Production of Lactic Acid and lysine from Cassava Starch and Cassava Waste

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Abstract

The present research deals with the production of lactic acid and lysine from cassava starch and cassava waste. Firstly,the starch (10.5 % yields) was extracted from cassava waste (pulp) and it wasthen converted to glucose syrup by enzymatic hydrolysis using α-amylase and aminoglucosidase enzymes.The concentration of glucose syrup(91.40 % yield, based onraw cassava starch), (9.96 % yield, based on cassava waste) could be determined as 3.02 mg in the prepared 5 mg/mL solution.From cheese, *Lactobacillus casei*was isolated and identified, andit was applied for the fermentation of cassava glucose syrup at pH 5.5 and 8 % inoculum level for72 h. The lactic acid was produced with different concentrations of cassava glucose syrup (2 %,4 %, 6 %, 8 % and 10 %) atselected optimum conditions. The highest lactic acid content (0.045 g/mL) and highest yield productivity (1.125 g/mL of based on glucose syrup) were observed by using 4% of cassava glucose syrup. On the other hand, *Corynebacterium glutamicum* was isolated from soil, and lysine was produced from the fermentation of glucose syrup withC. *glutamicum*.The highest lysine content (0.025 g/mL) and highest yield productivity (0.227 g/mL) based on glucose syrup were obtained.

Keywords: cassava, lactic acid, glucose syrup,*Lactobacillus casei*and *Corynebacterium glutamicum*

1. Introduction

Cassava (*Manihotesculenta*) is a woody shrub of the Euphorbiaceae (spurge) family, native to South Americaand extensively cultivated as an annual crop in tropical and subtropical regions for its edible starchytuberous root, a major source of carbohydrates. And it is also grown in all parts of Myanmar.Cassava is classified as sweet or bitter. Cassava is a starchy rich root. However, in the process ofCassava production,cassava peel and biogas wastes that contain a low nutrient content,crude fiber and nutrients in the form of antihydrogencyanide effect environmental pollution. Therefore, there are still emerging new value-added products from cassava waste,to reduce environmental pollution, such as biofuel production, lactic acid production and lysine production. Cassava is widely used as a raw material in many productions.

Lactic acid and lysine fermentation has been continuously investigated because of many industrial applications and almost all studies have been carried out with glucose or lactose as a carbon source but very few studies have been done using cassava hydrolysis. The advantage of cassava for starch production over other grain or root crop includes: high purity level, taste, desirable textural characteristics, is relatively cheap and it contains a high concentration starch (dry matter basis) (Masamba*et al.*, 2001). It is one of the most important starchy root crops of the tropics used for food and industrial purpose (Gunorubon, 2012).Lactic acid is used for a wide variety of industrial applications. It is applied in food industry, cosmetic industry, chemical industry, chemical feedstock and pharmaceutical industry (Wee *et al.*, 2006).

Lysine is generally recognized as the most deficient amino acid in the food supply of both man and domestic meat producing animals. Since animal feed, such as grain and defatted oil

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seeds, contain only a small quantity of lysine, poultry, cattle and other livestock are unable to synthesize this amino acid. As a result, it must be added to this feed stuff to provide an adequate diet (Tosaka, 1983).

Lactic acid and lysine can be produced by chemical synthesis, enzymatic method and microbial fermentation.Among them, Microbial fermentation is mostly used because of environmental concerns, uses of renewable sources instead of petrochemicals, low production temperature, low energy requirements and high purity (Vijayakumar*et al.*,2008).Microbial fermentation of lactic acid and lysine can be attractive if low cost waste materials are used asthe carbon source. Cassava is considered to be an attractive carbon source since it is cheap and it containsstarchthat can be converted to glucose which may be essential for the growth of themicroorganisms.

The present investigation was carried out to produce lactic acid and lysine from cassava by fermentation.The complete process includes four main steps such as production of starch and glucose as intermediate steps and, finally isolation of *L.casei* from cheese and *C. glutamicum* from soil and,fermentation using these bacteriawas performed.

