

# Effects of Glucose and Ferrous Supplements and Culture Conditions on Lipopeptide Biosurfactant from *Pseudomonas* spp.

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**Abstract**—To enhance lipopeptide biosurfactant production by *Pseudomonas* spp., the effects of medium and culture conditions were investigated. Nutrient broth (NB) supplemented with glucose and molasses as carbon sources were used to increase biosurfactant yield. Cultivation temperatures and initial pH were also studied. In this experiment, NB was used as a control to produce biosurfactant with the yield of 0.58 g/L. The lipopeptide biosurfactant yield was increased to 2.76 g/L when *Pseudomonas* spp. was cultured in NB supplemented with 1% w/v glucose, 500  $\mu$ M FeSO<sub>4</sub>, pH 9.0 and cultivation of 20 °C for 72 h. Its specific growth rate was 0.25 h<sup>-1</sup>.

**Index Terms**—*Pseudomonas* spp., lipopeptide biosurfactant, glucose, ferrous.

## I. INTRODUCTION

Surfactants consist of hydrophilic and hydrophobic moieties. According to their compositions, surface tension is able to be decreased by surfactants. These compounds play many rolls of human life in many fields not only agricultural, cosmetic, pharmaceutical applications, but also in food industries [1]. However, the synthetic chemical surfactants are toxic and difficult to degrade [2].

Biosurfactants are biological surface active compounds. Biosurfactants are produced by many living organisms, especially microorganisms. They are classified into 4 groups based on their chemical structures; glycolipids, lipopeptides, phospholipids and fatty acids, and polymeric biosurfactants [3]. In addition, biosurfactants exhibit important properties such as antimicrobial activity, anti-adhesion of microorganisms, biodegradability, emulsifying agents, and low toxicity. Therefore, biosurfactants have potentially applied in environmental, food industrial, agricultural, therapeutic and biomedical applications [1], [4].

To enhance biosurfactant yield, medium and culture conditions are investigated. Mouafi *et al.* [1] reported that lipopeptide biosurfactant production from *Bacillus brevis* was increased by glucose supplementation in medium. The lipopeptide biosurfactant yield of *Bacillus subtilis* B20 was

increased to 2.29 g/L when cultured with molasses [5]. Karanja oil was also able to increase the biosurfactant yield of *Pseudomonas aeruginosa* KVD-HR42 to 5.90 g/L [3].

Trace elements are important for bacterial growth by acting as co-factors for enzymes. The effect of metal ion concentration on biosurfactant production was studied by [6]. Kiran *et al.* [7] reported that iron concentration in culture medium was the critical factor on biosurfactant production by *Brevibacterium aureum* MSA13. Moreover, Surfactin, the lipopeptide biosurfactant from *Bacillus subtilis* ATCC 21332, was increased to approximately 3000 mg/L, when iron sulfate was added into culture medium [6].

Culture temperature and pH of culture medium are also affected to biosurfactant production. The biosurfactant yield from *Pseudomonas* sp. was increased after cultivation with optimal pH and temperature [3]. Moreover, Hemtala *et al.* [8] reported that culture temperature and pH was also the factor to enhance biosurfactant yield from *Stenotrophomonas maltophilia* NBS-11.

In this study, medium was investigated to enhance lipopeptide biosurfactant yield from *Pseudomonas* spp. by supplement nutrient broth (NB) with glucose and molasses as carbon sources and metal ion of ferrous. The effects of temperature and pH on biosurfactant yield were also studied.

## II. MATERIALS AND METHODS

### A. Microorganism and Inoculum Preparation

The microorganism *Pseudomonas* spp. was screened and studied from previous study [9]. This strain produced lipopeptide biosurfactant. The biosurfactant producing bacteria was kept in 20% glycerol solution and maintained at -20 °C.

Inoculum was prepared in 50 ml of NB (Difco), sterilized at 121 °C for 15 min, in 250 ml Erlenmeyer flask. The cultivation was shaken at 100 rpm at 30 °C for 24 h. All experiments of this study, 1% inoculum was added to be  $1 \times 10^6$  CFU/ml of medium.

### B. Biosurfactant Production and Extraction

*Pseudomonas* spp. was cultured by using NB with 200 ml working volume in 500 ml Erlenmeyer flask at 30 °C, 100 rpm for 72 h. The experiment was performed with triplication.

After cultivation, culture broth was centrifuged at 5500  $\times$ g, 4 °C for 15 min to collect cell-free culture broth (CFCB) and cell pellet.

