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Identification of Lactic Acid Bacteria Proteolytic Isolated from An Indonesian Traditional Fermented Fish Sauce Bakasang by Amplified Ribosomal DNA Restriction Analysis (ARDRA)

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Abstract: Lactic Acid Bacteria (LAB) is bacteria which has an important role in the process of fermentation of organic matter. 'Bakasang' is traditional fermented fish product made from the guts of fish mainly Kastsuwonus pelamis L as well as other small fish and fish eggs. 'Bakasang' is well know as typical food of North Sulawesi (Manado) LAB have proteolytic activity that degrade fish proteins into bioactive eptide that could performe as anthyhipertensive compound during fer,emtatopm. This study aimed to identify LAB that produce strong proteolytic abilities. This study revealed that two isolates that were desigh as pediococcus B3.5 and Pediococcus B9.7 based on phenothypic characterization have the strong proteolytic abilities. These strains were further identified by Amplified Ribosomal DNA Restriction Analysis (ARDRA) was carried out with one restriction endonuclesae enzyme (Hae3). Isolates LAB proteolytic from bakasang have a identical character with *Pediococcus acidilactici*.

Key word : Bakasang, Lactic Acid Bacteria Proteolytic, ARDRA.

Introduction

Bakasang' is traditional fermented fish product made from the guts of fish mainly *Katsuwonus pelamis* L as well as other small fish and fish eggs. 'Bakasang' is well known as a typical food of North Sulawesi (Manado). Traditional fermentation process generally takes place spontaneously that involve microbes present in the raw material so that there are different types of microbes that grow according to changes in its environment. Lactic acid bacteria are found as microbial dominant in the process of traditional fish fermentation and has an important role to hydrolyze proteins and accumulate the free amino acids during fermentation, lowers high blood pressure, cholesterol and glucose[1,2,3].

Fermentation that using lactic acid bacteria will produce metabolites that may provide health effects on the body. Several peptide that degradation protein of products such as wheat, soy, tuna, sardines, bonito, mackerel and milk by lactic acid bacteria that could performe as ACE inhibitors (Angiotensin I Converting Enzyme) and can serve as an antihypertensive[4,5,6]. Some studies have also been conducted on fish products indicating the antihypertensive peptides. Antihypertensive peptides found in fish by salting and fermentation[7], bonito fish[6]. Mackerel extract fermented with *Lactobacillus plantarum*, *Lactococcus lactis* subsp. *lactis*, Lactobacillus *helveticus* and *Pediococcus pentosaceus* capable of lowering blood pressure tukus SHR[8].

Fish as a raw material of bakasang is a source of protein that has the amino acid and sequence of certain amino acids in accordance with the structure of the peptide ACE inhibitor and the presence of lactic acid bacteria involved in the degradation of fish protein into an ACE inhibitor shows that bakasang found any ACE

inhibitor[9]. Objectives of the experiment was to identify lactic acid bacteria on bakasang that produce strong proteolytic abilities by Amplified Ribosomal DNA Restriction Analysis (ARDRA).

Material and Methods

Isolation of Lactic Acid Bacteria

Guts of Big fish, meat and egg fish were collected from local market in Manado. These samples were transported to the laboratory using cool box (4°C) and cut into small pieces and mashed. Salt and rinse reagent were added and mix thoroughly. The mixture was packed into bottles, corked and then incubated at 37ºC for 7 days. LAB were isolated from sample of bakasang. Ten gram of samples were taken aseptically and homogenized in 90 ml of NaCl solution. Serial dilutions up to 10^{-7} were prepared and appropriate dilutions were plated onto de Man Rogosa and Sharpe Agar supplemented with $CaCO_3$ 1%, Na Azida and Syclo-hexamide. All plates were incubated at 37°C for 48 hours. Only lactic acid producing bacterial colonies were selected. This can be observed from clear zones around the colonies which indicated the dissolving of $CaCO_3$ by an acid. Colonies with different morphology were counted, picked up and purified by restreaking on the same medium.

Cell morphology, Gram staining and catalase test, motility, non-spore forming were performed as a preliminary screening for lactic acid bacteria. The selected lactic acid bacteria were maintained as stock cultures at -80 °C in 10% skim milk and 20% glycerol.

