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# Characterization of the antioxidant and anti-inflammatory properties of a polysaccharide-based bioflocculant from *Bacillus subtilis* F9

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ABSTRACT

Microbial flocculants are versatile class of novel biomacromolecules with numerous potential industrial applications. This study sought to investigate the antimicrobial, antioxidant, and anti-inflammatory potential of a polysaccharide-based bioflocculant (PBB) extracted from *Bacillus subtilis* F9. To achieve this, the antioxidant activity of different PBB concentrations(100 μg/mL- 1000 μg/mL) was first examined *in vitro* using 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl radicals, and superoxide radical scavenging assays. Further, the anti-inflammatory activity of PBB against lipopolysaccharide (LPS; 1 μg/mL)-induced inflammatory mediators released from headkidney (HK)-derived macrophages of *Labeo rohita* was investigated. Our results revealed that the capacities of 800 μg/mL of PBB to scavenge DPPH, hydroxyl radicals, and superoxide radicals were 81.46 ± 1.37%, 66.34 ± 2.63%, and 78.03 ± 2.46%, respectively, which were slightly higher that observed following treatment with 400 μg/mL of the positive control (ascorbic acid). Further, the radical scavenging capacity of PBB was found to steadily increase with increasing concentrations of PBB. Pre-treatment with PBB also inhibited nitric oxide production in a dose-dependent manner. We next examined the effect of PBB on proinflammatory cytokines (TNF-α, and IL-1β) and anti-inflammatory cytokines (IL-10, TGF-β) via qRT-PCR and ELISA. We found that PBB markedly inhibited the LPS-induced mRNA and protein expression levels of TNF-α and IL-1β, while it significantly increased those of IL-10 and TGF-β. Further, PBB exhibited an antibacterial activity against multiple food-borne pathogens with minimal inhibitory concentration values in the range of 3-11 mg/mL. Importantly, PBB exhibited negligible cytotoxic effects against HK macrophages. Taken together these results suggest that PBB may serve as a natural antioxidant for application in functional therapies and may also be exploited for its anti-inflammatory potential.

## 1. Introduction

Microbial flocculants are metabolites composed of polysaccharides, proteins, glycoproteins, and proteoglycans [1]. They are a multifaceted group of bio-macromolecules with potential applications in cosmetology, pharmacology, wastewater treatment, textile manufacturing, and fermentation [2]. Bioflocculants has received key attention for their application in wastewater treatment. These microbial polysaccharides are complex in nature and are primarily composed of carbohydrate moieties. Thus, the degradation products from bioflocculants are harmless to the ecosystem. The non-toxic nature of bioflocculants has also been demonstrated in a mouse model [5]. Many bacterial polysaccharides are heteropolymers made up ofseveral copies of oligosaccharide units (heparin, xanthan, hyaluronic acid, etc.) [4]. Further, the economical large-scale production and recovery of bioflocculants from fermentation broth is feasible [3]. Microbial flocculants have also been employed as emulsifying, stabilizing, and viscofying agents [6]. Thus, recently, exopolysaccharides (EPSs) or bioflocculants from microbial sources have been receiving increasing attention for use in practical applications.

Reactive oxygen species (ROS) including hydrogen peroxide, superoxide radicals, and hydroxyl radicals are inevitably produced during normal and aberrant consumption of molecular oxygen. The damaging effects induced by ROS on biological molecules (e.g. lipids, proteins, and nucleic acids) are associated with numerous pathological processes including atherosclerosis, cancer, and rheumatoid arthritis [7]. Thus, antioxidants have the potential to effectively treat degenerative diseases and ageing caused by excessive free radicals [8]. Since synthetic antioxidants have been shown to possess safety issues, considerable focus has been given to defining the antioxidant capacity of natural

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**Nomenclature**

| | |
|---|---|
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethyl sulfoxide |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| EPS | Exopolysaccharides |
| FBS | Fetal bovine serum |
| HK | head-kidney |
| LAB | Lactic acid bacteria |
| MH | Mueller-Hinton |
| MIC | Minimal inhibitory concentration |
| NO | Nitric oxide |
| PBB | polysaccharide-based bioflocculant |
| qRT-PCR | Real-time quantitative reverse transcription polymerase chain reaction |
| ROS | Reactive oxygen species |
| SDS | Sodium dodecyl sulfate |

molecules. Recently, the antioxidant activities of microbial polysaccharides derived from lactobacilli or bacilli species has been investigated [4,9,10]. However, few studies have examined the antioxidant properties exhibited by novel microbial bioflocculants.

The biological actions of the bioactive substances are generally either proinflammatory or anti-inflammatory. Cytokines are immunological components shown to be associated with the pathogenesis of various diseases. Investigating the expression profiles of various cytokines and assessing their role in immune defense may, therefore, aid in identifying indirect immunological markers [11]. Recently, the anti-inflammatory properties of microbial EPSs have been investigated. For example, EPS derived from *Bacillus subtilis* was found to induce anti-inflammatory functions in M2 macrophages, thereby inhibiting T cell activation and suppressing T cell-dependent immune responses [12]. However, only a recent study by Zong et al. [13] has investigated the anti-inflammatory activities of PBB specifically extracted from the alkaliphilic bacterium *Paenibacillus jamilae*.

