Peertechz

Clinical Group

Journal of Clinical Microbiology and Biochemical Technology

DOI CC By

Caruso G[1]*, Caruso R[2], Monticelli LS[1], Maimone G[1] and Crisafi E[1]

[1]National Research Council, Institute for Coastal Marine Environment (CNR-IAMC), Messina, Italy
[2]Hospital Agency A.O.U. "G. Martino", Messina, Italy

Dates: Received: 25 November, 2017; Accepted: 12 December, 2017; Published: 16 December, 2017

***Corresponding author:** Gabriella Caruso, Graduate in Biological Sciences, Specialist in Applied Microbiology. Researcher at: CNR-IAMC, Spianata S. Raineri 86, 98122 Messina, Italy, Tel: +39-0906015423; Fax: +39-090669007; E-mail: gabriella.caruso@iamc.cnr.it

Keywords: Fecal pollution, Seawater, *Escherichia coli*, β-Glucuronidase, IBDG

https://www.peertechz.com

Research Article

β-glucuronidase activity determination as an indirect estimate of *Escherichia coli*: development of a miniaturized assay and its application to seawater samples

Abstract

Background: The search of rapid methods for the detection of *Escherichia coli* in coastal marine waters is a topic of scientific interest to evaluate potential risks to human health related to their low bacteriological quality.

The context and purpose of the study: A miniaturized assay for the analytical determination of β-glucuronidase activity in seawater as a selective marker of *Escherichia coli* was developed by using the chromogenic Indoxyl-β-D-glucuronide (IBDG) substrate. This compound is specifically cleaved by *E. coli*, releasing an insoluble chromophoric blue-indigo product that precipitates and is measured at 450 nm wavelength by a microplate reader.

Results: After its preliminary optimization, the enzymatic assay was applied to the analysis of seawater samples and enabled to discriminate them according to their pollution level.

Main findings: The first obtained data proved the suitability of the developed miniaturized enzymatic assay for fecal contamination monitoring. It can be used for the detection and indirect quantification of *E. coli*, without the need for confirmatory steps.

Conclusions: This study suggests that the proposed analytical protocol is suitable for *E. coli* monitoring in seawater, and provides in a short time (i.e. 2 hours from sampling) results which are in agreement with the standard culture counts.

Brief summary: The results obtained with the developed IBDG protocol encourage its use for environmental quality assessment.

Any potential implications: The possibility to obtain near "real-time" data on the occurrence and distribution of anthropogenic inputs makes this method a simple and quick tool for early warning detection of marine fecal pollution.

Abbreviations

E.coli: *Escherichia coli*; IBDG: Indoxyl-β-D-glucuronide; O.D: Optical Density; U: enzymatic units; CFU: Colony Forming Units, V_{max}: Maximal Velocity of the enzymatic reaction

Introduction

Multiple human activities that coexist in coastal areas cause significant threats to both the whole marine environment and human health [1]. The overall quality of bathing sites may be affected by many pollution sources such as urbanization, agricultural activities, harbour activities, waste discharges, industry; a complete environmental management needs the availability of specific and quick tools, able to provide integrated information on the level of contamination and pollution and its consequences for the health of coastal areas [2-4]. Since 2006 the new European Directive for the quality of bathing waters underlines the concept of active management of coastal areas vulnerable to contamination, paying attention to the responsibility for routine monitoring and remediation of such

Citation: Caruso G, Caruso R, Monticelli LS, Maimone G, Crisafi E (2017) β-glucuronidase activity determination as an indirect estimate of *Escherichia coli*: development of a miniaturized assay and its application to seawater samples. J Clin Microbiol Biochem Technol 3(1): 046-050.