The CFCB was then acidified to pH 2.0 by 6 N HCl and

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chilled overnight. The acidified CFCB was centrifuged at  $7300 \times g$ ,  $4^\circ\text{C}$  for 20 min to collect the sediment. 6 N NaOH was used to neutralize the sediment and then extracted with a mixture of chloroform:methanol (2:1) [9]. The organic phase was pooled and evaporated. Crude lipopeptide biosurfactant was purged with nitrogen gas to dryness and weighted.

The cell pellet was dried in hot air oven at  $105^\circ\text{C}$  until weight constant. The dried biomass was then weighted [10].

### C. Surface Tension (ST) Measurement

To measure the ST, 40 ml of CFCB were added into beaker and measured ST by tensiometer K6 (KRÜSS) with Du-Nouy Ring Method at  $25^\circ\text{C}$  [9] from triplicated experiments. Sterile NB and supplemented NB were used as controls.

### D. Effect of Carbon Sources and Ferrous Ion on Biosurfactant Production

#### 1) Carbon Sources

In this experiment, glucose (Difco) and molasses (50.47% total sugar) were individually added into NB. Their supplement concentrations of glucose and molasses were 0.5, 1.0 and 1.5% w/v of total sugar. The cultivation was incubated at  $30^\circ\text{C}$ , 100 rpm for 72 h. After cultivation period, culture broth was collected to analyze biomass, biosurfactant yield and ST.

#### 2) Ferrous Ion

The culture medium with the highest biosurfactant yield from experiment 2.4.1 was selected to study effect of ferrous ion on biosurfactant production by *Pseudomonas* spp.  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  was added into medium to be the final concentrations of 50, 100 and 500  $\mu\text{M}$ . After 72 h cultivation at  $30^\circ\text{C}$  with 100 rpm, biomass, biosurfactant yield and ST were determined.

### E. Effect of Temperature and pH on Biosurfactant Production

#### 3) Temperature

The temperatures of 10, 20, 30 and  $40^\circ\text{C}$  were used to culture *Pseudomonas* spp. with a selected ferrous concentration. The biomass, biosurfactant yield and ST were determined.

#### 4) pH

The culture temperature with the highest biosurfactant yield was used to vary initial culture medium pH of 5.0, 7.0 and 9.0. The biomass, biosurfactant yield and ST were also analyzed.

### F. Growth Kinetics of *Pseudomonas* spp. with Suitable Medium and Conditions

*Pseudomonas* spp. was cultured in the glucose and ferrous supplemented medium and culture conditions with the maximum biosurfactant yield. The cell viability, ST and biosurfactant yield were monitored every 3 h for 72 h. During exponential phase, the specific growth rate was determined [11].

### G. Experimental Design and Data Analysis

The experiments of this study were performed in triplicate with completely randomized design. Data was calculated for analysis of variance. One way – ANOVA was used to analyze data with 95% significant level ( $\alpha = 0.05$ ).

## III. RESULTS AND DISCUSSION

### A. Effect of Molasses and Glucose

All media of this experiment was able to culture *Pseudomonas* spp. and used to produce biosurfactant (Table I). The molasses supplement in NB showed the significant biomass increasing, but decreased biosurfactant yield. Molasses did not only significantly increase the biomass of bacterial cells, but it might also inhibit the biosurfactant production [10], [12]. The high biosurfactant yield was observed with 0.87 and 0.84 g/L, when *Pseudomonas* spp. was cultured in 0.5 and 1.0% glucose supplement, respectively. However, the ST of CFCB from 1.0% glucose supplement culture medium was significant lower than 0.5% glucose. Glucose is a good carbon source for organisms including microorganisms. It provides faster growth than other sugars, and is consumed first in sugar mixtures [8]. In contrast, high glucose concentration in the medium might inhibit the growth rate of microorganisms [8]. According to the results, the NB supplemented by 1% glucose was selected to use in the further experiments.

TABLE I: SURFACE TENSION VALUE OF CFCB, BIOMASS AND BIOSURFACTANT YIELD FROM PSEUDOMONAS SPP. WITH GLUCOSE AND MOLASSES SUPPLEMENT IN NB

