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Selection of lactic acid bacteria producing bacteriocin

Tuyển chọn vi khuẩn lactic có khả năng sinh bacteriocin

Research article

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Lactic acid bacteria were isolated from 10 samples of the traditionally fermented foods (5 samples of Vietnamese fermented pork roll and 5 samples of the salted field cabbage) and 5 samples of fresh cow milks collected from households in Vietnam. 22 strains of lactic acid bacteria were isolated for inhibition to *Lactobacillus plantarum* JCM 1149. Of these, only 2 strains including DC1.8 and NC1.2 have rod shape, the others have coccus shape. 7 strains showing higher antibacterial activity were selected for checking spectrum of antibacteria with indicator bacteria consistting of *Bacillus subtilis* ATCC 6633, *Enterococcus faecium* JCM 5804 and *Staphylococcus aureus* TLU. By which, 3 strains including NC3.5 (from Vietnamese fermented pork roll), DC1.8 (from salted field cabbage) and MC3.19 (from fresh cow milk) were selected because of their higher antibacterial ability. However, the antibacterial activity of the lactic acid bacteria can be based on their disposable compounds and some other antibacterial compounds produced during their growth (such as lactic acid, H_2O_2, bacteriocins, etc.). For seeking lactic acid bacteria with capability of producing bacteriocins, antibacterial compounds with protein nature, 3 above strains were checked sensitiveness to proteases (including protease K, papain, α – chymotrypsin and trypsin). Because bacteriocins are proteinaceous antibacterial compounds, so their antibacterial activity will be reduced if proteases are added. The result showed DC1.8 and MC3.19 were capable of producing bacteriocin during culture process. They were identified as *Lactobacillus acidophilus* and *Lactococcus lactis* and classified, respectively, based on analysis chemical characterisitcs by standard API 50 CHL kit and phylogeny relationship by 16s rRNA sequences.

Các chủng vi khuẩn lactic được phân lập từ 10 mẫu thực phẩm lên men truyền thống (5 mẫu nem chua, 5 mẫu dưa cải bẹ muối) và 5 mẫu sữa bò tươi được thu thập từ các hộ gia đình ở Việt Nam. 22 chủng vi khuẩn lactic đã được phân lập với tiêu chí có khả năng kháng lại vi khuẩn kiểm định Lactobacillus plantarum JCM 1149. Trong số đó, 2 chủng DC1.8 và NC1.2 có tế bào hình que, các chủng còn lại có tế bào hình cầu. 7 chủng thể hiện hoạt tính kháng khuẩn cao được lựa chọn để xác định phổ kháng khuẩn rộng hơn với ba loài vi khuẩn kiểm định Bacillus subtilis ATCC 6633, Enterococcus faecium JCM 5804 và *Staphylococcus aureus TLU. Từ đó lựa chọn được 3 chủng có hoạt tính kháng khuẩn cao hơn hẳn. Các chủng này gồm NC3.5 phân lập từ nem chua, DC1.8 phân lập từ dưa cải bẹ muối và MC3.19 phân lập từ sữa bò tươi. Tuy nhiên, hoạt tính kháng khuẩn của vi khuẩn lactic bao gồm những hợp chất nội tại có trong nó và cả những hợp chất được sinh ra trong quá trình phát triển của nó (như axit lactic, H_2O_2, bacteriocin, ...). Với định hướng tìm chủng vi khuẩn lactic có khả năng sinh bacteriocin, chất kháng khuẩn có bản chất protein, 3 chủng trên được kiểm tra độ nhạy cảm với các protease (gồm protease K, papain, α – chymotrypsin và trypsin). Do bacteriocin là chất kháng khuẩn có bản chất protein nên hoạt tính kháng khuẩn của chúng sẽ bị giảm nếu protease được bổ xung vào. Kết quả lựa chọn được chủng DC1.8 và MC3.19 có khả năng sinh bacteriocin. Hai chủng này được phân loại đến loài nhờ vào phân tích đặc điểm sinh hóa bằng kit API 50 CHL và mối quan hệ di truyền thông qua trình tự gen 16s rRNA. Kết quả phân loại đã xác định chủng DC1.8 thuộc loài Lactobacillus acidophilus và chủng MC3.19 thuộc loài Lactococcus lactis.*

Keywords: lactic acid bacteria, antibacterial activity, bacteriocins, Lactobacillus acidophilus, Lactococcus lactis, 16s rRNA

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1. Introduction

Food poisoning usually occurs simultaneously in many people due to eating foods that contain bacteria secreting toxins. Currently, the food preservation by using chemicals are restricted due to unwanted effects. Thus, people have been seeking natural compounds with capable of inhibiting microorganisms that cause food poisoning. These compounds can be lactic acid, H_2O_2, bacteriocin, etc.

