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HIGH LACTIC ACID PRODUCTION BY *LACTOBACILLUS* SP. V156 ISOLATED FROM VIETNAMESE FERMENTED MUSTARD GREENS

Doan Van Thuoc*, Pham Thi Huong, Tran Huu Phong

Department of Biotechnology and Microbiology, Faculty of Biology,
Hanoi National University of Education, 136 Xuan Thuy, Cau Giay, Ha Noi, Viet Nam

*Emails: *thuocdv@hnue.edu.vn*

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Abstract. Lactic acid, which can be produced by many bacterial groups through fermentation, is an interesting compound because it can be used in various fields such as in the food, pharmaceutical, material and chemical industries. In the present study, eight high lactic acid producing bacteria were isolated and screened from fermented mustard greens. The highest lactic acid producer (strain V156) was selected for further studies. Strain V156 is a Gram positive, non-motile, non-spore forming, catalase negative and rod-shaped bacterium. Based on the morphological and biochemical characteristics and 16S rRNA gene sequence, strain V156 was classified into genus *Lactobacillus*. The effect of two different neutralizing agents (calcium hydroxide and ammonium hydroxide) on bacterial growth and lactic acid production by *Lactobacillus* sp. V156 was investigated. The results indicated that ammonium hydroxide was a suitable neutralizing agent that could stimulate cell growth and lactic acid production by *Lactobacillus* sp. V156. High lactic acid concentration of 101.6 g/L and productivity of 2.82 g/L/h were efficiently achieved by *Lactobacillus* sp. V156 after 36 h of cultivation in fed-batch fermentation using ammonium hydroxide as the neutralizing agent. The production of high lactic acid during a short cultivation period demonstrates that *Lactobacillus* sp. V156 is a promising strain for L-lactic acid production.

Keywords: Fermented mustard greens, lactic acid, *Lactobacillus*

Classification numbers: 1.1.5, 1.3.2, 1.3.4.

1. INTRODUCTION

Lactic acid is an organic acid with a long history of use in food, pharmaceutical and cosmetics industries. Recently, it has been used for the production of biopolymer poly-lactic acid (PLA), which is a promising biodegradable and environmentally friendly alternative to plastics derived from petrochemicals [1, 2]. Lactic acid can be produced industrially in two ways, either through chemical synthesis or by microbial fermentation. However, the fermentation process has some advantages over chemical synthesis, e.g., low substrate cost, low production temperature,

low energy consumption, and high product specificity. For that reason, approximately 90 % of all lactic acid worldwide is produced by microbial fermentation [1, 2].

Lactic acid bacteria (LAB) can be classified into two groups: homofermentative LAB and heterofermentative LAB. While the homofermentative LAB catabolize glucose *via* the Embden-Meyerhof pathway and convert glucose almost exclusively into lactic acid; the heterofermentative LAB metabolize glucose *via* phosphoketolase pathway and convert glucose into lactic acid, CO_2, and either acetic acid or ethanol. Only the homofermentative LAB are available for the commercial production of lactic acid and most of the LAB used belongs to the genus *Lactobacillus* [3].

The demand for lactic acid has increased considerably due to its wide range of applications. In order to enhance the economics of the lactic acid fermentation process, it is necessary to increase the efficiency and yield of lactic acid production. Several factors were reported to increase lactic acid production efficiency such as lactic acid producer, fermentation substrate, and fermentation conditions [1, 2].

Batch fermentation has been used for lactic acid production. The best results obtained in batch fermentation with glucose as the substrate are 4.02 g/L/h for productivity and 58.3 g/L for final lactic acid concentration [4]. However, the major disadvantage of batch fermentation is that lactic acid concentration and productivity decreased due to the inhibition of high substrate concentration at the beginning and low substrate concentration at the end of the fermentation process. For that reason, fed-batch fermentation is commonly used for lactic acid production which avoids substrate-level inhibition by maintaining suitable substrate concentration for bacterial growth during the fermentation process. High lactic acid concentration of 91.4 g/L and productivity of 4.01 g/L/h were obtained in fed-batch fermentation under constant feeding conditions [4].

