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Biotechnological Micronutrient Production: Recombinant DNA Technology-Based Vitamin A Synthesis

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ABSTRACT

Vitamin A is an essential micronutrient and has important functions such as vision, growth, reproduction embryogenesis, cellular differentiation, and proliferation, immune function and epithelial protector in the organism. Biotechnological production of vitamins is increasing due to their advantages and significant advances. The vitreoscilla hemoglobin (VHb) gene is extremely effective in binding oxygen and conducting it under hypoxic conditions. In this study, the production of vitamin A in *E. herbicola* (wild type) and its recombinant strains was investigated in LB medium and M9 medium (containing high concentrations (1%) of different carbon sources). The maximum production of vitamin A of the recombinant strain with the hemoglobin gene (vgb+) was observed in including glucose and sucrose M9 medium and their total product levels in vgb+ recombinant strain were 0.14 µg/ml and 0.1 µg/ml, respectively. The vitamin A production in the M9 medium with glucose and sucrose were 2-fold and 1.4- fold higher than that of the wild strain, respectively. The extracellular product level (0.07 µg/ml) in LB was 7-fold higher than wild strain at 48 h. These results reveal that the expression of VHb in *E. herbicola* in the both LB and M9 medium (containing 1% glucose and 1% sucrose, specially) increase the vitamin A production.

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Introduction

Micronutrients are one of the major groups of nutrients. They include vitamins and minerals. Many studies on the worldwide nutrition reveal that there are vitamin deficiencies and the need for nutritional supplements. There are many symptoms caused by vitamin deficiency. In addition, it has been demonstrated that vitamins have a significant protective effect in neoplastic diseases such as cancer or degenerative diseases such as rheumatism (Berstenhorst, Hohmann and Stahmann, 2009). Insufficient vitamin intake is a serious problem, leading to increased demand for industrially produced supplemental vitamins worldwide. Also, the growing vitamin market demands cost-effective production processes using genetically engineered microorganisms as an alternative to chemical synthesis (Berstenhorst et al., 2009; Revuelta, Buey, Ledesma-Amaro and Vandamme, 2016). At the present time, vitamins are produced by chemical synthesis, extraction chemistry, biotechnical or biotechnical procedures combined with fermentation or biotransformation processes. In recent years, vitamin production by biotechnological means has been considered as a very promising approach. Especially, these biotechnological production methods attract great attention because they are environmentally friendly processes (Yuan et al., 2016).

Vitamin A (retinol, acceroftol, epithelial protective vitamin) is a colorless lipophilic biomolecule required to perform important metabolic functions. Vitamin A has important functions such as vision, growth, reproduction embryogenesis, cellular differentiation, and proliferation, immune function and epithelial protector in the organism. Vitamin A regulates gene transcription through interaction with the nuclear receptor (Dadon and Reifen, 2017). Also, the production of white blood cells is triggered by vitamin A, and this vitamin stimulates the activity of these cells. Due to the lack of rhodopsin formation in the retina, visual losses, bone growth disorders, reproductive disorders (failure of spermatogenesis in men, resorption of the fetus in pregnant women) and regression in growth are observed (Stephensen, 2001). Defects in the differentiation of epithelial tissues often result in keratinization.

Vitreoscilla hemoglobin (VHb) is the first microbial hemoglobin to be best characterized, exhibiting a classical globin fold, but unusual structures in both proximal and distal pockets (Anand et al., 2010; Chi, Webster & Stark, 2009; Sanny et al., 2010). Its unique structural organization and ability to stay in different conformational states allow VHb to perform more than one function. Vitreosilla hemoglobin, which acts as an oxygen source, increases the intracellular effect of dissolved oxygen concentration under microaerobic conditions, while the increase in dissolved oxygen increases both cytochrome o and cytochrome d activity. On the other hand, cytochrome increases this specific activity faster than cytochrome d and causes an increase in proton pump. ATP is therefore produced by this proton gradient via ATPase in the cytoplasm (Kallio et al., 1994). VHb is particularly useful for engineering in the energy metabolism of many heterologous hosts and functions as a versatile tool for a variety of biotechnological applications (Anand et al., 2010). This article focuses on vitamin A whose microbial production is currently commercially related and emphasizes on the production of vitamin A in recombinant bacteria whose vitreoscilla hemoglobin gene has been transferred.

