

BACTERIAL AMYLASE PRODUCTION BY THE L-LACTIC ACID-PRODUCING STRAIN USING CASSAVA STARCH SUPPLEMENTED WITH RICE BRAN

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Abstract

Amylase is a crucial starch-degrading enzyme, which is commercially available, provides benefit to starchy industry to overcome viscosity problem that caused by using high starch concentration, and to supply sugar residues. This study aimed to investigate the production of amylase by the potential optical pure L-lactic acid-producing *Lactococcus* sp. SUT 513 for further development of the optical pure acid fermentation process from the cheap cassava starch. Rice bran, the main byproduct in the process of rice milling, was found to provide the maximum amylase production when supplemented with 4.0 g/L (w/v) to the medium containing cassava starch (20.0 g/L, w/v), compared to the cassava medium added either the expensive nitrogen source, tryptone (5.0 g/L) and yeast extract (3.0 g/L, w/v); or the cheap alternatives, soy ptotein (8.0 g/L) or urea (2.0 g/L) combined with yeast extract (3.0 g/L). The amylase activity obtained from the developed medium was approximately 3 times higher than the original medium. This bacterium gave the highest crude amylase activity of 4.848 ± 0.237 U/mL in the suitable medium performed in a 7.5-L bioreactor with 3.0-L working volume for the optimal cultivation time of only 18 h under a control pH of 7.0, temperature at 35°C, agitation at 150 rpm, and without aeration. The results reveal the high potential production of amylase by *Lactococcus* sp. SUT 513 using the cheap cassava starch medium supplemented with rice bran, providing an opportunity to develop both amylase and optically pure L-lactic acid production processes.

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

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Introduction

Amylase is one of the starch-degrading enzymes that can randomly hydrolyze starch molecules into linear and branched oligosaccharides in various length composed of dextrin and finally glucose units (Gupta *et al.*, 2003), and can be obtained from various sources including plants, animals, and microorganisms (Pandey *et al.*, 2000). The available amylase is usually produced by bacteria, particularly several species in the genus *Bacillus* (Nigam and Singh, 1995), and has potential applications in a wide variety of industrial processes such as food production, fermentation, textile, paper, detergent, and pharmaceutical industries (Pandey *et al.*, 2000; Richardson *et al.*, 2002; Li *et al.*, 2012). Its application was mainly on the liquefaction and saccharification of the higher complexity of the carbohydrate structure to simpler molecules of diverse dextrans and oligosaccharides (Paul *et al.*, 2021) such as maltopentaose, maltotetraose, maltotriose, maltose, and the monosaccharide glucose. From the global alpha-amylase baking enzyme market, the trend of microbial enzyme usage is continuously increasing every year. According to the report by Grand View Research, Inc., the global alpha-amylase baking enzyme market size is expected to reach \$320.1 Million by 2024, increasing importance of enzymes as functional additives in food and beverage applications on account of attributing features including good emulsification properties and self-stabilization effects is expected to remain a crucial factor (James, 2016).

The high cost of amylase production medium has been continuously considered for decades. *Bacillus* sp. was noted to give a maximum raw starch digesting amylase in a medium containing lactose and yeast extract (Rahardjo *et al.*, 2005). Agricultural wastes consisting of carbon and nitrogen sources necessary for growth and metabolism of organisms, have been used for reducing the cost of cultivation media. 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). For nitrogen source, 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 the enzyme activity of *Bacillus* sp. The maximum α -amylase secreted by *Bacillus amylolyticus* was found in the medium supplemented with peptone (1.0%), yeast extract (0.5%), and maltose (0.5%) under vigorous

shaking conditions (Dettori-Campus *et al.*, 1992). Aiyer (2004) compared the influence of organic and inorganic nitrogen sources for enzyme production by *Bacillus licheniformis* SPT 278, and reported that peptone was a better nitrogen source than ammonium hydrogen phosphate, and 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*, and yeast extract resulted in significant α -amylase yield (Nguyen *et al.*, 2000). The supplementation of casein hydrolysate to the specific medium resulted in a 143% increase in α -amylase productivity by *Aspergillus oryzae* A1560 compared to ammonia, and yeast extract along with ammonium sulfate also gave the significant enzyme productivity 110% by the *Aspergillus oryzae* (Pedersen and Nielsen, 2000).

