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Semicontinuous Production of Red Pigment by Immobilized Cells of *Bacillus* sp. BH-99 Using Column Bioreactor

Beung-Ho Ryu*

Department of Food Science and Biotechnology, Kyungsung University, Busan 608-736, Korea

Abstract The semicontinuous production of red pigment by immobilized cells of *Bacillus* sp. BH-99 was investigated in comparison with free cells. The red pigment produced highest productivity under the conditions of aeration of 0.2 mL/min and 2 mm diameter of gel beads by using 3.0% sodium alginate. Semicontinuous production by immobilized cells showed the highest productivity with replacement of fresh production medium in every 72 h for fourth fermentation cycle following the conditions of red pigment productivity.

Keywords: red pigment, immobilized cell, colloidal chitin, *Bacillus* sp.

INTRODUCTION

Natural pigment is essential to restore an acceptable appearance and it plays a significant part in our enjoyment of food. These compounds are responsible for many of the brilliant red, oranges, yellow, blue colors of edible fruit and vegetables and mushrooms as well as flowers, insects, birds, marine algae, fishes and other animals [1]. Nevertheless, pigments cannot be used for food processing because these natural colors were easily decolorized during food processing, storages and transportation and pH, heat, ultraviolet and oxygens [2,3].

Instead of natural pigment, the artificial synthetic pigments are still of great importance to the food industry. But the toxicological status of all these colours has been already studied [4-6]. Therefore, there has been a continuing trend over the last few years to replace artificially [2]. Attempts were strongly required to produce the natural colors production through economic synthetic methods [7]. Natural pigments from several natural sources are used in food among carotenoid, anthocyanin [8], naphthoquinone [9], and anthraquinone [10], *etc*. Most of these pigments have significant disadvantages in use such as cost, stray characteristic odour, or peppery taste [11-13]. Their use is hence fairly limited to specialised outlets. To overcome this problem, hence much work has been produced naturally occuring pigments from their sources in nature as well as research in economic synthetic manufacturing routes. Natural pigments production of various microorganisms has been reported such as *Monascus* sp. [14,15], *Streptomyces californicus* sp. [16] and *Streptomyces propurpuratus* [17-19].

In a previous paper, optimal culture conditions for the production of red pigment already were investigated [20]. The red pigment production by *Bacillus* sp. BH-99 was found to have a possibility of mass production for use of good.

Those factors seemed to be important to increase yield of red pigment for given strain. The present paper describes the optimal conditions for efficient production of red pigment.

* **Corresponding author**
Tel: +82-51-620-4712 Fax: +82-51-622-4986
e-mail: bhryu@star.kyungsung.ac.kr

MATERIALS AND METHODS

Microorganism and Culture Conditions

The strain employed in this study was *Bacillus* sp. BH-99 [20]. The stock culture was maintained in a medium containing with 20 g of colloidal chitin, 0.25 g of peptone, 0.25 g of yeast, 0.5 g of NaCl, 0.1 g of KH_2PO_4, 0.01 g of $MgSO_4 \cdot 6H_2O$, 0.01 g of $ZnSO_4$, 0.01 g of $MnSO_4$, and 2.0 g of agar per 1 L of deionized water (pH 7.0). Cultivating of red pigment production was grown at 25°C for 3 days with rotary shaking at 150 rpm.

Assay for red pigment, a preculture (1 mL) of *Bacillus* sp. BH-99 was inoculated into 300 mL shake flask containing 50 mL of medium. After incubation at 25°C for 3 days, broth was sampled periodically for analysis. After centrifugation at 3,000 rpm for 10 min to separate the cells, the supernatant was determined colorimetically by measuring its absorbance at 510 nm.

