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Bioaugmentation of potent acidogenic isolates: A strategy for enhancing biohydrogen production at elevated organic load

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HIGHLIGHTS

- Augmentation with potent acidogenic isolates enhances biohydrogen production.
- *Bacillus subtilis* strain promoted a significant increase of the H_2 yield.
- Highest COD removal efficiency was observed with *Pseudomonas stutzeri*.
- Application of bioaugmentation strategy at higher loading was successfully evaluated.

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ABSTRACT

The efficiency of bioaugmentation strategy for enhancing biohydrogenesis at elevated organic load was successfully evaluated by augmenting native acidogenic microflora with three acidogenic bacterial isolates viz., *Bacillus subtilis*, *Pseudomonas stutzeri* and *Lysinibacillus fusiformis* related to phyla *Firmicutes* and *Proteobacteria* separately. Hydrogen production ceased at 50 g COD/l operation due to feed-back inhibition. *B. subtilis* augmented system showed higher H_2 production followed by *L. fusiformis*, *P. stutzeri* and control operations, indicating the efficacy of Firmicutes as bioaugmentation biocatalyst. Higher VFA production with acetic acid as a major fraction was specifically observed with B. *subtilis* augmented system. Shift in metabolic pathway towards acidogenesis favoured higher H_2 production. FISH analysis confirmed survivability and persistence of augmented strains apart from improvement in process performance. Bio-electrochemical analysis depicted specific changes in the metabolic activity after augmentation which also facilitated enhanced electron transfer. *P. stutzeri* augmented system documented relatively higher COD removal.

1. Introduction

Biological route of hydrogen (H_2) production is a potential alternative to fossil based production process. Biological process involves utilization of waste as major feed-stock for production of H_2 through acidogenic fermentative pathway along with simultaneous remediation of the waste (Venkata Mohan et al., 2007a Wang et al., 2008). This process has several advantages like lower cost, ease of control and simultaneous waste remediation associated with clean bioenergy generation in a unified and sustainable approach (Ren et al., 2010; Marone et al., 2012; Venkata Mohan et al., 2009a,b; Wang et al., 2008; Venkata Mohan and Pandey, 2013). The efficacy of acidogenic H_2 production depends on many biotic and abiotic factors. Among them, the nature of biocatalyst as well as composition and load of substrate (wastewater) plays a critical role in overall process efficiency (Vazquez and Valardo, 2009; Venkata Mohan 2008a,b).

Wastewater has a significant quantity of organic fraction, which can be metabolically converted to H_2 by acidogenesis (Venkata Mohan, 2009). However, the composition of wastewater and the concentration of organic load have a direct influence on the fermentation output. Increment in the organic load effects H_2 production positively to certain extent and after that acidification of system microenvironment by production of excess VFA and consequent reduction system buffer capacity gives negative impact (Mohanakrishna et al., 2010). The resulting drop in pH (<4.0) inhibits acidogenic bacteria (Venkata Mohan et al., 2008c; Wang et al., 2008). Thus to operate the bioreactor at higher organic load without undergoing processes inhibition bioaugmentation appears to be a feasible strategy operation.

Bioaugmentation implies addition of actively growing, desired and specialized microbial strains to native microbial community of the bioreactor in an effort to enhance the ability of the microbial

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community to withstand the process fluctuations and accomplish enhanced and improved treatment waste degradation. Bioaugmentation strategy has been used to improve the start up of a reactor (Ma et al., 2009), to enhance reactor performance (Venkata Mohan et al., 2005, 2009c; Raghavulu et al., 2013) and to protect the existing microbial community against adverse effects (Hajji et al., 2000; Venkata Mohan et al., 2005) or to compensate for organic or hydraulic overloading (Chong et al., 1997). Studies were performed to enhance H_2 production by means of bioaugmentation with different degrees of successes (Li and Fang, 2007; Venkata Mohan et al., 2007b; Wang et al., 2008; Ma et al., 2009; Kuo et al., 2012; Marone et al., 2012). The application of bioaugmentation strategy to enhance biological H_2 production by augmentation of native microflora by selectively enriched acidogenic consortia has been reported earlier (Venkata Mohan et al., 2007a). Recent studies demonstrated the effectiveness of bioaugmentation in improving H_2 production by *Clostridium acetobutylicum* (Wang et al., 2008). Fermentative bacteria capable of producing H_2 were added to the native mixed microflora to improve the yield (Marone et al., 2012). However in some cases, bioaugmented species failed to compete with indigenous population (Goldstein et al., 1985; Bouchez et al., 2000).

In the present communication, three potent acidogenic bacterial isolates viz., *Bacillus subtilis*, *Pseudomonas stutzeri* and *Lysinibacillus fusiformis* belonging to phylum Firmicutes and Proteobacteria were augmented to bioreactors inhibited due to operation at elevated organic load for enhancing process performance in terms of biohydrogen production. The viability of bioaugmentation strategy on process performance was studied by process monitoring in terms of H_2 production, substrate degradation, VFA production/composition, dehydrogenase (DH) activity along with bioelectrochemical and FISH analysis.

