-value	p-value	Result
Model	9	170.64	18.96	10.38	0.0005	Significant
A: Cassava starch	1	27.83	27.83	15.24	0.0029	
B: Defatted rice bran	1	2.17	2.17	1.19	0.3010	
C: Initial pH	1	68.29	68.29	37.39	0.0001	
AB	1	0.20	0.20	0.11	0.7496	
AC	1	9.81	9.81	5.37	0.0430	
BC	1	1.75	1.75	0.96	0.3503	
A^2	1	20.95	20.95	11.47	0.0069	
B^2	1	3.25	3.25	1.78	0.2115	
C^2	1	44.53	44.53	24.38	0.0006	
Residual	10	18.27	1.83			
Lack of fit	5	11.94	2.39	1.89	0.2514	Not significant
Pure error	5	6.33	1.27			
Cor total	19	188.90				

AB: Interaction of cassava starch and defatted rice bran concentration, AC: Interaction of cassava starch concentration and initial pH in the medium, BC: Interaction of defatted rice bran concentration and initial pH in the medium, A^2; B^2; C^2: Intra-factor interaction.

Table 4.10 Fit statistics for response 1: amylase activity.

Fit statistic	Result
Standard deviation	1.35
Mean	5.13
Coefficient of variation (%)	26.33
R^2	0.9033
Adjusted R^2	0.8163
Predicted R^2	0.4726
Adeq Precision	11.6028

In this case, the value of the determination coefficient (R^2= 0.9033) indicates that only 9.67% of the total variations were not explained by the model. The value of the adjusted R^2 (0.8163) was also high, which indicates a higher significance (p-value < 0.01) of the model. The predicted R^2 of 0.4726 was not as close to the adjusted R^2 of 0.8163 as one might normally expect; i.e. the difference was more than 0.2. This may indicate a large block effect or a possible problem with the model or data. Things to consider were model reduction, response transformation, outliers, etc. All empirical models should be tested by doing confirmation runs**.** Adequate precision (Adeq Precision) measures the signal to noise ratio. A ratio greater than 4 was desirable. The ratio of 11.603 indicates an adequate signal. This model can be used to navigate the design space. At the same time a relatively lower value of the coefficient of variation (C.V.= 26.33%). The model was selected with Quadratic process order and the second-

order polynomial equation for amylase production was generated the equation of factor and coefficient estimate that show in Table 4.11

Table 4.11 Coefficients in terms of code factors.

Factor	Coefficient estimate	Standard error	95% Confidence interval		VIF
			Low	High	
Intercept	7.480	0.551	6.25	8.71	
A: Cassava starch	1.430	0.370	0.61	2.24	1.00
B: Defatted rice bran	0.397	0.364	-0.41	1.21	1.00
C: Initial pH	2.240	0.366	1.42	3.05	1.00
AB	0.157	0.478	-0.91	1.22	1.00
AC	1.110	0.478	0.04	2.17	1.00
BC	0.468	0.478	-0.60	1.53	1.00
A^2	-1.210	0.357	-2.00	-0.41	1.02
B^2	-0.467	0.350	-1.25	0.31	1.02
C^2	-1.760	0.357	-2.56	-0.97	1.02

AB: Interaction of cassava starch and defatted rice bran concentration, AC: Interaction of cassava starch concentration and initial pH in the medium, BC: Interaction of defatted rice bran concentration and initial pH in the medium, A^2; B^2; C^2: Intra-factor interaction.

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors were held constant. The intercept in an orthogonal design was the overall average response of all the runs. The coefficients were adjustments around that average based on the factor settings. From Table 4.11, the coefficient estimate revealed the effect of factors and a combination of factors. The most affected factor for amylase production was initial pH at a coefficient estimate value of 2.24, then cassava starch concentration (1.43), and defatted rice bran concentration (0.3971). The strongest combination between 2 factors was AC-*Cassava starch concentration and initial pH* combination (1.11), and in the same factor was C^2 –*Square of initial pH* (-1.76). The defatted rice bran showed the smallest effect on amylase production that means we can change the defatted rice bran concentration with a little bit change of amylase production. Therefore, when combining term of the factor and coefficient estimate value, the final equation in terms of coded factors for the RSM data plot is:

$$\textit{Amylase activity } (R_1) = 7.48 + 1.43A + 0.3971B + 2.24C + 0.1568AB + 1.11AC + 0.4682BC - 1.21A^2 - 0.4671B^2 - 1.76C^2 \quad (1)$$

where, *A- Cassava starch concentration, B- Defatted rice bran concentration, and C- Initial pH,* the equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors were coded as +1 and the low levels were coded as -1. The coded equation was useful for identifying the relative impact of the factors by comparing the factor coefficients. The final equation in terms of actual factors is:

$$\textit{Amylase activity } (R_1) = 0.519807A - 1.16356B + 0.35164C + 0.522507AB + 0.492154AC + 1.56065BC - 0.536954A^2 - 11.67869B^2 - 0.782922C^2 - 36.34323 \quad (2)$$

where, *A- Cassava starch concentration, B- Defatted rice bran concentration, and C- Initial pH*, the equation in terms of actual factors can be used to make predictions about the response for given levels of each factor. Here, the levels should be specified in the original units for each factor. This equation should not be used to determine the relative impact of each factor because the coefficients were scaled to accommodate the units of each factor and the intercept was not at the center of the design space.

4.4.3 Diagnostic plots

Diagnostic plots were performed to check to a problem that might be found in the results of CCD with the condition and solution from Table 4.12 and 4.13. The normal probability plot (Figure 4.11a) was showing the data points followed a normal distribution, thus follow the straight line indicates with some scatter of blue (low amylase activity point) point that a transformation of the response may better analysis if the condition does not provide too small of amylase production.

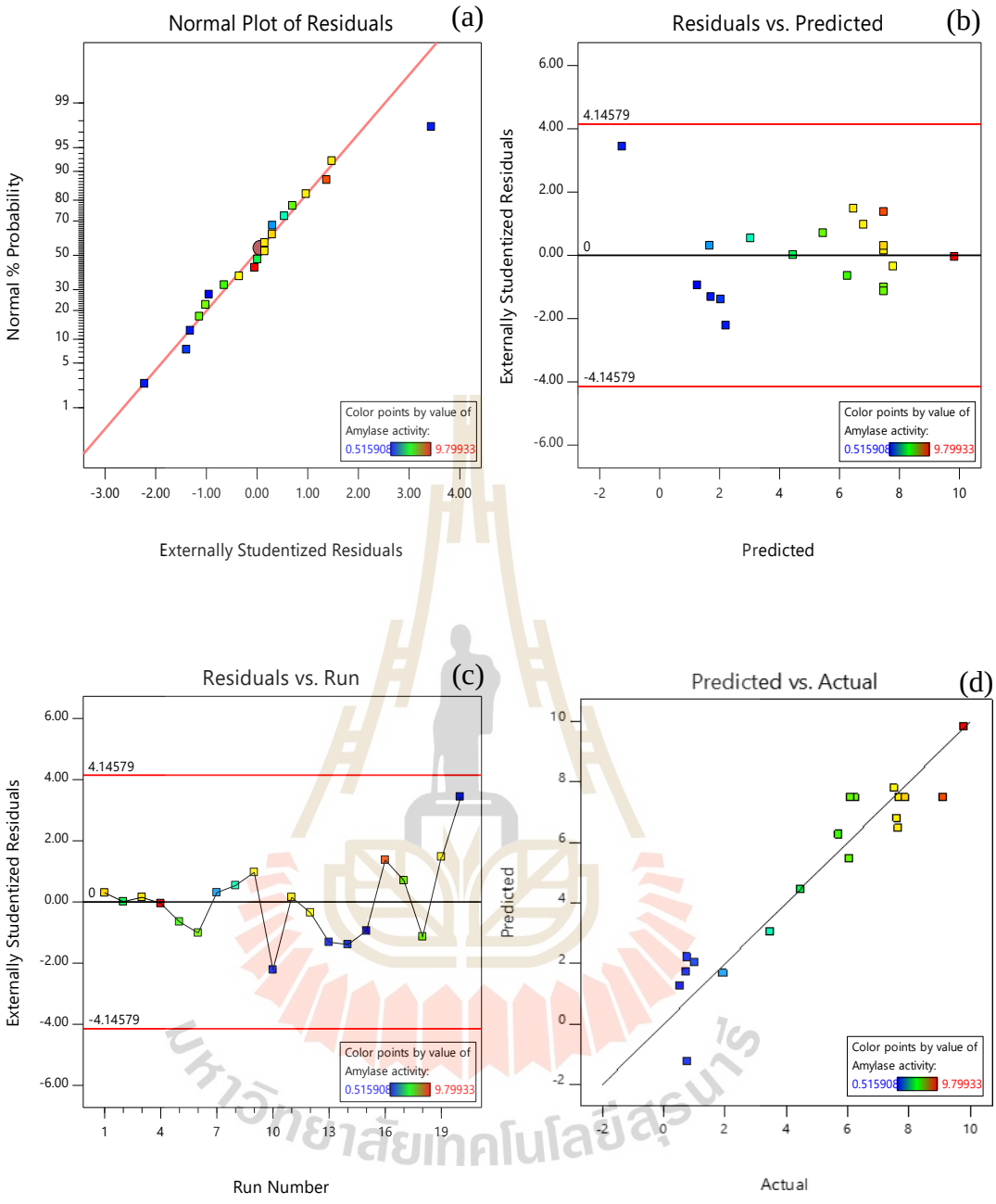


Figure 4.11 Diagnostic plots of amylase activity; the normal probability plot (a), the residuals versus the ascending predicted response values plot (b), the residuals versus the experimental run order plot (c), and the predicted response values versus the actual response values plot (d).

The residuals vs. predicted plot (Figure 4.11b), this was a plot of the residuals versus the ascending predicted response values. It tests the assumption of constant variance. The plot was showed a random scatter of data point in the limit line of the plot. Expanding variance in this plot indicated the need for a transformation. The residuals vs. run plot (Figure 4.11c), this was a plot of the residuals versus the experimental run order.

Table 4.12 Constraints of amylase production condition.

Name	Goal	Lower limit	Upper limit
A: Cassava starch	In range	0.48	5.52
B: Defatted rice bran	In range	0.06	0.74
C: Initial pH	In range	4.48	9.52
Amylase activity	Maximize	0.52	9.80

The residuals vs. run plot (Figure 4.11c) was used to check for lurking variables that may have influenced the response during the experiment. This plot was showed a random scatter of data points. The trends line showed a time-related variable lurking in the background. Blocking and randomization provide insurance against trends ruining the analysis. The predicted vs. actual plot (Figure 4.11d), a graph of the predicted response values versus the actual response values. The amylase production in lower levels than 0.515 U/ml was to detect a value, which was not easily predicted by this model.

Table 4.13 Top 5 solutions from 100 optimized solutions for amylase production**.**

Number	Factor			Amylase activity (U/ml)	Desirability	Result
	A	B	C			
1	5.034	0.620	8.535	9.809	1.000	
2	4.707	0.577	8.606	9.853	1.000	Selected
3	4.703	0.564	8.854	9.805	1.000	
4	4.589	0.682	8.945	9.819	1.000	
5	5.107	0.625	8.651	9.826	1.000	

A: Cassava starch concentration, B: Defatted rice bran concentration, C: Initial pH in the medium

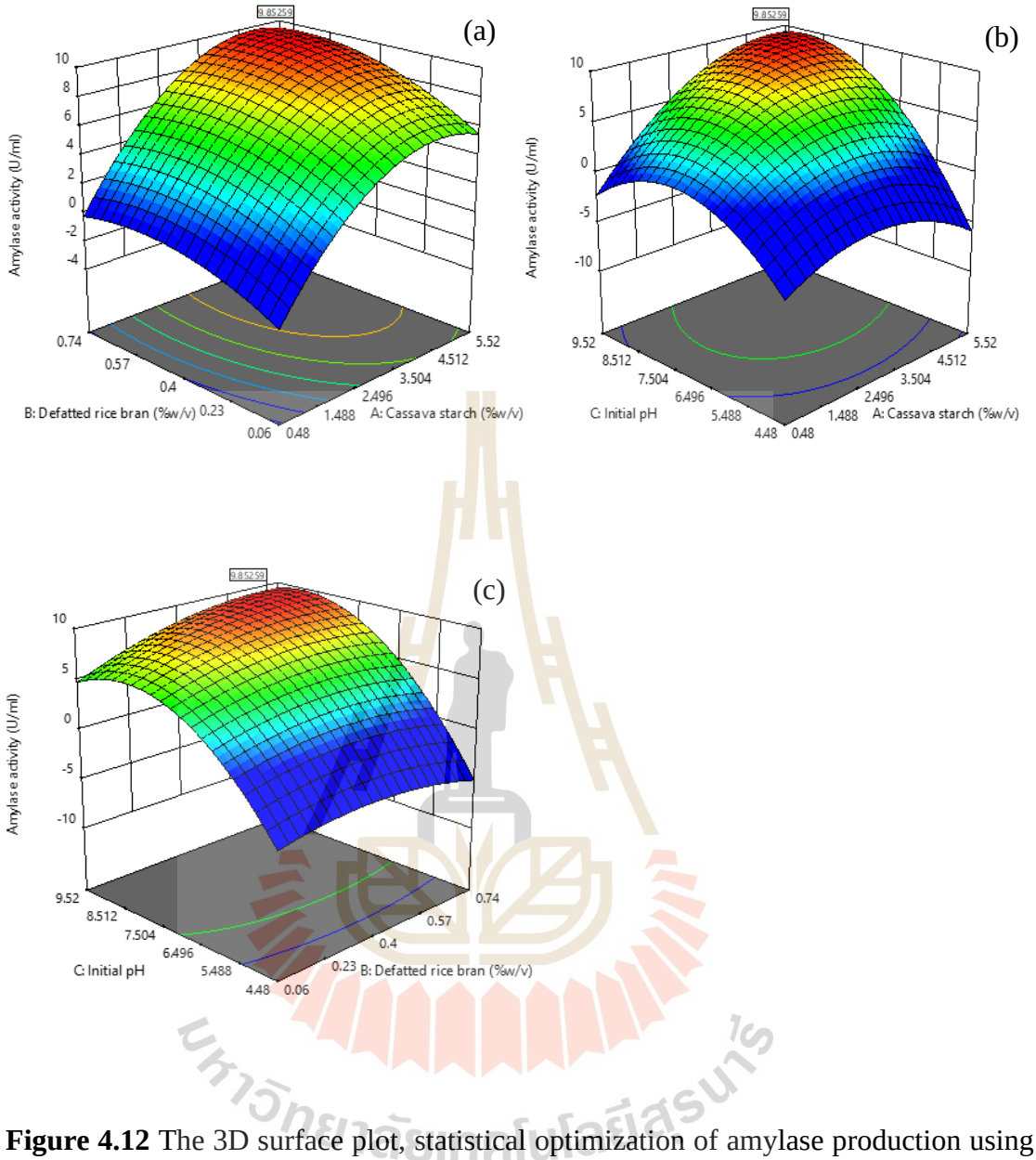


Figure 4.12 The 3D surface plot, statistical optimization of amylase production using RSM, cassava starch concentration, and defatted rice bran concentration (a); cassava starch concentration and initial pH (b); defatted rice bran concentration and initial pH (c).

