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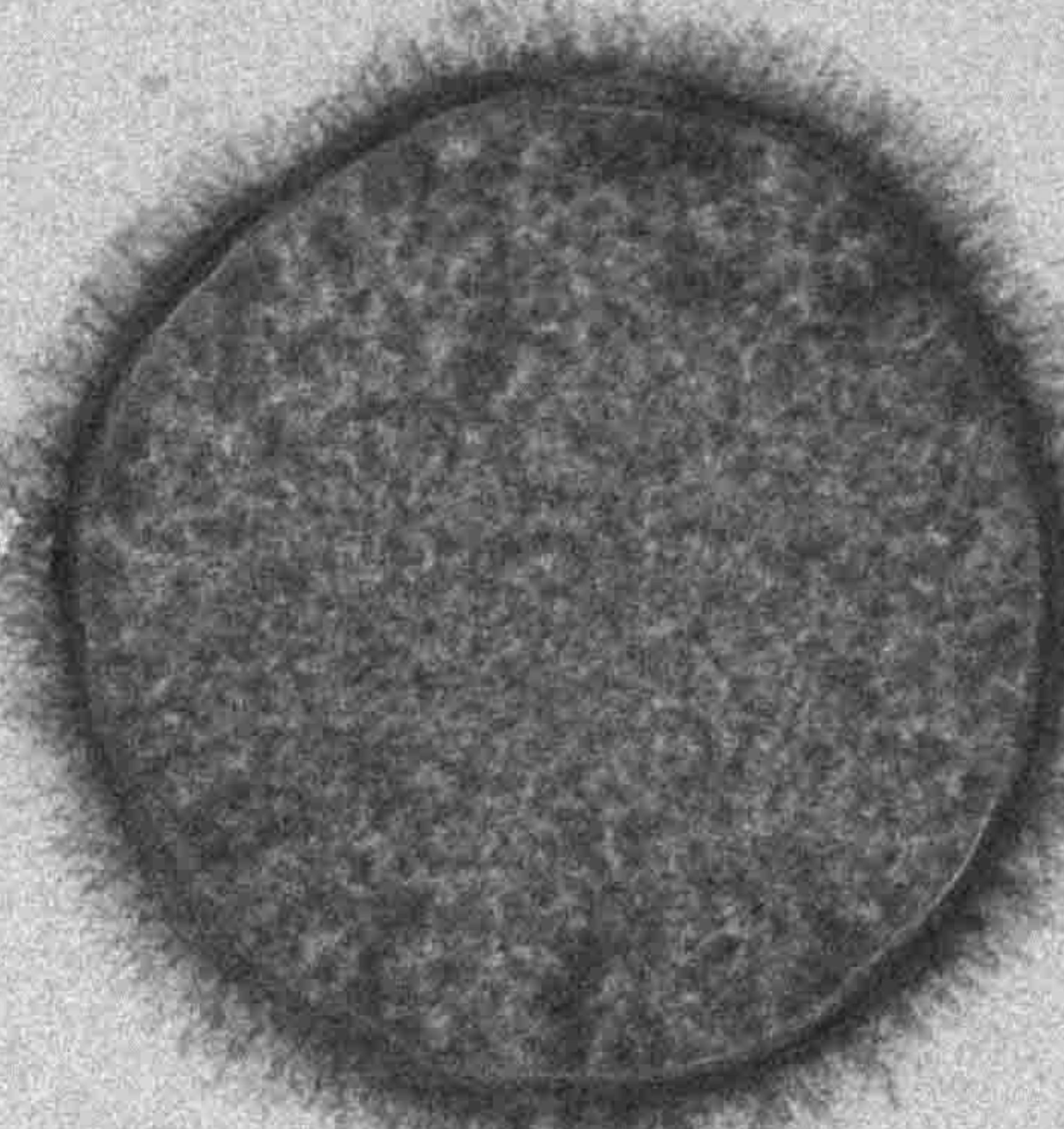

Bacillus subtilis photo by Allon Weiner

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Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta*. 50th Annual Symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

Proceeding:

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Phytochemical composition and antimicrobial activities of three selected spices seed extracts against food spoilage microorganisms

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Abstract. *Persaud C, Daniel R, Hemraj D. 2019. Phytochemical composition and antimicrobial activities of three selected spices seed extracts against food spoilage microorganisms. Bioteknologi 16: 41-47.* The study emphasizes on three selected spice extracts to be efficiently used as antimicrobial agents in food preservation, with the intention of developing antimicrobial agents capable of replacing synthetic chemical agents and natural agents weakened by antimicrobial resistance. The purpose of this study aims at stepping away from the artificial and exploring the extent to which natural substances, such as spices, provide antimicrobial effects in attempt to be formulated as natural food preservative. Antimicrobial screening of ethanolic and hexane extracts of *Cuminum cyminum* (cumin), *Sinapis alba* (mustard) and *Pimenta dioica* (all spice) against food spoilage bacteria *Bacillus subtilis* and *Pseudomonas aeruginosa* and fungi *Candida albicans* was attempted, with references to synthetics drugs Ciprofloxacin, Erythromycin and Fluconazole. Phytochemical analysis on the extracts showed that there were more secondary metabolites present in the ethanolic extracts. It was observed that ethanolic extracts of *C. cyminum* and *P. dioica* were more effective against the three microbes as compared to the hexane extracts of *C. cyminum* and *P. dioica*, while hexane extracts of *S. alba* was more effective against bacterial species. *S. alba* was not an effective antifungal agent. The results indicated that plant extracts possessing antibacterial and antifungal properties can be formulated as an ideal food preservative.

Keywords: All spice, antimicrobial activities, cumin, mustard, phytochemical analysis, spices

INTRODUCTION

Food spoilage and food poisoning is a complex naturally occurring process (Gram et al. 2002) and to understand how food spoil, the causes must first be assessed. The number one cause of food spoilage is because of microorganisms. In developing countries, food poisoning has been recorded as the one of the most common causes of illness and death; most cases are associated to Gram negative bacteria such as *Escherichia coli*, *Salmonella typhi* and *Pseudomonas aeruginosa*. Other Gram-positive bacteria that are known to be common causative agents in food poisoning and spoilage, includes *Staphylococcus aureus* and *Bacillus subtilis* (Mostafa et al. 2017). Fungal spores also contribute to food spoilage. Whereas, in undeveloped countries, food borne pathogens are the leading cause of illness and death with fungi being the major causative agent (Mohanka and Priyanka 2014). Ensuring microbial safety of food has become an important issue, but despite many technological advances and cultural techniques, food spoilage and food poisoning is still an ongoing phenomenon.

Prevention of food spoilage today is commonly achieved by chemical preservatives that prevent bacterial growth, but these chemicals cannot completely eradicate several pathogenic bacteria that spoil food products or delay the growth of these spoilage organisms (Liu et al. 2017). The effectiveness of most antimicrobial agents, which are currently being utilized today to extend shelf life and increase the safety of food products in the food industry, have been significantly weakened and reduced by microbial resistance. As a result, it is necessary that new antimicrobial agents that can overcome this resistance be discovered (Liu et al. 2017; Mostafa et al. 2017).

Antimicrobial compounds exist in many plants as secondary metabolites, where they provide natural defense mechanisms. Their broad activities against bacteria and fungi have led researchers to suggest that they can be used as natural preservatives in food (Kong et al. 2017). Natural products as substitutes of synthetic chemical preservatives are increasingly accepted because they are better tolerated by the human body and are less detrimental to human health, reducing the occurrences of side effects and eliminates chemical residues in food and feed chains (Liu et al. 2017; Mostafa et al. 2017).

As a result, scientific research began focusing on botanical condiments in attempt to combat food spoilage. In this study, the growth response of two common food spoilage bacteria (*B. subtilis* and *P. aeruginosa*) and fungus (*Candida albicans*) to the ethanolic and hexanic extracts of three spices will be investigated. Specifically, the effects of 25%, 50% and 100% extracts of *Cuminum cyminum* (cumin), *Sinapis alba* (mustard) and *Pimenta dioica* (all spice) on the growth of the microorganisms will be studied on the surface of Mueller-Hinton Agar by Agar disc diffusion method.

MATERIALS AND METHOD

Collection and preparation of seeds

Approximately 1360g each of dry seeds of *S. alba*, *C. cyminum* and *P. dioica* were purchased from N&S Mattai. Seeds were stored in airtight zip lock bags and containers, with suitable labelling after purchase. Seeds were incubated at 45°C until constant weight was achieved. Seeds were grounded with a stainless-steel manual hand mill until they became fine and powdery. They were then stored in airtight zip lock bags and containers with suitable labelling until further use.

Preparation of crude seed extracts by solvent extraction and rotary evaporation

Ethanol extract

Seed extract was prepared by solvent extraction method. 500g of each powdered seed material was extracted with 1000 mL of ethanol. The seeds and the solvent mixture were frequently agitated for 48 hours at 30°C. The mixtures were strained using a gauze and then filtered with Whatman's no.1 filter paper. The filtrates were evaporated in a rotary evaporator at 45°C. The extraction process continued until all the ethanol was removed and collected in the condensing flask and the extracts became thick and gel like. The extract was then collected and labelled, before being kept in sealed containers and wrapped in aluminum foil. The containers were stored at room temperature to prevent contamination until further use in phytochemical analysis and paper disc diffusion.

Hexane extract

Seed extract was prepared by solvent extraction method. 250g of each powdered seed material was extracted with 500 mL of Hexane. The seeds and the solvent mixture were frequently agitated for 48 hours at 30°C. The mixtures were strained using a gauze and then filtered with Whatman's no.1 filter paper. The filtrates were evaporated in a rotary evaporator at 45°C. The extraction process continued until all the hexane was removed and collected in the condensing flask, with the extracts being thick and gel like. The extract was collected, labelled and kept in sealed containers and wrapped in aluminum foil. The containers were store at room temperature.

Preparation of extract concentration

The extract was broken down into three concentrations, 25%, 50% and 100% which were all tested. For the Ethanolic extracts, ethanol was added to the extract to achieve desired concentrations. 10 mL of each extract was prepared. For 25% extract, 2.5 mL extract was added to 7.5 mL ethanol, 50% extract was prepared with 5 mL extract and 5 mL ethanol and 10 mL extract was used and treated as 100%. For the Hexane extracts, hexane was used in the extract to achieve desired concentrations. 10 mL of each extract was prepared. For 25% extract, 2.5 mL extract was added to 7.5 mL hexane, 50% extract was prepared with 5 mL extract and 5 mL hexane and 10 mL extract was used and treated as 100%. Extracts were stored in accurately labelled sealed glass vials at room temperature.

Preparation of 0.5 McFarland standard

McFarland 0.5 was prepared by mixing 0.05 mL 1% Barium Chloride ($BaCl_2$) and 9.95 mL 1% Sulphuric acid (H_2SO_4) in a test tube to obtain an estimated bacterial density $1.5x10^{8.}$ The McFarland was used to prepare the bacterial suspension.

Preparation of inoculum and bacterial suspension

Each bacterium (*B. subtilis* and *P. aeruginosa*) and fungus (*C. albicans*) were sub-cultured from cultures obtained from the Georgetown Public Hospital in Mueller-Hinton Agar petri dishes. Bacterial strains were incubated for 24 hours 30°C, while the fungus was incubated for 48 hours at 30°C. Bacterial suspension was prepared in test tubes containing 10 mL sterilized water. The microbial growth was harvested from the sub-cultured petri dishes with a sterile loop and inoculated the water. The turbidity of the suspension was compared to the turbidity of 0.5 McFarland standard in front of a Wickerham Card.

Paper disc diffusion

Paper disc diffusion was done in triplicates for each extract, at the three concentrations, with the three microbial species. The petri dishes containing Mueller-Hinton agar was swabbed with a sterilize swab to prepare a lawn of the microorganism. Sterile paper disc were soaked in the different concentrations of the extract for 24 hours, before being placed over the lawn. Four discs were placed to each plate. The petri dishes were sealed with transparent tape and incubated. Petri dishes containing bacteria was incubated for 24 hours at 30°C, while petri dishes containing fungi was incubated for 48 hours at 30°C. The formation of zones of inhibition were observed upon formation, which is a circular field around the paper discs. The zones were measured with a ruler in millimeter (Agabalogun et al. 2012).

Preparation of controls

Ciprofloxacin, Fluconazole and Erythromycin were used as the reference or positive control. 0.01g of each drug was mixed in 100 mL water. Paper discs were infused in the solution and was placed on the surface of the agar streaked with the microbes. Antibiotics, Ciprofloxacin and Erythromycin, were tested against *B. subtilis.* and *P. aeruginosa.* Antifungal drug Fluconazole was tested against *C. albicans.* The solvents Hexane and Ethanol were used as the negative control. Paper discs were infused in the solvents and were placed on the surface of the agar streaked with the microbes. Petri dishes containing bacteria was incubated for 24 hours at 30°C, while petri dishes containing fungi was incubated for 48 hours at 30°C.

Qualitative phytochemical analysis

The extracts were analyzed by the following tests to qualitatively test for phytochemical including steroids, phenols and tannins, alkaloids, saponins, volatile oils and terpenoids.

Test for steroids

5 mL of crude seed extract was placed in a test tube and mixed with 2 mL acetic anhydride. Followed by 2 mL chloroform, concentrated Sulfuric acid (H_2So_4) was added dropwise at the side of the test tube. A red color formation indicates the presence of steroids (Deshpande et al. 2014).

Test for phenols

In a test tube, 5 mL of crude seed extract was mixed with 2 mL of 2% iron tri-chloride ($FeCl_3$). A black-dark green color change indicates the presence of both phenols and tannins (Deshpande et al. 2014).

Test for tannins

In a test tube, 5 mL of crude seed extract was mixed with 2 mL of 2% iron tri-chloride ($FeCl_3$). A green color change indicates the presence of catecholic tannin, while a blue color indicates the presence of gallic tannins (Deshpande et al. 2014).

Test for alkaloids

2 mL of the crude extract was measured in a test tube, with picric acid added drop wise until an orange color persisted (Deshpande et al. 2014).

Test for saponins

0.5 mL of the crude extract was measured in a test tube and 2 mL water was added and shaken; a persistent foam for 10 minutes indicates the presence of saponins (Deshpande et al. 2014).

Test for volatile oils

2 mL of extracts was placed in a test tube, with 0.1 mL dilute NaOH and a small quantity of dilute Hydrochloric acid (HCL) being added. The formation of a white precipitate indicates the presence of Volatile oils (Deshpande et al. 2014).

Test for terpenoids

5 mL of crude seed extract was placed in a test tube and mixed with 2 mL acetic anhydride. Then 2 mL chloroform, concentrated Sulfuric acid (H_2So_4) is added dropwise at the side of the test tube. A red color formation indicates the presence of steroids (Deshpande et al. 2014).

Data analysis

Data recorded was analyzed by Analysis of Variance (ANOVA). Tukey Honest significant difference test was carried out to identify whether there was a significant difference between the different concentrations of spice extracts as well as comparing the concentrations between the different groups of extracts. There was a significant difference if the p-value obtained was below 0.05

RESULTS AND DISCUSSION

The yield of ethanol extracts was higher than hexane extracts, albeit they showed no significant difference when assessed statistically. These results are supported by Widyawati et al. (2014). Ethanol extracts were more effective compared to the hexane extracts, except for *S. alba,* where the hexane extracts were more effective (Table 1-5). These results are supported by Mohanka and Priyanka (2014) and Deshpande et al. (2014).

Microorganisms used in the study were chosen because of their dynamic roles in food poisoning and food spoilage. Bacterial strains *B. subtilis* (locally known as the Fried-Rice spoilage bacteria) and *P. aeruginosa,* are common bacterial species that produce toxins and other metabolites that induce human gastrointestinal diseases, in accordance with Mostafa et al. (2017). The antimicrobial activities generated by the extracts of these spices were more effective against bacterial strains than fungi, like the results of Erturk (2006).

Cuminum cyminum was the most useful spice against bacterial strains *B. subtilis* and *P. aeruginosa,* as compared to *P. dioica* and *S. alba*, to which there was a statistically significant difference. This was in accordance with Mostafa et al. (2017), who found *C. cyminum* to be effective against several strains of Gram-positive and Gram-negative bacteria implicated in food poisoning. *C. cyminum* inhibited the growth of *B. subtilis* at 25%, 50%, and 100% concentrations and there was a statistically significant difference between the *C. cyminum*-ethanol extracts and the *C. cyminum*-hexane extracts at all three levels (Figures 1-3), with *C. cyminum*-ethanol extracts at all three concentrations being the most effective against *B. subtilis*. *C. cyminum* inhibited the growth of *P. aeruginosa* at 50% and 100% concentrations for *C. cyminum*-hexane extract and 25%, 50% and 100% concentrations for ethanol extracts. Ethanol extracts of *C. cyminum* were more effective against *P. aeruginosa.* However, *C. cyminum* was not effective against the fungi *C. albicans.*

Pimenta dioica-hexane was not effective against Gram-negative bacteria *P. aeruginosa,* but *P. dioica*-ethanol was effective against *P. aeruginosa* at all three concentrations. Gram-positive bacteria *B. subtilis* and the fungi *C. albicans* were the most susceptible microorganisms to *P. dioica*. *P. dioica* could inhibit the growth of *B. subtilis* and *C. albicans* at 25%, 50% and 100 % concentrations for both ethanol and hexane extracts. Ethanolic extracts of *P. dioica*, however, were more effective and produced larger inhibitory zones compared to the hexane extracts. There was a statistically significant difference between the *P. dioica* in two solvents at all three concentrations (Figures 4-6). These results supported by Tajkarimi et al. (2010) and Azzouz et al. (1982).

Table 1. ANOVA analysis of the spice extracts in the ethanol versus hexane at the three concentrations.

Extract	P-value	Difference
C. cyminum 25 % (ethanol-hexane)	0.007734	+
C. cyminum 50 % (ethanol-hexane)	0.0000000303874	+
C. cyminum 100 % (ethanol-hexane)	0.0000336138	+
P. dioica 25 % ethanol-hexane)	0.000984	+
P. dioica 50 % (ethanol-hexane)	0.0000288718	+
P. dioica 100 % (ethanol-hexane)	0.000000414	+
S. alba 25 % (ethanol-hexane)	0.063133731	-
S. alba 50 % (ethanol-hexane)	0.02132	+
S. alba 100 % (ethanol-hexane)	0.000000274424	+

Note: +: Statistically significant difference; -: No statistical difference

Table 2. ANOVA analysis of the difference between the three spices used.

Spice	P-value	Difference
C. cyminum-P. dioica	0.0497385	+
S. alba-P. dioica	0.0000023	+
S. alba-C. cyminum	0.0000000	+

Note: +: Statistically significant difference

Table 3. ANOVA analysis of the difference between the three microorganisms.

Microorganisms	P-value	Difference
C. albicans -B. subtilis	0.0052063	+
P. aeruginosa-B. subtilis	0.7824462	-
P. aeruginosa-C. albicans	0.0356384	+

Note: +: Statistically significant difference; -: No statistical difference

Table 4. Phytochemicals detected in the extract of the three spices: *S. alba*, *C. cyminum* and *P. dioica*.

Spice extracts	P-value	Difference
C. cyminum ethanol-*P. dioica* ethanol	0.3597876	+
C. cyminum hexane -*P. dioica* ethanol	0.0000306	+
S. alba ethanol -*P. dioica* ethanol	0.0000000	+
S. alba hexane -*P. dioica* ethanol	0.0000000	+
C. cyminum ethanol-*P. dioica* hexane	0.0000000	+
C. cyminum hexane -*P. dioica* hexane	0.1879712	-
S. alba ethanol -*P. dioica* hexane	0.1046461	-
S. alba hexane -*P. dioica* hexane	0.9999923	-
S. alba ethanol -*C. cyminum* ethanol	0.00000000	+
S. alba hexane -*C. cyminum* ethanol	0.00000000	+
S. alba ethanol -*C. cyminum* hexane	0.0000896	+
S. alba hexane -*C. cyminum* hexane	0.0956633	-

Note: +: Statistically significant difference; -: No statistical difference

Table 5. ANOVA analysis of the difference between the spice extracts in solvents, ethanol, and hexane.

Phytochemicals	Hexane			Ethanol		
	C. cyminum	*P. dioica*	*S. alba*	*C. cyminum*	*P. dioica*	*S. alba*
Alkaloids	-	-	-	-	-	+
Tannins	-	+	-	+	+	-
Saponins	-	-	+	-	+	-
Volatile oils	-	-	+	-	+	-
Phenols	-	-	-	-	+	-
Steroids	-	-	+	-	+	+
Terpenoids	-	-	+	+	+	+

Sinapis alba was the least effective spice tested in this study. The *S. alba* extracts were not effective and completely susceptible to the fungal species *C. albicans*. However, bacterial strains *B. subtilis* and *P. aeruginosa* were inhibited by the *S. alba* extracts with *B. subtilis* being most sensitive to the *S. alba* extracts, specifically the *S. alba*-hexane extract (Figures 7-9). The Gram-positive *B. subtilis* was inhibited at 50% and 100% concentrations of the *S. alba*-hexane extract. These results are in accordance with Tajkarimi et al. (2010). *P. aeruginosa* was inhibited by *S. alba*-hexane at 25% and 100% and by *S. alba*-ethanol at 25% concentration only. *S. alba* ethanolic extracts were not effective against *B. subtilis*. and *C. albicans*. This result is like Widyawati et al. (2014), ethanol is a polar solvent and chemical compounds of *S. alba* seeds are likely mostly non-polar. Therefore, *S. alba* seeds are insoluble in the polar solvent ethanol hence, there were no strong antimicrobial effects exhibited. There was no statistically significant difference between the 25% *S. alba*-hexane extract and the 25% *S. alba*-ethanol extract in relation to the zone of inhibition produced and effectiveness because they were both only effective against *P. aeruginosa* at that concentration.

Table 6. The mean zone of inhibition of the spice extracts at concentrations 25%, 50% and 100% in solvents hexane and ethanol. Values are the mean ± SD.

Solvent	Spice extract	Concentration (%)	Inhibition zones (mm)		
			Microorganisms		
			B. subtilis	*P. aeruginosa*	*C. albicans*
Hexane	*C. cyminum*	25	10.25 ± 5.34	0 ± 0	0 ± 0
		50	13.75 ± 0	17 ± 1.38	0 ± 0
		100	21.25 ± 2.05	14.25 ± 3.50	0 ± 0
	P. dioica	25	2.83 ± 1.83	0 ± 0	5.16 ± 5.17
		50	8.41 ± 1.83	0 ± 0	11.16 ± 1.18
		100	10.5 ± 2.60	0 ± 0	4.33 ± 5.62
	S. alba	25	0 ± 0	6.5 ± 3.68	0 ± 0
		50	12.58 ± 6.23	0 ± 0	0 ± 0
		100	14 ± 1.94	9.08 ± 1.20	0 ± 0
Ethanol	*C. cyminum*	25	13.83 ± 3.47	27.1	19.8
		50	23.83 ± 1.01	24.6	11.5
		100	20.33 ± 0.73	21.2	15.25
	P. dioica	25	16.16 ± 1.92	18.25	10.3
		50	16.75 ± 2.47	14.66 ± 0.58	15.66 ± 0.36
		100	20.16 ± 0.65	10.16 ± 5.53	14.08 ± 1.37
	S. alba	25	0 ± 0	10.83 ± 5.42	0 ± 0
		50	0 ± 0	0 ± 0	0 ± 0
		100	0 ± 0	0 ± 0	0 ± 0

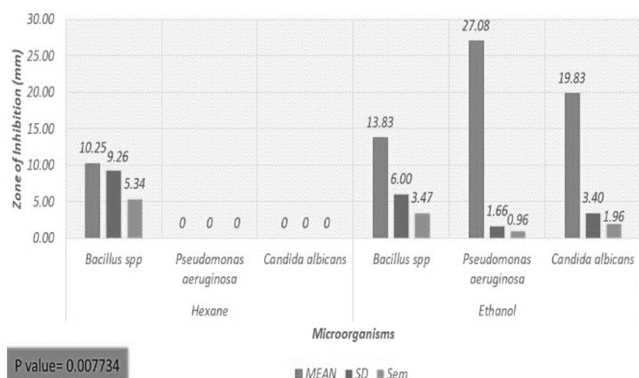

Figure 1. Zone of inhibition of 25% *C. cyminum* against *B. subtilis*, *P. aeruginosa* and *C. albicans*. There was a significant difference between the ethanol and hexane extracts of *C. cyminum* 25% in relation to the zones of inhibition.

