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Identification of a novel hydrogen producing bacteria from sugarcane bagasse waste

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ABSTRACT

A new hydrogen producing strain with potent cellulose degrading ability was isolated from soil sample collected from sugarcane bagasse (SCB) storage yard. Among the colonies screened, the newly identified strain, *Bacillus subtilis* AuChE413 has shown better performance in terms of hydrogen yield and cellulose degradation. Morphological, physiological, and biochemical traits of the isolated strain were studied. And its molecular characterization was studied by analyzing the sequence of 16S rRNA gene. The sequence of *Bacillus subtilis* AuChE413 was compared with other GenBank sequences using BLASTn to study its homology with other species. Phylogenetic tree was constructed using the neighbor joining method. The hydrogen production capacity of *Bacillus subtilis* AUChE413 was tested by employing the pretreated SCB and sweet sorghum stalk biomass (SSB) as substrates. In comparison, the substrate sorghum stalk has produced the highest hydrogen yield of 55.21 H_2 /kg SSB at pH 7 and 37 °C.

1. Introduction

Due to depletion of non- renewable energy resources such as coal, petroleum, oil and natural gas the search of an alternative energy source has got priority in the current research. Search of sustainable and renewable nature of energy source is the need of the hour. Hydrogen is found to be a better alternative because of its environmental friendly nature. Hydrogen has high energy content (142 KJ/g) and its conversion leads to the formation of water. Biologically, hydrogen could be produced by photosynthetic and fermentative routes which are more environmentally friendly and less energy intensive compared to thermo- chemical/ electrochemical processes (Levin et al., 2004). Dark fermentative method of bio- hydrogen production has become more attractive due to the utilization of renewable resources, especially cellulosic and lignocellulosic biomasses as substrates, simple in operation, no light source is required and the process can be carried out at ambient temperature and pressure (Das and Veziroglu, 2001). Among the different fermentative hydrogen producing microorganisms, the genus *Enterobacter* (Fabiano and Perego, 2002; Nakashimada et al., 2002), *Bacillus* (Kalia et al., 1994) and *Clostridium* (Chin et al., 2003; Chen et al., 2008; Md. Saiful Islam et al., 2017) were studied extensively.

At present, most of the studies on bio-hydrogen production were confined to pure carbohydrates and carbohydrate-rich waste water as substrates (Fan et al., 2004; Wu et al., 2008; Lo et al., 2008a; Lin et al., 2008). Bio-hydrogen production from cellulosic biomass seems to be a better option due to the abundant availability of cellulosic biomass in the earth (Xing et al., 2009). Direct conversion of cellulosic biomass to hydrogen could not be possible due to the complex structure of cellulose. And thus, the utilization of pretreated cellulosic substrate is essential which reduces time and overall cost of the process. Only few studies have been reported on the direct conversion of cellulosic biomass to hydrogen and by using pretreated substrates either by physical/ chemical methods (Fan et al., 2006a, 2006b; Levin et al., 2006; Antonopoulou et al., 2008; Ntaikou et al., 2008; Shi et al., 2010; Nagaiah et al., 2012; Kumar et al., 2016a; Fujita et al., 2016). Most of the studies were restricted only on the degradation of cellulosic biomass using thermophilic and strict anaerobic bacteria especially *Clostridium species*. Though, bio-hydrogen research has been carried out for more than two decades, there is no commercial hydrogen production processes established. This is due to the high maintenance costs involved and low H_2 yields obtained. Economically viable fermentative hydrogen production process could be possible only by utilizing cellulosic biomass as substrates. Identification of a strain with potent cellulose degrading and hydrogen yielding ability would be a success in hydrogen fermentation process in future. The strain capable of converting cellulose and hemicellulose part of the cellulosic biomass with larger percentage is the crucial step in biomass fermentations used in fuel and other chemicals production applications (Goncalves et al., 2013; Ajijolakewu et al., 2017).

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In this work, we identified a mesophilic, facultative anaerobic bacterium isolated from SCB waste and employed for hydrogen fermentation. The isolated species was biochemically and molecularly characterized. The phylogenetic analysis of the isolated species was carried out to analyze its evolutionary relationship. The isolated mesophilic strain has special characteristics and thus it has been attracted and was used in this study. The hydrogen production ability of the strain was tested with the future renewable energy resources such as sugar cane bagasse and sweet sorghum stalk biomass.

2. Methodology

2.1. Strain isolation

The strain used in this study was isolated from soil samples collected from sugarcane bagasse storage yard. One-gram soil sample in 50 ml LB medium was incubated at 30 °C for overnight. 0.5 ml of the supernatant was transferred to 50 ml of the minimal salt medium supplemented with 0.1% w/v carboxy methyl cellulose (CMC). The minimal salt medium contains the following components in a liter of medium: 5.0 g NH_4HCO_3, 1.0 g $MgSO_4{\cdot}7H_2O$, 0.1 mg $ZnCl_2$, 0.1 mg $MnSO_4$, 0.1 mg $CuSO_4$, 4.0 mg $FeSO_4{\cdot}7H_2O$, and 2.0 mg $NaMoO_4$. The culture was incubated at 30 °C for 48 h. 0.5 ml of the culture broth was diluted to 50 ml using the same minimal salt medium and incubated at 30 °C for 48 h. The same procedure was repeated for three times to enrich the culture. After enrichment, the culture broth was spread on agar plate which contains minimal salt medium. Pure culture was obtained by spreading each colony on agar plates by repeating the procedure for three times. Hydrogen producing ability of the strain was tested for each colony and the best strain was selected based on its high hydrogen yielding capacity.

