Production of Vitamin E in *Erwinia herbicola* Bearing the *Vitreoscilla* Hemoglobin Gene (vgb⁺)

Running Title: Production of Vitamin E in *Erwinia herbicola*

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Abstract: *Vitreoscilla* hemoglobin (VHb) is bacterial hemoglobin and has beneficial effects on heterologous organisms. The VHb/vgb system, in the advanced phases of the culture, transfers oxygen to the membrane transferases, giving the elderly cell the ability to better oxygen-uptake, grow and proliferate. In this study was investigated the intracellular and extracellular production of vitamin E in *Erwinia herbicola* and its recombinant strains (vgb⁻ and vgb⁺). There was a significant increase the production of vitamin E in the vgb⁺ strain in the LB medium. Production of total vitamin E in the vgb⁺ recombinant strain had 2-fold higher than its host. The wild strain produced 0.06 μg/mL total vitamin E, whereas the recombinant bacteria carrying the vgb gene produced 0.12 μg/mL the product. In addition, production of vitamin E was investigated in the minimal medium (M9) including 1% concentrations of different carbon resources such as glucose, sucrose and fructose. It was recorded that the vitamin E levels produced by the recombinant strain in the culture containing 1% fructose were higher than with 1% glucose and sucrose cultures. The findings showed that the presence of vgb may provide an effective biotechnological method to increase yield of vitamin E.

Key words: *Vitreoscilla* hemoglobin gene, *Erwinia herbicola*, vitamin E, pUC8, pUC8:15-plazmide.

1. Introduction

*Vitreoscilla* hemoglobin (VHb) is bacterial hemoglobin [1] and its characterization has been optimally performed [2-5]. The VHb protein binds the extracellular oxygen present in low concentrations to the terminal respiratory oxidases. Therefore, this protein has an important role in buffering the oxygen of the environment and thus contributes to cellular respiration under hypoxic conditions [2]. In the late stage of culture, the VHb/vgb system transfers oxygen to membrane transferases, enabling older cells to better breathe, grow, and proliferate. In this way, it is known that it helps the production of secondary metabolites [6-8]. In recent years, many bacteria have been used as microbial chemistry factories in metabolic engineering and synthetic biology [9].

Thanks to the developments in these areas, many metabolites are produced from the bacteria, thereby gaining momentum. *Erwinia herbicola*, is a gram-negative bacteria and is also used for biotechnological studies. Even if its main metabolism is similar to many other bacteria, it has its own metabolic pathways. *E. herbicola* is one of the rare bacteria that synthesize products found in various industries as part of its secondary metabolism. These products are important neurotransmitter agents (L-DOPA, dopamine) [10], various antibiotics (herbicilines, pantosins, phenazines, carbapenems) [11-15], carotenoids (lycopene, b-carotene) [16], vitamins [17-19] and plant hormone (indole-3-acetic acid (IAA)) [20-23]. Because of these properties of *E. herbicola*, it can be considered an important cell factory for use in metabolic engineering. In this current study, the effect on vitamin E production of the vgb gene transferred to *E. herbicola* was investigated.
Vitamin E is an important lipophilic antioxidant [24-29]. In addition, it has both anti-inflammatory effects and protective against cardiovascular diseases, cancer, neurodegenerative and other diseases [29]. In addition, vitamin E has an anti-aging effect that can maintain normal permeability, increase skin capillary resistance, improve blood circulation and adjust fertility function [24, 30]. Vitamin E is used for therapeutic purposes in conditions such as coronary heart disease, arteriosclerosis, muscular dystrophy, muscle spasm, newborn scleroma, lupus, dermatomyositis [30]. The production of vitamins by chemical synthesis has quite serious disadvantages. Therefore, the use of biotechnological processes in vitamin production has been increasing in recent years. For these processes, microbiological processes are available and are progressing rapidly. Different methods such as genetic engineering and biocatalyst conversion are used to improve vitamin production [31, 32]. Advances in genetic and genomic engineering accelerate the optimization of metabolic flows to increase the production of target biomass products. Metabolic engineering enables the development of microbial strains that efficiently produce chemicals and materials. Many microorganisms are used as host strains that produce by supporting them with new genetic tools and strategies [9, 32-36]. Microbial metabolism engineering enables microbial production of high-value molecules beyond their natural synthesis capacity. It uses genetic engineering as a powerful tool to do this, thereby manipulating metabolic pathways to achieve effective production. Recombinant DNA technology has also been used in strain improvement to enhance yield [28, 32]. There are limited studies on the production of vitamin E by making genetic modifications on microorganisms [32-36]. In this study, the production of vitamin E was investigated by using recombinant microorganisms containing VHb/vgb system. The production of vitamin E under various genetic (vgb) and environmental (oxygen, glucose, fructose, sucrose) conditions has been demonstrated in detail in *E. herbicola*.

