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ISOLATION, PREPARATION, AND PRODUCTION OF BIOFERTILIZER (*ENTEROCOCCUS CLOACA*, *ENTEROCOCCUS HOMACHEII*, AND *CLOSTRIDIUM BEIJERINCKII*)

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Abstract: The introduction of chemical fertilizers has significantly boosted agricultural yields, but their negative environmental impacts, such as water pollution, damage to ecosystems, and reduced soil fertility have raised concerns. In response, biofertilizers have gained attention as eco-friendly alternatives to chemical fertilizers. Biofertilizers, particularly those based on phosphate-solubilizing bacteria (PSB), offer advantages such as safety, lack of accumulation in the food chain, and minimal resistance development. This study isolates and produces a biofertilizer using three bacterial strains: *Enterococcus cloaca*, *Enterococcus homaecheii*, and *Clostridium beijerinckii*. AA total of 15 isolates were identified through 16S rRNA gene sequencing. Isolate AFS-A was identified as *Enterobacter hormaecheii* (96.57% sequence homology), AFS-B was identified as *Enterobacter cloacoa* (96.57% sequence homology), while isolate AFS-M was identified as *Clostridium beijerinckii* (97.88% sequence homology). The bacteria isolates were cultured and processed into biofertilizers using a carrier-based method. The biofertilizer's physicochemical properties, such as pH (6.8-7.2), moisture content (22.5-26.2 %), and particle size (1.2 mm), were within acceptable agricultural standards. It also contained balanced nutrients, including nitrogen (10.2-12.2 %), phosphorus (5.5-5.7 %), and potassium (10.5 %). With a microbial load of 8.2×10^8 CFU/g and a shelf life of 18 months, the biofertilizer also exhibited plant growth-promoting substances like indole-3-acetic acid (IAA). These findings highlight the potential of *E. cloaca*, *E. homaecheii*, and *C. beijerinckii* as biofertilizers for sustainable agriculture, offering an eco-friendly alternative to chemical fertilizers while promoting plant growth and soil health.

Keywords: biofertilizer, phosphate-solubilizing bacteria, *Enterococcus cloaca*, *Enterococcus homaecheii*, *Clostridium beijerinckii*.

INTRODUCTION

Soil is a dynamic living body and contains enormous numbers of diverse living organisms. Organic agricultural practices aim to enhance biodiversity, biological cycles, and soil biological activity so as to achieve optimal natural systems that are socially, ecologically, and economically sustainable. The increasing global demand for food and the need to sustain soil fertility have led to a growing interest in the development and use of biofertilizers (Ahamed and Jha, 2022; Igiebor *et al*., 2023). Biofertilizers are living microorganisms that, when applied to the soil, can promote plant growth and improve soil fertility by fixing atmospheric nitrogen, solubilizing phosphates, and producing plant growth-promoting substances (Chen *et al*., 2020). They offer a sustainable and environmentally friendly alternative to chemical fertilizers, which can harm the environment and human health (Bhattacharyya and Jha, 2020).

Biofertilizers differ from chemical and organic fertilizers in the sense that they do not directly supply any nutrients to crops and are cultures of special bacteria and fungi. The production technology for biofertilizers is relatively simple, and the installation cost is very low compared to chemical fertilizer plants. Bio-fertilisers are a low-cost source of plant nutrients, environment-friendly, and have an auxiliary role with chemical fertilizers (Gaind, 2020)

Phosphate-solubilizing microbes (PSM) are different in their habits and functionalities on the basis of the content of phosphorus present in the soil, and accordingly, the response of these communities towards the phosphorus availability varies. That is why the management strategies toward these communities are dependent on their response. The mode of action of these PSBs includes increasing the surface area of the plant roots, increasing the availability of the nutrients in the soil to the plants, assisting the N_2 fixation, and enhancing other beneficial effects symbiotically. PSB solubilizes phosphate by the production of organic acids. These acids can either dissolve the phosphorus directly by lowering the pH of the soil, which can help in the ion exchange of PO_4^{2-} by acid ions, or they can chelate heavy metal ions such as calcium, aluminium, and iron and release associated phosphorus with them (Alam *et al*., 2022). It is reported that PSB culture increased yield up to 200-500 kg/ha, and thus 30 to 50 kg of superphosphate can be saved.

