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Comment 1

Abstract is not prepared according to the journal format and it does not provide basic content of the paper. Please note that the journal requires the Abstract to be divided into the following sections: Background/Objective, materials and methods, results and Conclusion.

Background/Objective: In this section, a brief description of the context and purpose of the study is presented.

Materials and Methods: In this section author briefly describe how the study was performed and which statistical tests were being used.

Results: Author presents the summary of main findings of the study.

Conclusion: In this section author states the conclusions of the study clearly and concisely.

The following example will serve to illustrate the structure of an Abstract.

Abstract

Background: The cecum of the chicken gut may be susceptible to pathogens because it is readily colonized by microbes. The lower segment of the gut is also the primary tissue that permits the invasion of microorganisms from the external environment and the cloaca. Mucins, which are composed of glycoproteins, play significant roles in forming the barrier against infection on the mucosal surface. **Objective:** The aim of this study was to determine the differences in the mucosal barrier of the lower segment of the gut between Indonesian naked neck chickens and normal feathered chickens. **Methodology:** The lower segments of the gut (rectum, colon and cecal tonsil) of Indonesian indigenous naked neck chickens and normal chickens were collected. The expression of the mucin 2 gene in the gut mucosa was analyzed by reverse-transcription-polymerase chain reaction (RT-PCR). The localization and molecular sizes of the mucosal glycoproteins were analyzed by Western blot. Wheat Germ Agglutinin (WGA) and jacalin lectins were used for Western blot analysis. **Results:** The mucin 2 gene was expressed in the mucosal gut of the rectum, colon and cecal tonsil in both naked neck chickens and normal chickens. Western blot analysis showed a single band for both WGA and jacalin from the mucosal gut of the rectum, colon and cecal tonsil in both naked neck chickens and normal chickens. **Conclusion:** These results suggest that the mucin 2 gene and glycoproteins containing WGA and jacalin positive sugars cover the surface of mucosal gut in both naked neck chickens and normal chickens, most likely to form a mucosa barrier.

Comment 2

Significance Statement (120 words maximum) (Compulsory)

A statement about the significance of this research work should be included in the manuscript. The significance statement should provide the novelty aspect and significance of this research work with respect to the existing literature and more generally to the society. It should be a short summary which describe what this paper adds to and what was already known.

Start this statement with the following words:

This study discover the ---------- that can be beneficial for ……

And the last sentence of this statement could be such as:

This study will help the researcher to uncover the critical areas of ---------- that many researchers were not able to explore. Thus a new theory on ---------- may be arrived at.

A Model Significance Statement:

This study discovers the possible synergistic effect of vitamin E, calcium, and vitamin D combination that can be beneficial for osteoporosis-induced ovariectomized rats. This study will help the researcher to uncover the critical area of postmenopausal bone loss that many researchers were not able to explore. Thus, a new theory on these micronutrients combination, and possibly other combinations, may be arrived at.

Comment 3

Statement on Conflicts of Interest

A statement on conflicts of interest should be included in the manuscript. Either mention: 'none declared', or specify the authors' financial or other interests which should be known to the readers.

You are requested that please modify your article according to the above instructions and according to the side notes given below in the text and re-submit it as early as possible for further processing.

SCREENING AND CHARACTERIZATION OF KERATINOLYTIC BACTERIA FROM PUFFER FISH SKIN WASTE

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ABSTRACT

Puffer fish skin tannery is an alternative to substitute the production of hide and animal skin in Indonesia that has been decreasing. To improve the quality of puffer fish leather, keratinase was needed by removing the thorns. The purpose of this study is to screen bacteria that show keratinolytic activity. The result of the screening was that 3 of 5 kinds of strains exhibited caseinolytic activity (by showing the clear zone) when put into the media of skim milk agar. Three Bacillus bacteria that were newly isolated from puffer fish waste—using a feather enrichment technique—were identified on the basis of l6S ribosomal RNA gene sequence analysis, physiological and carbohydrates assimilation tests. They were revealed as the strains of *Bacillus thuringiensis* for BRAW_PT isolate, *Bacillus aerius* for BRAW_PB isolate, and *Bacillus firmus* for BRAW_PI isolate. The results of proteolytic enzymes assay showed that *Bacillus firmus* BRAW_PI has the highest protease and keratinase activity, which was 37.52±0.96 U/mg and 6.781±0.479 U/mg consecutively. All bacteria obtained were the superior bacteria that can be used for the removal of thorns from puffer fish skin in the tanning process.

Key words: Screening, Characterization, Microbia, Keratinolytic, Pufferfish

INTRODUCTION

Puffer fish (*Arothon reticularis*) is an underutilized fish in any fishing. It can be superior product for the surrounding communities. One of the utilization of puffer fish skin is for tannery business. The tanning of puffer fish leather can be an alternative to tanning industry that is currently limited to the production of cattle leather. The leather industry in Indonesia was only able to produce 350 million sqft/year, while the demand for the footwear industry and the finished goods amounted to 673 million sqft/year so there was a shortage of 323 million sqft per year[1]. Additionally, puffer fish also has a uniqueness that lies in its rounded body shape.

The quality of puffer fish leather can be improved by removing the thorns. One of the ways to destroy the thorns is to degrade the protein keratin in it with the keratinase enzyme. Keratinase is important for the pre-tanning process in leather industry so the skin tanning can be an eco-friendly process by reducing the use of sodium sulfate[2].

Keratinase that is produced by microbes is an enzyme capable of degrading the structural protein that is generally found in feathers, hair, and wool. The types of bacteria that have been screened to produce extracellular keratinase that can degrade fur and keratin, are for example *Bacillus licheniformis, Bacillus subtilis, Bacillus cereus and Bacillus pseudofirmus*[3]. Therefore, the screening of bacteria keratinolytic is one of the innovative solutions in tannery and interesting to be developed and studied in depth.

