

The lactic acid fermentation process can be classified into heterofermentation and homofermentation. The type of the process is determined by the microorganisms which are used in the fermentation.

The homofermentative process is the important method in producing pure lactic acid and it is an anaerobic process (Lasprilla *et al.*, 2012). *Lactobacillus delbrueckii*, *L. amylophilus*, *L. bulgaricus*, and *L. leichmannii* are examples of homofermentative lactic acid bacteria (Garlotta, 2001).

Even though *Streptococcus thermophilus* a well-known lactic acid bacterium and corn starch to lactic acid conversion has been studied in depth, the studies related to the conversion of corn starch to lactic acid by *Streptococcus thermophilus* were not explained in the literature.

In this study, glucose was obtained from powdered corn kernels via hydrolysis by α -amylase. The hydrolysis was optimized by varying the stirring time, temperature and corn starch concentration. The presence of glucose in the samples were determined using 3,5-Dinitrosalicylic acid (DNSA) method and Fourier transform infrared spectroscopy (FTIR) analysis. Glucose to lactic acid conversion was done using *Streptococcus thermophiles* as a fermenting organism. UV spectrometer and FTIR were used to determine the Lactic acid presence of the samples.

2. MATERIALS AND METHODS

2.1 Materials

Sri Lankan corn kernels, α -amylase enzyme from porcine pancreas and DNSA (3,5-Dinitrosalicylic acid) were purchased from the Sigma Aldrich Co. (St.Loui,MO). Iodine solution, Conc. Sulfuric acid, Monopotassium phosphate, Di-potassium hydrogen phosphate, Sodium chloride, Sodiumhydroxide, Potassium sodium tartrate, Iron(III) chloride, Calcium hydroxide, Lactic acid, Tin(II)Chloride dehydrate and Chlorobenzene used were of analytical grade.

Commercial Glucose was purchased from the State Pharmaceuticals Corporation of Sri Lanka. Starter culture of *Streptococcus thermophilus* was obtained from CHR Hansen.

2.2 Methodology

2.2.1 Conversion of corn starch to glucose

Starch hydrolysis by α -amylase: The corn kernels were ground and sieved to obtain micrometer size powder particles. The required amount of corn starch powder (Table 1) was properly mixed with 125.0 mL of distilled water to prepare the corn starch solution. The corn starch solution was dissolved in the microwave oven for 5 minutes, followed by cooling to room temperature. Then the equal volumes (125.0 mL) of α -amylase solutions and the corn starch solutions were mixed separately and stirred at a given temperature for given period (Table 1).

The FTIR analysis: The FTIR spectra of the samples were recorded using an FTIR spectrophotometer (500-4500 cm^{-1} , resolution of 4 cm^{-1}) (Bruker, USA). Reference spectrum of water was also recorded and deducted from the sample spectrum to obtain the glucose spectrum (since the glucose concentration of the sample is low, water peaks are prominent and glucose peaks have been hidden) using the OPUS 7.5 software in the instrument.

Quantitative analysis of glucose yield: The glucose yield of the enzymatically hydrolyzed corn starch samples was quantitatively determined by slightly modifying the method described by Gonçalves *et al.*, (2010) as below. A 100 μl of the hydrolyzed corn starch sample was mixed with 100 μl of pre-prepared 3,5-Dinitrosalicylic acid (DNSA) solution in a microtiter plate reader well and the microtiter plate was placed in a water bath (85 $^{\circ}\text{C}$) for 15 minutes. The absorbance was measured at 540 nm by Microplate Reader (Thermo Scientific - MULTISKEN GO) against the reference solutions (100 μL DNSA and 100 μl of water).

Table 1: Reactant (corn starch powder in 125.0 mL of water) concentrations and reaction conditions for trials

Trial number	Weight of corn starch powder (g)	Concentration of corn starch solution. (% w/v)	Stirring temperature (°C)	Stirring time (hours)
1	0.6250	0.5	37	2.0
2	0.6250	0.5	37	1.5
3	0.6250	0.5	37	1.0
4	0.6250	0.5	37	0.5
5	0.6250	0.5	37	0.0
6	0.3125	0.25	37	1.5
7	0.9375	0.75	37	1.5
8	1.2500	1.00	37	1.5
9	0.9375	0.75	27	1.5
10	0.9375	0.75	47	1.5
11	0.9375	0.75	57	1.5

The absorbance of the sample was measured for each trial. Three replications were done for each trial.

The concentration of glucose in each sample was determined using glucose calibration, ranging from (0.01-0.07) %. The experimental trials 1-5, 2 and 6-8, 7 and 9-11 were used to determine the optimum stirring time, optimum starch concentration, and optimum stirring temperature, respectively as given in Table 1.

