Asia-Pacific Journal of Science and Technology :Volume :30 Issue :04 Article ID :APST-30-04-05 Research Article

APST

Asia-Pacific Journal of Science and Technology

https//:www.tci-thaijo.org/index.php/APST/index

Published by Research and Innovation Department,
Khon Kaen University, Thailand

Screening for high-purity L-lactic acid-producing thermotolerant *Bacillus* capable of utilizing sucrose and starch in an energy-efficient production process

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Received 1 July 2024
Revised 14 November 2024
Accepted 3 December 2024

Abstract

This study aimed to isolate and characterize thermotolerant *Bacillus* strains capable of utilizing sucrose and starch for efficient production of optically pure L-lactic acid. High-purity L-lactic acid is a valuable bio-based chemical with applications in bioplastics and other industries. Thailand's abundant sucrose and tapioca starch byproducts present an opportunity to produce L-lactic acid from low-cost feedstocks using thermotolerant bacteria like *Bacillus coagulans*. Twenty-eight thermotolerant *Bacillus* isolates were screened for their capability to grow on glucose, sucrose, and cassava starch at 50 °C. Four strains (NF17, N24A2t, NF11, N47B2) could utilize all three carbon sources. These were further evaluated for growth, acid production profile, and L-lactic acid yield. Strain NF11, identified as *B. coagulans*, produced the highest L-lactic acid titter, 108 g/L from molasses, with a yield of 1.12 g/g at 50 °C under aerobic conditions. The effects of aeration, initial sugar concentration and sterilization method on L-lactic acid production were investigated using the NF11 strain. Moderate aeration at 100 rpm improved sugar utilization and lactic acid productivity over that of static conditions. An initial 100 g/L total sugar concentration from molasses was optimal. Remarkably, NF11 could produce high L-lactic acid titers without conventional heat sterilization, using only chemical sterilization with potassium metabisulfite or no pretreatment at all. This energy-efficient bioprocess employing a thermotolerant *B. coagulans* strain enables valorization of sugary byproducts like molasses into optically pure L-lactic acid, a valuable bio-based chemical feedstock. Eliminating energy-intensive pasteurization/sterilization offers significant cost advantages for large-scale L-lactic acid biorefineries.

Keywords: *Bacillus coagulans*, Energy-saving bioprocess, optically pure lactic acid, Sucrose utilizing *Bacillus*

1. Introduction

Global warming is an issue that increasingly affects all forms of life, especially humans. Its impacts include the emergence of warm-climate pathogens, water scarcity, and unpredictable weather patterns. These challenges have spurred global awareness and mitigation efforts. The primary cause is atmospheric carbon dioxide accumulation, exacerbated by ongoing human-induced pollution. Plastic waste, which is difficult to decompose naturally, presents a significant problem. Incineration as a disposal method further contributes to environmental pollution. Consequently, there is growing interest in biodegradable polymers derived from natural materials [1].

Various bioplastics are now used as environmentally friendly alternatives to plastics derived from fossil fuel. Polylactic acid (PLA) has gained attention in agriculture and packaging due to its availability, suitable chemical

properties, and excellent biodegradability in natural environments like soil or compost [2]. PLA is synthesized by fermenting agricultural raw materials into lactic acid, which is then chemically processed into a polymer. Lactic acid production involves lactic acid bacteria (e.g., *Lactobacillus* spp.) and fungi (e.g., *Aspergillus* spp.), digesting starch or sugar for energy. These microorganisms can directly utilize sugar or starch without prior hydrolysis [3]. The lactic acid yield varies based on the nutrient composition, especially the carbon and nitrogen content [4].

Sakai et al. [5] demonstrated *Bacillus coagulans*' potential in lactic acid production, achieving 97% L-lactic acid purity and a yield of 86 g/L (using 74% of the total sugars) from an unsterilized community food waste with no optimization. *B. coagulans* offers several advantages over traditional lactic acid producers like *Lactobacillus*, *Lactococcus*, and *Aspergillus* for industrial applications. It thrives at temperatures up to 65 °C [5-12], produces optically pure L-lactic acid [9,11,13,14], and forms endospores for easy maintenance [7,15]. As a facultative anaerobe, it ferments various substrates including xylose, starch, and cellobiose under both aerobic and anaerobic conditions [7,16,17]. It selectively produces L-lactic acid from xylose without growth inhibition at high concentrations [17]. Some strains can directly ferment unsterilized food waste [7,18], potentially reducing production costs.

These properties make *B. coagulans* an attractive candidate for L-lactic acid production, due to its product purity and thermotolerance. High-temperature fermentation is advantageous in the context of global warming and offers several benefits over mesophilic processes. It prevents contamination by mesophilic microorganisms [12,19,20], potentially eliminates the need for active temperature control [12,19] and avoids viscosity issues in high-starch feedstocks [12,20]. It also circumvents the formation of microbial inhibitors from conventional sterilization [3]. Using thermophilic microorganisms can simplify bioprocessing and expand the range of usable low-cost feedstocks. However, *B. coagulans* strains vary in their capability to metabolize different nutrient sources. For instance, *B. coagulans* M21, previously used for lactic acid production from food waste [10], performed poorly with sugarcane juice, likely due to ineffective sucrose metabolism.

