# The effect of physico-chemical parameters and chemical compounds on the activity of β-D-galactosidase (B-GAL), a marker enzyme for indicator microorganisms in water

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## Abstract

The presence of coliforms in polluted water was determined enzymatically (i*n situ)* by directly monitoring the activity of β-D-galactosidase (B-GAL) through the hydrolysis of the yellow chromogenic subtrate, chlorophenol red beta-D-galactopyranoside (CPRG), which produced a red chlorophenol red (CPR) product. The objectives of this study were to monitor the effect of compounds commonly found in the environment and used in water treatment on a B-GAL CPRG assay and to investigate the differences between the environmental B-GAL enzyme and the pure enzyme. B-GAL was most optimally active at

pH 7.8. Two temperature optima were observed at 35 and 55$^{o}$C, respectively. B-GAL activity was strongly inhibited by silver and copper ions. While calcium and ferrous ions at lower concentrations (50-100 mg $l^{-1}$) increased the enzyme activity, a reduction was observed at higher concentrations (200 mg $l^{-1}$). Sodium hypochlorite, normally used in rural areas to disinfect water gradually decreased B-GAL activity at concentrations between 0 and 5600 ppm for both the commercial and environmental enzymes. B-GAL from the environment behaved differently from its commercially available and pure counterpart.

*Keywords:* β-D-Galactosidase; Chlorophenol red (CPR); Chlorophenol-red-β-D-galactopyranoside (CPRG); Coliforms; Faecal

## 1. Introduction

Coliforms are a group of bacteria used by the water industry to assess the microbiological quality of both drinking and recreational water. These bacteria, though not generally pathogenic (disease-causing) themselves, serve as indicators for the potential presence of organisms which may be pathogenic. Most coliforms are present in large numbers among the intestinal flora of human and other warm-blooded animals, and are thus commonly found in faecal wastes. As a consequence, coliforms (such as *Escherichia coli*) detected in higher concentrations than pathogenic bacteria are used as an index of the potential presence of enteric-pathogens in the aquatic environment (Rompré et al., 2002). Various methods for determining and quantifying the presence of indicator organisms exist.

These can be classified into (a) cultural or classical, (b) enzymatic and (c) molecular methods (Frampton and Restaino, 1993; De Boer and Beumer, 1999; Venter, 2000; Rompré et al., 2002). The classes; however, overlap and improvements have seen combinations of these methods, which tend to increase the sensitivity and rapidity of the assays. Coliforms secrete an enzyme, β-galactosidase (B-GAL) which can be monitored to measure activity of the coliforms.

B-GAL catalyses the breakdown of lactose and structurally related compounds, yielding galactose and glucose or a structurally related product (Davies and Apte, 1999; George et al., 2002). Examples of chromogenic substrates for B-GAL include *o*-nitrophenyl-β-D-galactopyranoside (ONPG), chlorophenol red β-galactopyranoside (CPRG), *p*-nitrophenyl-β-D-galactopyranoside (PNPG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). The application of direct enzymatic detection of coliforms by monitoring β-D-galactosidase activity has been demonstrated by several authors including Davies and Apte (1999) and George et al. (2002). Previous comparative studies (in our laboratory) of different chromogenic substrates (with regards to cost, sensitivity and kinetic considerations) showed that CPRG was a better substrate. Studies with MUGal, although very sensitive, suffered from a high level of interference to several compounds, and therefore was not suitable for direct "*in situ*" assays on environmental water samples.

A wide variety of metal ions are found in the environment. Metal ions play important roles in the biological function of many enzymes and can have a variety of effects on

68 enzyme systems. Enzymes in dilute solutions function best under limited conditions of
69 temperature, pH and salt concentration (Berg et al., 1986; Singh et al., 1990).

70 The various modes of metal-protein interaction include metal-, ligand- and enzyme-
71 bridge complexes. Metals can also serve as electron donors or acceptors (Tryland et al.,
72 1997). For some enzymes, the presence of metal ions is crucial and required for activity.
73 Some enzymes require the assistance of metal ions in order to perform catalysis. Even in
74 cases where metals are required, very high metal concentrations or the incorrect metal can
75 inhibit the enzyme's activity (Den Blanken, 1985). A large number of enzymes have been
76 found to be dependent on alkali metal ions for activity. Of the alkali metal ions, sodium and
77 potassium are commonly found in living systems. Metal ions such as $Zn^{2+}$, $Mg^{2+}$, $Mn^{2+}$,
78 $Fe^{3+}$, $Cu^{2+}$, $K^{+}$ and $Na^{+}$ can be employed as cofactors of enzymes (Den Blanken, 1985). B-
79 GAL is a well studied enzyme in its purified form but few studies have looked at this
80 enzyme's activity *in situ* in the environment such as a river.

