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Optimization of sugarcane juice as a culture medium for scale up of *Lactobacillus plantarum* TISTR 2083 production

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Abstract

Lactobacillus plantarum TISTR 2083 is one of the industrially relevant lactic acid bacteria vastly used as a probiotic. The culture of *L. plantarum* TISTR 2083 in sugarcane juice was studied to optimize the culture medium and growth conditions for the highest viable cell production at a low cost. The effect of sugar content (5 - 20°Bx), amount of seed culture (1 - 10%), different nitrogen sources (ammonium sulfate, diammonium phosphate, yeast extract and peptone) with different concentrations (0 – 1.5%), and different agitation speeds (0 – 150 rpm) were investigated in the sugarcane juice growth media for higher cell growth of *L. plantarum* TISTR 2083. The concentration of sugarcane juice was optimized with 5, 10, 15, and 20°Bx. The highest cell viability was found in the 20°Bx sugarcane juice as 8.90-9.24 log CFU/mL. Moreover, seedlings with a 5% (v/v) seed culture maximized the viable cell (9.07 log CFU/mL) than 1, 3, 7, and 10% seed cultures, respectively. Among different nitrogen sources, high cell viability was obtained using 1.5% yeast extract (8.76-9.11 log CFU/mL). With all other optimized parameters, the agitation speed of 100 rpm resulted in a higher viable cell count. Optimized sugarcane juice can be used as a low-cost medium and a simple bottle fermenter for the scale-up of *L. plantarum*.

1. Introduction

At present, lactic acid bacteria (LAB) hold the principal role as a starter culture in different fermented food industries. These industries include dairy, meat, and vegetables. Besides, LAB also improves food qualities such as taste, texture, palatability, digestibility, and storability (Carr *et al*., 2002). Furthermore, some LABs are essential probiotics with health-promoting benefits. These benefits include interactions of the gastrointestinal (GI) tract and microflora, bifidogenic activity, immune-simulation properties, prevention of cancer, antipathogenic, and antioxidant effects (Regina *et al*., 2016; Cirrincione *et al*., 2018). *Lactobacillus plantarum* is one of the most widespread species among the LAB group that is used in fermentation technology and for the development of different probiotic formulations (Russo *et al*., 2017; Seddik *et al*., 2017). The demand for commercial production of *L. plantarum* is significantly increasing because this bacterium is considered to be "generally recognized as safe" (GRAS) status (Tsai *et al*., 2017). *Lactobacillus plantarum* grows in medium rich in sugar content with other nutrients. De Man, Rogosa and Sharpe (MRS) broth is the most common growth medium for lactobacilli use in the lab scale, which contains nitrogen sources, meat, and yeast extract. However, the MRS broth is not recommended for commercial application due to its high costs and the risk concerns relating to the occurrence of bovine spongiform encephalopathy (Horn *et al*., 2005).

In general, an ideal culture medium for the commercial production of food bacteria should be cheap and also contain essential nutrients necessary for growth. The main concern of this study is the optimization of the culture medium for *L. plantarum* from sugarcane juice. The production cost of a starter culture is affected by the price of raw materials and other substrates. Thus, the use of cheap raw materials provides an alternative medium to help reduce the commercial production of starter culture. Sugarcane is recognized as a prominent crop that grows faster than other commercial crops. Sugarcane juice contains 13-16% (w/v) sucrose as disaccharides as well as glucose and fructose as monosaccharides. Thus,

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making sugarcane juice an excellent substrate for the cultivation of *L. plantarum* (Sikder *et al*., 2014; Silalertruksa and Gheewala 2018). Nevertheless, there is limited research carried out on the sugarcane juice medium for different microorganisms or fermentation by -products. Furthermore, to the best of our knowledge, there is no research study on the optimization of sugarcane juice for high cell density cultivation of *L. plantarum*. Sugarcane juice can be an abundant, renewable, and economical substitute for common substrate raw materials. However, it requires pre-treatment that is related to the factors involved in starter culture cultivation, such as sugar content, nitrogen, and cultivation conditions. Therefore, it is essential to investigate the interaction between the parameters that optimize the condition for the growth of *L. plantarum.* On the other hand, different factors are also related to the growth of viable cell density such as oxygen availability, fermentation time and temperature which are related to fermenters (Shah, 2000). The fermenter is equipment that is used to maintain the desired condition for scale-up like high cell density cultivation, fermentation, and other commercial productions. Overall, this study aimed to optimize sugarcane juice as a culture medium for scale-up cultivation of *L. plantarum* by using a simple fermenter.

2. Materials and methods

2.1 Bacterial strain

Lactobacillus plantarum TISTR 2083 strain was collected from the Thailand Institute of Scientific and Technological Research (TISTR). The strain is formerly isolated from rice cake starter (Loog-pang Koawmak) used for traditional Ka-nom Tuay-fu production in Pattani province, Thailand (Maneesri *et al*., 2018). It is inoculated in MRS agar, incubated at 30°C for 24 hrs, subsequently stored at 4°C and sub-cultured every month.