Lactic acid bacteria are traditionally used as starter cultures involved in the fermentation of foods and beverages, because they contribute to flavour and aroma development and to spoilage retardation (Gilliland S.E., 1986). The preservative efficiency is mainly due to acidic conditions that these bacteria create in food during their development, but they are capable of producing antimicrobial compounds, including bacteriocins (De Vuyst L., Vandamme E.J., 1994).

Bacteriocins are proteinaceous antibacterial compounds that inhibit the development of Gram-positive bacteria and closely related species (De Vuyst L., Vandamme E.J.,1994). That is why bacteriocins of lactic acid bacteria are of particular interest as natural preservatives in foods safely and effectively (Stiles M.E.,1996). In Vietnam, the generation of lactic acid bacteria appear mainly in the traditional fermented foods, a resource for providing lactic acid bacteria abundantly.

This study was carried out with the aim of selection lactic acid bacteria that are capable of producing antibacterial compounds as bacteriocin from fermented foods and fresh milk of Vietnam to apply for food preservation.

2. Materials and methods

2.1. Materials

Sample sources: Five samples of Vietnamese fermented pork roll and five samples of salted field cabbage were collected from the households in Ve village, Hanoi, Vietnam, named NH1 to NH5 and DH1 to DH5, respectively. Five samples of fresh cow milks were from the households in Ba Vi, Hanoi, designated MH1 to MH5.

Indicator bacreria: *Lactobacillus plantarum* JCM1149*, Bacillus subtilis* ATCC 6633, *Enterococcus faecium* JCM 5804 and *Staphylococcus aureus* TLU were received from Institute of Biotechnology, Vietnam Academy of Science and Technology.

Bacteria culture media: MRS, MRS agar (De Man, Rogosa and Sharpe).

2.2. Methods

Isolation of lactic acid bacteria: 1 g of each sample were homogenized in 9 ml sterilized saline, diluting the samples to reach the concentration of $10^{-1} \div 10^{-8}$. To distinguish the acid-producing bacteria from other bacteria, 1% $CaCO_3$ was added to the MRS agar. 50ml of each concentration was spread directly onto the surface of MRS agar plates containing 1% $CaCO_3$. Incubation of these plates at 30°C within 24-36 hours. Colonies of lactic acid bacteria were identified by a clear zone around each colony, and were randomly selected from MRS plates and purified by replacing on MRS agar plates. Colonies were reselected and initially examined for Gram staining and production of catalase. Only Gram-positive, catalase-negative strains were selected.

Detection of antibacterial activity:

Method 1: The isolates were cultured in MRS media overnight. Using sterile toothpicks to move cultured solution onto MRS agar plates. The MRS plates were incubated for 24 hours at 30°C. After incubation, a layer of 15ml of MRS media containing 0,7% agar and 0,5% indicator strain of *L. plantarum* JCM1149 was coated on surface of the above MRS plates, kept at 4°C for 4÷6 hours, continued incubation at 37°C for 24 hours. Antibacterial activity of the lactic acid bacteria was determined based on inhibition zones around each colony (zones of no bacteria, mm) (Schillinger et al., 1989).

Method 2: The isolates were cultured in MRS at 30°C in 14 ÷ 16 hours, centrifuged to obtain cell free supernatant. The indicator strains were incubated in MRS media at 37° C overnight. 0,5% of the each indicator strain was added in MRS containing 0,7% agar, mixed well and poured into different plates. Creatting round wells on the plates with 10mm in diameter (d, mm). 100µl of the each cell free supernatant were dropped in the wells. Keepping at 4°C for 4 hours and then incubating at 37°C for 20 hours. Mesuring of inhibition zones through d (D, mm). Antibacterial activity was calculated by D-d (mm).

