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Integrative approach to produce hydrogen and polyhydroxybutyrate from biowaste using defined bacterial cultures

Sanjay K.S. Patel [a,b,1], Prasun Kumar [a,c,1], Mamtesh Singh [a], Jung-Kul Lee [b], Vipin C. Kalia [a,*]

[a] Microbial Biotechnology and Genomics, CSIR – Institute of Genomics and Integrative Biology (IGIB), Delhi University Campus, Mall Road, Delhi 110007, India
[b] Department of Chemical Engineering, Konkuk University, 1 Hwayang-Dong, Gwangjin-Gu, Seoul 143-701, Republic of Korea
[c] Department of Biotechnology, Pune University, Pune 411007, India

HIGHLIGHTS

- Integrative approach for efficient biowaste utilization by defined mixed cultures.
- A maximum of 75 L H_2/kg total solids and 2.1 g PHB/L obtained in continuous culture.
- Enhanced H_2 and PHB yields by bacteria immobilized on ligno-cellulosic wastes.

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ABSTRACT

Biological production of hydrogen (H_2) and polyhydroxybutyrate (PHB) from pea-shell slurry (PSS) was investigated using defined mixed culture (MMC4, composed of *Enterobacter*, *Proteus*, *Bacillus* spp.). Under batch culture, 19.0 L H_2/kg of PSS (total solid, TS, 2% w/v) was evolved. Using effluent from the H_2 producing stage, *Bacillus cereus* EGU43 could produce 12.4% (w/w) PHB. Dilutions of PSS hydrolysate containing glucose (0.5%, w/v) resulted in 45–75 L H_2/kg TS fed and 19.1% (w/w) of PHB content. Under continuous culture, MMC4 immobilized on coconut coir (CC) lead to an H_2 yield of 54 L/kg TS fed and a PHB content of 64.7% (w/w). An improvement of 2- and 3.7-fold in H_2 and PHB yields were achieved in comparison to control. This integrative approach using defined set of bacterial strains can prove effective in producing biomolecules from biowastes.

1. Introduction

Production of hydrogen (H_2) as a clean fuel and bioplastics (polyhydroxyalkanoates) as alternatives to petroleum based non-biodegradable plastics are possible solutions for a cleaner and sustainable society (Kumar et al., 2013; Nissila et al., 2014; Omar et al., 2001; Patel et al., 2012a,b; Reddy et al., 2003; Singh et al., 2009). Among various options for H_2 production, dark fermentative process has been recognized as the suitable and energy efficient (Kalia and Purohit, 2008). *In silico* genomic studies along with the screening of a large bacterial diversity have led to the identification of bacterial strains, each having an ability to produce both H_2 and polyhydroxybutyrate (PHB) under dark fermentative conditions (Kalia et al., 2003a,b; Porwal et al., 2008). Biological processes driven by a single bacterial isolate are invariably at the risk of getting contaminated. The contaminant may grow rapidly and eventually eliminate the original culture. In addition, each bacterial strain invariably can operate within a narrow range of physiological conditions. To surmount these problems, use of a mixture of well defined bacterial cultures with high and diverse metabolic activities has been found to be effective (Lin et al., 2011; Patel et al., 2010, 2012b, 2014).

A promising possibility for producing H_2 and PHB in a sustainable manner is to exploit biowastes as feeds (Arumugam et al., 2014; Nissila et al., 2014; Singh et al., 2009). Among a large number of biowastes used for producing these biomolecules, the prominent ones have been damaged wheat grains, cheese whey, Taihu blue algae, olive oil mill wastewater and pea-shells (PS) slurry (Kalia et al., 1994; Ntaikou et al., 2009; Patel and Kalia, 2013; Rosa et al., 2014; Venkata Mohan et al., 2010; Yan et al., 2010). Another aspect which needs attention is to produce these biomolecules in a continuous culture mode. Here, it is difficult to retain a large population of free floating bacteria within the bioreactor. Thus, the need was to immobilize the bacterial cultures. The use of biological materials such as ligno-cellulosic materials – banana leaves, coconut coir (CC), groundnut shells (GS) and PS has proved to be more suitable than synthetic materials as supports, for

* Corresponding author. Tel.: +91 11 27666156x132; fax: +91 11 27667471.
E-mail addresses: vckalia@igib.res.in, vc_kalia@yahoo.co.in (V.C. Kalia).
[1] These authors have contributed equally to this work.

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immobilization due to their high biocompatibility and eco-friendly nature (Kumar and Das, 2001; Patel et al., 2010, 2014).

In this study, an integrative approach was developed by employing bacteria with diverse and well defined high metabolic activities for performing specific processes. PS slurry (PSS) was hydrolyzed by defined mixed hydrolytic bacterial cultures (Patel et al., 2012b). The PS hydrolysate was subjected to defined mixed bacterial culture to produce H_2 in batch mode. The effluent from H_2 production stage was exploited by *Bacillus cereus* EGU43 for producing PHB. This multi-stage integrative approach has been shown to have the potential to improve the efficiency and sustainability of these processes.

2. Methods

2.1. Organisms and growth conditions

Bacterial strains used in this study were isolated previously in our laboratory (http://www.ncbi.nlm.nih.gov/). All the strains were maintained on nutrient agar at pH 7 containing 0.5% NaCl. Strains were grown in nutrient broth (HiMedia, Mumbai) at 37 °C at 200 rpm for 16–20 h (Patel et al., 2012b).

