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ISOLATION, SELECTION OF *LACTOBACILLUS* STRAINS WITH PROBIOTIC AND ANTIOXIDANT ACTIVITIES

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ABSTRACT

This study was conducted to collect Lactobacillus strains which having probiotic and antioxidant activities from traditional Vietnamese fermented food. After isolation, screening and identification, we selected two strains, Lactobacillus plantarum and Lactobacillus brevis with probiotic activity and high antioxidant activity. These strains would be the microbiological agent for further studies about probiotic functional food with antioxidant and other valuable activities.

Keywords: Antioxidant***,*** *Lactobacillus*, probiotic.

TÓM TẮT

Phân lập, tuyển chọn chủng Lactobacillus có hoạt tính probiotic và khả năng kháng oxi hóa

Nghiên cứu này được thực hiện nhằm thu nhận chủng Lactobacillus có hoạt tính probiotic, đồng thời có khả năng kháng oxi hóa từ nguồn thực phẩm lên men truyền thống của Việt Nam. Sau quá trình phân lập, sàng lọc và định danh, chúng tôi chọn được hai chủng gồm Lactobacillus plantarum và Lactobacillus brevis có hoạt tính probiotic và khả năng kháng oxi hóa cao. Các chủng vi khuẩn này sẽ là tác nhân vi sinh vật cho những nghiên cứu sâu hơn về khả năng trở thành thực phẩm chức năng probiotic với hoạt tính kháng oxi hóa và những hoạt tính quý khác.

Từ khóa: *Lactobacillus*, kháng oxi hóa, probiotic.

1. Introduction

Oxidative stress is the result of imbalance between oxidants and antioxidant in body, which plays an importance role in disorder metabolic disease. Reactive oxygen species (ROS) are produced as a result of normal cellular metabolism of living organisms. Under various stress conditions, ROS were produced and highly reactive and reacted with cell structure such as proteins, DNA and lipids, leading to cell structure damages and metabolic disorder [1].

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [2]. Probiotics do not produce and also not contain toxins, tolerate conditions of the intestines. In particular, *Lactobacillus* is popular bacterium that has been shown to be safe, plays a major role in many important probiotics in the body, such as maintaining intestinal micro balance, digestive support, prevent intestinal disease and enhance metabolism [3]. Beside many functional foods have

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antioxidant activity, several studies have shown many probiotic bacteria, include *Lactobacillus*, have antioxidant activity such as free radical scavenging, metal chelating, lipid peroxidation antioxidant, increase antioxidant compounds in the fermentation products and enhance the antioxidant activity in cell [4], [5], [6].

Traditional Vietnamese fermented products are rich and varied in materials as well as fermentation [7]. *Lactobacillus* isolated from these sources showed different probiotic activity. In order to get a source of microbiological material for further studies on the antioxidant capacity of *Lactobacillus* strains, we isolated *Lactobacillus* from some traditional fermented food. The probiotic activity such as tolerance to acid and bile salts, adhesion and antibacterial activity. After screening for basic probiotic activity was test, selected strains were screened for a number of antioxidant activities including total reduction capacity and DPPH radical scavenging response. Experiments were taken *in-vitro* condition.

2. Material and method

2.1. Isolation of Lactobacillus

Some fermented foods like 'kim chi', 'ca phao', 'cai chua', 'nem chua', 'gia chua' were collected from markets and supermarkets in Ho Chi Minh City. Samples were pre enrichment in MRS broth (20 g/l glucose, 5 g/l yeast extract, 10 g/l meat extract, 10 g/l peptone, Sodium acetate 5 g/l, tri ammonium citrate 2 g/l, K_2HPO_4 2 g/l, $MgSO_4.7H_2O$ 0.2 g/l, $MnSO_4.H_2O$ 0.2 g/l, Tween 80 1 g/l) overnight before isolate. The isolation was performed by tenfold serial dilution, up to 10^{-6} and 10^{-7}, spread on MRS agar and incubated at 37^{o}C for 24 to 48 hours. After the incubation period, colonies were picked on the plate and sub_cultured. Some biochemistry test were used to select *Lactobacillus* strains, The selection criterias were Gram positive, catalase negative, aerobic and non-motif. Selected strains were kept in MRS slant agar at 4^{o}C.

