Production and Optimization of Polyhydroxyalkaonoate Obtained from *Bacillus megaterium* JHA

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Abstract
Introduction: The last four decades have recognized the critical environmental issues pertaining to the use of non-degradable synthetic plastics. Currently, the synthesis of biodegradable polymers, like polyhydroxyalkaonoates (PHA) has been gaining considerable interest as a sustainable approach to overcome these issues. The current study was carried out with an objective to optimize the PHA production from *Bacillus megaterium* JHA.
Materials and Methods: Various nutritional and physico-chemical parameters were optimized using the 'one factor at a time' approach. The findings were analysed statistically by one-way ANNOVA and linear regression using the open-source R software.
Results: The optimum physico-chemical parameters for maximum PHA production were attained when 5% inoculum size of *B. megaterium* JHA was added to the JHA medium (pH 8) and incubated at 28 °C for 96 h under shaker conditions (120 rpm). The nutritional parameters were further optimized by addition of 0.6 g% K_2HPO_4, 0.4 g% KH_2PO_4, 21 g% glucose, 12.5 mM microcosmic salt, 0.04 g% KNO_3, 1 mM $MgSO4$, and 2 ml trace elements to the JHA medium. The C:N and C:P ratio was adjusted to 70:1 and 20:1 respectively. The growth of bacteria under these optimized parameters allowed 13.71 g/L accumulation of PHA leading to a yield of 54.51%. This resulted in 66.88% increase in yield as compared to the original medium. The scale-up study using a 2 L fermenter further increased PHA accumulation by 4.39% under optimized conditions.
Conclusions: The above results clearly indicate that *B. megaterium* JHA is a promising isolate that can be exploited for the industrial production of PHA.
Keywords: Biopolymer, Fermenter, JHA medium, Optimize, Sustainable
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Introduction

The convenience in using plastic products is apparent with its global demand in the industrial as well as consumer sectors. The properties like adaptability, durability and high flexibility along with cost-effective nature allow its feasible application in packaging, insulation, electronics as well as modern construction materials.[1] Hence, building a life without plastic is unimaginable. However, the non-biodegradable nature of plastics has created problems with its disposal for over decades. Statistically, it is estimated that over 340 million tons of plastics are accumulated every year.[2] Moreover, the amount of fossil fuels consumed while manufacturing plastics is equal to the amount utilized in the aviation industry, globally.[3] The resulting 'white pollution' entails significant environmental and human health hazards, if left unchecked.[4] Numerous initiatives like recycling, re-use, degradation and alternatives of plastics have been considered in the last four decades.[5] However, the malleable and pliable property of plastics makes it practically irreplaceable. Although challenging, implementing a sustainable approach towards the production of bio-based plastics, which are degradable and biocompatible

as compared to commercial plastics, appears to be a prospective solution to the existing problem.

Till date, various bio-based plastics including polymers have been produced from starch, cellulose and proteins. Few examples include casein, polylactic acid, polyglycolic acid, polycaprolactone, polydioxone, polyhydroxyalkanoate (PHA) and poly-3-hydroxybutyrate-co-3-hydroxyvalerate.[6,7] The PHAs are energy reserves accumulated intracellularly by a wide variety of microorganisms under surplus carbon, and nitrogen/phosphate limiting conditions.[8] The type of produced PHA is dependent on the substrate utilised during the process of biosynthesis,[9] and the properties may differ depending on the monomer composition.[10] They are steadily gaining importance due to their eco-friendly nature and their ability to degrade under both aerobic and anaerobic conditions.[11]

Various gram negative and gram positive microorganisms such as *Pseudomonas* sp., *Alkaligenes* sp., *Bacillus* sp., *Azotobacter* sp., *Escherichia coli*, *Klebsiella* sp., *Clostridium* sp. and many more have been identified and explored for the biosynthesis of PHA.[10,12-14] The gram positive organisms

such as *Bacillus* sp. show a higher potential for PHA biosynthesis, over gram negative organisms. This is due to their ability to utilize diverse substrates, including inexpensive or agro-waste carbon sources, and subsequently, produce a diverse range of monomers. They can also thrive in nutrient limiting and stringent fermentation conditions, and synthesize PHA. However, for commercial production of PHA, the major advantage of using gram positive organisms is the lack of lipopolysaccharide layer in their cell membrane. This prevents the use of vigorous extraction procedures, and thus decreases the production cost.[15] The present study takes into consideration all these factors to optimize the yield of PHA from *Bacillus megaterium* JHA, and hence will make a significant contribution to the existing knowledge on microbial production of biopolymers like PHA.

The current study was carried out with an objective to optimize the extraction protocol as well as the nutritional and physicochemical parameters for maximizing the yield of PHA from *B. megaterium* JHA that was previously isolated from soil sample.

Materials and Methods
Chemicals and Media
All the chemicals, sugars, nutrient sources and growth factors used in the current study were analytical grade and procured from Loba chemie (Mumbai, India).

PHA Producer and Growth Conditions
A potential PHA producer, *B. megaterium* JHA (accession no. LC201962), isolated in an earlier study from oil contaminated soil, was used for optimization studies.[16] It was grown in 60 ml modified E2 broth supplemented with 2 g% glucose as carbon source for biomass estimation and PHA extraction, under previously described incubation conditions.[17]

Optimization of Method for Extraction of Polyhydroxyalkanoate
A 10 ml volume of culture broth was used for biomass estimation and the pellet obtained after centrifugation of 50 ml broth was subjected to different extraction methods, mentioned below, to study enhanced PHA recovery. The white precipitate of PHA obtained by various extraction methods was estimated quantitatively by the Slepecky and Law method.[18]

Hypochlorite Method
The PHA was extracted using a rapid, and a modified hypochlorite method. The pellet obtained from the above culture broth was suspended in 5 ml sodium hypochlorite solution (containing 4% active chlorine) and vortexed. For the rapid hypochlorite method,[19] the suspension was incubated at 37 °C for 10 min and centrifuged at 8000 rpm for 15 min. The supernatant was discarded and the obtained

pellet was dissolved in 5 ml cold Diethyl ether. For the modified hypochlorite method,[20] the pellet was suspended in sodium hypochlorite solution, vortexed and the suspension was incubated at 37 °C for 30 min. The obtained PHA pellet was washed with 5 ml alcohol: acetone mixture (1:1 v/v) and centrifuged at 8000 rpm for 12 min. The pellet was thus dissolved in 5 ml chloroform.

The solvents were allowed to evaporate at 28 °C by pouring the solution in a sterile glass petri plate to obtain a white precipitate of PHA.

