Recovery of laccase-producing gammaproteobacteria from wastewater

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ABSTRACT

Wastewater environment is a rich source of microorganisms with the capability for the degradation of malicious aromatic pollutants. Although wastewater could be regarded as both a resource and a problem, we intended to elucidate its beneficial aspect in this study sourcing for laccase-producing proteobacteria. Different wastewater samples, from selected wastewater treatment plants (WWTPs), were selectively enriched with some model compounds (vanillin, lignin and potassium hydrogen phthalate) to screen out bacterial isolates that possess excellent degradation potentials. Thereafter, positive isolates were screened for the production of laccase and degradation on phenolic (guaiacol, α-naphthol and syringaldazine) and non-phenolic (ABTS; 2,2 azino-bis -(3-ethylbenzothiazoline 6 sulphonic acid) and PFC; potassium ferrocyanoferrate) substrates characteristic of laccase oxidation. Remarkable laccase producers were identified based on their 16 S rRNA sequences and their secreted enzymes were subjected to substrate specificity test, employing laccase substrates; ABTS, PFC, guaiacol, α-naphthol, 2,6-dimethoxyphenol and pyrogallol. Results showed that wastewater and selective enrichment, in tandem, produced the gammaproteobacteria *Pseudomonas* aeruginosa DEJ16, *Pseudomonas* mendocina AEN16 and *Stenotrophomonas* maltophila BIJ16, which preferably oxidized the non-phenolic substrates. Units of extracellular laccase activity ranging between cca. 490 and cca. 600 U/mL were recorded for ABTS whereas outputs recorded from PFC catalysis ranged from cca. 320 to cca. 430 U/mL. *Stenotrophomonas maltophila* BIJ16 presented an unparalleled high laccase activity and had a responsive substrate specificity to aromatic and inorganic substrates, thereby suggesting its employment for in situ biodegradation studies. In conclusion, wastewater serves as an ideal milieu for the isolation of laccase producing bacteria.

1. Introduction

Wastewater, as somewhat agreeably communicated by Corcoran et al. [1] to be a blend of effluent cocktails from municipal and industrial settlements, either as dissolved or particulate matter, has a significant consequence on environmental well-being. Hanjra and his colleagues [2] have purported it to be "a resource and a problem" due to its capricious importance in the environment. This is because, as it derives benefits in serving as an inexhaustible supply of water and nutrients for agriculture, so also does it contain toxic, recalcitrant, surreptitious microcontaminants and also serve as a habitat for disease vectors and pathogenic microorganisms, which deride its desirability. One salient reason for its demerits could be attributed to the design of most municipal wastewater treatment plants (WWTPs) which can only afford the almost utmost elimination of basic, regular organic pollutants. Despite the potential dangers most municipal wastewater are generally believed to pose, a careful exploitation of its microbial guild might serve as a treasure chest for environmentally astute bacteria of beneficial significance in the environmental and industrial applications.

WWTPs being physicochemically well defined, inimitable synthetic microbial ecosystems, could be considered as ideal mesocosms [3], since it is customary that they provide suitable environmental conditions to permit and preserve the copiousness of microbial populations, particularly, the environmentally astute species, within regular levels for an unabated running. The microbial consortia and associated guilds on the other hand, whose distribution patterns and metabolism are influenced by deterministic (niche-specific) and stochastic factors (unsystematic events) [4], as well as the type and modus operandi of the WWTP or geographic site [5,6], consequently play a pivotal role in the structure-strata routine, which may comprise biodegradation and mineralization or sequestration of heterogenous organic pollutants at basic nutrient limiting conditions, and ensure mineral and

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nutrient redistribution and cycling, and ultimately, ecosytem stability, through proliferation and interactions of vastly disparate microbial populations. Consequently, these routines may entail the secretion of highly desirable metabolites of biotechnological relevance, hence, there is little or no bewilderment that WWTPs afford the largest applications of bioprocess engineering apposite for municipal wastewater treatment.

One notable metabolite of the several secreted while bacteria go about their degradation and transformation procedure is 'laccase'(EC 1.10.3.2), which has gained prominence due to its resilience in an extensive mode of application, and its ecological friendliness, since it requires the readily available molecular oxygen as co-substrate for catalysis, and yields water as the sole byproduct, post-catalysis. Out of the growing number of laccase-producing bacteria species being discovered, the gammaproteobacteria are a distinctive class which are ubiquitously represented in many nutrient limiting, xenobiotic environments, where their degradative instincts are displayed. However, little is known about their distribution in different wastewater mesocosms of the WWTPs, and the role selective enrichment of wastewater could play in their extraction and speciation. Therefore, this study serves to give an early account of the strategic isolation of laccase producing gammaproteobacteria from different WWTP samples within the Eastern Cape, South Africa.

2. Materials and methods

2.1. Sampling sites and sample collection

Wastewater treatment facilities located in Adelaide (S32°42′343″S and 26°18′790″E), Seymour (32°32′873″S and 26°49′926″E), Dimbaza (32°51′274″S and 27°14′167″E), and Berlin (32°50′700″S and 27°37′049″E) were chosen for this study (Fig. 1). They are recipients of wastewater from domestic as well as industrial sewerages; except for Seymour which receives exclusively domestic sewage, and also has the lowest capacity of bulk volumes being treated, daily (0.25 mega litres). It was observed on sampling that the facilities available per WWTP were of modest sophistication and tertiary treatment was scarcely applied. The final effluents of the

respective treatment plants are usually discharged into adjacent tributaries which serves as a means of livelihood to the inhabitants of the suburbs. Samples were collected in pre-washed and autoclaved polythene bottles at wastewater treatment plants as influents, effluents as well as upstream and downstream sources around treatment plants in Adelaide, Dimbaza, Berlin and Seymour respectively, all within the Eastern Cape Province, South Africa. They were transported on ice to the AEMREG laboratory for further analysis within 6 h of collection.

2.2. Physicochemical evaluation of samples

Field equipments were appropriately calibrated according to manufacturers' instructions, parameters like temperature, pH, total dissolved solid (TDS), and dissolved oxygen (DO), were determined on-site with the aid of a multi-parameter ion specific meter (Hanna-BDH laboratory supplies). Concentrations of ortho-phosphate (PO_4) and nitrate (NO_3), and chemical oxygen demand (COD) were evaluated in the laboratory by standard photometric method using the Spectroquant Pharo 100 Photometer (Merck Pty. Ltd, South Africa). Prior to analysis, samples for COD analysis were digested with a thermoreactor (Model TR 300, Merck Pty. Ltd., South Africa), whereas, phosphate and nitrate were analyzed in the laboratory using standard methods as described by the supplier (Merck, VWR International, Poole, UK).

2.3. Selective enrichment

Samples were enriched following a modified method of Benslama and Boulahrouf [7]. About 5 mL of each vigorously shaken sample was added to 95 mL of microbiological saline supplemented with 1 g/L lignin, 0.02 g/L potassium hydrogen phthalate (PHP) and 0.02 g/L vanillin respectively at 30 °C under shaking condition (140 rpm) for seven days. Thereafter, 1 mL aliquots were serially diluted five folds in microbiological saline under aseptic conditions and a 500 μL volume was spread on plates of the following chemical composition:

