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# Isolation and Identification of PHA-Producing Haloalkaliphilic Bacteria from Bledug Kesongo Mud Volcano

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**Abstract:** Bledug Kesongo, a geothermal mud volcano in Indonesia, provides an extreme hypersaline and hyperalkaline environment that supports diverse haloalkaliphilic bacteria with potential for biopolymer production. This study explored the diversity of haloalkaliphilic bacteria inhabiting this site and evaluated their ability to synthesize polyhydroxyalcanoates (PHAs), intracellular polymers that serve as compatible solutes for osmotic balance. A total of 35 bacterial isolates were obtained, predominantly Gram-positive cocci exhibiting moderate halophicity and alkaliphicity. PHA screening revealed that 14 isolates were capable of accumulating PHAs, with isolates BK25 and BK30 showing the highest productivity, yielding 12.91±2.48% and 18.66±1.43% PHA content, respectively. Molecular identification based on 16S rRNA gene sequencing identified the isolates as *Priestia flexa* (BK25) and *Bacillus flexus* (BK30). These findings highlight Bledug Kesongo as a promising source of haloalkaliphilic bacteria with potential application in sustainable bioplastic production.

**Keywords:** Kesongo mud volcano; haloalkaliphilic; polyhydroxyalkanoates; 16S rRNA.

## 1. Introduction

Indonesia, situated along the Pacific Ring of Fire (ROF), experiences intense volcanic activity and possesses vast geothermal energy potential [1]. Among its diverse geothermal features are mud volcanoes, geological formations that expel mud, fluids, and gases from subsurface overpressured zones [2,3]. One notable example is the Bledug Kesongo mud volcano in Blora Regency, Central Java, characterized by active mud eruptions and extensive saline, alkaline deposits [4].

Mud volcanoes serve as habitats for diverse and potentially exploitable microorganisms. However, the microbial potential of Bledug Kesongo remains largely unexplored compared to other mud volcanoes. Previous studies on microbial communities in various mud volcanoes have revealed diverse potentials, such as sulfur and methane oxidation by microbes through metagenomic approaches in the Kerch Peninsula mud volcano [5]; the potential of halotolerant bacteria in polyethylene microplastic biodegradation in Bledug Kuwu [6]; and the production of antibacterial and antimalarial compounds by bacteria from the Lapindo mud volcano [7].

The microbial potential of Bledug Kesongo can be further explored through bioprospecting. Bioprospecting is a systematic process that allows researchers to investigate biodiversity and identify novel molecules, microorganisms, and enzymes for further study and industrial application [8,9]. One promising area of microbial bioprospecting at Bledug Kesongo is the potential of haloalkaliphilic bacteria to produce polyhydroxyalkanoates (PHAs). PHAs are synthesized as carbon storage and can maintain the cell's osmotic pressure due to their ability as compatible solutes [10–15]. Over 300 bacterial species have been identified as PHAs producers, although only a few are utilized in industry, such as *Ralstonia eutropha* [16,17]. Haloalkaliphiles are promising PHA producers due to their contamination-resistant growth, ease of cell lysis, and ability to utilize low-cost substrates [18–20].

Several previous studies have explored potential PHA-producing microbes, with microbial isolation conducted in various locations, such as sewer water [21] and household, garden, gas station, and agricultural waste [22]. An exploration of haloalkaliphilic bacteria as PHA producers was conducted in Lake Simbi Nyaima, a hypersaline lake located in western Kenya. The results led to the discovery of *Halomonas alkalicola* as a haloalkaliphilic bacterial producer of PHAs under high salinity and alkaline conditions [23]. However, research on haloalkaliphilic bacteria producing PHAs in mud volcano habitats remains limited. Thus, the exploration of haloalkaliphilic bacteria from the Bledug Kesongo mud volcano is warranted.

This study aims to isolate haloalkaliphilic bacteria for analysis of their macro- and microscopic diversity, assess their ability to produce PHAs, and identify them using the 16S rRNA gene to determine species.

## 2. Materials and Methods

### *2.1. Sample collection.*

The sampling site was Bledug Kesongo, Central Java, Indonesia. Mud samples were collected from an active erupting dome at a depth of 10–20 cm. Environmental parameters, including temperature, pH, and salinity, were measured using a thermometer, pH meter, and refractometer, respectively.

### *2.2. Isolation of haloalkaliphilic bacteria.*

Bacteria were isolated from mud samples following the method of Asy'ari *et al.* [24], with modifications to the Luria Bertani (LB) medium to adjust the pH to 9.0 and salinity to 4% to simulate the original environment in Bledug Kesongo (composition provided in supplementary file table S1). Enrichment was performed by mixing 2 g of the mud sample with 200 mL of modified Luria Bertani Broth (LBB) at 0.1× and 0.01× concentrations, followed by incubation at 37°C for 72 hours in a shaker incubator. After incubation, serial dilutions of the enriched samples were prepared in modified LBB (0.1× and 0.01×) up to a $10^{-7}$ dilution. From each dilution, 1 mL was spread-plated in duplicate onto modified Luria Bertani Agar (LBA) plates (0.1× and 0.01×) and incubated at 37°C for 120 hours. The resulting colonies were observed for morphological characteristics and subsequently purified on modified LBA (0.1× and 0.01×). Pure isolates were then maintained on standard LB medium. The composition of standard LB medium was based on Atlas [25].

### *2.3. Isolate characterization.*

The pure isolates of haloalkaliphilic bacteria were characterized macroscopically by observing the colony morphology, including colony shape, margin, color, texture, and elevation. The bacterial isolates were also characterized microscopically by observing cell shape and performing Gram staining.

### *2.4. PHA's screening.*

Primary screening was performed by cultivating haloalkaliphilic bacterial isolates on carbon-enriched agar medium [26], modified to pH 9.0 and 4% salinity (composition provided in supplementary file table S2). The isolates were incubated at 37°C for 48 hours. After incubation, the agar plates were flooded with 0.02% ethanolic Sudan Black B solution and left for 20 minutes. The dye solution was then discarded, and the plates were rinsed with absolute ethanol to remove excess stain. A positive result was indicated by the appearance of dark bluish-black bacterial colonies [27]. For secondary screening, bacterial isolates were inoculated onto modified carbon-enriched agar supplemented with Nile Blue A dye. The cultures were incubated at 37°C for 48 hours and observed under UV light at a wavelength of 365 nm. Positive PHA-producing isolates were identified by the presence of fluorescent colonies [27–30].

