

<https://doi.org/10.48047/AFJBS.6.14.2024.1987-1991>



African Journal of Biological Sciences

Journal homepage: <http://www.afjbs.com>



Research Paper

Open Access

Evaluation of biosurfactants derived from *Bacillus* spp. as immunostimulant in red hybrid tilapia (*Oreochromis* spp.)

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Article History

Volume 6, Issue 14, Aug 2024

Received: 15 July 2024

Accepted: 7 August 2024

Published: 8 August 2024

doi: [10.48047/AFJBS.6.14.2024.1987-1991](https://doi.org/10.48047/AFJBS.6.14.2024.1987-1991)

Abstract—Microbial biosurfactants exhibit various beneficial properties, including antimicrobial, anti-inflammatory, and immunomodulatory activities. Several studies have reported the efficacy of biosurfactants in increasing the immune responses and disease resistance of fish. Among various microbial surfactants, lipopeptides, glycolipids, and phospholipids are predominantly investigated. The present study aims at extracting and characterizing the biosurfactants derived from *Bacillus* spp. as a potential immunostimulant in the diet of red hybrid tilapia fish (*Oreochromis* spp.). Biosurfactants were obtained from *Bacillus subtilis* (ATCC®6051C) and *Bacillus licheniformis* (ATCC®12759) using the solvent extraction method. The extracted biosurfactants were confirmed by CTAB-methylene blue agar based on the formation of a blue halo ring. The collected biosurfactants were further analyzed for antimicrobial properties against two selected bacterial pathogens (*Streptococcus agalactiae* and *Aeromonas hydrophila*) via disc diffusion tests and the minimum inhibitory concentration (MIC) determination. Five experimental feeds containing purified biosurfactant were prepared and designated as BS0 (formulated feed without biosurfactant), BS-1 and BS-2 (formulated feed containing 1% and 2% of biosurfactant from *B. subtilis*), and BL-1 and BL-2 (formulated feed containing 1% and 2% of biosurfactant from *B. licheniformis*). The experimental feed was given to the red hybrid tilapia fish (*Oreochromis* spp.) at a mass of 5% of the body weight and fed twice daily for 8 weeks. The growth performance of treated tilapia fish was determined, and the white blood cell (WBC) compositions were analyzed. Biosurfactants derived from *B. licheniformis* demonstrated better antimicrobial activities and immunomodulatory activities besides being able to enhance growth performance in red hybrid tilapia.

Index Terms—biosurfactant, *Bacillus* spp., immunostimulant, tilapia

I. INTRODUCTION

Tilapia is well known for its hardiness characteristics, including the ability to adapt to a variety of environmental conditions, rapid growth, and high quality of life. These characteristics render it suitable to be farmed extensively, and it is currently one of the important aquaculture industries in Malaysia. However, intensive culturing activities of this species have exposed them to the risks of infection caused by bacterial or viral pathogens. Several common infectious diseases in farmed tilapia include streptococcal infections [1], columnaris caused by *Flavobacterium columnare* [2], francisellosis caused by *Francisella noatunensis* subsp. *orientalis*, edwardsiellosis caused by *Edwardsiella ictaluri* [3] and hemorrhagic septicemia caused by motile aeromonads (*Aeromonas hydrophila*, *Aeromonas sobria*, *Aeromonas veronii*, and *Aeromonas jandaei*) [4].

Tilapia culture in Malaysia is commonly affected by *Streptococcus agalactiae* and *A. hydrophila* [5]. Recently, the Tilapia Lake virus (TiLV), a novel RNA virus, has been reported in three continents (Asia, Africa, and South America), including Thailand [6] and Malaysia [7]. In Malaysia, a case of natural co-infection of TiLV and *A. veronii* has been reported in a Malaysian red hybrid tilapia (*Oreochromis niloticus* × *Oreochromis mossambicus*) farm [8]. This newly emerging virus is affecting wild and farmed tilapines, which is now highlighted as a global threat to the tilapia industry worldwide.

Currently, measures to control microbial diseases in aquaculture are limited. Furthermore, no effective and affordable vaccines are yet available to control the infections. As an alternative, a preventive approach has been proposed through the enhancement of immunity and disease resistance properties in tilapia species by the employment of immunostimulants, such as biosurfactants, which have been proven to boost immunity and provide better protection against pathogenic microbes in several fish species.