Phosphate-solubilising bacteria (PSB), include *Pseudomonas*, *Bacillus*, *Enterobacter*, *Azospirillum,* and *Rhizobium*, also called Rhizobacteria because they colonize the plant roots and promote plant growth (Glick, 2020). These are free-living bacteria that enrich the nutrient quality of the soil and are beneficial to crops.

Phosphate-solubilizing bacteria (PSB) biofertilizer are one of the most important types of biofertilizers, as

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they can solubilize insoluble phosphates, making them available to plants (Kumar *et al.*, 2020). Among the various microorganisms used as biofertilizers, bacteria are the most widely used due to their ability to promote plant growth and soil fertility. *Enterococcus* spp. and Clostridium spp. are two genera of bacteria that have been reported to have plant growth-promoting properties.

Enterococcus cloaca, *Enterococcus homaecheii*, and *Clostridium beijerinckii* are three bacterial species that have been isolated from various environmental sources and have been reported to have plant growth-promoting properties. These bacteria have been shown to produce plant growth-promoting substances such as indole-3-acetic acid (IAA), ammonia, and siderophores. The use of biofertilizers produced from *Enterococcus cloaca*, *Enterococcus homaecheii*, and *Clostridium beijerinckii* has several benefits, including: plant growth promotion, Soil fertility improvement, and Environmental benefits. (Tiwara *et al*., 2018)

The production of biofertilizer using Enterococcus cloaca, Enterococcus homaecheii, and Clostridium beijerinckii can be scaled up for commercial production using various biotechnological approaches such as fermentation and formulation (Kumar *et al*., 2020). The quality control of biofertilizer is essential to ensure its safety, efficacy, and quality, and can be achieved using various methods such as microbiological analysis, physicochemical analysis, and contamination testing (García-Fraile *et al*., 2020).

The use of these bacteria as biofertilizers has the potential to promote sustainable agriculture by reducing the use of chemical fertilizers and promoting soil fertility. However, the production of biofertilizers using these bacteria requires careful consideration of various factors, such as the isolation and characterization of the bacteria, the preparation and formulation of the biofertilizer. These are critical steps in the development of effective biofertilizers.

This study will focus on the isolation and characterization of *Enterococcus cloaca*, *Enterococcus homaecheii*, and *Clostridium beijerinckii*, and the preparation and formulation of a biofertilizer using the isolated bacteria. This study has the potential to contribute to the development of sustainable agriculture by promoting the use of biofertilizers as an eco-friendly alternative to chemical fertilizers. The use of *Enterococcus cloaca*, *Enterococcus homaecheii*, and *Clostridium beijerinckii* as biofertilizers has the potential to promote plant growth and soil fertility, reducing the need for chemical fertilizers and promoting sustainable agriculture.

MATERIALS AND METHODS

Experimental Conditions

The experiments were carried out at the Faculty of Agriculture farm, University of Benin, Benin City, Edo state, located on Latitude 6º 20′1.32ºN and Longitude 5 º 36′ 0.53ºE and 239.16 meters (784.65 feet) above sea level. Benin City has a tropical or savannah climate with a yearly temperature of 28.78ºC (83.80F), which is -0.68% lower than Nigeria's average. Relative humidity is 81.51 %.

Soil sample collection

Rhizosphere soil samples were randomly collected from the rhizosphere of three (3) economically important plants at the Faculty of Agriculture Research farm, University of Benin, Ugbowo main campus. The plants were *Vernonia amygdalima* (Bitter leaf), *Occimum gratissimum* (Scent leaf), and *Moringa oleifera.* The soil samples were collected from a depth of 0-30cm, packaged in sterile disposable bags, and transported to the Soil Science Research Laboratory, Faculty of Agriculture, and stored at -4ºC for further analysis (Mahantesh and Patil, 2022; Igiebor *et al*., 2019)

Isolation and Purification of phosphate solubilizing Bacteria from Rhizosphere soil samples

