Identification of Lactic Acid Bacteria from Papuan Red Fruit (*Pandanus conoideus* Lam.) with Potential as Probiotics and Antibacterials

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DOI: 10.36347/sajb.2020.v08i09.006 | Received: 06.09.2020 | Accepted: 14.09.2020 | Published: 30.09.2020

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### Abstract

Lactic acid bacteria (LAB) are mostly explored for probiotic activities. These organisms improve the organoleptic characteristics of food, inhibit microbial activities, and consequently prevent possible diseases. The aim of this study, therefore, is to investigate organisms with possible inhibitory action against pathogenic bacteria. Besides, Papuan red fruits obtained from Sorong (West Papuan) and Timika (Papuan) were estimated to contain LAB, hence samples were selected and isolated using de Man Rogosa Sharpe selective medium equipped with 1% CaCO₃. The organisms were then analyzed based on acid sensitivity (pH 2, 3, and 4) and bile tolerance (0.3%; 0.5% and 1%). The antibacterial activity was evaluated using well diffusion method on the Muller Hilton Agar medium against pathogenic bacteria *Staphylococcus aureus* ATCC 25923, *Salmonella typhi* BPE 122.4 CCA, and *Salmonella typhi* NCTC 786. The identity of LAB isolates was confirmed based on the API 50CHL test, and a total of 25 isolates were successfully selected. These were characterized based on the cellular properties of rod-shaped, Gram-positive, homofermentative, non-motile, catalase-negative, as well as the capacity to grow at 10°C and 45°C. Furthermore, five of the isolates (strains S1B1, S2B1, S1T2, S2T4, and S1T1) were identified as *Lactobacillus plantarum*, with the ability to survive at pH 2 and in 1% bile salts. The antibacterial activity of the S2T4 strain was strong against *S. aureus* ATCC 25923, *S. typhi* BPE 122.4 CCA, and *S. typhi* NCTC 786. Besides, LAB S2B1 significantly restrained the growth of *Salmonella typhi* BPE 122.4 CCA, while LAB S1T1 inhibited *S. aureus* ATCC 25923. Therefore, it is possible to further explore *Lactobacillus plantarum* strains S2T4, S2B1, and S1T1 for the inherent health and nutritional values.

**Keywords:** Lactic acid bacteria, *Pandanus conoideus* Lam., Probiotics, Antibacterial, *Lactobacillus plantarum*.

### INTRODUCTION

Lactic acid bacteria (LAB) are known to play a crucial role in the preservation and fermented foods. These organisms have been applied as natural microflora or as starter culture introduced under controlled conditions [1]. According to Gutiérrez-Cortés *et al.* [2], LAB metabolites can improve the organoleptic characteristics of fermented food and inhibit the growth of microorganisms responsible for food spoilage. These bacteria are classified as probiotics, considering the positive impact on human health. Meanwhile, there are numerous advantages derived from the consumption, including to increase immunity, suppress pathogenic bacteria, balance intestinal microbiota, and reduce serum cholesterol [3].

According to Allen *et al.* [3], LAB is used as a probiotic, particularly due to the inherent resistance against acids and bile. This microorganism can produce antibacterial substances required to suppress pathogenic enteric bacteria growth. Besides, the antifungal property produced is known to extend the shelf life of food, hence the potential for application as a natural preservative in various edible products [4].

Several studies have successfully obtained LAB strains from various soured beverage ingredients, including yogurt and traditional fermented foods, specifically tape, growol and gatot [5]. The microorganism yield is naturally dominant in dairy products, whole grains, meat, fish, fruit, juices drinks, pickled vegetables, and sourdough batter. Meanwhile, numerous reports are indicating the minute quantities present in all plant materials, while abundant amounts have been observed in decaying varieties, especially rotting fruits. The species isolated from various plant sources include *Lactobacillus plantarum*, *L. brevis*, *L. cerevisiae*, *L. rhamnosus*, and *L. delbrueckii*. Address for correspondence: Charis Amarantini, Department Biology, Faculty of Biotechnology, Duta Wacana Christian University, Jl. Dr. Wahidin Sudirohusodo No.5-25, Kotabar, Gondokusuman, Yogyakarta, Daerah Istimewa Yogyakarta 55224, Indonesia.
**Pandanus conoideus** also more, the fied using 50 CHL determined. Besides, an 8. Moreover, the inulin content in pedicel RS medium adjusted to pH 2, followed by incubation at 37°C for 24 to 48 hours [13].

