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Antibacterial Activity Test of Indigenous Yeast from Sapodilla Fruit against *Staphylococcus aureus* and *Escherichia coli*

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The research aimed to identify indigenous yeast antibacterial activity from sapodilla fruit against *Escherichia coli* and *Staphylococcus aureus*, which conducted by experimental methods and followed by descriptive analysis. This study was done by the isolation of indigenous yeast, macroscopic and microscopic identification, yeast identification using RapID Yeast Plus System, antibacterial test by measuring the clear zone diameter, testing of pathogenic bacteria viability against indigenous yeast and identification of organic acid produced by yeast. The results of yeast isolation obtained 1 isolate (*S.cereviseae* 1) from fruit and 3 isolates form sapodilla skin (*S.cereviseae* 2, *Candida famata*, and *Pichia anomala*) which had antibacterial activity against *E. coli* and *S. aureus* except *C. famata* isolates. Isolates with the largest antibacterial activity against *E. coli* and *S. aureus* based on the clear zone diameter were *S. cerevisiae* (2) isolates. The results of organic acid analysis by HPLC found that *S.cerevisiae* (2) isolate produced the highest organic acid namely acetic acid as much as 2.442 mg mL^{-1}.

Key words : antibacterial, organic acid, sapodilla fruit, yeast

Penelitian ini bertujuan untuk mengidentifikasi aktivitas antibakteri khamir *indigenous* buah dan kulit buah sawo terhadap bakteri *Escherichia coli* dan *Staphylococcus aureus* yang dilakukan dengan metode eksperimental dan data yang diperoleh dianalisis secara deskriptif. Tahapan penelitian dilakukan dengan isolasi khamir *indigenous*, pengamatan khamir secara makroskopis dan mikroskopis, identifikasi khamir dengan RapID *Yeast Plus System,* pengujian aktivitas antibakteri khamir dengan pengukuran diameter zona hambat, pengujian viabilitas sel bakteri patogen terhadap khamir, dan pengujian metabolit asam organik yang dihasilkan oleh khamir. Hasil isolasi khamir didapatkan 1 isolat pada bagian buah dan 3 isolat pada bagian kulit buah sawo. Setelah diidentifikasi didapatkan 2 isolat *Saccharomyces cerevisiae,* dimana isolat *S.cerevisiae* (1) merupakan hasil isolasi pada bagian buah sawo, 1 isolat *Candida famata,* dan 1 isolat *Pichia anomala* yang memiliki aktivitas antibakteri terhadap bakteri *E. coli* dan *S. aureus* kecuali isolat *C. famata. S. cerevisiae* (2) merupakan isolat khamir *indigenous* yang menghasilkan aktivitas antibakteri tertinggi terhadap bakteri *E. coli* dan *S. aureus* karena menghasilkan asam-asam organik jenis laktat, asetat, sitrat, dan malat dimana asam asetat adalah jenis asam organik tertinggi dengan jumlah 2,442 mg mL^{-1}.

Kata kunci : antibakteri, asam organik, buah sawo, khamir

Sapodilla (*Achras zapota L.*) is one type of potential fruit plant that grows in Indonesia. Sapodilla fruit consumption in Indonesia is growing rapidly along with the easy of planting sapodilla and sapodilla plants which can produce fruit throughout the year (Ying *et al.* 2017). Sapodilla fruit known as an herbal medicine that can cure various diseases, one of which is diarrhea.

Sapodilla fruit contains flavonoids, saponins and tannins, besides sapodilla fruit also contains organic acids such as citric acid and malic acid (Murnisyazwani and Rabeta 2019). These compounds are known to have antibacterial properties. According to Jenie (1996) organic acids show antimicrobial activity against many pathogenic microorganisms, including pathogenic bacteria that cause diarrhea (Gómez-García *et al.* 2019; Utama *et al.* 2015). Some bacteria that cause diarrhea like *Staphylococcus aureus* and *Eschericia coli*. These bacteria are found in food and can potentially cause infection and food intoxication so that the food consumed can cause diarrhea and even poisoning for consumers (Putri *et al.* 2015).

