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Antibiofilm and antimicrobial activities of papaya (*Carica papaya* L.) and stevia (*Stevia rebaudiana* Bertoni) leaf extracts against three biofilm-forming bacteria

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Abstract

Biofilm is a structural form of a microbial group that is protected by the Extracellular Polymeric Substance (EPS) matrix. The biofilm is considered as the main mediator of infection, and plays a major role in the occurrence of drug resistance. This study was aimed at determining the antimicrobial and antibiofilm activities of papaya (*Carica papaya* L.) and stevia (*Stevia rebaudiana* Bertoni) leaf extracts against three biofilm-forming bacteria. The antimicrobial assay showed that papaya leaf extract exhibits higher activity compared to stevia leaf extract in inhibiting the growth of the biofilm-forming bacteria. The optimum condition of papaya leaf extract to inhibit biofilm-forming bacterial growth occurred at 45% and 75% concentrations of the extract (pH 7). A 100% biofilm degradation by papaya leaf extract occurred at pH 6 and pH 9.

Keywords: antibiofilm, bacteria, plant extracts, 16S rRNA

Introduction

A biofilm is a structural form of a microbial group that is protected by an extracellular matrix called Extracellular Polymeric Substance (EPS) (Prakash *et al.* 2003). Biofilms are currently considered as the primary mediator of infection by various species of pathogenic microorganisms, with about 80% of infections related to the biofilm formation (Archer *et al.* 2011). The biofilm handling can be done through physical, chemical, or biological treatments. In the present time, the handling of biofilms tends to be mostly carried out biologically, such as using various plant extracts because they have a low level of toxicity and are more environmentally friendly. Several natural materials from plants with potential as antibiofilm include the terpenoid, steroids, saponins, carotenoids, phenolics, furanones, alkaloids, peptides, tannins, and lactones (Viju *et al.* 2013).

Papaya (*Carica papaya* L., Caricaceae) is a common medicinal plant in Indonesia to treat various diseases. Papaya contains protease enzyme (papain) and several phenolic compounds, such as protocatechuic acid, p-coumaric acid, 5,7-dimethoxycoumarin, caffeic acid, kaempferol, quercetin, chlorogenic acid (Vuong *et al.* 2013, Mohamed *et al.* 2018). these compounds have anti-inflammatory, antimicrobial, and antibiofilm activity (Romasi *et al.* 2011, Mohamed *et al.* 2018). Besides papaya, leaf extract from stevia plant (*Stevia rebaudiana* Bertoni, Asteraceae) has also been reported to have anti-microbial and antibiofilm activity (Barba *et al.* 2014, Sichani *et al.* 2012, Ortiz-Viedma *et al.* 2017). The

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antimicrobial activity of *S. rebaudiana* were reported against *Escherichia coli*, *Lactobacillus acidophilus*, *Streptococcus mutans*, *Corynebacterium diphtheriae*, and *Candida albicans*, among others (Gamboa & Chaves 2012, Mali *et al.* 2015, Kishta-Derani *et al.* 2016). In this study, we examined the antimicrobial and antibiofilm activities of papaya (*Carica papaya* L.) and stevia (*Stevia rebaudiana* Bertoni) leaf extracts against three biofilm-forming bacteria.

Materials and methods

Microorganisms

Samples of biofilm-forming bacteria were obtained from a washbasin in the Health Microbiology Laboratory, Research Center for Biology (LIPI). Three isolates of the biofilm-forming bacteria (strains SA 1-2, SA 1-3, and SA 1-5) were isolated using Mueller Hinton Congo Red Agar (MHCRA) medium (16 g agar powder, 3 g beef extract, 17.5 g casein hydrolysate, 1.5 g starch, 1000 mL distilled water, amended with 0.8 $g.L^{-1}$ congo red) [Modification from Bridson (1998)]. Biofilm formation assay was carried out using Trypticase Soy Broth (TSB) medium with 5% sucrose (Mathur *et al.* 2006). The TSB medium composed of 17 g peptone casein, 2.5 g K_2HPO_4, 2.5 g glucose, 5 g NaCl, 3 g soya peptone in 1000 mL distilled water (Bridson 1998).

