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Enterobacter hormaechei: an endophytic bacterium found in Avocado Peel (*Persea americana* Mill.) with antioxidant properties

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ABSTRACT

Avocado peels (AVP) are renowned for their potent antioxidant properties, making them highly effective in preventing oxidation and free radical formation. Endophytes, microorganisms residing within plant tissues, have demonstrated the ability to produce novel compounds with remarkable biological activities. These bioactive compounds are sometimes even more potent than those found in their host plants. This study explores the potential of endophytes from avocado peels as rich sources of antioxidant compounds. AVP samples are collected, surface-sterilized, and segmented before being cultured in growth media. The bacteria are then isolated, purified, and subjected to ethyl acetate extraction to evaluate their antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) microassay. Samples demonstrating favorable antioxidant properties undergo molecular identification through 16S rRNA gene sequencing. Four bacterial strains are successfully isolated, with only the APK4 strain exhibiting significant antioxidant activity with an IC_{50} value of 302.3 μg/mL. Molecular analysis and phylogenetic tree construction reveal that APK4 is closely related to the *Enterobacter hormaechei* species, with a percent identity value of 99.93%. These findings highlight the potential of active metabolites from endophytic bacteria in AVP extracts as promising lead compounds for the development of novel drugs, nutraceuticals, and cosmetic ingredients.

Keywords: Avocado peel (AVP), Endophytic bacteria, *Enterobacter hormachei*, antioxidant activity

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INTRODUCTION

Free radicals are unstable molecules that harm cells, leading to aging, cancer, and cardiovascular diseases. Antioxidants neutralize these molecules, protecting cells. Natural antioxidants from fruits and vegetables are safe and beneficial, while synthetic ones in supplements and processed foods can be harmful. Excessive synthetic antioxidants may disrupt the body's balance, causing toxicity and increasing disease risk. Thus, obtaining antioxidants from natural sources is essential (Rautiainen et al., 2016).

Avocado peels (AVP) are known for their potent antioxidant properties, containing over phenolic compounds including hydroxycinnamic acids, flavonols, and flavan-3-ols (Akan, 2021). Quercetin derivatives, the main flavonols in avocado peels, are known for their antioxidative (Feng et al., 2019). Endophytes, microorganisms residing within plant tissues, are emerging as a promising source of natural products for oxidative stress and bioactive agents. These endophytes influence plant metabolite production through microbial-host interactions, potentially enhancing antioxidant compound production. They produce bioactive compounds, sometimes the derivates are more potent than those in their host plants and have shown the ability to generate both plant-associated and novel compounds with remarkable biological activities. For example, phenazine-1-carboxylic acid from *Pseudomonas aeruginosa* effectively scavenges free radicals and inhibits oxidative processes (Denning et al., 2003). *Methylobacterium radiotolerans*, from *Combretum erythrophyllum* seeds, showed an IC_{50} of 5.65 µg/mL in the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay (Photolo et al., 2020). Endophytes, which can be cultivated rapidly under controlled conditions and in a short time, are a viable source of natural bioactive molecules.

This study is interested in the potential of endophytes of avocado peel to be an abundant source of antioxidant compounds. It focuses on the development of more potent and sustainable antioxidant agents that can replace synthetic alternatives in the food and nutraceutical industry. This is the first study that will explore endophytes from avocado peels as a source of antioxidant, hence not yet explored.

MATERIALS AND METHOD

Sample preparation and surface sterilization

Avocados (*Persea americana* Mill.) were harvested In January 2023 from Kuranji in Padang City, West Sumatra. These plants were verified at Universitas Andalas Herbarium. The avocados were then prepared by peeling and sterilizing them through a process involving soaking in 1% NaOCl for two minutes followed by treating with 70% alcohol for two minutes and rinsing five times with water. To ensure sterilization a sample of the avocado skin rinse was cultured on agar (NA) media. After two days of incubation, at 37°C the NA medium was examined to confirm if any microbial growth had taken place (Anjum & Chandra, 2015; Rustini et al., 2023).