Medium 1 (g/L): Agar; 13.0, Kraft lignin; 1, $NaNO_3$; 2.6, K_2HPO_4; 0.4, KH_2PO_4; 0.6, $MgSO_4.7H_2O$; 0.5, NaCl; 0.5. Medium 2 (g/L):
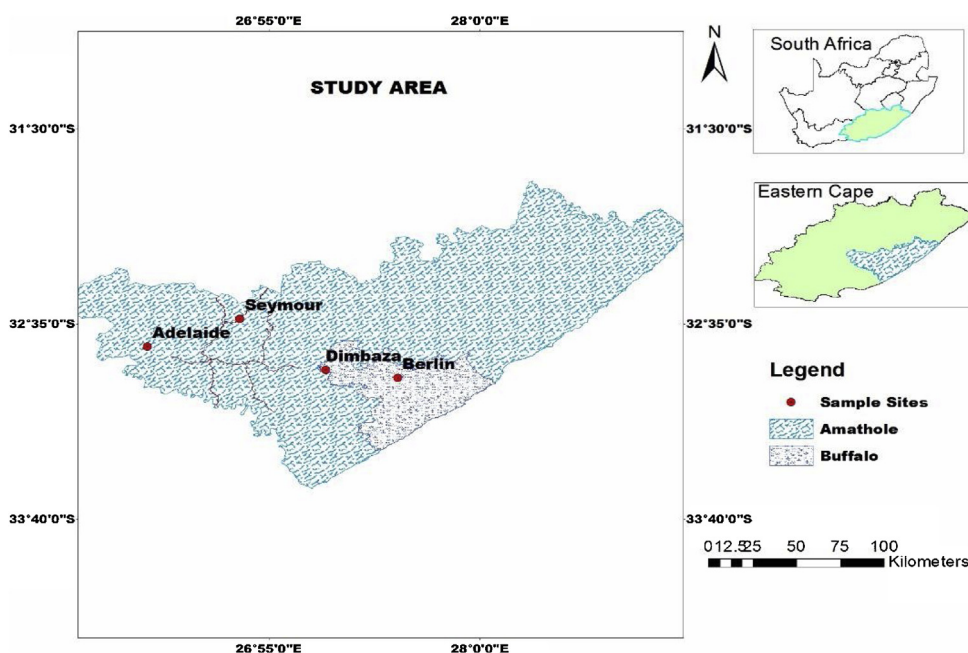

Fig. 1. Map showing the location of the wastewater treatment plants (www.demarcation.org.za).

Agar; 13.0, Potassium hydrogen phthalate; 0.04, $NaNO_3$; 2.6, K_2HPO_4; 0.4, KH_2PO_4; 0.6, $MgSO_4.7H_2O$; 0.5, NaCl; 0.5. Medium 3 (g/L): Agar; 13.0, Vanillin; 0.04, $NaNO_3$; 2.6, K_2HPO_4; 0.4, KH_2PO_4; 0.6, $MgSO_4.7H_2O$; 0.5, NaCl; 0.5. The plates were incubated at 30 °C for seven days and distinct colonies were labelled, streaked out to ensure purity and stored at 4 °C.

2.4. Screening for laccase positive isolates

Isolates with positive activity from preliminary screening were pooled and subjected to comprehensive qualitative determination of laccase activity using a medium for lignin modifying enzymes (LME) as described by Pointing [8], with slight modifications. The medium comprised (g/L) agar; 13.0, Yeast Extract; 0.01, KH_2PO_4; 1.0, $KNaC_4H_4O_6.4H_2O$; 0.5, $MgSO_4.7H_2O$; 0.5, $CaCl_2.2H_2O$; 0.01, $CuSO_4.5H_2O$; 0.001, $FeCl_3.7H_2O$; 0.001, $MnSO_4.H_2O$. One litre each of this medium was supplemented with 0.02% w/v 2,2 azino-bis -(3-ethylbenzothiazoline 6 sulphonic acid) (ABTS), syringaldazine and guaiacol, 0.3% w/v lignin and 0.005% w/v α-naphthol and were autoclaved at 121 °C for 15 min. Furthermore, 10% w/v separately autoclaved aqueous glucose solution was aseptically added to the different media and were thereafter poured in Petri plates. The presumptive laccase-positive isolates were inoculated on the plates and then incubated at 30 °C. Colour appearances in the medium indicated laccase activity viz; 2,2 azino-bis –(3-ethyl-benzothiazoline 6 sulphonic acid) (ABTS) (green), syringaldazine (shade of pink), α-naphthol (bluish to violet), guaiacol (reddish brown) while non green portions of a green-stained plate due to flooding with equal parts of $FeCl_3$ and ${K_3[Fe(CN)_6]}$ solutions sufficed for ligninolytic activity on lignin-ammended plates.

2.4.1. Quantitative screening

Quantitative screening was carried out in two modes, in order to compare their potentials to catalyze the oxidation of the model compounds evaluated in our study: (i) the direct inoculation of cell pellets in broths containing the substrates, and (ii) the use of cell-free culture supernatants for laccase assay. Choice isolates were grown in nutrient broth, harvested and twice washed in sterile standard microbiological saline solution, and thereafter standardized with 0.5 McFarland solution. For the first mode, 50 μL inocula were aseptically transferred into 400 μL media, with cell free broths as control, and they were read at 96 h using wavelegnths requisite for each oxidation process (ABTS; 420 nm, Syringaldazine; 530 nm, α-naphthol; 520 nm, Guaiacol; 470 nm; potassium ferrocyanoferrate (II) $[K_4(FeCN_6)]$ (PFC); 420 nm. For the second mode, 150 μL standardized inocula were aseptically transferred into individual 60 mL basal salt media containing (g/L) glucose; 5.0, Yeast Extract; 2.0, KH_2PO_4; 0.8, K_2HPO_4; 0.5, $CaCl_2.2H_2O$; 0.01, $CuSO_4.5H_2O$; 0.001, $MnSO_4.H_2O$; 0.15, $MgSO_4.7H_2O$; 0.5, $KNaC_4H_4O_6.4H_2O$; 0.5, vanillin; 0.02. The flasks were incubated at 30 °C, 140 rpm for 96 h. Subsequently, 1.5 mL aliquots of samples were harvested post-incubation, centrifuged at 15,000 rpm for 12 min and varying aliquots were pipetted out for laccase activity.

2.5. Enzyme activity and substrate specificity assay

Laccase-catalyzed oxidations of ABTS, guaiacol, syringaldazine (SGZ), α-naphthol and PFC in a phosphate buffer (100 mM; pH6) were monitored with a Synergy MX microtitre plate reader (BioTek™) where their respective activities were estimated at 420 nm ($\varepsilon = 36{,}000$ M^{-1} cm^{-1}), 470 nm ($\varepsilon = 26{,}600$ M^{-1} cm^{-1}), 530 nm ($\varepsilon = 65{,}000$ M^{-1} cm^{-1}), 520 nm ($\varepsilon = 57{,}490$ M^{-1} cm^{-1}) and 420 nm ($\varepsilon = 1023$ M^{-1} cm^{-1}). Briefly, 2 mM substrates in pH 6 potassium phosphate buffer were incubated at room temperature with 100 μL crude laccase, except for ABTS which was reacted with 50 μL of appropriately diluted laccase at 30 °C, allowed to react for

10 min and thereafter stopped with 40 μL 20% TCA. The control contained all reaction conditions, except that the crude laccase was replaced with a thermally deactivated enzyme. One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 μmol of substrates per minute under their respective conditions. Specificity studies was conducted under the same experimental conditions except that 2,6 – Dimethoxyphenol (468 nm; $\varepsilon = 49{,}600$ M^{-1} cm^{-1}) and pyrogallol (510 nm; $\varepsilon = 4400$ M^{-1} cm^{-1}) were added to the list of substrates respectively.

2.6. Molecular Identification and phylogenetic analysis of laccase positive isolates

Axenic bacterial cultures which had emerged best laccase producers had their genomic DNA extracted using the ZR Fungal/ Bacterial DNA kit™ (Zymo Research). Their 16S target region was amplified using Dream Taq DNA polymerase (Thermo Scientific™) and the universal primers, 27F: 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1429R: 5′-CGGTTACCTTGTTACGATT-3′. Amplicons obtained from PCR run were gel extracted (Zymo Research, Zymoclean™ Gel DNA Rocover Kit), and sequenced in the forward and reverse directions on the ABI PRISM™ 3500xl Genetic Analyzer. Purified sequencing products (Zymo Research, ZR-96 DNA sequencing clean-up kit™) were analyzed using CLC Main Workbench 7 followed by a BLAST search (NCBI database) for most probable strain identification. Thereafter, sequences were submitted to Genbank for accession numbers, while the Molecular Evolutionary Genetics Analysis software (MEGA 7) was used to elucidate the phylogenetic relationships between isolates per aquatic milieu, with reference to isolates in the NCBI library.

