

EFFECTS OF MEDIA COMPOSITIONS ON AMYLASE AND D-LACTIC ACID PRODUCED BY A POTENTIAL STARCH-UTILIZING BACTERIUM

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Abstract

The high demand for amylase and lactic acid in various industries made enzyme and acid production from cheap substrates very attractive. This study investigated the effect of major medium constituents on amylase and D-lactic acid production by the potential starch-utilizing and optical pure D-lactic acid-producing *Lactobacillus* sp. SUTWR 73, to provide data for the efficient application of the lactic acid bacterium. Its amylase and D-lactic acid production was considerably affected by the addition of organic nitrogen tryptone to certain cassava starch concentrations as a carbon-source. While the other medium compositions: yeast extract and cheap nitrogen sources (soy protein, defatted rice bran, and spent brewery yeast) compared to tryptone as equal total nitrogen contents, did not have any effect on either metabolite production. The medium containing cassava starch (4.0%, w/v) and tryptone (4.5%, w/v) can be satisfactorily used for both products. The initial pH of the production medium was another factor affecting metabolite production. A suitable initial pH was 8.0 and 7.0-10.0 for amylase and D-lactic acid production, respectively. Amylase activity was detected at the early log growth phase while the D-lactic acid was produced at the late log phase. Understanding the relationship between amylase and D-lactic acid production from this study is important for designing an economical operating system for the direct production of D-lactic acid from cheap cassava starch without adding any commercial amylase as a substrate in pretreatment resulting in reduction costs of the production process.

Keywords: Amylase, D-lactic acid, Cassava starch, Lactic acid bacteria, Starch-utilizing bacterium

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Introduction

Amylase, the commercial amylolytic enzyme, has been applied in various industries. Its application was mainly in 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. This enzyme has received attention due to its high demand in various applications. Broadly speaking, it is used in a range from pulp and paper, textile, laundry detergents, dairy, baking, juice, and the cosmetic industries (Li *et al.*, 2012). A few lactic acid bacteria such as some species of *Lactobacillus* (Xu *et al.*, 2020) and *Enterococcus* (Unban *et al.*, 2017) can produce amylase. Generally, lactic acid bacteria are Gram-positive, fastidious, non-sporulating, microaerophilic or aerotolerant, cocci or rods producing lactic acid as the main metabolite (Von Wright and Axelsson, 2011). The bacteria lack catalase and cytochrome, and are either homofermentatives or heterofermentatives based on the physiology of their lactic acid fermentation. The homofermentatives produce only lactic acid as the end product via the Embden-Meyerhof-Parnas pathway while the heterofermentatives produce lactic acid, the main product, and also some other acids, particularly acetic acid, ethanol, and CO₂ through the phosphoketolase pathway (Abdel-Rahman *et al.*, 2013). 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 the food, cosmetic, and pharmaceutical industries as well as the bioplastic industries resulting in high global lactic acid production of around 270,000 tons per year which tends to increase annually by 10% (Alexandri *et al.*, 2019a). For the biodegradable plastic production, poly (L-lactic acid) (PLLA) is used as the main polymer. However, the biopolymer containing only L-lactic acid has the drawback of a low melting point. The addition of poly (D-lactic acid) can efficiently improve biopolymer thermostability, enhance mechanical properties, and prolong the shelf-life of PLLA by stereocomplex formation (Alexandri *et al.*, 2019b). Lactic acid can be produced by either chemical or biotechnological processes. Approximately 90% of the acid is produced by a biotechnological or a microbial fermentation process due to their high optical purity yield, low-cost substrates, and environmental care (Lübeck and Lübeck, 2019). Lactic acid bacteria can produce either D- or L-lactic acid or a racemic mixture of D- and L-lactic acid. The acid isomer is

dependent upon the stereospecificity of D- or L-lactate dehydrogenase possessed by the bacteria. There are very few bacterial strains which produce optically pure D-lactic acid. The bacteria which produce L-lactic acid and the mixture of D- and L-lactic acid have been identified (Vos *et al.*, 2009). D-lactic acid is generally produced from glucose by some species of lactic acid bacteria, particularly in the genus *Lactobacillus*, such as *Lactobacillus lactis* (Joshi *et al.*, 2010) and *Lactobacillus plantarum* S 21 (Unban *et al.*, 2019). Lactic acid can also be produced from starch, a high complexity carbohydrate molecule. Usually, starch is gelatinized at a high temperature followed by saccharification using commercial amylolytic enzymes to hydrolyze the gelatinization product to glucose, and subsequently convert glucose to lactic acid (Reddy *et al.*, 2008). Amylolytic lactic acid bacteria have, therefore, the advantage of performing a single-step lactic acid fermentation.

The use of agricultural residues such as straw, sugarcane, bagasse, and corn stover would be an alternative method of lactic acid fermentation due to its lower cost. However, these carbon sources contain complex structures composed of hemicellulose and cellulose. The structure complexity requires a series of enzymes for pretreatment, and contains a lactic acid production inhibitor which has the crucial limitation of using agricultural residues (Cubas-Cano *et al.*, 2018).

