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Research article

Screening of potential bioremediation enzymes from hot spring bacteria using conventional plate assays and liquid chromatography - Tandem mass spectrometry (Lc-Ms/Ms)

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ABSTRACT

The search for an eco-friendly, non-toxic, economical and efficient means of cleaning water through bioremediation is not only more favourable but critical to maintaining water quality globally especially in water-scarce countries. Thermophilic bacteria including *Bacillus* species are an important source of novel enzymes for biotechnology applications. In this study, 56 bacterial isolates which were cultured from five hot springs in South Africa were identified predominantly as *Bacillus* sp. or *Bacillus*-related *spp* by 16S rDNA gene sequencing. These isolates were screened for potentially useful enzymes for water bioremediation. Using conventional agar plate assays, 56% (n = 43), 68% (n = 38) and 16% (n = 31) were positive for amylase, protease and bromothymol blue decolorisation respectively. In liquid starch culture, three amylase-positive isolates differentially degraded starch by 34% (isolate 20S) to 98% (isolate 9T). Phenol degradation revealed that five out of thirty reduced phenol up to 42% by colorimetric assay. A thermophilic strain of *Anoxybacillus rupiensis* 19S (optimal growth temperature of 50 °C), which degraded starch, protein and phenol, was selected for further analysis by tandem LC-MS/MS. This newer technique identified potential enzymes for water bioremediation relating to pollutants from the food industry (amylase, proteases), polyaromatic hydrocarbons and dye pollutants (catalase peroxidase, superoxide dismutase, azoreductase, quinone oxidoreductase), antibiotic residues (ribonucleases), solubilisation of phosphates (inorganic pyrophosphatase) and reduction of chromate and lead. In addition, potential enzymes for biomonitoring of environmental pollutants were also identified. Specifically, dehydrogenases were found to decrease as the level of inorganic heavy metals and petroleum increased in soil samples. This study concludes that bacteria found in South African hot springs are a potential source of novel enzymes with tandem LC-MS/MS revealing substantially more information compared with conventional assays, which can be used for various applications of water bioremediation.

1. Introduction

Bioremediation of wastewater (WW) has made use of microbially produced enzymes with advantages of lower costs, green footprint, decreased toxicity to the environment, ease of application, specificity and flexible applications to a variety of possible substrates. The two main categories of enzymes useful for bioremediation are hydrolytic enzymes including lipase, cellulase, amylase, protease, phosphatases and beta-lactamases, and oxidoreductases which include laccase and peroxidase (Karigar and Rao, 2011; Facchin et al., 2013). Microorganisms from extreme environments like hot springs are investigated for their enzymes that remain active at harsh environmental conditions of pH and temperature (Demirjian et al., 2001; de Miguel Bouzas et al., 2006). The genera *Bacillus* spp. and related bacteria predominate when bacteria are cultured from hot spring sites (Derekova et al., 2008; Panda et al., 2016). This group of bacteria are well known for their industrial applications and production of useful enzymes (Kumar et al., 2013). Amylase (Zhang et al., 2015; Acer et al., 2016), protease (Panda et al., 2013; Bekler et al., 2015) and phosphatase (Sen and Maiti, 2014) producing *Bacillus* sp have been previously isolated from hot spring sites globally. Even though this is well studied, novel enzymes that are active at 110 °C are still being discovered. Recently, novel enzymes were described in *Geobacillus* spp isolated for Tunisian hot springs (Thebti et al., 2016). More modern techniques of protein or enzyme

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detection including LC-MS/MS has been useful in expediting the identification and discovery of microbial enzymes (Fandi et al., 2012) although the conventional plate assays have their place in low resource laboratories. An indirect application to water bioremediation has been the use of microbial enzymes for biomonitoring (Logar and Vodovnik, 2007) in aquatic environments (Li et al., 2010). For example, lead (Pb) is highly toxic in the environment because of its ability to mimic biologically important metals and produce membrane damage. Delta-aminolevulinate dehydratase (ALAD) is a conserved metalloprotein in many organisms including bacteria and is very sensitive to Pb (Konuk et al., 2010). Monitoring of this enzyme using *Pseudomonas* spp (Korcan et al., 2007) has been used to detect Pb concentrations in contaminated water environments.

Water temperature of hot springs in Limpopo, South Africa, can range from 42 °C to 68 °C and pH of 7–9 (Olivier et al., 2011) thereby introducing the possibility of obtaining alkaliphilic and/or thermophilic enzymes. Metagenomic studies of hot springs in Limpopo have been investigated, showing a great diversity of bacteria (Tekere et al., 2013, 2012). The aim of this study was to screen isolated and identified bacteria from these for oxidoreductase and hydrolytic enzymes that could be potentially useful in water bioremediation biotechnology by conventional biochemical assays, and LC-MS/MS, and enzymes potentially useful for biomonitoring.

