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Spore of Aspergillus niger photo by Michael Walther (CRANN NanoArt)

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Chapter in book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds) Tropical Forest Community Ecology. Wiley-Blackwell, New York.

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:

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.) Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

Thesis, Dissertation:

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Characterization of antibacterial activity produced by *Bacillus* spp. isolated from honey and bee-associated products against foodborne pathogens

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Abstract. *Magdalena S, Anggelia, Yogiara. 2020. Characterization of antibacterial activity produced by* Bacillus *spp. isolated from honey and bee-associated products against foodborne pathogens. Bioteknologi 17: 51-59.* Four potential isolates (*Bacillus velezensis* Y12, *Bacillus amyloliquefaciens* Y21, *Bacillus amyloliquefaciens* Y23, and *Bacillus velezensis* Y33) isolated from honey, propolis, and bee pollen from West Java, Indonesia showed antifungal and enzymatic activities. This study aimed to assay antibacterial activity against foodborne pathogens, determine the optimum condition for antibacterial compounds production, observe the effect of thermal and pH treatment on the antibacterial compounds, and detect the presence of antibacterial peptide biosynthesis gene. In this research, bacterial isolates showed antibacterial activity against *Bacillus cereus* ATCC 14579, *Salmonella enterica* 51741, and *Salmonella* Typhimurium ATCC 14028. The growth condition for antibacterial production of isolate *B. velezensis* Y12 was at the range of 25-60°C and pH 7-9. Meanwhile, the other three isolates showed the same pattern, also at the range of 37-60 °C. Antibacterial compounds against *B. cereus* were found heat stable at 4-90°C and active over pH 3-9. Cultivation of the isolates in BHIB and TSB did not significantly increase the antibacterial activity against all pathogens as compared to an LB medium. On the contrary, the antibacterial activity against *S. typhimurium* of the isolates cultivated in optimized medium two (yeast extract 32.5 g/L, glucose 33.4 g/L, $MnSO_4$ 0.042 g/L, $CaCl_2$ 0.031 g/L, KH_2PO_4 0.5 g/L, K_2HPO_4 0.5 g/L, $(NH_4)_2SO_4$ 1.0 g/L, and $MgSO_4$ 4.0 g/L) was significantly increased. Furthermore, seven antibacterial peptide biosynthesis genes primers were amplified from the genomic DNA of isolates *B. velezensis* Y12 and *B. velezensis* Y33 by PCR analysis. These genes were *fenD, srfAA, bacA, bmyB, ituC, ituD,* and *bmyD*. Meanwhile, there was no presence of *ituC* gene from the other isolates. This result suggests that isolate *B. velezensis*Y12 might be the most potential isolate for antibacterial compounds production.

Keywords: Antibacterial activity, *Bacillus*, foodborne pathogens, honey

INTRODUCTION

Microbes are potential resources of antimicrobial compounds that have novel mechanisms (Martinez-Klimova et al. 2017). Antimicrobial properties of honey and other bee-associated products such as a beehive, pollen, propolis, and royal jelly have been widely studied against pathogenic microorganisms, including bacteria and fungi. The inhibition of these microorganisms is due to the high osmolarity of sugar content, and high hydrogen peroxide compounds found in honey. The presence of some bacteria, mold, and yeast may also contribute to this activity in honey and other bee-associated products. The diversity of microorganisms found in honey can be expected because honey is not processed food. The microorganisms can originate from the environment like dust, dirt, air, pollen, flowers, other plants, and the digestive tracts of honeybee (Lee et al. 2008). Among all microorganisms, *Bacillus* spp. is one of the bacteria mostly found in honey samples. *Bacillus* has many potential antimicrobial compounds against bacteria or fungi plant pathogens, as reported by previous studies (Zhao et al. 2013). However, there has been no research on microorganisms in Indonesian honey and their potential microbial activities; even though Indonesia has various types of honey produced in different locations.

In our previous research, four potential isolates were isolated from honey, beehive, pollen, and propolis obtained from West Java, Indonesia (Purnawidjaja 2018). These bacterial isolates were identified as *Bacillus velezensis* and *Bacillus amyloliquefaciens*. The isolates had enzymatic and antifungal activities against *Candida albicans* ATCC 10231 and *Aspergillus fumigatus* ATCC 204305. However, there are still many potential antibacterial activities that could be assayed from these isolates. Characteristics of the antibacterial properties are considered to obtain information about the optimum growth conditions for antibacterial production. One of antimicrobial agents that recently emerged is an antimicrobial peptide (Mahlapuu et al. 2016). The antimicrobial peptide can potentially be used to combat the rapid increase in conventional antibiotics. In *Bacillus* strain, several antimicrobial peptides have been identified, such as fengycin, bacyllomycin, iturin, surfactin, bacilysin, subtilin (Mora et al. 2011; Sumi et al. 2015). The presence of those antimicrobial peptides can be detected by the presence of the gene *fenD, bmyB, ituC, srfA, bacA, spaS* (Mora et al. 2011).

This research aims to obtain potential antibacterial activity against foodborne pathogens, to obtain the optimum condition for cell growth and antibacterial production, to evaluate the stability of the extracellular antibacterial compounds, and to detect antibacterial peptide biosynthesis genes from genomic DNA of each isolate.

MATERIALS AND METHODS

Bacterial isolates and growth condition

Four *Bacillus* isolates used in this study were obtained from the culture collection of the Department of Food Technology, Atma Jaya Catholic University of Indonesia, Indonesia. These bacteria had been isolated from honey and other bee-associated products in previous research (Purnawidjaja 2018). The isolates were cultivated on Luria Agar (LA) and incubated for 24 h at 37°C. Gram-positive bacteria, *Bacillus cereus* ATCC 14579; and Gram-negative bacteria, *Salmonella enterica* subsp. *enterica* ATCC 51741 and *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 were used as bacterial indicators in antibacterial activity assay. All bacteria used as bacterial indicators were grown in Luria Broth (LB) for 24 h at 37°C.

Antibacterial activity assay

The antibacterial activity against bacteria was determined by the agar well diffusion method. Suspension of indicator bacteria was prepared according to the Clinical and Laboratory Standard Institute protocol (CLSI 2018) for the determination of antimicrobial activity. The cell suspensions were equivalent to 0.5 McFarland based on the absorbance value of 0.132 at 600 nm. The amount of 100 µL of bacterial suspensions was spread on the Mueller-Hinton Agar (MHA) plates. 6mm diameter wells were made by using a sterile cork borer.

Bacteria isolates were grown at 37ºC for 24 h in LB medium. The crude extract (cell-free supernatant) was obtained by centrifugation at 7800× g for 20 minutes. Each supernatant with the amount of 100 µL was loaded into the well and incubated at 37ºC for 24 h. The assay was carried out in triplicate. Streptomycin (25 µg) and Aztreonam (30 µg) antibiotic disc were used as a positive control. An uninoculated LB medium was used as a negative control. The inhibition was measured by the diameter of the clear zone (Lertcanawanichakul and Sawangnop 2008).

Optimization of cell growth for antibacterial compounds production

Bacteria isolates were cultivated in LB for 24 h and measured to reach 0.5 McFarland (OD_{600} = 0.132). For growth temperature optimization, the isolates were grown in LB medium at different temperatures ranging from 25°C, 37°C, and 60°C for 24 h. The antibacterial activity was determined by the agar well diffusion method, as previously mentioned. The growth of each isolate was verified by plating after 4 h, 8 h, and 24 h incubation. The optimum growth temperature was chosen based on the highest antibacterial production (Iqbal et al. 2018).

For pH optimization, bacterial isolates were grown in LB medium at different pH values (citrate buffer pH 4, phosphate buffer pH 7, and Tris buffer pH 9) and incubated at the optimum temperature. Antibacterial activity and plating of isolates were determined to evaluate the optimum pH value (Song et al. 2013).

Antibacterial compounds stability assay

The thermal stability of the crude extract of each bacterial isolate was determined by different temperatures ranging from 4, 37, 70, 90, and 121°C for 30 minutes. The pH stability was determined by adjusting the pH value with 0.1 M buffers: citrate buffer (pH 3 and 5), phosphate buffer (pH 7), and Tris buffer (pH 9) at 37°C for 2 h (Ramachandran et al. 2014). Enzymatic sensitivity was tested by treating the crude extract with the enzymes: papain (phosphate buffer pH 6.5), proteinase K (Tris buffer pH 8.5), and amylase (phosphate buffer pH 6.1) at final concentration 2 mg/mL for each enzyme. The crude extract was incubated for 2 h at different temperatures, depending on each enzyme treatment. Proteinase K and amylase were incubated at 37°C, bromelain at 55°C, and papain at 65°C. Each treatment was carried out in triplicate. The antibacterial activity was assayed after treatments by agar well diffusion methods against indicator strain (Alfonso et al. 2012).

Detection of antibacterial peptide biosynthesis genes

Genome isolation was carried out by using a DNA kit from SolGent™ Genomic DNA Prep Kit for Bacteria (Solution type) (Solgent Co., Ltd., South Korea). Isolated genomic DNA was used as a template for PCR reaction. Peptide biosynthesis gene was detected using PCR amplification method with specific primers (Table 1). The composition of PCR mixture was 1 µL DNA template, 1 µL of each forward and reverse primer, 22 µL of nuclease-free water, and 25 µL of 2X GoTaq Green master mix (Promega, USA). Amplification of no template control (NTC) was also included. PCR condition consisted of the initial denaturation step at 95°C for 4 minutes, 40 cycles of denaturation at 94°C for 1 minute, annealing step for 1 minute, elongation at 70°C for 1 minute, followed by final extension at 70°C for 5 minutes. The annealing temperature was set at 55°C for *bmyB*; 58°C for *ituC, bacA, fenD, srfAA,* and *bmyD*; and 50°C for *ituD*. PCR results were visualized by agarose gel electrophoresis at 1.5% agarose, 90 V for 60 minutes (Ramarathnam et al. 2007; Mora et al. 2011).

Media variation for antibacterial compounds production

Bacteria isolates were cultured in different liquid media such as Luria Broth (LB, 10 g/L tryptone, 5 g/L yeast extract, and 0.05 g/L NaCl), Brain Heart Infusion Broth (BHIB, 12.5 g/L brain infusion, 5 g/L beef heart infusion, 10 g/L peptone, 2 g/L glucose, 5 g/L NaCl, and 2.5 g/L Na_2HPO_4), Trypticase Soy Broth (TSB, 17 g/L pancreatic digest of casein, 3 g/L soybean meal, 5 g/L NaCl, 2.5 g/L dextrose, and 2.5 g/L K_2HPO_4), and two optimized media with different compositions.

Table 1. Gene primers for PCR amplification.

Primer	Sequence (5' → 3')	Gene	Product	Size (bp)	Source
FEND-F	GGCCCGTTCTCTAAATCCAT	*fenD*	Fengycin	269	Mora et al. 2011
FEND-R	GTCATGCTGACGAGAGCAAA				
SRFA-F	TCGGGACAGGAAGACATCAT	*srfAA*	Surfactin	201	Mora et al. 2011
SRFA-R	CCACTCAAACGGATAATCCTGA				
BAC-F	CAGCTCATGGGAATGCTTTT	*bacA*	Bacilysin	498	Mora et al. 2011
BAC-R	CTCGGTCCTGAAGGGACAAG				
BMYB-F	GAATCCCGTTGTTCTCCAAA	*bmyB*	Bacillomycin L	370	Mora et al. 2011
BMYB-R	GCGGGTATTGAATGCTTGTT				
ITUC-F	GGCTGCTGCAGATGCTTTAT	*ituC*	Iturin A	423	Mora et al. 2011
ITUC-R	TCGCAGATAATCGCAGTGAG				
ituD-F	ATGAACAATCTTGCCTTTTTA	*ituD*	Iturin A	1203	Hsieh et al. 2008
ituD-R	TTATTTTAAAATCCGCAATT				
BACC1-F	GAAGGACACGGCAGAGAGTC	*bmyD*	Bacillomycin D	875	Ramarathnam et al. 2007
BACC1-R	CGCTGATGACTGTTCATGCT				

Note: F-Forward Primer, R-Reverse Primer

Optimized medium 1 was adopted from Akpa et al. (2001). The medium was composed of sucrose 20 g/L, peptone 30 g/L, yeast extract 7 g/L, KH_2PO_4 1.9 g/L, $MgSO_4$ 0.45 g/L, and trace elements 9 mL/L. The composition of trace elements was 0.001 g of $CuSO_4$, 0.005 g of $FeCl_3$, 0.004 g of $NaMnO_4$, 0.002 g of KI, 0.014 g of $ZnSO_4$, 0.01 g of H_3BO_3, 0.0036 g of $MnSO_4$, 10 g of citric acid. Optimized medium 2 from Mosquera et al. (2014) was composed of yeast extract 32.5 g/L, glucose 33.4 g/L, $MnSO_4$ 0.042 g/L, $CaCl_2$ 0.031 g/L, KH_2PO_4 0.5 g/L, K_2HPO_4 0.5 g/L, $(NH_4)_2SO_4$ 1.0 g/L, and $MgSO_4$ 4.0 g/L.

All bacteria isolates were incubated at the optimum temperature for 24 h, and the antibacterial activity of the crude extract was assayed by the agar well diffusion method and carried in triplicate (Iqbal et al. 2018).

Statistical analysis

All data obtained from the research were analyzed by one way ANOVA with posthoc Tukey test using SPSS application to determine the significant differences between treatments ($P<0.05$).

RESULTS AND DISCUSSION

Antibacterial activity

Antibacterial activity assay showed that all isolates exhibited antibacterial activity against *B. cereus*, *S. enterica*, and *S. typhimurium* (Table 2). The highest activity was *B. amyloliquefaciens* Y21 against *B. cereus*, with a 10.3 mm zone of inhibition, among other tested strains.

Optimization of cell growth for antibacterial compounds production

Bacteria isolates were grown in various temperatures and pH levels to achieve the optimum condition for antibacterial compounds production. The selection of optimum range depended on cell growth and the inhibitory zones against pathogens.

It was shown that the optimum growth *B. velezensis* Y12 and *B. amyloliquefaciens* Y21,was achieved at 37°C, while *B. amyloliquefaciens* Y23 and *B. velezensis* Y33 was at 25°C. The highest antimicrobial activity at this optimum growth was achieved by *B. amyloliquefaciens* Y23, with a 13.0 mm of inhibition zone against *B. cereus*. All isolates could grow at 60°C even though the growth was lower than bacterial growth at 37°C.We can still observe antimicrobial activity against all pathogens, except for *B. amyloliquefaciens* Y21 (Figure 1). In *B. amyloliquefaciens* Y21, the antimicrobial compounds lose the activity against *S. enterica* and *S. typhimurium.*

Nevertheless, there is no significant difference between antimicrobial activity in various growth temperatures of *B. velezensis* Y12. In contrast, the antimicrobial compound that isolated from bacteria grown at 60°C showed significantly lower activity compared to other antimicrobial compounds from bacteria grown at 25 and 37°C. This might be due to the decrease in cell numbers that affected antimicrobial compound production.

As for pH study, the neutral condition (pH 7) exhibited the highest growth and antibacterial activity than the alkaline condition (pH 9). However, there was no significant difference between the results (Table 3). These conditions indicate that the pH condition was at the range of pH 7-9 for all isolates. Moreover, the acidic condition of pH 4 showed the lowest growth with no antibacterial activity was observed against all pathogenic bacteria.

Antibacterial compounds stability

Antibacterial compounds of each isolate remained stable over various heat treatments ranging from 4-90°C and various pH levels ranging from pH 3-9 against *B. cereus* (Figure 2). However, the antibacterial compounds showed no activity against *S. enterica* and *S. typhimurium* at heat treatment above 70°C and below pH 7. The antibacterial compound activity remained stable at 4-70°C and pH 7-9 for isolates *B. amyloliquefaciens* Y21 and *B. amyloliquefaciens* Y23. Antibacterial activity for the other two isolates significantly declined when exposed to higher pH values (Figure 3).

Table 2. Antibacterial activity of bacteria isolates against foodborne pathogens.

Isolates	Zone of inhibition (mm)		
	B. cereus **ATCC 14579**	***S. enterica*** **ATCC 51741**	***S. typhimurium*** **ATCC 14028**
B. velezensis Y12	9.3	6.5	6.5
B. amyloliquefaciens Y21	10.3	5.3	6.0
B. amyloliquefaciens Y23	9.3	4.7	5.3
B. velezensis Y33	8.0	5.3	7.5

Table 3. Antibacterial activity of cell culture grown in various pH levels.

Isolates	Growth condition	Zone of inhibition (mm)		
		B. cereus **ATCC 14579**	***S. enterica*** **ATCC 51741**	***S. typhimurium*** **ATCC 14028**
B. velezensis Y12	pH 4	-	-	-
	pH 7	11.5[a]	6.0[a]	7.0[a]
	pH 9	8.7[a]	5.0[a]	5.0[a]
B. amyloliquefaciens Y21	pH 4	-	-	-
	pH 7	10.3[a]	5.7[a]	7.0[a]
	pH 9	8.3[a]	4.7[a]	5.0[a]
B. amyloliquefaciens Y23	pH 4	-	-	-
	pH 7	13.7[a]	5.7[a]	6.3[a]
	pH 9	11.3[a]	4.7[a]	6.3[a]
B. velezensis Y33	pH 4	-	-	-
	pH 7	11.7[a]	7.0[a]	7.3[a]
	pH 9	10.0[a]	6.0[a]	6.3[a]

Note: [a,b] represent a significant difference between treatment (P<0.05)

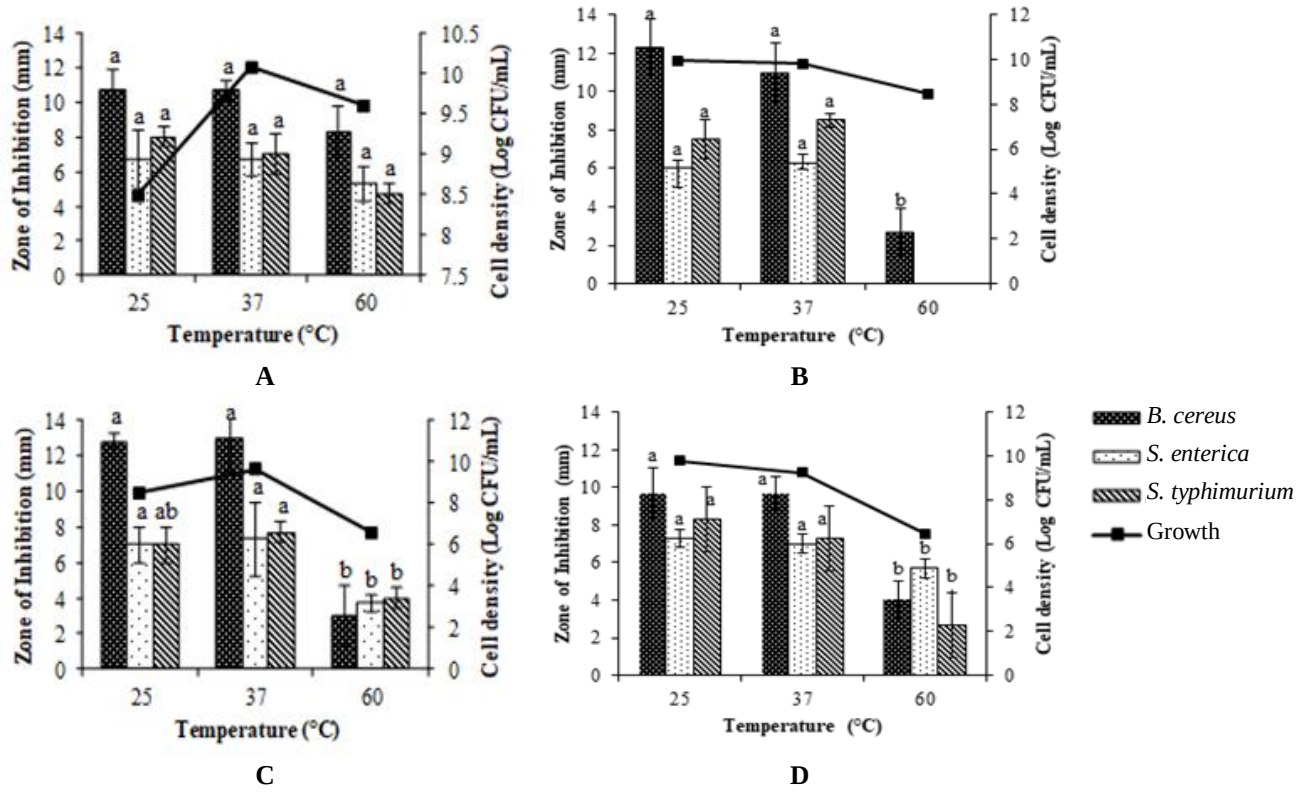

Figure 1. Effect of temperature variation on *Bacillus velezensis* Y12 (A), *B. amyloliquefaciens* Y21 (B), *B. amyloliquefaciens* Y23 (C), *B. velezensis* Y33 (D), growth (line) and antibacterial activity (column) against *B. cereus, S. enterica,* and *S. typhimurium.*

The significant decline of antibacterial activity was found after all antibacterial compounds being treated by papain and bromelain enzyme against *B. cereus* (Figure 4.A), except for *B. velezensis* Y33. The antibacterial compound isolated from *B. velezensis* Y33 lost its activity after being treated by papain and bromelain. Hence, these papain and bromelain treatment caused the loss of antibacterial activity of all antibacterial compounds against *S. enterica* and *S. typhimurium* (Figure 4.B). There was no effect after exposure to α-amylase and proteinase-K enzyme compared to control treatment without the addition of enzymes.

