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Characterization of newly isolated thermotolerant lactic acid bacteria and lactic acid production at high temperature

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Abstract

This study aimed to select a number of thermotolerant lactic acid bacteria for lactic acid fermentation at high temperature, and to identify these LAB strains by using 16S ribosomal RNA sequences. The fermentative ability of 16 selected strains was examined at different temperatures (37ºC, 39ºC and 41ºC), of which, the lactic acid concentration produced by these LAB strains at 37ºC ranged from 6.0 g/L to 14.1 g/L. Ten LAB strains (L2, L7, L9, L11, L21, L26, L30, L36, L37 and L52) with effective lactic acid fermentation at 37ºC were chosen for identification. The selected strains were characterized as *L. plantarum, L. casei, L. acidophilus,* and *L. delbrueckii, L. casei* L9 was the superior lactic acid producing strain at both 39ºC (18.9 g/L) and 41ºC (18.0 g/L). Based on statistical analysis, appropriate conditions for lactic acid production by *L. casei* L9 at 39ºC were defined as follows: pH 6.51, glucose concentration 6% (w/v) and inoculation level 2.37% (v/v). At these conditions, the validated lactic acid concentration and the yield were recorded at 21.15 g/L and 80.84%, respectively.

Introduction

Lactic acid is a widely used organic acid discovered by Scheele in 1780. It is applied in different fields such as pharmaceuticals, cosmetics and food industries with the annual world production is approximately 259,000 metric tons. Lactic acid could be produced by fermentation or chemical synthesis. However, lactic acid manufactured via natural fermentation is preferred due to its environmental friendliness since many low cost agricultural wastes could be recycled (Castillo *et al*., 2013).

In the world of global environmental changes, thermotolerant lactic acid bacteria have increasingly attracted both researchers and producers because of some benefits. Thermotolerant LAB could ferment effectively at high temperature conditions, so using them for mass lactic acid production would reduce cooling expenses and carbon dioxide production. Also, the fermentation management and the biomass utilization are optimized. Furthermore, utilization of thermotolerant lactic acid bacteria could be a promising solution for a stable lactic acid industry in tropical areas. Therefore, the objectives of this study were to select a number of thermotolerant LAB and to investigate appropriate conditions for lactic acid fermentation at high temperature as well as to characterize the gene sequencing of the selected superior LAB strains.

Materials and Methods

Cultures

Sixteen selected lactic acid bacterial strains isolated from different sources (e.g. fermented products, fermented milk products, agricultural wastes and fruits) were stored in the Food Biotechnology Lab, Biotechnology Research and Development Institute, Can Tho University. *Lactobacillus thermotolerans* obtained from Kyushu University, Japan was used as a control strain.

The bacterial suspension of each LAB strain was prepared by inoculating a colony of bacteria grown on a MRS agar plate into 5 mL sterilized MRS Broth medium (De Man *et al*., 1960) contained in a 10 mL test tube. The bacteria were cultured for around 48 hours with continuous shaking (120 rpm) at 37ºC until the cell concentration reached at 10^7 cells/mL.

Fermentation media

MRS Broth medium was employed in all experiments, including peptone (10.0 g/L), meat extract (8.0 g/L), yeast extract (4.0 g/L), D(+) glucose (20.0 g/L), di-potassium hydrogen phosphate (2.0 g/L), Tween 80 (1.0 g/L), di-ammonium hydrogen citrate (2.0 g/L), sodium acetate (5.0 g/L), magnesium sulfate (0.2 g/L) and manganese sulfate (0.04 g/L).

Study of appropriate conditions

The experiment was set up in a factorial design

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(three factors) at three levels: pH (5, 6, and 7), glucose concentration (40 g/L, 50 g/L, and 60 g/L) and inoculation level (1%, 2%, and 3% v/v) with the bacterial cell concentration was 10^7 cells/mL. The surface plotting and the contour were constructed by the software Statgraphics Centurion XV (ver. 15.1.02) based on the identified regression equation. The favorable results were then verified using 1.0 L scale lactic acid fermentation in MRS medium.

Analytical methods

The lactic acid production was daily monitored by using titration method. The remained reducing sugar concentration was measured by dinitrosalicylic colorimetric method (DNS) (Sumner, 1921), and the pH meter was used in measuring the final pH. The yield and the rate of glucose conversion were calculated based on the lactic acid production and consumed glucose. Experimental data were analyzed using Statgraphics Centurion XV (ver. 15.1.02).

The 16S rRNA gene sequencing and phylogenetic tree construction

The 16S rRNA gene of 10 selected thermotolerant LAB strains were extracted and amplified by polymerase chain reaction (PCR) in a thermal cycler. The primers 1492R (5'-TACGGTTACCTTGTTACGACT - 3') and 27F (5' - AGAGTTTGATCCTGGCTC - 3') were used for PCR (William *et al*., 1991). The alignment of 16S rRNA sequences with database of GenBank (NCBI) was conducted by Nucleotide Blast tool. The phylogenetic tree of 10 LAB strains was constructed by software MEGA (ver. 6.06) using maximum likelihood method, and the bootstrap program with 1000 replicates was applied to assess the reliability.

