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Selection of thermotolerant lactic acid bacteria producing high antibacterial activity and production of biomass from tofu sour liquid

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ABSTRACT

The objectives of this study were to select a number of thermotolerant lactic acid bacteria (LAB) for their application in biomass production at high temperature and to study the genetic relation of these selected strains by using 16S ribosomal DNA sequences. All 16 tested strains of thermotolerant LAB were found to possess the antibacterial ability and the capability of bacteriocin production against Bacillus subtilis. As a result, all 16 LAB strains had an antibacterial ability and produced bacteriocin against indicator. Ten selected strains having the strongest antibacterial ability were identified as Lactobacillus plantarum, L. casei, and L. delbrueckii. The L. plantarum L54 was selected for the experiment of the optimum conditions for biomass production because of its strongest antibacterial ability. The diameter of inhibitory zone in "agar spot test" and "well-diffusion agar" were 13.76 mm and 17.33 mm, respectively. Based on statistical analysis, the optimum conditions for biomass production by L. plantarum L54 at 39°C were 5.99% (w/v) of glucose concentration, 6.37% (v/v) of bacterial inoculum concentration, and pH 6.0.

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1 INTRODUCTION

Lactic acid bacteria (LAB) are ubiquitous microorganisms that can be beneficial in crop and livestock production. The primary antimicrobial effect exerted by LAB is the production of various antimicrobial compounds, which can be classified as low-molecular-mass compounds such as hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl (2,3-butanedione), and high-molecular-mass compounds like bacteriocins (Piard and Desmazeaud, 1991; Ouwehand, 1998). In recent years, interest in these compounds has grown substantially due to their potential usefulness as a natural substitute for chemical food preservatives in the production of foods with enhanced shelf life and/or safety (Cleveland *et al.*, 2002). The problem of contamination during lactate production can be effectively minimize by raising the fermentation temperature (Liu *et al.*, 2010; Calabia *et al.*, 2011). Therefore, finding and applying thermotolerant LAB in production for fermented food and lactic acid are momentous. It orientates a new solution to mitigate the problem of pathogen contamination in lactic acid production which is seriously risky to human health and production yield. Besides, tofu has long been an essential component in Asia cuisine and culture, particularly in Vietnam, brought many benefits to health (Ying and Meng, 2017). Tofu sour liquid released after pressing into tofu cakes

has not been collected and handled properly. Therefore, it caused bad odours and pollution to surface and ground waters (Sudiyani *et al.*, 2007). In contrast, tofu sour liquid is known as a good source of nutrients for bacterial growth. This study was carried out to select thermotolerant LAB strains that have strong antibacterial activity and assess conditions of biomass production by utilizing tofu sour liquid. Thus, biomass of these strains may be produced and applied in various fields such as aquaculture, agriculture, and food preservation industry.

2 MATERIALS AND METHODS

2.1 Preparation of bacterial cultures and tofu sour liquid

Sixteen strains of selected LAB isolated from different sources (e.g. fermented meat products, fermented milk products, agricultural wastes, and fruits) were stored in the Food Biotechnology Laboratory, Biotechnology Research and Development Institute, Can Tho University (Bui Hoang Dang Long, 2016). *Lactobacillus thermotolerans* obtained from Kyushu University (Japan) and proved to have high thermoterant properties at Hokkaido University (Japan) was used as a control strain (Niamsup *et al.*, 2003). The bacterial suspension was prepared in sterilized de Man, Rogosa & Sharpe broth (MRS broth) medium for 48 hours. Tofu sour liquid was collected in Vinh Tran tofu production facility in Can Tho City.

2.2 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) (De Man *et al.*, 1960).

2.3 Testing the antibacterial activity

The antibacterial activity of LAB was tested by using *agar spot test* and *well-diffusion agar test* (Herna´ndez *et al.*, 2004). *Bacillus subtilis* isolated from Biosubtyl II was used as an indicator for testing the antibacterial activity.