Screening of Lactic Acid Bacteria for Proteolytic Ability

Isolates which gives the characteristics of lactic acid bacteria was tested proteolytic activity by using the medium of skim milk agar (composition: 2% skim and 2% agar). Inoculation is done by inserting isolates on medium skim agar with a sterile toothpick, then incubated at room temperature for 24 hours. Proteolytic LAB isolates characterized by the formation of clear zones in the medium skim agar and proteolytic activity was measured by the ratio of the diameter of clear zone/the diameter of colony (R)[10].

Identification of Lactic Acid Bacteria Isolated

Characterization of LAB proteolytic isolates performed on isolates that have relatively high proteolytic activity ($R \geq 1,5$ mm). The isolates LAB showing the highest proteolytic abilities were identified based on phenotypic and genotypic characterization. Phenotypic characterization was performed by examining cell morphology, motility and spore forming of isolate. Isolate was gram stained and tested for catalase production, and were preliminarily identified based on the phenotypic properties such as gas production from glucose, growth at different temperatures as well as the ability to grow in different concentrations of sodium chloride and pH in De Man, Ragosa, Sharpe (MRS) broth[11]. Genotypic characterization was conducted by amplified ribosomal DNA restriction Analysis (ARDRA).

Genomic DNA Extraction

Extraction of genomic DNA were done as described by Pospiech and Neumann (1995) in Lawalata et al.[12]. Bacteria were cultivated on MRS broth. After 2-3 days of cultivation at 30 °C, 1.5 mL of biomass was collected by centrifugation (5 min, 13.000 rpm). The pellet was resuspended in 400 µL SET buffer (75mM NaCl, 25mM EDTA, 20 mM Tris, pH 7,5), 50 µL Lysozyme (10 mg/mL), 20µL Proteinase K (15mg/mL) were added and incubated at 37ºC for 1 h. 50µl SDS 10% was added and incubated at 65ºC for 1 h. 400µL cooled Chloroform was added and centrifuged (13.000 rpm 10 min). The aqueous layer was re-extracted with isopropanol (1:1v/v) and then incubated at -20ºC overnight. DNA was centrifugated and washed with cooled ethanol 70% and then supernatant was removed. The purify of DNA solution was resuspended with TE buffer and checked spectrophotometrically at λ_{260} and λ_{280} nm.

16S rRNA gene Amplification

The 16S rRNA genes were amplified from purified DNA of the strain using a commercial kit (Mega Mix Blue ® and universal primers 27f(5'-AGAGTTTAGTCCTGGCTCAG-3') and 1492r(5'-GGTTACCTTGTTACGACTT-3') for 16S rDNA by Thomas[13]. The condition of amplified gene fragment : pre-denaturation of the target DNA at 96ºC for 4 min followed by 30 cycles at 94ºC for 1 min, primer annealing at 51,5ºC for 1min and 30 s and primer extension at 68ºC for 8 min. PCR was completed with 10 min

elongation at 68 °C followed by cooling to 4 °C. PCR product was visualised by electrophoresis on a 2 % (w/v) agarose gels, stained with ethidium bromide in the presence of a 1 kb ladder. The parameters for the electrophoresis were 90 V for 30 min[12] .

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Result of 16S rRNA gene purification with Microclean Kit (Microzone Ltd) were then separated with *Hae*III enzyme endonuclease. DNA phragments produced were electrophorised in 2% agarose gel with 90 voltage 30 min using electrophoresis buffer solution TAE 0,5X [14]. Differences pf DNA polymorphism pattern were analysed using *Multi Variate Statistical Package* (MVSP) Plus-version 2.0 with UPGMA (*Unweighted Pair Group Method with Arithmatic Averages)* algoritm[15].

Result and Discussion

Isolation of Lactic Acid Bacteria

Samples of bakasang were used for isolation of lactic acid bacteria. 50 isolates of LAB in which production clear zone around theirs colonies were obtained from *bakasang*. The clear zone appearance is due to the dissolution of $CaCO_3$ on MRS medium by acid agent[16]. Among the 50 isolates were rearrange and confirmed as LAB in amount of 30 isolates. All these isolates were gram positive, rods and cocci, appeared singly, in pair, chain, tetrad. Cell were non motile and non sporing, they gave negative reaction for catalase. These strains were then classified into genus level using profile matching method. Based on the profile matching method that 30 isolates separated into four groups. Group I were putatively identified as genus *Lactobacillus*. Group II were represented as cocci (tetrad) homofermentative which were identified as genus *Pediococcus*, Group III were represented as cocci (pair, chain) homofermentative which were identified as genus *Streptococcus/ Enterococcus*, Finally, group IV were identified as genus *Leuconostoc*. It was concluded that lactic acid bacteria isolated from *bakasang* are dominated by *Lactobacillus, Pediococcus*, *Streptococcus/Enterococcus* and *Leuconostoc*.

