International Biodeterioration & Biodegradation 120 (2017) 216–223

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Evidence of emerging challenge of chlorine tolerance of *Enterococcus* species recovered from wastewater treatment plants

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ARTICLE INFO

Article history:
Received 3 November 2016
Received in revised form
10 February 2017
Accepted 11 February 2017

Keywords:
Chlorine tolerance
Lethal dose
Inactivation kinetics
Enterococcus sp.
Wastewater treatment
Public health

ABSTRACT

This study assessed the survival of some *Enterococcus* species recovered from the clarifier of two selected wastewater treatment plants in the Eastern Cape Province, South Africa, in the presence of different concentrations of chlorine disinfectant. The bacterial survival, lethal dose and inactivation kinetics at lethal doses were examined. Inactivation of the test bacteria (n = 20) at the recommended dose of 0.5 mg l^{-1} progressively increased from 3.0–5.8 log reduction within the range of chlorine residual of 0.14–0.45 mg l^{-1} after 30 min. The bactericidal activity of chlorine increased at higher chlorine dosages with a significant reduction in viability of the bacteria and complete sterilization of the bacterial population was achieved at a lethal dose of 0.75 mg l^{-1} and 1.0 mg l^{-1} in 30 min. For the inactivation kinetics, bactericidal activity of chlorine increased with time showing a 3.0–6.8 log reduction in 10 min, 4.0–8.3 log reduction in 20 min and above 7.9 log reduction in 30 min for all test *Enterococcus* isolates used in this study. Chlorine dose of 0.75 mg l^{-1}–1.0 mg^{-1} showed a better disinfecting capacity to effectively reduce the tolerance of *Enterococcus* species.

1. Introduction

Water quality is important for the well-being of the environment, society and economy (UNEP, 2010). Over the years, increasing demand for water to meet agricultural, industrial, environmental and municipal needs has extended the requirements for improvement in water and wastewater treatment (Levantesi et al., 2010). As such, a major concern is the microbiological state of the water which can lead to the spread of waterborne disease causing pathogens when inadequately disinfected. Therefore, it is paramount that water undergoes satisfactory disinfection treatment that guarantees appropriate water quality (Valero et al., 2015).

Various disinfectants have been employed for water treatment processes. These include ozone which is highly efficient but expensive, requires special operation, and potentially forms bromate as a by-product in waters containing bromide (Werschkun et al., 2012). Although, ultraviolet (UV) radiation does not form disinfection-by-products (DBPs) (Werschkun et al., 2012), it requires large amount of energy and frequent maintenance such as replacement of UV lamps (Lloyd's Register, 2012). Chlorine, on the other hand, is a commonly used water disinfectant around the world (Rauen et al., 2012), due to its potent oxidizing capacity in destroying nucleic acid, causing membrane damage of microbial species, ease of handling and cost-effectiveness (Anastasi et al., 2013; Gregg et al., 2009) but its application at high doses can generate potential DBPs (Tsolaki and Diamadopoulos, 2010). Chlorine has been used as a disinfectant for drinking water, wastewater, wash water in food processing in order to reduce microbial population on fresh-produce and to increase shell life (Stopforth et al., 2008; Luo et al., 2011; Van Haute et al., 2013) as well as surface cleaning disinfectants in hospitals (Guimaraes et al., 2000). Various studies have reported the disinfectant efficiency of chlorine in the removal of microbial pathogens in water. Helbling and Vanbriesen (2007) showed that 0.2 mg l^{-1} chlorine concentration at 0.5 min caused greater than 99% reduction of *E. coli* isolates in drinking water. Another study also reported a significant removal of pathogenic and wild strains of *E. coli* 0157:H7 at four orders of magnitude when chlorine dose of 1.1 mg l^{-1} for 1 min was applied (Rice et al., 1999).

However, other studies have reported chlorine tolerance of microbial pathogens, indicating that microorganisms are surviving

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http://dx.doi.org/10.1016/j.ibiod.2017.02.016
0964-8305/

at chlorine dosages applied for water/wastewater treatment (Fong et al., 2010; Coronel-Olivares et al., 2011; Li et al., 2013). Consequently, this presents a potential threat to human health and environmental safety.

For microbial control and difficulties of pathogens quantification in water samples, regulatory standards have stipulated a minimum free chlorine concentration of 0.5 mg l^{-1} after 30 min at pH below 8, with turbidity of less than 1 NTU for treatment of potable water (WHO, 2011) and a maximum contaminant level (MCL) of zero for total coliforms including *E. coli* (WHO, 2001; DWAF, 2013).

The Gram-positive bacteria, *Enterococcus* sp. are leading causes of nosocomial infections and have been found to be the third most commonly reported pathogen causing health care acquired infections in the United States (Hidron et al., 2008). Enterococci are facultative, anaerobic oval cocci that form chains of various lengths; they are sturdy and versatile, with a particular ability to grow under harsh conditions of 6.5% NaCl at a wide range of temperatures between 10 °C and 45 °C at a pH of 9.6 and can survive at 60 °C for 30 min (Fisher and Phillips, 2009; Byappanahalli et al., 2012; Staley et al., 2014). Enterococci generally display low levels of virulence, as evidenced by their presence as natural colonizers of the gastrointestinal (GI) tract in most humans and animals and by the fact that they have been used safely for decades as probiotics in humans and farm animals (Arias and Murray, 2012).

