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Inhibitory spectrum of diverse guaiacol-producing *Alicyclobacillus acidoterrestris* by poly dimethyl ammonium chloride disinfectant

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ABSTRACT

Alicyclobacillus acidoterrestris is a soil-borne, thermo-acidophilic, endospore-forming bacterium which causes the spoilage of pasteurized fruit juices with the production of guaiacol taints. In this study the antimicrobial activity of poly dimethyl ammonium chloride (PDAC), an emerging disinfectant, against *A. acidoterrestris* was investigated. Initially, four bacteria isolates obtained from orchard soils were identified as *A. acidoterrestris* by 16S ribosomal RNA (rRNA) gene sequencing followed by analysis of random amplified polymorphic DNA (RAPD) fragments, which grouped the isolates into two genotypes. High performance liquid chromatographic (HPLC) analysis revealed the activity of vanillic-acid decarboxylase (*vdc*) in the *A. acidoterrestris* strains, as measured by the production of guaiacol from vanillic acid (VA), indicating the isolates' spoilage potential. Subsequently, representative strains from the two RAPD types of *A. acidoterrestris* were treated with PDAC. Disc diffusion assay on YSG agar revealed that *A. acidoterrestris* strains ULAG14, ULAG15 and the type strain DSM 3922^T were sensitive to PDAC at concentrations ranging from 0.5% to 20% and inhibition of endospore germination was observed after 5 min exposure. PDAC has an inhibitory effect against the vegetative cells and endospores of *A. acidoterrestris* under laboratory conditions; it may be potentially useful for industrial application in cleaning of fruits and production surfaces for quality control during the processing of tropical fruit juices.

1. Introduction

Alicyclobacillus acidoterrestris is a thermo-acidophilic, endospore-forming bacterium which possesses characteristic ω-alicyclic fatty-acids as a major component of its cell membrane (Deinhard, Blanz, Poralla, & Altan, 1987; Wisotzkey, Jurtshuk, Fox, Deinhart, & Poralla, 1992). These characteristics and the ability to produce off-flavor taints make *A. acidoterrestris* a challenge to the fruit juice industry, where it evades product pasteurization through endospore formation, germinate in acidic fruit juices and subsequently produce off-flavour taints that may result in product rejection and recalls (Splittstoesser, Churey, & Lee, 1994; Borlinghaus & Engel, 1997; Pettipher, Osmundson, & Murphy, 1997; Jensen & Whitfield, 2003). The off-flavour taints produced by *A. acidoterrestris* have been identified as guaiacol (2-methoxyphenol) and the halophenols (2, 6-dibromophenol and 2, 6-dichlorophenol) (Pettipher et al., 1997; Gocmen et al., 2005). Guaiacol being the predominant taint chemical produced by *A. acidoterrestris* and other spoilage *Alicyclobacillus* species is a product of the non-oxidative decarboxylation of vanillic-acid, a reaction catalysed by the vanillic-acid decarboxylase (vdc) enzyme system (Chow, Pope, & Davies, 1999; Niwa and Kawamoto, 2003; Witthuhn, Van der Merwe, Venter, & Cameron, 2012; Álvarez-Rodríguez et al., 2003).

The main challenge posed by *A. acidoterrestris* is the heat resistance of its endospores, enabling the bacterium to survive the commercial pasteurization of fruit juices at 88–96 °C for 2 min or 90–95 °C for about 30–60 s (Tianli, Jiangbo, & Yahong, 2014). Consequently, many studies have focused on the use of alternative non-thermal control methods or measures combining heat treatments and other forms of treatments for the inactivation of *A. acidoterrestris* (Alberice, Funes-Huacca, Guterres, & Carrilho, 2012; Bevilacqua, Sinigaglia, & Corbo, 2008; Pei, Yue, & Yuan, 2014).

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A. acidoterrestris has been detected at various stages of fruit juice production, including raw materials, ingredients, processing environments and finished products (Danyluk et al., 2011; Durak, Churey, Danyluk, & Worobo, 2010; Groenewald, Gouws, & Witthuhn, 2009). However, the bacterium is soil-borne and soil contamination of fruits and production surfaces has been reported as the primary route of *A. acidoterrestris* contamination during fruit juice production (Brown, 1995; Takahashi et al., 2007). Therefore the application of effective disinfectants for the cleaning of fruits and production surfaces is recommended as a first-line of defence against spoilage of fruit juices caused by *A. acidoterrestris* (Chang & Kang, 2004).

Previous studies have reported on the effectiveness of commercial disinfectants against *A. acidoterrestris* (Danyluk et al., 2011; Lee, Gray, Dougherty, & Kang, 2004; Orr & Beuchat, 2000). However, several emerging commercial disinfectants have demonstrated antimicrobial effectiveness against a wide range of bacteria and are increasingly being applied for general disinfection of environmental surfaces.

PDAC (120 g/L of poly dimethyl ammonium chloride) is an emerging liquid disinfectant, indicated for use as a broad spectrum disinfectant for surfaces against all poultry viruses, bacteria, fungi, Mycoplasma, yeasts and algae. It is marketed as Virukill™ (ICA Laboratories, Stellenbosch, South-Africa). Virukill is also recommended for the disinfection of drinking water for poultry, hatching eggs and the air in poultry houses, indicating the chemical's low level of toxicity against living tissues. The Virukill solution is becoming widely used for general disinfection beyond the poultry applications and therefore may be useful in the fruit juice industry for the control of *A. acidoterrestris* contamination. The susceptibility of diverse strains and endospore of *A. acidoterrestris* to PDAC has not been reported. Therefore, the objective of this study is to determine *in vitro* antimicrobial activity of PDAC against the vegetative cells and endospores of soil isolates of *A. acidoterrestris*.

