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Effect ethyl acetate extract of *Lasiodiplodia pseudotheobromae* Industrial Biotechnology Research Laboratory OS-64 on growth and cell morphology of a foodborne bacterium, *Bacillus cereus*

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ABSTRACT

Background: A foodborne bacterium, *Bacillus cereus* is one of the common food contaminants that could cause food poising with several sickness symptoms to humans. **Objective:** This study was undertaken to evaluate the effect of ethyl acetate extract of endophytic fungus, *Lasiodiplodia pseudotheobromae* Industrial Biotechnology Research Laboratory (IBRL) OS-64 towards a test bacterium, *B. cereus* with emphasis on bacterial growth reduction and cell morphological changes. **Materials and Methods:** The ethyl acetate (fermentative broth) and methanolic (fungal biomass) extracts were screened for their antibacterial activity against test bacteria via agar disc diffusion assay. **Results:** The results demonstrated that there is a significant antibacterial activity of the ethyl acetate extract with a diameter zone of inhibition in the value of 22.0 ± 0.6 mm. However, no antibacterial activity was observed from the methanolic extract. In broth microdilution assay, the minimum inhibitory concentration and minimum bactericidal concentration values of the extract were 250 and 500 μg/mL, respectively. The results revealed that the extract exhibited a bactericidal effect on the test bacterium. A time-kill study was then performed to determine the efficacy of the extract and the results showed that the antibacterial activity of the extract was dose and time-dependent. The morphological changes of the bacterial cells exposed to the extract were observed under a scanning electron microscope and transmission electron microscope. The results revealed the abnormalities of the treated bacterial cells compared to the control and thus led to ultimate death beyond repair. **Conclusion:** The present study suggested the potency of the ethyl acetate extract from an endophytic fungus, *L. pseudotheobromae* IBRL OS-64 could be further used as an antibacterial agent.

Keywords: *Bacillus cereus, Lasiodiplodia pseudotheobromae,* disc diffusion assay, microdilution broth assay, time-kill study, scanning electron microscope, transmission electron microscope

INTRODUCTION

Bacillus cereus is a Gram-positive, spore-forming, aerobic to facultative and motile rod bacterium that is commonly found in many types of environments. Fresh and marine waters, the intestinal tract of invertebrates, vegetables and fomites, decaying organic matter, soils, and foods are known as the natural environmental reservoir of *B. cereus*.[1] The strain is a common food contaminant and a well-known human pathogen that can cause emetic and diarrheal syndromes.[2] Emetic syndrome generally happens after consumption of food containing a preformed toxin namely emetic toxin or cereulide.[3] Meanwhile, diarrheal illnesses are caused by ingestion of *B. cereus* cells with the contaminated food that lead to several sickness symptoms including diarrhea, moderate nausea, rectal tenesmus, vomiting, and abdominal

pain.[4] *B. cereus* is easily spread from its natural environment to any kind of food due to its resistance to endospores to various stress conditions and their long-term survival capacity. Its spores and vegetative forms are frequent inhabitants of many food types including rice, vegetables, spices, herbs, additives, milk, dairy products, cereals, eggs, raw meat, processed foods, and ready to eat foodstuffs.[5]

Nowadays, there are several reports regarding the prevalence and antimicrobial resistance of *B. cereus*. The outbreaks become a serious burden and main concern in public health and food safety. For instance, Shawish[6] reported the multidrug resistance of *B. cereus* isolated from beef products in Egypt. They reported that the isolated strains of *B. cereus* were resistant to penicillin G and sensitive to ceftriaxone, ciprofloxacin, clindamycin, erythromycin, gentamicin, oxacillin, and vancomycin. On the other hand, there are also reports revealed the occurrence of antibiotic resistance strains found in fresh vegetables.[7] It was reported that 35 isolates from various salad vegetables including cabbage, carrot, cucumber, long melon, radish, spinach, and tomato exhibited high resistance to ß-lactam antibiotics such as ampicillin, cefepime, oxacillin, and penicillin. According to Chauhan,[8] the excessive usage of antibiotics including tetracycline, oxytetracycline, and sulfadimidine in livestock production, especially in treating sick animals is one of several factors that leads to antibiotic resistance among bacterial strains. Therefore, new effective antibiotics need to be discovered to combat the bacterial resistance strains.

