Research article

Insights into the microbial composition and potential efficiency of selected commercial biofertilisers

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A B S T R A C T

This study investigated 13-commercial biofertilisers for their microbial contents and potential functional capabilities using a culture-based approach. Isolates obtained were identified by sequencing the partial I6S rRNA gene and ITS 1 and 2 regions and screened for plant growth-promoting capabilities. A total of 58 bacterial and three fungal isolates were obtained from all biofertilisers, with major genera being *Bacillus, Rhizobium, Pseudomonas, Candida* and *Aspergillus*. Five of the biofertilisers had the microbes (all or some) listed in the label detected while eight products had none detected. All the products had more microbes than that declared in the labels, suggesting the presence of potential contaminants. Generally, all the identified microbes, including the potential contaminants, had different beneficial capabilities. Approximately 40% of the isolates showed potential for nitrogen-fixation, while 27% exhibited high phosphate-solubilisation ability. Additionally, 87% of the isolates produced indole acetic acid in the range of 0.1–114.4 µg/mL. High levels of siderophore production were mainly observed amongst *Bacillus* and *Pseudomonas* genera. The potential of the microbes, including those not listed in the label, to fix nitrogen and produce acid phosphatase, indole acetic acid and siderophore, was highest in four products. This suggests the products have multiple functional abilities in improving crop productivity. However, other qualities of biofertiliser, such as viable cell count and level of contamination, must always be within the acceptable standards. This will guarantee high product quality as well as efficiency when applied in the field. Overall, the results show that there is a high correlation between microbial compositions and potential capability of biofertilisers for plant-growth promotion.

1. Introduction

Over the years, the application of agrochemicals such as chemical fertilisers and pesticides has been a major agricultural management practice used for improving soil nutrient content and crop productivity. However, leaching and poor management of chemical fertiliser have contributed to environmental pollution, soil quality degradation, agronomic inefficiencies and economic losses of farmers (Savci, 2012). Considering the adverse effects of chemical fertiliser application, more sustainable, cost-effective and eco-friendly techniques are being explored for improving crop productivity (Gliessman, 2016; Lesueur et al., 2016; Suyal et al., 2016). A potential solution to this challenge is the use of beneficial microbes such as bacteria, fungi and blue-green algae as a method for improving agricultural productivity. Microbial-based formulations such as biofertilisers are now widely employed in sustainable agriculture (Lesueur et al., 2016; Majeed et al., 2015). Biofertilisers are ready-to-use preparations that comprise beneficial microbes, which when applied to plant surfaces or roots and soil cause increase in crop yield by improving the supply and availability of essential plant nutrients and growth-promoting substances (Raimi et al., 2017; Suyal et al., 2016; Vessey, 2003). In addition, the use of biofertiliser generates increased revenue amongst farmers through improved crop productivity and reduced use of highly-priced agrochemical inputs, which is a major objective of sustainable agriculture (Raimi et al., 2017).

The promotion of sustainable agriculture has caused an increase in biofertiliser development and farmers awareness on the possible use of biofertilisers, and consequently, commercial products being introduced into the agro-markets are on the rise. Biofertiliser may consist of either a

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Table 1. Culture media used for microbial isolation.

Culture media	Microorganisms	Incubation °C	References
Congo red yeast extract mannitol agar	Rhizobia	30°C ± 2 °C	Sobti et al. (2015)
N-free semi-solid bromothymol blue Nfb	*Azospirillum*	30°C ± 2 °C	Baldani et al. (2014)
Burks N-free medium	*Azotobacter*	30°C ± 2 °C	Revillas et al. (2000)
Potato Dextrose Rose Bengal agar	Fungal species	25°C ± 2 °C	Rao et al. (2007)
Nutrient agar	*Bacillus, Pseudomonas,* and others	36°C ± 2 °C	Sigma-Aldrich, India

Table 2. Biofertiliser quality as determined by the microbial contents.

Parameters	High quality	Medium quality	Low quality	Poor quality
Declared	All present	All present	At least one present	Absent
Undeclared	Absent	Present	Present	Present

Table 3. Total viable cell and spore count of biofertiliser samples.

Samples	CB1L	CB2S	CB3L	CB4L	CB5S	CB6L	CB7L	CB8L	CB9L	CB10L	CB11S	CB12L	CB13S
Declared (10^9)	1.0	3.0	Not stated	1.0	4.0	8.0	12.0	4.0	4.0	16.0	4.0	4.0	≥100 spores/g
Observed (10^7)	1.53	5.40	16.8	360	NCO	NCO	NCO	NCO	NCO	NCO	NCO	NCO	≥152 spores/g

Colony-forming unit (CFU) per g or mL of the sample; NCO- no viable count observed.

single or consortium of microbes with similar or different functional capabilities (Lesueur et al., 2016; Pindi and Satyanarayana, 2012). These abilities include biological nitrogen fixation (BNF), phosphate solubilisation and production of plant growth-promoting substances such as indole acetic acid (IAA), gibberellin, cytokinins and siderophore (Suyal et al., 2016). The N-fixing microbes may be symbiotic (*Bradyrhizobium, Rhizobium* and *Sinorhizobium*) or free-living bacteria (*Azospirillum* and *Azotobacter*) while major phosphate solubilisers, which occur freely in the soil include *Pseudomonas, Bacillus, Enterobacter, Aspergillus* and *Penicillium* (Elmerich and Newton, 2007; Raimi et al., 2017).

The success of biofertiliser application is generally affected by the products' quality. Poor-quality products cannot be effective in improving crop yield when applied in the field (Lupwayi et al., 2000; Simiyu et al., 2013) because they lack the necessary microbial element needed for field action (Herrmann et al., 2015; Raimi et al., 2019). Studies have shown that poor-quality mycorrhizal biofertilisers failed to form associations with host plants, thus causing economic losses to farmers when such products are purchased and applied in the field (Corkidi et al., 2004; Faye et al., 2013). Moreover, several commercial biofertilisers have been reported to have contaminants instead of the microbes declared in the product's label (Herrmann et al., 2015; Olsen et al., 1996). As a result, the need for an efficient quality control system during the production process is essential. It has been advocated that proper quality-control system and regulatory frameworks be put in place to ensure the production and supply of good-quality biofertiliser to the agro-market, especially in developing countries (Herridge et al., 2002; Simiyu et al., 2013). Developed countries such as China, Australia, the United States, and the United Kingdom have some regulations in place. For instance, India and China use quality parameters such as the total viable cell (TVC), water content, pH, shelf life, contamination level and particle sizes (Malusá and Vassilev, 2014). Primarily, acceptable TVC depends on the type of microbes used for biofertiliser production. For bacteria, the required TVC ranges between $>5 \times 10^7$ CFU/g or $>10^8$ CFU/mL and $>0.2 \times 10^9$ CFU/g or $>1.5 \times 10^9$ CFU/mL for solid and liquid products, respectively. In arbuscular mycorrhizal biofertilisers, at least a gram of the product must contain 100 viable propagules (Malusá and Vassilev, 2014).