The results of research by Mukhriani *et al.* (2017), sapodilla fruit extract was able to inhibit the growth of *S. aureus* with an optimum concentration of 1500 ppm or 1.5 mg mL^{-1}, while the results of Arsyad and Annisa (2016) the minimum inhibition concentration of sapodilla fruit extract which could inhibit total *E. coli* growth is 22.5% (v / v).

The antibacterial properties are not only from the compounds contained in the fruit, but can also come from microorganisms such as yeast contained in the fruit. Romano *et al.* (2019) states that yeast can be

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found in a place that is rich in sugar content, for example in fruit. Sapodilla fruit has a sugar content of 14%(Jadhav 2018). The sugar content in sapodilla fruit can act as a substrate for yeast growth. The optimum sugar concentration for yeast growth is 14-18% (Ranalli 2007). It is suspected that sapodilla fruit has yeast which can inhibit the activity of *S. aureus* and *E. coli* bacteria like antibacterial activity of sapodilla fruit extract.

The antibacterial properties of indigenous yeast from sapodilla fruit are derived from the results of the yeast's own metabolism. According to Raftari *et al.* (2009), the effect of inhibition on pathogenic bacteria by yeast is largely due to the accumulation of organic acids, where acid will cause a decrease in pH to below the pH range of bacterial growth where these acids are not dissociated and can diffuse rapidly into in pathogenic cells that cause cells to become damaged. Different types of yeast can produce organic acids with different antibacterial activity. Therefore, in this study we aimed to identify indigenous yeast antibacterial activity from sapodilla fruit against *Escherichia coli* and *Staphylococcus aureus*, which conducted by experimental methods and followed by descriptive analysis.

MATERIALS AND METHODS

Chemical Materials. The raw materials used in this study include the skin and sapodilla fruit which are 3 months old, *S. aureus*, *E. coli*, NA media (Nutrient Agar), NB media (Nutrient Broth), YMA media (Yeast and Mold Agar), Yeast Extract (Kraft Food), antibiotics (Amoxicillin 500 mg), Chloramphenicol 500mg, aquades, Physiological NaCl,70% alcohol, MSA (Mannitol Salt Agar), EMB (Eosin Methylene Blue), 1% BaCl2, 1% H2SO4, citric acid, tartaric acid, maleic acid, oxalic acid, lactic acid, and acetic acid. The research method used is the experimental method using descriptive analysis.

Yeast Isolation. Yeast is isolated from the skin and sapodilla fruit. 1 gram of sample is diluted using physiological NaCl solution of 0.85% until dilution to 10^{-3} 100 μL of each dilution was inserted into the petri dish then poured Yeast and Mould Agar (YMA) media with a composition consisting of 3 g L^{-1} malt extract agar and 3 g L^{-1} yeast extract agar and incubated for 48 hours at room temperature. Characterization of yeast isolates was carried out by observing the physical characteristics macroscopically and microscopically from yeast isolates (Balia *et al.* 2018; Ruriani *et al.* 2012).

Yeast Identification. Identification of yeast types is done using the RapID Yeast Plus System. The results of the color change data are inputted into the website and found yeast isolates(Utama *et al.* 2016).

Antimcrobial Activity Test. Yeast colony swabs in 20 mL of Yeast and Mold Agar (YMA) media aseptically and incubated for 48 hours at room temperature. Swab Liquid culture *Staphylococcus aureus* and *Escherichia coli* on Nutrient Agar (NA) evenly. Plug aseptically yeast agar plate, use a sterile forceps or needle to carefully pick up the plug and place them onto each NA plates. Incubate the NA plates at 30°C for 48 h then diameter of the clear zones were measured at 24 and 48 hours(Roostita *et al.* 2011).