Results and Discussion

Fermentative ability of LAB at 37ºC

The lactic acid production of 16 selected LAB strains and the control strain *L. thermotolerans* shown in Table 1 reveals that seven strains coded as L7, L9, L11, L21, L26, L30 and L37 produced the most lactic acid after 7 days of fermentation with the lactic acid production ranged from 9.9 to 14.1 (g/L). Markedly, strains L7, L9, L11 and L30 produced the highest lactic acid (12.3-14.1 g/L). Meanwhile, the lactic acid production of the control strain was 12.0 (g/L).

In the previous study of Karin *et al.* (2000) about lactic acid production at 37ºC, *L. acidophilus* CRL 640 has been reported to produce lactic acid at 14 g/L, which was equivalent with the lactic acid production of strain L11 (14.1 g/L). Similarly, strains L7, L9 and L30 had the comparable lactic acid concentrations (12.3-12.9 g/L) with *L. delbrueckii* sp. *bulgaricus* CRL 870 (12 g/L). Furthermore, different LAB strains resulted in the variation of the yield (31.45-72.97%) of 16 selected LAB strains. Dissimilar carbon sources also led to the divergence of the precursor conversion rate, for instance, strain *L. acidophilus* L11 at 37ºC using glucose as the precursor had the glucose conversion rate at 0.73 g acid/g glucose (equivalent to 73% yield) which was higher than of *L. acidophilus* ATCC 43121 (0.61 g/g) in the research using a hydrolyzed lignocellulosic waste (brewer's spent grain) as the main substrate (Rossana *et al.*, 2015).

Table 1. Lactic acid production of 16 LAB strains at 37°C

LAB	Lactic acid (g/L)[1]	Yield (%)[1,2]
L2	9.3^{cdefg}	47.65^{cdefg}
L6	6.0^{h}	31.45^{h}
L7	12.3^{abc}	63.90^{abc}
L9	12.9^{ab}	68.39^{ab}
L10	7.2^{fgh}	38.46^{fgh}
L11	14.1^{a}	72.97^{a}
L20	7.2^{fgh}	37.26^{fgh}
L21	11.4^{abcde}	58.57^{abcde}
L26	9.9^{bcdef}	51.60^{bcdef}
L27	8.4^{efgh}	43.35^{efgh}
L30	12.6^{ab}	64.63^{ab}
L36	9^{defgh}	47.11^{defgh}
L37	10.8^{bcde}	55.06^{bcde}
L38	6.3^{gh}	32.84^{gh}
L52	8.7^{efgh}	45.68^{efgh}
L54	7.2^{fgh}	37.96^{fgh}
Control	12^{abcd}	62.52^{abcd}

[1]Values having different alphabet are significant different at the 95% confidence level;
[2]Calculated based on the ratio of practical lactic acid production to theoretical lactic acid production.

Fermentative ability of LAB at 39ºC and 41ºC

Strains L7, L9, L11, L21, L26, L30 and L37 were selected for lactic acid fermentation at higher temperature levels (39ºC and 41ºC). The strain *L. thermotolerans* was also used for comparison. Experimental data in Figure 1 show that strain L9 had the highest lactic acid production at both 39ºC (18.9 g/L) and 41ºC (18.0 g/L). Noticeably, fermentation

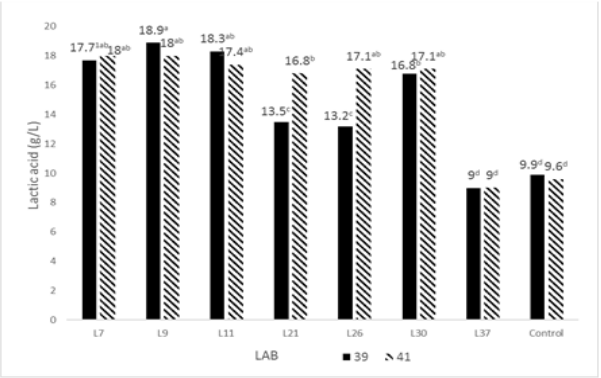

Figure 1. Lactic acid production of 7 tested LAB at 39°C and 41°C

[1]Values having different alphabet are significant different at the 95% confidence level.

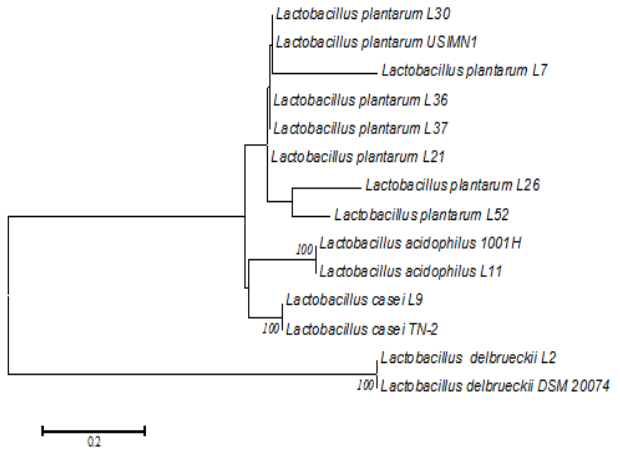

Figure 2. Phylogenetic tree of 10 selected LAB strains, as inferred by the maximum likelihood method with 16S rRNA gene sequences. The bar indicates 20% sequence divergence.

temperature at 41ºC was more favorable for most thermotolerant LAB strains (except L9 and L11) than at 39ºC. Temperature is a vital factor that influences cell enzymes and the rate of nutritional metabolism. Cell density and enzyme activities are optimized when temperature is adjusted at optimum levels (Panesar *et al*., 2010).