Other quality parameters used for evaluating potential field efficiency of biofertilisers including types, effectiveness and functional capabilities of microbe(s) have been reported in different studies (Lupwayi et al., 2000; Vessey, 2003). However, this study aims to investigate the

reliability of the information provided in the product label through *in vitro* evaluation of the actual quality of the biofertiliser products. Therefore, 13 commercially available biofertilisers in South Africa were analysed for their microbial community using enumeration and identification techniques as well as their functional properties. The present study also sought to establish the link between the microbial contents of available commercial biofertilisers and their potential field efficiencies. This study may bring about a better understanding of potential efficiency and quality of biofertilisers available to farmers in the agro-market.

2. Materials and methods

2.1. Commercial biofertiliser samples

Samples of 13 commercial biofertilisers were analysed in the present study. Ten of the samples were liquid, while three were carrier-based. Codes assigned to the products were CB1L, CB2S, CB3L, CB4L, CB5S, CB6L, CB7L, CB8L, CB9L, CB10L, CB11S, CB12L, and CB13S (Table 4). CB being commercial biofertiliser, followed by a number code and the type, either solid (S) or liquid (L) form.

2.2. Analysis of biofertilisers

2.2.1. Total viable count

Total viable cell count was estimated by dilution plate technique using a ten-fold serial dilution with saline solution (0.85% (w/v) NaCl) as the diluent (Motsara and Roy, 2008). The biofertiliser-saline solution was agitated on a rotary shaker (United Scientific, South Africa) at 150 rpm for 25 min prior to further dilution up to 10^{-9}. Subsequently, 0.1 mL of dilution 10^{-5} to 10^{-9} was spread on different culture media plates (Table 1) in triplicate. Colonies were enumerated after incubation for 2–5 days, and the microbial count was expressed as colony-forming units (CFU g^{-1} or mL^{-1}).

2.2.2. Most probable number

Most probable number (MPN) technique was used to enumerate *Azospirillum* spp. A 0.1 mL aliquot of dilutions 10^{-4} to 10^{-8} was transferred into 25 ml McCartney bottles containing 10 mL of semi-solid N-free (Nfb) media and incubated at 32 °C for four days. The experiment was conducted in five replicates per dilution (Alexander, 1982).
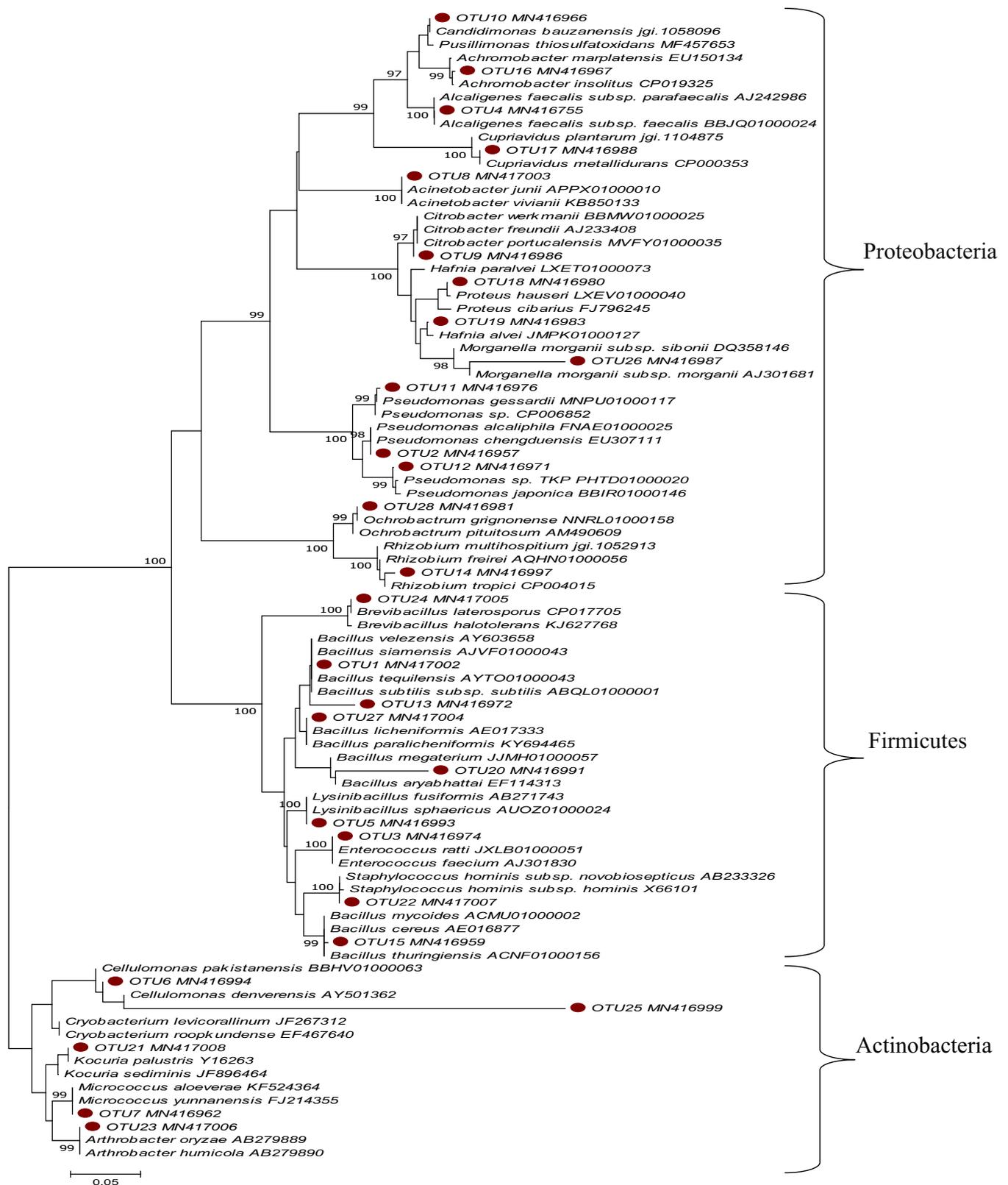
Figure 1. Phylogenetic tree showing identified bacterial OTUs with their closest relative based on the 16S rRNA gene sequences. The maximum likelihood phylogeny based on the Tamura 3-parameter model was used to infer the evolutionary history of the sequences in MEGA 7.

2.2.3. Arbuscular mycorrhizal fungal spore count and viability test

Arbuscular mycorrhizal fungi (AMF) spores were extracted using the wet-sieving and decanting method while the viability was determined using the technique of Habte and Osorio (2001). Briefly, distilled water

was added to 20 g biofertiliser in a beaker, and the suspension was thoroughly agitated for 30 min to release spores from the dispersed aggregates. The resultant solution was passed through nested sieves with mesh sizes of 0.75, 0.50, 0.25, 0.10 and 0.05 mm arranged in descending

Table 4. The microbial community of commercial biofertilisers as revealed by Sanger sequences.