A 10 g sample from each soil was suspended in 90ml of sterilized water in 250 Erlenmeyer flasks to make a 1:10 dilution. These were agitated on a rotary shaker at 125 RPM for 30 minutes to break clogs (Ngwoke and Igiebor, 2024). A ten-fold serial dilution was made to obtain an appropriate dilution factor. From dilutions 10^{-4}, 0.1ml suspension was transferred to Petri dish containing Pikovskaya's medium which consists of glucose (10g), $Ca_3(PO_4)_2$ (5g), $(NH_4)S0_4$ (0.5g), Yeast extract (0.5g), $MgS0_47H_20$ (0.1g), $MnSO_4$(0.002g), $FeSO_4$(0.002g), NaCl (0.2g), Agar (15g), Distilled water (1000ml), pH (7.0) (Pikovskaya, 1948) Incubation was done at 30ºC for 7days. Colonies of bacteria were counted on the 5th to the 7th day. Isolates showing discreet halo zones around colonies were assumed to be phosphate solubilizers. These were selected, purified, and preserved on agar slants at -4ºC for further use.

Identification and Characterization of Bacterial Isolates

All selected isolates were examined for morphological and microscopic (Gram staining) characteristics with reference to (Ngwoke and Igiebor, 2025; Ngwoke and Igiebor, 2024; Krieg and Holt, 2022; Osarumwense et al., 2019; Kucey, 2018).

Biochemical Tests

In order to completely identify the bacterial isolates, biochemical tests were carried out. These tests include oxidase test, indole test, starch hydrolysis, catalase test, methyl red, Voges-Proskauer test, citrate utilization, oxidative/fermentative, hydrogen sulphide production, Nitrate reduction, glucose, manitol, xylose, maltose, galactose, lactose, dextrose, sucrose, fructose, and Urease tests (Ngwoke and Igiebor, 2025; Igiebor et al., 2024; Duguid *et al*., 2020). The bacterial isolates were then identified with reference to the 8th edition of Bergey's Manual of Determinative Bacteriology by Buchanan and Gibbons (2022).

Molecular Identification by 16S rRNA Sequence analysis

Genomic DNA Extraction

50-100mg (wet weight) bacterial cells that have been resuspended in up to 200µl of isotonic buffer (PBS) were added to a ZR Bashing™ Lysis tube.

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750µl Lysis Solution was used for the tube. A bead fitted with a 2ml tube holder was secured and processed at a maximum speed for 5 minutes. The ZR BasingBead™ Lysis Tube in a microcentrifuge was centrifuged at 10,000 x g for 1min. 400µl of supernatant was transferred to a Zymo-Spin™ IV Spin Filter in a collection tube and centrifuged at 7,000 x g for 1min. The base of the Zymo-Spin™ Spin filter was snapped off prior to use. 1,200µl of Bacterial DNA Binding Buffer was added to the filtrate in the collection Tube from step 4. 800 µl of the mixture from step 5 was transferred to a Zymo-Spin™ IIC Column in a Collection Tube and centrifuged at 10,000 x g for 1 minute. The flow was discarded from the Collection Tube, and step 6 was repeated. 200ul of DNA Pre-Wash Buffer was added to the Zymo-Spin™ IIC Column in a new Collection tube and centrifuged at 10,000 x g for 1min. 500µl bacterial DNA Wash buffer was added to the Zymo-Spin™ IIC column and centrifuged at 10,000 x g for 1min. The Zymo-Spin™ IIC Column was transferred to a clean 1.5ml microcentrifuge tube, and 100µl (35µl minimum) DNA Elution Buffer was added directly to the column matrix. Centrifuge at 10,000 x g for 30 seconds to elute DNA.

Polymerase Chain Reaction (PCR) amplification of 16S rDNA and Sequencing.

2 µl of the extracted DNA was used as a template for PCR amplification. The 25µl PCR reaction generally contained 0.4 µl 10mM dNTP, 2.5µl 10x PCR buffer, 2.5µl 25mM $Mgcl_2$, 0.2µl (5"-AGAGTTTGATCCTGGCTCAG-3') and 1µl (10mM) of reverse primer 1492r(5'-GGTTACCTTGTTACGACTT-3'). The PCR program included a denaturation step of 5min at 95°C, followed by 30cycles of 95°C for 30s, 50°C for 45s, 72°C for 1hr, 30min, and a final extension step of 10min at 72°C. PCR products were Sanger-sequenced using the forward primer 27F at the International Institute of Tropical Agriculture, Ibadan. The sequences were edited using the Bioedit programme. A blast search on the NCBI Gene Bank Database was used to identify the isolates.