.MATERIALS AND METHODS

Materials: Materials used in this research were 30 pieces of 14-days-rotten puffer fish skin. The skin was taken randomly from the fish market in TPI Tanjungsari of Marine and Fisheries Agency of Rembang. It is used as raw material for the isolation and identification of microbes.

149 The growth medium for the isolation process was the same as the one used by Macedo
150 *et al*.[4] with modification: puffer fish skin flour as the sole carbon and nitrogen sources as
151 much as 10 g; and as mineral sources are: 0.5 g of NaCl, 0.3 g of K_2HPO_4, and 0.4 g of
152 KH_2PO_4. The medium for the stock solution includes 1 g of yeast extract, 1 g of biological
153 peptone, 0.5 g of NaCl and 100 mL of H_2O.

154 The materials for microbial identification are: (a) simple staining including 70%
155 alcohol, safranin; Gram staining including, gram A (crystal violet), gram B (Lugol iodine),
156 H_2O, gram C (acetone alcohol washing solution), gram D (safranin); Ziehl Neelsen's stain
157 includes ZN A carbol fuchsin solution as the primary stain, ZN B acid alcohol solution (37%)
158 as a laxative, and ZN C Loeffler's methylene blue solution. The materials for molecular
159 identification consist of tris-HCl, NaCl, EDTA, SDS, proteinase K, phenol, chloroform, TE,
160 ethanol, RNAase, PCR extraction kit (Genaid), sequencing kit, primary.

161 The equipments used in this research are: *HIRAYAMA* autoclave-Japan, *LABCONCO*
162 laminar air flow, *MEMMERT* oven, *MEMMERT* water bath, *SOCOREX* macro and
163 micropipettes, *HANNA* pH meter, *THERMOLYNE* stirrer, *OHAUS* analytical balances,
164 *MOTIC* test tubes, loopful, microscopes, *SNE 4500M* SEM microscope, *PERKIN ELMER*
165 *Lambda 25* UV/Vis Spectrophotometer, *ROTOFIX 32* coolbox, and centrifuge, *EPPENDORF*
166 *5804R* refrigerated centrifuge, *PCR BIO-RAD T100 Thermal Cycler* sequencer, *STUART*
167 shaker, *Jouan Maxi Artic* refrigerator, Glove BOX, *MEMMERT* and *HERAEUS* incubators,
168 *IWAKI* and *HERMA* glasswares, such as Erlenmeyer, Petri dishes, glass slide, bunsen, glass
169 beaker, measuring glass, and flask.

170 **Screening of keratinolytic bacteria:**The isolated bacterial strains were screened for the
171 production of extracellular keratinase using skim milk agar medium. The pure cultures were
172 streaked on the skim milk agar plates, and the plates were incubated at 37˚C for 48 h. After
173 incubation, the formations of a clear zone around the bacterial growth were observed[5].

Caseinolytic Activity: Skim milk agar medium was sterilized at 121°C for 15 min at 15 lbs pressure. The isolates were streaked on the medium. The zone formed around the colonies due to the production of caseinase enzyme was considered as a positive result. The organisms screened with skim milk agar medium were subcultured by growing the bacterium in nutrient broth medium at 37°C for 24 h[6].

Morphological Test: The identification of bacteria can be conducted by viewing the colony morphology both macroscopically and microscopically, including simple, Gram, Zn and spore staining as well as viewing on the bacterial biochemical test. The morphology of bacteria includes the shape, size, texture, colony color, and motility. The biochemical test was conducted to ensure the species of bacteria; it included the test of catalase, oxidase, and fermentation of carbohydrates.

Production of keratinolytic enzymes: The enzyme production was based on the method of Hoq *et al.*[7] with slight modification. Each of the isolates was cultivated in a basal medium (per liter of the solution: NaCl, 0.5 s: 0.06 s: K_2HPO_4, 0.3 g: K_2HPO_4, 0.4 g) containing keratinous substrates: (10.0 g of puffer fish; pH 7.5) as the only source of nitrogen, carbon, and sulfur. The supplementation of 1.0 g of nitrogen (yeast extract) and 1.0 g of carbon sources (bacteriological peptone) together was also tested in presence or absence of keratinous substrates under identical conditions. Cultivation was done with 5 mL of 24 h grown inoculum (in nutrient broth) of the respective bacterial cultures (100 mL) on the liquid medium in a 500 mL Erlenmeyer flask at 37˚C under shaking (120 rpm) for 24 h. The samples were withdrawn at 24 h and centrifuged at 4500 rpm at 6˚C for 20 min. The supernatants were preserved at 4˚C and assayed for protein and enzymes.

One ose pure isolates was cultured in 5 mL of sterile Pre-Culture medium (Stock solution) and incubated at 30˚C for 48 h in a shaker incubator at 120 rpm. After incubation,

198 the broth was centrifuged at 4500 rpm for 20 minutes, and the supernatant was used to study
199 the keratinolytic activities.

200 **Protease Activity:** Protease activity was determined with the method of Bergmeyer *et al.*[8].
201 One unit (U) of proteolytic enzyme activity is defined as the amount of enzyme that releases 1
202 µmol tyrosine per millilitre per minute at standard test condition. Specific activity is stated in
203 enzyme activity unit per milligram of protein.

204 **Keratinolytic Activity:** Keratinolytic activity was determined with the method of Wang *et*
205 *al.*[9]. The substrate used was Keratin Azure. The keratinase enzyme activity was measured by
206 using several types of keratin substrates such as feather flour[10,11,12], and keratin azure[13,14,9].
207 Keratin azure is pure keratin derived from wool that is stained with azo dyes. Keratin azure
208 that is added with water and is reacted with specific enzyme will produce a blue-colored
209 reaction product. The amount of keratinase enzyme activity is determined by the unit, where
210 one unit (U) of keratinase activity is defined as the amount of enzyme required to improve the
211 absorbance of 0.01 between the sample and the control at a wavelength of 595 nm in
212 accordance with the test conditions[15,9]

213 **Scanning Electron Microscope (SEM):** The fixation solution was made by using 0.2145 g of
214 sodium cacodylic, 1.0081 g of NaCl, 630 µl of HCl 0.2 M, 1 mL of glutaraldehyde and was
215 added with H_2O for a volume of 100 mL using a Volumetric flask. After all of the ingredients
216 were dissolved and mixed together until the mixture was homogenous, the solution was put
217 into a dark bottle and stored in the refrigerator.

