Antifungal Prospect of *Bacillus cereus* Postbiotic on Crustacean Pathogen, *Lagenidium thermophilum*

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Received 9 February, 2021/Accepted 25 May, 2021

Pathogenic marine fungi, *Lagenidium thermophilum* is known causative agent in the crustacean industry. Current disinfection practice in hatchery has risks and negative impacts which prompts suitable substitute to synthetic antifungal agents. Thus, this study was conducted to evaluate the antifungal potential of postbiotic from four potential probiotics towards marine oomycetes, *L. thermophilum* IPMB 1401. The screening test showed that the *Lactobacillus plantarum* GS12 and *Bacillus cereus* GS15 postbiotics were positive for antifungal activity on *L. thermophilum* IPMB 1401. These two bacterial extracts have minimum inhibitory concentration (MIC) at 50%. The toxicity assay on MIC level of the postbiotic revealed that the cumulative mortality of brine shrimp nauplii exposed to *B. cereus* postbiotic was significantly lower compared to *L. plantarum* GS12 postbiotic and formalin. This indicates a high potential of *B. cereus* GS15 as a prospect for alternative control method for fungal infections in the crustacean culture industry.

**Key words**: Aquaculture / Disease control / Alternative treatment method / Biocide.

Mass mortalities of larvae in crustacean culture such as mud crab and shrimp cultures are common problems when fungal infections occurred (Jithendran et al., 2010). There are plenty of reports on fungal infection cases regarding both the mud crab and shrimp cultures, and most of the causative agents responsible for the infections are originally from a class of lower fungi, the oomycetes, called Oomycota (Jithendran et al., 2010). Fungal infections by *Lagenidium* genera had been reported to affect the penaeid shrimps and the mud crab species, which both are important crustacean culture products (Lee et al., 2016, 2017). Pathogenic marine fungi, *L. thermophilum* isolated back in 1993 was notoriously responsible for the low larval production in tiger prawns, *Penaeus monodon* at a hatchery in Thailand (Muraosa et al., 2006). Similar with the *L. callinectes*, seed production of mangrove crab, *Scylla serrata* was affected greatly by this fungus (Hatai et al., 2000).

Presently, fungal infections are controlled by most hatcheries as a prevention method via the use of synthetic chemicals. Due to concern regarding food security, issues on chemical safety when chemicals are used in food products remain a global health attention (Fung et al., 2018). Hence, with a view to engage this problem, an option of using bacterial extracts (postbiotics) as a potential antifungal candidate is proposed. Postbiotics are non-living metabolites that is the by-product of probiotics which is obtained either from secretion of live bacteria or released from bacterial breakdowns (Aguilar-Toalá et al., 2018; Ang et al., 2020). A previous study from Nguyen et al., (2019) showed that heat-killed probiotics (postbiotics) retains...
the same remunerative effects of their living counterpart whereby the bacterial extracts of *Lactobacillus plantarum* strain L137 were given to test the growth performance, immunity and stress of Nile tilapia (*Oreochromis niloticus*). Thus, these recent breakthroughs highlights postbiotics carries a hefty number of beneficial characteristics including clear chemical structure, safety doses, longer shelf-life, anti-inflammatory, immunomodulatory, anti-obesogenic, antihypertensive, hypolipidemic, anti-proliferative and antioxidative (Agular-Toalá et al., 2018), which can justify the need to conduct this antifungal potential of the postbiotic. Besides, the usage of bacterial extract as sources of antifungal agent is relatively new and scarce.

There is a need to conduct this study as the complications that results from the absence of viable treatments in salvaging infected cultured crustaceans from fungal diseases is vital. Thus, this study is aimed to determine the antifungal potential of postbiotics from four different bacteria on marine oomycetes, *L. thermophilum* IPMB 1401 and assess its toxicity on brine shrimp. Four identified potential probiotic bacteria from the case study of Ang and Lai (2019) were used in this study. They were identified as *Bacillus thuringiensis* GS11, *Lactobacillus plantarum* GS12, *B. cereus* GS15 and *Shewanella* sp. WS5. All four probiotic bacteria were stored in glycerol stock at -80°C at the concentration of 25% (v/v). *B. thuringiensis* GS11, *L. plantarum* GS12 and *B. cereus* GS15 were maintained in MRS Broth with 1% NaCl supplementation, while *Shewanella* sp. WS5 was maintained in Tryptic Soy broth (TSB) with 1% NaCl supplementation. Pure isolate of *L. thermophilum* IPMB 1401 from the Borneo Marine Research Institute culture collection was selected as the fungal strain. This fungal strain was maintained at 25°C using Peptone Yeast Glucose Seawater (PYGS) agar media throughout the experimental period. The media was prepared according to the recipe of 0.125% of peptone, 1.25% of yeast extract, 0.3% of glucose in filtered seawater (Saito and Lai, 2019).