Many studies have sought to examine the production of bioflocculants by various microorganisms, including *Aspergillus*spp., *Bacilli*spp., *Halomonas* spp., and *Pseudomonas* spp [14]. Organisms from the class, *Bacilli*, are capable of producing larger quantities of EPS compared to lactic acid bacteria (LAB) [15]. In a previous study, we isolated a thermotolerant bioflocculant-producing *Bacillus subtilis* F9 species from wastewater sludge [16]. Using optimal conditions, we purified 2.32 g/L of bioflocculant from these cultures. The total carbohydrate and total protein of *B. subtilis* F9 bioflocculant were determined to be 88.3% and 10.1%, respectively, indicating a primarily polysaccharide-based bioflocculant (PBB) [16]. Further, the total sugar composition of the bioflocculant was found to be 38.4% neutral sugar, 2.86% uronic acid, and 2.1% amino sugar. The molecular weight was $5.3 \times 10^4$ Da as revealed through HPLC analysis [16].

The aim of the current study was to characterize the biological functions of PBB purified from *Bacillus subtilis* F9, focusing on its antioxidant activity and cytotoxic activity against *Labeo rohita* (*L. rohita*) head-kidney (HK) macrophage cells. Further, we examined the effect of PBB on the expression of proinflammatory and anti-inflammatory cytokines in HK macrophage cells.

## 2. Materials and methods

### 2.1. Bacterial strain and culture conditions

*Bacillus subtilis* F9 was previously isolated from wastewater sludge [16] and stored at -80 °C with 15% (v/v) glycerol. The strain was thawed and used for the production of bioflocculants. The bacteria were cultured in media composed of sucrose (20 g/L), yeast extract (3.5 g/L), $K_2HPO_4$ (5 g/L), and NaCl (0.1 g/L); the pH was adjusted to 7.0. The media was inoculated with 2% (v/v) young *B. subtilis* F9 culture and maintained at 40 °C for 60 h with continuous agitation at 160 g [16].

### 2.2. Purification of bioflocculants

The bioflocculants were purified following the method described in our previous study [16]. Briefly, the culture broth was centrifuged at 5000 × *g* for 30 min, and the supernatant was concentrated and dialyzed overnight at 4 °C in deionized water. Three volumes of cold anhydrous ethanol (4 °C) were poured onto the dialyzed broth. The precipitate was collected and dissolved in deionized water and 10% cetylpyridinium chloride (Sigma-Aldrich, USA) was added with continuous stirring. The mixture was centrifuged at 5000 × *g* for 15 min and the precipitate was dissolved in 0.5 M NaCl. Cold anhydrous ethanol (three volumes) was again added and the precipitate was gently washed thrice with 75% ethanol. The precipitate was lyophilized to obtain the crude bioflocculants. The partially purified bioflocculant solution (0.1%) was further purified using column chromatography [16] and lyophilized until further use. A working solution of 4 mg/mL in deionized water was prepared for experimental purposes.

### 2.3. Detecting antioxidant properties of polysaccharide-based bioflocculant

The antioxidant activities of purified PBB were evaluated using 2,2-diphenyl-1-picrylhydrazyl(DPPH), hydroxyl radicals, and superoxide radical scavenging assays. Ascorbic acid (Sigma-Aldrich) was employed as a positive control.

The DPPH-scavenging activities of PBB were determined following the method described by Chen et al. [17]. Briefly, 1 mL of PBB sample (appropriate dilution) was mixed with 1 mL of DPPH solution (Sigma-Aldrich, USA) (0.2 mM) and incubated in the dark for 30 min. Deionized water was used as a blank sample. The percentage of scavenged DPPH radicals was calculated as per Equation (1):

$$\text{Scavenging effect (\%)} = [1 - A_{517}\ (\text{sample}) / A_{517}\ (\text{blank})] \times 100 \quad (1)$$

The hydroxyl radical scavenging properties of PBB were quantified as previously described by Zhai et al. [18]. Briefly, 2 mL of PBS (pH 7.4), 1 mL of 2.5 mM of 1, 10-Phenanthroline (Sigma, USA), 1 mL of appropriately diluted PBB sample, and 1 mL of 2.5 mM $FeSO_4$ were combined. We then added 1 mL of 20 mM $H_2O_2$to initiate the reaction. The reaction mixture was incubated at 37 °C for 90 min.

