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1 **The effect of physico-chemical parameters and chemical compounds on the activity of**

2 **β-D-galactosidase (B-GAL), a marker enzyme for indicator microorganisms in water**

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12

13

14 **Abstract**

15

16 The presence of coliforms in polluted water was determined enzymatically (i*n situ)* by

17 directly monitoring the activity of β-D-galactosidase (B-GAL) through the hydrolysis of the

18 yellow chromogenic subtrate, chlorophenol red beta-D-galactopyranoside (CPRG), which

19 produced a red chlorophenol red (CPR) product. The objectives of this study were to

20 monitor the effect of compounds commonly found in the environment and used in water

21 treatment on a B-GAL CPRG assay and to investigate the differences between the

22 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°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.

46 These can be classified into (a) cultural or classical, (b) enzymatic and (c) molecular
47 methods (Frampton and Restaino, 1993; De Boer and Beumer, 1999; Venter, 2000; Rompré
48 et al., 2002). The classes; however, overlap and improvements have seen combinations of
49 these methods, which tend to increase the sensitivity and rapidity of the assays. Coliforms
50 secrete an enzyme, β-galactosidase (B-GAL) which can be monitored to measure activity
51 of the coliforms.
52
53 B-GAL catalyses the breakdown of lactose and structurally related compounds, yielding
54 galactose and glucose or a structurally related product (Davies and Apte, 1999; George et
55 al., 2002). Examples of chromogenic substrates for B-GAL include *o*-nitrophenyl-β-D-
56 galactopyranoside (ONPG), chlorophenol red β-galactopyranoside (CPRG), *p*-nitrophenyl-
57 β-D-galactopyranoside (PNPG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-
58 Gal). The application of direct enzymatic detection of coliforms by monitoring β-D-
59 galactosidase activity has been demonstrated by several authors including Davies and Apte
60 (1999) and George et al. (2002). Previous comparative studies (in our laboratory) of
61 different chromogenic substrates (with regards to cost, sensitivity and kinetic
62 considerations) showed that CPRG was a better substrate. Studies with MUGal, although
63 very sensitive, suffered from a high level of interference to several compounds, and
64 therefore was not suitable for direct "*in situ*" assays on environmental water samples.
65

66 A wide variety of metal ions are found in the environment. Metal ions play important
67 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.

110 ***2.3. B-GAL enzyme assay***

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

117

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

124

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

127

128 ***2.4. pH and temperature optimization***

129

130 The pH dependence of B-GAL activity was studied within the range of pH 5-11 and
131 the effect of temperature between 20-65°C. GAL activity was assayed at various pH values

132 (5 – 11) using the following buffer systems: pH 5.0-6.0, sodium acetate; pH 6.0-8.0,
133 sodium phosphate; pH 7.0-9.0, Tris-HCl and pH 9.0-11.0, carbonate-bicarbonate. All
134 buffers used in the assays were at a concentration of 0.1 M.
135
136 ***2.5 Effect of environmental water samples on commercial B-GAL activity***
137
138 In order to establish the total contribution of compounds already present in the
139 environmental water samples on the enzyme assay, varying volumes ranging between 0-50
140 percent of the total assay volume were pre-incubated with the commercial B-GAL assay for
141 30 min prior to determining the enzyme activity.
142
143 ***2.4 Effect of ions and compounds on 'in situ' B-GAL activity***
144
145 With the exception of sodium hypochlorite, appropriate volumes of commercial B-
146 GAL, buffer and effector concentration ranging between 0 and 200 mg l^{-1} were pre-
147 incubated at room temperature (20 ± 2ºC) for 30 min, after which the reaction was initiated
148 by the addition of CPRG and monitored spectrophotometrically at 575 nm. Effectors
149 studied included sodium sulphate, sodium chloride, sodium carbonate, cadmium chloride,
150 magnesium chloride, calcium chlorite, ferrous chloride, potassium chloride, ferulic acid,
151 edta, potassium nitrate, sodium hypochlorite, citric acid, copper sulphate, calcium sulphate
152 and sodium sulphite. The feasibility of a study of the effects of these compounds on B-GAL
153 was justified by the fact that these compounds are commonly found in the water
154 environment and are also used in water treatment processes. Appropriate controls for each

