Bioaugmentation process of secondary effluents for reduction of pathogens, heavy metals and antibiotics

A. A. Al-Gheethi, R. M. S. R. Mohamed, A. N. Efaq, I. Norli, Abdullah Abd Halid, H. K. Amir and M. O. Ab Kadir

ABSTRACT

The study probed into reducing faecal indicators and pathogenic bacteria, heavy metals and β-lactam antibiotics, from four types of secondary effluents by bioaugmentation process, which was conducted with *Bacillus subtilis* strain at 45 °C. As a result, faecal indicators and pathogenic bacteria were reduced due to the effect of thermal treatment process (45 °C), while the removal of heavy metals and β-lactam antibiotics was performed through the functions of bioaccumulation and biodegradation processes of *B. subtilis*. Faecal coliform met the guidelines outlined by WHO and US EPA standards after 4 and 16 days, respectively. *Salmonella* spp. and *Staphylococcus aureus* were reduced to below the detection limits without renewed growth in the final effluents determined by using a culture-based method. Furthermore, 13.5% and 56.1% of cephalexin had been removed, respectively, from secondary effluents containing 1 g of cephalexin L^{-1} (secondary effluent 3), as well as 1 g of cephalexin L^{-1} and 10 mg of Ni^{2+} L^{-1} (secondary effluent 4) after 16 days. The treatment process, eventually, successfully removed 96.6% and 66.3% of Ni^{2+} ions from the secondary effluents containing 10 mg of Ni^{2+} L^{-1} (secondary effluent 2) and E4, respectively. The bioaugmentation process improved the quality of secondary effluents.

Key words | *Bacillus subtilis* 2012WTNC, bioaccumulation, biodegradation, cephalexin, nickel, pathogenic bacteria

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INTRODUCTION

Proper handling of sewage effluents has gained heightened recognition due to the presence of many pollutants, including pathogenic bacteria, viruses and parasites, as well as heavy meals and antibiotics (Sano *et al.* 2003; Ji *et al.* 2012; Al-Gheethi *et al.* 2015). In fact, for many years, different techniques, such as adsorption, ion exchange, ozonation, UV irradiation and filtration, have been used to remove those contaminants from sewage effluents (Dutta *et al.* 1999; Koivunen *et al.* 2003; Dodd *et al.* 2006; Batt *et al.* 2007; Putra *et al.* 2009). Nevertheless, despite the numerous claims pertaining to their effectiveness, it was revealed that there are inherent setbacks in the application of these technologies, including insufficiency, as well as in producing toxic by-product and carcinogenic compounds, as in the case of the oxidation processes of ozonation and chlorination (Xu *et al.* 2002; Putra *et al.* 2009; Wang & Chen 2009; Vital *et al.* 2010; Wang *et al.* 2012).

In terms of the disinfection process, the oxidation process of ozonation has led to the increase of assimilable organic carbon, and thus, increases the pathogen growth potential, as recorded for *Escherichia coli* O157 and of *Pseudomonas aeruginosa* (Vital *et al.* 2010). In addition, the UV irradiation of secondary effluents might be insufficient due to the presence of high suspended solids that protect the bacterial cells from UV damage. Therefore, regrowth and resistance among these bacteria have been observed, such as the regrowth and the repair potential, as well as the resistance among *E. coli*, faecal coliform (FC),

doi: 10.2166/wh.2016.046

Bacillus subtilis and *Clostridium* sp. in disinfected waters (Alonso *et al.* 2004; Ting *et al.* 2011; Wang *et al.* 2012).

Also, in regard to heavy metals, both adsorption and ion exchange techniques have become ineffective in achieving the removal level acquired to meet the standard limits if the heavy metals' concentrations are between 1 and 100 mg L^{-1} (Putra *et al.* 2009; Wang & Chen 2009). These techniques are designed to remove one pollutant, such as heavy metals alone; therefore, the secondary effluents have to be subjected to further treatment process to reduce other pollutants, such as pathogenic bacteria. On the other hand, the antibiotics could not be removed completely by sewage treatment processes, and the removal efficiency ranged between −34 and 72% (Gao *et al.* 2012). β-lactam antibiotics enter sewage streams through faeces and urine because the main removal of β-lactam antibiotics from the body is by secretion into the urine (Pauwels & Verstraete 2006). In fact, the discovery of antibiotics in sewage effluents has been reported by numerous authors in the literature: trimethoprim and ciprofloxacin in Sweden (Olofsson 2004); erythromycin, tetracycline, cephalexin and norfloxacin in Hong Kong (Gulkowska *et al.* 2008); fleroxacin, ofloxacin, sulfapyridine and sulfamethoxazole in China (Gao *et al.* 2012); as well as amoxicillin, ampicillin and ciprofloxacin in Tanzania (Kihampa 2014).

As mentioned earlier, it appears that in order to remove more than one pollutant, such as pathogenic bacteria, heavy metals and antibiotics, a series of treatment stages is required. Nevertheless, the treatment process has to be a simple system with only a few requirements of chemical additives, besides possessing the ability to remove a wide range of the challenging contaminants within one stage (Lim *et al.* 2002).

In addition, the increased loads of antibiotics and heavy metals in sewage might induce the bacterial cells to acquire resistance/tolerances, either by mutation or by the transfer of resistant genes among the bacteria. This results in the bacterial strain to have the potential to resist antibiotics and heavy metals simultaneously (Silva *et al.* 2006). For instance, *Bacillus* spp. have developed strategies for survival in unfavourable environments. Other than that, many decomposing bacteria are present during sewage treatment in the stage concerning the biological process (secondary process). In this work, *B. subtilis* 2012WTNC, which was isolated from secondary effluents, displayed the potential to biodegrade cephalexin by β-lactamase. The ability of β-lactamase (EC 3.5.2.6) produced from bacteria to hydrolyse the β-lactam ring of β-lactam antibiotics, thus, had been found to further inactivate the compounds (Liao *et al.* 2010). Therefore, the bioaugmentation process, including adding selected indigenous bacteria strains into sewage effluents, might be efficient in degrading antibiotics, as well as in removing heavy metals via biodegradation and biosorption processes simultaneously. Moreover, bioaugmentation of wastewater by adding selected strains' cultures can improve the catabolism of specific compounds and it is indeed a promising technique to solve practical problems in wastewater treatment plants, and to enhance removal efficiency (Herrero & Stuckey 2015). This process, nonetheless, would be more efficient if conducted at a temperature above that required for pathogenic bacterial growth, which does mean that this process would contribute in reducing pathogens.

For instance, a previous work that employed treatment of sewage at 45 °C exhibited high efficiency in reducing pathogenic bacteria (Al-Gheethi *et al.* 2013). *B. subtilis* had been found to be effective in removing antibiotics and heavy metals from sewage effluents at 37 °C (Al-Gheethi & Norli 2014; Adel *et al.* 2015). In this study, a strain of *B. subtilis*, which was grown during the thermal treatment of sewage, was applied as reference in a previous work (Al-Gheethi *et al.* 2013). This strain was found to be thermotolerant and exhibited resistance against heavy metals and antibiotics. Therefore, the bioaugmentation with the thermotolerant strain of *B. subtilis* was used in this study to remove heavy metals and β-lactam antibiotics from different types of secondary effluents in one stage, and this emphasizes the novelty of the current work. Faecal indicator (FC and Enterococci) and pathogenic bacteria (*Staphylococcus aureus* and *Salmonella* spp.) were reduced as a result of the thermal treatment conducted at 45 °C.

