Isolation and Screening of Hydrogen Producing Bacterial Strain from Sugarcane Bagasse Yard Soil

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Abstract: The aim of this study is to isolate a highly competent bacterium with potent cellulose degrading capability and a better hydrogen producer. Soil sample from sugarcane bagasse yard was isolated, serially diluted and plated on cellulose specific nutrient agar plate. Four colonies have been isolated in which a single colony has potent cellulose degrading ability and the highest hydrogen productivity of 275.13 mL H_2 L^{-1}. The newly isolated bacterium was morphologically and biochemically characterized. The molecular characterization of the bacterium was carried out using 16S rDNA sequencing and the organism was identified as *Bacilllus subtilis* AuChE413. Proteomic analysis such as MALDI-TOF was carried out to differentiate the isolated *Bacillus subtilis* from *Bacillus thuringiensis* and *Bacillus amyloliquefaciens*. Phylogenetic tree was constructed to analyze the evolutionary relationship among different genus and species with the newly isolated strain.

Keywords: Hydrogen producer, cellulose degrader, biochemical and molecular characterization, phylogenetic analysis

1. INTRODUCTION

Global sustainable development is focused in our present condition due to environmental degradation and energy crisis. Nearly 80% of energy consumption mostly depends on the fossil fuels such as coal, oil and natural gas, which results in the rapid exhaustion of fossil fuels [1]. In addition to that, the process involved during the conversion of fossil fuels to energy results in global warming and drastic climate change problems in the environment. Energy gap should be filled with the new, alternative energy source around the world. In that way, the first generation biofuels like bioethanol and biodiesel have been progressed by the researchers using food crops such as corn, sugar crops and palm oil as organic substrates. This leads to indirect impact on the rise of food price and thus contributes to global food crisis. Further, the researchers are much focused in developing the second generation biofuels by utilizing the agricultural residues as organic substrates and resulting in the production of biofuels [2].

"Environmental biorefinery" is the optimization of process conditions for the production of wide range of products from biomass through designing the specific installations [2]. The suggestion of hydrogen is more advantageous due to its harmless nature to environment and the mammals [3,4,5,6]. At present, fossil fuels are mainly employed to generate 88% of H_2 production [7]. Hydrogen is the ideal and best alternative to fossil fuels due to its environmental friendly nature and zero green gas emissions. Its oxidative combustion can lead only to the formation of water and it has its higher energy content of 142 KJ g^{-1}[6]. At present, the different chemical methods have been employed for the production of H_2 such as water electrolysis, steam reforming by methane and thermocatalytic reformation of H_2 rich substrates [8,9,10,11,12]. The mostly used method for the synthesis of H_2 are water electrolysis and steam reformation

by methane. About 4% of H_2 from the total hydrogen production have been produced by water electrolysis method. All the chemical methods of H_2 production are energy intensive and unsustainable but the operation of the process is simple. Now a-days, the energy source of H_2 is utilized for refining diesel by using H_2 as one of the chemical reactants and also in the synthesis of ammonia. But the commercialization of H_2 has not yet implemented due to problems in storage facilities and transportation methods and highest production costs [5,13,14,15] but the research on optimization is under progress [16]. The main emerging drawback of H_2 is the storage facility due to lesser volumetric density at normal condition is 3.00 k Wh m^{-3} compared to 9.97 kWh m^{-3} for methane [17].

Hence, the alternative best method for the synthesis of H_2 is through biological routes that require less energy, carry out the operations at ambient temperature and pressure and can be highly developed based on performance and design [7,10]. The different routes of biohydrogen production are direct and indirect biophotolysis by green algae and cyanobacteria respectively, photo fermentation using photosynthetic bacteria and dark fermentation using obligate or facultative anaerobic bacteria [8, 10, 11, 18, 19, 20, 21, 22, 23]. In contrast to different biohydrogen production processes, dark fermentative method is a key technology due to its no requirement of light source for operation, higher H_2 evolution rate (mmol $L^{-1}h^{-1}$) and the complex lignocellulosic wastes could be degraded biologically by cellulose and hemicellulose degrading bacteria resulting in an effective hydrogen production [12, 14, 24]. Major drawback of dark fermentation is the low yield (less mole of H_2 per mole of substrate consumed) which is mainly due to the poor knowledge on the fundamentals of metabolism of each microorganism [25].

