Article

Biosynthesis of Polyhydroxyalkanoates from Defatted *Chlorella* Biomass as an Inexpensive Substrate

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Abstract: Microalgae biomass has been recently used as an inexpensive substrate for the industrial production of polyhydroxyalkanoates (PHAs). In this work, a dilute acid pretreatment using 0.3 N of hydrochloric acid (HCl) was performed to extract reducing sugars from 10% (*w/v*) of defatted *Chlorella* biomass (DCB). The resulting HCl DCB hydrolysate was used as a renewable substrate to assess the ability of three bacterial strains, namely *Bacillus megaterium* ALA2, *Cupriavidus necator* KCTC 2649, and *Haloferax mediterranei* DSM 1411, to produce PHA in shake flasks. The results show that under 20 g/L of DCB hydrolysate derived sugar supplementation, the cultivated strains successfully accumulated PHA up to 29.7–75.4% of their dry cell weight (DCW). Among the cultivated strains, *C. necator* KCTC 2649 exhibited the highest PHA production (7.51 ± 0.20 g/L, 75.4% of DCW) followed by *H. mediterranei* DSM 1411 and *B. megaterium* ALA2, for which a PHA content of 3.79 ± 0.03 g/L (55.5% of DCW) and 0.84 ± 0.06 g/L (29.7% of DCW) was recorded, respectively. Along with PHA, a maximum carotenoid content of 1.80 ± 0.16 mg/L was produced by *H. mediterranei* DSM 1411 at 120 h of cultivation in shake flasks. PHA and carotenoid production increased by 1.45- and 1.37-fold, respectively, when HCl DCB hydrolysate biotransformation was upscaled to a 1 L of working volume fermenter. Based on FTIR and ^1H NMR analysis, PHA polymers accumulated by *B. megaterium* ALA2 and *C. necator* KCTC 2649 were identified as homopolymers of poly(3-hydroxybutyrate). However, a copolymer of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) with a 3-hydroxyvalerate fraction of 10.5 mol% was accumulated by *H. mediterranei* DSM 1411.

Keywords: Acid pretreatment; *Bacillus megaterium*; Carotenoids; *Cupriavidus necator*; Defatted *Chlorella* biomass; *Haloferax mediterranei*; Polyhydroxyalkanoates

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1. Introduction

Petrochemical-based materials have gained particular attention in various fields due to their excellent properties, such as good mechanical resistance and high thermal stability. However, being non-biodegradable, the accumulation of these materials may produce adverse effects on environmental and public health [1]. Recently, biodegradable polymers derived from microbial fermentation have been investigated as promising alternatives to help reduce plastic waste disposal in the environment. Among a variety of bioplastics, polyhydroxyalkanoates (PHAs) produced through microbial fermentation have received particular interest owing to their low toxicity, biocompatibility, complete biodegradability, and competitive properties compared with conventional plastics [2]. Despite their excellent properties and environmental benefits, the industrial production of PHA is somehow restricted due to the use of highly purified substrates that may entail high costs. Therefore, many studies have investigated the use of residue raw materials such as rice bran [3], lignocellulosic biomass [4], waste cooking oils [5], and coffee waste [6] as inexpensive fermentation substrates to reduce the PHA production cost.

Among a variety of waste materials, biomass from macro- and microalgae has shown great potential as a promising candidate for the industrial production of bioplastics due to

its high carbohydrate content and negligible lignin amount giving rise to high reducing sugar recovery without any costly lignin removal [7]. Moreover, the utilization of algal biomass as a substrate for PHA production may reduce food waste utilization, which may entail ethical issues related to the interference with human nutrition and animal feeding.

Dilute acid hydrolysis has been widely used for the pretreatment of algal biomass owing to its ability to enhance the conversion efficiency of carbohydrates into reducing sugars [8]. Previously, high fermentable sugar contents of 29 g/L and 52 g/L were respectively reported by Alkotaini et al. [9] and Khomlaem et al. [10] as a result of subjecting either *Laminaria japonica* or defatted *Chlorella* biomass to dilute acid hydrolysis using 94 mM of sulfuric acid (H_2SO_4) and 0.3 N of hydrochloric acid (HCl), respectively.