For nitrogen-sources considered as interesting low-cost sources from agricultural by-products, soy protein and defatted rice bran were reported to support lactic acid production. Soy protein was by-product from soybean oil extraction. It enriched with protein and peptides that were desirable for lactic acid bacterium cultivation (Li *et al.*, 2020). Defatted rice bran was by-product of milling rice and extracting oil from the rice bran. It composed of protein as high as 17%, and was reported to be used for fermenting lactic acid due to its low cost (Alexandri *et al.*, 2019b).

In this study, cassava starch, one of the main low-cost agricultural products in Thailand, was used as the raw material for both amylase and D-lactic acid production by the potential D-lactic acid-producing bacterium *Lactobacillus* sp. SUTWR 73. The strain belonged to a rod-shaped Gram-positive lactic acid bacterium group, and could directly produce optically pure D-lactic acid from cassava starch (Pramkaew and Rodtong, 2010). The direct production of both amylase and D-lactic acid from cassava starch using the efficient lactic acid bacteria would be of great benefit to use low-cost starch

substrate and to reduce steps and time for the acid production process. This study aims to clarify the effect of major constituents of the media on lactic acid and amylase produced by the SUTWR 73 strain, in order to provide data for the efficient application of the bacterium.

Materials and Methods

Microorganism

The starch-utilizing lactic acid bacterium *Lactobacillus* sp. SUTWR 73, a potential strain for the direct production of optical pure D-lactic acid from cassava starch, was used in this study. Its amylase activity was attractive because of its applications as well as the production of D-lactic acid. The SUTWR 73 was kept at the Microbial Culture Collection and Applications Research Center, Suranaree University of Technology. Its 16S rRNA gene sequence was deposited in the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank>) with the accession number of JN035217. The starch-utilizing and D-lactic acid-producing bacterium was cultured on a modified MRS medium containing 1.0% cassava starch (Sanguan Wongse Industries Co., Ltd., Nakhon Ratchasima, Thailand) to replace glucose, and the following constituents: tryptone (pancreatic digest of casein) and yeast extract HiMedia, HiMedia Laboratories, Mumbai, India), K_2HPO_4 , tri-ammonium citrate, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$, and $FeSO_4 \cdot 7H_2O$ (Carlo Erba Reagenti, Milan, Italy) at concentrations of 5.0, 6.0, 3.0, 1.0, 0.57, 0.12, and 0.03 g/L, respectively (Rogasa agar modified medium or RAM, Rodtong and Ishizaki, 2003). The bacterial cultivation was performed in anaerobic conditions using an anaerobic chamber (Shel Lab, Sheldon Manufacturing Inc, Oregon, U.S.A.). The stock culture was kept in 5% skimmed milk solution at -20°C.

Bacterial Inoculum Preparation

For the preparation of bacterial inoculum for amylase and D-lactic acid production, two loopfuls of the 48-h *Lactobacillus* sp. SUTWR 73 culture on starch agar medium (medium no. 1 in Table 1 with the addition of 1.5% agar, HiMedia) was transferred to 5 mL of the same broth medium. The broth was then incubated at 35°C in an anaerobic chamber for overnight to obtain approximately 10^6 - 10^7 cells/mL to be used as the bacterial inoculum.

Bacterial Amylase Production

A series of media of different compositions (Table 1, 2 and 3) was used to investigate their effects on the production of amylase by

Lactobacillus sp. SUTWR 73. Cassava starch was used as the main carbon source in all the media studied. The effect of cassava starch concentration on amylase production was also investigated. Various concentrations (1.0-5.0%, w/v) of the starch were added to the base medium (medium no. 1, Table 1), then gelatinized, adjusted to the initial pH of the starch medium of 7.0, and 50-mL aliquote was dispensed to each 125-mL Erlenmeyer flask. Starch concentrations higher than 4.0% were so viscid after gelatinization that they were not suitable for this investigation. The prepared media were subsequently sterilized by autoclaving at 121°C for 15 min prior to use for cultivating the SUTWR 73 strain. Bacterial amylase activity was detected after 24-h cultivation at 35°C in an anaerobic chamber.

In order to test effect of nitrogen sources on amylase production by the *Lactobacillus* sp. SUTWR 73, different kinds and concentrations of both the commercial microbiological nitrogen sources: tryptone and yeast extract (HiMedia), and the cheap alternative nitrogen sources: defatted rice bran (Surin Bran Oil, Surin, Thailand), soy protein (Food grade soy protein, Food EQ Co., Ltd, Bangkok, Thailand), and spent brewery yeast (Boonrawd Brewery Co., Ltd., Khon Kaen, Thailand), were investigated using the same base medium composed of a suitable concentration of cassava starch as the result of the above study. An attempt to use alternative nitrogen sources was made partially and fully to replace tryptone and yeast extract using the same amount of total nitrogen contents (medium nos. 3 to 6, Table 2). In this study, the defatted rice bran, soy protein, and spent brewery yeast were found to consist of 12.94, 92.63, and 32.21% (w/v) total nitrogen; and 9.67, 8.69, and 3.66% (w/v) moisture contents, respectively.

A test on the effect of the initial pH of the media on amylase production was also performed concurrently. The inoculum (2%, v/v) was prepared as mentioned above and it was transferred into 50 mL of each medium (Table 3) in a 125-mL Erlenmeyer flask. The cultivation was accomplished in an anaerobic chamber at 35°C for 24 h.