3. Results and discussion

Wastewater treatment plants (WWTPs) may have been designed from an engineering inclination and perspective, nonetheless, their biological systems cannot be overlooked since they actively contribute to the efficiency of wastewater treatment. However, specifically speaking, only resilient species of micro-organisms, which have over a considerable period of time acclimatized to the physico-chemical status of their substratum, could thrive where survival entails the metabolism of unorthodox forms of nutrients, concomitant with the secretion of some unspecified enzymes (peroxidases, oxidases, hydrolases etc.) or cofactors during their microbial transformation [9]. Consequently, there might be a decrease in the indwelling bacterial diversity, albeit this study was not intently designed to evaluate the bacterial diversity in the wastewater community. Therefore, this investigative study was premised on the hypothesis that selected WWTPs in the Eastern Cape Province, South Africa might serve as repositories of proteobacteria, which are able to secrete environmentally resilient laccases.

3.1. Physicochemical properties of wastewater samples

A list of regulatory guidelines for the physicochemical properties of wastewater has been compiled by an earlier study in our research group [10], which is congruent with the limits (general and special) established by The National Water Act, Republic of South Africa in 1998.

Wastewater qualities highlighted in Table 1 suggest that almost all the treatment plants conformed to set guidelines for most parameters, except for dissolved oxygen (DO) and chemical oxygen demand (COD). The non-compliance of some WWTPs in the Eastern cape with set standards for DO and COD has been consistently reported earlier by investigators in our research group [11,12]. The disquieting statistics portrayed by these two

Table 1
Physicochemical properties of the samples collected from different wastewater treatment plants.

Location	Sample	pH	Temp	TDS	DO	COD	Phosphate	Nitrate
Adelaide	Primary effluent	7.13	23.2	220.0	3.11	62.0	1.6	8.8
(Industrial & Domestic)	Downstream	7.67	21.9	116.9	7.96	87.3	1.0	4.7
Berlin (Industrial & Domestic)	Influent	7.14	22.0	368	0.38	ND	ND	ND
Dimbaza (Industrial & Domestic)	Secondary effluent	6.36	17.87	0.12	0.08	73	9.12	15.5
Seymour (Domestic)	Influent	8.10	21.9	223.6	6.6	80.0	4.8	12.7

ND = not determined.

parameters suggest a liable increase in the sample's organic matter content [13]. Furthermore, this outcome might foreshadow two scenarios: the elevated levels of nutrients in the wastewater, and the associated presence of toxic compounds, which might as well include biologically active compounds, since their outfalls are mostly heterogenous, i.e. domestic and industrial setting.

Although the evaluation of presence, nature and possible abundance of putative aromatic pollutants in the abstracted samples was outside the scope of this investigation, further studies, which are necessary, should be conducted to answer the rising curiosity on the presence of aromatics cocktails in the named WWTPs. Notwithstanding, we can hypothetically surmise that the wastewater constitutents might not only instigate a shift in bacterial composition in the matrix, but also decreases their diversity and species opulence. This might be supportive of the phenomenon of biotic homogenization, which purports human modifications of the environment (in our case, the WWTP mesocosm) could lower the phylogenetic variations in any given ecosystem [14].

3.2. Potent bacterial biodegraders of aromatics

Isolates were selected based on their ability to utilize all supplemented model aromatic substrates; potassium hydrogen phthalate (PHP), vanillin and lignin) as sole sources of nutrient and energy, thereby suggesting their degradation proficiency (Table 2). The model aromatic compounds were chosen based on their analogy to contaminants implicated in municipal wastewater.

It was observed that about 39 isolates (72%), were considered active degraders based on their ability to utilize at least two of the model aromatic substrates supplemented, while the excellent degraders (22%) utilized all three subsrates employed (Fig. 2).

This categorization however could be based on their chemo-tactic responses to the respective compounds, which had been identified as an important determinant in the biodegradation efficiency of bacterial strains [15]. Although genomic evaluation has disclosed that a considerable large portion of bacterial genomes are occupied by chemotaxis genes [16], the phenomenon, 'chemotactic responses' is mostly applicable to motile bacterial species, mostly pseudomonads and associated nonenteric bacteria in aquatic matrices. It could either be metabolism-dependent, in which chemotactic behaviour is only exhibited toward metaboliz-able or co-metabolizable xenobiotic aromatic compounds, or metabolism-independent, where chemotactic behaviour is dis-played notwithstanding the presence of more propitious metabo-lizable compounds in the surrounding medium [17]. Furthermore, chemotactic responses could also be exhibited as chemoattraction or chemorepellation and with respect to the aromatics employed in our study, it could be deduced that all the isolates exhibited metabolism-independent chemotaxis, since they contained (to some extent) traces of nutrients made available in their natural environment from which they were abstracted, prior to their introduction to a new medium.

However, based on the potential toxicity of aromatic com-pounds to microbial life, owing to their ability to liquefy in the

bacterial membrane thereby instigating its disorientation and may ultimately cause mortality [18], tactic responses such as chemo-attraction or chemorepellation, which attracts microorganisms to a particular carbon source or repels them therefrom have been imparted by diverse bacterial species to manage the presence of toxic compounds [19]. This therefore explains our outcome, sequel to selective enrichment, however, other possible reasons could be: suboptimal levels of expression of the enzymes and cofactors of the degradation pathways, and the physical state of the examined chemical compound [20]. Bearing these in mind, it should be considered that some chemicals, whilst being chemoattractants for a particular bacterial species may not necessarily be so for another [21]. The likely chemoattraction, which was proposed in this study coincides with earlier investigations on benzoic and chlorobenzoic acids [21], chloronitroaromatics and explosives [22], and herbicides [23], just as our chemorepellation claim, is consistent with Ortega-Calvo et al. [24].

Further categorization of bacterial isolates based on their wastewater environment showed that influent samples accounted for the highest amount of heterotrophic isolates (52%), as well as active (59%) and excellent (41%) biodegraders (Fig. 3).

The secondary effluent was next ranked in terms of abundance of excellent biodegraders (25%), although this outcome was not from as many isolates as that of the primary effluent. However, it was observed that the number of viable and culturable bacteria reduced on successive sampling along the course of the WWTP. Ye and Zhang [25], likewise observed that bacteria diversities in different WWTP samples varied, as well as their viable counts, given that bacterial populations in the influent may not thrive in activated sludge [26], or may be removed during the sedimenta-tion process. Although pyrosequencing or any other related molecular analysis had not been conducted at this stage in our investigation, we presumed that only the free-swimminmg bacteria population might be able to maneuver their way into successive communities. This may imply that wastewater charac-teristics, mode of handling and geographic location indeed dictate the changes between particular bacterial populations [5,6].

3.3. Laccase-producing bacteria

Our rationale behind the screening for laccase production amongst the enzyme batteries that have been so far implicated in the treatment of organic and aromatic contaminants in wastewater was based on the premise that its application and mechanism of action is less complicated due to its requirement of readily available atmosheric O_2 as the vehicle for reaction and the release of H_2O molecules as sole by product during its oxidative transformation of a range of aromatic and inorganic substrates, while the assessment of isolate proficiency of laccase production was to painstakingly sift out isolates that could produce laccases with broad substrate specificity as this will, to some extent, treat the heterogenous aromatic pollutants in wastewater. Kiiskinen et al. [27] employed this method when they were screening for novel laccase-producing fungi, although they included a tradition-al laccase screening compound, tannic acid as part of their

Table 2
Evaluation of degradation potentials of isolates from WWTP samples based on their ability to metabolize lignin, vanillin and phthalate in selectively enriched media.

