Bacterial biosensors for rapid and effective monitoring of biodegradation of organic pollutants in wastewater effluents

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Significant amounts of toxic substances which are hazardous to animals, plants, microorganisms, and other living organisms including humans are released annually into aquatic and terrestrial environments, mostly from improper wastewater discharges. Early detection of such pollutants in wastewater effluents and proper monitoring before their final release into the environment is therefore necessary. In this study, two whole-cell bacterial biosensors were constructed by transforming competent cells of *Shigella flexneri* and *Shigella sonnei* with pLUX plasmids and evaluated for their potential to monitor wastewater samples undergoing degradation by measuring bioluminescence response using a microplate luminometer. Both bacterial biosensors were found to be extremely sensitive to the wastewater samples, with different patterns, concomitant with those of the COD removals demonstrated at the different days of the degradation. Generally higher bioluminescence values were obtained at the later days of the degradation period compared to the initial values, with up to 571.76% increase in bioluminescence value obtained at day 5 for 0.1% (v/v) effluent concentration. Also, a steady decrease in bioluminescence was observed for the bacterial biosensors with increasing time of exposure to the wastewater effluent for all the sampling days. These biosensor constructs could therefore be applicable to indicate the bioavailability of pollutants in a way that chemical analysis cannot, and for *in situ* monitoring of biodegradation. This has great potential to offer a risk assessment strategy in predicting the level of bioremediation required during municipal wastewater treatment before their final discharge into the aquatic milieu.

Introduction

The continuous addition of industrial, municipal, and agricultural effluents to the environment has led to a great increase of toxic pollutants.[1] Significant amounts of these toxic substances, which are hazardous to animals, plants, microorganisms, and other living organisms including humans, are released annually into aquatic and terrestrial environments.[2] There is, therefore, a continuing need for early detection and monitoring of pollutants, including various hydrocarbons, aromatic substances, chlorine compounds, and toxic metals released from industrial effluents.[3]

To evaluate the impact of toxic agents in effluents, bioassays have been developed and applied to complement chemical and physical detection methods.[4] Once toxic compounds have been detected, various industries try to reduce their toxicity before release to the environment by biodegradation.[5] However, the biodegradation process may result in more toxic products than the parent compound, or products with different toxicity targets, and these possibilities would not be detected by normal analytical methods.[5] It is, therefore, important that further processes of detection and monitoring of the wastewater effluents be developed to determine whether the toxic pollutants have been totally mineralized or at least degraded to their stable intermediates that pose no threat to the environment.[2] Accordingly, understanding the biological activity or toxicity of the end products and stable intermediates of biodegradation is crucial.[2]

Analytical techniques, apart from being very costly and time consuming, only measure and quantify the concentration of compounds before and after degradation, but do not measure their degree of bioavailability to living organisms.[6] The use of biosensors has found a place in detecting, monitoring and evaluating industrial effluents and wastewater samples for their bioavailability to microorganisms. A variety of luminescent bacterial bioassays have therefore been developed, which detect a wide range of pollutants while simultaneously evaluating their bioavailability in environmental and industrial effluents,[7] including; naphthalene and salicyclate,[8,9] mercury,[3] and short-chain alkanes.[10] The majority of these biosensors are based on bioluminescence upon contact of whole cell constructs with specific pollutants or general toxic compounds in the environment.[11] Whole-cell microbial bioassays and lux-marked (*lux CDABE*) bioassays offer great environmental relevance for luminescent-based testing[12] and non-destructive tracking of a specific organism, or monitoring of the presence of toxic compounds during biodegradation without exogenous addition of aldehyde substrates.[11] Currently, a large spectrum of microbial biosensors have been developed that enable the monitoring of pollutants by measuring bioluminescent light production, fluorescence color, or electrochemical detection.[13]

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Improper disposal of poorly treated industrial, municipal, and agricultural effluents to the environment may lead to the contamination of water resources, drinking water supplies, soil, and other environments.[2] Most wastewater treatment plants spend huge amount of money on the laborious and time-consuming analytical methods of detecting pollutants in the effluents before discharge. In this study, whole-cell bacterial biosensors were developed, using *Shigella flexneri* and *Shigella sonnei*, for rapid detection and monitoring of wastewater samples undergoing degradation and compared to the routine chemical measurements of effluent quality. It is believed that these biosensors will complement the analytical techniques currently in use and provide a faster way of monitoring the degradation process.

