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Improved microbial conversion of de-oiled Jatropha waste into biohydrogen via inoculum pretreatment: process optimization by experimental design approach

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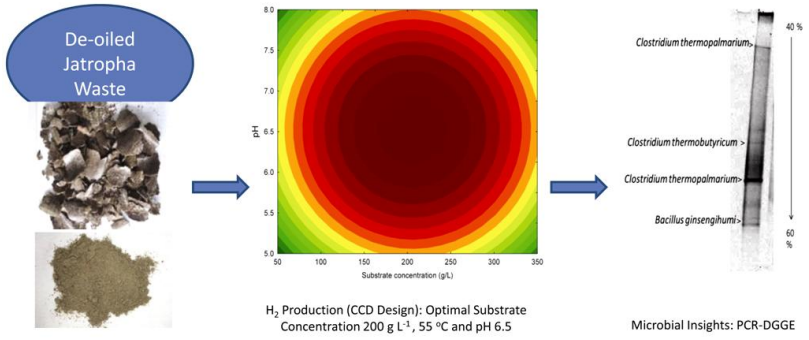
HIGHLIGHTS

- *Enhanced hydrogen fermentation via heat-treated inoculum and statistical optimization.*
- *Production was increased by nearly 4 folds through using pretreated inoculum.*
- *Peak hydrogen production rate of 1.42 ± 0.03 L H2/L-d was achieved.*
- *An insight into the microbial aspects of the process was achieved bt PCR-DGGE.*

GRAPHICAL ABSTRACT



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ABSTRACT

In this study various pretreatment methods of sewage sludge inoculum and the statistical process optimization of de-oiled jatropha waste have been reported. Peak hydrogen production rate (HPR) and hydrogen yield (HY) of 0.36 L H_2/L-d and 20 mL H_2/g Volatile Solid (VS) were obtained when heat shock pretreatment (95 ^oC, 30 min) was employed. Afterwards, an experimental design was applied to find the optimal conditions for H_2 production using heat-pretreated seed culture. The optimal substrate concentration, pH and temperature were determined by using response surface methodology as 205 g/L, 6.53 and 55.1 ^oC, respectively. Under these circumstances, the highest HPR of 1.36 L H_2/L-d was predicted. Verification tests proved the reliability of the statistical approach. As a result of the heat pretreatment and fermentation optimization, a significant (~ 4 folds) increase in HPR was achieved. PCR-DGGE results revealed that *Clostridium* sp. were majorly present under the optimal conditions.

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1. Introduction

The facts of ever increasing energy consumption, diminishing reservoirs of fossil fuels and threatening environmental problems awaked the scientists to find acceptable fuel alternatives both from the ecological and energetic points of views. As a result, various green energy carriers have been proposed, among which hydrogen is a promising candidate due to its unique characteristics (Akil and Jayanthi, 2014). However, the majority of hydrogen is currently derived from non-renewable sources such as methane conversion and oil/naphtha (Ewan and Allen, 2005). Consequently, clean technologies should be developed to make hydrogen a more attractive energy carrier. For this purpose, biological approaches are among the emerging opportunities (Hallenbeck, 2009). In fact, the dark fermentative way to produce hydrogen is one of the most extensively studied fields and is currently the most promising one when practicality is considered (Han et al., 2012; Wang et al., 2012; Diamantis et al., 2013; Sarma et al., 2013). However, utilization of abundant and inexpensive lignocellulosic wastes, such as de-oiled Jatropha waste (DJW) needs adequate microbial consortia (Fan et al., 2006). To obtain such bacterial populations, the seed pretreatment is often a key step. Nevertheless, the most appropriate technique to enrich the hydrogen-producing strains has to be found specifically for each case due to the diversities in the microbial population structure of seed sources of different origins (Mohammadi et al., 2011; Wang et al., 2011). In addition to the importance of seed pretreatment, it is also known that biotechnological hydrogen formation is influenced by the environmental conditions applied. Among them, temperature, pH and substrate concentration are most crucial (Hawkes et al., 2007; Wang and Wan, 2009a). Therefore, these factors must carefully be optimized to improve hydrogen generation e.g. by the experimental design approach (Wang and Wan, 2009b).

