

Isolation and identification of lactic acid bacteria producing β glucosidase from Indonesian fermented foods

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Abstract

Indonesia has many kind of fermented foods such as tempe, fermented rice, fermented cassava, and fermented vegetable. Lactic acid bacteria that can produce β glycosidase most likely in the fermented foods from plants materials. The objectives of the research were to isolate lactic acid bacteria producing β glucosidase enzyme from Indonesian fermented foods and identify the isolates to their species. Twenty one isolates were identified using 3 different methods, including phenotypic and genotypic identification from Bergey's manual of Systematic Bacteriology, API 50 CHL Kits, and 16S rRNA sequence based methods. Finally, 20 isolates were close related as *Pediococcus acidilactici*, *P. pentosaceus* and *P. lolii* AB362985.1 and one isolate identified as *Lactobacillus pentosus-plantarum* group.

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Introduction

Beta glucosidase(s) (β -D-glucoside glucohydrolase, EC 3.2.1.21) are enzymes that hydrolyse glucosidic bonds between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety. One of bioactive compounds, with β glucoside linkage, which naturally present in fruits and vegetables was anthocyanin. This bioactive compound is polyhydroxy or polymethoxy derivatives of 2-phenyl-benzopyrylium. Most of them are present in plants attached to sugars as mono, di, or triglycosides by α - or β - linkage that most frequently at C-3 of the aglycons (anthocyanidins).

Experimental data indicate that dietary anthocyanins may protect against cardiovascular disease, anti-inflammatory, therapeutic effect for diabetic, and also act as anticancer (Kano *et al.*, 2002; Oki *et al.*, 2002; Bagehi *et al.*, 2004; Wang and Stoner, 2009). As an antioxidant, anthocyanin could scavenge metal ion such as Fe and Cu and also could prevent lipoprotein oxidation and platelet aggregation (Ghiselli *et al.*, 1998).

Deglycosylation of anthocyanin to its aglycone enhances chemopreventive impact by removing the bulky sugar groups which could cause steric hindrance, replacing the glucosidic linkage by another antioxidant-scavenging hydroxyl group and facilitating transport independent cell uptake (Hu *et al.*, 2009). Previous studies by Walle *et al.* (2005) showed that key responsible enzyme for metabolizing flavonoid glycosides such as genistin was β -glycosidase. Additional studies by Fleschhut *et*

al. (2006) showed that gastrointestinal flora not only metabolize monoglycosylated anthocyanins (such as cyanidin 3-glucoside) but also more complex deglycosylate form, i.e: di- or tri glycosylated and acetylated anthocyanins.

Hydrolysis of anthocyanin glycosides is proposed as the initial step for subsequent bacterial degradation and the formation of a set of new metabolites (Keppler and Humpf, 2005). Some experimental data showed that some genera of lactic acid bacteria show β -glucosidase activity and participate in the hydrolysis of plant β -glucosides (Matsuda *et al.*, 1994; Jeon *et al.*, 2002; Otieno *et al.*, 2005; Avila *et al.*, 2009).

Lactic acid bacteria (LAB) belongs to a group of Gram-positive bacteria that produce lactic acid as their main fermentation product into the culture medium and generally recognized as safe (Konings *et al.*, 2000). Recently, LAB is important for the food and dairy industries, because the lactic acid and other organic acids produced by these bacteria act as natural preservatives and flavor enhancers. Several species of LAB are also regarded as probiotics which able to stimulate immune responses and prevent infections against enteropathogenic bacteria (Reid, 1999). Thus, LAB could contribute to food safety and exist in several Indonesian fermented foods, such as tempe, tapai, growol, gatot, terasi, and fermented fish products (Rahayu, 2012).

The purpose of this study is to isolate LAB producing β glycosidase from Indonesian fermented foods and identify the isolates using phenotypic and genotypic identification methods. Furthermore, the

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other purpose of this study is to find the potential starter for designing glycoside based products, such as anthocyanin, and to construct a phylogenetic tree of those organisms.

Materials and Methods

Isolation and screening of LAB producing β glycosidase from Indonesian fermented foods

Seven samples of Indonesian fermented foods (plant materials, such as: black/white glutinous fermented rice, tempe, cassava tapai, and fermented vegetable) were collected from the local market in Yogyakarta, Indonesia. Isolation of LAB which produce β -glycosidase were done as describe by Chun *et al.* (2007) with some modifications. Samples (Indonesian Fermented Foods) were serially diluted with 1% of peptone water, pour plate in modified MRS (de Man *et al.*, 1960) agar medium (Oxoid Ltd., Basingtoke, Hampshire, England) supplemented with 1% of CaCO_3 to define acid producers.

