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Semicontinuous Production of Blue Pigment from Gardenia Fruit by Immobilized Cells of *Bacillus subtilis* KS-380 Using Air Bubble Column Reactor

Beung-Ho Ryu* and Kyung-Ja, Cho**

**Department of Food Science and Engineering, Kyungsung University, Pusan 608-736, Korea.*
***Department of Home Economics, Kyungsung University, Pusan 608-736, Korea*

Abstract

The semicontinuous production of blue pigment from gardenia fruit by immobilized cells of *Bacillus subtilits* KS-380 which excreted β-glucosidase was investigated in comparison with free cells. The blue pigment produced highest productivity under the conditions of aeration of 0.2mℓ/min and 2mm diameter of gel beads by using 3.5% sodium alginate. Semicontinuous production by immobilized cells showed the highest productivity with replacement of fresh production medium in every 24hr for fourth fermentation cycle following the conditions of blue pigment productivity.

Introduction

Gardenia fruits contain three major groups of pigment such as carotenoids, iridoids and flavonoids[1]. The carotenoid and related compounds develop in the fruit of gardenia during the 8-23 weeks of growth, but the iridoid pigment develop 1-6 weeks after flowering. The iridoid pigments are known gardenoside, geniposide, shanzhiside, gardoside, methyldeacetylasperuloside, genipingentiobioside, geniposidic acid, acetylgeniposide and scandoside methylester[2]. The third group comprises a series of flavonoid compounds as illustrated by the five flavonoid colorant of gardenia fruits tend to be similar in closely related yellow[3,4], but their contribution to the overall color of gardenia preparation is unclear. Although the production of food colorants from gardenia is being investigaed at the present time, the mechanism is unclear [5]. A series of colorants to be produced which vary from yellow to green, red, violet or blue. Most gardenia colorants involved by hydrolysis of β-glucosidase. Several patents involved culturing preparations of pigment by gardenia fruit with microorganisma such as *bacillus subtilis*[6], *aspergillus japonicus or rhizopus* sp[7]. The blue pigment also produced to react with genipin and amino acids, and the genipin hydrolyze from geniposide of gardenia by treatement of β-glucosidase which excreted from microorgnisms[9,10,11]. Especially, the blue pigment produced from gardenia fruits is very stable under various conditions[8]. Recently, there has been much interest in the application of immobilized microorganism to food production because of shorter time of fermentation, high production efficiency, and conventional operation[12-16].

In this papers, in order to development of blue pigment by using materials of gardenia fruit, an attempts were performed to immobilized to the growing cells excreated β-glucosidase from *bacillus subtilis* KS-380 and immobilized systems applied to the produce the blue pigment from gardenia

fruit by using air bubble column fermentor.

Materials and Methods

Microorganism

Bacillus subtilis KS−380 from the stock culture of our laboratory was used and maintained with LB broth.

Materials

Gardenia fruit was obtained commercially.

Isolation of geniposide and genipin

Commercially obtained gardenia fruit was extracted with water. After extracts loaded into charcoal column, eluted with 100% methanol from charcoal column and geniposide was eluted with 85% chloroform in methanol from silica gel column[17, 18].

Genipin in gardenia fruit was hydrolzed with β-glucosidase extracted from *bacillus subtilis* KS−380 and its extracted with ether and then recrystalized with methanol[18]

Analytical methods

The concentrations of geniposide and genipin were measured by high performance liquid chromatography(HPLC) on ODSA column with UV detector(240nm)[9]. Elution was carried out with 43% methanol at a flow rate of 1mℓ/min. The level of blue pigment was measured by optical density at 590nm using spectrophotometer[11](Shimadzu, UV 240).

The cell mass, genipioside and genipin concentration in the gel bead were measured by the method described above after the beads were dissolved in phosphated buffer(pH 7.5) with stirrer bar.