2. Methods

2.1. Biocatalyst

2.1.1. Anaerobic consortia

Anaerobic consortium collected from a full scale anaerobic reactor treating composite wastewater was used as parent inoculum. After sampling, the parent anaerobic consortium was sieved to separate the grit by nylon filter, resulting in thick sludge (3.6 g VSS/l) which then centrifuged (1500 g; 20 °C) and washed thrice with saline buffer. The resultant pellet was used for the inoculum preparation by applying different pretreatment methods separately. The performance of the bioreactors operated with augmented inoculums was evaluated with the reactors operating with untreated inoculums served as control.

2.1.2. Bioaugmentation strains and identification using 16S rRNA gene sequence

Three strains isolated from long term operated acidogenic bioreactors producing biohydrogen were used as biocatalyst for bioaugmentation. Genomic DNA of the three isolates was extracted as described earlier (Venkata Mohan et al., 2010). Total extracted genomic DNA was used as a template to amplify 16S rRNA by using universal primers with PCR (Thermal cycler, Eppendorf). The nucleotide sequences of the universal primers were as follows: primer 28F, 5′-AGA GTT TGA TCC TGG CTC AG-3′; primer 1542R, 5′-AAG GAG GTG ATC CAG CCG CA-3′ (Ueno et al., 2001). PCR reaction mixture (50 μl) consisted of Taq polymerase (5 U), 1.2 ml, 28F and 1542R (10 pmol), 1 ml DNA template (1 ng), 4 ml of PCR buffer (10×), 5 ml of $MgCl_2$ (25 mmol/l) and 37.6 ml of sterile water. PCR was operated for 30 cycles at 95 °C (50 s), 52 °C (40 s) and 72 °C (40 s). The amplified product was identified based on molecular mass by agarose gel electrophoresis using 1.2% agarose gel. The nucleotide sequences of 16S rRNA gene of the isolates were compared with other related sequences available in GenBank by BLAST programme. Further, the nucleotide sequence of isolates was aligned with closely related sequences (that were downloaded from GenBank) using ClustalW and pair wise evolutionary distances were computed. BLAST analysis confirms isolates belong to *B. subtilis* (Strain IICTSVMH13), *P. stutzeri* (strain IICTSVMHi) and *L. fusiformis* (strain IICTSVMH10) and the sequences of these strains is deposited with in the public domain (GenBank). The GenBank accession numbers of these strains are *B. subtilis* (FR849706), *P. stutzeri* (HE586324) and *L. fusiformis* (HE586325). Phylogenetic analysis was performed using MEGA version 4 and the phylogenetic trees were constructed using neighbourhood joining method of analysis. Confidence in the tree topologies was evaluated by re-sampling 100 bootstrap trees. Phylogenetic tree of three augmented strains depicts 100% sequence similarity with *B. subtilis*, *P. stutzeri* and *L. fusiformis* clusters with the type strains (Supplementary Fig. 1). Based on BLAST search programme and phylogenetic analysis revealed that all the isolates belonged to two major phyla *Firmicutes* and *Proteobacteria*.

Prior to augmentation, the strains were cultured overnight in specific growth media (LB broth; yeast extract-5 g/l, casein peptone-10 g/l, sodium chloride-10 g/l; pH 7). A loop full of overnight grown culture was inoculated into nutrient broth. After 12 h of incubation at 37 °C, the culture was centrifuged and washed twice in saline buffer (6500*g*, 20 °C), resuscitated in nutrient solution and augmented through wastewater feeding.

2.2. Experimental methodology

Four identical bench-scale anaerobic batch reactors (AnSBR) were fabricated using borosilicate-glass with total/working volume of 1.2/0.84 l and gas holding capacity of 0.36 l. The reactors were operated in suspended growth mode configuration continuously for 50 cycles. Each cycle comprised 48 h (hydraulic retention time, HRT) accounted by 20 min of FILL, 47 h of REACT (anaerobic), 20 min of SETTLE and 20 min of DECANT phases. The reactors were kept in suspension mode during the REACT phase. Predefined volume (0.72 l) of real field food wastewater was fed during the FILL phase at the beginning of each cycle and contents were subjected to continuous mixing (100 rpm). Appropriate measures were taken to maintain the reactors under anaerobic conditions. All the bioreactors were operated under similar conditions. Prior to bioaugmentation, the reactors were operated with real field food wastewater (the characteristics of the food waste prior to adjusting OLR are summarized in Supplementary Table 1) with COD load of 5 g/l, 10 g/l, 20 g/l and 30 g/l at room temperature (28 ± 2 °C) in sequencing batch mode operation (total cycle period of 48 h) and adjusting the influent pH to 6 (acidophilic conditions). At higher load (50 g/l COD) operation the system showed process inhibition and cessation of H_2 production. The bioreactors were then augmented with H_2 producing strains [Reactor 1 (Control, without augmentation), Reactor 2 (*B. subtilis*), Reactor 3 (*P. stutzeri*) and Reactor 4 (*L. fusiformis*)] through wastewater during feeding and operated under the same conditions until a stable performance was achieved. The four bioreactors were operated simultaneously for 50 cycles accounting for 100 days.

2.3. Analysis

Quantification of H_2 gas was carried out using a microprocessor based pre-calibrated H_2 sensor (ATMI GmbH Inc.).The fraction of H_2 in the biogas was determined by a gas chromatograph (Nucon) equipped with a thermal conductivity detector (TCD) and a 2.1/8″ × 2 m SS column with a molecular sieve (size 60/80 mesh)

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