Due to abundant availability of low cost effective feedstocks of lignocellulosic biomass such as grasses, water plants, soft woods, hard woods and agricultural residues like bagasse, straw and reeds, it can be effectively utilized for the conversion of biomass to biofuels and other chemicals with environmental, strategic and economic advantages [26]. Cellulose occupies a major component in the lignocellulosic biomass along with the smaller quantities of hemicellulose and lignin. Cellulose is a homopolymer composed of the linkage of 1→4 β D- glucose residues in a native form and these type of cellulose is classified as cellulose I. Each of the polymer chains of cellulose are assembled together as bundles which is called as microfibrils or fibrils [27]. The two phases of cellulose of cellulose I_a and I_b are present in the structure of cell wall of the plants along with amorphous cellulose [28,29,30]. The rare form of cellulose I_a is available only in few green algae [30] but the higher availability of cellulose I_b is present in the cell wall of higher plants and in some of the green algae.

Due to highest proportion of cellulose in the lignocellulosic wastes, it is used as a raw material for the production of pulp, paper and other value added products [31]. Hence for the utilization of complex lignocellulosic waste as organic streams, the microorganism must be competent in degrading cellulose and thus the bacterium must have an inbuilt cellulose degrading systems. The hydrolytic enzymes named as cellulases that perform the degradation of complex cellulose systems in nature. The complex systems of enzymes present in the cellulolytic systems such as endo- 1,4 β- D- glucanase (endoglucanase, EC3.2.1.4), 1,4- β-D- glucan- cellobiohydrolase (exoglucanase, EC 3.2.1.91) and β-D- glucoside glucanohydrolase (cellobiase, β-glucosidase, EC 3.2.1.21) which acts together for the degradation of cellulosic substrate [32, 33. Enzymatic cellulose degrading systems are available only in few of the bacterial species.

In this article, the challenging effective biohydrogen producing bacteria with inbuilt cellulose degrading ability was isolated from the soil and was further characterized. The basic microbiological investigations of the bacterium was carried out to improve the overall strain performance and further it will be helpful in the bioprocess engineering research during the optimization of different operating parameters in biohydrogen productivity.

2. MATERIALS AND METHODS
2.1 Medium components and analytical instruments used
Nutrient broth and other chemicals used for mineral salt medium (MSM) preparation, genomic DNA isolation, MALDI-TOF analysis were procured from Himedia Limited, Mumbai, India. The gas products mainly hydrogen was analyzed using Chemito 7610 gas chromatography, Chemito Instruments private limited, India. UV spectral analysis were carried out using UV/Vis Biospectrophotometer (EliCo private limited, India) to read the absorbance. MALDI-TOF mass spectral analysis was done using Microflex, Bruker Daltonics Inc., USA. The forward and reverse primers for the amplification of template DNA were procured from Eurofins, Bangalore,

India. Purification and sequencing of PCR product was carried out using a wizard PCR DNA purification system (Promega, Madison, Wis.,) and ABI PRISM 310 automated sequencer (PERKIN- ELMER, Conn.) as described in the manual for the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit respectively [34].