New sources of amylase are still desired. For example, Al-Agamy *et al.* (2021) found a *Streptomyces rochei* strain that could efficiently produce the starch hydrolytic enzyme, α -amylase, under the most four significant parameters; starch, K_2HPO_4 , pH, and temperature. A few lactic acid bacteria such as some species of *Lactobacillus* (Xu *et al.*, 2020) and *Enterococcus* (Unban *et al.*, 2017) could produce amylase. Lactic acid is an organic acid having a molecular weight of around 90.08 g/mol, water-soluble, and two isomers, L- and D-forms. The acid is very useful for applications in food, cosmetic, and pharmaceutical as well as bioplastic industries causing the high global lactic acid production around 270,000 tons per year and tends to increase annually 10% (Alexandri *et al.*, 2019). Lactic acid can also be produced from starch, the high complexity of carbohydrate molecule. Usually, starch is gelatinized at high temperature followed by saccharification using commercial amylolytic enzymes to hydrolyze the gelatinization product to glucose, and subsequently converting glucose to lactic acid. Amylolytic lactic acid bacteria are, therefore, advantages to perform the single-step lactic acid fermentation.

This study aimed 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 pure L-lactic acid production, and could potentially be used for amylase enzyme production to reduce or replace commercial amylases when using the high concentration of cassava starch resulting in the high viscosity of the acid production medium.

Materials and Methods

Microorganism and Cultivation Conditions

The lactic acid bacterium *Lactococcus* sp. SUT 513, the potential strain for the direct production of optical pure L-lactic acid from cassava starch, was used in this study. Its amylase activity was attractive to produce for application as well as the production of L-lactic acid. The *Lactococcus* sp. SUT 513 strain was obtained from our L-lactic acid production project carried out at the Microbial Culture Collection and Applications Research Center, Suranaree University of Technology, Thailand. Its 16S rRNA gene sequence was deposited in the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank>) with the accession number of FJ611789. The starch-utilizing and L-lactic acid-producing bacterium was cultured on the modified Lactobacilli MRS medium containing 1.0% cassava starch (Sangan Wongse Industries Co., Ltd., Nakhon Ratchasima, Thailand) to replace glucose, and the following components: tryptone (pancreatic digest of casein; HiMedia, HiMedia Laboratories, Mumbai, India), yeast extract (HiMedia), K_2HPO_4 (Carlo Erba Reagenti, Milan, Italy), tri-ammonium citrate, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$, $FeSO_4 \cdot 7H_2O$ (Carlo Erba Reagenti) at concentrations of 5.0, 6.0, 3.0, 1.0, 0.57, 0.12, and 0.03 g/L, respectively (Rodtong and Ishizaki, 2003). The bacterial cultivation was performed in anaerobic conditions using the anaerobic chamber (Shel Lab, Sheldon Manufacturing Inc, Oregon, U.S.A.). The stock culture was kept in 5% skim milk solution at -20°C.

Preparation of Bacterial Inoculum

For the preparation of bacterial inoculum for investigating amylase production, a loopful of the 48-h *Lactococcus* sp. SUT 513 culture on the modified MRS medium (Rodtong and Ishizaki, 2003). was transferred to 5 mL of the same broth medium. The broth was then incubated at 35°C in an anaerobic chamber (Shel Lab) for overnight to obtain approximately 10^6 - 10^7 cells/mL to be used as the bacterial inoculum.