Sample code	Declared microbes in the product	OTUs from Sanger sequences	Quality category
CB1L	*Enterobacter, Bacillus, Rhizobium, Pseudomonas, Trichoderma, Stenotromonas*	*Bacillus wiedmannii*, Pseudomonas alcaliphila, Micrococcus aloeverae, Lysinibacillus fusiformis, Candidimonas bauzanensis, Achromobacter marplatensi, Candida ethanolica*	Low
CB2S	*Rhizobium tropica*	*Rhizobium tropici*, Bacillus velezensis, Lysinibacillus fusiformis, Cellulomonas denverensis, Acinetobacter junii*	Medium
CB3L	*Azotobacter chrococcum, Bacillus subtilis, Bacillus thuringiensis, Saccharomyces cerevisiae, Pseudomonas florescense, Trichoderma harzianum, Lactobacillus sp.,*	*Bacillus velezensis*, B. megaterium*, Cupriavidus metallidurans, Aspergillus fumigatus, Candida ethanolica*	Low
CB4L	*Bacillus* sp.	*Bacillus velezensis*, Bacillus paralicheniformis, Acinetobacter junii,*	Medium
CB5S	*Bradyrhizobium japonicum*	*Brevibacillus laterosporus, Arthrobacter oryzae, Staphylococcus hominis* subsp. *novobiosepticus, Kocuria palustris*	Poor
CB6L	*Azospirillum brasilense, Azospirillum lipoferum*	*Enterococcus ratti, Alcaligenes faecalis* subsp. *faecalis, Pseudomonas gessardi*	Poor
CB7L	*Azospirillum brasilense, Azospirillum lipoferum, Azotobacter chrococcum*	*Enterococcus ratti, Bacillus velezensis,*	Poor
CB8L	*Bradyrhizobium japonicum*	*Pseudomonas japonica, Proteus hauser, Ochrobactrum grignonense*	Poor
CB9L	*Pseudomonas florescense*	*Enterococcus ratti, Hafnia paralvei, Alcaligenes faecalis* subsp. *faecalis,*	Poor
CB10L	*Bravibacillus laterosporous, Paenibacillus chitinolyticus, Lysinibacillus sphaericus, Sporolactobacillus laevolacticus,*	*Enterococcus rattii, Citrobacter portucalensis*	Poor
CB11L	*Bacillus* sp.	*Morganella morganii* subsp. *sibonii, Citrobacter portucalensis*	Poor
CB12L	*Rhizobium phaseolus*	*Pseudomonas japonica, Bacillus subtilis* subsp. *subtilis Alcaligenes faecalis* subsp. *faecalis*	Poor
CB13S	*Gigaspora gigantea, Funneliformis mosseae, Claroideoglomus etunicatum, Paraglomus occulum Rhizophagus clarus* (AMF)	*Bacillus valezensis, Acinetobacter junii,* Cryobacterium levicorallinum, *Bacillus licheniformis, Aspergillus fumigatus, Candida ethanolica,*	Medium

Note: (*) The declared genus detected. The genus taxa level was used in comparing the microbes listed in the labels and the detected OTUs. The spore count and viability test were used for AMF product quality using the acceptable standard parameters.

order of sizes. The trapped spores in 0.05-, 0.10- and 0.25 mm sieves were centrifuged (JP Selecta Centrifuge, Barcelona, Spain) at 7 000 rpm for 3 min after suspending in distilled water. The sediment was centrifuged after being re-suspended in 50% sucrose solution. The supernatant was washed with distilled water to release the spores. Subsequently, the spores were examined and counted under a light microscope (Motic BA210, Spain). The results were expressed in numbers of counted spores per gram of biofertiliser.

Sterilised soil (autoclaved at 121 °C for 30 min) contained in a Petri dish was moistened to its maximum water-holding capacity with 0.1% (w/v) Trypan blue solution. This solution increases the visibility of hyphal growth (Habte and Osorio, 2001). Pieces of membrane filter of 10 × 10 mm (pore size 0.45 μm) were placed on another membrane filter of pore size 50 μm already positioned on the soil surface in the Petri dish. The membrane filters were pre-sterilised in 70% alcohol for 5 min and rinsed with water before use. A spore was placed on each of the filters, and after that, the Petri dish was covered and incubated in the dark at 20 °C. After 14 days of incubation, the spores were examined under a light microscope (Motic BA210, Spain). The viability is indicated by the germination (subtending hyphae) of the spores.

2.3. Molecular analysis of biofertilisers

2.3.1. DNA extraction

For bacterial isolates, a colony of an overnight culture on nutrient agar was placed in 30 μl of sterile polymerase chain reaction-grade water

and heated for 2 min in a microwave set at 1400 Watts (Defy, model DMO351, China) to lyse the cells and release the nucleic materials. Subsequently, the microwaved cell suspension was centrifuged (JP Selecta Centrifuge, Barcelona, Spain) at 10 000 rpm for 1 min and 2 mL of the supernatant was used as a template for PCR reactions. For the fungal isolates (not including the arbuscular mycorrhizal fungi), DNA was extracted from a 5-day old fungal growth on PDA using PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., CA, USA), following the manufacturer protocol. The DNA yield was quantified using Qubit® 2.0 fluorometer (Invitrogen, California, USA).

2.3.2. PCR amplification of 16S rRNA gene of bacteria and ITS region of fungi

The V3–V4 regions of the 16S rRNA gene of bacterial isolates were amplified using universal primer set 27F (5′-AGAGTTTGATCCTGGCT-CAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) while the fungal Internal Transcribed Spacer (ITS) regions 1 and 2 were amplified with primer set ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) (forward) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (reverse) (Ma et al., 2015). The PCR was performed in a T100™ (BioRad, USA) Thermal cycler. Each PCR reaction mixture contained 12.5 μL of one *Taq* 2x Master Mix with Standard Buffer (New England, Biolabs Inc. USA), 1 μL of DNA template (~50 ng), and 0.5 μL (10 μM) each of forward and reverse primers and nuclease-free water to a final volume of 25 μl. The thermocycling conditions were as follows: initial denaturation for 30 s at 94 °C, 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 50 s and extension at

Table 5. Estimation of functional abilities of isolates.