In our previous study, it was shown that the DJW could be successfully converted into hydrogen and methane by untreated, mixed microbial flora (Kumar and Lin, 2013). In this work, firstly, the selection of feasible seed pretreatment was attempted. Subsequently, the enhancement of hydrogen production was aimed by optimizing the substrate (i.e. DJW) concentration, pH and temperature using the experimental design approach.

2. Materials and methods

2.1. Inoculum and DJW

The seed inoculum was collected from a municipal wastewater treatment plant located in central Taiwan. The main features of the inoculum were previously described elsewhere (Kumar and Lin, 2013). The cellulose (14.1%), hemicellulose (24.2%) and lignin (30.4) contents of the DJW were measured by the FIBERTEC™ analyzer as indicated in our previous study (Kumar et al., 2012).

2.2. Inoculum pretreatment methods

Several methods were tested for inoculum pretreatment. The heat shock treatment was conducted by heating the sludge in a water bath at 95 °C for 30 min. The acid pretreatment was performed by adjusting the pH of the sludge to 3.0 for 24 h. During base pretreatment, the pH of the sludge was increased to 11.0 and maintained for 1 d. The chemical treatment was carried out by adding KNO_3 (1000 mg/L) to the sludge. The combination of acid and heat pretreatment involved acidification and subsequent heat shock, meanwhile the base and heat strategy was also established by exposing the sludge firstly to base and then to heat, according to the procedure mentioned above.

2.3. Experiment I: selection of efficient inoculum pretreatment method

Batch vials (holding capacity of 225 mL) with a working volume of 150 mL were used for the fermentation. The bottles contained 30 mL of seed sludge (pretreated by various ways), dried DJW substrate to a final concentration of 50 g/L, 5 mL of the nutrient solution and drops of either 1N HCl or NaOH to adjust the pH of the solution to neutral. The final working volume was made up with tap water. The composition of the nutrient solution used can be found elsewhere (Kumar and Lin, 2013). The vials were purged with argon for 5 min to provide a fully anaerobic environment. Afterwards, the batch bottles were placed in a reciprocal air-bath shaker at 150 rpm agitation rate and temperature of 55 °C. The volume and composition of the biogas formed were measured periodically.

2.4. Experiment II: statistical parameter optimization

The batch tests were performed by following the procedure given in Section 2.3, except that the batch vials used had a total and working volume of 125 mL and 60 mL, respectively. This change was made in order to reduce the amount of chemicals and substrate needed. The initial measurement conditions –substrate concentration, temperature and pH – were set according to the experimental design matrix (**Table 1**). A five-level central composite design (CCD) and response surface methodology (RSM) were used to optimize substrate concentration, pH and temperature. After optimization, verification experiments were conducted in parallel.

Table 1.
Central composite design for optimizing the hydrogen production rate.

Substrate concentration		Temperature		Initial pH		HPR*
X_1 code	X_1 (g/L)	X_2 code	X_2 (°C)	X_3 code	X_3 (1)	(L H_2/L-d)
-1	100	-1	45	-1	5.5	0.33
-1	100	-1	45	1	7.5	0.35
-1	100	1	65	-1	5.5	0.36
-1	100	1	65	1	7.5	0.35
1	300	-1	45	-1	5.5	0.47
1	300	-1	45	1	7.5	0.48
1	300	1	65	-1	5.5	0.46
1	300	1	65	1	7.5	0.45
-1.682	32	0	55	0	6.5	0.32
1.682	368	0	55	0	6.5	0.34
0	200	-1.682	38	0	6.5	0.34
0	200	1.682	72	0	6.5	0.38
0	200	0	55	-1.682	4.8	0.37
0	200	0	55	1.682	8.2	0.5
0	200	0	55	0	6.5	1.3
0	200	0	55	0	6.5	1.34
0	200	0	55	0	6.5	1.38
0	200	0	55	0	6.5	1.4
0	200	0	55	0	6.5	1.38

* HPR: Hydrogen production rate

2.5. Analytical procedures

The soluble metabolic products (SMPs) including volatile fatty acids (VFAs) and alcohols (e.g. ethanol an butanol) were determined by GC-FID (Shimadzu GC-14, Japan). The volume of biogas was measured by a glass syringe at room temperature and 1 atm. The gas composition was analyzed by GC-TCD (China Chromatograph 8700T). All the measurement conditions are available in our previous work (Kumar and Lin, 2013). Kinetic analysis using the modified Gompertz equation was performed as previously reported (Kumar and Lin, 2013). Microbial community analysis (PCR-DGGE) was conducted by following the protocol described by Sivagurunathan et al., (2014).