Investigation of Cassava Starch Concentration for Amylase Production

Cassava starch was used as the main carbon source in this study. The suitable concentration of cassava starch for supporting amylase production by *Lactococcus* sp. SUT 513 was studied by varying the starch concentration at 1.0 to 2.0% (w/v) in the same basal medium as the modified MRS medium as previously mentioned. Cassava starch was added to the base medium, then gelatinized, adjusted the initial pH of the starch medium to 7.0, and dispensed

50-mL aliquote to each 125-mL Erlenmeyer flask. The prepared media were subsequently sterilized by autoclaving at 121°C for 15 min prior to use for cultivating the *Lactococcus* sp. SUT 513 strain. The bacterial amylase activity was detected at 24-h cultivation at 35°C in an anaerobic chamber (Shel Lab). The medium formula that provided the highest amylase activity and good bacterial growth were selected for nitrogen source investigation.

Type and Concentration of Alternative Nitrogen Sources

The suitable cassava starch concentration was selected to prepare the medium for investigating nitrogen source. Rice bran (Surin Bran Oil, Surin, Thailand), soy protein (Food grade soy protein, Food EQ Co., Ltd, Bangkok, Thailand), and urea (AR grade, KemAus™, Cherrybrook, NSW, Australia) were the cheap alternative nitrogen sources tested to either replace or combine with the commercial tryptone and yeast extract (HiMedia) as equal total nitrogen contents. The rice bran and soy protein had 12.94 and 92.63% (w/v) total nitrogen; and 9.67 and 8.69% (w/v) moisture contents, respectively. The inoculum (2%, v/v) prepared as mentioned above was transferred into 50 mL of each medium in 125-mL Erlenmeyer flask. The cultivation was accomplished in the anaerobic chamber (Shel Lab) at 35°C for 24 h.

The optimal concentration of the suitable type of nitrogen source was then studied by varying concentrations. The medium that gave the highest amylase activity was selected to investigate time courses of amylase production.

Production of Amylase

The optimal complex medium was prepared and applied in a 7.5-L glass bioreactor (BioFlo®/CelliGen® 115, New Brunswick, Eppendorf, Edison, NJ, U.S.A.) with a 3-L working volume. The *Lactococcus* sp. SUT 513 inoculum (2.0%, v/v) was prepared and inoculated to the complex medium. The cultivation conditions for amylase production were 150 rpm agitation speed, no aeration, pH control at 7.0, temperature control at 35°C for 36 h. Cultivated broth samples were taken during cultivation every 2 h for monitoring the bacterial growth, then centrifuged at 8,000 rpm for 10 min to collect supernatant for analyzing amylase activity and reducing sugars.

Enumeration of Bacterial Growth

The bacterial growth was monitored as viable cell counts (colony forming units, CFU) according to the standard microbiology method of viable cell counts. The cultured broth was serially diluted with

Butterfield's buffered phosphate diluent (Butterfield, 1932), and spread onto the modified MRS medium containing 1.0% cassava starch (Rodtong and Ishizaki, 2003), and incubated at 35°C in an anaerobic chamber (Shel Lab) for 48 h. The bacterial enumeration was reported as CFU/mL medium.

Measurement of pH

pH of all bacteriological media and cultures was measured using pH meter (CCMD 510 pH meter, WPA, Biochrom Ltd., Holliston, MA, U.S.A.).

Determination of Protein

The supernatant protein concentration was measured by Bradford's method 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 (Fluka Chemie GmbH, Buchs, Switzerland; Bradford, 1976) and incubating at least 5 min but not longer than 1 h. The absorbance was reading at 595 nm.

Assay of Amylase Activity

Amylase activity was determined by incubating the supernatant, the crude enzyme with soluble starch (Carlo Erba Reagenti). The starch hydrolysis product was determined in terms of reducing sugars using the method as described by Bernfeld (1955). The reaction contained equal volume of 1% (w/v) soluble starch in 50 mM of sodium phosphate buffer (pH 6.8) and the crude enzyme. The reaction was left at 35°C for 30 min, then measured for reducing sugars. The enzyme activity was then calculated. One unit of amylase activity was defined as the amount of enzyme releasing 1 µmol of reducing sugars (as maltose equivalents) per min under the assay condition.

