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ORIGINAL ARTICLE

Plant growth promoting potentials of novel phosphate-solubilizing bacteria isolated from rumen content of White Fulani cattle, indigenous to Nigeria

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Abstract

Phosphate-solubilizing bacteria (PSB) with plant growth promoting (PGP) traits enhance sustainable agriculture. Due to this, bacteria capable of promoting the growth of plants were isolated from rumen content of indigenous White Fulani cattle. The phosphate solubilizing potentials of the bacterial isolates and their plant growth promoting activities were assessed following standard techniques. The total bacterial population in the rumen contents ranged from $5.20 \pm 0.36 \times 10^5$ to $4.00 \pm 0.01 \times 10^6$ CFU g^{-1}). Out of 23 isolates, 61% exhibited varying P-solubilization efficiency which ranged between 144 µg mL^{-1} and 687.75 µg mL^{-1}. Other plant growth promoting activities differed among bacterial isolates. The 16 S rRNA gene sequencing analysis confirmed 57% of the isolates that expressed multiple PGP ability as either *Pseudomonas aeruginosa* or *Escherichia coli*. Among the genus *Escherichia*, *E. coli* AKRC16 showed most promising result by liberating 345 µg mL^{-1} soluble P. Overall, *P. aeruginosa* AKRC7 showed highest PGP activity (687.75 µg P mL^{-1}) at pH 4.2 after 120 h, IAA (39.27 µg mL^{-1}), ammonia (3.89 µmol mL^{-1}) and also displayed ability to fix nitrogen. *P. aeruginosa* AKRC7, *E. coli* AKRC16 and *E. coli* AKRC4 consistently, in that order, exhibited highest productivity for both enzymes at the same pH (6), incubation time (24 h), and optimal source of nitrogen and carbon for phosphatase (tryptone and starch) and phytase (peptone and glucose) correspondingly. Furthermore, *P. aeruginosa* AKRC7 and *E. coli* AKRC16 enhanced 100% germination of tomato seeds with significantly high vigour index (2177 and 2114.33) and P content (309.04 ± 0.05 mg g^{-1} and 301.01 ± 0.02 mg g^{-1}) of seedlings respectively. The findings revealed rumen content as potential reservoir of prominent PGPB and presented *P. aeruginosa* AKRC7 (MK332569) and novel strains of *E. coli* AKRC4 (MK332563) and AKRC16 (MK332576) as promising candidate for bio-inoculation.

Keywords Rumen PSB · Phosphate-solubilizing bacteria (PSB) · Tricalcium phosphate (TCP) · Seed bacterization · Biofertilizer · Bio-inoculation

Abbreviations

P	Phosphorus
Pi	Inorganic phosphate
PSB	Phosphate Solubilizing Bacteria
PSM	Phosphate Solubilizing Microorganisms
PGP	Plant Growth Promoting
PGPM	Plant Growth Promoting Microorganisms
NBRIP	National Botanical Research Institute's Phosphate
TCP	Tricalcium phosphate
CFU	Colony Forming Unit
SI	Solubilization Index
IAA	Indole Acetic Acid
HCN	Hydrogen Cyanide
SVI	Seed Vigour Index
SGR	Seedling Germination Ratio
PCR	Polymerase Chain Reaction
UV	Ultraviolet
DW	Distilled Water
CMC	Carboxymethyl cellulose
SD	Standard Deviation
SE	Standard Error

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Background

The relevance of phosphorus (P) as the second most important macronutrient cannot be overemphasized (Gupta et al. 2018). This P stimulates plant growth and maturity (Wortmann et al. 2014), whose deficiency limits plant growth (Yu et al. 2011). Despite its importance, P is much less abundant in soil globally. This is because out of the reserve of total P (typically within 0.2-5 g P kg^{-1} with an average of 0.6 g P kg^{-1}) in agricultural soil (Zhang et al. 2017), a significant fraction is locked in an insoluble form in various organic and inorganic substrates (Zhang et al. 2018; Tang et al. 2020). Consequently, only < 10% of total P enters the plant-animal cycle (Panhwar et al. 2014; Zhang et al. 2017), making it a limiting plant nutrient. Therefore, it is quite common to apply phosphatic fertilizers to agricultural soil to ensure substantial phosphorus is available for plants. Only a small portion of total P in phosphatic fertilizers applied on agricultural soil is utilizable by plants, as majority (75–90%) of it, rapidly form complexes with soil constituents like Al, Fe and/or Ca (Toro 2007; Cao et al. 2018), making it highly unavailable for plant. Meanwhile, apart from the cost, accumulation of complex compounds resulting from excessive application of phosphatic fertilizer pose several environmental threats (Zhang et al. 2018).

Natural agricultural practices such as application of organic fertilizer (Pare et al. 2000; Dittmar et al. 2009), plant health management system using antagonists of phytopathogens (Zaidi et al. 2014, 2016) and plant growth promoting microorganisms (PGPM) as biofertilizers (Rodrigues et al. 2016; Zhang et al. 2017) that promise environmentally sustainable development, have currently been considered among diverse biotechnological approaches to enhance plant growth (Khan et al. 2013; Rizvi et al. 2014; Tang et al. 2020). More interestingly, promoting growth and nutrient absorption in plant by biofertilizers has recently been reported to have potential of reducing the use of chemical fertilizers by half without loss (Pereg and McMillan 2015), and also increasing plant tolerance to abiotic and biotic stresses through biocontrol (Olanrewaju et al. 2017).

Many studies have documented the isolation of PSB from different natural environment, among which include; rhizosphere soil (Zaidi et al. 2009; Sadiq et al. 2013; Wang et al. 2017), acid sulfate soil (Panhwar et al. 2014) and mushroom residues (Zhang et al. 2017). Despite the complexity of rumen as a habitat, reports suggests that rumen of cattle could harbour PSM. Considering that Africa's ruminants graze on grass (Cohen 1980) and the P constituent of grass has been estimated to range from 0.2 to 1.5 g P kg^{-1} (Tenikecier and Ates 2018), phosphate-solubilizing microbes are hence required to hydrolyse or enhance the metabolism of phosphorus in their rumen. Meanwhile, most of the microorganisms associated with the plant rhizosphere soil, classified as PGPM, particularly PSB are not only capable of solubilizing mineral phosphates, among other nutrients, they also promote associative/atmospheric nitrogen fixation; synthesize or induce production of some plant hormones such as indole-3-acetic acid (IAA) and gibberellic acid; siderophores; hydrolytic enzymes; and antimicrobial agents (Goswani et al. 2014; Olanrewaju and Babalola 2018).

Since production of certain organic acids (such as acetic acid), hydrolysing enzymes and regulating molecules (such IAA, HCN and siderophore) by bacteria are responsible for solubilizing insoluble phosphate (Dutta et al. 2015; Wang et al. 2017), we hypothesized that PSB with such traits in this study, promise good PGP performance. The study was therefore design to isolate and characterize PSB with plant growth promoting (PGP) potentials from rumen content of White Fulani cattle.

Results

Isolation of microorganisms from rumen content of White Fulani cattle

The bacterial population in the rumen content in Table 1 ranged from $5.20 \pm 0.36 \times 10^5$ CFU g^{-1} to $4.0 \pm 0.07 \times 10^6$ CFU g^{-1} while the total fungal count (TFC) ranged between $0.14 \pm 0.02 \times 10^5$ CFU g^{-1} and $0.06 \pm 0.01 \times 10^6$ CFU g^{-1}. A total of 31 microbes; 74% bacterial and 26% fungal isolates, were recovered from the rumen content.