Determination of Antibacterial Indigenous Yeast Activity on Viability of Test Bacteria. Fresh cultures of yeast and bacteria were inoculated as follows: 190 μL at a concentration of 1×10^4 cells mL^{-1} of *S. aureus* and *E. coli* with 10 μL at a concentration of 3×10^6 cells mL^{-1} of each yeast, including the following controls: (i) 200 μL of bacterial culture at a concentration of 1×10^4 cells mL^{-1}, (ii) 198 μL of bacterial culture at a concentration of 1×10^4 cells mL^{-1} with 2 μL of amoxicilin at a concentration of 100 mg mL^{-1}. Incubation at 30 C for 48 hours. 1 mL suspension of E. coli bacteria was platted in 20 mL of EMB (Eosin Methylene Blue Agar) media while the suspension of *S. aureus* bacteria in MSA (Mannitol Salt Agar) media at t = 0, 12, 24, 36, and 48 hours. Incubation was carried out at 37C for 24 hours and TPC calculations were carried out(Acuña-Fontecilla *et al.* 2017).

Identification of Indigenous Yeast Organic Acid Production using HPLC. Yeast samples that have been dissolved in NB media added with yeast extract are filtered and inserted through the injector. The data is a chromatogram that displays retention times in the form of sums of peaks and surface area compared between standard organic acids and organic acids in the sample(Kim *et al.* 2018).

RESULTS

Indigenous Yeasts Identification. Based on the results of the yeast isolation of indigenous sapodilla fruit, 1 yeast isolate from sapodilla fruit and 3 yeast isolates from the sapodilla skin were observed macroscopically and microscopically. All data from macroscopic observations have characteristics such as yeast, which is white, round (circular) with prominent elevation in the middle of the colony (ambonate),

Table 1 Microscopic and macroscopic characteristics of sapodilla indigenous yeasts

Code of isolates	Macroscopic image	Macroscopic morphology	Microscopic morphology	Microscopic image
S.1		Yellowish white, round shape (circular), full edge (entire), ambonate elevation, rough texture (rough)	Round shape, measuring 4-10 µm	
S.2		White, round (circular), ambonate elevation, entire edge (full) , glistening.	Ovoid shape, measuring 3–7 µm	
S.3		white, irregular shape, flat elevation, full edge (entire), smooth texture	Oval shape, measuring 4–9 µm	
S.4		Yellowish white, round (circular), ambonate elevation, full edge (entire), glossy texture (glistening)	Oval shape, measuring 4–10 µm	

smooth or glossy texture (glistening) and full (entire) edge on S.1, S.2, and S.4, while in S.3 has a flat elevation. Based on microscopic observations, the four yeast isolates have a round to oval shape with a range of 3-10 µm. Yeast isolates were then purified to form pure colonies which could be used to identify the types of yeast isolates by biochemical tests using the RapID Yeast Plus Kit. Based on the results of identification, three indigenous yeast species from four isolated isolates were obtained. There are two yeast isolates of *Saccharomyces cerevisiae* which isolated from fruit (S.1) and sapodilla skin (S.4), other isolates were *Candida famata* (S.2), and *Pichia anomala* (S.3) that isolated from sapodilla skin.

Indigenous Yeasts Antibacterial Activities. The diameter of clear zone formed is classified into weak (d=0-3 mm), medium (d=3-6 mm), and strong (d>6 mm) (Pan *et al.* 2009). Based on the Fig. 1, it known the yeast that has strong antibacterial activity against *E. coli* is S.1 (*S. cerevisiae* 1) and S.4 (*S. cerevisiae* 2) isolates. S.3 isolates (*P. anomala*) have weak antibacterial activity, whereas P.2 (*C. famata*) isolates do not form clear zone diameters, so it can be said that these isolates do not have antibacterial activity. The antibacterial activities towards for *S. aureus* has shown *S. cerevisiae* (1) and *S. cerevisiae* (2) had moderate antibacterial activity (d=3-6 mm), *C. famata* has no antibacterial activity because there is no clear zone diameter formation, whereas *P. anomala* isolates have weak antibacterial activity (d = 0-3 mm) against *S. aureus*.

