Thesis for the Degree Master of Science

Enhancement of the astaxanthin production in *Paracoccus haeundaensis*

by

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The Graduate School

Pukyong National University

February 2011

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*Paracoccus haeundaensis*로부터 Astaxanthin 생산 최적화 연구

Advisor : Prof. Young Tae Kim

by

Hee Ju Hwang

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ABSTRACT

Astaxanthin represents a group of valuable molecules for pharmaceuticals, food and feed industries. In order to enhance the production of astaxanthin in *Paracoccus haeundaensis*, we have used the change of the culture conditions that change culture temperature, addition of carbon/nitrogen sources and

hydrogen peroxide on *Paracoccus haeundaensis*. The changes of culture temperature were not very much influenced by the production of astaxanthin. When carbon sources (lactose, fructose, glucose and mannitol), and nitrogen sources (sodium nitrate and potassium nitrate) were used, the production of astaxanthin was increased. The optimal C/N ratio was 3% carbon and 1% nitrogen, the astaxanthin production was 1.5 folds increased (641.17μg/g to 973.12μg/g dry cell weight (DCW)). The cultures were supplemented with eight levels of hydrogen peroxide and carried out in shaken flasks and 3 L aerated fermentors, respectively. When hydrogen peroxide was 1%, the product of astaxanthin was the highest. When H_2O_2 and C/N sources were the simultaneous added, approximately 1.83 folds (1019.16μg/g DCW) the production of astaxanthin was increased. Cultures of *Paracoccus haeundaensis* were exposed to mutagens such as UV and ethylmethanesulfonate (EMS). The results showed that the survival rate of the cells was decreased with the increase in UV exposure time and obtained in EMS concentration. The astaxanthin hyperproduction mutant was PUE by UV irradiation (20 min exposure) with EMS treatment (0.4M concentration). The isolated mutants exhibited an increase of 3.82 folds in astaxanthin contents compared to the production of original strain. A genetically stable mutant stain obtained using mutagen (UV and EMS) may be a suitable candidate for the industrial scale of astaxanthin production.

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INTRODUCTION

Carotenoids are group of over 600 molecules which can be found in most life forms and fulfill diverse functions, ranging from their original evolutionary role as photosynthetic or light-quenching pigments to antioxidants, precursors of vitamin A, or pigments in the visual attraction of animals such as flower pollinators or mating partners [1].

Astaxanthin (3,3'-dihydroxy-b,b-carotene-4,4'-dione) is a pigment that belongs to the family of the xanthophylls oxygenated derivatives of carotenoids. Astaxanthin is a natural carotenoid pigment which has powerful biological antioxidant properties exhibiting strong free radical scavenging effect [2, 3, 7]. Astaxanthin has been widely used as a pigment in aquaculture and food industries and has applications in pharmaceuticals and nutraceuticals due to its beneficial effects on human health such as functions of antioxidants [4, 14], an involvement in cancer prevention [5] and enhancers of immune responses [6]. Previously, we reported that the isolation of a new marine bacterium, *Paracoccus haeundaensis*, which produces carotenoids, mainly astaxanthin [11] and the characterization of the astaxanthin biosynthesis gene cluster, consisting of six carotenogenic genes, isolated from this organism [12]. In addition, the molecular characteristics including enzymatic activities were also reported [13]. The biosynthetic pathways for the production of astaxanthin from FPP (Farnesyl pyrophosphate) were shown in Figure 1.

The controlling factors of carotenogenesis in organism are various, such as light, temperature, carbon/nitrogen (C/N) source ratio, solvent, metal ions

and salts, etc. Carotenogenesis in many organisms are regulated by light [17, 18, 19]. Also, temperature is an important factor affecting the production of carotenoids. It brings about changes in carotenoid biosynthesis pathways [20, 21]. The presence of a suitable carbon source is important for carotenoid biosynthesis during stationary phase, and the astaxanthin production was enhanced by a high initial C/N ratio in the medium, whereas a lower C/N ratio was suitable for cell growth [22, 23].

Induction and selection of mutants have been widely employed for strain improvement and used to unravel pathways [9]. Mutant strain with improved growth rate and enhanced astaxanthin accumulation makes the commercial process of astaxanthin production more feasible. A mutant of *Haematococcus pluvialis* and *Phaffia rhodozyma* has been obtained by UV exposure and by EMS treatment for hyperproduction of astaxanthin [8, 14]. However, there is little information available on astaxanthin-hyperproducing mutants of *Paracoccus haeundaensis*. In the present study, in order to enhance the production of astaxanthin for the wild-type strain, an attempt was made to obtain mutants of *Paracoccus haeundaensis* by exposure to UV and by EMS treatment. A genetically stable mutant stain obtained from this study may be applied for upscale production of astaxanthin.

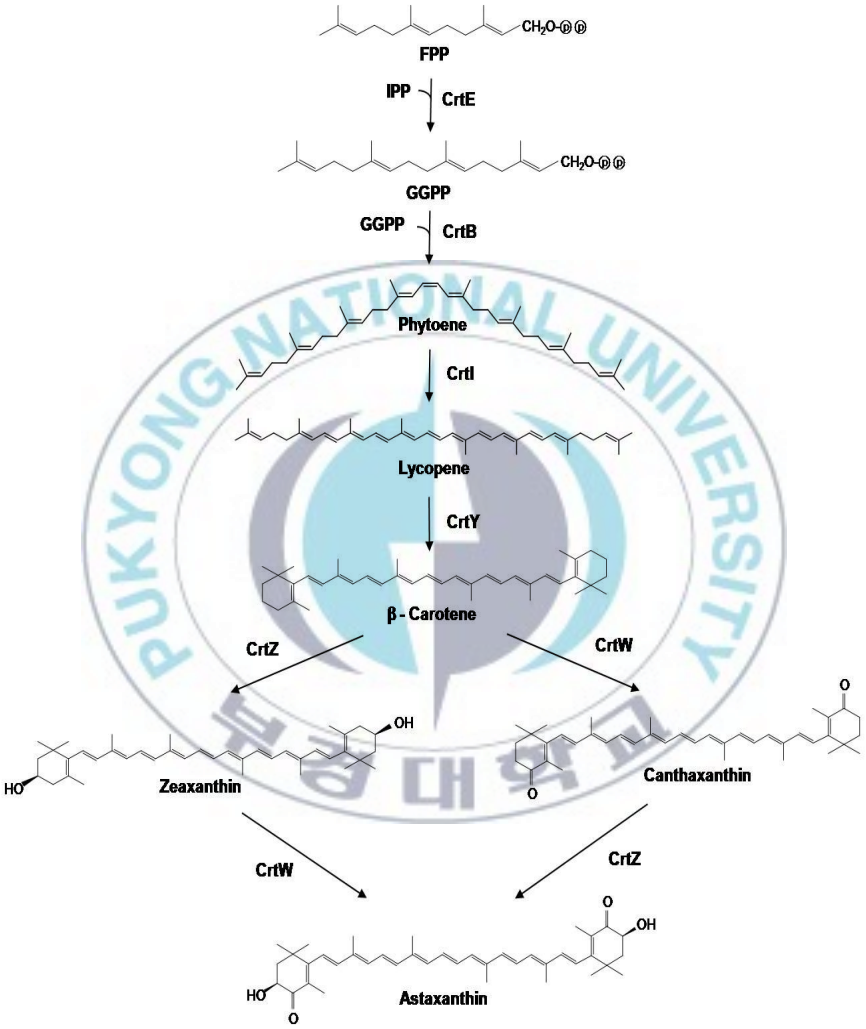


Figure 1. The astaxanthin biosynthesis pathways.

