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Investigation Antibacterial Bioprocess Kinetics of Wood Acid against *Pseudomonas Aeruginosa*, *Enterococcus Faecium*, *Clostridium Perfringens* and Faecal Bacteria

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Abstract: The accumulation of various infectious diseases and contaminations caused by bacteria has become a critical issue in all over the world. Besides, artificial chemical-based disinfectant also brings adverse effects on the environment, flora, and fauna. This research focuses on the study of wood acid as an alternative environmentally preferable disinfectant agent. A quantitative suspension test (EN 1040) was chosen to evaluate the antibacterial activity. On the obtaining results, the bioprocess kinetics of a variety of bacterial strains to pyroligneous acid-based disinfectants were further explored. This study resulted that the bioactivity reduction percentage and mean log reduction: *Pseudomonas aeruginosa* ATCC 9027 (100%, 6.0792), *Enterococcus faecium* ATCC 35667 (100%, 6.0000), Faecal bacteria (100%, 5.8921), and *Clostridium Perfringens* NCTC 8237 (100%, 5.9494) respectively. For bioprocess kinetics analysis, the specific reduction rate and halve rate: *Pseudomonas aeruginosa* (2.7996, 4.0389), *Enterococcus faecium* (2.7631, 3.9863), Faecal bacteria (2.7134, 3.9146), and *Clostridium Perfringens* (2.7398, 3.9527) respectively. Wood acid is a natural and environmentally friendly material and research results show that wood acid-based disinfectants can significantly reduce the number of bacteria (100% reduction for 5 minutes).

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1. Introduction

A variety of diseases, infections, and contaminations such as food, animal feed, and water contaminations by harmful microorganisms such as *Pseudomonas aeruginosa*, *Enterococcus faecium,* Faecal bacteria, and *Clostridium Perfringens* have become a dangerous issue to human health and animal life. For example, around 9.4 million illnesses cases related to food-born disease per annual that happen in the USA [6]. Also, the economic implications of wide-ranging recalls, a loss of consumer confidence, and widespread public panic can be crippling for any firm [22]. Besides, in 2015, 671,689 infections with antibiotic-resistant harmful bacteria (63.5% of nosocomial) in the European Union. These infections caused 33,110 deaths and 874,541 disability-adjusted life years [4].

Clostridium perfringens is a gram-positive and anaerobic bacterium that is widely distributed in nature that easily found in dust, soil, animal production environments, for example, litter and contaminated feed, and humans and animals' gastrointestinal tract [19, 31]. It is normally found in spore form, which is usual thermal treatments, resistant [32]. Due to these characters, *Clostridium perfringens* is one of the most common strains of human foodborne illness [3]. Besides, *Clostridium perfringens* may cause serious life-threatening on gas gangrene of the uterus and it may rarely encounter in gynecologic oncology also [12]. This harmful microorganism also causes necrotic enteritis. This disease even causes 33% of economic losses on chicken production in a farm due to nutrient absorption decreasing lead to lower feed conversion and weight gain [17, 24].

Enterococcus faecium is members of the gastrointestinal biota of animals and humans, and is considered opportunistic harmful microorganism that is mainly responsible for nosocomial infections in humans and a lot of types of infections in fauna, for example, septicemic diseases in poultry, mastitis in cattle, and diarrhea in cattle and swine [20, 37]. *Enterococcus faecium* can produce potential virulence factors that may increase its pathogenicity, evenly causing serious diseases [13, 38]. Also, its cause of multidrug-resistant infections in the USA [11] and when more antibiotic-resistant than *Enterococcus faecalis* will cause a variety of infections.

Pseudomonas aeruginosa will cause a lot of health problem and infection such as 7.5-9.2% of healthcare-associated infections happen in Taiwan [33], around 51,000 healthcare infections happen in the United States of America [16, 26], microbial keratitis, which can cause corneal perforation, endophthalmitis and eventually blindness [1] and induce infections in burn patients [25].

In addition, *Pseudomonas aeruginosa* is also a of life-threatening infection. It is quite frequent cause of nosocomial gram-negative pneumonia with mortality rate reaches 60% [8, 23]. In cystic fibrosis, this harmful bacterium chronically colonizes the lung and lead to respiratory failure and death [35]. 75-90% of patients with cystic fibrosis will face chronic *Pseudomonas aeruginosa* infection problem. It is the major reason in pulmonary function weakening and early mortality. A relationship has been found between missing or mutant cystic fibrosis transmembrane conductance regulator in lung epithelial cell membranes and a failure in innate immunity leading to initiate of *Pseudomonas aeruginosa* infection [9].

