Isolation and identification of indigenous lactic acid bacteria on corn flour BISI-16 during spontaneous fermentation process

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Abstract. Lactic acid bacteria (LAB) is one of the dominant bacteria species that have an important role during spontaneous fermentation process of corn flour BISI-16. LAB are known to change the chemical structure of natural corn starch through the activity of enzyme and lactic acid produced during fermentation process. This change of structure causes the modification of physicochemical properties of corn flour. The objectives of this study were to isolate and identify indigenous LAB involved in spontaneous fermentation process of BISI-16 cornflakes. Isolation and identification of early LAB was done by isolation morphology characterization including cell shape, Gram staining, catalase test, and endospora staining. Furthermore, isolates LAB were identified by their genotypes using Polymerase Chain Reaction (PCR) method and 16S rRNA sequencing analysis. The results showed two isolates (ASN3 and ASN5) that had been isolated from corn flour during spontaneous fermentation and were grown on MRSA media having rounded colonic and creamy, rod-shaped cells, Gram (+), catalase (-), and endospores (-). The initial identification stage shows both isolates as candidate LAB. The results of the 16S rRNA sequence analysis showed both isolates were genotypically similar with *Lactobacillus fabifermentans* with 97% similarity for ASN3 isolates and ASN5 isolates of 98%.

1. Introduction
The use of corn into food products is still very low. The condition of hard corn seeds with large seed shape causes the processing to take longer. Therefore, processing corn into corn flour products is needed. When compared with corn shaped, corn flour will be more easily applied to food products, although the application of corn flour is highly dependent on its physicochemical properties.

Physicochemical properties are one of the properties associated with viscosity and gelatinization of corn flour during the heating process, namely the peak viscosity and hot paste viscosity, where these parameters are describes the ability of a granule to experience maximum development during heating [1]. Breakdown viscosity or changes in heat paste are physicochemical properties that describe the resistance of a granule to the heating process and mechanical treatment during processing, while the cold paste viscosity and setback parameters are physicochemical properties that describe the ability of the granule to retrograde during cooling [2].

Corn flour as natural starch still has a non-uniform gel viscosity, is not resistant to high temperatures, cannot stand acid conditions, cannot withstand mechanical treatment, has limited...
solubility, and is still susceptible to cirrhosis. This causes the application of corn flour to food products is still very limited, so we need an effort to modify corn flour which is expected to modify the physical properties of corn flour so that the potential of the application becomes greater.

Modification of the characteristics of corn flour can be done by fermentation. Modification of flour with fermentation has the potential to be developed because of low operating costs. The fermentation process is defined as the decomposition of starch by an enzyme produced by microorganisms on a substrate, so that the fermentation of corn starch is the decomposition of corn starch carried out by the amylase and amyloglucosidase enzymes produced by indigenous microbes. Starch degradation process depends on the composition of amylose and amylopectin corn flour which also affects the performance of the enzyme, the amylase enzyme will degrade more starch with high amylose content [3]. Corn fermentation process has been investigated by [4], who reported that the fermentation of corn flour can increase the protein content and amino acid lysine which has been the inhibiting factor of corn flour. [5] also found that corn fermentation can reduce total acid from corn so that the quality of corn flour becomes better. Likewise with the discovery of [6], which shows that the fermentation process can increase globulin and albumin from corn flour. In addition, [7] reported that the process of spontaneous fermentation of corn can improve food safety because the fermentation process can reduce M1 aflatoxin, citrine, and cyclopiazonic acid.

Corn flour which had been spontaneously had better nutritional content compared to unfermented corn flour [8]. [9] also reported that two modified types of corn flour (BISI-2 and pop corn) using spontaneous fermentation caused a decrease in enthalpy value, reduced crystalline characteristics of starch, and decreased value of peak viscosity, reverse viscosity, initial temperature and corn starch peak temperature.

Modification of corn flour using the method of spontaneous fermentation is considered to have weaknesses, namely the type of living microbes that can vary and are very dependent on conditions and environment so that it is difficult to control. Several studies have reported that the dominant bacteria involved during the spontaneous fermentation process of corn flour is from the group of lactic acid bacteria (LAB). Lactic acid bacteria are beneficial bacteria in the fermentation process and are always involved in spontaneous fermentation because they are indigenous. Lactic acid bacteria are able to inhibit the growth of pathogenic and decay bacteria due to this bacteria capable of producing several anti-bacterial compounds such as bacteriocin, hydrogen peroxide, fatty acids, reuterin, diasetil and lactic acid.

Research studies on the involvement of LAB during the process of spontaneous fermentation of corn flour have been reported by several researchers. However, studies on BISI-16 corn which are hybrid corn have not been reported. Corn BISI-16 is a corn that has been given genetic engineering technology. The process of genetic engineering allows indigenous microbes in BISI-16 corn to differ from other types of corn. Therefore, this study focused on the study of LAB involved during the spontaneous fermentation process of BISI-16 corn. The purpose of this study was to isolate and identify LAB involved in the spontaneous fermentation process of BISI-16 corn flour.