Isolation and selection of Lactic Acid Bacteria
A total of 25 g of red fruit were inoculated into 225 mL de Mann Rogosa Sharpe (MRS, Oxoid) liquid medium and incubated at 37°C for 48 hours. Furthermore, step dilution was carried out by inoculation through the pour plate method into the MRS agar medium for 48 hours. These cultures were then selected based on phenotypic properties through Gram stain, catalase, gas production, motility test, and survival at 10°C or 45°C [11, 12].

Probiotic Selection
LAB potential as a probiotic candidate was selected through a tolerance test for acidic pH and bile salts, along with antibacterial assessment.

**Acid pH tolerance test**
The LAB isolates were grown in 5 ml of liquid MRS medium for 48 hours, and the cells were harvested by centrifugation at 13,500 rpm for 15 minutes. Furthermore, the cell pellets were washed twice with Phosphate Buffer Saline (PBS, Merck) and dissolved in 100 µl of PBS solution. These globsules were then inoculated into a liquid MRS medium adjusted to pH 2, 3, and 4 for 4 hours at 37°C. Subsequently, the respective culture growth results were determined by growing on MRS agar using the streak plate method. This was followed by incubation at 37°C for 24 to 48 hours [13].

**Bile salts tolerance test**
Approximately, 1ml of LAB culture aged 48 hours was inoculated into 5 ml liquid MRS medium containing bile salts (Oxoid) with concentrations of 0.3%, 0.5%, and 1%. Furthermore, growth tests were carried out after 4 hours incubation at 37°C by inoculating on the MRS agar medium using streak plate method and incubated at 37°C for 24 to 48 hours [13].

**Potential Test as Antibacterial Solution preparation of Cell-Free Culture Supernatant (CFCS)**
The LAB cultures were obtained by aseptically, inoculated in 5 mL of liquid MRS medium, and incubated at 37°C for 18 hours. Besides, the CFCS solution was harvested by centrifugation at 13,500 rpm for 15 minutes. Meanwhile, the supernatant was neutralized at pH 6.5 with 1 N NaOH solution and refrigerated before being used for antibacterial assessment [14].

**Pathogenic bacteria strain preparation**
The pathogenic bacteria used were Gram-positive, specifically *Staphylococcus aureus* ATCC 25923. However, *Salmonella typhi* 122.4 CCA [15] and *Salmonella typhi* NCTC 786 obtained from PT. Biofarma were equally used. These bacterial cultures were obtained by aseptically and inoculated into liquid BHI medium at 37°C for 18 hours before applying antibacterial analysis.

**Antibacterial activity test**
The potential of LAB as an antibacterial was tested using the CFCS solution based on the well-diffusion agar method [14, 16, 17]. Besides, an 8 mm well was created in Mueller Hinton agar (MHA, Oxoid) medium, then indicator bacteria swab was performed using sterile cotton on the surface. Therefore, each well was filled with 100 µl of CFCS solution and allowed to cool, to permit a more rapid diffusion, before the petri dishes were incubated at 37°C for 24 hours. Furthermore, antibacterial activities were determined based on the assessment of clear zone (mm) formed around the well, where bacterial growth was reduced to a well diameter (8 mm) [18, 19].