Immobilization of Growing Cells

Immobilized beads were prepared using White and Portno [21] method with some modifcations. The cells were harvested in the late exponential phase of growth by centrifugation at 400 rpm for 5 min. The harvested cells (2.4 g) were mixed with 36 mL of 2.5% Na-alginate suspension (Hayashi Co, Japan), and mixed with 21.6

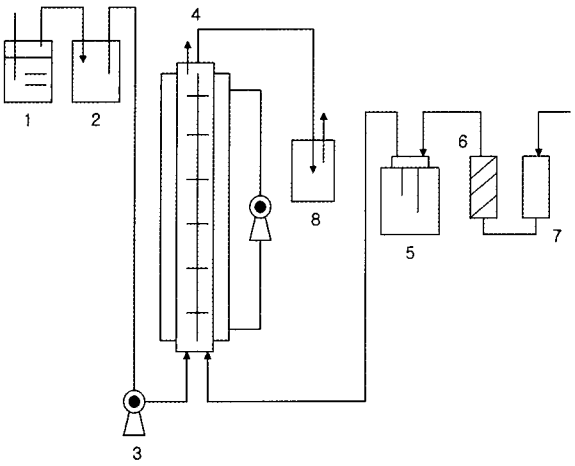

Fig. 1. Semiconscious production of red pigment by immobilized cells of *Bacillus sp.* BH-99 using column bioreactor.

1. Bottle with conc-H_2SO_4
2. Medium reservoir
3. Peristaltic pump
4. Fermentor
5. Humidifier
6. air filter
7. Rotameter
8. Product reservoir

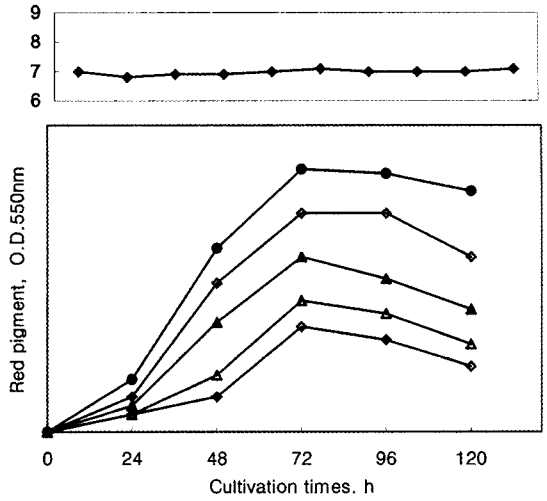

Fig. 2. Red pigment production by *Bacillus sp.* BH-99 at various concentration of colloidal chitin at 120 h. Incubation was carried out in the production medium contanning colloidal chitin. ◇, None; ○, 10% colloidal chitin; ●, 20% colloidal chitin; ▲, 30% colloidal chitin; △, 40% colloidal chitin.

mL of distilled water for immobilized beads. The total suspension was extruded into gentle stirred 0.1 M $CaCl_2$ solution using a 22 gauze syringe. The mean diameter of resulting Ca-alginate gel beads was 2-3 mm. The beads were stored at 4°C for solidification before use.

Semicontinuous Production of Red Pigment by Immobilized Cells

Semicontinuous fermentation was carried out in a column bioreactor(150 × 20 cm) with 1,000 mL working volume. Calcium alginate beads containing immobilized cells was transferred to the sterilized bioreactor in Fig. 1 [22]. For semicontinuous fermentations, cultivations medium was fed semicontinuously at a flow rate of 10 mL per hours. Products were pumped out through a plastic filter (0.5 mm opening, 4 cm diameter). Temperature was maintained at 25°C. The fermenter was stirred by sterilized air bubble using pump.

RESULTS AND DISCUSSION

Growth and Pigment Production during Batch Cultivation

Fig. 2 showed the results of red pigment production at various concentration of colloidal chitin. The amount of red pigment increased gradually with increasing concentrations of colloidal chitin. The pigment showed the highest level of productivity when 20% colloidal chitin was contained in the medium. When culture broth incubated under the optimum conditions, the red pigment appeared after 48 h, and highest yield of pigment showed on 72 h after incubation. Red pigment gradually decreased for 96 h. On the other hand, red pigment concentration increased continuously for 72 h and began to decrease gradually after 72 h. *Bacillus* sp. BH-99 was probably released the hydrolytic enzyme of chitin and then utilized the hydrolyzed of chitin oligmer. This result indicated that it released red pigment under the condition of 20% colloidal chitin.