Chloroform Method
The pellet was washed twice with sterile phosphate buffered saline (pH 7.2) and centrifuged at 8000 rpm for 12 min. The pellet was then suspended in 10 ml of chloroform and vortexed. The mixture was incubated at 37 °C for 24 h and then centrifuged at 8000 rpm for 12 min. The supernatant containing the PHA was poured in sterile petri plates, and chloroform was allowed to evaporate at 28 °C to obtain a white precipitate of PHA.[21]

Extraction with Chloroform Followed by Precipitation by Ice Cold Methanol
The pellet was suspended in chloroform at 37 °C for 3 h in screw cap tubes. The suspension was centrifuged at 8000 rpm for 12 min. The dissolved polymer was precipitated from chloroform solution with chilled methanol (1:3) added drop wise. The polymer was once again dissolved in chloroform and precipitated in methanol in order to obtain highly purified polymer.[22]

Dispersion Method
The pellet was suspended in a tube containing 10 ml of sodium hypochlorite and chloroform mixture (1:1 v/v), and incubated at 37 °C for 1 h. The suspension was vortexed and then placed for separation in the separating funnel where three layers were obtained. The top layer consisted of sodium hypochlorite solution, the interphase showed the presence of cell debris and the bottom layer consisted of PHA dissolved in chloroform. The bottom layer was carefully removed and placed in sterile petri plates. Chloroform was allowed to evaporate at 28 °C to obtain a white precipitate of PHA.[23]

Optimization of Media and Physicochemical Parameters for Accumulation of Polyhydroxyalkanoate
The optimization of PHA biosynthesis was initiated by determining a suitable media. The 12 different media used in this study and its composition have been presented in Table 1. The sterile medium (60 ml) was inoculated with 2.5% w/v of culture suspension and was incubated at 28 °C for 72 h under shaker conditions (120 rpm). The PHA was extracted using the optimized extraction method and estimated quantitatively by the Slepecky and Law method.[18] The

medium which gave maximum PHA accumulation was selected for further studies.

The different physico-chemical components were optimized for PHA accumulation by altering one factor at a time, keeping the other variables constant, at a specific set of conditions. All experiments were run in triplicates. The varying factors included pH (5.0, 6.0, 7.0, 8.0 and 9.0), temperature (28 °C, 37 °C, 45 °C and 55 °C), optical density (0.2, 0.4, 0.6, 0.8, 1, 1.2 and 1.4), inoculum size (1%, 2%, 3%, 4%, 5%, 6%, 7% ,8%, 9% and 10%), rate of aeration (static condition, 100 rpm, 120 rpm, 140 rpm and 160 rpm) and time (24 h, 48 h, 72 h, 96 h and 120 h).[24-27]