Determination of β-glucosidase activity

Bacillus subtilis was preincubated in YPD medium(1% yeast extract, 2% pepton, 2% dextrose, 0.02% $CaCl_2$) for 24hr at 30℃. After preincubation, the cells were suspended with 1.0×10^7/mℓ in YPG medium(1% yeast extract, 2% peptone, 2mM geniposide, 0.02% $CaCl_2$) and then incubated at 30℃ for 30min.

The culture broth was filtered through a millipore filter(0.45μm). Geniposide was added to produce a concentration of 20mM in 1mℓ broth. Reduced geniposide was measured by HPLC after 60 min at 30℃. One unit of β-glucosidase activity was defined as the amount of enzyme degrading 1 μmole of geniposide per min at 30℃[11].

β-glucosidase activity in resting cell

The culture broth was centrifuge at 3000rpm for 15min. Cell were washed twice with sterilized water and then 0.05M phosphated buffer(pH 5.5) containing geniposide(20mM) was added to the cells. The decrease in geniposide was measured by HPLC after 60min at 30℃.

Immobilization of *Bacillus subtilis* KS−380

The cells were harvested in the late exponential phase of growth by centrifuge at 3000rpm for 5min. The havested cells were mixed with appropriate concentration of each matrices in suspension. The total suspension was extruded into a gently stirred 0.1M $CaCl_2$ solution using a syringe. The mean diameter of the resulting each matrices gel beads was 2~6mm. The beads were stored at 4℃ for solidification before use.

Semicontinuous production of blue pigment by immobilized *Bacillus subtilis* KS−380 cells

Semicontinuous fermentation was carried out in a air bubble column fermentor with 200mℓ working volume(Fig. 1). Calcium alginate beads(200 mℓ) containing immobilized *B. subtilis* KS−380 was transferred to the sterilized fermentor. For semicontinuous fermentation, YPD medium containing 35mM gardenia fruit of water extract was fed semicontinuously at a flow rate of 80mℓ per

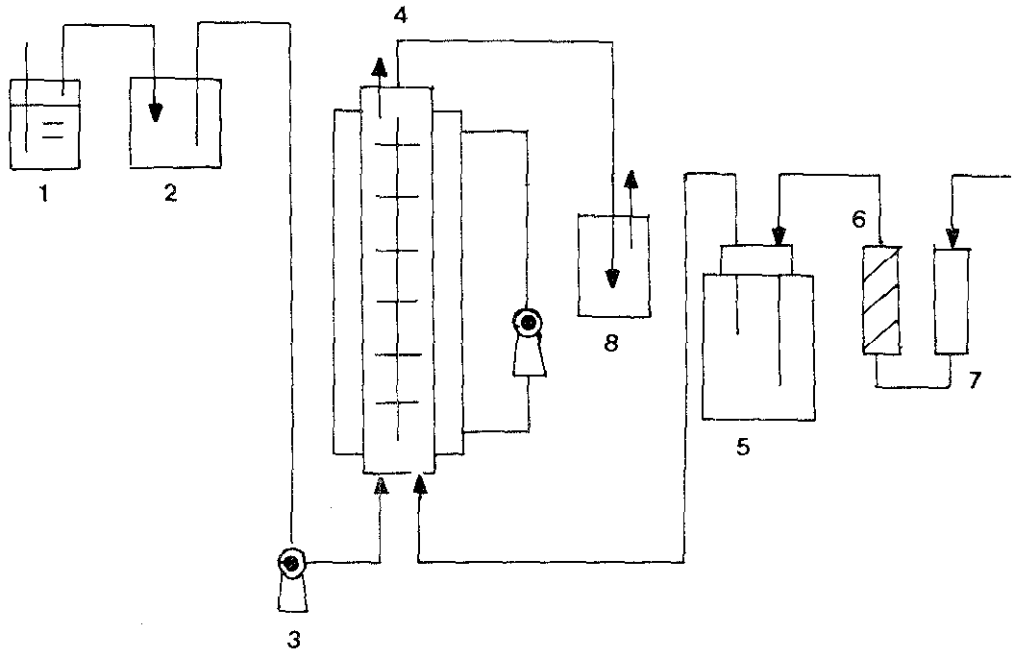

Fig. 1. Schematic diagram of continuous-air-lift tube fermentor.
1. Bottle with conc-H_2SO_4
2. Medium reservoir
3. Peristaltic pump
4. Fermentor
5. Humidifier
6. air filter
7. Rotameter
8. Product reservoir

day. Products were pumped out through a plastic filter(0.5mm opening, 4㎝ diameter). Temperature was maintained at 30℃. The fermentor was stirred by sterilized air bubble using pump.