In Vietnam, *Dưachua* is a sour fermented vegetable made from mustard greens or beet. The vegetable is cleaned and air dried, and then soaked in brine containing 2-7 % salt and 1-3 % sugar. The fermentation is carried out at a temperature of above 25 ^{o}C for 1-3 days in tight containers to achieve a pH value of lower than 5.0 [5]. LAB play a dominant role in the fermentation process. They convert sugars into lactic acid and metabolize the other components of the raw materials that reduce the pH value and also contribute to the nutritive value of the products [5, 6]. Recently, many LAB species such as *Lactobacillus pentosus, L. plantarum, L. fermentum, L. pentosaceus* have been isolated from fermented mustard greens (*dưa cải bẹ*) or fermented beet (*dưa củ cải*) [5]. The aim of this study is to isolate a new high potential lactic acid producer from Vietnamese fermented mustard greens. The selected strain was identified by molecular method and the conditions for lactic acid production by selected strain were also investigated. In addition, a fed-batch process was developed to attain high lactic acid productivity.

2. MATERIALS AND METHODS

2.1. Isolation of bacteria

Fermented mustard greens juices were collected and serially diluted with 3 % NaCl solution, and then 100 µL of the diluted solution was spread on MRS medium, containing (g/L): glucose, 20; K_2HPO_4, 2; $MgSO_4{\cdot}7H_2O$, 0.2; $MnSO_4{\cdot}4H_2O$, 0.05, meat extract, 8; peptone, 10; yeast extract, 4; $CH_3COONa{\cdot}3H_2O$, 5; triammonium citrate, 2; tween 80, 1 mL; agar, 20. After

48 h of cultivation at 35 ^{o}C, more than 200 colonies were isolated and streaked onto fresh MRS plates to obtain pure cultures.

2.2. Screening of lactic acid bacteria

The collected colonies were grown on MRS medium supplemented with 0.5 % $CaCO_3$. After 24 h of cultivation at 35 ^{o}C, the colonies with clear zone were counted as LAB. The LAB with clear zone diameter of above 20 mm were collected and grown on MRS liquid medium. Samples were taken after 48 h of cultivation for lactic acid analysis.

2.3. Phylogenetic characterization of the selected lactic acid-producing bacterium

The genomic DNA of the selected strain was extracted by Thermo Scientific GeneJET Genomic DNA Purification Kit according to the manufacturer's recommendations. The 16S rRNA gene was amplified using the universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGCTT-3') and the genomic DNA as template. Sequencing of the amplified DNA fragment was performed at 1st Base (Singapore), and GenBank database was used to search for 16S rRNA gene similarities. Phylogenic analysis based on 16S rRNA gene sequences was performed with the aid of MEGA6 software [7] using the neighbor-joining distance correlation method [8].

2.4. Effect of different carbon sources on lactic acid production

The selected strain was initially grown at 37 °C in 100 mL flask containing 20 mL of MRS medium for 13 h. Subsequently, 2.5 mL of the culture was inoculated in 250 mL flask containing 50 mL of MRS medium with 50 g/L of different carbon sources including glucose, maltose, xylose, fructose, sucrose, and lactose. The culture was incubated at 37 °C for 48 h, samples were then taken for cell dry weight (CDW) and lactic acid analysis.

2.5. Lactic acid production in flasks

The selected strain was initially grown at 37 °C in 100 mL flask containing 20 mL of MRS medium for 13 h. Subsequently, 2.5 mL of the culture was inoculated in 250 mL flask containing 50 mL of MRS medium with 50 g/L glucose. The culture was incubated at 37 °C, samples were then withdrawn at various time intervals for cell dry weight (CDW) and lactic acid analysis.

2.6. Batch fermentation for lactic acid production in bioreactor

The selected strain was initially grown at 37 °C in 250 mL flasks containing 50 mL of MRS medium for 13 h. Three hundred of culture medium was then used to inoculate 2.7 L of MRS medium with 50 g/L glucose as carbon source in a 10 L bioreactor. The cultivations were performed in batch mode during which temperature was kept constant at 37 °C and pH was maintained at 6.5 by adding 15 % $Ca(OH)_2$. Stirring velocity was set at 50 rpm during the fermentation. Samples were taken every 3 h for CDW, lactic acid, and glucose analysis.