Chlorella (chlorophyte, Trebouxiophyceae) is considered to be one of the most cultivated microalgae in the world, providing more than 2000 tons of annual biomass production, and has been widely used as feed and food raw material owing to its high content of proteins, short-chain polyunsaturated fatty acids, vitamins, and minerals [11]. In the last few years, defatted *Chlorella* biomass (DCB) has received a particular interest as an excellent feedstock for bioethanol production [12,13] owing to its high content of carbohydrates either present in the form of hemicellulose and cellulose in the cell wall or accumulated in the form of starch in the chloroplast [14]. Recently, our research group has claimed the potential use of *Chlorella* biomass as a promising substrate for the production of PHA owing to its high carbohydrate content (69.5%) and has reported a maximum PHA production of 3.62 g/L (39.4% of dry cell weight) when *Paracoccus* sp. LL1 was cultivated in a minimal medium supplemented with 20 g/L of DCB-hydrolysate-derived sugars [10].

Among a variety of PHA-accumulating bacteria, *Bacillus megaterium* ALA2, *Cupriavidus necator* KCTC 2649, and *Haloferax mediterranei* DSM 1411 strains have shown great potential in producing high PHA contents through the bioconversion of algae biomass [7,9,15]. In this work, a dilute acid pretreatment using 0.3 N of HCl was performed to extract reducing sugars from 10% (*w/v*) of DCB. The resulting HCl DCB hydrolysate was investigated as a sustainable substrate to assess the ability of three bacterial strains, namely *B. megaterium* ALA2, *C. necator* KCTC 2649, and *H. mediterranei* DSM 1411 to produce PHA in shake flasks. Along with PHA, the ability of *H. mediterranei* DSM 1411 in producing carotenoids was also investigated at both flask and fermenter scale.

2. Materials and Methods

2.1. Chemicals and Microalgae Biomass

Chemicals including astaxanthin, poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (P (3HB-co-3HV)), and poly(3-hydroxybutyrate) (PHB) were purchased from Sigma-Aldrich, while DCB was obtained from the Korea Research Institute of Bioscience and Biotechnology. Algal biomass used as a substrate in this work was prepared according to Khomlaem et al. [10].

2.2. Microbial Strains

Three microbial strains, namely *Bacillus megaterium* ALA2 (NRRL B-21660) (originally isolated from a soil sample collected from McCalla, AL [16]), *Cupriavidus necator* KCTC 2649 (purchased from the Korean Collection for Type Cultures (Daejeon, Korea)), and *Haloferax mediterranei* DSM 1411 (purchased from the DSMZ culture collection (Braunschweig, Germany)) were evaluated for their PHA-accumulation ability when using HCl DCB as a fermentative substrate. The strains grown on Luria-Bertani (LB) agar were stored at 4 °C.

2.3. Media Preparation

A minimal medium [10] composed of 9 g/L of $Na_2HPO_4 \times 12H_2O$, 0.5 g/L of KH_2PO_4 , 1 g/L of $(NH_4)_2SO_4$, 1 g/L of $MgSO_4 \times 7H_2O$, 20 mg/L of $CaCl_2 \times 2H_2O$, 10 g/L of NaCl, and 2 mL/L of trace element solution under 20 g/L of fermentable sugar supplementation from 0.3 N HCl DCB hydrolysate was used for *B. megaterium* ALA2 cultivation in shake

flasks. The same medium devoid of NaCl was used for *C. necator* KCTC 2649 to accumulate PHA in shake flasks. The trace element solution was composed of 0.44 g/L of $\text{ZnCl}_2 \times 7\text{H}_2\text{O}$, 4.98 g/L of $\text{FeSO}_4 \times 7\text{H}_2\text{O}$, 0.78 g/L of $\text{CuSO}_4 \times 5\text{H}_2\text{O}$, 0.81 g/L of $\text{MnSO}_4 \times 4\text{H}_2\text{O}$, and 0.24 g/L of $\text{Na}_2\text{MoO}_4 \times 2\text{H}_2\text{O}$ dissolved in 1 N HCl solution [10].