Results and Discussion

β-Glucosidase activity of *Bacillus subtilis* KS-380

B. subtilis KS-380 was cultured aerobically at 30℃ in the standard medium containing geniposide at a concentration of 30mM. Fig. 2 showed the growth and β-glucosidase activity excreted from *Bacillus subtilis* KS-380 as a function of incubation periods. β-glucosidase activity of the growing cells calculated from the time course of the rate of decrease of geniposide in the medium.

The maximum cell growth was reached after 15hr of incubation and this level was retained for the following 24hr. At that time, β-glucosidase acti-

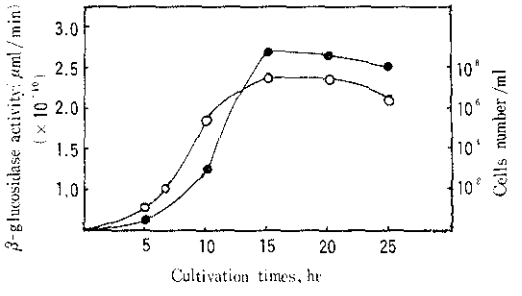

Fig. 2. β-Glucosidase activity and cell growth of *B. subtilis* KS-380 during incubation periods at 30hr.
Incubation was carried out in the YPG medium containing 30mM geniposide.
○——○ : β-Glucosidase
●——● : Cell mass

vity was appeared at the highest level of 2.7×10^{-10} μmol/cell on the 15hr.

Prolonged incubation failed to cause a further increase in the enzymatic activity. This apparent increase in β-glucosidase activity was due to cell caltivation[19].

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[20-22].

In order to select the most suitable matrices for the immobilization of *B. subtilis* KS-380 cells, the productivities by immobilized cells of various matrices were investigated. The same amounts of cells(0.5g wet weight) were immobilized in agar, polyacrylamide, calcium alginate and K-carrageenan gel. As shown in Table. 1, the amounts of blue pigment showed the highest productivity by cells immobilization in calcium alginate. It is well known that the enzyme activity in cells entrapped in calcium alginate is generally high and stable[22]. Calcium alginate in generally very stable with standard packing pressures without extensive damage[21, 23].

Therefore, calcium alginate was chosen as the most favorable matrice for immobilized cells.

Effect of particle sizes of immobilized gel of *B. subtilis* KS-380

The mechanical stability of the immobilized particles, it affected by the entrapped microorganisms as well as by the cultivation method[23, 24].

Table 1. Blue pigment productivity of immobilized *B. subtilis* KS-380 cells by various polymer metrices

Wet cells (g)	Concentration of matrices for immobilization %		Blue pigment (*O. D. at* 590*nm*)
0.5	Ca-alginat,e	3.5	0.74
0.5	Agar,	2.0	0.42
0.5	Carrageenan,	2.5	0.48
0.5	Polyacrylamide,	6.0	0.60

As shown in Fig. 3, effects of immobilized gel sizes of immobilzed cells on the blue pigment production was investigated. This experiment was carried out for efficiency of blue pigment production by the gel sizes of immobilized cells which made the diameter of 2mm, 4mm, and 6mm, respectively. The rate of blue pigment production with the 2mm diameter of gels was higher than 4mm and 6mm during fourth fermentation cycles. Recent results indicated that metabolic activities of immobilized microorganisms may depended on the particle surface[23-25]. Calcium alginate bead of immobilized cells was found more productivity at particle sizes diameters of 3mm and 1.5mm[26].