Screening of Lactic Acid Bacteria for Proteolytic Ability

Thirty LAB isolates obtained from bakasang, screened by its ability proteolytic to use media skim milk (2%). Isolates were forming a clear zone on skim milk medium that is could performe as lactic acid bacteria that have the ability proteolysis.

Based on the screening results, obtained 3 kinds of characteristics: 1) isolates that grow and form clear zones, 2) isolates that grow but does not form a clear zone and, 3) isolates that did not grow. Isolates LAB grow and establish a clear zone indicates that the isolates of LAB has the ability to hydrolyze casein in skim milk, while the isolates that grew but did not establish a clear zone allegedly showed that this isolate able to utilize other nutrients contained in the medium skim milk but do not have the ability to degrade casein milk in incubation conditions (room temperature). Isolates that do not grow LAB isolates showed that they could not grow on media and incubation temperatures are applied. Data proteolytic LAB isolates screening results are presented in Table 1.

Table 1. The Number of Lactic Acid Bacteria (LAB) and Number of Lactic Acid Bacteria (LAB) proteolytic isolated from bakasang

Type of Isolates	Number of Isolates
LAB acid forming	50
LAB	30
LAB Proteolytic	10
LAB Proteolytic R $\geq$ 1,5	2
LAB non Proteolytic	20

Identification of Strain LAB proteolityc

Identification of strain B3.5 and B9.7 which have highest proteolytic activity were done based on phenotypic and genotypic characterization. The phenotypic properties of strain B3.5 and B9.7 were summarized in Table 2 and Table 3.

Table 2. Identification genus level (*generic assignment*) LAB proteolytic isolated from bakasang based on *Profile Matching* Method

Key Character*	*Enterococcus*	*Lactobacillus*	*Leuconostoc*	*Pediococcus*	*Streptococcus*	B3.5	B9.7
Cell Shape Coccus	+	-	+	+	+	+	+
Gram Stain	+	+	+	+	+	+	+
Cell Arrangement Ttetrad	-	-	-	+	-	+	+
Spore Formation	-	-	-	-	-	-	-
Catalase	-	-	-	-	-	-	-
Gas Production	-	-/+	+	-	-	-	-
Motility	-	-	-	-	-	-	-
Homofermentatif	+	+	-	+	+	+	+
Heterofermentatif	-	-	+	-	-	-	-

*Key Character description genus *Lactobacillus, Enterococcus/Streptococcus, Leuconostoc* dan *Pediococcus* berdasarkan *Bergey's manual Systematics of Bacteriology* [18].

Table 3. Phenotypic characteristics of the LAB Proteolytic from Bakasang Sample

Characteristic	*P.acidilactici* FNCC 0110	*P.pentosaceus* FNCC 0019	B3.5	B9.7
Gram stain	+	+	+	+
Shape	Cocci	Cocci	Cocci	Cocci
Cell arrangement	Tetrad	Tetrad	Tetrad	Tetrad
Production gas from glucose	-	-	-	-
Catalase	-	-	-	-
Spore formation	-	-	-	-
Motility	-	-	-	-
Fermentation type	Homo	Homo	Homo	Homo
Growth on :				
- 10ºC	+	+	+	+
- 40ºC	+	+	+	+
- 45ºC	+	+	+	+
- 50ºC	+	-	+	+
Growth on :				
- pH 4,5	+	+	+	+
- pH 8,0	+	+	+	+
- pH 9,0	+	+	+	+
Growth on :				
- 6,5% NaCl	+	+	+	+
- 10% NaCl	+	+	+	+
- 18% NaCl	-	-	-	

From the morphological examination, strains of B3.5 and B9.7 which have highest proteolytic activity were gram positive and coccus (tetrad). These isolates were catalase negative, facultative anaerobes, non endospora forming, non motil. Based on these characteristics, the isolates were phenotypically identified as

member of Genus *Pediococcus* and member of species *P.acidilactici* [18]. Genotypic characterization study was carried out to identify strains B3.5 and B9.7 after studying phenotypic characteristics.