2.7. Fed-batch fermentation for lactic acid production

Fed-batch fermentation was carried out in a 10 L bioreactor containing 3 L of MRS medium under the same conditions as described above (temperature of 37 °C and stirring velocity of 50

rpm). Glucose concentration was analyzed using DNS method. A feed solution containing MRS medium and 500 g/L glucose was used for maintaining the concentration of carbon source in the range of 10 - 20 g/L. pH during the cultivations was maintained at 6.5 by adding15 % calcium hydroxide or 25 % ammonium hydroxide. Samples were taken at various time intervals for CDW, lactic acid, and glucose analysis.

2.8. Analytical method

CDW was determined by centrifuging 3 mL of the culture samples at 5000 ×g for 10 min in a pre-weighed centrifuge tube, and the pellet was washed with 3 mL of distilled water, centrifuged, and dried at 105 ^{o}C until a constant weight was obtained. The centrifuge tube was weighed again to calculate the CDW.

Lactic acid content in the culture broth was determined by HPLC analysis using a Thermo Finnigan Surveyor HPLC system with a Cosmosil 5C18-PAQ column (Nacalaitesque, Inc., Japan) and a UV detector at 30 ^{o}C. The compounds were monitored at 210 nm, and 20 mM phosphate buffer (pH 2.5) was used as mobile phase at a flow rate of 1.0 mL/min [9]. L-lactic acid from Sigma was used for a standard curve.

Residual glucose concentration in the culture broth was determined by the dinitrosalysilic acid (DNS) method [10]. The mixtures of 0.5 mL of diluted culture broth and 0.75 mL of DNS solution were placed in a boiling water bath for 5 min. Samples were cooled to ambient temperature and then absorbance was read at 540 nm. Glucose from Sigma was used for a standard curve.

The yield (g/g, product/substrate) was calculated as the ratio between lactic acid formation and glucose consumption.

3. RESULTS AND DISCUSSION

3.1. Isolation and screening of lactic acid producer

From Vietnamese fermented mustard greens, about 216 bacterial colonies were isolated. The isolates were then grown on MRS medium containing 0.5 % $CaCO_3$, 155 isolates showed clear zone around their colonies after 24 h of cultivation. Among them, eight isolates with clear zone diameters above 20 mm were selected and grown on MRS liquid medium containing 50 g/L glucose (Figure 1).

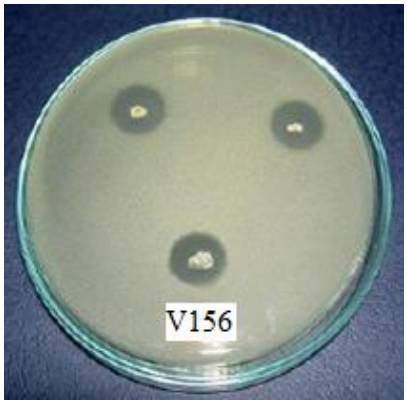


Figure 1. The lactic acid bacterium (strain V156) produced transparent circle in MRS medium containing 0.5 % calcium carbonate.

The results of final pH, lactic acid concentration, glucose consumption and production yield (g lactic acid/g glucose) obtained from eight selected strains are shown in Table 1. At the beginning of the experiment, the pH of the culture medium was 6.5 and it decreased to 3.36 - 3.48 after 48 h of cultivation. The decrease in pH of the culture medium was due to the formation of lactic acid, as shown in Table 1. All eight selected strains could produce L-lactic acid with concentrations ranging from 13.8 g/L to 19.3 g/L. The yield of lactic acid production ranged from 69 % to 80 % depending on the lactic acid bacterial strains. Among the eight selected bacterial isolates, the strain V156 with the highest lactic acid concentration of 19.3 g/L and a production yield of 80 % was selected for further studies.

Table 1. L-lactic acid production by eight selected strains.

