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*Full Length Research Paper*

# Production and characterization of heavy-metal removing bacterial bioflocculants

**J. Lin* and C. Harichund**

School of School of Life Sciences,University of KwaZulu-Natal (Westville)
Private Bag X 54001, Durban, Republic of South Africa.

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**Bioflocculants produced by *Herbaspirillium* sp. CH7, *Paenibacillus* sp. CH11, *Bacillus* sp. CH15 and *Halomonas* sp. are potential flocculanting agents in the treatment of industrial wastewater effluents. Up to 250% increases in bioflocculant production were achieved by manipulation of the media composition. Increases in peptone and glycerol contents, up to 2 and 3%, respectively enhanced the bioflocculants production. With the exception of *Herbaspirillium* sp. CH7, all isolates preferred higher yeast extract content for bioflocculant production. Physicochemical analysis showed most of bioflocculants produced appeared to contain high protein content, then carbohydrate. All bioflocculants contained similar hexosamine and uronic acid contents in the range of 0.0115 to 0.0150 mM and 0.0054 to 0.0068 mM, respectively. Purified bioflocculants produced under optimized conditions possessed higher flocculating activities (up to 14-fold increases) in comparison to those under the control conditions with the exception of that from *Herbaspirillium* sp. CH7. A decrease in bioflocculant concentration from 10000 ppm to 1 ppm resulted in a significant increase in the removal percentages of $Pb^{+2}$, $Zn^{+2}$ and $Hg^{+2}$ with the optimal dosage of 1 to10 ppm. Bioflocculants in this study removed $Cd^{+2}$ effectively only at 10000 ppm, but not at the lower concentrations. This $Cd^{+2}$-removing capacity of bioflocculant could be further stimulated by an increase in temperature. The pH requirement for maximum flocculating activity seems to be varied for different strains of microorganisms.**

**Key words:** Bioflocculant, heavy metal removal, physiochemical properties, pH, temperature.

## INTRODUCTION

Flocculation has been applied to a wide range of fields, such as dredging, wastewater treatment and fermentation. Due to their economic advantage and potency, synthetic organic and inorganic flocculants are widely used. However, serious environmental pollutions caused by these materials have been reported in the literature (Nakata and Kurane, 1999; Ahluwalia and Goyal, 2007; Chakraboti et al., 2008). Bioflocculants as an alternative choice have gained more attention recently since they are environmentally friendly, biodegradable and nontoxic (Salehizadeh and Shojaosadati, 2001).

Bioflocculants have been used to flocculate inorganic solid suspensions (Natarajan and Das, 2003; Lu et al., 2005; Yim et al., 2007; Zhang et al., 2007), humic acids (Zouboulis et al., 2004), dyes solution (Deng et al., 2005; Gao et al., 2009; Liu et al., 2009), and others (Salehizadeh and Shojaosadati, 2003; Deng et al., 2003; Vijayalakshmi and Raichur, 2003; Lu et al., 2005; Aguilera et al., 2008). A number of studies have also demonstrated the potential for the use of bioflocculants in heavy metal removal (Quintelas et al., 2008; Salehizadeh and Shojaosadati, 2003; Wu and Ye, 2007; Gong et al., 2008). Gong et al. (2008) demonstrated that bioflocculant produced by *Serratia ficaria* was more effective in removing chemical oxygen demand and the color of pulp effluent than traditional chemical flocculants. Lin and Harichund (2011a) have previously demonstrated the ability of bacterial bioflocculants simultaneously in

*Corresponding author. E-mail: linj@ukzn.ac.za. Tel: +27-31-2607407. Fax: +27-31-2607809.

**Abbreviations: CPC,** Cetyl pyridinium chloride; **NRF,** national research foundation

removing bacterial populations, heavy metals and turbidity from 3 industrial effluents.

A number of bioflocculant-producing bacteria have been discovered (Jiyoung et al., 2007). Wu and Ye (2007) conclude that microbial bioflocculant are largely composed of high molecular weight biopolymers such as polysaccharide flocculants (*Alcaligenes latus* B-18, *Alcaligenes cupus* KT201 and *Bacillus* sp. Dp-152), glycoprotein flocculants (*Arthrobacter* sp.) and protein flocculants (*Rhodococcus ertyropolis* and *Bacillus* sp. Dp-152). Kumar et al. (2004) reported that the molecular weight and functional groups in the molecular chains of bioflocculant are important determinants for the flocculating activity. However, the high production costs and low yield incurred to produce bioflocculants have been the rate-limiting steps for their applications (Smith and Miettinen, 2006). Research is needed to identify and select new bioflocculant-producing microorganisms and to learn how to optimize fermentation conditions to enhance bioflocculant yields (He et al., 2002; Yang et al., 2007; Wang et al., 2007). The purpose of this present study was to investigate bioflocculant production by selected microorganisms. In addition, the physic-chemical properties of purified bioflocculants were characterized as well as their flocculating properties in removing heavy metals and solid suspension.

## MATERIALS AND METHODS

### Bacterial strains and media

Bioflocculant-producing strains of *Pseudomonas* sp. CH6, *Bacillus* sp. CH15, *Herbaspirillium* sp. CH7, and *Paenibacillus* sp. CH11 isolated previously from an industrial effluent sample containing heavy metals in a previous study (Lin and Harichund, 2011a) and *Halomonas* sp. obtained from the stock cultures of the Department of Microbiology at UKZN-Westville were used in this study. Pure cultures were maintained on YMPG agar containing 0.3% Yeast extract, 0.3% malt extract, 0.5% peptone, 1% D-glucose and 2% bacterio-logical agar at pH 7 (Nakata and Kurane, 1999). Stock cultures were subjected to cryopreservation for long-term storage. All assays were conducted in triplicate and the values were presented as mean ± standard deviation.