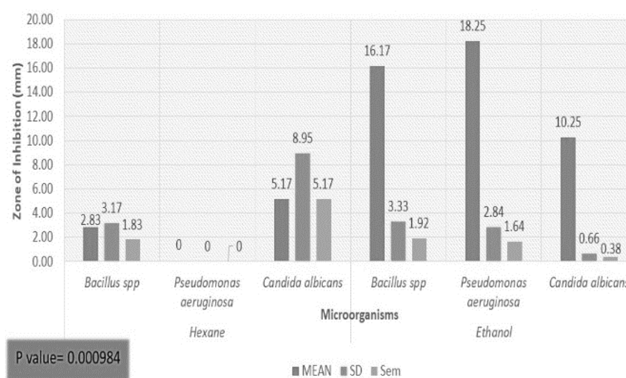

Figure 4. Zone of inhibition of *P. dioica* 25% against *B. subtilis*, *P. aeruginosa* and *C. albicans*. There was a significant difference between the ethanol and hexane extracts of *P. dioica* 25% in relation to the zones of inhibition.

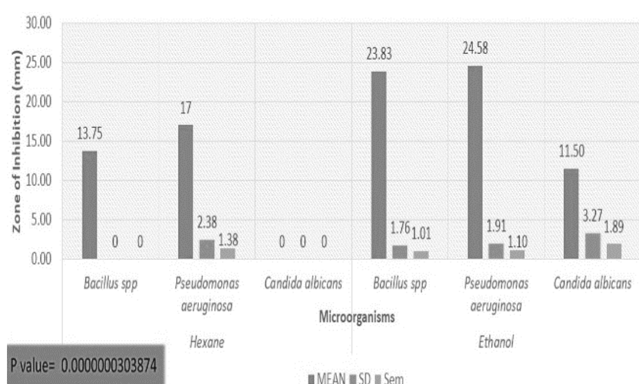

Figure 2. Zone of inhibition of 50% *C. cyminum* against *B. subtilis*, *P. aeruginosa* and *C. albicans*. There was a significant difference between the ethanol and hexane extracts of *C. cyminum* 50% in relation to the zones of inhibition.

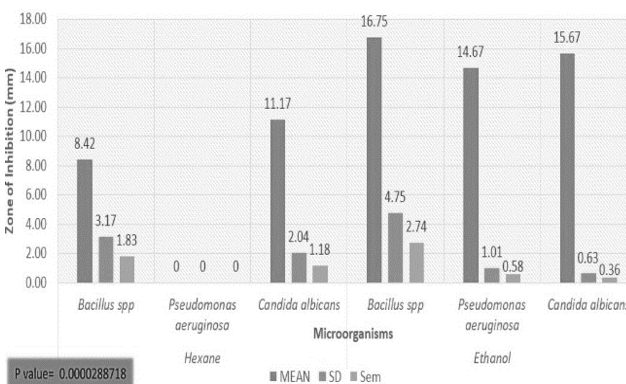

Figure 5. Zone of inhibition of *P. dioica* 50% against *B. subtilis*, *P. aeruginosa* and *C. albicans*. There was a significant difference between the ethanol and hexane extracts of *P. dioica* 50% in relation to the zones of inhibition.

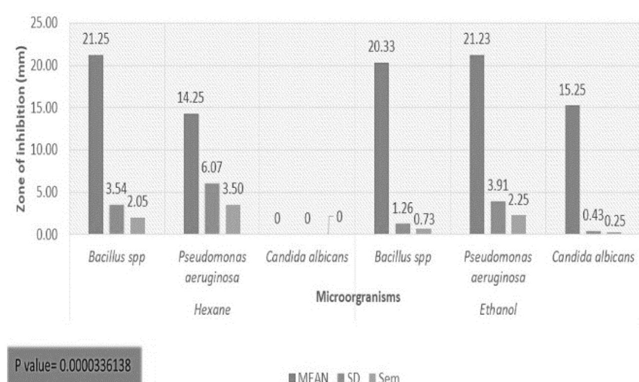

Figure 3. Zone of inhibition of 100% *C. cyminum* against *B. subtilis*, *P. aeruginosa* and *C. albicans*. There was a significant difference between the ethanol and hexane extracts of *C. cyminum* 100% in relation to the zones of inhibition.

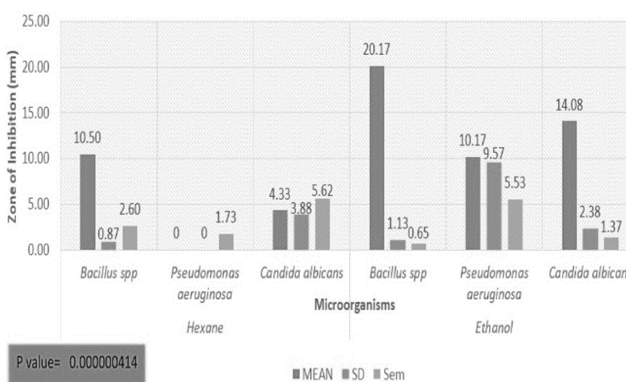

Figure 6. Zone of inhibition of *P. dioica* 100% against *B. subtilis*, *P. aeruginosa* and *C. albicans*. There was a significant difference between the ethanol and hexane extracts of *P. dioica* 100% in relation to the zones of inhibition.

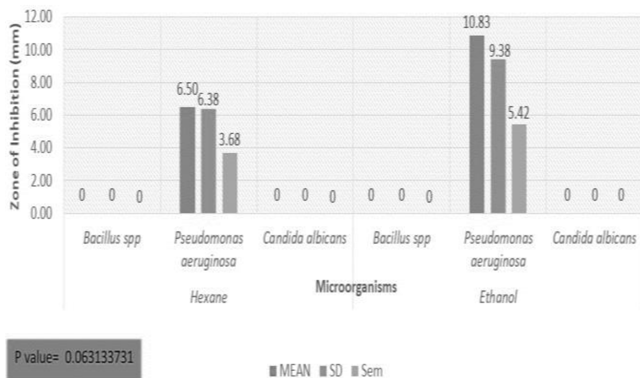

Figure 7. Zone of inhibition of *S. alba* 25% against *B. subtilis*, *P. aeruginosa* and *C. albicans*. There was no significant difference between the ethanol and hexane extracts of *S. alba* 25% in relation to the zones of inhibition.

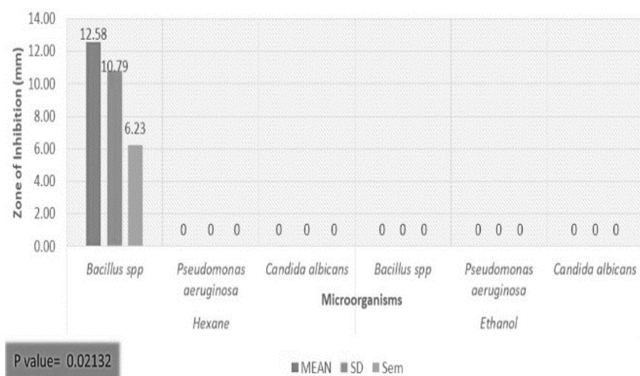

Figure 8. Zone of inhibition of *S. alba* 50% against *B. subtilis*, *P. aeruginosa* and *C. albicans*. There was a significant difference between the ethanol and hexane extracts of *S. alba* 50% in relation to the zones of inhibition.

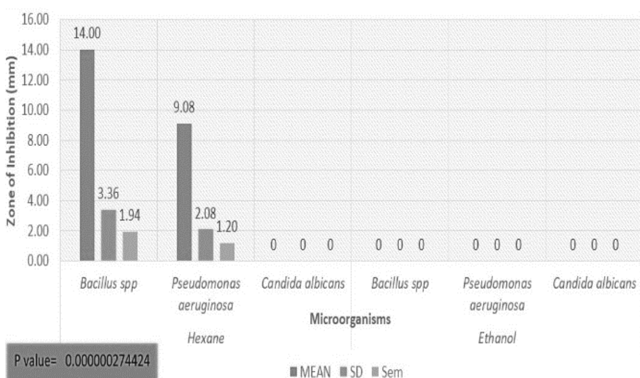

Figure 9. Zone of inhibition of *S. alba* 100% against *B. subtilis*, *P. aeruginosa* and *C. albicans*. There was a significant difference between the ethanol and hexane extracts of *S. alba* 100% in relation to the zones of inhibition.

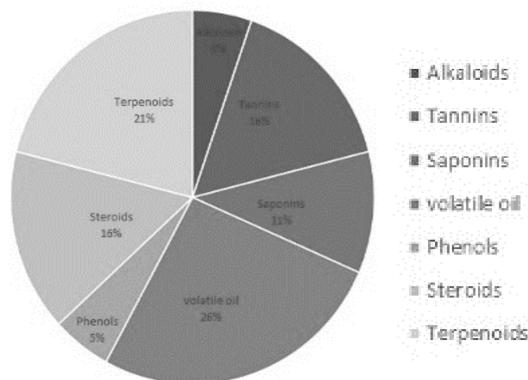

Figure 10. Total percent of phytochemicals found in the extracts of *S. alba*, *P. dioica* and *C. cyminum* in solvents hexane and ethanol.

Ethanol was able to extract most chemical compounds from *C. cyminum*, *P. dioica*, and *S. alba;* including alkaloids, tannins, saponins, volatile oils, phenols, steroids and terpenoids than the hexane extracts where alkaloids and phenols were not extracted (Figure 10). In *P. dioica*-ethanol all phytochemicals tested were detected, except for alkaloids. While *C. cyminum*-ethanol tested positive for tannins, volatile oils and terpenoids. *S. alba*-ethanol tested positive for the presence of alkaloids, volatile oils, steroids, and terpenoids. *C. cyminum*-hexane and *P. dioica*-hexane tested positively only for volatile oils and tannins, respectively. *S. alba*-hexane has the most chemical compounds present for hexane extracts, which tested positively for saponins, volatile oils, steroids and terpenoids. These results are in accordance with that of Widyawati et al. (2014). Hexane, a non-polar compound was ineffective in extracting phenols and alkaloids because they are semi-polar compounds that are easily dissolved in polar solvents such as ethanol. Compounds present in seeds of *C. cyminum*, *P. dioica*, and *S. alba* were mostly polar than non-polar.

All factors were tested and observed against three references ciprofloxacin, erythromycin, and fluconazole; however, the zones of inhibition produced by these drugs were higher than the zones produced by the spices. The three spices tested in this study all exhibited some range of antimicrobial effectiveness. *C. cyminum* was the most effective spice and inhibited *B. subtilis, P. aeruginosa*, and *C. albicans*. *P. dioica* was the second most effective spice and inhibited *B. subtilis, P. aeruginosa*, and *C. albicans*. *S. alba* was the least effective spice, it slightly inhibited the growth of *B. subtilis,* and *P. aeruginosa* at varying concentrations but was ineffective in inhibiting the mycelial growth on *C. albicans.* Therefore, *C. cyminum* and *P. dioica* can effectively be used as a natural food preservative to control food poisoning and spoilage. The effectiveness of these spices was because of the antimicrobial compounds present in the seeds of each spice, which were detected by phytochemical analysis. It was suggested that these chemical compounds present in the seeds of each spice interact with enzymes and proteins

of the microbial cell membrane causing disruption and dispersal of protons inducing cell death or inhibited enzymes necessary for amino-acids biosynthesis (Mostafa et al. 2017). This study indicated that spice extracts which proved to be potentially effective could be used as natural preservatives to control food poisoning and food spoilage, avoiding application of hazardous chemical preservatives.

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Tannins inhibition of tea leaves (*Camellia sinensis*) against *Escherichia coli* diarrhea-causing bacteria

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Abstract. *Cahyani RK, Susilowati A, Sari SLA. 2019. Tannins inhibition of tea leaves (*Camellia sinensis*) against* Escherichia coli *diarrhea-causing bacteria. Bioteknologi 16: 48-52.* Diarrhea is often suffered by people and has become the biggest cause of death in developing countries. Diarrhea can be caused by *Escherichia coli* infection. Treatment with antibiotics can lead to resistance, so herbs like tea leaves can be used as an alternative to overcome diarrhea. This research aims to determine the effect of tannins in ethanol extract of tea leaves from Kemuning Estate against diarrhea-causing bacteria. Tea leaves powder was macerated using ethanol 70% to obtain a paste extract for an antibacterial test. The antibacterial test was conducted using contact bioautography methods, which combine thin-layer chromatography with the response of the bacteria test by antibacterial activity. The test results showed that the antibacterial activity of tannins in ethanol extract of tea leaves with a concentration of 30% and 50% could inhibit the growth of *E. coli*. The strongest concentration to inhibit the *E. coli* bacteria is 50% which contains 13.8% of tannins and has a 14 mm inhibition zone diameter. In comparison, a concentration of 30%, which contains 8.28% of tannins and an inhibition zone diameter of 10 mm, is included in moderate categorized inhibition. Tannins analysis by Thin Layer Chromatography (TLC) using $FeCl_3$ reagent formed blue ink or black colors, which means tannins in tea leaves are classified as the hydrolyzed tannins. Analysis of quality tannins with the Folin-Ciocalteu method showed that the tannins content in ethanol extract of tea leaves from Kemuning Estate was 27.6%.

Keywords: Bioautography, diarrhea, *Escherichia coli, Camellia sinensis*, tannin

INTRODUCTION

Diarrhea is derived from the Greek and Latin words dia, which means to pass, and rhein, which means to flow. Diarrhea occurs when stools become much watery in consistency and occur more frequently than usual (Nuratmi et al. 2006). Diarrhea is a very prevalent disease in children and adults alike. Until now, diarrhea has been one of the leading causes of death in children under five years. According to Alexander et al. (2013), diarrhea is responsible for 15% of child mortality. Adults in Indonesia experience over 100 million episodes of diarrhea per year. Simultaneously, the WHO estimates that over 4 billion cases of diarrhea occur each year, with a mortality rate of 3-4 million (Zein et al. 2004). Diarrhea is caused by contaminated beverages and rotten food contaminated with bacteria, fungus, or poisonous or harmful microorganisms. Diarrhea can be caused by germs such as *Escherichia coli*. Enteropathogenic bacteria produce diarrhea by attaching to epithelial cells with or without mucosal injury, invading the mucosa, and producing enterotoxins or cytotoxins.

If antibiotics such as ciprofloxacin, chloramphenicol, or tetracycline are taken continuously, resistance will develop. Bacterial resistance occurs as a result of a mutation in a bacterial gene. According to Ameri (2014), the proliferation of antibiotic resistance genes among different disease-causing bacteria has increased, lowering an antibiotic's effectiveness.

The importance of traditional medicine is becoming more apparent now, as is the growing understanding of the usefulness of numerous medicinal plants in Indonesia. Antidiarrheal properties of medicinal plants such as tea leaves are well established. Traditionally, tea has been used to help alleviate or even stop diarrhea. Tea is an indigenous plant to Indonesia and has a long history of widespread distribution. Tea cultivation is concentrated in the Surakarta area, specifically at the Kemuning tea estate in the Ngargoyoso District, Karanganyar, Indonesia. This plantation's tea has been processed and marketed domestically and internationally. Tea is a versatile plant utilized in various ways, including as an ingredient in refreshing beverages and a component of pharmaceutical and cosmetic products. The health advantages of tea are derived from the presence of key components such as caffeine, tannins, essential oils, vitamins, and minerals (Loto 2011). Tannins have an astringent flavor and are phenolic, which means they can be employed as antibacterial agents, antioxidants, and tumor inhibitors (Vitanti et al., 2012; Mailoa et al., 2014). Tannins are considered to be antibacterial due to their ability to build complex compounds with proteins via hydrogen bonds; when tannins and proteins establish hydrogen bonds, protein denaturation interferes with bacterial metabolism.

According to Sung et al. (2012), tannins are categorized into two types according to their molecular structure: condense tannins (catechins) and hydrolyzed tannins (gallic acid). Hydrolyzed tannins have the ability to contract bigger tissues in response to irritation-induced diarrhea. The broken tannate protein will attach to hydrolyzed tannins that pass through the colon, reducing small

intestinal secretion and constipation. Tannin condensed has a protective function. Tannins can be used as diarrhea medications because they help to minimize fluid loss from the gastrointestinal tract and restore the intestinal flora's equilibrium (Defrin et al. 2010). Tea leaf extract has been shown in earlier research to prevent the growth of *E. coli* bacteria, which is one of the bacteria that causes diarrhea. According to Widiana (2012), tea leaf extract has a minimum inhibitory concentration of 3.125% against *E. coli* bacteria. However, it is unknown which individual chemicals in tea leaves can suppress *E. coli* bacteria based on the results of these investigations. As a result, this study focused on the unique properties of tea leaf extract, specifically tannins, which limit the growth of diarrhea-causing bacteria such as *E. coli*. The tannins from tea leaves were studied for their ability to prevent the growth of *E. coli* bacteria. Tea leaves contain tannins, which may be used as an alternative diarrhea medication.

MATERIALS AND METHODS

Material

The materials used were tea leaves (*Camellia sinensis* (L.) Kuntze) from the Kemuning plantation, Karanganyar, Indonesia, isolates of *E. coli* bacteria obtained from diarrhea patients and cultured in the Microbiology Laboratory of the Faculty of Medicine, Airlangga University Surabaya, nutrient broth (NB), nutrient agar (NA), gallic acid, Folin-ciocalteu, Na_2CO_3 15%, acetic acid, butanol, aquadest, $FeCl_3$, and 70% ethanol.

Method

Ethanolic extraction of tea leaf

To make a thick paste for antibacterial testing, the tea leaf powder was macerated with 70 % ethanol for 3 x 24 hours with regular stirring (Harborne 1996), then filtered through Whatman filter paper no.42 and evaporated in a rotary evaporator at 50°C.

Analysis of tannin content

Analysis of tannin content was based on the Folin-ciocalteu method with a gallic acid standard. Standard solutions of 0, 10, 25, 50, 100, and 250 ppm gallic acid were taken as much as 2 mL and added 0.2 mL of Folin-Ciocalteu reagent and 1 mL of 15% Na_2CO_3 solution, then shaken until smooth. The solution was allowed to stand for 2 hours at room temperature, and its absorbance was measured using a UV-Vis spectrophotometer of 765 nm. For the sample analysis, 100 mg of tea leaf extract was taken, then 0.2 mL of Folin-Ciocalteu reagent was added, 1 mL of 15% Na_2CO_3, and 2 mL of distilled water were shaken until smooth. The solution was allowed to stand for 2 hours at room temperature, and its absorbance was measured using a UV-Vis spectrophotometer of 765 nm (Yulia 2006).

Thin-layer chromatography (TLC) analysis

TLC analysis of tannin compounds in tea leaf extract with concentrations of 10%, 30%, and 50% used silica gel GF254 as a stationary phase and a mobile phase in the form of butanol-acetic acid-water (2:0,5:1,1 v/v). Spot stain detection was carried out using UV254 and UV365 light and an Rf value of 0.22-0.92 (Yamuna et al. 2012). Qualitative analysis of tannins was carried out by detecting 1% $FeCl_3$ spray (Chavan and Amarowicz 2013).

Antibacterial activity test with bioautography method

Researchers used the contact bioautography approach to determine antibacterial activity. The test bacteria were cultured in a petri dish using a 10 mL sterile NA medium using the swab method. Following drying, the TLC plate was mounted to the surface of the NA medium for 20-30 minutes with a 10%, 30%, or 50% extract solution. After removing the TLC plate, the petri dish was incubated for 24 hours at 37°C (Akhyar 2010). The inhibitory zone was seen by checking for a bright area that was not overrun by bacteria. A caliper or ruler is used to measure the diameter of the inhibitory zone vertically, horizontally, and diagonally and then averaged in millimeters (Dharmawati 2011). The positive control plate included 13.8% gallic acid, while the negative control plate contained butanol-acetic acid-water.

RESULTS AND DISCUSSION

The tannin content of tea leaf extract

Folin-Ciocalteu reagent and gallic acid standard were used to determine the tannin content. The standard gallic acid curve (Figure 1) was designed to determine the regression equation for determining the tannin content in tea leaf extract via phenolic analysis (Sultana et al., 2012).

According to the regression equation derived from the standard curve, the tannin concentration in tea leaf extract was 27.6%, implying 0.276 grams of tannin in 1 gram of tea leaf extract. Table 1 shows the tannin content of various concentrations of ethanol extract from tea leaves.

Table 1. The concentration of tea leaf ethanol extract and tannin content

Tea leaf ethanol extract concentration (%)	Tannin content (%)
10	2.76
30	8.28
50	13.8

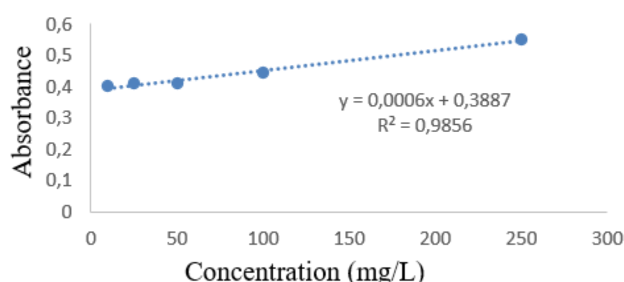

Figure 1. Gallic acid standard curve

The tannin content of the tea leaves obtained from the Kemuning Plantation was 27.6%, while the tannin content obtained from the Nusantara VIII Plantation, Sukabumi, was 0.13%. The Kemuning Plantation produced tea leaves with a tannin content of 27.6%, while the Nusantara VIII Plantation produced tea leaves with a tannin content of 0.13% (Yulia 2006). The tannin concentration in tea leaves from Kemuning Plantation is quite high, demonstrating that the plantation has a high tannin content.

TLC tannins tea leaf extract

Tea leaf extract was subjected to TLC to separate tannin components. This method is used to separate one substance from another. It is based on the difference in distribution between two phases, which are the stationary phase and the mobile phase. Silica gel GF254 is used as the stationary phase to carry out this TLC procedure. The solvent is butanol: acetic acid: water eluent in the ratio of 2:0.5:1.1 (v/v), with a swelling distance of 5 cm between the stationary phases. However, according to Hayati et al. (2010), the butanol: acetic acid: water (BAA) mixture is the most effective eluent for separating tannin compounds because the eluent composition is particularly polar, allowing it to separate tannin compounds that are also polar.

TLC chromatogram of tea leaf ethanol extract at a concentration of 50% (Figure 2) showed there were 3 separation spots with Rf values of 0.22, 0.72, and 0.92. Of the three stains formed, the second stain was suspected to be a hydrolyzed tannin compound or error, which had an Rf value of 0.72, and the color of the stain, when irradiated with UV254 light, became violet. In contrast, UV365 light produced two colors: purple and reddish-purple. This result is reinforced by Harborne (1996) that UV254 can detect tannins in the form of purple stains, and according to Hayati et al. (2010), color chromatogram stains with UV365 light produced two colors at different Rf, namely greenish-brown and reddish-purple. The eluted ethanol extract of tea leaves, when sprayed with 1% $FeCl_3$, becomes ink blue or black, indicating the presence of hydrolyzed tannins (Sa'adah 2010).

