Effect of *Vitreoscilla* hemoglobin on production anti-tumor enzyme methionine –γ-lyase, by *Citrobacter freundii*

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Abstract Methionine, a sulfur amino acid, is the first amino acid that is required for many proteins, during synthesis. Our preliminary studies showed that this compound was produced during the late (post-stationary) secondary phase of growth. Therefore, restriction of methionine may be a useful strategy in limiting cancer growth. The bacterial strain used in this study was *Citrobacter freundii* (NRRL B-2643) and their *vgb*$^{+}$ recombinant strain. A 1/100 inoculum of overnight cultures grown in LB was made in 50 ml LB in 150 ml Erlenmeyer flasks. Inocula in flasks were grown for 24 h at 30 ℃ in a 200 rpm water-bath. For MGL production, 250 µL of this O/N culture was then inoculated into 150 mL conical flask containing 50 mL of sterile mineral salts medium supplemented with 1 % or 0.1 % (w/v) glucose, respectively. This was incubated for 96 h at 30 ℃, 200 rpm on an orbital shaker. The highest MGL concentration (2,02) was reached by the recombinant strain of Cf[pUC8:15] 72 h after the start of incubation MM+0,1% glucose source. In comparison, the wild type strain produced 3,14 of MGL concentration 72 h was reached MM+0,1% glucose source. The poor media and secondary phase (72 h and up) was used to for MGL production. This is more appropriate. Plasmid is disadvantages in the secondary stage.

Keywords: *Citrobacter freundii*, Methionine γ-lyase, *Vitreoscilla* hemoglobin

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Introduction

Cancer is a class of diseases characterized by out-of-control cell growth. There are over 100 different types of cancer, and each is classified by the type of cell that is initially affected. Cancer is characterized by uncontrolled cellular growth as a result of changes in the expression of tumor promoting and tumor suppressing genes. A common feature of some cancers is the absolute requirement for methionine, a phenomenon known as 'methionine dependence. Therefore, restriction of methionine may be a useful strategy in limiting cancer growth[1].

Studies found that the methionine γ-lyase (MGL, EC 4.4.1.11) can specifically split the methionine of extracellular and intracellular, so it can strongly inhibit the growth of tumor cells and induce apoptosis of tumor cells[1-2]. Many cancer cells have an absolute requirement for plasma methionine, whereas normal cells are relatively resistant to the restriction of exogenous methionine[3]. This allows one to consider MGL as a potential target for novel antibiotics [4]. Thus, therapeutic exploitation of C. freundii MGL to deplete plasma methionine has been extensively investigated[2, 5]. Thus, when these tumor cells are deprived of methionine in homocysteine-containing medium *in vitro*, they reversibly arrest in the late S/G_2 phase of the cell cycle. On another hand, MGL helps to inhibit the growth of mouse and human tumors in rodents and prolongs their survival[6]. Methionine- γ -lyase utilizes a pyridoxal-5'-phosphate-cofactor and catalyzes the degradation of L-methionine to α-ketobutyrate, methanethiol and ammonia[2]. MGL is proposed to be a potential drug target for treating anaerobic, parasitic infections[6-7]. Anaerobic bacteria and parasitic protozoa that possess MGL, rely on glycolysis and amino acid degradation for energy generation[2]. At the same time methionine also is required for the formation of the polyamines, spermine, and spermidine, which are necessary for cell proliferation[8]. The prospects of application of the enzyme as an antitumor agent were also demonstrated *in vitro* and *in vivo*. The possibility of using MLG for the therapy of Parkinson's disease, atherosclerosis, aging, and obesity is discussed in work[7].

MGL has been detected *Clostridiums porogenes*, *Pseudomonas ovalis*, *Pseudomonas putida*, *Aeromonas sp.*, *Citrobacter intermedius*, *Brevibacterium linens*, *Citrobacter freundii*, *Porphyromonas gingivalis*, and *Treponema denticola*, parasitic protozoa such as *Trichomonas vaginalis*, *Entamoeba histolytica*, and a model plant Arabidopsis thaliana. MGL activity was also detected from archae *Ferroplasma acidarmanus*, cheese surface bacteria such as *Micrococcus luteus*,

Arthrobacter sp., *Corynebacterium glutamicum*, and *Staphylococcus equorum*. Crystal structures have been reported from *Pseudomonas putida*, *Citrobacter freundii*, *Trichomonas vaginalis*, and *Entamoeba histolytica*. MGL is absent in mammalian cells[2-3, 7, 9-11].