Isolation and fermentation of endophytic bacteria

Sterile AVP was cut into 1x1 cm lengths and then cultured on Nutrient Agar (NA) media. It was purifying the bacteria that grew around the avocado skin involved picking each colony that grew and inoculating it on fresh NA media until a pure isolate was obtained. The colour and surface texture of the colonies were observed. Fermentation of endophytic bacteria in AVP skin was carried out using Nutrient Broth (NB). The substrate was separated by pipetting during 24, 48, 72, and 96 hours of fermentation in a shaker incubator set at 37°C and 120 rpm. Next, the mixture was centrifuged for 15 minutes at 5000 rpm, and then the filtrate and residue were separated (Beal et al., 2020; Yati et al., 2018).

Extraction and antioxidant assay

Endophytic bacterial fermentation products were extracted with ethyl acetate solvent in 1:1 v/v, then macerate for 3 days. The product was then pumped into a seperatory funnel, stirred, and allowed to settle until two separate layers were formed. After distillation, the organic extract was separated and finally was evaporated to give widely-used and easily transferable concentrated organic extract for more

fractionation and isolation. In this experiment, the antioxidant activity of this extract was tested using DPPH assay at the concentration of 24mg/100 mL of methanol. The stock solution was further filtered using methanol and further diluted to get a concentration approximately yielding a mixture with an absorbance of 0.1713 at 517 nm. Eighty microliters of the DPPH solution was mixed with 50 µL of the extract at different concentrations in test tubes; triplicate extractions of each concentration were made for the assay A standard solution of DPPH at 12.5 mg dissolved in 100 mL methanol was prepared with ascorbic acid as a positive control. Incubation in the dark lasted for 30 min. Subsequently, absorbance was read at 517 nm using a microplate reader to get the IC_{50} value. The percentage of antioxidant activity was calculated using the following equation (Mohamed et al., 2013).

$$\% \text{ of antioxidant activity= = } [(Ac-As) \div Ac] \times 100 \quad \text{.....(1)}$$

where:
Ac=Control reaction absorbance;
As=Testing sampel absorbance

Molecular identification

A 35-cycle PCR protocol was used with denaturation at 95°C for 45 s, annealing at 56°C for 45 s, and extension at 72 °C for 1 min. The PCR protocol included an initial 2-minute denaturation step at 95°C, and the last extension step was at 72°C for 5 minutes. After the PCR cycles, the samples were assessed using electrophoresis on a 1% agarose gel. Loading the 1 kb marker in the leftmost well (2 µL) and the remaining wells containing 5 µL each of PCR reaction mixture. Sanger Sequencing of the 16S rRNA Gene PCR Products of Bacteria. The following primers for sequencing: 16SrRNA_27F (5' AGA GTT TGA TCM TGG CTC AG 3') and 16SrRNA_1525R (5' AAG GAG GTG WTC CAR CC 3') had the product was a 1,498 bp long sequence. Then, it aligned children sequences derived from applying forward and reverse primers using the SeqMan (Kumar et al., 2018).

Phylogenetic analysis

The base sequence of the 16S rRNA was aligned using the BLAST program from the NCBI Gene Bank to identify similarities with closely related organisms. Based on the BLAST results, bacterial sample sequences were chosen from the Gene Bank for further analysis. These sequences were aligned, and a phylogenetic tree was constructed. Genetic distances were determined using the MEGA software with the Kimura 2-Parameter method. The reliability of the phylogenetic tree was validated with a bootstrap value of 1,000. Finally, genetic distances were analyzed using the Pairwise Distances Method (Saitou & Nei, 1987; Tamura et al., 2011).

RESULT AND DISCUSSION

In this study, four isolates of endophytic bacteria APK1, APK2, APK3, and APK4 were successfully obtained from the avocado peel (*Persea americana* Mill.) with different shapes, colors, and textures (Figure 1). APK1 colony is almost clear white, with a ratty surface and edges, whereas APK2 is structurally and morphologically similar to APK1 but has a milky white colony color. In contrast, APK3 has a yellowish colony color with a slimy and ratty surface while the APK4 colony is yellowish, with a raised surface, wavy edges, and a slightly dull/cloudy appearance.