Strain	Final pH	L-lactic concentration (g/L)	Glucose consumption (g/L)	Yield (g/g)
V130	3.36	16.10 ± 0.32	22.02	0.73
V156	3.43	19.35 ± 0.35	24.18	0.80
V165	3.42	14.30 ± 0.40	20.70	0.69
V166	3.37	16.32 ± 0.30	22.90	0.71
V167	3.37	15.65 ± 0.25	21.23	0.74
V171	3.43	14.97 ± 0.28	19.39	0.77
V190	3.40	15.20 ± 0.15	20.31	0.75
V214	3.48	13.85 ± 0.28	19.88	0.70

3.2. Phenotypic characterization of the selected strain

The cells of strain V156 were Gram positive, non-motile, non-spore forming, catalase negative and rod-shaped, usually occurring singly or in pairs. Colonies on MRS agar were round with entire margins, smooth, convex, creamy white and approximately 2 mm in diameter after 2 days of incubation. Strain V156 was a strictly homofermentative LAB. Lactic acid was produced from simple carbon sources such as glucose, sucrose, maltose, lactose, fructose, and xylose. Among six tested carbon substrates, glucose was found to be the most suitable carbon source for lactic acid production (Figure 2). The ability to use various carbon sources suggests that the selected bacterial strain can use inexpensive carbon sources such as agricultural by-products to produce lactic acid, and it can help to reduce the cost of lactic acid production.

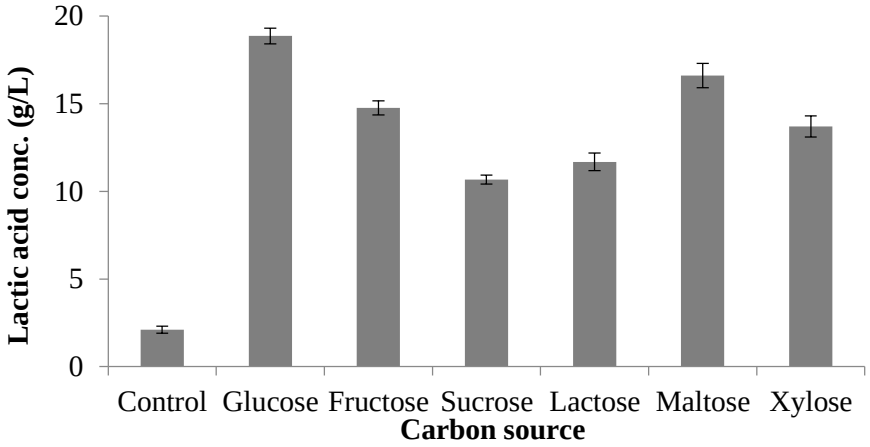


Figure 2. Effect of different carbon sources on lactic acid production by *Lactobacillus* sp. V156.

The bacterial strain can grow over a wide pH range of 4 to 10 and is optimal at pH 6-6.5. Strain V156 grew rapidly at 35 ^{o}C and 40 ^{o}C, and grew slowly at 20 ^{o}C and 45 ^{o}C. The phylogenetic characterization of strain V156 was analyzed using its 16S rRNA gene sequence (about 1422 bp). The 16S rRNA gene sequence of strain V156 was closely related to those of *Lactobacillus* spp., and showed the closest similarity to *Lactobacillus suntoryeus* NCIMB 14055 (99.3 %), *Lactobacillus helveticus* DSM 20075 (98.8 %) and *Lactobacillus gallinarum* ATCC 3319 (97.2 %) (Figure 3).