Isolate ID	Closest 16S rRNA gene relatives in the GenBank	IAA (μg/mL)	PSI (mm)	Acid phosphatase (μg/mL)	Siderophore (mm)
AN1	*Enterococcus faecium*	49,6	4,1 ± 0.8	36,8	-
AN2	*Alcaligenes faecalis* subsp. *faecalis*	38,1	-	25,9	-
ANP1	*Enterococcus ratti*	37,5	3,1 ± 0.1	23,7	47,3 ± 6.1
ANP3	*Bacillus wiedmannii*	63,3	-	18,7	-
BC1	*Bacillus velensis*	114,4	-	7,1	16,7 ± 1.5
BC3	*Acinetobacter junii*	70,0	1,7 ± 0.3	7,8	-
BC5	*Cryobacterium levicorallinum*	30,9	1,8 ± 0.0	7,1	15,7 ± 0.6
BC7	*Bacillus paralicheniformis*	9,3	-	8,9	16,3 ± 1.53
BN	*Ochrobactrum grignonense*	nd	1,9 ± 0.0	nd	-
NS1	*Pseudomonas japonica*	-	3,6 ± 0.3	33,6	56,3 ± 3.8
NS3	*Proteus hauseri*	49,7	-	33,9	30,7 ± 3.1
CP1	*Citrobacter werkmanii*	5,5	4,1 ± 0.3	37,1	16,0 ± 2.0
CP3	*Morganella morganii* subsp. *sibonii*	2,9	3,0 ± 0.1	36,8	14,0 ± 1.0
HS2	*Brevibacillus laterosporus*	6,3	1,5 ± 0.1	7,4	16,7 ± 3.2
HS3	*Arthrobacter oryzae*	23,6	-	7,6	16,3 ± 1.5
HS4	*Staphylococcus hominis* subsp. *novobiosepticus*	43,6	-	7,7	-
HS5	*Kocuria palustris*	14,8	-	6,6	16,0 ± 2.0
LF2	*Bacillus paralicheniformis*	17,7	1,4 ± 0.0	8,9	27,7 ± 2.5
LF4	*Acinetobacter junii*	54,4	2,0 ± 0.3	9,4	-
LF5	*Bacillus velezensis*	16,6	-	9,5	24,7 ± 2.5
NB1	*Pseudomonas veronii*	39,0	3,9 ± 0.1	21,1	28,3 ± 2.1
NB2	*Alcaligenes faecalis* subsp. *parafaecalis*	36.0	4,0 ± 0.3	24,8	16,3 ± 2.5
NB4	*Pseudomonas japonica*	25,1	4,7 ± 0.8	15,5	48,0 ± 2.7
RNB1	*Bacillus tequillensis*	nd	4,4 ± 0.1	-	-
NP1	*Bacillus megaterium*	0,3	-	23,7	-
NP2	*Hafnia paralvei*	1,4	3,7 ± 0.1	27,9	51,7 ± 4.0
NP3	*Enterococcus ratti*	0,16	4,3 ± 0.1	18,7	-
NP4	*Alcaligenes faecalis* subsp. *faecalis*	-	-	25,7	-
NT1	*Bacillus velezensis*	-	-	9,6	-
NT3	*Lysinibacillus sphaericus*	-	1,5 ± 0.0	10,2	-
NT4	*Cellulomonas denverensis*	7,8	1,3 ± 0.1	9,3	-
NT5	*Cellulomonas pakistanensis*	-	1,2 ± 0.1	8,1	-
NT6	*Acinetobacter junii*	19,3	1,7 ± 0.1	9,6	-
RN1	*Rhizobium multihospitium*	nd	2,4 ± 0.1	nd	-
O10	*Bacillus thuringiensis*	42.0	-	7,6	-
O12	*Lysinibacillus sphaericus*	17,3	2,9 ± 0.1	14,5	-
O14	*Gordonia humi*	11,6	-	7,7	-
O15	*Lysinibacillus sphaericus*	25,7	1,4 ± 0.0	8,6	-
O17	*Micrococcus aloeverae*	18.0	1,3 ± 0.1	10,3	-
O18	*Pseudomonas oleovorans* subsp. *lubricantis*	19,6	1,5 ± 0.1	11,6	25,7 ± 2.1
O19	*Serratia sp.*	14,5	-	9,5	-
O2	*Pseudomonas stutzeri*	52,2	1,9 ± 0.1	10,7	25,0 ± 2.7
O23	*Pseudomonas stutzeri*	14,6	1,5 ± 0.1	9,4	-
O3	*Micrococcus aloeverae*	17,2	1,7 ± 0.3	7,7	-
O4	*Micrococcus yunnanensis*	44,6	-	8,0	-
O5	*Pseudomonas alcaliphila*	64.0	1,5 ± 0.1	5,3	59,3 ± 3.8
O7	*Pseudomonas chengduensis*	56.0	1,7 ± 0.0	7,7	50,3 ± 3.5
SF2	*Enterococcus ratti*	4,2	3,5 ± 0.6	36,8	-
SF3	*Escherichia coli*	65,3	3,2 ± 0.7	21,8	21,7 ± 2.5
VQ2	*Cupriavidus metallidurans*	11,6	-	10,0	-
VQ3	*Bacillus velezensis*	-	1,4 ± 0.0	8,9	57,7 ± 4.0
VQ4	*Bacillus siamensis*	101,2	1,4 ± 0.0	9,3	59,0 ± 2.7
LVQ	*Bacillus megaterium*	-	1,9 ± 0.0	-	-

The standard deviations for PSI and siderophore production represent a triplicate number (n = 3). nd = Not determined. Isolates that showed no functional ability were not presented. Similar code represents isolates from the same biofertiliser.
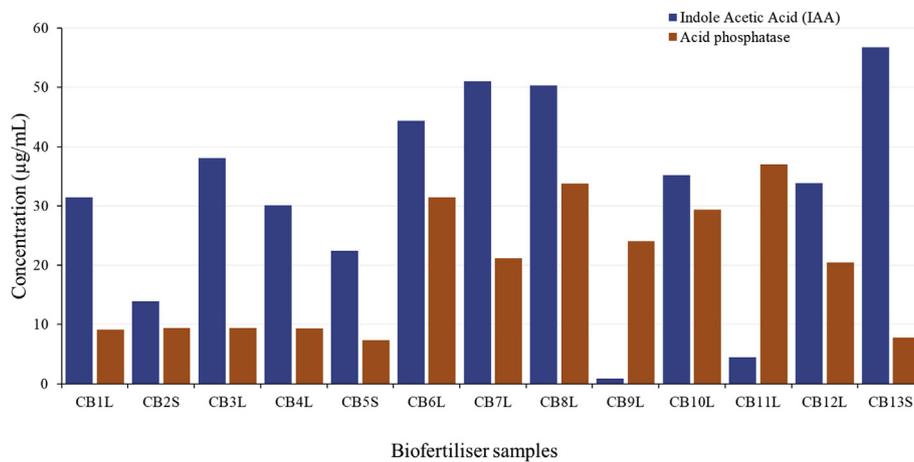
Figure 2. The average indole acetic acid and acid phosphatase production of the isolates contained in each biofertiliser. The collective contributions of all the isolates in each of the biofertilisers suggests the potential ability of the biofertiliser product for IAA and acid phosphatase production.
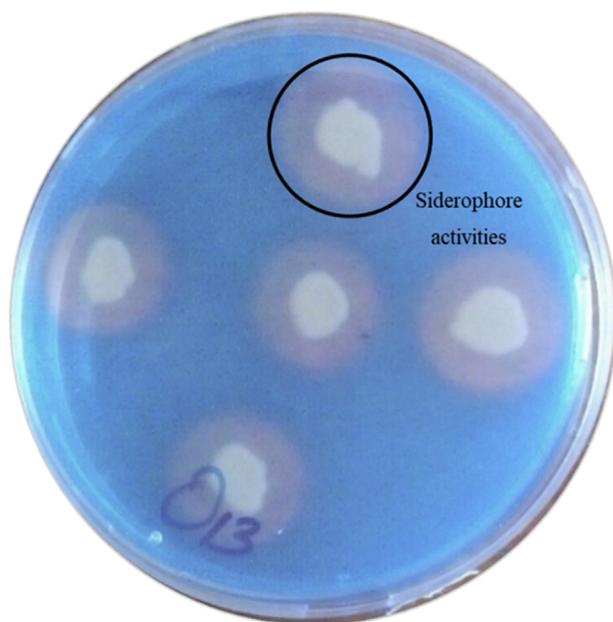

Figure 3. Chrome Azurol S (CAS) agar plate showing siderophore production by isolates. The appearance of orange halo zones suggests the activity of siderophore produced by the isolates.

68 °C for 60 s. The final extension was at 68 °C for 5 min. An aliquot of 2 μL PCR products was run on a 1% agarose gel at 80 V for 45 min to verify the integrity and sizes of the PCR amplicons.