Material and Methods

Microorganisms, Media, and Culture Conditions

The bacterial strains used in this study were *Erwinia herbicola* (NRRL B-3466) and its vgb+ (Eh[pUC8:15]) and vgb– (Eh[pUC8]) recombinants. Wild-type bacterial strain (NRRL B-3466) was provided by Dr. Alejandro Rooney, the curator of bacterial stock cultures at the United States Department of Agriculture (USDA, Peoria, IL, USA). In our previously studies, the plasmids pUC8 and vgb-carrying recombinant plasmid of pUC8 (pUC8:15) were transformed to *E. herbicola* in our laboratory (Giray, 2020; Kurt, Aytan, Ozer, Ates & Geckil, 2009). Wild-type strain was protected on LB agar, while the recombinants strains were grown with ampicillin plates. The growth medium used for vitamine A production was both LB broth (pH 7.0): 10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl and M9 medium (6 g/L Na2HPO4; 3 g/L KH2PO4; 0.5 g/L NaCI; 1 g/L NH4CI; 10 ml/L 0.01 M MgSO4.7H2O; 10 ml/L 0.01 M CaCI2; 1% carbon sources which are glucose, fructose and sucrose). The final pH values of broth media were adjusted to 7.0 in the experiments. EDTA, Na2HPO4, KH2PO4, NaCI, NH4CI, MgSO4.7H2O, CaCI2, potassium phosphate, NaCI, NaOH, methanol, glucose, sucrose, fructose, HPLC-grade vitamine A, chemicals for HPLC and all other chemicals were purchased from Sigma-Aldrich and Merck. Agar, yeast and peptone were purchased from Mast Diagnostics.

Vitamin A Levels of E. Herbicola and Its Recombinant Cultures

The bacterial strains were inoculated into 50 ml of the medium in 150 ml volume erlenmeyer flasks. Cells were incubated at 37°C in a 200 rpm and then were harvested at 24h. Harvested cells were centrifuged at 6,000 rpm for 10 min at +4°C. The supernatans were stored for measurement of extracellular vitamin A. The pellets were taken into a clean tube for intracellular vitamin A measurement and then were suspended by adding 3 ml of 0.05 M KPi buffer (pH 8.6). The suspension freeze-dried at -4°C and then re-dissolved. After thawing, 250 µl of 15% TCA and 730 µl 70% HClO4 were added to the suspension and vortexed. After vortexing, 3 ml of ethyl-alcohol and 1.5 ml of n-hexane were added onto the extract and vortexed for 2 min and centrifugated at 4500 rpm for 8 min. The hexane phase formed in the tube was transferred to a clean tube. 250 µl of n-hexane was added again and vortexed for 2 min, centrifugated at 4500 rpm for 8 min. The hexane phase at the top of the tube was transferred to the same tube. The hexane phases which were then transferred to the clean tube were evaporated with nitrogen gas. 150 µl of mobile phase was added to the tubes and vortexed for 4-5 sec. The solution in the tube was transferred to the insert vials. The inserts were placed in the HPLC vials and then placed in the HPLC apparatus for injection. Vitamin A was read at a length of 326 nm for 5 min (Parlak, Celik, Karatepe & Koparir, 2015).

Assaying Vitamine A by HPLC

The concentration of vitamin A was determined by HPLC (Shimadzu DGU- 20A5 HPLC); Nucleosil C18 (4,6 x 150 M9,5 m) CBM-20A and SPD-M20A Diode Array Dedector (DAD). Chromatographic conditions for the HPLC method were determined (Mobile phase: 1L Dicholoromethane, 350 ml Acetonitrile, 350 ml methanol, 8 ml 20 mM Ammonium acetate; Mobile phase rate (ml/min): 5.0; Column temperature: 30°C). Vitamin A levels were read at a length of 326 nm for 5 min. In calibration of vitamin A was prepared 1000 ppm stock solution. This stock solution was dilueted at 0,3125; 0,625; 1,25; 2,5; 5,0 ppm concentrations (Parlak, Çelik, Karatepe & Koparir, 2015). Calibration curves were obtained by using the external standard. The correlation coefficients for the calibration curves were generally at ≥ 0.99 level. In the analyzes, three replicates of each sample were prepared, and the arithmetic averages of the obtained data and the results were calculated.