Fungal endophytes from medicinal plant origin are one of the natural antimicrobial agent producers. Many reports revealed the potency of the endophytic fungi isolated from the medicinal plant as the source of antimicrobial agents. For instance, Dos Santos[9] reported the antimicrobial potential of endophytic fungi isolated from the leaves of the Brazilian medicinal plant, *Indigofera suffruticosa* Miller. They found that the methanolic and ethyl acetate extracts of *Nigrospora sphaerica* exhibited significant antibacterial activity towards Gram-positive and Gram-negative bacteria. *Lasiodiplodia pseudotheobromae* is one of the fungal endophytes that have the capability of antibacterial agents. Wei[10] reported the potency of *L. pseudotheobromae* F2 isolated from healthy flower *Illigera rhodantha* (Hernandiaceae) as a good antibacterial agent. They reported that the lasiodiplines E of the fungal endophytic isolate exhibited significant antibacterial activity against the clinical strains including *Bacteroides vulgates*, *Peptostreptococcus* sp. *Streptococcus* sp., and *Veillonella parvula*. Besides that, Taufiq[11] reported the efficacy of ethyl acetate extract of *L. pseudotheobromae* IBRL OS-64 against Methicillin-resistant *Staphylococcus aureus* by altering bacterial cells and reducing its growth. The present study was designed to evaluate the effect of ethyl acetate extract of *L. pseudotheobromae* IBRL OS-64 on *B. cereus*, a food contaminant bacterium.

MATERIALS AND METHODS

Microorganisms and Cultural Maintenance

A fungal endophyte, *L. pseudotheobromae* IBRL OS-64 and a test bacterium, *B. cereus* ATCC 10876 were provided by the Industrial Biotechnology Research Laboratory (IBRL), School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. The fungal culture was grown on potato dextrose agar (PDA) with the addition of powdered host plant (2 g/L) whereas the bacterial culture was cultured on nutrient agar (NA). Both fungal and bacterial cultures were incubated at 30°C (for 7 days) and 37°C (for 24 h), respectively. Both cultures were kept at 4°C prior to further use and subculturing was done every month to ensure their purity and viability.

Preparation of Bacterial Suspension

The bacterial suspension was prepared by transferring five single colonies from 24 h old culture into 5 ml of 0.85% (w/v) sterile normal saline. The turbidity was adjusted using 0.5 McFarland standards solution to obtain approximately 1×10^8 colony unit per (CFU/mL) of the bacterial suspension.

Culture Medium

Yeast extract sucrose (YES) broth was used as a culture medium in the present study. The medium was prepared by boiling 2 g of host plant powder in 1000 mL distilled water for 30 min. The mixtures were then filtered using Muslin cloth to obtain host plant water extract. An amount of 20 (g/L) yeast extract (Merck, Germany), 40 (g/L) sucrose, and 0.5 (g/L) ammonium sulfate were then added into the host plant water extract, mixed them well and the pH of the medium was calibrated using pH meter to 6.0. The culture medium was subsequently autoclaved at 121°C for 15 min.

Fermentation and Extraction

Two agar plugs of 3 days old of fungal culture were introduced into 250 mL Erlenmeyer flasks containing 100 mL YES broth (containing 20 [g/L] yeast extract [Merck, Germany], 40 [g/L] sucrose, 0.5 [g/L] ammonium sulfate, and host plant water extract) and incubated at 30°C for 16 days in the dark at static condition. The fermentative broth and fungal biomass were separated using Muslin cloth, filtered with filter papers (Whatman, No.1) and the filtered broth was then extracted thrice with an equal volume of ethyl acetate (1:1; v/v). Meanwhile, the fungal biomass was freeze-dried for 3 days and then macerated with methanol (1:50; w/v). Both ethyl acetate and methanol extracts were collected, concentrated using a rotary evaporator and left to dryness in a fume hood to obtain a crude paste.

Disc Diffusion Assay

The antibacterial activity of the extract was determined following the method described by CLSI.[12] An amount of 20 µL of the fungal extract (1 mg/ml) was impregnated to the sterile antibiotic disc (Whatman, 6.0 mm diameter) and then placed on the surface of MHA that was previously seeded with test bacteria. Five percent of (v/v) methanol (solvent used for dissolving the extract) was used as a negative control whilst chloramphenicol (30 µg/mL) was set as a positive control. The plate was then inversely incubated at 37°C for 24 h. The diameter zone of inhibition formed surrounding the disc was measured and recorded. The experiments were carried out in triplicates on different occasions.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Detection

The MIC and MBC values of the extract were determined following the method described by CLSI.[13] Briefly, 50 µl of bacterial suspension with a density of 1 × 10^8 CFU/ml was added into each of the wells that previously loaded with 50 µL Muller Hinton Broth (MHB) and 100 µl of the extract (at different concentration levels) to give a final volume of 200 µL and final bacterial density of 5 × 10^5 CFU/mL. The microtiter plate was then incubated at 37°C for 24 h. A volume of 5% (v/v) methanol was set as a control to detect the effect of solvent. Forty microliters of p-iodonitrotetrazolium violet (INT) were loaded to each well as a growth indicator after the incubation period. The lowest dilution of the extract showing no bacterial growth (indicated by no changes of broth color after loaded with an INT) was recorded as the MIC value of the extract. The MBC was determined by a subculturing aliquot sample from each well on a fresh MHA and followed by incubating at 37ºC for 18–24 h. The lowest concentration of the MIC well with no visible bacterial growth on MHA was regarded as the MBC value of the extract.

