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RESEARCH ARTICLE

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β-Galactosidase activity of *Escherichia coli* under long-term starvation, alterations in temperature, and different nutrient conditions in lake water

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Abstract β-Galactosidase activity of *Escherichia coli* was investigated in response to long-term starvation, changes in temperature and the presence of certain nutrient sources in lake water. β-Galactosidase activity decreased markedly in filtered-autoclaved lake water at 25 °C and 37 °C, whereas it remained almost constant at 4 °C and 15 °C for 60 days. Increases in β-galactosidase activity were observed in response to the following nutrient sources: glycine, serine, methionine and ammonium sulfate at 4 °C; glycine and ammonium sulfate at 15 °C; glycine, serine, methionine and ammonium sulfate at 30 °C. Glycine addition led to an increase in β-galactosidase activity of almost five and seven orders of magnitude at 15 °C and 30 °C, respectively. In addition, L-methionine had the strongest influence on β-galactosidase activity, which was detected as an increase of seven and eleven orders of magnitude at 4 °C and 30 °C, respectively. The effect of several amino acids and other nitrogen sources depended on the concentration of the nutrient source and the temperature. The results showed that, in lake water, long-term starvation, temperature change, and variations in nitrogen sources alter β-galactosidase activity. Those effects should be taken into account when monitoring coliforms from the environment.

Keywords *Escherichia coli* · β-Galactosidase activity · Long-term starvation · Nutrient sources effect

Introduction

Like other bacteria, under nutrient deprivation and different growing conditions, *Escherichia coli* undergoes physiological modifications involving, among others, enzyme activity [8], protein synthesis [15], and changes in the cell envelope [18]. Such cellular alterations under starvation, stress and excess nutrient conditions could enable microorganisms to adapt to survive in the surrounding environment [7].

β-Galactosidase is a catabolic enzyme (EC 3.2.1.23) that cleaves lactose into galactose and glucose [2]. It is a typical cytosolic periplasmic protein that is released to the cell membrane but not to periplasm [9]. Warren et al. [17] suggested the importance of this enzyme for the detection of fecal coliforms. There was a linear correlation between fecal coliforms most probable numbers (MPNs) and the substrate *o*-nitrophenyL-β-D-galactoside (ONPG) hydrolysis times. In addition, Munro et al. [8] speculated that the disappearance of *E. coli* β-galactosidase activity in seawater could have significant implications for the enumeration of these bacteria by standard culture methods.

β-Galactosidase activity measured in cultivable coliforms or fecal coliform bacteria in environmental samples is higher than that measured in pure cultures. However, loss of culturability does not necessarily lead to loss of β-galactosidase activity [14]. Thus, the activity of this enzyme can be used as an indicator of fecal pollution and to determine the "real" amount of bacteria in the environment. This may be of great importance to human health. However, there is a possibility that *E. coli* β-galactosidase activity is affected by environmental factors, such as the lack of nutrients, temperature changes, or the presence of certain nutrients in the aquatic environment. Many surface waters are monitored for the presence of total coliforms and fecal coliforms by means of a β-galactosidase assay that has been developed as one of the faster detection methods, e.g. the Autoanalysis Colilert method [2, 6]. This assay method is based on the utilization of fluorogenic and chromogenic substrates. The enzymatic hydrolysis of ONPG is carried out by β-galactosidase in fecal coliforms [3, 17]. Therefore, the effects of different factors in lake water on the induction of β-galactosidase activity is important.

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It was reported that β-galactosidase activity is also influenced by the nutritional status of the medium. Moreover, β-Galactosidase activity levels depend on the physiological status of the bacteria, and anywhere from 10 to 90% of coliforms may be stressed [1]. It was shown that β-galactosidase activity of *E. coli* was reduced by increasing starvation time and became undetectable after 13 days of incubation. *E. coli* β-galactosidase activity decreased gradually with incubation period even though other enzyme activities, such as alkaline phosphatase, increased [8]. The presence of certain nutrients also affects β-galactosidase activity. It was reported that the highest β-galactosidase activity occurred under succinate and glycerol limitation. On the other hand, there was no β-galactosidase activity under histidine starvation [13]. In addition, temperature might be another major factor affecting *E. coli* β-galactosidase activity. β-Galactosidase activity of *E. coli* was compared at 25 °C, 35 °C and 44.5 °C, and maximum activity was observed at 44.5 °C. The same study also revealed large variations in the enzyme levels of different β-D-galactosidase- and β-D-glucuronidase-positive bacteria [16]. In addition, it was reported that β-galactosidase synthesis in *relA*$^-$ mutants of *E. coli* was more sensitive to temperature and that the activity of the enzyme was lower than in wild-type cells [13].