Total Cell Mass

Total cell mass was determined as growth indicators in cell cultures. Bacterial growth change was spectrophotometrically monitored. The optical density of the culture was recorded at a wavelength of 600 nm in the culture phases determined for this. When OD600 was > 0.5 and the cultures were diluted appropriately (1/5 or 1/10) and their values recorded. Three replicates of each sample were prepared in the analyzes of total cell mass and the arithmetic averages were obtained and recorded.

Results and Discussion

The Vhb Gene Leads to İncreased Production of Vitamin A in Rich Medium (LB)

The production of vitamine A was studied in *E. herbicola* and in its recombinant harboring the vgb in rich medium (LB). The intracellular (cytoplasmic) and extracellular (cell-free growth medium) vitamin A levels were determined in cultures harvested at 12, 24 and 48 h. In LB-grown cultures, the extracellular vitamine A level of vgb+ recombinant strain of *E. herbicola* was substantially higher than that of its wild strain at 48 h (Figure 1). Vgb+ recombinant strain showed a more than about 7-fold higher

vitamin A production (about 0.07 µg/ml) compared to its wild-type strain at 48 h. Results revealed that under low aeration conditions vitamin A production increased in the vgb+ strain. These results concur with the role for *vgb* gene done by other scientists (Kurt, Aytan, Ozer, Ates & Geckil, 2009**;** Mejía, Viniegra-González & Barrios-González, 2003; Du, Shen, Huang, Huang & Zhang, 2016; Wang et al., 2019).

The two strains (wild-type and vgb⁻ recombinant strain) determined a liear correlation with respect to the extracellular vitamin A production. On the other hand, the product formation of vgb+ recombinant strain showed different correlation between cell mass and extracellular vitamin A levels (Fig. 3). The same three strains, however, had an inversed type of correlation for the intracellular and extracellular vitamin A level (Figure 1 and Figure 2). In *E. herbicola* and its vgb+ recombinant, there was an inverse relationship between the cytoplasmic and extracellular product level; the highest level of vitamin A formation was observed in the cell-free growth medium (extracellular) at 48 h (Figure 1). Conversely, intracellular vitamin A production for vgb + recombinant strain was quite low (Figure 2). Therefore, it has been demonstrated that vitamin A was released into the extracellular environment in the LB medium. The amount of intracellular Vitamin A of the wild strain at 48 hours was determined to be 15-fold and 3-fold higher, respectively, compared to *E. herbicola* [pUC8: 15] carrying the vgb gene and *E. herbicola* [pUC8] recombinant without the vgb gene. In other words, the amount of intracellular Vitamin A of the wild strain increased significantly compared to the vgb + and vgb- recombinant strains. The low cytoplasmic level of vitamin A in vgb+/vgb⁻ recombinants and substantially higher level extracellular of this compound in vgb+ recombinant may be due to its differential use in cell metabolism. It is known that the presence of the VHb gene differently affect the growth and metabolit production of bacteria (Kurt, Aytan, Ozer, Ates & Geckil, 2009**;** Sanny, Arnaldos, Kunkel, Pagilla & Stark, 2010; Mejía, Luna, Fernández, Barrios-Gonzalez, Gutierrez et al., 2018). These results show that the *E. herbicola* carrying the vgb gene releases the vitamin A out of the cell after producing it in LB medium.