Following cultural, morphological and biochemical characterization, 56% of the total bacterial isolates were identified as gram negative and the rest 44% were gram positive. Bacterial isolates were tentatively identified as belonging to either of six genera with varying distribution including; Pseudomonas (6), Staphylococcus (4), Escherichia (4), Bacillus (3), Micrococcus (3), Klebsiella (2) and Streptococcus (1).

Phosphate solubilizing potentials of bacterial isolates

The ability of bacterial isolates recovered from the rumen content to solubilize P was qualitatively enumerated solubilization index (SI) (Fig. 1). Of the 23 bacterial isolates, only 61% could solubilize P on NBRIP medium and were grouped as Phosphate Solubilizing Bacteria (PSB). The isolates exhibited varying SI ranging from 0.43 ± 0.02 by *P. aeruginosa* RC22 to 1.32 ± 0.43 by *P. aeruginosa* RC7. The solubilization index exhibited by *P. aeruginosa* RC7 was significantly higher than those from other isolates at $p \leq 0.05$.

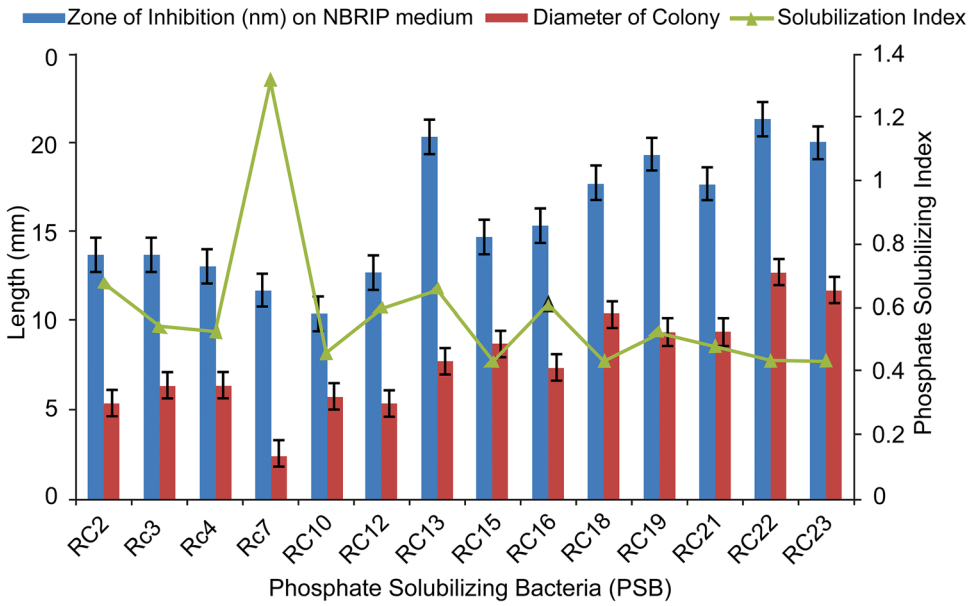

Fig. 1 Estimation of Phosphate Solubilization Index (SI) of bacteria isolated from rumen content of White Fulani cattle. SI were estimated after 4 d of incubation in a triplicate experiment recorded as Mean ± SE; Error bars indicates significant differences at $p \leq 0.05$ with One-way ONOVA by Duncan's test

Plant growth promoting trait of PSB isolates

The plant growth promoting substances released by PSB isolated from rumen content varied considerably, according to results obtained from in-vitro assessment, presented in Online Resource Table S1. The test for siderophore production ability (Fig. 2) revealed that isolates *P. aeruginosa* RC7, *E. coli* RC13, *E. coli* RC16 and *P. aeruginosa* RC22 produced three forms of siderophore including catecholate, hydroxymate and carboxylate, while isolates *Staphylococcus* sp.RC3, *P. aeruginosa* RC10 and *Staphylococcus* sp.RC21 were not able to produce siderophore.

Molecular characterization of rumen PSB with PGP traits

Among the 14 PSB isolated from rumen content, 10 strains with prominent PGP traits were selected and tagged rumen PSB isolates for further identification based on 16 S rRNA gene sequence. The BLAST results show that eight (8) of the PSB have high similarities with different strains four of which are *Pseudomonas aeruginosa* and other four were *Escherichia coli*. The sequence data were deposited in public domain and the Accession Numbers allocated to these rumen PSB strains are presented in Table 2.

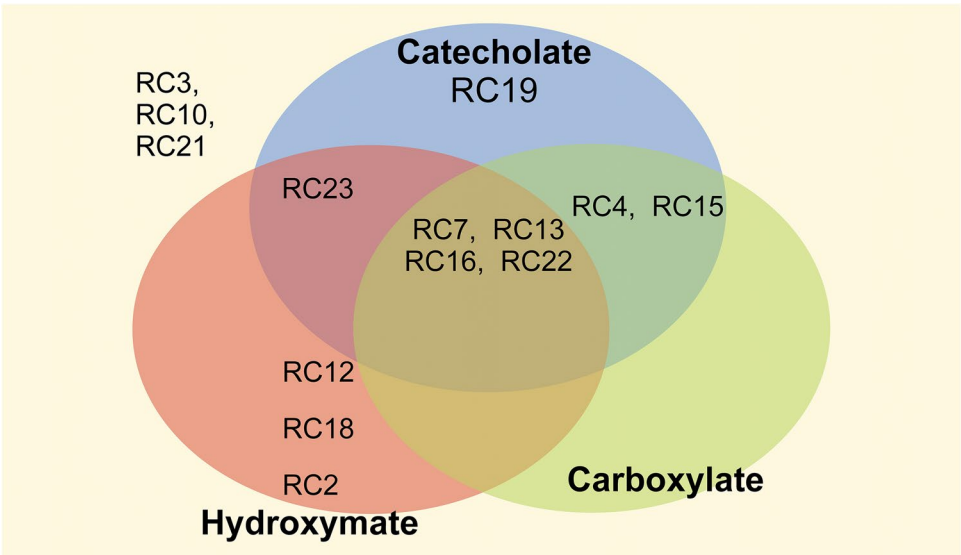

Fig. 2 Venn chart showing the distribution of siderophore producing PSB isolated from rumen content. Hydroxymate and catecholate siderophore determined by ferric chloride test and carboxylate siderophore determined by phenolphthalein test

Concentration of PGP metabolites produced by rumen PSB

The result of quantitative assay for organic phosphate production by rumen PSB for a period of 7 days is depicted in Fig. 3. During incubation, there was a progressive increase in the concentration of phosphate released by the 10 rumen PSB strains with the highest concentration of 687.75 µg mL^{-1} produced by *P. aeruginosa* AKRC7 on day 4. *E. coli* AKRC16 liberated the least concentration of 207.75 µg mL^{-1} P after day 7. *E. coli* AKRC16 however released maximum (345 µg mL^{-1} P) phosphate among *Escherichia* spp. after 5 days of growth.

The pH of media used to assess phosphate solubilizing potential of strains of rumen PSB showed constant decrease in value, indicating increasing acidity (Fig. 4). Furthermore, highest initial pH value for all cultured broth was 6.8 but decreased through day 7 of incubation to the least value of 4.2.

The concentration of IAA produced by rumen PSB after 5 days of incubation period depicted in Fig. 5 shows that the 10 isolates were able to produce varying amount of IAA. *E. coli* AKRC15 produced the least concentration of 25.05 µg mL^{-1} IAA while *P. aeruginosa* AKRC7 liberated the highest concentration of 39.27 µg mL^{-1} IAA. *E. coli* AKRC16 however produced the highest concentration of 30.52 µg mL^{-1} IAA among the genus of *Escherichia*.