The suitable amylase production medium was used for scaling up the enzyme production process by using *Lactobacillus* sp. SUTWR 73 with 3 L of the selected medium no. 12 (Table 3) in a 7.5-L bioreactor (BioFlo®/ CelliGen® 115, New Brunswick, Eppendorf, Edison, NJ, U.S.A.). Three liters of the starchy medium were prepared by gelatinizing cassava starch (50 g/L) in 2.8 L of water, and heating to 70-90°C for 20 min without the addition of commercial α -amylase to decrease the viscosity of the starch. Then, external sources of micro - and macro - nutrients for bacterial growth were added and made up to the final volume of the

cultivation medium adjusted to a suitable pH of 8.0 using 2 N NaOH and transferred to the bioreactor (7.5 L in capacity, BioFlo®). The bacterial inoculum for 3-L amylase production medium was prepared in 50 mL of RAM (medium no. 1, Table 1) in a 125-mL Erlenmeyer flask and cultivated at 35°C overnight in an anaerobic chamber to obtain approximately 10^6 - 10^7 cells/mL. The inoculum (2%, v/v) was transferred to 3 L of the amylase production medium (medium no. 12, Table 3) in a 7.5-L bioreactor (BioFlo®). The cultivation conditions for amylase production were 150 rpm agitation speed with no aeration, and without pH being controlled at 35°C for 48 h. Cultivated broth samples were taken during cultivation for monitoring the bacterial growth and pH, then centrifuged at 8,000 rpm for 10 min to collect supernatant for an analysis of amylase activity, and also total lactic acid, D-lactic acid concentration and isomer, and total sugars.

D-lactic Acid Production

The effects of the carbon source cassava starch concentration, and type and concentration of nitrogen source on D-lactic acid production by *Lactobacillus* sp. SUTWR 73 were concurrently investigated in all the media used for amylase production as described above. Then the batch culture for lactic acid production was performed in a 7.5-L bioreactor (BioFlo®) using 3 L of a suitable medium composed of cassava starch (Sangan Wongse Industries Co., Ltd.), tryptone (Hi-Media), K_2HPO_4 , $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$, and $FeSO_4 \cdot 7H_2O$ (Carlo Erba Reagenti) at concentrations of 60.0, 45.0, 6.0, 0.57, 0.12, and 0.03 g/L, respectively, (medium no. 2, Table 1) obtained from our previous study. A high concentration (6.0%) of cassava starch was used in order to obtain a high D-lactic yield which would be economically suitable for the acid purification, so the starch was gelatinized, then its viscosity was reduced using α -amylase (α -amylase from *Bacillus amyloliquefaciens*, A7595, Sigma-Aldrich, Inc., Søborg, Denmark) according to the manufacturer's method. The inoculum culture (2%) was prepared and inoculated into 3 L of the lactic acid production medium in a 7.5-L bioreactor. Simple batch fermentation was performed at 35°C, 150 rpm agitation speed, and without aeration for 48 h. The measurements of bacterial growth, pH, total lactic acid, D-lactic acid concentration and isomer, and the remaining starch (substrate) were measured

in terms of total sugars, and were monitored during cultivation.

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 RAM agar medium (medium no. 1a, Table 1), and incubated at 35°C in an anaerobic chamber (Shel Lab) for 48 h. The bacterial enumeration was reported as CFU/mL medium.

pH

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

Total Lactic Acid and D-lactic Acid

The cultured broth was centrifuged at 8,000 rpm, at 4°C for 15 min to obtain a supernatant to be used for total lactic acid and D-lactic acid analyses. The total lactic acid was determined by a colorimetric assay according to Kimberley and Taylor (1996). The concentration, isomer, and optical purity of the D-lactic acid were determined by high-performance liquid chromatography (HPLC, Agilent 1200 Series LC System and Module, Agilent Technologies International sael, Lausanne, Switzerland) equipped with a chiral column (Astect CLC-L column, Sigma-Aldrich, Inc., St. Louis, U.S.A.) and a UV (254 nm) detector according to the method described by Yang and Chung (2007).

Amylase Activity

Amylase activity was determined by incubating the supernatant, and the crude enzyme with soluble starch (Carlo Erba Reagenti). The starch hydrolysis product was determined in terms of reducing sugars using the method described by Bernfeld (1955). The reaction contained an equal volume of 1% (w/v) soluble starch in 50 mM of sodium phosphate buffer (pH 6.8) and 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 that produced 1 μ mol of reducing sugars per min.

Reducing Sugars

The method of Bernfeld (1955) was used for measuring the concentration of reducing sugars. One hundred microliters of sample were added to 100 μ L dinitrosalicylic acid reagent (10.0 g of 3,5-dinitrosalicylic acid (Fluka Chemie GmbH, Buchs, Switzerland), 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 800 μ L of water was added. The absorbance of the solution was measured at 540 nm.