218 Agar medium was made using 3.5 g of agar, 0.1 g of gelatin, and added with 1/100
219 stock solution for dilution that consisted of 1 mL of stock solution added with 99 mL of H_2O.
220 The 100% stock solution was made by putting 1 g of meat extract, 1 g of microbiological
221 peptone, 0.5 g of NaCl and 80 mL of H_2O into a glass beaker. It was then stired well until it
222 dissolved evenly and the pH was adjusted to 7.2. If it is too acidic, it can be added with 0.1 N

of NaOH and if it is too alkaline, it can be added with 0.1 N of H_2SO_4. Then, the solution was poured into the Erlenmeyer and added with H_2O to get 100 mL volume. It was then boiled on the stove and stirred constantly until it dissolved evenly. After that, it was sterilized with an autoclave.

The steriled agar medium was then poured into the petri dish. A special filter paper that had been cut to the size of 5x5 mm (previously sterilized) was put on the un-ossified agar, and we waited until it was cool and ossified. The isolates which had previously been grown on preculture medium and shaken for one night was taken 1 µl and dripped on the filter paper that had been placed on the agar, then it was incubated for four days.

Filter paper that had been overgrown by isolates on the agar was then taken and put in microcentrifuge tube, added with 1 mL of fixation solution, and incubated at 4˚C for 30 minutes. The fixation solution was then taken using a pipette until it was empty, added with 1 mL of acetone 60%, and incubated at room temperature for 15 minutes. The acetone 60% was then taken until it was empty and replaced by 1 mL of acetone 80%. The acetone 80% was then taken until it was empty and replaced by 1 mL of acetone 100%; the process with acetone 100% was repeated twice. After the acetone had been removed and replaced by 1 mL of pentyl acetate and stored at room temperature for 20 minutes, it was dried using filter vacuum. When the fluid was gone, the sample had reached the critical point drying. It was then coated and ready to be observed using a SEM[16].

Molecular Identification: The molecular identification was conducted using the 16S rRNA gene sequence. The sequencing of 16S-rRNA gene consisted of several stages, including DNA extraction, amplification between 16S-rRNA gene and PCR, and sequencing using Sequencer machine.

Amplification of 16S rRNA gene was conducted using thermal cycler. The Primers used were the couple of 8F (5' - AGAGGTTGATCCTGGCTCAG-3'), primer 1492R (5' -

GTTTACCTTGTTACGACTT-3'). The PCR process began with initial denaturation stage at a temperature of 94˚C for 5 minutes, and continued with the process of as many as 30 cycles consisted of denaturation process at a temperature of 94˚C for 1 second, primer attachment at a temperature of 55˚C for 1 minute, and elongation at 72˚C for 1 minute. After the 30 cycles had finished, it was followed by lengthening process on the temperature of 72˚C for 10 minutes, and the PCR process stopped at a temperature of 12˚C. PCR results were then viewed by electrophoresis on agarose gel of 0.8%.

Phylogenetic Analysis: The identical 16S rDNA sequences were identified by phylogenetic tree analysis and manual comparison, where the sequences with a similarity of more than 90% were described as identical, and these sequences were used for further phylogenetic analysis as Operational Taxonomic Unit (UTO). The evolutionary tree was based on distances compiled using the nearest neighbor algorithm.

Data Analysis: Bacterial Isolation and Identification were conducted with descriptive method. The microbial activity tests were analyzed using a Completely Randomized Design. Furthermore, if there are significant differences, the analysis will be followed by Duncan's New Multiple Range Test.

RESULTS

Totally five isolates were found from puffer fish waste. All the isolates were subjected to primary screening on Milk Agar plate, and 3 of 5 isolates formed the clear zone, which supported the degradation and utilization of casein (Skim Milk) by the respective isolates. Those organisms were named as BRAW_PT, BRAW_PB, and BRAW_PI strains (BRAW – Buntal Rembang Ari Wibowo). The macroscopic observation on bacterial isolates was shown in Table 1.

They capable of growing and degrading puffer fish skin at 35°C within 14 days. The BRAW_PT, BRAW_PB, and BRAW_PI strains which appeared single or in the chain had

straight rods. They were Gram-positive, endospore-forming organisms, aerobic, motile, strong oxidase, and catalase positive. The additional morphological, physiological, and biochemical test were shown in Table 2.

Caseinolytic Activity: Table 3 showed the results of clear zone diameter in 72 h of observations, BRAW_PT strains showed the largest colony diameter (3.06 mm), followed by BRAW_PI strain (2.40 mm). The smallest colony diameter (1.34 mm) belonged to BRAW_PB strain. The results of clear zone diameter and the diameters of colonies from the largest to the smallest were then sorted and tested to determine the protease activity.

Proteolytic Activity: The results of proteolytic enzyme assay showed that BRAW_PI had a specific activity of 37.52±0.96 U/mg (Table 4). This was the highest activity of the three isolates. The test showed significantly different results among the treatments. A further test using Duncan's Multiple Range Test (DMRT) showed that BRAW_PI had a significantly different proteolytic enzyme activity ($P<0.05$) than other isolates. Proteinase enzyme activity of BRAW_PT and BRAW_PB had no significant differences ($P<0.05$).

Keratinase Activity: Keratinase activity assay was carried out on all of 3 isolates. The test results of keratinase activity were shown in Table 5. Based on the table, BRAW_PI strain had the highest specific keratinase activity of 6.78 U/mg. BRAW_PI had a significantly different proteolytic enzyme activity ($P<0.05$) than other isolates. The proteinase enzyme activity of BRAW_PT and BRAW_PB hadno significant differences ($P<0.05$).

