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ISOLATION AND CHARACTERIZATION OF LACTIC ACID BACTERIA FROM INASUA

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ABSTRACT

Inasua is a traditionally product of wet salt fish fermentation produced by Teon, Nila and Serua (TNS) Communities in Central Maluku, Indonesia. The community made this fermented fish to anticipate the lean time when fisherman could not go to sea. The fish that used as inasua raw material is demersal fishes that live around coral reefs, such as Samandar fish (Siganatus guttatus), Gala-gala fish (Lutjanus sp.) and Sikuda fish (Lethrinus ornatus). The objective of the research was to isolate and characterize of bacterial indigenous in Inasua from three producers in Seram Island. The measurement of pH from inasua samples were 5.9, 5.0 and 5.8, respectively. The highest number of lactic acid bacteria was found from Gala – gala inasua was 2,5x10^7 cfu/g sample. Isolation of all isolates bacteria from inasua showed that a total of 7 isolates of bacteria was obtained from Samadar inasua, 9 isolates from Gala-gala inasua, and 7 isolates from Sikuda inasua. From a total of 23 isolates, only 6 isolates had characteristic as lactic acid bacteria that were Gram positive, negative catalase, and cocci shape. The microscopic characteristics of the isolates are coccid in pairs or uniforms which combine to form tetrads. Carbohydrate utilization test of selected isolate by using API 50 CHB kit indicated that 13 carbohydrates are fermented by these isolates after incubation for 48 hours. The research was concluded that the dominant bacteria in inasua sample are cocci-lactic acid bacteria.

1. Introduction

Inasua is a traditionally product of wet salt fish fermentation produced by Teon, Nila and Serua (TNS) Communities in Central Maluku, Indonesia (Nara *et al*., 2013) The community made this fermented fish to anticipate the lean time when fisherman could not go to sea (Walalohun, 2014).

Inasua is fermented fish in a salt solution that is often called wet salt fish (Nendissa, 2013). The fish used is demersal fish that live around coral reefs (Nara *et al*., 2013). Beside demersal fish, coconut sap is also added to the inasua fermentation to extend the shelf life of this product until more than a year.

Fermentation of inasua takes place spontaneously (without the addition of inoculum) and involves various microbes. In general the main role microbial in the fish fermentation is lactic acid bacteria (LAB) (Noonpakdee *et al*., 2009). In food safety, lactic acid bacteria are included in the category of Generally Recognized as Safe (GRAS) so it is safe to be in a food product (Pringsulaka *et al*., 2012). The composition of lactic acid bacteria in traditionally fermented fish is very determined by the type of carbohydrate and the amount of salt added (Saisithi, 1994).

Lactic acid bacteria involved in the inasua fermentation not only from fermented fish that took place anaerobically, but also derived from coconut sap as a source of carbohydrates are added. Coconut sap contains sugar about 5-10 % at neutral pH. Coconut sap sugar will be converted into alcohol by yeast under anaerobic conditions and further oxidized to acetic acid by acetic acid bacteria (Borse *et al*., 2007) during

inasua fermentation. In addition, the sugar contained in coconut sap can be also converted into lactic acid by lactic acid bacteria (Atputharajah *et al*., 1987). Fermented coconut sap is a combination of alcoholic, lactic acid, and acetic acid fermentation (Njoki *et al*., 2015).

Shelf life of inasua can last for a long time. Good processing can extend the shelf life of this fermentation product to more than a year. Long shelf life of a product fermented fish very influenced the diversity of microbes, especially lactic acid bacteria. The diversity of microbes in the inasua fermentation using coconut sap has not been done. Inasua processing that utilizes various demersal fish species may allow the identification of lactic acid bacteria involved in the fermentation of fish. Based on the description above, it is necessary to isolation and characterization of lactic acid bacteria in inasua.

2. Materials and methods

2.1. Sample Collection

Inasua samples were processed using three different types of fish and has undergone fermentation for 5 months is taken from the people at TNS-Waipia District , Ceram Island. The three sample is Samandar inasua/beronang (Siganatus guttatus), Gala - gala inasua (Lutjanus sp), and inasua sikuda/ lencam (Lethrinus ornatus). Each of samples was taken 250 g in an Tupperware and then transported to the Laboratory of Microbiology IPB to measure pH, calculations of total plate count, isolation and characterization of lactic acid bacteria in inasua.