Moreover, Ni^{2+} ions and cephalexin were chosen as the model because previous work had found that there was a correlation between bacterial resistance against nickel and cephalexin (Al-Gheethi & Norli 2014). Furthermore, the cephalexin was resistant against degradation during the sewage treatment process, while nickel removal had been limited during the treatment process in comparison to other heavy metals (Leung *et al.* 2001). Chua & Hua (1996) found that the nickel concentration of 25 mg L^{-1} caused

serious upsets in the biological processes of the sewage treatment. This concentration could not be removed via chemical and physical techniques. As well, Ni^{2+} at low concentrations plays the role of cofactor for some bacterial enzymes, and a previous study had indicated that the production of β-lactamase increased in the presence of nickel ions (Al-Gheethi & Norli 2014).

Hence, the theoretical hypothesis for this treatment is given as follows: the treatment process conducted at 45 °C enhances the ability of *B. subtilis* 2012WTNC as a thermotolerant strain to grow, as well as reducing faecal indicators and pathogenic bacteria. The ability of *B. subtilis* 2012WTNC to tolerate cephalexin and to produce β-lactamase leads to the biodegradation of cephalexin and the production of bacterial biomass yield. Thus, the resulting biomass would remove nickel ions as a result of the affinity of metal ions towards bacterial surfaces (Abdel-Monem *et al.* 2010; Al-Gheethi *et al.* 2014).

MATERIALS AND METHODS

Source of secondary effluent samples

The secondary effluents were collected from the Jelutong Sewage Treatment Plant (JSTP) located at Penang, Malaysia. The sewage that flowed to this plant comprised residential, commercial and institutional wastes. The plant was designed in 2008 and received 270,000 m^3/day from 1.2 million population equivalent in 2014 (Saad & Zahera 2013, personal communication, Jelutong Sewage Treatment Plant, Penang, Malaysia). The JSTP was classified as a typical sewage treatment plant; the treatment of sewage in the plant was based on sequenced batch reactors (Saad & Zahera 2013, personal communication, Jelutong Sewage Treatment Plant, Penang, Malaysia). The waste treatment facility was designed to generate effluent quality to meet the EAQ 1974, regulation 2009 (sewage) Standard A requirements, which should be less than 20 mg L^{-1} of BOD (biochemical oxygen demand), 50 mg L^{-1} of TSS (total suspended solids) and 10 mg L^{-1} of total ammonia nitrogen, plus nitrate nitrogen with a limit of 5 mg L^{-1} of ammonia nitrogen. As well, the plant used UV irradiation for the disinfection process. However, the microbiological quality of secondary effluents was not determined before final disposal into the receiving sea water.

Glass bottles (1 L) used for the collection of samples were sterilized in an autoclave (high-pressure steam sterilization, Model ES-315, Tomy-Japan) at 15 psi and 121 °C for 15 min. The collection of samples (1 L) was carried out from the point of disposal of secondary effluents. For the heavy metals' determination, 2.4 mL of a 15% solution of ethylene di-amine-tetra acetic acid tetra-sodium salt (R & M Marketing, Essex, UK), was added as a chelating agent to the samples. The samples were transported to the laboratory in a cooler box and the microbiological analysis was carried out within 2 h of collection.

Bacterial strain

B. subtilis 2012WTNC (CP003492.1) was selected as described in previous works (Al-Gheethi & Norli 2014; Adel *et al.* 2015). The culture characteristics, as well as the microscopic morphology and the Gram stain reaction, were determined according to Benson (2005). A pure culture of bacterial isolate 2012WTNC was sent to Vivantis Technologies Sdn. Bhd. at Selangor, Malaysia for identification through the analysis of 16S rRNA sequencing via polymerase chain reaction (PCR) technique (Figure 1; Appendix A, available with the online version of this paper). The bacterial strains used in this study possessed the following criteria. They were isolated from the sewage treated effluent, were multiresistant to both heavy metals and antibiotics, as well as tolerant to high concentration of heavy metal ions. In addition, they were able to accumulate and biosorp heavy metals. Other than that, they were tolerant to β-lactam antibiotics, produced β-lactamase in the presence of heavy metals, had the ability to biodegrade β-lactam antibiotics in sewage effluent samples, non-pathogenic and thermotolerant bacteria. The experiments with heavy metals and antibiotics' resistance, as well as the production of β-lactamase and biosorp of nickel ions, were conducted as detailed in Al-Gheethi *et al.* (2014), Al-Gheethi & Norli (2014) and Adel *et al.* (2015).

Bioaugmentation studies

Four secondary effluent samples were prepared in the laboratory, including secondary effluents (E1), secondary effluents

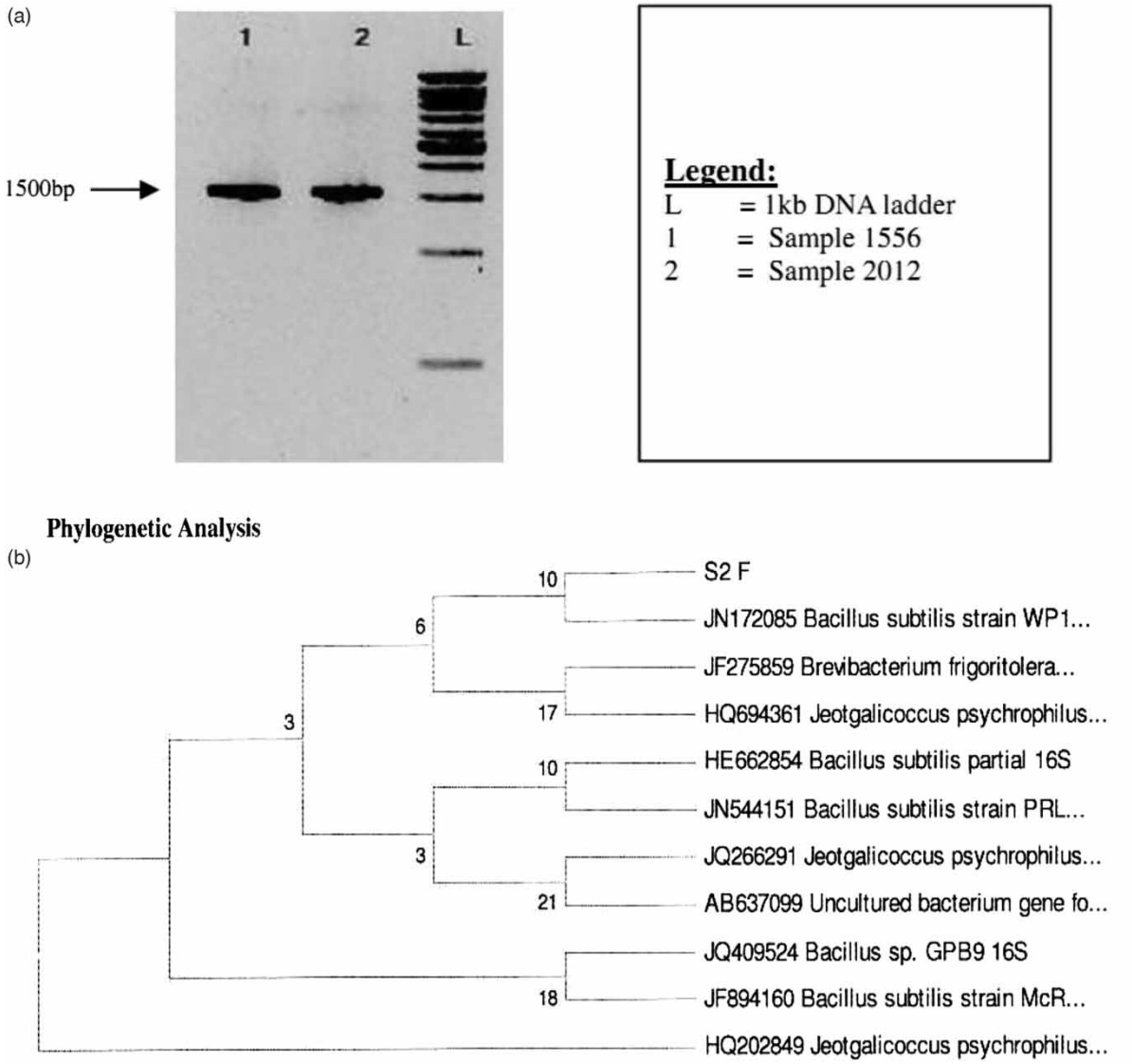

Figure 1 | Identification of *Bacillus subtilis* 2012WTNC (CP003492.1) based on molecular technique: (a) gel electrophoresis photo for the purified samples; (b) phylogenetic tree showing the interrelationship between sample 2012WTNC (CP003492.1) and the top 10 BLAST hits from National Centre for Biotechnology Information (NCBI).