2.2 Media preparation
The composition of MSM is as follows (g L^{-1}): Na$_2$HPO$_4$, 3.6; KH$_2$PO$_4$, 1.6; (NH$_4$)$_2$ SO$_4$, 1.0; Mg SO$_4$, 1.0; CaCl$_2$. 2H$_2$O, 0.10; Fe (NH$_4$) citrate, 0.01; agar, 15.0 and trace elements solution, 10 ml. The trace element solution contains the following constituents (mg L^{-1}): ZnSO$_4$. 7H$_2$O, 10.0; CoCl$_2$. 6H$_2$O, 1.0; MnCl$_2$. 4H$_2$O, 3.0; NO$_2$MoO$_4$. 2H$_2$O, 3.0; NiCl$_2$. 6H$_2$O, 2.0; CuCl$_2$. 2H$_2$O, 1.0 and H$_3$BO$_3$, 3.0 and pH of the medium was adjusted to 7.0 ± 0.05. Nutrient broth contains the following composition in g L^{-1}: beef extract, 1.0; yeast extract, 2.0; peptone, 5.0 and sodium chloride, 5.0 and pH of the medium was adjusted to 7.0 ± 0.2.

2.3 Strain isolation
The strain used in this study was isolated from soil samples collected from sugarcane bagasse storage yard. One gram of soil sample was mixed with 50 ml sterile distilled water. The supernatant was serially diluted upto 10^5 dilution and spread plated on CMC agar (MSM supplemented with 0.6% (w/v) CMC). The plates were incubated under aerobic condition at 37 ℃ for 48 h. The grown colonies were isolated and inoculated separately in fermentation medium supplemented with 0.6% CMC as sole carbon source. The percent hydrogen content present in the biogas was estimated by Gas Chromatography. It was equipped with thermal conductivity detector (TCD) connected with packed column. 20μL of the gas was taken from the headspace with the help of air tight syringe and injected into the column. Nitrogen was used as a carrier gas at a flow rate of 2 ml/min. The operational temperature of the injection port, detector and oven were maintained at 150℃, 200℃ and 80℃ respectively. The highest hydrogen producing strain was selected for further studies.

2.4 Identification of the bacterial strain using 16S rDNA sequencing
Morphological and biochemical characterization of the isolated strain was carried out by following the standard procedures [36]. An isolated strain was inoculated in 1.5 ml Luria broth (LB) medium and grown overnight in a shaking incubator at 37 ℃. The grown culture was centrifuged at 10,000 rpm for 10 minutes. From the pellet, chromosomal DNA was isolated using the following procedure. The procedure includes disruption of cells by cell-lysing solution, RNase treatment, phenol/chloroform extraction and ethanol precipitation [37]. The extracted DNA was run in agarose gel electrophoresis and visualized under UV light. The quantity and the purity of the extracted DNA were estimated by measuring the UV absorbance at 260 and 280 nm spectra.

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 1522R (5"- AAG GAG GTG WTC CAR CC-3"). PCR amplification was carried out using the master mix containing dNTP at a concentration of 200 μM, each of the primers at a concentration of 4 μM, 100 ng of template DNA, and 2.5U DNA polymerase in a total volume of 50 μL. PCR reaction conditions were as follows: (i) 2 min at 95 ˚C, (ii) 30 cycles of 30 s at 95 ˚C, 30 s at 50-55 ˚C, and 5 min at 72 ˚C, (iii) 5 min at 72 ˚C. Further, the PCR product was purified and sequenced. After sequencing, the closest known relatives of the new isolate were determined using the Basic Local Alignment Search Tool (BLAST) algorithm (BLAST tool) available at National Center for Biotechnology Information (NCBI) by performing a nucleotide database search.

2.5 Confirmation of the species using MALDI-TOF analysis

Strain identification was carried out by MALDI-TOF MS analysis. The procedure is as follows: A portion of strain at the exponential growth phase was smeared onto a 96-well target plate. After drying, 1 μl of α- cyano- 4- hydroxy cinnamic acid (CHCA) matrix solution was used to cover the surface of the microorganism. The dried target plate was loaded into the machine, which was equipped with a 337-nm nitrogen laser. In the mass range of 2 to 20 kDa, the spectra were recorded in the linear mode and subsequently analyzed using MALDI Biotyper Automation Control and Biotyper 2.0 software.