### Production of bacterial bioflocculants

Isolates were cultivated in 250 ml Erlenmeyer flasks containing 30 ml YMPG media for 20 h at 28°C at 220 rpm (Nakata and Kurane, 1999). Aliquots (0.7 ml) of cultivated bacterial isolates were then inoculated into a 250 ml Erlenmeyer flask containing 70 ml of production medium (0.5% yeast extract, 0.5% polypeptone, 2% ethanol, 1% glycerol, 0.05% $K_2HPO_4$, 0.05% $MgSO_4$. $7H_2O$, 0.2% NaCl, and 0.2% $CaCO_3$) (Nakata and Kurane, 1999). The flasks were incubated for three days at 28°C at 220 rpm. To determine the optimal production of bioflocculants of each isolate, the composition of production medium was modified with various concentrations of carbon sources (ethanol and glycerol), nitrogen sources (peptone and yeast extract) and NaCl as shown in Table 1.

30 ml of the cell-free supernatant, obtained after centrifugation (6000 × *g* for 15 min), was mixed with 60 ml of ethanol and the mixture was stored overnight at 4°C for precipitation of bioflocculants. Bioflocculant pellets were recovered by centrifugation at 6000×*g* for 15 min. Bioflocculant pellets were dried in a desiccator containing anhydrous cobalt chloride under reduced pressure at room temperature (~25°C) and the dry mass of bioflocculants was obtained. Recovered bacterial cells were re-suspended in 5 ml 0.9% NaCl and filtered using No. 1 filter paper (Whatman). Cells present on the filter paper were dried at 110°C in an oven. The cell mass was recorded after the weight of the dry cell did not change during consecutive measurements (Nakata and Kurane, 1999).

### Purification of bacterial bioflocculants

Bioflocculants were further purified using the method of Xia et al. (2008). Dried bioflocculants were resuspended in sterile distilled water (50 ml) followed by the addition of 25 ml of 2% cetyl pyridinium chloride solution (CPC) with constant stirring for 2 h. The bioflocculant and CPC complex was obtained by centrifugation at 4000 × *g* for 30 min, was resuspended in 50 ml of 0.5 M NaCl. Two volumes (100 ml) of cold ethanol were added to the mixture. Purified bioflocculants were obtained through centrifugal separation as described above. Precipitated bioflocculants were washed twice with 100% ethanol and the precipitate, vacuum dried in a dessicator as described above. After purification of the bacterial bioflocculants, the mass of the purified bioflocculant was measured. The bioflocculants were standardized to 10000 ppm as a stock solution and were used throughout the study.

### Flocculating activity of purified bacterial bioflocculants

Flocculating activity of the bioflocculants was determined using the method of Nakata and Kurane (1999) using kaolin clay. One ml of bacterial bioflocculant (1000 ppm) was mixed with 9 ml of kaolin solution (5.5 g/l) and 100 µl of $CaCl_2.2H_2O$ (30 g/l). The mixture was vortexed for 30 s and rested for 5 min. The upper layer of the solution (3 ml) was then removed for further analyses. The flocculating activity was calculated based on $OD_{550nm}$ as described by Kurane et al. (1994). The turbidity of

**Table 1.** Media composition for optimal bioflocculant production by selected bacterial isolates

| Isolate | Media composition | | | | | Bioflocculant production | | |
|---|---|---|---|---|---|---|---|---|
| | Ethanol | Glycerol | Yeast extract | Peptone | NaCl | Standard concentration (g/L) | Optimal concentration (g/L) | Ratio increase (%) |
| *Pseudomonas* sp. CH6 | 2% | 3% | 2% | 1% | 0.3% | 0.715 | 1.43 | 200 |
| *Herbaspirillium* sp. CH7 | 2% | 2% | 0.5% | 1% | 0.5% | 0.571 | 1.00 | 175 |
| *Paenibacillus* sp. CH11 | 2% | 3% | 2% | 2% | 0.4% | 0.857 | 1.86 | 217 |
| *Bacillus* sp. CH15 | 2% | 3% | 1% | 2% | 0.5% | 0.571 | 1.42 | 250 |
| *Halomonas* sp. | 2% | 2% | 1% | 1% | 0.3% | 1.714 | 3.57 | 208 |

Standard controlled conditions. 2% ethanol, 1% glycerol, 0.5% yeast extract, 0.5% peptone, 0.2% NaCl, 0.05% $K_2HPO_4$, 0.05% $MgSO_4.7H_2O$ and 0.2% $CaCO_3$ @ 28°C, 220 rpm for 3 days.

samples (A) and control (B) was measured at 550 nm using a spectrophotometer (Varian Cary 50 UV-Visible Spectrophotometer). The control experiment was performed using 1 ml of water (Millipore Elix purification system, 17 megaΩ) without bioflocculant. The flocculating activity and removal rate was determined using the method of Nakata and Kurane (1999).

### Heavy metal removal efficiency of purified bacterial bioflocculants

Heavy metal removal efficiency of bacterial bioflocculants was determined using the modified method of Nakata and Kurane (1999) as described by Wu and Ye (2007) using heavy metal solutions without kaolin clay. Different bioflocculant concentrations ranging from 1 ppm to 10000 ppm were suspended in the various concentrations of heavy metal solutions (0.5, 1 and 10 ppm, respectively) for 5 min. The heavy metal concentrations of the upper layer of the solution (3 ml), as described above, were also measured using ICP-OES.