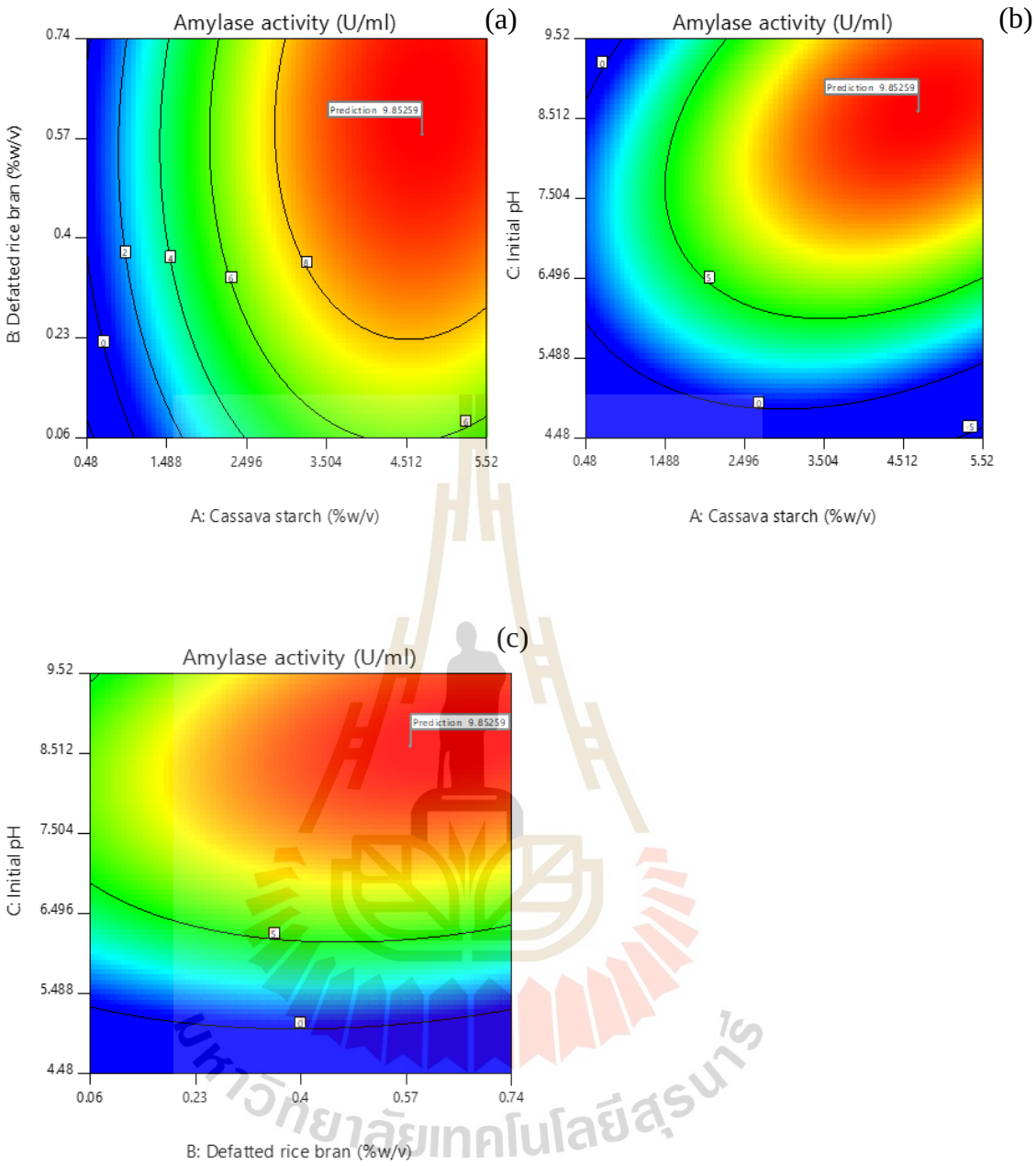


Figure 4.13 The contour plot, statistical optimization of amylase production using RSM, cassava starch concentration, and defatted rice bran concentration (a); cassava starch concentration and initial pH (b); defatted rice bran concentration and initial pH (c).

4.4.4 Point prediction and confirmation of the RSM model for amylase production

Point prediction was calculated to predict the amylase production by using the actual equation (Equation 2) and compared with the actual amylase activity from the experiments that showed in Table 4.15. The 20 runs were calculated the predict mean and then, compared with actual mean that use to building this model. The paired t-test analysis result was shown in Table 4.15. The paired t-test was performed (Table 4.14) to compare mean of 20 runs and reveal the result of the p-value (0.787) that higher than α (0.05) so that the data retained in the null hypothesis that were no differences between the two conditions, that means this model could be predicted amylase production in the condition that used to build the model by CCD.

Table 4.14 Paired sample t-test of the 20 samples of 50 ml in 125 ml flask.

	Paired differences							
				95% confidence interval				
	Mean	Standard deviation	Standard error mean	Lower	Upper	t	df	Sig. (2-tailed)
Pair 1 Predict-Actual	0.060	0.981	0.219	-0.399	0.519	0.274	19	0.787

To confirm amylase production from the independent condition of model, The solution of medium compositions and condition came from the space in the model that could be predicted the amylase production. The runs 1, 2, and 3 were performed in low initial pH to confirm and check the problem that diagnosis in the four of the diagnostic plot.

Table 4.15 Prediction of amylase activity from actual equation (2) versus actual value.

Run	Amylase activity (U/ml)		95% Confidence interval	
	Actual mean	Predicted mean	Lower	Upper
1	7.87	7.54	5.45	9.63
2	4.46	4.62	2.53	6.71
3	7.68	7.54	5.45	9.63
4	9.80	9.89	7.80	11.98
5	5.70	6.43	4.34	8.52
6	6.23	7.54	5.45	9.63
7	1.94	1.80	-0.29	3.89
8	3.47	3.20	1.11	5.29
9	7.62	6.86	4.77	8.95
10	0.75	2.17	0.08	4.26
11	7.68	7.54	5.45	9.63
12	7.51	7.85	5.76	9.94
13	0.72	1.77	-0.32	3.86
14	1.01	2.01	-0.08	4.10
15	0.52	1.31	-0.78	3.40
16	9.10	7.54	5.45	9.63
17	6.06	5.52	3.43	7.61
18	6.10	7.54	5.45	9.63
19	7.65	6.42	4.33	8.51
20	0.77	-1.25	-3.34	0.84

When The runs 1, 2 and, 3 were considered the actual and predicted mean, it was found that actual means were out of the lower limited as the diagnostic plot in Figure 4.11 that revealed the prediction which might be not precise if the amylase production was performed by the condition that provides amylase activity around 0.50 U/ml.

Table 4.16 The confirmation of 12 independent runs.

Run	Factor			Amylase activity (U/ml)		95% Confidence interval	
	A	B	C	Actual mean	Predicted mean	Lower	Upper
1	4.49	0.58	5.62	0.49	2.84	0.75	5.15
2	4.49	0.58	5.62	0.41	2.84	0.75	5.15
3	4.49	0.58	5.62	0.47	2.84	0.75	5.15
4	4.49	0.58	7.00	10.62	7.82	5.73	10.13
5	4.49	0.58	7.00	9.95	7.82	5.73	10.13
6	4.49	0.58	7.00	9.67	7.82	5.73	10.13
7	4.49	0.58	8.43	10.93	9.85	7.76	12.16
8	4.49	0.58	8.43	11.71	9.85	7.76	12.16
9	4.49	0.58	8.43	13.53	9.85	7.55	12.16
10	4.49	0.58	8.43	13.76	9.85	7.55	12.16
11	4.49	0.58	8.43	11.48	9.85	7.55	12.16
12	4.49	0.58	8.43	7.08	9.85	7.55	12.16

A: Cassava starch, B: Defatted rice bran, C: Initial pH

Table 4.17 Paired sample t-test of the 12 independent runs of 50 ml in 125 ml flask.

	Paired differences							
				95% confidence interval				
	Mean	Standard deviation	Standard error mean	Lower	Upper	t	df	Sig. (2-tailed)
Pair 1 Predict-Actual	-0.752	2.520	0.727	-2.353	0.849	-1.033	11	0.324

However, the paired t-test was performed (Table 4.17) to confirm the overall prediction by this model, when compared to the mean of 12 independent runs and analyzed the result of the p-value of 0.324 that higher than α of 0.05. Therefore, the data retained in the null hypothesis that were no differences between the two conditions, which means this model could be predicted amylase production in the independent point of the surface of RSM.

Table 4.18 The confirmation of RSM in the 3-liter working volume.

Run	Cultivation time (h)	Amylase activity (U/ml)		95% Confidence interval	
		Actual mean	Predicted mean	Lower	Upper
1	20	8.03	9.89	6.82	12.85
2	24	9.78	9.89	6.82	12.85
3	23	8.80	9.89	6.82	12.85
4	18	8.36	9.89	6.82	12.85

The confirmation of amylase production in the fermenter was shown in Table 4.18. The fermentation was started with the same condition (Factors, A= 4.5; B= 0.60; C= 8.50). The duplicate 4 points of time of 18, 20, 23, and 24 h were selected to confirm together with 20 runs of RSM.

Table 4.19 Paired sample t-test of the 4 samples from 3 liters in 7.5-liter the fermenter.

	Paired differences							
				95% confidence interval				
	Mean	Standard deviation	Standard error mean	Lower	Upper	t	df	Sig. (2-tailed)
Pair 1 Predict-Actual	0.241	1.020	0.208	-0.190	0.672	1.158	23	0.259

The paired t-test was performed (Table 4.19) to compare mean and revealed the result of the p-value of 0.259 that higher than α of 0.05, therefore, the data retained in the null hypothesis that there was no difference between the two conditions, that means this model could be used to predict amylase production in the 3-liter working volume of the fermenter.

A little experiment to study the effect of essential elements on amylase production in the optimal medium was performed in 125 ml flask with 50 ml of the optimal medium, cultivated for 24 h and crude amylase was assayed amylase activity, For the result, The medium containing essential element had amylase activity 3.636±0.76 U/ml, and medium without essential element revealed amylase activity 4.208±1.02 U/ml, the paired t-test was performed to compare mean and revealed the result of the p-value of 0.631 that higher than α of 0.05, therefore, the data retained in the null hypothesis that there were no differences between the two conditions, that means the essential element

source in amylase production medium were enough for amylase production and bacterial growth. Manano et al. (2018) reported that cassava starch contained element likes calcium, iron, zinc, magnesium, and copper, and this element could support bacterial growth and amylase production of *Lactococcus* sp. SUT 513. In the element of rice bran as was reported by Oluremi et al. (2013). The essential element could be obtained from 2% inoculum of the bacterium in a RAM medium (Rodtong and Ishizaki, 2003). For further studies on the defatted rice bran and rice bran, the protein extraction method might be introduced to increase the homogeneity of component and medium. The microwave method that had been studied in Indian defatted rice bran meal by using microwave 800 W for 20-90 s, pH 8.0, the result shows the protein recovery of 67-70% (Bandyopadhyay et al., 2012)

4.5 Amylase production in 3-liter RSM optimized medium in the 7.5-liter bioreactor

This is the results of the amylase activity, pH, and bacterial growth monitoring for 36 h in 3-liter working volume in the 7.5-liter bioreactor with an agitation speed of 150 rpm without aeration (Figure 4.14). One of the optimal conditions from RSM with 4.5% (w/v) of cassava starch concentration, 0.6% (w/v) of defatted rice bran concentration, all of the essential elements were eliminated, and initial pH at 8.50 was used to cultivate The bacterium had been cultivated start with 2% of inoculum size that provides the bacterial growth at 7.14 log CFU/ml, then the lag-phase only around 2 h cultivation, the bacterial growth starts the log-phase estimate at 4 h, and the highest bacterial growth was numbered of 9.90 log CFU/ml at 12 h within the late log-phase, then bacterial showed the stationary at 14-26 h with bacterial growth 9.82-9.37

log CFU/ml, and the death phase started between 26-28 h so the bacterial growth drops to 7.05-6.94 log CFU/ml at 28-36 h. The amylase activity showed a positive correlate trend with bacterial growth. The bacterium was produced amylase activity of 0.634 U/ml at 0 h cultivation, then increase to the maximum activity of 8.360-8.617 U/ml at 16-18 h and show the stationary phase of amylase production until 36 h cultivation, For the pH, started with pH 7.58 in the optimal medium, then pH reduces while the bacterial growth increase at 0-12 h cultivation and the stationary phase 12-36 h cultivation, pH still drops with a slower rate than log-phase, and finally pH 4.51 at 36 h that show in Figure 4.14 because of pH represent L-lactic acid production as a primary metabolite.

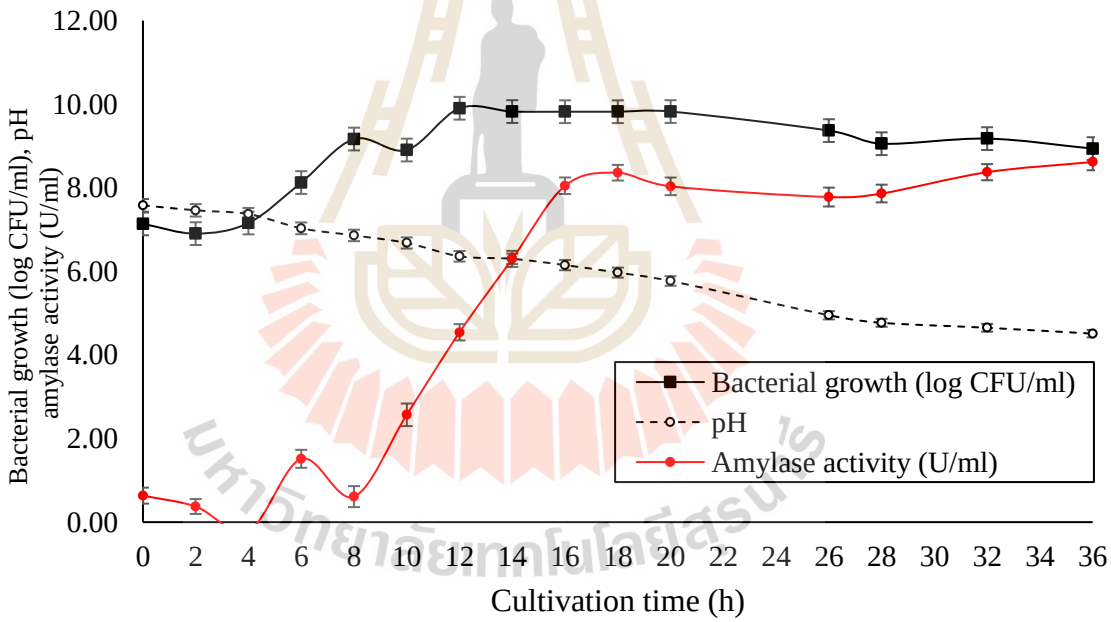


Figure 4.14 Time courses of growth of *Lactococcus* sp. SUT 513 (filled squares), pH (blank circle), and the production of amylase (filled circles) in 3-liter RSM optimized medium during cultivation at 35℃ for 36 h.

The reducing sugars-*product* and cassava starch concentration-*substrate*, and amylase activity were also monitored in the fermenter that showed in Figures 4.15 and

4.19 that obtained from the fermentation with agitation 150 rpm (Figure 4.15) and without agitation (Figure 4.16). The result reveals the positive correlation of amylase activity and amount of reducing sugars, these 2 parameters were a negative correlation with cassava starch concentration, which represented the hydrolysis of cassava starch and turns into the reducing sugars in the broth by amylase activity.

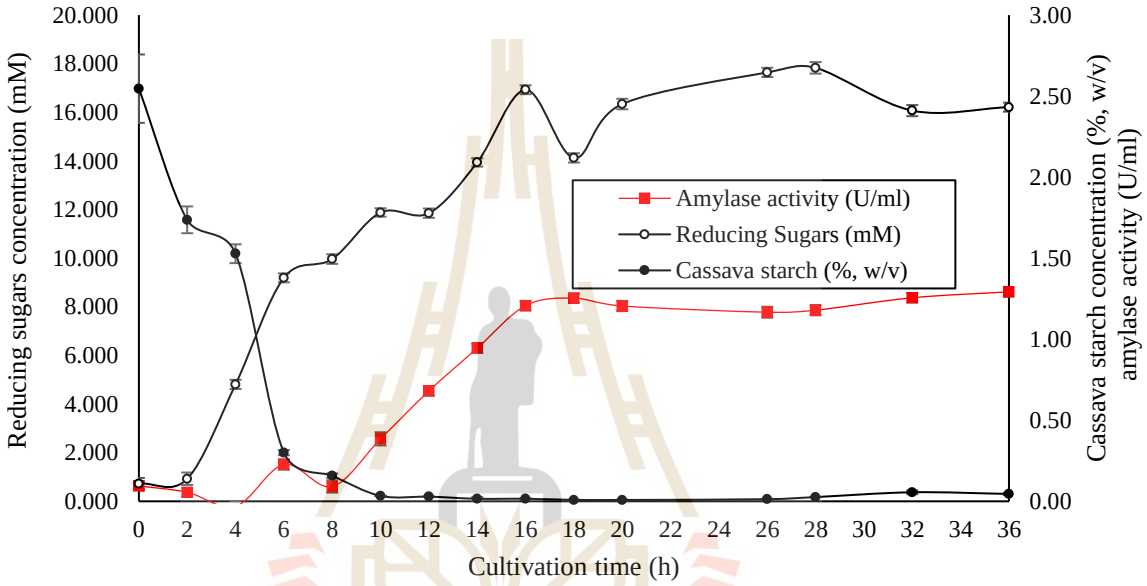


Figure 4.15 Time courses of amylase activity (filled squares), reducing sugars (blank circle), and cassava starch concentration in crude amylase (filled circles) in 3-liter RSM optimized medium during cultivation at 35°C for 36 h.