Determination of Reducing Sugars

The method of Bernfeld (1955) was used for measuring the concentration of reducing sugars. One hundred microliters of sample were added with 100 µL dinitrosalicylic acid reagent (10.0 g of 3,5-dinitrosalicylic acid (Fluka Chemie GmbH), 19.0 g of sodium hydroxide (Carlo Erba Reagenti), 306.0 g of potassium sodium tartate (Ajax Fine Chem, Thermo Scientific, New South Wales, Australia), 7.6 g of phenol (Carlo Erba Reagenti), and 8.3 g of sodium bisulphite (Carlo Erba Reagenti) in 1,416.0 mL of de-ionized water). The mixture was boiled for 10 min before adding 800 µL of water. The solution was measured absorbance at 540 nm.

Results and Discussion

The Amylolytic L-Lactic Acid Bacterium, *Lactococcus* sp. SUT 513

The bacterium, *Lactococcus* sp. SUT 513, was confirmed for its amylase production capability on the starch agar medium using the point inoculation technique and iodine solution detection. After 48-h incubation, the large clear zones resulting from hydrolyzed starch were detected (Figure 1).

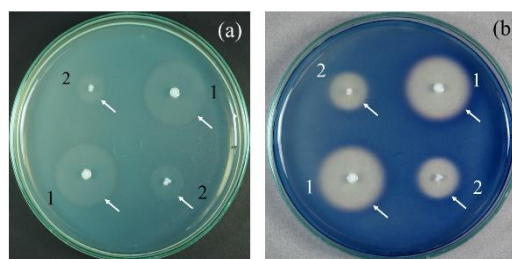


Figure 1. Confirmation of amylase production on an agar medium by point inoculation of *Lactococcus* sp. SUT 513 (1) compared to another strain (2) having less amylase activity, on modified MRS agar containing 1% cassava starch and incubating at 35°C in an anaerobic chamber for 48 h, then overlaid the bacterial colonies (arrows, a) with 1.0% iodine solution for observing clear zones (arrows, b) around the colonies

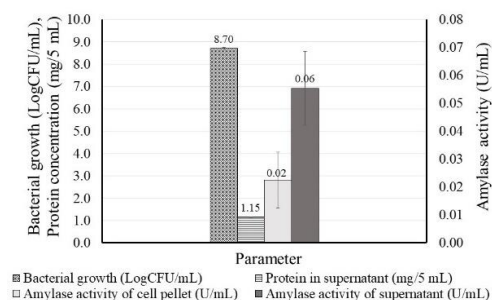


Figure 2. Detection of extracellular and cell-bound amylase produced by *Lactococcus* sp. SUT 513 after cultivating in the modified MRS broth containing 1% cassava starch and incubating at 35°C in an anaerobic chamber for 48 h. The enzyme activity was detected from 30 mL of the cultured broth

Lactococcus sp. SUT 513 produced both cell-bound and extracellular amylases (Figure 2). The enzyme activity in the supernatant was around 5 times higher than the cell pellet (Figure 2).

Cassava Starch Concentration for Amylase Production

The highest amylase activity was exhibited in the medium containing 2.0% (w/v) of cassava starch (Figure 3). The starch concentration higher than 2.0% (w/v) was not designed to investigate in this study because of the limitation of growth ability and the low pH of the cultured broth. However, the starch concentration parameter is considered to verify in the future. The bacterial growth decreased when the concentration of cassava starch increased (Figure 3), which might be the effect of lactic acid production and starch viscosity.