2.3.3. Sequencing and taxonomic assignment

The PCR amplicon samples were sequenced at the Central Analytical Facilities, Stellenbosch University using universal primers 27F and 1492R for bacteria and, ITS1 and ITS4 for fungi. Sequences obtained were manually inspected, edited, and bidirectional sequences merged using BioEdit Sequence Alignment editor to generate a contiguous consensus sequence. For the taxonomic assignment, contiguous sequences were matched against available sequences in the National Centre for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) and the most similar hit from the GenBank database was extracted. A phylogenetic tree of the obtained sequences with relatives above 97% similarity was constructed using MEGA software 7.0.25 (Kumar et al., 2016). The evolutionary history of the sequences was computed using the Neighbor-Joining method. The

percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter model and are in the units of the number of base substitutions per site. The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). All positions with less than 95% site coverage were eliminated (Kumar et al., 2016).

2.4. Plant growth-promoting potential of isolates

The N-fixing potential of isolates was examined on Burks medium, and nitrogen-free bromothymol blue medium (Nfb) supplemented with 15 g agar (Table 1) (Rodrigues et al., 2016). The phosphate-solubilisation ability was determined by plate assay using National Botanical Research Institute's Phosphate medium (NBRIP) (glucose 10 g, Ca$_3$(PO4)$_2$ 5 g, MgCl$_2$.6H$_2$O 5 g, MgSO$_4$.7H$_2$O 0.25 g, KCl 0.2 g, (NH$_4$)$_2$SO$_4$ 0.1 g/L, pH 7) supplemented with 15 g Bacto-agar (Difco Laboratories, Detroit, MI, USA), following the method of Bello-Akinosho et al. (2016). The inorganic phosphate (Pi) solubilisation indices were calculated using the formula in Eq. (1).

$$Pi\ Solubilisation\ index(PSI) = \frac{diameter\ of\ halo\ zone + well(mm)}{diameter\ of\ well(mm)} \qquad (1)$$

The production of IAA by the isolates was estimated using Salkowski's reagent (1 mL of 0.5 M FeCl$_3$ in 50 mL 35% (v/v) HClO$_4$ solution), following the method of Patten and Glick (2002). The absorbance of the IAA mixture was measured at 540 nm and the concentration was calculated by comparing with a standard curve. The acid phosphatase assay was analysed using ρ-nitrophenyl phosphate (ρNPP) (Sigma-Aldrich, India) as the organic phosphorus substrate by employing the summarised methods of Behera et al. (2017). Siderophore activity on chrome azurol S (CAS) blue agar with hexadecyltrimethylammonium bromide (HDTMA) as a colour indicator was performed according to the method of Schwyn and Neilands (1987).

2.5. Biofertiliser quality evaluation

For biofertiliser quality evaluation, this study considers any microbe not declared by the manufacturer but detected in the product as a potential contaminant (undeclared microbes). Based on declared and undeclared microbes being present or absent, the products were categorised as high, medium, low or of poor quality (Table 2). The comparison between the detected isolates and that declared on the label was done at the genus taxa level.
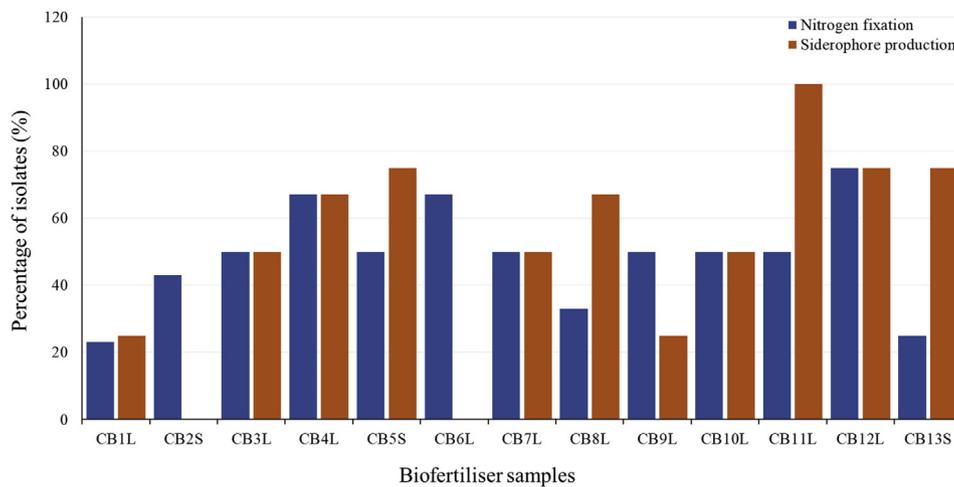
Figure 4. Nitrogen fixation and siderophore production potential of biofertilisers. The potential is indicated by the percentage of isolates that can fix nitrogen or produce siderophore in the biofertiliser samples.
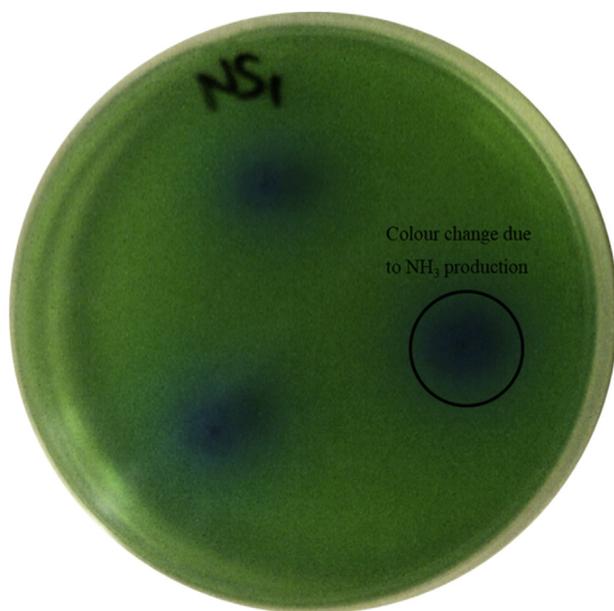

Figure 5. Nitrogen fixation potential of isolates on nitrogen-free bromothymol blue agar. The change in colour of the medium from green to blue as a result of ammonia production suggests the potential of the isolates to fix nitrogen.

2.6. Statistical analysis

Canonical correspondence analysis (CCA) was used for evaluating the plant growth-promoting (PGP) capabilities of the biofertilisers (ter Braak and Šmilauer, 2012). The approach related microbial communities isolated from each biofertiliser to their PGP capabilities. The microbes declared in the label of each biofertiliser were scored 1 when present and 0 if absent. The CCA technique was then employed to assess the PGP capability of each biofertiliser using the result already obtained from the *in vitro* assessment of the PGP potential of the isolates. Data were coded using positive (+ve) or (1) if isolates have PGP attributes and negative (-ve) or (0) if it did not. The CCA technique was applied in Canoco version 5.0 (ter Braak and Šmilauer 2012), extracting ordination axis that (i) maximised the separation of microbes in space in relation to their abilities to produce specific growth-promoting attributes as well as (ii) maximised the separation of biofertilisers with respect to their capabilities for plant-growth promotion. The resulting CCA biplots showed different PGP attributes such as phosphate solubilisation, N-fixation, siderophore activity, or production of IAA or acid phosphatase, as functional arrows. The arrows were plotted in the direction of their maximum change. Thus, long arrows indicated high capability for such a function. Species occurring in positions close to or beyond the tip of the specific arrow were strongly and positively correlated with that function.