Indigenous Yeasts Viability towards *E.coli* and *S.aureus*. Based on Fig. 2, it is known that almost 100% of *E.coli* has decreased from the 0 until 12 hour. The effectiveness of the decrease in the number of *E.coli* cells by *S. cerevisiae* (2) was 23.7% at 0 hour, while the number of viability of *S. aureus* decreased by 76.2% at 0 to 12 hours.

Organic Acid Production. Based on the Table 3, *S. cerevisiae* can produce acetic, citric, malic, and lactic acid compounds as indicated by an increase in the amount of organic acid. While oxalic and tartaric acid compounds experience a decrease in the amount of organic acid.

DISCUSSION

Indigenous Yeasts Identification. The *S. cerevisiae* colonies are yellowish white, have a circular edge shape, and the surface glistening. *S.*

Table 2 The results of RapID Yeasts Plus System with ERIC analysis

Test	S.1	S.2	S.3	S.4
Glucose	+	+	+	+
Maltose	-	-	+	-
Sucrose	+	+	+	+
Trehalose	-	-	+	-
Raffinose	-	-	+	-
Lipid	-	-	-	-
NAGA	-	-	-	+
αGlucoside	+	+	+	+
bGlucoside	+	+	+	+
ONPG	-	-	-	-
αGalactoside	-	-	-	-
bFucoside	+	-	+	-
PHS	-	-	-	-
PCHO	-	-	-	-
Urea	-	-	-	-
Prolyne	-	+	-	-
Histidine	+	-	-	+
Leucyl-Glycine	+	-	-	+
Yeast Name	***S.cereviseae (1)***	***C.famata***	***P.anomala***	***S.cereviseae (2)***

Table 3 Organic acid production by potential indigenous yeast *S.cereviseae* (2)

	Blank	*S.cereviseae*	Metabolite
Oxalic	2.650	2.489	-0.161
Tartaric	1.862	1.206	-0.656
Lactic	3.968	4.186	0.218
Acetic	1.677	4.119	2.442
Citric	9.542	`0.664	1.122
Malic	0.023	0.044	0.021

cerevisiae cells are round (spherical), sometimes ellipsoidal (oval, elongated) to cylindrical, and produce pseudomycelium. The cell size of *S. cerevisiae* ranges from 5-12×5-10µm (Kurtzman *et al.* 2011). *P. anomala* is white, smooth with fine serrated edges. *P. anomala* has round, elliptical or elongated cells. The cell size of *P. anomala* ranges from 2-7×2-5µm (Passoth *et al.* 2006). *C. famata* forms yellowish white colonies, smooth and shiny texture. *C. famata* is elongated round (ovoid) with cell size 2.0-3.5×3.5-5.0µm (Dmytruk and Sibirny 2012).

Based on observations, it is known that all isolates can hydrolyze substituted glycosides, namely αGLU and β-GLU compounds enzymatically. All isolates are known to hydrolyze glucose and sucrose types of carbohydrates, but only S.3 isolates can hydrolyze maltose and trehalose carbohydrate compounds. HIST and LGY compounds which are amino acid groups can only be hydrolyzed by isolates S.1 and S.4.

S. cerevisiae can hydrolyze simple sugars such as glucose and fructose, and can hydrolyze disaccharides like sucrose because it produces the enzyme sukrase (invertase) which converts sugar to be easily fermented (Sainz-Polo *et al.* 2013). *P. anomala* can utilize all types of sugar such as glucose, maltose, sucrose, galactose,