A *Halobacterium* medium composed of 13 g/L of $\text{MgCl}_2 \times 6\text{H}_2\text{O}$, 156 g/L of NaCl, 20 g/L of $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 4 g/L of KCl, 1 g/L of $\text{CaCl}_2 \times 6\text{H}_2\text{O}$, 0.5 g/L of NaBr, 0.2 g/L of NaHCO_3 , and 5 g/L of yeast extract [17] supplemented with 20 g/L of DCB-hydrolysate-derived sugars was used to assess the ability of *H. mediterranei* DSM 1411 to co-produce PHAs and carotenoids at both shake flask and fermenter scale.

2.4. Algal Biomass Pretreatment and Reducing Sugar Quantification

A dilute acid pretreatment using 0.3 N of HCl was performed to extract reducing sugars from 10% (*w/v*) of DCB according to the method used by Khomlaem et al. [10]. After centrifugation at $10,950 \times g$ for 20 min, the resulting supernatant was used for reducing sugar quantification based on the 3,5-dinitrosalicylic acid (DNS) method.

Acid type and concentration were selected based on our previous study reporting the efficacy of this acid pretreatment to achieve a maximum fermentable sugar level of 52 g/L. The recovered reducing sugars were identified by HPLC analysis as glucose (46 g/L) and cellobiose (5.94 g/L) [10].

2.5. PHA Production via Batch Cultivation in Shake Flasks

The shake flask cultivation of three bacterial strains including *B. megaterium* ALA2, *C. necator* KCTC 2649, and *H. mediterranei* DSM 1411 was performed to screen their PHA-accumulating ability.

Flask cultures were carried out in 200 mL of a minimal medium under 20 g/L of reducing sugar supplementation from sterilized HCL DCB hydrolysate. The temperature was set at 30 °C for *B. megaterium* ALA2 and *C. necator* KCTC 2649, while a temperature of 42 °C was used for the cultivation of *H. mediterranei* DSM 1411. Flask cultures were shaken at 200 rpm in a rotary incubator.

2.6. Analytical Techniques

2.6.1. Determination of Cell Growth and Dry Cell Weight (DCW)

Microbial growth was determined by optical density at 600 nm. DCW was determined according to Khomlaem et al. [10]. For each bacterial culture, DCW was measured gravimetrically at a 24-h interval.

2.6.2. Reducing Sugar Quantification during the Fermentation Process

The DNS method was used to quantify residual reducing sugar content as previously described by Khomlaem et al. [10].

2.6.3. Quantification of Intracellular PHA and Carotenoid Content

A methanolysis–gas chromatography (Agilent model 6890N, Agilent, Santa Clara, CA, USA) analysis was performed to quantify PHA as previously detailed by Khomlaem et al. [10]. Benzoic acid and P (3HB-*co*-3HV), containing 88 mol% of 3HB and 12 mol% of 3HV, respectively, were used as standards.

Carotenoid content was quantified based on the method described by Khomlaem et al. [10].

2.7. PHA Characterization

PHAs accumulated inside bacterial cells were characterized by FTIR and ^1H NMR analysis.

A Nicolet model Magna-IR 200 FTIR spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to collect FTIR spectra of the accumulated PHA polymers in a spectral range of 4000–400 cm^{-1} .

A ^1H NMR (Bruker Avance 500 MHz, Bruker BioSpin, Rheinstetten, Germany) analysis was performed to identify the molecular structure of the produced PHA as previously described by Muhammad et al. [15].

2.8. Upscaling of PHA and Carotenoid Co-Production by *H. mediterranei* in a Fermenter

H. mediterranei DSM 1411 batch cultivation was performed in a 1 L working volume fermenter to further improve PHA and carotenoid production. For this purpose, 20 g/L of fermentable sugar derived from HCl DCB hydrolysate was added to the medium. The inoculum size was 10% *v/v*. The agitation speed was adjusted to 300 rpm. The dissolved oxygen (DO) level was maintained beyond 20%. The temperature and pH were respectively adjusted to 42 °C and 7.2.