Selection of Matrices for Immobilization

The advent of immobilized whole cells technology has led to increasing effect of replace the conventional fermentative processes with immobilized system [23,24]. In order to select of the most suitable matrices for productivities of *Bacillus* sp. BH-99 cells, the productivities by immobilized cells of various matrices were investigated. The same amount of cells (0.5 g wet weight) were immobilized in agar, polyacrylamide, calcium alginate and *K*-carrageenan gel. As shown in Table 1, the amounts of red pigment showed the highest productivity by cells immobilization in calcium alginate. It is well known that the activity of cells entrapped in calcium alginate in generally high and stable [24]. Calcium alginate was generally very stable with standard packing pressures without extensive damages [23,24]. Therefore, calcium alginate was chosen as the most favorable matrices for immobilized cells.

Effects of Particle Sizes by Immobilized Gel of *Bacillus* sp. BH-99

The mechanical stability of the immobilized particles, is affected by the entrapped microorganisms as well as by the cultivation method [25,26].

As shown in Fig. 3, effects of immobilized gel sizes of

Table 1. Red pigment productivity of immobilized *Bacillus* sp. BH-99 cells by various polymer metrices

Wet cells (g)	Concentration of matrices for immobilization (%)	Red pigment (O. D. at 550 nm)
0.5	Ca-alginat,e 3.0	0.7.
0.5	Agar, 2.0	0.48
0.5	Carrageenan, 2.5	0.50

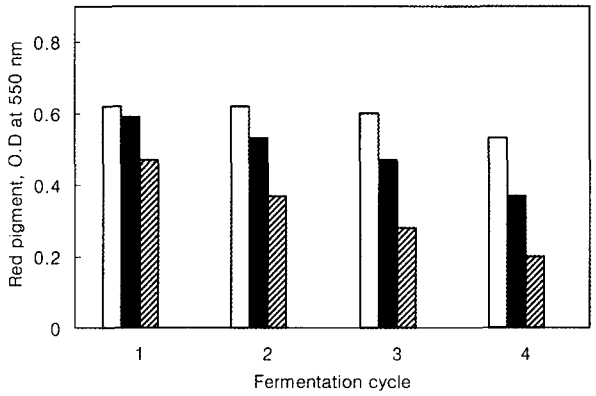

Fig. 3. Effects of immoboilized cells sizes in red pigment production by immoboilized *Bacillus sp.* BH-99. One fermentation cycle took on every 72 h. □, 2 mm; ■, 4 mm; ▨, 6 mm.

Immobilized cells on the red pigment production was investigated. This experiment was carried out for efficiency of red pigment production by the gel sizes of immobilized cells which made the diameter of 2 mm, 4 mm, and 6 mm, respectively. The rates of red pigment production with the 2 mm diameter of gels was higher than 4 mm and 6 mm during fourth fermentation cycles. Recent results indicated that metabolic activities of immo-bilized microorganisms may depended on the particles surface [23,26]. Calcium alginate bead of immobilized cells was found more productivity at particle sizes diameters of 3 mm and 1.5 mm [24].

Eikemeir and Rehm [27] indicated much more productivity that calcium alginate entrapped cells at 3 mm and 1.5 mm particle sizes. From this results, and increased particle sizes surface may occur a diffusional limitation of the gel lattice with component of substrates. For the production of red pigment, 2 mm diameter of gels has easily to contact with substrates. The mass transformation of substrates and oxygen into gel shapes and sizes is very important and effective diffusivities of substrates depended on the gel size [24,25].