Detection of antibacterial peptide biosynthesis genes

As Figure 5 indicates, all DNA amplicon bands were visible, showing that *fenD, srfAA, bacA, bmyB, ituC, ituD,* and *bmyD* genes were present in genomic DNA of isolate *B. velenzensis* Y12. The same results were obtained for isolates *B. velezensis* Y33. Meanwhile, the other two isolates results showed no indication of *ituC* gene.

Media variation for antibacterial compounds production

All four isolates exhibited antibacterial activity on different media. The results showed that cultivation on BHIB and TSB media did not alter much the antibacterial activity against *B. cereus, S. enterica,* and *S. typhimurium* as compared to LB medium. Meanwhile, cultivation on optimized medium 1 (Akpa et al. 2001) lowered the activity of all isolates. Another optimized medium with different compositions adopted from Mosquera et al. (2014) study showed slightly increased activity against *S. typhimurium* (Figure 6).

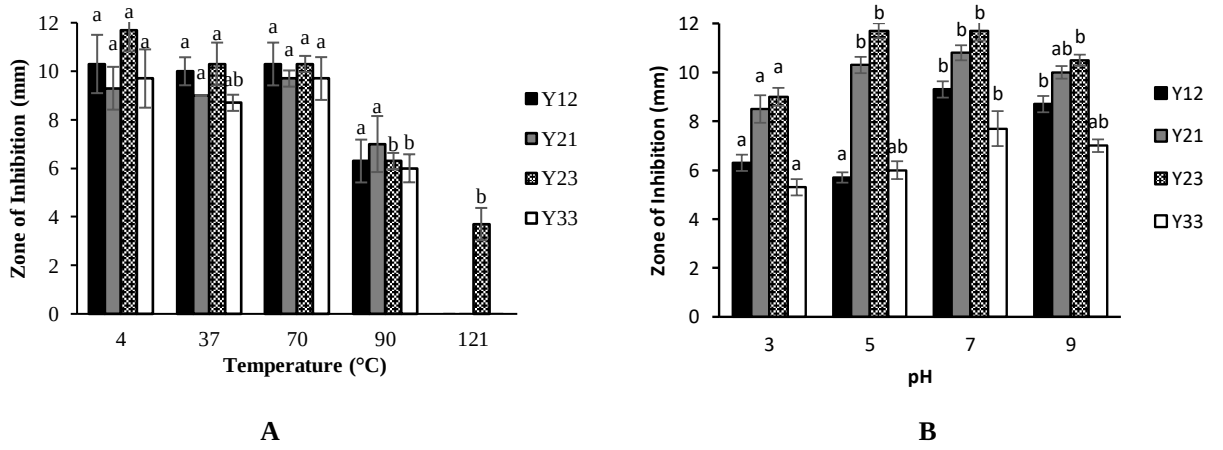

Figure 2. Heat (A) and pH (B) treatment on antibacterial activity against *Bacillus cereus.*

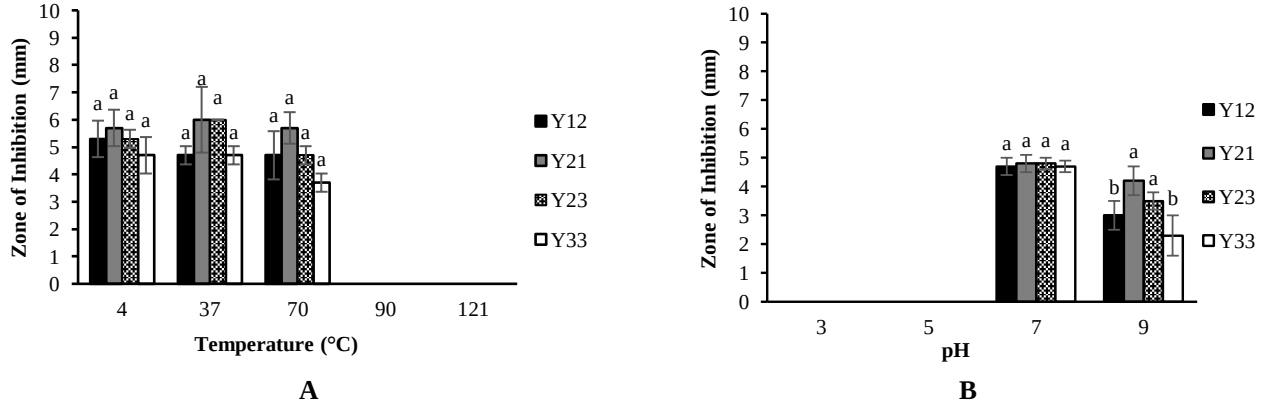

Figure 3. Heat (A) and pH (B) treatment on antibacterial activity against *Salmonella enterica.*

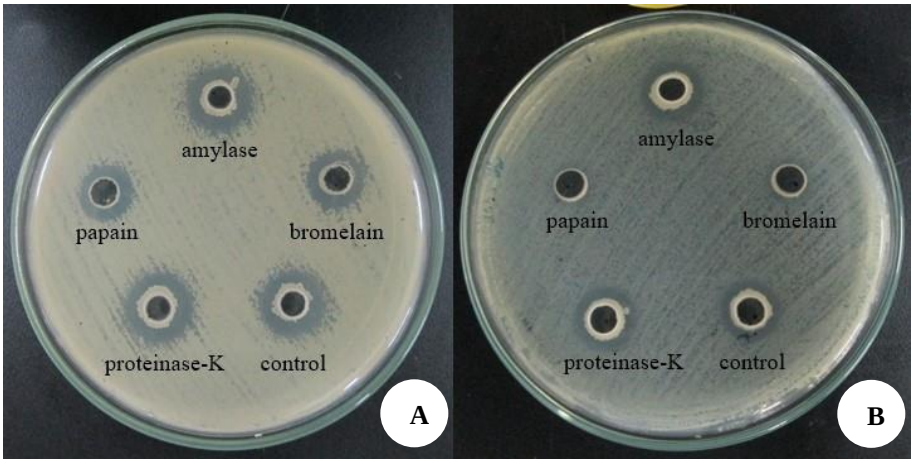

Figure 4. The results of enzymatic sensitivity assay of the antibacterial compound as represented by antibacterial compound isolated from *Bacillus amyloliquefaciens* Y23 against *B. cereus* (A) and *Salmonella enterica* (B).

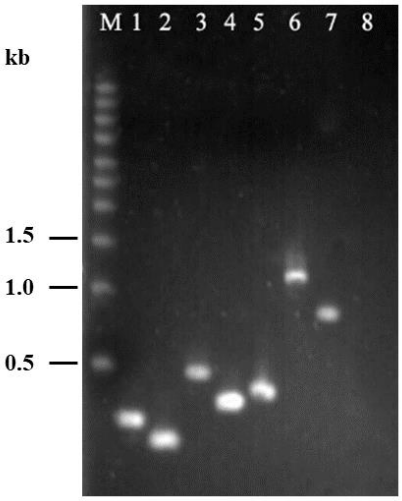

Figure 5. Representative gel image of PCR amplification results for isolate *Bacillus velezensis* Y12. Lane M is 1 kb DNA ladder, Lane 1-8, *fenD, srfAA, bacA, bmyB, ituC, ituD, bmyD,* and NTC, respectively.

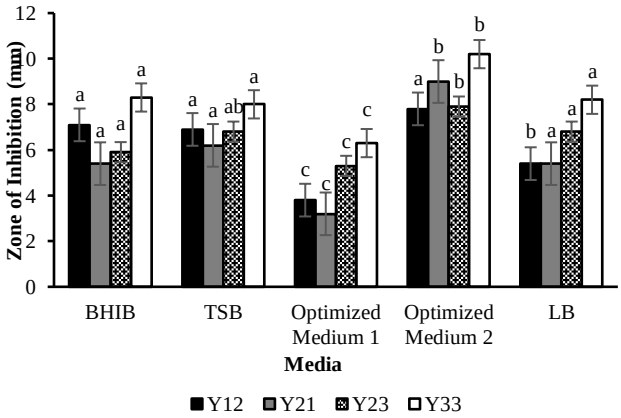

Figure 6. Antibacterial compounds production against *Salmonella typhimurium* in various media.

Discussion

The isolates of *B. velezensis* Y12, *B. velezensis* Y33, *B. amyloliquefaciens* Y21, and *B. amyloliquefaciens* Y23 exhibited antibacterial activity against all tested foodborne pathogens. *Bacillus* spp. is known as a cosmopolitan species due to its ability to survive in an adverse environment (Lee et al. 2008). This genus also produced beneficial compounds like antibacterial compounds with various action mechanisms. The activity of these compounds, especially antibacterial peptides, has been extensively explored against plant pathogens as one of the biocontrol agents (Li et al. 2016). *B. subtilis*, isolated from the honey sample and bee gut, showed activity against important honeybee pathogens, which were *Paenibacillus larvae* and *Ascophaeraapis* (Sabate et al. 2009). Several strains of *Bacillus* isolated from soil also produced compounds against pathogenic and food-spoilage bacteria such as *B. cereus* and *Listeria monocytogenes* (Lisboa et al. 2006).

Cell growth in various conditions, like temperature and pH medium, affected the production of antibacterial compounds. Various studies on incubation temperature revealed that *B. velezensis* Y12 showed the highest growth at 37°C yet showed no difference in terms of antibacterial compounds production at lower and higher temperatures. This indicates that production could be achieved regardless of optimum temperature. Meanwhile, the result of three isolates *B. amyloliquefaciens* Y21, *B. amyloliquefaciens* Y23, and *B. velezensis* Y33 showed the same pattern. As reported by Iqbal et al. (2018), temperature affected the antimicrobial activity in the way of denaturing the compounds to some extent. This might cause loss of antibacterial activity during heat exposure. Moreover, high temperature also decreased cell growth of isolate Y23 (Figure 1). It was indicated that antibacterial compounds were produced along with the cell growth of each isolate.

The condition of pH in the cultivation medium also affected the production of the antibacterial compound. The treatment of the acidic medium was not suitable for the growth of every isolate. There was no activity shown by isolate which was cultivated in pH 4 medium (Table 3). The cell growth was also inhibited, showing that the production of antibacterial compounds occurred in line with the bacterial cell growth. Both pH 7 and pH 9 of media were the conditions in which bacterial isolate could grow and produce antibacterial compounds better than acidic conditions. The result of each treatment indicated no significant difference. Thus, the medium of antibacterial compounds production could be adjusted to higher pH values.

In Moshafi et al.'s (2011) study, *Bacillus* sp. strain FAS_1 was isolated from soil produced antibacterial compounds at alkaline condition pH 8.4. There were changes in pH value as the biomass of cells increased. This condition shows that antibacterial compounds might not be associated with organic acids as the pH value continued to increase during cell growth. A similar finding was also found in Motta et al.'s (2004) study demonstrating that the inhibitory effect of *Bacillus* sp. isolated from the Amazon basin was not caused by organic acids, as the pH value increased during antibacterial production. The preferable condition was at 37°C with a neutral pH cultivation medium for further steps.

Antibacterial compounds were heat-stable, starting from 4°C until 70°C against all foodborne pathogens. Moreover, antibacterial compounds were stable until 90°C against *B. cereus* (Figure 2). In a study by Teixeira et al. (2013), antibacterial peptides produced by *Bacillus subtilis* could retain their activity until exposure at 80°C. The ability to retain antibacterial activity was also reported by Risoen et al. (2004), stating that the compounds had stable structures that could resist heat treatment. On the contrary, the present study suggests that exposure to extreme heat might cause the loss of antibacterial activity due to different characteristics of each compound produced by bacterial isolates. However, heat-stable antibacterial compounds were suitable for food applications which could be adapted to a harsh environment including those with higher temperatures (Sutyak et al. 2008).

Treatment of antibacterial compounds with various pH conditions showed no loss of activity against *B. cereus*. The compounds were stable over pH 3-9, with a slight decrease in activity for the treatment of pH 3 (Figure 2). A previous study stated that bacteriocin-like inhibitory substance (BLIS) from *B. amyloliquefaciens* LBM 5006 against *B. cereus* was stable over pH 3-8 (Lisboa et al. 2006). As for the activity against *S. enterica* and *S. typhimurium*, there was no loss of activity for the treatment of pH 7 and pH 9; however, there was loss of activity when the compounds were exposed to lower pH conditions, such as pH 3 and pH 5 (Figure 3). Another study by Ramachandran et al. (2014) reported that *B. subtilis* RLID 12.1 could retain 100% activity between pH 6 and pH 8. This ability indicates that bacteria isolates produced more than one compound to attack different kinds of pathogenic bacteria, which had different tolerance range for heat and pH treatment. Consequently, antibacterial compounds should be handled in suitable conditions to prevent loss of activity.

Proteolytic enzyme sensitivity test represented proteinaceous compounds of antibacterial compounds produced. The results of the papain and bromelain treatment show significant reduction against *B. cereus* (Figure 4). In the same treatment, antibacterial compounds showed no activity against *S. enterica* and *S. typhimurium*. Based on Sabate and Audisio (2013), antibacterial compounds were found active after treatment with proteinase-K. In agreement with that study, the other proteolytic enzyme which was proteinase-K did not eliminate or decrease the antibacterial activity of each isolate. This might happen due to the enzyme activity or the compounds itself. Proteolytic enzymes are divided based on their mode of action. Papain and bromelain actively cleave peptide bonds by the presence of cysteine at the active site. Proteinase-K is the group of serine protease whose activity is determined by the presence of serine (Walsh 2002).

Furthermore, *Bacillus* spp. produced cyclic peptides with unusual amino acids. This relatively rigid structure caused protease resistance due to the inaccessible cleavage site (Bizani and Brandelli 2002; Korenblum et al. 2005). The same results were obtained from glycolytic (α-amylase) enzyme treatment of antibacterial compounds that retained the antibacterial activity. This result indicates that there was no carbohydrate moiety involved in antibacterial activity. According to Zhao et al. (2013), inhibition of various pathogen bacteria and fungi is detected because *Bacillus* spp. can produce different kinds of antibiotics, enzymes, amino acids, or peptide.

A further step to detect the presence of antibacterial peptides biosynthetic genes was conducted using PCR analysis. The result showed that specific primer bands matched the sizes of PCR products conducted in previous research. Antimicrobial peptides, which are mostly cyclic lipopeptides, are non-ribosomally synthesized by enzymes called non-ribosomal peptide synthetases. Each gene encoded different products of peptides, including *fenD* for fengycin synthetase, *srfA* for surfactin synthetase subunit 1, *bacA* for bacilysin biosynthesis protein, *bmyB* for bacillomycin L synthetase B, *bmyD* for bacillomycin D synthetase C, *ituC* and *ituD* for iturin A synthetase (Ramarathnam et al. 2007; Hsieh et al. 2008; Mora et al. 2011).

Fengycin is cyclic decapeptide, whose biosynthesis consists of five genes (*fenA-E)*, including *fenD* conducted in this study. The mechanism of antagonistic activity was shown by the pore formation of the cell membrane (Gong et al. 2015). Surfactin is a cyclic lipoheptapeptide which alters membrane integrity and permeability by channel formation mechanism against several microorganisms (Meena and Kanwar 2015). Another lipoheptapeptide compound, iturin, consists of iturin A, C, D, and E, along with bacillomycin D, F, and L. Bacillomycin D has *bmy*gene clusters including *bmyD*, *bmyA*, *bmyB*, and *bmyC*. Iturin operon has four open reading frames, specifically, *ituA*, *ituB*, *ituC*, and *ituD*. Similar tofengycin, iturin also had pore formation activity against target bacteria (Maget-Dana and Peypoux 1994). The application of iturin has widely spread in food or pharmaceutical products. Meanwhile, bacilysin is a dipeptide compound composed of L-alanine and L-

anticapsin as an inhibitor of cell wall biosynthesis. It also has (*bacA-E*) gene clusters (Arguelles-Arias et al. 2009; Chen et al. 2009).

PCR amplification results of *B. velezensis* Y12 and *B. velezensis* Y33 align with a study by Palazzini et al. (2016), showing that fengycin, surfactin, iturin, bacillomycin, and bacilysin were identified in the genome sequence of *B. velezensis* RC 218 isolated from wheat anthers. As for *B. amyloliquefaciens* Y21 and *B. amyloliquefaciens* Y33, the lack of *ituC* gene shows that iturin A might not be present as one of the antibacterial peptides. Moreover, *ituD* gene is an essential gene in the biosynthesis of iturin A because its disruption caused iturin A deficiency (Tsuge et al. 2001).

Many factors influence the ability of bacteria to produce antibacterial compounds, such as the conditions of cultivation and nutrition of medium. These conditions include temperature, pH, and incubation period. Based on the results, the preferable condition was at 37°C with pH 7. The medium composition along with the metabolic capacity of each bacterium also played a significant role in the biosynthesis of antibacterial compounds (Kiranmayi et al. 2011). As reported in the study from Muhammad et al. (2015), nitrogen-rich medium (BHIB and TSB) enhanced both cell growth and antimicrobial compounds production than simple nutrient medium. In this study, cultivation in such media resulted in no significant enhancement of antibacterial activity. This might happen due to the complex genetic regulation of each bacterial isolates. Lisboa et al. (2006) also stated that the condition of cultivation might induce different peptide antibiotics production. Therefore, the selection of carbon and nitrogen source was critical, depending on bacteria strain (Ripa et al. 2009).

Besides, the incubation time influenced antibacterial compounds production. Jin et al. (2015) found that the production of iturin by *Bacillus subtilis* gradually achieved after the exponential growth phase and reached to maximum level during stationary phase after 24 h. Different lipopeptides, surfactin, and bacillomycin production were induced during stationary phase (Chen et al. 2009). A study by Coutte et al. (2010) showed that fengycin was produced at the end of exponential phase and continued until the next phase. Cyclic lipopeptides produced by *Bacillus* spp. showed the same tendency because those compounds are non-ribosomal peptides. The metabolites are more likely to be produced at the late phase of bacterial growth (Kumar et al. 2012).

Bacterial isolates cultivated in optimized medium 1 showed a reduction in activity (data not shown) as opposed to the previous study by Iqbal et al. (2018), showing that *Bacillus safensis* MK-12 grown in the optimized medium had the highest activity among other cultivation media. Akpa et al.'s (2001) study stated that cultivation of *B. subtilis* NT02 in the same optimized medium yielded homologous products identified as bacillomycin in vigorous intensity. These findings suggest that the production of antibacterial compounds depends on bacteria strain and its ability to produce different compounds by utilizing nutrients in the medium.