The facultative anaerobic *Citrobacter freundii*, Gram-negative and long rod-shaped bacterium belongs to the family of *Enterobacteriaceae*, and can be found in water, food and the intestinal tracts of animals and humans. As an opportunistic pathogen, *C. freundii* is responsible for a number of significant opportunistic infections, such infections of the respiratory tract, urinary tract and blood[12].

The obligate aerobic bacterium, *Vitreoscilla*, synthesizes elevated quantities of *Vitreoscilla* hemoglobin (VHb) under hypoxic growth conditions. Expression of VHb in heterologous hosts often enhances growth and product formation. A role in facilitating oxygen transfer to the respiratory membranes is one explanation of its cellular function[13].

In this study, the production of MGL by *C. freundii* was studied in different culture medium. The goal of this work was to obtain insight into how MGL stimulates of different culture conditions by *C. freundii* and pUC8:15.

Materials and Methods

Materials

Methionine (Aldrich); PLP, mercaptoethanol (Sigma), EDTA (Applichem), TCA (Across), 3-MBTH (Merck); sodium acetate (Riedel-de Haen), potassium phosphate and di-potassium phosphate (Sigma). All other chemicals used in this study were of analytical reagent grade or the highest grade commercially available.

Bacterial strain and culture conditions

The bacterial strain used in this study was *Citrobacter freundii* (NRRL B-2643) and their *vgb*$^+$ recombinant strain. *C. freundii* was transformed with *vgb* carrying recombinant plasmid pUC8:15, the cells were maintained on LB agar plates at 4 ℃ with transfers at monthly intervals. The liquid media used throughout the study were Luria-Bertani (LB) broth and M9 minimal medium (MM) containing (per liter); 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl. The final pH values of both media were adjusted to 7.0.

Growth conditions

Cells were maintained on Luria- Bertani (LB) (Miller, 1972) agar plates at 4 ℃ with transfers at monthly intervals. A 1/100 inoculum of overnight cultures grown in LB was made in 50 ml LB in 150 ml Erlenmeyer flasks. Inocula in flasks were grown for 24 h at 30 ℃ in a 200 rpm water-bath. *C. freundii* strains were routinely grown on Luria- Bertani medium (LB). Minimal medium (M9 Minimal media (MM)) contained 1 % and 0.1 % glucose, added from a stock solution respectively, autoclaved. LBG medium contained 1 % and 0.1 % glucose. The growth characteristics of both wild *C. freundii* and its recombinants in the rich (LB) and in the nutrient restricted nutritional (MM) media were monitored at the given intervals during the course of the experiments. For MGL production, 250 µL of this O/N culture was then inoculated into 150 mL conical flask containing 50 mL of sterile mineral salts medium supplemented with 1 % or 0.1 % (w/v) glucose, respectively. This was incubated for 96 h at 30 ℃, 200 rpm on an orbital shaker.

For MGL production and assay

For MGL production, 250 µl of this O/N culture was then inoculated into 150 ml conical flask containing 50 ml of sterile mineral salts medium supplemented with 1% or 0.1% (w/v) glucose, respectively. This was incubated for 96 h at 30 ℃, 200 rpm on an orbital shaker. Recombinant MGL activity was determined by the method described[14-15]. The absorbance measured at 320 nm a unit's enzyme. One unit of enzyme activity was determined as the amount of enzyme catalyzing transformation of 1 µmol of L-methionine per minute pH 8.0 at 37 ℃.

Results

The intracellular MGL level was determined cultures harvested at 96 h. Our preliminary studies showed that this compound was produced during the late (post-stationary) secondary phase of growth (Figure 1 b, 2 b, 3 b, 4 b, 5 b). On further incubation (e.g., 72 h and up), a substantial decrease in MGL production was observed. In LB and LBG-grown cultures, the intracellular MGL level of recombinant strain Cf [pUC 8:15] was substantially higher than that of wild type bacteria, especially 48 h and up. The cell density in MM was significantly lower than in complex LB medium. When glucose was used as a carbon source, the recombinant strain was observed decrease both cell density and specific activity. In MM-grown cultures, the intracellular MGL level of wild-type strains of *C. freundii* was substantially higher than that of their recombinant Cf [pUC 8:15] strain (Figure 3-5 b). Although to a lesser extent, the intracellular levels of MGL for the wild-type strain of were also higher than for their recombinant counterparts.

