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Production and Characterization of Polyhydroxyalkanoate from Lignin Derivatives by *Pandoraea* sp. ISTKB

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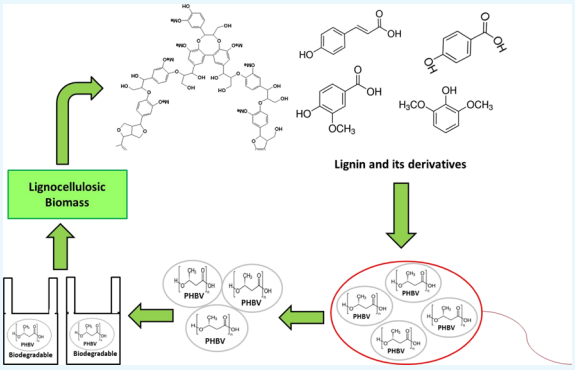
Supporting Information

ABSTRACT: The present study investigates polyhydroxyalkanoate (PHA) production from lignin and its derivatives by a previously reported lignin-degrading bacterial strain *Pandoraea* sp. ISTKB. PHA production was screened by fluorescence microscopy and flow cytometry using a Nile red stain. PHA and biomass accumulation, while screening, was found to be maximum on 4-hydroxybenzoic acid followed by *p*-coumaric acid, vanillic acid, 2,6-dimethoxyphenol, and kraft lignin after 96 h. Monomer composition was analyzed by gas chromatography−mass spectrometry (GC−MS) and was followed by Fourier transform infrared and ^{1}H NMR analysis, indicating PHA to be a copolymer of P(hydroxybutyrate-*co*-hydroxyvalerate). Genomic analysis of *Pandoraea* sp. ISTKB also complemented the results of GC−MS and NMR, and the relevant genes responsible for the synthesis of small chain length PHA were discovered in the genome. Process parameters were optimized by response surface methodology for enhanced production of PHA and biomass on 4-hydroxybenzoate. Optimization results showed 30 and 66% increase in the biomass and PHA production, respectively. The results obtained were promising and indicated that if lignin is depolymerized into low-molecular-weight intermediates, then it can easily be utilized and converted into value-added products like PHA by microbes.



INTRODUCTION

Polymers derived from petroleum sources are nonbiodegradable, and as a result, these are accumulated in the environment as landfills or in water bodies. Their dumping leads to health hazards and further intensifies the problem of their disposal. Finite petroleum resources, climate change, and concern toward environment compelled us to search renewable alternatives for fuel and chemicals. Polyhydroxyalkanoate (PHA) can be a potential replacement of petroleum-based plastics because of its biodegradability and biobased, carbon neutral, nontoxic, and environment friendly nature. It is estimated that a complete substitution of a petroleum-based polymer with PHA can possibly reduce greenhouse gas emission and fossil energy use by 200 and 95%, respectively.[1,2]

PHA is a group of biopolyesters having more than 150 different monomers synthesized as granules inside cells by various microorganisms. Microbes accumulate PHA as carbon and energy reserve material under stress and nutrient imbalance conditions, that is, in the presence of the excess carbon source followed by limiting nitrogen, phosphorous, or oxygen in the medium.[3−5] They are linear polymers of hydroxyl fatty acid monomers. The PHA synthesis occurs by the concerted action of genes such as 3-ketothiolase, acetoacetyl-CoA reductase, and PHA polymerase or by diverting fatty acid metabolites into PHA synthesis pathways with the help of several enzymes. The PHA metabolism is carried out by the action of numerous enzymes, and the genes responsible for PHA metabolism are tightly regulated.[4,6]

The physical and mechanical properties of PHA are comparable to polyalkenes-based plastic, and their properties can be controlled by choice of microbial strain, carbon source, and fermentation conditions. On the basis of the number of carbon atoms present in the monomer, PHA is classified as short chain length (Scl, C3−C5) and medium chain length (Mcl, C6−C14).[5] Bacteria can also produce a mixed copolymer of Scl or Mcl or Scl−Mcl by feeding on a mixed or complex substrate. Production of copolymers provides scope for further improvement in the properties of PHA. PHA has got several applications in various sectors, such as packaging, plastic processing, cosmetics, food and agriculture, medical, and biomaterials, as a precursor for fuel and chemicals.[4,6,7]

PHA production at an industrial scale grew rapidly after years of research with limited success, despite its market share being still very low. The main reasons are high production cost, high price, and policy framework. Raw materials used for production constitute about 50% of the total production cost, and the carbon source constitutes 70−80% of the total raw material's cost.[8] Utilization of lignocellulosic biomass as renewable raw