Carbon Sources	ST (mN/m)	Biomass (g/L)	Biosurfactant Yield (g/L)
NB (control)	$28.83 \pm 0.29^a$	$0.77 \pm 0.02^a$	$0.58 \pm 0.01^b$
NB + 0.5% Glucose	$37.17 \pm 1.04^d$	$1.02 \pm 0.01^{bc}$	$0.87 \pm 0.06^c$
NB + 1.0% Glucose	$32.83 \pm 0.29^c$	$0.97 \pm 0.03^b$	$0.84 \pm 0.02^c$
NB + 1.5% Glucose	$32.33 \pm 0.76^c$	$1.12 \pm 0.05^c$	$0.06 \pm 0.01^a$
NB + 0.5% Molasses	$29.50 \pm 0.50^{ab}$	$1.75 \pm 0.13^d$	$0.16 \pm 0.09^a$
NB + 1.0% Molasses	$30.00 \pm 0.00^b$	$1.90 \pm 0.04^e$	$0.06 \pm 0.03^a$
NB + 1.5% Molasses	$30.17 \pm 0.58^b$	$2.18 \pm 0.06^f$	$0.08 \pm 0.10^a$

The superscript of <sup>a,b,...</sup> with different letter in the same column is significantly difference ( $p \leq 0.005$ ).

### B. Effect of Ferrous Ion

TABLE II: SURFACE TENSION VALUE, BIOMASS AND BIOSURFACTANT YIELD FROM PSEUDOMONAS SPP. BY USING 1% GLUCOSE IN NB WITH DIFFERENT CONCENTRATIONS OF FERROUS ION

Ferrous Ion ( $\mu\text{M}$ )	Surface Tension (mN/m) <sup>ns</sup>	Biomass (g/L)	Biosurfactant Yield (g/L)
0	$33.00 \pm 0.00$	$0.95 \pm 0.02^b$	$0.84 \pm 0.01^a$
50	$32.33 \pm 0.29$	$0.69 \pm 0.03^a$	$1.55 \pm 0.02^b$
100	$33.00 \pm 1.32$	$0.64 \pm 0.02^a$	$1.58 \pm 0.07^b$
500	$33.00 \pm 1.00$	$0.65 \pm 0.01^a$	$2.08 \pm 0.12^c$

The superscript of <sup>a,b,...</sup> with different letter in the same column is significantly difference ( $p \leq 0.005$ ).

The superscript of <sup>ns</sup> with different values in the same column is significantly no difference ( $p \leq 0.005$ ).

Metal ions are the cofactor in living organisms. In this experiment, ferrous ion showed the remarkable effect on biosurfactant yield by *Pseudomonas* spp. (Table II). The maximum biosurfactant yield with 2.08 g/L was derived by using 1% glucose in NB coupled with 500  $\mu\text{M}$   $\text{FeSO}_4$ . The increase of biosurfactant yield was reported previously. Surfactin production of *B. subtilis* was increased

approximately to 3.5 g/L, when added 2 mM FeSO<sub>4</sub> into culture medium [6], [13]. To enhance biosurfactant production by *P. aeruginosa*, iron and other metal ions were supplemented in the media [14]. The raised biosurfactant by ferrous ion was not only presented by bacterial cultivations, but biosurfactant productions by *Aspergillus ustus* and *Pleurotus djamor* were also promoted by this ion [2], [15]. From the results, 1% glucose in NB coupled with 500 μM FeSO<sub>4</sub> was used for study of cultivation temperature.

### C. Effect of Cultivation Temperature

Culture temperature caused effect on growth and bioprocessing by microorganisms. The cultivation of *Pseudomonas* spp. at 20 °C by using 1% glucose in NB added with 500 μM FeSO<sub>4</sub> represented the maximum biosurfactant yield of 2.39 g/L (Table III). In contrast, the biosurfactant production was not observed at 40 °C, and ST was as same as control with 48 mN/m. According to the biomass, temperature at 40 °C might suppress *Pseudomonas* spp. growth. Kiran *et al.* [2] and Velioglu and Urek [15] also reported that temperature was a critical factor on microbial growth and biosurfactant production. On next experiment, *Pseudomonas* spp. was cultured at 20 °C by using NB added with 500 μM FeSO<sub>4</sub> as medium.

TABLE III: SURFACE TENSION VALUE, BIOMASS AND BIOSURFACTANT YIELD FROM PSEUDOMONAS SPP. BY USING 1% GLUCOSE IN NB ADDED WITH 500 μM FeSO<sub>4</sub> AT VARIOUS CULTURE TEMPERATURES

Culture Temperature (°C)	Surface Tension (mN/m)	Biomass (g/L)	Biosurfactant Yield (g/L)
10	31.50 ± 0.50 <sup>a</sup>	0.80 ± 0.02 <sup>c</sup>	1.62 ± 0.11 <sup>a</sup>
20	33.00 ± 1.00 <sup>ab</sup>	1.08 ± 0.01 <sup>d</sup>	2.39 ± 0.09 <sup>b</sup>
30	50.17 ± 0.76 <sup>c</sup>	0.68 ± 0.01 <sup>b</sup>	2.07 ± 0.09 <sup>c</sup>
40	48.50 ± 0.50 <sup>c</sup>	0.04 ± 0.01 <sup>a</sup>	N/A

The superscript of <sup>a,b,c,d</sup> with different letter in the same column is significantly difference ( $p \leq 0.005$ ).