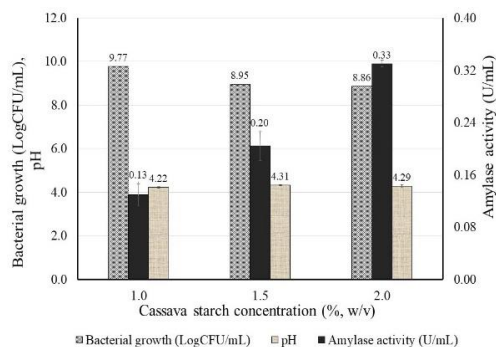


Figure 3. Amylase activity in supernatant and pH of the modified MRS medium containing 1.0, 1.5, and 2.0% (w/v) of cassava starch after cultivating *Lactococcus* sp. SUT 513 at 35°C in an anaerobic chamber for 24 h

Table 1. Media used for investigating the stimulation of amylase production by *Lactococcus* sp. SUT 513

Component	Medium no. and component concentration (g/L)				
	1 ^a	2	3	4	5
Cassava starch	20.0	20.0	20.0	20.0	20.0
Tryptone	5.00	5.00	-	-	-
Yeast extract	3.00	3.00	-	3.00	-
K ₂ HPO ₄	6.00	6.00	6.00	6.00	6.00
tri-Ammonium citrate	1.00	-	-	-	-
MgSO ₄ ·7H ₂ O	0.57	0.57	0.57	0.57	0.57
MnSO ₄ ·4H ₂ O	0.12	0.12	0.12	0.12	0.12
FeSO ₄ ·7H ₂ O	0.03	0.03	0.03	0.03	0.03
Rice bran	-	-	8.00	-	-
Soy protein	-	-	-	-	8.00
Urea	-	-	-	2.00	-

^a, the original medium developed by our previous study (Rodtong and Ishizaki, 2003) with increasing cassava starch from 1.0 to 2.0%

Effect of Alternative Nitrogen Sources on Amylase Production

The cheap alternative nitrogen sources, rice bran, soy protein, and urea combined with yeast extract, were compared between each other and to

tryptone and yeast extract (Table 1). All media contained cassava starch 2.0% (w/v) as the main carbon source.

The amylase production using tryptone as a nitrogen source has been reported for decades about its stimulatory effect. Kumar and Khare (2015) reported that tryptone was highly affected amylase production from bacteria. Yeast extract was one of the main components generally used in various microbiological media for providing growth factors. The positive effect of yeast extract on amylase production was observed in *Bacillus amyloliquefaciens* (Sharma and Satyanarayana, 2012). However, in this study, the starch medium containing rice bran exhibited the highest amylase activity (Figure 4). The rice bran had the potential for supporting the *Lactococcus* sp. SUT 513 strain amylase production better than tryptone and yeast extract, the expensive nitrogen sources, and the rest of cheap alternatives. Rice bran is a nutrient-rich by-product of the rice milling process. It consists of pericarp, seed coat, nucellus, and aleurone layer. RB is a rich source of a protein, fat, dietary fibers, vitamins, minerals, and phytochemicals (mainly oryzanols and tocopherols), and is currently mostly used as animal feed (Sapwarobol *et al.*, 2021) that could serve as sufficient growth nutrient and factors for the lactic acid bacterium. Sreekanth *et al.* (2013) found that the increase in wheat bran hydrolysate concentration supported alpha-amylase production by *Bacillus* sp. CFR 67.

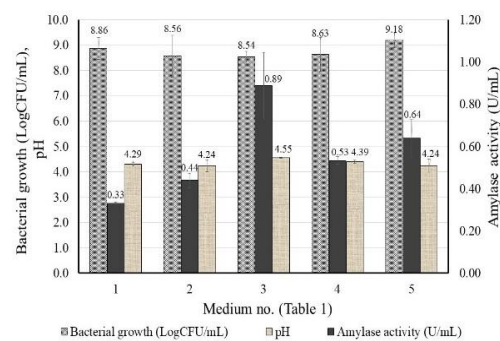


Figure 4. Growth and amylase production by *Lactococcus* sp. SUT 513 in 5 media (Table 1) containing 2.0% (w/v) of cassava starch with different types and concentration of nitrogen-source at 35°C in an anaerobic chamber for 24 h