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materials is the main focus nowadays because of their abundance, low cost, and easy availability.[9] Lignocellulosic biomass-based biorefineries utilize cellulose and hemicellulose while lignin is discarded as waste. Lignin is recalcitrant to degradation with very limited usage, that is, combustion in the paper industry and precursor generation for vanillin and dimethyl sulphoxide synthesis.[10−13] Recently, a smart composite material for packaging was produced from lignin and PHA having viscoelastic and gas barrier properties.[14] Closing the loop by utilizing the waste generated from biorefinery and industry will validate the model of circular economy. Intense research is needed to develop new processes for conversion of lignin into value-added products for sustainable biorefinery.[11] Fungi and bacteria can degrade lignin in the natural environment. They secrete various enzymes such as oxidases and peroxidases to degrade lignin into various aromatic intermediates.[15] Aromatic intermediates were further degraded and utilized by bacteria through peripheral and central pathways with the concerted action of various enzymes for growth.[10,16]

The present study investigates screening, characterization, and optimization of PHA production by a previously reported lignin-degrading bacterium *Pandoraea* sp. ISTKB while growing on lignin and its derivatives. Genomic analysis was performed to identify the relevant genes responsible for PHA metabolism and substrate utilization.

RESULTS AND DISCUSSION

Screening for PHA Accumulation on Lignin and Its Derivatives. A lignin-degrading bacterial strain *Pandoraea* sp. ISTKB was screened for PHA accumulation. This strain has been studied earlier for lignin degradation and sugarcane bagasse pretreatment.[12,17] The genome sequence has also been reported, and analysis indicated the presence of genes responsible for degradation of lignin and its derivatives.[16] For PHA production, this strain was cultured aerobically on kraft lignin and various lignin derivatives such as 4-hydroxybenzoic acid (4-HBA), *p*-coumaric acid, vanillic acid, 2,6-dimethoxyphenol, or syringol as carbon sources followed by incubation at 30 °C, pH 8, and 185 rpm, under nitrogen limitation for six days. Microbes tend to accumulate PHA inside the cell as carbon and energy reserve under nutrient imbalance conditions. Bacterial growth, dry biomass, and PHA content were monitored at regular intervals for all substrates under investigation.

Fluorescence microscopy was performed to establish the PHA accumulation property by staining the cells with Nile red.[18] The cells harvested after 96 h were stained with Nile red and visualized under a confocal microscope. The cells displayed strong orange red fluorescence indicating accumulation of lipid or PHA granules inside cells as shown in Figure S1. Transmission electron microscopy (TEM) analysis was performed to further confirm granule accumulation inside cells. The TEM image visibly showed the PHA granules accumulated inside the *Pandoraea* sp. ISTKB as depicted in Figure S2. This strain was cultured under nitrogen-free, nitrogen-limited, and nitrogen-excess media, and results indicated that higher PHA was accumulated under the nitrogen-limited condition.

The biomass and PHA accumulation increased with time from 0 to 96 h and was found to be 4-HBA (523 mg/L; 246 mg/L), *p*-coumaric acid (417 mg/L; 170 mg/L), vanillic acid (215 mg/L; 72 mg/L), 2,6-dimethoxyphenol (200 mg/L; 69 mg/L), and least by kraft lignin (86 mg/L; 18 mg/L) as shown in Figures 1 and 2. The percent accumulation of PHA observed

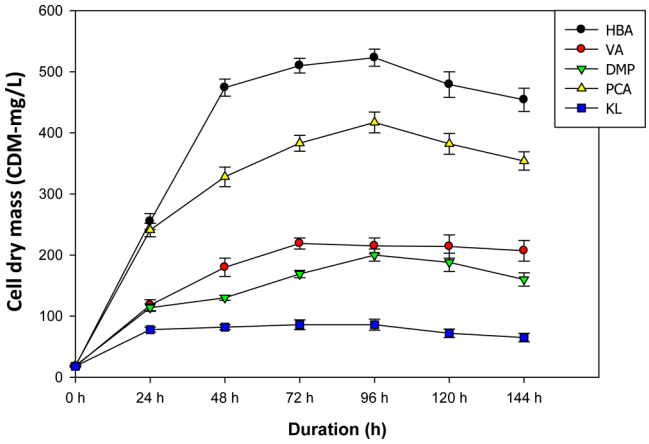

Figure 1. Growth profile and quantification of biomass accumulation measured after 24 h for six days on different carbon sources 4-HBA, vanillic acid (VA), *p*-coumaric acid (PCA), 2,6-dimethoxyphenol (DMP), and KL by *Pandoraea* sp. ISTKB. The experiment was performed in triplicate.

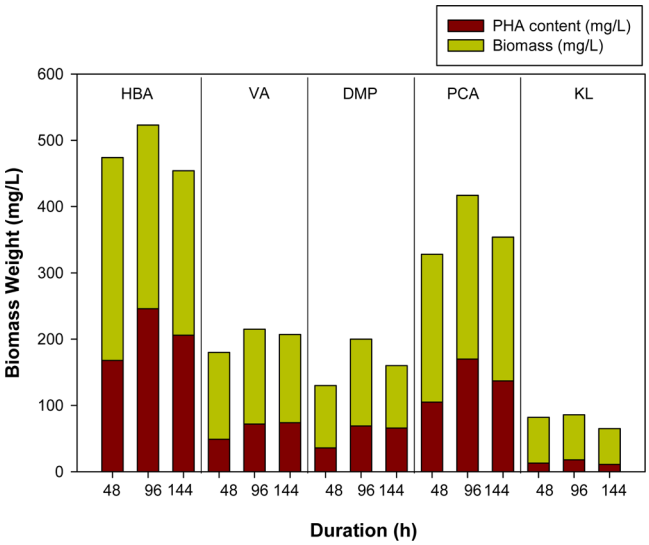

Figure 2. Biomass and PHA accumulation by *Pandoraea* sp. ISTKB grown on different carbon sources 4-HBA, vanillic acid (VA), *p*-coumaric acid (PCA), 2,6-dimethoxyphenol (DMP), and KL estimated after 48, 96, and 144 h. The experiment was done in triplicate.