Estimation of Indole-3-acetic acid (IAA) production

Indole-3-acetic acid production was detected by a modified method as described by Brick *et al.* (1991). Quantitative analysis of IAA was performed using the method of Loper and Scroth 1986. Cultures of the isolates were grown in Pikovskaya broth medium supplemented with 5µL tryprophan for 48 hours. The cultures were centrifuged at 10,000 rpm for 15min. 2ml supernatant was transferred to test tubes to which 100µL of O-phosphoric acid and 4ml of Salkowaski reagent (1ml of 0.5m $FeCl_3$ in 50ml 35% $HCLO_4$) were added. The mixture was incubated at room temperature for 25 minutes. The tubes were observed for the development of pink colour. Quantification of IAA was carried out using spectrometric analysis at 530nm. The 1AA concentration was determined against a standard curve constructed from different concentration of indole-3-acid.

Preparation and Production of PSB Biofertilizer

Starter Cultures for Phosphate-Solubilizing Bacteria

Following the screening of phosphate-solubilizing bacterial species, the PSB isolates with the highest solubilization potential were used for the preparation of the biofertilizer. After screening of PSB bacterial strains from pure culture slants, the bacterial strains were transferred to the liquid broth, which served as the production media and starter culture for cell growth. The production medium is essential for increasing the viable bacterial cell count, as the bacteria specifically thrive and proliferate in this medium. (Patel *et al*., 2022)

Formulation of PSB Biofertilizer

A 100ml PVK broth was prepared in 3 separate conical flasks and autoclaved. In the laminar airflow, pure cultures in the pure culture slants (starter culture) were transferred to the PVK production media in the conical flasks, using a sterilized inoculating loop. The conical flasks were put in the rotary BOD shaker for 7days. When the viable cell count in the production media or liquid broth reached 10^9Cfu/ml, a loopful of starter culture for each strain was inoculated into a 100ml conical flask containing Pikovskaya broth. When growth reached a maximum point of 10^9Cfu/ml, the culture was transferred to 500ml broth. The process was repeated in a 1000ml conical flask. Continuous agitation and proper aeration were done for 1 week for mass multiplication in a BOD shaker. The flasks were checked from time to time for the growth of cell mass and to ensure they were free of any contaminants. After 1wk, the cell population increased to 10^9 cells /ml or 10^9Cfu/ml load in the larger conical flasks. The conical flasks were stored at a cool temperature so that they could be mixed with proper carrier materials. (Patel *et al*., 2022)

Preparation of carrier material

4kg of black charcoal made at home, sundried, crushed into powder using a pestle and mortar. It was then sealed in a sterile polythene bag and brought into the Microbiology laboratory at University of Benin, Benin City. After it was brought into the laboratory, the powdered charcoal was transferred into a sterile beaker for sterilization. The charcoal was sterilized following the standard sterilization method in Microbiology, 121°C for 15 minutes. The procedure maintained sterility throughout and was conducted within a laminar airflow environment. (Patel *et al*., 2022)

Mixing of Carrier Material with PSB Inoculum

The mass-produced bacterial cell cultures of the 3 PSB isolates were taken out of storage, and then the cell cultures were mixed with the sterilized carrier material in individual beakers. The mixing of the carrier material and the production media was in the ratio of 2:1, where 1 part of the production media was mixed with 2 parts of carrier material. It was done manually and under aseptic conditions. The cell count

of the carrier mixed culture was 10^8Cfu/gm. The biofertilizers were packed in polythene bags and left at room temperature for curing. The polythene packets containing biofertilizers were stored in a cool place away from direct sunlight. (Patel *et al*., 2022)

Physicochemical Properties of Biofertilizer

The physicochemical properties of the biofertilizer were analyzed using various instruments following standard procedures (Igiebor *et al*., 2019).