Another study by Mosquera et al. (2014) reported that higher cell density led to a higher activity of *B. subtilis*, which was achieved by optimizing the medium with a high dose of glucose and yeast extract. Glucose was the preferable source of carbon and energy. Meanwhile, yeast extract contained amino acids and nucleotides as precursors of cell biomass and antibacterial compounds. In this study, the results of antibacterial activity against *S. typhimurium* and *S. enterica* showed enhancement. Modification of nutritional factors in cultivation medium might promote the possibility of a new antibacterial compound biosynthesis. The selection of carbon source also played a significant role. As discussed in Liu et al. (2012), surfactin, a lipopeptide antimicrobial compound, was produced optimally by *Bacillus* sp. MB199 in medium with fructose as the carbon source and ammonium nitrate as the nitrogen source. The regulation of carbon and nitrogen source differed from each bacterial strain, as stated by previous studies.

Different sources of carbon (fructose, maltose, dextrose, mannitol, sucrose, and lactose) and sources of nitrogen (meat peptone, beef extract, yeast extract, tryptone, meat infusion extract, and malt extract) were also used in Kumar et al.'s (2012) study. The result showed that antimicrobial activity differed significantly when the sources were changed. In addition, the study also revealed that eliminating carbon source caused the reduction of antimicrobial compounds production. Therefore, further research is required to manipulate nutrients and determine the best source of carbon and nitrogen in the cultivation medium to achieve a higher activity in the inhibition of foodborne pathogens.

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Identification, aflatoxin content, and antagonistic test of spoilage fungi in bread to *Aspergillus niger*

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Abstract. *Fendiyanto MH, Satrio RD. 2020. Identification, aflatoxin content, and antagonistic test of spoilage fungi in bread to Aspergillus niger. Bioteknologi 17: 60-66.* The major problem in shelf storage life in bread is contamination by molds, fungi and mycotoxins. The growth of spoilage fungi indicates that the bread has structural damage. Infection from this fungus will affect the shelf life of the bread to be shorter. Contaminated bread may influenced human health in the future. One of the many solutions to overcome the fungi-contamination of bread is the use of biological agents. *Aspergillus niger* has the ability, as a biological agent, to suppress food-destroying pathogens in many foods, including bread. However, there are few reports about the antagonistic test in bread, particularly between *A.niger* as biological control agent to spoilage fungi. Therefore, this study aimed to analyze the growth antagonistic test of *A. niger* against food spoilage fungi, expecting the storage life of bread could be extended and mycotoxin contamination avoided. The antagonistic test was in the form of a test that utilizes the properties of microorganisms that grow faster than pathogens or produce antibiotic compounds. The methods used in this study included isolation and identification of fungi, water content analysis, aflatoxin test, and antagonistic test. Interestingly, the antagonist test results showed *A. niger* can inhibit the growth of spoilage fungi on bread. In conclusion, the species of fungus with the highest inhibition value was *Hyphopichia burtonii*, while the lowest was *Saccharomyces cerevisiae*. These findings indicate that *A.niger* can be used as a biological control in extending bread shelf storage in the future.

Keywords: Antagonist test, *Aspergillus niger*, spoilage fungi

INTRODUCTION

The success of global development is determined by the availability of quality human resources (HR) which includes strong minds and excellent health, in addition to mastery of science and technology. The quality of a country's human resources will be low if there is malnutrition; as people whose health is disturbed, their intelligence decreases. Therefore, the main step in creating superior human resources is by improving the quality of nutrition. The continued impact of the decline in the nutritional status or health of these nutrient vulnerable groups in the long term will reduce the quality of Indonesia's human resources (Syarief 1997). FAO classifies Indonesia as a low income and food shortage country. The number of poor people in Indonesia is close to 50 million, of which, 32.7 million live in rural areas (BPS 2001). Fulfilling food needs for energy sources in activities. Food provides energy and stamina. Consuming food will increase vitality so that it is possible to live longer, not tire easily, increase immunity and help keep the body fit and healthy (Marshal 2005).

Carbohydrates are one of the most common macronutrients and the cheapest source of calories (Handewi and Sallem 2002). These nutrients are the main source of calories for much of the world's population, especially residents of developing countries. Carbohydrates have an important role in determining the characteristics of food ingredients such as taste, color, and texture. Whereas in the body, carbohydrates are useful for preventing ketosis, breaking down excessive body protein, losing minerals, and for helping metabolize fat and protein (Winarno 2008). It is recommended that carbohydrate foods be consumed by 6-11 servings per day. Example food sources of carbohydrates include bread, rice, cereals, and pasta (Astawan and Kasih 2008).

Bread is a group of carbohydrate foods that should be consumed in about one-third of the diet. Bread, like pasta, potatoes, rice, and other cereals, is the best food source of energy. These foods also contain complex B vitamins to release energy from food and maintain a healthy digestive and nervous system. The recommended amount of carbohydrate foods is 6 or more servings per day (Marshal 2005). Bread is made from wheat flour that is spread with yeast and baked. Bread has become one of the staple foods for Indonesians, even among teenagers and children. The position of this food is starting to shift from rice as the main source of carbohydrates, because of its nutritional content compared to rice or noodles.

Bread is a flour product that is easily damaged, especially due to fungi attack. The types of fungi that often contaminate food are mold and yeast. Fungi are microbes consisting of more than one cell in the form of fine threads called hyphae, a collection of hyphae is called mycelium, reproducing by spores, or dividing (SNI 7388; 2009). As a result of growing fungi on bread, that bread cannot be

consumed. This is unfortunate considering that bread is a food that contains carbohydrates, and can be the main source of energy. In addition, the fast growth of fungi on bread often causes bakers to experience losses due to the low storage capacity of bread (BSN 2009).

The major problem for the shelf life of bread is mold fungal infection. Commonly, bread is contaminated by mold spoilage during storage, especially being contaminated after spreading of the fungi spores during storage (El Sheikha 2016). The mold will grow rapidly in the bakery products as well, particularly *Aspergillus, Fusarium*, and *Penicillium*. The environment sterility in the bakery is low, as usually the room contains mold spores and the dust containing spores spreads easily in the air (El Sheikha 2016). According to the report by El Sheikha (2016), there are 8000 mold spores per 1 gram of flour. Therefore, the crucial problem in the bakery industry is the presence of fungi spores and its potentially dangerous mycotoxins (El Sheikha 2016).

Microorganisms that cause food spoilage are bacteria, molds, and yeasts (Aryulina et al. 2005; Shehata et al. 2008; Pundir and Jain 2011). They are always present in specifically in the air and water in bread. They can activate many enzymes and furthermore, cause changes in color, flavor, and texture of the bread. Mold fungi can attack many loaves of bread, spread mycotoxins, and influence human health (El Sheikha and Mahmoud 2016). Mycotoxins of *Aspergillus, Fusarium*, and *Penicillium* are generated during the growth of molds on loaves of bread. Some of the mycotoxins are only present in the mold tissues, while most of them are excreted into the environment like loaves of bread (El Sheikha and Mahmoud 2016). Mold growth can be prevented by storing bread in low humidity conditions. The first molds that can colonize are of genus *Eurotium*, followed by *Aspergillus*, *Penicillium*, and *Fusarium.* The mold will produce mycotoxins and change the bread's texture. Filamentous molds are more easily recognized than other microbes like yeast or bacteria (El Sheikha and Mahmoud 2016).

Control of fungal contamination in bread can use several methods, i.e., reformulation to reduce bread Aw, freezing, preservatives, and the effect of bio preservatives. The use of biological controls in bread was never performed; this method is necessary to avoid spoilage fungi and to explore novel compounds against spoilage fungi in bread (El Sheikha and Mahmoud 2016). Nowadays, to detect the mycotoxigenic fungi in food has progressed, i.e., the use of molecular markers (Fendiyanto et al. 2019). The molecular markers to detect the toxins in the broad application are PCR-DGGE (El Sheikha 2019), RAPD, SSR, ISSR (Pratami et al. 2020), and ITS (El Sheikha et al. 2018). All the methods are able to detect many spoilage fungi species in food, including bread (El Sheikha et al. 2018). Besides molecular markers, there are many other markers, i.e., morphological markers (Pratami et al. 2019), metabolomics markers (Fendiyanto et al. 2020), and EST markers (Satrio et al. 2019).

According to El Sheikha (2015), food is safe if it is properly handled at all steps of production, processing, distribution, from retail and foodservice business through consumption. Safe food is unlikely to cause illness or injury. A major challenge in the food industry is food supply chains (El Sheika 2015). Therefore, this study aimed to analyze the growth of *Aspergillus niger* against spoilage fungi on bread as an effort to increase the shelf life of bread.

MATERIALS AND METHODS

Fungi isolation

Isolation media is a medium that contains nutrients for fungal growth. The isolation medium used to isolate postharvest fungi that were commonly used is DG18 media. Dichloran 18% Glycerol Agar (DG18) media is a medium for isolating xerophilic fungi. Xerophilic fungi are fungi that grow and develop on substrates with low water content, such as cereals, nuts, flour, nutmeg, and other spices. The selective or differential isolation media used to isolate *Aspergillus flavus*, *A.parasiticus*, and *A.nomius* were *Aspergillus flavus* and *Parasiticus* Agar (AFPA) media (Pitt et al. 1983; Zummo and Scott 1990). DG18 media is suitable for the growth of fungi *Eurotium* sp., *Aspergillus restrictus*, *A. penicillioides*, *Wallemia sebi*, *Trichoderma*, and *Endomyces*. DG18 media contained chloramphenicol and dichloran. Chloramphenicol was used to avoid contamination by bacteria, while dichloran was used to limit the growth of fast-growing fungi such as *Mucor* and *Rhizopus*. The composition of DG18 media included glucose 10 g, peptone 5 g, KH_2PO_4 1 g, $MgSO_4.7H_2O$ 0.5 g, glycerol A.R. 220 g, 15 g agar, 2 mg dicloran, 100 mg chloramphenicol, and 1 liter of distilled water. All the ingredients were mixed simultaneously and cooked and sterilized using an autoclave.

Fungi identification

Fungi identification was performed by two methods, namely the method of identification media and the method of fungi morphology on the microscope. The media used to identify were CYA, CY20S, MEA, and G25N media. The fungi isolated on DG18 media were transferred to identification media. Fungi were transferred using an inoculation loop and the fungi transferred had to be pure cultures. The pure cultures obtained were then grown on standard media, namely CYA, MEA, and G25N with an incubation temperature of 25°C and 37°C for seven days (Pitt et al. 1992). Fungi identification was carried out after seven days of incubation. The observations included: colony diameter, colony character, and making wet preparations. The colony diameter was determined using a ruler in mm. The diameter of the fungus was calculated on the reverse side of the Petri dish. Observations of colony characteristics included colony color, surface texture, and color of exudate produced by fungi (Pitt and Hocking 2009).

Water content test

The method of analyzing moisture content was performed by using the oven or distillation method (BSI 1980). In this study, the analysis of water content in bread

used the distillation method. The principle of this method was the difference in the boiling point of water and xylol. The equipment used for the analysis of moisture content included a 500 ml boiling flask, Aufhauser device, electric bath, and analytical balance. A sample of 5-10 grams was weighed and put in a boiling flask, with an addition of 300 ml of xylol and a boiling stone. The boiling flask was connected to the Aufhauser appliance and heated over an electric bath for one hour from the time it started to boil. After just one hour, the electric bath was turned off and the Aufhauser appliance was allowed to cool. The coolant was rinsed using pure xylol or toluene. The amount of water volume in the instrument was then read. The calculation of water content was done using the following formula adopted from Satrio et al. (2019):

Water content = w/v x 100%
Where:
w: weight of sample in grams
v : volume of water read on the Aufhauser device in ml

Aflatoxin test

The aflatoxin analysis method used was high-performance liquid chromatography (HPLC). Bread samples that were already in the form of a solution mixed with xylol, were homogenized using a vortex device and then injected into the HPLC device. The principle of separation of materials by HPLC was to determine the presence of aflatoxins based on the retention time and the area of the curve produced by the standard aflatoxin curve. If there was a curve that has the same retention time as the standard, then the sample contains aflatoxin. The retention time and curve width was detected using a computer that was integrated with the HPLC tool (Ambarwati et al. 2011).

Antagonistic test

The antagonist test was carried out by growing the *Aspergillus niger* fungus and other types of fungi that had been isolated in one plate. Inhibition diameter was calculated by measuring the diameter of the inhibition zone on the isolated fungi based on the results of the isolation of the fungus on bread (Nakkeeran and Zhang 2005; Khalimi and Wirya 2008). The antagonistic test was carried out by comparing the results of the inhibition zone between one fungus and another. Calculations were made by calculating the diameter of hyphae that did not grow on fungi (Pitt et al. 1992). The calculation of the inhibition of fungi was done by using the following formula:

Inhibition (%) = (a-b)/a x 100%

Where:
a: length from the center of the fungus to the edge of the fungus on the side opposite the side that is blocked.
b: length from the center of the fungus to the edge of the fungus on the inhibited side

RESULTS AND DISCUSSION

Isolation and identification of fungi on bread

Bread that has been stored for a long time, or has passed its shelf life, will have many microorganisms that appear on it. Fungi and bacteria can grow on bread that has passed its expiration date. Variable *Trichosporon*, *Saccharomyces*, *Pichia*, and *Zygosaccharomyces* are fungi that are usually found in bread. Contamination of *Saccharomyces* sp. will cause the bread to have white spots, this is called chalk bread (Saranraj 2011; Amadi and Adeniyi 2009). Similar to Saranraj (2011), *Saccharomyces cerevisiae* was found in this fungal isolation of our bread samples (Figure 1). According to dominantly fungi identification, we simultaneously found *Aspergillus tamarii* on sample RT1, *Saccharomyces cerevisiae* on sample RT2, *Cladosporium sphaerospermum* on sample RT3, *Aspergillus flavus* on sample RT4, and *Aspergillus flavus* on sample RT5 (Figure 1). Many species of *Aspergillus* were found in many foods, particularly rice, sorghum, and wheat. Traditionally, bread was made from wheat. Therefore, on seven-day shelf life of bread in this study, spoilage fungi were commonly found in samples RT1, RT4, and RT5. Surprisingly, we successfully identified *Aspergillus flavus* on sample RT4, indicating bread in seven-day shelf life has potentially mycotoxins, particularly aflatoxin. This toxin was reported to periodically affect vital organs, i.e., liver, lung, heart attack (Probst et al. 2007; Ambarwati et al. 2011). Therefore, it is necessary to study mycotoxin activity on bread to avoid the health risk of the toxin (Amadi and Adeniyi 2009). Regarding fungi identification, *Aspergillus tamarii* had white abaxial hyphae and green-yellowish spores. According to this study, *Saccharomyces cerevisiae* had a texture resembling bacteria, as it was white and did not have hyphae. *Cladosporium sphaerospermum* had greenish-white spores and grew in many colonies in RT3 samples (Figure 1). *Aspergillus flavus* had a light-yellow texture with white abaxial hyphae. The colony diameter of *Aspergillus flavus* in RT5 was relatively larger compared to RT4 (Figure 1).

In general, the fungi that appeared on the identification results of the fungi at the seven-day shelf life were *Saccharomyces cerevisiae, Cladosporium sphaerospermum, Hyphopichia burtonii,* and *Aspergillus tamarii* (Table 1). *Cladosporium sphaerospermum, Hyphopichia burtonii,* and *Aspergillus tamarii* included postharvest destructive fungi (Amadi and Adeniyi 2009). Postharvest destructive fungi, based on Saranraj (2011) and Ambarwati et al. (2011), was called as spoilage fungi.

Saccharomyces cerevisiae is not a destructive fungus, so there are no disadvantages with the presence of this fungus in the bread sample (Ahmad 2005). In bread RT3, the fungus that grew was more varied. The most dominant fungus in the bread RT3 was *Saccharomyces cerevisiae*. In bread RT5, the dominant growing fungus was *Saccharomyces cerevisiae*.

Many of the post-harvest destructive eukaryotes-fungi (spoilage fungi) grew in all samples, except for RT5. It was periodically caused by the differences in water content

among samples (Saranraj 2011; Ambarwati et al. 2011). Therefore, it is important to check the water content in each sample.

The dominant spoilage fungi in the seven-day bread sample was *Cladosporium sphaerospermum*. In our hypothesize, the growth of the fungus can be inhibited with *Aspergillus niger*. Therefore, it is necessary to have an antagonist test to determine the inhibitory level between *Aspergillus niger* and the postharvest fungal *Cladosporium sphaerospermum*, and other fungal spoilage from bread samples stored for seven days after expiration.

In general, the most common fungi that appear in the results of the experiment to identify fungi at a shelf-life of thirty days are *Saccharomyces cerevisiae*, *Cladosporium sphaerospermum*, *Aspergillus flavus*, *Eurotium chevalieri*, and *Aspergillus tamarii*. The most important thing that was obtained from the experimental results on the bread with a shelf life of thirty days, was the appearance of the *Aspergillus flavus*. This fungus is an aflatoxin-producing fungus which is very dangerous for the body and can cause chronic disease (Probst et al. 2007; Ambarwati et al. 2011).

The species of fungi were isolated and identified from bread stored for seven days with thirty different days. The diversity of destroying fungi was more dominant in the bread samples that were stored for seven days. This could have happened because of the moisture of the bread that was seven days old was higher. However, the type of fungi isolated from bread in thirty days old had more potential to produce aflatoxins. It was indicated by the growth of *Aspergillus flavus* in thirty-day-old bread samples. In addition, bread RT3, RT4, RT5 had a wider variety of spoilage fungi than bread accession RT1, and RT2 (Table 1,2). This can be demonstrated in either a seven-day or thirty-day sample of bread (Table 1, 2).

Water content analysis

The highest water content in the samples stored for seven days belonged to bread RT2. While the lowest water content was owned by bread accession RT5. In general, the water content of RT1 and RT2 was higher than that of bread accessions RT3, RT4, and RT5 (Table 3).

Table 1. Fungi identification of bread with a shelf life of seven days.

Sample code	Sample weight (g)	Species	Number of colonies			1/dilution factor	Fungi population (colony / g wet weight)
			Replication				
			1	2	3		
RT 1	25.02	*Aspergillus tamarii*	4	1	2	10^4	1.30×10^4
		Hyphopichia burtonii	6	7	7	10^4	6.67×10^4
		Saccharomyces cerevisiae	70	69	-	10^5	6.95×10^6
RT 2	25.00	*Hyphopichia burtonii*	14	21	17	10^5	1.73×10^6
		Paecilomyces variotii	4	4	6	10^5	4.67×10^5
		Saccharomyces cerevisiae	508	644	-	10^5	5.76×10^7
RT 3	25.03	*Cladosporium sphaerospermum*	132	263	206	10^5	2.00×10^7
		Hyphopichia burtonii	9	6	6	10^5	7.00×10^5
		Paecilomyces variotii	14	10	7	10^2	1.03×10^3
		Saccharomyces cerevisiae	184	240	-	10^5	2.12×10^7
RT 4	25.03	*Hyphopichia burtonii*	16	18	24	10^3	1.93×10^4
		Saccharomyces cerevisiae	400	460	-	10^5	4.30×10^7
RT 5	25.00	*Saccharomyces cerevisiae*	-	448	416	10^5	4.32×10^7

Table 2. Fungi identification of bread with a shelf life of thirty days.