Moreover, strains of rumen PSB produced variable amount of ammonia after day 5 of incubation ranging from 2.86 µM mL^{-1} to 3.89 µM mL^{-1} (Fig. 6). Among the genera of *Pseudomonas*, *P. aeruginosa* AKRC7 secreted the highest concentration (3.89 µM mL^{-1}) of ammonia while *E. coli* AKRC16 liberated the highest concentration (3.73 µM mL^{-1}) of ammonia among the genus *Escherichia*.

All rumen PSB exhibited varying productivity of phosphate solubilizing enzymes; phosphatase (ranging from 4.51 to 35.08 mmol mL^{-1}) and phytase (ranging from 0.32 to 2.98 mmol mL^{-1}), under different optimal conditions (Table 3). Overall, *P. aeruginosa* AKRC7 consistently exhibited highest productivity for both enzymes at the same temperature (35°C), pH (6), incubation time (24 h), and optimal source of nitrogen and carbon for phosphatase (tryptone and starch) and phytase (peptone and glucose) correspondingly. Similarly, among the genera, *E. coli* AKRC16 and *E. coli* AKRC4 exhibited, in that order, highest enzyme activities at same conditions, except for the former with higher optimal temperature (60°C) and the latter with low optimal temperature (35°C). *E. coli* AKRC16 recorded high optimum temperature of 60°C with phosphatase activity and phytase activity of 25.20 mmol mL^{-1} and 0.52 mmol mL^{-1}.

Growth promoting potential of rumen PSB on seeds of *Solanum lycopersicum*

Table 4 showed that 70% of rumen PSB in this study displayed promoting potential on the growth of tomato seeds through seed bacterization, in varying degree compare to the control. *P. aeruginosa* AKRC7 and *E. coli* AKRC16 enhanced 100% germination of tomato seeds with significantly high vigour index (2177 and 2114.33), fresh weight (1.42 ± 0.01 and 1.37 ± 0.02 g) and P content (309.04 ± 0.05 mg g^{-1} and 301.01 ± 0.02 mg g^{-1}) of seedlings respectively. Only about 62, 63 and 65% of total P content (8.74% w/w) of the pot soil were respectively accumulated and solubilized by *E. coli* AKRC16, *P. aeruginosa* AKRC7 and *E. coli* AKRC16 for seed growth, compared to control treatment which resulted in seeds with 199.59 ± 0.04 mg P g^{-1} after accumulating 69.34% of P content of the pot soil. The pH values of pot soils after the greenhouse experiment were not significantly different at $p = 0.05$ but decreased by 7–12% of the initial pH of the soil before sowing.

Discussion

Microorganisms with phosphate solubilizing potential from rumen content of White Fulani cattle

In this study, a high microbial load was recorded (Table 1) which is in accordance with the findings reported by Miron et al. (2001) and Preedaa et al. (2016). The high microbial composition of the rumen content in this study could be attributed to the conducive condition of rumen with high nutitional composition, relatively constant temperature range, water and food ingested and the exocrine secretion, and saliva Nagaraja (2016). Several researcher have also reported the predominance of bacteria in rumen belonging to different genera including *Methanobacteriun*, *Methanobrevibacter*, *Ruminobacter*, *Ruminococcus*, *Butyrivibrio*, *Treponema*, *Pseudomonas*, *Micrococcus*, *Staphylococcus*, *Streptococcus*, *Klebsiella*, *Clostridium*, *Bacillus* and

Table 1 Microbial composition of rumen content from White Fulani cattle

Microorganism	Concentration (CFU g^{-1}) (Mean ± Std Dev.)			Number of Isolate (%)
	10^5	10^6	10^7	
Bacteria	5.20 ± 0.36^{d}	1.28 ± 0.07^{c}	0.40 ± 0.07^{b}	23 (74)
Fungi	0.14 ± 0.02^{ab}	0.06 ± 0.01^{a}	NC	8 (26)

Values with different letter (superscript) are significantly different at $p \leq 0.05$ with One-way ONOVA by Duncan's test

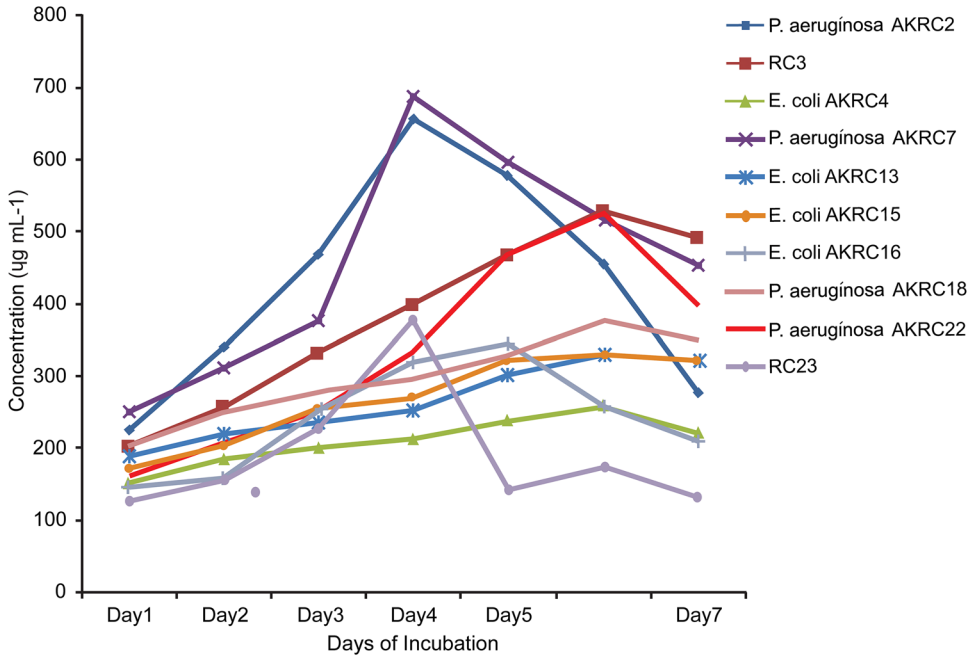

Fig. 3 Concentration of soluble phosphate produced in vitro by rumen PSB for seven days of incubation. Quantity of soluble P in NBRIP broth supplemented with 0.5% $Ca_3(PO_4)_2$, assessed by molybdenum blue method and measured at 650 nm

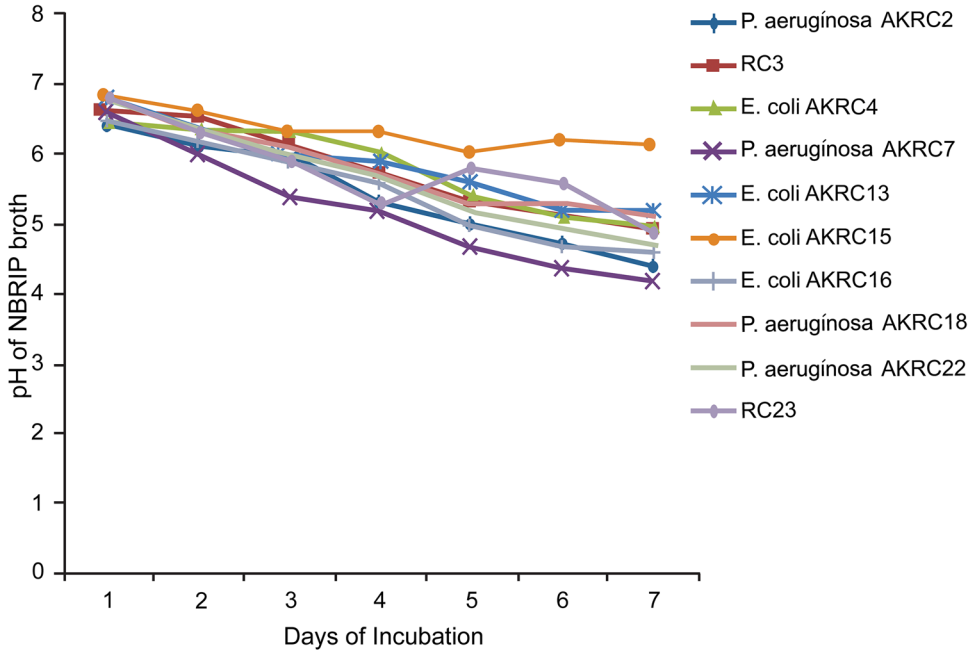

Fig. 4 Change in pH during in vitro solubilization of phosphate by rumen PSB. Change in pH of NBRIP broth inoculated with PSB, determined at 24 h interval for 168 h incubation period

