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Effects of Carbon Source and *Vitreoscilla* Hemoglobin (VHb) on the Production of β-Galactosidase in *Enterobacter aerogenes*

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Abstract. At fixed concentration (0.5%), lactose and galactose acted as inducers while glucose and other tested carbon sugars showed repression effects on β-galactosidase production in *Enterobacter aerogenes* strain. The expression of *Vitreoscilla* hemoglobin gene (*vgb*) in this bacterial strain managed to overcome the repression effects as well as improving the induction of β-galactosidase formation by carbon sources. In parallel, the bacterial O_2 consumption was increased correspondingly to the *vgb* induction of β-galactosidase synthesis. When *Enterobacter aerogenes* strains were grown at the incubation temperature 42°C, about 5-fold higher enzyme productivity was obtained than with a similar incubation at 37°C. The bacterial growth expressed as biomass yield had a different optimum temperature and was not influenced to the same extent by variations in the carbon sources. These data are discussed in terms of proposed enhancement in β-galactosidase productivity by *vgb* expression as well as its significance to improve the technology of whey processing.

Since the discovery of bacterial hemoglobin (VHb) in gram-negative bacteria *Vitreoscilla* [1], more research interest is still arising on the biotechnological applications of this protein [2]. Recently, a gene encoding the VHb part of *Vitreoscilla* hemoglobin (*vgb*) has been cloned and expressed in a wide range of bacterial strains [2–4]. The main physiological function of VHb protein seems to be confined to its efficient supply of oxygen to terminal oxidases under limited aerobic conditions [2]. Therefore, its expression inside various heterologous hosts often yields an enhancement of cell density, oxidative metabolism, engineered product formation, and bioremediation [5–12].

The disposal of whey, which is rich in lactose, has become a major problem for the dairy industry. Therefore, one of the promising methods in whey disposal is the hydrolysis of lactose into glucose and galactose by β-galactosidase. Such unique degradation ability gives this enzyme tremendous application impacts in nutrition, medicine, and the food industry [13, 14]. So far, little information is available on the production of β-galactosidase by *Enterobacter aerogenes*. The present work represents an attempt to investigate the influence of *Vitreoscilla* hemoglobin gene transformation on β-galactosidase synthesis in *Enterobacter* strains growing under various carbon sources.

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Materials and Methods

***Enterobacter* Strains Growth and Transformation.** The parental strain *Enterobacter aerogenes* was previously characterized [9]. Cells were transformed with two different plasmids and maintained in either LB or LB media plus 100 μg ml^{-1} ampicilline (Ap) The first plasmid (pUC8: 16) contains *Vitreoscilla* hemoglobin gene *vgb* inserted on 1.4-Kb fragment and has been cloned into pUC8 [3]. It was obtained from the Biology Department, Illinois Institute of Technology (Chicago, IL, USA). The second plasmid (pUC9) was similar to pUC8: 16 but lacks the *Vitreoscilla* hemoglobin gene, *vgb* [8, 9, 15]. This plasmid was a gift from Prof. Khaled Altarawneh, Biology Department, Mutah University, Jordan. The resulting transformed recombinant strains were denoted as *Enterobacter*: pUC8: 16 and *Enterobacter*: pUC9, respectively. The expression of VHb in *Enterobacter*: pUC8: 16 strain was confirmed by CO-difference spectra as described previously [9]. For examining the effects of carbon source on the production of β-galactosidase, various carbon compounds were supplemented to LB medium at a final concentration of 0.5% as described by Shaw et al. [16]. Then these cultures were inoculated with the corresponding *Enterobacter* strain. A control culture without the carbon source was incubated under similar conditions.