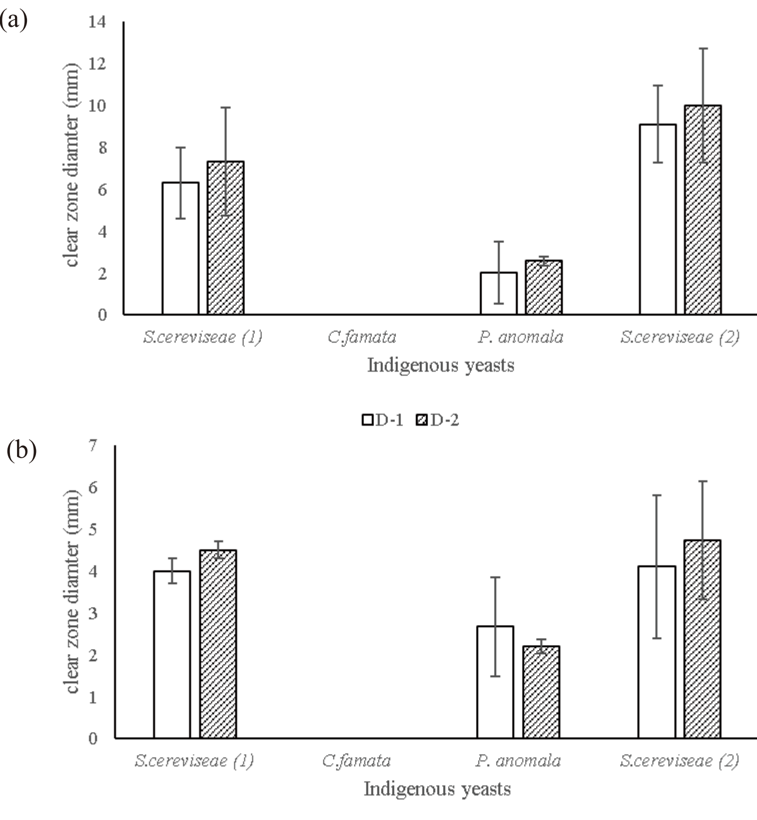

Fig 1 Sapodilla indigenous yeasts antibacterial activities towards (a) *E. coli*; (b) *S. aureus*.

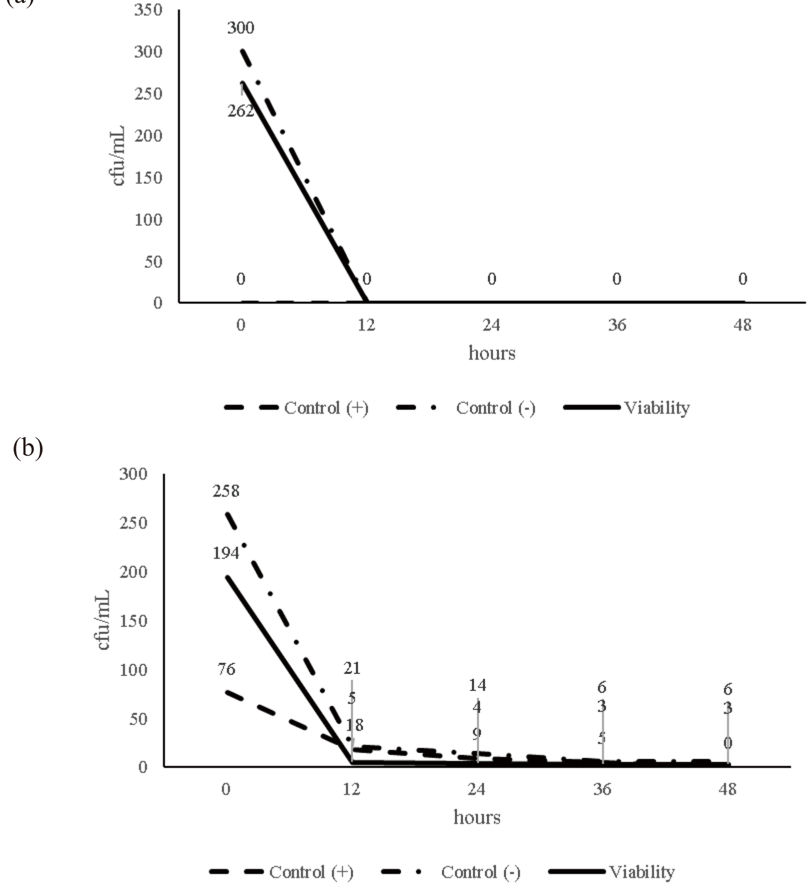

Fig 2 Indigenous yeasts viabity towards (a) *E. coli*; (b) *S. aureus*.

and raffinose, except lactose (Tao *et al.* 2011). *C. famata* can ferment glucose, sucrose, and trehalose (Gientka *et al.* 2016).