2. Materials and Methods

2.1 Chemical and Reagents

Potassium phosphate, NaCl, NaOH, methanol, chemicals for HPLC, glucose, sucrose, fructose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Yeast and peptone were purchased from Mast Diagnostics. Restriction enzymes were from Roche Diagnostics (Mannheim, Germany). The HPLC-grade vitamin E was purchased from Merck. All other chemicals used were of analytical grade and supplied by Merck or Sigma-Aldrich.

2.2 Plasmid Isolation and Transformation

Plasmid DNA pUC8 and pUC8:15 were isolated from *Enterobacter aerogenes* NRRL B-427 and were performed the alkaline lysis method [10, 37]. The transformation of the plasmids into bacteria (*E. herbicola*) was used with CaCl₂ heat-shock method [10, 37-40]. The inoculation was done into 20 mL LB medium and grown overnight at 37 °C with 200 rpm. A 1/100 inoculum (0.2 mL) of each culture was transferred into a fresh 20 mL LB medium and grown at 37 °C in 200 rpm. After cells were centrifuged in 5,000 rpm, for 5 min at 4 °C, the pellet was resuspended in 0.5 mL ice-cold 0.1 M CaCl₂ and left on ice for 20 min. The pellet was gently mixed with 0.5 mL ice-cold 0.1 M CaCl₂ and left on ice 20 min. After centrifugation (5,000 rpm, 5 min at 4 °C) 150 μL ice-cold 0.1 M CaCl₂ was added to the pellet. DNA of 2 μL 10 mM Tris-HCl pH 8.0 with 1 mM EDTA was added to suspensions of competent cells, which were left on ice for 1 h. The cells were then heat shocked into a 42 °C water bath for 2 min and then placed on ice for 10 min. LB medium (1 mL) was added to mixtures and was incubated at 37 °C for 1 h. Each of these mixtures (200-μL aliquot) was plated on LB agar plates with ampicillin (100 μg/mL) and was incubated for 24 h.
2.3 Bacterial Strains and Culture Conditions

In this study was used *Erwinia herbicola* (NRRL B-3466) and its vgb⁺ (Eh[pUC8:15]) and vgb⁻ (Eh[pUC8]) recombinants. Each of plasmids pUC8 and pUC8:15 (vgb-carrying recombinant plasmid of pUC8) (Fig. 1) was transformed to *E. herbicola* [10]. The presence of pUC8 and pUC8:15 plasmid was confirmed by restriction mini preparation of a satellite-forming colony on LB ampicillin agar (Fig. 2).

Cells were maintained on LB agar (for wild type) and ampicillin plates (for recombinants) at 4 °C with transfers at monthly intervals. It was used with ampicillin plates for recombinants strains. In this study, it was used LB broth (rich growth medium) and minimal medium (M9) for vitamin E production. LB broth includes 10 g/L peptone, 5 g/L yeast extract and 10 g/L NaCl. On the other hand, M9 medium includes (6 g/L Na₂HPO₄; 3 g/L KH₂PO₄; 0.5 g/L NaCl; 1 g/L NH₄Cl; 10 mL/L 0.01 M MgSO₄·7H₂O; 10 mL/L 0.01 M CaCl₂; 1% carbon source). Glucose, fructose and sucrose were used as carbon sources. The final pH value of broth media was adjusted to 7.0. Cells were inoculated into 50 mL of the medium in 150 mL volume flasks and were incubated at 37 °C in a 200 rpm and were harvested at 24 h.

