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Peroxidase production and ligninolytic potentials of fresh water bacteria *Raoultella ornithinolytica* and *Ensifer adhaerens*

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Guaiacol (PubChem CID: 460)
Pyrogallol (PubChem CID: 1057)
RBBR, Remazol Brilliant Blue R (PubChem CID: 17409)
Veratryl alcohol (PubChem CID: 7118)

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ABSTRACT

Interest in novel ligninolytic bacteria has remained topical due to, in part, the maneuverability of the bacterial genome. Conversely, the fungal genome lacks the dexterity for similar maneuverability thus, posing challenges in the fungal enzyme yield optimization process. Some impact of this situation includes the inability to commercialize the bio-catalytic process of lignin degradation by fungi. Consequently, this study assessed some fresh water bacteria isolates for ligninolytic and peroxidase properties through the utilization and degradation of model lignin compounds (guaiacol and veratryl alcohol) and the decolourization of selected ligninolytic indicator dyes; Azure B (AZB), Remazol Brilliant Blue R (RBBR) and Congo Red (CR). Bacterial strains with appreciable ligninolytic and peroxidase production potentials were identified through 16S rDNA sequence analysis and the nucleotide sequences deposited in the GenBank. About 5 isolates were positive for the degradation of both guaiacol (GA) and veratryl alcohol (VA) thus, accounting for about 17% of the test isolates. Similarly, AZB, RBBR and CR were respectively decolorized by 3, 2 and 5 bacterial strains thus, accounting for 10%, 7% and 17% of the test isolates. Two of the test bacterial strains were able to decolourize AZB, RBBR and CR respectively and these bacterial strains were identified as *Raoultella ornithinolytica* OKOH-1 and *Ensifer adhaerens* NWODO-2 with respective accession numbers as KX640917 and KX640918. Upon quantitation of the peroxidase activities; 5250 ± 0.00 U/L was recorded against *Raoultella ornithinolytica* OKOH-1 and 5833 ± 0.00 U/L against *Ensifer adhaerens* NWODO-2. The ligninolytic and dye decolourization properties of *Raoultella ornithinolytica* OKOH-1 and *Ensifer adhaerens* NWODO-2 marks for novelty particularly, as dyes with arene substituents were decolourized. Consequently, the potentials for the industrial applicability of these test bacterial strains abound as there is a dearth of information on organisms with such potentials.

1. Introduction

Lignin; the aromatic, non-carbohydrate, component of lignocellulose is recalcitrant to degradation. Thus, effective degradation of lignin is of prime importance to the industrial sectors utilizing lignocellulose as raw materials for various value-added products [1]. More so, the recalcitrance of lignin to degradation constitutes an undesirable barrier to the efficient and optimum utilization of the abundant lignocellulosic materials. On the same note, the large amount of lignin generated during industrial production of ethanol, pulp and paper making processes, accumulates and, thus, constitutes serious environmental challenge hence, the need for effective and eco-friendly lignin degradation techniques [2].

The biological means of lignin degradation involves microbial or/and microbial enzymes degradative activities. This technique is advocated over the physical and chemical methods which are generally expensive and saddled with lots of other limitations [3,4].

Fungal degradation of lignin, particularly, the white-rot basidiomycetes have been studied extensively [5–7] and, white-rot fungi have been reported as the most effective microbial lignin-degrader. Effectiveness in lignin degradation has been attributed to some extracellular enzymes produced by the white-rot fungi [8]. These extracellular enzymes include laccases (EC 1.10.3.2), some heme-peroxidases such as lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13), versatile peroxidase (EC 1.11.1.16) and dye-decolourizing peroxidase (EC 1.11.1.19). Nonetheless, industrialization of white-rot fungal bio-catalytic/extracellular enzyme process for the de-polymerization of lignin is yet to be achieved. Perhaps, the insufficiency in

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the maneuverability of the white-rot fungal genome for optimum extracellular enzyme yield, as a function of production cost to commercial value quotient may have constituted an important factor impeding industrialization of the process [9,10].

Bacteria, on the other hand, hold very strong potential considering their striking resilience in diverse environments and, as well, their biotechnological significance following, faster growth rate and high dexterity in genome maneuverability [10,11]. Hence, the imperativeness in the exploration of bacteria species for lignin depolymerization potentials. Besides, the evolving significance of bacteria in the degradation of lignin has been severally documented [9]. Bacteria species classed into the actinomycetes, α-proteobacteria and γ-proteobacteria have been reported to possess lignin degrading ability [9,12,13]. Documented ligninolytic bacteria includes *Streptomyces viridosporus* T7A, *Rhodococcus* sp, *Nocardia autotrophica* [14], *Microbacterium* sp, *Brucella melitensis*, *Ochrobactrum* sp, *Sphingomonas* sp [15], *Streptomyces coelicolor, Arthrobacter globiformis, Rhodococcus jostii RHA1, Pseudomonas putida mt-2* [16], *Serratia* sp. JHT01, *Serratia liquefacien* PT01, *Pseudomonas chlororaphis* PT02, *Stenotrophomonas maltophilia* PT03 and *Mesorhizobium* sp. PT04 [11].

Ligninolytic bacteria similarly produce extracellular oxidative enzymes including peroxidases which have been implicated in lignin degradation. Besides the association of these extracellular peroxidases in lignin degradation, they have applications in the removal of phenolic pollutants [17], synthetic dye decolourization [18], and the synthesis of natural aromatic flavours [19,20]. Other applications have likewise included deodourization of manure [21], applications in peroxidase biosensors [22], analysis and diagnostic kits [23] and development of skin lightening agents [24,25]. Given, the diverse applications of peroxidases in different industrial sectors, the exploration of bacteria species with novel ligninolytic abilities and high potentials for peroxidase production is of prime importance. Consequently, the reported study evaluated bacterial isolates from fresh water milieu of the Raymond Mhlaba Municipality, Eastern Cape, South Africa for peroxidase production potentials and ligninolytic activities.

