

Research Article

Optimized Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) **Production by Moderately Haloalkaliphilic Bacterium** *Halomonas alkalicola* Ext

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Polyhydroxyalkanoates (PHAs) are biodegradable and biocompatible polymers that are produced by microorganisms as storage materials under limited nutrition and excess carbon. These PHAs have been found to be ideal for replacing synthetic plastics for use in packaging and biomedical applications. In this study, an alkaliphilic and moderately halophilic bacterium *Halomonas alkalicola* Ext was isolated from Lake Simbi Nyaima in western Kenya and investigated for PHA production. Sudan Black B and Nile Red A staining showed that bacterium had distinct ability for accumulation of PHAs. To optimize PHA production, the bacterium was grown in submerged fermentation under varying culture conditions and different sources and concentrations of carbon and nitrogen. With one-factor-at-a-time (OFTA) approach, optimal PHA yields were obtained after 72 hours at a pH of 10.0, temperature of 35°C, and 2.5% (*w*/*v*) NaCl. The bacterium yielded the highest biomass, and PHA amounts on 2% galactose and 0.1% ammonium sulfate as sources of carbon and nitrogen, respectively. A record PHA yield of 0.071 g g⁻¹ with a titer of 1.419 ± 0.09 g/L was achieved from 3.397 g/L of biomass, equivalent to 41.8% PHA content. Using response surface methodology, PHA titer was increased by 1.5% to 1.44 g/L, while PHA content was improved 1.1-fold to 45.57%. Polymer analysis revealed that the extracted PHA was a poly(3-hydroxybutyrate-co-3-hydroxybutyrate (3-HV). (3 - HB : 3 - HV = 92 : 8) with two copolymer subunits of 3-hydroxybutyrate (3-HB) and 3-hydroxybutyrate (3-HV). *Halomonas alkalicola* Ext attained efficient galactose conversion into PHBV under high salinity and alkalinity conditions.

1. Introduction

Petroleum-derived polymers have numerous applications in packaging, transportation, construction, manufacturing, and other industries. As a result, the amount of plastic generated and used globally has risen rapidly over the years, reaching over 380 million metric tons annually [1]. However, a significant amount of plastics produced in the world ends up as waste. Every year, approximately 80 million metric tons of plastic wastes are released into the environment, endangering both the ecosystem and human health [2, 3]. The nondegradable nature of the polymers in the synthetic plastic have led to severe soil and water pollution, thus making plastic pollution a major global issue of concern [4]. Hence, efforts aimed at developing alternative materials to synthetic polymers may help in resolving the global environmental pollution problem associated with the synthetic plastics.

Biopolymers from biological sources are simple to produce and purify and are ecofriendly, thus suitable substitutes for polymers made from petrochemicals [5]. Polyhydroxyalkanoates (PHAs) are a class of biopolymers that possess various advantages including being produced by diverse groups of microorganisms and physiochemical characteristics similar to those of petroleum-based polymers, biodegradability and biocompatibility [6]. These advantages have increased the utilization of products made from PHAs in scientific community including food packaging, agriculture, and medicine [6–8].

PHAs are thermoplastic, aliphatic polyesters with linear polymer chains that are typically accumulated inside the cells of different microorganisms in an abundance of carbon sources and a deficiency of microelements such as nitrogen [9]. Inside the bacterial cell, they can serve as energy and carbon reserve molecules [10]. Polyhydroxyalkanoates have also been shown to play a role in enhancing the bacterial tolerance to stresses and survival [11]. PHAs can be divided into three groups based on the number of carbon atoms in the molecule, namely, the short chain length (SCL) that has 3-hydroxy acids with 3 to 5 carbon atoms, the medium chain length (MCL) whose 3-hydroxy acids have 6 to 16 carbon atoms, and the long chain length (LCL) that is composed of 3-hydroxy acids with more than 16 carbon atoms [12].

Despite the attractive sustainability potential, commercial production of polyhydroxyalkanoates is limited by high production costs [13]. The market price of PHAs ranges from US\$ 2.4 to 5.5/kg while that of petroleum-based plastics may be as low as US\$ 1.2/kg [14]. The high cost of PHA production is often associated with the price of the carbon substrate. In a PHA production process, the carbon substrate may contribute up to 40% of the total cost [15]. As a result, recent research efforts have sought to address the high-cost bottlenecks in PHA production by exploring different types of cheap carbon sources [13, 15-17]. Methane and volatile fatty acids have also been explored as cheap and highly available feedstocks for production of polyhydroxyalkanoates [18-20]. Methanotrophs have been shown to have efficient abilities for the conversion of methane into polyhydroxyalkanoates [20]. For instance, Methylcystis sp. MJC1 has been found to accumulate poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer from methane as a sole carbon source [19]. Wastewater could also provide a cheap feedstock for PHA production. For example, halophilic bacterium Halomonas sp. has been found to have the capacity for treatment of wastewater and production of polyhydroxyalkanoates from sludge [21]. Thus, the optimization of fermentation and production bioprocesses including careful selection of carbon sources is one of the key strategies of reducing the production costs of PHAs since slow growth of microorganisms may contribute to the high cost of PHA production [22, 23]. Thus, the current work sought to optimize the production of PHAs by a newly isolated strain, Halomonas alkalicola Ext. In this work, optimization included the evaluation of physiochemical factors like pH, temperature, salinity, and the types and concentrations of carbon and nitrogen sources.

Extremophiles such as halophilic bacteria can accumulate high amounts of polyhydroxyalkanoates. A major advantage of halophiles is their resistance to contamination due to extreme culture conditions; also, they have various mechanisms that enable them to survive in high-salinity environments that other microorganisms cannot [24]. Besides, halophilic microorganisms have genetic mechanisms for enhanced survival in high-salinity environments, due to possession of genes that regulate uptake and efflux of salt ions for enhanced osmoregulation [25]. Moreover, PHA accumulation may also be used as a strategy for protecting halophiles from hypertonic and osmotic regulations [24]. Halophilic bacteria of the genus *Halomonas* have shown great potential in the production of polyhydroxyalkanoates [26–28]. Thus, this study sought to optimize the production of PHAs by a moderately halophilic bacterium *Halomonas alkalicola* Ext isolated from Lake Simbi, a hypersaline-alkaliphilic lake in Kenya.

2. Materials and Methods

2.1. Sampling and Bacteria Cultures. A total of 24 samples comprising eight each from soil, sediments, and water were collected from eight sampling points in Lake Simbi Nyaima, a hypersaline lake located in western Kenya (0.3676° S, 34.6290° E) as previously described and reported in our earlier work [29]. Screening for polyhydroxyalkanoates production was conducted using Sudan Black B plate assay [30], microscopy [31], and Nile Red A staining [32]. Pure cultures were maintained in 20% (*w*/*v*) glycerol in nutrient broth at -80°C at the Institute for Biotechnology Research of Jomo Kenyatta University of Science and Technology.

2.2. Culture Conditions and PHA Production in Shake Flasks. Bacteria cells preserved in 20% (w/v) glycerol were cultured in nutrient agar media supplemented with 3% (w/v) NaCl for 24 h. Precultured cells were used to prepare seed cultures for subsequent PHA production. Seed cultures were prepared by growing a single colony of 24-hour-old cultures in 10 mL nutrient broth in culture bottles. One milliliter of seed culture was transferred to a 250 mL Erlenmeyer flasks, and 50 mL of sterilized PHA production media was added to the flask. The PHA production media was prepared using the following components (g/L): glucose 10g, Na₂HPO₄.2H₂O 4g, NaCl 30 g, $(NH_4)_2SO_4$ 5 g, MgSO₄ 0.39 g, and KH₂PO₄ 3.7 g/L and the pH adjusted to 9.0. Sterilization of PHA production media was achieved through steam autoclaving at 121°C and 15 psi for 15 minutes. PHA production was conducted in submerged fermentation. Isolates were grown in an incubator at 150 rpm and 30°C for 72 hours after which the cells were harvested and the PHAs extracted and measured.