2.2. Calculation of Microbes Number in Inasua

A total of 25 g sample was mixed with 225 ml of sterile peptone (0.1 % w /v peptone) is then homogenized using a stomacher at 10 x g for 1 minute. A total of 100 mL of sample inasua homogenized further diluted serially and spreaded linearly on the de Man, Rogosa and Sharp Agar (MRSA) medium, which has been added 1 % (w /v) of calcium carbonate ($CaCO_3$) and Nutrient Agar (NA) medium. Incubation was performed at room temperature for 48 hours. Dilutions used are 10^{-2}, 10^{-3}, and 10^{-4}. Colonies that grew on NA media is used to determine the total plate count (TPC) while colonies grown on media MRSA+$CaCO_3$ is used to determine the amount of lactic acid bacteria

2.3. Isolation and Characterization of Microbial in Inasua

Colonies that have different morphological characteristics were scratched at MRSA+ $CaCO_3$ to obtain pure cultures which are considered as isolates. Further isolates characterized macroscopically. All isolates were stained with Gram stain and catalase test using hydrogen peroxide 3 % (v/v). Formation of bubbles indicates isolates tested are positive catalase. Isolates are Gram positive and negative catalase subsequently observed under a light microscope (1000x magnification) to determine the microscopic characteristics of lactic acid bacteria (Fan et al. 2013; Kusmarwati et al .2013). One selected bacteria were tested for physiological characteristics to hydrolyze the carbohydrates using API 50 CHB kit. Incubation was performed for 24 hours and 48 hours and then analyzed using softwere API bioMérieux.

3. Results

3.1. The Number of Microbes in Inasua

The measurement of pH from inasua samples were 5.9, 5.0 and 5.8, respectively. The measurement of total plate count of bacterial isolates from inasua by using Nutrient Agar (NA) medium, and for lactic acid bacteria by using de Man, Rogosa dan Sharp Agar (MRSA) + $CaCO_3$ medium. The highest number of lactic acid bacteria was found from Gala – gala inasua was $2{,}5x10^7$ cfu/g sample (Table 1).

Table 1. Total plate count of the third inasua that grow on NA and MRSA+CaCO3 medium

Sources	Total plate count (cfu/g)	
	NA Media	MRSA+$CaCO_3$ Media
Samandar inasua	$1.4 \times 10^6 \pm 2.68$	$1.2 \times 10^6 \pm 1.64$
Gala-gala inasua	$2.8 \times 10^7 \pm 0.10$	$2.5 \times 10^7 \pm 0.70$
Sikuda inasua	$3.5 \times 10^5 \pm 1{,}94$	$6.2 \times 10^4 \pm 3.10$

3.2. Morphological Characteristics of Microbes in Inasua

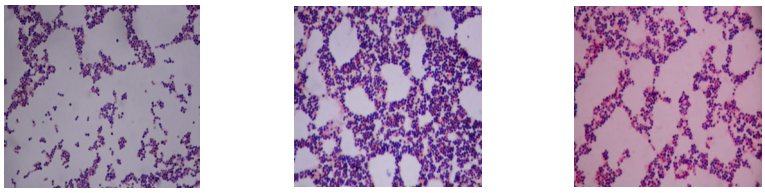
Isolation of all isolates bacteria from inasua showed that a total of 7 isolates of bacteria was obtained from Samadar inasua, 9 isolates from Gala-gala inasua, and 7 isolates from Sikuda inasua. From a total of 23 isolates, only 6 isolates had characteristic as lactic acid bacteria that were Gram positive, negative catalase, and cocci shape (Table 2).