Eikemeir and Rehm[27] indicated much more productivity that calcium alginate entrapped cells at 3mm and 1.5mm 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 blue pigment, 2mm 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.

Effect of air volume

Effects of aeration volume on blue pigment production were carried out by varying the aeration volume of 0.2mℓ/1, 0.5mℓ/1, 1.5mℓ/1 and 2.0mℓ/1 for fourth fermentation cycles. As shown in Fig. 3, increases in the air flow rate were increased in blue pigment production by the immobilized cells through the fermentation cycles. But increase of blue pigment prodution was not observed while aeration increased over 2.0mℓ/min. When aeration were passed through 2.0mℓ/min, the immobilized gels surface were damaged by powerful ciculation through increasing the aeration rate in the ferme-

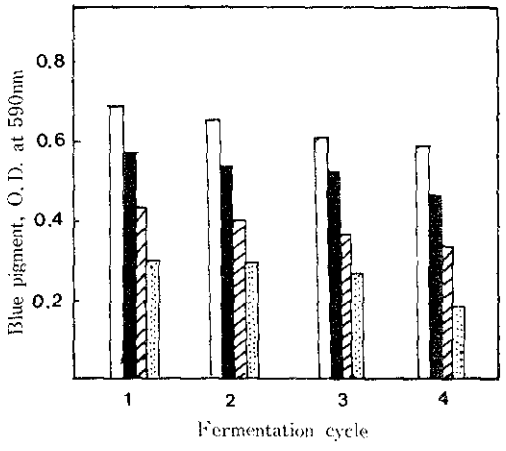

Fig. 3. Effect of aeration volume in the semicontinuous fermentation of blue pigment protuction from gardenia fruit by immobilized *B. subtilis* KS−380.
One fermentation cycle took on every 24hr.
□ ; 0.2mℓ/min ■ ; 0.5mℓmin
■ ; 1.5mℓ/min ■ ; 2.0mℓ/min

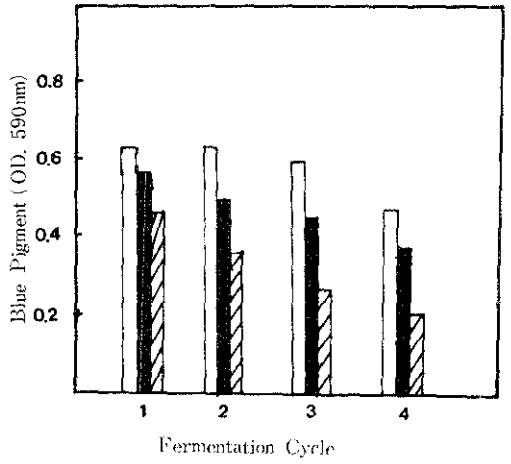

Fig. 4. Effect of immobilized cells sizes in blue pigment production from gardenia fruit by immobilized *B. subtilis* KS−380. One fermentation cycle took on every 24hr.
□ ; 2mm ■ ; 4mm ■ ; 6mm

ntor[25,27]. This results were accordance with immobilized cells systems[22,25,26].

Time courses of blue pigment production and geniposide hydrolysis by immobilized cells of *B. subtilis* KS−380

1.0×10^8cells/mℓ of *B. subtilis* KS−380 were immobilized in calcium alginate and cultivated aerobically in YPG medium. Fig. 4 showed the time courses of cell number and blue pigment production, genipin and geniposide concentration in the medium and gel beads. The living cell number increased gradually in the cells of calcium alginate beads. Particularly, cell number showed highest level on the 16h after begining fermentation. When culture broth incubated under the optimum conditions, the blue pigment appeared after 4hr, and highest yield of pigment showed on 14hr after incubation. The geniposide concentration and gradually decrease for 24hr. On the other hand, genipin concentration increased continuously for 16hr and began to decrease gradually after 16hr. This