Identification and genetic relation of selected thermotolerant LAB

The alignment results of the 16S rRNA sequences of 10 selected LAB strains (L2, L6, L7, L9, L11, L21, L36, L37 and L52) with the database of GenBank (NCBI) indicate that all strains belonged to species of *Lactobacillus* genus in which *Lactobacillus plantarum* was the most prevalent strain, representing 70% of aligned LAB strains (L7, L21, L26, L30, L36, L37 and L52). Meanwhile, strains L2, L9 and L11 shared high identity with *Lactobacillus delbrueckii, Lactobacillus casei* and *Lactobacillus acidophilus,* respectively.

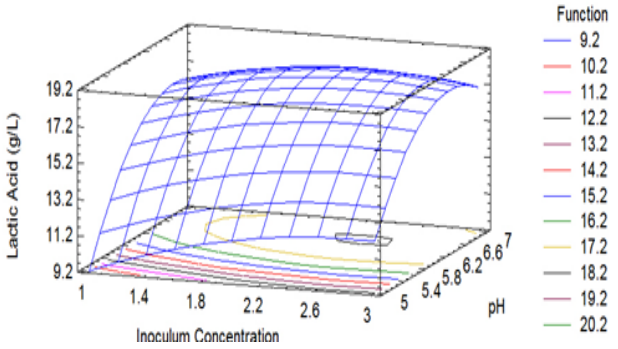

Figure 3. The surface plotting analysis of condition effect on lactic acid production

The phylogenetic tree (Figure 2) shows that strains identified as *L. plantarum* (L7, L21, L26, L30, L36, L37 and L52) had close molecular relation as all 7 strains grouped with the type strain *L. plantarum* USIMN1 (accession no. KT962237.1). Also, strains L2, L9 and L11 were respectively monophyletic with *L. delbrueckii* DSM 20074 (accession no. AJ616219.1), *L. casei* TN-2 (accession no. KF648599.1) and *L. acidophilus* 1001H (accession no. JQ031741.1) at 100% bootstrap. In addition, molecular analytic result reveals L2 *(L. delbrueckii)* owned higher genetic divergence compared to other mentioned strains.

Previous studies concluded that *Lactobacillus* was a popular genus that could be found abundantly in fermented food, silages because of their acid tolerant ability (Lars, 2004). According to Rodolphe *et al.* (2012), genomic differences in species of *Lactobacillus* genus were originated from the diversity of environmental niches of bacteria. *L. plantarum* was the most diverse species because of their large genome with genes encoding for metabolic enzymes which allow bacteria to grow on several substances and in unfavorable conditions. Also, new genes emerged through gene duplication had led to the divergence of *L. plantarum* and *L. casei* (Kira and Eugene, 2007).

Appropriate conditions of lactic acid fermentation at 39ºC

The analyses of the contour and the surface plotting (Figure 3) were constructed using the multivariable regression equation with the glucose concentration was fixed at 6% (w/v) whereas initial pH (X, 5-7) and inoculum concentration (Y, 1-3% v/v) were variables. The appropriate conditions for lactic acid fermentation of *L. casei* L9 were determined at initial pH of 6.51, 2.37% (v/v) of inoculum concentration and 6% (w/v) of glucose concentration. In the experimental validation using a fermentation volume (1.0 L), lactic acid concentration of 21.15 g/L and

theoretical yield of 80.84% were obtained.

In the study of Serna *et al.* (2006) on favorable conditions for lactic acid fermentation, the glucose concentration at 6% (w/v) was found as the most effective concentration for the fermentation of *Lactococcus lactis* subs *lactis* isolated from sugarcane. In addition, Panesar *et al*. (2010) in a research about *L. casei* has concluded that inoculation level around 2% (v/v) and pH at 6.5 were the most appropriate conditions for lactic acid production. Also, the optimum pH for the lactic fermentation of the thermophilic LAB strain *Enterococcus faecium* QU50 was determined at 6.5 in the study of Mohamed *et al*. (2015).

Conclusion

A number of the selected thermotolerant LAB strains were found to be able for the lactic acid production at high temperatures. *L. casei* L9 was defined as the most effective fermentation strain at 39ºC and 41ºC with the lactic acid production could reach at 18.9 g/L and 18.0 g/L, respectively. Lactic acid concentration of 21.15 g/L and theoretical yield of 80.84% were obtained at 39ºC with the determined appropriate conditions. This finding can indicate the promising application of such strains for the controlled lactic acid production at high temperatures.

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