The supply of oxygen is the critical factor for growth and production when the bacteria are cultivated after post-stationary secondary phase in the LB broth and M9 cultures in flasks. Such an oxygen supply process is inefficient; both the substrate filled in bottles and the substrate speeds are all greatly limited. The oxygen content in the medium in the culture is rapidly reduced in the early stage of cultivation, and then remains stable until the bacteria fully spread throughout the culture in the flasks (Wilson, Page, Welch & Robeck, 2016). Correspondingly, the recombinant strains grew fast in the early stages and then slow with a low maintenance level. The recombinant strains growth rate was maximized 24 h and then lowered (Figure 3). In this study, the maximum growth rates of the vgb recombinant were significantly higher than those of the wild strain. In addition, the maximum growth rate appeared to be delayed to later hours. The growth rate decreased after the maximum growth rate, indicating that the oxygen utilization efficiency of the recombinant strain is increased at low oxygen levels compared to the wild type.

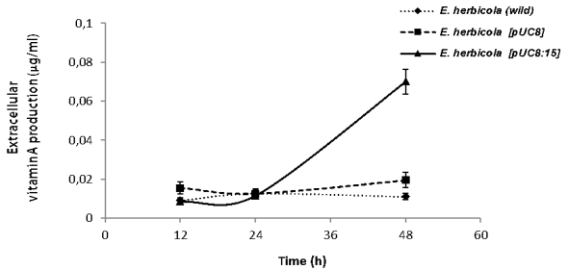

Figure 1. Extracellular vitamin A production of *E. herbicola* (wild), E. herbicola [pUC8] and E. herbicola [pUC8: 15] in different culture phases in LB medium.
Each data point is the average of three separate experiments made in duplicates and error bars indicate standard deviations (σ_n -1).

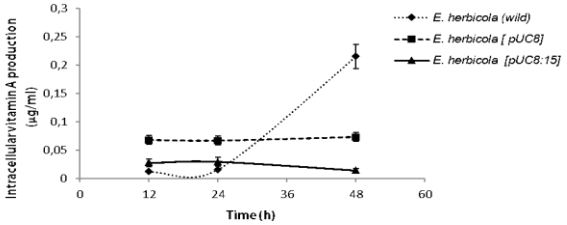

Figure 2. Intracellular (cytoplasmic) vitamin A production of *E. herbicola* (wild), E. herbicola [pUC8] and E. herbicola [pUC8: 15] in different culture phases in LB medium.
Each data point is the average of three separate experiments made in duplicates and error bars indicate standard deviations (σ_n -1).

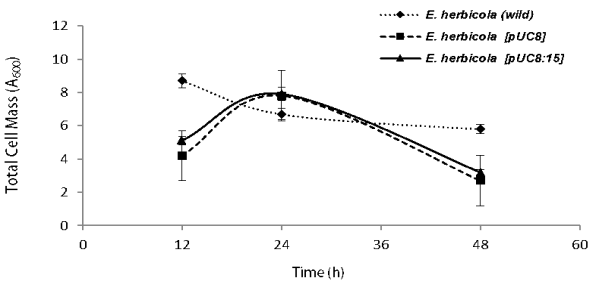

Figure 3. Total cell mass levels in *E. herbicola* (wild) and in its vgb⁺ (Eh[pUC8:15)] and vgb⁻ (Eh[pUC8)] recombinants grown in LB.
Each data point is the average of three separate experiments made in duplicates and error bars indicate standard deviations (σ_{n-1}).

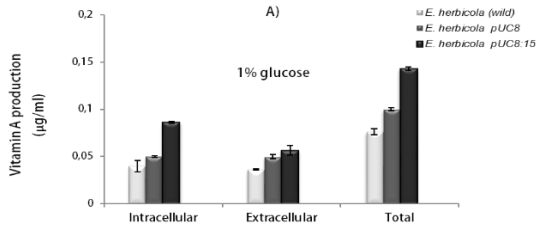

Figure 4A. The analysis of extra- and intracellular vitamin A production in *E. herbicola* (wild type) and its recombinant strains under 1% glucose (A) and 1% sucrose (B) conditionals in M9 medium at 24 h.
Each data point is the average of three separate experiments made in duplicates and error bars indicate standard deviations (σ_n -1).