ARDRA

An about 1.5-kb portion of the 16S rRNA gene from 4 cultures of lactic acid bacteria was amplified using the universal primers 27f and 1492r. The result of 16S rRNA gene amplification were then separated by using *Hae*III restriction enzyme. Polymorphism patterns as a result of 16S rRNA gene cleavage is shown in Figure 1 and 2.

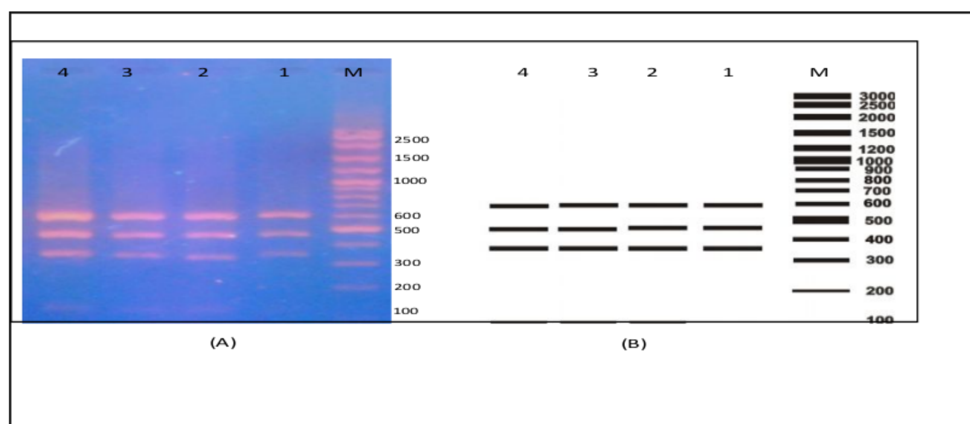

Figure 1. ARDRA profiles of 16S rRNA gene LAB proteolytic and reference strains were digested with *Hae3 enzyme* and representative diagrams. (A) ARDRA profiles of 16S rRNA gene LAB proteolytic and reference strains were digested with *Hae3 enzyme,* (B) Representative diagrams of ARDRA profile16S rRNA gene LAB proteolytic and refence strains wrere digested with *Hae3 enzyme* . Lane 1 (*P. pentosaceus* FNCC 0019), Lane 2 (*Pediococcus acidilactici* FNCC 0110, Lane 3 (B3,5), Lane 4 (B9.7), M (Marker 100 bp Ladder).

According to the given results of ARDRA in Figure 1, 16S rRNA gene cleavage from analyzed bacterial isolates shows two different patterns of DNA polymorphism. Each DNA polymorphism pattern consists of 3-4 DNA band with molecule weight varies from 100bp – 600bp. DNA polymorphism pattern derived from ARDRA method was not only used to differentiate bacterial in species level and higher taxonomy but also to define the familial relationship between analyzed bacterial isolates[19]. Familial relationship of analyzed bacterial isolates in this research was performed by construction dendrogram form (Figure 2).

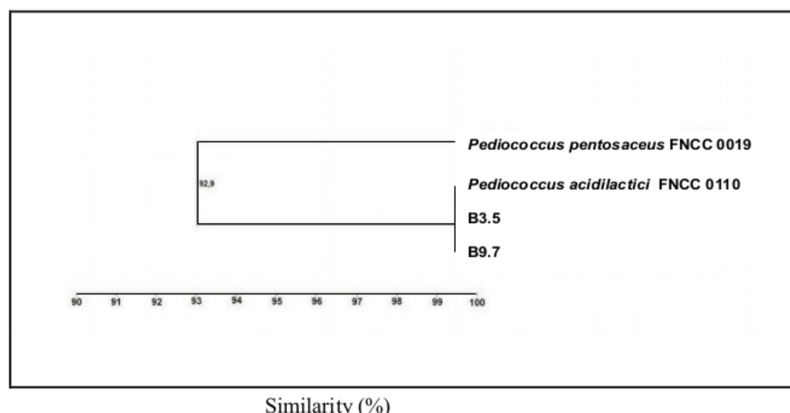

Figure 2. Dendrogram obtained by ARDRA profile of 2 strains lactic acid bacteria proteolytic and 2 reference strains member of *Pediococcus* genus generated from restriction by *Hae*III enzyme restriction.