The optimum growth time of isolates is revealed by fermentation. The Optical Density (OD) calculation is used to calculate the fermentation time. Fermentation occurs at the beginning of the stationary phase, as indicated by the bacterial growth curve because secondary metabolites are produced during this phase. Metabolite synthesis occurs as a result of a lack of available nutrients, which causes an accumulation of metabolite enzyme inducers and the release of genes that can trigger metabolite synthesis. The induction of enzymes that can be obtained from the cell's metabolism is linked to biosynthesis. APK1 bacteria reach their optimum time in the fermentation process close to 24 hours, then significantly decrease entering the death phase, which is similar to APK2, which enters the

stationary phase after 24 hours, with isolates reaching their optimum time approaching 40 hours. APK3 discovered that the optimum growth time was close to 40 hours, which was relatively similar to the APK4 isolate (Figure 2).

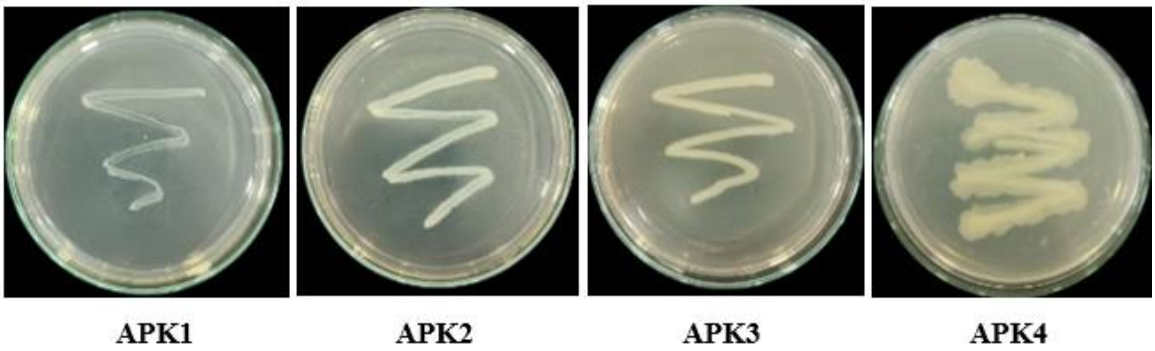

Figure 1. Endophityc bacteria colony isolated from Avocado peel *Persea americana* Mill.

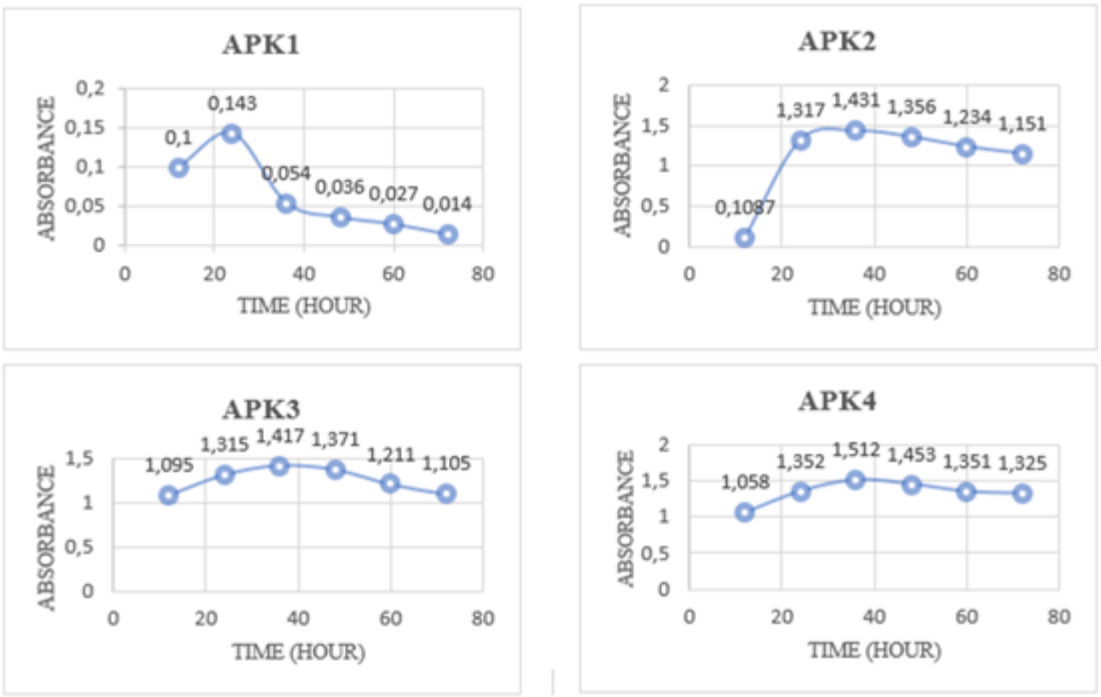

Figure 2. Optical density measurements of bacterial isolates' growth curves

The antioxidant testing results of the APK4 bacterial isolate extract demonstrated the highest antioxidant activity, evidenced by the lowest IC_{50} value of 302.3 µg/mL. This activity was significantly high when compared to the other isolates, APK1, APK2, and APK3, which exhibited IC_{50} values of 19.737 µg/mL, 18.972µg/mL, and 31.217 µg/mL, respectively (Table. 1).