Molecular identification of biofilm-forming bacteria

DNA extraction, PCR amplification and sequencing

Bacterial DNA extraction was carried out directly from the 48-h colony on the Nutrient Agar (NA) medium. A total of 1-2 bacterial colonies grown on the NA medium was taken using a sterile ose, and then put into 50-100 µL nuclease free water and then homogenized. The bacterial suspension is considered as a DNA template. The DNA extraction was carried out using KAPA 3G Plant PCR Kit® (KAPA Biosystems, USA) with the following mix solution composition per sample: 18.9 µL nuclease free water, 25 µL KAPA 3G Plant PCR Buffer (2x), 1.5 µL Primer 27F (5'-AGAGTTGATCMTGGCTCAG-3'), 1.5 µL Primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), 0.6 µL KAPA 3G Plant dNTPs, 0.5 µL KAPA enhancers, 0.5 µL $MgCl_2$, and 1.5 µL DNA template. The PCR reaction was conducted using the following condition: initial denaturation at 95°C for 3 min, followed by 35 cycles consisting of denaturation at 95°C for 20 s, annealing at 50°C for 15 s, extension at 72°C for 30 s. A final extension at 72°C for 30 s was conducted after 35 cycles.

Phylogenetic analysis

A nucleotide sequence from the 27F and 1492R primer pair were edited and trimmed using the ChromasPro version 1.7.5 software (Technelysium Pty ltd, Australia). The ambiguous nucleotide base was checked to ensure that there is no readable base error. Homologous sequences were searched from the GenBank database using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences were further aligned in the FASTA format using MUSCLE (MUltiple Sequence Comparison by Log-Expectation) (Edgar 2004) in MEGA (Molecular Evolutionary Genetics Analysis) version 6 (Tamura *et al.* 2013).

Bacterial identification was carried out using the Phylogenetic Species Concept (PSC), where species are defined as sequences that form independent clades. The phylogenetic tree was constructed using Neighbor-Joining (NJ) method in MEGA 6. The Maximum Composite Likelihood was used to estimate evolutionary distances between all pair of sequences simultaneously. Partial deletion was used to treat indels or missing data. The branch strength of the phylogenetic tree was tested by 1000 replications of bootstrap method.

Papaya (*Carica papaya* L.) and Stevia (*Stevia rebaudiana* Bertoni) leaves extraction

A total of 50 g of papaya leaf powder (< 80 mess) was extracted using 250 mL ethanol absolute, and then incubated in the rotary shaker at room temperature for 2 days at 120 rpm. The solution was further filtered and then concentrated in a rotary vacuum evaporator at a temperature of 40°C. The same procedure was used to extract stevia leaf powder.

Antimicrobial activity assay

Antimicrobial test was carried out using the method described by Ulyah *et al.* (2015) with a modification. Leaf extracts were prepared in several concentrations, namely 45%, 75% and 90%. The most optimum concentration in inhibiting biofilm-forming bacteria was carried out by agar diffusion method. The MHA (Mueller Hinton Agar) medium with different pH conditions (6, 7, and 9) were prepared. Three biofilm-forming bacterial isolates were prepared in 5 mL of Luria Bertani (LB) medium and incubated at 37°C for 24 h. After incubation, each bacterial suspension was swabbed on the surface of MHA medium and then incubated for 10 min. Three agar wells (3 mm in diam.) were prepared in the MHA medium. A total of 50 µL of each leaf extract concentration (45%, 75%, 90%) was inserted into the holes in the MHA medium. Chloramphenicol and sterile distilled water were used as positive control and negative control, respectively. Incubation was carried out at different temperatures (30°C and 37°C) and different times (24 h, 48 h, 72 h). The inhibition zone was measured as follows:

Inhibition zone (mm) = clear zone diameter (mm) - diameter of the hole (mm)

Inhibition effectiveness parameter was set as follows:

- Diameter of inhibition zone > 20 mm: very strong inhibition activity
- Diameter of inhibition zone > 10-20 mm: strong inhibition activity
- Diameter of inhibition zone > 5-10 mm: moderate inhibition activity
- Diameter of inhibition zone > 0-5 mm: weak inhibition activity

Antibiofilm activity assay

Biofilm inhibition assay was conducted using the microtiter assay method (Ulyah 2015) with a modification. First, the biofilm was formed on the microplate by growing biofilm forming bacteria for 24 h, then a total 50 µL of papaya leaf extract or stevia leaf extract was added into the microplate. This assay was conducted at pH (6, 7, 9)), temperatures (30, 37°C) and incubation times (24, 48, 72 h).