Table 1. pH Values of 96th h.

	LB	LB+0,1% Glucose	LB+1% Glucose	MM+0,1% Glucose	MM+1% Glucose
Cf Wild	9,01	9,09	4,27	6,76	5,74
Cf [pUC 8:15]	9,14	9,14	4,39	6,71	5,03

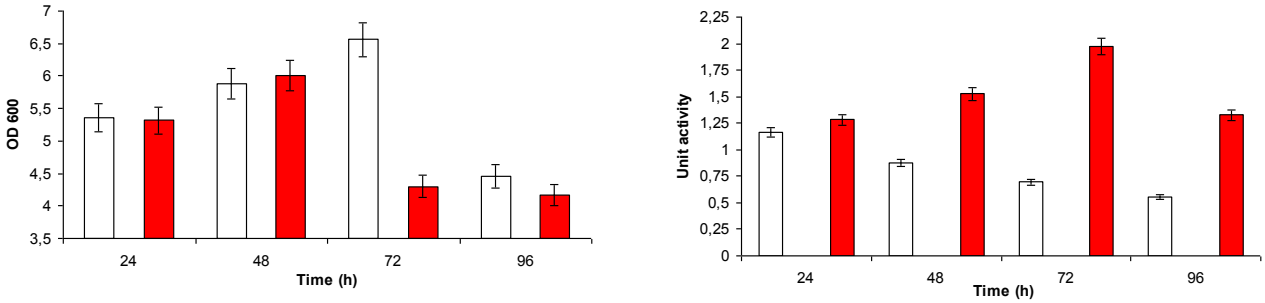

Figure 1. Total cell mass (a) and (b) intracellular MGL levels of *C. freundii* and their Cf [pUC 8:15] harboring recombinant grown in LB medium for 24-96 h. Each value is the average of three independent experiments.

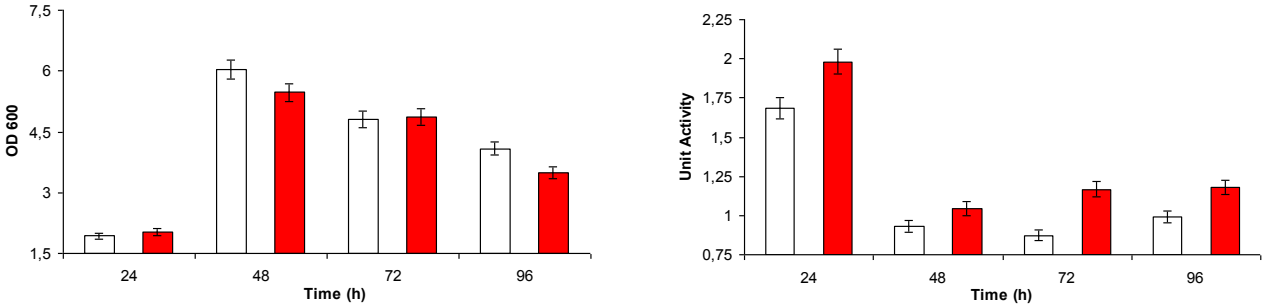

Figure 2. Total cell mass (a) and (b) intracellular MGL levels of *C. freundii* and their Cf [pUC 8:15] harboring recombinant grown in LB+0.1% glucose medium for 24-96 h. Each value is the average of three independent experiments.