### Composition analysis of purified bacterial bioflocculants

The composition of each purified bioflocculant was determined using a variety of analyses. The carbohydrate, uronic acid and hexosomine contents of bioflocculants were determined using the phenol-sulfuric acid, carbozole assay and Morgan-Elson assays, respectively (Chaplin, 1994). The protein composition of bioflocculants was determined using the Folin-Lowry method (Plummer, 1978).

### Effect of pH and temperature on heavy metal removal of purified bioflocculants

The effect of pH and temperature on heavy metal removal by bioflocculants was assayed under the specific concentrations of heavy metal and bioflocculant for which the optimal removal has been achieved. A pH range of 3 to 9 was chosen to assay the effect of pH on removal of heavy metals. A temperature range of 4 to 45°C was selected to determine the effect of temperature on heavy metal removal using bioflocculants.

### Statistical analysis

The experiments were conducted in triplicate and the results were expressed as mean ± S.D. Paired *t*-tests, using SPSS version 15, were used to examine the statistical significance between different treatments. Probability was set at 0.05 as significance.

## RESULTS

### Optimization of bioflocculants productions

Experiments were conducted to optimize bioflocculant production, which involved the variation of carbon source concentration (ethanol and glycerol), nitrogen source concentration (yeast extract and peptone) and NaCl concentrations. Table 1 shows the media composition for the optimal bioflocculant production by selected bacterial isolates. By modifying carbon, nitrogen and NaCl compositions, bacterial bioflocculant production was increased by 175 to 250% (Table 1). The different bacterial isolates had different combination preferences for their optimal bioflocculant production. All isolates produced the highest amounts of bioflocculants using the same ethanol content (2%) as in the controlled conditions. Most of these isolates preferred higher contents of glycerol, yeast extract, peptone and NaCl in order to achieve a better production compared to the corresponding compositions under the controlled conditions. The highest amount of bioflocculant production prior to and after optimization was observed for *Halomonas* sp. (Table 1). No trend was observed between accumulated cell mass and bioflocculant productions (data not

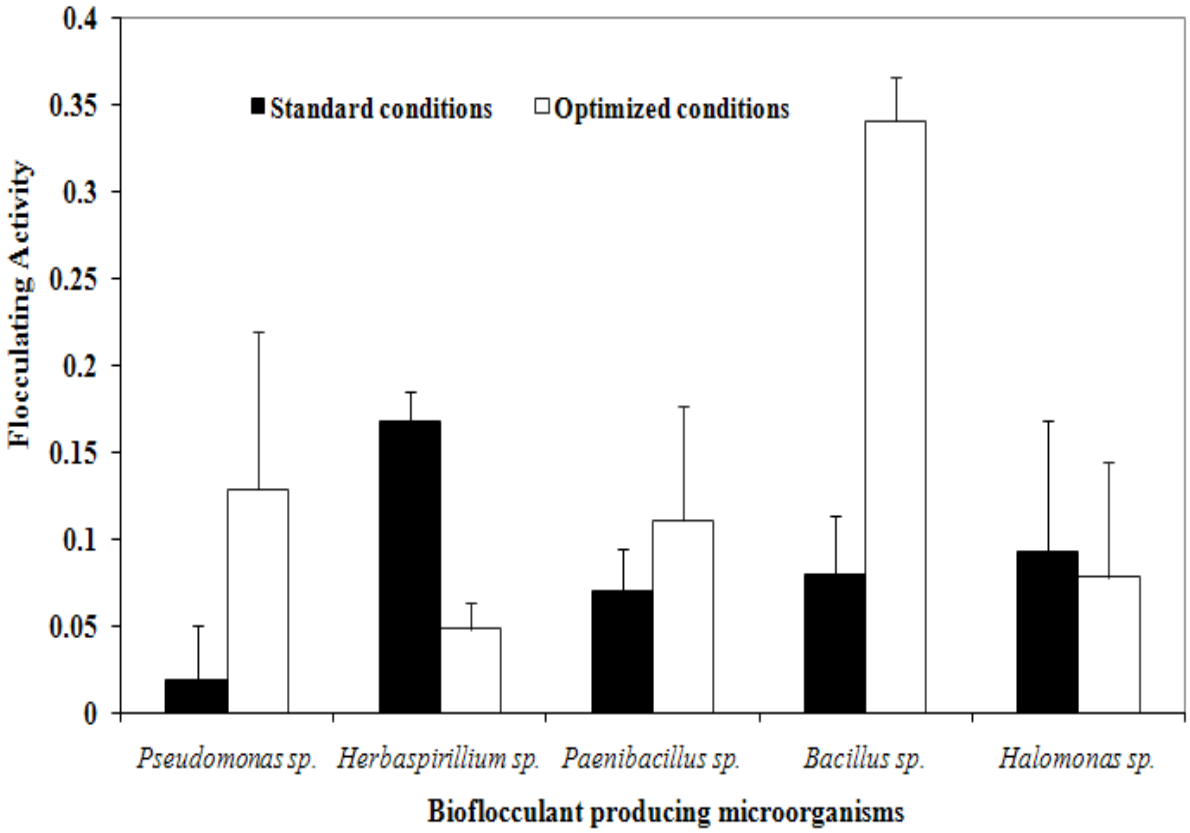


**Figure 1.** Flocculating activity of purified bacterial bioflocculants (1000 ppm) obtained under the standard and optimized production conditions.

**Table 2.** Physicochemical analysis of partially purified bacterial bioflocculants.

| Isolate | Carbohydrate (mM) | Protein (mM) | Hexosamine (mM) | Uronic acid (mM) |
|---|---|---|---|---|
| *Pseudomonas* sp. CH6 | 0.1755 | 0.1775 | 0.0134 | 0.0057 |
| *Herbaspirillium* sp. CH7 | 0.1995 | 0.1305 | 0.0115 | 0.0054 |
| *Paenibacillus* sp. CH11 | 0.2741 | 1.1012 | 0.0145 | 0.0057 |
| *Bacillus* sp. CH15 | 0.3446 | 1.5780 | 0.0150 | 0.0060 |
| *Halomonas* sp. | 0.0054 | 0.1775 | 0.0119 | 0.0068 |

shown).