**Identification of Lactic Acid Bacteria**
The LAB isolates with probiotic and antibacterial potential were identified using 50 CHL Analytical Profile Index (API) (BioMerieux) and grown in MRS agar medium for 48 hours. These cultures were obtained aseptically and inoculated into ampoules containing 50 CHL API medium, with turbidity equalized to the 2 McFarland. Furthermore, the specimen was inoculated into a microtube strip at an API 50CHL kit and incubated for 48 hours according to a predetermined procedure [20]. Also, the respective identity was determined using an API web software and

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RESULTS AND DISCUSSION
Isolation and Selection of Lactic Acid Bacteria from Red Fruit

Figure 1 depicts the growth of LAB colonies in the MRS agar medium equipped with CaCO₃. This is characterized by the formation of a clear zone, due to a reaction between the chemical and lactic acid compounds where dissolved calcium lactate (Ca-lactate) is produced [21]. The colonies with clear zones are then selected through phenotypic tests to determine the bacteria main character. Table 1 illustrates 25 LAB isolates obtained in the form of rods, Gram-positive, homofermentative, non-motile, and catalase-negative cells, grown at 10°C and 45°C, according to the digestive tract temperature range. These are the predominant bacteria traits [22], used in the selection of potential probiotics.

| No | Isolate | Acid pH tolerance test | Bile salts tolerance test (%) | Temperature test (ºC) | Gas Production |
|----|---------|-----------------------|-----------------------------|----------------------|----------------|
|    |         | 2  | 3  | 4  | 0,3 | 0,5 | 1  | 10 | 45 |                     |
| 1  | S1A1    | -  | -  | +  | +   | -   | -   | +  | +  | Homofermentative    |
| 2  | S1A2    | -  | -  | -  | +   | -   | -   | +  | +  | Homofermentative    |
| 3  | S1A3    | -  | +  | +  | +   | +   | +   | +  | +  | Homofermentative    |
| 4  | S1A4    | -  | -  | -  | +   | +   | +   | +  | +  | Homofermentative    |
| 5  | S1T1    | -  | +  | +  | +   | +   | +   | +  | +  | Homofermentative    |
| 6  | S1T2    | +  | +  | +  | +   | +   | +   | +  | +  | Homofermentative    |
| 7  | S1T3    | +  | +  | +  | +   | +   | +   | +  | +  | Homofermentative    |
| 8  | S1T4    | +  | +  | +  | +   | +   | +   | +  | +  | Homofermentative    |
| 9  | S1B1    | +  | +  | +  | +   | +   | +   | +  | +  | Homofermentative    |
| 10 | S1B2    | -  | +  | +  | +   | +   | +   | +  | +  | Homofermentative    |
| 11 | S1B3    | -  | -  | +  | +   | +   | +   | +  | +  | Homofermentative    |
| 12 | S2A1    | -  | -  | +  | -   | -   | -   | +  | +  | Homofermentative    |

According to Table 1, the 25 selected LAB isolates were grown in homofermentative conditions at 10°C and 45°C. This means there was no gas production during the growth phase. These results make it possible to determine the potential to isolate technology in food industry applications [2]. Based on the tolerance test, 24 lived on 0.3% while 18 out of the total survived with 1% bile salts. Moreover, 8 lived in acidic environments of up to pH 2. The probiotic microbe requires strength to survive against various extreme conditions in the human digestive tract, including acidic pH and bile salts [23]. These characteristics are important for the organism to reach the digestive tract alive, adhere to the mucosal layer and other components of the extracellular matrix. Also, the formation of a fast-microbial community triggers a reduction in pH and competition for adhesion with pathogenic bacteria, therefore preventing colonization [13]. The ability to fight stomach acid is considered as the main condition during screening because these bacteria pass through the stomach before digestion. Moreover, gastric acid in the human body has a pH of 3 with a digestion time between 1-3 hours. Therefore, the tolerant LAB is assumed to be a potential probiotic candidate [24].
Determination of Lactic Acid Antibacterial Activity based on agar well diffusion method

The Agar well diffusion method was used to determine the antibacterial activity produced by LAB isolates as potential probiotic candidates. This ability was tested against three pathogenic organisms, including \textit{S. aureus} ATCC 25923 representing Gram-positive bacteria, while \textit{S. typhi} BPE 122.4 CCA and \textit{Salmonella typhi} NCTC 786 as Gram-negative. The results in table 2 showed a different inhibition spectrum for each bacterium. According to a report by [18], the activity against the pathogenic bacteria is grouped into 3 categories, including weak (0-3mm), moderate (3-6mm), and strong (> 6mm) activity.