2.2. Screening for basic probiotic activities

2.2.1. Acid and bile salt tolerance

The acid and bile salt tolerance assay was performed according to Shokryazdan et al., 2014 with some modifications [8]. *Lactobacillus* cells after secondary hyperplasia at 37^{o}C for 24 hours were collected by centrifugation at 4.000 rpm for 15 min and the palets were suspended in 5 ml phosphate buffer pH 6.5. 1 ml biomass of each strain was tranfered into 10 ml of MRS pH 2.5 medium supplemented with 0.3% bile salts. MRS medium pH 6.5 was used as a control. We incubated these samples at 37^{o}C for 3 hours, and then 100 µl of dilution from each samples of 10^{-6} and 10^{-7} was spread on MRS agar plates. These plates were incubated at 37^{o}C for 24 hours. After incubation, colonies on the plates were counted and enumerated as CFU/ml. The percentage survival of the cell was determined according to the formula: $(M_1/M_0) * 100\%$ where: M_1: colonies count (CFU/ml) at pH 2.5 and bile salt 0.3%, M_0: colonies counts (CFU/ml) at pH 6.5.

2.2.2. Auto aggregation assay

Auto aggregation assay was performed according to Kos et al., 2003 [9]. *Lactobacillus* cells after incubated overnight with MRS broth at 37ºC were harvested by centrifugation at 4.000 rpm for 15 minutes, pellets were washed twice and re_suspended in phosphate buffered saline (PBS). Cell suspensions (4 ml) were mixed by vortexing for 10 s and auto aggregation was determined during 5 hours of incubation at room temperature. Every hour, 0.1 ml of upper suspension was transferred to another tube with 3.9 ml PBS and absorbance was measured at 600 nm. The auto aggregation percentage was expressed as: $[1-(A_t/A_0)]*100\%$, where A_t: absorbance at time t = 1, 2, 3, 4, 5 hour and A_0: absorbance at t = 0.

2.2.3. Antimicrobial activity

Two pathogens, *Salmonella typhimurium* (Gram negative) and *Listeria monocytogenes* (Gram positive) from Biotechnology Department, University of technology were used to test antimicrobial activity of *Lactobacillus*. *Lactobacillus* bacteria were grown in 20 ml of MRS broth overnight at 37ºC, after that the culture were centrifuged at 4000 rpm for 15 minutes. The supernatant of each strain was used to test the antimicrobial activities of *Lactobacillus* strains.

After *Salmonella typhimurium* and *Listeria monocytogenes* were grown overnight in LB strains for 24 hours on LB broth medium (1 g/l peptone, 0.5 g/l NaCl, 0.5 g/l yeast extract), the culture were diluted to 10^{-4} and spread on LB agar plates. 40 µl supernatant of each *Lactobacillus* strains were spotted on the plates, and then incubated at 37^0C. Measurements the antibacterial zones of the strains of *Lactobacillus* to be tested were taken after 48 hours.

2.3. Antioxidant activity

2.3.1. 1, 1 - Diphenyl-2-picryl hydrazyl (DPPH) Free Radicals Scavenging Assay

Compounds with free scavenging activities will reduce the color of the DPPH solution (1, 1-diphenyl-2-picrylhydrazyl). This capability is determined by measuring the absorption at 517 nm. *Lactobacillus* strains were grown on 10 ml MRS broths at 37ºC for 24 hours. Bacteria cells were removed by cold centrifugation at 13.000 rpm for 5 minutes. The reaction mixture was prepared by mixing 40 µl supernatant and 460 µl ethanol and 1.5 ml DPPH 250 µM. Mixture was incubated in the dark for 30 minutes. The activity was determined by the decrease in absorbance at 517 nm. Blank sample only have solvent and reagent [10]. The percentage radical scavenging activity was calculated using the formula: $[(A_{blank} - A_{sample})/A_{blank}]*100\%$, where A_{blank} is absorbance of blank sample, A_{sample} is absorbance of sample.