Location	Sample	Isolate Code	Lignin	Vanillin	Phthalate
Adelaide (Industrial and Domestic)	Primary effluent	$AP_2{}^1a_2$	–	+	+
		$AP_2{}^1a_3$	–	–	+
		$AP_2{}^1a_{12}$	+	–	+
		$AP_2{}^1a_{13}$	+	–	–
		$AP_2{}^1a_{14}$	+	–	+
		$AP_2{}^1a_{15}$	+	+	+
		$AP_2{}^1a_{16}$	+	+	–
		$AP_2{}^1b_1$	–	+	+
		$AP_2{}^1b_2$	+	–	+
		$AP_2{}^1b_3$	+	+	+
		$AP_2{}^1b_4$	+	–	+
	Downstream	**$AP_4{}^2c_1$**	+	+	+
		$AP_4{}^2c_2$	–	+	–
		$AP_4{}^2c_3$	+	+	–
		$AP_4{}^2c_4$	–	–	+
		$AP_4{}^2c_6$	–	–	+
		$AP_4{}^2c_7$	+	+	+
		$AP_4{}^2c_8$	–	+	–
Berlin (Industrial and Domestic)	Influent	$Berl1^1a_1$	–	+	+
		$Berl1^1a_2$	–	+	+
		$Berl1^1a_3$	–	+	+
		$Berl1^1a_4$	+	–	+
		$Berl1^1a_5$	+	+	–
		$Berl1^1a_6$	+	–	+
		$Berl1^1a_7$	–	+	+
		$Berl1^1a_8$	–	+	–
		$Berl1^1b_1$	–	–	+
		$Berl1^1b_1$	+	+	–
		$Berl1^1b_2$	+	+	+
		$Berl1^1b_3$	–	+	+
		$Berl1^1b_4$	–	+	–
		$Berl1^1c_1$	+	+	+
		$Berl1^1c_2$	+	+	+
		$Berl1^1c_3$	+	–	+
		$Berl1^1c_4$	–	+	+
		$Berl1^1c_6$	+	–	+
Dimbaza (Industrial and Domestic)	Secondary effluent	**$Dbza_3$**	+	+	+
		$Dbza_4$	+	+	+
		$Dbza_5$	–	–	+
		$Dbza_6$	+	–	+
		$Dbza_7$	+	+	+
		$Dbzb_1$	–	+	–
		$Dbzb_3$	+	–	–
		$Dbzb_5$	+	–	+
Seymour (Domestic)	Influent	$SY1b$	–	+	+
		$SY1b_1$	+	+	–
		$SY1b_6$	–	+	+
		$SY1b_2$	+	–	+
		$SY1b_4$	+	–	+
		$SY1b_5$	–	+	+
		$SY1c_1$	–	+	–
		$SY1c_2$	+	+	+
		$SY1c_3$	+	–	–
		$SY1c_4$	+	+	+

Fig. 3. Overall percentage distribution of aromatics degrading bacteria from different wastewater environments. The concentric outline represents the total number of isolates (outer circle), the active biodegraders (middle circle), and the excellent biodegraders (inner circle). Fractions with shades of a certain color are grouped as the same wastewater environment.

substrates. Therefore, production proficiency of the isolates was judged based on the ability of isolates to elicit the colour development or halos of oxidation on at least three of the substrates evaluated (Fig. 4), and the degradation of syringaldazine in particular, since it is widely recognized as a laccase specific substrate [28].

According to Table 3, 10 of the 12 isolates screened yielded positive results on at least three substrates tested. Furthermore, as observed in the laccase positive isolates in Fig. 4, only the lignin based screening (PFC) showed an extensive halo around the colony as opposed to the reaction on other plates. Bearing this in mind, and in line with the critical opinions of Wang et al. [29,30], it was supposed that the oxidation zones, which were just about the size of the colony, might be evident of mineralization of the substrates, rather than cometabolism, since the substrates were in their monomeric units and were probably oxidized to yield less organic products and CO_2, as compared to lignin, a heterogenous polymer consisting of an assortment of phenolic and aromatic units, which on oxidation might elicit the production of diverse organic compounds that may further initiate other nonenzymatic reactions along the course of the agar base. Therefore, substrates of known lower molecular weights may be mineralized at a given concentration, whereas higher molecular weights might only be cometabolized, or mineralized at much slower rates. Furthermore, such action could be attributed to the chemotactic response of the isolates on their growth substratum as demonstrated by Zhang et al [31].

Contrariwise, the lignin plate flooding with potassium ferric cyanide was meant to bind to the phenolic fraction of undegraded lignin thereby staining it blue-green. Consequently, halo formation would mean that the phenolic component of that part has been oxidized [8]. However, it is noteworthy that the principle behind this particular screening is that the secretion of laccase only initiates the reaction by splitting the chemical bonds of lignin, all other reactions thereafter might be non-enzymatic.

Quantitation assays of cell-free culture supernatant and axenic cultures (Fig. 5a-d) were intended for comparative monitoring of substrate oxidation or transformation, and also prefigure their suitability for some time-related real life environmental applications. It was revealed that the culture supernatant, that is, our supposed laccase, elicited higher yields of measurable laccase activity on all substrates assayed, when compared to the axenic cultures. Furthermore, the non-phenolic substrates (ABTS and PFC) were more rapidly oxidized than their phenolic counterparts (guaiacol, syringaldazine and 1-naphthol). The readiness of oxidation of the substrates by crude laccase may be ascribed to its unabated production of copious volumes, since it was produced
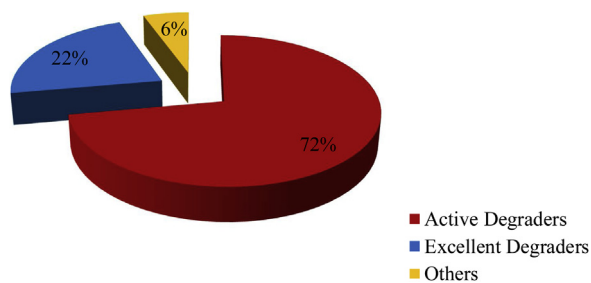