Time-kill Study

A volume of 0.1 mL of bacterial suspension with a density of 1 × 10^8 CFU/mL was transferred into 50 mL Erlenmeyer flask containing 18.9 mL MHB and 1.0 mL of the extract (at ½ × MIC, MIC, and 2 × MIC levels) to give a final volume of 20 mL and bacterial density approximately 5 × 10^5 CFU/mL.[14] A growth control was prepared by replacing the extract with 1.0 mL 5% methanol (v/v). The cultures were then homogenized and incubated at 37°C for 48 h in an orbital shaking incubator with an agitation speed of 150 rpm. A volume of 1.0 mL of aliquot sample was taken out from each flask every 4 h during an incubation time interval of 0 to 48 h to determine viable cell counts. The samples were then diluted using micro serial dilution by spread plating onto fresh Muller Hinton agar (MHA) and incubated at 37°C for 24 h. The plates with the number of colonies ranging from 30–300 were only counted to determine the CFU/mL. A time-kill curve ($\log_{10}$ CFU/mL vs. time) was constructed for each level of extract concentrations and the control. In the meantime, the time needed to reduce 50%, 90% 99%, and 99.9% of bacterial cells growth were determined according to the equation described by Taufiq and Darah.[11]

Scanning Electron Microscopy (SEM) and Transmission Electron Microscope (TEM)

A volume of 0.1 mL of bacterial suspension with a density of 1 × 10^8 CFU/mL was loaded into a 50 mL Erlenmeyer flask containing 18.9 mL MHB and 1.0 mL of the extract at 2 × MIC level to give a final volume of 20 mL and bacterial density of 5 × 10^5 CFU/mL. A growth control was set by replacing the extract with 1.0 mL 5% methanol (v/v). The cultures were then incubated at 37°C for 36 h in an orbital shaking incubator with an agitation speed of 150 rpm. The cultures were harvested at every 12 h of incubation time interval and centrifuged to obtain the bacterial cell pellet. The primary fixation, post-fixation, and dehydration process of the bacterial pallet were carried out according to procedures described by Borges.[15] The specimen was then viewed under a SEM (Leica Cambridge, S-360, UK). The bacterial samples for TEM were prepared the same as the SEM procedures. The bacterial cell pellets were processed according to the method described by Ibrahim.[16] The cell samples were placed on the copper grids and then viewed under a SEM (LIBRA 120 EFTEM, Germany).

Statistical Analysis

All the experiments were performed in triplicates (n = 3) and the experimental data were expressed as mean ± standard deviation (SD). The data were analyzed employing of the One-Way ANOVA using SPSS 15.0 and Duncan test was used to access the differences between means. The results were considered statistically significant if $P < 0.05$.

RESULTS

Antimicrobial Activity

The ability of the ethyl acetate and methanol extracts to inhibit bacterial cells of *B. cereus* was screened by disc diffusion assay and the results are illustrated in Table 1. Based on the findings, the ethyl acetate extract from the fermentative broth showed significant antibacterial activity with a diameter of inhibition zone in the value of 22.0 ± 0.6 mm. However, no inhibitory effect was observed on the bacterial cells treated with methanol extract that was previously obtained from fungal biomass. The results suggested that the bioactive compounds that have ability to inhibited *B. cereus* were secreted extracellularly.

Determination of MIC and MBC

Table 2 shows MIC and MBC values of the ethyl acetate extract of *L. pseudotheobromae* IBRL OS-64. The MIC value of the extract was 250 µg/mL whereas the MBC value was 500 µg/mL. The results revealed that the extract exhibited a bactericidal effect toward test bacteria, *B. cereus* since the MBC/MIC ratio was <4.