β-Galactosidase activity also depended on growth conditions and medium composition [6]. It was higher in samples collected from water containing high concentrations of organic and inorganic nutrients [17]. For cellular economy, microorganisms synthesize certain enzymes only when they are needed, i.e. in the presence of substrate [4]. Induction of β-galactosidase activity of *E. coli* may be a metabolic burden with respect to the survival of cells, since it has been shown that mRNA appears and disappears quickly during starvation. The quantity of specific β-galactosidase mRNA present in the cells is proportional to the amount of enzyme synthesized [9].

While a few studies on *E. coli* β-galactosidase activity under starvation stress have been carried out, little is known regarding long-term starvation stress of these bacteria in aquatic environments. Changes in β-galactosidase activity may reflect changes in the metabolic activity of *E. coli* in response to different nutritional conditions and different temperatures. Therefore, monitoring the changes in enzyme activity may help in elucidating the factors affecting the survival and detection of *E. coli* in lake water. The main goal of this study was to investigate long-term starvation stress, temperature and some nutrient sources on β-galactosidase activity of *E. coli* in lake water.

Material and methods

Escherichia coli ML30 was used for all experiments. The strain was provided by the University of Warwick University Culture Collection (and originally obtained from Queen Elizabeth College, London). Cultures were maintained on nutrient agar (Oxoid) slopes incubated at 37 °C for 24 h and were stored at 4 °C. The organism was then subcultured and transferred to the lake water microcosms.

β-Galactosidase assay

β-Galactosidase activity of *E. coli* in lake water was assayed according to the method of Pardee et al. [12]. Solutions were prepared in 0.05 M sodium phosphate buffer (pH 7.5), except for 0.01 M ONPG (*o*-nitrophenyl-β-D-galactopyranoside), 1 M sodium carbonate, and 0.1% w/v deoxycholate which were prepared in distilled water; 0.032 M glutathione (reduced form) was made up in distilled water and stored at 4 °C.

Experimental procedure

Cultures of *E. coli* were grown overnight at 30 °C. The cells were harvested and washed with 0.05 M phosphate buffer. Lake water samples (100 ml) (Tocil Lake, Warwick University Campus, England) were inoculated with *E. coli* to give an initial viable count of approximately 10^7 colony-forming units (cfu)/ml.

One ml of the culture was removed from the flasks and added to 4 ml of 0.05 M phosphate buffer. To this mixture, a drop of toluene and 0.1% sodium deoxycholate were added. To prevent false coloration, 0.2 ml glutathione was added to the solution. All samples were incubated at 30 °C for 4 h. The reaction was stopped by adding 0.2 ml of sodium carbonate. Total activity was expressed as the change in absorbance at 420 nm/4 h and was based on the total mass of bacteria in the samples. Specific enzyme activity was calculated as total activity per unit of viable-cell count.

Nutrient sources amendments

Samples (100 ml) of filtered-autoclaved lake water microcosms were individually amended with different concentrations of amino acids (proline, serine, glycine and methionine, 2.0–6.0 mg/l) and other nitrogen sources (ammonium sulfate, 10–30 mg/l; ammonium nitrate, 5.0–15 mg/l).

Long-term starvation experiments

E. coli was grown overnight at 30 °C in basal medium containing glycerol as the sole source of carbon and energy. Aliquots (100 ml) of sterile lake water microcosms were inoculated to give an initial viable-cell count of approximately 10^7 cfu/ml. The flasks were incubated in the dark without shaking at 4 °C, 15 °C, 25 °C, and 30 °C. Viable-cell counts of *E. coli* were determined by the surface spread-plate technique. Samples were taken immediately after inoculation to determine initial number of bacteria at the time of inoculation (T_0), and at regular intervals (up to 60 days). Culturability was assessed by standard plate-counting; 0.1 ml of the diluted sample was spread on triplicate nutrient agar plates. Plates were incubated at 37 °C for 24 h and then were counted manually; results were expressed as cfu/ml.

Results

Survival and β-galactosidase activity

Figure 1 shows the survival of *E. coli* at different temperatures in filtered-autoclaved lake water. Cells lost viability after 7 days at 37 °C.