Microplate was rinsed with distilled water three times, then a 125 mL of 0.1% crystal violet was added, and the microplate was incubated at room temperature for 15 minutes. The microplate was rinsed again using distilled water three times, and then dried. A total 200 mL of ethanol was added into the microplate wells and was incubated at room temperature for 15 minutes. After the incubation, the absorbance was measured in the microplate reader at λ = 595 nm. The optical density (OD) was calculated as follows:

% biofilm inhibition = 1 – (a corrected sample / a corrected blank) x 100%

Notes:
A corrected sample = fungal extract + plant leaf extract
A corrected blank = DMSO 20% + plant leaf extract

Results

Molecular identification of biofilm-forming bacteria

The BLAST results of the three bacterial isolates showed that the sequence from strain SA 1-3 had a close relationship with type sequences of the genus *Bacillus* (data not shown). The NJ tree showed that SA 1-3 sequence nested in the same clade with *B. thuringiensis* strain ATCC 10792, *B. marcorestinctum* strain LQQ, *B. toyonensis* strain BCT-7112, *B. wiedmannii* strain FSL W8-0169, *B. mycoides* strain NBRC 101228, *B. weihenstephanensis* strain DSM 11821, and *B. cereus* strain CCM 2010 with 92% bootstrap

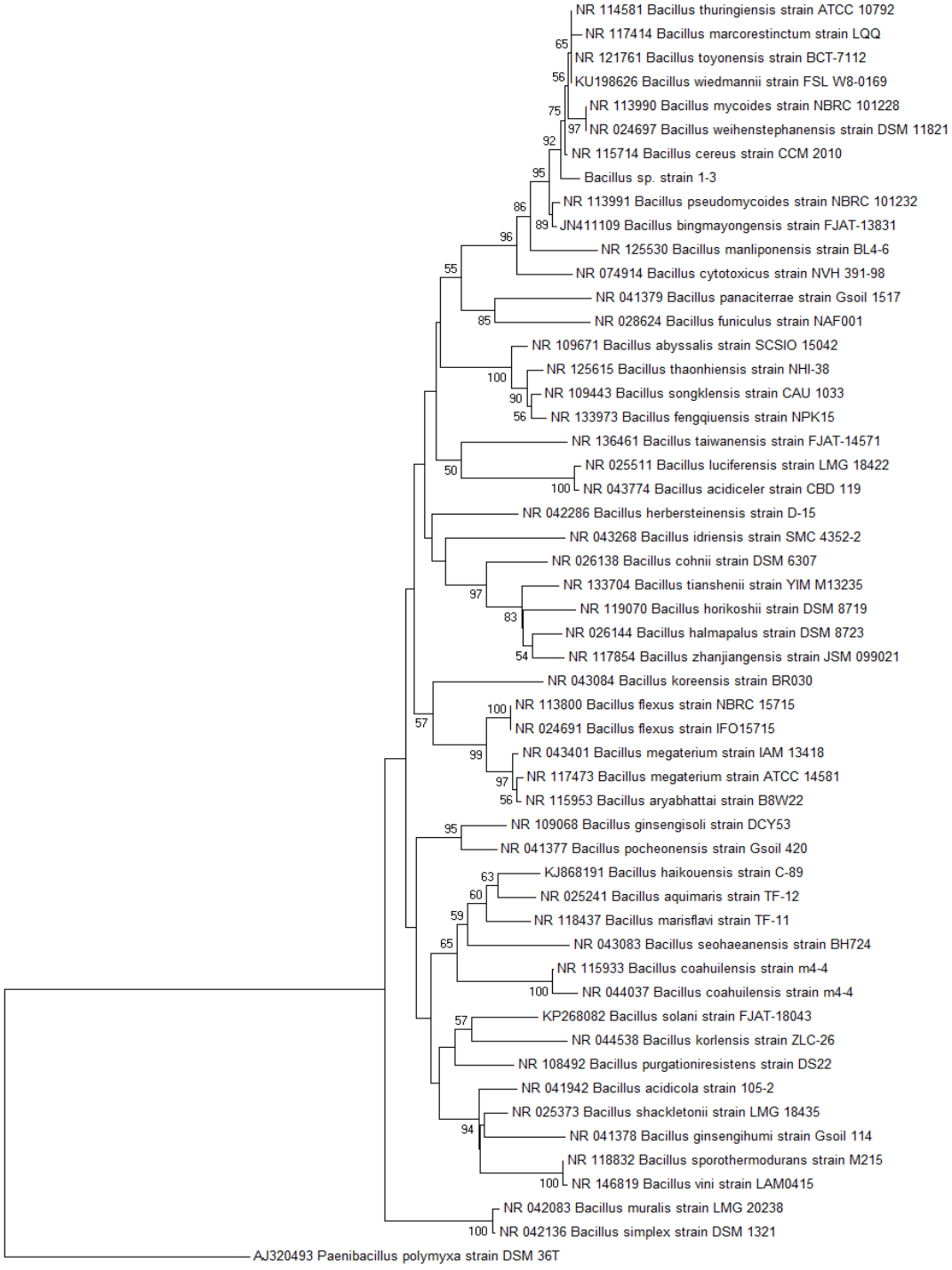

Figure 1. A phylogenetic tree generated from the NJ analysis showed a relationship of *Bacillus* sp. strain 1-3 with closely related sequences (1000× BS)

support (BS) (Fig. 1). However, the SA 1-3 sequence was named as *Bacillus* sp. strain 1-3, due to formed an independent lineage within this clade.

The BLAST results showed that the nucleotide sequence of strain SA 1-5 showed a close relationship with type sequences of the genus *Enterobacter* (data not shown). The phylogenetic tree generated from the NJ analysis showed that the SA 1-5 sequence nested in the same clade with *E. hormaechei* subsp. *steigerwaltii* strain EN-5624, and *E. cloacae* strain ATCC 13047 with 67% BS (Fig. 2). Sequence of SA 1-5 was named as *Enterobacter* sp. strain 1-5 strains due to paraphyletic in this clade.