Table 1. Cell biomass g l^{-1} [wet weight] of strains *Enterobacter*, *Enterobacter*: pUC9 and *Enterobacter*: pUC8:16 in LB as a function of the added carbon source type

Carbon source used at 0.5% concentration	Cell biomass g l^{-1} (wet weight)					
	Parental cells		Enterobacter: pUC9		Enterobacter: pUC8:16	
	37°C	42°C	37°C	42°C	37°C	42°C
Control	6.4 (0.10)	5.8 (0.10)	6.0 (0.10)	5.6 (0.10)	7.2 (0.35)	5.9 (0.43)[a]
Glucose	6.5 (0.30)*	5.2 (0.25)	5.5 (0.30)	4.8 (0.20)	7.8 (0.39)	6.0 (0.25)*
Fructose	6.0 (0.11)	5.4 (0.24)	4.45 (0.15)	4.0 (0.20)	6.4 (0.50)	6.0 (0.35)*
Mannitol	6.5 (0.22)*	5.9 (0.15)*	6.2 (0.22)*	5.6 (0.17)*	8.2 (0.30)	7.0 (0.14)
Lactose	8.0 (0.22)	6.0 (0.09)*	7.2 (0.30)	5.5 (0.22)*	9.6 (0.3)	7.0 (0.45)
Maltose	7.0 (0.27)	6.0 (0.10)*	8.2 (0.14)	6.7 (0.15)	8.6 (0.20)[a]	7.2 (0.14)[a]
Sucrose	6.3 (0.09)*	4.2 (0.10)	6.0 (0.11)*	4.0 (0.15)	7.4 (0.09)*	5.4 (0.12)
Succinate	6.0 (0.20)*	4.0 (0.12)	5.5 (0.16)	3.5 (0.12)	7.5 (0.20)*	4.5 (0.22)
Pyruvate	4.0 (0.15)	3.0 (0.10)	3.75 (0.07)	3.2 (0.15)	5.9 (0.22)	4.8 (0.1)
Citrate	3.0 (0.08)	2.5 (0.10)	2.8 (0.07)	2.5 (0.10)	5.0 (0.20)	3.2 (0.1)
Glycerol	4.0 (0.22)	4.3 (0.15)	5.5 (0.22)	4.0 (0.17)	6.9 (0.12)*	4.85 (0.15)
Galactose	7 (0.08)	5.5 (0.17)	6.1 (0.15)*	5.0 (0.20)	7.4 (0.25)*	5.4 (0.30)[a]*

The times of sampling were at 14 h (early stationary phase). Values are the average of three individual experiments; standard deviations are in parentheses. The t-test was first compared between biomass values in the presence of each carbon source with the corresponding values of the carbon lacking control. All data are significant ($P < 0.05$) except the values with asterisks (*). A second t-test comparison was made between the biomass in pUC8:16 versus the corresponding values of the pUC9 strains. All data are significant ($P < 0.05$) except the values that contain the letter ([a]).

To prepare inoculum, cells were inoculated from freezer stocks (originated from single colony) into 25 ml fresh LB medium [17] in case of untransformed strains; or LB plus 100 μg ml^{-1} ampicilline and growth overnight at 37°C and 150 rpm for transformed strains. These cultures were diluted 1:50 by the same medium used for each culture and grown for 3-h recovery period at 37°C and 120 rpm. A sample of 1 ml from each strain culture (volume adjusted to give a constant $A_{600} = 0.120 \pm 0.04$ of cells) was then harvested by centrifugation, washed twice with fresh antibiotic-free LB medium at 37°C, and inoculated into 200 ml of fresh antibiotic-free LB medium. All cultures were incubated for 14 hours either at 37° or 42°C using pH 7.0 and an agitation rate of 150 rpm.

Enzyme Assay. Late log phase cells ($A_{600} = 0.6–0.7$) were harvested by centrifugation (5000 rpm), washed, and resuspended in ice-cold 0.1 M sodium phosphate buffer at pH 7.0 to obtain $A_{600} = 10$. After mixing, the suspension was treated with 25 μl of lysozyme enzyme (25,000 IU) for 1 hour at 30°C. The treated mixture was centrifuged at 7000 rpm for 10 min and the supernatant was collected to be used as a source of the enzyme.

The assay of β-galactosidase was determined calorimetrically using *O*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate [18]. The reaction mixture was incubated at 37°C for 15 minutes and the produced yellow colour was measured spectrophotometrically at 420 nm. One unit of enzyme activity was expressed as the amount of enzyme that produced 1 μmol of orthonitrophenol (ONP) in 1 minute under the assay conditions.