was 47, 33, 35, 41, and 21% of cell dry mass for 4-HBA, vanillic acid, 2,6-dimethoxyphenol, *p*-coumaric acid, and kraft lignin, respectively. There was a slight decrease in the biomass and PHA content measured on 120 h (Figure 2). A decrease in fluorescence was observed at 120 h which may be due to the metabolic recycling of PHA as a carbon and energy source. The *p*-coumaric acid degradation pathway leads to formation of intermediate 4-HBA, and it can be inferred from the results that biomass as well as PHA accumulation decreases in the case of *p*-coumaric acid because of complexity in the structure. Thus, lignin needs to be depolymerized before it can be utilized efficiently by microbes.

4-HBA was found to be the preferred substrate for biomass and PHA production by *Pandoraea* sp. ISTKB. The biomass

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and PHA production profile obtained by gravimetric analysis was further confirmed by flow cytometry after staining cells with Nile red cultured on 4-HBA for six days. The mean fluorescence intensity was found to be in accordance with the PHA production profile, and maximum fluorescence was detected in the 96 h sample as depicted in Figure 3a,b. Flow cytometry analysis indicated that the accumulation of PHA in *Pandoraea* sp. ISTKB started from the beginning of growth and strongly followed the growth pattern.

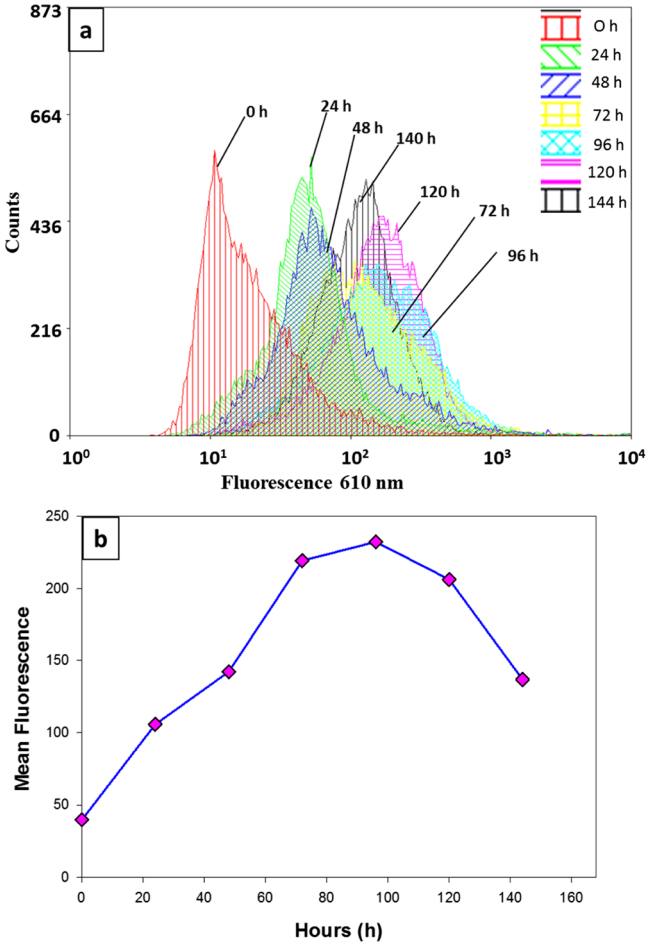

Figure 3. (a) Time course analysis of Nile red-stained cells of *Pandoraea* sp. ISTKB. The cell count was plotted with respect to fluorescence intensity grown on 4-HBA as a substrate. (b) The graph shows mean fluorescence intensity with respect to time.