2.3.1 Agar spot test

After 16 hours of incubation at 30°C, aliquots (2 µL) of the LAB cultures were spotted onto agar plates containing 10 mL of MRS medium. After incubation at 30°C for 18 hours, the plates were overlaid with 5 mL of the appropriate soft agar (1% w/v agar) inoculated with the cell suspension of the indicator strain (10^7 CFU/mL checked with Hemacytometer). The plates were incubated at 35ºC for 18 hours to observe the inhibitory zones.

2.3.2 Well-diffusion agar test

After incubation for 24 hours in the petri dishes, colonies of indicator strain were added to sterile distilled water to prepare the suspension at a density of 10^9 CFU/mL. The wells (6 mm diameter) were made with a sterile metal cylinder in the medium containing 10% (v/v) indicator suspension and fish sauce-peptone-agar (2% w/v agar). LAB strains were grown in 2 mL of MRS broth, under anaerobic conditions in order to avoid H_2O_2 formation, up to stationary phase (48 hours). Cultures were centrifuged at 8,000 rpm for 10 minutes at 4ºC, the supernatants were collected, adjusted to pH 6.5. A volume of 80 µL of crude bacteriocin solution was placed into each well of the plates containing indicator strain. The plates were incubated for 15 minutes for the well diffused solution. Then, plates were incubated at 35ºC for indicator growth.

The antibacterial ability of LAB was calculated by the diameter of the inhibitory zone around the colonies or around the wells in the petri dishes. Inhibition was scored positive if the diameter of the inhibition zone was wider than 2 mm (Herna'ndez *et al.*, 2004).

2.4 Sequencing of 16S rRNA gene and construction of phylogenetic tree

The 16S rRNA genes of 10 selected thermotolerant LAB strains were extracted and amplified by polymerase chain reaction in a thermal cycler. The universal primers F (5'-TACGGTTACCTTGT TACGACT-3') and R (5'- AGAGTTT-GATCCTGGCTC-3') were used for Polymer Chain Reaction (PCR) (William *et al.*, 1991). The alignment of 16S rRNA sequences of selected strains to those of other bacterial species on GenBank of National Center for Biotechnology Information (NCBI) was conducted by Nucleotide Blast tool to identify the scientific name. The phylogenetic tree was constructed by MEGA 6 software (Tamura *et al.*, 2013) by using maximum likelihood. The bootstrap program with 1,000 samples was applied to assess the reliability.

2.5 Study of the optimum conditions for biomass production

The experiment was set up in a factorial design (three factors) at three levels: pH (5.0, 6.0, 7.0), glucose concentration (3%, 6%, 9% w/v) and inoculum concentration (1%, 5%, 10% v/v). Tofu sour liquid was prepared and sterilized at 121ºC for 20

minutes. 30 mL of media were inoculated with LAB suspension at 10^7 CFU/mL and incubated at 39ºC for the LAB biomass increasing in 72 hours. The biomass, reducing sugar content and the final pH were determined.

2.6 Data analysis

Data were processed by using Microsoft Excel 2013 software. Statgraphics Centurion XV was used to test for the least significant difference with the confidence interval of 95%. The optimal condition was determined by Surface and Contour Plotting function of Statgraphics program.

3 RESULTS AND DISCUSSION

3.1 The antibacterial activity of thermotolerant lactic acid bacteria

3.1.1 Agar spot test

Sixteen strains of LAB were examined for their primary antibacterial activity by agar spot test (Figure 1). Diameters of inhibition zones were recorded after 48-hours incubation and were presented in Table 1. All 16 bacterial strains were found to perform the antibacterial activity. Of which, 7 strains gave the strong antibacterial activity (inhibition zone >10.0 mm), 8 strains had intermediate antibacterial activity (5.0< inhibition zone <10.0 mm), strain L38 had weak antibacterial activity (<5.0 mm).