81 This study investigated the effect of several ions and compounds on B-GAL in an *in*
82 *situ* assay on the potential inaccuracies in coliform determination in polluted waters using
83 direct enzyme methods. The effects of physico-chemical parameters (e.g. pH and
84 temperature) were also investigated. The significance of this study is in that it will aid in
85 the identification of potential limitations in the use of chromogenic substrates for *in situ* B-
86 GAL assays for the rapid enzymatic determination of coliforms. This study therefore has
87 both important public health and research and development significance.

88

## 2. Materials and methods

### *2.1. Materials*

Chlorophenol red B-D-galactopyranoside (CPRG), dihydrogen sodium phosphate, disodium hydrogen phosphate, sodium hydroxide, sodium acetate and glacial acetic, sodium sulphate, sodium chloride, sodium carbonate, cadmium chloride, magnesium chloride, calcium chlorite, ferrous chloride, potassium chloride, ferulic acid, EDTA, potassium nitrate, sodium hypochlorite, citric acid, copper sulphate, calcium sulphate and sodium sulphite were all obtained from Merck (Darmstadt, Germany). The buffers and effectors were prepared using water purified with a Milli-Q system (Millipore, Milford, CT, USA). All reagents were of the highest analytical grade available.

### *2.2. Sampling*

Water collection was performed in accordance with the standard procedures outlined in Frampton and Restaino (1993). Water samples were collected from the Bloukrans River, Grahamstown in the Eastern Cape of South Africa aseptically in 250 ml sterile pyrex glass bottles (Schott Duran, Germany), placed on ice and transported immediately to the laboratory and analysed within an hour. Two sampling points, stagnant and running, were selected. The water temperature was measured on site, and on arrival in the laboratory the pH was determined. Samples were collected in triplicate between 08h00 and 08h30 on each sampling morning.

### *2.3. B-GAL enzyme assay*

The B-GAL assay was performed according to modified protocols of Seeber and Boothroyd (1996) and Pelisek et al. (2000) as described by Wutor et al. (2007). CPRG (80 µg in 20 µl water) was added to 90 µl 0.1 M sodium phosphate buffer (pH 7.8), and 90 µl of environmental (water) sample was added to initiate the reaction. The change in absorbance per min was determined at 575 nm on a PowerWave$_x$ (Bio-Tek Instruments, USA). Enzyme activity was then calculated from the mean of triplicate results.

In all the environmental assays performed, two sets of controls were set up; an enzyme control which entailed a reaction mixture of enzyme and substrate and a substrate control containing only the substrate and buffer (no enzyme). These controls allowed the quantification of background reactions or contributions from the environment. All assays were performed in triplicate, unless stated otherwise. Results were reported as means ± standard deviations (SD).

Analysis of variance (ANOVA) was performed using Microsoft Excel 2003 statistical tool at 5% level of significance.

### *2.4. pH and temperature optimization*

The pH dependence of B-GAL activity was studied within the range of pH 5-11 and the effect of temperature between 20-65$^{o}$C. GAL activity was assayed at various pH values

(5 – 11) using the following buffer systems: pH 5.0-6.0, sodium acetate; pH 6.0-8.0, sodium phosphate; pH 7.0-9.0, Tris-HCl and pH 9.0-11.0, carbonate-bicarbonate. All buffers used in the assays were at a concentration of 0.1 M.

***2.5 Effect of environmental water samples on commercial B-GAL activity***

In order to establish the total contribution of compounds already present in the environmental water samples on the enzyme assay, varying volumes ranging between 0-50 percent of the total assay volume were pre-incubated with the commercial B-GAL assay for 30 min prior to determining the enzyme activity.

***2.4 Effect of ions and compounds on 'in situ' B-GAL activity***

With the exception of sodium hypochlorite, appropriate volumes of commercial B-GAL, buffer and effector concentration ranging between 0 and 200 mg $l^{-1}$ were pre-incubated at room temperature (20 ± 2ºC) for 30 min, after which the reaction was initiated by the addition of CPRG and monitored spectrophotometrically at 575 nm. Effectors studied included sodium sulphate, sodium chloride, sodium carbonate, cadmium chloride, magnesium chloride, calcium chlorite, ferrous chloride, potassium chloride, ferulic acid, edta, potassium nitrate, sodium hypochlorite, citric acid, copper sulphate, calcium sulphate and sodium sulphite. The feasibility of a study of the effects of these compounds on B-GAL was justified by the fact that these compounds are commonly found in the water environment and are also used in water treatment processes. Appropriate controls for each

effector concentration were also prepared. Environmental water samples were sent to the Nelson Mandela Metropolitan Municipality Scientific Services for analysis of ion content. All assays were performed in triplicate.