Fig. 1 These maps are physical maps of plasmids pUC8 and pUC8:15. While pUC8 is a derivative of plasmid pBR322 and filamentous phage M13, plasmid pUC8:15 bear a 2.3-kb *Vitreoscilla* chromosomal fragment (Hind IIIHind III) containing the *Vitreoscilla* hemoglobin gene (vgb); ori, origin of replication of plasmid pMB1; Amp R, ampicillin resistance gene; lacZa, β-galactosidase gene encoding alpha monomer (peptide) of the enzyme; plac, β-galactosidase gene promoter.

Fig. 2 Restriction minipreps of plasmids from vgb⁺ and vgb⁻ recombinant of *E. herbicola*. In panels: Hind III digest of λ DNA (1), Eh[pUC8] (2) and Eh[pUC8:15] (3).
2.4 Intracellular Vitamin E Levels of Cultures

Harvested cell was centrifuged at 6,000 rpm for 10 min at +4 °C. The pellets were taken into a clean tube for vitamin E measurement. The pellets were used for suspension. The suspension was prepared by adding 3 mL of 0.05 M KPi buffer (pH 8.6) onto the pellets. And 2 mL of this suspension was transferred to a clean tube and freeze-dried at -4 °C. The pellets were taken into a clean tube for vitamin E measurement. The pellets were used for suspension. The suspension was prepared by adding 3 mL of 0.05 M KPi buffer (pH 8.6) onto the pellets. And 2 mL of this suspension was transferred to a clean tube and freeze-dried at -4 °C and re-dissolved. After thawing, 250 μL of 15% TCA was added to the suspension. And 730 μL 70% HClO₄ was added to the extraction and vortexed. After vortexing, 3 mL of ethyl alcohol was added onto the extract. And 1.5 mL of n-hexane was added and vortexed for 2 min and centrifugated at 4,500 rpm for 8 min. The hexane phase at the top of the mixture in the tube was transferred to a clean tube. And 250 μL of n-hexane was added and vortexed for 2 min and centrifugated at 4,500 rpm for 8 min again. 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. In total 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 E was read at a length of 296 nm for 5 min [41].

2.6 Assaying Vitamin E by HPLC

The intracellular concentration of vitamin E was determined by HPLC (Shimadzu DGU-20A5 HPLC); Nucleosil C18 (4.6 × 150 M 9.5 m) CBM-20A and SPD-M20A Diode Array Detector (DAD). Chromatographic conditions for the HPLC method were determined (mobile phase: 1 L 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 E levels were read at a length of 296 nm for 5 min. In calibration of vitamin E was prepared 1,000 ppm stock solution. This stock solution was dilueted at 1.5; 3.0; 6.0; 12.0; 24.0 ppm concentrations. Calibration curves were obtained using the external standard. The correlation coefficients for these calibration curves were generally at 0.99 levels. In the analysis, three replicates of each sample were prepared and the arithmetic averages of the obtained data and the results were calculated.

3. Results & Discussion

Currently, microbiology has an important place in the global industry, especially in the pharmaceutical, food and chemical industries [25, 28]. The production of vitamin E was studied in E. herbicola and in its recombinant harboring the vgb. The intracellular (cytoplasmic) and extracellular (cell-free growth medium) vitamin E levels were determined in cultures harvested at 24 h. Preliminary studies showed that this compound was produced during the late (post-stationary) secondary phase of growth. When incubation continued in the later phases (e.g. 48 and
72 h), a significant decrease in vitamin E production was observed. The effect of various conditions on the production of vitamin E was also investigated. In addition, 1% glucose, fructose and sucrose were added to M9 cultures, and our preliminary studies revealed that vitamin E production at concentrations less than 1% was much lower.