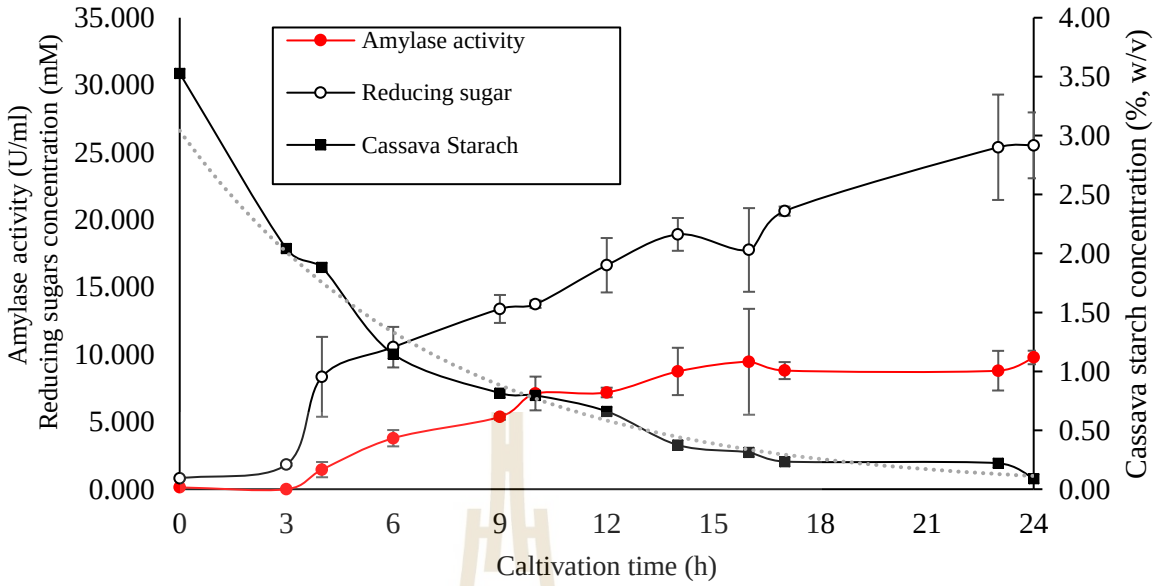


Figure 4.16 Time courses of amylase activity (filled squares), reducing sugars (blank circle), and cassava starch concentration in the fermenter (filled circles) in 3-liter RSM optimized medium during cultivation at 35℃ for 24 h, in 2nd fermentations.

Figures 4.15 and 4.16 showed the same pattern of the trendline of 3 parameters, but the cassava starch concentration in Figure 4.15 dropped very fast because it was determined in crude enzyme and lowering of cultivated broth caused some of the starch packed with the pellet after centrifugation but in Figure 4.16 the cassava starch in a fermenter, it was determined to start with 3.528% (w/v) at 0 h and showed reduction of concentration as exponential trend line to 0.089% (w/v) at 24 h cultivation. *Lactococcus* sp. SUT 513 can hydrolyze starch from 3.5% (w/v) to 0.089% (w/v) and produce the reducing sugars 25.26 mM within 24 h at the condition without aeration and agitation. Dextrose 4.41-5.625 g/h of dry cassava starch under fermentation condition for 24 h. The production of amylase and bacterial growth in the fermenter with 150 rpm agitation

were stable than in without agitation cultivation that might be caused by sampling and homogeneity of cultivation broth. The duration of fermentation is an important factor in the fermentation process.

4.6 Partial purification of α-amylase produced by *Lactococcus* sp. SUT 513

In this study focusing on purification with a simple and fast step to screening or elimination the other protein and obtain the enzyme suspension with higher concentration and purification folds that without reducing sugars, were used to performed enzyme characterization in terms of pH and temperature profile.

4.6.1 Ammonium sulfate precipitation

For ammonium sulfate precipitation was screening for finding the range of ammonium sulfate saturation (Figure 4.17). The saturation that provided the highest relative total activity was 90%, but from many trials to do the precipitation, the ammonium sulfate was not stable to purify this enzyme with the very low of activity recovery.

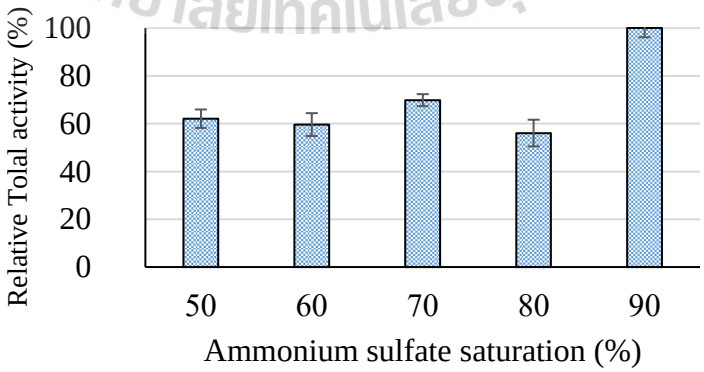


Figure 4.17 The relative total amylase activity (%) from ammonium sulfate precipitation screening.

Sometimes, the enzyme recovery was very high of 96% recovery (Table 4.20) by resuspending the partially purified enzyme by half of the crude amylase volume with Na-phosphate buffer pH 6.8, 50 mM. The ratio of dilution was played an important role in enzyme recovery and solubility.

Table 4.20 Purification table of ammonium sulfate precipitation of α-amylase.

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Volume (ml)	Folds	Recovery (%)
1) Crude amylase	275.20	5.140	53.534	20	1.00	100
90% $(NH_4)_2SO_4$	265.54	1.71	154.501	9	2.89	96
2) Crude amylase	86.64	-	-	25	-	100
90% $(NH_4)_2SO_4$	26.73	-	-	10	-	31
3) Crude amylase	4,591.6	-	-	400	-	100
80% $(NH_4)_2SO_4$	203.82	-	-	30	-	4
Dialysis 10kDa	7.29			2.5		0
4) Crude amylase	1473.9	-	-	100	-	
90% $(NH_4)_2SO_4$	8.94			2		1

4.6.2 Isoelectric precipitation

From the study of ammonium sulfate precipitation, we found the concentration and pH of the buffer that using to resuspend, and dialysis is important. The crude amylase with pH below 5.0 was observed in the brown pellet after setting in 4°C. Accidentally, in the step of diafiltration 10 kDa cut-off with the 50 mM maleate

buffer pH 4.0 was usage instead of pH 6.8. After centrifugation, a lot of brown pellets were found to be precipitated and packed beside the tube. Then the isoelectric precipitation screening (Figure 4.18) began with the results in Table 4.21.

Table 4.21 Purification table of isoelectric precipitation of α-amylase.

Step		Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Volume (ml)	Folds	Recovery (%)
1) Crude amylase		17.25	15.62	1.104	40	1.00	100
pH	3.0	23.77	5.29	4.491	5	4.07	138
	3.5	25.92	7.90	3.280	5	2.97	150
	4.0	26.77	6.86	3.905	5	3.54	155
	4.5	12.79	5.89	2.172	5	1.97	74
	5.0	12.00	3.40	3.532	5	3.20	70
Fraction purification study							
2) Crude amylase		844.37	56.432	14.963	100	1.00	100
pH	3.5	57.98	14.950	3.878	6	0.26	7
90% $(NH_4)_2SO_4$		-	-	-	6	-	-
Diafiltration 10kDa		23.16	9.805	2.362	6	0.12	3

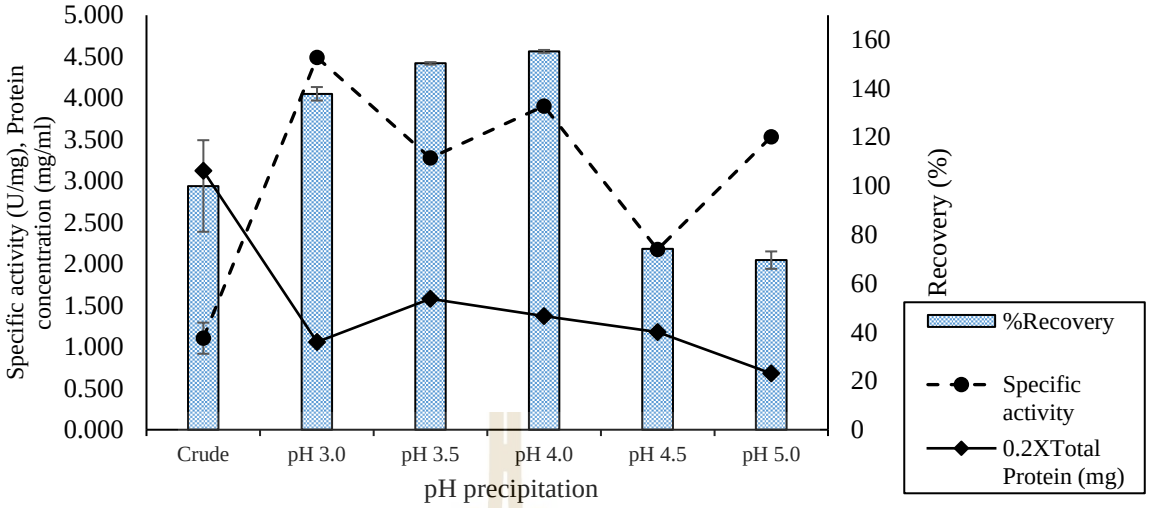


Figure 4.18 The recovery of total amylase activity (%) from isoelectric precipitation screening.

The advantage of isoelectric precipitation was to reduce the usage of ammonium sulfate and this method, and this method could separate protein from reducing sugars in supernatant without starch precipitation that found in one problem of ammonium sulfate precipitation. When comparing the partially purified amylase by pH and ammonium sulfate precipitation, that showed in Table 4.21, this crude might have 2 types of amylase with the different value isoelectric point. The isoelectric point of α-amylase from lactic acid bacteria, such as *Lactobacillus fermentum* Ogi E1, *Lactobacillus manihotivorans*, and *Lactobacillus plantarum* was equaled to pH 3.6 that was reported by Talamond et al. 2002.

However, the protein that precipitate might be the rice bran protein that was reported by Fabian et al. (2010), with the high amount of rice bran protein was found in pH precipitation by using alginate and carrageenan at pH 3.5. The buffer and condition that supply a higher recovery of this amylase could be further study.

4.7 Characterization of partially purified α-amylase

4.7.1 Effect of pH on the enzyme activity and stability

The partially purified amylase by 90% ammonium sulfate precipitation was studied the amylase activity (Figure 4.19). The enzyme reaction incubated 35°C, 30 min with 1% of soluble starch in 50 mM pH 3.0-9.0 buffers. The reaction of 50 mM pH 6.8 sodium phosphate buffer was set as control. The amylase activity revealed the optimal pH in the range of pH 6 to 6.8, which was estimated to 7.0 with amylase activity in a range of 25.158 to 30.840 U/ml as 82-100% when compared with the control (pH 6.8 Na-P). This result also shows the effect of the buffer with the same value of pH and concentration that means this enzyme needs the optimal buffer to providing the highest amylase activity.

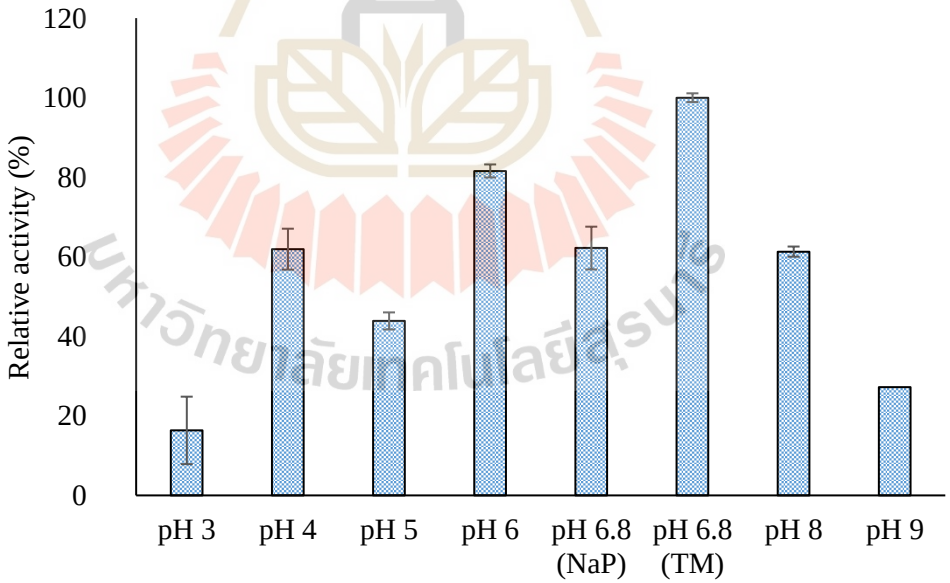


Figure 4.19 Amylase activity of partially purified amylase by 90% ammonium sulfate precipitation (Na-P is sodium phosphate buffer, TM is tris-maleate buffer).

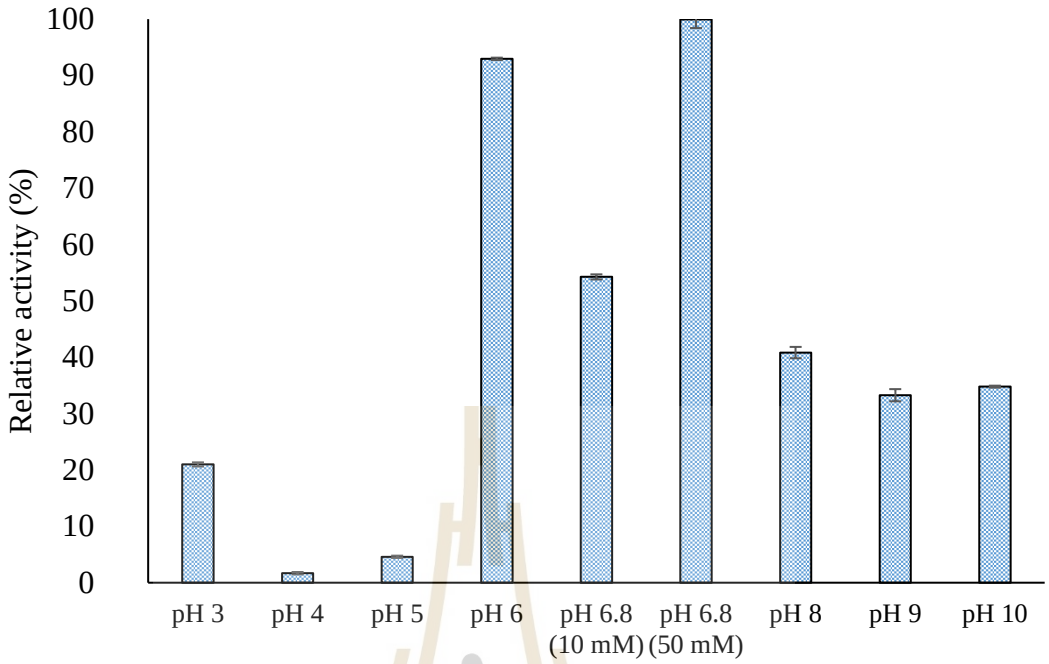


Figure 4.20 The stability of partially purified amylase by pH 3.0 precipitation.

For the pH stability study (Figure 4.20), the partially purified amylase by pH 3.0 isoelectric precipitation was selected. This fraction was diluted 10 folds with the 50 mM pH 3.0-10.0 buffers for 30 min at 4℃, then after pre-incubation, the stability of enzyme was represented by amylase activity at the same condition. The control was 50 mM pH 6.8 tris-maleate buffer because of these buffers used from the purification step and storage with tris-maleate buffer. The result revealed, that this fraction showed low stability at acid and basic condition of pH 3.0, 4.0, and 5.0 with 2, 5, and 21% and pH 8.0, 9.0. pH 6 and 6.8 supplying the highest enzyme stability at 93 and 100%. However, the concentration of buffer on the enzyme was interested when compared between10 mM and 50 mM of pH 6.8 tris-maleate buffer the stability in 10 mM was 54%, while 50 mM was 100% of relative activity. The trend of enzyme stability might be related to the precipitation of protein, the solubility of the enzyme, and protein folding.

The effect of pH on amylase activity of crude amylase from 18 h cultivation in the bioreactor was selected to study the characteristic of pH and temperature. It was found that the amylase had high activities between pH 7.0 and 9.0, with maximal activity at pH 7.0-8.0 that comes from different storage time with 1 and 7 days (Figure 4.21) at 4°C. From two experiments, the amylase activity of 1-day crude amylase increased from pH 4.0 to 6.0 and decreased from pH 8.0 to 9.0. The amylase activity decreased at pH below 5.0.