Molecular taxonomy, sequencing, and phylogenetic analysis: Results of PCR with 16S rRNA primer was then viewed by electrophoresis on a 0.8% agarose gel as shown in Figure 3. The isolates had a very close genetic relationship with *Bacillaceae* family based on the 16S rRNA gene sequencing method. BRAW_PT was closed to *Bacillus thuringiensis* by showing 99% similarity, BRAW_PB was closed to *Bacillus aerius* by showing 99% similarity, and BRAW_PI was closed to *Bacillus firmus* by showing 99% similarity. The phylogenetic tree (Figure 2) was constructed by the neighbor-joining method using Molecular Evolutionary Genetics Analysis 6 (MEGA6) program.

DISCUSSION

Skim milk contains casein, a milk protein which will be degraded by proteolytic microorganisms into dissolved nitrogen compounds so the colony will be surrounded by a clear area. It showed that these microbes had proteolytic activity[17]. Based on this test, there were only three isolates that had the ability to degrade casein isolates while the other two did not.

Three of five isolates that showed protease activity were later corroborated by the skim milk agar and they were used for further research. Sivakumar *et al*.[19] confirmed that the zone formed around colonies was due to the formation of the casein enzyme. It was considered as a positive result. The caseinolytic ability of bacteria could be used to select the initial keratinolytic bacteria because most keratinolytic bacteria that derived from nature also had a good caseinolytic activity. The results obtained showed that the BRAW_PB, BRAW_PT, and BRAW_PI isolates were able to degrade the casein because casein is the main protein in milk. Benson[18] stated that the media became clear due to the caseinase exoenzymes produced by bacteria.

Based on the method of Gupta and Ramnani[4], the chosen casein agar media was related to the most reported keratinase enzyme derived from nature. The isolates were grown in an incubator at 30°C for 72 h. The statistical analysis was conducted with variance analysis that showed that there were significant differences among the treatments. A further test was conducted using Duncan's Multiple Range Test (DMRT). It showed that the clear zone diameter of all isolates had been significantly affected by the incubation time. All isolates had the largest clear zone diameter in 72 h. It can be seen in Table 3.

Brandelli *et al*.[20] stated that the hydrolysis ability of casein depended on the species and environment of the bacterial isolation place. Furthermore, in order to ensure the ability of bacteria in hydrolyzing protein, a protease activity test was conducted. The screening was

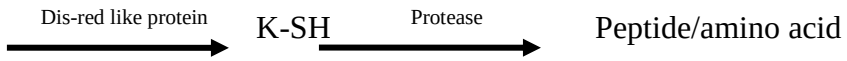
conducted on 3 isolates using qualitative (the formation of the clear zone) and quantitative (enzymes activity) methods[21].

Protease is also called peptidase or proteinase. It is a hydrolase-class enzyme that will break down proteins into simpler molecules such as short oligopeptides or amino acids, with hydrolysis reaction on the peptide bond. Proteolytic bacteria are the bacteria that are able to produce extracellular protease enzyme. The enzyme breaks protein that is produced in the cell and releases it out of the cell.

Some researcher found keratinase activity in some bacterial enzymes such as *Bacillus subtilis* that has keratinase activity of 1.8 U/mL[22]; *Aspergillus sp.* 1.7 U/mL, *Cladosporium sp.* 1.9 U/mL, and *Periconia sp* 1.5 U/mL[23], as well as *Streptomyces gulbargensis* that has keratinase activity of 1.5 U/mL[14]. Nevertheless, the keratinase activity of those three strains is lower than the one produced by *Bacillus megaterium* F7-1 by 58 U/mL[24], *Chryseobacterium sp.* by 40 U/mL[25], and *Flavobacterium sp.* by 7 U/mL[26].

Some keratinolytic microorganisms have been reported, including several species of fungi such as *Microsporum*[27], *Trichophyton*[28], *Streptomyces*[29,30] and *Actinomycetes*[31,32]. Recently, keratinase activity was also reported for coccus that was rod-shaped Gram-positive.

Yamamura *et. al.*[33] reported a joint action between the similar protein disulfide reductase and proteases produced by *Stenotrophomonas sp*. to degrade deer-hair keratin. The mechanisms of keratin degradation occurred as follows:



Native keratin *Reduced keratin*

There were two types of morphological forms of microbes in the form of short bacilli, while four types of microbes are long-shaped bacilli. SEM was useful to clarify the simple coloring using safranin dye, and Gram and Zn staining indicated that all of the isolates belonged to Bacilli class.

The results of amplification of the encoding 16S rRNA gene of bacterial isolate were then determined for its DNA base sequences. The process of determining the base sequence of DNA was conducted by 1st BASE, Malaysia. The results of the determination was then read with DNA baser. Then, the DNA's base sequence obtained was used to search for the comparison of DNA sequence in various similar microorganisms or those that have a close genetic relationship to the NCBI (National Center for Biotechnology Information) GenBank through the BLAST (Basic Local Alignment Search Tool) method (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The results of the matching using BLAST method was then selected to find the one that had the closest genetic relationship and sequenced for the phylogenetic tree using the Molecular Evolutionary Genetics Analysis 6 (MEGA6) program. The sequence of 16S rRNA gene has been determined for many strains. Genbank is the largest data bank for nucleotide sequences, saving over 20 million nucleotide sequences and almost more than 90.000 of them are the 16S rRNA gene. It shows that many previously saved nucleotide sequences are compared with the sequence of a newly known strain. In addition, the universal 16S rRNA gene in bacteria can be used to analyze the phylogenetic relationship between the bacteria from the genus level of many phyla to the level of strains that are species and subspecies.

