Effects of Various Phosphates on Biosurfactant Fermentation by *Ochrobactrum Intermedium* XY-1

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Abstract. Phosphates are important components in the fermentation medium for biosurfactant production. In the present study, *Ochrobactrum intermedium* strain XY-1 was used as the experimental strain, the effects of various phosphates used in the biosurfactants fermentation media was preliminarily investigated on cell growth, and surface tension, and emulsifying activity of fermentation broth. Under the same total phosphorus concentration of 0.035 mol/L, KH$_2$PO$_4$ + Na$_2$HPO$_4$, KH$_2$PO$_4$, and Na$_2$HPO$_4$ was respectively used as the phosphorus source of biosurfactant fermentation at 30°C and 160 rpm. The results showed that the bacteria grew slowly and the maximum emulsifying activity of fermentation broth reached 52.95% in the fermentation with KH$_2$PO$_4$ + Na$_2$HPO$_4$ as phosphorus source, and larger bacterial growth and 35.9% maximum emulsifying activity exhibited in the fermentation with KH$_2$PO$_4$, smaller bacterial growth and 24.54% maximum emulsifying activity exhibited in the fermentation with Na$_2$HPO$_4$. The phosphate mixture of KH$_2$PO$_4$ and Na$_2$HPO$_4$ benefits the biosurfactant fermentation by limiting the growth of *Ochrobactrum intermedium* strain XY-1, which might be the earliest report on the phosphorous source of biosurfactant fermentation by *Ochrobactrum intermedium*.

Keywords: *Ochrobactrum intermedium*; biosurfactant; fermentation; phosphate; emulsifying activity.

1. Introduction

Biosurfactant has many advantages such as low toxicity, and high biodegradability and ecological acceptability, therefore, is potential for applications in the production of medicine, cosmetics and food, petroleum recovery and the environmental bioremediation. But its commercial production is limited by low yield and high production cost. Some strategies to overcome the economic obstacles in commercialization of biosurfactants have been suggested, including the isolation of microbe with high yield, the optimization of medium components and the utilization of low-cost substrates [1]. At present, researches mainly enhance its yield and reduce costs through optimizing the upstream fermentation process and downstream purification process.

Microorganisms producing biosurfactant conclude bacteria, fungi, and yeast. *Ochrobactrum* is an aerobic, Gram-negative bacterium of the family Brucellaceae. The investigation on *Ochrobactrum* producing biosurfactant has been not so well as other bacteria such as *Pseudomonas* spp., *Acinetobacter* spp., *Bacillus* sp., *Streptomyces* spp., *Rhodococcus* spp., *Achromobacter* spp., *Brevibacterium* spp., and *Arthrobacter* spp [2]. Phosphorus element is required for the growth and metabolism of microorganisms, and their pathway of EMP and HMP [3], which will also affect the growth and biosurfactant production of bacteria in biosurfactant fermentation. There are few studies on the effect of phosphate on biosurfactant fermentation worldwide so far, mainly focusing on...
optimizing the amount of phosphate in the medium of \textit{Pseudomonas aeruginosa}. \textit{Pseudomonas aeruginosa} ATCC9027 had the highest rhamnolipid yield in phosphate-limited media (peptone was the only source of phosphate), but the yield was very low in the broth and the media rich in inorganic phosphorus [4]. In the process of metabolic conversion, the reduction of phosphate induces the activity of APase and reduces the activity of PATH, thus inducing the production of biosurfactant and affecting biosurfactant yield. Rhamnolipid accumulation started only under the condition of limiting phosphate concentration, indicating that phosphorus limitation promoted the production of rhamnolipid by \textit{Pseudomonas aeruginosa} [5].

In the present study, the effect of phosphates on biosurfactant fermentation by \textit{Ochrobactrum intermedium} strain XY-1 was investigated by analyzing cell growth, and surface tension and emulsifying activity of fermentation broth.

2. Materials and Methods

2.1. Microorganism

The strain XY-1 identified as \textit{Ochrobactrum intermedium} was maintained in a 20% glycerol medium tubule and stored at -80°C. A loopful of the strain was inoculated on medium slopes at 30°C for 24 h, and stored at -4°C for routine use. Before fermentation, the culture was transferred into another fresh medium slopes and incubated at 30°C for 24 h for strain activation. The medium composed of NaCl 15 g, beef extract 5 g, peptone 10 g, and agar 20 g per liter.