MATERIALS AND METHODS

Microorganisms and seed culture condition

Paracoccus haeundaensis was isolated from Haeundae coast of Korea and described in the previous study [11]. The strains were cultivated under 12 h light/12 h dark illumination conditions at 25℃ on the PPES Ⅱ medium, In order to test the optional production of astaxanthin, we have also used LB (Luria-Bertani) medium containing 3% NaCl.

The basic culture condition

In the flask batch culture, 3 ml of the preculture broth ($Absorbance_{600}$ 0.5~0.6) was inoculated into a 250 ml Erlenmeyer flask containing 100 ml of LB medium (3% NaCl) at pH 8.0. The flask batch culture was performed with shaking at 150 rpm on shaking incubator at 25°C.

In the batch culture, the preculture broth (90 ml) was inoculated into a 5 L jar fermentor containing 3 L of LB medium (3% NaCl) and initial pH controlled at 8.0. The temperature was controlled at 25°C. An aeration rate was 1.0 vvm and an agitate speed was 150 rpm [19].

The variation of incubation temperature in culture condition

The basic medium for fermentation of *Paracoccus haeundaensis* was LB medium containing 3% NaCl at pH 8.0. Exponentially growing cells (3 ml) tube were inoculated into the 250 ml Erlenmeyer flask containing 100 ml of LB medium (3% NaCl). Agitation speed was maintained at 150 rpm.

In some of microorganism that produced astaxanthin, cultured higher temperature after growth phase, before cultured on optimal temperature, that produced more astaxanthin than optimal culture condition [24].

The initial culture temperature was controlled at the set 25℃, 30℃, 37℃. Cell was changed after 1 day or 2 days of incubation from 25 to 30 or 37 to investigate the effect of temperature shift.

The addition of carbon sources

The basic medium for *Paracoccus haeundaensis* fermentation was LB medium (3% NaCl) at pH 8.0. To investigate effects of adding various carbon and nitrogen sources on the astaxanthin production, added 1% of various carbon sources, such as lactose, mannitol, fructose, maltose, sucrose, glucose, raffinose and galactose, on medium, respectively. After, different concentration of carbon sources, were added to test the effecting different concentration of carbon sources on the LB medium.

The addition of nitrogen sources and C/N ratio

1% of nitrogen sources added on LB medium. The nitrogen sources were used ammonium carbonate, ammonium sulfate, potassium nitrate, sodium nitrate and potassium nitrate. After the test of various concentration of nitrogen source to ues selected one nitrogen source. After that, for the effect of addition carbon/nitrogen ratio, supplemented variable carbon/nitrogen ratio that used carbon source, glucose and nitrogen source, sodium nitrate.

The addition of hydrogen peroxide

Hydrogen peroxide is a reagent inducing reactive oxygen species. To prevent the oxidative damage contain cells produce related antioxidants. Astaxanthin was one of the powerful antioxidant to scavenge the oxidative damage [25].

To further enhance the astaxanthin production of perfusion culture, we have tested a perfusion experiment with the feeding of different concentration of hydrogen peroxide (H_2O_2) to the culture broth at 2days after initial culture. And then this culture sustain during 2days to recover the cell damage induced by hydrogen peroxide.

Mutagenesis and screening of *Paracoccus haeundaensis*

The cell culture (5 ml) of the *Paracoccus haeundaensis* with cell count of 52 x 10^5 cells/㎖ in logarithmic growth phase was exposed to UV irradiation (254 nm) for different periods of time (10, 15, 20, 25, and 30 min) at a

distance of 20 ㎝. The irradiated cultures were kept at 4℃ overnight in the dark prior to the plating. UV irradiation treated cell was spread onto LB medium and incubated at 25℃ temperature during 2 days. Alternatively, the bacterial cells were treated separately with different concentration of EMS solution (0.01 to 0.4 M, v/v) in sterile distilled water and incubated for 15 min at 25℃. After the incubation, the individually treated cell cultures were thoroughly washed twice with 0.2 M phosphate buffer and refrigerated in the dark overnight prior to plating. Suitable dilutions were made and the appropriate dilutions were plated on LB medium and incubated for 2 days at 25℃. The UV exposure time and EMS concentration were selected to get 20% survival. The largest bright orange colonies were selected for investigation of growth rate and astaxanthin content.

Analytical method

The extraction and quantitation of carotenoids from harvested cells were carried out substantially by the methods of An *et al*. [3]. Acetone was used to extract the carotenoid pigments. The acetone extracts were evaporated to dryness and were further extracted with chloroform/methanol (9:1, v/v). After being dissolved in a small volume of methanol, the astaxanthin was analyzed by HPLC using a Nova-Pak HR 6U C_{18} column (3.9 mm X 300 mm) with acetonitrile/methanol/2-propanol (85:10:5, by vol.) at 1 ml/min.

RESULTS

The effect of culture temperature

The optimal culture temperature of *Paracoccus heaundaensis* is 25℃. Cell growth was better on 25℃ and 25℃ to 30℃ shift on last 1and 2days, but cells did not grow well on 37℃ (Figure 2). The result of extracted pigment analysis was also similar to cell growth density (Figure 3).

Some microorganisms, such as yeast (*Phaffia*) and algae (*Haematococcus*), that made astaxanthin produced more astaxanthin when the temperature was shift to higher temperature for period of production. But *Paracoccus haeundaensis* was not effected on the aastaxanthin production when temperature was shifted to higher temperature. Figure 2 and 3 show the cell density and astaxanthin production according to temperature shift, respectively. But the cell density and astaxanthin production was very similar to cultured on optimal temperature one, resulting that *Paracoccus haeundaensis* was not effected on the temperature shifts.