Gallic acid as a positive control only formed 1 spot stain with an Rf value of 0.72 (Figure 3).

Antibacterial activity in the bioautographic method

Contact bioautography is used to determine the antibacterial activity of tannin components found in the tea leaf extract. This method was chosen due to the clarity of the data obtained without using methyl thiazole tetrazolium (MTT). The tea leaf ethanol extract concentrations utilized to determine the bioautography method's antibacterial efficacy were 10%, 30%, and 50%. Figure 4 illustrates the results of the bioautography test.

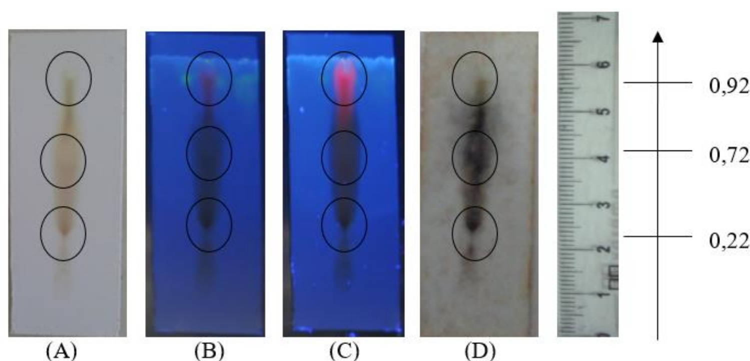

Figure 2. TLC chromatogram of tea leaf ethanol extract detection by (A) visible light, (B) UV254 light, (C) UV365 light and (D) 1% $FeCl_3$ spray detection

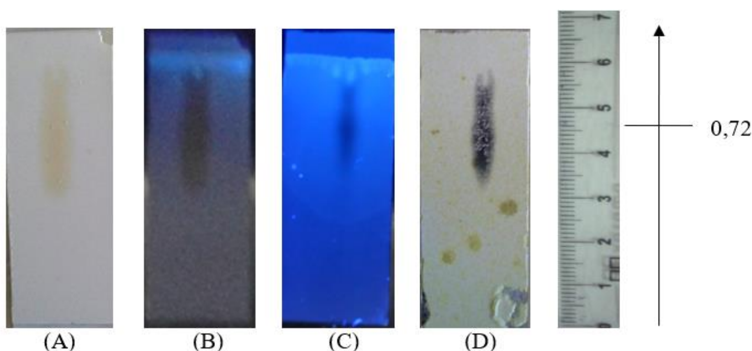

Figure 3. TLC chromatogram of gallic acid 13.8% detection by (A) visible light, (B) UV254 light, (C) UV365 light and (D) 1% $FeCl_3$ spray detection

The results indicated that the tannin compound from the ethanol extract of tea leaves had a maximum inhibition zone diameter of 14 mm at a 50% concentration. The 30% concentration had an inhibition zone diameter of 10 mm (Table 2) as a comparison. However, the tannin component from a 10% ethanol extract of tea leaves did not generate an inhibitory zone. This condition is most likely because the concentration of tannin compounds is so low. According to Ristiningsih (2009), the concentration increases the size of the inhibitory zone. The active ingredients in it are becoming more concentrated, increasing the amounts of bacteria inhibited.

As a positive control, gallic acid (13.8%) exhibited a bigger and more distinct inhibitory zone than the tea leaf tannin complex. It is most likely because gallic acid is a pure chemical, but the tannins in tea leaf ethanol extract are more complicated. The inhibitory zone was not generated when butanol: acetic acid: water was used as a negative control. It demonstrates that the TLC mobile phase does not affect the suppression of *E. coli* bacterial growth.

The study's results indicated that the tannin components in the ethanol extract of tea leave possessed moderate to strong inhibitory activity against the *E. coli* bacteria that cause diarrhea. According to Davis and Stout in Nurrakhman (2011), the provisions for the strength of antibacterial power are as follows: an inhibition area of 20 mm or greater is considered very strong, an inhibition area of 10-20 mm is considered strong, an inhibition area of 5-10 mm is considered medium, and an inhibition area of 5 mm or less is considered weak.

According to Mailoa et al. (2014), tannins exert antibacterial action by entering the bacterial cell wall. Gram-negative bacteria have thinner cell walls, allowing tannins to more easily destroy the proteins found in the cell walls of *E. coli* bacteria. Because protein is a component of bacteria's cell walls and plasma membranes, substances that induce bacterial metabolic problems can easily enter proteins in damaged or denatured cell walls. Tannins can generate hydrogen bonds with proteins found in bacterial cells; when these hydrogen bonds form, protein denaturation occurs, interfering with bacterial metabolism. Tannins are hypothesized to limit the growth of *E. coli* bacteria based on this interaction. Tannins can also impede the growth of germs and destroy them via interactions with cell membranes. Tannins react with proteins in cell membranes to produce hydrogen bonds, denaturing the proteins. Additionally, tannins can react with phospholipids, damaging cell membranes and allowing for the leaking of compounds that inactivate bacterial enzyme systems. Damage to cell membranes prevents the admission of food items or nutrients required for bacteria to make energy, inhibiting bacterial growth and perhaps cell death.

Table 2. The concentration of ethanol extract in tea leaves, along with their tannin content (grams) and the diameter of the inhibition zone (mm) for the growth of *E. coli* bacteria

Test sample	Tannin content (g)	Inhibition zone diameter (mm)
Tea leaf ethanol extract 10%	0.0276	-
Tea leaf ethanol extract 30%	0.0828	10
Tea leaf ethanol extract 50%	0.138	14
Positive control	0.138	20
Negative control	0	-

Note: Positive control: gallic acid 13.8%, Negative control: mobile phase (butanol-acetic acid-water)

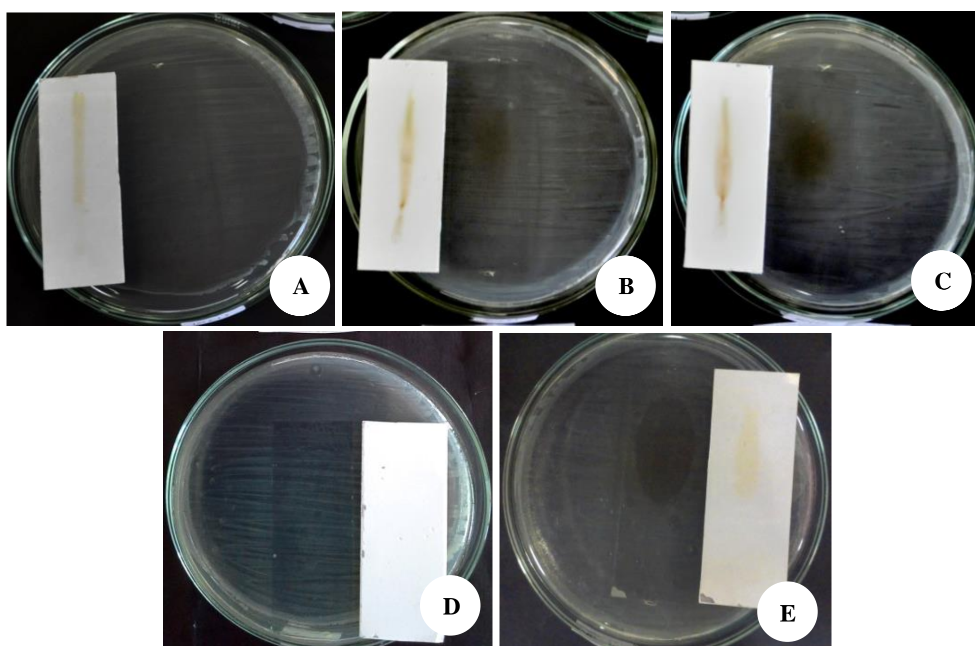

Figure 4. Antibacterial inhibition zone of tea leaf ethanol extract with concentration (A) 10%, (B) 30%, (C) 50%, (D) negative control (butanol: acetic acid: water) and (E) control positive (gallic acid 13.8%)

In comparison to the tannin extract of akway leaves (*Drimys Piperita* Hook.f.), the tannin extract of tea leaves (*C. sinensis*) has greater potential as antibacterial inducing diarrhea. According to Situmorang (2009), akway leaf tannin extract with a 12.62% tannin content inhibits *E. coli* bacteria with a 3.93 mm clear zone diameter. In this investigation, the tannin extract of tea leaves with a tannin level of 13.8 % inhibited *E. coli* bacteria with a clear zone diameter of 14 mm.

To conclude, Tannin compounds in the ethanol extract of tea leaves from the Kemuning plantation have antibacterial activity against *E. coli* bacteria that cause diarrhea. The tannin content in the ethanol extract of tea leaves from the Kemuning plantation is 27.6%. The strongest inhibitory concentration of tannin compounds in the ethanolic extract of tea leaves that could inhibit *E. coli* bacteria was 50%, with a tannin content of 13.8% and an inhibition zone diameter of 14 mm.

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Effect of fermentation duration and $Ca(OH)_2$ concentration for immersion on the characteristics of modified cassava flour (mocaf) of bitter cassava variety (*Pandemir* L-2)

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Abstract. *Kurniawan S, Amanto BS, Utami R. 2019. Effect of fermentation duration and $Ca(OH)_2$ concentration for immersion on the characteristics of modified cassava flour (mocaf) of bitter cassava variety (*Pandemir *L-2). Bioteknologi 16: 53-61.* Due to the high HCN content (>100 ppm) in bitter cassava varieties, a technology is needed to remove the content so that this cassava starch can be used as a food ingredient. One technology to remove HCN is by processing bitter cassava into mocaf (modified cassava flour). Mocaf is cassava flour made by modifying the cassava cell using the fermentation method. The fermentation process of cassava results in flour with a neutral (tend to be aromatic) smell characteristic, soft texture, and whiter color. In addition, the fermented cassava flour also has an advantage over the ordinary cassava flour, including digestibility, viscosity, gelation ability, rehydration power and solvability, the capability of binding water, lower HCN, wide application, easier dispersion to the food product, and easily forming 3 dimensions between the components so that the product consistency become better. The objective of the research is to find out the effect of Calcium Hydroxide $Ca(OH)_2$ and fermentation duration on the chemical (water, acid, protein, HCN levels, and viscosity) and physical (whiteness degree) characteristics of bitter cassava mocaf. The experimental design employed was a Completely Random Design (CRD) with two factors: $Ca(OH)_2$ concentration variation (0%, 5%, 10%, 15%) and fermentation duration (0 hour, 24-hours, 48-hours, 72-hours). The research data was analyzed using ANOVA at a significance level $\alpha = 5\%$ and followed by DMRT with a significance level of 95%. The research results show a higher concentration of Ca(OH)2, lower total acid, protein, and HCN content and higher water containt, viscosity levels, and whiteness degree of mocaf. Longer fermentation duration, higher water content, total acid levels, whiteness degree intensity, and lower HCN content and protein levels of mocaf.

Keywords: $Ca(OH)_2$, fermentation duration, HCN, mocaf, modified cassava flour

INTRODUCTION

Indonesia has tubers that can potentially be a source of carbohydrates and raw materials for local flour, which is equivalent to wheat, namely *canna*, *gembili*, sweet potato, arrowroot, cassava, and so on (Subagyo 2006). Until now, the use of cassava in Indonesia is still very limited. Cassava is generally planted simultaneously at the beginning of the rainy season on dry or rainfed land. Therefore, the harvest time is also simultaneous, from July to September. As a result, overproduction occurs, which is repeated every year so that cassava prices become very low at harvest time. The low price of cassava is also influenced by the nature of fresh cassava, which is easily damaged if post-harvest handling is not immediately carried out due to the high moisture content of fresh cassava, the presence of cyanide acid (HCN), which causes poisoning, the presence of polyphenol compounds that cause browning, and the limited technology of cassava processing. Flour technology is one of the recommended alternative processes for semi-finished products because it is more resistant to storage, easy to mix (made composites), can be enriched with nutrients (fortified), easy to shape, and can be cooked faster according to the demands of modern life which wants practicality.

The cassava processing technology is generally traditional: boiling, frying, *gaplek* making, flouring (cassava flour, *tiwul* flour), and extracting tapioca starch. However, one of the diversification efforts in cassava processing currently being developed is mocaf (modified cassava flour). Mocaf is cassava flour made using the principle of cell modification of cassava by fermentation. The cassava fermentation process produces flour with a characteristic neutral smell (which tends to be fragrant), soft texture, and whiter color. In addition, fermented cassava flour also has advantages over ordinary cassava flour, namely digestibility, viscosity, gelation ability, rehydration and ease of dissolving, water binding ability, lower HCN, wide application, easier dispersion into food products, and easy to form 3 dimensions between components, so that product consistency is better. The technology of the making process of fermented cassava flour was first introduced in West Africa, especially in Nigeria. The flour produced is used as a staple food known as *gari* flour (Wahjuningsih et al. 2009).

However, some cassava contains cyanide compounds that can cause poisoning for those who consume them. Cyanide compounds decompose to produce cyanide acid (HCN), which can inhibit oxygen absorption in the respiratory system, resulting in throat spasms followed by shortness of breath, loss of consciousness, and even death.

The lethal dose of cyanide is 0.5-3.5 mg per kg of body weight. Types of cassava that contain cyanide compounds generally have tightly-packed large tubers with no stem on the tree and contain more starch.

HCN in bitter cassava is a solid material that is more than 100 ppm in size. The process of peeling and washing cassava does not eliminate all HCN toxins. Therefore, to remove the HCN content in cassava, it is necessary to carry out special treatments, including fermentation and adding $Ca(OH)_2$. Hydrogen cyanide present in wood tubers is a weak acid, which, theoretically, an acidic compound can be neutralized with an alkaline solution, which will form salt and water. The addition of alkaline $Ca(OH)_2$ is expected to reduce or eliminate the HCN content in cassava. According to Wahjuningsih (1990), cassava fermentation can help hydrolysis of cyanogenic glucosides and HCN.

Bitter cassava is usually only used as animal feed. However, increasing utilization of bitter cassava can be done by turning it into mocaf. Therefore, it is necessary to research to determine the effect of the addition of $Ca(OH)_2$ and fermentation on the chemical characteristics of the resulting mocaf. In this study, the type of cassava used was *Pandemir* L2 variety with fermentation duration of 0-hour, 24-hours, 48 hours, and 72-hours and used $Ca(OH)_2$ solution with concentrations of 5%, 10%, and 15%. This research is expected to provide scientific information to the public on processing bitter cassava into mocaf as a safe food for consumption.

This study aims (i) to determine the effect of adding calcium hydroxide ($Ca(OH)_2$) to the chemical characteristics (moisture content, acid content, protein, HCN, viscosity) and physical characteristics (degree of whiteness) of mocaf produced from bitter cassava. , (ii) to determine the effect of fermentation duration on chemical characteristics (moisture content, acid content, protein, HCN, viscosity) and physical characteristics (degree of whiteness) of mocaf produced from bitter cassava.

MATERIALS AND METHODS

Materials

This research was conducted at the Food and Nutrition Laboratory of the Faculty of Agriculture, Universitas Sebelas Maret, Surakarta, Central Java, Indonesia, and the Food Laboratory, Universitas Gajah Mada, Yogyakarta, Indonesia.

The main material in this study was cassava of the *Pandemir* L2 variety aged 10-12 months obtained from a farmer from Nglengkong Hamlet, Sendang Village, Purwantoro Sub-district, Wonogiri District, Central Java, Indonesia, with specifications of 10-12 months of age, the water content of 63.2961% w/w, the starch content of 28.875% w/w, HCN content of 186.55 ppm. Meanwhile, the materials for soaking and fermenting are well water (whose hardness has been identified) and a solution of $Ca(OH)_2$ with concentrations of 5%, 10%, and 15%.

Research design

This study used a completely randomized design with two factors, namely the effect of the use of $Ca(OH)_2$ (P) and the effect of variations in fermentation duration (F) and the design was as follows (i) Factor I: Immersion concentration of $Ca(OH)_2$ (P) consisting of 4 levels, namely: P1 = 0%, P2 = 5%, P3 = 10%, P4 = 15%, (ii) Factor II: Fermentation duration (F) consisting of 4 levels, namely: F1 = 0 days, F2 = 1 day, F3 = 2 days, F4 = 3 days, so there would be 16 treatments.

Research procedure

Material preparation

This sample preparation stage begins by sorting cassava according to the freshness state (not deformed/rotten), the size uniformity (not too small), and the color.

Preparation of $Ca(OH)_2$ solution

$Ca(OH)_2$ is dissolved using water (well water of identified hardness). The concentrations of $Ca(OH)_2$ were 5%, 10%, and 15%.

Mocaf making

The process of mocaf making in this study was according to Wahjuningsih et al. (2009). In this study, the manufacture of mocaf was carried out by pretreatment with 5%, 10%, 15% $Ca(OH)_2$ immersion and then followed by the fermentation for 0-hour, 24-hours, 48-hours, and 72-hours.

Mocaf analysis

All mocaf samples were then analyzed chemically (HCN, protein, viscosity, moisture content, acid content) and physically (whiteness). Each analysis was carried out on samples of fermented mocaf at 0, 24-hours, 48 hours, 72-hours, and at immersion concentrations of 5%, 10%, and 15% $Ca(OH)_2$. The methods for analyzing the chemical properties and physical properties of the mocaf are as follows: for the moisture content test using thermogravimetry (Sudarmadji et al. 1997), for the acid level test using titration (AOAC 1995), for the protein content test using the Kjeldahl method (AOAC 1995), for the HCN test using a Spectrophotometer (AOAC 1995), for the Viscosity test using a Stromer viscometer (Fardiaz et al. 1992), for the degree of whiteness using a photometer (Fardiaz et al. 1992).

Data analysis

To determine the effect of each treatment on mocaf, statistical test analysis of variance (ANOVA) was used. If there were a significant difference between treatments, it was continued with Duncan's Multiple Range Test (DMRT) with a significance level of 95%.

RESULTS AND DISCUSSION

Chemical properties of mocaf

Water content (%) db

Water content is one of the most important characteristics of foodstuffs because water can affect the appearance, texture, and taste of foodstuffs. The water content in foodstuffs determines the freshness and durability of these foodstuffs. High water content makes it easier for bacteria, fungi, and yeasts to breed, so food ingredients will change (Winarno 2002). In this study, with the treatment of fermentation duration (0. 24, 48, 72-hours) and immersion concentration (0%, 5%, 10%, 15% $Ca(OH)_2$), the water was calculated as the water content (%) in the material, and it can be seen in Table 1.

The purpose of mocaf water content analysis is to determine the water content in the final product because it relates to the product's resistance to microorganism attacks (Winarno 2002). When the free water content is reduced, the growth of microorganisms can be controlled.

The results of statistical analysis in Table 1 show that the variation of the immersion concentration of 5%, 10%, and 15% $Ca(OH)_2$ give no significantly different effect on the water content of mocaf at 0-hour, 24-hours, 48-hours and 72-hours when compared to the control / 0% $Ca(OH)_2$. It is shown in the mocaf sample with Ca(OH)2 immersion variations having the same notation behind the numbers.

The treatment of fermentation duration in the manufacture of mocaf had a significantly different effect on the water content. Based on Table 1, the length of fermentation duration increases the moisture content of the mocaf. Mocaf samples with 24-hour, 48-hour and 72-hour fermentation on variations of 0%, 5%, 10% and 15% $Ca(OH)_2$ immersion did not show a significant difference, but at 0-hour fermentation on variations of 0%, 5%, 10% and 15% $Ca(OH)_2$ immersion showed significant differences when compared with 72-hours fermentation.

Table 1 shows the water content of mocaf with variations in the $Ca(OH)_2$ immersion treatment and fermentation duration. In this study, the water content of mocaf tends to increase from 0-hour to 72-hours of fermentation, both with the initial treatment of 0%, 5%, 10%, and 15% $Ca(OH)_2$ immersion.

Table 1 shows that the length of fermentation treatment affects the water content of the mocaf. The length of time of fermentation affects the water content of the mocaf. This result is from research carried out by Wahjuningsih et al. (2009), which shows that the water content will increase in proportion to the length of fermentation in the making of *gari*.

Total acid

The degree of acidity is a certain concentration required to neutralize an alkaline solution. According to Kusmanto (2009), microbes that grow during fermentation will produce enzymes that hydrolyze starch into sugar and convert it into organic acids, especially lactic acid. According to Wahjuningsih (1990), microbes that grow during gari fermentation will produce pectinolytic and cellulolytic enzymes that can destroy cassava cell walls so that starch granules' liberation occurs. In this study, with the treatment of fermentation duration (0, 24, 48, 72-hours) and immersion concentration of Ca(OH)2 (0%, 5%, 10%, 15%), the acid content was calculated as the acid content contained in the ingredients. The amount of this content can be seen in Table 2.

Based on Table 2, the total acid content of mocaf with the treatment of fermentation duration and concentration of $Ca(OH)_2$ immersion ranged from 0.11072% to 0.49472%. From the results of statistical analysis, it can be seen that the $Ca(OH)_2$ immersion treatment with concentrations of 5%, 10%, and 15% had no significant effect on acid levels in the mocaf sample but was significantly different from the control / 0% $Ca(OH)_2$ at 0-hour, 24-hours and 48-hours of fermentation. While at 72-hours fermentation, variations of immersion of 5%, 10% and 15% $Ca(OH)_2$ gave no significant effect on the control / 0% $Ca(OH)_2$.

The long fermentation treatment had a significantly different effect on the total acid content of mocaf. Based on Table 2, all treatments of 0%, 5%, 10%, and 15% $Ca(OH)_2$ samples showed a significant increase in total acid after 24-hours of fermentation. The increase in total acid will increase with the longer fermentation. The mocaf sample with variations of 5%, 10%, and 15% $Ca(OH)_2$ immersion at 48-hours of fermentation did not significantly differ from 24-hour fermentation, but 72-hours of fermentation had a significant effect on the total acid content of mocaf. While the mocaf sample with 0% $Ca(OH)_2$ / control immersion will significantly affect fermentation from 24-hours to 72-hours. It can be seen from the different notations on the numbers (Table 2).

Table 2 shows the total acid content of mocaf with variations of $Ca(OH)_2$ immersion treatment and fermentation duration. In this study, the total acid content of mocaf tended to increase from 0-hour fermentation to 72-hours fermentation, both with pre-soaking treatments of 0%, 5%, 10%, and 15% $Ca(OH)_2$. From Table 2, it can be seen that the highest total acid content was in the mocaf within 48-hours of fermentation treatment and 0% $Ca(OH)_2$ immersion or with no immersion, namely, 0.49472. On the contrary, the lowest acid content was in mocaf with 0-hour of fermentation or no fermentation and immersion of 15% $Ca(OH)_2$, namely, 0.11072 (Table 2).

In Table 2, it can be seen that the acid content of the mocaf with 0% $Ca(OH)_2$ immersion treatment was higher than the 5%, 10%, and 15% immersion; it was due to the presence of $Ca(OH)_2$, which is a strong base. When used for immersion, it will inhibit the growth of acid-producing bacteria, and disturb the environmental conditions in the fermentation location, because these acid-producing bacteria can only grow and develop in an acidic environment with a pH range of 3.6. These bacteria have an optimum pH of about 6.5-7.5 (Fardiaz et al. 1992). The process of cassava tuber fermentation produces lactic acid (pH 3.8), and the dominant microbe is lactobacillus (Kobawila et al 2005), so the amount of acid produced will be inversely proportional to the concentration of $Ca(OH)_2$ used in the immersion. $Ca(OH)_2$ solution is also a binder of vegetable acids.