Time-kill Study

The time-kill studies were performed throughout 48 h with the *B. cereus* cells being exposed to ½ × MIC, MIC, and 2 × MIC values of the extract and the results are shown in Figure 1. The bacterial cells of the control were observed to increase over the incubation period, and they are followed the normal bacterial growth pattern with lag, log, stationery, and death phase. At ½ × MIC values, the extract exhibited a slightly dropped of bacterial growth after 4 h of incubation period and the bacterial growth was in a stagnant line

Table 1: Diameter zone of inhibition of extract against test bacterium

Test bacterium	Diameter zone of inhibition (mm)			
	Fermentative broth	Fungal biomass	Positive control	Negative control
Bacillus cereus	22.0±0.6	-	30.0±0.8	-

Positive control was Chloramphenicol (30 μg/mL) and negative control was 5% methanol

thereafter. The results revealed that the bacterial reduction and regrowth have occurred at the same rate. The bacterial cell reduction was observed after the cells were exposed to the extract at the MIC level. The cells were slightly reduced after 4 h of exposure time but, the extract produced a drastic cell eradication after 8 h of the incubation period. The bacterial cells were continuously dropped thereafter and at 48 h of exposure time, only 8.3×10^3 CFU/mL of bacterial density was observed compared to 3.1×10^7 CFU/mL of bacterial cells in control. At 2 × MIC value, a sharp bacterial reduction pattern was observed and the bacterial cells were completely killed after 36 h of exposure time. Table 3 shows the time needed by the extract to reduce bacterial cells at several levels. At ½ × MIC, 90% of the bacterial reduction was achieved after 20–24 h of exposure time but, the 99 and 99.9% was not achieved. However, at MIC and 2 × MIC, 99.9% of the bacterial reduction was observed after 20–24 h and 16–20 h of exposure time, respectively. Overall, kill-time studies revealed that the extract exerts bactericidal effect against test bacterial with a dose and time-dependent. Therefore, the present study suggested the potency of ethyl acetate extract of endophytic fungus, *L. pseudotheobromae* IBRL OS-64 as an antibacterial agent against food contaminant bacterium, *B. cereus*.

Morphological Changes of the Bacterial Cells

Figure 2 shows the SEM micrographs of the *B. cereus* cells exposed to the extract. Figure 2a shows the untreated bacterial cells with typical morphology of Gram-positive bacteria. Most bacterial cells were presented round smooth shape without any cavity in good condition. After 12 h treated with 2 × MIC value of the extract, some of the bacterial cells were started to shrink as indicated by red arrows [Figure 2b]. However, most of the cells were still regular. The formation of dents was observed on the surface of bacterial cells after 24 h exposed to the extract [Figure 2c]. Some of the cells experienced the impact and burst and the cavities were formed on a few cells (indicated by red arrows). Figure 2d shows the bacterial cells after 36 h treated with the extract. At this level, the cells exhibited more severe impacts compared to 24 h of exposure time. Most of the cells were shrunken, with more dents on their outer surface and it seems that the cells were completely collapsed and thus lead to ultimate death beyond repair.

Figure 3 shows the TEM micrographs of the bacterial cells exposed to the extract. As for control, the cell wall of the Gram-positive bacteria was observed in a good condition with organelles resembles accordingly. However, after 12 h exposed to the extract [Figure 3b], the leakage of bacterial cells was observed (indicated by red arrow). Cell leakage was observed after 24 h exposed to the extract indicated by white spot (indicated by black arrows) and at this level, the cells were thicker compared to the control [Figure 3c]. Finally, the

Table 2: MIC and MBC values of ethyl acetate extract against *Bacillus cereus*

Test bacterium	Extract concentration (mg/mL)		Ratio
	MIC	MBC	MBC/MIC
Bacillus cereus	250	500	2

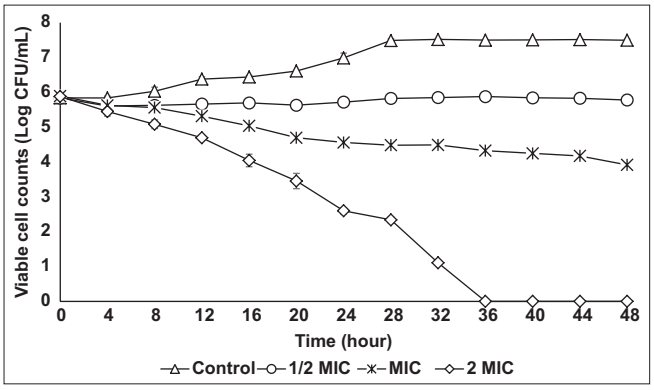

Figure 1: Time-kill curve of ethyl acetate extract of *Lasiodiplodia pseudotheobromae* IBRL OS-64 against *B. cereus* at different extract concentrations