The advantages of *Bacillus coagulants* in lactic acid production have been reported [6-12,18]. Application of this microorganism for lactic acid production using molasses and cassava pulp, is of significant interest. Molasses, a by-product of the sugar industry, and cassava pulp, a waste material from tapioca starch production, represent abundant and inexpensive raw materials for lactic acid fermentation. Utilization of these waste streams for lactic acid production not only provides an environmentally friendly solution for waste management but also adds value to the sugar and tapioca industries. Applying *B. coagulants* M21, which can utilize food waste for lactic acid production according to Tongpim et al. [10], revealed that lactic acid production from sugar cane juice and molasses was unsuccessful. This was possibly because this strain could not utilize sucrose, which is the primary sugar in sugarcane juice. A *B. coagulans* strain capable of utilizing both sucrose (the main sugar in molasses) and cassava starch would be valuable for adding value to these raw materials. However, few studies have focused on screening for thermotolerant *Bacillus* strains with this dual metabolic capability.

This research aims to screen thermotolerant *Bacillus* strains from the stock culture of Assoc. Prof. Dr. Saowanit Tongpim for their capability to metabolize both sucrose and starch. The selected strains will be further characterized for low-cost and energy-efficient lactic acid production using molasses as a feedstock. This approach can be used to develop an economically viable and environmentally friendly process for lactic acid production, contributing to the broader goal of developing sustainable bioplastic manufacturing.

2. Materials and methods

2.1 Microorganisms and culture conditions

Thermotolerant Bacillus strains were kindly provided from stock cultures by Assoc. Prof. Dr. Saowanit Tongpim, the Department of Microbiology, Faculty of Science, Khon Kaen University, Thailand. One milliliter of each stock culture of thermotolerant Bacillus strains was transferred into 100 mL of Glucose Yeast Extract Peptone (GYP) medium (2% glucose, 0.5% yeast extract, 0.5% peptone [15]) and incubated at 50 °C and 100 rpm (Shaking incubator, Model VS-8480SFN, Vision Scientific, Korea) for 24 h. This culture was reactivated in the same medium until reaching the exponential growth stage (18-24 hours) before use as inoculum in further experiments.

2.2 Molasses preparation

Molasses was kindly supplied by the Mitr Phol Phu Wiang Sugar Factory, Khon Kaen, Thailand. It was diluted with distilled water in a 1:1 ratio before filtering through a plate and frame filter press (Type 20/17, Seitz Enzinger Noll, Germany) to remove sediment before use. The total sugars concentration was analyzed using a phenol-sulfuric acid method [21]. The total sugars level of the molasses was 75.04 g/100 g or about 75% (w/w).

2.3 Selection of lactic acid-producing thermotolerant Bacillus strains capable of utilizing glucose, sucrose, and starch

Twenty-eight isolates of thermotolerant Bacillus strains from stock cultures, as discussed earlier in session 2.1, shown in Table 1, were used in the selection step. A 20 µl inoculum of these isolates was dropped onto agar plates containing 1% of $CaCO_3$ and 1% of various carbon sources, glucose, sucrose, and cassava starch, and incubated at 50 °C. After 24 h, growth and acid formation were reported from the colony appearance and clear zones around the colonies. For the cassava starch plate, the clear zones were visualized by staining with an iodine solution.

Table 1 Twenty-eight isolates of thermotolerant Bacillus used in the screening step.

1)	NF17	8)	N26A2.2.2	15)	N52AM	22)	N50At
2)	SD27	9)	NF11	16)	N58A1M	23)	N39At
3)	SD9	10)	N61AO	17)	NF15sl	24)	NF5
4)	SF1	11)	N47B2	18)	SD10	25)	N15t
5)	T191TP1	12)	N46At	19)	N54At	26)	N59At
6)	N61At	13)	N24BM	20)	N43A2t	27)	N38At
7)	N24A2t	14)	N58A2t	21)	N53At	28)	N28A3.2

2.4 Growth and acid production of the selected strains

Selected strains were evaluated for their growth and acid production to obtain the best bacterium for further studies. One percent seed of the selected strain, which was taken from a culture having an optical density at 660 nm (OD_{660}) of 0.6, was transferred to a production medium (modified from [7], PD medium consisting of glucose 2%, yeast extract 1.5%, KH_2PO_4 0.1%, $(NH_4)_2SO_4$ 0.1% $MgSO_4.7H_2O$ 0.02%, $MnSO_4.4H_2O$ 0.005% and $FeSO_4.7H_2O$ 0.001%) and incubated under static conditions at 50 °C. Samples were collected every 4 h for 24 h. The samples were evaluated for growth using turbidity at a 660 nm wavelength. The total acidity of the sample supernatant was measured by titration [22]. Lactic, formic, and acetic acids were quantified using HPLC equipped with an Aminex HPX-87H column and UV detector operating at 210 nm using 5 mM H_2SO_4 as a mobile phase at a 0.6 mL/min flow rate and 40 °C [7].

