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Feasibility study on production of biodegradable polymer and wastewater treatment using *Aeromonas* strains for materials recycling

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ABSTRACT

With consideration of wastewater treatment and materials recycling for cradle-to-cradle (C2C) sustainable development, this treatability study analyzed the capability of poly 3-hydroxybutyrate (PHB) production in wastewater-laden media using indigenous dye-decolorizing *Aeromonas hydrophila* NIU01, KB23, *Aeromonas salmonicida* 741. Compared to paper-container, frozen food, wine manufacturing wastewater, wastewater generated from printing and dyeing industry was found to be more appropriate to efficiently produce PHB for materials recycling. Due to lack of sufficient essential inorganic nutrients provided for cell propagation, dye-decolorized wastewater with augmented MR media in different ratios was used to explore toxicity potency of mixed media and to present PHB-producing capability of cells. In particular, when MR media were completely replaced by decolorized culture broth, significant stimulating effect on PHA-production was shown (ca. 52.5% PHB content). This study clearly revealed the promising feasibility of simultaneous wastewater treatment and biopolymer production for cradle-to-cradle sustainable development.

1. Introduction

As known, most of popularly-used plastics are not biodegradable and approx. 25 billions tons plastics significantly accumulated in worldwide natural environment. That is why "plastic pollution has become a man-made global catastrophe" (*e.g.*, "plastic ocean" in North Pacific and Atlantic Ocean) nowadays [1]. To significantly attenuate accumulation of non-biodegradable plastic waste for remediation of worldwide "waste disaster", using biodegradable polymers as one of environmentally friendly biomaterials is inevitably to environmental protection. In fact, poly-hydroxyalkanoates (PHAs) as one of such biomaterials to replace plastics are intracellular energy storage linear polyesters produced in nature by bacterial fermentation of sugar and lipids. Due to biocompatibility and biodegradability of biologically-produced PHA with promising physical and chemical characteristics (*e.g.*, viability to be changed by blending, modified the surface), PHAs could be applicable to be biodegradable plastics for green technology of sustainable development [2]. As a matter of fact, several naturally-occurring microbes have capabilities for biosynthesis of PHAs at conditions of physiological stress (*e.g.*, nitrogen, magnesium-limiting conditions; [3]). Moreover, among diverse chemically-structured PHAs, polyhydroxybutyrate (PHB) could be used for myriads of biomedical applications [4]. In particular, several indigenous PHA-producing microbes evolved in contaminated environments could simultaneously degrade pollutants and produce PHAs, since cellular metabolism of PHA production could be simultaneously induced in hostile environments. For example, Tian et al. [5] and Yao et al. [6] mentioned that with supplementation of different carbon sources *Pseudomonas mendocina* and *P. nitroreducens* isolated from petroleum contaminated soil could produce PHAs in contents of 29–45 wt% and 6–70 wt%, respectively. Lee et al. [7] also indicated that *Aeromonas hydrophila* could synthesize ca. 5–35 wt% PHAs using glucose, gluconic acid, acetic acid and lauric acid as the sole carbon substrate under nitrogen and phosphorous-limiting conditions. Recently, Chen et al. [8–10] used dye-decolorizing bacterium *A. hydrophila* NIU01 isolated from northeast Taiwan to produce PHB at ca. 32–34 wt% in azo dye reactive red 141 (RR141)-bearing cultures.

To consider cradle-to-cradle (C2C) sustainable development, this study tended to demonstrate the feasibility of simultaneous wastewater treatment and bioresource recycling by using model industrial wastewaters as culture media. In addition, indigenous dye-decolorizing bacteria *A. hydrophila* NIU01, KB23 and *A. salmonicida* 741 were used as model bacteria to explore the characteristics of microbial growth and intracellular PHA accumulation. As shown in Zhang et al. [10], toxic intermediates (*e.g.*, decolorized amines) might be generated during reductive

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decolorization. Chen et al. [9] showed that the presence of some model dye intermediates (*e.g.*, aminophenols) might somehow repress PHA production of dye-decolorizing bacteria. For the sake of simultaneous materials recycling and wastewater treatment, this study compared capabilities of PHA generation of dye-decolorizing microbes with different wastewaters (*e.g.*, wine manufacturing wastewater) as culture media. The findings indicated that dye-associated wastewater seemed to be more biologically-viable for such purposes of C2C design of materials recycling and reuses. This finding may account for the fact that these strains were originally isolated from dye-bearing environments [8–11].

2. Materials and methods

2.1. Microbial cultures

Dye-decolorizing bacterial strains *A. hydrophila* NIU01, KB23 and *A. salmonicida* 741 were isolated from Taiwan and identified via 16S rRNA phylogenetic-tree analysis [8–11]. All of the strains were cultured in LB broth medium for 12–16 h and then the cultures were mixed with 40% (v/v) glycerol to store at −80 °C for long-term use.

2.2. Culture media and chemical reagents

MR medium [7] used for batch cultures (per liter) contained 4.0 g $(NH_4)_2HPO_4$, 0.8 g $MgSO_4{\cdot}7H_2O$, 0.8 g citric acid and 6.67 g KH_2PO_4, 10 mL TES-I and 1 mL TES-II. Trace-element solution I (TES-I) (in 1.0 L) contained 5.0 g Fe(III)–NH_4–citrate and 2.0 g $CaCl_2{\cdot}2H_2O$ [11]. Trace-element solution II (TES-II) (prepared in 1.0N HCl) contained 100 mg $ZnSO_4{\cdot}7H_2O$, 30 mg $MnCl_2{\cdot}4H_2O$, 300 mg H_3BO_3, 200 mg $CoCl_2{\cdot}6H_2O$, 10 mg $CuSO_4{\cdot}5H_2O$, 20 mg $NiCl_2{\cdot}6H_2O$ and 30 mg $Na_2MoO_4{\cdot}2H_2O$.