2.2. Identification of the strain

A large fragment of 16S rRNA gene was amplified by PCR using universal primers as 27F (5′- AGA GTT TGA TCM TGG CTC AG- 3″) and 1492 R (5″- TAC GGY TAC CTT GTT ACG ACT T-3″). PCR amplification conditions are 94 °C for 45 s, 55 °C for 60 s and 72 °C for 60 s performed for 35 cycles. The PCR product was purified using a Montage PCR clean up kit (Millipore). PCR product was sequenced using 518 F/800 R (518F- CCA GCA GCC GCG TAA TACG; 800R- TAC CAG GGT ATC TAA TCC) primers. Sequencing reactions were performed using an ABI PRISM BigDyeTM Terminator Cycle Sequencing kit with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems) (Gutell et al., 1990). The 16S rRNA gene was sequenced in Synergic scientific services, Chennai.

2.3. Phylogenetic tree construction

Using the BLASTn search tool, the sequence of *Bacillus subtilis* AuChE413 was compared. The obtained sequences were further aligned by multiple sequence alignment (MSA) using the CLUSTAL X program (Tompson et al., 1997). Phylogenetic tree construction by the neighbor joining method was performed using the Mega3.1 program (Kumar et al., 2004, 2016).

2.4. Feed stock preparation and treatment

Fresh sugarcane bagasse was obtained from nearby sugar industry (MRK Sugar Mills Pvt. Ltd., Cuddalore, India). It was cleaned by removing dirt and other materials and stored at - 20 °C. The fresh sorghum stalk was collected from farm land near Salem, India. The leaves of sorghum stalk were removed and cut into 15–20 cm length and stored at − 20 °C. The contents of pretreated SCB and sweet sorghum stalk were analyzed using standard procedure (Ververis et al., 2007). The compositions are as follows: SCB- cellulose 34.5%, hemicelluloses 21.5% and lignin 5.57%; Sorghum stalk- cellulose 45.3%, hemicelluloses 26.3% and lignin 15.2%. The biomass was ground using the laboratory mixer grinder to an average particle size of 2–3 mm. Pretreatment of biomasses were carried out using 0.5% HCl by

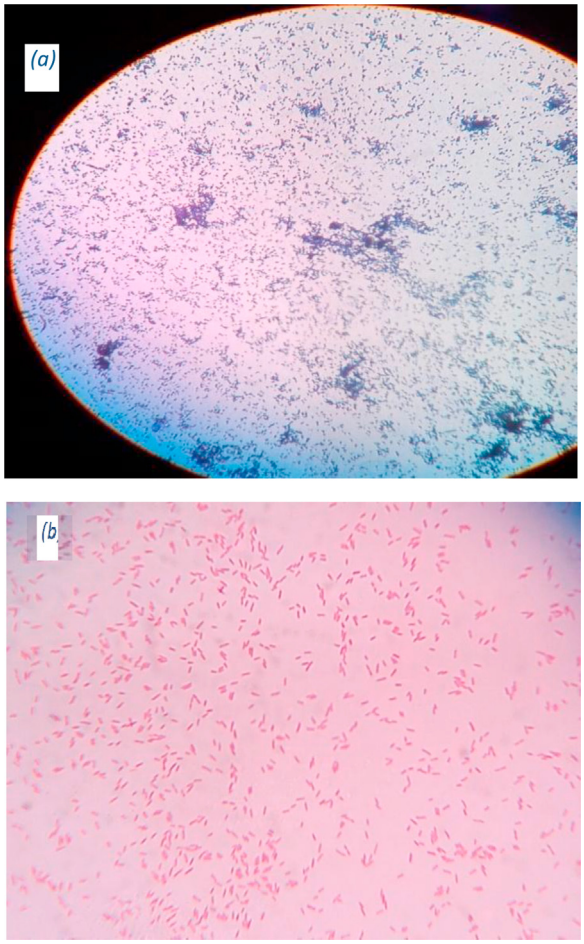

Fig. 1. Phase contrast micrograph of *Bacillus subtilis* AuChE413 conforming the morphological tests. (a) Gram staining; (b) Endospore staining.

Table 1
Comparison of related hydrogen producing strains with newly identified strain using MSA.

Microorganism	Strain	Accession no.	% Similarity
Bacillus subtilis	AuChE413	JX471147	100
Brevibacterium halotolerans	DSM_8802	NR042638	97
Bacillus mojavensis	IFO15718	NR024693	97
Bacillus subtilis subsp.spizizenii	NRRL_B-23049	NR024931	97
Bacillus vallismortis	DSM11031	NR024696	97
Bacillus atrophaeus	JCM9070	NR024689	97
Bacillus amyloliquefaciens	NBRC15535	NR041455	96
Bacillus sonorensis	SMPGMb7	JX280500	96
Bacillus licheniformis	NBRC 12200	NC_006270.3	95
Bacillus sonorensis	NRRL B-23154	NR025130	95
Bacillus aerius	UAC-16	JX475117	94
Bacillus pumilus	NBRC 12092	NR_112637.1	93
Bacillus mycoides	NBRC 101228	NR_113990.1	91
Bacillus cereus	PG2	KF804071.1	91
Clostridium butyricum	TM-9B	FR734080.1	82
Bacillus thuringiensis	BHUVB22	FJ905907.1	81
Clostridium sp.,	HR1	NC_0012665.1	80
Escherichia coli	EGY	JF412031.1	75
Rhodoplanes serenus	SS-10	JX282404.1	75
Pantoea agglomerans	SS -10	NZ_ JNGC01000001.1	74