Production of PHA from genus *Pandoraea* has not been explored, and there is only a single report available on PHA production from crude glycerol by this organism. There are very limited reports available on PHA production from lignin and its derivatives. PHA accumulation reported by *Oceanimonas doudoroffii* was less than 3% on all lignin and its derivatives tested; however, *p*-coumaric acid and vanillate significantly inhibit the cell growth.[13] *Ralstonia eutropha* H16 was grown on 18 lignin derivatives, and among these, only 3-HBA, 4-HBA, 2,5-dihydroxybenzoic acid, and 3,4-dihydroxybenzoic acid showed poly-3-hydroxybutyrate (P3HB) production. The growth and PHA accumulation was not observed on vanillate and *p*-coumaric acid.[15] Compared to the above-reported strains, *Pandoraea* sp. ISTKB can utilize *p*-coumaric acid, vanillic acid, and other derivatives with a greater ease. A study reported a PHA content of 34 and 32% on *p*-coumaric acid and alkaline-pretreated liquor (APL), respectively, by *Pseudomonas putida* KT2440, which is less compared to the present study.[10]

Salvachúa et al.[19] reported that screening of PHA accumulation using fluorescent microscopy and flow cytometry from APL by *P. putida* KT2440, *P. putida* mt-2, *Cupriavidus necator*, *Ralstonia jostii*, and *Amycolatopsis* sp. was found to be 52, 60, 168, 288, and 13 mg/L, respectively. Among the tested strains mentioned above, only *C. necator* and *R. jostii* showed higher PHA accumulation than *Pandoraea* sp. ISTKB on kraft lignin. Also, APL is a favorable substrate for growth compared to kraft lignin. Production of PHA from kraft lignin has been recently reported from *Cupriavidus basilensis* B-8 and was found to be 15−18.5%.[20] Production of biomass by *C. basilensis* B-8 was observed to be higher on kraft lignin (KL), but the PHA accumulation percentage was lower compared to *Pandoraea* sp. ISTKB; this is possibly due to the higher initial inoculum size. This is the first report available so far of PHA production from lignin and its derivatives by the genus *Pandoraea*. These findings clearly indicated that if lignin can be converted to low-molecular-weight intermediates (4-HBA, vanillate, and *p*-coumaric acid), then bacteria can efficiently synthesize PHA by utilizing these derivatives.

Confirmatory Analysis of PHA Production. Fourier transform infrared (FTIR) spectroscopy was performed to identify the functional groups present in the PHA polymer extracted from *Pandoraea* sp. ISTKB. The FTIR spectrum was scanned in the range of 4000−400 cm^{-1}, and the graph of the polymer is represented in Figure S3. Characteristic bands of the PHA polymer were observed in the FTIR spectrum. The band around 3436 cm^{-1} (O−H stretching vibration) indicated the presence of the hydroxyl group in the polymer.[12] Spectra observed around 2970 cm^{-1} denotes asymmetric methyl stretching, and this CH_3 stretching can form interaction with the carbonyl (C═O) group and union of C−H−O.[5] The band at 2921 cm^{-1} signifies the asymmetric methylene (CH_2) group and is responsible for lateral chain formation of monomeric units. The intense band observed around 1721 cm^{-1} is the marker band of the polymer, and it represents carbonyl (C═O) stretching vibration of ester bonds. The presence of the amide group [amide I (−CO−N−) carbonyl stretching vibration and amide II (N−H) bending vibration] near 1650 and 1462 cm^{-1} was observed.[21] These signals would have appeared because of interference while extraction by bacterial intracellular components and also from protein-coated PHA granules. The signal of terminal CH_3 present in the polymer can be traced at 1380 cm^{-1}. Various prominent signals observed between 1400 and 600 cm^{-1} were assigned to C−O−C, C−O, C−H, and C−C vibrations.[5,21] Hence, it can be inferred from the functional group analysis that the extracted polymer is PHA.

Gas chromatography−mass spectrometry (GC−MS) analysis was performed to characterize the monomeric composition present in the extracted polymer accumulated by *Pandoraea* sp. ISTKB. The presence of 4 and 5 carbon monomers, that is, 3-hydroxybutanoate (3HB) and 4-hydroxyvalerate (4HV), was observed. The total ion chromatogram (TIC) and the electron ionization mass spectrum are depicted in Figure S4. Peaks obtained at retention time (10.205 and 13.207) and 16.283 were identified as 3HB and 4HV, respectively, and the percent area acquired by these peaks in TIC is 36.38 and 6.85%, respectively. The major monomer constituent of the PHA polymer is 3HB followed by 4HV. The characteristic ionic mass

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fragmentation pattern of 3HB and 4HV, that is, m/z 87, 59, 117, 115, 101 and 131 were detected.[3,22] The analysis of the fragmentation pattern indicated the presence of 3HB and 4HV components in PHA.

The monomeric composition of extracted and purified polymers was further analyzed by proton nuclear magnetic resonance (^{1}H NMR). The spectrum of ^{1}H NMR of the PHA copolymer (HB-*co*-HV) has been depicted in Figure 4. The