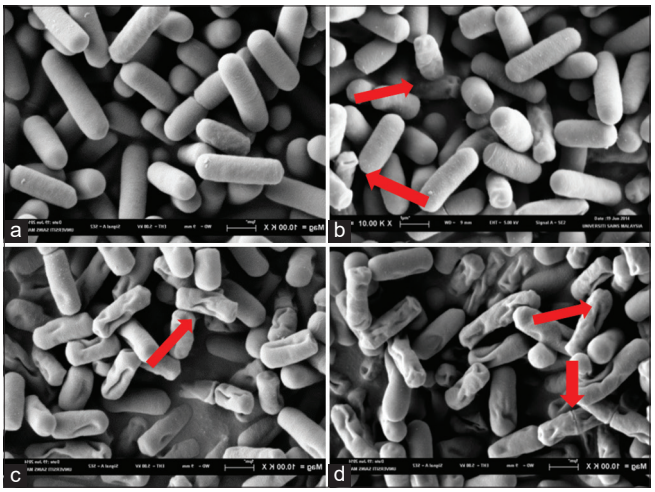

Figure 2: Scanning electron microscopy micrographs of *Bacillus cereus* treated with 500 µg/mL of ethyl acetate crude extract at different exposure time. (a) 0 h (control) (b) 12 h (c) 24 h (d) 36 h. Scale bars: 200 nm

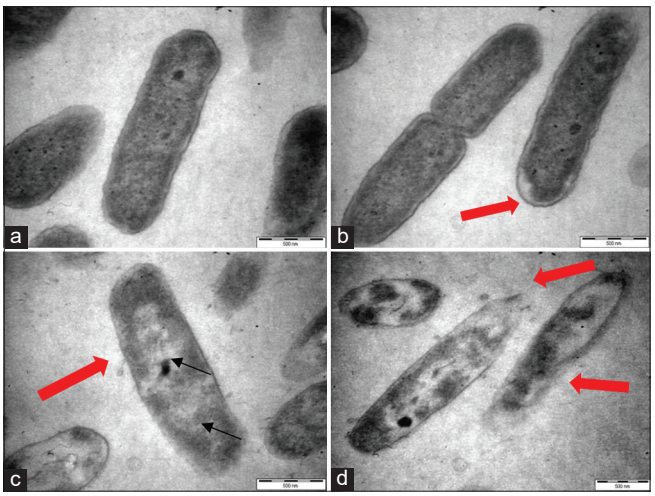

Figure 3: Transmission electron Microscope micrographs of *Bacillus cereus* treated with 500 µg/mL of ethyl acetate crude extract at different exposure time. (a) 0 h (control) (b) 12 h (c) 24 h (d) 36 h. Scale bars: 100 nm

Table 3: The time to achieve 50, 90, 99, and 99.9% growth reduction in initials inoculum of *Bacillus cereus*

Percentage of reduction (%)	Time (h)			
	Control	½ MIC	MIC	2 MIC
50	NR	4–8	4–8	0–4
90	NR	20–24	8–12	8–12
99	NR	NR	20–24	12–16
99.9	NR	NR	24–28	16–20

NR: Not reached

cells were burst after 36 h treated with the extract [Figure 3d] whereby the internal structure such as cytoplasm and organelles were found outside the cells. Moreover, there were some holes formed on the cell wall (indicated by red arrows) that reflect the cell leakages. At this stage, the bacterial cells had lost their original rod shape compared to the cells in the control.

DISCUSSION

Nowadays, the emergence of multidrug-resistant strains has become a worldwide problem and main concern in public health as well as food safety. The rapid outbreaks of resistant strains endangering the efficacy of the available antibiotics and the crisis have been attributed to overuse and misuse of the antibiotics as well as a lack of development of new drugs by the pharmaceutical industry due to reduced financial support and challenging regulatory requirements.[17] In the food industry, foodborne bacteria which can be caused food poisoning may be attributed to food illnesses, hospitalizations and may lead to death.[18] Therefore, a new antimicrobial agent needs to be discovered to overcome the increasing problems. There are several studies regarding on antibacterial activity of fungal endophytes isolated from various medicinal plants against *B. cereus*. For instance, Marcellano[19] reported that six fungal isolates from the bark of *Cinnamomum mercadoi* including *Cunninghamella* sp., *Rhizoctonia* sp., *Aspergillus* sp., *Fusarium* sp., and *Mycelia sterilia* can inhibit *B. cereus* with the diameter zone of inhibition in the range between 8.3 ± 0.6 mm and 19.7 ± 5.9 mm. On the other hand, Desale[20] found that ethyl acetate extract of *Phomopsis* sp. isolated from the leaves of *Vitex negundo* Linn exhibited significant activity against *B. cereus* with a diameter zone of inhibition was 16 mm. However, the detailed study on antibacterial activity of *L. pseudotheobromae* isolated from *Ocimum sanctum* leaves is none. To the best of our knowledge, this is the 1st time that antibacterial activity of *L. pseudotheobromae*, an endophytic fungus isolated from the leaf of medicinal plant, *O. sanctum* against *B. cereus* is described.