Table 1. Composition of Media Used for Optimizing PHA Production

Sr. No.	Medium	Composition (g/L)	Reference
1	Medium 1	Na_2HPO_4 (7.8), KH_2PO_4 (6.8), $MgSO_4$ (0.2), $NaNO_3$ (0.085), $ZnSO_4.7H_2O$ (0.05), $ZnCl_2$ (0.02), $Ca(NO_3)_2.4H_2O$ (0.05), dextrose (20), pH 7	28
2	Medium 2	$Na_2HPO_4.12H_2O$ (9.0), KH_2PO_4 (1.5), $MgSO_4.7H_2O$ (0.2), NH_4Cl (1.0), $CaCl_2.2H_2O$ (0.02), Fe(III)NH_4-citrate (0.0012), glucose (20), 1ml trace element solution containing EDTA (50.0), $FeCl_3$ (8.3), $ZnCl_2$ (0.84), $CoCl_2.6H_2O$ (0.1), $MnCl_2.6H_2O$ (0.016) and H_3BO_3 (0.1), pH 7.3	29
3	Medium 3	CH_3COONa (5.0), $(NH_4)_2SO_4$ (0.1), K_2HPO_4 (0.1), NaH_2PO_4 (0.2), $MgSO_4.7H_2O$ (0.2), NaCl (0.05), $CaCl_2$ (0.05), $FeCl_3.6H_2O$ (0.0083), $MnCl_2.4H_2O$ (0.0014), Na_2MoO_4 $2H_2O$ (0.00117), $ZnCl_2$ (0.001), glucose (20), pH 7	30
4	Medium 4	$Na_2HPO_4.2H_2O$ (2.2), KH_2PO_4 (1.5), $(NH_4)_2SO_4$ (1.5), $MgSO_4.7H_2O$ (0.2), glucose (20), pH 7	31
5	Medium 5	Glucose (20), KH_2PO_4 (0.5), 0.2 g $MgSO_4$ $.7H_2O$ (0.2), NaCl (0.1), tryptone (2.5), peptone (2.5), yeast extract (2.5), pH 7	32
6	Medium 6	Glucose (20), $(NH_4)_2SO_4$ (1.4), KH_2PO_4 (1.5), Na_2HPO_4 (1.8), $MgSO_4.7H_2O$ (0.2), 1ml trace elements solution containing Fe(III)NH_4-citrate (6), $CaCl_2.2H_2O$ (10), H_3BO_3 (0.3), $CoCl_2.6H_2O$ (0.2), $ZnSO_4.$ $7H_2O$ (0.1), $MnCl_2.4H_2O$ (0.03), $Na_2MoO_4.2H_2O$ (0.03), $NiSO_4.$ $7H_2O$ (0.02), $CuSO_4.5H_2O$ (0.01), pH 7	33
7	Medium 7	Tryptone (17.0), phytone peptic digest of soya meal (3), NaCl (5), K_2HPO_4 (2.5), glucose (20), pH 7	34
8	Medium 8	Glucose (20), yeast extract (10), peptone (10), Na_2HPO_4 (1), $MgSO_4$ (0.2), pH 7	35
9	Medium 9	Glucose (20), $MgSO_4$ (4), $CaCl_2$ (1.1), Na_2HPO_4 (37), $(NH_4)_2SO_4$ (2), K_2HPO_4 (20), Fe(III)NH_4-citrate (6), pH 7	36
10	Medium 10	$NaNH_4HPO_4.4H_2O$ (3.5), $K_2HPO_4.3H_2O$ (7.5), KH_2PO_4 (3.7), 100mM $MgSO_4.7H_2O$ (10 ml) and 1ml microelements stock solution containing $FeSO_4.7H_2O$ (2.78), $MnCl_2·4H_2O$ (1.98), $CoSO_4.7H_2O$ (2.81), $CaCl_2.2H_2O$ (1.47), $CuCl_2.2H_2O$ (0.17) $ZnSO_4.7H_2O$ (0.29), yeast extract (0.04 g%), glucose (20), pH 7	24
11	Medium 11	Na_2HPO_4 (6), KH_2PO_4 (3), NaCl (5), NH_4Cl (1), 1M $MgSO_4.7H_2O$ (1mL), dextrose (20), pH 7	37
12	Medium 12	$(NH_4)_2SO_4$ (1.0), KH_2PO_4 (13.3), $MgSO_4$ (1.3), citric acid (1.7), trace elements (10 ml) containing $FeSO_4·7H_2O$ (10), $ZnSO_4·7H_2O$ (2.25), $CuSO_4·5H_2O$ (1), $MnSO_4·5H_2O$ (0.5), $CaCl_2·2H_2O$ (2.0), $Na_2B_4O_7·10H_2O$ (0.23), $(NH_4)_6MO_7O_{24}$ (0.1), HCl (0.1N), glucose (20), pH 7	21
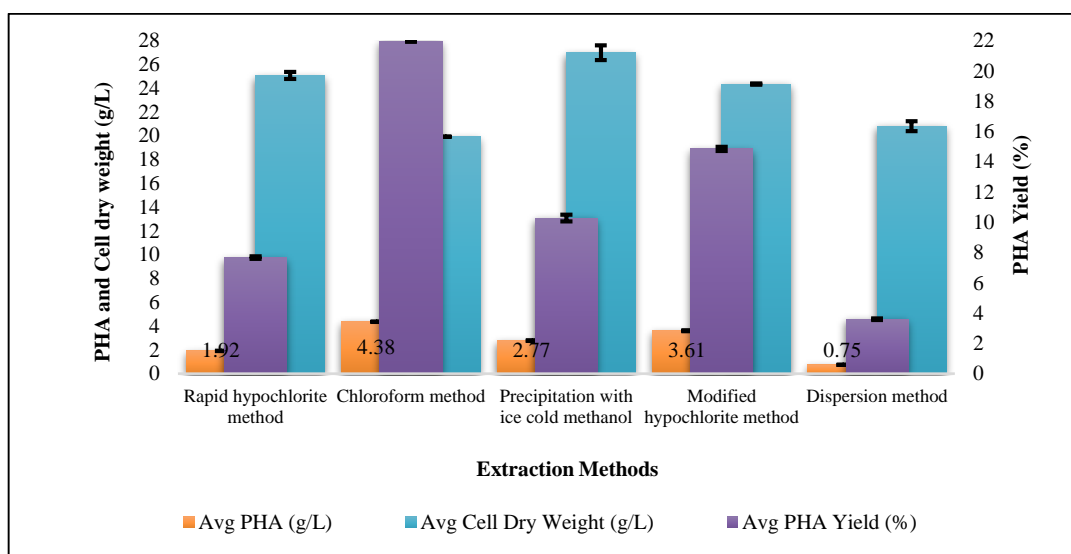
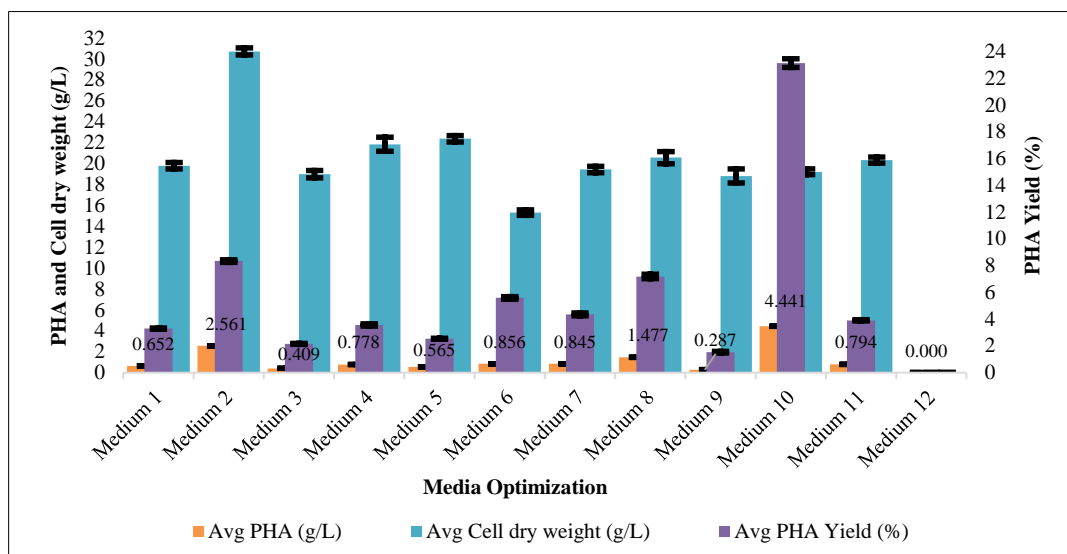**Figure 1.** Optimization of Extraction Method for Maximum PHA Yield.

Figure 2. Optimization of Media for Maximum PHA Accumulation.

Optimization of Nutritional Parameters for Accumulation of Polyhydroxyalkanoate

In addition to the above parameters, the effect of carbon (2 g% w/v glucose, lactose, sucrose, mannitol, maltose, fructose, xylose, galactose, raffinose, glycerol, starch, palmitic acid, lactic acid, glutamic acid, lactic acid, stearic acid, gelatin, sodium acetate and sodium citrate), inorganic nitrogen (0.35 g% w/v urea, sodium nitrate, potassium nitrate, ammonium dihydrogen phosphate, ammonium chloride, ammonium sulphate, ammonium acetate, ammonium per sulphate, diammonium hydrogen phosphate and microcosmic salt) and organic nitrogen sources (soya meal, peptone, yeast extract, beef extract, tryptone, peptic digest of soymeal, protein powder and meat extract) were also studied under previously optimised conditions. After optimization of the nutrient sources, the respective concentrations of carbon (0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10%),

inorganic (5 mM, 10 mM, 12.5 mM, 15 mM, 17.5 mM, 20 mM and 25 mM) and organic nitrogen (0.01 g% - 0.08 g%) sources were also optimized.[24,38-41]

We further studied the effect of replacing the optimized organic nitrogen source with an inorganic source. Other optimized nutritional parameters in our study included phosphate sources (K_2HPO_4, KH_2PO_4) and its concentrations (0.1 g% - 1 g%), carbon nitrogen ratios (3.3:1, 5:1, 6.6:1, 10:1, 15:1, 20:1, 25:1, 30:1, 40:1, 50:1, 60:1, 70:1 and 80:1), carbon phosphate ratios (1:1, 2:1, 4:1, 6:1, 8:1, 10:1, 15:1, 20:1 and 25:1), volumes of trace elements (1-8 ml with a variance of 1 ml in 1 L medium) and concentrations of $MgSO_4$ (1-10 mM) at an interval on 1 mM) on PHA accumulation.[24,40,42-45]

Finally, the growth profile of test bacteria with respect to cell dry weight and PHA yield was studied by comparing original medium and optimized medium.