2.5 Effect of agitation on growth and lactic acid production of the selected strain

The selected strain was inoculated in culture broth containing molasses with a total sugars concentration of 120 g/L, 20 g/L of yeast extract, and 60% of $CaCO_3$ based on the sugar concentration with an initial pH of 6.4 [17]. The working volume was 150 mL with an inoculum size of 2% in a 250 mL Erlenmeyer flask. All cultures were incubated at 50 ºC for 72 h under 3 different agitation conditions, shaking continuously at 100 rpm, shaking for 10 min prior to sampling, and shaking for 10 min once every 24 h. Samples were taken every 6 h for the first 24 h and after that collected every 12 h until the end of the experiment, except those shaken once every 24 h. In this case, samples were taken after shaking. The experiments were conducted in duplicate. In each experiment, 5 mL of the samples were taken and centrifuged at 10,000 rpm (KINTARO-18, TOMY, Japan) for 5 min. Supernatant pH was measured using a pH meter (LAQUAtwin, HORIBA, Japan). Total sugars were determined using a phenol-sulfuric acid method [21], and lactic acid by HPLC [7].

2.6 Effect of sugar concentration on growth and lactic acid production of the selected strain

The selected Bacillus strain was cultured in the same culture broth composition and conditions as the previous experiment except the total sugars concentration of molasses was varied to 25, 50, 100, and 150 g/L. All cultures were incubated at 100 rpm, 50 ºC for 72 h with samples taken every 6 h for analysis during the first 24 h and then every 12 h until the end of the experiment. Samples were analyzed for total sugars and lactic acid concentration as previously described.

2.7 Energy saving of lactic acid fermentation by the selected Bacillus

In order to reduce energy costs associated with medium sterilization, four different processes were evaluated which were sterilization (110 ºC for 28 min), pasteurization (80 ºC for 15 min), chemical sterilization overnight (200 ppm of potassium metabisulfite or KMS), and unsterilized media. The culture broth contained molasses with 100 g/L of total sugars, 20 g/L of yeast extract, and 60% $CaCO_3$ based on the sugar concentration with an initial pH of 6.4. A 2% inoculum was added and the culture incubated at 50 ºC and 100 rpm for 72 h. Samples were analyzed for total sugars and lactic acid concentrations.

2.8 L- and D- Lactic acid analysis

The optical form of lactic acid was analyzed by HPLC using a Sumiciral OA5000 (UV254) column with 1 mM $CuSO_4$ as the mobile phase at a 1 mL/min flow rate [7]. The standard L- and D-lactic acid concentrations were 20 g/L. Sample supernatants were mixed with standard L- and D-lactic acid in the ratio of 1:1 before injection.

3. Results and discussion

3.1 Selection of lactic acid-producing thermotolerant Bacillus strains capable of utilizing glucose, sucrose, and starch

Twenty-eight isolates of acid-producing thermotolerant Bacillus were obtained from the stock cultures of Assoc. Prof. Dr. Saowanit Tongpim, as shown in Table 1. Three substrates (glucose, sucrose, and starch) were used to select the desired strains which were indicated by positive results for growth and acid production at 50 °C. The results are shown in Table 2. From the experimental results in this table, all tested Bacillus strains could use glucose as a carbon source to grow at high temperatures. However, it was found that there were only 13 Bacillus strains that could form clear zones around the colonies on glucose agar plates, including NF17, SF1, T191TP1, N61At, N24A2t, NF11, N47B2, N46At, N52AM, SD10, N43A2t, NF5, and N38At. These 13 strains, thus, could use glucose as a carbon source to grow and produce acid. Among these strains, only 4, NF17, N24A2t, NF11, and N47B2 could utilize glucose, sucrose, and cassava starch for growth and acid production at high temperatures. These 4 strains, thus, were used in a further selection step.

Table 2 Growth and acid formation of the thermotolerant *Bacillus* strains on the test media.

Strain	Glucose agar plate		Sucrose agar plate		Cassava starch agar plate	
	Growth	clear zone	Growth	clear zone	Growth	clear zone
NF17	+	+	+	+	+	+
SD27	+	-	+	-	+	-
SD9	+	-	-	-	-	-
SF1	+	+	+	-	+	+
T191TP1	+	+	+	-	+	+
N61At	+	+	+	-	+	-
N24A2t	+	+	+	+	+	+
N26A2.2.2	+	-	+	-	+	-
NF11	+	+	+	+	+	+
N61AO	+	-	+	-	+	-
N47B2	+	+	+	+	+	+
N46At	+	+	+	-	+	-
N24BM	+	-	+	-	+	-
N58A2t	+	-	+	-	+	-
N52AM	+	+	-	-	-	-
N58A1M	+	-	+	-	+	-
NF15sl	+	-	+	-	+	-
SD10	+	+	-	-	-	-
N54At	+	-	+	-	+	-
N43A2t	+	+	+	+	+	-
N53At	+	-	+	-	+	-
N50At	+	-	+	-	+	-
N39At	+	-	+	-	+	-
NF5	+	+	+	-	+	+
N15t	+	-	+	-	+	-
N59At	+	-	+	-	+	-
N38At	+	+	+	-	+	+
N28A3.2	+	-	+	-	+	-