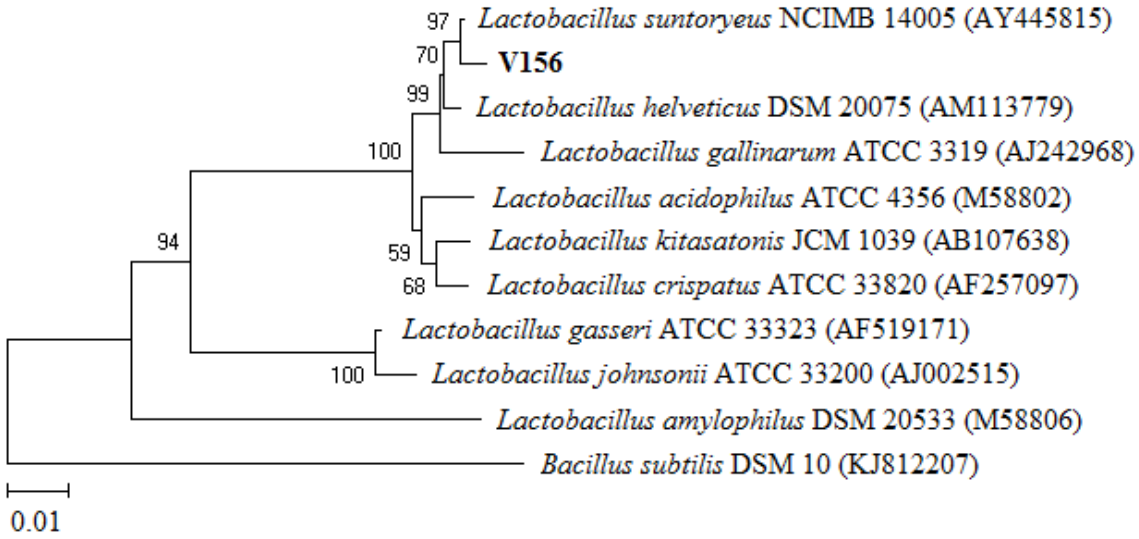
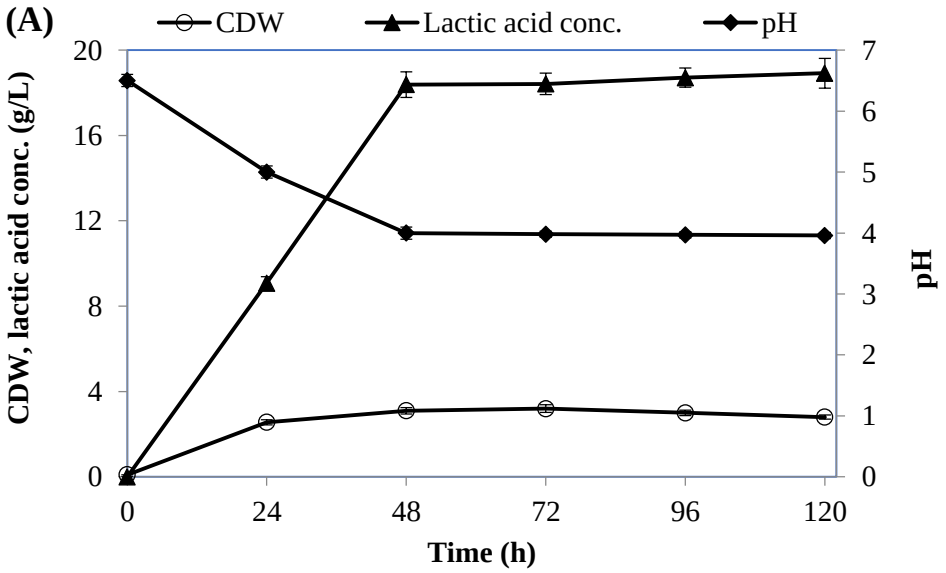


Figure 3. Neighbor-joining phylogenetic tree based on the comparison of 16S rRNAgene sequences, showing the relationships between the selected strain and other strains of the genus *Lactobacillus*. Bar, 0.01 substitutions per nucleotide position. Bootstrap value of 1000 replicates. *Bacillus subtilis* DSM 10 (KJ812207) was used as an outgroup to root the tree.

3.3. Lactic acid production by the selected strain in batch fermentation

The production of lactic acid by strain *Lactobacillus* sp. V156 was first investigated in flask experiments using glucose as a carbon source. As shown in Figure 4A, CDW and lactic acid concentration increased rapidly and reached high values of 3.1 g/L and 18.4 g/L, respectively, after 48 h of cultivation. In contrast, the pH value decreased from 6.5 to 4.0 within the first 48 h of cultivation. After that, the cell mass, lactic acid concentration and pH value seemed to be constant. During the fermentation process, glucose was converted into lactic acid as a major metabolic end-product by strain *Lactobacillus* sp. V156. The formation of lactic acid led to a decrease in the pH value and this would inhibit the metabolism of strain V156. For that reason, the growth rate and lactic acid formation by strain V156 were completely inhibited when the pH value in the culture medium was below 4.0. The inhibitory effect of lactic acid on LAB growth has been mentioned by previous studies [11 - 13].