Sample code	Sample weight (g)	Species	Number of colonies			1/dilution factor	Fungi population (colony / g wet weight)
			Replication				
			1	2	3		
RT 1	19.00	*Aspergillus tamarii*	10	4	9	10^4	7.67×10^4
		Cladosporium sphaerospermum	20	22	23	10^4	2.17×10^4
		Saccharomyces cerevisiae	4	2	-	10^4	3.00×10^4
RT 2	19.00	*Saccharomyces cerevisiae*	20	11	11	10^5	1.40×10^6
RT 3	25.00	*Aspergillus flavus*	3	5	6	10^2	4.67×10^2
		Cladosporium sphaerospermum	30	28	22	10^5	2.67×10^6
		Eurotium chevalieri	14	10	12	10^4	1.20×10^5
		Saccharomyces cerevisiae	41	41	-	10^6	4.10×10^7
RT 4	25.03	*Aspergillus flavus*	1	5	3	10^5	3.00×10^5
RT 5	25.01	*Aspergillus flavus*	2	3	4	10^3	3.00×10^3
		Saccharomyces cerevisiae	324	392	-	10^3	3.58×10^5

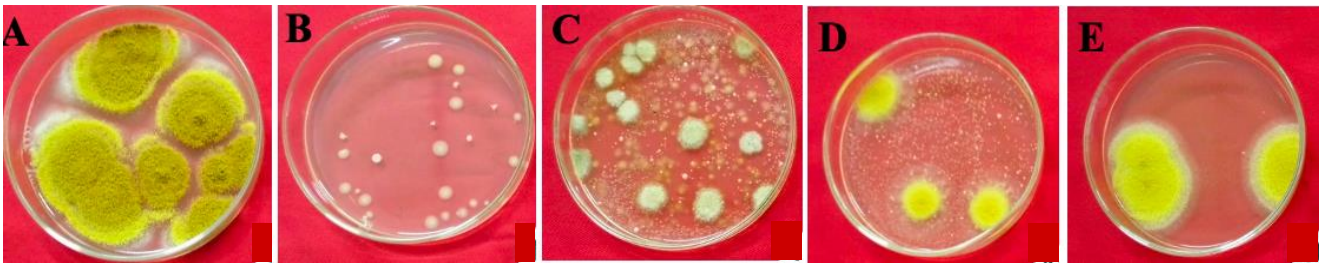

Figure 1. Isolation of the bread's fungus; *Aspergillus tamarii* on plain bread 1 (RT1) (A); *Saccharomyces cerevisiae* on plain bread 2 (RT2) (B); *Cladosporium sphaerospermum* on plain bread 3 (RT3) (C); *Aspergillus flavus* on plain bread 4 (RT4) (D); *Aspergillus flavus* on plain bread 5 (RT5) (E).

Table 3. Water content analysis of bread samples at seven and thirty days after storage.

Number	Bread Samples	Shelf life (days)	Water Content (%)	Method
1	RT 1	7	39,86	Gravimetry
2	RT 2	7	40,77	Gravimetry
3	RT 3	7	25,13	Gravimetry
4	RT 4	7	29,20	Gravimetry
5	RT 5	7	21,21	Gravimetry
6	RT 1	30	37,44	Gravimetry
7	RT 2	30	37,29	Gravimetry
8	RT 3	30	21,15	Gravimetry
9	RT 4	30	21,59	Gravimetry
10	RT 5	30	23,15	Gravimetry

Table 4. Biotoxicology test of bread samples based on aflatoxin B1, B2, G1, and G2.

Number	Bread's Samples	Aflatoxin content (ppb)			
		B1	B2	G1	G2
1	RT 1	< 1	< 2	< 1	< 2
2	RT 2	< 1	< 2	< 1	< 2
3	RT 3	< 1	< 2	< 1	< 2
4	RT 4	< 1	< 2	< 1	< 2
5	RT 5	< 1	< 2	< 1	< 2

The highest water content in the samples stored for seven days was bread RT2. While the lowest water content was in bread RT5. In general, the water content of fresh bread was higher than that of sandwiches. The moisture content between samples stored for seven days and samples stored for thirty days was relatively different. The moisture content of the samples at the shelf life of thirty days was lower than the moisture content of the samples at the shelf life of seven days. This is because the water vapor pressure in bread food is higher than the water vapor pressure in the environment, so the water in food will move outside the environment so that the water content will decrease further. The longer the storage period, the moisture content in the bread will decrease until at a certain point the water vapor pressure in the bread is equal to the water vapor pressure in the outer environment (Gibson et al. 1994; Addy 2007).

The water content suitable for the growth of *Aspergillus flavus* in the samples at the shelf life of seven days and the samples at the shelf life of thirty days, is owned by the sandwich samples. However, the fungus *Aspergillus flavus* only grew on samples stored for thirty days. This difference is caused by the growth period of the *Aspergillus flavus* fungus which takes a long time (Hedayati et al. 2007; Ambarwati et al. 2011).

Aflatoxin and antagonistic test of spoilage fungi to *A.niger*

The results of the aflatoxin test research on bread had the same levels for different types of aflatoxins. For aflatoxin B1 and G1 toxins, the aflatoxin content is lower than 1 ppb. Whereas aflatoxin B2 and G2, the aflatoxin content is lower than 2 ppb. The aflatoxin content was the same for all bread samples. The aflatoxin content in the sample bread was still below the recommended standard. Low aflatoxin content has shown to not cause toxins for humans (Brown et al. 1999; Ambarwati et al. 2011).

The results of the antagonist test showed that fungus was inhibited by *Aspergillus niger*. The type of fungus with the highest inhibition value was *Hyphopichia burtonii*, while the lowest was *Saccharomyces cerevisiae* (Table 6). Thus, *Aspergillus niger* has the potential to be used to inhibit destructive fungi in bread food.

A mycotoxin is a toxin that comes from fungi, one of which can be in the form of aflatoxins. Aflatoxins synthesized by *A. flavus* have four types of toxins, namely aflatoxins B1, B2, G1, and G2. The four types of aflatoxins are reported to be found in several foods such as '*gado-gado', 'karedok', 'ketoprak'* and in nutmeg and rice (Ambarwati et al. 2011). In this study, we confirmed that *A. flavus* can also be found in expired bread. On the other hand, the use of *A. niger* as a biocontrol agent can have a significant effect on some staple foods such as grain and nut foods. Therefore, antagonistic testing is necessary to understand the mechanism of biocontrol agent in various fungi species. According to the antagonistic test, *A.niger* effectively inhibits *Hyphopichia burtonii, Paecilomyces variotii, Aspergillus tamari, Aspergillus flavus,* and *Eurotium chevalieri*.

Table 5. Antagonistic analysis of spoilage fungi to *A.niger* as biocontrol.

Number	Species of spoilage fungi	*A. niger* inhibition (%)	Spoilage fungi inhibition (%)	Total inhibition (%)
1	*Aspergillus flavus*	0.00	51.23	51.23
2	*Aspergillus tamari*	7.96	59.44	51.48
3	*Paecilomyces variotii*	0.00	60.00	60.00
4	*Saccharomyces cerevisiae*	0.00	34.72	34.72
5	*Hyphopichia burtonii*	0.00	70.02	70.02
6	*Eurotium chevalieri*	0.00	45.15	45.15
7	*Cladosporium sphaeospermum*	0.00	nd	nd

Note: nd was similar to "not determined"

In conclusion, *A. niger* can be used as a biocontrol agent against various spoilage fungi with broad species inhibition on bread. The species of fungus with the highest inhibition value was *Hyphopichia burtonii*, while the lowest was *Saccharomyces cerevisiae*. In future studies, this finding can provide preliminary information for the metabolomics approach to understand many novel compounds in *A. niger* against spoilage fungi in the cell level in various foods.

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Isolation and identification of Ice Nucleation Active (INA) bacteria causing *embun upas* (frost injury) on leaves of potato plant in the Dieng Plateau, Central Java, Indonesia

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Abstract. *Rajasa AK, Susilowati A, Suranto. 2020. Isolation and identification of Ice Nucleation Active (INA) bacteria causing embun upas (frost injury) on potato plant leaves in the Dieng Plateau, Central Java, Indonesia. Bioteknologi 17: 67-74. Embun upas* (frost injury) is one of the symptoms of ice crystal formation that occurs on the leaves of potato plants (*Solanum tuberosum* L.) in the Dieng area, Central Java, Indonesia. Symptoms of *embun upas* occur on a large scale and cause a decrease in potato yields of up to 6 tons per hectare. The cause of the symptoms of *embun upas* on potato plants is suspected to be the activity of ice-core-forming bacteria. This study aimed (i) to obtain isolates of Ice Nucleation Active (INA) bacteria from potato plants in Batur District, Dieng, (ii) to determine the INA bacterial species which can be identified using the gene encoding 16S rRNA, and (iii) to determine the population density of INA bacteria in one gram of plant leaves. Sampling used the purposive sampling technique by taking potato leaves at three different altitudes: ±1,800 m above sea level (Bakal Village), ±2,000 m above sea level (Diengkulon Village), and ±2,200 m above sea level (Sembungan Village) with two repetitions. Bacterial isolation was carried out using the spread plate method on Nutrien Agar media containing 2.5% glycerol (NAG). Colonies with different morphology were taken and purified on slanted agar media. Ice nucleation activity was determined by the tube nucleation test method. INA bacteria were estimated using the multiple-tube nucleation test 3,3,3 method. Identification of INA bacteria was carried out by amplifying the gene encoding 16S rRNA by the PCR method. The gene encoding 16S rRNA amplicon was purified using PCR/DNA Fragments Extraction Kit (Genaid) and continued with determining the concentration and purity of DNA using a biophotometer. Sequencing was carried out by sending samples to 1st BASE Singapore, and the data from the sequencing results were analyzed using the BLAST Nucleotide device on the NCBI website. Analysis of the data used is descriptive analysis. The isolation results obtained in this study were six isolates of INA bacteria in all sampling villages. The bacterial species obtained were *Arthrobacter sulfonivorans* (98%), *Curtobacterium luteum* (98%), and *Pseudomonas azotoformans* (99%). The total bacterial population on potato leaves was 1.44 x 102 MPN INA/gram in Bakal Village, 3 x 103 MPN INA/gram in Diengkulon Village, and 1.824 x 105 MPN INA/gram in Sembungan Village.

Keywords: 16S rRNA, Dieng, *embun upas*, INA bacteria, potato

INTRODUCTION

The Dieng Plateau is an area located in the province of Central Java, Indonesia. Dieng is mainly located between Banjarnegara District and Wonosobo District and Temanggung District and Kendal District. Most of the Dieng area is fertile land with cold temperatures. Dieng Plateau is 1,500-2,500 meters above sea level (m asl). Dieng has an average rainfall of 4,000-7,000 mm in October-May, but in June-September, the rainfall decreases by 2,000-3,500 mm (Nijman and Van Balen 1998).

The air temperature in the Dieng area with an altitude below 1,500 m asl is different from the area with an altitude above 1,500 m asl. At an altitude below 1,500 m asl, the air temperature ranges from 22-34°C with an average of 26°C. In areas above 1,500 m asl, the average air temperature is 14°C. In the Dieng Plateau, an area above 1,500 m asl, frost can form, especially during the dry season (Arwiyanto 1996).

Potato (*Solanum tuberosum* L.) is one of the primary commodities cultivated in the Dieng highlands. Many people have been cultivating this plant since 1974. The productivity of potato plants from 1975 to 1990 has increased very significantly, where yields can reach 20-25 tons per hectare. In the year 2000, potato production experienced a drastic decline. The yield obtained is only 16 tons per hectare (Sularso 2009). It is due to the many land problems in the Dieng Plateau. These land management problems do not apply soil and water conservation principles only; potato plants are very susceptible to environmental and temperature balance in the Dieng Plateau and plant diseases that attack cultivated plants (Bondansari et al. 2011).

The main problem with potato plantations in the Dieng area is the phenomenon of frost injury or *embun upas* called by local people. It has a detrimental impact on potato farmers. According to Arwiyanto (1996), *embun upas* occurs during the dry season, and it is recorded that more than 35 ha of potato plantations are affected by the *embun upas* phenomenon. The loss value of potato farmers reaches hundreds of millions of rupiah.

Embun upas not only occurs in Indonesia but also in several countries in the world. In the United States, it occurs on maize, tomatoes, wheat, and some lignosus crops

such as mangoes, oranges, and apples. *Embun upas* is also found in Japan, namely cabbage, broccoli, and mulberry plants (Goto et al. 1988). Lindow (1983a) proved that *embun upas* that occurred in some of these plants resulted from the activity of epiphytic bacteria on the leaf surface. These bacteria are harmful to plants and can cause plant death.

According to Gurian-Sherman and Lindow (1993), bacteria that can cause *embun upas* can be Ice Nucleation Active (INA) bacteria. INA bacteria have active proteins located on the cell surface, which function to increase the temperature of ice core formation. The protein in INA bacteria is called INP (Ice Nucleation Protein). Several species of bacteria that are INA bacteria are *Pseudomonas syringae, Pseudomonas fluorescens, Pseudomonas viridiflava, Xanthomonas campestris, Erwinia herbicola* (Lindow 1983b), and *Erwinia ananas* (Goto et al. 1989).

In the Americas, INA strains of *P. syringae* and *E. herbicola* bacteria have been detected on the leaf surface of corn plants. INA bacteria can cause *embun upas* at -2°C to -4°C. Leaf surfaces that do not detect the presence of INA bacteria will experience *embun upas* at a temperature of around -20°C. Detection and identification of INA bacterial species can be made by comparing the biochemical activity between isolates of one bacterial species and another (Arwiyanto 1996). However, along with advances in science and technology, researchers use molecular methods, namely identification with the gene encoding 16S rRNA. This molecular method has a higher accuracy and validity level than the comparison of biochemical activity between bacterial species. The time required for molecular identification is faster and shorter, with a lower error rate than other methods (Lindow et al. 1978; Goto et al. 1989; Chakravorti et al. 2007). Therefore, research related to INA bacteria suspected as the cause of *embun upas* on potato plants in the Dieng Plateau and identification using the 16S rRNA encoding gene needs to be carried out.

In addition to the activity of INA bacteria on the leaf surface, leaf damage is also influenced by the large population of INA bacteria on the leaf surface. The more the people of INA bacteria on the leaf surface, the faster the plant damage will occur. The calculation of the bacterial population on the plant surface can be done using the Most Probable Number (MPN) method. The MPN method determined the bacterial population per gram of plant leaves (APHA 1975).

Based on these problems, the research objectives are (i) obtaining INA bacterial isolates from potato plants in Batur District, Dieng, (ii) knowing the INA bacterial species from potato plants in Batur District, Dieng, to be identified using the gene encoding 16S rRNA, (iii) knowing the population density of INA bacteria in one gram of potato plant leaves.

MATERIALS AND METHODS

Research site

This research was carried out from November 2014 to February 2015 at the laboratory of the Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Central Java, Indonesia, and the Biotechnology Laboratory of Universitas Katolik Atmajaya (Unika), Jakarta, Indonesia. A sampling of potato plants (*S. tuberosum*) was carried out in November 2014 at the potato plantation, Dieng Plateau, Central Java, Indonesia.

Ingredient

The leaf samples were potato plant leaves originating from Dieng Plateau and growing above 1,500 m asl. The leaves taken were old leaves of the third order from the shoots, which had brownish spots on the leaf surface and leaf with a slightly dry texture. Materials for culture media include nutrient agar (NA) weighing 6 grams, 1 L aquadest, and 25 mL glycerol.

Materials for bacterial isolation and pure bacterial culture include potato leaves weighing 5 grams that have been cut into small pieces, phosphate buffer pH 7 0.1 m, pure bacterial isolate, and 0.1% peptone meat. DNA extraction materials and amplification of the 16s rRNA encoding gene include DNA templates, presto™ mini gDNA bacteria kit, Kapa 2g fast ready mix, reverse primer, forward primer, PCR mix, tae buffer, and ddh2o.

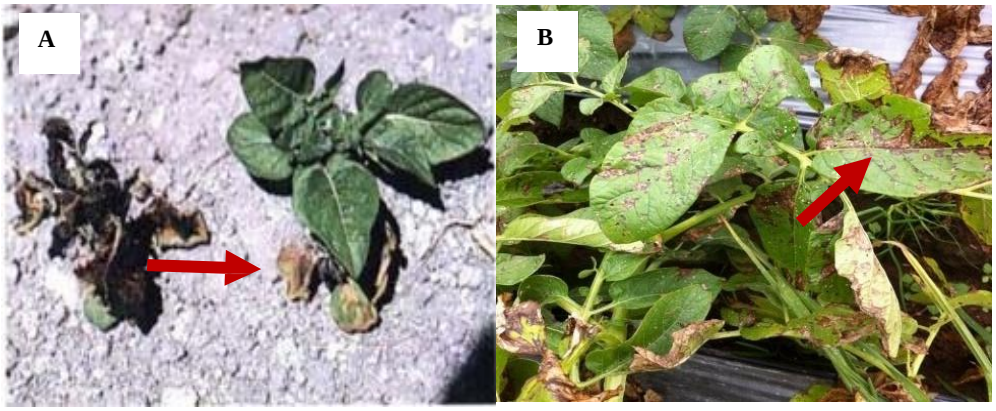


Figure 1. A. Damage to potato leaves infected with INA bacteria based on research by Lindow (1983a). B. Potato leaves suspected of being infected with INA bacteria in the Dieng Plateau, Central Java, Indonesia

Procedure

*Potato leaf sampling (*Solanum tuberosum *L.)*

A sampling of potato leaves was carried out in November 2014 in potato plantations in Batur District, Dieng Plateau, with a purposive sampling method based on altitude. Potato leaf samples were taken at an altitude above 1,500 m asl. Sampling was carried out in three villages with different altitude ranges, namely Bakal Village with a height of ± 1,800 m asl, Dieng Kulon Village with an altitude of ± 2,000 m asl, and Sembungan Village with an altitude of ± 2,200 m asl. Potato leaves are cut using a cutter at an angle to the stem, then put in a plastic clip. Leaf samples that have been taken must be immediately brought to the laboratory using a cooler box for isolation at <24 hours. While in the laboratory, the sample was put in a refrigerator at a temperature of about 5°C so that it could be isolated for the next few days.

Sterilization of tools and materials

Petri dishes, test tubes, Erlenmeyer, NA, and other tools that will be used are sterilized first. Petri dishes are sterilized first before being packaged using old newsprint. The sterilization process was carried out by wet sterilization using an autoclave at a pressure of 1 atm and a temperature of 121°C for 20 minutes.

Preparation of nutrient agar with 2.5% glycerol (NAG) media and inclined media

NA weighing 6 grams was put into a 250 mL Erlenmeyer tube, then added 200 mL of distilled water into the Erlenmeyer. Stir until homogeneously mixed. The homogeneously mixed solution is added with 5 mL of glycerol, then stirring is done again until blended. Erlenmeyer covered with cotton and aluminum foil, then heated using a microwave for 2 minutes. Erlenmeyer was then sterilized by autoclave at 121°C, 1 atm, for 20 minutes. The sterilized media was then poured into a petri dish aseptically in a laminar airflow cabinet (LAFC). The dry and solid nag media cup was then wrapped in old newsprint and stored upside-down at room temperature or in an incubator. If the media is not contaminated, then the media is ready for use (Kartika 2009).

The first process carried out in the manufacture of inclined media is that nag is inserted into the test tube up to a quarter of the tube. Then the test tube was closed using cotton and aluminum foil and put in an autoclave for sterilization. The nag in a sterile test tube is then placed at an angle of 35° or tilted until the nag medium reaches half of the tube. The slanted media is left at room temperature, and if there is no contamination and it has solidified, it can be used (Kartika 2009).

Preparation of 0.1 m phosphate buffer pH 7 with 0.1% peptone meat

Phosphate buffer pH 7.0 was made with 0.6 grams of monosodium phosphate ($NaH_2PO_4.2H_2O$) and 1.6 grams of disodium phosphate heptahydrate ($Na_2HPO_4.7H_2O$) dissolved in 1 L of sterile distilled water and added with 1 gram of peptone meat. Stirring is done so that the solution is homogeneously mixed. Furthermore, the phosphate buffer medium was sterilized using an autoclave at a temperature of 121°C and a pressure of 1 atm for 20 minutes.