3. Results and Discussion

3.1. PHA Production via Batch Cultivation in Shake Flasks

The cell growth profile, PHA production, and substrate consumption by *B. megaterium* ALA2 during the fermentation process are shown in Figure 1. The reducing sugar concentration in DCB HCl hydrolysate-supplemented media decreased with increasing biomass production, giving rise to a maximum DCW of 2.81 ± 0.05 g/L at 72 h of cultivation. PHA production occurred concomitantly with cell growth and achieved a maximum of 0.84 ± 0.06 g/L (29.7% of DCW). Lower DCW (1.80 g/L) and PHA (0.40 g/L, 21% of DCW) levels were previously reported by Muhammad et al. [15] when *B. megaterium* ALA2 was cultivated in minimal media supplemented with *Laminaria japonica* hydrolysate. Such a difference in DCW and PHA yield can be explained by the difference in reducing sugar composition between the two hydrolysates. Previously, Gouda et al. [18] investigated the effect of different carbon sources, including maltose, fructose, Na-gluconate, glucose, lactose, xylose, and sucrose, on PHA accumulation by *B. megaterium*. The maximum production in terms of PHA was recorded in media supplemented with 2% glucose, which indicates the suitability of this carbon source for *B. megaterium*. Based on our previous study, glucose was identified as the main reducing sugar in DCB HCl hydrolysate [10], which may explain, in part, the higher level of PHA that accumulated inside *B. megaterium* ALA2 cells in this study, compared with that produced from *Laminaria japonica* hydrolysate [15] for which mannitol was identified as the main carbohydrate [19].

Figure 1. Time profile of optical density (OD) at 600 nm, dry cell weight (DCW), polyhydroxyalkanoate (PHA) accumulation, and substrate utilization by *Bacillus megaterium* ALA2 using 0.3 N HCl defatted *Chlorella* biomass (DCB) hydrolysate as the carbon source in a flask culture. Data are shown as mean values $\pm$ standard deviation ($n = 3$).

Figure 2 shows the cell growth profile, PHA production, and substrate consumption by *C. necator* KCTC 2649 during batch cultivation in shake flasks. Reducing sugar consumption increased with increasing bacterial growth. PHA accumulation started during the exponential phase and gradually increased during the stationary phase, giving rise to a maximum polymer content of 7.51 ± 0.20 g/L (75.4% of DCW) at 120 h of cultivation due to PHA accumulation inside bacterial cells. In fact, during the stationary phase, cells cease division but remain metabolically active and start accumulating PHA under the excess of carbon source and nitrogen-limiting conditions [20]. The PHA level decreased after this time due to PHA utilization for cell maintenance purposes. PHA accumulation by *C. necator* in this work was significantly higher than that reported by Muhammad et al. [15] when cultivating *C. necator* NCIMB 11599 in a minimal medium supplemented with 2% of *L. japonica* acid hydrolysate (1.58 g/L; 32% of DCW). Likewise, a PHA production of only 0.62 g/L was previously reported by Verlinden et al. [21] when *C. necator* was cultivated in a basal salt medium supplemented with waste rapeseed oil. Interestingly, the PHA content accumulated by *C. necator* in this work was significantly higher than that produced by the same strain cultivated in a mineral salt medium supplemented with 1% glucose, for which a maximum PHA production of only 1.61 g/L (57.07% of dry cell weight) was recorded [22]. This result underlines the importance of using DCB hydrolysate as a promising low-cost substrate for industrial bioplastics production.

Figure 2. Time profile of OD at 600 nm, DCW, PHA accumulation, and substrate utilization by *Cupriavidus necator* KCTC 2649 using 0.3 N HCl DCB hydrolysate as the carbon source in a flask culture. Data are shown as mean values $\pm$ standard deviation ($n = 3$).