RESULTS AND DISCUSSION

Results of the halo zone activity on Pikovskaya agar medium (Fig. 1) showed the presence of microorganisms capable of solubilizing TCP in vitro. The highest halo zone formation was obtained from microorganisms in the rhizosphere of *Mangifera indica* (Mango), *Cocos nicifera* (coconut), *Vernonia amygdalina* (Bitterleaf), and *Moringa oleifera* (moringa). Table 1 showed that the three isolates (AFS-A, AFS-B, and AFS-M) all exhibited viable growth on PVK medium over 5–7 days, with AFS-B and AFS-M showing relatively higher counts initially, while AFS-A increased progressively to its peak by day 7.

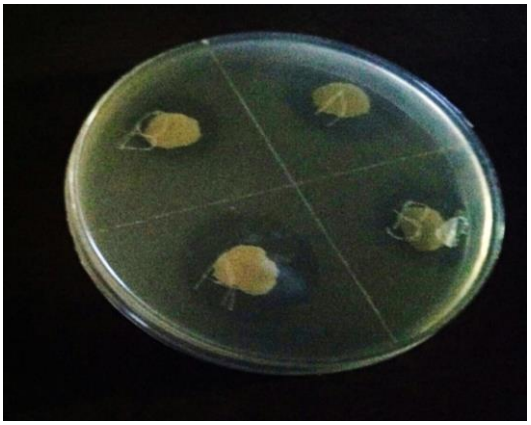

Fig. 1. Halo zone formation of some of the isolates.

Table 1.

Total viable bacterial count (log_{10}cfu/ml) on PVK agar at 37^{O}C for 5-7 days

Isolate	Day 5	Day 6	Day 7
AFS-A	1.57±1.0^d	2.64 ±1.0^d	2.98 ± 0.2^d
AFS-B	8.3 ±0.2^b	1.76 ±1.0^b	2.00 ±0.3^b
AFS-M	7.2±0.0^a	1.52 ±1.0^a	1.96 ±0.1^a

Values are mean ± standard deviations of triplicate counts.

Table 2 shows that there was a significant difference in the solubilization index of the isolates after incubation for 7days. Maximum PSI was observed by *AFS-A* with a solubilization index of 3.22 ± 0.1.

This was followed by *AFS-B* with a solubilization index of 2.14 ± 0.1, and *AFS-M* had a weak PSI of 0.25 ± 0.0 after incubation for 7days.

Table 2.

Qualitative estimation of phosphate solubilization index (PSI) of PSB isolates after 7 days of incubation

Plant source	Inferred PSB	Rate of Solubilisation	Solubilisation Index
Vernonia amygdalima	*AFS-A*	Strongly	3.22 ± 0.1^a
Moringa oleifera	*AFS-B*	Moderate	2.14 ± 0.0^b
Cocos nicifera	*AFS-M*	Weak	0.25 ± 0.0^d

Means followed by different letters are different at 5% level of significance.

Phosphate solubilization efficiency of the isolates was confirmed by quantitative analysis of available phosphorus in PVK liquid medium (Table 3). All the isolated microorganisms solubilized TCP, though they varied in their ability and the growth period. The phosphate solubilization efficiency of the isolates varied from 4.6±0.1µg/ml to 239.0 ± 1.0 µg/ml from day 4 to day 12. Among the 3 isolates, *AFS-A* showed the highest phosphate solubilization efficiency on day 12 (2.39.0 ± 1.0 µg/ml) while *AFS-B* showed the least PSE of 98.0 ± 1.01µg/ml after 12 days of incubation. pH varied from an initial value of 5.0± 0.1 to 4.7 ± 0.1

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for both *AFS-A and AFS-B*. The progressive decrease in pH was the same for all the isolates within the 12-day incubation period.

Table 4 further confirmed that each isolate possesses a distinct biochemical profile, allowing preliminary identification at the genus level through differences in oxidase activity, carbohydrate utilization, motility, and other metabolic reactions. These identifications were validated in Table 5 through 16S rRNA molecular characterization, which precisely classified AFS-A as *Enterobacter hormaecheii*, AFS-B as *Enterobacter cloacae*, and AFS-M as *Clostridium beijerinckii*, each showing high sequence similarity to known reference strains.