+ indicates growth or clear zone formation around colonies growing on agar plates
- indicates no growth or no clear zone formation around colonies growing on agar plates

3.2 Growth and acid production of the selected strains

Four strains that could utilize glucose, sucrose, and cassava starch, were evaluated for their growth and acid production to select the best one for further studies. These results are shown in Figures 1A-D. The growth patterns and acid production of all 4 *Bacillus* strains showed the same trend. All four *Bacillus* strains could grow directly with a short lag phase and entered the stationary phase after 8 hours. Acid production correlated with these growth patterns. The total acids produced by *Bacillus* strains NF11, NF17, N24A2t, and N47B2 were 4.1, 4.5, 4.1, and 4.1 g/L, respectively.

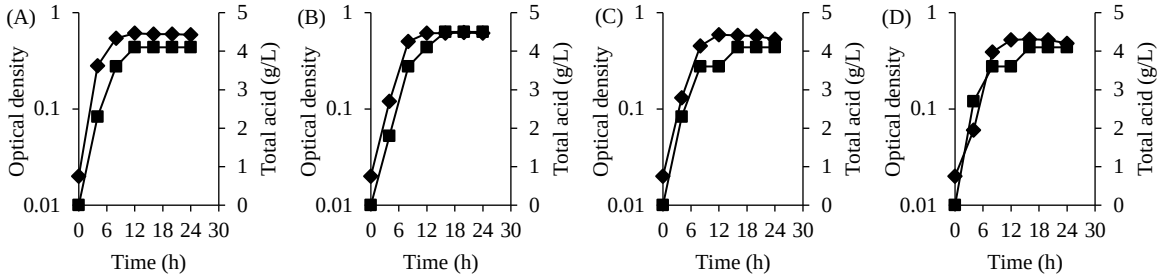

Figure 1 Growth (♦) and total acid concentration (■) of *Bacillus* NF11 (A), NF17 (B), N24A2t (C) and N47B2 (D).

Acid types were also evaluated using HPLC at 24 and 48 h. These results are shown in Figure 2. At 24 h, all strains produced more than one type of acid, with lactic acid production being the most prevalent. At 48 h, however, the amount of acetic acid significantly increased. It is possible that when the primary carbon source was exhausted, lactic acid was converted into other acids [23,24]. In this case, formic and acetic acid were produced. Therefore, fermentation time should not be excessive, and research should be done to determine the appropriate

harvesting time. Interestingly, strain NF 11 still showed the highest lactic acid concentration at 48 h. Thus, this strain was used for further studies. It was identified by conventional methods and API 50 CHB test kits as *Bacillus coagulants*, similar to NF 17, as reported earlier [25]. The molecular identification techniques, however, have to be confirmed.

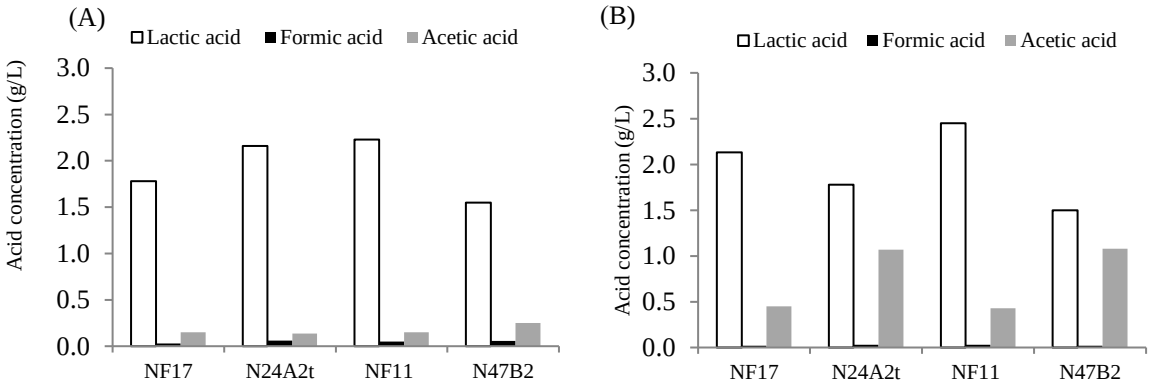

Figure 2 Concentration of each acid produced by *Bacillus* NF17, N24A2t, NF11 and N47B at 24 (A) and 48 h (B).