## Flocculating activity of crude and purified bioflocculants

Crude bacterial bioflocculants produced under the controlled and optimal conditions were further purified using 2% CPC. The flocculating activities of purified bioflocculants from both conditions are shown in Figure 1. Flocculating activities of bioflocculants produced by *Pseudomonas* sp. CH6 and *Bacillus* sp. CH15 were increased, but not significant ($p>0.05$), up by 1400 and 350%, respectively while that of *Paenibacillus* sp. CH11 increased by 140% (Figure 1). However, the flocculating activity decreased from 0.168 to 0.049 of the purified bioflocculant of *Herbaspirillium* sp. under controlled conditions to those for optimal conditions (Figure 1).

## Physicochemical analysis of purified bacterial bioflocculants

Physicochemical analyses of purified bioflocculants were conducted to determine the composition of the bacterial bioflocculants (Table 2). Bioflocculants produced by *Paenibacillus* sp. CH11, *Bacillus* sp. CH15 and *Halomonas* sp. comprised of protein as the major component while that of *Pseudomonas* sp. CH6 contained carbohydrate and protein in almost equal

amounts. *Herbaspirillium* sp. CH7 differed in that it possessed more carbohydrate content. All bacterial bioflocculants in this study had similar levels of uronic acid and hexosamine conposition. Bioflocculants produced were found to have the uronic acid and hexosamine contents in the range of 0.0115 to 0.0150 mM and 0.0054-0.0068 mM, respectively (Table 2).

### Effect of purified bioflocculant concentrations in heavy metal removal

The effect of purified bioflocculant concentrations on heavy metal removal is presented in Figure 2. In general, decreases in bioflocculant concentration resulted in a significant increase in the percentages of metal removal except in the case of cadmium. As shown in Figure 2a, bioflocculant produced by *Pseudomonas* sp. dramatically increased the efficiency of $Zn^{+2}$ removals from 24 to 46% as the concentration dropped from 10000 to 1000 ppm, respectively and slowly increased the efficiency as the concentration continued to decrease. The same trends were observed when removing $Pb^{+2}$ and $Hg^{+2}$ using the same bioflocculant (Figure 2b and 2c) as well as the bioflocculants produced by other bioflocculant-producing bacteria in this study. Metal-removing capacity of the biofloculant produced by *Halomonas* sp. was more sensitive to changes in concentrations, and $Hg^{+2}$ in particular (Figure 2c). Eighty percentages of mercury (1 ppm) was efficiently removed by one ppm of the bioflocculant from *Herbaspirillium* sp as shown in Figure 2c. However, a reverse trend was observed when these bioflocculants were used to remove $Cd^{+2}$. The $Cd^{+2}$ removal efficiency of *Pseudomonas'* bioflocculant was significantly decreased from 66 to 19% while that of the *Herbaspirillium sp.* bioflocculant decreased from 66 to 10% when the bioflocculant concentration was reduced from 10000 to 1000 ppm, respectively (Figure 2d). Bioflocculant produced by *Halomonas sp.* had a lower $Cd^{+2}$ removal capacity compared to other isolates in this study.

### Effects of pH and temperature on heavy metal removal using bacterial bioflocculants

The effect of pH on heavy metal removal efficiency of purified bioflocculants at the optimal removal concentrations is demonstrated in Figure 3. In general, the bacterial bioflocculants in this study had better heavy-metal removing capacity for all metals tested at pH 5 and 7 (Figure 3). At pH 3, the efficiency of all bioflocculants for binding $Zn^{+2}$ decreased from 40 and 50% to 10 and 20%. The efficiency in binding Zn was dropped from around 46% to 10 and 20% when the pH was adjusted to 3 for all bioflocculants. At pH 9 and 10 and 10000 ppm, binding efficiencies of $Cd^{+2}$ and $Hg^{+2}$ decreased in pH 9 for all tested bioflocculants with the exception of *Bacillus* sp. (Figure 3c and 3d). Bioflocculant produced by *Paenibacillus* sp. removed only 5% of $Cd^{+2}$ at pH9 compared to 87% at pH3. However, bioflocculant produced by *Bacillus* sp. was capable of removing 64% of $Cd^{+2}$ at pH7, compared to 4.4% at pH 3. Changes in pH had less impact in removing $Pb^{+2}$ using bioflocculants (Figure 3b). The results in Figure 4 showed the percentage of heavy metal removal at different temperatures (4 and 45°C) by *Paenibacillus* sp. CH11 biofloculant at the optimal removal concentrations observed in Figure 2. No trend was observed in the relationship between temperature and heavy metal-removing ability of this bioflocculant at low concentrations in the case of $Zn^{+2}$, $Pb^{+2}$ and $Hg^{+2}$. Altering the temperature within the 4 and 45°C range did not produce any significant changes on flocculating activities of bioflocculants in this study. However, there was an increase in the $Cd^{+2}$ removal from 20% up to 93% as the temperature increased from 4 to 45°C using a high concentration (10000 ppm) of bioflocculant (Figure 4). Similar results were obtained with other bioflocculants in this study (data not shown).

