Optimization and Molecular Characterization of Syngas Fermenting Anaerobic Mixed Microbial Consortium TERI SA1

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ABSTRACT. The present study focused on the optimization and molecular characterization of anaerobic mixed consortium TERI SA1 that can utilize synthesis gas as sole carbon source for volatile fatty acids production. Optimization study using Box-Behnken design and RSM methodology was carried out in order to investigate the effect of three medium factors on metabolite formation from synthesis gas bioconversion: (yeast extract (0.0–2.0 g/L), ammonium chloride (0.0–1.5 g/L) and corn steep liquor (0.0–10 g/L). Optimized parameters enhanced the production of volatile fatty acids upto 3.9 g/L, which indicated an increase of around 289 % from the non-optimized conditions. Furthermore, two approaches were used for isolation and phylogenetic identification of anaerobic consortium TERI SA1 involving 16S rRNA sequencing of culturable bacterial isolates as well as metagenomic approach (by making a 16S rRNA gene library of total community DNA). Based on similarity search with NCBI database selected positive clones were most closely related with acetogenic microorganisms \textit{Clostridium scatologenes}, \textit{Clostridium carboxydivorans}, \textit{Clostridium drakei} and Uncultured \textit{Clostridium sp}. and strains isolated by culturable method (ASH051 and ASH 052) with \textit{Clostridium scatologenes}, and \textit{Clostridium drakei}. These strains have previously been reported for acetic acid production from syngas bioconversion.

Keywords: Synthesis gas; Consortium; Volatile fatty acids; Optimization; Characterization.

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1. Introduction

Bioethanol production from lignocellulosic biomass by using synthesis gas fermentation is a relatively new technology. This technology combines gasification of nearly all the components of biomass into synthesis gas (also called syngas) which mainly consists of (CO, H\textsubscript{2} and CO\textsubscript{2}) and fermentation of syngas components into acetic acid, ethanol and other valuable metabolites using homo-acetogenic organisms. Syngas fermentation process have several advantages than chemical catalytic process such as, higher substrate specificity of biocatalysts, lower energy costs, greater resistance to catalyst poisoning and the lack of requirement for a fixed CO:H\textsubscript{2} ratio (Bredwell et al., 1999). Acetogenic microorganisms can reduce CO\textsubscript{2} to acetate via the Wood-Ljungdahl pathway or acetyl-CoA pathway. These bacteria are strictly anaerobic and produce acetate as the major fermentation product ( Muller, 2003). Homoaacetogenic bacteria is capable to produce ethanol from syngas fermentation includes \textit{C. autoethanogenum}, \textit{Mesophilic bacterium P7}, \textit{Alkalibaculum bacchi}, \textit{Clostridium ljungdahlii}, \textit{Clostridium drafkei}, \textit{Butyrribacterium methylotrophicum}, \textit{Clostridium ragsdalei}, \textit{Eubacterium limosum} and mixed consortium TERI SA1 among others (Liu et al., 2012; Mohammadi et al., 2012; Singla et al., 2014). So, far syngas bio-conversion was mainly studied using pure cultures or defined co-cultures (Lundie and Drake, 1984; Hurst and Lewis, 2010; Kundiyanag et al 2011). However, Liu et al (2013) reported the ethanol and acetic acid production from

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syngas bioconversion using defined co-culture of strain CP15 and Clostridium propionicum. Hitherto, mixed culture approaches for bioconversion of syngas in to valuable products has been received little attention and microbial community diversity linked to CO conversion was never assessed. Further, optimization of medium is very important step for enhancing metabolites production through microbial interventions (Demain and Davies, 1999; Kennedy and Krouse, 1999). Studies revealed the importance of medium composition in enhancing the overall metabolite production (Klasson et al., 1992; Cotter et al., 2009; Abubackar et al., 2011). Cotter et al., (2009), examined the effect of nitrogen-limited media on ethanol and acetate production using bacterial strains C. autoethanogenum and C. ljungdahlii. Gaddy and Clausen, (1992) analyzed the effect of yeast extract concentration on product formation during the batch fermentation of C. ljungdahlii. A molar ethanol to acetate ratio of 0.11 was achieved at low levels of yeast extract (0.005, 0.01 and 0.05%), whereas this ratio was 0.05 at higher yeast extract concentrations (0.1 and 0.2%). Moreover, VFAs are valuable chemical compounds and have diverse uses in the market and offers an economically as well as ecologically sustainable platform. They are building blocks for the various valuable organic compounds such as alcohols, aldehydes, ketones, esters and olefins (Fig. 1).