The modified medium containing 1% cellobiose was prepared on the basis of the formula of Difco™ lactobacilli MRS broth (Becton Dickinson and Co.). Dextrose, a carbon source in the MRS broth, was replaced by cellobiose (Sigma, St. Louis, MO, U.S.A) to isolate LAB with β -glycosidase activities. The prepared medium consisted of: peptone (10.0 g/L), beef extract (10.0 g/L), yeast extract (5.0 g/L), polysorbate 80 (1.0 g/L), ammonium citrate (2.0 g/L), sodium acetate (5.0 g/L), magnesium sulfate (0.1 g/L), manganese sulfate (0.05 g/L), dipotassium phosphate (2.0 g/L), and cellobiose (10.0 g/L). Plates were incubated for 2 d at 37°C. After incubation, typical colonies with different morphologies and those which formed a clear zone were randomly isolated. Three to ten LAB isolates of each plate were randomly selected and cultured in MRS Broth for further purification (Chen *et al.*, 2006). Then, the isolates were checked by using Gram staining and catalase assay (Adnan and Tan, 2007). Only Gram-positive bacteria and catalase-negative colonies were chosen for further analysis. Isolates were stored at 0-4°C in MRS broth for further analysis and at -40°C in skim milk (10%) medium containing 10% glycerol prior to molecular analysis (Benito *et al.*, 2008).

One hundred and eighty eight colonies were selected based on their color and size. Twenty one isolates were finally selected according to their Gram test, catalase assay, and β glycosidase activity. From the 21 isolates, 4 of them were preliminary identified as *Lactobacillus* sp and complete for API 50 CHL kit (BioMerieux, France) identification. All of strains were performed to 16S rDNA sequence analysis. Beta

glycosidase activity was measured by determine the rate of hydrolysis of *p*-nitrophenol- β -glucopyranoside (Sigma, St. Louis, MO, U.S.A) presented by Jeon *et al.* (2002).

Characterization and identification of LAB isolates to species level

Morphological characteristics and Gram staining of LAB were examined after 24 h incubation on MRS agar. Catalase activity and gas production from glucose, as carbon source, were determined by the method of Kozaki *et al.* (1992). All isolates assigned to a particular LAB genus or species were identified on API 50 CHL kit strips (bioMe'rieux, France) by using carbohydrate assimilation and fermentation of 49 different compounds (and one as control treatment). Isolates were incubated at 30°C for 3 to 6 d. The organisms were identified by using the APILAB Plus software version 3.3.3 from bioMe'rieux. Identification to species level were determined according to Holzapfel *et al.* (2009).

Genotypic characterizations

Cell preparation for genotypic identifications

Total DNA was extracted from 2 mL of cells which is growth in MRS broth for 18-24 hours. Briefly, DNA samples were centrifuged (12000g, 4°C, 1 min) to collect cells pellet. The cells pellet was then suspended with 180 μL of enzymatic lysis buffer (2mM Tris HCl, 2 mM EDTA-2Na, 1,2% Triton X-100, pH 8,0, 20 mg/mL lysozyme) and incubate for at least 30 min at 37°C. After incubation, the cells suspension were treated with 25 μL of Proteinase K and 200 μL of Buffer AL and mix by vortexing. After incubation at 70°C for 30 min, the dilution were ready for DNA preparation.

DNA preparation for genotypic identification

DNA preparation were done by adding 200 μL of ethanol (96-100%) to the sample (cells pellet). Cells pellet was homogenized and added to the DNeasy Mini Spin Column placed in a 2 mL collection tube. The suspension was centrifuged at 6,000g for 1 min at 4°C. Then discard flow-through and placed in new 2 mL tube. The suspension was centrifuged again at 6,000g for 1 min at 4°C after 500 μL of buffer AW1 were add to the column and discard flow-through and the column was placed into new 2 mL tube. After 500 μL of buffer AW2 were add, the column was centrifuged at 6,000g for 3 min at 4°C and discard flow-through. The column was placed into the same 2 mL tube, then centrifuged at 20.000g for 1 min at 4°C to remove ethanol in buffer AW2 completely and

discard flow-through. DNA were isolated by adding 100 μ L of distilled water (or buffer AE) directly on the DNeasy membrane, then incubated at room temperature for 1 min, and centrifuged at 6,000g for 1 min. DNA was in the eluent. DNA purity were determined by nanospectrometer (NanoDrop ND-1000 spectrophotometer), then dilute the DNA solution till 10 ng/ μ L to PCR amplification.