Figure 2 shows that β-galactosidase activity of *E. coli* remained relatively constant for 60 days in filtered-

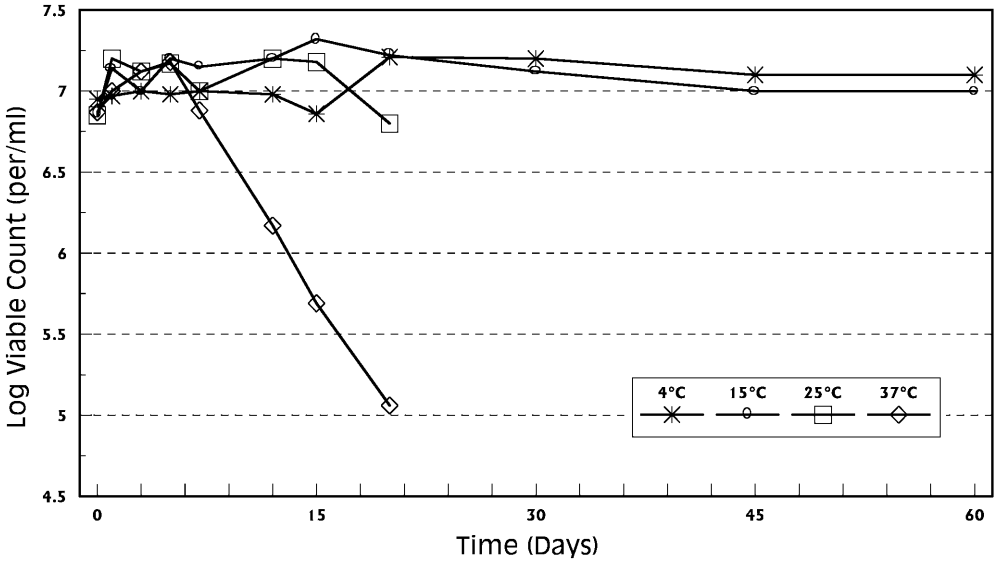

Fig. 1. Survival of *Escherichia coli* at different temperatures in filtered-autoclaved lake water. The samples were incubated in the dark without shaking at 4 °C (*asterisks*), 15 °C (*open circles*), 25 °C (*open squares*), 37 °C (*open diamonds*). Viable-cell counts were determined on surface-spread plates after overnight incubation at 37 °C

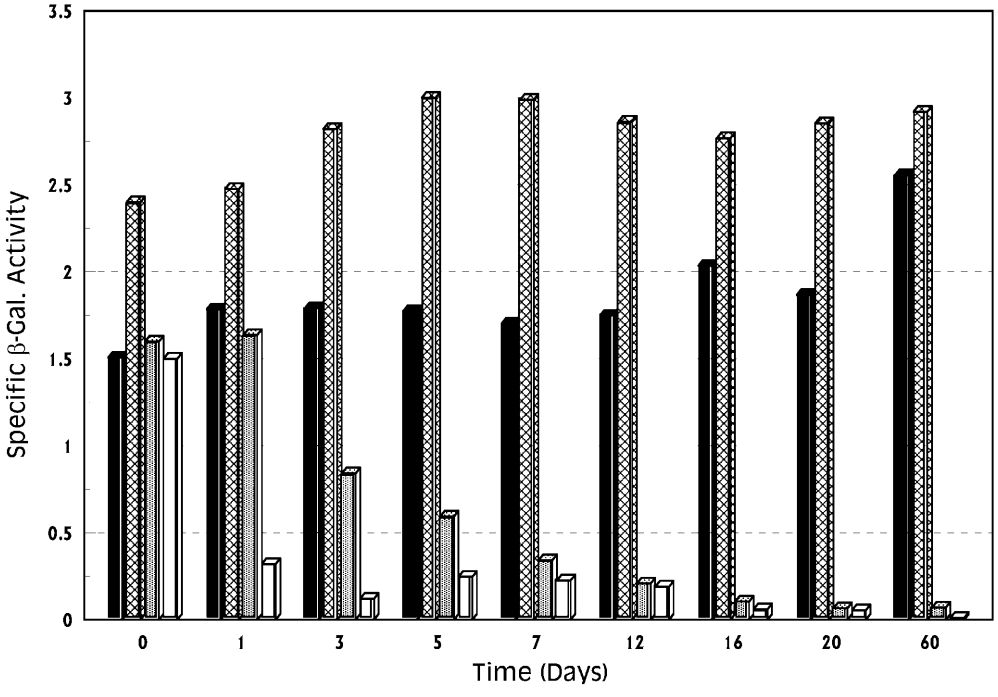

Fig. 2. *β*-Galactosidase activity of *E. coli* at different temperatures in filtered-autoclaved lake water. The samples were incubated in the dark without shaking at 4 °C (*black columns*), 15 °C (*cross-hatched columns*), 25 °C (*striped columns*), 37 °C(*open columns*). Activity assay were carried out at 30 °C for 4 h. Specific activity is expressed as the change in absorbance at 420 nm/4 h per cfu