The present research was focused on optimization and phylogenetic identification of syngas fermenting mixed microbial consortium TERI SA1 for valuable organic compounds production (i.e., volatile fatty acids) from syngas bioconversion. The effect of different combination of nitrogen sources such as: yeast extract (YE), corn steep liquor (CSL) and ammonium chloride (NH4Cl) concentration, on syngas bioconversion to ethanol and other valuable products such as volatile fatty acids was also investigated. A two level, Box-Behnken RSM (response surface methodology) experimental design was employed to estimate the optimum concentration of these parameters that affects syngas bioconversion for enhanced VFAs production. Furthermore, two approaches culturing under anaerobic conditions as well as metagenomic approach (by making 16S rRNA gene library) was used for the isolation and phylogenetic identification of the consortium members.
2. Experimental Section

2.1. Microorganism and medium composition

Mixed anaerobic microbial consortium used for this study was enriched from chicken faecal material by growing on Pfennig’s basal medium and using syngas as sole carbon source (Singla et al., 2014). Pfennig’s basal media (PBM) medium containing (per liter) 50 ml mineral stock solution, 10 ml trace metal stock solution, 10 ml vitamin stock solution, 1 g yeast extract, 1 ml resazurin (0.1%) and 20 ml L-cysteine HCl (2.5%) was used for the initial enrichment of microbial strains. The composition of the mineral, vitamin and trace metal stock solutions has been previously reported (Gaddy and Clausen, 1992). Experimentation work was performed in serum bottles of total volume 130 mL with 40 mL of the liquid medium. Anaerobic condition during media preparation was maintained as previously reported by Singh et al. (2014). B-Vitamins solution was added after sterilization. The initial pH of the medium was adjusted at 6.0±0.1 with 2 N KOH and 2 N HCl solutions. However, solid medium was prepared by adding 1% glucose as carbon source and 1.5% agar as solidifying agent in the PBM medium.

2.2. Experimental system and bioconversion studies adopted for medium optimization

Anaerobic bottles with a total volume of 130 mL with 40 mL working volume were used for experimental purposes. Around one third of the total space of reactor bottle was dispensed with medium and remaining two third was pressurized with syngas. Higher headspace volume allows more gaseous substrate to be available for microbes to utilize. The experimental set-up and the method used for media preparation have been previously described (Singla et al., 2014). The consortium was grown under anaerobic conditions at 150 rpm and 37°C in an orbital shaker. Synthetic syngas (Laser gases, New Delhi, India) was provided as carbon source for bacterial growth. 10% (v/v) of actively growing culture (grown with only syngas as sole carbon source) was used as inoculum and aseptically transferred to each experimental bottle. 0.5 mL of gaseous sample collected from reactor bottle headspace was used for analyzing syngas utilization and 2 mL of liquid sample was periodically withdrawn from each culture bottle (once every 72 h) up to 360 h in order to measure pH, optical density and for analyzing the changes in ethanol, acetic acid and other VFAs concentrations. After collecting liquid as well as gaseous sample, culture bottles were resparged with fresh syngas and maintained with the same headspace pressure of 1 kg/cm² and incubated under the same conditions.

2.3. Experimental design and statistical analysis for medium optimization studies

Present study also focused on estimating the optimum concentration of nitrogen source that enhances ethanol and VFAs production from syngas bioconversion. A two-level Box-Behnken RSM (response surface methodology) experimental study was done to analyze the effects of different concentration of nitrogen sources; YE (low 0.0 g/L and high 2.0 g/L), CSL (low 0.0 g/L and high 10 g/L) and NH4Cl concentration (low 0.0 g/L and high 1.5 g/L) on VFAs and ethanol production from the syngas by mixed consortium TERI SA1 (Table 1).

In this study, experimental design and data analysis have been performed using software package Minitab 17 (Minitab Inc. State College, PA, USA) (Table 4). Furthermore, in this study three different variables (CSL, Yeast extract and NH4Cl) were studied with VFAs and ethanol as the response factors. The relationship and interrelationship of the variables was determined by fitting the second-degree polynomial equation to data obtained from 15 run order experiments using mean values of the triplicates (Table 3). All these experiments were set for observations at 3rd, 6th, 9th, 12th and 15th days. The parameters analyzed were ethanol production and VFAs production. The data was analyzed using multiple regressions and a second order polynomial model as given in Equation (1).

\[ Y = \beta_0 + \sum \beta_n X_n + \sum \beta_{nm} X_n^2 + \sum \beta_{nm} X_n X_m \]  