Table 1. DPPH assay results for antioxidant activity of bacterial isolate extracts

Isolate (extract)	Standard Concentration (µg/mL)	Average Absorbance ± SD	Sample Absorbance	Inhibition (%)	IC_{50} (µg/mL)
APK1	1000	0.1033 ± 0.047	0.0636	35.906	19.737
	500	0.1476 ± 0.009	0.1080	-8.724	
	250	0.1513 ± 0.009	0.1116	-12.416	
	125	0.1510 ± 0.021	0.1113	-12.080	
	62.5	0.1546 ± 0.002	0.1150	-15.771	
	31.25	0.1553 ± 0.005	0.1156	-16.443	
APK2	1000	0.1596 ± 0.003	0.1180	12.807	18.972
	500	0.1636 ± 0.005	0.1220	9.852	
	250	0.2006 ± 0.010	0.1590	-17.487	
	125	0.2103 ± 0.004	0.1686	-24.630	
	62.5	0.2140 ± 0.001	0.1723	-27.339	
	31.25	0.2153 ± 0.003	0.1736	-28.325	
APK3	1000	0.1610 ± 0.004	0.0996	37.184	31.217
	500	0.1703 ± 0.004	0.1090	31.302	
	250	0.1680 ± 0.008	0.1066	32.773	
	125	0.1786 ± 0.006	0.1173	26.050	
	62.5	0.1806 ± 0.003	0.1193	24.789	
	31.25	0.1836 ± 0.002	0.1223	22.899	
APK4	1000	0.1010 ± 0.008	0.0393	75.105	302.3
	500	0.1260 ± 0.002	0.0643	59.282	
	250	0.1606 ± 0.003	0.0990	37.341	
	125	0.1656 ± 0.003	0.1040	34.177	
	62.5	0.1863 ± 0.003	0.1246	21.097	
	31.25	0.1843 ± 0.004	0.1226	22.362	
Ascorbic acid	1000	0.0397 ± 0.000	0.000	100	3.54
	10	0.0496 ± 0.000	0.0100	86.238	
	5	0.0616 ± 0.002	0.0220	69.724	
	2.5	0.0850 ± 0.002	0.0453	37.614	
	1.25	0.0930 ± 0.003	0.0533	26.605	
	0.625	0.0963 ± 0.004	0.0566	22.018	

The bacterial isolates containing antioxidant properties in APK4 were subsequently identified molecularly. The genomic DNA of the bacterial isolate APK4 was confirmed by the presence of a single band on the gel (Figure 3). A λ DNA marker with a concentration of 100 µg/mL was used as a reference. The clear, singular DNA band indicates that there are no contaminating substances, such as RNA or proteins, in the sample. This result demonstrates the purity and integrity of the extracted DNA, which is crucial for accurate downstream molecular analyses.

DNA concentration is measured in µg/mL, while purity is assessed using absorbance ratios at 260/230 nm and 260/280 nm. A pure DNA sample has a 260/230 ratio between 2.0 and 2.2. The bacterial isolate APK4 has a 260/230 ratio of 2.197, indicating minimal contamination. Similarly, a pure DNA sample should have a 260/280 ratio between 1.7 and 2.0. The APK4 sample has a 260/280 ratio of 2.000, confirming high purity. Thus, the DNA from the bacterial isolate APK4 is very pure and suitable for further analysis. The estimated size of the amplified 16S rRNA gene product is 1,498 bp. For bacterial isolate APK4, the target band, indicated by a red arrow, matches this estimated size (Figure 3). Additionally, several faint bands of various sizes were observed. These faint bands likely result from the

presence of multiple copies of the 16S rRNA gene in the bacterial genome, causing the primers to anneal to other regions of the genome.