areas. In seawater quality monitoring, rapid microbiological methods relying on biochemical or molecular approaches offer new interesting perspectives for the determination of *Escherichia coli*, which is universally recognised as the most suitable indicator of fecal contamination [5-9]. Typically, 90 to 95% of *E. coli* strains possess the gene for β-glucuronidase, and therefore this enzyme activity has been traditionally regarded as a selective marker for rapid detection of this microorganism [10]. Since the last decade, a variety of chromogenic and fluorogenic substrates have been formulated and proposed in the bacterial diagnostic as an attractive tool to quickly identify specific microorganisms [11-12], by their inclusion as components in both solid and liquid culture media. They proved to be successful for the analysis of water, food and clinical samples, providing sensitive and specific results [13]. Like fluorogenic substrates, widely applied in the monitoring of aquatic environmental health [7, 14-15], chromogenic substrates produce a visible signal when cleaved by the specific enzyme that is present or is being expressed by a specific microorganism. In the case of chromogenic compounds, however, the reaction product precipitates in the assay mixture and can be easily quantified by a photometric method; this results in a significant advantage, because it avoids background fluorescence due to the spreading of the fluorescent reaction product into the culture medium, which is frequently reported as a source of biases when using fluorogenic substrates [16-18].

Experiments were carried out at the laboratory of the CNR-IAMC, with the aim of developing a miniaturized method for the determination of β-glucuronidase activity rates as an indirect estimate of *E. coli*, by using the chromogenic compound Indoxyl-β-D-glucuronide (IBDG). This compound is specifically cleaved by *E. coli*, producing after reaction a water-insoluble blue-indigo aglycone product that precipitates and is measured at 450 nm wavelength; this makes the observation of the reaction product easy, also under normal daylight [19].

Materials and Methods

Preliminary trials for the set up and optimisation of the analytical protocol

For the development of the microassay analytical protocol, disposable 96-well, flat bottomed, white polystyrene plates (CellStar, Greiner Bio-One Inc., Longwood, FL, USA) and an axenic culture of *E. coli* O125 as the reference strain were used.

The chromogenic IBDG compound as the enzymatic substrate and the β-Glucuronidase enzyme (EC 3.2.1.31) were purchased from Sigma-Aldrich, Milan (Italy). In order to find the optimal concentrations of both enzyme and substrate, preliminary trials were performed, in which the amount of IBDG was kept constant (0.5 mmoles) and serially diluted amounts of β-Glucuronidase (from 100 to 0.78 Units) were added, or, vice-versa, the amount of enzyme was kept constant (0.78 Units) and serially diluted amounts of substrate (0.5 mmoles to 5 µmoles) were added. The combination between the concentrations of substrate and enzyme yielding the maximum amount of precipitate as the reaction product in such preliminary trials was chosen as the working solutions (50 µmoles and 0.78 U for IBDG and β-Glucuronidase, respectively) in further trials performed using a bacterial suspension of *E. coli* at a concentration of 2.86 x 10^2 cells/ml in sterile physiological saline solution. Enzymatic measurements were performed by using a Microplate Reader ELx 808 (Biowhittaker, Walkersville, MA, USA) at 44.5°C and at 450 nm as the excitation wavelength. Optical Density (OD) readings were taken immediately after substrate addition, namely at time 0, and at increasing incubation times (2, 3.5, 5, 7, 21 hours), in order to evaluate the effects of the incubation period on the enzyme expression. The microplate wells containing the blue chromophoric product were scored as positive by the automatic reader. Taking into account the direct molar relationship between the amount of the released reaction product and the amount of hydrolysed substrate (one mole of product released per one mole of hydrolysed substrate), the mean value of OD obtained from two replicate wells per each enzyme-substrate combination was converted into the amount of enzyme present in the analysed sample volume (20 µl). However, the first results obtained using this approach were unclear and scarcely reproducible; this led us to adopt an alternative procedure, in which multiple concentrations of the IBDG substrate were added to the sample.

Protocol of the enzyme assay

A constant volume (20 µl) of *E. coli* suspension (2.86 x 10^2 cells/ml) and variable volumes of IBDG substrate (stock solutions from 5 µmoles to 0.5 mmoles) were dispensed into each well of a microplate, so obtaining final concentrations ranging from 0.5 to 50 µmoles. Phosphate buffered saline (PBS) pH 7.0 was added to each well in variable amounts, until a final volume of 200 µl *per* well was reached. The scheme of the optimized enzymatic assay protocol is reported in table 1. All reactions were performed in duplicate wells. A sample control (culture+ PBS buffer) was added; known amounts of substrate (from 0.5 mmoles to 5 µmoles in PBS) were used for the calibration of the microplate reader. Incubation conditions (both temperature and times) were the same as those applied in the preliminary trials. The increase in OD values *per* hour was converted into V max and expressed in micromoles of product released *per* 100 ml of sample and *per* hour.