3. Results and discussion

3.1. Influence of various pretreatment methods on hydrogen yield and formation rate

In **Figure 1A**, it can be seen that both biogas and hydrogen generation were highly dependent on the applied seed sludge pretreatment,

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demonstrating that selection of appropriate pretreatment is a key step for hydrogen generation. The obtained production performances are listed in **Table 2**, where it is shown that heat treatment was the most efficient method in comparison with the other methods employed. On the other hand, chemical, base and acid pretreatments as well as the control produced not only hydrogen but also methane. Thus, these methods were not suitable for efficient hydrogen production due to the co-generation of methane (Wang et al., 2012).

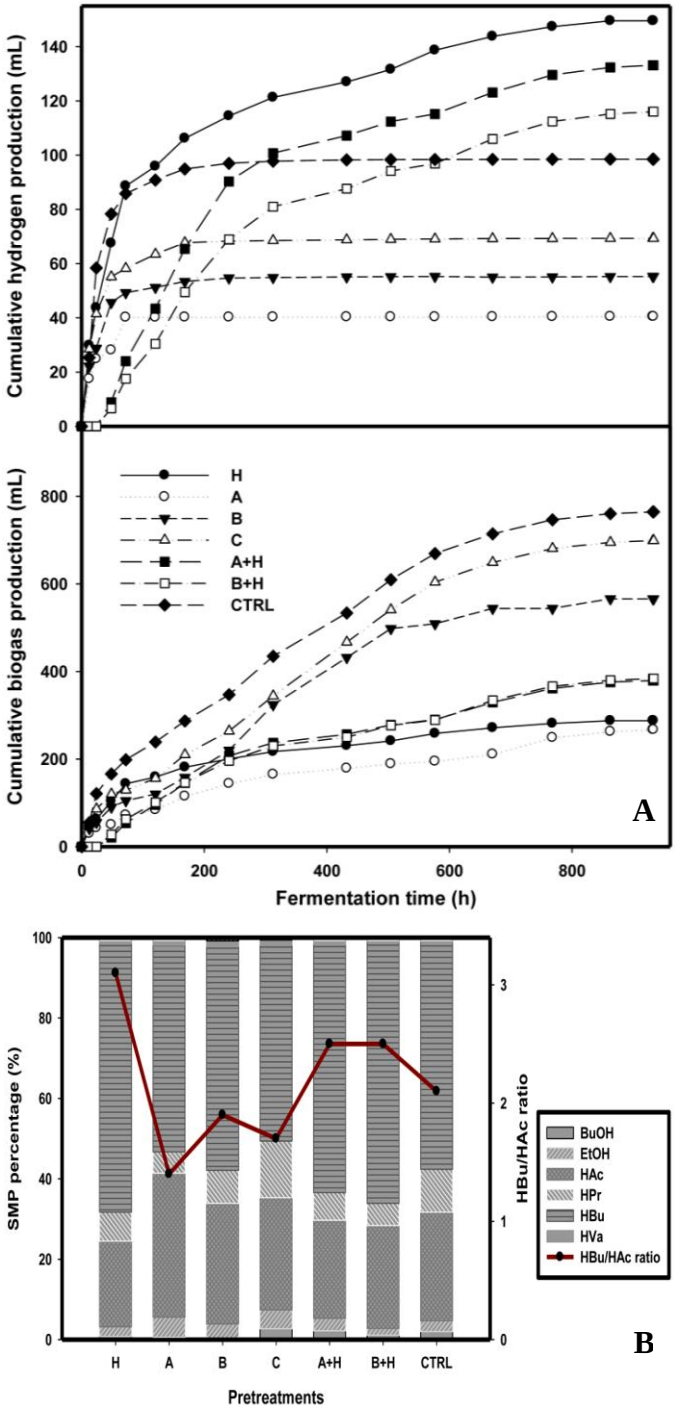

Fig.1. A: Progress curves of biogas and hydrogen fermentation for the various pretreatment methods, and B: SMP distribution of hydrogen fermentation for the various pretreatment methods (BuOH: butanol; EtOH: ethanol: HAc: acetate; HPr: propionate; HBu: butyrate; and HVa: valerate).