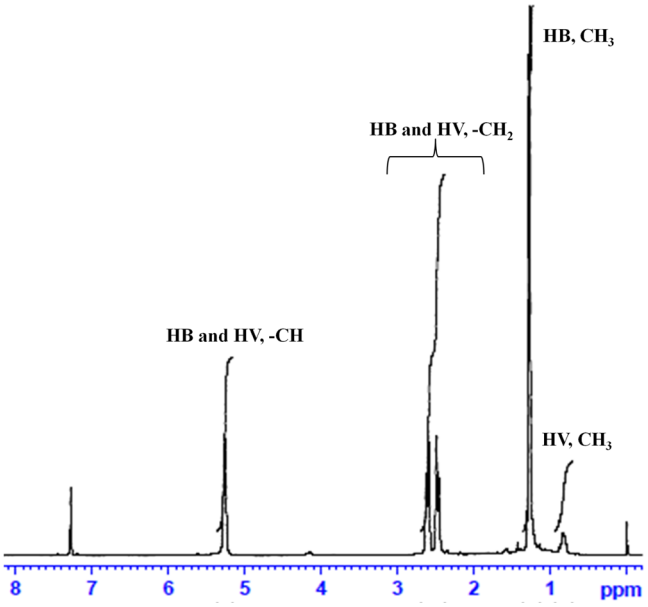

Figure 4. ^{1}H NMR spectra of PHA produced and extracted from *Pandoraea* sp. ISTKB cells grown on 4-HBA.

resonance signal indicated the presence of different types of protons surrounding the carbon atom. The resonance signals around 1.25−1.43 and 0.83−0.89 ppm designates the presence of methyl group proton (CH_3) in HB and HV. The existence of methylene proton (CH_2) in PHA monomers gives multiple resonances around 1.58, 1.68, and 2.17−2.64 ppm. Multiple resonances around 5.24−5.28 ppm showed the presence of the methane proton in PHA and PHV.[23−25] The signals obtained are similar to the earlier report of pure P(HB-*co*-HV) polymers.[24,25] The results indicate synthesis of the heteropolymer by *Pandoraea* sp. ISTKB. Production of a similar copolymer P(3HB-*co*-3HV) from crude glycerol plus propionic acid by *Pandoraea* sp. MA03 has been reported recently.[3] Production of the 4HV monomer from *C. necator* ATCC 17699 on levulinic acid and *Cupriavidus* sp. USMAA2-4 on γ-valerolactone has been reported earlier.[26,27]

Poly(3-hydroxybutyrate) is the most common and extensively studied polymer having properties similar to petroleum-based plastics, but its high crystallinity and less flexibility limit its application range. These undesired properties can be improved by incorporation of co-monomers with PHB such as HV, 4-hydroxybutanoate (4HB), or hydroxyhexanoate (HHx) to form copolymers. Synthesis of copolymer P(HB-*co*-HV) by this strain will take care of limitations and provide stiffness with flexibility to PHA. PHA synthesized from lignin and its derivatives by different strains was reported recently. PHA synthesized from 4-HBA by *O. doudoroffii* was a copolymer of P(HB-*co*-HV), and a similar polymer was obtained from *Pandoraea* sp. ISTKB on 4-HBA.[13] The PHA synthesized by *R. eutropha* H16 on 4-HBA was the homopolymer P3HB.[15] The PHA monomer composition obtained on kraft lignin by *C. basilensis* B-8 was the heteropolymer of C4 carbon, that is, R3HB, 3HB, and S3HB, but *Pandoraea* sp. ISTKB produced C4 and C5, that is, R3HB, 3HB, and 4HV.[20] Analysis of PHA by GC−MS, FTIR, and ^{1}H NMR indicated the presence of the copolymer P(HB-*co*-HV).

Genomic Analysis of PHA Metabolism and Substrate Utilization. We have earlier reported the genome sequence of *Pandoraea* sp. ISTKB (accession number: MAOS00000000.1), and the important genes related to lignin or aromatic compound degradation as well as stress, and the detoxification mechanism has been highlighted.[16] However, the genes responsible for PHA synthesis have not been reported earlier. After screening and characterization for PHA production, we further analyzed the genome of this strain for the identification of candidate genes responsible for PHA synthesis. The various genes responsible for PHA synthesis were identified in the genome, and the predicted genes have been shown in Table S1. The PHA synthesis genes identified in the genome are responsible for short chain length PHA production (C3−C5). The absence of enzyme (*R*)-3-hydroxyacylACP: CoA transacylase (PhaG) in *Pandoraea* sp. ISTKB genome limits the synthesis of medium or long chain PHA.[28] There are several copies of important genes discovered in the genome related to the Scl PHA metabolism such as 8 of 3-ketothiolase (acyltransferase), 5 acetyl-CoA acetyltransferase, 12 acetoacetyl-CoA reductase (dehydrogenase), 3 PHA synthases, 15 enoyl CoA hydratase, and one copy each of phasin (PHA-granule-associated protein), PHA synthesis repressor PhaR, and PHA depolymerase. Other relevant genes related to fatty acid biosynthesis, degradation, and fatty acid transporters are also annotated in the genome. 3-Ketothiolase performs the first reaction by condensation of two acetyl-CoA molecules or acetyl CoA and propionyl CoA to form intermediates that undergo reduction into 3-hydroxybutyryl-CoA or 3-ketovaleryl-CoA by the action of acetoacetyl-CoA reductase followed by their polymerization into the P(HB) or P(HB-*co*-HV) polymer by PHA synthases.[26] The 3-hydroxyalkanoate synthetase (locus tag A9762_23655) belongs to class III, PHA polymerase (A9762_13630) and PHB polymerase (A9762_18785) to class I. The PHA polymerase and PHB polymerase showed 40 and 32% identity with poly(3-hydroxyalkanoic acid) synthase (class II) of *Pseudomonas aeruginosa* PAO1, which needs to be further investigated. The genomic analysis of PHA metabolism genes further validated the results of GC−MS and NMR. The presence of two PHA synthases has also been reported in the genome of beta proteobacterium *R. eutropha* H16 and *Cupriavidus baseliensis* B-8, but one additional PHA polymerase was observed in the genome of *Pandoraea* sp. ISTKB that needs to be studied further.[20,29] There is only one report available on PHA production by *Pandoraea* genus from glycerol plus propionic acid, but the genes responsible for PHA production was not studied.[3] We report for the first time about the important genes responsible for PHA production in genus *Pandoraea*.