2.2 Inoculum preparation and seed culture

To prepare the inoculum, the microorganism from the stored vial was transferred to a 250 mL flask containing 50 mL MRS broth, incubated at 30°C and shaken at 100 rpm for 24 hrs. Then it was inoculated in MRS agar to preserve the pure culture for a month. Seed culture was prepared by transferring one loop from the stored culture in a 500 mL flask containing 200 mL of MRS broth. After that, it was incubated at 30°C and shaken at 100 rpm for 18-24 hrs to measure the Optical Density at 600 nm (OD_{600}). The OD_{600} of seed culture broth was adjusted to 0.5±0.05 before experimental inoculation.

2.3 Culture media

Sugarcane juice was used as a culture medium for the cultivation of *L. plantarum* TISTR 2083. The fresh sugarcane juice was extracted from a clean sugarcane stalk, then the juice was pasteurized at 75°C and stored for a short period at 4°C and for longer periods at -20°C. The composition of sugarcane juice is shown in Table 1.

2.4 Optimization of culture condition for the growth of Lactobacillus plantarum TISTR 2083

2.4.1 Determination of sugar content effect on cell growth

To optimize the culture conditions, the sugar content of sugarcane juice was standardized to 5, 10, 15, and 20° Bx by adding commercial sugar (D-Glucose) or diluted by distilled water. Then the experimental media was adjusted to a pH of 6.0±0.05 by either HCl 0.1 N or NaOH 0.1 N and subsequently autoclave at 121°C for 15 mins. The autoclaved sugarcane juice was inoculated by 5% (v/v) seed culture and incubated at 30°C and shaken at 100 rpm for 24 hrs. The sampling was carried out every 4 hrs over the 24 hrs duration to determine viable cell count, pH, and cell dry weight.

2.4.2 Amounts of seed culture for optimum cell growth

Then the experimental media was inoculated with 5 different concentrations of 1, 3, 5, 7, and 10% (v/v) seed cultures to optimize cell growth.

2.4.3 Nitrogen source for cultivation of Lactobacillus plantarum TISTR 2083

Different nitrogen source such as $(NH_4)_2SO_4$ (ammonium sulfate), $(NH_4)_2HPO_4$ (diammonium phosphate), yeast extract and peptone was added into the

Table 1. Composition of sugarcane juice before and after autoclave.

Test item	Result (Unit)		Test method
	Fresh juice	After autoclave	
TSS (total soluble solids)	17.2°Bx	17.1°Bx	Hand refractometer
Total Sugar	15.29 g/100 g	15.32 g/100 g	AOAC (Lane and Eynon)
Reducing Sugar	3.16 g/100 g	2.44 g/100 g	AOAC (Lane and Eynon)
Protein	0.21 g/100 g	0.15 g/100 g	AOAC (Kjeldahl)
Acidity (as citric acid)	0.14 g/100 g	0.09 g/100 g	AOAC (Titrimetric)
Color (RGB)	R = 75, G = 71, B = 46	R = 103, G = 93, B = 63	Colour value apps by iPhone 8-plus

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culture media with 0.5% (w/v) before autoclaving. MRS broth media was also applied as a standard to compare the results. After that, different concentrations of 0, 0.5, 1, and 1.5% (w/v) of a single nitrogen source from above, which resulted in higher cell growth were added to the experimental media.

2.4.4 Investigation of agitation speed for Lactobacillus plantarum cultivation

Four different shaking speeds of 0, 50, 100, 150 rpm were applied to examine the outcome of shaking speed on bacterial growth.

2.5 Use of simply assembled fermenter

The high cell density cultivation of *L. plantarum* TISTR 2083 was conducted in the 10 L bottle fermenter equipped with an agitator. This bottle fermenter is made from polymer, non-permeable, transparent glass (Figure 1). The fermenter body has a scale in litre (L) and it contains an agitator, operated by magnetic agitation, for mixing purposes. The fermenter was sterilized by autoclaving and the agitator with 70% alcohol.

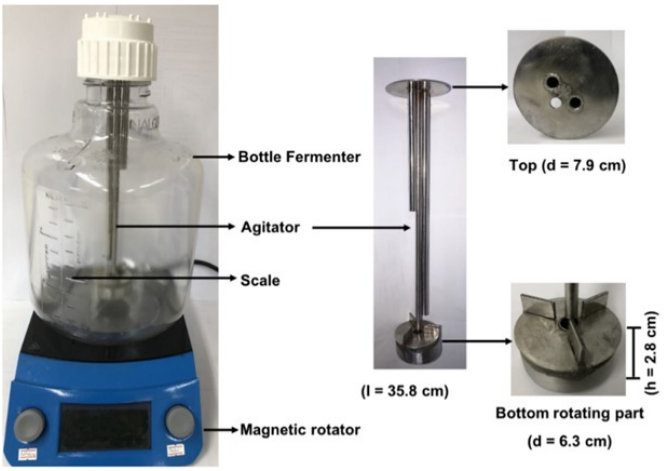

Figure 1. Lab scale simple bottle fermenter (l = length, d = diameter, h = height).