Experimental

Sample collection and biodegradation set-up

Raw wastewater effluent sample was collected from the Northern Wastewater Treatment Plant in KwaZulu-Natal region of South Africa and stored at 4 °C prior to use. Five hundred millilitres of the effluent was transferred into a 1 litre Erlenmeyer flask in triplicate and was allowed to undergo degradation on a shaking incubator at 150 rpm. Two sets of experimental set-ups were performed; one at predetermined optimum conditions (1% activated sludge inoculum, pH 6 and temperature of 25 °C) and the other one without inocula addition and at 28 °C. Samples were collected at days 0, 1, 3, and 5 and used in the bioassay. The chemical oxygen demand (COD) values of the different samples were determined using Aqua Lytic AL 282-Photomer (Germany) following the manufacturer's instruction. Samples (5 ml) were dispensed into COD bottles [containing potassium dichromate, mercury(II) sulfate and sulfuric acid] and vortexed for about 15 seconds to ensure thorough mixing of sample with the COD reagents. The samples were then digested in a thermoreactor at 148 °C for 2 hours, and allowed to cool down before measuring the COD using the control cuvette to zero the machine.

Bacterial isolates and culture conditions

The bacterial isolates used in the construction of the biosensor systems; *Shigella flexneri* and *Shigella sonnei* were obtained from the Culture Collection of the Department of Microbiology, University of KwaZulu-Natal (Westville Campus), Durban, South Africa. The isolates were grown and maintained in Luria–Bertani (LB) agar slants (10 g NaCl, 10 g tryptone and 5 g yeast extract per litre of distilled water) and stored at 4 °C and sub-cultured regularly on LB agar plates as working stocks.

Bacterial biosensor constructs

Electrocompetent cells of both *S. flexneri* and *S. sonnei* were successfully prepared using standard procedures[14] and 50 μL aliquots were dispensed into 1.5 mL microfuge tubes, snap-frozen and stored at −70°C until required. Plasmids pUC19 (2686 bp) harbouring the *Lux*CDABE operon (8649 bp), thus forming the pLUX plasmid (11 335 bp) also obtained from the Department of Microbiology, University of KwaZulu-Natal (Westville Campus) was used in the transformation process. The pLux plasmid was successfully introduced into the electro-competent cells of *S. sonnei* and *S. flexneri* using the Gene Pulser apparatus (BioRad) set at 25 μF and 2.5 kV for 0.2 cm cuvettes (BioRad) and the Pulse controller (BioRad) to 200 Ω. Recombinant cultures of both organisms were obtained by plating out transformants on LB agar supplemented with 100 μg mL^{-1} of ampicilin. Since the pLUX plasmid exhibit an ampicilin resistant gene (amp^r) between the *Sal* I restriction sites, it therefore confers this resistance to the *S. flexneri* and *S. sonnei* for easy expression and maintenance. Biosensor constructs were freeze-dried and resuscitated just before use.

Resuscitation and harvesting of freeze-dried cells

Cells were inoculated into 10 mL LB broth containing 100 μL of ampicillin (10 mg mL^{-1}), and incubated at 28 °C with shaking for 2–2.5 hours. Cells were evaluated for bioluminescence in a Bio-Orbit 1254 luminometer for cell-overload (500 000). Cells were then centrifuged at 13 000 rpm for 2 minutes and the pellet re-suspended in 1 mL of 0.1 M KCl.