2. Materials and methods

2.1. Isolation, identification and vdc activity assay

The test strains of *A. acidoterrestris* were isolated from soil samples collected from a lemon orchard in Ilishan-remo, South-Western Nigeria, using a modified protocol obtained from Groenewald, Gouws, and Witthuhn (2008). The sample enrichment was done in acidified YSG broth (pH 3.5) containing 100 μg/ml amphotericin B (Sigma-Aldrich, USA) and the enrichments were incubated aerobically at 45 °C. Following 5 d of incubation, samples were streaked on YSG agar plates (pH 3.5) followed by incubation at 45 °C for 3 d. The isolated colonies were re-streaked until pure cultures were obtained. Stock cultures of isolates were prepared in YSG broth plus 30% (v/v) glycerol and stored at −80 °C. Working cultures of isolates were kept on YSG agar at 4 °C and maintained by by-weekly transfers to fresh agar plates.

Genomic DNA was extracted from the isolates using a modified method obtained from Than (2006) and described elsewhere (Osopale, Witthuhn, Albertyn, & Oguntoyinbo, 2016). The extracted DNA was PCR-amplified using primers 27F (5′-AGAGTTTGATYMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Eden, Schmidt, Blakemore, & Pace, 1991; Edwards, Rogall, Blöcker, Emde, & Böttger, 1989; Weisburg, Barns, Pelletier, & Lane, 1991). PCR conditions are described elsewhere (Osopale et al., 2016) and the amplicons were sequenced using the BigDye terminator v3.1 cycle sequencing Kit (Applied Biosystems, USA) according to manufacturer's instruction. The obtained sequences were aligned in Geneious 6.0.3 and identified using the BLAST algorithm (Altschul, Gish, Miller, Myers, & Lipman, 1990) to compare with existing DNA sequences in the NCBI/GenBank database (www.ncbi.nlm.nih.gov). All 16S rRNA gene sequences obtained in this study have been deposited in Genbank through BankIt.

The genetic diversity of the isolates identified as *A. acidoterrestris* was established by RAPD-PCR using primers Ba-10, F-61 and F-64 (Yamazaki, Okubo, Inoue, & Shinano, 1997). The adopted PCR conditions are described in Osopale et al. (2016) and each PCR was performed in duplicate to ensure reproducibility of RAPD profiles. RAPD fragments were analysed by electrophoresis (90V for 1 h) on 1.5% agarose gels (Seakem® LE, USA) stained with 5 mg/mL ethidium bromide (Sigma-Aldrich, Germany), using the O'GeneRuler™ DNA ladder (Fermentas, USA) as molecular weight standard. Gel images were captured under ultraviolet (UV) illumination in a Bio-RAD Chemidoc XRS (BioRad laboratories, USA) and banding patterns were analysed by visual comparison. The type strains *A. acidoterrestris* DSM 3922^T and *Alicyclobacillus acidocaldarius* DSM 446^T served as reference strains for the RAPD analysis.

The vanillic-acid decarboxylase (*vdc*) activity in the isolated *A. acidoterrestris* strains was measured by guaiacol production from vanillic acid using the protocol also described in Osopale et al. (2016). Guaiacol was detected in the samples after 24 h incubation of YSG-vanillic acid samples by HPLC according to the method of Witthuhn et al. (2012), using HPLC Discovery HS C18 column (Sigma-Adrich, USA) and UV detection at 272 nm. Vanillic-acid and guaiacol and concentrations were measured by peak area determination using the Shimadzu LC solution calibration curve. YSG-vanillic acid samples lacking *A. acidoterrestris* inoculums or YSG lacking vanillic acid served as experimental controls, which were analysed for vanillic-acid and guaiacol after 168 h. The guaiacol production experiments were performed in duplicate.

2.2. Determination of antimicrobial activity of PDAC against A. acidoterrestris

PDAC was tested against *A. acidoterrestris* at test concentrations of 0.5%, 1%, 2%, 5%, 10%, and 20%. Initially antimicrobial activity was determined using a modified disc diffusion method on YSG agar seeded with ca. 10^6 cfu/mL of the cells or endospores of *A. acidoterrestris* strains ULAG14, ULAG15 or DSM 3922^T. Endospores suspensions were prepared according to the method described by Pettipher et al. (1997) in which 5 d old cultures of the test strains were heated at 80 °C for 10 min followed immediately by cooling on ice. Plates were incubated at 45 °C and examined for zones of inhibition after 24–48 h of incubation.

For disinfectant contact time study, cells and endospore suspensions of the test strains were treated with the different test concentrations of PDAC for 5 min, 15 min and 30 min. The cells and endospore suspensions at optical density (OD) of 1.0 were washed twice in sterile distilled water and pelleted in 1.5 ml Eppendorf tubes. The different test concentrations of PDAC solutions were prepared in sterile distilled water to final volume of 1 ml and added to the tubes containing the cells and endospore pellets. The samples were mixed and incubated at room tmperature for the various contact times. After exposure, the treated samples were separated from the disinfectant by centrifuging at 3000×*g* for 2 min and then washed thrice in sterile distilled water. The washed samples were incubated in 50 ml YSG broth (pH 4) at 45 °C for 144 h (6 d) during which growth was measured by turbidity (OD_{540}) using spectrophotometer (Jenway 6400, United Kingdom). The cells and endospores suspensions of *A. acidoterrestris* not exposed to the disinfectant served as experimental control. Growth data was expressed as inhibition index, evaluated according to the method of Maldonado, Aban, and Navarro (2013) and Bevilacqua, Ciuffreda, Sinigaglia, and Corbo (2014). All analyses were performed in