Table 1. Results of water content analysis (%)

Fermentation duration	$Ca(OH)_2$ concentration			
	0% (P1)	5% (P2)	10% (P3)	15% (P4)
0 hour (F1)	7.028[ab]	7.023[ab]	7.016[a]	7.015[a]
24-hours (F2)	7.186[abc]	7.188[abc]	7.185[abc]	7.185[abc]
48-hours (F3)	7.227[c]	7.198[abc]	7.208[bc]	7.214[c]
72-hours (F4)	7.327[c]	7.261[c]	7.279[c]	7.283[c]

Note: *) different notation indicates significant difference ($p < 0.05$)

Table 2. Results of analysis of total acid levels (%)

Fermentation duration	$Ca(OH)_2$ concentration			
	0% (P1)	5% (P2)	10% (P3)	15% (P4)
0 hour (F1)	0.151[b]	0.114[a]	0.111[a]	0.110[a]
24-hours (F2)	0.406[d]	0.334[c]	0.332[c]	0.332[c]
48-hours (F3)	0.494[f]	0.363[c]	0.358[c]	0.353[c]
72-hours (F4)	0.458[e]	0.469[ef]	0.436[de]	0.435[de]

Note: *) different notation indicates significant difference ($p < 0.05$)

Table 2 shows that the length of fermentation will increase the acid content of mocaf. EL Tinay et al. (1984) state that fermented bitter cassava resulted in a decrease in pH from 6.0 to 3.8 and an increase in acidity from 0.111% to 0.802% during 192 hours (8 days) fermentation. Gari usually only undergoes fermentation for approximately 96 hours (4 days), so the pH reaches around 4.75, and the acidity reaches 0.422%.

Wahjuningsih (1990) added that the gari flavor was due to lactic acid produced in the first stage of fermentation. In contrast, ketones and aldehydes were produced in the second stage. Also mentioned by Akinrele in Wahjuningsih (1990) is that two types of organic acids have been identified in cassava fermentation, namely lactic acid, and formic acid, but only lactic acid is dominant in mocaf. It is due to the breakdown of formic acid to form carbon dioxide and possibly hydrogen. The gas will cause an anaerobic atmosphere on the substrate. Further research has found lactic, oxalic, and succinic acids in *gari*. However, lactic acid is still dominant in the *gari* (Dougan et al. 1983).

Furia (1980) stated that lactic acid is not a volatile acid, so it can be assumed that the low acidity of mocaf produced by drying can be caused by evaporation of other organic acid components contained in mocaf. Dougan et al. (1983) added that lactic acid (CH_3CH (OH)COOH), succinic acid ($HOOCH_2C$-CH_2COOH), and oxalic acid (HOOCCOOH) in mocaf were 0.4%, 0.04%, and 0.04% respectively. Oxalic acid begins to evaporate at temperatures below 100ºC (Kirk and Othmer 1967), while succinic acid evaporates at room temperature (Furia 1980), and lactic acid has a boiling point of 122ºC (Anonymous 2010).

Lactobacillus plantarum bacteria produce lactic acid, which becomes dominant after 48-hours of fermentation. On the other hand, oxalic acid is known to be produced by a fungus of the type *Geotricum,* which appears after 3 days of fermentation. In mocaf fermentation, sooner or later, this acid becomes anaerobic. Under these conditions, oxalic acid can be metabolized and depleted as the fermentation continues, so only a small amount is left in the mocaf. Likewise, succinic acid is produced from the metabolic process of fungus (Wahjuningsih 1990).

Further research conducted by Ejiofor and Okafor (1980) stated that it was possible to identify microorganisms that play a role in *gari* fermentation, namely *Leuconostoc*, *Lactobacillus*, *Bacillus* sp., and *Geotrichum* sp. *Corynebacterium* manihot will break down starch into glucose in the early stages of fermentation. Then lactic acid bacteria will convert glucose into lactic acid and other organic acids, aldehydes, and ketones, resulting in the distinctive aroma of *gari*. *Leuconostoc* sp. can be isolated as soon as fermentation begins, and the amount continues to increase until 72-hours of fermentation. After that, the growth was reduced, and no growth was observed after 96 hours of fermentation.

Protein level

In this study, the protein content determination test was carried out using the Kjeldahl method to determine the total protein content, which was calculated as total N. The total protein content of mocaf with variations in fermentation treatment and immersion concentration with $Ca(OH)_2$ can be seen as in Table 3.

Table 3 shows that the treatment variation of the fermentation duration and the concentration of $Ca(OH)_2$ immersion in mocaf gave various effects on the protein content, expressed as N-total. From these data, it can be seen that the protein content of mocaf with various treatment variations of fermentation duration and $Ca(OH)_2$ immersion concentration ranged from 0.9306% to 2.3610% (Table 3).

Immersion treatment with 5%, 10%, and 15% $Ca(OH)_2$ at 0-hour, 24-hours, 48 hours, and 72-hours had a significant effect on the total protein content of mocaf samples when compared to the control (0 % $Ca(OH)_2$).

While the 5%, 10%, and 15% $Ca(OH)_2$ treatment variations did not have a significantly different effect on 0-hour, 24-hours, 48 hours, and 72-hours of fermentation. It is shown in Table 3. The protein content of mocaf samples soaked with 0%, 5%, 10%, and 15% $Ca(OH)_2$ at 0-hour fermentation were 2.3610%; 1.5646%; 1.5528%; 1.5239%, respectively.

The long fermentation treatment had a significantly different effect on the total protein content of the mocaf sample. Long fermentation will lower the protein content of mocaf. Table 3 showed that the protein content of mocaf decreased significantly at the 24 hour fermentation when compared to the control in all samples, namely, 1.5229% (0% $Ca(OH)_2$); 1.3719% (5% $Ca(OH)_2$); 1.3707% (10% $Ca(OH)_2$); 1.3547% (15% $Ca(OH)_2$), respectively. The decrease in protein content occurred along with the increase in fermentation duration. A significant decrease in protein occurred up to 72-hours of fermentation in all samples of 0%, 5%, 10%, and 15% $Ca(OH)_2$. Until 72-hours of fermentation, total protein content in mocaf in sample of 0% $Ca(OH)_2$ was 1.2950%, 5% $Ca(OH)_2$ was 0.9285%, 10% $Ca(OH)_2$ was 0.9314%, 15% $Ca(OH)_2$ was 0.9306%.

Table 3 indicates the total protein content of mocaf decreased in line with fermentation. Wahjuningsih (1990) stated that the protein content of fresh cassava is low. In this study of making mocaf, the value of the protein content produced was also low. In this study, the highest total protein content was shown by mocaf with 0-hour fermentation treatment and 0% $Ca(OH)_2$ immersion or control, which was 2.3610%. Meanwhile, the lowest total protein content was shown by mocaf with 72-hours fermentation treatment and 15% Ca(OH)2 immersion, which was 0.9306% (Table 3).

In this study, a protein content determination test was carried out using the Kjeldahl method to determine the total protein content, calculated as total N. The protein content of mocaf was related to the amount of HCN. Therefore, the smaller the HCN mocaf, the smaller the protein content. Therefore, the N element in HCN will decrease along with the length of fermentation treatment and the variation of $Ca(OH)_2$ immersion.

The fermentation in the manufacture of mocaf is a wet fermentation that uses water as the medium. According to Hidayat (2009), most types of protein can be dissolved in water, especially methionine. Cassava is an energy source rich in carbohydrates but low in protein. The source of protein found in cassava is the amino acid methionine (Panggih 2009). Based on this description, soaking cassava with water can reduce protein content because the type of protein in cassava can dissolve in water.

According to Ezeala (1984), *gari* fermentation can cause a protein reduction of approximately 3%. It is proven by the low value of protein content in the fermentation treatment of 24-hours, 48 hours, and 72-hours and by immersing 5%, 10%, and 15% $Ca(OH)_2$. Even the presence of immersion during the fermentation process greatly influences the reduction of protein content.

HCN Level

HCN is naturally found in cassava as a cyanogenic glycoside. Cyanogenic glycosides are potentially toxic compounds because they can decompose and produce HCN. The cyanogenic glycoside found in cassava is called Linamarin with the chemical name acetone cyanohydrin glycoside (Winarno 2002). Meanwhile, according to Waspodo (1980), cyanogenic glycosides in cassava are linamarin and lotaustralin, with 93% and 7% of the total cyanogenic compound content. Mocaf HCN levels with treatment variations in fermentation and immersion concentration with $Ca(OH)_2$ can be seen in Table 4.

Table 3. Results of analysis of mocaf protein content (%) with various treatments

Fermentation duration	$Ca(OH)_2$ Concentration			
	0% (P1)	5% (P2)	10% (P3)	15% (P4)
0 hour (F1)	2.36^{f}	1.56^{e}	1.55^{e}	1.52^{e}
24-hours (F2)	1.52^{e}	1.37^{cd}	1.37^{d}	1.35^{cd}
48-hours (F3)	1.41^{d}	1.29^{bc}	1.28^{b}	1.27^{b}
72-hours (F4)	1.29^{bc}	0.92^{a}	0.93^{a}	0.93^{a}

Note: *) different notation indicates significant difference ($p < 0.05$)

Table 4. Analysis results of HCN levels (ppm) on mocaf with various treatments

Fermentation duration	$Ca(OH)_2$ Concentration			
	0% (P1)	5% (P2)	10% (P3)	15% (P4)
0 hour (F1)	91.97^{j}	62.63^{i}	50.76^{gh}	47.80^{fg}
24-hours (F2)	65.79^{i}	55.55^{h}	40.20^{de}	37.04^{cd}
48-hours (F3)	47.78^{fg}	42.72^{ef}	34.40^{bcd}	31.69^{bc}
72-hours (F4)	43.09^{ef}	31.69^{bc}	23.91^{a}	19.18^{a}

Note: *) different notation indicates significant difference ($p < 0.05$)

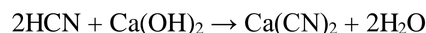
Based on Table 4, it can be seen that the immersion treatment with $Ca(OH)_2$ in the manufacture of mocaf has a significantly different effect on the HCN content when compared to the control. It can be seen from the different notations behind the HCN numbers (Table 4). The high concentration of $Ca(OH)_2$ will lower the HCN content of mocaf. Table 4, showed that HCN levels on mocaf with 0-hour fermentation at immersion of 0%, 5 %, 10 %, 15% $Ca(OH)_2$ concentrations were 91.97 ppm, 62.6349 ppm, 50.7659 ppm, 47.8048 ppm, respectively. A significant decrease in HCN occurred at 5%, 10% and 15% $Ca(OH)_2$ concentrations when compared with 0% $Ca(OH)_2$ concentrations, while at 10% $Ca(OH)_2$ and 15% $Ca(OH)_2$ concentrations was not significantly different in HCN levels.

On the contrary, the length of fermentation treatment also significantly affected the HCN levels of mocaf. HCN levels of mocaf also decreased along with the length of fermentation compared to the control. In 0% $Ca(OH)_2$ treatment, HCN decreased significantly and significantly differed during the 24-hour fermentation compared to 0-hour fermentation. Meanwhile, at 48-hour and 72-hour fermentation, the decrease in HCN was not significantly different, but when compared with control or 0-hour fermentation and 24-hours fermentation, there was a significant difference. In 5% $Ca(OH)_2$ treatment, HCN will experience a significant decrease and give a significant difference in 24-hour, 48-hour, and 72-hour fermentation compared to control / 0-hour fermentation. This condition is shown in Table 4.

Table 4 indicates that in this study, the 10% $Ca(OH)_2$ treatment did not give a significant difference at 0-hour, 24-hours, 48 hours, and 72-hours of fermentation compared to the 15% $Ca(OH)_2$ treatment. The 24-hour fermentation treatment compared to 48-hour fermentation in 10% and 15% $Ca(OH)_2$ immersion did not give a significant difference but gave a significant difference with 0-hour and 72-hour fermentation.

Table 4 shows that the HCN content of mocaf with treatment variations of $Ca(OH)_2$ immersion and fermentation duration tends to experience a significant decrease from 0-hour fermentation to 72-hours fermentation, with the initial treatment of 0%, 5%, 10%, 15% $Ca(OH)_2$ immersion (Table 4). Based on Table 4, it can be concluded that the length of fermentation duration will reduce the HCN level of mocaf. Table 4 shows that the duration of fermentation has a significantly different effect from 0-hour fermentation to 72-hours fermentation. Based on Table 4, the level of HCN at the treatment of 72-hours fermentation with 0%, 5%, 10%, and 15% $Ca(OH)_2$ immersion was 43.0917 ppm; 31.6915 ppm; 23.9138 ppm; 19.1809 ppm, respectively.

The decrease in HCN levels in this mocaf study is in accordance with Wahjuningsih et al. (2009) that hydrogen cyanide is an acid that is easily soluble in water so that it can be reduced. In addition, cyanide compounds will be reduced if they react with one of the basic compounds, for example, calcium hydroxide ($Ca(OH)_2$). This is because it will cause the CN- ions in the HCN structure to bind with calcium hydroxide to form a complex salt, i.e., the cyanide salt. For example, the following is the reaction of the HCN compound when reacted with $Ca(OH)_2$ to form $Ca(CN)_2$:



$$2HCN + Ca(OH)_2 \rightarrow Ca(CN)_2 + 2H_2O$$

Lactic acid fermentation, commonly used in processing cassava products, also aids in the hydrolysis of cyanogenic sugar glucosides and HCN and can be removed further in processing by heating (Akingbala et al. 2005). Winarno (2002) revealed that the processing of bitter cassava is in the form of drying, soaking before cooking, or fermentation for several days. With this treatment, much linamarin was damaged, and the HCN content dropped to 10 to 40 mg/kg of peeled cassava. HCN is easily removed by boiling as long as the lid of the pot is not tightly closed. With heating, the linamarin cleavage enzyme becomes inactive so that HCN is not formed.

Bourdoux et al. (1982) also stated that soaking cassava for one day will reduce HCN levels by 45%; if it is continued for 96-hours (4 days), HCN levels drop to 90%, and if continued for five days, HCN levels will disappear 100%, but the cassava will rot. EL Tinay et al. (1984) in Wahjuningsih (1990) stated that fermentation primarily aimed to reduce or eliminate HCN levels from cassava at low pH.

According to Wahjuningsih (1990), the allowable HCN content in traditional *gari* is a maximum of 19 ppm. However, according to FAO, cassava with a maximum HCN content of 50 ppm is still safe for human consumption (Winarno 2002). Therefore, the mocaf in this study is safe for human consumption.

Viscosity

Viscosity is one of the most important physical properties of flour. Viscosity is the internal frictional force that occurs in a fluid or fluids. The purpose of the viscosity test was to determine the level of mocaf viscosity. Based on the standard method (FAO 1990) cited in Samsuari (2006), flour viscosity was measured at 29ºC with a concentration of 2%. Mocaf viscosity was measured using a stormer viscometer. Table 5 exhibits the viscosity of mocaf samples with variations in fermentation duration and $Ca(OH)_2$ immersion concentration ranging from 19.5817 to 26.0733 c poise.

From the statistical analysis results, it can be seen that the immersion treatment with $Ca(OH)_2$ had a significantly different effect on the viscosity of the mocaf sample. It can be seen from the different notations behind the viscosity numbers (Table 5). The high concentration of $Ca(OH)_2$ will increase the viscosity of the mocaf. Table 5 shows that the fermentation treatments at 0-hour, 24-hours and 48-hours with 0% $Ca(OH)_2$ and 5% $Ca(OH)_2$ immersion did not significantly affect the viscosity in mocaf but were significantly different at 72-hours fermentation.

On the contrary, the 10% $Ca(OH)_2$ immersion treatment compared to the 15% $Ca(OH)_2$ immersion treatment mostly differed significantly. The 0-hour fermentation treatment at 10% $Ca(OH)_2$ immersion compared to 15% $Ca(OH)_2$ immersion did not significantly differ, but it would

significantly differ in the 24-hour, 48-hour, and 72-hour fermentation.

The long fermentation treatment also had a significantly different effect on the mocaf. The length of fermentation will make the viscosity higher. It can be seen in Table 5 that compared to 0-hour fermentation, the 24-hours fermentation had a significantly different effect on the 0%, 5%, and 15% $Ca(OH)_2$ treatments except for the 10% $Ca(OH)_2$ treatment. Compared to the 48-hours fermentation, at the 24-hours fermentation, samples with the 5%, 10%, and 15% $Ca(OH)_2$ immersion treatment did not have a significantly different effect, except for the samples with 0% $Ca(OH)_2$ immersion treatment which gave a different effect. Compared with 72-hours of fermentation, 24-hours of fermentation gave significantly different effects on the $Ca(OH)_2$ treatment of 0%, 5%, 10%, and not significantly different from the 15% $Ca(OH)_2$ treatment.

Table 5 shows that $Ca(OH)_2$ immersion treatment will increase the viscosity of mocaf. In this study, the increase in viscosity value will be directly proportional to the immersion treatment with $Ca(OH)_2$. It is because the starch content in cassava tubers, especially amylopectin, will bind to calcium ions and function to strengthen the cell wall structure. Calcium's ability to form insoluble complexes with amylopectin and free carboxyl groups on the amylopectin chain will form a strong formation so that the viscosity value of the mocaf sample will increase.

Table 5 also shows that the viscosity of mocaf with variations in the length of fermentation treatment will increase. In this study, the viscosity of mocaf tended to increase significantly at 0-hour fermentation to 72-hours fermentation, both with the initial treatment of 0%, 5%, 10%, and 15% $Ca(OH)_2$ immersion (Table 5). In addition, it is shown in Table 5 that the viscosity at 0-hour, 24-hours, 48 hours, and 72-hours of fermentation experienced a significant increase at the immersion of 10% $Ca(OH)_2$ concentration.

Wahjuningsih et al. (2009) stated that adding water to the fermentation will make the starch molecules absorb water to break the amylose crystals and break the structural bonds of the molecules. As a result, amylose will begin to diffuse out of the tissue, eventually, the tissue only consists of mostly amylopectin (Harper 1981). Winarno (2002) as well as Widaningrum and Purwani (2006) stated that the amylose content of a food ingredient has an effect on its amylograph properties.

In Table 5, it can be seen that the fermentation treatment has a major influence on the viscosity of the mocaf. It can be seen that the longer the fermentation is carried out, the higher the viscosity value produced (Table 5). It is due to the presence of fermentation, microbes that grow during fermentation will produce pectinolytic and cellulolytic enzymes that can destroy cassava cell walls in such a way that starch granules liberation occurs. This liberation process will cause changes in the characteristics of the flour produced in the form of an increase in viscosity, gelation ability, rehydration power and dissolving easiness (Wahjuningsih 1990).

As a glucose polymer, amylose and amylopectin are the two largest components of starch. Amylose has a linear structure with 1,4-α-D-glucoside bonds and forms the amorphous part of starch, while amylopectin has branched chains that meet with linear chains at 1,6-α-D-glucoside bonds and forms the crystalline part of starch. The composition of amylose and amylopectin is different for each type of starch which also affects the characteristics of starch (Belitz and Grosch 1999; McWilliams 2001).

In the presence of fermentation, microbes will degrade cell walls which cause damage to the structure and integrity of starch granules. Damage to the integrity of starch causes starch granules to absorb water so that some fractions separate and enter the medium (Greenwood 1979). Starch tends to absorb more water with either the smaller the amylose content or the higher the amylopectin content (Tjokroadikusoemo 1986). With this description, the starch fractions in cassava, amylose and amylopectin will also be damaged due to fermentation and water used for the fermentation media. This causes the amylose fraction to be dissolved in water and the amylopectin fraction to be insoluble in water. With the release of the amylose fraction from the medium, the content of the amylopectin fraction increased.

The starch composition, which mostly consists of amylopectin makes the starch structure more open so that water will more easily enter, penetrate the starch granules and cause the starch granules to swell (swollen), which is indicated by the increasing value of viscosity. According to Wuzburg, the presence of branching in amylopectin will hinder the movement and tendency to approach each other in forming hydrogen bonds. It causes amylopectin to be more stable and more resistant to changes than amylose. It affects the viscosity of the mocaf, namely the more amylose that comes out, the greater the viscosity.

Table 5. Results of mocaf viscosity analysis (c poise) with various treatments

Fermentation duration	$Ca(OH)_2$ Concentration			
	0% (P1)	5% (P2)	10% (P3)	15% (P4)
0 hour (F1)	19.58^{a}	19.85^{a}	23.86^{f}	24.33^{fg}
24-hours (F2)	20.81^{b}	21.19^{bc}	24.20^{f}	25.76^{h}
48-hours (F3)	21.52^{cd}	21.69^{cd}	24.07^{f}	25.98^{h}
72-hours (F4)	21.91^{d}	22.72^{e}	24.80^{g}	26.07^{h}

Note: *) different notation indicates significant difference ($p < 0.05$)

According to Subagyo (2006), when compared to tapioca starch, the viscosity of mocaf is lower. It is because, in tapioca, the starch component covers almost all of the dry matter, while in mocaf, the components other than starch are still in significant amounts. However, within 72-hours of fermentation, a mocaf product with a viscosity close to tapioca will be obtained. Therefore, it can be understood that, with long fermentation, more and more cassava cells will be broken so that the starch granule liberation becomes very extensive.

Physical properties of mocaf

Whiteness level

The whiteness level of flour depends on the basic ingredients that are processed. The color of the *Pandemir* L-2 cassava variety was classified as white so that the whiteness value was higher. In general, consumers prefer white flour.

In this study, the whiteness level on the mocaf was tested using the L*a*b* system using the *Color Reader CR-100* (Minolta, Japan), to determine the intensity of the color produced by mocaf with variations in fermentation duration and immersion concentration with $Ca(OH)_2$ which can be seen in Table 6. In Table 6, the whiteness level of the mocaf samples with variations in fermentation duration and immersion concentration with $Ca(OH)_2$ ranged from 85.195 - 90.1567 (Table 6).

From the results of statistical analysis showed that the treatment of soaking $Ca(OH)_2$ with different concentrations had an effect on the degree of whiteness of the mocaf (Table 6). This is shown in samples with $Ca(OH)_2$ immersion with different concentrations having different notations behind the numbers on the degree of whiteness. The concentration of $Ca(OH)_2$ used will increase the degree of whiteness of the mocaf. This can be seen in Table 6. Mocaf soaked with $Ca(OH)_2$ at a concentration of 5%, 10%, 15%, when compared to the control, the degree of whiteness was greater, namely: 85.1950 for 0% $Ca(OH)_2$, 86.5717 for 5 % $Ca(OH)_2$, 87.0867 for 10% $Ca(OH)_2$, 87.2300 for 15 % $Ca(OH)_2$, respectively (Table 6).

Table 6 shows that 5% $Ca(OH)_2$ treatment, when compared with 10% $Ca(OH)_2$ treatment, mostly showed significant differences. Compared to 10% $Ca(OH)_2$ treatment, the 5% $Ca(OH)_2$ treatment at 0-hour, 24-hours, and 72-hours gave a significant difference, except for 48-hours fermentation, which did not give a significant difference. On the contrary, compared to 15% $Ca(OH)_2$ treatment, the 10% $Ca(OH)_2$ treatment did not have a significant effect on 0-hour, 24-hour and 48-hour fermentation, except for 72-hour fermentation which gave a significant difference.

In Table 6, it is shown that the length of fermentation also has a significantly different effect on the mocaf sample. The length of fermentation will increase the degree of whiteness produced. When compared with the 24-hour fermentation, the 0% $Ca(OH)_2$ treatment with 0-hour fermentation was significantly different, namely 85.9517 for 24-hour fermentation and 85.1950 for 0-hour fermentation, respectively. On the other hand, when compared with control / 0-hour fermentation, the treatment of 5%, 10%, and 15% $Ca(OH)_2$ at 24-hour fermentation was not significantly different. It is shown in Table 6 by the same notation behind the number on the intensity of the whiteness level.