## 3. Results and discussion

### *3.1. pH and temperature optimisation*

A gradual increase in environmental B-GAL activity was observed between pH 5 and 7.5 after which a sharp rise appeared at 7.8 (Figure 1). An approximate increase of 40% in activity occurred when the pH was increased from 7.5 to 7.8 and a similar level of reduction occurring between 7.8 and 9 (Figure 1). A similar trend was observed with both stagnant and running water samples. The temperature profiles for the environmental samples were particularly interesting (Figure 2). Two peaks at 35 and 55$^{o}$C were observed. A sudden drop in activity at 40$^{o}$C for both samples was also observed. The pH and temperature optima of the commercial enzyme were 8 and 37.5 respectively.

B-GAL enzymes exhibit a wide range of temperature optima, depending on the source or organism. Tryland and Fiksdal (1998) observed that *E. coli* B-GAL performed optimally at 45.5$^{o}$C, while the enzymes from other coliforms exhibited a range of temperature optima. B-GAL activities of *Enterobacter cloacae, Klebsiella pneumoniae* subsp. p*neumoniae, Yersinia intermedia* and *Ranunculus aquatilis* were however unstable

178 at 45.5$^{o}$C and less than the activities observed at 35$^{o}$C. Some fungal B-GAL enzymes have
179 been found to perform optimally at 60$^{o}$C (Maheshwari et al. 2000). *Aspergillus oryzae*
180 exhibited a temperature optimum at 55$^{o}$C while *Kluyveromyces lactis* performed most
181 efficiently at 37$^{o}$C. The source of coliforms in this study was confirmed as *E. coli* through
182 tryptic mapping and MALDI-TOF and subsequent bioinformatic analysis (data not shown).
183 From the results obtained, it can be assumed that there were two major types of coliforms
184 with temperature optima of 37 and 60$^{o}$C, respectively. Similar enzymes, but from different
185 environments can also have different pH and temperature conditions. Wakabayashi and
186 Fishman (1961) noted variations in pH optima for GUD from different sources using
187 phenolphthalein-β-D-glucuronide (PHEG) as substrate.
188
189 ***3.2 Effect of environmental water sample on commercial B-GAL activity.***
190
191 At a 50% (v/v) level, the environmental water samples reduced the relative activity
192 of B-GAL by about 15% (Figure 3). This implied that there was some level of
193 underestimation of the amount of B-GAL determined *in situ.* The highest decrease in
194 activity was observed between 0 and 10% (v/v) sample volumes. Little or no change
195 occurred between 10 and 50% (v/v) additions of environmental water sample (Figure 3).
196 The activity of B-GAL was underestimated by about 15% at 50% (v/v) of
197 environmental water sample volume (expressed as a % of the total assay volume). This
198 could possible be explained by the complex interaction between certain ions which
199 increased and others that inhibited B-GAL activity.
200

### *3.3. Effect of ions and compounds on an 'in situ' B-GAL activity*

The effect of various ions and compounds on commercial and environmental B-GAL over a concentration range of 0 – 200 mg $l^{-1}$ is presented in Table 1. Table 2 shows the result of the analysis of the environmental water samples from the Nelson Mandela Metropolitan Municipality Scientific Services Department, P.E., South Africa. Metal ions had varying effects on B-GAL. Monovalent sodium cations at 50 mg $l^{-1}$ increased the activity of the commercial enzyme (139%) while reducing the activity of the environmental enzyme (87.6 and 91.8%) for stagnant and running water samples, respectively. Potassium ions had a stronger activation effect at lower concentrations than did sodium ions. Silver ions inhibited the activity of B-GAL in both commercial and environment samples at concentrations between 50-150 mg $l^{-1}$.

With the exception of $Mg^{+2}$ divalent cations generally inhibited the enzyme activity at higher concentrations. Calcium ($CaCl_2$) at 200 mg $l^{-1}$ inhibited B-GAL activity, while an activated effect was observed lower concentrations. A similar trend was observed with copper ions. Ferrous ions increased enzyme activity between 50 and 100 mg $l^{-1}$ but inhibited the activity at 150 mg $l^{-1}$ and above. The addition of ferrous ions also resulted in a strong reduction in pH (data not shown).