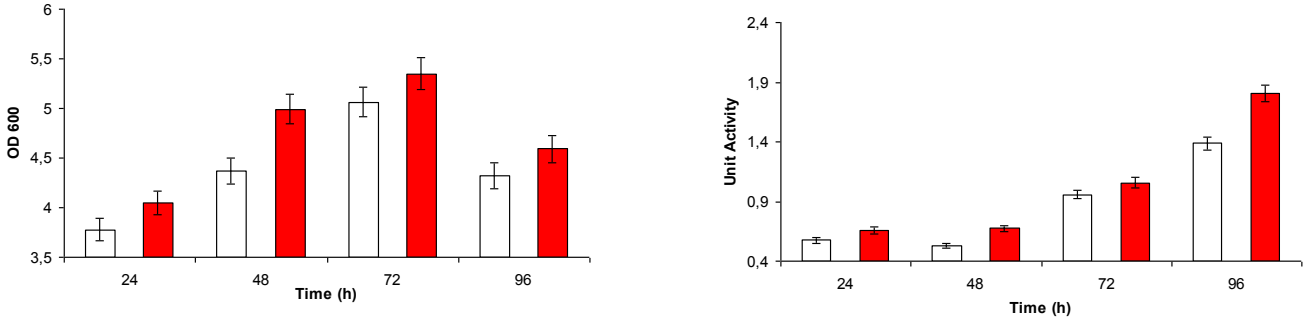

Figure 3. Total cell mass (a) and (b) intracellular MGL levels of *C. freundii* and their Cf [pUC 8:15] harboring recombinant grown in LB+1% glucose medium for 24-96 h. Each value is the average of three independent experiments.

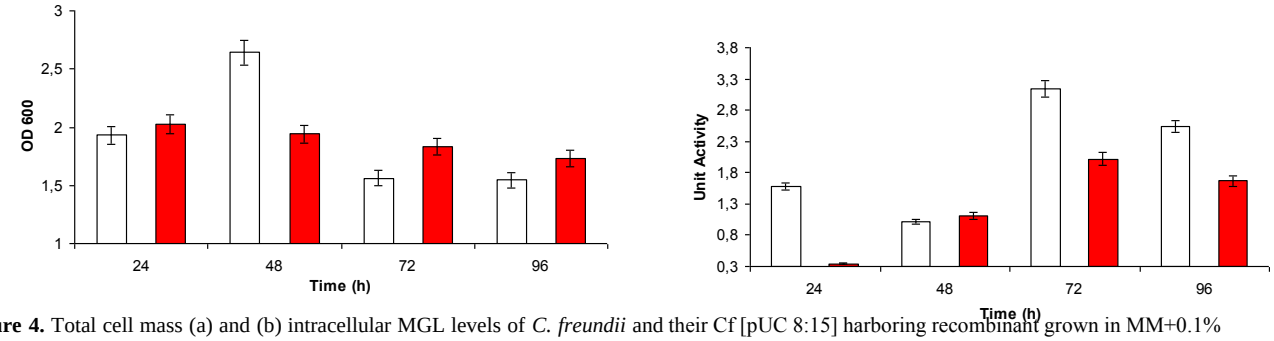

Figure 4. Total cell mass (a) and (b) intracellular MGL levels of *C. freundii* and their Cf [pUC 8:15] harboring recombinant grown in MM+0.1% glucose medium for 24-96 h. Each value is the average of three independent experiments.

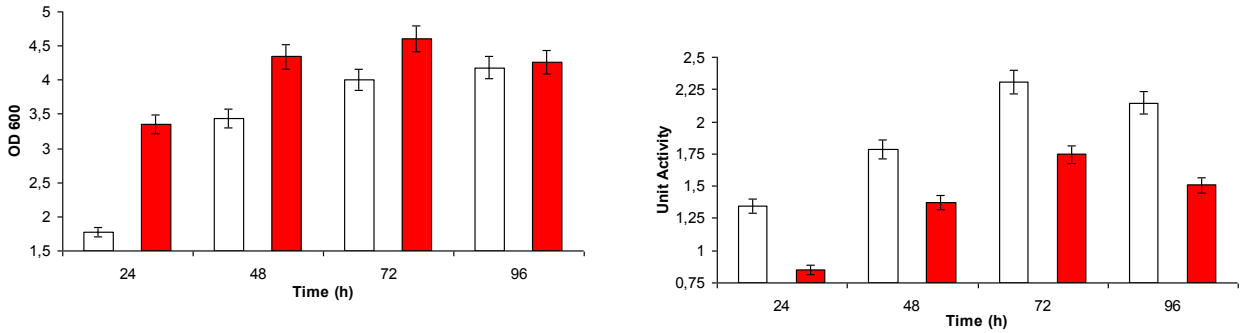

Figure 5. Total cell mass (a) and (b) intracellular MGL levels of *C. freundii* and their Cf [pUC 8:15] harboring recombinant grown in MM+1% glucose medium for 24-96 h. Each value is the average of three independent experiments.