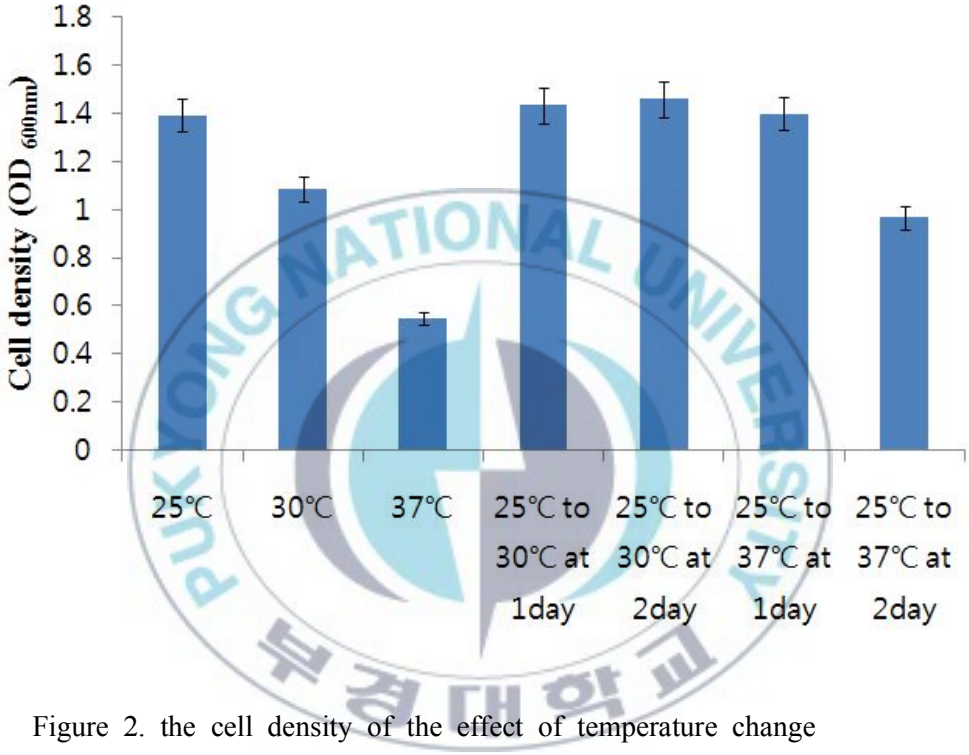


Figure 2. the cell density of the effect of temperature change

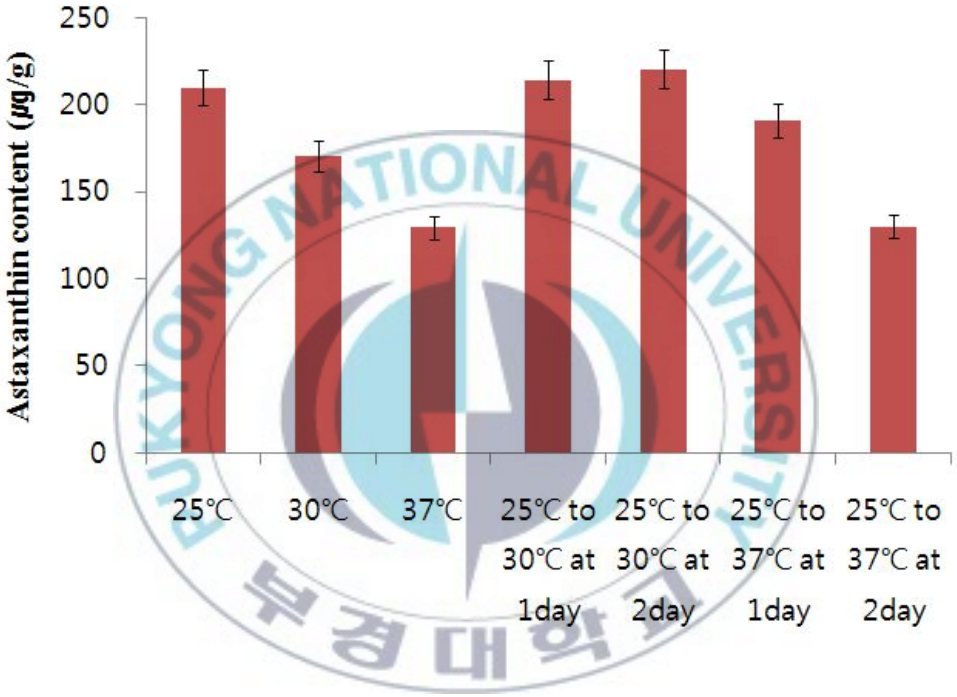


Figure 3. the astaxanthin contents of the effect of temperature change.

The addition effect of carbon/nitrogen sources

Various carbon and nitrogen sources were used to investigate their effects on the growth and astaxanthin production of *Paracoccus haeundaensis*. The total amounts of carbon and nitrogen sources were added 1% up to 10 g/L into the LB medium. The effects of added various carbon sources were shown in Figure 4 and 5. The cell growth was better when the carbon sources (lactose, galactose, sucrose and raffinose) were added but all of these were not to enhance astaxanthin production. The suitable carbon sources were lactose, fructose, glucose and mannitol. The effects of added various nitrogen source were shown in Figure 6 and 7. The potassium nitrate (KNO_3) is better nitrogen source for the culture but the astaxanthin production was better when sodium nitrate ($NaNO_3$) was added.

The effects of carbon and nitrogen sources added were summarized in Table 1 and Table 2. In the carbon source, the addition of glucose concentration up to 3% was able to produce the highest biomass yield, but the highest production of astaxanthin was achieved when the total amount of glucose added 2%. As shown in Table 2, the highest biomass yield was 2% $NaNO_3$ addition, but the best astaxanth production was achieved at 1% $NaNO_3$.

The effects of mixed different ratio of carbon and nitrogen sources were summarized in Table 3. The highest biomass yield was produced when 1% glucose and 4% $NaNO_3$ mixture were added, but the astaxanthin production was lower than that of 1% glucose and 3% $NaNO_3$ addition.