Specificity test

In order to exclude false-positive results due to the

Table 1: Scheme of a miniaturised β-glucuronidase assay by using the chromogenic IBDG substrate.

E. coli suspension (µl)	20	20	20	20	20	20	20
IBDG substrate (µl)	20*	40*	100*	20§	40§	100§	20**
PBS buffer (µl)	160	140	80	160	140	80	160
Total volume (µl)	200	200	200	200	200	200	200
Final substrate concentration (µmol l^{-1})	0.5	1	2.5	5	10	25	50

* at a 5 µmol l^{-1} concentration
§ at a 50 µmol l^{-1} concentration
**at a 0.5 mmol l^{-1} concentration

Citation: Caruso G, Caruso R, Monticelli LS, Maimone G, Crisafi E (2017) β-glucuronidase activity determination as an indirect estimate of *Escherichia coli*: development of a miniaturized assay and its application to seawater samples. J Clin Microbiol Biochem Technol 3(2): 046-050.

possible interference of not-coliform bacteria [18] on the right interpretation of the data, a specificity test was carried out using axenic cultures of Enterobacteriaceae strains other than *E. coli*, such as *Klebsiella pneumoniae*, *Shigella flexneri*, *Enterobacter sp.*, *Salmonella gallinarum*, *S. typhi*, *S. typhimurium*, *Proteus mirabilis*, as well as an environmental isolate of *Vibrio sp.* Bacterial suspensions in sterile physiological saline with an initial cell concentration of 10^6 cells ml^{-1} (as estimated after staining with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, Milan, final concentration 10 µg ml^{-1}according to Porter and Feig [20]) were assayed using the miniaturized assay.

On field application

The developed protocol was applied to the analysis of seawater samples. Surface samples were collected from selected sites of Messina shoreline differently impacted by urban wastes (Tremestieri, Pagliarisi, Annunziata, Tennis), filtered through a 0.45 µm pore size mixed cellulose esters filtering membrane (Millipore Corporation, Bedford, USA) and 10 times concentrated by resuspension in sterile physiological saline, in order to obtain a signal detectable by the microplate reader. The β-glucuronidase activity rates were reported as the maximal velocity of enzymatic reaction (V_{max}), which was calculated through the Lineweaver Burke transformation of each substrate concentration plotted *versus* the corresponding velocity of hydrolysis of IBDG substrate. V_{max} values were multiplied by the initial concentration factor and reported *per* 100 ml of sample.

Plate counts

Contextually to the enzymatic assay, standard plate counts were carried out by culture method on m-FC agar medium [21], in order to compare and relate the measured β-glucuronidase activity rates with *E. coli* abundance. Blue colonies grown on m-FC agar were confirmed to be *E. coli* after addition of James indole reagent (Biomérieux, Marcy l'Etoile, France).

Results

Specificity test

No positive results with the strains assayed in the specificity test were obtained, suggesting that the substrate IBGD was specifically metabolised by *E. coli* and that the enzymatic assay was specific for the detection of this microorganism.

On field application to marine samples

The substrate concentration ranges used here were the lowest concentrations able to give detectable OD values using a small sample volume (20 µl). The plots of the microplate measurements taken over time are shown in figure 1. The obtained microplate readings pointed out clearly the different range of magnitude of the OD values between the most (Tremestieri and Pagliarisi) and the least polluted (Annunziata and Tennis) stations; particularly, at the sites suffering more pollution, all the OD values were lower than 0.150, except for