Fig. 2. Distribution of Isolates based on their ability to degrade model aromatic compounds: lignin, vanillin, PHP. Active degraders utilized at least two substrates while excellent degraders utilized all three model compounds.
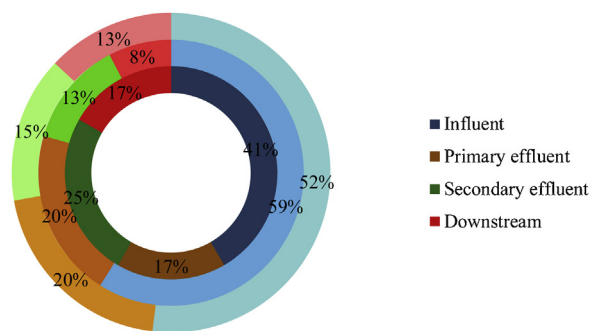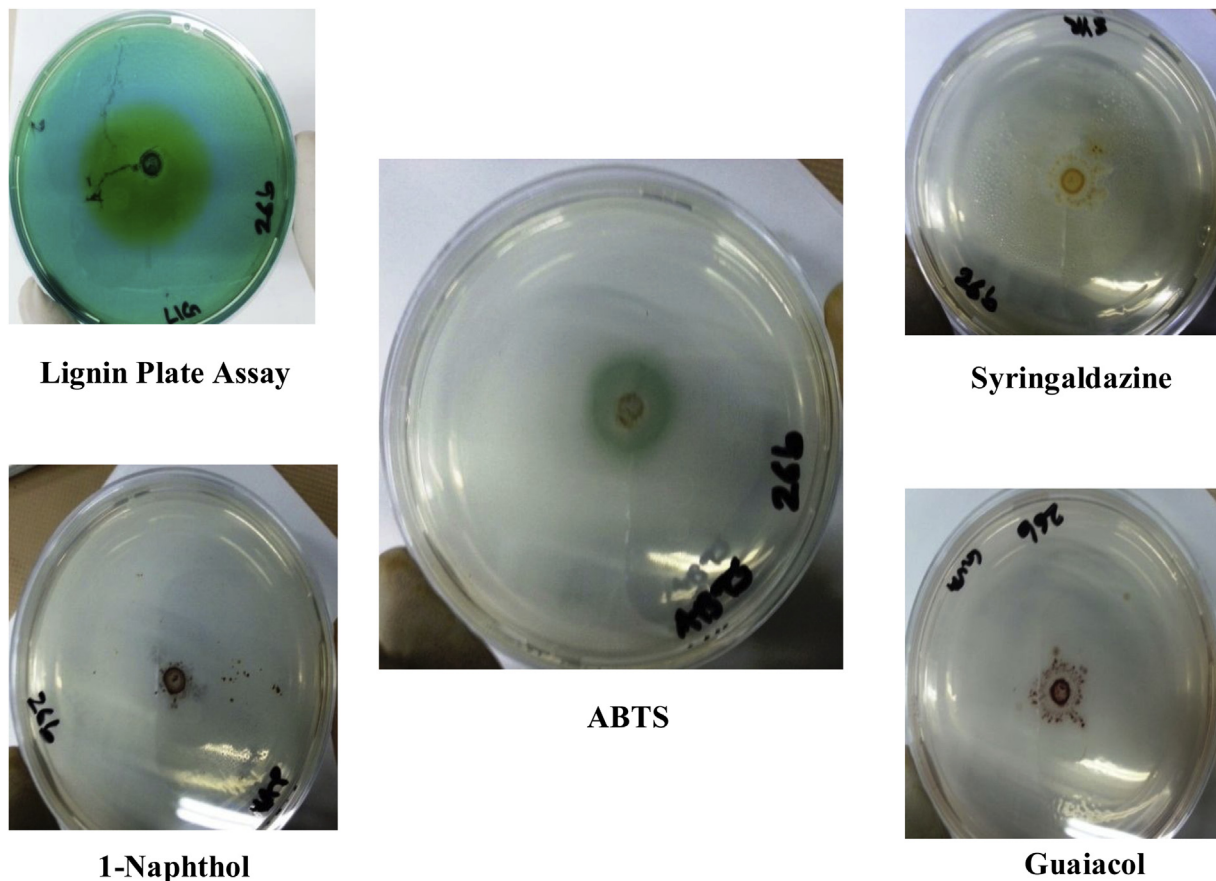
Fig. 4. The degradation of different laccase substrates by ligninolytic bacteria with their corresponding chromogenic reactions.

in a medium that delivered a simpler carbon source, glucose, coupled with the depletion of nitrogen in the spent broth. Nitrogen limiting conditions could elicit a greater laccase yield [32]. Although in our time controlled investigation the cell-free culture supernatant catalyzed a higher reaction turnover, which has been ascribed to the availability of simple nutrients. This might not be entirely so, especially from perspective of real life detoxification of aromatic and inorganic pollutants analogous to the model compounds evaluated. This is because a biostimulation of the matrix intended for detoxification with nutrients might produce an overshoot in reaction turnover, when compared to the culture fitrates, due to the likelihood of possessing a broader metabolic capacity and synergy.

Table 3
Assessment of selected aromatics degrading bacteria for laccase activity.

Isolate Code	Lignin Plate Assay	Guaiacol	Syringaldazine	ABTS	1-Naphthol
$AP_2^1a_{15}$	+	+	+	+	+
$AP_2^1b_3$	+	+	+	–	+
$AP_4^2c_1$	+	–	–	na	–
$AP_4^2c_7$	+	+	+	+	+
$Berl1^1b_2$	+	+	+	+	+
$Berl1^1c_1$	–	+	+	na	+
$Berl1^1c_2$	+	+	+	+	+
$Dbza_3$	+	+	+	na	–
$Dbza_4$	+	+	+	–	+
$Dbza_7$	+	+	+	–	+
SY_1c_2	+	+	+	na	+
SY_1c_4	–	+	+	–	–

na = not accessible.

The preference of nonphenolic substrates over the phenolic ones could be because they are electron rich nucleophiles, from which electrons are readily asbtracted to generate stable cation radicals almost instataneously [33]. The predilection of laccases for electron transfer has afforded them the use of inorganic substrates like iodide [34] and potassium ferrocya-noferate (II) [35]. Three isolates (Dbza$_4$, Berl1^{1}b$_2$ and AP$_2^1$a$_{15}$) were selected based on their relative performances in the quantitative assays performed (Fig. 5a-d). Ultimately, the biotechnological prospects of the secreted laccases were pronounced after comparison of their respective activities (Dbza$_4$; cca. 7011 U/L, Berl1^{1}b$_2$; cca. 6938 U/L, AP$_2^1$a$_{15}$; cca.5758 U/L) with notable results such as the commercial *Trametes versicolor* laccases, which had maximum activities within the range of 508–945 U/L as reported by Margot et al. [36], as well as the 20–30 U mg^{-1} correspondingly reported by Ihssen et al. [37] on the same fungus. Furthermore, the economy of laccase production reported in this present study make them a desirable means for largescale real life applications since they encourage the adoption of aromatic pollutants as inducers, thereby assuaging the harmful effects of these sinister compounds to natural environments when the spent wastewater based media are discharged.

3.4. Phylogenetic relationship among the laccase-producing bacteria

Analysis of 16 S rRNA sequences disclosed that they shared similarities with *Pseudomonas aeruginosa* (99%), *Stenotrophomonas maltophilia* (99%) and *Pseudomonas mendocina* (97%) respectively. Their nucleotide sequences have however been deposited in
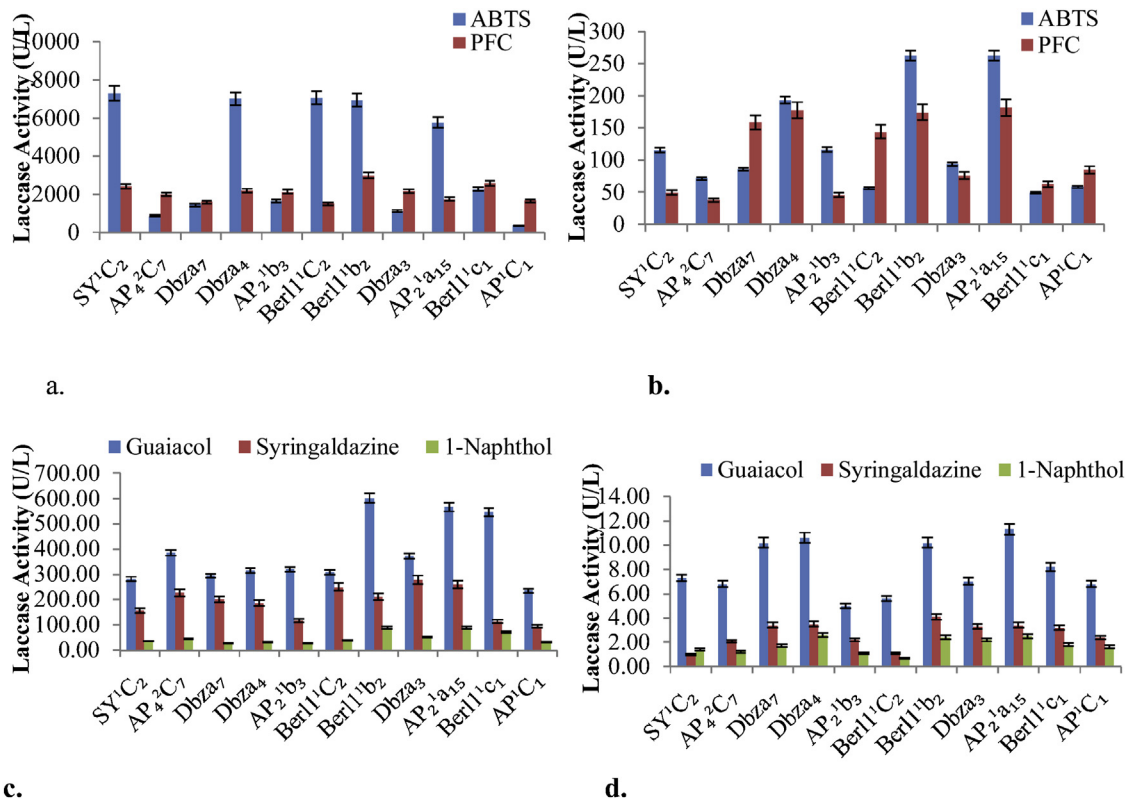
Fig. 5. Quantitative evaluation of wastewater isolates for laccase production (a&c represent assays using cell-free culture supernatant whereas b&d represent assays using cell biomass).