Total Sugars

The concentration of total sugars was determined by the colorimetric (phenol-sulphuric acid) method as described by Dubois *et al.* (1956). Two millilitres of sugar solution containing between 10 to 70 μ g were transferred into a colorimetric tube, and one millilitre of 5% phenol (in water, Carlo Erba Reagenti) was added. Then 5 millilitres of concentrated sulphuric acid (Ajax Fine Chem) were rapidly added, and allowed to stand for 10 min. The mixture was well mixed and placed for 10 to 20 min in a water bath at 25-30°C before the absorbance was recorded at 485 nm. The remaining substrate of cassava starch was detected in terms of total sugars by the sulphuric acid hydrolysis of the residues to sugars,

Results and Discussion

Results from our previous studies suggested that the key factor affecting D-lactic acid production by a potential starch-utilizing lactic acid bacterium strain was the cultivation of the medium composition. To further investigate the starch-degrading bacterium for the worthwhile production of D-lactic acid, factors affecting the amylase and D-lactic acid production by *Lactobacillus* sp. SUTWR 73 were focussed on, such as the concentration of cassava starch used as the main carbon source substrate, and the type and concentration of the nitrogen source in this study.

Effect of Medium Compositions on Bacterial Amylase Production

In terms of cassava starch as the main carbon source for growth and amylase production, it was found that 4.0 and 5.0% (w/v) of the starch supported similar production of amylase (21.47 $\pm$ 2.31 and 21.53 $\pm$ 2.53 U/mL, respectively) which were the maximum enzyme production by

Lactobacillus sp. SUTWR 73 (Figure 1) in the starch medium containing 0.5% tryptone and 0.3% yeast extract as the nitrogen source and growth factors. The medium containing cassava starch at concentrations higher than 5.0% (w/v) was so viscous after gelatinization that it was not used in this step. Growth of the bacterium was also similar around 10⁸ CFU/mL in the medium containing 2.0-5.0% (w/v) cassava starch (Figure 1). The bacterial cell counts were higher than in the medium containing 1.0% of the starch. The SUTWR 73 was able to produce amylolytic enzyme to digest the high viscosity of cassava starch up to the 5.0% concentration tested. Sreekanth *et al.* (2013) also mentioned that amylase could be induced by the high complex carbohydrate like starchy biomass. The amylase production varied from substrate to substrate. The higher complex of substrate tended to induce enzyme production better than the smaller molecules like maltose, lactose, galactose, fructose, and glucose (Ensari *et al.*, 1995). The monosaccharide as glucose was also reported to be an amylase production repressor. The increase in high starch concentration caused the reduction of the water activity of the medium, which led to the repression of bacterial growth, and lowered the rate of starch hydrolysis. Consequently, the maximum concentration of cassava starch had its effect on amylase production by providing the highest amylase activity at 4.0-5.0% (w/v) starch without pretreatment with commercial α -amylase to reduce the medium viscosity in this study.

The effects of the nitrogen source on amylase production by *Lactobacillus* sp. SUTWR 73, were tested for both type and concentration using the medium containing 3.0-5.0% (w/v) cassava starch (Table 2 and Table 3). The amylase production using tryptone as a nitrogen source was first reported a decade ago for its stimulatory effect. Sharma and Satyanarayana (2012) showed the use of organic nitrogen sources like tryptone having an effect on enzyme production, and also suggested that the concentration of the nitrogen source was important. The lower levels of nitrogen and also excess nitrogen were equally detrimental causing enzyme inhibition. Kumar and Khare (2015) reported that tryptone highly affected amylase production from bacteria. To investigate the effect of the tryptone concentrations, medium nos. 2, 7, and 8 (Table 2) containing 0.5, 4.5, and 7.0% (w/v) of tryptone, respectively, were used. Tryptone (4.5%, w/v) provided the highest amylase production, 12.23 $\pm$ 0.80 U/mL (Figure 2).

Yeast extract was one of the main constituents which is generally used in various microbiological media for providing growth factors. The results from using media are shown in Table 2 in this study:

yeast extract showed its effect on amylase production when combined with soy protein (medium no. 5, Table 2). The bacterial amylase activity decreased dramatically when using only soy protein (medium no. 6, Table 2). Yeast extract seemed to have a slight effect on amylase production stimulation when combined with tryptone compared with medium nos. 1 (with yeast extract) and 2 (without yeast extract) in Table 2, which presented the amylase production of 10.86 ± 1.65 and 10.58 ± 2.87 U/mL, respectively (Table 2 and Figure 2). The positive effect of yeast extract on amylase production was observed in *Bacillus amyloliquifaciens* (Sharma and Satyanarayana, 2012).

Neither the combination nor the replacement of tryptone and yeast extract with soy protein, the alternative nitrogen source (medium nos. 4, 5, and 6, Table 2) did not support the amylase production (Table 2 and Figure 2). Medium no. 6 (Table 2) contained only soy protein as the nitrogen source. It clearly showed that the amylase activity (7.37 ± 0.12 U/mL) was much lower than the media containing tryptone and soy protein, and yeast extract and soy protein (medium nos. 4 and 5, Table 2) having the amylase production of 11.02 ± 0.03 and 10.45 ± 0.37 U/mL, respectively.