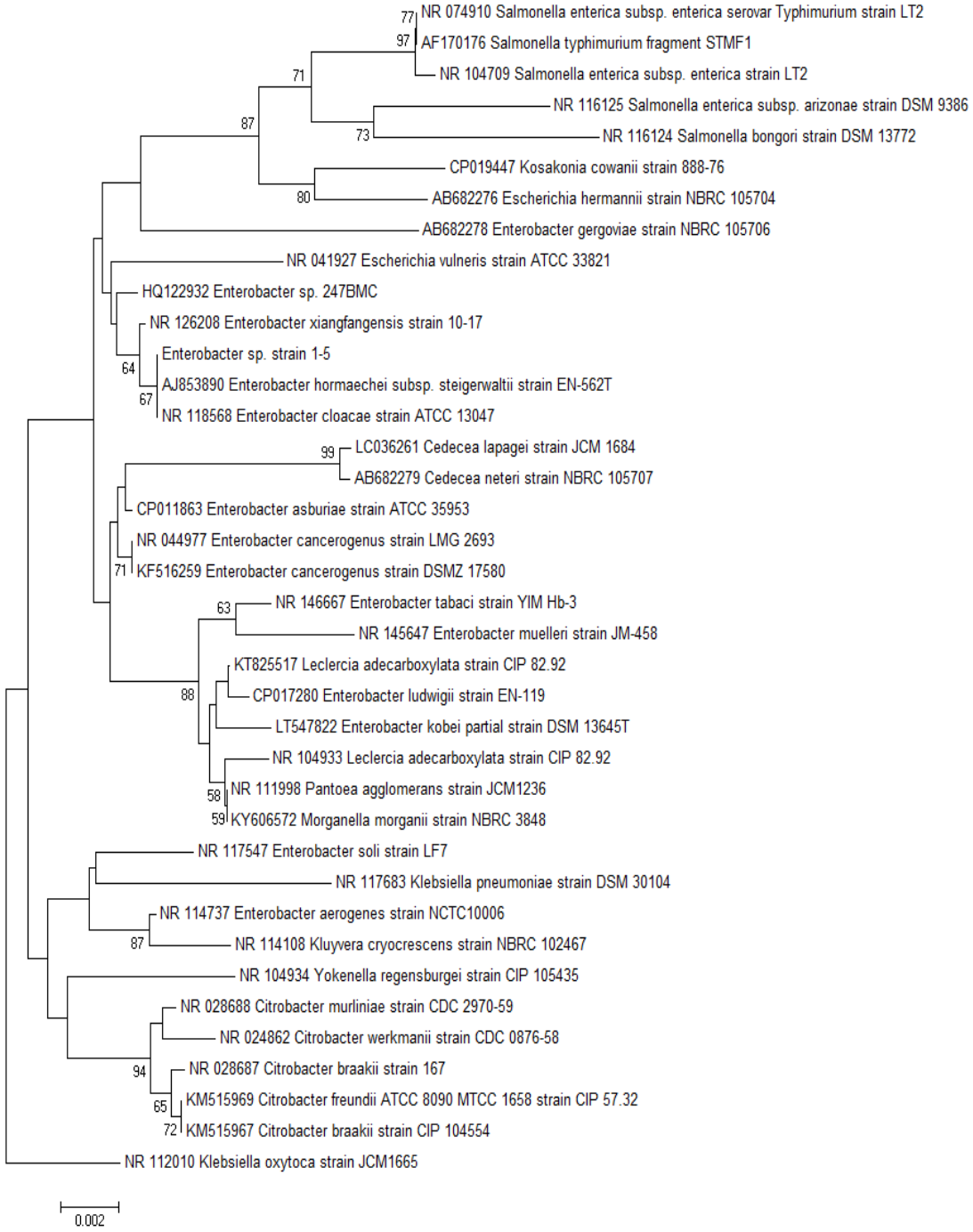

Figure 2. A phylogenetic tree generated from the NJ analysis showed a relationship of *Enterobacter* sp. strain 1-5 with closely related sequences (1000× BS)

Antimicrobial activity assay

The results of the antimicrobial assay showed that stevia leaf extract at 75% and 90% inhibited biofilm-forming bacterium *Enterobacter* strain 1-5 at 37°C with 15 mm and 10 mm inhibition zones, respectively (Table 1). In addition, highest inhibition zones from papaya leaf extract against *Bacillus* sp. strain SA 1-3 and *Enterobacter* sp. strain SA 1-5 were found at 45% concentration (30°C) and 75% (37°C), respectively (Table 1).

Table 1. The antimicrobial assay of stevia leaf extract and papaya leaf extract at different concentrations

Bacterial strain	Concentration (%)	Clear zone diameter (mm)			
		Temperature 30°C		Temperature 37°C	
		Stevia extract	Papaya extract	Stevia extract	Papaya extract
Bacillus sp. strain SA 1-3	45	-	10	-	-
	75	-	5	-	-
	90	-	-	-	-
Enterobacter sp. strain SA 1-5	45	-	25	-	-
	75	-	15	15	30
	90	-	10	10	-