Table 3.

pH and quantitative estimation of phosphate solubilization efficiency (PSE) of PSB isolates from Day 4 -12

PSB	$Ca_3(PO_4)_2$					
	Day 4		Day 8		Day 12	
	pH	p(µg/ml)	pH	p(µg/ml)	Ph	p(µg/ml)
AFS-A	5.6±0.1a	4.8±0.1a	5.1±0.1a	284.0±1.0d	4.7±0.1a	239.0±1.0a
AFS-B	5.5±0.1a	4.6±0.1a	5.0±0.1a	136.0±1.0a	4.7±0.1a	98.0±1.0d
AFS-M	5.8±0.1b	5.3±0.1 a	5.2±0.1a	164.0±1.0c	5.0±0.1b	146±1.0c

Means followed by different letters are different at 5% level of significance.

Table 4.

Biochemical characteristics of bacterial isolates

Biochemical Tests	AFS-A	AFS-B	AFS-M
Oxidase	-	+	-
Motility	+	+	+
Nitrate	+	+	+
H2s	+	-	+
Indole	+	-	+
Urease	-	+	-
V/p	+	-	-
Citrte	+	+	-
Catalase	+	+	-
Glucose	+	+	+
Manitol	+	+	+
Xylose	+	+	+
O/f test	F	O	F
Maltose	+	+	+
Galactose	+	+	+
Lactose	+	-	+
Dextrose	+	+	+
Sucrose	+	+	+
M/red	+	-	+
Fructose	+	+	+
Most probable organism	***Enterobacter sp***	***Clostridium sp***	***Alcaligene sp***

Key: +VE = Positive, -VE = Negative, O = Oxidative and F = Fermentative

Table 5.

Molecular Characterization of PSB Isolates

Sample code	Source	Query cover (%)	Percent identity	Accession number	Identity
AFS-A	Rhizosphere	82	96.57	CP017180.1	*E. hormaecheii*
AFS-B	Rhizosphere	82	96.57	CP026850.1	*E. cloacae*
AFS-M	Rhizosphere	90	97.88	MK522142.1	*C. beijerinckii*

All the PSB isolates produced IAA (Table 6). The amount of IAA produced by the isolates ranged from 12.3 ± 0.2 to 44.0 ± 0.0 µg/ml. *E. cloacae* produced the maximum amount of IAA (44.0 ± 0.0 µg/ml). This was followed by *C. beijerinckii* with a total IAA production of 19.1 ± 0.0 µg/ml. The minimum amount of IAA was produced by *E. hormaecheii* (12.3 ± 0.2 µg/ml). Table 7 indicated that the formulated biofertilizers produced from these isolates meet acceptable physicochemical standards, with near-neutral pH (6.8–7.2), moderate moisture content, uniform particle size, stable nutrient composition (notably 10–12% nitrogen and ~5–6% phosphorus), and a high microbial load (8.2×10^9 CFU/g), confirming their suitability and effectiveness for agricultural application.

Table 6.

Indole-3-Acetic Acid (IAA) production efficiency of PSB isolates

PSB	IAA (µg/ml)
E.hormaecheii	12.3 ± 0.2[d]
E. cloacae	44.0 ± 0.0[a]
C. beijerinckii	19.1 ± 0.0[c]

Means followed by different letters are different at 5% level of significance.

Table 7.

Physicochemical Analysis of Biofertilizer

Parameter	*E. hormaecheii*	*E. cloacae*	*Clostridium beijerinckii*
pH	**7.2 ± 0.1**	**6.8 ± 0.1**	**7.0 ± 0.1**
Moisture content (%)	**22.5 ± 1.0**	**24.5 ± 1.0**	**26.2 ± 1.0**
Temperature (°C)	**25.0 ± 0.5**	**27.0 ± 0.5**	**28.0 ± 0.5**
Texture	Moist	Moist	moist
Particle size (mm)	**1.2 ± 0.1**	**1.2 ± 0.1**	**1.2 ± 0.1**
Bulk density (g/cm³)	**1.1 ± 0.1**	**1.1 ± 0.1**	**1.1 ± 0.1**
Water holding capacity (%)	**35.0 ± 2.0**	**36.0 ± 2.0**	**36.0 ± 2.0**
Nitrogen content (%)	**10.2 ± 0.5**	**12.2 ± 0.5**	**10.2 ± 0.5**
Phosphorus content (%)	**5.5 ± 0.2**	**5.7 ± 0.2**	**5.6 ± 0.2**
Potassium content (%)	**10.5 ± 0.5**	**10.5 ± 0.5**	**10.5 ± 0.5**
Microbial Load (CFU/g)	**$8.2 \times 10^9 \pm 1.0$**	**$8.2 \times 10^9 \pm 1.0$**	**$8.2 \times 10^9 \pm 1.0$**