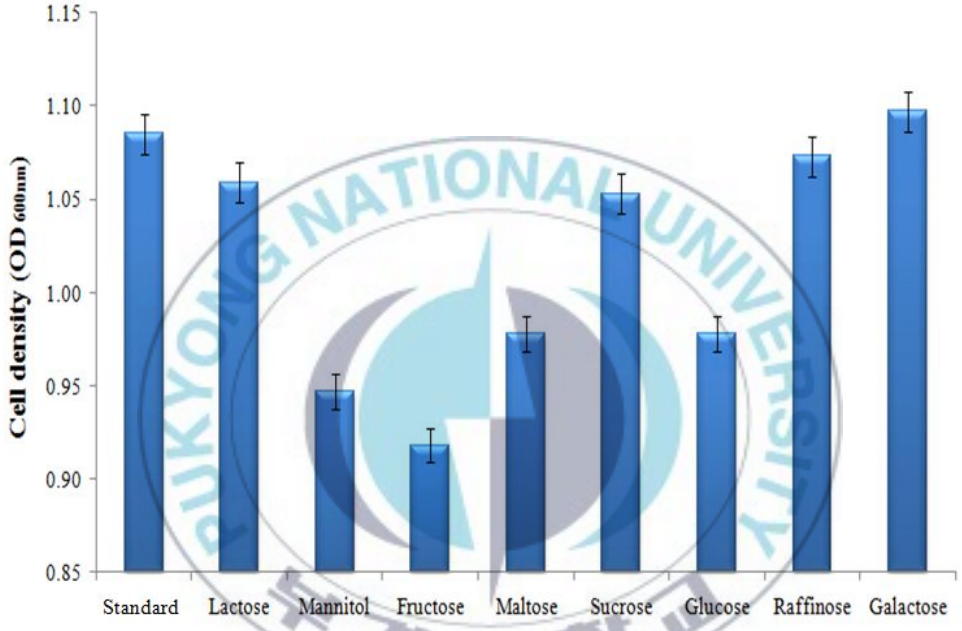


Figure 4. The cell density with addition of various carbon sources

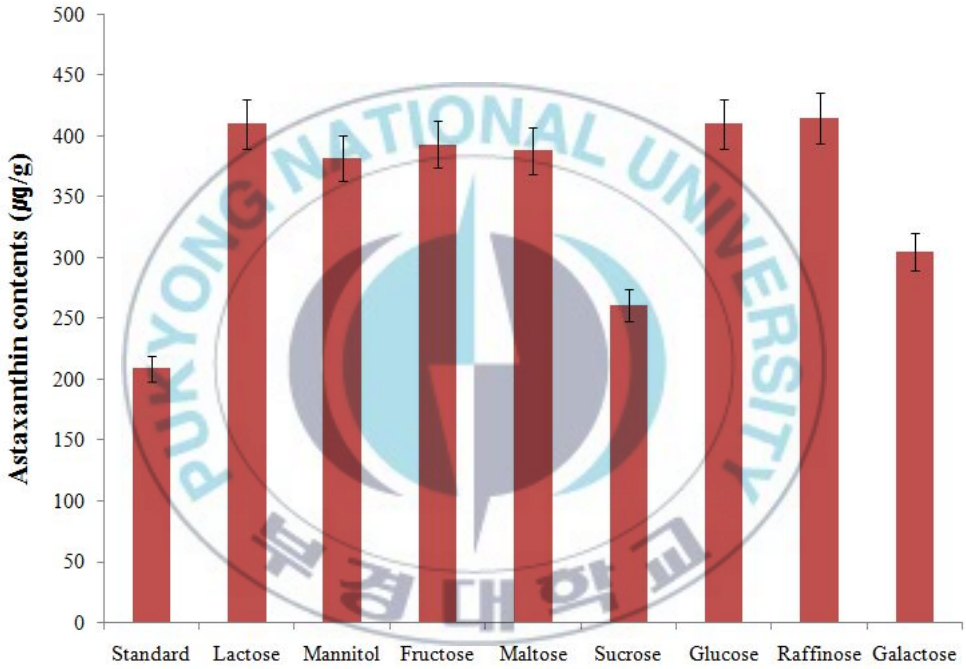


Figure 5. The astaxanthin contents of various carbon sources

Table 1. The cell density and astaxnathin contents with addition of various glucose concentration.

	Dry cell weight (㎎/㎖)	Astaxanthin content (㎍/g)
standard	12.94	639.04
1% glucose	10.62	764.55
2% glucose	9.82	1002.75
3% glucose	8.95	824.74
5% glucose	7.53	749.18
7% glucose	5.30	540.91
9% glucose	1.30	141.11

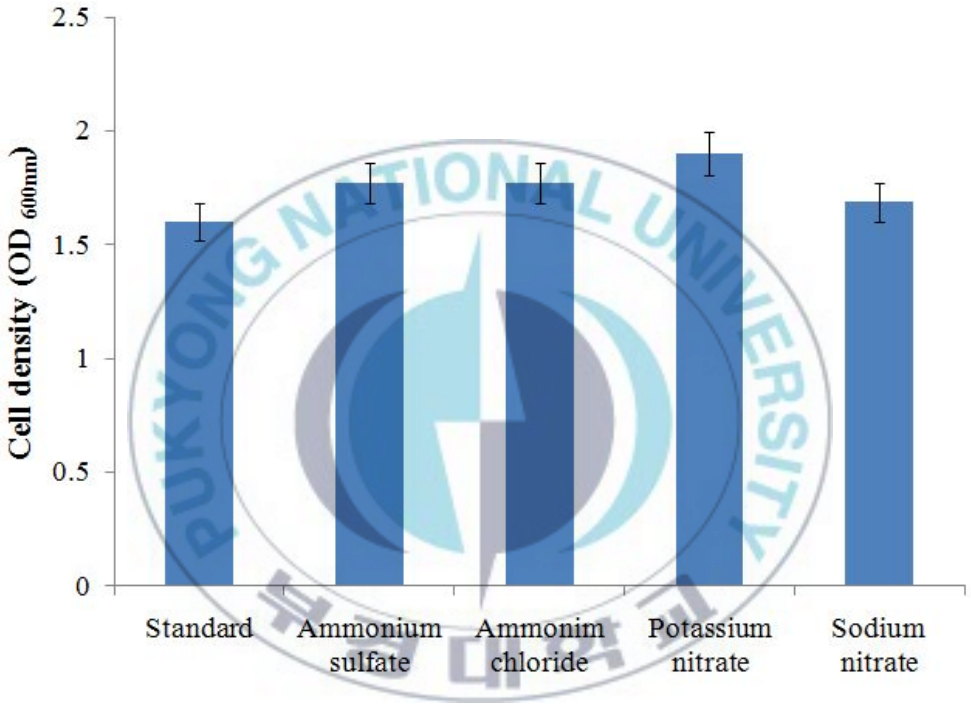


Figure 6. The cell density with addition of various nitrogen sources

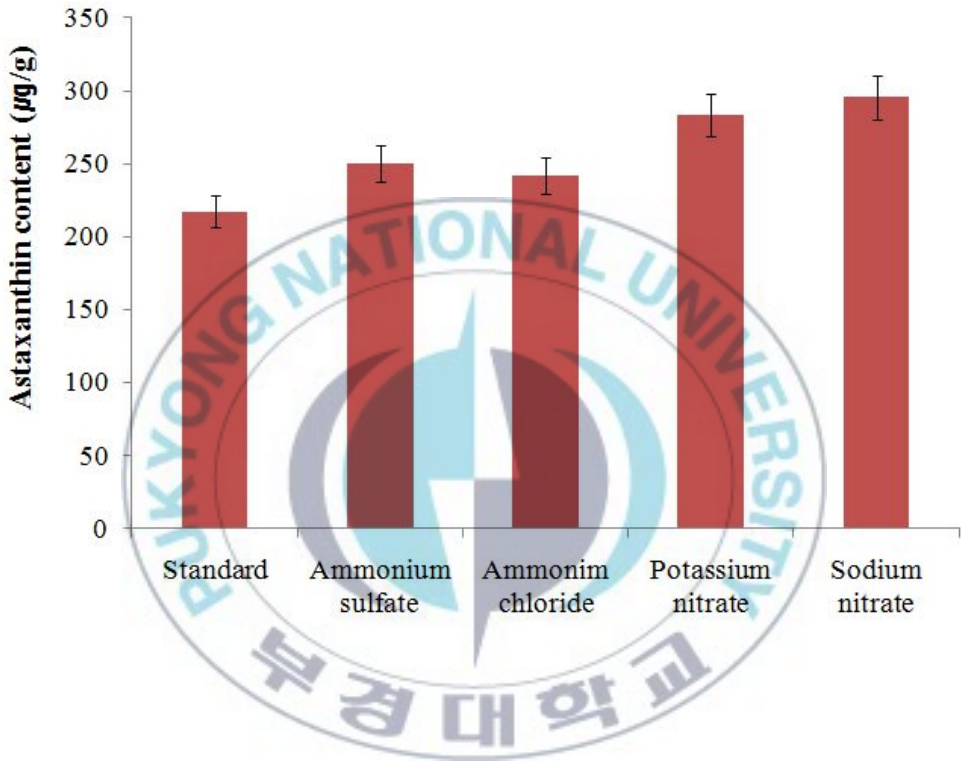


Figure 7. The astaxanthin contents with addition of various nitrogen sources.