PCR amplification for genotypic identification

Sixteen S rDNA amplification method were adopted from the previously published procedure (Nakagawa *et al.*, 2002; Miyashita *et al.*, 2012). Twenty microliter of PCR mixture consist of 2 x Ampdirect Plus 10 μ L, KAPATaq (or ExTaq) 0,07 μ L, Primer F (10 μ M) 1 μ L; Primer R (10 μ M) 1 μ L; Sample (supernatant) 0,5 μ L; and distilled water 7.43 μ L. The thermocycle program was as follow: 94°C for 3 min; 35 cycles at 94°C for 15 sec, annealing temperature 15 sec, and 72°C for 1 min, and final extension step at 72°C for 1 min. Agarose gel electrophoresis were performed to examine the purity and amount of 16S rDNA which is done by mixing 5 μ L of dye (loading buffer) and 1 μ L of Amplified PCR product and pipet 6 μ L the amplified DNA to the prepared 1% agarose gel contained SYBR Safe DNA Gel Stain. Electrophoresis run for 15 min and read the results by Imagesaver AE-6905c (Bioinstrument ATTO). DNA purified by Monofus (Cat Nr. 5010.21500) size 1000 μ L. Pure DNA were then add 200 μ L buffer A (2 fold from DNA volume) to membrane filter and put gently all of the sample to the column. After homogenized by slowly and gently mixing, centrifuge at 9000g, 30 sec and add 500 μ L buffer B. Buffer B were then remove the by centrifuge at 9000g for 30 sec and add 30 μ L of distilled water to the membrane filter in the column to eluate the pure DNA by centrifuge at 9000g for 1 min.

For phylogenic identification, the target regions of 16S rDNA were partially sequenced by using a BigDye Terminator V3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and an ABI 3730 PRISM genetic analyzer (Applied Biosystems, USA) and 3 primers (9F, 915F, 1510R) were used.

The 16S rDNA sequence homologies were examined by comparing the sequences obtained with those in the National Center for Biotechnology Information (NCBI) GenBank database and by using the online analysis ATGC. For phylogenetic analysis, sequences were aligned using the CLUSTAL X software (Thompson *et al.*, 1997; Larkin *et al.*, 2007). A phylogenetic tree was constructed by the neighbor-joining method, njplot (Saitou and Nei, 1987) with 1000 bootstrap replicates (Felsenstein, 1985).

Results and Discussion

Phenotypic identification

One hundred and eighty eight of acid-producing bacteria were isolated from 8 samples of fermented food. Seventy nine strains isolated from black glutinous rice tapai (BGRT), 74 strains from white glutinous rice tapai (WGRT), 10 strains from half fermented tempe, 1 strain from over fermented tempe, and 24 strains from cassava tapai. From 188 strains, only 123 strains were negative catalase and Gram positive. Further screening procedure were performed for the isolates by inoculating cell in modified MRS medium and motility test, and results showed that only 21 isolates were capable to grow and non motile.

According to Bergey's Manual for LAB identification, isolates were divided into three 3 groups: 3 isolates in Group I Lactobacilli 3 isolates), Group II Streptococci/Lactococci (1 isolate), and Group III Pediococci (17 isolate). Isolates from Group I and II identified by using API 50 CHL Kit (Table 2). Table 2 show phenotypic characteristics of the isolates and preliminary identification of the isolates according to the Bergey's manual, meanwhile Table 2 show probable genera of the isolates from group I and II identified using API 50 CHL Kit.