2.6 Scale-up

For scale-up of *L. plantarum* TISTR 2083, 3 L of the sugarcane juice culture media with optimized conditions (sugar content, nitrogen content, seed culture and agitation speed) was incubated at room temperature for 24 hrs. Sampling was carried out every 4 h over the 24 hrs duration to determine viable cell count, pH and cell dry weight.

2.7 Analysis of samples

2.7.1 Viable cell count

Viable cells in the cultured sugarcane juice medium were determined by the plate counting method on MRS agar containing 0.5% $CaCO_3$. The process was carried out by serial dilution in 0.85% (w/v) physiological saline and the plates were incubated at 30°C for 48 hrs (Hou *et al*., 2016).

2.7.2 pH

The pH of the collected sample media was determined with a pH meter.

2.7.3 Cell dry weight

To measure the cell dry weight, samples of cultured media were centrifuged at 9600g for 10 mins at 4°C and washed twice with distilled water. Then the washed cells were dried in a hot air oven at 105°C for 24 hrs.

2.8 Statistical analysis

All the experimental steps were accomplished with 3 replications and the output of ANOVA was expressed as mean±SD. Comparison between the data was analysed by Duncan's multiple range test and the statistical significance level was set at $p \leq 0.05$.

3. Results and discussion

3.1 Effect of sugar concentration on the growth of bacteria

Sugarcane juice with different sugar contents was used as culture media to study the effect of sugar concentration on the growth of *L. plantarum* TISTR 2083. The growth of *L. plantarum* TISTR 2083 was found in almost all the culture mediums with different sugar content from 6 h of incubation (Figure 2). Among all the 4 mediums, the sugarcane juice with 20°Bx showed the highest viable cell count of 9.07 log CFU/mL after 12 hrs of incubation. The cell growth of *L. plantarum* TISTR 2083 in the early stationary phase was observed, and the cell growth was slower in the high-sugar medium than in the low-sugar medium. However, the cell viability later gained at least 9.21 log CFU/mL with high sugar content. Bacteria consumed the sugar to increase the bacterial population and at the same time, lactic acid production by converting sugar leading to the lowering of the pH medium. Among the four different mediums, the final pH was lower than the initial for 15 and 20 °Bx sugarcane juice culture medium (Figure 2). Furthermore, the amount of lactic acid production increased due to the increase in sugar content in the medium (Timbuntam *et al*., 2006). The initial pH of 5, 10, 15, and 20°Bx medium were 4.42, 4.63, 5.12, and 5.20, respectively, those decreased (from initial pH 6) after autoclaving the sugarcane juice medium. All four sugarcane juice culture media showed a rapid decrease of pH in the first 12 hrs (pH 3.4 - 3.65) and slightly decreased till the end of incubation (3.35 - 3.55). Biomass production was determined by measuring cell dry weight produced by bacterial cells. In Figure 2, the

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final biomass of all the treatments was higher than the initial stage. The optimum production of biomass showed by 20°Bx sugarcane juice with the final biomass was 1.52 g/L. For the high biomass yield, *L. plantarum* is highly dependent on carbohydrate feeds where glucose was used as the primary energy source (Manzoor *et al*., 2017). In general, CFU (colony forming unit) represents the viable cell while cell dry weight may show the dead cells also.