Concentration of Rice Bran for Amylase Production

Concentrations of rice bran at 0.4, 0.8, 1.2, 1.6% (w/v) were tested for amylase production by *Lactococcus* sp. SUT 513. The cassava starch

medium containing 0.4% (w/v) of rice bran exhibited the highest amylase activity at 1.054 U/mL (Figure 5a). Actually, the amylase activity in the crude enzyme was not significantly different among the four rice bran concentrations, but there were different protein concentrations. The starch medium with rice bran (0.4%, w/v) contained the lowest protein concentration (Figure 5a), thus, there was the highest of specific activity (18.88 U/mg) of the enzyme.

This bacterium grew very well in all concentrations of rice bran tested, and performed the acid production making the pH of all media dropping from 7.0 to around 4.5 (Figure 5b).

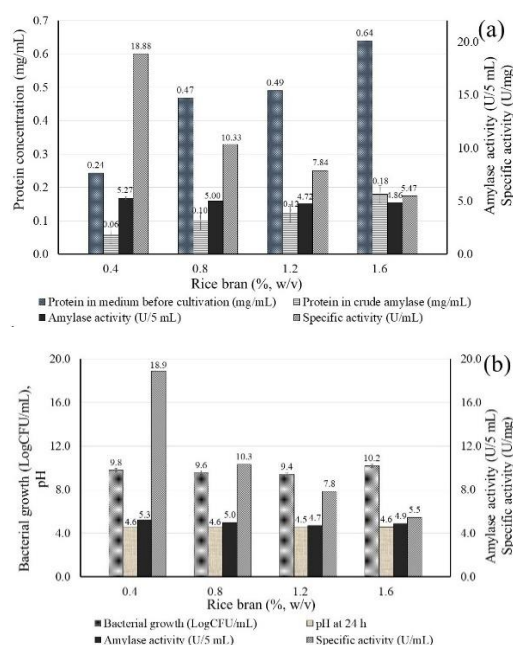


Figure 5. Amylase activity, and protein in broth medium before and after cultivation (crude amylase) (a), and amylase activity, the bacterial growth and pH before and after cultivation (b) of *Lactococcus* sp. SUT 513 in medium no. 3 (Table 1) with various rice bran concentrations at 35°C in an anaerobic chamber for 24 h

Lactic acid bacteria being well-known for their complex nutritional requirements, organic nitrogen source-related cost can be as high as 38% of total operational costs, being its nature and concentration critical factors in the growth and productivity of the selected strain (De la Torre *et al.*, 2018). In this study, the developed medium was concluded as

shown in Table 2. Its composition was simple and much cheaper than the original medium (medium no. 1, Table 1) by cutting off yeast extract, tryptone, and tri-ammonium citrate. The suitable medium developed is thus cheaper than the original medium around 79%.

Amylase production by *Lactococcus* sp. SUT 513

The production of bacterial amylase was performed in 3 L of optimal complex medium (Table 2) in a 7.5-L bioreactor (BioFlo®). The amylase produced by *Lactococcus* sp. SUT 513 was a growth-dependent metabolite. Growth of the bacterium showed its lag phase at the first 2 h of cultivation (Figures 6(a) and 6(b)). Amylase production was initiated during the exponential growth phase of the strain and began to increase along the exponential growth phase. The amylase production reached its maximum of 4.848 U/mL and specific activity of 21.81 U/mg at 18 h of cultivation, which corresponds to the end of the exponential growth phase. The maximum growth of 13.26 LogCFU/mL was at 18 h cultivation (Figures 6(a) and 6(b)). The liberation of reducing sugars was quite related to the growth (Figure 6(b)).