### *2.5. PHA's production.*

Two media were used for bacterial cultivation: a modified LBB medium for starter growth and a modified Mineral Salt Medium (MSM) for PHA fermentation. The modified LBB contained LB powder and glucose, while the MSM consisted of urea, $KH_2PO_4$, $Na_2HPO_4$, $MgSO_4 \cdot 7H_2O$, $CaCl_2$, glucose, and a trace element solution (composition provided in supplementary file table S3). Glucose and trace elements were sterilized separately and added aseptically [31]. Both media were adjusted to pH 9.0 and 4% salinity.

The best PHA-producing isolate was grown in 50 mL of modified LBB medium in 250 mL Erlenmeyer flasks, then incubated at 37°C for 48 hours. Growth was monitored by measuring optical density (OD 600) every 4 h to construct growth curves in both LBB and MSM media, allowing determination of the logarithmic (starter) and stationary (harvest) phases. For PHA production, 2% (v/v) of logarithmic-phase culture was inoculated into 50 mL of sterile modified MSM medium in 250 mL Erlenmeyer flasks and incubated under the same conditions. Each fermentation was conducted in duplicate, with uninoculated MSM as a control.

### *2.6. Extraction of PHAs.*

Polyhydroxyalkanoates (PHAs) were extracted from bacterial fermentation cultures using a modified sodium hypochlorite–chloroform dispersion method [32]. Briefly, 10 mL of the fermentation broth was centrifuged at 8500× g for 15 min, and the supernatant was discarded. The resulting cell pellet was washed with phosphate-buffered saline (PBS, pH 7.4), air-dried for 2 h, and weighed to determine the dry cell weight (DCW).

For PHA extraction, the dried pellet was suspended in chloroform and 4% sodium hypochlorite solution (12.5 µL each per mg of pellet weight) and incubated at 37°C for 90 min with shaking to digest non-PHA cellular material. The mixture was centrifuged at 6500×g for 10 min, and the lower chloroform phase containing PHA was collected. This phase was mixed with five volumes of methanol–water (7:3 v/v) to precipitate the polymer, followed by centrifugation (8500×g, 15 min) and air-drying for 1 h. The dry weight of the PHA precipitate was recorded as the extracted PHA weight.

Biomass residue was calculated as the difference between DCW and extracted PHA weight, while PHA accumulation (%) was determined as the ratio of PHA weight to DCW × 100 [27]. The formulas can be written as follows:

$$BR = DCW - DWP \qquad (1)$$

$$\%PHA\ Accumulation = \frac{DWP}{DCW} \times 100 \qquad (2)$$

Where BR is biomass residue (g/L), DCW is the dry cell weight of the cell pellet (g), and DWP is the dry weight of extracted PHA (g/L).

All experiments were performed in duplicate, then analyzed in Microsoft Excel, and the results were expressed as mean ± standard deviation (SD).

### *2.7. Molecular identification by 16S rRNA gene.*

Genomic DNA from the bacterial isolate was extracted using the Instagene Matrix according to the procedure recommended by Bio-Rad Laboratories. The concentration and purity of the extracted DNA were analyzed using the NanoDrop 2000 spectrophotometer (Thermo Scientific). The 16S rRNA gene was amplified using the universal primers 27F (5’-AGA GTT TGA TCC TGG CTC AG - 3’) and 1492R (5’- GTT TAC CTT GTT ACG ACT T - 3’) [33]. A 50 µL PCR master mix solution was prepared containing 2 µL of primer 27F, 2 µL of primer 1492R, 2 µL of DNA template, 25 µL of MyTaq polymerase, and 19 µL of ddH$_2$O. PCR was performed with an initial denaturation at 96°C for 2 minutes, followed by denaturation at 96°C for 1 minute, annealing at 51°C for 1 minute, extension at 72°C for 38 seconds, and final extension at 72°C for 7 minutes. The PCR amplicon was analyzed by electrophoresis on a 1% agarose gel, then visualized using gel documentation. The amplicon was then sequenced to obtain the nucleotide sequence, which was compared with sequences in GenBank using BLAST on the NCBI website.

### *2.8. Phylogenetic analyses.*

Phylogenetic analysis was conducted by constructing a phylogenetic tree to determine the genetic relationship of the haloalkaliphilic bacteria. Phylogenetic tree reconstruction was performed using the Neighbor-Joining method with 1000 Bootstrap replications in MEGA XI software, and the resulting tree was evaluated to identify the species with the closest genetic relationship [34-37].

## 3. Results and Discussion

### 3.1. Isolation and characterization of bacteria.

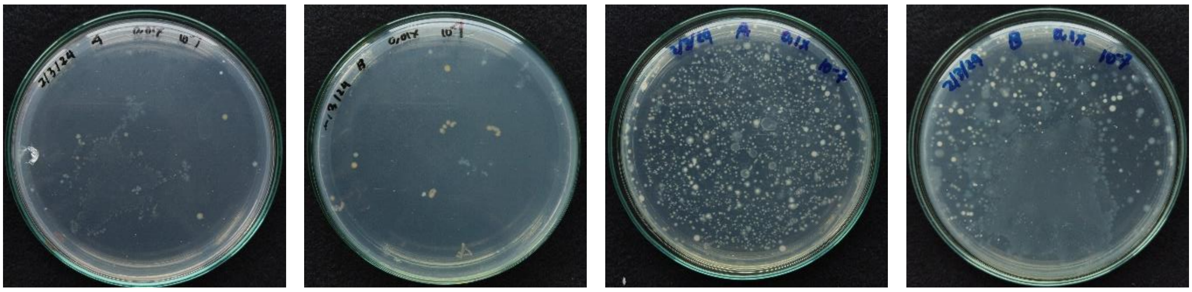
The mud emitted from the Bledug Kesongo dome structure can be characterized through measurement of its environmental parameters (Figure 1). Measurement of environmental parameters at Bledug Kesongo mud volcano resulted in the following: temperature 34°C, pH 9, and salinity 4%. Environmental parameters can vary at different mud volcanoes depending on their characteristics and geographic location. A study by Sepanian *et al*. [38] showed that the Ain mud volcano, located in Iran, had a temperature of 20°C and a pH of 9. Frolova *et al*. [39] measured the environmental parameters at the Gnilaya Gora mud volcano, located on the Taman Peninsula, Krasnodar Krai, Russia, where the temperature was 21°C and pH 8.5. Sabdaningsih and Lunggani [40] measured environmental parameters at Bledug Kuwu, Grobogan, which included temperature and salinity. The results showed that the mud fluid of Bledug Kuwu had a temperature of 20°C and a salinity of 2%.