Seventeen isolates showed round shape and gram-positive cells. Colonies from MRS broth showed variable-size round cocci that tended to form tetrads. Almost all of the isolates in group III were able to grow well in tested growth condition except for isolate code 20.7 which has identified as *P. halophilus*. The optimum growth temperature was 40°C and all of them did not grow at 50°C after 5 days incubation. One of phenotypic differences between *P. pentosaceus* and *P. acidilactici* was tolerance to grow at 50°C. After 3 times tested, the 16 isolates failed to grow in this temperature. According to the phenotypic identification methods, we identify the 17 isolates as *P. pentosaceus* rather than *P. acidilactici*. Pediococci acidilactici would grew slowly in 10% NaCl, tolerated 50°C, preferred 40°C, and were unable to use maltose. The isolates could grow well in 10% NaCl, fail to grow in 50°C, and able to use maltose as a carbon source. Isolate code 20.7 identified as *P. halophilus* because it couldn't grow in pH 4.2 and also tolerance in 10% of NaCl. *Pediococcus halophilus* has been reclassified as *Tetragenococcus halophilus*.

Genus differentiation of lactic acid bacteria are usually based on Gram staining, catalase test, and determination of carbohydrate utilization using tube or API 50 CHL kit. However data overlapping occasionally exist and need more prove to identify

Table 1. Phenotypic identification of Group III.

Isolate Code	Shape	Growth condition					Suspected Species
		pH 8,5	pH 4,2	5% NaCl	40°C	10% NaCl	
P4.A	Cocci, tetrad	+	+	+	+	+	<i>P. pentosaceus</i>
N1.5	Cocci, tetrad	+	+	+	+	+	<i>P. pentosaceus</i>
21.46	Cocci, tetrad	+	+	+	+	+	<i>P. pentosaceus</i>
20.7	Cocci, tetrad	+	-	+	+	+	<i>P. halophilus</i>
P10.3	Cocci, tetrad	+	+	+	+	+	<i>P. pentosaceus</i>
24.9	Cocci, tetrad	+	+	+	+	+	<i>P. pentosaceus</i>
P13.1	Cocci, tetrad	+	+	+	+	+	<i>P. pentosaceus</i>
24.1	Cocci, tetrad	+	+	+	+	+	<i>P. pentosaceus</i>
27.9	Cocci, tetrad	+	+	+	+	+	<i>P. pentosaceus</i>
N11.16	Cocci, tetrad	+	+	+	+	+	<i>P. pentosaceus</i>
P12.c	Cocci, tetrad	+	+	+	+	+	<i>P. pentosaceus</i>
20.10	Cocci, tetrad	+	+	+	+	+	<i>P. pentosaceus</i>
P6.1	Cocci, tetrad	+	+	+	+	+	<i>P. pentosaceus</i>
11.10B	Cocci, tetrad	+	+	+	+	+	<i>P. pentosaceus</i>
T2.12	Cocci, tetrad	+	+	+	+	+	<i>P. pentosaceus</i>
P9.5	Cocci, tetrad	+	+	+	+	+	<i>P. pentosaceus</i>
Ta.2	Cocci, tetrad	+	+	+	+	+	<i>P. pentosaceus</i>

(+) good growth; (-) no growth

Table 2. API 50 CHL kit identification from Group I and II

Isolate Code	Shape	Catalase	Gram	Fermentation Type	API 50 CHL Kit	
					identify as:	% ID
T2.11	Short Rod 0.3 x 0.4 µm	-	+	Heterofermentative	<i>L. plantarum</i> 1	69.2
					<i>L. pentosus</i>	16.1
					<i>L. brevis</i> 1	14.5
T1.8	Rod	-	+	Homofermentative	<i>L. brevis</i> 1	56.1
					<i>L. pentosus</i>	30.7
					<i>L. mesentroides</i> ssp <i>mesentroides</i> / <i>dextranicum</i> 1	8.7
					<i>L. plantarum</i> 1	4.2
Ta.1	Cocci	-	+	Homofermentative	<i>L. plantarum</i> 1	99.9
					<i>L. pentosus</i>	0.1
T2.9	Short Rod 0.3 x 0.5 µm	-	+	Heterofermentative	<i>L. plantarum</i> 1	99.9
					<i>L. pentosus</i>	0.1

the isolate using other methods, such as 16S rRNA sequence analysis. Considering from the metabolic, morphological, and 16S rRNA sequence similarities between *P. acidilactici* and *P. pentosaceus* and ability to grow that they are able to grow in the same selective media for isolation of lactate-producing bacteria; it is possible that *P. acidilactici* has been misidentified with *P. pentosaceus*.