The present study on disc diffusion assay has shown that the ethyl acetate extract of fermentative broth of *L. pseudotheobromae* IBRL OS-64 had promising antibacterial activity. However, methanolic extract of fungal biomass did not exhibit antibacterial activity towards *B. cereus*. The findings revealed that the bioactive compounds of the fungal endophyte isolate were secreted extracellularly. This may be due to the secretion of bioactive compounds by fungal endophytes as a defense mechanism against other pathogens. The current study was consistent with Ibrahim[21] who reported a broader spectrum of antimicrobial activity of the ethyl acetate extract of fermentative broth compared to the methanolic extract of fungal biomass. They also suggested the main constituents of bioactive compounds by *N. sphaerica* were produced extracellularly. In contrast, Prabavathy[22] reported that ethyl acetate mycelial mat of fungal endophytes, *Aspergillus* sp. showed prominent activity compared to the ethyl acetate extract of culture broth. They found that the intracellular bioactive compounds exhibited a significant inhibitory effect against all test bacteria such as *Escherichia coli*, *S. aureus*, *Klebsiella pneumoniae,* and *Pseudomonas aeruginosa*. However, it was suggested that the ability to secrete secondary metabolites either intra or extracellularly is depending on the microbial strains and the internal factors that induce secretion of the metabolites.

Broth microdilution assay is an alternative method to determine the antimicrobial activity of natural extracts. This assay is more sensitive compared to disc diffusion assay and able to give most reproducible results especially on minimal inhibition concentration and thus, it was recommended as a general standard for the testing of natural products.[23] The results of the present study showed the MIC and MBC values of the extract were 250 and 500 µg/mL, respectively. The mode of action of the extract was proved to bactericidal based on MBC/MIC ratio (MBC/MIC = 2). According to Krishnan,[24] antimicrobial compounds are considered as bacteriostatic agents when the MBC/MIC ratio ≤4 whilst bactericidal agents if the MBC/MIC ratio >4. Furthermore, the extract exhibited a bacteriostatic effect at low concentration, but a bactericidal effect was observed at higher extract concentration. The bacteriostatic effect of the extract could be an advantage especially in combating the strains that can produce toxic shock syndrome such as staphylococci and streptococci because bacteriostatic agents are able to inhibit protein synthesis in resting slow-growing bacteria which not affected by bactericidal antibiotic agents such as ß-lactams.[25]

The potency and efficacy of the extract could be determined via a time-kill assay. According to Ferro,[26] the time-kill assay provides important pharmacodynamic information regarding antibiotics including the killing rate of antibiotics at different concentration levels, the dose-dependent and time-dependent bactericidal activities of antimicrobial agents. The present study analyzed bacterial viability overtime during treatment with the fungal extract at different concentration levels. At the sub-inhibitory level (½ × MIC), the growth of bacterial cells was consistent with a stagnant growth curve and this indicated that the extract possesses bacteriostatic activity against the test bacteria. However, at a higher concentration level (2 × MIC), the extract showed a rapidly bactericidal activity toward bacterial cells and achieving complete elimination of the cells within 32–36 h of exposure time. The findings were consistent with Marliyana[27] who found that bioactive compound, 4-hydroxypanduratin A isolated from *Kaempferia pandurata* showed a bacteriostatic activity at low extract concentration against foodborne pathogen bacteria, *B. cereus* but bactericidal activity at higher extract concentration. Besides that, the results revealed that the antibacterial activity of the ethyl acetate extract of *L. pseudotheobromae* IBRL OS-64 demonstrated a dose and time-dependent.

The SEM and TEM studies were performed to have a clearer view of what happening in the time-kill assay. The results revealed that the extract produced considerable morphological changes in the bacterial cells. It was suggested that the effects of the extract exerted on the peptidoglycan layer of Gram-positive bacteria and thus leads to the disintegration of the cell wall. According to Malanovic,[28] the antibiotic targeting peptides such as antimicrobial peptides (AMPs) need to traverse through the cell wall composed of peptidoglycan and lipoteichoic acid before they can reach the cytoplasmic membrane of Gram-positive bacteria. The AMPs-peptidoglycan interaction might facilitate penetration of AMPs while its interaction with anionic teichoic acid might act as either a trap for AMPs or a ladder for a route to the cytoplasmic membrane. The interaction of AMPs with the cytoplasmic membrane leads to lipid segregation affecting membrane organization, which affects membrane permeability, inhibits cell division processes, or leads to delocalization of essential peripheral membrane proteins. Some classes of antibiotics such as ß-lactams are able to interfere in specific steps of cell wall biosynthesis.[29] For instance, penicillin can block the cross-linking of peptidoglycan units by inhibiting peptide bond formation reactions catalyzed by transpeptidases (penicillin-binding protein).