**Figure 1.** The eruption area and mud volcano dome structure of Bledug Kesongo.

The high salinity of the Bledug Kesongo mud volcano results from the mineral salt content of the mud and the fluids generated by geological processes. The fluid exhibits high salinity due to the dominance of $Na^+$ and $Cl^-$ ions, characteristic of mud volcano geochemistry [41,42]. These ion concentrations are primarily controlled by water-source mixing, surface evaporation, and upward fluid migration rather than by biological activity. The salinity originates from connate water—ancient seawater trapped within sedimentary rock pores—that mixes with the mud. In addition to $Na^+$ and $Cl^-$, the mud fluid contains various minerals, including K, Mg, Ca, $SO_4$, Sr, Li, Fe, and B, as well as gases such as $CO_2$, $N_2$, $CH_4$, $O_2$, Ar, He, and $H_2$ [43]. The alkaline pH of the mud is attributed to high bicarbonate ($HCO_3^-$) and sulfate ion concentrations, which influence the overall geochemical composition [44].

The high salinity and alkaline pH of the Bledug Kesongo environment create favorable conditions for the growth of haloalkaliphilic bacteria. The bacterial isolates inhabit a habitat with approximately 4% salinity, classifying them as moderate halophiles according to Kushner [45], who defined halophiles as slightly (3%), moderately (3–15%), and extremely (12–25%) halophilic. With an environmental pH around 9, these bacteria are also categorized as alkaliphiles. Microorganisms are grouped based on optimal growth pH as acidophiles (pH <5), neutrophiles (pH 5–9), and alkaliphiles (pH >9) [46, 47].

Isolation of haloalkaliphilic bacteria was carried out using LB media with modifications to pH and salinity. LB media is commonly used in microbiology to grow nearly all types of microbes. The modified LB media composition was diluted to 0.1x and 0.01x to facilitate bacterial adaptation to artificial media. According to Lee *et al*. [48] and Sun *et al*. [49], LB media at a 0.1% dilution can be used as a growth medium for cultures isolated from the environment. Diluted LB media is effective for cultivating bacteria that are difficult to culture on undiluted LB media. Research by Yamamoto *et al*. [50] showed that bacterial communities on 10% LB were more diverse than on LB, indicating that the concentration of LB media is crucial for supporting colony formation and isolating beneficial bacterial strains.The isolation resulted in bacterial colonies growing on modified LBA 0.1x and 0.01x, with varying colony counts. The colony count on LBA 0.1x media was 9.5 x $10^9$ CFU/mL, while on LBA 0.01x media it was 6 x $10^7$ CFU/mL. The bacterial colonies were most clearly visible at the $10^{-7}$ dilution (Figure 2). According to Elisanti *et al*. [51], Schoeborn *et al*. [52], and Yan *et al*. [53], serial dilution aims to reduce the bacterial load in the liquid suspension. Serial dilution effectively reduces microbial diversity in each dilution, making it easier to grow specific bacterial groups. Serial dilution has proven successful in enhancing the culture capacity and facilitating the isolation of bacteria and archaea from environmental samples.



**(a)** **(b)**

**Figure 2.** The growth of haloalkaliphilic bacterial colonies after 10-7dilution on LBA media. The colonies appear to grow clearly and are spread out across the petri dish: **(a)** LBA 0.01x modified media; **(b)** LBA 0.1x modified media.

Isolation of haloalkaliphilic bacteria from Bledug Kesongo yielded 35 pure bacterial isolates (BK1–BK35). Colony morphology varied among the isolates, but most exhibited round shapes with smooth margins, raised elevation, moist texture, and brown-cream pigmentation (Table 1). These features are consistent with observations from similar hypersaline environments, such as the Ain mud volcano in Iran [38]. The observed variation in colony form, color, and texture likely reflects adaptive responses to environmental pressures and represents evidence of evolutionary divergence among the isolates.

**Table 1.** Macroscopic and microscopic characterization of haloalkaliphilic bacteria isolates on modified LBA media.

| **No.** | **Code** | **Macroscopic characteristic** | | | | | **Microscopic characteristic** | |
|---|---|---|---|---|---|---|---|---|
| | | **Shape** | **Margin** | **Elevation** | **Texture** | **Colour** | **Cell shape** | **Gram** |
| 1 | BK1 | Round | Smooth | Raised | Moist | Brown with transparent edges | Bacil | + |
| 2 | BK2 | Rhizoid | Irregular | Raised spreading edge | Moist | Brownish cream | Coccus | + |
| 3 | BK3 | Round | Smooth | Raised | Moist | Brown | Coccus | + |
| 4 | BK4 | Round | Smooth | Convex | Moist | Orange | Bacil | - |
| 5 | BK5 | Round | Smooth | Convex | Moist | Dark orange | Coccus | - |
| 6 | BK6 | Round | Smooth | Raised | Moist | White cream | Coccus | + |
| 7 | BK7 | Round | Smooth | Convex | Moist | Dark orange | Bacil | + |