Identification of LAB from Group I and II (API 50 CHL Kit)

In API CH assay, all of tested LAB isolates were screened for their performance regarding growth characteristics using 49 of carbon source and then the results of the analytical data of carbohydrates fermentation were calculated using API software (Table 2). The result showed that 2 isolates had high % ID (99.9), that was Ta.1 and T2.9 and also always in pairs (two cell formation) but when there was no tetrad configuration. This configuration was also done in Ta.1. According to Bergey's manual procedure, *L.*

Table 3. Identity of isolates based on different identification method

Isolate Code	Identity of Isolates according to different method			
	Bergey's Manual	API 50 CH kit	Blast Test	16S rRNA
T2.9	<i>Lactobacillus</i> sp	<i>L. plantarum</i> 1	<i>P. acidilactici</i>	<i>P. acidilactici</i>
P4.A	<i>P. pentosaceus</i>	<i>P. pentosaceus</i>	<i>P. acidilactici</i>	<i>P. acidilactici</i>
N1.5	<i>P. pentosaceus</i>	<i>P. pentosaceus</i>	<i>P. acidilactici</i>	<i>P. acidilactici</i>
21.46	<i>P. pentosaceus</i>	<i>P. pentosaceus</i>	<i>P. acidilactici</i>	<i>P. acidilactici</i>
20.7	<i>P. halophilus</i>	<i>P. halophilus</i>	<i>P. acidilactici</i>	<i>P. acidilactici</i>
T1.8	<i>Lactobacillus</i> sp	<i>L. plantarum</i> 1	<i>L. pentosus</i>	<i>L. plantarum</i>
Ta.1	<i>Lactobacillus</i> sp	<i>L. plantarum</i> 1	<i>P. acidilactici</i>	<i>P. acidilactici</i>
P10.3	<i>P. pentosaceus</i>	<i>P. pentosaceus</i>	<i>P. acidilactici</i>	<i>P. acidilactici</i>
24.9	<i>P. pentosaceus</i>	<i>P. pentosaceus</i>	<i>P. acidilactici</i>	<i>P. acidilactici</i>
P13.1	<i>P. pentosaceus</i>	<i>P. pentosaceus</i>	<i>P. acidilactici</i>	<i>P. acidilactici</i>
24.1	<i>P. pentosaceus</i>	<i>P. pentosaceus</i>	<i>P. acidilactici</i>	<i>P. acidilactici</i>
27.9	<i>P. pentosaceus</i>	<i>P. pentosaceus</i>	<i>P. acidilactici</i>	<i>P. acidilactici</i>
N11.16	<i>P. pentosaceus</i>	<i>P. pentosaceus</i>	<i>P. acidilactici</i>	<i>P. acidilactici</i>
P12.c	<i>P. pentosaceus</i>	<i>P. pentosaceus</i>	<i>P. acidilactici</i>	<i>P. acidilactici</i>
20.10	<i>P. pentosaceus</i>	<i>P. pentosaceus</i>	<i>P. acidilactici</i>	<i>P. acidilactici</i>
P6.1	<i>P. pentosaceus</i>	<i>P. pentosaceus</i>	<i>P. acidilactici</i>	<i>P. acidilactici</i>
T2.11	<i>Lactobacillus</i> sp	<i>L. plantarum</i> 1	<i>P. acidilactici</i>	<i>P. acidilactici</i>
11.10B	<i>P. pentosaceus</i>	<i>P. pentosaceus</i>	<i>P. acidilactici</i>	<i>P. acidilactici</i>
T2.12	<i>P. pentosaceus</i>	<i>P. pentosaceus</i>	<i>P. acidilactici</i>	<i>P. acidilactici</i>
P9.5	<i>P. pentosaceus</i>	<i>P. pentosaceus</i>	<i>P. acidilactici</i>	<i>P. acidilactici</i>
Ta.2	<i>P. pentosaceus</i>	<i>P. pentosaceus</i>	<i>P. acidilactici</i>	<i>P. acidilactici</i>

plantarum and *L. pentosus* have homofermentative type while *L. brevis* obligatorily heterofermentative (Table 2). But the results show that T2.9 and T2.11 which were identified as *L. plantarum*/*L. pentosus* were heterofermentative. Strain T2.9 and T2.11 had low %ID, that was 69.2 and 56.1. Strain 24.1 and N11.16 were identified by using API 50 CHL kit as *P. pentosaceus*.