autoclaved lake water without any nutrient amendments at 4 °C and 15 °C. However, *β*-galactosidase activity declined rapidly in cells incubated at 37 °C, with more than 80% of enzyme activity lost in the first 24 h. The decrease in activity was also faster at 25 °C than at 4 °C and 15 °C, with 80% of activity lost in 7 days. This sudden decline in *β*-galactosidase activity in *E. coli* is similar to the loss of respiratory enzymes under starvation stress at 37 °C [11]. Although the activity of *E. coli* *β*-galactosidase began to decline after 3 days of incubation, there was no decline in the viability of the cells in filtered-autoclaved lake water at 25 °C until after 15–17 days (Fig. 1). With respect to temperature, *β*-galactosidase activity was highest at 15 °C and remained relatively constant for up to 60 days. The activity of the enzyme increased 60% after 60 days at 4 °C and 20% after 5 days at 15 °C. There was no decline in the number of viable *E. coli* cells at either temperature. These results suggest that metabolic changes occur in *E. coli* cells under starvation stress at 25 °C and 37 °C in filtered-autoclaved lake water.

β-Galactosidase activity in water amended with nutrient sources

Previous studies have shown that the addition of certain nutrients increased the survival of *E. coli* in lake water. Based on those results, several nutrients were chosen in order to monitor *β*-galactosidase activity and its

relationship to survival. The results showed that carbon sources did not significantly influence either survival or β-galactosidase activity with the exception of lactose (data not shown).

β-Galactosidase activity of *Escherichia coli* in filtered-autoclaved lake water amended with amino acids

β-Galactosidase activity of *E. coli* was also followed after the addition of single amino acids and other nitrogen sources to filtered-autoclaved lake water and subsequent incubation at several different temperatures. The addition of amino acids to filtered-autoclaved lake water led to an increase in the total and specific activity of β-galactosidase at 4 °C, 15 °C and 30 °C (Tables 1, 2, and 3. It was observed that the effect of amino acids on enzyme activity depended on both incubation temperature and the concentration of the amended nutrient. As shown in Table 1, the specific activity of the enzyme was significantly enhanced at 4 °C, 15 °C and 30 °C after the addition of glycine. Although changes in the specific activity ratio (compared to non-amended control) increased at all incubation temperatures, different glycine and serine concentrations showed an almost similar impact on specific activity at 4 °C. However, glycine led to an increase in specific activity that was proportional to the concentration added at 15 °C and 30 °C. Serine additions resulted in an increased activity at 4 °C for all concentrations and also at the highest concentration at 30 °C. On the other hand, proline did not critically influence β-galactosidase activity of *E. coli* at all incubation temperatures. The effect of the amino acid

Table 1. The effect of nutrient sources on β-galactosidase activity of *Escherichia coli* in filtered-autoclaved lake water at 4 °C

Nutrient sources concentrations(mg/l)	Total β-galactosidase activity	Specific β-galactosidase activity	Changes in specific activity ratio (compared to non-amended control)
Control	0.780	0.11	1.00
Glycine (2.0)	1–500	0.42	3.81
Glycine (4.0)	1.690	4.16	3.78
Glycine (6.0)	1.140	0.36	3.27
L-Proline (2.0)	1.060	0.18	1.63
L-Proline (4.0)	0.970	0.13	1.18
L-Proline (6.0)	0.750	0.18	1.63
Serine (2.0)	1.460	0,27	2.45
Serine (4.0)	1.120	0.36	3.27
Serine (6–0)	1.100	0.36	3.27
L-Methionine (2.0)	0.850	0.23	2.09
L-Methionine (4.0)	0.910	0.81	7.36
L-Methionine (6.0)	0.980	0.35	3.18
Ammonium sulfate (10.0)	1.020	0.27	2.45
Ammonium sulfate (20.0)	1.140	0.38	3.45
Ammonium sulfate (30.0)	1.730	0.65	5.90
Ammonium nitrate (5.0)	0.930	0.36	3.27
Ammonium nitrate (10.0)	0.759	0.18	1.63
Ammonium nitrate (15.0)	0.800	0.11	1.00