Table 1: Diameters of the inhibition zones in agar spot test

No	Strain	Inhibitory zone (mm)[1]	No	Strain	Inhibitory zone (mm)
1	L52	13.67^{a2}	10	L7	9.33^{abcd}
2	L54	13.67^{a}	11	L37	9.00^{bcd}
3	Control	11.67^{ab}	12	L30	8.67^{bcd}
4	L2	11.00^{ab}	13	L11	7.33^{bcde}
5	L9	11.00^{ab}	14	L26	7.33^{bcde}
6	L21	11.00^{ab}	15	L27	6.33^{cde}
7	L10	10.67^{abc}	16	L20	5.00^{de}
8	L36	10.67^{abc}	17	L38	3.33^{e}
9	L6	9.67^{abc}			

[1]Values are mean of triplicates; [2]means with different superscripts are statistically different at the 95% confidence level.

Herna'ndez *et al*. (2004) reported that only 20% of 180 LAB strains isolated from cheese Tenerife had primary antibacterial activity. Dung and Phong (2011) indicated that only 23 of the 46 LAB strains isolated from the fermentation products had anti bacterial activity as well as only 7 strains exhibited strong antibacterial activity with diameters of the inhibitory zone wider than 10.0 mm.

Fig. 1: The primary antibacterial activity of LAB strains tested by using agar spot test

According to data on Table 1, strains L52 and L54 were dominant for their largest inhibitor zone as 13.67 mm in agar spot test. The antibacterial ability of LAB is mainly due to lactic acid production from the fermentation process which reduces the pH. Moreover, LAB cells contained the compounds such as reuterin, reutericyclin, acid 2-pyrrolidone-5-carboxylic that have antibacterial activity. During their growth, they produce other antibacterial components, namely the low-molecular-weight compounds as hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl (2,3-butanedione) and the high-molecular-weight compounds as bacteriocin (Piard and Desmazeaud, 1991; Ouwehand, 1998).

3.1.2 Well-diffusion agar test

To investigate whether the antibacterial activity of the selected strains involved the production of bacteriocins, the well-diffusion agar assay was utilized (Figure 2). It has been indicated from the experiment that 15 strains produced bacteriocin strongly (inhibitory zone >10.0 mm) (Table 2). Strain L26 was capable of intermediate bacteriocin production (9.33 mm), within the range of 5.0 to 10.0 mm. Strain L30 produced bacteriocin weakly with diameters of inhibitory zone of 4.0 mm.

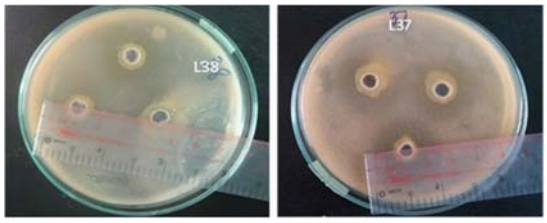

Fig. 2: The antibacterial activity by producing bacteriocin of thermotolerant LAB strains tested by using well-diffusion agar test

Table 2: Diameters of the inhibition zones in well-diffusion agar test

No	Strain	Inhibitory zone (mm)[1]	No	Strain	Inhibitory zone (mm)
1	L6	19.33[a2]	10	Control	14.00[cde]
2	L37	19.33[a]	11	L21	13.33[def]
3	L10	17.67[ab]	12	L20	12.67[ef]
4	L54	17.33[ab]	13	L11	12.00[efg]
5	L2	16.33[bc]	14	L36	12.00[efg]
6	L52	16.33[bc]	15	L27	10.67[fg]
7	L9	16.00[bcd]	16	L26	9.33[g]
8	L38	15.67[bcd]	17	L30	4.00[h]
9	L7	14.00[cde]			

[1]Values are mean of triplicates; [2]means with different superscripts are statistically different at the 95% confidence level