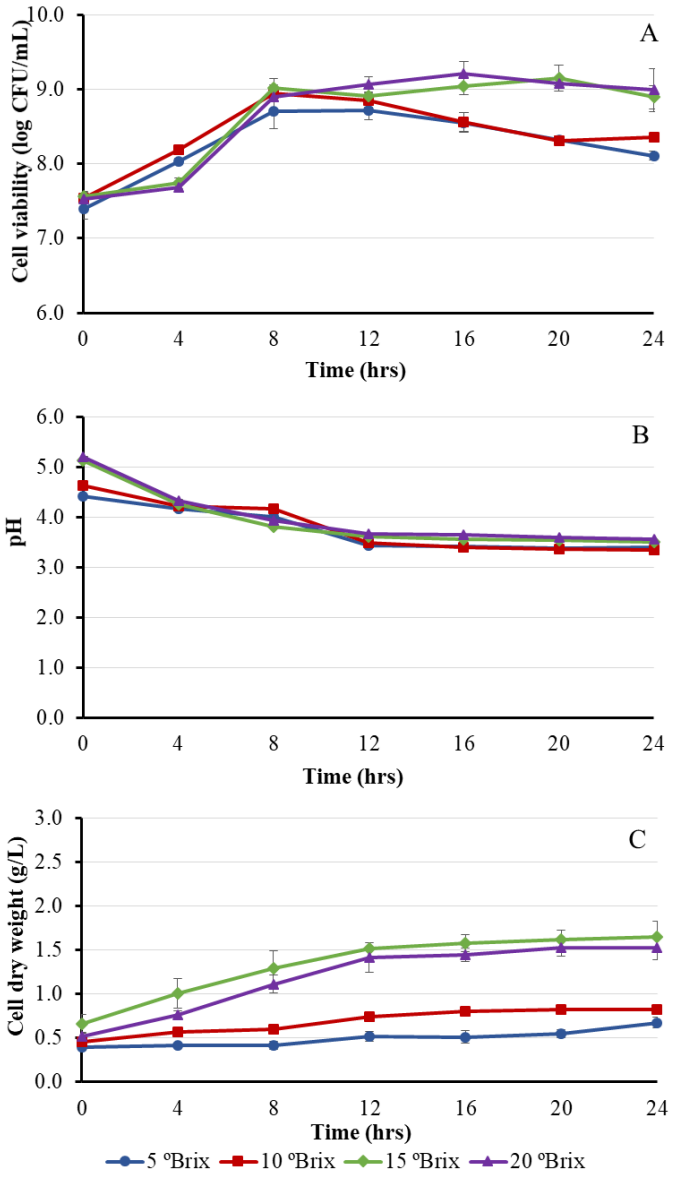

Figure 2. The cell viability (A), pH change (B) and cell dry weight (C) of *L. plantarum* TISTR 2083 in sugarcane juice culture medium of different sugar content.

3.2 Effect of amount of seed culture on the growth of Lactobacillus plantarum TIRTR 2083

In this experiment, sugarcane juice culture media was inoculated with five different inoculum sizes that affected the initial bacterial population in the culture medium. The initial population of *L. plantarum* TISTR 2083 in sugarcane juice culture media with different inoculum sizes of 1, 3, 5, 7, and 10% (v/v) were 6.26, 6.70, 7.52, 7.34 and 7.57 log CFU/mL respectively (Figure 3). The multiplication rate of *L. plantarum* TISTR 2083 was very slow in the case of 1% (v/v) seed culture and the bacterial population was significantly increased with the addition of 3 and 5% (v/v). On the other hand, the bacterial population growth was slower, with the addition of 7 and 10% (v/v) inoculum. After 12 hrs incubation time, the viable cell of *L. plantarum* TISTR 2083 with inoculum sizes of 1, 3, 5, 7 and 10% (v/v) were 7.47, 8.47, 9.07, 8.87 and 8.79 log CFU/mL (Figure 3) respectively. Wardani *et al*., (2017) showed that the population number increased with increasing inoculum size by 1, 3, 5, and 10% during milk fermentation. This result is similar to the conclusion made in the study of Shu *et al*., (2015) where it reported that an increase in the viable cell on goat milk media leads to an increase in the inoculum sizes 3, 4, 5, 6 and 7%. The amount of seed culture or inoculum size plays a significant role in the commencement of bacterial

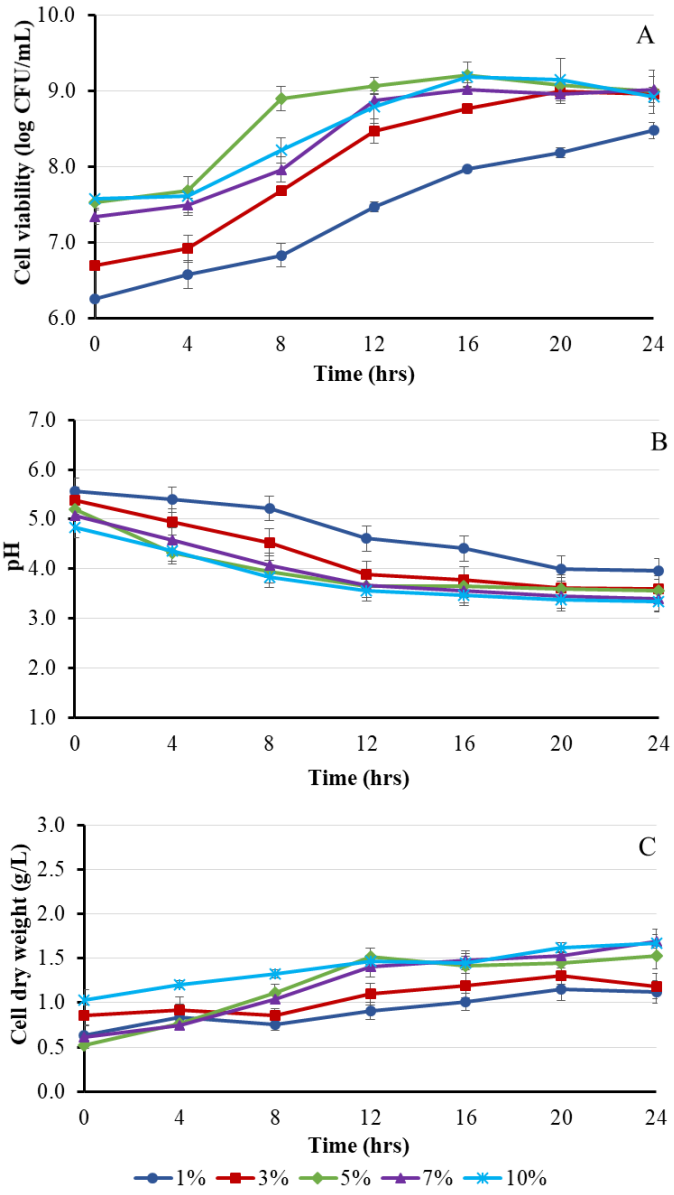

Figure 3. Cell viability (A), pH change (B) and cell dry weight (C) of *L. plantarum* TISTR 2083 with different amount of seed.