Table 2. The effect of nutrient sources on β-galactosidase activity of *Escherichia coli* in filtered-autoclaved lake water at 15 °C

Nutrient source concentrations(mg/l)	Total β-galactosidase activity	Specific β-galactosidase activity	Changes in specific activity ratio (compared to non-amended control)
Control	0.69	0.17	1.00
Glycine (2.0)	0.82	0.24	1.41
Glycine (4.0)	0.97	0.47	2.76
Glycine (6.0)	1.11	0.92	5.41
L-Proline (2.0)	0.68	0.20	1.17
L-Proline (4.0)	0.71	0.22	1.29
L-Proline (6.0)	0.71	0.18	1.05
Serine (2.0)	0.68	0.15	0.88
Serine (4.0)	0.66	0.12	0.70
Serine (6–0)	0.67	0.21	1.23
L-Methionine (2.0)	0.78	0.20	1.17
L-Methionine (4.0)	0.82	0.17	1.00
L-Methionine (6.0)	0.85	0.20	1.17
Ammonium sulfate (10.0)	0.86	0.24	1.41
Ammonium sulfate (20.0)	0.80	0.36	1.11
Ammonium sulfate (30.0)	0.83	0.31	1.82
Ammonium nitrate (5.0)	0.83	0.21	1.23
Ammonium nitrate (10.0)	0.78	0.25	1.47
Ammonium nitrate (15.0)	0.78	0.33	1.94

Table 3. The effect of nutrient sources on β-galactosidase activity of *Escherichia coli* in filtered-autoclaved lake water at 30 °C

Nutrient source concentrations(mg/l)	Total β-galactosidase activity	Specific β-galactosidase activity	Changes in specific activity ratio (compared to non-amended control)
Control	1.09	0.22	1.00
Glycine (2.0)	1.53	0.30	1.36
Glycine (4.0)	1.84	0.84	3.81
Glycine (6.0)	1.82	1.54	7.00
L-Proline (2.0)	0.92	0.82	3.72
L-Proline (4.0)	1.10	0.13	0.59
L-Proline (6.0)	1.13	0.24	1.09
Serine (2.0)	0.65	0.10	0.45
Serine (4.0)	0.52	0.06	0.27
Serine (6–0)	1.05	0.87	3.95
L-Methionine (2.0)	1.55	1.41	6.40
L-Methionine (4.0)	1.92	2.42	11.00
L-Methionine (6.0)	1.75	1.96	8.90
Ammonium sulfate (10.0)	1.39	0.55	2.50
Ammonium sulfate (20.0)	1.32	1.36	6.18
Ammonium sulfate (30.0)	1.10	2.76	12.50
Ammonium nitrate (5.0)	1.31	0.52	2.36
Ammonium nitrate (10.0)	1.52	0.56	2.54
Ammonium nitrate (15.0)	1.26	0.33	1.50

methionine on β-galactosidase activity of *E. coli* in lake water was also studied. Addition of methionine did not influence specific β-galactosidase activity at 15 °C, whereas it was effective at 4 °C and 30 °C. The highest specific enzyme activity occurred following the addition of 4 mg methionine/l at 4 °C and 30 °C, and was, respectively, seven and eleven orders of magnitude higher than in the control. In each case, there was an increase in total activity that led to an increase in specific activity rather than a decrease in viable-cell count, which would have the same effect. This fact suggests a further induction of β-galactosidase under starvation conditions even in the absence of the inducer isopropyl β-D-thiogalucopyranoside (IPTG) . Even at 4 °C there was an increase in total activity. As protein synthesis was considered very improbable to occur in *E. coli* at 4 °C, this increase was probably due to permeability changes in the organisms induced by the reduction in temperature, starvation, or both. The increased specific activity of the enzyme was often apparent within 24 h of addition of the amino acids. After 7 days or longer, the increase was often four-fold or more, which is not explainable by simple permeability changes but must be due to an increase in enzyme activity.

β-Galactosidase activity of *Escherichia coli* in filtered-autoclaved lake water amended with ammonium sulfate and ammonium nitrate

Özkanca (1993) showed that the disappearance of *E. coli* in the presence of the natural microflora of lake water was delayed by the addition of ammonium sulfate and ammonium nitrate [10]. Therefore, the effects of ammonium sulfate and ammonium nitrate on β-galactosidase were examined at 4 °C, 15 °C, and 30 °C in filtered-autoclaved lake water. Tables 1–3 show that the specific β-galactosidase activity of *E. coli* increased by the addition of 10, 20 and 30 mg ammonium sulfate/l to lake water at 15 °C. A similar increase was observed at 4 °C and 30 °C. The rate of increase was proportional to the ammonium sulfate concentration added at 4 °C and 30 °C but this relationship was conclusive at 15 °C. Similar results were obtained after the addition of ammonium nitrate at the same incubation temperatures. Again the increase was due to an increase in total activity, which suggests that either cells were more permeable or that there was an increased production of β-galactosidase.