Biosurfactants derived from selected bacterial species, such as poly-3-hydroxybutyrate (PHB), demonstrated antimicrobial effects and also acted as an immunostimulant to enhance immunity and disease-resistant properties in aquatic organisms. Recently, several researchers have used bacterial secondary metabolites to control diseases in aquaculture species. The immunomodulatory activities of biosurfactants extracted from *Bacillus licheniformis* VS16 and *Bacillus subtilis* VSG4 have been demonstrated in fish [9]. Biosurfactants at 200 µg/mL (intraperitoneally, i.p) positively influenced the immune responses (lysozyme, alternative complement pathway [ACP], phagocyte, respiratory bursts, and serum bactericidal activities), exerted resistance against *Aeromonas* challenge, and stimulated the expression of immune-related genes in *Labeo rohita* [10].

The present study was conducted to investigate the antimicrobial and immunomodulatory properties of biosurfactants derived from *Bacillus* spp. in red hybrid tilapia (*Oreochromis* spp.). The effect of biosurfactants incorporated into the daily diet of tilapia was evaluated based on the growth performance and analysis of differential leukocyte or white blood cell (WBC) counts.

II. MATERIALS AND METHODS

A. Preparation of bacterial inoculum

Two selected bacterial strains, *B. subtilis* (ATCC®6051C) and *B. licheniformis* (ATCC®12759), were carefully streaked on nutrient agar plates and incubated for 24 h at 37°C. After an 18-h incubation, a loopful culture was added to 50 mL of culture medium (LB) and incubated at 37°C for 18 h. About 200 µL broth was inoculated into a 250 mL Erlenmeyer flask containing LB medium (50 mL). The culture broth was incubated at 37°C using an orbital shaker at 150 rpm and used as an inoculum for all experiments.

B. Extraction of biosurfactants

The bacterial cells were removed by centrifugation (10 × *g* for 10 min), and the proteins and lipids from cell-free extract were precipitated by adding concentrated hydrochloric acid (HCl, pH 2.0) [11]. Finally, a grey-white pellet obtained after precipitation was isolated by centrifugation at 4°C at 10 × *g* for 10 min. It was lyophilized and used in the quantification assay. Then, 50 mL of chloroform-methanol mixture (2:1) was added to the sample to a final chloroform/methanol/water ratio of 2:1:0.75. The mixture was centrifuged (10,000 rpm, 10 min), the organic layer was carefully collected, and the sample was evaporated to dryness under N₂ at 37°C for 30 min [11].

C. Analysis of biosurfactants

The extracted biosurfactants from *B. subtilis* (BS) and *B. licheniformis* (BL) were streaked onto CTAB-methylene blue agar for the confirmation of their properties. The CTAB agar test was done according to Liu [12] with some modifications to identify the type of surfactant. The medium was prepared by adding 5 g CTAB, 0.02 g methylene blue, 20 g glucose, 10 g peptone, 1 g beef extract, 0.5 g yeast extract, and 17 g agar. Then, 0.1 mL of biosurfactant suspension was inserted into the hole made on the CTAB-methylene blue agar. The CTAB-methylene blue agar was then incubated at 37°C for 7 days. The presence of a halo around the hole inoculated with biosurfactant suspension indicates the presence of biosurfactant produced by the selected bacteria. The collected biosurfactants were further analyzed for their antimicrobial properties against two selected bacterial pathogens, *S. agalactiae* (ATCC®27956) and *A. hydrophila* (ATCC®7966), by conducting disc diffusion test and minimum inhibitory concentration (MIC) determination.

D. Preparation of experimental feed

Five experimental feeds containing an optimized concentration of *Lemna minor* at 10% [13] and purified biosurfactant were prepared and designated as BS0 (formulated feed without biosurfactant), BS-1 and BS-2 (formulated feed containing 1% and 2% of biosurfactant from *B. subtilis*), and BL-1 and BL-2 (formulated feed containing 1% and 2% of biosurfactant from *B. licheniformis*). The basic ingredients of the experimental feed contained 25% fishmeal, 10% *L. minor* meal, 20% soybean meal, 10% rice bran, 10% corn meal, 10% wheat meal, 3.5% multivitamin, 7.5% yeast, and 4% vegetable oil.