Estimation of bacterial count INA

The number of INA bacteria was estimated using the multiple tube nucleation method (Cazorla et al. 1995). The test tube containing 9 mL of sterile phosphate buffer was cooled at -10°C for 30 minutes. Then the tubes were shaken, and all the frozen tubes were separated. The tube containing the unfrozen phosphate buffer is heated to 5°C. Potato plant samples weighing 2 grams were homogenized in 20 mL phosphate buffer media and 0.1% peptone meat, then 1 mL was taken and put in a test tube containing 9 mL sterile phosphate buffer. Furthermore, three dilutions series were carried out, namely 10^{-1}, 10^{-2}, 10^{-3}, into a tube containing unfrozen phosphate buffer. Each dilution series was carried out in three replications so that a series dilution of 3.3.3 tubes was obtained. The diluted bacterial suspension was put in an alcohol bath at a temperature of -5°C for 10 minutes (Cazorla et al. 1995).

The number of frozen test tubes was counted at each dilution. The total population of INA bacteria/gram fresh weight of the sample was estimated by measuring the number of frozen tubes in each dilution (Cazorla et al. 1995), then matched to the Most Probable Number (MPN) table according to the Thomas formula series 3.3.3 so that the table value was obtained (APHA 1975).

Amplification of 16S rRNA encoding gene using PCR method

Genomic DNA was extracted with the Presto™ Mini gDNA Bacteria Kit. Then the gene encoding the bacterial 16S rRNA was amplified using Polymerase Chain Reaction (PCR). The PCR reaction was carried out by mixing 25 µL *Kapa2G Fast Ready Mix*, one µL 63 forward primers (63f: 5'-CAGGCCTAACACATGCAAGTC-3') with a concentration of 10 pmol, one µL 1387 reverse primer (1387r: 5'-GGGCGGAWGTGTACAAGGC-3') with a concentration of 10 pmol, one µL DNA template, and 9.5 µL ddH_2O. Before entering the PCR cycle, pre-denaturation was conducted at 94°C for 2 minutes. One cycle of PCR carried out consisted of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 1 minute, and finalizing at 72°C for 20 minutes, and then stopped for storing at 4°C. The sample can then be electrophoresed. The denaturation, annealing, and elongation processes consist of 25 cycles (Marchesi et al. 1998).

16S rRNA coding gene sequencing

The results of the PCR gene encoding 16S rRNA for INA bacteria were purified using the PCR/DNA Fragments Extraction Kit (Genaid) and continued with DNA concentration treatment using a Biophotometer. The DNA was then sent to 1st Base Singapore for sequencing using the ABI PRISM 310 Genetic Analyzer. The DNA sequences were then analyzed using bioinformatics techniques, namely, the BLAST Nucleotide device on the

NCBI website (www.blast.ncbi.nlm.nih.gov/blast.cgi) (Waturangi et al. 2008; Arifin 2014).

Data analysis

Analysis of the data used for the results of the isolation of INA bacteria is descriptive data analysis. Bacterial population data per gram of leaf was analyzed descriptively. INA bacterial DNA sequences obtained from DNA extraction and 16S rRNA gene amplification were analyzed using the BLAST Nucleotide device on the NCBI website (www.blast.ncbi.nlm.nih.gov/blast.cgi) (Arifin 2014).

RESULTS AND DISCUSSION

Sampling location

Based on the results of surveys and field observations conducted in the Dieng Plateau, it is known that potato plants are mostly found at an altitude between 1,800-2,200 m asl. Therefore, in this study, samples of potato plants used as the main source for isolating INA bacteria that cause *embun upas* were taken between 1,800-2,200 m asl. Potato crop sampling stations are presented in Table 1.

Sampling station one is in Bakal Village (sampling code: BK) with an altitude of 1,800 m asl. The location for the collection is a potato plantation located in the vicinity of residents' housing. The potato planting system in Bakal Village is intercropping, where potato plants are planted with several other crops such as chili, cabbage, and shallots. Not many cases of *embun upas* were found at station one because the air temperature tends to be warm and does not change rapidly and significantly.

Sampling station two is located in Diengkulon Village (sample code: DK) with an altitude of 2,000 m asl. The collection location is a potato plantation in the Mount Prau hiking trail area. The potato planting system in Diengkulon village is single planting, i.e., only potato plants are grown in one garden plot. There are several cases of *embun upas* that occurred in Diengkulon Village. *Embun upas* occurred at 04.00 West Indonesian Time (WIB), when the air temperature was lowest.

Sampling station three is located in Sembungan Village (Sampling code: SB) with an altitude of 2,200 m asl. Station three is the sampling station with the highest altitude. The sampling location is a potato plantation located around the PT. Geodipa Energi, more precisely, in front of the Dieng gas station. The case of *embun upas* in Sembungan Village occurred during the dry season, namely July-October. *Embun upas* occurred between 24.00-05.00 WIB. The air temperature in Sembungan village can reach 2°C at night at 23.00 WIB.

Bacterial isolates of Ice Nucleation Active (INA) from potato plant leaves in Dieng Plateau

INA bacteria were isolated using NA media with 2.5% glycerol (NAG). The addition of glycerol to NA media is beneficial for the growth and development of cultured bacteria. Lindow (1990) stated that media containing glycerol is a common medium used to isolate ice-core bacteria because it can optimize the growth of the culture. On the other hand, Lindow et al. (1982) explained that the growth of INA bacteria culture on media containing polyalcohols such as glycerol, sorbitol, mannitol, and the like could increase the frequency of ice core formation. Waturangi and Tjhen (2009) explained that NA media enriched with 2.5% glycerol was also expected to grow INA bacteria and increase ice core formation activity.

Bacterial colonies obtained from the plates were then used as pure cultures on inclined NAG media. Pure cultures were incubated at room temperature at 25-27°C for 4-6 days. According to Kieft and Ruscetti (1990), the INA bacterial culture with the highest ice core formation activity is cultured at 4-6 days of age. Pure cultures that are either too young or old do not have high ice core-forming activity. Samples were taken at each station with two repetitions, and each repetition was carried out three times with dilutions so that 18 cultures were obtained on NAG media in Petri dishes. From 18 cultures on plates, 76 isolates were obtained, presented in Table 2.

A total of 76 bacterial isolates with different morphological appearances and pigmentation were then purified before testing the ice nucleation activity. Six isolates were frozen after testing the ice nucleation activity on all isolates. The ice nucleation activity of INA bacterial isolates is presented in Figure 2.

Table 2. Bacterial isolates obtained from each village

Name of village	Number of obtained isolates	The number of frozen isolates
Bakal	28	3
Diengkulon	23	2
Sembungan	25	1

Table 1. Potato sampling station in Dieng Plateau, Central Java, Indonesia

Station	Altitude (asl)	Morning (06.00) air temperature (°C)	Daytime (11.00) air temperature (°C)	Night (01.00) air Temperature (°C)	Name of village
1	1.800	17	21	12	Bakal Village
2	2.000	15	17	9	Diengkulon Village
3	2.500	11	16	2	Sembungan Village

Samples that experience freezing are thought to be the result of the activity of the INA bacteria. Colonies that can be categorized as positive for INA bacteria are colonies that have ice nucleation activity. The ice nucleation activity can be seen when the bacterial suspension in the microtube freezes after being put into a circulating alcohol bath at a temperature below -5°C for 10 minutes. This condition indicated that the microtube with the suspension contained INA bacteria which had a single protein as an initiator of ice core formation (Morris et al. 2008). On the other hand, if the bacterial suspension does not freeze, then the suspension is categorized as negative for INA bacteria.

Tests were carried out at -5°C, -7°C, and -10°C. Tests at different temperatures were carried out because not all samples at each station experienced freezing activity at the same temperature. The temperature difference was due to differences in the activity of each bacterial suspension that was tested. It depends on the protein activity of the INA bacteria that live on the leaves. It shows several bacteria in each sampling station with different activities, even in one station. In the ice nucleation activity test, it was found that 1 sample was frozen at -7°C, while five samples froze at -10°C (Table 3).

Kieft and Ruscetti (1990) stated that the formation of an ice core indicated a positive test for INA bacteria after an incubation period of 5 minutes. Tests were carried out at different temperatures. Based on their activity, INA proteins were divided into three main classes, namely classes A, B, and C. Class A was active in forming ice cores at temperatures > -2°C to -5°C, class B was active at temperatures ≥ -5°C to -7°C, and class C is active at ≥ -7°C to -10°C (Baertlein et al. 1992). Based on the tests on the ice nucleation activity of bacterial isolates, it was found that one bacterial isolate was classified into class B. In contrast, the other five isolates were class C. Class A bacteria were not found in this study.

INA bacterial species in potato plant leaves based on the 16S rRNA coding gene sequence

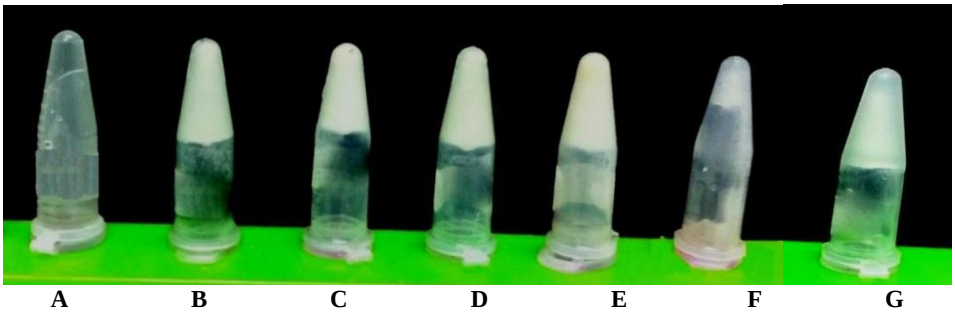
Ice core-forming bacteria or known as INA bacteria, have been widely studied and tested for ice nucleation activity by several researchers in various countries, such as Lindow (1983b), Gurian-sherman, and Lindow (1993), Arwiyanto (1996), and Waturangi et al. al. (2008). Identification has also been carried out to find out the species of INA bacteria that develop in an area. Based on Waturangi and Tjhen's (2009) research, the phenotype of INA bacteria has been found in the genera of three bacteria, namely *Pseudomonas, Xanthomonas,* and *Erwinia*. Still, other bacterial species may be found that are also included in the INA bacterial species. Several species of bacteria included in the INA bacteria are *E. ananas* (Arwiyanto 1996); *Chryseobacterium* sp., *Pseudomonas lurida, Enterobacter aerogenes, P. syringae* (Hendrawan 2014); *Pantoea* sp., *Pseudomonas* sp., *Rahnella* sp. (Arifin 2014); *P. viridiflava, P. fluorescens,* and *X. campestris* (Gurian-Sherman and Lindow 1993).

The identification carried out to determine the species of INA bacteria has different techniques and methods, such as standard methods (Hayward 1990; Arwiyanto 1996), biochemical test methods (Cappucino and Sherman 1978; Fu'adah 2014), and 16S rRNA gene sequencing methods (Castrillo et al. 2000; Arifin 2014). Identification using the 16S rRNA gene sequencing method is effective because the 16S rRNA gene is not easy to mutate and has the same function from one bacterium to another, with a length of ± 1,500 bp which is sufficient for identification purposes (Pangastuti 2006; Janda and Abbott 2007). Identification using the 16S rRNA gene sequencing has the highest data validity among other methods (Hauben et al. 1997; Janda and Abbott 2007).

Several species were obtained at each collection station based on identifying INA bacteria isolated from potato plants in the Dieng Plateau. Of the 76 samples purified based on their colony morphological characters, six isolates showed ice nucleation activity and were identified using the 16S rRNA gene sequencing method.

Table 3. Classification of INA bacteria based on differences in ice nucleation activation temperature from potato plant leaves

Isolate code	Freezing temperature	INA protein class
BK-2a2	-7°C	B
BK-1a6	-10°C	C
BK-1a5	-10°C	C
DK-1b1	-10°C	C
DK-2b3	-10°C	C
SB-3a2	-10°C	C



A B C D E F G

Figure 2. Ice formed from the activity of INA bacteria on potato leaves in microtubes. Note: A. Control, B. DK-1b1, C. DK-2b3, D. PW-3a2, E. BK-1a6, F. BK-1a5, G. BK-2a2

Amplification was carried out using the PCR method. The resulting PCR product was 1,324 bp. The amplification results were then carried out by electrophoresis and presented in Figure 3. All positive samples that were successfully electrophoresed were then identified by sending samples to 1st Base Singapore for sequencing. There were three species of bacteria suspected of causing *embun upas* on potato plants in the Dieng Plateau, presented in Table 4.

Each sampling station contained ice nucleation activity indicated by the freezing of the isolate in the previous test. Based on research and tests conducted by Waturangi and Tjhen (2009), gram-negative bacteria with the genus Pseudomonas is one of the bacteria that has ice nucleation activity. In other words, it can be said that this genus of bacteria is included in the category of INA bacteria. INA bacteria similar to *Pseudomonas azotoformans* were found in Sembungan and Diengkulon villages and are INA bacteria belonging to class C bacteria, which freeze between temperatures of -7°C to -10°C. In Bakal Village, two species of bacteria were found that were similar to *Arthrobacter sulfonivorans* and *Curtobacterium luteum*.

Pseudomonas azotoformans can express the gene encoding the protein INA (INP). The gene that bacteria express to initiate the occurrence of *embun upas* is the inaW gene. These genes can control the mechanism of ice crystal formation by activation of INPs located on the surface of bacterial cell membranes. INP will catalyze the formation of ice crystals called *embun upas* (Castrillo et al., 2000; Morris et al., 2008). No one has stated that *A. sulfonivorans* and *C. luteum* are INA bacteria. Still, these two bacteria have specific mechanisms in response to cold temperatures around their environment to tolerate cold temperatures. Arthrobacter sulfonivorans has cold shock protein (CSP) which helps the bacteria to tolerate cold temperatures. One way is by the mechanism of ice crystal formation (Mishra et al. 2010).

Estimation of the population density of INA bacteria in one gram of leaf using the multiple tube nucleation method

The calculation of the bacterial population was carried out using the multiple tube nucleation method, which is a method for estimating the number of ice nuclei from bacterial suspensions and the population of INA bacteria on plant parts. This method focuses on diluting bacterial suspensions in liquid media and is calculated based on the number of tubes undergoing freezing (Cazorla et al. 1995; Watson and Lawrence 2003). According to Watson and Lawrence (2003), the tube nucleation test method can calculate the population of INA bacteria associated with plants and is based on and developed from the MPN (Most Probable Number) method. The MPN method is used to determine the amount of coliform in water. According to Montesinos and Vilardell (1991), using a tube assay to determine the number of ice-core-forming bacteria is based on the number of tubes that freeze in each dilution series.

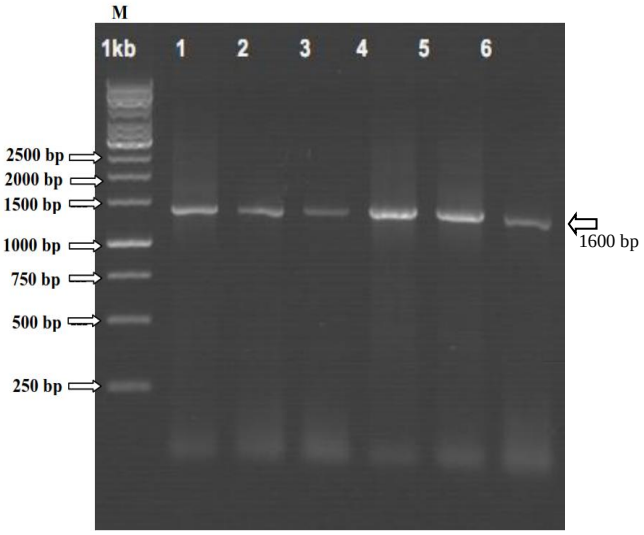


Figure 3. Electrophoresis of INA bacteria that ice nucleation activity. Description: M: Marker

Table 5. MPN values based on the Thomas formula MPN table series 3.3.3

Village	Dilution 10^{-1}	Dilution 10^{-2}	Dilution 10^{-3}	MPN INA/gram
Bakal	1	0	0	1,44 x 102
Diengkulon	3	1	1	3,00 x 103
Sembungan	3	3	1	1,824 x 105

Table 4. Species of INA bacteria found at each sampling station

Isolate code	Isolate description	Query cover	Ident.
BK1-a5	*Arthrobacter sulfonivorans* Strain All16S ribosomal RNA gene Partial Sequence	98%	98%
BK-1a6	*Curtobacterium luteun* Strain DSM 20542 16S ribosomal RNA genepartial sequence	99%	98%
BK-2a2	*Arthrobacter sulfonivorans* Strain All16S ribosomal RNA gene Partial Sequence	98%	98%
DK-1b1	*Pseudomonas azotoformans* Strain NBRC 12693 16S ribosomal RNAgene partial sequence	97%	98%
DK-2b3	*Pseudomonas azotoformans* StrainNBRC 12693 16S ribosomal RNA gene partial sequence	99%	99%
SB-3a2	*Pseudomonas azotoformans* Strain NBRC 12693 16S ribosomal RNAgene partial sequence	99%	99%

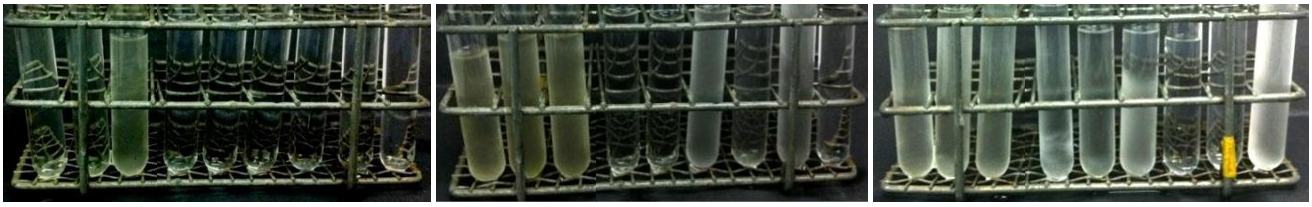


Figure 4. Freeze-positive tube in the MPN test. Note: A. Tube combination 1-0-0; B. 3-1-1 tube combination; C. 3-3-1 tube combination

Several researchers have carried out several studies on estimating the population of INA bacteria using the Thomas formula series 3.3.3. Hirano et al. (1985) stated that the optimum temperature for the estimation test was -5ºC. This study is supported by a test carried out by Baertlein et al. (1992) and Cazorla et al. (1995). At a temperature of -5ºC, it was sensitive enough to detect the activity of ice core formation by INA bacteria. In several other studies conducted by Lindow et al. (1982), Hirano and Upper (1986), and Olive and McCarter (1988), it is stated that a temperature of -5ºC for routine testing in determining the activity of INA bacteria is the right and optimum temperature. From the research conducted, each sampling station was found to have frozen positive tubes (Figure 4).

The bacterial population was estimated by matching the results obtained with the MPN table according to the Thomas series 3.3.3 formula with the number of frozen test tubes. The MPN method assumes that a frozen tube contains at least one ice core (Oblinger and Koburge 1975; Govindarajan and Lindow 1988). The output of the MPN method is the MPN value.

The MPN value estimates the number of growth units or colony-forming units in the sample. However, the MPN value can generally be interpreted as an estimate of the number of individual bacteria. The obtained freezing series were then matched with the MPN table and calculated for different MPN results from each retrieval station (Table 5).

Based on the tests carried out, the results were that freezing occurred at all sampling stations. In sampling station 1, namely Bakal Village (1,800 m asl), the number of frozen positive tubes with a combination of 1-0-0 was then matched to the MPN table, and a calculation was made, showing the results of 1.44 x 102 MPN/gram. In sampling station 2, namely Diengkulon Village (2,000 m asl), the number of frozen positive tubes with a combination of 3-1-1 was then matched to the MPN table, which shows the results of 3.00 x 103 MPN/gram. In sampling station 3, namely Sembungan Village (2,200 m asl), the number of frozen positive tubes with a combination of 3-3-1 was then matched with the MPN table, which showed the results of 1.842 x105 MPN/gram.