3.3 Effect of agitation on lactic acid production of the selected strain

Several studies investigated the influence of agitation on the growth and lactic acid production of *B. coagulans* [8,9,26]. Agitation plays a crucial role in fermentation processes, driving dissolved oxygen levels and mass transfer within the fermentation medium. Three different agitation conditions described in the Materials and Methods section were evaluated. The results are presented in Figure 3 and Table 3.

The experimental results in Figure 3A indicate that the average initial pH for all agitation conditions ranged from 6.1 to 6.4. As fermentation progressed, pH decreased across all conditions due to acid production. After 72 h, the pH values were 5.0, 5.7, and 5.8 for the three conditions. Sugar utilization (Figure 3B) is correlated with acid production, with the 100-rpm continuous shaking condition exhibiting faster sugar utilization compared to intermittent shaking (at sampling times) and 24-hour interval shaking.

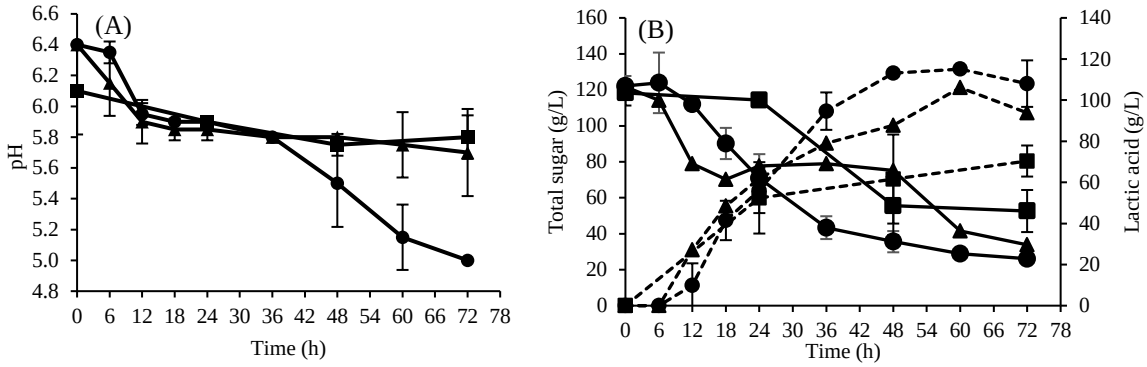

Figure 3 (A) pH and (B) total sugars (solid lines) and lactic acid (dashed lines) concentrations with shaking at 100 rpm (●), before sampling (▲) and every 24 h (■).

Table 3 presents the yield and productivity of lactic acid production by *Bacillus* NF11 under various agitation conditions at 72 h. The sugar consumed in each condition was 96.2, 58.2, and 65.7 g/L, while lactic acid production was 108.0, 61.9, and 70.4 g/L, respectively, for shaking at 100 rpm, shaking before sampling and shaking every 24 h. Corresponding yield values were 1.12, 1.06, and 1.07 g/g, with production rates of 1.5, 0.86, and 0.98 g/L.h, respectively.

The 100-rpm continuous shaking condition resulted in higher sugar utilization and lactic acid production compared to intermittent and 24-h interval shaking. This agrees with Kotzamanidis et al. [27], who reported superior lactic acid production in shaken flasks (150 rpm) versus static conditions when fermenting beet sugar. Continuous shaking likely enhances nutrient diffusion into cells, promoting faster growth and shorter fermentation times. *Bacillus* NF11 demonstrated optimal growth and sugar utilization for lactic acid production under continuous agitation. The agitation rate significantly influenced lactic acid production, with continuous shaking at 100 rpm yielding the best results. Consequently, this agitation rate at 50 °C incubation and 60% calcium carbonate addition (relative to the total sugar concentration) were selected for subsequent studies. The lactic acid yield surpassed theoretical predictions, likely due to the diverse nutrient profile of molasses [27]. Molasses contains various sugars beyond just sucrose, including glucose, fructose, and other oligosaccharides. It also contains organic acids and amino acids. These additional carbon sources can be metabolized to produce lactic acid, contributing to yields that exceed calculations based solely on the primary sugar content.

Table 3 Yield and productivity at 72 h of lactic acid production by *Bacillus* NF11 under various agitation conditions

Agitation condition	Consumed sugar (g/L)	Lactic acid (g/L)	Yield (g/g)	Productivity (g/L.h)
Shaking 100 rpm	96.15 ± 2.03	108.02 ± 2.16	1.12 ± 0.02	1.50 ± 0.03
Shaking before sampling	58.16 ± 1.16	61.86 ± 0.43	1.06 ± 0.01	0.86 ± 0.01
Shaking every 24 h	65.65 ± 2.50	70.36 ± 0.76	1.07 ± 0.03	0.98 ± 0.01

3.4 Effect of initial sugar concentration on lactic acid production of the selected strain

Substrate concentration significantly influenced cell growth and product formation. The impact of initial molasses concentration on lactic acid production by *Bacillus* NF11 was investigated, with these results presented in Figure 4 and Table 4.