Compared to 24-hours fermentation, the 0% and 5% $Ca(OH)_2$ treatments at 48-hours fermentation gave a significant difference, meanwhile the 10% and 15% $Ca(OH)_2$ treatments at 48-hours fermentation compared to 24-hours fermentation did not give any significant difference. Meanwhile, the 48-hour and 72-hour fermentation for all treatments of 0%, 5%, 10%, and 15% $Ca(OH)_2$ gave significantly different effects.

Table 6 depicts the degree of whiteness of the mocaf with variations in the $Ca(OH)_2$ immersion treatment and the duration of fermentation. In this study, the degree of whiteness of mocaf tended to increase significantly (Table 6) from the 0-hour to 72-hour fermentation, both with the initial treatment of soaking 0%, 5%, 10%, 15% $Ca(OH)_2$. The highest degree of whiteness was in the mocaf sample immersed with 15% $Ca(OH)_2$ and fermented for 72-hours at 90.1567. While the intensity of the lowest level of whiteness was in the mocaf without the treatment of $Ca(OH)_2$ immersion and fermentation (control) at 85.1950 (Table 6).

The duration of immersion in the $Ca(OH)_2$ solution has an effect on increasing the color quality of the flour produced. This means that the longer the $Ca(OH)_2$ solution is immersed, the clearer the resulting color will be. Chemically, quicklime solution emits a lot of heat, is alkaline, and easily attracts carbon dioxide gas from the air, so that the water is easily cloudy. It causes the color pigments in the material to dissolve in the $Ca(OH)_2$ solution. Thus the soaked cassava becomes clear and will allow the resulting mocaf to be white.

Table 6. Whiteness levels of mocaf

Fermentation duration	$Ca(OH)_2$ Concentration			
	0% (P1)	5% (P2)	10% (P3)	15% (P4)
0 hour (F1)	85.19^{a}	86.57^{c}	87.08^{def}	87.23^{efg}
24-hours (F2)	85.95^{b}	86.82^{cde}	87.44^{fgh}	87.52^{fghi}
48-hours (F3)	86.63^{cd}	87.37^{fg}	87.70^{ghi}	88.01^{i}
72-hours (F4)	87.36^{fg}	87.88^{hi}	88.72^{j}	90.15^{k}

Note: *) different notation indicates significant difference ($p < 0.05$); L = brightness of color, L (0) = dark, L (100) = bright

In Wahjuningsih et al. (2009), it is stated that the fermentation in making *gari* uses the wet method, meaning that when the fermentation takes place, soaking in water will prevent the material from browning. According to Kusmanto (2009), the protein content in cassava flour can cause a brown color when drying or heating. Cassava is an energy source rich in carbohydrates but low in protein. Based on this description, soaking cassava with water can reduce protein content because the type of protein contained in cassava can dissolve in water. From this description, the protein content of the ingredients greatly affects the whiteness of the mocaf.

In the manufacture of flour products, the needed cassava is the one that does not contain much protein because flour containing more than 2% protein will become less white in color and has a "musty" smell and cannot be stored for a longer period of time. From this description, the protein content of the ingredients greatly affects the whiteness of the mocaf. Subagyo (2006) stated that the bright color of the mocaf was due to the absence of a protein hydrolysis process, so the browning caused by the Mailard reaction did not take place intensively. While the mocaf without fermentation treatment has a lower color intensity, this is due to the hydrolysis process so that the protease enzyme breaks the peptide bond to produce an amine group which is the Mailard reaction material, where in this condition, the amine group of the protein reacts with the aldehyde or ketone group of reducing sugar to produce a brown color (Subagyo 2006).

The conclusions from the research on the effect of fermentation duration and concentration of $Ca(OH)_2$ for immersion on the characteristics of mocaf (modified cassava flour) of bitter cassava variety (*Pandemir* L2) are as follows: (i) Treatment variation of fermentation duration (0-hour, 24-hours, 48-hours, and 72-hours) and immersion with $Ca(OH)_2$ (0%, 5%, 10%, 15%) had an effect on the chemical properties of mocaf. The higher the $Ca(OH)_2$ concentration, the lower the water content, viscosity, acidity, HCN and protein content. The longer the fermentation, the higher the water content, acid content and viscosity, but the HCN and protein content decreased. (ii) Variations in the treatment of fermentation duration (0-hour, 24-hours, 48-hours, and 72-hours) and $Ca(OH)_2$ immersion (0%, 5%, 10%, 15%) affected the physical properties of mocaf. The high concentration of $Ca(OH)_2$ and the length of fermentation increased the whiteness level of the mocaf.

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Xylanolytic and cellulolytic enzymes extracellular characterization on organic waste degrading bacteria

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Abstract. *Ochieng OR, Njagi EN, Orinda GO, Otieno MF. 2019. Xylanolytic and cellulolytic enzymes extracellular characterization on organic waste degrading bacteria. Bioteknologi 16: 62-73.* The disposal of untreated municipal wastes is a global problem that challenges public and environmental health. In Kenya, composting, a microbial process with minimal environmental impact, has been given very little attention as an alternative waste treatment technology. Although bacteria can easily be genetically manipulated and therefore are good candidates for industrial use, very few have been researched. Furthermore, the optimum conditions for biodegrading solid wastes are not well understood. This study aimed at extracting and characterizing the biodegradative enzymes from 15 bacterial isolates obtained from dumpsite soil. The optimum period for extracellular enzyme production, pH, temperature, and the influence of selected ions on the activities of the enzymes was studied. The isolates were grown in four different growth media, and their enzymes were purified using ammonium sulphate precipitation. The substrate utilization pattern of the individual isolates was investigated using spectroscopic methods. Most of the isolates were bacterial of the genusbacillus. All the isolates were secreting extracellular proteins into their growth media. Most isolates had optimum protein production between 48-96 hours. Enzymes produced by most isolates were acting on all three commercial substrates (CM- cellulose, xylan, and cellobiose) [P>3.23 (F=3.88)]. Cellulases, xylanases and cellobioses had activities at broad temperature ranges 27ºC ([P< 3.23 (F=0.21)], 50ºC [P<3.23(F=0.14)] and 80ºC [P<3.23 (F=0.06)], respectively). From the study, cellulases and cellobioses were acting within a large pH range (pH 4.8) (P> 3.23 (F=3.24] and (pH 6.8) [P>3.23 (F= 6.33)], respectively, while xylanases were acting within a narrow pH range (pH 4.8) [P< 3.23 (F=1.91]). Magnesium and calcium ions had a stimulatory effect on cellulases and cellobioses, while Silver and Copper ions inhibited the enzymes. *Bacillus clausii* (293), *Bacillus* sp. NER (117) and *Bacillus* sp. CSS-8 strain (108) had highactivities on all the substrates and biodegrades at high pH and temperature ranges and canbe used to facilitate biodegradation of waste when used with the correct ion concentration.

Keywords: Composting, degrading bacteria, extracellular cellulolytic, organic waste, xylanolytic enzyme

INTRODUCTION

Due to urbanization, rapid industrialization, and economic development, the population grows with the generation of large quantities of heterogeneous solid waste. Sustainable management of solid waste is a major challenge faced by municipal authorities worldwide, both in the North and South. In developing countries, the urban waste remains a serious problem that causes contamination of soil and water bodies and endangers human health and the environment. In Africa, Asia, and Latin America, sustainable waste management is a major challenge for municipal authorities (Qizilbash 2002). Municipal authorities have insufficient financial, technical, and institutional capacities to collect, transport, and safely treat and dispose of municipal wastes.Consequently, waste management remains one of the major urban problems (Drechsel and Kunze 2001). In Ghana, for example, 58 percent of solid waste generatedis dumped by households in designated dumping sites, 25 percent is dumped elsewhere in non-designated sites, and only 5 percent s actually collected (Asumani-Boateng and Haight 1999). The situation in other African cities is hardly different. In many city households, waste collection is restricted to wealthy neighborhoods, while in the remaining areas, wastes are dumped along roadsides, illegal dumps, and in stormwater drains (Mbuyi 1989). The city authorities in Tanzania collect only 24 percent of the refuse, while in Nigeria, 35 percent of Ibadan's households, 33 percent of Kaduna's, and44 percent of Enugu's do not have access to the waste collection (Asumani-Boateng and Haight 1999). In India, about 50 percent of the refuse generated is collected. As much as 90 percent of the MSW collected in Asian cities end up in open dumps (Medina 2002).

The situation in Kenya's capital city, Nairobi, is no exception. A report by NEMA reveals that Nairobi generates approximately 2000 tonnes of daily waste. Of this, 68% is household waste (East African Standard 2004). About 50 percent of the Nairobi residents live below the poverty line and are concentrated in Peri-urban and slum areas characterized by limited amenities and unhygienic living conditions (Republic of Kenya 2001b). Such rapid urban growth has caused deterioration of the city's solid waste management services, resulting in environmental pollution (Kibwage 2002). The Nairobi City Council, the legal authority responsible for waste management, has no capacity because only 40 percent of the amount of solid waste generated by the city is collected and disposed of. This poor state of solid waste management services in Nairobi is attributed to insufficient financial outlays, a

shortage of equipment, and unfavorable institutional and organizational arrangements. Furthermore, there is an absence of a systematic and integrated approach to tackling the waste management problem. The attitudes of poorer city residents towards environmental cleanliness are also a contributing factor (Slavin et al. 1981). An urgent need exists for new methods of waste handling and promoting fuller environmental awareness.

As a city with critical waste management problems and a burgeoning informal sector, Nairobi possesses both the need and potential for an innovative approach to its waste problems. Composting technology, in contrast to the above-mentioned conventional techniques, has been shown, where feasible, as cost-effective and environmentally friendly. The process is best achieved by providing optimal conditions for the microorganisms, primarily fungi, and bacteria, through the best combination of air, moisture, temperature, and organic materials (Taylor 2003). Although bacteria are considered easier to manipulate genetically than fungi, most researchers are biased toward the latter. Their ability to secrete extracellular enzymes into their growth media is little known. Moreover, theincubation period the microorganisms require for maximum enzyme secretion is largely unknown. Although enzymes are known to be substrate-specific, several bacteria have been found to secrete enzymes that degrade more than one substrate. Determination of such bacteria would therefore be of great benefit for the selection of decomposers. Further, the natural conditions under which the bacteria biodegrade solid wastes at the dumping sites are largely uncontrolled. The rapid pH and temperature changes witnessed under such conditions influence the rate of enzymatic activities and need to be fully investigated. Similarly, there is a need to understand the role of ions that forms the ecosystem where the biodegrades thrive. Some of these ions may directly affect the enzyme's active site, whereas others may cause mutations in the organisms. Based on the strength of these limitations, the study was conducted to determine the most appropriate organism and the best conditions for biodegradation of solid organic wastes. The study will therefore contribute to the millennium development goal number 7, which aims at "ensuring environmental sustainability."

MATERIALS AND METHODS

Collection, isolation, and identification of bacteria

Two sets of soil samples were collected from dumping sites at Uthiru and Dandora of Nairobi, Kenya. The soils were stored at room temperature in axenic conditions and protected from direct sun and light until the isolation was performed. Bacteria were extracted by suspending 1 g of soil sample in 20 mL saline solution, Ringer (2.25 g NaCl, 0.105 g KCl, 0.12 g $CaCl_2$ per liter, pH 7.2, ADSA Micro, Pharmafaster SA, Barcelona, Spain). After 10min vigorous stirring and additional sedimentation, two samples of 8 mL of the aqueous phase were collected. One of the samples was treated for 10 mins at 80°C to isolate only spore-forming bacteria. The other sample remained untreated to isolate both spore-forming and non-spore-forming micro-organisms (Ruiz et al. 2005).

These samples were used to isolate aerobic and facultative micro-organisms by serial dilutions. 0.1 mL of each dilution was spread on agar plates containing four different culture media, and the resulting plates were incubated in duplicate at different temperatures (27°C, 42°C, and 50°C). The four culture media used were: Luria Bertani (LB) agar plates [10 g bactotryptone, 5 g yeast extract,10 g NaCl, 15 g agar per liter, pH7.0]; Nutrient broth(NB) agar plates [5 g peptone, 2 g yeast extract, 5 g NaCl, 1 g meat extract, 15 g agar, per liter, pH 7.4]; Horikoshi-1- Agar (H1A) plates [10 g D(+) glucose, 5 g yeast extract, 5 g bactotryptone,1 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 10 g $NaCO_3$, 20 g agar per liter, pH 7.0]; and polycarboxymethyl Sodium salt (PCS) plates [10 g Cellulosic material-Sodium salt, 2.5 g NaCl, 2.5 g $CaCO_3$, 2.5 g peptone, 2.5 g yeast extract, 10 g agar, per liter, pH 8] (Sorano et al. 2000).

A total of 308 bacterial colonies were isolated. Identification of the colonies was performed morphologically and physiologically. Further identification based on full 16S rRNA sequence length was made to ascertain the safety of the isolates (Shirai et al. 1997). The fifteen safe bacterial isolates with high hydrolytic activities on the solid media considered for further characterization were as shown in Table 1.

Table 1. Selected bacterial isolates were incubated on different solid media (H1A, PCS, NB, and LB) at 27°C, 42°C, and 50°C

Organism S/no.	Name	Incubation temp (°C)	Media
31	*Bacillus cohnii*	42	H1A
35	*Bacillus* sp. YASI	27	H1A
108	*Planococcus* sp.	27	H1A
117	*Bacillus* sp. NER	27	H1A
119	*Bacillus flexus* strain KSC SF. 9C	27	H1A
195	*Bacillus clausii* KSM - K16	27	H1A
160	*Planomicrobium* sp. EP22	27	PCS
145	Bacillaceae bacterium KVD - 1982	27	PCS
203	*Bacillus* sp. YAS 1	27	PCS
207	*Bacillus psychrodurans* strain	27	PCS
217	Uncultured bacterium clone Y2	27	PCS
262	*Bacillus* sp. YY	27	NB
267	*Bacillus subtilis* strain JM4	27	NB
293	*B. clausii* strain	50	NB
153	*Bacillus* sp. CSS -8	27	LB

Protein production and purification

Each bacterial isolate was harvested and used to inoculate a 6 mL liquid media at previously identified temperature conditions (Table 1) for 30 hours in a centrifuge tube. Two (2) mL of each inoculated media was centrifuged for 10 minutes, and 6 mL of Acetone was added to pellet the proteins (Harris and Angal 1994). The pellets were then redissolved in 50 mM citrate buffer (pH 4.8) enriched with Tween-20 to reduce the adsorption of proteins by glassware. Soluble protein was measured by the Biuret method, using bovine albumin as the standard. Uninoculated media was used as a control (Harris and Angal 1994).

Period for optimum protein production

Freshly grown bacterial isolates were harvested and used to inoculate 14.5 mL of semi-solid media in 15 mL plastic centrifuge tubes. The inoculated media were left to standfor 6 hours to allow the bacteria to start growing. The tubes were then shaken vigorously using a vortex mixer to obtain a uniform bacterial distribution. The mixture was then divided into seven 2 mL portions to be used every 24 hours interval. The protein concentration was determined for each isolate for seven consecutive days using the Biuret method with bovine albumin as the standard. Uninoculated media was used as a control.

Substrate specificity

Characterizing the extracted extracellular enzyme(s) that formed part of the protein was done using commercial substrates (CM cellulose, larch wood xylan and cellobiose). Six (6) mL of concentrated ammonium sulphate was added to 2 mL of supernatant to precipitate the proteins. Ammonium sulphate helps retain enzyme activity (Harris and Angal 1994). The precipitate is redissolved in buffer at the ratio of 3:1 to form enzyme preparation. The tests were duplicated and averaged across to minimize experimental error. Controls were prepared using the substrates without enzyme preparation. Buffers were used as blanks in each case. Enzyme concentration was determined for each treatment for each isolate. The activities of the enzymes were expressed as micromoles of glucose and xylose equivalence liberated per minute per mg protein for cellulases and xylanases, respectively (Pratimaba 1997).

Cellulases

With CM cellulose as the substrate, 1 mL of enzyme preparation was incubated in 1 mL of 50 mM citrate buffer (pH 4.8) containing 10 mg of CM- cellulose for 30 minutes at 50°C. The reaction was halted, and the reducing sugars were determined by adding 3 mL of DNS acid reagent (Miller 1959; Chaplin 1986). The absorbance at 540 nm values was read with glucose as the standard (Domingues et al. 2000).

Xylanases

With xylan as the substrate, 1 mL of enzyme preparation was incubated with 1 mL of 50 mM citrate buffer (pH 4.8) containing 10 mg of Larchwood xylan for 30 minutes at 50°C. The reaction was halted, and the reducing sugars were determined by adding 3 mL of DNS acid reagent. The xylose liberated was measured spectrophotometrically with absorbance at 540 nm using xylose as the standard (Chaplin 1986).

Cellobioses

With Cellobiose as the substrate, 1 mL of enzyme preparation was incubated with 1 mL of 50 mM citrate buffer (pH 4.8) containing 2 mM cellobiose for 30 minutes at 50°C. The reaction was halted, and the reducing sugars were determined by adding 3 mL of DNS acid reagent. The liberated glucose was determined spectrophotometrically with absorbance at 540 nm using xylose as the standard (Chaplin 1986).

The activity of the extracted extracellular enzymes at different temperatures

The activity of the extracted and purified extracellular enzyme(s) from the bacterial isolates was determined by incubating 1 mL of enzyme preparation with 1 mL of each substrate (CM cellulose, larchwood xylan, and cellobiose as described previously) at different temperatures (27°C, 50°C, 80°C) for 30 minutes. The tests were carried out in duplicate and averaged across. Controls were prepared using the substrates without enzyme preparation. Each case used Buffers as blanks (Bischoff et al. 2006).

The activity of the extracted extracellular enzymes at different pH values

The activities of the extracted and purified extracellular enzymes from the bacterial isolates were determined by incubating 1 mL of enzyme preparations with 1 mL of the substrates (CM cellulose, Larch wood xylan, and Cellobiose) dissolved in citrate buffers (pH 4.8, 6.8 and 8.6) at 50°C for 30 minutes (Shikata and Nisizawa 1975). The tests werecarried out in duplicate and averaged across. Controls were prepared using the substrates without enzyme preparation. Each case used Buffers as blanks (Singh et al. 2001a,b). The liberated monosaccharides were estimated as outlined earlier.

Influence of metal ions on the activities of the purified extracellular enzymes

The activities of the extracted and purified extracellular enzymes from the bacterial isolates were determined by incubating 1 mL of enzyme preparation with 1 mL of the substrates (CM cellulose, Larch wood xylan, cellobiose) containing 1mM of the metal ions (Ca^{2+}, Mg^{2+}, Cu^{2+}, Ag^{+}) at 50°C for 30 minutes. The buffer used in this test was supplemented with EDTA (metal ion scavenger) to chelate the metal ions (Elling 1995). The products were estimated as outlined previously. The tests were carried out in duplicate and averaged across controls made up of substrates lacking the identified metal ions were also set up.

Data management and statistical analysis

The spectrophotometer readings for treatments, controls, and standards were recorded, tabled, and averaged across the three samples to minimize experimental error.

The spectrophotometric analysis relies on the interaction of electromagnetic radiation (light) with the matter of interest. Strictly speaking, every compound has a distinct absorption spectrum which allows its identification, in many cases, in the presence of other compounds. In addition, it is also possible to determine the concentration of that compound quantitatively.

A standard curve was generated by measuring the absorbance of a series of samples for which the concentration was known. Since the Beer-Lambert Law shows a linear relationship between absorbance and concentration, a linear least-squares fit of the standard curve yielded a mathematical relationship between absorbance and concentration. This relationship, in turn, was used to determine the concentration of the unknown samples.

Laboratory analysis

Determination of total protein-Biuret Method

The method relies on the complexation of Cu^{2+} by the functional groups involved with the peptide bond. A minimum of two peptide bonds is needed for the complexation to occur. Upon complexation, purple color is observed. Relatively few substances interfere with the method. The characteristic wavelength at which the colors are read is 540 nm.

Determination of concentration of sugar- DNS colorimetric method

The method tests for the presence of a free carbonyl group (C=O) called reducing sugars. It involves the oxidation of the aldehyde or ketone functional groups in reducing sugars. Simultaneously, 3, 5-dinitrosalicylic (DNS) is reduced to 3-amino, 5- nitrosalicylic acid under alkaline conditions. The reaction leads to the development of color whose optical density (OD) depends on the reducing sugar type and concentration.

RESULTS AND DISCUSSION

Extraction and purification of proteins

All the bacterial isolates secreted extracellular proteins in their growth media after 30 hours. There was a relation between the growth media and the protein level produced in the study period, as shown in Figure 1. Bacterial isolates grown on low molecular weight (HIA) media (*Bacillus cohnii*, *Bacillus* sp. YASI) recorded higher protein production than those from high molecular weight media (PCS, NB, and LB). However, Uncultured bacterium clone Y2 (207) grown on PCS produced the highest protein contentduring the 30-hour growth period.

Time for maximum protein production

All the bacterial isolates had secreted proteins into their growth media after 24 hours. The *B. cohnii* (31), *Bacillus subtilis* strain JM4 (267), and *Bacillus flexus* strain KSC SF. 9C (119) had two distinct production peaks, while the rest had single peaks. Most bacterial isolates had peaks falling between 48 hrs and 96 hrs except the *Bacillus clausii* strain (293), which peaked at 24 hrs. There was a gradual drop in the concentration of protein produced after 120 hrs except in *B. cohnii* (31) and *B. subtilis* strain JM4 (267). *Bacillus* sp. YAS 1 had very low protein production throughout the growth period, as shown in Figure 2.

Substrate specificity

All bacterial isolates secreted cellobioses except *Planomicrobium* sp. EP22 (160). All bacterial isolates biodegrade CM cellulose substrate at a relatively low rate. Uncultured bacterium clone Y2 (217), *Bacillus* sp. YY (262), *B. subtilis* strain JM4 (267) and *B. clausii* KSM K16 (195) did not secrete xylanases. *Planococcus* sp. (108), *Bacillus* sp. YASI (35) and *B. flexus* strain KSC SF. 9C (119) secreted enzymes with moderate activities on Cellobiose, while enzymes from *B. clausii* (293) and *Bacillus* sp. CSS-8 strain (153) had exceptionally high activity on the substrate, as shown in Figure 3.

Effect of temperature changes on the activity of cellulases

All the bacterial isolates secreted cellulases with activities at 27°C except Bacillaceae bacterium KVD-182 (145), *Bacillus* sp. CSS-8 (153), *Bacillus psychrodurans* strain (207), and *Planomicrobium* sp. EP22 (160). The *B. cohnii* (31) cellulases had very activity at this temperature. All bacterial isolates produced cellulases with activities at 500C. *Planococcus* sp. (108) and *Planomicrobium* sp. EP22 (160) produced cellulases with high activities.

