Antibacterial activities of indigenous yeasts isolated from pomegranate peels (*Punica granatum* L.)

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**Abstract**

Pomegranate peels (PGPs) are known to have the potential as antibacterial not only from their nutrient content but also the microflora. The activities might be caused by the existence of indigenous yeast that can be utilized to inhibit the growth of pathogenic bacteria. This study aims to identify antibacterial and antioxidant activity of indigenous yeast isolated from PGP. The research was conducted by experimental methods and followed by descriptive analysis. The study was done by the isolation of indigenous yeast from PGPs, which was identified using the rRNA sequence analysis of internal transcribed spacer (ITS) region with the primers of ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and then compared with Basic Local Alignment Search Tools (BLAST) algorithm toward the GenBank. Antibacterial activities of indigenous yeast were tested with agar plug diffusion and time kill test toward *Escherichia coli* and *Staphylococcus aureus*. The yeast identification obtained two isolates similar to *Hanseniaspora uvarum* CBS 314 and two isolates of *Pichia kudriavzevii* ATCC 6258 which have antibacterial activity against *E. coli* and *S. aureus*. *P. kudriavzevii* PGP D4 have best antimicrobial activities with a strong activity against *E. coli* (±9 mm) and medium activity against *S. aureus* (±3.1 mm).

**Key words:** Antibacterial, pomegranate peels, yeast

**INTRODUCTION**

Pomegranate peels (PGPs) are the outer and inedible part of the fruit and also being the byproduct of pomegranate processing with the amount of 60% of the total fruit.¹ PGP has demonstrated an expansive range of antimicrobial activities, especially toward antibiotic-resistant microorganisms.² Alkaloids, flavonoids, sterols, triterpenes, tannins, saponins, and phenols have been found in PGP which have shown antibacterial and antioxidant properties.³ Besides the chemical compound, antibacterial activity may originate from indigenous microorganisms such as yeasts.

PGPs contain several chemical compounds including protein, carbohydrates, fats, fiber, and phenols. PGPs contain several compounds needed by yeast to support its growth, such as about 66% carbohydrates (dry basis).⁴ Total yeasts population of $7.73 \times 10^8$ cfu/g was found in PGP, which is higher than others.⁵

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Many research found various mechanisms in indigenous yeast inhibitory activities toward other microorganisms. Antagonistic activities are shown between yeasts that entangle mycocins that have been known as second metabolites.[9] Meanwhile, the main metabolites such as organic acids, volatile acids, and H₂O₂ create stress conditions for other microorganisms surround the yeast’s growth.[9] The formation of secondary metabolites by yeast is caused by the reduced nutritional condition which causes the accumulation of cell excretion, so that more free radicals start to enter cells and cells begin to form antioxidant compounds to capture free radicals produced by cell metabolism.[10,11]

Yeast antimicrobial activity can inhibit the growth of harmful bacteria and molds.[12] The antimicrobial potential can be expanded to microbiological control that influenced quality control and food safety. Escherichia coli and Staphylococcus aureus as two of the food safety indicator were used to determine the antibacterial activity in occasion to achieve the food quality control and safety systems objectives.[13,14] Wide scope utilization of yeasts in the food industry is promising. Similarly, yeasts have a positive endurance toward a stress environment while shown their antibacterial properties against pathogenic or spoilage bacteria.[15,16] Hence, it is necessary to do research on the antibacterial and antioxidant activity of indigenous yeast isolated from PGP.

MATERIALS AND METHODS

The raw materials used in this study include the inner skin of a local red pomegranate (6 months old) taken from a local market in Bandung City, while S. aureus ATCC 25923 and E. coli ATCC 25922 were taken from the Department of Biology, Universitas Padjadjaran.

Isolation and identification of indigenous yeast from pomegranate peels
One gram of PGP is diluted using 9 ml physiological NaCl (Merck) solution of 0.85% until dilution to 10⁻¹. 100 μl of each dilution was inserted into the Petri dish and then poured yeast and mold agar/YMA (Merck) and incubated for 48 h at room temperature (25°C–28°C) and then replicated three times. Yeast isolates were sequenced according to internal transcribed spacer (ITS) region with the primers of ITS1 and ITS4, aligned by MEGA X, and analyzed with Basic Local Alignment Search Tools (BLAST) algorithm.