However, enterococci may become an opportunistic pathogen in individuals with compromised immune systems and in patients who have been hospitalized for prolonged periods or who have received broad-spectrum antimicrobial therapy (Castillo-Rojas et al., 2013). Indeed, enterococci are well documented as the leading causes of a variety of infections in the health care including pelvic infections, neonatal infections and urinary tract infections (UTIs), as well as infective endocarditis (Sievert et al., 2013).

Faecal enterococci are also used as index of faecal pollution of water because they do not multiply in the environment, but are able to survive longer than coliforms and *E. coli,* and are still numerous enough to be detected after significant dilution (WHO, 2006; Teklehaimanot et al., 2014). For example, *E. faecalis* and *E. faecium* sp. were recovered from chlorinated wastewater treatment plants in Mexico (Castillo-Rojas et al., 2013), and vancomycin-resistant enterococci were recovered in the final effluents of four wastewater treatment plants in the United States (Goldstein et al., 2014). Reports from other parts of the world including South Africa, have shown that microbial pathogens are increasingly surviving chlorine disinfection in many wastewater treatment plants (Momba et al., 2009; Munir et al., 2011; Chern et al., 2014) and *Enterococcus* sp. have been recovered from final effluents of wastewater treatment plants having as low as 0.01 mg l^{-1} residual chlorine (Samie et al., 2009). In the Eastern Cape Province, several reports have also detailed the detection of microbial pathogens including *Enterococcus* species in the final effluents of wastewater treatment plants and have highlighted the health implications to humans and the environment (Odjadjare et al., 2012; Iweriebor et al., 2015; Adefisoye and Okoh, 2016). However, there is paucity of information on the chlorine disinfectant tolerance limits of *Enterococcus* species, as well as an effective chlorine dose for microbial inactivation. This paper reports for the first time on the efficacy of chlorine disinfectant on the survival of *Enterococcus* species recovered from two wastewater treatment plants in the Eastern Cape Province, South Africa.

2. Materials and methods

2.1. Sources of bacteria and processing of samples

Effluent samples were collected from the clarifier of two wastewater treatment plants in the Eastern Cape Province of South Africa and processed for isolation of *Enterococcus* sp. following standard procedures (DWAF, 1996; APHA et al., 1999) with some modifications. Briefly, twenty millilitres of wastewater samples were filtered through sterile cellulose–nitrate membrane filter (0.45 μm pore size, 47 mm diameter, Millipore filters) under partial vacuum in five replicates. The membrane filters were immediately placed in Petri dishes containing Bile Esculin Azide (BAE) agar (Merck, Darmstadt, Germany) with sterile forceps. Following incubation at 37 °C for 24 h, isolates that showed black colonies (typical of *Enterococcus* sp.) were recovered, purified and stored in 20% glycerol at −80 °C.

2.2. Preparation of stock chlorine solution

A stock of chlorine solution (1% w/v) containing 7000 mg l^{-1} of free chlorine was prepared from calcium hypochlorite granules in accordance with the method described by APHA et al. (1999). The stock solution was diluted to final free chlorine concentrations of 0.5 mg l^{-1} to 1.5 mg l^{-1} and then quantified by the N, N-diethyl-ρ-phenylenediamine (DPD) method. Free chlorine concentration was determined using free chlorine kit (Hanna instruments Inc. Woonsocket, USA). The photometer has an accuracy of measurement of ±3% of reading at 25 °C and a sensitivity of 0–5.00 mgl^{-1}.

2.3. Molecular identification of presumptive Enterococcus sp. test isolates

2.3.1. DNA extraction

Genomic DNA was extracted by the boiling method as described by Lopez-Saucedo et al. (2003) and Maugeri et al. (2004) with modifications. Briefly, previously stored glycerol stocks of *Enterococcus* isolates were resuscitated in Tryptic soy broth at 37 °C for 24 h. The isolates were then purified and cultured on nutrient agar at 37 °C for 24 h. Single colonies from presumptive *Enterococcus* sp. and a positive control of *Enterococcoccus faecalis* (ATCC 19433) were inoculated into 200 μl of sterile distilled water in sterile Eppendorf tubes. Cells were lysed at 100 °C for 15 min and centrifuged at 15,000 rpm for 10 min at 4 °C. The supernatant was collected and stored at −20 °C for further use.

2.3.2. Molecular identification

Identities of the presumptive *Enterococcus* sp. were confirmed by amplification of the *tuf* –gene (product size 112 bp) in combination with the cultural characteristic of the isolate as previously described by Ke et al. (1999). Reference strain of *Enterococcus faecal* ATCC 19433 was used as the positive control. The *tuf* gene amplification was performed in a final volume of 25 μl mixture containing 12 μl of TaqMan DNA polymerase Master Mix (BIOLAB Inc., New England), 6 μl of nuclease free water, 1 μl of each primer *tuf* F:5′-AAAACGG-CAAGAAAAAGCAG-3′ and *tuf* R: 5′-ACGCGTGGTTAACAGTCTTGCG-3′ and 5 μl of DNA template. The PCR mixture was subjected to a 3 min denaturation step at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s for 55 °C and 60 s at 72 °C and final elongation step for 7 min at 72 °C (Ke et al., 1999). PCR products were confirmed in 1% Agarose gel electrophoresis in 1 × Tris-Borate-EDTA (TBE) buffer at 100 V for 60 min, visualized after staining with Ethidium bromide in ALLIANCE 4.7 UV transilluminator and photographed. Thereafter, three *Enterococcus* sp. with the highest survival were sequenced using the 16S rDNA sequencing.

2.4. Chlorine disinfection assay of Enterococcus sp. suspensions at the recommended free chlorine concentration (0.5 mgl^{-1})

Chlorine disinfection assay was performed using twenty