RESEARCH ARTICLE

Aspergillus oryzae attenuates quorum sensing -associated virulence factors and biofilm formation in *Klebsiella pneumoniae* extended-spectrum beta-lactamases

[version 1; peer review: 1 approved with reservations]

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v1 **First published:** 07 Oct 2022, **11**:1148
https://doi.org/10.12688/f1000research.123391.1
Latest published: 07 Oct 2022, **11**:1148
https://doi.org/10.12688/f1000research.123391.1

Abstract

Background: *Klebsiella pneumoniae* communicate between and among species using quorum sensing (QS). Biofilm formation and virulence factors are regulated by QS. This QS is indirectly responsible for *K. pneumoniae* pathogenicity. Inhibiting QS is a novel and highly effective method for controlling *K. pneumoniae* extended-spectrum beta-lactamases (KP-ESBL) infections. This study aimed to investigate how *Aspergillus oryzae* extracellular protein (AOEP) affected QS and KP-ESBL virulence factors.
Methods: Methods used included minimal inhibitory concentration (MIC) through the microdilution method, biofilms with crystal violet staining, extracellular polysaccharides using the Congo Red assay, quantifying the expression of genes coding for capsular polysaccharide (*wzI* gene) and adhesion (*mrkA* gene) through quantitative reverse-transcription polymerase chain reaction (RT-qPCR), siderophore level measurement using Chrome Azurol sulphonate assay (CAS assay), biofilm morphology using a scanning electron microscope (SEM), and confirmation using the life span killing assay method on *Caenorhabditis elegans* (*C. elegans*).
Results: *In vitro* studies revealed that AOEP inhibited biofilms and exopolysaccharides (EPS) in KP-ESBL at the sub-MIC level. In addition, AOEP inhibited the expression of the *mrkA* gene, which is involved in the adhesion process. Furthermore, an *in vivo* study revealed that AOEP levels of 75 and 150 µg/mL respectively increased *C. elegans* survival rates by 72.67% and 80.76% against *K. pneumoniae* infection.
Conclusions: Our findings suggest that the extracellular protein of *A.*

Open Peer Review

Approval Status ?

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version 1 07 Oct 2022	? view

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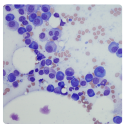
oryzae may be an effective QS inhibitor and a novel anti-virulence agent to control bacterial pathogens.

Keywords
A. oryzae, QS, Virulence factors, Biofilm, KP-ESBL.

This article is included in the Pathogens gateway.



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Author roles: Rachma LN: Conceptualization, Formal Analysis, Investigation, Methodology, Validation, Writing – Review & Editing; **Fitri LE**: Formal Analysis, Investigation, Methodology, Supervision, Writing – Review & Editing; **Prawiro SR**: Conceptualization, Methodology, Supervision, Writing – Review & Editing; **Mardining Raras TY**: Investigation, Methodology, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: We thank the Faculty of Medicine for financial support through the Lektor Kepala Grant.

How to cite this article: Rachma LN, Fitri LE, Prawiro SR and Mardining Raras TY. ***Aspergillus oryzae* attenuates quorum sensing - associated virulence factors and biofilm formation in *Klebsiella pneumoniae* extended-spectrum beta-lactamases [version 1; peer review: 1 approved with reservations]** F1000Research 2022, **11**:1148 https://doi.org/10.12688/f1000research.123391.1

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Introduction

The severity of infectious diseases caused by bacterial strains that are resistant to treatment has made their advent a global problem today, including *Klebsiella pneumoniae* extended-spectrum beta-lactamases (KP-ESBL).[1] KP-ESBL can hydrolyze beta-lactam antibiotics in addition to producing biofilms that hinder medications from penetrating cells.[2] Biofilms comprise an extracellular matrix (polysaccharides, proteins, and extracellular doxyribonucleic acid [DNA]) that acts as a strong barrier for bacteria and makes them more resilient to environmental stress than planktonic cells.[3] Additionally, biofilms aid KP-ESBL in spreading the infection and provide an environment that fosters the development of antibiotic resistance.[4–6] Finding novel compounds that can suppress KP-ESBL virulence factors and biofilms is therefore urged in order to assist in fighting these bacteria.

Quorum sensing (QS) molecules, adhesion molecules, iron, and exopolysaccharides (EPS) can affect the formation of biofilms.[7–9] Because QS is a regulator for the expression of capsule polysaccharides, the development of this biofilm is inversely related to the polysaccharide capsule's virulence factor.[10] It is now crucial to find antibiofilm and antivirulence chemicals, especially those derived from natural sources. Biofilms have been shown to be inhibited by bioactive substances obtained from nature.[11–14]

Aspergillus sp., a filamentous fungus, is well known for its potent antibacterial properties.[15–17] Inhibition of virulence factors and direct harm to the *K. pneumoniae* are known antibacterial mechanisms of *Aspergillus* sp. However, little is known about *Aspergillus* sp ability's to combat KP-ESBL biofilms. Our preliminary research proves *Aspergillus* crude protein which has the greatest biofilm inhibition is *Aspergillus oryzae* extracellular crude protein (AOEP). The aim of this study was to evaluate any potential antibiofilm properties of *Aspergillus oryzae* extracellular protein against KP-ESBL.

Methods

Preparation of *Klebsiella pneumoniae* extended-spectrum beta-lactamases, *Aspergillus oryzae* and *Caenorhabditis elegans*

KP-ESBL (ID.100029) were obtained from the Laboratory of Microbiology, Faculty of Medicine, Brawijaya University, Malang, Indonesia. Luria Broth (LB) medium was used to cultivate the KP-ESBL strain, which was then incubated at 37°C for 16–18 hours. Sterile saline was diluted 100 times after being equalized with the Mc. Farland standard to produce a concentration of 106 CFU/mL. This bacterial suspension was then ready for testing. The fungal strain *Aspergillus oryzae* was provided by the Indonesian Culture Collection (Ina-CC). Preparation of *Aspergillus oryzae* begins with sub-culture and preservation of previously isolated *Aspergillus oryzae*. Sub-cultures were carried out from cryo to Luria Bertani solid medium. The culture was then treated in the form of H_2O_2 and without glucose, then stored at 37°C in an incubator. The nematode *Caenorhabditis elegans* was maintained on agar medium for nematode growth media (NGM) fed with *Eschericia coli* OP50. Gravid *C. elegans* were treated with hypochlorite to synchronize *C. elegans* culture at the first larval stage. Before being employed for infection, the *C. elegans* were then reared at 25°C until they reached the young adult stage.