2. Materials and Methods

2.1. Materials and Tools
The main ingredient is BISI-16 hybrid corn obtained from Jeneponto Regency, South Sulawesi. The microbial growth media used were PCA media, NA media, and MRSA media. The materials used for analysis were water and distilled water, NaCl, 95% ethanol, immersion oil, crystal violet solution, 70% alcohol, methylated spirits, cotton, aluminum foil and safranin solution.

The equipment used for analysis includes blenders, test tubes, centrifuge tube racks, vortices, hot plates, glass beakers, volumetric flasks, claspers, analytical scales, measuring cups, volumetric pipettes, drop pipettes, PCR (polymerase chain reaction), pH meter, Water bath, Erlenmeyer, Petri dish, incubator, autoclave, burette, 9FZ-23 type disc mill and PPK N 70 type corn seed disposal machine.

2.2. Research Methods
2.2.1. Isolation and Identification Indigenous Bacteria
Corn seeds that have been cleaned from dirt and defective seeds are soaked for 1 hour at room temperature with a ratio of corn and the amount of water 1: 2 (b / v). Furthermore, the seeding of corn kernels was carried out using PPK N 70 type buffering machine. The corn grain which was silo was added with water that had been cooked in a ratio of 1: 2 (b / v) fermented spontaneously for 48 hours using the microaerophilic method. During the fermentation process, the fermentation liquid is inoculated into PCA, NA, and MRSA growth media. Inoculation and analysis are carried out at fermentation time 24 and 48 hours.

Indigenous bacteria on PCA, NA, and MRSA media were isolated using quadrant scratching techniques. After obtaining isolates with separate colonies a simple identification was made consisting of typical colonies, morphological forms (cocci or bacil), Gram staining test (+/-), catalase test , and Endospora staining test. After that, the pure isolates obtained with LAB characteristics were tested for their genotypic properties (16sRNA coding DNA sequence analysis, 16sRNA encoding DNA amplification, 16sRNA coding DNA sequencing).

2.2.2. Genotitive Identification Using PCR and Analysis of 16S rRNA Coding DNA Sequences
Genotification of bacterial culture was carried out by extracting 16s rRNA encoding DNA which was then amplified and sequenced. Isolates to be analyzed are classified as Gram positive (+) bacteria. Therefore, DNA extraction uses the GenAid method to extract DNA from Gram positive (+) bacteria with the Geneaid Presto™ Mini gDNA Bacteria Kit Protocol. Culture cells as much as 10° cells/ml were centrifuged at a speed of 14,000 g for 1 minute. Theformed supernatant is removed. Furthermore, pellets were added 200 µl buffer GT and lysozyme (4 mg/ml), occasionally distorted, and incubated at 37°C for 30 minutes. Supernatant was added with 20 µl proteinase K, then reexorted until mixed, and incubated at 60°C for 10 minutes. After incubation, the supernatant was added 200 µl of buffer GB, vortexed occasionally, and incubated at 70°C for 10 minutes. After that, 200 µl of absolute ethanol was added, distilled for 10 seconds, put into GD column, and centrifuged at 14,000 g for 2 minutes. The GD column holder is replaced with a new one. The next process, W1 buffer of 400 µl was added, centrifuged at a speed of 14,000 g for 30 seconds, the liquid contained in the container GD column was removed. Next, 600 µl of W1 buffer was added, centrifuged at 14,000 g for 30 seconds, the GD column was transferred to sterile eppendorf, the liquid contained in the GD column container was discarded. Centrifugation was carried out again for 3 minutes to dry the GD column. The next step, EB 100µl solution was added, it was transferred from the GD column to the new eppendorf, then centrifuged again for 30 seconds with a speed of 14,000 g. DNA samples are produced.

2.2.3. DNA amplification of 16s rRNA encoders with PCR
The amplification reaction of DNA samples was carried out in a 0.2 ml PCR tube. Each tube of PCR reaction was added with RBC Taq (5 units / ml) as much as 0.25 µL, 10 x buffer Taq (containing Mg 2+ ) as much as 5 µl, dNTP 2.5 mM as much as 4 µl. The primers used were universal primers, namely 63F (5'-CAGGCCTAACACATGCAAGTC) and 1387R (5'-GGGCGGWGTGTACAAGGC-3'), respectively 1.25 µl (20 pmol). The genome extract was 2.5 µl (100 ng) and added with ddH2O until the volume became 50 µl. PCR amplification was carried out at an initial denaturation temperature of 95°C for 5 minutes, primer attachment at 94°C for 30 seconds with 30 cycles, and extension at 50°C for 1 minute, 72°C for 2 minutes, and the final stage 72°C for 2 minutes. PCR products are taken and stored at 4°C. Furthermore, PCR products (DNA amplification as much as 5 µl) were put into wells of 1.5% agarose gel and TAE buffer solution submerged in the tank. Agarose gel 1.5% and TAE buffer solution consisted of 1.5 g agarose powder and 100 ml TAE buffer and 8 µl ethidium bromide. Electrophoresis is run for 1 hour with a constant voltage of 100 Volts. DNA tape (gel formed) is observed under UV light.