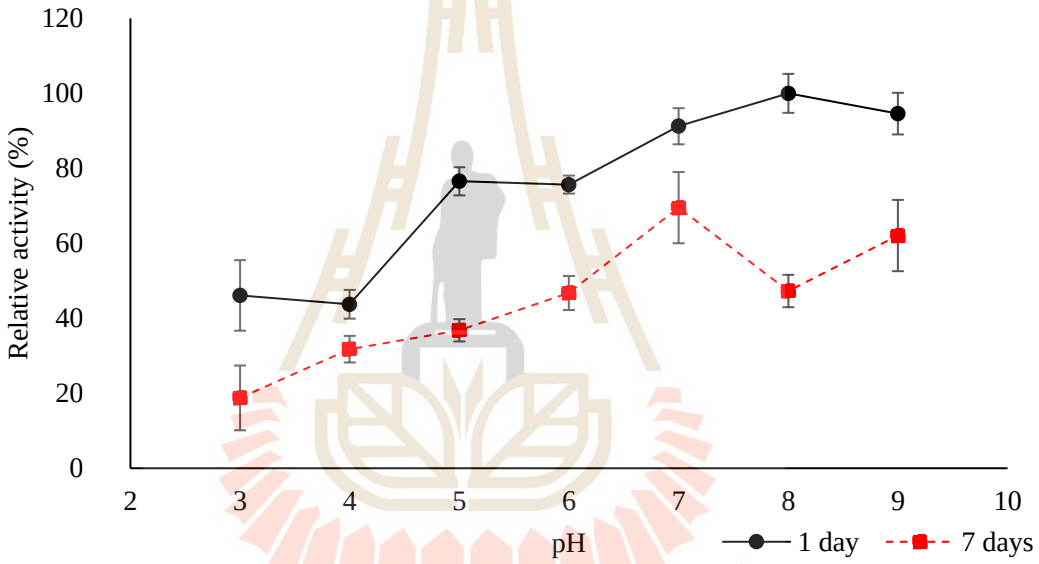


Figure 4.21 From the 1st day (circle) and 7 days (square) of the experiment, the effect of pH on crude amylase activity produces by *Lactococcus* sp. SUT 513, cultivated for 18 h.

The first day experimental of the effect of pH on amylase activity at pH 8.0 showed a maximum of 0.648 U/ml (Figure 4.21 circle). After that at 7 days, the second experiment (Figure 4.21) was performed to study the stability of crude enzyme after thaw 1 time to confirm the optimum point, pH 7.0 Na-phosphate buffer exhibited 0.451

U/ml of amylase activity while pH 8.0 exhibited 0.306 U/ml that decrease about 2.12 folds. It was possible if this amylase had an optimum pH at 7.0- 8.0.

Besides, the effect of pH and buffer concentration was studied. For the results, the effect of pH on hydrolysis of soluble starch. Soluble starch with 200 mM buffer pH 3.0-9.0 in 1:1 ratio did not significantly show hydrolysis of starch after incubating in boiling water bath for 10 min for color developing of DNS assay.

The effect of buffer concentration on the chromatic of DNS assay. when using reducing sugars sample with more than 100 mM of the buffer that affects less coloring of DNS assay in the range of pH from 3.0-9.0, so that cannot detect the concentration of reducing sugars. That also means low respect results. But the concentration of buffers in our sample were below 100 mM.

4.7.2 Effect of temperature on the enzyme activity and stability

The partially purified amylase by 90% ammonium sulfate precipitation and isoelectric pH 3.0 precipitation was studied the amylase activity. The enzyme reaction incubates from 0-100°C, 30 min with 1% of soluble starch in 50 mM pH 6.8 tris-maleate buffer. The reaction of 35°C 50 mM pH 6.8 tris-maleate buffer was set as control. The amylase activity reveals the optimal temperature of 2 fractions were different for 90%$(NH_4)_2SO_4$ fraction (Figure 4.23a) in range of temperature 50 to 70^oC with amylase activity in the range of 5.581 to 5.679 U/ml as 98-100%, and pH 3.0 precipitation (Figure 4.23b) in range of 25 to 35°C with amylase activity in the range of 7.274 to 7.843 U/ml as 93-100%. This result was correlated with the purification profile (Table 4.18; 2) that show to fraction of amylase with the different isoelectric point, from this part we found it show different of amylase activity temperature profile. The temperature activity profile the amylase can apply in a wide range of temperatures.

90% $(NH_4)_2SO_4$ was interested in a high-temperature profile so that the thermostability of the enzyme was studied. The control of the temperature stability profile was amylase activity at 35°C (Figure 4.22) without pre-incubation in 50 mM pH 6.8 tris-maleate buffer for 30 min. The results of stability showed this fraction most stable at temperature 4 to 25, and 80 to 90°C, show the relative activity at 99-100% with amylase activity 5.302 to 5.410 U/ml.

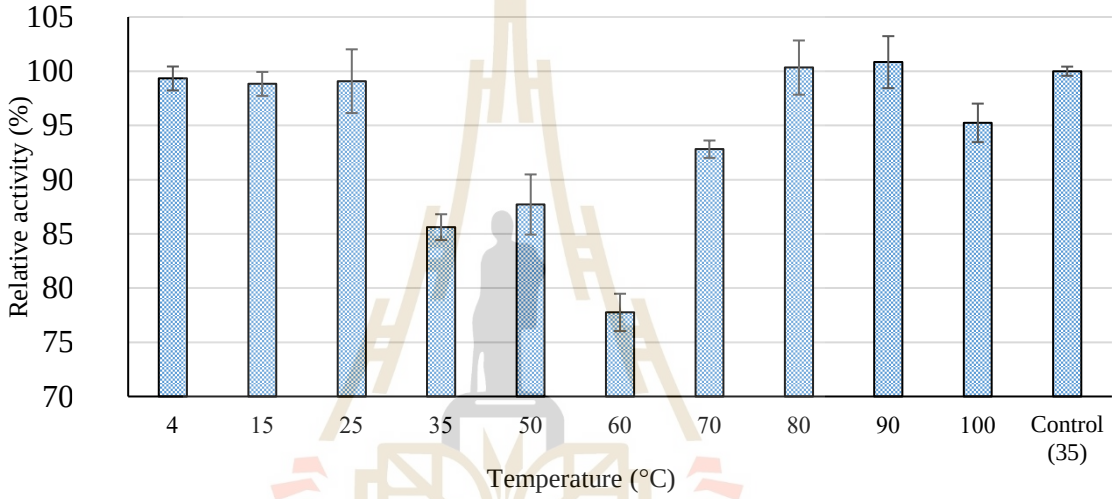


Figure 4.22 Effect of temperature on the stability of partially purified amylase by 90% ammonium sulfate precipitation.

For the effect of temperature on amylase activity of the crude amylase by *Lactococcus* sp. SUT 513, cultivation for 18 h. The amylase activity exhibited in the range of 30-80°C. However, the crude amylase was observed as the higher of reducing sugars along the time at temperature 4°C that might be the activity of the enzyme still active but at the slow rate.

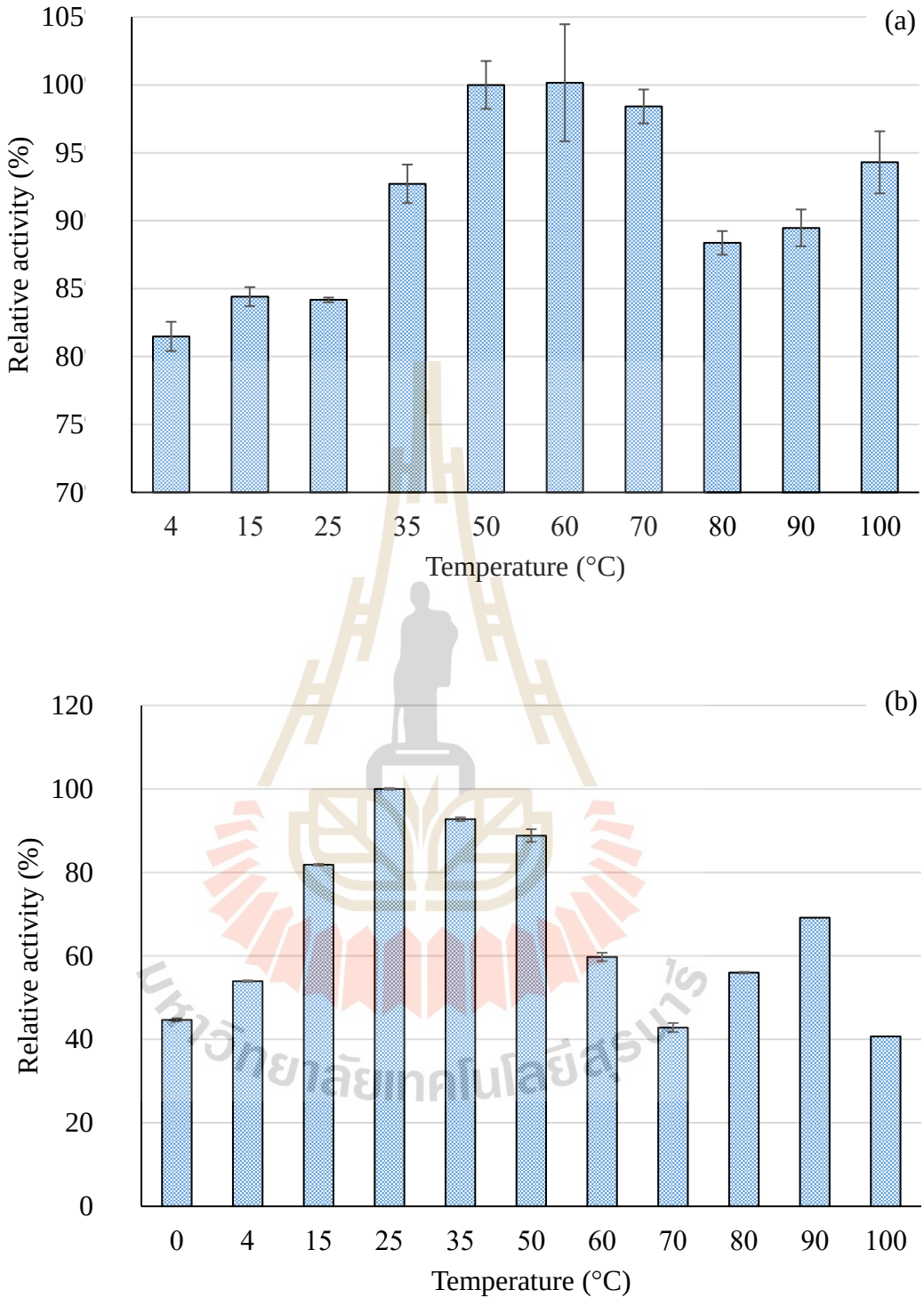


Figure 4.23 The effect of temperature on amylase activity of partially purified amylase by 90% ammonium sulfate precipitation (a), and pH 3.0 precipitation (b).

That relates to the temperature profile of partially purified amylase. The optimal reaction temperature for the amylase activity was about 50°C of 0.556 U/ml (Figure 4.24), the amylase activity decreased sharply at temperatures above 70°C. The enzyme activity was stable when the temperature was below 50°C. However, the enzyme was almost inactivated at temperatures over 80°C with 30 min incubation.

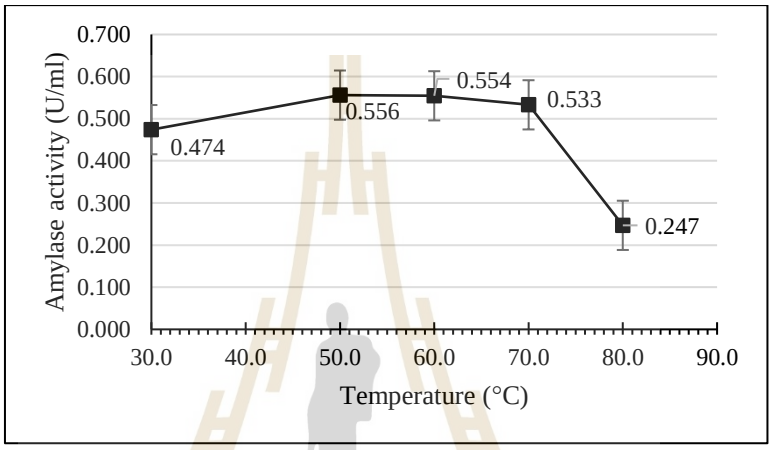


Figure 4.24 Effect of temperature on amylase activity of the crude amylase by *Lactococcus* sp. SUT 513, cultivation for 18 h.

The reducing sugars and residual starch were removed from partially purified enzyme provided the data more accurately than crude enzyme, However, the problem that could be found in both experiments of pH and temperature profile was enzyme still active at high temperature and cassava starch as a substrate also remained in the crude amylase. So that starch hydrolysis was active between incubating in boiled water that caused the level of reducing sugars in the background, which were increased, then amylase activity would be decreased. From this problem, the truly optimum point of the enzyme could not confirm. Therefore, the study of the inhibitor to stop the enzyme reaction or developed the amylase activity assay to heat and stop the reaction of enzyme-substrate faster must be studied.

4.7.3 Effects of metal ions and chelating agent on amylase activity

The control of this study uses the only enzyme with 1%soluble starch in 50 mM of tris-maleate buffer pH 6.8 not adding any cation ions or chelating agents. The concentration of metal ion and the chelating agent was 5 mM in the reaction.

Table 4.22 Effect of metal cations and chelating agent 5 mM on amylase activity of partially purified by using 90% ammonium sulfate precipitation.

Metal ion and chelating agent	Concentration (mM)	Relative activity (%)
Control	-	100
NaCl	5.00	63
$MgSO_4$	5.00	198
$CaCl_2$	5.00	37
$FeSO_4$	5.00	218
$MnSO_4$	5.00	-47
EDTA	5.00	139

For the results, the effect of Alkaline ion as Na^+ in 5 mM NaCl showed inhibition effect with drop activity to 63%. The effect of Alkaline earth ions as Mg^{2+} and Ca^{2+} was different from Mg^{2+} show activation affect the activity up to 198% but for Ca^{2+} show, the inhibition affects the activity drop to 37%. The effect of transition ions as Fe^{2+} and Mn^{2+} was different with Fe^{2+} provide activity up to 218% but Mn^{2+} the activity was minus 47%, that comes from the interfere of transition metal ion to DNS reagent that similar to the effect of a buffer made the color faded while Fe^{2+} made the

color browner. For Ca^{2+} was interested because most of the amylase was metalloenzyme for this fraction the Ca^{2+} show inhibition effect (Table 4.21 and Figure 4.25) that could confirm with EDTA was provided activity up to 139% that means the EDTA could be chelated the Ca^{2+} or another inhibition ion in reaction, so amylase can active more.

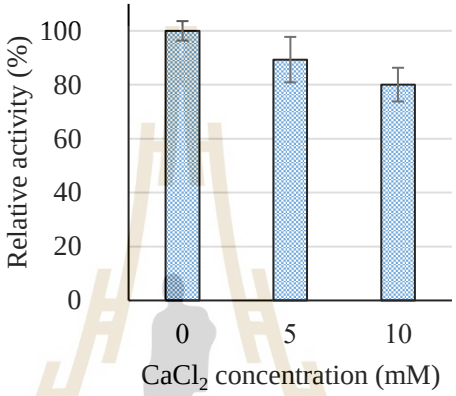


Figure 4.25 Effect of calcium ion on amylase activity.

4.7.4 Stability of amylase produced by *Lactococcus* sp. SUT 513 during storage at 4°C and effect of thawing

The reduction of amylase activity in crude when storage at 4°C or freeze and thaw that came from enzyme denature or just increasing of reducing sugars that came from starch hydrolysis. The results of the amylase activity of the crude enzyme were shown in Figure 4.26. From several times of experiments, the reducing sugars in crude amylase were increasing around 20-50% at each time of the thawing experiment, when it was stored for a long time or thaw several times. While reducing sugars in the reaction of crude enzyme-substrate did not change much. This event could be drop amylase activity around 5-15% at each time of the thawing experiment because more background value to negate.

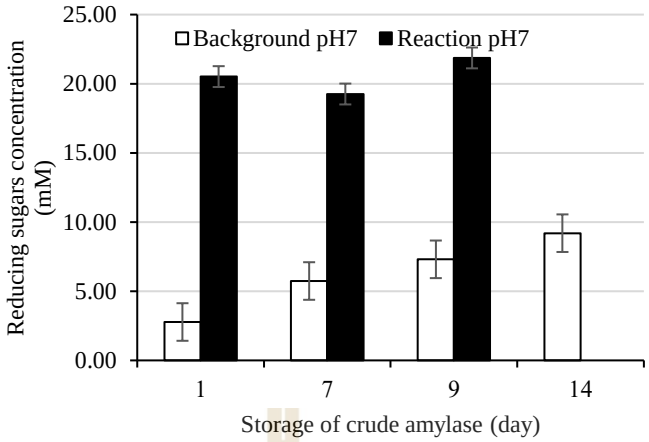


Figure 4.26 Effect of thawing and storage of crude amylase, the level of reducing sugars in crude amylase (white), and in the reaction of amylase (black).