Preparation of *Aspergillus oryzae* extracellular protein (AOEP)

100 mL of potato dextrose broth (PDB) medium were inoculated with 8-mm (diameter) *Aspergillus oryzae* mycelium and placed in a 250 mL Erlenmeyer flask containing 2% glucose. The flask was incubated for 72 hours at 27°C in a shaker incubator under static conditions (OD600 = 1.2). The culture was filtered using 0.22-micron filter paper after incubation (Whatman, Sigma Aldrich). As a source of extracellular protein, the supernatant was centrifuged at 12.000 rpm for 15 minutes at 4°C. Ammonium sulfate was used to precipitate extracellular proteins at saturation values of 80%. After one hour of stirring in the ice bath, ammonium sulfate was added to the supernatant. The crude protein extract was centrifuged at 4°C for 15 minutes at 12,000 rpm the next day after being maintained at 4°C overnight. After that, the complete protein precipitate underwent a twenty four hour-dialysis in a 0.01 M phosphate buffer at pH 7 using a 10×-sample volume. After that, the *Aspergillus oryzae* extracellular protein (AOEP) was prepared for the assay.

Determination of MIC

A growth inhibition test was conducted using microdilution broth. Briefly, fresh cultures were inoculated on LB medium at turbidity equivalent to 0.5 McFarland standard, 500 µL of each bacterial culture were added to a 96-well polystyrene flat-bed microtiter plate. The samples were added to the bacterial suspension in each well at final concentrations ranging from 0 to 150 g/mL. The growth control wells only contained bacteria on LB media and kanamycin as positive control. Double serial dilution of the *Aspergillus oryzae* extracellular protein (AOEP) tested sample was made starting from the first well by adding 50 µL of the tested sample, dissolved at 150 µg/mL. After incubation at 37°C for 24 hours, the absorbance was measured at 600 nm. The lowest absorbance value of the sample that could reduce more than 90% of the absorbance of the negative control was recorded as the MIC value. All experiments were performed in triplicate. Minimum bacterial concentration (MBC) for each sample was calculated by coating the contents of the first three wells, which showed no visible bacterial growth on the LB plate, and incubated for 24 hours.

Biofilm assay

The test well on a 96-well microplate received a total of 100 µL of *Aspergillus oryzae* extracellular protein (AOEP) at various concentrations (18.75, 37.5, 75, and 150 µg/mL). The negative control wells received 200 µL of mixed LB media and 1% glucose, while the positive control wells received 64 µg/ml of kanamycin. Each well was then filled with 100 µL of the KP-ESBL suspension. For 24 hours, the microplate was wrapped and kept at 37°C in an incubator. The microplate's contents were taken out the following day, thoroughly cleaned with sterile distilled water three times, and then dried. 200 µL of 0.1 % crystal violet dye was added to each well once the microplate had dried, and it was air dried at room temperature for 15–20 minutes. The microplate's contents were then cleaned with sterile distilled water and dried. After 15 minutes of incubation at room temperature, 200 µL of a 96% ethanol solution were added to each well, and the results were measured at 570 nm with a microplate reader.

Biofilm growth inhibition was calculated using the following formula:

$$\%\text{biofilm adhesion prevention} = \frac{\text{OD control} - \text{OD test} \times 100\%}{\text{OD control}}$$

(OD Control = Optical density control negative. OD Test = Optical density test)

Analysis of biofilm structure using SEM

Visual evaluation of AOEP's impact on KP-ESBL morphology was conducted using a scanning electron microscope (SEM, model Zeiss 224 EVO 50 VP, Germany). KP-ESBL bacteria were cultivated in LB broth and incubated for 24 hours in an aerobic environment at 37°C. A 1-mL volume of the bacterial suspension was obtained and treated with AOEP for two hours once it reached around 1×10^8 CFU/ml. Another 1 mL sample was taken from the culture and left untreated. These two bacterial samples were centrifuged after two hours for three minutes at 1400 rpm, and the pellets were then cleaned twice with 0.1 M phosphate buffer saline (PBS). KP-ESBL cells were exposed to 2.5% glutaraldehyde for two hours at 4°C for the SEM analysis. Samples were exposed to each concentration after fixation for one to two minutes in order to dehydrate them. The samples were then centrifuged at 1400 rpm for 10 min, after which the pellets were re-dispersed in 100% ethanol and air dried. The samples were coated with gold and palladium in an 80:20 ratio prior to examination under SEM at 20 kV. The working magnification was kept at less than 10 mm for better focusing.

Production of cell-free EPS

Cultures of bacterial isolates left overnight were inoculated to 9.5 mL of LB broth along with 0.5 mL of cell lysate and incubated at 30°C for 24 hours. The late-log phase cells attached to the test tube walls were harvested by centrifugation at 8500 rpm for 30 min at 2°C. The filtered supernatant was added with three volumes of cold ethanol and incubated overnight at 2°C to precipitate the released EPS. The precipitated EPS were then collected by centrifugation at 5000×g for 30 min and dissolved in 1 mL of deionized water. Enzyme-free media culture added with PBS served as a control. The bacterial cells were removed, resuspended in sterile PBS, and read at 600 nm. The collected EPS was quantified using the phenol-sulfuric acid method.

C. elegans life span killing assay

Similar to the anti-infection screen, the liquid-based survival test was carried out with a few minor adjustments. A total of 30 young adult *C. elegans* were used in place of the N2 young adults that received treatment. As a result, the *C. elegans* were kept at 16°C to create gravid *C. elegans*, and they were given a hypochlorite treatment to develop eggs. In order to conduct an infection assay, eggs were sown on NGM agar and developed into sterile young adults of *C. elegans* at 25 °C. Every four hours following infection, both alive and dead *C. elegans* were counted. Each extract was examined in three wells, each representing about 100 *C. elegans.* In control wells, dimethyl sulfoxide (DMSO) was used in place of the extract, and *Escherichia coli* OP50 were fed. To examine the impact of AOEP on KP-ESBL pathogenicity to *C. elegans*, we performed a slow-killing survival experiment. On a 48-well microplate, KP-ESBL were first cultured for an overnight period at 37°C in the presence of AOEP (18.75, 37.5, 75, and 150 µg/mL). When 100 sterile young adult *C. elegans* were put into the well, the infection began. KP-ESBL was given DMSO treatment as a negative control in place of AOEP. After 48 hours, the *C. elegans* that were still alive were counted under microscope with a magnification of 100×.

