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Acidogenic spent wash valorization through polyhydroxyalkanoate (PHA) synthesis coupled with fermentative biohydrogen production

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HIGHLIGHTS

- PHA production from *B. tequilensis* and SWE was evaluated for the first time.
- Produced co-polymer P (3HB-co-3HV) showed higher fraction of HB.
- Integration of PHA and H_2 production reduces overall production and treatment cost.

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ABSTRACT

The production of polyhydroxyalkanoates (PHAs) by *Bacillus tequilensis* biocatalyst using spent wash effluents as substrate was evaluated to increase the versatility of the existing PHA production process and reduce production cost. In this study, spent wash was used as a substrate for biohydrogen (H_2) production and the resulting acidogenic effluents were subsequently employed as substrate for PHA production. Maximum H_2 production of 39.8 L and maximum PHA accumulation of 40% dry cell weight was attained. Good substrate removal associated with decrement in acidification (53% to 15%) indicates that the VFA generated were effectively utilized for PHA production. The PHA composition showed presence of copolymer [P (3HB-co-3HV)] with varying contents of hydroxybutyrate and hydroxyvalerate. The results obtained suggest that the use of spent wash effluents as substrate can considerably reduce the production cost of PHA with simultaneous waste valorization. PHA synthesis with *B. tequilensis* and spent wash effluents is reported for the first time.

1. Introduction

Hydrogen is regarded as a sustainable and viable alternative energy source whose consumption does not lead to any CO_2 emissions. With respect to sustainable development and waste minimization issues, bio-hydrogen (H_2) production from renewable sources has received considerable attention in recent years (Kim et al., 2012; Venkata Mohan et al., 2013). Production of clean energy with simultaneous valorization of waste materials makes biological hydrogen production process a promising approach for meeting the mounting energy needs of the world. Extensive research has been carried out on H_2 production using different types of wastewaters like food waste, municipal waste, distillery effluents etc. Venkata Mohan et al. (2008) and Searmsirimongkol et al. (2011) have reported H_2 production from distillery spent wash with concurrent wastewater treatment. H_2 production was reported to improve when a co-culture (*Citrobacter freundii* 01, *Enterobacter aerogenes* E10 and *Rhodopseudomonas palustris*) was used with sugar cane distillery effluent as substrate (Vatsala et al., 2008). However, one of the major drawbacks of using organic waste as a substrate is lower H_2 yield and existence of about 60–70% of the original organic matter as residue in the wastewater (Venkata Mohan et al., 2010, 2013). In addition, large amounts of soluble acid metabolites e.g. volatile fatty acids (VFA) are generated that mandate further treatment to evade the negative environmental impacts associated with their disposal. VFAs are the precursors of polyhydroxyalkanoates (PHA) in microbial metabolism. Production of PHAs under defined microaerophilic conditions using the VFA rich effluents from the biohydrogen producing reactor, as main substrate is an alternate and highly sustainable valorization technique (Venkata Mohan et al., 2010; Passanha et al., 2013; Reddy et al., 2013).

In an increasingly globalized world, plastics have turned out to be one of the most widely used materials in all facets of life such as packaging, home appliances, computer equipment, bottles,

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disposable items and automobiles (Jin et al., 2013; Lee and Gilmore, 2005). The usage of these non-biodegradable plastics will have potentially damaging ramifications on the environment and hence considerable emphasis has to be laid on the development of biopolymer materials like PHA that are eco-friendly (Akaraonye et al., 2010) and biodegradable (Jin et al., 2013). PHAs are linear aliphatic polyesters that accumulate as cytoplasmic inclusions in various bacteria and archaea under certain nutrient deprived growth conditions such as excess supply of carbon and lack of one or more essential nutrients, e.g. oxygen, nitrogen, phosphorus, sulfur and trace elements such as magnesium, calcium, and iron (Hazer and Steinbuchel, 2007). Poly-β-hydroxybutyrate (PHB) and poly-β-hydroxyvalerate (PHV) are the most common forms of PHA accumulated by microorganisms (Luengo et al., 2003).

Over the past few years, research has been focused on the use of synthetic fatty acids and low cost agro-industrial surplus feed stocks (Albuquerque et al., 2011) and fermented paper mill effluents (Bengtsson et al., 2008) as prime substrates for PHA production. The integration of PHA production with simultaneous valorization of acidogenic effluents is a fairly recent innovation (Ntaikou et al., 2009; Passanha et al., 2013). Santimano et al. (2009) have reported use of cane molasses as substrate for the growth of *Bacillus sp* COLI/A6 (biomass content 6 g l^{-1} dry cell weight (DCW)) and subsequent PHA production (54.68% DCW). Omar and co-workers also achieved 50% (DCW) of P(3HB) using *Bacillus megaterium* grown on beet molasses (Omar et al., 2001). PHA production using synthetic acids and food waste, by *Bacillus tequilensis* has also been reported (Reddy et al., 2013). In view of the emerging interest in the use of fermented substrates as feedstocks for PHA production this work has been aimed at evaluating the possibility of using VFA rich spent wash effluents from biohydrogen production process as substrate and *B. tequilensis* as the biocatalyst for PHA production. This study also helps in understanding the influence of different types of VFAs on polymer composition. This integration approach can dramatically reduce production costs with simultaneous waste valorization, for a cleaner and more sustainable society. There are no reports in literature till date that describe the production of PHA with *B. tequilensis* using spent wash effluents (SWE) as substrate.