E. Experimental fish

This work was approved by the Research Ethics Committee of Universiti Selangor (Ref no: UNISEL/CRIL/EC/0025), which follows the general guidelines of the Canadian Council on Animal Care with the code number (R/8). Healthy red hybrid tilapia (*Oreochromis* spp.) between 4.0–5.0 g was obtained from a private farm in Sungai Besar, Selangor, Malaysia. The health status of the fish was assessed through observation of clinical signs during the acclimation period (2 weeks).

F. Experimental feeding in tilapia fish

The experiments were conducted in five tanks, with the control group labeled as BS-0 (control), BS-1, BS-2, BL-1, and BL-2 according to different experimental feeds. Each tank can accommodate 20 fish supplied with continuous aeration. The experimental feed was given to the tilapia fish at a mass of 5% of the body weight and fed twice daily for 8 weeks. The growth performance of the treated tilapia was determined based on parameters such as weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), feed conversion efficiency (FCE), and survival rate (SR) at the end of the experimental feeding. The growth parameter was calculated as follows:

$$\begin{aligned} \text{Weight gain (WG)} &= \text{Final weight} - \text{Initial weight} \\ \text{Specific growth rate (SGR)} &= \frac{\ln \text{ final weight} - \ln \text{ initial weight}}{\text{days}} \times 100 \\ \text{Feed conversion rate (FCR)} &= \frac{\text{Feed intake (g)}}{\text{Weight gain (g)}} \\ \text{Feed conversion efficiency (FCE)} &= \frac{\text{Total weight gain (g)}}{\text{Total feed intake (g)}} \\ \text{Survival rate (SR)} &= \frac{\text{No of survived (fish)}}{\text{No of leased (fish)}} \times 100 \end{aligned}$$

G. Total and differential leukocyte counts

After 8 weeks of treatment, the blood samples were collected from each group of the treated fish. Blood samples were obtained by venipuncture of the caudal vessels and placed into microtubes containing sodium heparin for further analysis. The blood samples were diluted with Natt-Herricks solution, and the number of leukocytes (WBC) was counted manually using a hemocytometer. Differential leukocyte count was performed by preparing blood smears stained with Wright-Giemsa stain. The number of leukocytes (WBC), including neutrophils, monocytes, and lymphocytes, was determined manually [14].

H. Statistical analysis

The experiments were carried out in triplicates. One-way ANOVA using SPSS ver. 25 were used to analyze the experimental data, and the value of $p < 0.05$ is considered statistically significant. The results are expressed as mean $\pm$ SD (standard deviation).

III. RESULTS

3.1 Analysis of biosurfactants

Analysis of the extracted biosurfactants on CTAB-methylene blue agar demonstrated the presence of a

blue halo surrounding the biosurfactant suspension column (Fig. 3.1). The blue halo indicates that the biosurfactant was successfully extracted from *B. subtilis* and *B. licheniformis*. Meanwhile, bacterial extract from *Escherichia coli*, a non-producing biosurfactant bacteria, was used as the negative control, with the absence of the blue halo. The antimicrobial activity of the extracted biosurfactants at different concentrations (1% and 2%) was demonstrated by the disk diffusion test. Table 3.1 summarizes the inhibition zones developed by *B. subtilis*- and *B. licheniformis*-derived biosurfactants against selected pathogens. The biosurfactant from *B. licheniformis* (2% BL) inhibited the growth of test pathogens at an intermediate range: *S. agalactiae* (13.67 mm) and *A. hydrophila* (17.33 mm). Meanwhile, Table 3.2 indicates that the biosurfactant derived from *B. licheniformis* (BL-2) has the highest MIC against both pathogens.