In LB-grown cultures, cytoplasmic and extracellular vitamin E levels of vgb⁺ strain of *E. herbicola* were substantially higher than of its wild-type and the vgb⁻ strain. Total vitamin E level of vgb⁺ strain was essentially 2-fold higher compared to its wild strain. On the other hand, there was a similar trend for *E. herbicola* and its vgb⁻ (Eh[pUC8]) (Fig. 3). *E. herbicola* and its recombinants showed a linear correlation between total cell mass (recorded as A600 values) and the product formation. The highest product formation of vgb⁺ strain was observed in LB-grown culture with the highest total cell mass (Fig. 4). Dissolved oxygen-limiting conditions affect not only cell growth but also the production of metabolite. It has been reported previously that VHb enhances ATP production and respiratory activity [38, 42]. This study has shown that the expression of VHb increased the yield of vitamin E, especially under oxygen-limiting conditions.

![Figure 3](image1.png)

**Fig. 3** Cytoplasmic (intracellular) and extracellular vitamin E levels in *E. herbicola* (Eh[wild]) and in its vgb⁺ (Eh[pUC8:15]) and vgb⁻ (Eh[pUC8]) recombinants grown in LB medium for 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 4](image2.png)

**Fig. 4** Total cell mass levels in *E. herbicola* (Eh[wild]) and in its vgb⁺ (Eh[pUC8:15]) and vgb⁻ (Eh[pUC8]) recombinants grown in LB and in M9 medium (with 1% glucose, fructose and sucrose, respectively) for 24 h. Each data point is the average of three separate experiments made in duplicates and error bars indicate standard deviations (σ n-1).
The inclusion of 1% glucose into the M9 medium resulted in almost similar intra- and extracellular vitamin E production in the wild type. However, the effect on recombinant strains was quite lower (Fig. 5). *E. herbicola’s* vgb⁺ strain had intracellular and extracellular vitamin E levels 20-fold and 3-fold lower than the host, respectively. These values were about 1.4 and 2 fold lower for the vgb⁻ recombinant of *E. herbicola*. Low intracellular and extracellular levels in vitamin E in recombinants (vgb⁻ or vgb⁺ strains) may be due to plasmid burden on cells. It is assumed that these cells may use as a resource of their amino acids and their derivatives (e.g., vitamin E) in the cell instead of releasing them into the growth medium because it may need a higher demand for metabolism and growth. There have been some studies related to metabolic burden of plasmids and their effects on the expression of host genes [43-45]. It was shown that this metabolic burden associated with plasmid presence limited the cell growth, the metabolites studied and the recombinant protein production. Therefore, in this study, the load exerted on the cells by the plasmid may exceed the useful effect of VHb. Lower intracellular and extracellular vitamin E levels of vgb⁺ and vgb⁻ recombinants may also be due to a higher metabolic burden inflicted by the presence of these high numerous-copy plasmids. This, however, was the case for only with 1% glucose cultures. In recombinant strains may have occurred readily reversible under appropriate conditions such as high growth demand.

VHb increases the performance of oxidative phosphorylation of cells by coordinating the activity of the proper respiratory oxidase and thus changing the central carbon metabolic pathway, and finally initiating cell growth and protein expression under hypoxic conditions. In addition, many heterologous hosts protect against oxidative stress by expressing vgb gene due to peroxidase activity of VHb [46]. On the basis of these observations, VHb expression may promote production of vitamine, thanks to the increasing of respiratory activity and ATP procurement. In addition, another prominent finding was that VHb expression significantly changed the Vitamin E yield in fructose medium. The findings show that the intracellular and extracellular vitamin E levels in 1% fructose-supplemented medium were substantially higher in vgb⁺ (Eh[pUC8:15]) and vgb⁻ (Eh[pUC8]) (Fig. 6). *E. herbicola* and its vgb⁻ and vgb⁺ recombinants showed higher vitamin E levels both