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multiplication as well as the culture condition (Baka *et al*., 2015). The amount of seed culture also affects the pH of the culture media. The initial pH with inoculum sizes of 1, 3, 5, 7 and 10% (v/v) were 5.57, 5.38, 5.20, 5.07 and 4.84 respectively. However, after 24 hrs of cultivation, the pH dropped to 3.95, 3.59, 3.55, 3.39, and 3.34, respectively. A large amount of seed culture resulted in low pH media because *L. plantarum* utilizes the glucose for growth and metabolic activity that produced the acid (Wardani *et al*., 2017). From Figure 3, the trend of cell dry weight result was also a similar pattern of viable cell count. After 12 hrs of cultivation, the cell dry weight with an inoculum size of 1, 3, 5, 7 and 10% (v/v) were 0.91, 1.10, 1.52, 1.41 and 1.47 g/L respectively. Based on the cell growth and viable cell count of *L. plantarum* TISTR 2083 in sugarcane juice media, an inoculum size of 5% was selected for further study. The 5% chosen for further study have been previously used in some research. Timbuntam *et al*. (2006) used a 5% (v/v) seed culture of *Lactobacillus* sp. for lactic acid production in sugarcane juice and Hou *et al*., (2016) used a 5% (v/v) seed culture for high vitality *L plantarum* starter culture production.

3.3 Effect of nitrogen source on growth of Lactobacillus plantarum TIRTR 2083

The higher viable cell count obtained from the media with yeast extract as the nitrogen source, 9.11 log CFU/mL after 12 hrs, where $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, peptone and MRS showed 8.76, 8.95, 8.73 and 8.50 log CFU/mL respectively (Figure 4). Yeast extract is used in culture medium for cell growth and is suitable for *Lactobacillus* cultivation and identified as the best nitrogen source for *L. plantarum* (De Carvalho *et al*., 2009; Coghetto *et al*., 2016). From Figure 4, the pH of the different culture media did not have any significant change after the 12 hrs period. This result also has a similar trend with Solval *et al*., (2019), where MRS had a pH value lower than 3.75 after 24 hrs. *Lactobacillus plantarum* has the ability to produce compounds like lactic acid, acetic acid, ethanol, and mannitol. Those compounds can lead to a decrease in the pH of the cultivation media (Camu *et al*., 2007). From Figure 4, the media containing yeast extract resulted in a higher cell dry weight of 2.35 g/L after 24 hrs, while other nitrogen sources $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and peptone were 1.53 g/L, 1.83 g/L and 1.38 g/L respectively. The use of yeast extract resulted in an improved growth rate and increased viable cells of the *L. plantarum* TISTR 2083. Therefore, yeast extract was chosen as a nitrogen source for further study.

Different concentrations of yeast extracts of 0, 0.5, 1, and 1.5% (w/v), were performed for the optimization of yeast extract concentration. From the overall result (Figure 5), the highest viable cell was achieved using 1.5% yeast extract between 8-16 hrs of the incubation period (9.03-9.24 log CFU/mL), while the lowest viable cell count was recorded for the culture media without yeast extract (0%). From Figure 5, the pH of culture media with different concentrations of yeast extract was lower than the media without yeast extract. At the same time, the cell dry weight increased with an increase in yeast extract concentration. After 24 hrs of cultivation, the cell dry weight of sugarcane juice culture media contained 0, 0.5, 1, and 1.5% (w/v) yeast extract, respectively 1.52, 2.35, 2.63, and 2.83 g/L respectively. Yeast extract has a positive influence on Lactobacillus growth as it serves nitrogen, vitamin, and mineral sources needed to satisfy the growing requirements of microorganisms (Timbuntam *et al*., 2006; Li and Ma, 2014). Moreover, from Figures 4 and 5, respectively, the growth and viable cell of *L. plantarum* TIRTR 2083 was