Merih *et al.* (2009) reported that only 33 strains of 45 LAB strains isolated from 10 beer samples in Turkey inhibited *B. subtilis* with diameters of inhibitory zones were in the range of 11.1 to 16.0 mm. One study about the antibacterial activity of LAB strains isolated from the Japanese Miso (Onda *et al.*, 1999) showed that only 1 of 125 LAB strains inhibited *B. subtilis.* This result was also compatible and better than the study of Lu Nguyen Bich Ngoc (2014) which selected a strain could create 10.33 mm inhibitory zone (compare to 17.33 mm of L54 in this study). Through two methods, it can be concluded that all 16 strains had primary antibacterial activity and produced bacteriocin. Strain L38 had weak primary antibacterial activity (3.33 mm) but produced bacteriocin strongly (15.67 mm). In contrast, strain L30 produced bacteriocin weakly (4.00 mm) but had intermediate primary antibacterial activity (8.67 mm). To sum up, strain L54 was dominant and had the best antibacterial properties in both agar spot test and well-diffusion agar test.

3.2 Identification of selected thermotolerant lactic acid bacteria

Ten LAB strains selected based on their strong antibacterial activity were sent to 1st Base Company (Singapore) and Kyushu University (Japan) for sequencing and identifying at the species level. 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. There were 2 species (L2 and L6) belonging to *Lactobacillus delbrueckii*. Strains L9 and L10 were identified as *L. casei*. Six remained strains (L7, L21, L36, L37, L52 and L54) were *L. plantarum.*

Lactobacillus is an important genus of LAB that includes many species used in food production and preservation. LAB strains belonging to this genus are used in the final stage of vegetable and fodder fermentation because of their acid resistance (Axelsson, 2004). All 10 selected strains were closely identified with species of *Lactobacillus* genus, so it can be explained thank their living environment and the diversity of *Lactobacillus* species. Particularly, *L. plantarum* has been reported to be a dominant naturally occurring bacterial species in vegetables such as cabbage and lettuce. Therefore, a major of 10 selected strains (L7, L21, L36, L37, L52 and L54) were identified into this species. *L. casei* is typically the dominant species used in industrial, specifically for dairy production. Thus, L9 and L10 strain isolated from milk and dairy product belonged to this species.

3.3 Study on the genetic relation of selected thermotolerant LAB

The genetic relation of 10 selected LAB strains was reflected in the phylogenetic tree that was built by using MEGA 6 software and is presented in Figure 3. The phylogenetic tree shows that strains identified as *L. plantarum* (L7, L21, L36, L37, L52 and L54) had close molecular relation as all 6 strains grouped with the type strain *L. plantarum* CJG1 (accession no. JQ446466.1). Strain L2 and L6 were monophyletic with *L. delbrueckii* DSM 20074 (accession no. AJ616219.1). Also, L9 and L10 shared close molecular relation with *L. casei* TN2 TN-2 (KF648599.1) at 100% bootstrap.