2.6 Phylogenetic tree construction

For making evolutionary studies, the sequence homology was obtained by nucleotide- nucleotide BLAST (BLASTn) search tool. Phylogenetic analysis was performed with the Clustal X program [38]. Phylogenetic tree construction and bootstrap analysis were performed using the Mega 7 program. Phylogenetic tree was constructed using neighbour-joining method available in the Mega 7 program [39,40].

3. RESULTS AND DISCUSSION

The soil sample was serially diluted and spread plated on mineral salt medium containing CMC as sole carbon source and further the plates were incubated at 37 ℃. In 10^5 dilution, the four colonies were grown on the specific growth media and each of the colony showed different morphologies. The four colonies can effectively degraded the cellulosic substrate, CMC. After isolation, the hydrogen productivity of the four colonies was analyzed by using glucose as carbon source. Among the different colonies, cellulose degrading single colony with highest hydrogen yield was selected for further studies.
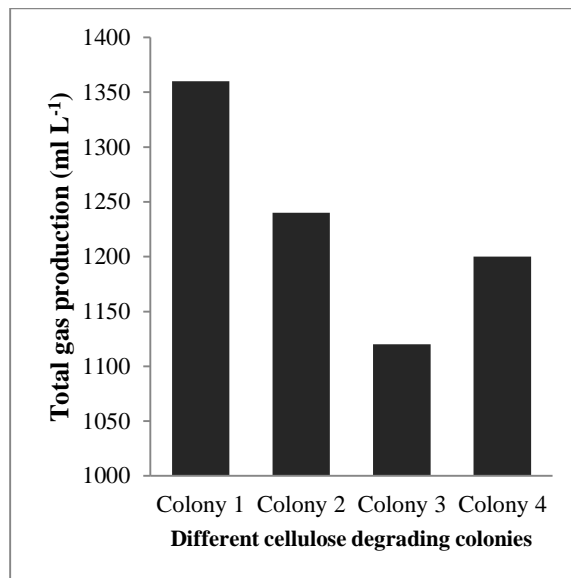

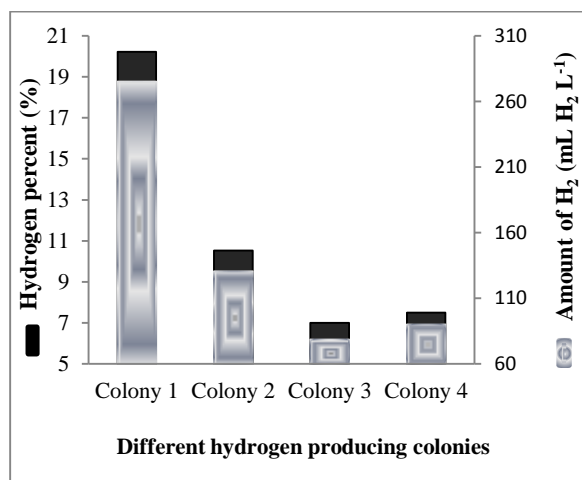**Figure 1 Total gas production of different cellulose degraders and hydrogen producers**

Figure 2 Hydrogen percent in total gas production and hydrogen productivity of the different isolated colonies

The isolated four colonies have the potent to degrade cellulose and capable of producing hydrogen. Among the four colonies, colony 1 showed the highest hydrogen productivity of 275.13 mL H_2 L^{-1}. 20.23% of H_2 was present in total gas produced of 1360 mL L^{-1} (Figure 1 and 2). The highest hydrogen produced strain was further biochemically and morphologically characterized.