RT-qPCR analysis

The hot phenol method was used to extract total ribonucleic acid (RNA), where the DNA was removed using TURBO DNA-free (Ambion, Inc.), and the RNA quality was determined using a NanoDrop (ND-1000; Thermo Scientific) and an Agilent 2100 bioanalyzer with a Picochip (Agilent Technologies). After 35 qPCR cycles, the absence of contaminating DNA was determined by the absence of amplification products. A 1 µg of RNA, random hexamer primers (0.2 µg/L), and M-MulV-RT (20 U/L, Moloney murine leukemia virus reverse transcriptase; Thermo Fisher Scientific) were used to synthesize cDNA. Specific primers for *mrkA* 5′-CGGTAAAGTTACCGACGTATCTTGTACTG-3′, and

wzI 5′-GCTTAYGCRGCYGGGTTAGTRGT-3′ designed with the Primer3Plus software (Primer3Plus is an open alternative). A master mix of the following components was prepared for light cycler reactions: 3.0 mL PCR-quality water, 1.0 µL (10 M), 10 µL 2× SYBR Green I Master Mix, 10 µL reverse primer, and 5.0 µL cDNA (50–100 ng). A multi-well plate was sealed with sealing foil, centrifuged for two minutes at 1500 g, and loaded into the LightCycler 480 instrument (Roche). For each sample examined, amplification was carried out in triplicate wells. All reactions had control reactions with no template (water) and minus-reverse transcriptase (RNA). Cycling conditions were as follows: denaturation (95°C for 10 minutes); amplification and quantification repeated for 45 cycles (95°C for 10 seconds, 57°C for 20 seconds, 72°C for 30 seconds with a single fluorescence measurement); melting curve (95°C for 10 seconds, 65°C for one minute with continuous fluorescence measurement at 97°C); and finally, a cooling step at 40°C for 10 seconds. After each run, a melting curve analysis was performed to confirm the specificity of the primers. For normalization, 16S rRNA was used as a reference gene, and relative gene expression was calculated using the 2Ct method.

Results

AOEP inhibited the growth of KP-ESBL

The antimicrobial activity of AOEP was quantitatively assessed by measuring the turbidity at a wavelength of 600 nm. The results in Figure 1 represent crude proteins' MIC and MBC values in various concentrations with kanamycin as positive control. The concentration of AOEP, which could inhibit > 90% of the bacterial population, represented MIC and was 300 µg/mL. The concentration used in the growth inhibition test of antibiofilm activity was sub-MIC, namely at 1/8 and 1/16 × MIC. This study used a 64-µg/mL dose of kanamycin as a positive control.

The highest concentration used for the AOEP inhibition test against KP-ESBL biofilms was 150 µg/mL, which was the MIC (p-value < 0.05). The Tukey Post Hoc test showed that there were significant differences between the overall treatment group and the negative control. The linear regression test results showed a R-value of 0.797, reflecting that AOEP could inhibit the growth of KP-ESBL in a dose-dependent manner. Furthermore, measurement of the inhibitory ability of biofilms and virulence factors used sub-MIC concentrations of 1/8 and 1/16 MIC, there were 18.75, 37.5, 75, and 150 µg/mL.

AOEP inhibited the formation of KP-ESBL biofilms at MIC

The microdilution method was used to test the inhibitory activity of the AOEP biofilm against KP-ESBL. Figure 2 displays the AOEP biofilm's inhibitory efficacy against KP-ESBL.

AOEP was administered relative to the MIC. The MIC value of the *K. pneumoniae* strain was 300 µg/mL, so the highest concentrations for the anti-biofilm test were 1/8, 1/16 MIC. The biofilm inhibition value [100-(sample ABS/control ABS × 100)] for each AOEP concentration can be seen in Figure 2. In the crystal violet staining assay, KP-ESBL biofilms were significantly inhibited at concentrations of 1/4 × MIC (75 µg/mL) and 1/2 × MIC (150 µg/mL) (p < 0.05). Interestingly, AOEP inhibited biofilm formation at concentrations below the MIC. The ability of AOEP to inhibit biofilm formation in KP-ESBL exceeded the ability of the kanamycin (69.46 µg/mL and 37.9 µg/mL). The positive control had a biofilm inhibitory value of 37%, which was lower than the AOEP biofilm inhibitory level of 150 and 75 µg/mL. The negative control (KP-ESBL bacteria without AOEP exposure) showed the lowest biofilm inhibition value (0), which

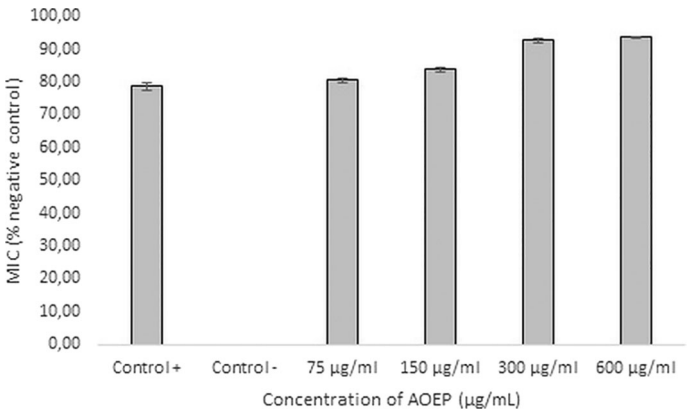

Figure 1. Minimal inhibitory concentration (MIC) of *Aspergillus oryzae* extracellular protein (AOEP) against *Klebsiella pneumonia* extended-spectrum beta-lactamases (KP-ESBL). MIC at a concentration of 300 µg/mL was 92.74% (bold). Kanamycin as a positive control, was only able to inhibit KP-ESBL 78.51%. The negative control of KP-ESBL without AOEP exposure was 0%. Bars indicate the standard error, and the sign (*) above the bars indicates a significant difference (p < 0.05).