2.2.2 Conversion of glucose to lactic acid

Fermentation of glucose: Glucose was fermented by *Streptococcus thermophilus* culture (STI 12 Freeze dried 50U). *Streptococcus thermophilus* starter culture (1.000 g) was mixed with the 250.0 mL of glucose solution in the fermenter and incubated at 37 °C for 4 days. The pH of the glucose solution was maintained between 5.4 and 6.4 throughout the fermentation period by using Ca(OH)₂ solution. This pH range is the optimum pH range that *Streptococcus thermophilus* have high metabolism activity.

Quantitative analysis of lactic acid yield: The produced lactic acid after the fermentation was quantified using the method explained by Borshchevskaya and coworkers (Borshchevskaya *et al.*, 2016). A 2.0 mL of fermentation broth was

diluted with 38.0 mL of distilled water to obtain 20-fold diluted sample. The microbial cells were removed by centrifuging the sample at 5000 rpm for 10 minutes. A 50 µL of supernatant was mixed with 2 mL of 0.2% iron (III) chloride solution and stirred. The absorbance was measured at 390 nm against the reference solution (2 mL of a 0.2% FeCl₃ solution and 50 µL of distilled water) using UV visible spectrophotometer (Thermo Scientific - Evolution 201). The concentration of lactic acid in each sample was determined using lactic acid standard calibration, ranging from (0.2 -2.0)% lactic acid concentration.

Purification of crude lactic acid: The fermentation broth was centrifuged at 5000 rpm for 10 minutes and microbial cells were removed. Conc. Sulfuric acid (50.0 mL) was added to the total volume of cell free broth to precipitate calcium and filtered out. Methanol was added to the filtrate with 2 drops of sulfuric acid. Methanol-to-filtrate volume ratio was maintained at 1:4. The filtrate-methanol mixture was distilled and fractionated at 98 °C. The remaining solution was distilled again and fractionated at 122 °C.

FTIR analysis of lactic acid: FTIR spectra of the collected fraction and commercial lactic acid were recorded by FTIR spectrophotometer (Bruker, USA) within the range of (500 – 4000 cm⁻¹) with

a resolution of 4 cm^{-1} and compared. Spectra were collected and analyzed by OPUS 7.5 software.

Data analysis: The glucose concentrations obtained under three conditions such as stirring time, starch concentration and the temperature was analyzed by ANOVA. The lactic acid yield was also analyzed by ANOVA. The statistical analysis was performed in Minitab® 17.1.0.

3. RESULTS AND DISCUSSION

3.1 Enzymatic conversion of starch to glucose

Alpha-amylase enzyme was used to convert the starch into glucose. Required pH and salt concentrations were maintained to facilitate the enzymatic hydrolysis process.

The peaks in the FTIR spectrum of the hydrolyzed starch solution were expressed by the characteristic peaks of glucose at 2920 cm^{-1} (asymmetric vibration of CH), 3350 cm^{-1} (O–H stretching), 2850 cm^{-1} (symmetric vibration of CH₂) and 1450 cm^{-1} (bending vibration of CH) proving the presence of glucose in the hydrolyzed corn starch sample (Figure 1).

As the followed method could produce glucose, method parameters such as stirring time, stirring temperature and starch concentration were optimized to enhance the glucose yield. DNSA method was used to compare the glucose yield because the DNSA method is intended to be used to determine the reducing sugars. In the presence of glucose, DNSA is reduced to 3-amino-5-nitrosalicylic acid while the aldehyde group of glucose is oxidized to carboxylic acid. The color of the reagent changes from yellow to orange or red depending on the concentration of reducing sugar. The reddish brown colored complex has an absorbance maximum at 540 nm.

3.1.1 Optimum stirring time

Optimum stirring time was determined based on the absorbance data recorded in half an hour intervals of stirring. As shown in Figure 2, the glucose concentration has increased with the

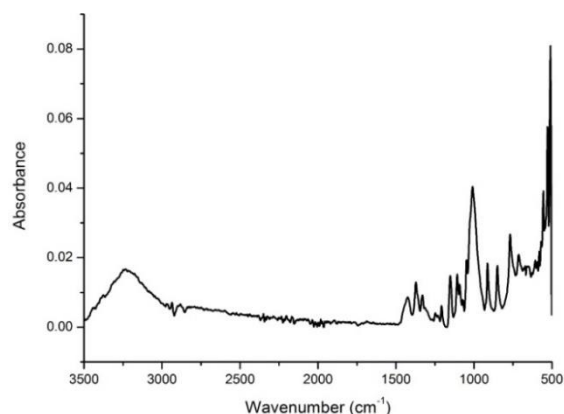


Figure 1: FTIR spectrum of the hydrolyzed corn starch sample

stirring time until 1.5 hours, and at 2 hours it has reduced compared to 1.5 hours. The significantly improved ($a \leq 0.05$) glucose concentration was also observed after 1.5 hours of stirring time. Therefore, 1.5 hours can be considered as the optimum stirring time.