The total sugars content decreased over time for all tested concentrations. At lower sugar concentrations (25 and 50 g/L), sugars were almost completely exhausted after 36 h. However, at higher concentrations (100 and 150 g/L), sugar utilization was incomplete, with residual sugars remaining at the end of the experiment (72 h), especially at 150 g/L.

Bacillus NF11's sugar utilization appeared to decrease with greater molasses concentrations. This phenomenon may be attributed to osmotic pressure effects, as reported by Roukas [28]. Higher sugar concentrations can reduce water activity due to water loss, causing cell shrinkage and consequently decreasing sugar utilization.

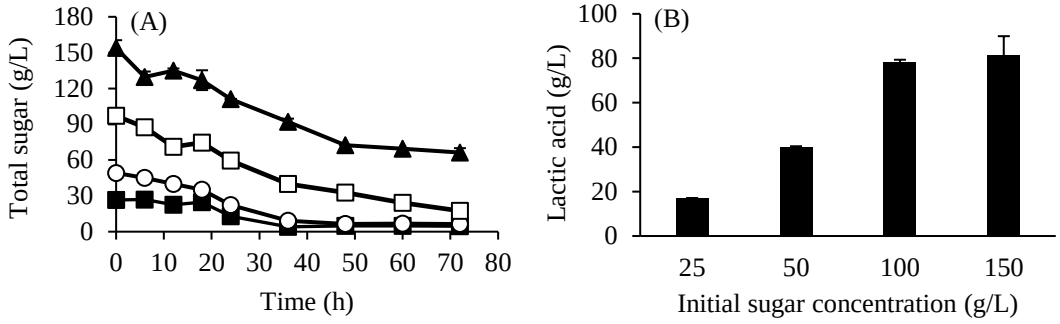

Figure 4 Time course of total sugar levels of each initial sugar concentration (g/L: 25 (■), 50 (○), 100 (□) and 150 (▲)) (A) and lactic acid concentration at 72 h (B).

Figure 4 and Table 4 show that at molasses concentrations of 100 and 150 g/L, lactic acid concentrations reached 78 and 81.11 g/L, respectively. The corresponding yields were 0.98 and 0.93 g/g, with production rates of 1.08 and 1.13 g/L.h. These results were not significantly different despite the 50% difference in initial sugar concentrations. Based on these findings, an initial total sugar concentration of 100 g/L and 3.5% yeast extract as a nitrogen source were determined to be optimal for lactic acid production by *Bacillus* NF11.

Table 4 Yield and productivity of lactic acid production of *Bacillus* NF11 at 72 h of each tested initial sugar concentration

Initial sugar (g/L)	Consumed sugar (g/L)	Lactic acid (g/L)	Yield (g/g)	Productivity ($g/L/h^{-1}$)
25	22.12 ± 0.25	16.90 ± 0.17	0.76 ± 0.08	0.24 ± 0.03
50	42.83 ± 1.58	40.00 ± 0.40	0.93 ± 0.02	0.56 ± 0.01
100	79.61 ± 3.96	78.01 ± 1.34	0.98 ± 0.07	1.08 ± 0.02
150	87.57 ± 0.25	81.11 ± 0.81	0.93 ± 0.01	1.13 ± 0.12

However, to fully optimize lactic acid production by *Bacillus* NF11, further investigation is required. Additional factors that need examination include a broader range of initial sugar concentrations, alternative nitrogen sources and concentrations, and varying initial pH levels. These studies will provide a more comprehensive understanding of the optimal conditions for lactic acid production. The results of these investigations, along with their application at a larger scale, will be reported in future work.

3.5 Energy savings of lactic acid fermentation by the selected Bacillus

Traditional lactic acid (LA) fermentations typically involve energy-intensive steps such as medium sterilization and cooling, which contribute significantly to overall production costs [3,29,30]. However, alternative approaches to medium sterilization, such as chemical methods similar to those used in wine fermentation, could potentially reduce energy costs. Moreover, given that *Bacillus* NF11 thrives at high temperatures, it may outcompete other microorganisms during fermentation. This suggests the possibility of using unsterilized or minimally treated media, such as pasteurization to eliminate heat-sensitive microorganisms, for lactic acid production. These media preparation methods, instead of sterilization before inoculation of the starter, could reduce energy costs.

An experiment was conducted comparing four media preparation methods to evaluate these energy-saving approaches. This included moist heat sterilization, pasteurization, chemical sterilization using potassium metabisulfite (KMS), and no sterilization. Figures 5 and 6 present the pH, total sugars level, and lactic acid concentrations in these experiments.

Figure 5 shows that total sugar concentrations (A) and pH values (B) decreased over time under all four conditions, indicating sugar conversion to acid. After 72 h, 46.0, 39.6, 57.3, and 44.6 g/L of total sugars were consumed, while lactic acid production reached 67.0, 63.5, 69.3, and 66.1 g/L for the treatments shown in Figure 6. Notably, lactic acid concentrations did not differ significantly across the four conditions. The incomplete sugar consumption observed in all treatments may be attributed to the inoculum not being in an active state. Consequently, a longer fermentation time might be required to achieve complete sugar utilization.

