



## Microbiology Research Journal International

30(8): 15-22, 2020; Article no.MRJI.60654

ISSN: 2456-7043

(Past name: British Microbiology Research Journal, Past ISSN: 2231-0886, NLM ID: 101608140)

# Isolation, Characterization, and Molecular Identification of Indigenous Bacteria from Fermented Almonds (*Prunus dulcis*)

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### Authors' contributions

This work was carried out in collaboration among all authors. Author SN designed the study, wrote the protocol, and wrote the first draft of the manuscript. Author EF managed the analyses of the study. Author NEL managed the literature searches. All authors read and approved the final manuscript.

### Article Information

DOI: 10.9734/MRJI/2020/v30i830247

#### Editor(s):

(1) Dr. Zahid Anwar, University of Gujrat, Pakistan.

#### Reviewers:

(1) B. Sathya Priya, Bharathiar University, India.

(2) Amit Verma, S. D. Agricultural University, India.

(3) Basil A. Abbas, University of Basrah, Iraq.

Complete Peer review History: <http://www.sdiarticle4.com/review-history/60654>

Original Research Article

Received 26 June 2020

Accepted 03 September 2020

Published 10 September 2020

## ABSTRACT

**Aims:** This study aims to isolate and identify the indigenous bacteria of almonds fermentation.  
**Methods:** Characterization of the indigeneous bacteria are using gram staining, biochemical tests, 16SrRNA gene sequencing, and the antimicrobial activity against *Escherichia coli* bacteria.  
**Results:** Approximately  $28 \times 10^6$  CFU / mL bacteria were obtained from almonds fermentations with 14 isolates from enrichment results. Three randomly selected isolates were gram-positive rod-shaped with a negative catalase and positive fermentation test. However, one isolate showed positive results on the motility test. The antimicrobial test results from the three randomly selected isolates using the disk diffusion method obtained inhibition zones of 7 mm, 6.7 mm, and 7 mm, respectively. Therefore, by using 16S rRNA gene sequencing, three different microorganisms were found, namely *Bacillus subtilis* strain IAM 12118, *Bacillus Piscis* strain 16MFT21, and *Bacillus licheniformis* strain BaDB27.  
**Conclusion:** It was found that *Bacillus subtilis* strain IAM 12118, *Bacillus Piscis* strain 16MFT21, and *Bacillus licheniformis* strain BaDB27 in almonds fermentation and also can be used as probiotic bacteria.

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**Keywords:** Fermentation; almonds; probiotics; indigenous bacteria; antimicrobials.

## 1. INTRODUCTION

Probiotics are supplements in the form of live microorganisms that can provide beneficial effects for the host by increasing the balance of microorganisms in the digestive tract [1]. Substances produced by the metabolism of probiotic bacteria such as hydrogen peroxide, lactic acid, bacteriocin are antimicrobial and antibiotic, which suppresses the growth of pathogenic bacteria [2]. One characteristic of probiotic bacteria is its high resistance to acids [3].

Good probiotics survive in various environmental conditions and can be packaged in the form of a stable life on an industrial scale with a prolonged storage period capable of surviving in the field conditions, with a good effect on intestine [4].

Public awareness in determining safe and cost-effective alternative treatments for antibiotic resistance led to the development of the current therapeutic patterns. From a large number of suggested options, probiotic therapy is the most feasible, based on prolonged historical usage and guaranteed safety. Lactic acid and Bifidobacterium species are two groups of bacteria that are globally recognized as safe to be consumed with potential health benefits. Other bacteria used for probiotics include strains of *Enterococcus*, *Streptococcus*, and *Bacillus sp* [4].

Almonds (*Prunus dulcis*) contain many antioxidants and many substances such as  $\alpha$ -tocopherol and Mono Unsaturated Fatty Acid (MUFA). It also contains fibers, protein, phosphorus, potassium, selenium, iron, vitamin E, biotin, manganese, tryptophan, magnesium, vitamin D, calcium, copper, and riboflavin [5]. Almonds also contain 0.3 grams of unsaturated fat as well as a mixture of phenol and polyphenol that are flavonoid compounds [6]. Research on the microbiological and chemical aspects of almonds is considered essential because it is rarely found. The microbiological attributes discuss the microbes' properties, therefore it is necessary to isolate and identify bacteria. Microbial isolation is to separate one type of microbe from others to produce a pure culture of only one microbe. Microbial identification is the process of obtaining morphological, biochemical, and molecular characteristics from microbes.