| No. | Code | Macroscopic characteristic | | | | | Microscopic characteristic | |
|---|---|---|---|---|---|---|---|---|
| | | Shape | Margin | Elevation | Texture | Colour | Cell shape | Gram |
| 8 | BK8 | Irregular | Lobate | Raised spreading edge | Moist | Brown | Coccus | + |
| 9 | BK9 | Lobate | Irregular | Raised spreading edge | Mucoid | Cream | Bacil | + |
| 10 | BK10 | Round | Smooth | Convex | Mucoid | Brown white | Coccus | + |
| 11 | BK11 | Filamentous | Irregular | Raised | Moist | Cream | Coccus | + |
| 12 | BK12 | Filamentous | Filamentous | Raised | Moist | Cream | Coccus | + |
| 13 | BK13 | Round | Smooth | Raised | Moist | Yellow | Bacil | + |
| 14 | BK14 | Round | Lobate | Raised | Moist | White cream | Coccus | + |
| 15 | BK15 | Round | Smooth | Raised spreading edge | Dry | Cream with transparent edges | Coccus | - |
| 16 | BK16 | Filamentous | Filamentous | Raised | Dry | Cream | Coccus | + |
| 17 | BK17 | Round | Smooth | Umbonate | Moist | Brown with transparent edges | Coccus | + |
| 18 | BK18 | Round | Smooth | Raised | Mucoid | Cream with white edges | Bacil | - |
| 19 | BK19 | Round | Loabte | Raised | Moist | Yellow | Bacil | + |
| 20 | BK20 | Rhizoid | Irregular | Raised | Dry | Cream | Coccus | + |
| 21 | BK21 | Irregular | Lobate | Umbonate | Moist | Orange | Coccus | - |
| 22 | BK22 | Irregular | Irregular | Umbonate | Dry | White cream | Coccus | + |
| 23 | BK23 | Filamentous | Lobate | Raised spreading edge | Moist | Brown with white edges | Bacil | + |
| 24 | BK24 | Filamentous | Filamentous | Raised | Dry | Cream | Coccus | + |
| 25 | BK25 | Round | Irregular | Raised | Moist | White cream | Bacil | + |
| 26 | BK26 | Filamentous | Filamentous | Raised | Dry | Cream | Coccus | + |
| 27 | BK27 | Round | Smooth | Raised | Moist | Cream | Coccus | + |
| 28 | BK28 | Irregular | Irregular | Flat | Dry | Transparent cream | Coccus | + |
| 29 | BK29 | Irregular | Lobate | Umbonate | Moist | Brown with cream edges | Bacil | + |
| 30 | BK30 | Round | Irregular | Raised | Moist | Yellow cream | Bacil | + |
| 31 | BK31 | Rhizoid | Rhizoid | Flat raised margin | Dry | White | Coccus | + |
| 32 | BK32 | Round | Irregular | Convex | Moist | Pink with cream edges | Coccus | + |
| 33 | BK33 | Round | Smooth | Flat | Dry | White with transparent edges | Bacil | + |
| 34 | BK34 | Round | Irregular | Umbonate | Moist | Transparent brown | Coccus | + |
| 35 | BK35 | Round | Irregular | Raised | Moist | Light brown | Coccus | + |

Microscopic analysis revealed two main cell morphologies—bacilli and cocci—with a predominance of Gram-positive bacteria. The prevalence of Gram-positive isolates suggests that cell wall composition plays an important role in adaptation to the high salinity and alkalinity of Bledug Kesongo. The thick peptidoglycan layer of Gram-positive bacteria enhances structural stability and helps prevent osmotic lysis, providing a selective advantage in hypersaline environments. As noted by Aono [54] and Uma *et al*. [55], the presence of teichuronic acid (TUA) and teichurono peptide (TUP) within the cell wall further contributes to maintaining cell integrity under stress conditions.

### *3.2. Screening of PHAs.*

Screening was conducted to evaluate the ability of bacterial isolates to produce PHA. Out of 35 isolates tested, 31 exhibited positive results (Table 2), as indicated by the color change of the colonies to black (Figure 3). Colonies that do not possess affinity for, or the ability to bind, Sudan Black B appear white, whereas positive colonies are characterized by a distinct black coloration. Sudan Black B is soluble in triglycerides, making it a useful stain for detecting lipid inclusions such as phospholipids, sterols, and neutral fats [56–59].

However, Sudan Black B is non-specific for PHA, as it can also bind to other hydrophobic substances, including lipids. Therefore, a secondary screening using more specific dyes, such as Nile Blue or Nile Red, is required to confirm PHA production. These dyes exhibit higher specificity toward PHA compounds compared to Sudan Black B, which may also stain other lipid substances and inclusion bodies [60–64].

**Table 2.** Results of PHA's screening of haloalkaliphilic bacteria from Bledug Kesongo.

| **Isolate Code** | **Screening** | | **PHA** | **Isolate Code** | **Screening** | | **PHA** |
|---|---|---|---|---|---|---|---|
| | **Primary** | **Secondary** | | | **Primary** | **Secondary** | |
| BK1 | +++ | - | - | BK19 | +++ | - | - |
| BK2 | + | - | - | BK20 | +++ | + | + |
| BK3 | +++ | - | - | BK21 | - | - | - |
| BK4 | + | - | - | BK22 | +++ | + | + |
| BK5 | + | - | - | BK23 | +++ | - | - |
| BK6 | + | - | - | BK24 | +++ | - | - |
| BK7 | + | - | - | **BK25** | **++** | **++++** | **+** |
| BK8 | + | - | - | BK26 | +++ | + | + |
| BK9 | ++ | - | - | BK27 | - | - | - |
| BK10 | - | - | - | BK28 | +++ | + | + |
| BK11 | ++ | + | + | BK29 | +++ | ++ | + |
| BK12 | + | + | + | **BK30** | **+++** | **+++** | **+** |
| BK13 | + | - | - | BK31 | ++ | - | - |
| BK14 | ++ | + | + | BK32 | ++ | - | - |
| BK15 | ++ | + | + | BK33 | - | - | - |
| BK16 | ++ | + | + | BK34 | ++ | - | - |
| BK17 | + | - | - | BK35 | ++ | + | + |
| BK18 | ++ | + | + | K | - | - | - |
| Total | | | | | 31 | 14 | 14 |

Primary screening is performed using Sudan Black B, while secondary screening is done using Nile Blue A. The symbol "+" indicates the intensity of the darkening of the black color in the primary screening and the fluorescence level in the secondary screening. The symbol "–" indicates a negative result in the screening. The screening data presented were obtained at 48 hours of bacterial age on carbon-enriched agar media.

**(a)** **(b)**

**Figure 3.** Results of PHA primary screening using Sudan Black B dye at 48 hours. **(a)** Isolate before staining; **(b)** Isolate after staining. A color change to black in the colonies was observed in BK 19, 20, 22, 23, and 24, while the colonies remained white in BK 21.

Secondary screening was performed using Nile Blue A dye. Based on this secondary screening, 14 haloalkaliphilic bacterial isolates showed positive results (Table 2), indicated by fluorescence observed in the colonies (Figure 4). The ability of Nile Blue A to stain PHA is related to the structural characteristics of PHA, which is stored in the bacterial cytoplasm as granules measuring 0.2–0.5 µm in diameter and encapsulated by a phospholipid monolayer. This monolayer interacts with lipophilic dyes, resulting in fluorescence under UV illumination (312/365 nm) or upon excitation at wavelengths of 480–490 nm and 510–560 nm, depending on the degree of hydrophobicity [65–69].

**(a)** **(b)**

**Figure 4.** Results of secondary PHA screening using Nile Blue A dye at 48 hours. The colonies on the Petri dish were illuminated with UV light at a wavelength of 365 nm using instruments **(a)** gel documentation; **(b)** UV transilluminator. Fluorescence or luminescence was observed with varying intensities in the haloalkaliphilic bacterial isolates BK25, BK26, BK28, BK29, and BK30, while no fluorescence was observed in isolate BK27.