## DISCUSSION

Bioflocculants have been actively studied as an alternative choice in various industrial waste treatments recently (Aguilera et al., 2008; Gao et al., 2009; Liu et al., 2009; Yim et al., 2007) including heavy metal removal (Iyer et al., 2005; Wu and Ye, 2007; Gong et al., 2008; Lin and Harichund, 2011a). Due to their extensive capacity for metals, bioflocculants are recommended as surface-active agents for the removal of heavy metals (Morillo et al., 2006). However, bioflocculant production costs are high compared to conventional approaches (Lian et al., 2008). Therefore, optimal bioflocculant production and high flocculating activity of bioflocculant at a lower dosage are crucial for applications.

### Optimization of bioflocculants productions

Elements such as culture medium and culture conditions are known to affect bioflocculant production (Noghabi et al., 2007; Xia et al., 2008). Previous studies on optimazation of production yield revealed optimum bioflocculant production occurred in complex media such as yeast extract and peptone (Liu et al., 2010; Nakata and Kurane, 1999; Xia et al., 2008; Zheng et al., 2008). Decreased bioflocculant production of *Bacillus licheniformis* due to the presence of peptone in the production medium has also been reported (Shih et al., 2001). In the current study, carbon source (ethanol and glycerol), nitrogen source (yeast extract and peptone) and NaCl concentrations were varied and the effect of these

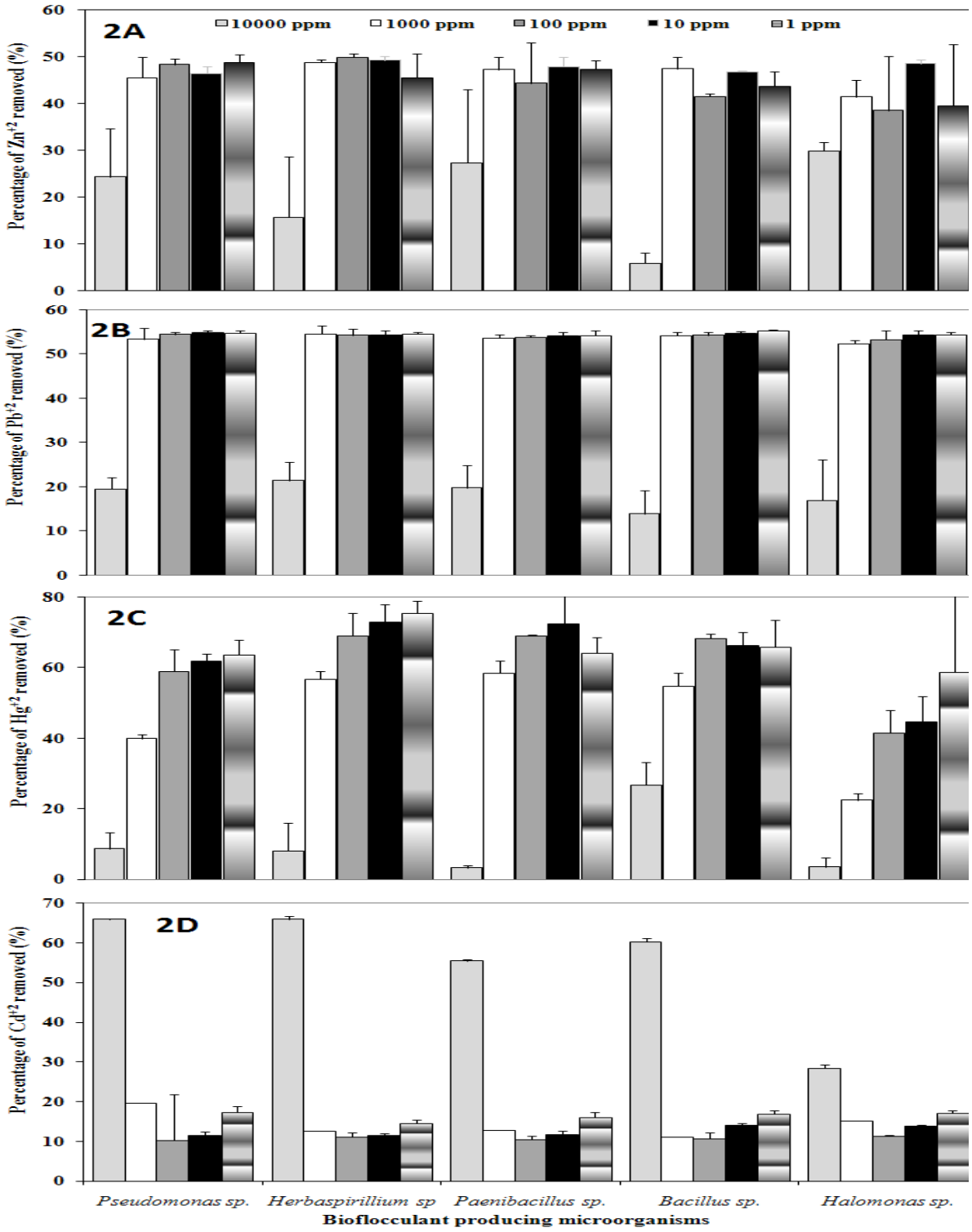


**Figure 2.** Percentage removal of heavy metal (**A**, zinc; **B**, lead; **C**, mercury; **D**, cadmium at 1 ppm) through bioflocculation using a range of bioflocculant concentrations (1 to 10000 ppm).

variations on bioflocculant production was assessed. Our results further support previous studies, since organic nitrogen such as yeast extract and peptone (He et al., 2004; Xia et al., 2008, Zheng et al., 2008) were better sources than inorganic nitrogen for bioflocculant production. Increasing in the yeast extract and peptone