Escherichia (Pukall et al. 2009; Kelly et al. 2010; Morgavi et al. 2013; Nagaraja 2016. These reports support the diversity of bacterial isolates recovered and identified as belonging to different genera (*Pseudomonas, Staphylococcus, Escherichia, Bacillus, Micrococcus, Klebsiella and Streptococcus*) in this study. It is a fact that the nutritional composition of every habitat determines the types of microbes that proliferate in them. Daily turn over of phosphorus (77%) in the rumen of cattle by their saliva and total P of the grass grazed by Africa's ruminant contributes to the solubilizing ability of bacteria isolated from the rumen content of the cattle. The assay for phosphate solubilizing ability of rumen PSB was done using TCP as the inorganic phosphate. This is in support of the work by Son et al. (2006) that reported $Ca_3(PO_4)_2$ as better inorganic phosphate (Pi) for PSB solubilization.

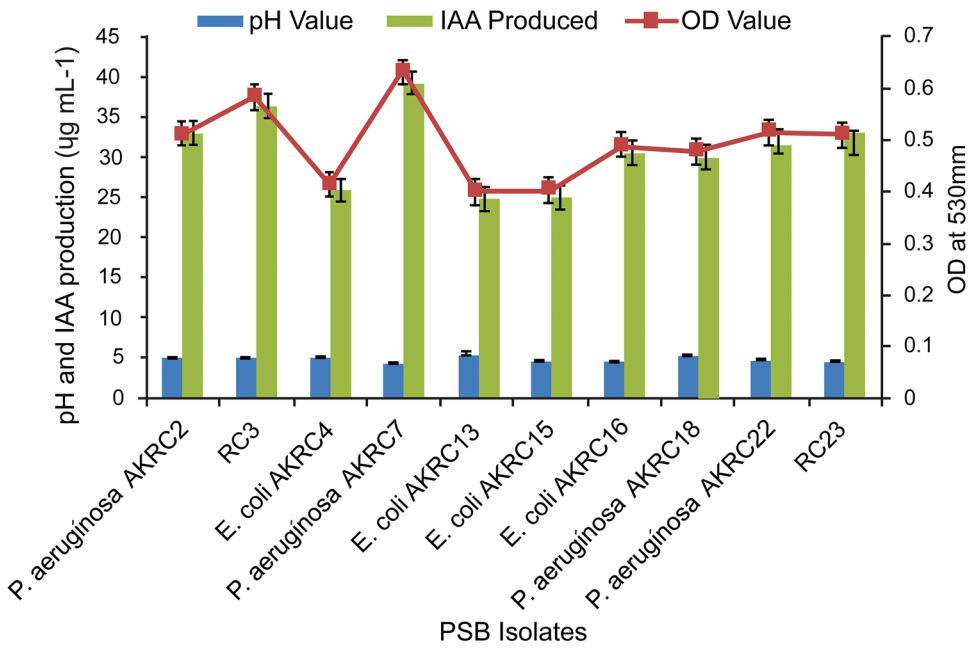

Fig. 5 Concentration of Indoleacetic acid produced in vitro by rumen PSB. Data were recorded as Mean ± SE of three independent replicates; Error bars indicates significant differences at $p \leq 0.05$ with One-way ONOVA by Duncan's test

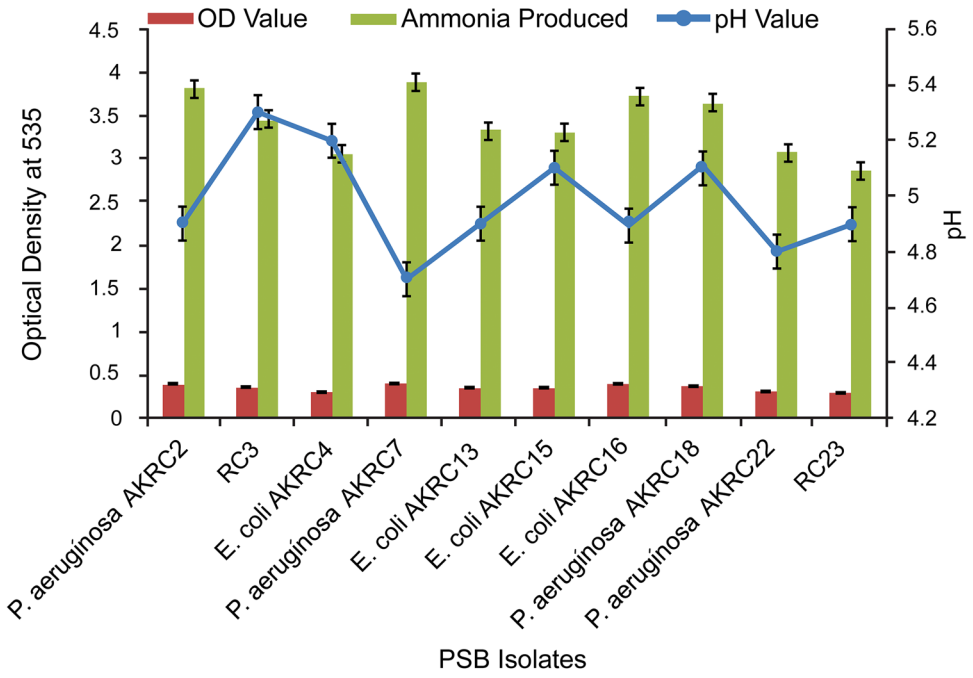

Fig. 6 Concentration of Ammonia produced in vitro rumen PSB. Data were recorded as Mean ± SE of three independent replicates; Error bars indicates significant differences at $p \leq 0.05$ with One-way ONOVA by Duncan's test

PGP traits of PSB from rumen content

Production of PGP metabolites that influence the physiology and productivity of plants by controlling some specific metabolic activities is one of the major attributes of PSB with great potentials as biofertilizers (Davona et al. 2012; Khan et al. 2014). The rumen PSB isolated here possess varying potentials to promote plant growth (Table 2). Particularly, the ability of PSB to produce siderophores of one or more forms, which can be used by the organisms to solubilize irons from minerals or organic compounds during iron starvation (Indiragandhi et al. 2008) is in support of the report of Khan et al. (2014). According to Dutta et al. (2015), protease, cellulase and chitinase production by the PSB isolates is also an indication of the ability to promote plant growth. While cellulase aid nutrient mineralization and degradation of organic matter; protease and chitinase hydrolyse the proteins in the cell wall of other organisms thereby serving as biocontrol. Hence, production of these enzymes (chitinase, proteins and cellulose) coupled with secreation of HCN and

Table 2 Identity of rumen PSB based on 16 S rRNA gene with respective accession number in GenBank