Antibacterial activity test
Yeast colony is aseptically swabbed on YMA and incubated for 48 h at room temperature. The liquid culture of S. aureus and E. coli was swabbed evenly on nutrient agar/NA (merck). Yeast agar plate was plugged aseptically using a sterile forceps or needle and placed onto each NA plate. Incubate the NA plates at 30°C for 72 h and the diameter of the clear zones was measured at 24, 48, and 72 h (modification of[7]); all the treatments were replicated three times.

Viability of Escherichia coli and Staphylococcus aureus
The test bacterial suspension which has been standardized with McFarland 0.5 was diluted using 0.85% physiological NaCl. S. aureus or E. coli was grown in nutrient broth/ NB (Merck) and then added 10 μl of yeast indigenous for 72 h at 30°C. 1 ml of bacterial suspension was plated in eosin methylene blue/EMB (Merck) for E. coli and mannitol salt agar/MSA (Merck) for S. aureus and then incubated at 37°C for 24 h. For positive (+) control treatment, 200 μl of bacterial culture of 1 × 10⁴ CFU/mL + 2 μL amoxicillin 100 mg/mL were used, while 200 μL bacterial culture of 1 × 10⁴ CFU/mL used as negative (-) control treatment. The population of E. coli and S. aureus were count every 24 h; all the treatments were replicated three times.

RESULTS AND DISCUSSIONS

Isolation and identification of indigenous yeast from PGP
Based on the results of yeasts isolation from PGP, 4 types of indigenous yeast isolates were obtained. The BLAST identification results obtained two indigenous yeast species from four isolates [Table 1]. There are two isolates of Hanseniaspora uvarum and two isolates of Pichia kudriavzevii.

Antibacterial activity of indigenous yeasts
Based on Figure 1, it is known that the yeast which has the highest antibacterial activity against E. coli bacteria

Table 1: Basic local alignment search tools results of pomegranate peels yeast isolates

| Isolate name | Yeast species | Percent identify | Accession |
|--------------|---------------|------------------|-----------|
| D1           | H. uvarum CBS 314 | 99.75            | NR_130660.1 |
| D2           | H. uvarum CBS 314 | 99.83            | NR_130660.1 |
| D3           | P. kudriavzevii ATCC 6258 | 100             | NR_131315.1 |
| D4           | P. kudriavzevii ATCC 6258 | 100             | NR_131315.1 |

H. uvarum: Hanseniaspora uvarum, P. kudriavzevii: Pichia kudriavzevii, CBS: Centraal Bureau voor Schimmelcultures, ATCC: American Type Culture Collection
is *P. kudriavzevii* PGP D4. Meanwhile, *Hanseniaspora uvarum* PGP D1 and D2 form a clear zone diameter with a size range below 1 mm. The antibacterial activity of *Hanseniaspora uvarum* PGP D1 and D2 was classified in the weak category (0–3 mm), while the yeast isolates of *P. kudriavzevii* PGP D3 and D4 belong to the strong category (≥6 mm).

The antibacterial metabolites produced by yeast can be originated from primary and secondary metabolites. Primary metabolites produced by yeast that could role as an antibacterial compound can be in the form of organic acids and proteins. Secondary metabolites produced by yeast can be in the form of flavonoid, phenolic, alkaloid, and polyketide compounds. As shown in Figure 1, the diameter of clear zones tends to increase until 72 h. The results showed that the increase of antibacterial activity were shown the logarithmic and stationary phase of yeasts growth occurred which produce both primary and secondary metabolites that gave an antibacterial effect against *E. coli*.