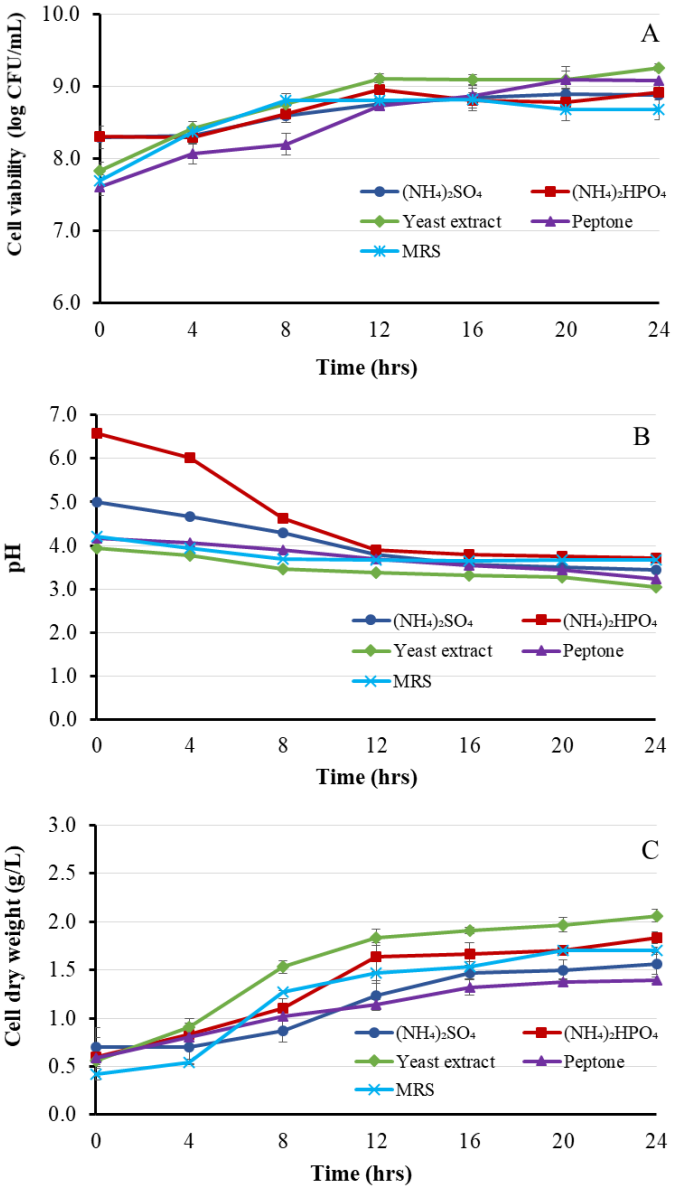

Figure 4. Cell viability (A), pH change (B) and cell dry weight (C) of *L. plantarum* TISTR 2083 with the different nitrogen source.

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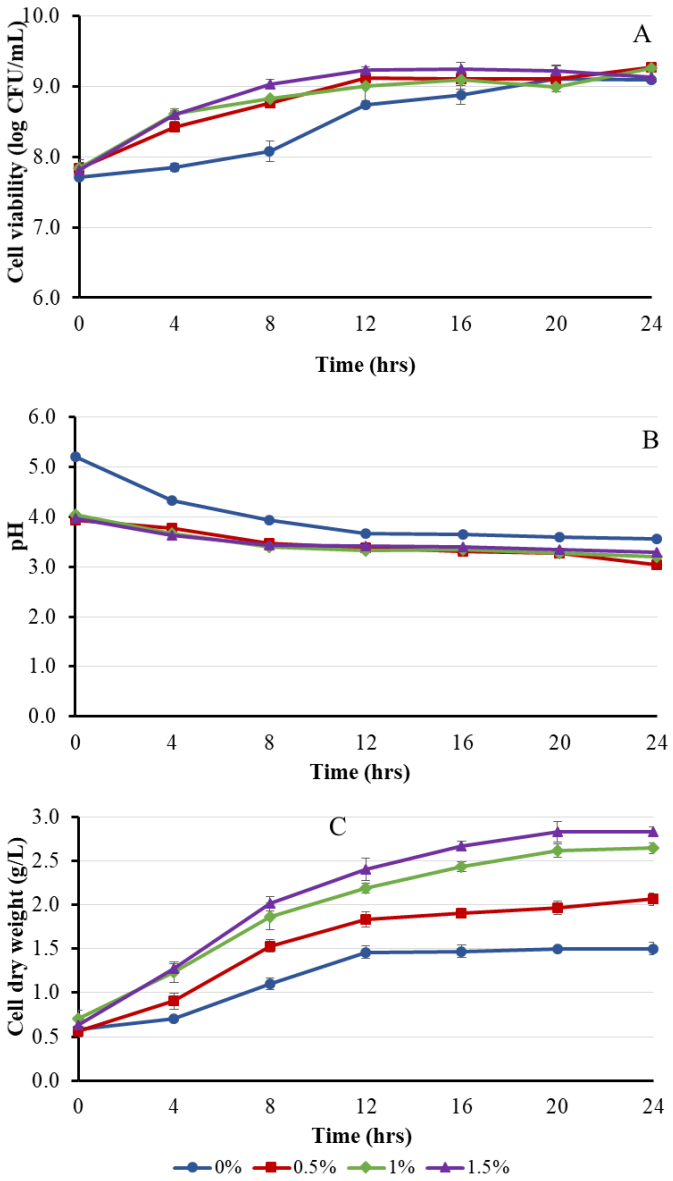

Figure 5. Cell viability (A), pH change (B) and cell dry weight (C) of *L. plantarum* TISTR 2083 with different amount of yeast extract.

favoured at a higher concentration of yeast extract. Thus, 1.5% yeast extract was chosen for the next step of the experiment.