N/A means no biosurfactant detected.

### D. Effect of pH

The biomass and biosurfactant yield represented with the low values at initial pH 5 of *Pseudomonas* spp. cultivation by using 1% glucose in NB, 500 μM FeSO<sub>4</sub> at 20 °C (Table IV). The biomass from pH 7 and 9 was not significantly different, but their biosurfactant yields were increased from 2.36 to 2.76 g/L, respectively. The result agreed with other reports [15]. For *P. aeruginosa* KVD-HR42, the biosurfactant production was raised by cultivation at pH 8.5 [3]. The optimal pH for extracellular biosurfactant produced by *Yarrowia lipolytica* NCIM 3589 was 8.0 [16]. In contrast, the low pH culture medium was suitable for biosurfactant production by fungi [2], [15].

### E. Pseudomonas spp. Growth Kinetics

When *Pseudomonas* spp. was cultured in NB supplemented with 1.0 % w/v glucose, 500 μM FeSO<sub>4</sub>, pH 9.0 at 20 °C for 72 h, the surface tension was significantly decreased at the exponential growth phase (Fig. 1). After 24 h culture time, the surface tension was mostly constant at 30 mN/m. The result was agreed with *P. aeruginosa* MA01 [17]

and *Candida albicans* [18]. Also, Rufino *et al.* [19] reported that *C. lipolytica* UCP0988 produced rufisan biosurfactant during exponential growth phase. By this medium and culture conditions, the biosurfactant yield was enhanced up to 2.76 g/L. While biosurfactant yield from NB was 0.58 g/L. During exponential phase, the specific growth rate of *Pseudomonas* spp. was 0.25 h<sup>-1</sup>.

TABLE IV: SURFACE TENSION VALUE, BIOMASS AND BIOSURFACTANT YIELD FROM PSEUDOMONAS SPP. BY USING 1% GLUCOSE IN NB, 500 μM FeSO<sub>4</sub> AT 20 °C WITH DIFFERENT INITIAL pHs

pH	Surface Tension (mN/m)	Biomass (g/L)	Biosurfactant Yield (g/L)
5.0	32.67 ± 1.15 <sup>b</sup>	0.64 ± 0.03 <sup>a</sup>	0.03 ± 0.01 <sup>a</sup>
7.0	31.50 ± 0.50 <sup>ab</sup>	1.06 ± 0.03 <sup>b</sup>	2.36 ± 0.06 <sup>b</sup>
9.0	30.67 ± 0.29 <sup>a</sup>	1.08 ± 0.05 <sup>b</sup>	2.76 ± 0.03 <sup>c</sup>

The superscript of <sup>a,b,c</sup> with different letter in the same column is significantly difference ( $p \leq 0.005$ ).

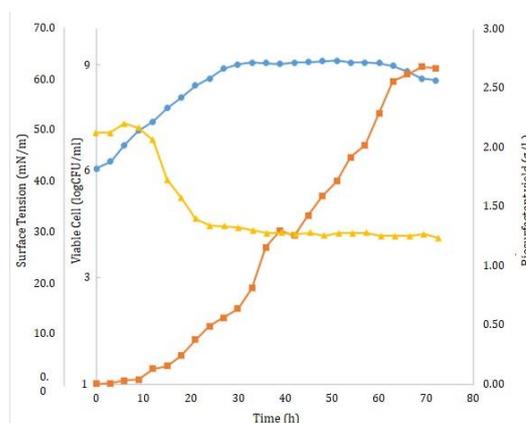


Fig. 1. Growth Kinetics of *Pseudomonas* spp. when cultured with selected conditions. ST (triangle), Viable cell (circle) and Biosurfactant yield (square).

## IV. CONCLUSION

The effects of NB supplemented by carbon sources and ferrous ion, temperature and initial pH were investigated to enhance the biosurfactant production form *Pseudomonas* spp. with single factor experimental design. Its biosurfactant yield was increased with 4.5 times when cultured by using NB supplemented with 1.0 % w/v glucose, 500 μM FeSO<sub>4</sub>, pH 9.0 at 20 °C. Furthermore, *Pseudomonas* spp. will be carried out for chemical structure identification and investigation on biological activity of its biosurfactant.

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