Table 2. The cell density and astaxanthin contents with addition of $NaNO_3$ concentrations.

	Dry cell weight (㎎/㎖)	Astaxanthin content (㎍/g)
standard	12.13	662.09
0.25% $NaNO_3$	12.87	665.94
0.50% $NaNO_3$	12.84	674.90
1% $NaNO_3$	13.88	785.68
2% $NaNO_3$	14.25	747.26
3% $NaNO_3$	13.79	637.76
5% $NaNO_3$	1.60	170.33

Table 3. The cell density and astaxnathin content with addition of different C/N ratio.

	Dry cell weight (㎎/㎖)	Astaxanthin content (㎍/g)
standard	11.87	641.17
N1C1	19.25	754.24
N1C2	17.42	943.12
N1C3	17.77	821.70
N2C1	15.90	758.10
N2C3	9.19	792.79
N3C1	11.29	600.05
N3C2	10.02	660.44

standard : not added

N1C1 : 1% sodium nitrate and 1% glucose

N1C2 : 1% sodium nitrate and 2% glucose

N1C3 : 1% sodium nitrate and 3% glucose

N2C1 : 2% sodium nitrate and 1% glucose

N2C3 : 2% sodium nitrate and 3% glucose

N3C1 : 3% sodium nitrate and 1% glucose

N3C2 : 3% sodium nitrate and 2% glucose

The addition effect of hydrogen peroxide

In order to enhance the production of astaxanthin, various chemicals and solvents were added into the culture medium. Hydrogen peroxide was one of chemical that led to oxidative reactive species in the cell.

The effect of different concentrations of hydrogen peroxide (H_2O_2) were shown in Figure 8 and 9. Different concentrations (0% to 3%) of H_2O_2 were tested, but the biomass yield was decreased while H_2O_2 concentration was increased and cell damage by H_2O_2 was not able to restore when H_2O_2 concentration was over 0.3%. The highest astaxanthin yield was achieved when 0.3% H_2O_2 was added. The cell density was decreased about 10% , but the yield of astaxanthin was increased 1.5 folds (641.17μg/g to 973.12μg/g).

Both hydrogen peroxide and carbon and nitrogen sources were simultaneously added, the results were shown in Figure 10. The hydrogen peroxide was decreased cell density by inducing the oxidative reactive species. But the astaxanthin content was higher than not added state, that yield 1.83-fold-increased astaxanthin production.

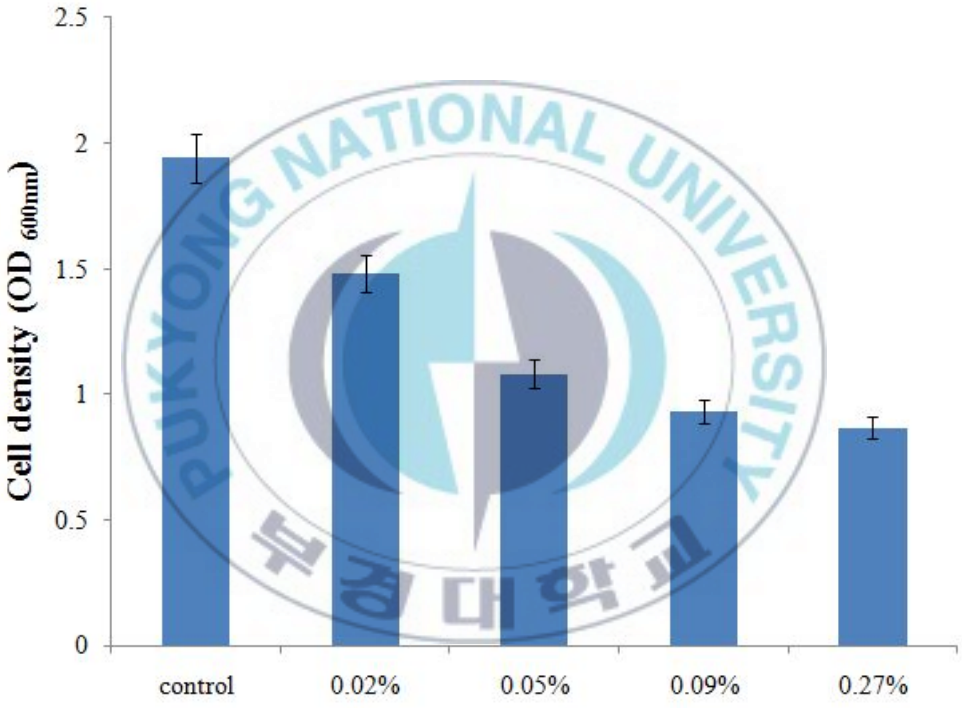


Figure 8. The cell recovery after hydrogen peroxide treatment.

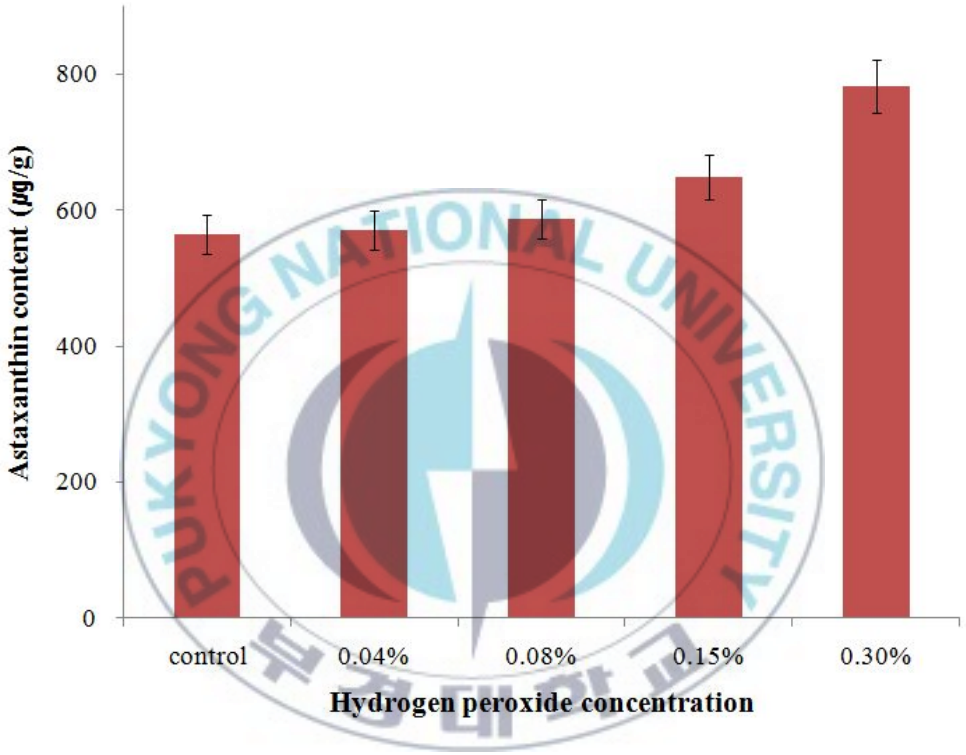
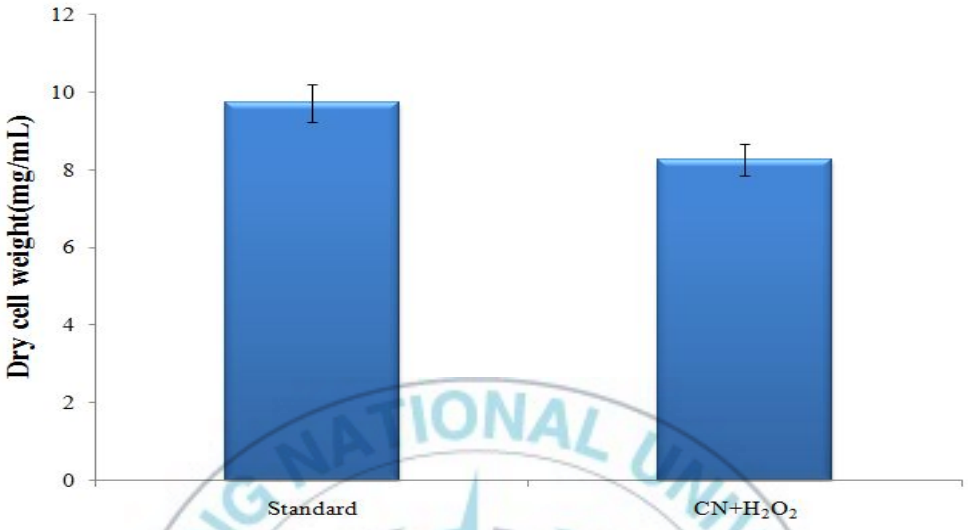