Hydroxyl radical scavenging effect (%) was calculated according to Equation (2):

$$[\{A_{536}\ (\text{sample}) - A_{536}\ (\text{blank})\} / \{A_{536}\ (\text{control}) - A_{536}\ (\text{blank})\}] \times 100 \quad (2)$$

The superoxide radical scavenging assay was performed according to the method described by Li et al. [9]. Briefly, 2 mL of Tris-HCl buffer (pH 8.0, 150 mM), 1.0 mL of 1,2,3-phentriol (1.5 mM dissolved in 10 mM HCl), and 0.5 mL of PBB sample was added sequentially to a tube and combined thoroughly. The reaction tube was incubated at 25 °C for 30 min. The absorbance was measured at 325 nm and the quantity of superoxide radicals generated by 1,2,3-phentriol auto-oxidation was calculated according to Equation (3).

$$\text{Superoxide scavenging activity (\%)} = [1 - (A_{1} - A_{10}) / (A_{0} - A_{00})] \times 100 \quad (3)$$

Where, $A_{00}$ is the absorbance of the sample in the absence of PBB and 1,2,3-phentriol, $A_{01}$is the absorbance of samples containing1,2,3-phentriol in the absence of PBB, $A_{10}$is the absorbance of samples

containing PBB in the absence of 1,2,3-phentriol, and $A_{11}$is the absorbance of samples containing PBB and 1,2,3-phentriol.

### 2.4. Examining antibacterial activities of polysaccharide-based bioflocculant

The minimal inhibitory concentration (MIC) of PBB against various pathogenic bacterial strains was assessed using a broth micro-dilution method in 96-well plates (FALCON, USA) [19]. The bacterial strains (*Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes, Salmonella typhimurium,* and *Escherichia coli*) were inoculated in 3 mL of Mueller-Hinton (MH) broth and incubated overnight in a shaker incubator at 37 °C. Broth cultures were diluted two-fold in 3 mL of MH broth and incubated for 4 h at 37 °C. The first column on the plate contained only 0.1 mL of MH broth, which acted as a negative growth control. Columns 2–11 contained increasing concentrations of PBB (100 μg/mL-13,000 μg/mL). The dilutions of PBB were prepared at concentrations 2x higher than the desired final concentrations followed by addition of the same volume of inoculum (total volume 100 μL/well). The bacterial cell concentrations were adjusted to $10^6$ CFU/mL. Column 12 contained a positive growth control (without EPS), containing 50 μL of MH broth and 50 μL of bacterial inoculum. The plates were incubated overnight at 37 °C and MIC was determined. The OD was measured at 600 nm in a microplate reader (Molecular Devices, USA).

### 2.5. Macrophage cell culture and MTT assay

Isolation and culturing of head-kidney (HK) macrophages from *L. rohita* was carried out as previously described [20]. Cell number was adjusted to $1 \times 10^6$ viable cells/mL. Purified HK macrophages were used for the establishment of macrophage cell lines. Cells were maintained in an atmosphere of 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, NY, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 μg/mL penicillin.

Cell viability assays were performed in 96-well microplates using the MTT assay system (abcam, Cambridge, USA). Briefly, cells were seeded into plates at $1 \times 10^6$ cells/well and cultured with different concentrations of PBB (100 μg/mL, 200 μg/mL, 400 μg/mL, 600 μg/mL, or 800 μg/mL) for 24 h at 37 °C, 5% $CO_2$. Untreated cells were considered to be negative controls. Cells were then stimulated for 4 h with LPS (1 μg/mL) and 100 μL of MTT was then added into each well for an additional 4 h. The formazan product was solubilized by adding 150 μL of Dimethyl sulfoxide (DMSO) and 100 μL of 10% Sodium dodecyl sulfate (SDS) in 0.01 M HCl. Viability was estimated by measuring the absorbance at 570 nm using an ELISA plate reader.

### 2.6. Quantifying nitric oxide production

Production of nitric oxide (NO) was quantified by measuring the nitrite concentration using the Griess assay [21]. A microplate reader was employed to measure the absorbance at 550 nm. Nitrite concentrations were calculated from a sodium nitrite standard reference curve [22].

### 2.7. Effect of the polysaccharide-based bioflocculation the inflammatory response in HK macrophages

HK microphage cells were incubated with 100 μg/mL, 200 μg/mL, 400 μg/mL, or 600 μg/mL of PBB for 1 h at 26 °C, and stimulated with LPS (1 μg/mL; *Escherichia coli*0111: B4) for 24 h. The expression levels of TNF-α, IL-1β, IL-10, and TGF-β in the culture supernatants were measured by employing a fish specific ELISA kit (MyBioSource, San Diego, USA) according to manufacturer's instructions.

The mRNA expression levels of the inflammatory cytokines were measured by Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). Briefly, HK macrophages were stimulated with different concentrations of PBB for 1 h and treated with LPS for 24 h. Total RNA was extracted from the stimulated HK macrophages using TRIzol reagent (Invitrogen, USA). The extracted RNA was reverse transcribed to cDNA using a SuperScript® cDNA synthesis kit (Life Technologies), according to the manufacturer's instructions. qRT-qPCR analysis of IL-1β, IL-10, TNF-α, TGF-β, and β-actin was performed with CFX96™ Real-Time PCR (Bio-Rad Laboratories Inc., Hercules, CA, USA) following the standard protocols. The primer sequences and thermocycling conditions are mentioned in Table 1. All samples were run in triplicate. Melting curve analysis was performed after amplification to verify the accuracy of each amplicon. Gene expression results were analyzed using the $2^{-\Delta\Delta CT}$ method after verifying that the primers effectively amplified the target genes with an efficiency of approximately 100% [24].