The colony characteristics of the six isolates on MRSA-$CaCO_3$ medium were round with smooth edges, convex elevation, and creamy white. The microscopic characteristics of the isolates are coccid in pairs or uniforms which combine to form tetrads (Figure 1)

Table 2. Microscopic characteristics and catalase test of microbes in inasua

Isolate	Resources	Cell shape	Gram stain	Catalase test
Sm 01	Samandar inasua	rod	negative	positive
Sm 02*	Samandar inasua	cocci	positive	negative
Sm 03*	Samandar inasua	cocci	positive	negative
Sm 04	Samandar inasua	rod	negative	positive
Sm 05	Samandar inasua	rod	negative	positive
Sm 06	Samandar inasua	cocci	positive	positive
Sm 07	Samandar inasua	rod	negative	positive
Gg 01*	Gala-gala inasua	cocci	positive	negative
Gg 02	Gala-gala inasua	rod	negative	positive
Gg 03	Gala-gala inasua	rod	positive	positive
Gg 04	Gala-gala inasua	rod	negative	positive
Gg 05	Gala-gala inasua	cocci	positive	positive
Gg 06	Gala-gala inasua	rod	negative	positive
Gg 07	Gala-gala inasua	rod	negative	positive
Gg 08*	Gala-gala inasua	cocci	positive	negative
Gg 09	Gala-gala inasua	cocci	positive	positive
Si 01*	Sikuda inasua	cocci	positive	negative
Si 02	Sikuda inasua	rod	negative	positive
Si 03	Sikuda inasua	rod	positive	positive
Si 04	Sikuda inasua	rod	positive	positive
Si 05	Sikuda inasua	rod	negative	positive
Si 06*	Sikuda inasua	cocci	positive	negative
Si 07	Sikuda inasua	batang	positive	positive

* Isolates that have characteristics such as LAB



(a) (b) (c)

Figure 1. Microscopic characteristic of lactic acid bacteria in Samandar inasua (a), Gala-gala inasua (b), and Sikuda Inasua (c).

3.3 Characteristics of Microbial Physiology in Inasua

Catalase test using 3% hydrogen peroxide showed that six isolates were proposed as lactic acid bacteria. All isolates did not form bubbles of gas on catalase test, so it can be concluded that these isolates are negative catalase. Carbohydrate hydrolysis test using API 50 CHB kit showed that 13 kinds of carbohydrates are fermented by selected isolate after incubation for 48 hours (Table 3). The results of bacterial identification kit API 50 CHB can not show a species of lactic acid bacteria.

Table 3 The test results of carbohydrate utilization of selected isolates using API 50 CHB kit after incubation for 48 hours

Types of carbohydrate	Test results	Types of carbohydrate	Test results
Control	-	Esculin	-
Glycerol	+	Salicin	-
Erythritol	-	Cellobiose	-

D-Arabinose	-	Maltose	+
L-Arabinose	-	Lactose	+
Ribose	-	Melibiose	-
D-Xylose	-	Sucrose	+
L-Xylose	-	Trehalose	+
Adonitol	-	Inuline	-
Methyl xyloside	-	Melezitose	-
Galactose	+	Raffinose	-
Glucose	+	Starch	-
Fructose	+	Glycogen	-
Mannose	+	Xylitol	-
Sorbose	-	Gentobiose	+
Rhamnose	-	D-Turanose	+
Dulcitol	-	D-Lyxosa	-
Inositol	-	D-Tagatose	-
Mannitol	+	D-Fucose	-
Sorbitol	-	L-Fucose	-
Met-D-Mannoside	-	D-Arabitol	-
Met-D-Glucoside	-	L-Arabitol	-
NAc-Glukosamine	+	Gluconate	-
Amygdalin	-	2-Kt-gluconate	-
Arbutin	-	5-Kt-gluconate	-

4. Discussion

The number of microbes in the gala - gala inasua higher than the number of microbes that grow on other samples. The number of microbes that grow on the MRSA+$CaCO_3$ medium as much as 2.5 x 10^7 cfu/g indicate that Gala–gala inasua has a number of lactic acid bacteria higher than Samandar and Sikuda inasua. The number lactic acid bacteria of Gala – gala inasua correlated with a decrease of pH to 5.0. The pH decreasing was caused by the accumulation of acidic compounds produced by microbes, especially lactic acid bacteria. The pH decreasing gives a specific flavor to the fermented fish (Kopermsub and Yunchalard, 2008). This correlates with sour aroma specific of Gala-gala inasua that are not found in other inasua.