Sensitive capability with proteases: Determination antibacterial activity of the isolates by bacteriocin or other compounds, protease K, papain, α – chymotrypsin and trypsin were added to the cell free supernatants. Detection of antibacterial activity of the cell free supernatent was carried out by method 2. Positive control: the cell free supernatant was not added protease. *L. plantarum* JCM1149 was used as indicator strain in this experiment.

Classification of lactic acid bacteria: Shape and physiological analysis was carried out as described by Teuber M. (2003). Standard API 50 CHL kit was used for assessment of sugars fermentation according to the procedures described by the manufacturer.

Genetic tests: Sequence analysis of 16S rDNA were used to classify and identify the lactic acid bacterial isolates. PCR was carried out by using primers:

16SF: 5'-AGAGTTTGATCCTGGCTCAG-3'
16SR: 5'-TACGGTTACCTT GTTACGACTT-3'

The components of PCR: Buffer for Taq polymerase 10x: 5µl; dNTPs 10 mM: 2µl; Dream Taq polymerase 5000U/ml: 0,3µl; Primer 16SF 10pmol: 1µl; Primer 16SR 10pmol: 1µl; ADN template 20ng: 2 µl; DI water: 38,7 µl.

The process of PCR: set up at 95 ^oC in 3 minutes.; 95 ^oC in 1 minute; 55^oC in 1 minute; 68^oC in 1 minute 15 seconds; 70^oC in 7 minutes; keeping at 4 ^oC; repeatting 30 cycles. PCR products were checked by agarose gel electrophoresis and purified by Kit GeneJET™ Gel Extraction (Fermentas, Canada). PCR products were sequenced by ABI-377 Perkin Elmer machine.

Software MEGA3 was used to determine phylogenetic relationships of strains.

3. Results and discussions

3.1. Isolation and detection of antibacterial activity of lactic acid bacteria

Lactic acid bacteria were selected based on characteristics as follows: coccus or rod-shaped, gram-positive, catalase-negative, resolving $CaCO_3$ and having antibacterial activity to *Lactobacillus plantarum* JCM1149.

A total of 22 acid-producing bacteria were isolated from the samples (Table 1). Of these, 7 strains were isolated from Vietnamese fermented pork roll, 9 strains were isolated from salted field cabbage, others were isolated from fresh cow milk. All of 22 strains were capable of resistance to the development of *L. plantarum* JCM1149. There are 7 strains with high antibacterial activity (diameter of no bacteria zone >10mm), including NC1.2, NC3.5, DC1.8, DC2.10, DC4.13, MC2.18, MC3.19. These strains were mainly isolated from NH1, NH3, DH1, DH2, DH4, MH2 and MH3. Of the above 7 strains, only NC1.2 and DC1.8 were rod shape, the others were coccus shape.

Table1. Shape characteristics and antibacterial activity of the isolates

Strain number	Sample source	Shape	Antibacterial activity for *L. plantarum* JCM1149
NC1.1	NH1	C	+[1]
NC1.2	NH1	R	+++
NC2.3	NH2	C	++
NC3.4	NH3	R	++
NC3.5	NH3	C	+++
NC4.6	NH4	C	+
NC5.7	NH5	C	++
DC1.8	DH1	R	+++
DC1.9	DH1	C	+
DC2.10	DH2	C	+++
DC3.11	DH3	R	+
DC4.12	DH4	C	+
DC4.13	DH4	C	+++
DC4.14	DH4	R	++
DC5.15	DH5	C	++
DC5.16	DH5	C	+
MC1.17	MH1	C	++
MC2.18	MH2	C	+++
MC3.19	MH3	C	+++
MC3.20	MH3	C	++
MC4.21	MH4	C	++
MC5.22	MH5	C	+

C: coccus; R: rod; [1]diameter of no bacteria zone (+) d < 5mm; (++) d= 5÷10mm; (+++): d > 10mm

The 7 above strains were selected to check for inhibition to *Bacillus subtilis* ATCC 6633, *Enterococcus faecium* JCM 5804 and *Staphylococcus aureus* TLU as described by Method 2 (Figure 1). Among these 7 strains, DC1.8, NC3.19 and NC3.5 strains had highest inhibition to 3 indicator strains. These three strains were chosen to test sensitive capability with proteases.