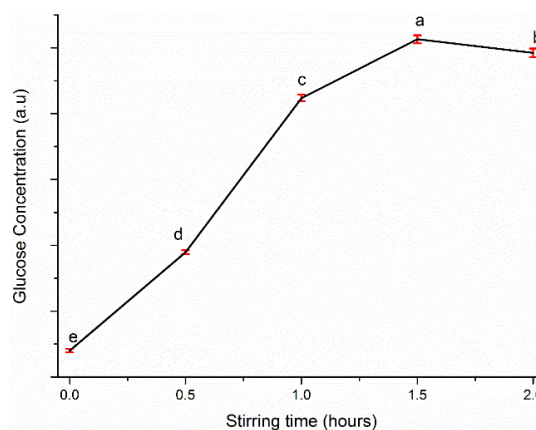


Figure 2: Glucose concentration, yield from the hydrolysis of corn starch samples for different stirring times

3.1.2 Optimum starch concentration

The variation of glucose concentration with the starch concentration is illustrated in Figure 3. According to that, the significant ($a \leq 0.05$) glucose yield was obtained from 0.75% and 1% starch solution. As shown in Figure 3, glucose concentration has increased with the starch concentration until 0.75%, and at 1.00% it has reduced compared to 0.75%. Therefore 0.75% can be considered as optimum starch concentration.

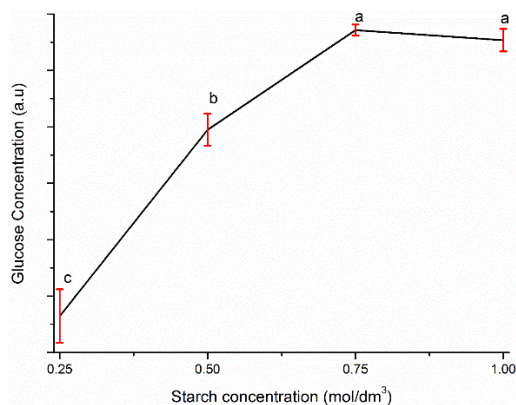


Figure 3: Glucose concentration, yield from the hydrolysis of corn starch samples at different initial corn starch concentrations

3.1.3 Optimum temperature

The stirring temperature was varied from 27-57 °C and glucose concentration vs. temperature was graphed (Figure 4). The significantly ($\alpha \leq 0.05$) improved glucose concentration was obtained from the sample stirred at 47 °C. As shown in Figure 4, glucose concentration has increased with the temperature until 47 °C and at 57 °C it has reduced compared to 47 °C.

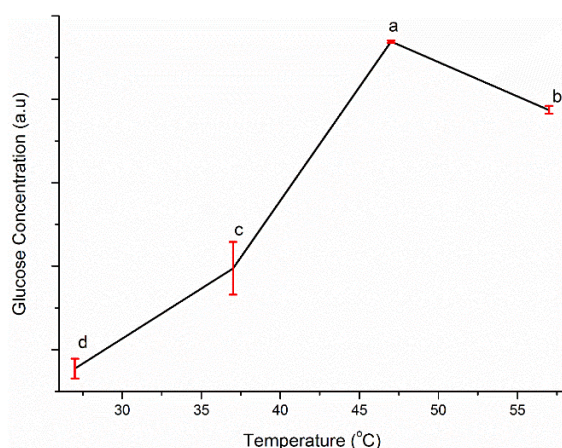


Figure 4: Glucose concentration, yield from the hydrolysis of corn starch samples at different hydrolyzing temperatures

3.1.4 Glucose yield under optimized conditions

With the identified optimum parameters, hydrolysis was carried out to determine the maximum glucose yield. A calibration curve was plotted using a series of glucose solutions with known concentrations from 0.01-0.07%. Glucose

concentration of the sample, which was carried out under optimum reaction parameters, was calculated using the calibration curve and triplicated. The average glucose concentration of the 10 fold diluted sample was determined as 0.02846 % (w/v). Therefore, actual concentration was back-calculated as 0.2864% (w/v), in other words 2.86 g/L.

3.2 Conversion of glucose into lactic acid

Glucose fermentation was carried out using *Streptococcus thermophilus* bacterium. According to the literature we could not find any studies of converting corn starch into lactic acid using this *S. thermophilus*. During the fermentation period $\text{Ca}(\text{OH})_2$ was added to neutralize the fermentation broth. Due to the formation of lactic acid, the medium becomes acidic. In the acidic medium *Streptococcus thermophilus* reaction is inhibiting (Reddy *et al.*, 2008).