According to Tomasz,[30] the treatment with a cell wall synthesis inhibitor could result in changes in cell size and shape, induce cellular stress responses and thus, lead to cell lysis. This was consistent with the current findings via SEM and TEM. Besides that, the morphological changes of bacterial cell treated with antibiotics occurs when the antimicrobial agent attacked the cell membrane.[31] The bioactive compounds in the ethyl acetate extract of *L. pseudotheobromae* IBRL OS-64 might attach and lock on the surface structure of bacterial cells and induce the permeability of the cell membranes. This phenomenon was consistent with Darah[32] who reported the methanol extract of *Wedelia chinensis* Osbeck leaves exerted its inhibitory effect on the cell wall of *B. cereus* which led to collapsed cells and complete cell damage. According to Ibrahim,[33] any disruption in the integrity of the cell wall would have a great influence on cell growth. Hyde[31] suggested that there will be loss of membrane potential, failure in cellular uptake of ethidium bromide, and leakage of potassium ions and ATP and thus resulted in cell wall disintegration. The SEM micrographs also revealed that the formation of holes and crumpled cells. These damages may be due to the loss of cellular materials and organelles from the cell cytoplasm.[34] As shown by the TEM micrographs where the cell became thicker and completely burst thereafter. This might be due to the water flow into the cell as a result of cell leakage. Black[35] stated that a cell with non-rigid, non-sturdy, and abnormal usually tends to burse when exposing to low osmotic pressure. These unstable and altered cells were completely collapsed and resulted in ultimate cell death.

CONCLUSION

Based on the results reported in this study, it was concluded that the ethyl acetate crude extract of *L. pseudotheobromae* IBRL OS-64 isolated from the leaf of *O. sanctum* exhibited significant antibacterial activity test foodborne bacterium, *B. cereus*. It was also suggested that the fungal endophytic strain is promised as a potential candidate in bioprospecting for antimicrobial drugs. This fungal extract also may be used as a food preservative due to its antimicrobial properties. However, the toxicity test must be first carried out to investigate its safety.

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CONFLICT OF INTEREST

Authors have declared that no competing interests exist.

REFERENCES

1. Bottone EJ. *Bacillus cereus*, a volatile human pathogen. Clin Microbiol Rev 2010;23:382-98.
2. Sanchez JA, Correa MM, Aceves-Diez AE, Castaneda-Sandoval LM. Direct detection of toxigenic *Bacillus cereus* in dietary complement for children and cassava starch. Rev Colomb Quimica 2014;43:5-9.
3. Bhunia A. *Bacillus cereus* and *Bacillus anthracis*. In: Heldman D, editor. Foodborne Microbial Pathogens: Mechanisms and Pathogenesis. West Lafayette, IN: Springer; 2008. p. 135-48.
4. Pal M, Asefa M, Deressa A, Muzein R. Processed foods and *Bacillus cereus* poisoning. Beverage Food World 2014;41:41-3.
5. Tewari A, Abdullah S. *Bacillus cereus* food poisoning: International and Indian perspective. J Food Sci Technol 2014;52:2500-11.
6. Shawish R, Tarabees R. Prevalence and antimicrobial resistance of *Bacillus cereus* isolated from beef products in Egypt. Open Vet J 2017;7:337-41.
7. Sood B, Sahota PP, Hunjan M. Multidrug resistant *Bacillus cereus* in fresh vegetables: A serious burden to public health. Int J Curr Microbiol Appl Sci 2017;6:649-61.
8. Chauhan AS, George MS, Chatterjee P, Lindahl J, Grace D, Kakkar M. The social biography of antibiotic use in smallholder dairy farms in India. Antimicrob Resist Infect Control 2018;7:60.
9. Dos Santos IP, da Silva LC, da Silva MV, de Araujo JM, Cavalcanti M, Lima VL. Antibacterial activity of endophytic fungi from leaves of *Indigofera suffruticosa* Miller (*Fabaceae*). Front Microbiol 2015;6:350.
10. Wei W, Jiang N, Mei YN, Chu YL, Ge HM, Song YC, *et al*. An antibacterial metabolite from *Lasiodiplodia pseudotheobromae* F2. Phytochemistry 2014;100:103-9.
11. Taufiq MM, Darah I. Anti-MRSA of the ethyl acetate crude extract from *Lasiodiplodia pseudotheobromae* IBRL OS-64, an endophytic fungus isolated from leaf of *Ocimum sanctum* Linn. Int J Pharm Pharm Sci 2018;10:50-5.
12. CLSI. Performance Standards for Antimicrobial Disk Susceptibility Tests, CLSI Standard M02. 13th ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2018.
13. CLSI. Methods for Determining Bactericidal Activity of Antimicrobial Agents. Approved Guideline CLSI Document M2-A11. Wayne, Pennsylvania: Clinical Laboratory and Standards Institute; 1999.
14. Jalil MT, Ibrahim D, Suhaimi NS. Time-kill study and morphological changes of *Proteus mirabilis* cells exposed to ethyl acetate crude extract of *Lasiodiplodia pseudotheobromae* IBRL OS-64. Malays J Microbiol 2020;16:219-28.
15. Borges M, de-ven MA, van-Cutsem J. Structural degeneration of *Aspergillus fumigatus* after exposure to saperconazole. J Med Vet Mycol 1989;27:381-9.
16. Ibrahim D, Lee CC, Yenn TW, Zakaria L, Lim SH. Effect of the extract of endophytic fungus, *Nigrospora sphaerica* CL-OP 30,