The genetic relationship of bacteria was known from the base sequence analysis of 16S rRNA gene nitrogen. The base sequence of isolates' nitrogen obtained from the sequencing of the 1st BASE was analyzed with DNA Baser program to get the nitrogen's sequence that can be compared with the nitrogen's base sequence of reference strain from Genbank National Center for Biotechnology Information (NCBI) with Basic Local Alignment Search Tool (BLAST). The base sequences of the isolates' nitrogen and the base sequences of the reference strain nitrogen or comparators were used to analyze the genetic relationship in the form of a phylogenetic tree. The phylogenetic tree on this analysis was performed with

Molecular Evolutionary Genetics Analysis 6 (MEGA6) program and neighbor-joining method. The formed phylogenetic tree was evaluated with the bootstrap method (1000 replications) to determine the level of robustness and accuracy of the analysis. The bootstrap value was indicated by the number contained in the branches of the phylogenetic tree. According to Hall[34], the higher value bootstraps, the more reliability or trustworthiness. The results of phylogenetic tree analysis are shown in Figure 2.

Based on the formed phylogenic tree, all isolates had a very close genetic relationship with different species of Bacillaceae family. BRAW_PT was close to *Bacillus thuringiensis* with 99% similarity, BRAW_PB was close to *Bacillus aerius* with 99% similarity, and BRAW_PI was close to *Bacillus firmus* with 99% similarity.

CONCLUSIONS

The results of the screening showed that 3 of 5 bacterial strains presented keratinolytic activity. They are *Bacillus thuringiensis BRAW_PT, Bacillus aerius BRAW_PB* and *Bacillus firmus BRAW_PI.* The proteolytic enzyme test showed that BRAW_PI bacterial strain had the highest protease and keratinolytic specific activity, which was 37.52±0.96 U/mg and 6.781±0.479 U/mg consecutively.

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FIGURE LEGEND

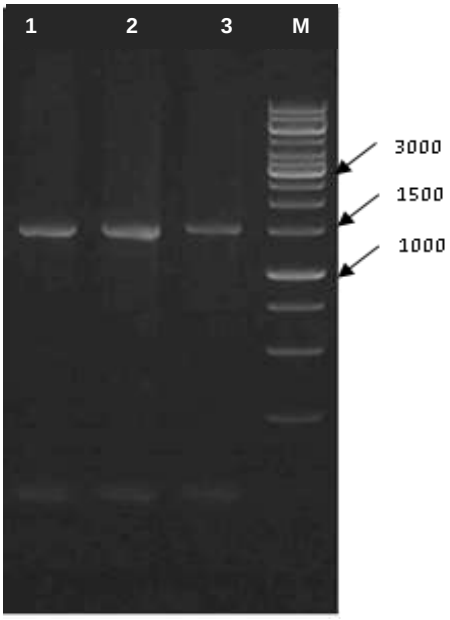

Figure 1. Agarose gel eclectrophoresis of Amplified products. 1 : BRAW_PI; 2 : BRAW_PB; BRAW_PT; M : Broad range marker

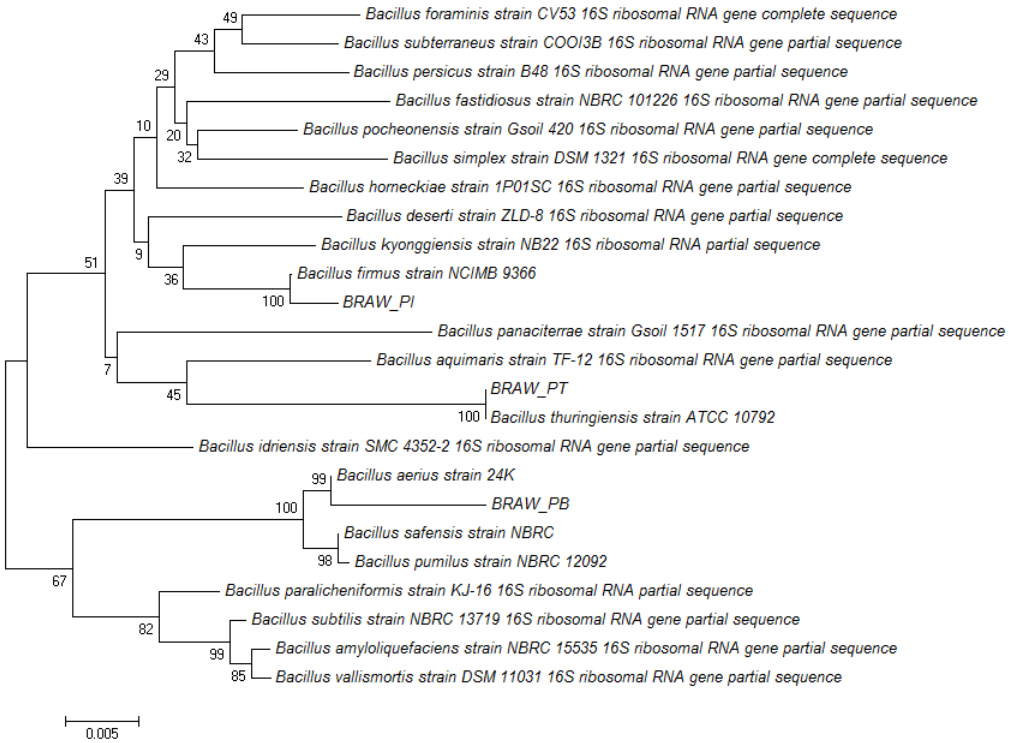

Figure 2. The Phylogenic Tree of 3 Isolates

TABLES

Table 1. Observation of keratinase bacterial isolates

Isolate Code	Colony Color	Colony Shape
1	Milk (Ps)	Round, convex, glossy
2	White (Pb)	Round, convex, wavy