containing 10 mg L^{-1} (w/v) of Ni^{2+} ions (E2), secondary effluents containing 1 g L^{-1} (w/v) of cephalexin (E3), and secondary effluents containing 10 mg L^{-1} (w/v) of Ni^{2+} and 1 g L^{-1} (w/v) of cephalexin (E4). These samples were prepared to represent different types of secondary effluents. The cephalexin was added into the secondary effluents with high concentrations to simulate pharmaceutical and hospital effluents due to the difficulty of obtaining these samples in the study area. The concentrations of cephalexin were added based on previous studies reported for antibiotics (Qiting & Xiheng 1988; Li *et al.* 2008). Furthermore, the nickel ions concentrations used in this study were less and could be removed by chemical technologies, such as adsorption and ion exchange processes, as mentioned above.

Additionally, FC, Enterococci, *S. aureus* and *Salmonella* spp., as well as enzymatic biodegradation and sorption

processes in the secondary effluents treated at 45 °C, were evaluated. Sewage treated effluents without *B. subtilis* 2012WTNC were used as control. Moreover, in order to avoid false results, the concentration of Ni^{2+} ions metals in the original sewage treated effluents was determined and used as a blank. Another secondary effluent sample containing cephalexin without bacterial inoculum was also used as a blank.

One litre of each effluent sample (in triplicate) was placed in a glass bottle (1 L), inoculated with 10 mL (v/v) of *B. subtilis* 2012 WTNC (~1.8×10^6 CFU mL^{-1}). The effluents were incubated in an incubator (JE10 TECH, Model SI.300R-Korea) at 45 °C. The sample containing bottles were covered with aluminium foil to prevent photodegradation of antibiotics in E3 and E4, while E1 and E2 were covered to provide equal treatment conditions for all samples. The samples were taken at days 0, 1, 2, 4, 8, 16, 32 and 64, whereby a fixed volume of 100 mL of each sample was taken for bacteriological, chemical and enzymatic analyses.

FC, Enterococci, *S. aureus* and *Salmonella* spp. were enumerated by using membrane filtration techniques (Sartorius, Germany) with a sterilized membrane filter (Millipore cellulose or nylon membrane filters; 45 mm diameter, 0.45 mm pore size) on the appropriate selective media. In brief, the sample bottle was shaken vigorously at least 25 times to distribute the bacteria uniformly. One mL of each effluent was filtered, and the membrane filter was removed from the filter base with forceps (sterilized by alcohol flaming) and placed onto the Endo agar for FC, bile esculin iron agar (BEIA) for Enterococci, xylose lysine deoxycholate agar (XLD) for *Salmonella* spp. and mannitol salt agar (MSA) for *S. aureus*. The plates were closed and sealed using a Para-film, then transferred into a plastic bag containing wet cotton to prevent dryness, and incubated for 24–48 h at 37 °C for *E. faecalis* and *Salmonella* spp., and *S. aureus* at 44.5 °C for FC.

Confirmation tests for faecal indicator and pathogenic bacteria

The colonies of FC grew with a greenish metallic sheen or pink colour on the Endo-agar and EMB agar. In order to confirm the FC colonies, APHA (1999) was employed. In brief, a loopful of a single colony grown on the Endo agar and the EMB agar incubated at 44.5 °C was transferred and inoculated to an EC medium broth tube containing Durham tubes. After 24 h, the tubes were examined for growth and gas bubbles in the EC Medium (Merck, Germany) broth tube to confirm the presence of FC.

The colonies of *E. faecalis* grown on BEIA had brown centres, while the colonies of *S. aureus* on MSA grew with a gold colour, and the *Salmonella* spp. was red in colour with black centres. *Salmonella* spp., *S. aureus* and *E. faecalis* were identified based on their morphological and cultural characteristics, as well as biochemical tests by using API 20 Strep (Ref 07226 B) and API 20NE (Ref 07224 B) (BioMerieux, SA-France). Furthermore, a catalase test was performed by adding a drop of 3% H_2O_2 (BioMerieux, SA-France) to the bacterial slant tubes and oxygen bubbles indicated that the test was positive. Other than that, oxidase production was tested by using the wet filter paper methods with oxidase test reagents (BioMerieux, SA-France), where violet colour indicated that the test was positive.

Next, the number of bacteria was calculated and reported in terms of CFU of the bacteria per 100 mL of the sewage effluent sample. In order to ensure that the reduction of faecal indicators and pathogenic bacteria had been less than the detection limits, 10 mL of the treated effluent was filtrated through the membrane filtration and then, the filter was transferred and placed on the surface of a brain–heart infusion medium as an enriched medium and incubated at 37 °C for 48 h; the absence of growth indicated the total elimination of bacteria (Al-Gheethi *et al.* 2013). If there was no growth of bacteria, the number of colonies were considered as 10 CFU/100 mL.

Indigenous bacterial growth and nickel ions removal

The growth of *B. subtilis* 2012WTNC in the secondary effluents during the treatment process was estimated by using the direct plating technique on Nutrient agar and expressed in CFU mL^{-1}. Bacterial biomass yield was determined based on the dry weight (Al-Gheethi & Norli 2014). Nickel ions were extracted from the samples by employing the nitric acid digestion method and were determined by an atomic absorption spectrophotometer (Model P.E.A. ANALYST 100, HGA-800 and MHS-10, Perkin Elmer, USA) (APHA

1998). The removal percentage was calculated based on Equation (1) (Adel *et al.* 2015):

$$E = \frac{C_i - C_f}{C_i} \times 100 \quad (1)$$

where E is the removal efficiency, C_i is the initial concentration of nickel ions (mg L^{-1}) and C_f is the final concentration of nickel ions (mg L^{-1}).

Biodegradation of cephalexin

The biodegradation of cephalexin by *B. subtilis* 2012WTNC was determined based on the determination of β-lactamase in the secondary effluents. β-lactamase (EC 3.5.2.6) was identified by determining the hydrolysis of cephalexin substrate according to Çelik & Çalik (2004). In brief, after the incubation period at intervals of 0, 1, 2, 4, 8, 16, 32 and 64 days, a fixed volume (10 mL) of the secondary effluent samples (E3 and E4) were harvested via centrifugation at 13,500 (rpm) for 10 min and the supernatant was tested for β-lactamase. The benzyl penicillin was replaced by cephalexin as substrate according to Sawai *et al.* (1978). Furthermore, the optimal wavelength of the cephalexin absorbance among different wavelengths was determined, and was 340 nm with a spectrophotometer (Model DR 2800, HACH, Germany (Appendix B, available with the online version of this paper)).The calibration curve of the different cephalexin concentrations in 0.1 M phosphate buffer had been determined at 30 °C and pH = 7.0 (λ = 340 nm) (Appendix C, available with the online version of this paper). In order to determine the β-lactamase concentrations in the secondary effluents, fresh substrate solutions were prepared daily by dissolving 0.4 mg mL^{-1} (w/v) of cephalexin in 0.1 M phosphate buffer (pH 7) and maintained at 30 °C. A fixed volume (0.1 mL) of centrifuged culture supernatant was added to 3 mL (v/v) of substrate solution. Immediately after that, the changes in absorbance over 1 min were recorded.