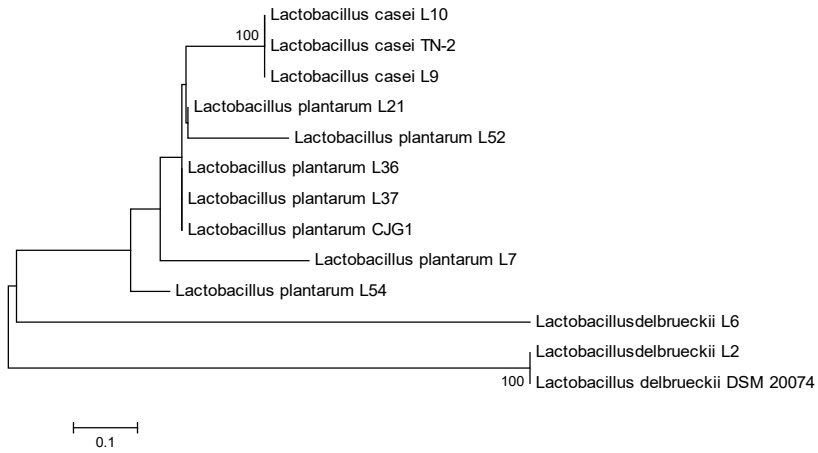

Fig. 3: The phylogenetic tree of 10 selected LAB

3.4 Examination of the optimum conditions for biomass production

The highest increasing biomass yield, 0.705 (g biomass/g substrate), peaked at initial pH 7.0, glucose concentration of 6% (w/w) and inoculum concentration of 1% (w/w); while the lowest one 0.010 (g biomass/g substrate) bottomed out at initial pH 5.0, glucose concentration of 3% (w/w) and inoculum concentration of 1% (w/w). The average value of final pH of the fermentation solution decreased and reached 4.3 (Table 3). The pH will reduce significantly during the exponential stage and reach at about pH 4.0 for the remaining phases during the incubation time (Elmarzugi *et al.*, 2010).

Table 3: The results of assessing increasing biomass condition of thermotolerant LAB at 39°C

Treatment No	Glucose (% w/v)	Initial pH	Inoculated strain (% v/v)	Biomass (g)	Final pH	Utilized Glucose (g L^{-1})	Biomass (g L^{-1})	Biomass yield (g biomass/ g substrate)
1	3	5	1	0.0102	4.75	34.512	0.3422	0.010
2	3	5	5	0.0196	4.43	24.705	0.6533	0.039
3	3	5	10	0.0105	4.26	6.476	0.3489	0.403
4	3	6	1	0.0179	5.34	7.711	0.5978	0.184
5	3	6	5	0.0246	4.73	18.866	0.8200	0.078
6	3	6	10	0.0233	4.45	33.651	0.7778	0.024
7	3	7	1	0.0123	3.58	11.117	0.4089	0.035
8	3	7	5	0.0187	3.96	24.780	0.6233	0.026
9	3	7	10	0.0152	4.51	15.347	0.5078	0.033
10	6	5	1	0.0377	4.85	69.212	1.2556	0.018
11	6	5	5	0.0613	4.47	43.983	2.0444	0.047
12	6	5	10	0.0543	4.29	24.480	1.8089	0.079
13	6	6	1	0.0294	5.35	21.299	0.9800	0.047
14	6	6	5	0.0459	4.64	33.052	1.5300	0.046
15	6	6	10	0.0415	4.29	21.523	1.3833	0.065
16	6	7	1	0.0235	3.53	1.123	0.7844	0.705
17	6	7	5	0.0299	4.46	21.973	0.9978	0.046
18	6	7	10	0.0262	4.57	1.609	0.8744	0.615
19	9	5	1	0.0161	4.57	9.246	0.5367	0.058
20	9	5	5	0.0299	4.45	6.251	0.9978	0.192
21	9	5	10	0.0247	4.20	8.198	0.8222	0.102
22	9	6	1	0.0157	3.78	16.732	0.5233	0.033
23	9	6	5	0.0159	4.11	47.277	0.5300	0.011
24	9	6	10	0.0142	4.29	4.641	0.4745	0.133
25	9	7	1	0.0057	3.51	3.893	0.1911	0.049
26	9	7	5	0.0142	3.42	18.716	0.4745	0.025
27	9	7	10	0.0119	3.42	17.930	0.3955	0.023

The analyses of the surface plotting (Figure 4) and the contour (Figure 5) were constructed using the multivariable regression equation with the initial pH was fixed at 6 whereas the glucose concentration (X, 3-9% w/v) and inoculum concentration (Y, 1-10% v/v) were variables.

Cell biomass = -0,133951 + 0,0352226 * X + 0,00424481 * Y + 0,0245063 * 6 – 0,00246152 * X * X – 0,00037716 * Y * Y – 0,00194259 * 6 * 6 + 0,000318094 * X * Y – 0,000986248 * X * 6 + 0,0000637523 * Y * 6 – 0,0000480647 * X * Y * 6

Base on the surface plotting and contour of LAB biomass analyzed by statistical software Statgraphics Centurion XV, it can be concluded that supplemental glucose concentration of 5.99% (w/v) (cell density of 10^7 CFU/mL), inoculum concentration of 6.37% (v/v), and initial pH at 6.0 were the optimum conditions for increasing of LAB biomass.