3	Sharp-White (Pt)	Round, convex, jaggy
4	Viscous-White (Pk)	Round, convex,
5	Pink (Pi)	Round, convex, jaggy

Table 2. Morphology of keratinolytic isolates

Morphology	Bacteria strains		
	BRAW_PT	BRAW_PB	BRAW_PI
Catalase	Positive	Positive	Positive
Oksidase	Positive	Positive	Positive
Upright media	Rhizoid	Beaded	Beaded
Slant media	Echinulate	Echinulate	Echinulate
Elevation	Convex rugose	Convex	Convex papilate
Deep Struktur	Opaque	Opaque	Finely granular
Colony Form	Curied	Circulair	Circulair
Motility	Positive	Negative	Negative
Spora Staining	Positive	Positive	Positive
Gram Staining	Positive	Positive	Positive
Acid Staining (Zn)	Acid negatif	Acid negatif	Acid negatif
Carbohidrate fermentation			
Glucose	Positive	Positive	Positive
Fructose	Positive	Positive	Positive
Sucrose	Positive	Positive	Positive
Lactose	Positive	Positive	Positive

Table 3. Diameters of clear zone (mm)

Bacterial Strain	Incubation Time		
	24 h	48 h	72 h
BRAW_PT	$0.242^{a}\pm0.01$	$2.029^{b}\pm0.01$	$3.063^{c}\pm0.06$
BRAW_PB	$0.000^{a}\pm0.00$	$1.018^{b}\pm0.01$	$1.339^{c}\pm0.03$
BRAW_PI	$0.058^{a}\pm0.07$	$1.992^{b}\pm0.01$	$2.404^{c}\pm0.04$

^{a,b,c} Different superscripts in the same column indicate significant differences ($P<0.05$)

Table 4. Proteinase enzyme activity (U/mg)

Strain	Enzyme activity (U/mg)
BRAW_PT	$33.44^{a}\pm0.56$
BRAW_PB	$32.49^{a}\pm1.18$
BRAW_PI	$37.52^{b}\pm0.96$

$^{a, b}$ Different superscripts in the same column indicate significant differences ($P<0.05$)

Table 5. Keratinase enzyme activity (U/mg)

Strain	Enzyme activity (U/mg)
BRAW_PT	$4.867^{a}\pm0.326$
BRAW_PB	$4.349^{a}\pm0.634$
BRAW_PI	$6.781^{b}\pm0.479$

$^{a, b}$ Different superscripts in the same column indicate significant differences ($P<0.05$)