Based on the results of primary and secondary screening, the two best haloalkaliphilic bacterial isolates for qualitative production of PHA compounds are BK25 and BK30. The determination of the best PHA-producing isolates was based on the darkness of the bacterial colony color in the primary screening and the intensity of fluorescence in the secondary screening (Table 2).

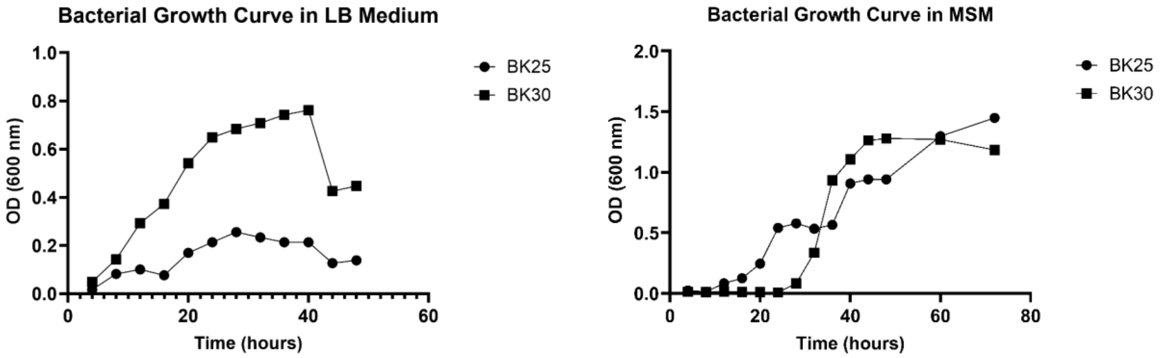
### *3.3. PHAs production.*

PHA production by the haloalkaliphilic bacterial isolates BK25 and BK30 was carried out using the shake flask fermentation method. The process consisted of three main stages: observation of the bacterial growth curve, preparation of the inoculum, and fermentation. Monitoring bacterial growth helps determine the optimal phase for inoculum harvesting to ensure efficient PHA synthesis. According to Hall *et al*. [70], the growth rate reflects the change in cell number per unit time. It is typically estimated by measuring optical density (OD) at 600 nm using a spectrophotometer, which quantifies culture turbidity as an indicator of cell concentration.

Based on the growth rate analysis, the optimal inoculum ages selected for fermentation were 24 hours for isolate BK25 and 20 hours for isolate BK30, corresponding to their respective exponential growth phases (Figure 5). According to Risna *et al*. [71], Siswati *et al*. [72], and Yarinsa *et al*. [73], the exponential or logarithmic phase is the second growth phase, marked by an increase in bacterial or microbial activity characterized by rapid cell shape changes and number growth. The starter culture used is generally in the mid-exponential or logarithmic growth phase. The exponential growth phase is influenced by biological factors, such as the characteristics and morphology of the microbes, as well as non-biological factors, such as growth medium, pH, and temperature.

Cell harvesting was conducted at 72 hours for BK25 and 60 hours for BK30, coinciding with the stationary phase when PHA accumulation typically peaks (Figure 5). According to Garcia *et al*. [74] and Norhafini *et al*. [75], PHA starts accumulating at the end of the exponential phase and increases during the stationary phase. Sharma *et al*. [76] added that PHA is a secondary metabolite produced during the stationary phase, regulated by the transcription and translation of specific genes, such as the rpoS gene in *Pseudomonas putida*, which positively regulates PHA synthesis and is expressed during the stationary phase.

PHA production by both isolates was carried out through batch culture fermentation using MSM production media. Batch culture fermentation is the simplest method for initial laboratory-scale production with minimal contamination. According to Yang and Sha [77], in batch fermentation, microorganisms are inoculated into the media in the fermenter, and no additional nutrients are added during fermentation. Nutrients in the medium are consumed by the microorganisms, and products are accumulated. The advantages of batch culture fermentation are its ease of use and low contamination risks.

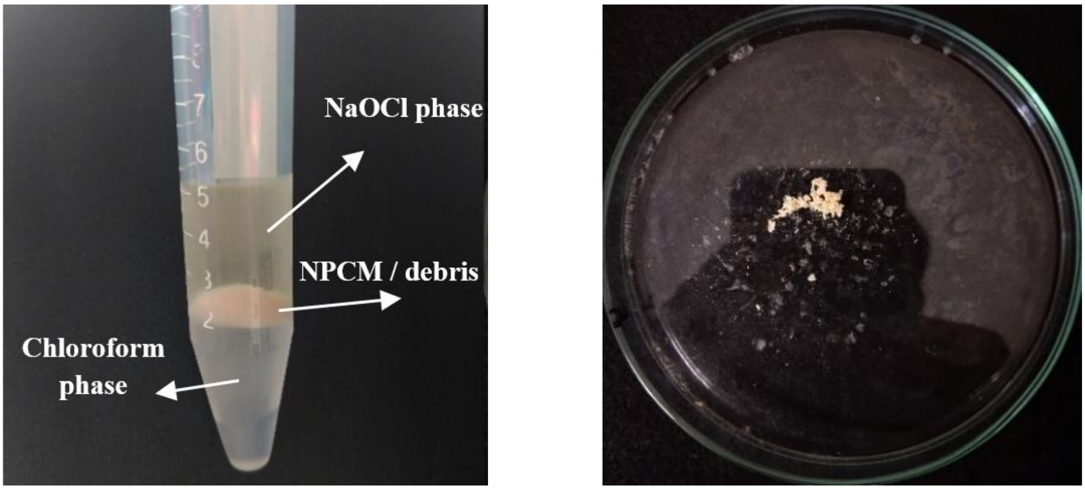


**(a)** **(b)**

**Figure 5.** The growth curve of haloalkaliphilic bacteria isolates BK 25 and 30 on media **(a)** Luria Bertani; **(b)** Mineral Salt Medium. The curve on LB media is used to determine the starter age in the fermentation process based on the bacterial log phase. The curve on MSM media is used to determine the harvest time based on the bacterial stationary phase.

### *3.4. PHAs extraction.*

PHA extraction was conducted at 60 hours for isolate BK25 and 72 hours for BK30 using the sodium hypochlorite (NaOCl)–chloroform dispersion method. This technique combines cell digestion and solvent extraction to recover intracellular PHA granules efficiently. NaOCl serves as a digestion reagent that lyses bacterial cells, releasing PHA granules from the cytoplasm, while chloroform acts as a solvent to dissolve and separate the granules from other cellular components.