3.1 Characterization of the highest hydrogen producing strain

During primary identification, the colony morphology was thoroughly analyzed. Colony forming units (CFU) of the isolated bacterium were appeared as cream coloured, the

edges are circular, flat and it formed an undulate margins. The shape of the colony was appeared as like bacilli when cultured on the nutrient agar plate. In the nutrient broth culture, the isolate was in the form of pellicle with little or no turbidity. The isolated highest hydrogen yielding strain is a Gram positive and rod-shaped bacterium (Fig. 1.3) which was visualized under 1000X total magnification (100X lens × 10X eyepiece magnification) oil immersion light microscope. Differential staining technique such as endospore staining is mainly to differentiate the vegetative cells from endospore formation. After staining, both vegetative cells and endospore cells were observed under 1000X total magnification (100X lens × 10X eyepiece magnification) oil immersion light microscope (Fig. 1.4). The spore formation was occurre at the end of the exponential growth phase or during the time of substrate depletion. From the motility test, it was observed that the isolate is motile.
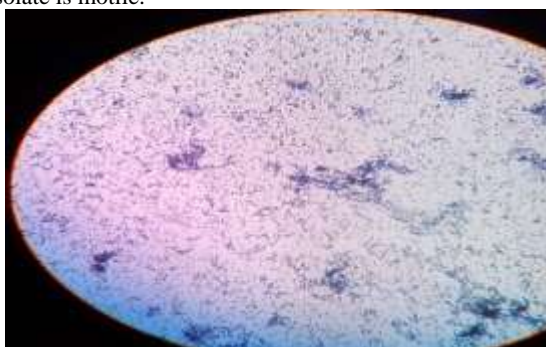

Figure 3 Gram staining of the highest hydrogen producing colony

Figure 4 Endospore staining of the highest hydrogen yielding strain

The different secondary biochemical characterization methods such as IMViC (Indole, methyl red (MR), Voges-Proskauer (VP), citrate utilization) tests, sugar utilization, nitrate reduction, starch, urea and gelatin hydrolysis, oxidase and catalase tests were carried out to identify the exact genus and species (Table 1).

The isolate has the ability to produce organic acids by utilizing different sugars such as glucose, sucrose, lactose and mannitol. Nitrate could be reduced by the isolate to nitrite. The isolated microorganism can hydrolyze starch which was identified by the formation of black colour in the agar plate containing minimal medium with starch as sole carbon source. Urease and gelatinase were produced by the

microorganism that was confirmed through the hydrolysis of urea and gelatin. The organism showed negative result in the oxidase test. Catalase is the enzyme responsible for the detoxification of hydrogen peroxide into water and oxygen gas (Eq. 1). The isolate showed positive result in the catalase test.
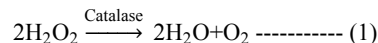
$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2 \text{ ----------- (1)}$$

Table 1 Biochemical characterization of the highest hydrogen producing isolate

S. No.	Biochemical tests	Results observed
01.	Gram staining	Gram positive
02.	Endospore staining	Spore formation occurs
03.	Motility test	Motile
04.	Indole test	Negative
05.	MR test	Negative
06.	VP test	Positive
07.	Citrate Utilization test	Positive
08.	Starch hydrolysis	Positive
09.	Nitrate reduction test	Positive
10.	Gelatin hydrolysis	Positive
11.	Oxidase test	Negative
12.	Catalase test	Positive
11.	Glucose fermentation	Acid formation occurs
12.	Sucrose fermentation	Acid formation occurs
13.	Lactose fermentation	Acid formation occurs after 48 h of growth
14.	Mannitol fermentation	Acid formation occurs after 48 h

3.2 Growth curve of an isolate, *B. subtilis* AuChE413

The viable cells present at different time intervals were analyzed using cell count and optical density measurements. The growth curve of the bacteria was plotted using incubation time and its corresponding optical density at 600 nm (Figure 5). The cells grew favorably at 37 °C and there is a minimum lag phase in the simple substrate, glucose.
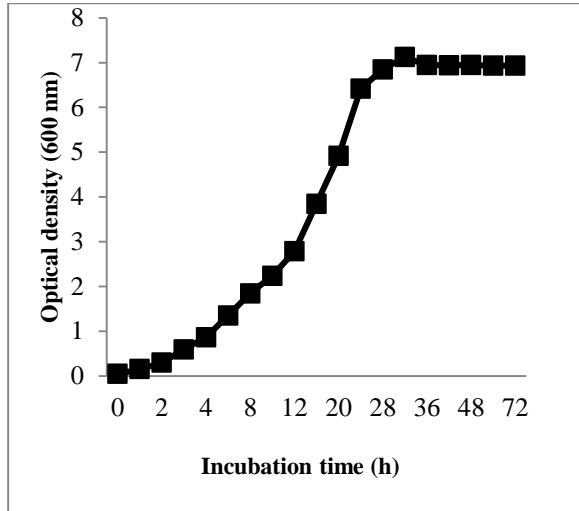
Figure 5 Growth curve of an isolate