Table 2. Statistical Representation of One Way ANNOVA Based on Observed PHA Yield Under Optimized Parameters

Variables	t value	*p* value	F value	R squared
pH	4.9506	0.0385	12.4712	0.9258
O.D	2.8635	0.0458	9.3424	0.8237
Inoculum size	1.5420	0.1670	1.2073	1.2073
Aeration rate	4.0235	0.0566	17.9332	0.9472
Incubation time	2.4097	0.0434	9.9874	0.9385
Temperature	-2.5305	0.1271	6.4034	0.762
Glucose concentration	-0.7988	0.4430	2.1971	0.3053
Microcosmic salt concentration	2.3376	0.0452	9.8415	0.9576
Yeast extract concentration	1.5422	0.0693	7.1482	0.9374
K_2HPO_4	2.7555	0.0513	9.9284	0.9313
KH_2PO_4	2.7659	0.0508	10.3789	0.9282
C:N ratio	2.8071	0.0171	7.8801	0.4174
C:P ratio	2.4587	0.0435	6.0454	0.4634
Trace elements	1.1639	0.2886	1.4795	0.3303
$MgSO_4$ concentration	-0.4720	0.6482	0.2228	0.0242

Statistical Analysis

The standard deviation values obtained for optimization of above parameters, on the amount of PHA (g/L), cell dry weight (g/L) and PHA yield (%), were analysed statistically using one way ANNOVA test. The significance of the outcome obtained for all the experiments was determined by linear regression analysis using the open source R software.

Scale up Studies

The scale up studies were done by using a lab scale fermenter. Fermentation was carried out using 2 L Zenith BioFERMA bioreactor (Zenith Engineers, USA) to increase PHA yield. The optimized JHA medium (1500 ml) was sterilized along with the glass fermenter unit and its accessories at 121 °C at 15 psi for 20 min. A stock of glucose (100 g%) was sterilized separately. The entire unit was allowed to come to 28 °C overnight. The medium was supplemented with 100 mM of $MgSO_4.7H_2O$ and trace elements. Seed culture was prepared by the method mentioned earlier during optimization studies and was inoculated into the sterilized medium supplemented with glucose, $MgSO_4$ and trace elements. Parameters used for operation were pH (8), air cycle (60 min ON and 60 min OFF), agitation speed (120 rpm), temperature (28 °C) and time period (96 h). The pH was maintained robotically by the addition of H_2SO_4 and NaOH. Paraffin oil mixed with water (1:1) was used as the anti-foaming agent.[46]

Results and Discussion

Optimization of Mthod for Extraction of Polyhydroxyalkanoates

In the current study, the chloroform method was found to be the most effective extraction technique followed by the modified hypochlorite method. These extraction methods gave a PHA yield of 4.38 g/L and 3.61 g/L respectively (Figure 1). Other methods like precipitation with ice cold methanol (2.77 g/L), rapid hypochlorite method (1.92 g/L) and dispersion method (0.75 g/L) also enabled extraction of PHA from the cell but were not as effective as the chloroform method.

For the extraction of intracellular polymer from *Bacillus* sp., the most routinely used methods include PHA solubilisation using chloroform and removal of cellular material with the help of sodium hypochlorite solution.[47,48] The use of chloroform for the extraction of PHA is considered to be a simple and effective technique since it has an affinity for PHA that enables its solubilisation giving a highly refined product without any degradation within the cell.[49] The sodium hypochlorite, on the other hand, acts on the cellular components, except PHA, thus enabling polymer extraction.[50] However, many reports have mentioned the fact that longer exposure of the sodium hypochlorite to PHA granules reduces the PHA yield due to polymer degradation to low molecular weight. This makes it

inappropriate for various applications.[51,52] A higher yield of PHA using chloroform as compared to dispersion and soxhlet methods and better quality compared to hypochlorite method has also been previously reported.[53,54] Moreover, the chloroform extraction method efficiently extracted polymer with 92% purity from freeze-dried *Bacillus cereus* SPV cells.[53]

Optimization of Media for Accumulation of Polyhydroxyalkanoate

The maximum PHA accumulation of 4.41 g/L and PHA yield of 23.13% was observed in medium 10 (E2 medium), followed by medium 2 (Mineral salt medium) that showed the PHA accumulation of 2.56 g/L and PHA yield of 8.34% (Figure 2). Other media were mostly utilized for biomass production rather than for polymer synthesis.

Similar to our findings, the use of the E2 medium for maximum biopolymer accumulation has been reported by organisms such as *Bacillus* sp. COL1/A6 (65.25%),[55] *Bacillus* sp. NQ-11/A2 (61%),[56] *Bacillus* sp. 87I (70.04%),[15] *Bacillus* sp. 112A (67.73%),[57] *B. megaterium* SW1-2 (36%),[58] *Pseudomonas aeruginosa* LMG 1242 (57.77%)[59] and *Pseudomonas putida* BET001 (34.9%).[60] The mineral salt medium, i.e. the second best medium in our study, has been reported to be optimum for the production of PHA by *P. aeruginosa* NCIB 40045 (2.3 g/L),[61] *Burkholderia* sp. USM JCM15050 (1.5 g/L),[62] *Paenibacillus durus* BV-1 (0.9 g/L),[63] *Bacillus cereus* PW3A (0.325 g/L),[64] *B. cereus* RCL02 (1.5 g/L),[65] *Bacillus aryabhattai* PHB10 (3.264 g/L)[66] and *Pseudomonas* sp. PAMC 28620 (7.95 g/L).[8]

Among other nutrient media, Wagle et al.[67] reported maximum PHA yield of 43.04 ± 1.09%, 47.38 ± 1.31% and 49.06 ± 0.75% in *Bacillus flexus*, *Brucella melitensis* and *P. aeruginosa* respectively using Luria Bertani medium. *Bacillus thuringiensis* E101, *Burkholderia thailandensis* and *Pseudomonas hydrogenovora* showed maximum PHA yield of 2.12 g/L, 7.5 g/L and 1.27 g/L when grown on modified Rhizobium culture medium, nutrient broth and H3 production medium respectively.[10,68,69]

Optimization of Physicochemical Parameters for Accumulation of Polyhydroxyalkanoate

In the present study, *B. megaterium* JHA showed optimum growth at pH 9 but maximum PHA yield (25.58%) was observed at pH 8. A steady increase in PHA yield was observed up to an optical density of 1 at 540 nm (29.72%) and 5% v/v inoculum size (32.17%), beyond which there was no significant effect on bacterial growth and PHA accumulation. Thus, it can be concluded that the increase in cell density was utilized towards biomass formation rather than PHA accumulation. The biomass formation was quite constant at different aeration rates but the PHA accumulation in *B. megaterium* JHA was significantly influenced by the same. We were not able to accumulate PHA under static

conditions but there was a steady increase in its yield as the rate of aeration increased, reaching a maximum at 120 rpm (33.61%). Also, the PHA accumulation began in the exponential phase and continued in the stationary phase showing maximum PHA yield of 34.65% in 96 h. Beyond 96 h, a gradual decrease in PHA accumulation was observed which may be due to the metabolic depolymerase activity of *B. megaterium* JHA that enabled utilization of polymer as a substrate for survival.[70] The optimal temperature of 28 °C supported both the growth and PHA accumulation which was 19.58 g/L and 5.21 g/L respectively. The increase of temperature beyond 37 °C did not affect growth but had a negative impact on PHA accumulation which may be due to low enzyme activity at this temperature. Table 2 represents the statistical significance of the observed results at $p < 0.05$. The negative t value (for temperature) indicates the non-linear relation between temperature and PHA yield. Other physico-chemical parameters showed a linear relation between the respective increasing values and PHA yield.