Indigenous Yeasts Antibacterial Activities. *S.cerevisiae* isolates can produce antimicrobial metabolites such as organic acids, phenolic compounds, besides *S. cerivisiae* is known to produce several proteins that have antimicrobial properties (Roostita *et al.* 2011). *S. cerevisiae* produces high concentrations of ethanol which are toxic to many microbial species and are capable of producing volatile compounds such as aromatic alcohols involved in inhibiting microorganisms (Jouhten *et al.* 2016). The antibacterial activity of *S. cerevisiae* with an incubation time of two days caused by the primary metabolites produced by yeast, namely organic acid compounds. Organic acids produced by *S. cerevisiae* such as acetic acid, malic acid, succinic acid, and lactic acid have strong antimicrobial activity (Fakruddin *et al.* 2017).

Based on research by Younis *et al.* (2017), *S. cerevisiae* showed moderate antibacterial activity against *E. coli* bacteria and showed weak antibacterial activity against *S. aureus*. *E. coli* population decreased after exposed acetic acid, lactic acid, propionic acid, and formic acid, where the reduction in the *E. coli* population increased with an increase in the concentration of organic acids (Raftari *et al.* 2009).

P. anomala produces ethanol under limited oxygen conditions and produces acetic acid in aerobic conditions. *P. anomala* produces volatile compounds such as ethyl acetate, ethyl propanoate, phenyl ethanol, and 2-phenylethyl acetate (Passoth *et al.* 2006). *P. anomala* can reduce fungal growth in several ways: production of killer poisons, secretion of β-1-3-glucanase, ethyl acetate production or by competition for nutrition (Muccilli and Restuccia 2015).

P. anomala is known to produce acetic acid where acetic acid has an inhibitory effect on bacteria. Raftari *et al.* (2009) stated that *E. coli* and *S. typhimurium* had a high susceptibility to lactic acid and acetic acid. *P. anomala* had the most influential antimicrobial activity on *E. coli* growth (Walker 2011).

The absence of a clear zone indicates no antibacterial activity in *C. famata* isolates. This can be caused because most of the inhibition of bacteria by *C. famata* is with produce the optimal killer toxin at pH 4.5 with a temperature of 20 °C. Above pH 4.5 and 20 °C killer toxin activity decreases (Muccilli and Restuccia 2015). Tests are carried out above 20 °C, so the production of killer toxin is inhibited and antibacterial activity cannot take place.

The antibacterial activity of indigenous yeast to sapodilla fruit against *S. aureus* bacteria is lower when compared to *E. coli*, this can occur because test bacteria have different resistance to different types of organic acids. *S. aureus* test bacteria have high acid resistance when compared to *E. coli*, this is caused by differences in the cell wall structure of the two types of bacteria (Lopez-Romero *et al.* 2015). *E. coli* bacteria grow at pH 7.0-7.5 while *S. aureus* bacteria grows at pH 4.0-9.8 (Padan *et al.* 2005).

Indigenous Yeasts Viability towards *E. coli* and *S. aureus*. The optimum temperature for the growth of *E. coli* and *S. aureus* is 35-37 °C, so that antibacterial testing of yeast carried out to determine the effectiveness antibacterial origin of yeast that could be use as disinfectant for *E. coli* and *S. aureus* in the environment. Temperature greatly influences the growth and physiological activities of microorganisms. Temperature differences can affect the speed of bacterial enzyme synthesis, enzyme inactivation, changes in metabolic processes and cell shape, and bacterial growth rate (Suriani *et al.* 2013).