In order to improve lactic acid concentration during fermentation, batch fermentation was carried out using a 10-L bioreactor system and the pH value of the culture medium was maintained at 6.5 by adding 15 % $Ca(OH)_2$. The highest CDW of 6.3 g/L, lactic acid concentration of 34.5 g/L and yield of 0.71 g/g were obtained after only 15 h of cultivation (Figure 4B). The highest lactic acid productivity of 2.6 g/L/h was obtained after 12 h of cultivation (Table 2), 6.8 times higher than that obtained in the flask experiment (0.38 g/L/h after 48 h of cultivation).



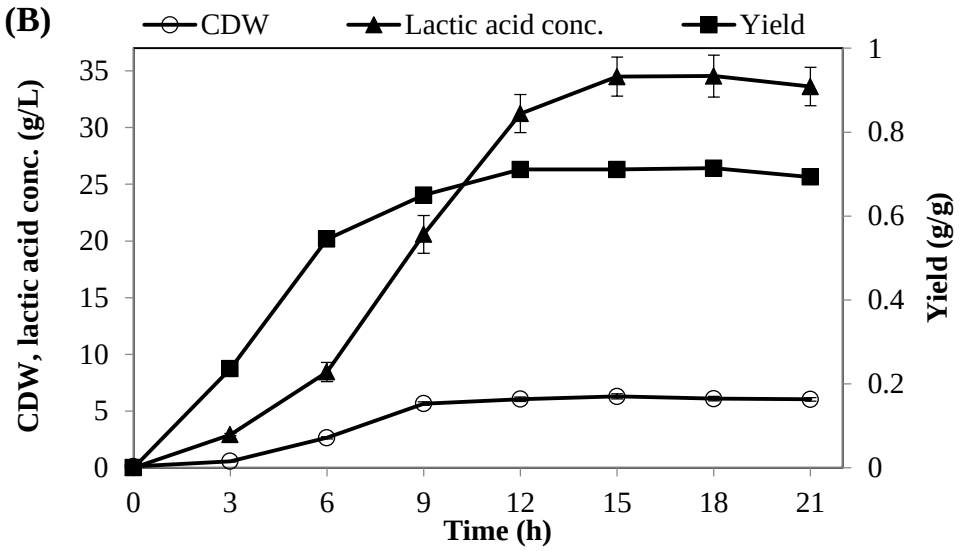
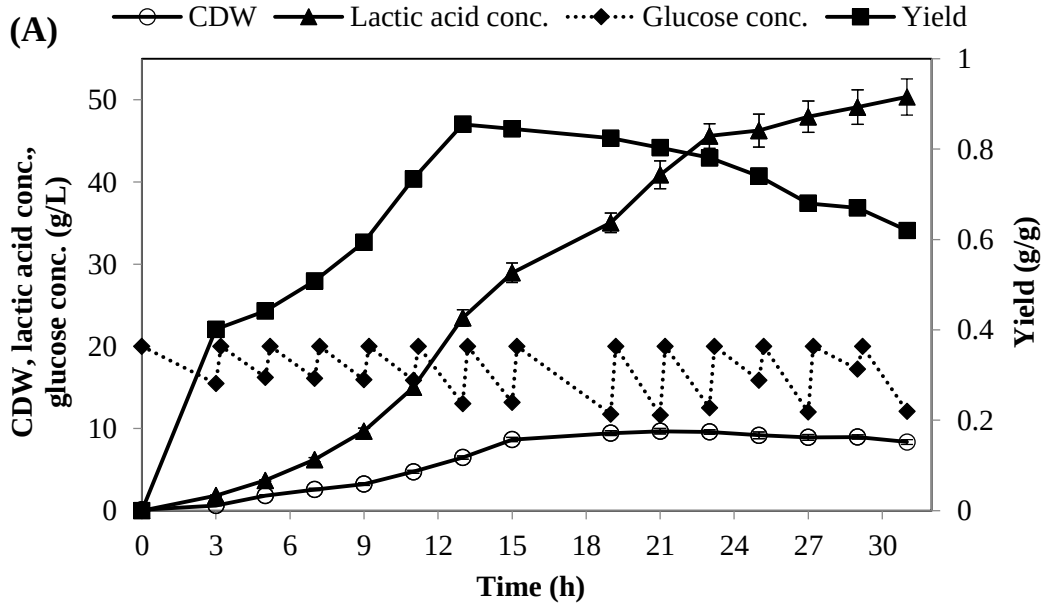


Figure 4. Batch fermentation for lactic acid production by *Lactobacillus* sp. V156 in flasks (A) and in a 10-L bioreactor system (B).