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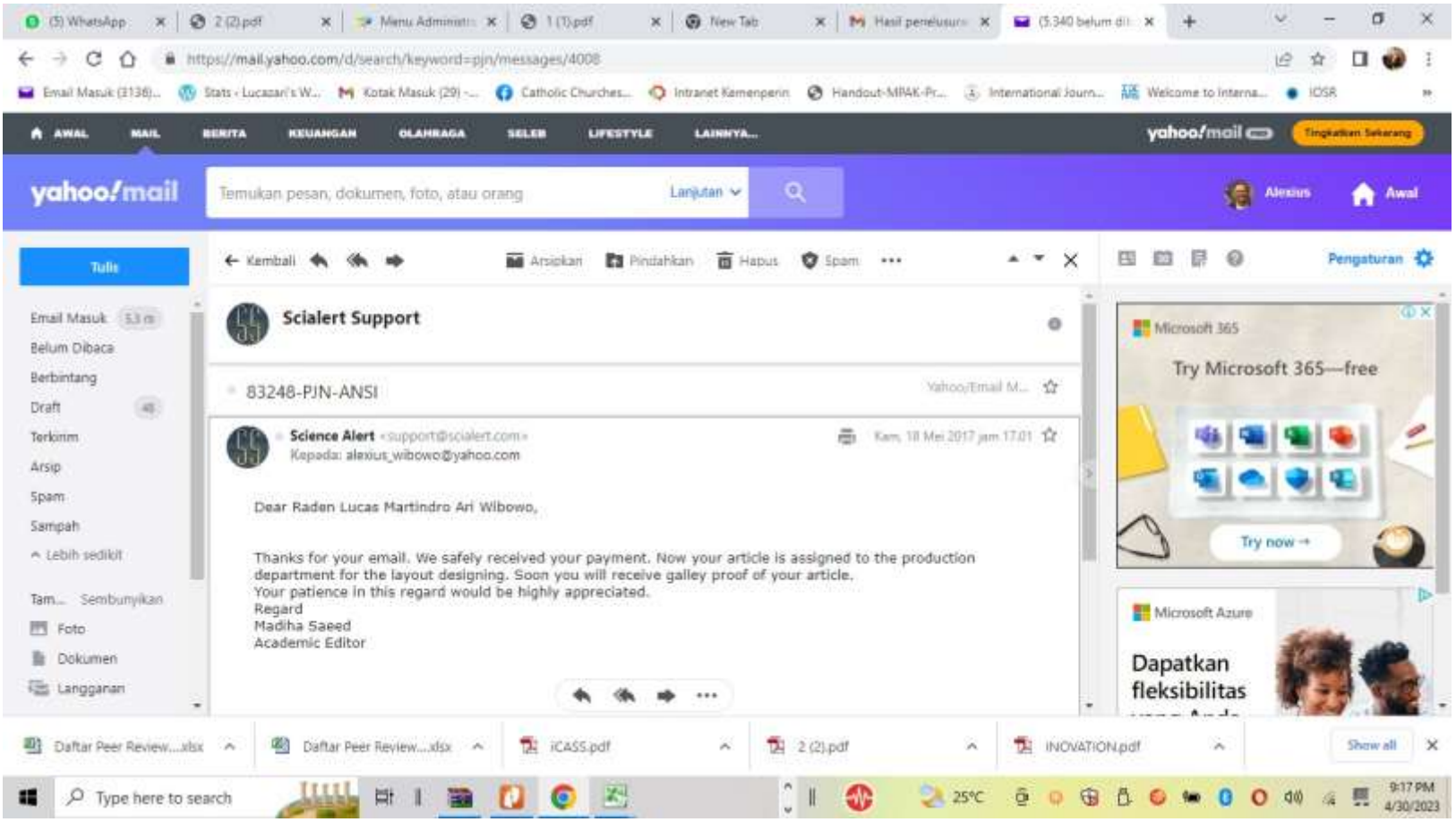
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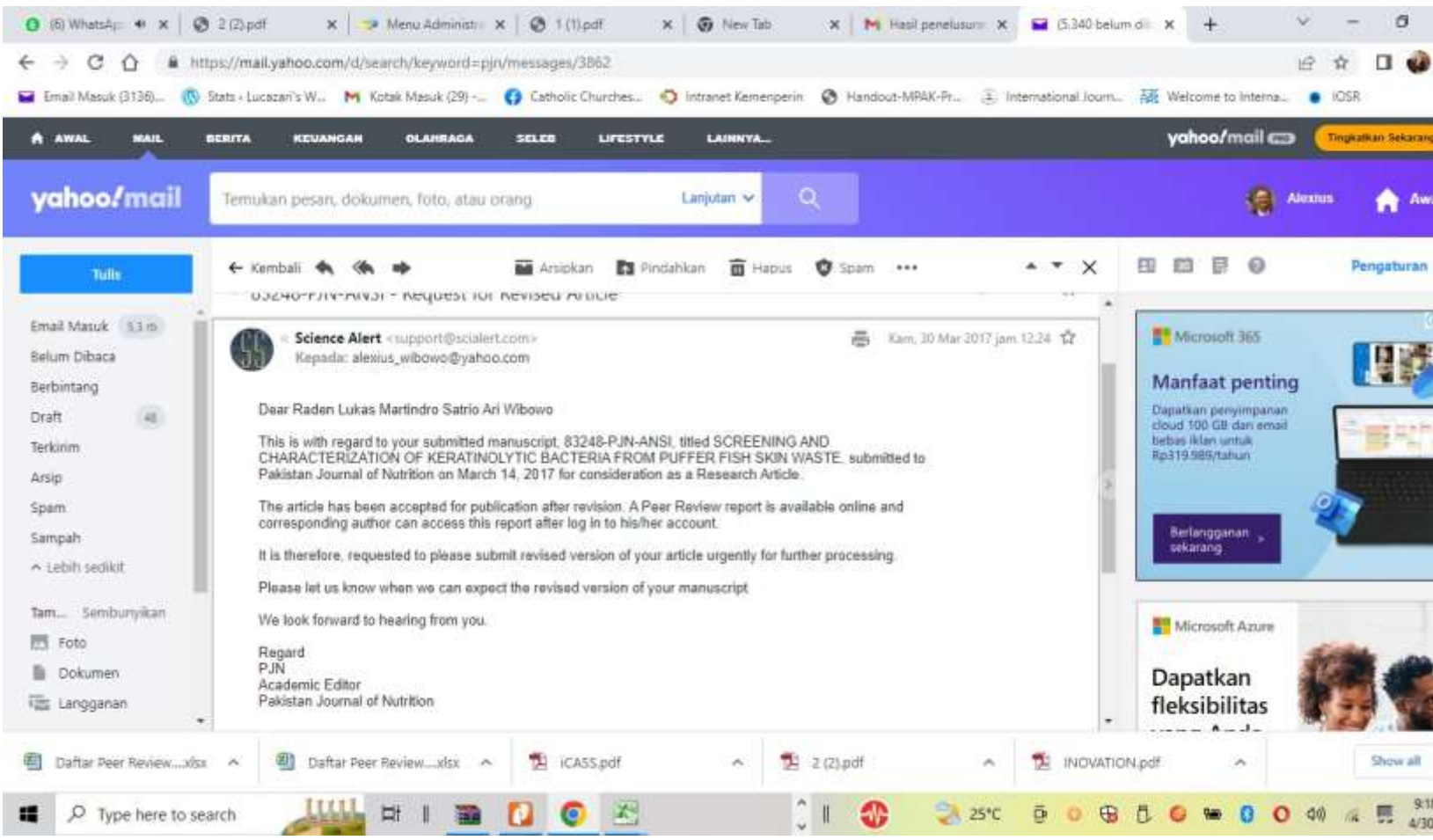
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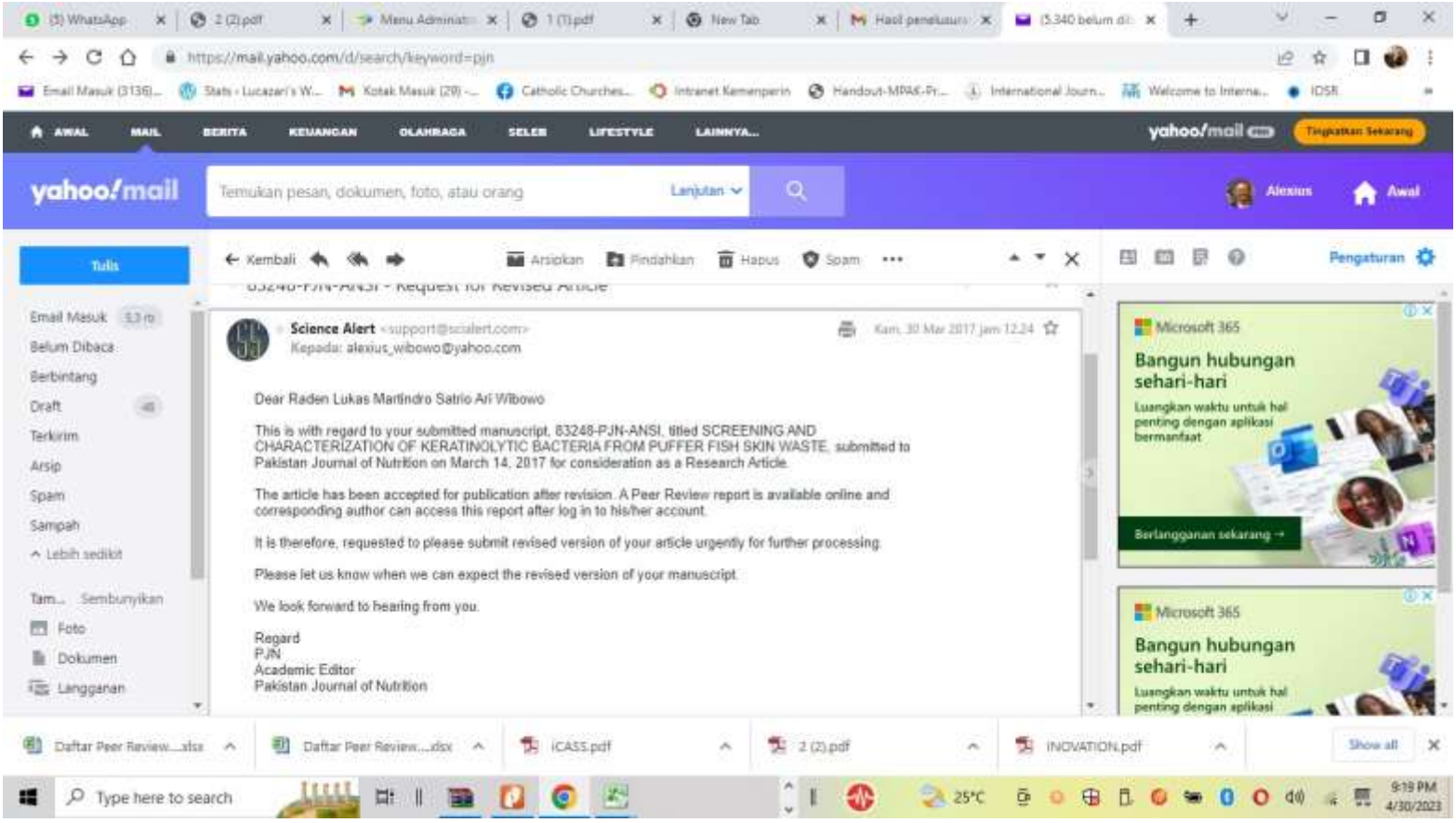