Determination of Oxygen Utilization. Measurement of oxygen utilization was performed according to the method described by Khosravi et al. [19]. Respiration of the whole cells was measured polarographically with a Yellow Springs Instruments Model 53 oxygen monitor at room temperature using air-saturated 0.1 M potassium phosphate buffer at pH 7. Before measuring the oxygen uptake, the buffer was stirred for 2 h to be air saturated (DO = 250 μM). One milliliter of bacterial culture (cfu was simultaneously determined by plating on LB and LB-Ap) was centrifuged at 10,000 rpm for 5 min and washed twice with 0.1 M potassium phosphate, pH 7, and the resulting pellet was added quantitatively to 4 ml air-saturated buffer. The remaining concentration of oxygen in cells containing buffer was monitored by an oxygen electrode and recorded for 5 min.

Results

Effect of Carbon Sources on the Cell Biomass of *Enterobacter* Strains. The effect of 11 carbon sources on cell mass of *Enterobacter* strains are shown in Table 1. Based on g.l^{-1} measurements, all strains exhibited an increase in bacterial growth when the LB medium was supplemented with 0.5% lactose or maltose. Such elevation in growth was particularly observed at the incubation temperature 37°C. Under these conditions, glycerol, pyruvate, and citrate caused a reduction in bacterial growth while other carbon sources had little effect. The decrease in growth yield might be related to the less efficient metabolic turnover of these three carbon sources in LB medium. In general, the *vgb*-bearing cells showed a significant advantage over the *vgb*-lacking strains in biomass production. This growth advantage was more pronounced at 37°C than at 42°C.

Effect of Carbon Sources on *Enterobacter* β-Galactosidase Production. The thermodependency of β-galactosidase production was determined by incubating *Enterobacter* strains at a range of different incubation temperatures (20°, 25°, 30°, 37°, and 42°C). Before adding carbon sources at fixed supplementation,

Table 2. Effect of different carbon sources on the β-galactosidase production level by *Enterobacter, Enterobacter*: pUC9, and *Enterobacter*: pUC8:16 grown at (a) 37°C and (b) 42°C

Carbon source used at 0.5% concentration	Parental cells	*Enterobacter*: PUC9	*Enterobacter*: pUC8:16
a			
Control	0.36 (0.07)	0.34 (0.04)	0.5 (0.08)
Citrate	0.09 (0.01)	0.06 (0.007)	0.15 (0.01)
Fructose	0.05 (0.01)	0.025 (0.005)	0.102 (0.01)
Galactose	0.39 (0.08)*	0.30 (0.07)*	0.60 (0.10)*
Glucose	0.05 (0.01)	0.06 (0.005)	0.11 (0.02)
Glycerol	0.06 (0.01)	0.043 (0.07)	0.102 (0.02)
Lactose	0.43 (0.09)*	0.39 (0.07)*	0.75 (0.12)
Maltose	0.175 (0.02)	0.25 (0.05)	0.22 (0.05)[a]
Mannitol	0.102 (0.01)	0.115 (0.009)	0.153 (0.09)
Pyruvate	0.18 (0.02)	0.13 (0.02)	0.36 (0.07)
Succinate	0.18 (0.03)	0.15 (0.03)	0.34 (0.08)
Sucrose	0.108 (0.02)	0.090 (0.06)	0.25 (0.07)
Lactose + glucose	0.08 (0.01)	0.07 (0.002)	0.13 (0.01)
b			
Control	2.57 (0.19)	2.56 (0.28)	2.57 (0.22)[a]
Citrate	0.26 (0.06)	0.20 (0.03)	0.36 (0.07)
Fructose	0.70 (0.13)	0.52 (0.10)	2.20 (0.40)
Galactose	2.7 (0.25)*	2.44 (0.60)*	3.45 (0.90)
Glucose	0.90 (0.20)	0.70 (0.14)	2.20 (0.13)
Glycerol	0.06 (0.01)	0.043 (0.07)	0.102 (0.02)
Lactose	2.9 (0.33)*	1.35 (0.20)	4.10 (0.85)
Maltose	0.51 (0.22)	0.44 (0.10)	0.66 (0.18)
Mannitol	1.55 (0.16)	1.35 (0.07)	2.70 (0.60)*
Pyruvate	0.32 (0.07)	0.14 (0.07)	0.40 (0.07)
Succinate	0.34 (0.15)	0.20 (0.07)	0.83 (0.11)
Sucrose	0.16 (0.07)	0.11 (0.07)	0.70 (0.14)
Lactose + glucose	1.1 (0.17)	0.82 (0.18)	2.35 (0.55)*