The dominant microbes in Samandar inasua are Gram negative bacteria. These bacteria do not form spores and able to ferment lactose produce acid and gas in TSI agar media (data not shown). One group of bacteria that have these characteristics are coliforms. Coliform are bacteria that are commonly found as spoilage bacteria and pathogens in food (Jay 1996). Inasua is a product of fermented fish in a salt solution. Although salt can inhibit the growth of spoilage bacteria and pathogens in inasua but the fermentation takes place under water for a long time allowed coliform bacteria can grow in inasua. The presence of coliform bacteria in Sabandar inasua also affected by a decrease in pH, which only reached 5,91. According to Owen and Mendosa (1985), pH can inhibit spoilage and pathogenic bacteria in fermented fish ranged between 4.5 – 5.0.

As Samandar inasua, the dominant microbes in the Gala-gala inasua are Gram negative bacteria. Other microbial characteristics that are also found in Gala – gala inasua is Gram-positive cocci bacteria and catalase positive. One of the bacteria that have these characteristics is Staphylococcus. Most of the bacteria are halotoleran and often found in products of fermentation are added salt. In some high salt fermented fish product from Thailand was found *Staphylococcus carnosus* and *S. pisciferm*entans that play arole in the fermentation process (Tanasupawat *et al.,* 1992). The addition of salt about 20-25 % in the inasua fermentation allows *Staphylococcus* can grow in Gala–gala inasua. High salt content can inhibit the growth of spoilage bacteria. On the other hand, the addition of salt at high levels in fermented fish causes fermentation rate becomes slow (Panda *et al.,* 2011). Slowing the rate of fermentation due to high salt content also inhibits the growth of lactic acid bacteria that play a major role in the fish fermentation process and support the growth of Staphylococcus (Paludan-Muller *et al.,* 2002).

The dominant microbes in Sikuda inasua are Gram-positive rods bacteria and positive catalase.

One group of bacteria that have these characteristics is *Bacillus*. These bacteria can be found in soil and water, including sea water. The ability to form spores causing Bacillus can survive in conditions of low pH and high salinity. The decreasing pH that reach only 5.80 allows these bacteria can grow in Sikuda inasua. Bacillus intolerant below pH 5-5.5 in fermented fish (Paludan-Muller, 2001). This bacterium is the dominant microbe in inasua (without coconut sap) which has undergone fermentation for 3 months (Nara *et al.,* 2013).

Lactic acid bacteria are found in all samples of inasua are Gram-positive cocci bacteria. These bacteria are often found in fermented fish such as Streptococcus, Leuconostoc, Lactococcus, Pediococcus and Weissella (Kimhamanon, 1994; Kopermsub and Yuchalard, 2010). Cocci shaped lactic acid bacteria are generally less tolerant of acidic than rod-shaped lactic acid bacteria (Kopermsub and Yuchalard, 2010). The presence of Gram positive-cocci lactic acid bacteria in inasua also supported by the decrease of pH fermented fish product is not less than 5.00. Cocci lactic acid bacteria are generally have the ability to produce acid that is lower than the rod-shaped lactic acid bacteria, particularly Lactobacillus (Kopermsub and Yuchalard, 2010; Saithong *et al.,* 2010).

Lactic acid bacteria in inasua are generally uniformly shaped cocci in pairs or form a tetrad. Lactic acid bacteria that have these characteristics are Pediococcus. (Holzapfel *et al.,* 2006) Pediococcus is a lactic acid bacterium that is often found in fermented fish (Saisithi, 1994). Unlike other cocci lactic acid bacteria, *Pediococcus* has a pH optimum of 5.0 and is able to grow below pH 4.5 so that the bacteria are commonly found in late stages of a fermentation product. *Pediococcus* can grow under aerobic conditions, microaerophilic and anaerobic (Carr *et al.,* 2002; Holzapfel *et al.,* 2006). Most Pediococcus can hydrolyze fructose, mannose, sucrose, galakstosa, and mannitol (Simpson and Taguchi, 1995; Holzapfel *et al.,* 2006).

5. Conclusion

Gala–gala inasua has the highest number of microbes, especially lactic acid bacteria per gram samples compared to inasua samandar and inasua sikuda. Lactic acid bacteria found in inasua has the characteristics of Gram positive cocci shaped, negative catalase. As microscopi- cally, these bacteria form uniform cocci in pairs or combine to form tetrads.

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