The reason assumed beyond the superiority of heat-associated treatment is that it preferentially enhances the growth of the endospore-forming *Clostridium* species, which are considered as good hydrogen-producers (Lin et al., 2006; Li and Fang, 2007). The kinetic analyses of the various pretreatment methods are tabulated in **Table 2**, where it could be observed that treatment conditions significantly influenced the bacterial lag phase time. This can be explained by the distinct adaptation capabilities of the microbes to the changes in the environmental conditions caused by the pretreatments applied.

3.2. SMP distribution

Monitoring the SMPs during anaerobic fermentation is suggested to be used to judge the bioreactor's performance and consequently the appropriateness of the available hydrogen producing cultures (Dabrock et al., 1992; Khanal et al., 2004).

The concentrations of VFAs and solvents formed in the hydrogen evolution step for different sludge pretreatment methods are summarized in **Figure 1B**, where it is indicated that the SMPs were dominated by butyric- and acetic acids accounting for 76.6-90.7% of the total SMPs.

The other side products such as propionate, valerate, and alcohols showed less significant contributions (9.3-23.4%) to the total SMPs. The high HBu/HAc ratio indicated an efficient biohydrogen generation system, since efficient hydrogen production is usually associated with high HBu production. Similar findings were reported in other studies as well (Lin and Chang, 1999; Chen et al., 2002; Lin et al., 2006; Demirbas, 2007; Hawkes et al., 2007).

According to **Figure 1B**, it can be pointed out that the highest HBu/HAc ratio (3.1) could be attained via applying heat pretreatment. These research findings demonstrate that metabolic activity (e.g. SMPs release) of the microbes is related to the pretreatment method used to promote the growth of the reliable hydrogen-evolving organisms.

3.3. Optimization of process parameters affecting biohydrogen production

The main operational parameters in biohydrogen production are substrate concentration, temperature and pH. In order to evaluate their effects on hydrogen fermentation of DJW, central composite design and response surface methodology were employed.

During these experimental runs, the heat-pretreated sludge was used. The experimental design matrix along with the corresponding response values of the dependent variable HPR are shown in **Table 1**.

The levels of the independent variables were chosen based on the preliminary experimental results. Analysis of Variance (ANOVA) was carried out by using Statistica 8 software to get the significance of each process variable. The results of ANOVA are listed in **Table 3**. The impacts of the parameters scoped were ranked based on the obtained *P-values*.

Basically, a smaller *P-value* stands for higher influence and only factors having *P-values* <0.05 can be considered as statistically important ones (Guo et al., 2009).

Accordingly, as it can be seen in **Table 3**, all the input variables studied in this work could affect biohydrogen formation in a statistically significant manner. As a result of the statistical evaluation, a mathematical model describing the hydrogen production, more specifically the HPR in connection with substrate concentration, pH and temperature could be established (**Eq. 1**):

$$HPR = 1.356 + 0.037X_1 - 0.345X_1^2 + 0.004X_2 - 0.334X_2^2 + 0.017X_3 - 0.308X_3^2 - 0.009X_1X_2 - 0.001X_1X_3 - 0.006X_2X_3. \quad \textbf{(Eq. 1)}$$

In the present study, according to the ANOVA results, the R^2 value of 0.986 suggested that there was a reliable agreement between the experimental data and the values predicted by the model (Chong et al., 2009). Using the results obtained the contour plots could be constructed with the two-dimensional projections of the fitted three-dimensional surfaces (**Fig. 2**).

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Table 2.
Biogas production performance under various pretreatment conditions.