The secondary effluent sample containing cephalexin without bacterial inoculum was used as a blank. One unit of β-lactamase concentration was defined as the amount of enzyme that could hydrolyse 1 μmol of cephalexin at 30 °C and pH 7.0 in 1 min. The product of the hydrolysis reaction, cephalosporanic acid, also displayed absorbance at 340 nm, and therefore, the difference of the slopes was $y_1 - y_2 = 0.2135\ A_\lambda$.

The concentrations of enzyme, U mL^{-1}, is given by $1U = A_\lambda \times 0.2135 \times Dilution\ factor$.

The percentage of biodegradation was calculated based on Equation (2) (Al-Gheethi & Norli 2014):

$$\text{Biodegradation}(\%) = \frac{\mu \text{ mol of antibiotic hydrolyzing}}{\mu \text{ mol of antibiotic substrate}} \times 100 \quad (2)$$

Statistical analysis

The data were subjected to a one-way analysis of variance (ANOVA) with three determinations. The results were compared by employing the one-way ANOVA post hoc multiple comparison (LSD). The ANOVA tests were performed to determine the significance of the differences among the results. The differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

In the present work, the bioaugmentation process for four types of secondary effluents using indigenous *B. subtilis* 2012WTNC were carried out at 45 °C. The efficiency of this process to reduce faecal indicators (FC and Enterococci) and pathogenic bacteria (*Salmonella* spp. and *S. aureus*), enzymatic biodegradation of cephalexin, as well as removal of Ni^{2+} ions, was determined. *B. subtilis* 2012WTNC was chosen based on specific criteria, which are described earlier and presented in Tables 1–5. *B. subtilis* 2012WTNC exhibited high growth at 45 °C than thermotolerant bacteria. Therefore, in addition to the effectiveness of the treatment process at 45 °C to reduce indicators and pathogenic bacteria, this process had successfully removed both heavy metals and antibiotics.

The initial concentration of the FC present in the secondary effluents was 5×10^5 CFU/100 mL. The counts of FC decreased to meet the WHO guidelines (1989) (1,000 CFU/100 mL) after 2 days in E3 (Figure 2). In contrast, E1, E2 and E4 met the WHO guidelines after 8 days. After 16 days,

Table 1 | Characteristics of *Bacillus subtilis* 2012WTNC (CP003492.1) obtained from secondary effluents. Culture and morphological characteristics

			Culture characteristics						
Gram stain	**Cell morphology**	**Spore forming**	**Configuration**	**Elevation**	**Margins**	**Colour**	**Consistency**	**Opacity**	**Surface of colony**
+ve	bacilli	+	Round with scalloped margin	Umbonate	Irregular (Erose)	Beige	Mucoid	Opaque	Shine

+ve, Gram positive; +, spore forming.

Table 2 | Characteristics of *Bacillus subtilis* 2012WTNC (CP003492.1) obtained from secondary effluents. Heavy metals (Cu^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+} and Cd^{2+}) tolerance and antibiotics (cephalexin, cefuroxime, ampicillin, ciprofloxacin and amoxicillin) resistance

Heavy metals tolerance					Antimicrobial resistance				
Cu^{2+} (1 mM)	**Ni^{2+} (1 mM)**	**Pb^{2+} (0.5 mM)**	**Zn^{2+} (2 mM)**	**Cd^{2+} (0.2 mM)**	**Cephalexin**	**Cefuroxime**	**Ampicillin**	**Ciprofloxacin**	**Amoxicillin**
T	T	T	S	T	R	R	R	S	R

Heavy metals resistance: s (susceptible, <2 $\log_{10}$ CFU mL^{-1}); M (moderate, 2–3 $\log_{10}$ CFU mL^{-1}); T (tolerate, >4 $\log_{10}$ CFU mL^{-1}). For antibiotics S = susceptible, diameter of the inhibition zone >1.2 cm; M = moderate, diameter of the inhibition zone between 1.0 and 1.2 cm; R = resistance, the diameter of the inhibition zone between 0.7 and 1.0 cm.

FC decreased to meet the standard limits recommended by US EPA (2004) (14 CFU/100 mL) in all types of secondary effluents. The results indicated that the composition of secondary effluents affected the inactivation of FC. For example, the inactivation of FC in the secondary effluents that contained 1 g of cephalexin L^{-1} (E3) was higher than those of secondary effluents (E1) and secondary effluents that contained 10 mg Ni^{2+} L^{-1} (E2), as well as the one that contained 1 g of cephalexin L^{-1} and 10 mg of Ni^{2+} L^{-1} (E4).

The initial concentration of Enterococci presented in the secondary effluents at the beginning of the bioaugmentation process was 2.3×10^5 CFU/100 mL (Figure 3). The counts of Enterococci in E3 declined to less than 100 CFU/100 mL after 4 days, while there were still more than 1,000 CFU/100 mL in E1 and E4. In E2, Enterococci was still more than 3.2×10^2 CFU/100 mL at 30 days.

The reduction of *Salmonella* spp. during the bioaugmentation process of E3 was more than that for the other effluents. Moreover, *Salmonella* spp. decreased to less than the detection limits ($\leq$10 CFU/100 mL) after 8 days of treatment, before it increased again after 16 days, but to insignificant levels ($p < 0.05$) (16.98 CFU/100 mL) (Figure 4). The reduction of *Salmonella* spp. in E1, E2 and E4 showed different behaviours compared to FC; after 16 days of the bioaugmentation process, the concentrations of *Salmonella* spp. in E2 was 1.1×10^2 CFU/100 mL and more than that in E1 and E4. However, the presence of *Salmonella* spp. had been less than the detection limits ($\leq$10 CFU/100 mL) in E1, E2 and E3 after 32 days, as determined by the enrichment medium.

The ability of *S. aureus* to survive in the domestic treated effluent samples (E1) was more than those in industrial (E2), hospital (E3) and mixed effluents (E4). The inactivation of *S. aureus* in E2, E3 and E4 occurred rapidly after 16 days of the bioaugmentation process, where the concentrations were less than the detection limits, while it was 2.5×10^2 CFU/100 mL in E1. Also, *S. aureus* was eliminated from E2 and E4 after 32 days, but it was not eliminated from E1 and E3 during the same period. However, it was less than the detection limits ($\leq$10 CFU/100 mL) after 64 days (Figure 5).

Comprehension of the growth requirements of pathogenic bacteria in aquatic ecosystems is essential for a holistic approach to microbial risk assessment, as well as for improving effluents' treatment design and operation. In fact, several factors determine the growth of pathogenic bacteria in the aquatic environment. Vital *et al.* (2010) identified the environmental conditions and competition with the indigenous microbial flora as the main consideration in reducing pathogens in water systems.