Dendrogram regerated from restriction by *Hae*III enzyme restriction in Figure 2 show that strains of B3.5 and B9.7 could performe to be member of the same species with similarity index 100%. Those isolates showed that they have the taxonomy or very close relationship. These two strains are within one cluster with reference strains of *Pediococcus acidilactici* 0110 FNCC with similarity index 100%, its means that the two strains are identical to the reference strain of *Pediococcus acidilactici* FNCC 0110. These results are consistant with data of phenotypic characteristics.

Fingerprint analysis ARDRA based on differences in DNA polymorphism pattern formed due to differences in the introduction of a restriction enzyme *Hae*III position on the 16S rRNA gene. Differences in the pattern of DNA poliforfisme indicate a difference in the nucleotide sequence of 16S rRNA gene proteolytic LAB isolates were analyzed. Differences in the nucleotide sequences also showed that rRNA gene proteolytic LAB isolates analyzed have different taxonomic position. Although the DNA fingerprint profile generated by cutting with the restriction enzyme *Hae*III can not distinguish the two rRNA proteolytic LAB isolates selected.

The ARDRA technique, as applied to bacteria, is based on 16S ribosomal genes as DNA targets, which may increase the sensitivity of this molecular biotechnique[14]. DNA fingerprint profiles from restriction analysis of 16S rRNA gene with *Hae*III enzyme indicate that the reference strain can be clearly distinguished by differences in the DNA bands. Thus, this analysis shows that based on the DNA fingerprint profiles of all test strains in contrast to the reference strain *P. pentosaceus* FNCC 0019 but very similar to the reference strain of *Pediococcus acidilactici* FNCC 0110.

Fingerprints ARDRA is a method that is fast, simple and to compare the 16S rDNA fragments do not require information that was amplified DNA sequences [20,21,22]. In addition, DNA polymorphism pattern obtained through ardra fingerprint method can be used to differentiate bacteria at the species level and higher taxonomic levels[23].

Conclusion

ARDRA is an accurate and reliable method capable of discriminating among the test strains (B3.5 and B9.7) and reference strains (*P. pentosaceus* FNCC 0019 and *Pediococcus acidilactici* FNCC 0110). The two strains (B3.5 and B9.7) are identical to the reference strain of *Pediococcus acidilactici* FNCC 0110. These results are consistant with data of phenotypic characteristics.

References

1. Hu, Y., W. Xia and C. Ge. 2007. Characterization of Fermented Silver Carp sausages Inoculated with Mixed Starter Culture. *Food Science and Technology*
2. Yin, L.J., C.L and S.T. Hang. 2002a. New Technology for Producing Paste-Like Fish Products Using Lactic Acid Bacteria Fermentation. *Food Science.* 76. No.8. p. 3114-3118.
3. Yin, L-J.,C-L Pan., dan S-T Jiang. 2002b. Effect of Lactic Acid Bacterial Fermentation on The Characteristics of Minced Mackerel. *Journal of Food Science.* Vol. 67.(2) : 766-792.
4. Seppo L., Jauhininen, T., Poussa, T., dan Korpela. R. 2003. A Fermented Milk High in Bioactive Peptides has Blood Pressure-Lowering Effect in Hypertensive Subject. *American Journal of Clinic Nutrition*. 77 (2); 326-330.
5. Itou, K, and Akahane, Y. 2004. Antihypertensive Effect of *heshiko*, a Fermented Mackarel Product, on Spontaneously Hypertensive Rat. *Fisheries Science*. (70); 1121. *Design.* 9 : 1309-1323.
6. Yoshikawa, M., Keichi, Y., Masayosi, H., Ryouchi, Y., Hiroyuki, F. 1994. Method of Producing an Angiotensin Converting Enzyme Inhibitor-Containing Compound. United State Patent 5369015. www.freepatentsonline.com/5369015.html.
7. Kim S.B., Lee, T-G., Park, Y-B., Yeum, D-M., Kim, O-K., Gyun, H-S., Park, Y-H. 1993. Characteristic of Angiotensin Inhibitory Converting Enzyme Derived From Fermented Fish Product (Salted and Fermented Anchovy). *Bulletin Korean Fishery Society* 26 (4), 321-329.
8. Yin,L-J.,Tong,Y-L.,and Jiang,S-T. 2005. Effect of Combining Proteolysis and Lactic Acid Bacteria Fermentation on the Characteristics of Minced Mackerel. *Journal of Food Science.* Vol. 70.(3) : 186-192.