Isolate	Closest type strain in NCBI data base	E value	16 S rDNA identity (%)	Isolates' identity	Accession number
RC2	*Pseudomonas aeruginosa* strain 268	0.0	100	*Pseudomonas aeruginosa* AKRC2	MK330001
RC3	-	-	-	ND	-
RC4	*Escherichia coli* genome assembly FH189	0.0	100	*Escherichia coli* AKRC4	MK332563
RC7	*Pseudomonas aeruginosa* strain BA7823	0.0	100	*Pseudomonas aeruginosa* AKRC7	MK332569
RC13	*Escherichia coli* strainWCHEC025943	0.0	98	*Escherichia coli* AKRC13	MK332572
RC15	*Escherichia coli* E2865	0.0	97	*Escherichia coli* AKRC15	MK332574
RC16	*Escherichia coli* E2863	0.0	99	*Escherichia coli* AKRC16	MK332576
RC18	*Pseudomonas aeruginosa* strain 268	0.0	100	*Pseudomonas aeruginosa* AKRC18	MK332578
RC22	*Pseudomonas aeruginosa* strain YB01	0.0	100	*Pseudomonas aeruginosa* AKRC22	MK332579
RC23	-	-	-	ND	-

Key: ND: Not Done

Table 3 Phosphate solubilizing enzymes produced by rumen PSB

Isolates	Enzyme activity (EA) (mmol mL^{-1}) at optimal growth condition				
	EA/Temperature (°C)	EA/pH	EA/Incubation time (h)	EA/Nitrogen source (Tryptone)	EA/Carbon source (Starch)
Phosphatase					
Pseudomonas aeruginosa AKRC2	23.21 / 35	26.41 / 4	25.00 / 24	25.62	23.11
Escherichia coli AKRC4	24.86 / 35	34.70 / 6	26.23 / 24	24.45	22.54
Pseudomonas aeruginosa AKRC7	24.99 / 35	35.08 / 6	28.12 / 24	25.73	25.86
Escherichia coli AKRC13	12.84 / 40	11.20 / 7	10.72 / 36	5.40	10.93
Escherichia coli AKRC15	9.67 / 40	13.25 / 7	11.89 / 36	8.47	4.51
Escherichia coli AKRC16	25.20 / 60	26.43 / 6	23.70 / 24	23.91	24.18
Pseudomonas aeruginosa AKRC18	21.17 / 40	16.21 / 4	21.81 / 36	20.77	18.18
Pseudomonas aeruginosa AKRC22	17.17/ 60	18.92 / 7	19.81 / 48	21.85	19.88
RC3	12.12 / 60	14.42 / 7	12.62 / 48	10.81	9.29
RC23	11.98 / 40	13.62 / 4	11.18 / 42	6.63	8.06
Phytase					
				Peptone	Glucose
Pseudomonas aeruginosa AKRC2	2.38 / 50	2.98 / 7	2.44 / 24	1.78	1.45
Escherichia coli AKRC4	0.58 / 35	0.63 / 6	0.51 / 24	0.50	0.46
Pseudomonas aeruginosa AKRC7	2.19 / 35	2.98 /6	2.47 / 24	1.80	2.09
Escherichia coli AKRC13	0.40 / 40	0.46 / 7	0.50 / 48	0.52	0.41
Escherichia coli AKRC15	0.48 / 50	0.46 / 5	0.49 / 36	0.49	0.39
Escherichia coli AKRC16	0.52 / 60	0.69 /6	0.66 / 24	0.54	0.49
Pseudomonas aeruginosa AKRC18	0.94 / 70	0.85 / 4	1.53 / 36	0.98	0.94
Pseudomonas aeruginosa AKRC22	1.59 /30	1.39 / 5	1.47 /48	0.90	1.52
RC3	0.41 / 60	0.40 / 6	0.49 / 48	0.38	0.95
RC23	0.64 / 70	0.67 / 5	0.61 / 48	0.40	0.32

siderophore could confer the isolates the ability to inhibit the growth of plant pathogens (Olanrewaju and Babalola 2018). Furthermore, it has been shown by Goswani et al. (2014) that several PSB that produced IAA, HCN, chitinase and siderophores also possessed biocontrol ability.

Identity of PSB with PGP traits isolated from rumen content

Molecular identification of the PSB that showed consistent PGP traits, revealed that eight (8) of them were strains

Table 4 Promoting effect of rumen PSB on growth response of *Solanum lycopersicum* seed and phosphorus content of soil in greenhouse experiment

Isolates	Growth response of seedlings						Pot soil composition	
	P Content** (mg g^{-1})	SGR (%)	Weight (g)		Length (cm)		SVI**	pH I = 7.0	P Content (%) (w/w)
			Fresh	Dried	Root**	Shoot**			I = 8.74%
Pseudomonas aeruginosa AKRC2	271.03 ± 0.15	90	1.23 ± 0.02^{e}	0.37 ± 0.00^{cde}	14.61 ± 0.01	6.09 ± 0.01	1863.00	6.80^{ab}	2.98 ± 0.02^{e}
Escherichia coli AKRC4	260.41 ± 0.20	95	1.10 ± 0.01^{d}	0.43 ± 0.08^{ef}	13.97 ± 0.02	5.48 ± 0.00	1848.38	6.80^{ab}	3.30 ± 0.43^{f}
Pseudomonas aeruginosa AKRC7	309.04 ± 0.05	100	1.42 ± 0.01^{f}	0.40 ± 0.01^{def}	14.91 ± 0.01	6.86 ± 0.00	2177.00	6.79^{a}	3.28 ± 0.20^{f}
Escherichia coli AKRC13	145.06 ± 0.08	55	0.91 ± 0.02^{bc}	0.34 ± 0.11^{cd}	10.67 ± 0.01	3.97 ± 0.00	805.01	6.82^{b}	1.98 ± 0.02^{a}
Escherichia coli AKRC15	139.62 ± 0.02	50	0.85 ± 0.07^{b}	0.30 ± 0.01^{c}	12.10 ± 0.01	4.00 ± 0.01	805.00	6.82^{ab}	2.05 ± 0.03^{ab}
Escherichia coli AKRC16	301.01 ± 0.02	100	1.37 ± 0.02^{f}	0.46 ± 0.02^{f}	14.30 ± 0.01	7.10 ± 0.00	2114.33	6.79^{a}	3.03 ± 0.00^{e}
Pseudomonas aeruginosa AKRC18	243.20 ± 0.08	70	0.64 ± 0.06^{a}	0.10 ± 0.00^{a}	8.10 ± 0.01	3.71 ± 0.01	827.17	6.80^{ab}	2.29 ± 0.03^{bc}
Pseudomonas aeruginosa AKRC22	198.07 ± 0.17	80	0.71 ± 0.02^{a}	0.20 ± 0.00^{b}	7.21 ± 0.01	3.63 ± 0.00	867.47	6.80^{ab}	2.42 ± 0.00^{cd}
RC3	206.31 ± 0.01	60	0.96 ± 0.06^{c}	0.39 ± 0.00^{def}	12.09 ± 0.01	3.80 ± 0.01	953.20	6.79^{a}	2.43 ± 0.02^{cd}
RC23	201.03 ± 0.02	50	0.71 ± 0.10^{a}	0.37 ± 0.00^{cde}	9.37 ± 0.00	3.67 ± 0.01	652.00	6.80^{ab}	2.63 ± 0.01^{d}
Control	199.59 ± 0.04	75	0.93 ± 0.09^{bc}	0.40 ± 0.00^{def}	8.96 ± 0.00	4.96 ± 0.01	1044.50	6.79^{a}	2.68 ± 0.02^{d}

Keys: SVI - Seed Vigour Index (Sum of the mean of root length and shoot length, multiplied by 100); SGR - Seedling Germination Ratio; I - Initial mean value of pot soil content

Values presented as Mean ± Standard Deviation in column with the same superscripted alphabet are not significantly different at $p = 0.05$

** Mean of values on the column are significantly different at $p = 0.05$, hence no superscripted alphabet were alocated to any of the values

of either *Pseudomonas aeruginosa* or *Escherichia coli* (Table 4). Our finding is consistent with previous reports of bacteria found in the rumen (Nagaraja 2016). However, while we report *Pseudomonas* with PSP from rumen, most previous reports (Muleta et al. 2013; Dutta et al. 2015) documented Phosphate-solubilizing *Pseudomonas* from plant rhizosphere, soil and river. As regards *E. coli*, the authors are not aware of any study that has reported with phosphate solubilizing potential. It is therefore novel and very interesting that these strains were isolated from rumen content of cattle.