2. Materials and Methods

2.1 Materials

Cassava waste (pulp)was collected from cassava mill, Hinthada Township, Ayeyarwady Region, Cheddar cheese (Brand–PRESIDENT) was purchased from City Mart Supermarket, Thamine Branch and soil samples were collected from Yangon division, Myanmar. The commercial enzymes (α-amylase and amyloglucosidase)were purchased from MY Associates Co., Ltd., Dagon Center, Yangon, Myanmar. All other chemicals were from Merck, Germany. All of the experiments were carried out independently in triplicates and repeated twice.

2.2 Production of raw cassava starch

Raw cassava starch was extracted from cassava waste (pulp), according to Laderia*et al.*, 2013.Firstly, Cassava waste was grated.The grated cassava waste (100 g) was then mixed with 1000 mL of water, ground by blender and filtered. Settling of the starch was allowed for 2 h to take place until the suspension forms a clear liquid. Dewatering was carried out in a clean cloth-bag and the raw starch paste was dried in the oven at 60°C for about 4 h and ground with blender to obtain white granule powder and it was stored in an air-tight bottle to be used for further work. The raw starch yield wasthen calculated.

2.3 Optimal conditions and the maximum activity of α-Amylase and amyloglucosidase enzymes

The optimum pH, the optimum temperature and the maximum activity of the enzymes were studied by the colour changes in comparison with the standard dextrin iodine solution(0.2 mL of 1 % dextrin in 10 mL of iodine solution) (Rourke, 2002). Raw cassava starch solution (1 % w/v) was prepared with 0.2 M acetate buffer.The effect of pH on the activity of enzymes was investigated withdifferent pH ranges (pH 3 to 7) at 90 °C for α-amylase and at 60 °C foramyloglucosidase and with different temperature ranges (30 °C to 110 °C) at pH 6 forα-amylase and pH 4 for amyloglucosidase.

2.4 Enzyme activity by paper disc plate assay

Starch agar plates (2 % produced raw cassava starch and 1.5 % agar) were prepared. The paper discs were sunk in the prepared enzyme solutions(1:0, 1:4, 1:9 and 1:14) for 1 minute and serially put on the starch agar plates. The plates were then incubated at 28 °C in incubator. After 24 h incubation, the plate was smeared with freshly prepared 1 % iodine solution (10 % of KI and 1 % of I_2 in 100 mL of distilled water). The observation was made to see the diameter of the hydrolytic zone around the paper disc(Senthilkumar *et al.*, 2012).

2.5 Preparation of glucose syrup from raw cassava starch

The produced raw cassava starch (50 g in 150 mL of distilled water) was left to gelatinize at 80 °C on the thermo shaker water-bath. Then, it was allowed to liquefy for 1 h after adding 5 mL of α-amylase at pH 6 and 90 °C.After that,the same amount of aminoglucosidasewas added at pH 4 and60 °Cand the condition was kept to complete saccharification process for 4 h. Finally, the syrup was centrifuged (2000 rpm, 30 minutes), the supernatant was collected and evaporated by rotatory (40 °C, 30 minute), and heated on a water-bath (Ayoola*et al.*, 2012). The glucose syrup was pre-identified by Benedict's test, Molisch test, Fehling's test and Rapid furfural test and the glucose content in 5 % (w / v) syrup was determined by DNSA method at 575 nm at UV-vis Spectrometer.

2.6 Isolation and identification of *L. casei*and*C. glutamicum*

Isolation of*L.casei*and *C.glutamicum*were performed by the serial dilution and plating techniques (Dubey and Maheshwari, 2009). For *L.casei,* the 10 g of cheese sample was homogenized in 90 mL of Ringer's solution and tomato agar medium (tomato juice 10 mL, peptone 1.5 g, yeast extract 0.5 g, glucose 1 g, $KH_2PO_4$0.2 g, tween-80 1 mL, agar 2 g, distilled water 100 mL and pH 6-6.5) was prepared. The 10 g of soil sample was suspended in 90 mL of sterilized water and Nutrient medium (meat extract 1 g, peptone 1 g, NaCl 0.5 g, agar 2 g, distilled water 100 mL and pH 7) was for the isolation of *C. glutamicum*.Well grown bacterial colonies were picked and further purified by streaking and then stored in medium slants. The isolated pure cultures were identified by the characteristics of their biochemical activities.