Based on the research, the following conclusions were obtained: (i) INA bacteria could be isolated from potato plants in Batur District, Dieng, as many as six isolates. All isolates were found at all sampling stations, namely Bakal Village (station 1: 1,800 m asl) with three isolates, Diengkulon Village (station 2: 2,000 m asl) with two isolates, and Sembungan Village (station 3: 2,200 m asl) with one isolate. (ii) The INA bacterial species found in potato plants in Batur District, Dieng were *A. sulfonivorans* in sample BK-1a5 (98%), *C. luteum* in sample BK-1a6 (98%), *A. sulfonivorans* in sample BK-2a2 (98). %), *P. azotoformans* in sample DK-1b1 (98%), *P. azotoformans* in sample DK-2b3 (99%) and *P. azotoformans* in sample SB-3a2 (99%). (iii) The population of INA bacteria on potato leaves in Batur District, Dieng Plateau was 1.44 x 102 MPN INA/gram in Bakal Village, 3.00 x 103 MPN INA/gram in Diengkulon Village, and 1.824 x 105 MPN INA/gram in Sembungan Village.

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Bioethanol production from banana's tuber (*Musa paradisiaca*) with hydrolysis using α-amylase and glucoamylase enzyme

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Abstract. *Utami IP, Mahajoeno E, Susilowati A. 2020. Bioethanol production from banana's tuber (*Musa paradisiaca*) with hydrolysis using α-amylase and glucoamylase enzyme. Bioteknologi 17: 76-80.* Energy consumption has increased in lockstep with economic expansion and population development, resulting in the depletion of fossil fuel supplies. Bioethanol is a non-fossil fuel that may be produced from rich biological resources in Indonesia, one of which is the banana tuber (*Musa paradisiaca* L.). The purpose of this study is to find the maximum reducing sugar levels in the banana tuber hydrolysis using a concentration ratio of α-amylase and glucoamylase enzymes, as well as to create the highest amount of ethanol utilizing a variety of baker's yeast concentrations (*Saccharomyces cerevisiae* Meyen ex E.C. Hansen). A Completely Randomized Design (CRD) with two components was used in this study. The first component was the ratio of α-amylase and glucoamylase concentrations, while the second element was the concentration of baker's yeast. The banana tuber was hydrolyzed to a maximum of 0.2 grams using α-amylase and glucoamylase at various concentration ratios (0:0; 100:0; 75:25; 50:50, 25: 75; 0: 25), followed by fermentation with baker's yeast (7.5 mg, 10 mg and 12.5 mg). The amount of reducing sugar created during hydrolysis was determined using the DNS method, whereas the amount of ethanol produced was determined using AOAC tables. ANOVA evaluated the data, and significant differences were found using Duncan's Multiple Range Test (DMRT) at a 95% confidence level. The results indicated that a 75% α-amylase ratio to 25% glucoamylase resulted in 26.17 mg/mL maximum sugar reduction. The highest ethanol concentrations were obtained by combining 75% α-amylase and 25% glucoamylase with 12.5 mg 7.98% baker's yeast. A 7.5 mg baker's yeast produced substantially more ethanol than 10 mg or 12.5 mg baker's yeast.

Keywords: α-amylase, banana's tuber, bioethanol, glucoamylase, hydrolysis

INTRODUCTION

Fossil energy, particularly oil, is the country's primary energy source and a source of foreign exchange. Energy consumption increases with economic and population expansion (Tambunan 2008). Thus, natural resources capable of producing fossil energy will continue to diminish, as most fossil energy sources are non-renewable, such as oil, gas, and coal. Indonesia has seen a reduction in national oil output over the previous few decades due to depleting oil reserves in natural production wells. Population growth will also increase the need and consumption of fuel oil for transportation facilities and industrial activities (Triwahyuningsih and Rahmat 2006).

Bioethanol is a non-fossil fuel alternative produced by the fermentation of carbohydrates-containing biomass using microorganisms. Bioethanol can be produced in Indonesia using the country's extensive biological resources (Hambali 2007). Therefore, bioethanol development as an alternative energy source has the potential to be used in Indonesia. Furthermore, bioethanol raw materials are plentiful and obtained from various carbohydrates-containing plants (Anonymous 2012).

In 2006, Indonesia produced 4.3 million tons of bananas (*Musa paradisiaca* L.) (Gusmawarni et al. 2009). Large production will also generate a great deal of garbage. One of the wastes is the banana tuber; after the fruit is picked, this banana tuber is rarely reused. The banana tuber comprises 76% carbohydrate, 20% water, and the remainder contains protein and vitamins (Yuanita 2008). The starch content of this enormous banana tuber has the potential to be utilized as a raw material for bioethanol production.

Converting starch into bioethanol consists of two stages: hydrolysis and fermentation. The first stage, hydrolysis, involves the conversion of starch to glucose. It can be accomplished biochemically using the α-amylase enzyme or chemically using an acid/base solution. α-amylase enzyme is an endoamylase enzyme, which means that it can degrade starch randomly from the center or inside the glucose molecule. The optimal temperature range for the enzyme α-amylase is between 4.5 and 70°C., and it is active at a pH of 5.2 to 5.6. The glucoamylase enzyme is an exoamylase enzyme, which means that it can convert the starch chain to glucose molecules in the non-reducing region of the molecule. The optimal pH range for the glucoamylase enzyme is between 4.5 and 5.0, but this value varies according to the source of the enzyme. Therefore, the optimal temperature ranges between 40 and 50°C. Enzymatic hydrolysis of starch with α-amylase enzyme rapidly breaks α-1,4 glycosidic bonds in gelatinized starch solutions. In contrast, although slower, glucoamylase

enzymes can break α-1,4 glycosidic and α-1,6 glycosidic bonds in starch molecules. The second stage, fermentation, involves the conversion of the produced glucose to ethanol and carbon dioxide by the yeast *Saccharomyces cerevisiae* Meyen ex E.C. Hansen (Jumari et al. 2009). To generate ethanol, Baker's yeast can be used in place of *S. cerevisiae* isolates during the fermentation process. It is due to *S. cerevisiae* isolates in fermenting yeast and baker's yeast. Baker's yeast is widely available and does not require special preparation (Reed and Nagodawithana 1991).

According to Komarayati et al. (2011), the greatest ethanol content for sago starch was obtained using a combination of 0.18 mL α-amylase enzyme; 0.15 mL glucoamylase enzyme; 0.48 grams baker's yeast, and three days of fermentation. The highest ethanol content for sago pith was 44.84% when 0.13 mL of α-amylase enzyme; 0.11 mL of glucoamylase enzyme; and 0.33 grams of yeast were combined, while the highest ethanol content for fiber was 8.26% when 0.13 mL of -amylase enzyme; 0.11 mL of glucoamylase enzyme; and 0.33 grams of yeast were combined. Mucaramah (2012) used rice bran substrate hydrolyzed with 0.09 mL of the glucoamylase enzyme and fermented with 2 mg of baker's yeast to achieve the highest ethanol concentration of 6.80%. Risnoyatiningsih (2011) did research on 50 grams of yellow sweet potato hydrolyzed for five days using 0.1 gram α-amylase enzyme, resulting in a glucose level of 5.64%. The substrate to enzyme ratio is 1:0.002. Rahmi et al. (2007) conducted a study employing sorghum substrate that had been hydrolyzed using α-amylase enzyme at a concentration of 0.2%, resulting in an 18 mg/mL reducing sugar content and an 8.22% ethanol content. Hasanah et al. (2012) conducted a study using 5 grams of banana tuber substrate hydrolyzed with 0.09 mL of the glucoamylase enzyme and fermented for three days with 50 mg of tape yeast. The resulting ethanol level was 3.12%.

The objectives of this study were (i) to compare the concentrations of α-amylase and glucoamylase enzymes that produced the maximum concentrations of reducing sugars during the banana tuber hydrolysis process and (ii) to compare the concentrations of α-amylase and glucoamylase enzymes required to create the maximum quantities of bioethanol using baker's yeast.

MATERIALS AND METHODS

Preparation of tools and materials

All tools and media used were sterilized by autoclaving at 121ºC for 15 minutes.

Preparation of *Dinitrosalycilic acid* (DNS) Reagent

Ten grams of *dinitrosalycilic acid* (DNS), 2 g phenols, 0.5 g Na-sulphite or Na-bisulphite, and 10 g NaOH were dissolved in 300 mL H_2O. Further, the solution was diluted into 1 L. The solution was stored in the refrigerator.

Standard curve setup

To begin, the researchers created a typical glucose solution (10 mL anhydrous glucose in 100 mL). The solution was diluted five times to create glucose solutions with concentrations of 2, 4, 6, 8, and 10 (mg/100 mL). Six sterile test tubes have been prepared. Each was 0.5 mL with a typical glucose solution. One tube was loaded with 1 mL of pure water as a blank. Each solution was placed in a test tube, followed by the addition of 3 mL of DNS reagent. Five minutes were spent heating the solution in boiling water and then cooling it to room temperature. After adding 1 mL of potassium sodium tartrate, the absorbance was measured at a wavelength of 550 nm (Miller 1959).

Making banana tuber pulp

100 g cleaned banana tuber was blended with 400 mL distilled water and then poured into a 500 mL Erlenmeyer. After adjusting the pH to 5.5 with 0.1% HCl, aquadest was added until the volume reached 500 mL.

Hydrolysis process

In the hydrolysis of banana tuber pulp, α-amylase enzyme was added. It was heated at approximately 55ºC for 2 hours (Retno et al. 2009), cooled to 45ºC, adjusted to pH 4.5, and added with glucoamylase enzyme for one hour (Mucaramah 2012).

Measurement of reducing sugar level

After hydrolyzed, the reducing sugar concentration was measured based on the DNS method. Glucose solution was used as the standard solution (Miller 1959).

Fermentation process

The hydrolyzed banana tuber pulp was combined with 7.5, 10, and 12.5 mg of baker's yeast (Jayanti 2011). The Erlenmeyer was then sealed with a cork stopper and left at room temperature (28-30ºC) for three days. The ethanol concentration was determined following incubation.

Ethanol level measurement

We created a distillation tube and a 250 mL volumetric flask. Then, 50 mL of liquid sample from fermented banana tuber starch mixed with 100 mL of distilled water was combined with 100 mL of distilled water and distilled until 50 mL of distillate was obtained.

The pycnometer is calibrated during distillation. The pycnometer was filled and closed with distilled water. Weighing the pycnometer and distilled water yielded the value C. After emptying the pycnometer, it was dried in an oven. Weighing the dry pycnometer yielded the value B. We estimated the weight of distilled water (W) using the C-B method.

Transferring the distillate to a dry beaker was performed. It was agitated to ensure homogeneity before filling the pycnometer with the distillate. The dry pycnometer is filled with distillate and then dried and weighed. The obtained results yielded the value A.

A-B = L is the weight of the distillate. The distillate's weight (L) is determined by its "specific gravity," or spg = L/W. The ethanol percentage was estimated after determining the spg value using the AOAC (Association of Official Analytical Chemists) chart (Horwits and Franklin 1975).

Data analysis

The analysis was conducted using the statistical technique known as analysis of variance (ANOVA). However, there was a significant difference in this research. Thus, it was continued with the DMRT test at a 5% significance level.

RESULTS AND DISCUSSION

Hydrolysis of banana tuber starch

Hydrolysis of starch is when starch molecules are broken down into simpler constituents, such as dextrin, isomaltose, maltose, and glucose (Rindit et al. 1998 in Purba 2009). According to Musanif (2008), starch hydrolysis is when starch polymer chains are broken down into dextrose or monosaccharide units, specifically glucose ($C_6H1_2O_6$). The enzymatic approach is used to hydrolyze banana tuber starch because enzyme hydrolysis results in a higher conversion rate than acid hydrolysis. Additionally, because the nature of the enzyme catalyst is so specialized, it can preserve the flavor and aroma of the basic ingredients (Winarno 1995).

The hydrolysis of banana tuber starch occurs in three stages: gelatinization, liquefaction by the α-amylase enzyme, and saccharification by the glucoamylase enzyme. The results indicated that the proper ratio of α-amylase and glucoamylase enzymes would result in a high concentration of reducing sugars (Table 1). The function of the α-amylase enzyme in hydrolyzing the α-1,4 glycosidic bond during the liquefaction process, followed by hydrolysis with the glucoamylase enzyme, will cut the α-1,4 glycosidic bond and the α-1,6 glycosidic bond at a lower frequency, allowing the hydrolysis process on starch to run more efficiently. The reducing sugar concentration (y= 0.138x+0.003) was determined using a standard curve (Figure 1). The absorbance value obtained is placed in the formula y= 0.138x+0.003, where x denotes the amount of reducing sugar (appendix 2 p. 44). The maximum reducing sugar levels were obtained by hydrolyzing with 75% α-amylase and 25% glucoamylase enzymes (26.17 mg/mL), while the lowest reducing sugar levels were obtained by hydrolyzing with 100 % glucoamylase (15.067 mg/mL).

In the hydrolysis reaction of banana tuber starch, the inclusion of α-amylase and glucoamylase enzymes acts as a catalyst. With the presence of this enzyme, the reaction will be accelerated, resulting in the production of more products. Reducing sugar content can be generated optimally with a precise hydrolysis procedure and an optimal enzyme dosage. According to Aransiola (2006), increasing the concentration of the enzyme accelerates the enzymatic reaction. To a certain extent, the rate of the enzymatic reaction is directly proportional to the enzyme concentration, indicating that the reaction is in equilibrium. Increases in enzyme concentration had no effect at equilibrium.

Additionally, the inclusion of enzymes can enhance the amount of glucose converted. This rise was attributable to the chance of starch coming into contact with enzymes, increasing proportion to increased starch concentration. It is consistent with the general rule that the rate of an enzymatic reaction increases proportionally to the concentration of the enzyme catalyzing the reaction (Sukandar et al., 2010). It is also supported by Yunianta et al. (2010); the higher the rate of enzyme reaction, the more starch is hydrolyzed, but the enzyme reaction rate decreases when practically all starch is hydrolyzed.

ANOVA analysis revealed a significant difference ($P<0.05$) between the ratio of enzyme concentrations to lowering sugar production. This data demonstrates that the ratio of the enzyme's concentrations considerably affects the amount of reducing sugar generated. The enzymes α-amylase and glucoamylase function as biocatalysts to accelerate the rate of the banana tuber starch hydrolysis reaction.

Without the addition of enzymes (control), hydrolysis produced a reducing sugar concentration of 8.14 mg/mL. It could be because water can also hydrolyze starch into glucose when heated to high temperatures. Additionally, the sterilization procedure at elevated temperatures and pressures will alter the raw components, one of which is starch. When water is added to the starch particles, they swell and lose their link cohesion; specifically, some amylose diffuses out under heat, allowing the starch to be hydrolyzed to release glucose (Winarno 2002).

Table 1. Reducing sugar levels at different enzyme concentrations

Comparison of the concentration of α-amylase and glucoamylase enzymes (0.20 g/500 mL)	**Reducing sugar concentration (mg/mL)**	**Reducing sugar level** (%)
A0	08.14[a]	0.81
A1	20.94[e]	2.09
A2	26.17[f]	2.61
A3	19.40[d]	1.94
A4	17.68[c]	1.77
A5	15.06[b]	1.56

Note: a-f: numbers followed by different letters indicate significant differences in the 5% DMRT test for the addition of α-amylase and glucoamylase enzymes. A0=control; A1=100% α-amylase; A2=75% α-amylase:25% glucoamylase; A3=50% α-amylase:50% glucoamylase; A4=25% α-amylase:75% glucoamylase; A5 = 100% glucoamylase.

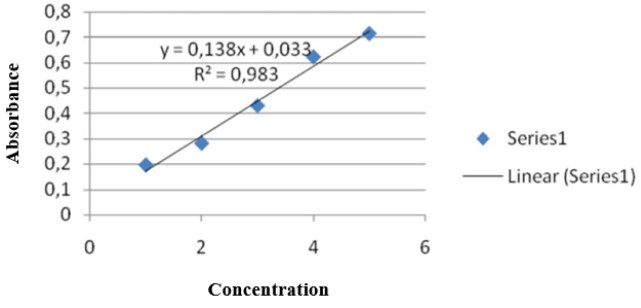

Figure 1. Glucose Standard Curve 10 mg anhydrous glucose/100 mL aquadest

Ethanol content fermented using baker's yeast

Ethanol fermentation is a biological process that converts carbohydrates like glucose, fructose, and sucrose into cellular energy and metabolic waste products like ethanol and carbon dioxide. Fermentation's fundamental premise is to stimulate the activity of microbes to alter the character of raw materials to produce a product. For example, the sugar produced by the hydrolysis of banana tuber starch is transformed into ethanol by *S. cerevisiae* present in baker's yeast. Zimase invertase is produced by *S. cerevisiae*. The invertase enzyme breaks down the remaining polysaccharides (starch) into monosaccharides (glucose) during hydrolysis. In contrast, the zymase enzyme further transforms monosaccharides into alcohol during fermentation (Stark in Underkofler and Hickey 1954). The higher the concentration of dissolved sugar, the more alcohol is produced, as more sugar must be converted to ethanol by yeast (Judoamidjojo et al. 1992).

Three days of ethanol fermentation were used in this study. According to Kusmiyati's (2010) research, the microorganism (*S. cerevisiae*) was most active or in the logarithmic phase at 72 hours. The logarithmic phase is the phase during which the biggest ethanol product is formed. After 72 hours, the bacteria enter a stationary phase. The number of growing microorganisms equals the number of dead microorganisms, ensuring that no more microorganisms convert the substrate to ethanol, so the amount of ethanol created tends to be constant. After a stationary phase, the bacterium enters the death phase. The ethanol produced at 24 and 48 hours was suboptimal due to *S. cerevisiae* being in the lag and exponential phases. The lag phase is when microorganisms adjust to their environment, while the exponential phase is when they begin to increase. As a result, the activity for the synthesis of ethanol products is suboptimal. According to Azizah et al. (2012), *S. cerevisiae* can produce up to 2% alcohol after 72 hours of fermentation.

After fermentation, the fermentation result is distilled to separate the ethanol from other components. Distillation is evaporating and condensing a vapor at a specified pressure and temperature. Distillation is used to purify liquids at their boiling points. It can separate liquids from solids or combinations with different boiling points. The separation will begin with the components with the lowest boiling points. Pure ethanol has a boiling point of 78°C, while water has a boiling point of 100°C under typical conditions. Distillation at a temperature of 70°C because Sari et al. (2008) state that running the distillation process at a temperature of 70-80°C causes the majority of the ethanol to evaporate and pass through the condensation unit, resulting in the production of ethanol.

The results indicated that the hydrolysis treatment with 75% α-amylase enzyme, 25% glucoamylase enzyme, and 12.5 mg baker's yeast resulted in the highest ethanol content of 7.98% (Table 2). Conversely, the hydrolysis treatment with 100 % glucoamylase enzyme and 5 mg baker's yeast resulted in the lowest ethanol content of 2.7%.

The higher the enzyme concentration added, the more sugar is generated, which results in a larger ethanol content produced by yeast fermentation. Roukas (1996) asserts that the amount of ethanol produced is highly dependent on the amount of available substrate. Yeast uses reducing sugar to grow, and during metabolism, it produces ethanol.

According to the statistical analysis (Appendix 4, p. 46), there is a substantial difference between the ratios of the enzymes α-amylase, glucoamylase, and baker's yeast and the ethanol concentration. It demonstrates that the concentration ratios of the amylase and glucoamylase enzymes and baker's yeast substantially affect the sugar content of the ethanol generated. The greatest ethanol level is 7.98% when added by 75% α-amylase enzyme, 25% glucoamylase enzyme, and 12.5 milligrams of baker's yeast. Each addition of enzymes during hydrolysis alters the concentrations of reducing sugars utilized as substrates in ethanol fermentation, whereas baker's yeast ferments glucose to ethanol. Therefore, the more reducing sugars present, the more ethanol is created.

The addition of baker's yeast affects the ethanol output. Adding baker's yeast to the ideal limit increases ethanol production because the more yeast present, the more microorganisms capable of converting glucose to ethanol. Admianta et al. (2001) reported that the higher the dose of yeast *S. cerevisiae* used, the higher the bioethanol content. It is because yeast is the primary generator of bioethanol. However, the amount of yeast given must be appropriate, as a tiny amount of yeast used to convert glucose to ethanol will diminish the yeast's ability to ferment. Similarly, excessive yeast use will hinder the fermentation process, resulting in a delayed growth phase (Sari et al. 2008).