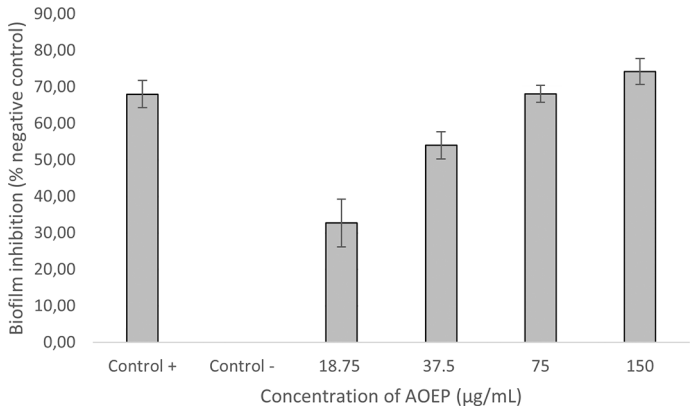

Figure 2. Inhibition of *Aspergillus oryzae* extracellular protein (AOEP) against *Klebsiella pneumonia* extended-spectrum beta-lactamases (KP-ESBL) biofilms. Inhibitory effect of AOEP on biofilms after co-incubation for 24 h with different concentrations of AOEP. The concentration of AOEP is given relative to MIC KP-ESBL. The AOEP biofilm inhibition concentrations of 18.75, 37.5, 75, and 150 µg/mL were 32.94%, 39.17%, 68.14%, and 72.18%, respectively. Bars indicate the standard error, and the sign (*) above the bars indicates a significant difference ($p < 0.05$).

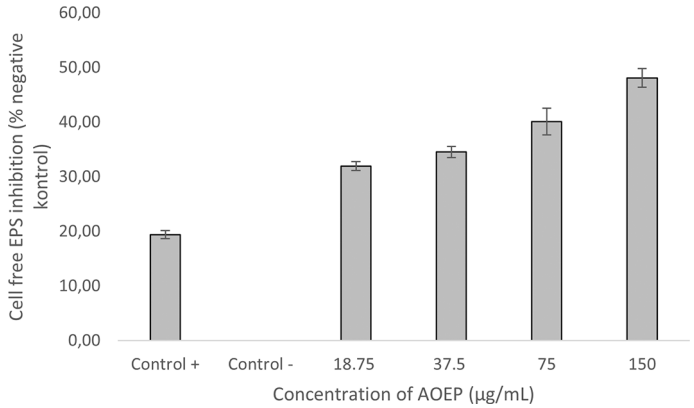

Figure 3. Reduction of *Aspergillus oryzae* extracellular protein (AOEP) against cell-free *Klebsiella pneumonia* extended-spectrum beta-lactamases (KP-ESBL) exopolysaccharide. Inhibitory effect of AOEP on cell-free exopolysaccharide KP-ESBL after being incubated together for 24 hours with different concentrations: 18.75, 37.5, 75, and 150 g/mL. Bars indicate the standard error and the sign (*) above the bars indicates a significant difference ($p < 0.05$). KP-ESBL

indicated that biofilm production was not inhibited at all. When administering the four concentrations of AOEP, the resulting OD value decreased significantly as the dose increased when compared with the OD of the negative control. This indicates AOEP inhibition of the KP-ESBL biofilm. Tukey test results indicated that there were significant differences between the overall treatment group against the negative control. The linear regression test results show that the R-value (0.957) that represented AOEP could inhibit the growth of KP-ESBL biofilm in a dose-dependent manner.

AOEP reduced cell-free exopolysaccharide of KP-ESBL

The assay was performed to test the ability of AOEP to reduce cell-free exopolysaccharide KP-ESBL.

KP-ESBL treated with AOEP 150 µg/mL could reduce the bond matrix with Congo Red dye by as much as 49% after staining and assessment with a spectrophotometer. As the dose of AOEP was reduced (75 µg/mL, 37.5 µg/mL, 18.75 µg/mL), its inhibition ability decreased (42%, 38%, 37%). Cell-free EPS might be reduced by 19% using AOEP 150 µg/mL and it surpassed the kanamycin (30 %). There was a significant difference, according to the one way ANOVA test (p-value < 0.05). The results of the Tukey Post Hoc test revealed that the overall treatment group and the unfavorable control group differed significantly. The findings of the linear regression test indicated that AOEP might inhibit the cell-free EPS KP-ESBL in a dose-dependent manner, and the R-value for this test was 0.896.

AOEP disrupted fimbriae through downregulation of the merized fimbrial shaft (mrkA) genes

The delta-delta Ct method (2 DDCt) was used to quantify RT-qPCR results. Results are represented as "Target/adh3 fold change." The results of gene expression analysis via RT-qPCR (Figure 4) showthat AOEP downregulated the gene expression for fimbriae *mrkA*, which acts as an adhesion molecule. Meanwhile, capsular EPS as measured by the *wzI* gene expression was increased.

AOEP disturbed the biofilm structure of KP-ESBL on observation under SEM

The impact of AOEP on cellular alterations was examined using SEM analysis. To supplement the information of the quantity of the biofilm, observation of the architecture of the biofilm mass using SEM was conducted. The impact of AOEP on the KP-ESBL biofilm structure was demonstrated by SEM data (Figure 5).

The negative control group showed bacterial colonies along with thick biofilms evenly distributed on the adhesion surface (Figure 5A). This was different from when the bacteria were treated with the 150 µg/mL AOEP (Figure 5B), the cells failed to aggregate, and there was a highly significant decrease in biofilm mass. In this group, the bacterial colonies were separated and became planktonic bacteria, and the adhesion surface was free of bacterial biofilms. Biofilm mass was also decreased in the AOEP 75 µg/mL (C) group, while the positive control group (Kanamycin, F) showed partial inhibition of the KP-ESBL biofilm. It can be seen that the biofilm structure of KP-ESBL was impaired due to the addition of AOEP when compared to the control. The control group was KP-ESBL which was not exposed to AOEP, as shown in Figure 5A. When bacteria stick together, the attachment of bacteria is more clearly facilitated by a thick mass of biofilm surrounding the bacterial colony. In this group, the biofilm appeared to cover all bacterial colonies on the surface of the adhesion medium. This appearance differed significantly from the group treated with 150 µg/mL of AOEP (Figure 5B) provides a clearer picture of the dispersal in the bacterial colonies treated with AOEP reflecting the impair the biofilm. The inhibition of biofilm mass formation in the group exposed to AOEP at a dose of 75 µg/mL (Figure 5C) also shows that the bacterial colony dispersed. However, the number of bacteria was higher than for a concentration of 150 µg/mL. At an AOEP dose of 37.5 µg/mL (Figure 5D), it was seen that some bacteria were separated, and some bacteria were attached (left). At 10000× magnification, a biofilm mass began to surround the bacteria and facilitated adhesion between bacteria and the adhesion medium.