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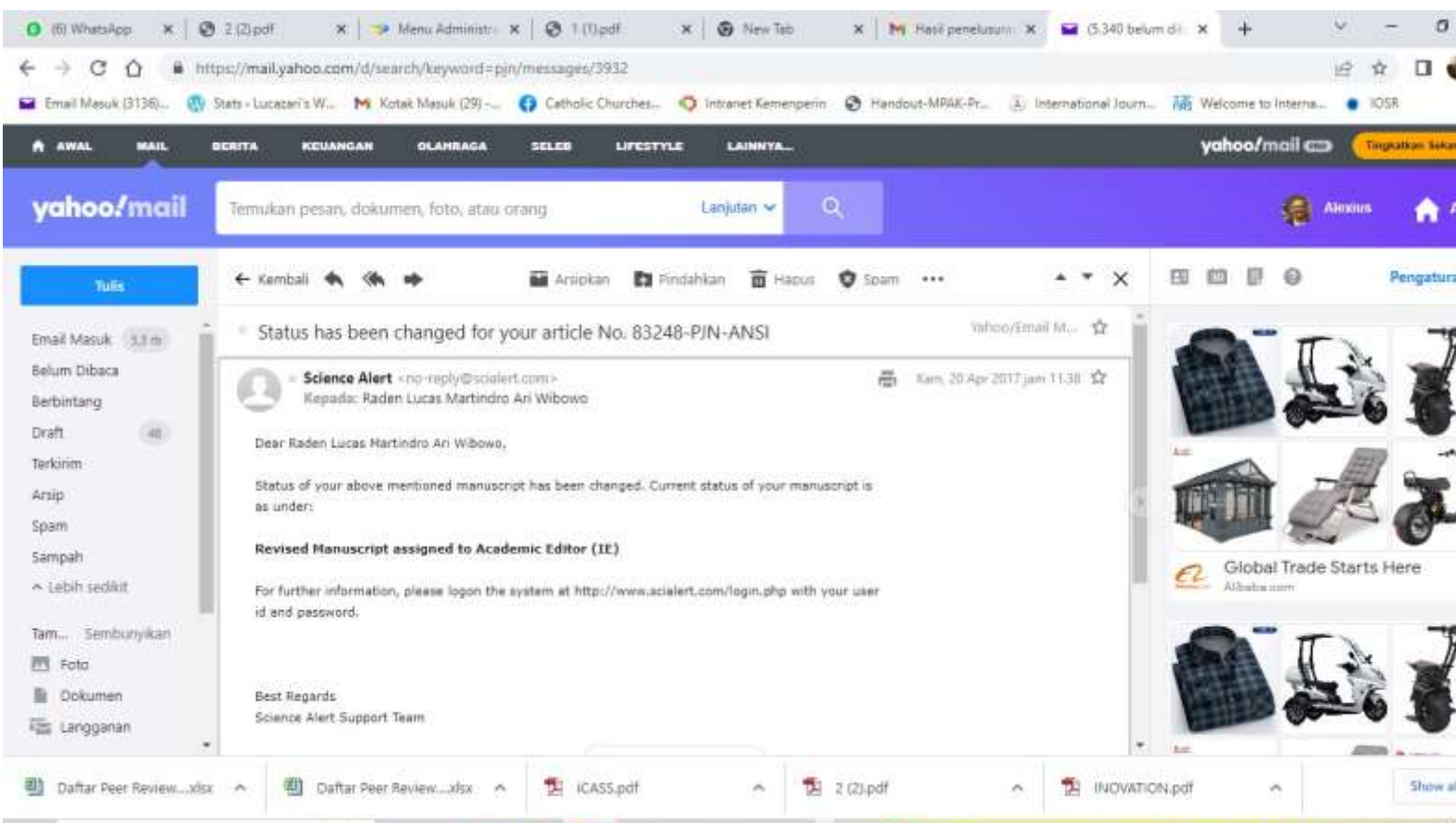

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