2.3. Characterization of Selected Isolate. Based on preliminary experiments, Gram-negative bacteria Halomonas alkalikola Ext isolated from Lake Simbi Nyaima was identified as the most promising candidate as it had the highest initial PHA production. The selected isolate was characterized phenotypically using morphological and biochemical features. The morphology of the bacteria colonies on nutrient agar was observed delineate the colony features including colour, opacity, shape, size, elevation, and surface characteristics. Microscopy was used to study Gram staining and cell arrangement of the isolate. Various biochemical tests were conducted including catalase, oxidase, H₂S production, Voges-Proskauer, urease activity, indole production, and citrate utilization. The ability of the bacteria to ferment carbon such as glucose, sucrose, xylose, lactose, starch, maltose, galactose, fructose, mannose, cellobiose, glycerol, and mannitol was also assessed. The selected isolate was also

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Variables	Symbol	Unit	$-\alpha$ (-1.68)	Low (-1)	Middle (0)	High (1)	$+\alpha$ (+1.68)
Galactose concentration	А	g/L	5.86	10.00	20	30.00	34.14
NaCl concentration	В	g/L	3.79	10.00	25	40.00	46.21

TABLE 1: Test variables and levels of CCD for optimizing NaCl and galactose concentrations for optimized PHA production.

characterized using 16S rRNA sequencing. Genomic DNA was extracted using the Invitrogen PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific) in accordance with the manufacturer's instructions.

The gene encoding for the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the primers 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492 (5' TACGGYTACCTTGTTACGACTT 3'). The PCR protocol involved initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1.5 min, and final extension at 72°C for 5 min. Amplified products were resolved on 1.2% agarose gel electrophoresis and visualized on a UV transilluminator. The PCR products were sent to Inqaba Biotec (Pretoria, South Africa) for sequencing, and the sequences analyzed using ChromasPro software. The identification of similar sequences was done using the BLAST tool of the National Center for Biotechnology Information (NCBI) and phylogenetic tree constructed using MEGA 7.0.

2.4. Polyhydroxyalkanoate Extraction, Recovery, and Quantification. The biopolymer was extracted using the detergent-based method of Hahn et al. [33] with modifications. Bacteria cells were harvested by centrifugation at 10,000 q for 15 minutes and rinsed thrice with distilled water. The cells were dried overnight to obtain a constant dry weight. The harvested cells were incubated at 50°C in 10% (v/v) sodium hypochlorite for 1 hour with constant homogenization. PHAs were extracted by centrifugation of the detergent solution at 10,000 g for 10 minutes and then serially washed with water, acetone, and ice-cold methanol. PHAs were dissolved in hot chloroform and then dried at room temperature for 48 hours. The purity of the recovered PHA was assessed using the rapid spectrophotometric crotonic acid-based assay of Law and Slepecky [34]. Briefly, 5 mg of the extracted polymer was mixed with 10 mL concentrated (95%) H₂SO₄ and heated at 100°C for 30 minutes with periodic vortexing to convert the PHA into crotonic acid. The sample was cooled, and its absorbance at 235 nm was determined using the UV spectrophotometer. A standard calibration curve was used to estimate the percentage purity from 5 mg of initial sample with comparison of a standard PHA comprising the commercial poly[R-3-hydroxybutyric acid] purchased from Sigma-Aldrich. In all experiments, constant dry weights were taken for the cell dry weight (CDW) and PHA concentration (g/L). The PHA yield (gg⁻¹) was calculated as the content of PHAs for each gram of carbon source used. Finally, the PHA content extracted per liter of media was calculated as the proportion of PHA weight per liter of production media to the cell dry weight.

TABLE 2: Parameters investigated and the variation ranges.

Parameters	Units	Variation range
Cell dry weight (CDW)	g/L	2.412-3.34
PHA concentration	g/L	0.397-1.44
PHA content	%	16.46-48.15
PHA yield	g/g	0.022-0.072

$$PHA \text{ content } (\%) = \frac{PHA \text{ weight}}{Cell \, dry \, weight} \times 100,$$

$$PHA \text{ yield } (g/g) = \frac{PHA \text{ weight } (g/L)}{Amount \text{ of carbon source } (g/L)}.$$
(1)

2.5. Optimization of PHA Production. A one-factor-at-atime (OFAT) approach was adopted in optimizing PHA production in this study. In this method, the design of experiments involved the testing of independent variables, one factor at a time, while all other factors were held constant. The approach is effective in determining the most important factors in fermentation experiments and the effective ranges [35]. The production of PHAs was conducted under varying fermentation conditions of pH, temperature, carbon source, nitrogen source, and incubation times. For pH optimization, fermentations were carried out in triplicates with variable initial pH levels of 6, 7, 8, 9, 10, and 11 on media containing 1% (w/v) glucose as substrate, a temperature of 35°C, 3% NaCl, and 0.5% (w/v) ammonium sulfate for 72 hours. The effect of temperature on PHA production was assessed by cultivating the bacteria on 1% glucose, 3% NaCl, and 0.5% ammonium sulfate for 72 hours while altering the incubation at different temperatures, viz., 25°C, 30°C, 35°C, 40°C, 45°C, and 50°C. Halomonas alkalikola Ext was also cultivated under varying concentrations of NaCl on constant of pH, temperature, glucose, and nitrogen sources at 72-hour incubation period. The PHA production media was altered with NaCl concentrations of 1%, 2%, 3%, 4%, and 5%. To determine the effects of incubation time, cell dry weight and PHA content were measured at intervals of 24h, 48h, 72h, and 96h.

Halomonas alkalikola Ext was also cultivated on PHA production media containing 1% (w/v) of different carbon sources including glucose, fructose, galactose, glycerol, xylose, sucrose, and lactose. For the carbon source with the highest productivity, further optimization was conducted by cultivating the bacteria on different concentrations of 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, and 4.0%. PHA production was conducted with various types of nitrogen sources, namely, peptone, yeast powder, (NH₄)₂SO₄, NH₄Cl, and

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Isolate ID	Identity	Accession number	CDW (g/L)	PHA titer (g/L)	PHA content (%)
SW 36	Halomonas alkalicola	MK478810.1	2.412	0.397	16.46%
SO32	Bacillus pumilus strain 37	MK327816.1	1.772	0.235	13.26%
SW38	Bacillus sp. MEB205	MN809475.1	1.942	0.189	9.73%
SO62	Bacillus subtilis QD9	EF488088.1	3.52	0.188	5.34%
SE93	Bacillus sp. ISO_06_Kulunda	EU676884.1	0.916	0.1	10.92%
SE89	Paracoccus sp. TMN-21-1	JX950033.1	1.082	0.085	7.86%
SO75	Bacillus sp. strain FA2-253	KY476210.1	1.232	0.082	6.66%
SO13	Bacillus sp. 01105	EU520307.1	1.194	0.078	6.53%
SE83	Exiguobacterium sp. QZS4_8	KX364032.1	1.411	0.062	4.39%
SO31	Bacillus subtilis OTPB28	KT265083.1	1.401	0.052	3.71%
SE42	Paracoccus sp. TMN-21-1	JX950033.1	0.613	0.047	7.67%

TABLE 3: Identities and PHA accumulation by halophilic and halotolerant bacteria isolated from Lake Simbi under nonoptimized conditions.

 $(NH_4)_3PO_4$ to a final concentration of 5 g/L. Finally, PHA production was assessed on different concentrations of $(NH_4)_2SO_4$ including 0.1%, 0.2%, 0.3%, 0.4%, and 0.5%.