The method of supernatant extraction was based on the procedure performed by Lv et al., (2017) with several modifications. The extraction procedure was performed firstly for each probiotic bacterium inoculated in their respective media broth overnight (24 hours) at 25°C with agitation. After 24 hours, the bacteria cells were centrifuged at 4400 rpm to obtain its supernatant. The supernatant for each bacterium was filtered using sterile syringe filter with pore size of 0.45 μm. Prior to adding the organic solvent into the test tube containing the supernatant, the pH was adjusted to 7. After pH adjustment, ethyl acetate was added into the supernatant fluid with the ratio 1:1 (v/v). The mixture was gently and thoroughly mixed and left for a few minutes. Then, when two layers formed in the test tube, the upper layer was collected and transferred into the rotary evaporator vial. After removing the organic solvent, Tris buffer (0.1 M, 7.0 pH) was added (1/5 volume of the supernatant) into the vial containing the crude extracts of postbiotics. From now on, the postbiotic was regarded as 100% concentrations. The postbiotic collected was stored at -5°C freezer until further used.

The presence of antifungal activity of the postbiotics was screened using a modified version of the agar diffusion method based from Laipuria et al. (2013). The screening test was conducted in duplicates per each postbiotic. There were four treatment wells per agar media of which consisted of one positive control well (150 ppm formalin diluted in Tris buffer), two postbiotic wells, and one negative control well (Tris buffer). After *L. thermophilum* IPMB 1401 growth radius reaches the 14 mm diameters, 30 μL of the treatments was place inside the well and the plates were incubated at 25°C for the period of 7 days with daily observations. The antifungal effect were determined via observing the inhibition of fungal growth occurs towards the postbiotic treatments wells.

Postbiotics that showed the inhibition of hyphal growth from the screening test were carried forward to determine their MIC. Modified version of the broth dilution method from the National Committee for Clinical Laboratory Standards (NCCLS) (Chryssanthou & Cuenca-Estrella, 2006) was used to test for the minimum inhibitory concentration of the postbiotics against *L. thermophilum* IPMB 1401. The assay consisted of postbiotic concentrations at 10%, 20%, 30%, 40% and 50% of extract to PYGS broth. PGYS broth served as negative control. As for positive control, formalin was added into PYGS broth (150 ppm). The test was conducted in triplicates. A 5 mm x 5 mm fungal block was added into each treatment. The test was performed at 25°C for the period of 7 days with daily observations. The tube with no mycelia growth from agar was regarded as positive.

The brine shrimp toxicity test bioassay described by McLaughlin & Rogers (1998) was used in this study. The cyst-hatching method was obtained from Libralato et al. (2016). Brine shrimp was hatched and allowed to reach instar II or III stages for the toxicity assay (Carballo et al., 2002). There were three types of treatment for this challenge test which are, sterilized filtered seawater as a negative control, a positive control (commercial antifungal, 150 ppm formalin diluted in seawater) and the postbiotics with their minimum inhibitory concentrations diluted in seawater. The experiment was conducted in five replicates, where each treatment was prescribed in 5 mL containing 30 brine shrimp nauplli of instar II or III. The conditions for each treatment were same and incubated at room temperature of
The signiﬁcant difference was subjected to one-way ANOVA and Tukey’sHonestly Significant Difference (HSD) test at the 5% level of significance. Post-Hoc test at the 5% level of signiﬁcance.

FIG. 1. Representative of negative and positive antifungal effect of postbiotic against L. thermophilum. (A) The fungal growth is not inhibited by postbiotic from B. thuringiensis GS11; (B) The fungal growth is inhibited toward well by postbiotic from B. cereus GS15.

TABLE 1. Antifungal screening tests of postbiotics against Lagenidium thermophilum IPMB 1401

| Postbiotic                | Antifungal activity |
|---------------------------|---------------------|
| Bacillus thuringiensis GS11 | -                   |
| Lactobacillus plantarum GS12 | +                   |
| Bacillus cereus GS15      | +                   |
| Shewanella sp. WS5        | -                   |

* + = Positive; - = Negative

Cumulative mortality (%) = 

Cumulative number of dead brine shrimp nauplii

Initial number of brine shrimp nauplii X 100%

Statistical analysis was performed in R studio version 1.2.33. Prior to running statistical analysis, all percentage data were normalized using arc sine square root transformation before analysis. The signiﬁcance of the data was subjected to one-way ANOVA and Tukey’s Honestly Significant Difference (HSD) was performed as the Post-Hoc test at the 5% level of signiﬁcance.