Cadmium at all the studied concentrations inhibited B-GAL activity. It must however be noted that the toxic effect of cadmium on living cells will also result in the absence of the microbes which produce the enzymes. Ferrous chloride, which is used in

224 water treatment, may present serious problems when a direct enzyme assay method is
225 adopted. Tryland et al. (1997) observed that low concentrations of chlorine and divalent
226 cations such as magnesium increased B-GAL activity. In this study; however, the addition
227 of sodium hypochlorite resulted in an inhibition of the activity of both environmental and
228 commercial B-GAL enzymes. Sodium chloride at the concentrations studied generally
229 reduced the activity of B-GAL. Magnesium, however, increased the activity of B-GAL.
230
231 Chloride ions caused a reduction in B-GAL activity at concentrations between 0 and
232 100 mg $l^{-1}$. Divalent anions also showed varying effects on the activities of B-GAL.
233 Sulphate ions generally increased the enzyme activity over the range of concentrations used
234 in this study. In contrast, carbonate ions decreased the activity of B-GAL over the range of
235 concentrations studied. There was also an increase in pH as the carbonate concentration
236 increased (data not shown).
237
238 Sodium hypochlorite, which is normally used as a disinfectant, especially in remote
239 rural regions, reduced the activities of B-GAL by approximately 40-60% at 5600 ppm
240 (Figure 4). Similar trends were observed with EDTA and ferulic acid (Table 1).
241
242 Ferulic acid is a phenolic compound produced during lignin degradation and its
243 ability to inhibit the enzyme activities could imply that the use of direct enzyme assays in
244 eutrophic water bodies may yield a false negative result. In such water bodies, there will be
245 heavy decomposition of plant matter leading to the release of this phenolic compound.
246 Carbonates chelate phenolic compounds, thereby preventing their interaction with enzymes

(Wetzel, 1991). Even though the impact of phenolic compounds in hard waters may be countered by the carbonates, the presence of carbonates in water samples could yield false negative results. The availability of phenolic compounds as well as carbonates in a water environment thus presents a very severe limitation for the determination of coliforms based on the B-GAL assay.

Despite the possible contribution of B-GAL activity by *Vibrio* and *Aeromonas* species, a strong correlation between enzyme activity and coliform CFU was established for all the environmental water samples (Fig 5), hence the use of 'unfiltered' raw water samples in this study. A clean-up step was therefore not required and is an advantage in the application of this enzyme assay for use in rural communities. A further advantage of using an "*in situ*" assay is the application of biosensor and other similar technology for the rapid on-line and direct detection of coliforms in water intended for drinking purposes (Wutor et al., 2006).

## 4. Conclusions

Several compounds commonly found in the environment were able to affect B-GAL activity in the study. Results of enzymatic quantification of coliforms in polluted waters should therefore be stated with caution, especially when working with water samples which could potentially contain any of the metal ions studied. It may be prudent to remove some of these compounds prior to assaying for B-GAL. The possibility of obtaining false

positive or negative readings in the enzymatic detection of B-GAL in contaminated water should be cautiously anticipated and verified using more traditional microbiological methods. The direct enzyme assays, however, still serve as an excellent early warning sign for the potential presence of faecal material in the water. The possibility of using an internal reference or standard (i.e. commercial B-GAL) should also be explored in order to assess and correct (calibrate) for chemical (ion) interference when testing environmental samples.

Due to the complex nature of the interactions between ions and other compounds in polluted waters, pH and temperature optima may vary from that of the commercially available and pure B-GAL. Environmental enzymes exhibited different kinetics from their commercial counterpart.

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**Captions to Figures**

**Figure 1.** Effect of pH on commercial and environmental B-GAL activity using CPRG (100% relative activity is defined as the activity at the pH optimum for each enzyme). Values represent the means ± SD, n = 3.

**Figure 2.** Effect of temperature on commercial and environmental B-GAL using CPRG. (100% relative activity is defined as the activity at the temperature optimum for each enzyme). Values represent the means ± SD, n = 3.

**Figure 3.** Effect of environmental water sample on commercial B-GAL activity. Values represent the means ± SD, n = 3.

**Figure 4.** Inhibitory effect of sodium hypochlorite on B-GAL activity. Values represent the means ± SD, n = 3.

**Figure 5.** Correlation between B-GAL activity and coliform CFU (on CM 1046 media, Oxoid).