Values are expressed as mean ± standard deviation (SD).
Standard values are based on industry standards and regulatory requirements.
CFU/g = Colony-Forming Units per gram.

DISCUSSION

In the present study, the rhizosphere regions of 15 plants were selected for the isolation of phosphate-solubilizing bacteria. This habitat was selected for isolation because of the greater possibility of occurrence of phosphate-solubilizing bacteria. *Panhwar et al*. (2019) found considerably higher numbers of PSB population in the rhizosphere in comparison with non-rhizospheric or bulk soil. The population level varied in different rhizosphere soils. This is supported by the findings of Kucey (1918), who reported that PSB isolates have been found in almost all soils tested, although the number varies with soil, climatic, and cropping history. This large variation in the distribution of PSB in different soils may be due to the differences in organic carbon content of the soil (Yadav and Singh 2022).

Goenadi et al. (2020) also screened several rhizospheric bacteria for phosphate-solubilizing potential. In this study, 8 bacterial isolates produced a halo zone around colonies in Pikovskaya agar medium. These were selected and purified on the same medium. This is in line with the findings of Gaind (2020), who reported that PSB strains were isolated using the Pikovskaya medium based on the formation of a halo zone around these microorganisms. These findings were also supported by Ahamed and Jha (2022). These organisms grew exponentially in PVK agar at 37°C for 5-7days (Table 1). Sanjotha and Sudheer (2020) also isolated microbial colonies from the soil of Karwar Coastal Region, which showed clear zones around the microbial growth and were considered as phosphate solubilization. Three isolates (AFS-A, AFS-B, and AFS-M) were selected from the 8 bacterial isolates based on their phosphate solubilization index (Table 2), Phosphate Solubilization Efficiency, and pH in liquid medium (Table 3).

pH of the medium turned acidic, indicating that production of organic acids by PSB can facilitate the solubilization of phosphate. (Singh *et al*., 2020 and Kumar *et al*., 2020). Maximum decline in pH was recorded with *Enterobacter cloacoa*, from 5.6 ± 0.1 to 3.7 ± 0.1 at the end of the 12-day incubation period. *C. beijerinckii* had the least decline in pH from 5.8 ± 0.1 to 5.0 ± 0.1 from day 4 to day 12. A fall in pH accompanied phosphate solubilization, but due to the production of organic acids, no correlation could be established between acidic pH and the quantity of Phosphate liberated (Patel *et al*., 2022). Medium pH tended to decrease in all cases of growth; however, no constant relationship was found between amounts of P released and the drop in pH. The lack of relationship between pH drop and P solubilized may be due to the liming effect of rock P and the production of other metabolites by the microbes (Kucey, 1918)

Morphological and Biochemical tests carried out on the PSB isolates led to their probable identification up to the genus level. Results for some of the common tests performed are listed in Table 4. The results of the

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morphological and biochemical tests performed were in line with the descriptions of *Barnett et al*. (2017)

The assessment of the bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique as it can better identify weakly described, rarely isolated or phenotypically aberrant strains. (*Panhwar et al*., 2019). In this study, a molecular phylogenetic approach based on 16S rRNA sequences to identify pure potential isolates was used. According to the sequence of the 16S rRNA gene, 2 isolates belong to the *Enterobacter sp* and one to the *Clostridium sp*. Sequences from the 3 isolates were almost 97-98% similar to other 16S rRNA sequences from the NCBI database, as shown in Table 5. Bacterial isolate AFS-A were identified as *Enterobacter hormaecheii* (96.57% sequence homology), AFS-B was identified as *Enterobacter cloacoa* (96.57% sequence homology), while isolate AFS-M was identified as *Clostridium beijerinckii* (97.88% sequence homology). This is in line with the findings of *Yadav and Singh (2022)*, who isolated 7 *Enterobacter* strains, including *E. hormaecheii,* from the roots of the Quinoa plant. *Maria-Micaela et al.* (2020) reported that *Enterobacter sp* was one of the most efficient strains isolated from the rhizosphere with the potential to enhance tomato plant performance. In addition, *Liu et al*. (2013) also noted that *E. cloacae* species are an extremely diverse group of bacteria that are associated with plants, soils, and humans.