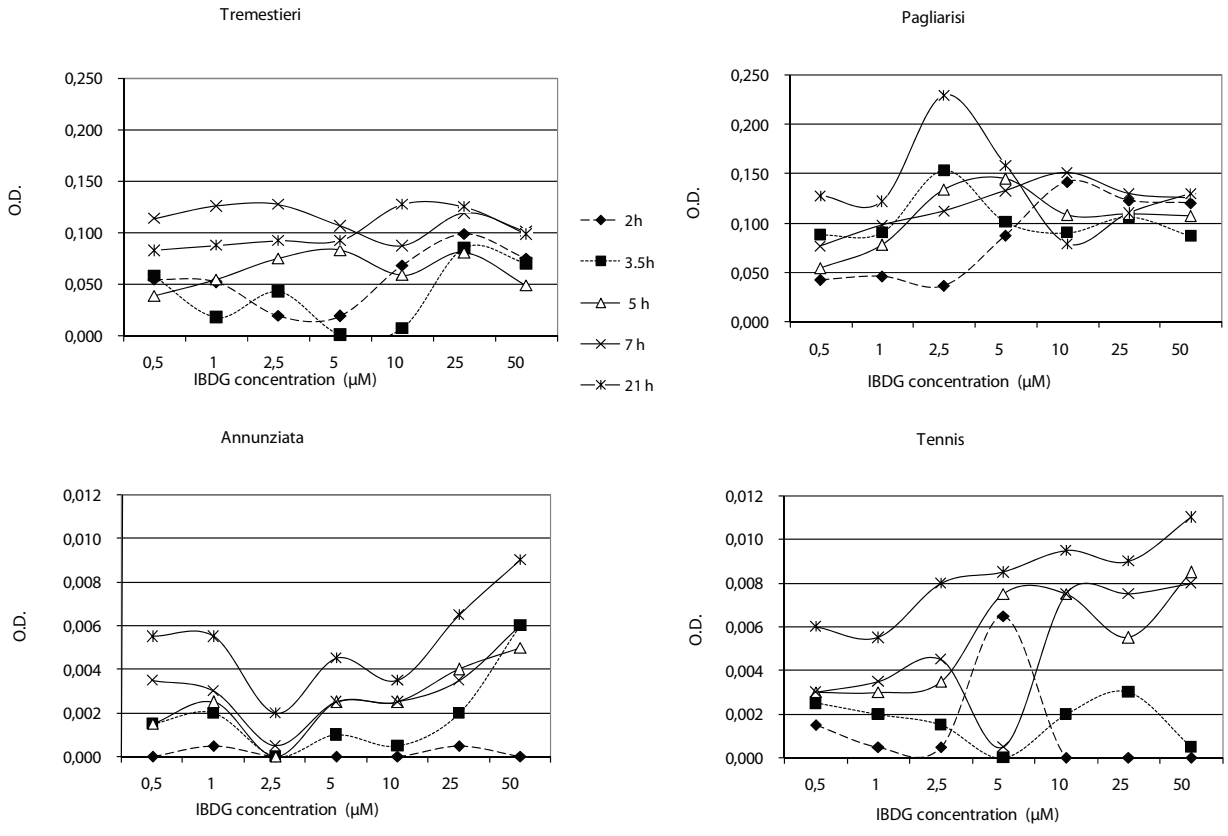

Figure 1: Beta-glucuronidase activity measurements in natural samples: plots of the Optical Density (O.D.) values over time.

Citation: Caruso G, Caruso R, Monticelli LS, Maimone G, Crisafi E (2017) β-glucuronidase activity determination as an indirect estimate of *Escherichia coli*: development of a miniaturized assay and its application to seawater samples. J Clin Microbiol Biochem Technol 3(2): 046-050.

a peak recorded in Pagliarisi after incubation for 21 h. From the obtained results, it was observed that already after 2 of incubation and even using a small volume of sample, the miniaturized method made a screening of the samples at different fecal pollution level possible. Moreover, for weakly polluted samples, the extension of the incubation period could increase the intensity of the enzymatic response.

The different levels of enzymatic activity recorded at the examined stations were even more evident when the averaged substrate colour development (ASCD) was calculated, using the formula ASCD = Σ ((R – C)/number of measurements), where R is the averaged absorbance of the duplicate wells with IBDG substrate and C is the averaged absorbance of the control wells (without IBDG substrate). The colour development for each sample (Figure 2) highlighted the ability of the IBDG miniaturized assay to discriminate heavily from weakly polluted samples.