The doubling time (or) the generation time of the bacteria is calculated by using Eq. (2) and (3).

$$k = \frac{\log N_t - \log N_0}{0.301\,t} \text{ ----------- (2)}$$

$$g = \frac{1}{k} \text{ ------------- (3)}$$

Based on the growth rate constant (k), the mean generation time or doubling time was calculated [40]. The doubling time of an isolate is 120 min i.e., 120 min/ gen or 0.5 generations h^{-1}. Researchers have also reported that the doubling time of *B. subtilis* is 120 min [41,42]. The microorganism attains the sporulation stage 12 hrs after the end of exponential phase which was observed through phase contrast microscope. During this stage, the spores are mainly formed to compensate the unfavorable conditions created in the medium due to the production of primary and secondary metabolites and the depletion of medium components [43]. The isolate, *B. subtilis* AuChE413 grows well in cellulose and starch medium rather than in glucose medium. It grows well in aerobic as well as in anaerobic environment. Thus, the strain is a facultative anaerobic microorganism.

3.3 Specific growth rate of an isolate at different temperatures and pH

The specific growth rate of the isolate at different temperatures was obtained from the viable cells at different time intervals corresponding to different temperatures. Specific growth rate (h^{-1}) was calculated for each temperature using Eq. (4).

$$log_{10} \frac{N_2}{N_1} = K . \frac{(t_2 - t_1)}{2.303} \text{ ------ (4)}$$

Table 2 Growth parameters of the isolate incubated at different temperatures at pH 7

Temperature (℃)	Specific growth rate (h^{-1})	Regression (R^2)
20	0.160	0.999
30	0.188	0.987
32	0.215	0.989
35	0.297	0.992
37	0.347	0.994
40	0.184	0.991
45	0.182	0.972
50	0.174	0.986

Based on the specific growth rate at different temperatures, the isolate exhibited significant growth upto 40-50 ℃ and the maximum growth falls between 30-35 ℃ [44]. The maximum growth of the bacterium was achieved at an incubation temperature of 35 ℃ (Table 2).

Table 3 Growth parameters of the isolate at different pH conditions with a constant temperature of 37 ℃

pH	Specific growth rate (h^{-1})	Regression analysis (R^2)
4	0.180	0.987
5	0.196	0.996
6	0.204	0.995
6.5	0.297	0.990
7	0.347	0.992
8	0.189	0.986
9	0.147	0.991

Maximum specific growth rate was achieved at pH 7 and hence it is considered as an optimum pH for the growth of the strain (Table 3). Compared to pH 6.5, there was 14% more growth rate was obtained at pH 7. Hence, pH plays an important role in obtaining the maximal growth.

3.4 Molecular characterization of the highest hydrogen yielding strain

To identify the exact species, the isolated highest hydrogen yielding strain was molecularly characterized using 16S rRNA sequencing.

Figure 6 shows the FASTA format of the gene sequence. The obtained gene sequence of the isolate was compared with the known gene sequence of the microorganism found in the National Center for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST). To find the similar sequences, BLAST uses heuristic method in which the short matches between query and known database sequences has been find out. After seeding, local alignment between the sequences was carried out. Finally based on the Expectation or expect value "E" (also identified as E- score), the comparison results were analyzed which signifies the high- scoring segment pair (HSP). Through BLAST analysis, the gene sequence of the isolate showed 99% identity with *Bacillus subtilis* and the novel strain was named as *Bacillus subtilis* AuChE413. The obtained gene sequence was uploaded in NCBI with the reference identification number JX471147.1 .

