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Characterization of Chitinolitic Bacteria from *Hermatia illucent* Larvae Waste: Antifungal Activity, Hydrolytic Enzyme, and Phosphate-Potassium Solubilization

Qorisha Lutfia Prameselly, Bowo Sugiharto, Umi Fatmawati*

[1]Biology Education Study Program, Faculty of Teacher Training and Education, Universitas Sebelas Maret. Jl. Ir. Sutami No 36A , Kentingan, Surakarta, 57126, Indonesia

*Corresponding author: umifatmawati@staff.uns.ac.id

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Abstract. *Hermetia illucens* larvae, also known as the black soldier fly larvae, are best known as the decomposers of organic matter. There are many potential microbes found in the feces of BSF larvae. This research aimed to isolate the chitinolytic bacteria from chicken manure maggot waste and identify the antifungal activity, hydrolytic enzyme activity, phosphate and potassium solubilization, and bacterial species through 16S rRNA gene analysis. The initial screening focused on bacteria with chitinolytic ability. Antifungal activity tests were performed against phytopathogenic fungi, *Colletotrichum* sp. Isolate MKP02 showed the highest activity in inhibiting the growth of *Colletotrichum* sp. up to 100% and produced protease and cellulase enzymes, along with the ability to solubilize potassium. Furthermore, the potential isolate MKP04, the isolate shows the highest cellulolytic activity with a percentage of 300%. It can inhibit *Colletotrichum* sp. fungi, as well as having lipase enzyme content, and being able to dissolve potassium. The results of 16S rRNA gene amplification on the two isolates showed that both isolates were close to bacteria of the genus *Lysinibacillus* and *Brevibacterium*. This research is expected to provide valuable information about the bacterial content, levels of hydrolytic enzymes, and the ability to solubilize phosphate and potassium in BSF.

Keywords: antifungal; decomposer agent; hydrolytic enzyme; maggots

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INTRODUCTION

Maggot is a common term used to refer to fly larvae. Maggots are often found on decaying organic matter and can decompose such material. One frequently discussed species is the larva of the *Hermetia illucens* fly, also known as the black soldier fly (BSF) belongs to the order Dipterans, family Stratiomydae, subfamily Hermetiinae, and genus *Hermetia*. BSF has several functions, such as being a source of biological agents, a protein source comprising 45-50%, capable of rapidly decomposing organic waste, mitigating odors, and promoting sustainability. Additionally, it serves as an alternative feed that can be directly converted into pellets (Masrufah et al., 2020; Putra et al., 2020). Through the utilization of maggots in bioconservation technology, it is possible to address critical environmental issues, specifically the reduction of organic waste originating from households, restaurants, industries, markets, and locations generating substantial organic materials like food scraps, leaf litter, and branches or wood. This is achieved by degrading the waste efficiently and rapidly. Furthermore, it contributes to improving the local economy through the sale of sustainable protein sources and the production of high-quality feed by maggots (Handayani et al., 2021).

Derived from the role of BSF maggots as a source of biological agents, this is due to their ability to transform various organic materials into proteins, fats, and calories, due to the protease, lipase, and amylase enzymes in their digestive system. Additionally, according to the study conducted by Iber et al. (2022) BSF has chitin content that was successfully isolated through demineralization. In research conducted by Nguyen (2010), BSF maggots can consume varied foods, this can occur due to factors such as the type of food and the size of BSF maggot. Based on these factors, it also affects the bacteria found in the digestive system, some of the bacteria found in the research of Zheng et al. (2012), include *Lactobacillus spp.*, *Clostridium* sp., *Bacillus spp.*, *Stenotrophomonas* sp., *Nocardiopsis* sp., *Clavibacter* sp., *Proteus spp.*, *Lysobacter*, *Pseudomonas spp.* and others.

In the research of Arabzadeh et al. (2023), it was reported that the black soldier fly (BSF) contains bacteria with antifungal properties, such as *Bacillus velenzensis*. The research revealed that *Bacillus velenzensis* is capable of inhibiting the growth of seven types of fungi, classified into three categories based on their effectiveness in inhibiting growth: *Sclerotinia sclerotiorum*, *Botrytis cinerea*, and *Alternaria solani* (very strong category); *Phytophthora ulmimum* and *Rhizoctonia solani* (strong category); and *Fusarium oxysporum* and *Phytophthora capsici* (moderate category). These fungi are recognized as significant plant-pathogenic fungi. Additionally, according to Xia et al. (2024), it was found that the bacterium *B. velenzensis* contains enzymes such as amylase, protease, cellulase, and lipase, which contribute to its ability to combat plant-pathogenic fungi.