GenBank under the accession numbers MF073264, MF073263 and MF073256 as *Pseudomonas aeruginosa* DEJ16, *Stenotrophomonas maltophila.* BIJ16 and *Pseudomonas mendocina* AEN16 correspondingly. Therefore, a phylogenetic tree was constructed to compare the relationship of selected laccase producers in this study to other laccase-producing bacteria (Fig. 6).

The evolutionary history was determined using the neighbour joining method using the stated scale. Furthermore, the evolutionary distances were computed using the Maximum Composite Likelihood method with the analysis involving 29 nucleotide sequences; evolutionary analysis were conducted in MEGA7 [38].

The molecular identification of our choice isolates classify them as proteobacteria, a diverse group of bacteria, which have been implicated in a lot of aquatic matrices, including wastewater [25]. Specifically, the gammaproteobacteria have been associated with the removal of micropollutants in wastewater [26,39]. This could be through the secretion of a *mélange* of enzymes, however, the role played by laccase cannot be overemphasized. Studies on Pseudomonas species have highlighted their importance in the treatment some of the putative contaminats in wastewater [40,41].

Conversely, *Stenotrophomonas maltophilia* has been consistently reported for its antibiotic resistance [42]. Hence, it is not surprising that it was found in the influent of domestic discharge in our study, perhaps it indicates the imbalanced patronage of pharmaceuticals and personal care products (PPCPs) among the residents of the adjacent suburb. Notwithstanding, their extraordinary range of activities have made them somewhat indispensable in the breakdown of natural and anthropogenic pollutants [43,44]. Furthermore, laccase from *Stenotrophomonas maltophilia* have been shown to be of benefit in environmental cleanups [45].

3.5. Substrate specificity of the laccases

A brief evaluation of the substrate specificities of the laccases secreted by the gammaproteobacteria included two additional substrates, 2,6- Dimethoxyphenol (DMP) and pyrogallol (Fig. 7).

Results showed that Berl1[1]b[2] *Stenotrophomonas* sp. BIJ16 elicited the highest activity on the non-phenolic substrates, with an estimate of 600 U/mL on ABTS, and cca. 400 U/mL on PFC, though laccase ouputs by the other bacteria screened were in recognizable limits. This outcome confirms its tenacity and predilection for bioremediation of pollutants. However, amongst the phenolic substrates assayed, pyrogallol presented the highest oxidation coefficient ($\leq$ cca. 250 U/mL), followed by DMP. This could be becuase of their respective solubilities, structural conformations, substitution patterns, assay pH and temperature, and other conditions that might affect the enzyme kinetics and substrate transformation. Comparative studies, using commercial laccases, showed that ABTS catalytic oxidation was 1.3-, 2- and 12-times more than DMP, syringaldazine and guaiacol, respectively, across certain pH regimes [36], at individual optima [46]. Correspondingly, ABTS and syringaldazine were observed to present high affinities and catalytic efficiencies in other studies conducted with bacterial laccases [47,48]. Riess et al. [49] discovered that substitution patterns of phenols is linked to laccase activity, and the rate of oxidation could also be ascribed to disparities in redox or ionization energy [50,51]. Overall, with respect to substrate specificity, our findings that the non-phenolic substrates are more readily oxidized than their phenolic equivalents, and the variations in affinities among the phenolic substrates are congruent with various studies [35,52–54].
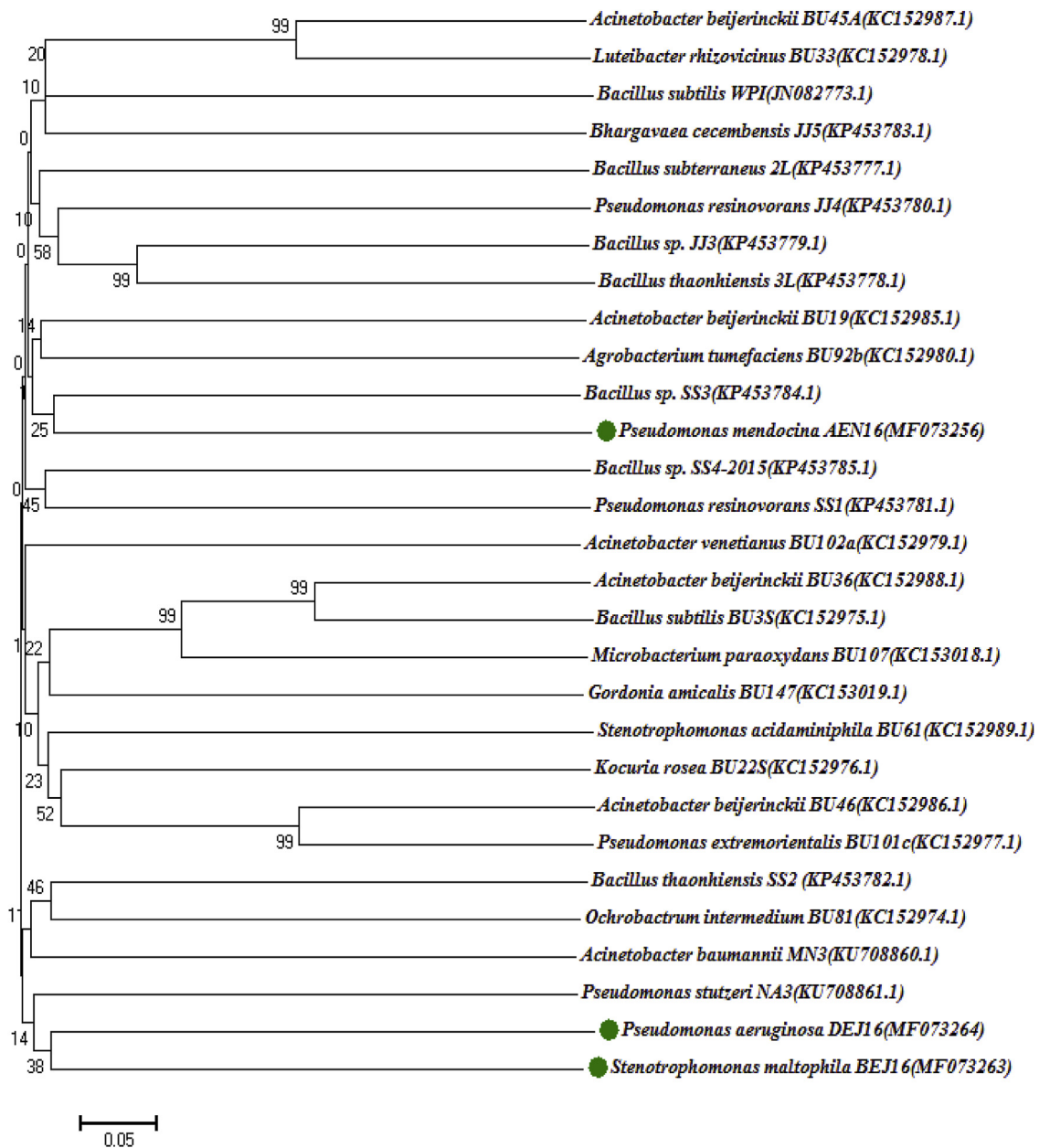
Fig. 6. Phylogenetic tree showing the evolutionary relationships between laccase producing isolates and their related bacterial species. Isolates reported in the study are labelled with green tips.
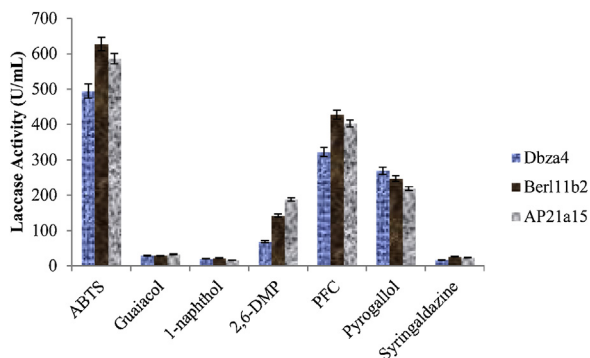