On further incubation a substantial increase in total cell masses (OD_{600} values) were observed (Figure 1 a, 3-5 a). On the other hand, the lower levels of intracellular MGL production and total cell mass in recombinant bacteria Cf [pUC 8:15] may be due to plasmid burden on cells. Especially *vgb* bearing recombinant strain Cf [pUC 8:15] had higher total cell mass levels than the wild type strain (Figure 3-5a). These findings are in agreement with those from previous studies showing that the dramatic effect of VHb on cell metabolism and production formation is found under limited oxygen conditions where an elevated expression of VHb occurs[16]. In *C. freundii* and its recombinants, there was inverse relationship between the total cell mass and intracellular product level; the highest product formation was observed in cultures with lowest total cell mass (Figure 2 a, b and 3 a, b). In LB and LB +1% glucose-grown cultures, the intracellular MGL level of recombinant strain Cf[pUC8:15] was substantially higher than that of their respective wild- strain C. freundii (Figure 1-3 b) On other hand in MM- grown cultures, the intracellular MGL level of wild-type strains of C. freundii was substantially higher than that of their respective recombinant strain Cf[pUC8:15] (Figure 4-5 b). The highest MGL concentration (2,02) was reached by the recombinant strain of Cf[pUC8:15] 72 h after the start of incubation MM+0,1% glucose source (Figure 4 b). In comparison, the wild type strain produced 3,14 of MGL concentration 72 h was reached MM+0,1% glucose source (Figure 4 b). In *C. freundii* and its recombinants, there was inverse relationship between the total cell mass and intracellular product level; the highest product formation was observed in cultures with lowest total cell mass (Figure 4 a, b). Glucose rate is increased, the pH is reduced. The presence of glucose 1% decreases pH values (Table 1). Low pH values in MM may reduce the enzyme activity, in particular recombinant bacteria.

Discussion

Industrial biotechnology is becoming increasingly important as the unique metabolic capacities of microbes offer new enzymes. MGL is one of a few microbial enzymes with high therapeutic value in certain kinds of cancers, where it is used to "starve" cancer cells of amino acid L-methionine. A characteristic feature of many solid tumors is their requirement for exogenous methionine in order to proliferate whereas normal cells are generally methionine independent[17]. Therefore, restriction of methionine may be a useful strategy in limiting cancer growth[1]. On further incubation (e.g., 48 h and up), a substantial increase in MGL production was observed. All in growth medium after 48 hours there is an increase in enzyme activity. Lower intracellular MGL levels of Cf[pUC8:15] may also be due to a higher metabolic burden inflicted by the presence of high copy-number this plasmid[16]. In *C. freundii* and its recombinant strain Cf[pUC8:15], there was inverse relationship between the total cell mass and intracellular product level; the highest product formation was observed in cultures with lowest total cell mass (Figure 2-4 b). MGL production of all media started after the all phase and generally leveled at 72 h. However, the MGL production was affected by the rich media LB and LB + Glucose. The lower levels of intracellular MGL in recombinant bacteria Cf [pUC 8:15] may be due to plasmid burden on cells. In addition, glucose rate in culture conditions significantly affected the rate of cell mass and MGL production. Especially, low pH values in MM may reduce the enzyme activity, in particular recombinant bacteria (Table 1). Oxygen deficiency that develops in the fermentation with a low oxygen transfer rate has significant effects, such as low viable cell number which results low product yield[16].

Conclusion

This is indicated the poor media and secondary phase (72 h and up) was used to MGL production. Short term production of MGL recombinant bacteria is advantageous. Plasmids are disadvantages in the secondary stage.

In MM-grown cultures, the intracellular MGL level of wild-type strain and their recombinant was substantially higher than that their culture conditions. In addition, glucose rate in culture conditions significantly affected the rate of cell mass and MGL production. The effect of plasmid-mediated metabolic burden on the expression of the host genes has been the subject of some studies, in which biosynthetic burden associated with plasmid presence was shown to limit the cell growth, the metabolites studied and the recombinant protein production.

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