Regarding an environment supporting bacterial growth, the strength of the treatment process in disinfecting secondary effluents depends on the inactivation of pathogenic bacteria and the constitution of the secondary effluents. It has been well-documented that pathogenic bacteria grow in a range

Table 3 | Characteristics of *Bacillus subtilis* 2012WTNC (CP003492.1) obtained from secondary effluents. Tolerance of high concentrations of nickel ions and the association with the resistance to antibiotics

Concentration of Ni^{2+} (mM)				Sensitivity test																	
				Control					Tolerance at 2 mM of Ni^{2+}						Tolerance at 4 mM of Ni^{2+}						Tolerance at 6 mM of Ni^{2+}	
1 mM	2 mM	4 mM	6 mM	Cephalexin	Cefuroxime	Ampicillin	Ciprofloxacin	Amoxicillin	Cephalexin	Cefuroxime	Ampicillin	Ciprofloxacin	Amoxicillin	Cephalexin	Cefuroxime	Ampicillin	Ciprofloxacin	Amoxicillin	Cephalexin	Cefuroxime	Ampicillin	Ciprofloxacin	Amoxicillin
+++	+++	+	+	R	R	R	S	R	ND	ND	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

+++, good; ++, +, excellent. S = susceptible, diameter of the inhibition zone >1.2 cm; M = moderate, diameter of the inhibition zone between 1.0 and 1.2 cm; R = resistance, diameter of the inhibition zone was between 0.7 and 1.0 cm. ND, not detected if the bacteria has exhibited resistance for antibiotics.

Table 4 | Characteristics of *Bacillus subtilis* 2012WTNC (CP003492.1) obtained from secondary effluents. Production of β-lactamase by *B. subtilis* 2012WTNC

Medium	Bacterial growth (CFU mL^{-1})	Enzyme production (U mL^{-1})
Culture medium	3.2×10^6	0.31
Secondary effluents	4.6×10^6	0.21
Secondary effluents containing with nickel ions	2.4×10^6	0.31

CFU, colony forming unit.

Table 5 | Characteristics of *Bacillus subtilis* 2012WTNC (CP003492.1) obtained from secondary effluents. Biosorptive capacity of nickel and biodegradability of *B. subtilis* 2012WTNC for cephalexin in the secondary effluents

Biosorptive capacity	Biodegradation efficiency of cephalexin
143.2 mg Ni^{2+} g^{-1}	4.32%

between 10 and 40 °C and with the best growth at a moderate temperature or close to the human temperature (37 °C) (Frey & Price 2003). In this work, the treatment process was conducted at 45 °C. The retention time and the temperature are the most influential factors for microorganism removal (Alcalde *et al.* 2003). Lucero-Ramirez (2000) reported that pathogenic bacteria in sewage reduced to less than detectable levels in properly operated heat-drying systems.

Nonetheless, interestingly, the bacterial species investigated in this study exhibited different responses to the treatment process. Gram-positive bacteria (Enterococci and *S. aureus*) survived more than Gram-negative bacteria (FC and *Salmonella* spp.). Similar findings were also reported in previous work where *E. faecalis* exhibited more resistance than *E. coli* during the treatment process at different temperatures (55 and 65 °C). These differences are explained based on the cell wall structure. Gram-positive bacteria displayed a cell wall with greater strength than Gram-negative bacteria. Celico *et al.* (2004) also indicated that Enterococci are a more reliable indicator than FC for the detection of microbial pollution because Enterococci are more resistant in the environment than FC. In fact, Enterococci have been frequently considered as a reference microorganism for thermal treatments to be applied in pasteurized foods (Smith *et al.* 1990; Ghazala *et al.* 1991). However, WHO (1989) and US EPA (2004) did not regulate standard limits

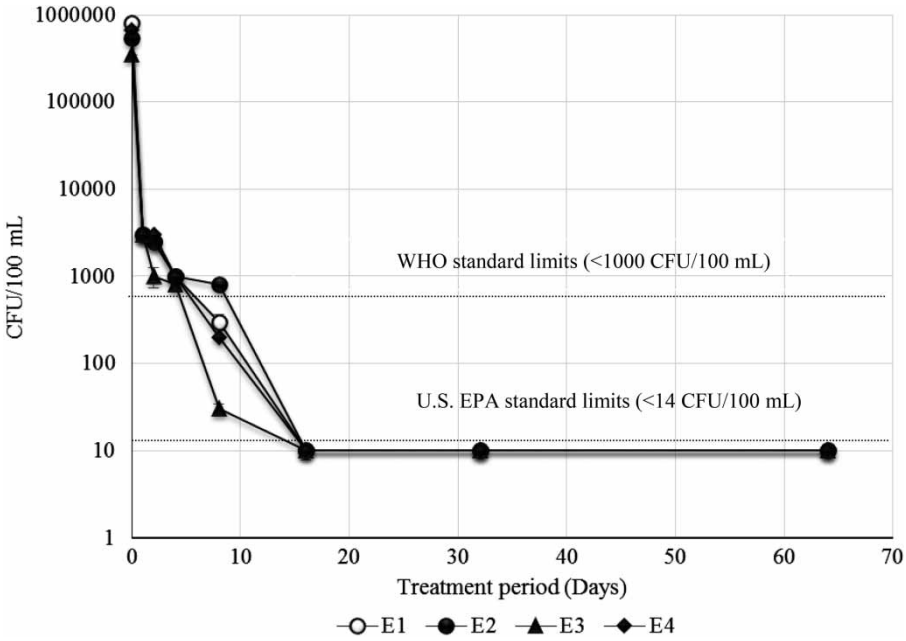

Figure 2 | Concentration of fecal coliforms during the bioaugmentation process of four types of secondary effluents conducted at 45 °C for 64 days: E1, secondary effluent; E2, secondary effluent containing 10 mg Ni^{2+} L^{-1}; E3, secondary effluent containing 1 g cephalexin L^{-1}; E4, secondary effluent containing 10 mg Ni^{2+} L^{-1} and 1 g cephalexin L^{-1}. Error bars represent the standard division from the mean.

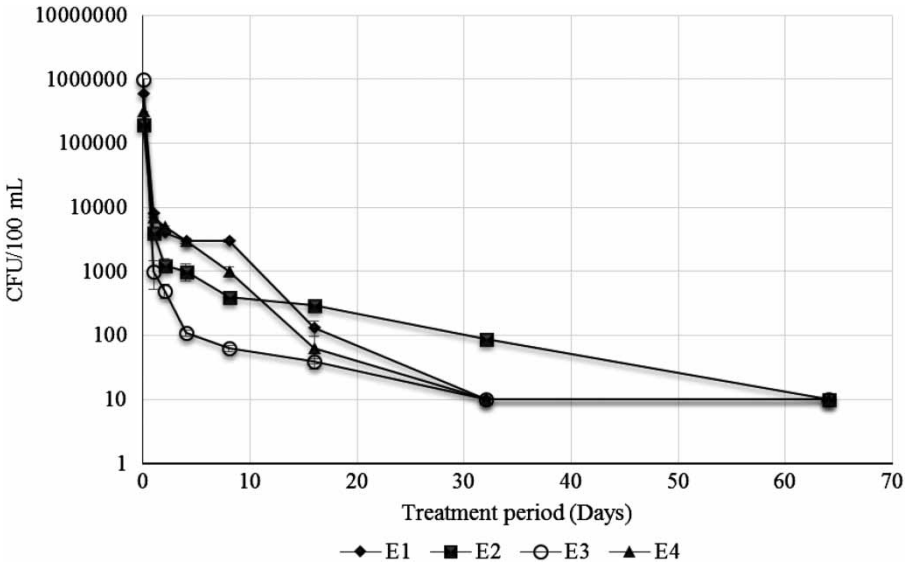

Figure 3 | Concentration of Enterococci during the bioaugmentation process of four types of secondary effluents conducted at 45 °C for 64 days: E1, secondary effluent; E2, secondary effluent containing 10 mg Ni^{2+} L^{-1}; E3, secondary effluent containing 1 g cephalexin L^{-1}; E4, secondary effluent containing 10 mg Ni^{2+} L^{-1} and 1 g cephalexin L^{-1}. Error bars represent the standard division from the mean.