Pathogenic fungi are detrimental organisms that can cause diseases in plants, manifesting symptoms such as wilting, tissue damage, and even death in the affected plants. One of the pathogenic fungi that is often found in plantations is *Colletotrichum* sp. This fungus induces anthracnose disease affecting various parts of the plant, including leaves, stems, flowers, and fruits. Targeted crops include strawberries, tomatoes, chili peppers, mangoes, string beans, roses, banana trees, and coconut trees. Anthracnose disease is characterized by the appearance of small dark spots, causing the fruit flesh to shrink, dry, and ultimately decay, leading to a decline in quality ranging from 20-90% (Zakaria, 2021). In managing the onslaught of *Colletotricum* sp. fungal diseases, several researchers have conducted research, including controlling the plant environment first such as managing sanitation, selecting disease-resistant plant varieties, cleaning infected plants by destroying them, the enhancement of beneficial organisms, and implementing appropriate cultivation techniques (Syahbana et al., 2022; Hsieh et al., 2023). Additionally, biological control can involve the utilization of living organisms or products of living organisms to control the population of pathogenic organisms. In this case, it is utilizing antifungal bacteria as biocontrol agents to inhibit the growth of the fungus *Colletotrichum* sp. Some examples of bacteria that can control the fungus are *Bacillus* sp., *Escherichia* sp., *Streptomyces* sp., and *Lysinibacillus* sp. (Jamal & Ahmad, 2022). Furthermore, chitinolytic bacteria are also able to inhibit growth and control these pathogenic fungi, which is proven by research that has been conducted by experts (Ali et al., 2020; Herdyastuti et al., 2021).

Bacteria found in the digestive tract are also called gut bacteria, playing a crucial role in the digestion and breakdown of organic matter consumed by a species. Some other species that have positive bacteria in their digestive tract are cockroaches, centipedes, lizards, and rodents. This phenomenon is triggered by their environment so that the animal species develops specific defense mechanisms to stay alive. Derived from the formation of these specific defense mechanisms, the ability of antibacterial and antifungal activity is formed (Akbar et al., 2018; Ali et al., 2017). Maggot BSF is one type of gut bacterial, but the information about the potency of bacterial from the digestive tract as an antifungal agent has not been widely carried out, so researchers conducted several tests to determine the types of bacteria present in the digestive tract of maggot waste, assay the antagonistic activity pathogenic fungi, and identify their potential hydrolytic enzyme produced.

Bacteria containing hydrolytic enzymes play a significant role in agriculture, as these microbes can hydrolyze macromolecules such as carbohydrates, proteins, and lipids. These macromolecules are among the compounds found in organic matter that are difficult to degrade (Muharram et al., 2021); Meng et al., 2023). If the black soldier fly (BSF) harbors bacteria containing effective hydrolytic enzymes, it can be utilized as a decomposer. Additionally, bacteria with the ability to solubilize phosphate and potassium are crucial in agriculture and environmental contexts as they can break down organic matter containing these nutrients, thereby accelerating the nutrient cycle in ecosystems, ultimately benefiting plants and other organisms in the food chain (Agustian & Salsabila, 2021; (X. Chen et al., 2023). Therefore, this research is expected to provide valuable information about the bacterial content, levels of hydrolytic enzymes, and the ability to solubilize phosphate and potassium in BSF. It can be a reference for researchers interested in developing bacterial content for decomposition or other agricultural products.

METHODS

The research was conducted at PT Biotek Cipta Kreasi and Microbiology Laboratory, Biology Education, Faculty of Education and Science, Sebelas Maret University. Isolates were taken from chicken manure maggot waste (*Hermetia illucens*) from PT Biotek Cipta Kreasi, Yogyakarta.

Isolation of bacteria

Samples of chicken manure maggot waste were collected in the amount of 1 gram for dilution up to 5 times, i.e., 10^{-1} to 10^{-5}, with only three dilution results chosen for spreading on chitin media, specifically 10^{-3}, 10^{-4}, and 10^{-5}. After spreading all the samples onto respective Petri dishes containing chitin media (4 g colloidal chitin and a mixed mineral gram consisting of 0.5 g $MgSO_4.5H_2O$; 0.7 g K_2HPO_4; 0.3 g KH_2PO_4; 20 g agar; 1 mL trace element; 1 L H_2O), they were incubated for approximately three days (Herdyastuti et al., 2009). The purpose of using chitin media is to obtain bacteria that can break down or use chitin as a source of nutrients or can be referred to as bacteria with chitinolytic activity (Rahma et al., 2019).