Values are the average of three individual experiments; and standard deviations are presented in parentheses. Double t-test comparisons were made on the values of enzyme activities as described in Table 1.

a progressive elevation in enzyme synthesis was observed as the temperature increased, until a peak enzyme activity was reached at 42°C (data not shown).

In the presence of either lactose or galactose as carbon sources, all *Enterobacter* strains growing at 37°C produced higher levels of β-galactosidase activity than the corresponding controls (Table 2a). However, except for the lactose induction in *vgb*-bearing cells ($P < 0.05$), the enzyme activities recorded in other strains were not statistically different from the controls ($P > 0.05$). The addition of glucose or other tested carbon sources at this incubation temperature generally lead to poor levels of enzyme synthesis. As the incubation temperature was shifted to 42°C, there was a more than 5-fold elevation in β-galactosidase production even in the absence of carbon source supplementation (Table 2b). Under these conditions, lactose and galactose exhibited significant induction of β-galactosidase synthesis in *vgb*-bearing and parental cells but not in the pUC9-containing strain. The incubation at 42°C temperature also showed repression of β-galactosidase synthesis by glucose and other carbon sources similar to that observed under previous temperatures. However, such enzymic repression in *vgb*-bearing cells was not as severe as the degree of repression detected in the *vgb*-lacking strains. Therefore, when *vgb*-bearing cells were supplemented with either glucose or fructose or mannitol carbon sources, the β-galactosidase activity almost recovered a repressor-free control level (Table 2b). Further comparison between pUC8:16 and pUC9 harboring cells indicated that the enzyme induction by lactose and galactose at either 37° or 42°C (Table 2a,b) was statistically significant ($P < 0.05$).

Effect of Carbon Sources on the Aerobic Respiration of *Enterobacter* Strains. Measurement of oxygen uptake suggested that the consumed amounts of oxygen were not significantly different when determined either at the incubation temperature of 37° or 42°C (data not shown). Relatively, the consumption of O_2 by *vgb*-bearing was significantly higher ($P > 0.05$) than *vgb*-lacking cells and the demand for this consumption was

Table 3. Effect of different carbon sources on the respiration level by *Enterobacter*, *Enterobacter*: pUC9 and *Enterobacter*: pUC8:16

Carbon source used at 0.5% concentration	Parental cells	*Enterobacter*: pUC9	*Enterobacter*: pUC8:16
Control	0.95 (0.05)	0.90 (0.04)	1.10 (0.02)
Citrate	0.39 (0.03)	0.30 (0.02)	0.5 (0.06)
Fructose	0.63 (0.09)	0.57 (0.09)	0.78 (0.04)
Galactose	0.65 (0.05)	0.55 (0.04)	1.16 (0.01)
Glucose	0.65 (0.06)	0.60 (0.06)	0.70 (0.08)[a]
Glycerol	0.35 (0.05)	0.30 (0.04)	0.50 (0.05)
Lactose	1.1 (0.10)*	0.96 (0.03)*	1.20 (0.01)
Maltose	0.60 (0.09)	0.40 (0.03)	0.66 (0.03)
Mannitol	0.30 (0.04)	0.28 (0.03)	0.34 (0.02)
Pyruvate	0.42 (0.05)	0.33 (0.02)	0.45 (0.01)
Succinate	0.44 (0.05)	0.40 (0.04)	0.58 (0.03)
Sucrose	0.72 (0.08)	0.60 (0.04)	0.95 (0.05)

Piror to oxygen measurements 0.1 M potassium phosphate buffer was stirred for 2 h to be air saturated (DO = 250 μM). One milliliter of bacterial culture (cfu was simultaneously determined by plating on LB and LB-Ap) was centrifuged at 10,000 g for 5 min and washed twice with 0.1 M potassium phosphate, pH 7, and the resulting pellet added quantitatively to 4 ml air-saturated buffer. The remaining concentration of oxygen in the cell-containing buffer was monitored by an oxygen electrode and recorded for 5 min. Values are the averages of two independent experiments. Double t-test comparisons were made on the values of oxygen uptake as described in Table 1.

associated more with lactose and galactose supplementation than other tested carbon sources (Table 3).