Ecotoxicological bioassay procedure

The ecotoxicological bioassay was conducted for both biosensors in triplicate at the different biodegradation conditions. A stock sample of the effluents was collected at the different sampling times and diluted 10-fold. Two hundred and seventy microlitres of the appropriate 10-fold dilutions of the effluent samples were used to inoculate the wells of white round-bottomed, 96-well microtitre plate (Nunc, ThermoLabsystems), followed by the addition of 30 μL of the biosensor cells resuscitated as described above. Bioluminescence was measured periodically as relative light units (RLUs) using a temperature-controlled (30 °C) Fluoroscan Ascent FL (ThermoLabsystems) with 15 minute integration time for 1 hour.

Results

Wastewater effluent biodegradation

The biodegradation profile of the wastewater effluent at both the optimized and non-optimized conditions was monitored by the determination of the COD values at the different days during the degradation period. Results, as shown in Fig. 1, revealed that the wastewater raw effluent sample was degraded to varying levels at the different treatment conditions. After day 1, a lower COD reduction of 28.28% and 21.22% was observed for the optimized and non-optimized degradation conditions, respectively. The highest COD removal was observed in the wastewater sample undergoing degradation under optimized conditions, resulting in 57.46% and 73.07% degradation after day 3 and day 5, respectively (Fig. 1). However, a degradation of 48.86% and 61.05% was observed in the non-optimized effluent sample at the same period (Fig. 1).

Response of bacterial biosensor constructs to the wastewater effluent undergoing biodegradation

The list of the bacterial biosensor constructs made in the study is shown in Table 1. Both organisms were successfully transformed

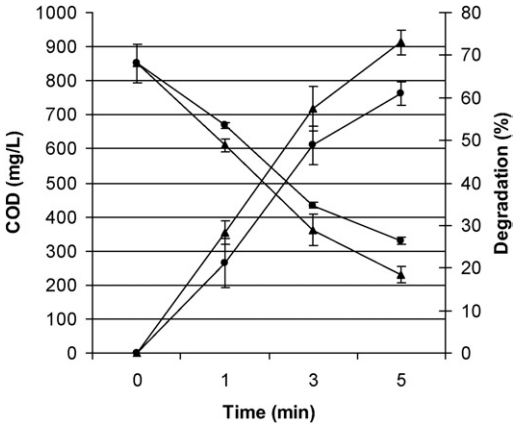

Fig. 1 Degradation and COD profiles of the wastewater effluent for both optimized (▲) and non-optimized (●) conditions. Bars indicate the average of triplicate samples while the error bars show the standard deviation.

Table 1 Bacterial biosensors constructed in this study and the plasmid used

Biosensors constructed	Features incorporated		
	Organism	Plasmid	Plasmid properties
S. flexneri pLux	*S. flexneri*	pLUX	11 335 bp, *Lux*CDABE operon, *amp*^{r}
S. sonnei pLux	*S. sonnei*	pLUX	11 335 bp, *Lux*CDABE operon, *amp*^{r}

