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IDENTIFICATION OF CATALASE-PRODUCING BACTERIA IN ECOENZYMES FROM FOOD WASTE USING GEN 16S rRNA

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ABSTRACT

Food waste is a global problem that has a negative impact on the environment and society as a whole because it endangers food security and the sustainability of the food supply chain in the long term. One of the efforts made to overcome the problem of kitchen waste and food waste is by making ecoenzymes made from the fermentation of organic kitchen waste. Ecoenzymes are naturally produced through the fermentation of fruit and vegetable waste by anaerobic bacteria. Among the components of ecoenzymes is the enzyme catalase, which helps break down excess hydrogen peroxide. One source of catalase is microorganisms such as bacteria and fungi. In identifying bacteria in ecoenzymes, molecular testing using the 16S rRNA gene can be employed, as it can determine the specific type of bacteria. The objectives of this study are to determine the number of bacterial isolates producing catalase enzymes in ecoenzymes from food waste and to identify the bacterial species in ecoenzymes from food waste that produce catalase enzymes using the 16S rRNA DNA marker. This study employs an experimental research design with both qualitative and quantitative approaches. This study showed that there were three isolates (A, B, and C) that were positive for producing catalase enzymes. Based on molecular testing using the 16S rRNA gene, the three isolates were most closely related to the species B. subtilis. This study demonstrated the presence of catalase enzyme activity in ecoenzymes from food waste and the presence of B. subtilis bacteria, which play a role in disease control in plants.

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Introduction

Food waste is a significant global issue that negatively affects the environment, food security, and the food supply chain (Saputri et al., 2018). In Indonesia, food waste amounts to 1.3 million tons per year, equivalent to IDR 27 trillion, while millions of people continue to suffer from malnutrition (Usman, 2020). According to data from BAPPENAS (2022), 38.3% of food waste originates from households, heavily influenced by consumption behavior (Parfitt et al., 2010). Additionally, SIPSN reported that in 2023, food waste accounted for 41.60% of the total 69.9 million tons of national waste.

One viable solution for reducing organic waste involves the production of an ecoenzyme derived from the fermentation of fruit and vegetable waste by anaerobic microorganisms (Mahali et al., 2022). This product encompasses enzymes such as amylase, protease, catalase, and peroxidase, all of which offer beneficial properties (Galintin et al., 2021; Larasati, 2020).

Catalase is an enzyme resulting from the ecoenzyme production process. Various bacteria can produce this enzyme, including Enterobacter, Streptococcus, Pseudomonas, Bacillus, Streptomyces, and Neisseria (Sayuti et al., 2016). Molecular methods are now commonly employed to identify bacteria that produce this enzyme, particularly through analysis of the universal 16S rRNA gene, which effectively uncovers phylogenetic relationships among bacteria (Bottger, 1989).

Based on this background, the identification of bacteria producing catalase enzyme in the ecoenzyme from food waste using the 16S rRNA gene has not been widely done. The use of the 16S rRNA gene can be used to identify bacteria with more certainty. The research conducted aims to identify bacteria producing the catalase enzyme using the 16S rRNA gene.

Research Methods

The tools that will be used in the study are test tubes, petri dishes, Erlenmeyer flasks, measuring cups, beakers, flasks, vortex, analytical balance, autoclave, hot plate magnetic stirrer incubator, oven, freezer, refrigerator, cooled incubator, Bio Safety Cabinet, electrophoresis kit, UV-Vis spectrophotometer, micropipette, tip (1000 µl, 200 µl, and 10 µl), refrigerated micro centrifuge, laminar air flow (LAF), thermal cycler, gel documentation, spatula, spreader rod, round ose, 50 ml conical tube, cutter, blender, mortal. The materials used are ecoenzyme derived from food waste, NA (Nutrient Agar), NB (Nutrient Broth), aquades, peroxide reagent (H2O2), Prestotm Mini GDNA Bacteria Kit (Geneaid Lot No: FH36208-S, glycerol, ladder 3000 bp, bromothymol blue, hydrochloric acid (HCL), sodium hydroxide (NaOH), sodium hypochlorite (NaOCL), 1.5 ml microtube, proteinase K, lysozyme, gram + buffer, GT buffer, GB buffer, elution buffer, GD column, W1 buffer, wash buffer, collection tube 1.5 ml, gel red, ddH2O, Tris-Borate-Edta (TBE 1x), loading dye, primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R('-GGTTACCTTGTTACGACTT-3'PCR mix, agarose powder, nuclease free water.