The cell growth profile, PHA accumulation, carotenoid production, and substrate utilization by *H. mediterranei* DSM 1411 cultivation during the fermentation process are shown in Figure 3. The reducing sugar content gradually decreased with time due to cell growth, denoting the ability of the cultivated strain to assimilate reducing sugars from HCl DCB hydrolysate. A continuous increase in DCW and PHA content was noticed during the exponential phase, achieving a maximum of 6.84 ± 0.15 g/L and 3.79 ± 0.03 g/L (55.5% of DCW) respectively at 120 h of cultivation. Along with PHA accumulation, a maximum carotenoid content of 1.80 ± 0.16 mg/L was obtained. The PHA production achieved by *H. mediterranei* DSM 1411 in this work was significantly higher than that accumulated by the same strain cultivated in a minimal synthetic medium (MSM) supplemented with emulsified volatile fatty acids, for which a maximum PHA content of 0.93 g/L (37.4% of DCW) was recorded [23]. Likewise, the DCW and PHA levels recorded in this study were respectively 71% and 156% higher than those obtained by Khomlaem et al. [10]

when using the same HCl DCB hydrolysate as a carbon source by *Paracoccus* sp. LL1. Among halophiles, haloarchaeon *H. mediterranei* has been reported to be the most efficient in accumulating PHA at high cell densities owing to its fast growth rate, genetic stability, and efficient metabolic capacity [24]. Several studies have reported the ability of this strain to produce high PHA levels through the conversion of different waste products, including cheese whey [25], rice-based ethanol stillage [26], and macroalgal biomass [7].

Figure 3. Time profile of OD at 600 nm, DCW, PHA accumulation, carotenoid production, and substrate utilization by *Haloferax mediterranei* DSM 1411 using 0.3 N HCl DCB hydrolysate as the carbon source in a flask culture. Data are shown as mean values $\pm$ standard deviation ($n = 3$).

3.2. Characterization of PHA Polymers

3.2.1. FTIR Analysis

The FTIR spectra of PHA polymers accumulated by *B. megaterium* ALA2, *C. necator* KCTC 2649, and *H. mediterranei* DSM 1411 are shown in Figure 4. PHA polymers produced by the different bacterial strains showed characteristic bands of ester bonding present in PHB homopolymer at 1734 and 1185 cm^{-1} [27]. The FTIR spectrum of PHA polymers produced by *H. mediterranei* DSM 1411 showed additional peaks between 1450 and 1000 cm^{-1} corresponding to CH_2 wagging, CH_3 bending, C–O, C–C and C–O–C stretching, confirming the P (3HB-*co*-3HV) nature of the sample [28]. Raho et al. [29] reported the same bands for P (3HB-*co*-3HV) produced from ricotta cheese exhausted whey.

Figure 4. The FTIR spectra of PHA produced by (a) *Bacillus megaterium* ALA2, (b) *Cupriavidus necator* KCTC 2649, and (c) *Haloferax mediterranei* DSM 1411 using 0.3 N HCl DCB hydrolysate as the carbon source.

With respect to PHA polymers produced by *H. mediterranei* DSM 1411, the ^1H NMR spectrum clearly shows two additional peaks at 0.9 ppm and 1.6 ppm respectively corresponding to the methyl and methylene groups of the valerate monomer, denoting that the produced PHA is a P (3HB-co-3HV) copolymer (Figure 5c). Similar peaks were identified in the ^1H NMR spectra of the P (3HB-co-3HV) copolymer produced from macroalgal biomass hydrolysate [31]. The 3HV molar fraction in the P (3HB-co-3HV) copolymer was calculated from the area ratio of methyl group peaks corresponding to 3HB and 3HV units (1.27 and 0.9 ppm, respectively). Based on this calculation, the 3HV molar fraction was present in a ratio of 10.5 mol%. This 3HV ratio was higher than that recorded for the P (3HB-co-3HV) copolymer accumulated by *H. mediterranei* from different carbon sources, including *Ulva* sp. macroalgae hydrolysate [7], hydrolyzed whey permeate [32], and glucose and yeast extract [33]. Likewise, the 3HV content obtained in this study was 68.8% higher than that recorded in our previous work when the same carbon source (DCB hydrolysate) was used by *Paracoccus* sp. LL1 [10]. Comparable 3HV content was previously recorded for the P (3HB-co-3HV) copolymer accumulated by *Paracoccus* sp. LL1 cultivated in a minimal medium supplemented with glucose [10]. These results underline the ability of *H. mediterranei* to use DCB hydrolysate as an inexpensive and sustainable carbon source for the accumulation of P (3HB-co-3HV) copolymers without any precursor supplementation.