Antifungal Antagonist Assay

The antagonist test was conducted to determine the potential of bacteria to attack fungi. The fungi used in this test is *Colletotrichum* sp. For the antagonistic tests, the test bacteria need to be cultured for approximately three days before inoculating the fungus on both sides of the bacteria, a technique known as dual culture based on the dual culture method proposed by Sathish et al. (2012).

Hydrolytic Enzyme Activity

For cellulolytic assay, bacterial inoculation is performed on Carboxymethyl cellulose (CMC) media with point inoculation and incubated for 2-3 days at room temperature. Positive results are characterized by the presence of a clear zone after being given iodine liquid, if formed then the bacteria can be said to have the ability to hydrolyze cellulose (Gupta et al., 2012).

To investigate the lipolytic activity, bacteria are cultured in Tween 80-Pepton agar media to select bacteria with the potential to produce lipase enzymes capable of hydrolyzing or digesting fats (Utomo & Shovitri, 2014). This test is performed by point inoculation and incubated for 4-6 days at room temperature. A clear zone will form if the bacteria have this potential and will be calculated using the SI formula.

To assay the proteolytic activity, the selected potential isolates are inoculated on skim milk agar (SMA) media that had been sterilized in UV. Bacteria are planted in agar by point inoculation and incubated for 1-2 days (Simarmata et al., 2020). Positive results are indicated by the formation of a clear zone around the bacterial colony and measured by the SI formula. This test is conducted to determine bacteria that produce protease enzymes capable of digesting or hydrolyzing proteins into peptides or amino acids.

Bacteria are inoculated on starch agar or media that has been supplemented with starch. The incubation process is carried out for 2 days, and to visualize the clear zones formed by bacteria, an iodine solution is added. If a clear zone is formed, it indicates that the bacteria produce amylase enzymes capable of hydrolyzing starch or polysaccharides into simpler compounds such as glucose or maltose (Wulandari, 2021). The Solubilization Index (SI) is calculated using the following formula, SI = $\frac{XI-X2}{X2}$, with XI = clear zone diameter and X2 = colony zone diameter (Lau et al., 2020).

Bacterial Gram Identification

This test is conducted to determine the type of bacteria, whether negative (-) or positive (+), by taking 1-2 ose of bacteria to be touched with 1 drop of 3% KOH and mixed evenly on an object glass. After mixing, press the colonies and lift them slowly. If there is an unbroken thread of mucus when lifted, the bacteria are classified as gram-negative. However, if no thread is formed, the bacteria are considered gram-positive (Ogolla & Neema, 2019)

Potassium and Phosphate solubilization assay

The isolation of bacteria with the K solubilization test aims to determine bacteria that can dissolve potassium (K). The media used is Alexandrov media with point inoculation and incubation for 1 week or 7 days at room temperature (Safriani et al., 2020). Bacteria can be categorized as potassium solvents if they produce a clear zone around the isolate. The potassium solubilization index is measured based on the SI (Solubilization Index) formula, SI = $\frac{XI-X2}{X2}$, with XI = clear zone diameter and X2 = colony zone diameter (Lau et al., 2020).

The P solvent test was conducted to determine the bacteria capable of dissolving phosphate. The media used in this test is Pikovskaya media (PK) with point inoculation and incubation for 6-7 days at room temperature (Safriani et al., 2020;Putri et al., 2020). Bacteria are considered to have the ability to solubilize phosphate if they can produce a clear zone around the isolate. The index used to calculate the ability of bacteria to dissolve phosphate is the formula,