2.2.4. Analysis of 16s rRNA encoding DNA sequences
DNA sequencing of 16s rRNA coding was carried out in 1st Singapore BASE sequencing facilitated by PT. Genetics Science Indonesia. Analysis of sequencing results was carried out with the BLASTN
2.5.1+ program, which is by matching the nucleotide sequence from the 16S rRNA sequencing results with the data base available at the site www.ncbi.nlm.nih.gov

3. Results and Discussions

3.1. Isolation and identification of lactic acid bacteria

The results showed that indigenous microbes involved during the spontaneous fermentation process of cornstarch-BISI 16 were mold, yeast, bacteria, and LAB. The results of isolation and identification of mold using slide culture techniques found 7 types of mold involved in the spontaneous fermentation process of BISI-18 corn flour, namely Aspergillus fumigatus, A. flavus, 3 classified species of Aspergillus sp, Cunninghamella elegans, and Dendryphiopsisatra [10].

The dominant indigenous microbes in the spontaneous fermentation of corn flour BISI-16 is LAB, so that during the fermentation there is a change in the pH value (the more acidic) the total acid content increases with the fermentation time interval. LAB produces lactic acid and other organic acids as the main metabolite products. Identification data on mold and yeast are not shown.

The results of the simple identification of 6 isolates of indigenous bacteria that had been isolated showed that there were four isolates of LAB bacteria, namely isolates ASN3, ASN4, ASN5, and ASN12. ASN9 and ASN11 bacterial isolates have been reported as Enterobacter cloacae subsp. cloacae [11]. Both of these bacteria were found to be involved spontaneously during the fermentation process of BISI-16 corn flour. The results of simple identification of isolates are presented in Table 1.

| No. | Bacterial Isolates | Characteristics |   |
|-----|--------------------|-----------------|---|
|     | Gram               | Catalase        | Endospora | Cell Form | Typical Colonies |
| 1   | ASN3               | +               | -         | Short stem | Creamy round   |
| 2   | ASN4               | +               | -         | Stem       | Creamy round   |
| 3   | ASN5               | +               | -         | Stem       | Round, large beige colonies |
| 4   | ASN9               | -               | +         | Stem       | Round white, the edges of the surface are slimy |
| 5   | ASN11              | -               | +         | Stem       | White round    |
| 6   | ASN12              | +               | -         | Short stem | Creamy round   |

The isolates of LAB ASN3 and ASN5 were further analysed in the genotypic characterization of LAB so that the two species of isolate were known. Genotypic characterization analysis of LAB was only carried out on these two isolates because the isolates of LAB ASN4 and ASN12 had similar cell form and typical colonies of isolates LAB ASN3 and ASN5.

Isolates LAB ASN3 and ASN5 are included in Gram (+) bacteria which have catalase (-) properties and do not produce endopora. Both of these isolates belong to the LAB group, because LAB is included in the Gram (+), catalase (-), and endospore groups. (-). ASN3 isolates were obtained after spontaneous fermentation of BISI-16 corn flour for 24 hours, while ASN5 isolates were obtained at the end of fermentation time, which was 48 hours. Typically, the two isolates of this bacteria have similarities, namely cream-colored bacterial colonies. However, ASN5 isolates have the characteristics of a larger colony.

3.2. Genotypic characterization of indigenous lactic acid bacteria

Characterization of indigenous LAB bacterial genotypes was carried out using 16S rRNA coding DNA. Primers used in the DNA amplification process were 63F (5'-CAGGCTAACACATGCAAAGTC-3') and 1387R (5'-GGGCAGGTGTCACAAGGC-3'). The primary specificities of 63F and 1387R have been systematically tested with various types of bacteria and environmental samples, this primer is better used for 16S rRNA gene amplification, both ecologically and systematic studies compared to PCR amplitude that is more commonly used.
The results of the 16S rRNA encoding sequence alignment analysis of the ASN3 isolates are presented in Figure 1, while ASN5 isolates can be seen in Figure 2.

![Figure 1. Alignment of DNA base sequences of the 16S rRNA code for ASN3 isolates](image-url)
The results of identification of 16S rRNA DNA sequence analysis using the BLASTN 2.5.1 + program are presented in Table 2. Identification of DNA strand sequences showed ASN3 isolates had
similar levels to \textit{L. fabifermentans} DSM 21115 NODE\_181, whole genome shotgun sequence, which was 97\%, while ASN5 isolates had similarity level of 98\%.