2.3.2. Reducing power assay

Compounds with reducing power indicate that they are electron donors and can reduce oxidants. Reducing power assay was done according to Jayanthi et al., 2011 [11].

Lactobacillus strains were grown on 10 ml MRS broths at 37ºC for 24 hours. Bacteria cells were removed by centrifugation at 4.000 rpm for 20 minutes. 2.5 ml supernatant after centrifuged was mixed with phosphate buffer (2.5 ml) and potassium ferricyanide (2.5 ml). The mixture was kept at 50ºC for 20 minute. After cooling down, 2.5 ml of 10% trichloroacetic acid was added and centrifuged at 3000 rpm for 10 minutes. The upper layer of solution (2.5 ml) was mixed with 2.5 ml and then added 0.5 ml ferric chloride 1% solution. The absorbance was measured at 700 nm. Control sample is sterilized MRS broth. Increase absorbance of the reaction mixture indicates increase in reducing power.

2.4. Identification

The potential strains were identified by 16S rRNA sequencing method at Nam Khoa Service and Trade Co., Ltd.

3. Result and discussion

3.1. Isolation Lactobacillus

Different types of traditional Vietnamese fermented foods were used to isolate *Lactobacillus*. 29 *Lactobacillus* strains which 6 from Cai Chua, 2 from Ca Phao, 2 from Dua Gia, 17 from Kimchi, 1 from Nem Chua and 1 from commercial products were isolated. These strains were used in the investigation of basic probiotic activities, in order to find strains that can tolerate intestinal conditions.

Lactobacillus is one of the most important microorganisms in human intestinal and food industry. Some strains of *Lactobacillus* reside on the surface of intestinal mucosa. The ability to tolerate basic intestinal conditions such as low pH, bile salt as well as adhesion and antimicrobial activity are essential when choosing a probiotic microorganism, these characteristics are often assessed *in-vitro* in the preliminary selection of a probiotic strain. Although in-vitro experiments do not completely simulate actual conditions in the intestinal tract, these results could contribute to the basis of *in-vivo* experiments.

3.2. Basic probiotic activities

3.2.1. Acid and bile salt tolerance

To conduct acid tolerance test, we chose pH 2.5 because this is the average pH in human gastric. We chose bile salt at concentration 0.3% because although bile salt concentration in small intestinal is depending on the diet, pancreatic fluid concentration, 0.3% is normal level encountered in human intestine and in many studies, bile salt 0.3% has been used to determine probiotic *Lactobacillus* activity [8]. In one study about of 29 isolated strains of *Lactobacillus* isolated from traditional Greek fermented products, the results showed that most of the strains were not affected by pH 3 and 0.3 % bile salts [12]. Similarly, 23 lactic acid bacteria isolated from human enzyme and human milk are also well tolerated under pH 3 for 3 hours [13].

All 29 strains isolated *Lactobacillus* showed good tolerance to acid (pH 2.5) and bile salts 0.3%, survival rates over 60%. 20 *Lactobacillus* strains had a survival percentage greater than 85% and we selected those strain to test auto aggregation and antibacterial capacity. *Lactobacillus* strain Lac 29 was the best tolerance, with survival percentage over 94%.

3.2.2. Auto aggregation and antimicrobial activity

Investigation of self-adhesion is an important test to determine the adhesion activity of probiotic strains [9]. The 5-hour test self-adhesion of 29 strains of *Lactobacillus plantarum* in Tuo et al., 2013 showed differences between strains, suggesting that membrane proteins and proteins on cell surface play an important role in self-adherence and are also characteristic of each microbial species [14]. Adhesion to the intestinal wall helps long-term colonization in the gut, and is also a way of inhibiting the entry of pathogenic bacteria. In addition, the probiotic bacteria also secrete many antimicrobial compounds such as organic acids, bacteriocin, small molecule compounds such as diacetyl, hydrogen peroxide, etc., which inhibit and destroy invading bacteria [15].