Therefore, this study aims to isolate and identify indigenous bacteria from fermented almonds.

## 2. MATERIALS AND PROCEDURES

### 2.1 Samples Preparation

For sample preparation, 50 grams of almonds are soaked in boiled water and covered in an airtight container for eight days. Furthermore, the fermented almonds are mashed with 100 mL of boiled water in the blender with a 1: 1 ratio for 2 minutes.

### 2.2 Isolation of Bacteria

1 ml of almond fermentation is weighed and then dissolved in 90 ml MRS broth solution and stirred until homogeneous. MRS media (De Man, Rogosa and Sharpe) consist of Oxoid peptone 10 g, Lab-Lemco (Oxoid) 10 g, Yeast extract (Difco or Oxoid) 5 g, glucose 20 g, polyoxyethylene sorbitan monooleate (Tween 80) 1 ml,  $K_2HPO_4$  2 g,  $CH_3COONa \cdot 3H_2O$  5 g, triammonium citrate 2 g,  $MgSO_4 \cdot 7H_2O$  200 mg,  $MnSO_4 \cdot 4H_2O$  50 mg, distilled water 1 litre [7]. The next step is to make dilutions with concentrations of  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$ . The suspensions are pipetted at 0.1 ml and spread on a petri dish containing MRS agar supplemented with 2%  $CaCO_3$  using the spread technique. The inoculums are then inserted into the incubator for 48 hours at  $37^{\circ}C$ . After that, the growing of bacteria colonies are seen using the colony counter. Furthermore, 14 isolates are randomly selected and etched into new MRS agar while determining the Gram staining, tests of catalase, gelatin hydrolysis, fermentation type, citrate, motility, starch hydrolysis, and  $H_2S$  tests.

### 2.3 Characterization of Bacteria

#### 2.3.1 Gram staining

The culture results were flattened on the slide glass surface using a loop needle, then fixed using a spirit lamp. Dropped with crystal violet on the fixed culture, left for 60 seconds after that washed with running water. Then drop the iodine solution and leave it for 30 seconds then washed and drop the decolorizer solution. The last step is to drop safranin and let it sit for 20 seconds then wash it with running water, then examined under a microscope.

### 2.3.2 Catalase test

The culture results were flattened on the slide glass surface using a loop needle. Drop 3% H<sub>2</sub>O<sub>2</sub> by using a dropper to the LAB colony which is smeared on the glass object and then observed the presence of air bubbles.

### 2.3.3 Gelatin hydrolysis test

Bacterial isolates were inoculated into nutrient gelatin medium in a test tube aseptically incubated at 37°C for 24-48 hours. Then the culture was placed in a cooler at 4°C for 30 minutes, and the consistency of the medium was observed.

### 2.3.4 Type of fermentation test

Observations were made by growing isolate cultures in liquid MRS media in a test tube. Furthermore, the incubation is carried out for 24-48 hours at a temperature of 30°C. if the tested bacteria produce gas that is accommodated in the tube, then the bacteria is declared as heterofermentation, otherwise it is declared as homofermentation.

### 2.3.5 Citrate test

Isolates were inoculated on Simmon's Citrate agar medium in a vertical test tube, then incubated at 37°C for 24-48 hours and observed the changes that occurred, the test was positive if there was a change in the color of the medium from green to blue.

### 2.3.6 Motility test

Bacterial isolates were inoculated on SSM medium (Semi Solid Medium) in the test tube aseptically and punctured on the upright agar then incubated at 37°C for 24-48 hours. The test will be positive indicated by the widening of the puncture marks on the media.

### 2.3.7 Starch hydrolysis test

Starch agar is put in a petri dish, then inoculated the bacterial isolate in the middle of the petri dish then incubated for 24-48 hours at 37°C. After incubation, a few drops of iodine solution are added to the surface of the growing bacterial isolate colonies. If a clear zone is formed around the colony, the test is positive.

### 2.3.8 H<sub>2</sub>S production test

Isolates were inoculated into TSIA (triple sugar iron agar) medium then incubated for 24-48

hours at 37°C. If a black deposit forms on the medium market, the test is positive.