Figure 9. The astaxanthin content with the addition of different hydrogen peroxide concentrations.

(A)



(B)

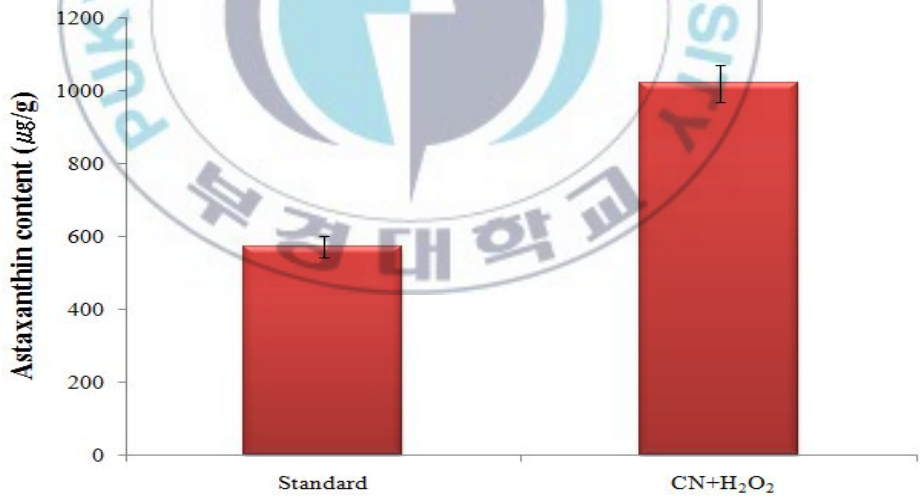


Figure 10. The mixed effects of H_2O_2 and C/N ratio

(A) dry cell weight of *Paracoccus haeundaensis*

(B) comparison of astaxanthin contents

Mutantation on *Paracoccus haeundaensis*

For UV irradiation (254 nm), cultured on 7 levels times. the survival rates according to exposure time were shown in the figure 11. The survival rate was decreased as time passes by and only 4% cell were survived after 30 minute exposure (Figure 11). The highest production of carotenoid and astaxanthin was 229.20 ㎍/g DCW (dry cell weight) and about 31% when exposure time was 20 min (Figure 12).

The effect of EMS solution treatment was shown in Figure 13 and 14. the results showed a maximum survival rate of 95.1% when the cells treated with EMS concentration of 0.1M and the lowest survival rate of 24.1% with 0.4 M (Figure 13). The production of carotenoid and astaxanthin was maximum 209.93 ㎍/g DCW (dry cell weight) and about 27% when EMS concentration was 0.4 M (Figure 14).

The wild type strain and mutant strain were cultured at the optimum culture conditions for 72 h. The results showed that the entire cells were inhibited growth at 24 h but astaxanthin production continuously increased after cell growth stopped (Figure 15).

In this study, we have isolated *Paracoccus haeundaensis* mutants by UV irradiation and EMS treatment, respectively. The astaxanthin hyperproduction mutant was designated to when the cell was mutated with the UV irradiation (20 min exposure) with EMS treatment (0.4 M concentration). The isolated mutant exhibited an increase of 3.82 folds in astaxanthin content compared to the wild type strain. The carotenoid contents was dependent on the level of intracellular activated oxygen species which may induce or activate the enzymes involved in carotenoids biosynthesis [16]. The result indicates that the

mutant producing more carotenoids is more resistant to oxygen radicals than the initial strain by scavenging oxygen radicals. The *Paracoccus haeundaensis* mutant strain (PUE) with a high yield of astaxanthin was obtained by UV irradiation with EMS treatment. This strain might be a suitable candidate for the production of astaxanthin on an industrial scale.

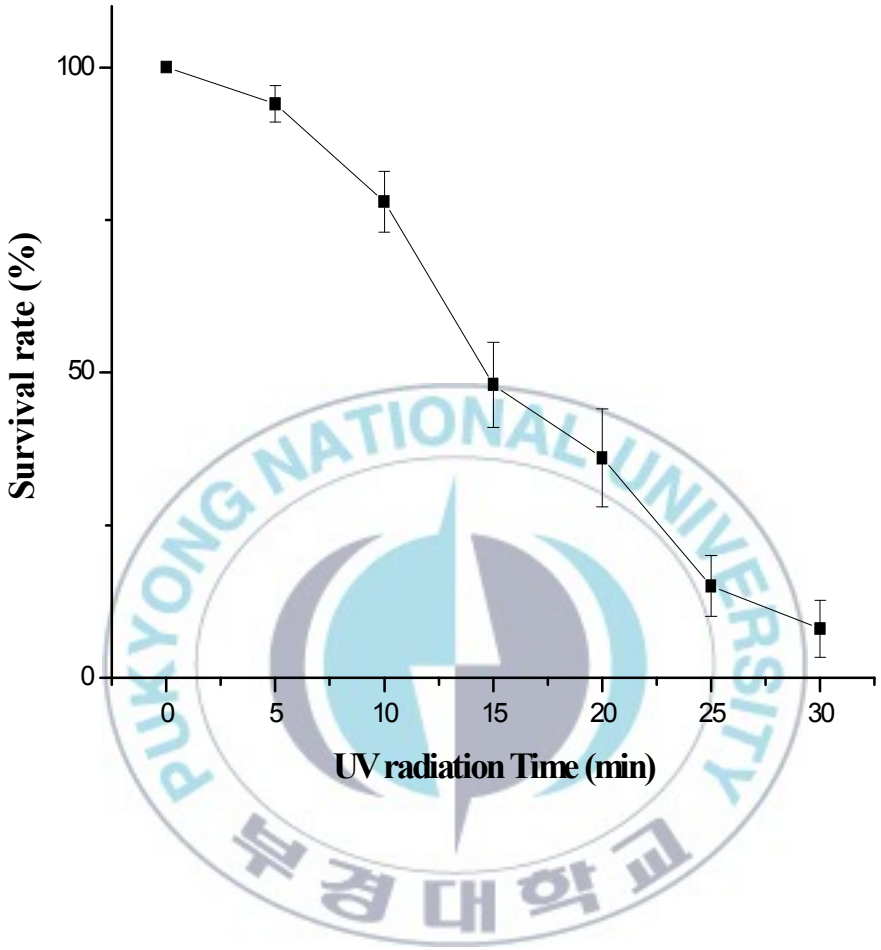


Figure 11. Effect of UV irradiation on cell survival.