3.3. Upscaling of PHA and Carotenoid Co-Production in a Fermenter

Although *C. necator* KCTC 2649 exhibited the highest PHA production in shake flask culture, *H. mediterranei* DSM 1411 was selected for batch cultivation in the fermenter owing to its ability to produce P (3HB-co-3HV) copolymers. In fact, P (3HB-co-3HV) copolymers with a high 3HV molar ratio are usually required for the processing of bioplastic materials with high mechanical and thermal properties. Moreover, along with P (3HB-co-3HV) copolymer accumulation, *H. mediterranei* DSM 1411 was able to produce carotenoids as high-value products, which may reduce the overall cost of PHA production.

Batch fermentation of *H. mediterranei* DSM 1411 was performed in a 1 L working volume fermenter under specific conditions of temperature, pH, aeration, and agitation. Figure 6 shows the cell growth profile, carotenoid production, PHA accumulation, and substrate utilization by *H. mediterranei* DSM 1411 during 144 h of cultivation in the fermenter. PHA production began during the exponential growth phase and continuously increased as cell biomass increased, reaching a maximum level of 5.50 ± 0.21 g/L (58.4% of DCW) at the beginning of the stationary phase with a carotenoid co-production of 2.48 ± 0.18 mg/L. In fact, during the stationary phase, cell division ends and cells start the process of polymer accumulation induced by the excess of carbon source and nitrogen-limiting conditions [34]. The DCW and PHA levels recorded in this study were respectively 148% and 150% higher than those obtained by Ghosh et al. [7] when the same strain was cultivated in the presence of 25% (*w/w*) of *Ulva* sp. hydrolysate. This difference in terms of PHA production can be attributed to the difference in carbohydrate composition between the two algal biomasses as well as to the presence of toxic fermentation inhibitors in *Ulva* sp. biomass hydrolysate (1.3 mg of 5-hydroxymethylfurfural per gram of dried macroalgal biomass) that may affect cell growth and thus PHA accumulation. Although a higher carotenoid concentration of 11.7 mg/L was reported by Khomlaem et al. [10] when upscaling the bioconversion of HCl DCB hydrolysate to a 3 L working volume fermenter by *Paracoccus* sp. LL1, PHA content accumulated by *H. mediterranei* in this work was 1.5-fold higher. Our results demonstrate the high potential of *H. mediterranei* in converting DCB-hydrolysate-derived sugars into P (3HB-co-3HV) without any precursor supplementation. These findings are of great importance, as the cultivated strain was able to convert reducing sugars from an inexpensive substrate into a P (3HB-co-3HV) copolymer with higher functionality compared with a PHB homopolymer.

Figure 6. Time profile of OD at 600 nm, DCW, PHA accumulation, carotenoid production, and substrate utilization by *Haloferax mediterranei* DSM 1411 using 0.3 N HCl DCB hydrolysate as the carbon source in the fermenter. Data are shown as mean values $\pm$ standard deviation ($n = 3$).

4. Conclusions

In this work, DCB-hydrolysate-derived sugars were successfully converted into PHA by *Cupriavidus necator* KCTC 2649, *Bacillus megaterium* ALA2, and *Haloferax mediterranei* DSM 1411. *C. necator* exhibited a maximum PHA production of 7.51 ± 0.20 g/L (75.4% of DCW) at 120 h of cultivation. Along with PHA accumulation, a maximum carotenoid content of 1.80 ± 0.16 mg/L was produced by *H. mediterranei* DSM 1411 at 120 h of cultivation in shake flasks. PHA and carotenoid production respectively increased by 1.45- and 1.37-fold when HCl DCB hydrolysate biotransformation was upscaled to a 1 L working volume fermenter. Taking into account the overall results of this study, DCB can be investigated as a sustainable substrate to reduce the high production cost related to bioplastic production, along with limiting environmental problems arising from waste disposal.

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Abbreviations

DCB	defatted <i>Chlorella</i> biomass
HCl	hydrochloric acid
PHA	polyhydroxyalkanoate
PHB	poly(3-hydroxybutyrate)
3HB	3-hydroxybutyrate
3HV	3-hydroxyvalerate
P (3HB-co-3HV)	poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
OD	optical density
DCW	dry cell weight

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