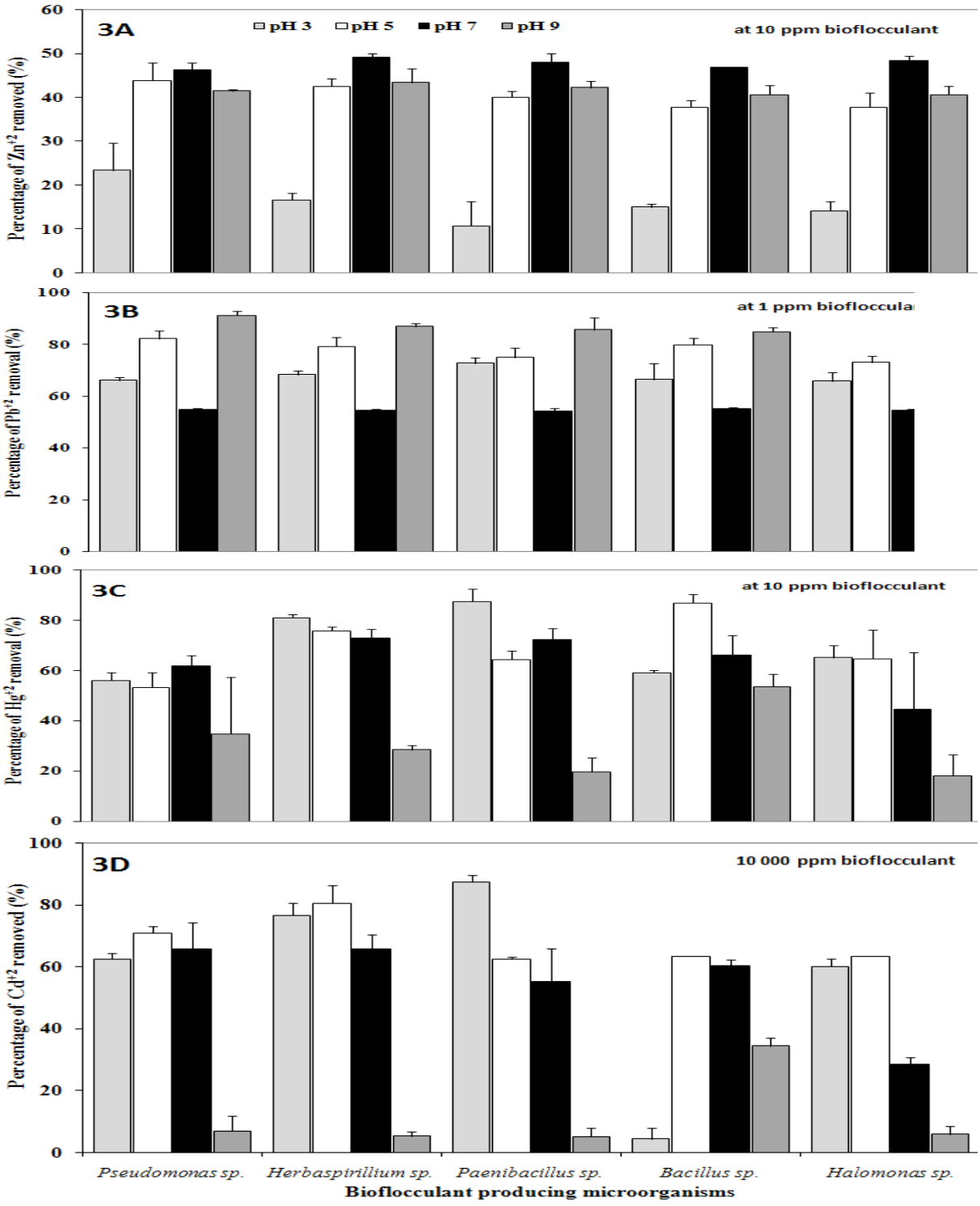


**Figure 3.** Percentage removal of heavy metal at 1 ppm (A, $Zn^{+2}$; B, $Pb^{+2}$; C, $Hg^{+2}$; D, $Cd^{+2}$) at different pH using bacterial bioflocculants at the optimal removal concentrations.

contents from the standard compositions recommended by Kurane et al. (1994) enhanced the bioflocculant productions by all bacterial isolates (Table 1) except for *Herbaspirillium* sp. CH7. Similar to the reports by Noghabi et al. (2007) and Freitas et al. (2009), high glycerol concentration in the medium also stimulated the bioflocculation production. Up to 250% increases in bioflocculant production was obtained in this study. He et