Where Y was the predicted response, \( \beta_0 \) was the intercept coefficient, \( \beta_n \) was linear coefficient, \( \beta_{nm} \) was quadratic coefficient and \( \beta_{nm} \) was interaction coefficient. The interactive effects of significant variables were represented in form of contour plots. The response surface interaction and contour plots were generated to understand the interaction among various variables. The statistical significance of the regression coefficient was calculated by Student’s t-test. The second order model equation was determined by Fischer’s test and the quality of the fit for the polynomial model equation was evaluated by the coefficient of determination R² (Dalal et al., 2013).
2.4. Analytical methods

The cultures were tested for syngas utilization using gas chromatograph (Agilent-7890A, USA) equipped with thermal conductivity detector (TCD) and a molecular siev stainless steel packed column (2 mm i.d. NUCON, INDIA). Argon gas with the flow rate of 5 mL/min along with nitrogen at flow rate of 3 mL/min was used as makeup gas. The operational temperatures of the oven, injector and detector port were kept at 50, 100 and 150 °C respectively. Prior to the analysis, a calibration curve for gases was prepared and R² value was found to be 0.998 (Singh et al., 2014). The concentrations of VFAs in liquid phase were analysed using same GC equipped with flame ionization detector and DB-WAX column (30 m × 500 µm × 1 µm) as described by Singh et al., (2014). Helium was used as the carrier gas. The injector and detector temperatures were 220 and 230 °C respectively. VFA method was calibrated and the calibration curve of each VFA was prepared and R² value was 0.998. The bacterial cell growth was monitored by measuring the optical density (OD) at 600 nm using UV visible spectrophotometer (UV-2450, Shimadzu, Japan). Initial and final pH was checked by pH meter (Mettler Toledo, India). The protocol followed for analysis has also been previously described by Singla et al., (2014).

2.5. Isolation of bacterial strains from mixed consortium TERI SA1

The authors have enriched mixed consortium TERI SA1 from chicken fecal sample procured from Gwal pahari (Gurgaon, Haryana, 28°28′N 77°1.9′E) India (Singla et al., 2014). To isolate individual bacterial strains from syngas fermenting anaerobic mixed consortium TERI SA1, the enriched culture was serially diluted up to 10⁻⁵ times and 100-μl aliquots of the diluted bacterial suspension was spread on the specific PBM (Pfennig’s basal medium) medium plates with 1% glucose as carbon source and 1.5% agar as solidifying agent. Spreading was done in triplicates. Incubation was then carried out for 96 h at 37 °C under anaerobic condition in an anaerobic chamber (Bactron-IV, Shell lab USA). The two distinct bacterial colonies thus obtained were further purified by sub-culturing thrice using same medium and was subsequently identified using 16S rRNA gene analysis based molecular techniques.

2.6. Molecular characterization using 16S rRNA gene analysis based techniques

Two approaches were simultaneously used for molecular characterization and phylogenetic identification of syngas fermenting anaerobic mixed consortium TERI SA1. One was the 16S rRNA gene sequencing of bacterial strains isolated using culturable method and another was by making 16S rRNA gene library of 33 clones isolated from mixed consortium TERI SA1 for entire bacterial diversity study.

DNA extraction was done using a standard CTAB method for bacterial genomic DNA isolation (Winnepenninckx et al., 1993). 16S rRNA gene was amplified using polymerase chain reaction (PCR) with Taq Polymerase and primers M13F 5′- GTAAAACGACGGCCAGT-3′ and M13R 5′- AACAGCTATGACCATG-3′ (Sigma, USA). Gene cloning and selection of positive clones was done using blue/white screening approach. The PCR (polymerase chain reaction) products were subjected to electrophoresis in 0.8 % agarose gels in 1X TAE buffer (91 V for 30 min), and were then analyzed using a Gel Doc system (UVITECH Cambridge, UK). PCR products were purified with the gel purification kit (RBC). Alternatively, the recombinant vector was identified by performing a restriction enzyme digestion to determine the presence of the correct insert.

Nucleotide sequencing was performed using di-deoxy termination method (Macrogen, Korea). Identification of bacterial strains was carried out by homology search of the 16S rRNA sequences with the Nucleotide Sequence Data Library (NCBI) using the BLAST n program. The 33 nucleotide sequences obtained from mixed consortium TERI SA1 have been submitted to the Genebank under the accession numbers KR997801 to KR997835 (Table 1). All the sequences were aligned using the Clustal W program.