Quantitative estimate of PGP metabolite produced by PSB from rumen content

In this study, *Pseudomonas aeruginosa* AKRC7 and *Escherichia coli* AKRC16 exhibited the highest phosphate solubilizing efficiency amoung their respective genera with 687.75 µg mL^{-1} and 345 µg mL^{-1} soluble phosphate released after fourth and fifth day respectively. The efficiency exhibited by these isolates could be responsible for their better growth properties when compared to other isolates and consequently resulted in decrease in pH of the medium (Fig. 5) and increased productivity of phosphate solubilizing enzymes, phosphatase and phytase (Table 4). This is in support of the result of Panhwar et al. (2014) and Gen-fu and Xue-Ping (2005) who observed a linear relationship between soluble phosphorus and microbial growth. Soluble phosphate liberated by *E. coli* AKRC16 at 5th day of incubation (345 µg mL^{-1}) and by *P. aeruginosa* AKRC7 at 4th day of incubation (687.75 µg mL^{-1}) in this study was much higher than the highest produced by a strain of *Bacillus megaterium* reported by Zheng et al. (2018). This could be an indication that the *P. aeruginosa* AKRC7 isolated from rumen content of White Fulani cattle in this study appears more promising as a potential bioinoculant based on its attributes (Panhwar et al. 2014). Further corroborating this, is their production of significantly high concentration of IAA and ammonia both of which are among important metabolites

that stimulate plant growth and yield (Wang et al. 2018; Zheng et al. 2018).

Secretion of enzymes phosphatase and phytase, by some of our rumen PSB strains especially under feasible optimal conditions, is an added advantage, as it enhances hydrolysis of insoluble P to a more available and utilizable P content for plant usage (Wang et al. 2017). The source of our PSB strains could have contributed to the difference in the activities of enzymes they produced, when compared with those reported by other studies of different sources (Singh et al. 2014; Behera et al. 2017; Obidi et al. 2018). Moreso, the prospective application of above said enzymes in agricultural, biotechnological, pharmaceutical and other industries (Vohra and Satyanarayana 2003; Obidi et al. 2018; Handa et al. 2020), has also scored our rumen PSB as prospective candidate for industrial exploration.

Phosphate solubilization by bacterial isolates was accompanied by decline in pH of media which is in line with findings documented by other researchers (Paul and Sinha 2016; Zheng et al. 2018; Tang et al. 2020). Production of organic acid which accounts for acidification of the medium (decrease in pH) is one of the major mechanisms used by PSB to mineralise available phosphorus, hence the negative correlation of pH with concentration of phosphate released (Chen et al. 2006; Khan et al. 2007; Zaidi et al. 2009; Zheng et al. 2018).

Greenhouse study of rumen PSB on seeds of *Solanum lycopersicum*

Seed bacterization has been tremendously used in the recent time, to evaluate the plant growth promoting potential of several microorganisms (Singh et al. 2014; Kumar et al. 2015; Qessaoui et al. 2019; Arkhipova et al. 2019). Growth parameters of *Solanum lycopersicum* seeds bacterized with our rumen PSB were better enhanced coupled with increased soluble P than those of control, which is apparently indicative of potential of our isolates to provide better nutrient uptake (Tahir et al. 2015; Majeed et al. 2015). Growth promoting effects recorded by *Pseudomonas aeruginosa* AKRC7 on tomato seed in our study corroborates those reported of rhizobacteria *Pseudomonas* isolated by (Qessaoui et al. 2019). Remarkable improvement in growth response of tomato seeds in this study further justifies the plant growth promoting traits exhibited in vitro by our rumen PSB.

Conclusion

This study demonstrated that the rumen content of White Fulani cattle, indigenous to Africa served as a reservoir of novel PSB which exhibited plant growth promoting traits making them potential bioinoculants. Furthermore, *Pseudomonas aeruginosa* AKRC7 showed promising potential which could be exploited in sustainable agricultural production systems for optimizing the crop production. Also, the discovery of new strains of *Escherichia coli*, AKRC16 which is thermophilic and AKRC4, both exhibiting plant growth promoting potential under feasible conditions, is promising. Further works like molecular delineation PGP potentials, evaluation for biocontrol and large scale insitu application of these isolates for plant growth promotion, are however required to further elucidate their potential as biofertilizers.

Materials and methods

Sample collection

The sample (rumen content) used in this study was aseptically collected from the rumen of a White Fulani cattle (Online Resource Figure S1), using a pre-sterilized metal hand trowel into a sterile 20 mL sample bottle and transported in ice-chest within 2 h, to the Laboratory for analysis.

Isolation of microbial isolates from rumen content

With slight modification, serial dilution technique described by Olutiola et al. (2000) and pour plate method of Oje et al. (2016) were adopted to culture bacteria and fungi from the rumen content. Ten grams (10 g) amount of rumen content sample was homogenized for about 1 h in 90 mL of sterile physiological saline solution using manual agitation. The homogenate was ten-fold serially diluted and 1 mL aliquot of appropriate dilutions were inoculated into petri dish and overlaid with sterile molten Nutrient Agar (Oxoid, Basingstoke Hampshire, England) and Potato Dextrose Agar (Hi-Media Lab, India). The plates were gently swirled and allowed to set before incubated aerobically at 37°C (for 24 h) and 45°C (for 48 h) for bacteria and fungi, respectively. After incubation, microbial colonies were examined and counted with illuminated colony counter (Gallenkamp, England). The microbial counts were expressed as colony forming unit per gram (CFU g^{-1}) of the sample homogenate (Olutiola et al. 2000). Discrete colonies were sub-cultured to obtain pure culture which were stored in sterile slanted agar (at 4°C), until subsequently identification and further studies (Oje et al. 2016).

Phenotypic identification of bacterial isolates from rumen content

Bacterial isolates were tentatively identified using cultural characteristics on solid media, cellular morphology through microscopy of Gram-stained smear and biochemical

reactions. The results were interpreted according to Holt et al. (1994).