Concern the cost of medium compositions for growth and desirable product production from the *Lactobacillus* sp. SUTWR 73 strain, defatted rice bran (medium no. 3, Table 2) and soy protein (medium no. 4, Table 2) were used to replace yeast extract with a total equal to the nitrogen content in this study. The defatted rice bran contained 12.94% total nitrogen content which was less than soy protein (92.63%) and spent brewery yeast (32.21%). It was found that the replacement with both defatted rice bran and soy protein supported the amylase production (11.40 ± 0.11 and 11.02 ± 0.30 U/mL, medium nos. 3 and 4, Figure 2, respectively). The soy protein supported the enzyme production when combined with tryptone (11.02 ± 0.30 U/mL, medium no. 4, Table 2) but it did not support the bacterial amylase production in the medium either combined with yeast extract (10.45 ± 0.37 U/mL, medium no. 5, Table 2 and Figure 2) or when replaced both tryptone and yeast extract (7.37 ± 0.12 U/mL, medium no. 6, Figure 2). De la Torre *et al.* (2018) also reported the use of orange peel waste hydrolysate supplemented with corn steep liquor due to the high cost of yeast extract for growth and the D-lactic acid productivity of the selected *Lactobacillus delbrueckii* ssp. *delbrueckii* strain. Since lactic acid bacteria are well-known for their complex nutritional requirements, organic nitrogen source-related costs can be as high as 38% of the total operational costs, because of their nature

and the degree of concentration which are critical factors in the growth and productivity of the selected strain (De la Torre *et al.*, 2018).

The enzyme production medium containing tryptone without the addition of yeast extract (medium no. 2, Table 2) showed a lower amylase production (10.58 ± 2.87 U/mL) than the same medium component with either defatted rice bran or soy protein added (11.40 ± 0.11 and 11.02 ± 0.30 U/mL, medium nos. 3 and 4, Table 2 and Figure 2). This suggests that defatted rice bran and soy protein could be used as an alternative component to replace yeast extract when using a medium containing tryptone as the main nitrogen source for amylase production. There is some evidence that defatted rice bran and tryptone might act as the source of activator for amylase production (Kumar and Khare, 2015). In order to improve amylase production, the pretreatment of defatted rice bran is an option. Sreekanth *et al.* (2013) found that the increase in rice bran hydrolysate concentration supported alpha-amylase production by *Bacillus* sp. However, rice bran can contain some ingredients, such as zinc, copper, iron, nickel ions, and phenolic compounds which were reported as amylase inhibitors (Quan *et al.*, 2019).

In this study, spent brewery yeast was considered as another cheap alternative nutrient source to be used in combination with tryptone and replace yeast extract as shown in Table 3. In this table, there were three sets of experiments (medium nos. 1 to 6, 7 to 11, and 12) designed on the basis of the results from our previous studies which confirmed various concentrations of carbon and nitrogen sources, varied the initial pH, and tried to replace yeast extract with spent brewery yeast (32.21% total nitrogen content). Jaeger *et al.* (2020) mentioned that spent brewery yeast contained high nitrogen content (40%) and high amounts of growth particularly vitamin B. It was found that, after 24 h of cultivation, the amylase production in the media (medium nos. 1 to 6, Table 3) with added tryptone and spent brewery yeast, depended very much on the initial pH of each medium. The amylase production by the strain SUTWR 73 in the medium with an adjusted pH of 8.0 (medium no. 6, Table 3) showed better result (5.32 ± 0.07 U/mL) than the other five media (medium nos. 1 to 5, Table 3) and with adjusted pH ranging from 5.0 to 7.0 (Table 3). The results of medium pH were confirmed by medium nos. 9 and 12 (Table 3) in which pH at 8.0 was found to be a suitable initial pH of the medium used for amylase production (12.96 ± 1.23 and 15.99 ± 1.87 U/mL from medium nos 9 and 12, Table 3, respectively). Elmansy *et al.* (2018) reported that the maximum yield of α -amylase was 15.15 ± 0.47 U/ml from *Bacillus* sp. NRC 22017. This strain was

characterized by high temperature and high salinity in a cultivated culture, and achieved maximum yield of α -amylase in a starch medium adjusted to pH at 7.0.

The production of bacterial amylase was scaled up to perform in 3 L of the starchy medium no. 12 (Table 3) which was developed from the results of investigating medium nos 7 and 8 (Table 2). The medium was prepared by gelatinizing cassava starch (50 g/L) without the addition of commercial α -amylase in a 7.5-L bioreactor. The bacterial strain SUTWR 73 produced sufficient amylase for hydrolysis of a high concentration of cassava starch substrate. Growth of the bacterium showed that its lag phase was approximately in the first 12 h of cultivation (Figure 3). The long lag phase would be from a high starch substrate concentration without pretreatment. After 12 h of cultivation, the amylase production (7.61 ± 1.20 U/mL, Figure 3) increased rapidly to the highest level after 18 h (17.00 ± 0.71 U/mL), and the enzyme production reached steady state till 22 h of cultivation. The amylase activity dropped after 22-h cultivation to become stable during 24 to 28 h (5.99 ± 0.46 to 5.43 ± 0.01 U/mL), then went up to 15.99 ± 1.87 U/mL after 48-h cultivation (Figure 3). These results would have been affected by the inhibition of the high accumulation of sugars as stated by Pranay *et al.* (2019). In this study, it was found that the highest amount of reducing sugars (9.43 ± 0.68 g/L, Figure 3) after 18-h cultivation resulted in the accumulation of D-lactic acid (11.26 ± 0.81 g/L) after the cultivation time. For the concurrent D-lactic acid detection, the acid slowly increased after 2 h (0.01 ± 0.00 g/L), and dramatically increased after 12 h (4.18 ± 0.07 g/L) of cultivation. The highest concentration of D-lactic acid was obtained after 48 h of cultivation (22.16 ± 0.52 g/L). It was found that amylase production from both lactic acid and amylase systems, was mainly affected by the concentration of the initial substrate and reducing sugars at an early state of cultivation. As mentioned earlier, the amylase was induced by starch. The higher the starch concentration the less accessible the bacterium was to the substrate. The bacterium was able to produce a high amount of amylase to increase substrate accessibility. After the amylase had accumulated, the product from the starch hydrolysis by the enzyme became the substrate for lactic acid production by the starch-hydrolyzing bacterium. Hence, the lactic acid production was also affected by the amount of amylase in this cultivation (Figure 3).