Meanwhile, at the lowest concentration of AOEP, a dose of 18.75 µg/mL (Figure 5E), the presence of a thick biofilm was seen that matched the negative control. Interestingly, the sub-MIC ability of AOEP to reduce biofilm mass formation produced stronger effect than the kanamycin (Figure 5F). Overall, SEM results showed the highest reduction in biofilm mass formation occurred with a treatment of AOEP 150 µg/mL, which had a stronger effect than the kanamycin. These results indicate that AOEP can be used as a candidate antibiofilm agent at concentrations lower than MIC, especially against biofilm formation by KP-ESBL.

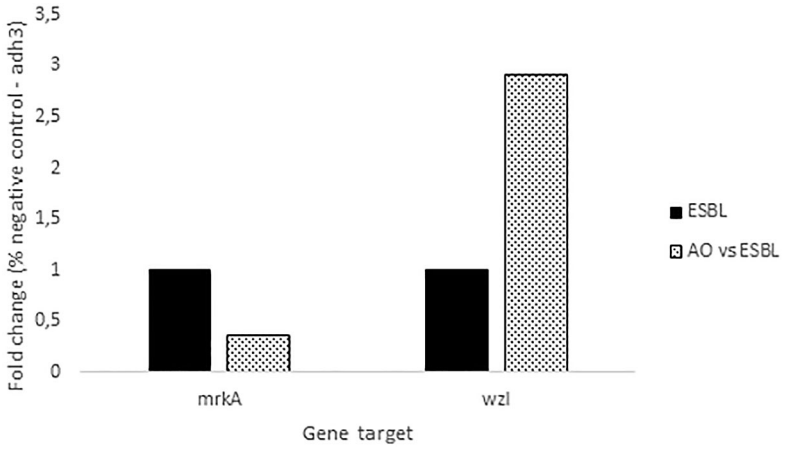

Figure 4. Effect of *Aspergillus oryzae* extracellular protein (AOEP) on the gene expression coding for the fimbriae and capsule of *Klebsiella pneumonia* extended-spectrum beta-lactamases (KP-ESBL). The expression of these two genes was measured in response to AOEP: *mrkA* (type 3 fimbrial shaft) and *wzI* (surface assembly of capsule). The expression of the *adh3* house-keeping gene was used as an internal control for each sample. The concentration of AOEP treatment was 150 µg/mL, while the control group was KP-ESBL without AOEP exposure.

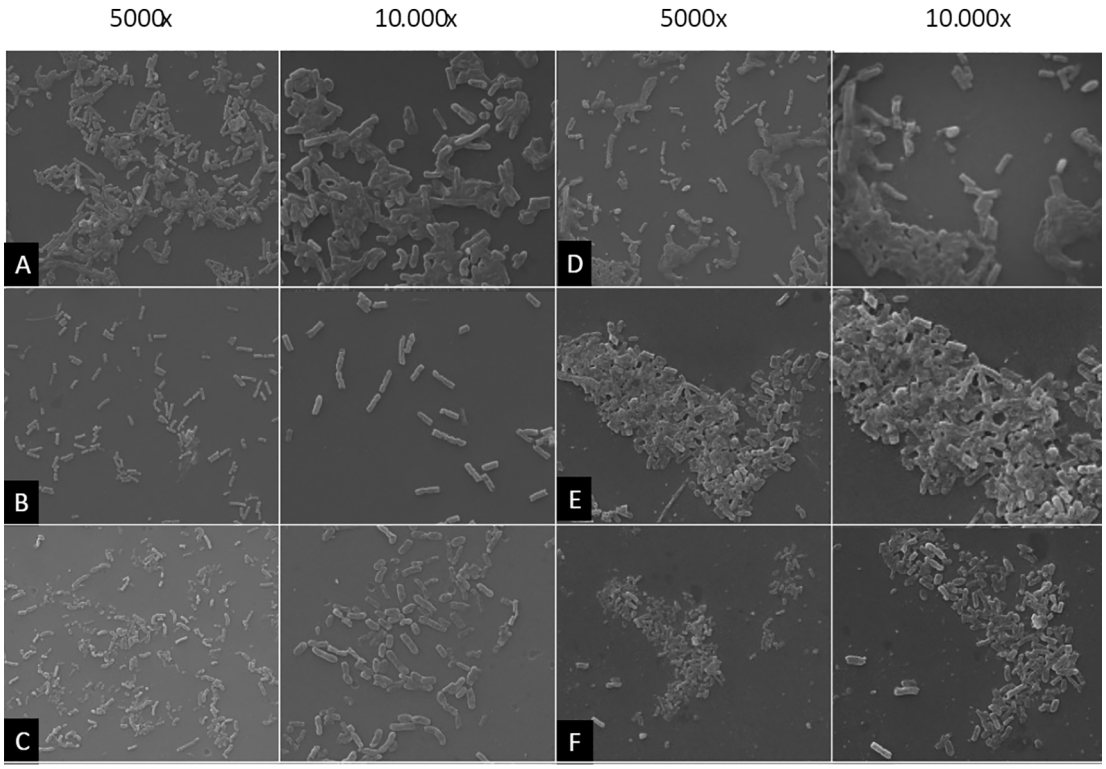

Figure 5. Scanning electron microscope (SEM) micrograph image of *Klebsiella pneumonia* extended-spectrum beta-lactamases (KP-ESBL) cells exposed to *Aspergillus oryzae* extracellular protein (AOEP) sub-minimum inhibitory concentration (MIC, 5000× magnification left, 10,000× magnification right). *K. pneumoniae* biofilms grew after incubation for 24 hours. AOEP was added with different concentrations: 150 µg/mL (B), 75 µg/mL (C), 37.5 µg/mL (D), 18, 75 µg/Ml (E) Positive control with the addition of the Kanamycin (F).