2. Materials and methods

2.1. Chemicals and reagents

Hydrogen peroxide, Pyrogallol, Magnesium sulphate, Manganese II sulphate, Sodium chloride, Ammonium nitrate, Potassium dihydrogen phosphate, Dipotassium hydrogen phosphate, Nystatin, Nalidixic acid, yeast extract, Agar bacteriological, Azure B, Remazol Brilliant Blue R and Congo red were sourced from Merck KGaA, Darmstadt, Germany while Guaiacol, Veratryl alcohol and Kraft lignin were sourced from Sigma-Aldrich, South Africa. Unless stated otherwise, all other chemicals are of analytical grade while the water was glass distilled.

2.2. Sample collection and sampling site

Water samples and sediments (Table 1) were collected from Tyhume river courses in Raymond Mhlaba Municipality, Eastern Cape, South Africa and transported on ice to Applied and Environmental Microbiology Research Group (AEMREG) Laboratory, University of Fort Hare, Alice, South Africa for analysis.

Tyhume River is situated in the Raymond Mhlaba Municipality of the Eastern Cape Province, South Africa. It originates from the Amathole mountains in Hogsback and flows through the lower coastal escarpment down to Alice, a small town with several suburbs. Sample collection was on the courses of Tyhume River, with reduced anthropogenic activities, in Alice.

2.3. Isolation and preliminary screening of ligninolytic bacteria

Ligninolytic bacteria were isolated by enrichment method as described by Sasikumar et al. [26] with minor modifications. Briefly, a phosphate buffered (pH 7) minimal salts medium (MSM) containing 4.55 g/L K_2HPO_4, 0.53 g/L KH_2PO_4, 0.5 g/L $MgSO_4$ and 5 g/L NH_4NO_3 [27] was supplemented with 1 g/L of commercially available kraft lignin (Sigma-Aldrich, South Africa) and 0.1 g/L yeast extract (MSM-L). The components were mixed and the resulting broth autoclaved at 121 °C for 15 min. Enrichment culture was performed in 250 mL Erlenmeyer flask by placing 5 g of sample in 95 mL MSM-L and the culture was incubated in an orbital shaking incubator at 30 °C and 140 rpm for 168 h [26]. Enriched sample of 1 mL was transferred to 9 mL of sterile normal saline and stirred vigorously at room temperature. Using 1 mL of the liquid mixture, serial dilutions was prepared. Thereafter, 100 µL of serially diluted sample was spread on MSM-L agar plate amended with 50 mg/L nystatin (to inhibit the growth of fungi) and the plates were incubated at 30 °C for 168 h until colonies developed. Subsequently, the cultures were purified and presumptive ligninolytic bacteria stored in 20% glycerol at −80 °C for further analysis.

Table 1
Isolated ligninolytic bacteria from fresh water milieu of Raymond Mhlaba Municipality, Eastern Cape, South Africa.

S/N	Isolate code	Isolation source	Guaiacol degradation	Veratryl alcohol degradation
1.	T1CS3[1]	Sediment	−	−
2.	T1CS3[2]	Sediment	−	+
3.	T1CS3[3]	Sediment	−	−
4.	T1CS3[4]	Sediment	−	−
5.	T1B2S3[1]	Sediment	−	+
6.	T1B2S3[2]	Sediment	−	+
7.	T1B2S3[3]	Sediment	−	+
8.	T1B1S3[1]	Sediment	+	+
9.	T1B1S3[2]	Sediment	−	−
10.	T1B1S3[3]	Sediment	−	−
11.	T1B1S3[4]	Sediment	+	+
12.	T1B1S3[5]	Sediment	+	−
13.	T1B1W3[1]	Water	+	+
14.	T1B1W3[2]	Water	−	+
15.	T1B2W3[1]	Water	−	−
16.	T1B2W3[2]	Water	−	−
17.	T1B2W3[3]	Water	−	−
18.	T1CW3[1]	Water	−	−
19.	T1CW3[2]	Water	−	−
20.	T1CW3[3]	Water	+	−
21.	T2BS2[1]	Sediment	−	+
22.	T2BS3[1]	Sediment	−	+
23.	T2BS3[2]	Sediment	−	−
24.	T2BW3[1]	Water	+	+
25.	T2BW3[2]	Water	−	+
26.	T2BW3[3]	Water	−	−
27.	T1CS3[B]	Sediment	−	−
28.	T1CS3[C]	Sediment	−	−
29.	T1CS3[D]	Sediment	+	+
30.	T1CS3[A]	Sediment	−	−

2.4. Evaluation of ligninolytic activity

The ligninolytic activities of isolates were assessed through the utilization and degradation of model lignin compounds (guaiacol and veratryl alcohol) using the modified method of Taylor et al. [28]. Briefly, 5 µL of standard inoculum of the bacterial suspension (O.D. 600 nm ≈ 1.0) was aseptically inoculated onto guaiacol and veratryl alcohol plates composed of the following: K_2HPO_4 (4.55 g/L), KH_2PO_4 (0.53 g/L), $MgSO_4$ (0.5 g/L), NH_4NO_3 (5 g/L), yeast extract (0.1 g/L), guaiacol or veratryl alcohol (0.1% v/v), and agar (15 g/L). The plates were incubated at 30 °C for 168 h and subsequently flooded with Gram's iodine solution to determine the zone of degradation.