2.7. Data accessibility

The sequences obtained in the present study have been deposited in the GenBank of the National Centre for Biotechnology Information (https://www.ncbi.nlm.nih.gov) under the accession numbers MN414326-MN414328 and MN416954-MN417008 for fungi and bacteria, respectively.

3. Results

3.1. Total viable cell and spore count

An important criterion to determine the quality of biofertiliser is the total viable count. This is because biofertiliser must supply a substantial amount of live microbes to the field for a guaranteed field efficiency. Hence, a good quality biofertiliser should have a total viable count within the acceptable quality standard or that tallies with those declared by the manufacturer on the product label. Sample CB4L had a total viable count

Table 6. Canonical Correspondence analysis (CCA) axis and eigenvalues.

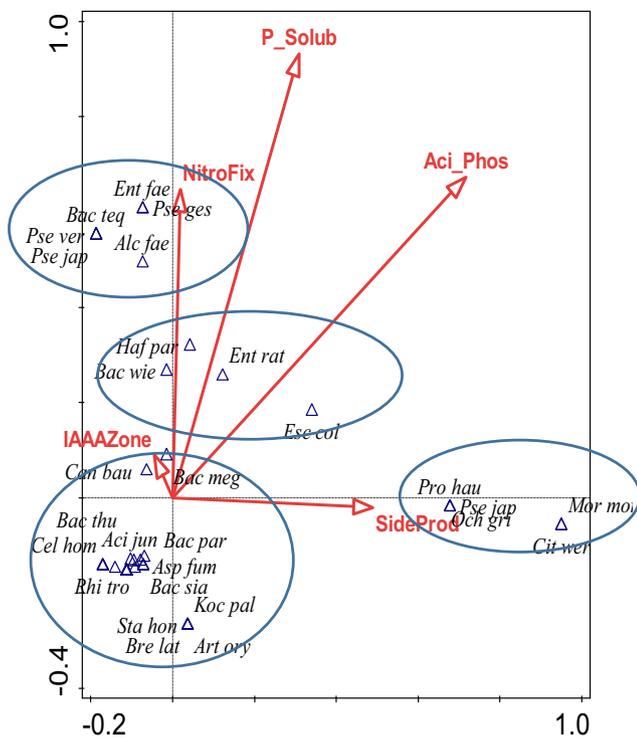| Parameters	Axis 1	Axis 2	Axis 3	Axis 4
Eigenvalues	0.9685	0.9380	0.7940	0.5658
Explained variation (cumulative)	11.19	22.03	31.21	37.75
Pseudo-canonical correlation	0.9954	0.9965	0.9650	0.9322
Explained fitted variation (cumulative)	25.67	50.53	71.57	86.56
Permutation Test results (All axes)	pseudo-F = 1.1	$P = 0.094$		

Figure 6. Canonical Correspondence analysis (CCA) Biplot: points (triangles) represent species isolated with respect to their positions along a particular functional arrow [Solubilisation of phosphate (P_Solub); nitrogen fixation (NitroFix); siderophore production (SideProd); indole acetic acid production (IAAzone) and acid phosphatase production (ACi_Phos)].

Figure 7. CCA Biplot: points (circles) represent biofertilisers (1–7) with respect to their positions along a particular functional arrow. [Solubilisation of phosphate (P_Solub); nitrogen fixation (NitroFix); siderophore production (SideProd); indole acetic acid production (IAA zone) and acid phosphatase production (ACi_Phos)].

of 3.6×10^9 CFU/mL that is more than what was reported in the label (1.0×10^9), while samples CB1L (1.53×10^7) and CB2S (5.40×10^7) had lesser count when compared to the information reported in the product labels, 1.0×10^9 and 3.0×10^9, respectively. Eight of the biofertilisers did not have viable cell count for the microbes listed in the labels (Table 3). The MPN technique revealed the products tested contained no *Azospirillum* sp. In addition, the AMF spore count for sample CB13S showed 194 spores/g of sample, of which 78% developed germ tubes.

3.2. Microbial isolation and sanger sequencing of 16S rRNA gene and ITS regions 1 and 2

A total of three fungal and 58 bacterial isolates were obtained from all the culture media types. The 16S rRNA gene sequences of bacterial isolates were clustered into 28 OTUs while the ITS region sequences of fungal isolates clustered into two OTUs. The evolutionary association of the sequences with their close relatives in the GenBank are depicted in the phylogenetic tree (Figure 1). The *Rhizobium* and *Bacillus* species present in the labels of CB2S and CB4L, respectively, were detected by our analysis. Of the six genera declared to be present in samples CB1L and CB3L, only *Bacillus* and *Pseudomonas* were detected for CB1L while only *Bacillus* was detected for CB3L. Other microbial species detected but not declared in the label included bacterial species *Acinetobacter junii, Brevibacillus laterosporus, Alcaligenes* sp. and *Enterococcus ratti* and fungal species *Aspergillus fumigatus* and *Candida ethanolica*. Though CB13S (AMF product) contained the above fungal species that are not known to be AMF species, the present study did not identify the isolated spores.

3.3. Determination of biofertiliser quality and level of contamination

The OTUs obtained from Sanger sequences revealed that all the biofertilisers had more microbes than those declared by the
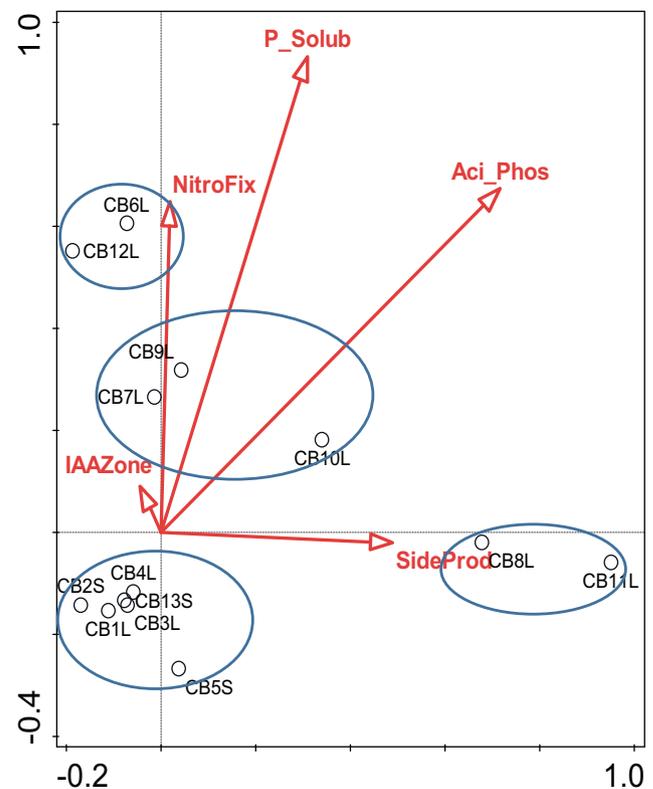
manufacturers, implying the presence of potential contaminants in the products (Table 4). Consequently, applying the criteria defined in Table 2, none of the biofertilisers can be regarded as a high-quality product. Three products were categorised as medium quality (CB2S, CB4L and CB13S, containing rhizobia, PGPRs and AMF, respectively) because they also had other microorganisms that were not declared in the labels. The consortium products (CB1L and CB3L), which had a lower number of the declared species and several other species not listed in the labels were regarded as low-quality products. Other products including all the free-living N-fixing biofertilisers and three of each rhizobia and "other PGPR" products were regarded as poor quality because none of the microbes declared in the label was amongst the OTUs detected (Table 4).