3. Result and Discussion

3.1. Optimization of medium parameters for enhancing volatile fatty acids production

Syngas bioconversion using mixed consortium approach has also been previously studied (Liu et al., 2013; Singla et al., 2014). Nutrients such as nitrogen source, vitamins and sodium have been shown to affect acetogenic metabolism (Lundie and Drake, 1984; Yang and Drake, 1990; Muller, 2003; Saxena and Tanner, 2012). Some of the main medium parameters that enhance metabolite production from syngas bioconversion process are yeast extract, corn steep liquor and ammonium chloride concentration (Gaddy and Clausen, 1992; Guo et al., 2010; Maddipati et al., 2011). Limited availability of some of the essential medium components can cause obstructions in cell metabolism, cofactor formation and intracellular enzyme production. Such condition favors non-growth conditions which divert the metabolic pathway towards solvent formation from acidogenic phase (Klasson et al., 1992; Cotter et al., 2009).

RSM was used to determine the optimum concentration of three factors affecting bioconversion of syngas, maintaining the rest of the variables at a constant level. According to current experimental design, 15 set of experiments were conducted containing three replications at the center point for
estimating the experimental uncertainty variance in triplicates (Dalal et al., 2013). In present study, three key variables with two concentration levels were studied. Table 1, revealed the three variables and their concentrations at the different coded levels. Table 2 indicated the three variables and their concentrations at different levels of the experimental design and the corresponding response.

### Table 2
The Experimental data for the tested process variables at the end of 9th day (n=3)

| Run Orders | Yeast extract (g/L) | Corn steep liquor (g/L) | Ammonium chloride (g/L) | Ethanol (g/L) | VFAs (g/L) |
|------------|---------------------|-------------------------|-------------------------|---------------|------------|
| 1          | 2                   | 0                       | 0.75                    | 0.3           | 3.9        |
| 2          | 2                   | 10                      | 0.75                    | 0.2           | 2.4        |
| 3          | 1                   | 0                       | 1.5                     | 0.15          | 2.6        |
| 4          | 0                   | 5                       | 0                       | 0.06          | 2.1        |
| 5          | 1                   | 5                       | 0.75                    | 0.05          | 3          |
| 6          | 0                   | 5                       | 1.5                     | 0.1           | 2          |
| 7          | 1                   | 5                       | 0.75                    | 0.05          | 3          |
| 8          | 0                   | 0                       | 0.75                    | 0.009         | 0.13       |
| 9          | 1                   | 10                      | 1.5                     | 0.09          | 3.3        |
| 10         | 1                   | 0                       | 0                       | 0.05          | 2          |
| 11         | 2                   | 5                       | 0                       | 0.07          | 3.8        |
| 12         | 0                   | 10                      | 0.75                    | 0.06          | 2.3        |
| 13         | 1                   | 10                      | 0                       | 0.06          | 3.2        |
| 14         | 2                   | 5                       | 1.5                     | 0.08          | 3.4        |
| 15         | 1                   | 5                       | 0.75                    | 0.05          | 3          |

### 3.2. Main effects plot
VFAs and ethanol are valuable metabolites synthesized from syngas bioconversion by microbial means. The main effects plot for the studied medium components was shown in Fig. 2. Significant improvement in VFAs production was observed when increasing the YE concentration and concentration of CSL up to 5 g/L, whereas increasing NH₄Cl concentration and CSL concentration above 5 g/L did not make a significant impact (Fig. 2a). Similar syngas bioconversion studies have been previously performed suggesting that increasing the YE concentration and CSL concentration results in the increased VFAs production (Maddipati et al., 2011; Gao et al., 2013; Shenkman, 2014). The metabolites production pattern used to stay in the acidogenic phase and did not shift towards solventogenic phase when there was sufficient amount of nutrients or nitrogen source available. As the metabolite, volatile fatty acids (acetic acid, butyric acid, Isobutyric acid, Isovaleric acid, valeric acid and hexanoic acid ), are growth related product and produced in the maximum amount when there was sufficient amount of nutrients available for the growth of bacterial cells (Liggett and Koffler, 1948) and hence resulted in increased production of VFAs than ethanol. Increasing CSL up to a limited concentration was found to enhance the VFAs production using C.autoethanogenum (Maddipati et al., 2011). In similar way, for selected consortium TERI SA1 higher concentration of CSL (above 5 g/L) negatively affected and was associated with decreased VFAs production (Fig. 2a). One other medium factor studied that effect metabolites production from syngas bioconversion was NH₄Cl, acting as essential mineral component and nitrogen source. But with the studied mixed consortium TERI SA1 there was no significant effect of varying NH₄Cl concentration on VFAs production, as there was similar amount of VFAs production observed with and without NH₄Cl (Fig. 2a). This was due to the reason that YE and CSL were capable to fulfill all the nitrogen source and mineral requirements of the bacterial cells and there was no more requirement of NH₄Cl.