of Enterococci in secondary effluents. In addition, in comparison between FC and *Salmonella* spp. (both are Gram-negative bacteria), the results revealed that *Salmonella* spp. survived more than FC. It demonstrated that *Salmonella* spp. was resistant and projected the potential to survive under hostile environmental conditions (Espigares *et al.* 2006; Alvarez-Ordonez *et al.* 2011). Therefore, US EPA (1995) and US EPA (2003) suggested it as an indicator of

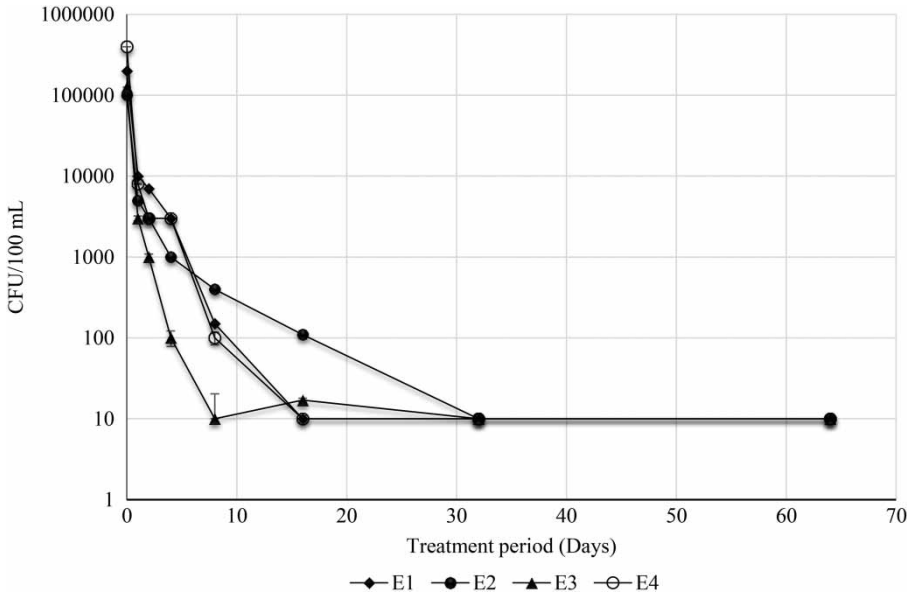

Figure 4 | Concentration of *Salmonella* spp. during the bioaugmentation process of four types of secondary effluents conducted at 45 °C for 64 days: E1, secondary effluent; E2, secondary effluent containing 10 mg Ni^{2+} L^{-1}; E3, secondary effluent containing 1 g cephalexin L^{-1}; E4, secondary effluent containing 10 mg Ni^{2+} L^{-1} and 1 g cephalexin L^{-1}. Error bars represent the standard division from the mean.

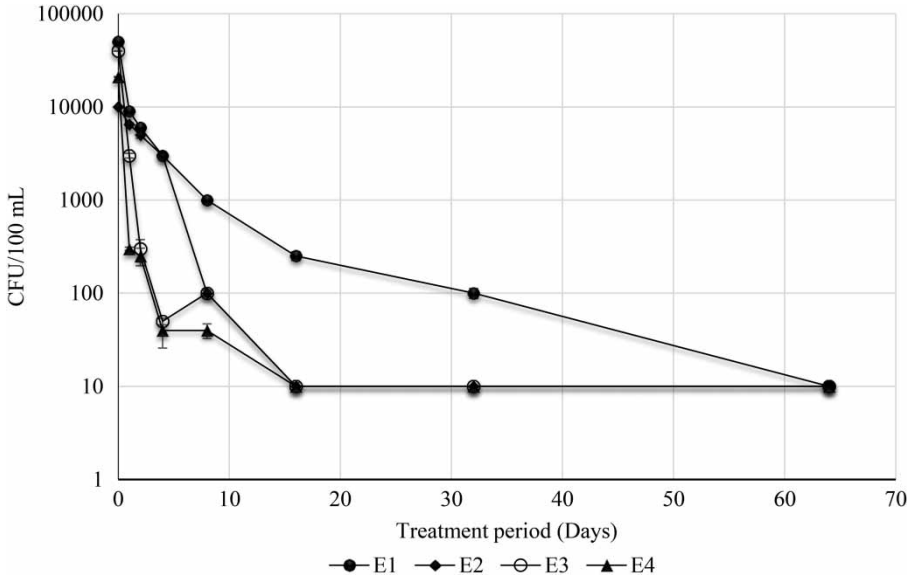

Figure 5 | Concentration of *Staphylococcus aureus* during the bioaugmentation process of four types of secondary effluents conducted at 45 °C for 1, 2, 4, 8, 16, 32 and 64 days: E1, secondary effluent; E2, secondary effluent containing 10 mg Ni^{2+} L^{-1}; E3, secondary effluent containing 1 g cephalexin L^{-1}; E4, secondary effluent containing 10 mg Ni^{2+} L^{-1} and 1 g cephalexin L^{-1}. Error bars represent the standard division from the mean.

choice for monitoring the effectiveness of sewage pathogen reduction. In contrast, Enterococci displayed more resistance than *S. aureus* (both are Gram-positive bacteria) during the treatment process. Enterococci showed the ability to grow at both 10 °C and 45 °C, at high pH (9.6) and in a medium containing 6.5% of NaCl (Cooper & Olivieri 1998; Carvalho & Teixeira 2002). Enterococcus is an environmental organism and it has been suggested as useful for

indicating the presence of viruses, particularly in sludge, sea water and biosolids (Bitton 2005). *S. aureus* has been detected previously in sewage and hospital wastewater (Rusin *et al.* 2003; Thompson *et al.* 2013) and has been proposed as an indicator of hospital hygiene for microbiological standards (Dancer 2004). For instance, Jin *et al.* (2013) used *S. aureus* as an indicator to be evaluated in a hydrothermal treatment process for achievement of hygienic safety for food waste. US EPA (1988) classified this bacterium in the minor list, which concerns opportunistic pathogens that only cause disease in debilitated or immunologically compromised individuals, but the evaluation of the indicator value of *S. aureus* requires further studies.

Hence, the considerations for the use of inoculated microorganisms in secondary effluents are insufficiency of substrate, competition between the introduced species, and the indigenous biomass. Hence, the choice of organisms is particularly important due to the differences in their ability for growth and survival under extreme conditions, as well as their affinity for pollutants. The selected bacteria must be able to survive under extreme conditions (e.g., redox, moisture, nutrient, osmotic factor and pH), besides competing with indigenous microbial populations and predators (Morikawa 2006). In the present work, *B. subtilis* 2012WTNC was indigenous in the secondary effluents and further exhibited the ability to survive and grow at 45 °C in the presence of pathogenic bacteria, indicating the ability of this strain to compete with these pathogens. *B. subtilis* 2012WTNC increased to around 4 $\log_{10}$ CFU/100 mL in different secondary effluents after 4 days (Figure 6).

It is worth noting that the reductions in faecal indicators and pathogenic bacteria were in response to the treatment process at 45 °C and not as a result of competition with *B. subtilis* 2012WTNC, where the reductions of faecal indicators and pathogenic bacteria in the control samples (without *B. subtilis* 2012WTNC) were quite similar and did not differ significantly in comparison to the *bioaugmented* ones. In previous work, the concentrations of faecal indicators and pathogenic bacteria were reduced to less than the detection limits after 1 week of the treatment process at 45 °C, without bioaugmentation with *B. subtilis* 2012WTNC (Al-Gheethi *et al.* 2013). Moreover, in this study, *B. subtilis* 2012WTNC had grown to be thermotolerant bacteria, while faecal indicators and pathogenic bacteria were reduced to less than the detection limits, as determined by using the culture-based method on the enrichment medium.