**(a)** **(b)**

**Figure 6.** The extraction process of polyhydroxyalkanoates (PHAs) compounds using the sodium hypochlorite-chloroform solvent method. **(a)** The fractions formed after digestion and centrifugation; **(b)** The PHA extract powder after drying. PHA is present in the chloroform phase.

PHAs are synthesized intracellularly as granules surrounded by specific proteins, necessitating cell disruption for their isolation [78–81].

The downstream process of PHA production involves separating the polymer from cellular components through centrifugation. Centrifugation of the cell–reagent mixture produces three distinct phases: the NaOCl phase, the chloroform phase, and the cell debris (Figure 6). The chloroform phase contains the PHA compound, as chloroform, a non-polar solvent, is effective in dissolving organic, non-polar polymers such as PHA [32, 82].

The extraction results showed that isolate BK30 had an average PHA content of 18.66±1.43%, which was higher than that of BK25, with 12.91±2.48% (Table 3). Under non-optimized conditions, these results are comparable to those reported for other bacteria, including *Halomonas alkalicola* (16.46%), *Bacillus pumilus* strain 37 (13.26%), *Bacillus* sp. strain FA253 (10.92%), *Bacillus subtilis* OTPB28 (3.71%) [83], *Cupriavidus* VK-15 (40±0.19%), and *Pseudomonas* VK-10 (18±0.11%) [84]. The genus *Bacillus* can produce PHA due to its tolerance to various environmental conditions, including stressors such as carbon excess and nutrient limitation. Ray and Kalia [85] reported that *B. cereus* and *B. thuringiensis* achieved up to approximately 71% PHA yield based on dry cell mass under favorable conditions.

**Table 3.** PHA extraction using the NaOCl – chloroform dispersion method at harvest times of 60 hours (BK25) and 72 hours (BK30). The data were measured in duplicate and reported as mean ± SD.

| Isolates | Rep | DCW | DWP | RB | PHA Content (%) | Average (%) |
|---|---|---|---|---|---|---|
| BK25 | 1 | 0.060 ± 0.021 | 0.009 ± 0.004 | 0.051 ± 0.017 | 14.67 ± 1.89 | 12.91 ± 2.48 |
| | 2 | 0.098 ± 0.031 | 0.011 ± 0.031 | 0.085 ± 0.035 | 11.16 ± 2.82 | |
| BK30 | 1 | 0.055 ± 0.018 | 0.011 ± 0.002 | 0.044 ± 0.016 | 19.67 ± 2.49 | 18.66 ± 1.43 |
| | 2 | 0.060 ± 0.004 | 0.011 ± 0.001 | 0.049 ± 0.003 | 17.64 ± 0.14 | |

DCW: dry cell weight (g/L); DWP: dry weight of extracted PHA (g/L); RB: residual biomass (g/L).

The production and extraction of PHA compounds by bacteria are influenced by both internal and external factors. Internal factors include the number of strains used and the catalytic activity of PHA synthase. According to Zhu *et al*. [86] and Tsuge [87], the use of mixed microbial cultures (MMC) can enhance PHA synthesis capacity compared to single-strain cultures through horizontal gene transfer between bacteria. The molecular weight of PHA is also affected by the catalytic activity of PHA synthase during metabolism, as demonstrated in experiments using mutant strains of *Ralstonia eutropha*. These mutant strains produced PHA with a higher molecular weight compared to the wild type.

External factors influencing PHA production include the types of carbon and nitrogen sources, as well as environmental conditions in the production medium. Muigano *et al*. [83] optimized several parameters for PHA synthesis by the haloalkaliphilic bacterium *Halomonas alkalicola* strain Ext pH2, including temperature, pH, NaCl concentration, incubation time, and nutrient sources, using a one-factor-at-a-time (OFAT) approach. Optimal PHA accumulation was achieved at 35 °C, pH 10, 3% NaCl, and 72 hours of incubation. Among the tested substrates, ammonium sulfate was identified as the most effective nitrogen source, yielding a PHA content of 38.72%, while galactose was the most favorable carbon source, producing 39.7% PHA.

In this study, PHA production was conducted as a preliminary, non-optimized experiment, which explains the relatively low PHA yield. Optimization of external factors is essential to determine the ideal conditions that maximize PHA accumulation. Muigano *et al*. [83] reported that *H. alkalicola* isolated from Lake Simbi produced 16.46% PHA under non-optimized conditions, which increased to 45.57% following optimization using the OFAT approach combined with response surface methodology (RSM). Optimization is also critical for scaling up PHA production for commercial applications, where high yields must be achieved using cost-effective substrates and processes. According to Soni *et al*. [88], the main challenges in industrial PHA production can be addressed through microbial strain improvement, optimization of fermentation and extraction processes, and the utilization of low-cost carbon sources such as agricultural residues.

### *3.5. Molecular identification by 16S rRNA.*

The results of the DNA quality test using the Spectrophotometer Nanodrop showed that isolate BK25 had a DNA concentration of 442.9 ng/µL with a purity ratio of 1.97, while isolate BK30 had a DNA concentration of 782.5 ng/µL with a purity ratio of 1.84 (Table 4). These values indicate good DNA purity, as they fall within the acceptable range of 1.8 to 2.0 ng/µL. According to Latif and Osman [89], high-quality DNA has an A260/280 ratio between 1.8 and 2.0, indicating minimal contamination by substances such as polysaccharides or phenol. Bunu *et al*. [90] also state that a 260/280 ratio is used to assess the purity of DNA and RNA. A ratio of around 1.8 ng/µL typically indicates pure DNA, while a ratio close to 2.0 ng/µL suggests pure RNA. Ratios below 1.8 ng/µL indicate contamination with proteins, phenol, or other substances that strongly absorb at 280 nm.

**Table 4.** The results of DNA quality testing on the NanoDrop spectrophotometer.

| **Isolate** | **DNA Concentration** | **λ 260** | **λ 280** | **260/280** | **Sample Type** |
|---|---|---|---|---|---|
| BK 25 | 442.9 | 8.858 | 4.491 | 1.97 | DNA |
| BK 30 | 782.5 | 15.650 | 8.498 | 1.84 | DNA |

Amplification of the 16S rRNA gene from the purified DNA of haloalkaliphilic isolates BK25 and BK30 was performed using polymerase chain reaction (PCR) with universal primers 27F and 1492R. The 16S rRNA gene is widely used for bacterial identification and phylogenetic classification, providing a foundation for modern bacterial taxonomy based on genetic similarity [91]. The PCR products were analyzed by agarose gel electrophoresis using a 1 kb DNA ladder as a size marker. Clear, distinct bands of approximately 1500 bp were observed (Figure 7), confirming successful amplification of the 16S rRNA gene. Gel electrophoresis enables verification of amplicon size by separating DNA fragments based on their migration through an agarose matrix under an electric field [92].