2.6. Process Optimization with Central Composite Design (CCD). One-factor-at-a-time experiments have the limitation of inability to evaluate multiple factors and the associated interactions [35]. As such, statistical experimental designs are useful for investigating simultaneous interactions of several independent variables. In the present study, the widest range in PHA accumulation was noted for two variables, viz., galactose and NaCl concentrations. While definitive optimal conditions of PHA production were determined for pH, temperature, nitrogen source concentration, and incubation time, galactose and NaCl concentrations showed a wide range for which the bacteria accumulated high amounts of PHAs, hence the need to optimize these variables through response surface methodology. As a result, the two variables were selected for further optimization through response surface methodology. In this study, central composite design was used to design fermentation experiments for optimal PHA production. The Design-Expert software, trial version 13.0.0 (Stat-Ease, Inc., MN, USA) was used for statistical design of the experiment. The design included 13 experiments with two factors and 5 replicates at center points to optimize the effects of the two most important factors of PHA production by the halophile, sodium chloride, and galactose concentration. The five levels were set at -1.68, -1, 0, +1, and+1.68 as shown in Table 1. Experiments were conducted in triplicates in 250 mL flasks containing 0.1% (w/v) (NH₄)₂SO₄ and 10% (v/v) inoculum at 30°C, initial pH of 9.0, and an incubation period of 72 hours. The results were fitted to a second-order polynomial equation with multiple regression and response surface plotted on 3D surface plots and contour plots. The performance of the model was validated by comparing the predicted values with actual result.

2.7. Characterization of Extracted Polymer. Extracted polymers were characterized by Fourier-transform infrared (FTIR) spectroscopy on a Bruker Alpha I spectrometer

(Bruker Corporation, Billerica, Massachusetts, USA). 10 mg of the sample was finely ground and mixed with FTIR grade potassium bromide and pressed into a pellet. The infrared spectra were recorded on the spectrometer at wavelengths of between 400 and 4000 cm⁻¹. The FTIR spectra were visualized and analyzed using the Bruker OPUS software version 8.0.1. In addition, gas chromatography-mass spectroscopy (GC-MS) was used to analyze the profile of extracted PHA. The GC measurements were used for qualitative analysis of the polymer produced and quantitative determination of the monomeric units of the copolymers. About 2 mg of the sample was processed into a paste with 2 mL methanol and the sample separated with a CE GC 8000 top MSMD Fyson instrument with a Db 35 mr column (10 m \times 0.5 mm, 0.25 μ m film thickness) (CE Instruments Ltd., Wigan, UK). The heating program was maintained as described in literature [36].

2.8. Statistical Analysis. All measurements were conducted in triplicates for biomass yields, PHA concentration, and PHA content of the different experimental setups. Statistical analysis involved making comparisons of the means of the triplicates of cell dry eight (CDW), PHA concentration, and PHA content at a 95% level of confidence. Table 2 shows the list of parameters investigated in this study and the corresponding variation ranges. The means and standard deviations were calculated for each experiment using IBM SPSS Statistics V.22.0 (IBM SPSS Inc., Chicago, IL, USA). Means were separated using Tukey's HSD. Production of PHAs by Halomonas alkalicola Ext was optimized with Design-Expert v.13.0. The central composite design (CCD) suggested a total of 13 experiments with five replicates of the central point. The experiments were conducted to optimize PHA production by two independent variables, namely, galactose concentration (A) and salinity (B). The fitness of the statistical model was evaluated with analysis of variance (ANOVA) at 95% significance level.

3. Results

3.1. Isolation and Screening for PHA Production. In this study, 96 isolates were isolated from Lake Simbi and

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Mannitol	+	
Glycerol	+	
Cellobiose		
Mannose	+	
Fructose	+	
Galactose	+	
Maltose	ī	
Starch		
Lactose 3	+	
Xylose	+	
Sucrose	+	
Glucose	+	
Citrate utilization	ı	
Indole production	1	
Urease activity	ı	
Voges- Proskauer	1	
H ₂ S production	1	
Oxidase	+	
Catalase	+	
Gram reaction	ı	
Cell shape	Rod	
Pigmentation	White	
Colony shape	Circular	
Characteristic	Results	



FIGURE 1: Phylogenetic analysis for Halomonas alkalicola Ext.

screened for PHA production using the Sudan Black B staining and Nile Red A fluorescence. Of these isolates, 11 had the abilities to accumulate PHAs as indicated in Table 3. The sample included eight halophilic bacteria belonging to the genera *Bacillus, Halomonas, Paracoccus*, and *Exiguobacterium*. The sample also included three halotolerant bacteria recovered from the soils on the shores of the lake, namely, *Bacillus pumilus* strain 37, *Bacillus subtilis* QD9, and *Bacillus subtilis* OTPB28. Isolate SW36 had the highest PHA concentration of 0.397 g/L, equivalent to a PHA content of 16.46% with a CDW of 2.412 g/L when cultured in a nonoptimized PHA production medium. As such, the isolate was selected for further optimization studies.

3.2. Characterization of Selected Isolate. Isolate SW36 was recognized as an ideal PHA producer. Observation of its colony morphology revealed that it had an off-white coloration with small, circular-shaped, and opaque colonies with entire margins. Microscopic examination revealed that the bacterium was Gram-negative with rod-shaped cells. The isolate showed positive tests for catalase and oxidase tests but was negative for Voges–Proskauer, citrate utilization, indole production, and urease activity. As shown in Table 4, the isolate was capable of fermenting a wide range of sugars including glucose, sucrose, xylose, lactose, fructose, galactose, mannitol, and glycerol but not starch, maltose, and cellobiose.

Identification of the isolate through 16S rRNA sequencing and BLAST search on NCBI revealed isolate SW36 had 97.49% similarity with *Halomonas alkalicola* Ext (accession number: MK478810.1). A phylogenetic analysis (Figure 1) revealed that the closest relatives are various strains of *Halomonas campisalis*.

3.3. Optimization of PHA Production

3.3.1. Effects of Culture Conditions. The physical growth parameters greatly affected the amount of biomass produced and the PHA accumulated by Halomonas alkalicola Ext. As shown in Figure 2, the bacterium grew in a wide temperature range. However, optimal biomass and PHA accumulation of 2.62 g/L and 0.9133 g/L, respectively, were achieved at 35°C. No significant difference was noted for the production at 30°C and 35°C. The bacteria further tolerated a wide pH range, but optimal growth was observed at pH of 9.0 and 10.0. At a pH of 10.0, the bacteria accumulated 0.963 g/L of PHAs from a biomass of 2.653 g/L (Figure 2). The duration of incubation was also found to affect PHA production outcomes with optimal production of 0.975 g/L being achieved after 72 hours of incubation from 2.63 g/L of biomass. However, incubation durations of 48 h and 72 h had no significant differences in PHA accumulation and biomass production. After 72 hours, the biomass yield remained constant while the PHA yield declined. Finally, the salinity of the fermentation media was found to have profound impacts on PHA production. The bacteria produced optimally at NaCl of 30 g/L (3% w/v) with 1.045 g/L of PHAs from



FIGURE 2: Continued.



FIGURE 2: Effects of temperature (a), initial pH (b), NaCl concentration (c), and incubation time (d) on cell dry weight and PHA production under shake flask fermentation. Error bars represent the standard deviations from means of triplicates.

Carbon sources	Cell dry weight (g/L)	PHA concentration (g/L)	PHA content (%)	PHA yield (g g ⁻¹)
Glucose	2.64 ± 0.16^{a}	$0.92\pm0.05^{\rm b}$	34.66 ± 1.16^{b}	0.046
Fructose	2.48 ± 0.12^{ab}	$0.93\pm0.02^{\rm b}$	37.64 ± 1.21^{a}	0.047
Sucrose	2.55 ± 0.14^{ab}	$0.82\pm0.11^{\rm b}$	$32.26\pm0.95^{\rm b}$	0.041
Xylose	1.87 ± 0.08	0.51 ± 0.07	$27.48 \pm 0.71^{\circ}$	0.026
Galactose	3.14 ± 0.23	1.25 ± 0.09^{a}	39.68 ± 1.13^{a}	0.063
Lactose	2.67 ± 0.51^{a}	1.03 ± 0.19^{a}	38.57 ± 1.11^{a}	0.052
Mannitol	$2.31 \pm 0.19^{\circ}$	0.32 ± 0.05	13.77 ± 0.58	0.016
Glycerol	$2.27 \pm 0.15^{\circ}$	0.61 ± 0.06	$26.87 \pm 0.19^{\circ}$	0.031

TABLE 5: Production of PHAs by Halomonas alkalicola on different carbon sources.