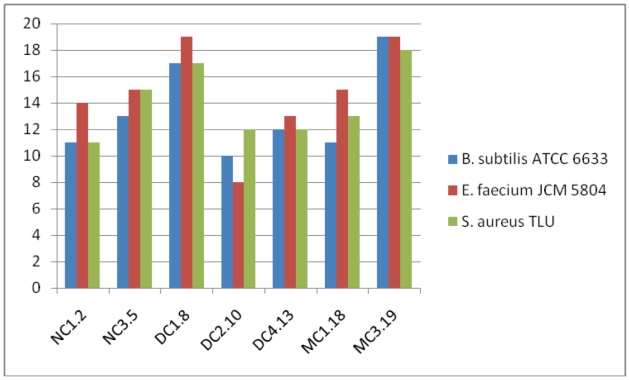

Figure 1. Ability of inhibiton to *B. subtilis* ATCC 6633, *E. faecium* JCM 5804 and *S. aureus* TLU

3.2. Sensitive capability with proteases

The antibacterial activity of the lactic acid bacteria can be based on disposable compounds, such as reuterin, reutericyclin, acid 2-Pyrrolidone-5-carboxylic. During of their growth, some other antibacterial compounds are produced as lactic acid, H_2O_2, bacteriocin, etc. (Ouwehand and Satu Vesterlund, 2004). According to Ladzinski et al (2000), bacteriocins are synthesized in ribosome and rapidly resolved by proteases in the human digestive system. Thus, sensitive capability with protease of these antibacterial compounds of the NC3.5, DC1.8 and MC3.19 were checked for seeking strains producing bacteriocin.

Table 2 showed that antibacterial activity of DC1.8 and MC3.19 strains was reduced comparing to positive control (C) when protease K (K), papain (P), α – chymotrypsin (α) and trypsin (T) were added to the supernatant of MRS media after culturing (example in Figure 2). While NC3.5 strain had antibacterial activity similar to positive control.

It indicated that antibacterial compounds produced by DC1.8 and MC3.19 strains were bacteriocins.

Table 2. Sensitive capability with protease of NC3.5, DC1.8 and MC3.19 strains

Protease	Inhibition to *L. plantarum* JCM1149 (D-d±2mm)		
	NC3.5	DC1.8	MC3.19
Protease K	15	3	4
Papain	13	5	5
α– chymotrypsin	14	2	0
Trypsin	14	4	4
Positive Control	15	18	19

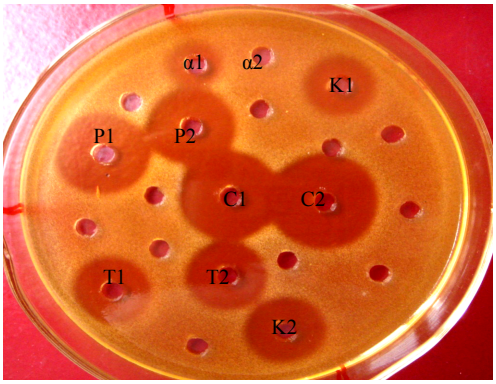

Figure 2. Sensitive capability with protease of DC1.8 (K1, P1, α1, T1) and MC3.19 (K2, P2, α2, T1); C1, C2: positive control

3.3 Fermentation of sugars of DC1.8 and MC3.19 strains

Biochemical properties were confirmed by fermentation efficacy of sugars based on standard API 50 CHL Kit (Table 3). Comparison with the database of APILAB Plus version 4.0 software revealed 97.9% homology of DC1.8 with *Lactobacillus acidophilus*, and 99.9% homology of MC3.19 with *Lactococcus lactis*.

Table 3. The fermentation efficacy of sugars of DC1.8 and MC3.19 strains based on standard API 50 CHL Kit