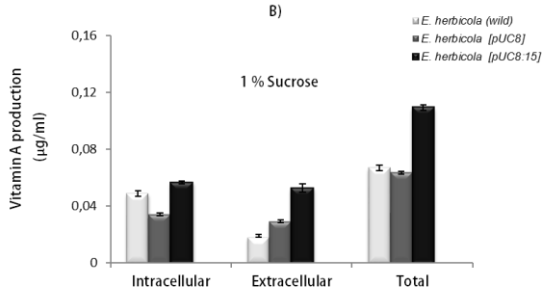

Figure 4B. The analysis of extra- and intracellular vitamin A production in *E. herbicola* (wild type) and its recombinant strains under 1% glucose (A) and 1% sucrose (B) conditionals in M9 medium at 24 h.

Each data point is the average of three separate experiments made in duplicates and error bars indicate standard deviations (σ_n -1)

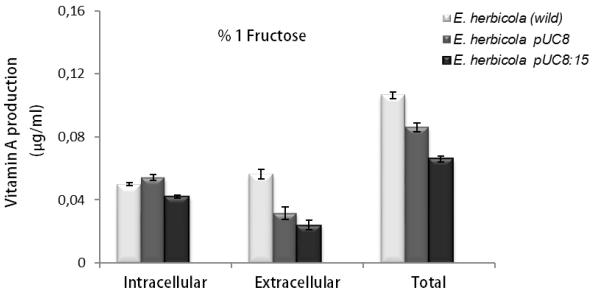

Figure 5. Differences in vitamin production between recombinant strains and wild-type strain in including fructose medium at 24 h.

Each data point is the average of three separate experiments made in duplicates and error bars indicate standard deviations (σ_n -1).

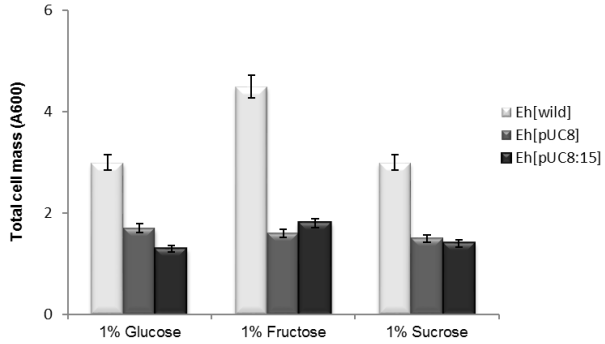

Figure 6. Total cell mass levels in *E. herbicola* (wild) and in its vgb$^+$ (Eh[pUC8:15)] and vgb$^-$ (Eh[pUC8)] recombinants grown in M9 medium (including 1% glucose, fructose and sucrose).

Each data point is the average of three separate experiments made in duplicates and error bars indicate standard deviations (σ_{n-1}).

Glucose and Sucrose Induces Production of Vitamin A in the Presence of Vhb

Many bacteria and fungi have been genetically engineered to express the vgb gene to improve metabolite production including metabolites such as L-DOPA, dopamine (Kurt, Aytan, Ozer, Ates & Geckil, 2009), vitamine E (Giray, 2020), phenazine (Kahraman, Aytan, Giray & Özcan, 2013) arachidonic acid (Zhang, Feng, Cui & Song, 2017), penicillin (Li, Li, Zhou, Zhao & Zhu, 2006), hypocrellin and amylase production (Gao, Deng, Guan, Liao & Cai, 2018), flavones (Zhu, Sun & Zhang, 2011), ganoderic acid (Li et al., 2016a), cellulase (Lin et al., 2017), protease and chitinase (Zhang et al., 2014). In this study, the effect of both VHb gene and various environmental conditions such as glucose, fructose, and sucrose on the production of vitamin A was investigated. For this, high concentrations of 1% glucose, 1% fructose and 1% sucrose were added separately to the M9 medium. The high concentration for production of vitamin A was determined as 1%. The intra- and extracellular vitamin A levels were determined in cultures harvested at 24h. Our studies showed that vitamin A was produced during post-stationary secondary phase (e.g., 24 h) of growth in M9 medium. On further incubation (e.g., 48 h), a decrease in vitamin A production was observed.