*Measurements with similar letters in superscripts in a column are not statistically significant based on Tukey's HSD (p > 0.05).

2.728 g/L of biomass. The findings further revealed that the bacteria biomass was not significantly different for three NaCl concentrations of 1.0%, 2.0%, and 3.0%. Moreover, the PHA concentrations were not significantly different for NaCl concentrations of 1% and 2% but a concentration of 3% produced significantly higher yields (p < 0.05). Thus, the optimal culture conditions were identified as temperature of 35°C, pH of 10, 72 hours incubation time, and NaCl concentration of 30 g/L.

3.3.2. Effects of Carbon and Nitrogen Sources. Having optimized the physical parameters, the effects of carbon and nitrogen sources were explored under varying concentrations. The bacterium showed an ability to accumulate PHAs from diverse carbon sources including glucose, fructose, sucrose, xylose, galactose, and glycerol. As shown in Table 5, the highest biomass and PHA accumulation were achieved with galactose as a sole carbon source. The bacterium produced 1.25 ± 0.09 g/L of PHAs from 3.143 g/L of biomass, accounting for 39.7% PHA content. High yields were also recorded for media containing lactose as a sole carbon source with biomass and

PHA concentration of 1.03 g/L and 2.67 g/L, respectively. With fructose as a sole carbon source, the bacterium produced 0.93 g/L of PHAs from 2.48 g/L of cellular biomass. For media containing glucose as a sole carbon source, 0.92 g/L of biopolymer was produced from 2.64 g/L of biomass. Glucose, fructose, and sucrose had comparable yields with no significant differences in their biomass and PHA production (p < 0.05). Galactose was selected as the most ideal substrate for production of PHAs from *Halomonas alkalicola*.

The bacterium was capable of accumulating PHAs exceeding 0.5 g/L from a variety of nitrogen sources. However, ammonium sulfate and peptone had the highest productivity values. The bacterium was able to convert 38.72% of biomass into PHAs using ammonium sulfate as a nitrogen source. For media containing ammonium sulfate, 1.313 g/L of PHAs was produced from 3.39 g/L of cellular dry weight (Table 6). Peptone produced an equally high amount of PHAs at 1.052 g/L from 3.083 g/L of biomass, accounting for 34.12% PHA content.

The concentrations of carbon and nitrogen sources in the growth media were found to have significant effects on PHA accumulation. Thus, optimal yields were evaluated

Nitrogen sources	Cell dry weight (g/L)	PHA concentration (g/L)	PHA content (%)	PHA yield (gg^{-1})
Ammonium chloride	2.55 ± 0.26^{b}	0.96 ± 0.05^{ab}	37.66 ± 1.17^{a}	0.048
Ammonium sulfate	3.39 ± 0.11^{a}	1.31 ± 0.03	38.72 ± 0.92^a	0.066
Ammonium phosphate	$2.78\pm0.17^{\rm b}$	0.89 ± 0.04^{bc}	$31.87 \pm 0.25^{\circ}$	0.045
Ammonium nitrate	2.53 ± 0.68^b	$0.83 \pm 0.02^{\circ}$	32.80 ± 1.15^{bc}	0.042
Peptone	3.08 ± 0.650^a	1.05 ± 0.04^a	34.12 ± 0.52^{ab}	0.053
Beef extract	2.67 ± 0.62^{b}	0.59 ± 0.03	22.12 ± 0.14	0.030
Yeast extract	2.53 ± 0.52^{b}	0.43 ± 0.06	16.82 ± 0.15	0.022

TABLE 6: Production of PHAs by Halomonas alkalicola on different nitrogen sources.

*Measurements with similar letters in superscripts in a column are not statistically significant based on Tukey's HSD (p > 0.05).



FIGURE 3: PHA production on different concentrations of galactose (a) and ammonium sulfate (b). Controls had 0.0% concentrations of galactose and ammonium sulfate. Error bars represent standard deviations from means of triplicates.

using different concentrations of ammonium sulfate as a nitrogen source and galactose as a sole carbon source for production of PHAs from *Halomonas alkalicola*. The highest productivity was recorded at galactose concentration of 2.0% and 2.5%. An optimal production of 1.28 g/L of PHAs

was achieved from 3.38 g/L of biomass at a galactose concentration of 2.0% (Figure 3). For nitrogen sources, the bacterium was found to accumulate 41.8% PHAs from 0.1% (1 g/L w/v) ammonium sulfate. A record output of 1.419 ± 0.09 g/L of PHAs was obtained from 3.397 g/L of biomass, equivalent to

Dun andan	A: Calastana (a/I)	$\mathbf{P} \cdot \mathbf{N} = C \mathbf{I} \left(\mathbf{a} / \mathbf{I} \right)$	CDW	(g/L)	PHA concen	tration (g/L)	PHA cont	ent (%)
Kun order	A: Galactose (g/L)	B: NaCI (g/L)	Actual	Predicted	Actual	Predicted	Actual	Predicted
1	20	25	3.11 ± 0.25	3.12	1.41 ± 0.04	1.42	45.34 ± 1.15	45.57
2	20	3.79	2.19 ± 0.07	2.23	0.63 ± 0.05	0.66	28.77 ± 0.55	28.59
3	20	25	2.97 ± 0.13	3.12	1.43 ± 0.08	1.42	48.15 ± 1.61	45.57
4	5.86	25	1.92 ± 0.09	1.86	0.45 ± 0.06	0.46	23.44 ± 0.28	22.80
5	30	40	2.25 ± 0.05	2.20	0.51 ± 0.00	0.55	22.67 ± 0.25	21.98
6	34.14	25	3.04 ± 0.05	3.14	1.17 ± 0.09	1.15	38.49 ± 0.77	39.22
7	20	46.21	0.78 ± 0.00	0.95	0.03 ± 0.00	-0.04	3.85 ± 0.35	0.27
8	30	10	3.02 ± 0.04	2.92	1.07 ± 0.02	1.05	35.43 ± 0.18	35.06
9	10	40	1.27 ± 0.06	1.30	0.04 ± 0.00	0.06	3.15 ± 0.12	3.43
10	20	25	3.18 ± 0.10	3.12	1.44 ± 0.11	1.42	45.28 ± 2.28	45.57
11	10	10	1.98 ± 0.07	2.02	0.59 ± 0.05	0.56	29.80 ± 0.40	30.40
12	20	25	3.18 ± 0.05	3.12	1.43 ± 0.00	1.42	44.97 ± 0.97	45.57
13	20	25	3.15 ± 0.10	3.12	1.39 ± 0.08	1.42	44.13 ± 0.44	45.57

TABLE 7: Predicted and actual results of the central composite design for CDW (g/L), PHA (g/L), and PHA (%).

TABLE 8: Analysis of variance (ANOVA) for the quadratic model of interactive effects of galactose concentration (A) and NaCl concentration (B) on PHA concentration (g/L).

Source of variation	Sum of squares	Degrees of freedom	Mean square	F value	p value
Model	3.48	4	0.8699	668.78	<0.0001 S
A-Galactose concentration	0.4842	1	0.4842	372.27	<0.0001 S
B-NaCl concentration	0.4857	1	0.4857	373.41	<0.0001 S
A^2	0.6634	1	0.6634	510.01	<0.0001 S
B^2	2.11	1	2.11	1624.01	<0.0001 S
Residual	0.0104	8	0.0013		
Lack of fit	0.0088	4	0.0022	5.50	0.0637 NS
Pure error	0.0016	4	0.0004		
Cor total	3.49	12			

 $R^2 = 0.9970$, adjusted $R^2 = 0.9955$, predicted $R^2 = 0.9871$, coefficient of variation (CV%) = 4.05%, adequate precision = 64.8530. *S: significant; NS: not significant.

a yield of 0.071 g g^{-1} (Figure 3). Higher production levels exceeding 1.0 g/L were also recorded at nitrogen source concentrations of 0.3% and 0.4%. A notable observation was that there were no significant differences in the PHA and biomass yields for ammonium sulfate concentrations of 0.2%, 0.3%, 0.4%, and 0.5% (p < 0.05).