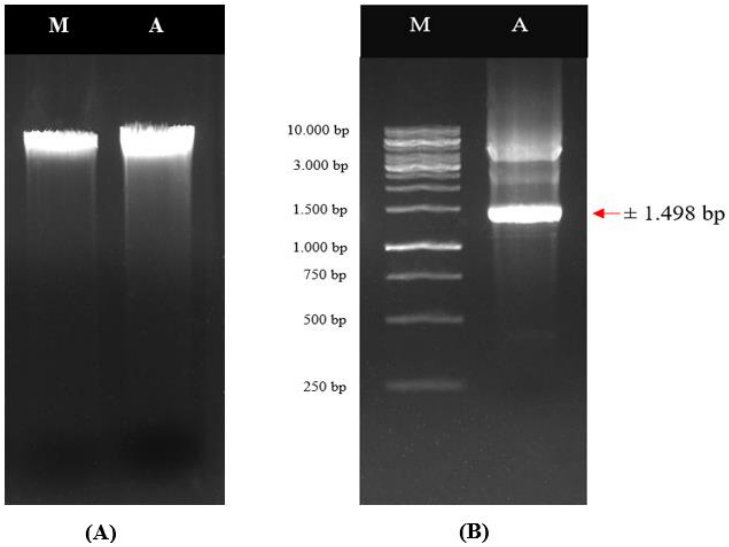

Figure 3. (A) Visualization of genomic DNA and (B) 16S rRNA gene amplification results for bacterial isolate APK4, M (Marker), A (DNA sample genome)

The PCR product of the 16S rRNA gene from the bacterial sample was sequenced in both directions. The electropherogram of the 16S rRNA gene from bacterial isolate APK4 was analyzed. The amplicon, amplified using primers 16SrRNA_27F and 16SrRNA_1525R, was 1,498 bp long. The forward primer (16SrRNA_27F) produced a 757 bp read, while the reverse primer (16SrRNA_1525R) produced a 1,197 bp read. The 5' and 3' ends of both electropherograms had overlapping, unclear, and low-quality peaks, which required trimming. Specifically, the forward primer read was trimmed by 40 bp at the 5' end and 39 bp at the 3' end, and the reverse primer read was trimmed by 38 bp at the 5' end and 94 bp at the 3' end. After editing, the final sequence length of the 16S rRNA gene fragment for isolate APK4 was 1,389 bp Figure 4).

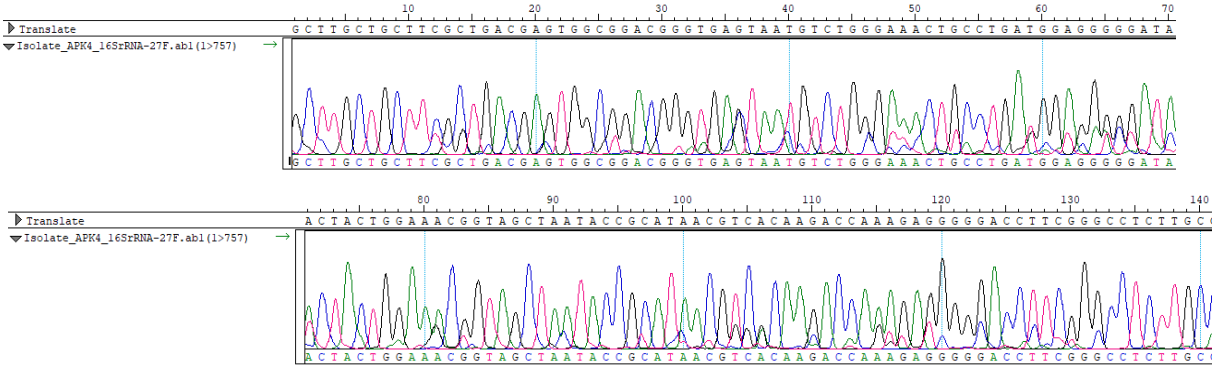

Figure 4. Electropherogram of 16S rRNA gne sequencing for bacterial isolate APK4

Based on the phylogenetic tree construction, two main branches are dividing the bacteria into two clusters. Cluster A comprises 12 bacteria, while Cluster B consists of 4 bacteria. Bacterial isolate APK4,

highlighted by a red box, is located in Cluster B. The closest branch to isolate APK4 includes *Enterobacter hormaechei* subsp. *xiangfangensis* strain FJAT-30604, followed by *Enterobacter hormaechei* isolate EC-TO80 and *Enterobacter hormaechei* strain A1. According to BLAST results, alignment, genetic distance calculations, and phylogenetic tree construction, it is concluded that bacterial isolate APK4 belongs to the species *Enterobacter hormaechei* with percentage of similarity value of 99.93% with 100% query cover (Figure 5).