Table 2. The ethanol content of fermented banana tuber starch using baker's yeast

baker's yeast (mg)	Comparison of enzymes administration of α-amylase and glucoamylase (g/500 mL)					
	A0	**A1**	**A2**	**A3**	**A4**	**A5**
B1	$0,40^{a}$	$3,70^{de}$	$6,57^{h}$	$3,12^{bc}$	$2,91^{bc}$	$2,70^{b}$
B2	$0,53^{a}$	$4,91^{f}$	$7,27^{i}$	$3,33^{cd}$	$3,19^{bcd}$	$3,12^{bc}$
B3	$0,73^{a}$	$5,58^{g}$	$7,98^{j}$	$3,94^{e}$	$3,40^{cd}$	$3,33^{cd}$

note: a-j: numbers followed by different letters showed significant differences in the 5% DMRT test for the addition of α-amylase and glucoamylase enzymes with baker's yeast to the ethanol content (α=0.05). A0=control; A1=100% α-amylase; A2=75% α-amylase: 25% glucoamylase; A3=50% α-amylase:50% glucoamylase; A4=25% α-amylase:75% glucoamylase; A5= 100% glucoamylase and B1= 5 mg baker's yeast; B2= 7.5 mg baker's yeast; B3 = 12.5 mg of baker's yeast

The maximum level of ethanol generated by banana tuber starch substrate was 7.98% in this investigation. This ethanol level is consistent with the percentage range determined by Wijaya et al. (2012), which indicates that the ethanol content of products created through fermentation ranges between 3 and 10%, depending on the type of product fermented. Therefore, around 8% to 12% bioethanol will render yeast inactive due to an overabundance of ethanol, which is harmful to yeast (Retno et al. 2009).

The lower the overall sugar concentration, the less substrate *S. cerevisiae* can consume. The amount of accessible simple sugars are very limited at low concentrations. Simple carbohydrates such as glucose and fructose are critical throughout *S. cerevisiae*'s early development phases. However, if the substrate concentration is too high, the fermentation period will be prolonged, and more sugar will be left unused (Sari et al. 2008).

According to Schlegel (1994), ethanol, or ethyl alcohol in industry, can be used as a fuel, a solvent, a medicine, a detergent, an oil, a candle, or gasohol. In addition, it is possible to increase the alcohol concentration of alcohol by distilling it in stages.

The cost of producing bioethanol from banana tuber at a concentration of 7.98% in 1L is Rp. 52,000.00 (Appendix 6, p. 49), whereas the cost of unsubsidized gasoline and diesel is Rp. 8,400.00. Depending on the purity of the bioethanol, it can be used in place of fuel oil. Bioethanol with a concentration of 95-99% can be utilized as a premium material (gasoline), whereas bioethanol with a concentration of 40% can be used in place of kerosene.

This study indicates that a 75% α-amylase ratio to 25% glucoamylase produces the highest reducing sugar content of 26.17 mg/mL during the banana tuber hydrolysis process. A ratio of 75% α-amylase to 25% glucoamylase produces the highest ethanol content of 7.98% compared to a ratio of 75% α-amylase to 12.5 mg baker's yeast.

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Enhancing performance and fatty acid composition of Nile tilapia (*Oreochromis niloticus*) from housefly maggot (*Musca domestica*) feeds

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Abstract. *Lobina AA, Mkupasi EM. 2020. Enhancing performance and fatty acid composition of Nile tilapia (*Oreochromis niloticus*) from housefly maggot (*Musca domestica*) feeds. Bioteknologi 17: 81-89.* This study was conducted to compare the effect of three substrates on the yield and composition of housefly (*Musca domestica*) maggots (HFM); assessed the growth performance of *Oreochromis niloticus* (Linnaeus, 1758) fed HFM were mixed with other ingredients, and investigated the enhancement of omega 3 fatty acids composition in the produced *O. niloticus*. The substrates for HFM production were poultry manure (HFMChick), *Lemna* species of freshwater macrophytes (HFMLemn), and *Eucheuma* species of marine macrophytes (HFMEuch). The HFM was then used to formulate eleven diets: nine isonitrogenous diets with 35% crude protein, one diet of 5% fish meal, and the other with soybean meal. Diets were named SBM, FM, HFMChick, HFMLemn, and HFMEuch, denoting soybean meal, fish meal, and HFM cultured on poultry manure, HFM cultured on *Lemna* species of freshwater macrophyte, and HFM cultured on *Eucheuma* species of marine macrophyte, respectively. A feeding trial was carried out on triplicate groups of ten fish (1.9-2.2 g) in recirculation aquaculture systems (RAS). The fish were fed up to 5% of their body weight twice daily throughout the experimental period. The effect of inclusion levels of HFM and other diets on fish growth feed utilization and ω-3FAs were determined. A gas chromatography-mass spectrometer (GC-MS) was used to analyze the composition of Omega 3 fatty acids. Results showed that the yields of HFM from poultry manure and *Eucheuma* species of marine macrophytes (HFMEuch) substrates were significantly higher ($P<0.05$) than those from *Lemna* species of freshwater macrophyte. The protein content of HFM from *Eucheuma* species of macrophyte was significantly higher ($P<0.05$) than those from poultry manure and *Lemna* species of freshwater macrophyte. Fish fed on diets containing HFMEuch and FM had significantly higher ($P<0.05$) growth performance compared to fish fed on HFMChick, HFMLemn, and SBM diets. Thirty-two (32) types of FAs with different saturation levels were detected. The fish-fed HFM cultured on *Eucheuma* species had the highest composition of FAs (32) compared to others. In conclusion, poultry manure substrates showed better yield results for culturing HFM than other substrates. *Eucheuma* species can be used to culture HFM as an alternative feed ingredient to improve the performance and composition of ω-3FAs in cultured *O. niloticus.*

Keywords: Aquatic macrophytes, fatty acid, gas chromatography-mass spectrometer, Nile tilapia

INTRODUCTION

Globally, there is an increased demand for protein for human and animal consumption. Fisheries and aquaculture contribute up to 17% of the global population's intake (FAO 2014). Approximately 85% (130.8 million MT) of total fish production in 2011 was used for human consumption, while the remaining (23.2 million MT) was used for non-human uses (Barbaroux et al. 2012). According to FAO (2014), from 2006 to 2012, global capture fisheries production remained stagnant at around 90 million MT, and it is believed the trend will remain the same until 2030. Thus aquaculture has been identified as an alternative to sustaining demand for fish as global aquaculture production is expected to increase to approximately 120 million MT by 2030 (FAO 2014).

Limiting factors in the growth of the aquaculture industry may include both the scarcity and high cost of key ingredients used in making fish feeds (Aniebo et al. 2009; Bureau et al. 2009; Huntington and Hasan 2009; Dedeke et al. 2013). For aquaculture to meet the future demand for fish protein, quality ingredients must be available in the required quantities. Conventionally, fishmeal (FM) and/or legumes and cereals have been used as protein and energy sources, respectively (Craig and Helfrich 2002; Huntington and Hasan 2009; Chapman and Miles 2015). However, in formulating nutritionally balanced fish diets, FM is the preferred dietary protein source because of its nutritional quality and palatability properties (Tacon and Metian 2008; Huntington and Hasan 2009; Mohanta et al. 2013).

Tilapia is one of the world's most important farmed freshwater fish species, as it is an important source of human food (FAO 2014). Global tilapia production is expected to double from 4.3 million tons to 7.3 million tons between 2010 and 2030 (FAO 2012). Furthermore, the species is expected to contribute 60% to total freshwater aquaculture production in 2025 (FAO 2016).

In Tanzania, aquaculture production accounts for a small proportion of the total fish produced at the national level. However, it contributed about 2.1 billion tons to the national GDP in 2018/2019 (MLFD 2019). In Tanzania, aquaculture is dominated by extensive and semi-intensive small-scale freshwater fish farming (Chenyambuga et al. 2014). The most popular cultured with high economic value species are Nile tilapia (*Oreochromis niloticus* (Linnaeus, 1758) and, to a lesser extent African

catfish (*Clarias gariepinus* Burchell, 1822) (Kaliba et al. 2006; Novebrianto et al. 2011). *O. niloticus* is often used in aquaculture due to its desirable characteristics, like fast growth and high fecundity (Fitzsimmons 2000a,b; Negroni 2013; FAO 2014). In addition, *O. niloticus* tolerate a wide range of environmental parameters and have resistance to parasites, and of their suitability in a wide range of farming systems, they are becoming increasingly popular worldwide (Allanson and Noble 1964; Behrends et al. 1990; Fitzsimmons et al. 2014).

Fish are typically a good source of omega 3. However, freshwater fish, including tilapias, do not contain significant amounts of ω-3 fatty acids, especially nutritionally important docosahexaenoic acid (DHA) and eicosatetraenoic acid (EPA) (Silva et al. 2014). Earlier studies revealed that supplementing Tilapia feed with ω-3 fatty acids can increase the amount of these fatty acids in the muscle tissue (Tiffany et al. 2016). EPA and DHA are essential fatty acids that must be obtained from food (Whelan 2009). The fish feeds currently in use do not significantly improve the levels of omega 3 in farmed fish, hence alternative feeds must be identified.

This study aimed to: (i) Assess the effect of different substrates on yield and composition of cultured housefly (*Musca domestica*) maggots (HFM) meal; (ii) Evaluate the growth and feed utilization of *O. niloticus* fed on feed with HFM meal cultured on selected aquatic macrophytes; (iii) Assess the ω-3 fatty acids (PUFAs) content in *O. niloticus* fed diets containing HFM meals cultured on selected aquatic macrophytes.

MATERIALS AND METHODS

Description of the study area

The study was conducted at Aquaculture Research Facility within the Department of Animal, Aquaculture, and Range Science of Sokoine University of Agriculture (SUA), Morogoro, Tanzania. The present study was conducted from February to July 2018. SUA is located about 2.5 km South of Morogoro Municipality at an altitude of 550 m above sea level, with monthly mean minimum and maximum temperatures being 14.2°C to 35.5°C, respectively. Morogoro receives approximately 880 mm of bimodal rainfall annually, ranging from 29% to 96%.

Macrophyte collection and preparation

Two species of aquatic macrophyte, *Lemna* and *Eucheuma*, were collected from Lake Victoria and the Indian Ocean, respectively. *Lemna* was sorted to remove unwanted materials and debris and fermented for three days to obtain an offal odor to attract houseflies. *Eucheuma* was cleaned with fresh water to remove sand and reduce salt content. Fresh poultry manure was collected and transported to SUA.

Maggots production

Maggots were produced using three different substrates in triplicates. The substrates were poultry manure (control), marine macrophyte (*Euchaema*), and freshwater macrophyte (*Lemna*). The culture chamber was in a 2.7 L plastic container, were 2.5 kg of each substrate and 0.5 kg of poultry offal as the attractant was added and left open for eight hours to allow houseflies to lay eggs. Culturing was done indoors in triplicates for each substrate, as done by Nzamujo (2001) and Devic et al. (2014). The mixture was wrapped with a net with a mesh size of 1.2 mm to allow for the easy harvesting of maggots. Observations on the development of the maggots were recorded daily. Eggs hatched within two days and were left for an additional two days to develop into mature maggots. The mature maggots were harvested according to Sogbesan et al. (2006) and blanched with hot water at 100°C for 10 seconds. Thereafter, they were weighed to determine the total wet weight per harvest. The maggots were oven-dried at 60°C for 24 hours to constant weight, cooled, and ground into powdery form as maggot meal using a grinder machine (sieve 1mm). The samples for maggot meal were taken to a laboratory for proximate analysis.

Setup of the feeding experiment

The experiment was conducted at the SUA aquaculture research facility. This is a recirculation system that has two large tanks and five medium water tanks. The upper tank receives clean water from a major water pipe to the inner system. Used water from the inner system passed through several pipes to the filtering tanks sequentially. From the lower large tank, a pumping machine pumps treated water to the upper tank and repeatedly to the inner system for reuse.

Five diets were formulated and randomly allocated in triplicates of 15 rearing tanks (Table 1). Each rearing tank was stocked with 10 fingerlings with an initial mean weight of 2.07±0.12 g, totaling 30 fingerlings per treatment. Experimental fish were fed twice a day from 0930 and 0945 hrs and 1630 to 1645 hrs according to their feeding response, while limited to 5% of their body weight. The amount of feed was adjusted in response to changes in fish's body weights. Rearing tanks were siphoned twice daily to enhance aeration, remove uneaten food, and avoid the risks of infection and diseases.

Proximate analysis of diets formulated

The nutrient contents of all diets were determined using procedures described by AOAC (2005). First, dry matter and ash were determined by weighing 1 gram of the samples by using a 160 g capacity analytical weighing balance (Precisa 180A, Oerlikon, Switzerland), oven-drying (E 115, WTB binder 7200, Germany) at 700 C to constant weight, re-weighing, and ashing the samples in Muffle furnace (N31R, Nabertherm, West Germany) at 5000 C for three hours. The crude protein was determined by weighing the samples (Presica 180A, Oerlikon, Switzerland) followed by three stages of the Kjeldahl system, namely digestion (Digestion System 12 1009, Digester, Tecator, Sweden), distillation (2200 Kjeltec Auto Distillation, Foss Tecator, Sweden) and titration (Digitrate, Tecator, Sweden). This was multiplied by the factor of 6.25 to get the amount of crude protein.

Table 1. Diet formulation and grouping

	SBM	FM,	HFMChick	HFMLemn	HFMEuch
SBM	51.49	43.90	12.72	10.80	7.50
FM	0.00	5.00	0.00	0.00	0.00
MM	37.70	40.33	40.15	43.33	44.75
HMM	0.00	0.00	35.00	35.00	35.00
CRM	2.49	2.48	3.90	2.53	4.33
CGM	0.00	0.00	0.00	0.00	0.00
SFO	4.32	4.29	0.46	0.47	0.65
Premix	1.00	1.00	1.00	1.00	1.00
Meth	1.00	1.00	1.00	1.00	1.00
Lysine	1.00	1.00	1.00	1.00	1.00
MCP	1.00	1.00	1.00	1.00	1.00
Total	100.00	100.00	100.00	100.00	100.00

Note: SBM: Soya bean meal 35%, FM: Soya bean meal with 5% fish meal, HFMEuch:Housefly maggot meal *Eucheuma* 35%, HFMLemn: Housefly maggot meal *Lemna* 35%, HFMChick: Housefly maggots poultry 35%. 35%: Amount of protein on each diet

Crude fat was determined by weighing the samples (Presica 180A, Oerlicon, Switzerland), and fat was extracted by the Soxhlet extraction method (Soxtec system HT 1043 Extraction unit, Tecator, Sweden). After that, the extraction cups containing fat material were dried at 105°C for 30 minutes to remove traces of moisture. Then, the cups containing fat material were cooled in a desiccator for about 10 minutes and weighed to calculate the amount of crude fat in the feeds.

The crude fiber was obtained by weighing samples (Presica 180A, Oerlicon, Switzerland) into filter bags, digesting the sample in weak sulfuric acid and rinsing in weak NaOH solution at 100°C for 30 minutes in an Ankom machine (ANKOM220, ANKOM Technology, USA), and followed by washing and rinsing using distilled water. Then weak sodium hydroxide (alkaline) solution was added and heated at 100°C for another 30 minutes to remove acids in the samples. It was then rinsed with distilled water before acetone was added to remove the fat remaining in the residues. The samples were then dried and ashed. The difference between the residues and ash weight gave the amount of crude fiber.

Growth trials using maggots diet cultured on different substrates were conducted, involving fifteen (15) treatments whereby nine (9) treatments were experimental diets using maggots cultured on three different substrates, namely poultry manure (control, treatment one), *Eucheuma* species of marine macrophyte (treatment two), and *Lemna* species of freshwater macrophyte (treatment three). The other six (6) treatments were other diets most fish farmers commonly use. One diet contained only a soybean meal, while the other contained a soybean meal with 5% fishmeal. Feed utilization and growth rate were measured. Subsequent body weights were weighed and recorded after every seven days. Before weighing, fish were starved for a day. The body weights of fish from each replicate were recorded, and mean weights were calculated. Performance characteristics were calculated using the following formulae by Olvera-Novoa et al. (1990).

Average daily weight gain (ADWG)

$$ADWG = \frac{Final\ bodyweight\ (g) - Initial\ bodyweight(g)}{\text{Time (days)}}$$

Specific Growth Rates (SGR %)

$$\%SGR = \frac{FLN(Final\ bodyweight) - LN(Initial\ bodyweight)X100}{\text{Experimental period (days)}}$$

Survival Rate (SR)

$$SR = \frac{Final\ number\ of\ fish\ harvest \times 100}{Initial\ number\ of\ fish\ at\ stocking}$$

Protein Efficiency Ratio (PER)

$$PER = \frac{Bodyweight\ gain\ (g)}{Crude\ protein\ intake\ (g).}$$

Feed Conversion Ratio (FCR)

$$FCR = \frac{Feedsuppied\ (g)}{Bodyweight\ gain\ (g)}$$

Fish sample collection

At the end of the experiment, 150 fish were collected from 15 rearing tanks. Ten (10) individuals were collected per rearing tank. After collection, each sample was stored in a plastic bag, preserved using dry ice, and later frozen at -20°C before lipid analysis. Frozen samples were transported to the Zoology laboratories at the University of Dar es Salaam, Tanzania, for lipid extraction and fatty acids analysis.

Sample analysis

Sample preparation

The fish samples were freeze-dried to remove excess water. For each sample, a piece weighed between 10-20 g was grinded to soften the muscles.

Lipid extraction

Lipid extraction was done using a 2:1 methanol and chloroform ratio and minced with a vortex machine for 2 minutes, as Folch et al. (1957) described. Samples were stored in a refrigerator for 48 hours to speed up lipid extraction. Filtration was done using filter paper (GF/F-

glass fiber filter) to separate tissues from obtaining filtrate solution. The addition of 1:1 methanol and chloroform was done to extract the remaining lipids from the tissues. The two layers formed by fish tissues (lipids and aqueous solution) were separated by using a separating funnel to obtain lipids. This was followed by adding sodium sulfate to remove traces of water from the lipids. Evaporation to remove chloroform was done in an air-conditioned room at 16°C for 24 hours.

Preparation of Fatty Acid Methyl Esters (FAMEs)

Methylation was done by using concentrated sulfuric acid methods to obtain FAMEs. Before derivatization, five (5) mg of lipid was suspended in 1ml of toluene. Then, 2 mls of methanoic sulfuric acid (1%v/v) was added to each sample in vials and sealed. The samples were heated in a stopper tube at a temperature of 50°C overnight for 16 hours. This was followed by adding 2 mls of water containing sodium bicarbonate (2%: w/v) to each sample to neutralize the acid. Next, extractions were done by adding a hexane/diethyl ester (1:1, by Vol; 2×5 m). Finally, evaporated locally in an air-conditioned room at 16°C for three days to remove the acid.

Analysis of fatty acids

Determination of types and levels of ω-3 PUFAs was done using a Gas chromatograph Mass Spectrometer (GC MS-QP2010 Ultra), which is equipped with a flame ionization detector, (FID). 1ULofFAME in hexane was injected into the GCMS in a split ratio -1.0. Helium was used as a carrier gas at a 2 Ml/min flow rate. The injector temperature was 250°C. The temperature was then programmed as follows: column oven was set at 90°C, held for two minutes, and then increased to 260°C, held for five minutes, with a total 41 minutes. The ω-3 PUFAs (EPA, ALA, and DPA) were identified by comparing their retention time with those of commercial standards. These ω-3 PUFAs commercial standards were brought from Fluka –United States of America (USA).

Water quality monitoring

Water quality is an important factor in pond fish production, throughout the experiment period, water quality parameters, including temperature, pH, and dissolved oxygen (DO) were monitored weekly. These were measured using a GWQ-DO280 Dissolved Oxygen Meter.