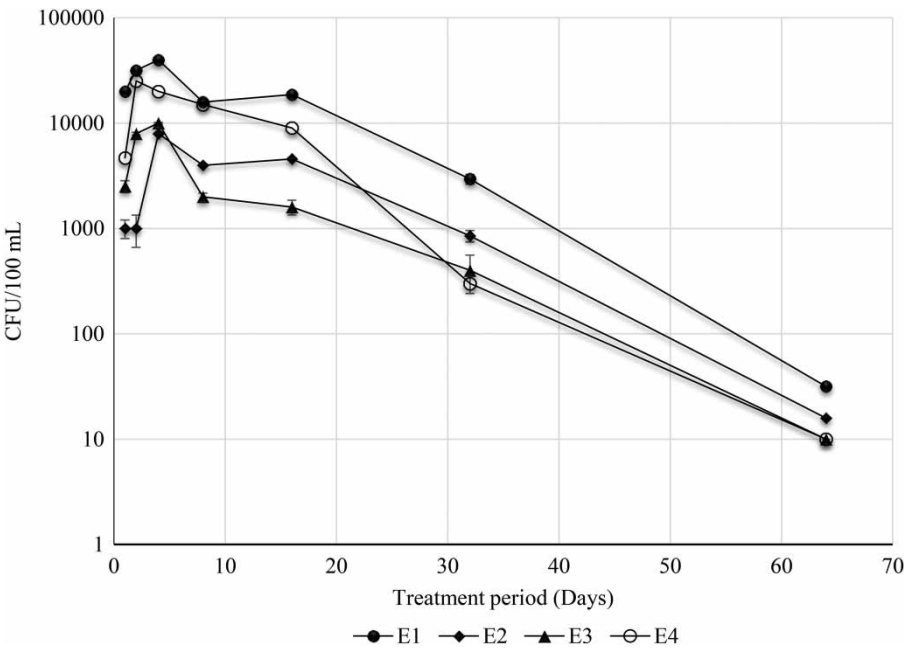

Figure 6 | Concentration of *Bacillus subtilis* 2012WTNC in four types of secondary effluents during the bioaugmentation process conducted at 45 °C for 64 days: E1, secondary effluent; E2, secondary effluent containing 10 mg Ni^{2+} L^{-1}; E3, secondary effluent containing 1 g cephalexin L^{-1}; E4, secondary effluent containing 10 mg Ni^{2+} L^{-1} and 1 g cephalexin L^{-1}. Error bars represent the standard division from the mean.

With regard to that, for the composition of the secondary effluents, it was observed that the reductions of FC and Enterococci in E3 were significantly more than those in E1, E2 and E4, as determined by the paired t-test ($p < 0.05$). The growth of *B. subtilis* 2012WTNC in E3 was also less than in E1, E2 and E4. This might be due to the presence of cephalexin, which inhibited the bacterial cell wall synthesis, thus enhancing the reduction levels. *B. subtilis* 2012WTNC was less sensitive to cephalexin as it was acclimatized to tolerate high concentrations of cephalexin during the screening process. E4 also contained cephalexin, but the presence of nickel ions might limit the effect of cephalexin on the bacterial cell growth.

As previously stated, it was noted that the presence of FC, Enterococci, *Salmonella* spp. and *S. aureus* of the secondary samples after 64 days of the bioaugmentation process were less than the detection limits, in comparison to the concentrations at zero day, which indicated that the quality of the secondary effluents had improved.

The second objective of this treatment was to biodegrade cephalexin by *B. subtilis* 2012WTNC. The results are presented in Figure 7. It was observed that *B. subtilis* 2012WTNC had the ability to biodegrade cephalexin after 16 days. Biodegradation of cephalexin in E4 was more significant (as determined by the paired t-test, $p < 0.05$) than that in E3 (13.5 versus 56.1%), respectively. The degradation of cephalexin in E3 and E4 without *B. subtilis* was very low (less than 5%), and therefore, the degradation recorded in this study was due to the bacterial enzyme. In fact, *Bacillus* spp. is industrially employed to produce enzymes under extreme (pH and temperature) conditions (Banik & Prakash 2004). In relation to the effect of pH values on the biodegradation of cephalexin, it was noted that they ranged from 6.5 to 7.5 at the maximum biodegradation percentage. These findings were similar to those observed in previous works (Mitchell *et al.* 2013; Al-Gheethi & Norli 2014).

As a result of the thermal treatment at 45 °C, which enhanced the rapid growth of *B. subtilis* 2012WTNC, the bacterial biomass increased during the first week of the treatment to the maximum value recorded after 8 days in all secondary effluent samples (Figure 8). Accordingly, the removal of nickel increased significantly (as determined by the paired t-test, $p < 0.05$) to 95.85 and 53.12% in E2 and E4, respectively, with maximum removal achieved after 16 days (96.63 and 66.33%; 0.96 and 0.66 mg Ni^{2+} L^{-1} in E2 and E4, respectively) (Figure 9). The explanation of the increase of nickel ions removal within the period of 8 to

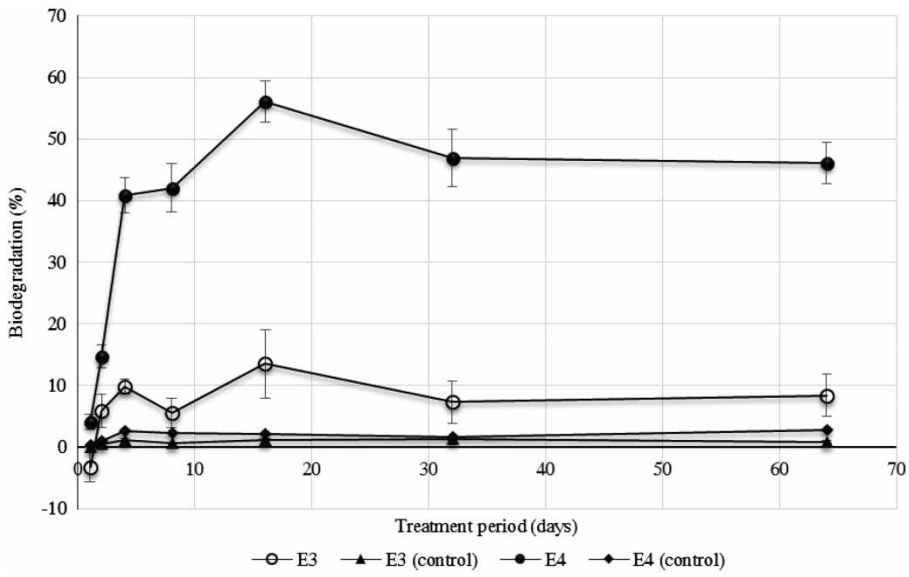

Figure 7 | Biodegradation of cephalexin in E3 and E4 by *Bacillus subtilis* 2012WTNC during the bioaugmentation process of four types of secondary effluent conducted at 45 °C for 64 days: E3, secondary effluent containing 1 g cephalexin L^{-1}; E4, secondary effluent containing 10 mg Ni^{2+} L^{-1} and 1 g cephalexin L^{-1}. Error bars represent the standard division from the mean.