2.7 Fermentation medium and conditions for lactic acid production

The lactic acid production was designed using produced cassava glucose syrup instead of commercial glucose and optimizing the conditions of fermentation was carried out. MRS broth was used as a fermentation medium at 28 °C and 200 rpm in shaker incubator.The effect of fermentation period (with8 % inoculum, 4 % cassava glucose syrup and pH 6),inoculum level (with4 % cassava glucose syrup,pH 6 and 72 hfermentation period)and pH (with4 % cassava glucose syrup, 8 % inoculum and 72 h fermentation period)were investigated. Lactic acid productivity wasdetermined at different cassava glucose syrup contents (2 %, 4 %, 6 %, 8 % and 10 %).

2.8 Lactic acid content in fermented MRS broth

The lactic acid content in fermented medium was determined according to British Pharmacopeia (1986).The 5 mL of supernatant was applied after being centrifuged at 2000 rpm for 30 minutes. The actual lactic acid content was calculated. The results of fermented media were subtracted from the un-inoculated medium result. The real titrated volume of

lactic acid (mL) with NaOH was multiplied by the lactic content factor 0.00908 g. The lactic acid content in fermented media was expressed as g/mL and the productivity was shown as the yield percent of consumption of produced cassava glucose syrup in fermented broth.The produced lactic acid was identified by thin layer chromatography (TLC), comparison with the standard lactic acid.

2.9 Fermentation condition in lysine production

Cassava glucose syrup instead of commercial glucose was used as a carbon source in media. Oneloopful of *C.glutamicum* inoculated in 25 mL seed culture (glucose 3 g, peptone 1 g, yeast extract 0.5 g, NaCl 0.3 g, $MgSO_4.7H_2O$ 0.5 g, $MnSO_4.H_2O$ 0.5 g, K_2HPO_4 0.15 and KH_2PO_4 0.05 g and biotin 0.25 μg in 100 mL distilled water at pH 7.2) and incubated at 200 rpm and 30 °C for 1 day in shaker incubator and then, 1 mL of seed culture was inoculated into the flask containing 25 mL main medium (glucose 11 g, peptone 1 g, yeast extract 0.5 g, Meat extract 0.5 g, $MgSO_4$ 0.025 g, $MnSO_4$ 0.001 g, $(NH_4)_2SO_4$ 1.5 g, K_2HPO_4 0.15 g and KH_2PO_4 0.05 g, $CaCO_3$ 1 g, $FeSO_4$ 0.001 g, CH_3COONa 0.2 g, vitamin B1 0.125 μg and biotin 0.25 μg in 100 mL distilled water at pH 7.2) and incubated at 200 rpm and 30 °C for 6 daysin shaker incubator.

2.10 Quantitative analysis of L lysine

The broth medium was centrifuged at 2000 rpm for 20 mins and the supernatant was collected. Lysine content was determined by Ninhydrin ferric reagent method(Hsieh *et al.*, 1995). 1 mL of sample wasmixed in 3.35 mL of reagent A (the mixture of 373mLof Methylecellosolve,30 mLof 50% ferric chloride solution and 600 mlof 0.1M KCl solution) and 1.85 mL ofreagent B (1% Ninhydrin in 0.1MKCl solution). This solution was then subjected to heatingup to 100°C for 20 min in a water bath. Then,it was cooled at room temperature. The 0.1 mL of DMSOand 3 mL of distilledwater wereadded to the mixture and absorbance was measured at 470nm. The lysine content wascalculated against the lysine standard curve.

3. Results and Discussion

3.1 Starch extraction from cassava waste

Raw cassava starch (10.5 %) was extracted from 100 g of cassava waste using 1000 mL distilled water, at 2 h setting time and 4 h drying periods.

3.2 Activities of α-Amylase and Aminoglucosidase

The activities of α-amylase and amyloglucosidase were determined at the different pH and different temperatures and the corresponding results are shown in Figures 1,2, 3, 4 and Table 1. It could be detected that the maximum power of α-amylase was 60×10^4 U/mL atpH 6 and 90 °C. However, that of amyloglucosidase was 2.57×10^4 U/mL at pH 4 and 60 °C. Therefore, it was found thatα-amylase has more potent enzymatic activity than amyloglucosidase.