3.4. Plant growth-promoting attributes of isolates

3.4.1. Production of indole acetic acid

Eighty-seven percent of the isolates produced IAA in the presence of tryptophan as a precursor. Isolate BC1 from sample CB13S produced the highest amount of IAA, about 114.4 μg/mL while isolate NP3 from CB9L produced a relatively smaller quantity, about 0.1 μg/mL (Table 5). The production of IAA was not detected in seven of the isolates, including NS1, NP4, NT1, NT3, NT5, VQ3 and LVQ, which are *Pseudomonas japonica, Alcaligenes faecalis* subsp. *faecalis, Bacillus velezensis, Lysinibacillus sphaericus, Cellulomonas pakistanensis, Bacillus velezensis* and *Bacillus megaterium,* respectively. The average concentrations of IAA produced by all the isolates in each of the biofertilisers are presented in Figure 2. Biofertiliser sample CB13L had the highest average IAA production, followed by sample CB7S, while sample CB9L was reported with the least average IAA production (Figure 2).

3.4.2. Phosphate solubilisation ability

Sixty percent of the isolates exhibited high phosphate-solubilisation ability, as indicated by the halo-zone formation on NBRIP agar. The halo zone ranged from an average minimum of 1.5 mm occurring with isolate NT5 to a maximum of 18.3 mm with isolate NB4 (P-solubilisation index (PSI) of 1.20 and 4.67, respectively) (Table 5). Seventeen isolates including AN2, ANP3, BC1, BC7, NS3, HS3, HS4, HS5, LF5, NP1, NP4, NT1, O10, O14, O19, O4 and VQ2 showed no sign of solubilisation ability on NBRIP agar.

3.4.3. Acid phosphatase assay

Some of the isolates produced acid phosphatase, which was evidenced by the release of phosphorus from the metabolism of ρNPP, an organic phosphorus source. Highest enzyme activity was observed in isolates CP1 and CP3 from sample CB11L with a concentration of 37.1 μg/mL and 36.8 μg/mL, respectively, while the least production occurred in isolate HS5 with 6.6 μg/mL from sample CB5L (Table 5). The average enzyme activity in each of the biofertiliser products is shown in Figure 2. Seven of the biofertiliser samples, including CB11L, CB8L, CB6L, CB10L, CB9L, CB7 and CB12L had an average concentration above 20.4 μg/mL compared to other samples with an average concentration below 9.4 μg/mL (Figure 2).

3.4.4. Detection of siderophore production

Forty percent of the isolates produced the iron-chelating agent, siderophore, as indicated by the orange halo zone on CAS agar (Figure 3). Isolates O5 and VQ4 from samples CB1L and CB3L, respectively had the highest siderophore production capability (Table 5). All the isolates in CB11L and 25% of isolates in samples CB1L and CB9L had the potential for siderophore production (Figure 4). However, the production of siderophore was not detected in isolates from samples CB2S and CB6L.

3.4.5. Nitrogen-fixing potential

The analysis showed that about 40% of the isolates had the potential to fix atmospheric N, as shown by the change in colour of the nitrogen-free medium from green to blue due to the production of ammonia (Figure 5). Sample CB12L had the highest percentage of isolates with the potential to fix nitrogen while CB1L had the least (Figure 4).

3.5. Correlation between isolates and plant growth-promoting capabilities

3.5.1. Isolated microorganisms and their growth-promoting capabilities

Four main clusters of microbial species were identified through the CCA by plotting their PGP attributes. The CCA four-axis accounted for most of the variation in the data (44%) indicating eigenvalues as follows (Axis 1 = 096875, Axis 2 = 0.9380, Axis 3 = 0.7940 and Axis 4 = 0.56587, pseudo-F = 11, P = 0.094) (Table 6).

The first cluster consisted of *Enterococcus faecium* (Ent fae), *Bacillus tequilensis* (Bac teq), *Alcaligenes faecalis* subsp. *faecalis* (Alc fae), *Pseudomonas gessardii* (Pse ges), *Pseudomonas veronii* (Pse ver) and *Pseudomonas japonica* (Pse jap). Species in this cluster exhibited a high ability for N fixation and IAA production but were unrelated to siderophore production. The same cluster showed fairly high capabilities for P solubilisation and acid phosphatase production (Figure 6). The second cluster, which consisted of *Escherichia coli* (Esc col), *Enterococcus ratti* (Ent rat), *Candidimonas bauzanensis* (Can bau), *Bacillus wiedmannii* (Bac wie), *Hafnia paralvei* (Haf par) and *Bacillus megaterium* (Bac meg) exhibited high IAA production but lower ability for N-fixation, P solubilisation and acid phosphatase production in comparison to the first cluster. This cluster was also unrelated to siderophore production. A third cluster consisted of *Pseudomonas japonica* (Pse jap), *Ochrobactrum grignonense* (Och gri), *Morganella morganii* subsp. *sibonii* (Mor mor), *Citrobacter werkmanii* (Cit wer) and *Proteus hauseri* (Pro hau) (Figure 6). This cluster had the highest ability for siderophore production and some moderate levels of acid phosphate production as well as P solubilisation but was largely unrelated to N-fixation and even showed some levels of a negative association

with IAA production. The rest of the isolates including *Kocuria palustris* (Koc pal), *Staphylococcus hominis* subsp. *novobiosepticus* (Sta hon), *Brevibacillus laterosporus* (Bre lat) and *Arthrobacter oryzae* (Art ory) were grouped into a fourth cluster (Figure 6). Although cluster 4 was associated with minimal IAA production, largely there was insufficient evidence that showed capabilities for other growth-promoting attributes.

3.5.2. Biofertiliser capabilities for plant growth-promoting attributes

With respect to the capabilities of biofertilisers to exhibit PGP attributes, four clusters were evident (Figure 7). The first cluster consisted of biofertilisers CB6L and CB7L, which showed high capabilities for N-fixation and IAA production but was unrelated to siderophore production (pseudo-F = 11), P = 0.094 (Table 6). In addition, both biofertilisers exhibited fairly high capabilities for P solubilisation as well as acid phosphatase production. The second cluster consisted of biofertilisers CB12L, CB7L and CB6L. This cluster was fairly high in IAA production but relatively lower in the ability for N-fixation, P solubilisation, and acid phosphatase production in comparison to the first cluster. This cluster was also unrelated to siderophore production. A third cluster which consisted of biofertiliser CB12L and CB7L exhibited the highest ability for siderophore production. Both these biofertilisers also showed some levels of acid phosphatase production as well as P solubilisation but was largely unrelated to N-fixation and exhibited some level of a negative association with IAA production. Biofertilisers CB12L, CB7L and CB6L occurred in all clusters that exhibited high levels of PGP attributes under investigation (Figure 7).