3.3.3. Process Optimization with Response Surface Methodology. The optimal galactose and NaCl concentrations in the fermentation medium for PHA production were determined using a five-level CCD design. Table 7 shows the experimental and predicted results for cell dry weight, PHA concentration, and PHA content. A maximum production of $1.44 \,\mathrm{g\,L^{-1}}$ with 45.07% PHA content was achieved through statistical optimization with fermentation conditions of 2% galactose and 2.5% NaCl concentrations. Conversely, negligible amounts of PHAs were produced with 4.62% salinity.

The statistical model was analyzed using ANOVA. The results showed that the model was adequately fitted with F

value of 668.78 and p value of <0.0001, indicating that there was only a 0.01% chance that the F value could be due to noise (Table 8). Similarly, the models for cell dry weights and PHA content were significant with F values of 115.11 and 315.85, respectively, and p values of <0.0001 for both variables as shown in Supplementary Materials Table S1 and Table S2. The predicted and experimental values of cell biomass, PHA concentration, and PHA content were nearly identical as shown in Supplementary Materials Figures S1, S2, and S3, indicating that the response values were consistent with the projected ones.

Figures 4 and 5 show the interactive effects of galactose and NaCl concentrations on PHA production and cell dry weights, respectively. As shown in Figures 4 and 5, the concentrations of galactose and NaCl in the growth media had profound effects on biomass accumulation and PHA production. The results of the experimental trials were then fitted to a second-order polynomial model. The response of PHA concentration and content to NaCl and galactose concentration are shown in 40

34

PHA concentration (g/L)





2

1.5

1

FIGURE 4: Statistical optimization of fermentation parameters through central composite design showing the contour plots and 3D surface plots for (a) PHA concentration (g/L) and (b) PHA content (%CDW).

the following equations. The model predicted a maximum PHA production of 1.493 g/L as shown in Supplementary Materials Figure S4. However, actual results produced a maximum PHA titer of 1.44 g/L.

$$Y_1 = 1.427 + 0.246A - 0.2464B - 0.309A^2 - 0.55B^2,$$

$$Y_2 = 45.57 + 5.80A - 9.54B + 3.47AB - 7.45A^2 - 15.07B^2,$$
(2)

where Y_1 is the PHA concentration (g/L); Y_2 is the PHA content (%CDW); A and B are the concentrations of galactose and NaCl, respectively.

3.4. Polymer Characterization. As shown in Figures 6 and 7, the extracted polymer showed a prominent peak at 1733.83 cm⁻¹ and 1735.73 cm⁻¹ for galactose and glucose substrates, corresponding to the carbonyl (C=O) group. Strong absorption peaks at 1281 cm⁻¹ and 1283 cm⁻¹ for PHAs produced on galactose and glucose substrates were indicative of an ester linkage of C-O groups. The absorption peaks at 1378 for both polymers reflected a stretching mode for the methyl group (CH₃). Absorption peaks at 2979 cm^{-1} for galactose-based polymer and 2983 cm⁻¹ for the glucosebased polymer reflect a methine (-CH) group. A hydroxyl (-OH) was evident at absorption peaks of 3589 cm⁻¹ and 3435 cm⁻¹ for PHA derived from galactose and glucose as carbon sources, respectively. The FTIR profiles match closely with those of commercial poly(3-hydroxybutyrate-



FIGURE 5: Contour plot and 3D surface plot for the interactive effects of carbon source concentration and salinity for cell dry weight optimization.



FIGURE 6: FTIR profile of PHA produced by Halomonas alkalicola on a galactose substrate.

co-3-hydroxyvalerate) (PHBV) standard and earlier studies [37–40]. The GC-MS chromatography revealed that the extracted polymer had two monomeric units, namely, poly(3-hydroxybutyrate) (P3-HB) and 3-hydroxyvalerate (3-HV). Gas chromatography revealed a prominent peak with a retention time of 5.728 minutes, corresponding to the polyhydroxybutyrate fraction of the polymer. As shown in Figure 8, another peak was recorded at 7.659 minutes, which was indicative of the hydroxyvalerate (3-HV) fraction. Thus, the polymer extracted from *Halomonas alkalicola* Ext is poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) copolymer (3 – HB : 3 – HV = 92.23 : 7.77).

The purity of the polymer extracted from *Halomonas alkalicola* was estimated using a spectrophotometric assay. Using the crotonic acid standard curve (Figure 9), the concentration of PHAs produced by *H. alkalicola* was determined as 4.28 mg from 5.0 mg of sample. As such, the purity

of the extracted PHA was estimated at 85.6% in comparison with the standard.

The polymer extracted in this study contained 7.77% 3-HV fraction. This was lower than the 3-HV fraction reported in some earlier studies (Table 9). According to Hammami et al. [41], a 3-HV fraction of about 10-20% is desirable for PHBV to have some practice use but the attainment of high 3-HV fraction often demands the incorporation of chemical precursors. In the present study, PHBV precursors were not incorporated in the growth media, hence the low 3-HV fraction in the extracted polymer.

4. Discussion

Plastic waste pollution is one of the major challenges facing the world today. Biodegradable polymers have emerged as suitable alternatives to synthetic plastics in resolving the



FIGURE 7: FTIR spectrum in absorbance units of PHA produced by Halomonas alkalicola on a glucose substrate.



FIGURE 8: Gas chromatogram of PHA synthesized by Halomonas alkalicola Ext.



FIGURE 9: Standard curve for determining the purity of PHAs. The curve was obtained for 0-5 mg of commercial PHA with a total volume of 10 mL.

global plastic waste problem [45]. Among the available natural polymers, polyhydroxyalkanoates are attractive due to their desirable physical, chemical, and thermoplastic properties [7, 46-49]. Screening for PHA production by bacteria often involves the use of rapid phenotypic methods such as Sudan Black B staining and Nile Red A screening. However, Sudan Black B and Nile Red A stains are lipophilic and may detect lipid granules that are not PHAs [16]. Thus, amplification of the PhaC gene is an important confirmatory test for detecting PHA production ability. In the present study, 16S rRNA gene sequence analysis revealed that the isolates belonged to well-known groups of PHA producers. The isolates screened in this study included both halophilic and halotolerant bacteria. Of these isolates, Halomonas alkalicola emerged as the most effective PHA producer in moderate salinity and was selected for further optimization studies.

The production of polyhydroxyalkanoates is influenced by multiple factors including cultural conditions and

PHBV (g/L)	PHBV (%)	PHA (g/g)	3-HV fraction (%)	Reference
1.44	45.34	0.071	7.77	This work
1.5	68.18	*ND	52.04	[41]
0.27	17	ND	2.83	[42]
0.42	20.1	ND	60.59	[43]
4.1	65	0.44	25	[44]

TABLE 9: Properties of the extracted polymer.