The production of PHAs is dependent on various factors such as the type of organism, cell density, the constituents present in the nutrient medium, and physico-chemical parameters like pH, temperature, aeration and incubation period. Thus, the optimization of these factors is a crucial step to maximize the yield of PHA from micro-organisms. For a medium to be selected as an optimal production medium, it should enable cell biomass as well as PHA accumulation. The pH and temperature have an impact on metabolite production and microbial growth. An increase in pH beyond optimum may cause changes in solubility of media components and cell permeability.[71] It may also lead to degradation of biopolymers resulting in PHA depletion at the same rate as its production.[72] Temperature affects the growth of bacteria by regulating the metabolic activities, biochemical composition and enzymatic functions of a cell and hence affects PHA yield.[73] Elevated temperatures may cause thermal inactivation of the enzymes responsible for the biosynthesis of PHA.[74] The size of the inoculum also influences the length of the lag phase of the culture which successively affects the growth, the efficiency of biopolymer production and the incubation time. Hence, the optimum PHA production can also be achieved using a two stage strategy, whereby the cells are grown in a suitable medium initially to increase biomass followed by exposing them to stress conditions that ensures PHA accumulation.[75] Apart from these factors, oxygen is essential for sustenance of the reducing power. Minor difference in oxygen availability affects the tricarboxylic acid cycle (TCA cycle) leading to significant changes in the metabolite distribution, including biopolymer accumulation, in various micro-organisms.[76] The high cell mass leading to oxygen depletion during growth phase hinders the TCA cycle resulting in accumulation of NADPH that inhibits citrate synthase. The accumulated

cofactor is then used by various organisms for PHA synthesis.[77]

A similar study reported optimum PHA production from *B. megaterium* at 30 °C, pH 6 and with the presence of either 30 g/L glucose or xylose. In another study, *Klebsiella* sp. NCCP-138 showed optimum PHA production at pH 7.5 and 35 °C in 72 h.[26] A high biopolymer content at pH 8 has been reported in *B. cereus* NRRL-B-3711 (8.4 ± 0.06 g/L),[39] *Rhodobacter sphaeroides* (25.2%),[78] *Alcaligenes eutrophus* MTCC 1285 (11 g/L),[79] *Pannonibacter phragmitetus* ERC8 (1.36 g/L PHA)[80] and *Wickerhamomyces anomalus* VIT-NN01 (19.50 ± 0.3 g/L PHA).[41] A pH 7 and temperature between 28-30 °C has been reported to be optimum for biopolymer production in *Brevibacillus invocatus* MTCC 9039 (52%),[81] *Bacillus subtilis* ATCC 6633 (0.011 g/L)[82] and *B. cereus* FA11 (76.40%).[83] However, biopolymer synthesis in *Bacillus* sp. is commonly reported between 30-38 °C.[15,84-86] Recent studies have reported a maximum yield at 30 °C in *B. subtilis* 6833, *B. melitensis* AUH2, *Microbacterium aurum* TPL18 and *Paracoccus* sp. LL1 showing 35.97 ± 1.64%, 38.37 ± 0.06%, 25.6 ± 0.95% and 30.89% PHA production respectively.[67,87] In contrast to our findings, Maximum PHA production at high temperatures of 40-45 °C was observed in *Haloferax mediterranei*, *Bacillus* sp. INT005 and *B. subtilis* NG220 showing PHA accumulation of 3.09 g/L, 0.166 g/L and 5.201 g/L respectively.[74,88,89]

Optimal PHA production in *Enterobacter* sp. SU16 (1.5 g/L) was observed in mineral medium (pH 8) when incubated at 35 °C for 48 h.[79] Another isolate *W. anomalus* VIT-NN01 produced 19.50 g/L PHA in production medium (pH 8) when incubated at 37 °C and 120 rpm for 96 h. Pandian et al.[22] reported optimum PHA production from *Brevibacterium casei* SRKP2 (2.940 g/L) in mineral media (pH 8) when inoculated with 5% inoculum at 37 °C in 48 h.

Our findings are also in agreement with studies on PHA production in *Methylobacterium extorquens* ATCC 55366,[90] *Alcaligenes latus*[91] and *B. thuringiensis* IAM 12077,[92] that reported an increase in the PHA yield with increasing cell density. A 5% (v/v) inoculum size for maximum biopolymer accumulation has been observed in *Azomonas macrocytogenes* P173, *B. subtilis* MSBN17 and *Pseudomonas* sp. PAMC 28620 showing a yield of 24%, 58% and 1.10 g/L respectively.[8,93,94] Also similar to our results, a 15-16 h old inoculum was used for maximum biopolymer production by *Azohydromonas lata* DSMZ 1123,[95] *Bacillus sphaericus* NCIM 5149 and *Ralstonia eutropha*.[45,96] Maximum PHA accumulation, in *Corynebacterium* sp., has been reported at 100 rpm for PHA accumulation of 1.4 g/L.[38] In another study, *B. flexus* WY2, *B. melitensis* AUH2, *P. aeruginosa* VSS6, *B. subtilis* 6833 and *P. aeruginosa* S164S showed a maximum PHA yield of 32-42% at an agitation rate of 150 rpm.[67] Higher agitation rate of 180 and 200 rpm has been reported for maximum PHA yield in *Microbacterium aurum* TPL18 and *Vibrio nereis*.[38,67] In contrast, PHA production in

Klebsiella sp. NCCP-138 (0.31 g/L) was reported under static conditions.[26] A 96 h incubation period for maximum biopolymer production, similar to our study, was recorded in *Agrobacterium radiobacter* (60%),[97] *Burkholderia cepacia* ATCC 17759 (8.72 g/L),[98] *H. mediterranei* DSM 1411 (70%),[99] *B. subtilis* and *E. coli* (54.1% and 47.16% respectively),[40] *P. phragmitetus* ERC8 (1.36 g/L),[80] *Paracoccus* sp. LL1 (30.89%)[87] and *B. cereus* suaeda B-001 (43.1%).[100]