Therefore, from the main effects graphical representation it could be interpreted that, YE at the concentration of 2 g/L slightly enhanced ethanol production using mixed consortium TERI SA1 (Fig. 2b). This might be due to the minimum growth requirements of bacterial cells for ethanol production. The nutritional requirement by a particular microbial community depends on their metabolic pathway and varies from species to species. Acetogenic bacteria
develop different metabolic capabilities which might be associated with different medium requirement (Frostl et al., 1996; Silveira et al., 2001). NH₄Cl showed only a slight change in metabolites concentration due to availability of other two nitrogen sources in sufficient amount. Similar medium optimization studies have been previously done by Gao et al., (2010) who studied the effect of NH₄Cl on ethanol production with Clostridium autoethanogenum. CSL did not make a significant effect on ethanol production at the concentration of 5 g/L and 10 g/L (Fig. 2b) as, it has been reported that ethanol is a non-growth related product (Klasson et al., 1992). This might be due to the high cell growth achieved at increased concentrations of CSL due to the presence of additional nutrients such as sugars, vitamins, amino acids, minerals and trace metals in CSL (Liggett and Koffler, 1948). Similar trends have been previously shown by Saxena and Tanner (2012), with Clostridium ragsdalei which observed that using CSL at the concentration of 10 g/L leads to reduced ethanol production. Higher concentration of CSL (50 and 100 g/L) was also inhibitory for cell growth and ethanol production (Savage and Drake, 1986).

3.3. Interaction effects plot

Graphical representation of interaction effect revealed the mean response at all possible combinations of each two factors studied. For VFAs and ethanol production interaction plot represented that there was a significant positive interaction between YE and CSL as well as between YE and NH₄Cl (Fig. 3a and 3b). However, NH₄Cl did not show any significant interaction with CSL (Fig. 3a and 3b). Maximum amount of VFAs production was observed at yeast extract concentration of 2.0 g/L and CSL concentration of around 5.0 g/L, at both (minimum and maximum) concentrations of NH₄Cl (Fig. 3a). Highest concentration of CSL (10 g/L) resulted in decreased VFAs production than at CSL concentration of 5 g/L, revealing the effect of YE (2 g/L) and limited concentration of CSL (up to 5 g/L) for enhancing VFAs production. It was possible that sufficient amount of nutrients for VFAs production and cell growth were available at higher YE concentration (2.0 g/L) and concentration of CSL up to 5.0 g/L, therefore further increment in any of the nitrogen source did not make significant positive effect on metabolites production (Fig. 3a and 3b). Similar type of study has been previously reported by Kundiyana et al (2010), where maximum acetic acid and ethanol concentration was 4.9 g/L and 0.6 g/L respectively at midst value of studied CSL concentration. However, higher concentration of VFAs was also observed to be produced at maximum concentration of YE, at midst value of NH₄Cl (0.75 g/L). Therefore, the following interpretation could be make from the study that at highest concentration of YE (2 g/L), NH₄Cl (0.75 g/L) and CSL (5 g/L) were playing supplementary role for each other means NH₄Cl was no longer required in the presence limited concentration of CSL (5.0 g/L) or vice versa.

At high YE concentration, an increase in CSL concentration from 5.0 g/L to 10 g/L and NH₄Cl concentration from minimum (0.0 g/L) to maximum (1.5 g/L) leads to no significant improvement in VFAs production and only slight improvement in ethanol concentration (Fig. 3a and 3b). It could be easily observed from the interaction plot that at maximum YE concentration of 2.0 g/L, there was significant improvement in ethanol production with no CSL in the medium (Fig. 3b), confirming that optimum nitrogen requirement of bacterial cell was fulfilled by only increasing the concentration of YE. Interaction between YE and NH₄Cl at their highest concentrations, had a significantly positive effect on ethanol production.
Similar trends have been previously reported by Guo et al. (2010) with *C. autoethanogenum* where optimization of NH$_4$Cl enhanced final ethanol concentration from 0.06 g/L to 0.25 g/L. Furthermore, increased concentration of NH$_4$Cl above mid value (0.75 g/L) as well as interaction between CSL and NH$_4$Cl did not make any significant impact on ethanol production (Fig. 3b). It could be interpreted by the fact that increase in the CSL concentration (up to 5 g/L) resulted in more supply of nitrogen sources, therefore increment in only VFA concentration not ethanol concentration (Fig. 3a and 3b).