After 5 hours take auto aggregation experiments, the ability to self-adhere of bacterial strains increased over time and change according to strain, Lac 28 showed the highest adhesion, reaching 71%. The remaining strains exhibited self-adhere activity from 20% to 50%. In term of antimicrobial activities of *Lactobacillus* strains in our collection, after taking antimicrobial experiments with two pathogens, *Salmonella typhimurium* (Gram-positive) and *Listeria monocytogenes* (Gram-negative), we found that *Lactobacillus* strains had different resistance. As a result, 11 strains were resistant to both pathogens, 3 were resistant to one of them, and the rest were resistant to none. Lac 16 showed the best resistance to both pathogenic bacteria.

After investigating basic probiotic activity, we selected the 8 strains that have highest probiotic activity, including Lac 4, Lac 8, Lac 9, Lac 16, Lac 19, Lac 28, Lac 29 and Lac 33. The results were summarized in Table 1. These strains showed were used to test antioxidant activity.

Table1. *Summarized probiotic activities of 8 Lactobacillus strains*

STT	Strains	Source	Survived in low acid and bile salts (%)	Auto aggregation (%)	Inhibit zone *Salmonella (mm)*	Inhibit zone *Listeria (mm)*
1	Lac 4	Cai Chua	86.45±2.13	28.45±0.03	16±0.17	4.97±0.65
2	Lac 8	Ca Phao	88.07±0.99	29.68±1.20	15.67±0.12	4.82±0.35
3	Lac 9	Ca Phao	88.44±0.20	34.30±1.66	11.67±0.15	4.25±0.57
4	Lac 16	Kimchi	87.28±0.98	47.31±1.29	21±0.14	5.64±0.12
5	Lac 19	Kimchi	86.64±2.79	43.38±0.47	9.67±0.15	3.70±0.71
6	Lac 29	Kimchi	94.42±2.84	38.55±1.71	6.5±0.64	2.86±0.07
7	Lac 28	Dua Gia	89.22±0.09	71.35±2.59	11.33±0.35	3.15±0.06
8	Lac 33	Kimchi	87.50±0.32	48.36±0.29	8.5±0.21	3.55±0.35

3.3. Antioxidant activity

After screening, 8 highest probiotic *Lactobacillus* strains were obtained. We investigated the antioxidant capacity of these strains, through DPPH Free Radicals Scavenging activity and total reduction assays. Results showed that all 8 strains had antioxidant activity and the antioxidant compounds of each strain were susceptible to different oxidative stresses, as shown in figure 1 and figure 2.

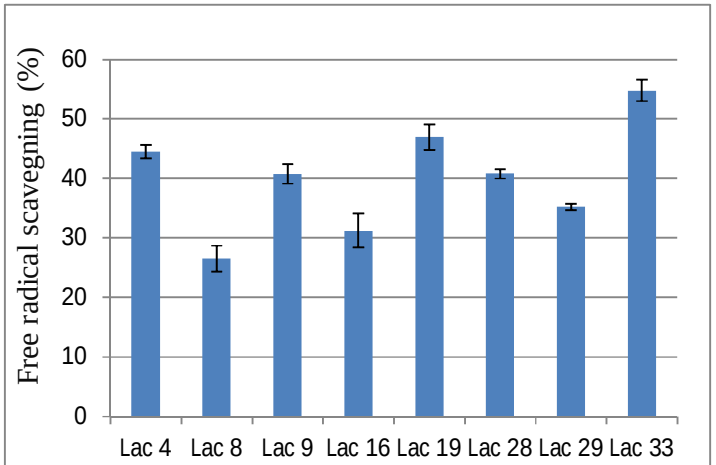

Figure 1. *DPPH radical scavenging activity*

We found that the strain Lac 33 showed the highest free radical scavenging activity, as its percentage scavenging activity was about 55%. Strain Lac 28 showed the highest total reduction capacity. Although Lac 33 shows the highest DPPH, the total reduction of the elimination reaction was not the highest. However, antioxidant differences are most apparent in strains such as Lac 8, Lac 16, Lac 4. These results showed that bacteria have capable of responding to different oxidizing agents. Lac 28 and Lac 33 have high probiotic activity and show the highest antioxidant potential for in these antioxidant assays.