Fig. 5  In M9 medium with 1% glucose, intra- and extracellular vitamin E levels in *E. herbicola* (Eh[wild]) and in its vgb⁺ (Eh[pUC8:15]) and vgb⁻ (Eh[pUC8]) recombinants for 24 h. Each data point is the average of three separate experiments made in duplicates and error bars indicate standard deviations (σ n-1).
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Intracellular and extracellular culture compared to wild type. At this culture phase, intracellular vitamin E levels were 0.08 and 0.04 μg/mL for the vgb<sup>+</sup> and the vgb<sup>-</sup> strains of *E. herbicola*, respectively, while the level of the product was 0.01 μg/mL for wild-type strain. This effect provided by presence of vgb gene was more obvious, when the cultures aged. The vgb-bearing *E. herbicola* (vgb<sup>+</sup>) after 24 h of culture had 6-fold higher total vitamin E level than its host. The effect of VHb is more effective on product yield under limited oxygen conditions. It was strongly demonstrated that the concentration sugar was greatly enhanced by over expression of the VHb and on the other hand, the cell growth was almost the similar [47]. Some studies showed that expression of vgb gene was effective on the production several metabolites [10, 38, 48-50]. In addition, vgb<sup>-</sup> strain showed more or less similar results with vgb<sup>+</sup> in both intra- and extracellular vitamin production. The presence of plasmids in 1% fructose medium did not have a negative effect on product formation; on the contrary, it was observed a positive result in product formation in lower total cell mass. It can be assumed that it stimulates product formation while metabolizing fructose in the pathway that the bacteria use for the production of vitamin E.

It was determined that the vitamin E levels (intracellular and extracellular) produced by vgb<sup>+</sup> (*Eh*pUC8:15) and vgb<sup>-</sup> (*Eh*[pUC8]) were almost similar including 1% sucrose in M9 medium. On the other hand, higher product formation was recorded in its host than recombinant strains (Fig. 7). The intracellular and extracellular vitamin E levels in wild type were 0.04 and 0.05 μg/mL, respectively. Under these culture conditions, including 1% sucrose, the total vitamin E production of the wild strain was about 2-fold higher than recombinant strains that total cell mass was dependent. Total cell mass showed differences in LB and M9 culture (including glucose, fructose and sucrose) conditions. Total cell mass in M9 medium containing different carbon sources was quite low, but vitamin E production in these culture conditions was not lower, especially in 1% including fructose (Fig. 6). In fact, it was recorded that the vitamin E levels produced by the recombinant strain in the environment containing 1% fructose were higher than with 1% glucose and sucrose cultures. These results show that the growth characteristic determined for higher product formation in one bacterium may not be applied to another, so conditions for product formation must be investigated independently according to the situation.

**Fig. 6** The levels of vitamin E in *E. herbicola* (*Eh*[wild]) and in its vgb<sup>+</sup> (*Eh*[pUC8:15]) and vgb<sup>-</sup> (*Eh*[pUC8]) recombinants grown in M9 medium with 1% fructose for 24 h. Each data point is the average of three separate experiments made in duplicates and error bars indicate standard deviations (σ n-1).
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Fig. 7 The levels of vitamin E in *E. herbicola* (Eh[wild]) and in its vgb⁺ (Eh[pUC8:15]) and vgb⁻ (Eh[pUC8]) recombinants grown in M9 medium with 1% fructose for 24 h. Each data point is the average of three separate experiments made in duplicates and error bars indicate standard deviations (σ n-1).

4. Conclusions

Vitamin E, playing an important role as an antioxidant against environmental stress, is used for therapeutic purposes in conditions such as coronary heart disease, arteriosclerosis, muscular dystrophy. Advances in DNA technology have expanded the options available for the exploitation of biotechnology in vitamin production. Moreover, biotechnological processes and products often have a positive environmental impact and a positive appeal to people. The purpose of this study was to determine whether the vgb/VHb system provides advantage for production of vitamin E. The production of vitamin E of vgb⁺ strain was observed in LB-grown and including 1% fructose culture. The findings show that the presence of vgb gene may provide an effective biotechnological method to increase yield of vitamin E.

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Conflict of Interest

The author declares that she has no conflict of interest.

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References


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