This study uses a combined form of research, particularly with a qualitative and quantitative approach. The qualitative approach is by looking at the morphology and microscopy of bacteria producing catalase and peroxidase enzymes, in addition, qualitative approaches are used to see positive results of bacteria producing catalase enzymes by examining the presence or absence of bubbles. The quantitative method involves measuring the absorbance value of bacteria that produce peroxidase enzymes using a UV-Vis spectrophotometer. Additionally, a molecular quantitative method use 16S rRNA DNA markers to identify bacteria that produce catalase peroxidase enzymes.

Isolation of bacterial isolates from ecoenzyme

Bacteria were isolated from ecoenzyme samples using the spread plate method. Prior to bacterial isolation, a multistage dilution was performed, yielding dilutions 10-1, 10-2 and 10-3 with codes A, B, and C. The isolation results were refined to produce pure isolates.

Catalase Activity

Catalase enzyme testing is qualitative, based on the presence of gas bubbles created by each

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bacterial isolate in the ecoenzyme. The catalase test, which involves using an ose needle to scrape one loop of bacterial isolate on a glass item and then dripping it with hydrogen peroxide, can detect groups of bacteria capable of producing catalase enzyme. Positive results have air bubbles, while negative results do not cause the formation of air bubbles (Lay, 1994).

Identification of Bacteria Using Gen 16S rRNA

The results of isolation of catalase enzyme-producing bacteria were then amplified using the 16S rRNA gene with primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R ('-GGTTACCTTGTTACGACTT-3') (Weisburg *et al*., 1991). These primers were chosen because they are universal, the amplification process can amplify 16S rRNA genes which are usually 1500 bp long (Ethica et al., 2018). The PCR process will be conditioned starting from pre denaturation (94°C, 5 minutes), denaturation (94°C, 30 seconds), annealing (55°C, 45 seconds), extension (72°C, 1 minute 30 seconds), and final extension (72°C, 10 minutes). Next is electrophoresis at 100-volt voltage for 30 minutes, after the electrophoresis process is complete the results will be observed under UV light using a UV transilluminator.

Result and Discussion

Bacterial isolation aims to select bacteria that produce catalase and peroxidase enzymes starting with serial dilution or multilevel dilution with the aim of reducing the number of microbes in the sample (Yunita, 2015). The isolation method used is the streak plate or spread technique which has the advantage of being able to estimate the number of bacteria that grow (Damayanti, 2020). undefinedthree isolates with the code A, B, and C obtained different results in each isolate that was grown which was then purified to obtain pure isolate results.



Figure 1. Results of Bacterial Isolate Isolation in ecoenzyme from food waste

Catalase testing is done using hydrogen peroxide (H_2O_2) reagent, the test is done to identify bacteria capable of producing peroxidase catalase enzyme. This enzyme detoxifies hydrogen peroxide by breaking it down into water and oxygen gas (Rahmi et al., 2015).

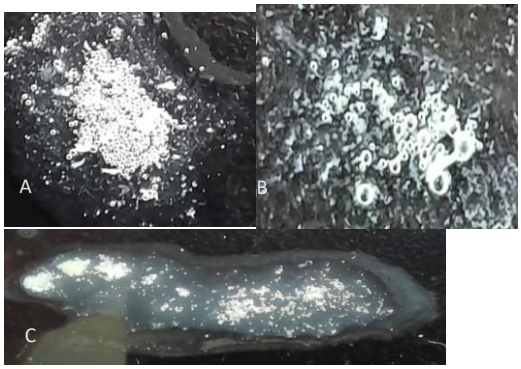


Figure 1. Positive catalase test results on isolates in ecoenzymes from food waste with isolate codes A, B, and C.

The bacterial enzyme catalase functions to break down (H_2O_2) formed from the aerobic respiration process of bacteria, into water (H_2O) and oxygen (O_2) (Purba, 2013). undefinedso that they are not harmful to bacteria because they can cause toxicity so that they cannot live in an environment where hydrogen peroxide is present (Hadioetomo, 1990). In Figure 2, it can be seen that there are gas bubbles that prove the ability of bacteria to convert hydrogen peroxide into water and oxygen.

Positive results in the catalase test are evidenced by the presence of gas bubbles which

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means the ability of bacteria to convert hydrogen peroxide into water, it is under Dewi (2014) which states that bacteria that produce oxygen bubbles include aerobic bacteria, hydrogen peroxide compounds are formed during aerobic metabolism so that bacteria grow in an environment where there is oxygen.