The antimicrobial assay at different pH conditions (pH 6, 7, and 9) showed that stevia extract exhibited antimicrobial activities against *Enterobacter* sp. strain SA 1-5 at 75% concentration (37°C, pH 7) (Table 2). In addition, the highest antimicrobial activities of the papaya extract against *Bacillus* sp. strain SA 1-3 and *Enterobacter* sp. strain SA 1-5 were found at 45% (30°C, pH 7) and 45% (30°C, pH 9) (Table 2).

Table 2. The antimicrobial assay of stevia leaf extract and papaya leaf extract at different pH conditions

Bacterial strain	Extract (%)	Temperature (°C)	Clear zone diameter (mm)		
			pH 6	pH 7	pH 9
Bacillus sp. strain SA 1-3	Papaya 45	30	8	25	5
	Papaya 75	37	8	20	-
Enterobacter sp. strain SA 1-5	Papaya 45	30	-	8	15
	Stevia 75	37	-	10	-

Antibiofilm activity assay

The microtiter assay showed that the best antibiofilm activities from stevia leaf extract against *Bacillus* sp. strain SA 1-3 and *Enterobacter* sp. strain SA 1-5 were found at 75% (37°C, pH 6) and 75%/90% (37°C, pH 9), respectively (Table 3, Fig. 3).

In addition, the papaya leaf extract at all concentrations showed a high antibiofilm activities (Table 4). The microtiter assay showed that the best antibiofilm activities from the papaya leaf extract against *Bacillus* sp. strain SA 1-3 and *Enterobacter* sp. strain SA 1-5 were found at 45% (37°C, pH 6) and 90% (37°C, pH 9), respectively (Table 3).