Data analysis

Data were analyzed using Statistical Package for Social Sciences program version 10 (SPSS Richmond, VA, USA) as described by Dytham (2013). Data were tested for normality and homogeneity of variance before being analyzed using One-way analysis of variance (ANOVA). Treatment means were considered significant at $P<0.05$. Post–hoc analysis was also done where significant differences existed between treatment means, using Tukey's Honest Significant Difference Test (Steele and Torrie 1980). The model was:

$$Yij= \mu+Ti+Lij+ Eij$$

Where:

μ : General means.
Ti : the effects of treatment (I:1, 2)
Eij : Residual Error
Lij : Levels within treatments (j:1, 2, 3, 4, 5)
Yij : Observation value (nutritional composition of experimental meals, fish growth performance and feed utilization)

RESULTS AND DISCUSSION

Yield and nutritional composition of HFM produced from different media

The yield of HFM differed significantly among substrate treatments ($P<0.05$). Yield from poultry manure was significantly higher than from *Eucheuma* species and *Lemna* species of macrophyte, as shown in Table 2. There was a significant difference in the protein content of HFM harvested from three culturing substrates ($P<0.05$). HFM from *Eucheuma* marine macrophyte species had significantly higher crude protein content ($P<0.05$) than those from poultry manure and *Lemna* species of freshwater macrophyte substrate. Crude fiber and ether extracts of the maggots produced from all substrates had no significant differences ($P>0.05$). The maggots from poultry manure had significantly higher ($P<0.05$) ash content than those from *Lemna* and *Eucheuma* substrates (Table 2).

The chemical composition of formulated diets is shown in Table 3. Crude fiber content was between accepted ranges for *O. niloticus* production.

Feed intake, growth performance, and feed utilization of cultured *Oreochromis niloticus*

The diet consumption (feed intake) of HFMEuch and FM were good throughout the experiment, while the feed intake was poor in fish-fed SBM, HFMChick, and HFMLemn. No feed-related mortality was observed during the experiment. A significant ($P<0.05$) increase in the FBWT and ADG was observed in fish-fed FM and HFMEuch, compared to those fed with the control diet (SBM). However, fish-fed diets HFMLemn and HFMChick had comparable FBWT and ADG to those fed the SBM diet. There was no significant difference in IBWT and SGR among the treatment groups. HFMEuch showed lower FCR among other treatments. PER was found to be significantly different ($P<0.05$) in the fish fed with HFMEuch and FM compared to the SBM diet, but not those fed HFMChick and HFMlemn. When compared to the control diet (SBM), significantly high FI and BWTG were observed in the fish fed with all diets ($P<0.05$) (Table 4).

Composition of fatty acids in cultured *Oreochromis niloticus*

A total of 32 FAs were identified in *O. niloticus* fed five formulated diets (Table 5). The unsaturated FAs were relatively more (26) than saturated ones (6). Of the 26 unsaturated FAs, 17 were PUFAs, and 9 were MUFAs.

Among the 17 types of PUFAs, the Omega 6 PUFAs were (12), followed by Omega 3 PUFAs (4), and Omega 9 PUFAs was (1). The most dominant saturated fatty acids (SAFAs) were palmitic acid, pentadecanoic acid, stearic (octadecanoic) acid, tetracosanoic acid and heptadecanoic acid.

The dominant omega 3 PUFAs were docosatrienoic acid, docosapentanoic acid, docosahexaenoic acid and eicosatetraenoic acid. The principal Omega 6 PUFAs were gamma linoleic acid and arachidonic acid. Omega 9 PUFAs was eicosadienoic acid. Oleic acid was the dominant MUFA. Alfa linolenic and gamma linoleic FAs were also found. The ratio of PUFAs to SAFAs was 2:2:1, and the ratio of Omega 6 to Omega 3 PUFAs in five diets was 1:1:1.

The results showed that fish-fed HFMEuch had the highest FA composition of FAs (32) compared to other diets (Table 6). Some FAs found in fish-fed HFMEuch included 7, 10-Hexadecadienoic acid, alfa linolenic acid, 9-Octadecenoic acid, and 10, 13-Octadecadienoic acid.

Water quality parameters

There was no significant difference between dissolved oxygen (DO) and temperature. However, a significant difference ($p<0.05$) was shown in pH among different treatments (Table 7).

Discussion

The results showed differences in yield and composition of HFM cultured in different substrates. The high yield of HFM from poultry manure probably was due to the substrate's long-lasting odor, which strongly attracted more flies. A similar observation was made by Nzamujo (1999) and Agbeko et al. (2014), who reported that the more the quantity and long-lasting odor of substrate, the greater number of flies and the greater the number of maggots produced. Calvert (1979) and Patricia and Salas (2007) also reported a similar observation, where chicken manure produced many maggots compared to cow dung manure.

Table 2. Yield (g/kg) and chemical composition of HFM (% dry matter) (mean ± SD)

Item	Substrate		
	Eucheuma	Lemna	Poultry manure
Yield	610 ± 15.4 ^{a}	584.8 ± 30.7 ^{c}	857.0 ± 2.0 ^{b}
Dry matter	97.52 ± 0.47^{a}	96.42 ± 1.46^{a}	95.71 ± 0.08^{a}
Crude protein	53.55 ± 0.81^{a}	40.43 ± 0.21^{c}	42.61 ± 0.22^{b}
Crude fiber	5.01 ± 0.26^{a}	6.00 ± 0.25^{a}	5.71 ± 0.25^{a}
Ether Extract	20.40 ± 0.42^{a}	19.07 ± 0.46^{a}	20.01 ± 0.06^{a}
Ash	10.70 ± 0.48^{a}	11.13 ± 0.23^{a}	10.45 ± 0.18^{a}

Note: Means with different superscripts within a row are significant different at ($p<0.05$).

Table 3. Chemical composition of formulated diets

Ingredient (%)	SBM	FM	Diets		
			HFMChick	HFMLemn	HFMEuch
Dry matter	90.75	90.89	88.42	91.86	91.40
Crude protein	41.73	46.01	40.66	46.03	50.00
Ether extract	18.98	19.20	20.00	19.07	20.40
Crude fiber	1.45	1.90	1.22	0.86	1.08
Ash	7.57	7.66	7.31	7.69	7.73

Table 4. Growth performance and nutrient utilization of *Oreochromis niloticus* fed different diets (mean±SE)

Parameter	SBM	FM	Diets		
			HFMChick	HFMLemn	HFMEuch
INBWT (g)	2.09 ± 0.12^{a}	2.23 ± 0.12^{a}	1.89 ± 0.12^{a}	2.07 ± 0.12^{a}	2.09 ± 0.12^{a}
FBWT (g)	5.62 ± 0.26^{c}	6.63 ± 0.26^{ab}	6.21 ± 0.26^{abc}	6.15 ± 0.26^{bc}	7.01 ± 0.26^{a}
BWTG (g)	3.53 ± 0.28^{b}	4.40 ± 0.28^{ab}	4.33 ± 0.28^{ab}	4.08 ± 0.28^{ab}	4.91 ± 0.28^{a}
ADG (g/day)	0.063±0.005^{b}	0.078±0.005^{ab}	0.077±0.005^{ab}	0.073±0.005^{ab}	0.087±0.005^{a}
SGR (%day)	1.76 ± 0.12^{a}	1.95 ± 0.12^{a}	2.13 ± 0.12^{a}	1.95 ± 0.12^{a}	2.15 ± 0.12^{a}
FI (g/fish/day)	0.159±0.004^{b}	0.183 ±0.004^{a}	0.160±0.004^{b}	0.166±0.004^{b}	0.192±0.004^{a}
FCR	1.41 ± 0.22^{a}	1.31 ± 0.22^{a}	1.62 ± 0.22^{a}	1.33 ± 0.22^{a}	0.11 ± 0.22^{b}
PER	1.26 ± 0.08^{c}	1.64 ± 0.09^{ad}	1.49 ± 0.05^{b}	1.35 ± 0.01^{b}	1.81 ± 0.03^{a}
SR (%)	96.66 ± 4.47^{a}	96.66 ± 4.47^{a}	90.00 ± 4.47^{a}	93.33 ± 4.47^{a}	90.00 ± 4.47^{a}

Note (Table 1-7): INBWT: Initial body weight, FBWT: Final body weight, BWTG: Body weight gain, ADG: Average daily gain, SGR: Specific growth rate, FI: Feed intake, FCR: Feed conversion ratio, PER: Protein efficiency ratio, SR: survival rate, FM: Fish Meal, SBM: Soybean Meal, HFMchick: Housefly maggots diets cultured on poultry manure, HFMLemn: Housefly maggot diets cultured on *Lemna*species, HFMEuch: Housefly maggot diets cultured on *Eucheuma* species. Means with different superscripts within a row are significant different at ($p<0.05$)

Table 5. Fatty acids composition in *Oreochromis niloticus* fed five diets including HFM

Fatty acids	SBM	FM	HFMChick	HFMLemn	HFMEuch	Level of saturation
Nonadecanoic acid	+	+	+	+	+	saturated
Tricosanoic acid	-	+	+	-	+	saturated
Myristic acid	+	+	+	+	+	saturated
Palmitic acid	+	+	+	+	+	saturated
Stearic acid	+	+	+	+	+	saturated
Heptadecanoic acid	+	+	+	+	+	saturated
Tetradecenoic acid	-	+	-	-	+	MUFAs
9-Octadecenoic acid	-	-	-	-	+	MUFAs
11-Octadecenoic acid	-	-	-	-	+	MUFAs
Heptadecenoic acid	+	+	+	+	+	MUFAs
Hexadecenoic acid	+	+	+	+	+	MUFAs
11-Eicosenoic acid	-	+	+	-	+	MUFAs
Oleic acid	+	+	+	+	+	MUFAs
Tetradecenoate	-	-	+	+	+	MUFAs
Eicosadienoic acid	-	+	-	-	+	MUFAs
11,13- Eicosadienoic acid	-	+	+	-	+	PUFAs
11,14- Eicosadienoic acid	-	+	+	-	+	PUFAs
Linoleic acid	+	+	+	+	+	PUFAs
Arachidonic acid	+	+	+	+	+	PUFAs
Eicosatrienoic acid	-	+	-	-	+	PUFAs
Docosatetraenoic acid	-	+	+	+	+	PUFAs
Docosahexaenoic acid	+	+	+	+	+	PUFAs
Alfa Linolenic acid	-	-	-	-	+	PUFAs
Eicosatrienoic acid	-	+	+	+	+	PUFAs
Eicosapentanoic acid	+	+	+	+	+	PUFAs
4,7,10,13,16-Docosapentaenoate	+	+	+	+	+	PUFAs
Docosapentaenoic acid	-	+	+	+	+	PUFAs
Eicosatetraenoic acid	+	+	+	+	+	PUFAs
8,11-Octadecadienoic acid	+	+	+	+	+	unsaturated
10,13-Octadecadienoic acid	-	-	-	-	+	unsaturated
Eicosadienoic acid	-	+	+	+	+	unsaturated
7,10-Hexadecadienoic acid	-	-	-	-	+	unsaturated

Table 6. Accumulation of Omega-3 PUFAs and Omega-6 PUFAs found in *Oreochromis nilotucus*

Parameters	SBM (D1)	FM (D2)	Diets		
			HFMChick (D3)	HFMLemn (D4)	HFMEuch (D5)
ΣPUFAS	1.99 ± 0.01^{a}	2.84 ± 0.10^{b}	0.88 ± 0.12^{a}	4.81 ± 0.05^{c}	9.52 ± 0.82^{c}
Σω-3 PUFAs	1.54 ± 0.06^{a}	2.83 ± 0.16^{b}	0.69 ± 0.29^{a}	2.73 ± 0.38^{b}	4.07 ± 0.91^{c}
Σω-6 PUFAs	0.43 ± 0.05^{a}	0.33 ± 0.0^{a}	0.13 ± 0.04^{a}	1.09 ± 0.0286^{a}	4.54 ± 0.37^{c}

Note: ΣPUFAS: sum of polyunsaturated fatty acids, Σω-3 PUFAs: sum of Omega 3 polyunsaturated fatty acids, Σω-6 PUFAs: sum of Omega 6 polyunsaturated fatty acids. Means with different superscripts within a row are significant different at ($p<0.05$)

Table 7. Water quality parameters recorded during the feeding experiment

Parameter	SBM	FM	Diets		
			HFMChick	HFMLemn	HFMEuch
DO (mg/L)	7.42 ± 0.04^{a}	7.47 ± 0.04^{a}	7.34 ± 0.04^{a}	7.46 ± 0.04^{a}	7.49 ± 0.04^{a}
pH	7.28 ± 0.09^{ab}	7.40 ± 0.09^{a}	7.09 ± 0.09^{bc}	7.02 ± 0.09^{bc}	6.98 ± 0.09^{c}
Temperature (°C)	24.17 ± 0.06^{a}	24.22 ± 0.06^{a}	24.14 ± 0.06^{a}	24.22 ± 0.06^{a}	24.27 ± 0.06^{a}

Note: DO: Dissolved oxygen. Means with different superscripts within a row are significant different at ($p<0.05$).

The results showed that a mean crude protein of 45.53% of HFM was produced from the three substrates. There was no significant difference in crude protein contents of produced maggots from different substrates. However, *Eucheuma* marine macrophyte species produced maggots with a higher crude protein content of 53.55% compared to others. The mean of the produced maggots was 55.2%, as previously reported (Nzamujo 1999; Adeniji 2007; Odesanya et al. 2011). The amount of crude protein in the produced maggots reported depended on the nutrients present in the substrate and the ability of the organisms to feed and assimilate them (Patricia and Salas 2007; Agbeko et al. 2014).

Despite a high yield of HFM from poultry manure, the maggots had a relatively low crude protein content of 42.61%. The poultry manure used in this study could have been of low quality, which might be attributed to the loss of nitrogen due to its conversion to ammonia. Similarly, the negative impact of manure storage time on its nutrient content and subsequent nutritional quality of maggots cultured there has been previously described (Horn 1998). Regarding crude protein contents, *Eucheuma* species of marine macrophyte proved to be a better substrate for culturing HFM.

The number of ether extracts of maggots was not significantly different among the substrates. The mean level was within the recommended level of 10-25 (Ogunji et al. 2006). Present results were nearly equal to 19.3%EE reported by (Nzamujo 1999) but higher than those reported in other studies (Okah and Onwujiariri 2012).

Regarding ash content in produced HFM meals, there was no significant difference among the substrates. The average ash content of the experimental HFM diets in the present study was 10 to 19%. These results agree with other studies' results (Nzamujo 1999; Yaqub 1999; Okah and Onwujiariri 2012).

Regarding fish performance, the fish fed on the HFMEuch diet were superior to those fed on other diets. Higher growth performance of *O. niloticus* fed on HFMEuch diets reflects palatability of maggots cultured in *Eucheuma* species of the marine macrophyte. In addition, the high acceptability of HFM meal made it a suitable ingredient for fish feed leading to increased feed utilization and growth performance (Makkar et al. 2014; Ogunji et al. 2008). This shows that *Eucheuma* species can produce more nutritious maggots than other substrates, such as poultry manure and *Lemna* of freshwater macrophytes.

Feed conversion ratio (FCR) is used to measure feed utilization efficiency. The present study obtained better FCR from a fish-fed HFM diet. This was similar to findings previously reported (Ogunji et al. 2006; Ogunji et al. 2008; Jabir et al. 2012; Omoyinmi and Olaoye 2012; Mekhamar et al. 2015). However, the FCR of the HFMEuch diet was relatively lower than those reported in previous studies (Mohanta et al. 2013; Olaniyi and Salau 2013) whose values ranged from 3.13 to 5.07. This resulted in higher body weight gain of fish fed HFMEuch. The FCR of the present study was better than that reported by Yaqub (1999), who fed fish with HFM from different substrates.

The present results are supported by previous findings, which showed that a diet with maggot and fishmeal was well accepted and well utilized by the fish (Jonathan 2012; Monebi and Ugwumba 2012; Omoyinmi and Olaoye 2012). Furthermore, according to Coyle et al. (2004), the inclusion of HFM in the fish diet produced the highest PER, likely due to good protein and other nutrients such as fatty acids. This agrees with previous findings that recommended the use of HFM in the diets of the fish to improve feed intake and utilization for better growth performance (Stafford and Tacon, 1988; Dedeke et al. 2010; Hasanuzzaman et al. 2010; Sogbesan 2014).

The slow growth performance of fish fed on HFMLemn and HFMChick diets could be attributed to factors such as the presence of anti-nutritional factors and the unpalatability of the diets. However, the present results are like observations made by Ogunji et al. (2006), who reported different growth performances of *Carassius auratus* (Linnaeus, 1758) when fed HFM cultured from different substrates. The overall weight of the fish fed HFMEuch and FM-based diets were higher than those fed SBM, HFMChick, and HFMLemn-based diets. This reflects the better condition of fish fed on FM and HFMEuch-based diets, regardless of the protein sources.

Fish-fed diets with HFMEuch and FM showed high feed intake, probably due to the high palatability of the diets. Meena (2015) reported that palatability of the diet is a factor that largely impacts fish acceptance of the feed. In addition, the palatability of these diets mighty is attributed to the nutrient content and good odor, as previously reported (Hilton 1989; Sogbesan et al. 2003; Makkar et al. 2014). Nevertheless, fish-fed HFMChick and HFMLemn diets had the same feed intake as those fed the SBM diet.

Thirty-two types of FAs with different saturation levels were found in *O. niloticus* in this study. These results are comparable to Mohamed and Al-Sabahi (2011) results, who identified 33 FAs of different saturation levels in commercial *O. niloticus.* This study registered that the saturated (SAFAs) were 6 and unsaturated were 26, including 17 PUFAs and 9 MUFAs. Similarly, Mwanja et al. (2010) observed more categories of unsaturated FAs than saturated FAs. The more unsaturated than saturated FAs observed in the present study is probably due to the type of substrates used to culture the HFM. Henderson (1996) reported that aquatic plants and invertebrates such as HFM contain more unsaturated FAs than SAFAs.

The availability of Omega-3 PUFAs such as EPA, DPA, and DHA was higher in fish fed HFMEuch and FM diets than in those fed with SBM, HFMChick, and HFMLemn diets. This might be contributed to the de novo synthesis from alfa linolenic acid found in the diets. Bachok et al. (2006) reported that alfa linolenic acid is a short-chain FA that animals, including fish, do not synthesize. This is like reports by Zenebe et al. (1998) and Cintra et al. (2012), who described the different levels of FAs in *O. niloticus*, such as EPA and DHA, according to diets. Therefore, the higher availability of EPA, DPA, and DHA in *O. niloticus* is probably due to the elongation of ALA. These findings show that *O. niloticus* can be a good source of Omega-3 PUFAs to consumers when fed with good Omega-3 fatty acids. This further proves *Eucheuma* species of marine macrophyte to be superior in producing HFM with high Omega 3 content and transferring them to fed *O. niloticus*.

Water quality parameters from this study showed that the water temperature ranged from 24.17°C to 24.22°C. This temperature range has been reported as the optimum range for tilapia growth and yield (El-Sayed 2006). Other studies reported that the temperature range for normal tilapia development, reproduction and growth is between 20°C and 35°C (El-Sayed 2006). Similarly, pH and dissolved oxygen were within optimum ranges for tilapia

growth. Other studies have shown that tilapia can improve survival at pH ranging from 5 to 10, but they do best if the pH ranges from 6 to 9 Cintra et al. (2012). Dissolved oxygen levels should be maintained above 5.0 ppm for best growth (Siddiqui et al. 1989). Dissolved oxygen levels between 3.0 and 5.0 ppm feeding should be reduced, and feeding should be stopped at dissolved oxygen levels below 3.0, Ogunji et al. (2006).

This study found that poultry manure substrate supports high yields of HFM, while *Eucheuma* of marine species macrophyte is a good substrate to produce HFM with high omega 3 fatty acids and higher crude protein level. The inclusion of HFM in fish feeds improved the performance of cultured *O. niloticus*, and *the Eucheuma* diet supported the high performance of *O. niloticus*. Fish produced from diets with HFM from *Eucheuma* of marine macrophytes had higher levels of fatty acids and proteins.

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