3.4. Lactic acid production in fed-batch fermentation with different neutralizing agents

In order to enhance the concentration of lactic acid produced by strain *Lactobacillus* sp. V156, we carried out fed-batch fermentation using two different neutralizing agents (calcium hydroxide and ammonium hydroxide). Using calcium hydroxide, the maximum CDW of 9.65 g/L and lactic acid concentration of 50.3 g/L were obtained after 21 h and 31 h of cultivation, respectively (Figure 5A), higher than those obtained in batch fermentation. However, the maximum lactic acid productivity obtained in fed-batch fermentation after 23 h of cultivation was 1.98 g/L/h, lower than that obtained in batch fermentation (2.6 g/L/h) (Table 2).



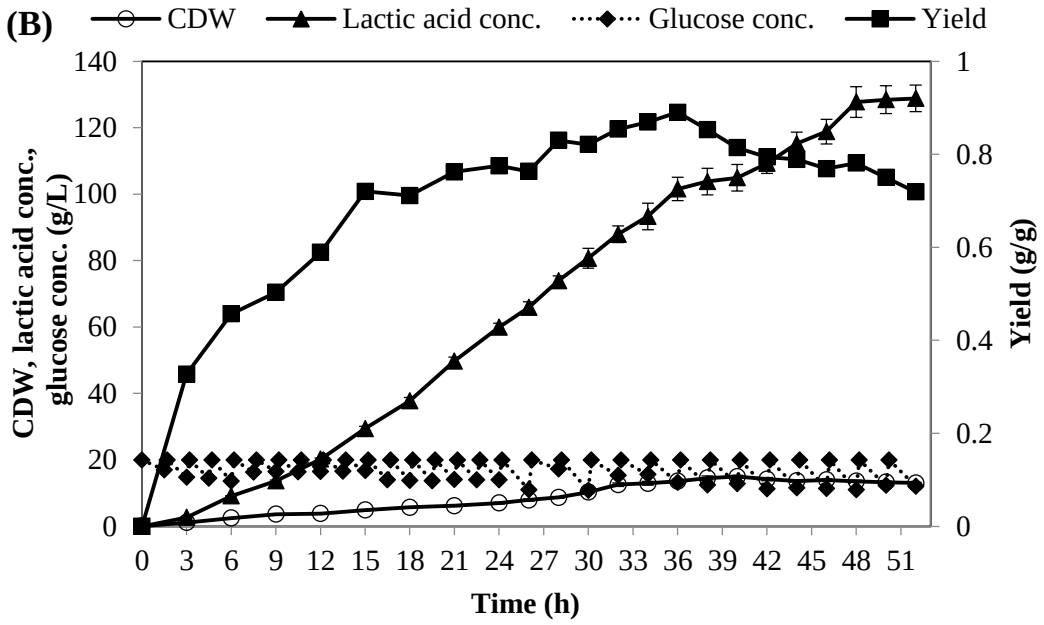


Figure 5. Fed-batch fermentation for lactic acid production by *Lactobacillus* sp. V156 in 10-L bioreactor. pH was maintained at 6.5 by using $Ca(OH)_2$ (A) and NH_4OH (B).

Previous studies demonstrated that bacterial cell growth and lactic acid productivity were enhanced by using ammonium hydroxide compared with sodium hydroxide due to the utilization of ammonium ion as nitrogen source [12, 14]. These trends were also observed in this study. As shown in Figure 5, the maximum CDW increased from 9.65 g/L using calcium hydroxide to 14.96 g/L using ammonium hydroxide, and lactic acid concentration was enhanced from 50.3 g/L with calcium hydroxide to 128 g/L with ammonium hydroxide. In addition, the maximum lactic acid productivity was also increased from 1.98 g/L/h using calcium hydroxide to 2.82 g/L/h using ammonium hydroxide, and the yield of conversion from glucose to lactic acid was increased from 0.78 to a maximum value of 0.89 (Table 2). These results indicated that ammonium hydroxide was a suitable neutralizing agent that could stimulate cell growth and lactic acid production by *Lactobacillus* sp. V156.