AOEP increased survival rates of *C. elegans* when challenged against KP-ESBL

To observe the effect of AOEP on the infection caused by KP-ESBL, an *in vivo* study was conducted on *C. elegans.*

Figure 6 shows the percentage of *C. elegans* survival after 48 hours of exposure to KP-ESBL. Only about 4% of *C. elegans* infected with KP-ESBL survived up to 48 hours, while *C. elegans* exposed to *E. coli* OP50 88% survived until the end of the test. Surprisingly, the *C. elegans* that were exposed to AOEP and KP-ESBL (18.75, 37.5, 75, and

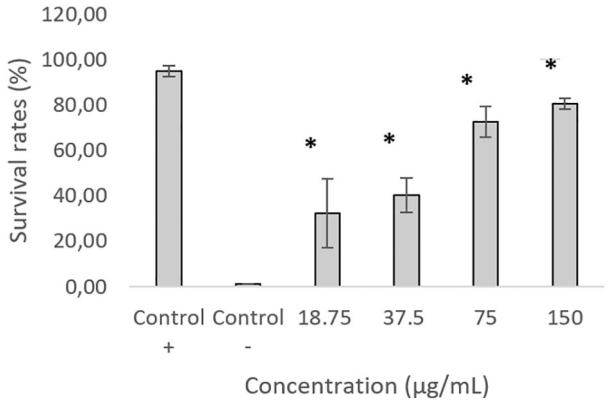

Figure 6. Survival rates of *C. elegans* after being inoculated with *Klebsiella pneumonia* extended-spectrum beta-lactamases (KP-ESBL) both with and without *Aspergillus oryzae* extracellular protein (AOEP) by slow killing assay method. The graph shows the percentage survival rates when the test was carried out without exposure to AOEP with four different concentrations. KP-ESBL without AOEP was a negative control, while KP-ESBL with *Escherichia coli* OP50 (non-pathogenic) was a positive control. Results are expressed as mean ± SD.

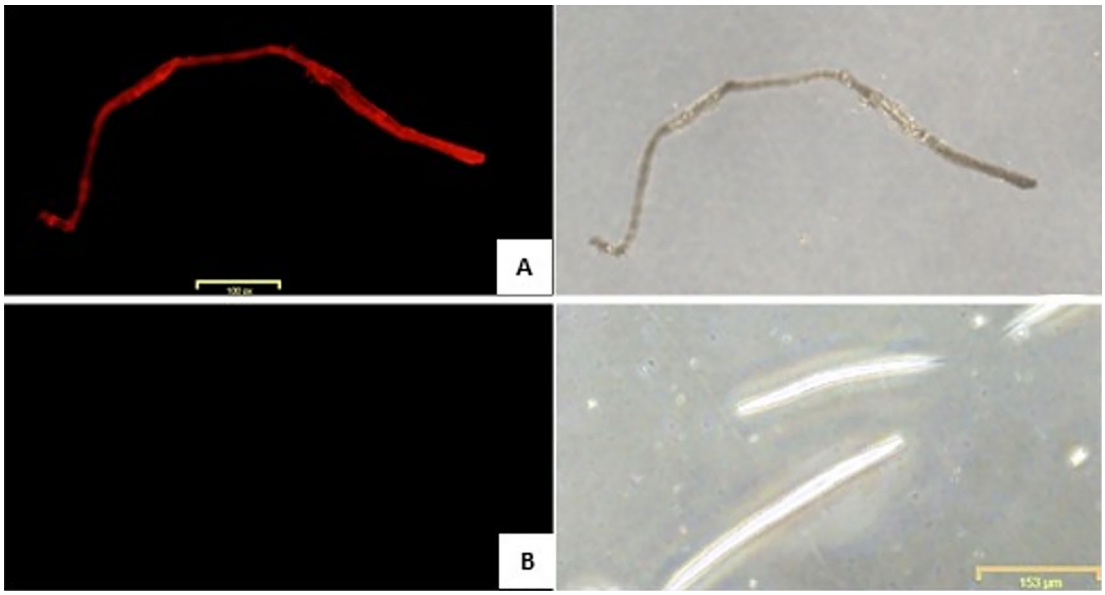

Figure 7. Fluorescence micrograph analysis using propidium iodide on *C. elegans* survival assay (the right image is colored with PI, the left image is without PI, 100× magnification) after being challenged with *Klebsiella pneumonia* extended-spectrum beta-lactamases (KP-ESBL) both with and without *Aspergillus oryzae* extracellular protein (AOEP) by slow killing assay method. (A) *C. elegans* infected with KP-ESBL without AOEP administration. (B) *C. elegans* infected with KP-ESBL with AOEP administration. Propidium iodide fluorescence micrograph of *C. elegans* (100× magnification). *C. elegans* infected with KP-ESBL without AOEP died and showed positive red fluorescence when cultured (increased fluorescence intensity of propidium iodide indicated the death of the nematode parasiteof *C. elegans*).

150 µg/mL) had significantly increased survival rates (17 – 68%) compared to the group of *C. elegans* that were only infected with KP-ESBL. The highest survival was in the 150 µg/mL group (68.25 ± SD 4.6). the one-way ANOVA test showed that there was a significant difference between negative control and treated groups (p-value < 0.05). The linear regression test results showed the R-value was 0.958. The analysis showed that AOEP could reduce the ability of KP-ESBL to infect *C. elegans* in a dose-dependent manner. Figure 7A shows the propidium iodide fluorescence micrograph of *C. elegans* (10× magnification) and infected with KP-ESBL. The *C. elegans* showed negative PI fluorescence when cultured under standard conditions with OP50 as a food source. There was an increase in the fluorescence intensity of propidium iodide when the *C. elegans* were infected with KP-ESBL and treated with AOEP (Figure 7B). The results of the *in vivo* survival assay showed that AOEP was able to reduce the virulence of KP-, which could be observed from the increased survival of *C. elegans* that were infected with KP-ESBL.

AOEP used a competitive antagonistic mechanism by producing QS-like Gram Negative bacteria molecule to interfere with KP-ESBL virulence

A liquid chromatography-mass spectrometry LC-MS/MS study against AOEP was carried out in order to identify the *A. oryzae* molecule that contributes to KP-ESBL virulence and biofilm suppression. The results were displayed as a chromatogram, which showed the peak height and molecular weight of the sample substance. Figure 8 and Table 3 show the outcomes of the LC-MS/MS study.

The chromatograms showed a number of substances with various peaks and molecular weights. Six compounds had prominent and high peaks (Figure 8). Based on the precursor ion (m/z), ion product (m/z), cone voltage, and impact energy, the six peaks were identified. The six peaks contained substances with properties resembling those of the QS substance *K. pneumoniae.* The six substances were NHQ, NQNO, 3-OH-C12-HSL, 3-oxo-C6-HSL, and C10-HSL. The six compounds were found to match the typical precursor parameters 3-oxo-C6-HSL, 3-OH-C6-HSL, C10-HSL, NHQ, NQNO, and 3-OH-C12-HSL (see Table 1).