Fig. 7. Laccase substrate specificity of selected wastewater isolates.

4. Conclusions

Wastewater samples proved to be resourceful in producing active biodegraders which belong to the phylum proteobacteria. The selective enrinchment of the samples helped to adapt the gammaproteobacteria to screening substrates tested. However, with the ominous activities displayed by the bacteria studied on some substrates, a good knowledge of the metabolic capacity of the natural environments from which the isolates are abstrated would be a good feat. Hence, future studies should be channelled towards the detection of metabolic diversities and pathways responsible for the degradation of related aromatic toxicants in wastewater matrices through XeDetect-mediated MetaCyc analysis [39], as this would inform investigators on the most apposite cultural method for a seamless laccase producing bacteria.

The presence of a supposedly antibiotic resistant isolate should be a caveat on the mode of handling and consumption of pharmaceuticals. *Stenotrophomonas maltophila* BIJ16, though skeptically accepted due to its clinical implications, if mishandled, presented the best statistics among its counterparts. We hereby submit that it is not only apposite for environmental applications, but could also be an invaluable tool in the advancement of industrial bioeconomy. Therefore, further intensive exploration of the wastewater mesocosms is suggested.

Conflict of interest

The authors have declared no conflict of interest

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References

[1] E. Corcoran, C. Nellemann, E. Baker, R. Bos, D. Osborn, H. Savelli (eds), Sick Water? The Central Role of Wastewater Management in Sustainable Development. A Rapid Response Assessment UNEP/UNHABITAT, (2010) .

[2] M.A. Hanjra, J. Blackwell, G. Carr, F. Zhang, T.M. Jackson, Wastewater irrigation and environmental health: implications for water governance and public policy, Int. J. Hygiene Environ. Health 215 (2012) 225–269.

[3] H. Daims, M.W. Taylor, M. Wagner, Wastewater treatment: a model system for microbial ecology, Trends Biotechnol. 24 (2006) 483–489.

[4] I.D. Ofiteru, M. Lunn, T.P. Curtis, et al., Combined niche and neutral effects in a microbial wastewater treatment community, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 15345–15350.

[5] A. Cydzik-Kwiatkowska, M. Zielińska, I. Wojnowska-Baryła, Impact of operational parameters on bacterial community in a full-scale municipal wastewater treatment plant, Pol. J. Microbiol. 61 (1) (2012) 41–49.

[6] J. Ma, Z. Wang, Y. Yang, et al., Correlating microbial community structure and composition with aeration intensity in submerged membrane bioreactors by 454 high-throughput sequencing, Bioresour. Technol. 179 (2013) 436–443.

[7] O. Benslama, A. Boulahrouf, Isolation and characterisation of glyphosate-degrading bacteria from different soils of Algeria, Afr. J. Microbiol. Res. 7 (49) (2013) 5587–5595.

[8] S.B. Pointing, Qualitative methods for the determination of lignocellulolytic enzyme production by tropical fungi, Fungal Divers. 2 (1999) 17–33.

[9] K. Fischer, M. Majewsky, Cometabolic degradation of organic wastewater micropollutants by activated sludge and sludge-inherent microorganisms, Appl. Microbiol. Biotechnol. 98 (15) (2014) 6583–6597.

[10] O. Osuolale, A.I. Okoh, Assessment of the Physicochemical Qualities and Prevalence of *Escherichia coli* and Vibrios in the final effluents of two wastewater treatment plants in South Africa: ecological and public health implications, Int. J. Environ. Res. Pub. Health 12 (2015) 133999–134412.

[11] M.N.B. Momba, A.N. Osode, M. Sibewu, The impact of inadequate wastewater treatment on the receiving water bodies – case study: Buffalo City and Nkokonbe Municipalities of the Eastern Cape Province, Water Sa 32 (2009) 687–692.

[12] E.E.O. Odjadjare, A.I. Okoh, Physicochemical quality of an urban municipal wastewater effluent and its impact on the receiving environment, Environ. Monit. Assess. 170 (2010) 383–394.

[13] T. Verma, P.W. Ramteke, S.K. Garg, Quality assessment of treated tannery wastewater with special emphasis on pathogenic E. coli detection through serotyping, Environ. Monit. Assess. 145 (2008) 243–249.

[14] B. Drury, E. Rosi-Marshall, J.J. Kelly, Wastewater treatment effluent reduces the abundance and diversity of benthic bacterial communites in urban and suburban rivers, Appl. Environ. Microbiol. 79 (6) (2013) 1897–1905.

[15] J. Lacal, J.A. Reyes-Darias, C. Garcia-Fontana, J.-L. Ramos, T. Krell, Tactic responses to pollutants and their potential to increase biodegradation efficiency, J. Appl. Microbiol. 114 (2012) 923–933.

[16] K. Wuichet, I.B. Zhulin, Origins and diversification of a complex signal transduction system in prokaryotes, Sci. Signall. 3 (128) (2010) ra50.

[17] G. Pandey, R.K. Jain, Bacterial chemotaxis toward environmental pollutants: role in bioremediation, Appl. Environ. Microbiol. 68 (12) (2002) 5789–5795.

[18] J.L. Ramos, E. Duque, M.T. Gallegos, P. Godoy, M.L. Ramos-Gonzalez, A. Rojas, W. Teran, A. Segura, Mechanisms of solvent tolerance in gram-negative bacteria, Annu. Rev. Microbiol. 56 (2002) 743–768.

[19] G. Vardar, P. Barbieri, T.K. Wood, Chemotaxis of *Pseudomonas stutzeri* OX1 and *Burkholderia cepacia* G4 toward chlorinated ethenes, Appl. Microbiol. Biotechnol. 66 (2005) 676–701.

[20] J. Hanzel, H. Harms, L.Y. Wick, Bacterial chemotaxis along vapor-phase gradients of naphthalene, Environ. Sci. Technol. 44 (2010) 9304–9310.

[21] V. Tremaroli, C. Vacchi Suzzi, S. Fedi, H. Ceri, D. Zannoni, R.J. Turner, Tolerance of *Pseudomonas pseudoalcaligenes* KF707 to metals, polychlorobiphenyls and chlorobenzoates: effects on chemotaxis-, biofilm- and planktonic-grown cells, FEMS Microbiol. Ecol. 74 (2010) 291–301.

[22] J. Pandey, N.K. Sharma, F. Khan, A. Ghosh, J.G. Oakeshott, R.K. Jain, G. Pandey, Chemotaxis of *Burkholderia* sp. Strain SJ98 towards chloronitroaromatic compounds that it can metabolise, BMC Microbiol. 12 (2012) 19.