For the shelf-life of the crude enzyme in the refrigerator with chill condition temperature 2-8℃ was estimate the trend line of amylase of activity and reducing sugars in Figure 4.27. The crude amylase from 36 h of cultivation in 3-liter working volume bioreactor was monitored amylase activity and amount of reducing sugars by amylase activity assay for 39 days. For the results, the amylase activity was showed a negative correlation with the amount of reducing sugars at the first-day amylase activity of 8.617 U/ml and reducing sugars of 32.40 mM. Both of parameter was stable for 26 days in refrigerator temperature 2-4°C with amylase activity of 5.061 U/ml and reducing sugars of 36.72 mM until we change the sample to a new refrigerator that lower stability of temperature control with temperature 4-8°C for 14 days. The final day showed a remarkably high amount of reducing sugars and extremely low amylase activity. That meant at the first phase from 1-day to 26-day amylase activity reduced 41% of the 1st day in the rate 1.5% per day in 2-4°C storage condition but in second phase amylase activity reduced 3.8% per day in 4-8°C.

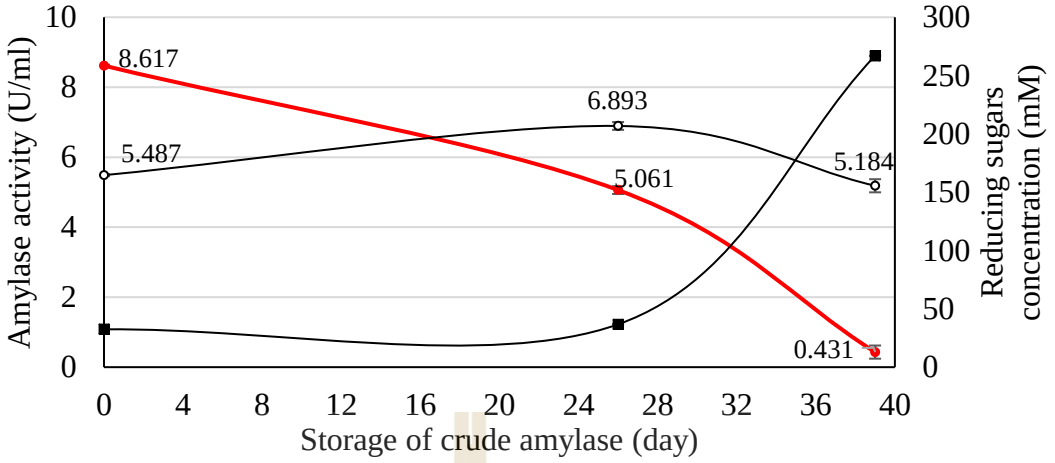


Figure 4.27 The trend of amylase activity in crude amylase (filled circle), amylase activity from partial precipitation (white circle), and reducing sugars concentration in the supernatant (square) that storage in temperature 2-8°C for 39 days.

4.7.5 SDS-PAGE and molecular weight estimation

The partially purified amylase by 90% ammonium sulfate precipitation was performed SDS-PAGE (Figure 4.28). The coomassie brilliant blue staining showed many bands of protein. Because of zymogram did not successfully, so the diafiltration process of protein purification was the one clue that used to estimate the molecular weight of amylase. Amylase activity was found in the permeate supernatant of the 30 kDa cut-off membrane with and in the retentate supernatant of the 10 kDa cut-off membrane. This amylase enzyme could be passed the 30 kDa cut-off membrane, but could not be passed through the 10 kDa cut-off membrane. Therefore, this showed that the possibility of amylase molecular weight might be in the range between 16-52 kDa.

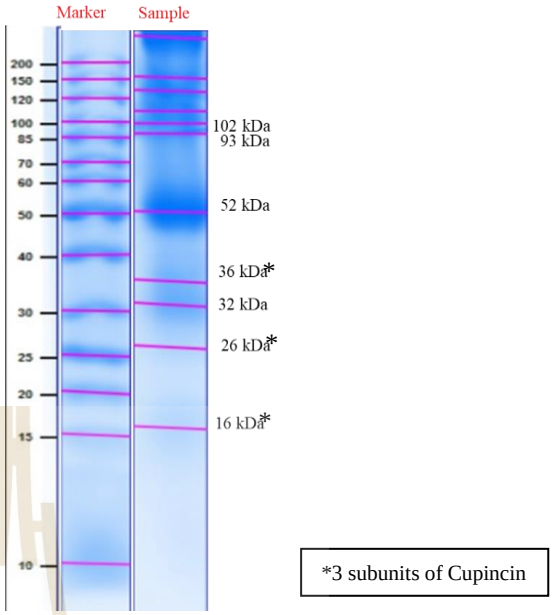


Figure 4.28 SDS-PAGE of partially purified amylase by 90% ammonium sulfate precipitation (Lanes 2 and 3) and PageRuler™ Unstained Protein Ladder marker (Lane 1).

The 16 kDa fraction of SDS-PAGE was selected for sequencing by using LC-MS: short gradient and LC/MS/MS: In-gel digestion. Then, the sequence of 16 kDa fraction was searched on Swissport 2019 database with bacteria (eubacteria) (334059 sequences) and Oryza sativa (rice) (5,064 sequences) taxonomy. From the Mascot search of Eubacteria taxonomy (Figure 4.29a), the result did not show the similarity of 16 kDa fraction with amylase in eubacteria so the amylase might be in another band such as 26, 32, 36, and 52 kDa. However, 16 kDa fraction showed some part of protein sequence similarity with 60 kDa chaperonin of *Bacillus thuringiensis* strain Al Hakam of 78-score and *Geobacillus stearothermophilus* **(***Bacillus stearothermophilus*) with a score of 65. The 60 kDa chaperonin of *Geobacillus stearothermophilus* was translated from gene *groL* with **s**ubunit structure of oligomer of 14 subunits composed of two

stacked rings of 7 subunits that usually found in the cytoplasm (Schon and Schumann, 1993).

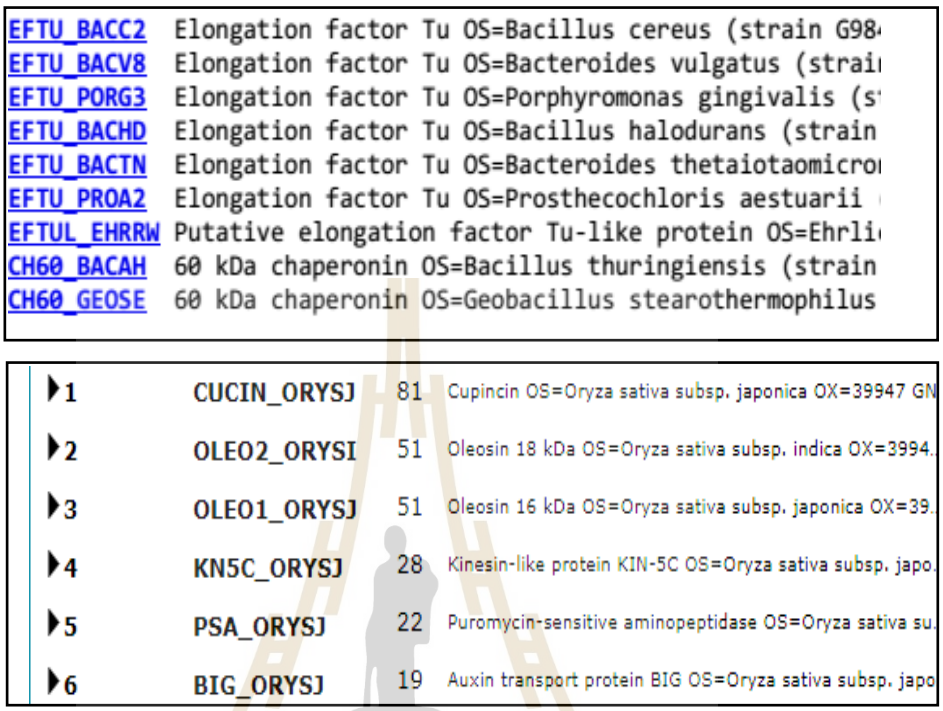


Figure 4.29 Mascot search results of 16 kDa with Eubacteria (a) and *Oryza sativa* taxonomy (b) from www.matrixscience.com database.

The function of 60 kDa chaperonin was prevents miss-folding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions such as heat, so that this may be one factor that helps the enzyme from *Lactococcus* sp. SUT 513 had thermostability of 90-100% relative activity at 50-100°C. When search with *Oryza sativa* taxonomy (Figure 4.29b), The 16 kDa fraction was showed similarity with 16 and 18 kDa of rice structural protein (Olesin) and a subunit of protease (cupintin) from *Oryza sativa* subsp. *japonica* (Fabian et al., 2010).

From the literature, Sreedhar and Tiku (2016) mentioned that cupincin was found to be a homotrimer, consisting of 3 distinct subunits with molecular masses of

33.45 kDa, 22.35 kDa, and 16.67 kDa. Therefore, the protein bands of partially purified amylase in SDS-PAGE (Figure 4.29) were concluded to bo 16 kDa, 24 kDa, and 36 kDa might be cupincin, the protease from rice bran.

The 14-to-16-kDa rice salt-soluble protein from *RAG2* gene was found in embryo and seed grain (Zhou et al., 2017) that can be α-amylase/trypsin inhibitor. This protein might be found in the rice bran and defatted rice bran, which consists of a pericarp, aleurone layer, and embryo that causes the lowering of amylase activity when using rice bran at too high concentration. This result was related to the coefficient of defatted rice bran in Table 4.11 with B^2 equal to -0.467 and from the coefficient equation (- 11.67869B^2).

Also, if the amylase remains in the 30 kDa cut-off membrane, but not exhibited the amylase activity, which could be caused by the low recovery of the enzyme (Table 4.17). The molecular weight of the amylase could be in the band of 93 and 102 kDa. From literature, most α-amylase produced by lactic acid bacteria had molecular weight near to 100 kDa. Talamond et al. (2002) reported that the molecular mass of α-amylase was obtained from *Lactobacillus fermentum* Ogi E1 of 106 kDa, *Lactobacillus manihotivorans* of 100 kDa, and *Lactobacillus plantarum* of 99.5 kDa. Kanpiengjai et al. (2015) also mentioned that the molecular mass of α-amylase was obtained from *Lactococcus lactis* IBB500 of 121 kDa, *Lactobacillus plantarum* A6 of 135 kDa, *Lactobacillus manihotivorans* LMG18010 of 140 kDa, and *Lactobacillus amylovorus* NRRL B-4540 of 150 kDa. The result of SDS-PAGE (Figure 4.28) showed the possibility of the a-amylase protein bands at 93 and 103 kDa. In a further study of α-amylase production, the pre-treatment of defatted rice bran with alkaline and heat might be used in the medium that could be supported more amylase production than un-

treatment defatted rice bran. For α-amylase purification, to reduced unnecessary protein in crude amylase, the hydrolyzed nitrogen source as tryptone could be applied instead of defatted rice bran in the α-amylase purification medium.

CHAPTER V

CONCLUSION

Commercial amylase was used to solve the high viscosity problem of cassava starch-containing medium for L-lactic acid production which had high concentration (12%, w/v). The high viscosity caused low water activity in the medium that not suitable for bacterial cultivation. This research aims to investigate amylase production by *Lactococcus* sp. SUT 513, a specific strain of L-lactic acid-producing bacteria. The strain actively uses cassava starch as the main substrate for optically purified L-lactic acid production. The bacterium could potentially be used for amylase enzyme production to reduce or replace commercial amylases. The extracellular amylase production from *Lactococcus* sp. SUT 513 was determined by amylase activity assay. The amylase activity appeared in every formula of cultivated broth. This study aimed to optimize medium composition and cultivation conditions for amylase production by *Lactococcus* sp. SUT 513 on amylase in the supernatant, after screening of medium composition, 3 factors of cassava starch concentration, defatted rice bran concentration, and initial pH were optimized and build the model by using Central composite design (CCD) with Response surface methodology (RSM). The optimal amylase production medium formula composing of cassava starch, defatted rice bran, and di-potassium hydrogen phosphate at concentrations of 45, 6, and 6 g/l, respectively, was developed from the original medium composing of cassava starch, tryptone, yeast extract, tri-ammonium citrate, di-potassium hydrogen phosphate, magnesium sulfate, manganese

sulfate, ferrous sulfate at concentrations of 10, 5, 3, 1, 6, 0.57, 0.12 and 0.03 g/l. When cultivated *Lactococcus* sp. SUT 513 for 24 h in the optimal medium with an initial pH 8.5, the amylase activity of 9.508±0.022 U/ml with protein concentration 0.305±0.053 mg/ml was obtained. The cost of the optimal medium was 79.0% cheaper than the original medium. The optimal and shorted cultivation time for amylase production by *Lactococcus* sp. SUT 513 was at 16 to 18 h when cultivated in a 3-liter working volume in the 7.5-liter bioreactor. The bacterium produces enzyme exhibiting 9.459±0.219 U/ml of amylase activity. After enzyme production, we aimed to partially purify and characterize amylase produced by *Lactococcus* sp. SUT 513. The partially purified amylase by 90% ammonium sulfate precipitation showed enzyme 4-96% recovery and specific activity of 118-154.5 U/mg and showed the characteristic of enzyme activity with optimal pH 6.0-7.0 and temperature 50-60°C, and showed thermostability when incubated the enzyme at 70-100°C for 30 min with 88-95% relative activity. The crude enzyme had precipitated to pellet at pH 3.0-5.0, but stable at pH 6.0-7.0. The pellet suspension had shown a specific activity of 3.53-4.07 U/mg with characteristics of optimal pH 6.0-7.0 and temperature 25 to 35°C. For the partially purified amylase by 90% ammonium sulfate precipitation, the metal ions and chelating agent that act as the activators on amylase activity were Mg^{2+} (198%), Fe^{2+} (218%), and EDTA (139%). The metal ions that act as the inhibitors on amylase activity were Mn^{2+} (-47%), Ca^{2+} (37%), and Na^{2+} (63%), and amylase molecular weight might be in the range between 16-52 kDa. After freezing and thawing crude enzyme, the reducing sugars in crude amylase were increasing around 20-50% at each time of the thawing experiment. While reducing sugars in the reaction of crude enzyme-substrate did not change much. The amylase activity was decreasing around 5-15%. The activity of crude enzyme reduced in the rate

of 1.5% per day during storage at 2-4°C for 26 days, and amylase activity reduced in the rate 3.8% per day during storage at 4-8°C for 14 days. In a further study of α-amylase production, the pre-treatment of defatted rice bran with alkaline and heat might be used in the medium that could be supported more amylase production than un-treatment defatted rice bran. For α-amylase purification, to reduced unnecessary protein in crude amylase, the hydrolyzed nitrogen source as yeast extract could be applied instead of defatted rice bran in the amylase medium. The results of this study would take advantage of future application and production of the enzyme production from waste to wealth. However, purification and characterization of the enzyme will need to be further investigation.

REFERENCES

REFERENCES

Anto, H., Trivedi, U., and Patel, K. (2006). α-Amylase production by *Bacillus cereus* MTCC 1305 using solid-state fermentation. **Food Technology and Biotechnology.** 44(2): 241-245.

Aiyer, P. D. (2004). Effect of C: N ratio on alpha amylase production by *Bacillus licheniformis* SPT 27. **African Journal of Biotechnology.** 3(10): 519-522.

Arnesen, S., Eriksen, S. H., ørgen Olsen, J., and Jensen, B. (1998). Increased production of α-amylase from *Thermomyces lanuginosus* by the addition of tween 80. **Enzyme and Microbial Technology.** 23(3): 249-252.

Ashraf, H., Qadeer, M., and Iqbal, J. (2005). Pearl millet, a source of alpha amylase production by *Bacillus licheniformis*. **Bioresource Technology.** 96(10): 1201-1204.

Bandyopadhyay, K., Chakraborty, C., and Barman, A. K. (2012). Effect of microwave and enzymatic treatment on the recovery of protein from Indian defatted rice bran meal. **Journal of Oleo Science.** 61(10): 525-529.