9. Lawalata, H.J and Gedoan S.P. 2014. Bakteri Asam Laktat Pada Fermentasi Bakasang Sebagai Penghasil *Angiotensin Converting Enzyme* Inhibitor. Penelitian Hibah Bersaing (Desentralisasi), Lembaga Penelitian Unima. Manado.
10. Vichasilp, C., Sanjindavong, M., Wilaipun,P. 2008. The Use of Selected Lactic Acid Bacteria Isolates for Acceleration of Fermented Fish (*Pla-ra*) Process. *Kasertsart University Fisheries Research Bulletin.* 32:3
11. Dykes, G.A., Britz, T.J., von Holy, A., 1994. Numerical taxonomy and identification of lactic acid bacteria from spoiled, vacuum packaged Vienna sausages. *Journal of Applied Bacteriology* 76, 246–252.
12. Lawalata, H.J., Sembiring, L.,and Rahayu, E.S. 2011. Molecular Identification of Lactic Acid Bacteria Producing Antimicrobia Agents From Bakasang, An Indonesian Traditional Fermented Fish Product. *Indonesian Journal of Biotechnology*, 16 (2), 93-99.
13. Thomas, P. 2007. Isolation and Identification of Five Alcohol-defying Bacillus spp. Covertly Associated with In Vitro Culture of Seedless Watermelon. *Current Science* 92 (7) 983-986.
14. Mendoza-Espinoza, A., Koga, Y, and Zavaleta, A.I. 2008. Amplified 16S Ribosomal DNA Restriction Analysis for Identification of Avibacterium paragallinarum. Avian Diseases, 52:54–58.
15. Sembiring, L. & Goodfellow, M. 2001. The Application of Molecular Biology in The Development of Streptomycete Systematics. *Biology.* 2(11) : 629-653.
16. Panthavee, W., Pramuan, S and Nisakornn W. 2007. Identification and Evaluation of Lactic Acid Bacteria for Pla-Som (Fermented Fish) Starter. The 2nd International Conference on “Fermentation Technology For Value Added Agricultural Products” May 23-25, at Kosahotel, Kohn-Kaen, Thailand
17. Sneath, D.H.A., Nicholas, S.M., Elisabeth, M & Holt, J.G. 1987. *Bergey`s Manual of Systematics Bacteriology* Vol 2. Williams and Wilkins. USA.
18. Holt J.G., Krieg N.R., Sneath, P.H.,Stanley, J.T. and Williams, S.T. 1994. Bergey`s Manual of Determinative Bacteriology. Ninth Edition. Williams and Wilkins.USA.
19. Cruz, L.M., Souza, E.M., Weber, O.B., Baldani, J.I., Dobereiner, J, and Pedrosa, F.O. 2001. 16S ribosomal DNA charactetization of Nitrogen-fixing bacteria isolated from Banana (*Musa* spp.) and Pineapple (*Ananas comosus* (L) Merril). *Applied and Environmental Microbialogy* 2375-2379.
20. Laguerre, G., Allard, M., Revoy, F.& Amarger, N. 1994. Rapid Identification of Rhizobia by Restriction Fragment Length Polymorphism Analysis of PCR-Amplified 16S rRNA Genes. *Applied and Environmental Microbiology.* 60: 56-63.
21. Liu, W., T.L. Marsh, H. Cheng., L.J. Forney. 1997. Characterization of Microbial Diversity by Determining Terminal Restriction Fragment Length Polymorphisms of Genes Encoding 16S rRNA. *Applied and Environmental Microbiology,* 4516-4522.
22. Rodas, A.M., Ferrer, S. & Pardo, I. 2003. 16S-ARDRA, A Tool For Identification of Lactic Acid Bacteria Isolated from Grape Must and Wine. *Systematic & Applied Microbiology*. 26: 412-422.
23. Reinhardt, E.L., Ramos, P.L., Manfio, G,P., Barbosa, H.R., Pavan, C. & Filho, C.A.M. 2008. Molecular Identification of Nitrigen-fixing Bacteria Isolated fron Brazilian Agriculture Plants at São Paulo State. *Brazilian Journal of Microbiology.* 39, 414-422

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