3.4 Effect of shaking speed on growth of Lactobacillus plantarum TIRTR 2083

The effect of agitation speed was also observed since other factors to optimize the sugarcane juice resulted in better growth of *L. plantarum* TISTR 2083. The highest viable cell count was recorded at 100 rpm agitation speed, which was increased to 9.03 log CFU/mL after 8 hrs and 9.24 log CFU/mL after 12 hrs incubation. While the lowest growth was recorded for the treatment at 0 rpm or without agitation (Figure 6). Agitation plays a vital role in mixing and shearing during microbial culture. Agitation further helps the growth of culture on account of additional ATP generation by allowing aeration or oxygen supply and enhancing fluid-to-particle mass transfer, which results in higher biomass production (Mustafa *et al*., 2019). Therefore, the result also shows a similar conclusion of the cell growth pattern of *L. plantarum* TISTR 2083 rose as the agitation speed was increased from 0 to 100 rpm. On the other hand, excessive agitation speed could damage the cell physically, raise oxidative stress, and sometimes oxygen inhibition related to superoxide. These might be the reason for low viable cells but higher biomass (Fu and Mathews, 1999; Gupta *et al*., 2011). As a result, the cell dry weight of 150 rpm treatment was higher than other speeds, while cell viability of 150 rpm was not the highest one (Figure 6). The pH of all treatments had a similar pattern of slightly decreasing with time, while the maximum decrease was 100 rpm treatment from pH 3.9 to pH 3.2. The decrease of pH or increasing acidic environment occurred because LAB utilizes the carbon source for its growth and converts it to lactic acid as a fermentation process, ensuring microbial cell stability

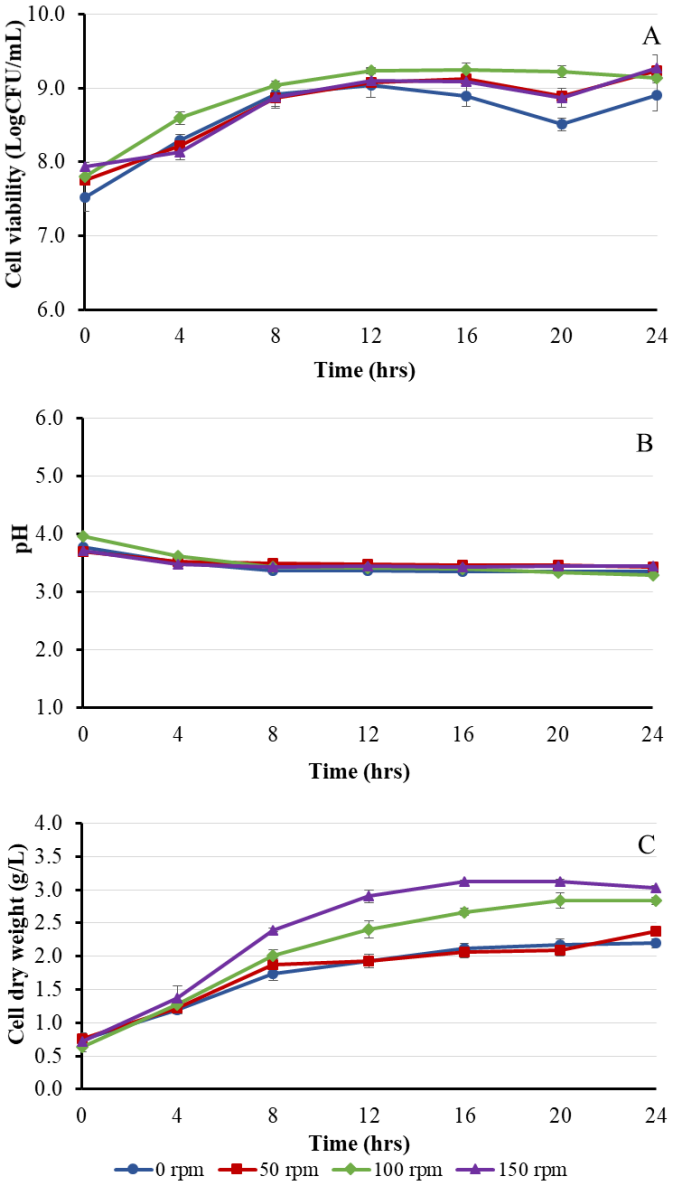

Figure 6. Cell viability (A), pH change (B) and cell dry weight (C) of *L. plantarum* TISTR 2083 with different shaking speed.

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(Gupta *et al*., 2011).