Discussion

The antibacterial ability of antibiofilm derived from natural sources can come from the production of enzymes, the formation of secondary metabolites or compounds that inhibit QS signals.[18] QS inhibition can be mediated by receptor antagonists or quorum quenching enzymes.[19] In this study, we searched for QS inhibitor compounds derived from the fungus *A. oryzae*. *A. oryzae* was harvested at a stationary phase in order to obtain the dominant secondary metabolite.[20]

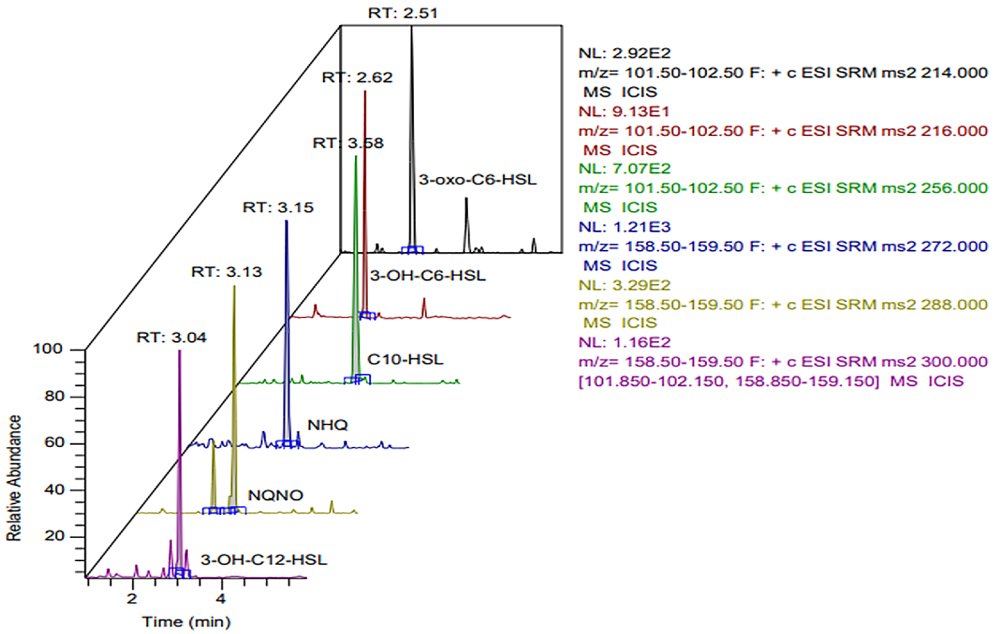

Figure 8. Ion-extracted liquid chromatography-mass spectrometry (LC-MS/MS) chromatogram of a mixture of standard AHL and AQ molecules showing separation. In order (from top to bottom), 3-oxo-C6-HSL, 3-OH-C6-HSL, C10-HSL, NHQ, NQNO, 3-OH-C12-HSL.

Table 1. *Aspergillus oryzae* extracellular protein (AOEP) against cell-free exopolysaccharide (EPS) of *Klebsiella pneumoniae* extended-spectrum beta-lactamases (KP-ESBL).

	Control (+)	Control (-)	18.75	37.5	75	150
%Inhibition*	18.68	0.00	31.00	34.68	37.90	48.67
	20.14	0.00	32.50	35.45	39.66	49.42
	19.32	0.00	32.29	33.45	42.75	46.15
Average	19.38	0.00	31.93	34.53	40.11	48.08
Standard error	0.74	0.00	0.81	1.01	2.46	1.71

*The inhibitory effect of AOEP on cell-free EPS of KP-ESBL after incubation for 24 hours with different concentrations of AOEP exhibited inhibitory effect on cell-free EPS of KP-ESBL after incubation for 24 hours. The reduction of cell-free EPS by AOEP at concentrations of 18.75, 37.5, 75, and 150 µg/mL was 31.93%, 34.53%, 40.11%, and 48.08%, respectively. The kanamycin was only able to reduce 19.38% of cell-free EPS, while the negative control of KP-ESBL without AOEP exposure was 0%. The inhibition value of cell-free EPS by AOEP was better than that by kanamycin (48.08% versus 19.38%).

Table 2. Survival rates of *C. elegans* after being challenged against *Klebsiella pneumoniae* extended-spectrum beta-lactamases (KP-ESBL) with *Aspergillus oryzae* extracellular protein (AOEP) by slow killing assay method.

	Control (+)	Control (-)	18.75	37.5	75	150
Survival rate	95	2	31	47	66	83
	97	1	28	34	77	79
	93	1	38	40	75	80
Average	95.00*	1.33	32.33*	40.33*	72.67*	80.67*
Standard error	2.00	0.58	5.13	6.51	5.86	2.08

Table shown the percentage survival rates of C elegans infected with KP-ESBL treated with AOEP. KP-ESBL without AOEP served a negative control, while KP-ESBL with *Escherichia coli* OP50 (non-pathogenic) was a positive control. Results are expressed as mean ± SD.
*$p < 0.001$ showed a significant difference in the percentage of survival rates among *C. elegans* not exposed to AOEP and those exposed to AOEP 150 µg/mL.

Table 3. QSSM analytes from *Aspergillus oryzae* secondary metabolites. QSSM: quorum sensing-like molecule.