Pretreatments	Final pH	Total biogas (mL)	Cumulative H_2 (mL)	Cumulative CH_4 (mL)	HPR_{max} (L H_2/L-d)	HY_{max} (mL H_2/g VS)	MPR_{max} (L CH_4/L-d)	MY_{max} (mL CH_4/g VS)	λ (h)	
									H_2	CH_4
CTRL	6.8	764.5	98.5	258.5	0.35	13.1	0.09	34.5	0.4	114
A	6.7	267.0	40.5	86.1	0.12	5.4	0.02	11.5	6	66
B	6.5	566.0	55.3	242.0	0.18	7.4	0.12	32.3	5	159
C	6.4	699.0	69.3	239.9	0.23	9.2	0.09	33.0	3	120
A+H	5.4	379.5	133.2	ND	0.25	17.8	ND	ND	29	ND
B+H	5.8	384.0	116.0	ND	0.29	15.5	ND	ND	27	ND
H	5.4	287.5	149.5	ND	0.36	20.0	ND	ND	37	ND

A : Acid B : Base C : Chemical H : Heat treatment HY : expressed in terms of VS_{added} ND : not detected

Table 3.
ANOVA table for hydrogen production rate (HPR).

	Response variable: HPR (L H_2/L-d)				
Factor	***SS***	***df***	***MS***	***F-value***	***P-value***
X_1	0.019	1	0.019	11.608	0.027
X_1^2	1.622	1	1.622	1014.016	<0.001
X_2	<0.001	1	<0.001	0.150	0.718
X_2^2	1.524	1	1.524	952.582	<0.001
X_3	0.004	1	0.004	2.392	0.197
X_3^2	1.292	1	1.292	807.396	<0.001
X_1X_2	0.001	1	0.001	0.383	0.570
X_1X_3	<0.001	1	<0.001	0.008	0.934
X_2X_3	<0.001	1	<0.001	0.195	0.681
Pure Error	0.006	4	0.002	-	-
Total SS	3.506	18	-	-	-

SS : sum of squares df : degree of freedom MS : mean square

These graphs were intended to illustrate the effects of two independent variables on HPR while keeping the level of the third factor at its center value (**Fig. 2A-C**). As it appears in **Figure 2A-C**, all the contour plots demonstrated a round ridge running around the center point implying that the interactions between the factors had only low importance (Kim et al., 2004). This is confirmed by the data presented in **Table 3** as well, where it can be observed that the interactive effects (X_1X_2, X_1X_3, X_2X_3) are insignificant (*P-value*>0.05).

Moreover, it is to be concluded that the maximum HPR value could be achieved within the design boundaries. Hence, the optimal conditions yielding the highest HPR could be derived by RSM as follows: substrate concentration of 205 g/L, initial pH of 6.53 and temperature of 55.1 ^oC, where peak HPR value of 1.36 L H_2/L-d was estimated.

Although experimental design methods have definite benefits, the statistically estimated optimum conditions need to be validated (Mu et al., 2006). Under the predicted optimal conditions, the HPR value was found as 1.42±0.03 LH_2/L-d. This proved that the actual optimal HPR satisfactorily matched its statistically-forecasted value (1.36 L H_2/L-d) with only 4% difference between them. Therefore, the results obtained confirmed the feasibility of using the experimental design and RSM for biohydrogen process development.