The β-glucuronidase activity rates, reported as V_{max} values, correlated significantly with plate counts, which showed concentrations of 5.52x10², 6.0x10², 3.24x10² and 5.40x10² CFU 100 ml^{-1} at the stations Tremestieri, Pagliarisi, Annunziata and Tennis, respectively (Figure 3).

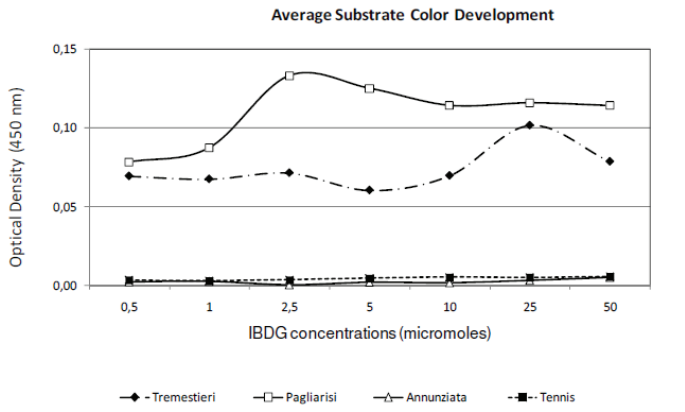

Figure 2: Average substrate color development obtained in the natural samples with variable pollution levels.

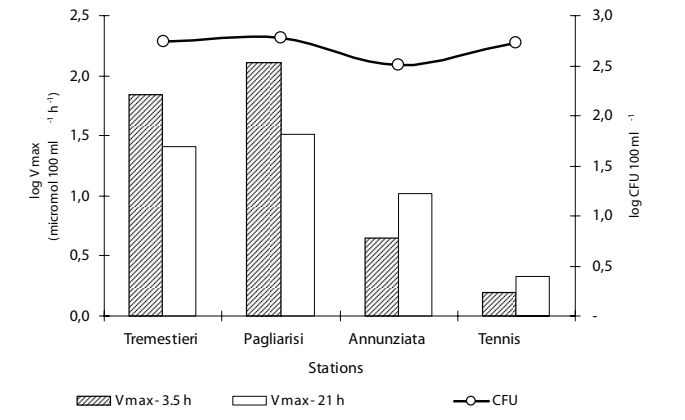

Figure 3: Comparison between enzymatic activity rates (as Vmax, in µmol 100 ml-1 h-1) and abundance (in Colony Forming Units/100 ml of sample).

Discussion

This study can be considered as a further contribute in the field of rapid analytical methods for environmental quality monitoring. In this context, the set-up of laboratory procedures allowing us to detect and quantify in short times (within a few hours) *E. coli* abundance, can play a strategic role. The developed protocol was applied to the analysis of natural samples collected from coastal sites more or less affected by fecal pollution, in comparison with the plate count method, for the quantitative determination of *E. coli* in seawater. The assay also included a preliminary step of concentration, which was necessary to overcome possible difficulties encountered in detecting *E. coli* in the case that this bacterium was present in low abundances in natural seawater.

Previous experiments, performed through the incubation of a concentrated sample with the fluorogenic 4-metilumbellyferil-β-d-glucuronide (MUG) substrate, showed the suitability of the enzymatic method as a rapid tool for the screening of marine environments according to their pollution degree [7]. Following the same methodological approach (i.e. addition of multiple concentrations of the substrate), but modified through the use of the chromogenic IBDG substrate and automatic readings of the hydrolysis product, a new experimental protocol for the estimation of β-glucuronidase present in natural seawater samples was developed.

Such method has been conceived with the final objective of miniaturizing the enzymatic assay and automating their OD measurements. Using the proposed procedure, it is possible to monitor indirectly the presence and distribution of *E. coli* in seawater. Compared to other technologies developed for the rapid detection of bacteria in recreational waters, the developed protocol offers also the advantages of working with low volumes of sample, disposable microplates, and simple instrumentation, therefore it is cheaper than other commercial diagnostic kits for *E. coli* detection based on molecular approaches [22-23].