Effect of Various Carbon and Nitrogen Sources on PHA Accumulation

The carbon and nitrogen sources play a vital role in PHA biosynthesis. The PHA production, in the range of 2.7 to 4.5 g/L, was obtained using carbon sources like maltose, raffinose, glycerol, lactic acid and groundnut oil. However, *B. megaterium* JHA did not metabolize other carbon sources used in this study. Among the diverse nutrient sources analyzed, 2 g% glucose (6.99 g/L), 12.5 mM microcosmic salt (7.46 g/L) and 0.04 g% yeast extract (7.46 g/L) was found to be the best carbon and nitrogen sources respectively for PHA synthesis by *B. megaterium* JHA. The PHA accumulation was found to increase with an increase in the concentration of the above nutrients, until it reached a constant, after which there was a significant decline. The regression analysis also confirmed the above findings (Table 2). The observed results were found to be statistically significant at $p<0.05$, except for the concentration of glucose (Table 2). The negative t value indicates the non-linear relation between (higher than optimum) glucose concentration and PHA yield.

PHA accumulation of 6.53 g/L and 6.18 g/L was also obtained using potassium nitrate and sodium nitrate respectively. Interestingly, the most ideally used inorganic nitrogen source, i.e. ammonium acetate, showed the least PHA production of 0.78 g/L in our study. Among the organic nitrogen sources, the least PHA production was recorded with soya peptone and peptic digest of soymeal. Thus, it can be suggested that these organic compounds contain certain factors that enhance cell growth but affect PHA accumulation. Our results showed that nitrogen limitation plays an important role in PHA accumulation, and higher concentration of nitrogen is diverted towards biomass production.

Glucose, as a carbon source, can be easily assimilated. Hence, under adequate nutrient conditions it is utilised by bacteria for cell growth and maintenance and under nutrient-limited conditions, excess glucose is converted into an intermediate substance like acetyl coenzyme A, via the glycolysis pathway, thus enabling biopolymer synthesis.[14] The nitrogen deficient conditions decreases the NADPH consumption and thus prevents amino acid synthesis, especially of glutamate from α-ketoglutarate. The accumulation of NADPH in the cells, in turn, triggers biopolymer synthesis.[101] Yeast extract is a rich source of amino acids, sugars, vitamin B and minerals that help reduce the lag phase to enhance biomass production. Hence, it allows dynamic biomass production, initially, in the growth medium, and later biopolymer accumulation, under nitrogen deficient conditions.[102]

Our findings, that showed glucose as the most capable substrate, are supported by several previous reports on biopolymer production by *B. megaterium* sp. By using 1 g% of glucose, *B. cereus, B. subtilis, B. megaterium* and *B. thuringiensis* GVP were able to accumulate 0.04 g/L, 0.1 g/L, 0.08 g/L and 0.72 g/L of PHA respectively.[103-105] *B. cereus* suaeda B-001 and *B. cereus* MM7 showed 43.1% and 55% yield of PHA using 1.5% and 3% glucose as carbon substrate respectively.[106,107] A high concentration of 5% glucose was utilized by *B. cereus* PW3A to produce 0.325 g/L biopolymer.[64] The use of microcosmic salt as an inorganic nitrogen source for maximum PHA accumulation has been observed in *Vibrio* sp. 85/6.[38] However, more commonly, studies have reported the use of ammonium sulphate or ammonium chloride for efficient biopolymer production by *Bacillus* sp.[33,45,108-110] Yeast extract, on the other hand, was utilised for optimum biopolymer yield by other *Bacillus* sp.[65,67,111,112]

Medjeber et al.[113] reported producing PHA from *B. megaterium* by using 3% beet molasses as carbon sources and 0.05% ammonium chloride as a nitrogen source to yield 41% PHA in 48 h. In another study, Shenoy et al.[26] reported optimum PHA production in medium containing 2.5% mannitol, 0.5% yeast extract and 1% peptone by *Klebsiella* sp. NCCP-138. Interestingly, a mutant culture of *Bacillus licheniformis* showed optimum production using 3% palm oil mill effluent at 45 °C and pH 7, with no additional trace elements.[114]

The Effect of Replacing an Organic Nitrogen Source with an Inorganic Nitrogen Source on PHA Accumulation

Cost-effectiveness is a critical factor in any production process. In general, organic nitrogen sources are much more expensive compared to inorganic nitrogen sources. Hence, we decided to replace yeast extract with KNO_3 (i.e., the second best inorganic nitrogen source). Interestingly, it was observed that E2 medium containing microcosmic salt and KNO_3 was more efficient, allowing 8.03 g/L PHA accumulation, than the same containing microcosmic salt and yeast extract that showed 7.74 g/L PHA accumulation. Moreover, a steady increase in PHA production was observed with the increase in KNO_3 concentration up to 0.04 g% (8.36 g/L). Thus, a novel production medium for PHA accumulation was formulated and was named as JHA production medium.

Similar to our study, that showed 41.53% PHA yield, an enhanced PHA production using 0.01 g% to 0.5 g% KNO_3

has also been observed in *Vibrio* sp. BTKB33 (0.16 g/L),[115] *A. macrocytogenes* P173 (42%),[93] *B. subtilis* (48.07%),[40] *E. coli* (52.31%)[40] and *Rhodotorula mucilaginosa* KUG15 (34.87%).[116]

Effect of Various Concentrations of K₂HPO₄ and KH₂PO₄ on PHA Accumulation

In the present study, an enhanced PHA yield of about 45% showing 8.51 g/L and 8.57 g/L PHA was observed in the presence of 0.6 g% K_2HPO_4 and 0.4 g% KH_2PO_4 respectively. The observed results were found to be statistically significant at $p < 0.05$ (Table 2). The regression analysis confirmed the linear relation between the increase in concentration of phosphate sources and PHA yield.