155 effector concentration were also prepared. Environmental water samples were sent to the
156 Nelson Mandela Metropolitan Municipality Scientific Services for analysis of ion content.
157 All assays were performed in triplicate.
158
159 **3. Results and discussion**
160
161
162 ***3.1. pH and temperature optimisation***
163
164 A gradual increase in environmental B-GAL activity was observed between pH 5
165 and 7.5 after which a sharp rise appeared at 7.8 (Figure 1). An approximate increase of 40%
166 in activity occurred when the pH was increased from 7.5 to 7.8 and a similar level of
167 reduction occurring between 7.8 and 9 (Figure 1). A similar trend was observed with both
168 stagnant and running water samples. The temperature profiles for the environmental
169 samples were particularly interesting (Figure 2). Two peaks at 35 and 55°C were observed.
170 A sudden drop in activity at 40°C for both samples was also observed. The pH and
171 temperature optima of the commercial enzyme were 8 and 37.5 respectively.
172
173 B-GAL enzymes exhibit a wide range of temperature optima, depending on the
174 source or organism. Tryland and Fiksdal (1998) observed that *E. coli* B-GAL performed
175 optimally at 45.5^{o}C, while the enzymes from other coliforms exhibited a range of
176 temperature optima. B-GAL activities of *Enterobacter cloacae, Klebsiella pneumoniae*
177 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°C. Some fungal B-GAL enzymes have
179 been found to perform optimally at 60°C (Maheshwari et al. 2000). *Aspergillus oryzae*
180 exhibited a temperature optimum at 55°C while *Kluyveromyces lactis* performed most
181 efficiently at 37°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°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

201 ***3.3. Effect of ions and compounds on an 'in situ' B-GAL activity***

202

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

213

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

220

221 Cadmium at all the studied concentrations inhibited B-GAL activity. It must
222 however be noted that the toxic effect of cadmium on living cells will also result in the
223 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

247 (Wetzel, 1991). Even though the impact of phenolic compounds in hard waters may be
248 countered by the carbonates, the presence of carbonates in water samples could yield false
249 negative results. The availability of phenolic compounds as well as carbonates in a water
250 environment thus presents a very severe limitation for the determination of coliforms based
251 on the B-GAL assay.
252
253 Despite the possible contribution of B-GAL activity by *Vibrio* and *Aeromonas*
254 species, a strong correlation between enzyme activity and coliform CFU was established
255 for all the environmental water samples (Fig 5), hence the use of 'unfiltered' raw water
256 samples in this study. A clean-up step was therefore not required and is an advantage in the
257 application of this enzyme assay for use in rural communities. A further advantage of using
258 an "*in situ*" assay is the application of biosensor and other similar technology for the rapid
259 on-line and direct detection of coliforms in water intended for drinking purposes (Wutor et
260 al., 2006).
261
262
263 **4. Conclusions**
264
265 Several compounds commonly found in the environment were able to affect B-GAL
266 activity in the study. Results of enzymatic quantification of coliforms in polluted waters
267 should therefore be stated with caution, especially when working with water samples which
268 could potentially contain any of the metal ions studied. It may be prudent to remove some
269 of these compounds prior to assaying for B-GAL. The possibility of obtaining false

270 positive or negative readings in the enzymatic detection of B-GAL in contaminated water
271 should be cautiously anticipated and verified using more traditional microbiological
272 methods. The direct enzyme assays, however, still serve as an excellent early warning sign
273 for the potential presence of faecal material in the water. The possibility of using an internal
274 reference or standard (i.e. commercial B-GAL) should also be explored in order to assess
275 and correct (calibrate) for chemical (ion) interference when testing environmental samples.
276
277 Due to the complex nature of the interactions between ions and other compounds in
278 polluted waters, pH and temperature optima may vary from that of the commercially
279 available and pure B-GAL. Environmental enzymes exhibited different kinetics from their
280 commercial counterpart.
281
282 **Acknowledgements**

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294 **References**

295

296 Berg, J.D., Robert, P.V., Martin, A., 1986. Effect of Chlorine dioxide on selected

297 membrane functions of *Escherichia coli.* J. Appl. Bacteriol. 29, 186-194.

298

299 Davies, C.M., Apte, S.C., 1999. Field evaluation of a rapid portable test for monitoring

300 fecal coliforms in coastal waters. Environ. Toxicol. 14, 355-359.

301

302 DeBoer, E., Beumer, R.R., 1999. Methodology for detection and typing of foodborne

303 microorganisms. Int. J. Food Microbiol. 50, 119-130.

304

305 Den Blanken, J.G., 1985. Comparative disinfection of treated sewage with chlorine and

306 ozone. Water Res. 19, 1129-1140.