The lactic acid yield was compared by measuring the absorbance using iron(III) chloride as a reagent. The reaction of iron(III) chloride with lactic acid in an aqueous solution results in the yellowish-green iron(III) lactate which shows maximum absorption at 390 nm (Borshchevskaya *et al.*, 2016)

According to Figure 5, significantly high lactic acid concentration was obtained after four days of fermentation. Lactic acid concentration has increased with the time until day 4, and at 4.5 days it has reduced compared to day 4. Therefore, optimum fermentation time can be considered as 4 days.

3.2.1 Determination of Lactic acid concentration

A calibration curve obtained from known series of lactic acid solutions (0.2-2.0 % (w/v)) was used to determine the Lactic acid concentration. Concentration of the sample with the highest yield which was obtained after 4 days was measured. The average lactic acid concentration of the 20 fold diluted sample was determined as 0.642 %

(w/v). Therefore, actual concentration was back calculated as 12.84 % (w/v) in other words 128.4 g/L. Therefore, it can conclude that *S. thermophilus* can be used to obtain a significant amount of lactic acid. The similar study has recognized that fermentation of the wheat and rice bran by *Lactobacillus sp.* can yield around 129 g/L of lactic acid which was very much close to the lactic acid yield of the present study (Nampoothiri *et al.*, 2010).

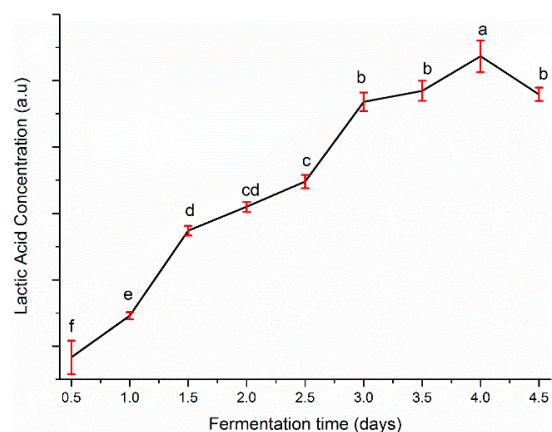


Figure 5: Lactic acid concentration, yield from the fermentation of glucose using *Streptococcus thermophilus* at different fermentation times

3.2.2 Purification of crude lactic acid

As the first step, microbial cells were removed by centrifugation. Then concentrated sulfuric acid was added to the system to convert the calcium lactate into lactic acid. Sulfuric acid was selected because it easily removes calcium. After all, calcium sulphate is a precipitate and can filter out.

The separation of lactic acid was a multi-step process. Methanol was added to the filtrate to esterify the lactic acid into methyl lactate. At first distillation, the methyl lactate was collected (Komesu *et al.*, 2017b). Then Methyl lactate was hydrolyzed to convert the methyl lactate into lactic acid and separated by second distillation.

FTIR data proved that purified product is pure lactic acid because the FTIR spectrum of the purified lactic acid has overlapped with the FTIR spectrum of commercial lactic acid (Figure 6).

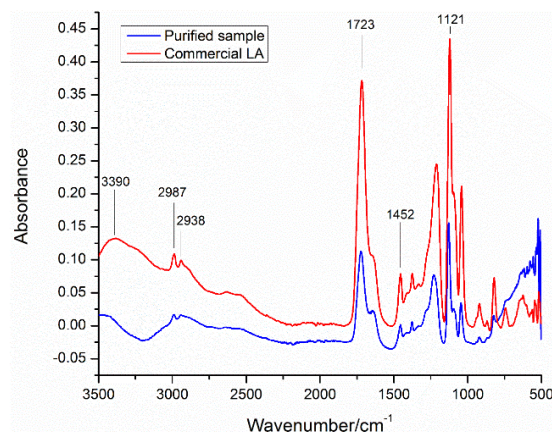


Figure 6: FTIR spectra of commercial Lactic acid and the purified lactic acid product of the present study

The characteristic peaks of Lactic acid at 1722 cm^{-1} (C=O stretching), 2600–3200 cm^{-1} (O–H stretching), 1200–950 cm^{-1} (C–C and C–O stretching) and 1200 cm^{-1} (C–H, C–O, and CH_3 vibrations) proved the purity of Lactic acid (Paucean *et al.*, 2017).

4. CONCLUSIONS

Hydrolysis of corn starch using alpha-amylase enzyme was optimized and identified that the maximum glucose yield could be obtained by stirring a 0.75% (w/v) of corn starch solution for 1.5 hours at 47 °C. Lactic acid was successfully produced by fermentation of glucose obtained from corn starch using *Streptococcus thermophilus*, a lactic acid bacterium. The highest concentration of lactic acid was obtained after 4 days of fermentation at 37 °C, which was around 128 g/L. Therefore, it was concluded that *S. thermophilus* could be used to extract lactic acid from corn starch with a considerable yield.

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