Phosphate is an integral part of cell structures like nucleic acids, phospholipids, proteins and coenzymes and also plays a vital role in energy metabolism. Various biochemical processes are also dependent on phosphates. In the production medium, phosphates influence the cell density as well as the PHAs production.[117] It also acts as a buffer.[118] It has been reported that improved PHA production can be observed under conditions of limiting nitrogen, phosphorus and potassium and excess carbon source.[9,119] Similar to our findings, maximal PHA accumulation of 23.11 g/L and 15.74 g/L has been recorded in *B. subtilis* and *E. coli* respectively when a combination of 0.2 g% of KH_2PO_4 and $Na_2HPO_4.2H_2O$ were used in the medium.[40] In another study, accumulation of 2.19 g/L was observed in *Natrinema ajinwuensis* RM-G10 when 0.005 g% KH_2PO_4 was incorporated in the production medium.[120] Mukhopadhyay et al.[121] also reported enhanced PHA yield at suboptimal phosphate concentration (0.06-0.08 g%). However, in contrast to our findings, PHA yield in *P. putida* was enhanced under phosphate limited conditions whereas nitrogen, sulphur and oxygen limitation did not accelerate PHA biosynthesis.[117]

Effect of Carbon Nitrogen and Carbon Phosphate Ratios on PHA Accumulation

In the present study, the increase in C:N ratio increased PHA accumulation, but the cell mass was relatively constant. The optimum PHA yield of 50.13% was observed when 70:1 C:N ratio was incorporated in the medium. This was equivalent to a 17.11% increase in PHA accumulation and 9.14% increase in yield as compared to previously optimized media. The optimum C:P ratio was 20:1 giving a PHA yield of 50.14%. From the obtained data, it can be concluded that more than phosphate limitation, the PHA accumulation in *B. megaterium* JHA is dependent on nitrogen limitation. The statistically insignificant values reported for the above observations at $p<0.05$ (Table 2) was due to the non-linear relation between the increasing values and PHA yield observed in our study.

At low C:N ratio, carbon is converted to acetyl-CoA which is used for cell metabolism and cell growth rather than PHA accumulation. As the C:N ratio increases, depletion of nitrogen source stops the synthesis of protein which then inhibits the enzymes of the TCA cycle. This allows the excessive acetyl-CoA to be utilised for the production of PHA.[122] The increase of PHA under phosphate limited conditions may be due to surplus reducing power and reduced production of ATP.[123] These factors make the process of PHA accumulation unique to the bacterial strain and availability of nutrients.

As observed in our study, *B. cereus* SPV also showed a diverse PHA accumulation under conditions of potassium, nitrogen, sulphur and phosphate limitation. However, the maximum PHA yield of 38% was reported under nitrogen deficient conditions.[119] Also high C:N ratio of 68.9:1 and 100:1 has been reported for biopolymer production by *Azotobacter chroococcum* 6B and *Pseudomonas nitroreducens* AS 1.2343.[122,124] A higher C:N ratio of over 140:1 has been recorded for enhanced PHA production by micro-organisms in activated sludge.[125,126] Similarly, a positive effect of phosphate limitations for biopolymer production has been reported in several studies.[102,127,128]

Effect of Various Concentrations of Trace Elements on PHA Accumulation

Trace elements are metal ions that are required for the normal functioning of the cell in very low concentrations. Our study clearly depicted the failure to produce PHA in the absence as well as excess of trace elements. PHA accumulation of 10.26 g/L was recorded at an optimum concentration of 2 ml in one litre of the production medium. The insignificant p value (for concentration of trace elements, Table 2) was due to the comparatively high variation observed between the peak (obtained at 2 ml volume) and its adjacent values for PHA yield.

Since the accumulation of biopolymers depend on diverse enzymes, that many require trace elements as co-factors, the choice of an appropriate supplement of trace elements is vital for high PHA production. This further adds to the unique ability of individual bacterial strains to produce biopolymers. The presence of metal ions like K^+, Mg^{2+}, Fe^{3+}, Ca^{2+}, Mo^{6+}, Mn^{2+}, Co^{2+} and Zn^{2+} in the medium has been reported to have a positive effect on PHA accumulation.[33,118] Our findings are in accordance with Grothe et al.[33] where high PHA production was observed only at optimum concentration of trace elements. The production of biopolymer like PHB in *Pseudomonas* sp. K, *M. extorquens* and other methylotrophs were also affected by trace elements like $CaCl_2$, $FeSO_4$, $MnSO_4$ and H_3BO_3.[129] Moreover, another study has also reported that the increasing amount of trace element solution negatively affected both biomass and PHB production.[109] Thus, it can be further concluded that, in addition to nitrogen and phosphate limitation, sulphur and trace elements like magnesium, calcium, and iron also influences

the accumulation of PHA granules.

Effect of Various Concentrations of MgSO₄ on PHA Accumulation

The supplementation of 1 mM MgSO₄ in the production medium enabled PHA accumulated of 11.26 g/L. Beyond 1 mM concentration of MgSO₄, a decline in the PHA biosynthesis was observed but the cell growth continued even at a maximum MgSO₄ concentration of 10 mM. The insignificant p value (for concentration of MgSO₄, Table 2) was due to the comparatively high variation observed between the peak (obtained at 1 mM concentration) and its adjacent values for PHA yield.

The Mg^{2+} ions control membrane stability and acts as a cofactor for various enzymes. They also affect membrane fluidity. It has been identified that due to ionic contact of Ca^{2+} and Mg^{2+} ions with phosphonyl groups there is reduction in membrane mobility.[130] This reduced stability has a profound impact on glucose uptake across the membrane ultimately affecting acetyl-CoA necessary for PHA synthesis.[131] The role of magnesium in PHA biosynthesis has been confirmed in *Cupriavidus taiwanensis* 184 where the biomass increased from 7 g/L to 10 g/L.[42] *Acinetobacter nosocomialis* RR20 also showed significant improvement in the PHB production of 4.17 g/L after addition of MgSO₄ in the medium.[132] *Bacillus endophyticus* showed maximum PHA yield of 69.9% in the presence of 2 g/L of MgSO₄ in the production medium.[133] These observations are in accordance with the present study. In contrast, there are reports showing no significant role of MgSO₄ on the cell growth or PHA production in *Bacillus subtiltis, E. coli* and *R. sphaeroides* N20.[40,118, 134]

Comparative Studies of Original and Optimized Media

On the basis of the results obtained by the 'one factor at a time' approach, Table 3 enlists the parameters considered and the optimized values to obtain maximum PHA accumulation in *B. megaterium* JHA.

While comparing the original E2 and the optimized JHA

medium, a 66.88% increase in PHA biosynthesis was observed. The PHA accumulated in the original E2 media after 96 h incubation was 4.54 g/L and 23.5% PHA yield. After optimization, the JHA medium accumulated 13.71 g/L of PHA that accounts to 54.51% yield (Figure 3). Moreover, there was a noticeable increase in the biomass of *B. megaterium* JHA. Thus, *B. megaterium* JHA can be considered as an ideal organism for PHA production on a large scale.