**Table 2.** Results of 16S rRNA DNA sequence analysis using the BLASTN 2.5.1+ program

| Isolate | Description (Homolog) | Maximum score | Total Score | Query Cover | value E | Identification | Access Code |
|---------|-----------------------|---------------|-------------|-------------|---------|----------------|-------------|
| ASN3    | \textit{L. fabifermentans} DSM 21115 NODE\_181, whole genome shotgun sequence | 2228 | 2228 | 96\% | 0.0 | 97\% | NZ\_AYGX0 2000121.1 |
| ASN5    | \textit{L. fabifermentans} DSM 21115 NODE\_181, whole genome shotgun sequence | 2266 | 2266 | 94\% | 0.0 | 98\% | NZ\_AYGX0 2000121.1 |

\textit{L. fabifermentans} is included in \textit{Lactobacillus}. \textit{Lactobacillus} is the most diverse genus among LAB, \textit{Lactobacillus} is a group of Gram positive bacteria that produce lactic acid as the main end product of fermentation, this group of bacteria is often involved in food fermentation. Several studies have reported the involvement of LAB in spontaneous fermentation of corn. LAB involved during spontaneous fermentation of grits, including \textit{L. plantarum}, \textit{Pediococcus pentosaceus}, \textit{L. brevis}, and \textit{L. paracasei} \textit{paracasei} [13]. Other types of LAB involved during spontaneous fermentation of corn have also been reported, including \textit{L. cellobiosus}, \textit{L. pentosus}, \textit{Leuconostoc mesenteroides} [14] and \textit{L. fermentum} and \textit{P. acidilactici} [15].

Unlike previous studies, LAB which was successfully isolated during BISI-16 corn flour fermentation was \textit{L. fabifermentans}. \textit{L. fabifermentans} is one of the bacteria that has been proposed as a new species. Initially, \textit{L. fabifermentans} was associated with spontaneous fermented cocoa beans. In addition, this bacterium was also isolated from Marc grapes collected after a prolonged storage period to allow spontaneous alcohol fermentation in northeastern Italy [16].

\textit{L. fabifermentans} is included as a species of \textit{L. plantarum} group, this group is very homogeneous in terms of metabolic features, this group is facultative heterofermentative, with GC content, which ranges between 44 and 47 mol\% [17]. The \textit{L. plantarum} group consists of 5 species, of which two species are \textit{L. fabifermentans} and \textit{L. xiangfangensis}.

\textit{L. fabifermentans} is involved in spontaneous fermentation of corn because this type of LAB is capable of producing carbohydrate-breaking enzymes, especially glycoside-binding enzymes which are the main bonds in carbohydrates. \textit{L. fabifermentans} is one of the LAB capable of producing glycoside hydrolase (GH) enzymes [18].

General characteristics of \textit{L. fabifermentans} have been reported by [19]. \textit{L. fabifermentans} is Gram positive, catalase negative, facultative and non motile anaerobe. \textit{L. fabifermentans} cells are long rod shaped (1.0–3.0 mm wide and 10.0 mm long) that appear singly, in pairs or in short chains. The grayish colonies are white (creamy), opaque, smooth and round with convex elevation and the entire margin (diameter of about 1.0 mm). Cell growth can be observed at temperatures from 10 °C (from 8th day of incubation) to 37 °C (direct growth from day 1 of incubation). The ratio of production of D- and L-lactic acid isomers is 80: 20. \textit{L. fabifermentans} does not produce gas and dehydrated arginine. Acids are produced from L-arabinose, ribose, D-xylose, galactose, glucose, fructose, mannose, mannotol, N-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, selobiose, maltose, sucrose, trehalose and gentiobiose. Acid is not produced from glycerol, erythritol, D-arabinose, L-
xylose, adonitol, methyl βD-xylopyranoside, sorbose, rhamnose, dulcitol, inositol, sorbitol, methyl αD-mannopyranoside, methyl αD-glucopyranoside, lactose, melibiose, inulin, melezitose, raffinose, starch, glycogen, xylitol, turanose, D-lyxose, D-tagatose, D- or L-fucose, D- or L-arabitol, gluconate or 2- or 5-ketogluconate.

4. Conclusions
The spontaneous fermentation of BISI-16 corn flour involved LAB, namely ASN3 and ASN5 isolates, with characteristic creamy colonies, rod-shaped cells, Gram (+), catalase (-), and endospores (-). Genotypic properties based on sequencing analysis of 16S rRNA DNA strands showed that both isolates had homologous similarities with *Lactobacillus fabifermentans DSM 21115 NODE_181 ie* 97% (ASN3) and 98% (ASN5).

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