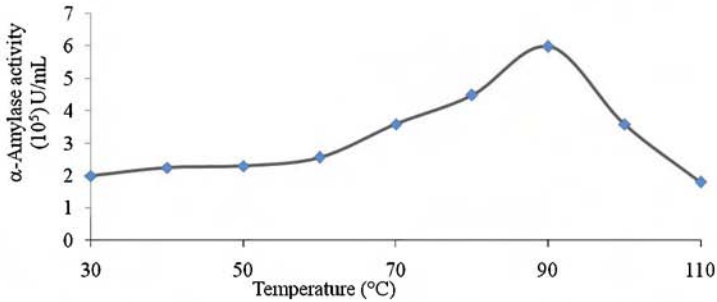

Figure 1. Effect of temperature on α-amylase activity at pH 6

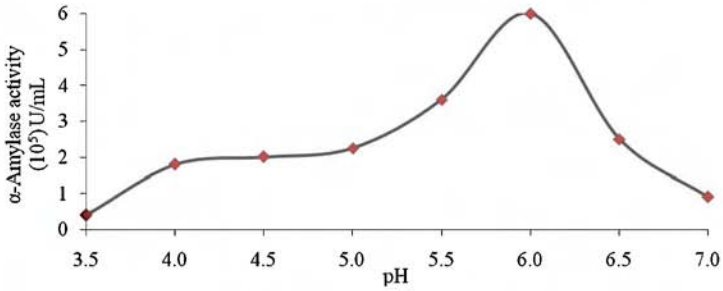

Figure 2.Effect of pH on α-amylase activity at 90 °C

The enzyme activity was also performed on starch agar medium by paper disc plate assay. The results are summarized in Table 2. The α- amylase enzyme (1:0 dilutions) exhibited slightly larger hydrolytic zone (36 mm) than that of amyloglucosidase (33 mm). According to results, the hydrolysis power of different dilution ratioswas observed nearly to be similar to each other.

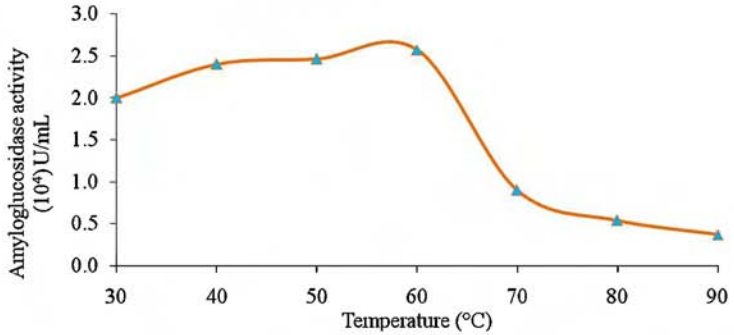

Figure 3. Effect of temperature on amyloglucosidase activity at pH 4

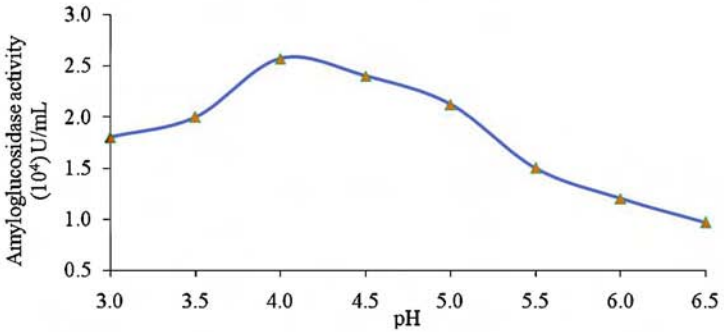

Figure 4. Effect of pH on amyloglucosidase activity at 60 °C

Table 1. Optimum conditions and enzyme power of selected enzyme

Enzymes	pH	Temperature (°C)	Enzyme power (U/mL)
α-Amylase	6	90	60×10^4
Amyloglucosidase	4	60	2.57×10^4

Table 2. Results for α-Amylase and amyloglucosidaseactivity by paper disc plate assay