2.3. Culture conditions and chemical analysis

2.3.1. Batch fermentation

A loopful of bacterial seed taken from single colony on a Luria-Bertani (LB)-streak plate was inoculated into 50 mL sterilized LB broth medium for 12–16 h overnight (O/N) preculture. Then, 5.0 mL culture broth was inoculated into 100 mL MR medium containing TES-I and TES-II and wastewater in different ratios at 30 °C, 200 rpm for 72 h culture. After batch fermentation, for analysis cultures were aseptically cooled to 4 °C in order to completely solidify lauric acid. Next, filter papers (ADVANTEC, NO. 5A, 7 μm) were used to separate biomass from solid lauric acid. To remove residual abiotic ash from biomass, biomass solutions were three-times washed with distilled water, and then centrifuged at 6000 rpm for 10 min. The supernatants of each wash step were discarded. The harvested biomass was then dried at 100 °C over time until time-invariant weight was achieved for PHA-content analysis.

Regarding toxicity assessment via dose-response evaluation, toxicity effects of supplemented wastewater on cellular respiratory characteristics associated to microbial growth of cell cultures were tested individually using automated Columbus Micro-Oxymax Respirometer equipped with CO_2 sensors [12].

2.3.2. Analytical methods

The GC analysis (Gas chromatography; SRI 8610C, USA: GC Column-Zebron ZB-5 in length = 30 m, I.D. = 0.25 mm, film thickness = 0.25 μm with FID detector) for PHB content was performed after methanolyzing the polymer in sulfuric acid and methanol [13]. Temperatures for GC detection in splitless mode for injection, detector and oven temperature were 250, 300 and 75 °C, respectively. Initial temperature was at 75 °C holding 1 min at rate of 25 °C/min and final temperature at 200 °C holding 4 min at carrier flow of 3 mL/min (retention times of PHB and internal standard were ca. 2.2 and 4.6 min, respectively). Benzoic acid was used as an internal standard to measure PHB concentration for quantitatively comparing PHB peaks [14]. The purity of the standard PHB (purchased from Sigma Inc.) is defined as 100%. Based upon this definition, the relative purity of the purified PHB, as aforementioned in GC analysis, was quantified to be ca. 95%.

3. Results and discussion

3.1. PHB production using Aeromonas strains

Prior to consideration of operation feasibility of PHB production and wastewater (WW) treatment for C2C sustainable development, the toxicity potency of the test WW onto model dye-decolorizing bacteria *A. hydrophila* NIU01, KB23 and *A. salmonicida* 741 should be explored. First, 0.2× LB broth supplemented with 200 mg/L azo dye reactive red 141 (RR141) was used as the growth medium for bacterial cultures. Once microbial cell cultures were aerobically grown in late exponential growth phase, RR141 was then supplemented to be decolorized via static incubation until ca. 5% residual dye was achieved. The RR141 decolorized broth (DB) was then used as model WW for the feasibility study. To explore the viability of using decolorized WW as culture medium, MR media supplemented with 10 g/L lauric acid and DB in various ratios were used for quantitative assessment of their toxicity potency via 5-day data of CO_2 biorespirometric evaluation of *Aeromonas* spp. (*e.g.*, strains NIU01, KB23, 741; Fig. 1). The toxicity rankings of these mixed WWs (in increasing order) for the 5-day cumulative CO_2 production (unit: mg CO_2; Table 1) were shown as follows:

A. hydrophila NIU01: 0% (725.48) > 20% (594.37) > 40% (515.69) > 60% (513.70) > 80% (395.74) > 100% (300.61),
A. hydrophila KB23: 0% (515.75) > 20% (505.41) > 40% (493.02) > 60% (425.53)> 80% (378.10) > 100% (307.75),
A. salmonicida 741: 0% (532.90) > 20% (487.49) > 40% (437.44) > 60% (419.72)> 80% (388.16) > 100% (297.82).

As shown, when lauric acid-bearing MR culture medium was completely replaced by DB, CO_2 cumulative amount decreased ca.

Table 1
Comparison on CO_2 cumulative amounts of dye-decolorizing bacteria using RR141 decolorized broth in different ratios as culture media.[a]

Dye-decolorizing bacterium	MR medium: waste LB medium	CO_2 accumulation (mg)	
		3 days	5 days
A. hydrophila NIU01	100:0	476.17	725.48
	80:20	464.95 ± 4.92	594.4 ± 11.6
	60:40	307.81	515.69
	40:60	297.99	513.70
	20:80	222.8 ± 10.9	395.7 ± 18.7
	0:100	202.73	300.61
A. hydrophila KB23	100:0	300.26	515.75
	80:20	288.02	505.41
	60:40	284.64	493.02
	40:60	303.49	425.53
	20:80	283.2 ± 1.3	378.1 ± 4.2
	0:100	253.0 ± 4.9	307.8 ± 10.4
A. salmonicida 741	100:0	317.79	532.90
	80:20	330.64	487.49
	60:40	320.4 ± 11.5	437.4 ± 2.3
	40:60	310.9 ± 2.6	419.7 ± 4.9
	20:80	289.89	388.16
	0:100	245.63	297.82

[a] Data statistics with random replication were implemented based upon EPA QA/QC protocol.