2.2. Biosurfactant Fermentation

The basic fermentation medium contained sucrose 10 g, NaNO$_3$ 4.0 g, MgSO$_4$·7H$_2$O 0.2 g, yeast extract powder 0.2 g per liter, which was adjusted to pH 7 and sterilized by autoclaving at 115°C for 20 min. All the fermentation were carried out in 50 mL Erlenmeyer flasks containing 20 mL medium, incubated at 30°C for 7 days in a shaker at 160 rpm, under which the culture as fermentation starter was obtained by inoculating two loops of the strain activated on the slope medium. Inoculation of 8% was applied for fermentation.

In the basic fermentation medium, KH$_2$PO$_4$, Na$_2$HPO$_4$ and KH$_2$PO$_4$+Na$_2$HPO$_4$ (at equal molar ratio) was applied as phosphorous source, respectively, maintaining at 0.035 mol/L of the final total phosphorus content in the medium. The fermentation broth were sampled every day for measuring the pH, bacteria density, surface tension, emulsifying activity, reducing sugar content and total sugar content.

2.3. Measurement of Parameters

Throughout the whole fermentation process, the surface tension (ST), emulsifying activity and cell density of fermentation broth were periodically measured, respectively, by Du Nouy ring method, and by measuring E$_{24}$ index and optical density at 600 nm (OD$_{600}$). The diluted fermentation broth was directly used to measure OD$_{600}$, the cell-free supernatant obtained by centrifuging the fermentation broth at 4°C, 13300 g for 20 min was used as the sample for measurement of ST and E$_{24}$ index. The E$_{24}$ index was expressed as the percentage of the height of the emulsified layer to the total length of the mixture of sample and liquid paraffin. The height of the emulsified layer was formed after the mixture of 1.5 mL sample and 0.9 g liquid paraffin was vortexed for 2 min and subsequently standing for 24 h.

2.4. Statistical Analysis

All the fermentation and measurement were performed in triplicates, the values were expressed as means ± standard deviation from three independent experiments. Significant differences were determined by analysis of variance (ANOVA), followed by Duncan at a level of $p<0.05$. Data were analyzed by using IBM SPSS Statistics software version 17.0.
3. Results and Discussion

3.1. Effect of Phosphate on Microbial Growth and pH in Fermentation

![Figure 1](image1.png)

Figure 1. The variation in cell density and pH during biosurfactant fermentation with various phosphates. (A) cell density; (B) pH.

In order to explore the effects of various phosphates on cell growth and pH of biosurfactants fermentation, the cell density and pH value of the fermentation broth was measured under the condition of different phosphorus sources. As shown in figure 1A, the cell density in the fermentation broth with KH$_2$PO$_4$ exhibited the highest throughout the entire fermentation, the cell density in the fermentation broth with Na$_2$HPO$_4$ was significantly higher than that with KH$_2$PO$_4$ + Na$_2$HPO$_4$ in the first three days of fermentation, in which differed significantly between various groups. However, the cell densities in both fermentation broth with Na$_2$HPO$_4$ and KH$_2$PO$_4$ + Na$_2$HPO$_4$ were almost the same during the fermentation from day 3 to day 7. It was indicated that KH$_2$PO$_4$ as phosphorus source is more conducive to the rapid and great growth of the tested strain, followed by Na$_2$HPO$_4$ in growth rate.

In the fermentation process, the pH of fermentation broth with different phosphorus sources all increased, but slightly fluctuated after 3 days (figure 1B). The pH of fermentation broth with KH$_2$PO$_4$ as phosphorus source increased significantly higher than those with the other two phosphorus sources. The pH of fermentation broth with Na$_2$HPO$_4$ and KH$_2$PO$_4$ + Na$_2$HPO$_4$ was close, and the former was slightly higher in the late stage of fermentation. The pH variation was just similar with the bacteria growth in the fermentation with various phosphates, implying that pH variation should be directly caused by the growth and metabolism of bacteria, moreover, somewhat related to the buffering effect of various phosphates.

3.2. Effect of Phosphate on Substrates Consumption in Fermentation

![Figure 2](image2.png)

Figure 2. The variation in contents of total sugar and reducing sugar during biosurfactant fermentation with various phosphates. (A) reducing sugar content; (B) total sugar content.