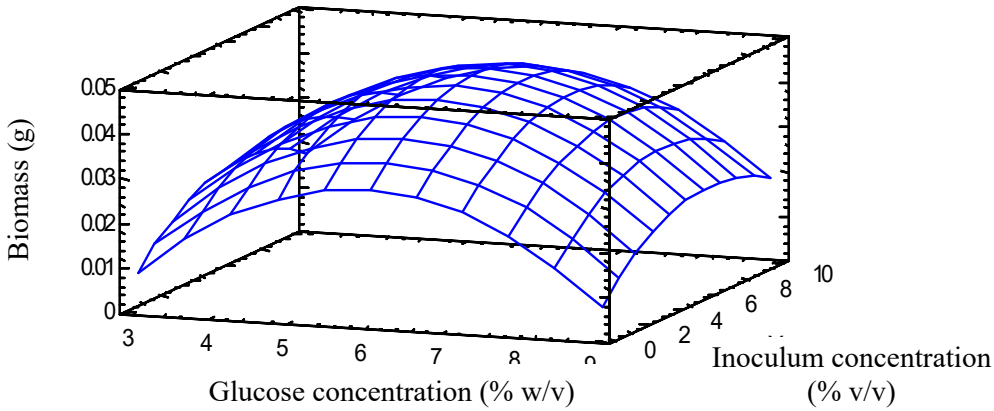

Fig. 4: The surface plotting analysis of condition effect on biomass production

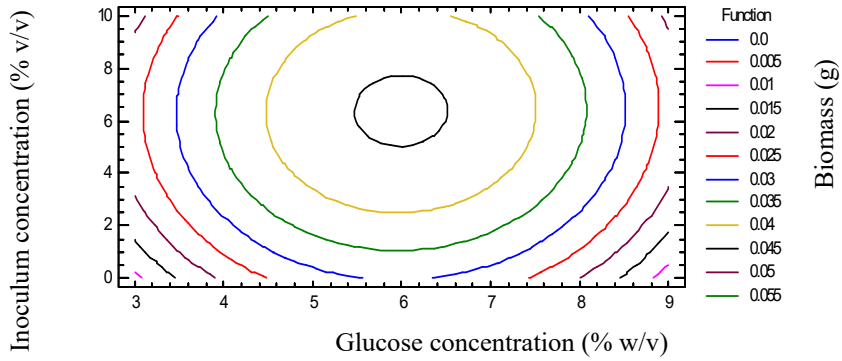

Fig. 5: The contour analysis of condition effect on biomass production

LAB, specifically, *Lactococcus lactis* strain, develop optimally at pH 6.5 (Andersen *et al.*, 2009). Thus, the optimum pH of this experiments is compatible with this study. In addition, the highest LAB biomass (2.04 g/L) reached at initial pH 5.0, glucose concentration of 6% (w/w), and inoculum concentration of 5% (v/v) is better than the research of Dung and Phong (2011). The highest biomass of this study was only 0.4 g/L with using the cheap medium of tofu sour liquid added with 10% brewer's grains. However, when culturing LAB in the MRS broth supplemented with glucose as the main substrate at 40°C, the average biomass was 4.38 g/L (Bai *et al.*, 2003). Therefore, the cheap medium of tofu sour liquid supplemented with glucose as the main substrate can be used to culture LAB to achieve high biomass.

4 CONCLUSIONS

All 10 strains selected based on the strongest antibacterial activity were identified into the *Lactobacillus* genus. Particularly, strain L2 and L6 belonged to *L. delbrueckii* while L9 and L10 were identified into *L. casie*. Six remained strains (L7, L21, L36, L37, L52 and L54) shared a high identity with *L. plantarum*. The genetic relations

between thermotolerant LAB strains were also determined based on the branching in phylogenetic trees of 16S rRNA gene. The favorable conditions for biomass production of thermotolerant LAB (L54 strain) were 5.99% (w/v) of glucose concentration, 6.37% (v/v) of inoculum concentration and initial pH at 6.0.

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