Among other *B. megaterium* strains, maximal biopolymer production of 40% and 48% is reported by Macrae and Wilkinson,[135] and Shahid et al,[136] respectively. *B. megaterium* OU303A, uyuni S29 and 12 showed 62.43%, 70% and 77% PHA yield respectively.[27,137,138] A comparatively low PHA yield of 29.7% was observed in *B. megaterium*.[139] A considerable PHA yield has also been reported in other micro-organisms like *B. cereus* NRRL-B-3711 (14.2 g/L),[39] *B. flexus* WY2 (7.93 g/L),[114] *Bacillus licheniformis* M2-12 (19.55 g/L),[94] *B. subtilis* MSBN17 (19.08 g/L),[140] *B. melitensis* AUH2 (7.84 g/L)[39] and *Burkholderia cepacia* ATCC 17759 (8.72 g/L).[67]

PHA yield of greater than or equal to 60% has been considered as potential strains for the industrial production of the polymer. PHA yield of greater than equal to 60% has been reported in various *Bacillus* sp. such as *B. cereus*, *B. megaterium* uyuni S29, *B. subtilis* and *B. thuringiensis* IAM12077.[53,112,141-144]

Scale up Studies

In order to further improve the PHA accumulation by *B. megaterium* JHA, the optimized parameters were implemented in the batch fermentation process using a 2 L fermenter. This resulted in an additional 4.39% (14.34 g/L) increase in PHA accumulation using glucose (21 g%) in 96 h. This increase, however, was not very significant as expected in the lab scale fermentation. Thus, in order to enhance PHA yield, standardization of various fermentation parameters is necessary. The type of operation strategy like carbon source, fermentation mode and type of bioreactor also affects the PHA accumulation

Table 3. Optimized values of various parameters for maximum PHA accumulation

Factors	Original E2 medium	Optimized JHA medium
pH	7.0	8.0
Temperature	28 °C	28 °C
Aeration	120 rpm	120 rpm
Time of incubation		96h
Culture OD$_{540 nm}$	0.5	1.0
Inoculum size	2.5% (v/v)	5% (v/v)
Carbon source	Glucose	Glucose
Inorganic nitrogen sources	Microcosmic salt and KNO₃	Microcosmic salt and KNO₃
Carbon: Nitrogen		70:1
Carbon: Phosphate		20:1
Concentration of Glucose	2g%	21 g%
Concentration of Microcosmic salt	3.5 g/L	12.5 mM
Concentration of KNO₃		0.4 g/L
Concentration of K₂HPO₄	7.5 g/L	6 g/L
Concentration of KH₂PO₄	3.7 g/L	4 g/L
Volume of 100 mM MgSO₄.7H₂O	10 ml	10 ml
Volume of Trace elements	1 ml	2 ml
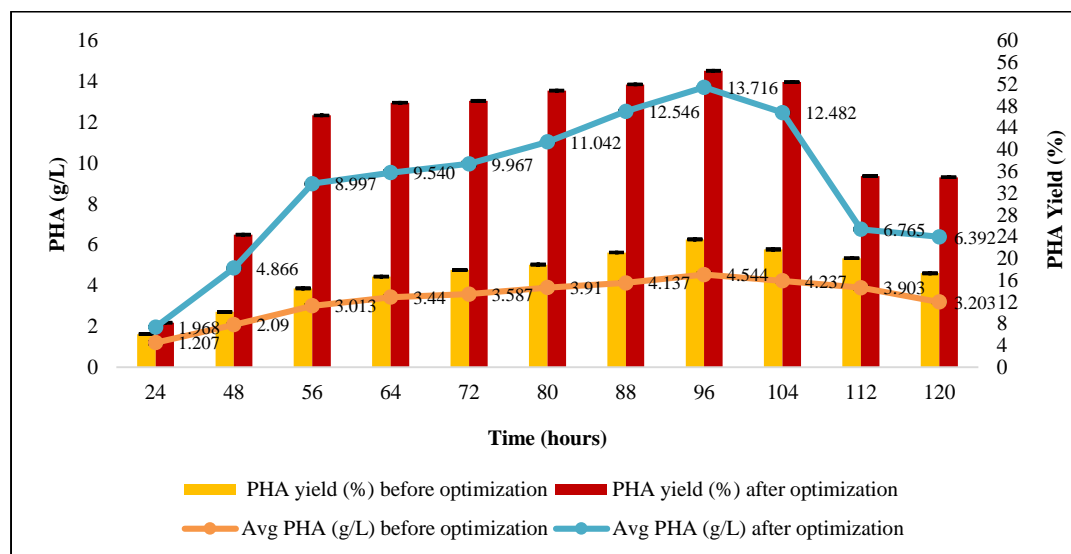
Figure 3. Comparison of the PHA Accumulated Using Original and Optimized Media.

in bacterial cell.[145,146] PHA biosynthesis using the batch fermentation is a flexible process and is less expensive. The only disadvantage of this method is that once the carbon feedstock is utilized, the bacterial cells begin to degrade the accumulated PHA, thus reducing PHA yield.[14] Hence, this can be overcome by using fed-batch fermentation method, where one nutrient is limited whereas the carbon feedstock is supplied continuously to the bioreactor. Verlinden et al,[147] proposed a combination of batch and feed-batch method of fermentation and these techniques are commonly used for the commercial production of PHA.

Our results are in corroboration with the results observed by Valappil et al.[53] They reported a drop in pH of the fermentation medium of *B. cereus* SPV with time that negatively affected the PHB production. They obtained a PHB yield of 29% which was similar to the values obtained by the shake flask method. Similarly, while studying polymer production by *B. cereus* SPV Akaraonye et al,[148] observed maximum PHA accumulation of 6.63 g/L by shake flask method using molasses as carbon feedstock. When molasses were used as a carbon source in batch fermentation, a maximum PHA yield of 51% and 54.68% was reported in *Bacillus* sp. COLI/A6 and *B. cereus* SPV respectively.[55,148]

Conclusion

The bacterial strain, *B. megaterium* JHA, used in our study was studied for PHA production. Generally, biopolymer biosynthesis is a complex process and various factors are involved in its increased accumulation. In the current study, we optimized all the common nutritional as well as physico-chemical parameters for increased PHA production. It was clearly observed that optimizing every parameter had a positive effect on PHA accumulation. Moreover, not only the basic nutrient sources but also the trace elements and micronutrients are equally essential for PHA accumulation

inside the bacterial cell. As compared to similar published studies, the potential of *B. megaterium* JHA to produce 13.71 g/L of PHA in JHA medium is encouraging. Although a slight increase was noted after applying the optimized parameters in the fermentation process, it can be suggested that the bacterial strain is practically stable under circumstances of minor fluctuation in the growth conditions that may have occurred in the fermentor. This observation is further encouraging since previously reported studies have indicated over 30% increase in biopolymer accumulation under fermentation process. Considering the PHA yield of 54.51%, and the fact that over 66% increase was observed after optimization studies in the current study, it can be indicated that *B. megaterium* JHA can be exploited for industrial production of PHA.

Authors' Contributions

Both the authors made equal contribution to the study design, analysis and literature review. Author JM wrote the first draft of the manuscript and carried out statistical analysis. Authors JM and KA made appropriate changes to finalise the manuscript.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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