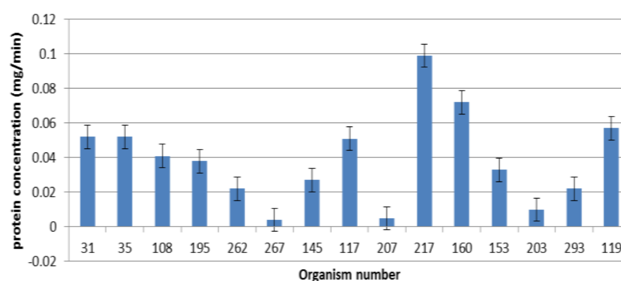

Figure 1. Proteins (mg/mL) produced by bacterial isolates grown in liquid media for 30-hour

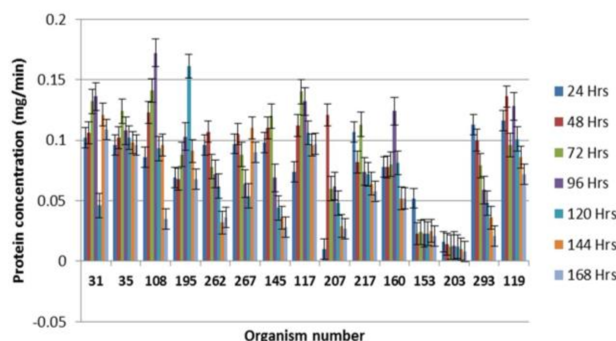

Figure 2. The concentration of proteins produced (mg) from 1 mL of enzyme extract when bacterial isolates were grown on liquidmedia at the 24-hour interval for seven days

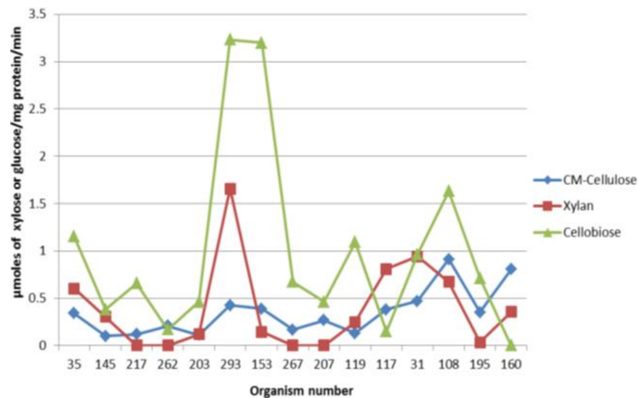

Figure 3. Activities of enzymes from each bacterial isolate on three substrates. BothCM cellulose and cellobiose biodegraded to glucose while xylan forms xylose (µmole/mgprotein/min) at 50°C and pH 4.8

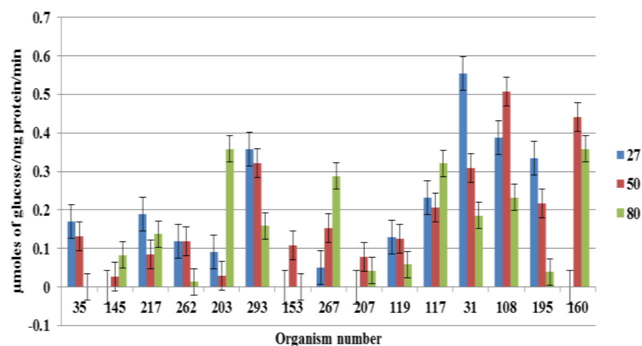

Figure 4. Enzyme activity (µmole glucose/mg protein/min) on CM cellulose at 27°C, 50°C, and 80°C and ph 4.8

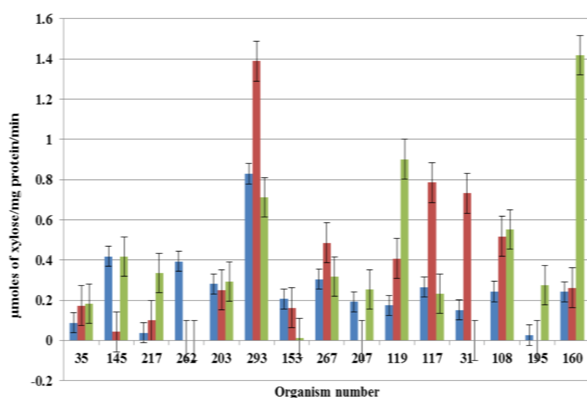

Figure 5. Enzyme activity (µmole xylose/mg protein/min) on xylan producing xylose at27°C, 50°C, and 80°C and pH 4.8

However, enzymes from Bacillaceae bacterium KVD - 182(145) and *Bacillus* sp. YASI (203) showed exceptionally low activities at this temperature. *Bacillus* sp. CSS- 8 produced enzyme activity at 50°C only, and 13 (86 %) organisms produced cellulases with activity at 80°C. Enzymes produced by *Bacillus* sp. YAS 1 (35) and *Bacillus* sp. CSS-8 (153) showed no activities at this temperature. *Planomicrobium* sp. EP22 produced cellulases with high activities at the said temperature, as outlined in Figure 4.

Effect of temperature changes on the activity of xylanases

All organisms produced xylanases with low activities at 27°C except the *B. clausii* strain (293). Xylanases from the isolate showed relatively high activities at all temperatures. Enzymes from 12 (80%) isolate had activities at 50°C. Of these, 3 (20%) had very low activities. *Bacillus sp* YY (262), *B. psychrodurans* strain (207), and *B. clausii* KSM-16 (195) had no activity at this temperature. Enzymes from 12 (80%) isolate had activities at 80°C. Enzymes from *Bacillus* sp. YY (262), *Bacillus* sp. CSS-8 (153), and *B. cohnii* (31) had no activity on xylan, while those from 7 (47%) isolates had minimal activity at this temperature. *Planomicrobium* sp. EP22 (160) produced xylanases with high activity at this temperature, as shown in Figure 5.

Effect of temperature changes on the activity of cellobioses

Most isolates (86%) secreted enzymes with very low activities on cellobiose at all temperatures except *B. clausii* strain (293) and *Bacillus* sp. CSS-8(153), which showed relatively high activities. Enzymes from the two isolates showed comparatively high activities at 27°C. Cellobioses secreted by *Planomicrobium* sp. EP22 (160), and Uncultured bacterium clone Y2 (217) had no activity at the said temperature. All isolates secreted cellobioses with low activities at 50°C except *Planomicrobium* sp. EP22 (160) and Uncultured bacterium clone Y2 (217). Cellobioses from 9 (60 %) isolates showed relatively higher activities at this temperature than at 27°C and 80°C. Only 2 (13 %) isolates produced enzymes with high activities at 80°C. Enzymes secreted by *B. clausii* strain (293) and *Bacillus* sp. CSS-8 (153) showed activities at all the experimental temperatures, as in Figure 6.

Effect of pH changes on the activity of cellulases

All isolates produced cellulases with activities at a pH of 4.8. Cellulases from *Bacillus* sp. YAS 1(203), *Bacillus bacterium* KVD-1982 (145), and *B. psychrodurans* strain (207) had minimal activities, while those from *Planoccocus* sp. (108) and *Planomicrobium* sp. EP22 (160) had high activities at this pH. Enzymes from Bacillaceae bacterium KVD-1982 (145) and *B. psychrodurans* strain (207) had low pH values. Cellulases secreted from *B. subtilis* strain JM4 (267) showed high activity, those from *Bacillus* sp. NER (117) had moderate, and the rest had minimal activities at a pH of 6.8. Forty percent of the isolates secreted enzymes that had activities at pH 8.6. Enzymes from *Bacillus* sp. NER (117) expressed an outstandingly high activity, while the rest secreted cellulases with low activities at the pH, as expressed in Figure 7.

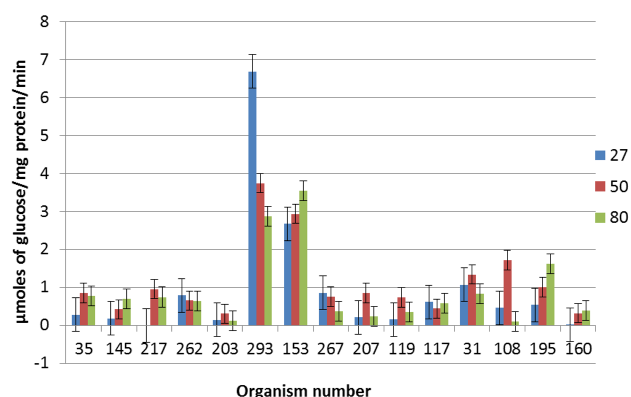

Figure 6. Enzyme activity (µmole glucose/mg protein/min) on cellebiose producingglucose at 27°C, 50°C, and 80°C and pH 4.8

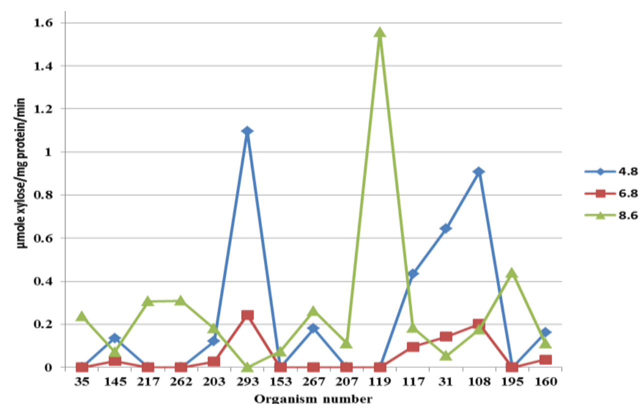

Figure 8. Activity of xylanases (µmole xylose/mg protein/min) xylan at 50°C under pHof 4.8, 6.8, and 8.6

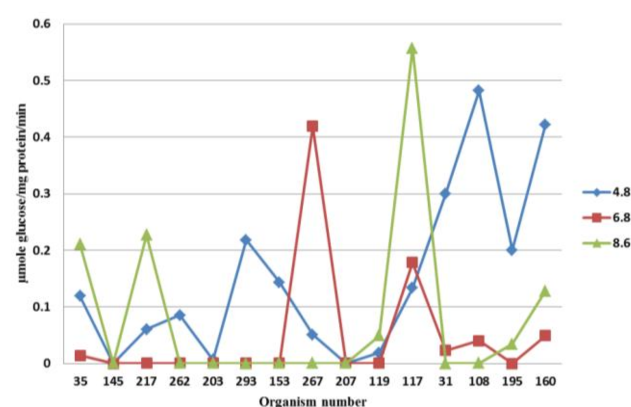

Figure 7. The activity of cellulases (µmole glucose/mg protein/min) on CM-cellulose at 50°C under pH of 4.8, 6.8, and 8.6

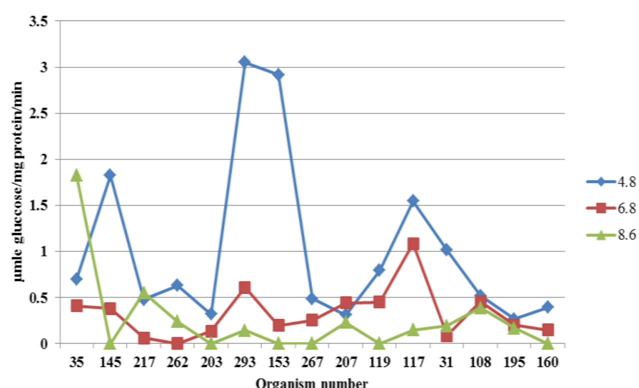

Figure 9. The activity of cellobioses (µmole glucose/mg protein/min) on cellobiose at 50°C under pH of 4.8, 6.8, and 8.6, producing glucose

Effect of pH changes on the activity of xylanases

High xylanase activities were found at pHs 4.8 and 8.6; however, enzymes from all the isolates had very low activities at a pH of 6.8. The *B. clausii* strain (293) and *Planococcus* sp. (108) produced enzymes with relatively high activities at a pH of 4.8. Enzymes from *B. flexus* strain KSC SF. 9C (119) showed very high activities at a pH of 8.6. Bacillaceae bacterium KVD-1982 (145), *Bacillus* sp. CSS-8 (153), and *B. clausii* KSM-K16 (195) had low xylanase activity at all pH, as shown in Figure 8.

Influence of pH on cellobioses

Nearly all isolates produced enzymes with activities within a wide pH range. Enzymes from all the organisms showed activities at a pH of 4.8. The *B. clausii* strain (293) and *Bacillus* sp. CSS-8 (153) produced enzymes with very high activities at this pH. Enzymesfrom all the isolates showed minimal activities at a pH of 6.8. Enzymes obtained from all isolates showed very low activity at a pH of 8.6. Only *Bacillus* sp. YASI (35) produced enzymes with moderate activity at this pH, as shown in Figure 9.

Influence of metal ions on cellulases

Calcium ion (Ca^{2+}) stimulated the activities of enzymes from 11 (73 %) isolates but inhibited enzymes from the rest. The stimulatory effect was moderate on *B. subtilis* strain JM4 (267) and very low on the rest. Magnesium ions (Mg^{2+}) stimulated enzymes from 67 percent of the isolates, while the rest were inhibited. The stimulatory effect was pronounced in *B. subtilis* strain JM4 (267), *B. flexus* strain KSC SF. 9C (119) and *B. psychrodurans* strain (207). Copper ions (Cu^{2+}) inhibited cellulases from most isolates with limited stimulation on enzymes from a few isolates. Eighty percent of the isolates produced enzymes stimulated by silver ions (Ag^{+}), while the ion inhibited enzymes produced by the rest. Enzymes from *B. flexus* strain KSC SF. The ion highly stimulated 9C (119). All the tested metal ions stimulated cellulases from the *B. subtilis* strain. Enzymes from *Bacillus* sp. CSS-8 (153), *Planococcus* sp. (108), and *Planomicrobium* sp. EP22 (160) were inhibited by all the ions except Calcium ions (Ca^{2+}) as in Figure 10.

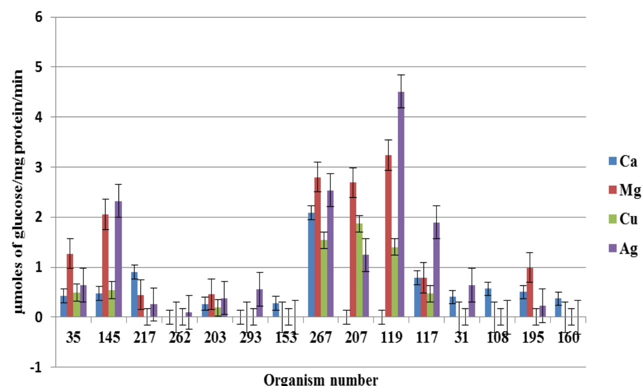

Figure 10. The activity of cellulases (µmole/mg protein/min) on cellulose substrate dissolvedin a buffer containing 50 mM of four metal ions (Ca^{2+}, Mg^{2+}, Cu^{2+}, Ag^{+}) at 50°C and pH of 4.8

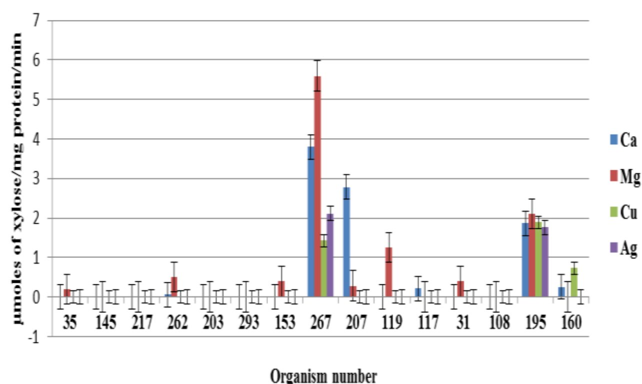

Figure 11. Activity of xylanases (µmole xylose/mg protein/min) on xylan substrate dissolved in buffer containing 50 mM of four metal ions (Ca^{2+}, Mg^{2+}, Cu^{2+}, Ag^{+}) at 50°C and pH of 4.8

Influence of metal ions on xylanases

All tested metal ions highly stimulated xylanases from *B. subtilis* strain JM4 (267) and *B. clausii* KSM-K16 (195) . Calcium ions (Ca^{2+}) stimulated enzymes from 6 (40 %) isolates, while enzymes from other bacteria showed low activities. A remarkable stimulation was shown by xylanases from *B. subtilis* strain JM4 (267). A similar trend was expressed by Magnesium ion (Mg^{2+}). The highest stimulation by the ion was seen in enzymes from *B. subtilis* strain JM4 (267). Xylanases from 12 (80%) organisms showed low activities under the influence of copper ions (Cu^{2+}) with minimal stimulation on enzymes obtained from the rest of the isolates. Xylanases from most isolates had very low activities when treated with silver ions (Ag^{+}) except those from *B. subtilis* strain JM4 (267) and *B. clausii* strain (195), which were slightly stimulated. Xylanases from Bacillaceae bacterium KVD-1982 (145), uncultured bacterium clone Y2 (217), *Bacillus* sp. YAS and *B. clausii* strain (293) had low activities when treated with all the metal ions, as shown in Figure 11.

Influence of metal ions on cellobioses

Cellobioses from 10 (67%) isolates were minimally stimulated by Calcium ions (Ca^{2+}). However, enzymes from the rest showed low activities when incubated by the ion. Magnesium ions (Mg^{2+}) stimulated enzymes from 10 (67%) isolates with Uncultured bacterium clone Y2 (217) and *Bacillus* sp. NER (117) showed moderate stimulation while other isolates produced enzymes with very low activity under the influence of the ion. Cellobioses from *Bacillus* sp. CSS-8 (153) were highly stimulated by copper ion (Cu^{2+}), while six produced cellobioses with low activity. Seven (47%) isolates produced enzymes that were moderately stimulated by silver ions (Ag^{+}), while those from 5 (20%) isolates had very low activities. Very high stimulation by the ion was shown by enzymes from the *B. clausii* strain (293), as shown in Figure 12.

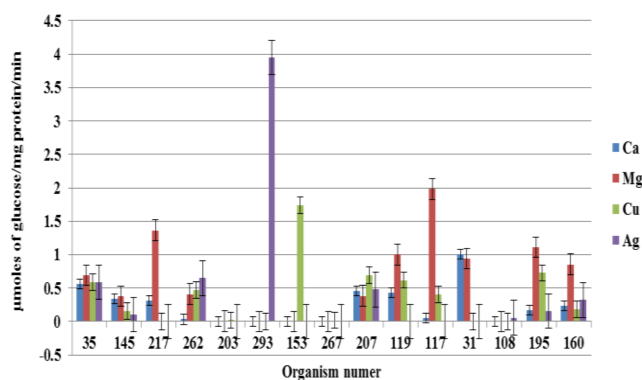

Figure 12. The activity of cellobioses (µmole glucose/mg protein/min) on cellobiose substrate dissolved in a buffer containing 50 mM of four metal ions (Ca^{2+}, Mg^{2+}, Cu^{2+},Ag^{+}) at 50°C and pH of 4.8

Discussion

Microorganisms decompose organic wastes to generate energy and nutrients for their growth by producing intra and extracellular enzymes. A great significance for the process of composting represents the cell wall of microorganisms through which mass transfer is possible. Low molecular weight and water-soluble molecules can easily pass through the cell wall where they take part in the cell metabolism, providing energy and being built into larger polymers with the help of intracellular enzymes. To attack high molecular weight components, which cannot pass through the cell wall, microorganisms secrete extracellular enzymes, which break molecules down into fragments that can beassimilated. At the same time, the rest is converted into a stable product, humus or compost (Haug 1980).

The results showed that all bacterial isolates used in the study secreted extracellular enzymes into their growth media within 24 hours period. The production was higher in theisolates grown on high molecular weight media than those grown in the low molecular weight media. Isolates grown on high molecular weight media secretes enzymes earlier tobreak the substrates to generate soluble products that are catabolized to produce energy (Deshpande et al.

1978). However, isolates grown in low molecular weight media obtain soluble products that readily diffuse into the cell for assimilation hence delayed enzyme production (Biddlestone and Gray 1985).

Several previous researchers have reported the production of extracellular cellulolytic enzymes by Bacillus bacteria into their growth media (Priest 1977; Robson and Chambliss 1984). James and Barko (2004) reported that 115 bacilli grown on Horikoshi-I medium for 24 hours at 37°C using eight different substrates: birchwood xylan, carboxymethylcellulose, casein, citrus pectin, polygalacturonic acid, soluble starch, and Tween 20 and 80 produced extracellular enzymes. These strains were identified as producers of extracellular proteases (Singh et al. 2001a,b) and can be cultivated under extreme temperatures and pH conditions to give rise to products that are, in turn, stable in a wide range of harsh environments (Han and Damodaran 1997).

Bacterial isolates grown on H1A (containing readily available carbon source; glucose) showed maximum enzyme production between 72-120 hours. However, isolates grown on media where free carbon and Nitrogen (CMC and NB, respectively) lack secretes their enzymes early (24-48 hours) to digest the complex materials to obtain the raw materials for growth. The maximum enzyme production stage by different bacterial species is quite variable. It largely depends on the type of microbial strains and their genetic make-up on cultural and environmental conditions employed during the organism's growth. Easily metabolizable and utilizable substrates induce lower enzyme production (due to catabolite repression) than the complex and slowly utilizable substrates. Isolates grown on CM cellulose media secretes cellulases digesting the substrate to an insoluble disaccharide (cellobiose). This is followed by the production of cellobioses that finally breaks the sugar into soluble glucose. *Bacillus* sp. mostly produces two groups of proteases, alkaline and neutral (Rao et al. 1998), of which the neutral proteases are active in a narrow pH range (pH 5.0-8.0) and have relatively low thermotolerance (Barrett 1994). For most isolates, the level of enzyme production diminished towards the end of the seven days. This may be attributed to the accumulation of proteases that degrades the proteins or product inhibition (Shevelev and Hubscher 2002). The products of exoglucanases and cellobiohydrolases that are cellobiose and cellodextrans, respectively, are inhibitory to their activity (Miranda et al. 2009).

The results showed that most bacterial isolates' enzymes secreted into the growth media biodegrade more than one type of substrate. Given the mixed composition of the solid wastes in the field, most isolates produce enzymes that biodegrade across a section of substrates as a survival strategy. In addition, plant tissues are formed from several different monomers covalently linked. The isolates must secrete corresponding enzymes to biodegrade the plant tissues to obtain energy. In cellulase synergism, the reaction between endoglucanases and exoglucanases was considered a mechanism in which endoglucanases initially nicked the crystalline cellulose surface, followed by exoglucanases liberating cellobiose from these nicks (Henrissat et al. 1985). Finally, beta-glucosidase is required to hydrolyze cellobiose into individualmonosaccharides for use by the isolate. A novel thermophilic, a cellulolytic bacterium isolated from swine waste, *Brevibacillus* sp. Strain JXL used a broad spectrum of substrates such as crystalline cellulose, carboxymethyl cellulose, xylan, cellobiose, glucose, and xylose (Liang et al. 2009). A bifunctional endoglucanase/ endoxylanase isolated from *Cellulomonas flavigena* had cellulase and xylanase activity (Pérez-Avalos et al. 2008). Similarly, in 2007, a multifunctional enzyme was produced by *Terendinibacter turnerae* T7902, a bacterial symbiont isolated from the wood-boring marine bivalve *Lydrodus pedicellatus*. According to Moo-Young (1992), the actions of cellulases and xylanases are synergetic over substrates, especially for microorganisms isolated from environments where wood and agro-residues are biodegraded.

These results suggest that some isolates may have secreted enzymes that produce promiscuous secondary metabolites that act on a relatively broad range of different substrates, which is important for the evolution of new biosynthetic pathways (Firn 2006). Cellulases from strains like *C. flavigena* and *T. turnerae* have been cited to produce multifunctional cellulases with broad substrate utilization (Firn 2006). Some purported xylanases, for example, also appear to have activity against substituted carboxymethyl cellulose. When present, they can reduce cellulose viscosity by attacking the amorphous regions (Miranda et al. 2009). Some degree of cross-specificity was reported from numerous fungal and bacterial 1, 4-β glucanases and xylanases (Shikata and Nisizawa 1975; Tods et al. 1975; Kanda et al. 1976; Hurst et al. 1978; John et al. 1979; Pettipher and Latham 1979: Uchino and Nakane 1981; Peiris et al. 1982).