Table 2. Composition of the suitable medium for amylase production by *Lactococcus* sp. SUT 513 obtained from this study

Component	Concentration (g/L)
Cassava starch	20.0
Rice bran	4.0
K ₂ HPO ₄	6.0
MgSO ₄ ·7H ₂ O	0.57
MnSO ₄ ·4H ₂ O	0.12
FeSO ₄ ·7H ₂ O	0.03
Initial pH	7.0

Kanpiengjai *et al.* (2016) reported that *Lactobacillus plantarum* S21 produced amylase activity of 2.1 U/mL at 36 h of incubation. *Lactobacillus acidophilus* GV6 exhibited 0.28 U/mL at 24 h of cultivation (Vishnu *et al.*, 2000), and *Bacillus methylotrophicus* strain P11-2 exhibited 117.0 U/mL at 72 h of cultivation (Xie *et al.*, 2014). This study found the high amylase activity of *Lactococcus* sp. SUT 513 at the short cultivation time (18 h) in the low-cost complex medium. These would be advantages for both the production of amylase and application of the bacterium to produce the optical purity of L-lactic acid from cheap agricultural product and byproduct.

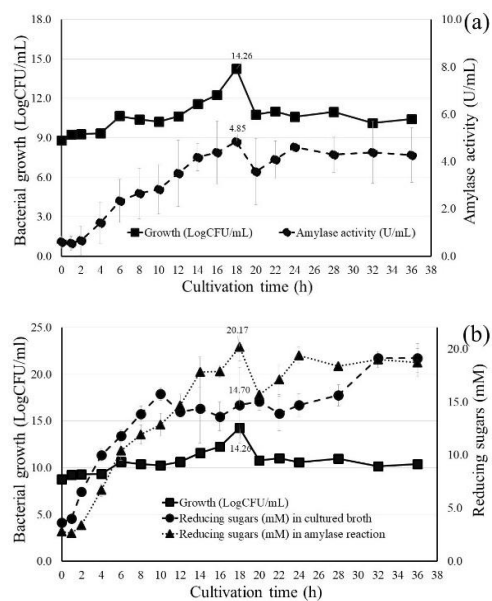


Figure 6. Time courses of growth and the production of amylase (a), and growth and reducing sugars (b) in the suitable medium (Table 2) during cultivating *Lactococcus* sp. SUT 513 in a 7.5-L bioreactor (Bioflo®) with 3-L working volume at 150 rpm agitation speed, no aeration, pH control at 7.0, temperature control at 35°C for 36 h

Conclusions

The lactic acid bacterium *Lactococcus* sp. SUT 513 could produce both cell-bound and extracellular amylases. The lactic acid bacterium showed amylase activity in all test media for amylase production containing cassava starch (10.0, 15.0, or 20.0 g/L, w/v) supplemented with either rice bran (4.0, 8.0, 12.0, or 16.0 g/L, w/v), or the expensive nitrogen source, tryptone (5.0 g/L, w/v) and yeast extract (3.0 g/L, w/v), or the cheap alternatives, soy protein (8.0 g/L, w/v) or urea (2.0 g/L, w/v) combined with yeast extract (3.0 g/L) based on the equal nitrogen content of each medium. All media tested contained the same base medium comprising of K_2HPO_4 , $MgSO_4$, $MnSO_4$, and $FeSO_4$ at contrations of 6.0, 0.57, 0.12, and 0.03 g/L, respectively, with the initial pH of 7.0. The *Lactococcus* sp. SUT 513 gave the highest crude amylase activity in the suitable medium containing cassava starch (20.0 g/L, w/v) added with rice bran (4.0 g/L, w/v). The amylase activity obtained from the developed medium was approximately 3 times higher than the original medium. The cost of the develop medium was cheaper than the original medium around 79.0%. Results from the investigation of amylase

production by the potential optical pure L-lactic acid-producing *Lactococcus* sp. SUT 513 in this study are very useful for future production of the enzyme and further application of the enzyme to develop the optical pure acid fermentation process from the cheap cassava starch by increasing the cassava starch to the high concentration as requiring for obtaining L-lactic yield at the level of economical acid purification. This study results also are advantages to the amylase production from the safe lactic acid bacterium. The purification and characterization of the enzyme need to be further investigated.

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