In addition to Phosphate solubilization, PSB also produced other secondary metabolites like indole-3-acetic acid(IAA) (Table 6). Several evidence have demonstrated plant growth promotion by PSB through the production of IAA (Sanjotha and Sudheer, 2020). The result of IAA production from the PSBs tested here supports the observations of Selvi et *al.* (2017), who reported the production of PGPS-like IAA, gibberelins, cytokinins, or their combinations from 50 phosphate-dissolving bacteria. Several workers, like Ahemad and Kibret (2020), Gulati *et al*. (2020), and Singh *et al*. (2021), have also reported on the production of IAA by PSBs.

Enterobacter cloacoa produced the maximum quantity of IAA (44.0 ± 0.0), while *Clostridium beijerinckii* produced the least amount (19.1 ± 0.0). The amount and range of IAA produced in general in this study by the PSB isolates were comparable to those reported by several authors about different PSB strains (Singh *et al*., 2021; Xu *et al*., 2020), and Rodriguez *et al*. (2022), and also supported the view that auxin production is common among rhizosphere bacteria.. The large variation in the amount of IAA produced by different strains has been attributed to the variability in the metabolism of the different strains of PSB (Tamura *et al*., 2013). Auxins produced by PSBs can influence several plant growth components, including nutrients, thus increasing plant growth (Tiwara *et al*., 2020). Thus, it is possible that the strains tested here can also provide additional growth-promotional activity apart from releasing P into the rhizosphere.

The physicochemical properties of any biofertilizer play a crucial role in determining its effectiveness, stability, and safety. The optimal physicochemical properties of biofertilizer can vary depending on the type of microorganisms and the carrier material used.

The physicochemical properties of the biofertilizer were found to be within the acceptable range for agricultural use.

The pH of the biofertilizer was found to be in the range of 6.8-7.2. The moisture content of the biofertilizer was found to be less than 10%. The biofertilizer was found to contain a high concentration of nitrogen (10-12%), phosphorus (5-10%), and potassium (5-10%). The biofertilizer was found to contain a high concentration of viable bacterial cells (10^8 CFU/g). The biofertilizer was found to contain a diverse range of beneficial microorganisms, including nitrogen-fixing bacteria, phosphate-solubilizing bacteria, and plant growth-promoting rhizobacteria. The biofertilizer was found to be effective in promoting plant growth and increasing crop yields. The biofertilizer was found to be effective in improving soil fertility and reducing soil pollution. The biofertilizer was found to be safe for use on a wide range of crops, including cereals, legumes, and vegetables.

The isolation, preparation, and production of biofertilizer using *Enterococcus cloaca, Enterococcus homaecheii,* and *Clostridium beijerinckii* is a promising approach for sustainable agriculture. The use of these microorganisms as biofertilizers has several benefits, including plant growth promotion, soil fertility improvement, environmental benefits, and economic benefits.

CONCLUSIONS

The results of this study demonstrate the potential of using *Enterococcus cloaca, Enterococcus homaecheii*, and *Clostridium beijerinckii* as biofertilizers for sustainable agriculture. The biofertilizer produced in this study was found to be effective for promoting plant growth, improving soil fertility, and reducing soil pollution. The biofertilizer was also found to be safe for use on a wide range of crops. Further research is needed to scale up the production of this biofertilizer and to promote its widespread adoption in sustainable agriculture.

AUTHORS CONTRIBUTIONS

*C*onceptualization: A.C.N and F.A.I.; methodology, A.C.N.; data collection A.C.N.; data validation, A.C.N., and F.A.I.; data processing F.A.I.; writing—original draft preparation, A.C.N. and F.A.I.; writing—review and editing, A.C.N. and F.A.I.

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CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

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