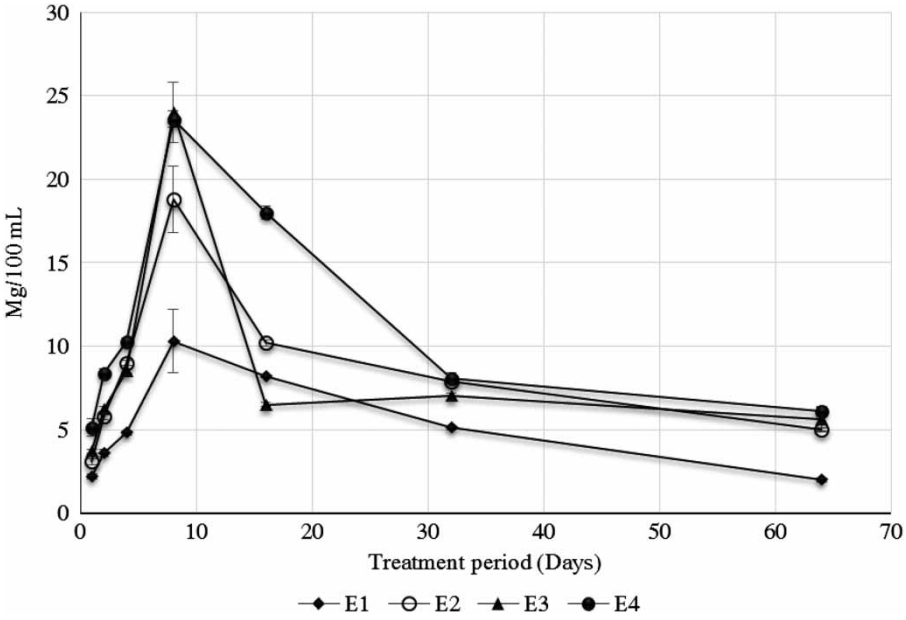

Figure 8 | Biomass yield in four types of secondary effluent samples during the bioaugmentation process conducted at 45 °C for 64 days: E1, secondary effluent; E2, secondary effluent containing 10 mg Ni^{2+} L^{-1}; E3, secondary effluent containing 1 g cephalexin L^{-1}; E4, secondary effluent containing 10 mg Ni^{2+} L^{-1} and 1 g cephalexin L^{-1}. Error bars represent the standard division from the mean.

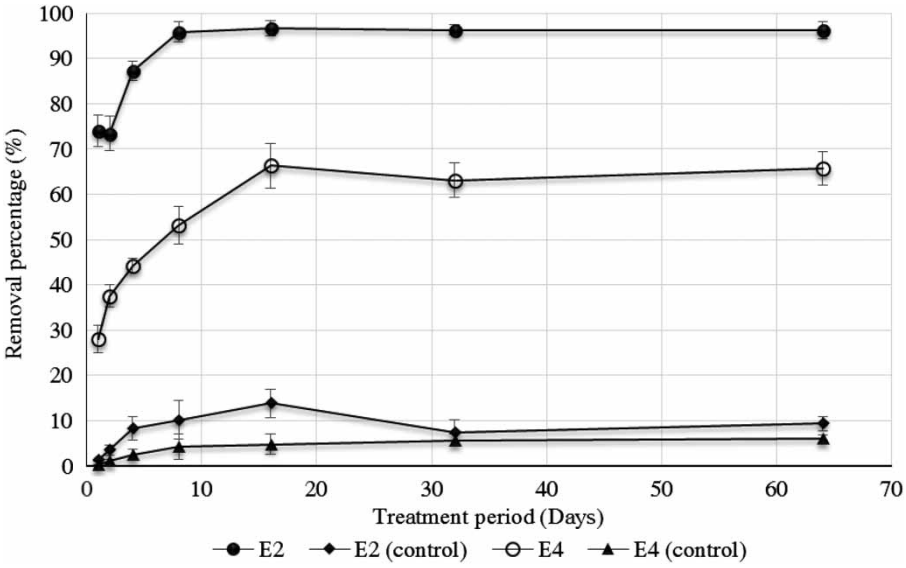

Figure 9 | Removal of Ni^{2+} ions in E2 and E4 by *Bacillus subtilis* 2012WTNC during the bioaugmentation process of four types of secondary effluents conducted at 45 °C for 64 days: E2, secondary effluent containing 10 mg Ni^{2+} L^{-1}; E4, secondary effluent containing 10 mg Ni^{2+} L^{-1} and 1 g cephalexin L^{-1}. Error bars represent the standard division from the mean.

16 days was due to the high *B. subtilis* 2012WTNC biomass yield. The transport system of Ni^{2+} ions acts by both specific and unspecific systems (Fath & Kolter 1993; Fagan & Saier 1994). *B. subtilis* 2012WTNC was found to be tolerant towards nickel, therefore, with a high concentration of nickel in the secondary effluents, the nickel ions were transported by chemiosmotic and diffusion system (unspecific system) into the cytoplasm, in spite of its high concentration,

because these unspecific transporter systems were constitutively expressed. Thus, the gate cannot be closed (Nies & Silver 1995). Temperature and pH also had a role in the bioaccumulation of nickel ions, since the diffusion process reflected the thermodynamic process.

Microorganisms, in many ways, can take up heavy metals through biosorption on the bacterial surface or via bioaccumulation, where the metal crosses through the membrane to enter the bacterial cytoplasm (Brierley 1990). In the present work, it was found that the removal of Ni^{2+} ions by *B. subtilis* 2012WTNC took place within the period of 1 to 4 days via bioaccumulation, where the bacterial cells exhibited the maximum growth and accumulated nickel ions (Figure 6). The removal process after 8 days occurred through the biosorption process, where the biomass yield was at the maximum level (Figure 8). The removal of nickel ion in the E4 was less than in E2, 96.63 versus 66.33% (Figure 9). This may occur as the nickel ions were adsorbed to the functional group of cephalexin, insoluble in mixed effluents and formed complexes, and thus, inhibited the removal process by bacterial biomass, as in the case of Al^{2+} and Fe^{2+} oxides that formed ciprofloxacin–surface complexes (Gu & Karthikeyan 2008).

According to EQA 1974, Regulation 2009 (Sewage) Standards A and B, the pH value of secondary effluents should be in the range of 5–9. Therefore, the best treatment process is the one that does not change the pH of the treated effluents to less than 5 or more than 9. In this study, the pH was in the range of 5–9 at the end of the treatment process.

B. subtilis 2012WTNC displayed the potential to remove both β-lactam antibiotics and heavy metals from secondary effluents that had been heavily contaminated with heavy metals, as well as to confront any contamination in secondary effluents obtained from hospital sewage or industrial wastewaters. *B. subtilis* 2012WTNC was chosen in this study based on its specific criteria determined in the section 'Bacterial strain'. These criteria were applied in this work and were suitable to choose the bacterial isolates to remove heavy metals from secondary effluents.

CONCLUSION

It can be concluded that the bioaugmentation process investigated in this study projected the potential to reduce faecal indicators and pathogenic bacteria, heavy metals, as well as β-lactam antibiotics in one stage. Two major treatment processes were combined into one stage, temperature control and the addition of *B. subtilis*. As a result, FC was reduced to meet the standard limits set by the US EPA, and nickel ions were decreased to meet EQA1974, Regulation 2009 (Sewage) Standard B. Other than that, the pH levels were still in the range recommended by these regulations. This process would be able to improve the quality of secondary effluents. The sewage treated effluents generated from this process were suitable for safe disposal or to be reused in agriculture. However, it is important to indicate that the process had been proven to be efficient for inactivation of pathogenic bacteria in those specified conditions. Moreover, more studies with other types of pathogens, such as viruses, should be carried out.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the Third World Organization for Academic Science (TWAS), Ministry of Higher Education of Malaysia for the research project financial support under fundamental research grant scheme (FRGS) No. 1453 and FRGS (203/PTEKIND/6711328).

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First received 1 March 2016; accepted in revised form 20 April 2016. Available online 27 May 2016