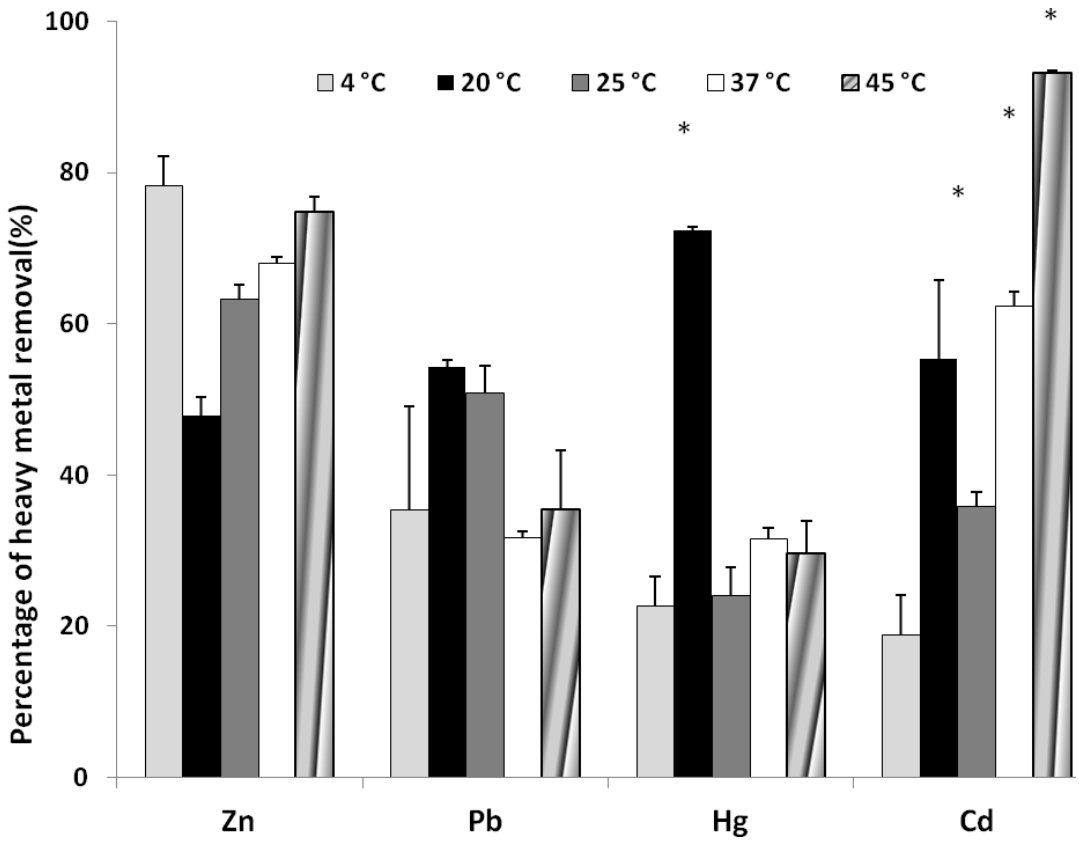


**Figure 4.** Percentage removal of heavy metal ($Zn^{+2}$; $Pb^{+2}$; $Hg^{+2}$; $Cd^{+2}$) at 1 ppm at different temperature using *Paenibacillus* sp. CH11 bioflocculant at the optimal removal concentrations (Figure 2). *: significant, $p<0.05$.

al. (2009) reported that edible glucose was found to be one of the significant factors affecting bioflocculant production by *Halomonas* sp.

## Flocculating activity of bioflocculants

Although, an increase in flocculation was observed using purified bioflocculants produced by *Pseudomonas* sp. CH6, *Paenibacillus* sp. CH11 and *Bacillus* sp. CH15 under the optimal conditions compared to those from the controlled conditions, there were not significant ($p> 0.05$). A significant decrease in flocculating activity was observed for the bioflocculant from *Herbaspirillium* sp. CH7. A direct correlation between increased bioflocculant production and increased/decreased flocculating activity was not evident. Wu et al. (2005) reported that different microorganisms produce bioflocculants using different compositions resulting in different flocculating activities. Bacteria and fungi, belonging to different species, have been reported to produce different EPS types that possess different characteristics and function optimally at various media concentrations (Subramanian et al., 2007). Changes to media composition may have positively or negatively affected the contents of functional groups such as carboxyl and hydroxyl groups of bioflocculants produced resulting in increased (*Pseudomonas* sp. CH6, *Paenibacillus* sp. CH11 and *Bacillus* sp. CH15) or decreased (*Herbaspirillium* sp. CH7) flocculation. Another possibility that arises to explain fluctuations in flocculating activity is that cations ($CaCl_2$) may affect flocculating activity by neutralizing and stabilizing the residual negative charge of functional groups by forming bridges between particles (Wu and Ye, 2007). Our previous study (Lin and Harichund, unpublished results) showed that the bioflocculants used in this study had better flocculating activities when other heavy metal ($Pb^{+2}$, $Zn^{+2}$ or $Hg^{+2}$) were used instead of $Ca^{+2}$. Several groups also found that $Ca^{+2}$ had little effect on the flocculating activity of produced bioflocculant (Sheng et al., 2006; Yim et al., 2007; Zheng et al., 2008).

## Physicochemical analysis of purified bacterial bioflocculants

Bioflocculants have been classified into four types based on their main chemical constituent, that is, polysac-

charide (Wu and Ye, 2007, 2004; Xia et al., 2008; Yu et al., 2009; Zheng et al., 2008), protein (Liu et al., 2010; Park and Novak, 2007), lipid (Kurane et al., 1994; Guiband et al., 2005) and/or nucleic acid/nucleoprotein bioflocculants (Wang et al., 1994). Polysaccharides and proteins offer numerous active sites capable of binding metal ions (Saiffudin and Raziah, 2007). Most of the bioflocculants in the present study can be considered as protein bioflocculants since their main components were proteins, with the exception of the *Herbaspirillium* sp. CH7 bioflocculant which was polysaccharide. All the bioflocculants contained similar contents of hexosamine and uronic acid. Bioflocculants produced by *Halomonas eurihalina* and by *Bacillus subtilis* DYU1 contained 11.1% (Bejar et al., 1998) and 2.7% uronic acid (Wu and Ye, 2007), respectively. All these compositions are involved in the flocculating ability. Microbial bioflocculants involved multiple adsorption points/structures for their flocculating abilities so that bioflocculants are capable of forming the floc with different varieties of organic, inorganic particles as well as microorganisms from the environments as demonstrated by our previous study (Lin and Harichund, 2011b) and others (Gong et al., 2008; Quintelas et al., 2008).