Table 3 An antibiofilm activities of the stevia leaf extracts at different temperatures and pH conditions

Incubation temperature (°C)	Concentration of extract (%)	Antibiofilm activity (%)					
		Bacillus sp. strain SA 1-3			*Enterobacter* sp. strain SA 1-5		
		pH 6	pH 7	pH 9	pH 6	pH 7	pH 9
30	45	48.5	61.7	32.7	54.2	67.4	38.5
	75	55	41.7	22.6	56.3	53	28
	90	17.6	66.7	30.3	26.8	68.3	30
37	45	82.5	72.2	1.5	60.8	76.7	97.6
	75	95	65.9	0	51.4	43	98
	90	29.1	79.1	42.2	65.4	67.8	98

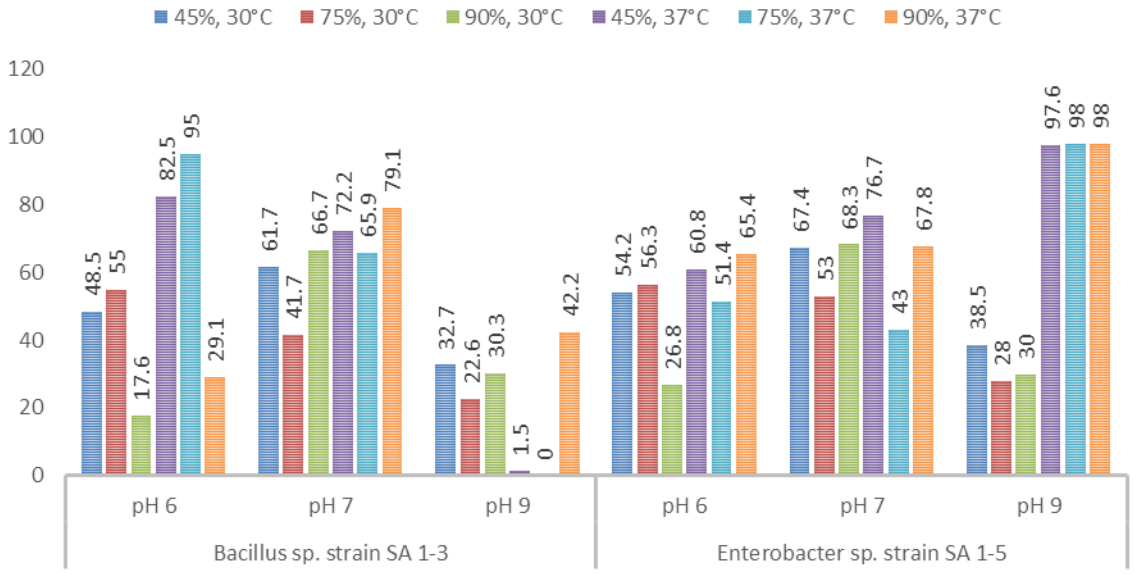

Figure 3. Comparison of the antibiofilm activities of the stevia leaf extracts at different temperatures and pH conditions

Table 4. An antibiofilm activities of the papaya leaf extracts at different temperatures and pH conditions

Incubation temperature (°C)	Concentration of extract (%)	Antibiofilm activity (%)					
		Bacillus sp. strain SA 1-3			*Enterobacter* sp. strain SA 1-5		
		pH 6	pH 7	pH 9	pH 6	pH 7	pH 9
30	45	97.9	97.5	98.3	98.7	98.3	97.7
	75	97.3	98.5	99	97.9	99	98.4
	90	93.5	98.9	98.5	96.2	99.2	98.4

Incubation temperature (°C)	Concentration of extract (%)	Antibiofilm activity (%)					
		Bacillus sp. strain SA 1-3			*Enterobacter* sp. strain SA 1-5		
		pH 6	pH 7	pH 9	pH 6	pH 7	pH 9
37	45	100	99.3	98.6	98.5	99.3	99.1
	75	99.4	99.5	99.1	98.9	98.6	98.6
	90	99.1	97.4	98.4	97.7	98.9	99.4