Qualitative screening of rumen bacteria for phosphate-solubilizing potential

The method of Zhang et al. (2017) with little modification was adopted to screen the bacteria isolated from rumen content for ability to solubilize phosphate. Isolates were separately streaked on a National Botanical Research Institute's Phosphate (NBRIP) growth (agar) (g L^{-1}: glucose (10); $MgCl_2 \cdot 6H_2O$ (5); $MgSO_4 \cdot 7H2O$ (0.25); KCl (0.2) and $(NH_4)_2SO_4$ (0.1)) incorporated with 0.5% $Ca_3(PO_4)_2$ as an insoluble source of phosphorus (P) (Nautiyal 1999; Chauhan et al. 2017) and incubated at 30°C for 96 h (4 d). Solubilization Index (SI) which indicated phosphate solubilizing ability of the isolates was calculated using the following formular. Bacterial isolates with better solubilizing potential tagged "rumen PSB" were subjected to further studies.

$$SI = \frac{\text{Diameter of CH}}{\text{Diameter of colony (mm)} \times \text{Number of IP (days)}}$$

Where

SI is the solubilization index
CH is the clear halo around colony on NBRIP
IP is the incubation period

Quantitative assay of phosphate solubilizing potential by rumen PSB

Tricalcium phosphate (TCP) solubilization assay

Phosphate solubilizing potentials of PSB isolates were quantified following the method of Zhang et al. (2017) with slight modification on the volume of the media and inoculum used. A single colony of 24 h old PSB was inoculated into 3 mL NBRIP broth containing 5 g L^{-1} $Ca_3(PO_4)_2$ and incubated under agitation (190 rpm) at 30 ± 1 °C for 20 h. One millilitre (1 mL) of 20 h old broth culture of the PSB was then introduced into a 250 mL Erlenmeyer flask containing 120 mL medium in triplicate, incubated in the dark in a shaker incubator (New Brunswick Scientific Co., Inc., USA) (working at 190 rpm) at 30 ± 1 °C for 168 h (7 d), using sterile NBRIP medium as control. Subsequently, the pH of 20 mL aliquot of cultured broth was measured using portable pH meter (Model HI-96,107, Hanna Instrument) and afterwards spun in a centrifuge (Beckman Coulter Massachusetts, USA) at 13,000 rpm for 10 min. Molybdenum-blue method described by Chen et al. (1956) was used to assess the soluble P in the supernatant as the absorbance of resultant blue solution was measured using UV/Visible spectrophotometer (WPA Linton Cambridge, UK) at 650 nm. The value of total soluble phosphorus was estimated and expressed in µg mL^{-1} using the regression equation of standard curve described by (Adebayo 2019).

Phosphate solubilizing enzymes (phosphatase and phytase) production assay

Quantitative assessment of phosphatase and phytase production by rumen PSB were carried out following the method of Obidi et al. (2018) and Gontia-Mishra et al. (2013) with slight modification respectively. Isolates (24 h old) were aseptically introduced into sterile 100 mL nutrient broth and incubated under agitation (150 rpm) for 24 h at 28°C. Five millimeters (5 mL) of culture suspensions were mixed with 100ml of sterile basal medium (consisting (L^{-1}) of 0.9 g K_2HPO_4; 0.2 g KCl; 0.2 g $MgSO_4.7H_2O$; 1.0 g NH_4NO_3; 0.002 g $ZnSO_4$; 0.002 g $MnSO_4$; 0.002 g $FeSO_4.7H_2O$; 2 g yeast extract; incorporated with 1 g of specific substrate (sodium phosphate for phosphatase and sodium phytate for phytase)), and incubated for 48 h at 150 rpm and 28°C. Cell-free supernatants were obtained at 6 h interval through centrifugation of 5 mL of resultant suspension at 6000 rpm at 4°C for 20 min.

Determination of phosphatase activity

A reaction solution of modified universal buffer (consisting of 3.025 g tris-hydroxymethyl-aminomethane, 2.9 g maleic acid, 3.5 g citric acid, 1.57 g boric acid, 122 mL of 1 M sodium hydroxide; in distilled water to a final volume of 250 mL; pH 6.5), cell-free supernatant and 0.115 M p-nitrophenyl phosphate (*p*-NPP), was prepare at 1:4:1 v/v ratio. Toluene (0.1 mL) was added to the solution (to stop bacterial growth) before incubated at 37°C for 1 h. Mixture of 4 mL of 0.5 M NaOH and 1 mL of 0.5 M $CaCl_2$ was added to the solution to discontinue the reaction. Absorbance of the solution indicative of P-nitrophenol (*p*-NP) constituent was then measured with UV-Visible spectrophotometer (WPA Linton Cambridge, UK) at 450 nm and the value was extrapolated from standard curve of p-NP (Behera et al. 2017).

Determination of phytase activity

Reaction mixture of 0.2 mL of cell (resultant) suspension from production assay and 0.8 mL of acetate buffer (0.2 M at pH 5.5, containing 1 mM sodium phytate) were incubated at 37°C for 30 min. The mixture was added with 0.5 mL of stop solution (5% (w/v) trichloroacetic acid) and colour solution (10% ascorbic acid; 2.5% ammonium molybdate; 5 N H_2SO_4; and distilled water, at 1:1:1:2 v/v ratio) and incubated for 10 min at room temperature (El-Toukhy et al.

2013). Concentration of phosphate released was measured by correlating absorbance value in a UV-Visible spectrophotometer (WPA Linton Cambridge, UK) at 650 nm with standard curve of KH_2PO_4 (Adebayo 2019).

Optimization of growth conditions for enzyme production

Optimal conditions for production of phosphatase and phytase by rumen PSB were determined by evaluating the activities of the enzyme under such different varietal conditions as; pH (3–11) temperature (25-70°C); incubation time (0–60 h) and carbon sources. A unit of enzyme activity is the amount of such enzyme required to liberate 1 mmol Pi per minute under the assay condition (Obidi et al. 2018).

In-vitro assessment of rumen PSB for plant growth promoting traits

Cellulase and protease production assay

Casein Yeast Extract Agar (containing g L^{-1}: casein 5.0; yeast extract 2.5; glucose 1.0; agar 15.0 dissolved in distilled water) amended with 1% carboxymethyl cellulose (CMC) as described by Teather and Wood (1982) was inoculated with a single colony of 24 h old pure PSB culture and incubated for 72 h at 28 °C. After incubation time, the cultured agar was flooded with aqueous solution of Congo red (1 mg mL^{-1}) and left on bench for 15 min. Clear halo around bacterial colonies after 15 min reaction with Congo red indicated cellulase production by PSB isolates.

As described by Kumar et al. (2005), Casein Yeast Extract Agar supplemented with 7% skimmed milked powder was inoculated with PSB and incubated at 28 °C for 72 h to test for their Proteolytic activity. Protease production was indicated by presence of clear halo zone around bacterial colonies.

Hydrogen cyanide (HCN) production assay

Picrate assay described by Castric (1975) was adopted with little modification to assess test isolates for HCN production. Bacterial isolates were streaked on Nutrient agar plates supplemented with 4.4 g/% glycine. Whatman filter paper No. 1 soaked in solution of 2% Na_2CO_3 and 0.5% picric acid was sealed with parafilm in-between base and lid of the cultured plate and incubated for 96 h at 27 ± 2°C. Change in colour of filter paper from yellow to orange-brown indicated HCN production.

Chitinase production assay

PSB isolates were spot-inoculated on Chitin Agar plate (containing in g L^{-1}: chitin (4), K_2HPO_4 (0.7); KH_2PO (0.3); $MgSO_4$-$5H_2O$ (0.5); $FeSO_4.7H_2O$ (0.01); $ZnSO_4$ (0.001); $MnCl_2$ (0.001); and 15 g of agar) amended with 2% phenol red and incubated at 27 ± 2°C for 72 h. Presence of clear zones around culture spots (inoculants) indicated chitinase production (Wang et al. 2008).

Nitrogen fixation assay

Burk's Nitrogen-free Medium (comprising g L^{-1}: glucose (10); KH_2PO_4 (0.41); K_2HPO_4 (0.52); Na_2SO_4 (0.05); $CaCl_2$ (0.2); $MgSO_4.7H_2O$ (0.1); $FeSO_4.7H_2O$ (0.005); $Na_2MoO_4.2H_2O$ (0.0025); and agar (15); pH: 7 ± 1) described by Wilson and Knight (1952) was adopted to test nitrogen-fixing potential of isolated PSB. Media were inoculated with a 24 h old pure PSB and incubated at 37°C for 120 h. Only the PSB isolates with potential of nitrogen-fixation were able to grow after 120 h of incubation.