Bendelow, V. M. (1963). Modififed procedure for the determination of diastatic activity and α-amylase activity. **Journal of The Institute of Brewing.** 69(6): 467-472.

Bernfeld, P. (1955). Amylases, α and β. **Methods in Enzymology.** 1: 149-158.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Analytical Biochemistry.** 72: 248-254.

Bunni, L., McHale, L., and McHale, A. (1989). Production, isolation and partial characterization of an amylase system produced by *Talaromyces emersonii* CBS 814.70. **Enzyme and Microbial Technology.** 11(6): 370-375.

Burgess, R. R. (2009). Chapter 20 protein precipitation techniques. In Burgess, R. R. and M. P. Deutscher (eds.). **Methods in Enzymology** (pp. 331-342). Cambridge, Massachusetts: Academic Press.

Cao, X., Wen, H., Li, C. and Gu, Z. (2009). Differences in functional properties and biochemical characteristics of congenetic rice proteins. **Journal of Cereal Science.** 50(2): 184-189.

Carlsen, M., Nielsen, J., and Villadsen, J. (1996). Growth and α-amylase production by *Aspergillus oryzae* during continuous cultivations. **Journal of Biotechnology.** 45(1): 81-93.

Chandra, A., Medda, S., and Bhadra, A. (1980). Production of extracellular thermostable α-amylase by *Bacillus licheniformis*. **Journal of Fermentation Technology.** 58(1): 1-10.

Chanput, W., Theerakulkait, C. and Nakai, S. (2009). Antioxidative properties of partially purified barley hordein, rice bran protein fractions and their hydrolysates. **Cereal Science.** 49(2): 422-428.

Chrambach, A., and Rodbard, D. (1971). Polyacrylamide gel electrophoresis. **Science.** 172(3982): 440-451.

Cripwell, R. A., Rose, S. H., Viljoen-Bloom, M., and van Zyl, W. H. (2018). Improved raw starch amylase production by *Saccharomyces cerevisiae* using codon optimization strategies. **FEMS Yeast Research.** 19(2): foy127.

Dettori-Campus, B., Priest, F., and Stark, J. (1992). Hydrolysis of starch granules by the amylase from *Bacillus stearothermophilus* NCA 26. **Process Biochemistry.** 27(1): 17-21.

Du, R., Song, Q., Zhang, Q., Zhao, F., Kim, R.-C., Zhou, Z., and Han, Y. (2018). Purification and characterization of novel thermostable and Ca-independent α-amylase produced by *Bacillus amyloliquefaciens* BH072. **International Journal of Biological Macromolecules.** 115: 1151-1156.

Erdal, S., and Taskin, M. (2010). Production of alpha-amylase by *Penicillium expansum* MT-1 in solid-state fermentation using waste Loquat (*Eriobotrya japonica* Lindley) kernels as substrate. **Romanian Biotechnological Letters.** 15(3): 5342-5350.

Fabian, C. B., Huynh, L. H., and Ju, Y. H. (2010). Precipitation of rice bran protein using carrageenan and alginate. **LWT - Food Science and Technology.** 43(2): 375-379.

Fabiana Guillen, M., Francieli Arrias de, L., Sophia Renata Fazzano, P., Lenartovicz, V., Cristina Giatti Marques de, S., and Peralta, R. (1999). Production of amylases by *Aspergillus tamarii*. **Revista de Microbiologia.** 30(2): 157-162.

Feller, G., Le Bussy, O., and Gerday, C. (1998). Expression of psychrophilic genes in mesophilic hosts: assessment of the folding state of a recombinant α-amylase. **Applied and Environmental Microbiology.** 64(3): 1163-1165.

Fossi, B. T., and Tavea, F. (2013). Application of amylolytic *Lactobacillus fermentum* 04BBA19 in fermentation for simultaneous production of thermostable α-amylase and lactic acid. **Lactic Acid Bacteria-R and D for Food, Health, and Livestock Purposes** (pp. 633-658). London, United Kingdom: IntechOpen.

Francis, F., Sabu, A., Nampoothiri, K. M., Ramachandran, S., Ghosh, S., Szakacs, G., and Pandey, A. (2003). Use of response surface methodology for optimizing process parameters for the production of α-amylase by *Aspergillus oryzae*. **Biochemical Engineering Journal.** 15(2): 107-115.

Ganesh Karanth, N., and Eugene Raj, A. (2005). Fermentation technology and bioreactor design. **Food Biotechnology** (2nd ed., pp. 35-57). Florida, USA: CRC Press

Gangadharan, D., Sivaramakrishnan, S., Nampoothiri, K. M., and Pandey, A. (2006). Solid culturing of *Bacillus amyloliquefaciens* for α-amylase production. **Food Technology and Biotechnology.** 44(2): 269-274.

Ghaffar, T., Irshad, M., Anwar, Z., Aqil, T., Zulifqar, Z., Tariq, A., Kamran, M., Ehsan, N., and Mehmood, S. (2014). Recent trends in lactic acid biotechnology: A brief review on production to purification. **Journal of Radiation Research and Applied Sciences.** 7(2): 222-229.

Gomes, I., Gomes, J., and Steiner, W. (2003). Highly thermostable amylase and pullulanase of the extreme thermophilic eubacterium *Rhodothermus marinus*: production and partial characterization. **Bioresource Technology.** 90(2): 207-214.

Goyal, N., Gupta, J., and Soni, S. (2005). A novel raw starch digesting thermostable α-amylase from *Bacillus* sp. I-3 and its use in the direct hydrolysis of raw potato starch. **Enzyme and Microbial Technology.** 37(7): 723-734.

Gupta, R., Gigras, P., Mohapatra, H., Goswami, V. K., and Chauhan, B. (2003). Microbial α-amylases: a biotechnological perspective. **Process Biochemistry.** 38(11): 1599-1616.

Hamada, J. S. (1997). Characterization of protein fractions of rice bran to devise effective methods of protein solubilization. **Cereal Chemistry.** 74(5): 662-668.

He, F. (2011). Laemmli-SDS-PAGE. **Bio-Protocol.** 1(11): e80.

Helm, R. M., and Burks, A. W. (1996). Hypoallergenicity of rice bran protein**. Cereal Foods World.** 41(11): 839–843.

Hayashida, S., and Teramoto, Y. (1986). Production and characteristics of raw-starch-digesting α-amylase from a protease-negative *Aspergillus ficum* mutant. **Applied and Environmental Microbiology.** 52(5): 1068-1073.

Hayashida, S., Teramoto, Y., and Inoue, T. (1988). Production and characteristics of raw-potato-starch-digesting alpha-amylase from *Bacillus subtilis* 65. **Applied and Environmental Microbiology.** 54(6): 1516-1522.

Ilmén, M., Koivuranta, K., Ruohonen, L., Suominen, P., and Penttilä, M. (2007). Efficient production of L-lactic acid from xylose by *Pichia stipitis*. **Applied and Environmental Microbiology.** 73(1): 117-123.

Jensen, B., and Olsen, J. (1992). Physicochemical properties of a purified alpha-amylase from the thermophilic fungus *Thermomyces lanuginosus*. **Enzyme and Microbial Technology.** 14(2): 112-116.

Jeon, H.-Y., Kim, N.-R., Lee, H.-W., Choi, H.-J., Choung, W.-J., Koo, Y.-S., Ko, D.-S., and Shim, J.-H. (2016). Characterization of a novel maltose-forming α-amylase from *Lactobacillus plantarum* subsp. *plantarum* ST-III. **Journal of Agricultural and Food Chemistry.** 64(11): 2307-2314.

Kammoun, R., Naili, B., and Bejar, S. (2008). Application of a statistical design to the optimization of parameters and culture medium for α-amylase production by

Aspergillus oryzae CBS 819.72 grown on gruel (wheat grinding by-product). **Bioresource Technology.** 99(13): 5602-5609.

Kanpiengjai, A., Lumyong, S., Nguyen, T.-H., Haltrich, D., and Khanongnuch, C. (2015). Characterization of a maltose-forming α-amylase from an amylolytic lactic acid bacterium *Lactobacillus plantarum* S21. **Journal of Molecular Catalysis B: Enzymatic.** 120: 1-8.

Kelly, D., Smyth, J. A., and McCracken, K. J. (1991). Digestive development of the early-weaned pig. 2. effect of level of food intake on digestive enzyme activity during the immediate post-weaning period. **British Journal of Nutrition.** 65(2): 181-188.

Knox, A. M., du Preez, J. C., and Kilian, S. G. (2004). Starch fermentation characteristics of *Saccharomyces cerevisiae* strains transformed with amylase genes from *Lipomyces kononenkoae* and *Saccharomycopsis fibuligera*. **Enzyme and Microbial Technology.** 34(5): 453-460.

Kunamneni, A., Permaul, K., and Singh, S. (2005). Amylase production in solid state fermentation by the thermophilic fungus *Thermomyces lanuginosus*. **Journal of Bioscience and Bioengineering.** 100(2): 168-171.

Kundu, A. K., and Das, S. (1970). Production of amylase in liquid culture by a strain of *Aspergillus oryzae*. **Applied Microbiology.** 19(4): 598-603.

Laderman, K. A., Davis, B. R., Krutzsch, H. C., Lewis, M. S., Griko, Y. V., Privalov, P. L., and Anfinsen, C. B. (1993). The purification and characterization of an extremely thermostable alpha-amylase from the hyperthermophilic archaebacterium *Pyrococcus furiosus*. **The Journal of Biological Chemistry.** 268(32): 24394-24401.

Le, L. T., van Ierland, E. C., Zhu, X., and Wesseler, J. (2013). Energy and greenhouse gas balances of cassava-based ethanol. **Biomass and Bioenergy.** 51: 125-135.

Lévêque, E., Janeček, Š., Haye, B., and Belarbi, A. (2000). Thermophilic archaeal amylolytic enzymes. **Enzyme and Microbial Technology.** 26(1): 3-14.

Liu, B., Wang, F., Zhang, B., and Bi, J. (2013). Energy balance and GHG emissions of cassava-based fuel ethanol using different planting modes in China. **Energy Policy.** 56: 210-220.

Lonsane, B. K., and Ramesh, M. V. (1990). Production of bacterial thermostable alpha-amylase by solid-state fermentation: a potential tool for achieving economy in enzyme production and starch hydrolysis. **Advances in Applied Microbiology.** 35: 1-56.

Machius, M., Wiegand, G., and Huber, R. (1995). Crystal structure of calcium-depleted *Bacillus licheniform* is α-amylase at 2.2 Å Resolution. **Journal of Molecular Biology.** 246(4): 545-559.

Manano, J., Ogwok, P., and Byarugaba-Bazirake, G. (2018). Chemical composition of major cassava varieties in Uganda, targeted for industrialisation. **Journal of Food Research.** 7(1): 1-9.

McMahon, H. E. M., Kelly, C. T., and Fogarty, W. M. (1999). High maltose-producing amylolytic system of a *Streptomyces* sp. **Biotechnology Letters.** 21(1): 23-26.

Mendu, D. R., Ratnam, B., Purnima, A., and Ayyanna, C. (2005). Affinity chromatography of α-amylase from *Bacillus licheniformis*. **Enzyme and Microbial Technology.** 37(7): 712-717.

Mielenz, J. R. (1983). *Bacillus stearothermophilus* contains a plasmid-borne gene for alpha-amylase. **Proceedings of the National Academy of Sciences.** 80(19): 5975-5979.

Mihajlovski, K. R., Radovanović, N. R., Veljović, Đ. N., Šiler-Marinković, S. S., and Dimitrijević-Branković, S. I. (2016). Improved β-amylase production on molasses and sugar beet pulp by a novel strain *Paenibacillus chitinolyticus* CKS1. **Industrial Crops and Products.** 80: 115-122.

Mishra, S., Noronha, S., and Suraishkumar, G. (2005). Increase in enzyme productivity by induced oxidative stress in *Bacillus subtilis* cultures and analysis of its mechanism using microarray data. **Process Biochemistry.** 40(5): 1863-1870.

Møller, K., Sharif, M. Z., and Olsson, L. (2004). Production of fungal α-amylase by *Saccharomyces kluyveri* in glucose-limited cultivations. **Journal of Biotechnology.** 111(3): 311-318.

Mukherjee, R., Paul, T., Soren, J., K. Halder, S., Mondal, K., R. Pati, B., and Das Mohapatra, P. (2019). Acidophilic α-amylase production from *Aspergillus niger* RBP7 using potato peel as substrate: a waste to value added approach. **Waste and Biomass Valorization.** 10 (4):851-863.

Nguyen, Q., Rezessy-Szabó, J. M., and Hoschke, Á. (2000). Optimisation of composition of media for the production of amylolytic enzymes by *Thermomyces lanuginosus* ATCC 34626. **Food Technology and Biotechnology.** 38(3): 229-234.

Nigam, P., and Singh, D. (1995). Enzyme and microbial systems involved in starch processing. **Enzyme and Microbial Technology.** 17(9): 770-778.

Oluremi, O. I., Solomon, A. O., and Saheed, A. A. (2013). Fatty acids, metal composition and physico-chemical parameters of Igbemo Ekiti rice bran oil. **Journal of Environmental Chemistry and Ecotoxicology.** 5(3): 39-46.

Okudoh, V., Trois, C., Workneh, T., and Schmidt, S. (2014). The potential of cassava biomass and applicable technologies for sustainable biogas production in South Africa: A review. **Renewable and Sustainable Energy Reviews.** 39: 1035-1052.

Pandey, A., Nigam, P., Soccol, C., Soccol, V., Singh, D., and Mohan, R. (2000). Advances in microbial amylases. **Biotechnology and Applied Biochemistry.** 31: 135-152.

Patel, A. K., Nampoothiri, K. M., Ramachandran, S., Szakacs, G., and Pandey, A. (2005). Partial purification and characterization of alpha-amylase produced by *Aspergillus oryzae* using spent-brewing grains. **Indian Journal of Biotechnology.** 4(4): 336-341.

Pedersen, H., and Nielsen, J. (2000). The influence of nitrogen sources on the α-amylase productivity of *Aspergillus oryzae* in continuous cultures. **Applied Microbiology and Biotechnology.** 53(3): 278-281.

Rahardjo, Y. S., Sie, S., Weber, F. J., Tramper, J., and Rinzema, A. (2005). Effect of low oxygen concentrations on growth and α-amylase production of *Aspergillus oryzae* in model solid-state fermentation systems. **Biomolecular Engineering.** 21(6): 163-172.

Raimbault, M. (1998). General and microbiological aspects of solid substrate fermentation. **Electronic Journal of Biotechnology.** 1(3): 26-27.

Ramachandran, S., Patel, A. K., Nampoothiri, K. M., Francis, F., Nagy, V., Szakacs, G., and Pandey, A. (2004). Coconut oil cake: A potential raw material for the production of α-amylase. **Bioresource Technology.** 93(2): 169-174.

Raul, D., Biswas, T., Mukhopadhyay, S., Kumar Das, S., and Gupta, S. (2014). Production and partial purification of alpha-amylase from *Bacillus subtilis* (MTCC 121) using solid state fermentation. **Biochemistry Research International.** 2014: 5.

Richardson, T. H., Tan, X., Frey, G., Callen, W., Cabell, M., Lam, D., Macomber, J., Short, J. M., Robertson, D. E., and Miller, C. (2002). A novel, high performance enzyme for starch liquefaction discovery and optimization of a low pH, thermostable α-amylase. **Journal of Biological Chemistry.** 277(29): 26501-26507.

Rodríguez Couto, S., and Sanromán, M. A. (2005). Application of solid-state fermentation to ligninolytic enzyme production. **Biochemical Engineering Journal.** 22(3): 211-219.

Rodtong, S., and Ishizaki, A. (2003). Potential microorganism for the direct production of L-lactic acid from cassava starch without carbon dioxide production. **Macro Review.** 16(1): 332-336.

Sanchez, S., and Demain, A. L. (2010). Enzymes and bioconversions of industrial, pharmaceutical, and biotechnological significance. **Organic Process Research and Development.** 15(1): 224-230.

Sánchez, A. S., Silva, Y. L., Kalid, R. A., Cohim, E., and Torres, E. A. (2017). Waste bio-refineries for the cassava starch industry: New trends and review of alternatives. **Renewable and Sustainable Energy Reviews.** 73: 1265-1275.

Saunders, R. M. (1990). The properties of rice bran as a foodstuff. **Cereal Foods World.** 35(7): 632, 634-636.

Schon, U., and Schumann, W. (1993). Molecular cloning, sequencing, and transcriptional analysis of the *groESL* operon from *Bacillus stearothermophilus.* **Journal of Bacteriology.** 175(8): 2465-2469.