### 2.8. Statistical analysis

Data were analyzed using one-way ANOVA. For comparison between two groups, Student's t-tests were used. The OriginPro (version 8; OriginLab Corporation, Northampton, USA) was used for all statistical analyses. Level of significance was set at $P < 0.05$, and results were expressed as mean ± standard deviation (SD).

## 3. Results

### 3.1. Polysaccharide-based bioflocculant exhibits significant antioxidant activities

The free radical scavenging capacity for PBB was measured using DPPH radical scavenging activity and was compared with that of the positive control, ascorbic acid (Table 2). Results revealed that PBB exhibited high capacity for scavenging radicals and this ability increased steadily with increasing PBB concentrations with 62.43% activity observed following treatment with 100 μg/mL and 81.46% with 800 μg/mL. However, the DPPH radical scavenging capacity of PBB was

**Table 1**
Real-time primer sequences and thermocycling conditions.

| Target Gene | Primer sequence (5′ to 3′) | Thermocycling condition | Reference |
|---|---|---|---|
| IL-1β | ATCTTGGAGAATGTGATCGAAGAG<br>GATACGTTTTTGATCCTCAAGTGTGAAG | 95 °C 30 s, 40 cycles of 95 °C 5 s, 60.5 °C 30 s and 72 °C 30 s | [23] |
| IL-10 | AAGGAGGCCAGTGGCTCTGT<br>CCTGAAGAAGAGGCTCTGT | 95 °C 30 s, 40 cycles of 95 °C 5 s, 60.5 °C 30 s and 72 °C 30 s | [23] |
| TNFα | CCAGGCTTTCACTTCACG<br>GCCATAGGAATCGGAGTAG | 95 °C 30 s, 40 cycles of 95 °C 5 s, 60.5 °C 30 s and 72 °C 30 s | [23] |
| TGF-β | ACGCTTTATTCCCAACCAAA<br>GAAATCCTTGCTCTGCCTCA | 95 °C 30 s, 40 cycles of 95 °C 5 s, 60.5 °C 30 s and 72 °C 30 s | [23] |
| β-actin | AGACCACCTTCAACTCCATCATG<br>TCCGATCCAGACAGAGTATTTACGC | 95 °C 30 s, 40 cycles of 95 °C 5 s, 60.5 °C 30 s and 72 °C 30 s | [23] |

**Table 2**
DPPH radical scavenging, hydroxyl radical scavenging, and superoxide radical scavenging activities (%) of various concentration (100–1000 μg/mL) of polysaccharide-bioflocculant and positive control vitamin C (ascorbic acid). Data are presented as mean ± S.E.M. One way ANOVA was used for data analysis (n = 3; P < 0.05). Data with superscript letter "a" denotes significant differences with correspond value of ascorbic acid.

| Material | Concentration (μg/mL) | DPPH radical scavenging | Hydroxyl radical scavenging | superoxide radical scavenging |
|---|---|---|---|---|
| Bioflocculant | 100 | 62.43 ± 2.63 | 37.56 ± 1.40[a] | 53.29 ± 1.8[a] |
| | 200 | 65.17 ± 1.57[a] | 42.92 ± 0.86[a] | 56.03 ± 1.27 |
| | 400 | 67.32 ± 1.82[a] | 52.33 ± 2.42[a] | 63.6 ± 2.14[a] |
| | 600 | 78.14 ± 2.46[a] | 57.82 ± 1.74[a] | 72.4 ± 1.63[a] |
| | 800 | 81.46 ± 1.37[a] | 66.34 ± 2.63[a] | 78.03 ± 2.46[a] |
| | 1000 | 83.72 ± 2.11[a] | 64.08 ± 1.86[a] | 81.74 ± 2.26[a] |
| Ascorbic acid | 100 | 65.8 ± 0.88 | 43.7 ± 2.16 | 48.1 ± 1.73 |
| | 200 | 69.04 ± 1.32 | 49.4 ± 0.86 | 57.3 ± 1.12 |
| | 400 | 74.11 ± 1.68 | 61.02 ± 3.26 | 69.9 ± 3.17 |
| | 600 | 84.3 ± 1.14 | 68.6 ± 1.53 | 80.2 ± 2.19 |
| | 800 | 90.62 ± 2.13 | 72.1 ± 2.74 | 88.5 ± 2.46 |
| | 1000 | 91.84 ± 1.86 | 75.6 ± 1.36 | 92.3 ± 1.34 |

found to be lower ($p < 0.05$) than the positive control, except 100 μg/mL PBB. In fact, the DPPH radical scavenging capacity of ascorbic acid was seen to increase steadily up to 800 μg/mL at which point it began to plateau. Further, the scavenging activity of 1000 μg/mL of PBB (83.72 ± 2.11%) was similar to that of 600 μg/mL of ascorbic acid (84.3 ± 1.14%).