Temperature affects the speed of molecules, the catalytic reaction's activation energy, and the enzyme's and substrate's thermal stability. Generally, the enzyme reaction rate is very slow at low temperatures as the molecules have low kinetic energy, and collisions between them are less frequent. Even if they collide, the molecules do not possess the minimum activation energy required for the reaction. It can be said that the enzymes are deactivated at low temperatures (Abrahams and Katherine 2011). An increase in temperature increases the enzyme activity since the molecules now possess greater kinetic energy. The rate of enzyme activity is highest between 0-40°C, and this increase is almost linear. After 40°C, the rate of reaction starts to decrease. This is because the increase in temperature after 40°C does not increase the kinetic energy of the enzyme but instead disrupts the forces maintaining the molecule's shape. The enzyme molecules are gradually denatured, causing the active site's shape to change. Temperatures above 65°C completely denature the enzymes (Abrahams and Katherine 2011).

The study showed that cellulases and xylanases and cellobioses were acting within large temperature range ([$P < 3.23$ ($F=0.21$)], [$P<3.23$ ($F=0.14$)] and [$P<3.23$ ($F=0.06$)] respectively). Temperature increases with depth at the dump site, and isolates at the lower depths need to produce thermophilic enzymes that tolerate high

temperatures. The stability of xylanase at high temperatures might be due to the protection caused by any compatible solute, such as the polymeric substrate xylan and/or xylooligosaccharides resulting from the hydrolysis, which could exert a protective effect on the enzymes (Damaso et al. 2000). These protection effects related to disaccharides and trisaccharides and also polymeric ficoll on the restriction enzyme PstI was described by Colaço et al. (1992). Kumar et al. (2000) observed that both thermophilic and mesophilic proteins have similar hydrophobicities, oligomeric states, and hydrogen bonds. On the other hand, salt bridge numbers are higher in most thermophilic proteins. This fact can be explained since salt bridges, and their networks rigidify protein structures. A higher concentration of salt bridges, particularly networks, "stitches" the protein structure, making it more resistant to local deformation/melting or unfolding at high temperatures. Previous studies reported that xylanase was active over a wide range of temperatures and pH values, with the optimum at 75ºC and 6.0, respectively (Uchino and Nakane 1981). Xylanase from thermophilic *Bacillus* exhibited a temperature profile with a sharp peak of maximal activity at 90°C and showed activity between 40-100°C (Cordeiro et al. 2002).

Cellulases from thermophilic fungi showed optimum activities at 50°C (Cooney and Emerson 1964). Similarly, the optimum temperature for cellulase activity with carboxymethyl cellulose substrate varied between 58-77°C with 10 hrs incubation time (Eriksen and Goksoyr 1976). *Bacillus* sp. 3M exhibits cellulase retained high activity at 90^0C, making it attractive in the pulp and paper industry (Marques et al. 1997). Effect of temperature on endoglucanase activity showed that the enzyme was highly active over a broad temperature range (50-100°C). Cellulases from different *Bacillus* spp. have been reported to possess optimum activity at 40-50°C. However, *Bacillus licheniformis* cellulase was found to be moderately thermostable with optimum activity at 65°C and retained 90% of the original activity for 1 hr at 60°C (Bischoff et al.1987). The highest mean activity of *Bacillus* sp. C14 endoglucanase enzyme was observed as 88 and 96% between 20-60°C and had a thermal stability average of 71 and 60% for 15 and 30 min, respectively, between 20-90°C (Oyekola et al. 2007).

Most enzymes are sensitive to pH changes and have specific activity ranges and an optimum pH. The pH change can stop enzyme activity by denaturation (altering) the three-dimensional shape of the enzyme by breaking weak bonds such as ionic and hydrogen. The isolates are subjected to drastic pH changes. Anaerobic decomposition of organic matter increases the acidity of the soil. This is compounded by the acidic industrial wastes, including sulphuric acid from used batteries. This condition is neutralized by stone debris from construction sites leaving the pH to stabilize around a neutral value. These abrupt pH changes at the dump sites can only be tolerated by isolates that secrete enzymes acting within a large pH range (Saini 2007).

From the research, cellulases and cellobioses were acting within a wide pH range ($P>3.23$ ($F=3.24$] and [$P>3.23$ ($F= 6.33$)]) respectively, while xylanases were acting within a narrow pH range [$P< 3.23$ ($F=1.91$]). Most enzymes function between a pH of 6 and 8 (Fukumori et al. 1985). Bacteria that thrive at lower pH values are acidophilic (acid loving) (Robson and Chambliss 1984). *Bacillus* sp. JB-99 was also able to grow over a wide range of pH (6-12), and it required an alkaline pH (8-10) for growth and enzyme secretion (Johnvesly and Naik 2001). Alkaliphilic Bacillus strains often produce various alkaline enzymes, including alkaline cellulases (Horikoshi and Akiba 1982; Horikoshi 1996). Horikoshi and colleagues found that Alkaliphilic Bacillus strains N-4 and NO. 1139 (Horikoshi et al. 1984), both of which had first been described in Japanese patent, produced alkaline cellulases (Carboxymethylcellulose (CMC) hydrolyzing enzymes CMCase). A bifunctional endoglucanase/ endoxylanase isolated from *C. flavigena* was found to have optimum cellulase and xylanase activity at pH 6 and 9, respectively (Miranda et al. 2009). Enzymes stably work in a wide range, including an alkaline side. Their activity is shown even at low temperatures (cellulase K, CMCase I, and CMCase II) were isolated from a culture product of *Bacillus* sp. KSM-635 (Japanese patent Application Laid-open No. 61-19483). *Bacillus* sp. C14 showed growth at a wide range of pH from 6 to 12. *Bacillus* sp. JB-99 was also able to grow over a wide range of pH (6-12), and it required an alkaline pH (8-10) for growth and enzyme secretion (Johnvesly and Naik 2001). Seven strains of *B. subtilis* group, isolated from fermented African locust bean (iru), compared based on growth and extracellular enzyme production in media with and without locust bean, had their optimum pH for growth between 7.0 and 9.0 (Aderibigbe and Odunfa 1990).

Enzymes produced by the isolates at the dump sites are subjected to various metal ions derived from household wastes. Calcium ions are readily obtained from stone wall debris, while magnesium ion is abundant in the dumping soil. Copper ions constitute 80% of the electrical wastes, while silver ions come as impurities from industries' oil discharge. The survival of such organisms depends on the ability of their enzymes to circumvent the adverse effects the ions have on their structure. Many reports are available about different activation and the inhibition pattern of the metal ions on enzymes. Calcium (Ca^{2+}) and Magnesium (Mg^{2+}) ions had a stimulatory effect on cellulases as they might be involved in the protection of the enzyme or strengthening of the active site, thereby maintaining the conformation of the enzyme in its active state. Metal ions have been reported to influence enzyme production by increasing their activity in microorganisms (Rani and Nand 2000; Rani et al. 2004). Cu^{2+}, Cd^{2+}, and Ni^{2+} have been reported as heavy metals which are generally toxic to some organisms (Sunkar et al. 2003; Rani et al. 2004). According to Mawadza et al. (2000), most metal ions such as K^+, Na^+, Ca^{2+}, and Zn^{2+} did not actually influence the enzyme activity.

However, Singh reported an increased enzyme activity in the presence of Na^+ (Singh et al. 2001a,b; Singh et al. 2004). The inhibition of *Bacillus* sp. C14 by Zn^{2+} (27 percent) concur with other work due to the inhibitory

effects of heavy metals on enzymes. The obstructive activity of Zn^{2+} on cellulases was also reported by Voget et al. (2006) and Huang and Monk (2004). Mercuric and cupric ions have been found to inactivate the cellulolytic activity of the cellulase (1, 4- (1.3; 1, 4)-β-d-glucan-4-glucanohydrolase; endoglucanase; EC 3.2.1.4) from *Schizophyllum commune* in a time-dependent manner (Anthony and Lenley 1987). Inhibition of cellulase activity by the metal ions Ag^{+} and Hg^{2+} was also reported and was ascribed to interaction with tryptophan residues rather than with thiol groups (Hurst et al. 1977). Of the numerous metal ions examined, Ca^{2+} and Co^{2+} at 0.1 mM concentration were slightly activated under the assay conditions, while 1.0 mM Pb^{2+} and Hg^{2+} were the most inhibiting ions (Ferchak and Pye 1983). Ca^{2+} ions have earlier been reported to be required by cellulosome enzymes, with the former enhancing the substrate binding affinity of the enzyme and stabilizing the conformation of the catalytic site (Mansfield et al. 1998). These results suggest that the metal ions such as Ca^{2+}, Na^{+}, and Zn^{2+} apparently protected the enzyme against thermal denaturation and played a key role in continuing the enzyme's active conformation at high temperatures (Donagy and Mckay 1993). A study by Wang and his colleagues showed that Fe^{2+} might activate cellulase even in lower concentrations, while Mg^{2+} showed an inhibitory effect in higher concentrations. The other seven kinds of metal ions all showed the inhibitory character, and the inhibitory effect of Hg^{2+} was the most potent, even inlower concentrations (Wang et al. 2009). Ca^{2+} ion has long been known to promote the formation of active trypsin from the inactive trypsinogen and stabilizes trypsin against autolysis (Zoltan et al. 2003).

Mark and his colleagues found that the activity of a purified enzyme shown to be exclusively xylanolytic was significantly enhanced following treatment with manganese and potassium chlorides but significantly reduced by calcium, cobalt, and iron (Mark et al. 2009). Annamalai et al. (2008) also reported the activity of xylanases produced by *B. subtilis* isolated from the marine environment, which MgSO4 enhanced, $CaCl_2$, $FeCl_2$, and $FeSO_4$ in both 1 and/or 10 mM concentrations. While $NiSO_4$ and $ZnSO_4$ showed slight inhibition in the enzyme activity, $CuCl_2$, $CoCl_2$, $CoSO_4$, $CuSO_4$, $MnSO_4$, and $ZnCl_2$ showed moderate inhibition, and $HgCl_2$ expressed severe inhibition at 10 mM concentration (Annamalai et al. 2008). Similarly, Monica (2002) noted that 5 mM of Zn^{2+}, Ca^{2+}, and Ba^{2+} caused slight inhibition of the xylanase activity. Yet the ions of Mg^{2+} and Fe^{3+} at the same concentration led to an inhibition of approximately 25 percent. On the other hand, NaCl concentrations at 3 mM or 5 mM resulted in slight enzyme activity stimulation.

Mohammed identified three cellobioses from *Aspergillus niger* A20, A, B, and C. Ca^{2+} ions were found to stimulate cellobioses B and C, while Co^{2+} and Mg^{2+} ions stimulated A (Fareeha et al. 2011). The activity of β-glucosidase from thermophilic fungus *Melanocarpus* sp. microbial type culture collection (MTCC) 3922 was positively influenced by metal ions such as Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, and Zn^{2+} but inhibited by the presence of $CuSO_4$ (Jatinder et al. 2001). The studies on two extracellular β-glucosidase from *A. niger* USDB 0827 and *A. niger* USDB 0828 showed that both enzymes were relatively unaffected by Ca^{2+}, Cu^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} (Yin et al. 1992). β-glucosidase from *A. ornatus* has also been inhibited by Ag^{+} and Fe^{2+} (Yeoh et al. 1986), whereas β-glucosidase from *A. terreus* was relatively unaffected by metal ions (Workman and Day 1982). In the study of unique inhibitors and inducers of the protease by Siddalingeshwara et al. (2010), Cu^{2+}, Mn^{2+}, Ca^{2+}, Hg^{2+}, Na^{2+}, and $MgCl_2$ were shown to be potent inducers while Zn^{2+} and Fe^{2+} were potent inhibitors.

In conclusions, (i) *B. clausii, Bacillus* sp. NER and *Bacillus* sp. CSS-8 strain with broad substrate spectrum, high activities at large temperatures, and pH ranges can form a good consortium for biodegradation of solid organic waste, (ii) All the studied bacterial isolates showed significant production of extracellular enzymes, (iii) There was a significant difference between the times taken for maximum enzymeproduction by the individual isolates, (iv) Enzymes produced by different isolates had broad range specificity on the substrates, (v) Temperature changes influenced the activities of cellobioses. At the same time, cellulases and xylanases act over a wide temperature range, (vi) pH changes influenced the activities of cellulases and cellobioses but not on xylanases, and (vii) Cellulase and cellobioses were stimulated by Ca^{2+} and Mg^{2+}. At the same time, Cu^{2+} and Ag^{+} inhibited the enzymes. However, xylanases were inhibited by the metal ions.

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Biological characterization of isolates of the *Rhizoctonia solani* fungus in rice (*Oryza sativa*) from Karanganyar District, Indonesia

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Abstract. *Itsnaini NR, Supyani, Gutomo HS. 2019. Biological characterization of isolates of the* Rhizoctonia solani *fungus in rice (*Oryza sativa*) from Karanganyar District, Indonesia. Bioteknologi 16: 74-81.* This research aims to study the biological character of *Rhizoctonia solani* J.G.Kühn isolates, evaluate the virulence of *R. solani* isolates, analyze the relationship between biological characters and the virulence level of the collected *R. solani* isolates, and study the biological character of *R. solani,* which has a low virulence level. This research was conducted at the Laboratory of Plant Pests and Diseases Faculty of Agriculture, Universitas Sebelas Maret, Surakarta, Central Java, Indonesia, at an altitude of 99 m above sea level, while *Rhizoctonia solani* fungus in rice (*Oryza sativa*) was collected from Karanganyar District, Indonesia. The researchers designed this research as exploratory research in the field and conducted it in the laboratory and greenhouse. Research variables included growth rate, colony phenotypic character (including color, surface structure, and the presence or absence of air hyphae), the diameter of damage to apples, percentage of spot area, and disease intensity. Data analysis of test results on the growth rate, virulence test on apples, and percentage of spot area using the mean and standard deviation presented in graphical form while the analysis of disease intensity on the host plant using analysis of variance based on the F test at 5% level and if there was a significant difference, it was continued with Duncan's test. The results showed that the *R. solani* isolates from the Karanganyar area had a variety of biological characteristics (color, air mycelium, colony profile, and colony diameter) and virulence levels. Isolates that were categorized as hypovirulent were isolates K+ (P6); P28; P17; P16; and P26, each caused a spot area of 35.93%; 36.94%; 42.16%; 45.28%; 45.99% in host plants and the isolate which was considered virulent was isolate P7 which caused 56.57% of the spot area. The disease intensity in isolate K+ (P6); P28; P17; P16; and P26, respectively, were 53.33%; 46.67%; 55.55%; 59.26%; 62.96%, and the disease intensity of P7 isolate, which was virulent was 73.33%.

Keywords: Biological characterization, *Oryza* sativa, *Rhizoctonia solani*

INTRODUCTION

The need for rice is increasing as the population increases. Therefore, it causes the high fulfillment of rice consumption needs. Rice is the staple food for most of Indonesia's population. However, disturbances from pests and diseases that attack rice plants can hinder the efforts to fulfill rice consumption needs.

Midrib blight is an important disease in rice plants. Rice midrib blight causes plants to fall easily. This disease causes the grain to be less filled or even empty. Midrib blight usually occurs when the plant begins to form tillers until just before harvest. However, this disease can also occur in young plants when the rice plants are 20 DAP (Days After Planting). The *Rhizoctonia solani* J.G.Kühn fungus causes this disease with early symptoms of pale white oval or round spots on the midrib. In humid conditions, this disease can reach the flag leaf. Pathogens survive and spread with the help of resistant structures called sclerotia (Center for Research and Development for Food Crops 2007).

Rhizoctonia can be found in all parts of Indonesia (environmental conditions allow) where the host plant is located. The severity of the infection can vary; for heavily infected patches, the severity can be very detrimental to the farmer. Some of these consequences are large yield losses (from 25% to 100%), increased soil tare (due to soil adhering to fungal mycelium), and poor quality of industrial plants based on increased sodium, potassium, and nitrogen levels. In addition, the host number of pathogen attacks causes various consequences and harms various plants. Midrib blight caused by this pathogen is the second deadliest disease after the rice blast (Molla et al. 2013).

High temperature, humidity, nitrogen fertilizers, and planting of susceptible varieties strongly favor disease development in the field. In addition, wet leaves and inter-plant and leaf-to-leaf contact favor the spread of disease. Pathogens can also be spread through irrigation water or soil displacement during planting preparation. In addition to rice, the pathogen can survive on citrus, cabbage, vegetables, beans, pumpkin, peanuts, chilies, carrots, soybeans, cotton, barley, celery, tomatoes, sorghum, wheat, tulips, and corn. (Suparyono 2009).

Plant pathogenic fungi (including *R. solani*) have many strains in the field. These strains have different virulence levels, including the damage or loss caused. For example, *R. solani,* which attacked rice during the planting season in 1997 at South Sulawesi, Indonesia; caused 0.5 ha of damage (high category) in Tana Toraja District, in 1997-1998, it caused damage of 1 ha (light category) in Soppeng District, during the 2000 planting season, this fungus caused damage of 4 ha in Enrekang District and 11 ha in

Tana Toraja District. During the 2001 planting season, this fungus caused 1 ha of damage in Luwu District (Syatrawati 2005). So far, only fungicides have been used to control damping off diseases.

Pesticides not only have a detrimental impact on human health and the environment but also on agricultural land and make agricultural products unsafe for consumption. In addition, the use of pesticides in agricultural ecosystems has resulted in various environmental damage and pollution, which impacts the destruction of species diversity in the ecosystem. Therefore, the impact that occurs requires limiting the use of pesticides and biological control to restore the ecological function of an agro-ecosystem.

This research was conducted to (i) study the biological character of *R. solani* isolates from Karanganyar District, Central Java, Indonesia, (ii) evaluate the virulence of *R. solani* isolates from Karanganyar, (iii) analyze the relationship between biological characteristics and the virulence level of *R. solani* isolates from Karanganyar, and (iv) studied the biological character of *R. solani* from Karanganyar which has a low virulence level.

MATERIALS AND METHODS

Materials

The main ingredients used were plant parts infected with the *R. solani*, collected from Karanganyar District, Central Java, Indonesia, and apples for comparison. The research was conducted at the Laboratory of Plant Pests and Diseases, Faculty of Agriculture, Universitas Sebelas Maret, Surakarta, Central Java, Indonesia, at an altitude of 99 m above sea level,

Research design

This research is designed as exploratory research in the field conducted in a laboratory and a greenhouse. The virulence test on apples was conducted in the laboratory. In contrast, the virulence test on the host rice plant was conducted in a greenhouse using a completely randomized design (CRD).

Collection of *R. solani* isolates

The *R. solani* isolates were collected from the endemic area of the *R. solani* in the Karanganyar area. Researchers collected these isolates by visiting the area. Rice plants that showed symptoms of midrib blight are oval spots on the midrib cut at the base. Parts of the plant, especially the stem and base of the stem, were examined for signs of disease, such as spots, wounds, or signs of disease in the form of sclerotia. Plant parts that showed symptoms or signs of the disease were then put in a cooler. Furthermore, the plant samples were immediately transferred to a refrigerator at 4°C to be further cultured in a PDA medium.

Culture of *R. solani* isolates on artificial medium

Cultured of *R. solani* isolates were conducted at LAF (Laminar Air Flow) according to the Streets (1972) method. Tissue surfaces showing symptoms of spots or sores were sterilized with 90% alcohol. A small section of the border area between diseased and healthy plant tissue was cut, removed, and placed in the center of a 90 mm diameter sterile petri dish containing 20 ml of PDA (Potato Dextrose Agar). The preparations were incubated on the experimental rack under standard conditions at 22-26°C for 7-10 days. All isolates were assigned an identification number according to the identity on the label when they were isolated from the field. Photographs for documentation were conducted when the culture was 1 week old.

At the time of the photo shoot, each isolate was stocked by culturing it on a regeneration medium in a 4 cm diameter petri dish, according to Hillman et al. (1990). Making this stock began with inoculating 3x3x3 mm cubic agar which was taken from the edge of the culture, then placed in the middle of the provided medium. The preparations were incubated on the experimental rack under standard conditions at 22-26°C for 1 week. After that, the preparation was stored in a refrigerator at 4°C as stock for subsequent tests. Each of these stocks was assigned an appropriate identification number or derived from the isolated identity number on the PDA medium above.

Phenotype characterization of *R. solani* isolates

Morphological characterization was conducted following Hillman et al. (1990). The experiment was started by inoculating 3x3x3 mm^3 agar, taken from the edge of a 1-week-old stock culture, in the center of an 85 mm diameter petri dish containing 20 mL of PDA. The petri dish was then incubated on a test rack under standard conditions of 22-26°C. Cultures were observed on days 3, 5, 7, and 9. The observed and recorded characters were the colony growth rate and colony phenotype. If different characters were found in the observed isolates, such as colony growth rate indicated by smaller colony diameter, colony phenotype with a darker or lighter color, and non-smooth colony surface, then the isolates concerned were marked or selected and documented. These isolates had a high chance of low virulence (hypovirulence). Furthermore, the selected isolates were tested by the next test, which was the virulence test using apples and rice seeds.

Apple virulence test

The test was designed using Completely Randomized Design (CRD) and repeated three times. This virulence test used selected hypovirulent isolates based on the characterization of the isolates. The virulence test was conducted on selected isolates following the Elliston (1985) method. Ripe apples were disinfected with 90% alcohol. The researchers assigned 4 points around the fruit with a balanced spread position.

Then, each point was inoculated with fungal isolates on the wounded area. Next, an inoculum was inserted into each wound in an inward position, then pressed with a sterile spatula until complete contact with the apple tissue was made. Then, the inoculated part was wrapped with

parafilm to prevent drying. Furthermore, the apples were incubated in a plastic tray of 35x25x7 cm at room temperature. The diameter of the lesion was measured on days 3, 5, 7, and 9.

Testing on host plants

A test on the host plant was conducted to determine the intensity and percentage of *R. solani* attacks on the real host. The selection of isolates to be tested was based on the results of the virulence test on apples. The virulence test for this host plant used the CRD (Completely Randomized Design).

From these treatments, there was 1 control as a comparison, namely a positive control (high virulence). Then, each stem was repeated 3 times, and each stem was taken from each midrib which was taken on average at the end of the observation. Researchers used Memberamo varieties of rice seedlings aged 36 DAP in this virulence test. This test will inoculate the selected fungal isolates by slipping the inoculated pieces between the rice midribs. Observations were made once a week after inoculation (MSI) for one month. The results of this virulence test show that the fungal isolates showed a lower virulence level (hypovirulent) than the control (virulent).

Preparation of planting media

The media used was latosol soil previously sterilized by autoclaving at a temperature of 121°C and a pressure of 1.5 atm for 2 hours. After sterilization, the soil was placed in a 20 cm diameter pot, which had previously been mixed with urea 1 g/plant and 6 g/plant of compost.

Nursery

Seedling of rice plants was to sow the seeds in a square container previously soaked for 24 hours so that seed imbibition occurred. Then, the soaked seeds were planted in square tubs that had been given sterile soil media and a mixture of compost in a ratio of 1:1 and sown for 14 days. After sowing, the rice seeds were transferred to the media provided.

Plant maintenance

Watering was conducted twice to maintain the humidity and temperature of the plants inoculated with the *R. solani* to prevent disease development. Fertilizer was given to meet the nutritional needs of plants, namely Urea, at a dose of 6 g/plant. Fertilizer was applied before planting, 21 DAP, 45 DAP, 60 DAP, and 90 DAP.

Observed variables

Growth rate

The growth rate of *R. solani* was observed by measuring the diameter of the fungal colonies on the 90 mm diameter petri dish. The growth rate measurement data is calculated with the standard deviation using the following formula:

$$SD = \sqrt{\frac{\sum (X_1 - \bar{X})^2}{n-1}}$$

Colony phenotype characters

The phenotypic characters of the fungal isolate colonies included color, surface structure, and the presence or absence of air hyphae.