2. Materials and methods

2.1. Sampling from hot springs, isolation and identification of bacteria

Fig. 1 provides a summary of the methods used in this study. Water and sediment samples were taken from hot springs in Limpopo Province, South Africa at the following locations: Tshipise (T), Siloam (S), Mphephu (M), Lekkerrus (Le) and Libertas (Li). Geographical locations, site descriptions of the sampling sites, sample processing and isolation and culture of bacteria are described in Jardine et al. (2017). The 16S rDNA gene was amplified by polymerase chain reaction (PCR) using Universal primers and sequenced. Identification of isolates was made by comparison with a public database, GenBank as outlined in Jardine et al. (2017).

2.2. Plate assay for screening of potential enzymes for bioremediation for amylase, protease and bromothymol blue decolorisation

Hydrolytic activities of pure cultures were tested qualitatively on diffusion agar plates containing the various substrates: starch for amylase (http://www.bd.com/europe/regulatory/assets/ifu/difco_bbl/272100.pdf) and skim milk (http://microbiologyonline.org/teachers/preparation-of-media-and-cultures) for protease, where a clearing around the colonies after growth at 37 °C or 53 °C for 24–48 h would indicate a positive presence of an enzyme. The degradation of starch was detected by flooding the plate with Lugol's iodine post incubation to observe the clearing zones around the colonies. For the detection of dye decolorisation, bromothymol blue (BB) nutrient agar (Tekere et al., 2001) was used, again with a clearing denoting positive for enzyme production.

2.3. Concentration of amylase produced

A liquid assay was used to determine starch concentration by generating a standard curve made with soluble starch (boiled for 1.5hrs) 1% stock starting from 0.00001% to 0.1%, stained with 10% Lugol's iodine. The optical density (OD) was read at 660 nm in a Biorad 96 well iMark plate reader. The method and standard curve are described in Appendix 1.

Three thermophilic isolates (amylase producers) were selected (Table 1), that had a range of clearing widths on starch agar plates of 3 mm (isolate 9T), 5 mm (isolate 20S) and 10 mm (isolate 13S). Conical flasks were inoculated with the selected isolates 9T, 13S and 20S into 50 mL 1% starch broth and incubated at 53 °C for 24 h without agitation. The negative control was starch broth that was not inoculated. Supernatants were serially diluted and tested for starch as in the liquid assay described in Appendix 1 after 24 h.

2.4. Biochemical tube assay for phenol reduction

Ficoll-Ciocalteau (FC) reagent was used for the quantitation of phenol in phenol red broth base media containing 0.018 g phenol red per litre (Merck1.10987) (Agbor et al., 2014). The method was modified to increase sensitivity by diluting the sample 1: 1 instead of 1:16 as described by Strong and Burgess (2008). Phenol red broth base was mixed in a 1: 1 ratio with overnight cell-free culture supernatant (CFCS) and incubated at room temperature for 3 h, to see if the concentration of phenol was reduced. Green tea was used as a positive control, and sterile uninoculated nutrient broth (NB) was used as a negative control.

2.5. Identification of protein bands by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

10 mls of NB was inoculated with cultures (Isolates 19T, 76S, 77S & 85Li) and incubated at 37 °C or 53 °C for 24hrs. The cultures were centrifuged at 10000 rpm to remove the bacteria, and the supernatant (CFCS) was further processed. 2 mls of each culture was dehydrated by freeze drying under vacuum and resuspended in loading buffer for SDS-PAGE. 200 µl was loaded into each well. Post run, the gel was stained with Coomassie blue dye.

Protein bands of interest were in-gel trypsin digested as per the protocol described in (Shevchenko et al., 2006). In short, gel bands

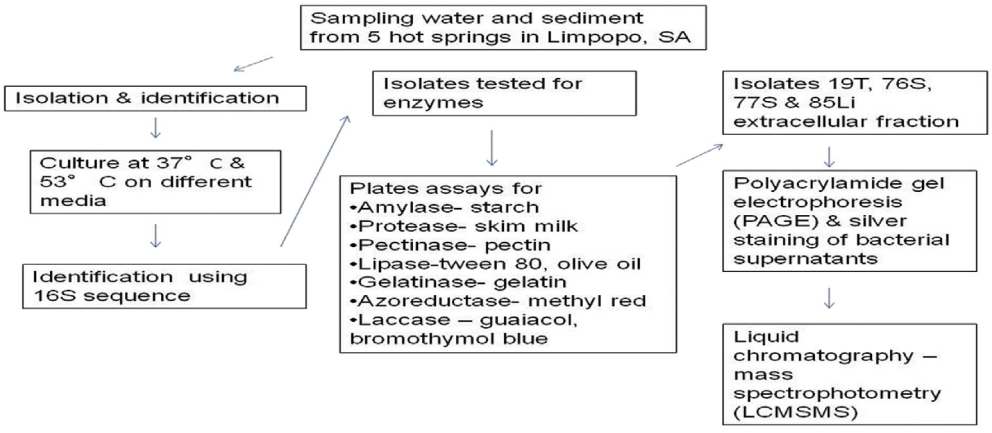

Fig. 1. Summary of methods used in the study.