Table 2. Lactic acid production by different lactic acid bacteria in bioreactor system.

Strain	C[a]	FM[b]	Lactic acid (g/L)	CT[c] (h)	Yield (g/g)	P_{LA}[d] (g/L/h)	Neutralizing agent	Ref[e]
Lactobacillus sp. V156	G	Batch	31.2	12	0.71	2.6	$Ca(OH)_2$	This study
	G	Fed-batch	45.6	23	0.78	1.98	$Ca(OH)_2$	This study
	G	Fed-batch	101.6	36	0.89	2.82	NH_4OH	This study
	G	Fed-batch	128.8	52	0.719	2.48	NH_4OH	This study

L. casei CCDM 198	G	Batch	58.3	14.5	0.973	4.02	NaOH	[4]
	G	Fed-batch	116.5	72	0.984	1.62	NaOH	[4]
	G	Fed-batch	91.4	23	0.984	4.01	NaOH	[4]
E.mundtii QU 25	G+X	Fed-batch	113	168	0.809	0.67	NaOH	[12]
	G+X	Fed-batch	129	168	0.785	0.77	NH_4OH	[12]
S.nakayamae	S	Fed-batch	122.4	54	-	2.27	NaOH	[15]
L. lactis	G	Batch	50	32	1.0	1.56	$CaCO_3$	[16]
	G	Fed-batch	210	96	0.97	2.19	$CaCO_3$	[16]
L. casei	G	Fed-batch	180	84	0.903	2.14	NH_4OH	[17]
L. rhamnosus B103	L	Batch	57	48	-	1.18	NaOH	[18]
	L	Fed-batch	143.7	72	-	2.00	Whey and CSL	[18]

G, glucose; X, xylose; L, lactose; S, sucrose; CLS, corn step liquor; [a]Carbon source; [b]Fermentation mode; [c]Cultivation time; [d]Productivity of lactic acid; [e]Reference.

The lactic acid concentration and productivity obtained in this study by *Lactobacillus* sp. V156 are comparable to that of the high producers reported so far (Table 2). The lactic acid concentration obtained in this study (128 g/L) is higher than that obtained by *L. casei* CCDM 198 (116.5 g/L) [4], in the same range as *Enterococcus mundtii* QU 25 (129 g/L) [12] and *Sporolactobacillusnakayamae* (122,4 g/L) [15], but lower than that reported for other *Lactobacillus* species such as *L. lactis* (210 g/L) [16], *L. casei* (180 g/L) [17], and *L. rhamnosus* B103 (143.7 g/L) [18]. The lactic acid productivity (2.82 g/L/h) is lower than that obtained by *L. casei* CCDM 198 [4], but higher than that by other bacterial strains (Table 2), it is due to the short cultivation time required for strain *Lactobacillus* sp. V156. This work has placed *Lactobacillus* sp. V156 as a new attractive option for the production of lactic acid.

4. CONCLUSIONS

A high lactic acid producing bacterium belonging to *Lactobacillus* genus was isolated and identified from Vietnamese fermented mustard greens. The lactic acid concentration of only 34.5 g/L was obtained in batch fermentation. The concentration of lactic acid was enhanced by fed-batch fermentation using ammonium hydroxide as a neutralizing agent, reaching 101.6 g/L after 36 h of cultivation. The lactic acid productivity obtained in fed-batch fermentation was 2.82 g/L/h, which can be comparable to that produced by other *Lactobacillus* species published in literature to date.

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CRediT authorship contribution statement. Doan Van Thuoc: conceived and designed the experiments, performed some part of the experiments, prepared figures and tables and wrote the manuscript. Pham Thi Huong: performed main part of the experiments and analyzed the data. Tran Huu Phong: designed the experiments, performed some part of the experiments and analyzed the data.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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