From the results of isolates of catalase enzyme-producing bacteria, then continued with molecular identification using the 16S rRNA gene. Molecular identification is done through four stages, namely DNA isolation, PCR, electrophoresis, and sequencing analysis. DNA isolation aims to separate DNA from cells to obtain pure DNA. Isolation is done by damaging the cell wall of microorganisms so that DNA can come out of the cell (Harvianti, 2017).

DNA isolation followed the protocol of the presto TM ^{mini} gDNA Bacteria Kit with the addition of proteinase K which functions in helping to stop RNase / DNase so as to prevent nucleic acid degradation (Gautam, 2022). The PCR process in this study used the 16S rRNA gene because it is universal for bacteria so that it can be used to measure the phylogeny of bacterial species (Woese et al., 1985).

Fewer stages, namely PCR using the 16S rRNA gene which was carried out as many as 30 cycles, this is because if using more than 30 cycles will not increase the accuracy of the primer and if less than 30 will reduce the quantity level of PCR results (Lorenz, 2012). The primer used in this amplification is primer 27F-1492R (Weisburg *et al.,* 1991).

After the amplification process is carried out, electrophoresis is used to see the resulting DNA band, and confirmation of the results shows the results parallel to the ladder, namely at a size of 1500 bp, then compared with DNA markers as a comparison in the analysis. PCR results from three isolates A, B and C get good results with thick bands and parallel to the ladder used, which is around 1,500 bp, so it is in accordance with Yuenleni (2019), namely the characteristics of good PCR results, namely the results of thick (clear) bands or bands, no smears, and the size according to DNA.

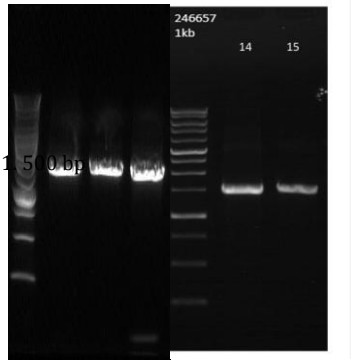


Figure 2. Visualization results of DNA isolation from isolates A, B, and C. PCR results for isolates A and B.

But isolate C still has a smear so it is not said to be good and appropriate because there is still a smear at the bottom of the band. undefinedthat arise during visualization can be caused by two factors, namely the degradation of DNA and the contamination of the resulting product (Mulyani *et al.,* 2011).

The results of molecular isolation of three isolates producing catalase and peroxidase enzymes A, B and C that have been visualized using electrophoresis produce thick and single bands. Isolates that are good and feasible to enter the sequencing stage are isolates A and B with the results of clean and thick bands. The amplification results that have been repeated then enter the sequencing stage with the Sanger method. undefinedforward and reverse in A and B formats. The results of sequencing analysis of isolate samples A and B are obtained in the form of digital data which contains two files, namely *forward* and *reverse* in A and B formats.

Undefined good results are with high graphs and separated from each other in isolate B while the results are not good in isolate A, because there are many peaks that overlap and are not formed due to the presence of double sequence templates that can interfere with the determination of the position of DNA bases (Naipospos *et al.,* 2014).

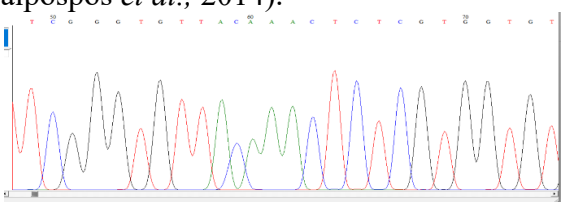


Figure 3. Good sequencing peak pattern

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To get contig sequences, which are the results of overlapping sequencing reads that have been made into a continuous whole (Mount, 2004). The contig sequences were used in BLASTn or sequence sequencing on the NCBI website.

After being entered into the NCBI database, BLASTn was then carried out on the NCBI website (BLASTn), which will look for similarities with the same species that already exist in the database. From the BLASTn data, we found 10 species that have similarities with Bacillus subtilis. undefinedSelection of the same 10 species is based on a high percent query cover value with a value of 100%, a percent identity value in the 98.80% value range, and a low expectation value with a value of 0. The following BLASTn data results that show similarities with B. subtilis species are seen in Table 1.