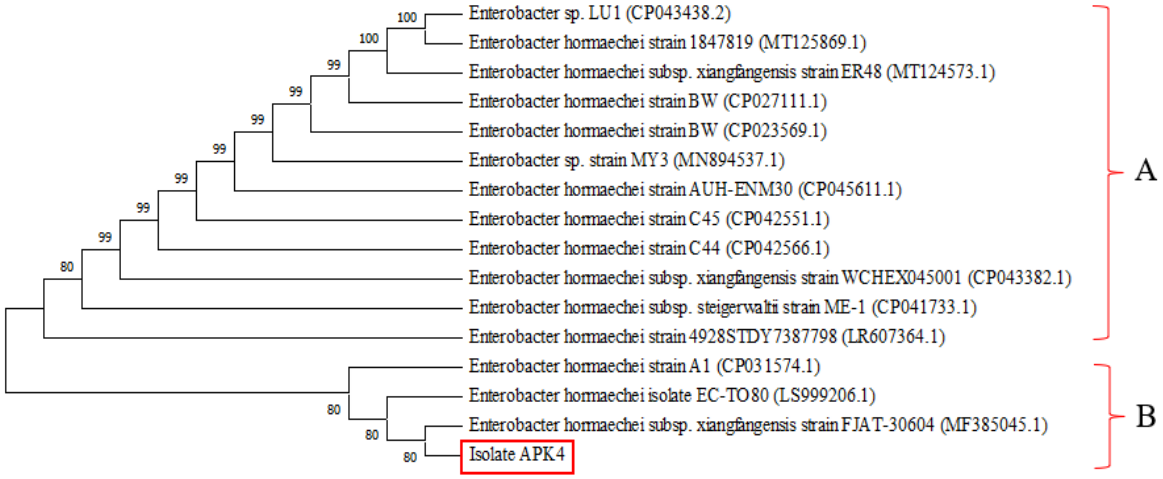

Figure 5. Phylogenetic tree analysis of the isolate APK4-comparison bacteria relationship

The name of the endophyc bacteria in this study is *Enterobacter hormaechei*. It is a Gram-negative bacterium within the enterobacter cloacae complex (ECC), was first identified and described in 1989 (O'Hara et al., 1989). Like other Enterobacter species, *E. hormaechei* has been found in diverse natural settings, including plants, water, soil, and the digestive tracts of insects and animals (Rizwan et al., 2023) (Martins et al., 2020). Previous research has shown that this bacterial isolate extract has strong anti-larvicidal, antimicrobial, and fungicidal properties. However, there are still no specific reports on its antioxidant potential (Wang et al., 2019).

This study highlights an interesting link to the natural habitat of avocados (*Persea americana* Mill.). It suggests that avocados and their endophytes, including *E. hormaechei*, may have developed increased antioxidant activity to survive in tough conditions. This idea fits with the broader understanding that endophytic bacteria play a crucial role in helping plants adapt to and thrive in difficult environments. The ability of *E. hormaechei* to handle and reduce oxidative stress in avocados emphasizes the importance of these bacteria in the plant's resilience. Investigating the antioxidant properties of *E. hormaechei* further, particularly in relation to avocados, could shed light on the complex relationship between endophytes and their host plants in challenging habitats.

CONCLUSION

This research successfully identified four distinct endophytic bacteria from avocado peel, each with unique characteristics. Four isolates were obtained, namely APK1, APK2, APK3, and APK4. Through fermentation analysis, we determined that APK1 and APK2 have optimal growth times of 24 hours, while APK3 and APK4 peak at around 40 hours. Only APK4 exhibited antioxidant properties. We conclusively identified APK4 as a member of the *Enterobacter hormaechei* species using molecular identification with percentage of similarity of 99,93%.

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