Identification based on 16S rRNA sequence

Species identification of pediococci from foods by conventional culture techniques is time-consuming and often unreliable. Since molecular methods are fast, reliable, and culture-independent, they are indispensable to day. In this study, we also report the sequencing of 16S rRNA gene of all isolates. New sequence data was provided for taxonomic discrimination and phylogenetic studies. The phylogenetic tree was constructed by the neighbour-joining method. As can be seen in Figure 1 and Figure 2, all of the Pediococci were closely related to *Pediococci lolli* AB362985.1 and *P. acidilactici* AJ305320 from the Genbank reference strain. While *Lactobacillus* sp T1.8 was closed to *P. pentosus*. Bacterial identification based on molecular data could produce ambiguity. Depend on isolate reference, sequence data in Genbank, or maybe from another data analysis methods.

The comparison result for all identification procedures show some differences (Table 3). Such as, strain T2.9 which was identified as *L. plantarum* by phenotypic and morphological identification, and also was identified as *P. acidilactici* by molecular identification method, using BLAST test or sequence analysis. Since, this isolate had pair configuration in morphological test. Otherwise, all of isolates which has identified as *P. pentosaceus* in morphologic and biochemical test, have identified as *P. acidilactici* in molecular basic test methods. Even molecular basic identification test was more valid than morphological test, according to Riebel and Washington (1990). In

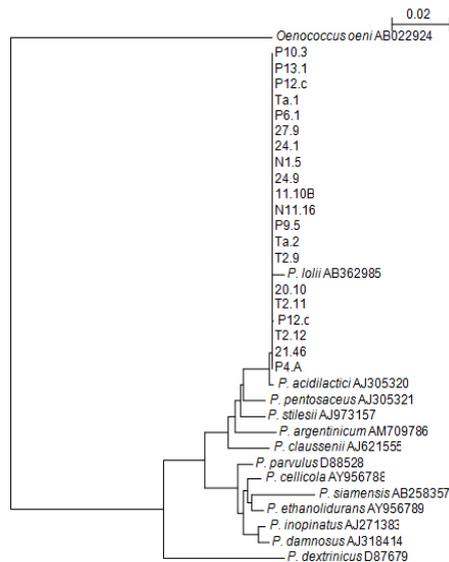


Figure 1. Neighbour-joining phylogenetic tree for *Pediococci* based on the 16S rRNA gene sequences. The relationship between isolated strains and related species were shown. *Oenococcus oeni* AB022094 was used as an outgroup. Bootstrap value based upon 1000 replicates are included at the major branch points. Bar, 0.02 nucleotide substitutions per site

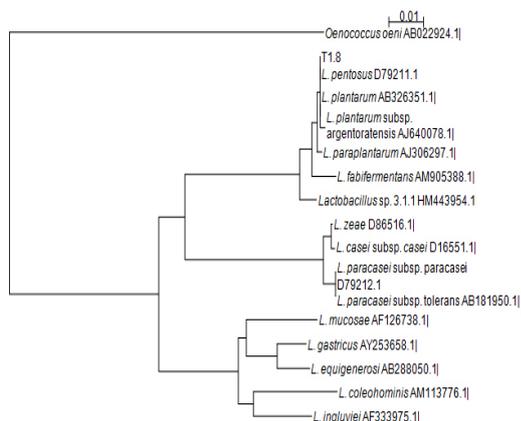


Figure 2. Neighbour-joining phylogenetic tree for *Lactobacilli* based on the 16S rRNA gene sequences. The relationship between *Lactobacillus pentosus* T1.8 and related species were shown. *Oenococcus oeni* AB022094 was used as an outgroup. Bootstrap value based upon 1000 replicates are included at the major branch points. Bar, 0.01 nucleotide substitutions per site

addition *P. acidilactici* was not produce β glycosidase enzyme. The results suggested that all of the strain has potential to be use as starter in the development of fermented food products anthocyanin-based. All of the strain were potent to be use as starter in the development of anthocyanin-based fermented food products.

Conclusions

Sixteen S rRNA based identification methods showed that 20 isolates were closely related

to *Pediococcus acidilactici* AJ305320 and *P. lolii* AB362985.1 and one isolate identified as *Lactobacillus pentosus*. Twenty isolates showed β glycosidase activity and identified as *P. pentosaceus*. Strain identification based on molecular data especially 16S rRNA sequence was not always giving the same results with phenotypic and genotypic methods. *Pediococcus pentosaceus* N11.16 could be used for construction of specific starter cultures for anthocyanin-based fermented food products.

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