3.2 Growth performance of treated tilapia

Table 3.3 shows the growth performance of red hybrid tilapia (*Oreochromis* spp.) following treatment with the experimental feeds incorporated with 1% and 2% of BS and BL. The fish growth was observed every seven days by measuring the WG. The highest WG can be seen in BS-1 (482.33 g), and the lowest WG is in BS-2 treatment at 412.00 g. Excellent SGR is demonstrated in the BL2 treatment at 41.78%, while the lowest SGR is noted in BS2 (40.64%). Meanwhile, BS-2 shows the lowest FCR at 0.64, while the highest FCR is observed in BS-1 (0.86). BS-1 demonstrates the highest FCE, with 27.46, and BL-2 represents the lowest FCE at 25.83. High SRs are observed in all treatments and control experiments with no mortality. BS-0 (0% biosurfactant) shows a significant difference ($p < 0.05$) to all treatments in WG, while BS-0 and BS-2 demonstrate a significant difference ($p < 0.05$) to other treatments for SGR and FCR. No significant difference ($p < 0.05$) is noted in all treatments for FCE and SR.

3.3 Total and Differential Leucocyte Counts

Table 3.4 summarizes the results of total and differential leucocyte counts from red hybrid tilapia (*Oreochromis* spp.) analyzed treatment with the experimental feed. The highest value of total leucocyte count (TLC) is recorded by BL-2 ($40.33 \times 10^3/\mu\text{L}$), while the lowest TLC is shown by BS-0 ($25.00 \times 10^3/\mu\text{L}$). The neutrophil count is increased in BL-1 at $17.33 \mu\text{L}$, while the lowest is C-0 with $5.33 \mu\text{L}$. BL-2 shows a higher monocyte count of $4.67 \mu\text{L}$, while C1 and BS2 show the lowest count ($0.67 \times 10^3/\mu\text{L}$). The lymphocyte count is higher in BL-2 ($11.33 \times 10^3/\mu\text{L}$) and the lowest in BS-2 ($1.67 \times 10^3/\mu\text{L}$). There is no significant difference ($p < 0.05$) in the TLC, neutrophil, and monocyte counts in all treatments and controls. BL-2 shows a significant difference ($p < 0.05$) to other treatments and controls in the lymphocyte count.

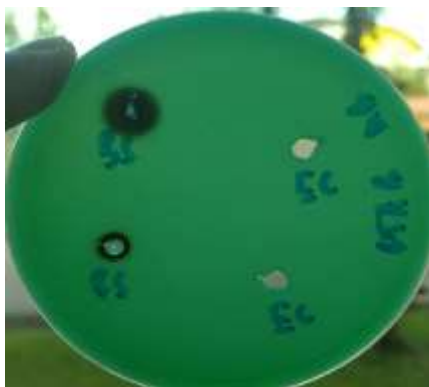


Fig. 3.1. Formation of a blue halo surrounding the column of biosurfactant suspension of BS and BL (left) and no reaction demonstrated for the bacterial extract derived from *E. coli* (EC)

Table 3.1: Antimicrobial activity of different concentration of biosurfactant extracted from *B. subtilis* and *B. licheniformis* against *A. hydrophila* and *S. agalactiae* using disk diffusion method

Selected m/o	Conc (%)	Inhibition zone (mm)			
		BS	BL	C(+)	C(-)
<i>A. hydrophila</i>	1	8.67±2.31 ^a	8.00±2.65 ^a	37.00±3.61 ^b	7.33±1.15 ^{ab}
	2	13.33±5.77 ^a	17.33±4.16 ^b	34.33±3.06 ^b	7.67±2.08 ^{ab}
<i>S. agalactiae</i>	1	9.33±2.08 ^a	13.67±5.86 ^{ab}	15.00±0.00 ^a	9.33±1.53 ^b
	2	9.67±3.21 ^a	9.67±3.21 ^{ab}	16.67±1.53 ^a	6.00±0.00 ^a

Note: Inhibition zone (mm); Resistant: ≤12; Intermediate: 13-17; Susceptible: ≥18 Inhibition zone (mm) are expressed as mean ± STD of replicate sample (n=3). (Horizontal) the values with different superscript (^a, ^b, ^{ab}) denotes with significantly different (p<0.05). Note: BS(*Bacillus subtilis*), BL(*Bacillus licheniformis*), C(+) (Chloramphenicol) and C(-) (control negative).