Substance	DC1.8	MC3.19	Substance	DC1.8	MC3.19
Control	-	-	Esculine	+	+
Glycerol	-	-	Salicine	+	+
Erythritol	-	-	Cellobiose	+	+
D-Arabinose	-	-	Maltose	+	+
L- Arabinose	-	+	Lactose	+	+
Ribose	-	+	Melibiose	-	+
D-Xylose	-	+	Saccharose	+	+
L-Xylose	-	-	Trehalose	+	+
Adonitol	-	-	Inuline	-	-
β-Methyl-xyloside	-	-	Melezitose	-	-
Galactose	+	+	D-Raffinose	+	w
D-Glucose	+	+	Amidon	+	+
D-Fructose	+	+	Glycogene	-	-
D-Mannose	+	+	Xylitol	-	-
L-Sorbose	-	-	β-Gentiobiose	+	+
Rhamnose	-	-	D-Turanose	-	-
Dulcitol	-	-	D-Lyxose	-	-
Inositol	-	-	D-Tagatose	+	w
Mannitol	w	+	D-Fucose	-	-
Sorbitol	-	-	L-Fucose	-	-
α-Methyl-D-mannoside	-	-	D-Arabitol	-	-
α-Methyl-D-glucoside	-	+	L-Arabitol	-	-
N-Acetyl glucosamine	+	+	Gluconate	-	+

Substance	DC1.8	MC3.19	Substance	DC1.8	MC3.19
Amygdaline	+	+	2-ceto-gluconate	-	-
Arbutine	+	+	5-ceto-gluconate	-	-

-: negative; +: positive; w: weak

3.4 Analysis of 16s rRNA gene of DC1.8 and MC3.19 strains

PCR products of 16S rRNA gene of DC1.8 and MC3.19 were approximately 1500bp. Sequences of 16S rRNA gene were analyed and compared to some homologous sequences in GENBANK. MEGA3 sofware was used for assessment phylogeny relationship of survey sequences (Figure 3).

The phylogeny relationship between groups of organism are often presented in the form of phylogenetic tree. The ends of the branches show exists of organism groups, the branching points of the tree denotes our ancestors, and length of branches indicates evolution time or differentiation of DNA sequences (Nei M. and Kumar S., 2000).

Figure 3 specified DC1.8 strain closely related to *Lactobacillus acidophilus* JCM2121. MC3.19 strain closely related to *Lactococcus lactis* NIRD712 and *Lactococcus lactis* JCM7638.

Furthermore, bootstrap values in branched position were very high, and bootstrap values >70% would be equivalent to 95% reliability (Hillis D.M. and Bull J.J., 1993). That proved the reliability in phylogeny analysis result.

The result of classification by genetic analysis of 16s rRNA gene was same to result of chemical characteristics. That also coincided with shape characteristic, that's supposed genus *Lactobacillus* is rod-shape and genus *Lactococcus* is coccus-shape (Salminen S. et al., 2004).

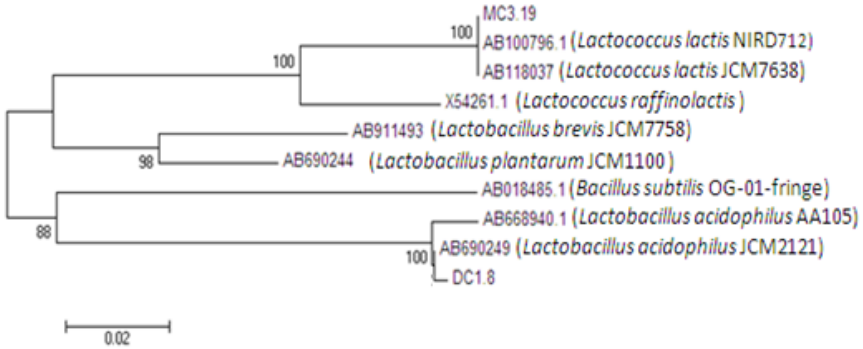

Figure 3. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences. Numbers at the root of the branch are the bootstrap value.

4. Conclusion

From 15 samples (consist of 5 samples of Vietnamese fermented pork roll; 5 samples of salted field cabbage and 5 samples of fresh cow milks) 22 strains of lactic acid bacteria were isolated. Of 22 isolates, 3 strains were selected from 3 different sample sources because of high inhibition to *Lactobacillus plantarum* JCM1149, *Bacillus subtilis* ATCC 6633, *Enterococcus faecium* JCM 5804 and *Staphylococcus aureus* TLU. However, only 2 strains (DC1.8 isolated from salted field cabbage and MC3.19 isolated from fresh cow milk) were sensitive with proteases. That means antibacterial compounds of them are bacteriocin.

These strains were classified base on physicochemical properties and genetic relationship of 16s rRNA gene. The results showed that DC1.8 strain belong to *Lactobacillus acidophilus,* MC3.19 strain belongs to *Lactococcus lactis*.

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