Disinfectants are commonly used in most countries and are important agents to maintain health and quality standards in the food processing industry, the health care settings, the agricultural industry, the cosmetics industry, domestic households, and pharmaceutical products [15]. And, the only effective way to prevent unwanted bacterial growth is through the appropriate application of disinfectant and good biosecurity [2]. In addition, seeking less hazardous and environmentally preferable disinfectants is in focus, for example, castile soap, distilled white vinegar, and plant essential oils are common ingredients. It is because these ingredients create a comfort and safety sense to users due to, they are natural products, users may be supposed to be safer than artificial chemicals while still thought to be effective disinfectant [10]. Pyroligneous acid, potentially consider as an environmentally friendly, and less harmful compare than chemical anti-fungal agents [34].

Pyroligneous acid, also called "wood acid, is the highly oxygenated, complex and a brown, yellowish liquid which obtained from the pyrolysis process (slow pyrolysis or fast pyrolysis) or thermochemical breakdown of biomass such as hemicellulose, cellulose, and lignin [18, 36]. It is acidic and contains volatile organic compounds, for example, phenols, organic acids, alcohols, aldehydes, ketones, polyphenolic compounds, and pyran furan derivates [28]. Pyroligneous acids proved potentially apply in a variety of industries such as antimicrobial, antioxidant, and anti-inflammatory agents also act as a source of commercial chemicals and imparts a smoky flavour and food preservatives [18]. Besides, antifungal properties, pyroligneous acid also reported as a variety of inhibitory properties such as pesticide, termiticide, synergistic insecticide in plant protection [21, 30] and against some plant pathogens [5, 14]. Pyroligneous acids has high potential as an antimicrobial agent due to each element have a different action mechanism making it difficult for harmful microorganisms to evolve or develop wall-to-wall resistant ability [28].

However, recent researches regard antimicrobial efficacy of the environmentally preferable disinfectant agent against *Pseudomonas aeruginosa*, *Enterococcus faecium*, Faecal bacteria, and *Clostridium Perfringens* has not been investigated further. Therefore, in this study, the three strains that major caused diseases, contaminations, or infections (*Pseudomonas aeruginosa* ATCC 9027, *Enterococcus faecium* ATCC 35667, Faecal bacteria, and *Clostridium Perfringens* NCTC 8237) and dilute pyroligneous acid as potential greenbased disinfectant were selected. The bactericidal effects of pyroligneous acid-based disinfectant on the selected harmful microbial strains' percentage of reduction and its bioprocess kinetics were explored.

2. Materials and Methods

2.1 Microbial selection and inoculum preparation

Pseudomonas aeruginosa (ATCC 9027), *Enterococcus faecium* (ATCC 35667), Faecal bacteria, and *Clostridium Perfringens* (NCTC 8237) were selected. The bacteria had been incubated on suitable agar until it was ripe and fruiting at 36-37ºC.

2.2 EN 1040:2005 antimicrobial analysis

A quantitative suspension test (EN 1040:2005) was selected in this testing. Dilute pyroligneous acid as a target functional sample is added to a test suspension of microbial strain. The mixture is kept at (20 ± 1) °C for 5 minutes. At the end of this contact time, an aliquot is taken; the antimicrobial activity in this portion is instantly neutralized. The numbers of surviving microorganisms are determined, and the reduction is calculated.

2.3 antibacterial activity analysis

2.3.1 Mean number of cells, mean log, the percentage of reduction, log reduction, and log growth

The antibacterial activity was determined by the plate count method with the colony counter (Funke Gerber, Colony Star 8502-3952). Each experiment was performed in duplicates to ensure reproducibility of results. Then, bacterial strains' bioactivity and its bioprocess kinetics were calculated with the equation 1 - 7:

$$Mean\ nuumber\ of\ cell\ (m), CFU/g = \frac{Sample\ 1 + Sample\ 2}{2} \quad (1)$$

$$Mean\ log = log_{10}\, m \quad (2)$$

$$Percentage\ of\ reduction, \% = \frac{Initial\ value - Sample\ value}{Initial\ value}\ X\ 100\% \quad (3)$$

$$log\ growth = \log_{10} Sample\ value - \log_{10} Initial\ value \quad (4)$$

$$log\ reduction = \log_{10} Initial\ value - \log_{10} Sample\ value \quad (5)$$

$$Specific\ growth\ rate\ (y) = \frac{\ln(N_2 - N_1)}{t_2 - t_1} \quad (6)$$

$$Specific\ reduction\ rate\ (z) = \frac{\ln(N_1 - N_2)}{t_2 - t_1} \quad (7)$$

Where N_2 and N_1 represent bacterial number concentrations at time t_2 and t_1, respectively, whereas k_1 represents the time taken to duplicate the bacterial division rate for control and k_2 represents the time take to halve the bacterial halve rate for a sample, evaluated according to the equation. 8 and 9.