Sivaramakrishnan, S., Gangadharan, D., Nampoothiri, K. M., Soccol, C. R., and Pandey, A. (2006). α-Amylases from microbial sources: An overview on recent developments. **Food Technology Biotechnology.** 44(2): 173-184.

Sodhi, H. K., Sharma, K., Gupta, J. K., and Soni, S. K. (2005). Production of a thermostable α-amylase from *Bacillus* sp. PS-7 by solid state fermentation and its synergistic use in the hydrolysis of malt starch for alcohol production. **Process Biochemistry.** 40(2): 525-534.

Sreedhar, R., and Kaul Tiku, P. (2016). Cupincin: A unique protease purified from rice (*Oryza sativa* L.) bran is a new member of the Cupin superfamily. **PloS One.** 11(4): e0152819-e0152819.

Srivastava, R., and Baruah, J. (1986). Culture conditions for production of thermostable amylase by *Bacillus stearothermophilus*. **Applied and Environmental Microbiology.** 52(1): 179-184.

Sudan, S. K., Kumar, N., Kaur, I., and Sahni, G. (2018). Production, purification, and characterization of raw starch hydrolyzing thermostable acidic alpha-amylase from hot springs, India. **International Journal of Biological Macromolecules.** 117: 831-839.

Sundarram, A., and Murthy, T. P. K. (2014). α-Amylase production and applications: A review. **Journal of Applied and Environmental Microbiology.** 2(4): 166-175.

Syu, M.-J., and Chen, Y.-H. (1997). A study on the α-amylase fermentation performed by *Bacillus amyloliquefaciens*. **Chemical Engineering Journal.** 65(3): 237-247.

Takenaka, S., Miyatake, A., Tanaka, K., Kuntiya, A., Techapun, C., Leksawasdi, N., Seesuriyachan, P., Chaiyaso, T., Watanabe, M., and Yoshida, K. (2015). Characterization of the native form and the carboxy-terminally truncated halotolerant form of alpha-amylase from *Bacillus subtilis* strain FP-133. **Journal of Basic Microbiology.** 55(6): 780-789.

Tanyildizi, M. S., Özer, D., and Elibol, M. (2005). Optimization of α-amylase production by *Bacillus* sp. using response surface methodology. **Process Biochemistry.** 40(7): 2291-2296.

Thippeswamy, S., Girigowda, K., and Mulimani, V. (2006). Isolation and identification of α-amylase producing *Bacillus* sp. from dhal industry waste. **Indian Journal of Biochemistry and Biophysics.** 43(5): 295-8.

Talamond, P., Desseaux, V., Moreau, Y., Santimone, M., and Marchis-Mouren, G. (2002). Isolation, characterization and inhibition by acarbose of the α-amylase from *Lactobacillus fermentum*: Comparison with *Lb. manihotivorans* and *Lb. plantarum* amylases. **Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology.** 133(3): 351-360.

Van Der Maarel, M. J., Van Der Veen, B., Uitdehaag, J. C., Leemhuis, H., and Dijkhuizen, L. (2002). Properties and applications of starch-converting enzymes of the α-amylase family. **Journal of Biotechnology.** 94(2): 137-155.

Vieille, C., and Zeikus, G. J. (2001). Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. **Microbiology and Molecular Biology Reviews.** 65(1): 1-43.

Vishnu, C., Seenayya, G., and Reddy, G. (2000). Direct conversion of starch to L(+) lactic acid by amylase producing *Lactobacillus amylophilus* GV6. **Bioprocess Engineering.** 23(2): 155-158.

Viswanathan, P., and Surlikar, N. R. (2001). Production of α-amylase with *Aspergillus flavus* on amaranthus grains by solid-state fermentation. **Journal of Basic Microbiology.** 41(1): 57-64.

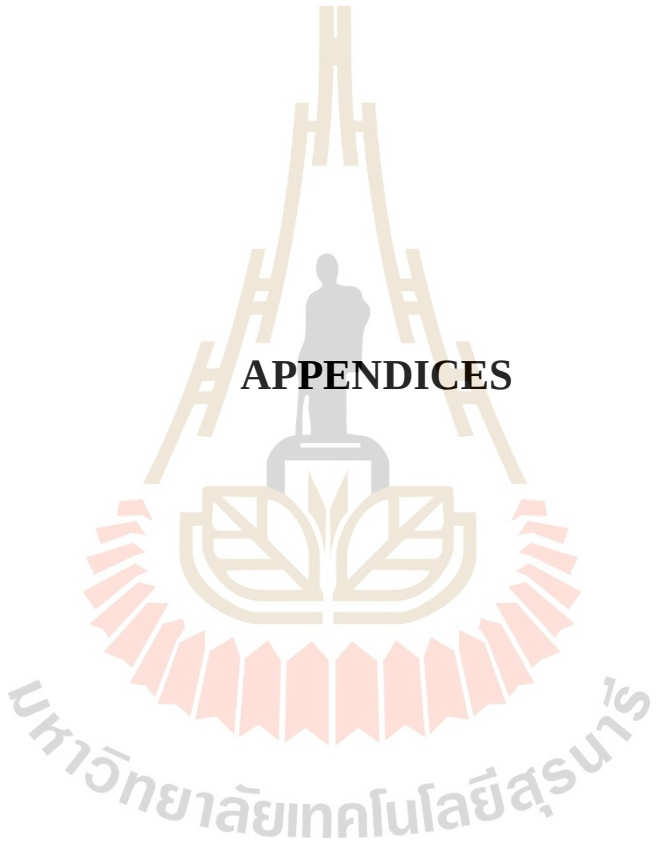
Xia, N., Wang, J. M., Gong, Q., Yang, X. Q., Yin, S. W., and Qi, J. R. (2012). Characterization and *in vitro* digestibility of rice protein prepared by enzyme-assisted microfluidization: Comparison to alkaline extraction. **Journal of Cereal Science.** 56(2): 482-489.

Xiao, Z., Storms, R., and Tsang, A. (2006). A quantitative starch? iodine method for measuring alpha-amylase and glucoamylase activities. **Analytical biochemistry.** 351(1): 146-148.

Xie, F., Quan, S., Liu, D., Ma, H., Li, F., Zhou, F., and Chen, G. (2014). Purification and characterization of a novel α-amylase from a newly isolated *Bacillus methylotrophicus* strain P11-2. **Process Biochemistry.** 49(1): 47-53.

Zhou, W., Wang, X., Zhou, D., Ouyang, Y., and Yao, J. (2017). Overexpression of the 16-kDa α-amylase/trypsin inhibitor RAG2 improves grain yield and quality of rice. **Plant Biotechnology Journal.** 15(5): 568-580.

APPENDICES



APPENDIX A

REAGENT AND CULTURE MEDIUM PREPARATIONS

A1. REAGENTS USED FOR EXPERIMENTS

1.1 Reagents for Gram-staining of bacterial cells

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
Adjustment by distilled water	300.00	ml

1.2 Reagent for protein determination

1.2.1 Bradford reagent

Coomassie blue	100.00	mg
Ethanol (95%, v/v)	50.00	ml
Phosphoric acid	100.00	ml

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

1.3 Reagent for amylase activity determination

1.3.1 Dinitrosalicylic reagent

3,5-Dinitrosalicylic acid	10.00 g
Sodium hydroxide	19.00 g
Potassium sodium tartrate	306.00 g
Phenol	7.60 g
Sodium metabisulfite	8.30 g

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

A2. CULTURE MEDIA FOR LACTIC ACID BACTERIUM CULTIVATION AND AMYLASE PRODUCTION

2.1 Modify Lactobacillus MRS medium (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°C

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°C.

2.2 L-lactic producing medium

Pancreatic digest of casein or Tryptone	2.50	g
Yeast extract	10.0	g
Soy-protein isolate	2.50	g
Ammonium sulfate	1.00	g
Dipotassium phosphate	6.00	g
$MgSO_4.7H_2O$	0.30	g
$MnSO_4.4H_2O$	0.30	g
$FeSO_4.7H_2O$	0.03	g
Tapioca starch	120.00	g

Final pH 7.0 ± 0.2 at 25°C

For prepare carbon source in this medium calcium chloride 0.12 g was added in to tapioca starch while gelatinization and add α-amylase 0.12 g. 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°C.

A3 SDS-PAGE GEL PREPARATION

3.1 SDS-PAGE Gel

1) Preparation of the separation gel (10%). Mix in the following order:

Milli-Q water	4.1 ml
Acrylamide/bis (30% 37.5:1)	3.3 ml
Tris–HCl (1.5 M, pH 8.8)	2.5 ml

SDS (10%)	100 µl
N,*N*,*N*′,*N*′-tetramethylethylene-diamine (TEMED) (Bio-Rad)	10 µl
Ammonium persulfate (APS, 10%)	32 µl

After adding TEMED and APS to the SDS-PAGE separation gel solution, the gel will polymerize quickly, so add these two reagents when ready to pour.

2) Pour gel, leaving 2 cm below the bottom of the comb for the stacking gel. Make sure to remove bubbles.

3) Layer the top of the gel with isopropanol. This will help to remove bubbles at the top of the gel and will also keep the polymerized gel from drying out.

4) Remove the isopropanol and wash out the remaining traces of isopropanol with distilled water.

5) Prepare the stacking gel (4%). Mix in the following order:

Milli-Q water	6.1 ml
Acrylamide/bis (30%, 37.5:1)	1.3 ml
Tris–HCl (0.5 m, pH 6.8)	2.5 ml
SDS (10%)	100 µl
TEMED	10 µl
Ammonium persulfate (APS, 10%)	100 µl

6) Pour stacking gel on top of the separation gel.

7) Add combs to make wells. In ~30 min, the stacking gel should become completely polymerized.

8) Clamp gel into apparatus, and fill both buffer chambers with gel running buffer according to the instructions for the specific apparatus.

9) Load samples and molecular mass protein markers into wells for separation by electrophoresis.

3.2 10X Running buffer

Tris base	30.0	g
Glycine	144.0	g
SDS	10.0	g

The compositions were dissolved in 1000 ml of H_2O. The pH of the buffer should be 8.3 and no pH adjustment was required. Store the running buffer at room temperature and dilute to 1X before use.

3.3 2X SDS protein sample buffer

1 M Tris-HCl (pH 6.8)	1.25	ml
SDS (10%)	4.0	ml
Glycerol	2.0	ml
EDTA (0.5 M)	0.5	ml
Bromophenol blue	4.0	mg
β-mercaptoethanol (14.3 M)	0.2	ml

Bring the volume to 10 ml with H_2O.

3.4 30%T (2.6%C) Acrylamide stock solution

Acrylamide	29.22 g
Bisacrylamide	0.78 g

The compositions were dissolved in 100 ml of H_2O, then filtered the stock solution through Whatman filter paper and stored at 4°C. The fresh stock acrylamide solution was prepared every few weeks.

3.5 Gel staining solution (SDS-PAGE)

Coomassie blue R-250	1.0	g
Methanol	460.0	ml
Acetic acid	8.0	ml

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

3.6 Gel destaining solution (SDS-PAGE)

Methanol	200.0	ml
Acetic acid	5.0	ml

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

APPENDIX B

STANDARD CURVES AND TABLES

Table B1 Standard protein (bovine serum albumin, BSA) concentration determined by Bradford protein assay (Bradford, 1976).

Concentration of BSA (mg/ml)	Absorbance at 595 nm			
	1	2	3	Average
0.1	0.172	0.196	0.148	0.172
0.3	0.300	0.356	0.319	0.325
0.5	0.594	0.597	0.555	0.582
0.7	0.876	0.811	0.825	0.837

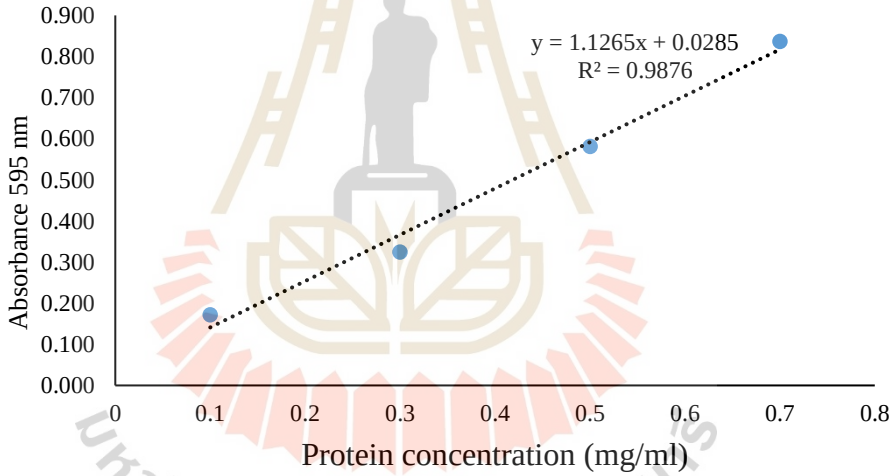


Figure B1 The standard curve plotted between standard protein (BSA) concentration and absorbance 595 nm.

Table B2 Standard maltose concentration determined by the DNS method (Bernfeld, 1955), measured at absorbance 540 nm.

Concentration of maltose (mg/ml)	Absorbance at 540 nm			
	1	2	3	Average
0.00	0	0	0	0
0.05	0.004	0.01	0.005	0.0063
0.10	0.045	0.046	0.051	0.0473
0.25	0.204	0.197	0.199	0.2000
0.50	0.468	0.449	0.453	0.4567
0.75	0.628	0.687	0.745	0.6867
1.00	0.934	0.95	0.916	0.9333

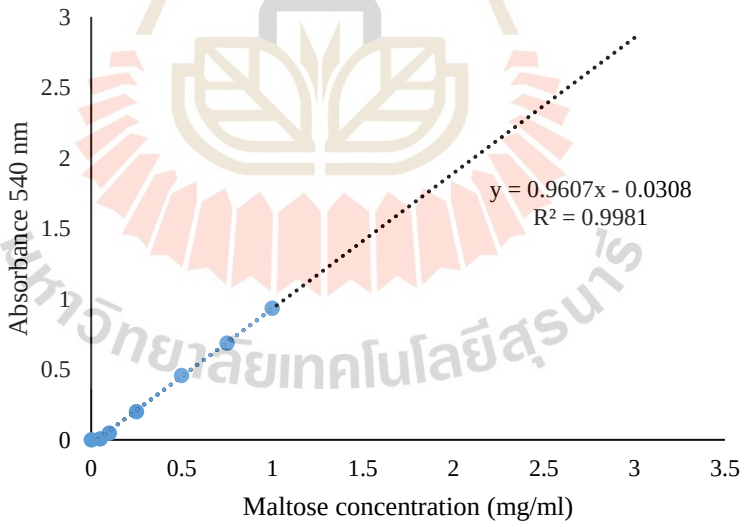


Figure B2 The standard curve of maltose concentration by DNS method (Bernfeld, 1955).

Table B3 Standard maltose concentration by DNS assay in 96-well plate.

Concentration of maltose (mg/ml)	Absorbance at 540 nm				
	1	2	3	Average	SD
0	0.000	0.001	0.002	0.001	0.001
0.1	0.008	0.007	0.007	0.007	0.079
0.25	0.058	0.061	0.068	0.062	0.082
0.5	0.225	0.208	0.206	0.213	0.049
0.75	0.373	0.374	0.35	0.366	0.037
1	0.528	0.522	0.477	0.509	0.055
1.25	0.683	0.696	0.63	0.670	0.052
1.5	0.942	0.868	0.815	0.875	0.073

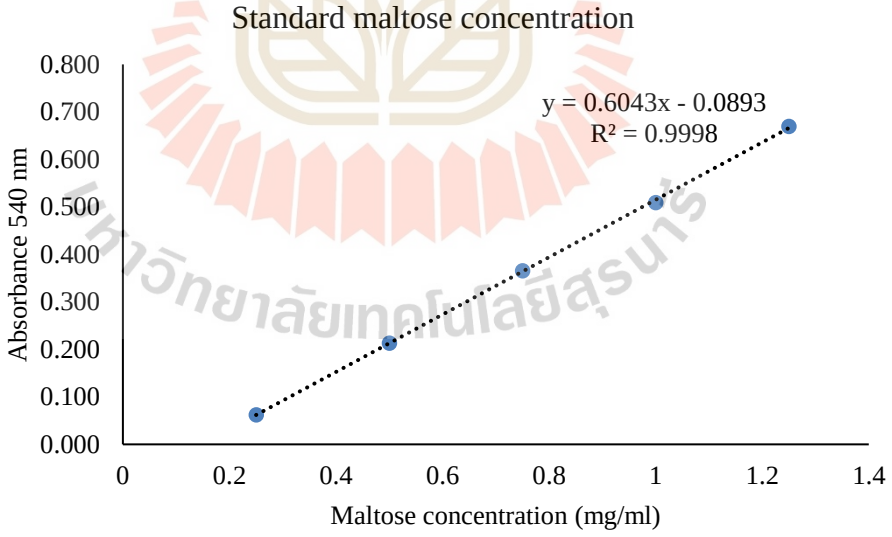


Figure B3 The standard curve of maltose concentration by DNS method (Bernfeld, 1955).