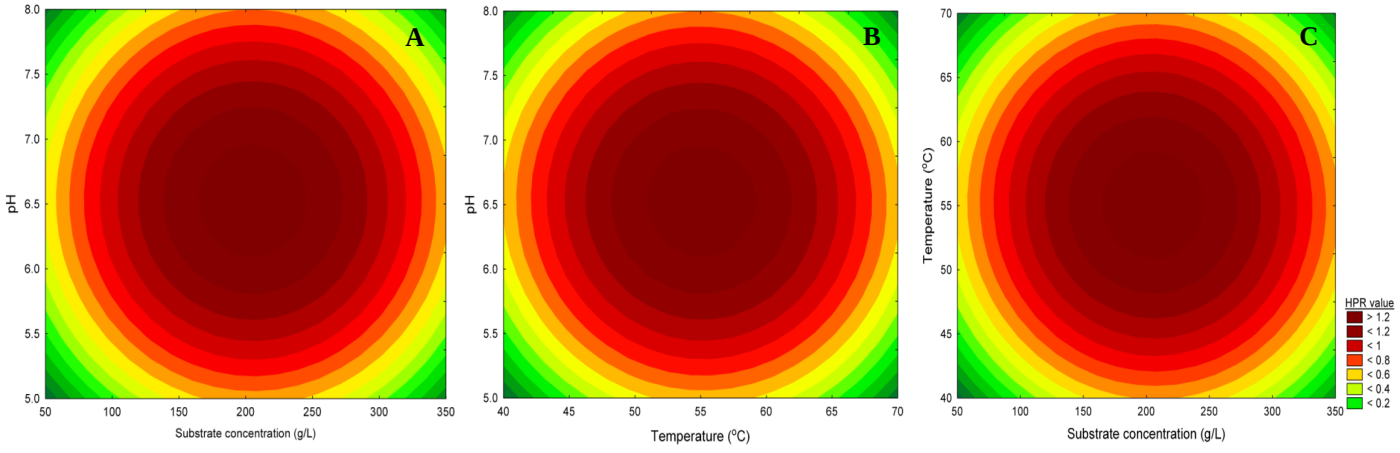

Fig.2. (A) Contour plots showing the effects of initial pH and substrate concentration on hydrogen production rate (HPR), (temperature: 55 ^oC), (B) Contour plots showing the effects of initial pH and temperature on hydrogen production rate (HPR), (substrate concentration: 200 g/L), (C) Contour plots showing the effects of temperature and substrate concentration on hydrogen production rate (HPR), (pH: 6.5).

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Table 4.
Comparison of the results with relevant literature data.

Substrates	Inoculum	Range studied			Optimal conditions	Hydrogen production index	References
		Substrate Concentration index	Temperature (^{0}C)	pH			
Glucose	Potato & soybean oil soil, HT-Compost	1.5-44.8 g COD/L	NA	4.5-7.5	SC:7.5 g COD/L, T:NS, pH:5.5	HPR:74.7 mL/L-h	Van Ginkel et al., 2001
POME	*C. butyricum* EB6	60-100 g COD/L	32-42	5.3-6.7	SC: 94 g COD/L, T: 36 ^{o}C, pH:6.05	HPR: 849.5 mL/h	Chong et al., 2009
Sucrose	HT-sludge	10-30 g COD/L	NA	5.5-8.5	SC:20g COD/L, T:NS, pH:7.5	HPR :745 mL/L-h	Wang et al., 2006
Mushroom waste	HT-cow dung	NA	NA	NA	SC: 20 g COD/L, T: 55 ^{o}C, pH:8.0^a	HY: 0.68 mol/g COD	Lay et al., 2012
DJW	HT-Sludge	40-240 g/L	45-65	5.5-7.5	SC: 205 g/L, T:55 ^{o}C, pH:6.5	HPR: 59.2 mL/L-h	This study

DJW : De-oiled Jatropha Waste
T : temperature (in ^{0}C)
POME : palm oil mill effluent
NA : not available
HT : heat treated
a : given values
SC : substrate concentration (specified)

A comparison with other works focusing on substrate concentration, temperature and pH for enhanced biohydrogen production is given in **Table 4.** As a conclusion, it can be pointed out that most of these investigations were performed using the classic, so-called "one-factor-at-a-time" method. On the other hand, only a few studies were dedicated to the experimental design. The optimum value obtained in the current research is quite comparable with the results of other reports employing complex (lignocellulosic) substrate materials.

3.4. Microbial community pattern

The DGGE profile obtained using the bacterial primer set EUB968gc–UNIV1392r revealed the structural composition of the microbial communities based on the V6 region of the 16s rRNA. The DGGE profile of the hydrogen producing microbial community using DJW under optimal condition are depicted in **Figure 3A.** As shown, four individual distinct bands patterns representing four different strains were observed. The phylogenetic tree distribution was established using the bootstrap neighbor joining method (Saitou and Nei, 1987) as shown in **Figure 3B**.

The microbial load in the seed inocula enriched with de-oiled Jatropha waste was made up by *Clostridium sp.*, and *Lactobacillus sp*. According to the major bands shown in **Figure 3A**, *Clostridium sp.* was the most abundant followed by *Bacillus sp*. The bacterial species *C. thermopalmarium*, *C. thermobutyricum*, and *B. ginsengihumi* were previously reported as potential hydrogen producing bacteria (Wiegel et al., 1989; Geng et al., 2010; Walton et al., 2010). *Clostridium Sp.*, a low G+ C content bacterium, is known to generate hydrogen along with butyrate and acetate as major SMPs (Chen et al., 2002; Levin et al., 2004).