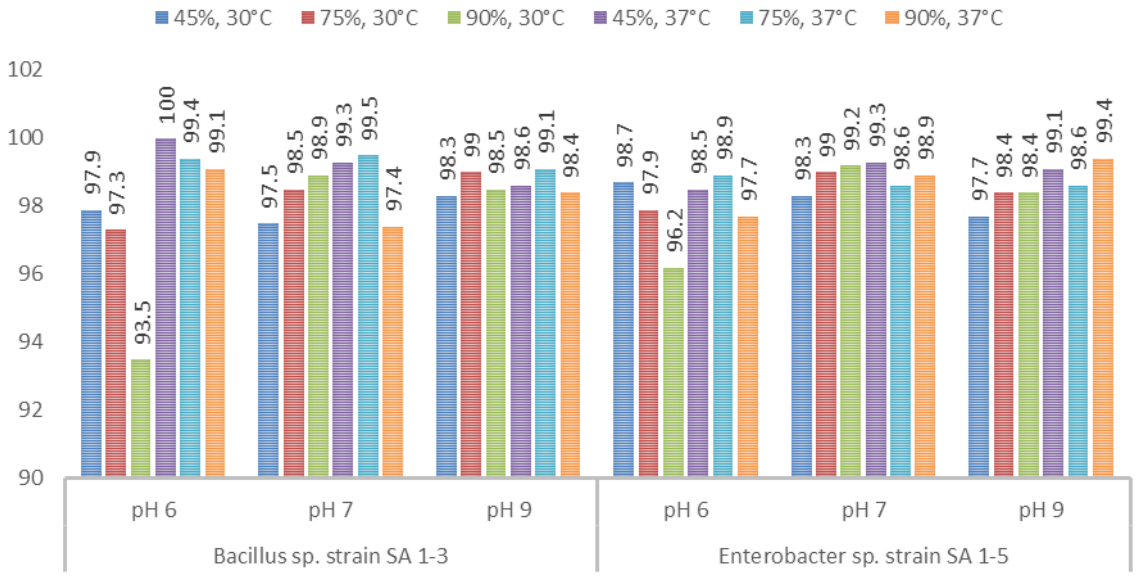

Figure 4. Comparison of the antibiofilm activities of the papaya leaf extracts at different temperatures and pH conditions

Discussion

Biofilm is the main mediator of an infection, with an estimated 80% of microbial infections related to the biofilm formation (Tarver 2009, Archer *et al.* 2011). This is because the biofilm can occur on surfaces of the organs of organisms (human, animal, and plant) and affect the function of the colonized organs. In general, biofilm can be treated using physical, chemical, and biological methods. The biological methods include application of microbial and plant metabolites (Hamieh *et al.* 2015, Sánchez *et al.* 2016, Bazargani & Rohloff 2016). Among the plant extracts, papaya and stevia leaves extracts were reported to have antimicrobial activities (Romasi *et al.* 2011, Gamboa & Chaves 2012, Miranda-Arámbula *et al.* 2017). Papaya leaf contains active substances such as carbainine alkaloids, carpaine, vitamin C and E, choline, proteolytic enzyme (papain), flavonoids, and tannins (Milind & Gurdita 2011). Meanwhile, stevia leaf contains active substances such as alkaloids, tannins, flavonoids, and phenol (Lamonthe *et al.* 2009).

The results of this study showed that both stevia and papaya leaves extracts exhibited antimicrobial and antibiofilm activities, but the papaya leaf extract showed higher activities than that of stevia leaf extract (Tables 1-4). This might be due to the activity of the papain enzyme from the papaya leaf extract. Papain is the proteolytic enzyme in the papaya leaves that has an antibiofilm and antimicrobial activities (Cynthya *et al.* 2014, Blanchette & Wenke 2018, Mohamed *et al.* 2018). Another important antimicrobial compound from papaya is the carpaine alkaloid (Julianti *et al.* 2014). Alkaloid compounds can inhibit bacterial cell wall

synthesis through lysis activities of the bacterial cell wall. In addition, alkaloid compounds can interfere with the formation of peptidoglycan constituents in the bacterial cells, causing failure in the bacterial cell wall layer formation.

In the mechanism of antibiofilm formation by natural compounds such as papain, the biofilm degradation usually occurs in the Extracellular Polymeric Substance (EPS) layer. The EPS composition varies between microbial species and growth conditions (Borlee *et al.* 2008). This study showed that the bacterial biofilm responses to the stevia and papaya leaves extracts varies at different temperatures and pH conditions (Tables 3-4). Biofilm degradation ability of metabolites is related to their ability to penetrate into the EPS layer or the mucous layer that envelops the bacteria. Many antimicrobial compounds fail to penetrate biofilms because EPS, which acts as a barrier, protects bacterial cells inside. The formation of EPS is signaled by the secretion of Quorum Sensing (QS) (Tan *et al.* 2014). The QS is a mechanism used by the bacterial cells to determine the condition of their extracellular environment and takes a major role in the biofilm formation (Borlee *et al.* 2008). Therefore, disrupting the secretion of QS and preventing the formation of EPS is the main concern to prevent the biofilm formation (Ardani *et al.* 2010).

Conflict of Interest

The authors state no conflict of interest from this manuscript.

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