Table 1. BLAST results for isolate B

Spesies	% ID	Query Cover	E Value	Acc.Number
B. subtilis	98.80 %	100 %	0. 0	MN704480.1
B. subtilis	98.80 %	100 %	0. 0	PP809306.1
B. tequilensis	98.80 %	100 %	0. 0	PQ813782.1
B. subtilis	98.80 %	100 %	0. 0	ON329106.1
B. tequilensis	98.80 %	100 %	0. 0	PP935364.1
B. tequilensis	98.80 %	100 %	0. 0	PQ813762.1
B. subtilis	98.80 %	100 %	0. 0	OQ847923.1

The BLASTn results showed that sequence B has similarities with the genus B. subtilis, this is evidenced by the percent identity value of 98.80%, so that similarities of more than 97% can be said to be similar at the species or genus level (Andersen, 2019).

The species of BLAST results were 10 sequences, then entered into MEGA 11 and also included one species as an outgroup, namely E. coli. The use of outgroups is needed because in making phylogenetic trees to provide polarization of kinship or characters between apomorphic and plesiomorphic (Hidayat, 2008). undefinedresults of the alignment were followed by the creation of a phylogenetic tree.

undefinedanalyzes the phylogenetic tree using the maximum likelihood method with a bootstrap of 1000 using the Kimura 2-parameter model. The results of the tree formed are divided into 2 types, namely original and with bootstrap results (Anafarida, 2020). The Kimura 2- model was chosen because it can determine the similarity between sequences and is fast and easy in analyzing sequence groups (Bhambri, 2012).

Maximum likehood with 1000 bootstraps was chosen because using a bootstrap of 1000 replicates can increase confidence in making phylogenetic trees (Hall, 2001). In addition, according to Ubadillah (2009), the greater the bootstrap value, can increase the level of confidence in the results of tree reconstruction. undefinedthat proves that the level of confidence is very high in accordance with Hidayat (2008) statement that the bootstrap value is high if it is more than 50.

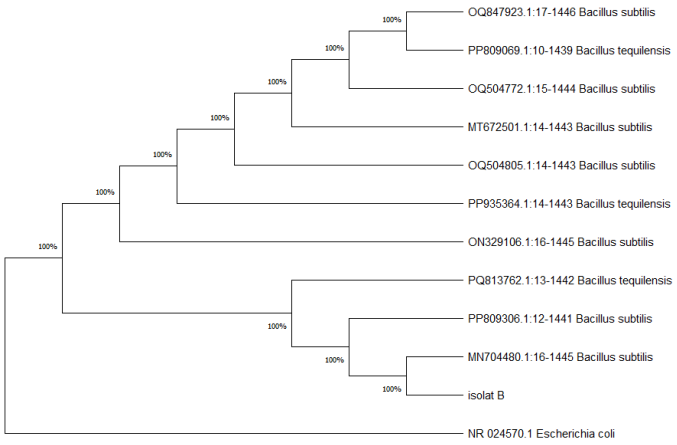


Figure 4. Phylogenetic tree results using the method Maximum likehood

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The results of the phylogenetic tree are in the B sequence one clad with B. subtilis species, namely with a bootstrap value of 100%, thus proving that the species still have a very close kinship, besides that it is also reinforced by a high percent query cover value, namely with a value of 100%, a percent identity value in the 98.80% value range and a low expectation value, namely with a value of 0 so that it can strengthen that bacterial isolate B has a close kinship with the identified reference species (Altschul, 1990). The results of the phylogenetic tree are by Ludwig (2012), which states that the presence of two isolates with the same branch indicates the similarity of species.

Based on the results of the catalase test and reinforced by sequencing using the 16S rRNA gene and data analysis, it was found that the bacterial species in the ecoenzyme isolate B is *B. subtilis*, which is a bacterium of the genus *Bacillus* that has the characteristics of being catalase positive (Awais, 2010). Meanwhile, based on molecular tests, isolate B has a close relationship with the species B. subtilis because it has a high percent query cover value with a value of 100%, a percent identity value in the 98.80% value range and a low expectation value with a value of 0 so that it can strengthen that bacterial isolate B has a kinship with B. subtilis, besides that it is also reinforced by the results of the phylogenetic tree which shows a bootstrap value of 100% and a genetic distance value of 0.00 which proves that the genetic distance is very close.

Conclusion

This study proves the identification of bacterial species producing catalase enzymes in ecoenzyme from food waste using the 16S rRNA DNA marker, namely the sequence B has kinship with the species B. Subtilis because it has a bootstrap value on the phylogenetic tree of 100%, proving that the confidence is very high. Subtilis because it has a bootstrap value on the phylogenetic tree that is 100% which proves that the level of confidence is very high.

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