Table 3.2: Minimal inhibition concentration (MIC) of different concentration of biosurfactant extracted from *B. subtilis* and *B. licheniformis* against *A. hydrophila* and *S. agalactiae* using microtiter plate method.

Sample	Conc. (mg/ml)	1:02	1:04	1:08	1:16	1:32	1:128	1:256	1:512	1:1024	1:2048	1:4096
<i>A. hydrophila</i>	BS1	+	-	-	-	-	-	-	-	-	-	-
	BS2	+	+	-	-	-	-	-	-	-	-	-
	BL1	+	+	-	-	-	-	-	-	-	-	-
	BL2	+	+	+	+	+	-	-	-	-	-	-
<i>S. agalactiae</i>	BS1	+	-	-	-	-	-	-	-	-	-	-
	BS2	+	+	-	-	-	-	-	-	-	-	-
	BL1	+	+	+	+	-	-	-	-	-	-	-
	BL2	+	+	+	+	-	-	-	-	-	-	-

Note: Inhibition zone (mm); Resistant: (-); Susceptible: (+). (Vertical) Note: BS1(*Bacillus subtilis* 1%), BL1 (*Bacillus licheniformis* 1%), BS2 (*Bacillus subtilis* 2%), BL2 (*Bacillus licheniformis* 2%),

Table 3.3: Growth performance of red hybrid tilapia (*Oreochromis* spp.) fed with the experimental diet containing 0%, 1%, and 2% BS and BL for 8 weeks.

	BS0	BS1	BS2	BL1	BL2
WG, g	536.67±50.20 ^a	482.33±16.50 ^a	412.00±172.66 ^a	450.67±117.59 ^a	456.33±38.11 ^a
SGR, %	41.32±1.43 ^{ab}	41.67±0.36 ^{ab}	40.64±1.41 ^a	41.33±1.30 ^{ab}	41.78±0.81 ^{ab}
FCR	0.52±0.03 ^{ab}	0.86±0.03 ^c	0.64±0.02 ^{bc}	0.67±0.05 ^{bc}	0.71±0.06 ^{bc}
FCE	33.06±1.03 ^a	27.46±0.31 ^a	27.28±3.81 ^a	26.00±2.26 ^a	25.83±0.72 ^a
SR, %	1.00±0.00 ^a	1.00±0.00 ^a	1.00±0.00 ^a	1.00±0.00 ^a	1.00±0.00 ^a

SGR=(lnW2-lnW1/T) x 100, FCR=food conversion rate, FCE=Feed conversion efficiency

All values are expressed as mean ± STD of replicate sample (n=3). (Horizontal) the values with different superscript (^a and ^b) denotes with significantly different (p<0.05). Note: BS0 (0% treatment), BS1 (1% treatment), BS2 (2% treatment), BL1 (1% treatment) and BL2 (2% treatment).

Table 3.4: Total and differential leucocyte counts of red hybrid tilapia (*Oreochromis* spp.) fed with the experimental diet containing 0%, 1%, and 2% BS and BL for 8 weeks.

	BS0	BS1	BS2	BL1	BL2
Total Leucocyte Count (10 ³ /μl)	25.00±6.00 ^a	29.67±3.21 ^a	31.67±17.62 ^a	25.00±13.11 ^a	40.33±16.62 ^a
Neutrophil (10 ³ /μl)	7.00±8.19 ^a	10.67±0.58 ^a	10.00±6.24 ^a	17.33±10.97 ^a	12.33±8.62 ^a
Monocyte (10 ³ /μl)	0.67±0.58 ^a	1.33±2.31 ^a	0.67±0.58 ^a	2.00±2.65 ^a	4.67±2.52 ^a
Lymphocyte (10 ³ /μl)	13.33±11.93 ^a	2.33±2.52 ^a	1.67±0.58 ^a	2.00±1.00 ^a	11.33±5.77 ^a

All values are expressed as mean ± STD of replicate sample (n=3). (Horizontal) the values with different superscript (^a and ^b) denotes with significantly different (p<0.05). Note: BS0 (0% treatment), BS1 (1% treatment), BS2 (2% treatment), BL1 (1% treatment) and BL2 (2% treatment).