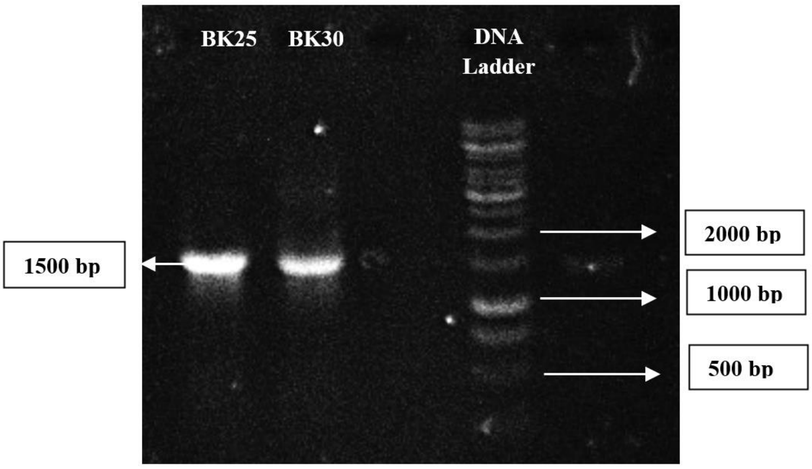


**Figure 7.** The gel electrophoresis results of 16S rRNA gene amplification for isolates BK 25 and BK30. The bands formed are clearly visible at a length of 1500 bp.

The PCR products were subsequently subjected to sequencing to determine their nucleotide composition. Sequencing enables the identification of the precise order of nitrogenous bases within the amplified DNA fragments. The obtained sequences were compared with reference sequences in the GenBank database using the BLAST algorithm available on the NCBI platform. BLAST analysis revealed that isolate BK25 shared 99.21% similarity with *Priestia flexa* strain NCBR 15715, while isolate BK30 showed 99.90% similarity with *Bacillus flexus* strain cifa_chp39 (Table 5). These high similarity values confirm the species-level identification of both isolates, as sequence identities above 97% indicate conspecific relationships, above 95% indicate the same genus, and above 80% denote affiliation to the same higher taxonomic group [93].

**Table 5.** The results of the nucleotide sequence BLAST for isolates BK 25 and BK 30.

| Isolate code | Nucleotide size (bp) | Accession number | Proximity of species | Percentage identification (%) |
|---|---|---|---|---|
| BK 25 | 1023 | OQ331053.1 | *Priestia flexa* strain NCBR 15715 | 99.21 |
| BK 30 | 1000 | KC895903.1 | *Bacillus flexus* strain cifa_chp39 | 99.90 |

*Priestia flexa* belongs to the Bacillaceae family and was previously known as *Bacillus flexus*, but since 2020, it has been reclassified as *Priestia flexa*. This change in genus was based on a study by Gupta *et al*. [94], which conducted a reconstruction or mapping of evolutionary relationships within the Bacillaceae family. Phylogenomic analysis based on conserved signature indels (CSIs) and whole-genome sequences resulted in the formation of new clades within the Bacillaceae family, one of which was the Megaterium clade. The Megaterium clade proposed a new genus called *Priestia*, consisting of seven members, including *B. megaterium*, *B. abyssalis*, *B. aryabhattai*, *B. endophyticus*, *B. filamentosus*, *B. flexus*, and *B. koreensis*.

The 16S rRNA gene sequence data of isolates BK25 and BK30 were then submitted to the NCBI database. Isolate BK25 was assigned the accession number PV076978, while isolate BK30 was assigned the accession number PV076977.

### *3.5. Phylogenetic analyses.*

The phylogenetic analysis of isolates BK25 and BK30 can be observed through the construction of a phylogenetic tree.

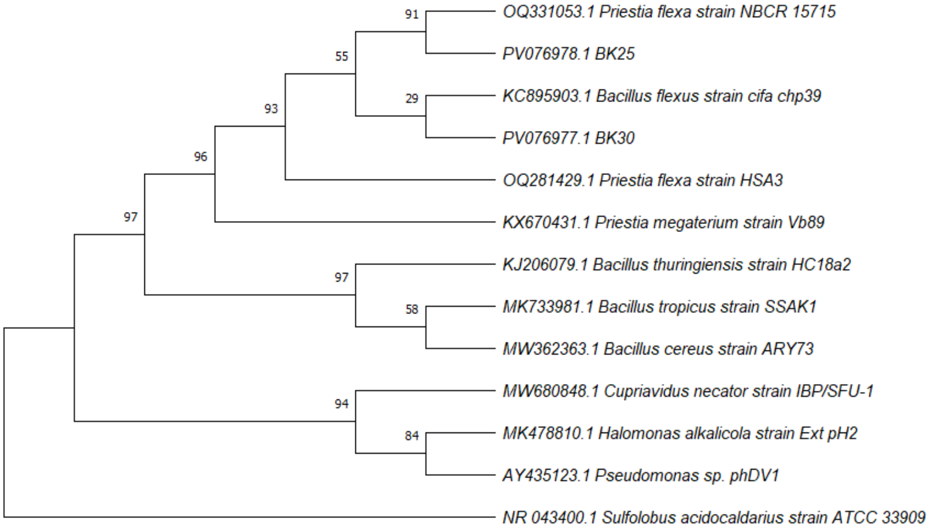


**Figure 8.** The results of the phylogenetic tree reconstruction for the haloalkaliphilic bacterial isolates using MEGA XI software. The evolutionary history was inferred using the Neighbor-Joining method, with a bootstrap percentage of 1000.

The in-group used includes *Halomonas alkalicola* strain Ext pH2 [83]; *Bacillus tropicus* strain SSAK1 [31], *Cupriavidus necator* strain IBP/SFU [95]; *Bacillus thuringiensis* strain HC18a2 [96]; *Bacillus cereus* strain ARY73 [97]; *Pseudomonas* sp. phDV1 [98], *Priestia megaterium* strain Vb89 [99], *Priestia flexa* strain HSA3 [100], *Priestia flexa* strain NCBR 15175, and *Bacillus flexus* strain cifa_chp39. The outgroup used was the extreme thermophilic and acidophilic bacterium *Sulfolobus acidocaldarius* strain ATCC 33909 [101]. The phylogenetic tree of haloalkaliphilic bacteria from Bledug Kesongo is shown in Figure 8.