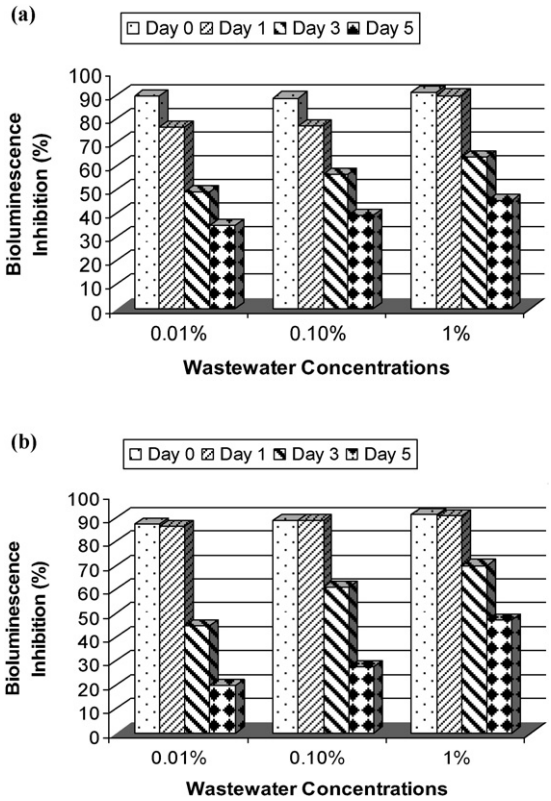

Fig. 2 Bioluminescent response of *S. flexneri* pLux biosensor to varying concentrations of wastewater effluent undergoing degradation under (a) optimized and (b) non-optimized conditions after 30 minutes exposure. Bioluminescent value in the presence of non-polluted water was taken as 100% and used as control.

and harboured the pLUX plasmid. Both bacterial biosensors were found to be extremely sensitive to the wastewater samples, with different patterns demonstrated at the different days of the degradation. Generally higher bioluminescence values were obtained at days 3 and 5 of the degradation period compared to the initial values and those obtained at day 1 (Fig. 2 and 3). Also, a steady decrease in bioluminescence inhibition was observed for the two bacterial biosensors as the degradation of the wastewater effluent progresses (Fig. 2 and 3). The observed bioluminescence inhibition observed for both bacterial biosensors was slightly higher at higher concentrations of the wastewater effluent samples compared to the lower concentrations.

For the *S. flexneri* pLux biosensor, a general reduction of 76.37–90.97% in bioluminescence was observed after day 1, while only 19.91–47.19% bioluminescence reduction was observed after day 5 for the varying concentrations of both optimized and non-optimized samples (Fig. 3a and b). The lowest bioluminescence intensity was observed at day 1 of the degradation process, with only a 10.20% and 0.69% increase in bioluminescence observed at 0.01% and 0.1% concentrations, respectively, for non-optimized wastewater effluent (Fig. 2b) after 30 minutes exposure. A bioluminescence increase of 4.97% and 11.47% was observed at 0.01% and 0.1% concentration, respectively, for the same sample after 15 minutes exposure (results not shown). However, a bioluminescence increase of 347.36% and 549.57% was obtained at days 3 and 5 for the non-optimized effluent at 0.01% (Fig. 2b), and as high as 553.91% and 521.58% increase in bioluminescence value was obtained at day 5 for 0.1% and 1% effluent concentrations, respectively (Fig. 2b), after 30 minutes exposure. For the optimized samples, a bioluminescence increase of 395.70% and 534.20% was obtained at days 3 and 5, respectively, after 30 minutes at 0.01% effluent concentration (Fig. 2a), and up to 450.54% and 539.29% increase observed for 0.1% and 1% effluent concentrations, respectively (Fig. 2a).

Similarly, for the *S. sonnei* pLux biosensor, a steady increase in bioluminescence was observed as the degradation of the wastewater progresses for both optimized and non-optimized effluent samples (Fig. 3a and b). For example, an increase of 579.65% and 760.89% in bioluminescence was observed at days 3 and 5, respectively, for the optimized sample at 0.01% effluent concentration (Fig. 3a), while an increase of 348.06% and 755.40% in bioluminescence was observed for the non-optimized effluent sample at the same period (Fig. 3b) after 30 minutes. Also, up to 438.73% and 454.89% increase was observed after 15 minutes for optimized and non-optimized effluent samples, respectively, at 0.1% concentration (results not shown). However, a higher increase in bioluminescence was observed after 30 minutes, with up to 579.65% increase at day 3 and 760.89% increase at day 5 observed for the optimized effluent sample at 0.01% concentration (Fig. 3a). Also, 755.40% and 534.59% increase in bioluminescence was observed at day 5 for the non-optimized effluent sample at 0.01% and 0.1%, respectively, (Fig. 3b). A reduction in bioluminescence of 22.59% and 35.16% was observed for the non-optimized samples at 0.01% and 0.1% concentrations,