>gi|406822415|gb|JX471147.1| *Bacillus subtilis* strain
AuChE413 16S ribosomal RNA gene, partial sequence
CGGGCGATTGGGGCGGCGGTGGCCCTTATACAT
GCAAAGTCGAGCGGACAGATGGGAGCTTGCTC
CCTGATGTTAGCGGCGGACGGGTGAGTAACACG
TGGGTAACCTGCCTGTAAGACTGGGATAACTCC
GGGAAACCGGGGCTAATACCGGATGGTTGTTTC
AACCGCATGGTTCAAATATAAAAGGTGGCTTCG
GCTACCACTTACAGATGGACCCGCGGCGCATTA
ACTAGTTGGTGAGGTAACGGCTCACCAAGGGA
ACGATGCGTAGCCGACCTGAAAGGGTGATCGG
CCACACTGGGACTGAAACACGGCCCAAACTCCT
ACGGGAGGCAGCAGTAGGGAATCTTCCGCAAT
GGACGAAAGTCTGACGGAACAACGCCGCGTGA
GTGATGAAGGTTTTCGGATCGTAAAACTCTGTT
GTTAGGGAAGAACAAGTACCGTTCGAATAGGG
CGGTACCTTGACGGTACCTAACCAGAAAGCCAC
GGCTAACTACGTGCCAGCAGCCGCGGTAATACG
TAGGTGGCAAGCGTTGTCCGGAATTATTGGGCG
TAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGAT
GTGAAAGCCCCGGCTCAACCGGGGAAGGTCA
TTGGAAACTGGGGAACTTGAGTGCAGAAGAAG
AGAGTGGAATTCCACGTGTAGCGGTGAAATGCG
TAGAGATGTGGAAGAACACCAGTGGCGAAGCG
ACTCTCTGGTCTGTAACTGACGCTGAAGAGCGA
AAGCGTGGGGGAGCGAACAAGATTAGATACCC
CTGGTAGTCCACGCC

**Figure 6 16S rDNA sequencing of the newly isolated
bacterium**

3.5 Confirming the isolated strain by MALDI-TOF analysis

Ample of literature studies have reported on difficulty in
differentiating *B. subtilis* from *B. amyloliquefaciens* due to
similarity in 16S rRNA sequences (Wang et al., 2007;
Fernandez- No et al., 2013). The complementary method
through phylo proteomic analysis to the genetic analysis
such as 16S rRNA sequencing is Matrix- assisted laser
desorption ionisation- time of flight mass finger printing
(MALDI- TOF) analysis. This method can accurately
identify and differentiate the *Bacillus* at its species level
particularly *B. cereus* and *B. subtilis* from *B. thuringiensis*
and *B. amyloliquefaciens* (Fernandez-No et al., 2013). The
isolated bacterium was confirmed at its species level using
MALDI- TOF analysis. The spectral result of the isolate was
obtained and it was shown in Figure 7. By comparing the
mass profiling ratio (m/z) and intensity of the peak with the
reference database, score value was provided by the Real-
Time Classification Software (RTC) installed in Bruker
Daltonics MALDI- TOF which confirms the microorganism
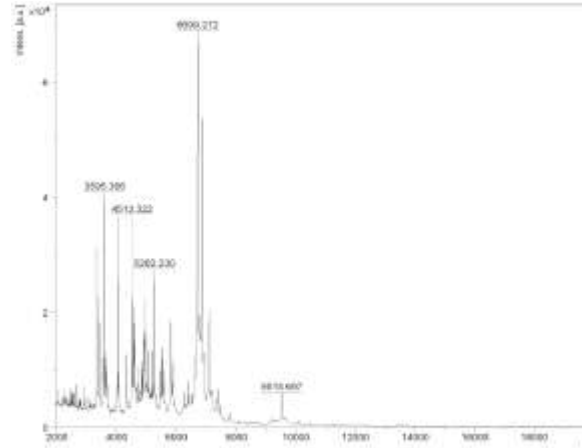as *Bacillus subtilis*.