The antibacterial activity of *S. cerevisiae* with an incubation time of two days was caused by the primary metabolites produced by yeast, namely in the form of organic acid compounds. Organic acids produced by *S. cerevisiae* such as acetic acid, malic acid, succinic acid, and lactic acid have strong antimicrobial activity (Fakruddin *et al.* 2017). Organic acids have a bactericidal effect whose effects increase with increasing concentration (Abbott *et al.* 2009). Differences bacterial viability against organic acids can caused by the ability of strains to adapt in different acidic environments. Gram-positive bacteria such as *S. aureus* have murein compounds that cause cell wall resistance in gram negative bacteria to be lower than gram-positive bacteria (Nazzaro *et al.* 2013). The ability of *S. aureus* to survive in acidic environment is with the phase of adaptation to the acidic environment , namely by pumping protons out of the cell to maintain normal pH and also by increasing the concentration of alkaline compounds in cells to prevent cytoplasmic acidification, repair and degradation mechanisms damaged protein (Bore *et al.* 2007).

Organic Acid Production. Organic acids produced by yeast result in the accumulation of acidic end products and a decrease in pH which will inhibit the growth of both gram-positive and gram-negative bacteria (Kim *et al.* 2018). The decrease in organic acid is caused by the use of organic acids by yeast for the fermentation process (Walker and Stewart 2016).

Changes in the concentration of organic acids illustrate that yeast can freely use organic acids as a source of energy and provide organic acids as intermediate compounds in cell metabolism. During fermentation *S. cerevisiae* can utilize acid because of the hydrolysis process by enzymes derived from yeast cells (Azhar *et al.* 2017). *S. cerevisiae* cannot metabolize tartaric acid, where tartaric acid is the most commonly used acid for pH adjustment in the wine industry (Jolly *et al.* 2014). Oxalic acid hydrolysis is carried out with enzymes produced by microorganisms (Pal *et al.* 2016).

The highest type of organic acid produced by *S. cerevisiae* isolates is acetic acid followed by citric acid. *S. cerevisiae* produced acetic acid where acetic acid production increased with increasing fermentation temperature, where the optimum temperature of acetic acid production was at 30 °C (Shang *et al.* 2016). The fermentation temperature corresponding to the optimum temperature of acetic acid production allows the highest production of acetic acid by *S. cerevisiae*. Acetic acid formed from glucose fermentation under aerobic conditions (Gomes *et al.* 2018). The synthesis of citric acid by *S. cerevisiae* is higher with dissolved oxygen in the media that is getting higher (Walker and Stewart 2016). Yeast can produce small amounts of lactic acid. Lactic acid produced at 28−30 °C. Lactic acid formed by the reduction of pyruvic acid and the transformation of malic acid (Vilela 2019). The best temperature for producing malic acid is 18−25 °C with optimum fermentation time for 2 to 3 days. Based on observations of *S. cerevisiae* yeast produce small amounts of malic acid, this can be caused because most of the malic acid produced has been transformed into lactic acid or used as an energy source.

Several studies have reported the inhibitory effects of various organic acids on pathogenic or destructive microbes, including Blom *et al*. (1997) which states that the use of organic acid in the form of 2.5% lactic acid and 0.25% acetic acid can extend the shelf life of roast pork for up to 5 weeks (Silano *et al.* 2018). Other studies have also been reported by Castilo *et al*. (2001) who used lactic acid solution at a concentration of 4% (v / v) by spraying on beef carcass turned out to be effective in reducing pathogenic microbes such as *E. coli (*Castillo *et al.* 2001*)*. Giving of citric acid 5.5%, 0.75% lactic acid and 0.5% malic acid can inhibit the growth of *S. aureus* and *E. coli* (Al-Rousan *et al.* 2018).

The inhibition of bacterial growth with organic acids depends on the form of non- dissociating acids and the ability to donate hydrogen ions. Non-dissociated organic acids can diffuse through the plasma membrane and acidify the cytoplasm. Cells tend to neutralize this intracellular acidification by extruding protons through the plasma membrane ATPase, but this is done at the expense of ATP causing bacterial cell death (Andrés and Fierro 2010).

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