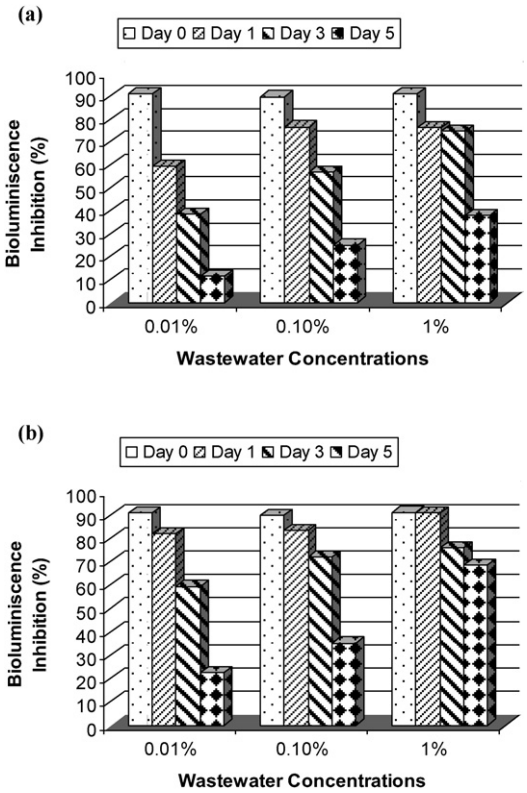

Fig. 3 Bioluminescent response of *S. sonei* pLux biosensor to varying concentrations of wastewater effluent undergoing degradation under (a) optimized and (b) non-optimized conditions after 30 minutes exposure. Bioluminescent value in the presence of non-polluted water was taken as 100% and used as control.

respectively (Fig. 3b), with only 11.46% and 24.95% inhibition observed for the optimized samples at the respective concentrations (Fig. 3b).

Discussion

Apart from the reproducible bioluminescence results generated, the integration of the entire *lux*CDABE operon into the *S. flexneri* and *S. sonnei* pLux is advantageous in many ways. It is economical as it by-passes the need for the complex enzymatic sub-processes involved in the production and recycling of the natural luciferase substrate.[15] Actively growing bacteria have been reported to have limiting capabilities of meeting the requirements of a portable toxicity biosensor, such as on-site evaluation of environmental pollution. Thus, various approaches of maintaining bioluminescent biosensors have been used, including the immobilization of exponentially growing cells to a wide variety of matrices, *i.e.*, calcium-alginate beads[16] and glass beads in agar.[17] Bioluminescent-based Microtox™ kits have previously been used for pollutant detection in wastewater, based on *Vibrio fischeri*, without any genetic manipulation.[18] However, since this organism is a marine bacterium not found naturally in freshwater samples, they are not ecologically representative of freshwater systems[19] and its application is very limited because wastewater samples could not in any way mimic the natural habitat that *V. fischeri* requires. Short-term exposure of the toxicants to fish and water flea (*Daphnia pulex*), also currently in use, was found to be time consuming while the use of higher organisms as test species have also raised ethical issues.[20]