Effect of Medium Compositions on D-lactic Acid Production

As mentioned above, the batch culture for the detection of D-lactic acid by the starch-utilizing *Lactobacillus* sp. SUTWR 73 was performed concurrently with amylase production. The D-lactic acid concentration increased from 2.96 ± 0.01 to 3.22 ± 0.17 g/L (Figure 1) in the medium containing the increased concentration of cassava starch from 1.0 to 2.0% (w/v), respectively. The growth of the SUTWR 73 strain was stable in the medium which contained 2.0-5.0% cassava starch after 24 h of cultivation (Figure 1). But the bacterial amylase production increased as the concentration of cassava starch increased from 1.0-4.0% (Figure 1). The bacterial enzyme hydrolyzed cassava starch into available sugars so that it was able to gradually produce D-lactic acid under static incubation in an anaerobic chamber. The higher amylase production represented the degree of hydrolysis which provided a high yield of available sugars for lactic acid production. However, as previously mentioned, the increase in high starch concentration caused a reduction in the water activity of the medium, which led to the repression of bacterial growth, and lowered the rate of starch hydrolysis.

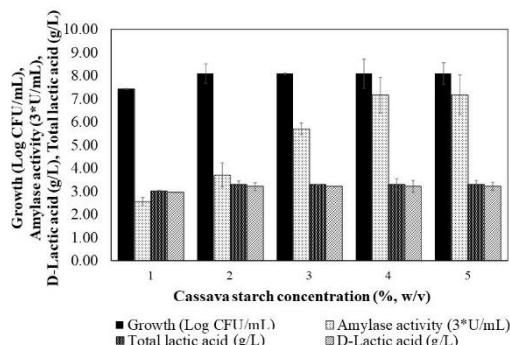


Figure 1. Effect of different cassava starch concentrations in the same base medium (medium no. 1, Table 1) on amylase and D-lactic acid production by *Lactobacillus* sp. SUTWR 73

The lactic acid production was also affected by the imbalance of the C:N ratio at higher carbon-source concentrations. A high C:N ratio was related to high acid (Carsanba *et al.*, 2019) and amylase production (Aiyer, 2004). Even at 4.0% (w/v) of cassava starch without pretreatment by the commercial α -amylase, it was favorable for amylase production by the bacterial strain SUTWR 73.

In the nitrogen-source investigation, only tryptone and a combination of tryptone with either yeast extract or defatted rice bran or soy protein supported D-lactic acid production better than using only soy protein as an alternative nitrogen-source (Table 2 and Figure 2). Both the acid production as well as the amylase production and growth dramatically decreased in the cassava medium containing soy protein which replaced both tryptone and yeast extract (medium no. 6, Table 2). But the cassava medium composed of tryptone and soy protein (medium no. 4, Table 2) provided the best acid production (3.34 ± 0.14 g/L) from the 8 media tested. Hence, the D-lactic acid production was sensitive to the type of nitrogen source preferring a complex organic source. Huang *et al.* (2005) mentioned that lactic acid bacteria required substrates with high nitrogen content for growth and fermentation of saccharified potato starch wastewater to lactic acid. The acid from the production also directly affected cell weakness because of the concentration of excess protons in the cultured medium which penetrated the cells. Each metabolic pathway inside the cells had their individual pH. A high number of protons caused a motive force between the inside and outside of the bacterial cells which led to changes. If the balance of the protons is broken, the homeostasis of the cell membrane fails and leads to cell deaths (Padmavathi *et al.*, 2018).

Yeast extract and tryptone enriched with amino acid and peptides did not lack growth factors compared to the inorganic nitrogen sources (De La Torre *et al.*, 2018). Some reports showed that the inorganic nitrogen source inhibited urea and the α -amylase activity of *Bacillus subtilis* (Das *et al.*, 2004). So the urea was not included in the study. Yeast extract had little effect on stimulating either D-lactic acid or amylase production in the cassava medium containing tryptone (medium nos. 1 and 2, Table 2; and nos. 7 and 8, Table 3). Medium no. 1 (Table 2) showed higher D-lactic acid production (3.15 ± 0.10 g/L, Figure 2) than medium no. 2 (3.13 ± 0.06 g/L, Figure 2). Paul *et al.* (2020) mentioned that the addition of organic compounds, especially yeast extracts had effects on lactic acid production.