$$k_1 = \frac{y}{ln2} \quad (8)$$

$$k_2 = \frac{z}{ln2} \quad (9)$$

3. Results and Discussions

3.1 Effect of environmentally preferable disinfectant agent against different bacterial strains

Antibacterial activity of pyroligneous acid-based disinfectant with a variety of bacteria strains within 5 minutes contact time showed in figure 1. The control showed a negative percentage of reduction (*Pseudomonas aeruginosa* ATCC 9027: -3%, *Enterococcus faecium* ATCC 35667: -2%, Faecal bacteria: -2% and *Clostridium Perfringens* NCTC 8237: -3%) represented that the bacteria strain test in this study is valid and able to grow under without disinfectant condition (control). The pyroligneous acid-based disinfectant achieved 100% of bacteria reduction to *Pseudomonas aeruginosa* (ATCC 9027), *Enterococcus faecium* (ATCC 35667), Faecal bacteria, and *Clostridium Perfringens* (NCTC 8237). In similar result reported that pyroligneous acid as a highly efficient antimicrobial agent with the reason of the synergistic activity of the various compounds and several sites of the mechanism of each of chemical mixtures, it is assumed that it would be additional hard for the harmful microorganisms to develop resistant ability [28]. In Souuza et al. (2017) also found that the antimicrobial activity of pyroligneous acid from *Eucalyptus urograndis* and *Mimosa tenuiflora* against *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, and *Cryptococcus neoformans* due presence of high contents of guaiacol and related derivatives along with other furfural and phenols [27].

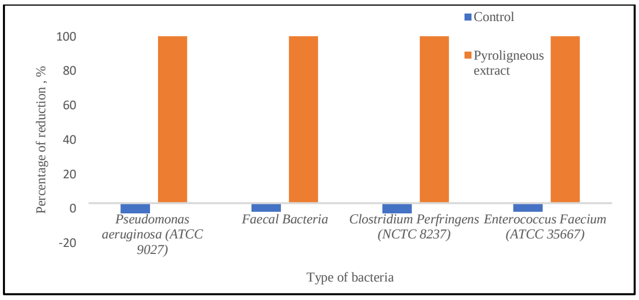

Fig. 1 - Bacterial reduction percentage of pyroligneous acid against *Pseudomonas aeruginosa* (ATCC 9027), *Enterococcus faecium* (ATCC 35667), Faecal bacteria, and *Clostridium Perfringens* (NCTC 8237).

Figure 2 represents the mean log reduction of pyroligneous acid-based disinfectant against *Pseudomonas aeruginosa* (ATCC 9027), *Enterococcus faecium* (ATCC 35667), Faecal bacteria, and *Clostridium Perfringens* (NCTC 8237). The mean log reduction to *Pseudomonas aeruginosa* (ATCC 9027), *Enterococcus faecium* (ATCC 35667), Faecal bacteria, and *Clostridium Perfringens* (NCTC 8237) are 6.0792, 6.0000, 5.8921, and 5.9494 respectively which proved that this natural disinfectant can reduce the bacterial population significantly. Any antimicrobial agents have to reach 3-log reduction in microorganisms for non-food contact surfaces and 5-log reduction for food contact surfaces effectiveness [7] and achieved a "6-log reduction" means lowering the number of bacteria by 1 x 10^6 fold, that is, if a surface has 1 x 10^6 pathogenic microbial strain on it, a 6-log reduction would decrease the number of bacteria to one and 99.9999% of reduction.