*ND: not determined.

nutrition. Several studies have shown that carbon and nitrogen sources as well as their concentration in fermentation media have profound impacts on PHA production [4, 50–52]. Thus, this study sought to optimize the fermentation conditions for Halomonas alkalicola Ext, a moderately halophilic bacterium isolated from Lake Simbi, a hypersaline lake in Kenya. The accumulation of polyhydroxyalkanoates was found to be optimal at 30-35°C, pH of 10, and an incubation period of 48-72 hours. An exploration of PHA production on different carbon sources revealed that Halomonas alkalicola Ext produced the highest amount of PHAs on a galactose substrate with a yield of 1.28 ± 0.04 g/L. Galactose concentrations of between 2.0% and 2.5% yielded the optimal PHA concentrations and content. Our findings are similar to those of Jung et al. [28] who reported a preference for 2% galactose as a carbon source for polyhydroxybutyrate production by a halophilic bacterium Halomonas sp. YK44. The present study revealed that ammonium sulfate at a concentration of 0.1% was the most ideal nitrogen source for PHA production by Halomonas alkalicola Ext whereby 1.4188 ± 0.09 g/L of PHAs was obtained from 3.397 g/L of biomass under optimized conditions, corresponding to 41.8% PHA content. Through statistical modeling, the optimal production was increased by 1.5% to 1.44 g/L with 2% galactose and 2.5% salinity. In the present study, Halomonas alkalicola Ext showed a commendable potency for accumulation of polyhydroxyalkanoates. Furthermore, it portrayed a wide variety of growth conditions including ability to accumulate PHAs at temperatures of between 20°C and 50°C, a pH range of between 6.0 and 11.0, and salinity levels of 0-5%. Members of the genus Halomonas have been shown to have a strong potential for accumulation and production of various types of polyhydroxyalkanoates [26, 50, 53-57]. For example, El-malek et al. [26] reported PH accumulation by Halomonas salifodiane ASL11 isolated from salt lakes in Egypt which exhibited a production of up to 6.2 g/L while Dubey and Mishra [27] recorded a PHA production of 2.6 g/L from 3% glycerol. Stanley et al. [58] reported PHA accumulation from 2.66 g/L, accounting for 70.56% content from moderately halophilic bacteria Halomonas venusta KT832796 in shake flask fermentation.

The identity of PHAs produced by *Halomonas alkalicola* Ext was determined by FTIR spectroscopy and GC-MS. The FTIR spectra of PHAs produced on glucose and galactose substrates revealed similar patterns with prominent absorption peaks at 1733.83 cm⁻¹ and 1735.73 cm⁻¹, respectively, indicating the presence of an ester carbonyl group characteristic of PHB. The observed bands had similar signals of P(3-HB-co-3-HV)

copolymer reported in earlier studies. Sinaei et al. [59] have reported a strong absorption peak at 1735 cm⁻¹ for poly(3hydroxybutyrate-co-3-hydroxyvalerate) synthesized by Bacillus cereus. Polymer analysis with GC-MS revealed that the extracted biopolymer comprised of two subunits, namely, 3hydroxyvaryrate (3-HB) and 3-hydroxybutyrate (3-HV) in the ratio of 92.23%: 7.77%. Other biopolymers synthesized by Halomonas bacteria have varied 3-HV fractions. For instance, Hammami et al. [41] reported a maximum incorporation of 52.04% 3-HV fraction from biopolymer synthesized by Halomonas desertis G11 while Mozejko-Ciesielska et al. [42] showed that Halomonas alkaliantarctica could synthesize PHBV with 60.59% HV fraction. On their part, Tan et al. [57] synthesized PHBV copolymers with 4-6% 3-HV fraction from Halomonas TD01. Our findings are consistent with earlier reports that showed the prominence of PHBVs in PHAs extracted from bacteria of the genus Halomonas [26, 41, 60, 61]. However, short-chain polyhydroxyalkanoates have also been recovered from members of the Halomonas genus [62, 63]. The production PHBV by Halomonas alkalicola Ext shows a potential for production of biocompatible copolymer. However, PHBV is often limited by its poor mechanical properties including brittleness, fragility, and low impact strength [64-66]. Thus, there is a need for exploration of chemical and physical strategies of improving the utility of this useful biomaterial.

5. Conclusion

This study sought to enhance the production of polyhydroxyalkanoates by a moderately halophilic bacterium *Halomonas alkalicola* isolated from Lake Simbi, an alkaliphilic, hypersaline lake in Kenya. The highest PHA production was achieved after 72 h fermentation at an initial pH of 10.0, temperature of 35°C, and 3% NaCl with 2% galactose and 0.1% ammonium sulfate as carbon and nitrogen sources, respectively. A maximum PHA concentration of 45.57% was achieved with a yield of 1.44 g/L under optimized cultural and growth conditions. FTIR and GC-MS analysis revealed that the extracted biopolymer was a copolymer of 3-hydroxyvaryrate (3-HV) and 3hydroxybutyrate (3-HB). The findings of this study show the potential for technological development, bioprocess engineering, and exploration of low-cost feedstocks for improved PHBV production by *Halomonas alkalicola*.

Data Availability

The data used to support the findings of this study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supplementary Materials

Table S1: Analysis of variance (ANOVA) for the quadratic model of the interactive effects of galactose concentration (A) and NaCl concentration (B) on CDW (g/L). Table S2: Analysis of variance (ANOVA) for the quadratic model of the interactive effects of galactose concentration (A) and NaCl concentration (B) on PHA content as a % of CDW. Figure S1: Correlation between predicted and actual results for CDW (g/L). Figure S2: Correlation between predicted and actual results for PHA concentration (g/L). Figure S3: Correlation between predicted and actual results for PHA concentration (% of CDW). Figure S4: Three-dimensional interactions between galactose concentration (A), NaCl concentration (B), and PHA production (g/L) for the highest possible yield. (*Supplementary Materials*)

References

- [1] L. Gross and J. Enck, "Confronting plastic pollution to protect environmental and public health," *PLoS Biology*, vol. 19, no. 3, article e3001131, 2021.
- [2] T. M. Adyel and P. I. Macreadie, "Plastics in blue carbon ecosystems: a call for global cooperation on climate change goals," *The Lancet Planetary Health*, vol. 6, no. 1, pp. e2–e3, 2022.
- [3] P. Moodley and C. Trois, "Circular closed-loop waste biorefineries: organic waste as an innovative feedstock for the production of bioplastic in South Africa," *South African Journal* of Science, vol. 118, pp. 1–6, 2022.
- [4] F. Muneer, I. Rasul, M. Qasim, A. Sajid, and H. Nadeem, "Optimization, production and characterization of polyhydroxyalkanoate (PHA) from indigenously isolated novel bacteria," *Journal of Polymers and the Environment*, vol. 30, no. 8, pp. 3523–3533, 2022.
- [5] F. Muneer, H. Nadeem, A. Arif, and W. Zaheer, "Bioplastics from biopolymers: an eco-friendly and sustainable solution of plastic pollution," *Polymer Science-Series C*, vol. 63, no. 1, pp. 47–63, 2021.
- [6] A. Anjum, M. Zuber, K. M. Zia, A. Noreen, M. N. Anjum, and S. Tabasum, "Microbial production of polyhydroxyalkanoates (PHAs) and its copolymers: a review of recent advancements," *International Journal of Biological Macromolecules*, vol. 89, pp. 161–174, 2016.
- [7] M. Singh, P. Kumar, S. Ray, and V. C. Kalia, "Challenges and opportunities for customizing polyhydroxyalkanoates," *Indian Journal of Microbiology*, vol. 55, no. 3, pp. 235–249, 2015.