$$SI = \frac{X1-X2}{X2}.$$

Molecular identification using 16S rRNA gene analysis

The selected potential isolates are bacteria with antibacterial and antifungal abilities, then the bacteria are purified by the quadrant method and are streaked onto chitin slant agar media. After that, identification is carried out using the 16S rRNA gene and isolated using a special bacterial isolation tool on the total genome that appears with the procedure according to the instructions listed in the manufacturer's instructions. PCR was performed using primers 63f (5'-CAGGCCTAACACATGCAAGTC-3') and 1387r (5'-GGGCGGWGTGTACAAGGC-3') for amplification of the 16S rRNA consensus gene of 1,300 bp (Marchesi et al., 1998). The PCR reaction is carried out with a volume of 40 µL composed of 20 µL DNA polymerase MyTaq™ HS Red Mix (Bioline), 2 µL of each primer, 12 µL nuclease-free water (NFW), and 2 µL DNA template. The PCR steps include pre-denaturation (95°C, 4 minutes), denaturation (95°C, 30 seconds), annealing (57°C, 30 seconds - for MKP02; 55°C, 30 seconds - for MKP04), elongation (72°C, 1 minute), and post-elongation (72°C, 7 minutes) for 30 cycles. The initial sequencing data results are trimmed and assembled using the Bioedit program and Seq trace 0.9, then further analyzed using the BLAST tool from the National Center for Biotechnology Information (NCBI). Furthermore, the data is aligned with MEGA 11 and will get a phylogenic tree that serves to show the relationship of isolates MKP02 and MKP04 with the Neighbor-Joining method (Fatmawati et al., 2018).

RESULTS AND DISCUSSION

Chitinolytic Bacterial Isolated from Maggot Waste (*Hermetia illucens*)

The isolates obtained after cultivation in chitin media are 11 types of isolates selected from maggot waste samples. The results of observations of isolates grown on LB medium showed three different types of colony colors, namely white, cream, and yellow. The white color is formed in isolates MKP01-05 and MKP01-02, except for MKP04 the colonies are yellow. In addition, yellow colonies are found in MKP11 and MKP12, and there is one isolate that has beige colonies, namely MKP06. Bacteria that can produce the enzyme chitinase are screened using chitin agar media (Kotb et al., 2023). For a variety of harmful fungal species, the chitinase enzyme that bacteria produce can function as an antifungal agent (Ekundayo et al., 2022) Chitinases are chitin-degrading enzymes that play an important role in biological control and plant defense mechanisms against phytopathogens by split the chitin polymer into 1,4-β-linked N-acetyl-D-glucosamine (GlcNAc) oligomers and monomers (Jadhav et al., 2017).

Characterization of isolates through various tests

Eleven isolates of chitinolytic bacteria were obtained from the previous stage, then each isolate was characterized regarding the activity of the hydrolytic enzymes produced. The identified hydrolytic enzyme tests were carried out qualitatively, including cellulolytic, amylolytic, proteolytic, and lipolytic enzyme tests. The test results can be seen in Table 1.

Table 1. Activity and index of each test on eleven isolates of chicken manure maggot waste.

Isolates	Hydrolytic Ability (%)				Solubilization Index		
	Cellulolytic	Proteolytic	Lipolytic	Amylolytic	Potassium	Phosphate	Gram Test
MKP01	66.6	-	-	-	-	-	-
MKP02	100	83.3	-	-	33.3	-	-
MKP03	-	-	-	100	11.1	-	+
MKP04	300	-	75	-	40	-	-
MKP05	-	80	-	-	-	-	+
MKP06	200	-	-	125	14.3	-	+
MKP08	133.3	60	-	180	-	20	+
MKP09	155.6	-	13.3	33.3	-	-	-
MKP10	40	-	40	-	-	85.7	+
MKP11	216.6	-	20	-	40	42.8	+
MKP12	200	-	75	225	33.3	50	-

Based on the results provided, the higher the percent index, the better the ability of an isolate (Sun et al., 2020). Concluding each test, in the cellulolytic test the best isolate was MKP04, proteolytic test MKP02, lipolytic test MKP04 and MKP12, amylolytic test MKP12, K solvent test MKP04 and MKP11, and P solvent test MKP10. The ability of bacteria to produce various types of hydrolytic enzymes such as cellulolytic, proteolytic, and amylolytic can also play a role in inhibiting the growth of pathogens in plants by degrading and lysing bacterial and fungal cell walls so that they can potentially act as biocontrol agents for plant pathogens (Shaikh et al., 2016).

6 of the 11 isolates were detected as having the ability to dissolve phosphate *in vitro* on Pikovaskaya Agar media, characterized by the presence of a clear zone around the colony. Meanwhile, 4 of the 11 bacterial isolates were also detected as having the ability to dissolve potassium as indicated by the presence of a clear zone around the colony on Alexandrov media. The presence of this isolate in the soil can help plants obtain P and K easily so that it can increase plant growth and be resistant to biotic and abiotic stress (Bakhshandeh et al., 2017).