Table 2: Antibacterial activity of LAB isolates from Papua red fruit (\textit{Pandanus conoideus} Lam.) against pathogenic bacteria based on agar well diffusion method.

| Isolate | Inhibition zone (mm) against indicator bacteria |
|---------|-----------------------------------------------|
|         | \textit{Salmonella typhi BPE 122.4 CCA} | \textit{Salmonella typhi NCTC 786} | \textit{Staphylococcus aureus ATCC 25923} |
| S1A1 | 2.00±1.00 | 2.33±0.58 | 2.33±0.58 |
| S1A2 | 2.33±0.57 | 3.67±0.58 | 2.00±0.00 |
| S1A3 | 2.33±0.58 | 1.33±0.58 | 3.33±0.58 |
| S1A4 | 2.00±1.00 | 4.00±1.00 | 2.67±0.57 |
| S1T1 | 2.33±0.57 | 5.53±0.57 | 6.67±0.57* |
| S1T2 | 3.00±0.00 | 1.67±0.57 | 2.33±0.57 |
| S1T3 | 2.33±0.58 | 1.33±0.57 | 1.67±0.57 |
| S1T4 | 2.67±0.58 | 1.00±0.00 | 3.33±0.57 |
| S1B1 | 3.00±0.00 | 2.67±0.57 | 2.33±0.57 |
| S1B2 | 1.00±0.00 | 1.67±0.57 | 2.67±0.57 |
| S1B3 | 2.00±1.00 | 3.00±1.00 | 2.00±1.00 |
| S2A1 | 2.00±0.00 | 2.33±0.57 | 1.33±0.57 |
| S2A2 | 3.00±1.00 | 2.67±0.57 | 1.00±0.00 |
| S2A3 | 2.33±0.57 | 2.67±0.57 | 2.33±0.57 |
| S2A4 | 1.67±0.57 | 2.67±0.57 | 1.33±0.57 |
| S2T1 | 2.00±0.00 | 3.67±0.57 | 2.00±1.00 |
| S2T2 | 2.67±0.57 | 1.00±0.00 | 1.00±0.00 |
| S2T3 | 2.33±0.57 | 3.00±1.00 | 2.67±0.57 |
| S2T4 | 6.33±0.57* | 6.67±0.57* | 6.00±0.00* |
| S2T5 | 1.67±0.57 | 2.67±0.57 | 1.33±0.57 |
| S2T6 | 2.33±0.57 | 1.67±0.57 | 2.00±0.00 |
| S2B1 | 6.33±0.57* | 2.67±1.15 | 0 |
| S2B2 | 3.00±0.00 | 2.67±0.57 | 3.33±0.57 |
| S2B3 | 2.00±1.00 | 2.33±0.57 | 2.67±0.57 |
| S2B4 | 3.3±0.57 | 2.67±0.57 | 3.67±1.15 |

Description: *strong inhibition against indicator bacteria
According to Table 2 the LAB isolates S1T1 have stronger inhibition against Gram-positive indicator (S. aureus ATCC 25923) than Gram-negative (S. typhi BPE 122.4 CCA and S. typhi NCTC 786), while strain S2B1 has the reverse effect. However, the S2T4 variant had strong inhibition with broad-spectrum against all the microbes investigated. Furthermore, some of the organisms produce antibacterial effects and prevent the growth of pathogenic bacteria, therefore protecting the host's defense against infections in the intestinal lumen [13]. The LAB is more profitable compared to other microorganisms, due to the antibacterial substances produced [25]. This characteristic is important for assessing the potential as a probiotic and producer of antimicrobial compounds [26]. Besides, tolerance to acidic pH and bile salts, with the ability to inhibit pathogenic bacteria are of benefit to human health.