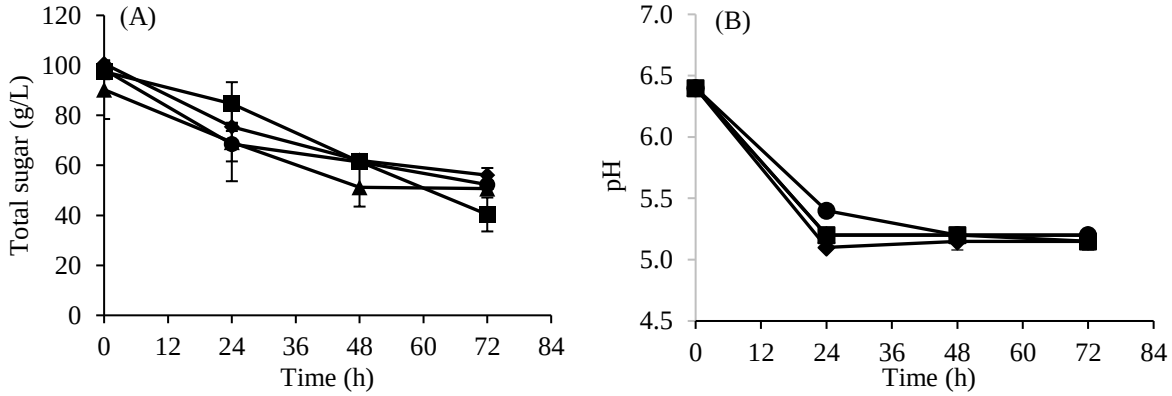

Figure 5 Time course of total sugar consumed (A) and pH (B) of each tested sterilization condition. Sterilization (●), pasteurization (▲), chemical sterilization (■), and unsterilized media (♦).

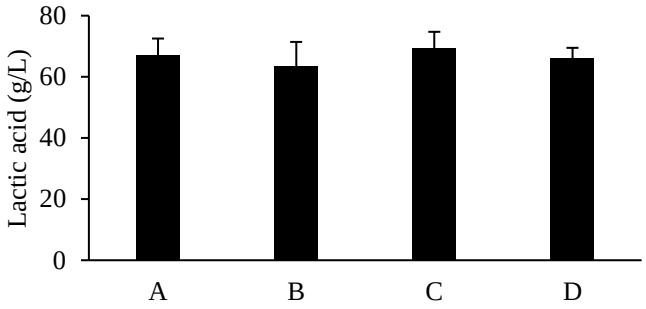

Figure 6 Lactic acid concentration at 72 h of each tested sterilization condition. (A) Sterilization, (B) pasteurization, (C) chemical sterilization, and (D) unsterilized media

These results suggest that chemical sterilization or even unsterilized molasses could be viable alternatives to energy-intensive sterilization or pasteurization methods for lactic acid production using thermotolerant *Bacillus* NF11. This is advantageous since high-temperature sterilization may cause the breakdown of important nutrients or minerals in the raw materials, while also requiring costly equipment, along with high energy consumption and greater operating costs.

The capability to produce lactic acid without sterilizing the raw materials is especially valuable for low-cost production processes aiming to maintain or increase L-lactic acid yields [4]. Similar approaches using unsterilized raw materials have been reported for lactic acid production from various substrates, including food waste [7,15], [18,31,32], rice bran [11], excess sludge [9], and cheese whey powder [33].

3.6 Type of lactic acid produced

Lactic acid exists in two isomeric forms, D- and L-lactic acid. Chemical synthesis typically produces a mixture of both forms, complicating downstream processing and separation. In contrast, fermentation can yield high-purity D- or L-lactic acid. HPLC analysis was conducted by comparing the fermentation supernatant with D- and L-lactic acid standards to determine the isomeric form produced by *Bacillus* NF11.

Figure 7 presents experimental HPLC results. The samples had a retention time of 8.563 minutes, while the D-lactic acid standard showed a retention time of 9.711 minutes (Figure 7(A)), indicating that the sample was not D-lactic acid. Conversely, Figure 7(B) revealed that both the sample and L-lactic acid standard had identical retention times of 8.228 minutes, confirming that *Bacillus* NF11 produces L-lactic acid.

This finding aligns with other studies on lactic acid production by *B. coagulans* [9,13,14]. Production of pure L-lactic acid simplifies purification. Wang et al. [34] reported that NAD-dependent lactate dehydrogenases play a crucial role in high optical purity L-lactic acid production by thermophilic *B. coagulans*.