Antifungal Activity of Bacteria from Maggot Waste (*Hermetia illucens)*

Antagonistic tests were carried out on eleven isolates that were selected using phytopathogenic fungi *Colletotrichum* sp. This fungi were selected due to their ability to attack a wide range of crops in agricultural fields. This test was carried out with dual-culture techniques and dual-culture techniques with four quadrants, employing potato dextrose agar (PDA) as the medium. Based on the results obtained, none of the isolates were able to inhibit *Phytophthora* sp. and *Fusarium* sp. fungi, but four types of isolates were able to inhibit the development of *Colletotrichum* sp., namely MKP 01-04 as shown in Table 2.

Table 2. Inhibitory ability of isolates on *Colletotrichum* sp.

Bacteria	Diamater Control (mm)	Diameter Treatment (mm)	Performance Index (%)
MKP 01	40	25	60
MKP 02	40	20	100
MKP 03	40	21	90.4
MKP 04	40	27	48.1

After conducting the research, based on the characterization results and antagonistic assays against three fungi, two isolates, MKP02 and MKP04, were found to be potential. MKP02 demonstrated potential as an antifungal agent against *Colletotrichum* sp. with a performance index of 100%, while MKP04 showed 48.1%. This index indicates that the higher the percentage index, the better the bacteria's ability to inhibit *Colletotricum* sp. fungi (Sharma et al., 2021). This suggests that these isolates can function as antifungal agents against *Colletotrichum* sp. and can directly contribute to agriculture by protecting and saving plants from this pathogenic fungus (Syahbana et al., 2022). Additionally, MKP02 and MKP04 exhibited superior cellulolytic activity and the ability to solubilize potassium, thus they can serve as decomposer agents and organic fertilizers (Phitsuwan et al., 2013). This is corroborated by Zahroh et al. (2023) and Menino et al. (2021) who found that black soldier fly maggots produce ‘kasgot’, a residue useful for organic fertilizers containing various potassium

(K), nitrogen (N), and phosphate (P) elements.

Bacterial molecular identification using 16S rRNA gene analysis

Molecular identification was carried out on isolates that had the best antagonistic activity and characterization, namely MKP02 and MKP04. Analysis was conducted using 16S rRNA, which involves identifying bacteria by sequences to determine the sequence of their bases with more accurate results (Kusharyati et al., 2020). Isolates of the 16S rRNA gene were compared with genes in the GenBank using the BLAST-N (Basic Local Alignment Search Tool-Nucleotide) program to know the bacteria that have similarities with the isolates under study (Simarmata et al., 2020). The comparison results show that both isolates are similar to the species *Lysinibacillus xylanilyticus* and *Brevibacterium sediminis* (Table 3). Consistently, these results are also confirmed by their position in the phylogenetic tree (Figure 1).

Table 3. Percentage similarity of 16S rRNA gene sequences of isolates MKP02 and MKP04

Isolates	Species Affiliation (GenBank)	Query Cover (%)	E-Value	Similarity (%)	Accession Number
MKP02	*Lysinibacillus xylanilyticus* strain T2G-1	99	0.0	89.69	MT605496.1
MKP04	*Brevibacterium sediminis* strain J8A3RI	97	0.0	100	MT409544.1

Based on the research of Yu et al. (2011) BSF maggot has a diverse bacterial symbiosis, one of which is *Bacillus* sp. which can act as a plant pathogen control agent and be beneficial as rhizobacteria for plants. Before the discovery of new species of the genus *Lysinibacillus*, all *Lysinibacillus* bacteria belonged to the *Bacillus* genus based on their peptidoglycan composition, physiology, and molecular phylogenic position, so the two types of bacteria still maintain a relatively close relationship (Ahsan & Shimizu, 2021). This affects its usefulness, it is proven that after characterization with various tests, the bacterium *Lysinibacillus xylanilyticus* has the same role as a decomposer as *Bacillus* sp. and this ability has been tested by Diener et al. (2011) that BSF can reduce organic waste by 65.5% - 78.9% per day through digestion. Genus *Lysinibacillus is* also found in the centipedes gastrointestinal tract and has been proven to have antibacterial activity against *S. pyogenes* and *P. aeruginosa* (Akbar et al., 2020).