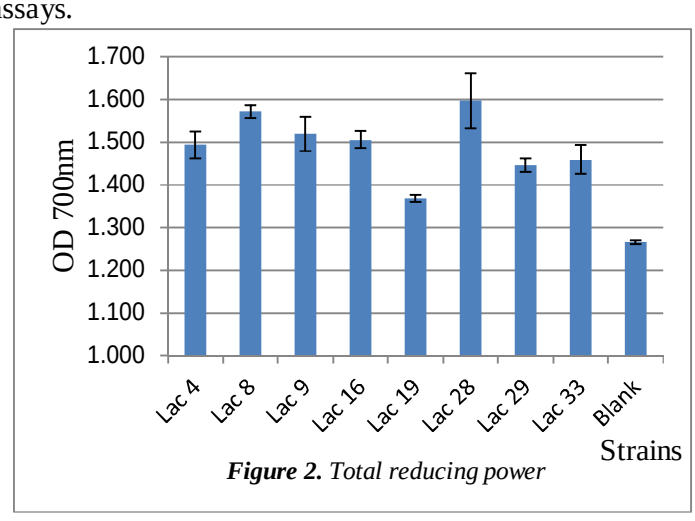

Figure 2. *Total reducing power*

Many studies have demonstrated antioxidant capacity in *Lactobacillus* strains. One strain of *Lactobacillus brevis* exhibited 48% DPPH radical-scavenging activity [16]. *Lactobacillus plantarum* strains isolated from traditional Indian fermented products resulted in a DPPH scavenging from 25 to 70% and ratio with cellular density [17]. Reduced capacity indicates a potential compound donor the electron and reduce the body's oxidative activitiy. High reduction potential suggests high antioxidant activity [11]. Exopolysaccharide (EPS) derived from the fermentation of some strains of *Lactobacillus* showed high antioxidant activity, capable of free radical scavenging, chelating metal, stimulate body's antioxidant

system [18]. Short peptides are hydrolyzed by *Lactobacillus*'s protease during milk fermentation showed high antioxidant activity [19]. *Lactobacillus* helps to increase the amount of antioxidant compounds released during fermentation, such as soybeans milk fermented with the beta-galactosidase activity of *L. rhamnosus*, resulted in higher isoflavone levels, antioxidant activity, free radical scavenging, and DNA protection, higher than non-fermented soybean milk [20].

Based on our result about probiotic and antioxidant activities, we selected two strains, Lac 28 and Lac 33, for further identification by 16S rRNA sequencing technique, to determine names and initial identification strain information.

3.4. Identification Lactobacillus

Identification by 16S rRNA sequencing and blast search resulted in Lac 28 isolated from Dua Gia and Lac 33 isolated from Kimchi with 100% homology were *Lactobacillus plantarum* and *Lactobacillus brevis*, respectively. The 16S rRNA sequence of the two strains and the comparison results were shown in figures 3.

Fugure 3. *Identification by 16S rRNA sequence of Lac 28 and Lac 33*

Two *Lactobacillus* strains we have identified are also found in many traditional fermented products in the word with many other activities. *L plantarum* isolated from traditional Chinese fermentation products exhibited high antioxidant capacity, both extracellular and intracellular, *in-vitro* and *in-vivo* trials [21]. *L. brevis* strain has been shown high probiotic activities against fungi and antioxidant activity, which enhanced antioxidant system in the body [16].

4. Conclusion

From traditional fermented foods, we isolated, screened and selected *Lactobacillus* has probiotic and antioxidant, we obtained two strains which high probiotic and antioxidant capacity, identified as *Lactobacillus plantarum* and *Lactobacillus brevis*. These strains could provide microbial agents for further studies on the antioxidant capacity of probiotic bacteria and functional food applications.

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