## 2.4 Antimicrobial Test against Pathogenic Bacteria

Antimicrobial effect towards *E.coli* bacteria is carried out using the disk diffusion method. Approximately 8 colonies were put into a saline tube and then homogenized to obtain a concentration of 0.5 Mc Farland. Sterile disc paper with a 5 mm diameter is inserted into the solution and placed on the nutrient agar surface. After incubating for 24 hours, the inhibition zone is calculated using calipers.

## 2.5 DNA Extraction and 16S rRNA Gene Amplification

The process of identifying bacterial isolates is carried out through molecular techniques based on partial genetic analysis on 16S rRNA. Pitcher et al. (1989) modified Guanidium thiocyanate Extraction method was used to determine the RNA extraction. Meanwhile, the PCR amplification process on 16S rRNA uses Primary 27 F: 5'-- AGA GTT TGA TCC TGG CTC AG -- 3' and primary 1492 R: 5'-- GGT TAC CTT GTT ACG ACT T --3' (White et al., 1990; O'Donnell, 1993). According to Hiraishiet al. (1995) PCR purification process is carried out using the PEG precipitation method. This continues to the sequencing process which uses Primary 27 F : 5'-- AGA GTT TGA TCC TGG CTC AG -- 3', Primary 518 F: 5' -- CCA GCA GCC GCG GTA ATA CG --3', Primary1492 R: 5'-- GGT TAC CTT GTT ACG ACT T --3', and Primary 800 R: 5'-- TAC CAG GGT ATC TAA TCC --3' are also used. The product from sequencing is then purified again using Ethanol, the purification method. After that, the sequence of nitrogen bases was determined using the automated DNA sequencers method (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems).

## 2.6 Nucleotide Sequences

The sequencing data is then trimmed and assembled by the BioEdit program (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). It was further synchronized with genome data registered at the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/LABST/>) to determine species with the largest and closest molecular homology.

### 3. RESULTS

#### 3.1 Isolation, Determination of Colonies Number and Biochemical Characteristics

Fig. 1 shows the colony growth results from almond milk fermentation, which obtained a bacteria value of  $28 \times 10^6$  CFU/ml. Most of the colonies were round, white or cream and smooth. In accordance with the isolation products that have been carried out, 14 isolates grow and form colonies on MRSA media. From these 14 growing isolates, 3 were randomly selected.

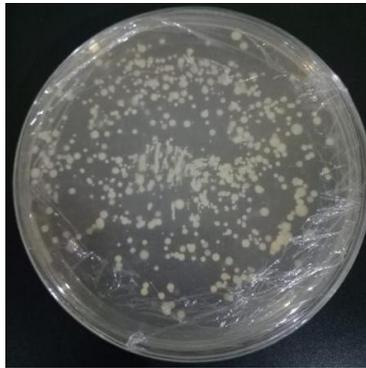


Fig. 1. Bacteria isolates from almonds fermentation at MRS agar media

Table 1 shows the gram staining results and biochemical tests on three isolates capable of surviving until the end of the study. Fermentation test results showed positive results on all 3 isolates, with different types of fermentation. SA3 and SA8 isolates show a hetero fermentation picture, while in SA7, it shows a homo fermentation picture. Catalase test results for all isolates showed negative results.

#### 3.2 Antimicrobial Activity of Bacteria

Antimicrobial activity against disease-causing bacteria (pathogens) is carried out by the disk diffusion technique with 5 mm disc diameter. The pathogenic bacteria used are *Escherichia coli* with the antimicrobial effects of the isolate as shown in Table 2.

#### 3.3 Amplification of 16S rRNA Gene by PCR and Molecular Identification

Based on the results of the 16S rRNA gene sequence and carrying out adjustment with the NCBI database, three types of bacteria were identified, namely *Bacillus subtilis* strain IAM 12118 Accession no: MK267098.1, *Bacillus Piscis* strain 16MFT21 Accession no: NR\_165685.1, and *Bacillus licheniformis* strain BaDB27 Accession no: JX237861.1.