PBB also exhibited concentration-dependent scavenging activities against hydroxyl radicals (Table 2). This specific scavenging activity of PBB was 37.56 ± 1.4% at the concentration 100 μg/mL and increased to 66.34 ± 2.63% when the concentration reached 800 μg/mL. Alternatively, ascorbic acid exhibited 43.7 ± 2.16% to 72.1 ± 2.74% hydroxyl radical scavenging capacity at concentrations of 100 μg/mL and 800 μg/mL, respectively. These values obtained for the positive control were determined to be significantly higher than corresponding values in PBB. Moreover, the hydroxyl scavenging activity exhibited by 800 μg/mL of PBB (66.34 ± 2.63%) was slightly lower than that observed for 600 μg/mL of ascorbic acid (68.6 ± 1.53%).

The scavenging activities of PBB and ascorbic acid against superoxide radicals are presented in Table 2. The superoxide radical scavenging capacity of PBB was determined to be 53.29 ± 1.8% at 100 μg/mL and increased to 81.74 ± 2.26% when the concentration reached 1000 μg/mL. Alternatively, at the same concentrations of ascorbic acid, 48.1 ± 1.73% and 92.3 ± 1.34% superoxide radical scavenging was observed. Interestingly, the superoxide radical scavenging activity observed at 1000 μg/mL of PBB (81.74 ± 2.26%) was similar to that of 600 μg/mL of ascorbic acid (80.2 ± 2.19%).

### 3.2. Antibacterial properties of polysaccharide-based bioflocculant

PBB exhibited antibacterial properties against all of the tested bacterial strains as revealed by MIC values ranging from 3 mg/mL to 9.5 mg/mL (Table 3). Our study found that *E. coli*, *S. aureus*, and *S. typhimurium* were significantly inhibited by PBB concentrations of 3 mg/mL, 4.5 mg/mL, and 7 mg/mL, respectively. However, *L. monocytogenes* was only inhibited following treatment with PBB at a concentration of 9.5 mg/mL.

### 3.3. Cell viability and nitric oxide production

The effect that PBB elicited on cell viability is shown in Fig. 1. PBB concentrations between 100 μg/mL and 800 μg/mL demonstrated no significant effect on cell viability. Within the control, cell viability was found to be 99.4%, whereas the viability in cultures exposed to 800 μg/mL of PBB was 98.2%. Results from the MTT assay confirmed that the observed inhibitory effect elicited by PBB was not associated with cytotoxicity.

To determine if PBB exhibited anti-inflammatory functions, we analyzed NO production by LPS-stimulated macrophages. We found that PBB functioned to attenuate the LPS-induced NO production in a concentration-dependent manner (Fig. 2). At a concentration of 600 μg/mL PBB exhibited maximum inhibition of LPS-induced NO production (approximately 60% inhibition compared to the control).

### 3.4. Effect of PBB on inflammatory cytokine production in LPS-stimulated carp head-kidney macrophages

To investigate the anti-inflammatory effect exhibited by PBB on LPS-stimulated macrophages, the production of pro-inflammatory and anti-inflammatory cytokines were evaluated. Cell supernatants were analyzed by ELISA to quantify cytokine protein secretion, and qRT-PCR analysis was performed to examine the transcriptional response (Fig. 3). Our results show that the levels of TNF-α and IL-1β increased in LPS-stimulated macrophage cultures; however, pretreatment with PBB acted to significantly prevent this over expression of TNF-α and IL-1β in a concentration-dependent manner (Fig. 3A–D). Alternatively, the expression level of IL-10 and TGF-β were found to be slightly increased in the LPS-stimulated macrophage culture media, however, these levels were further augmented by pretreatment with PBB (Fig. 3E–H). Overall, PBB pretreatment inhibited the expression of pro-inflammatory cytokines while enhancing the expression of anti-inflammatory cytokines (Fig. 3).

## 4. Discussion

EPSs are primarily produced by the metabolic pathways of microorganisms [19] and are largely polyanionic since they are composed of sulfate, pyruvate, phosphate and uronic acids. The physical properties of EPSs are affected by monosaccharide configuration as well as the aggregation of polymer chains [25]. In the present study, we

**Table 3**
Antimicrobial activity of polysaccharide-bioflocculant (PB) against pathogenic bacterial strains.

| Bacterial strains | MIC (mg/mL) of PB |
|---|---|
| *Bacillus cereus* MTCC 6629 | 6 |
| *Staphylococcus aureus* MTCC 737 | 4.5 |
| *Listeria monocytogenes* 1143 | 9.5 |
| *Salmonella typhimurium* ATCC14028 | 7 |
| *Escherichia coli* MTCC 443 | 3 |

PB exhibited antibacterial activities against all the test bacterial strains as revealed by MIC values ranging from 3 mg/mL to 11 mg/mL (Table 3). Our study found that *E. coli*, *S. aureus*, and *S. typhimurium* were inhibited with concentration of 3, 4, and 7 mg/mL, respectively. However, *L. monocytogenes* was inhibited with a PB concentration of 11 mg/mL.