**Figure 7 MALDI- TOF analysis to confirm the
species level**

3.6 Comparison of new isolate with different *Bacillus* strain

The isolate was compared with different other *Bacillus* sp.,
using 16S rRNA sequencing by multiple sequence
alignment.

**Table 4 Comparison of 16S rRNA sequencing of different
Bacillus sp., strains with *B. subtilis* AuChE413 (new
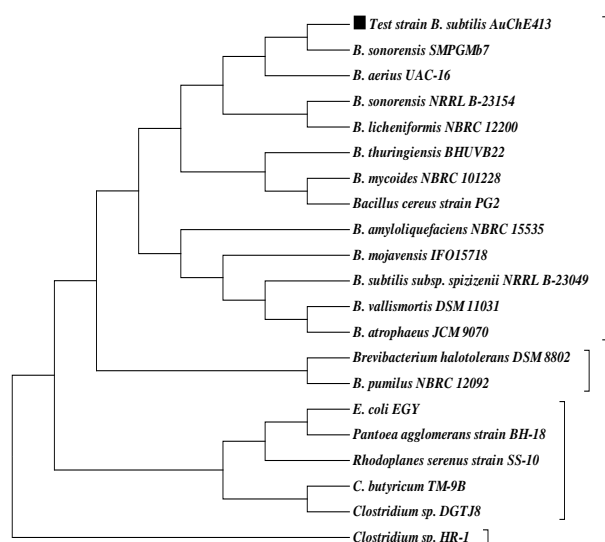isolate) by Multiple Sequence alignment (MSA)**

Microorganism	Strain	Accession No.	% similarity
New isolate, *B. 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 sonorensis	NRRL_B-23154	NR025130	95
Bacillus aerius	UAC-16	JX475117	94

The isolated soil bacterium was highly correlated with *B.
subtilis* strains and with other *Bacillus sp.,*. It showed 97%
similarity with *B. mojavensis, B. vallismortis, B. subtilis
subsp. spizzenii, B. atrophaeus* and *Brevibacterium
halotolerans.* The organism showed 96% similarity with *B.*

amyloliquefaciens and *B. sonorensis* and 94% similarity with *B. aerius* (Table 4).

3.7 Phylogenetic analysis

The phylogenetic analysis of the isolated strain was compared with different genus such as different *Bacillus sp., Clostridium sp., E. coli, Pantoea sp.,* and *Rhodoplanes sp.,* and is represented in Figure 8. Initially, the 16S rRNA sequence of all the strains were collected from the database NCBI. The multiple sequence alignment was carried out using CLUSTAL W and the phlyogenetic tree was constructed using maximum likelihood method. The isolated strain was closely related with some of the *Bacillus sp.,* such as *Bacillus sonorensis, aerius and licheniformis.* The isolated strain was much different from few of the microorganisms such as *Clostridium, Rhodoplanes sereus, Pantoea agglomerans and E. coli.*

Figure 8 Phylogenetic relationship of an isolate with other *Bacillus sp.,*

CONCLUSION

A competent strain with the ability of hydrogen producing and cellulose degrading was the major target during isolation of bacterium from the soil. Among the different colonies, a single colony with an ability of degrading the complex substrate, cellulose obtained the highest hydrogen productivity of 275.13 ml H_2 L^{-1}. The isolated bacterium is a Gram positive, *Bacillus sp.,* which was inferred from the morphological and biochemical characterization. Further, the bacterium was molecularly characterized using 16 S rDNA analysis. The obtained sequence was compared with the known gene sequence available in the NCBI database using BLAST tool and named as *Bacillus subtilis* AuChE413. Further, the bacterium was confirmed using proteomic analysis by MALDI-TOF. Phylogenetic tree was constructed to compare the evolutionary analysis between the species and different genus.

Conflict of Interest

The authors did not declare any conflict of interest.

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