Different levels of initial pH in the cultivation medium were applied for D-lactic production (medium nos. 1 to 6, nos. 7 to 11, and no. 12 in Table 3). It was found that lactic acid production was affected by the initial pH of the media during cultivation without control of pH. The D-lactic acid tended to increase with the initial pH increment by yielding high acid production at pH 7.0 to 10.0 (Table 3) which was generally found in lactic acid production (Hofvendahl and Hahn-Hägerdal, 2000).

Lactobacillus sp. SUTWR 73 (medium no. 12, Table 3) provided suitable cultivation for amylase production with the highest amount of D-lactic acid (22.16 ± 0.52 g/L) detected after 48-h cultivation.

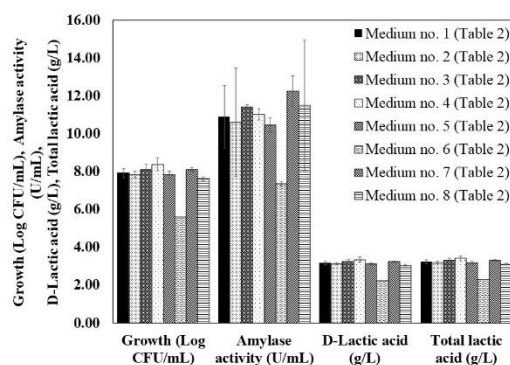


Figure 2. Effect of nitrogen sources on amylase and D-lactic acid produced by *Lactobacillus* sp. SUTWR 73 using media as described in Table 2

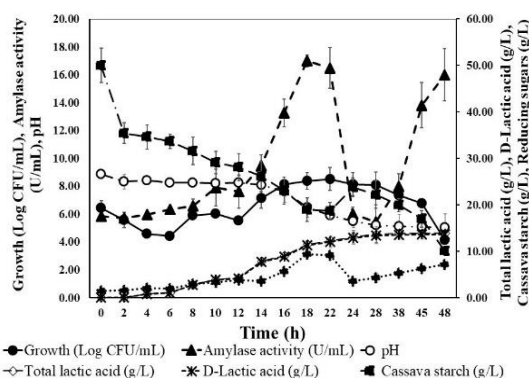


Figure 3. Amylase production by *Lactobacillus* sp. SUTWR 73 in the medium no. 12 (Table 3) in a 7.5-L bioreactor (Bioflo®) with a 3-L working volume

Medium no. 2 (Table 1) was used to investigate the scaling up of D-lactic acid production from *Lactobacillus* sp. SUTWR 73. The cultivation was performed in a 7.5-L bioreactor with a 3-L working volume. Because a large amount of pretreated cassava starch was added to the acid fermentation process, no lag phase of growth occurred (Figure 4). Amylase production was detected together with bacterial growth. The optical pure D-lactic acid formation (Figure 5) was detected after 4-h cultivation (0.41 ± 0.15 g/L at 4 h) and reached its maximum concentration (30.59 ± 0.47 g/L) after 48 h of cultivation. The highest amylase production was found after 16 h (5.14 ± 0.04 U/mL)

with the growth of 10.51 Log CFU/mL. The maximum growth (10.64 Log CFU/mL) was detected after 20-h cultivation. A restriction of amylase activity was caused by the accumulation of the available sugars in the medium which acted as inhibitors. Conversely, the high number of available sugars in the early production stage were good for lactic acid production (Abdel-Rahman *et al.*, 2013). The trend in lactic acid production was the opposite to that of amylase production after 16-h cultivation. This is because of the effect of the acid inhibition of the amylase activity (Jukonyte *et al.*, 2018). Thus, this finding is evidence that the enzyme was secreted at the early log phase due to the induction of starch similar to that referred to in report of Ensari *et al.* (1995), who noted that there was a parallel increase with the growth of the bacteria. The amylase produced by SUTWR 73 was a growth-

dependent metabolite. Coincidentally, the amylase activity detected from the supernatant perhaps suggested that there was an inhibition of the product at the late log phase. Finally, the lowest amylase activity was found at the maximum D-lactic acid production (0.96 ± 0.06 U/mL). In comparison to the growth in the amylase production medium, the lactic acid production medium did not exhibit any lag phase but a shorter log phase because the substrate was gelatinized and reduced viscosity by commercial amylase.

Effect of Initial pH of Media on Amylase and D-lactic Acid Production

After the bacterial metabolites were grown in media the different compositions resulted in changing the pH of the media, the effect of the initial pH of the media on amylase and D-lactic acid production was further investigated. It was found that the initial pH of the different media compositions showed nearly the same effect on the amylase production. The highest amylase production was at pH 8.0 which was better than the lower pH production (Table 3). With regard to lactic acid production, the higher initial pH of the media caused a higher buffer capacity that neutralized the D-lactic acid produced by the *Lactobacillus* sp. SUTWR 73. The optimum initial pH of the cassava starch medium for lactic acid production was 7.0-10.0. The initial pH had an impact on both amylase and D-lactic acid production by the bacterial strain SUTWR 73.