Bioassays developed with pathogenic bacteria, especially *Shigella* sp have been widely shown to be efficient in monitoring pollutants in wastewater samples,[13] thus showing the potential of this bacterial group for use as biosensors for pollutant detection in wastewater samples. However, because of the complex nature of wastewater effluents, bacterial biosensor constructs with the ability to show a general trend in metabolic functioning as a result of exposure to such wastewater effluents are of great interest. An understanding of the acute toxicity imposed by combinations of pollutants (as in complex wastewater effluent samples) is essential in interpreting the fate of environmental contaminants during bioremediation.[19] In this study, two bacterial biosensors were developed using *S. flexneri* and *S. sonnei* for use as environmental toxicity determinants and in monitoring the biodegradation of wastewater effluents. The two bacterial biosensors demonstrated a great potential for monitoring biodegradation of the wastewater effluent as different trends were observed as the degradation increases. The higher bioluminescence values obtained with increasing degradation of the wastewater effluent could be attributed to the formation of degradation products which are less and/or non-toxic to the bacterial biosensor, compared to the original raw effluent, thus acting as inducers of the bioluminescence in these organisms.[18] A panel of stress-responsive luminous bacteria has also been previously found to be applicable in determining the toxic nature of samples since the ecotoxicity bioassays of some strains in the panel are based on a decrease in bioluminescent response.[21] Furthermore, a *Synechocystis* sp. with a *glnA::lux* fusion[22] and a *Synechocystis* sp. with a *nblA::lux* fusion,[23] both of which are sensitive reporters of available nitrogen in wastewater, have been previously described. The *Synechocystis glnA* reporter was responsive to ammonia, nitrate, nitrite and organic nitrogen, while the *Synechocystis nlbA* reporter was characterized mostly for nitrate detection. A highly sensitive phosphorus bioavailability sensor based on a *pho::lux* fusion in *Synechocystis* sp. was also reported.[24]

During freeze-drying and preservation of these genetically engineered biosensor cells, the production, maintenance and metabolic functions of the *lux*CDABE genes was of primary concern. From previous studies (results not shown), freeze-drying in skimmed milk did not consistently yield uniform freeze-dried products and also required longer than one hour for resuscitation with agitation to create a homogenous test solution. For this reason the biosensor constructs freeze-dried in skimmed milk did not indicate a practical application as a potential on-site biosensor. Alternatively, freeze-drying in trehalose consistently yielded uniform freeze-dried products and only required thirty minutes resuscitation without agitation. It therefore seemed that trehalose maintained the viability and biosensing activity of these biosensors, thus producing high bioluminescence values after resuscitation in LB broth. This may be because trehalose increases the tolerance of the biosensor membrane phase transition, and maintains the general protein structure in the dry state.[25] Hence, both bacterial biosensors were freeze-dried in trehalose and not skimmed milk.

The stability of these *S. flexneri* and *S. sonnei* pLux biosensor constructs at room temperature of 20–26 °C after resuscitation is advantageous for their potential on-site applications for the detection of pollutants in wastewater effluent samples, since the ambient temperature falls within this temperature range. Although whole-cell bacterial pLux biosensors are unlikely to completely replace the well accepted methods of instrumental analysis that provide the basis for environmental monitoring and regulation, findings from this study indicate their usefulness in detecting pollutants in wastewater samples. Continued improvements of these biosensor constructs could thus meet the urgent need not only to indicate the bioavailability of pollutants in a way that chemical analysis cannot and quantify bioavailable pollutants, but to also perform *in situ* monitoring of biodegradation.[26] This has great potential to offer a risk assessment strategy in predicting the level of bioremediation required during municipal wastewater effluent treatment before the final discharge into the receiving water bodies.

Conclusions

Two bacterial biosensors were successfully constructed and applied as a wastewater biodegradation monitoring system. Although, these bacterial biosensors may not be able to quantitatively analyze the different toxic substances present in the wastewater at the different degradation days like other conventional methods, it possessed many advantages in practical applications such as: (i) generation of consistent patterns useful as indicators of the level of degradation; (ii) real-time monitoring every 15 minutes; (iii) rapid warning; (iv) indication of the bioavailabilty of pollutants in the environment; (v) rapid and reliable bioluminescence response; (vi) low costs and the ability to cope with large sample runs; and (vii) portability and possibility for use in on-line monitoring at wastewater treatment plants. Thus, these biosensor constructs could be very useful for monitoring the biodegradation of toxic pollutants in wastewater effluents during municipal wastewater treatment processes. Further optimization is ongoing for a wider application of these bacterial biosensors.

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