The contour plots drawn with the help of Minitab 17 software revealed the effect of interaction among the studied components on bioconversion of syngas into ethanol and VFAs. In the present study three sets of contour plots were obtained as presented in Fig. 4 and 5. Figure 4 suggest significant interaction between CSL and YE, CSL and NH$_4$Cl and NH$_4$Cl and YE. Similar variable patterns were also found in case of ethanol production (Fig. 5).

From the shape of the contour plots, the interaction between the variables could be predicted. Therefore, an elliptical and saddle nature of contours suggested that there was a significant interaction among the variables whereas, circular plots showed negligible interactions (Murthy et al., 2000).

The statistical software was used to evaluate the observed experimental results to derive a regression equation by using an ordinary least square method. From regression equation the statistical significance, direction and magnitude of the relationship between an effect and the response could be predicted (Table 3 and 4). The sign of each regression coefficient indicate the direction of the relationship among the variables. The effects with low p-values are statistically significant (Table 4) (Montgomery, 2003).
The regression models proposed were as follows:

Ethanol (g/L) = 0.007 + 0.003 x₁ - 0.0111 x₂ + 0.111 x₃ + 0.0574 x₁ x₁ + 0.00220 x₂ x₂ - 0.0264 x₃ x₅ - 0.01055 x₁ x₂ - 0.0100 x₁ x₅ - 0.0040 x₂ x₃

VFAs (g/L) = 0.056 + 2.631 x₁ + 0.4462 x₂ - 0.257 x₃ - 0.384 x₁ x₁ - 0.01735 x₂ x₂ + 0.371 x₃ x₅ - 0.1835 x₁ x₂ - 0.100 x₁ x₅ - 0.0333 x₂ x₃

These regression models were confined for each variable within the following range: (A) YE = 0.0-2.0 g/L, (B) CSL = 0.0 - 10 g/L, (C) NH₄Cl = 0.0 - 1.5 g/L.

### Table 3
Regression analysis for Ethanol (g/L) versus NH₄Cl, CSL and YE and YE and VFAs versus NH₄Cl, CSL and YE.

| Term       | Total VFAs | Total Ethanol |
|------------|------------|---------------|
| **Effect** | **Coeff**  | **SE Coeff**  | **t-Value** | **p-Value** | **Effect** | **Coeff**  | **SE Coeff** | **t-Value** | **p-Value** |
| Constant   | 3.00       | 0.172         | 17.45       | 0.000       | 0.0600     | 0.0523      | 1.15        | 0.303       |
| YE         | 1.743      | 0.871         | 8.28        | 0.000       | 0.1153     | 0.0576      | 1.80        | 0.132       |
| CSL        | 0.643      | 0.321         | 3.05        | 0.028       | -0.0272    | -0.0136     | -0.43       | 0.688       |
| NH₄Cl      | 0.050      | 0.025         | 0.24        | 0.822       | 0.0625     | 0.0313      | 0.98        | 0.374       |
| YE*YE      | -0.768     | -0.384        | 0.155       | -2.48       | 0.056      | 0.1148      | 0.0574      | 0.471       | 1.22        | 0.278       |
| CSL*CSL    | -0.868     | -0.434        | 0.155       | -2.80       | 0.038      | 0.1098      | 0.0549      | 0.471       | 1.17        | 0.297       |
| NH₄Cl*NH₄Cl| 0.417      | 0.209         | 0.155       | 1.35        | 0.236      | -0.0297     | -0.0149     | 0.471       | -0.32       | 0.765       |
| YE*CSL     | -1.835     | -0.918        | 0.149       | -6.16       | 0.002     | -0.1055     | -0.0527     | 0.453       | -1.17       | 0.296       |
| YE*NH₄Cl  | -0.150     | -0.075        | 0.149       | -0.50       | 0.636      | -0.0150     | -0.0075     | 0.453       | -0.17       | 0.875       |
| CSL*NH₄Cl | -0.250     | -0.125        | 0.149       | -0.84       | 0.439       | 0.0471       | 1.17        | 0.297       |