The main function of VHb is thought to be contributing to cellular respiration in hypoxic conditions by binding extracellular (extracellular) oxygen at low concentrations to deliver it to terminal respiratory oxidases (Dikshit & Webster, 1988). Indeed, the *vgb* gene has a promoter that is negatively regulated by oxygen, and the expression level of the gene increases by up to 50-fold in environments with low oxygen levels (containing approximately 2% O_2) (Dikshit, Spaulding, Braun & Webster, 1989). Under these conditions, oxygen uptake levels of vgb recombinant bacteria have been reported to increase 5-10-fold. It is known that vitreoscilla hemoglobin (VHb) is expressed in various recombinant microorganisms by providing a controlled intake and release of oxygen and providing advantages in the synthesis of products that require a microaerophilic environment (Buddenhagen, Webster & Stark, 1996; Chen et al., 2007; Chien, Chen, Yang & Lee, 2006; Chien & Lee, 2007).

The strains were cultivated in M9 which contain 1% glucose and 1% sucrose and then were harvested from M9 shaking culture after for 24 h. The production of vitamin in strains were analyzed by using HPLC. Level of vitamin A was detected in the all strains, especially the highest production level was observed in the vgb+ recombinant strain (0.14 µg/ml), in which the vgb express (Figure 4A). It was observed that the VHb activated production of both intracellular and extracellular vitamin in the media with glucose and sucrose. Inclusion of glucose and sucrose in M9 medium resulted in about 2-fold induction of total vitamin production in the vgb+ recombinant strain (Figure 4A and 4B). The effect on the vgb$^-$ recombinant strain (Eh pUC8) and wild strain, however, were lower. These findings are consistent with the findings of our previous study (Giray, 2021).

The Vitreoscilla Hemoglobin (Vhb) Did Not Induce Vitamin A Production in M9 Medium Including Fructose

Production of many metabolites are increased by the expression of VHb (Gao, Deng, Guan, Liao & Cai, 2018; Giray, 2020; Kahraman, Aytan, Giray & Özcan, 2013; Kurt, Aytan, Ozer, Ates & Geckil, 2009; Li et al., 2016b; S. Wang et al., 2020; X. Wang et al., 2019). However, there could be dissimilar effects of VHb towards different product in different medium. There are studies reported in the literature that the expression of VHb enhanced production of various products, while the other product

activities did not increase (Mora-Lugo, Madrigal, Yelemane & Fernandez-Lahore, 2015). In this study, extracellular vitamine production were significantly improved in wild type strain in M9 culture which contains 1% fructose but, intracellular vitamin production did not increase in the VHb expressing strain (Figure 5). Plasmid burden on cells may have caused the lower levels of intra- and extracellular vitamin in recombinant bacteria (vgb+ strain). On the other hand, under fructose-containing conditions, the strain expressing VHb grew slower than the wild strain (Figure 6).

In summary, the recombinant strain expressing VHb grew more slowly (Figure 6) in the M9 medium (with 1% glucose and sucrose) where oxygen was hypoxic and produced more vitamin A compared to the wild strain. These findings show that more product can be obtained with fewer cells. These are important factors for high vitamin yield. The expression of VHb in *E. herbicola* could help to produce high vitamin A yield strategy in the M9 cultures.

Conclusion

Biotechnological production of vitamins is increasing due to their advantages and significant advances. In recent years, high vitamin A yields have been achieved through genetic engineering. In future research, microbial production of some vitamins will be constructed to enable industrial production based on well-reported biosynthetic pathways. It is possible to develop microbial strains that can produce vitamins. Moreover, there are many strategies to improve the productivity of secondary metabolites, including selecting high-yielding organisms, genetic engineering, and optimizing environmental conditions. Vitamin A can be produced both economic and sustainable by using recombinant DNA technology. VHb is particularly useful for engineering in the energy metabolism of many heterologous hosts and functions as a versatile tool for a variety of biotechnological applications.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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