Indole Acetic Acid (IAA) production assay

Production of Indole acetic acid (IAA) was assayed using colorimetric method described by Glick (1995) with slight modification. The PSB isolates were cultured in IAA Minimal Medium (IAA-MM) (g L^{-1}: KH_2PO_4 (1.36); Na_2HPO_4 (2.13); $MgSO_4.7H_2O$ (0.2) and trace elements) supplemented with 100 µg mL^{-1} of L-tryptophan and incubated at 37°C for 72 h in a shaker incubator (New Brunswick Scientific Co., Inc., USA). After incubation, 1.5 mL of bacterial suspension was spun at 10,000 rpm for 10 min in a centrifuge (Beckman Coulter, Massachusetts, USA). The supernatant was added to Salkowski's reagent (2% 0.5 M $FeCl_3$ in 35% $HClO_4$) at 1:2 proportion before incubating in dark for 30 min at 25 °C. Absorbance of the final solution was then measured with UV/Visible spectrophotometer (WPA Linton Cambridge, UK) at 530 nm and IAA produced by PSB isolates was determined against standard curve (Zhang et al. 2017; Adebayo 2019).

Ammonia production assay

PSB were cultivated in 10 mL Peptone Water Broth and incubated at 27 ± 2°C for 120 h, afterwards 5ml of bacterial suspension was spun at 10,000 rpm for 10 min in a centrifuge (Beckman Coulter, Massachusetts, USA). The supernatant (0.2 mL) of the cultured broth was then mixed with 1mL Nessler's reagent (10 g Mercuric chloride, 7 g Potassium iodide and 16 g Sodium hydroxide in 100 mL distilled water (DW) (ammonia free); pH 13.2 ± 0.05) and the volume of this mixture was made up to 8.5 mL with ammonia free distilled water. Development of brown to yellow colour was indicative of ammonia production and the absorbance of the solution was measured with spectrophotometer (WPA Linton Cambridge, UK) at 450 nm. Concentration of ammonia

was estimated with standard curve of ammonium sulphate (0.1-1 µM mL^{-1} range) (Adebayo 2019).

Siderophore production assay

Production of siderophore was determined following the method described by Goswami et al. (2014) with little modification. The PSB isolates were inoculated into the Fiss-Glucose Mineral Medium and incubated in a shaking incubator (New Brunswick Scientific Co., Inc., USA) at 25 °C for 3 d at 174 rpm. After incubation, 5 mL of bacterial suspension was spun at 10,000 rpm for 10 min in a centrifuge (Beckman Coulter, Massachusetts, USA). The cell-free supernatants of the cultured broth were assayed for siderophore production using ferric chloride test for hydroxamate and catecholate nature of the siderophore following Neilands spectrophotometric assay (Neilands 1981); and phenolphthalein test described by Furniss et al. (1989) for carboxylate nature of the siderophore. Formation of red/pink colour indicated the presence of siderophore in the solution which was measured with a UV/Visible spectrophotometer (WPA Linton Cambridge, UK). The absorbance peak between 420 and 450 nm of ferrated siderophores indicated its nature as hydroxamate while absorption peak at 495 nm indicated the presence of catecholate siderophore. The disappearance of pink colour on the addition of phenolphthalein to the solution indicated its nature as carboxylate.

Molecular identification of PSB from rumen content

Bacterial isolates of interest were further characterised by amplification and sequencing of the 16S rRNA gene (~ 1500 bp). Firstly, genomic DNA was extracted using Bacterial Genomic DNA Isolation Kit (Jena Bioscience, Germany) following manufacturer's instructions. Subsequently, the 16S rRNA gene was amplified from genomic DNA by polymerase chain reaction (PCR) using universal bacterial primers; 27F (5'-AGAGTTTGATYMTGGCTC AG-3') and 1390R (5'-ACGGGCGGTGTGTRCAA-3') (Mao et al. 2012). Each 30 µL PCR reaction contained 6 µL of RedLoad (Jena Bioscience, Germany) PCR mix, 0.3 µL of each primer, 18.4 µL of PCR grade water and 5 µL of DNA template. PCR was done with GeneAmp 9700 (Applied Biosystems, USA) thermal cycler as follows; 94 °C for 3 min, 30 cycles at 94 °C for 30 s, 54 °C for 30 s, 60 °C for 90 s and a final 7 min extension at 72 °C, after which product was held at 4°C till terminated. The PCR products were subsequently resolved on 1% electrophoresis gel stained with ethidium bromide and viewed using UV transilluminator (Fotodyne Incorporated, USA). Amplicons were shipped to Macrogen Inc., South-Korea, for purification and Sanger sequencing.

To identify the PSB isolates, the sequences were compared using the BLASTn programme (http://www.blast.ncbi.nlm.nih.gov) with other publicly available nucleotide sequences in GenBank (http://www.ncbi.nlm.gov/genbank/). The nucleotide sequences of the 16 S rDNA of PSB recovered from this study were submitted to GenBank and have been assigned Accession Numbers (Table 3).

Greenhouse evaluation of rumen PSB for growth promotion in tomato plant

Seed bacterization method described by Singh et al. (2014) was adopted with slight modification to evaluate plant growth promoting potential of rumen PSB on pre-authenticated tomato (*Solanum lycopersicum* L.). Briefly, 1 g of plant seeds, surface-sterilized according to Qessaoui et al. (2019), were treated with 10 mL bacterial suspension (1.0×10^8 CFU mL^{-1}) incorporated with 200 mg CMC for 2 h on a rotary shaker at 150 rpm. Control (non-bacterized) treatment were similarly prepared with sterile DW (10 mL) in place of bacterial suspension. Moisture content of seed preparation were drained off and air-dried for 12 h in a laminar hood. Bacterized and non-bacterized seeds (20 pieces) were separately sown in sterilized and triplicated pot soils (with 3:1 ratio of soil to decomposed cow dung manure), arranged (randomly) in a greenhouse under 18 – 6 h at 25-20°C (day-night) cycle and relative humidity of 70 ± 5%. Farmed pot soils were watered every 2 d for the period (21 d) of experiment. Growth responses such as; relative germination rate (determined at 5 d after sowing); height (shoot and root) and weight (both fresh and dried) after 21 d, of the seedlings were determined and used to calculate seedling vigour index (SVI) according to (Sankar et al. 2017).

Phosphorus content and pH of soil before the experiment and individual treatment soils after the experiment, were determined by Olsen's method; and the phosphorus content of 21 d old seedlings were determined by ammonium-vanado-molybdate method (Estefan et al. 2013).

Statistical analysis

All experiments were performed in triplicate and data obtained was subjected to Analysis of Variance (ANOVA). Values were presented in Mean ± SD/SE (Standard Deviation/ Standard Error) computed with Duncan's multiple range tests using SPSS package (version 22.0). Differences at $P \leq 0.05$ level was considered significant.

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Author contributions AAA, IAA and NEE conceptualized the research; AAA, TOCF, OMA and NEE design the work; AAA; TOCF, OMA acquired and analysed data relating to isolation, identification and invitro screening; AAA, and TOCF acquired and analysed all molecular data; AAA, TOCF, OMA and NEE interpreted all data; AAA wrote the first manuscript; AAA, TOCF, OMA, IAA and NEE revised the manuscript critically; and AAA, TOCF, OMA, IAA and NEE approved the final manuscript for submission.

Data availability All data generated or analysed during this study are included in this article.

Declarations

Ethical approval and consent of participation Not applicable.

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