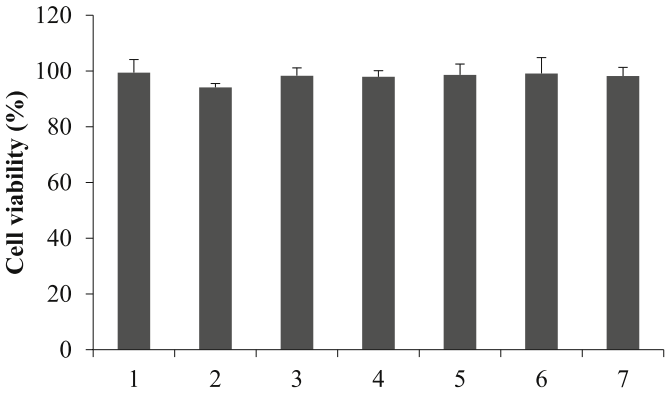


**Fig. 1.** Effects of PBB on cell viability in carp macrophages. Cells were pretreated with PBB for 1 h, and then stimulated with LPS (1 μg/mL). Cell viability was assayed by MTT assay. Data are presented as mean ± SEM.

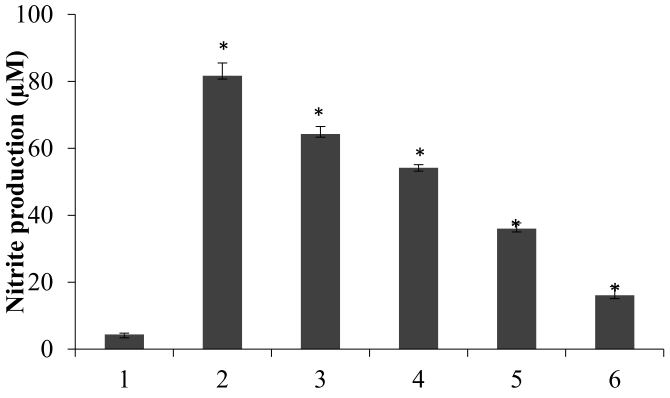


**Fig. 2.** Effect of PBB on LPS-induced NO production in carp macrophages. Data are presented as mean ± SEM ($n = 3$).

investigated the antioxidant properties of PBB using three different methods. DPPH free radical is a stable radical containing an unpaired valence electron on one of the nitrogen bridge atoms. These free radicals must scavenge to donate an electron or an active hydrogen atom from surrounding antioxidant compounds [26]. In the present study, the scavenging activity exhibited by 1000 μg/mL of PPB (83.72 ± 2.11%) was similar to that of 600 μg/mL of the positive control, ascorbic acid (84.3 ± 1.14%). Furthermore, the DPPH scavenging activity of PBB was found to be higher than that of the EPS isolated from *B. subtilis*, which exhibited 61.9% DPPH antioxidant activity at a concentration 800 μg/mL [27]. However, EPS isolated from *B. amyloliquefaciens* exhibited a higher DPPH radical scavenging activity (99.39 ± 0.63%) at 1000 μg/mL [28].

Hydroxyl radicals function as a strong oxidant that can react with almost all biological molecules, including proteins, lipids, and carbohydrates and leads to biological damage [29]. In addition, free radical ·OH can function to accelerate aging by oxidizing most biomacromolecules, thereby leading to the development of related oxidative stress disease [30]. Thus, eliminating ·OH is vital for protection of cells and food systems against oxidative stress. In our study, the purified PBB demonstrated an upward trend for hydroxyl radical scavenging with increasing concentrations. Specifically, at 800 μg/mL, 66.34 ± 2.63% scavenging was observed, which was similar to that of 600 μg/mL of ascorbic acid (68.6 ± 1.53%). This scavenging activity was higher than that exhibited by EPS from *Bifidobacterium bifidum* or *Lactobacillus plantarum* [9]. However, EPS from *Bacillus* sp. S-1 elicited higher hydroxyl radical scavenging than PBB [10]. In agreement with the results of the present study, EPS extracted from *B. amyloliquefaciens* GSBa-1 [4] and from *B. amyloliquefaciens* 3MS 2017 [28] exhibited strong hydroxyl radical scavenging capacity. The antioxidant mechanism of PBB may involve the electrons or hydrogen atoms from polysaccharide molecules chelating with $Fe^{2+}$ thereby preventing radical chain reactions [31].