2.2. Bacterial cultures used for hydrolysis, H_2 and PHB production

Bacteria used here were selected on the basis of their performance as mixed culture with: (i) high hydrolytic enzyme activities (MHC2) – *Bacillus sphaericus* EGU542, *Bacillus thuringiensis* EGU378, *Bacillus* sp. EGU85, *Bacillus* sp. EGU367, *Bacillus* sp. EGU447, and *Proteus mirabilis* EGU30, and (ii) high H_2 producing abilities (MMC4) – *Enterobacter aerogenes* EGU16, *P. mirabilis* EGU21, *B. cereus* EGU43, *B. thuringiensis* EGU45, *B. pumilus* HPC464, *Bacillus* sp. HPC459, as reported earlier (Patel et al., 2010, 2012b). In each combination, different bacteria were mixed in equal proportions amounting to a final protein concentration of 10 μg/mL (Patel et al., 2010, 2012b). *B. cereus* EGU43 was used for PHB production from the effluents of the H_2 production stage.

2.3. Batch culture hydrolysis of PS

For batch-culture digestion, green PS (10% TS and 9.5% organic solids) collected from local market of Delhi (India) were cut into small pieces (1–2 cm). PSS (2 L) (1%, 3%, 5%, and 7% total solids (TS)) were prepared using distilled water in 5 L flask and inoculated with MHC2 and incubated at 37 °C for 2 days (Patel et al., 2012b). After hydrolysis, fibers and cells were removed by centrifugation at 4000 rpm for 30 min at 20 °C and the PSS hydrolysate so obtained was used for further studies.

2.4. Batch culture H_2 production from PSS hydrolysates

For all batch culture H_2 production, 250 mL of MHC2 hydrolysates of PSS (1–7% TS) obtained in the previous section were inoculated with MMC4 and incubated in 300 mL BOD bottles for H_2 production. The pH of the reactors was checked and adjusted to 7 using 2 N NaOH or 2 N HCl daily. Anaerobic conditions were maintained in the reactor by flushing with argon gas. The evolved gases were collected by water displacement method (Patel et al., 2012b) and their analyses were done until H_2 production ceased. The values presented here are based on three sets of experiments.

2.5. Effect of dilutions of PSS hydrolysate with nutrient medium

Different combinations – 9:1, 7:3, 1:1, and 3:7 of PSS hydrolysate (2% TS) to GM-2 or M-9 medium (final concentration of 0.1×) were used for the H_2 production. Supplementation of different combinations of PSS and media with glucose (0.5% w/v) was done to evaluate its effect on the H_2 and PHB yields and to improve the overall bioconversion process.

2.6. Continuous culture H_2 production from PSS hydrolysate by immobilized mixed bacterial culture (MMC4)

Dried ligno-cellulosic materials CC or GS were packed in polyvinylchloride (PVC) tube (length: 3 cm and diameter: 2 cm) and tied with a 10 cm^2 nylon net to develop a cartridge for immobilizing bacteria, as described in the previous study (Patel et al., 2010). PSS hydrolysate supplemented with 0.5% w/v of glucose was mixed with GM-2 (0.1×) medium, to achieve dilutions of 3:7, 1:1, 7:3 and 9:1. Continuous culture H_2 production was carried out at a hydraulic retention time (HRT) of 4 days over a period equivalent to 10 cycles (40 days), as described previously (Patel et al., 2010).

2.7. PHB production from effluent of H_2 production stage

Effluents (200 mL) from batch and continuous culture H_2 production stages were centrifuged at 6000 rpm, 4 °C for 20 min. Supernatant (adjusted to pH 7.2) was inoculated with PHB producing strain *B. cereus* EGU43 at the rate of 10 μg cell protein/mL and incubated at 37 °C for 48 h at 200 rpm. Effect of nutrient supplementation was studied with effluent of continuous culture stage (1:1) by the addition of fresh GM-2 (to a final concentration of 0.1×) with and without glucose (0.5% w/v). The values presented here are based on three sets of experiments.

2.8. Analytical methods

2.8.1. Gas analysis

The gas composition was determined using gas chromatography (Nucon GC5765, India) equipped with a thermal conductivity detector and argon as the carrier gas at a flow rate of 30 mL/min. Gas collection and analyses were done daily and H_2 production was calculated from the head space measurement of the total biogas produced (Porwal et al., 2008). Although no CH_4 was expected to be evolved in the absence of any added methanogens at any stage of experiments, however, all gases were evaluated for the presence of CH_4 produced by any contaminating methanogen.

2.8.2. PHB analysis

Samples (200 mL) were analyzed for dry cell mass (DCM) and PHB production. PHB produced was analyzed with GC–FID fitted with 10% Reoplex 400. Gravimetric estimation of the polymer yield was done from DCM by extraction of the polymer using chloroform and methanol as described in previous reports (Kumar et al., 2009; Patel et al., 2012b).

2.9. Characterization of produced PHB from the H_2 effluent

2.9.1. Fourier transform infrared spectroscopy (FTIR)

The chloroform extract of PHB (4 mg) was mixed thoroughly with KBr (Spectroscopic grade) and dried at 100 °C for 4 h. Infrared spectra of the PHB sample was recorded and analyzed on a single beam Perkin Elmer (Spectrum BX series, Sweden), with the following scan parameters: scan in the range of 4000–400 cm^{-1}; number of scans, 16 and resolution, 4.0 cm^{-1}.

2.9.2. Transmission electron microscopy

B. cereus EGU45 grown on the effluents of H_2 production stage (2 mL) were harvested after 48 h at 12,000 × g for 10 min and washed thrice with 100 mM sodium phosphate buffer (pH 7.4).