**Effect of bioflocculant concentrations in removal of heavy metals**

Bioflocculants in this study were effective as heavy metal flocculating agents with low dosage requirement as the optimal bioflocculant concentration in removing $Zn^{+2}$, $Pb^{+2}$ and $Hg^{+2}$ in the range of 1 to10 ppm (Figure 2). Yim et al. (2007) demonstrated that sedimentation of flocculated particles was inhibited by the viscosity generated at high concentrations of bioflocculants. Over-addition of negatively charged bioflocculant caused the repulsion of each other and poor stability. The relationship between bioflocculant dosage and flocculating rate was similar to that of the bioflocculants produced by other pure strains (Zheng et al., 2008; Gong et al., 2008; Yim et al., 2007; Liu et al., 2009). The optimal bioflocculant dosage was often reported in the similar range (He et al., 2009; Yim et al., 2007; Zheng et al., 2008). The amino and carboxyl functional groups of bioflocculant can form a floc with heavy metals by neutralizing and stabilizing the residual charge as the binding distance is shortened. The physical and chemical properties of the metals, the availability of appropriate binding sites present to metal, as well as the tertiary structure of bioflocculant may all contribute to metal-binding interactions (Kachlany et al., 2001). Surprisingly, high concentrations (10000 ppm) of bioflocculants were needed to effectively remove $Cd^{+2}$ from the medium (Figure 2). It is the first report demonstrating the binding of heavy metals and bioflocculants at this high dosage. It is generally believed that bioflocculants have a low metal binding capacity or flocculating activity at higher dosage due to the repulsion and low stability (Zheng et al., 2008; Yim et al., 2007; Liu et al., 2009). All the bioflocculant studies in the literature conducted the experiments using less than 2000 ppm of bioflocculants. It is possible that other metal-binding motifs might exist for the bioflocculant. In biological systems, amino acids, like Cys residues of the metal binding CXXC motif, are responsible of $Cd^{+2}$ tolerance (Banci et al., 2006; Wu et al., 2006). Bioflocculants in this study comprised of high protein contents. It will be interesting to study the $Cd^{+2}$ binding motifs of these bioflocculants. However, the application to remove $Cd^{+2}$ using bioflocculants at the high dosages may be limited.

**Effect of pH on heavy metal removal using bacterial bioflocculants**

The pH of the suspension had an extreme effect on the function of flocculating agent including bioflocculant because the surface charge of the dispersed phase changed according to various pH values (Sheng et al., 2006). Cations such as heavy metal ions can neutralize negatively-charged functional groups of bioflocculant molecules and weaken the static repulsive force, and promote floc formation (Li et al., 2008; He et al., 2010). These results could be attributed to double layer compression effects of divalent cation (Zheng et al., 2008; Li et al., 2008, 2009). Bioflocculants may either enhance or decrease heavy metal removal due to the change of pH (Kaewchai and Prasertsan, 2002; Kiran and Kaushik, 2008; Panicker et al., 2006). Gupta et al. (2000) demonstrated that binding of metals to bioflocculants may be reversed by pH adjustments indicating that the type of binding is pH-dependent. Depending on the functional groups of bioflocculants that are responsible of binding the heavy metals, microbial bioflocculants were likely to absorb hydrogen ions ($H^{+}$) at low pH, which might weaken the forming of complexes between bioflocculant and heavy metals. By the same token, hydroxide ions ($OH^{-}$) interfered with the combi-nation of the flocculant molecules and heavy metals at high pH, resulting in lower flocculating activity. As observed previously by other researchers (Li et al., 2008; Liu et al., 2009; He et al., 2010), flocculating effect of most bioflocculants appeared to be the strongest at neutral pH values in general. Bioflocculants produced by *Bacillus* sp. F19 and *Gyrodinium impudicum* KG03 had the highest flocculating activity at pH 2.0 and pH 4.0, respectively (Zheng et al., 2008; Yim et al., 2007). Bioflocculants from *Agrobacterium* sp. M-503 had the optimal flocculating activity between pH 8-12 (Li et al., 2010). The pH requirement for maximum flocculating activity seems to be varied for different strains of microorganisms (Xia et al., 2008).

**Temperature factor**

In biological wastewater treatment processes, tempera-

ture fluctuations occur, due to seasonal variation. This temperature shift results in alteration in the efficiency of the treatment process. Flocculation has been found to be affected by temperature shifts (Sagastume and Allen, 2003). Pure protein bioflocculants, where the amino and carboxyl groups are the effective groups for bioflocculation (Kurane et al., 1994), usually not heat-stable as protein destroyed upon heating (Takeda et al., 1991). If the major component of a bioflocculant is glycoprotein that has many functional groups (Kurane et al., 1991), its stability will depend on the relative content of protein and polysaccharide. The results obtained in the present study indicated no correlation between the metal removal patterns by bioflocculants and specific temperatures using low concentration of bioflocculants. At higher dosages, bioflocculants removed $Cd^{+2}$ effectively and this was increased with an increase in temperature under the optimal conditions. At high concentrations of bioflocculants, the binding of $Cd^{+2}$ and bioflocculants might neutralize negatively-charged functional groups of bioflocculant molecules partially. Further increases in temperature might weaken the static repulsive force and promote floc formation (Li et al., 2008; He et al., 2010). Further studies are needed.

## Conclusions

The present study has shown that up to 250% increases in bioflocculant productions were achieved by manipulation of media components. Purified bioflocculants produced under the optimal conditions also possessed higher flocculating activities (up to 14-fold increases) compared with those produced the controlled conditions. The results also showed that the optimal dosages of purified bioflocculants in removing $Pb^{+2}$, $Zn^{+2}$ and $Hg^{+2}$ was in the range of 1 to10 ppm. Surprisingly, high dosages of bioflocculants were needed in $Cd^{+2}$ removal, which was also stimulated by an increase in temperature. The pH requirement for maximum heavy metal removal activity seems to be varied for different strains of microorganisms.

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