3.5 High cell density cultivation of Lactobacillus plantarum TIRTR 2083 in a fermenter

Optimized sugarcane juice culture medium was used for high cell density cultivation and the overall optimized condition of different individual variables (Table 2) was applied to scale up in this study. The growth curves of *L. plantarum* TISTR 2083 were determined in the batch culture fermentation, which includes viable cell counts (log CFU/mL), pH value and cell dry weight (g/L) (Figure 7). The cell growth was increased with time by the consumption of sugar present in culture media resulting in the fermentation of sugarcane juice. The viable cell count during the log phase increased to 9.07 log CFU/mL after 8 hrs and 9.15 log CFU/mL after 12 hrs. During the stationary phase, the maximum viable cell count was 9.22 log CFU/mL at 20 h of fermentation. It was observed that the pH value of the culture media decreased from its initial pH value to pH 3.27 during the fermentation period. The decrease in pH is likely due to a large amount of lactic acid production during fermentation.

After maximum cell growth was achieved, the cell growth was decreased at the death phase (at 24 hrs of fermentation). From the result of cell dry weight, it is clearly seen that the cell dry weight was increased gradually from the initial to the end of fermentation. The cell dry weights during the log phase were 1.65 g/L (after 8 hrs) and 1.9 g/L (after 12 hrs). The maximum dry weight was 2.35 g/L (after 24 hrs) during the death phase, indicating that cell lysis and subsequent cell death occurred. During the high cell density cultivation of *L. plantarum* TISTR 2083, the cell growth will be arrested and the culture condition enters into the stationary phase. That can happen for a lack of nutrients and a hostile environment for microorganisms like the production of organic acids. The growth was decreased during the decline phase and the pH value descent during fermentation suggested that the sugar was mostly used

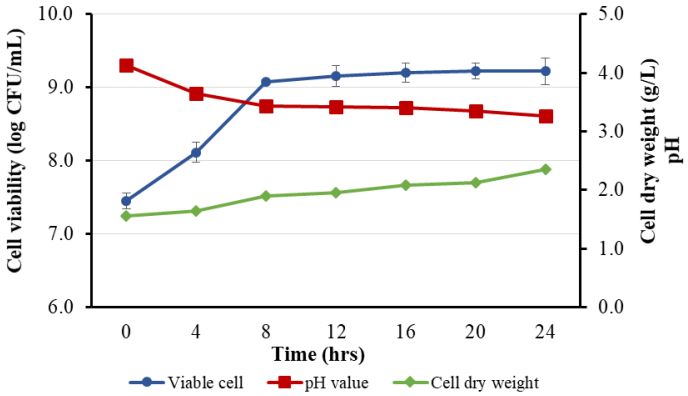

Figure 7. The growth of *L. plantarum* TISTR 2083, pH change and cell dry weight in sugarcane juice medium in fermenter.

Table 2. Trial of all independent variables for optimization.

Trial	A	B	C	D	E	F	G	VCC
1	5	5	0	0	0	0	100	8.72
2	10	5	0	0	0	0	100	8.85
3	15	5	0	0	0	0	100	8.91
4	20	5	0	0	0	0	100	9.07
5	20	1	0	0	0	0	100	7.47
6	20	3	0	0	0	0	100	8.47
7	20	5	0	0	0	0	100	9.07
8	20	7	0	0	0	0	100	8.87
9	20	10	0	0	0	0	100	8.79
10	20	5	0	0	0	0	100	9.07
11	20	5	0.5	0	0	0	100	8.76
12	20	5	0	0.5	0	0	100	8.96
13	20	5	0	0	0.5	0	100	8.74
14	20	5	0	0	0	0.5	100	9.12
15	20	5	0	0	0	0	100	9.07
16	20	5	0	0	0	0.5	100	9.12
17	20	5	0	0	0	1	100	9.00
18	20	5	0	0	0	1.5	100	9.24
19	20	5	0	0	0	1.5	0	9.04
20	20	5	0	0	0	1.5	50	9.07
21	20	5	0	0	0	1.5	100	9.24
22	20	5	0	0	0	1.5	150	9.10

A: sugar content (°Bx), B: seed culture (%), C: $(NH_4)_2SO_4$, D: $(NH_4)_2HPO_4$, E: peptone, F: yeast extract (w/v), G: agitation (rpm), VCC: viable cell count (log CFU/mL).

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for biomass growth and lactic acid production (Xiong *et al*., 2011; Hou *et al*., 2016).

4. Conclusion

The optimum sugar concentration of Sugarcane juice was 20°Bx, which produced the highest viable cell of *L. plantarum* TIRTR 2083 (9.24 log CFU/mL, after 12 hrs). The inoculum size of 5% was employed to maximize cell growth. In regards to improving the cultural condition of sugarcane juice, 1.5% yeast extract resulted in high population growth. The agitation speed of 100 rpm showed a higher viable cell growth with optimized culture conditions. Optimized sugarcane juice, as a cheap raw material, can be used as a low-cost medium for further high-density cultivation to produce dried starter culture of *L. plantarum* TIRTR 2083.

Conflict of interest

The authors declare no conflict of interest.

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