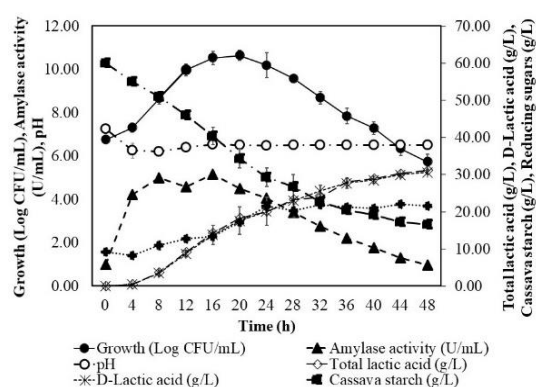


Figure 4. D-lactic acid production by *Lactobacillus* sp. SUTWR 73 in medium no. 2 (Table 1) in a 7.5-L bioreactor (Bioflo®) with a 3-L working volume

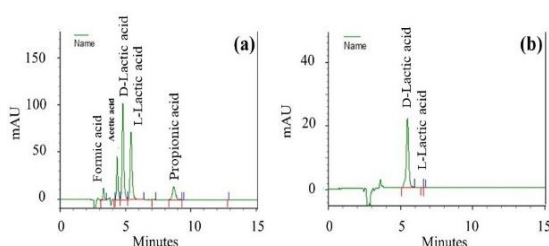


Figure 5. HPLC chromatograms of organic acid standards: formic, acetic, D-lactic, L-lactic, and propionic acids (a); fermentation medium after cultivating the bacterial strain SUTWR 73 (b)

Table 1. D-lactic acid production by *Lactobacillus* sp. SUTWR 73 in medium no. 2 (Table 1) in a 7.5-L bioreactor (Bioflo®) with a 3-L working volume

Component (g/L)	Medium no.	
	1 ^a	2 ^b
Cassava starch	10.0	60.0
Tryptone	5.0	45.0
Yeast extract	3.0	-
K ₂ HPO ₄	6.0	6.0
tri-Ammonium citrate	1.0	1.0
MgSO ₄ ·7H ₂ O	0.57	0.57
MnSO ₄ ·4H ₂ O	0.12	0.12
FeSO ₄ ·7H ₂ O	0.03	0.03
Initial pH	7.0	7.0

^a, RAM, the medium developed from our study (Rodtong and Ishizaki, 2003)

^b, D-lactic acid production medium designed from our previous study results

Table 2. The composition of eight media composed of the same base medium as medium no. 1 (Table 1), designed for stimulating *Lactobacillus* sp. SUTWR 73 to produce amylase and concurrently to detect D-lactic acid

Medium no.	Medium composition (g/L)				
	Cassava starch	Tryptone	Yeast extract	Defatted rice bran	Soy protein
1	40.0	5.0	3.0	-	-
2	40.0	5.0	-	-	-
3	40.0	5.0	-	11.9	-
4	40.0	5.0	-	-	2.5
5	40.0	-	3.0	-	5.0
6	40.0	-	-	-	8.0
7	40.0	45.0	-	-	-
8	40.0	70.0	-	-	-

Table 3. Different media composed of the same base medium as mentioned in Table 2, but with an adjustment of the different initial pH for investigating amylase production and concurrently to detect the D-lactic acid produced

No.	pH	Medium composition (g/L) ^a				A ^b (U/mL)	LA ^c (g/L)
		Cas	Try	Yex	Sby		
1	5.0	30.0	3.0	-	3.0	3.58	1.29
2	5.0	30.0	5.0	-	3.0	4.67	1.41
3	6.0	30.0	3.0	-	3.0	4.86	1.46
4	6.0	30.0	5.0	-	3.0	5.02	1.54
5	7.0	30.0	3.0	-	3.0	5.15	1.83
6	8.0	30.0	3.0	-	3.0	5.32	1.75
7	7.0	40.0	45.0	-	-	12.38	3.03
8	7.0	40.0	45.0	3.0	-	12.40	3.03
9	8.0	40.0	45.0	3.0	-	12.96	3.05
10	9.0	40.0	45.0	3.0	-	6.57	3.07
11	10.0	40.0	45.0	3.0	-	6.00	3.11
12	8.0	50.0	47.0	-	-	15.99	22.16

Conclusions

This study showed the effect of the main nutrients, carbon and nitrogen sources, on the production of amylase and D-lactic acid by the starch-utilizing lactic acid bacterium *Lactobacillus* sp. SUTWR 73. Both metabolite productions were critically affected by tryptone as the main nitrogen-source in the cassava starch medium with very low concentration of minor elements. The main components, cassava starch (4.0%, w/v) and tryptone (4.5%, w/v), of the medium perfectly supported both enzyme and the acid production by *Lactobacillus* sp. strain SUTWR 73. Yeast extract seemed to have an insignificant effect on both amylase and D-lactic acid production when a sufficient concentration (C:N ratio balance) of tryptone was added to the cassava starch medium. Results from this study revealed the possibility for further development of simple media and the bacterial cultivation conditions and duration for both amylase and D-lactic acid production.

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