- [8] C. J. Brigham and A. J. Sinskey, "Applications of polyhydroxyalkanoates in the medical industry," *International Journal of Biotechnology for Wellness Industries*, vol. 1, no. 1, p. 52, 2012.
- [9] S. M. Hamdy, A. W. Danial, S. M. F. Gad El-Rab, A. A. M. Shoreit, and A. E.-L. Hesham, "Production and optimization of bioplastic (polyhydroxybutyrate) from Bacillus cereus strain SH-02 using response surface methodology," *BMC Microbiol*ogy, vol. 22, no. 1, p. 183, 2022.
- [10] B. Singh Saharan, A. Grewal, and P. Kumar, "Biotechnological production of polyhydroxyalkanoates: a review on trends and latest developments," *Chinese Journal of Biology*, vol. 2014, Article ID 802984, 18 pages, 2014.
- [11] S. Tienda, J. A. Gutiérrez-Barranquero, I. Padilla-Roji, E. Arrebola, A. de Vicente, and F. M. Cazorla, "Polyhydroxyalkanoate production by the plant beneficial rhizobacterium *Pseudomonas chlororaphis* PCL1606 influences survival and rhizospheric performance," *Microbiological Research*, vol. 278, article 127527, 2024.
- [12] G. Y. A. Tan, C. L. Chen, L. Li et al., "Start a research on biopolymer polyhydroxyalkanoate (PHA): a review," *Polymers*, vol. 6, no. 3, pp. 706–754, 2014.
- [13] L. O. B. Cardoso, B. Karolski, L. H. Gracioso, B. B. Borrego, C. A. O. . Nascimento, and E. A. Perpetuo, "Enrichment of *Methylosinus*-dominant consortia from mangroves for polyhydroxybutyrate (PHB) production," *Journal of Environmental Chemical Engineering*, vol. 10, no. 5, article 108490, 2022.
- [14] D. Crutchik, O. Franchi, L. Caminos et al., "Polyhydroxyalkanoates (PHAs) production: a feasible economic option for the treatment of sewage sludge in municipal wastewater treatment plants?," *Water*, vol. 12, no. 4, p. 1118, 2020.
- [15] R. Chandra, A. Thakor, T. H. Mekonnen, T. C. Charles, and H. S. Lee, "Production of polyhydroxyalkanoate (PHA) copolymer from food waste using mixed culture for carboxylate production and *Pseudomonas putida* for PHA synthesis," *Journal* of Environmental Management, vol. 336, article 117650, 2023.
- [16] W. N. Chaudhry, N. Jamil, I. Ali, M. H. Ayaz, and S. Hasnain, "Screening for polyhydroxyalkanoate (PHA)-producing bacterial strains and comparison of PHA production from various inexpensive carbon sources," *Annales de Microbiologie*, vol. 61, no. 3, pp. 623–629, 2011.
- [17] L. H. Gracioso, A. Bellan, B. Karolski et al., "Light excess stimulates poly-*beta*-hydroxybutyrate yield in a mangrove-isolated strain of *Synechocystis* sp.," *Bioresource Technology*, vol. 320, no. Part B, article 124379, Article ID 124379, 2021.
- [18] C. Amabile, T. Abate, S. Chianese, D. Musmarra, and R. Muñoz, "The co-conversion of methane and mixtures of volatile fatty acids into poly(3-hydroxybutyrate-co-3-hydroxyvalerate) expands the potential of an integrated biorefinery," *Bioresource Technology*, vol. 387, article 129699, 2023.
- [19] O. K. Lee, S. G. Kang, T.-R. Choi, Y.-H. Yang, and E. Y. Lee, "Production and characterization of a biodegradable polymer, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), using the type II methanotroph, *Methylocystis* sp. MJC1," *Bioresource Technology*, vol. 389, article 129853, 2023.
- [20] C. Amabile, T. Abate, S. Chianese, D. Musmarra, and R. Muñoz, "Assessing the performance of synthetic cocultures during the conversion of methane into poly(3-hydroxybutyrate)," *Chemical Engineering Journal*, vol. 476, article 146646, 2023.
- [21] L. Wang and Y.-W. Cui, "Simultaneous treatment of epichlorohydrin wastewater and polyhydroxyalkanoate recovery by halophilic aerobic granular sludge highly enriched by *Halomonas*

sp.," *Bioresource Technology*, vol. 391, no. Part A, article 129951, 2024.

- [22] H. Wu, Z. Fan, X. Jiang, J. Chen, and G. Q. Chen, "Enhanced production of polyhydroxybutyrate by multiple dividing *E. coli*," *Microbial Cell Factories*, vol. 15, no. 1, p. 128, 2016.
- [23] G. Q. Chen, X. Y. Chen, F. Q. Wu, and J. C. Chen, "Polyhydroxyalkanoates (PHA) toward cost competitiveness and functionality," *Advanced Industrial and Engineering Polymer Research*, vol. 3, no. 1, pp. 1–7, 2020.
- [24] P. Aytar Celik, D. Barut, B. M. Enuh et al., "A novel higher polyhydroxybutyrate producer *Halomonas halmophila* 18H with unique cell factory attributes," *Bioresource Technology*, vol. 372, article 128669, 2023.
- [25] Y. H. Chen, C. W. Lu, Y. T. Shyu, and S. S. Lin, "Revealing the saline adaptation strategies of the halophilic bacterium *Halomonas beimenensis* through high-throughput omics and transposon mutagenesis approaches," *Scientific Reports*, vol. 7, no. 1, 2017.
- [26] F. A. El-malek, A. Farag, S. Omar, and H. Khairy, "Polyhydroxyalkanoates (PHA) from *Halomonas pacifica* ASL10 and *Halomonas salifodiane* ASL11 isolated from Mariout salt lakes," *International Journal of Biological Macromolecules*, vol. 161, pp. 1318–1328, 2020.
- [27] S. Dubey and S. Mishra, "Natural sea salt based polyhydroxyalkanoate production by wild *Halomonas hydrothermalis* strain," *Fuel*, vol. 311, article 122593, 2022.
- [28] H. J. Jung, S. H. Kim, D. H. Cho et al., "Finding of novel galactose utilizing *Halomonas* sp. YK44 for polyhydroxybutyrate (PHB) production," *Polymers*, vol. 14, no. 24, p. 5407, 2022.
- [29] M. N. Muigano, S. E. Anami, J. M. Onguso, and G. M. Omare, "The isolation, screening, and characterization of polyhydroxyalkanoate-producing bacteria from hypersaline lakes in Kenya," *Bacteria*, vol. 2, no. 2, pp. 81–97, 2023.
- [30] M. Liu, J. E. González, L. B. Willis, and G. C. Walker, "A novel screening method for isolating exopolysaccharide-deficient mutants," *Applied and Environmental Microbiology*, vol. 64, no. 11, pp. 4600–4602, 1998.
- [31] A. Mohan Nair, K. Annamalai, S. Kamala Kannan, and S. Kuppusamy, "Utilization of sugarcane molasses for the production of polyhydroxyalkanoates using Bacillus Subtilis," *Malaya Journal of Biosciences*, vol. 1, no. 1, pp. 24–30, 2014.
- [32] P. Spiekermann, B. H. A. Rehm, R. Kalscheuer, D. Baumeister, and A. Steinbüchel, "A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds," *Archives of Microbiology*, vol. 171, no. 2, pp. 73– 80, 1999.
- [33] S. K. Hahn, Y. K. Chang, B. S. Kim, and H. N. Chang, "Optimization of microbial poly(3-hydroxybutyrate) recover using dispersions of sodium hypochlorite solution and chloroform," *Biotechnology and Bioengineering*, vol. 44, no. 2, pp. 256–261, 1994.
- [34] J. H. Law and R. A. Slepecky, "Assay of poly-β-hydroxybutyric acid," *Journal of Bacteriology*, vol. 82, no. 1, pp. 33–36, 1961.
- [35] C. Trakunjae, A. Boondaeng, W. Apiwatanapiwat et al., "Enhanced polyhydroxybutyrate (PHB) production by newly isolated rare actinomycetes *Rhodococcus* sp. strain BSRT1-1 using response surface methodology," *Scientific Reports*, vol. 11, no. 1, p. 1896, 2021.
- [36] A. Gurunathan, J. Senguttuvan, and S. Paulsamy, "Evaluation of mosquito repellent activity of isolated oleic acid, eicosyl

ester from *Thalictrum Javanicum*," *Indian Journal of Pharmaceutical Sciences*, vol. 78, no. 1, p. 103, 2016.