Superoxide radicals are precursors for most ROS including singlet oxygen, hydrogen peroxide, and hydroxyl radicals, all of which induce tissue damage or cell death [9,29]. Although the superoxide scavenging activity of PBB (81.74 ± 2.26% at 1000 μg/mL) was found to be significantly lower than that of the same concentration of ascorbic acid (92.3 ± 1.34%), and EPS from *B. amyloliquefaciens* 3MS 2017 exhibited higher superoxide radical scavenging activity (91.44 ± 2.51% at 1000 μg/mL) [28]. It was higher than that exhibited by EPSs from *B. amyloliquefaciens* GSBa-1 (44.86% at 5000 μg/mL) [4], EPS from *Bacillus* sp. (68.43 ± 3.21% at 7000 μg/mL) [10], EPS from *Bifidobacterium bifidum* (59.75 ± 8.12% at 1000 μg/mL) or *Lactobacillus plantarum* (65.45 ± 8.48% at 1000 μg/mL) [9]. The presence of more electron withdrawing groups on polysaccharides results in a weaker dissociation energy of the O–H bond; and the presence of only a few electrophilic groups, such as aldehyde or keto, in PBB may account for its relatively lower scavenging activity compared to that of ascorbic acid [32]. Further, the pH of the culture media was 7.0 and while, PBB has demonstrated optimal activity at neutral pH [16], it has been previously reported that acidic polysaccharides exhibit stronger superoxide anion scavenging activity compared to neutral polysaccharides [33]. Nevertheless, overall, PBB in our study, exhibited strong antioxidant properties. The presence of reducing sugars, monosaccharides, proteins, amino acids, uronic acids and other organic molecules may have contributed to the observed scavenging activities [27].

In addition to its antioxidant properties, purified PBB also exhibited antimicrobial activity against various Gram-positive and Gram-negative bacteria, which agrees with results from an earlier study that demonstrated inhibitory activity in EPS from probiotic *L. plantarm* for the growth of various food-borne pathogenic bacteria [19]. Similarly, EPS produced by *L. kefiranofaciens* DN1 was seen to inhibit the growth of various food-borne bacterial pathogens [34]. Moreover, EPS from *B. bifidum* and *L. plantarum* exhibited antibacterial effects against various bacterial pathogens such as *Listeria monocytogens* CMCC54007, *Staphylococcus aureus* CGMCC26003, *Bacillus cereus* ATCC14579, *Salmonella typhimurium* ATCC13311, *Shigella sonnei* ATCC25931, and *E. coli* O157:H7 [9]. Thus, further exploitation of PBB may serve to further elucidate the mechanism responsible for its observed antimicrobial activity against pathogenic bacteria.

Macrophages play a vital role in acute and chronic inflammatory responses [35]. To determine whether PBB exhibit cytotoxic effects, isolated carp HK macrophage cells were incubated with or without PBB pretreatment (Fig. 1). We found that at concentrations between 100 μg/mL-800 μg/mL PBB did not significantly affect cell viability. Following 24 h of treatment, cell viability was determined to be 98.2% in cultures with 800 μg/mL of PBB. This negligible cytotoxic effect confirms the possible use of this PBB for biological uses. Similarly, in a recent study, bioflocculants isolated from *P. jamilae* were also described as being non-cytotoxic [13]. In accordance with ISO-1009935, 2009, cell viability above 80% is considered to be non-toxic [36].

Further, PBB functioned to attenuate the LPS-induced NO production in a concentration-dependent manner (Fig. 2). NO is a signaling molecule that has important functions in the pathogenesis of inflammation. It appears as an anti-inflammatory in normal state, however NO functions as a pro-inflammatory mediator thereby promoting inflammation through its overproduction in abnormal physiological states [28]. Therefore, NO inhibitors have been shown to play a vital role in controlling inflammatory diseases [37]. Our results show that 600 μg/mL of PBB resulted in maximum inhibition of LPS-induced NO production, which was in agreement with a previous study, that reported on bioflocculant isolated from *Paenibacillus jamilae*having the ability to effectively reduce the level of LPS-induced NO production in peripheral blood mononuclear cells [13].

Inflammatory cytokines, particularly TNF-α, IL-1β, and IL-6 play key roles in the induction and perpetuation of inflammation caused by