4. Discussion

The quality and potential field efficiency of biofertilisers are greatly affected by the genetic and functional diversity, as well as the total number of viable cells of the microbes declared to be in the products (Lupwayi et al., 2000; Raimi et al., 2019). Several quality assessments had shown that many biofertilisers evaluated do not have the manufacturers' declared microbial composition (Herridge et al., 2002; Herrmann et al., 2015; Singleton et al., 1997). In some situations, biofertilisers sold globally have been reported to contain no microbial species (Lupwayi et al., 2000; Olsen et al., 1995). These observations are corroborated by the results obtained in this study where some of the biofertilisers sampled did not have the expected microbes. Where the bacteria or fungi were detected, their total viable counts were below the manufacturers' specification and the acceptable quality standard (Herridge et al., 2002; Malusá and Vassilev, 2014). For the AMF biofertiliser tested, adequate viable spores within the acceptable standard (>100 spores/g) was observed, implying the product may be of good quality. Microbial viability is essential for initial infectivity or colonisation of the host plant as well as the subsequent exhibition of functional abilities (Habte and Osorio, 2001; Rodríguez-Navarro et al., 2010). This accentuates the need for biofertilisers to contain viable cells and spores that are metabolically and physiologically competent for field efficiency.

The microbial community as revealed by the 16S rRNA gene and ITS region sequences showed *Bacillus*, *Rhizobium* and *Pseudomonas* as the only genera represented amongst the microbes declared in the product labels. Similar to other studies, these genera have been reported in different types of biofertilisers (Herrmann et al., 2015; Raimi et al., 2017). Besides, other genera such as *Alcaligenes, Morganella, Hafnia, Citrobacter, Candida* and *Aspergillus*, which were not declared in the labels, were also detected as part of the products' contents. Some of the products, especially the consortium, had less or none of the declared microbes, suggesting the products may not be efficient when applied in the field. For a guaranteed field effectiveness, a substantial quantity of the viable microbe(s) listed on the label must be supplied to the field (Lupwayi et al., 2000; Herridge et al., 2002). This is necessary to increase the ability of biofertiliser species to exhibit their functional abilities and outcompete the indigenous microbes (Faye et al., 2013; Herridge et al., 2002). Formulation of consortium products with microbes of better

competitive capabilities over the diverse native microbes is essential for field efficiency (Pindi and Satyanarayana, 2012). However, carrier formulation for consortium products is very challenging. Most carriers for consortium products are less selective and hence promote the growth of diverse microbes, including undesired microbes that may cause product damage (El-Fattah et al., 2013). A similar observation was reported in this study where the consortium products had more microbes than what was declared by the manufacturer. This may be linked to formulation challenges.

The biofertilisers analysed had diverse microbial contaminants with major ones found in the liquid products. Liquid medium offers an abundance of readily available substrates such as amino acids, sugars, minerals and salts for microbial growth and development (Pindi and Satyanarayana, 2012). This consequently suggests the reason for the detection of diverse microbes not declared in the label of the liquid products. Some of the contaminants have been reported as opportunistic pathogens in human, animal and plant (Herrmann et al., 2015; Olsen et al., 1996). Though this study did not assess the pathogenicity of the microbial community, several studies have elucidated on the ability of some of these microbes to cause diseases. For example, *Acinetobacter junii, Arthrobacter oryzae* and *Alcaligenes faecalis* subsp. *faecalis* found in this study have been reported as human and animal pathogens (Saffarian et al., 2017; Tille, 2013). Other previously reported pathogenic strains isolated in the present study included *Cellulomonas denverensis, Escherichia coli, Enterococcus ratti* and *Staphylococcus hominis* subsp. *Novobiosepticus* (Brown et al., 2005; Chaves et al., 2005; Rivas et al., 2015). Therefore, the quality control system should be improved to reduce, if not eliminate, the incidence of microbial contaminants in biofertilisers.

Interestingly, many of the isolates, including those not declared in the label considered to be potential contaminants, exhibited diverse PGP characteristics. Hence, the presence of undeclared species with multiple broad-spectrum PGP activities may reduce the risk of limited efficacy of the products. Moreover, not declaring the species on the label may also be the manufacturers' strategy to conceal the identity of the microbial contents of the products. Such undeclared strains may be specifically added to avoid risk from control issues, especially when viable cells of the main species are not in a sufficient amount.

The results of past *in vitro* analyses of microbial functions offer an insight into the potential efficiency of the isolated microbes especially as it relates to N-fixation, P solubilisation and the production of phosphatase, IAA and siderophore (Ahmad et al., 2005; Baldani et al., 2014 Solanki et al., 2014). Similar to the observations in this study, the ability of *Pseudomonas, Bacillus* and *Alcaligenes* to fix N, solubilise P and as well synthesise IAA, have been widely reported (Beneduzi et al., 2008; Elmerich and Newton, 2007). In P-deficient soils, P solubilising biofertiliser is a good alternative to increase the available form of soil P for crop use (Adeleke et al., 2010; Saiyad et al., 2015). In the present study, *Citrobacter, Alcaligenes, Bacillus* and *Pseudomonas* exhibited high P solubilisation ability. Previous studies have reported similar observations using the formation of halo zone on phosphate agar plate when microbes use organic acids such as gluconic, oxalic and citric acids to solubilise the inorganic P in the media (Adeleke et al., 2010, 2017; Behera et al., 2017). In addition, acid phosphatase has a huge potential in the solubilisation and biogeochemical cycle of P. Acid phosphatase activities similar to that obtained in the present study, which employed para-nitrophenyl phosphate as an organic phosphate in culture media have also been reported. For example, Fitriatin et al. (2011), observed a range of 2.0–4.96 μg/ml of phosphatase production in *Pseudomonas, Micrococcus* and *Flavobacterium* while Saiyad et al. (2015) reported a range of 0.061–0.164 μg/ml in *Bacillus* species. Rhizosphere beneficial microbes produce most phosphatases found in the soil. Hence, isolates with high P solubilising capability can be used in the formulation of phosphate biofertiliser (Ribeiro and Cardoso, 2012).

The production of IAA has been reported in various microbes such as *Trichoderma, Bacillus* and *Pseudomonas* (Ahmad et al., 2005; Dixit et al., 2015). The present study showed *Hafnia paralvei* and *Bacillus velensis* produced the lowest and the highest concentrations at 1.6 and 115.3 μg/ml, respectively. The possible factors responsible for the variations in IAA production ability of the isolates may be attributed to the position of concerned genes, biosynthetic pathways and the presence of specific enzymes that convert active IAA to other forms (Patten and Glick, 2002). Predominantly, *Pseudomonas, Alcaligenes, Enterococcus* and *Bacillus* showed high siderophore production ability in this study. This is similar to the observations by Ribeiro and Cardoso (2012) and therefore confirming the ability of these organisms in the production of efficient biofertilisers.

5. Conclusion

The total viable count for most of the biofertilisers analysed did not meet the manufacturers' specification and the quality standard for biofertilisers. More so, over 60% of the products did not contain the microbes declared in the labels, which implies the products are possibly of low quality. In spite of the aforementioned results, the majority of the isolates, including the contaminants have the potential for N-fixation, P solubilisation and, IAA and siderophore production. Four clusters of biofertiliser were correlated with different functional capabilities, suggesting these products have PGP traits that can effectively improve crop productivity. In general, efficient quality control systems that support a regular assessment of biofertiliser quality, from production point to marketplace are necessary for the production and marketing of good quality products. In general, the present study investigated a limited sample size; therefore, broader surveys are necessary to investigate a larger number of samples, following standardised legal sampling and analytical procedures. This will provide a broader perspective and contribute to the growing need for the development of efficient biofertilisers.

accession numbers MN414326-MN414328 and MN416954-MN417008 for fungi and bacteria, respectively.

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