2. Methods

2.1. Biocatalyst

Acidogenic H_2 production experiments were carried out using anaerobic consortium as inoculum collected from a full scale anaerobic reactor treating composite wastewater. Prior to inoculation, the parent culture was washed in phosphate saline buffer (5000 rpm, 20 °C) and enriched with designed synthetic wastewater (DSW) [glucose – 3 g/l; NH_4Cl – 0.5 g/l, KH_2PO_4 – 0.25 g/l, K_2HPO_4 – 0.25 g/l, $MgCl_2$ – 0.3 g/l, $CoCl_2$ – 25 mg/l, $ZnCl_2$ – 11.5 mg/l, $CuCl_2$ – 10.5 mg/l, $CaCl_2$ – 5 mg/l, $MnCl_2$ – 15 mg/l, $FeCl_3$ – 25 mg/l, $NiSO_4$ – 16 mg/l] under anaerobic microenvironment at pH 6.0 in conical flask under shaking (80 rpm; 48 h). *B. tequilensis* was used as biocatalyst for PHA production studies and the organism was isolated from PHA producing bioreactor by direct enrichment techniques (Reddy and Venkata Mohan, 2012a). The genomic DNA was extracted and purified from the isolated pure colonies using phenol–chloroform method. The variable V3 region of 16S rDNA was amplified by PCR using primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1542R (5′-AAGGAGGTGATCCAGCCGCA-3′). The nucleotide sequence was deposited in the GenBank database under accession number HE612876 (Reddy and Venkata Mohan, 2012a). Stock culture was preserved on nutrient agar slants overlaid with 20% (v/v) glycerol and kept at −20 °C.

2.2. Wastewater

Distillery spent wash (pH, 4.2; VFA, 27 g/l; chemical oxygen demand (COD), 126 g/l; Total dissolved solids (TDS), 112 g/l; and Total solids (TS), 113 g/l) was used as substrate for acidogenic H_2 production. The outlet of H_2 producing reactor viz., spent wash effluents (SWE) (pH, 3.83; VFA, 12 g/l; COD, 16.80 g/l; carbohydrates, 13.75 g/l) was used as substrate for PHA production. Prior to loading, the effluent was adjusted to required organic loading rates (OLR1, 0.66; OLR2, 1.32; OLR3, 1.98; OLR4, 2.64 kg COD/m^3-day) with tap water. After adjusting the organic load the SWE was autoclaved and used as substrate for PHA production.

2.3. Experimental methodology

2.3.1. Biohydrogen

Anaerobic bioreactor (working volume, 18 L; gas holding capacity, 4.51 L) operated in periodic-discontinuous batch mode with a total cycle period of 72 h was used to produce biohydrogen. The bioreactor was operated at ambient temperature (29 ± 2 °C) under anaerobic microenvironment with spent wash by adjusting the COD load to 30 g/l and pH to 6.0. The effluents (rich in VFA, SWE) collected from the bioreactor after 48 h were used as primary-substrate for PHA production.

2.3.2. Bioplastics

The feasibility of bioplastics production in the form of PHA was evaluated using *B. tequilensis* as biocatalyst in presence of SWE as substrate. The pH was adjusted to 7.0 ± 0.1 using 1 N NaOH. After adjusting the organic load and pH, the wastewater was sterilized by autoclaving (121 °C; 15 lb; 15 min) and then transferred to reactors with a total/working volume of 250/100 ml. Prior to start-up, the reactors containing 100 ml of substrate were inoculated with 4% of overnight grown *B. tequilensis*. The reactors were subjected to continuous mixing (120 rpm) and were maintained at ambient room temperature (29 ± 2 °C). In order maintain aerobic microenvironment and to ensure the exchange of gases, the reactors were closed with cotton plugs.

2.4. Extraction and quantification of bioplastics

Extraction and estimation of PHA was performed following the procedure reported elsewhere (Law and Slepecky, 1960; Reddy et al., 2012b). The biomass pellet was collected and washed with acetone and ethanol separately to remove unwanted materials. The pellet was suspended in 4% sodium hypochlorite and incubated at room temperature for 3 h. The resulting mixture was centrifuged (3000*g* for 30 min at 10 °C) and the pellet (with lysed cells) was again washed with acetone and ethanol prior to dissolving in hot chloroform. To separate the polymer from cell debris the pellet was passed through glass fiber filter (0.45 μm pore size). The chloroform filtrate thus obtained was used to estimate PHA colorimetrically. The chloroform from the filtrate was evaporated and 10 ml of sulphuric acid (36 N) was added, which converts the polymer to crotonic acid. The resultant solution was heated at 100 °C on a water bath for 10 min. The solution was cooled and its absorbance was measured at 235 and 285 nm for determining the hydroxyl butyrate (HB) and hydroxyvalerate (HV) concentration respectively, against a sulfuric acid blank. Standard curve was prepared using pure poly (3hydroxybutyrate-*co*-3hydroxyvalerate), P (3HB-*co*-3HV) (co-polymer; natural origin, Aldrich). FTIR spectroscopic analysis was also performed to confirm the presence of PHA.