against the growth of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Klebsiella pneumonia* cells. Trop J Pharm Res 2015;14:2091-7.
17. Ventola CL. The antibiotic resistance crisis: Part 1: Causes and threats. P T 2015;40:277-83.
18. Painter JA, Hoekstra RM, Ayers T, Tauxe RV, Braden CR, Angulo FJ, *et al*. Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998-2008. Emerg Infect Dis 2013;19:407-15.
19. Marcellano JP, Collanto AS, Fuentes RG. Antibacterial activity of endophytic fungi isolated from the bark of *Cinnamomum mercadoi*. Pharmacogn J 2017;9:405-9.
20. Desale MG, Bodhankar MG. Antimicrobial activity of endophytic fungi isolated from *Vitex negundo* Linn. Int J Curr Microbiol Appl Sci 2013;2:389-95.
21. Ibrahim D, Lee CC, Sheh-Hong L. Antimicrobial activity of endophytic fungi isolated from *Swietenia macrophylla* leaves. Nat Prod Commun 2014;9:247-50.
22. Prabavathy D, Nachiyar CV. Study on the antimicrobial activity of *Aspergillus* sp. isolated from *Justicia adathoda.* Indian J Sci Technol 2012;5:3317-20.
23. Scorzoni L, Benaducci T, Almeida AM, Silva DH, Bolzani VS, Mendes-Giannini MJ. Comparative study of disk diffusion and microdilution methods for evaluation of antifungal activity of natural compounds against medical yeasts *Candida* spp and *Cryptococcus* sp. Rev Cienc Farm Basica Apl 2007;28:25-34.
24. Krishnan N, Ramanathan S, Sasidharan S, Murugaiyah V, Mansor SM. Antimicrobial activity evaluation of *Cassia spectabilis* leaf extracts. Int J Pharmacol 2010;6:510-4.
25. Pankey GA, Sabath LD. Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial infections. Clin Infect Dis 2004;38:864-70.
26. Ferro BE, van Ingen J, Wattenberg M, van Soolingen D, Johan W, Mouton JW. Time-kill kinetics of antibiotics active against rapidly growing mycobacteria. J Antimicrob Chemother 2015;70:811-7.
27. Marliyana SD, Mujahidin D, Syah YM, Rukayadi Y. Time-kill assay of 4-hydroxypanduratin A isolated from *Kaempferia pandurata* against foodborne pathogens. Molekul 2017;12:166-73.
28. Malanovic N, Lohner K. Antimicrobial peptides targeting Gram-positive bacteria. Pharmaceuticals 2016;9:59.
29. Kohanski MA, Dwyer DJ, Collins JJ. How antibiotics kill bacteria: From targets to networks. Nat Rev Microbiol 2010;8:423-35.
30. Tomasz A. The mechanism of the irreversible antimicrobial effects of penicillins: How the beta-lactam antibiotics kill and lyse bacteria. Annu Rev Microbiol 1979;33:113-37.
31. Hyde AJ, Parisot J, Mcnichol A, Bonev BB. Nisin-induced changes in *Bacillus* morphology suggest a paradigm of antibiotic action. Proc Natl Acad Sci USA 2006;103:19896-901.
32. Darah I, Lim SH, Nithianantham K. Effects of methanol extract of *Wedelia chinensis* Osbeck (*Asteraceae*) leaves against pathogenic bacteria with emphasise on *Bacillus cereus*. Indian J Pharm Sci 2013;75:533-9.
33. Ibrahim D, Hong SH, Kuppan N. Potent anticandidal activity of the methanolic extract of *Wedelia chinensis* leaves (Osbeck) against pathogenic *Candida albicans*. Int J Pharm Bio Sci 2013;4:120-30.
34. Al-Adham IS, Dinning AJ, Eastwood IM, Austin P, Collier PJ. Cell membrane effects of some common biocides. J Ind Microbiol Biotechnol 1998;21:6-10.
35. Black JG. Microbiology. 7th ed. New York, United States: John Wiley and Sons, Inc.; 2008. p. 80-110, 367-91.