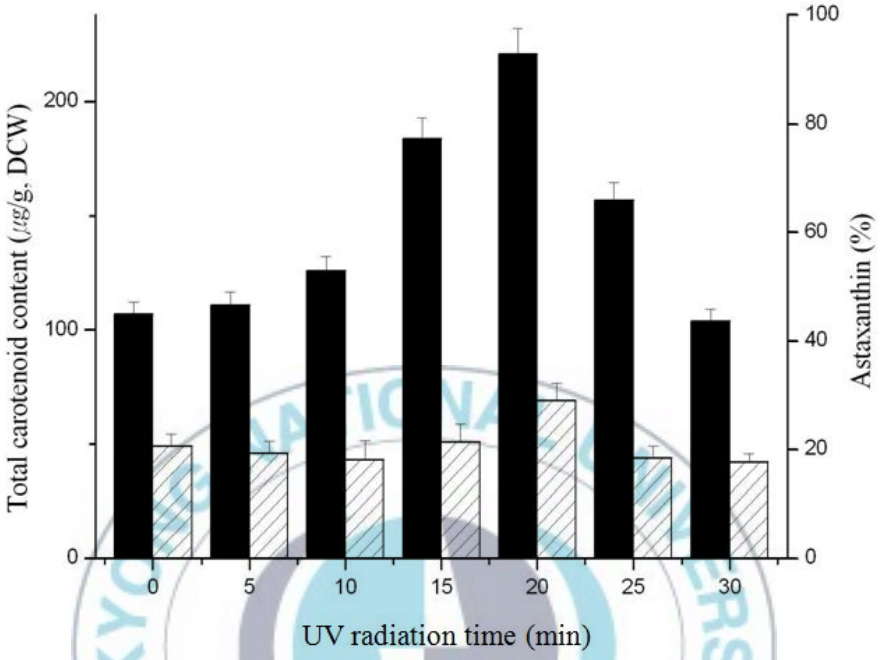


Figure 12. Total carotenoid and astaxanthin accumulation in *Paracoccus haeundaensis*. Total carotenoid(■), astaxanthin % (▨).

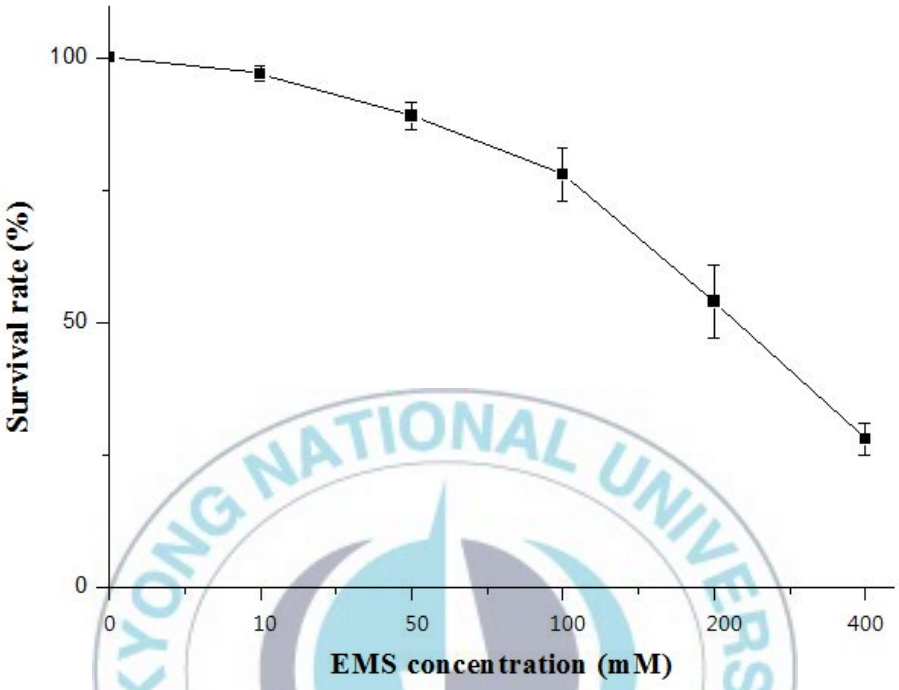


Figure 13. Effect of EMS treatment on cell usurvival

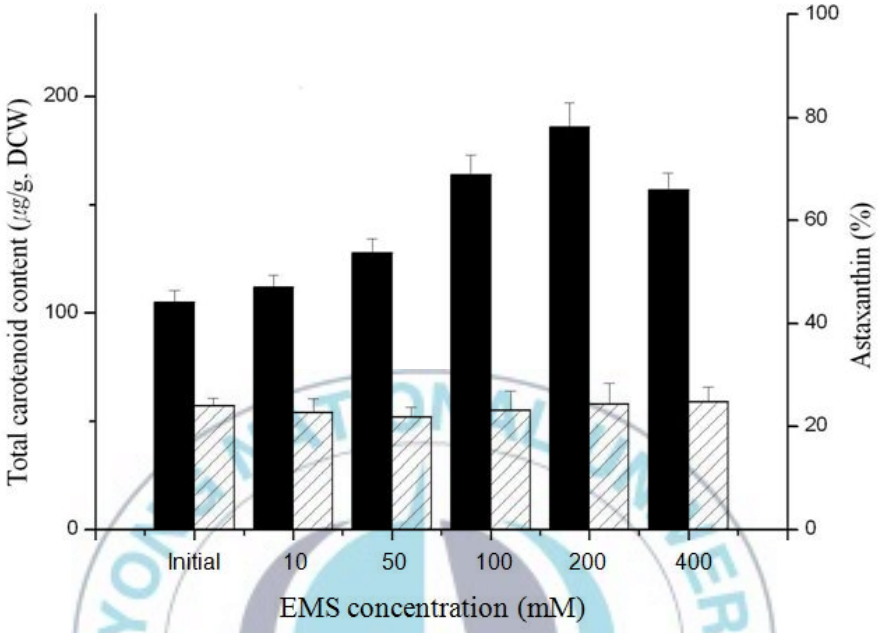


Figure 14. Total carotenoid and astaxanthin accumulation in *Paracoccus haeundaensis*. Total carotenoid (■), astaxanthin % (▨).