Discussion

The present data pointed to an effective role played by transformed VHb protein to enhance the β-galactosidase production in *Enterobacter aerogenes.* This was clearly manifested by the VHb improvement in lactose and galactose dependent induction of the enzyme synthesis. Also, there was general relief of enzyme activity from the repression caused by other carbon sources being tested. In particular, the VHb protein almost completely managed to overcome the repression effects of glucose, fructose, and mannitol on β-galactosidase formation. The *Enterobacter aerogenes* wild type or cells containing the same plasmid but lacking the VHb-expressing gene failed to achieve similar positive effects on β-galactosidase synthesis. Therefore, an overestimation of the observed enzyme induction by VHb protein that may arise from artifact binding of some molecules to the plasmid or *lac Z* gene would be rather remote. In an analogous study, Shaw et al. [16] isolated a gene encoding β-galactosidase from *Bacillus megaterium* that was either induced or repressed when the LB medium was supplemented with 2% of lactose or glucose, respectively. Base substitutions within certain elements of this gene caused partial relief of β-galactosidase repression.

Further work is still needed to elucidate the mechanism by which VHb protein induces the level of β-galactosidase formation in *Enterobacter aerogenes*. However it is possible to speculate on three targeting sites of intervention: (1) The level of Lac Z operon system; (2) The posttranscriptional level, such as mRNA stability; (3) The level of catabolite repression control. Since our data revealed better uptake of oxygen by *vgb*-bearing cells particularly under conditions of lactose and galactose enzyme induction, it is likely that the mechanism of VHb effects is mediated through an oxygen-trapping process. This may lead to enrichment in ATP generation, which is probably needed for higher productivity of β-galactosidase. The *vgb*-containing cells are known to have a high rate of electron flux through the respiratory chain and produce 65% more amounts of ATP than non-*vgb*-expressing cells [6]. *E. coli* was reported to have multiple regulatory mechanisms that sense changes in oxygen availability [18]. Also it was found that the promoter for the *Vitreoscilla* hemoglobin gene, *vgb*, is mainly induced under microaerobic but not anaerobic conditions. Irrespective of the operative VHb mechanism, the present data indicated a marked difference in the optimum temperature between cell biomass and β-galactosidase productivity. All *Enterobacter* strains being tested had an optimum temperature of 37°C for biomass yield and optimum temperature of 42°C for enzyme production. The peptide elongation in *E. coli* was found to increase as a function of temperature elevation from 37° to 44°C, while the growth rate was declining under these conditions [7]. This phenomenon was attributed to the maintenance of a ribosome pool, which at high temperature is readily available to meet future bacterial demands for protein synthesis but becomes inaccessible for cell growth.

It is worth mentioning that the high productivity of β-galactosidase under the influence of *vgb* transformation may have a great impact on the dairy industry. The low solubility of lactose increases the opportunity of its crystallization in dairy food, which may affect its texture and causing deposit formation as well as protein denaturation [20]. Moreover, the major byproduct of the dairy industry cheese whey contains about 70% of its solid as lactose and is considered a hazardous pollutant that cannot be discharged without expensive treatments. The present biotechnology of enhancing the β-galactosidase production may provide a valuable tool that can help to improve the dairy products by reducing the lactose concentration to acceptable levels. Also, through lactose hydrolysis it can maintain an environmental protection against the pollution effects of cheese whey accumulation. Though other chemical methods may also carry out the hydrolysis of lactose, the enzymatic method has the advantage of lowering the lactose contents without adversely affecting the nutritional value of whey or milk products [21].

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