In addition, *C. thermopalamarium* was reported to efficiently utilize cellulose (a major component of the DJW) to produce hydrogen. Moreover, *Bacillus sp.* was also reported to produce lactic acid from hemicellulose extracts (Walton et al., 2010). The inoculum source i.e. the sewage sludge selected in the present study was a rich source of hemicelluloytic and cellulolytic bacteria (Sleat et al., 1984).

Under the optimum conditions employed in the present study, *Clostridium sp.* and *Bacillus sp.* were the predominant microorganisms. while the former was more abundant. *Clostridium* species are known hydrogen producers with butyrate type fermentation in which acetic acid and alcohols are the minor metabolites (Levin et al., 2004). This was in agreement with the results of the SMPs analysis, suggesting that butyrate was the major metabolite observed during the fermentation, followed by acetic acid and ethanol. The PCR-DGGE based sequence analysis also revealed the presence of dominant butyrate-mediated hydrogen-producing bacteria present in the mixed cultures.

4. Conclusion

Various sludge pretreatment methods were tested for biohydrogen production using de-oiled Jatropha waste as feedstock. It turned out that among the methods studied, heat pretreatment was most effective in order to enrich efficient hydrogen-producing microorganisms. Moreover, central composite design was successfully used for the optimization of the most crucial operational parameters. The distribution pattern of the SMPs showed that the fermentation mainly followed the acetate-butyrate pathway. Moreover, PCR-DGGE results revealed that *Clostridium* sp. were majorly present under the optimal conditions.

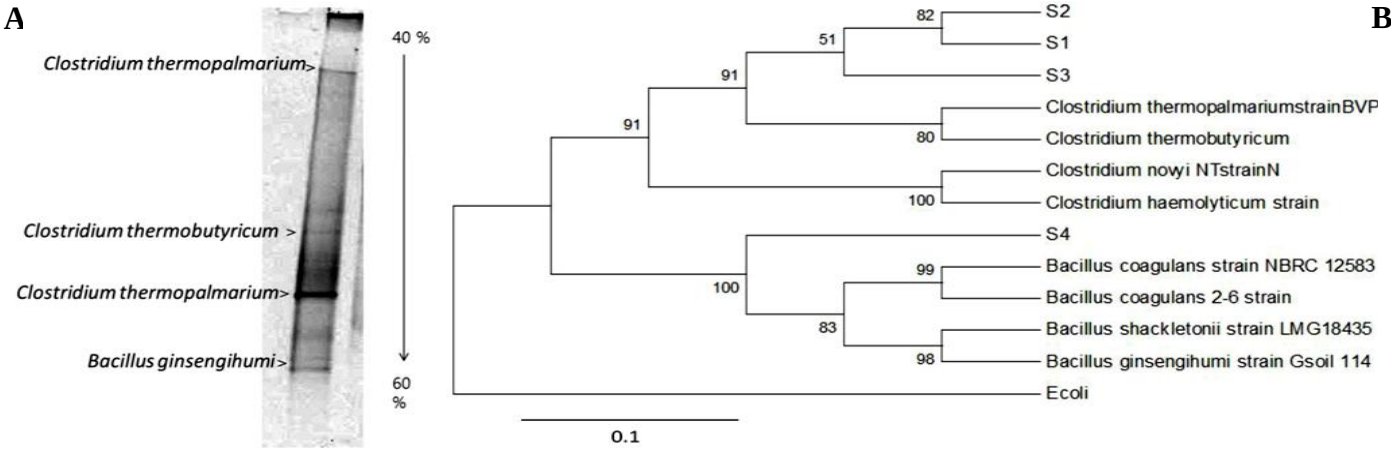

Fig.3. A: The DGGE profile of the hydrogen-producing microbial community under optimum conditions using DJW, and B: Phylogenetic tree showing the relatedness of the sequences identified in the mixed cultures. The tree was constructed based on maximum composite likelihood method using the neighbor-joining algorithm with 1,000 bootstrapping. *E.coli* was selected as the outgroup species. The scale bar represents 0.1 substitutions per nucleotide position. Numbers at the nodes are the bootstrap values.

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