The microplate reader can run a fully automated analysis of the substrate hydrolytic reaction and the β-glucuronidase activity rates can be available in short times, typically within a few hours. The frequency of automatic readings can be regulated according to the operator needs, but normally an incubation period of 2 hours is required to obtain a detectable signal. The use of IBDG in a liquid mixture also involves a significant decrease in the response time compared with its incorporation into a solid medium, for which at least 18 hours of incubation are normally needed to appreciate bacterial growth. Another important aspect is the high incubation temperature (44.5°C) set up in the assay, which increases the specificity of the reaction for *E. coli*. All these features make our assay particularly suitable to match with the requirements (speed and specificity of detection) for early warning of bacterial pollution in coastal monitoring.

Conclusion

The results obtained from the application of the developed enzymatic assay to naturally polluted samples are encouraging.

Citation: Caruso G, Caruso R, Monticelli LS, Maimone G, Crisafi E (2017) β-glucuronidase activity determination as an indirect estimate of *Escherichia coli*: development of a miniaturized assay and its application to seawater samples. J Clin Microbiol Biochem Technol 3(2): 046-050.

The high β-glucuronidase activity rates measured in samples collected from the most polluted coastal areas are in agreement with the wide occurrence of *E. coli* as the main component of fecal coliform population in these environments. Moreover, the high content of organic matter available where anthropogenic inputs are present supports the active metabolism of bacterial cells which express their enzymatic activity. Taking into account these considerations, and not least its easiness of execution, the miniaturized enzymatic assay using IBDG can be proposed as a simple tool to detect β-glucuronidase activity as an indirect proxy of *E. coli*; the developed protocol can find a wide application in the screening of the microbiological quality of seawater and in the evaluation of potential risks to human health.

Acknowledgments

Experimental trials with IBGD started within the "Advanced Systems for Monitoring"- SAM Cluster 10 Project (2000-2004), funded by Italian Ministry for Scientific Research (MIUR).

References

1. Bonamano S, Piermattei V, Madonia A, de Mendoza FP, Pierattini A, et al. (2016) The Civitavecchia Coastal Environment Monitoring System (C-CEMS): A new tool to analyse the conflicts between coastal pressures and sensitivity areas. Ocean Sci 12: 87-100. Link: https://goo.gl/6xxGsQ

2. Zappalà G, Bonamano S, Madonia A, Caruso G, Marcelli M (2012) Microbiological risk assessment in a coastal marine environment through the use of mathematical models. WIT Trans Ecol Environ 164: 3-14. Link: https://goo.gl/zAffZj

3. Bonamano S, Madonia A, Borsellino C, Stefanì C, Caruso G, et al. (2015) Modeling the dispersion of viable and total Escherichia coli cells in the artificial semi-enclosed bathing area of Santa Marinella (Latium, Italy). Mar Pollut Bull 95: 141-154. Link: https://goo.gl/QkKMgs

4. Zappalà G, Caruso G, Bonamano S, Madonia A, Piermattei V, et al. (2016) A multi-platform approach to marine environment assessment in the Civitavecchia (Rome) area. J Oper Oceanogr 9: 131-143. Link: https://goo.gl/XLPDcR

5. Ashbolt NJ, Grabow WOK, Snozzi M (2001) Indicators of microbial water quality. In World Health Organization (WHO). Water Quality: Guidelines, Standards and Health. Edited by Fewtrell L and Bartram J. London, IWA Publishing 289-316. Link: https://goo.gl/u6qRZq

6. Caruso G, Zaccone R, Crisafi E (2000) Use of the indirect immunofluorescence method for detection and enumeration of Escherichia coli in seawater samples. Lett Appl Microbiol 31: 274-278. Link: https://goo.gl/4BXkUW

7. Caruso G, Crisafi E, Mancuso M (2002) Development of an enzyme assay for rapid assessment of Escherichia coli in seawaters. J Appl Microbiol 93(4): 548-556. Link: https://goo.gl/1WGkqj