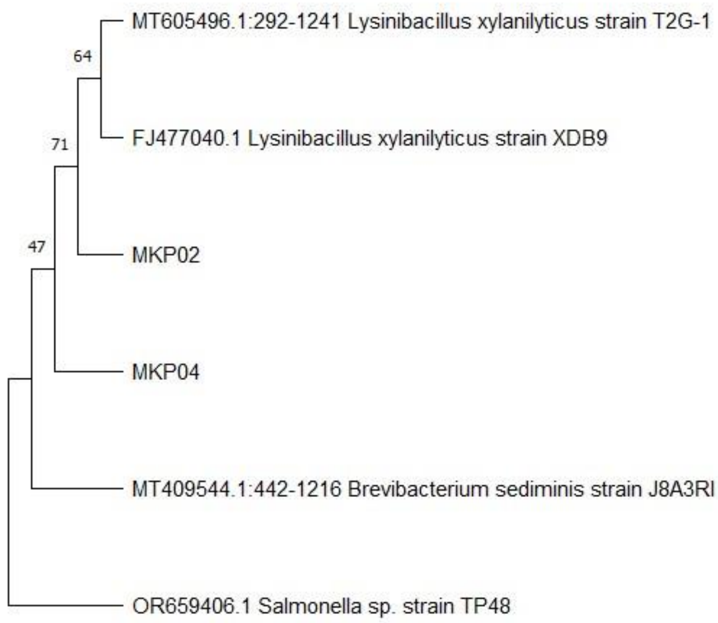

Figure 1. Neighbor-joining tree based on 16S rRNA gene sequence, showing the phylogenic relationship of isolates MKP02 and MKP04 with the genus *Lysinibacillus* and *Brevibacterium*.

Brevibacterium sediminis is a bacterium from the genus *Brevibacterium* which has a cylindrical shape without spores and is gram-positive (Chen et al., 2016). The bacteria have potential as antifungal and antimicrobial, according to research conducted by Faisal and Hasnain (2006) *Brevibacterium* is classified as a rhizospheric bacterium found in plant roots and has a mutually beneficial relationship. The rhizosphere is a biological agent that is rich in exudates containing carbohydrates, amino acids, organic acids, enzymes, and other compounds that can function as antifungal because it can secrete the enzyme chitinase, which can decompose the cell wall of pathogens (Singh et al., 2023). Additionally, research by Soyer & Tunali (2020) indicates that *Brevibacterium* sp. has antimicrobial ability against *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, *Bacillus subtilis*, and *Colletotrichum* sp. This aligns with the findings of the study, where MKP04, which has a close relationship with *Brevibacterium sediminis*, can inhibit the growth of *Colletotrichum* sp. fungi by forming an inhibition zone of 48.1% and has high cellulose activity, thus playing a role in the decomposition of organic matter. According to Klemm et al. (2006), agricultural waste has a cellulose content of up to 40% and this content is difficult to degrade naturally and requires 4-5 months. Therefore, as suggested by Adimpong et al. (2012) cellulolytic bacteria are needed to hydrolyze cellulose into simpler products such as glucose. The results of this study indicate that the two types of bacteria *Lysinibacillus* sp. and *Brevibacterium* sp. isolated from maggot feces, have the potential as an antifungal against *Colletotrichum* sp and are supported by their ability to produce hydrolytic enzymes such as chitinase, cellulase, protease and lipase which can degrade the cell walls of fungi and pathogenic bacteria in plants. Apart from that, this isolate also can dissolve K and P so that it can help growth and increase resistance to environmental stress so that these microbes can be developed as sustainable agricultural biocontrol.

CONCLUSION

From this research, 11 isolates were able to grow well after initial screening in chitin media and purification on ISP 2 media. These eleven isolates underwent characterization and antagonistic testing, resulting in 4 isolates that had antifungal activity against *Colletotrichum* sp. For further analysis, isolates with the best capabilities were reselected, namely MKP02 and MKP04. MKP02 has antifungal activity against *Colletotrichum* sp. at 100% and contains 83.3% protease enzyme, 100% cellulose enzyme, and the ability to solubilize potassium at 33.3%. Meanwhile, MKP04 could inhibit *Colletotrichum* sp. fungus at 48.1%, forming a clear zone in the cellulolytic test at 300%, the potassium solubilization test at 40%, and the lipolytic test at 75%. Based on the 16S rRNA gene analysis, it showed that the two isolates show that MKP02 is close to *Lysinibacillus xylanilyticus* and MKP04 is close to *Brevibacterium sediminis*, with a similarity index shows 89.69% and 100% respectively. These findings suggest that MKP02 and MKP04 isolated from chicken manure maggot waste have the potential as an antifungal agent and biodecomposer agent.

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