Diameter of damage to apple

Observations were made by observing the diameter size of the lesion on the apple. Data on the diameter of the lesion in apples was calculated with the standard deviation using the following formula:

$$SD = \sqrt{\frac{\sum (X_1 - \bar{X})^2}{n-1}}$$

Disease intensity

Disease intensity was observed from the beginning of inoculation until harvest, starting two weeks after inoculation (MSI). Assessment to determine the intensity of plant disease using a scale of 0, 1, 2, 3, 4, and 5 as follows in Table 1.

Then, the plant disease intensity scale is used to calculate the disease intensity using the formula:

$$IP = \frac{\sum (n.v)}{N.V} x100\%$$

Where:
IP : Disease Intensity
n : the Number of plants that show symptoms with a certain category
v : scale value with a certain symptom category
N : Number of sample plants
V : The highest score for the symptom category

Data analysis

Growth rate and virulence test on apples: the data were analyzed with the mean and standard deviation presented as a graph. Virulence test to host plants: the virulence test data on the host plant used analysis of variance based on the 5% level F test, and if there was a significant difference, it was continued with Duncan's test.

Table 1. Plant disease intensity scale

Scale value	Symptoms of spotting
0	No spots/healthy
1	1-10% of spots
2	11-30% of spots
3	31-60% of spots
4	61-90% of spots
5	>90% or dead plants

RESULTS AND DISCUSSION

The *R. solani* isolate collection in the field

The location for collecting *R. solani* isolates in the field was Karanganyar Regency. The purpose of taking isolates

was to obtain information about the symptoms caused by the fungus as well as to study the biological character and level of damage to rice plants. Therefore, this information was used as a first step in determining isolates that were hypovirulent or high virulence. Moreover, the isolation results of hypovirulent fungi can be used as biological control agents for the disease.

Symptoms in the field, namely on the surface of the midrib necrotic wounds, appeared like an ellipse. At first, these patches were oval, gray-green in color, and measured 1-3 cm in length. Then, the center of the spot became grayish white with brown edges. In severe cases, spotting may occur on leaves, including flag leaves. Plants that were attacked by this fungus easily fell and produced empty grain.

Then the researchers isolated the fungus based on the information found in the field. The isolating method of the *R. solani* was to take 1/3 of the diseased plant parts, and the healthy parts grew in PDA media. However, PDA media was still mixed with other fungi, so pure culturing was necessary. In pure culture, the researchers selected fungi that were believed to have the morphology and characteristics of *R. solani*. The isolation results produced 45 isolates which were then observed and cultured in PDA media.

The isolation results produced 31 isolates whose biological characteristics were studied, such as growth rate, presence or absence of air mycelium, and their virulence to the host plant. From these results, it will be known how the relationship between morphological characters to the level of pathogenicity in the host plant and the lowest level of damage to the host plant.

Macroscopic characterization of *R. solani* isolates

Some *R. solani,* which are pathogenic to rice, can produce sclerotia with thick outer walls so that they can float and survive in water. This fungus also survives as a mycelium employing a saprophyte colonizing soil organic matter, especially due to plant pathogenic activity. Sclerotia and/or mycelia, which are in the soil or plant tissue, grow and form hyphae that can attack several types of plants. This pathogen is suitable for poor soil structure and high soil moisture (Ceresini 1999).

According to Yulianti and Suhara (2010), the first step to determining effective control methods is to study the bio-ecology of these fungi. This morphological appearance can be used as an initial grouping of these isolates. Macroscopic observations of *R. solani* isolate colonies were conducted on the 3rd to 7th day after isolation. Macroscopic observations were conducted directly by looking at the development of each colony, such as colony diameter, colony color, air mycelium, and colony profile. This macroscopic observation determined the character of isolates with high or low virulence levels. The observation results of *R. solani* isolates are presented in Table 2.

Table 2. Macroscopic characterization of *R. solani* isolates on the 3rd day

Isolates	Colony diameter (cm)	Colony color	Air mycelium	Colony profile
P1	7.85	Dirty white	Plentiful	Like a ring and already formed sclerotia, rough
P2	7.90	Dirty white	Plentiful	Circlet, rough
P3	8.05	Dirty white	Plentiful	Sclerotia have formed, like a circlet
P4	7.70	Dirty white	Plentiful	like a circlet, rough
P5	7.30	Dirty white	Few	Circlet, rough
P6	7.60	Dirty white	Plentiful	Circlet, rough
P7	8.20	Dirty white	Few	Plenty of sclerotia, rough
P8	7.65	Dirty white	Plentiful	Circlet
P9	7.25	Dirty white	None	Smooth as it settled
P10	7.25	Dirty white	Plentiful	Sclerotia began to appear
P11P12	0	-	-	-
	6.30	Dirty white	Plentiful	Irregular wavy colony
P13	3.45	Green white	Plentiful	Rough
P14	8.15	Beige	Plentiful	Circlet
P15	3.65	White in the middle of the green	Plentiful	Rough
P16	8.25	Dirty white	Plentiful	Circlet, rough
P17	8.15	Dirty white	Plentiful	Circlet
P18	7.20	Dirty white	Plentiful	The circlet had formed sclerotia
P19	3.00	Green white	Plentiful	Rough
P20	7.05	Dirty white	Plentiful	Rough
P21	6.5	Dirty white	Plentiful	Rough
P22	6.55	Dirty white	Plentiful	Rough
P23	7.15	Dirty white	Plentiful	Rough, settled
P25	5.45	Dirty white	Plentiful	like a circlet, rough
P26	5.70	Dirty white	Plentiful	Formed a circle like a ring
P28	4.80	Dirty white	Plentiful	Rough, formed a circle
P29	4.95	Dirty white	Plentiful	Rough
P31	5.00	Dirty white	Plentiful	Rough, formed a circle
P32	2.35	Dirty white	Sparse	Rough, a light-dark circle was formed
P33	5.20	Dirty white	Plentiful	Rough, like a circlet
P39	5.1	Dirty white	Plentiful	Rough

This morphological appearance can be used as an initial grouping of these isolates. The isolation results in Table 2 show various variations in the morphological characters of the *R. solani* isolates, which were then identified according to color, hyphae, and profile. The results of the isolation produced 45 isolates, and 31 isolates were selected, which were suspected to be the *R. solani* for further identification. The results of this study found the diversity of *R. solani* isolates.

In general, the colony color of all isolates was dirty white. However, if the incubation period of each isolate on PDA media were extended, the color would be brown, and a sclerotium-like structure would form the stroma. According to Holliday (1989) cit. Irawati (2010), the color of the colonies of the isolates was related to the melanin content produced by the isolates. Melanin usually does not play a role in growth but increases endurance and competitiveness, especially in fungi.

According to Yulianti and Suhara (2010), the hyphae pigment of *Rhizoctonia* sp. generally varied, with the main color brown. Young colonies on artificial media are usually white or close to white, but with increasing age, the colonies will become dark brown. According to Danersen and Rasmussen (1996) cit. Irawati (2010), the formation of sclerotium is not the main feature of the *Rhizoctonia* genus. The formation of sclerotium is often influenced by the type of substrate in which this fungus grows, whereas monyloid cells are the precursors of sclerotia formation in the Rhizoctonia genus. The results of this study (Table 2) showed that, in general, the isolates grown in PDA formed sclerotium.

The research observations (Table 2) showed that all isolates had a lot of air mycelium. This result follows the opinion of Sneh et al. (1991) cit. Irawati (2010), that mushrooms grown in bright conditions will have more air mycelium than in other conditions. It is due to fungal hyphae growing following the direction of light (phototropy). Light plays an important role in the formation of the teleomorph phase of the *Rhizoctonia* sp. Sporulation occurs at night, and light stimulates hymenium formation but inhibits basidium maturation.

The effect of light on the growth of fungal vegetative hyphae is usually in the form of inhibiting or triggering their growth. According to Semangun (1988), mushrooms are generally hyaline pigmented (colorless). If the fungus is colored, then the fungus is pigmented, generally a melanin pigment bound to the cell wall of the hyphae.

The *R. solani* isolates grown in the PDA medium had different colony appearances from one another. The results showed that the isolates had the appearance of rings, rough and smooth. These results follow Irawati (2010), that light may affect the concentration of pigment production. With these two light-influenced factors, if a fungal culture is treated with alternating dark and light conditions, a colony morphology will form light-dark concentric circles. Therefore, concentric circles generally formed in continuous darkness and light-dark conditions are not visible. The isolation results found the presence of concentric circles such as rings with dark and light.

Observation of the colony growth rate showed that the colony growth rate of each isolate was different. Observation of this growth rate by growing isolates on PDA media was observed and measured on days 3, 5, 7, and 9 DAI (Days after Isolation). In general, the growth of *R. solani* was very fast. One isolate could grow to cover a 90 mm petri dish in three days. This fungus can live for several years by producing sclerotia in soil and plant tissues. The results of observations of colony growth rates of *R. solani* isolates are presented in Figure 1.

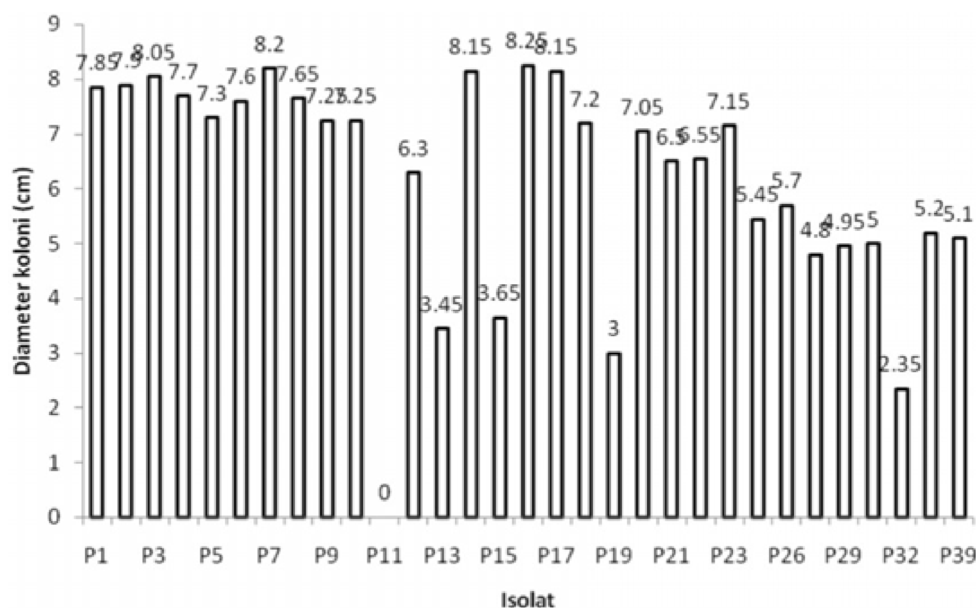

Figure 1. Bar chart of *R. solani* colony diameter on PDA day 3

The study's results on the colony growth rate (Figure 1) showed that each isolate had a different diversity of colony growth. The diameter measurement results of the observed isolates on the 3rd day showed that the highest isolate colony diameter was found in isolate P16 at 8.25 cm. In comparison, the lowest diameter of the isolated colony was found in isolate P11 at 0 cm. According to Irawati (2010), there is a relationship between the morphological character of the colony with the growth rate and level of pathogenicity of *Rhizoctonia*. The grouping of *Rhizoctonia* can be done based on the appearance of the colony morphology, but this is rather difficult to do because of the high diversity (variability) of this species.

The *R. solani* virulence assay on apples

The factors that influence the development of epidemic diseases are the level of virulence, the Number and type of inoculum approaching the host, the reproductive cycle (generation time), the environment in which the inoculum is formed, the resistance of the inoculum to the environment, the spread of the pathogen and its potential inoculum (Purnomo 2010)

The isolates that had been identified macroscopically were tested for virulence to determine the ability of the *R. solani* isolates to cause both symptoms and damage to apples. Testing on apples was the first step in selecting isolates suspected to have low virulence levels to be tested on host plants and one isolate suspected to have high virulence levels to be used as control. The parameter observed was the diameter of apple damage caused by *R. solani* isolates after inoculation. Virulence test observations were conducted on days 3, 5, 7, and 9. The results of virulence testing on apples were presented in the form of a bar chart with a standard deviation (Figure 2).

The observations of damage to apples on the ninth day after inoculation showed that the isolates of *R. solani* in each colony had different levels of infecting ability. These results indicate that the isolates tested gave the most serious damage to the apple fruit lesion in isolate P14 of 2.76 cm. Isolates that gave the lowest level of damage were isolates of P13, P22, P10, Control, P4, P7, and P16, each of which was 0 cm; 0.1 cm; 0.2 cm; 0.3 cm; 0.3 cm; 0.3 cm; 0.4 cm. Furthermore, several researchers, such as Syaifudin (2010), stated that fungi that received culture treatment would lose their pathogenicity after being transferred several times in the medium or after being stored for a long time.

According to Syaifudin (2010), various strains or origins (isolates) of a type of pathogen can vary in severity (virulence), depending on the genes contained in the nucleus or the material that acts as the nucleus. Furthermore, Gene arrangement can change due to various processes, so the virulence of a particular type of pathogen can change from time to time. For example, these changes can occur due to hybridization, heterochrony, and parasexuality. In addition, the pathogenicity of a pathogen is influenced by internal factors such as age and physical condition and external factors such as climate, environmental conditions, and agronomic measures, especially the use of antifungal and antimicrobial materials.

Furthermore, the researchers selected 20 isolates from the results of the virulence test on apples that were thought to have the lowest level of damage. One isolate with the highest damage rate as control was then tested on the host plant.

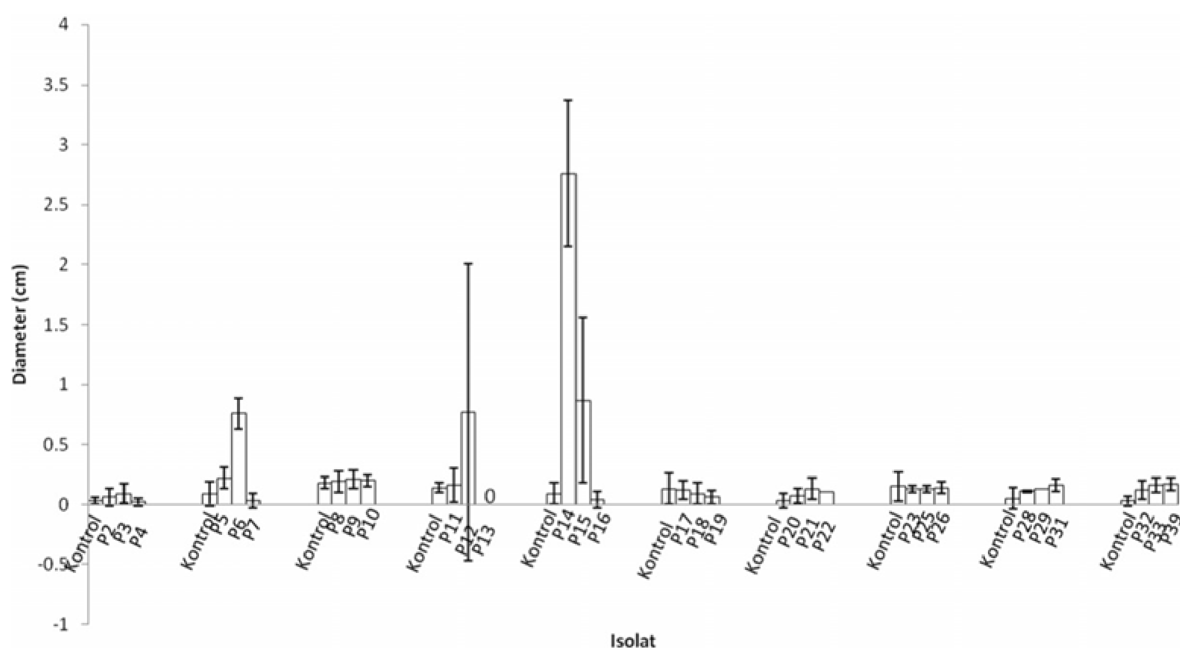

Figure 2. Diameter of damage to apples on day 9 (data were analyzed by means and standard deviation)

The *R.solani* virulence test on host plants

The results of the virulence test on apples were used as the first step in selecting isolates that were thought to have low virulence levels compared to positive controls. Then, 20 isolates from the test results on apples were selected to be tested on host plants. The test on the host plant was to determine the extent of damage to the host plant caused by the *R. solani* isolates, which had been characterized by color, hyphae, and colony diameter.

The virulence test on this host plant was conducted in a greenhouse with an average daily temperature of 25-40°C. This test used Memberamo rice varieties at 36 DAP (Days after Planting). The test on this host plant was to inoculate sclerotia on the rice midrib by inserting the sclerotia on the rice midrib by opening the rice midrib a little and then inserting the sclerotia clumps into it so that infection was expected between the pathogen and the host plant being tested. The data on the results of the virulence test on the host plant is presented in Figure 3.

Figure 3 shows that the isolates tested on the host plant had different pathogenicity in each isolate. This test on host plants aims to determine the relationship between morphological characters and the level of damage to the test plants. The results of virulence testing on 4 MSI (weeks after inoculation) host plants showed that the isolates suspected to be hypovirulent were isolates of K+ (P6); P28; P17; P16; and P26, each caused a spot area of 35.93%; 36.94%; 42.16%; 45.28%; 45.99%. While the isolate, which was initially suspected to be a hypovirulent isolate, when tested on the host plant, giving the highest spot area, which was isolate of P7 with 56.57%. Those follow Wakman (2004), who states that the extent of a disease attack is influenced by pathogens, hosts, and the environment. Pathogens with high pathogenicity that attack sensitive hosts with favorable environmental conditions will expand the symptoms of the attack. However, if one of these factors is not appropriate, the occurrence of the disease will be hampered.

According to Prayudi (2000), if the disease progresses to the flag leaf, the yield reduction can reach 20%. The higher the intensity of leaf midrib blight, the more it will disrupt yield stability. Efforts to control rice disease caused by *R. solani* are experiencing obstacles because the pathogen has a very diverse host and can survive for a long time in the form of sclerotium. In subtropical areas such as Japan, a sclerotium is a survival tool and a source of initial inoculum (X_0) for subsequent crops (Prayudi 2000). It happens because no other form of pathogen other than sclerotium can survive in winter. In the following season, sclerotium emerges on the soil surface from tillage and is ready to become a source of early inoculum for planting. In the tropics, other forms besides sclerotium are always available, so the role of sclerotium as a source of initial inoculum for planting is not dominant.

The *R. solani* that infects the host cell wall causes lesions on the midrib, which causes it to fall, and the exudate of the pathogen joins the flow of water transport, causing widespread attacks and increasing the intensity of the disease. This research follows Hadi et al. (1975) in Rosnawati (1991) cit. Winarni et al. (2004), where the high intensity of the disease was influenced by incubation, conidium density, and the ability of the pathogen to attack the bundle vessels in ginger plants which were closely related to water transport in plants because these pathogens were in the xylem and the conidium was transported by transpiration flow. The speed at which the transport is conducted also affects the speed at which the disease symptoms occur. The intensity of the disease is presented in Table 3.

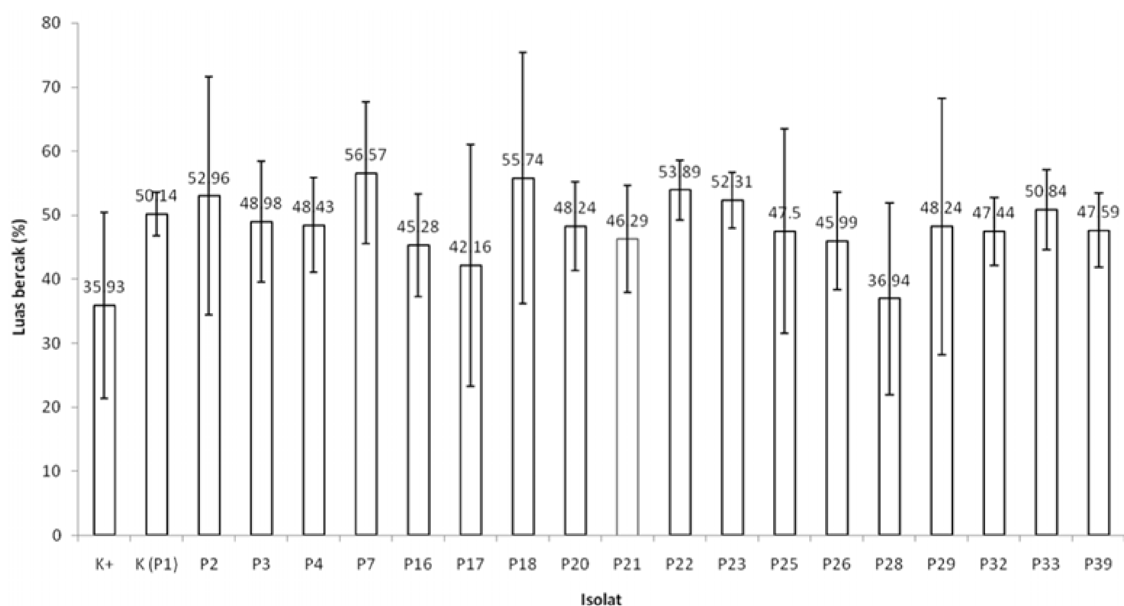

Figure 3. Percentage of *Rhizoctonia solani* spot area on host plants at 4 weeks after inoculation

Table 3. Disease intensity at 4 weeks after inoculation

Isolates	4th Week
K+	53.33ab
K (P1)	65.93 ab
P2	63.70 ab
P3	60.74 ab
P4	65.92 ab
P7	73.33 b
P16	59.26 ab
P17	55.55 ab
P18	69.63 ab
P20	59.26 ab
P21	62.96 ab
P22	63.70 ab
P23	65.18 ab
P25	62.22 ab
P26	62.96 ab
P28	46.67a
P29	62.96 ab
P32	62.96 ab
P33	69.63 ab
P39	62.96 ab

Information: Numbers followed by the same letter are not significantly different according to Duncan's Test 5 %

The results showed that the intensity of the disease caused by *R. solani* isolates fluctuated. The isolates suspected to be hypovirulent were isolates of K+(P6); P28; P17; P16; and P26 had disease intensity of 53.33%; 46.67%; 55.55%; 59.26%; 62.96%, respectively while isolate of P7 which was suspected to have high virulence had a disease intensity of 73.33%.

Research on *R. solani* isolates which were characterized macroscopically, virulence tests on apples and host plants were conducted to determine the virulence level of each isolate. The results of this study showed that isolates with relatively low virulence levels were found in isolates of K+(P6), P28, P17, P16, and P26 which had biological characteristics such as having a dirty white color, lots of air mycelium, a ring-like colony profile or forming concentric circles. Meanwhile, isolates with a high virulence level were found in isolate P7, which had biological characteristics such as dirty white color, little air mycelium, colony profile with lots of sclerotia, and roughness.

The results showed: (i) *R. solani* isolates from Karanganyar had a variety of biological characteristics (color, air mycelium, colony profile, and colony diameter). (ii) *R. solani* isolates from Karanganyar had various virulence levels. (iii) Isolates that were classified as hypovirulent were isolates of K+ (P6); P28; P17; P16; and P26, each caused a spot area of 35.93%; 36.94%; 42.16%; 45.28%; 45.99% in host plants and the isolate which was considered virulent was isolate of P7 which caused 56.57% of the spot area. (iv) Disease intensity in isolates of K+(P6); P28; P17; P16; and P26, respectively by 53.33%; 46.67%; 55.55%; 59.26%; 62.96%, and the disease intensity of P7 isolate which was virulent was 73.33%.

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