[23] X. Liu, R.E. Parales, Bacterial chemotaxis to atrazine and related s-triazines, Appl. Environ. Microbiol. 75 (2009) 5481–5488.

[24] J.J. Ortega-Calvo, A.I. Marchenko, A.V. Vorobyov, R.V. Borovick, Chemotaxis in polycyclic aromatics hydrocarbon-degrading bacteria isolated from coal-tar and oil-polluted rhizospheres, FEMS Microbiol. Ecol. 44 (2003) 373–381.

[25] L. Ye, T. Zhang, Bacterial communities in different sections of a wastewater treatment plant revealed by 16 rDNA 454 pyrosequencing, Environ. Biotechnol. 97 (2013) 2681–2690.

[26] N.M. Shchegolkova, G.S. Krasnov, A.A. Belova, A.A. Dmitriev, S.L. Kharitonov, K. M. Klimina, N.V. Melnikova, A.V. Kudryavtseva, Microbial community structure of activated sludge in treatment plants with different Wastewater Compositions, Front. Microbiol. 7 (2016) 90.

[27] L.L. Kiiskinen, M. Rättö, K. Kruus, Screening for novel laccase-producing microbes, J. Appl. Microbiol. 97 (2004) 640–646.

[28] C.F. Thurston, The structure and function of fungal laccases, Microbiology 140 (1994) 19–26.

[29] Y.-S. Wang, R.V. Subba-Rao, M. Alexander, Effect of substrate concentration and organic and inorganic compounds on the occurence and rate of mineralization and cometabolism, Appl. Environ. Microbiol. 47 (6) (1984) 1195–1200.

[30] Y.-S. Wang, E.L. Madsen, M. Alexander, Microbial degradation by mineralization of cometabolism determined by chemical concentration and environment, J. Agric. Food Chem. 33 (1985) 495–499.

[31] J. Zhang, Y. Xin, H. Liu, S. Wang, N. Zhou, Metabolism-independent chemotaxis of *Pseudomonas* sp. strain WBC-3 toward aromatic compounds, J. Environ. Sci. 20 (2008) 1238–1242.

[32] G.F. Leatham, T.K. Kirk, Regulation of ligninolytic activity by nutrient nitrogen in white-rot basidiomycetes, FEMS Microbiol. Lett. 16 (1) (1983) 65–67.

[33] G. Gramss, Reappraising a controversy: formation and role of the azodication (ABTS²⁺) in the laccase-ABTS catalyzed breakdown of lignin, Fermentation 3 (2017) 27, doi:http://dx.doi.org/10.3390/fermentation3020027.

[34] J. Ihssen, M. Schubert, L. Thöny-Meyer, M. Richter, Laccase catalysed synthesis of iodinated phenolic compounds with antifungal activity, PLoS One 9 (3) (2014)e89924.

[35] P. Durão, I. Bento, A. Fernandes, E.P. Melo, P.F. Lindley, L.O. Martins, Perturbations of the T1 copper site in the CotA laccase from *Bacillus subtilis*: structural, biochemical, enzymatic and stability studies, J. Biol. Inorg. Chem. 11 (2006) 514–526.

[36] J. Margot, C. Bennati-Granier, J. Maillard, P. Blánquez, D.A. Barry, C. Holliger, Bacterial versus fungal laccase: potential for micropollutant degradation, AMB Exp. 3 (2013) 63.

[37] J. Ihssen, M. Schubert, L. Thöny-Meyer, M. Richter, Laccase catalyzed synthesis of iodinated phenolic compounds with antifungal activity, PLoS One 9 (3) (2014)e89924.

[38] S. Kumar, G. Stecher, K. Tamura, MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets, Mol. Biol. Evol. 33 (7) (2016) 1870–1874.

[39] A. Cydzik-Kwiatkowska, M. Zielińska, Bacterial communities in full-scale wastewater treatment systems, World J. Microbiol. Biotechnol. 32 (2016) 66.

[40] A.A. Telke, D.C. Kalyani, U.U. Jadhav, G.K. Parshetti, S.P. Govindwar, Purification and characterisation of an extracellular laccase from *Pseudomonas* sp. LBC1 and its application for the removal of bisphenol A, J. Mol. Catal. B: Enzym. 61 (2009) 252–260.

[41] M. Neifar, H. Chouchane, M. Mahjoubi, A. Jaouani, A. Cherif, *Pseudomonas extremorientalis* BU118: a new salt-tolerant laccase-secreting bacterium with biotechnological potential in textile azo dye decolourization, 3 Biotech 6 (2016) 107.

[42] Y.-T. Chang, C.-Y. Lin, Y.-H. Chen, P.-R. Hsueh, Update on infections caused by *Stenotrophomonas maltophilia* with particular attention to resistance mechanism and therapeutic options, Front. Microbiol. 6 (2016) 893.

[43] R.P. Ryan, S. Monchy, M. Cardinale, S. Taghavi, L. Crossman, M.B. Avison, G. Berg, D. van der Lelie, J.W. Dow, The versatility and adaptation of bacteria from the genus Stenotrophomonas, Nat. Rev. Microbiol. 7 (2009) 514–525.

[44] P. Murkherjee, P. Roy, Genomic potential of *Stenotrophomonas maltophilia* in bioremediation with an assessment of its multifaceted role in our environment, Front. Microbiol. 7 (2016) 967.

[45] S. Galai, Y. Touhami, M.N. Marzouki, Response surface methodology applied to laccase activities exhibited by *Stenotrophomonas maltophilia* AAP56 in different conditions, BioResour 7 (1) (2012) 706–726.

[46] L.M.P. Sampaio, J. Padrão, J. Faria, J.P. Silva, C.J. Silva, F. Dourado, A. Zille, Laccase immobilization on bacterial nanocellulose membranse: antimicrobial, kinetic and stability properties, Carbohydr. Polym. 145 (2016) 1–12.

[47] A. Kumari, N. Kishor, P. Guptasarma, Characterization of a mildly alkalophilic and thermostable *Thermus thermophilus* laccase with applications in decolourization of dyes, Biotechnol. Lett. 20 (2018) 285–295.

[48] Q. Yang, M. Zhang, M. Zhang, C. Wang, Y. Liu, X. Fan, H. Li, Characterization of a novel, cold-adapted, and thermostable laccase-like enzyme with high tolerance for organic solvents and salts and potent dye decolourization ability, derived from a marine metagenomic library, Front. Microbiol. 9 (2018) 2998.

[49] R. Reiss, J. Ihssen, M. Richter, E. Eichlom, B. Schilling, L. Thöny-Meyer, Laccase versus laccase-like multi-copper oxidase: a comparative study of similar enzymes with diverse substrate spectra, PLoS One 8 (6) (2013)e65633.

[50] F. Xu, Oxidation of phenols, anilines, and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition, Biochem 35 (1996) 7608–7614.

[51] F. Medina, S. Aguila, M.C. Baratto, A. Martorana, R. Basosi, et al., Prediction model based on decision tree analysis for laccase mediators, Enzyme Microb. Technol. 52 (2013) 68–76.

[52] D. Kalyani, M.K. Tiwari, J. Li, S.C. Kim, V.C. Kalia, Y.C. Kang, J.-K. Lee, A highly efficient recombinant laccase from the yeast *Yarrowia lipolytica* and its application in the hydrolysis of biomass, PLoS One 10 (3) (2015)e0120156.

[53] L.I. Trubitsina, S.V. Tishchenko, A.G. Gabdulkhakov, A.V. Lisov, M.V. Zakharova, A.A. Leontievsky, Structural and functional characterisation of two-domain laccase from *Streptomyces viridochromogenes*, Biochimie 112 (2015) 151–158.

[54] D.C. Kalyani, L. Munk, J.D. Mikkelsen, A.S. Meyer, Molecular and biochemical characterisation of a new thermostable bacterial laccase from *Meiothermus ruber* DSM 1279, RSC Adv. 6 (2016) 3910–3918.