*P. kudriavzevii* PGP D4 showed the highest has antibacterial activity that shown by the diameter of the clear zone. The clear zone formed by *P. kudriavzevii* PGP D4 occurred at 24-72 h; this indicates the presence of antibacterial metabolites produced by isolates. The antibacterial activity is derived from primary metabolites and secondary metabolites produced. *P. kudriavzevii* can produce primary metabolites in the form of organic acids, namely, acetic acid, pyruvic acid, and lactic acid. Lactic acid and acetic acid resulted from yeasts metabolism have been proven can control the growth of *E. coli*. *P. kudriavzevii* also can produce ethanol as a secondary metabolite, so that a clear zone in the *E. coli* is formed.

The antibacterial activity of *Hanseniaspora uvarum* PGP D1 and D2 is relatively weak because the diameter of the clear zone formed is <1 mm. *H. uvarum* has the ability to produce killer toxin which can against several pathogenic including *Staphylococcus aureus* and *Escherichia coli*. pH and temperature are factors which give a significant effect on the development of killer toxin.

Based on Figure 1, the yeast which has the highest antibacterial activity against *S. aureus* is *P. kudriavzevii* PGP D4. *Hanseniaspora uvarum* PGP D2 and *P. kudriavzevii* PGP D3 did not show antibacterial activity against *S. aureus* because there were no clear zone diameters showed. The antibacterial activity of *H. uvarum* PGP D1 was classified in the weak category (0–3 mm), while the yeast isolate *P. kudriavzevii* PGP D4 was in the moderate category (3–6 mm).

The formation of primary and secondary metabolites of *P. kudriavzevii* PGP D4 such as organic acids, alcohols, and phenolic compounds, results in clear zone diameter against *S. aureus*. An increase in the diameter of the clear zone occurred at 72 h; it was marked because of the additional activity of the formation of secondary metabolites in the form of phenolic compounds. Phenol compounds were very sensitive to the single-cell wall *S. aureus* because can change the permeability characteristics of bacterial cell membranes and causes leakage of essential constituents of cells so that bacteria dead. Meanwhile, *H. uvarum* produces a small amount killer toxin which influences by environmental factors. The optimum conditions for *H. uvarum* to produce killer toxin and against *S. aureus* are at pH 4 and temperature 25°C with 4% of NaCl.

Based on antibacterial activity, *P. kudriavzevii* PGP D4 has the highest activity against *Escherichia coli* and *Staphylococcus aureus*. So that *P. kudriavzevii* PGP D4 was taken as a candidate isolate that has antibacterial activity. The positive control used was amoxicillin, which has antibacterial activity and the negative control was without amoxicillin nor yeast isolate.

The ability of *Staphylococcus aureus* cells to survive is higher than that of *Escherichia coli* bacteria. This can be seen from

![Figure 1: The diameter of clear zone against *Escherichia coli* and *Staphylococcus aureus*](image-url)
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Figure 2, where the number of *E. coli* bacteria cells decreased drastically at 12 h, while the number of *S. aureus* bacteria cells decreased slowly. *E. Coli* and *S. aureus* are significantly decreased in the range of time 0-24 h. The effectiveness of *P. kudriavzevii* PGP D4 in reducing *E. coli* was 18.58%. Meanwhile, the decreasing effectiveness in reducing *S. aureus* reached 85.18%. This can be caused by several factors including temperature, nutrients, and antibacterial compounds.

The decrease in the number of *E. coli* and *S. aureus* also indicates the formation of antibacterial compounds by *P. kudriavzevii* PGP D4 which can be derived from the metabolites produced. The existence of antioxidant activity in yeast, such as flavonoid, phenolic, isoprenoid, alkaloid, and polyketide compounds, can induce antibacterial activity.\[23,30,31]\ These compounds can cause a decrease in cell surface tension and denature cell proteins so that the number of pathogenic bacteria reduced.\[29,32,33]\

**CONCLUSIONS**

Identification of indigenous yeast isolated from PGP resulting in two isolates 99.83%–99.75% identical with *Hanseniaspora warorum* CBS 314 and two isolates 100% identical with *P. kudriavzevii* ATCC 6258. All isolates showed antibacterial activity against *E. coli* and *S. aureus*, with *P. kudriavzevii* PGP D4 being the highest against *E. coli* (9 mm) and *S. aureus* (3.1 mm).

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**Conflicts of interest**

There are no conflicts of interest.

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