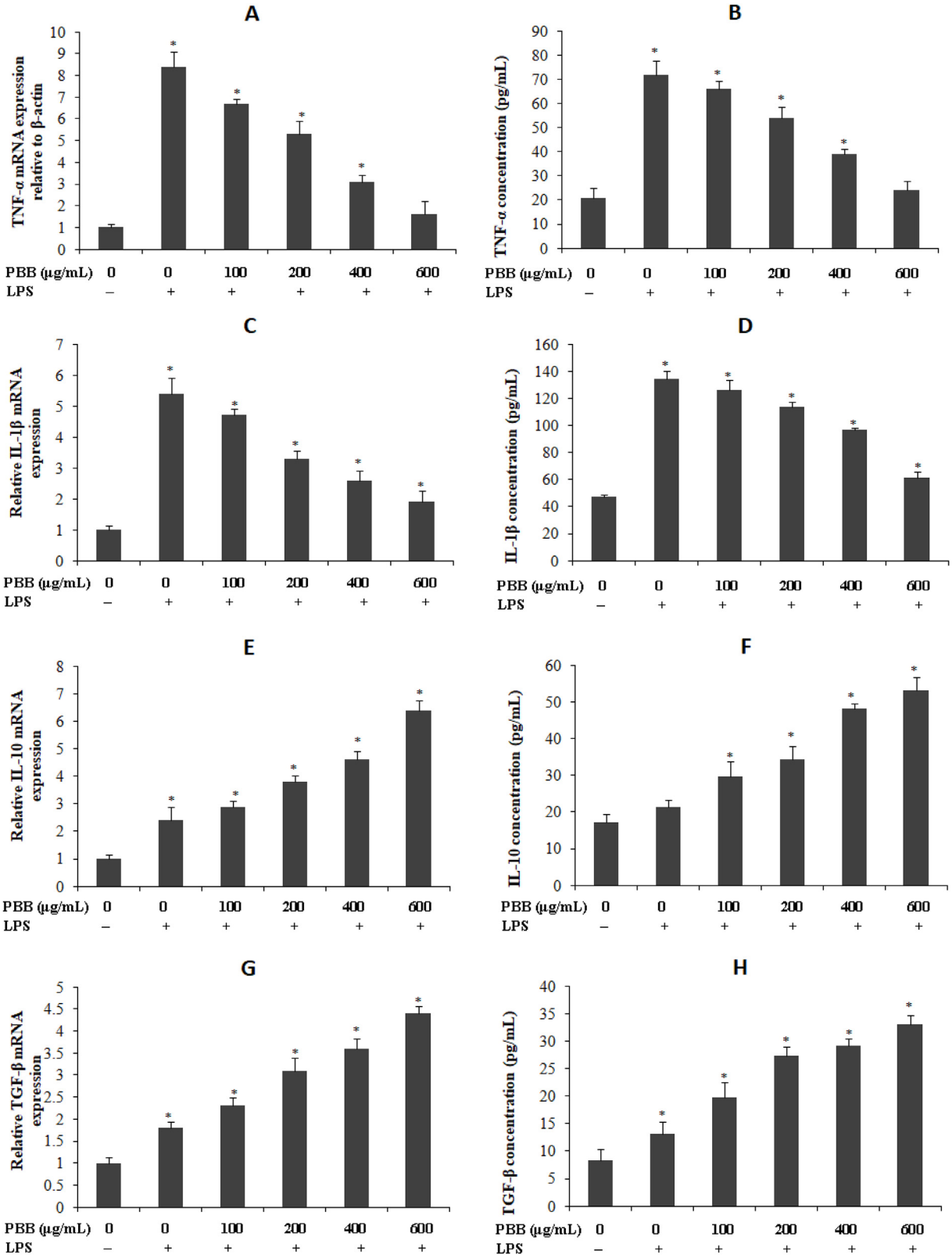


**Fig. 3.** Effect of PBB on the production of TNF-α, IL-1β, IL-10, and TGF-β in LPS-stimulated carp macrophage cells. Levels of cytokines were measured by ELISA. The mRNA levels of cytokines were measured by RT-PCR. The figures show the representative results of three independent experiments and data are presented as mean ± S.E.M.

macrophages. Elevated levels of TNF-α has the potential to cause tissue damage or even sepsis and death [38]. High doses of LPS (μg/mL) have been shown to induce strong inflammatory responses in fish. Furthermore, LPS is a strong stimulator of macrophages and activates several signal transduction pathways producing various proinflammatory cytokines in humans and animals, including fish [20,39].

Our results demonstrated that treatment with PBB served to significantly reduce the expression of TNF-α and IL-1β at the mRNA level, which was confirmed by quantifying the corresponding protein levels via ELISA. Conversely, PBB was found to significantly augment the production of specific anti-inflammatory cytokines, namely, TGF-β and IL-10 compared to the control group (Fig. 3). IL-10 elicits anti-inflammatory effects, which contributes to its role in inhibiting monocyte/macrophages to secrete inflammatory mediators such as TNF-α, IL-1β, and IL-6 [40]. These findings suggest that PBB may suppress the production of pro-inflammatory cytokines, however the detailed mechanisms need to be further elucidated. EPS from *B. amyloliquefaciens* 3MS2017 exhibited anti-inflammatory activity [28]. Previously, bioflocculant from *Paenibacillus jamilae* was shown to exhibit anti-inflammatory activity through the inhibition of LPS-induced TNF-α and IL-1β production in PBMCs, and through enhanced production of IL-10 and TGF-β [13].

## 5. Conclusions

The PBB extracted from *B. subtilis* F9 was characterized for antioxidant and anti-inflammatory properties. PBB exhibited potent scavenging capacity against DPPH, hydroxyl, and superoxide radicals, which suggests that it has the potential to be exploited further as a natural source of antioxidants for nutraceuticals or functional food development. It also elicited strong antibacterial properties against multiple pathogenic bacteria; and acted to inhibit the pro-inflammatory response while increasing the anti-inflammatory response of LPS-stimulated macrophages. Although, PBB exhibited potential beneficial biological activities, further studies are required to determine its structure-function relationship and better understand the antioxidant, anti-inflammatory, and antibacterial nature of *B. subtilis* F9 PBB. Also, scale up of bioflocculant production and economic production is warranted for its viable application.

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## Conflicts of interest

There is no conflict of interest to declare.

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