Effects of Air Volume

Effects of aeration volume on red pigment production were carried out by varying the aeration volume of 0.2, 0.5, 1.5, and 2.0 mL/L for fourth fermentation cycles. As shown in Fig. 4, increases in the air flow rate were increased in red pigment production by the immobilized cells through the fermentation cycles. But increase of

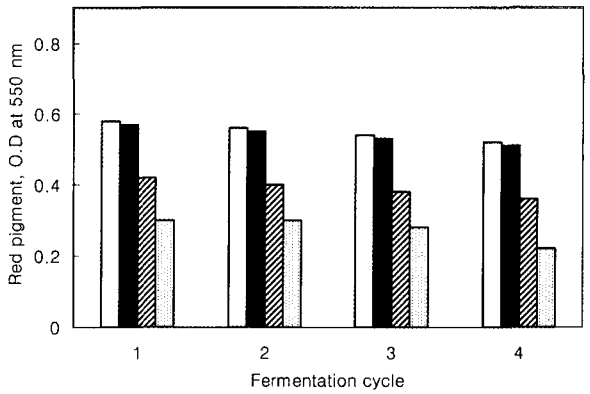

Fig. 4. Effects of aeration volume in the semicountinuous fermentation of red pigment protuction by immoboilized *Bacillus.* BH-99. One fermentation cycle took on every 72 h. □, 0.2 mL/min; ■, 0.5 mL/min; ▨1.5 mL/min; ⊡.0 mL/min df.

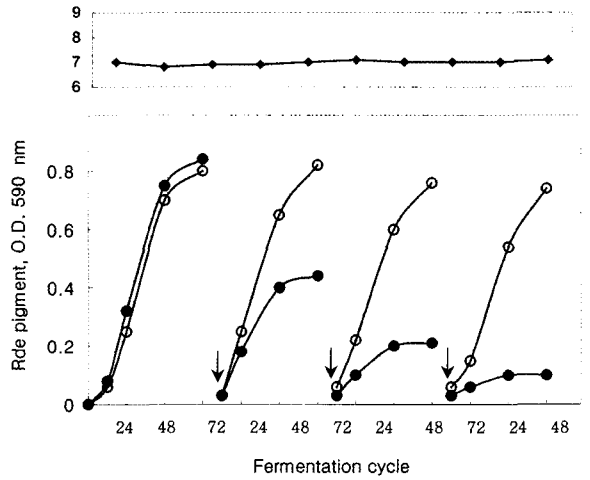

Fig. 5. Semicountinuous fermentation of red pigment production by free cells and immoboilized cells of *Bacillus* sp. BH-99. ○, free cells; ●, immoboilized cell; ↓, exchange of fresh production.

red pigment production was not observed while aeration increased over 2.0 mL/min. When aeration were passed though 2.0 mL/min, the immobilized gels surface were damaged by powerful circulation through increasing the aeration rate in the fermentor [26,27]. This results were accordance with immobilized cells systems [23,24].

Semicontinuous Red Pigment Production by Immobilized *Bacillus* sp. BH-99

Red pigment production was carried out by semicontinuous fermentation using immobilized *B. subtilis* BH-99 cells. In this experiment, red pigment production were performed the fermentation cycles of fourth steps. Each fermentation cycle took for 72 h and then fresh production media was exchanged at each 72 h during fermen. Fig. 5 showed semicontinuous fermentation by *Bacillus* sp. BH-99 free cells or in calcium alginate beads

in the bioreactor. The productivity of the red pigment by *Bacillus* sp. BH-99 which immobilized gels in comparision with the same amount of free cells. The immobilized cells produced almost same levels of red pigment production as the free cells, but in case of free cells, red pigment rapidly and signigicantly decreased the second, third and fourth fermentation cycle except for first fermentation cycle. Immobilized cells could be kept at a minimum growing state within the gel matrice by continuous supply suitable nutrient and also could have retained enzyme activities for a long time. Generally, immobilization cells system is very useful technique than other free cell system in fermentation. It follows from results obtained that the immobilized *Bacillus* sp. BH-99 cell retained both a sufficient activity of basic synthetic pathway and physiological stability required for the production of red pigment production even after a long term semicontinuous cultivation.

Immobilized *Bacillus* sp. BH-99 cells grew relative continuously and the elasticity of the alginate beads could resist the pressure of the growing cells which maintained stable beads when four fermentation cycles were investigated for red pigment prodction.

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