Table 1. Gram staining and biochemical characteristics of isolate bacteria from fermented almonds

Isolates	Gram Staining	Fermentation test	Catalase test	Starch hydrolysis test	Gelatine test	motility test	citrate test	H <sub>2</sub> S test
SA3	positive gram rods	Hetero fermentation	negative	negative	negative	negative	negative	negative
SA7	positive gram rods	Homo fermentation	negative	negative	negative	negative	negative	negative
SA8	positive gram rods	Hetero fermentation	negative	positive	negative	negative	negative	negative

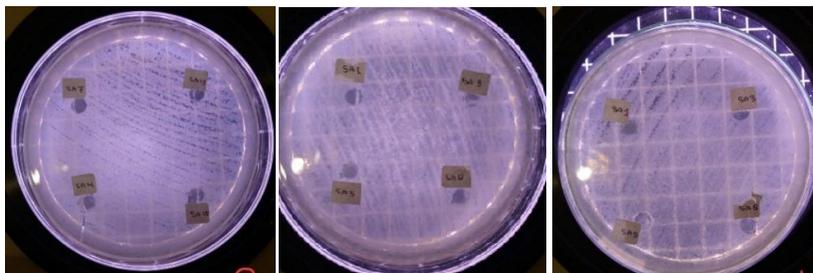


Fig. 2. Inhibitory zone magnitude in BAL isolates on agar nutrient media

**Table 2. Inhibition zone diameter of BAL isolates on agar nutrient media**

Isolates	Inhibition zone diameter (mm)
SA 3	7
SA 7	6,7
SA 8	7

#### 4. DISCUSSION

A total of  $28 \times 10^6$  CFU / ml bacteria was obtained from 1 ml of fermented almonds using MRSA + CaCO<sub>3</sub>. The colonies number obtained from the research can meet the probiotic food standard because it contains at least  $2 \times 10^3$  CFU / mL of the substance [8]. The isolation results showed that 14 isolates can grow on MRSA media, however, only 3 survived until the end of the study.

Catalase test for all isolates shows negative results, which are similar to the study carried out by Fachrial et al. (2018) regarding bacteria isolated from palm oil juice. From the research, 6 isolates that show a negative catalase test were obtained [9]. The catalase test is used to identify the microorganism's ability to produce the catalase enzyme produced by various types of bacteria to prevent free radicals oxidation that damage or kill the bacteria. The principle of catalase test is associated with the production of the compound that is highly toxic to cells, namely hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and superoxide radicals (O<sub>2</sub><sup>-</sup>) produced from the direct transfer of electron directly by flavoprotein, which is a carrier molecule in electron transfer chain. Facultative aerobic and anaerobic bacteria produce enzymes capable of detoxifying these compounds [10].

In this study, the isolated bacteria from fermented almonds could not hydrolyze gelatin or starch. Palumbo et al. (2006) also obtained similar results in a study conducted on isolates from almonds [11]. The gelatin test aims to see the ability of bacteria to produce gelatinase enzymes that are able to hydrolyze gelatin. Some microorganisms can break down gelatin molecules into amino acids and are then able to use them as a source of nutrients used in metabolism. The starch hydrolysis test was carried out to determine the presence of the amylase enzyme which functions to break down starch into simpler components. Starch is a polysaccharide consisting of several glucose saccharides. When starch is hydrolyzed by amylase exoenzyme, the

compound will be broken down into maltose and glucose [12].

The results of the fermentation type test in this study showed different results among the three isolates. This result is different from the results obtained by Fachrial et al. (2017) who conducted research on bacterial isolates in cow's milk products, the results of this study found homofermentative type of all isolates [13]. Homofermentation bacteria only produce lactic acid as the main product of fermentation. while heterofermentation bacteria besides lactic acid also produce ethanol, other acids such as acetic acid and CO<sub>2</sub> gas [14].

In this study, the results of citrate, motility and H<sub>2</sub>S tests were negative. In a study conducted by Setti and Bencheikh (2013) also found negative results on citrate and H<sub>2</sub>S tests on all isolates from almonds, but the results of the motility test showed positive results [15].