The results of the phylogenetic analysis show that isolate BK25 has the closest relationship with the clade *Priestia flexa* strain NCBR 15175, with a bootstrap value of 91%. A bootstrap value of 91% means that 91% of the branches of the phylogenetic tree will generate similar branches. Isolate BK30 has the closest relationship with the clade *Bacillus flexus* strain cifa_chp39, with a bootstrap value of 29%. According to Larasati *et al*. [102] and Subari *et al*. [103], the bootstrap method can be used to determine the quality of the data model. A phylogenetic tree is considered good if it has a bootstrap value above 70%.

The phylogenetic tree results show a close relationship between isolates BK25 and BK30 and other PHA-producing bacteria. Research by Chathalingath *et al*. [104] found that *Priestia flexa* is a halotolerant bacterium with the ability to produce PHA compounds, particularly PHB specifically. Research by Adnan *et al*. [100] confirmed the PHA production ability of *Bacillus flexus* bacteria isolated from a landfill in Ha'il, Saudi Arabia.

*Bacillus flexus* (syn. *Priestia flexa*) is a halotolerant and facultative alkaliphilic bacterium [105]. The macroscopic characteristics of isolates BK25 and BK30 include round colonies with irregular margins, raised elevation, and a moist texture; BK25 appears cream-white, while BK30 is cream-yellowish. Microscopically, both isolates are Gram-positive, rod-shaped bacteria. These characteristics are consistent with the findings of Bhimani *et al*. [106], who reported that *P. flexa* isolated from the sugarcane rhizosphere exhibits Gram-positive, bacillus-shaped cells and colonies that are round, undulate, cream-colored, and convex in elevation.

*Priestia flexa* has potential applications in various fields. In the material industry, it can be used as a producer of the biopolymer polyhydroxybutyrate (PHB) for bioplastics. According to Sheeks *et al*. [107], *Bacillus flexus* can produce PHB on a large scale using inexpensive molasses as a carbon source. The enzymes produced by *Priestia flexa* are resistant to high pH and salinity, making them useful in detergent industries. According to Niyonzima and More [108], *Bacillus flexus* can produce alkaline α-amylase, alkaline lipase, and alkaline protease, which are useful in detergent industries. *Priestia flexa* can also be used as an agent to produce free amino acids and short-chain peptides in agricultural fertilizer industries. According to Hongjun *et al*. [109], *Bacillus flexus* acts as a plant growth-promoting bacterium by producing free amino acids and peptides (FAPS), which are important for plant nutrition.

## 4. Conclusions

Haloalkaliphilic bacteria isolated from Bledug Kesongo mud volcano were identified as *Priestia flexa* and *Bacillus flexus*, both capable of producing polyhydroxyalcanoates (PHAs). This study represents the first report of PHA-producing haloalkaliphilic bacteria from this unique geothermal environment, highlighting Bledug Kesongo as a promising source of extremophiles with biotechnological potential. Future studies should focus on optimizing fermentation conditions and characterizing the physicochemical properties of the produced PHAs to assess their suitability for future applications.

## Author Contributions

Conceptualization, A.B., and W.W.; methodology, A.B., D.W., I.D.N., and M.A.; data curation, A.B., D.W., I.D.N., W.W., and M.A.; formal analysis, A.B., D.W., and I.D.N.; writing—original draft preparation, A.B., D.W., I.D.N., W.W., and M.A.; writing—review and editing, A.B., D.W., I.D.N., W.W., and M.A. All authors contributed equally to this work and have read and agreed to the published version of the manuscript.

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Not applicable.

## Informed Consent Statement

Not applicable.

## Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

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## Conflicts of Interest

The authors declare no conflict of interest.

## Abbreviations

| Abbreviation | Definition |
|---|---|
| BK | Bledug Kesongo |
| BLAST | Basic Alignment Search Tool |
| CFU | Colony Forming Unit |
| DCW | Dry Cell Weight |
| DMSO | Dimethyl Sulfoxide |
| DNA | Deoxyribonucleic Acid |
| LAF | Laminar Air Flow |
| LB | Luria Bertani Medium |
| LBA | Luria Bertani Agar Medium |
| LBB | Luria Bertani Broth Medium |
| MSM | Mineral Salt Medium |
| NCBI | National Center for Biotechnology Information |
| OD | Optical Density |
| PCR | Polymerase Chain Reaction |

| Abbreviation | Definition |
|---|---|
| PHA | Polyhydroxyalcanoate |
| PHB | Polyhydroxybutirate |
| ROF | Ring of Fire |
| rRNA | Ribosomal Ribonucleic Acid |
| SD | Standard Deviation |
| UV | Ultraviolet |

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## Publisher’s Note & Disclaimer

# Supplementary materials

**Table S1.** Composition of standard Luria Bertani (LB) medium modification.

| Component | Concentration (g/L) |
|---|---|
| Trytptone | 10 |
| Yeast extract | 5 |
| Agar | 3 |
| NaCl | 10 |

Final pH was adjusted to 9 and salinity 4%. The medium was autoclaved at 121 $^{o}$C for 15 minutes.

**Table S2.** Composition of Carbon-Enriched Agar modification.

| Component | Concentration (g/L) |
|---|---|
| $Na_2HPO_4$ | 6 |
| $KH_2PO_4$ | 3 |
| $NH_4Cl$ | 1 |
| NaCl | 0.5 |
| Yeast extract | 0.05 |
| Agar | 17 |
| Glucose | 10 |

Final pH was adjusted to 9 and salinity 4%. The medium was autoclaved at 121 $^{o}$C for 15 minutes.

**Table S3.** Mineral Salt Medium modification.

| Medium Composition | |
|---|---|
| Component | Concentration (g/L) |
| Urea | 1 |
| $KH_2PO_4$ | 1.52 |
| $Na_2HPO_4$ | 4 |
| $MgSO_4.7H_2O$ | 0.52 |
| $CaCl_2$ | 0.02 |
| Glucose | 40 |
| Trace element solution | |
| Trace element solution | |
| Component | Concentration (g/L) |
| $ZnSO_4.7H_2O$ | 0.13 |
| $FeSO_4.7H_2O$ | 0.02 |
| $(NH_4)_6MO_7O_2.4H_2O$ | 0.06 |
| $H_3BO_3$ | 0.06 |

Final pH was adjusted to 9 and salinity 4%. The medium was autoclaved at 121 $^{o}$C for 15 minutes.