IV. DISCUSSION

The present study highlights the potential of biosurfactants

derived from two *Bacillus* spp. as immunostimulants in red hybrid tilapia (*Oreochromis* spp.). Biosurfactants extracted from *B. subtilis* (ATCC®6051C) and *B. licheniformis* (ATCC®12759) demonstrated the formation of blue halo rings on CTAB-methylene agar, confirming the presence of biosurfactant. Coagulation of cationic and anionic surfactants can be detected due to the presence of CTAB in the medium [15]. The antimicrobial properties of both extracts were analyzed against two selected aquatic pathogens, *S. agalactiae* (ATCC®27956) and *A. hydrophila* (ATCC®7966). Disk diffusion test and MIC analysis on both biosurfactants indicated that the biosurfactant derived from *B. licheniformis* (BL) had better antimicrobial properties than *B. subtilis*. A study reported that *B. licheniformis* produced several classes of antimicrobial substances, including bacteriocins, particularly licheniformins and bacitracins, which possess strong antimycobacterial activity [16]. The effectiveness of *B. licheniformis* as a probiotic is associated with its ability to produce a large number of substances with antimicrobial, antioxidant, and immunomodulatory activities [17]. *B. licheniformis* showed a protective effect in zebrafish (*Danio rerio*) against *Vibrio parahaemolyticus* infection. Due to the antagonistic activity of this probiotic, the complete survival of infected fish was observed in contrast to untreated fish [18]. The present study demonstrated the ability of biosurfactant derived from *B. licheniformis* to inhibit the growth of bacterial pathogens that cause diseases in tilapia fish, *S. agalactiae* (ATCC®27956) and *A. hydrophila* (ATCC®7966) [19].

The effect of biosurfactants on the growth performance of red hybrid tilapia was evaluated through oral administration of the biosurfactants incorporated in the formulated feed. Feeding of red hybrid tilapia with experimental feed incorporated with biosurfactants derived from *B. subtilis* (BS) and *B. licheniformis* (BL) resulted in significant improvement in the SGR demonstrated in fish treated with BL2 (2% biosurfactant from *B. licheniformis*). Analysis of TLC or WBC count indicated that fish treated with BL2 demonstrated the highest TLC than other treatments. Fish treated with BS2 (2% biosurfactant from *B. subtilis*) demonstrated the second-highest value of TLC (Table 3.4). This finding indicates that the addition of biosurfactants in fish diet may influence the immunomodulatory activity in treated fish.

The immunomodulatory activities of biosurfactants extracted from *B. licheniformis* VS16 have been demonstrated in fish [20]. *L. rohita* fingerlings were intraperitoneally (i.p) injected with various concentrations of BS. Immunological parameters (e.g., lysozyme levels, ACP, and phagocytic activities) and digestive enzymatic activities were higher (p < 0.05) in the BS-treated groups. In a similar study, *B. subtilis* VSG4 derived BS at 200 μg/mL (i.p) positively influenced the immune responses (lysozyme, ACP, phagocyte, respiratory bursts, and serum bactericidal activity), resistance against *Aeromonas* challenge, and stimulated the expression of immune-related genes in *L. rohita* [21]. In the present study, a different approach has been employed to introduce biosurfactants in fish. Instead of an injection method, the biosurfactant was delivered through oral administration by incorporating it into the experimental feed. The administration of biosurfactants through an oral

feeding approach is much more practical to be applied in fish, as this approach can save time and reduce stress in fish.

V. CONCLUSION

The present study revealed the ability of biosurfactants derived from *Bacillus* spp. as a potential immunostimulant in red hybrid tilapia (*Oreochromis* spp.). Biosurfactant extracted from *B. licheniformis* (BL) demonstrated antibacterial activity against two selected fish pathogens (*S. agalactiae* and *A. hydrophila*). Fish treated with BL through feeding experiments demonstrated better growth performance and appropriate immunomodulation activity indicated by total leucocyte count (TLC).

ACKNOWLEDGMENT

This work was supported by the Ministry of Higher Education, Malaysia, under the Fundamental Research Grant Scheme (FRGS/1/2021/WAB04/UNISEL/02/1) and Universiti Selangor (UNISEL).

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