Four postbiotics were used for the screening test (Fig. 1) which resulted in two positives for antifungal and two negatives for antifungal against the marine fungi, L. thermophilum IPMB 1401. The postbiotics that showed positive antifungal effect were from the bacterial extract of L. plantarum GS12 and B. cereus GS15, while the postbiotics that showed negative antifungal effect were B. thuringiensis GS11 and Shewanella sp. WS5 extracts (Table 1).

Postbiotic that showed positive antifungal effect from the screening test was carried out in MIC assay. The result of the MIC of both postbiotic (L. plantarum GS12 and B. cereus GS15) against L. thermophilum IPMB 1401 was at 50% (Table 2).

The test for toxicity on brine shrimp showed varied results between treatments (Fig. 2). At 24 hours, the cumulative mortalities of brine shrimp nauplii were at 16.00±7.72% in seawater treatment, 88.67±8.84% in formalin, 94.00±8.00% in the L. plantarum GS12 postbiotic, and 44.67±18.21% in the B. cereus GS15 postbiotic. At 12 hours, all treatment exhibited mortalities with less than 20%. There was a signiﬁcant difference between the treatments at 24 hours after incubation. The comparison of means using Tukey’s HSD test showed that B. cereus GS15 postbiotic treatment showed a signiﬁcant difference between formalin (P < 0.05; 0.003) and L. plantarum GS12 postbiotic treatments (P < 0.05; 0.001), while there was no signiﬁcant difference between L. plantarum GS12 postbiotic and formalin treatments (P > 0.05; 0.97).

Antimicrobial effects of postbiotics had already been tested on bacterial-based pathogens. Bacterial extracts obtained from six different strains of L. plantarum showed positive results in inhibiting various bacterial pathogens including Pediococcus acidilactici, Listeria monocytogenes, Salmonella enterica, Escherichia coli and Vancomycin Resistant Enterococci (Kareem et al., 2014). However, currently there is no reports on the antifungal activities of bacterial extracts against fungal pathogen. Thus, this study was conducted to evaluate the potential of postbiotic as antifungal agent. As of date, this is the first known study of using bacterial extracts to inhibit the growth of pathogenic fungus, L. thermophilum.

Two postbiotics from B. cereus and L. plantarum strains in this study possessed the antifungal activity against L. thermophilum. The ﬁnding of this study is comparable to the ﬁnding of Kareem et al. (2014) where the extract induced inhibition zone on the pathogens. Crude extract from postbiotic composed with various compound such as peptides, exopolysaccha-
rides and organic acids which have antibacterial properties (Ang et al., 2020). The antibacterial effect of postbiotic’s components might also suggest it also possessed antifungal properties. This outcome was further supported by Kerr (1999) which described that bacterial genus Lactobacillus and Bacillus may produce antifungal molecules that inhibit fungal growth. The antifungal molecules which might be responsible were azoxybacilin, bacereutin, cispentacin and mycocerein for B. cereus (Kerr, 1999), whilst the antifungal molecules from Lactobacillus are still unknown.

Two postbiotics showed similar effect as formalin at concentration of 150 ppm. Formalin concentration at 150 ppm was a suggested concentration for disinfection of mud crab culture (De Pedro et al., 2007). Thus, this prompts the notion of testing the toxicity of formalin and the postbiotic on a model organism to see if there is a potential alternative means to substitute the formalin with the postbiotics. The toxicity assays revealed varied results. Both postbiotic and formalin showed negative effect towards brine shrimp after 24 hours treatment. However, due to the B. cereus postbiotic’s mortality count was lower than the formalin, it might be more suitable as an alternative solution to treat fungal infections in the near future aside from using synthetic chemicals.

L. plantarum GS12 and B. cereus GS15 postbiotic were found to have antifungal effect to L. thermophilum IPMB 1401 which was similar to formalin. This study also showed a comparable toxicity effect of the postbiotic with the formalin. B. cereus GS15 postbiotic seems promising as antifungal agent to substitute to chemical agent. This study concludes that B. cereus GS15 has a prospect of becoming an antifungal agent for the control of fungal infections in the crustacean industry. However, further tests on various life cycle of fungus, the content of B. cereus postbiotic and optimization of the production of postbiotic are needed to be conducted. The evaluation of the time kill assay against the fungal pathogen and dose respond of the toxicity of postbiotic should also be further studied. These information are needed to enhance the feasibility of postbiotic utilization as aquaculture fungal control strategies in the future.

ACKNOWLEDGMENTS

The study was supported by the Ministry of Education Malaysia under the Fundamental Research Grant Scheme (FRGS) No.: FRG0502-1/2019. This study was also received supports from the JSPS Core-to-Core Program “Building up an international research network for successful seed production technology development and dissemination leading South-East Asian region”.

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