- [37] H. Xiang, S. Wang, R. Wang, Z. Zhou, C. Peng, and M. Zhu, "Synthesis and characterization of an environmentally friendly PHBV/PEG copolymer network as a phase change material," *Science China Chemistry*, vol. 56, no. 6, pp. 716–723, 2013.
- [38] J. Bai, J. Dai, and G. Li, "Electrospun composites of PHBV/ pearl powder for bone repairing," *Progress in Natural Science: Materials International*, vol. 25, no. 4, pp. 327–333, 2015.
- [39] G. P. S. Aguiar, C. D. Magro, J. V. Oliveira, and M. Lanza, "Poly(hydroxybutyrate-co-hydroxyvalerate) micronization by solution enhanced dispersion by supercritical fluids technique," *Brazilian Journal of Chemical Engineering*, vol. 35, no. 4, pp. 1275–1282, 2018.
- [40] N. Pramanik, R. Das, T. Rath, and P. P. Kundu, "Microbial degradation of linseed oil-based elastomer and subsequent accumulation of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer," *Applied Biochemistry and Biotechnology*, vol. 174, no. 4, pp. 1613–1630, 2014.
- [41] K. Hammani, Y. Souissi, A. Souii et al., "Extremophilic bacterium Halomonas desertis G11 as a cell factory for poly-3-hydroxybutyrate-co-3-hydroxyvalerate copolymer's production," Frontiers in Bioengineering and Biotechnology, vol. 10, 2022.
- [42] J. Możejko-Ciesielska, K. Moraczewski, and S. Czaplicki, "Halomonas Alkaliantarctica as a platform for poly(3-hydroxybutyrate-co-3-hydroxyvalerate) production from biodieselderived glycerol," *Environmental Microbiology Reports*, vol. 2023, 2023.
- [43] J. Mozejko-Ciesielska, K. Moraczewski, S. Czaplicki, and V. Singh, "Production and characterization of polyhydroxyalkanoates by *Halomonas alkaliantarctica* utilizing dairy waste as feedstock," *Scientific Reports*, vol. 13, no. 1, article 22289, 2023.
- [44] Y. Chen, X.-Y. Chen, H.-T. Du et al., "Chromosome engineering of the TCA cycle in *Halomonas bluephagenesis* for production of copolymers of 3-hydroxybutyrate and 3hydroxyvalerate (PHBV)," *Metabolic Engineering*, vol. 54, pp. 69–82, 2019.
- [45] T. Narancic and K. E. O'Connor, "Plastic waste as a global challenge: are biodegradable plastics the answer to the plastic waste problem?," *Microbiology*, vol. 165, no. 2, pp. 129–137, 2019.
- [46] S. A. Acharjee, P. Bharali, B. Gogoi, V. Sorhie, B. Walling, and Alemtoshi, "PHA-based bioplastic: a potential alternative to address microplastic pollution," *Water, Air, and Soil Pollution*, vol. 234, no. 1, p. 21, 2023.
- [47] R. A. Ilyas, S. M. Sapuan, A. Kadier et al., "Properties and Characterization of PLA, PHA, and Other Types of Biopolymer Composites," in Advanced Processing, Properties, and Applications of Starch and Other Bio-based Polymers, pp. 111–138, Elsevier B.V., 2020.
- [48] R. Muthuraj, O. Valerio, and T. H. Mekonnen, "Recent developments in short- and medium-chain-length polyhydroxyalkanoates: production, properties, and applications," *International Journal of Biological Macromolecules*, vol. 187, pp. 422–440, 2021.
- [49] N. Gómez-Gast, M. D. R. L. Cuellar, B. Vergara-Porras, and H. Vieyra, "Biopackaging potential alternatives: bioplastic composites of polyhydroxyalkanoates and vegetal fibers," *Polymers*, vol. 14, no. 6, p. 1114, 2022.
- [50] J.-M. Jeon, Y. Son, L. Chang, Y.-H. Yang, and J.-J. Yoon, "Polyhydroxyalkanoate production by *Halomonas* sp. YLGW01

using volatile fatty acids: a statistical approach to apply for food-waste water," *Biomass Conversion and Biorefinery*, vol. 2022, 2022.

- [51] F. Ahmadi, A. A. Zinatizadeh, and A. Asadi, "The effect of different operational strategies on polyhydroxyalkanoates (PHAs) production at short-term biomass enrichment," *Journal of Environmental Chemical Engineering*, vol. 8, no. 3, article 103531, 2020.
- [52] N. Ojha and N. Das, "A statistical approach to optimize the production of polyhydroxyalkanoates from *Wickerhamomyces anomalus* VIT-NN01 using response surface methodology," *International Journal of Biological Macromolecules*, vol. 107, no. Part B, pp. 2157–2170, 2018.
- [53] R. Shen, Z. Y. Ning, Y. X. Lan, J. C. Chen, and G. Q. Chen, "Manipulation of polyhydroxyalkanoate granular sizes in *Halomonas bluephagenesis*," *Metabolic Engineering*, vol. 54, pp. 117–126, 2019.
- [54] W. Tao, L. Lv, and G. Q. Chen, "Engineering Halomonas species TD01 for enhanced polyhydroxyalkanoates synthesis via CRISPRi," *Microbial Cell Factories*, vol. 16, no. 1, p. 48, 2017.
- [55] J. M. Cervantes-Uc, J. Catzin, I. Vargas et al., "Biosynthesis and characterization of polyhydroxyalkanoates produced by an extreme halophilic bacterium, *Halomonas Nitroreducens*, isolated from hypersaline ponds," *Journal of Applied Microbiology*, vol. 117, no. 4, pp. 1056–1065, 2014.
- [56] I. Pernicova, D. Kucera, J. Nebesarova et al., "Production of polyhydroxyalkanoates on waste frying oil employing selected *Halomonas* strains," *Bioresource Technology*, vol. 292, article 122028, 2019.
- [57] D. Tan, Q. Wu, J. C. Chen, and G. Q. Chen, "Engineering Halomonas TD01 for the low-cost production of polyhydroxyalkanoates," *Metabolic Engineering*, vol. 26, pp. 34–47, 2014.
- [58] A. Stanley, H. N. Punil Kumar, S. Mutturi, and S. V. N. Vijayendra, "Fed-batch strategies for production of PHA using a native isolate of Halomonas Venusta KT832796 strain," *Applied Biochemistry and Biotechnology*, vol. 184, no. 3, pp. 935–952, 2018.
- [59] N. Sinaei, D. Zare, and M. Azin, "Production and characterization of poly 3-hydroxybutyrate-co-3-hydroxyvalerate in wheat starch wastewater and its potential for nanoparticle synthesis," *Brazilian Journal of Microbiology*, vol. 52, no. 2, pp. 561–573, 2021.
- [60] X. Chen, J. Yin, J. Ye et al., "Engineering Halomonas bluephagenesis TD01 for non-sterile production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate)," Bioresource Technology, vol. 244, Part 1, pp. 534–541, 2017.
- [61] T. Thomas, K. Sudesh, A. Bazire et al., "PHA production and PHA synthases of the halophilic bacterium *Halomonas* sp. SF2003," *Bioengineering*, vol. 7, no. 1, p. 29, 2020.
- [62] D. Tan, Y. S. Xue, G. Aibaidula, and G. Q. Chen, "Unsterile and continuous production of polyhydroxybutyrate by *Halomonas* TD01," *Bioresource Technology*, vol. 102, no. 17, pp. 8130–8136, 2011.
- [63] B. Kim, S. J. Oh, J. H. Hwang et al., "Polyhydroxybutyrate production from crude glycerol using a highly robust bacterial strain *Halomonas* sp. YLGW01," *International Journal of Biological Macromolecules*, vol. 236, article 123997, 2023.
- [64] E. Bugnicourt, P. Cinelli, A. Lazzeri, and V. Alvarez, "Polyhydroxyalkanoate (PHA): review of synthesis, characteristics, processing and potential applications in packaging," *Express Polymer Letters*, vol. 8, no. 11, pp. 791–808, 2014.

- [65] K. E. Mazur, P. Jakubowska, A. Gaweł, and S. Kuciel, "Mechanical, thermal and hydrodegradation behavior of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) composites with agricultural fibers as reinforcing fillers," *Sustainable Materials and Technologies*, vol. 31, article e00390, 2022.
- [66] B. S. Kushwah, A. V. S. Kushwah, and V. Singh, "Retracted Article: Towards understanding polyhydroxyalkanoates and their use," *Journal of Polymer Research*, vol. 23, no. 8, 2016.