8. Caruso G, Mancuso M, Crisafi E (2003) Combined fluorescent antibody assay and viability staining for the assessment of the physiological rates of Escherichia coli in seawaters. J Appl Microbiol 95: 225-233. Link: https://goo.gl/ETfduV

9. Caruso G, Denaro R, Genovese M, Giuliano L, Mancuso M, et al. (2004) New methodological strategies for detecting bacterial indicators. Chem Ecol 20: 167-181. Link: https://goo.gl/XAGdPb

10. Hartman PA (1989) The MUG (Glucuronidase) test for Escherichia coli in food and water. In Rapid methods and automation in microbiology and immunology. Edited by Balows A, Tilton RC and Turano A. Brescia: Brixia Academic Press 290-308.

11. Manafi M, Kneifel W, Bascomb S (1991) Fluorogenic and chromogenic substrates used in bacterial diagnostics. Microbiol Rev 5: 335-348. Link: https://goo.gl/FUEK2a

12. Frampton EW and Restaino L (1993) Methods for Escherichia coli identification in food, water and clinical samples based on beta-glucuronidase detection. J Appl Bacteriol 74: 223-233. Link: https://goo.gl/xezuVQ

13. Manafi M (2000) New developments in chromogenic and fluorogenic culture media. Int J Food Microbiol 60: 205-218. Link: https://goo.gl/NJFPXV

14. George I, Petit M, Servais P (2000) Use of enzymatic methods for rapid enumeration of coliforms in freshwaters. J Appl Microbiol 88: 404-413. Link: https://goo.gl/7uBR6b

15. Farnleitner AH, Hocke L, Beiwl C, Kavka GG, Zechmeister T, et al. (2001) Rapid enzymatic detection of Escherichia coli contamination in polluted river water. Lett Appl Microbiol 33: 246-250. Link: https://goo.gl/eH5eAC

16. Davies CM, Apte SC, Peterson SM, Stauber JL (1994) Plant and algal interference in bacterial beta-D-galactosidase and beta-D-glucuronidase assays. Appl Environ Microbiol 60: 3959-3964. Link: https://goo.gl/DRvkXU

17. Fiksdal L, Pommepuy M, Caprais MP, Midttun I (1994) Monitoring of fecal pollution in coastal waters by use of rapid enzymatic techniques. Appl Environ Microbiol 60: 1581-1584. Link: https://goo.gl/B8PLim

18. Tryland I and Fiksdal L (1998) Enzyme characteristics of beta-D-galactosidase and beta-D-glucuronidase-positive bacteria and their interference in rapid methods for detection of waterborne coliforms and Escherichia coli. Appl Environ Microbiol 64: 1018-1023. Link: https://goo.gl/85Mji3

19. Ley AN, Bowers RJ, Wolfe S (1988) Indoxyl-β-D-glucuronide, a novel chromogenic reagent for specific detection and enumeration of Escherichia coli in environmental samples. Can J Microbiol 34: 690-693. Link: https://goo.gl/w8vpHp

20. Porter KG, Feig YS (1980) The use of DAPI for identifying and counting aquatic microflora. Limnol Oceanogr 25: 943–948. Link: https://goo.gl/1EbRv9

21. APHA (American Public Health Association) (1992) Standard methods for the examination of water and wastewater. APHA, Washington, DC, 18th edition.

22. Noble RT, Weisberg SB (2005) A review of technologies for rapid detection of bacteria in recreational waters. J Water and Health 3: 381-392. Link: https://goo.gl/LxSMYT

23. Briciu-Burghina C, Heery B, Regan F (2017) Protocol for the recovery and detection of Escherichia coli in environmental water samples. Anal Chim Acta 964: 178-186. Link: https://goo.gl/qzYVqW

Citation: Caruso G, Caruso R, Monticelli LS, Maimone G, Crisafi E (2017) β-glucuronidase activity determination as an indirect estimate of *Escherichia coli*: development of a miniaturized assay and its application to seawater samples. J Clin Microbiol Biochem Technol 3(2): 046-050.