Identification of Lactic Acid Bacteria Isolates

Furthermore, five LAB isolates tolerant of acidic pH and 1% bile salts, with strong inhibition against pathogenic bacteria were selected and identified using 50 CHL Analytical Profile Index (API) (BioMérieux). These include S1B1, S2B1, S1T2, S2T4, and S1T1. Based on the Gram staining results in Figure 2, the Gram-positive characteristics of long rod cells were identified and arranged in groups or chains. These features are initial indications for the classification of isolates suspected to be Lactobacillus.

Table 4 shows the results of the LAB isolates identification test performed using the API 50 CHL kit. Also, S1B1, S2B1, S1T2, S2T4, and S1T1 strains were able to ferment 23 carbon sources. These include L-arabinose, ribose, galactose, glucose, fructose, mannose, mannitol, sorbitol, methyl-D-monoxide, N-acetyl-glucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melezitose, raffinose, β-gentiobiose, and D-turanose. Besides, data confirmation was performed using API web software, where 99.9% of the discovered isolates were similar to Lactobacillus plantarum 1.
Table-4: Identification of lactic acid bacterial isolates from Papuan red fruit selected as probiotic candidates using Analytical Profile Index (API) 50 CHL (BioMérieux)

| No | Carbon sources | Isolate S1B1 | Isolate S1T2 | Isolate S2B1 | Isolate S2T4 | Isolate S1T1 |
|----|----------------|--------------|--------------|--------------|--------------|--------------|
| 0  | Control        | -            | -            | -            | -            | -            |
| 1  | Glycerol       | -            | -            | -            | -            | -            |
| 2  | Erythritol     | -            | -            | -            | -            | -            |
| 3  | D-Arabinose    | -            | -            | -            | -            | -            |
| 4  | L-Arabinose    | +            | +            | -            | +            | +            |
| 5  | Ribose         | +            | +            | +            | +            | +            |
| 6  | D-Xylose       | -            | -            | -            | -            | -            |
| 7  | L-Xylose       | -            | -            | -            | -            | -            |
| 8  | Adonitol       | -            | -            | -            | -            | -            |
| 9  | B-Methyl-D-Xylo| -            | -            | -            | -            | -            |
| 10 | Galactose      | +            | +            | +            | +            | +            |
| 11 | Glucose        | +            | +            | +            | +            | +            |
| 12 | Fructose       | +            | +            | +            | +            | +            |
| 13 | Mannose        | +            | +            | +            | +            | +            |
| 14 | Sorbose        | -            | -            | -            | -            | -            |
| 15 | Rhamnose       | -            | -            | -            | -            | -            |
| 16 | Dulcitol       | -            | -            | -            | -            | -            |
| 17 | Inocitol       | -            | -            | -            | -            | -            |
| 18 | Mannitol       | +            | +            | +            | +            | +            |
| 19 | Sorbitol       | +            | +            | +            | +            | +            |
| 20 | Methyl-D-Mannox| +            | +            | +            | +            | +            |
| 21 | Methyl-D-Glucoxi| -            | -            | -            | -            | -            |
| 22 | N-Acetyl-Glucosamine | +      | +            | +            | +            | +            |
| 23 | Amygdalin      | +            | +            | +            | +            | +            |
| 24 | Arbutin        | +            | +            | +            | +            | +            |
| 25 | Esculin        | +            | +            | +            | +            | +            |
| 26 | Salicin        | +            | +            | +            | +            | +            |
| 27 | Cellobiose     | +            | +            | +            | +            | +            |
| 28 | Maltose        | +            | +            | +            | +            | +            |
| 29 | Lactose        | +            | +            | +            | +            | +            |
| 30 | Melibiose      | +            | +            | +            | +            | +            |
| 31 | Sucrose        | +            | +            | +            | +            | +            |
| 32 | Trehalose      | +            | +            | +            | +            | +            |
| 33 | Inulin         | -            | -            | -            | -            | -            |
| 34 | Melezitose     | +            | +            | +            | +            | +            |
| 35 | Raffinose      | +            | +            | +            | +            | +            |
| 36 | Starch         | -            | -            | -            | -            | -            |
| 37 | Glycogen       | -            | -            | -            | -            | -            |
| 38 | Xylitol        | -            | -            | -            | -            | -            |
| 39 | β-Gentiobiose  | +            | +            | +            | +            | +            |
| 40 | D-Turanose     | +            | +            | +            | +            | +            |
| 41 | D-Lyxose       | -            | -            | -            | -            | -            |
| 42 | D-Tagatose     | -            | -            | -            | -            | -            |
| 43 | D-Fucose       | -            | -            | -            | -            | -            |
| 44 | L-Fucose       | -            | -            | -            | -            | -            |
| 45 | D-Arabinol     | -            | -            | -            | -            | -            |
| 46 | L-Arabinol     | -            | -            | -            | -            | -            |
| 47 | Gluconat       | -            | -            | -            | -            | -            |
| 48 | 2-Keto-Gluconat| -            | -            | -            | -            | -            |
| 49 | 5-Keto-Gluconat| -            | -            | -            | -            | -            |