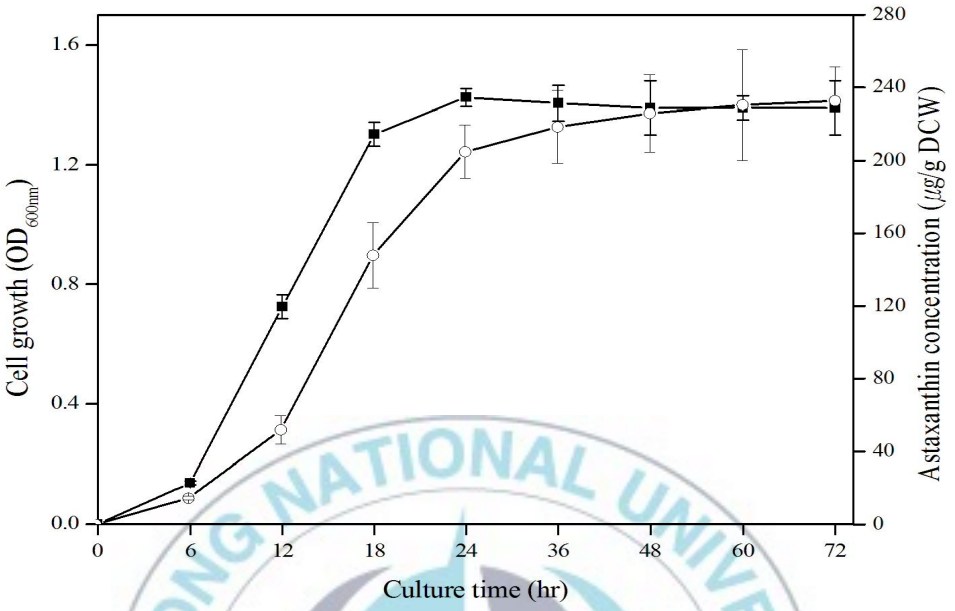


Figure 15. Cell growth curve and astaxanthin production of PUE mutant. (○), astaxanthin production; (■), Cell growth in LB medium.

Table 4. Total coretenois and astaxanthin content of *Paracoccus haeundaensis**

*Bacteria were grown in LB broth at 25℃ for 72 hrs. Analysis of astaxanthin and total carotenoid were performed under subdued light. All the analyses were repeated in triplicate and their averages were employed.

	Total carotenoid (μg/g, DCW)	**Astaxanthin Content (μg/g, DCW)**	**Ratio (%)**
Initial strain[a]	106.20	53.57	50.4
P_u20[b]	229.20	158.40	69.1
P_E400[c]	209.93	121.50	57.9
PUE[d]	261.72	204.84	78.3

[a] Initial strain was *P. haeundaensis*.

[b] Pu20 was obtained by screening UV irradiation for 20 minutes.

[c] P_E400 was obtained by screening EMS treatment (0.4 M).

[d] PUE was obtained by treating the mutant P_E400 with UV irradiation.

DISCUSSION

Astaxanthin is a pigments in nature, that has diverse bioactivity. The high commercial demand for many carotenoids have long been met by chemical synthetic technology, but this compounds that made by chemical synthetic technology has undesirable side effects. So, In now and then this compounds from microbial source has been focus of extensive process.

This research was one focused on the enhanced production of astaxanthin. To enhance the production of astaxanthin various researches were performed such as controling culture condition, addition on culture condition and mutational techniques.

The temperature was a important factor on enzymes in carotenogenesis. The optimal temperature of *Paracoccus haeundaensis* is at 25℃. The results produced by the temperature shift, were not much difference the production of astaxanthin.

The addition of carbon and nitrogen sources was influenced the cell growth and astaxanthin production. (Figure 4-7). When lactose, raffinose and galactose were added to the culture medium, the highest biomass was produced But the astaxanthin production was lower their yield when using lactose, raffinose and glucose as carbon source. When the nitrogen source (potassium nitrate (KNO_3)), was used the highest biomass yield was achieved. But the astaxanthin was quite lower than that of $NaNO_3$ for using nitrogen source. It is indicated that the yield of astaxanthin was not proportional to the biomass. When glucose concentration was increased, the dry cell weight was decreased.

As glucose concentration was increasedover 5%, cell growth was decreased up to 50%. The addition of 2% glucose produced about 0.3 times higher astaxanthin production (Table 1). And the addition 2% of $NaNO_3$, was shown the highest dry cell weight, but astaxanthin content was best on 1% $NaNO_3$ addition (Table 2). When both nitrogen source ($NaNO_3$ 1%) and carbon source (glucose 3%) added, astaxathin production was achieved about 150% higher (Table 3). The result of the addition of H_2O_2 (1%) was more than 150% increase. The simultaneous addition of H_2O_2 and C/N ratio was decreased cell weight by hydrogen peroxide effect, but approximately 1.83 times increased astaxathin production.

The optimal condition for mutagenesis on *Paracoccus haeundaensis* was UV 20J/20min and EMS 400 mM. (Figure 11-14). The initial strain and mutant strains (PUE) were cultivated at the optimum culture conditions for 72 h. The results showed that the cells growth was to stopped at 24 h but astaxanthin production continuously increased after cell growth stop (Figure 14). The astaxanthin contents of PUE was 204.84 ㎍/g DCW. The mutant strains exhibited about 3.82 folds astaxanthin production compared to the initial strain (Table 4).

국문초록

아스타잔틴은 의약, 식품, 사료 사업에서 중요한 물질 중 하나이다. 아스타잔틴을 생산하는 *Paracoccus haeundaensis*로부터 아스타잔틴의 생산량을 증진시키기 위해서 온도의 변화, 탄소원/질소원의 첨가, 과산화수소수의 첨가 등과 같은 배양조건을 변화시켰다. 온도의 변화를 주었을 때에는 아스타잔틴의 생산량의 증진을 거의 볼 수가 없었다. 탄소원으로 lactose, fructose, glucose, mannitol을, 질소원으로 sodium nitrate, potassium nitrate를 사용하였을 때 아스타잔틴 생산량이 가장 높았다. 탄소원/질소원의 비율을 보았을 때 탄소원인 glucose 3%, 그리고 질소원인 sodium nitrate 1%일 때 생산량이 1.4배 증가하였다. 그리고 산소종 유발물질인 과산화수소수를 0%에서 3%까지 8단계의 농도로 첨가하였는데, 0.3% 첨가하였을 때 가장 높은 생산량을 보였다. 이를 통해 glucose 3%, sodium nitarate 1% 첨가하여 배양중일 때 0.3% 과산화수소수를 투여한 결과 기본배지에서 키운 것에 비해서 아스타잔틴의 생산량이 1.83배 증가한 것을 볼 수 있었다. 배양조건의 변화외 다른 방법인 돌연변이 종 생산을 위해 UV와 ethylmethanesulfate (EMS)를 사용하였다. UV 조사와 EMS 처리 시 균주의 생존율은 감소함을 볼 수 있었다. UV조사 20J/20min, EMS 0.4 M처리를 통하여 PUE란 아스타잔틴 생산량이 증진된 돌연변이종을 선별하였고, 이 결과 3.82배의 증가를 볼 수 있었다. 유전적으로 돌연변이 유도체는 향후 산업적으로 아스타잔틴을 대량 생산 시 적합한 방식의 하나일 것으로 사료된다.

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