Enzyme : Water	Hydrolytic Zone (mm)	
	α-amylase	Amyloglu-cosidase
1:0	36	33
1:4	30	26
1:9	28	25
1:14	26	24

3.3 Cassava glucose syrup

The glucose syrup was produced from raw cassava starch by enzymatic hydrolysis under the optimum conditions of α-amylase (pH 6, 90 °C) and amyloglucosidase (pH 4, 60 °C) enzymes and thephotograph of produced glucose syrupis shown in Figure 5. The pre-identification tests showed all positive results.As mentioned in Table 3, the amount of glucose syrup,91.40 % yield based on raw cassava starch and 9.96 % based on cassava waste, was obtained at pH 4 and 4 h processing time. The glucose content in produced glucose syrup was determined to be 3.02 mg in the prepared 5 mg/mL solution.

Table 3. Results of the yield percentage of glucose syrup at pH 4,60 °C and 4 h of processing time and glucose concentration

No.	Yield % of Glucose Syrup		Glucose Concentration (mg/mL)
	A	B	
1	91.40	9.96	3.02

A= yield percent based on raw cassava starch

B=yield percent based on cassava waste

Figure 5. Photograph of produced glucose syrup

3.4 Isolation and identification of *L. casei* and *C. glutamicum*

*L.casei*was isolated from cheese and *C. glutamicum*was isolated from soil. They both are rod, non-motive and gram positive bacteria and no produce gas during sugar fermenting, Table 4 and 5 give the information on comparative results of some biochemical tests of the test culture and the reported data. According to the results, it could be confirmed the isolated test culture was to be *L. casei*and *C. glutamicum*because the experimental tests results were identical to those presented in Bergey's Manual of Determinative Bacteriology (Buchannan and Gibbons, 1974).

Table 4. Comparative results of some biochemical tests of *L. casei*

Tests performed	IsolatedTest Culture	Buchannan and Gibbons, (1974)
Gram's stain	+	+
Shape	rod	rod
Motility	–	–
Indole	–	–
Citrate	+	+
Gelatin	–	–
Catalase	–	–
Nitrate	–	–
TSI	+	+
Glucose Broth	+ and no gas	+ and no gas
Fructose Broth	+ and no gas	+ and no gas
Sucrose Broth	+ and no gas	+ and no gas
Lactose Broth	–	–
Mannose Broth	+ and no gas	+ and no gas
Sorbitol Broth	+ and no gas	+ and no gas

(+) positive test

(–) negative test

Table 5. Comparative results of some biochemical tests of *C. glutamicum*

Tests performed	IsolatedTest Culture	Buchannan and Gibbons, (1974)
Gram's stain	+	+
Shape	Rod	rod
Motility	–	–
Spore stain	–	–
Acid fast stain	–	–
Indole	–	–
VP	+	+
MR	–	–
Catalase	–	–
Indole	–	–
Starch	–	–
Citrate	–	–
Urase	+	+
Gelatin	+	+
Nitrate	+	+
Glucose Broth	+ and no gas	+ and no gas
Fructose Broth	+ and no gas	+ and no gas
Sucrose Broth	+ and no gas	+ and no gas
Lactose Broth	–	–
Maltose Broth	+ and no gas	+ and no gas

(+) positive test
(–) negative test

3.5 Optimization of the conditions of lactic acid fermentation

The results of lactic acid contents in different fermentation periods (24 h, 48 h, 72 h, 96 h and 120 h) were revealed to be 0.025, 0.032, 0.040, 0.030 and 0.012 g/mL, respectively (Table 5 and Figure 7).The highest lactic acid content (0.040 g/mL) was investigated at 72 h fermentation period. In addition, the results of lactic acid contents in different inoculum levels were determined to be 0.030, 0.027, 0.025, 0.040 and 0.038 g/mL, respectively and it was found that using 8 % inoculum produced more lactic acid then other inoculum levels (2 %, 4 %, 6 % and 10 %) (Table 6 and Figure 8), and the highest content was found to be 0.040 g/mL.Table 7 and Figure 9 illustrate the results for effect of pH on lactic acid production. The results of lactic acid contents in different pH levels (4.5, 5, 5.5, 6