* YE - yeast extract, CSL- corn steep liquor, NH₄Cl- ammonium chloride

### Table 4
Analysis of variance for Ethanol (g/L) versus NH₄Cl, CSL and YE and YE and VFAs versus NH₄Cl, CSL and YE.

| Source            | For VFAs | For Ethanol |
|-------------------|----------|-------------|
| **DF** | Seq. SS  | Adj SS | Adj MS | F-Value | P-Value | DF | Seq. SS  | Adj SS | Adj MS | F-Value | P-Value |
| Model             | 9        | 11.7725  | 11.7725 | 1.30806 | 14.76   | 0.004 | 9        | 0.071687 | 0.071687 | 0.007965 | 0.97 | 0.544 |
| Linear            | 3        | 6.9032  | 6.9032  | 2.30108 | 25.96   | 0.002 | 3        | 0.035863 | 0.035863 | 0.011954 | 1.46 | 0.331 |
| YE                | 1        | 6.0726  | 6.0726  | 6.07261 | 68.50   | 0.000 | 1        | 0.026565 | 0.026565 | 0.026565 | 3.24 | 0.132 |
| CSL               | 1        | 0.8256  | 0.8256  | 0.82561 | 9.31    | 0.028 | 1        | 0.001485 | 0.001485 | 0.001485 | 0.18 | 0.688 |
| NH₄Cl             | 1        | 0.0850  | 0.0850  | 0.08500 | 0.06    | 0.822 | 1        | 0.007813 | 0.007813 | 0.007813 | 0.95 | 0.374 |
| Square            | 3        | 1.4171  | 1.4171  | 1.41713 | 5.33    | 0.051 | 3        | 0.023569 | 0.023569 | 0.007856 | 0.96 | 0.480 |
| YE*YE             | 1        | 0.5047  | 0.5047  | 0.50473 | 6.13    | 0.056 | 1        | 0.011096 | 0.011096 | 0.012155 | 1.48 | 0.278 |
| CSL*CSL           | 1        | 0.7515  | 0.6947  | 0.69467 | 7.84    | 0.038 | 1        | 0.011656 | 0.011656 | 0.011119 | 1.36 | 0.297 |
| NH₄Cl*NH₄Cl       | 1        | 0.1609  | 0.1609  | 0.16090 | 1.82    | 0.236 | 1        | 0.000817 | 0.000817 | 0.000817 | 0.10 | 0.765 |
| 2-Way Interaction | 3        | 3.4522  | 3.4522  | 3.45223 | 12.98   | 0.009 | 3        | 0.012255 | 0.012255 | 0.004085 | 0.50 | 0.699 |
| YE*CSL            | 1        | 3.3672  | 3.3672  | 3.36723 | 37.99   | 0.002 | 1        | 0.011130 | 0.011130 | 0.011130 | 1.36 | 0.296 |
| YE*NH₄Cl          | 1        | 0.0225  | 0.0225  | 0.02250 | 0.25    | 0.636 | 1        | 0.000225 | 0.000225 | 0.000225 | 0.03 | 0.875 |
| CSL*NH₄Cl         | 1        | 0.0625  | 0.0625  | 0.06250 | 0.71    | 0.439 | 1        | 0.009090 | 0.009090 | 0.009090 | 0.11 | 0.754 |
| Error             | 5        | 0.4432  | 0.4432  | 0.44323 | 1.4477  | 5.004958 | 0.049585 | 0.081192 |
| Lack of Fit       | 3        | 0.4432  | 0.4432  | 0.00000 | 0.00000 | 0.00000 | 0.00000 |
| Pure Error        | 2        | 0.0000  | 0.0000  | 0.00000 | 0.00000 | 0.00000 | 0.00000 |
| Total             | 14       | 12.2158 | 12.2158 | 12.2158 | 14      | 0.112645 | 0.112645 | 0.112645 |

* YE - yeast extract, CSL- corn steep liquor, NH₄Cl- ammonium chloride
3.6. Isolation and characterization of culturable bacterial strains from mixed consortium TERI SA1

Analysis of sequences by comparing with known sequences from NCBI database revealed that the two isolates obtained through culturable method under anaerobic conditions had sequence similarities of more than 98.0% to sequences with Clostridia species. One out of two sequences was 99% similar to Clostridium scatoralgenes and the sequence of the second strain was 99% similar to that of Clostridium drakei (Table 1). They could be classified according to taxonomic status such as Firmicutes, Clostridia, Clostridiales, Clostridiaceae, and Clostridium. There are previous reports on Clostridium isolated from soil and waste samples that have also been reported for syngas bioconversion into ethanol and acetic acid (Kusel et al 2000; Liu et al 2005).