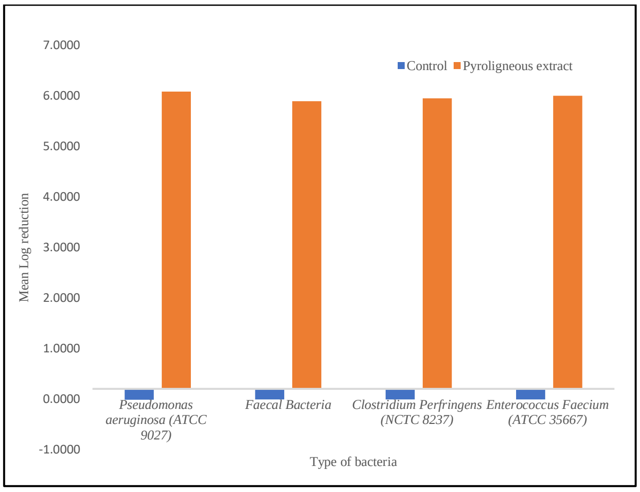

Fig. 2 - Mean log bacterial reduction of pyroligneous acid against *Pseudomonas aeruginosa* (ATCC 9027), *Enterococcus faecium* (ATCC 35667), Faecal bacteria, and *Clostridium Perfringens* (NCTC 8237).

3.2 Antibacterial bioprocess kinetics analysis of environmentally friendly-based disinfectant

Table 1 summarizes the antibacterial bioprocess kinetics of pyroligneous acid-based disinfectants against selected strains. The pyroligneous acid showed the significant reduction rate and halve rate to each bacterial strain within 5 minutes exposure time: *Pseudomonas aeruginosa* ATCC 9027 (2.7996, 4.0389), *Enterococcus faecium* ATCC 35667 (2.7631, 3.9863), Faecal bacteria (2.7134, 3.9146), and *Clostridium Perfringens* NCTC 8237 (2.7398, 3.9527) respectively. Pyroligneous acid contains furfural, vanillin, dihydrofuranone, acetic acid, cresol, and phenol which known as microbial growth inhibit agents. Among the specific reduction rate and halve rate, *Pseudomonas aeruginosa* (ATCC 9027) showed the highest rate compare to the *Enterococcus faecium* (ATCC 35667), Faecal bacteria, and *Clostridium Perfringens* (NCTC 8237). It is proved that pyroligneous acid-based disinfectant able to reduce *Pseudomonas aeruginosa* (ATCC 9027) population faster than *Enterococcus faecium* (ATCC 35667), Faecal bacteria, and *Clostridium Perfringens* (NCTC 8237) within the same contact time and condition. *Pseudomonas aeruginosa* as one of harmful microorganism is the major causes of multidrug-resistant nosocomial infections and consequently belong to the high concern group, against which there is a requirement for alternative antimicrobial agent according to World Health Organization report [29]. Also, *Enterococcus faecalis* is discovered to cause nosocomial infection with high death rates and drug-resistant and contaminants in packaged meat products, so an alternative antimicrobial agent would be required [28].

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Table 1 - Antibacterial bioprocess kinetics of pyroligneous extract-based disinfectant against *Pseudomonas aeruginosa* (ATCC 9027), *Faecal bacteria*, *Clostridium perfringens* (NCTC 8237), and *Enterococcus faecium* (ATCC 35667)

		Pseudomonas aeruginosa (ATCC 9027)		Faecal bacteria		*Clostridium perfringens* (NCTC 8237)		*Enterococcus faecium* (ATCC 35667)	
	Time, min	**Specific growth rate [a]**	**Division rate [c]**	**Specific growth rate [a]**	**Division rate [c]**	**Specific growth rate [a]**	**Division rate [c]**	**Specific growth rate [a]**	**Division rate [c]**
	0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Control	**5**	2.0618	2.9745	1.9232	2.7745	2.0253	2.9219	1.9232	2.7745
		Specific reduction rate [b]	**Halve rate [d]**	**Specific reduction rate [b]**	**Halve rate [d]**	**Specific reduction rate [b]**	**Halve rate [d]**	**Specific reduction rate [b]**	**Halve rate [d]**
	0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Pyroligneous extract	**5**	2.7996	4.0389	2.7134	3.9146	2.7398	3.9527	2.7631	3.9863

[a] Specific growth rate, min^{-1}
[b] Specific reduction rate, min^{-1}
[c] Division rate, min^{-1}
[d] Halve rate, min^{-1}

4. Conclusion

The antimicrobial activity of pyroligneous acid as an environmentally preferable disinfectant agent determined by a quantitative suspension test (EN1040). In this research, pyroligneous acid from *Rhizophora apiculata* showed significant and equivalent bacterial reduction effect to *Pseudomonas aeruginosa* ATCC 9027, *Enterococcus faecium* ATCC 35667, Faecal bacteria, and *Clostridium Perfringens* NCTC 8237. Besides, within five minutes of contact time, the pyroligneous acid able to eliminate all harmful bacteria. These in vitro test studies showed the pyroligneous based disinfectant consider a potential antimicrobial agent.

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