Because we observed the growth and PHA production maximum on 4-HBA, we looked into the genome for the identification of candidate genes responsible for 4-HBA metabolism. We discovered six 4-HBA transporters and one 4-hydroxybenzoate monooxygenase gene that would have directly facilitated in utilization of 4-HBA by *Pandoraea* sp. ISTKB. The microorganisms that belong to the Burkholderiaceae family are known to be metabolically versatile. The

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genomic and functional analysis of *Burkholderia xenovorans* LB400 while growing on 4-HBA showed that 4-HBA can be funneled through both protocatechuate and gentisate central pathways.[30] This bacterium contains both protocatechuate and gentisate central pathways and also belongs to the Burkholderiaceae family that further supports this strain's potential for efficient utilization of 4-HBA, but this needs to be functionally validated in *Pandoraea* sp. ISTKB.

Process Optimization by Response Surface Methodology. In the screening experiments, lignin and its derivatives were tested as a carbon source for biomass and PHA production. It was found that 4-HBA, a degradation product of lignin, was most suitable, and hence it was selected for further experiments. To maximize the biomass and PHA production, various process parameters (carbon content, pH, and duration) were optimized using Box−Behnken design (BBD). Other process parameters such as nitrogen content, rpm, inoculums size, and temperature were kept constant throughout the experiment. The result of 17 experiments designed according to the BBD for maximizing biomass and PHA production is given in Table 1.

Table 1. Set of 17 Experiments with 3 Factors and 2 Responses Suggested by the BBD for Optimization

	variable factors			responses	
	factor 1	factor 2	factor 3	response 1	response 2
run	A: pH	B: 4-HBA (g/L)	C: duration (h)	biomass (mg/L)	PHA (mg/L)
1	8	5	144	610	301
2	8	3	96	642	391
3	6	5	96	435	190
4	10	1	96	243	120
5	6	3	48	264	100
6	10	3	144	440	195
7	10	3	48	250	98
8	8	3	96	634	383
9	6	1	96	273	150
10	8	3	96	627	380
11	8	1	48	306	104
12	8	3	96	639	385
13	8	3	96	650	395
14	8	1	144	410	156
15	6	3	144	425	221
16	10	5	96	401	232
17	8	5	48	399	131

Statistical Analysis and Interactive Effects of Factors on Responses. The data of the 17 experiments were analyzed by analysis of variance (ANOVA), and results are given in Table S2 for biomass and PHA. The *F* value of the model is the ratio of the mean square value and residual mean square values of model coefficients. The significance of the model and its coefficients as well as mutual interactions was determined on the basis of *p* values. The smaller the *p* value, the more significant is the corresponding regression. *p* value less than 0.05 indicates that the model term is significant at the 95% confidence limit.

The model for the prediction of biomass has lack of fit (*F* value) 273.59 and *p* value < 0.0001 (Table S2). Thus, the model is statistically significant and lack of fit is insignificant. There is only a 12.86% chance that an *F* value (3.50) this large could occur because of noise. The *p* values given in Table S2 show that B (4-HBA), C (duration), A^2 (pH × pH), B^2 (4-HBA × 4-HBA), C^2 (duration × duration), and BC (4-HBA × duration) model terms are significant. 4-HBA content ($p < 0.0001$) and the duration of the experiment ($p < 0.0001$) strongly influenced biomass production. The multiple correlation coefficient, r^2 (also known as the coefficient of determination), is 0.99. This implies that the model can explain 99% variation in response. Thus, this model can be used to navigate the design space.

The results given in Table S2 indicated that the model is statistically significant for PHA also with *F* value 416 and *p* value < 0.0001. Lack of fit ($F = 2.26$; 22.38%) for the model is insignificant. The *p* values given in Table S2 show that B (4-HBA), C (duration), AB (pH × 4-HBA), BC (4-HBA × duration), A^2 (pH × pH), B^2 (4-HBA × 4-HBA), and C^2 (duration × duration) model terms are significant for response 2, that is, PHA content. 4-HBA content ($p < 0.0001$) and the duration of the experiment ($p < 0.0001$) strongly influenced PHA production. The multiple correlation coefficient, r^2, is 0.99; therefore, this model can be used for predicting optimum values for 4-HBA content, pH, and duration. For both biomass and PHA, the amount of 4-HBA and the duration of the experiment were more important than pH of the culture media. 4-HBA content and the duration were the most important interaction for both biomass and PHA. Correlation analysis of biomass and PHA content obtained after prediction experiments are strongly and positively correlated ($r^2 = +0.95$).