Table B4 Standard cassava starch concentration by starch-iodine complex assay in 96-well plate.

Cassava starch concentration (%, w/v)	Absorbance at 580 nm				
	1	2	3	Average	SD
0.00	-0.004	-0.003	0	-0.002	0.002
0.01	0.169	0.174	0.179	0.174	0.005
0.02	0.312	0.321	0.325	0.319	0.007
0.03	0.473	0.475	0.483	0.477	0.005
0.04	0.617	0.611	0.516	0.581	0.057
0.05	0.767	0.762	0.758	0.762	0.005

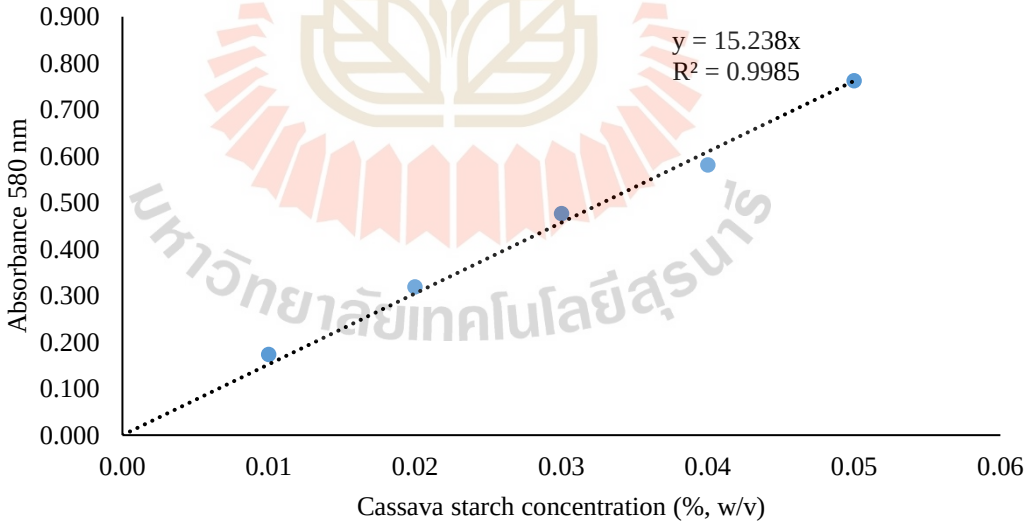
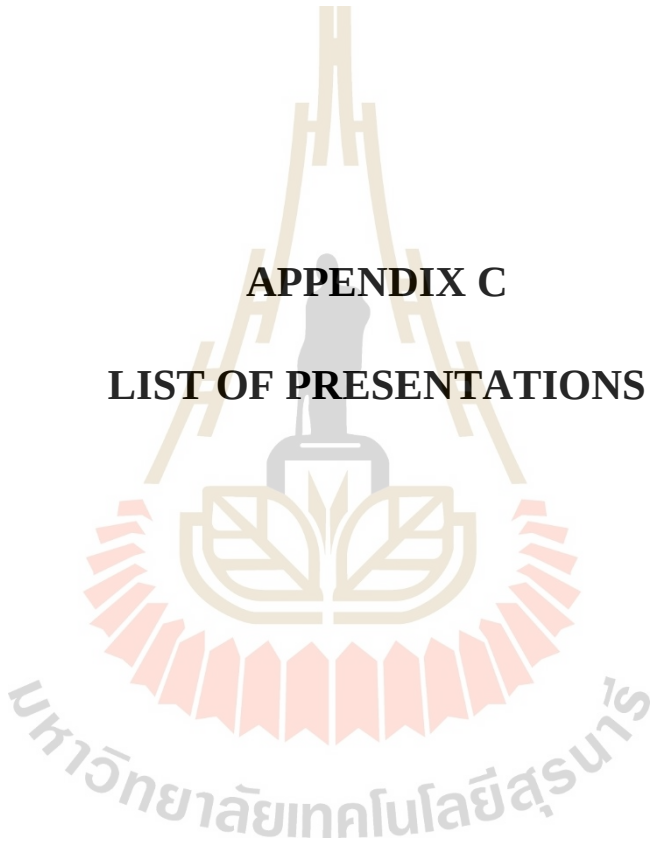


Figure B4 The standard curve of cassava starch concentration by starch-iodine complex assay in 96-well plate.

APPENDIX C

LIST OF PRESENTATIONS



Poster and oral presentation

Sumalu, R., Rodtong, S., Nopanitaya, T., and Pan-anu, A. (2015). Investigation of Ma Sang, *Feroniella lucida* (Scheef.) Swingle, fruit for developing food product. Abstracts of The 41st Congress on Science and Technology of Thailand (STT41), 6-8 November, 2015. Suranaree University of Technology, Nakhon Ratchasima, Thailand. p. A216.

Sumalu, R., Songsiriritthigul, C., and Rodtong, S. (2017). Potential production of amylase by starch-utilizing and L-lactic acid-producing bacterium. Abstracts of The 12st Conference on Science and Technology for Youths, 3-4 June, 2017. Bangkok International Trade and Exhibition Centre (BITEC), Bangna,Bangkok, Thailand. p. 85.

Sumalu, R., Songsiriritthigul, C., and Rodtong, S. (2019). Amylase production by the potential Strain of L-lactic acid-producing bacterium in cassava starch medium containing rice bran. Abstracts of The 21st Food Innovation Asia Conference 2019, 3-15 June, 2019. Bangkok International Trade and Exhibition Centre (BITEC), Bangna,Bangkok, Thailand. p 53-54 .

these bacterial strains have the pathway for the catabolism of amino acids. Interestingly, two key enzymes for flavor formation, including alcohol dehydrogenase (adh) and aldehyde dehydrogenases (aldh) were also identified. However, genes encoding histidine decarboxylase (hdc) were not detected to confirm the non-histamine-producing strains. These draft genome sequences have been deposited as the first version in DDBJ/EMBL/GenBank under accession numbers JPVT00000000 (*Tetragenococcus muriaticus* 3MR10-3) and JPVU00000000 (*Tetragenococcus muriaticus* PMC-11-5). (abstract only)

I_I0012: INVESTIGATION OF MA SANG, *Feroniella lucida* (SCHEFF.) SWINGLE, FRUIT FOR DEVELOPING FOOD PRODUCT

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Abstract: Ma Sang or wood apple, *Feroniella lucida* (Scheff.) Swingle, is a native plant in Asia. It grows wild as a small tree having 5-10 m high in North-eastern Thailand or Isaan region, particularly in deciduous dipterocarp forest, dry evergreen forest, and rice field. Fruits and leaves are sour taste using as seasoning for soup by local people. Chemical investigation and biological activity of this plant leaves have been reported by a small group of investigators. The leave oil contains anti-mycobacterial activity, such as against *Mycobacterium tuberculosis*. Also, there is very limited information in literature on its edible fruit. This investigation was thus considered to be performed both field survey of the fruit yield and preliminary study of the composition of the ripen fruit to obtain data for developing food product at least food for local communities in Isaan region where its climate is hot and dry and its output lags behind other parts of the country. For investigating yield of ripe Ma Sang fruit, the estimation based on a community in Isaan (Satuek District, Buriram Province) was come up with approximately 700 kg for each tree. If the plant is expanded to cultivate in a certain area, it could yield around 625 tons/acre/year. The ripe fruits of Ma Sang were collected. The fruit was about 70 mm in diameter with a tough, woody skin around 7-10 mm thick, and comprised a lot of seeds. The pulp of the fruit is normally eaten as a vegetable. The woody skin and flesh contained the average moisture contents of 57.09±0.87 and 75.94±0.06%, respectively. The ripen fruit was one of the good sources of vitamin C as well as a good source of calcium. The essential oil composition of the fruit skin, from hydrodistillation and chromagraphy analyses was similar to that has been reported in leaf. The major components were β-caryophyllene, dodecanal, decanal, and decyl acetate at approximately 25, 17, 16 and 12%, respectively. The ethanol extract of the ripe fruit skin displayed antimicrobial activity against the standard strain of Gram-positive *Staphylococcus aureus* (approximately 7 mm inhibition zone diameter) by agar diffusion technique. Characterization of organic acid and nutrient composition of the ripen Ma Sang fruit is under investigation. Results provide data for developing food product to support at least local community in Isaan region, Thailand. (abstract only)

I_I0013: INVESTIGATION OF RIPE FRUITS OF THE WILD PLANT HUAT KA, *Lepisanthes rubiginosa* (ROXB.) LEENH, FOR WINE PRODUCTION

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ศักยภาพในการผลิตอะไมเลสของแบคทีเรียผลิตกรดแอล-แล็กติกได้โดยตรงจากแป้ง

รัฐพร สุมาลุย์,[1] ชมภูนุช ส่งสิริฤทธิกุล[2] และ สุรีลักษณ์ รอดทอง[3,*]
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บทคัดย่อ: เอนไซม์อะไมเลสทางการค้ามีประโยชน์ในอุตสาหกรรมที่ต้องย่อยแป้งเพื่อแก้ปัญหาความหนืดสูงจากวัตถุดิบที่มีแป้งความเข้มข้นสูง แบคทีเรีย *Lactococcus* sp. SUT 513 ที่ศึกษาครั้งนี้ สามารถผลิตกรดแอล-แล็กติกได้โดยตรงจากแป้ง มีศักยภาพสูงในการผลิตอะไมเลส อาจสามารถลดการใช้เอนไซม์อะไมเลสทางการค้า จึงได้พัฒนาสูตรอาหารเลี้ยงเชื้อเพื่อผลิตอะไมเลส และได้ส่วนประกอบเหมาะสมที่มีแป้งมันสำปะหลัง รำข้าว K_2HPO_4, $MgSO_4$, $MnSO_4$, $FeSO_4$ ความเข้มข้น 20, 4, 6, 0.57, 0.12 และ 0.03 กรัมต่อลิตร ตามลำดับ จากส่วนประกอบหลักเริ่มต้นที่มีแป้งมันสำปะหลัง ทริปโตน สารสกัดจากยีสต์ K_2HPO_4 และไตร-แอมโมเนียมซิเตรท ปริมาณ 10, 5, 3, 6 และ 1 กรัมต่อลิตร ตามลำดับ เมื่อเลี้ยงแบคทีเรียเป็นเวลา 24 ชั่วโมง พบว่าในอาหารเลี้ยงเชื้อเหมาะสมมีกิจกรรมของอะไมเลส 1.05±0.11 หน่วยต่อมิลลิลิตร (มากกว่าสูตรเริ่มต้นถึง 3.18 เท่า) มีโปรตีน 0.57±0.06 กรัมต่อลิตร และสามารถลดต้นทุนค่าอาหารเลี้ยงเชื้อลงจากเริ่มต้นร้อยละ 87 ผลการศึกษาแสดงถึงศักยภาพสูงในการผลิตอะไมเลสโดย *Lactococcus* sp. SUT 513

คำสำคัญ: อะไมเลส, *Lactococcus* sp., แบคทีเรียผลิตกรดแอล-แล็กติก

Potential Production of Amylase by Starch-Utilizing and L-Lactic Acid-Producing Bacterium

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Abstract: The commercial amylases give benefit to starchy industry for fixing viscosity problem that caused by starch high concentration. The study of the potential amylase production from *Lactococcus* sp. SUT 513 used in this study, could directly produce L-lactic acid from cassava starch at high concentrations. This will reduce the application of commercial amylases. The suitable cultivation medium for amylase production comprising cassava starch, rice bran, K_2HPO_4, $MgSO_4$, $MnSO_4$, and $FeSO_4$; at 20, 4, 6, 0.57, 0.12, and 0.03 g/l, respectively, was developed from the original medium mainly composing of cassava starch, tryptone, yeast extract, K_2HPO_4, and tri-ammonium citrate at 10, 5, 3, 6, and 1 g/l). When cultivated the SUT 513 for 24 h, the amylase activity of 1.05±0.11 U/ml (3.18 times higher than the original medium) with 0.057±0.06 g/l of protein. The medium provides the cost reduction of 87%. These results reveal the high potential production of amylase by *Lactococcus* sp. SUT 513.

Keywords: Amylase, *Lactococcus* sp., L-Lactic acid-producing bacteria, Amylolytic lactic acid bacteria.

BIOLOGY

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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Commercial amylases give benefit to the starch hydrolysis process in the food industry as well as to L-lactic acid production to solve the viscosity problem caused by high starch concentrations. This study aimed to investigate amylase production by the lactic acid bacterial strain *Lactococcus* sp. SUT 513. The bacterium could directly produce L-lactic acid from cassava starch at high concentrations (12-15% starch), which reduced the usage of commercial amylases. The suitable amylase production medium was developed from the expensive standard medium De Man, Rogosa and Sharpe medium (MRS medium) that used for cultivation of lactic acid bacteria. The developed amylase production medium was studied by varying concentration of cassava starch, and rice bran as a nitrogen source. Finally, The medium was found to mainly comprises of cassava starch (2%) and rice bran (0.4%). When cultivated the lactic acid bacterium strain SUT 513 in 50 ml of the developed cassava starch medium containing rice bran in 125 ml Erlenmeyer flask under anaerobic incubation at 35°C without shaking for 24 h, the amylase activity of 1.05±0.11 U/ml (3.18 times higher than the original medium) and protein concentration of 0.057±0.06 g/l was achieved. The developed medium provides a cost reduction of 79% compared to MRS medium. The potential bacterial strain produced the highest amylase activity of 4.848±0.237 U/ml when cultured in the cassava starch medium increased its volume to 3 l in 5 l bioreactor with agitation speed 150 rpm and without aeration at 35°C for 18 h. For the preliminary separation of amylase from the developed medium, the 90% ammonium sulfate precipitation was suitable and could provide the alpha-amylase purification around 2.89 folds. These results revealed the high potential production of amylase by *Lactococcus* sp. SUT 513, in a simple and cheap medium, which provides an opportunity to scale up the production of amylase to replace the expensive commercial enzyme.

Keywords: *Amylase production, Lactococcus* sp., *Cassava starch, L-lactic acid- producing bacteria*

DPB163

Postharvest Application of Organic Acids and Inorganic Salts to Control Tomato Postharvest Fungi *Penicillium* sp.: *In Vitro* Study

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Postharvest losses during storage and transportation of tomatoes are mainly caused by fungal plant pathogens. In order to maintain postharvest quality, physical and chemical treatments have been used to achieve this goal. This study examined the inhibitory activity of several organic acids (acetic acid, citric acid, propionic acid and oxalic acids) and inorganic salts (sodium carbonate, sodium hydrogen carbonate, sodium chloride, potassium chloride, potassium carbonate, potassium bicarbonate and ammonium carbonate) on growth rate and spore germination of isolated fungi, *Penicillium* sp., strain was isolated from tomatoes in Thailand. In vitro treatment trials were performed by supplementing cultured medium with studied chemicals at different concentrations. Radial growth rate and spore germination for each studied treatment were determined. Statistical analysis indicated that both studied chemical treatments significantly affected growth rates and spore germination at conditions used ($P<0.05$). Overall, *Penicillium* sp. exhibited more noticeable sensitive response to organic acids than inorganic salts at nearly all organic acid levels used. Supplementing the media with acetic acid, propionic acid and oxalic acids at 0.5-1% (w/v) gave the significant strong inhibitory effect on growth where *Penicillium* sp. unable to grow. For inorganic salts, significant growth control was observed with 1-3% (w/v) ammonium carbonate and potassium bicarbonate. Whereas a moderate control was achieved with sodium carbonate, sodium hydrogen carbonate, sodium chloride and potassium chloride, where extended lag phases prior to growth was also obtained. The average spore germination and germ tube length of *Penicillium* sp. were significantly affected when treated with 0.5-1% (w/v) of acetic acid, propionic acid and oxalic acids. These organic acids significantly reduced ability to form germ tubes of fungi, where the average germ tube length was decreased 1.8 fold (5 hours) comparing to control. This finding will be beneficial for identifying organic acids and inorganic salts for improvement of postharvest quality during storage and prolong the shelf-life of tomatoes.

Keywords: *Chemical treatment, Inorganic salts, Organic acids, Penicillium, Tomato*

DPP40

Identification of Halophilic Bacteria Isolates from salty fermented foods and Characterization of their Pigment Production

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