307

308 Frampton, E.W., Restaino, L., 1993. Methods for *Escherichia coli* identification in food,

309 water and chemical samples based on beta-glucuronidase detection. J. Appl. Bacteriol. 74,

310 223-233.

311

312 George, I., Crop, P., Servals, P., 2002. Faecal coliform removal in wastewater treatment

313 plants studied by plate counts and enzymatic methods. Water Res. 36, 2607-2617.

314

315 Maheshwari, R., Bharadwaj, G., Bhat, M.K., 2000. Thermophilic fungi; their physiology
316 and enzymes. Microbiol. Mol. Biol. R. 64, 461-488
317 Pelisek, J., Engelmann, M.G., Fuchs, A.G.A., Armeanu, S., Shimizu, M., Mekkaoui, C.,
318 Rolland, P.H., Nikol, S, 2000. Optimization of nonviral transfection: variables influencing
319 liposome-mediated gene transfer in proliferating vs. quiescent cells in culture and in vivo
320 using a porcine restenosis model. J. Mol. Med. 80, 724 - 736
321
322 Rompré, A., Servais, P., Baudart, J., De-Raubin, M.R., Laurent, P., 2002. Detection and
323 enumeration of coliforms in drinking water: current methods and emerging approaches. J.
324 Microbiol. Meth. 49, 31-54.
325
326 Seeber F, Boothroyd J.C. 1996. Escherichia coli beta-galactosidase as an in vitro and in
327 vivo reporter enzyme and stable transfection marker in the intracellular protozoan parasite
328 Toxoplasma gondii, Gene 1, 39-45
329
330 Singh, A., Yu, F.P., McFeter, G.A., 1990. Rapid detection of chlorine-induced bacteria
331 injury by the direct viable count method using image analysis. Appl. Environ. Microb. 56,
332 389-394.
333
334 Tryland, I., Fiksdal, L. (1998). Enzyme Characteristics of β-D-Galactosidase- and β-D -
335 Glucuronidase-Positive Bacteria and Their Interference in Rapid Methods for Detection of
336 Waterborne Coliforms and *Escherichia coli*. Appl. Environ. Microb. 64, 1018-1023,
337

338 Tryland, I., Pommmepuy, M., Fiksdal, L., 1997. Effect of chlorination on beta-D-
339 galactosidase activity of sewage bacteria and *Escherichia coli.* J. Appl. Microbiol. 85, 51-
340 60.
341

342 Venter, S.N., 2000. Rapid microbiological monitoring methods: the status quo. IWA, blue
343 pages. London
344

345 Wakabayashi, M., Fishman, W.H., 1961. The comparative ability of b-glucuronidase
346 preparations (liver, *Escherichia coli*, *Helix pomatia* and *Patella vulgate*) to hydrolyse
347 certain steroid glucosiduronic acids. J. Biol. Chem. 236, 996-1001.
348

349 Wetzel, R.G., 1991. Extracellular enzymatic interactions: storage, redistribution and
350 interspecific communication. In: Chrŏst, R.J., (Ed) *Microbial enzymes in acquatic*
351 *environments*. Springer –Verlag, Berlin. pp 6-28.
352

353 Wutor, V.C., Togo, C.A., Pletschke, B.I., 2007. Comparison of the direct enzyme assay
354 method with the membrane filtration technique in the quantification and monitoring of
355 microbial indicator organisms- seasonal variations in the activities of coliforms and *E. coli*,
356 temperature and pH. Water SA 33, Ref No 2049. Water Research Commission of South
357 Africa.
358

359 Wutor, V.C., Togo, C.A., Limson, J.L., Pletschke, B.I., 2006. A novel biosensor for the
360 detection and monitoring of β-D-galactosidase of faecal origin in water. Enzyme Microb.
361 Tech. (doi:10.1016/j.enzmictec.2006.10.039).
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382 **Captions to Figures**
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385
386 **Figure 1.** Effect of pH on commercial and environmental B-GAL activity using CPRG
387 (100% relative activity is defined as the activity at the pH optimum for each enzyme).
388 Values represent the means ± SD, n = 3.

389

390 **Figure 2.** Effect of temperature on commercial and environmental B-GAL using CPRG.
391 (100% relative activity is defined as the activity at the temperature optimum for each
392 enzyme). Values represent the means ± SD, n = 3.

393

394

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

397

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

400

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

403

404

405