In the antimicrobial activity test, three isolates were randomly selected from the growing isolates. In this study, *Escherichia coli* is used as a pathogenic bacterium for antimicrobial activity testing. *E. coli* is a negative gram rod-shaped bacterium sized 0.4 - 0.7 × 1.4 μm, motile, and without spores. The bacterium is actually one of the normal flora in the digestive tract that participates in the intestine's metabolic functions. It comprises the synthesis of vitamin K. *E. coli*, an opportunistic and pathogenic bacterium located in tissue outside the intestine of a habitat [16]. SA3 and SA8 have the largest inhibition zone of 7 mm while SA7 is 6.7 mm. A study carried out by Sari et al. (2018) found that bacteria isolated from "bekasam" food, a fermented fish from Aceh, were used to inhibit the *E. coli*, *S. aureus*, and *S.typhi*. They also stated the antagonistic tests using the disk diffusion method, with inhibition zones of MS2 isolates against *E.coli*, *S. aureus*, and *S.typhi* with values of 13.1 mm, 12.7 mm and 7.3 mm. In general, the biggest inhibitory zone is owned by *S.aureus* bacteria [17]. Bacteriocin compounds produced by Lactic Acid Bacteria (LAB) can interfere with membrane permeability and damage the prototype motive force (PMF) capable of interfering with energy metabolism and protein biosynthesis. PMF serves to maintain the pH balance of intracellular acid. The process of bacteriocin bactericidal activity occurs through direct contact of bacteriocytes with cell membranes. It is capable of disrupting membrane destabilization, thereby leading to

perforation through PMF interference. One of PMF disorders is a reduction that enables the excessive protons derived from extracellular substances entering the cell. It disrupts metabolic function, thereby preventing the cells from transporting nutrients and maintaining the concentration of cofactor molecules that causes the death of pathogenic bacteria cells [18]. The large amount of LAB positively correlates with the number of antibacterial compounds and the inhibitory zones formed. Acetic and lactic acid is important in the formation of antibacterial compounds. Antibacterial compounds produced by all bacteria in multi-strain probiotics are associated with each other, therefore, in inhibiting pathogenic bacteria broad spectrum of inhibition is produced [19].

The adjustment results of 16S rRNA gene sequencing with a database in NCBI found three types of bacteria, namely *Bacillus subtilis* strain IAM 12118, *Bacillus Piscis* strain 16MFT21, and *Bacillus licheniformis* strain BaDB27. *Bacillus subtilis* is a type of bacteria that grows in diverse environments, including the digestive tract of animals. *Bacillus subtilis* can be isolated from various environments, making it appear like the species is everywhere and widely adapted to grow in various environments. However, like all members of *Bacillus* genus, *Bacillus subtilis* is able to form active endospores that are resistant in facing nutritional deficiencies and other environmental stresses [20]. *Bacillus subtilis* has also been studied to produce bacteriocin, namely subtilin and subtylosin, which are active against many strains of gram-positive bacteria [21].

*Bacillus Piscis* is a bacterium that was first identified in the Dissostic Husmawsoni fish in 2016. Therefore, there is limited information regarding this species [22]. *Bacillus licheniformis* has been proven to be a multipurpose organism and widely known along with *Bacillus subtilis*. *Bacillus licheniformis* is most often found in soil and in feathers of birds that live on the ground. *Bacillus licheniformis* has the ability to produce bacteriocin under aerobic and anaerobic conditions against anaerobic microorganisms [23]. Some bacteriocin reported that *Bacillus licheniformis* has limited antibacterial activity in Gram-positive organisms. The bacillocin produced by *Bacillus licheniformis* from dairy products has antibacterial activity against the species *Geobacillus stearothermophilus*, *B. subtilis*, *B. smithii*, *B. cereus*, *B. licheniformis*, and *B. anthracis*. Similar to the LAB, the genus *Bacillus* is known as 'generally recognized as

safe' (GRAS), such as *B. licheniformis* and *B. subtilis*. Therefore, it indicates that *Bacillus subtilis* and *Bacillus licheniformis* can be used as probiotic bacteria [24].

Palumbo, Baker, and Mahoney (2006) stated that the isolation in ripe almonds is most frequent in *Bacillus* bacteria [11]. It further reinforces the research results that the bacteria are indigenous in fermented almonds.

## 5. CONCLUSION

Based on the research, it can be concluded that in the fermentation of almonds, there are a number of bacteria that have anti-microbial effects. Therefore, from the identification results, it was found that *Bacillus subtilis* strain IAM 12118, *Bacillus Piscis* strain 16MFT21, and *Bacillus licheniformis* strain BaDB27. Further research needs to be carried out on the content of bacteriocin compounds produced by the lactic acid bacteria.

## ACKNOWLEDGEMENTS

The authors are grateful to Prima Indonesia University (UNPRI) and LIPI for their assistance. The authors are also grateful to all the reviewers that provided criticism and suggestions for the completion of this paper.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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