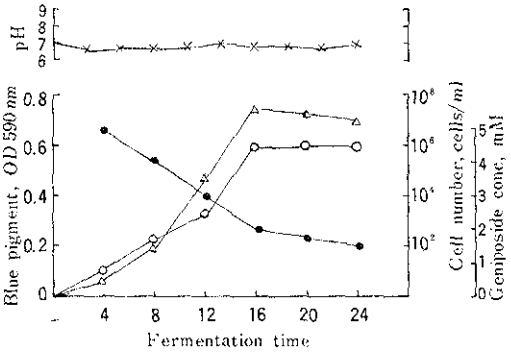

Fig. 5. Time course of blue pigment concentration, cell number and geniposide during fermantation times of immobilized *B. subtilis* KS−380 cells.
○——○ ; Blue pigment concentration
●——● ; Geniposide conc.
△——△ ; Cell number

result indicated that this cell converted to glucose and genipin by hydrolysis of geniposide in gardenia fruit by β-glucosidase[9,10].

Semicontinuous blue pigment production by immobilized *B. subtilis* KS−380

Blue pigment production was carried out by se-

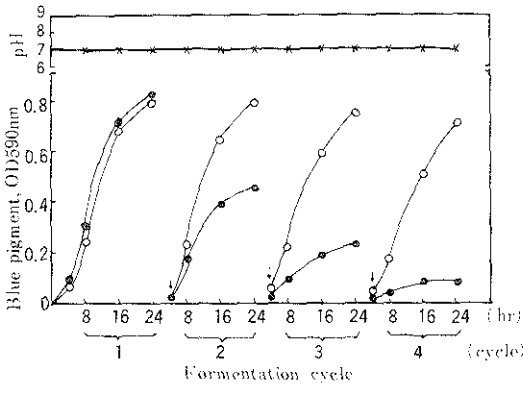

Fig. 6. Semicontinuous fermentation of blue pigment production by free cells and immobilized cells of *B. subtilis* KS-380.
○——○ : free cells
●——● : immobilized cell
↓ : Exchange of fresh production

micontinuous fermentation using immobilized *B. subtilis* KS-380 cells. In this experiment, blue pigment production were performed the fermentation cycles of four steps. Each fermentation cycle took for 24hr and fresh production media was exchanged every 24hr. Fig. 6 showed semicontinuous fermentation of *B. subtilis* KS-380 with or without entrapment in calcium alginate beads in the air lift fermentor. The productivity of the blue pigment from gardenia fruit by *B. subtilis* KS-380 which immobilized in gels comparision with to the same amount of free cells, The immobilized cells produced almost same levels of blue pigment production as the free cells, but in case of free cells, blue 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 of suitable nutrient and also could have retained enzyme activities for a long time. Generally, immobilization cells system in very useful technique than other free cells system in fermentation.

This result was accordance with some other reports[20,23,25,27]. It follows from results obtained that the immobilized *B. subtilis* KS-380 cell retained both a sufficient activity of basic synthetic pathway and physiological stability required for the production of blue pigment production even after a long term semicontinuous cultivation.

Immobilized *B. subtilis* KS-380 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 blue pigment production.

Acknowlegement

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Air Bubble Column Reactor를 이용하여 *Bacillus Subtilis* KS-380의 고정화에 의한 치자로 부터 청색 색소의 생산

류병호*, · 조경자**

*경성대학교 공과대학 식품공학과
**경성대학교 이과대학 가정관리학과

요 약

치자로 부터 청색 색소를 생산하기 위하여 β-glucosidase를 분비하는 *Bacillus subtilis* KS-380을 고정화시켜 air bubble column reactor를 이용하여 free cells와 고정화 세포로서 반연속식 발효를 실시하였다. 청색 색소의 생산은 *Bacillus subtilis* KS-380 균체를 3.5% 알긴산 소다로 고정화시키고 gel bead는 2mm크기로서 0.2㎖/min로 aeration 하였을 때 생산성이 가장 우수하였다. Air bubble column을 이용하여 최적 생산 조건에서 매회 24시간 간격으로 발효시 마다 새로운 생산 배지를 공급하면서 4회 반연속적으로 생산한 결과 free cells 보다는 매우 높은 생산성을 나타내었다.