Confirmatory Experiment. The conditions suggested by the model are pH (7.99), 4-HBA concentration (3.6 g/L), and duration (111.44 h). The optimum conditions for the production of biomass and PHA are shown in Figure S5. The expected yield for biomass is 671 mg/L and for PHA is 399 mg/L. Confirmatory experiments were done in triplicate. The biomass production was 682 ± 17 mg/L, and PHA content was 409 ± 12 mg/L. The maximum percent accumulation was 60 ± 2%. The results of confirmatory experiments are in good agreement with the values predicted by the model. Variables parameters and responses before and after optimization have been shown in Table 2. After optimization, bacterial biomass increased by 30% and PHA content by 66% as compared with screening experiments. In screening experiments, maximum biomass (523 mg/L) and PHA (246 mg/L) were produced at 96 h using 4-HBA as a substrate. The maximum percent accumulation during screening was 47% which increased to 60% after optimization. The increase in PHA content was mainly due to the increase in biomass as well as increased percent accumulation. Few studies have used bacterial strains for PHA production using lignin or its derivatives.[15] Biomass

Table 2. Value of Biomass and PHA Content after Optimization as Compared with Screening Experiments

variable factors and responses		screening conditions	after optimization	(%) increase
		Variable Factors		
factor 1	pH	8	7.9	almost same
factor 2	4-HBA (g/L)	2	3.6	80
factor 3	duration (h)	96	111.4	16
		Responses		
response 1	biomass (mg/L)	523	682 ± 17	30
response 2	PHA (mg/L)	246	409 ± 12	66

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production in the case of *R. eutropha* H16 was similar to that in our study with PHA accumulation of 63 ± 2%. Biomass production and PHA production were very low in the case of *P. putida* JCM 13063 and *P. putida* Gpo1.[15] Another study compared PHA accumulation by two-step cultivation of *O. doudoroffii* with lignin and its derivatives as a sole carbon source. In this study, the biomass production on 4-HBA was comparable (620 mg/L), but PHA accumulation was very low 0.8%.[13] Optimization of process parameters to maximize PHA content has yielded varied results with the increase in PHA content as low as 11%[31] to as high as 100%.[32] In this study, optimization of the process increased PHA content by 66%, thereby making the process more robust and economically feasible.

The findings highlight the application of *Pandoraea* sp. ISTKB in conversion of lignin and its derivatives such as 4-HBA into a value-added product, that is, PHA. 4-HBA-rich wastewater stream from industries (that depends on 4-HBA as a substrate for production of antiseptics, polymers, preservatives, and pesticides) can be utilized for the PHA production using this strain. The PHA production result obtained after optimization under flask conditions is promising. Taking clue from the initial flask-level optimization results, the PHA production can be further improved in a bioreactor where each and every parameter can be monitored in real time and the limiting factors can be identified and further optimized.

MATERIALS AND METHODS

All chemicals and reagents used in the present study were of analytical grade procured from Sigma-Aldrich (St. Louis, MO, USA), Merck (Darmstadt, Germany), or HiMedia unless stated otherwise.

Microorganism and Culture Condition. The earlier described lignin-degrading strain *Pandoraea* sp. ISTKB (accession no. KM609459) was examined for PHA production under shake flask conditions.[12] The composition of mineral medium (MM) for PHA production contains the following (g/L): 6.78Na_2HPO_4; 3KH_2PO_4; 0.5NaCl; 2 mL/L 1 M $MgSO_4$; 0.13$(NH_4)_2SO_4$; 0.1 mL/L 1 M $CaCl_2$; 1 mL/L trace metal solution consisting of (g/L): 0.15$FeSO_4{\cdot}7H_2O$; 0.1$ZnSO_4$; 0.3H_3BO_3; 0.006$CuSO_4$; 0.020$NiCl_2{\cdot}6H_2O$; 0.030$Na_2MoO_4{\cdot}2H_2O$; and 0.25$MnCl_2{\cdot}2H_2O$. A seed culture of 250 mL was prepared by inoculating a single colony of *Pandoraea* sp. ISTKB in a lysogeny broth (LB) and cultured overnight at 30 °C. The LB culture was centrifuged, washed once with phosphate-buffered saline (PBS), and then used to inoculate the 500 mL flask containing 150 mL MM (nitrogen limiting) supplemented with different carbon sources in triplicates, that is, 4-HBA (2 g/L), *p*-coumaric acid (2 g/L), kraft lignin (2 g/L), vanillic acid (2 g/L), and 2,6-dimethoxyphenol or syringol (2 g/L). The flasks were inoculated having a final optical density of ∼0.06 at 600 nm measured using a spectrophotometer (Cary 100 Bio UV−visible). Cultures were incubated at 30 °C and 185 rpm for 6 days. Appropriate volumes of samples were drawn periodically for further analysis.