In order to explore the effects of various phosphates on carbon source consumption of biosurfactants
fermentation, the reducing sugar content and total sugar content of the fermentation broth was measured under the condition of different phosphorus sources. As shown in figure 2A, in the whole fermentation process, the reducing sugar contents of fermentation with different phosphates were extremely low, only reducing sugar in the fermentation with KH$_2$PO$_4$+Na$_2$HPO$_4$ slightly increased after day 4. It was inferred that the tested strain could directly use sucrose for growth, or the reducing sugar from decomposing sucrose by the bacteria was almost all used rapidly.

The total sugar content (figure 2B) showed a downward trend during the entire fermentation process. The total sugar content decreased during day 1 to day 4 of fermentation with KH$_2$PO$_4$, but was relatively stable during day 4 to day 7. The total sugar content always decreased during the whole process of fermentation with Na$_2$HPO$_4$. The total sugar was consumed in large quantities at day 1 for the massive growth of bacteria. The content of total sugar slightly varied during day 2 to day 5 of fermentation with KH$_2$PO$_4$+Na$_2$HPO$_4$, meanwhile, the reducing sugar content increased and the bacteria still grew greatly, indicating that the slow consumption of sugar resulted in the increase of reducing sugar content, or some products containing sugar was produced by metabolism.

3.3. Effect of phosphate on biosurfactants production in fermentation

Figure 3. The variation in surface tension and emulsifying activity during biosurfactant fermentation with various phosphates. (A) surface tension (ST); (B) emulsifying activity.

In order to explore the effects of various phosphates on biosurfactant production of biosurfactants fermentation, the surface tension and emulsifying activity of the fermentation broth was measured under the condition of different phosphorus sources. As shown in figure 3A, during the fermentation process, the surface tension of fermentation broths with different phosphates changed in the same way. The surface tension of fermentation with KH$_2$PO$_4$ and Na$_2$HPO$_4$ simultaneously decreased for the first time to 56.3 mN/m and 51.2 mN/m, respectively, with a decrease of 14.40% and 18.16%. On the other hand, the surface tension of KH$_2$PO$_4$+Na$_2$HPO$_4$ was the lowest (52.8 mN/m) after 2 days of fermentation, with a decrease of 16.17%. The second decrease in surface tension was observed on day 6 of the fermentation with various phosphates. The difference of surface tension was small between the starting and ending, and the range of decrease was small (p>0.05).

As shown in figure 3B, the emulsifying activity of fermentation broth with different phosphates all increased to the maximum on day 5 of fermentation, and decreased successively in the order of KH$_2$PO$_4$+Na$_2$HPO$_4$, KH$_2$PO$_4$, and Na$_2$HPO$_4$, which were 52.95%, 35.9% and 24.5% respectively, and then decreased. It was shown that the type of phosphate had a significant effect on the emulsification activity of fermentation broth, biosurfactant production with KH$_2$PO$_4$+Na$_2$HPO$_4$ as phosphorous source was significantly superior to that with KH$_2$PO$_4$ or Na$_2$HPO$_4$ (p<0.05).

The surface tension of fermentation broth with various phosphates decreased at a smaller extent, demonstrating that a small quantity of biosurfactant decreasing surface tension was produced by fermentation. High emulsifying activity of fermentation broth implied that a large quantity of bioemulsifier was produced by fermentation. Therefore, it can be believed that KH$_2$PO$_4$+Na$_2$HPO$_4$ is more conducive to the biosurfactant production by metabolism. According to its higher content of total
sugar and reducing sugar, it can also be speculated that the biosurfactant generated may contain sugar.

4. Conclusion
The results of this study showed that KH$_2$PO$_4$+Na$_2$HPO$_4$ was not conducive to the rapid growth of bacteria, but conducive to biosurfactant formation; KH$_3$PO$_4$ was conducive to the large growth of bacteria, but not conducive to biosurfactant formation; Na$_2$HPO$_4$ was not conducive to neither the rapid growth of bacteria nor biosurfactant formation. The difference between the effects may be related to both the amount of K$^+$ and Na$^+$ in the medium and the different pH buffering ability of different phosphates. It is demonstrated that phosphate kinds affects the growth and biosurfactant production of the strain, rapid and great growth might be not beneficial to biosurfactant production. Therefore, KH$_2$PO$_4$+Na$_2$HPO$_4$ as phosphorus source can enhance biosurfactant yield by the fermentation using *Ochrobactrum intermedium* strain XY-1. In the future, we should investigate the specific reasons of different phosphates effect on biosurfactants fermentation and the influence of phosphate concentration on biosurfactants fermentation.

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