Table 5
Sequence similarity analysis of the isolates obtained from consortium TERI SA1 based on BLASTn comparison to the Genebank database.

| Culturable method          | Isolates     | Closest relative          | Accession No |
|----------------------------|--------------|----------------------------|--------------|
| Metagenomics approach      | ASH051       | Clostridium drakei        | KR997801     |
|                            | ASH052       | Clostridium scatoralgenes | KR997802     |
|                            | Clones No.   | Closest relative          | Accession No |
| 1                          | Clostridium scatoralgenes | KR997804     |
| 2                          | Clostridium scatoralgenes | KR997805     |
| 3                          | Clostridium scatoralgenes | KR997806     |
| 4                          | Clostridium scatoralgenes | KR997807     |
| 5                          | Clostridium drakei       | KR997808     |
| 6                          | Clostridium scatoralgenes | KR997809     |
| 7                          | Clostridium scatoralgenes | KR997810     |
| 8                          | Clostridium drakei       | KR997811     |
| 9                          | Clostridium scatoralgenes | KR997812     |
| 10                         | Clostridium scatoralgenes | KR997813     |
| 11                         | Clostridium scatoralgenes | KR997814     |
| 12                         | Clostridium drakei       | KR997815     |
| 13                         | Clostridium scatoralgenes | KR997816     |
| 14                         | Clostridium scatoralgenes | KR997817     |
| 15                         | Clostridium scatoralgenes | KR997818     |
| 16                         | Clostridium scatoralgenes | KR997819     |
| 17                         | Clostridium scatoralgenes | KR997820     |
| 18                         | Clostridium scatoralgenes | KR997821     |
| 19                         | Clostridium scatoralgenes | KR997822     |
| 20                         | Clostridium scatoralgenes | KR997823     |
| 21                         | Clostridium scatoralgenes | KR997824     |
| 22                         | Clostridium scatoralgenes | KR997824     |
| 23                         | Clostridium scatoralgenes | KR997825     |
| 24                         | Clostridium scatoralgenes | KR997826     |
| 25                         | Clostridium scatoralgenes | KR997827     |
| 26                         | Clostridium scatoralgenes | KR997828     |
| 27                         | Clostridium scatoralgenes | KR997829     |
| 28                         | Clostridium scatoralgenes | KR997830     |
| 29                         | Clostridium scatoralgenes | KR997831     |

Fig. 5. Contour plots for ethanol production by anaerobic mixed culture TERI SA1, (a) effects of yeast extract and corn steep liquor, (b) effects of yeast extract and ammonium chloride, (c) effects of ammonium chloride and corn steep liquor.
3.7. Molecular Characterization and Identification of consortium TERI SA1 by culture independent method

Thirty three positive clones were selected using a 16S rRNA clonal library of the community DNA of consortium TERI SA1 by culture independent method. 16S rRNA sequences from selected positive clones were amplified using M13F and M13R primers and further identified using bioinformatics tools. Comparative analysis of 16S rRNA sequences from an entire community with sequences from Genebank indicated that 31 out of 33 sequences were 99% similar to Clostridium scatologenes. However, the remaining two sequences revealed 99% similarity to Clostridium drakei (Table 5). Several recent reports have documented Clostridium strains from soil and waste samples, which are capable of syngas bioconversion into ethanol and acetic acid (Kusel et al., 2000; Liou et al., 2005). C. scatologenes isolated from soil in 1925, generated acetic acid as the major end product with an optimal growth temperatures of 37 °C - 40 °C. Furthermore, C. drakei, originally classified as C. scatologenes SL1, was isolated from an acidic coal mine pond (Kusel et al., 2000; Liou et al., 2005), was similar to C. carboxidivorans and C. scatologenes and found to have an optimal growth temperature of 37 °C.

As a result, isolation and phylogenetic identification of individual strains from the consortium TERI SA1 indicated that the mixed consortium TERI SA1 either contained only two categories of bacterial strains or there could be additional consortium members which were not discovered from culturable as well as non culturable method used for bacterial identification. Majority of strains from identified members were related to Clostridium scatologenes while the remainder yielded similarities to Clostridium drakei (Table 5). Moreover, these strains have been previously reported for syngas bioconversion to ethanol and acetic acid (Kusel et al., 2000; Liou et al., 2005; Hurst and Lewis, 2010; Ukpong et al., 2012).

4. Conclusion

The present study demonstrated that mixed consortium TERI SA1 was well capable of utilizing syngas for VFAs production. Higher VFAs production was favored at combination of lower NH4Cl concentration and higher concentrations of YE and CSL. A maximum VFAs concentration of 3.9 g/L was obtained under the following conditions: YE = 2.0 (the maximum value tested), CSL = 5.0 g/L (the midst of value tested) and NH4Cl = 0.0 g/L (the minimum value tested). VFAs, in turn can be a renewable feedstock and building block for production of various important biofuels and biochemicals for industrial uses. Moreover, phylogenetic identification of the individual strains isolated from consortium TERI SA1 along with molecular characterization of the consortium using culture independent methods indicated the presence of Clostridium scatologenes and Clostridium drakei strains.

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