Screening for PHA Accumulation. PHA accumulation in *Pandoraea* sp. ISTKB was visualized by staining the cells with Nile red using confocal microscopy. An appropriate volume of culture was harvested postinoculation at different time intervals (i.e., 0, 24, 48, 72, 96, 120, and 144 h), and cells were pelleted by centrifugation at 10 000 rpm for 10 min. The cell pellets obtained were washed twice with PBS and processed further for microscopy as described earlier.[10] The cell pellets were also processed for TEM to visualize the PHA granules accumulated inside the cells as described earlier.[32] The sample was visualized on TEM 2100F (JEOL, Japan) at 120 kV.

PHA accumulation within the cell was evaluated by staining the cells with Nile red and assayed using flow cytometry (FACS; Beckman Coulter, MoFlo Cytomation). An appropriate volume of culture was harvested at different time points, centrifuged, and washed twice in PBS. The cells were stained with 0.15 mg/mL Nile red dissolved in dimethyl sulfoxide and incubated in the dark for 20 min at room temperature. Then, the cells were washed twice in PBS and loaded into FACS for Nile red fluorescence measurement using 488 nm laser coupled with 610/20 nm detection. A histogram was generated by recording 20 000 events for each sample. A change in the intensity of fluorescence with time indicates the presence and the amount of PHA within cells.

Extraction and Characterization of PHA. For cell biomass and PHA extraction, the cultures were harvested and pelleted by centrifugation at 10 000 rpm for 10 min. Cell pellets were processed for cell dry mass and PHA content as described previously.[32] The functional group composition of extracted PHA was studied by FTIR. A thin pellet was prepared and scanned as described earlier.[12]

The chemical structure of PHA was analyzed by ^{1}H NMR spectroscopy using a Varian Mercury Plus NMR spectrometer. The extracted and dried PHA was dissolved in deuterated chloroform ($CDCl_3$; chloroform is toxic and proper precaution was taken during experiments) at a concentration of 10 mg/mL with tetramethylsilane as an internal reference. The 400 MHz ^{1}H NMR spectrum was recorded at 10 330.578 Hz, with a pulse width of 3.17 s, 45.2° pulse angle, recycle delay of 1 s, and 16 scans.

GC−MS was used to study the monomeric composition of the polymer. The composition of PHA was analyzed by methanolysis of dried cells with a solution of 2 mL chloroform, 0.3 mL of 98% sulfuric acid, and 1.7 mL methanol heated at 100 °C for 140 min to convert the constituents into methyl esters. Then, the reaction mixture was allowed to cool at room temperature followed by the addition of 1 mL water to induce phase separation. The lower chloroform layer was used for GC−MS analysis as described earlier.[32]

Genomic Analysis for PHA Production and Substrate Utilization. The genome of *Pandoraea* sp. ISTKB was sequenced using the Illumina MiSeq platform, and the raw reads were filtered to obtain high-quality filter reads and assembled into scaffolds as described earlier.[16] Genes and pathways were annotated from the draft genome using NCBI prokaryotic genome annotation pipeline, Pfam, and KEGG automatic annotation server.[16] The important genes known for PHA metabolism and degradation of lignin and its derivatives were further identified.

Optimization of Media Components for Biomass and PHA Production. The optimization was performed by Design-Expert version 10 using response surface methodology. The variable carbon concentration, duration, and pH were selected as factors for optimization of maximum biomass and PHA production, keeping inoculum size, temperature, nitrogen content, and shaking condition constant. BBD was used for optimization of three independent parameters (carbon concentration, pH, and duration) at three levels (minimum, medium, and maximum). On the basis of the experimental design suggested by the software, seventeen experiments were conducted with carbon concentrations (1, 3, and 5 g/L), pH (6,

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8, and 10), and duration (48, 96, and 144 h) to obtain the maximum response for bacterial dry biomass (mg/L) and PHA content (mg/L). The experimental design suits a quadratic response surface, and it generated a second-order polynomial regression model. The equation is given below

$$y = \beta_o + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_{ii}^2 + \varepsilon \quad (1)$$

where y is the predicted response, β_o is the intercept, X_i and X_j are independent variables, β_i is ith linear coefficient, β_{ij} is interaction coefficient, β_{ii} is ith quadratic coefficient, and ε is the random error term. This equation was used to fit the experimental data of 17 experiments. The data obtained after experiment were evaluated statistically using ANOVA ($p < 0.05$). Confirmatory experiments were performed at the predicted parameters, and the responses obtained after analysis were compared with the predicted values to confirm the validity of the model.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b01615.

Nile red fluorescence and TEM images of PHA; FTIR spectra of PHA; TIC and electron ionization mass spectra of PHA; annotation of PHA metabolism genes; predicted optimized condition ramps for PHA and biomass; and ANOVA results of quadratic model response PHA and biomass (PDF)

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Notes

The authors declare no competing financial interest.

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