Identification Results: L. plantarum 1  L. plantarum 1  L. plantarum 1  L. plantarum 1  L. plantarum 1
Similarity Index: 99.9%  99.9%  99.9%  99.9%  99.9%
The LAB isolate identification proves *L. plantarum* to be naturally present in plant materials and also its fermentation products [27, 28]. Furthermore, the prebiotic inulin content in red fruits is also considered a factor supporting LAB growth. Murtiningrum et al. [10] conducted an *in vitro* evaluation, and the results showed the ability for inulin present in the pedicle extract to supports *Lactobacillus casei* development.

The *Lactobacillus plantarum* strain S2T4 grows under acidic pH conditions and are also tolerant to bile salts. These microorganisms have strong inhibitory power with varying spectra against *S. aureus* ATCC 25923, *S. typhi* BPE 122.4 CCA, and *S. typhi* NCTC 786 indicator bacteria. Furthermore, the strain S1T1 had stronger inhibition against Gram-positive species (*S. aureus* ATCC 25923) than the Gram-negative. However, the inverse was reported for the S2B1 strain, while S2B1 and S1T1 significantly hinder the growth of *S. aureus* ATCC 25923, respectively. Besides, three isolates, including S1B1, S2B1, and S1T1 also have the potential for further development as probiotic candidates for use as antibacterial and preservative agents.

CONCLUSIONS

The lactic acid bacteria strains S1B1, S2B1, S1T2, S2T4, and S1T1 isolated from Papuan red fruit were identified as *Lactobacillus plantarum* and determined to survive at pH 2 and bile salt 1%. These isolates have the potential for further development as probiotic candidates. Specifically, the S2T4 strain has a strong inhibitory capacity against *S. aureus* ATCC 25923, *S. typhi* BPE 122.4 CCA, and *S. typhi* NCTC 786, while S2B1 and S1T1 significantly hinder the growth of *Salmonella typhi* BPE 122.4 CCA and *S. aureus* ATCC 25923, respectively. Besides, three isolates, including S2T4, S2B1, and S1T1 also have the potential for further advancement as biopreservatives.

ACKNOWLEDGEMENTS

Our gratitude goes to the Faculty of Biotechnology Universitas Kristen Duta Wacana which is funding this research in 2020 with a project number 118/D.01/Bio/2020.

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