QSSM analyte	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Cone voltage	Collision energy (V)
3-oxo-C6-HSL	3.1	214.1	102.1	26	15
3-OH-C6-HSL	3.1	216.1	102.1	26	15
C10-HSL	4.6	256.1	102.1	31	17
NHQ	4.6	272.1	159.1	81	27
NQNO	4.7	288.1	159.1	96	20
3-OH-C12-HSL	4.5	300.1	102.1	35	19

A. oryzae produces secondary metabolites, such as asperfuranon, aspyridone, penicillin, isocoumarin, aspercryptin, and indole diterpene.[21] However, from the LC-MS/MS analysis, we did not find any secondary metabolites or quorum quenching enzyme compounds from *A. oryzae.* This result is different from the LC-MS analysis from extract of *A. meleus* that produce AHL acylase, which can inhibit *P. aeruginosa* biofilms.[22] In another study, *A. niger* produced cellobiose dehydrogenase which reduced the biofilm of Gram-negative bacteria.[23]

We discovered three new substances that are similar to the QS molecules of Gram-negative bacteria, which is interesting because we did not uncover secondary metabolites or quorum quenching enzymes. We propose those molecules, *i.e.*, C10-HSL, 3-oxo-C6-HSL, 3-OH-C6-HSL and suggest them as QS molecules because, despite the fact that the three chemicals resemble the QS molecules found in Gram-negative bacteria, their activity is inversely related. We believe that *A. oryzae*'s QS molecules function as a competitive adversary. When it comes to attaching to AHL binding sites in *LuxR*, QS molecules compete with native AHL. One of the genes regulated by QS, the biofilm-encoding gene, is downregulated as a result of QS molecules binding to *LuxR.* According to this investigation, AOEP significantly reduced the KP-ESBL bacterial biofilm (74.24%). Aside from preventing the growth of biofilms, AOEP has also been demonstrated to lower EPS levels. EPS make up a robust biofilm matrix.[3,22,24] The decrease in EPS synthesis was consistent with the structure of the KP-ESBL biofilm as determined by SEM. In the presence of AOEP, the bacterium cells were unable to aggregate. The matrix that holds bacteria together was also obviously thinner. Therefore, the absence of QS barriers may be the cause of the decline in biofilms. QS inhibitors (QSI) function by obstructing the binding sites for autoinducers. It also interferes with the formation of pili types 1 and 3 and cyclic diguanylate mono phosphate (c-di-GMP).[25] As a result, QSI's inhibition will cause the expression of pili types 1 and 3 to be suppressed. Our findings are consistent with this notion. After exposure to AOEP, the expression of the pili type 3 gene (*mrkA*) was significantly reduced. This suggests that AOEP include QSI, which lowers *mrkA* expression. Sadly, there is no research to support our findings.

The expression of the *wzI* gene was assessed in order to support the mechanism through which AOEP inhibits KP-ESBL QS. Because QS controls the formation of capsular polysaccharides (CPS)[10,26,27], inhibiting QS will impair its regulator role and cause the CPS to continue to be produced.[26] The administration of AOEP in this investigation had no effect on the excretion of CPS by KP-ESBL. Through QS inhibition, it was discovered in this work that AOEP has antibacterial and antibiofilm activities against KP-ESBL. Because it synergistically reduces the expression of numerous virulence factors controlled by QS, QS inhibition in KP-ESBL is particularly helpful in the management of pneumonia.[19]

We chose *C. elegans* as a model because it is ideal for assessing QS inhibitors in order to further examine the therapeutic potency of AOEP on the infection caused by KP-ESBL. AOEP showed antibacterial and anti-QS action against KP-ESBL in *in vitro* experiments. The protective effect of AOEP against *K. pneumoniae* infection on *C. elegans* lends weight to these findings. The survival of infected *C. elegans* was generally increased by AOEP. We verified the anti-QS activity of AOEP in our investigation. Results from the *C. elegans* pneumoniae infection model demonstrate how AOEP can successfully reduce virulence by obstructing KP-QS ESBL's activity in *in vitro* investigations. It is possible to create new medicines for infectious diseases using *K. pneumoniae* QS inhibitors.

Conclusions

This study showed that the extracellular protein of *A. oryzae* posseses antimicrobial and antibiofilm activity against KP-ESBL. QSI is an AOEP compound that inhibits QS and degrade biofilms, EPS, and mrkA (type 3 pili). AOEP could protect *C. elegans* from KP-ESBL infection. AOEP is a potential source of natural antibiofilm agents against KP-ESBL.

Data availability

Underlying data

Figshare: Aspergillus oryzae attenuates quorum sensing -associated virulence factors and biofilm formation in Klebsiella pneumoniae extended-spectrum beta-lactamases raw data, https://doi.org/10.6084/m9.figshare.20290929.[28]

This project contains the following underlying data:

- Biofilm inhibition.xlsx
- c. elegans survival rates.xlsx
- Exopolysaccharide.xlsx
- Minimum Inhibitory concentration.xlsx
- qRT-PCR.xlsx

Acknowledgements

We thank the Central Laboratory of Biomedik, Brawijaya University particularly to Suci Megasari for technical assistance.

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Current Peer Review Status: ?

Version 1

Reviewer Report 21 August 2024

https://doi.org/10.5256/f1000research.135493.r307666

?

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1. The introduction is too brief and should provide more details on the clinical significance and global impact of KP-ESBL, quorum sensing signaling, and its role in biofilm formation. Additionally, some background on fungal proteins and their role in biofilm inhibition is needed.
2. Provide a stronger rationale for why *Aspergillus oryzae* was chosen for this study. Mention any relevant previous studies on Aspergillus sp. that support its potential as an antibiofilm agent.
3. The shift from discussing general biofilm inhibition to focusing on *Aspergillus oryzae* is abrupt. Introduce the relevance of *Aspergillus sp.* more smoothly.
5. The authors have used ammonium sulfate precipitation to isolate extracellular proteins, but the number of proteins present in this preparation is unclear. Details are missing.
6. The authors must provide SDS-PAGE analysis of the AOEP and mention the molecular weight of the proteins. The total yield of the protein and the concentration of the protein used in each experiment should be carefully mentioned.
7. The authors must cite the papers if they are following a method, for example, a biofilm assay or SEM.
8. Statistical analysis is not performed in various results; many bar charts do not show statistical significance. In Figure 6, the * symbols are not placed on top of the bars.
9. The *C. elegans* morphology under the microscope is not satisfactory. Are these adult worms or larval stages? The *C. elegans* images should be replaced with better images. Panel B in Figure 7 is completely dark. The authors should also show merged images to justify the experiments.
10. Ion-extracted liquid chromatography-mass spectrometry results are shown in Figure 8. The authors need to provide details of these signaling molecules and their presence (with citations) in KP-ESBL. Also, mention their role in quorum sensing pathways.
11. The authors are trying to show that the fungus produces molecules similar to the signal molecules of KP-ESBL, thereby potentially causing competitive inhibition of quorum sensing. However, this is confusing, as the study focused on the extracellular proteins of KP-ESBL.
12. Why was an ESBL-producing strain used in the study without performing antibiotic susceptibility testing? The authors should include more strains in the study.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Quorum sensing, Biofilm, Pseudomonas aeruginosa, Antibiotic Resistance, Bacteriophages, Microbiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.