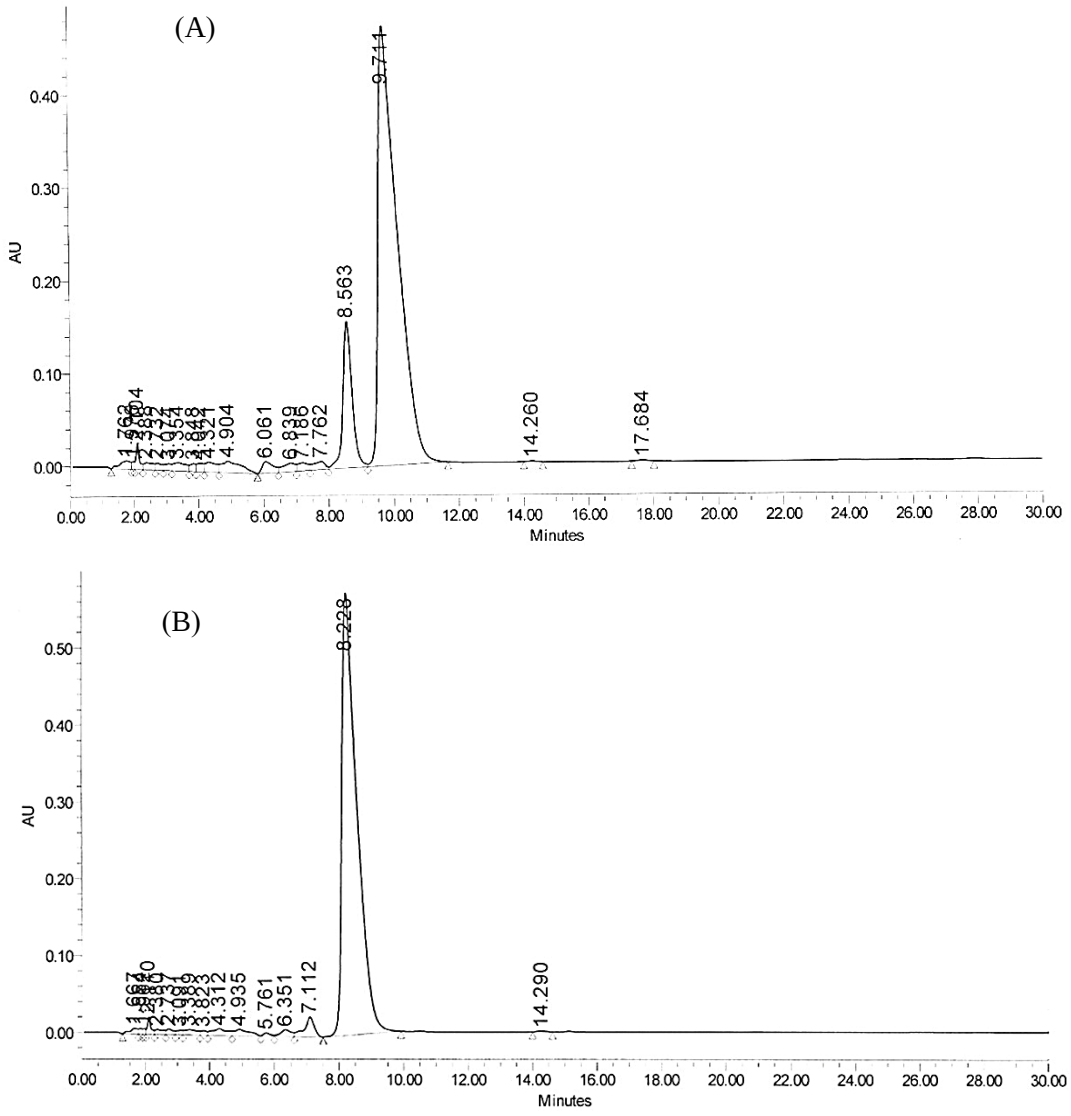

Figure 7 Analytical results of the mixture of supernatant and the D-lactic acid standard (A) and the L-lactic acid standard (B) by HPLC.

4. Conclusions

This study successfully isolated and characterized a thermotolerant *Bacillus coagulans* strain (NF11) capable of efficiently producing high-purity L-lactic acid from molasses under energy-saving conditions. The strain's capability to utilize both sucrose and starch, coupled with its thermotolerance, makes it particularly suitable for valorizing agricultural by-products. The capacity for lactic acid production under non-sterile conditions represents a significant advantage in process economics and energy efficiency. Furthermore, exclusive production of L-lactic acid simplifies downstream processing and enhances product value. These findings suggest that *B. coagulans* NF11 is a promising candidate for industrial-scale, cost-effective L-lactic acid production from agricultural waste streams.

5. Acknowledgments

This study was funded by the Fundamental Fund of Khon Kaen University, which received support from the National Science, Research and Innovation Fund, Thailand (Research grant number 161846). The authors extended their gratitude to the Fermentation Research Center for Value Added Agricultural Products (FerVAAP) for providing access to essential instruments. We also thank Mitr Phol Phu Wiang Sugar Factory for their consistent and generous supply of raw materials troughout the research process.

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