Discussion

Bacterial enzymes are strongly affected by the organism's nutritional status and its chemical and physiological environment. As a result, a wide range of changes in the physiological activities of bacterial cells may make their detection in environmental samples difficult, especially under starvation stress. Therefore, it is important to know what factors affect bacterial enzyme activity and how activity is affected in bacterial cells in natural environments under nutrient limitation.

One enzyme activity assay is based on the enzymatic hydrolysis of ONPG by fecal coliforms [17]. This method is very sensitive in terms of accuracy and allows faster detection of bacteria directly from the environment than traditional culture methods [2, 3, 6, 17]. It has been reported that β-galactosidase activity or the rate of hydrolysis of ONPG is proportional to the quantity of fecal coliforms in the inoculum [1,17]. Warren et al. [17] suggested that there was a linear correlation between ONPG hydrolysis time and fecal coliform MPN values. However, our results demonstrate that β-galactosidase activity is not related to the viable-cell count in filtered-autoclaved lake water under starvation stress. Although *E. coli* remained viable (as determined by plate counts)

and could be further cultured after 20 days at 25 °C and 37 °C, β-galactosidase activity declined markedly in filtered-autoclaved lake water. This suggests that long-term starvation of *E. coli* in the natural environment might affect their detection when measured by assaying β-galactosidase activity.

One explanation for the decrease in enzyme activity is that β-galactosidase is less stable than other bacterial enzymes. It is also possible that proteolytic activity is directed towards less essential enzymes such β-galactosidase rather than towards those involved in the alleviation of starvation stress, such as alkaline phosphatase and other nutrient-scavenging enzymes. However, the viability of *E. coli* cells and β-galactosidase activity remained almost constant at 4 °C and 15 °C during the 60 days of the experiment. The increased enzyme stability at the lower temperatures could be attributed to a decrease proteolytic activity or an increase in enzyme stability.

These results imply that certain nutrient sources should lead to an increase in *E. coli* β-galactosidase activity in filtered-autoclaved lake water at different temperatures. Warren et al. [17] had previously reported that β-galactosidase activity increased in water samples containing high concentrations of both organic and inorganic nutrients. The data here demonstrate that, whereas carbon sources did not significantly influence *E. coli* β-galactosidase activity, amino acids did. For instance, glycine amendments led to an increase in β-galactosidase activity at all incubation temperatures. In addition, although serine did not have any significant influence on β-galactosidase activity in lake water at 15 °C and 30 °C, it did have an effect at 4 °C under the same conditions. However, L-methionine showed the strongest effect at 30 °C and at 15 °C, but did not cause any changes in activity at 4 °C. Furthermore, the effect of some amino acids was concentration-dependent, with greater increases in specific activity as the amino acid concentration added to the lake water was increased.

Other nitrogen sources such as ammonium sulfate and ammonium nitrate also caused increases in β-galactosidase activity in filtered-autoclaved lake water. In this environment, both nutrient sources are known to be effective in prolonging the survival of *E. coli* [5, 10]. Neidhardt et al. [9] reported that, in the presence of inducer, the increase in β-galactosidase activity in a growing culture of *E. coli* reflects de novo enzyme synthesis. It is possible that *E. coli* utilize nitrogen sources for protein synthesis; if so, then β-galactosidase activity could be increased by the addition of ammonium sulfate and ammonium nitrate to filtered-autoclaved lake water.

To summarize, β-galactosidase activity is affected by different factors in lake water such as nitrogen source and temperature. Moreover, starvation stress influences β-galactosidase activity, which could lead to problems in the detection and identification of fecal coliforms. *E. coli* enter a dormant state in order to survive long-term starvation stress. Under this condition, it is unlikely that the bacteria synthesize β-galactosidase . The loss of activity in starving cells reflects denaturation of the enzyme by proteolysis thereby generating a nitrogen source for the cells in order to survive nutrient-limited conditions. The increased activity after the amendment of lake water with a nitrogen source may be a reflection of the decreased level of denaturation of existing enzymes, which would occur to fulfil the cells' nitrogen requirements.

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